Sylvatic Maintenance of *Borrelia burgdorferi* (Spirochaetales) in Northern California: Untangling the Web of Transmission

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ABSTRACT Lyme borreliosis is associated with several genospecies of *Borrelia burgdorferi* sensu lato (s.l.) (Spirochaetales), but human disease has been associated only with *Borrelia burgdorferi* sensu stricto (s.s.) Johnson, Schmid, Hyde, Steigerwalt & Brenner in the western United States. Restriction fragment length polymorphism (RFLP) analysis of *rrf-rrl* amplicons from 124 tick and mammalian isolates from various habitats yielded 13 RFLP patterns. Of these patterns, six were patterns previously associated either with *Borrelia bissettii* Postic, Marti Ras, Lane, Hendson & Baranton or *Borrelia burgdorferi* s.s., and the remaining seven patterns belonged to diverse and previously uncharacterized *Borrelia* spp. Uncharacterized *Borrelia* spp. were cultured most frequently from *Ixodes spinipalpis* Hadwen & Nuttall and California kangaroo rats, *Dipodomys californicus* Merriam, inhabiting grasslands, and *B. bissettii* from *I. spinipalpis* and dusky-footed woodrats, *Neotoma fuscipes* Baird, associated with oak woodlands or chaparral. *B. burgdorferi* s.s. typically was isolated from host-seeking *Ixodes pacificus* Cooley & Kohls collected in dense oak woodlands, woodland–grass, or redwood forests. Although some isolates of *B. burgdorferi* s.s. were cultured from woodrats, there was no clear association of this human pathogen with any vertebrate host. These findings, along with recent evidence indicating that the western gray squirrel, *Sciurus griseus* Ord, may be an important reservoir of *B. burgdorferi* s.s. in Californian oak woodlands, suggest that our earlier hypothesis implicating an enzootic cycle involving woodrats and *I. spinipalpis* is insufficient to account for observed patterns of infection in nature.

KEY WORDS disease reservoirs, arthropod vectors, *Ixodes*, *Neotoma fuscipes*, *Borrelia burgdorferi*

Lyme disease is caused by tick-borne spirochetes belonging to the *Borrelia burgdorferi* sensu lato (s.l.) (Spirochaetales) complex. To date, this complex contains 12 named genospecies, 10 genospecies of which occur only in Europe and Asia (Richter et al. 2004, Steere et al. 2005) and an unknown number of "types" that remain unnamed. Three genospecies—*Borrelia burgdorferi* sensu stricto (s.s.) Johnson, Schmid, Hyde, Steigerwalt & Brenner; *Borrelia afzelii* Canica, Nato, du Merle, Mazie, Baranton & Postic; and *Borrelia garinii* Baranton, Postic, Saint Girons, Boerlin, Piffaretti, Assousc & Grimont—commonly are associated with human cases of Lyme disease (Steere 2001, Piesman 2002, Steere et al. 2005). *Borrelia bissettii* Postic, Marti Ras, Lane, Hendson & Baranton (Strle 1999), *Borrelia spielmani* Richter, Schlee, Allgower & Matuschka (Richter et al. 2004), and *Borrelia lusitaniae* Le Fleche, Postic, Girardet, Peter & Baranton (Collares-Pereira et al. 2004) have been isolated from a small number of human patients in Europe, but only *B. burgdorferi* s.s. has been associated with human cases of Lyme disease in North America. *B. burgdorferi* s.s. and *B. bissettii* have been isolated repeatedly from ticks and rodents in California (Lane and Pascocello 1989; Schwan et al. 1993; Zingg et al. 1993; Postic et al. 1998, 1999; Vredevoe et al. 2004). Additionally, considerable genomic variation exists among undescribed isolates of *B. burgdorferi* s.l. that have yet to be associated with disease (Postic et al. 1998), and such unnamed variants collectively have been referred to as *Borrelia* spp.

B. burgdorferi s.l. is transmitted throughout the Holarctic primarily by ticks of the *Ixodes ricinus* complex (Eisen and Lane 2002), including the primary vectors *Ixodes scapularis* Say and *Ixodes pacificus* Cooley & Kohls in North America (Piesman 2002). Additional North American species of the *I. ricinus* complex include *Ixodes affinis* Neumann, *Ixodes angustus* Neumann, *Ixodes cookei* Packard, *Ixodes jellisoni* Cooley & Kohls, *Ixodes minor* Neumann, *Ixodes muris* Bishopp & Smith, and *Ixodes spinipalpis* Hadwen & Nuttall (Caporale et al. 1995, Norris et al. 1999, Xu et al. 2003). With the exception of *I. cookei* (Ryder et al. 1992), each of these species seems to have some degree of vector competence for *B. burgdorferi* s.l. (Brown and Lane 1992, Lane et al. 1999, Dolan et al. 2000, Peavey et al. 2000, Eisen et al. 2003, Oliver et al. 2003).

In the northeastern and north central regions of the United States, enzootic maintenance of *B. burgdorferi*

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s.s. most commonly involves a single vector, *I. scapularis,* and a single mammalian reservoir, *Peromyscus leucopus* Rafinesque (Piesman 2002, Steere et al. 2005). However, involvement of several additional reservoir hosts and vectors has been identified in enzootic cycles of local or regional importance (Telford and Spielman 1989, Slajchert et al. 1997, Oliver et al. 2003). In contrast, the ecology of Lyme borreliosis in California is more complex. At least three tick species, *I. pacificus, I. spinipalpis,* and *I. jellisoni,* and three rodent hosts, *Neotoma fuscipes* Baird (dusky-footed woodrat; hereafter referred to as woodrats), *Dipodomys californicus* Merriam (California kangaroo rat), and *Sciurus griseus* Ord (western gray squirrel), have been identified as key participants in sylvatic transmission cycles (Lane and Brown 1991; Brown and Lane 1992, 1996; Lane et al. 2005), and an isolate of *B. burgdorferi* s.l. was obtained from *I. angustus* collected in northeastern California (Gordus 1992). However, virtually all transmission of *B. burgdorferi* s.l. to people in California is thought to occur through the attachment of *I. pacificus* (Burgdorfer et al. 1985, Lane and Lavoie 1988, Clover and Lane 1995) because *I. angustus* and *I. spinipalpis* ticks seldom attach to people and *I. jellisoni* is not known to bite humans (Furman and Loomis 1984).

In comparison with the northeastern United States, the incidence of human cases of Lyme disease in California is low (CDC 2002). However, pockets of hyperendemicity exist in several northwestern counties, including Mendocino and Sonoma (Lane et al. 1992, Ley et al. 1994). The lower prevalence of Lyme disease in California has been attributed to 1) a parallel cycle involving nonhuman-biting maintenance vectors (Brown and Lane 1992); 2) the borreliacidal alternative complement pathway in the blood of the western fence lizard, *Sceloporus occidentalis* Baird & Girard, and the southern alligator lizard, *Elgaria multicarinata* Blainville, which serve as primary hosts for *I. pacificus* subadults (Lane and Quistad 1998, Wright et al. 1998, Kuo et al. 2000); and 3) a slightly lower vector competence of *I. pacificus* compared with *I. scapularis* (Piesman 2002).

Genotyping techniques, such as restriction fragment length polymorphism (RFLP) analysis of *rrf* (5S)-*rrl* (23S) ribosomal intergenic spacer amplicons, have provided insight into the diversity of *B. burgdorferi* s.l. in California. However, the number of isolates analyzed in individual studies has been relatively small (Lane and Pascocello 1989; Schwan et al. 1993; Zingg et al. 1993; Postic et al. 1994, 1998, 1999). Given the diversity of *Borrelia* spp. and the complexity of transmission cycles in California, a comprehensive genotypic analysis may help to explain the patterns of enzootic cycles and provide information necessary for efforts directed at minimizing human exposures to borreliae. The purpose of this study was to reexamine the sylvatic maintenance of *B. burgdorferi* s.l. spirochetes in northern California in relation to their tick vectors, small mammalian hosts, and vegetational types by RFLP analysis of *rrf-rrl* amplicons from 124 *B. burgdorferi* s.l. isolates. This is by far the largest series of *B. burgdorferi* s.l. isolates characterized from western North America.

Materials and Methods

Isolate Selection and Preparation. Isolates were selected from frozen stocks of *Borrelia* spp. originating from wild-caught ixodid ticks or small mammals in California between 1986 and 1999. These isolates were cultured during the course of ecological or epidemiological studies having varied goals and following different sampling designs (Burgdorfer et al. 1985; Lane and Burgdorfer 1988; Lane and Lavoie 1988; Lane and Brown 1991; Lane and Loye 1991; Brown and Lane 1992, 1996; Lane et al. 1992, 2001; Eisen et al. 2004). As a result, sampling of tick and rodent populations was not random nor was it standardized. In general, rodents were captured in live traps (H. B. Sherman Traps, Inc., Tallahassee, FL; Tomahawk Live Trap Company, Tomahawk, WI; and National Brand Live Trap Company, Tomahawk, WI), weighed, morphometrics were recorded, and they were identified to species. Each mammal was anesthetized with methoxyflurane, and both surfaces of the ear pinnae were cleaned by scrubbing with a 5% Betadine solution followed by 70% alcohol. Two 2-mm ear-punch biopsies were collected and cultured in ≈1 ml of modified Barbour–Stoenner–Kelly (BSK) II medium (Lane and Brown 1991; Brown and Lane 1992, 1996). Ticks were collected by flagging vegetation, by removing them from mammals directly with forceps, or by housing the host over water to collect replete ticks after they had finished feeding. All handling and sampling of mammals was conducted as described in protocols approved by the Institutional Animal Care and Use Committee at the University of California at Berkeley. The collection localities, all in northern California, included sites in the counties of Alameda, Contra Costa, Marin, Mendocino, Placer, Sonoma, and Yuba.

Low passage cultures of six or fewer passages were used whenever possible to minimize the influence of laboratory-induced selection. Frozen isolates were thawed rapidly and cultured in BSK II or BSK-H (Sigma-Aldrich, St. Louis, MO) medium at ≈34°C. Two milliliters of spirochetal suspension (density ≈100 spirochetes per 400× field) were washed twice with 0.01 M phosphate-buffered saline and 5 mM $MgCl_2$ and resuspended with 50–200 μl of LTE (10 mM Tris and 0.1 mM EDTA, pH 8.0) with 1% Tergitol (Sigma-Aldrich). The suspensions were boiled for 10 min and were stored at −20°C.

Polymerase Chain Reaction (PCR) of *rrf-rrl* Region. The *rrf* (5S)-*rrl* (23S) intergenic spacer was amplified using the primer set *rrf-rrl* one (5′-CTG CGA GTT CGC GGG AGA-3′) and *rrf-rrl* two (5′-TCC TAG GCA TTC ACC ATA-3′) (QIAGEN Operon, Alameda, CA) and reagents as described previously (Postic et al. 1994). *B. burgdorferi* s.s. isolate CA4 and a PCR grade water blank were included in all runs as positive and negative controls, respectively. A PTC200 thermal cycler (MJ Research, Waltham, MA) was used with an initial condition of 5 min at 95°C,

followed by 30 cycles of denaturation at 95°C for 1 min, annealing at 52°C for 1 min, and extension at 72°C for 2 min. A final elongation cycle at 72°C for 10 min was used. The reactions were loaded on a 10- by 12-cm 1.4% Tris-acetate-EDTA agarose gel containing ethidium bromide and run at 50 V for 30 min. A pBR322 HaeIII digest molecular size ladder (Sigma-Aldrich) was used as an amplicon size reference. The presence of 220–260-bp amplicons was confirmed under UV light.

Restriction Digest and Gel Electrophoresis. The *rrf-rrl* amplicons were digested overnight at 37°C using the restriction enzymes MseI and DraI with buffer conditions recommended by the manufacturer (New England Biolabs, Beverly, MA), as described previously (Postic et al. 1994). In total, 12 μl of PCR product was used for the DraI reaction. Twice as much volume was used with MseI to accommodate duplicate gels.

The endonuclease digests were run for 3.5 h on a 0.75-mm 16% acrylamide, 0.8% bisacrylamide Tris borate-EDTA minigel at 100 V. Each gel contained a negative control, a CA4 positive control, and HaeIII digested pBR322 (Sigma-Aldrich) for a molecular size reference. MseI gels were run in duplicate. The gels were stained in an ethidium bromide bath and photographed on an UV light box.

Analysis of RFLP Patterns. The distance from the bottom of the sample well to the middle of each of the pBR322 bands was measured on the photograph. A standard curve was created with Cricket Graph (Computer Associates, Islandia, NY) by plotting band size (in bp) versus distance (in mm) and employing a power fit. The equation for the standard curve was used to estimate the size of the restriction fragments for each isolate. RFLP groups were assigned primarily by MseI digest pattern and secondarily by DraI pattern.

Sequence Analysis. To validate fragment size estimates, representative isolates from each of the RFLP groups were selected for sequence analysis. The *rrf-rrl* amplicons were purified with the QIAquick PCR purification kit (QIAGEN, Valencia, CA) and prepared for cycle sequencing using Terminator Ready Reaction mix (PerkinElmer Life and Analytical Sciences, Boston, MA), as per the manufacturer's instructions. Thermal cycler conditions were set according to the instructions included with the sequencing reagent. The cycle sequencing products were purified with Centri·Sep columns (Princeton Separations, Adelphia, NJ) and dried with a speed-vacuum. Purified cycle sequencing products were run on an ABI 377 automated sequencer (Applied Biosystems, Foster City, CA) following the manufacturer's instructions.

Sequence and Phylogenetic Analyses. Sequences were aligned using Sequence Navigator software (Applied Biosystems). Additional sequences from other *B. burgdorferi* s.l. isolates were identified through GenBank and included in the analyses. PHYLIP 3.6 software (Felsenstein 1989) was used to construct phylogenetic trees using distance matrix-based neighbor-joining (NJ) (Saitou and Nei 1987), unweighted pair-group method with arithmetic mean (unweighted pair-group method with arithmetic average), and maximum likelihood (ML) (Felsenstein and Churchill 1996) analyses from single and multiple bootstrapped (100 replicates) sequence sets. The Jukes and Cantor model was used for distance matrix calculations (Jukes and Cantor 1969). Phylogenetic trees were formatted with TreeView 1.6.6. software (Page 1996).

Habitat Classification. Habitat associations obtained from field notebooks were classified as chaparral, grassland, oak woodland, woodland–grass, redwood forest, or unknown (when available data were inadequate for positive identification of habitat type). Chaparral was characterized by chamise, *Adenostoma fasciculatum* Hooker & Arnott; California lilac, *Ceanothus* spp.; oaks (*Quercus* spp., including scrub oak, *Quercus dumosa* Nuttall, and leather oak *Quercus durata* Jepson); and, occasionally, manzanita (*Arctostaphylos* spp.). Grasslands were pastures or fields with <10% trees or shrubs. In oak woodlands, canopies of oaks (*Quercus* spp., including interior live oak, *Quercus wislizenii* de Candolle, California black oak, *Quercus kelloggii* Newberry, and Oregon white oak, *Quercus garryana* Douglas); madrone, *Arbutus menziesii* Pursh; and California bay, *Umbellularia californica* Nuttall, were fairly closed and lacked extensive understory of shrubs or grass; these dense woodlands are associated with accumulation of ground leaf litter. Woodland–grass was either woodland composed of various species of oaks (*Quercus* spp.) and California bay with an open canopy and a grass understory, or the transitional ecotone between distinct patches of oak woodland and grass. Redwood forests contained primarily coast redwoods, *Sequoia sempervirens* (Don) Endlicher, with or without tan oaks, *Lithocarpus densiflorus* Hooker & Arnott, and tended to be moister and occur in closer proximity to the Pacific Coast than did the other habitats sampled.

Results

RFLP and Sequence Analyses. In total, 124 isolates produced 125 *rrf-rrl* amplicons ranging in size from 226 to 257 bp, and 13 RFLP patterns (groups A–M) (Table 1); one culture contained a mixture of borreliae categorized as groups J and L. Eleven MseI digest patterns, including the one mixed infection, were detected (Fig. 1). Although fragments <22 bp were not visually detectable, this exclusion did not affect determination of RFLP pattern by gel electrophoresis. The only patterns that required sequence analysis for differentiation were groups H and J. Their single base pair difference was difficult to detect by measurement of restriction fragments. Small differences (1 or 2 bp) within the grouped sequences were noted, and all variants were included in the phylogenetic analyses. Amplicon sequences were submitted to GenBank and assigned the accession numbers AY177630 to AY177643 and AY182037 to AY182061.

Phylogenetic Analyses. Phylogenetic trees were generated from unweighted pair-group method with arithmetic average, NJ, and ML analyses of 100 bootstrapped *rrf-rrl* amplicon-sequence replicates from

Table 1. RFLP groups of *B. burgdorferi* s.l. isolates displayed by genospecies, associated *Mse*I and *Dra*I digest patterns, and size of *rrf-rrl* amplicons

Isolate	RFLP group	*Mse*I fragment (bp)	*Dra*I fragment (bp)	Amplicon size (bp)
B. burgdorferi s.s.				
(CA4, CA5, CA6, CA7, CA16, CA17, CA18, CA30, CA57, CA58, CA59, CA129, CA359, CA361, CA382, CA417, CA418, CA419, CA420, CA470, CA518, CA521, CA528, CA529, CA530, CA531, CA533, CA534, CA535, CA536, CA537, CA538, CA541, CA543, CA544, CA551, CA553, CA567)	A	107, 52, 38, 29, 28	144, 53, 29, 28	254
(CA3, CA10, CA11, CA12, CA15, CA21, CA172, CA337, CA338, CA358, CA360, CA421, CA460, CA461, CA517, CA522, CA526, CA542, CA549, CA550, CA566)	B	107, 52, 40, 29, 28	146, 53, 29, 28	256
B. bissettii				
(CA370)	C	107, 53, 38, 28	144, 82	226
(CA24, CA25, CA41, CA44, CA53, CA54, CA128, CA136, CA323, CA369, CA374, CA375, CA377, CA379, CA389, CA390, CA391, CA392, CA403, CA438, CA439, CA441)	D	107, 52, 38, 29, 27	144, 53, 29, 27	253
(CA35, CA83, CA135, CA137, CA138, CA140, CA238, CA367, CA372, CA376, CA380, CA387, CA474)	E	107, 52, 38, 33, 27	144, 53, 33, 27	257
Borrelia spp.				
(CA400, CA401)	F	90, 52, 38, 29, 28, 17	144, 81, 29	254
(CA388)	G	90, 50, 38, 29, 28, 17	144, 108	252
(CA22, CA134, CA404, CA411, CA442, CA446, CA448, CA462, CA468, CA502, CA504, CA507, CA552, CA1107, CA1133)	H	107, 51, 37, 30, 28	173, 80	253
(CA402)	I	107, 53, 40, 16, 13	159, 70	229
(CA20, CA33)	J	107, 52, 37, 30, 28	173, 81	254
(CA393, CA399)	K	90, 51, 38, 29, 28, 17	144, 52, 29, 28	253
(CA142, CA445, CA547, CA1176)	L	90, 52, 37, 30, 28, 17	173, 81	254
(CA426)	M	90, 51, 38, 28, 22, 17, 7	144, 109	253

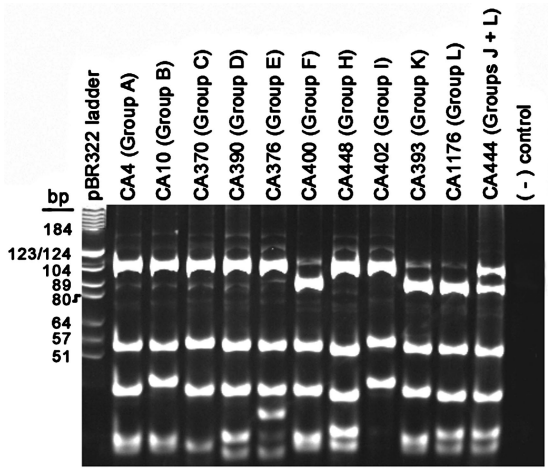

Fig. 1. Ethidium-bromide stained 16% acrylamide, 0.8% bisacrylamide, gel of MseI-digested *rrf-rrl* amplicons from 11 isolates of *B. burgdorferi* s.l. representing 109 digest patterns (groups A–F, H, I, K, and L) and one mixed infection (CA444). The molecular size ladder (pBR322 HaeIII digest) is shown in the lane on the far left, and the negative control is shown in the lane on the far right of the figure.

representative isolates and *B. burgdorferi* s.l. sequences identified on GenBank. A phylogenetic tree was produced with a representative sequence set by using the unweighted pair-group method with arithmetic average method (Fig. 2); GenBank accession numbers are shown after the isolate names, and RFLP group designations are displayed for isolates included in this study. In all trees, *B. burgdorferi* s.s. isolates clustered together as did *B. bissettii*, except for group C isolate CA370, which fell outside the main cluster in trees generated with the NJ and ML methods. Commonalities in *Borrelia* spp. groupings also were observed among the phylogenetic trees produced with the different methods. This included the clustering of groups F and K with *B. burgdorferi* s.s. isolates, groups G and M with isolate CA2, and groups H, J, and L. Within-group sequence variants clustered together, with the exception of pattern A isolate CA361, which clustered with group B.

Mean within-genospecies sequence variance of 0.8% (range 0.0–1.6%), 1.3% (range 0.0–2.9%), and 4.2% (range 0.0–7.6%) for *B. burgdorferi* s.s., *B. bissettii*, and uncharacterized *Borrelia* spp., respectively, were similar among RFLP groupings. The difference between mean sequence variances of *B. burgdorferi*

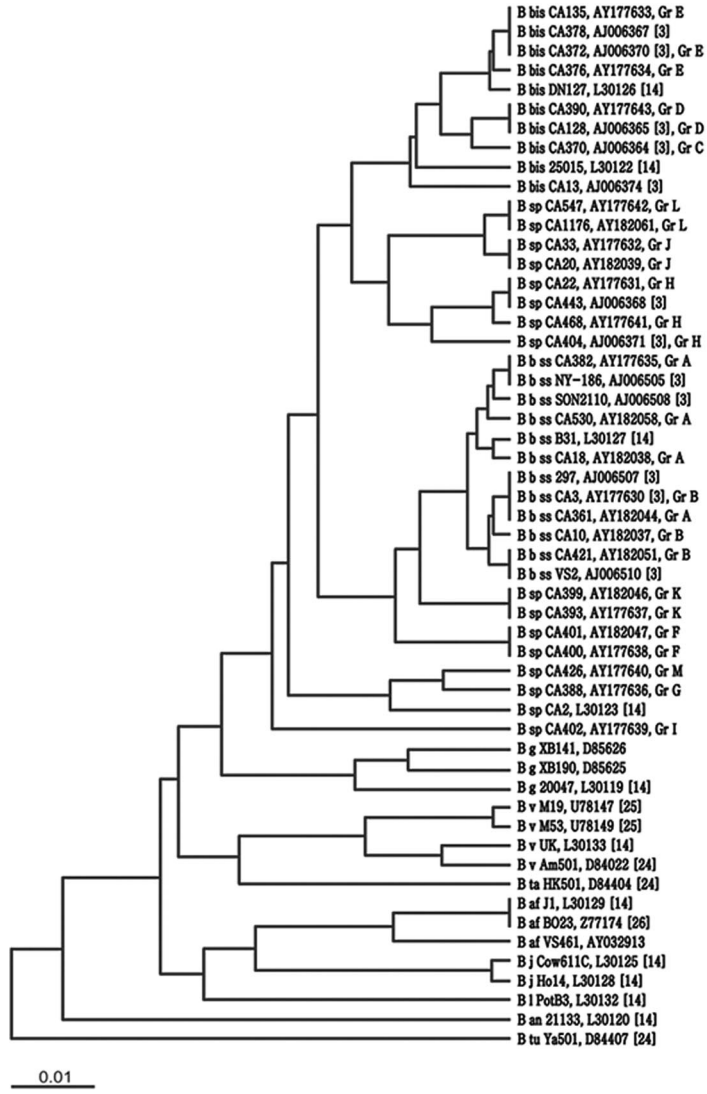

Fig. 2. Phylogenetic tree of *B. burgdorferi* s.l. *rrf-rrl* sequences, including isolates representative of RFLP groups and additional isolates identified on GenBank. The tree was produced with distance data generated by the unweighted pair-group method with arithmetic average method by using borrelial isolates representing *B. afzelii* (*Baf*), *B. andersonii* (*Ban*), *B. bissettii* (*Bbis*), *B. burgdorferi* s.s. (*Bbss*), *B. garinii* (*Bg*), *B. lusitaniae* (*Bl*), *B. tanukii* (*Bta*), *B. turdi* (*Btu*), and *Borrelia* sp. (*Bsp*). RFLP group (Gr) assignments are shown for isolates tested in this study, and the GenBank sequence accession number are shown after the isolate name. The reference bar represents 1.0% divergence.

s.s. and *B. bissettii* was 3.9% (range 1.8–5.8%), between *B. burgdorferi* s.s. and uncharacterized genospecies was 4.9% (range 1.6–7.7%), and between *B. bissettii* and uncharacterized genospecies was 4.0% (range 1.8–5 0.9%).

Host Species and Habitats. Seventy-five of the 124 isolates (Table 2) were cultured from tick tissues, including 57 (76.0%) from *I. pacificus*, 15 (20.0%) from *I. spinipalpis*, and three (4.0%) from *I. jellisoni.* Forty-nine isolates cultured from mammals included 23 (46.9%) from *N. fuscipes;* 20 (40.8%) from *D. californicus;* four (8.2%) from piñon mice, *Peromyscus truei* Shufeldt; and one each (2.0%) from a deer mouse, *Peromyscus maniculatus* Wagner, and a brush rabbit, *Sylvilagus bachmani* Waterhouse. One isolate from a *D. californicus* contained two different genospecies (one of RFLP group J and the other from RFLP group L); these are shown separately in Table 2. Isolates were obtained from ticks or mammals collected in habitats classified as oak woodlands ($n = 54$; 43.2%), chaparral ($n = 25$; 20.0%), grassland ($n = 21$; 16.8%), mixed woodland–grass ($n = 18$; 14.4%), redwood forest ($n = 4$; 3.2%), or in unspecified habitats ($n = 3$; 2.4%).

Fifty-two of the 59 isolates classified as *B. burgdorferi* s.s. were from *I. pacificus,* and five were from

Table 2. Number of isolates of *B. burgdorferi* sensu lato characterized as RFLP groups A–M stratified by hosts, vectors, and habitats from which samples were obtained

Host species	RFLP group													Total
	B. burgdorferi s.s.		*B. bissettii*			Uncharacterized *Borrelia* spp.								
	A	B	C	D	E	F	G	H	I	J	K	L	M	
Mammals														
N. fuscipes	3w; 1c	1w	1w	5w; 6c	6w									23
D. californicus		1c						1c; 12g		2c; 1g		4g		21
P. truei				3c	1u									4
P. maniculatus				1w										1
S. bachmani													1c	1
Ticks														
I. pacificus	18w; 1c; 4r; 10wg	8w; 1c; 8wg; 1 g; 1u		4w				1c						57
I. spinipalpis				2c; 1w	4c; 1w	2w	1u		1w		2w	1c		15
I. jellisoni	1 g				1g			1g						3
Total	38	21	1	22	13	2	1	15	1	3	2	5	1	125

Numbers refer to the number of isolates characterized from each host or vector species and the letters designate the type of habitat from which the samples were collected: c, chaparral; g, grassland; u, unknown or a sampling site about which we lack confidence in assigning a habitat classification; w, oak woodland; and wg, mixed woodland grass or ecotonal transition from grass to woodland.

woodrats. This genospecies was recovered also from a single *I. jellisoni* and a single California kangaroo rat. Of these, the majority (30 and 18, respectively) were from oak woodland or woodland–grass habitats with the remaining coming from redwood forest ($n = 4$), chaparral ($n = 4$), or grassland ($n = 2$). The habitat association of one isolate from an *I. pacificus* was not available.

Eighteen of the 35 *B. bissettii* isolates were from woodrats, and eight were from *I. spinipalpis*. *B. bissettii* also was cultured from four piñon mice, one deer mouse, four *I. pacificus*, and one *I. jellisoni*. Nineteen of the 35 *B. bissettii* isolates were collected from oak woodlands, 15 were from chaparral, and one was from grassland.

Twenty of the 30 isolates classified as unnamed *Borrelia* spp. were obtained from California kangaroo rats, and seven were from *I. spinipalpis*. Additionally, *Borrelia* spp. isolates were cultured from one brush rabbit, one *I. pacificus*, and one *I. jellisoni*. Eighteen of the 30 isolates were obtained from grassland, six were from chaparral, five were from oak woodlands, and one was from an *I. spinipalpis* from an unidentified habitat.

Five of 23 isolates cultured from woodrats were *B. burgdorferi* s.s., and no unnamed *Borrelia* spp. were characterized from this host. In contrast, 20 of 21 isolates from kangaroo rats were characterized as *Borrelia* spp., whereas only one was characterized as *B. burgdorferi* s.s. Thus, *B. bissettii* and *Borrelia* spp. were associated with different rodent host species (Fisher exact text; $P < 0.001$).

All isolates characterized from *I. spinipalpis* were either *B. bissettii* ($n = 8$) or unnamed *Borrelia* spp. ($n = 7$), whereas 52 of the 57 isolates characterized from *I. pacificus* were *B. burgdorferi* s.s. We conclude that the human pathogen *B. burgdorferi* s.s. is associated more frequently with *I. pacificus* than with *I. spinipalpis*, whereas *B. bissettii* and *Borrelia* spp. are associated more frequently with *I. spinipalpis* than with *I. pacificus* (Fisher exact test; $P < 0.001$). The three *I. jellisoni* isolates were characterized as one each of *B. burgdorferi* s.s. (group A), *B. bissettii* (group E), and *Borrelia* sp. (group H).

Most of the 59 isolates characterized as *B. burgdorferi* s.s. were from oak woodland ($n = 30$) or woodland grass ($n = 18$), and only 11 were collected from other habitats. *B. bissettii* was collected most frequently from either woodland ($n = 19$) or chaparral ($n = 15$); one isolate was from grassland. In contrast, 18 of 30 isolates characterized as *Borrelia* spp. were from grassland versus 11 from oak woodland ($n = 5$) or chaparral ($n = 6$). *B. burgdorferi* s.s. was associated more often with oak woodland or mixed woodland–grass, whereas the other genospecies were associated more often with chaparral or grassland (Fisher exact test; $P < 0.001$).

Discussion

RFLP analysis identified 13 different genomic groups of *B. burgdorferi* s.l. that were cultured from rodents and ticks collected in northern California. Six of the genomic groups (groups A–E and H) were described previously (Postic et al. 1994, 1998) and seven (groups F, G, and I–M) are newly described herein. The combination of these RFLP groups into different genospecies is supported both by the phylogenetic analysis and the greater degree of genomic variation reported between groups than within groups. The phylogenetic analysis is generally consistent with previous reports of borrelial relatedness (Postic et al. 1998, Wang et al. 1999, Lee et al. 2003), and it facilitates comparison of relatedness of isolates from within California to isolates of other genospecies from other regions. Evaluation of phylogenetic relatedness in the context of known or suspected maintenance cycles may provide insight into expected patterns of cycles yet to be described. Consistent results between our NJ, unweighted pair-group method with arithmetic average, and ML phylogenetic analyses suggest that RFLP groups F and K are closely related to, or may be identical with, *B. burgdorferi* s.s. Confirmation with other genotyping methods, however,

will be required to confirm this assertion. The analysis supports the generally held belief that *B. burgdorferi* s.s. and *B. bissettii* are monophyletic groups but that unnamed *Borrelia* spp. from California represent two or more polyphyletic genospecies. Groups J, H, and L cluster as a distinct sister group related to, but distinct from, *B. bissettii.* Groups F and K seem closely related to *B. burgdorferi* s.s., and groups L and M (along with previously reported CA 2) seem to represent an unaffiliated genospecies of *Borrelia* sp.

Previous studies of mammalian hosts of *B. burgdorfe*ri s.l. in northern California reported the primary importance of the dusky-footed woodrat and possibly a secondary role in some habitats for California kangaroo rats (Lane and Brown 1991, Brown and Lane 1992). Similarly, this woodrat, and the deer mouse were identified as potential reservoir hosts of *B. burgdorferi* s.l. in Oregon (Burkot et al. 1999). Most of our isolates from woodrats were characterized as *B. bissettii*, a genospecies not known to cause disease in humans in North America; *B. bissettii*, or a related spirochete, has been implicated as a human pathogen in Slovenia (Strle 1999, Maraspin et al. 2002). Isolates of *B. bissettii* also were obtained from woodrats, including *N. fuscipes* and the desert woodrat, *Neotoma lepida* Thomas, trapped in coastal mountains of central California (Vredevoe et al. 2004). Interestingly, Burkot et al. (1999) did not identify *B. bissettii*-like spirochetes among their isolates from rodents, including *N. fuscipes*, in Oregon, and further molecular analysis will be required to clarify the geographic and host ranges of *B. bissettii* in the Pacific Northwest.

We characterized five isolates from woodrats as *B. burgdorferi* s.s., and woodrats remain a potential reservoir species in certain oak-dominated, and possibly redwood forest, habitat types. Woodrats are fed upon by *I. pacificus* immatures, they become infected in nature, they were found to be reservoir competent during long-term studies in captivity (Brown and Lane 1992), and they may harbor primarily *B. burgdorferi* s.s., or as yet unclassified spirochetes, in Oregon (Burkot et al. 1999). Our previous studies reported that 102 of the first 105 isolates of *B. burgdorferi* s.l. obtained from wildlife in California were cultured from woodrats, and all isolates were identified by their reactivities to six monoclonal antibodies by using indirect immunofluorescence assays and by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Lane and Brown 1991, Brown and Lane 1992). Nonetheless, the use of PCR and sequencing analysis have allowed us to more fully evaluate isolates obtained from different host species, and the cycle involving woodrats and *I. spinipalpis* seems to be less involved in the regional maintenance of *B. burgdorferi* s.s. than was implied previously.

Sylvatic maintenance of *B. burgdorferi* s.l. in northern California has been suggested to rely upon a complex web of transmission involving multiple tick vectors and mammalian reservoirs (Lane and Brown 1991; Brown and Lane 1992, 1996; Lane et al. 1999). Our findings, based on RFLP analysis of *rrf-rrl* intergenic spacer amplicons from borrelial isolates collected from different species of hosts and vectors, reveal the potential existence of genospecies-specific enzootic maintenance cycles with potentially unique combinations of vectors, mammalian hosts and habitats. The nonhuman biter *I. spinipalpis* participates in multiple cycles involving *B. bissettii* and *Borrelia* spp., but it may not be important in the enzootic maintenance of *B. burgdorferi* s.s. (Lane and Brown 1991; Brown and Lane 1992, 1996; Lane et al. 1999). Unfortunately, borrelial isolates were available from only five mammalian species, and of these isolates only six isolates were obtained from *P. truei*, *P. maniculatus*, and *S. bachmani.* It now seems of special importance that our isolates were obtained from such a limited number of mammalian species because *B. burgdorferi* s.s. recently was detected in eight of 10 western gray squirrels and in 47% of *I. pacificus* larva attached to them (Lane et al. 2005). Furthermore, western gray squirrels occur commonly throughout the oak woodlands in which *I. pacificus* has been found to be infected with *B. burgdorferi* s.s. Although the sample of squirrels was small, no other vertebrate species has fulfilled consistently the requirements for reservoir competence of this human pathogen in the far-western United States. The current report strengthens this suggestion of a squirrel reservoir by reiterating that *B. burgdorferi* s.s. is closely associated with oak woodlands and by the apparent lack of frequent association of the pathogen with woodrats or kangaroo rats.

The role of *I. jellisoni* in the enzootic cycle of *B. burgdorferi* s.l. is less certain because only three isolates were available for study and the genetic diversity detected was high. Interestingly, the isolates characterized from this tick encompassed each of the three genospecies, *B. burgdorferi* s.s., *B. bissettii*, and *Borrelia* sp. A cycle involving *D. californicus*, *I. jellisoni*, and at least two uncharacterized *Borrelia* spp. was identified previously from a grassland biotope in northwestern California (Brown and Lane 1996, Lane et al. 1999). These findings are supported by our characterization of isolates from kangaroo rats associated with grasslands as being primarily *Borrelia* spp. This association further supports a potential role for *I. jellisoni* in one or more of the cycles because *I. jellisoni* specializes on heteromyid rodents (Furman and Loomis 1984), and most of our collections of this tick were from *D. californicus* (Brown and Lane 1996, Lane et al. 1999).

We used descriptive statistics and contingency tables to associate patterns of *rrf-rrl* amplicons with hosts and vectors sampled during previous field studies. We were unable to use more powerful statistical methods to evaluate interactions among variables because sampling was nonrandom and nonstandardized across studies. Thus, although we report associations for the largest number of isolates ever assessed from the far west, our data can only be used to infer cause-and-effect relationships. Interactions among variables must be evaluated using multiple regression or other multivariate methods to model interaction within a large randomly sampled community of hosts and vectors because the variables are interdependent. We also

have grouped our habitat data into broad categories to provide adequate sample sizes for comparisons. Optimally, habitat evaluations should be conducted at multiple scales including vegetational surveys at micro-, meso-, and macroscales to allow ecological modeling of associations. The current study represents a necessary, albeit first, step toward achieving such a goal.

In conjunction with human exposures and data concerning enzootic prevalence, knowledge of genospecies-specific transmission cycles can be used to increase the efficacy of environmentally or behaviorally-based efforts to prevent Lyme disease. In contrast to the initial ecological studies of *B. burgdorferi* s.l. in northern California that suggest a single, complex, transmission web, we now suggest that genospecies-specific transmission cycles are driven interdependently by their vertebrate hosts, vectors, and affiliated habitats. *B. burgdorferi* s.s. is associated with *I. pacificus* in oak woodland, redwood, and woodland-grass biotopes. Oak woodland has been shown previously to support high acarological risk (Clover and Lane 1995, Talleklint-Eisen and Lane 1999, Eisen et al. 2002), suggesting that people who work or recreate in oak woodlands may be at elevated risk of exposure to spirochete-infected ticks. Subsequent spirochetal surveys of vertebrate hosts and ticks from such potentially high-risk habitats will allow development of models for evaluating interactions among environmental variables and provide additional insights into the sylvatic transmission cycles of *B. burgdorferi* s.l.

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