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Phylogenetic Analysis of a Virulent *Borrelia* Species Isolated from Patients with Relapsing Fever[▽]

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Multilocus sequence analysis (MLSA) was used to clarify the taxonomic status of a virulent *Borrelia* organism previously isolated from patients with relapsing fever and from ticks in Spain that is designated the Spanish relapsing fever (SRF) *Borrelia*. This species has been used extensively in experimental infection models because of its continued virulence. Seven genes were amplified to analyze the phylogenetic relationships among several Spanish isolates of SRF *Borrelia* and other relapsing fever *Borrelia* species. The genes targeted in this study included *rrs* and *flaB*, which have commonly been used in phylogenetic studies; the *rrf-rrl* intergenic spacer (IGS), which is highly discriminatory; and four additional genes, *p66*, *groEL*, *glpQ*, and *recC*, which are located on the chromosome and which have therefore evolved in a clonal way. The species included in this study were *Borrelia duttonii*, *B. recurrentis*, *B. crocidurae*, and *B. hispanica* as Old World *Borrelia* species and *B. turicatae* and *B. hermsii* as New World *Borrelia* species. The results obtained by MLSA of the SRF *Borrelia* on the basis of 1% of the genomic sequence data analyzed confirmed that the SRF *Borrelia* isolates are *B. hispanica*. However, the prototype isolates of *B. hispanica* used in this study have an uncertain history and display unique phenotypic characteristics that are not shared with the SRF *Borrelia*. Therefore, we propose to use strain SP1, isolated from a relapsing fever patient in 1994 in southern Spain, as the type strain for *B. hispanica*.

The members of the *Borrelia* genus comprise two major groups: those causing Lyme disease and those causing relapsing fever (RF). Relapsing fever is distributed all over the world; and its agents are traditionally classified according to their geographic origins, vector, and infectivity in various animal species. In Spain, relapsing fever has been reported sporadically throughout the last century and has been associated with *Borrelia hispanica* (2, 11, 17, 19, 27, 28). From 1994 to 1996, spirochetes were isolated from the blood of two patients with RF symptoms and from *Ornithodoros erraticus*, and we have designated these spirochetes Spanish relapsing fever (SRF) *Borrelia*. This SRF organism is refractory to *in vitro* cultivation, and this represents a unique phenotypic characteristic not shared by *B. hispanica* (1). SRF *Borrelia* infects C3H/HeN, BALB/c, C57/B51, and Swiss outbred mice (1), and it is highly infectious. A murine model of SRF was developed (13) and has been used extensively in experimental studies on pathogenesis and the host response (5–7, 14, 16, 20). A preliminary genetic analysis comparing the *rrs* and *flaB* loci from *B. hispanica* to those from the Spanish isolates suggested that SRF could represent a new species (1).

To date, most of the ecological and epidemiological studies on relapsing fever *Borrelia* have used a single locus. Therefore, characterization of relapsing fever spirochetes has relied upon the amplification and sequencing of certain genes, such as *rrs* (24), *flaB* (12), and more recently, the noncoding intergenic spacer (IGS) *rrs-rrl* (4). However, the value of these approaches is limited. The *rrs* gene is highly conserved among relapsing fever *Borrelia* species, and it can discriminate among species but not among strains. The results with *flaB* have shown only minor differences among relapsing fever *Borrelia* species. On the other hand, although the intergenic spacer *rrs-rrl* is not able to discriminate between *B. recurrentis* and *B. duttonii* (29), it has been shown to discriminate between strains of other RF *Borrelia* species.

Multilocus sequence analysis (MLSA) is a powerful tool for the delineation of species and assignment of strains to defined species (23, 25, 26). The utilization of this method is increasing, especially with uncultured and slow-growing organisms that cannot be analyzed by DNA-DNA hybridization. MLSA has been used with the *Borrelia* genus in order to delineate or confirm species, such as *B. spielmanii* (25), *B. californiensis* (23), *B. carolinensis* (26), and *B. bavariensis* (21).

The purpose of the study described here was to clarify the taxonomic status of a group of SRF isolates from patients and ticks isolated in Spain. The selection of genes (*rrs*, *rrf-rrl*, *flaB*, *glpQ*, *groEL*, *recC*, *p66*) used for multilocus sequence analysis of this group of spirochetes was based on their suitability for phylogenetic purposes.

MATERIALS AND METHODS

Culture. The relapsing fever *Borrelia* strains used in this study are listed in Table 1. *B. duttonii*, *B. hermsii*, *B. crocidurae*, and *B. hispanica* were grown in Barbour-Stoenner-Kelly H (BSK-H) medium (Sigma-Aldrich, St. Louis, MO) supplemented with 6% rabbit serum (Pel-Freez, Rogers, AR) at 33°C. Cultures

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TABLE 1. RF *Borrelia* strains used in this study

Species	Strain	Geographic origin	Source
B. duttonii	1120K3	Congo	*Ornithodoros moubata*
B. duttonii	Ly	Tanzania	Human, CSF[a]
B. recurrentis	A1	Ethiopia	Human blood
B. crocidurae	Achema	Mauritania	*Ornithodoros sonrai*
B. crocidurae	CR2A[b]	Western Africa (?[c])	*Ornithodoros erraticus*
B. hermsii	HS1	USA	*Ornithodoros hermsi*
B. hermsii	DAH	USA	Human, CSF
B. turicatae	91E135	USA	*Ornithodoros turicatae*
B. hispanica	CR1	?	?
B. hispanica	ORIX	?	?
B. hispanica	JH	?	?
B. hispanica	Sp1	Spain	Human blood
B. hispanica	Sp2	Spain	Human blood
B. hispanica	Sp3	Spain	*Ornithodoros erraticus*

[a] CSF, cerebrospinal fluid.
[b] Proposed in this study to be reclassified as *B. duttonii* CR2A.
[c] ?, unknown or uncertain.

were harvested in the mid-log phase by centrifugation and washed with sterile phosphate-buffered saline (Gibco, Grand Island, NY).

Animal passages. Six- to 8-week-old C3H/HeN mice (Charles River Laboratories, Wilmington, MA) were inoculated with stocks of three SRF isolates frozen at −80°C and killed during the first peak of spirochetemia. Blood was obtained by cardiac puncture, and spirochetes were harvested from the plasma by centrifugation and washed with fresh BSK-H medium.

DNA extraction, primers, and PCR conditions. Genomic DNA was extracted using a DNeasy blood and tissue extraction kit (Qiagen, Valencia, CA), following the instructions of the manufacturer. Water was included as a negative control in every extraction to test for possible contamination. Primers were designed to amplify any relapsing fever *Borrelia* and are listed in Table 2. The conditions for the amplification of the *rrs*, *flaB*, *rrf-rrl*, *p66*, *groEL*, *glpQ*, and *recC* genes were as follows: an initial denaturalization step at 98°C for 30 s, followed by 35 cycles at 98°C for 10 s, 68°C for 15 s, and extension at 72°C for 15 s and with a final extension step at 72°C for 10 min. In the case of *rrs* fragment 2, *rrs* fragment 4, and *flaB* fragment 1, the annealing temperature was 57°C. The prevention of cross-contamination and false-positive results was achieved by the use of plugged tips, the performance of PCRs in a room separate from the room used for DNA extraction, and the use of specific separated areas dedicated for the production of reagents and analyses of the amplicons. A negative PCR control (water) was included in each run as well. The PCR products (20 μl) were separated in a 1% agarose gel and purified (30 μl) with a QIAquick PCR purification kit (Qiagen), following the manufacturer's recommendations. All the samples were submitted for direct sequencing to the DNA Sequencing Facility at Stony Brook University. Sequences were determined in both directions, using the same specific primers that were used in each PCR. All sequences were analyzed with the EditSeq module of DNAStar software (DNAStar, United Kingdom).

Phylogenetic analysis. Available sequences from different loci (*rrs*, *rrf-rrl*, *flaB*, *p66*, *recC*, *groEL*, *glpQ*) of *B. duttonii* strain Ly, *B. hermsii* strain DAH, *B. turicatae* strain 91E135, and *B. recurrentis* strain A1 were used in the phylogenetic analysis, together with the sequences obtained in this study. Subsequent analysis of the genes was performed using the software package MEGA (version 3.1) (15), after multiple-sequence alignment by the CLUSTAL_X program (30). Distance options were according to the maximum composite likelihood model, and clustering with the neighbor-joining method was performed by using bootstrap values based on 2,000 replications.

Nucleotide sequence accession numbers. The sequences generated in this study were deposited in GenBank under accession numbers GU350705 to GU350714 for the *rrs* gene sequences, GU350715 to GU350722 for the *rrf-rrl* gene sequences, GU357611 to GU357620 for the *flaB* gene sequences, GU357591 to GU357600 for the *p66* gene sequences, GU357601 to GU357610 for the *recC* gene sequences, GU357581 to GU357590 for the *groEL* gene sequences, and GU357571 to GU357580 for the *glpQ* gene sequences.

RESULTS

Analysis of partial 16S rRNA gene. Four sets of primers were used to obtain a partial sequence of the *rrs* gene. All the species showed the same amplicon length, without either deletions or insertions. Comparison of the 1,513-bp portion of the *rrs* gene sequences revealed that all the strains from the same species analyzed produced a single cluster, with the exception of *B. hispanica* and SRF *Borrelia*, which were different by a single nucleotide and which clustered separately from each other (Fig. 1A). Although the differences among species are minor and therefore the value of the discrimination of species for genotypic purposes is limited, it is still useful to discriminate between species, especially between *B. duttonii* and *B. recurrentis*.

TABLE 2. Primers used in this study

Gene	Forward primer (5′–3′)	Reverse primer (5′–3′)	Amplicon size (bp)	Reference or source
flaB fragment 1	TCATAAATCATAATACGTCAG	AATGTCCATGAAGCTTGTGA	514	This study
flaB fragment 2	CTGAAGAGCTTGGAATGCAAC	AGGTACTTGATTTGCTTGTGC	538	This study
rrs fragment 1	GTTTGATCCTGGCTTAGAAC	TTACAATCTTTCGACCTTCTT	417	This study
rrs fragment 2	CACACTGGAACTGAGATACGG	TTCGCCTCTGGTATTCTTCCT	419	This study
rrs fragment 3	TGCGTAAAATACCACAGCTCA	TGAGTCCCCATCTTTACATGC	547	This study
rrs fragment 4	TACCAGGGCTTGACATATACA	GAGGTGATCCAGCCACACTTT	561	This study
rrf-rrl outer	GTATGTTTAGTGAGGGGGGTG	GGATCATAGCTCAGGTGGTTAG	807[a]	3
rrf-rrl inner	AGGGGGGTGAAGTCGTAACAAG	GTCTGATAAACCTGAGGTCGGA	765[a]	3
recC fragment 1	AAGATATATAAAACAAACAAA	GTCAATTTCTCTAGTCTCTCC	623	This study
recC fragment 2	ATTGAAACAAAGAGAATAATA	ATTTATTCATTACTTTTGTGT	634	This study
recC fragment 3	GGAACGATTAGTAATTTTAA	TTTAAGGTATTGTATTTTTGT	474	This study
recC fragment 4	ATATGAATGGGCAGAAATAAT	TTATTGATATCTGGATTTAGA	495	This study
recC fragment 5	GCAAGCTCTGATAAAATTGAA	TTGATGTTTATGGGAATTATT	737	This study
groEL fragment 1	TGGCTAAGGACATATATTTTA	ATCTTTGCCAACTCTGTCCAT	512	This study
groEL fragment 2	TTCTGCAAATAATGATACTTC	AACATTCTCAAGAGTAAGTCC	487	This study
groEL fragment 3	ATTGCTATACTTACTGGAGG	TAAATAGAACTCTCAAATCCA	524	This study
groEL fragment 4	AAGGTTTTGAGATTGTGAAGA	TTACATCATTCCCATTCCAG	323	This study
p66 fragment 1	TTTAGATTTGATATGGATGA	GATATGTGTCCAAGTATAGA	899	This study
p66 fragment 2	TTCCTCAATAACATATGGTCT	ACACTTCCATTTTGATCTTT	945	This study
glpQ fragment 1	CATTAATTATAGCTCACAGAG	AACAAGCATTATCAATTTTCC	599	This study
glpQ fragment 2	TATGGCATAAACAACAAGGTA	AATCTGTAAATAGACCATCTA	453	This study

[a] The amplicon size may vary among species.

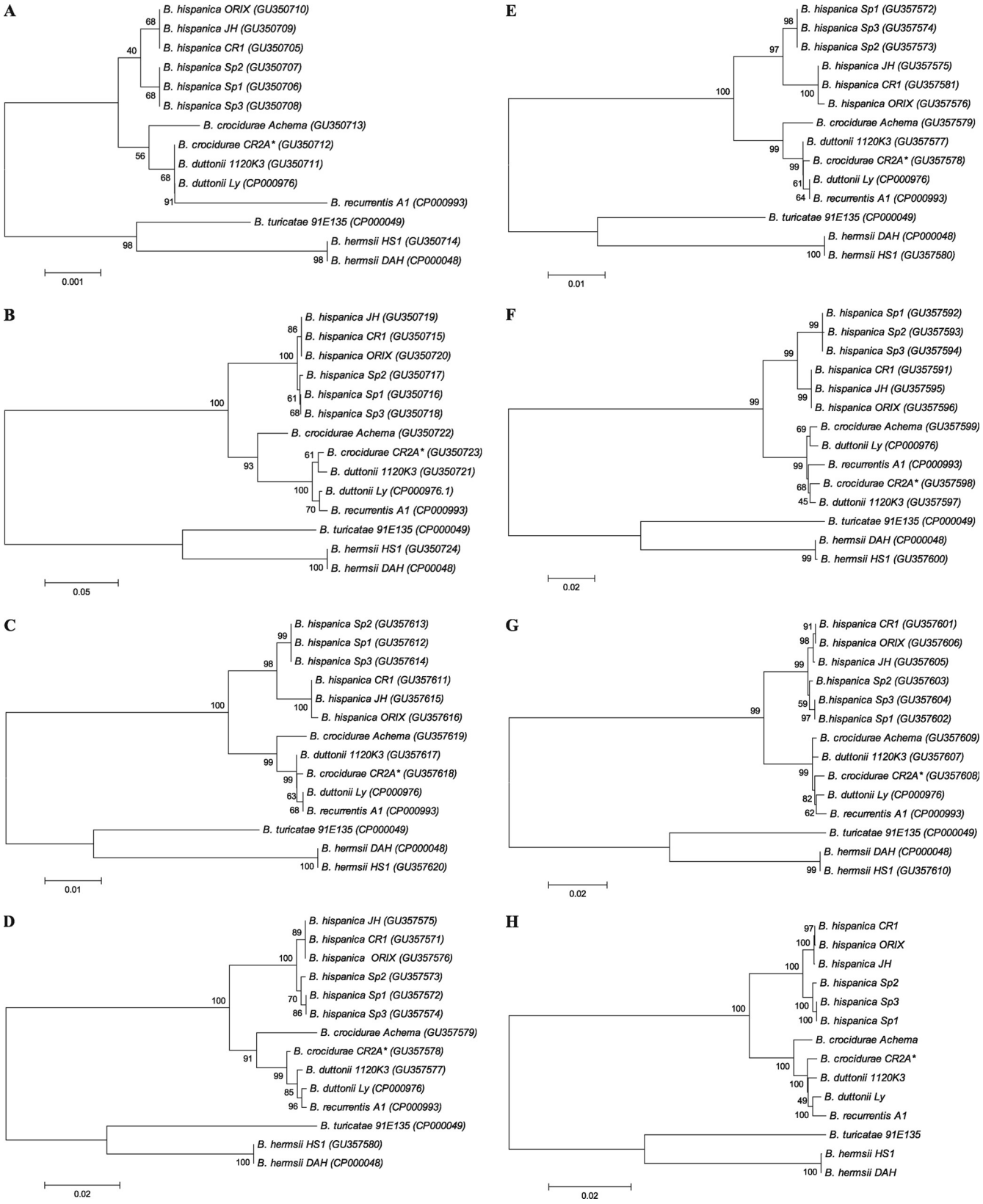

FIG. 1. Phylogenetic analyses of 16S rRNA (A), IGS (B), *flaB* (C), *glpQ* (D), *groEL* (E), *p66* (F), and *recC* (G). (H) Concatenated sequences. The GenBank accession numbers are indicated in parentheses in each tree. *, proposed in this study to be reclassified as *B. duttonii* CR2A. Scale bars, divergence, where 0.01 = 1% divergence.

Analysis of *rrf-rrl* IGS. The sizes of the *rrf-rrl* spacer regions of the *Borrelia* species tested were determined by direct sequencing of the purified amplicons amplified by the primers shown in Table 2. The New World relapsing fever *Borrelia* species have the largest intergenic sequences (over 700 bp), whereas the Old World *Borrelia* species are shorter (459 to 521 bp). The length varies among species, with the exception of *B. duttonii* Ly and *B. recurrentis* A1, which display the same IGS length. The *B. hispanica* amplicon was 459 bp for all the strains tested and also for the SRF *Borrelia*. This is the shortest IGS among the Old World relapsing fever *Borrelia* species analyzed. The IGS sequence of SRF *Borrelia* strains Sp1 and Sp3 exhibited 100% sequence identity and 99.8% identity with strain Sp2. All the *B. hispanica* strains exhibited 100% sequence identity at this locus, 99.6% identity with SRF *Borrelia* strains Sp1 and Sp3, and 99.3% identity with SRF *Borrelia* strain Sp2. Interestingly, among the other *Borrelia* species analyzed, *B. duttonii* Ly is closer to *B. recurrentis* A1 than to *B. duttonii* 1120K3, and this highlights further the limitations of this locus to discriminate between these two species (Fig. 1B).

Analysis of partial *flaB* gene. The size of the *flaB* amplicon was conserved among the Old World relapsing fever *Borrelia* species (937 bp), with the exception of *B. hispanica*, which showed a 3-bp deletion. All New World relapsing fever *Borrelia* species analyzed showed a 3-bp deletion, between positions 590 and 593 of the amplified fragment, compared with the sequences of the Old World RF species. The amplicons from SRF isolates showed 100% identity among themselves and 99% identity with *B. hispanica* strains. Interestingly, although this is a conserved locus, it showed more genetic differences between *B. hispanica* and SRF *Borrelia* than IGS. As happened with IGS, the partial amplification of the *flaB* gene failed to discriminate between *B. duttonii* strain Ly and *B. recurrentis* strain A1 (Fig. 1C).

Analysis of partial *glpQ* gene. An 829-bp fragment was amplified and sequenced. No insertions or deletions were found in any of the sequences analyzed. All *B. hispanica* strains analyzed showed 100% identity among themselves and 99.5% identity with the SRF *Borrelia*. Again, the differences between *B. duttonii* and *B. recurrentis* were minor, and it is reflected by the fact that *B. duttonii* Ly is closer to *B. recurrentis* A1 than to B. *duttonii* 1120K3 (Fig. 1D).

Analysis of partial *groEL* gene. Four set of primers were used to amplify and sequence a 1,596-bp fragment of the *groEL* gene. *B. hermsii* had a 3-bp insertion, for a total length of 1,599 bp. The *B. hispanica* strains showed 100% identity among themselves, whereas the identity with the different SRF isolates ranged from 99.81 to 99.74%. This locus cannot be used to discriminate between *B. duttonii* strains and *B. recurrentis* (Fig. 1E).

Analysis of partial *p66* gene. A fragment that ranged from 1,588 bp to 1,594 bp, depending on the species analyzed, was amplified using two set of primers. All the *B. hispanica* strains showed 100% identity among themselves and were clearly separate from the cluster formed by SRF isolates (Fig. 1F). Actually, the identity between these two was just 98.2%, which is a remarkable difference when it is taken into account that other African relapsing fever *Borrelia* species, such as *B. duttonii*, *B. recurrentis*, and *B. crocidurae*, showed *p66* gene sequence identity of greater than 98.8% among themselves. Also, all the Old World relapsing fever *Borrelia*, with the exception of *B. hispanica*, showed a 6-bp deletion (AACCAA) between positions 1333 and 1336 of the amplified fragment.

Analysis of partial *recC* gene. A 2,468-bp partial sequence of the *recC* gene was sequenced. All the *B. hispanica* strains clustered together, and strains CR1 and ORIX had identical sequences (Fig. 1G). The identity between them and the SRF strains ranged from 99.42% to 99.59%. The *recC* gene did not discriminate well among the *B. duttonii* and *B. recurrentis* species; also, the genetic differences were minor, with the identity between them being greater than 99.4%.

Analysis of concatenated sequences. The distance matrix for phylogenetic analysis was generated by alignment of the partial *rrs* gene, the partial *p66* gene, the partial *flaB* gene, the partial *groEL* gene, the partial *recC* gene, the IGS, and the partial *glpQ* gene. The concatenated sequences had ≈9,300 nucleotides, which represents about 1% of the chromosome. The phylogenetic tree of the concatenated sequences is shown in Fig. 1H. Analysis of the concatenated sequences of the SRF *Borrelia* with the six control species of relapsing fever *Borrelia* clearly exhibited the separation between the Old World and the New World relapsing fever *Borrelia*. Among the Old World relapsing fever species, it is clear that *B. hispanica* is well separated from the cluster formed by *B. crocidura*e, *B. duttonii*, and *B. recurrentis*. The nucleotide sequence difference for *B. crocidurae* versus *B. duttonii* and *B. recurrentis* is approximately 1%. Interestingly, this approach cannot distinguish between *B. duttonii* and *B. recurrentis*. The different isolates from the SRF *Borrelia* cluster together and form a cluster different from *B. hispanica*. Two of these isolates, Sp1 and Sp3, have the same concatenated sequence. The differences among the *B. hispanica* strains were minor. The similarities of the concatenated sequences of relapsing fever *Borrelia* species are shown in Table 3.

DISCUSSION

The species designation of relapsing fever *Borrelia* has historically been based upon the species names of their tick vectors (10), their geographical distribution, and their host range. In the last decade of the 20th century, the development of molecular tools enabled the common use of PCRs for phylogenetic purposes. Lately, MLSA approaches have been used for the species designation of isolates in the *Borrelia* genus, particularly with Lyme disease *Borrelia* (25, 26). In the present study, we have used an MLSA approach based on the sequences of seven different genes, *rrs*, *flaB*, *groEL*, *recC*, *rrs-rrl*, *glpQ*, and *p66*, to reveal the phylogenetic relationship between *B. hispanica* and SRF *Borrelia*. In these analyses, *B. hermsii* and *B. turicatae* were included as representative species of the American relapsing fever group and also served as outgroups for the Old World relapsing fever *Borrelia*.

Multilocus sequence analysis of the relapsing fever *Borrelia* showed that both groups, the Old World and the New World *Borrelia*, are well differentiated. The New World species included in the MLSA were clearly separated in different clusters for all the genes analyzed, and, as expected, for the concatenated sequences, too.

The MLSA analysis revealed that the SRF *Borrelia* cluster showed only minor genetic differences with the cluster of *B.*

TABLE 3. Similarities of concatenated sequences of relapsing fever spirochetes[a]

Strain	% similarity[a]								
	B. recurrentis A1	*B. duttonii*		*B. crocidurae*		*B. hispanica*		*B. turicatae* 91E135	*B. hermsii* HS1
		Ly	1120K3	CR2A[b]	Achema	CR1	Sp1		
A1	100								
Ly	99.49	100							
1120K3	99.39	99.46	100						
CR2A	99.36	99.43	99.54	100					
Achema	98.76	98.92	99.01	98.97	100				
CR1	96.5	96.64	96.76	96.73	96.87	100			
Sp1	96.55	96.63	96.75	96.72	96.88	99.39	100		
91E135	84.69	84.78	84.87	84.88	84.92	84.90	84.77	100	
HS1	84.74	84.90	84.95	84.98	84.95	84.97	84.94	91.28	100

[a] Similarities were calculated from the distance matrix (P-distance values) in a pairwise deletion procedure.
[b] Proposed in this study to be reclassified as *B. duttonii* CR2A.

hispanica. The identity between the two clusters determined using the seven concatenated genes is 99.4%. The SRF *Borrelia* consistently clustered together for all the loci analyzed, which it is not surprising, since these SRF isolates shared the same geographical origin. On the other hand, all three *B. hispanica* strains tested were very close to each other and showed a lack of variation. The nearly exact genetic identity among the strains of *B. hispanica* suggests that these strains have a common origin. Although the three isolates of *B. hispanica* (strains JH, CR1, and ORIX) have been stored for a long time in at least two laboratories in Europe, their history is not known; therefore, given their striking identity, it could be possible that these three strains of *B. hispanica* are actually the same isolate. Furthermore, the identity among the *B. hispanica* strains was higher than the identity among the SRF isolates, which were isolated at the same location during a short period of time.

The aim of this study was to clarify the taxonomic status of the SRF isolates that were thought to represent a new species (1). Interestingly, although the two studies reached a different conclusion, the results of both studies using the *rrs* and *flaB* genes were similar (1). These two genes are known to discriminate between species but not between strains because these two genes are well conserved among relapsing fever spirochetes. In both studies, *flaB* and *rrs* display differences, and in the case of *flaB*, this suggests that the SRF *Borrelia* could be a new species. The *p66* gene, a membrane protein that has a surface location and that would therefore be subjected to environmental pressures, also displayed noticeable sequence differences between the *B. hispanica* and the SRF *Borrelia* clusters. On the contrary, the noncoding intergenic spacer between the 16S and 23S genes that has been shown to be particularly valuable for characterizing relapsing fever spirochetes and the *glpQ* gene does not discriminate between strains, suggesting that these are the same species. The concatenated sequence, which takes into account all the loci analyzed, showed that while *B. hispanica* and the SRF *Borrelia* display some genetic differences, with an overall identity of 99.4%, the differences are not sufficient to justify a new species designation for the SRF *Borrelia*.

Apart from the minor genetic differences, the SRF *Borrelia* isolates have unique phenotypic characteristics that are not shared by *B. hispanica* strains. The SRF *Borrelia* has so far remained refractory to *in vitro* cultivation, and it is highly infectious for C3H/HeN, BALB/c, C57BL/6, and outbred mice, whereas the available strains of *B. hispanica* (strains ORIX, CR1, and JH) are cultivable and grow only in SCID mice. Nonetheless, it is possible that these phenotypic differences between *B. hispanica* and the SRF *Borrelia* are the result of an adaptation process in the laboratory through years of passage in guinea pigs and culture media. In summary, the SRF isolates have a better-documented history than the *B. hispanica* isolates. Therefore, we propose that an SRF isolate be used as the type strain for *B. hispanica*.

In addition to establishing the taxonomic status of the SRF *Borrelia*, this study has reached other conclusions as well. The Old World *Borrelia* species analyzed in this study were closely related. There are two main clusters; one cluster included *B. duttonii*, *B. recurrentis*, and *B. crocidurae* and the other cluster included *B. hispanica* and the SRF *Borrelia*. This is in agreement with the results of previous phylogenetic studies (8, 29). The MLSA scheme could not discriminate between *B. recurrentis* and *B. duttonii* with an identity greater than 99.39%. The close identity between the two species suggests that both species are indeed the same. This is not surprising, given that previous phylogenetic and genomic sequencing data suggest that either *B. recurrentis* has evolved from *B. duttonii* or they have a common ancestral origin (9, 18). Moreover, the *B. recurrentis* genome is a degraded subset of the genome of *B. duttonii* (18).

Our results also support the hypothesis that *B. duttonii*/*B. recurrentis* and *B. crocidurae* are clonal variants. However, all the genes analyzed, especially IGS and *glpQ*, showed greater differences between the *B. duttonii*/*B. recurrentis* complex and *B. crocidurae*. These results are in agreement with those described in previous reports (24, 29). Also, the fact that *B. duttonii* Ly and *B. recurrentis* A1 cluster more closely suggests that the Ly ancestor is closer to the *B. recurrentis* ancestor than to the *B. duttonii* 1120K3 ancestor. In our study, we included a second strain of *B. crocidurae* named CR2A, and the *rrs* gene of this organism was identical to the *rrs* sequences of other *B. duttonii* sequences. Actually, for all the loci analyzed, this strain clusters together with the *B. duttonii*/*B. recurrentis* complex, including when the IGS region, which has been reported to be valuable for the typing of relapsing fever spirochetes (4), is used for analysis. Consequently, it has been demonstrated in this study that *B. crocidurae* CR2A is actually a *B. duttonii*

strain and that it should be relocated to the correct taxon. Interestingly, *B. crocidurae* CR2A was isolated from an *O. erraticus* and was therefore named *B. crocidurae*, according to traditional phylogenetic criteria. Nowadays, this traditional phylogeny has largely been rewritten, with recent peculiarities including *B. duttonii* in Togo, East Africa (22), a *B. crocidurae*-like sequence in a patient and in an *Ornithodoros moubata* organism in the same geographical area (29), and the result obtained with *B. crocidurae* CR2A in this work, suggesting that these spirochetes may coexist in the same area and may have extended vector compatibility. We propose that it be renamed *B. duttonii* CR2A.

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