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COMPARATIVE ANALYSIS OF MICROSATELLITE AND MITOCHONDRIAL GENETIC VARIATION IN *IXODES SCAPULARIS*

by

CYNTHIA TAK WAN CHAN

(Under the Direction of Lorenza Beati)

ABSTRACT

Ixodes scapularis, the black legged tick, is a species endemic to North America with a range including most of the eastern-half of the United States and portions of Canada and Mexico. The tick is an important vector of diseases transmitted to humans and animals. Since its first description in 1821, the taxonomy of the species has been controversial. Biological differences have been identified in the northern and southern populations, yet no consensus exists on population structure and the causes of this disparity. Earlier molecular studies utilizing nuclear and mitochondrial genetic markers have revealed the occurrence of two distinct lineages: a genetically diverse southern clade found in the southern-half of the distribution area of *I. scapularis*, and a more genetically homogeneous American clade found throughout the *I. scapularis* range. Although mitochondrial markers have assisted in clarifying the population history of the tick, nuclear, bi-parentally inherited markers such as microsatellite loci can provide additional information at a finer scale. Furthermore, previous studies were based on either limited sampling, which did not represent the whole geographic range of the tick, or were based on single molecular markers. In this study, we **(a)** generated a new dataset by collecting samples throughout the distribution range of *I. scapularis*; **(b)** developed microsatellite markers for the study of the genetic structure of *I. scapularis*; **(c)** amplified and sequenced two different mitochondrial datasets and analyzed them phylogenetically in order to compare our data with previously published reconstructions; and **(d)** analyzed population genetics parameters and compared results obtained by analyzing mitochondrial vs. microsatellite markers. Our data confirm some of the earlier findings, but provide additional information on the geographically distinct genetic diversity of the species, and the evolutionary mechanisms that shaped its present structure. These data may further help our understanding of how pathogens circulate within *I. scapularis* populations.

INDEX WORDS: *Ixodes scapularis*, Mitochondrial markers, 12SrDNA, D-Loop (control region), Microsatellite markers, Phylogeography, Genetic structure

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CYNTHIA TAK WAN CHAN

B.A., Binghamton University, 2003

A Thesis Submitted to the Graduate Faculty of Georgia Southern University in Partial Fulfillment
of the Requirements for the Degree

MASTER OF SCIENCE

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CYNTHIA TAK WAN CHAN

Major Professor: Lorenza Beati

Committee: Lance A. Durden
William Irby

Electronic Version Approved:
May 2012

DEDICATION

I dedicate this thesis and the time I've spent in pursuit of my passions to my father, David Y. Chan, who has always inspired me to be interested in discovery, to ask questions, to find answers, and most importantly, to never give up.

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An enormous thanks goes to my advisor and mentor, Dr. Lorenza Beati, for believing in me and being a great guiding force in my success. I would also like to thank my committee members, Dr. Lance Durden and Dr. William Irby, who nurtured my interest in entomology and provided me with excellent feedback and assistance whenever I needed it. A special thanks goes to my close friend and field assistant, Cynthia Chin, without whom the collection trip in 2010 may have ended in tragedy. I am forever indebted to Heather Walker, Dr. Laura Regassa, Marcee Toliver, Phil Norman, the Porcaro Family, Jamie Kass, Jacob Lasala, Tim Sandlin, Kaetlyn Kerr, Dr. Scott Harrison, and numerous others that have helped in some way, either by providing localities to flag, deer to examine, insight on molecular techniques, or other means of support. And last, but not least, I am tremendously grateful to my family and friends including those at Georgia Southern University: Earlene Howard, Brett Rabeneck, Keely Duff, Jenny Dickson, Laquita Burton, Alysha Benn, Danielle Hibbs, Jaymin Patel, Bola Kushimo, Nina Griffin, and Stephanie Dold, who among other things, had the important task of helping me maintain my sanity throughout this entire process.

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Kempton, PA; and the Commonwealth of Virginia Department of Conservation and Recreation, Virginia State Parks.

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CHAPTER 1

Introduction

Taxonomic information: *Ixodes scapularis* Say, 1821, often referred to as the black legged tick, is a bloodsucking ectoparasite that feeds on a variety of vertebrate hosts, including: birds, mammals, and reptiles (Bishopp and Trembley, 1945; Keirans et al., 1996; Guzmán-Cornejo and Robbins, 2010; Bouchard et al., 2011). It is endemic to North America and its range includes the United States, Canada, and Mexico (Keirans et al., 1996; Dennis et al., 1998) (Figure I). Since its first description by Thomas Say (1821), the species has undergone periods of taxonomic ambiguity due in part to discontinuity in geographic distribution or clinal variations in morphological characters (Hutcheson and Oliver, 1996; 1998) and behavior (Kinsey et al., 2000; Clark et al., 2002). Nomenclature associated with *I. scapularis* is listed below.

Synonymy list:

Ixodes scapularis Say, 1821
Ixodes fuscous Say, 1821
Ixodes reduvius Neumann, 1899 (*pro parte*)
Ixodes pratti Banks, 1908 (*pro parte*)
Ixodes fuscus Neumann, 1911
Ixodes ricinus variation *scapularis* Say, 1821 *sensu* Nuttall and Warburton, 1911
Ixodes ozarkus Cooley, 1944
Ixodes muris Good, 1973
Ixodes dammini Spielman, Clifford, Piesman and Corwin, 1979

(From: *The ticks of the world. Nomenclature, described stages, hosts and distribution (Acarida, Ixodida)* Camicas et al., 1998; *Catalogue of life: 3rd February 2012*, www.catalogueoflife.org)

Due to the sexually dimorphic nature of the species, initial morphological descriptions resulted in a dual classification of adult forms: *Ixodes fuscous* and *I. scapularis*, referring

to males and females respectively (Say, 1821). Biological and morphological similarities to other ticks belonging to what is known as the *Ixodes ricinus* complex (Keirans et al., 1999; Xu et al., 2003), resulted in multiple re-classifications of *I. scapularis*. Neumann (1899) included specimens of both the European *I. ricinus* and the American *I. scapularis* in his description of *Ixodes reduvius*. In 1908, Banks described *Ixodes pratti* based on both, specimens of *Ixodes kingi* and *I. scapularis*; *I. kingi* formerly used as an ambiguous synonym of the latter (Camicas et al., 1998). In 1911, *I. scapularis* was reduced to a variation of *I. ricinus* Nuttall and Warburton, 1911. Cooley (1944) described a new species of *Ixodes* from Arkansas, *Ixodes ozarkus*, which he distinguished from *I. scapularis* based mainly on overall size and hypostomal variation. Spielman et al. (1979) considered the *I. ricinus*-like complex in North America, composed of three *Ixodes* species: *Ixodes pacificus* in the West, *I. scapularis* in the Southeast and *Ixodes dammini* in the Northeast and Upper Midwest; using morphology and distinct association with pathogens to distinguish between *I. dammini* and *I. scapularis*. More recently, Oliver et al. (1993) synonymized *I. dammini* with *I. scapularis* after establishing successful reciprocal crosses between colonies generated from southern and northeastern ticks, producing viable offspring through the F3 generation. These results were confirmed using ribosomal deoxyribonucleic acid (rDNA) genetic markers (Wesson et al., 1993).

Geographic distribution: *I. scapularis* has a widespread geographic range within North America. In the United States it is found throughout most of the eastern half of the country including: the Atlantic Coast (Maine to the southern tip of Florida), westward into the lower-lying elevations of eastern Texas, and in several north central states

(Michigan, Indiana, Illinois, Wisconsin, Minnesota, Iowa, North Dakota and South Dakota) (Keirans et al., 1996; Dennis et al., 1998) (Figure I). More recently, *I. scapularis* were collected in parts of Ohio (Needham personal communication). Endemic zones in Canada are believed to be isolated to specific locations in south-central and southeastern parts including southeastern Manitoba, southern and eastern Ontario, Nova Scotia, and more recently, southern New Brunswick and Quebec (Keirans et al., 1996; Ogden et al., 2006; Ogden et al., 2008b; Ogden et al., 2008c; Bouchard et al., 2011). In Mexico, the southernmost established range of *I. scapularis* extends into the states of Chiapas, Coahuila, Jalisco, Nuevo León, Oaxaca and Tamaulipas (Keirans et al., 1996; Guzmán-Cornejo and Robbins, 2010).

Geographic distribution is limited by a number of factors including: soil-type (Guerra et al., 2002; Bunnell et al., 2003), land cover (Mannelli et al., 1994; Stafford III et al., 1998; Guerra et al., 2002) and climate (Brownstein et al., 2003; Bunnell et al., 2003; Diuk-Wasser et al., 2010). In 2002, Guerra et al., created a habitat profile for *I. scapularis* in the north central United States and found habitat preference of *I. scapularis* to be deciduous, dry to mesic forests and alfisol-type soils of sandy or loam-sand textures overlying sedimentary rock. Bunnell et al. (2003) confirmed that there was a significant statistical correlation between areas with sandy soils, at lower elevations, and of moderate distance to forest and water and an increase in tick abundance. The same study also found a negative correlation between tick abundance and areas characterized as wetlands or areas at higher elevations. Climate and associated factors, particularly humidity, and elevation limit the range of *I. scapularis* (Brownstein et al., 2003) and its population density (Diuk-Wasser et al., 2010). A study conducted by Gern et al. (2008)

showed similar relationships in *I. ricinus*, with a negative correlation between increased altitude and tick questing density. In addition, they found that the prevalence of *Borrelia burgdorferi* infection in questing ticks decreased with increasing altitude. It is important that these factors be constantly re-evaluated, as recent climatic warming trends are believed to have resulted in range expansions into Western portions of Canada (Bouchard et al., 2011). Changes such as habitat alteration and decreases in pronounced seasonal variations due to global warming have already been shown to influence vectors and the pathogens that they transmit (Gubler et al., 2001).

Host associations: Host availability, along with environmental conditions play important roles in tick abundance (Oorebeek and Kleindorfer, 2008). *I. scapularis* is a generalist, found to feed on a variety of vertebrate hosts during its blood-feeding life cycles. Recorded host associations include several mammal species (i.e. domesticated and wild mammals, small to large in size), birds, reptiles and amphibians (Bishopp and Trembley, 1945; Keirans et al., 1994; Keirans et al., 1996; Clark et al., 2002; Durden et al., 2002; Guzmán-Cornejo and Robbins, 2010; Bouchard et al., 2011). By adopting a generalist strategy, species are readily able to exploit new hosts, which allows for possible range expansions under ideal environmental conditions (Dennis et al., 2011). Some hosts are important because they serve as competent reservoirs for vector-borne zoonotic pathogens. A competent host not only carries the pathogen, but also makes it available to the vector (Brunner et al., 2008). Brunner et al. (2008) confirmed the susceptibility of several host species to *Borrelia burgdorferi* sensu stricto and identified high (*Peromyscus leucopus*), intermediate (*Blarina brevicauda* and *Tamias striatus*) and low (*Odocoileus virginianus*, *Sciurus carolinensis* and *Tamiasciurus vulgaris*) level

competent reservoirs.

In the southeast, host preference of immature stages of *I. scapularis* have been found to include a variety of reptiles, most of which are not found in the north (Apperson et al., 1993). This was thought to be the reason why the prevalence of *Borrelia* was lower in the Southeast (Levin et al., 1996; Durden et al., 2002), as lizards were shown to be incompetent hosts along the Pacific coast (Lane, 1990; Manweiler et al., 1992). However, Levin et al. (1996) found that two eastern lizard species (*Eumeces inexpectatus* and *Anolis carolinensis*) could be infected in the laboratory through needle inoculation or tick bite. Xenodiagnosis with ticks showed that the infection with *B. burgdorferi* could last up to five weeks after a three-week incubation period. Clark et al. (2005) studied *B. burgdorferi* sensu lato infection rates in wild lizards in South Carolina and Florida and demonstrated high infection prevalence with three strains of the bacterium among nine species of wild caught lizard species. They also found evidence indicating significant genetic variability in southern strains of *B. burgdorferi* and individual lizards infected with multiple strains. Level of infectivity may be of particular importance to medical and veterinary practitioners when a high level competent reservoir is widely distributed and has an overlapping range with the vector. In a newly endemic zone in southeastern Canada, Bouchard et al. (2011) found in their survey of small mammals that 35% of all feeding ticks in their study fed on adult male mice (*Peromyscus leucopus* and *Peromyscus maniculatus*).

Territoriality and range size of the host also affect the geographic structure of *I. scapularis.* Recent *I. scapularis* range expansions in Canada have been associated with

migratory bird flyways and are believed to have caused an increase in infection rates for Lyme borreliosis and human granulocytic anaplasmosis (Ogden et al., 2008a).

Epidemiology and control: *I. scapularis* is the vector for several pathogens including three diseases that affect humans: Lyme disease (*B. burgdorferi* sensu stricto), human granulocytic anaplasmosis (*Anaplasma phagocytophilium*) and human babesiosis (*Babesia microti*). Due to high tick densities and prevalence rates of the etiologic agents, geographic areas of highest transmission risk are the Northeast and upper Midwest regions of North America (Varde et al., 1998).

Qiu et al. (2002) examined the co-evolution of *I. scapularis* and *B. burgdorferi* sensu stricto, the causative agent of Lyme disease, and the tick's influence on the geographic distribution of the disease. They found that the genetic structure of *I. scapularis* has played a crucial role in shaping the genetic structure of *B. burgdorferi* sensu stricto and also provided some evidence that a lack of tick genetic variability in the Northeast may be a cause of high infection rates.

All resources for vector control, including funding, have been allocated to the northern states due to noted differences of infection rate in the northern and southern populations. Northern *I. scapularis* are responsible for >80% of Lyme disease cases in North America (Barbour and Fish, 2003). Different control methods have been tested, including pesticides; e.g., fipronil (Dolan et al., 2004) and permethrin (Solberg et al., 2003; Curtis et al., 2011). Also, targeted applications of pesticides with a limited range of action have been attempted, such as the 4-Poster device, a passive feeding station that controls ticks on white-tailed deer (*Odocoileus virginianus*) (Solberg et al., 2003; Curtis et al., 2011) and

a prophylactic doxycycline bait formulated to prevent the transmission of *B. burgdorferi* to rodents (Dolan et al., 2008).

Molecular biology and *Ixodes scapularis* genetic diversity: Initial arguments over the classification of the *I. ricinus*-like ticks in North America relied on behavioral, morphological, and geographic inferences (Say, 1821; Cooley, 1944; Bishopp and Trembley, 1945). Biological differences have been identified in the northern and southern distributions (McLain et al., 1995; Rich et al., 1995; Hutcheson and Oliver, 1996; Keirans et al., 1996; Qiu et al., 2002; Rosenthal and Spielman, 2004), yet no consensus exists on population structure and the causes of this disparity. Earlier molecular studies that examined the population genetics of *I. scapularis* utilized nuclear and mitochondrial (mtDNA) genetic markers in phylogenetic analyses. Mitochondrial gene markers are maternally inherited, faster evolving than nuclear ribosomal genes, and are informative for resolving relationships between closely related populations and demographic evolutionary histories (Caporale et al., 1995; Rich et al., 1995; Norris et al., 1996; Xu et al., 2003). Similarly, the deoxyribonucleic acid (DNA) sequences of the nuclear internal transcribed spacer regions (non-functional ribonucleic acid (RNA) regions separating structural rRNAs) are variable enough to provide information at the intraspecific level (Wesson et al., 1993; McLain et al., 1995).

In 1993, a study involving reciprocal crosses, assortative mating, morphometrics, isozyme and chromosomal analyses indicated the conspecificity of *I. scapularis* and *I. dammini* (Oliver et al., 1993). Wesson et al. (1993) analyzed the two internal transcribed spacers of nuclear ribosomal DNA, ITS-1 and ITS-2, to investigate the validity of *I. dammini* in a comparative study, which

included samples of *I. pacificus* and *I. scapularis*. As they found *I. dammini* and *I. scapularis* sequences clustered in the same lineages, they deduced that the two species were conspecific. Based on 16SrRNA analyses, Caporale et al. (1995) examined the phylogeny of several members of the genus *Ixodes*, including *I. scapularis* and found the *I. ricinus* complex to be monophyletic, a conclusion that was later challenged by Xu et al. (2003). They also found *I. scapularis* and *I. dammini* to be more closely related to each other (2% genetic distance) than either of them to other recognized species (up to 12.8%). Nevertheless, they concluded that *I. dammini* and *I. scapularis* were different species, as the low divergence level between them, was comparable to genetic distance between distinct species of flies and beetles. Rich et al. (1995) included samples from a wider geographic range in a study also based on 16SrRNA analyses. Their work analyzed the taxonomic status of *I. dammini* and *I. scapularis.* Their analyses revealed support for the occurrence of two distinct lineages of recent divergence: a *southern clade* established in the southeast with its northernmost range in North Carolina and a *northern clade* found in the Northeast and north central states. They concluded that *I. scapularis* and *I. dammini* were two species, which originated through geographic isolation possibly during the last glacial cycles (20,000-12,000 years ago). As the two species co-exist in many areas, either sympatrically or as a mosaic, they attributed this pattern to the invasion of southerly sites by the northern ticks (Rich et al., 1995). They disputed prior support for synonymizing *I. dammini* with *I. scapularis* (Oliver et al., 1993; Wesson et al., 1993), citing inadequate sampling and misinterpreted data.

The same year McLain et al. (1995) evaluated genetic differentiation of ticks from the east coast with the internal transcribed spacer 1 (ITS-1) of nuclear ribosomal DNA. The results of their analyses did not support the existence of two distinct species. However, their data also suggest that gene flow between regions has been and possibly remains restricted.

A study by Norris et al. (1996) using both 16S and 12S mitochondrial rDNA sequences indicated the presence of at least two major mitochondrial lineages in *I. scapularis*: a strongly supported southern clade and a less well supported, broadly distributed American clade (Figure II), which is in agreement with the study of Rich et al. (1995). Shannon diversity analyses of tick collections indicated that haplotype diversity was least in the northeast and accounted for only 9.3% of the total. In addition, higher levels of genetic variation and basal rDNA lineages in the strictly southern population suggest that the species arose and diverged in the South (Norris et al., 1996). Since the American clade included specimens from the whole distribution area of *I. scapularis*, their conclusions were in agreement with those of Oliver et al. (1993).

Further research by Qiu et al. (2002) investigating the evolutionary relationship between *I. scapularis* and *B. burgdorferi* found an even greater complexity (larger number of haplotypes) in *I. scapularis*. Using 16SrRNA mitochondrial DNA (mtDNA), they also found higher levels of heterogeneity in the south, with the American clade being genetically so homogeneous that it suggested the occurrence of a population bottleneck at its origin. Their results showed, again, that there are two main groups, one distributed throughout the *I. scapularis* distribution area and one only found in the South. Their study also addressed the possible role of migrating birds in transport of northern ticks to the south during the fall to account for the presence of northern haplotypes in the south.

The studies of Rich et al. (1995), Norris et al. (1996), and Qiu et al. (2002) are consistent with each other, although they did not always reach similar conclusions. Their findings may explain the conclusions drawn from other publications. Oliver et al. (1993) and McLain et al. (1995), used colony ticks to study the difference between northern and southern populations, not

knowing that the southern colonies may have been a mixture of ticks from the southern and the American clades.

A majority of the most recent molecular studies on the genetic diversity of *I. scapularis* were based on the analysis of mitochondrial gene sequences, which are maternally inherited. ITS sequences occur in several copies in individual genomes and are, therefore, difficult to sequence directly from a PCR product without undergoing lengthy cloning processes. There was, therefore, a need to develop alternative bi-parentally inherited markers, informative at the intraspecific level. In the past decade, several studies have shown that microsatellite markers were useful tools for unraveling genetic relationships within *Ixodes uriae* (McCoy and Tirard, 2002; Kempf et al., 2009a), *I. ricinus* (Delaye et al., 1998; De Meeûs et al., 2004; Røed et al., 2006; Hasle et al., 2008; Kempf et al., 2011), and *Rhipicephalus (Boophilus) microplus* populations (Chigagure et al., 2000; Cutullé et al., 2010).

Research involving allozyme markers in *I. ricinus* revealed little polymorphism and were uninformative (Delaye et al., 1997). In order to provide more useful molecular tools to study the migratory capabilities and resulting gene flow in *I. ricinus*, Delaye et al. (1998) designed and tested several microsatellite primers. De Meeûs et al. (2002) examined population structure and dispersal behavior in *I. ricinus* using the microsatellite markers designed by Delaye et al. (1998). They found a significant geographic structure within their sample and sex-associated differences in dispersal ability (males being more genetically diverse at each given site, than females). They suggested a sex-associated difference in host preference, possibly favoring the dispersal of males. Additional markers were required for analysis of population genetics in *I. ricinus* because previously described markers (Delaye et al., 1998) had issues due to the possible presence of null

alleles related to DNA methylation (De Meeûs et al., 2002; De Meeûs et al., 2004). Røed et al. (2006) designed microsatellite primers for *I. ricinus*, of which, almost half were successfully amplified and polymorphic. However, not all loci conformed to Mendelian expectations. Kempf et al. (2009b) used microsatellite loci to investigate assortative mating in *I. ricinus*. In two out of four populations, they found a significant positive correlation between the mating status and the relatedness of pairs, implying the occurrence of assortative mating. Kempf et al. (2011) examined host-associated genetic structure in *I. ricinus* using microsatellite markers on ticks collected from wild animals at five sites in Europe. Their results showed genetic structure among individual hosts, among host types, and within local populations.

Chigagure et al. (2000) designed the first set of microsatellite primers for use with *R.* (*B.*) *microplus*. De Meeûs et al. (2010) investigated the sympatric speciation of *R.* (*B.*) *microplus* on cattle (*Bos taurus*) and rusa deer (*Cervus timorensis*) in New Caledonia. Their study found that there are significant genetic differences between ticks found on cattle and those found on deer. Evidence was provided for adaptive divergence having occurred in approximately 244 tick generations (the date of introduction of the exotic rusa deer in New Caledonia is known). McCoy and Tirard (2002) used microsatellite primers to investigate the reproductive strategies of *I. uriae*. They were able to show that multiple mating can result in multiple paternities in each brood. Kempf et al. (2009a) examined host-associated divergence in *I. uriae* using both microsatellite and mitochondrial markers in order to address both recent and historical events in the species evolutionary history. Microsatellite markers were used to analyze host-associated population structure in four seabird colonies and the mitochondrial marker was used to establish a relationship between host association and

the phylogeographic relationships among ticks. They found significant evidence supporting the evolution of host-associated races in *I. uriae*. However, nested-clade analysis showed that mitochondrial genetic diversity was more readily explained by geography than by host.

Although microsatellite loci had been detected in *I. scapularis* genomes, they had been deemed useless for population genetic analysis based on their alleged rarity within the *I. scapularis* genome (Fagerberg et al., 2001). Now that black legged tick genome has been sequenced and assembled (http://extension.entm.purdue .edu /igp/), we know that microsatellite loci are extremely frequent (Cat Hill, personal communication). Rosenthal and Spielman (2004) examined the genetic distribution of *I. scapularis* in eastern North America using a single microsatellite locus which had previously been developed for *I. ricinus* (IR27) by Delaye et al. (1998), to determine gene flow. Rosenthal and Spielman (2004) found the locus to be highly polymorphic for the 58 *I. scapularis* they tested. Their findings lead to the conclusion that *I. scapularis* found in the Northeast and Midwest were genetically isolated from those found in the Southeast and suggest that this homogeneity in the north is the result of a founder event.

GOALS FOR THIS PROJECT

The purpose of this project is to use samples collected from the different regions within the range of *I. scapularis* and do a comparative analysis of mitochondrial and microsatellite genes in order to investigate their differences and see whether both support similar population groupings. In studies similar to our own, researchers found that using only sequences of mtDNA was restrictive and failed to detect significant genetic differentiation between populations, most likely due to its depiction of only the maternal lineage (Brower and DeSalle, 1998; Brunner et al., 1998). By providing additional data

for analysis, results should generate a finer depiction of how, why, and where this differentiation is occurring.

OBJECTIVES

Objective 1- To gather a sampling of freshly collected *I. scapularis* from its whole distribution area.

Objective 2- To compare evolutionary and demographic histories resulting from the analyses of the two mitochondrial datasets (compare to each other and to previously published data).

Objective 3- Develop and characterize bi-parentally inherited informative molecular markers (microsatellite loci) for the analysis of the population structure of *I. scapularis.*

Objective 4- Verify whether or not microsatellite genotyping and analysis support the same genetic structure revealed by mitochondrial gene sequence analysis.

CHAPTER 2

Materials and methods

Sample collection: Sampling of ticks was representative of locations throughout the distribution range of *I. scapularis* (Keirans et al., 1996; Dennis et al., 1998). This range was broken into four regions and ticks were sampled from localities within each region (Table I). Sites for personal collection were selected based on distance to other designated sampling areas, accessibility to the researcher, and predicted likelihood of tick availability (Diuk-Wasser et al., 2010; *Centers for Disease Control and Prevention* website) (Figure I). Locations were visited in mid-October during anticipated periods of higher *I. scapularis* activity based on prior studies involving population density and seasonality (Falco et al., 1999; Brownstein et al., 2003). Personal field collection of ticks involved two methods: vegetation flagging and host surveys. Vegetation flagging required the use of a 1 m^2 piece of white flannel fabric attached to a wooden dowel. This tool was swept across ground cover to collect active, questing ticks. Host surveys involved direct removal of ticks from white-tailed deer (*O. virginianus*) at a deer check station (David W. Force Park, Ellicott City, MD). Ticks were removed from the fabric and host using forceps and placed directly into vials containing ethanol, where they remained until DNA extraction. A total of 65 additional ticks (31 collected in the south and 34 collected in the north) was used as test sample for microsatellite characterization.

DNA extraction: Specimens were identified using taxonomic keys for the genus *Ixodes* (Keirans and Clifford, 1978; Durden and Keirans, 1996; Kleinjan and Lane, 2008) and entered into a database prior to genetic analysis. The extraction method followed previously published protocols (Beati and Keirans, 2001; Beati et al., 2012). Qiagen DNeasy Blood and Tissue Kits (Qiagen Incorporated, Chatsworth, CA) supplemented with recombinant, PCR grade proteinase

K from Roche Applied Science (Roche Diagnostics Corporation, Indianapolis, IN) were used for DNA purification. Individual ticks were placed in separate, PCR grade, 2 mL Eppendorf vials and were set to dry in a vacuum dessicator for a minimum of ten minutes. A total of 180 μL of animal tissue lysis buffer (ATL) was added. Each tick was cut within the buffer with a flame-sterilized scalpel by one of two methods: (1) adults and nymphs had a small portion of the idiosoma excised and (2) larvae were prepared by puncturing the cuticle with the sharp point of the blade. These techniques allowed for conservative disruption of the exoskeleton and preservation of the cuticle for possible future morphological assessment (Beati et al., 2012). After each specimen was cut, 40 μL of proteinase K (14.3mg/ml) were added to digest proteins. The vials were vortexed and placed in a dry bath at 56°C overnight. A total of 220 μL of AL lysis buffer was added to each vial; vials were vortexed, and incubated in a dry bath at 72°C for 10 minutes. Molecular grade ethanol (250 μL) was added and the solution was mixed using a micropipette. All liquid contents of each vial were transferred to a DNA mini-spin column with an attached collection tube, while cuticles were left in their original vials and preserved in 70% ethanol. The columns were centrifuged for one minute at 12,000 rpm to bind the DNA to the spin column membrane and remove any of the unwanted digested material. The bottom of each vial was removed, discarded and replaced with a new collection tube. To remove denatured proteins, 500 μL of AW1 washing buffer were added to each vial and centrifuged as in the previous step. The collection tube was removed, discarded and replaced. A total of 500 μL of AW2 wash buffer was added and each vial was centrifuged, to remove any salts present. In order to guarantee complete removal of buffer residues, empty columns were centrifuged again for an additional two minutes at 12,000 rpm. A total of 100 μL of molecular grade water at 72°C was added to

each column, which was spun for one minute at 12,000 rpm for final elution of DNA. The eluted DNA was stored at 4°C until needed.

Characterization of microsatellite markers: Prior to this study, 67 primer pairs amplifying *I. scapularis* microsatellite loci were selected by examining parts of the *I. scapularis* genome (http://www.vectorbase.org/). PCR conditions were optimized through gradient testing and program modification. A total of 22 microsatellite loci was selected for further analyses, based on reliable amplification of the expected fragment length in *I. scapularis* and presence of polymorphism. In order to further characterize the set of 22 primer pairs, we evaluated them for cross-amplification of pooled DNA from three additional species belonging to the *I. ricinus* complex: *I. pacificus*, *Ixodes persulcatus*, *I. ricinus*, and *I. scapularis* (Keirans et al., 1999; Xu et al., 2003). In order to test for linkage disequilibrium (LD) and Hardy-Weinberg equilibrium (HWE), the 22 primer pairs were used to amplify DNA from our test sample. LD between pairs of loci was tested by genotypic randomization test for linkage disequilibrium implemented in GenePop (Raymond and Rousset, 1995) with 10,000 iterations. HWE exact test for heterozygosity deficit was also performed in GenePop for each locus (10,000 iterations). In order to evaluate the importance of null allelles in our data, an estimation of genotype amplification failure rate was performed by a maximum likelihood expectation maximization test with GenePop. Eight loci were chosen to test our sample for genetic structure.

PCR amplification and analysis: The DNA from each tick was amplified for two mitochondrial gene markers (12S small ribosomal subunit RNA gene (12SrRNA) and D-Loop, also called the control region) (Table II) and eight nuclear microsatellite loci (locus 1, 3, 11, 15, 16, 17, 18 and 19) (Table III). PCR master mix preparation for each mitochondrial gene marker and microsatellite locus, as well as their corresponding thermocycler programs are listed in Table

IV. Forward primers for microsatellite loci were fluorescently tagged (Table III). Different colored custom-designed fluorescent tags from Applied Biosystems (Life Technologies Corporation, Carlsbad, CA) were used for different loci to allow for multiplexing. Agarose gel electrophoresis was used to confirm the presence of gene fragments of the desired size. Confirmation of 12SrRNA and D-Loop amplifications was done using 1% agarose gels in 0.5 x Tris Borate-EDTA (TBE) buffer, stained with ethidium bromide. Either 4% NuSieve agarose gels in 1.0 x lithium borate (LB) buffer, stained with ethidium bromide or 2-4% E-gel® precast agarose gel cassettes prepared with molecular grade ethidium bromide from Invitrogen (Life Technologies Corporation, Carlsbad, CA) were used for the microsatellite loci.

Sequencing: Each mitochondrial non-purified amplicon (approximately 16 μL) was diluted with 10 μL molecular grade water and aliquoted in identical volumes in two wells of a 96-well plate. A total of 15 μl of each primer (2 pmole/μl) was placed in the corresponding wells of a second 96-well plate. The plates were sent to the University of Washington's High-Throughput Genomics Unit (UW HTSeq), Seattle, WA for sequencing. For each amplicon the two strands were assembled into a contig and verified for accuracy using Sequencher 4.10.1 (Gene Codes Corporation, Ann Arbor, MI). Corresponding primer sequences were removed and necessary base calls were made. The contigs (unique sequences obtained after assembling complementary strands) were exported into FASTA format for further analyses.

Alignment and phylogenetic analyses: Separate alignments of 12SrDNA and D-Loop gene sequences, and a concatenated alignment of 12SrDNA/D-Loop were prepared using MacClade 4.08 OSX (Sinauer Associates, Inc., Sunderland, MA). The uploaded sequences were first aligned automatically and the alignments were manually modified according to secondary structure (Hickson et al., 1996; Beati and Keirans, 2001). A total of five outgroup sequences was

used for 12SrDNA: *I. pacificus*, *Ixodes affinis*, *I. persulcatus* (Genbank accession #: JF758624), *I. ricinus* (Genbank accession #: AF150029), and *Ixodes brunneus*, all but the last species belonging to the *I. ricinus* complex. A total of four outgroup sequences was used for D-Loop: *I. pacificus*, *I. affinis*, *I. persulcatus* (Genbank accession #: NC004370), and *I. ricinus* (Genbank accession #: AY945473). The 12SrDNA and the D-Loop sequences were also concatenated in a single matrix, for total evidence analysis. The concatenated 12SrDNA/D-Loop alignment included four outgroup sequences: *I. pacificus*, *I. affinis*, *I. persulcatus* (Genbank accession #s: JF758624/NC004370) and *I. ricinus* (Genbank accession #s: AF150029/AY945473). The individual 12SrDNA and D-Loop alignments were exported in Phylip 3.6 format for use in TCS1.21, a network estimation tool that uses statistical parsimony (Clement et al., 2000). The simple and concatenated matrices were also exported in Phylip 3.6 format for analysis with PhyML (http://phylogeny.lirmm.fr/ phylo_cgi/index.cgi), an online tool that estimates substitution models based on a neighbor-joining starting tree, generates maximum likelihood (ML) phylogenetic trees, and evaluates branch support through bootstrapping (100 replicates) (Guindon and Gascuel, 2003; Dereeper et al., 2008).

Genotyping: Microsatellite non-purified amplicons were prepared for single or multiplex genotyping. Amplicons of the expected length were ranked according to a predetermined visual concentration scale. The strength of the band determined the amount of amplicon to be plated: strong band = 2.0 μL, medium band = 2.5 μL, and weak band = 3.0 μL. Using singleplexing, the determined amount of amplicon (1-3 μL) was then diluted with Hi-Di™ formamide (Applied Biosystems by Life Technologies Corporation, Carlsbad, CA) to a total of 10 μL and placed into a well of a 96-well plate. When multiplexing, the sum of the volume taken from each amplicon (up to three loci) would be diluted with enough Hi-Di™ formamide to a total of 10 μL or 15 μL,

depending on number and concentration of amplicons. The plates were sent to the Analysis Facility on Science Hill at Yale, New Haven, CT, where the samples were supplemented with molecular weight standards and genotyped with a capillary sequencer. Genotyping results received from Science Hill were reviewed using GeneMarker (SoftGenetics, LLC, State College, PA) version 2.2.0. Number of alleles and allele fragment lengths were recorded for each specimen at each locus. Data were standardized according to type of nucleotide repeat (di- or trinucleotide) in order to account for variability in base calls due to separate processing of plate batches. The Microsoft Excel add-in, Genetic Analysis in Excel (GenAlEx) (Peakall and Smouse, 2006) version 6.41, was used to export data into formats usable by Arlequin (Schneider et al., 2000; Excoffier and Lischer, 2010) version 3.5.1.3, and GenePop On The Web (http://genepop.curtin.edu.au/; Raymond and Rousset, 1995; Rousset, 2008).

Haplotype statistical analyses: Individual sequence alignments for 12SrDNA and D-Loop were imported into DnaSP (DNA Sequence Polymorphism) (Librado and Rozas, 2009) version 5.10.01 for analysis of nucleotide polymorphism. The program was used to calculate the haplotype diversity (Hd), number of segregating sites (*S*), average pairwise distance (π), population mutation rate (ϑ), and three neutrality tests: Tajima's *D* Tajima, 1989, Ramos-Onsin's and Roza's *R2* (Ramos-Onsins and Rozas, 2002), and Fu's F_S (Fu and Li, 1993; Fu, 1997). Graphs representing mismatch distribution (pairwise differences vs. haplotype frequencies) were also generated in DnaSP for the American (*American I + II*) and the southern (*South I + II + III*) clades (see phylogenetic analysis for clade designation) as was the Harpending's raggedness index of mismatch distribution (*r*) (Rogers and Harpending, 1992; Harpending, 1994). Differentiation between populations (FST) was estimated and the value was used to infer *Nm* (number of migrant/generation) in order to estimate gene flow between

populations (Hudson et al., 1992). The statistical significances of all tests performed with DnaSP were estimated by the coalescent method (with 95% confidence interval) with 5000 permutations.

Microsatellite analysis of our populations: Genic and genotypic differentiations between populations and between groups of populations were evaluated in GenePop (10,000 iterations). In addition, FST (genetic structure based on allele frequencies) and their significance values were also evaluated by using Arlequin 3.5 (Schneider et al., 2000; Excoffier and Lischer, 2010).

CHAPTER 3

Results

Sampling: A total of 323 ticks was collected from 20 locations among 15 states (Figure III, Table V). Regional distribution was as follows: north coastal (106), north inland (24), south coastal (125), and south inland (68). All ticks were identified as *I. scapularis*. The sampling included 24.5% of larvae, 10.5% of nymphs, 29.4% of males, and 35.6% of females (Table V).

Microsatellite development: Of the 22 primer pairs selected prior to this study, 15 successfully cross-amplified loci of at least one other species (Table III). All, but one of the 15 primer sets, were able to amplify both *I. scapularis* and *I. persulcatus* DNA. In addition, more than half of these primer sets succeeded in amplifying DNA from *I. pacificus* (eight loci) or *I. ricinus* (nine loci). When used to amplify the corresponding loci in the 65-test sample, Genepop revealed the occurrence of significant ($0.01 < p < 0.05$) linkage disequilibrium between loci 1 and 3, 3 and 16, 15 and 16, 16 and 17, and 15 and 19. Allele diversity was important in all loci and varied from 6 to 26 different alleles/loci (average of 14.91). No loci were at HWE as there was a significant heterozygous deficit encountered for each locus in the whole population and in the ticks belonging to the American clade. Within the southern clade there was a significant heterozygous deficit in all but locus 3 and locus 18. The estimation of genotype amplification failure rate was found to be non-significant.

DNA extraction, PCR, sequencing, and genotyping: The DNA of 323 ticks was extracted and successfully PCR-amplified for 12SrDNA and D-Loop. Both strands of each amplicon were sequenced and assembled unambiguously into contigs. Success rate for genotyping of resulting amplicons for the eight selected microsatellite markers varied according

to locus (percent success): locus 1 (100%), locus 3 (100%), locus 11 (97.8%), locus 15 (96.6%), locus 16 (99.7%), locus 18 (99.1.%), and locus 19 (98.1%).

Haplotype diversity:

12SrDNA: The alignment of the 12SrDNA sequences (323 total) resulted in a matrix of 356 characters. The matrix included a high proportion of sequences that differed from each other by single mutations (often indels). The alignment was reduced to a total of 104 unique haplotypes for phylogenetic analysis and 60 haplotypes for DnaSP analyses (gaps eliminated) (Table VI). The overall haplotype diversity was high (0.704 ± 0.03) as was the number of segregating sites (122). The average pairwise distance, however, was low (0.018). Appendix A shows the geographic distribution of the 12SrDNA haplotypes. Distribution of 12SrDNA haplotypes unique to each region is as follows: north coastal (33), north inland (6), south coastal (64), and south inland (21). Identical haplotypes found in multiple regions included: 7, 8, 15, 17, 22, 26, 28, 37, 39, 40, 58, and 87. Only three haplotypes were found in all four regions: 17, 26, and 28. The two most common haplotypes were 28 and 16, with 56 sequences distributed among all four regions and 17 sequences from south coastal states, respectively.

D-Loop: The alignment of the D-Loop sequences (323 total) resulted in a matrix of 521 characters. The alignment was reduced to a total of 155 unique haplotypes for phylogenetic analysis and 110 haplotypes for DnaSP analyses (gaps eliminated) (Table VII). The overall haplotype diversity was high (0.973 ± 0.004), as was the number of segregating sites (110). The average pairwise distance was low (0.029), but higher than that found in 12SrDNA analyses. Appendix B shows the geographic distribution of the D-loop haplotypes. Distribution of haplotypes unique to each region is as follows: north coastal (32), north inland (11), south coastal (63), and south inland (34). Identical haplotypes found in multiple regions included: 7,

26, 29, 43, 45, 47, 49, 57, 65, 66, 83, 85, 95, and 104. Only haplotype 43 was found in ticks collected from all four regions. The two most common haplotypes were 29 and 139, with 28 sequences from north coastal, north inland and south inland states and 14 sequences from south coastal states, respectively.

Phylogenetic analyses:

12SrDNA: Phylogenetic reconstructions were inferred by using ML (Figure IV). The 12SrDNA ML model best fitting the data selected by PhyML (Guindon and Gascuel, 2003) v3.0 was the Hasegawa-Kishino-Yano, 85 model (HKY85) with base frequencies of A = 0.40, C = 0.08, G = 0.12, and T = 0.40; transition/transversion rate = 3.562; and the proportion of invariable sites was 0.364, with equal rates. Among the outgroup taxa *I. affinis* appeared to be more closely related to *I. persulcatus* than to other species. All *I. scapularis* haplotypes were clustered in a well-supported, polytomic clade (bootstrap value (BS) 98%). The four unranked clades arising from the polytomy were all monophyletic. Based on branch length, clade *South III* (BS 100%) can be considered basal and consisted of strictly south coastal sequences. Two other monophyletic strictly southern clades arose from the polytomy: *South II* (BS 90%) and *South I* (BS 98%). The remaining and most recently evolving lineage was monophyletic (96%), and included a number of unranked lineages. Nevertheless, one of the groups (*American II*), weakly supported, included samples that are exclusively southern, unlike the rest of the cluster represented by samples collected throughout the distribution area of *I. scapularis.*

D-Loop: The D-Loop ML model best fitting the data by use of PhyML v3.0 (Guindon and Gascuel, 2003) was the generalised time reversable model (GTR) with base frequencies of A = 0.39, C = 0.10, G = 0.08, and T = 0.43; and the proportion of invariable sites was 0.368, with equal rates. The relative rate parameters for substitution were: A⇔C (0.47089), A⇔G

(5.04877), A⇔T (1.38835), C⇔G (0.25987), C⇔T (4.23489), and G⇔T (1.00000). All *I. scapularis* haplotypes were clustered in a well-supported, monophyletic clade (BS 100%) (Figure V). Once again, the ingroup was a polytomy including 4 well-supported lineages. The three southern clades were well resolved with bootstrap values of 92 % (*South I*), 100% (*South II*), and 100% (*South III*). *South III* consisted of only south coastal ticks (BS 100%). *South I* consisted of only south coastal ticks, whereas *South II* contained both south coastal and south inland ticks. The American clade was supported by 84% bootstrap value. Within the lineage, samples from diverse geographic origins were paraphyletic, while one clade containing only southern ticks (*American II*) distinguished itself clearly from everything else (BS 95%). Overall, the topologies of the D-Loop and the 12SrDNA phylogenetic trees were very similar. Samples assigned to the three main southern clades by 12SrDNA analysis were identically separated into three well-supported lineages in the D-Loop reconstruction.

Concatenated 12SrDNA and D-Loop sequences: The 12SrDNA/D-Loop matrix included 323 sequences (865 bp) corresponding to 213 unique haplotypes. The 12SrDNA/D-Loop ML model best fitting the data by use of PhyML v3.0 (Guindon and Gascuel, 2003) was the HKY85 model with base frequencies of A = 0.40, C = 0.09, G = 0.10, and T = 0.42; transition/transversion rate = 4.110; and the proportion of invariable sites was 0.474, with equal rates. The total evidence tree (Figure VI) was characterized by overall better resolution than the trees generated by analyzing genes separately. The ingroup was clustered in a well-supported, monophyletic clade (BS 100%). This clade was divided into two monophyletic and well-resolved sister groups: a lineage clustering all southern clades (BS 90%) and one clustering both American clades (BS 100%). *South I*, *South II*, and *South III* were all well supported, with BS values of 90%, 70%, and 100%, respectively. *South I* and *South II* were more closely related to each other than to

South III, and clustered in a monophyletic group (BS 80%). Within *South III*, a polytomic group characterized by short terminal branches diverges from the basal lineages. Within the American clade, *American I* was paraphyletic, while *American II* was placed in a basal position and supported by 100% BS.

TCS1.21 analyses:

12SrDNA: TCS1.21 generated four separate networks for 12SrDNA haplotypes (Figure VII): a combined *American I* and *American II* containing ticks from all four regions, *South I* containing ticks from the south coastal region, *South II* containing ticks from the south coastal and south inland regions, and *South III* containing ticks from the south coastal region. The networks remained separated even when the parsimony level cutoff was set to 90%, indicating that these lineages are distinct and that mitochondrial gene flow between them is reduced. The *American I* network was characterized by the presence of large loops connecting the predominant haplotypes. The number of mutations between most haplotypes was very low (1-2 bp, often represented by indels), particularly around the main haplotype (56 samples). Haplotypes were separated by a maximum of 17 steps. Only five specimens were represented in the *South I* network. The *South II* network had a linear structure, with haplotypes separated by up to 13 mutations. The *South III* network was represented by a predominant haplotype (13 samples) linked to other haplotypes by a large loop, and by few mutation steps on each branch.

D-Loop: TCS1.21 generated five phylogenetic networks for D-Loop haplotypes (Figure VIII): *American I* containing ticks from all four regions, *American II* containing ticks from south coastal, north inland, and south inland regions, *Southern I* containing ticks from the south coastal region, *Southern II* containing ticks from the south coastal and south inland regions, and *South III* containing ticks from the south coastal region. The D-Loop networks indicated a similar

pattern of relationships between individuals to that of 12SrDNA (Figure VII). However, with the D-Loop sequences, *American II* was separated from the *American I* network. The five networks remained separated even when the parsimony level cutoff was set to 90%, also indicating strong genetic differentiation and little genetic exchange. In addition, the *American I* network identified a number of clusters along its linear backbone. The number of mutations between most haplotypes in *American I* is higher for this mitochondrial marker than for 12SrDNA with up to 35 mutation steps. The *American II* network consisted of a linear structure with haplotypes separated by up to 12 mutations. The *South I* network was represented by only six specimens yet, it exhibited a linear structure with haplotypes separated by up to ten mutations. The *South II* network also had a linear structure, with haplotypes separated by up to 12 mutations. The *South III* network had a linear structure with a star formation around its main haplotype (14 samples), and with a maximum of ten mutations between haplotypes.

Haplotype demographic parameters:

12SrDNA: Overall the significant values revealed by all neutrality tests indicated that *I. scapularis* had undergone recent population growth (Table VI). When considering *South I, South II*, and combined *South I + II + III* separately, the non-significant neutrality tests are indications of relatively stable and older populations. *South III,* unlike the other southern populations, also showed signs of population growth. Not all neutrality tests reached the same conclusions about the *American* populations. Nevertheless, they consistently revealed population expansion for *American I.* Mismatch distribution (population expansion model) resulted in non-significant values for the raggedness index (*r*) for all populations. However, mismatch analysis graphs representing 12SrDNA haplotype frequency over pairwise differences, showed a unimodal distribution for the combined *American* populations consistent with population expansion

(Figure IXa) and a bimodal distribution for the combined southern populations (Figure IXb) which is indicative of older and stable populations.

D-Loop: Neutrality tests for the DL gene gave contradicting evaluations of the demographic history of the different populations. Nevertheless, the tests were significant for *American I* and indicated, as with the 12SrRNA analysis, that this particular population underwent population expansion. Raggedness indices (*r*) were all non-significant. However, the mismatch distribution graphs showed that the American clade was unimodal (Figure IXc), although not as clearly as for 12SrDNA, while the southern clade is markedly bimodal (Figure IXd).

Genetic differentiation:

12SrDNA: Differentiations between all populations (FST) were highly significant (Table VIII). FST values ranged from 0.39 to 0.95. The lowest value (0.39) was observed between *American I* and *American II*. The number of migrants per generation (Nm) was consistently low (0.03 to 0.78), with the highest value (0.78) found between *American I* and *American II*.

D-Loop: Genetic differentiation (FST) was highly significant between all populations (Table IX). FST values varied between 0.62 and 0.86 and Nm values varied between 0.10 and 0.31. As for the 12SrDNA analysis, the lowest FST value (FST = 0.62) and the highest Nm (Nm = 0.31), were recorded between *American I* and *American II*.

Microsatellites: Estimations of FST (Arlequin - Schneider et al., 2000; Excoffier and Lischer, 2010) yielded significant values between *American I* and all other populations (Table X). Contradictory to our prior analyses there was a lack of significance in the differentiation between *American II* and *South II*, as well as, between all southern populations.

CHAPTER 4

Conclusions

The main focus of this study was to examine the genetic structure of *I. scapularis* through more extensive sampling of the distribution range and by using different molecular tools. We also wanted to compare our results to previously published data on genetic diversity of *I. scapularis* (Wesson et al., 1993; McLain et al., 1995; Rich et al., 1995; Norris et al., 1996; Qiu et al., 2002). A total of 15 states was included in our study, representing all four regions within the *I. scapularis* distribution. We were unable to collect ticks from the southwestern-most states (Texas and Oklahoma) but our sampling covered most of the other areas where the tick is known to occur. To account for within and between site genetic variability, adequate sampling was necessary. The number of ticks collected in previous studies varied in sample size: 18 (Wesson et al., 1993), 26 (Rich et al., 1995), 196 (Norris et al., 1996), and 650 (Qiu et al., 2002). Although we tried to include as many specimens from each state as possible, samplings of some locations were limited due to differences in population density of questing ticks and number of times visited. Nevertheless, our sample (323 ticks) was larger than any sample considered in previous publications, with the exception of Qiu's (2002) study, which included 650 ticks. These, however, had been collected along the Atlantic coast and did not represent inland populations.

From the 323 ticks sequenced, a total of 104 and 155 unique haplotypes (indels included), were identified for 12SrDNA and D-Loop, respectively. These numbers were higher than those found in previous studies of *I. scapularis* involving 16SrDNA, a gene located close to 12SrRNA on the mitochondrial genome (11 haplotypes from 198 ticks throughout the distribution, Norris et al., 1996; 10 haplotypes from 650 ticks along the Atlantic Coast, Qiu et al., 2002). This difference could be due to a variety of factors, including the fact that 16SrDNA gene sequences

have lower mutation rates than 12SrDNA sequences (Norris et al., 1996). Indels may have been eliminated in other studies, whereas we counted each nucleotide difference at least for phylogenetic and TCS analyses. Our sample covered geographic areas not included in some of the previous studies. Also, we also obtained ticks from different sources and collected at different times while other authors may have obtained genetically more homogeneous samples from identical localities.

All of our ML phylogenetic reconstructions (12SrDNA, D-Loop, and concatenated 12SrDNA/D-Loop) support prior findings that indicated the existence of a well-supported American and a well-supported southern clade in *I. scapularis* (Wesson et al., 1993; Rich et al., 1995; Norris et al., 1996). The ML phylogenetic reconstruction for concatenated 12SrDNA/D-Loop provided the best, overall lineage resolution in comparison to the separate gene analyses. The southern clades contained samples collected in the South only and were found in a basal position in all trees, confirming the hypothesis by Qiu et al. (2002) that *I. scapularis* originated in the South. Unlike previous reconstructions, our phylogenetic analyses showed that the two monophyletic groups were further subdivided in distinct clades, varying in haplotype composition and regional distribution. The monophyletic southern clade included three ranked and well-resolved lineages: *South I*, *II*, and *III. South III* also contained a polytomy of unranked branches, whereas *South I* and *South II* appeared to be more structured. In the TCS minimum spanning network of *South III*, the star-shaped cluster corresponds to the polytomy found in the phylogenetic analysis. This type of structure indicates events of rapid radiation and population expansion. The neutrality tests confirmed these finding by providing a clear signature for population growth in *South III*, while indicating a comparatively more stable demographic history for *South II and South I* which evolved neutrally. The subdivision within the American

clade consistently revealed the occurrence of a paraphyletic *American I* lineage, which included a very large number of genetically, relatively homogeneous haplotypes collected throughout the distribution range of *I. scapularis*. The 12SrDNA network structure of this group of sequences was characterized by large loops joining the same two haplotypes through alternative paths differing greatly in the number of steps, which is typical of a young and recently radiating population. In this case, most nucleotide mutations are not yet fixed and are mostly represented by non-informative unique character changes (singletons). The relatively small number of mutations separating most haplotypes was also typical of an evolutionarily young assemblage. Nevertheless, by using D-Loop sequence, the *American I* network appeared to be more structured with clearly identifiable groups of haplotypes. The clusters, however, did not correspond to any geographic subdivision, as each of them contained sequences from all sampled areas. Therefore, if the observed clusters correspond to sequential evolution of new haplotypes along the linear backbone of the network, responding to adaptation to new environments, the haplotypes must have been subsequently reshuffled by migratory events (carried by birds or on deer). The hypothesis that *American I* is a recently expanding population is further confirmed by all neutrality tests. Our study identified an additional lineage within the *American* clade, *American II*, which only included sequences from specimens collected in the south, and more specifically in Florida, South Carolina, and Alabama. The basal position of *American II* within the American cluster, particularly well supported in the total evidence analysis, would provide additional evidence for a southern origin for the American clade, as hypothesized by Qiu et al. (2002) Overall, our work is showing that genetic diversity in the South is more important than previously assumed (Wesson et al., 1993; Rich et al., 1995; Norris et al., 1996; Qiu et al., 2002). Our ingroup was also involved in population growth, probably spearheaded by *American I.*

Although, different neutrality tests reached different conclusions, it has been shown that Fu's *FS* and *R2* statistics are superior tests when compared to Tajima's *D* (Ramos-Onsins and Rozas, 2002). Furthermore, evidence has confirmed that Fu's *Fs* test is more suitable for populations with large sample sizes, whereas *R2* performs best when used to test for neutrality in smaller populations (Ramos-Onsins and Rozas, 2002). This is consistent with the fact that neutrality is rejected by *R2*, but not by Fu's *Fs* in the combined American population (a comparatively large population). The mismatch distribution analyses of our 12S and D-Loop haplotypes also indicated that the combined *American I + II* clade is undergoing expansion, while the more genetically structured *South I + II + III* clade is older and more stable. Haplotype statistics on *South I* have to be considered very cautiously taking into account the very small sample size.

FST evaluations based on mitochondrial gene sequences strongly support the conclusions drawn from phylogenetic and TCS analyses, revealing highly significant genetic differentiation between the five groups. Interestingly, FST values between American and southern populations (0.72-0.95 for 12SrDNA and 0.75-0.84 for D-Loop) are similar to those recorded between southern clades (0.83-0.92 for 12SrDNA and 0.78-0.86 for D-Loop). This indicates that southern clades are as different among one another as they are from American clades. Branch lengths in the phylogenetic concatenated reconstruction are also longer among southern lineages than among American lineages.

In this study we also developed several polymorphic microsatellite primers for use in *I. scapularis*, some of which were also successful in amplifying the DNA of three other members of the *I. ricinus* complex (*I. pacificus*, *I. persulcatus*, and *I. ricinus*). The amplicons from the other taxa should be sequenced in order to verify whether or not they contain the same microsatellite locus. If the presence of microsatellite loci in the amplified fragments were

confirmed for all these additional species, these tools would show great potential for the study of their respective population structures. All loci were characterized by a large number of alleles indicating that all the markers were polymorphic. None of the 22 loci were at HWE and all showed a significant heterozygous deficit. Microsatellite homozygous excess has been observed in other tick species, and was usually explained by the occurrence of null alleles (De Meeûs et al., 2002; De Meeûs et al., 2004). In our case, homozygous excess is unlikely to be due to PCR issues such as the non-amplification of some alleles, because it is doubtful that all loci be diagnosed with from the same issue. Furthermore, an estimation of genotype amplification failure rate also confirmed that presence of null alleles was not the reason for homozygous excess. Homozygous excess can be interpreted in different ways, but usually it is an indication of either inbreeding or the Wahlund effect (Wahlund, 1928). The Wahlund effect (the occurrence of previously undetected genetically distinct sub-populations within a population) could apply to the southern clade, while inbreeding may be a more likely cause for homozygous excess in the *American I* population. The Wahlund effect was found to be responsible for homozygous excess in other tick species (Chevillon et al., 2007; Kempf et al., 2009a; Kempf et al., 2010). Additional tests will be needed to determine which of these may be at play in our populations.

Our findings showed that when using mitochondrial genes, the phylogenetically identified clades are genetically strongly differentiated with very significant FST values. The southern lineages are as distinct from each other as from the American lineages. Nevertheless, microsatellite data did not fully agree with the mitochondrial results. Results from Wright's F-statistics showed no significant genetic differentiation between groups within the southern clade and significant differentiation between American and southern clades (with the exception of *American II* and *South II*). Therefore, it appeared that when using nuclear markers, the southern

lineages were more homogeneous than revealed by mitochondrial analysis. The *American I* group remained well separated from everything else, while *American II* seemed to maintain some gene flow, at least with *South II.*

In summary, our study revealed the occurrence of important genetic structure within *I. scapularis*, a genetic structure maintained sympatrically in some southern localities where the five mitochondrial clades can be collected simultaneously. The reasons for this genetic subdivision may be found in host race formation or assortative mating, as was shown for other tick species (Oliver et al., 1993; McCoy and Tirard, 2002; Chevillon et al., 2007; Kempf et al., 2009a; Kempf et al., 2009b; De Meeûs et al., 2010; Kempf et al., 2011). Nevertheless, as the mitochondrial and the nuclear analyses revealed slightly different patterns, further analyses will be needed in order to evaluate the extent of gene flow among all these populations. Even so, all analyses appeared to indicate that *American I* is distinct from everything else, which would indicate that the identification of *I. dammini* as a separate species from *I. scapularis* (Spielman et al., 1979; Wesson et al., 1993; Rich et al., 1995), albeit based on incomplete and incorrect geographic assumptions may actually have been valid. Laboratory colonies of each mitochondrial clade have now been established and will help to determine, through cross-breeding experiments and morphological re-analysis, whether or not the *American I* population really corresponds to a separate species (*I. dammini*).

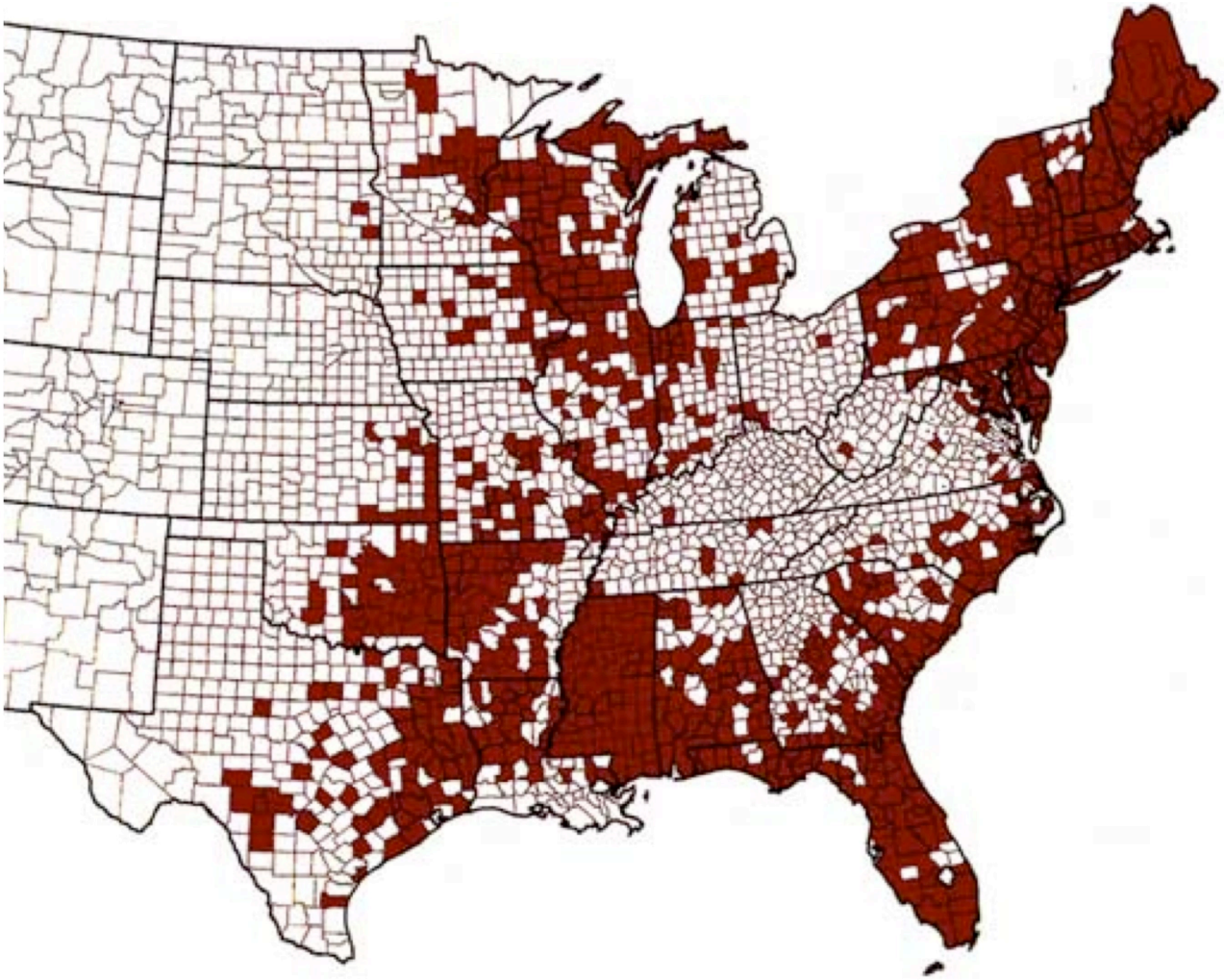

Figure I: Distribution of *I. scapularis* in the United States. Areas in brown represent counties where *I. scapularis* is either established or reported to occur <http://wildlifehealth.tennessee.edu/lyme_gradient> (September 2009).

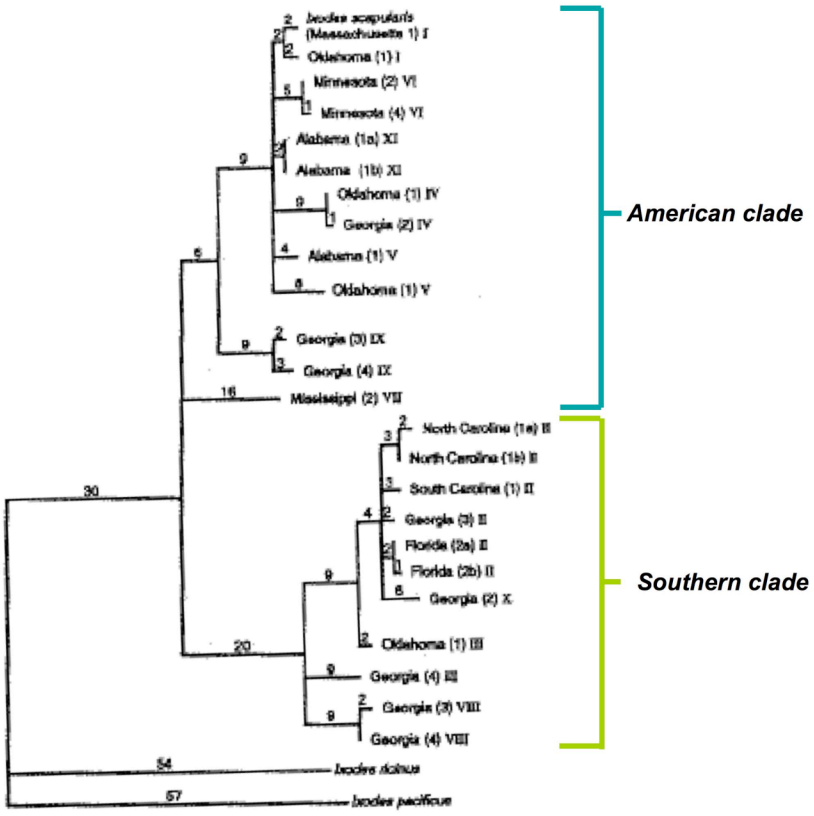

Figure II: Phylogenetic tree produced from combined 16S and 12S mitochondrial sequence data. A phylogram derived from maximum parsimony analysis. Branch lengths are proportional to the number of substitutions. As adapted from the results of Norris et al. (1996).

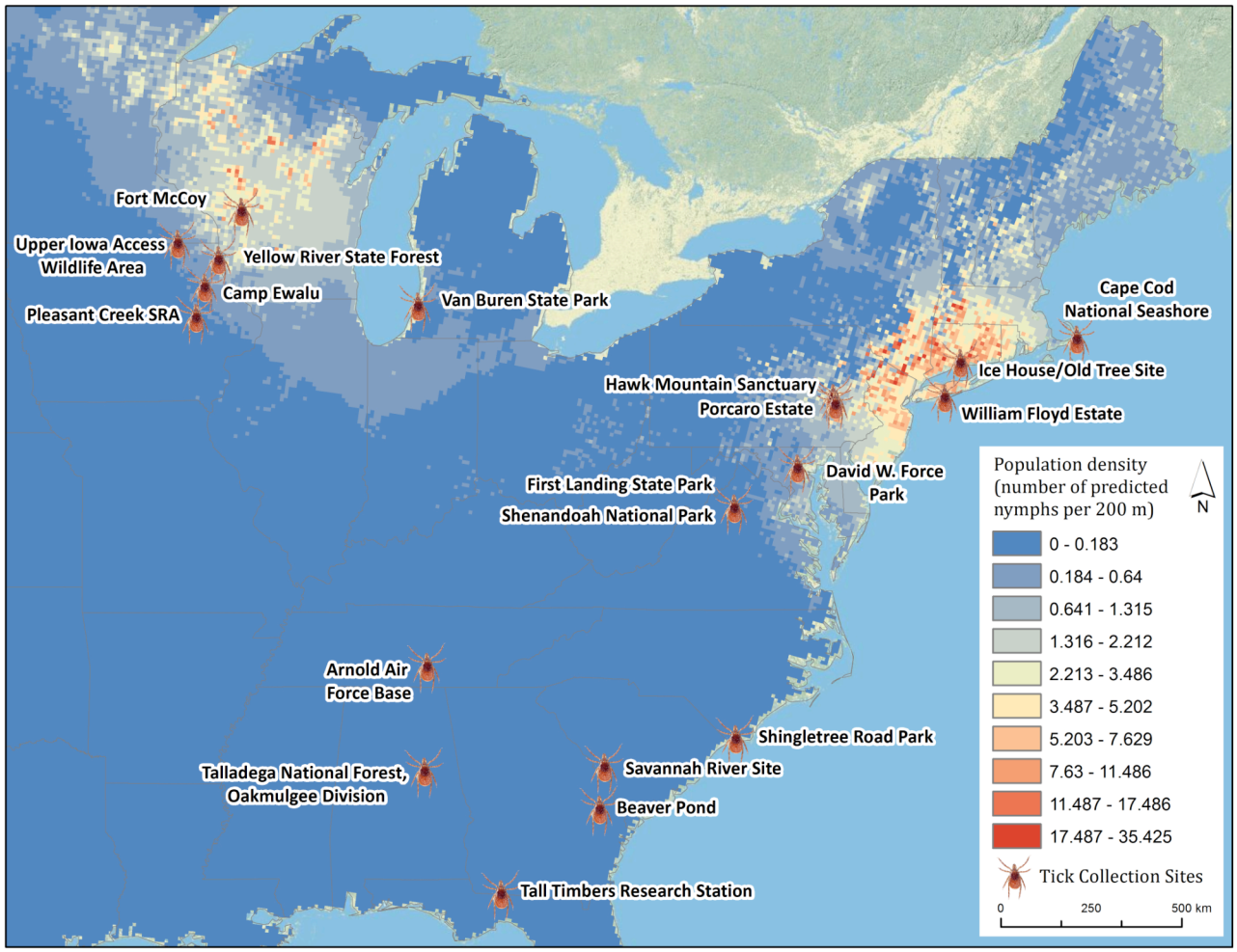

Figure III: Map of tick collection locations used for this project depicted relative to a field sampling/climate-based model of nymphal population density (Diuk-Wasser et al., 2010). Created by Jamie M. Kass (2011).

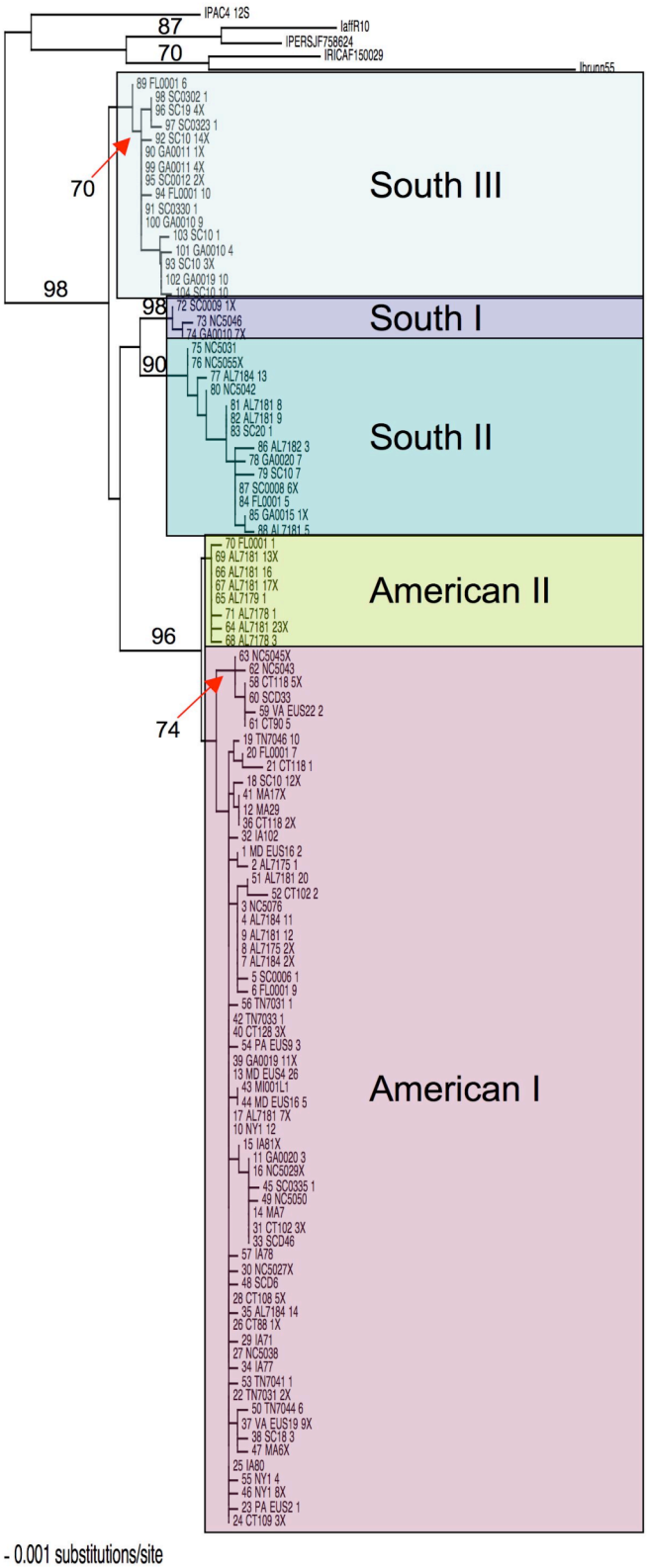

Figure IV: A phylogenetic tree inferred from maximum likelihood of the 12SrDNA gene fragments in studied populations of *I. scapularis*. Bootstrap values of significance are indicated to the left of branches.

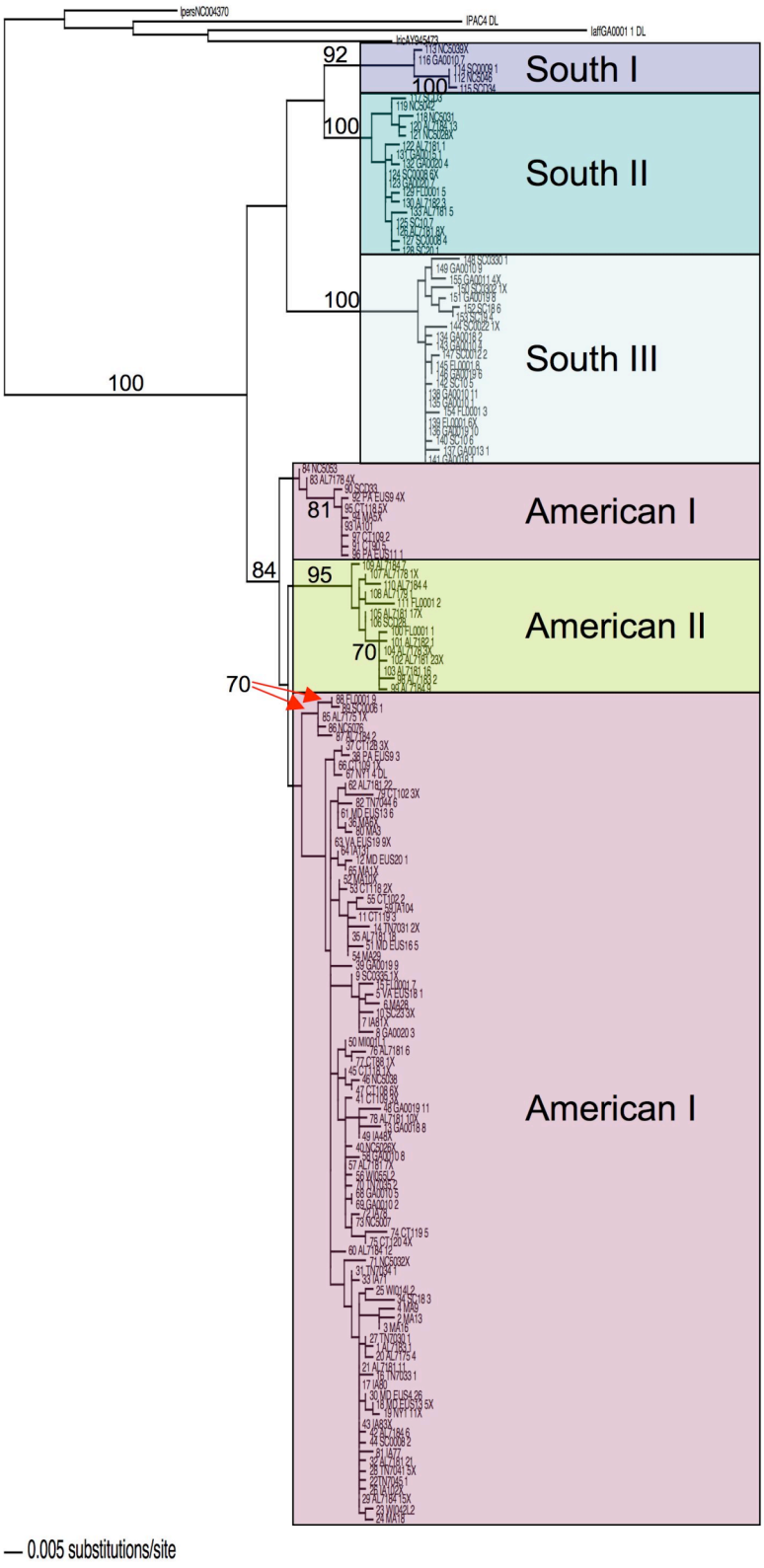

Figure V: Phylogenetic tree inferred from maximum likelihood of the D-loop gene fragments in studied populations of *I. scapularis*. Bootstrap values of significance are indicated to the left of branches.

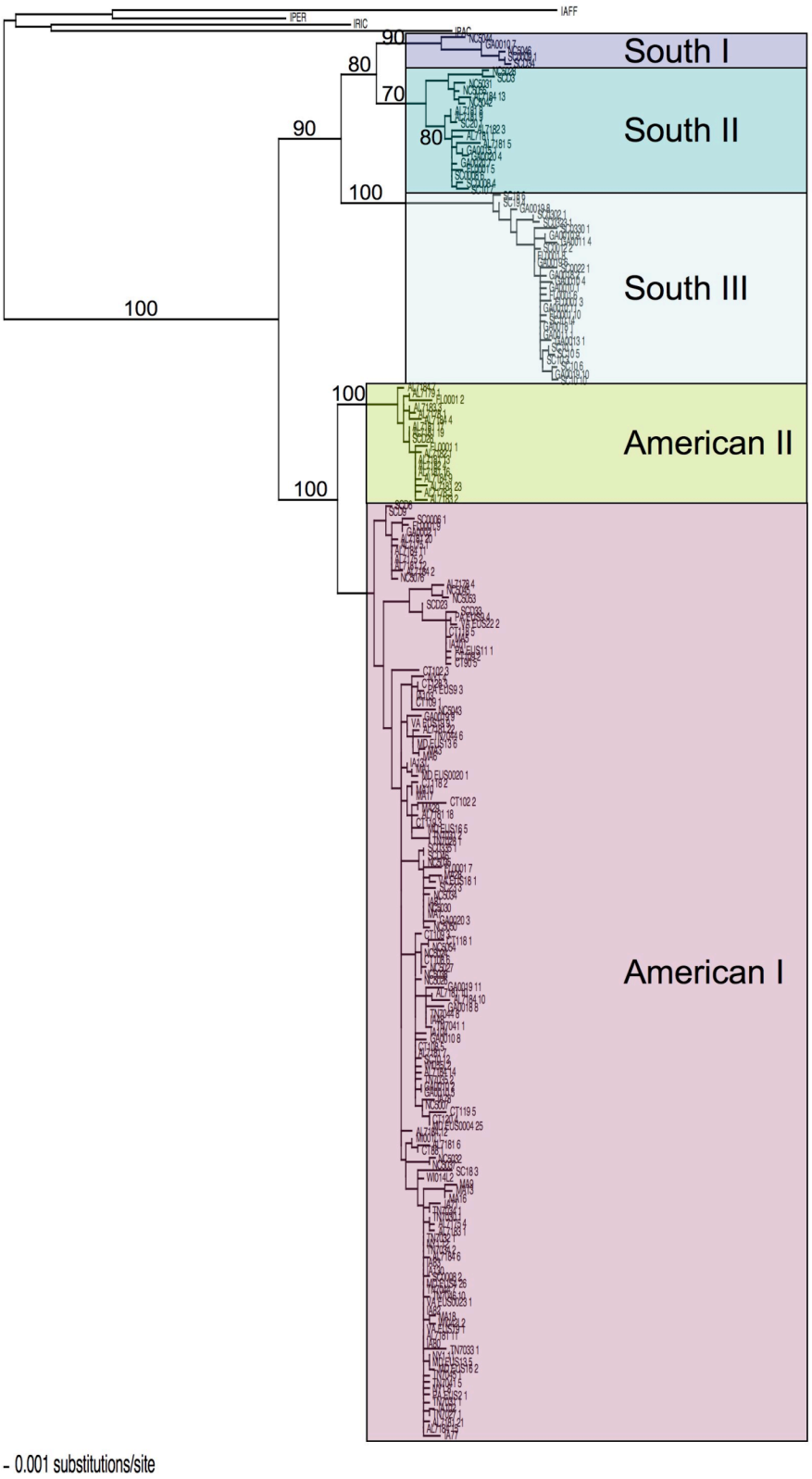

Figure VI: Phylogenetic tree inferred from maximum likelihood of the concatenated 12SrDNA/D-loop gene fragments in studied populations of *I. scapularis*. Bootstrap values of significance are indicated to the left of branches.

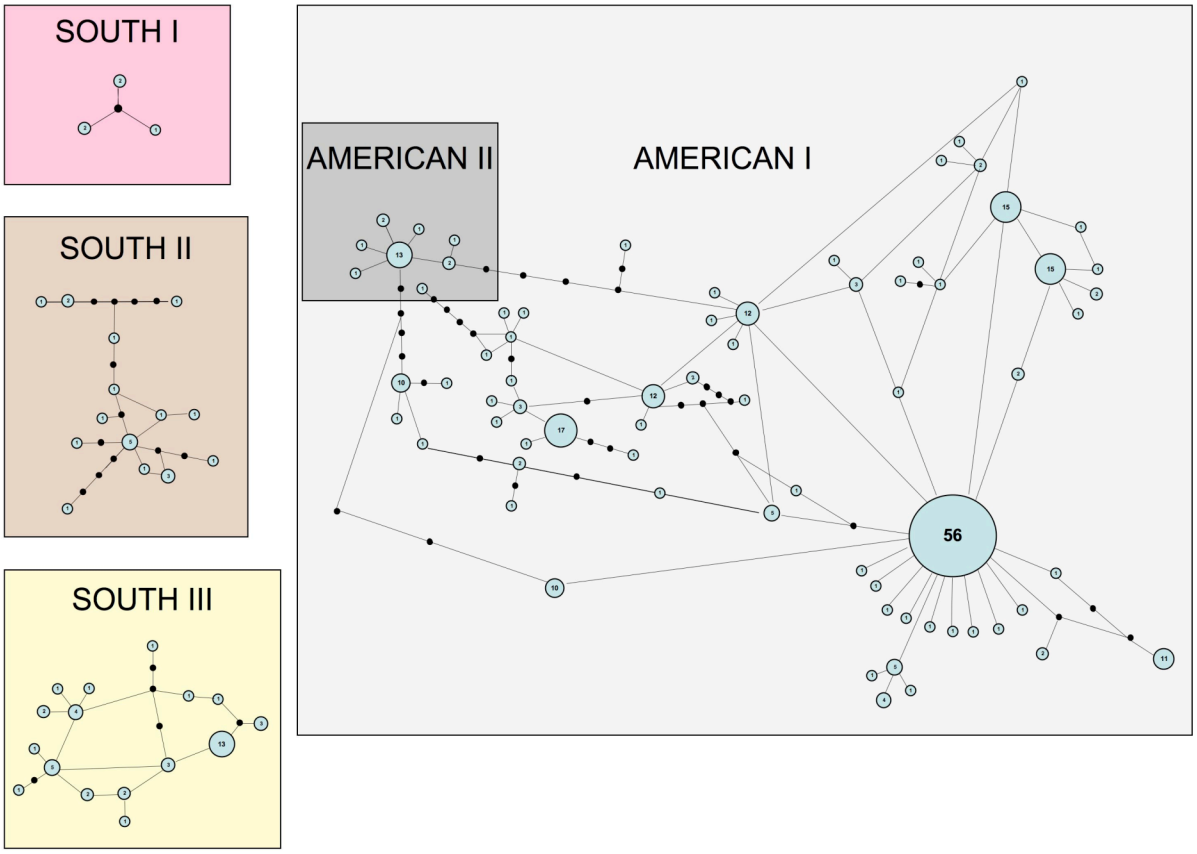

Figure VII: Unrooted minimum spanning networks with a 97% confidence interval of the *I. scapularis* 12SrDNA haplotypes, with numbers of identical sequences represented in each circle. Lines represent single mutations and dots represent unsampled intermediate haplotypes. Designations of clades are also noted.

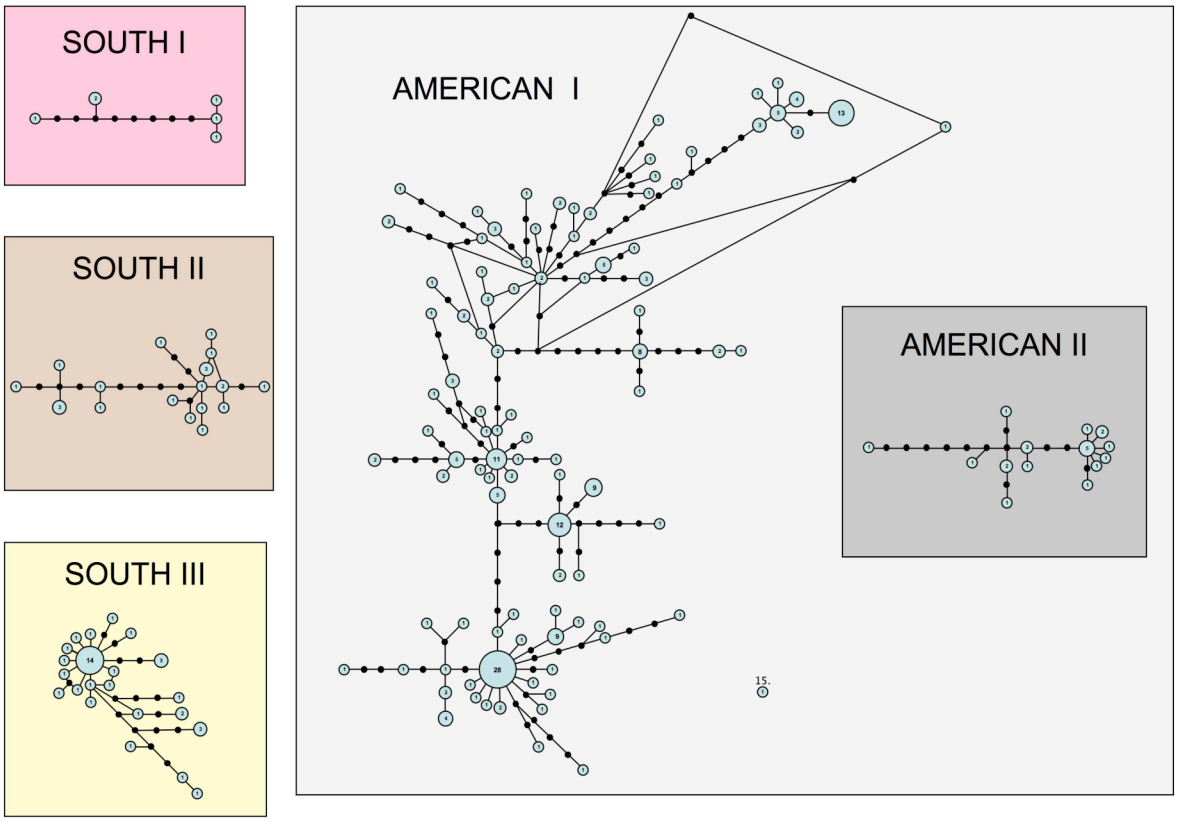

Figure VIII: Unrooted minimum spanning networks with a 95% confidence interval of the *I. scapularis* D-Loop haplotypes, with numbers of identical sequences represented in each circle. Lines represent single mutations and dots represent unsampled intermediate haplotypes. Designations of clades are also noted.

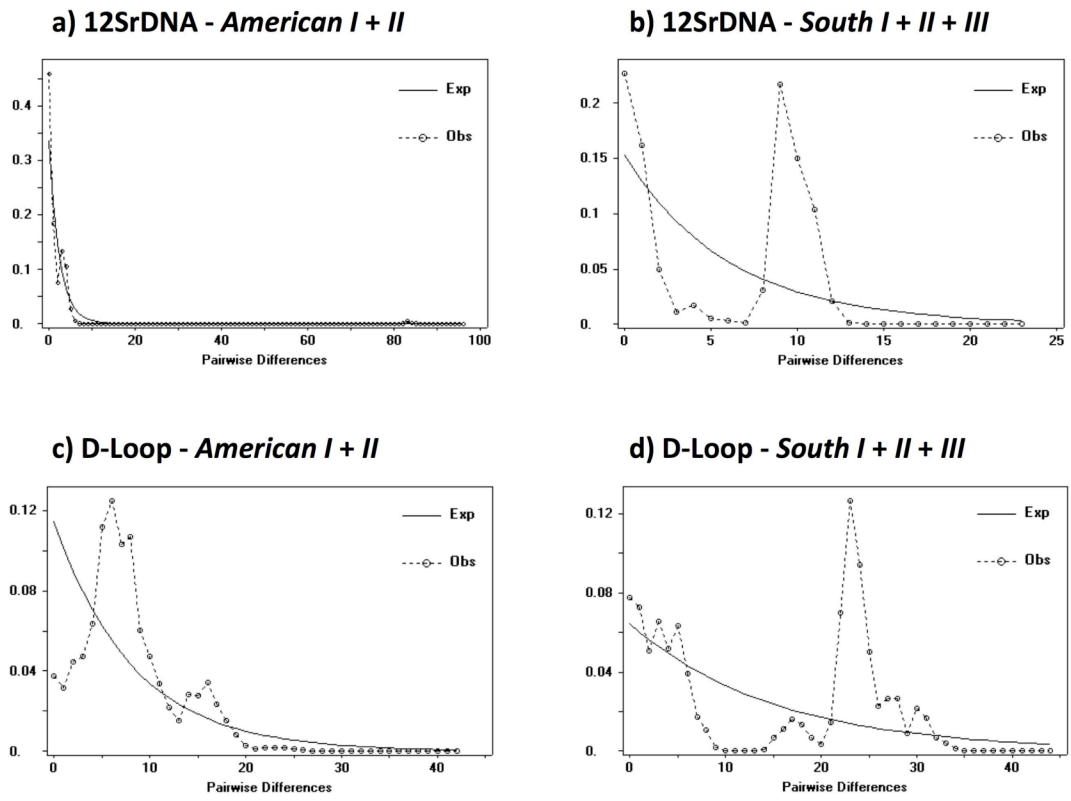

Figure IX: Mismatch distributions obtained from 12SrDNA gene sequence data for the American clade (*American I* and *American II*) (a) and southern clade (*South I*, *South II*, and *South III*) (b); and D-Loop gene sequence data for the American clade (c) and the southern clade (d). Expected distribution under the sudden expansion model and observed pairwise differences are as noted.

Table I: List of ticks used in this study, with locations of collection subdivided by: region, geographic coordinates of collection site, and source of ticks (✰ = obtained from researchers working together on NSF-EID Award EF-0914476; △ = specifically collected for this study; ♣ = obtained from Jon Oliver, Iowa State University).

Region	Location/State	Latitude	Longitude	No. of ticks	Source
North coastal	Ice House/Old Tree Site, CT	41.27944	-72.81555	31	L. Beati
	Cape Cod National Seashore, MA	41.71583	-69.92778	23	✰
	David W. Force Park, MD	39.29403	-76.88291	13	△
	William Floyd Estate, NY	40.76549	-72.83059	10	K. Kerr
	Hawk Mountain Sanctuary, PA	40.67023	-75.95793	3	△
	Porcaro Estate, PA	40.50328	-40.50328	9	△
	First Landing State Park, VA	36.90598	-76.01521	1	△
	Shenandoah National Park, VA	38.29276	-38.29276	16	△
North inland	Camp Ewalu, IA	42.67415	-91.59348	3	♣
	Pleasant Creek SRA, IA	42.11906	-91.80589	3	♣
	Upper Iowa Access Wildlife Area, IA	43.46802	-92.26184	10	♣
	Yellow River State Forest, IA	43.17183	-91.25136	1	♣
	Van Buren State Park, MI	42.33404	-86.30467	2	✰
	Fort McCoy, WI	44.03907	-90.67662	5	✰
South coastal	Tall Timbers Research Station, FL	30.65389	-84.22389	10	✰
	Beaver Pond, GA	32.44861	-81.78333	28	L. Durden
	Shingletree Road Park, NC	33.91195	-78.39564	30	M. Toliver
	Savannah River Site, SC	33.33223	-81.66362	57	✰
South inland	Talladega National Forest, AL	33.24386	-86.13447	44	✰
	Arnold Air Force Base, TN	35.37691	-86.07336	24	✰

Table II: Primers used for the amplification of mitochondrial gene sequences (Beati and Keirans, 2001; Beati et al., 2012).

Gene	Approx. length of PCR product	Primer name	Primer sequences (5' → 3')
12SrRNA	357 bp	T2A	F: AATGAGAGCGACGGGCGATGT
		T1B	R: AAACTAGGATTAGATACCCT
D-Loop	523 bp	D-Loop 3, 1X	F: TAACCGCTGCTGCTGGCACAA
		D-Loop 4, 1X	R: TAACCCTTTATTCAGGCAT

Table III: List of microsatellite loci used including: repeated motif, primer names and sequences for each locus, fluorescent tag used for amplification and genotyping, approximate expected size of the amplicons, number of observed alleles, observed heterozygosity (HO), expected heterozygosity (HE), statistical significance of the difference between expected and observed heterozygosity ($p < 0.001$ = highly significant), and results of cross amplification testing (PAC = I. pacificus, PER= I. persulcatus, RIC = I. ricinus).

Locus	Repeat array	Primer name	Primer sequences (5' → 3')	Dye	Size range (bp)	No. of alleles	HO	HE	p value	Cross-amplification
1	$(AG)_X$	Amy1-IsAG25a Amy2-IsAG25b	F: AAATGTCCGAACAGCCTTAT R: GCCCTTGAGTCTACCCACTA	6 FAM	93-193	17	25.00	58.92	< 0.001	PAC/PER/RIC
3	$(GTT)_5$	bac1d_a bac1d_b	F: GCAGATCTCTTGGGCTAG R: AAGCTAAGGCGTTCGTTG	VIC	76-100	7	29.00	38.72	< 0.001	
4	$(AT)_{21}$	bac1m_a bac1m_b	F: TGTCGGTTTGATGCCAA R: GGCTCCATTCACCAGTC	VIC	88-126	17	15.00	33.3	< 0.001	PAC/PER/RIC
5	$(CA)_9$	bac3dh_a bac3dh_b	F: TGCCTGTGACGAAACCA R: TCTCCCAAGAGATCTAGGTA	NED	62-140	17	14.00	43.95	< 0.001	
6	$(TA)_{10}$	bac1j_a bac1j_b	F: TCTCCCAAGAGATCTAGGTA R: ATCTGTTCAGTGGGCACA	VIC	100-186	13	27.00	52.72	< 0.001	PER
7	$(TA)_{11}$	bac1k_a bac1k_b	F: GGGACTGGACACACGA R: CTAGGTGGCGCAAGTC	VIC	48-170	26	29.00	47.53	< 0.001	
8	$(CA)_{14}$	bac3s_a bac3s_b	F: CGTTTCAAAGTCGGAGA R: GATGTGAGGGCGTGGT	PET	96-194	11	16.00	40.81	< 0.001	PER
9	$(AAAC)_5$	bac4cef_a bac4cef_b	F: CGCCTTTTGTCCCAACC R: GACTAACAGCATTGGAGCA	6 FAM	85-125	12	20.00	41.88	< 0.001	PER
10	$(TTA)_9$	bac5cf_a bac5cf_b	F: TCCCCCAACAAGATTGATG R: GAGACGACGTAGATTCTTG	6 FAM	77-137	15	16.00	48.77	< 0.001	
11	$(TTA)_6$	bac5g_a bac5g_b	F: GCTTTAGCGGGCTGGT R:TACGTGAATACGTCCTTGG	PET	81-165	12	26.00	58.39	< 0.001	PER
12	$(TA)_{43}$	bac6a_a bac6a_b	F: GCAAGCTTCGCTATTCTC R: CAGTAATTTCGCATCGGTT	6 FAM	111-229	26	10.00	35.26	< 0.001	
13	$(TA)_{22}$	bac6c_a bac6c_b	F: TAGGTACAAGAAAACGTGCT R: CAAGGTAATTGTTCTCGTCA	NED	37-91	17	30.00	55.93	< 0.001	
14	$(TA)_5$	bac6d_a bac6d_b	F: CCTTGCCTTACATGGTT R: CGTACCAAACCAAAGCAAG	HEX	57-105	13	15.00	42.02	< 0.001	PAC/PER/RIC
15	$(AT)_8$	bac6e_a bac6e_b	F: TATTGTAACCGACGCTAGG R: GACAATCTCTACGCAAATCC	NED	79-125	18	20.00	61.21	< 0.001	
16	$(CA)_8$	bac6f_a bac6f_b	F: CCCCCAAACACGCACA R: TTGCTTCATGCAGGGAAC	VIC	80-106	12	27.00	51.33	< 0.001	RIC
17	$(CA)_6$	bac7e_a bac7e_b	F: CCAGCATTTAACCCTCAAG R: TAGTGGGGTATGGCACTG	HEX	139-197	12	41.00	53.38	< 0.001	PER/RIC
18	$(TG)_6$	bac8a_a bac8a_b	F: GTAGGTACCCTAAGAAGGAT R: TTGAGGAAGCAGAATGTAGG	6 FAM	75-195	16	46.00	59.08	< 0.001	PER/RIC
19	$(CT)_7$	bac9a_a bac9a_b	F: AGAACCAGTTCAGCATTCC R: GAACATTTTCACGTGTTGC	PET	94-166	6	14.00	29.58	< 0.001	PER
20	$(GC)_9$	bac11a_a bac11a_b	F: CGCTCCCTTCGAAGTTC R: GAGAAGACAGTTTCCATCG	HEX	76-106	13	55.00	47.64	< 0.001	PAC/PER/RIC
21	$(ACG)_6$	bac11c_a bac11c_b	F: CGAATCGCGCACACTAG R: GCTGTGTTGCTGGTCAC	NED	109-251	14	40.00	49.21	< 0.001	PAC/PER
22	$(AC)_9$	ac18_a ac18_b	F: GATGAGTCCTGAGTAAAACACA R: CTTGCTGCGCCCATAGTCTC	VIC	110-136	12	25.00	43.65	< 0.001	PAC/PER/RIC
23	MIXED	e215_a e215_b	F: CCTTTCCTGGCCTTCTAATCC R: TCCTCTATGTCACCACCTAACCAG	6 FAM	76-121	22	24.00	55.09	< 0.001	PAC/PER

Table IV: PCR master mix solutions and thermocycler conditions for each of the markers used in this study (Beati and Keirans, 2001; Beati et al., 2012).

	12S		D-Loop		Microsatellite loci	
PCR master mix	dH2O	10.3 µL	dH2O	10.3 µL	dH2O	5.2 µL
	Taq buffer	2.5 µL	Taq buffer	2.5 µL	Taq buffer	1.3 µL
	Taq master enhancer	5 µL	Taq master enhancer	5 µL	Taq master enhancer	2.5 µL
	MgCl2	1.5 µL	MgCl2	1.5 µL	MgCl2	7.5 µL
	Primer T2A	1.25 µL	Primer DL3, 1X	1.25 µL	Forward primer	6.3 µL
	Primer T1B	1.25 µL	Primer DL4, 1X	1.25 µL	Reverse primer	6.3 µL
	dNTPs	0.5 µL	dNTPs	0.5 µL	dNTPs	0.3 µL
	Taq DNA polymerase	0.2 µL	Taq DNA polymerase	0.2 µL	Taq DNA polymerase	0.1 µL
	DNA sample	2.5 µL	DNA sample	2.5 µL	DNA sample	0.3 µL
Thermocycler program	5 Cycles of: Denaturation for 25sec at 94ºC Annealing for 35sec at 50ºC Elongation for 30sec at 68ºC 30 Cycles of: Denaturation for 25sec at 94ºC Annealing for 30sec at 53ºC Elongation for 30sec at 70ºC Final Elongation for 5min at 70ºC 4ºC ? End		8 Cycles of: Denaturation for 20sec at 93ºC Annealing for 25sec at 65ºC -1.5ºC/cycle Elongation for 45sec at 72ºC 10 Cycles of: Denaturation for 20sec at 93ºC Annealing for 30sec at 53ºC -0.4ºC/cycle Elongation for 45sec at 70ºC -0.2ºC/cycle 17 Cycles of: Denaturation for 20sec at 93ºC Annealing for 35sec at 51ºC Elongation for 40sec at 69ºC Final Elongation for 5min at 69ºC 4ºC ? End		5 Cycles of: Denaturation for 20sec at 93ºC Annealing for 20sec at 60ºC Elongation for 30sec at 72ºC 30 Cycles of: Denaturation for 20sec at 93ºC Annealing for 25sec at 50ºC Elongation for 30sec at 70ºC Final Elongation for 5min at 70ºC 4ºC ? End	

Table V: Total number of ticks by stage/sex and by geographic area (L=larva, N=nymph, M=adult male, F=adult female).

STATE	# OF TICKS				TOTAL/STATE
	L	N	M	F	
AL	0	0	24	20	44
CT	0	7	13	11	31
FL	0	0	4	6	10
GA	0	0	7	21	28
IA	0	14	3	0	17
MA	11	6	3	3	23
MD	3	2	2	6	13
MI	2	0	0	0	2
NC	0	0	8	22	30
NY	0	0	10	0	10
PA	3	4	5	0	12
SC	44	1	5	7	57
TN	0	0	8	17	24
VA	11	0	3	3	17
WI	5	0	0	0	5
TOTAL/LIFESTAGE	**79**	**34**	**95**	**116**	**323**

Table VI: Analyses of nucleotide polymorphism in 12SrDNA haplotype sequences (DnaSP - Rozas and Rozas, 1999; Librado and Rozas, 2009): n = number of samples, h = number of haplotypes, Hd = haplotype diversity, S = number of segregating sites, π = average pairwise distance, θ = population mutation rate, DT = Tajima's D, p (DT) = probability of DT, determined by coalescent simulation, p (FS) = probability of Fu's FS, r = Harpending's raggedness index for mismatch distribution, p (r) = probability of Harpending's raggedness index for mismatch distribution, R2 = Ramos-Onsin's & Rozas's R2, and p (R2) = probability of Ramos-Onsin's & Rozas's R2; significance levels: * = < 0.05; ** = < 0.01; *** = < 0.001 calculated by the coalescent method (95% confidence interval; 5000 replicates).

Population name	**n**	**h**	**Hd**	***S***	***π***	**θ**	**DT**	**p (DT)**	**Fu's FS**	**p Fu's FS**	**r**	***p* (r)**	***R2***	**p (R2)**
AM I	233	33	0.452±0.04	29	0.029	0.015	-2.215	***	-42.677	***	0.136	ns	0.017	***
AM II	23	6	0.395±0.13	87	0.023	0.071	-2.693	***	5.439	ns	0.179	ns	0.194	ns
AM I + AM II	256	39	0.541±0.04	107	0.006	0.056	-2.701	***	-30.696	**	0.098	ns	0.046	ns
SOUTH I	5	3	0.800±0.16	2	0.003	0.003	0.243	n/s	-0.475	ns	0.36	ns	0.25	ns
SOUTH II	21	10	0.886±0.05	10	0.008	0.008	-0.164	n/s	-3.088	ns	0.4	ns	0.124	ns
SOUTH III	42	8	0.452±0.09	7	0.002	0.005	-1.821	*	-6.165	*	0.12	ns	0.051	*
SOUTH I + II + III	68	21	0.773±0.05	27	0.016	0.017	-0.060	n/s	-2.697	ns	0.068	ns	0.098	ns
ALL	**324**	**60**	**0.704±0.03**	**122**	**0.018**	**0.065**	**-2.075**	***	**-21.902**	*	**0.058**	**ns**	**0.041**	*

.

Table VII: Analyses of nucleotide polymorphism in D-Loop haplotype sequences (DnaSP - Rozas and Rozas, 1999; Librado and Rozas, 2009): n = number of samples, h = number of haplotypes, Hd = haplotype diversity, S = number of segregating sites, π = average pairwise distance, θ = population mutation rate, DT = Tajima's D, p (DT) = probability of DT, determined by coalescent simulation, p (FS) = probability of Fu's FS, r = Harpending's raggedness index for mismatch distribution, p (r) = probability of Harpending's raggedness index for mismatch distribution, R2 = Ramos-Onsin's & Rozas's R2, and p (R2) = probability of Ramos-Onsin's & Rozas's R2; significance levels: * = < 0.05; ** = < 0.01; *** = < 0.001 calculated by the coalescent method (95% confidence interval; 5000 replicates).

Population name	**n**	**h**	**Hd**	***S***	**π**	**θ**	**DT**	**p (DT)**	**Fu's FS**	**p Fu's FS**	**r**	***p* (r)**	***R2***	**p (R2)**
AM I	232	85	0.955±0.007	89	0.013	0.031	-1.754	**	-75.671	***	0.009	*0.04	0.0346	**0.006
AM II	23	14	0.921±0.042	34	0.011	0.019	-1.551	*	-3.473	ns	0.018	ns	0.074	*0.02
AM I + AM II	255	99	0.962±0.006	98	0.016	0.033	-1.571	*	-82.261	***	0.007	*0.001	0.038	ns
SOUTH I	5	4	0.900±0.161	8	0.090	0.008	0.661	ns	0.212	ns	0.270	ns	0.197	ns
SOUTH II	21	15	0.952±0.032	31	0.011	0.018	-1.554	*	-5.826	* (0.016)	0.027	ns	0.101	ns
SOUTH III	42	18	0.962±0.060	42	0.010	0.020	-1.728	**	-4.288	ns	0.016	*0.04	0.055	**0.005
SOUTH I + II + III	68	37	0.922±0.062	55	0.313	0.024	0.877	ns	-4.799	ns	0.013	ns	0.128	ns
ALL	**323**	**136**	**0.973±0.004**	**110**	**0.029**	**0.042**	**-0.068**	**ns**	**-34.061**	**	**0.004**	**0.01**	**0.060**	**ns**

Table VIII: Measure of population differentiation based on 12SrDNA haplotype frequencies (FST), with values approaching 0 = identical populations and 1 = different populations; *** represent significance of FST < 0.001; estimation of gene flow is represented by number of migrants/generation (Nm) (DnaSP - Rozas and Rozas, 1999; Librado and Rozas, 2009).

FST(*p*)/Nm	AMI	AMII	SI	SII	ALLSOU
AMII	0.39***/0.78				
SI	0.95***/0.03	0.74***/0.18			
SII	0.91***/0.05	0.72***/0.20	0.83***/0.10		
SIII	0.94***/0.03	0.72***/0.19	0.92***/0.04	0.85***/0.09	
ALL AM					0.73***/0.40

Table IX: Measure of population differentiation based on D-Loop haplotype frequencies (FST), with values approaching 0 = identical populations and 1 = different populations; *** represent significance of FST < 0.001; estimation of gene flow is represented by number of migrants/generation (Nm) (DnaSP - Rozas and Rozas, 1999; Librado and Rozas, 2009).

FST(*p*)/Nm	**AMI**	**AMII**	**SI**	**SII**	**ALLSOU**
AMII	0.62***/0.31				
SI	0.82***/0.11	0.84***/0.10			
SII	0.75***/0.17	0.80***/0.12	0.78***/0.14		
SIII	0.79***/0.14	0.83***/0.10	0.86***/0.08	0.80***/0.13	
ALL AM					0.55***/0.40

Table X: Measure of population differentiation based on microsatellite allele frequencies (FST), with values approaching 0 = identical populations and 1 = different populations; significance values of FST: 0.001<**<0.01, 0.01<*<0.05.

FST(*p*)	**AMI**	**AMII**	**SI**	**SII**
AMII	0.021**			
SI	0.075**	0.068*		
SII	0.020*	0.015 ns	0.050 ns	
SIII	0.024***	0.024 **	0.051 ns	0.011 ns

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APPENDIX A:

Designation of 12SrDNA haplotypes according to clade, with state association and regional distribution.

Region	State	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30
North coastal	CT																	2				1	1		4		4		6		
	MA												1		1	1		1											9		
	MD	1												1											1		1		4		
	NY										1																		1		
	PA																							1			1		6		
	VA															1															
	TOT	**1**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**1**	**0**	**1**	**1**	**1**	**2**	**0**	**3**	**0**	**0**	**0**	**1**	**1**	**1**	**5**	**0**	**6**	**0**	**26**	**0**	**0**
North inland	IA															1		2								1	2		4	1	
	MI																												1		
	WI																										1		4		
	TOT	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**1**	**0**	**2**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**1**	**3**	**0**	**9**	**1**	**0**
South coastal	FL						1														1										
	GA							1	1			1						1											3		
	NC			1					1								5	1					1				1	1	2		2
	SC					1											12		2				2				3				
	TOT	**0**	**0**	**1**	**0**	**1**	**1**	**1**	**2**	**0**	**0**	**1**	**0**	**0**	**0**	**0**	**17**	**2**	**2**	**0**	**1**	**0**	**3**	**0**	**0**	**0**	**4**	**1**	**5**	**0**	**2**
South inland	AL		1		1			1	1	1								2					3				1		5		
	TN																	3		1			4				1		11		
	TOT	**0**	**1**	**0**	**1**	**0**	**0**	**1**	**1**	**1**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**5**	**0**	**1**	**0**	**0**	**7**	**0**	**0**	**0**	**2**	**0**	**16**	**0**	**0**
GRAND TOT		**1**	**1**	**1**	**1**	**1**	**1**	**2**	**3**	**1**	**1**	**1**	**1**	**1**	**1**	**3**	**17**	**12**	**2**	**1**	**1**	**1**	**11**	**1**	**5**	**1**	**15**	**1**	**56**	**1**	**2**

AMERICAN I

31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47	48	49	50	51	52	53	54	55	56	57	58	59	60	61	62	63	64	65	66
5					1				1												1						4			1					
					3					1						4											2								
						1			3				1																						
1					5										1									1											
									1														1				2								
5					1	2		1	5	1																		1							
11	**0**	**0**	**0**	**0**	**10**	**3**	**0**	**1**	**10**	**2**	**0**	**0**	**1**	**0**	**1**	**4**	**0**	**0**	**0**	**0**	**1**	**0**	**1**	**1**	**0**	**0**	**8**	**1**	**0**	**1**	**0**	**0**	**0**	**0**	**0**
	1		1						2																	1	1							
												1																							
0	**1**	**0**	**1**	**0**	**0**	**0**	**0**	**0**	**2**	**0**	**0**	**1**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**1**	**1**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**
						1		1																											
									3									1														1	2			
		1					1							1			1										1		1						
0	**0**	**1**	**0**	**0**	**0**	**1**	**1**	**1**	**3**	**0**	**0**	**0**	**0**	**1**	**0**	**0**	**1**	**1**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**1**	**0**	**1**	**0**	**1**	**2**	**0**	**0**	**0**
				1		1														1													2	1	1
											1								1			1			1										
0	**0**	**0**	**0**	**1**	**0**	**1**	**0**	**0**	**0**	**0**	**1**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**1**	**1**	**0**	**1**	**0**	**0**	**1**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**2**	**1**	**1**
11	**1**	**1**	**1**	**1**	**10**	**5**	**1**	**2**	**15**	**2**	**1**	**1**	**1**	**1**	**1**	**4**	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**10**	**1**	**1**	**1**	**1**	**2**	**2**	**1**	**1**

AMERICAN II					SOUTHERN I			SOUTHERN II														SOUTHERN III														
67	68	69	70	71	72	73	74	75	76	77	78	79	80	81	82	83	84	85	86	87	88	89	90	91	92	93	94	95	96	97	98	99	100	101	102
0	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**
0	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**
		2	1														1					1	1				1	1	1						
							1				1							2					8										3	1	1	1
						1	1	1	2				1					1					1													
		1			2							1				1				4			3	1	3	4		4	1	1	2				
0	**0**	**3**	**1**	**0**	**2**	**1**	**2**	**1**	**2**	**0**	**1**	**1**	**1**	**0**	**0**	**1**	**1**	**3**	**0**	**4**	**0**	**1**	**13**	**1**	**3**	**4**	**1**	**5**	**2**	**1**	**2**	**3**	**1**	**1**	**1**
3	1	10		1						1				1	1				1	1	1														
3	**1**	**10**	**0**	**1**	**0**	**0**	**0**	**0**	**0**	**1**	**0**	**0**	**0**	**1**	**1**	**0**	**0**	**0**	**1**	**1**	**1**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**
3	**1**	**13**	**1**	**1**	**2**	**1**	**2**	**1**	**2**	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**3**	**1**	**5**	**1**	**1**	**13**	**1**	**3**	**4**	**1**	**5**	**2**	**1**	**2**	**3**	**1**	**1**	**1**

103	104	TOT
		31
		23
		13
		10
		12
		17
0	0	106
		17
		2
		5
0	0	24
		11
		27
		30
2	1	57
2	1	125
		44
		24
0	0	68
2	1	323

APPENDIX B:

Designation of D-Loop haplotypes according to clade, with state association and regional distribution.

Region	State	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30
North coastal	CT											1																			
	MA		1	1	1		1	1																	1				4	
	MD												1						4											1
	NY																			1									1	
	PA																			1									4	
	VA					1																							5	
	TOT	**0**	**1**	**1**	**1**	**1**	**1**	**1**	**0**	**0**	**0**	**1**	**1**	**0**	**0**	**0**	**0**	**0**	**4**	**2**	**0**	**0**	**0**	**0**	**1**	**0**	**0**	**0**	**0**	**14**	**1**
North inland	IA							1										1								1			3	
	MI																													1	
	WI																							1		1				1	
	TOT	**0**	**0**	**0**	**0**	**0**	**0**	**1**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**1**	**0**	**0**	**0**	**0**	**0**	**1**	**0**	**1**	**1**	**0**	**0**	**5**	**0**
South coastal	FL															1															
	GA								1					1																	
	NC							7																							
	SC							3		2	9																				
	TOT	**0**	**0**	**0**	**0**	**0**	**0**	**10**	**1**	**2**	**9**	**0**	**0**	**1**	**0**	**1**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**
South inland	AL	1																			1	1								1	
	TN														2		1						1				1	1	2	8	
	TOT	**1**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**2**	**0**	**1**	**0**	**0**	**0**	**1**	**1**	**1**	**0**	**0**	**0**	**1**	**1**	**2**	**9**	**0**
GRAND TOT		**1**	**1**	**1**	**1**	**1**	**1**	**12**	**1**	**2**	**9**	**1**	**1**	**1**	**2**	**1**	**1**	**1**	**4**	**2**	**1**	**1**	**1**	**1**	**1**	**1**	**2**	**1**	**2**	**28**	**1**

AMERICAN I

31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47	48	49	50	51	52	53	54	55	56	57	58	59	60	61	62	63	64	65	66
						1				4				1		1						1		1		6								1
					3																4		1			1							1	
						3				1		1								1										1					1
												1									5															
						1	1																												
						1															1	1										2			
0	**0**	**0**	**0**	**0**	**3**	**6**	**1**	**0**	**0**	**5**	**0**	**2**	**0**	**1**	**0**	**1**	**0**	**0**	**0**	**1**	**10**	**2**	**1**	**1**	**0**	**7**	**0**	**0**	**0**	**1**	**0**	**2**	**0**	**1**	**2**
		1										2						2										1					1		1
																			1																
																		1							1										
0	**0**	**1**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**2**	**0**	**0**	**0**	**0**	**0**	**3**	**1**	**0**	**0**	**0**	**0**	**0**	**1**	**0**	**0**	**1**	**0**	**0**	**0**	**0**	**1**	**0**	**1**
								1									1										1								
									2					1	1	3																			1	1
			1									2	1													2									
0	**0**	**0**	**1**	**0**	**0**	**0**	**0**	**1**	**2**	**0**	**0**	**2**	**1**	**1**	**1**	**3**	**1**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**2**	**1**	**0**	**0**	**0**	**0**	**0**	**0**	**1**	**1**
	1			1							1															2			1		1				
1												3						2																		
1	**1**	**0**	**0**	**1**	**0**	**0**	**0**	**0**	**0**	**0**	**1**	**3**	**0**	**0**	**0**	**0**	**0**	**2**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**2**	**0**	**0**	**1**	**0**	**1**	**0**	**0**	**0**	**0**
1	**1**	**1**	**1**	**1**	**3**	**6**	**1**	**1**	**2**	**5**	**1**	**9**	**1**	**2**	**1**	**4**	**1**	**5**	**1**	**1**	**10**	**2**	**1**	**1**	**1**	**11**	**1**	**1**	**1**	**1**	**1**	**2**	**1**	**2**	**4**

67	68	69	70	71	72	73	74	75	76	77	78	79	80	81	82	83	84	85	86	87	88	89	90	91	92	93	94	95	96	97	98	99	100	101	102
							1	1		2		5												1				3		1						
													1														2								
								1																											
1												1																							
								1																	1				1						
												5													1										
1	**0**	**0**	**0**	**0**	**0**	**0**	**1**	**3**	**0**	**2**	**0**	**11**	**1**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**1**	**2**	**0**	**2**	**3**	**1**	**1**	**0**	**0**	**0**	**0**	**0**
					1									1												1									
0	**0**	**0**	**0**	**0**	**1**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**1**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**1**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**
																					1												1		
	1	1																1			1														
				2		1										1	1		1																	
																		2				1	1					2							
0	**1**	**1**	**0**	**2**	**0**	**1**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**1**	**1**	**3**	**1**	**0**	**2**	**1**	**1**	**0**	**0**	**0**	**0**	**2**	**0**	**0**	**0**	**0**	**1**	**0**	**0**
									1		2					1		5		1												1	1		1	2
			1												1																				
0	**0**	**0**	**1**	**0**	**0**	**0**	**0**	**0**	**1**	**0**	**2**	**0**	**0**	**0**	**1**	**1**	**0**	**5**	**0**	**1**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**1**	**1**	**0**	**1**	**2**
1	**1**	**1**	**1**	**2**	**1**	**1**	**1**	**3**	**1**	**2**	**2**	**11**	**1**	**1**	**1**	**2**	**1**	**8**	**1**	**1**	**2**	**1**	**1**	**1**	**2**	**1**	**2**	**5**	**1**	**1**	**1**	**1**	**1**	**1**	**2**

AMERICAN II									SOUTHERN I					SOUTHERN II																						
103	104	105	106	107	108	109	110	111	112	113	114	115	116	117	118	119	120	121	122	123	124	125	126	127	128	129	130	131	132	133	134	135	136	137	138
0	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**
0	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**
	1							1																		1										
													1							1									1	1		1	1	1	1	1
									1	2					1	1		3																		
			1								1	1		1							3	1		1	1											
0	**1**	**0**	**1**	**0**	**0**	**0**	**0**	**1**	**1**	**2**	**1**	**1**	**1**	**1**	**1**	**1**	**0**	**3**	**0**	**1**	**3**	**1**	**0**	**1**	**1**	**1**	**0**	**1**	**1**	**0**	**1**	**1**	**1**	**1**	**1**
1	4	2		2	1	1	1										1		1				2				1			1					
1	**4**	**2**	**0**	**2**	**1**	**1**	**1**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**1**	**0**	**1**	**0**	**0**	**0**	**2**	**0**	**0**	**0**	**1**	**0**	**0**	**1**	**0**	**0**	**0**	**0**	**0**
1	**5**	**2**	**1**	**2**	**1**	**1**	**1**	**1**	**1**	**2**	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**3**	**1**	**1**	**3**	**1**	**2**	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**1**

SOUTHERN III																	
139	**140**	**141**	**142**	**143**	**144**	**145**	**146**	**147**	**148**	**149**	**150**	**151**	**152**	**153**	**154**	**155**	**TOT**
																	31
																	23
																	15
																	10
																	10
																	17
0	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**106**
																	17
																	2
																	5
0	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**24**
2						1									1		10
3		1		1			1			1		1				2	28
																	30
9	1		1		3			1	1		3		1	1			57
14	**1**	**1**	**1**	**1**	**3**	**1**	**1**	**1**	**1**	**1**	**3**	**1**	**1**	**1**	**1**	**2**	**125**
																	44
																	24
0	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**68**
14	**1**	**1**	**1**	**1**	**3**	**1**	**1**	**1**	**1**	**1**	**3**	**1**	**1**	**1**	**1**	**2**	**323**