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# Population structure of the blacklegged tick Ixodes scapularis revealed by SSCP data using the mitochondrial Cyt b and the nuclear ITS1 markers *

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**Abstract** The population genetic structure of the blacklegged tick *Ixodes scapularis* was analyzed using 853 individual ticks collected from 12 sites along the eastern seaboard of the United States. PCR-based DNA single strand conformation polymorphism (SSCP) was used for analysis of one haploid locus, the mitochondrial cytochrome *b* (Cyt *b*), and one diploid locus, the internal transcribed spacer 1 (ITS1) of nuclear ribosomal RNA. Seven haplotypes in the Cyt *b* locus and 13 genotypes in the ITS1 locus were identified. Population differentiation and isolation by distance were found. Distribution of haplotype and genotype frequencies across geographic regions suggests that two distinct populations exist along the Eastern Coast of the US. However, genetic variation among individuals within regions was large. This degree of variation suggests frequent gene flow between regions. Moreover, overall genetic diversity among individuals was much higher in ticks from the southern population [*Acta Zoologica Sinica* 50 (2): 176 - 186, 2004].

**Key words** Blacklegged tick, Population genetics, Genetic variation, Gene flow, SSCP

# 利用 ITS1 和 Cyt b 位点的 DNA 单链空间构型多样性研究黑脚硬蜱的种群结构 *

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**摘 要** 黑脚硬蜱 (*Ixodes scapularis*) 是莱姆病的主要传毒媒介。本文利用从 12 个不同地点采集的 853 个样本, 采用 DNA 单连构型多样性的分子技术 (DNA single strand conformation polymorphisms) 对黑脚硬蜱的种群结构进行了分析。线粒体细胞色素 *b* (Cyt *b*) 和核糖体 rRNA 基因的内部转录空间 ITS1 被用为种群目标分子标记位点。在 Cyt *b* 位点上, 总共发现 7 个单倍基因型。在 ITS1 位点上, 共发现 13 个基因型。基因型频率分析结果显示, 沿美国东海岸分布的黑脚硬蜱隶属于两个不同的南北种群, 但是基因流在地理区域间频繁发生。尽管蜱自身的迁徙扩散能力有限, 但地理区域内个体间的遗传变异程度仍然较大, 这可能与黑脚硬蜱寄主动物的频繁迁移有关。另外, 本研究资料显示, 南方种群的遗传变异程度明显大于北方种群 [动物学报 50 (2): 176 - 186, 2004]。

**关键词** 黑脚硬蜱 种群遗传 遗传变异 基因流 SSCP

Blacklegged ticks *Ixodes scapularis* are obligate, nonpermanent ectoparasites that feed on mammals, birds, and lizards. They are well known for their ability to transmit the agents that cause Lyme dis-

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ease , babesiosis , and human granulocytic ehrlichiosis (Sonenshine , 1991 , 1993; Bakken et al. , 1994; Walker and Dumler , 1996; Durden and Oliver , 1999). Blacklegged ticks are expected to have a different pattern of genetic structure than many other arthropods because they have limited dispersal abilities and depend on their hosts for transport (Carroll and Schmidtmann , 1996). It might be expected that genetic variation among individuals would be small within geographic regions and larger between regions due to frequent inbreeding. In the present study , we investigate patterns of genetic variation among individuals within and between localities along the southeastern seaboard of the US using DNA single strand conformation polymorphisms (SSCP). We also attempt to understand how these patterns evolved over time.

*Ixodes scapularis* was described by Say in 1821 from Savannah , Georgia and was subsequently reported from many localities in the eastern half of North America , ranging from Florida to southeastern Canada , and parts of the midwestern United States and Mexico (Watson and Anderson , 1976; Keirans et al. , 1996; Wilson , 1998). Morphological and host preference variations in *I. scapularis* has been noted and formed the basis for Spielman et al. (1979) to describe a separate species *I. dammini* for the northern populations of *I. scapularis*. However , Oliver et al. (1993) argued that *I. dammini* was not a separate species and based their conclusions on morphometrics , genetic crosses , assortative mating , host preference analysis , chromosome comparisons , and isozyme data. Wesson et al. (1993) studied sequence variation of the two internal transcribed spacers (ITS1 and ITS2) of the ribosomal RNA gene among *I. scapularis* and *I. dammini* , plus a few closely related species and concluded that limited variation between northern and southern populations of *I. scapularis* did not suggest two distinct species. Similar conclusions were reached by McLain et al. (1995) , based on rDNA ITS2 spacer sequence variation. However , Caporale et al. (1995) and Rich et al. (1995) found that 16S rDNA sequence variation supported northern and southern clades within *I. scapularis*. Norris et al. (1996) examined variations in the 16S and 12S mitochondrial rDNA , using SSCP , RAPD , and sequence analysis , and found two clades : an American clade occurring in both the north and south and a Southern clade , restricted to the south (see also McLain et al. , 2001).

Previous studies used comparative or phylogenetic methods with few individuals from limited collection sites , and did not utilize population genetic approaches with gene frequencies. Extensive studies are needed to examine genetic variation and gene flow among large numbers of individuals within and across geographic regions to reveal the genetic structure of natural populations in this species , particularly in the southeastern US. Therefore , we investigated two loci , the haploid mitochondrial cytochrome *b* (Cyt *b*) and the diploid nuclear ribosomal internal transcribed spacer 1 (ITS1) with SSCP.

# 1 Materials and methods

## 1.1 Tick samples used

Ticks were collected from Bridgeport , Connecticut and the barrier islands of Florida (Big Talbot , Little Talbot , King George , and Anastasia) , Georgia (Sapelo and Jekyll Island) , South Carolina (Hunting , Edisto , Pinckney , and Pritchard's Islands) , and Maryland (Assateague Island). Collections were made on barrier islands and state parks along the coast because these areas are less disturbed and probably best reflect the natural population structuring of *I. scapularis*. The sample collection sites were plotted on Fig. 4.

Based on genetic variation reported in the literature , specimens from Connecticut and Maryland represent the northern clade while those from Florida , Georgia , and South Carolina represent the southern clade (Norris et al. , 1996). All specimens were collected from vegetation using a 1 m × 1 m drag cloth and were kept alive until returned to the laboratory , where they were placed at - 80 ℃ for storage or in 100 % ethanol (Assateague Island).

## 1.2 Nucleic acid isolation

DNA was isolated from ticks using a method modified from Doyle and Doyle (1990) and described by Whitlock et al. (2000). Individual ticks were ground with 250 μl of lysis buffer (4.5 mol/L guanidine thiocyanate , 25 mmol/L sodium citrate , 0.5 % lauryl sarcosine) and 250 μl of cetyltrime-thylammonium-bromide (CTAB) buffer (100 mmol/L Tris HCl , pH 8.0; 1.4 mol/L NaCl , 0.02 mol/L EDTA , 2 % (w/v) CTAB , and 0.3 % (v/v) 2-mercaptoethanol). The pellet was dried and resuspended in 50 μl of 10 mmol/L Tris-HCl (pH 8.0).

## 1.3 Polymerase chain reaction (PCR) amplification

DNA was amplified in a 25 μl volume which contained 1 × *Taq* DNA polymerase buffer (10 mmol/L Tris-HCl , pH 8.5 , 50 mmol/L KCl) , 2.5 mmol/L $MgCl_2$ , 200 μ mol/L of each of the four dNTPs , 2.5 U of *Taq* DNA polymerase , 0.2 μmol/L of each primer , and approximately 0.1 μg - 0.5 μg of DNA template. PCR was performed on a Perkin-Elmer 9600 Thermal Cycler with GeneAmp reagents (Perkin-Elmer , Foster City , California). The amplification conditions were 40 cycles with denaturation at 94 ℃ for 35 seconds , annealing at 55 ℃ for 45 seconds , and extension at 72 ℃ for 90 seconds. A hot-start method was used for all PCR amplifications.

To prevent cross-contamination, the PCR reaction mixture was combined and separated into 0.2 ml tubes under a sterile hood used only for PCR. All pipettes, tips, tubes, racks, water, centrifuge, etc. were used only for PCR and treated with UV light regularly. A negative control (sterile water in place of template) was always run with each PCR reaction. PCR products were visualized on 1% agarose gels with 1 kb standard DNA marker (Life Technologies, Rockville, MD).

Both Cyt *b* and ITS1 primers were developed from the available sequences of ticks and insects in GenBank and aligned using the software package of Genetic Data Environment (GDE) (Smith et al., 1994). Primer pair Cyt *b* 397F (5′-GCT TAT TTG GAA TTG CAC GAA A) and Cyt *b* 674RC (5′-ATG AAT AAA AAG CAC GTT) targets a 276 bp region of the cytochrome *b* gene in the mitochondrial genome. Primers ITS-6F (5′-TCT CCT TCA TTT TCA GCA TT) and ITS336RC (5′- TTC ATT TCA GCA TTG TTC C) target a 340 bp region of internal transcribed spacer 1 between the 5.8S and 18S ribosomal subunits.

**1.4 Single strand conformation polymorphism (SSCP)**

SSCP analysis was carried out on a 7% non-denaturing polyacrylamide (39∶1 acrylamide: bis-acrylamide) gel in 0.5 ×TBE buffer using Bio-Rad Protean Ⅱxi sub cell (Bio-Rad, Hercules, CA). Five microliters of PCR product was diluted three-fold with water and mixed with 5 μl of denaturing loading buffer (90% formamide, 2% (w/v) sodium hydroxide, and two dyes of 0.25% bromophenol blue and 0.25% xylene cyanol FF). To promote the formation of single strand conformation, 15 pmol forward and reverse primers were also added to the mix (Almeida et al., 1998). Before samples were loaded onto the gel, 25 μl of the mixture was denatured at 98 ℃ for five minutes on a PE 9 600 thermocyler and then quickly plunged into an ice bath where it remained for at least 10 minutes. All gels were run at 200 volts at 22 ℃ for 16 - 21 hours. Unique folding patterns were included on each gel as well as a 1kb standard marker for reference. Gels were stained for 15 minutes with 0.00025% (w/v) ethidium bromide TBE stain buffer. Bands were visualized using an UV transilluminator and photographed for analysis. To compare the banding pattern across gels, unique folding patterns found in the previous runs were always reloaded onto the next gel as controls. Each unique banding pattern was designated as a haplotype for the Cyt *b* locus and a genotype for the ITS1 locus.

The genotypes of ITS1 and the haplotypes of Cyt *b* were sequenced both as PCR products and as individual bands to verify the SSCP conformations. PCR products were purified for sequencing using QiaQuick PCR purification Kit (Qiagen, Valencia, CA) according to the manufacturer's protocols and resuspended in 35 μl of 2 mmol/L Tris-HCl. Individual genotypes were also run on a polyacrylamide gel then excised and purified using Wizard PCR preps purification kit (Promega, Madison, WI). Purified DNA was sequenced with an ABI 3100 Automated Sequencer at the Center of Agricultural Biotechnology (now Center for Biosystem Research), University of Maryland Biotechnology Institute. Sequences from PCR products were compared to that from individual bands excised from gel using software GAP4 in Staden Package (Staden et al., 2000) and, where there was double base calling, each nucleotide was assigned to different strands. Each strand was designated a pseudo-haplotype of one genotype following the descriptions given in Arlequin, a software for population genetics (Schneider et al., 2000). Degeneracy was also introduced for analysis in PAUP 4.0 (Swofford, 2000). A neighbor joining gene tree using distance options was derived to determine the relationships among genotypes.

**1.5 Statistical analysis**

We calculated frequencies of haplotypes for Cyt *b* and genotypes for ITS1. Population isolation was tested using G-tests of homogeneity of haplotype frequency distributions among all sites, among sites in each state, and between states using JMP (version 3.1 1995, SAS Institute Inc., Cary, NC). In cases where the expected frequency was less than 5, genotype/haplotype frequencies were combined into a category of rare genotypes/haplotypes. Cyt *b* haplotype and ITS1 genotype diversity indices (Nei, 1987) were calculated for each collection site using Arlequin. Nucleotide diversity indices (Nei, 1987) for ITS1 were also calculated using Arlequin.

**1.6 Population genetic structure**

Population structure was examined using Wright's $F_{ST}$ by analysis of molecular variance (AMOVA) (Excoffier et al., 1992; Michalakis and Excoffier, 1996) based on Cyt *b* and ITS1 using Arlequin software (Schneider et al., 2000). Significance of the fixation indices was determined using a non-parametric permutation approach described in Excoffier et al. (1992). The components of genetic variation associated with nesting sites were estimated by changing a group definitions a priori. For each grouping, populations were organized into regional groups and Weir and Cockerham's F-statistics (1984) were calculated. Pairwise $F_{ST}$ values were also calculated for each pair of sites, and significance was determined with a Markov chain analysis using 1 023 permutations (Schneider et al., 2000). Sequential Bonferroni adjustments were applied (Rice, 1989).

Isolation by distance was tested using a regression of $F_{ST}$ versus geographic distance (Reynolds et al., 1983; Slatkin, 1995) and significance was determined with Mantel's tests (1 000 permutations) using Arlequin (Schneider et al., 2000). Geographic distances were calculated using Distance Finder (http://www.indo.com/distance).

Nei's genetic similarity and distance (Nei, 1978), and coancestry similarity and distance (Reynolds et al., 1983) were calculated between each collection site using Genetic Data Analysis (GDA) (Lewis and Zaykin, 1999). Molecular Evolutionary Genetics Analysis (MEGA) (Kumar et al., 1993) was used to construct a neighbor-joining tree from the genetic distance matrix.

## 2 Results

### 2.1 Cyt b haplotype frequencies

A total of 674 samples were successfully analyzed with SSCP for Cyt *b*, where seven haplotypes (A to G) were identified (Figs. 1, 2; Appendix 1).

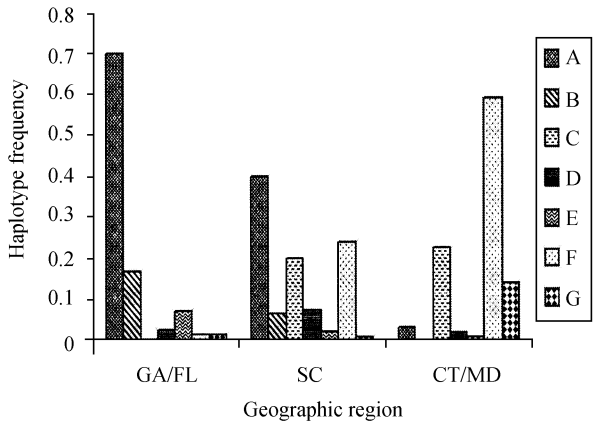


**Fig. 1 Distribution of Cyt b haplotypes by geographic region**

The most dominant, type A, was present in 46 % of the individuals analyzed and was the only one present at all collection sites. The second most frequent haplotype was F (20 %). Haplotypes D to G were scattered across all the collecting sites. Frequency of Cyt *b* haplotypes varied among all collection sites (Table 1). Cyt *b* haplotype diversities ranged from 0.20 in Florida to 0.76 at sites in South Carolina (Table 2). Clinal variation occurred from south to north in the pattern of frequency distributions of haplotypes (Fig. 1).

### 2.2 ITS1 genotype frequencies

In total, 592 samples were analyzed for the ITS1 region with 13 genotypes identified (types 1 to 13, Fig. 3; Appendix 2). The most dominant genotype, type 1, was present in 28.5 % of all individuals. The rarest genotype, type 13, was found in 0.8 % of individuals, all from a single island in Florida. Type 3 was the only one found at all collection sites. ITS1 frequencies varied significantly among all sites (Table 1). Genotype diversity indices for ITS1 ranged from 0.57 in Maryland to 0.89 in Florida (Table 2). Nucleotide diversities ranged from 0.051 at King George Island, Florida to 0.107 at Hunting Island, South Carolina (Table 2). Again, the genotype frequency distribution was clinal from north to south. The southern states possess more variations than that of north states.

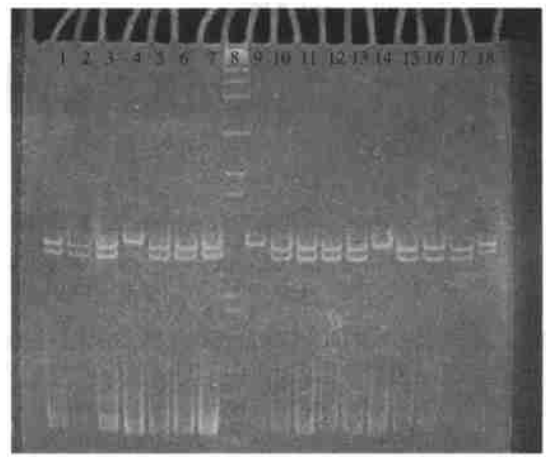


**Fig. 2 Polyacrylamide gel showing SSCP analysis of Cyt b haplotypes**

Lanes 1 - 7 represent individuals from Pinckney Island, lane 8 is standard 1 kb DNA ladder, lane 9 represents an individual from Pritchard's Island, and lanes 10 - 19 represent individuals from Anastasia Island. Lanes 1 - 3, 5 - 7, 10 - 13, and 15 - 17 are haplotype A, lanes 4, 9, and 14 are haplotype F, and lane 18 is haplotype C.

**Table 1 Results from a G test showing significance of haplotypes/genotypes among all collection sites, among states, and among populations within islands**

| Test sites | Cyt *b* | | ITS1 | |
|---|---|---|---|---|
| | *G* value | *P* value | *G* value | *P* value |
| Between all sites | 338.06 | < 0.001 | 259.22 | < 0.001 |
| Between states | 189.05 | < 0.001 | 85.87 | < 0.001 |
| Among Islands in Florida | 35.86 | 0.002 | 113.95 | < 0.001 |
| Among Islands in Georgia | 4.08 | 0.540 | 14.77 | 0.002 |
| Among Islands in S Carolina | 32.2 | 0.006 | 31.91 | 0.060 |

**Table 2 Haplotype, genotype and nucleotide diversity indices for all collection sites**

| Site | Cyt *b* Haplotype diversity | ITS1 Genotype diversity | ITS1 Nucleotide diversity |
|---|---|---|---|
| Anastasia | 0.27 +/- 0.08 | 0.86 +/- 0.02 | 0.081 +/- 0.040 |
| King George | 0.20 +/- 0.08 | 0.76 +/- 0.04 | 0.051 +/- 0.026 |
| Big Talbot | 0.53 +/- 0.08 | 0.79 +/- 0.04 | 0.075 +/- 0.037 |
| Little Talbot | 0.54 +/- 0.06 | 0.89 +/- 0.01 | 0.050 +/- 0.025 |
| Jekyll | 0.62 +/- 0.08 | 0.77 +/- 0.07 | 0.101 +/- 0.050 |
| Sapelo | 0.63 +/- 0.05 | 0.50 +/- 0.11 | 0.072 +/- 0.036 |
| Pinckney | 0.58 +/- 0.08 | 0.79 +/- 0.04 | 0.077 +/- 0.038 |
| Pritchard's | 0.72 +/- 0.03 | 0.79 +/- 0.04 | 0.066 +/- 0.033 |
| Hunting | 0.76 +/- 0.02 | 0.86 +/- 0.02 | 0.107 +/- 0.053 |
| Edisto | 0.60 +/- 0.05 | 0.82 +/- 0.03 | 0.099 +/- 0.049 |
| Maryland | 0.62 +/- 0.05 | 0.57 +/- 0.08 | 0.057 +/- 0.029 |
| Assateague | | | |
| Connecticut | 0.58 +/- 0.07 | 0.66 +/- 0.06 | 0.065 +/- 0.032 |
| Bridgeport | | | |

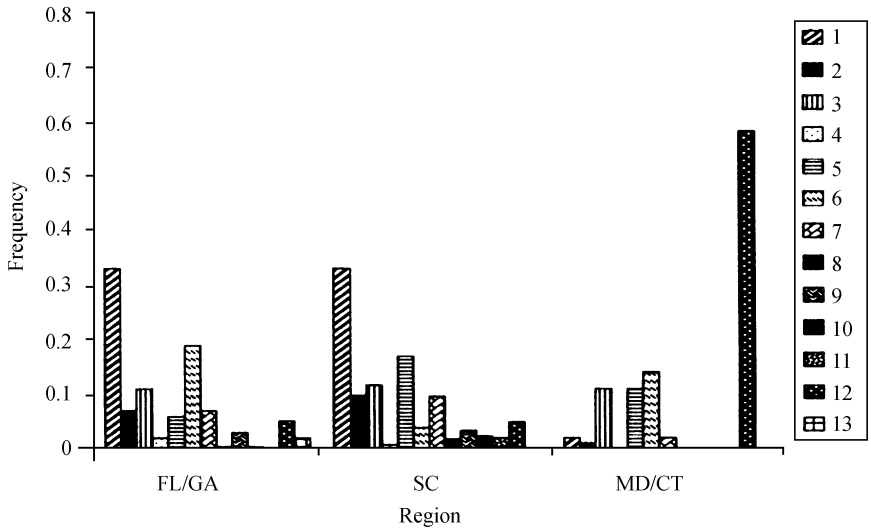


**Fig. 3 Polyacrylamide gel showing SSCP analysis of Cyt b haplotypes**
Lanes 1 - 7 represent individuals from Pinckney Island, lane 8: represents 1 kb DNA ladder, lane 9 represents an individual from Pritchard's Island, and lanes 10 - 19 represent individuals from Anastasia Island. Lanes 1 - 3, 5 - 7, 10 - 13, and 15 - 17 are haplotype A, lanes 4, 9, and 14 are haplotype F, and lane 18 is haplotype C.

**Table 3 AMOVA results for populations of** *I. scapularis* **for Cyt b and ITS1**

| Locus | Divisions | Variance component among | % total variance | *F*-statistics | *P* |
|---|---|---|---|---|---|
| Cyt *b* | South vs North | regions | 24.47 | $F_{ST}$ = 0.244 | 0.017 |
| | | localities/ regions | 10.95 | $F_{IS}$ = 0.144 | < 0.001 |
| | | individuals | 64.58 | $F_{IT}$ = 0.354 | < 0.001 |
| | GA, FL vs MD, CT | regions | 44.87 | $F_{ST}$ = 0.448 | 0.04 |
| | | localities/ regions | 2.80 | $F_{IS}$ = 0.051 | < 0.001 |
| | | individuals | 52.34 | $F_{IT}$ = 0.245 | < 0.001 |
| | SC vs GA, FL, MD, CT | regions | - 1.95 | $F_{ST}$ = - 0.012 | 0.002 |
| | | localities/ regions | 22.97 | $F_{IS}$ = 0.225 | < 0.001 |
| | | individuals | 78.98 | $F_{IT}$ = 0.210 | < 0.001 |
| ITS1 | South vs North | regions | 15.16 | $F_{ST}$ = 0.152 | 0.009 |
| | | localities/ regions | 4.12 | $F_{IS}$ = 0.049 | < 0.001 |
| | | individuals | 80.72 | $F_{IT}$ = 0.193 | < 0.001 |
| | GA, FL vs MD, CT | regions | 14.91 | $F_{ST}$ = 0.149 | 0.044 |
| | | localities/ regions | 4.85 | $F_{IS}$ = 0.057 | < 0.001 |
| | | individuals | 80.24 | $F_{IT}$ = 0.198 | < 0.001 |
| | SC vs GA, FL, MD, CT | regions | 0.57 | $F_{ST}$ = 0.006 | 0.176 |
| | | localities/ regions | 6.64 | $F_{IS}$ = 0.067 | < 0.001 |
| | | individuals | 92.79 | $F_{IT}$ = 0.072 | < 0.001 |

*P* values are based on 1 023 permutations.

### 2.3 Cyt b genetic population structure

Overall $F_{ST}$ values based on Cyt *b* were statistically significant (Table 3).

Pairwise comparisons of FST estimates were significant for most sites. Results from the AMOVA (Table 3) indicated that 64.58 % of the variation at the Cyt *b* locus was attributable to differentiation among individuals within sites, and 24.47 % of the variation was due to differentiation among regions (Assateague and Bridgeport versus all other populations). It is possible that South Carolina is boundary between southern and northern populations in our study. To see the differences between South Carolina and the southern and northern populations, genetic structuring was also tested by comparing all sites in South Carolina to all other sites. In this case, none of the variation was attributable to the between-region group variance. Isolation by distance (Table 4) indicated by a strong correlation between geographic distance and pairwise genetic differentiation ($r$ =

0.724 , $P < 0.0001$) . Because of the close proximity of three of the islands in Florida (Big Talbot , Little Talbot , and King George) , those haplotype frequencies were combined and treated as one locality in a separate analysis , with no effect on the significance of the correlation ( $r = 0.760$) . Geographical distance , therefore , explained 52 % of the variation at the Cyt *b* locus.

### 2.4 ITS1 genetic population structure

AMOVA of the ITS1 data analysis indicated that 80.72 % of the variation was attributable to differentiation among individuals within sites and 15.16 % to differentiation among regions ( Table 3) . Again , when all sites in South Carolina were tested against all other sites , there was no between-group variation. Mantel 's tests on the ITS1 locus indicated a strong positive correlation between geographic distance and $F_{ST}$ ( $r = 0.62$ , $P < 0.015$) . Combining the three Florida localities had no effect on the significance of the correlation ( $r = 0.69$) (Table 5) . Over 39 % of variation at the ITS1 locus was explained by geographical distance.

**Table 4 Pairwise $F_{ST}$ values and distance calculated from Cyt b allele frequencies used in the isolation by distance regression ( b = 0.0004 , $r^2 = 0.52$ , $P < 0.0001$)**

| | AN | KG | BT | LT | JK | SA | PI | PR | HU | ED | AS | BR |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| AN | 0 | 0.089 | 0.064 | 0.004 | 0.196 | 0.114 | 0.118 | 0.355 | 0.318 | 0.080 | 0.540 | 0.559 |
| KG | 53 | 0 | 0.001 | 0.104 | 0.019 | - 0.014 | 0.070 | 0.243 | 0.205 | 0.019 | 0.410 | 0.425 |
| BT | 56 | 3 | 0 | 0.103 | 0.016 | - 0.015 | 0.073 | 0.243 | 0.202 | 0.038 | 0.410 | 0.418 |
| LT | 58 | 5 | 2 | 0 | 0.240 | 0.157 | 0.137 | 0.375 | 0.338 | 0.087 | 0.564 | 0.589 |
| JK | 90 | 40 | 37 | 35 | 0 | - 0.012 | 0.117 | 0.204 | 0.160 | 0.080 | 0.355 | 0.368 |
| SA | 110 | 57 | 54 | 52 | 24 | 0 | 0.068 | 0.205 | 0.165 | 0.023 | 0.367 | 0.379 |
| PI | 163 | 120 | 117 | 115 | 84 | 70 | 0 | 0.118 | 0.111 | 0.019 | 0.288 | 0.287 |
| PR | 175 | 145 | 143 | 141 | 110 | 85 | 27 | 0 | 0.011 | 0.192 | 0.055 | 0.038 |
| HU | 180 | 150 | 147 | 145 | 115 | 90 | 32 | 5 | 0 | 0.160 | 0.094 | 0.098 |
| ED | 248 | 205 | 203 | 201 | 164 | 148 | 89 | 57 | 52 | 0 | 0.354 | 0.360 |
| AS | 690 | 655 | 658 | 660 | 620 | 600 | 535 | 525 | 520 | 498 | 0 | 0.003 |
| BR | 900 | 869 | 866 | 864 | 832 | 817 | 748 | 748 | 725 | 680 | 220 | 0 |

AN: Anastasia. KG: King George. BT: Big Talbot. LT: Little Talbot. SA: Sapelo. PI: Pinckney. PR: Pritchard's. HU: Hunting. ED: Edisto. AS: Assateague. BR: Bridgeport.

**Table 5 Pairwise $F_{ST}$ values and geographic distances calculated with ITS genotype frequencies used in the isolation by distance regression ( b = 0.0002 , $r^2 = 0.39$ , $P < 0.015$)**

| | AN | KG | BT | LT | JK | SA | PI | PR | HU | ED | AS | BR |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| AN | 0 | 0.131 | 0.017 | 0.110 | 0.045 | 0.007 | 0.016 | 0.029 | 0.029 | 0.011 | 0.239 | 0.185 |
| KG | 53 | 0 | 0.149 | 0.007 | 0.184 | 0.179 | 0.122 | 0.112 | 0.064 | 0.083 | 0.310 | 0.257 |
| BT | 56 | 3 | 0 | 0.129 | 0.074 | 0.059 | 0.015 | 0.024 | 0.047 | 0.026 | 0.260 | 0.194 |
| LT | 58 | 5 | 2 | 0 | 0.141 | 0.140 | 0.097 | 0.090 | 0.051 | 0.066 | 0.269 | 0.223 |
| JK | 90 | 40 | 37 | 35 | 0 | 0.096 | 0.068 | 0.031 | 0.031 | 0.030 | 0.076 | 0.036 |
| SA | 110 | 57 | 54 | 52 | 24 | 0 | 0.043 | 0.064 | 0.061 | 0.036 | 0.328 | 0.268 |
| PI | 163 | 120 | 117 | 115 | 84 | 70 | 0 | 0.024 | 0.022 | 0.007 | 0.256 | 0.196 |
| PR | 175 | 145 | 143 | 141 | 110 | 85 | 27 | 0 | 0.018 | 0.010 | 0.190 | 0.133 |
| HU | 180 | 150 | 147 | 145 | 115 | 90 | 32 | 5 | 0 | 0.004 | 0.164 | 0.123 |
| ED | 248 | 205 | 203 | 201 | 164 | 148 | 89 | 57 | 52 | 0 | 0.181 | 0.136 |
| AS | 690 | 655 | 658 | 660 | 620 | 600 | 535 | 525 | 520 | 498 | 0 | - 0.003 |
| BR | 900 | 869 | 866 | 832 | 832 | 817 | 748 | 730 | 725 | 680 | 220 | 0 |

Abbreviations are given in Table 4.

### 2.5 Combined genetic distance

Values for Nei's genetic identity ( *I*) and Nei's genetic distance ( *D*) were also calculated for all sites using combined data (Table 6). Genetic identity ranged from 0.06 to 0.98. Genetic distance ranged from 0.02 to 2.78. Finally, a neighbor-joining tree was generated from the combined distance data using MEGA (Fig. 4).

**Table 6 Matrix of genetic relatedness for 12 sites of** I. scapularis

| | AN | KG | BT | LT | JK | SA | PI | PR | HU | ED | AS | BR |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| AN | - | 0.08 | 0.06 | 0.21 | 0.10 | 0.16 | 0.07 | 0.62 | 0.54 | 0.11 | 1.96 | 1.55 |
| KG | 0.93 | - | 0.14 | 0.08 | 0.29 | 0.36 | 0.14 | 0.79 | 0.57 | 0.18 | 1.31 | 1.26 |
| BT | 0.95 | 0.87 | - | 0.19 | 0.03 | 0.12 | 0.08 | 0.61 | 0.47 | 0.15 | 2.07 | 1.55 |
| LT | 0.81 | 0.93 | 0.83 | - | 0.25 | 0.42 | 0.26 | 0.88 | 0.55 | 0.25 | 1.61 | 1.50 |
| JK | 0.91 | 0.75 | 0.97 | 0.78 | - | 0.20 | 0.13 | 0.58 | 0.48 | 0.19 | 2.78 | 1.74 |
| SA | 0.85 | 0.70 | 0.89 | 0.66 | 0.82 | - | 0.30 | 0.48 | 0.54 | 0.26 | 2.65 | 2.01 |
| PI | 0.93 | 0.87 | 0.93 | 0.77 | 0.87 | 0.74 | - | 0.35 | 0.26 | 0.03 | 0.98 | 0.72 |
| PR | 0.54 | 0.45 | 0.55 | 0.42 | 0.56 | 0.62 | 0.71 | - | 0.09 | 0.21 | 0.41 | 0.33 |
| Hu | 0.59 | 0.56 | 0.63 | 0.58 | 0.62 | 0.58 | 0.77 | 0.92 | - | 0.16 | 0.58 | 0.49 |
| ED | 0.89 | 0.84 | 0.86 | 0.78 | 0.83 | 0.77 | 0.97 | 0.81 | 0.85 | - | 0.93 | 0.74 |
| AS | 0.14 | 0.27 | 0.13 | 0.20 | 0.06 | 0.07 | 0.38 | 0.66 | 0.57 | 0.40 | - | 0.02 |
| BR | 0.21 | 0.29 | 0.21 | 0.22 | 0.18 | 0.13 | 0.49 | 0.72 | 0.61 | 0.48 | 0.98 | - |

Values of Nei's genetic distance ( *D*) are above the diagonal. Values of Nei's genetic identities ( *I*) are below the diagonal. Abbreviations are as given in Table 4.

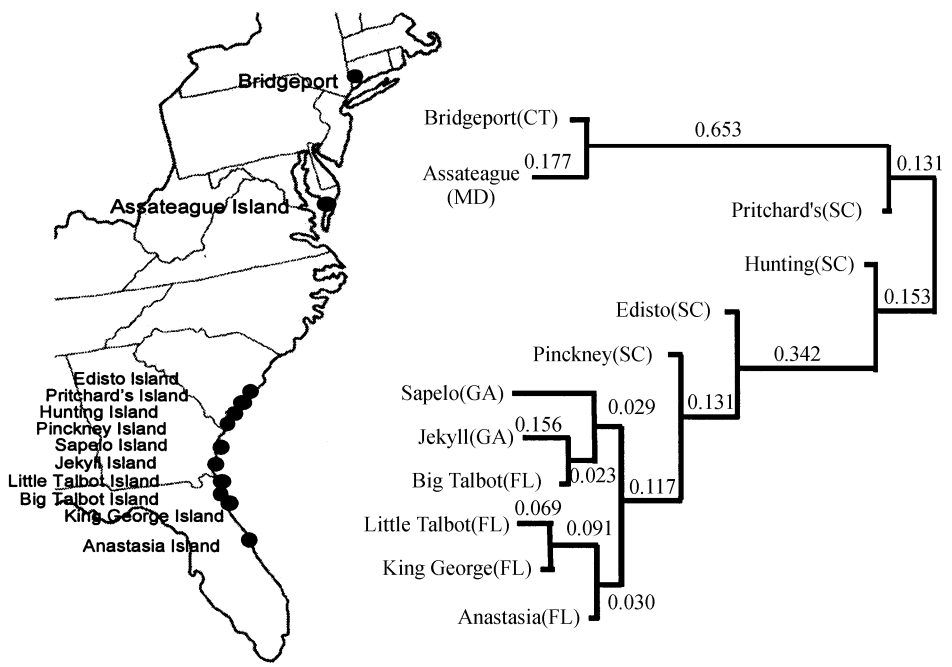


**Fig. 4 Neighbor-joining tree derived from Nei's distance based on combined data using software MEGA**

Numbers on the tree are branch lengths based on nucleotide substitutions.

The topology of the cladegram derived from coancestry distance and Nei's genetic distance were identical (data not shown). Ticks from Florida and Georgia islands were clustered together and separated from those from South Carolina and from the northeast. South Carolina sites were not part of a single clade.

# 3 Discussion

### 3.1 Evidence of population differentiation

Our data suggest that two distinct populations of *I. scapularis* exist along the eastern coast of the US. This has been recently suggested by Qiu et al. (2002) where patterns suggestive of separate evolutionary histories for northern and southern populations were found. Our conclusion is based on several lines of evidence. First, the distributions of Cyt *b* haplotypes are quite different between samples collected from northern (Connecticut & Maryland) and two southern (Florida & Georgia) regions. The dominant haplotypes in the southern region are types A and B, whereas the dominant types in the northern region are C, F, and G (Fig. 1). Second, the distributions of genotypes of ITS1 between sites and states are significantly different. The ITS1 results are consistent with that of Cyt *b*. Dominant genotypes in the south are types 1, 2, and 7, whereas the dominant genotype in the north was type 12 (Fig. 3). Third, AMOVA analysis showed that differences between northern and southern regions contribute significantly to the total variance. Fourth, pairwise $F_{ST}$ tests revealed significant differences between northern and southern sites. Fifth, pairwise genetic relatedness (represented by Nei's genetic distance and Nei's genetic identity) suggests divergence in concordance with isolation by distance. Finally, the NJ tree of phylogenetic analysis constructed from combined distance data placed northern and southern populations into two distinct clades. Differentiation of a clade (American) containing ticks from both northern and southern regions and a southern clade containing only southern ticks was reported previously (Rich et al., 1995; Norris et al., 1996). Interestingly, our data indicate that northern and southern populations share several haplotypes/genotypes in common. Thus, AMOVA analyses showed that 64.58 % (Cyt *b*) and 80.72 % (ITS1) of the total variances is shared among individuals across northern and southern populations.

Our study further suggested that northern and southern forms of *I. scapularis* overlap in South Carolina, where overall diversities of haplotypes and genotypes were highest. Unlike the northern and southern sites, where each possesses unique haplotypes (Cyt *b*) and genotypes (ITS1), sites in South Carolina share both haplotypes and genotypes with southern and northern regions. Moreover, South Carolina sites did not have their own unique haplotypes or genotypes. Furthermore, ticks from sites in South Carolina did not form a clade on the NJ genetree. Rich et al. (1995) suggested that the northern clade of *I. scapularis* was restricted to the north until only recently. Our data are consistent with range expansion and subsequent secondary contact of two populations. To the contrary, Norris et al. (1996) suggested that *I. scapularis* arose in the south and diverged in the north based upon the greater variability found within the southern region.

We found unique haplotypes on the most southern islands of Florida. This may suggest recent establishment and founder effects (Jaarola and Tegelstrom, 1996). Interestingly, the *Borrelia burgdorferi* s. l. spirochetes transmitted by *I. scapularis* are much more genetically diverse in the south than those found in the north (Oliver, 1996; Lin et al., 2001, 2002). Yet, consistent with Norris et al. (1996), analyses of both mitochondrial and ribosomal loci indicate that the southern populations exhibit the greatest diversity in haplotypes/genotypes. Genotypes 8 - 11 of ITS1 occurred only in the south. Further, nucleotide diversity based on ITS1 sequences is highest in the south. The pattern of the frequency distributions of both Cyt *b* haplotypes and ITS1 genotype suggests clinal genetic variation from south to north along the Atlantic coast. Clinal variation may be due to directional selection. Coan and Stiller (1986) found a cline of morphological variations in blacklegged ticks and Hutcheson et al. (1995) reported a morphometric cline among tick samples of *I. scapularis* collected along eastern coastal regions of the US.

### 3.2 Genetic variability within geographic regions

Surprisingly, we found a high level of genetic variability within geographic localities of *I. scapularis*. Diversities of Cyt *b* haplotypes, ITS1 genotypes, and nucleotides in ITS1 were all high within collection sites. In addition, AMOVA analysis of both loci revealed that the majority of variance is shared among the individuals across regions. Like other hard ticks, *I. scapularis* is a slow moving organism with limited intrinsic dispersal abilities (Carroll and Schmidtmann, 1996). Movement relies mostly on the movement of their hosts and is influenced by host density, host preference, and host mobility. Diversity indices and variances among individuals in each pairwise analysis were very high, implying that gene flow between geographic regions occurs frequently. We hypothesize that the high rates of gene flow in blacklegged ticks among localities may be due to the highly mobile bird hosts. Birds have been

recognized as hosts of *I. scapularis* and their role as hosts of ticks is well established (Giardina et al., 2000; Kinsey et al., 2000; Alekseev et al., 2001; Scott et al., 2001). We conclude that range expansion of members of northern and southern clades into South Carolina has been facilitated by vagile hosts and has resulted in clinal variation in genotype frequencies on a subcontinental scale.

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**Appendix 1 Haplotype frequencies of Cyt b among collecting sites of** I. scapularis

| Site | *n* | Haplotype | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | A | B | C | D | E | F | G |
| Florida | 204 | 0. 755 | 0. 127 | 0. 005 | 0. 025 | 0. 064 | 0. 015 | 0. 010 |
| Anastasia | 53 | 0. 849 | 0. 038 | - | - | 0. 094 | 0. 016 | - |
| King George | 47 | 0. 894 | 0. 043 | 0. 021 | 0. 021 | - | 0. 021 | - |
| Big Talbot | 40 | 0. 650 | 0. 225 | - | 0. 075 | - | - | 0. 050 |
| Little Talbot | 64 | 0. 641 | 0. 203 | - | 0. 016 | 0. 125 | 0. 016 | - |
| Georgia | 64 | 0. 531 | 0. 297 | - | 0. 031 | 0. 094 | 0. 016 | 0. 031 |
| Jekyll | 30 | 0. 567 | 0. 233 | - | 0. 067 | 0. 100 | - | 0. 033 |
| Sapelo | 34 | 0. 500 | 0. 353 | - | - | 0. 088 | 0. 029 | 0. 029 |
| S. Carolina | 304 | 0. 395 | 0. 066 | 0. 194 | 0. 076 | 0. 023 | 0. 237 | 0. 010 |
| Pinckney | 31 | 0. 613 | - | 0. 097 | 0. 065 | - | 0. 226 | - |
| Pritchard's | 82 | 0. 220 | 0. 049 | 0. 220 | 0. 061 | 0. 024 | 0. 427 | - |
| Hunting | 89 | 0. 236 | 0. 101 | 0. 315 | 0. 011 | 0. 045 | 0. 292 | - |
| Edisto | 102 | 0. 608 | 0. 069 | 0. 098 | 0. 147 | 0. 010 | 0. 039 | 0. 029 |
| Maryland Assateague | 57 | 0. 018 | - | 0. 246 | - | - | 0. 544 | 0. 193 |
| Connecticut Bridgeport | 45 | 0. 044 | - | 0. 200 | 0. 044 | 0. 022 | 0. 622 | 0. 067 |
| All sites | 674 | 0. 461 | 0. 10 | 0. 123 | 0. 050 | 0. 040 | 0. 200 | 0. 031 |

**Appendix 2 ITS1 genotype frequencies among collecting sites of** I. scapularis

| Site | *n* | Genotype | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 |
| Florida | 196 | 0.270 | 0.056 | 0.112 | 0.026 | 0.071 | 0.240 | 0.077 | - | 0.020 | 0.005 | - | 0.097 | 0.026 |
| Anastasia | 43 | 0.488 | 0.093 | 0.140 | - | 0.047 | - | - | - | 0.070 | - | - | 0.163 | - |
| King George | 50 | 0.140 | - | 0.040 | 0.040 | 0.10 | 0.460 | 0.120 | - | - | - | - | 0.100 | - |
| Big Talbot | 50 | 0.360 | 0.140 | 0.240 | 0.020 | 0.040 | 0.020 | 0.060 | - | 0.020 | 0.020 | - | 0.080 | - |
| Little Talbot | 53 | 0.132 | - | 0.038 | 0.038 | 0.094 | 0.434 | 0.113 | - | - | - | - | 0.057 | 0.094 |
| Georgia | 47 | 0.574 | 0.149 | 0.085 | 0.021 | 0.021 | - | 0.021 | 0.021 | 0.064 | - | - | 0.043 | - |
| Jekyll | 16 | 0.313 | 0.375 | 0.188 | - | - | - | 0.063 | - | 0.063 | - | - | - | - |
| Sapelo | 31 | 0.710 | 0.032 | 0.032 | 0.032 | 0.032 | - | - | 0.032 | 0.065 | - | - | 0.065 | - |
| S. Carolina | 264 | 0.330 | 0.098 | 0.117 | 0.008 | 0.170 | 0.038 | 0.095 | 0.019 | 0.034 | 0.023 | 0.019 | 0.049 | - |
| Pinckney | 45 | 0.378 | 0.067 | 0.244 | 0.022 | 0.089 | - | 0.067 | - | - | 0.044 | 0.022 | 0.067 | - |
| Pritchard's | 53 | 0.377 | 0.170 | 0.038 | 0.022 | 0.189 | 0.019 | 0.113 | 0.038 | - | - | - | 0.038 | - |
| Hunting | 82 | 0.244 | 0.085 | 0.098 | - | 0.207 | 0.085 | 0.134 | - | 0.061 | 0.037 | - | 0.049 | - |
| Edisto | 84 | 0.357 | 0.083 | 0.119 | - | 0.167 | 0.024 | 0.060 | 0.036 | 0.048 | 0.012 | 0.048 | 0.048 | - |
| Maryland Assateague | 40 | - | - | 0.025 | - | 0.175 | 0.150 | 0.025 | - | - | - | - | 0.625 | - |
| Connecticut Bridgeport | 45 | 0.044 | 0.022 | 0.222 | - | 0.044 | 0.125 | 0.022 | - | - | - | - | 0.533 | - |
| All sites | 592 | 0.285 | 0.076 | 0.115 | 0.014 | 0.117 | 0.117 | 0.073 | 0.010 | 0.027 | 0.019 | 0.008 | 0.137 | 0.008 |