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Comparative genetic diversity of Lyme disease bacteria in Northern Californian ticks and their vertebrate hosts

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ABSTRACT

Vector-borne pathogens are transmitted between vertebrate hosts and arthropod vectors, two immensely different environments for the pathogen. There is further differentiation among vertebrate hosts that often have complex, species-specific immunological responses to the pathogen. All this presents a heterogeneous environmental and immunological landscape with possible consequences on the population genetic structure of the pathogen. We evaluated the differential genetic diversity of the Lyme disease pathogen, *Borrelia burgdorferi*, in its vector, the western black-legged tick (*Ixodes pacificus*), and in its mammal host community using the 5S–23S rRNA intergenic spacer region. We found differences in haplotype distribution of *B. burgdorferi* in tick populations from two counties in California as well as between a sympatric tick and vertebrate host community. In addition, we found that three closely related haplotypes consistently occurred in high frequency in all sample types. Lastly, our study found lower species diversity of the *B. burgdorferi* species complex, known as *B. burgdorferi* sensu lato, in small mammal hosts versus the tick populations in a sympatric study area.

Introduction

Lyme disease, the most common vector-borne disease in North America, is caused by the bacterial spirochete, *Borrelia burgdorferi* (Spirochaetacae; Johnson et al., 1984). Though only described in 1984 (Johnson et al., 1984), molecular evidence suggests that North American populations of *B. burgdorferi* diverged from European populations several million years ago and have been well established in the northeastern, midwestern and far-western United States for some time (Margos et al., 2008). The disease vector and the bacterial pathogen feed on and cause infection in many vertebrate species, respectively (LoGiudice et al., 2003; Hanincova et al., 2006; Castro and Wright, 2007). The vector, *Ixodes* spp. ticks, feed on a suite of vertebrate hosts for blood meals including various species of mammals, birds and lizards (Furman and Loomis, 1984; Kurtenbach et al., 2006) and the bacteria is maintained and transmitted by many of these hosts back to the vector (Donahue et al., 1987; Mather et al., 1989; Telford et al., 1990; Lane and Brown, 1991; Brown and Lane, 1996; Lane et al., 2005; Brunner et al., 2008; Salkeld et al., 2008).

The life cycle of the pathogen requires both the tick vector and vertebrate host because there is no vertical transmission in either. The bacteria reside in the tick midgut until the tick attaches to a suitable host. The pathogen then replicates and migrates to the tick's salivary gland before entering a vertebrate host where it disseminates via the blood and causes multi-systemic infection in the host (Piesman et al., 1990, 1991; Barthold et al., 1991). The tick vector feeds three times, once during each of its life stages: larvae, nymph and adult. After each blood meal, the tick molts into the next stage or, in the case of adults, reproduces. In North America, nymphal stages of *Ixodes scapularis* Ixodidae in the East and *I. pacificus* in the West are the principal vectors of the pathogen to humans (Barbour and Fish, 1993; Clover and Lane, 1995).

Because *B. burgdorferi* infects many vertebrate species, it must contend with immunological factors in each host species it infects. Some host species, such as western fence lizards (*Sceloporus occidentalis*) and alligator lizards (*Elgaria multicarinata*) do not maintain the bacteria and kill the bacteria in the feeding tick (Lane and Quistad, 1998; Wright et al., 1998), whereas other pathogen reservoir hosts transmit the bacteria to feeding ticks with varying levels of efficiency (Donahue et al., 1987; Brown and Lane, 1996; Eisen et al., 2003; LoGiudice et al., 2003; Brunner et al., 2008). For

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instance, mule deer and birds have not been shown to be a viable pathogen reservoir in coastal woodlands of California (Ullmann et al., 2003; Eisen et al., 2004). Furthermore, certain pathogen genotypes may be better adapted to invade particular host species (Brisson, 2004; Brisson and Dykhuizen, 2006). Immunologically speaking, *B. burgdorferi* faces a heterogeneous host "landscape" that may lead to differential transmission and survival of particular bacterial genotypes or lineages (Kurtenbach et al., 2002a).

DNA sequence data from a neutral genetic marker can be used to compare the diversity of *B. burgdorferi* and has been used to determine population structure and infer gene flow within and between regions (Postic et al., 1994; Humphrey et al., 2010; Coipan et al., 2013). The 5S (*rrf*)–23S (*rrl*) intergenic spacer region is a tandemly repeated sequence that is unique to *B. burgdorferi* sensu lato and has been used to differentiate and identify different isolates of *Borrelia* spirochetes in many different sample types including ticks (Postic et al., 1994; Humphrey et al., 2010), blood meal hosts (Chu et al., 2008) and patients (Postic et al., 1998; Rijpkema et al., 2009). This is in contrast with other markers such as the *ospA* or *ospC* genes are not ideal for phylogenetic analyses of inferences because of frequent recombination events (Haven et al., 2011). Previous studies from the northeastern or midwestern US have examined the genetic structure of *B. burgdorferi* with other genes and found limited genetic structure at a local scale but evidence of regional barriers to gene flow (Qiu et al., 2002; Humphrey et al., 2010). This is the first study to comparatively examine haplotype diversity in *Ixodes pacificus* and their vertebrate hosts in the same spatiotemporal context. This study is unique in this regard and can thereby provide valuable insights into key elements of the population genetic history and ecology of pathogen transmission. The genotype diversity of *B. burgdorferi* present in ticks and its vertebrate hosts should theoretically match because they are linked in the reproduction and transmission of the pathogen. If they do not, then it suggests that there are unidentified pathogen reservoirs or differential transmission rates of particular isolates (Girard et al., 2009).

Within its North American distribution, Lyme disease is caused by *B. burgdorferi* sensu stricto (*B. burgdorferi* s.s.), the only confirmed cause of Lyme disease in North America (Margos et al., 2010; Wormser et al., 2008). In Europe, two other genospecies *B. afzelii* and *B. garinii* also cause disease in humans. These three genospecies, along with 14 other described genospecies and several additional, undescribed isolates are collectively referred to as the *B. burgdorferi* sensu lato (*B. burgdorferi* s.l.) complex (reviewed in Stanek and Reiter, 2011). In North America, members of the *B. burgdorferi* s.l. species complex that are not *B. burgdorferi* s.s. have, to date, not been shown to cause disease in humans in North America, but see Girard et al. (2011). It has been proposed that areas with greater genetic diversity of *B. burgdorferi* s.l. result from a higher number of closed enzootic cycles between ticks and particular host species that generate more habitat "niches" for the bacterial pathogen (Margos et al., 2010). This study examines the genetic diversity of a non-coding IGS locus in *B. burgdorferi* s.l. from a large sample of *I. pacificus* and its vertebrate host community in northern California and evaluates the ecological significance of these patterns.

Given the challenge of capturing all possible hosts in a community (and thus all pathogen reservoirs), we expected the genetic diversity of *B. burgdorferi* that we detect in tick hosts, to be a subset of the diversity that we find in the tick population.

Methods

Field collections

Collections of *I. pacificus* ticks took place in two adjacent northern California counties: Marin and Sonoma. Sampling in Marin County took place from 2006 to 2009 at two oak woodland sites, China Camp State Park (38°0′9.50″N, 122°28′2.53″W) and Marin Municipal Water District (37°58′5.39″N, 122°36′15.20″W). Field sampling in Sonoma County was conducted in 2006 and 2007 in several state parks and reserves, including Jack London State Historic Park (38°21′24.12″N, 122°32′38.4″W), Annadel State Park (38°27′7.2″N, 122°38′2.4″W), Sugarloaf Ridge State Park (38°26′16.30″N, 122°30′51.55″W), Sonoma State University Fairfield Osborn Preserve (38°20′56.12″N, 122°35′44.56″W) and Audubon Canyon Ranch Bouverie Preserve (38°21′51.69″N, 122°30′35.9″W) (Swei et al., 2011a). Sampled sites were in oak woodland habitat and chosen from a mixture of randomly selected plots (Meentemeyer et al., 2008). All ticks were collected by drag sampling using a 1 m^2 white flannel cloth attached to a wooden dowel (Swei et al., 2011a). Drag cloths were checked every 15 m and all attached ticks were removed and stored in 70% ethanol for lab identification and DNA extraction.

Tick host sampling targeted small mammals because local lizards are non-competent pathogen reservoirs of *B. burgdorferi* s.l. and birds have not been shown to be important reservoirs in north-coastal California (Eisen et al., 2004). Small mammal hosts of *I. pacificus* were collected from Marin County in 2006, 2007 and 2008 by live-trapping with extra-long Sherman traps (7.6 × 9.5 × 30.5 cm; H.B. Sherman Traps, Tallahassee, FL) on the tick collection plots (Swei et al., 2011b). Detailed animal handling methods are provided in Swei et al. (2011b). Briefly, all trapped animals were anesthetized with a 5% solution of isoflurane before two 2-mm ear punch biopsies were collected, one from each ear. Tissue samples were stored in 70% ethanol and refrigerated at −20 °C until extracted. Animals were allowed to revive before being released at the point of capture. Tick–host sampling was not conducted in Sonoma County.

Laboratory analyses

DNA from all *I. pacificus* nymphs and vertebrate tissue samples was extracted using a Qiagen DNeasy Kit (Qiagen, Foster City, CA, USA) following the manufacturer's instructions. All tick samples were then screened for infection with *B. burgdorferi* s.l. using real-time quantitative PCR techniques detailed in Swei et al. (2011b). Positive samples were PCR-amplified and sequenced at the 5S–23S *rrf–rrl* intergenic spacer (IGS) rDNA region (Lane et al., 2004). Much of the existing genetic work in western North America, including species descriptions, have used this neutral IGS marker for its utility to differentiate genospecies and reveal genetic differentiation at small geographic scales (Coipan et al., 2013; Postic et al., 1994; Rijpkema et al., 1995).

Cycle-sequencing reactions were performed in both forward and reverse directions using the ABI BigDye Terminator Kit v3.1 (Applied Biosystems, Foster City, CA). Cycle-sequencing reaction products were purified using Sephadex columns and then analyzed on an ABI 3730 automated sequencer (Applied Biosystems, Foster City, CA), following the protocol provided in Lane *et al.* (2004). Forward and reverse sequences were assembled and edited in Sequencher 4.7 (Gene Codes Corp., Ann Arbor, MI).

Alignment and phylogenetic analyses

Sequences were aligned using MAFFT (Katoh et al., 2009). Maximum likelihood (ML) analyses were performed using RAxML 2.2.3 (Stamatakis et al., 2008) via the CIPRES portal (Miller et al., 2009) to generate a topology with boostrap support values (100 pseudoreplicates). We considered a clade to be supported if bootstrap values were greater than 50%, and to be strongly supported if greater than 70% (Hillis and Bull, 1993). Phylogenetic trees were visualized using FigTree v1.0 (Rambaut, 2006). Examination of the

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alignment revealed one phylogenetially informative indel (4 bp deletion). We therefore re-coded the indel as a character in order to include it in the network analyses below.

Statistical parsimony haplotype networks were constructed using the program TCS version 1.21 (Clement et al., 2000). A 95% connection limit was used, and insertions and deletions were treated as missing data. This is an appropriate way to view the evolutionary relationship between closely related haplotypes where ancestry may not be strictly bifurcating (Posada and Crandall, 2001). Divergent haplotypes that could not be joined to the network within the 95% confidence limit were either placed on the network according to the topology recovered in the ML phylogenetic analyses if supported by bootstrap values greater than 50%, or not connected to the network if phylogenetic placement was uncertain. Prevalence with *B. burgdorferi* s.l. and s.s. were calculated and 95% Bayesian credible intervals for infection prevalence was determined. Calculations for credible intervals used the inverse of the cumulative distribution function of the beta distribution in *R*. Intervals were calculated in *R*.

The distribution of haplotype diversity was calculated for *B. burgdorferi* s.l. infections in ticks from Marin and Sonoma Counties separately because these sites were separated by over 100 km and the plot designs were different between the two counties (Swei et al., 2011a, 2012). The distribution of haplotype diversity identified from vertebrates sampled was also calculated for the two species with the highest sample sizes, *N. fuscipes* and *P. maniculatus*. Genetic differentiation between *B. burgdorferi* s.l. identified in ticks and mammals was assessed using the *F*-statistic (F_{ST}) in Arlequin (Excoffier et al., 2005) as well as Jost's *D* statistic, implemented in SMOGD (Crawford, 2010). Jost's *D* calculates the level of genetic differentiation between populations and ranges from 0 to 1, with 0 representing identical genetic composition and 1 indicating wholly differentiated populations (Jost, 2008). In addition, Jost's *D* allows for populations with high allelic diversity and permits populations to be have alleles that are exclusively fixed in one population or the other (Jost, 2008), which some authors (Jost, 2008; Crawford, 2010) argue provides more accurate estimates of genetic differentiation than the *F*-statistic.

Results

I. pacificus infection prevalence

We collected and tested a total of 7684 *I. pacificus* nymphal ticks between 2006 and 2009 from Sonoma ($n = 6048$) and Marin Counties ($n = 1636$) in northern California (Table 1). Across the two counties, total infection prevalence of *B. burgdorferi* s.l. was 7.81% and *B. burgdorferi* s.s. prevalence was 6.96%. Our genetic analyses found that *B. burgdorferi* s.s. accounted for 89.17% of all positively identified ticks with the remaining 10.83% of all samples belonging to lineages of the *B. burgdorferi* s.l. complex not known to cause disease in humans. We excluded 123 *B. burgdorferi* s.l.-positive samples from phylogenetic and haplotype network analyses (102 from Marin, 21 from Sonoma) due to difficulties in sequencing as a consequence of the very low yield of DNA. However, we were able to classify some of these individuals as either *B. burgdorferi* s.s. from shorter sequence fragments and these results are included in Table 1. The prevalence of both *B. burgdorferi* s.s. and *B. burgdorferi* s.l. positive ticks in Marin and Sonoma Counties ranged from a low of 3.30% in Marin County in 2009 to a high of 14.51% in Marin County in 2006 (Table 1). The total tick infection prevalence averaged over all years of data (Table 1) indicates that values for *B. burgdorferi* s.l. and s.s. are not significantly different between the two regions examined ($\chi^2 = 1.182$, df = 1, $P > 0.05$).

Mammal infection prevalence

We tested 1805 small mammal tick hosts for *B. burgdorferi* s.l. in Marin County between 2006 and 2008; 699 dusky-footed woodrats (*Neotoma fuscipes*), 1028 deer mice (*Peromyscus maniculatus*), 11 pinon mice (*Peromyscus truei*), 45 California voles (*Microtus californicus*), nine western harvest mice (*Reithrodontomys megalotis*), eight non-native black rats (*Rattus rattus*) and five shrews (*Sorex* sp.). All *B. burgdorferi* samples sequenced from mammals were *B. burgdorferi* s.s. and fell into subnetwork 1 of our haplotype network (Fig. 3). *B. burgdorferi* s.s. infections rate for *N. fuscipes* was 17.31%, *P. maniculatus* was 7.39%, *P. truei* was 9.09%,and *M. californicus* had the lowest infection prevalence at 4.44%. In all of our sampled vertebrate hosts, the only genospecies we detected was *B. burgdorferi* s.s.

Haplotype diversity

Tick and mammal analyses revealed 31 unique *rrf–rrl* IGS haplotypes in mammals and ticks in Marin and Sonoma Counties (Tables 2–4). Based on phylogenetic analyses and supporting bootstrap values of reciprocally monophyletic clades (Supplemental Fig. A), we classified haplotypes 1–19 as *B. burgdorferi* s.s. based on strong phylogenetic support for a monophyletic clade with 88% bootstrap value (Supplemental Fig. A). All other haplotypes were classified as *B. burgdorferi* s.l. including several unsupported clades that contain haplotypes 20–25. These haplotypes were not classified as *B. burgdorferi* s.s. based on their exclusion from the well-supported 88% clade that forms *B. burgdorferi* s.s. and because they aligned with other uncharacterized isolates. For instance, haplotype 20 grouped with CA400, an uncharacterized *Borrelia* sp. (Girard et al., 2011), with 98% bootstrap value. Haplotype 28 grouped with CA2, an unclassified *Borrelia* sp. that previous studies found forms a group distinct from *B. burgdorferi* s.s. based on DNA–DNA hybridization and sequencing analyses (Valsangiacomo et al., 1997; Postic et al., 1998). Haplotypes 29 and 30 grouped with an uncharacterized *Borrelia* sp. strain CA13 (92% bootstrap). We found marginal support that haplotype 31 grouped with this

Table 1
Summary of tick infection prevalence in Marin and Sonoma Counties in Northern California and sample sizes of total ticks tested for *B. burgdorferi* infection.

		% (95% BCI)				
		2006	2007	2008	2009	Total
Marin Co.	$N = 1440$	$N = 1668$	$N = 1210$	$N = 1730$	$N = 6048$	
	Bbss	11.32 (9.78–13.06)	7.73 (6.55–9.11)	5.04 (3.95–6.42)	3.30 (2.55–4.25)	6.80 (6.19–7.46)
	Bbsl	14.51 (12.80–16.43)	8.03 (6.83–9.43)	5.54 (4.39–6.97)	3.41 (2.66–4.27)	7.80 (7.16–8.51)
Sonoma Co.	$N = 515$	$N = 1121$			$N = 1636$	
	Bbss	9.51 (7.28–12.40)	6.70 (5.37–8.31)			7.60 (6.40–8.96)
	Bbsl	10.10 (7.78–13.00)	6.70 (5.37–8.31)			7.8 (6.62–9.22)

Sonoma County was sampled in 2006 and 2007, while Marin County was sampled each year between 2006 and 2009. Infection prevalences are given for *B. burgdorferi* sensu stricto (Bbss) and *B. burgdorferi* sensu lato (Bbsl) which includes *B. burgdorferi* s.s. In parentheses are 95% Bayesian credible intervals (BCI) for prevalence values.

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Table 2
Haplotype diversity in Marin County questing *I. pacificus* nymphs by year and site, China Camp State Park (CC) and Marin Municipal Water District (MW).

	2006			2007			2008			2009			All years		
	CC	MW	both	CC	MW	Both	CC	MW	Both	CC	MW	Both	CC (95% BCI)	MW (95% BCI)	Both (95% BCI)
Hap 01	19.35	**43.86**	**31.09**	**43.33**	**54.35**	**51.64**	15.79	24.14	19.40	6.90	**37.50**	20.75	20.75 (15.28–27.89)	**45.05 (38.40–51.95)**	**34.35 (29.64–39.39)**
Hap 02	6.45	8.77	7.56	36.67	6.52	13.93	10.53	**51.72**	28.36	**62.07**	**37.50**	**50.94**	23.27 (17.50–30.62)	17.33 (12.74–23.15)	19.95 (16.15–24.38)
Hap 03	**33.87**	24.56	29.41	3.33	11.96	9.84	**55.26**	3.45	**32.84**	17.24	8.33	13.21	**30.19 (23.75–37.96)**	13.86 (9.78–19.31)	21.05 (17.17–25.56)
Hap 04	0.00	0.00	0.00	0.00	26.09	19.67	0.00	20.69	8.96	0.00	12.50	5.66	0 (0–2.30)	16.34 (11.89–22.07)	9.14 (6.59–12.56)
Hap 05	0.00	1.75	0.84	3.33	1.09	1.64	0.00	0.00	0.00	6.90	4.17	5.66	1.89 (0.69–5.42)	1.49 (0.54–4.26)	1.66 (0.78–3.57)
Hap 19	0.00	0.00	0.00	0.00	0.00	0.00	2.63	0.00	1.49	0.00	0.00	0.00	0.63 (0.15–3.45)	0 (0–1.80)	0.28 (0.07–1.53)
Hap 20	1.61	0.00	0.84	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.63 (0.15–3.45)	0 (0–1.80)	0.28 (0.07–1.53)
Hap 21	1.61	1.75	1.68	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.63 (0.15–3.45)	0.50 (0.12–2.71)	0.55 (0.17–1.98)
Hap 22	14.52	15.79	15.13	3.33	0.00	0.82	0.00	0.00	0.00	0.00	0.00	0.00	6.29 (3.50–11.26)	4.46 (2.39–8.25)	5.26 (3.40–8.08)
Hap 23	1.61	0.00	0.84	0.00	0.00	0.00	2.63	0.00	1.49	0.00	0.00	0.00	1.26 (0.39–4.50)	0 (0–1.80)	0.55 (0.17–1.98)
Hap 24	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	3.45	0.00	1.89	0.63 (0.15–3.45)	0 (0–1.80)	0.28 (0.07–1.53)
Hap 25	3.23	0.00	1.68	0.00	0.00	0.00	0.00	0.00	0.00	3.45	0.00	1.89	1.89 (0.69–5.41)	0 (0–1.80)	0.83 (0.31–2.40)
Hap 26	3.23	3.51	3.36	6.67	0.00	1.64	0.00	0.00	0.00	0.00	0.00	0.00	2.52 (1.03–6.32)	0.99 (0.31–3.51)	1.66 (0.78–3.57)
Hap 27	3.23	0.00	1.68	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	1.26 (0.39–4.47)	0 (0–1.80)	0.55 (0.17–1.98)
Hap 28	4.84	0.00	2.52	0.00	0.00	0.00	10.53	0.00	5.97	0.00	0.00	0.00	4.40 (2.20–8.86)	0 (0–1.80)	1.94 (0.96–3.94)
Hap 29	3.23	0.00	1.68	3.33	0.00	0.82	0.00	0.00	0.00	0.00	0.00	0.00	1.89 (0.69–5.42)	0 (0–1.80)	0.83 (0.31–2.40)
Hap 31	3.23	0.00	1.68	0.00	0.00	0.00	2.63	0.00	1.49	0.00	0.00	0.00	1.89 (0.69–5.42)	0 (0–1.80)	0.83 (0.31–2.40)
Ticks (N)	776	664	1440	698	970	1668	621	589	1210	967	763	1730	3062	2986	6048
Bbsl+ (N)	62	57	119	30	92	122	38	29	67	29	24	53	159	202	361

The relative proportion of each haplotype by site and year is given. The most abundant haplotype at each site and/or year is highlighted in bold. Total sample size of the number of ticks tested and total number of ticks infected with *B. burgdorferi* s.l. are provided. In parentheses are 95% Bayesian credible intervals (BCI) for prevalence values across all years.

clade as well (54% bootstrap; Postic et al., 2007). In an ML analysis incorporating multiple loci, Postic et al. (2007) classified CA13 as *B. burgdorferi*, although our study found that this sample was well separated from the larger *B. burgdorferi* s.s. clade, although our genetic sampling was restricted to only one locus (Supplemental Fig. A). We did not find any haplotypes in our tick or vertebrate samples that grouped with *B. bissettii*, *B. californiensis* or *B. americana* previously described from northern California (Postic et al., 1998, 2007; Rudenko et al., 2009).

Haplotype diversity of B. burgdorferi s.l. in Marin ticks

Of the 31 unique haplotypes that we recovered, 17 were found in questing *I. pacificus* nymphs in Marin County between 2006 and 2009 (Table 2). A statistical parsimony network analysis recovered two unconnected sub-networks at the 95% confidence level (subnetworks 1 and 2, Fig. 1). As a consequence, we joined the two subnetworks using our phylogenetic results (Supplemental Fig. A and Fig. 1). Haplotypes 28 and 31 were more than 5 bp divergent from all the other haplotypes, and because of limited support for phylogenetic affinities in our maximum likelihood topology, these haplotypes could not be confidently placed in the network.

Numerically, the most common haplotypes were haplotypes 1, 3, 2, 4 in decreasing order. These four haplotypes collectively comprised 86.7% of all tick haplotypes sampled and 95% of all mammal haplotypes sampled (Fig. 1). The type strain for *B. burgdorferi* s.s. originally isolated from *I. scapularis* in New York (B31, Genbank Accession: L30127) was one point mutation different from haplotype 3 (Fig. 1). Marin nymphs had six closely related haplotypes we determined to be *B. burgdorferi* s.s. There was, on average, one point mutation between *B. burgdorferi* s.s. haplotypes (Fig. 1, subnetwork 1). The remaining 11 haplotypes were classified as *B. burgdorferi* s.l. and were, on average, separated by 2.5 point mutations (Fig. 1, subnetwork 2).

Haplotypes 1, 2 and 3 were the dominant haplotypes and were found in both of our Marin County study sites (China Camp (CC) and Marin Water (MW); Fig. 1). Some haplotypes were unique to particular sites. For instance, haplotype 4 was only found at Marin Water whereas haplotypes 20, 23, 24, 25, 29, 28 and 31 were only found at China Camp State Park (Fig. 1). The probability that haplotype 4 was missed at CC and the other haplotypes were missed at Marin Water is low (0–2.3 and 0–1.8%, respectively) based on Bayesian credible interval analysis (Table 2). The relative frequency of the three most common haplotypes varied depending on year and site. Site- and year-specific differences in haplotype prevalence are presented in Table 2. Averaged across all years, haplotype 1 was more common at Marin Water and haplotype 3 was more common at China Camp (Table 2). Overall, haplotype 1 was the most commonly encountered haplotype in Marin ticks (34.35%, Table 2).

Haplotype diversity of B. burgdorferi s.l. in Sonoma ticks

Seven unique haplotypes, all identified as *B. burgdorferi* s.s., were recovered from questing *I. pacificus* nymphs ($n = 1636$) in Sonoma County between 2006 and 2007 (Table 3, Fig. 1). The three dominant haplotypes from Marin County (haplotypes 1–3) were also found in Sonoma County. Haplotypes 1 and 3 were the most common, followed by haplotype 2. A few rare haplotypes were one point mutation divergent from either haplotypes 1 or 3 (Fig. 1).

Haplotype diversity of B. burgdorferi s.l. in Marin mammals

We identified 14 unique haplotypes in mammals sampled from Marin County. These were isolated from four species: *N. fuscipes*, *P. maniculatus*, *M. californicus* and *P. truei*. All haplotypes isolated from mammals trapped in Marin County were *B. burgdorferi* s.s. (Fig. 2). Similar to the tick populations, haplotypes 1–3 were the most common and each had several single, rare and closely related

Table 3
Distribution of haplotypes in Sonoma County *I. pacificus* nymphs by year.

	% (95% BCI)		
	2006	2007	All years
Sample size (*N*)	515	1121	1636
Bbsl (*N*)	41	64	105
Hap01	16.67 (7.45–33.73)	**47.83 (29.12–67.18)**	**52.38 (42.46–61.18)**
Hap02	3.33 (0.80–16.70)	30.43 (15.63–51.09)	8.57 (4.57–15.37)
Hap03	**73.33 (55.39–85.78)**	13.04 (4.74–32.36)	33.33 (24.80–42.45)
Hap05	3.33 (0.80–16.70)	0 (0–14.25)	2.86 (1.03–7.80)
Hap06	0 (0–11.22)	4.35 (1.03–21.12)	0.95 (0.23–5.10)
Hap13	0 (0–11.22)	4.35 (1.03–21.12)	0.95 (0.23–5.10)
Hap16	3.33 (0.80–16.70)	0 (0–14.25)	0.95 (0.23–5.10)

The proportion of the total haplotype population is shown as a percentage. The most prevalent haplotype is shown in bold. Total sample size of the number of ticks tested and total number of ticks infected with *B. burgdorferi* s.l. (Bbsl) are provided. In parentheses are 95% Bayesian credible intervals (BCI) for prevalence values.

Table 4
Distribution of haplotypes in small rodents in Marin County by site: China Camp State Park (CC) and Marin Municipal Water District (MW).

	N. fuscipes			*P. maniculatus*		
	CC (%)	MW (%)	Both [% (95 BCI)]	CC (%)	MW (%)	Both [% (95 BCI)]
Mammal (*N*)	642	57	699	281	747	1028
Bbsl (*N*)	82	7	89	24	35	59
Hap 01	23.17	28.57	23.6 (16.00–33.43)	33.33	**45.71**	40.68 (30.16–55.07)
Hap 02	23.17	28.57	23.6 (16.00–33.43)	4.17	11.43	8.47 (3.89–18.98)
Hap 03	**46.34**	**42.86**	**46.07 (36.07–56.40)**	**50**	37.14	**42.37 (31.74–56.77)**
Hap 05	1.22	0	1.12 (0.27–6.04)	0	0	0 (0.04–6.16)
Hap 06	0	0	0 (0.03–4.02)	0	2.86	1.69 (0.42–9.24)
Hap 07	1.22	0	1.12 (0.27–6.04)	0	0	0 (0.04–6.16)
Hap 08	1.22	0	1.12 (0.27–6.04)	0	0	0 (0.04–6.16)
Hap 09	0	0	0 (0.03–4.02)	4.17	0	1.69 (0.42–9.24)
Hap 10	0	0	0 (0.03–4.02)	0	2.86	1.69 (0.42–9.24)
Hap 11	1.22	0	1.12 (0.27–6.04)	0	0	0 (0.04–6.16)
Hap 12	0	0	0 (0.03–4.02)	4.17	0	1.69 (0.42–9.24)
Hap 14	1.22	0	1.12 (0.27–6.04)	0	0	0 (0.04–6.16)
Hap 15	0	0	0 (0.03–4.02)	4.17	0	1.69 (0.42–9.24)
Hap 17	1.22	0	1.12 (0.27–6.04)	0	0	0 (0.04–6.16)

Dusky-footed woodrats (*N. fuscipes*) and deer mice (*P. maniculatus*) were the most commonly infected vertebrates. The prevalence of each haplotype across all haplotypes for each species and site are shown as percentages. In parentheses are 95% Bayesian credible intervals (BCI) for prevalence values for each species across both sites. The most common haplotype relative to the total population of haplotypes is shown in bold. Total sample size of the number of *N. fuscipes* and *P. maniculatus* tested and total number of animals infected with *B. burgdorferi* s.l. are provided.

haplotypes, and were all isolated from the most common mammals in our small mammal surveys, *N. fuscipes* or *P. maniculatus* (Fig. 2). Similarly, haplotype diversity was found in *N. fuscipes* and *P. maniculatus* (Fig. 2), although haplotype 2 occurred in all five rodents sampled (Fig. 2). Haplotype 3 was the most common genotype isolated from *N. fuscipes* and *P. maniculatus* (Table 4).

Genetic differentiation of subpopulations

Genetic differentiation of *B. burgdorferi* s.l. subpopulations isolated from ticks from Marin and Sonoma County and from mammals trapped in Marin County was analyzed by Jost's *D* and F_{ST} statistics (Table 5). Pairwise comparisons between subpopulations found small but statistically significant genetic differentiation between ticks from Marin and Sonoma Counties, and between the two tick subpopulations and Marin mammals (Table 5). The degree of differentiation ranged from 0.019 to 0.023 with statistical significance (95% confidence internals not intersecting zero). Although the values differ slightly between F_{ST} and Jost's *D*, the ranked order of genetic differentiation was the same. From the lowest to the highest, the order of pairwise genetic differentiation are Sonoma tick and Marin mammal, Sonoma tick and Marin tick, and Marin tick and Marin mammal (Table 5).

Table 5
Jost's *D* and F_{ST} statistic for genetic differentiation of *B. burgdorferi* s.l. from ticks and mammals.

	D_{ST} (95% CI) and F_{ST} (95% CI)		
	Marin tick	Sonoma tick	Marin mammal
Marin tick	–	0.020 (0.010–0.040) 0.025 (0.012–0.049)	0.023 (0.010–0.045) 0.028 (0.012–0.055)
Sonoma tick		–	0.019 (0.002–0.050) 0.024 (0.002–0.065)
Marin mammal			–

Genetic differentiation as measured by Jost's *D* statistic calculates the level of subpopulation genetic distance relative to the total population's genetic diversity. Jost's *D* takes into account populations with high allelic diversity. Estimates of D_{ST} and the 95% confidence intervals are provided. F_{ST} is a standard measure of population similarity that is mathematically equivalent to D_{ST} but is not weighted by allelic differentiation.

Discussion

Comparative genetic diversity of zoonotic pathogens can reveal ecological and evolutionary patterns of public health concern. Lyme disease is the leading vector-borne disease in the United States but in many areas, the transmission dynamics and pathogen reservoirs are still unresolved. Our haplotype analyses focused on an IGS region (*rrf–rrl*, 5S–23S rRNA). This locus and another IGS regions (Bunikis et al., 2004; Humphrey et al., 2010) have been used extensively by researchers because they can differentiate between *B. burgdorferi* s.s. and other *B. burgdorferi* s.l. genospecies (Postic et al.,

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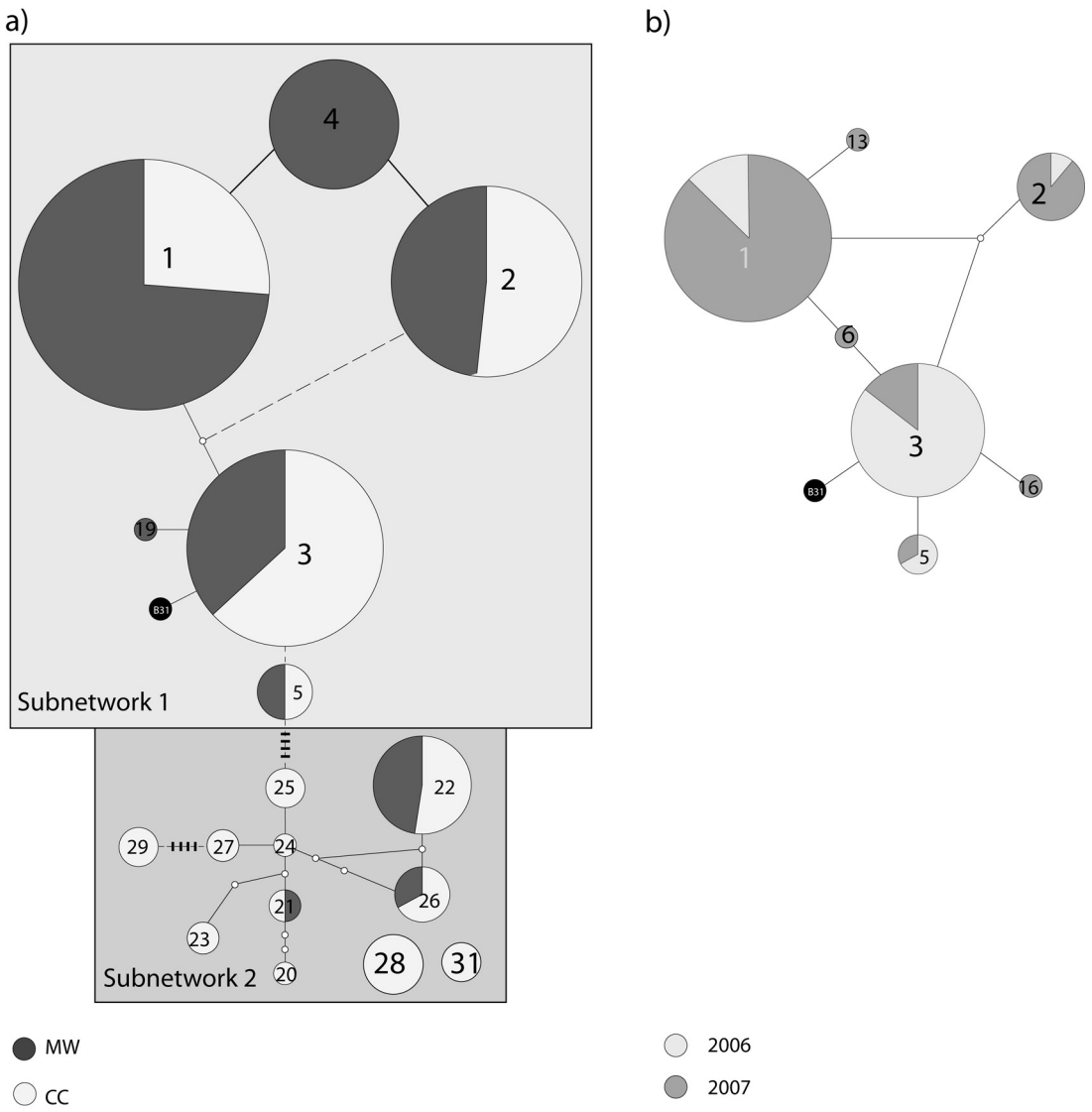

Fig. 1. Haplotype network of *B. burgdorferi* isolated from questing *I. pacificus* nymphs in (a) Marin County and (b) Sonoma County. The size of the haplotype circles indicates the frequency of the haplotype in the population and point mutations are indicated with a circle or dash. Each line segment represents one base pair mutation. The type strain (B31) is included for reference. Haplotypes are color-coded by sites for Marin County, China Camp State Park (CC) and Marin Municipal Water District (MW). Subnetworks 1 and 2 were joined by using our phylogenetic tree (Supplementary Fig. 1). Haplotypes that diverged by more than 5 bp were not joined in the network and are placed below subnetwork 2. The Sonoma County haplotype network is color-coded by year of collection.

1994; Chao et al., 2011; Rudenko et al., 2011). Many other loci such as the *ospA* or *ospC* genes are not ideal for phylogenetic analyses of inferences because of frequent recombination events (Anderson and Norris, 2006). Thus, for population and subpopulation level analysis of genetic diversity and differentiation, we consider the *rrf–rrl* IGS locus to be highly informative for studies investigating population dynamics at a local geographic scale.

We found three common *B. burgdorferi* s.s. haplotypes that occur in *I. pacificus* populations in two counties of Northern California and in small mammals in one of those counties, haplotypes 1–3. In the mammal population examined as well as the Sonoma County *I. pacificus* ticks, we only recovered *B. burgdorferi* s.s. from our genetic assays. Our recovery of three closely related *B. burgdorferi* s.s. haplotypes that occur in high frequency is in contrast to a study in the Northeast United States (Humphrey et al., 2010) that reported a more diverse, and on average, more distantly related population of *B. burgdorferi* haplotypes, although their study was based on a different IGS marker and spanned a greater geographic area. This pattern of reduced genetic diversity in California versus the northeastern US is supported by other studies that used other genetic markers and may reflect the lower frequency of *B. burgdorferi* or narrower ecological niche (Girard et al., 2009). The dominance of a few haplotypes found in this study may indicate a more recent establishment of *B. burgdorferi* s.l. in the western United States relative to the eastern United States, which supports the east–west movement of *B. burgdorferi* s.l. posited by other studies (Hoen et al., 2009). A notable difference of our study with other studies conducted in northern California was the absence of *B. burgdorferi* s.l. genospecies such as *B. bissettii* or *andersonii* (Brown et al., 2006; Eisen et al., 2003) that are present at high prevalence particularly in the reservoir host, *N. fuscipes*. Although there are sizable *N. fuscipes* populations in our Marin County sites, they were only found to be infected with *B. burgdorferi* s.s., demonstrating the high degree of geographic or habitat-related heterogeneity in *B. burgdorferi* diversity as well as the identity of pathogen reservoirs. It would be informative to expand haplotype analysis to other regions in Northern California with higher prevalence of other genospecies for more detailed analysis.

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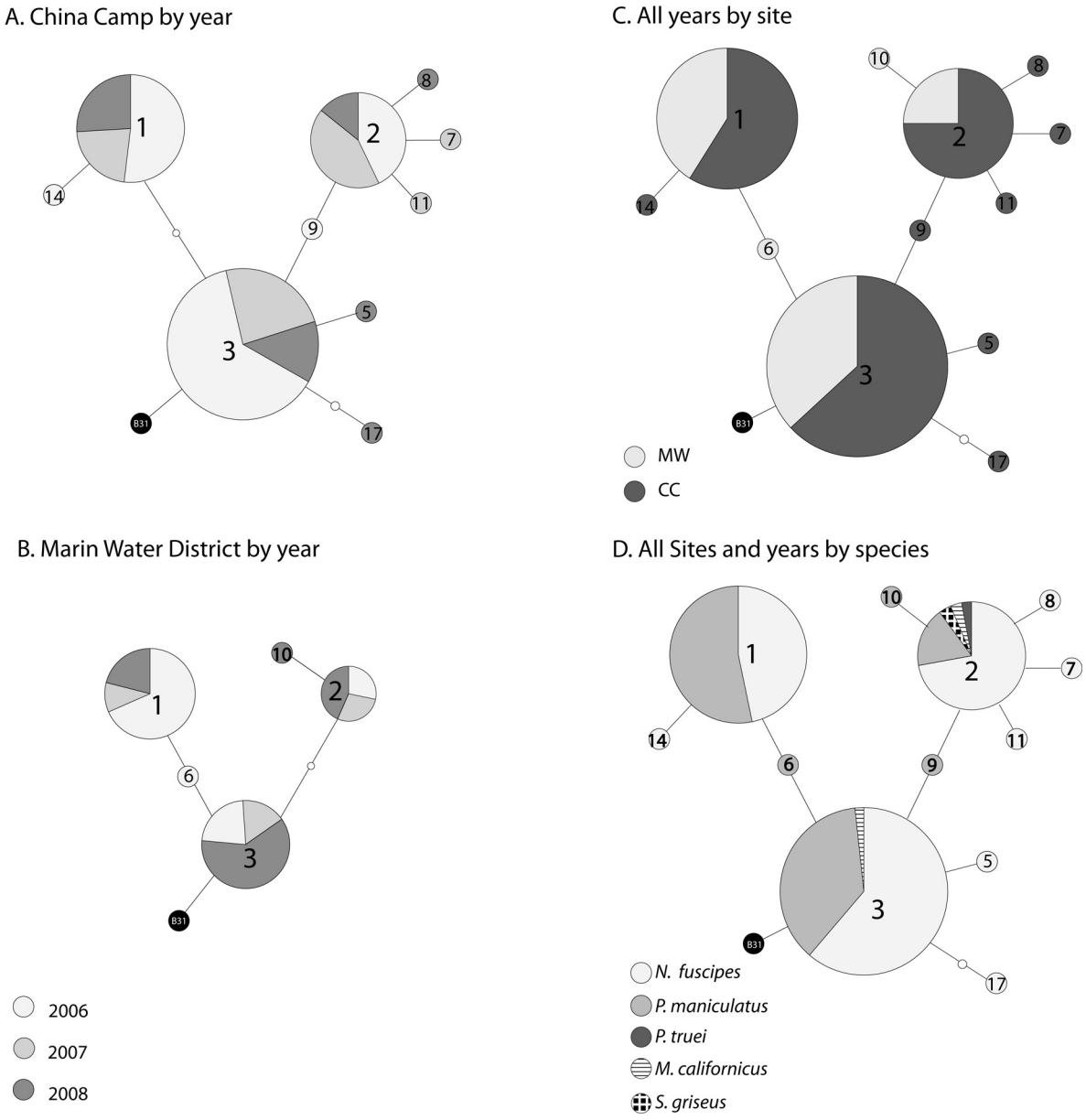

Fig. 2. Haplotype networks of *B. burgdorferi* isolated from vertebrate tick hosts in Marin County, and one sample from Mendocino County. Networks are shown illustrating the distribution of haplotypes at (a) China Camp State Park (CC) by year and (b) Marin Municipal Water District (MW) by year, (c) all years by site CC and MW, and (d) all sites and years by host species. The size of the haplotype circles indicates the frequency of the haplotype in the population. Each line segment represents one base pair mutation. The type strain (B31) is included for reference.

We found striking differences in the pattern of genetic diversity of *B. burgdorferi* s.l. lineages sampled in mammals and ticks from the same sites and years. *B. burgdorferi* s.l. lineages sampled from mammals (Fig. 2) had several singleton haplotypes, which were not recovered in ticks (Fig. 1, subnetwork 1). In contrast, tick subnetwork 2, which is comprised of genospecies of *B. burgdorferi* s.l. not known to cause disease in humans, is absent from mammals sampled in this study (Fig. 2). Nine Marin County tick haplotypes (Fig. 1) were placed outside the *B. burgdorferi* s.s. clade in subnetwork 2 (Supplementary Fig. A). In contrast, subnetwork 2 haplotypes were not found in Sonoma County ticks (Fig. 1). The absence of subnetwork 2 haplotypes from Marin County mammals and Sonoma County ticks may reflect a true ecological barrier or be a result of insufficient sampling. Although vertebrate community composition or abundance data are not available for our Sonoma County sites, it is possible that the observed lower diversity of *B. burgdorferi* s.l. diversity is due to lower vertebrate diversity and therefore fewer enzootic cycles to maintain pathogen diversity (Margos et al., 2011).

Statistical parsimony was used to construct our haplotype networks. Interpretation of our networks was based on three assumptions: (1) older or more frequent alleles are more likely to be interior in the network, (2) older (more common) alleles will have more connections and (3) "singletons" (haplotypes occurring only once) are not likely to be connected to each other and are expected to be connected to older alleles (Posada and Crandall, 2001). Based on these assumptions, haplotype 3 is most likely to be the ancestral haplotype because it occupies an interior position in the network (Crandall and Templeton, 1993). Isolate B31 was originally isolated from Shelter Island, New York, and is most closely related to haplotype 3 perhaps reflecting the east coast origin of *B. burgdorferi* sensu lato (Hoen et al., 2009; Fig. 1).

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Haplotypes 1–3 were found in multiple Marin County mammal species across both sites and multiple years, haplotype 1 is more prevalent in *P. maniculatus* from Marin Water and haplotype 3 is more prevalent in *N. fuscipes* at both sites and *P. maniculatus* overall, perhaps due to higher transmission success between *I. pacificus* and this species. Haplotype 2, on the other hand, is present in the greatest number of host species and could represent a haplotype with high transmission rate and persistence across a diversity of host species (Thomas et al., 2002).

We sampled considerably more ticks than mammals in this study ($n = 7684$ vs. $n = 1805$) and expected to find greater haplotype diversity in the tick population. Ticks did yield more haplotypes of *B. burgdorferi* s.l. but there was much less *B. burgdorferi* s.s. diversity in ticks relative to mammals (Fig. 1, subnetwork 1). The three common haplotypes (haplotypes 1–3) were present and common in both ticks and mammals, but mammals had many more singleton haplotypes identified as *B. burgdorferi* s.s. (Fig. 1 vs. Fig. 2). The chances that our Marin County tick surveys missed one of the rare haplotypes that we found in mammals is very low (0.06%). Other tick species may be the source of these low-frequency haplotypes in the mammals but in our 3-year survey of tick burdens we found little evidence of ticks other than *I. pacificus* on small mammals in Marin County (Swei, unpublished data). The Lyme disease spirochete readily disseminates to various tissues types in the host (Barthold et al., 1991) and can be maintained in the host for several years (Lane et al., 1999). We therefore speculate that replication of *B. burgdorferi* s.s. in the vertebrate hosts could be generating the mutations that result in the singleton haplotypes that we document in the host community and that *B. burgdorferi* with these mutations are not successfully transmitted to the tick population. However, although most *B. burgdorferi* with rare singleton haplotypes are not in found ticks, some, like Hap19, occasionally are (e.g. Fig. 1). When *B. burgdorferi* with these rare mutations that are successfully transmitted back to the tick population may be important in the generation of viable new strains or genospecies.

Our finding of multiple haplotypes of *B. burgdorferi* s.l. in ticks but not in the small mammals suggests that there are other hosts involved in the maintenance of these haplotypes that we did not sample or there was differential transmission of bacterial strains. The vertebrate species sampled in this study were only found to have haplotypes belonging to subnetwork 1, with a large proportion of their haplotype diversity originating from haplotypes 1–3. To the extent that our sampling effort captured the full haplotype diversity in the vertebrates, one explanation is that the haplotypes belonging to *B. burgdorferi* s.l. subnetwork 2 found in the Marin tick population suggests that those haplotypes originated from non-sampled taxonomic groups such as birds, insectivores or larger mammals such as carnivores (Kurtenbach et al., 2002a). Similarly, while we found similar haplotype diversity of *B. burgdorferi* s.s. (subnetwork 1) in *I. pacificus* ticks from Marin and Sonoma County, *B. burgdorferi* s.l. genospecies belonging to subnetwork 2 were absent in our sampling of Sonoma County ticks. The sample size of ticks tested in Marin was much higher than the Sonoma tick sample, which may explain the lack of haplotypes from subnetwork 2 in Sonoma or could reflect a difference in enzootic cycles. But differential transmission of *B. burgdorferi* strains may also explain the patterns found here.

We detected a small but statistically significant level of genetic differentiation based on 95% confidence intervals generated from both F_{ST} and Jost's D_{ST} statistic (Table 5). The greatest level of genetic differentiation was found between Marin tick and Marin mammal populations of *B. burgdorferi* s.l. (Table 5). This indicates that there are significant barriers to transmission of *B. burgdorferi* in its natural enzootic cycle. These barriers may be ecological or physiological. The causes and drivers of this genetic differentiation warrant more in-depth analysis.

Host species can vary considerably in their competency, or ability to act as pathogen reservoirs, and in extreme cases, some species are entirely non-permissive to particular *B. burgdorferi* s.l. genospecies such as western fence lizards (*S. occidentalis*) that are refractory to *B. burgdorferi* (Kurtenbach et al., 2002b). Similarly, there is evidence that compatibility and transmission efficiency can also vary with vector–pathogen associations (Dolan et al., 1998). Particular *B. burgdorferi* s.s. outer surface protein C (OspC) or *rrs–rrl* genotypes that are found only in certain vertebrate hosts may reflect the presence of host-specific pathogen compatibility as well (Brisson, 2004; Girard et al., 2009). Our study found that the distribution of genetic diversity was not homogenous between different tick populations or between ticks and their sympatric vertebrate hosts. In particular, we found very few *B. burgdorferi* s.l. genospecies in vertebrate hosts examined in Marin County as well as *I. pacificus* ticks collected from Sonoma County that were not *B. burgdorferi* s.s. This finding may reflect some of the underlying mechanisms that drive larger patterns of genetic diversity found in *B. burgdorferi* s.l. populations. We believe that this represents evidence that although ticks and mammals interact to maintain *B. burgdorferi* s.l. in enzootic transmission cycles, there are isolating host and vector-driven factors that generate genetic diversity and differentiate these components. For instance, genetic variability may be generated within the mammal hosts but most of this variability is not found in the tick population either because of differential transmission or maintenance of *B. burgdorferi* haplotypes within two disparate pathogen environments in the host and vector.

Conclusions

We conducted an extensive survey and analysis of the genetic diversity of a single neutral locus in *B. burgdorferi* s.l. in both ticks and vertebrate hosts in northern California (Coipan et al., 2013). We document a small but significant pattern of haplotype diversity in mammal and ticks that is distinct between ticks and their hosts. The genetic diversity that we found in *B. burgdorferi* s.l. likely reflects the complex host landscape the pathogen faces and stresses the need for more detailed understanding of the ecological and evolutionary processes that shape the genetic population structure of *B. burgdorferi* s.l., especially as it may apply to understanding the transmission biology and genetic patterns of *B. burgdorferi* in the far-western United States.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ttbdis.2015.03.011.

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