

**Delineation of *Borrelia burgdorferi* sensu lato species
by multilocus sequence analysis
and confirmation of the delineation of *Borrelia spielmanii***

Dania Richter¹, Danièle Postic², Natacha Sertour², Ian Livey³,

Franz-Rainer Matuschka¹, and Guy Baranton²

¹*Charité Universitätsmedizin Berlin, 12249 Berlin, Germany,*

²*Institut Pasteur, 75724 Paris, France, ³Baxter Vaccine AG, A-2304 Orth/Donau, Austria*

Corresponding author: Dania Richter, Abteilung Parasitologie, Institut für Pathologie, Charité
Universitätsmedizin Berlin, Malteserstraße 74-100, 12249 Berlin, Germany.
Phone: xx 49 30 838 70 372, fax: xx 49 30 776 2085, email: drichter@charite.de

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Abbreviations: MLEE – multilocus enzyme electrophoresis, MLSA – multilocus sequence analysis, MLST – multilocus sequence typing, WDDH – whole DNA-DNA hybridization

GenBank accession numbers for the sequences obtained in this study are available as supplementary data (Table 4) with the online version of this paper at <http://ijss.sgmjournals.org>

Abstract

To evaluate multilocus sequence analysis (MLSA) for taxonomic purposes in the delineation of species within *Borrelia burgdorferi* sensu lato, we sequenced seven relevant loci of various strains for which extensive DNA-DNA reassociation data were available. MLSA delineation proved to be fully concordant with conventional methods. Our analysis confirms the delineation of *Borrelia spielmanii* with the type strain PC-Eq17^T and, thereby, validates it as a distinct species.

Introduction

Borrelia burgdorferi sensu lato (*B. burgdorferi* s.l.) is a complex of at least 12 species, of which *Borrelia burgdorferi* sensu stricto (s.s.) (Johnson *et al.*, 1984), *Borrelia garinii* (Baranton *et al.*, 1992), and *Borrelia afzelii* (Canica *et al.*, 1993) cause Lyme disease in people. Particular species, such as *Borrelia japonica* (Kawabata *et al.*, 1993), *Borrelia andersonii* (Marconi *et al.*, 1995), *Borrelia bissettii* (Postic *et al.*, 1998), *Borrelia sinica* (Masuzawa *et al.*, 2001), *Borrelia turdi* and *Borrelia tanukii* (Fukunaga *et al.*, 1996), appear not to be pathogenic and have a restricted geographic distribution. *Borrelia valaisiana* (Wang, G. *et al.*, 1997), which is widely distributed in Europe and Asia, and *Borrelia lusitaniae* (Le Flèche *et al.*, 1997), which infects vector ticks in Europe and North Africa (Younsi *et al.*, 2005) are potentially pathogenic. DNA specific for *B. valaisiana* has been detected in human samples (Rijpkema *et al.*, 1997; Diza *et al.*, 2004) and a strain of *B. lusitaniae* has been isolated from a patient's skin (Collares-Pereira *et al.*, 2004). The latest species described within the complex of *B. burgdorferi* s.l. is *Borrelia spielmanii* (Richter *et al.*, 2004). Its exceptionally narrow specificity for a particular reservoir host, i.e. garden and hazel dormice, distinguishes it from all other Lyme disease spirochetes (Richter *et al.*, 2004). This unique biological relationship, together with its genotypic and phenotypic characteristics (Van Dam *et al.*, 1993; Wang *et al.*, 1999; Derdákóvá *et al.*, 2003;

Richter *et al.*, 2004) suggested that this dormouse-associated spirochete constitutes a distinct species. Due to the lack of DNA-DNA hybridization data, however, this species has not yet been validated. Current practice requires that validation of a novel bacterial species should be based on the level of DNA-DNA reassociation by means of whole DNA/DNA hybridization (WDDH), which is considered the gold standard in taxonomy (Wayne *et al.*, 1987). Few laboratories, however, can perform this technique, because it is non-cumulative and must include reference strains for all known species. Moreover, this method is not applicable for bacteria that cannot be cultivated and its application for slow-growing bacteria is limited. Also, WDDH data may not be reproducible (Stackebrandt *et al.*, 2002). Thus, alternative methods that replace DNA-DNA reassociation are required (Stackebrandt *et al.*, 2002).

The rapid development of sequencing techniques has made their application inexpensive and permits structural analysis of bacterial populations (Achtman, 2004). Multilocus sequence typing (MLST) is based on the identification of allelic mismatches of usually 7 housekeeping genes (Selander *et al.*, 1986; Gevers *et al.*, 2005). It has been used to reveal the population structure of diverse prokaryotic organisms at the intraspecific level, including *B. burgdorferi* s.s. and *B. afzelii* (Bunikis *et al.*, 2004; Qiu *et al.*, 2004). Recently, it was proposed that multilocus sequence analysis (MLSA), a phylogenetic characterization using sequences of alleles of several genes should be applied for species delineation (Gevers *et al.*, 2005). For MLSA, sequences are processed in a distance method procedure rather than in the cluster analysis procedure used in MLST. This tool may replace DNA-DNA reassociation, provided that both techniques demonstrate a sufficient degree of congruence (Stackebrandt *et al.*, 2002). We have previously accumulated values for DNA-DNA reassociation of all type strains and various other strains of *B. burgdorferi* s.l. by the S1-TCA method (Grimont *et al.*, 1980).

To validate the MLSA method for species delineation, we sequenced seven loci of numerous *B. burgdorferi* s.l. strains, including the type strains, and compared the results to our

extensive data collection of DNA-DNA similarity and thermal stability. MLSA was subsequently applied to several isolates of the recently described, but not yet validated, species *B. spielmanii* (Richter *et al.*, 2004).

Material and Methods

B. burgdorferi s.l. strains

The strains used in this study (Table 1) belong to five species endemic to Europe, *B. burgdorferi* s.s., *B. garinii*, *B. afzelii*, *B. valaisiana* and *B. lusitaniae*. Information regarding DNA-DNA reassociation was available for each of these strains (Postic *et al.*, 1990; Baranton *et al.*, 1992; Canica *et al.*, 1993; Le Flèche *et al.*, 1997; Wang *et al.*, 1997) and sequences at several of the selected loci were accessible from literature or databases (Postic *et al.*, 1994; Le Flèche *et al.*, 1997; Valsangiacomo *et al.*, 1997; Wang *et al.*, 1997; Casati *et al.*, 2004; Park *et al.*, 2004). For the delineation of *B. spielmanii* by MLSA, we used the first described isolate of this group, strain A14S, that was obtained from a patient's erythema migrans, three strains, including the type strain PC-Eq17^T, derived from *Ixodes ricinus* ticks that had fed on each of three garden dormice, *Eliomys quercinus*, live-trapped in Alsace, France (Richter *et al.*, 2004), as well as two isolates obtained from acute skin lesions of a Danish and a Hungarian patient. DNA of each of these strains was extracted from the centrifugation pellet of cultivated isolates, either by boiling at 100°C for 10 min. or by the QIAamp DNA Mini Kit.

MLSA

Seven loci, *rrs*, *hbb*, *groEL*, *recA*, *fla*, *ospA*, and *rrf-rrl* intergenic spacer, were selected for analysis (Table 2). All loci were amplified by a single PCR. Amplification reactions were performed in a final volume of 50 µl, comprising 0.2 µM of each primer of a primer pair, 200 µM of each deoxynucleoside triphosphate, 1.25 U or 1 U of *Taq* polymerase (Q.Bio gene or Qiagen,

respectively), and 1x *Taq* buffer (1.5 mM MgCl₂). The mixture was placed in a thermocycler, heated to 93°C for 1 min and subjected to 35 cycles of denaturation for 1 min at 93°C, annealing for 1 min at either 51°C for *rrs* and *groEL* loci, or at 59°C for the remaining loci, and extension for 1 min at 72°C, followed by a final extension step at 72°C for 5 min. Amplification products were sequenced either by Genome-Express (Meylan, France) or by the dideoxynucleotide chain termination method on a Licor DNA4200 sequencer using the same primers as for PCR.

Sequence analysis

The Clustal W (Thompson *et al.*, 1997) algorithm was used for sequence alignments and the Mega 3 (Kumar *et al.*, 1993) software for phylogenetic analyses of both individual and concatenated sequences. Distances were calculated by the Jukes and Cantor correction (Saitou & Nei, 1987) in a pairwise deletion procedure. A similarity table was generated from a distance matrix (P-distance values) with Excel. Both, Unweighted Pair Group with Mathematical Average (UPGMA) (Sneath & Sokal, 1962) and Neighbour Joining (NJ) (Saitou & Nei, 1987), methods were used to build phylogenetic trees. Percentage support values were obtained in a bootstrap procedure.

Results

MLSA of strains representative of previously validated *B. burgdorferi* s.l. species

To evaluate whether the seven loci, *rrs*, *hbb*, *groEL*, *recA*, *fla*, *ospA*, and *rrf-rrl* intergenic spacer, are appropriate for subsequent MLSA, we sequenced them or obtained published sequences from databases for each of 13 selected isolates representing *B. burgdorferi* s.s., *B. garinii*, *B. afzelii*, *B. valaisiana* and *B. lusitaniae* and analyzed their phylogenetic clustering. We generated a distance matrix from sequence alignments and constructed a phylogenetic tree for each locus. Independent of the method used for the phylogenetic analysis, such as UPGMA (Fig.

1) or NJ (data not shown), sequences from isolates belonging to each validated species constituted a single cluster on each individual tree. The *rrf-rrl* intergenic spacer provided greater strain diversity within a species than did other loci. *B. lusitaniae* strains PotiB1 and PotiB2 are particularly divergent at this locus, although their *rrf-rrl* sequences are clearly distinct from those of other species. Otherwise, *B. garinii* clearly appears to be the most diverse species, because the strain clustering varied according to the locus targeted.

For subsequent MLSA, sequences of the seven loci were concatenated for each of the 13 representative *B. burgdorferi* s.l. strains, resulting in a total length of each concatenated sequence of 2100 bp, and analysed comparatively. The sequences clearly segregated according to the established delineation of *B. burgdorferi* s.l. species (Fig. 2), supported by bootstrap analysis. Similar results were obtained by both NJ (Fig. 2a) and UPGMA (Fig. 2b) methods.

Correlation between MLSA and DNA-DNA reassociation

Genetic distances of concatenated sequences (Table 3, expressed as similarity table), generated in Mega software, were compared to those derived from WDDH performed previously (Postic *et al.*, 1990; Baranton *et al.*, 1992; Postic *et al.*, 1994). The clustering of strains obtained by MLSA strongly associated with the clustering inferred from values of DNA relatedness (Fig. 3). Within a species, defined by values of DNA relatedness above 70 % and a thermal stability estimated at ΔT_m below 5°C (Wayne *et al.*, 1987), values of sequence similarity deduced from genetic distances ranged from 97.9 to 99.8 % (Table 3). In contrast, comparing values of sequence similarity across species, they ranged from 92 to 94.9 % (Table 3). A slight interspecific discrepancy was observed, when comparing the strains *B. afzelii* VS461^T and *B. garinii* PBi. Although the similarity value was 94 %, their DNA relatedness reached 74%. A ΔT_m value of 9°C, however, demonstrated that these strains comprised two distinct species, as did the MLSA

similarity value determined in this study. An MLSA similarity value of 97.9 %, therefore, appears to be useful as a cut-off to differentiate *B. burgdorferi* s.l. species.

***B. spielmanii* characterization using MLSA**

B. spielmanii sequences clustered separately from all previously described *B. burgdorferi* s.l. species, independent of the locus targeted (Fig. 1). As for all other delineated species, the *rrf-rrl* locus varied most. Sequences scattered in three branches, when published *rrf-rrl* sequences of *B. spielmanii* isolates derived from either ticks (accession nos. AY147009 (PC-Eq17^T), AY573193 and AF497994) or human samples (accession no. U76616) and unpublished sequences of isolates obtained in our laboratories from *I. ricinus* (PC-Eq2/1, PC-Eq2r, 4AJL150) or human skin (2102, PZ30802, DK35 and DK38) were included in the analysis. In contrast, all *B. spielmanii* sequences were identical for the conserved *fla* locus and, more unexpectedly, virtually identical for the *ospA* locus. A very similar clustering resulted from the phylogenetic analysis of the *groEL*, *hbb* and *recA* loci.

When analyzing concatenated sequences, *B. spielmanii* isolates related closely to one another, but unambiguously diverged from all other species that were included in this study (Fig. 2). If we apply the MLSA similarity cut-off of 97.9 % to this non-validated species, all examined *B. spielmanii* isolates constitute a single and homogenous species, with similarities ranging from 99.2 to 100 %, while differing from other species by similarity values of 92.1 to 94.8 % (Table 3).

Discussion

Phylogenetic analysis of a single locus generally serves to evaluate the genetic diversity within a species. Ribosomal genes and spacers, however, have been used for species delineation (Marconi *et al.*, 1995). Moreover, several methodologies showed a good correlation with WDDH data. Analysis of *B. burgdorferi* s.l. strains by MLEE (Boerlin *et al.*, 1992), for example,

produced values strongly correlated to those of DNA-DNA reassociation (Baranton *et al.*, 1992). Even arbitrarily primed PCR (Welsh *et al.*, 1992) suggested that the isolate DN127 differs from other previously studied isolates and may be a member of a new species. Subsequently, DN127 has, indeed, been designated as the type strain of *B. bissetii* (Postic *et al.*, 1998). Methods involving multiple loci dispatched on the whole genome of an organism, however, provide a more valuable alternative to the laborious DNA-DNA reassociation (Stackebrandt *et al.*, 2002). MLST and recently, more appropriately, MLSA have been proposed as true alternatives to DNA-DNA reassociation for taxonomic purposes (Lan & Reeves, 2001; Stackebrandt *et al.*, 2002; Gevers *et al.*, 2005). Well-defined conditions for MLST have to be met; particularly, requiring a minimum of five housekeeping genes under stabilizing selection in order to obtain an informative level of data (Stackebrandt *et al.*, 2002). Whereas MLST characterizes genotypic relationships of prokaryotes at an intraspecific level by identifying allelic mismatches of housekeeping genes, MLSA permits phylogenetic characterization by yielding clusters of concatenated sequences of multiple genes (Gevers *et al.*, 2005). In our MLSA study, we used seven loci, whose relevance for taxonomic studies of *B. burgdorferi* s.l. had been demonstrated previously. Five of them are located on the chromosome and evolved, therefore, in a clonal way, while not being subjected to lateral transfer (Dykhuisen *et al.*, 1993): *rrs* gene (Le Flèche *et al.*, 1997; Wang *et al.*, 1997; Postic *et al.*, 1998), *fla* gene (Fukunaga & Koreki, 1996), *groEL* gene (Park *et al.*, 2004), *hbb* gene (Valsangiacomo *et al.*, 1997), and *recA* gene (Casati *et al.*, 2004). Instead of operational genes, we chose informational genes (*rrs*, *hbb*, *groEL*, *recA*) which are embedded in a network of interactions and are subjected only minimally to lateral transfer (Jain *et al.*, 1999). We added the *fla* gene encoding flagellin, because it constitutes the endoflagella of spirochetes, and therefore is not targeted by selective pressure of the host's immune response. The *ospA* locus, on the other hand, represents an adaptive, plasmid-encoded gene, which was previously employed for taxonomic purposes (Dykhuisen *et al.*, 1993; Bunikis *et al.*, 2004). Although this gene may be

subject to lateral transfer, it occurs only rarely (Dykhuizen *et al.*, 1993). In fact, the tree generated from the *ospA* gene appeared to evolve clonally for all sequences studied. Finally, we chose the non-coding locus, *rrf-rrl* intergenic spacer, its sequence correlates well taxonomically (Postic *et al.*, 1994). These last two loci represent the fast evolving part of the genome, contrasting with the remaining loci. Independent of the locus considered, the phylogeny reflects taxonomic associations, thereby, confirming that *B. burgdorferi* s.l. evolves clonally (Dykhuizen *et al.*, 1993).

MLST was originally designed for epidemiological purposes (Achtman, 2004). The related, but phylogenetic approach, which was recently termed MLSA (Gevers *et al.*, 2005), has been used successfully to delineate species in highly recombinogenic bacteria (Hanage *et al.*, 2005). We demonstrate here for *B. burgdorferi* s.l. that sequence similarities derived by MLSA strictly correlate with data inferred from WDDH. Relatedness deduced from MLSA, therefore, is applicable for distinguishing *B. burgdorferi* s.l. species, if performed with a sufficient number of appropriately selected loci. Species delineation by MLSA must be based on a cut-off in sequence similarity that is determined by comparing genetic distances issued from both DNA-DNA reassociation and MLSA. As the similarity cut-off, we selected the widest genetic distance corresponding to the lowest similarity percentage recorded within any of the previously validated *B. burgdorferi* s.l. species. The phylogenetic analyses of concatenated sequences in a bootstrap procedure provides additional support of robustness of MLSA. Therefore, MLSA constitutes a valuable alternative to replace the laborious DNA-DNA hybridization.

Using MLSA, we confirmed the status of a previously delineated species among the *B. burgdorferi* s.l. complex, *B. spielmanii*. This species is characterized by a peculiar reservoir relationship. In nature, *B. spielmanii* perpetuates in garden and hazel dormice (Richter *et al.*, 2004). No other member of the *B. burgdorferi* s.l. complex is restricted to such narrow host range. This unique ecological niche together with its genotypic and phenotypic characteristics (Van Dam *et al.*, 1993; Wang *et al.*, 1998; Derdákóvá *et al.*, 2003; Richter *et al.*, 2004) distinguish it from all

other Lyme disease spirochetes. The distinctiveness of *B. spielmanii* is fully reflected in our MLSA analysis.

Numerous questing *I. ricinus* ticks and those feeding on dormice have been found to harbor *B. spielmanii* in France (Richter *et al.*, 2004). Questing ticks infected by this dormouse-associated spirochete have also been collected in the Czech Republic (Derdáková *et al.*, 2003), Russia (sequence published with accession no. AY573193) and Germany (Rauter *et al.*, 2002). *B. spielmanii* causes erythema migrans in people and was isolated from or detected in patients' skin in the Netherlands (Van Dam *et al.*, 1993), in Slovenia (Ruzic-Sabljić, unpublished), in Hungary (Földvári *et al.*, 2005), twice in Denmark (Livey, unpublished), and twice in Germany (Michel *et al.*, 2004). Hitherto, *B. spielmanii* has been detected in ticks and patients solely in Europe, its distribution may correspond closely to that of dormice.

The description of *Borrelia spielmanii* sp. nov., N.L. gen. n. *spielmanii*, of Spielman, named in honour of Andrew Spielman, who described for the first time the life cycle and biological relationships of *B. burgdorferi* s.l., is given in Richter *et al.*, 2004. Note that the spelling of *B. spielmanii* has been corrected. The type strain PC-Eq17^T (strain number PC-Eq17N5^T) is deposited at the DSMZ, Braunschweig, Germany (strain number DSM 16813^T) and at the Collection of the Institut Pasteur, Paris, France (strain number CIP 108855^T).

Taken together, the results of MLSA fully agree with data obtained by WDDH. MLSA constitutes a valuable alternative for a reliable and precise delineation of *B. burgdorferi* s.l. species. This methodology results in solid sequence data with a highly discriminative power, that are not subject to experimental variation and may easily be shared, allowing interlaboratory comparison. It is desirable for the near future that this technique is widely adopted, because it facilitates studies on various bacterial isolates, which hitherto remain unclassified due to the impediment of cumbersome methods.

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Table 1. Characteristics of the *Borrelia burgdorferi* s.l. isolates used in MLSA.

Isolate	Species	Geographic origin	Biologic origin	Reference
B31 ^T	<i>B. burgdorferi</i> s.s.	USA	<i>I. scapularis</i>	Johnson <i>et al.</i> , 1984
IP1	<i>B. burgdorferi</i> s.s.	France	Human CSF*	Boerlin <i>et al.</i> , 1992
212	<i>B. burgdorferi</i> s.s.	France	<i>I. ricinus</i>	Postic <i>et al.</i> , 1994
VS461 ^T	<i>B. afzelii</i>	Switzerland	<i>I. ricinus</i>	Boerlin <i>et al.</i> , 1992
PGau	<i>B. afzelii</i>	Germany	Human skin	Wilske <i>et al.</i> , 1988
BO23	<i>B. afzelii</i>	Sweden	Human skin	Postic <i>et al.</i> , 1994
20047 ^T	<i>B. garinii</i>	France	<i>I. ricinus</i>	Valsangiacomo <i>et al.</i> , 1997
PBi	<i>B. garinii</i>	Germany	Human CSF	Preac-Mursic <i>et al.</i> , 1986
NT29	<i>B. garinii</i>	Japan	<i>I. persulcatus</i>	Postic <i>et al.</i> , 1994
VS116 ^T	<i>B. valaisiana</i>	Switzerland	<i>I. ricinus</i>	Wang <i>et al.</i> , 1997
UK	<i>B. valaisiana</i>	United Kingdom	<i>I. ricinus</i>	Wang <i>et al.</i> , 1997
PotiB2 ^T	<i>B. lusitaniae</i>	Portugal	<i>I. ricinus</i>	Le Fleche <i>et al.</i> , 1997
PotiB1	<i>B. lusitaniae</i>	Portugal	<i>I. ricinus</i>	Le Fleche <i>et al.</i> , 1997
PC-Eq17 ^T	<i>B. spielmanii</i>	France	<i>I. ricinus</i>	Richter <i>et al.</i> , 2004
PC-Eq2/1	<i>B. spielmanii</i>	France	<i>I. ricinus</i>	Richter <i>et al.</i> , 2004
PC-Eq2r	<i>B. spielmanii</i>	France	<i>I. ricinus</i>	Richter <i>et al.</i> , 2004
A14S	<i>B. spielmanii</i>	The Netherlands	Human skin	Wang <i>et al.</i> , 1999
DK35	<i>B. spielmanii</i>	Denmark	Human skin	Theisen <i>et al.</i> , 1995
PZ30802	<i>B. spielmanii</i>	Hungary	Human skin	This study

*CSF – cerebrospinal fluid; T – type strain

Table 2. Characteristics of the amplified fragments and corresponding primer sequences. Numbering derives from *Borrelia burgdorferi* s.s. strain B31^T.

Locus	Fragment length	Primers	Sequence analysed
<i>rrs</i>	522	F: AGAGTTTGATCCTGGCTTAG (10-29) R: CTTTACGCCCAATAATCCCGA (572-552)	30-551
<i>fla</i>	457	F: AACACACCAGCATCACTTTCAGG (475-497) R: GATTWGCRTGCGCAATCATTGCC (976-954)	497-844
<i>groEL</i>	268	F: TACGATTTCTTATGTTGAGGG (552-572) R: CATTGCTTTTCGTCTATCACC (861-841)	573-840
<i>hbb</i>	327	F: GCGAAGAATTCATAAAAATAAGGCTGC (--79 -53) R: TATAAGAATTCACGATATTA ACTGGC (End +26)	1-327
<i>recA</i>	156	F: GTGGATCTATTGTATTAGATGAAGCTCTTG (170-199) R: GCCAAAGTTCTGAAACATTA ACTCCCAAAG (391-362)	206-361
<i>ospA</i>	261	F: AATAGGTCTAATATTAGCCTTAATAGC (21-47) R: TTGATACTAATGTTTTGCCATCTTCTT (334-308)	48-308
<i>rrl-rrf</i> spacer	197	F: CTGCGAGTTCGCGGGAGAG (3' rrf) R: AAGCTCCTAGGCATTCACCATA (5' rrl)	197

Table 3. Similarity table of concatenated sequences of *Borrelia burgdorferi* s.l. Similarities were calculated from a distance matrix (p distance values) in a pairwise deletion procedure. [supplementary data]

	<i>B. burgdorferi</i> s.s.			<i>B. afzelii</i>			<i>B. garinii</i>			<i>B. valaisiana</i>		<i>B. lusitaniae</i>		<i>B. spielmanii</i>					
	B31 ^T	IP1	212	VS461 ^T	Pgau	BO23	20047 ^T	Pbi	NT29	VS116 ^T	UK	PotiB1	PotiB2 ^T	A14S	PCEq17 ^T	PCEq2/1	PCEq2r	PZ30802	
IP1	99.7																		
212	99.7	99.6																	
VS461 ^T	93.9	93.9	93.9																
PGau	94.0	94.1	94.0	99.6															
BO23	94.0	94.0	93.9	99.8	99.5														
20047 ^T	93.5	93.6	94.5	94.1	94.4	94.7													
Pbi	93.6	93.7	93.7	94.0	94.3	94.1	98.1												
NT29	93.8	94.0	93.9	93.9	94.3	94.0	97.9	98.4											
VS116 ^T	93.9	94.0	94.0	94.3	94.7	94.4	94.8	94.8	92.8										
UK	94.0	94.1	94.1	94.3	94.7	94.4	94.8	94.7	94.9	99.6									
PotiB1	92.0	92.1	93.7	91.5	92.0	92.7	92.3	92.0	92.4	92.5	92.6								
PotiB2 ^T	92.6	92.7	92.6	93.7	92.6	92.3	92.8	92.6	93.0	93.4	93.3	99.0							
A14S	93.4	94.5	93.4	94.3	94.6	94.5	93.7	93.9	94.3	94.2	94.2	92.4	93.0						
PC-Eq17 ^T	93.3	93.4	93.3	94.7	94.6	94.8	93.7	93.9	94.4	94.1	94.2	92.4	92.9	99.3					
PC-Eq2/1	93.2	93.3	93.2	94.7	94.6	94.8	93.6	93.8	94.3	94.0	94.1	92.3	92.8	99.3	100				
PC-Eq2r	93.3	93.4	93.3	94.7	94.6	94.8	93.7	93.9	94.4	94.1	94.2	92.4	92.9	99.3	100	100			
DK35	93.4	94.5	93.4	94.4	94.7	94.6	93.6	93.9	94.3	94.3	94.2	92.4	92.1	100	99.3	99.2	99.3		
PZ30802	93.3	93.4	93.3	94.7	94.7	94.8	93.5	93.8	94.3	94.2	94.1	92.2	92.9	99.3	99.5	99.5	99.5	98.3	

T - type strain

Table 4. Accession numbers of sequences derived in this study. [supplementary data]

Locus	<i>Borrelia burgdorferi s.l.</i>			
	Species	Isolate	Accession no.	
<i>fla</i>	<i>B. afzelii</i>	BO23	DQ111033	
	<i>B. burgdorferi s.s.</i>	212	DQ111031	
	<i>B. garinii</i>	NT29	DQ111032	
	<i>B. lusitaniae</i>	PotiB2 ^T	DQ111036	
		PotiB1	DQ111035	
	<i>B. spielmanii</i>	PC-Eq17 ^T	DQ133508	
		PC-Eq2/1	DQ133509	
		PC-Eq2r	DQ133510	
		A14S	DQ111034	
		DK35	AM055816	
		PZ30802	AM055817	
	<i>B. valaisiana</i>	VS116 ^T	DQ111037	
		UK	DQ111038	
	<i>groEL</i>	<i>B. afzelii</i>	PGau	DQ111043
			BO23	DQ286236
<i>B. burgdorferi s.s.</i>		212	DQ111045	
		IP1	DQ111046	
<i>B. garinii</i>		20047 ^T	DQ111041	
		NT29	DQ111042	
<i>B. lusitaniae</i>		PotiB1	DQ111039	
<i>B. spielmanii</i>		PC-Eq17 ^T	DQ133511	
		PC-Eq2/1	DQ133512	
		PC-Eq2r	DQ133513	
		A14S	DQ111040	
		DK35	AM055821	
		PZ30802	AM055820	
<i>B. valaisiana</i>		UK	DQ111044	
<i>hbb</i>		<i>B. afzelii</i>	PGau	DQ111050
	<i>B. burgdorferi s.s.</i>	212	DQ111048	
	<i>B. garinii</i>	20047 ^T	DQ111049	
	<i>B. spielmanii</i>	PC-Eq17 ^T	DQ133514	
		PC-Eq2/1	DQ133515	
		PC-Eq2r	DQ133516	
		A14S	DQ111047	
		DK35	AM055818	
	PZ30802	AM055819		

Table 4 continued

<i>ospA</i>	<i>B. afzelii</i>	BO23	DQ111055	
	<i>B. burgdorferi s.s.</i>	212	DQ111051	
		IP1	DQ111052	
		20047 ^T	DQ111053	
	<i>B. garinii</i>	NT29	DQ111054	
		<i>B. spielmanii</i>	PC-Eq17 ^T	DQ133517
	PC-Eq2/1		DQ133518	
	PC-Eq2r		DQ133519	
	DK35		AM055822	
	DK38		AM055823	
PZ30802	AM055824			
<i>recA</i>	<i>B. afzelii</i>		PGau	DQ111056
	<i>B. burgdorferi s.s.</i>	212	DQ111058	
	<i>B. garinii</i>	20047 ^T	DQ111059	
	<i>B. spielmanii</i>	PC-Eq17 ^T	DQ133520	
		PC-Eq2/1	DQ133521	
		PC-Eq2r	DQ133522	
		A14S	DQ111057	
		DK35	AM055826	
		PZ30802	AM055825	
<i>rrs</i>		<i>B. afzelii</i>	PGau	DQ111060
	BO23		DQ111061	
	<i>B. burgdorferi s.s.</i>	212	DQ111062	
		IP1	DQ111063	
		NT29	DQ111064	
	<i>B. garinii</i>	<i>B. spielmanii</i>	PC-Eq17 ^T	DQ133523
			PC-Eq2/1	DQ133524
			PC-Eq2r	DQ133525
			DK35	AM055831
			PZ30802	AM055830
			<i>rrf-rrl spacer</i>	<i>B. afzelii</i>
<i>B. lusitaniae</i>	PotiB1			DQ111065
<i>B. spielmanii</i>	PC-Eq17 ^T	DQ133526		
	PC-Eq2/1	DQ133527		
	PC-Eq2r	DQ133528		
	DK35	AM055827		
	DK38	AM055828		
PZ30802	AM055829			
2102	DQ286234			
4AJL150	DQ286235			

Figures

Figure 1. Phylogenetic analysis of *Borrelia burgdorferi* s.l. based on individual sequences of seven loci, *recA* (Fig. 1A), *hbb* (Fig. 1B), *groEL* (Fig. 1C), *ospA* (Fig. 1D), *fla* (Fig. 1E), *rrs* (Fig. 1F), or *rrf-rrl* intergenic spacer (Fig. 1G), available from databases (accession numbers given in parantheses) or obtained in this study. Trees were constructed after multiple alignment of data by Clustal W (Thompson *et al.*, 1997). Distances and clustering with the unweighted pair group with mathematical average (UPGMA) method (Sneath & Sokal, 1962) were performed by using the software package Mega 3 (Kumar *et al.*, 1993). Distances were calculated by the Jukes and Cantor correction (Saitou & Nei, 1987) in a complete deletion procedure. Percentage support values were obtained in a bootstrap procedure based on 500 replications.

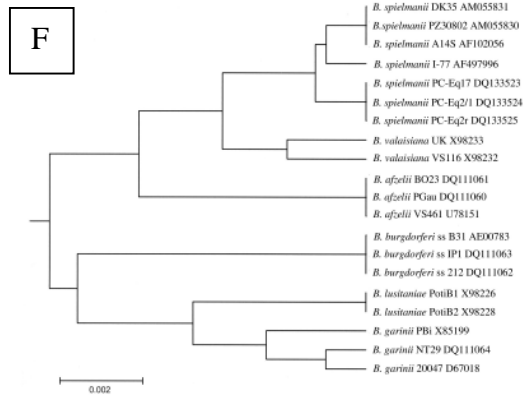
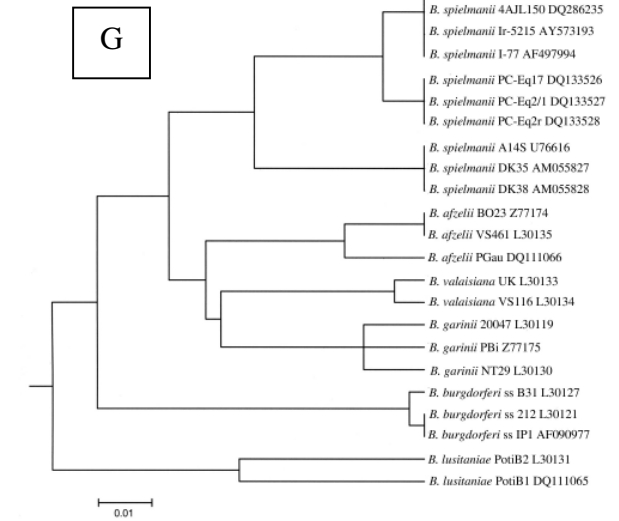
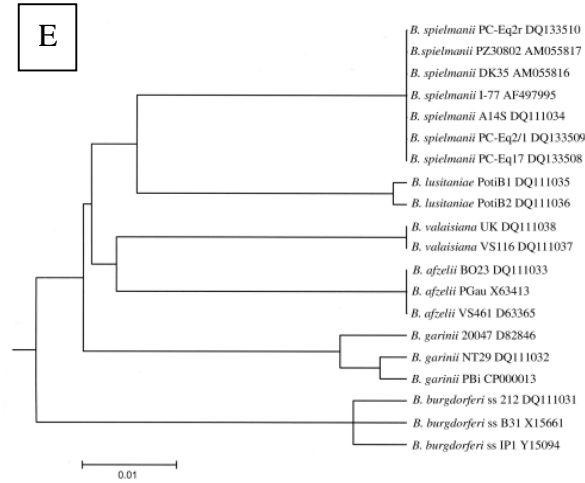
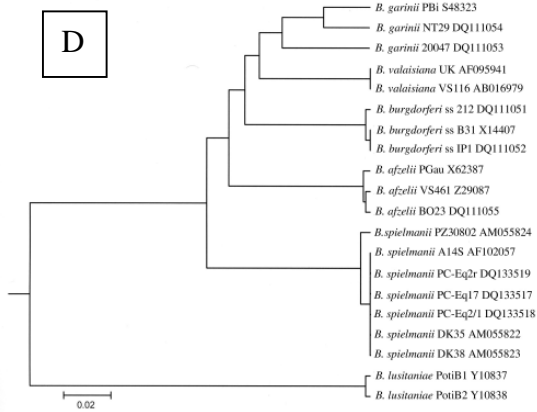
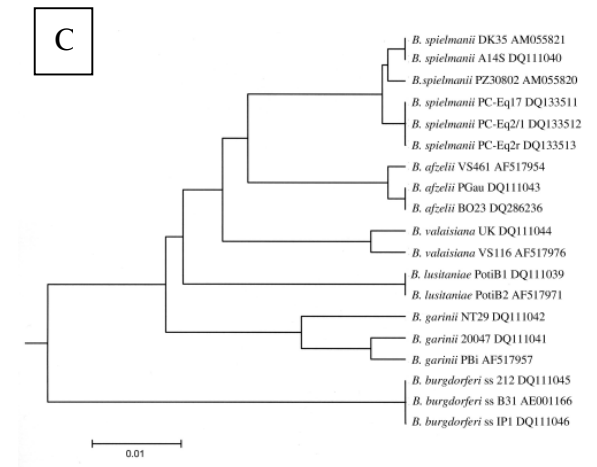
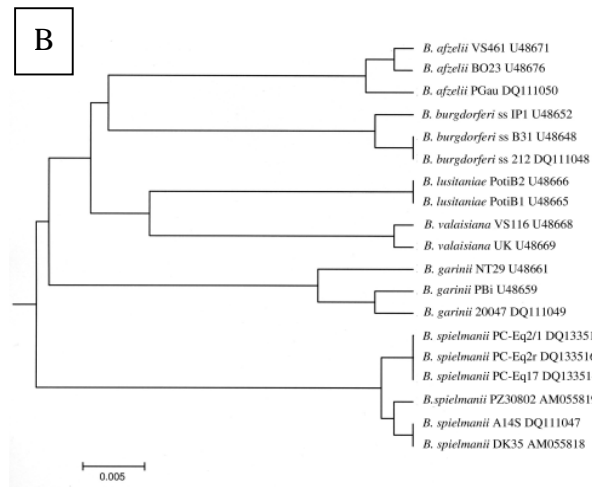
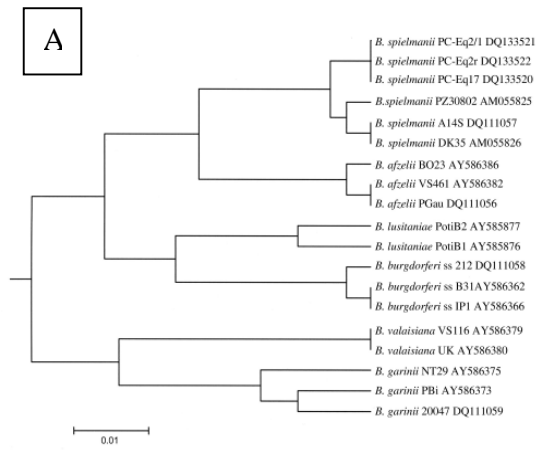
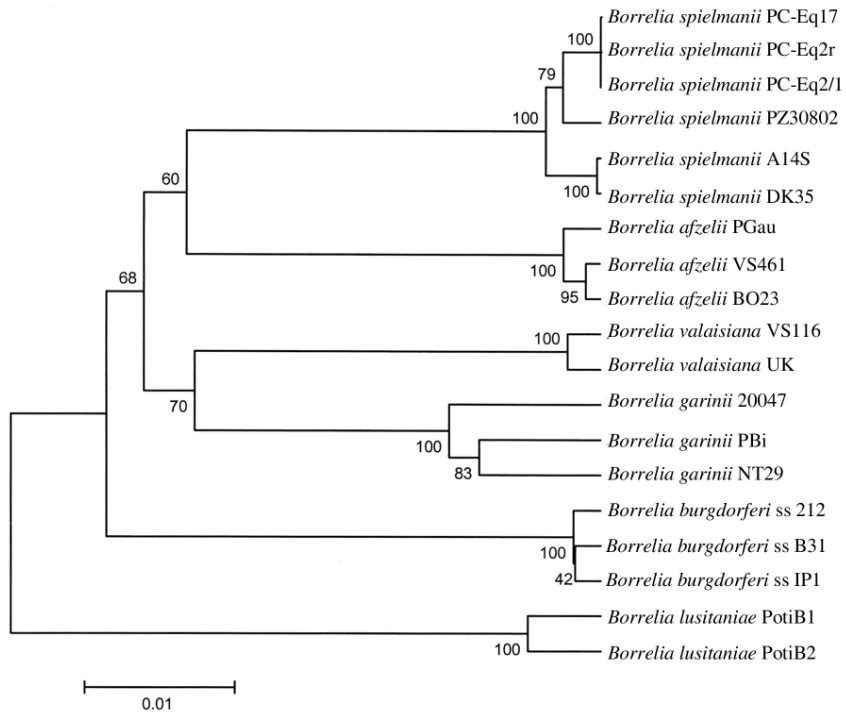


Figure 2. Phylogenetic analysis of *Borrelia burgdorferi* s.l. based on concatenated sequences of seven loci, *rrs*, *hbb*, *groEL*, *recA*, *fla*, *ospA*, and *rrf-rrl* intergenic spacer, available from databases or obtained in this study. Trees were constructed after multiple alignment of data by Clustal W (Thompson *et al.*, 1997). Distances and clustering using the unweighted pair group with mathematical average (UPGMA) method (Sneath & Sokal, 1962) (Fig. 2A) or using the neighbour-joining (NJ) method (Saitou & Nei, 1987) (Fig. 2B) were performed by means of the software package Mega 3 (Kumar *et al.*, 1993). Distances were calculated by the Jukes and Cantor correction (Saitou & Nei, 1987) in a complete deletion procedure. Percentage support values were obtained in a bootstrap procedure based on 500 replications.

A



B

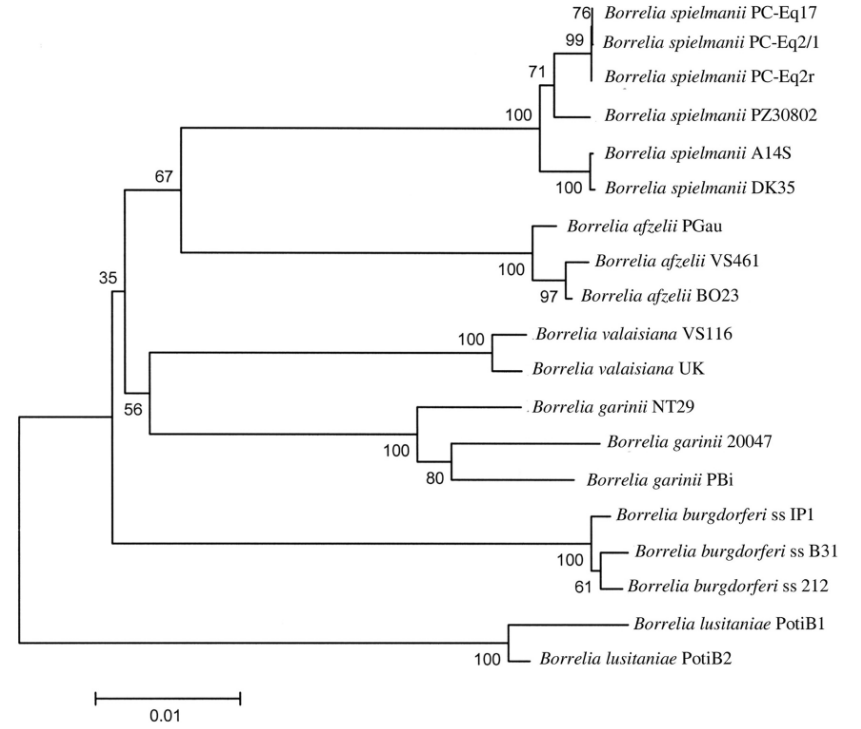


Figure 3. Correlation of genetic distances determined by whole DNA-DNA hybridization (WDDH) and by MLSA of concatenated sequences (Table 3). Levels of DNA relatedness, deriving from previous studies (Postic *et al.*, 1990, Baranton *et al.*, 1992, Postic *et al.* 1994 and Postic unpublished), were converted to genetic distances. Values for genetic distances obtained by either of the two methods correlate within (triangles) and between (circles) species. Values smaller than a genetic distance of 0.3 determined by WDDH generally designate an intraspecific relationship (Wayne *et al.*, 1987). The WDDH value of 0.26 genetic distance determined for the type strains *B. afzelii* VS461^T and *B. garinii* Pbi (open circle) constitutes an exception.

