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Molecular Typing of *Borrelia burgdorferi* Sensu Lato by Randomly Amplified Polymorphic DNA Fingerprinting Analysis

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To study whether pathogenic clusters of *Borrelia burgdorferi* sensu lato strains occur, we typed 136 isolates, cultured from specimens from patients ($n = 49$) with various clinical entities and from ticks ($n = 83$) or dogs ($n = 4$) from different geographic regions, by randomly amplified polymorphic DNA (RAPD) fingerprinting with four arbitrary primers. The RAPD patterns were reproducible up to the 95% similarity level as shown in duplicate experiments. In these experiments the purified DNAs prepared on different days, from different colonies, and after various passages were used as templates. With an intergroup difference of 55%, the 136 strains could be divided into seven genetic clusters. Six clusters comprised and corresponded to the established species *B. burgdorferi* sensu stricto ($n = 23$), *Borrelia garinii* ($n = 39$), *Borrelia afzelii* ($n = 59$), *Borrelia japonica* ($n = 1$), *Borrelia valaisiana* ($n = 12$), and genomic group DN127 ($n = 1$). One strain from a patient with erythema migrans (EM) did not belong to any of the species or genomic groups known up to now. The RAPD types of *B. burgdorferi* sensu stricto, *B. garinii*, and *B. afzelii* isolates, which may give rise to human Lyme borreliosis (LB), were associated with their geographic origins. A high degree of genetic diversity was observed among the 39 *B. garinii* strains, and six subgroups could be recognized. One of these comprised eight isolates from patients with disseminated LB only and no tick isolates. *B. afzelii* strains from patients with EM or acrodermatitis chronica atrophicans were not clustered in particular branches. Our study showed that RAPD analysis is a powerful tool for discriminating different *Borrelia* species as well as *Borrelia* isolates within species.

Lyme borreliosis (LB) is a tick-borne spirochetal disease endemic to regions in temperate climates throughout the world (7). The clinical spectrum of LB varies from cutaneous erythema migrans (EM) to severe arthritis, acrodermatitis chronica atrophicans (ACA), and cardiac and neurological manifestations (37). *Borrelia burgdorferi* sensu lato (19), the etiologic agent of LB, is genetically divergent. On the basis of DNA-DNA reassociation and the *Mse*I restriction enzyme patterns of the 5S-23S rRNA intergenic spacer, *B. burgdorferi* sensu lato can be divided into at least 10 different species or genomic groups: *B. burgdorferi* sensu stricto, *B. garinii*, *B. afzelii* (5, 11), *B. japonica* (20), *B. valaisiana* (43), *B. lusitaniae* (22), *B. andersonii* (25), *B. tanukii*, *B. turdi* (15, 26), and group DN127 (33). In North America, only *B. burgdorferi* sensu stricto, *B. andersonii*, and group DN127 have been identified (3, 5, 25), whereas *B. burgdorferi* sensu stricto, *B. garinii*, *B. afzelii*, *B. valaisiana*, and *B. lusitaniae* have been found in Europe (5, 11, 22, 43). *B. japonica*, *B. tanukii*, and *B. turdi* are limited to Japan (15, 20).

Not all strains from these species or genomic groups are pathogenic for humans. Up to now, only *B. burgdorferi* sensu stricto, *B. garinii*, *B. afzelii*, and group DN127 strains have been cultured from patients with LB (5, 32). Therefore, it may be that *B. japonica*, *B. valaisiana*, *B. lusitaniae*, *B. andersonii*, *B. tanukii*, and *B. turdi* are not pathogenic for humans. In addition, different species have been associated with distinct clinical manifestations of LB (4, 5, 11, 41). Arthritis is associated with *B. burgdorferi* sensu stricto infection and neuroborreliosis is associated with *B. burgdorferi* sensu stricto and *B. garinii* infection (4, 41), whereas *B. afzelii* has more frequently been cultured from skin biopsy specimens from patients with EM and ACA (10, 11, 41). However, it is not clear whether within species certain pathogenic clusters of strains which are responsible for the particular clinical syndromes of human LB occur and whether genetically distinguishable clusters causing EM, ACA, or neuroborreliosis can be found.

Randomly amplified polymorphic DNA (RAPD) analysis (46) or arbitrarily primed PCR (AP-PCR) (44), both of which use low-stringency PCR amplification with a single primer with an arbitrary sequence to generate strain-specific arrays of anonymous DNA fragments, has been used for the molecular typing of various microorganisms (39). In two studies, this technique was also used to type *B. burgdorferi*, although the procedure, including the use of radiolabelled nucleotides and analysis of the fragments on large sodium dodecyl sulfate-urea gels, was complex. In the first study, 29 *B. burgdorferi* isolates were divided by AP-PCR into three genospecific groups (45). In the second study, reported recently, the genetic diversity of 65 *B. burgdorferi* sensu stricto strains from various geographic locations in North America and Europe was also evaluated by AP-PCR (14).

In the present study we simplified and optimized RAPD analysis for the typing of *Borrelia* strains. Subsequently, we analyzed 136 *B. burgdorferi* sensu lato strains by using this method. The objectives of our study were to evaluate the usefulness of RAPD analysis as a molecular typing method for *Borrelia* strains, to identify pathogenic clusters of *Borrelia* strains causing particular clinical syndromes in patients with LB, and to assess the geographic diversity within different *Borrelia* species.

MATERIALS AND METHODS

Bacterial strains and DNA preparation. The 136 *B. burgdorferi* sensu lato strains used in this study are listed in Table 1. Forty-nine strains were isolated from human clinical specimens (of which 20 and 12 were cultured from skin

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TABLE 1. *B. burgdorferi* sensu lato strains used in this study

Species and strain	Biological source[a]	Geographic location	RAPD type[b]	RAPD cluster[c]	RFLP pattern[d]	Reference(s) or provider
B. burgdorferi sensu stricto (n = 23)						
M20	*I. ricinus*	The Netherlands	1	I	A	29
M26	*I. ricinus*	The Netherlands	1	I	A	29
M16	*I. ricinus*	The Netherlands	2	I	A	29
VS293	*I. ricinus*	Switzerland	3	I	A	30
M11	*I. ricinus*	The Netherlands	4	I	A	29
M24	*I. ricinus*	The Netherlands	4	I	A	29
M12	*I. ricinus*	The Netherlands	5	I	A	29
VS219	*I. ricinus*	Switzerland	6	I	A	30
M29	*I. ricinus*	The Netherlands	7	I	A	29
VS215	*I. ricinus*	Switzerland	8	I	A	30
M2	*I. ricinus*	The Netherlands	9	I	A	29
M48	*I. ricinus*	The Netherlands	10	I	A	29
VS130	*I. ricinus*	Switzerland	11	I	A	30
VS134	*I. ricinus*	Switzerland	11	I	A	30
A44S	Human skin (EM)	The Netherlands	11	I	A	41
A91-15	Dog	United States	12	I	A	S. Rijpkema
Hum3336	*I. pacificus*	United States	13	I	A	33
A92-1-2	Dog	United States	14	I	A	2
N40	*I. scapularis*	United States	15	I	A	13, 33
A91-10	Dog	United States	16	I	A	2
HB19	Human (blood)	United States	17	I	A	6
A91-9	Dog	United States	18	I	A	S. Rijpkema
B31	*I. scapularis*	United States	19	I	A	ATCC 35210
B. garinii (n = 39)						
NT29	*I. persulcatus*	Japan	20	IIa	C	33
Ip89	*I. persulcatus*	Russia	21	IIa	C	33
HT7	*I. persulcatus*	Japan	22	IIa	C	16
A91S	Human skin (NB)	The Netherlands	23	IIb	B	21, 41
A91C	Human (CSF)	The Netherlands	23	IIb	B	41
A76S	Human skin (CA)	The Netherlands	23	IIb	B	21, 41
A104S	Human skin (NB)	The Netherlands	23	IIb	B	This study
A94S	Human skin (MY)	The Netherlands	23	IIb	B	21, 41
A19S	Human skin (NB)	The Netherlands	24	IIb	B	21, 41
PBi	Human (CSF)	Germany	25	IIb	B	47
A01C	Human (CSF)	The Netherlands	26	IIb	B	41
HT55	*I. persulcatus*	Japan	27	IIc	B	16
JP5	*I. persulcatus*	China	28	IId	B	23
JP12	*I. persulcatus*	China	28	IId	B	Z. F. Zhang
JP2	*I. persulcatus*	China	28	IId	B	23
HT19	*I. persulcatus*	Japan	29	IId	B	16
M63	*I. ricinus*	The Netherlands	30	IIe	B	29
M45	*I. ricinus*	The Netherlands	31	IIf	B	29
PHei	Human (CSF)	Germany	32	IIf	B	47
A87S	Human skin (NB)	The Netherlands	33	IIf	B	21, 41
M3	*I. ricinus*	The Netherlands	34	IIf	B	29
TN	*I. ricinus*	Germany	35	IIf	B	47
M4	*I. ricinus*	The Netherlands	36	IIf	B	29
M64	*I. ricinus*	The Netherlands	36	IIf	B	29
M6	*I. ricinus*	The Netherlands	37	IIf	B	29
M44	*I. ricinus*	The Netherlands	38	IIf	B	29
A77S	Human skin (NB)	The Netherlands	39	IIf	B	21, 41
A77C	Human (CSF)	The Netherlands	39	IIf	B	41
VSBM	Human (CSF)	Switzerland	39	IIf	B	31
M41	*I. ricinus*	The Netherlands	40	IIf	B	29
VSBP	Human (CSF)	Switzerland	41	IIf	B	30, 31
20047	*I. ricinus*	France	42	IIf	B	33
VS102	*I. ricinus*	Switzerland	43	IIf	B	30
M8	*I. ricinus*	The Netherlands	44	IIf	B	29
T25	*I. ricinus*	Germany	45	IIf	B	47
M42	*I. ricinus*	The Netherlands	46	IIf	B	29
M59	*I. ricinus*	The Netherlands	47	IIf	B	29
PBr	Human (CSF)	Germany	48	IIf	B	47
VSDA	Human (CSF)	Switzerland	49	IIf	B	31
B. afzelii (n = 59)						
A51T	*I. ricinus*	The Netherlands	50	III	D	41
A48T	*I. ricinus*	The Netherlands	51	III	D	41
A49T	*I. ricinus*	The Netherlands	52	III	D	41
A57T	*I. ricinus*	The Netherlands	53	III	D	41
A50T	*I. ricinus*	The Netherlands	54	III	D	41
A53T	*I. ricinus*	The Netherlands	55	III	D	41
A105S	Human skin (EM)	The Netherlands	56	III	D	This study
A106S	Human skin (EM)	The Netherlands	57	III	D	This study

Continued on following page

TABLE 1—*Continued*

Species and strain	Biological source[a]	Geographic location	RAPD type[b]	RAPD cluster[c]	RFLP pattern[d]	Reference(s) or provider
A40S	Human skin (EM)	The Netherlands	58	III	D	41
A60T	*I. ricinus*	The Netherlands	59	III	D	41
A58T	*I. ricinus*	The Netherlands	60	III	D	41
A59T	*I. ricinus*	The Netherlands	61	III	D	41
A16S	Human skin (ACA)	The Netherlands	62	III	D	41
A95S	Human skin (ACA)	The Netherlands	62	III	D	This study
A116S	Human skin (EM)	The Netherlands	63	III	D	This study
A43S	Human skin (EM)	The Netherlands	64	III	D	41
A56T	*I. ricinus*	The Netherlands	65	III	D	41
PKo	Human skin (EM)	Germany	66	III	D	33, 47
A88S	Human skin (ACA)	The Netherlands	67	III	D	41
M10	*I. ricinus*	The Netherlands	68	III	D	29
A11S	Human skin (ACA)	The Netherlands	69	III	D	41
A86S	Human skin (ACA)	The Netherlands	69	III	D	41
A10S	Human skin (EM)	The Netherlands	70	III	D	41
A13S	Human skin (EM)	The Netherlands	71	III	D	41
A72T	*I. ricinus*	The Netherlands	72	III	D	41
A61T	*I. ricinus*	The Netherlands	72	III	D	41
A71T	*I. ricinus*	The Netherlands	72	III	D	41
A63T	*I. ricinus*	The Netherlands	72	III	D	41
A67T	*I. ricinus*	The Netherlands	73	III	D	41
A108S	Human skin (EM)	The Netherlands	74	III	D	This study
A64T	*I. ricinus*	The Netherlands	75	III	D	41
A68T	*I. ricinus*	The Netherlands	76	III	D	41
A65T	*I. ricinus*	The Netherlands	77	III	D	41
A20S	Human skin (EM)	The Netherlands	78	III	D	41
M55	*I. ricinus*	The Netherlands	79	III	D	29
A09S	Human skin (ACA)	The Netherlands	80	III	D	41
A02S	Human skin (EM)	The Netherlands	80	III	D	41
A84S	Human skin (EM)	The Netherlands	81	III	D	41
A03S	Human skin (EM)	The Netherlands	82	III	D	41
A21S	Human skin (EM)	The Netherlands	82	III	D	41
A69T	*I. ricinus*	The Netherlands	83	III	D	41
A70T	*I. ricinus*	The Netherlands	84	III	D	41
A62T	*I. ricinus*	The Netherlands	85	III	D	41
A110S	Human skin (ACA)	The Netherlands	86	III	D	This study
A114S	Human skin (ACA)	The Netherlands	86	III	D	This study
A117S	Human skin (EM)	The Netherlands	87	III	D	This study
A111aS	Human skin (ACA)	The Netherlands	88	III	D	This study
A111bS	Human skin (ACA)	The Netherlands	88	III	D	This study
A33S	Human skin (EM)	The Netherlands	89	III	D	41
A38S	Human skin (EM)	The Netherlands	90	III	D	41
VS461	*I. ricinus*	Switzerland	91	III	D	6, 33
A73T	*I. ricinus*	The Netherlands	92	III	D	41
A54T	*I. ricinus*	The Netherlands	93	III	D	41
A27S	Human skin (EM)	The Netherlands	94	III	D	41
A52T	*I. ricinus*	The Netherlands	95	III	D	41
A17S	Human skin (ACA)	The Netherlands	96	III	D	41
M7C[e]	*I. persulcatus*	China	97	III	D	23, 33
A66T	*I. ricinus*	The Netherlands	98	III	D	41
A26S	Human skin (ACA)	The Netherlands	99	III	D	41
B. japonica HO14 ($n = 1$)	*I. persulcatus*	Japan	100	IV	E	20, 33
B. valaisiana ($n = 12$)						
UK	*I. ricinus*	England	101	V	F	33, 43
M53	*I. ricinus*	The Netherlands	102	V	F	29, 43
M57	*I. ricinus*	The Netherlands	103	V	F	29, 43
AR-2	*I. ricinus*	The Netherlands	104	V	F	33, 43
M50	*I. ricinus*	The Netherlands	105	V	F	29, 43
M7	*I. ricinus*	The Netherlands	106	V	F	29, 43
VS116	*I. ricinus*	Switzerland	107	V	F	30, 43
M19	*I. ricinus*	The Netherlands	107	V	F	29, 43
M38	*I. ricinus*	The Netherlands	107	V	F	29, 43
M47	*I. ricinus*	The Netherlands	107	V	F	29, 43
M49	*I. ricinus*	The Netherlands	108	V	F	29, 43
M52	*I. ricinus*	The Netherlands	109	V	F	29, 43
Group DN127 25015 ($n = 1$)	*I. scapularis*	United States	110	VI	K	3, 33
Borrelia sp. strain A14S ($n = 1$)	Human skin (EM)	The Netherlands	111	VII	R	41

[a] For human skin isolates, the clinical syndromes of the patient are indicated in parentheses, as follows: NB, neuroborreliosis; CA, carditis; MY, myalgia. CSF, cerebrospinal fluid.
[b] RAPD types were defined at the 95% similarity level.
[c] RAPD clusters were defined at the 45% similarity level between genospecies, and the subclusters of *B. garinii* were defined at the 60% similarity level.
[d] All RFLP patterns except pattern R for strain A14S were described by Postic et al. (33).
[e] In earlier studies, this strain was designated M7.

biopsy specimens from patients with EM and ACA, respectively; 7 were cultured from skin biopsy specimens from patients with disseminated LB [21]; 9 were cultured from cerebrospinal fluid specimens; and 1 was from a blood specimen). Of the remaining 87 strains, 83 were isolated from *Ixodes* ticks and 4 were isolated from dogs. The origins of the isolates covered North America, Europe, and Asia. Most of these strains have previously been typed in our laboratory by reactivity with monoclonal antibodies and rRNA gene restriction analysis (41). Strains not included in previous analyses were identified as belonging to the species listed in Table 1 by PCR-restriction fragment length polymorphism (RFLP) analysis of the 5S-23S intergenic spacer (33, 43). Strains of *B. hermsii* and *B. anserina* were used as controls.

All spirochetal strains were grown in modified Kelly's medium (34) at 33°C. Three *B. burgdorferi* strains (strains A87S, A48T, and UK) were also incubated in solid culture medium to obtain pure colonies (12). DNA was extracted as described by Wilson (49), and the concentration of DNA was determined by spectrophotometry.

RAPD-PCR amplification. Initially, 16 arbitrary oligonucleotide primers, synthesized by Perkin-Elmer Applied Biosystems (Perkin-Elmer, Cheshire, United Kingdom) and available in our laboratory, were tested for their usefulness for the typing of *Borrelia* species. On the basis of the initial experiments, four primers, primers 1254 (5′-CCGCA GCCAA-3′), 1283 (5′-GCGAT CCCCA-3′), 1247 (5′-AAGAG CCCGT-3′) (1), and AP13 (5′-TTGTC TAGTG GCAAG GCT-3′), were selected and used for the typing of the *Borrelia* isolates collected.

RAPD-PCR was performed under a layer of mineral oil in a 25-μl reaction mixture containing 20 ng of purified DNA, 10 mM Tris-HCl (pH 8.8), 50 mM KCl, 4.0 mM $MgCl_2$, 0.1 mg of bovine serum albumin per ml, a 200 μM concentration of each deoxynucleotide triphosphate (Pharmacia Biotech), 1 U of AmpliTaq polymerase (Perkin-Elmer, Gouda, The Netherlands), and a 0.4 μM concentration of a single primer (0.8 μM for primer AP13). The PCR was carried out with a Biometra thermocycler (Westburg B.V., Leusden, The Netherlands) by using the following steps: initial denaturation at 94°C for 2 min, followed by 3 cycles of 94°C for 5 min, 36°C for 5 min, and 72°C for 5 min and then 30 cycles of 94°C for 1 min, 36°C for 1 min, and 72°C for 2 min and a final incubation at 72°C for 10 min. Duplicate experiments with either the same DNA template or DNA extracted on different days or from different colonies or passages were performed to assess reproducibility.

RAPD fingerprinting analysis. The amplified DNA fragments were separated on 1% (wt/vol) agarose gels (Boehringer, Mannheim, Germany) containing 10 μg of ethidium bromide per ml in the gel and in 1× Tris-acetate-EDTA buffer. For each experimental run, bacteriophage λ DNA digested with *Bst*EII and *Neisseria meningitidis* ET80 DNA amplified with primer 1254 by the same protocol were included and were used as a size marker for the amplified fragments and as a reference for the normalization of different gels, respectively. All pictures were digitized with an Iris video digitizer and were analyzed with computer software (GelCompar; Applied Math, Kortrijk, Belgium). The bands with a faint intensity which were not reproducible in duplicate experiments were excluded in the final analysis.

For each *Borrelia* strain, the DNA fingerprinting patterns obtained with the four primers were combined. These combined patterns were used for the similarity estimation and cluster analysis, in which the similarity among strains was estimated by means of the Dice comparison, and the clustering of strains was determined by the unweighted average pair group method (36) to facilitate the plotting of the dendrogram. Isolates with a level of similarity of more than 0.95 were assigned to the same RAPD type.

PCR amplification and sequencing of the 16S rRNA gene. PCR amplification and DNA sequencing of the 5′ end of the 16S rRNA gene were performed as described previously (43).

Nucleotide sequence accession numbers. The partial 16S rRNA gene sequences which we determined in this study have been assigned the following GenBank accession numbers: AF010163 (strain A01C), AF010164 (strain A76S), AF010165 (strain A87S), and AF010166 (strain M63). The accession numbers of the *B. burgdorferi* sensu lato strains which we used for comparison are as follows: U03396 (*B. burgdorferi* B31), D67018 (*B. garinii* 20047), X85199 (*B. garinii* PBi), and U78151 (*B. afzelii* VS461).

RESULTS

Reproducibility. In order to assess the reproducibility and the day-to-day variation of RAPD fingerprinting, we performed PCR reamplification with 54 of the 136 *Borrelia* strains. In these duplicate experiments we used either the same DNA template ($n = 36$) or DNA extracted on different occasions ($n = 18$). The DNA fingerprints of these strains were very reproducible. Occasionally, a discrepancy in the appearance of faint bands was seen. Usually, the intensities of these faint bands were below the limit for inclusion in the analysis. In addition, second DNA samples from 10 strains were tested by one of the investigators who had no knowledge of the origins of these samples. In these analyses, DNA samples from the same strain showed at least 95% similarity. No differences were seen between the RAPD patterns for two *Borrelia* strains (A38S and A87S) at low passage numbers (5 and 3) and high passage numbers (25 and 27) (data not shown). The RAPD patterns of four different colonies of strain A48T were identical to each other, as were the RAPD patterns of 10 different colonies of strain UK (data not shown). Four colonies of strain A87S were also highly similar to each other; however, two of these colonies lacked one band after amplification with primer 1247, leading to a similarity level of 95.8% (Fig. 1).

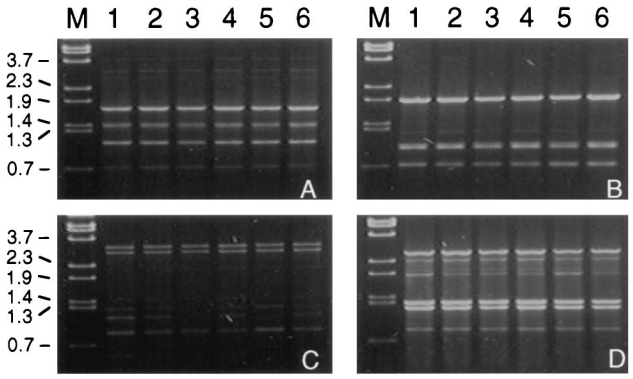

FIG. 1. Reproducibility of RAPD fingerprinting. The DNA fingerprints from different passages and different colonies of *B. garinii* A87S were obtained by PCR amplification with primers 1254 (A), 1283 (B), 1247 (C), and AP13 (D). Lanes 1 and 6, passages 3 and 27, respectively; lanes 2 to 5, four different colonies from passage 9, respectively; lane M, *Bst*EII-digested bacteriophage lambda DNA. The molecular sizes (in kilobases) are indicated on the left.

RAPD fingerprinting among species. In the combined gels obtained after four individual amplifications with different primers, the DNA fingerprints of *Borrelia* strains comprised 20 to 35 fragments with sizes ranging from 0.3 to 4.5 kb (Fig. 2). A total of 111 RAPD types, designated types 1 to 111, were identified for the 136 *B. burgdorferi* sensu lato strains (Table 1). The discrimination index of the RAPD technique calculated by application of the Simpson numerical index of diversity (18) was 0.996. Phylogenetic analysis showed that the 136 *B. burgdorferi* sensu lato strains used in this study could be divided into seven genetic clusters with an intergroup difference at least of 55% (Fig. 3). The RAPD fingerprints for representative strains of these seven clusters are included in Fig. 2. Clusters I, II, III, IV, and V corresponded to the well-known species *B. burgdorferi* sensu stricto ($n = 23$), *B. garinii* ($n = 39$), *B. afzelii* ($n = 59$), *B. japonica* ($n = 1$), and *B. valaisiana* ($n = 12$), respectively (5, 11, 20, 43). Cluster VI included one isolate from *Borrelia* genomic group DN127. The seventh cluster, with only one *Borrelia* isolate cultured from a sample from a patient in The Netherlands with EM who developed LB, has not been described previously.

All *Borrelia* strains from the three pathogenic species *B. burgdorferi* sensu stricto, *B. garinii*, and *B. afzelii* presented a 1.84-kb predominant band after amplification with primer 1254. This band was absent from *Borrelia* strains from species which were cultured only from nonhuman sources.

RAPD fingerprinting within species. Within the clusters of *B. burgdorferi* sensu stricto, *B. garinii*, and *B. afzelii*, a correlation between the geographic origins of the strains and their RAPD patterns was observed. Among the *B. burgdorferi* sensu stricto isolates, the European and North American isolates were in different clusters (Fig. 4). *B. garinii* strains from various sources exhibited high degrees of genetic diversity. At the 60% similarity level, the 39 *B. garinii* strains could be divided into six

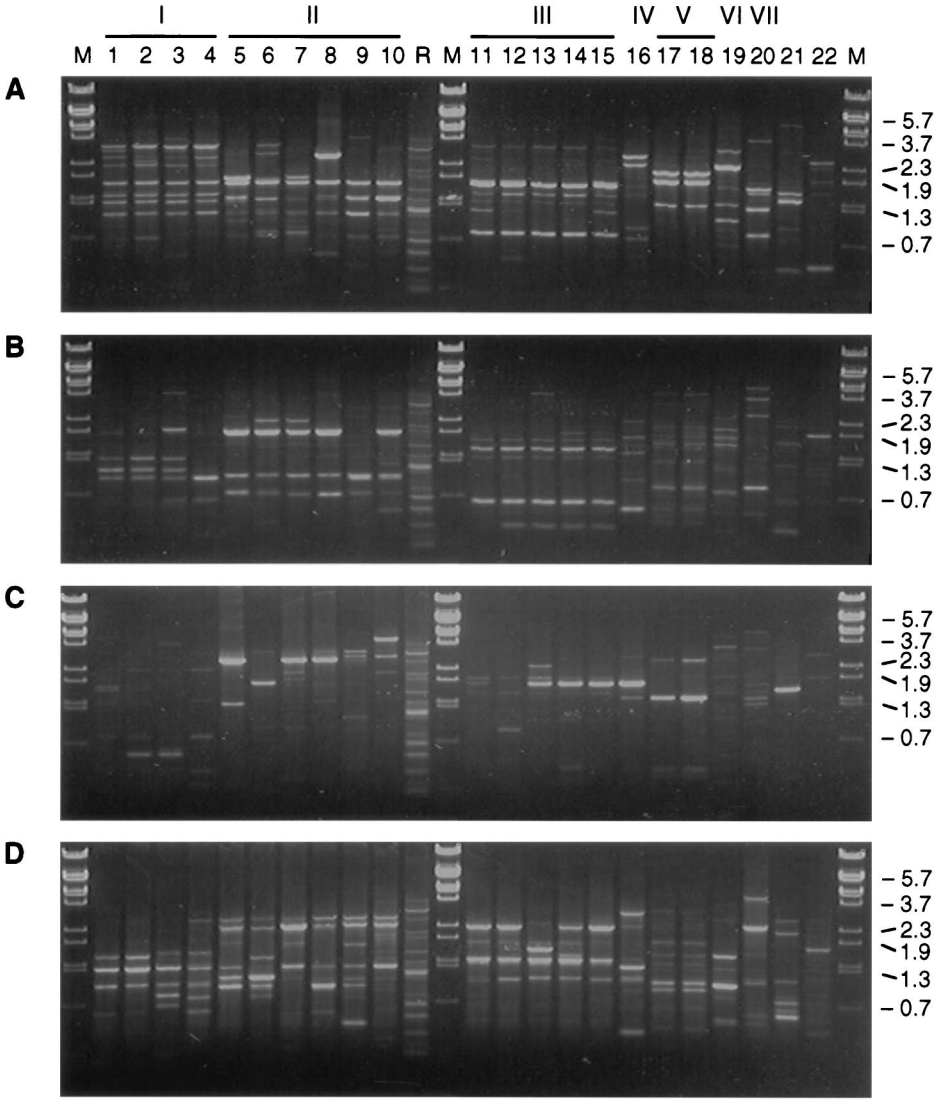

FIG. 2. RAPD fingerprints of the representative *B. burgdorferi* sensu lato strains from different species or genomic groups (RAPD clusters I to VII) obtained by using four primers as indicated in the legend to Fig. 1. Lanes 1 to 4, *B. burgdorferi* sensu stricto strains B31, A91-9, VS215, and A44S, respectively; lanes 5 to 10, *B. garinii* subgroup IIa to IIf strains NT29, PBi, HT55, HT19, M63, and 20047, respectively; lanes 11 to 15, *B. afzelii* VS461, A38S, A95S, A67T, and A71T, respectively; lane 16, *B. japonica* HO14; lanes 17 and 18, *B. valaisiana* VS116 and M19, respectively; lane 19, *Borrelia* group DN127 strain 25015; lane 20, *Borrelia* sp. strain A14S; lanes 21 and 22, *B. hermsii* and *B. anserina*, respectively; lane R, reference for normalization of different gels; lane M, *Bst*EII-digested bacteriophage lambda DNA. The molecular sizes (in kilobases) are indicated on the right.

subgroups. The eight *B. garinii* isolates from *Ixodes persulcatus* from the far eastern area of Russia, Japan, and China clustered into subgroups IIa, IIc, and IId, respectively, whereas the 30 European isolates from *Ixodes ricinus* as well as from patients were clustered into subgroups IIb and IIf (Fig. 5). Subgroup IIe contained only one strain, strain M63, which had previously been designated as an independent genomic group because of its unique rRNA restriction pattern (29). The majority (95%; 56 of 59) of the *B. afzelii* strains showed more than 70% identity in their RAPD fingerprints. However, strain M7C, originating from China (RAPD type 97), was rather different from the other strains, which were isolated from The Netherlands and Germany. Two other isolates from The Netherlands (RAPD types 98 and 99) were also distinct from the other strains (Fig. 6). Some of the *B. afzelii* tick isolates that had been collected from the same sampling site showed a tendency to cluster. Of the 12 tick isolates from Santpoort, in the dunes in the western part of The Netherlands, 9 clustered together into two groups (RAPD types 50 to 55 and 59 to 61, respectively), but 3 strains were divergent. A similar result was obtained for 13 tick isolates from a forest in Drenthe, in the northern part of The Netherlands: 11 isolates grouped into two subclusters (RAPD types 72 to 77 and 83 to 85, respectively) and 2 were unrelated (Fig. 6).

RAPD types among isolates from patients. Of the 49 *Borrelia* strains from human specimens, 48 were typed by RAPD analysis as *B. burgdorferi* sensu stricto ($n = 2$), *B. garinii* ($n = 16$), or *B. afzelii* ($n = 30$). These results were consistent with the classification of these strains as designated in Table 1. Only one strain, strain A14S, could not be classified by RAPD analysis into any of the six LB-related species or genomic groups included in this study. This strain also had a quite remarkable

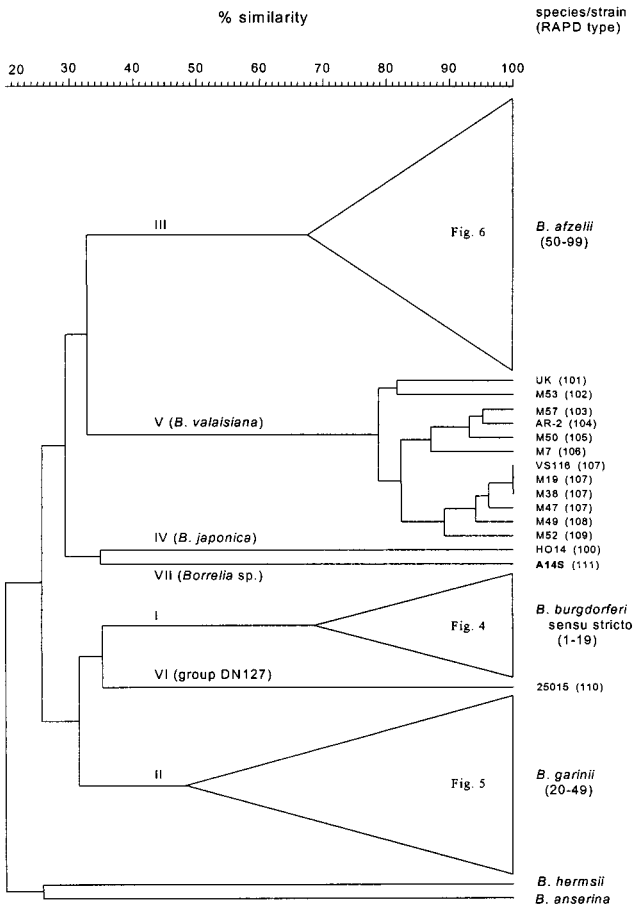

FIG. 3. Simplified dendrogram of *B. burgdorferi* sensu lato strains. The 136 LB-associated *Borrelia* strains used in this study could be divided into seven clusters with an intergroup difference of 55%. Six of them (clusters I to VI) corresponded to the indicated established species or genomic groups. One strain (branch VII) could not be classified into one of the described LB-related *Borrelia* species. Isolates from patients are in boldface. The numbers in parentheses indicate the corresponding RAPD type of each strain.

PCR-RFLP pattern: instead of a 246- to 255-bp fragment, a fragment of 225 bp was amplified. Digestion of this fragment with *Mse*I resulted in a unique pattern, which was designated pattern R (Table 1). This pattern was not consistent with any pattern produced by the 10 LB-related *Borrelia* species or genomic groups identified up to now (unpublished data). The 16 *B. garinii* strains, which were all isolated from patients with extracutaneous syndromes, were found in subgroups IIb and IIf. Isolates from human skin biopsy specimens and cerebrospinal fluid were evenly distributed among both subgroups. Interestingly, subgroup IIb included only eight isolates from patients with disseminated LB and showed little heterogeneity. Isolates from both humans and ticks were found in subgroup IIf, with no particular clustering for the isolates from humans. For *B. afzelii*, isolates from patients ($n = 30$) did not differ from those from ticks ($n = 29$). Furthermore, no significant subcluster was found among isolates recovered from patients with EM ($n = 18$) and ACA ($n = 12$).

Further characterization of *B. garinii* subgroup IIb. Since *B. garinii* subgroup IIb only contained isolates from humans, further characterization of this subgroup was performed by sequence analysis of the 16S rRNA gene. Partial 16S rRNA gene analysis of three isolates in this subgroup (isolates PBi, A01C, and A76S) showed the two conserved nucleotide substitutions at positions 469 and 635 (*B. burgdorferi* B31 numbering) (17) in comparison to the *B. garinii* consensus sequences (24) and to sequences from *B. burgdorferi* sensu stricto and *B. afzelii*, confirming that strains in this subgroup are genetically distinct from other *B. garinii* strains.

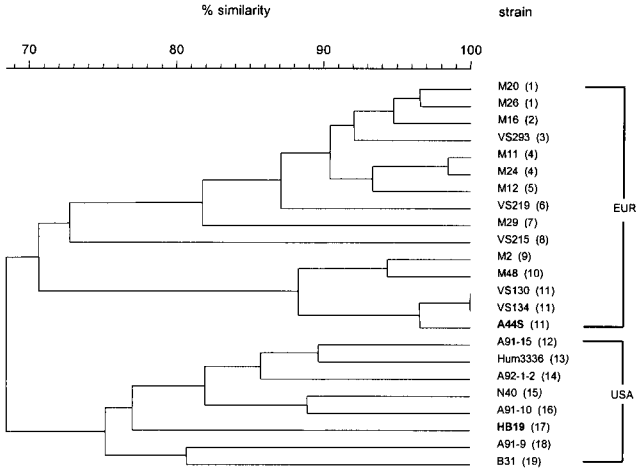

FIG. 4. Dendrogram of *B. burgdorferi* sensu stricto strains ($n = 23$). The *Borrelia* isolates derived from North America and Europe clustered into separate branches.

DISCUSSION

RAPD analysis has been used with increasing frequency as a method for the molecular typing and genetic characterization

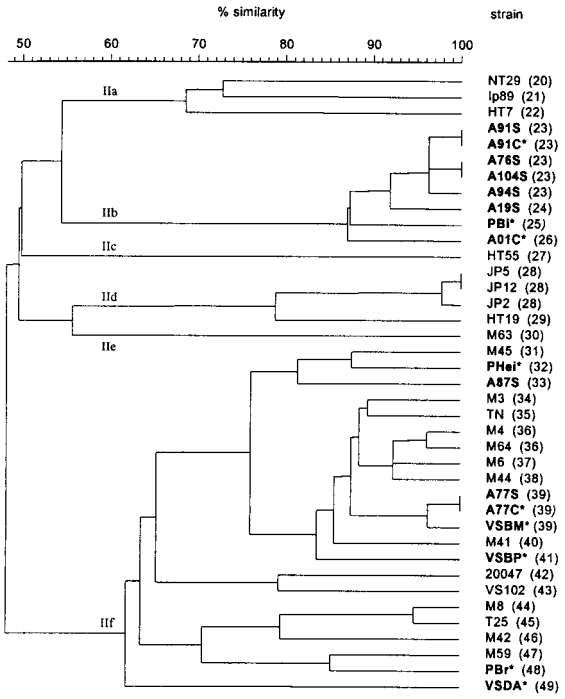

FIG. 5. Dendrogram of *B. garinii* strains ($n = 39$). The asterisks indicate strains from the cerebrospinal fluid of patients. See the legend to Fig. 3 for more information.

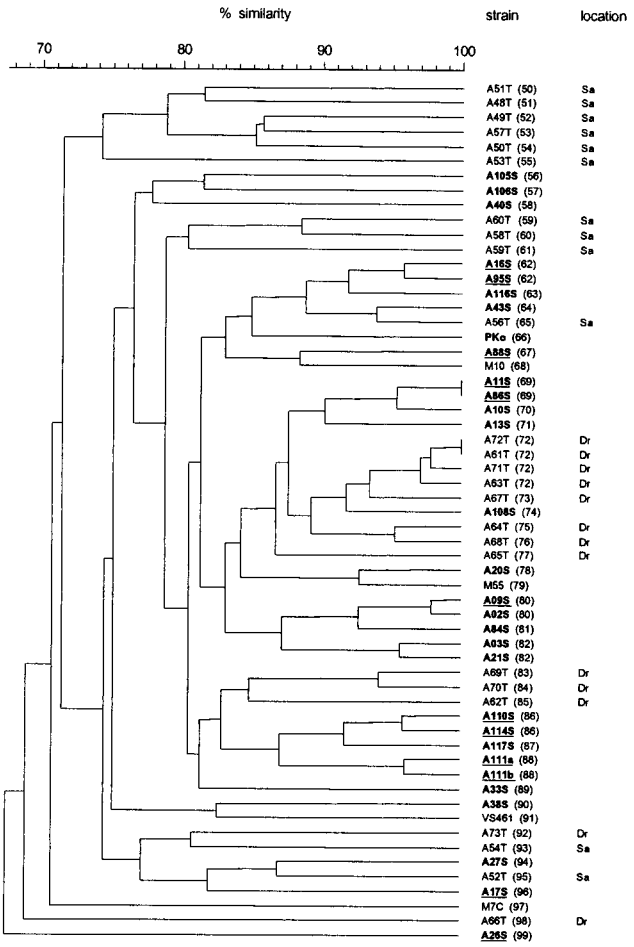

FIG. 6. Dendrogram of *B. afzelii* strains from different geographic and biological sources (n = 59). Tick isolates from two different regions in The Netherlands are indicated. Isolates from patients with ACA are underlined. Sa (Santpoort) and Dr (Drenthe) are two geographic regions in the western and northern parts of The Netherlands, respectively. See the legend to Fig. 3 for more information.

of various microorganisms (38, 39). However, considerable attention is directed to its reliability and reproducibility, since many studies have indicated that various factors can affect the results of RAPD fingerprinting (8, 28, 38, 40). In our experiments, optimal RAPD fingerprinting was found with the four primers that we selected and purified DNA. With these four different primers, the reproducibility of the RAPD fingerprints for a random subset of 54 of the 136 isolates was up to at least a 95% level of similarity. The number of passages did not affect the results of RAPD analysis. However, PCR amplification of different colonies of one strain resulted in a difference of one band, amplified by one of the primers. This band was absent from two colonies and was present in two other colonies. Whether this difference is caused by the presence of a mixed population of two related strains or by colonial variation within one strain must be elucidated. Since these RAPD fingerprints from different colonies still showed more than 95% similarity to each other and belonged to the same RAPD type according to our definition, we concluded that RAPD analysis is an appropriate method for the typing of uncloned *Borrelia* strains.

By RAPD fingerprinting 135 of the 136 *B. burgdorferi* sensu lato strains used in this study could be grouped into six different species or genomic groups. This result was consistent with our classification of these strains on the basis of rRNA gene restriction analysis (41) or PCR-RFLP. One strain, strain A14S, could not be classified into one of the six species or genomic groups included in this study and also had a highly divergent PCR-RFLP pattern. This strain has pathogenic potential, since it was originally isolated from a patient with EM.

In our study, strains belonging to the same species but originating from different geographical regions clustered into separate branches by RAPD analysis. Regarding *B. burgdorferi* sensu stricto, North American and European strains fell into separate subgroups. With the exception of one *B. garinii* strain, *B. garinii* strains from Europe clustered into two major subbranches. All eight *B. garinii* strains from far east Asia were clearly different from the European strains included in these two major subbranches. Among the *B. afzelii* strains, the heterogeneity was limited. Only 2 of the 56 Dutch strains and the one Chinese strain tested were more divergent than the majority of the Dutch strains. Since only one Chinese strain was studied, more strains from Asia should be typed by RAPD fingerprinting before a conclusion can be drawn as to whether *B. afzelii* strains from European and Asian sources differ. Regional differences among North American *B. burgdorferi* sensu stricto strains were also found with the use of pulsed-field gel electrophoresis and sequence analysis (27). Another recent study in which pulsed-field gel electrophoresis as well as the AP-PCR technique was used showed that *B. burgdorferi* sensu stricto strains could be subdivided into a number of subgroups, generally consisting of only North American or only European strains (14); however, a few North American strains, including strain B31, clustered with the European strains and vice versa. In contrast, in our study B31 clustered only with North American strains. Since Foretz et al. (14) studied different European and North American isolates, this may partly account for differences between the studies.

B. afzelii strains grown from ticks collected in a limited area were often quite similar to each other, although we also found some exceptions. This is in accordance with the assumption that the migration rate of the vectors of *B. afzelii*, presumably being ticks and rodents, is rather low. It would be interesting to study a large collection of *B. garinii* strains in the same way, since birds are thought to be involved in the transmission of these spirochete species (9), and much more diversity of the strains within one area may occur because of this route of transmission.

Among the *B. garinii* strains, one cluster of eight strains (subgroup IIb) consisted only of isolates from humans. The strains were closely related to each other and were markedly different from the other *B. garinii* strains tested. The three subgroup IIb strains for which 16S rRNA gene sequencing was performed differed from the other *B. garinii* strains. All eight isolates in subgroup IIb originated from patients with disseminated disease. In another study, we showed that these strains all belonged to OspA type 4, as defined by Wilske et al. (47), and that they were resistant to the activity of normal human serum, in contrast to other *B. garinii* strains (42). Interestingly, these type 4 *B. garinii* strains have only been recovered from human specimens until now (48), and therefore, they may have an increased pathogenic potential.

The RAPD patterns of *B. afzelii* strains from ticks and humans were randomly distributed. In addition, strains from patients with ACA were not different from strains from patients with EM. This is in accordance with a recent study in which pulsed-field gel electrophoresis analysis of strains from patients with EM or ACA did not reveal differences between these groups (10). Therefore, those *B. afzelii* strains causing

ACA have minor differences in their genomes in comparison with other *B. afzelii* strains. Alternatively, the persistence of these spirochetes, resulting in ACA, may mainly be determined by host factors.

In conclusion, RAPD analysis is a reliable technique for identifying the different *Borrelia* species, as well as for discriminating between strains within *Borrelia* species. Further use of this technique may lead to more information about the pathways of the geographic spread of the spirochetes. In addition, if more pathogenic subgroups exist, these may also be identified by this technique, which can be easily and rapidly performed after the isolation of a *Borrelia* strain.

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