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Research paper

Molecular identification of *Borrelia* genus in questing hard ticks from Portugal: Phylogenetic characterization of two novel Relapsing Fever-like *Borrelia* sp.

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ABSTRACT

In the last decades, several studies have reported pathogenic species of *Borrelia* related to those that cause Tick-borne Relapsing Fever (RF), but unexpectedly suggesting their transmission by hard ticks, known vectors of *Borrelia burgdorferi* sensu lato (*B. burgdorferi* s.l.) species, rather than by soft ticks. This study was designed to update the presence of *B. burgdorferi* s.l. species in ticks from several districts of mainland Portugal, where *Ixodes ricinus* had been previously described.

Ticks (a total of 2915 specimens) were collected in seven districts throughout the country, and analyzed using molecular methods. Three nested-PCR protocols, targeting the flagellin gene (*flaB*), the intergenic spacer region (IGS) located between 5S and 23S rRNA, and the *glpQ* gene, and a conventional PCR targeting the 16S rRNA, were used for *Borrelia* DNA detection.

Borrelia DNA was detected in 3% of the ticks from Braga, Vila Real, Lisboa, Setúbal, Évora and Faro districts. The obtained amplicons were sequenced and analyzed by BLASTn, and 15/63 (24%) matched with homologous sequences from *Borrelia lusitaniae* and 15/63 (24%) with *B. garinii*, being these the most prevalent species. DNA from *B. burgdorferi* sensu stricto (*s.s.*), *B. valaisiana* and *B. afzelii* were detected in 7/63 (11%), 6/63 (10%), and 2/63 (3%) of the specimens, respectively. Unexpectedly, DNA sequence (*flaB*) analysis from eight (13%) samples, two from *Rhipicephalus sanguineus* and six from *Haemaphysalis punctata* tick species, revealed high homology with RF-like *Borrelia*. Phylogenetic analyses obtained from three genetic markers (16S rRNA, *flaB*, and *glpQ*) confirmed their congruent inclusion in a strongly supported RF cluster, where they segregated in two subgroups which differ from the other Relapsing Fever species.

Therefore, the results confirm the circulation of multiple species of *B. burgdorferi* s.l. over a wide geographic range, covering most of the Portuguese mainland territory. Surprisingly, the obtained data also revealed two putative Relapsing Fever-like *Borrelia* species in different species of hard ticks, possibly disclosing the circulation of novel RF-like *Borrelia* species with different associated tick vectors.

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1. Introduction

The genus *Borrelia* is a group of helical-shaped, motile bacteria that form a monophyletic lineage within the phylum Spirochetes, and comprises two major clades. In some cases, associations of specific groups of bacteria with certain species of tick vectors have been postulated, but this is still open to debate.

Traditionally, spirochetes classified in the so-called *Borrelia burgdorferi* sensu lato (*B. burgdorferi* s.l.) complex are transmitted by hard ticks of *Ixodes* genus. On the other hand, the Tick-borne Relapsing Fever *Borrelia* (RFB) are usually described as being transmitted by soft *Argasidae* ticks (Cutler, 2015), with the exception for *B. theileri* that, despite being classified as a RFB, is usually associated with hard ticks (*Rhipicephalus* spp.) (McCoy et al., 2014).

Phylogenetic studies carried out in the last decades, whether based on 16S ribosomal RNA (rrs) or on flagellin gene (*flaB*) sequences have come to challenge the simplistic vector/host division, suggesting that a proposal of somewhat strict associations between certain species of *Borrelia* and their vectors might be more difficult to defend than initially

Abbreviations: RF, Relapsing Fever; *B. burgdorferi* s.l., *Borrelia burgdorferi* sensu lato; *flaB*, flagellin gene; IGS, intergenic spacer region; *B.*, *Borrelia*; *B. burgdorferi* s.s., *Borrelia burgdorferi* sensu stricto; RFB, Relapsing Fever *Borrelia*; STARI, Southern Tick-Associated Rash Illness; LD, Lyme Disease; ML, Maximum Likelihood; NJ, Neighbor-Joining; TOT, transovarial transmission.

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anticipated. Indeed, and as an example, many of these studies reported that several different species of *Borrelia* classified as RFB were, in fact, transmitted by hard ticks. These included *B. miyamotoi*, firstly found in *Ixodes persulcatus* in Asia (Fukunaga et al., 1995) but also present in other *Ixodes* species, *B. lonestari* detected in *Amblyomma americanum* in North America (Barbour et al., 1996), *Borrelia* sp. found in *Amblyomma geomydae* and in *Haemaphysalis* spp. in Japan (Takano et al., 2012), and *B. turcica* which was found to be transmitted among reptiles by *Hyalomma aegyptium* ticks (Güner et al., 2004; Kalmár et al., 2015). These findings also showed that *B. theileri*, *B. lonestari* and *B. miyamotoi* branch together as a single monophyletic group in phylogenetic trees, located deep within the Relapsing Fever spirochetes clade (Barbour, 2014), and clearly outside from *B. burgdorferi* s.l. complex. However, and despite their allocation to the RF-cluster of spirochetes, the metastriate-transmitted *Borrelia* spp. should not be assumed to be biologically equivalent to the RFB (maintained by argasid ticks), nor do they cause typical Relapsing Fever (Telford et al., 2015).

Up to the present day, the analysis of the different RFB has not been regularly updated, and with a few exceptions, they have not been as thoroughly studied as *B. burgdorferi* s.l. bacteria. As an exception, the biology of *B. theileri* has been relatively well examined, particularly the clinical aspects associated with bovine borreliosis and vector–pathogen interactions (Callow, 1967; Smith et al., 1985). *B. lonestari* has also been associated with a disease manifesting as what came to be known as Southern Tick-Associated Rash Illness (STARI) or Master's disease, although incrimination of *B. lonestari* as the etiologic agent of these diseases has not yet been demonstrated (Feder et al., 2011). Finally, *B. miyamotoi*, which has been found in different species of *Ixodes* ticks in different regions of North America, Europe and Asia (Geller et al., 2012; Cochez et al., 2015; Cosson et al., 2014; Crowder et al., 2014; Dibernardo et al., 2014; Hansford et al., 2014; Kiewra et al., 2014; Mukhacheva and Kovalev, 2014; Takano et al., 2014; Nunes et al., 2015; Venczel et al., 2015), has also been associated with human disease cases in Europe (Hovius et al., 2013; Jahfari et al., 2014), USA (Gugliotta et al., 2013; Krause et al., 2013), Russia (Platonov et al., 2011), and Japan (Sato et al., 2014).

Relapsing Fever has sporadically been reported in the Iberian Peninsula, mainly in Spain during the twentieth century (Sanchez-Yebra et al., 1997), but with an incidence that is most probably underestimated. In Portugal, reduction of human tick-borne RFB cases may have been an indirect consequence of African swine fever outbreaks from 1960 until 1993, resulting in decreasing numbers of Alentejano pig herds and traditional pigpens in this region (Boinas, 1994; Morais et al., 2007). Consequently, pig production housing was modified, with modern shelters being constructed with glass fiber or metal, unsuitable for tick survival. It became evident that traditional shelters constructed with stone and clay, often with cracks and crevices, were essential for *Ornithodoros erraticus* infestation, with no infestation being found in pigpens with smooth walls and floor (Palma et al., 2012). Nevertheless, *B. hispanica* was recently detected in *O. erraticus* (detection rate of 2.2%), from a swinery in the Alentejo region (in the south of Portugal), which proves that RF-causing bacteria still circulate naturally, and suggest that they may be responsible for the cases of fever-illness with an indeterminate etiology (Palma et al., 2012). In addition, very little is known about which RFB agents are potentially transmitted by hard ticks in Portugal. In fact there are only two recent studies reporting the molecular identification of *B. miyamotoi* at Tapada Nacional de Mafra in Lisboa region (≈35 km North of the capital). The first one in an *I. ricinus* nymph that had fed on a *Turdus merula* (Norte et al., 2012), and the second one in an *I. ricinus* nymph collected from the vegetation (Nunes et al., 2015).

In contrast to what is currently known for RFB, in the last decades, the incidence of Lyme Disease (LD) has been increasing in some countries of Europe (Hubálek, 2009). Consequently, LD is likely to become an increasingly relevant health risk in the near future due to complex interactions between diverse environmental and socio-economic factors, which will likely affect various aspects of disease ecology and epidemiology. Currently there are already 20 species of *B. burgdorferi* s.l. described (Margos et al., 2011), and six of them have already been reported in Portugal. The most prevalent species is *B. lusitaniae*, isolated for the first time from the vector in 1993 (Núncio et al., 1993), and lately from a patient skin biopsy in 2003 (Collares-Pereira et al., 2004). Despite the detection of *Borrelia* in several species of Ixodids in Portugal, the only tick species with proven vector competence is *I. ricinus*.

For two years, between 2012 and 2014, an extensive survey of ticks was carried out in several districts of Portugal, where *I. ricinus* ticks are present, with the aim of determining the prevalence of *B. burgdorferi* s.l., species. Thus, the results presented in this study not only confirmed the wide distribution of multiple species of *B. burgdorferi* s.l. throughout Portugal, but they also revealed the presence of two different species of RFB. One RFB DNA was found in questing *Haemaphysalis punctata*, while the other was detected in questing *Rhipicephalus sanguineus* hard ticks.

2. Material and methods

2.1. Study area and tick collection

Portugal, the westernmost country in continental Europe, has climatic conditions influenced by the Atlantic Ocean and the Mediterranean Sea (Information available at http://www.florestar.net. Access in 20-11-2015). Out of its 92,090 km^2 of land surface, 3.4 million ha correspond to forested areas, mainly localized north of the Tagus river, with agroforestry and forest grazing areas localized in the south of the country.

Between May 2012 and May 2014, questing ticks were collected from spring to fall on a monthly basis in Lisboa district, and one to three times during each season for the remaining districts, by flagging with a 1 × 1m cloth over low and high vegetation, with a similar time flag (30 min of sampling at each site). The 23 collecting sites were scattered throughout the country, and located in seven districts: Braga, Vila Real, Aveiro, Lisboa, Setúbal, Évora and Faro (Fig. 1). Collected ticks were identified at the species level using taxonomic keys (Estrada-Peña et al., 2004; Pérez-Eid, 2006) (Table 1), and then stored in vials with 70% ethanol until further use.

2.2. DNA extraction

Ticks were firstly washed in 70% ethanol and secondly in sterile distilled water, then dried on sterile paper and finally subjected to mechanical maceration. Genomic DNA was extracted by alkaline hydrolysis, with NH_4OH (0.7 M) as described by Wodecka et al. (2010), using a volume of 500 μl added to each adult ticks, or 100 μl added to immature ticks (larvae and nymphs). Adult and nymphal specimens were processed individually while larvae were pooled together by species and day of capture (ten specimens per pool). The obtained lysates were stored at −20 °C for further use.

2.3. PCR amplifications

2.3.1. DNA amplification from B. burgdorferi s.l. species

Detection of *B. burgdorferi* s.l. DNA was carried out using two different nested-PCR protocols. One of them targeted the intergenic spacer region (IGS), located between the 5S and 23S rRNA, using the 23SN1 and 23SC1 external primers (which amplify a 320 bp DNA fragment), and the 23SN2 and 5SC inner primers (which amplify a 280 bp DNA fragment), as described by Rijpkema et al. (1995). The nested-PCR protocol used included a denaturation step at 94.5 °C for 1 min, 25 cycles of amplification at 94 °C for 30 s, 52 °C for 30 s (outer primers), or 55 °C for 30 s (inner primers), and 72 °C for 1 min, followed by a 5 min extension phase at 72 °C. The second nested-PCR protocol used, targeted the

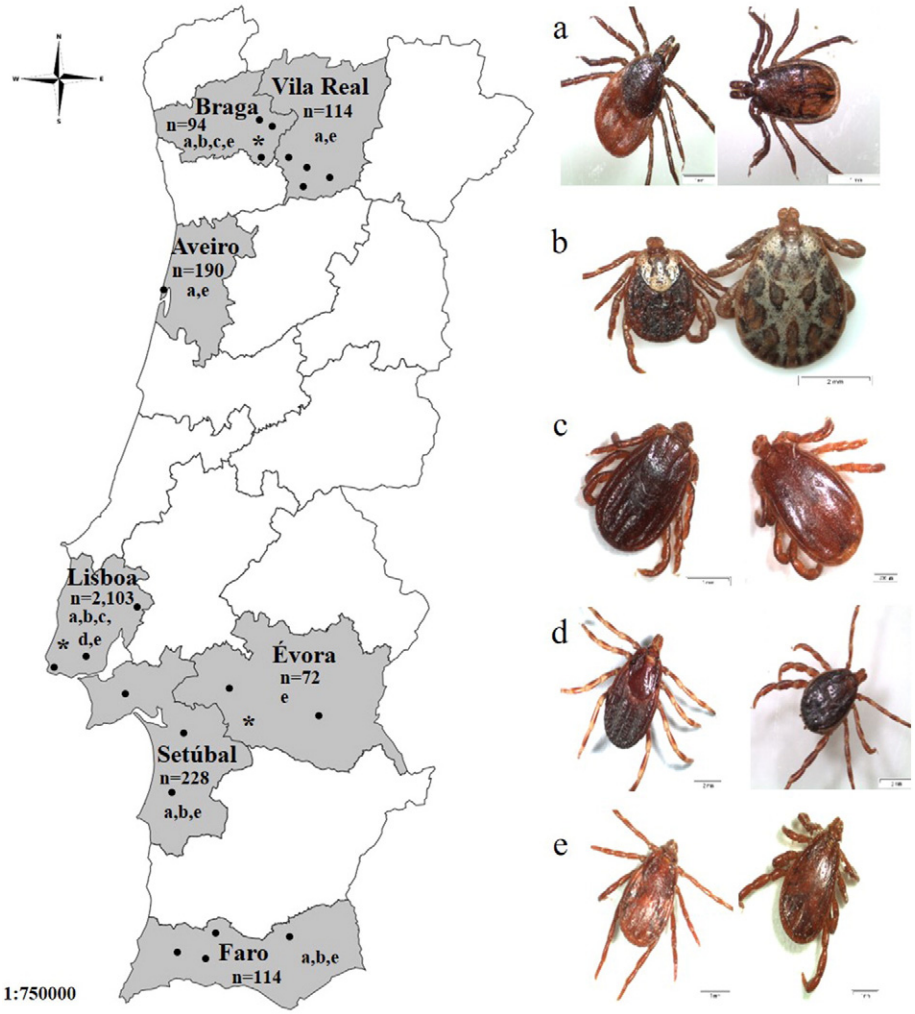

Fig. 1. Map of Portugal showing the total number of hard ticks collected by flagging per districts (Braga, Vila Real, Aveiro, Lisboa, Setúbal, Évora and Faro). Images of the different tick species collected are present (female specimens—left; male specimens—right). The black dots indicate the 23 collection sites; the genera of ticks identified in each district are indicated by letters (a — *Ixodes*; b — *Dermacentor*; c — *Haemaphysalis*; d — *Hyalomma*; e — *Rhipicephalus*); the asterisks indicate the districts were the two putative Relapsing Fever-like species were identified.

flagellin gene (*flaB*) (Wodecka et al., 2010). This included a first amplification reaction based on the use of outer primers 123f and 905r (which amplify a 774 bp DNA fragment), with a second amplification step using inner primers 220f and 824r (yielding an amplification product of 605 bp). The PCR conditions included an initial denaturation at 94 °C for 10 min, followed by 40 cycles of amplification, including denaturation at 94 °C for 30 s, annealing for 45 s at a temperature dependent on the primers used (outer primers—50 °C; inner primers—54 °C). An additional elongation step was carried out at 72 °C for 1 min with a final elongation at 72 °C for 7 min. PCR protocols were done in a separate vertical laminar flow bench using a different set of micropipettes, for PCR use-only as well filtered tips and sterilized material to ensure a contamination-free environment. *B. garinii* DNA was used as positive control and ultrapure water as negative control.

Nested-PCR products were detected by electrophoresis in 1.5% agarose gels stained with GreenSafe Premium (NZYTech), and visualized under UV light, using a Dolphin-1D Gel Image Analysis Software (Wealtec®) equipment.

2.3.2. DNA amplification from Relapsing Fever Borrelia species

For the characterization of the RF genospecies, two PCR protocols targeting the 16S rRNA, and *glpQ* genes were optimized using the primers described in Table 2. PCR conditions for the amplification of 16S rRNA (primer pair 16SB Fw/Rv) started with an initial denaturation at 94 °C for 3 min, followed by 35 cycles of amplification (denaturation at 94 °C for 30 s, annealing for 30 s at 60 °C, elongation at 72 °C for 2 min), with a final elongation at 72 °C for 7 min. Partial amplification of the *glpQ* gene was achieved using nested-PCR and a thermal profile comprising a denaturation step at 94 °C for 2 min, 40 cycles of amplification at 94 °C for 30 s, 56 °C for 1 min (outer primers: glpQ Fw1/Rv1), or 53 °C for 1 min (inner primers: glpQ Fw2/Rv2), and 72 °C for 45 s, followed by a 3 min extension phase at 72 °C. As in *B. burgdorferi* s.l. PCR protocols, negative control was prepared using ultrapure water and the same measures were taken to ensure the quality and prevent contamination of the biological material. As positive controls DNA from several RF species (*B. recurrentis*, *B. anserina*, *B. miyamotoi*, *B. parkeri*, *B. turicatae*, *B. duttoni* and *B. hispanica*), kindly provided by the German National Reference Centre for *Borrelia*, were used. PCR products were detected as described above.

2.4. DNA sequencing and analyses

The amplification products for *flaB* and IGS region were purified and directly sequenced on an Applied Biosystems 3730xl DNA Analyzer (Applied Biosystems, USA). The 16S rRNA and *glpQ* specific amplicons were sequenced with the oligonucleotide primers used for PCR, along with others, listed in Table 2. DNA sequences obtained in the course of this work were each assembled to generate single contigs using the CAP

Table 1
Species, stage, gender and number of collected ticks, analyzed for the presence of *B. burgdorferi* s.l. and Relapsing Fever *Borrelia* (RFB) spirochetes DNA.

		Collected and analyzed ticks					PCR-positive tick samples									
		Stage/gender					*B. burgdorferi* s.l. species					RF-like *Borrelia* sp.				
Districts	Tick species	Larva	Nymph	Female	Male	Total	Larva	Nymph	Female	Male	Total	Larvae	Nymph	Female	Male	Total
Braga	*Dermacentor marginatus*			6	4	10				1	1					
	Haemaphysalis punctata			2		2										
	Ixodes ricinus			3		3										
	Rhipicephalus sanguineus			41	38	79			1		1			1		1
Vila Real	*Ixodes ricinus*			8	7	15			2	2	4					
	Rhipicephalus sanguineus			40	59	99				1	1					
Aveiro	*Ixodes ricinus*				3	3										
	Rhipicephalus sanguineus			94	93	187										
Lisboa	*Dermacentor marginatus*			1		1										
	Haemaphysalis inermis			17	6	23										
	Haemaphysalis punctata	250	58	20	9	337	1	2		1	4	5	1			6
	Hyalomma lusitanicum		2	50	17	69		1			1					
	Hyalomma marginatum			2	16	18										
	Ixodes ricinus	440	947	32	58	1477	5	49	2		56					
	Rhipicephalus bursa			17	18	35										
	Rhipicephalus sanguineus	130	1	8	4	143										
Setúbal	*Dermacentor marginatus*			12	7	19										
	Ixodes ricinus			12	7	19			3	2	5					
	Rhipicephalus sanguineus			105	85	190										
Évora	*Rhipicephalus sanguineus*			37	35	72				2	2				1	1
Faro	*Dermacentor marginatus*			5	1	6										
	Ixodes ricinus			17	18	35			8	5	13					
	Rhipicephalus sanguineus			36	37	73										
	Total	820	1008	565	522	2915	6	52	16	14	88	5	1	1	1	8

Contig Manager tool available in BioEdit 7.0.9.0. (Hall, 1999). Nucleotide sequence (nt) similarity searches were carried out through the NCBI web server using BLASTn (megablast) and BLASTx tools (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The obtained data sequences were deposited in the public databases under accession numbers KR677086 to KR677091 and KT364297 (*flaB*), KT364298 to KT364305 (16S rRNA), and LC093500 to LC093507 (*glpQ*). The RFB and *B. burgdorferi* s.l. reference sequences used for the preparation of the different sequence-datasets were selected among those previously deposited in the GenBank database, on the proviso that they would be representative of (i) each of the previously described RFB and *B. burgdorferi* s.l. species with (ii) a significant sequence overlap with the sequences we have obtained, in order to maximize the number of unambiguously aligned nucleotide positions in each multiple-sequence alignment. When multiple reference sequences were available for each species, the 16S rRNA and *flaB* datasets included 1–2 sequences per species (randomly selected from those available in the databases), with the exception of (i) sequences described as *Borrelia* sp. and (ii) those more closely related to the ones obtained (e.g. *B. miyamotoi* and *B. lonestari*). In these cases, and when available, we chose to use from 3 to 5 references sequences. In the *glpQ* dataset, when available, each species was represented from 2 to 6 sequences.

Multiple alignments of nt sequences were performed using the iterative G-INS-i (*flaB* and *glpQ* sequences) or Q-INS-I algorithms (16S rRNA sequences) as implemented in MAFFT vs. 7 (Katoh and Standley, 2013). Editing of the alignments was done using the GUIDANCE guide-tree based alignment confidence (Penn et al., 2010), selecting columns with confidence levels above 0.9. For Maximum Likelihood (ML) and Bayesian phylogenetic analyses (see below), the choice of the best fitting evolutionary model was based on those defined using JModeltest2 (Darriba et al., 2012), on the basis of the AIC selection criterion. These models or the ones closest to them were used for phylogenetic reconstruction, depending on the software employed.

Phylogenetic trees were constructed using multiple approaches. Neighbor-Joining (NJ) tree reconstruction was carried out using the Mega 6.0 software (Tamura et al., 2013), and genetic distance matrixes corrected using the Tamura–Nei formula (Tamura and Nei, 1993). Mega 6.0 was also used for phylogenetic tree analysis using the ML optimization criterion. In this case the GTR + Γ (GTR—General Time Reversal; Γ—Gamma distribution) evolutionary model was chosen as being the one closest to those suggests by JModelstest (TIM3 + Γ and TPM2uf + Γ) for the analysis of *flaB*/*glpQ* and 16S rRNA sequences, respectively. Finally, a Bayesian approach to phylogenetic reconstruction was also undertaken using the GTR + Γ model and the MrBayes v3.0b4 software (Ronquist and Huelsenbeck, 2003). The analyses consisted of 5×10^7 generations starting from a random tree and four Markov chains with default heating values, sampled every 100th generation. Two separate runs were conducted for each analysis and the first 10% sampled trees discarded as burn-in. Finally, Maximum Clade Credibility trees were constructed using BEAST v1.7.5 (Drummond et al., 2012) GTR + Γ, and as coalescent priors, a Bayesian skyline plot for estimating demographic, under both strict and uncorrelated lognormal relaxed clock. These analyses were run for 1×10^8 generations starting from a random tree and sampling every 1000th generation. In each case,

Table 2
Primers used in this study for the specific analysis of Relapsing Fever *Borrelia*.

Gene	Primer designation	Sequence	Fragment size (bp)
16S rRNA	16SB Fw	5′-GAGGTGATCCAGCCACACTTTCCAG-3′	1324
	16SB Rv	5′-GCTTCGCTTGTAGATGAGTCTGCGTC-3′	
	16S Seq	5′-GAGCATACTCCCCAGGCGGCACACTTAAC-3′	
glpQ	glpQ Fw1	5′-TAGCTCAYAGRGGYGCHAGYG-3′	693
	glpQ Rv1	5′-ATCCAYGSVCCYATRCCYTC-3′	
	glpQ Fw2	5′-CCAGAACATACHYTAGARKCYAAAGC-3′	598
	glpQ Rv2	5′-TATTCATARTCYGTTGGKGMYTCDTYC-3′	

two separate runs were combined using LogCombiner (http://beast.bio.ed.ac.uk/logcombiner), and the first 10% discarded as bur-in. Convergence was monitored with Tracer v1.6 (available from http://beast/bio/ed.ac.uk/Tracer), with all ESS values were confirmed to be above 200. The phylogenetic trees were manipulated for display using Fig Tree v.1.4.2. (available at http://tree.bio.ed.ac.uk/software/figtree/). The genetic distances were calculated using the Tamura–Nei formula (Tamura and Nei, 1993), as implemented in the Mega 6.0 software (Tamura et al., 2013).

3. Results

In this study, 2915 ticks, representing both immature and adult stages, were collected across Portugal, in 23 collection sites located in seven districts. The ticks collected from each district were assorted by species based on morphological criteria, and separated by developmental stage and gender (male/female), as detailed in Table 1. Nymphs were the most prevalent development stage, followed by the larvae, and finally adults, including both females and males. The greater diversity of tick species was collected in the districts of Lisboa (the capital city), and Braga, in the north-west of Portugal. *I. ricinus* and *R. sanguineus* were the most frequently found species in these collections.

DNA of *B. burgdorferi* s.l. genospecies was detected by the two nested-PCR protocols (targeting *flaB* and IGS sequences), in 88 samples (3.3%), which included either single individuals (nymphs and adults), or pools of 10 larvae. Of these 7.3% of the total number of pools of larvae (6/82) were positive for *B. burgdorferi* s.l. DNA. On the other hand, 5.1% and 2.8%, respectively, of the total number of nymphs (52/1008), and adult specimens (30/1087), showed a positive amplification result. Sixty-three (72%) of the number of samples for which an amplicon was obtained were sequenced. The sequence data were blasted (megablast) against nucleotide references available in the GenBank/EMBL/DDBJ databases, and revealed matches with ≥97% identity with homologous sequences, all belonging to the *B. burgdorferi* s.l. complex. Fifteen (24%) of the sequences matched with *B. lusitaniae* and *B. garinii* (the most prevalent), from ticks collected at Lisboa, Setúbal, and Faro districts, followed by seven (11%) with *B. burgdorferi* s.s. from ticks collected at Vila Real, Lisboa and Setúbal districts, six (10%) with *B. valaisiana* and two (3%) with *B. afzelii*, both from ticks collected at Braga, Lisboa and Vila Real districts.

Unexpectedly, eight of the flagellin-specific amplicons revealed sequences with high identity (≥95%) with those from bacteria belonging to the RFB group. Since most of the obtained results disclosed matches to unknown species of *Borrelia* (*Borrelia* sp.), thorough genetic analyses of these strains were carried out, making use of different techniques for phylogenetic tree reconstruction.

In order to better understand the place held by the detected Portuguese *Borrelia* strains among RFB, two PCR protocols, targeting the 16S rRNA and *glpQ* genes from these bacteria, were additionally optimized, and the obtained amplification products (Fig. 2) were purified and sequenced. The determined 16S rRNA, *flaB*, and *glpQ* sequences were aligned with homologous sequences from a broad range of *Borrelia* species, including both RFB and *B. burgdorferi* s.l. downloaded from the public databases, and their phylogenetic relationships were inferred using complementary tree building methods (see Material and methods). Regardless of the analytic approach used, congruent trees

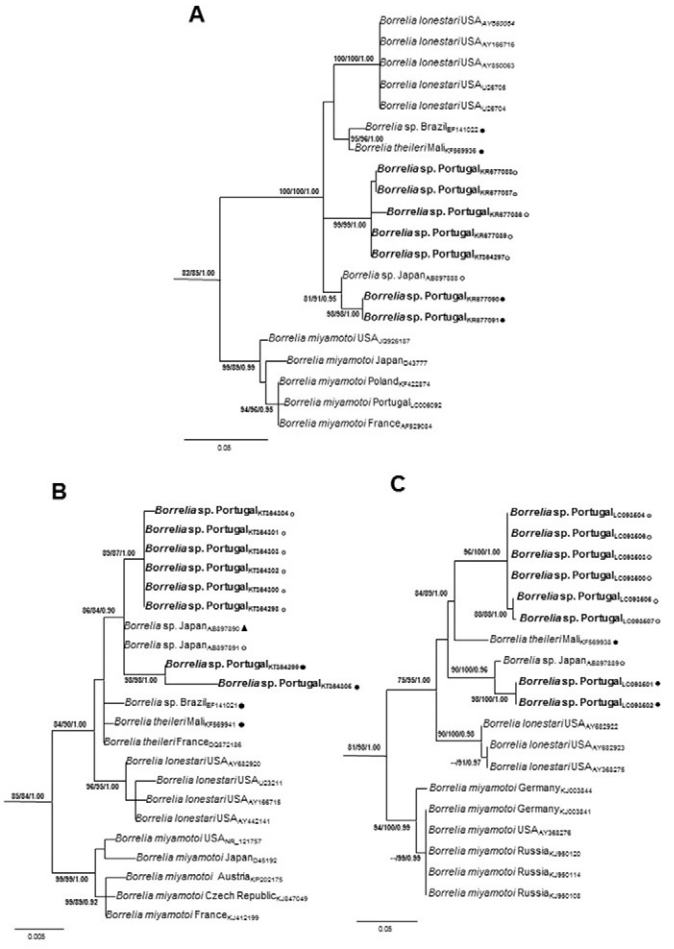

Fig. 3. Phylogenetic analysis of Relapsing Fever *Borrelia flaB* (A), 16S rRNA (B), and *glpQ* (C) sequences. At specific branch nodes bootstrap values (b) ≥75% or posterior probabilities (pp) ≥0.92 are indicated (bNJ/bML/ppBayes). Bootstrap/posterior probability values below these limits are indicated by "–". The size bar indicates the number of nucleotide substitutions per site. Open and closed circles represent, respectively, sequences amplified from *Haemaphysalis* spp. and *Rhipicephalus* spp. ticks. Triangles indicate sequences amplified from sika deer. The full-size phylogenetic trees can be found as Supplementary Fig. 1.

topologies were repeatedly obtained. As shown in Fig. 3 (and Supplementary Figs. 1, 2 and 3), the analyses of 16S rRNA, *flaB* or *glpQ* sequences showed that the majority (n = 6) of the Portuguese RFB strains (1 failed to produce a *flaB* amplicon compatible with DNA sequencing, as shown in lane 7 Fig. 2) clustered together in the RFB group, segregating away from any other reference sequences used. On the other hand, two others sequences (KT364299 and KT364305), amplified from DNA extracts of *R. sanguineus*, formed a separate cluster in all the trees. When shorter *fla*B reference sequences were analyzed (data not shown), the number of *Borrelia* sp. clustering with the two Portuguese strains increased. Curiously, these sequences of Asian (Japan) origin were either associated with *Haemaphysalis* spp. ticks or had been amplified directly from blood samples from sika deer

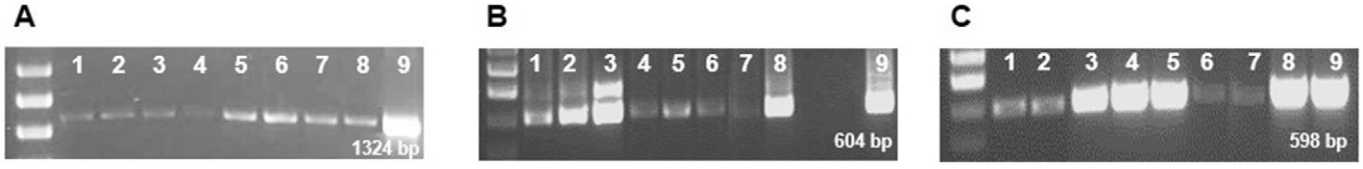

Fig. 2. Detection of RFB (Relapsing Fever *Borreliae*) DNA in extracts prepared from field-collected ticks. (A) Amplification of 16S rRNA (1324 bp amplicon), (B) *flaB* (604 bp amplicon), and (C) *glpQ* (598 bp amplicon). Lanes: 1–6: DNA amplified from *Haemaphysalis* punctata ticks; 7–8: DNA amplified from *Rhipicephalus sanguineus* ticks; 9: PCR positive control (*B. duttonii*).

(*Cervus nippon yesoensis*). In all the obtained phylogenetic trees, the unique character of the majority of the new RFB from Portugal, detect in *H. punctata* ticks, is suggested by their inclusion in a single monophyletic cluster. The position of this cluster within a larger one formed by other RFB (*Borrelia* sp., *B. lonestari* and *B. theileri*) changes slightly depending on the genetic marker used. For example, while the analysis of *glpQ* suggests they share a common ancestry with *B. theileri* (from Mali), this observation was not supported by any of the other trees. In all cases, these two clusters of Portuguese *Borrelia* sp. were reinforced by high bootstrap or posterior probability values. Despite the shared phylogenetic ancestry with both *B. theileri* and *B. lonestari*, the two clusters were clearly shown to be distinct from one-another, possibly representing two new species of RFB.

Regardless of the trees obtained (unrooted or rooted trees), and the sequence(s) eventually used as outgroup (including using *Leptospira interrogans* as outgroup in the analyses of *fla*B and 16S sequences, Supplementary Fig. 3) the topology of the trees remain essentially the same. This is especially true in what concerns the clustering of the sequences that define the RFB group, since they always form a statistically stable monophyletic cluster, where the different sequences segregate congruently in all the analyzed trees.

Additional support for the position of the strains found in our study was drawn from the calculation of genetic distances taking into consideration the two most polymorphic markers analyzed (*flaB* and *glpQ*). For both genes, the corresponding inter-group genetic distance matrixes are shown in Fig. 4. To assist the analysis, different genetic clusters defined in the *flaB* and *glpQ* trees were numbered (G1–G14), as graphically indicated in Supplementary Fig. 1.

The analysis of *flaB* distances not only clearly separated, as expected, the RFB from both the *B. turcica* (G12) and *B. burgdorferi* s.l. (G13), but also showed that some of the smallest genetic distance values were obtained upon comparisons of *B. theileri* vs. *B. lonestari* (G1 vs. G2 = 0.050). Since these are well established *Borrelia* species, it is worth mentioning that their *flaB* sequences were separated by a genetic distance value very similar to that calculated when the two clusters of Portuguese *Borrelia flaB* sequences were compared (G3 vs. G4 = 0.043). Similar observations when made upon analysis of *glpQ*. For this gene, the genetic distances between *B. hermsii* and *B. parkeri* (G9 vs G10 = 0.027) or *B. parkeri* and *B. persica* (G9 vs G11 = 0.044) were found to be smaller than those calculated when either the two groups of Portuguese *Borrelia* sequences were compared (G1 vs. G3 = 0.065), or when the former were compared with *B. theileri* (G1 vs. G2 = 0.090; G3 vs. G2 = 0.084). Therefore, both the topology of the phylogenetic trees and the analysis of genetic distances were compatible with the suggestion that the *Borrelia* detected in the course of our work may correspond to two putative RFB species.

4. Discussion

The number of publications in the literature describing *B. burgdorferi* s.l. bacteria, their distribution and association with human disease, is much larger compared to RFB. In Portugal, for example, several species of *B. burgdorferi* s.l., including *B. garinii*, *B. afzelii*, *B. burgdorferi* s.s., *B. valaisiana* and *B. lusitaniae*, have been detected over the years in *I. ricinus* as well as in other tick species (De Michelis et al., 2000; Baptista et al., 2004; Dietrich et al., 2010; Milhano et al., 2010; Norte et al., 2012; Maia et al., 2014). Furthermore, human cases with clinical symptoms compatible with LD were first identified in 1989 (Morais et al., 1989), and since then its number has increased (laboratory data not published). Nevertheless, it can be assumed that LD is underdiagnosed and underreported in Portugal. In sharp contrast, the slow pace at which the body of knowledge on RFB, transmitted by hard ticks, has built up is due, in part, to difficulties associated with their isolation under laboratory conditions using conventional cultivation techniques. As an example, regardless of being relatively well known, *B. theileri* has never been successfully cultivated in vitro despite multiple attempts, even when many spirochetes were found in tick tissues, as demonstrated by microscopic examination of Giemsa-stained tick organs (Smith et al., 1978). In any case, while the isolation of these bacteria may not be achievable on a routine basis, they are

***flaB* genetic distances analysis**

		G1	G2	G3	G4	G5	G6	G7	G8	G9	G10	G11	G12	G13
G1	*Borrelia lonestari*													
G2	*Borrelia* sp. Br + *Borrelia theileri*	0.050												
G3	*Borrelia* sp. Portugal ○	0.057	0.048											
G4	*Borrelia* sp. Japan + *Borrelia* sp.Portugal ●	0.050	0.041	0.043										
G5	*Borrelia miyamotoi*	0.110	0.099	0.111	0.104									
G6	*Borrelia hermsii*	0.147	0.129	0.140	0.129	0.116								
G7	*Borrelia anserina*	0.156	0.135	0.144	0.145	0.104	0.128							
G8	*Borrelia coriaceae*	0.151	0.127	0.151	0.146	0.094	0.111	0.084						
G9	*Borrelia persica*	0.146	0.129	0.145	0.143	0.112	0.117	0.091	0.091					
G10	*Borrelia hisp+mic+lat+cro+rec+dut*	0.139	0.118	0.125	0.117	0.090	0.095	0.063	0.077	0.095				
G11	*Borrelia turicatae + parkeri*	0.181	0.178	0.182	0.191	0.145	0.119	0.166	0.141	0.143	0.144			
G12	*Borrelia turcica*	0.247	0.210	0.234	0.212	0.217	0.212	0.193	0.193	0.207	0.178	0.230		
G13	*Borrelia burgdorferi* s.l.	0.226	0.202	0.208	0.205	0.160	0.169	0.160	0.145	0.181	0.155	0.194	0.195	

***glpQ* genetic distances analysis**

		G1	G2	G3	G4	G5	G6	G7	G8	G9	G10	G11	G12	G13	G14
G1	*Borrelia* sp. Portugal ○														
G2	*Borrelia theileri*	0.090													
G3	*Borrelia* sp. Portugal ● + *Borrelia* sp. Japan	0.065	0.084												
G4	*Borrelia lonestari*	0.093	0.095	0.072											
G5	*Borrelia miyamotoi*	0.118	0.110	0.111	0.088										
G6	*Borrelia turcica*	0.199	0.180	0.176	0.176	0.139									
G7	*Borrelia* sp.	0.180	0.169	0.178	0.174	0.146	0.093								
G8	*Borrelia turicatae*	0.178	0.175	0.184	0.170	0.135	0.097	0.017							
G9	*Borrelia parkeri*	0.223	0.199	0.203	0.186	0.171	0.156	0.176	0.172						
G10	*Borrelia hermsii*	0.222	0.201	0.200	0.184	0.178	0.164	0.180	0.175	0.027					
G11	*Borrelia persica*	0.198	0.191	0.188	0.170	0.157	0.148	0.165	0.157	0.044	0.046				
G12	*Borrelia hispanica*	0.213	0.213	0.205	0.200	0.181	0.150	0.159	0.153	0.113	0.116	0.099			
G13	*Borrelia crocidurae*	0.182	0.205	0.181	0.224	0.220	0.221	0.182	0.201	0.205	0.209	0.215	0.221		
G14	*Borrelia dut+rec+mic*	0.188	0.213	0.187	0.223	0.208	0.191	0.166	0.177	0.213	0.220	0.215	0.222	0.056	

Borrelia hisp+mic+lat+cro+rec+dut – *Borrelia hispanica. microti. latyschewii. crocidurae. recurrentis. duttonii; Borrelia dut+rec+mic* – *Borrelia duttonii. recurrentis. microti;*
Open and closed circles represent, respectively, *Haemaphysalis punctata* and *Rhipicephalus sanguineus* ticks.

Fig. 4. *glpQ* and *flaB* genetic distance analysis calculated using the Tamura–Nei as implemented in the Mega 6.0 software.

still important agents of animal disease, and therefore should be characterized more thoroughly.

The results presented in the current study confirm previous reports indicating a countrywide distribution of *B. burgdorferi* s.l. bacteria in questing ticks (De Michelis et al., 2000; Baptista et al., 2004). In one of these studies *B. lusitaniae* was the most prevalent species in the questing ticks (Baptista et al., 2004). Nevertheless, other *B. burgdorferi* s.l. species were also found throughout Portugal, even in ticks other than their "classical" known *I. ricinus* vector (De Michelis et al., 2000; Baptista et al., 2004; Núncio and Alves, 2014). The presence of spirochetes in these ticks does not necessarily mean that they are capable of transmitting them to new hosts. Tick species without the ability of transmission of the pathogen should not be recognized as vector competent. They can be called non-vector species. Examples of these non-vector are *Dermacentor reticulatus*, *D. andersoni*, *D. variabilis*, *D. occidentalis*, *Haemaphysalis concinna* and *H. punctata.* None of these ticks are recognized as vectors of *Borrelia* for their inability of transstadial transmission in natural conditions (Gern et al., 1991; Kahl et al., 1992; Barbour and Fish, 1993; Angelov et al., 1996; Dolan et al., 2000; Sun and Xu, 2003).

Somewhat unexpectedly, this study, backed by phylogenetic analysis of the DNA sequences of three independently amplified genetic markers (16S rRNA, *flaB* and *glpQ*), also suggested the existence of two possible new RF spirochetes, presently referred to as *Borrelia sp*., detected in *H. punctata* and *R. sanguineus* questing ticks.

H. punctata ticks comprised five pools of larvae collected in July 2012, as well as one nymph captured later in the same year (December) at Tapada Nacional de Mafra, near Lisboa. This is a protected area managed mainly for the sustainable use of natural ecosystems, composed by dense forests of deciduous oaks, pines, eucalyptus, chestnuts and *Platanus* trees, and inhabited by numerous mammal species. These include fallow deer (*Cervus dama*), red deer (*Cervus elaphus*), wild boar (*Sus scrofa*), foxes (*Vulpes vulpes*), wild-rabbits (*Oryctolagus cuniculus*), European hedgehogs (*Erinaceus europaeus*), as well as many bats and small rodents. This wide range of hosts allows the maintenance of permanent populations of numerous species of ticks, thus contributing to the persistence of several species of *Borrelia* in this protected biotope.

The data presented here report the detection of RFB-like *Borrelia* in ticks at two different development stages, most of which being larvae. Although this is compatible with the idea that these bacteria might be transmitted transovarially, how the larvae became infected in the first place remains undetermined. Spirochetes may have either originated from an interrupted blood meal (with the ticks failing further development) or may have already been present in the bodies of immature specimens at the moment of egg hatching due to transovarial transmission (TOT). The latter hypothesis has been explored for many tick-borne pathogens for maintenance in natural environment and can occur in both ixodid and argasid ticks (Rollend et al., 2013). The presence of RFB such as *B. miyamotoi* in larvae has not only been shown to result from direct transovarial acquisition from infected female ticks, but these larvae have also been proven competent for transmission of these spirochetes to vertebrates (Lee et al., 2014).

Two adult females of *R. sanguineus* have also been shown to harbor RFB-DNA. One of these ticks was captured in July of 2012 in the north of Portugal Braga district) in a wetland region surrounded by mountains with forests inhabited by several large mammal species like red deer, fallow deer, and wild boar. The other tick was captured in April of 2014 in the south of Lisboa (near Évora), in a biotope characterized by drier environmental conditions, with arid lands, high temperatures in the summer, and where roaming bovines are frequently observed. The fact that two ticks harboring RFB-DNA were caught at collection sites located hundreds of kilometers apart, suggest a possible wider distribution of these bacteria.

Interestingly, phylogenetic analyses of 16S rRNA, *flaB* and *glpQ* sequences have revealed generally congruent tree topologies in which the novel RFB sequences detected in this study form two independent clusters, consistently supported by bootstrap and posterior probability values. These clusters placed the analyzed sequences in a larger subgroup of RFB that included *B. theileri*, *B. lonestari*, and a number of unclassified spirochetes, referred to as *Borrelia* sp. Furthermore, they were clearly independent from one another, possibly revealing two different species of RFB. DNA of each of the two new RFB species was detected in a single tick species, either *H. punctata* or *R. sanguineus*. The *Borrelia* sequences amplified from *H. puntacta* was only detected in specimens collected at Tapada Nacional de Mafra. However, whether this apparent restricted distribution results from sampling bias, or suggests maintenance of these bacteria solely within the confinements of the Tapada Nacional de Mafra habitat, remains to be determined. Potentially, whether transmission restricted to certain tick species is also an open question for concerning *Borrelia sp.* detected in *R. sanguineus*. Due to the small number of positive detections of this type of RFB, it is not clear if these bacteria are restricted, or not, to the species of ticks where they have been found. However, phylogenetic analysis of *flaB* and *glpQ* sequences suggest a common ancestry between these bacteria and *Borrelia* sp. detected in *Haemaphysalis* spp. from Japan (Lee et al., 2014), which seems to contradict the idea of tick-restriction, but clearly deserves to be explored in the future. The observed genetic heterogeneity within the G1–G4 cluster (Supplementary Fig. 1), revealed by both phylogenetic and genetic distance analyses (Fig. 4), suggests that it may be formed by as many as four different *Borrelia* species (based on currently available data). Clearly, a more thorough genetic characterization of this cluster is worth being done in the near future, possibly involving new approaches such as multilocus sequence typing. These will be of importance to aid in the definition of clear-cut genetic limits for assignment of monophyletic groups of *Borrelia* sequences as individual species. Finally, despite the possibilities opened by the use of molecular approaches based on PCR, followed by phylogenetic analyses of the obtained DNA sequences, the characterization of these new RFB clearly calls for the isolation and in vitro cultivation of these bacteria. No attempts were made to isolate *Borrelia* from the ticks collected during this work as they were conserved in ethanol until further identification, and bacterial isolation was not one of the aims of this study when it was devised. Nevertheless, and despite the anticipated difficulties, the isolation of these bacteria in vitro, their characterization (both genetic and phenotypic) as well as their role in human or veterinary disease, will be the focus of future research.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.meegid.2016.03.008.

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