Am. J. Trop. Med. Hyg., 67(5), 2002, pp. 504–510

MOLECULAR CHARACTERIZATION OF LYME DISEASE SPIROCHETES (*BORRELIA BURGDORFERI* SENSU LATO) ISOLATED IN TAIWAN BY RESTRICTION FRAGMENT LENGTH POLYMORPHISM ANALYSIS OF 5S(*RRF*)–23S(*RRL*) INTERGENIC SPACER AMPLICONS

LI-LIAN CHAO AND CHIEN-MING SHIH

Department of Parasitology and Tropical Medicine, National Defense Medical Center, Taipei, Taiwan, Republic of China

Abstract. We analyzed the 5S (*rrf*)–23S (*rrl*) intergenic spacer amplicon gene of Lyme disease spirochetes (*Borrelia burgdorferi* sensu lato) for the first time in Taiwan. The genetic identities of these Taiwan isolates (TWKM1-7) were clarified by comparing their restriction fragment length polymorphism patterns and sequence similarities of the polymerase chain reaction–amplified intergenic spacer amplicon genes with 3 major genospecies of Lyme disease spirochetes. Amplified-spacer DNAs were purified further and subjected to the cleavage by nuclease DraI or MseI. Differential fragment patterns in relation to different genospecies of Lyme disease spirochetes were observed among tested *Borrelia* isolates, and all of these Taiwan isolates were closely related to the genospecies of *B. burgdorferi* sensu stricto. The phylogenetic analysis also revealed that the sequence similarity of polymerase chain reaction–amplified spacer genes of these Taiwan isolates was highly homogeneous (95.7–100%) within the genospecies of *B. burgdorferi* sensu stricto and can be distinguished clearly from other genospecies of Lyme disease spirochetes with a 4.1% sequence divergence. Based on the differential fragment patterns and sequence similarity among these Taiwan isolates, the genetic identity of these Taiwan isolates should be classified into the genospecies of *B. burgdorferi* sensu stricto.

INTRODUCTION

Lyme disease is a tick-borne spirochetal infection[1] that can cause multisystem human illness with varying degrees of clinical symptoms among infected persons, ranging from a relatively benign skin lesion to severe arthritic, neurologic, and cardiac manifestations.[2,3] The causative agent of Lyme disease, *Borrelia burgdorferi* sensu lato, is transmitted mainly by ticks of the *Ixodes ricinus* complex in North America and Europe[4,5] and by *Ixodes persulcatus* and *Ixodes ovatus* ticks in the countries of Far East Asia.[6–8] Although the first laboratory-confirmed case of human Lyme disease had been reported in Taiwan[9] and the spirochetes can be isolated from 6 species of rodents in the Taiwan area,[10] the genetic variety of spirochetes and the tick vector responsible for transmission in Taiwan remain undefined.

The heterogeneity of molecular and immunologic characteristics among isolates of *B. burgdorferi* sensu lato from different geographic and biologic origins has been shown previously.[11–18] On the basis of immunoreactivity with *B. burgdorferi*–specific monoclonal antibodies, plasmid profiles, and the clinical manifestations of the patient, the causative agents of Lyme disease can be classified into 3 major genospecies: *B. burgdorferi* sensu stricto, *Borrelia garinii*, and *Borrelia afzelii* (group VS461).[19,20] Genomic analysis of similarities among *Borrelia* isolates by differential polymerase chain reaction (PCR) primer reactivity analysis of a specific target gene encoding the outer surface proteins or 16S rRNA has been proved useful for the species identification and typing of *Borrelia* isolates from various biologic and geographic sources.[21–24] Our previous study had investigated the protein profiles, antigenic reactivities with monoclonal antibodies against *B. burgdorferi* sensu lato, and differential PCR primer reactivities of *Borrelia* isolates from Taiwan.[25]

The existence of 2 tandemly duplicated copies of *rrl* (23S) and *rrf* (5S) genes in *B. burgdorferi* sensu lato is unique and has not been found in other members of the genus *Borrelia* or in other eubacteria.[26–28] Taking advantage of this unique genomic character, the genetic heterogeneity of *B. burgdorferi* sensu lato isolates can be distinguished by analyzing the restriction fragment length polymorphism (RFLP) patterns of the 5S-23S intergenic spacer amplicon.[29] The objective of the present study was to characterize further the genetic identity of these Taiwan isolates by their differential restriction fragment patterns and sequence similarity of the PCR-amplified intergenic spacer region between the 5S (*rrf*) and 23S (*rrl*) rRNA genes.

MATERIALS AND METHODS

Bacterial strains and cultivation. Seven strains of Taiwan isolates (TWKM1-7) and 10 strains of *Borrelia* isolates belonging to the genospecies of *B. burgdorferi* sensu stricto (B31, JD1, N40, CT27985, TB, VS219, CT20004, and ECM-NY86), *B. garinii* (K48), and *B. afzelii* (VS461) were used in this study for PCR, RFLP, and phylogenetic analysis (Table 1). Briefly, spirochetes were cultured at 34°C in a humidified incubator (Nuaire, Inc, Plymouth, MN) with 5% carbon dioxide and maintained in BSK-H medium (catalog no. B3528; Sigma Chemical Co, St. Louis, MO) supplemented with 6% rabbit serum (catalog no. R7136; Sigma Chemical Co) as described previously.[10] All cultures were examined weekly for the optimal growth of spirochetes by dark-field microscope (model BX-60; Olympus Co, Tokyo, Japan).

Preparation of spirochete DNA. Total genomic DNA from all *Borrelia* strains was extracted as described previously.[25] Briefly, samples (3 mL) of cultured spirochetes were grown to a density about 2×10^8 cells per ml of medium and were centrifuged for 10 minutes at 12,000 × g to pellet the spirochetes. The pellets were washed twice with phosphate-buffered saline (pH 7.2) containing 5 mM of $MgCl_2$, resuspended in 150 μl of distilled water, and boiled for 10 minutes. After centrifugation at 10,000 × g for 10 seconds, the supernatant was collected, and the DNA concentrations were determined spectrophotometrically by using a DNA calculator (GeneQuant II; Pharmacia Biotech, Uppsala, Sweden).

Polymerase chain reaction and restriction fragment length polymorphism analysis. DNA samples extracted from the

TABLE 1
Spirochetal isolates of *Borrelia burgdorferi* sensu lato analyzed in this study

Genospecies and strain	Origin of isolation		*rrf-rrl* gene accession number
	Biologic	Geographic	
B. burgdorferi sensu stricto			
B31	Tick	United States	AY032903
JD1	Tick	United States	AY032911
N40	Tick	United States	AY032917
CT27985	Tick	United States	AY032915
TB	Tick	United States	AY032918
VS219	Tick	Switzerland	AY032919
CT20004	Tick	France	AY032914
ECM-NY86	Human skin	United States	AY032916
B. garinii			
K48	Tick	Czechoslovakia	AY032912
B. afzelii			
VS461	Tick	Switzerland	AY032913
Taiwan isolates			
TWKM1-7	Mouse ear	Taiwan	AY032904-10

Taiwan isolates and other spirochetes representative of the 3 major genospecies of *B. burgdorferi* sensu lato were used as a template to perform the PCR amplification for the intergenic spacer DNA. Primers set corresponding to the 3′ end of the 5S rRNA (*rrf*) (5′-CTGCGAGTTCGCGGGAGA-3′) and the 5′ end of the 23S rRNA (*rrl*) (5′-TCCTAGGCATTCAC-CATA-3′) as described previously[29] were synthesized by a custom oligonucleotide synthesis service (Gibco BRL, Taipei, Taiwan) and were used to amplify the variable spacer region between 2 conserved duplicate structures. All PCR reagents and TaqGold DNA polymerase were obtained from the GeneAmp kit (Perkin-Elmer Cetus, Taipei, Taiwan) and were used as recommended by the manufacturer.

Briefly, a total of 20-pmol of the appropriate primer set and various amounts of template DNA were used in each 50-μl reaction mixture. PCR amplification was performed with a Perkin-Elmer Cetus thermocycler (GeneAmp system 9700; Taipei, Taiwan), and amplification was set up for 35 cycles under the following condition: denaturation at 94°C for 1 minute, annealing at 52°C for 1 minute, and extension at 72°C for 2 minutes. PCR-amplified DNA products were electrophoresed on 2% agarose gels in Tris-Borate-EDTA (TBE) buffer and were visualized under UV light with ethidium bromide. The 1 kb plus DNA ladder (catalog no. 10787-018; Gibco BRL, Taipei, Taiwan) was used as the standard marker for comparison. After purification by a QIAquick PCR purification kit (catalog no. 28104; Qiagen, Taipei, Taiwan), the purified PCR products were subjected to digestion with restriction enzymes of DraI and MseI (New England Biolabs, Taipei, Taiwan). The digested DNA fragments of DraI and MseI were separated by electrophoresis on 3.5% agarose gels (Agarose-1000; Gibco BRL, Taipei, Taiwan), and the electrophoresed gels were visualized under UV light with ethidium bromide staining. DNA ladders of 25 bp (catalog no. 10597-011; Gibco BRL, Taipei, Taiwan) and 50 bp (10416-014, Gibco BRL, Taipei, Taiwan) fragments were used as the standard markers for comparison.

Sequence alignments and phylogenetic analysis. The nucleotide sequences of the intergenic spacer amplicon (*rrf-rrl*) of the *Borrelia* isolates used in this study have been sequenced by a dye-deoxy terminator reaction method using the bigdye terminator-Taq cycle sequencing kit under an ABI Prism 377-96 DNA sequencer (Applied Biosystems Inc, Foster City, CA). The determined sequences were aligned, and a similarity matrix and neighbor-joining phylogenetic tree were constructed using the DNASTAR program and the CLUSTAL V software package.[30,31]

Nucleotide sequence accession numbers. The nucleotide sequences of PCR-amplified intergenic spacer gene determined in this study have been assigned the following GenBank accession numbers: strains B31 (AY032903), TWKM1 (AY032904), TWKM2 (AY032905), TWKM3 (AY032906), TWKM4 (AY032907), TWKM5 (AY032908), TWKM6 (AY032909), TWKM7 (AY032910), JD1 (AY032911), K48 (AY032912), VS461 (AY032913), CT20004 (AY032914), CT27985 (AY032915), ECM-NY86 (AY032916), N40 (AY032917), TB (AY032918), and VS219 (AY032919).

RESULTS

In a preliminary experiment, PCR amplification of the spacer region located between the *rrf* (5S) and *rrl* (23S) genes of *B. burgdorferi* sensu lato was performed to generate the spacer DNA of all strains of Taiwan isolates and 10 other isolates belonging to 3 major genospecies of Lyme disease spirochetes. A DNA fragment of approximately 230–260 bp was generated and observed on a 2% agarose gel (Figure 1). These results show that the intergenic spacer amplicons within the duplicated *rrf-rrl* genes were highly conserved in all of the *Borrelia* isolates regardless of the isolation origin and genospecies of Lyme disease spirochetes.

To clarify the genomic identity of these Taiwan isolates, RFLP analysis of the spacer amplicon of *rrf-rrl* genes also was performed for further classification and differentiation of isolates belonging to the 3 major genospecies of Lyme disease spirochetes. After cleavage by DraI or MseI, the restriction site polymorphism of these spacer amplicons from the PCR-amplified *rrf-rrl* genes of 17 isolates was investigated by comparing their restriction patterns of digested DNA fragments. As revealed by the gel electrophoresis, all 17 isolates were classified into 3 different RFLP patterns in relation to 3 different genospecies of *B. burgdorferi* sensu lato (Figures 2 and 3). The restriction fragment pattern of DraI-digested spacer amplicons of the genospecies of *B. garinii* (strain K48) shows a specific pattern with DNA fragments of approximately 200 and 55 bp, and a specific pattern with DNA fragments of approximately 175 and 55 bp was observed on the spacer amplicon of *B. afzelii* (strain VS461) (Table 2). All 7 Taiwan isolates exhibit the same pattern as 8 other strains of *B. burgdorferi* sensu stricto with DNA fragments of approximately 146, 55, and 33 bp. The MseI-digested restriction fragment pattern of all 7 Taiwan isolates and the genospecies of *B. burgdorferi* sensu stricto represents a special pattern with DNA fragments of approximately 105, 55, 41, and 33 bp (Table 2). Differential restriction fragment patterns also were observed, however, in the MseI-digested spacer amplicons of *B. garinii* (DNA fragments of approximately 105, 100, and 50 bp) and *B. afzelii* (DNA fragments of approximately 105, 70, and 55 bp). These results reveal that the genomic identity of these Taiwan isolates was clarified to be related closely to the genospecies of *B. burgdorferi* sensu stricto.

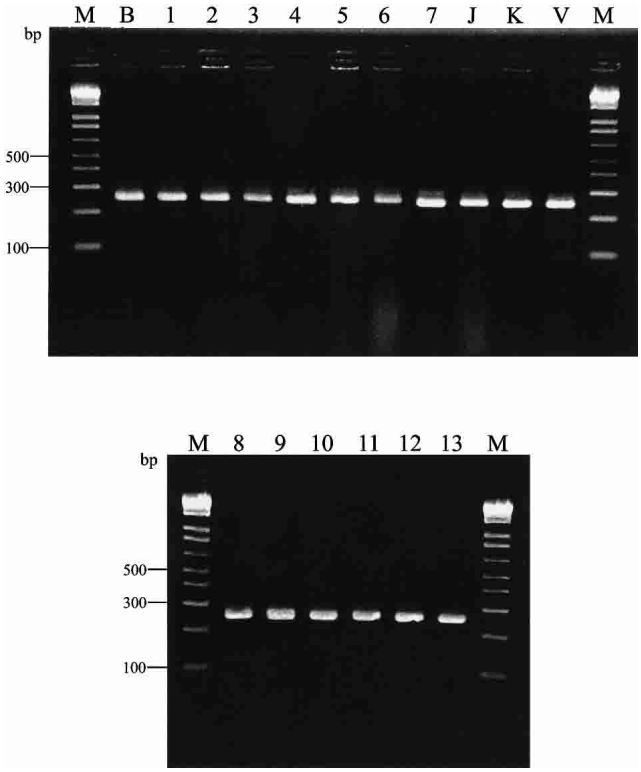

FIGURE 1. Polymerase chain reaction analysis with a primer set specific for the 5S (*rrf*)–23S (*rrl*) intergenic spacer amplicon genes of *B. burgdorferi* sensu lato. The amplified products of Taiwan isolates and other genospecies of *B. burgdorferi* isolates were electrophoresed on a 2% agarose gel (Agarose-LE; USB, Cleveland, OH), stained with ethidium bromide, and visualized by UV transillumination. Lane B, B31 isolate, lanes 1–7 represent the Taiwan isolates of TWKM1-7; lane J, JD 1 isolate of *B. burgdorferi* sensu stricto; lane K, K48 isolate of *B. garinii*; lane V, VS461 isolate of *B. afzelii*; lanes 8–13 represent the *B. burgdorferi* sensu stricto strains of CT20004, CT27985, ECM-NY86, N40, TB, and VS219; lanes M, 1-kb plus DNA ladder (Gibco BRL, Taipei, Taiwan).

The sequence similarity of intergenic spacer amplicons also was analyzed to identify the homogeneity of these Taiwan isolates in relation to the genospecies of *B. burgdorferi* sensu lato. As shown in Table 3, the *rrf-rrl* intergenic spacer amplicon sequences are highly homogeneous with a homogeneity ranging from 95.7–100% among Taiwan isolates and 8 other strains of *Borrelia* isolates within the genospecies of *B. burgdorferi* sensu stricto. When compared with the aligned sequence of B31 isolate, a highly variable nucleotide sequence was observed in the strains of K48 (*B. garinii*) and VS461 (*B. afzelii*). The differences of nucleotide sequence were located on the positions of 4, 40, 66, 69, 72, 73, 88, 91, 94, 95, 101, 130, 154, and 168 of the strain K48 and positions of 4, 40, 45, 69, 72, 73, 75, 78, 86–91, 94, 101, 114, 130, 154, and 168 of the strain VS461. The nucleotide sequence variations were observed, however, only on the positions of 4, 18, and 106 of the strain TWKM 3; position 102 of the strain TWKM 4; and position 101 of the strain N40. The phylogenetic relationships based on the gene sequences of intergenic spacer amplicons also were analyzed to construct the divergence among *Borrelia* isolates investigated in this study (Figure 4). These results reveal a high homogeneity of intergenic spacer amplicon genes among *Borrelia* isolates within the genospecies of *B. burgdorferi* sensu stricto.

DISCUSSION

This article describes the first genomic characterization and identification of the intergenic spacer amplicon (*rrf-rrl*) genes among Lyme disease spirochetes, *B. burgdorferi* sensu lato, isolated in Taiwan. In our previous investigations, the protein profiles of these Taiwan isolates were consistent with the major protein bands of other documented strains of Lyme disease spirochetes, and antigenicity was verified by their reactivities with monoclonal antibodies specific against *B. burgdorferi* sensu lato.[25] Although the heterogeneity among major protein bands and the immunoreactivity with *B. burgdorferi*–specific monoclonal antibodies had been used for the typing or species identification of Lyme disease isolates, the validity of these methods used for genospecies identification was not fully satisfied.[14,32] Genomic analysis based on the intergenic spacer amplicon (*rrf-rrl*) genes provides a simple and reliable method for genospecies identification of *Borrelia* spirochetes that may be discovered from various animal reservoirs and vector ticks in Taiwan.

Although genetic analysis based on the differential reactivities with genospecies-specific PCR primers had been rec-

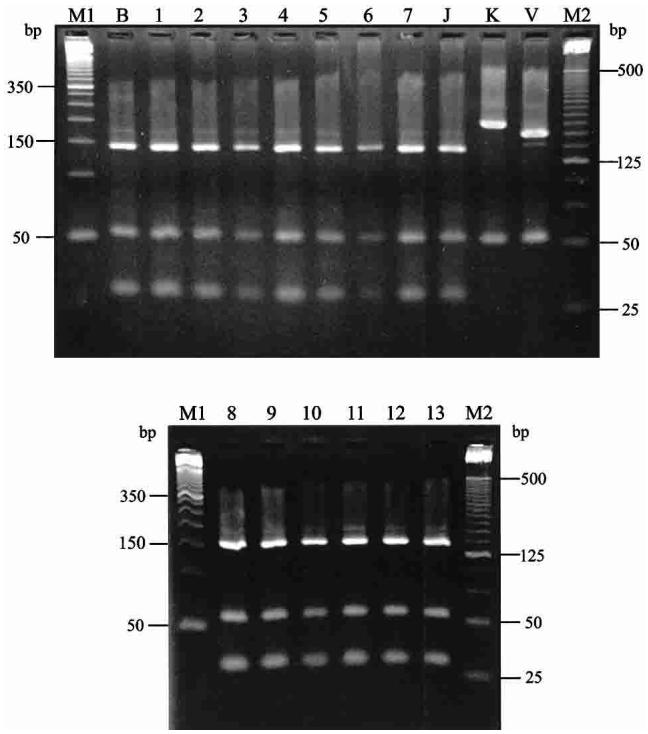

FIGURE 2. Polymerase chain reaction–restriction fragment length polymorphism analysis of the restriction patterns of 5S (*rrf*)–23S (*rrl*) intergenic spacer amplicon genes from Taiwan isolates and other genospecies of *B. burgdorferi* isolates. The amplification products were purified further by a QIAquick polymerase chain reaction purification kit (Qiagen, Taipei, Taiwan) and were subjected to digestion by nuclease DraI. The digested DNA fragments were electrophoresed on a 3.5% agarose gel (Agarose-1000; Gibco BRL, Taipei, Taiwan), stained with ethidium bromide, and visualized by UV transillumination. Lane B, B31 isolate, lanes 1–7 represent the Taiwan isolates of TWKM1-7; lane J, JD 1 isolate of *B. burgdorferi* sensu stricto; lane K, K48 isolate of *B. garinii*; lane V, VS461 isolate of *B. afzelii*, lanes 8–13 represent the *B. burgdorferi* sensu stricto strains of CT20004, CT27985, ECM-NY86, N40, TB, and VS219; lanes M1 and M2 represent the 50 bp and 25 bp DNA ladder (Gibco BRL).

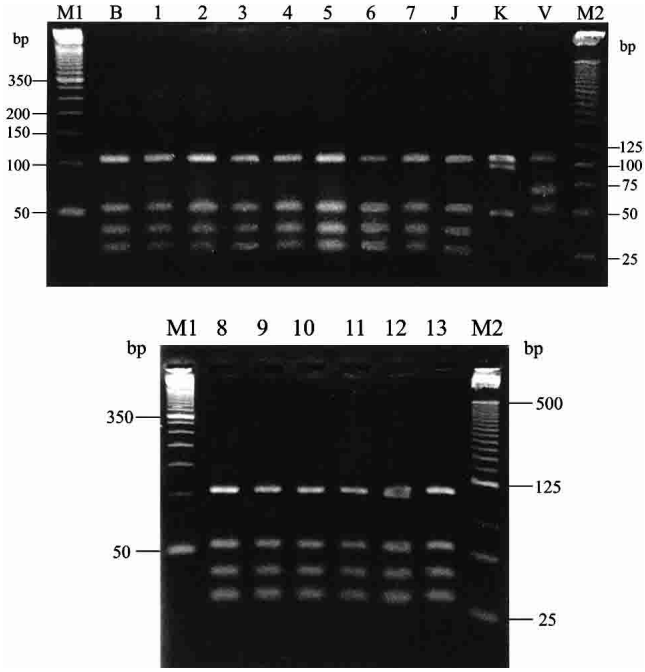

FIGURE 3. Polymerase chain reaction–restriction fragment length polymorphism analysis of the restriction patterns of 5S (*rrf*)–23S (*rrl*) intergenic spacer amplicon genes from the Taiwan isolates and other genospecies of *B. burgdorferi* isolates. The amplification products were purified further by a QIAquick PCR purification kit (Qiagen, Taipei, Taiwan) and were subjected to digestion by nuclease MseI. The digested DNA fragments were electrophoresed on a 3.5% agarose gel (Agarose-1000; Gibco BRL, Taipei, Taiwan), stained with ethidium bromide, and visualized by UV transillumination. Lane B, B31 isolate, lanes 1–7 represent the Taiwan isolates of TWKM1-7; lane J, JD 1 isolate of *B. burgdorferi* sensu stricto; lane K, K48 isolate of *B. garinii*; lane V, VS461 isolate of *B. afzelii*; lanes 8–13 represent the *B. burgdorferi* sensu stricto strains of CT20004, CT27985, ECM-NY86, N40, TB, and VS219; lanes M1 and M2 represent the 50 bp and 25 bp DNA ladder (Gibco BRL).

TABLE 2

Restriction fragment length polymorphism analysis of restriction fragment patterns of *rrf-rrl* intergenic spacer amplicons from various genospecies of *Borrelia burgdorferi* sensu lato

Genospecies and strain	DraI restriction fragment sizes (bp)	MseI restriction fragment sizes (bp)	RFLP type
B. burgdorferi sensu stricto			
B31	33, 55, 146	33, 41, 55, 105	A
JD1	33, 55, 146	33, 41, 55, 105	A
N40	33, 55, 146	33, 41, 55, 105	A
CT27985	33, 55, 146	33, 41, 55, 105	A
TB	33, 55, 146	33, 41, 55, 105	A
VS219	33, 55, 146	33, 41, 55, 105	A
CT20004	33, 55, 146	33, 41, 55, 105	A
ECM-NY86	33, 55, 146	33, 41, 55, 105	A
Taiwan isolates			
TWKM1-7	33, 55, 146	33, 41, 55, 105	A
B. garinii			
K48	55, 200	50, 100, 105	B
B. afzelii			
VS461	55, 175	55, 70, 105	C

RFLP, restriction fragment length polymorphism.

ognized as a rapid and distinguishable assay for the species identification of *Borrelia* spirochetes from various biologic and geographic origins,[14,21–24,33] the genetic diversity among *Borrelia* isolates was difficult to clarify at the intraspecies level.[34] Because of the uniqueness of the tandemly repeated 5S (*rrf*)–23S (*rrl*) intergenic spacer amplicon genes among Lyme disease spirochetes (*B. burgdorferi* sensu lato), the genetic heterogeneity can be classified further among *Borrelia* isolates that previously were identified as the same genospecies or atypical strains of spirochetes.[29,34,35] The new genomic groups and a new species of Lyme disease spirochetes had been described by analyzing the *rrf-rrl* intergenic spacer regions of *Borrelia* isolates.[29,34] In our previous study, the genetic identity of these Taiwan isolates had been clarified by their differential reactivities with genospecies-specific PCR primers based on the *OspA* gene of *B. burgdorferi* sensu lato.[25] Results from the present study further clarify the ge-

TABLE 3

Sequence similarity between *rrf-rrl* gene sequences from Taiwan isolates and other genospecies of *Borrelia* isolates*

	% Sequence similarity to																
Isolate	B31	Twkm1	Twkm2	Twkm3	Twkm4	Twkm5	Twkm6	Twkm7	JD-1	K48	VS461	CT20004	CT27985	ECM-NY86	N40	TB	VS219
B31	100.0	98.7	100.0	97.8	97.9	100.0	100.0	100.0	100.0	90.2	85.1	100.0	100.0	100.0	99.6	100.0	100.0
Twkm-1		100.0	99.7	97.8	96.6	98.7	98.7	98.7	98.7	89.4	84.2	98.7	98.7	100.0	98.3	100.0	98.7
Twkm-2			100.0	97.8	97.9	100.0	100.0	100.0	100.0	90.2	85.1	100.0	100.0	100.0	99.6	100.0	100.0
Twkm-3				100.0	95.7	97.8	97.8	97.8	97.8	87.9	81.6	97.8	97.8	97.8	97.4	97.8	97.8
Twkm-4					100.0	97.9	97.9	97.9	97.9	88.1	82.9	97.9	97.9	97.8	97.5	97.8	97.9
Twkm-5						100.0	100.0	100.0	100.0	90.2	85.1	100.0	100.0	100.0	99.6	100.0	100.0
Twkm-6							100.0	100.0	100.0	90.2	85.1	100.0	100.0	100.0	99.6	100.0	100.0
Twkm-7								100.0	100.0	90.2	85.1	100.0	100.0	100.0	99.6	100.0	100.0
JD-1									100.0	90.2	85.1	100.0	100.0	100.0	99.6	100.0	100.0
K48										100.0	90.8	90.2	90.2	90.1	90.6	90.1	90.2
VS461											100.0	85.1	85.1	83.8	85.5	83.8	85.1
CT20004												100.0	100.0	100.0	99.6	100.0	100.0
CT27985													100.0	100.0	99.6	100.0	100.0
ECM-NY86														100.0	99.6	100.0	100.0
N40															100.0	99.6	99.6
TB																100.0	100.0
VS219																	100.0

* Strains: B31, JD1, CT20004, CT27985, ECM-NY86, N40, TB, and VS219, *B. burgdorferi* sensu stricto; K48, *B. garinii;* VS461, *B. afzelii.*

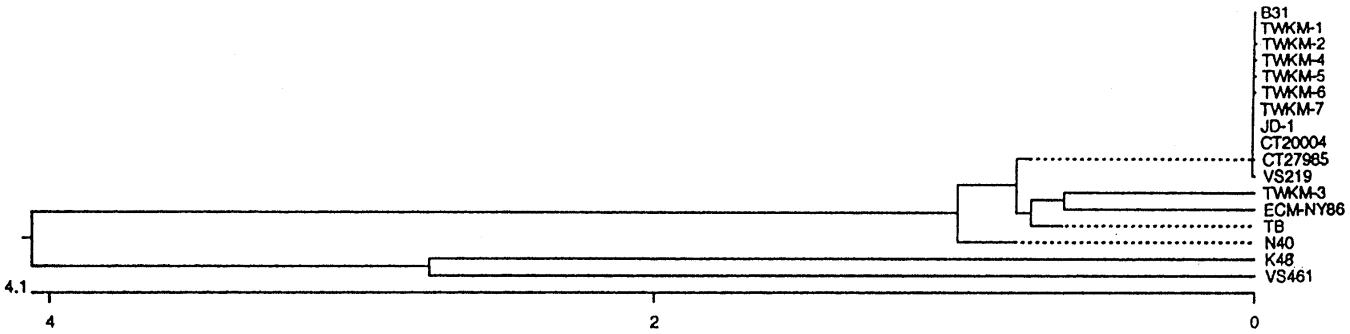

FIGURE 4. Phylogenetic tree based on a comparison of the 5S (*rrf*)–23S (*rrl*) intergenic spacer amplicon gene sequences from 7 Taiwan isolates and 10 strains of *B. burgdorferi* sensu lato. The bar represents the divergence beween sequences of *Borrelia* isolates.

netic identity of these Taiwan isolates by analyzing the intergenic spacer amplicon (*rrf-rrl*) gene, and the genospecies of these Taiwan isolates were classified genetically as *B. burgdorferi* sensu stricto.

The genetic relatedness of *Borrelia* isolates can be classified with their heterogeneity of restriction fragment patterns by RFLP analysis of specific target gene. Previous reports described that RFLP analysis of the 5S-23S rRNA genes, *OspA* or *OspC,* seems useful for detection of mixed spirochetal infections and classification of the genospecies of *Borrelia* isolates detected in various biologic specimens.[21,36,37] Results presented in this study also show that the genospecies of *Borrelia* isolates from rodents in Taiwan can be classified by their restriction fragment patterns of 5S (*rrf*)–23S (*rrl*) intergenic spacer amplicon gene, and all of the Taiwan isolates were genetically affiliated to the genospecies of *B. burgdorferi* sensu stricto according to the homogeneity of RFLP pattern within the same genospecies of *Borrelia* isolates (Figures 2 and 3). These observations suggest that the genomic identity of *Borrelia* isolates can be differentiated either interspecies or intraspecies of *B. burgdorferi* sensu lato by analyzing their restriction fragment polymorphisms of DraI- or MseI-digested intergenic spacer amplicons.

The phylogenetic relationships among *Borrelia* isolates can be constructed by analyzing their sequence homogeneity of a specific target gene. Sequence analysis of the intergenic spacer amplicon (*rrf-rrl*) gene among *Borrelia* isolates had been proved useful to evaluate the taxonomic relatedness of *B. burgdorferi* sensu lato derived from various geographic and biologic sources.[36,38,39] The phylogenetic analysis of *rrs* gene sequence of previously identified as atypical strains of *Borrelia* isolates also differentiated new genomic groups of Lyme disease spirochetes.[34] Although PCR amplification of the intergenic spacer region located between the *rrf* and *rrl* genes of *B. burgdorferi* sensu lato had been reported to generate a DNA fragment of approximately 226–266 bp long,[29] the variation of nucleotide sequence depends on the strain or group diversity of *Borrelia* isolates and may represent the genetic distance of phylogenetic divergence between or within the genospecies of *Borrelia* isolates.[29,34,38,40] In this study, the phylogenetic analysis of intergenic spacer amplicon (*rrf-rrl*) gene of these Taiwan isolates shows a high sequence homogeneity among *Borrelia* isolates within the genospecies of *B. burgdorferi* sensu stricto (Table 3) that can be distinguished from other genospecies (*B. garinii* and *B. afzelii*) of spirochetes with a 4.1% sequence divergence (Figure 4). Further application of these molecular tools to analyze the *rrf-rrl* genes of *Borrelia* strains isolated from various biologic and geographic sources may help to clarify the genetic divergence of *Borrelia* isolates in Taiwan.

The genetic divergence of Lyme disease spirochetes is relevant to the clinical presentation of patients, and the mechanism responsible for the nucleotide sequence variation of a target gene within the genospecies of *Borrelia* isolates remains undefined. The variable clinical syndromes of patients had been associated with different pathogenic species of *Borrelia* spirochetes,[41] and the genetic exchange by lateral transfer of the nucleotide sequence has been proposed for the relatively high levels of *OspC* gene diversity.[42] It is possible that the low level of sequence diversity (<1% sequence divergence) of 5S-23S intergenic spacer amplicon genes among these Taiwan isolates may correlate to the collection of these isolates from the same endemic site in Taiwan.[10] Another explanation for the low level of polymorphism indicated among these Taiwan isolates would be the possibility of recent, accidental importation of *B. burgdorferi* sensu stricto in Taiwan, which may lead to only one (or a few) genotype of *Borrelia* spirochetes observed in Taiwan. Confirmation by a large panel of *Borrelia* isolates collected in Taiwan and its correlation to the clinical presentations of patients would be beneficial to the discovery of the truth.

In conclusion, this article describes the first identification and characterization of intergenic spacer amplicon gene of *Borrelia* spirochetes isolated in Taiwan. On the basis of their differential RFLP patterns and sequence similarity of *rrf-rrl* gene, all of these Taiwan isolates were genetically classified into the genospecies of *B. burgdorferi* sensu stricto. Further investigations on the genetic variability of *rrf-rrl* gene among *Borrelia* isolates from the patients, reservoir hosts, and vector ticks would help to illustrate the significance of genetic diversity of *Borrelia* spirochetes in relation to the epidemiologic features and their pathogenicity for human Lyme borreliosis in Taiwan.

Financial support: This work was supported in part by grants from the National Science Council (NSC90-2320-B-016-068) and Center for Disease Control of the Department of Health (DOH90-DC-1063), Taipei, Taiwan, Republic of China.

Authors' address: Li-Lian Chao and Chien-Ming Shih, Department of Parasitology and Tropical Medicine, National Defense Medical Center, PO Box 90048-506, Neihu, Taipei, Taiwan, Republic of China.

Reprint requests: Chien-Ming Shih, Department of Parasitology and Tropical Medicine, National Defense Medical Center, PO Box 90048-506, Neihu, Taipei, Taiwan, Republic of China, Telelphone: 886-2-87921821, E-mail: cmshih@ndmctsgh.edu.tw

REFERENCES

1. Burgdorfer W, Barbour AG, Hayes SF, Benach JL, Grunwaldt E, Davis JP, 1982. Lyme disease—a tick-borne spirochetosis? *Science 216:* 1317–1319.
2. Steere AC, 1989. Lyme disease. *N Engl J Med 321:* 586–596.
3. Steere AC, Bartennagen NH, Craft JE, Hutchinson GJ, Newman JH, Rahn DW, Sigal LH, Spieler PN, Stenn KS, Malawista SE, 1983. The early clinical manifestations of Lyme disease. *Ann Intern Med 99:* 76–82.
4. Matuschka FR, Richter D, Fischer P, Spielman A, 1990. Subadult *Ixodes ricinus* (Acari: Ixodidae) on rodents in Berlin, West Germany. *J Med Entomol 27:* 385–390.
5. Spielman A, Wilson ML, Levine JF, Piesman J, 1985. Ecology of *Ixodes dammini*–borne human babesiosis and Lyme disease. *Annu Rev Entomol 30:* 439–460.
6. Ai CX, Wen YX, Zhang YG, Wang SS, Qui SS, Shi ZX, Li DY, Chen DQ, Liu XD, Zhao JH, 1988. Clinical manifestations and epidemiological characteristics of Lyme disease in Hailin county, Heilongjiang Province, China. *Ann N Y Acad Sci 539:* 302–313.
7. Kawabata M, Baba S, Iguchi K, Yamaguchi N, Russell H, 1987. Lyme disease in Japan and its possible incriminated tick vector, *Ixodes persulcatus. J Infect Dis 156:* 854.
8. Nakao M, Miyamoto K, Uchikawa K, Fujita H, 1992. Characterization of *Borrelia burgdorferi* isolated from *Ixodes persulcatus* and *Ixodes ovatus* ticks in Japan. *Am J Trop Med Hyg 47:* 505–511.
9. Shih CM, Wang JC, Chao LL, Wu TN, 1998. Lyme disease in Taiwan: First human patient with characteristic erythema chronicum migrans skin lesion. *J Clin Microbiol 36:* 807–808.
10. Shih CM, Chao LL, 1998. Lyme disease in Taiwan: Primary isolation of *Borrelia burgdorferi*–like spirochetes from rodents in Taiwan area. *Am J Trop Med Hyg 59:* 687–692.
11. Adam T, Gassmann GS, Rasiah C, Gobel UB, 1991. Phenotypic and genotypic analysis of *Borrelia burgdorferi* isolated from various sources. *Infect Immun 59:* 2579–2585.
12. Barbour AG, Heiland RA, Howe T, 1985. Heterogeneity of major proteins in Lyme disease borreliae: A molecular analysis of North American and European isolates. *J Infect Dis 152:* 478–484.
13. Bergstrom S, Bundoc VG, Barbour AG, 1989. Molecular analysis of linear plasmid-encoded major surface proteins, OspA and OspB of the Lyme disease spirochete *Borrelia burgdorferi. Mol Microbiol 3:* 479–486.
14. Jonsson M, Noppa L, Barbour AG, Bergstrom S, 1992. Heterogeneity of outer surface proteins in *Borrelia burgdorferi*: Comparison of osp operons of three isolates of different geographic origins. *Infect Immun 60:* 1845–1853.
15. Lane RS, Pascocello JA, 1989. Antigenic characteristics of *Borrelia burgdorferi* isolated from *Ixodid* ticks in California. *J Clin Microbiol 27:* 2344–2349.
16. Lefebvre RB, Lane RS, Perng GC, Brown JA, Johnson RC, 1990. DNA and protein analysis of tick-derived isolates of *Borrelia burgdorferi* from California. *J Clin Microbiol 28:* 700–707.
17. Masuzawa T, Okada Y, Yanagihara Y, Sato N, 1991. Antigenic properties of *Borrelia burgdorferi* isolated from *Ixodes ovatus* and *Ixodes persulcatus* in Hokkaido, Japan. *J Clin Microbiol 29:* 1568–1573.
18. Wilske B, Preac-Mursic V, Schierz G, Kuhbeck R, Barbour AG, Kramer M, 1988. Antigenic variability of *Borrelia burgdorferi. Ann N Y Acad Sci 539:* 126–143.
19. Baranton G, Postic D, Saint Girons I, Boerlin P, Piffaretti JC, Assous M, Grimont PAD, 1992. Delineation of *Borrelia burgdorferi* sensu stricto, *Borrelia garinii* sp. nov., and group VS461 associated with Lyme borreliosis. *Int J Syst Bacteriol 42:* 378–383.
20. Johnson RC, Schmid GP, Hyde FW, Steigerwalt AG, Brenner DJ, 1984. *Borrelia burgdorferi* sp. nov.: etiologic agent of Lyme disease. *Int J Syst Bacteriol 34:* 496–497.
21. Demaerschalck I, Messaoud AB, Kesel MD, Hoyois B, Lobet Y, Hoet P, Bigaignon G, Bollen A, Godfroid E, 1995. Simutaneous presence of different *Borrelia burgdorferi* genospecies in biological fluids of Lyme disease patients. *J Clin Microbiol 33:* 602–608.
22. Marconi RT, Garon CF, 1992. Development of polymerase chain reaction primer sets for diagnosis of Lyme disease and for species-specific identification of Lyme disease isolates by 16S rRNA signature nucleotide analysis. *J Clin Microbiol 30:* 2830–2834.
23. Marconi RT, Lubke L, Hauglum W, Garon CF, 1992. Species-specific identification of and distinction between *Borrelia burgdorferi* genomic groups by using 16S rRNA-directed oligonucleotide probes. *J Clin Microbiol 30:* 628–632.
24. Rosa PA, Hogan D, Schwan TG, 1991. Polymerase chain reaction analysis identifies two distinct classes of *Borrelia burgdorferi. J Clin Microbiol 29:* 524–532.
25. Shih CM, Chang HM, Chen SL, Chao LL, 1998. Genospecies identification and characterization of Lyme disease spirochetes of genospecies *Borrelia burgdorferi* sensu lato isolated from rodents in Taiwan. *J Clin Microbiol 36:* 3127–3132.
26. Fukunaga M, Sohnaka M, 1992. Tandem repeat of the 23S and 5S ribosomal RNA genes in *Borrelia burgdorferi*, the etiological agent of Lyme disease. *Biochem Biophys Res Commun 183:* 952–957.
27. Fukunaga M, Yanagihara Y, Sohnaka M, 1992. The 23S/5S ribosomal RNA genes (rrl/rrf) are separate from the 16S ribosomal RNA gene (rrs) in *Borrelia burgdorferi*, the aetiological agent of Lyme disease. *J Gen Microbiol 138:* 871–877.
28. Schwartz JJ, Gazumyan A, Schwartz I, 1992. rRNA gene organization in the Lyme disease spirochete, *Borrelia burgdorferi. J Bacteriol 174:* 3757–3765.
29. Postic D, Assous MV, Grimont PAD, Baranton G, 1994. Diversity of *Borrelia burgdorferi* sensu lato evidenced by restriction fragment length polymorphism of rrf(5S)-rrl(23S) intergenic spacer amplicons. *Int J Syst Bacteriol 44:* 743–752.
30. Higgines DG, Bleasby AJ, Fuchs R, 1992. CLUSTAL V: improved software for multiple sequence alignment. *Computer Appl Biosciences 8:* 189–191.
31. Saitou N, Nei M, 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol Biol Evol 4:* 406–425.
32. Anderson JF, Magnarelli LA, LeFebvre RB, Andreadis TG, McAninch JB, Perng GC, Johnson RC, 1989. Antigenically variable *Borrelia burgdorferi* isolated from cottontail rabbits and *Ixodes dentatus* in rural and urban areas. *J Clin Microbiol 27:* 13–20.
33. Park KH, Chang WH, Schwan TG, 1993. Identification and characterization of Lyme disease spirochetes, *Borrelia burgdorferi* sensu lato, isolated in Korea. *J Clin Microbiol 31:* 1831–1837.
34. Postic D, Ras NM, Lane RS, Hendson M, Baranton G, 1998. Expanded diversity among California *Borrelia* isolates and description of *Borrelia bissettii* sp. nov. (formally *Borrelia* group DN127). *J Clin Microbiol 36:* 3497–3504.
35. Mathiesen DA, Oliver JH Jr, Kolbert CP, Tullson ED, Johnson BJB, Campbell GL, Mitchell PD, Reed KD, Telford SR III, Anderson JF, Lane RS, Persing DH, 1997. Genetic heterogeneity of *Borrelia burgdorferi* in the United States. *J Infect Dis 175:* 98–107.
36. Liveris D, Gazumyan A, Schwartz I, 1995. Molecular typing of *Borrelia burgdorferi* sensu lato by PCR-restriction fragment length polymorphism analysis. *J Clin Microbiol 33:* 589–595.
37. Masuzawa T, Komikado T, Yanagihara Y, 1997. PCR-restriction length polymorphism analysis of the OspC gene for detection of mixed culture and for epidemiological typing of *Borrelia burgdorferi* sensu stricto. *Clin Diag Lab Immunol 4:* 60–63.
38. Li MQ, Masuzawa T, Takada N, Ishiguro F, Fujita H, Iwaki A, Wang H, Wang JC, Kawabata M, Yanagihara Y, 1998. Lyme

disease *Borrelia* species in northeastern China resemble those isolated from Far eastern Russia and Japan. *Appl Env Microbiol 64:* 2705–2709.
39. Rijpkema SGT, Molkenboer MJCH, Schouls LM, Jongejan F, Schellekens JFP, 1995. Simutaneous detection and genotyping of three genomic groups of *Borrelia burgdorferi* sensu lato in Dutch *Ixodes ricinus* ticks by characterization of the amplified intergenic spacer region between 5S and 23S rRNA genes. *J Clin Microbiol 33:* 3091–3095.
40. Fleche AL, Postic D, Girardet K, Peter O, Baranton G, 1997. Characterization of *Borrelia lusitaniae* sp. nov. by 16S ribosomal DNA sequence analysis. *Int J Syst Bacteriol 47:* 921–925.
41. Wang GQ, vanDam AP, Schwartz I, Dankert J, 1999. Molecular typing of *Borrelia burgdorferi* sensu lato: taxonomic, epidemiological, and clinical implications. *Clin Microbiol Rev 12:* 633–653.
42. Livey I, Gibbs CP, Schuster R, Dorner F, 1995. Evidence for lateral transfer and recombination in OspC variation in Lyme disease *Borrelia. Mol Microbiol 18:* 257–269.