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Population Structure of the Lyme Borreliosis Spirochete *Borrelia burgdorferi* in the Western Black-Legged Tick (*Ixodes pacificus*) in Northern California▽

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Factors potentially contributing to the lower incidence of Lyme borreliosis (LB) in the far-western than in the northeastern United States include tick host-seeking behavior resulting in fewer human tick encounters, lower densities of *Borrelia burgdorferi*-infected vector ticks in peridomestic environments, and genetic variation among *B. burgdorferi* spirochetes to which humans are exposed. We determined the population structure of *B. burgdorferi* in over 200 infected nymphs of the primary bridging vector to humans, *Ixodes pacificus*, collected in Mendocino County, CA. This was accomplished by sequence typing the spirochete lipoprotein *ospC* and the 16S-23S rRNA intergenic spacer (IGS). Thirteen *ospC* alleles belonging to 12 genotypes were found in California, and the two most abundant, *ospC* genotypes H3 and E3, have not been detected in ticks in the Northeast. The most prevalent *ospC* and IGS biallelic profile in the population, found in about 22% of ticks, was a new *B. burgdorferi* strain defined by *ospC* genotype H3. Eight of the most common *ospC* genotypes in the northeastern United States, including genotypes I and K that are associated with disseminated human infections, were absent in Mendocino County nymphs. *ospC* H3 was associated with hardwood-dominated habitats where western gray squirrels, the reservoir host, are commonly infected with LB spirochetes. The differences in *B. burgdorferi* population structure in California ticks compared to the Northeast emphasize the need for a greater understanding of the genetic diversity of spirochetes infecting California LB patients.

In the United States, Lyme borreliosis (LB) is the most commonly reported vector-borne illness and is caused by infection with the spirochete *Borrelia burgdorferi* (3, 9, 52). The signs and symptoms of LB can include a rash, erythema migrans, fever, fatigue, arthritis, carditis, and neurological manifestations (50, 51). The black-legged tick, *Ixodes scapularis*, and the western black-legged tick, *Ixodes pacificus*, are the primary vectors of *B. burgdorferi* to humans in the United States, with the former in the northeastern and north-central parts of the country and the latter in the Far West (9, 10). These ticks perpetuate enzootic transmission cycles together with a vertebrate reservoir host such as the white-footed mouse, *Peromyscus leucopus*, in the Northeast and Midwest (24, 35), or the western gray squirrel, *Sciurus griseus*, in California (31, 46).

B. burgdorferi is a spirochete species with a largely clonal population structure (14, 16) comprising several different strains or lineages (8). The polymorphic *ospC* gene of *B. burgdorferi* encodes a surface lipoprotein that increases expression within the tick during blood feeding (47) and is required for initial infection of mammalian hosts (25, 55). To date, approximately 20 North American *ospC* genotypes have been described (40, 45, 49, 56). At least four, and possibly up to nine, of these genotypes are associated with *B. burgdorferi* invasiveness in humans (1, 15, 17, 49, 57). Restriction fragment length polymorphism (RFLP) and, subsequently, sequence analysis of the 16S-23S rRNA intergenic spacer (IGS) are used as molecular typing tools to investigate genotypic variation in *B. burgdorferi* (2, 36, 38, 44, 44, 57). The locus maintains a high level of variation between related species, and this variation reflects the heterogeneity found at the genomic level of the organism (37). The IGS and *ospC* loci appear to be linked (2, 8, 26, 45, 57), but the studies to date have not been representative of the full range of diversity of *B. burgdorferi* in North America.

Previous studies in the northeastern and midwestern United States have utilized IGS and *ospC* genotyping to elucidate *B. burgdorferi* evolution, host strain specificity, vector-reservoir associations, and disease risk to humans. In California, only six *ospC* and five IGS genotypes have been described heretofore in samples from LB patients or *I. pacificus* ticks (40, 49, 56) compared to approximately 20 *ospC* and IGS genotypes identified in ticks, vertebrate hosts, or humans from the Northeast and Midwest (8, 40, 45, 49, 56). Here, we employ sequence analysis of both the *ospC* gene and IGS region to describe the population structure of *B. burgdorferi* in more than 200 infected *I. pacificus* nymphs from Mendocino County, CA, where the

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incidence of LB is among the highest in the state (11). Further, we compare the Mendocino County spirochete population to populations found in the Northeast.

MATERIALS AND METHODS

Tick collection, spirochete culture, and DNA extraction. Questing *I. pacificus* nymphs were collected from 78 dense woodlands in Mendocino County, CA, in 2004 during their peak activity period (late April to early June), as previously described (19). Up to 100 ticks were tested for the presence of *B. burgdorferi* at each site, and this target number of ticks was reached in 51 (65%) sites. Total DNA was extracted from individual ticks using a DNeasy Blood and Tissue Kit (Qiagen, Valencia, CA) according to the manufacturer's protocol for animal tissues. DNA was extracted similarly from scrapings of frozen *B. burgdorferi* cultures derived from questing *I. pacificus* nymphs and adults collected between 1987 and 1999 from Marin, Sonoma, and Mendocino Counties in California. These isolates are part of the *Borrelia* collection housed at the University of California, Berkeley. *B. burgdorferi* isolate CA8, passage 7, derived from an *I. pacificus* adult collected in Sonoma County, CA, in 1987 (32), was cultivated in Barbour-Stoenner-Kelly II medium (4). The DNA was extracted from passage 9 as previously described (8). Data presented throughout this report refer to *I. pacificus* nymphs collected in 2004 unless otherwise noted.

Sequence analysis. (i) 5S-23S rRNA spacer region, IGS, and *ospC*. Amplification of the 5S-23S rRNA spacer region of *B. burgdorferi* by PCR was performed as described earlier (34) with minor modifications. Cycling conditions included a 4-min denaturation step at 95°C, followed by 40 cycles consisting of 95°C for 40 s, 52°C for 40 s for outer primers (58°C for internal primers), and 72°C for 1 min, followed by a 10-min extension at 72°C. Only DNA from ticks determined by sequence analysis of the 5S-23S region to be infected with *B. burgdorferi* (n = 263) was subjected to further PCR amplification using primers for IGS and *ospC* as previously described (8) with minor modifications. IGS and *ospC* PCRs were conducted using Phusion DNA polymerase (New England Biolabs, Ipswich, MA) or Amplitaq DNA polymerase (Applied Biosystems, Foster City, CA). The *ospC* PCR was performed using 35 amplification cycles for both first and second reactions and an annealing temperature of 50°C. Products from positive tick samples were purified using either a QIAquick PCR Purification Kit (Qiagen) for 5S-23S PCR products or a ZR-96 DNA Clean and Concentrator (Zymo Research, Orange, CA) for IGS and *ospC* products.

Both strands of purified 5S-23S DNA were sequenced by the University of California, Berkeley, CA, DNA Sequencing Facility using internal PCR primers (34). Purified IGS and *ospC* PCR products were sequenced by Polymorphic DNA Technologies, Alameda, CA. Primers used for IGS sequencing included a forward internal (nested) PCR primer (8) and another, overlapping forward primer, 5′-TTT CGC TAA AGT GCA AGG A-3′ (B. Travinsky et al., unpublished data). *ospC* sequencing was carried out using internal PCR primers (8).

The 5S-23S, IGS, and *ospC* sequences were assembled and manually edited using Sequencher 4.6 (Gene Codes Corp, Ann Arbor, MI). 5S-23S contigs were aligned with sequences selected from the GenBank database (http://www.ncbi.nlm.nih.gov/GenBank/index.html) using Clustal X (version 1.83.1) (54) and edited to 158 bp using Mesquite (version 2.6; http://mesquiteproject.org). *B. burgdorferi* assignment was based on phylogenetic analysis using the neighbor-joining method implemented in PAUP* (version 4.0 beta; Sinauer, Sunderland, MA) (uncorrected *P* distances).

IGS contigs were manually edited in Sequencher, resulting in 805- to 812-bp sequences which were subsequently aligned using Clustal X. The alignment was manually edited for minor errors using MacClade (version 4; Sinauer, Sunderland, MA). IGS sequences were assigned to genotype based on both neighbor-joining distance analysis in PAUP* and direct sequence comparison to IGS sequences available in the GenBank. IGS genotypes, designated by their GenBank accession number, are defined by as little as a 1-nucleotide (nt) difference. Position 1 of the IGS alignment corresponds to position 2424 of *B. burgdorferi* B31, GenBank accession number U03396, and position 444532 of the B31 genome sequence NC_001318. *ospC* contigs were edited similarly in Sequencher and MacClade, resulting in a 549-bp alignment. *ospC* sequences were compared to those available in GenBank, and genotype assignments were based on direct sequence comparison. Position 1 of the *ospC* alignment corresponds to position 396 of *B. burgdorferi* B31 *ospC* gene sequence U01894 and position 16993 of the B31 cp26 genome sequence NC_001903.

Nomenclature for *ospC* genotypes follows the groups system described by Seinost et al. (49) and Wang et al. (56). Sequences were designated new *ospC* genotypes when two or more tick samples contained a nucleotide sequence that differed from other genotypes by >8% (56). When *ospC* sequences were <8% different in sequence identity, alleles were assigned a name including the genotype and polymorphic nucleotide position number, e.g., *ospC* A (nt 123). The prevalence of *ospC* alleles in *I. pacificus* nymphs was statistically compared to the prevalence in *I. scapularis* nymphs in a study by Wormser et al. (57) using Fisher's exact test (two-tailed).

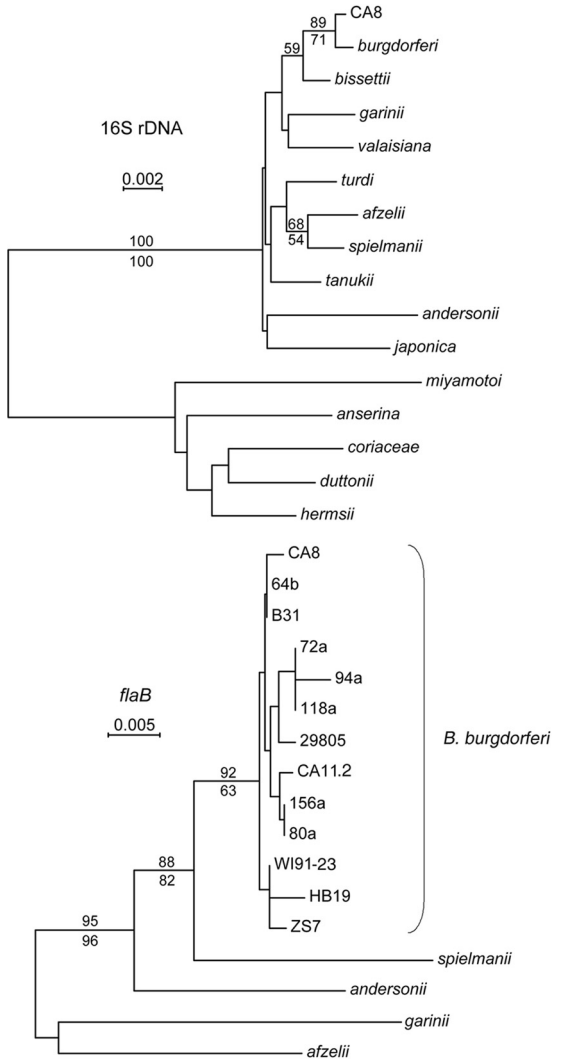

FIG. 1. Neighbor-joining distance phylograms for partial 16S rRNA gene (observed differences) and *flaB* (Tajima-Nei method) sequences of selected *Borrelia* species and *B. burgdorferi* strains. The upper panel includes relapsing-fever species as the outgroup. Nodes with bootstrap support (1,000 iterations) of ≥50% by distance criteria (above the line) and by maximum-likelihood criteria (below the line) are shown. CA8 is the representative strain expressing *ospC* H3. All *B. burgdorferi* strains, with the exception of CA8 and HB19, were subjected to whole-genome sequencing, and the sequences were obtained from the GenBank.

(ii) 16S rRNA and flagellin. To amplify regions of the 16S rRNA and flagellin (*flaB*) genes of California *B. burgdorferi* isolate CA8, PCR was performed as previously described (5) with some modification. Cycling parameters included an initial incubation for 3 min at 95°C and a final extension step of 75°C for 7 min using Phusion DNA polymerase. Primers used for *flaB* DNA amplification produced 641-bp products whose sequences were aligned using Clustal X, version 1.83, with other *B. burgdorferi* species (Fig. 1). 16S rRNA PCR produced 1,336-bp products whose sequences were aligned with other *Borrelia* species (Fig. 1). Phylogenetic analysis of alignments using neighbor-joining and maximum-likelihood criteria were performed with Phylo_win, version 2.0 (http://pbil.univ-lyon1.fr/software/phylowin.html) (23).

TABLE 1. *ospC* alleles identified in *B. burgdorferi*-infected *I. pacificus* ticks collected in 2004 and from 1987 to 1999 in Mendocino County

ospC genotype or allele	GenBank accession no.	Sequence length (nt; no gaps)	GenBank comparison data				Position of nucleotide change[b]	Nucleotide change	Amino acid change
			Best match accession no.	Strain identifier	% Similarity	Reference			
A		528	AY275213	B31	100	8	None		
B		531	AY275215	1-24	100	8	None		
B (nt 59)[a]	FJ932735	531	AY275215	1-24	99	8	59	C to T	T to M
D		531	L25413	CA-11.2A	100	56	None		
E3	FJ932732	531	EF592545	18.74/cp26	100	Unpublished	None		
F		525	L42896	27579	100	39	None		
I[c]		528	EU377752	CA92-1096	100[d]	40	None		
I3[a]	FJ932734	525	L42896	27579	94	39			
G		528	AY275223	2-43	100	8	None		
H3[a]	FJ932733	525	AY275221	NP40 (*ospC* E)	84	8			
H		528	EF053519	LDS79	100	17	None		
M (nt 348)[a]	FJ932736	534	AY275218	Bve	99	17	348	T to C	None
T		537	AY275222	10-33	100	17	None		

[a] Novel *ospC* genotype or allele.
[b] Based on 549-nt alignment (including gaps) unless otherwise indicated. Position number 1 of the alignment corresponds to position 396 of GenBank no. U01894 and position 16993 of the B31 cp26 genome sequence (GenBank no. NC_001903). For I3 and H3, see the text.
[c] Identified only in an *I. pacificus* male collected in 1992 (Table 3).
[d] Based on alignment of nt positions 4 to 487.

Habitat analysis of genotypes. Associations between habitat type and *ospC* genotypes were examined using Fisher's exact test (two-tailed). Collection sites were previously classified into seven habitat types based on field assessments (19), and we lumped six of these into two new broader categories: (i) hardwood-fir, composed of habitats that are suitable for LB reservoir western gray squirrels (46), and (ii) redwood-pine-tanoak, where western gray squirrels are not typically observed (31). The hardwood-fir category combined two habitat types: (i) hardwood with ≥90% *Quercus* sp. oaks, Pacific madrone (*Arbutus menziesii*), and California bay (*Umbellularia californica*) and (ii) mixed hardwood/conifer with ≥80% *Quercus* spp. oaks, Pacific madrone, California bay, Douglas fir (*Pseudotsuga menziesii*), and ponderosa pine (*Pinus ponderosa*). Tanoak (*Lithocarpus densiflorus*) represented 0 to 16% of trees at sites within these habitat types. The redwood-pine-tanoak category combined four habitat types: (i) redwood with ≥65% redwood (*Sequoia sempervirens*); (ii) coastal pine with ≥90% bishop pine (*Pinus muricata*), redwood, and Douglas fir; (iii) inland pine with ≥65% ponderosa pine and Douglas fir; and (iv) tanoak with >60% tanoak, redwood, and California bay. *Quercus* oaks were absent from or were rare in these habitat types. Four sites from a mixed habitat type previously referred to as tanoak-madrone-conifer (19) were excluded from the habitat analysis, meaning that 12 ticks infected with *B. burgdorferi ospC* genotype H3 ($n = 3$), *ospC* E3 ($n = 3$), *ospC* G ($n = 4$), *ospC* H ($n = 1$), and *ospC* M (nt 348) ($n = 1$) were eliminated.

To visualize the distributions of the three unique *ospC* genotypes in relation to habitat type (hardwood-fir versus redwood-pine-tanoak) throughout Mendocino County, site locations were coded by habitat type and displayed in map format using ArcGIS, version 9.3 (ESRI, Redlands, CA). The relative *ospC* genotype frequencies were calculated by using the number of ticks with a particular genotype at a site, divided by the total number of ticks containing that genotype across all sites ($n = 209$ ticks across 74 sites).

Nucleotide sequence accession numbers. DNA sequences are available in GenBank for *ospC* (FJ932732 to FJ932736) and IGS (EU886969 to EU886976 and FJ932731) loci of *B. burgdorferi* derived from *I. pacificus* and for the 16S rRNA (GQ247740) and *flaB* (GQ247741) loci of *B. burgdorferi* isolate CA8. Accession numbers for taxa used in the 16S rRNA gene-based phylogenetic analysis are U42298, AJ009753, AJ224141, D67024, NR_025861, NR_025983, M88329, L46696, U42292, U42284, L46701, NR_025874, EU135595, AM182229, and CP000395. With the exception of *B. burgdorferi* HB19 (X75200), *B. garinii* (L42885), *B. andersonii* (D83763), and *B. afzelii* (X75202), sequences used in the *flaB* gene phylogenetic analysis were taken from whole genomes (complete or in progress) available in GenBank (AE000783, NZ_ABKA00000000, NZ_ABGJ00000000, NZABGK00000000, NZ_ABGI00000000, NZ_ABJX00000000, NZ_ABJY00000000, NZ_ABCV00000000, NZ_ABJU00000000, NZ_ABJW00000000, CP001205, and NZ_ABKB02000003).

RESULTS

Frequency distribution of *ospC* genotypes. *ospC* DNA was successfully amplified in 227 (86.3%) of 263 *B. burgdorferi*-infected ticks collected in Mendocino County in 2004. Twelve *ospC* alleles, or unique sequences, belonging to 11 *ospC* genotypes were detected (Table 1). Exact matches to previously published sequences were found for eight of the *ospC* genotypes: A, B, D, E3, F, G, H, and T. We identified two new *ospC* genotypes, H3 and I3, and two new *ospC* alleles (sequence variants of genotypes): B (nt 59), which belongs to genotype B, and M (nt 348), which belongs to genotype M (Table 1). Because the *ospC* H3 nucleotide sequence was >8% different from *ospC* genotypes submitted to GenBank to date, we designated it a new *ospC* genotype (56). It was most similar (84%) to *ospC* genotype E (GenBank accession no. AY275221). Compared to AY275221, the H3 sequence had 90 nt changes throughout the 549-bp alignment, conferring 42 amino acid changes, as well as a deletion at positions 367 to 369 (amino acid 123) and a 3-bp insertion at positions 424 to 426. I3 is a hybrid of two previously described genotypes, F and A. I3 is nearly an exact match to genotype F (L42896) (39) from nucleotides 1 to 385, with two exceptions: a nucleotide change from A → G at position 184, conferring an N → D change in amino acids at position 62, and a nucleotide change from G → A at position 277, conferring a D → N amino acid change at position 93. I3 positions 386 to 549 are an exact match to sequences from genotype A (AY275213). Although *ospC* I3 is divergent from both F and A by less than 8%, its unique hybrid status and moderately high frequency in ticks (see below) led us to designate it a new genotype.

ospC sequences could not be fully typed in 22 (9.7%) of 227 amplicons, which presumably represented mixed *B. burgdorferi* infections. In 17 of these ticks, at least one *ospC* genotype was decipherable, and these genotypes were included in the overall *ospC* allele frequency given in Table 2. *B. burgdorferi* genotype H3 was the most frequently encoun-

TABLE 2. *ospC* allele frequency in *B. burgdorferi* DNA amplified from *I. pacificus* nymphs collected in Mendocino County in 2004

ospC allele	No. of ticks with allele		*ospC* allele frequency in tick population (%)
	−MI[a]	+MI[b]	
H3	51	53	24.0
E3	32	38	17.2
H	24	27	12.2
A	23	24	10.9
F	21	21	9.5
D	17	18	8.1
G	17	18	8.1
B	8	10	4.5
M (nt 348)	5	6	2.7
I3	5	5	2.3
B (nt 59)	1	1	0.5
T	1	1	0.5
Total	205	222	100.0

[a] −MI, not including mixed infections.
[b] +MI, including mixed infections.

tered (24%) *ospC* genotype detected in spirochete-infected *I. pacificus* nymphs (Table 2). The second-most-common genotype found was E3 (17.2%), followed by H (12.2%), A (10.9%), F (9.5%), D and G (8.1% each), B (4.5%), M (nt 348) (2.7%), I3 (2.3%), and B (nt 59) and T (0.5%) (Table 2).

We also determined the *ospC* sequence type for 35 *B. burgdorferi* frozen cultures derived from *I. pacificus* nymphs or adults collected in three northern Californian counties (Marin, Sonoma, and Mendocino) between 1987 and 1999 (Table 3). We then compared the genotype frequencies in Mendocino County nymphs collected in 2004 to the genotype frequencies in cultures derived solely from Mendocino County nymphs ($n = 14$). *ospC* A was detected in 50% of cultured nymphs and 10.9% of nymphs collected in 2004 (Fisher's exact test, $P < 0.01$). *ospC* H was detected in 28.6% of cultured nymphs compared to 12.2% of nymphs collected in 2004 ($P = 0.09$). *ospC* F had a similar prevalence in Mendocino County nymph cultures (14.3%) compared to DNA from nymphs collected in 2004 (9.5%; $P = 0.63$). We detected *ospC* I3 in 7.1% of cultures compared to 2.3% in ticks collected in 2004 ($P = 0.31$). A single example of *ospC* I was identified in culture isolate CA337 made from a male *I. pacificus* in Mendocino County in 1992, which matched 481 nt (positions 4 to 487) of isolate CA92-1096 made from a human skin biopsy in Sonoma County in 1992 (J. Piesman, personal communication) (40) (Table 1).

TABLE 3. *ospC* allele frequencies in California *B. burgdorferi* culture isolates from nymphal or adult *I. pacificus* ticks, 1987 to 1999

ospC genotype	No. of cultures positive for the allele (frequency [%])	California isolate no.	5S-23S RFLP type[a]	Tick life stage and sex[b]	County	Year of isolation
A	16 (45.7)	17	A	M	Mendocino	1990
		18	A	M	Mendocino	1990
		19	A	F	Mendocino	1990
		382	A	Unknown	Mendocino	1993
		535	A	N	Mendocino	1998
		536	A	N	Mendocino	1998
		537	A	N	Mendocino	1999
		4	A	M	Sonoma	1987
		5	A	F	Sonoma	1987
		6	A	M	Sonoma	1987
		538	A	N	Mendocino	1999
		543	A	N	Mendocino	1998
		544	A	N	Mendocino	1998
		3	B	F	Marin	1986
		9	Not typed	F	Marin	1988
		540	Not typed	N	Mendocino	1999
D	3 (8.6)	15	B	F	Mendocino	1990
		172	B	Unknown	Mendocino	Unknown
		338	B	M	Mendocino	1992
F	3 (8.6)	7	A	F	Sonoma	1987
		358	B	N	Mendocino	1992
		542	B	N	Mendocino	1998
I3	4 (11.4)	10	B	F	Mendocino	1988
		11	B	F	Mendocino	1989
		12	B	Unknown	Mendocino	1989
		360	B	N	Mendocino	1992
G	1 (2.9)	566	B	M	Sonoma	1999
H3	1 (2.9)	8	Not typed	F	Sonoma	1987
H	6 (17.1)	533	A	N	Mendocino	1998
		534	A	N	Mendocino	1998
		541	A	N	Mendocino	1998
		567	A	M	Sonoma	Unknown
		336	Not typed	M	Mendocino	1992
		569	Not typed	N	Mendocino	1999
I	1 (2.9)	337	B	M	Mendocino	1992

[a] Reference 7.
[b] M, adult male; F, adult female; N, nymph.

TABLE 4. Signature polymorphic nucleotides of 5S-23S IGS sequence types identified in *B. burgdorferi*-infected *I. pacificus* nymphs collected in Mendocino County, 2004

GenBank accession no.	Former IGS genotype or subtype name[b]	Residue at polymorphic position[c]															
		226	239	274	289	314	419	435	489	516	538	684	706	713	725	729	Δ[d]
AY275189[a]	1A	T	G	T	G	A	C	C	G	G	A	C	A	C	G	G	No
EU886973		T	G	T	G	A	C	C	G	G	A	T	A	C	G	G	No
AY275194[a]	2D	T	G	T	G	A	T	C	G	G	A	T	A	T	G	G	No
EU886969		T	G	T	G	A	T	C	G	G	A	T	A	C	G	G	No
AY275201	5	C	G	T	G	A	T	C	G	G	A	T	A	C	G	G	No
EU886970		C	A	T	G	A	T	C	G	G	A	T	A	C	G	G	No
FJ932731		C	G	T	G	A	T	C	G	G	G	T	A	C	G	G	No
EU886976		C	G	T	G	A	T	C	G	A	A	T	A	C	G	G	No
EU886975		C	G	T	G	A	T	C	G	G	A	T	A	C	A	G	No
EU886971		C	G	C	G	A	T	C	G	G	G	T	A	C	G	G	No
EU886972		C	G	C	G	A	T	T	G	G	G	T	A	C	G	G	No
EU886974	NG	T	G	T	A	T	C	C	A	G	A	T	G	C	G	T	Yes

[a] Sequence was not observed in this study and was used only for comparison.
[b] Reference 8. NG, new genotype.
[c] Based on 812-nt alignment (including gaps). Position 1 corresponds to position 2424 of *B. burgdorferi* strain B31 (GenBank no. U03396).
[d] Deletion at nt 794 to 798.

ospC genotype I was not detected in ticks collected in 2004. Only one example of the predominant *ospC* genotype detected in the nymphs collected in 2004, H3, was found in culture isolate CA8 obtained in 1987 from an *I. pacificus* female in Sonoma County.

We also evaluated the relationship between *ospC* genotype and RFLP sequence type of the 5S-23S rRNA IGS (7) (Table 3). Linkage between RFLP pattern and *ospC* genotype was observed in 28 out of 30 (93%) samples typed by both methods. With the exception of isolate CA3, all *ospC* genotype A and H cultures ($n = 16$ and 6, respectively) were 5S-23S RFLP pattern A. All other *ospC* genotypes (D, F, I3, G, G3, and I) were RFLP pattern B except for CA7 (RFLP type A).

Frequency distribution of IGS genotypes. Using an 812-bp alignment, 13 IGS alleles defined by as little as a 1-nt difference were identified in *I. pacificus* nymphs, nine of which have not been previously described (Table 4). Exact matches to sequences formerly named IGS genotype 5 (GenBank accession no. AY275201), subtype 3A (DQ437500), and subtype 6A (AY275202) (8) were found in addition to an exact match to GenBank sequence EF649786, which is most similar to genotype 4 described by Bunikis et al. (8) (Table 5). The most divergent Californian IGS allele (GenBank accession no. EU886974) had seven single nucleotide changes compared to the IGS sequence AY275201 and a 5-bp deletion at positions 794 to 798 (Table 4). Based on its nucleotide sequence divergence and its strict association with novel *ospC* genotype H3 (Table 5), we propose that this sequence represents a new *B. burgdorferi* IGS genotype.

***ospC* and IGS biallelic profiles.** Because regions of both IGS and *ospC* of *B. burgdorferi* were sequenced in individual ticks, we were able to investigate linkage between the two loci. IGS and *ospC* biallelic profiles could be analyzed in 192 ticks after mixtures and samples that were PCR negative for one of the two loci were eliminated (Table 5). *ospC* alleles D, H, F, I3, and M (nt 348) had strict associations with IGS alleles. *ospC* alleles H3, A, and B/B (nt 59) were associated with more than one IGS allele although a dominant allele linkage was evident (Table 5). The linked alleles H3 and EU886974 represented 21.9% of all ticks analyzed (Table 5). IGS allele EU886974 was not found associated with another *ospC* allele. The second-most-common biallelic profile was *ospC* H/IGS EU886969 (11.5%), followed by F/EF649786 (10.9%). *ospC* biallelic profiles E3/EU886975, A/EU886973, and D/EU886970 were all found at the same frequency (8.9%). The next most common

TABLE 5. *ospC*/IGS biallelic profiles found in *B. burgdorferi*-infected *I. pacificus* nymphs in Mendocino County in 2004[a]

ospC genotype or allele	IGS allele (accession no.)[b]	Former IGS genotype or subtype name[c]	No. of ticks positive for the allele (% of total)[d]
H3	AY275201	5	1 (0.5)
	EU886971	5	1 (0.5)
	EU886974	**NG**	**42 (21.9)**
	AY275202	6A	1 (0.5)
E3	**AY275201**	**5**	**11 (5.7)**
	EU886976	5	3 (1.6)
	EU886975	**5**	**17 (8.9)**
H	EU886969	2	22 (11.5)
A	**EU886973**	**1**	**17 (8.9)**
	AY275201	5	1 (0.5)
	EU886971	5	1 (0.5)
	AY275202	6A	2 (1.0)
F	EF649786	4	21 (10.9)
D	EU886970	5	17 (8.9)
G	**EU886971**	**5**	**8 (4.2)**
	AY275201	5	1 (0.5)
	EU886972	**5**	**7 (3.6)**
B	**DQ437500**	**3A**	**6 (3.1)**
	AY275201	5	1 (0.5)
I3	EF649786	4	5 (2.6)
M (nt 348)	AY275202	6A	5 (2.6)
B (nt 59)	AY275201	5	1 (0.5)
T	EU886975	5	1 (0.5)
Total			192 (100.0)

[a] Dominant biallelic profiles for each *ospC* genotype are in bold.
[b] GenBank accession numbers correspond either to newly submitted sequences (Table 4) or to sequences existing in the database.
[c] Based on an exact match or similarity to previously described sequences (8). NG, new genotype.
[d] Results from mixed infections are not included.

TABLE 6. Comparison of *ospC* allele frequency distribution in *I. pacificus* nymphs from Mendocino County (2004) versus in *I. scapularis* nymphs or adults from three studies conducted in the northeastern United States

ospC genotype	Presence of the genotype in ticks of the indicated region								Statistical comparison (*P*)[e]
	Shelter Island, NY (*n* = 40)[a]		Long Island and the coast from MA to SC (*n* = 203)[b]		Dutchess County, NY (*n* = 451)[c]		Mendocino County, CA (*n* = 205)[d]		
	No. positive	Frequency (% of total)	No. positive	Frequency (% of total)	No. positive	Frequency (% of total)	No. positive	Frequency (% of total)	
A	12	16.2	40	8.2	59	13.1	24	10.9	0.46
B	12	16.2	51	10.5	44	9.8	11	5.0	0.036
C	11	14.9	NA		0	0.0	0	0.0	
D	9	12.2	34	7.0	40	8.9	18	8.1	0.88
E	4	5.4	34	7.0	30	6.7	0	0.0	<0.001
F	6	8.1	38	7.8	31	6.9	20	9.0	0.35
G	5	6.8	40	8.2	25	5.5	18	8.1	0.24
H	7	9.5	39	8.0	24	5.3	27	12.2	0.003
I	1	1.4	NA		15	3.3	0	0.0	0.004
J	2	2.7	14	2.9	9	2.0	0	0.0	0.034
K	5	6.8	76	15.7	88	19.5	0	0.0	<0.001
L	0	0.0	0	0.0	0	0.0	0	0.0	
M	0	0.0	44	9.1	39	8.6	6	2.7	0.003
N	0	0.0	40	8.2	9	2.0	0	0.0	0.034
O	0	0.0	0	0.0	1	0.2	0	0.0	1
T	0	0.0	19	3.9	23	5.1	1	0.5	0.001
U	0	0.0	16	3.3	14	3.1	0	0.0	0.007
E3	0	0.0	0	0.0	0	0.0	38	17.2	<0.001
I3	0	0.0	0	0.0	0	0.0	5	2.3	0.004
H3	0	0.0	0	0.0	0	0.0	53	24.0	<0.001
Total	74	100.0	485	100.0	451	100.0	221	100.0	

[a] Adult *I. scapularis* ticks. Data are from reference 56.
[b] Nymphal and adult *I. scapularis* ticks. Data are from reference 45. NA, not available.
[c] Nymphal *I. scapularis* ticks. Data are from reference 57.
[d] Nymphal *I. pacificus* ticks.
[e] Data from reference 57 versus this study.

profile was E3/AY275201 (5.7%), followed by G/EU886971 (4.2%) and G/EU886972 (3.6%), B/DQ437500 (3.1%), I3/4C and M (nt 348)/AY275202 (2.6%), and T/EU886975 (0.5%) (Table 5). With the exception of *ospC* alleles G and T, all IGS linkages in *I. pacificus* ticks were similar to those reported from the northeastern and midwestern United States (8, 26, 40). Comparison of *ospC* E3 linkage to IGS alleles in other areas of *B. burgdorferi* transmission will be reported elsewhere (Travinsky et al., unpublished data).

***B. burgdorferi* population structure of Mendocino County versus the northeastern United States.** We compared the frequency distribution of *ospC* alleles found in *I. pacificus* nymphs collected in 2004 with that found in *I. scapularis* ticks collected in the northeastern United States in three studies (45, 56, 57) (Table 6). Eight *ospC* genotypes, C, E, I, J, K, N, O, and U, identified in *I. scapularis* were absent from *I. pacificus* nymphs collected in 2004. *ospC* K made up the majority of alleles found in *I. scapularis* in two out of three of the studies used for comparison (15.7% [45] and 19.5% [57]). In this comparison, three *ospC* genotypes, E3, H3, and I3, were found only in California.

Of the *ospC* alleles that the northeastern and Californian studies shared, A, D, F, and G occurred at similarly high frequencies in the two locations (10.9%, 8.1%, 9.0%, and 8.1%, respectively, for Mendocino County versus a range of 8.2 to 16.2%, 7 to 12.1%, 6.9 to 8.1%, and 5.5 to 8.2%, respectively, for northeastern studies) (Table 6). The frequency (5.0%) of *ospC* B in Mendocino County nymphs was about one-third to one-half that in ticks from the Northeast (range of 9.8 to 16.2%). *ospC* H, the third-most-frequent allele in California ticks (12.2%), was slightly less common in *I. scapularis* (range of 5.3 to 9.5%). *ospC* allele M was more common in *I. scapularis* (range of 0 to 9.1%) than in *I. pacificus* (2.7%). *ospC* T was less common in Mendocino County (0.5% versus a range of 0 to 5.1%) although only a single tick with this sequence type was identified during our study.

Because Wang et al. (56) and Qiu et al. (45) genotyped *B. burgdorferi* in adult ticks, we could directly compare *ospC* allele frequencies in our study only to those found in Dutchess County, NY, *I. scapularis* nymphs by Wormser et al. (57). This decision was made based on the assumption that allele frequencies may differ in nymphs and adults due to differences in host preference (12) and because adult ticks are likely infected with multiple alleles (45). The frequencies of *ospC* alleles A, D, F, and G were similar between studies (Table 6). All other shared alleles were in dissimilar proportions, including B, E3, H, H3, I3, M, and T. Only alleles H, E3, H3, and I3 were more common in Mendocino County than in Dutchess County.

Genetic characterization of Californian *B. burgdorferi* strain CA8. The genetic divergence and abundance of *ospC* H3 in Mendocino County nymphs prompted further investigation into the relationship between isolates containing the allele and other *B. burgdorferi* and *Borrelia* sp. strains. We analyzed partial sequences of the *B. burgdorferi* 16S rRNA and *flaB* genes

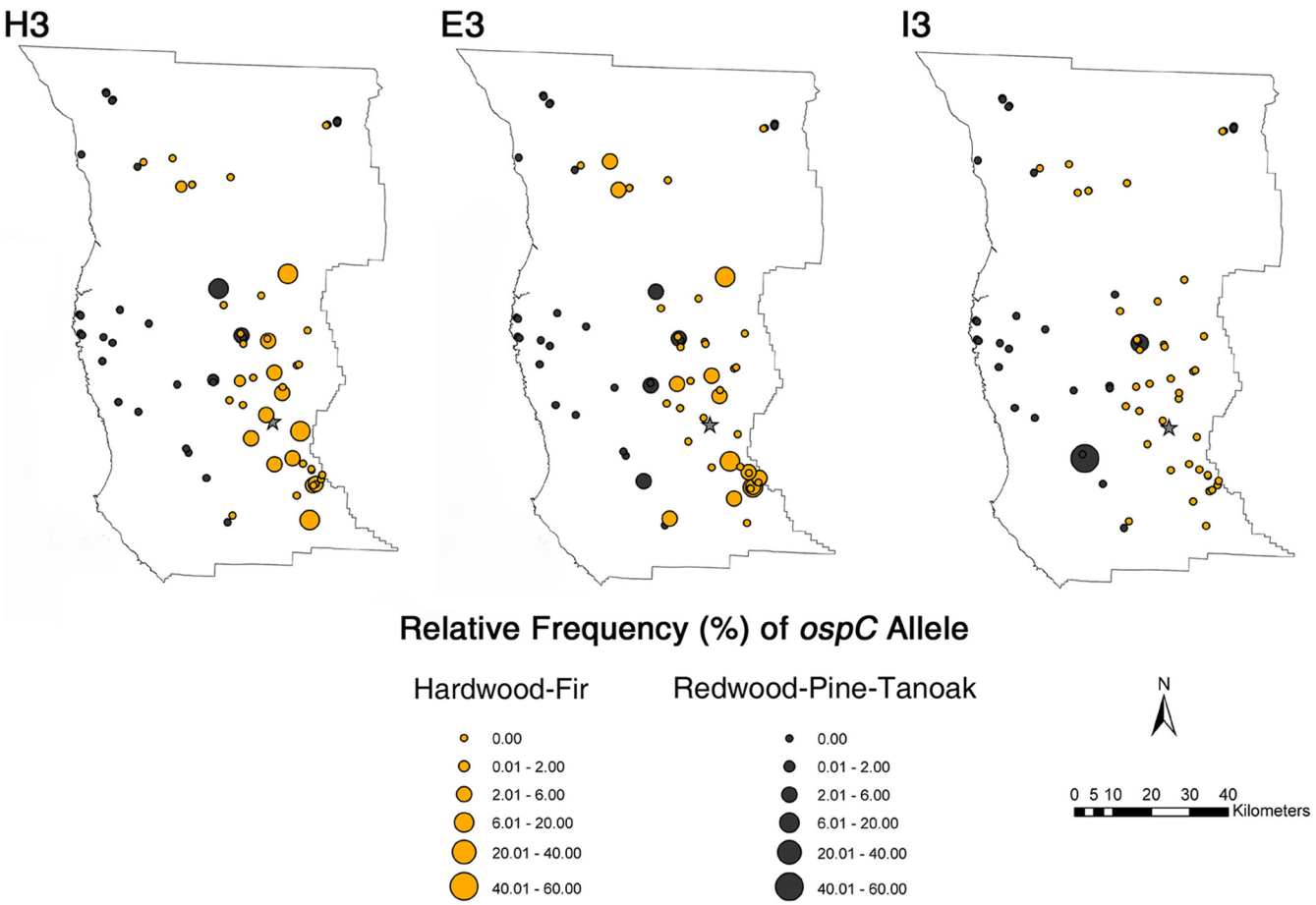

FIG. 2. Relative frequency distribution of *B. burgdorferi ospC* alleles H3, E3, and I3 found in *I. pacificus* nymphs across 74 woodland collection sites in Mendocino County. Circle diameters correspond to relative frequency values (displayed as percentages). The largest city in the county, Ukiah, is indicated by a star.

(Fig. 1) using DNA extracted from *B. burgdorferi* culture isolate CA8 whose *ospC* sequence belonged to genotype H3 (Table 3). The neighbor-joining distance phylograms comparing CA8 to select *Borrelia* species (16S rRNA locus) and to select *B. burgdorferi* strains (*flaB* locus) confirmed both its status as *B. burgdorferi* and its genetic divergence from previously characterized *B. burgdorferi* isolates.

Habitat associations of novel and abundant California *ospC* alleles. We analyzed the geographic distributions and relative frequencies of three *ospC* genotypes, H3, E3 and I3, which are unique and abundant in California (Fig. 2). The relative frequencies of *ospC* H3 and E3 were highest in the central and southeastern regions of the county, which overlapped previously reported areas of high predicted acarological risk of exposure to *I. pacificus* nymphs (22).

We hypothesized that *ospC* H3 and E3 would be significantly associated with hardwood-fir habitats because the primary LB reservoir in Mendocino County, the western gray squirrel, is commonly infected with *B. burgdorferi* in these habitats (20, 31, 46). In our analysis, tick collection sites that contained *ospC* H3 were more likely to be hardwood-fir habitats than redwood-pine-tanoak habitats (Fisher's exact test, $P < 0.05$). The same association was not found for *ospC* E3 ($P = 0.238$). Of the 164 *B. burgdorferi*-infected ticks in hardwood-fir habitats, 23.2% carried *ospC* H3, 17.1% were infected with E3, and none carried I3. Among the 45 *B. burgdorferi*-infected ticks found in redwood-pine-tanoak habitats, 26.7% carried *ospC* H3, 13.3% were infected with E3, and 11.1% carried I3. Redwood-pine-tanoak sites containing a high percentage of *ospC* H3 were located near hardwood-fir habitats in inland Mendocino County (Fig. 2). Redwood-pine-tanoak sites with little or no *ospC* H3 were located in the western, coastal region of Mendocino County.

DISCUSSION

California *B. burgdorferi* population structure and LB. We conducted the first large-scale, systematic survey of *B. burgdorferi ospC* and IGS sequence types in western North America. Our findings highlight differences between *B. burgdorferi* genotypes in *I. pacificus* ticks and those found in the eastern LB vector, *I. scapularis*. For example, the most abundant *B. burgdorferi ospC* allele in Mendocino County ticks, H3, had not been described in the Northeast, and E3 had been identified only in ticks from the north-central United States (Travinsky et al., unpublished). We identified seven *B. burgdorferi* strains defined by *ospC* alleles B, D, G, T, H3, I3, and E3, California representatives of which are lacking from a recent study of *B.*

burgdorferi evolution and geographic population structure in North America and Europe (40). Inclusion of these samples in future studies using methods suitable for inferring spirochete evolution, such as multilocus sequence typing based on housekeeping genes, may clarify the relationship between far-western *B. burgdorferi* populations and those found in the other parts of the United States and Europe (27, 40).

Sequence variation in the *ospC* locus of *B. burgdorferi* has been linked to variation in the probability of disseminated spirochete infection and in the clinical manifestations of LB (15, 17, 28, 49, 57). The lack of previous reports of *ospC* H3 in clinical studies enables us only to speculate about its pathogenic potential in humans. Although E3 has been identified only in ticks in the north-central United States, a sequence similar to the E3 allele (GenBank accession number EU482056) was recently described in a clinical isolate from the Northeast (26). Given the high frequency of H3 and E3 genotypes in *I. pacificus* and their location in areas of high acarological risk to humans (22), it is possible that humans in Mendocino County regularly are being exposed to these genotypes during a tick bite. However, further classification of tick collection sites by human usage is required to assess the likelihood of human exposure to particular genotypes.

ospC allele frequency distributions in tick populations do not always correspond to frequency distributions found in LB patient skin lesions or secondary sites of infection (49). This may be related to differences in the spirochete detection methods used for clinical samples (i.e., cultivation) versus ticks (i.e., PCR on extracted DNA), resulting in culture bias for particular genotypes. A limited amount of information exists regarding the relationship between *ospC* genotype and the pathogenesis of LB in California. To our knowledge, *B. burgdorferi* isolates derived from blood or cerebrospinal fluid of California patients have been *ospC* genotype A ($n = 3$ isolates), while this genotype and others including H, I, and M ($n = 1$ isolate each) have been found in the skin (40, 49). Thorough analysis of *ospC* sequence types in California LB patients is sorely needed and is essential to understanding the relationship between *ospC* allele frequency in ticks and human infections in the region.

I. pacificus nymphs lacked two *ospC* genotypes, I and K, which are considered to be highly invasive (15) or associated with disseminated human infection in the Northeast (49, 57). Genotype K is abundant in *I. scapularis* as it has been found in 6.8% (49, 56), 15.7% (45), and 19.5% of ticks (57) and has been isolated in about 30 to 40% of human skin and blood cultures in northeastern studies (49, 57). Although *ospC* genotype I was identified in a single tick from Mendocino County in 1992 (CA337), its absence, as well as the absence of genotype K, in the *B. burgdorferi*-positive ticks genotyped throughout the county in 2004 is notable. It is possible that the lack of genotypes I and K in *I. pacificus* is related to the lower incidence of human cases in the Far West, but extensive testing of California *B. burgdorferi* human isolates is needed to draw any conclusions regarding spirochete genetics and regional differences in disease.

Vertebrate-vector-spirochete interactions in California. California and the Northeast differ in climate and topography, as well as in reservoir and vector host species involved in enzootic LB transmission cycles. These transmission cycles are also geographically separated by about 3,000 miles. Therefore, it is not surprising that we detected major differences in *B. burgdorferi* population structures between the two regions. Approximately half of the *ospC* genotypes previously found in the Northeast were not detected in California ticks, meaning that the genetic diversity of *B. burgdorferi* is comparatively lower in the Far West. One possible explanation is that *B. burgdorferi*-refractory lizards (29, 30, 33, 59), the primary larval and nymphal hosts for *I. pacificus* in Mendocino County (18), are reducing the overall diversity of spirochetes. If so, only genotypes that can replicate well, or persist in, nonlizard tick hosts might be selected for.

Western gray squirrels, who account for the majority of infectious feeds to *I. pacificus* larvae in oak woodland habitats (20), appear to be particularly susceptible to *ospC* genotypes H3 and E3. In preliminary studies, over 50% of western gray squirrels collected in Mendocino County were infected with *B. burgdorferi ospC* genotypes H3 and E3 (S. Leonhard, Y. A. Girard, D. J. Salkeld and R. S. Lane, unpublished data). This prevalence is similar to the combined prevalence of *ospC* H3 and E3 in *I. pacificus* nymphs (41%). The positive association of *ospC* H3 with hardwood-fir habitats further supports the notion that these squirrels are members of an *ospC* H3 niche in Mendocino County (6).

Genotypic diversity of Californian *B. burgdorferi*. In 2007, three adjacent counties in north-coastal California, Trinity, Humboldt, and Mendocino, and one inland county, Amador, reported the highest incidence of LB cases in the state (≥5 per 100,000 person-years) (11). *B. burgdorferi* in other counties in California where Lyme disease is endemic may have different population structures than in Mendocino County, particularly where vertebrate reservoirs other than the western gray squirrel may maintain spirochetes. Variability in both the size (molecular mass) and the amount of *ospC* protein expressed by *B. burgdorferi* isolates from 26 counties throughout California (48) suggests that statewide spirochete diversity also may exist at a nucleotide level.

Strong linkage between the IGS and *ospC* loci indicate that *B. burgdorferi* is clonal (8, 26). When linkages between *ospC* and IGS were analyzed in our study, identification of nondominant biallelic profiles (Table 5) suggested the presence of mixed infections in which the IGS primers amplified the gene of one strain while the *ospC* primers amplified the gene of a different strain. This is a potential limitation of using PCR and DNA sequencing alone. Another explanation is that multiple IGS alleles are truly associated with a single *ospC* allele. Because our methods possibly underestimated the multiplicity of infection in *I. pacificus*, other methods such as reverse line blot or PCR–single-strand conformation polymorphism (45, 56) may be needed to further examine multiple infections in *I. pacificus* ticks. However, because the spirochete infection rate in *I. pacificus* in California was generally lower than in *I. scapularis* in the Northeast (53), we would expect to find a lower degree of multiplicity in *I. pacificus* (45).

Our report of *B. burgdorferi* genotypes in *I. pacificus* nymphs represents a snapshot of the spirochete population in Mendocino County vectors. Although we cannot directly compare the frequency distribution of alleles in *B. burgdorferi* culture isolates to the 2004 tick samples for a number of reasons (e.g.,

different life stages, multiple counties, multiple years of collection, and nonsystematic sampling), it is remarkable that, with the exception of CA8 from adjacent Sonoma County, the two predominant *ospC* genotypes in nymphs, H3 and E3, were absent in culture-derived DNA. It is possible that a temporal shift in allele frequency may have occurred in the spirochete population, as observed in areas of the Northeast (43, 45). More likely, differences in allelic frequencies between the sample sets are due to selection by culture, a phenomenon observed by others studying the allele frequencies of IGS, *p66*, and *ospA* (37, 41). Given the high frequency of *ospC* A in the culture isolates, this genotype may outgrow *ospC* H3 and E3 in a mixed infection.

PCR-RFLP typing using the 5S-23S or 16S-23S rRNA spacer regions has been used to sort *Borrelia* genospecies and genotypes within a genospecies (36, 42). Brown et al. (7), using PCR-RFLP to genotype northern Californian *B. burgdorferi* isolates based on MseI and DraI fragment patterns, defined only two genotypes, referred to as A and B. We improved upon this earlier effort by sequencing a portion of the *ospC* gene and were able to define seven different genotypes (A, D, F, I, I3, G, and H) within the same samples. Further characterization of northern California *B. burgdorferi* isolates by sequence analysis of other loci and through studies of animal pathogenesis and vector competence will continue to improve our understanding of spirochete diversity in the Far West.

Conclusions. Variation in the spirochete sequence types and abundance in California compared to the Northeast may be related to a number of factors including selection for genotypes within different tick vector species; differences in climate, habitat, and topography that affect the host-seeking phenology of such ticks; and differences in the type and abundance of reservoir hosts. Efforts to define the genetic background of *B. burgdorferi* in ticks, vertebrate hosts, and humans are important for developing effective LB diagnostics, vaccines, and public health interventions. Genetic variation in *B. burgdorferi* may influence both the severity of LB and the sensitivity of serological assays used to help diagnose it (58). Given the striking differences in spirochete population structure in *I. pacificus* compared to *I. scapularis*, it is likely that humans in California are exposed to a different subset of *B. burgdorferi* lineages than humans in the Northeast. Research is currently under way that should elucidate the role of California vertebrate reservoirs in *B. burgdorferi* transmission. This information, in conjunction with our current knowledge of high-risk habitats (13, 21, 22) and human behaviors that elevate exposure to infected ticks (34), will facilitate the design of interventions that target the unique *B. burgdorferi* transmission cycle in the Far West.

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