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Phylogenesis of Relapsing Fever *Borrelia* spp.

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The phylogenetic relationships of 20 relapsing fever (RF) *Borrelia* spp. were estimated on the basis of the sequences of *rrs* genes. Complete sequences were aligned and compared with previously published sequences, and the similarity values were found to be 97.7 to 99.9%. Phylogenetic trees were constructed by using the three neighbor-joining, maximum-parsimony, and maximum-likelihood methods. The results of the comparative phylogenetic analysis divided the RF *Borrelia* spp. into three major clusters. One cluster included *Borrelia crocidurae*, *Borrelia duttonii*, *Borrelia recurrentis*, and *Borrelia hispanica*. Another cluster comprised two main branches with *Borrelia coriaceae*, *Borrelia lonestari*, and *Borrelia miyamotoi* on one side and *Borrelia parkeri*, *Borrelia turicatae*, and *Borrelia hermsii* on the other side. *Borrelia anserina* constituted the third cluster. The phylogenetic position of *Borrelia persica* was more uncertain. These results suggested that the taxonomy of these spirochetes should be revised. To overcome the problems of culturing the spirochetes, RF *Borrelia* primers were defined. Following PCR amplification of the *rrs* gene, restriction length fragment polymorphism could be used to distinguish between RF *Borrelia* strains.

The genus *Borrelia* comprises the arthropod vector-transmissible spirochetes. These organisms are responsible for two groups of human disease: lyme borreliosis, which is transmitted by the hard *Ixodes* ticks (6), and relapsing fevers (RF), most of which are transmitted by soft ticks (Argasidae) (the exception is louse-borne RF, which is transmitted by *Pediculus humanus*) (5, 10, 24, 30).

Usually, when strictly parasitic organisms have been considered, their taxonomy has been based on the cospeciation concept. However, this does not apply to *Borrelia burgdorferi* sensu lato, since DNA-DNA reassociation studies have shown that *B. burgdorferi* sensu lato constitutes a complex of at least eight genomospecies (28), including five species which have been named (2, 7, 18, 21). The members of this complex have a broad spectrum of hosts and do not exhibit specific associations with vector species (for instance, *B. burgdorferi* sensu stricto can be transmitted by both *Ixodes scapularis* [formerly *Ixodes dammini*] and *Ixodes ricinus*, and the latter tick is also able to transmit *Borrelia garinii*, *Borrelia afzelii*, and genomic groups PotiB2 and VS116).

RF-associated *Borrelia* species have been named on the basis of the cospeciation concept, taking into account the geographic endemicity of the vectors. Some of these organisms have a worldwide distribution: *Borrelia recurrentis* is transmitted by human body lice (10), and *Borrelia anserina* is restricted to birds and *Argas* ticks. Other species are geographically restricted. The specificity of the association between *Borrelia* species and vectors has been questioned, as *Borrelia duttonii*, *Borrelia crocidurae*, and *Borrelia hispanica*, which are naturally transmitted in nature by *Ornithodoros moubata*, *Ornithodoros sonrai* (formerly *Ornithodoros erraticus sonrai*), and *Ornithodoros erraticus* (formerly *Ornithodoros erraticus erraticus*), respectively, could be experimentally adapted to lice (10). These RF *Borrelia* species have fastidious cultural requirements, and neither phenotypic characteristics nor genomic data (from DNA-DNA reassociation studies) are available for them, although cultivation of a strain of *B. recurrentis* was recently reported by Cutler et al. (9). In contrast, *Borrelia hermsii*, *Borrelia turicatae*, and *Borrelia parkeri* are easily cultivable. Characterization of RF *Borrelia* species by serotyping is not possible because of the phenomenon of antigenic variation (23, 27). To date, cross-immunity trials and the use of animal models have been the only methods for clarifying the identities of isolates (32). Taxonomic studies have not been possible previously.

Recently, the following new *Borrelia* species have been isolated from hard ticks: *Borrelia miyamotoi*, which was isolated from both *Ixodes persulcatus* and rodents in Japan (13); and *Borrelia lonestari*, which was isolated from *Amblyomma americanum* in the United States (4). Despite the association of these two *Borrelia* species with hard ticks, their *rrs* sequences appear to be more similar to the previously available RF *Borrelia rrs* sequences than *B. burgdorferi* sensu lato sequences are (4).

To study the phylogenetic relationships of RF *Borrelia* species, we sequenced the *rrs* genes from a variety of isolates. On the basis of the sequences obtained and restriction fragment length polymorphism profiles of PCR products, we were able to genetically identify RF *Borrelia* species.

MATERIALS AND METHODS

Culture. The RF *Borrelia* strains used in this study are listed in Table 1. *B. hermsii*, *B. parkeri*, *B. turicatae*, *B. coriaceae*, *B. anserina*, and *B. recurrentis* were grown in BSK II medium at 30°C (3).

Animal passages. All strains of *B. crocidurae* and *B. duttonii* were maintained by serial passage in 10-week-old female Swiss mice (obtained from IFFA-CREDO) weighing 28 to 30 g. The mice were inoculated intraperitoneally with 0.3 to 0.4 ml of infected blood preserved in liquid nitrogen with 10% dimethyl sulfoxide or with infected brain tissue from a mouse inoculated 1 month or more earlier. Spirochete counts were determined daily by using dark-field microscopy, until the optimal bacterial concentration was reached. The maximal yield of organisms, 10^6 to 10^7 organisms per ml of blood, was usually achieved 3 days after inoculation of infected blood or 6 days after inoculation of infected brain tissue. The animals were anesthetized with pentobarbital and bled immediately by puncture of the vena cava.

B. hispanica and *Borrelia persica* strains were maintained by passage in male guinea pigs (obtained from IFFA-CREDO) weighing 300 to 350 g. The animals were inoculated with 0.4 to 0.5 ml of infected blood preserved in liquid nitrogen

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TABLE 1. Sources and nucleotide sequence accession numbers of RF *Borrelia* strains used in this study

Species	Strain	Source	Geographic origin (date of isolation)	GenBank accession no.
B. hermsii	HS1^T (= ATCC 35209^T)	*Ornithodoros hermsi*	United States	U42292
B. parkeri	M3001	*Ornithodoros parkeri*	United States	U42296
B. turicatae	M2007	*Ornithodoros turicata*	United States	U42299
B. anserina	ES-1	*Argas persicus*		U42284
B. coriaceae	Co53^T (= ATCC 43381^T)	*Ornithodoros coriaceus*	United States	U42286
B. recurrentis	A1	Human blood	Ethiopia	U42300
B. persica	UESV/340	*Ornithodoros tholozani*	Iran (1974)	U42297
B. hispanica	UESV/246	*Ornithodoros erraticus*	Morocco	U42294
B. crocidurae	UESV/ACH	*Ornithodoros sonrai*	Mauritania (1968)	U42283
	UESV/626BAN	*Ornithodoros sonrai*	Senegal (1978)	U42285
	UESV/917BAR	Human blood	Mali (1988)	U42287
	UESV/1040DAK 24	*Mastomys erythroleucus*	Senegal (1989)	U42289
	UESV/1043DAK 28	*Mastomys erythroleucus*	Senegal (1989)	U42295
	UESV/1045DAK 33	*Mastomys erythroleucus*	Senegal (1989)	U42290
	UESV/MER	*Ornithodoros sonrai*	Morocco	U42291
	UESV/523SIS	Human blood	Mali (1977)	U42301
	UESV/1096TEN	Human blood	Senegal (1991)	U42302
B. duttonii	UESV/117DUTT	*Ornithodoros moubata*	Zaire (1949)	U42288
	UESV/334RWA	*Ornithodoros moubata*	Rwanda (1975)	U42298
	UR/BD94MIT	*Ornithodoros moubata*	Zaire (1994)	U42293

with 10% dimethyl sulfoxide. Blood was removed by intracardiac puncture approximately 3 days after infection by *B. persica* and 4 days after infection by *B. hispanica*.

When growth levels were low, multiple passages were required to reach the optimal concentration. Heparin, which interferes with the PCR (16), was avoided when infected blood samples were collected. Therefore, blood was allowed to clot, and the serum was separated by centrifugation and used for PCR experiments.

DNA preparation. DNAs from noncultivable RF *Borrelia* strains were extracted from 200 μl of serum by using a QIA ampBlood kit (Qiagen) according to the manufacturer's instructions.

Portions (2 ml) of cultures of *B. hermsii*, *B. turicatae*, *B. parkeri*, *B. anserina*, *B. coriaceae*, and *B. burgdorferi* sensu lato were centrifuged at 10,000 rpm, and the resulting pellets were washed in phosphate-buffered saline and resuspended in 100 μl of distilled water before the preparations were boiled at 95°C for 10 min. These suspensions were stored at −20°C until they were used for PCR experiments.

DNA from *B. recurrentis* was phenol extracted. Cells harvested from BSK II medium were incubated for 3 h at 56°C in lysis buffer containing 10 mM Tris (pH 8), 60 mM EDTA (pH 8), 5% sodium dodecyl sulfate, and 50 μg of proteinase K per ml. DNA was phenol extracted, ethanol precipitated, and resuspended in distilled water. Following a 30-min incubation at 37°C with RNase A (2 μg/ml), the DNA was again ethanol precipitated and resuspended in water, and then it was stored at −20°C until it was used.

PCR methods. All of the primer sets used in this study are listed in Table 2. Each PCR mixture (50 μl) contained 5 μl of heated bacterial suspension or 5 ng of DNA, 10 mM Tris-HCl, 1.5 mM $MgCl_2$, 50 mM KCl, 0.01% gelatin, each of the four deoxynucleoside triphosphates at a concentration of 200 μM, 1.25 U of *Taq* polymerase (Amersham International, Amersham, England), and each of the primers at a concentration of 5 μM. The reaction mixtures were overlaid with

TABLE 2. Primers used for PCR amplification and sequencing of RF *Borrelia* 16S ribosomal DNAs

Primer	Sequence (5′ to 3′)	Position (*E. coli* numbering)	Designed for:	Hybridization temp (°C)
PCR primers				
fD3[a]	AGAGTTTGATCCTGGCTTAG	8-27	Eubacteria	
UniB	T(AC)AAGGAGGTGATCCAGC	1582-1565	Eubacteria	
T50[b]	GTTACGACTTCACCCTCCT	1540-1522	Spirochetes	
REC4	ATGCTAGAAACTGCATGA	659-675	RF *Borrelia* spp.	
REC9	TCGTCTGAGTCCCCATCT	1191-1174	RF *Borrelia* spp.	
Sequencing primers				
fD3[a]	AGAGTTTGATCCTGGCTTAG	8-27		53
250F	CTTATTAGCTAGTTGGTAGG	263-282		50
250R	CCTACCAACTAGCTAATAAG	282-263		50
400F	GGAGCGACACTGCGTG	414-429		50
500R	CTGCTGGCACGTAATTAGCC	548-529		57
590R	ATATCCGCCTACTCA	616-602		40
800F	ATTAGATACCCTGGTAG	812-828		50
800R	CTACCAGGGTATCTAAT	828-812		50
900F	GAGTATGCTCGCAAGAGT	887-904		50
900R	ACTCTTGCGAGCATACTC	930-913		50
1050F	TGTCGTCAGCTCGTG	1095-1109		43
1050R	CACGAGCTGACGACA	1109-1095		43
1200F	TATGTCCTGGGCTACACACG	1248-1267		57
1200R	CGTGTGTAGCCCAGGACATA	1267-1248		57
rD1[a]	AAGGAGGTGATCCAGCC	1580-1564		50

[a] Data from reference 40.
[b] Data from reference 25.

mineral oil (50 μl). Amplification was carried out for 35 cycles consisting of denaturation at 93°C for 1 min, annealing at 4°C below the denaturation temperature of the primer used for 1 min, and extension at 72°C for 1 min, and there was a final extension step consisting of 7 min at 72°C.

Negative controls were included in all experiments. Reaction mixtures were prepared, and the samples were added with positive-displacement pipettes dedicated for the purpose. The PCR mixtures were prepared in a room where no DNA was handled.

The PCR products were detected by electrophoresis in a 0.8% agarose gel in TBE, followed by staining with ethidium bromide.

Direct sequencing of amplified fragments. The *rrs* gene was sequenced by the method of Sanger et al. (35).

DNA amplification was performed by using the primer set consisting of primers fD3 and UniB, which was chosen on the basis of previously published 16S ribosomal DNA sequences (40). The amplified product was purified by using Microspin S-400 HR columns (Pharmacia Biotech, Uppsala, Sweden) as recommended by the manufacturer.

The 5′ fluorescein-labeled primers (Table 2) chosen were primers in conserved regions and were selected after the known sequences of the *rrs* genes of *Borrelia* species were aligned.

Sequencing reaction mixtures were prepared by using an Amplicycle sequencing kit (Perkin-Elmer Cetus, Norwalk, Conn.) and the conditions recommended by the manufacturer. Sequencing reactions were carried out under two distinct conditions, depending on the hybridization temperature requirements of the primers (Table 2). When the hybridization temperature was >50°C, an initial denaturation step at 95°C for 3 min was followed by 25 cycles consisting of denaturation at 95°C for 30 s, annealing at the primer temperature for 30 s, and extension at 72°C for 1 min; the amplification reaction was completed by incubation for 5 min at 72°C to allow complete extension of the PCR products. When the hybridization temperature was <50°C, an initial denaturation step at 95°C for 3 min was followed by 30 cycles consisting of denaturation at 95°C for 30 s, annealing at the primer temperature for 30 s, and extension at 60°C for 2 min; 10 supplementary cycles of denaturation at 95°C for 10 min and extension at 60°C for 90 s were performed to increase polymerization.

A model 9600 thermocycler (Perkin-Elmer Cetus) and 6% polyacrylamide gels in a model ALF automatic sequencer (Pharmacia) were used for the sequencing reactions.

Sequence alignments and phylogenetic inferences. Sequences were aligned both manually with VSM software V.2.0 written by B. Lafay and R. Christen as described by Ruimy et al. (33) and with the multisequence alignment program Clustal V (14).

Phylogenetic relationships were analyzed by using three methods, and the results were compared. Parsimony analysis was performed with the PAUP program (phylogeny analysis using parsimony) (38). Evolutionary distances were computed with the Jukes-Cantor option (34) by using both the PHYLIP program package written by Felsenstein (11) and MEGA software (19). Phylogenetic trees were constructed by using the Kitsch+ genetic distance algorithm from distance matrix data and by using the neighbor-joining method (34) in MEGA. The robustness of the topologies was estimated through 100 bootstrap replications. For the maximum-likelihood analysis, we used the PHYLIP program package.

Restriction polymorphism of the amplified 16S DNA fragment. On the basis of the sequence data, enzyme restriction sites that could be used to rapidly identify RF *Borrelia* species were chosen. Endonucleases *Mse*I, *Bfa*I (New England Biolabs, Beverly, Mass.) (20 U/μg of DNA) and *Hph*I (Amersham) were used to digest 10 μl of PCR product.

Restriction fragments were resolved on a 10% acrylamide gel and/or a 16% acrylamide gel depending on the size of the expected DNA fragments. A molecular weight standard (pBR322 digested by *Hin*fI) was included in each gel.

Nucleotide sequence accession numbers. The 16S DNA sequences of *B. miyamotoi* HT31^T (T = type strain) (accession number D45192) *B. lonestari* (accession number U23211), and *B. burgdorferi* sensu stricto B31^T (accession number X57404) were obtained from the GenBank database. The accession numbers for 16S ribosomal DNA sequences determined in this study are shown in Table 1.

RESULTS

Sensitivity and specificity of the PCR. The sensitivity of the PCR assay was assessed by using serial dilutions of cultures containing 10^8 bacteria per ml. When primers fD3 and UniB were used, the PCR detected 15 spirochetes. Primers fD3 and T50 had a level of sensitivity of 500 bacteria. The sensitivity of the PCR with mouse sera was assessed by using 1 ml of mouse blood spiked with 10^8 *B. hermsii* cells. The results obtained were comparable to those obtained with bacterial cultures.

The specificities of the diverse primer sets were determined by using DNAs from the RF *Borrelia* species listed in Table 1, DNAs from representative strains of each species belonging to *B. burgdorferi* sensu lato, DNAs from *Serpulina hyodysenteriae*, *Leptospira interrogans*, and *Treponema denticola*, and DNAs from members of unrelated bacterial genera, including *Helicobacter pylori*, *Campylobacter fetus*, *Streptococcus* sp., *Staphylococcus aureus*, *Bacillus* sp., *Listeria monocytogenes*, *Corynebacterium* sp., *Salmonella typhimurium*, *Yersinia enterocolitica*, and *Escherichia coli*.

The use of primers fD3 and T50 resulted in amplification of a 1,487- or 1,489-bp DNA fragment specific for spirochetes, and the use of primers REC4 and REC9 led to amplification of a 523-bp DNA fragment specific for RF *Borrelia* species (data not shown).

Sequence analysis. Amplification of DNA with primers fD3 and UniB resulted in products that were about 1.5 kb long. These fragments were used to sequence the *rrs* gene. The sequences corresponding to positions 8 to 1,576 (*E. coli* numbering) were aligned manually and by using Clustal V software, with the same results. The levels of sequence similarity were calculated on the basis of 1,338 nucleotides common to all *rrs* sequences. The levels of similarity between RF *Borrelia* species ranged from 97.7 to 99.9% (Table 3). The levels of sequence similarity between *rrs* sequences of RF *Borrelia* species and *rrs* sequences of *B. lonestari* and *B. miyamotoi* ranged from 96.4 to 98.1%. The nine strains of *B. crocidurae* exhibited 100% sequence identity, as did three strains of *B. duttonii*. The highest level of similarity between "species" was the level of similarity between the sequences of *B. crocidurae* and *B. duttonii* (only one nucleotide difference). The lowest level of similarity, 97.7%, was the level of similarity between the sequences of *B. persica* and *B. anserina*.

Sequences were analyzed by using both distance methods and a character state method (parsimony analysis). Figure 1 shows the phylogenetic relationships among various taxa as determined by the neighbor-joining method. The RF *Borrelia* species were subdivided into three major clusters. One cluster included *B. crocidurae*, *B. duttonii*, *B. recurrentis*, and *B. hispanica*. *B. persica* appeared to be on the border of this group. A second cluster comprised two main branches, with *B. coriaceae*, *B. lonestari*, and *B. miyamotoi* on one side and *B. parkeri*, *B. turicatae*, and *B. hermsii* on the other side. However, *B. hermsii*, although recently shown by DNA relatedness studies to be closely related to *B. parkeri* and *B. turicatae* (15), was on a separate branch. *B. anserina* was a single distantly related group. The results obtained when the Kitch+ algorithm was used (data not shown) were consistent with the results obtained by other distance methods.

Figure 2 shows the 50% majority rule tree based on 12 trees obtained by using the parsimony analysis technique in the PAUP package. This tree was constructed by using the branch-and-bound option with *B. burgdorferi* as an outgroup. In this analysis the same main phylogenetic groups were distinguished, except that *B. persica* was more peripheral than it was with the distance method. The same topology was obtained by using the maximum-likelihood method (data not shown).

Restriction fragment length polymorphism. The PCR primers fD3 and T50 generated 1,487- to 1,489-bp DNA fragments specifically from spirochete DNA. These PCR products were digested by *Mse*I, *Bfa*I, and *Hph*I. The results of an analysis of the restriction polymorphism are shown in Fig. 3.

After restriction by *Mse*I, six patterns were observed (Fig. 4). *B. anserina*, *B. coriaceae*, *B. recurrentis*, and *B. persica* each exhibited a specific pattern. *B. duttonii*, *B. crocidurae*, and *B. hispanica* exhibited a common pattern. The strains of *B. hermsii*, *B. parkeri*, and *B. turicatae* similarly exhibited a common pattern.

B. hermsii could be differentiated from *B. parkeri* and *B. turicatae* after restriction by *Hph*I (Fig. 5). *B. parkeri* could also

TABLE 3. Levels of similarity for domains including all nucleotides aligned without ambiguity

Species	% Similarity											
	B. crocidurae	*B. duttonii*	*B. recurrentis*	*B. hispanica*	*B. persica*	*B. parkeri*	*B. turicatae*	*B. hermsii*	*B. coriaceae*	*B. anserina*	*B. lonestari*	*B. miyamotoi*	*B. burgdorferi*
B. crocidurae	100	99.9	99.7	99.8	98.7	99.2	99.2	98.9	98.9	98.5	97.7	97.3	89.9
B. duttonii		100	99.8	99.8	98.6	99.1	99.2	98.9	98.9	98.4	97.6	97.2	90
B. recurrentis			100	99.5	98.4	98.9	98.9	98.6	98.6	98.2	97.4	97	89.7
B. hispanica				100	98.7	99.2	99.2	98.9	98.9	98.5	97.7	97.3	90
B. persica					100	98.2	98.3	98	98.3	97.7	96.5	96.4	89.8
B. parkeri						100	99.9	99.1	98.9	98.3	98	97.6	90.2
B. turicatae							100	99.2	98.9	98.4	98	97.7	90.2
B. hermsii								100	98.6	98.1	97.5	97.5	90
B. coriaceae									100	98	97.8	97.4	89.7
B. anserina										100	96.8	97	84.1
B. lonestari											100	98.1	83.7
B. miyamotoi												100	83.7
B. burgdorferi													100

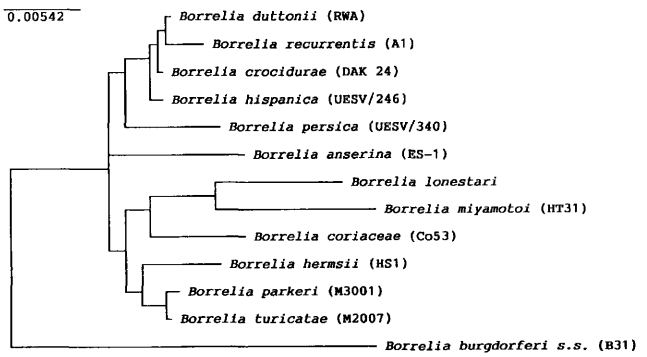

FIG. 1. Phylogenetic tree based on a comparison of the 16S rRNA sequences of RF *Borrelia* species. The branching pattern was generated by the neighbor-joining method.

be differentiated from *B. hermsii* and *B. turicatae* after restriction digestion by *Bfa*I (data not shown).

No restriction site which could be used to distinguish among the three species belonging to the *B. duttonii-B. crocidurae-B. hispanica* cluster was identified.

DISCUSSION

Phylogenetic relationships of RF *Borrelia* species. As traditional methods for bacterial characterization are not appropriate for RF *Borrelia* species, these spirochetes have been classified on the basis of geographical and epidemiological features. In addition, their fastidious culture requirements have prevented the use of DNA relatedness studies now used for taxonomic purposes. PCR amplification of *rrs* genes overcomes the need for large amounts of DNA, and subsequent sequencing studies provide the tools to review the phylogenetic position of these spirochetes.

To determine the evolutionary and taxonomic positions of RF *Borrelia* species, *rrs* gene sequences from 20 strains were compared. The levels of divergence among representative *rrs* sequences reflect the time taken by the strains studied to evolve from their common ancestor. The weak resolving power of 16S rRNA sequences for species delineation has been evident when closely related organisms have been compared (12, 37). DNA relatedness studies provide a measure of whole-genome similarity and therefore are a more accurate method

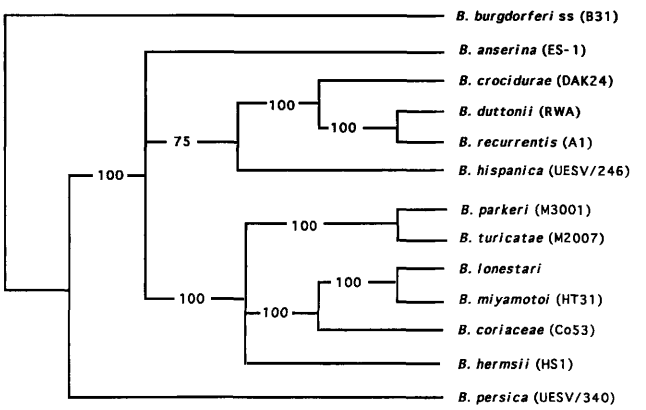

FIG. 2. Phylogenetic tree based on a comparison of the 16S rRNA sequences of RF *Borrelia* species. This 50% majority rule consensus tree based on 12 trees was obtained by using a maximum-parsimony method.

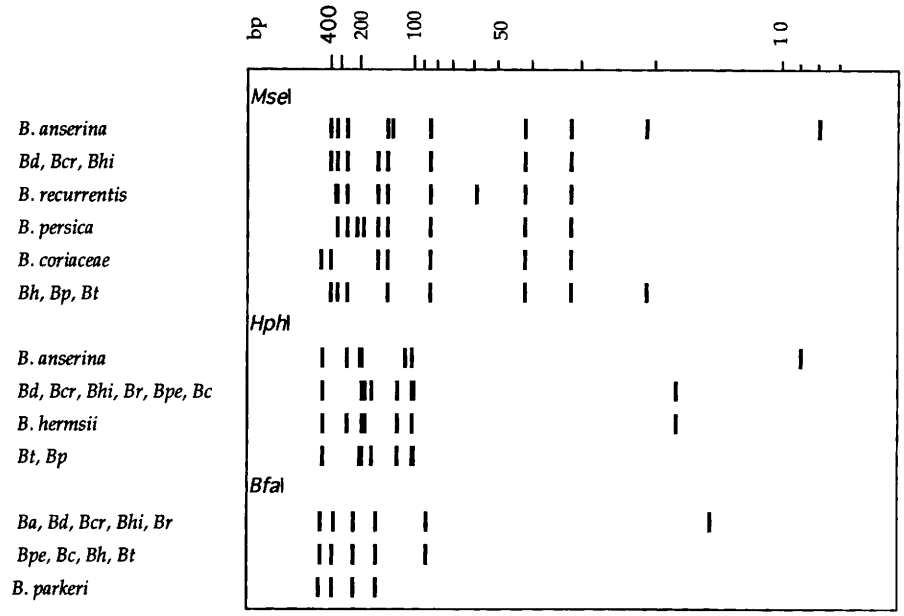

FIG. 3. DNA fragments obtained after restriction by *Mse*I, *Hph*I, and *Bfa*I of the *rrs* gene amplified with primers fD3 and T50. *Ba*, *B. anserina*; *Bd*, *B. duttonii*; *Bcr*, *B. crocidurae*; *Bhi*, *B. hispanica*; *Br*, *B. recurrentis*; *Bpe*, *B. persica*; *Bc*, *B. coriaceae*; *Bh*, *B. hermsii*; *Bt*, *B. turicatae*; *Bp*, *B. parkeri*.

for species identification (39). Although the two techniques mentioned above differ in their concepts, they are usually consistent in their results. However, it has been shown that if the level of 16S rRNA sequence similarity is greater than 97%, there is no constant correlation between levels of sequence similarity and DNA reassociation values (37). Thus, the status of RF *Borrelia* species, at the species level, cannot be deduced from an analysis of *rrs* sequences alone. However, despite certain controversial or ambiguous results (8), ribosomal units and flanking region studies have proven to be useful for delineating, identifying, or confirming *Borrelia* taxonomic units (1, 20, 22). For *B. lonestari*, *rrs* sequencing was the only basis for species definition (4).

Our results for the phylogenetic relationships of RF *Borrelia* species indicate that the taxonomy of the group should be revised. One distinct phylogenetic cluster comprises four species, *B. crocidurae*, *B. duttonii*, *B. recurrentis*, and *B. hispanica*. *B. hispanica* was previously placed in an intermediate position between *B. duttonii* and *B. recurrentis* (31). Interestingly, *B. recurrentis* could have evolved recently from a common ancestor with *B. duttonii*. Such a hypothesis has been proposed previously (32) on the basis of the common geographical endemicity of these organisms, their relatively high pathogenicities for humans, and successful experimental vector exchanges. It has even been suggested that each outbreak of louse-borne RF might be explained by the emergence of a new *B. recurrentis* strain from a tick RF *Borrelia* strain (5, 30). This suggestion is supported by the fact that tick-borne RF *Borrelia* strains can adapt to lice. However, the high number of nucleotide differences in *rrs* between the two groups of strains does not support this hypothesis. The hypothesis that *B. recurrentis* and *B. duttonii* belong to a single species is not supported by our findings which revealed four nucleotide differences in the *rrs* sequences of these two taxa. *B. recurrentis* could represent a clone from the cluster consisting of *B. crocidurae*, *B. duttonii*, and *B. hispanica*, which has adapted specifically to a human vector, *P. humanus*. *B. recurrentis* might therefore be classified at the subspecies taxonomic level because of the clinical severity and epidemic potential of louse-borne RF. The deeper branching of *B. persica* in the cluster consisting of *B. crocidurae*, *B. duttonii*, *B. hispanica*, and *B. recurrentis* may reflect distinct genospecies status.

In contrast, *B. anserina* was on a branch that was deeply separated from other *Borrelia* species. This may suggest that a putative ancestor of *B. anserina* was adapted to migratory birds, resulting in the distribution of clones throughout the world which later evolved into distinct taxa.

B. coriaceae is located in a heterogeneous cluster together with *B. lonestari* and *B. miyamotoi*, but the distances reflected by the branch lengths are large. *B. coriaceae* has been classified as a species that is distinct from *B. hermsii*, *B. anserina*, and *B. crocidurae* (17). Since the members of this group are widely distributed in different ecological niches, further study is

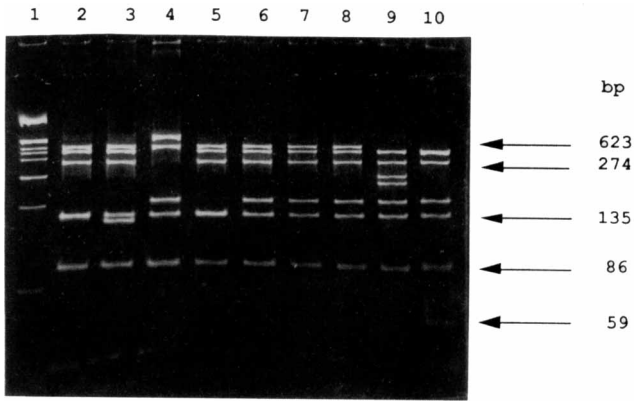

FIG. 4. Restriction patterns of RF *Borrelia* strains. DNAs amplified with primers fD3 and T50 were digested by *Mse*I. The DNAs were electrophoresed on a 10% acrylamide gel, stained with bromide ethidium, and UV illuminated. Lane 1, *Hin*fI-digested pBr molecular weight marker; lane 2, *B. hermsii*; lane 3, *B. anserina*; lane 4, *B. coriaceae*; lane 5, *B. parkeri*; lane 6, *B. duttonii*; lane 7, *B. crocidurae*; lane 8, *B. hispanica*; lane 9, *B. persica*; lane 10, *B. recurrentis*.

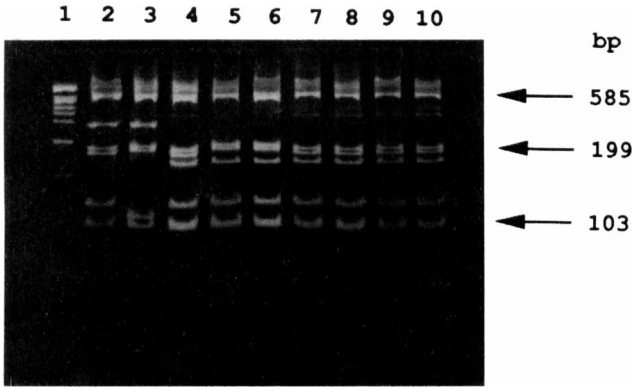

FIG. 5. Restriction patterns of RF *Borrelia* strains. DNAs amplified with primers fD3 and T50 were digested by *Hph*I. The DNAs were electrophoresed on a 16% acrylamide gel, stained with bromide ethidium, and UV illuminated. Lane 1, *Hin*fI-digested pBr molecular weight marker; lane 2, *B. hermsii*; lane 3, *B. anserina*; lane 4, *B. coriaceae*; lane 5, *B. parkeri*; lane 6, *B. turicatae*; lane 7, *B. duttonii*; lane 8, *B. crocidurae*; lane 9, *B. hispanica*; lane 10, *B. persica*.

needed to determine the exact taxonomic position of these organisms.

The taxonomic position of *B. hermsii* compared with the two other North American RF *Borrelia* species remains an open question. On the basis of DNA relatedness data, *B. hermsii*, *B. parkeri*, and *B. turicatae* have been assigned to the same genomospecies (15); despite this, they are still designated three separate species. Our phylogenetic analysis did not resolve the taxonomic status of *B. hermsii*. The levels of sequence similarity within this group (more than 99.1%) are consistent with the results of DNA relatedness studies. However, the most parsimonious tree obviously supports separation of *B. turicatae*, the *B. parkeri* cluster, and *B. hermsii*. Clearly, more work will be required to determine the exact position of *B. hermsii*.

Genetic diversity and applications to diagnosis. In the past, diagnosis of RF was based on the detection of *Borrelia* species in blood smears and on the pathogenicity of these organisms for laboratory animals. Identification of the responsible *Borrelia* species was based on the geographical location where exposure occurred and the suspected tick vector. Because of cross-reactivity and antigenic variations in surface proteins of RF *Borrelia* species, serological tests are not appropriate. Some methods to identify cultivable strains have been developed (26, 36); however, the remaining noncultivable strains are poorly characterized. A simple diagnostic method for identifying RF *Borrelia* species is clearly necessary, and such a method would allow workers to study the epidemiological significance of the various species.

A PCR-based strategy is the best method for this. We deduced from the 16S ribosomal DNA sequences several sets of primers that allowed amplification of DNAs from RF *Borrelia* species both from culture medium and from biological samples. As a first step, bacteria can be identified as spirochetes by using the specific primers fD3 and T50 and as RF *Borrelia* species by using the specific primers REC4 and REC9. Then, restriction digestion of the fD3-T50 PCR product by three endonucleases separately allows rapid identification of the main RF *Borrelia* species. As previously shown for *B. burgdorferi* sensu lato strains (29), the use of restriction site polymorphism of PCR products from *rrs* genes provides a simple, specific, and rapid way to identify RF *Borrelia* species without the need to culture the organisms.

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