Applied and Environmental Microbiology, Oct. 2011, p. 7088–7092 Vol. 77, No. 19
0099-2240/11/$12.00 doi:10.1128/AEM.05437-11

flaB Gene as a Molecular Marker for Distinct Identification of *Borrelia* Species in Environmental Samples by the PCR-Restriction Fragment Length Polymorphism Method[▽]

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Received 10 May 2011/Accepted 20 July 2011

A new protocol employing nested PCR-restriction fragment length polymorphism (RFLP) based on the *flaB* gene and two restriction enzymes was worked out. This protocol allows the identification of all *Borrelia* species transmitted by *Ixodes ricinus* in Europe, including *Borrelia miyamotoi* and 3 genetic variants of *B. garinii*. A dendrogram of *flaB* sequence similarity was in accordance with RFLP variants.

The *Borrelia* genus encompasses nearly 40 species of tick-transmitted pathogens, characterized by different host interactions and vector specificities. Those spirochetes are divided into two main groups: relapsing fever (RF) borreliae, transmitted by "soft ticks" (Argasidae), and the *Borrelia burgdorferi* sensu lato complex, also known as the Lyme disease (LD) borreliae, propagated by "hard ticks" (Ixodidae) (10). The host and vector specificities of the RF complex members facilitate their identification, with the exception of *B. miyamotoi*, which appears on three continents and which is transmitted by different tick species on each (3, 14, 15).

The spirochetes of the LD complex present host specificity (6), but they do not show strict vector requirements. In Europe, the main vector of all eight *B. burgdorferi* sensu lato species and of *B. miyamotoi* is *Ixodes ricinus*, whose hosts are at least 300 species of forest vertebrates (4, 5). It is therefore essential to precisely identify the *Borrelia* species spread on this continent by the same tick, since it determines correct risk assessment of different forms of borreliosis induced by different bacteria from the Lyme disease-inducing species.

Multiple markers are used for a DNA-based distinction of the *Borrelia* species, i.e., *rss*, encoding 16S rRNA, *ospA*, encoding surface protein A, and *flaB*, encoding flagellin, as well as noncoding genomic sequences *rrfA-rrlB* and *rrs-rrlA* for intergenic regions (1, 2, 8, 12, 13, 16). Some protocols for differentiation of *Borrelia* species involve the application of species-specific probes, but only five European species transmitted by *I. ricinus* have been identified using probes so far (9). Furthermore, the restriction analyses developed thus far permit the distinction of no more than 7 species of the *B. burgdorferi* sensu lato complex using 5 restriction enzymes, apart from *B. bissettii* and *B. miyamotoi* (8).

The aim of this study is the presentation of a possible application of the established PCR-restriction fragment length polymorphism (RFLP) protocol using two restriction enzymes for the identification of all *Borrelia* species transmitted by *I. ricinus* in biological material collected in Europe based on the *flaB* gene.

The examined material consisted of 52 *Borrelia* DNA isolates that had been obtained in previous studies from *I. ricinus* ticks collected from vegetation and removed from birds in west Poland and that were divided into 6 species of *Borrelia* (7, 17–20, 22).

The nested PCR method with genus-specific primer sets 132f/905r and 220f/823r used to detect the *flaB* gene fragment of *Borrelia* has been described earlier (21). DNA isolated from reference strains of 6 *Borrelia* species (Fig. 1 and 2) obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ; Germany) was used as positive controls. The PCR products were separated on 1.5% agarose gel (Prona, Spain) with addition of ethidium bromide (Sigma-Aldrich, Germany) at 5 V/cm for 1 h. A Nova 100-bp DNA ladder (Novazym, Poland) was applied for evaluation of the size of the obtained product. The results of the PCR were viewed under UV light and were archived in computer storage using BioCapt software (Vilber Lourmat, France).

The PCR-RFLP protocol with a single restriction enzyme, HpyF3I (Fermentas, Lituania), recognizing the CTNAG sequence inside a *flaB* gene fragment had been used previously for identification of *Borrelia* species (20, 21). This study constitutes an extension to that protocol with the use of an additional restriction enzyme, Ecl136II (Fermentas), which recognizes the GAGCTC sequence, allowing the identification of all *Borrelia* species vectored by *I. ricinus*. The procedure, based on analysis of unique sequences of the *flaB* gene from the European *Borrelia* species and strains obtained from GenBank (Table 1), was elaborated using DNAMAN 5.2.9 software (Biosoft, Canada), which performed *in silico* restriction analysis of the DNA sequences. Predicted restriction patterns are shown in Table 2. Digestion with HpyF3I generates 9 RFLP patterns, 6 of which are unique for 5 species: 2 for *B. afzelii* and 1 each for *B. valaisiana, B. bissettii, B. lusitaniae*, and *B. miyamotoi*. Therefore, no further analysis of the obtained results is necessary. The remaining 3 patterns require additional processing with the Ecl136II enzyme, which permits the distinction of *B. burgdorferi* sensu stricto from the unclassified strain *Borrelia* sp. SV1, of *B. spielmanii* from the south European strains of *B. lusitaniae*, and of *B. garinii* from *B. bavariensis*. The latter

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▽ Published ahead of print on 12 August 2011.

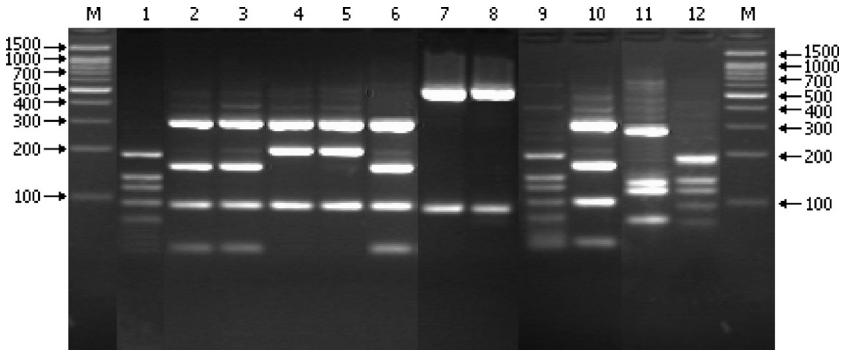

FIG. 1. HpyF3I restriction patterns of the amplified *flaB* DNA (604 or 598 bp) from *Borrelia* strains. Lanes: M, marker DNA (Nova 100-bp DNA ladder); 1, 9, and 12, *B. valaisiana*; 2, 3, 6, and 10, *B. afzelii*; 4 and 5, *B. lusitaniae*; 7 and 8, *B. miyamotoi*; 11, *B. bissettii*. Positive controls: *B. afzelii* VS461 (lane 10), *B. bissettii* DN127 (lane 11), and *B. valaisiana* VS116 (lane 12).

enzyme also allows strain differentiation within the heterogeneous species of *B. garinii*. The digestion products of both enzymes were analyzed on 3% agarose gel (Prona) and archived as described above.

Application of the presented protocol led to the confirmation of the presence of 6 *Borrelia* species in Poland, i.e., *B. burgdorferi* sensu stricto (Fig. 2, lanes 4 and 7) (pattern Ia), *B. afzelii* (Fig. 1, lanes 2, 3, and 6) (pattern II), *B. garinii* (Fig. 2, lanes 1 to 3, 5, 6, 8, and 9) (pattern V), *B. valaisiana* (Fig. 1, lanes 1 and 9) (pattern VI), *B. lusitaniae* (Fig. 2, lanes 4 and 5) (pattern VII), and *B. miyamotoi* (Fig. 1, lanes 7 and 8) (pattern IX). Digestion of the samples displaying restriction pattern V (*B. garinii*/*B. bavariensis*) with the second enzyme indicated also the presence of *B. bavariensis* in Poland (Fig. 2, lane 9). Sequence variability of the *flaB* gene among the Polish isolates of *B. garinii* was higher than the variability estimated from the sequences available in GenBank (patterns Va and Vb). An additional restriction pattern (Vd) (Fig. 2, lanes 6 and 8), composed of fragments of 397 and 217 bp and different from the pattern predicted for *B. garinii* and *B. bavariensis*, was individuated. Pattern Vd was found only in tick samples removed from birds, whereas pattern Va was characteristic of strains obtained from host-seeking ticks and pattern Vb was generated in strains obtained from distinct sources (human and animal samples and ticks collected from vegetation and removed from mammals and birds) (Table 1). Further examinations are required to confirm the host-dependent genetic diversity within *B. garinii* species observed in this study. Within *B. lusitaniae* isolates, different restriction patterns reflect the geographical versatility of south and central European strains.

Partial sequencing of the *flaB* gene with primers 220f and 823r was performed for 25 positive samples and gave restric-

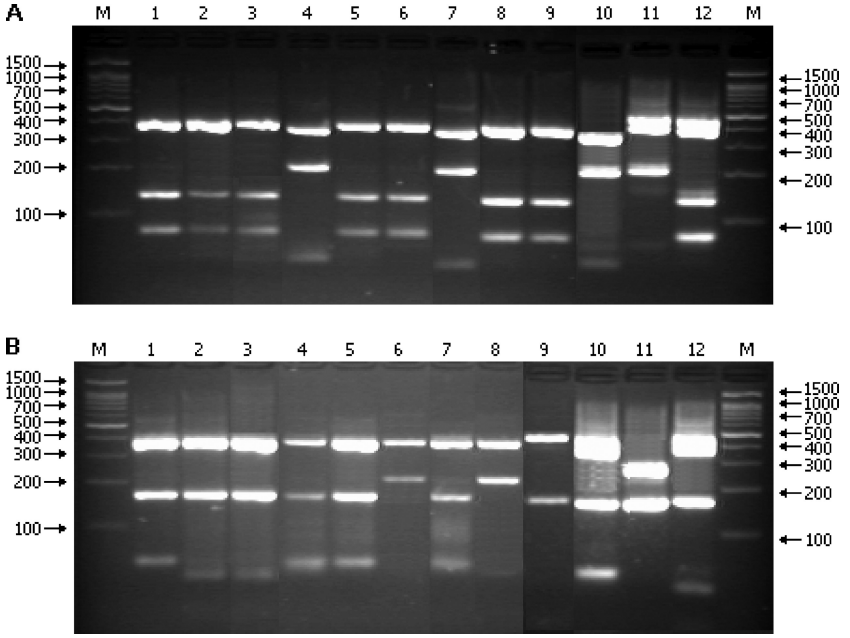

FIG. 2. HpyF3I (A) and Ecl136II (B) restriction patterns of the amplified *flaB* DNA (604 or 598 bp) from *Borrelia* strains. Lanes: M, marker DNA (Nova 100-bp DNA ladder); 1, 5, and 7, *B. garinii* type Vb; 2, 3, and 12, *B. garinii* type Va; 4, 7, and 10, *B. burgdorferi* sensu stricto; 6 and 8, *B. garinii* type Vd; 9, *B. bavariensis*; 11, *B. spielmanii*. Positive controls: *B. burgdorferi* sensu stricto IRS (lane 10), *B. spielmanii* PC-Eq17 (lane 11), and *B. garinii* 20047 (lane 12).

TABLE 1. *Borrelia* strains used in this study for working out the PCR-RFLP protocol based on the *flaB* gene for differentiation of *Borrelia* species and strains transmitted by *I. ricinus*

Borrelia species	Strain	Source	Country[a] (reference)	RFLP pattern[b] with:		Accession no.
				HpyF3I	Ecl136II	
B. burgdorferi sensu stricto	DG-1	*Canis lupus familiaris* (blood)	Poland (21)	I	Ia	DQ016625
	D69-04	*I. ricinus*	Poland (19)	I	Ia	DQ016620
	ZS7	*I. ricinus*	Germany	I	Ia	NC_011728
	GL56-07	*I. ricinus*	Poland†	I	Ia	HM345910
	T90-5-02	*I. ricinus* fed on *Turdus merula*	Poland†	I	Ia	HM345911
Borrelia sp. SV1	SV1	*I. ricinus*	Finland	I	Ib	NZ_ABJZ02000005
B. afzelii	9W10-04	*I. ricinus* fed on *Capreolus capreolus*	Poland (19)	II		FJ874924
	VS461	*I. ricinus*	Switzerland	II		D63365
	ZL109-07	*I. ricinus*	Poland†	II		HM345907
	ST19-05	*I. ricinus*	Poland†	II		HM345908
	OS17-07	*I. ricinus*	Poland†	II		HM345909
	P-Gau	Human	Germany	III		X63413
B. bissettii	DN127	*Ixodes pacificus*	United States	IV		D82857
B. garinii	20047	*I. ricinus*	France	V	Va	D82846
	PB35-99	*I. ricinus*	Poland†	V	Va	HM345897
	DB60-01	*I. ricinus*	Poland†	V	Va	HM345899
	ZL148-07	*I. ricinus*	Poland†	V	Va	HM345900
	D7-04	*I. ricinus*	Poland (19)	V	Vb	DQ016622
	D106-04	*I. ricinus*	Poland (19)	V	Vb	DQ016621
	T32-5-05	*I. ricinus* fed on *C. capreolus*	Poland (19)	V	Vb	DQ650336
	DB1F7-04	*I. ricinus*	Poland (19)	V	Vb	DQ650331
	DK29	Human	Denmark	V	Vb	X69608
	Far04	*Fratercula arctica*	Faroe Islands	V	Vb	NZ_ABPZ02000016
	HE			V	Vb	X69609
	K48	*I. ricinus*	Slovakia	V	Vb	X69610
	KL10			V	Vb	L42881
	PBr	Human	Germany	V	Vb	NZ_ABJV02000003
	DB74-01	*I. ricinus*	Poland†	V	Vb	HM345898
	T44-4-02	*I. ricinus* fed on *Turdus philomelos*	Poland†	V	Vb	HM345904
	ST12-05	*I. ricinus*	Poland†	V	Vb	HM345905
	RP54-05	*I. ricinus*	Poland†	V	Vb	HM345906
	T53-9-02	*I. ricinus* fed on *T. merula*	Poland†	V	Vd*	HM345901
	T40-10-02	*I. ricinus* fed on *T. philomelos*	Poland†	V	Vd*	HM345902
	T41-2-02	*I. ricinus* fed on *T. philomelos*	Poland†	V	Vd*	HM345903
B. bavariensis sp. nov.	PBi	Human	Germany	V	Vc	NC_006156
	TRO	Human	Slovenia	V	Vc	X69614
	DB18N6-04	*I. ricinus*	Poland†	V	Vc	DQ650333
B. valaisiana	BA9F9-05	*I. ricinus*	Poland (19)	VI		DQ650330
	D58-04	*I. ricinus*	Poland (19)	VI		DQ016624
	VS116	*I. ricinus*	Switzerland	VI		D82854
	T107-7-02	*I. ricinus* fed on *T. merula*	Poland†	VI		HM345913
	PR45-05	*I. ricinus*	Poland†	VI		HM345912
B. lusitaniae	D23-04	*I. ricinus*	Poland (19)	VII		DQ016623
	DB8-09-04	*I. ricinus*	Poland†	VII		HM345916
	SP6-09	*I. ricinus*	Poland†	VII		HM345914
	SP38-09	*I. ricinus*	Poland†	VII		HM345915
	PotiB2	*I. ricinus*	Portugal	VIII	VIIIa	D82856
B. spielmanii	A14S	Human	Netherlands	VIII	VIIIb	NZ_ABKB02000003
B. miyamotoi	WL10-6-04	*I. ricinus* fed on *C. capreolus*	Poland (19)	IX		DQ650338
	123T05-2	*I. ricinus* fed on *Cervus elaphus*	Poland (19)	IX		FJ823229
	D110-07	*I. ricinus*	Poland (20)	IX		FJ518804
	LB-M56	*I. ricinus*	France	IX		AF529084
	OS109-07	*I. ricinus*	Poland†	IX		HM345917
	PB111-09	*I. ricinus*	Poland†	IX		HM345918
	SP48-09	*I. ricinus*	Poland†	IX		HM345919
	ZL27-07	*I. ricinus*	Poland†	IX		HM345920

[a] †, this study.
[b] *, new restriction pattern obtained in this study.

TABLE 2. DNA fragments generated by digestion of the *flaB* gene amplified with 220f and 823r primers using HpyF3I and Ecl136II restriction enzymes

Borrelia species	HpyF3I		Ecl136II	
	Restriction fragment sizes (bp)	RFLP pattern[a]	Restriction fragment sizes (bp)	RFLP pattern
B. burgdorferi sensu stricto	359, 207, 38	I	387, 166, 51	Ia
Borrelia sp. SV1	359, 207, 38	I	387, 217	Ib
B. afzelii PKo type	305, 165, 92, 42	II*		
B. afzelii PGau type	397, 165, 42	III*		
B. bissettii	280, 135, 117, 72	IV*		
B. garinii 20047 type	388, 135, 72, 9	V	387, 166, 36, 15	Va
B. garinii PBr type	388, 135, 72, 9	V	387, 166, 51	Vb
B. bavariensis	388, 135, 72, 9	V	438, 166	Vc
B. valaisiana	188, 135, 117, 92, 72	VI*		
B. lusitaniae Polish type	305, 207, 92	VII*		
B. lusitaniae PotiB2 type	397, 207	VIII	387, 166, 51	VIIIa
B. spielmanii	397, 207	VIII	279, 166, 159	VIIIb
B. miyamotoi	512, 86	IX*		

[a] *, unique restriction pattern, further differentiation is not required.

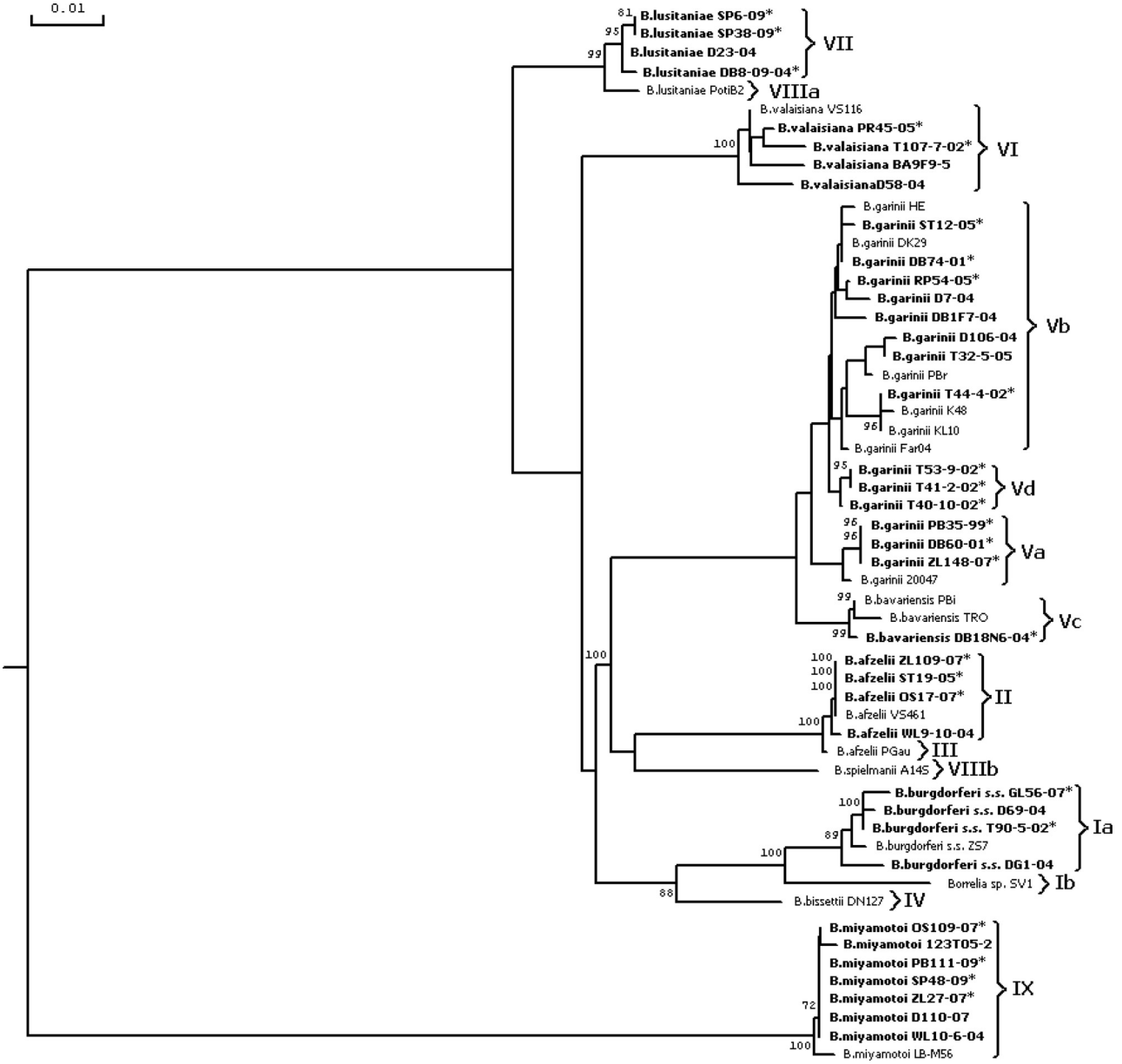

FIG. 3. Dendrogram of genetic relationships among the *flaB* DNA sequences of the European *Borrelia* strains based on PCR-RFLP analysis. The tree was constructed by a maximum-likelihood method. The values in the tree represent bootstrap results. Polish strains are in boldface. *, sequences used in this study; Ia to IX, restriction patterns.

tion patterns characteristic of different *Borrelia* species and strains. DNA sequencing was performed by dye termination cycle sequencing. Each strand was analyzed by using ABI fluorescence automated sequencers.

In order to compare the degrees of similarity of the examined samples within the restriction pattern groups, the abovementioned sequences were analyzed and presented in a dendrogram indicating the RFLP patterns. The dendrogram fully confirmed the correctness of the *Borrelia* species identification carried out using the PCR-RFLP analysis by grouping the isolates in accordance with their taxonomic classifications (Fig. 3). The analysis revealed the similarity of the strain-generated Vd pattern to *B. garinii*, as these strains made a compact cluster, distinct from *B. bavariensis* (Fig. 3). This is evidence of the conserved character of the mutations in *flaB* in each species from the *Borrelia* genus. The grouping was also coherent among the isolates identified as *B. garinii*, as all three groups constituted separate clusters.

In conclusion, the application of the discussed protocol permits 14 easily resolvable genetic variants to be obtained, based on the analysis of the *flaB* gene fragment produced with the 220f and 823r primers from the DNA of 9 *Borrelia* species transmitted by *I. ricinus*: 1 for *B. burgdorferi* sensu stricto, *B. valaisiana, B. bissettii, B. spielmanii, B. bavariensis, B. miyamotoi*, and strain *Borrelia* sp. SV1; 2 for *B. afzelii* and *B. lusitaniae*; and 3 for *B. garinii*. Thus, the method not only is a precise tool for distinguishing between the *Borrelia* species but also indicates their variability, especially in case of the most heterogeneous strains of *B. garinii*. Furthermore, the method is fast and simple and does not require the use of specialized equipment, and the results are clear and easy to interpret.

Nucleotide sequence accession numbers. *flaB* gene sequences obtained in this study were deposited in the GenBank/EMBL/DDBJ databases under accession numbers DQ650333 and HM345897 to HM345920 (Table 1).

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