Geographic Uniformity of the Lyme Disease Spirochete (*Borrelia burgdorferi*) and Its Shared History With Tick Vector (*Ixodes scapularis*) in the Northeastern United States

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ABSTRACT

Over 80% of reported cases of Lyme disease in the United States occur in coastal regions of northeastern and mid-Atlantic states. The genetic structure of the Lyme disease spirochete (*Borrelia burgdorferi*) and its main tick vector (*Ixodes scapularis*) was studied concurrently and comparatively by sampling natural populations of *I. scapularis* ticks along the East Coast from 1996 to 1998. Borrelia is genetically highly diverse at the outer surface protein *ospC*. Since Borrelia is highly clonal, the *ospC* alleles can be used to define clones. A newly designed reverse line blotting (RLB) assay shows that up to 10 Borrelia clones can infect a single tick. The clone frequencies in Borrelia populations are the same across the Northeast. On the other hand, *I. scapularis* populations show strong regional divergence (among northeastern, mid-Atlantic, and southern states) as well as local differentiation. The high genetic diversity within Borrelia populations and the disparity in the genetic structure between Borrelia and its tick vector are likely consequences of strong balancing selection on local Borrelia clones. Demographically, both Borrelia and *I. scapularis* populations in the Northeast show the characteristics of a species that has recently expanded from a population bottleneck. Major geological and ecological events, such as the last glacial maximum (18,000 years ago) and the modern-day expansion of tick habitats, are likely causes of the observed "founder effects" for the two organisms in the Northeast. We therefore conclude that the genetic structure of *B. burgdorferi* has been intimately shaped by the natural history of its main vector, the northern lineage of *I. scapularis* ticks.

THE evolutionary history of a species is in essence a coevolutionary process of species interactions (Thompson 1999) and this is particularly true of obligatory parasites (Price 1980). Our study infers the evolutionary history and population dynamics of *Borrelia burgdorferi*, the agent of Lyme disease, and its main tick vector, *Ixodes scapularis* (Acari: Ixodidae), from a comparative analysis of biogeographic patterns of the two species along the East Coast of the United States.

Lyme disease (also called Lyme borreliosis; Nadelman and Wormser 1998) is the most prevalent arthropod-borne disease in Europe and the United States (Baranton *et al.* 1998; Orloski *et al.* 2000). In the United States, >80% of Lyme disease cases occurred in the northeastern and mid-Atlantic seaboards (Orloski *et al.* 2000). *B. burgdorferi* (sensu stricto), one of 10 recognized genomic species in the species complex *B. burgdorferi* sensu lato distributed globally (Baranton *et al.* 1992; Canica *et al.* 1993; Kawabata *et al.* 1993; Balmelli and Piffaretti 1996; Le Fleche *et al.* 1997; Wang *et al.* 1997; Postic *et al.* 1998), is the predominant pathogenic species in North America (Mathiesen *et al.* 1997; Ras *et al.* 1997). The blacklegged tick *I. scapularis* (Keirans *et al.* 1996) is the primary vector of *B. burgdorferi* in North America (Dennis *et al.* 1998). The same tick species is also a vector of many other tick-borne diseases such as human babesiosis (Spielman *et al.* 1985), human granulocytic and monocytic ehrlichiosis (Schauber *et al.* 1998), and a tick-borne encephalitis (Telford *et al.* 1997).

The genome of *B. burgdorferi* has been sequenced (Fraser *et al.* 1997; Casjens *et al.* 2000) and shown to comprise a linear chromosome of ~0.91 Mb and at least 21 linear and circular plasmids with combined size of >0.5 Mb. *B. burgdorferi* is basically clonal, showing very little evidence for recombination or transfer of plasmids (Dykhuizen *et al.* 1993; Balmelli and Piffaretti 1996). What lateral transfer there is seems to be solely small piece (<1 kb) transfer (Dykhuizen and Baranton 2001).

Being a vector-borne, obligate parasite, *B. burgdorferi* undergoes severe population bottlenecks during both the host-to-tick and tick-to-host transmissions (for reviews of the transmission cycle see Lane *et al.* 1991; Sonenshine 1993). Thus, *B. burgdorferi* should have a small effective population size and an evolutionary his-

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tory closely associated with that of *I. scapularis*. Evolutionary relationships between the parasite and its vector may be revealed only through comparative genetic analysis of local populations of the two organisms. In a previous population genetic study of *B. burgdorferi*, little genetic differentiation was found among samples collected from different locations on eastern Long Island, New York (Qiu *et al.* 1997). The present study includes an analysis of the tick populations so that the population structures of the parasite and its vector can be compared. In addition, this study samples ticks and their infecting Borrelia along the East Coast from Massachusetts to South Carolina, giving a much-expanded survey. Previous surveys of *B. burgdorferi* and *I. scapularis* in North America (*e.g.*, Rich *et al.* 1995; Norris *et al.* 1996, 1999; Mathiesen *et al.* 1997) have examined the two organisms separately and have not analyzed within- and among-population variation.

MATERIALS AND METHODS

Study sites, tick processing, and determination of Borrelia infection: Blacklegged ticks (*I. scapularis*) were collected from vegetation by flagging during their host-seeking seasons (in the fall for adult ticks and early June for nymphs) during 1997–1998. The study area includes multiple sites in eastern Long Island, New York (Figure 1A) and 12 sites along the East Coast (Figure 1B). Samples of other tick species (*I. Pacificus* and *Dermacentor andersoni*) were collected from western North America and used as outgroups in phylogenetic analysis. The sources of the ticks are listed in Table 1. In the laboratory, frozen ticks were bisected individually. Total DNA was extracted using Chelex as described previously (Qiu *et al.* 1997). A nested-PCR procedure that targets the outer surface protein A locus (*ospA*) was used to determine whether the tick was infected with Borrelia (Guttman *et al.* 1996).

Cold single-strand conformation polymorphism analysis of tick mitochondrial 16S rRNA genes: Tick mitochondrial 16S ribosomal RNA genes were amplified using nested PCR. A 460-bp fragment was amplified for 15 cycles using primers 16S + 1 (primer sequences are listed in Table 2) and 16S − 1 in a 25-μl reaction mixture containing 5 μl of the tick DNA extract, 200 μm of each dNTP, 50 mm KCl, 10 mm Tris-HCl (pH 8.3), 2.5 mm $MgCl_2$, 0.1 μm of each external primer, and 1 unit of recombinant Taq polymerase (Life Technologies, Rockville, MD). The mixture was first heated to 96° for 1 min and then amplified for 15 cycles at 94° for 1 min, 48° for 1 min, and 72° for 1.5 min in a PTC-100 thermal cycler (MJ Research, Watertown, MA). Two microliters of this first amplification was used as a template for the second round of PCR. In the second round, a 300-bp portion of the 460-bp fragment was amplified using the same forward primer 16S + 1 and a reverse primer 16S − 2 in a 50-μl mixture containing 200 μm each dNTP, 50 mm KCl, 10 mm Tris-HCl (pH 8.3), 2.5 mm $MgCl_2$, 0.5 μm each primer, and 1.25 units of recombinant Taq polymerase. The amplification was run for 35 cycles at 94° for 1 min, 54° for 1 min, and 72° for 1 min. The final PCR products were electrophoresed on a 2% agarose gel (NuSieve:SeaKem 2:1; FMC BioProducts, Rockland, ME) to view the results. These same PCR primers have been used to amplify mitochondrial DNA from 36 species of hard- and soft-body ticks (Black and Piesman 1994), including *I. scapularis* ticks collected from eastern United States (Norris *et al.* 1996).

The PCR-amplified 300-bp fragment of 16S rDNA was subjected to cold single-strand conformation polymorphism (SSCP) analysis (Hongyo *et al.* 1993) as modified by Guttman *et al.* (1996) to distinguish alleles. Common alleles were included as standards in each gel to identify alleles. Fragments of the 16S gene amplified with the primer pair of 16 + 2 and 16 − 1 were unsuitable for SSCP analysis because they did not form well-defined bands.

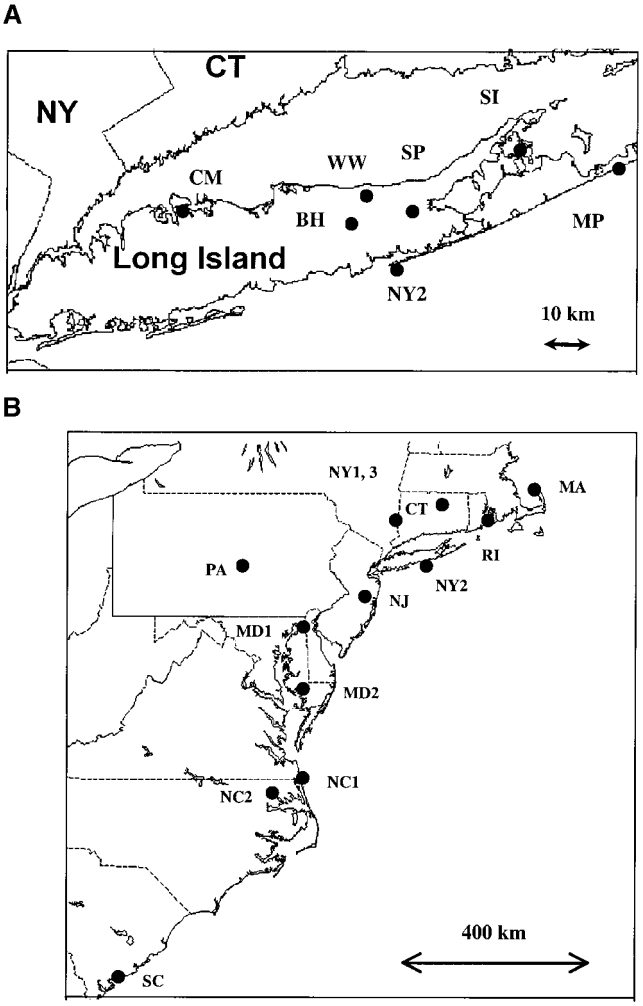

Figure 1.—Tick collection sites. (A) Long Island, New York; (B) East Coast.

Unique alleles, as recognized by distinct SSCP mobility patterns, were reamplified from the first amplification for an additional 30 cycles using 2 μl of the product of the first PCR reaction as template and primers 16S + 1 and 16 − 1. The 460-bp PCR product was purified using the Prep-A-Gene kit (Bio-Rad, Richmond, CA) according to the protocol recommended by the manufacturer. Each unique allele was then cycle sequenced from both directions using the PCR primers on a ABI 373S DNA sequencer (Perkin-Elmer, Norwalk, CT). The sequences were assembled and edited using the software Sequencher (Gene Code, Ann Arbor, MI). A total of 25 haplotypes were identified and their GenBank accession nos. are AF309008–AF309032.

Cold SSCP analysis of *ospA* variation: The genetic diversity of Borrelia was analyzed at the *ospA* locus (a single-copy gene located on a linear plasmid, lp54; see Fraser *et al.* 1997) for each infected tick. A PCR-amplified 345-bp fragment of *ospA* (from base 220 to 564) was subjected to cold SSCP analysis

TABLE 1

Tick source and Borrelia infection rate

Sample name	Location	Year	Stage	No. of ticks	Infection rate (SE) (%)	Collector/ collaborator
Long Island						
SI (LI1)	Shelter Island, NY	1996	Adult	50	64.0 (6.8)	J. Gebbia
WW (LI2)	Wildwood State Park, NY	1995, 1996	Adult	372, 199	44.7 (2.1)	E. Bosler
SP (LI3)	Swan Pond, NY	1995	Adult	70	42.9 (6.0)	G. Ugine
BH (LI4)	Brookhaven State Park, NY	1995	Adult	33	63.6 (8.5)	G. Ugine
CM (LI5)	Caumsett State Park, NY	1990	Adult	34	NA[c]	E. Bosler
NY2 (LI6)	Fire Island, NY	1997	Adult	44	22.7 (6.3)	H. Ginsberg
MP (LI7)	Montauk Point, NY	1998	Adult	43	41.9 (7.5)	M. Acosta
LI-nym	(Multiple sites)	1998	Nymph	380	4.2 (1.0)	M. Acosta
East Coast						
MA	Barnstable County, MA	1997	Adult	40	32.5 (7.4)	R. Mactier
RI	Washington County, RI	1997	Adult	40	30.0 (7.2)	R. Mactier
NY1	Millbrook, NY	1997	Adult	40	80.0 (6.3)	R. Ostfeld
NY3	Dutchess County, NY	1990, 1995	Adult, Nymph	33, 20	NA[c]	E. Bosler
CT	Tolland County, CT	1996	Adult	46	60.9 (7.2)	S. Bushmich
NJ	Monmouth County, NJ	1997	Adult	39	30.8 (7.4)	T. Schulze
PA	Lebanon County, PA	1998	Adult	39	2.6 (2.5)	M. Acosta
MD1	Cecil County, MD	1997	Adult	38	42.1 (8.0)	R. Mactier
MD2	Somerset County, MD	1997	Adult	11	81.1 (11.6)	R. Mactier
NC1	Currituck County, NC	1997	Adult	42	7.1 (4.0)	R. Mactier
NC2	Washington County, NC	1997	Adult	35	0	R. Mactier
SC	Beaufort County, SC	1997, 1998	Adult	52, 30	3.7 (2.1)	W. Golde
West Coast						
CA	Petaluma, CA	1997	Adult[a]	6	0	D. Taub
Van	Vancouver Island, BC, Canada	1997	Adult[b]	30	0	M. Morshed

[a] *I. pacificus.*
[b] Mixture of *I. pacificus* and *Dermacentor spp.*
[c] Not available.

as described above. (In this article, Borrelia sequences are numbered according to *ospA* or *ospC* sequences of strain B31.) Four SSCP mobility classes (five sequence haplotypes) of the amplified *ospA* fragments had previously been identified in Borrelia from eastern Long Island (Guttman *et al.* 1996; Qiu *et al.* 1997) and were used as controls for SSCP mobility classes. Any new mobility patterns indicating the presence of new *ospA* sequences were subsequently sequenced. GenBank accession numbers for the four *ospA* mobility classes (five haplotypes MC1a, MC1b, MC2, MC3, and MC4) and a new mobility class (MC5) are AF309002–AF309007.

Reverse line blotting assay of *ospC* variation: We also measured the genetic diversity of Borrelia populations using the outer surface protein locus, *ospC* (a single-copy gene located on a circular plasmid, cp26; see Fraser *et al.* 1997). Because of difficulties in interpreting the SSCP results when ticks are infected with multiple strains of *B. burgdorferi* , a method using reverse line blotting (Saiki *et al.* 1989; Kaufhold *et al.* 1994; Rijpkema *et al.* 1995; Kurtenbach *et al.* 1998) was designed for the direct detection and counting of *ospC* alleles in tick samples. The alleles of *ospC* of *B. burgdorferi* form "major sequence groups" (Wang *et al.* 1999). Sequences between major groups are ≥10% different from each other while sequences within groups are ≤1% different from each other (Wang *et al.* 1999). So far 21 major *ospC* groups (designated A through U) have been identified (Seinost *et al.* 1999; Wang *et al.* 1999). The advantages of reverse line blotting (RLB) over SSCP for the assay of *ospC* variation in natural population samples can be specified as the following:

1. RLB is more sensitive. The average number of *ospC* alleles in an infected tick was 1.5 using the SSCP (Wang *et al.* 1999), compared to 2.6 using RLB assays (see results).
2. Results of the RLB assay were generally less ambiguous since some *ospC* alleles show similar mobility patterns on a SSCP gel.
3. Individual bands tend to smear on SSCP gels when there is a high multiplicity of types.
4. The current RLB design identifies major groups but not sequence variation within a major group, while SSCP reveals DNA sequence change regardless of the number of bases changed. In other words, SSCP does not distinguish major groups from variation within these groups.

RLB has the following disadvantages: (1) Major groups, like group C, which are recombinants of other groups, can easily be misclassified (see below); and (2) unknown groups can escape detection (the universal probe can indicate a missing group only when that group is the only clone in a tick). Thus, the presence of some sequence groups like group C will sometimes have to be confirmed using other methods.

Design of ospC allele-specific probes: Among the 21 major *ospC* groups of *B. burgdorferi* identified (Seinost *et al.* 1999; Wang *et al.* 1999), four major groups (P, Q, R, and S) are found only in Europe and therefore were not included in this study. Groups L and O were also excluded in this study for lack of pure cultures of these two clones to be used as controls. Oligonucleotide probes specific for most major groups were designed according to sequences in a hypervariable gene re-

TABLE 2

Primers and oligonucleotide probes

Designation	Length	Sequence (5′ → 3′)
		PCR primers
16S + 1	22	CCGGTCTGAA CTCAGATCAA GT
16S − 1	29	CTGCTCAATG ATTTTTTAAA TTGCTGTGG
16S + 2	21	CGCTGTTATC CCTAGAGTAT T
16S − 2	22	TTGGGCAAGA AGACCCTATG AA
OA-ext (+)	23	AAAAAATATT TATTGGGAAT AGG
OA-ext (−)	29	GTTTTTTTAC TGTTTACAGT AATTGTTAA
OA-int (+)	16	GGAGTACTTG AAGGCG
OA-int (−)	18	GCTTAAAGTA ACAGTTCC
OC6 (+)	24	AAAGAATACA TTAAGTGCGA TATT
Fluo-OC6 (+)	24	Fluorescein-AAAGAATACA TTAAGTGCGA TATT
OC602 (−)	22	GGGCTTGTAA GCTCTTTAAC TG
OC623 (−)	24	TTAAGGTTTT TTTTGGACTT TCTGC
		RLB probes
OC-ALL	26	AGATTAGGCC CTTTAACAGA CTCATC
OC-A	22	ATTGTGATTA TTTTCGGTAT CC
OC-B	16	CTCGTTGCGA TTTGCT
OC-C		No specific probes (a recombinant between B, I, and E)
OC-D	36	ATGATTATTT AGAGTGCCTA AAGCATTGTT TTGATC
OC-E	39	TGTGTTTTTA CTCTGATTGG CCTCTAAACC ATTATTGCC
OC-F	27	CGCCTGAACG CCTAAACCAT TTGCATC
OC-G	16	GGTGTTGTGA TTCGCA
OC-H	30	GCCCCCATCG TCACCCAAAG TGCCATTTTG
OC-I	23	TTTGAAATTA AATATGCTCC TGA
OC-J	16	TCCGTTTTGA CCCACT
OC-K	33	CCCCGCTTCG CTACCTAAAC CAGCATTTTG TTG
OC-L	27	ATCGCTACCT AAAGTACCAC CTGCTTC
OC-M	31	ACCGGCATTT AAACCATTTT GGGCTATCAA A
OC-N	30	GTTTTGCACA TCATCTAAAC CATTATTATT
OC-O	22	TTGGTTAACT AAGCCATTTG CC
OC-T	18	ATGGCCTGCA TCGACACT
OC-U	19	CTGCCCTTGC AAGTCCTGT

gion (bases 237–285, Figure 2). Sequences of groups C and I are similar in this region and were distinguished using probes targeted at another region. A conserved 27-bp sequence (bases 70–96) shared by all alleles of *ospC* of *B. burgdorferi* was used as a control. Nucleotide sequences used in the RLB assay are listed in Table 2.

Fluorescein-labeled PCR amplification: A portion of the *ospC* gene was PCR amplified directly from infected ticks using a pair of primers OC6 (+) and OC602 (−) (Wang *et al.* 1999), one of which (OC6+) was commercially modified with fluorescein at the 5′ end. The 50-μl reaction mixture contained 10 μl of tick extract, 5 μl of 10× PCR buffer, 2.5 mM of $MgCl_2$, 200 μM of each dNTP, 1 μM of each primer, and 2.5 units of Taq polymerase. After initial denaturation at 96° for 1 min, the mixture was incubated for 45 cycles at 94° for 30 sec, 54° for 30 sec, and 72° for 1 min in a PTC-100 thermal cycler. The PCR product was examined afterward for purity and DNA concentration by electrophoresis on a 2% agarose gel. Fluorescein-labeled *ospC* genes were also amplified from cloned *ospC* preparation (Wang *et al.* 1999) or pure cultures of Borrelia strains to serve as positive controls in the RLB.

Tailing of oligonucleotide probes with poly(dT): Short stretches of oligonucleotides usually do not contain a sufficient number of thymine (T) residues to bind to a nylon membrane when activated by UV light (Saiki *et al.* 1989). Thus, between 400 and 600 thymine residues were added to the 3′ end of each oligonucleotide. The oligonucleotides were commercially synthesized and cartridge purified. After being reconstituted in water (not TE buffer, which inhibits terminal transferase activity), probes were tailed at 37° for 1 hr in a 50-μl mixture, containing 200 pmol oligonucleotides, 10 μl 5× reaction buffer (supplied by the manufacturer), 8 μl of 10 mM dTTP (nonsodium form, since sodium is an inhibitor of terminal transferase), and 4 units of terminal deoxynucleotidyl transferase [TdT (added last); Gibco BRL/Life Technologies]. The length of poly(dT) tails was examined by electrophoresis on a precast 20% TBE polyacrylamide gel (Novex, San Diego).

Membrane preparation: The tailed oligonucleotide probes were fixed onto a 15 × 15-cm nylon membrane (Stratagene, La Jolla, CA), using a MiniSlot 30 manifold (Immunetics, Cambridge, MA). The probes (40 pmol or 10 μl from 50-μl tailing product) were diluted in 2 ml of TE buffer and evenly applied into individual slots (0.8 mm × 13 cm) created by the manifold. The wet membrane was immediately UV crosslinked (125 mJ), briefly rinsed in water, and stored at 4° in 2× SSC. Once prepared, the membrane can be reused (up to 10 times) by stripping the membrane between uses (Boehringer Mannheim 1995).

Checkerboard hybridization assays: Up to 1350 individual hybridization assays (30 probes × 45 samples) can be performed

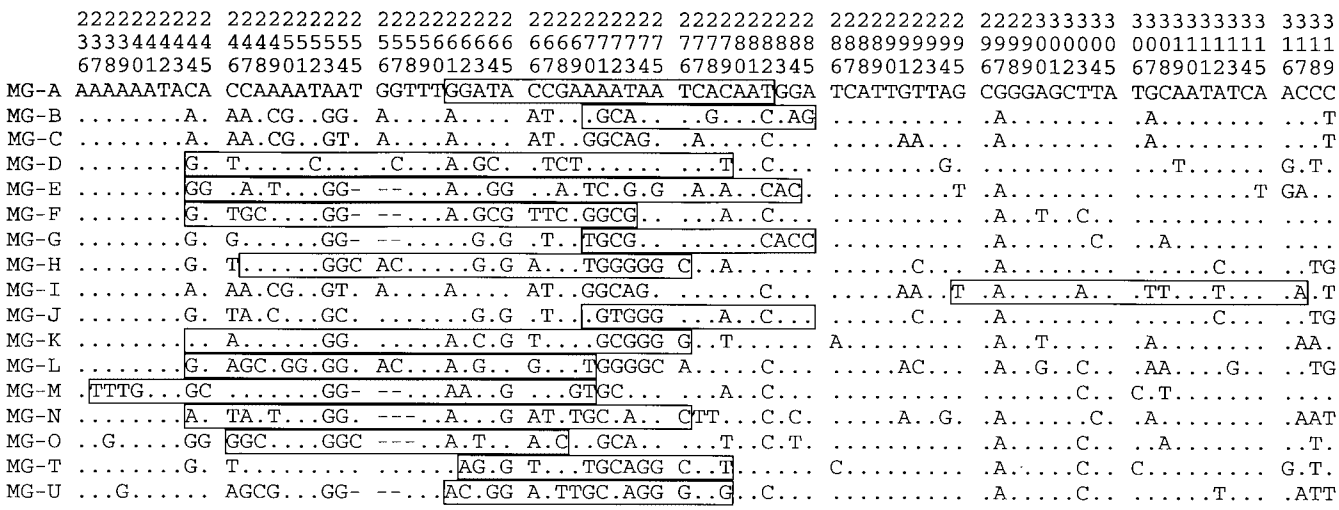

FIGURE 2.—The hypervariable region of *ospC* sequences used for designing oligonucleotide probes for the reverse line blotting (RLB) assay (B31 numbering). The probes used are boxed.

simultaneously using a checkerboard hybridization system (Checkerboard System 1; Immunetics; SOCRANSKY *et al.* 1994). The membrane was loaded into a 45-channel miniblotter. The lines in the miniblotter were perpendicular to the lines of the fixed probes. The channels were first filled with 100 μl of a hybridization solution (DIG Easy Hyb; Boehringer Mannheim). The hybridization solution contains 0.1 mg/ml Poly(A) (Sigma, St. Louis) to prevent nonspecific binding to the long poly(dT) tail of the probe. The miniblotter was incubated for 2 hr at 42° on a rocking platform. This solution was then aspirated and immediately replaced by 100 μl of hybridization solution containing also 1–10 μl (depending on concentration of amplified *ospC* genes) of denatured PCR products. The PCR products were denatured by heating in a boiling water bath for 10 min and chilling immediately on ice. The miniblotter was incubated again for 2 hr at 42° on a rocking platform. Subsequently, the channels were aspirated and washed twice at room temperature for 5 min with 2× SSC-0.1% SDS to remove unbound PCR products. The membrane was further washed stringently twice at 50° for 15 min with 0.1× SSC-0.1% SDS. Chemiluminescent detection was subsequently performed using a procedure suggested by the supplier (Boehringer Mannheim).

Statistical methods: *Gene frequency estimation:* The frequencies of the SSCP alleles of *ospA* in a Borrelia population were directly estimated from the occurrence of individual mobility classes in an SSCP assay. In the checkerboard hybridization assay of *ospC* variation using RLB, a positive signal produced on the membrane indicated the presence of a major *ospC* group in the tick. Numbers of positive signals were used as absolute gene frequencies of individual alleles in a population. The direct counting method (for both SSCP alleles and *ospC* major sequence groups) tends to underestimate the frequent alleles and overestimate rare ones, biasing toward an evenness in allele frequency distribution. RANNALA *et al.* (2000; see also ANDERSON and SCHEET 2001) developed an unbiased maximum-likelihood method for estimating gene frequencies in bacterial populations using molecular detection. For simplicity, the direct counting method was used in this study because the bias is very small and does not alter results of the analysis.

Tests of natural selection: The Ewens-Watterson-Slatkin test of neutrality (EWENS 1972; WATTERSON 1978; SLATKIN 1994), implemented in the computer program Arlequin Ver 1.1 (SCHNEIDER *et al.* 1997), was used for testing the presence of natural selection in maintaining the genetic diversity within Borrelia populations (on the basis of *ospA* and *ospC* frequencies) and tick populations (on the basis of frequencies of mitochondrial haplotypes).

Phylogenetic inference: The nucleotide sequences of all distinct haplotypes of the 460 bp of the tick mitochondrial 16S rRNA gene were aligned using CLUSTAL W (THOMPSON *et al.* 1994). Sequences of other haplotypes retrieved from GenBank (RICH *et al.* 1995; NORRIS *et al.* 1996) were included in the alignment. Unrooted minimum spanning trees (MSTs) were constructed manually to show sequence changes among haplotypes. Equal-length MSTs were discriminated using the approach of molecular variance parsimony (NEIGEL and AVISE 1993; EXCOFFIER and SMOUSE 1994). This method assumes that older alleles tend to be more frequent than new ones and new mutations are more likely to be found in the same population than in different ones. Missing links in the MSTs were filled with hypothetical haplotypes.

Genetic structure and spatial statistics: Genetic differentiation among local Borrelia populations was tested using the *G*-test of homogeneity of allele frequency distributions, using the biostatistical package BIOM (APPLIED BIOSTATISTICS 1996). For contingency tables with a small average number of entries (less than five), a nonparametric exact test (RAYMOND and ROUSSET 1995) was used. The population structure of *I. scapularis* along the East Coast was analyzed using analysis of molecular variance (AMOVA), a general framework for summarizing information on both nucleotide sequence variation and haplotype variation within and among geographic samples (EXCOFFIER *et al.* 1992). Three hierarchical levels were used: (1) within populations, (2) among populations within groups, and (3) among groups. Both the exact tests of population differentiation and AMOVA were performed using the molecular population genetic software package Arlequin Ver 1.1 (SCHNEIDER *et al.* 1997).

Demographic history: The program FLUCTUATE (KUHNER *et al.* 1998) was used to estimate population growth for both the tick and the Borrelia. On the basis of sequences of DNA haplotypes and frequencies of these haplotypes, the program coestimates the current population size (in the form of mutation parameter $\theta = 4N_e\mu$) and population growth rate (g, exponential growth rate, with the unit of $1/\mu$ individuals per generation).

RESULTS

***B. burgdorferi* population structure:** *Infection rate:* The infection rate as determined by PCR amplification of the Borrelia *ospA* gene was 20–80% in the Northeast and 0–7% in North and South Carolina (Table 1). One inland tick population from Pennsylvania (PA) also had low Borrelia infection rate (1 out of 39 ticks or 2.6%). All these adult ticks were collected in the same season (November 1997) and the density of adult *I. scapularis* ticks was as high in the South as in the Northeast. These results are in agreement with the findings of other field studies (Sonenshine *et al.* 1995; Piesman *et al.* 1999; Oliver *et al.* 2000).

Genetic diversity at ospA: Four SSCP mobility classes (five sequence haplotypes, MC1a, MC1b, MC2, MC3, and MC4) of *ospA* were identified in previous surveys of eastern Long Island, New York (Guttman *et al.* 1996; Qiu *et. al.* 1997). The frequency distributions of these classes were uniform across five populations of *B. burgdorferi* in adult ticks on eastern Long Island (Qiu *et al.* 1997). In this survey we wished to determine if populations along the East Coast of the United States are also uniform.

We determined the *ospA* alleles of the Borrelia from 109 infected *I. scapularis* ticks collected from 10 locations along the East Coast. Only one new *ospA* SSCP mobility class, MC5, was found in this much-expanded biogeographic survey. The new mobility class was observed only once in one of the three infected adult *I. scapularis* ticks from Pea Island, North Carolina. DNA sequencing revealed that the mobility shift was caused by a single third-base synonymous substitution at the nucleotide position 465 as compared with the B31 type. The creation of MC5 is most parsimoniously interpreted as a recombination between MC1a and MC4, not as a new mutation.

Not only did all but one Borrelia in infected ticks belong to one of the four previously identified mobility classes, but the frequencies of the mobility classes were uniform from Massachusetts to Maryland for 1997 (MA, RI, NY1, NJ, MD1, and MD2; $P = 0.1837$, Table 3). This geographic uniformity was also found for the 1996 samples from Long Island, New York (LI-96) and from Connecticut (CT; $P = 0.7170$, Table 3). The frequencies of mobility classes are temporally stable as well in the northeast region from 1995 to 1997 (LI-95, LI-96, CT, and NY1; $P = 0.207$), with the pattern of MC2 and MC3 relatively common and that of MC1 and MC4 relatively rare (Table 3). However, the frequencies of the mobility classes collected from Long Island, New York during the fall of 1994 were different from the pattern found in the later years (LI-94 *vs.* LI-95 and LI-96; $P < 0.001$). This difference between 1994 and the other years was confirmed by analysis of the frequencies of major *ospC* groups (Table 4).

Multiple infections of ospC clones: A much higher level of DNA sequence diversity exists at *ospC* than in *ospA*. Figure 3 shows the chemiluminescence image of an RLB assay for a highly infected population (NY1, from Millbrook, New York). The controls were amplified from cloned *ospC* alleles and should hybridize with their respective probes only. The off-diagonal signals in the figure were therefore caused by cross-hybridization among alleles. We were able to remove the cross-hybridization of A, G, and J probes by using shorter oligonucleotides as probes (Table 2 shows the revised probes). Since the group C allele is a recombinant of multiple alleles (B, I, and E; see Wang *et al.* 1999), we were not able to design a probe specific for the group C allele. Due to the cross-reactivity among B, C, and I probes, we were unable to estimate the frequencies of groups B, C, and I in some samples (Table 4).

Most of the infected adult ticks from the Northeast were infected with multiple Borrelia clones (Figure 3). The degree of multiplicity (the average number of distinct *ospC* clones per infected tick) is particularly high in localities with high Borrelia prevalence. For example, ticks from Millbrook, New York (NY1) had a multiplicity of 3.1 clones per infected tick. Some ticks from that sample (ticks 2 and 32) were infected with at least 8

TABLE 3

Frequency distributions of *ospA* SSCP mobility classes of *B. burgdorferi*

Sample	N[a]	Mobility classes of *ospA*[b] MC1	MC2	MC3	MC4	MC5
Long Island[c]						
LI-94	97	40	44	24	35	0
LI-95	301	38	126	96	95	0
LI-96	66	8	40	16	25	0
East Coast						
MA	16	1	8	5	2	0
RI	17	5	9	4	1	0
NY1	32	5	18	14	4	0
CT	25	5	13	8	9	0
NJ	11	2	3	5	2	0
MD1	16	4	7	5	4	0
MD2	9	5	2	6	0	0
NC1	3	1	0	0	1	1
Test of frequency homogeneity[d]						
Among 1997 populations (MA, RI, NY1, NJ, MD1, MD2): $P = 0.414$ (NS)						
LI-96, CT: $P = 0.717$ (NS)						
LI-95, LI-96, CT, NY1: $P = 0.207$ (NS)						
LI-94 *vs.* (LI-95 and LI-96): $P < 0.001$						

[a] Number of Borrelia-infected ticks.
[b] Number of SSCP bands observed in infected ticks in each sample. Because of multiple infections, the total number of SSCP alleles usually exceeds the number of infected ticks.
[c] Long Island data from Qiu *et al.* (1997).
[d] P values by Raymond and Rousset's (1995) exact test of population differentiation (NS, nonsignificant).

TABLE 4

Frequency distribution of ospC major groups of *B. burgdorferi*

Sample	N^a	Major groups of *ospC*[b] A	B	C	D	E	F	G	H	I	J	K	M	N	T	U	M^c
Long Island																	
SI-94	24	10	8	9	9	3	3	2	3	3	2	3	0	3	—	—	2.4
SI-96	32	3	8	—	12	4	6	6	9	2	6	14	9	12	0	9	3.1
MP	14	—	2	—	1	3	5	3	1	—	—	2	2	2	1	0	1.6
LI-nym	25	3	—	6	2	4	2	2	5	—	3	7	1	0	—	—	1.4
WW-96	21	—	6	—	4	6	3	5	2	—	—	7	3	3	13	5	2.7
East Coast																	
MA	11	5	7	—	0	3	1	4	2	—	0	6	5	2	0	0	3.2
RI	3	1	0	—	0	0	1	0	2	—	0	2	2	0	0	0	2.7
NY1	25	8	8	4	1	6	6	6	5	4	2	13	5	6	3	0	3.1
NY2	4	0	1	—	0	0	1	0	0	—	0	2	3	2	0	0	2.3
CT	14	—	3	—	0	2	1	6	2	—	—	5	3	4	2	0	2.0
NJ	4	2	1	—	0	1	2	0	1	—	0	2	2	1	0	0	3.0
MD1	13	4	6	—	5	2	6	3	3	—	0	9	7	2	0	1	3.7
MD2	9	5	1	—	0	0	0	2	4	—	1	5	2	2	0	1	2.6
NC1	3	0	1	0	0	0	0	2	0	—	0	0	0	0	0	0	1.0
SC	1	0	0	—	0	0	1	0	0	—	0	0	0	0	0	0	1.0
Total	203	40	51	—	34	34	38	40	39	—	14	76	44	40	19	16	

Average multiplicity

Adult ticks: 2.8 (clone/infected tick)
Nymph: 1.4 (clone/infected tick)
Adult ticks in NC1 and SC: 1.0 (clone/infected tick)

Frequency differentiation[d]

Among 1997 samples (MA, NY1, MD1, and MD2): $P = 0.570$ (NS)
Among 1996 samples (SI, WW, and CT): $P = 0.001$**
Between SI-94 and SI-96: $P = 0.00183$**

**$P < 0.01$.

[a] Number of Borrelia-infected ticks.

[b] Underlined entries are the four major groups (A, B, I, and K) that cause disseminated Lyme disease (Seinost *et al.* 1999). Dashes indicate data not available, due to either probes not included or probes not hybridizing to controls.

[c] Multiplicity. It is obtained by dividing the total number of signals by the total number of infected ticks.

[d] *P* values by Raymond and Rousset's (1995) exact test of population differentiation (NS, nonsignificant).

clones. On the other hand, all of the Borrelia-infected ticks from the southeast were infected with only a single clone (Table 4). Even in the Northeast, fewer nymphal ticks were infected with Borrelia and the multiplicity of infecting clones was lower in infected ticks. Infected nymphal ticks from Long Island were infected with about one-half the number of clones as the adults. To compare the results from an SSCP assay with RLB, the sample (SI-94) used in the original SSCP study (Wang *et al.* 1999) was reanalyzed using RLB. The RLB assay showed a higher sensitivity in detecting the presence of alleles in ticks than did SSCP. An average of 2.6 clones per infected tick was detected using RLB compared to 1.5 clones using SSCP. Three ticks were infected with group N clones, which had not been detected previously using SSCP. Also alleles with minor differences in this population were indistinguishable by RLB, but gave different mobility patterns using SSCP.

The five coastal (from Massachusetts to Maryland) samples (MA, NY1, SI-96, MD1, and MD2) were not significantly different in their frequency distribution of major *ospC* groups (samples with small numbers, such as RI, NY2, and NJ, were excluded in the test). Major group K, one of the four major *ospC* groups that cause disseminating Lyme disease in humans (Seinost *et al.* 1999), is one of the most common clones in all samples (Table 4).

Allele frequency distributions at both *ospA* and *ospC* in all the local Borrelia populations were significantly more than expected even from neutral distributions (results of Ewens-Watterson tests, not shown), suggesting balancing selection in the form of frequency-dependent selection. For examples of the actual distributions, see Qiu *et al.* (1997) and Wang *et al.* (1999).

Linkage between ospA and ospC: Because of the high degree of clonality of *B. burgdorferi* (Dykhuizen *et al.*

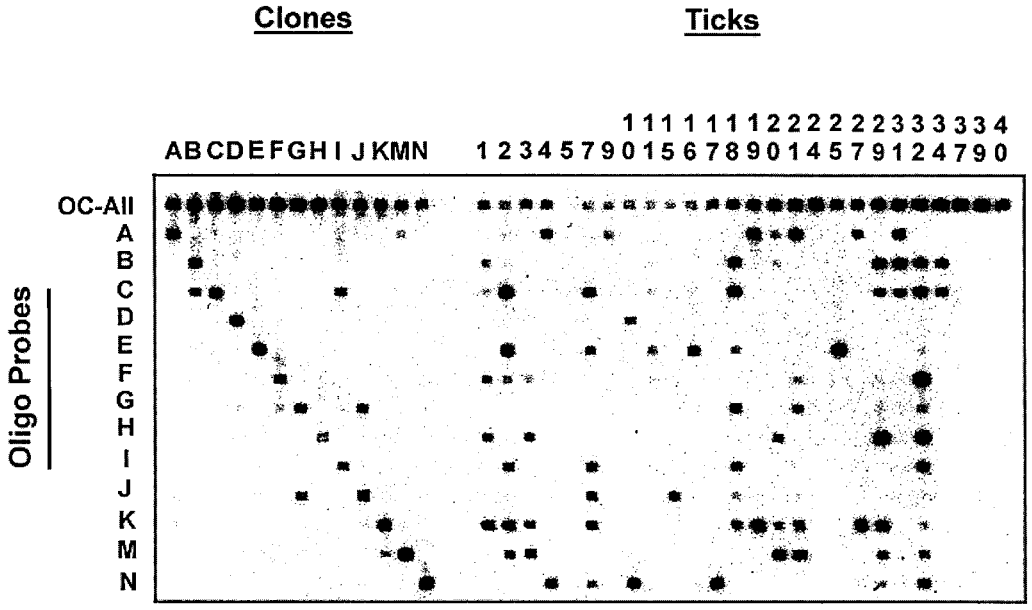

FIGURE 3.—Reverse line blotting assay of *ospC* variation. Oligonucleotide probes specific for *ospC* major groups (MG-A to MG-N, and OC-All, a probe for all *ospC* alleles) were UV fixed on the membrane in horizontal lines. Samples of fluorescein-labeled PCR amplicons were applied vertically to the probe lines. Samples include, on the right, PCR products amplified from cloned *ospC* genes (WANG *et al.* 1999), used as controls for probe specificity. PCR products amplified from individual Borrelia-infected ticks (NY1, from Millbrook, New York) were shown on the left of the membrane, one tick per vertical channel.

1993; BALMELLI and PIFFARETTI 1996), it is not surprising that results of analyses of Borrelia populations (such as tests of population differentiation and Ewens-Watterson tests of neutrality) are similar using either *ospA* or *ospC* as the genetic marker, even though the two genes are located on different plasmids. The pattern of linkage between the SSCP alleles of *ospA* and the major groups of *ospC* has been expanded from previous work (WANG *et al.* 1999) and is shown in Table 5. These associations of *ospA* and *ospC* alleles are inferred from the alleles that are present together in ticks from all samples. Almost all *ospC* major groups are in strict linkage with only one of the *ospA* SSCP alleles except groups G and K, which are associated with more than one *ospA* mobility class.

***I. scapularis* population structure:** *Divergence of northern and southern populations:* Cold SSCP analysis of the *I. scapularis* mitochondrial 16S rRNA gene revealed 10 common haplotypes (named A–J) in tick samples collected from the northeastern United States. Four rare haplotypes, each observed only once, were also identified. SSCP mobility patterns of the tick mitochondrial 16S haplotypes found in the northern populations are shown in Figure 4A. In the Southeast, three haplotypes, M, N, and F, dominated most of the ticks in two samples from Beaufort, South Carolina. The F allele was found in both the Southeast and the Northeast. Haplotypes found in tick samples from mid-Atlantic sites (MD2 from southern Maryland; NC1 and NC2 from North Carolina) carried mostly common alleles (*e.g.*, D and F) of the Northeast. Samples from the mid-Atlantic region, however, had a relatively large proportion of locally distinct haplotypes that were absent from either the northeastern or the South Carolina samples. SSCP mobility patterns of unique haplotypes from the North and South Carolina samples are shown in Figure 4B. A total of 31 distinct *I. scapularis* haplotypes were identified from this and two other studies (RICH *et al.* 1995; NORRIS *et al.* 1996).

Local populations of southern *I. scapularis* (*e.g.*, NC2 and SC) are genetically much more heterogeneous than northern populations (Table 6). Inland *I. scapularis* populations may also be much more diverse than coastal populations, as indicated by comparing two North Carolina samples (NC1 to NC2, Table 6). Variation of DNA sequences and allele frequencies of these tick samples do not deviate from neutral expectations, as analyzed by Ewens-Watterson tests (results not shown) and TAJIMA's (1989) tests (Table 6).

Minimum spanning tree (Figure 5) and mismatch distributions (Figure 6) clearly show differences in the genetic composition between the northern and southern *I. scapularis* populations. The minimum spanning tree revealed two major mitochondrial clades, in agreement with the findings of previous studies (RICH *et al.* 1995; NORRIS *et al.* 1996). Clade A is distributed mainly in the North, but also exists in the South (*e.g.*, SC). Clade B is found only in southern populations and not

TABLE 5

Linkage relationship between *ospA* and *ospC*

ospA mobility classes	*ospC* major groups	
	Linked with one *ospA* allele	Linked with more than one *ospA* allele
MC1	A, D	G
MC2	C, E, J, N, T	G, K
MC3	H, M	K
MC4	B, F, I	G, K
MC5		G

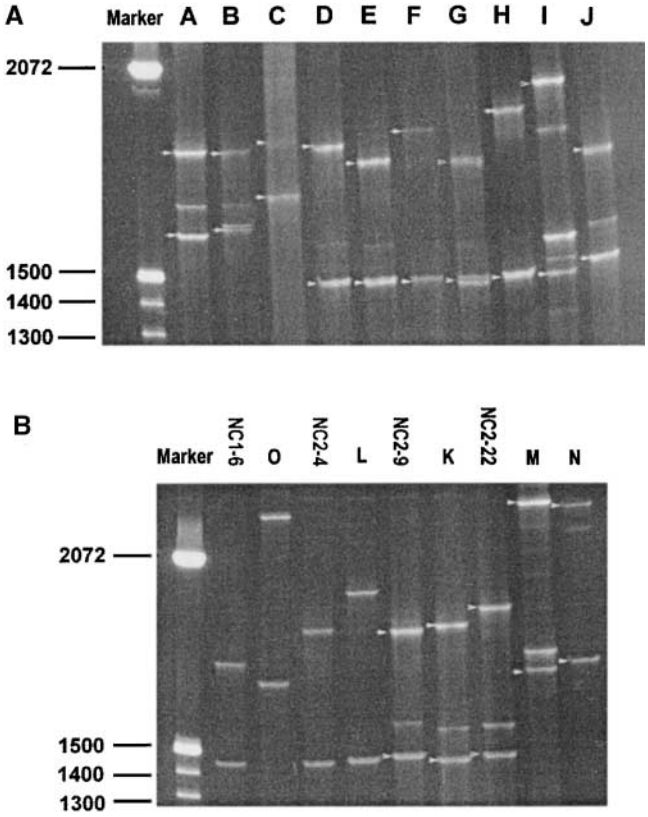

FIGURE 4.—SSCP analysis of tick mitochondrial (mt) 16S rRNA variation. A distinct haplotype is identified by a unique mobility pattern of a pair of single SSCP bands (indicated by arrows). Shown are common haplotypes found in the northern samples (A) and the southern samples (B) of *I. scapularis*.

in northern populations. Some haplotypes in the mid-Atlantic and South are genetically intermediate between the typical northern haplotype (F) and the common southern haplotypes (M and N). Northern tick samples typically show a unimodal mismatch distribution, while mid-Atlantic and southern samples show separated peaks (Figure 6). Because of possible population mixture in the South due to recent immigration from the North, the South Carolina sample was analyzed without clade A haplotypes (F and A). Including clade A sequences in the mismatch analysis of the SC sample would result in three peaks. For the mid-Atlantic sample (NC2), however, clade A haplotypes were too numerous and locally distinct to be attributed to immigrants from the North.

Temporal stability: Exact tests of population differentiation (RAYMOND and ROUSSET 1995) showed no significant temporal shift of genetic composition between pairs of samples collected in different years from the same site. One pair was from the North (Dutchess County, New York: NY3-90 (adults) *vs.* NY3-95 (nymphs), $P = 0.4299$) and the other was from the South (Beaufort, South Carolina: SC-97 *vs.* SC-98, $P = 0.5537$). Since there is no evidence of change of allele frequencies over these time spans, samples from the same sites were pooled.

AMOVA: The genetic composition of local tick populations shows enough local similarity that the populations can be subdivided into three localities (Table 7), as revealed by AMOVA (EXCOFFIER *et al.* 1992).

The three-group structure is caused by the genetic distinctness of *I. scapularis* in the mid-Atlantic region. The M and N haplotypes, which predominated samples from Beaufort, South Carolina, were absent from the tick samples from southern Maryland (MD2) and North Carolina (NC1 and NC2). Ticks from Washington County, North Carolina (NC2) contain mostly typical northern haplotypes (A, D, E, and F) but a large proportion of haplotypes in this sample (12 of 35 ticks or 34%) are distinct and found only in this region (6 O type ticks, 3 K type, 1 L type, and 2 singletons, NC2-22 and NC2-29; Table 6). Haplotype O belongs to clade B while K, L, and NC2-22 and -29 are all clade A (Figure 5), suggesting that these haplotypes had local origins and were not immigrants from either the northern or the southern populations. Moreover, haplotypes found in this region tend to be phylogenetically intermediate between clades A and B. The genetic distinctness of mid-Atlantic *I. scapularis* (especially inland populations) implies local origin of these populations and limited gene flow between distant tick populations.

Coastal vs. inland populations in North and South Carolina: The dissimilarity in the genetic composition of tick populations over a short distance is most striking between the two samples from North Carolina. The population NC1 was collected from a bird sanctuary on a coastal island (Pea Island National Wildlife Refuge, Cape Hatteras National Seashore, North Carolina) and NC2 from a nearby inland site ~80 km away (Washington County, North Carolina). Whereas all but one tick from the coastal site were northern types, the 35 ticks from the inland site were a mixture of northern and southern types. The coastal population was very much like the northeastern populations, implying a possible role of migratory birds in bringing the northern ticks south during the fall. Since no southern haplotypes were found in this coastal site, the long-distance gene flow mediated by migratory birds may be unidirectional (*i.e.*, from north to south only). The ongoing long distance transportation of ticks from north to south by birds was further supported by evidence from the South Carolina samples (SC 97 and 98), collected also from a coastal site (Spring Island, South Carolina). While the stable presence of the most common northern haplotype in these two samples (F, at ~20%) may be the result of mitochondrial introgression, the rarer northern haplotypes (A and G) that were found in the sample from November 1998 could be new immigrants from the North. More sampling from coastal and inland sites will be needed to test this hypothesis.

TABLE 6

Frequency distributions of mt 16S rDNA haplotypes of *I. scapularis*

Sample	No. of ticks	Haplotype															Mutation parameter π (SE) [a]	Tajima's D [b]
		A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	Singletons	
Long Island																		
LI1	39	10	3		7	2	17											1.014 (0.773)	0.140
LI2	53	4	3	5	6		30	1	1	3								1.108 (0.817)	−0.160
LI3	37	17	1			1	13	2		2	1							1.060 (0.798)	−1.322
LI4	33	12			5	2	10	1	2	2								1.285 (0.920)	−0.770
LI5	34	5			8	4	14	3										1.125 (0.835)	0.331
LI6	23	4			2		13	2									LI6-4, -9	0.696 (0.612)	−0.467
Northeast																			
MA	28	6			3		18										MA-1	0.700 (0.607)	−0.677
RI	27	6			5	1	15											0.802 (0.665)	0.043
NY3-90	33	8			1	1	20	2			1							0.922 (0.718)	−1.31
NY3-95	20	5		2			9	2			1						NY1-11		
NY1	30	13			4	4	9											1.171 (0.863)	1.226
CT	60	10		1	29		18		1	1								0.978 (0.746)	−0.611
NJ	27	6			6		15											0.726 (0.622)	0.812
PA	44	14	3		6		21											0.825 (0.668)	0.387
MD1	37	2			2		21	12										0.674 (0.586)	−0.179
Southeast																			
MD2	11				3		7						1					0.624 (0.596)	−0.290
NC1	24				8		14					1					NC1-6	0.722 (0.623)	−0.913
NC2	35	2			2		18	1				3	1			6	NC2-22, -29	5.470 (2.998)	0.364
SC-97	27						5							5	17			7.613 (4.000)	2.050*
SC-98	28	1					6	1						2	18				

*$P < 0.05$.

[a] Estimated using FLUCTUATE (Kuhner *et al.* 1998).

[b] Using Arlequin (Schneider *et al.* 1997).

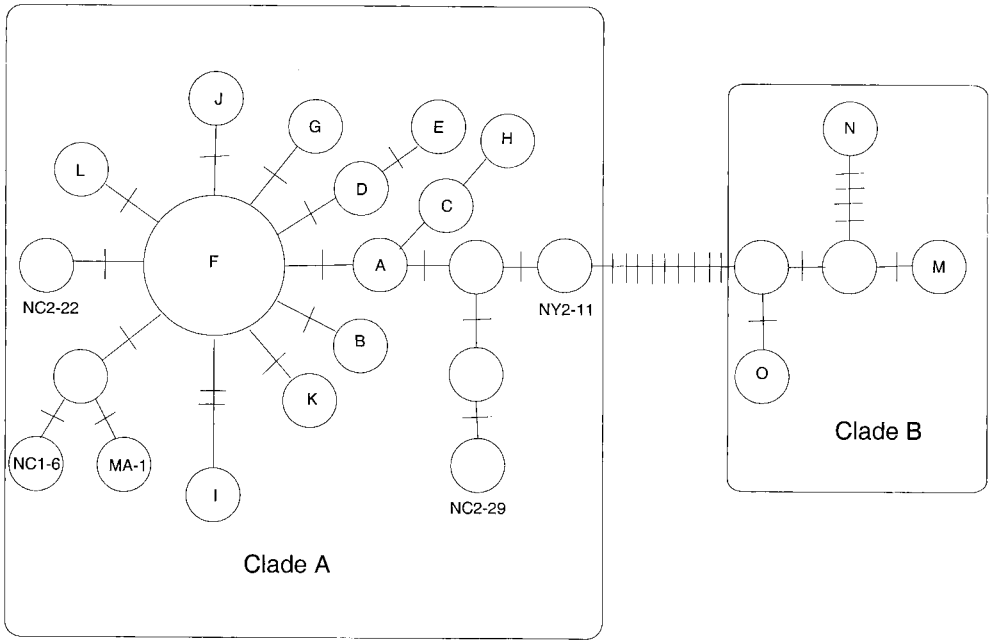

FIGURE 5.—Minimum spanning tree of mitochondrial 16S rDNA haplotypes of *I. scapularis*. Bars between two haplotypes indicate number of sequence differences.

High tick diversity in the New York-Connecticut region: Within the northern tick populations, the samples from New York and Connecticut showed higher levels of genetic diversity. The number of haplotypes in these populations ranged from six to eight (Table 6), while samples from Rhode Island, Massachusetts, New Jersey, Pennsylvania, and northern Maryland showed lower diversities with three to four haplotypes (Table 6). The higher levels of tick genetic diversity in the New York-Connecticut region imply that this region could be the source of the current geographic expansion of the northern ticks.

Comparisons between *I. scapularis* and *B. burgdorferi*: *Population structure:* Within the range of northern blacklegged ticks, local populations are differentiated. On the other hand, local populations of Borrelia are similar within this range. Noncorrespondence in the level of genetic differentiation among local geographic samples between the northern blacklegged ticks and Borrelia was shown by pairwise F_{ST} tests (Figure 7). As discussed below, we propose that balancing selection at *ospC* creates this nonconcordance. The balancing selection counters the effects of drift in *B. burgdorferi*, but, of course, not in the blacklegged tick. The other possibility for the geographic uniformity of Borrelia is that the uniformity is maintained by a high level of gene flow mediated by some long-ranging reservoir hosts, such as birds.

Demographic history: Maximum-likelihood tests of population growth (KUHNER *et al.* 1998) were performed

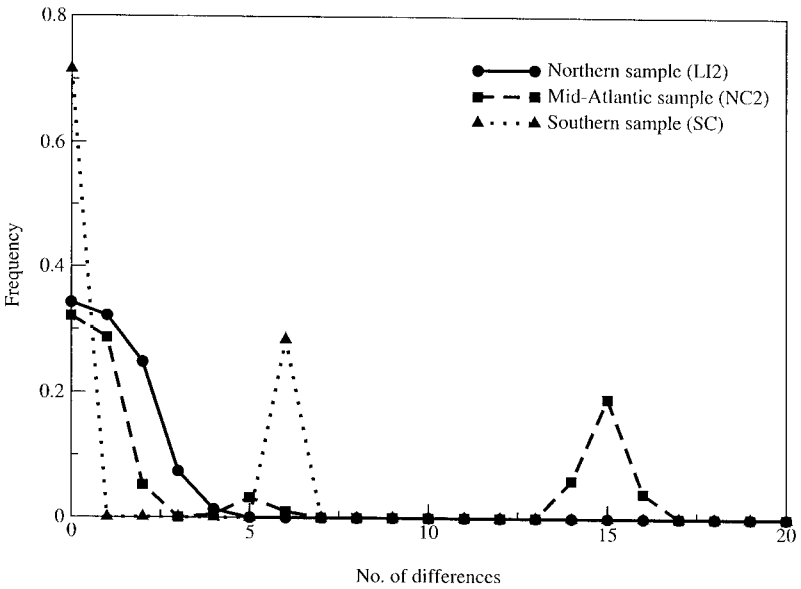

FIGURE 6.—Pairwise distributions of mt 16S rDNA haplotypes of *I. scapularis*. Northern, mid-Atlantic, and Southern tick populations are represented by LI2 (from Wildwood State Park, Long Island, New York), NC2 (from Washington County, North Carolina), and SC (from Beaufort County, South Carolina; clade A haplotypes are excluded), respectively.

TABLE 7

Results of AMOVA

	df	% variance	Fixation index	*P*
Two groups[a]				
Among groups	1	33.57	$\Phi_{CT} = 0.33569$	<0.005
Among populations within groups	10	26.96	$\Phi_{SC} = 0.40590$	<0.0001
Within populations	419	39.47	$\Phi_{ST} = 0.60533$	<0.0001
Three groups[b]				
Among groups	2	66.01	$\Phi_{CT} = 0.66009$	<0.005
Among populations within groups	9	1.59	$\Phi_{SC} = 0.04688$	<0.0001
Within populations	419	32.40	$\Phi_{ST} = 0.67603$	<0.0001

For methods and notations see Excoffier *et al.* (1992). Performed using Arlequin (Schneider *et al.* 1997).

[a] Two-group structure: Northeast (MA, RI, NY1, NY2, CT, NJ, PA, MD1) and Southeast (MD2, NC1, NC2, SC).

[b] Three-group structure: Northeast (MA, RI, NY1, NY2, CT, NJ, PA, MD1), mid-Atlantic (MD2, NC1, NC2) and South (SC).

on a tick population sample from Long Island (LI2, from Wildwood State Park in 1995), the Borrelia infecting this tick population (data from Qiu *et al.* 1997), a mid-Atlantic sample (NC2), and a southern sample (SC; Figure 8). The *B. burgdorferi* population in the Northeast and the northern *I. scapularis* share a remarkably similar demographic history, which is very different from the demographic history of the southern population. Clade A haploytpes were excluded from the South Carolina sample because these may belong to recent immigrant ticks from the North. Maximum-likelihood estimates of population growth, however, are not significantly different even if clade A haplotypes are included.

DISCUSSION

Contemporary patterns of biogeographic variation of a species are a result of both ongoing ecological and selective processes (such as gene flow and natural selection) and species histories (*e.g.*, range expansion and contraction; Templeton *et al.* 1995). It is therefore often necessary to separate these two sources of biogeographic variation (referred to as "ecological" and "historical" biogeography, respectively; see Morrone and Crisci 1995) when interpreting observed geographic patterns of a species. In this study, comparison of the population structure of Borrelia and *I. scapularis* suggests a common demographic history of the parasite and its tick vector along the northeast coast of the United States, resulting presumably from the same historical causes. This study also revealed significant differences in the genetic structures of the parasite and its tick vector, suggesting differential selective pressures on the Borrelia, causing differences in the population structure of the bacteria and tick host.

Geographic uniformity of Borrelia in the Northeast: This survey of local Borrelia populations along the East Coast showed that the clone frequencies of the Lyme disease bacteria in the northeastern and mid-Atlantic region of the country are uniform. Not only are the frequencies of *ospA* and *ospC* alleles the same in all localities (Tables 3 and 4), but the linkage relationships between the alleles of these two loci are also the same across localities.

Is the geographic uniformity of *B. burgdorferi* in the Northeast caused more by gene flow or by selection? We think that selection is more important than gene flow in maintaining the constant allele frequencies across Borrelia populations for the following reasons:

1. The gene-frequency uniformity across populations of Borrelia cannot be explained by gene flow via ticks since the tick populations show genetic differentiation (Figure 7). Movements of mammals and migratory birds infected with the spirochete can mediate gene flow among Borrelia (Sonenshine 1993). Long distance flow of Borrelia via migratory birds is used to explain the high genetic diversity of Borrelia in low-density southern populations. However, it is unlikely that the migration of infected small mammals or birds is at a high enough frequency to homogenize the spirochete populations.
2. Despite the geographic uniformity in the frequency distribution of *ospA* and *ospC* alleles, the frequency distribution is not temporally stable. Borrelia infecting tick populations from the same location (Shelter Island, New York) collected in 1994 are different from those collected in 1996 as measured at both the *ospA* and *ospC* loci (Tables 3 and 4). This sudden shift in allele frequencies of *ospA* between 1994 and 1996 happened both on Shelter Island and in Wildwood State Park on Long Island (Qiu *et al.* 1997), showing the change cannot be explained by migration, but must be explained by selection.
3. The gene frequency distributions of Borrelia in-

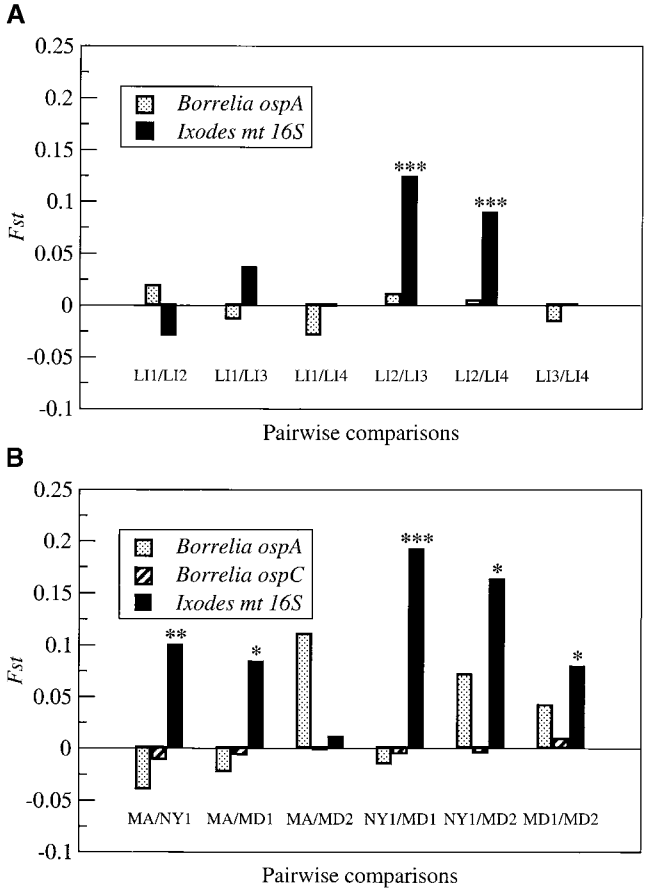

FIGURE 7.—Tests of population differentiation: *B. burgdorferi vs. I. scapularis.* Shown is the significant genetic structuring of the northern *I. scapularis* ticks within a small area (A, Long Island) and along the East Coast (B), in comparison to the gene-frequency uniformity of Borrelia populations from the same set of locations. The pairwise F_{ST} values were obtained using Arlequin (version 1.1; SCHNEIDER *et al.* 1997; $0.05 < * < 0.01$, $0.01 < ** < 0.001$, $*** < 0.001$; unannotated bars are not significant).

fecting nymphal ticks are significantly different from the distributions infecting the adults from the same cohort (QIU *et al.* 1997). Since larval and nymphal ticks feed on different hosts, this suggests selection is maintaining the gene frequency distributions.

4. The distribution is significantly different from that expected from neutrality, suggesting strong balancing selection. The balancing selection is evidenced by high local nucleotide sequence diversity at loci that are directly involved in establishing infection in mammalian hosts, such as *ospC* (GILMORE and PIESMAN 2000; SCHWAN and PIESMAN 2000). The effect of balancing selection is further displayed in the overdispersed allele frequency distribution at many nontargeted loci of selection (*e.g.*, silent variations of *ospA* SSCP alleles). Thus, we conclude that strong balancing selection caused by the host immune response is the major force maintaining the geographic uniformity of Borrelia across the Northeast.

Divergence among North American Borrelia: Compared with other Borrelia species found in North America (MATHIESEN *et al.* 1997; POSTIC *et al.* 1998; NORRIS *et al.* 1999), *B. burgdorferi* shows little genetic variation. The relative genetic homogeneity of *B. burgdorferi* is in agreement with an evolutionary history of recent population growth and rapid geographic range expansion of this species.

It is possible to learn more about the population biology of *B. burgdorferi* by cross-species comparisons. NORRIS *et al.* (1997, 1999) published data on 91 *B. bissettii* strains from Colorado. Since the same *ospA* primers were used in both studies, the variation within and between species can be compared. No northeastern *ospA* types were found in the Colorado study and vice versa. This divergence can be due to either adaptation to different hosts and vector or geographic isolation with the accumulation of neutral differences.

The hypothesis of adaptive divergence between *B. burgdorferi* and *B. bissettii* was tested using McDonald-Kreitman tests (MCDONALD and KREITMAN 1991; Table 8). Synonymous substitutions outnumber replacement changes in each species (3 to 1 in the northeastern sample and 23 to 5 in the Colorado sample). However, the majority of the fixed differences between the two species are replacement substitutions (15 fixed nonsynonymous substitutions *vs.* 4 fixed synonymous substitutions). Consequently, the neutrality index (N.I.; RAND and KANN 1996) for *ospA* is much smaller than one (N.I. = 0.064) and the McDonald-Kreitman test is highly significant (Table 8). The two housekeeping genes (*fla* and *hbb*) show no evidence of adaptive substitutions by the same test (Table 8). OspA is directly involved in tick-spirochete interaction (as opposed to OspC, which is more important in spirochete-host interactions) for establishing infection in Ixodes ticks (SCHWAN 1996; SCHWAN and PIESMAN 2000). These results suggest differential adaptation of *ospA* to genetically different ticks.

Separate histories of the northern and southern *I. scapularis* populations: Results of this study revealed substantial differences in genetic structure, evolutionary history, and epidemiological characters between the northern and southern blacklegged ticks.

First, it can be concluded that the species of *I. scapularis* is geographically structured across its range along the East Coast. Although the northern and southern blacklegged ticks are taxonomically now described as a single species (OLIVER *et al.* 1993; KEIRANS *et al.* 1996), two distinct mitochondrial lineages were identified from this and other studies (RICH *et al.* 1995; NORRIS *et al.* 1996). One clade is common in the South but not found in the North (the "Southern Clade," NORRIS *et al.* 1996), while the other is mainly found in the North (the "American Clade," NORRIS *et al.* 1996). Northern tick populations consist exclusively of clade A haplotypes, which are all only one or two mutational steps away from the most common haplotype (F), showing a star phylogeny

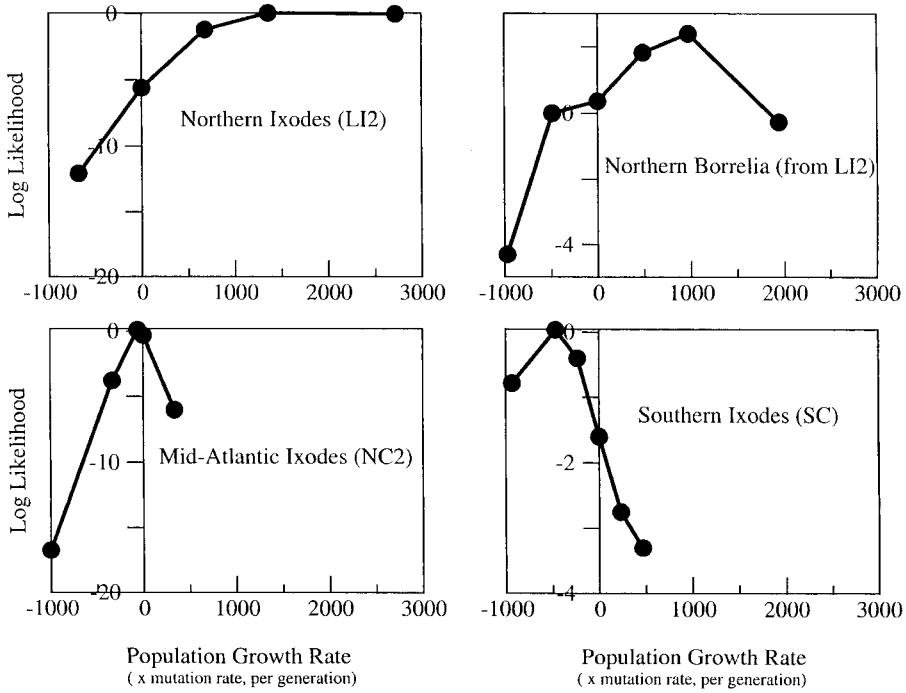

FIGURE 8.—Tests of population growth. Using the program FLUCTUATE (KUHNER *et al.* 1998), population growth rate (g) and population size (θ) were coestimated for a northern tick sample (LI2), a mid-Atlantic sample (NC2), a southern tick sample (SC, clade A haplotypes excluded), and Borrelia carried by the northern tick sample. The graphs are likelihood curves of population growth rate when the population size estimate is at its maximum-likelihood value. Estimates of population growth rate (g) for the northern tick sample and the Borrelia sample are both significantly greater than zero. Estimate of g for the mid-Atlantic and southern samples are not significantly different from zero. (The 95% confidence range of g is approximated by 2 log-likelihood units on each side of the maximum-likelihood estimates.)

(Figure 5). The average within-population sequence divergence is only ~0.2%. Ticks from the South consist of predominantly clade B haplotypes (mostly M and N) and the average within-population sequence diversity is much higher, ~1.5% (Figure 5). Studies of hybridization between the northern and southern blacklegged ticks using nuclear loci have so far failed to detect genetic discontinuity (WESSON *et al.* 1993; OLIVER *et al.* 1993; NORRIS *et al.* 1996). Morphological studies, however, showed North-South and East-West differences of blacklegged ticks (HUTCHESON *et al.* 1995; KEIRANS *et al.* 1996). Morphological and mitrochondrial data thus suggest *I. scapularis* ticks are geographically structured across its range of distribution due to lack of gene flow among populations. The strongest evidence for the lack of gene flow in this study is the extensive local differentiation of ticks collected from inland North Carolina (NC2, Table 6). Nevertheless, the presence of the most common clade A haplotypes in southern populations (F and A, Table 6; RICH *et al.* 1995; NORRIS *et al.* 1996) suggests secondary contact of the two previously separate lineages as a result of recent range expansion of northern ticks.

Second, the patterns of the genetic composition of populations suggested that the northern and southern *I. scapularis* populations have separate and different evolutionary demographic histories. The northern tick population samples showed evidence of exponential increase in effective population size, such as star phylogenies (Figure 5) and unimodal distributions of pairwise nucleotide differences (Figure 6) among haplotypes (SLATKIN and HUDSON 1991; ROGERS and HARPENDING 1992; HARPENDING *et al.* 1998). Corresponding genetic patterns of southern population samples were bifurcated (NC2) or trifurcated (SC) gene phylogenies (Figure 5), with bimodal mismatch distributions (Figure 6), in agreement with a long evolutionary history of con-

TABLE 8

McDonald and Kreitman tests

Locus		Fix.	Poly.	N.I.[a]	P
ospA[b]	Repl.	15	4		
	Synon.	6	25	0.064	3×10^{-5} (***)
hbb[c]	Repl.	2	4		
	Synon.	12	5	4.80	0.127 (NS)
fla[d]	Repl.	1	13		
	Synon.	17	42	5.14	0.066 (NS)

From MCDONALD and KREITMAN (1991). Tests were performed using the program DnaSP (ROZAS and ROZAS 1999). Fix., fixed changes; Poly., polymorphisms; Repl., amino acid replacement changes; Synon., synonymous changes. NS, not significant; ***$P < 0.001$.

[a] Neutrality index (RAND and KANN 1996).

[b] Comparison between six northeastern haplotypes (AF309002–AF309007) and seven Colorado haplotypes (U96254, U96256, U96258, U96259, U96261, U96263, and U96265; data from NORRIS *et al.* 1997).

[c] Comparison between two *B. burgdorferi* strains [B31 (U48648) and NY13-87 (U48649)] and four *B. bissettii* strains [25015 (U48685), CA128 (U48684), DN127 (U48683), and CA55 (U48665); data from VALSANGIACOMO *et al.* 1997].

[d] Comparison between two *B. burgdorferi* strains [B31 (X15661) and HB19 (X75200)] and two *B. bissettii* strains [CA127 (D82858) and DN127 (D82857); data from FUKUNAGA *et al.* 1996].

stant and stable population size. The difference in demographic history between the northern and southern populations is further supported by the maximum-likelihood estimates of population growth rates of these tick samples (Figure 8).

Third, the *B. burgdorferi* infection rate in *I. scapularis* ticks is markedly different between ticks collected from the Northeast and those from the Southeast (Figure 3). The localization of Lyme disease in the range of the northern lineage of *I. scapularis* suggests that genetic (as well as ecological) factors involving the ticks may play a part in the concentration of the disease in the North. The low Borrelia infection rate in ticks from the South is generally attributed to the lack of an efficient enzootic cycle of immature ticks and white-footed mice in the South compared to the one in the Northeast (Sonenshine *et al.* 1995; Oliver 1996; Ostfeld and Keesing 2000). The other possible cause of the low rate of Borrelia infection in southern *I. scapularis*, however, could involve the genetic heterogeneity of southern ticks. Piesman and Sinsky (1988) showed that *I. scapularis* from Alabama were highly efficient in both acquiring and transmitting Borrelia infection, using hamsters as experimental hosts. We observed in this study that ticks with southern (clade B) haplotypes can be naturally infected with *B. burgdorferi*. However, small differences in vector competence of different tick lineages could lead to large differences in Borrelia population size. On the basis of the geographic correspondence of the northern tick lineage and Lyme disease, and of the concurrence of the Lyme disease epidemic with range expansion of the northern *I. scapularis*, Telford (1998) argued that the species name *I. dammini* is epidemiologically justified.

Shared vicariant histories between *B. burgdorferi* and northern *I. scapularis*: Both *B. burgdorferi* and its vector, the northern lineage of *I. scapularis*, exhibit characteristics of a young evolutionary lineage, such as a low level of neutral genetic polymorphism within populations. Furthermore, statistical inference of population history on the basis of genetic compositions of local population samples suggested that the parasite and its vector share a similar demographic history of recent population growth and geographic expansion (Figure 8). The similar demographic histories between Borrelia and *I. scapularis* in the North may have the same evolutionary causes.

It has long been observed that species diversity of many animals and plants decreases with higher latitude (Laporte 1968). Besides ecological factors, the last glacial maximum, which was ~18,000 years ago, may have played an important part in creating such latitudinal gradients in species diversity. Fragmentation of a formerly continuous species distribution caused by geological events such as tectonic movements and glaciation is known as the "vicariance hypothesis" in biogeography (Nelson and Platnick 1981; Wiley 1988). A genetic consequence of population contraction during the glacial maxima and population expansion during the intervening warming periods is the loss of genetic variation of populations in the affected regions, relative to the genetic diversity of populations that were not affected (Hewitt 1996, 2000). Vicariance due to Pleistocene glaciation events seems to have caused parallel biogeographic patterns of reduced genetic variability in the North for a wide range of animal and plant species in North America (Avise 1992) and Europe (Sage and Wolff 1986; Magoulas *et al.* 1996; Marilä *et al.* 1996). These empirical studies often found short-spanning star phylogenies among haplotypes (differing from each other with one or two nucleotides) within northern populations, as observed for the northern *I. scapularis* ticks in this study. The New York-Connecticut region is a likely location of refugia populations of tick and Borrelia, suggested by its higher level of haplotype diversity in tick mitochondrial sequences. Northern blacklegged ticks in the Midwest may have originated from different refugia populations, as evidenced by the existence of locally distinct clade A haplotypes (*e.g.*, MN2-1 reported by Norris *et al.* 1996).

Conclusion: This study showed that the population structure of *B. burgdorferi*, a vector-transmitted parasitic bacterial species, is strongly shaped by the evolutionary history of its vector, the northern lineage of *I. scapularis*. We saw high infection rates in the Northeast where the genetic variability of the tick is low. There is also strong evidence of recent expansion of both the tick and Borrelia populations in the regions that were covered by glacier 18,000 years ago, as opposed to the tick populations in the South where the populations were not similarly destroyed. However, the population genetics of the northern tick and Borrelia are different, presumably because of the frequency-dependent selection on the Borrelia clones by the mammalian host. We postulate that the epidemic of *B. burgdorferi* in the Northeast (not only in humans but also in ticks and other mammals) is due to the biotic and genetic deprivation, induced by geological events like glaciations and modern human activities like deforestation and reforestation (Barbour and Fish 1993). As the ecosystem is reduced in species diversity, the remaining species will lose much of their genetic diversity due to population bottlenecks caused by human interference, followed by rapid expansion of the population. Disease organisms can evolve to specialize on the large number of very similar hosts, increasing virulence. Epidemics will then become both more frequent and more severe for the remaining species including humans (Ostfeld and Keesing 2000).

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LITERATURE CITED

Anderson, E. C., and P. A. Scheet, 2001 Improving the estimation of bacterial allele frequencies. Genetics **158:** 1383–1386.

Applied Biostatistics, 1996 *BIOMstat: Statistical Software for Biologists.* Applied Biostatistics, East Setauket, NY.

Avise, J. C., 1992 Molecular population structure and the biogeographic history of a regional fauna: a case history with lessons for conservation biology. Oikos **63:** 62–76.

Balmelli, T., and J.-C. Piffaretti, 1996 Analysis of the genetic polymorphism of *Borrelia burgdorferi* sensu lato by multilocus enzyme electrophoresis. Int. J. Syst. Bacteriol. **46:** 167–172.

Baranton, G., D. Postic, I. S. Girons, P. Boerlin, J.-C. Piffaretti *et al.*, 1992 Delineation of *Borrelia burgdorferi* sensu stricto, *Borrelia garinii* sp. nov., and Group VS461 associated with Lyme borreliosis. Int. J. Syst. Bacteriol. **42:** 378–383.

Baranton, G., N. M. Ras and D. Postic, 1998 Molecular epidemiology of the aetiological agents of Lyme borreliosis. Wien. Klin. Wochenschr. **110:** 850–855.

Barbour, A. G., and D. Fish, 1993 The biological and social phenomenon of Lyme disease. Science **260:** 1610–1616.

Black, IV, W. C., and J. Piesman, 1994 Phylogeny of hard- and soft-tick taxa (Acari: Ixodida) based on mitochondrial 16S rDNA sequences. Proc. Natl. Acad. Sci. USA **91:** 10034–10038.

Boehringer Mannheim, 1995 *The DIG System User's Guide for Filter Hybridization.* Boehringer Mannheim, Indianapolis.

Canica, M. M., F. Nato, L. D. Merle, J. C. Mazie, G. Baranton *et al.*, 1993 Monoclonal antibodies for identification of *Borrelia afzelii* sp. nov. associated with late cutaneous manifestations of Lyme borreliosis. Scand. J. Infect. Dis. **25:** 441–448.

Casjens, S., N. Palmer, R. van Vugt, W. M. Huang, B. Stevenson *et al.*, 2000 A bacterial genome in flux: the twelve linear and nine circular extrachromosomal DNAs in an infectious isolate of the Lyme disease spirochete *Borrelia burgdorferi.* Mol. Microbiol. **35:** 490–516.

Dennis, D. T., T. S. Nekomoto, J. C. Victor, W. S. Paul and J. Piesman, 1998 Reported distribution of *Icodes scapularis* and *Ixodes pacificus* (Acari: Ixodidae) in the United States. J. Med. Entomol. **35:** 629–638.

Dykhuizen, D. E., and G. Baranton, 2001 The implications of a low rate of horizontal transfer in *Borrelia.* Trends Microbiol. **9:** 344–350.

Dykhuizen, D. E., D. S. Polin, J. J. Dunn, B. Wilske, V. Preac-Mursic *et al.*, 1993 *Borrelia burgdorferi* is clonal: implications for taxonomy and vaccine development. Proc. Natl. Acad. Sci. USA **90:** 10163–10167.

Ewens, W. J., 1972 The sampling theory of selectively neutral alleles. Theor. Popul. Biol. **3:** 87–112.

Excoffier, L., and P. E. Smouse, 1994 Using allele frequencies and geographic subdivision to reconstruct gene trees within a species: molecular variance parsimony. Genetics **136:** 343–359.

Excoffier, L., P. E. Smouse and J. M. Quattro, 1992 Analysis of molecular variance inferred from metric distances among DNA haplotypes: application to human mitochondrial DNA data. Genetics **131:** 479–491.

Fraser, C. M., S. Casjens, W. M. Huang, G. G. Sutton, R. Clayton *et al.*, 1997 Genomic sequence of a Lyme disease spirochaete, *Borrelia burgdorferi.* Nature **390:** 580–586.

Gilmore, Jr., R. D., and J. Piesman, 2000 Inhibition of *Borrelia burgdorferi* migration from the midgut to the salivary glands following feeding by ticks on OspC-immunized mice. Infect. Immun. **68:** 411–414.

Guttman, D. S., P. W. Wang, I.-N. Wang, E. M. Bosler, B. J. Luft *et al.*, 1996 Multiple infection of *Ixodes scapularis* ticks by *Borrelia burgdorferi* as revealed by single-strand conformation polymorphism analysis. J. Clin. Microbiol. **34:** 652–656.

Harpending, H., M. A. Batzer, M. Gurven, L. B. Jorde, A. R. Rogers *et al.*, 1998 Genetic traces of ancient demography. Proc. Natl. Acad. Sci. USA **95:** 1961–1967.

Hewitt, G. M., 1996 Some genetic consequences of ice ages, and their role in divergence and speciation. Biol. J. Linn. Soc. **58:** 247–276.

Hewitt, G. M., 2000 The genetic legacy of the Quaternary ice ages. Nature **405:** 907–913.

Hongyo, T., G. S. Buzard, R. J. Calvert and C. M. Weghorst, 1993 'Cold SSCP': a simple, rapid and non-radioactive method for optimized single-stranded conformation polymorphism analyses. Nucleic Acids Res. **21:** 3637–3642.

Hutcheson, H. J., J. H. Oliver, Jr., M. A. Houck and R. E. Strauss, 1995 Multivariate morphometric discrimination of nymphal and adult forms of the blacklegged tick (Acari: Ixodidae), a principal vector of the agent of Lyme disease in eastern North America. J. Med. Entomol. **32:** 827–842.

Kaufhold, A., A. Podbielski, G. Baumgarten, M. Blokpoel, J. Top *et al.*, 1994 Rapid typing of group A streptococci by the use of DNA amplification and non-radioactive allele-specific oligonucleotide probes. FEMS Microbiol. Lett. **119:** 19–26.

Kawabata, H., T. Masuzawa and Y. Yanagihara, 1993 Genomic analysis of *Borrelia japonica* sp. nov. isolated from *Ixodes ovatus* in Japan. Microbiol. Immunol. **37:** 843–848.

Keirans, J. E., H. J. Hutcheson, L. A. Durden and J. S. H. Klompen, 1996 *Ixodes* (Ixodes) *scapularis* (Acari: Ixodidae): redescription of all active stages, distribution, hosts, geographical variation, and medical and veterinary importance. J. Med. Entomol. **33:** 297–318.

Kuhner, M. K., J. Yamato and J. Felsenstein, 1998 Maximum likelihood estimation of population growth rates based on the coalescent. Genetics **149:** 429–434.

Kurtenbach, K., M. Peacey, S. G. T. Rijpkema, A. N. Hoodless, P. A. Nuttall *et al.*, 1998 Differential transmission of the genospecies of *Borrelia burgdorferi* sensu lato by game birds and small rodents in England. Appl. Environ. Microbiol. **64:** 1169–1174.

Lane, R. S., J. Piesman and W. Burgdorfer, 1991 Lyme borreliosis: relation of its causative agent to its vectors and hosts in North America and Europe. Annu. Rev. Entomol. **36:** 587–609.

Laporte, L. F., 1968 *Ancient Environment.* Prentice-Hall, Englewood Cliffs, NJ.

Le Fleche, A., D. Postic, K. Girardet, O. Peter and G. Baranton, 1997 Characterization of *Borrelia lusitaniae* sp. nov. by 16S ribosomal DNA sequence analysis. Int. J. Syst. Bacteriol. **47:** 921–925.

Magoulas, A., N. Tsimenides and E. Zouros, 1996 Mitochondrial DNA phylogeny and the reconstruction of the population history of a species: the case of the European anchovy (*Engraulis encrasi0colus*). Mol. Biol. Evol. **13:** 178–190.

Marilä, J., M. Björklund and A. J. Baker, 1996 Genetic population structure and gradual northward decline of genetic variability in the greenfinch (*Carduelis chloris*). Evolution **50:** 2548–2557.

Mathiesen, D. A., J. H. Oliver, Jr., C. P. Kolbert, E. D. Tullson, B. J. Johnson *et al.*, 1997 Genetic heterogeneity of *Borrelia burgdorferi* in the United States. J. Infect. Dis. **175:** 98–107.

McDonald, J. H., and M. Kreitman, 1991 Adaptive protein evolution at the *Adh* locus in *Drosophila.* Nature **351:** 652–654.

Morrone, J. J., and J. V. Crisci, 1995 Historical biogeography: introduction to methods. Annu. Rev. Ecol. Syst. **26:** 373–401.

Nadelman, R., and G. P. Wormser, 1998 Lyme borreliosis. Lancet **352:** 557–565.

Neigel, J. E., and J. C. Avise, 1993 Application of a random walk model to geographic distributions of animal mitochondrial DNA variation. Genetics **135:** 1209–1220.

Nelson, G., and N. Platnick, 1981 *Systematics and Biogeography: Cladistics and Vicariance.* Columbia University Press, New York.

Norris, D. E., J. S. H. Klompen, J. E. Keirans and I. W. C. Black, 1996 Population genetics of *Ixodes scapularis* (Acari: Ixodidae) based on mitochondrial 16S and 12S genes. J. Med. Entomol. **33:** 78–89.

Norris, D. E., B. J. Johnson, J. Piesman, G. O. Maupin, J. L. Clark *et al.*, 1997 Culturing selects for specific genotypes of *Borrelia burgdorferi* in an enzootic cycle in Colorado. J. Clin. Microbiol. **35:** 2359–2364.

Norris, D. E., B. J. B. Johnson, J. Piesman, G. O. Maupin, J. L. Clark *et al.*, 1999 Population genetics and phylogenetic analysis of Colorado *Borrelia burgdorferi*. Am. J. Trop. Med. Hyg. **60:** 699–707.

Oliver, Jr., J. H., 1996 Lyme borreliosis in the southern United States: a review. J. Parasitol. **82:** 926–935.

Oliver, Jr., J. H., M. R. Owsley, H. J. Hutcheson, A. M. James, C. Chen *et al.*, 1993 Conspecificity of the ticks *Ixodes scapularis* and *I. dammini* (Acari: Ixodidae). J. Med. Entomol. **30:** 54–63.

Oliver, J. H., Jr., K. L. Clark, J. F. W. O. Chandler, L. Tao, A. M. James *et al.*, 2000 Isolation, cultivation, and characterization of Borrelia burgdorferi from rodents and ticks in the Charleston area of South Carolina. J. Clin. Microbiol. **38:** 120–124.

Orloski, K. A., E. B. Hayes, G. L. Campbell and D. T. Dennis, 2000 Surveillance for Lyme disease—United States, 1992–1998. Morb. Mortal. Wkly. Rep. **49:** 1–11.

Ostfeld, R. S., and F. Keesing, 2000 Biodiversity and disease risk: the case of Lyme disease. Conserv. Biol. **14:** 722–728.

Piesman, J., and R. J. Sinsky, 1988 Ability of *Ixodes scapularis, Dermacentor variabilis,* and *Amblyomma ammericanum* (Acari: Ixodidae) to acquire, maintain, and transmit Lyme disease spirochetes (*Borrelia burgdorferi*). J. Med. Entomol. **25:** 336–339.

Piesman, J., K. L. Clark, M. C. Dolan, C. M. Happ and T. R. Burkot, 1999 Geographic survey of vector ticks (*Ixodes scapularis* and *Ixodes pacificus*) for infection with the Lyme disease spirochete, *Borrelia burgdorferi*. J. Vector Ecol. **24:** 91–98.

Postic, D., N. M. Ras, R. S. Lane, M. Hendson and G. Baranton, 1998 Expanded diversity among Californian *Borrelia* isolates and description of *Borrelia bissettii* sp. nov. (Formerly *Borrelia* Group DN117). J. Clin. Microbiol. **36:** 3497–3504.

Price, P. W., 1980 *Evolutionary Biology of Parasites*. Princeton University Press, Princeton, NJ.

Qiu, W.-G., E. M. Bosler, J. R. Campbell, G. D. Ugine, I.-N. Wang *et al.*, 1997 A population genetic study of *Borrelia burgdorferi* sensu stricto from eastern Long Island, New York, suggested frequency-dependent selection, gene flow and host adaptation. Hereditas **127:** 203–216.

Rand, D. M., and L. M. Kann, 1996 Excess amino acid polymorphism in mitochondrial DNA: contrasts among genes from *Drosophila*, mice and humans. Mol. Biol. Evol. **13:** 735–748.

Rannala, B., W.-G. Qiu and D. E. Dykhuizen, 2000 Methods for estimating gene frequencies and detecting selection in bacterial populations. Genetics **155:** 499–508.

Ras, N. A., D. Postic, M. Foretz and G. Baranton, 1997 *Borrelia burgdorferi* sensu stricto, a bacterial species "Made in the U. S. A."? Int. J. Syst. Bacteriol. **47:** 1112–1117.

Raymond, M., and F. Rousset, 1995 An exact test for population differentiation. Evolution **49:** 1280–1283.

Rich, S. M., D. A. Caporale, I. S. R. Telford, T. D. Kocher, D. L. Hartl *et al.*, 1995 Distribution of the *Ixodes ricinus*-like ticks of eastern North America. Proc. Natl. Acad. Sci. USA **92:** 6284–6288.

Rijpkema, S. G. T., M. J. C. H. Molkenboer, L. M. Schouls, F. Jongejan and J. F. P. Schellekens, 1995 Simultaneous detection and genotyping of three genomic groups of *Borrelia burgdorferi* sensu lato in Dutch *Ixodes ricinus* ticks by characterization of the amplified intergenic spacer region between 5S and 23S rRNA genes. J. Clin. Microbiol. **33:** 3091–3095.

Rogers, A., and H. Harpending, 1992 Population growth makes waves in the distribution of pairwise genetic differences. Mol. Biol. Evol. **9:** 552–569.

Rozas, J., and R. Rozas, 1999 DnaSP version 3: an integrated program for molecular population genetics and molecular evolution analysis. Bioinformatics **15:** 174–175.

Sage, R. D., and J. O. Wolff, 1986 Pleistocene glaciations, fluctuating ranges, and low genetic variability in a large mammal (*Ovis dalli*). Evolution **40:** 1092–1095.

Saiki, R. K., P. S. Walsh, C. H. Levenson and H. A. Erlich, 1989 Genetic analysis of amplified DNA with immobilized sequence-specific oligonucleotide probes. Proc. Natl. Acad. Sci. USA **86:** 6230–6234.

Schauber, E. M., S. J. Gertz, W. T. Maple and R. S. Ostfeld, 1998 Coinfection of blacklegged ticks (Acari: Ixodidae) in Dutchess County, New York, with the agents of Lyme disease and human granulocytic ehrlichiosis. J. Med. Entomol. **35:** 901–903.

Schneider, S., J.-M. Kueffer, D. Roessli and L. Excoffier, 1997 *Arlequin*. University of Geneva, Geneva.

Schwan, T. G., 1996 Ticks and *Borrelia*: model systems for investigating pathogen-arthropod interactions. Infect. Agents Dis. **5:** 167–181.

Schwan, T. G., and J. Piesman, 2000 Temporal changes in Outer Surface Proteins A and C of the Lyme disease-associated spirochete, Borrelia burgdorferi, during the chain of infection in ticks and mice. J. Clin. Microbiol. **38:** 382–388.

Seinost, G., D. E. Dykhuizen, R. J. Dattwyler, W. T. Golde, J. J. Dunn *et al.*, 1999 Four clones of *Borrelia burgdorferi* sensu stricto cause invasive infection in humans. Infect. Immun. **67:** 3518–3524.

Slatkin, M., 1994 An exact test for neutrality based on Ewens sampling distribution. Genetics **64:** 71–74.

Slatkin, M., and R. R. Hudson, 1991 Pairwise comparisons of mitochondrial DNA sequences in stable and exponentially growing populations. Genetics **129:** 555–562.

Socransky, S. S., C. Smith, L. Martin, B. J. Paster, F. E. Dewhirst *et al.*, 1994 "Checkerboard" DNA-DNA hybridization. BioTechniques **17:** 788–792.

Sonenshine, D. E., 1993 *Biology of Ticks*. Oxford University Press, New York.

Sonenshine, D. E., R. E. Ratzlafe, J. Troyer, S. Demmerle, E. R. Demmerle *et al.*, 1995 *Borrelia burgdorferi* in eastern Virginia: comparison between a coastal and inland locality. Am. J. Trop. Med. Hyg. **53:** 123–133.

Spielman, A., M. L. Wilson, J. F. Levine and J. Piesman, 1985 Ecology of *Ixodes Dammini*-borne human babesiosis and Lyme disease. Annu. Rev. Entomol. **30:** 439–460.

Tajima, F., 1989 Statistical method for testing the neutral mutation hypothesis by DNA polymorphism. Genetics **123:** 585–595.

Telford, III, S. R., 1998 The name *Ixodes dammini* epidemiologically justified. Emerg. Infect. Dis. **4:** 132–134.

Telford, III, S. R., P. M. Armstrong, P. Katavolos, I. Forppa, A. S. O. Garcia *et al.*, 1997 A new tick-borne encephalitis-like virus infecting New England deer ticks, *Ixodes dammini*. Emerg. Infect. Dis. **3:** 165–170.

Templeton, A. R., E. Routman and C. A. Phillips, 1995 Separating population structure from population history: a cladistic analysis of the geographical distribution of mitochondrial DNA haplotypes in the tiger salamander, *Ambystoma tigrinum*. Genetics **140:** 767–782.

Thompson, J. D., D. G. Higgins and T. J. Gibson, 1994 CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res. **22:** 4673–4680.

Thompson, J. N., 1999 The evolution of species interactions. Science **284:** 2116–2118.

Wang, G., A. P. van Dam, A. L. Fleche, D. Postic, O. Peter *et al.*, 1997 Genetic and phenotypic analysis of *Borrelia valaisiana* sp. nov. (*Borrelia* Genomic Groups VS116 and M19). Int. J. Syst. Bacteriol. **47:** 926–932.

Wang, I.-N., D. E. Dykhuizen, W.-G. Qiu, J. J. Dunn, E. M. Bosler *et al.*, 1999 Genetic diversity of *ospC* in a local population of *Borrelia burgdorferi* sensu stricto. Genetics **151:** 15–30.

Watterson, G. A., 1978 The homozygosity test of neutrality. Genetics **88:** 405–417.

Wesson, D. M., D. K. McLain, J. H. Oliver, Jr., J. Piesman and F. H. Collins, 1993 Investigation of the validity of species status of *Ixodes dammini* (Acari: Ixodidae) using rDNA. Proc. Natl. Acad. Sci. USA **90:** 10221–10225.

Wiley, E. O., 1988 Vicariance biogeography. Annu. Rev. Ecol. Syst. **19:** 513–542.

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