

Population Genetics of *Amblyomma americanum* (Acari: Ixodidae) Collected From Arkansas

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ABSTRACT Lone star ticks, *Amblyomma americanum* L. (Acari: Ixodidae), infest multiple hosts such as birds, and mammals of various sizes (rodents to white-tailed deer) and can harbor human pathogens such as *Borrelia lonestari* and *Ehrlichiosis chaffeensis*. The population structure of 251 *A. americanum* ticks, collected from canines and two white-tailed deer in six Arkansas ecoregions, was examined using DNA sequences of a 247-bp region of the mitochondrial DNA ribosomal RNA 16S gene. Of the 247 nucleotide characters, 26 were variable. Thirty-three haplotypes were identified of which 25 haplotypes occurred once (10%). The most common haplotype was Aa25, occurring in 60% of the samples and found in all six ecoregions. The excess of low frequency haplotypes combined with the overall negative Tajima's D and Fu and Li statistics suggests population expansion. Phylogenetic relationships of the 33 *A. americanum* haplotypes were constructed with other *Amblyomma* species and identified *A. americanum* as a monophyletic species with two groups. The patterns of high nucleotide and haplotype diversity found in this study suggests that the *A. americanum* population is expanding perhaps due to its ability to survive in a variety of habitats and feed on multiple hosts. Given the gene flow in Arkansas, the spread of acaricide resistance and pathogens may be rapid.

KEY WORDS *Amblyomma americanum*, population genetics, 16S mitochondrial rDNA

Ticks of the genus *Amblyomma* Koch serve as vectors of several bacterial, viral, and protozoan pathogens (Goddard and Varela-Stokes 2009). The lone star tick, *Amblyomma americanum* (L.) (Acari: Ixodidae), is the principal vector for *Ehrlichiosis chaffeensis* (Stromdahl et al. 2000, Childs and Paddock 2003) and has been suggested as the vector of "*Rickettsia amblyommii*" (Apperson et al. 2008), a spotted fever-like rickettsia identified previously as nonpathogenic bacteria WB-8-2 (Burgdorfer et al. 1981). Additional bacteria have been isolated from several *Amblyomma* ticks (Burgdorfer et al. 1981, Goddard and Norment 1986, Kardatzke et al. 1992, Goddard et al. 2003, Paddock et al. 2003, Raoult 2004, Mixson et al. 2006, Stromdahl et al. 2008); consequently, members of the *Amblyomma* genus are a significant medical and veterinary concern. The combination of large population densities, reduced host specificity, utility of multiple habitats, and increased contact with humans has propelled *A. americanum*'s status from a nuisance pest to that of a vector (Childs and Paddock 2003). Moreover, the injury and damage caused by it is expected to increase dramatically over the next 15 yr due to the combination of biological and environmental factors that expanded its distribution and sociologic and demographic changes that increased the chance for tick encounters (Childs and Paddock 2003).

Population genetics of blood-feeding arthropods can provide insight into their dispersal patterns and population structure, thereby providing an estimate of associated pathogen dispersal and identify locations with a chance for high selection pressure (i.e., insecticide resistance) (Tabachnick and Black 1994). In addition, understanding genetic variation within and among tick populations will help predict vector competence by associating potential tick haplotypes with pathogens and within the environment. For example, patterns between tick lineages and pathogens have been demonstrated with Lyme disease and the black-legged tick, *Ixodes scapularis* Say, where northern lineages of the tick were more closely associated with Lyme disease than the southern lineages (Qiu et al. 2002). Conducting population genetic analyses on ticks has also provided "key elements for understanding pathogen transmission" (McCoy 2008). Population genetic data can provide information about cryptic species, random mating and gene flow and provide insights into applied entomology such as insecticide resistance, pathogen transmission, parasite–host interactions, pheromone use, and genetic control (Black et al. 2001). Also referred to as integrated genetic epidemiology, the study of population genetics of infectious diseases and vectors, we can identify the various ecological factors associated with the populations such as the population structure, the populations reproductive modes and their dispersal strategies (Mc-

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Coy 2008). In ticks, genetic variation is commonly associated with host abundance, host mobility, and their degree of host specificity (Hilburn and Sattler 1986b).

Both mitochondrial DNA (mtDNA) and nuclear ribosomal DNA (rDNA) have been used to identify phylogenetic relationships among ixodid ticks (McLain et al. 1995, Fukunaga et al. 2000). Although conserved nuclear markers are best for determining phylogenetics at higher levels (Norris et al. 1999), conducting population genetics at the species level requires only a single conserved gene such as the mitochondrial 16S rDNA to determine population variation (Nava et al. 2009). Several studies have used only the 16S mitochondrial gene to determine genetic variation within *Dermacentor andersoni* Stiles (Patterson et al. 2009), *Amblyomma parvum* Aragao (Nava et al. 2008), and *A. aureolatum* (Guglielmone et al. 2003). To date, genetic studies on *Amblyomma* ticks have been limited (Hilburn and Sattler 1986a, Lampo et al. 1998, Guglielmone et al. 2003, Reichard et al. 2005, Mixson et al. 2006, Marrelli et al. 2007, Nava et al. 2009), and these studies have not been conducted on ticks from Arkansas. Additionally there is a need for 16S rDNA sequences of ticks to be deposited in GenBank for further phylogenetic studies (Nava et al. 2009). Previous work on *A. americanum* and amplified regions of the mtDNA 16S gene identified 29 haplotypes across three regions (defined by nine sampling sites) in Georgia by using denaturing high-performance liquid chromatography (Mixson et al. 2006). Their study indicated population expansion and reduced genetic differentiation among *A. americanum* (Mixson et al. 2006). Price (1977) suggested both evolutionary and speciation rates should be high in ectoparasites due to founder effects and genetic drift. However, Hilburn and Sattler (1986b) found that genetic variation in ticks on livestock was determined by population size and migration rates such that species with small population sizes had decreased variability and those species with a large population size had increased variability (Hilburn and Sattler 1986a). They suggested that the combination of the host's population size and host's mobility would give chance for greater levels of genetic heterozygosity than previously expected with other parasites (Hilburn and Sattler 1986a).

We hypothesize that *A. americanum* will have high levels of genetic variation because this species is an abundant, aggressive ectoparasite of multiple host animals (birds, and small and large mammals) (Childs and Paddock 2003). In Arkansas, *A. americanum* is the most common tick infesting canines and the second most common infesting white-tailed deer (*Odocoileus virginianus*) (Trout and Steelman 2010). Because *A. americanum* are found on a number of hosts across the state, the tick population should be expanding due to their use of multiple hosts. We determined the extent of genetic variation within and among populations and regions of *A. americanum* ticks in Arkansas by using a portion of the 16S mitochondrial gene, which is often used to examine relationships among closely related

species. The data from this study provide insight into the degree of gene flow within Arkansas and identifies point mutations within the species.

Materials and Methods

Collection and Preservation. The University of Arkansas Veterinary Entomology laboratory assembled and mailed tick collection kits to 323 practicing veterinarians across Arkansas, which included instructions and vials containing 100% ethanol. Practicing veterinarians removed tick specimens from canines (*Canis lupus familiaris*) and stored the specimens in the provided 100% ethanol-filled vials to prevent DNA degradation. Two *A. americanum* from two different white-tailed deer were also collected from Washington county. Tick collections included collection information such as date, location, and host. Ticks were collected during 2007 and all collections came from the state of Arkansas and the portions of the Ozarks, which extend into Oklahoma and Missouri (Fig. 1). Collected ticks were identified to species by using keys from Arthur (1961), Lancaster (1973), and Goddard and Norment (1985). In addition, the sex, life stage, and specific location of each specimen were recorded. All tick specimens were maintained in 100% alcohol until DNA extraction. Voucher specimens are maintained in the University of Arkansas Arthropod Museum, Fayetteville, AR, and additional information on tick collections were reported previously (Trout and Steelman 2009).

DNA Preparation and Polymerase Chain Reaction (PCR) Amplification. To minimize DNA contamination, DNA extractions and PCR were conducted in different laboratories, negative controls were used, and supplies were dedicated to each procedure. *Amblyomma americanum* tick DNA was isolated in the Veterinary Entomology Laboratory at the University of Arkansas after each specimen had been dried on paper towels. Each specimen was longitudinally cut and subjected to the DNeasy insect protocol (QIAGEN, Valencia, CA) for DNA extraction. Samples were stored in a freezer at -20°C to delay DNA degradation until further analyses.

PCR was conducted in the Social Insect Genetics Laboratory at the University of Arkansas. Each PCR set included at least one negative control and a positive control. Negative controls (sterile water) were used to minimize contamination and positive controls (*I. scapularis* 16S rDNA) were used to make sure the PCR procedure worked properly. A master mix reaction was prepared according to the protocol of Szalanski et al. (1997) and a Flexigene thermocycler (Techne Duxford, Cambridge, United Kingdom) was used for all reactions. Negative controls substituted sterile water for template DNA. Reaction products were analyzed by agarose gel electrophoresis in 1% agarose gels stained with ethidium bromide and visualized and documented with a UVP biodoc-it system (UVP Inc., Upland, CA).

Amblyomma ticks were assessed by PCR with mitochondrial primers 16S+2 (5'-TTG GGC AAG AAG

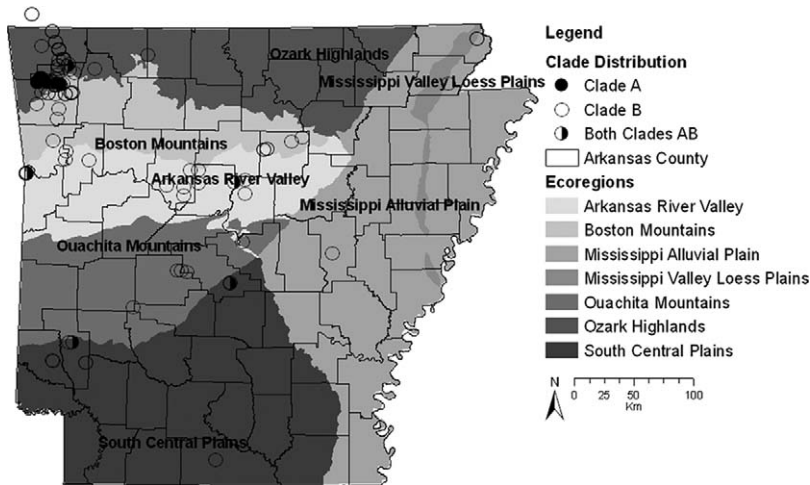


Fig. 1. *A. americanum* ticks sampled from 90 Arkansas hosts and population genetics analyses identified two clades (A and B) of *A. americanum* with the interaction of the two clades occurring between the Ozark Highlands and Boston Mountains (inset).

ACC CTA TGA A-3') and 16S-1 (5'-CCG GTC TGA ACT CAG ATC AAG T-3') (Black and Piesman 1994). Each reaction was initially denatured for 2 min at 94°C. Amplification included 35 cycles of denaturing at 95°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 60 s. At the end of the cycles, the samples were held for a final extension at 72°C for 5 min (Black and Piesman 1994). Reaction products were analyzed with gel electrophoresis in 1% agarose gels stained with ethidium bromide, and visualized with a UVP biodoc-it system (UVP Inc.). Amplified products were purified and concentrated with minicolumns according to the manufacturer's instructions (Wizard PCRpreps, Promega, Madison, WI). Samples were sent to the University of Arkansas Medical Sciences DNA Sequencing Facility (Little Rock, AR) for direct sequencing in both directions.

Data Analyses. Sequences were initially aligned using MAFFT v6 (Katoh et al. 2005), and PAUP* 4.0b10 (Swofford 2001) was used for phylogenetic analyses. Consensus sequences were derived from both directions of the DNA sequences using BioEdit 5.09 (Hall 1999) to verify nucleotide polymorphisms. Mitochondrial DNA haplotypes were aligned with MacClade version 4 (Sinauer Associates, Sunderland, MA). Haplotypes were assigned for each sequence and we defined each haplotype as an aligned set of nucleotides for the 16S mtDNA differing by one base from the other haplotypes in the population. A haplotype was defined as unique if it were found only once in the entire population.

DNAsp version 4.10.9 (Rozas and Rozas 1999) was used to determine haplotype diversity (H_d) and its variance (Nei 1987), nucleotide diversity (π) and its variance (Nei 1987), mean number of pairwise nucleotide differences (K) (Tajima 1983), number of synonymous and nonsynonymous mutations ($S+NS$), the parameters θ_s and θ_c , and the statistical tests (Tajima's D , Fu and Li's D , and Fu and Li's F). Nucleotide

diversity was interpreted as the average proportion of nucleotide differences between all possible pairs of sequences in the sample (Hartl and Clark 1997). The parameter θ is the proportion of nucleotide sites that are expected to be polymorphic in any suitable sample from this region of the genome (Hartl and Clark 1997) and because 16S is mitochondrial it is calculated as $\theta = 2N\mu$ ($2 \times \text{population size} \times \text{mutation rate}$) (Fu 1997).

The statistical tests of neutrality are based on θ . If the population has undergone recent mutations, Fu's F_s statistic will be negative and indicate an excess of rare alleles (or singleton haplotypes) (Fu 1997). Tajima's D statistic tests the neutral mutation theory to explain observed DNA polymorphisms, by comparing singleton and nonsingleton haplotypes in the population (Tajima 1989, Black et al. 2001). Negative Tajima D values may indicate either a population bottleneck or that the population has not yet reached equilibrium due to large insertions or deletions in the region (Tajima 1989). If Tajima's D is not significant then the neutral mutation hypothesis explains the polymorphisms, if the statistic is significant then the observed polymorphisms are not explained by the neutral mutation theory (Tajima 1989). A negative value indicates a large number of singleton haplotypes in the data segregating at a low frequency (Black et al. 2001). Fu and Li's statistical tests were conducted to compare independent estimates of heterozygosity per site, their D statistic is based on mutations, whereas the F statistic compares the average number of pairwise differences and the number of mutations in the external branches of the consensus tree (Black et al. 2001). Fu and Li's F and D statistics were calculated to determine whether background selection is affecting the population (Fu 1997). If only these are significant and Tajima's D is not, then background selection is significantly affecting the population (Fu 1997).

The number of migrants necessary to maintain the population was calculated using the formula ($Nm \approx$

Table 1. Species information and GenBank accession numbers used for phylogenetic analyses

Taxon	Family	GenBank	Reference
<i>Amblyomma americanum</i> Say	Ixodidae	L34314	Black and Piesman (1994)
<i>A. americanum</i>	Ixodidae	L34313	Black and Piesman (1994)
<i>A. aureolatum</i> (Pallas)	Ixodidae	AF541254	Guglielmo et al. (2003)
<i>A. cajennense</i> F.	Ixodidae	L34317	Black and Piesman (1994)
<i>A. dubitatum</i> Neumann	Ixodidae	DQ858954	Venzal et al. (2006)
<i>A. glauerti</i> Keirans, King & Sharrad	Ixodidae	U95853	Norris et al. (1999)
<i>A. hebraeum</i> Koch	Ixodidae	L34316	Black and Piesman (1994)
<i>A. maculatum</i> Koch	Ixodidae	AY375442	Scoles (2004)
<i>A. ovale</i> Koch	Ixodidae	AF541255	Guglielmo et al. (2003)
<i>A. parvum</i> Aragao	Ixodidae	EU306154	Nava et al. (2008)
<i>A. triste</i> Koch	Ixodidae	AY498563	Estrada-Pena et al. (2006)
<i>A. tuberculatum</i> Marx	Ixodidae	U95856	Norris et al. (1999)
<i>A. variegatum</i> F.	Ixodidae	L34315	Black and Piesman (1994)
<i>Argas brevipes</i> Banks	Argasidae	U95863	Norris et al. (1998)

$[(1/2 * F_{ST}) - \text{one-half}]$ assuming a discrete population (Lynch and Crease 1990). The F_{ST} value was calculated to evaluate the extent of genetic subdivision among sub-populations. Based on the F_{ST} value we determined the number of migrants (N_m) necessary to maintain the observed F value.

PAUP* 4.0b10 distance matrix option (Swofford 2001) was used to calculate maximum likelihood, unweighted parsimony analysis, and genetic distances according to the Kimura two-parameter model of sequence evolution (Kimura 1980). Bootstrap tests were conducted to test the reliability of the tree (Felsenstein 1985). We used Bayesian inference as described by Szalanski et al. (2008) to obtain phylogenetic trees with the GTR + G model by using Bayesian Evolutionary Analysis Sampling Trees (BEAST) version 1.4.2 software (Drummond and Rambaut 2003). To create the consensus tree, four Markov chains ran for 10^6 generations. Parsimony bootstrap analysis (Felsenstein 1985) and maximum likelihood analysis was conducted with the method by Tripodi et al. (2006), and genealogical relationships among mtDNA haplotypes were constructed using TCS (Clement et al. 2000) with the method described by Templeton et al. (1992). Once the consensus tree was constructed, PAUP* 4.0b10 was used to conduct maximum likelihood and unweighted parsimony analysis on the alignments, the Branch and Bound algorithm of PAUP* was used for parsimony bootstrap analysis, and a heuristic search was performed using the neighbor joining tree as the starting tree with PAUP*. In addition, to test the reliability of the tree, PAUP* was used to calculate the consistency index (minimal possible tree length/actual tree length) and bootstrap tests were conducted (Felsenstein 1985). The phylogenetic tree was constructed from the assigned "Aa" haplotypes and outgroup taxa were obtained from GenBank and are presented in Table 1. Representatives of each haplotype can be accessed with GenBank as accessions GQ368849–GQ368881.

Results

Two-hundred fifty one *A. americanum* from 88 canines (*C. lupus familiaris*) and two white-tailed deer

were analyzed for variation with population genetics. There was a mean of 2.77 ticks (± 0.13 SEM) analyzed per a host. These samples came from 20 counties across Arkansas representing six of the seven ecoregions (U.S. EPA 2004; Fig. 1). PCR amplification of the 16S rRNA gene resulted in a ≈ 300 -bp amplicon, and a 247-bp segment was used for analyses. Sequences were A/T rich, with adenine consisting of 36.24% and thymine representing 38.05% of the bases (guanine 15.66%, cytosine 9.67%). Of the 247 characters used for analysis, 157 characters were constant and 29 were mutations (Table 2). Thirteen sites were parsimony-informative characters, of which 10 sites were informative with two variants and three sites were informative with three variants. These 29 mutations defined 33 haplotypes (Table 3; Fig. 1). Haplotype Aa25 (60%) and Aa11 (23%) were the most common haplotypes, found in all ecoregions, and on canines and both deer. Twenty-five of the identified 33 haplotypes were singleton haplotypes (identified only once in the population). The number of sequences, haplotypes, haplotype diversity (H_d), and nucleotide diversity (π) per ecoregion are presented in Table 4. The observed haplotype diversity was 0.5829. Nucleotide diversity ($\pi = 0.005$) and the average number of pairwise nucleotide differences ($K = 0.918$) were low. Although a majority of the collections came from northwestern Arkansas (Boston Mountains, Ozark Highlands, and Arkansas River Valley), the haplotype diversity and nucleotide diversity was highest in the South Central Plains located in southern Arkansas ($H_d = 13.944$; $\pi = 0.0056$; Table 4).

A. americanum formed two distinct clades within the species represented by both the Bayesian tree (Fig. 2) and the TCS tree based on Templeton, Crandall, and Sing's algorithm (Fig. 3). Maximum parsimony analyses resulted in a single Bayesian tree with a total length of 193 steps and a consistency index (CI) value of 0.565 (Fig. 2). The CI is a measurement of character fit and compares the minimum number genetic "switches" to the actual number of genetic changes; our low value indicates weak support for the tree. The TCS spanning tree rooted *A. americanum* haplotype Aa11 as the ancestral haplotype (Fig. 3). Coalescence theory (Fu and Li 1993) predicts that the

Table 2. Haplotype assignment (Hap), ecoregion collected from (ER^a), number of sequences (n_s), and DNA mutation sites

Hap.	ER	n _s	Nucleotide mutation site																													
			5	9	10	20	21	22	26	46	47	59	63	77	84	87	88	89	90	92	93	99	124	125	127	131	185	194	195	233	247	
Aa25	A	151	G	A	C	T	A	T	A	G	C	T	T	C	T	T	-	-	-	A	A	A	A	C	C	G	A	A	T	A	A	
Aa11	A	59	A	
Aa28	RUO	3	A	.	A	
Aa26	RS	3	.	.	.	T	
Aa20	B	3	C	
Aa27	O	3	.	T	
Aa6	O	2	.	T	C	
Aa32	O	2	.	.	.	T	A	
Aa33	O	1	T	T	A	
Aa1	B	1	T	T	.	.	.	T	
Aa7	B	1	A	T	G	.	T	A	
Aa8	B	1	T	.	.	T	T	T	A	T	
Aa9	B	1	.	.	.	T	T	
Aa10	B	1	.	.	.	T	.	T	
Aa12	B	1	A	
Aa13	B	1	A	
Aa16	B	1	G	
Aa22	B	1	A	A	
Aa23	B	1	G	.	
Aa24	B	1	A	
Aa29	B	1	T	.	.	T	
Aa31	B	1	G	.	.	.	
Aa2	R	1	.	.	.	A	A	T	.	.	T	.	
Aa4	R	1	T	.	.	.	A	A	
Aa14	R	1	T	G	.
Aa17	R	1	G
Aa18	R	1	.	.	A	T	A	T	A	
Aa19	R	1	C	A	
Aa21	R	1	.	T	A	
Aa3	S	1	A	A	
Aa5	S	1	A	A	
Aa15	S	1	G	.
Aa30	S	1	G

^a A, all regions; O, Ozark Highlands; B, Boston Mountains; R, Arkansas River Valley; U, Ouachita Mountains; S, South Central Plains.

frequency and widespread distribution of the most common haplotype should be the ancestor, in this study the second most common haplotype (Aa11) was the common ancestor and the most common haplotype (Aa25) was derived from it.

Both trees were separated into two clades, designated as clade A and clade B. Clade A represented nine haplotypes from the Boston Mountains, Ozark Highlands, and South Central Plains. Clade A was found primarily in Washington County at the border of the Ozark Highlands and Boston Mountains. These haplotypes were found either by themselves (twice) or

concurrently with clade B (eight times) (Fig. 1). The root for clade A was Aa32 which derived eight of the 10 haplotypes in clade A. In addition, Aa32 was collected from the Ozarks Highlands and may have lead to a slight trend of geographic structuring where specific haplotypes were collected in the South Central Plains (Aa3, Aa26), Boston Mountains (Aa1, Aa8, Aa9, Aa10, Aa29), or Arkansas River Valley (Aa18, Aa26). Clade B represented 24 of the 33 haplotypes including the two most common (Aa11 and Aa25) and were found in all ecoregions. In clade B, haplotype Aa20 was ancestral to the 14 derived haplotypes and was originally derived from Aa11.

Statistical tests for neutral mutation (Fu and Li D⁺ and F⁺ and Tajima's D) had negative values (Table 4), and analyses on the entire *A. americanum* population were significant; Fu's F_S (-43.697), Fu and Li's D⁺ (-3.8515, P < 0.02), Fu and Li's F⁺ (-3.8229, P < 0.02), and Tajima's D (-2.2267, P < 0.001). These are based on the parameter θ, which was 13.944 in our study population. Negative values indicate an expanding population due to an excess of singleton haplotypes (Tajima's D) and that background selection in contributing to the genetic variation (Fu and Li's D⁺ and F⁺). Analyses at the ecoregion level were not significant and this may be due to the tick's host migration. Pairwise Tajima-Nei distances among *A. americanum* within the collected population ranged

Table 3. Descriptive results for mtDNA haplotype numbers for all *A. americanum* samples

Arkansas ecoregion	n _s	n _h	Aa haplotype (no. samples)
Ozark Highlands	70	8	6(2), 11(16), 23, 25(44), 27(3), 28, 32(2), 33
Boston Mountains	86	16	1, 7, 8, 9, 10, 11 (19), 12, 13, 16, 20 (3), 22, 23, 24, 25(51), 29, 31
Arkansas River Valley	54	11	2, 4, 11 (12), 14, 17, 18, 19, 21, 25(32), 26(2), 28
Mississippi Valley Loess Plain	7	2	11(2), 25(5)
Ouachita Mountains	14	3	11(4), 25(9), 28
South Central Plains	20	7	3, 5, 11 (4), 15, 25(10), 26(2), 30

n_s is number of sequences; n_h is number of haplotypes.

Table 4. Summary statistics for mtDNA polymorphisms in *A. ticks* among ecoregions

Arkansas ecoregion	n_s	h_n	$H_d (\pm SD)$	θ_s	$\Pi (k)$	Fu and Li's D^+ (P)	Fu and Li's F^+ (P)	Tajima's $D (P)$
Ozark Highlands	70	8	0.5565 (± 0.057)	2.957	0.0042 (0.771)	-1.2626 (ns)	-1.4504 (ns)	-1.1660 (ns)
Boston Mountains	86	16	0.6036 (± 0.052)	9.884	0.0055 (0.998)	-3.3.168 (<0.05)	-3.2831 (<0.02)	-2.0324 (<0.05)
Arkansas River Valley	54	11	0.6066 (± 0.065)	10.796	0.0059 (1.085)	-3.5901 (<0.02)	-3.6201 (<0.02)	-2.0306 (<0.05)
Mississippi Valley Loess Plain	7	2	0.4762 (± 0.171)	0	0.0026 (0.476)	0.9535 (ns)	0.9179 (ns)	0.5590 (ns)
Ouachita Mountains	14	3	0.5385 (± 0.115)	0.929	0.0035 (0.637)	-0.4458 (ns)	-0.3658 (ns)	0.0365 (ns)
South Central Plains	20	7	0.6790 (± 0.102)	3.8	0.0056 (1.032)	-1.5570 (ns)	-1.6961 (ns)	-1.2430 (ns)
Total	251	33	0.5829 (± 0.030)	13.944	0.0050 (0.918)	-3.8515 (<0.02)	-3.8229 (<0.02)	-2.2267 (<0.01)

n_s is number of sequences; h_n is number of haplotypes; $h_d (\pm SD)$ is haplotype diversity $\pm SD$; θ_s is theta per site; π is nucleotide diversity; k is mean number of nucleotide differences; Fu and Li's D^+ and F^+ statistics are detailed in the text (Fu and Li 1993); D is the Tajima and Nei (1984) statistic is detailed in the text.

from 0.005 to 5.71%, from 0.005 to 3.402% among all the *A. americanum* used in the phylogenetic analyses, and from 9.96 to 23.325% across all of the analyzed *Amblyomma* taxa. As mentioned, the F_{ST} value (0.02141) was calculated to evaluate the extent of genetic subdivision among sub-populations. Based on the F_{ST} value, the number of migrants necessary to maintain the observed F value was 0.4813 ($N_e m$) individuals per generation. The observed levels of heterozygosity ($H_o = 0.6614$) were higher than the expected levels of heterozygosity ($H_e = 0.6473$), and they were calculated as $1 -$ the sum of the squared gene frequencies (Weir 1996). Comparing the amount of genetic variation within and between populations (F_{ST} values) among the ecoregions ranged from 0.09128 (Mississippi Valley Loess Plain and Ouachita Mountains) to

0.00531 (Boston Mountains and Ozark Highlands) (Table 5).

Discussion

Our results indicate that the *A. americanum* population is actively intermixing among the ecoregions due to the large number of singleton haplotypes, excess of insertions and deletions, and consistency of singleton and multiple haplotypes across the ecoregions. The low F_{ST} and $N_e m$ values, the large population and the use of a relatively conserved gene indicate random drift is occurring in the population. Founders effect may have occurred in the population because many of the haplotypes were derived from a single haplotype and there were many singleton hap-

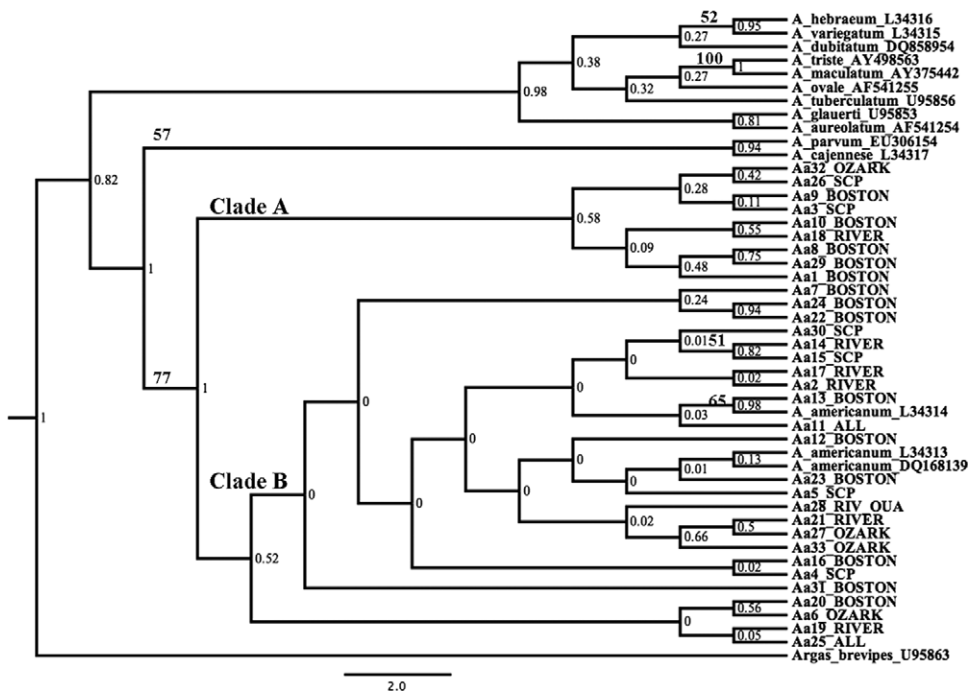


Fig. 2. Phylogenetic relationship of 33 mtDNA 16S *A. americanum* haplotypes. Numbers at the tree nodes indicate Bayesian posterior probabilities, and numbers above the nodes indicate bootstrap values obtained from 1,000 replicates by using maximum parsimony analysis.

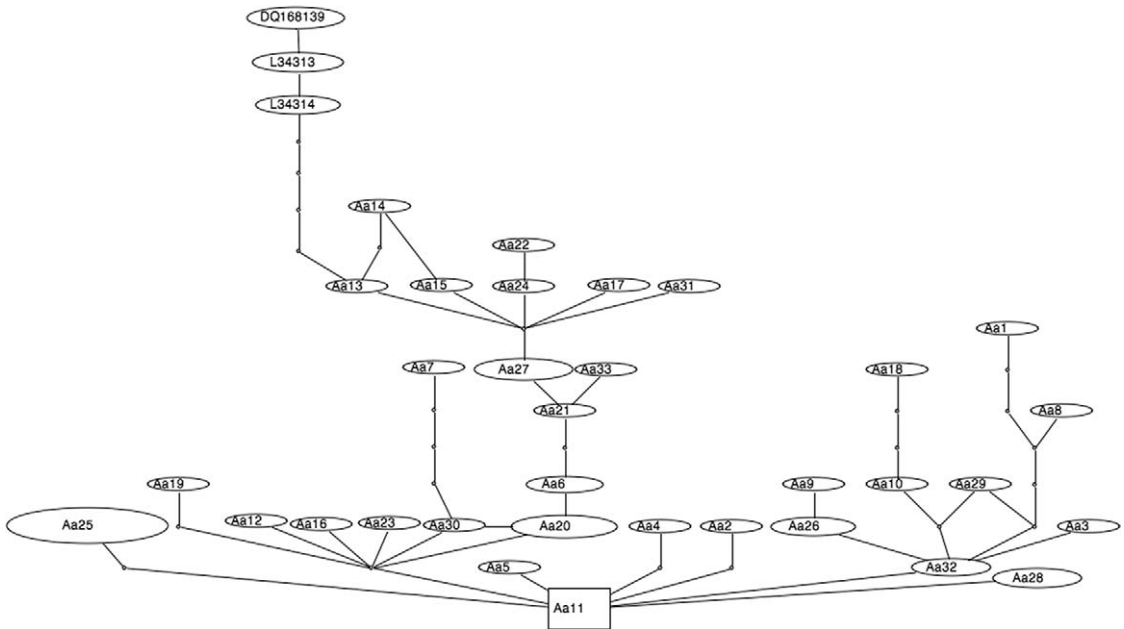


Fig. 3. Genealogical relationships among 33 haplotypes of *A. americanum* defined by nucleotide variability sites and estimated by TCS (Clement et al. 2000). A unit branch represents one mutation.

lotypes identified. However, we cannot be certain because the DNA sequence used for genetic analysis was relatively small, we had a limited sample size among the different regions, and there was not much variation with trends to identify distinct population groups. These findings may be due to *A. americanum* dependency on multiple vertebrate hosts for dispersal and their ability to live in various habitats (Childs and Paddock 2003).

Previous research on *Amblyomma* ticks has found varying degrees of genetic variation based on the molecular method, the markers used, and the species analyzed. Marrelli et al. (2007) combined PCR with direct sequencing to amplify the internal transcribed spacer (ITS)-2 of 15 Neotropical *Amblyomma* bird tick species and found high levels of genetic divergence (1.5% to 25.6%) among the different species. Genetic variation using allozyme analyses on a three-host tick from Venezuela, *Amblyomma dissimile* Koch, collected from different

hosts found the average mean heterozygosity per locus was $6 \pm 3.1\%$ per population (Lampo et al. 1998). Hilburn and Sattler (1986a) examined *A. americanum* for variations among 21 enzymes and found a homogenous population without a geographic structuring in genetic variation. Sequence variation of the nuclear rDNA ITS-1 region of *A. americanum* in Oklahoma State University's tick colonies were compared with field collected populations and results indicated two distinguishable groups based on rDNA ITS-2 (Reichard et al. 2005). Our findings of high heterozygosity may have been due to the large number of singleton haplotypes. Similar to our findings, Lampo et al. (1998) and Mixson et al. (2006) suggested that the tick genetic variability is closely associated with the host's dispersal ability. The majority of ticks used in this study were collected from canines, which may travel large distances with their owners allowing for increased dispersal. Previous research on another highly host-

Table 5. F_{st} values (above diagonal) and N_m values (below diagonal) for mtDNA comparing genetic variation within and between *A. americanum* ticks based on ecoregions

Ecoregion	Ozark Mountains	Boston Mountains	Arkansas River Valley	Mississippi Valley Loess Plain	Ouachita Mountains	South Central Plains
Ozark Mountains	—	0.0053	0.0040	0.0504	0.0152	0.0097
Boston Mountains	0.4974	—	0.0007	0.0364	0.0038	0.0105
Arkansas River Valley	0.4980	0.4997	—	0.04603	0.0234	0.0203
Mississippi Valley Loess Plain	0.4748	0.4818	0.4770	—	0.0913	0.0248
Ouachita Mountains	0.4924	0.4981	0.4883	0.4544	—	0.0554
South Central Plains	0.4952	0.4948	0.4899	0.4876	0.4723	—

^a F_{st} values were calculated from Hudson et al. (1992) with DNAsp version 4.10.9 (Rozas and Rozas 1999) and the number of migrants (N_m) were calculated from Lynch and Crease (1990).

dependent arthropod for dispersal, *Cimex lectularius* L. (bed bug), identified a highly variable population structure with 16S mitochondrial DNA and no variation within the ITS-2 nuclear gene of the same population (Szalanski et al. 2008). Using a nuclear marker in conjunction with the mitochondrial gene may provide further evidence for a recent intermixing of tick populations.

Our study identified northwest Arkansas as the most diverse region of the state. This region had 21 haplotypes of which 18 were unique. This area also accounted for a majority of the collections. Although we attempted to have an unbiased collection by sending collection kits across the state, our samples may have been biased. Due to the proximity of the veterinary clinics to the University of Arkansas, closer veterinarians may have been more likely to participate in the study than those further away. However, the recent fragmentation of the environment and dispersal by birds from other areas can account for the large number of singleton haplotypes. The northwest Arkansas areas of Springdale, Fayetteville, and Bentonville (cities in the Ozark and Boston Mountains) were identified as one of the top 10 fastest growing metropolitan areas in the nation (Anonymous 2001). Tick dispersal in Arkansas is probably due to immigration of pet owners into the state, travel across the state, and birds migration. In addition, the urban development in northwest and central Arkansas along with construction of new roads and highways is fragmenting the once continuous landscape. Fragmentation of the environment generally decreases effective population size; however, in this study we identified high levels of genetic variation due to the populations intermixing, the tick's use of multiple hosts (Barrett et al. 2008), and the differences in the host's environments (Klompen et al. 1996).

We believe the lack of host specificity and use of multiple habitats is responsible for the large amount of variation within *A. americanum* (Klompen et al. 1996, Barrett et al. 2008). *A. americanum* uses multiple hosts (Sonenshine 2005). This species typically reproduces on the third host, but the female deposits her eggs off the host allowing the environment to be "responsible" for egg hatch (Sonenshine 2005). Dispersal of this species is primarily via three different hosts (Sonenshine 2005), which may differ in distance from one host species to the next. For example, a bird's migratory pattern is generally greater than that of a ground animal and the dispersal pattern of a domesticated canine can vary by the owner. These life history factors may explain the high levels of genetic variation within *A. americanum*. The use of various hosts by *A. americanum*, their relationships with these hosts, and their interactions with bacteria should be investigated for dispersal and genetic differentiation.

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