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Comparative population genetics of two invading ticks: evidence of the ecological mechanisms underlying tick range expansions

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Abstract

Two species of ixodid tick, *Ixodes affinis* Neumann and *Amblyomma maculatum* Koch, are simultaneously expanding their ranges throughout the mid-Atlantic region of the US. Although we have some understanding of the ecology and life history of these species, the ecological mechanisms governing where and how new populations establish and persist are unclear. To assess population connectivity and ancestry, we sequenced a fragment of the 16S mitochondrial rRNA gene from a representative sample of individuals of both species from populations throughout the eastern US. We found that despite overlapping host preferences throughout ontogeny, each species exhibited very different genetic and geographic patterns of population establishment and connectivity. *Ixodes affinis* was of two distinct mitochondrial clades, with a clear geographic break separating northern and southern populations. Both *I. affinis* populations showed evidence of recent expansion, although the southern population was more genetically diverse, indicating a longer history of establishment. *Amblyomma maculatum* exhibited diverse haplotypes that showed no significant relationship with geographic patterns and little apparent connectivity between sites. Heteroplasmy was also observed in the 16S mitochondrial rRNA gene in 3.5% of *A. maculatum* individuals. Genetic evidence suggests that these species rely on different key life stages to successfully disperse into novel environments, and that host vagility, habitat stability and habitat connectivity all play critical roles in the establishment of new tick populations.

Keywords

Amblyomma maculatum; *Ixodes affinis*; population genetics; range expansion; tick dispersal; vector-borne disease

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1. Introduction

The spread of parasites is a major source of disease emergence for vertebrate taxa, including humans (George 2008, Mack et al. 2000, Williams et al. 2013). Climate change and anthropogenic landscape alterations, such as fragmentation and suburban sprawl, have been linked to tick range expansions worldwide, resulting in changes to tick community structure and altered tick-borne pathogen dynamics (Childs and Paddock 2003, Cumming and Van Vuuren 2006, Gage et al. 2008, George 2008, Léger et al. 2013).

In North America, the expanded ranges of the lone star tick, *Amblyomma americanum*, and the blacklegged tick, *Ixodes scapularis*, have received much attention because of the importance of these ticks to human health (Brinkerhoff et al. 2011, Childs and Paddock 2003, Ogden et al. 2008, Springer et al. 2014). More recently, *Ixodes affinis* and the Gulf coast tick, *Amblyomma maculatum*, have invaded the eastern United States (US), changing tick community and pathogen dynamics in the affected areas (Fornadel et al. 2011, Harrison et al. 2010, Nadolny et al. 2011, Paddock and Goddard 2015, Varela-Stokes 2011, Wright et al. 2011). Because of their small size and vulnerability to the environment when off-host, tick dispersal is intricately linked to movements of their hosts (Falco and Fish 1991). The ecological processes driving tick invasions require study in order to better predict and mitigate disease emergence and improve understanding of tick behavior.

Coalescent genetic analyses can reveal the ancestry of newly established populations, and offer clues to how organisms disperse over long distances. The use of molecular methods to trace the ancestry of invading species is well established (Templeton et al. 1995, Ibrahim et al. 1996, Le Roux et al. 2009). For more than 30 years, molecular methods have been used to investigate tick population genetic structure (Araya-Anchetta et al. 2015). Previous studies have documented the effects of host-mediated dispersal on genetic structure in the seabird tick *Ixodes uriae*, using microsatellite markers to determine how gene flow patterns changed depending on the host species exploited for dispersal (McCoy et al. 2001, McCoy et al. 2003). Other studies have used phylogenetic analyses to determine recent population expansion, identify founder effects, and examine population structure at the expansion fronts of *A. americanum* and *I. scapularis* (Kelly et al. 2014, Mechai et al. 2013, Ogden et al. 2011). Some broad conclusions reached by this body of work are that tick behaviors and life cycle strategies are as critical as host mobility in understanding tick population genetic structure, particularly as applied to different families of ticks (Araya-Anchetta et al. 2015).

The northward expansions of *I. affinis* and *A. maculatum* into the Mid-Atlantic region of the US have been documented over the last decade (Florin et al. 2014, Fornadel et al. 2011, Harrison et al. 2010, Nadolny et al. 2011, Varela-Stokes 2011, Wright et al. 2011). Both species are recent additions to a diverse assemblage of ticks and have the potential to significantly affect pathogen dynamics (Oliver 1996, Nadolny et al. 2011, Nadolny et al. 2014). *Ixodes affinis* is a competent sylvatic vector for *Borrelia burgdorferi* sensu stricto, the bacterial agent of Lyme disease, and *A. maculatum* is the major vector for *Rickettsia parkeri*, the agent of Tidewater spotted fever, and several significant veterinary pathogens (Oliver et al. 2003, Paddock et al. 2004, Paddock and Goddard 2015). Both species have expanded northward from historic ranges in the southern US and Central America and both

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parasitize avian and mammalian hosts throughout their ranges (Harrison et al. 2010, Paddock and Goddard 2015, Teel et al. 2010).

Despite some overlap in the vertebrates parasitized by these two tick species, *I. affinis* and *A. maculatum* are generally found in different habitat types and are expanding their ranges in different geographic patterns. The northern edge of the expansion front for *I. affinis* is currently in Virginia (Nadolny et al. 2011), and individuals can be consistently collected at low densities in woodland habitats (Gaff and Nadolny, unpublished data). *Amblyomma maculatum* appears to be expanding in isolated populations in ephemeral successional habitats, with large areas containing few to no *A. maculatum* separating established breeding groups (Gaff and Nadolny, unpublished data). These expansion patterns reflect the distributions of these ticks in their native ranges (Harrison et al. 2010, Teel et al. 2010, Varela-Stokes et al. 2011), but the implications of these two expansion types on population connectivity are largely unexplored.

Several genetic markers have been historically used to characterize population structure in tick populations, most commonly nuclear microsatellites and variation in mitochondrial genes. Microsatellites have been characterized for twelve tick species (Araya-Anchetta et al. 2015). Microsatellites, however, are often unable to achieve cross-species amplification (McCoy and Tirard 2000) and are also not universally abundant in all tick genomes (Fagerberg et al. 2001). The use of the 16S mitochondrial rRNA gene is pervasive in studies of tick population genetics and tick phylogeny (Black and Piesman 1994, Kelly et al. 2014, Mixson et al. 2006, Norris et al. 1996, Qiu et al. 2002). This gene mutates at a rate that is generally informative for species-level phylogenetics and broad biogeographic inferences (Araya-Anchetta et al. 2015).

In this study, we used single nucleotide polymorphisms (SNPs) in the 16S mitochondrial rRNA gene to examine patterns of genetic structure in *I. affinis* and *A. maculatum* ticks across the eastern US. This method enabled direct comparison of our results across our two species without the need to characterize two novel sets of species-specific microsatellites. Using a mitochondrial gene was also appropriate for the scale of our research, as we were investigating broad biogeographic patterns of connectivity across many US states (Araya-Anchetta et al. 2015). We used the resulting information on haplotype frequencies, geographic and genetic distance between new populations, and host and habitat associations of each tick species to describe likely routes by which these ticks are expanding their ranges.

Field-dwelling ticks and generalist ticks that parasitize a range of hosts are less likely to exhibit genetic structure than nidicolous (nest-dwelling) ticks, single-host ticks, or ticks that specialize on hosts with small home ranges (Araya-Anchetta et al. 2015). We compare the genetic structure of two non-nidicolous tick species that are expanding their ranges simultaneously across the eastern US, and that share host preferences for avian and mammalian hosts. We discuss how tick ecology and behavior may affect population structure and use this structure to infer likely invasion routes and important life history characters that have facilitated range expansion for each species. We hypothesized that both species would exhibit isolation by distance, with northern populations originating from related southern populations.

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2. Materials and methods

2.1 Study sites and tick collection

Questing adult ticks were collected from populations in Virginia, North Carolina, and South Carolina using standard tick collecting methods, as previously described (Nadolny et al. 2011, Wright et al. 2011). Ticks and/or tick DNA extracts from additional populations in states throughout the established US ranges of both ticks were included to provide a more complete analysis of population structure (Supplementary Table 1). After morphological species identification (Keirans and Litwak 1989, Sonenshine 1979), ticks were frozen at −80°C prior to DNA isolation.

2.2 Molecular methods

Prior to DNA extraction, individual ticks were placed in 2 mL microcentrifuge tubes containing 1 mm and 2.5 mm glass beads and homogenized in a bead-beater (BioSpec Products, Inc., Bartlesville, OK) for 30 seconds. DNA was then extracted from individual adult ticks using a DNeasy Blood & Tissue Kit (Qiagen, Inc., Valencia, CA), eluted in 50 μL of buffer AE and stored at −20°C until processing.

We used standard PCR to amplify a fragment of the tick mitochondrial 16S rRNA gene using primers 16S+1 (5′-CTGCTCAATGATTTTTTAAATTGCTGT-3′) and 16S-1 (5′-GTCTGAACTCAGATCAAGT-3′) (Macaluso et al. 2003, Nadolny et al. 2011). PCR reactions were performed in 15 μL reaction volumes, with 0.05 U/μL Taq DNA Polymerase (BioPioneer Inc., San Diego, CA), 1 μM each primer, 1.5 mM $MgCl_2$, 1X PCR buffer (Qiagen Inc., Valencia, CA) and 2 μL DNA template. The PCR protocol consisted of an initial 3-min denaturation step at 95°C followed by 30 cycles of 95°C for 30 seconds, 52°C for 45 seconds and 72°C for 1 minute, with a final extension at 72°C for 7 minutes. PCR products were visualized on 1.5% agarose gels, and purified using ExoSap-IT (Affymetrix Ltd., Santa Clara, CA). Sequencing reactions were performed using the BigDye Terminator v.3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA). Amplicons with overlapping peaks, indicating heteroplasmy, were cloned into the PCR 2.1 TOPO vector using the TOPO TA cloning kit (Invitrogen, Carlsbad, CA), and resulting plasmid inserts were sequenced, as above. DNA sequences were identified by BLAST search (Altschul et al. 1990). Successfully amplified DNA provided unambiguous bi-directional sequence data along the length of the sequence for all samples. All sequences were aligned and consensus sequences were constructed using Geneious R7 (http://www.geneious.com, Kearse et al. 2012).

2.3 Quantitative analysis

2.3.1 Sample size and haplotype variation—To determine the approximate sample size required to capture all haplotypes in a given population, the “vegan” package in R (R Core Team 2014) was used to compute rarefaction curves, following methods outlined by Lindblom et al. (2009). Rarefaction uses individual-based resampling curves to quantify the number of haplotypes found in each population (Gotelli and Colwell 2001) by randomly subsampling the community and plotting the number of observed haplotypes against the number of observations until an asymptote is reached. To assess whether our sample sizes

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provided total coverage of the haplotypes present in the populations sampled, we used the software EstimateS (Colwell et al. 2004) to apply two non-parametric methods, a Chao 1 richness estimator and an abundance-based coverage estimator (ACE) (Lindblom et al. 2009). EstimateS and the "vegan" package in R were also used to create haplotype accumulation curves to visualize the number of haplotypes detected in relation to the number of sites sampled (Lindblom et al. 2009). These methods allowed us to estimate how many haplotypes were likely missed by our sampling efforts.

2.3.2 Phylogenetic tree construction—The software MEGA v 6.06 (Tamura et al. 2007) was used to select the best-fitting molecular evolutionary models, and to create neighbor-joining and maximum likelihood phylogenies with 1000 bootstrap replicates. JModelTest (Darriba et al. 2012, Guindon and Gascuel 2003) was used to validate the model selections made by MEGA, and the MrBayes plugin in Geneious was used to create Bayesian trees (Huelsenbeck and Ronquist 2001, Ronquist and Huelsenbeck 2003).

2.3.3 Population genetic structure—We used AMOVA implemented in Arlequin v 3.5 (Excoffier and Lischer 2010) to explore patterns of population genetic structure within and among groups. We assessed population structure by generating pairwise Φ_{ST} values among sites. False discovery rate q values based on the Φ_{ST} values were used to minimize the probability of Type I error in multiple pairwise comparisons (Pike 2011). Minimum spanning trees (MSTs) of confirmed haplotypes were created using output from Arlequin visualized using HapStar (Teacher and Griffiths 2011).

A spatial analysis of molecular variance based on Φ statistics was calculated using SAMOVA, v 2.0 (Dupanloup et al. 2002) to define groups of populations that were geographically homogeneous and maximally differentiated. The method uses a simulated annealing procedure to maximize the proportion of total genetic variance as a result of differences between groups of populations. The analysis was run for 2–11 groups for each tick species, with 100 permutations.

To determine if there was significant genetic isolation by distance we used the "vegan" package and "ade4" library in R to perform Mantel and partial Mantel tests. These tests used distance matrices based on Φ_{ST} and the over-land distance between the latitude and longitude coordinates of each collection site. If clustering of haplotypes into clades was observed, partial Mantel tests were also conducted to determine if populations exhibited hierarchical clustering rather than true isolation by distance (Merimans 2012). Partial Mantel tests were conducted using a third matrix of zeroes and ones indicating the dominant clade of each population. Using Arlequin, we tested mismatch distributions (τ) within individual sites as well as among all samples for departure from models representing spatial and demographic expansion. We tested for departures from selective neutrality using Tajima's *D* and Fu's F_S statistics, both of which assess the difference between observed and expected numbers of alleles based on observed nucleotide diversity (Tajima 1989, Fu 1997).

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3. Results

3.1 Ixodes affinis

3.1.1 Sample size and haplotype variation—*Ixodes affinis* were collected from 19 sites in the US from Georgia to Virginia. The number of *I. affinis* collected and sequenced from each site varied from 1 to 55 over the span of 4 years, totaling 236 individuals (Supplementary Table 1). No sequences exhibited heteroplasmy. Haplotype rarefaction curves were generated for all sites, treating all years as independent samples, with most curves approaching an asymptote, indicating no new haplotypes being discovered, at 5 or fewer individuals (Fig 1A). A population haplotype accumulation curve failed to reach an asymptote, indicating that haplotype sampling was not nearing completeness when all samples and sites were included, which was supported by ACE and Chao 1 richness estimators (Supplemental Figure 1).

3.1.2 Phylogeny—We identified 18 unique 16S haplotypes (Genbank accession numbers TBD), one of which had been previously reported in the US by two research teams (AFF5, Genbank accession numbers IAU95879 from Sapelo Island, GA [Norris et al. 1999] and AF549834 from an unspecified US collection location [Xu et al. 2003]). *Ixodes affinis* haplotypes grouped in two mitochondrial clades, and are labeled numerically as AFF1 to AFF12 (Clade A) and AFF13 to AFF18 (Clade B)(Fig. 2A). One additional complete 16S sequence reported in Genbank (Genbank accession number AF549861, from Colombia [Xu et al. 2003]) was not included in our analyses; therefore, all analyses focused on US samples. The 18 haplotypes detected in this study were visualized on a MST, where the two clades observed in our phylogenetic tree were clearly separated by six base pair changes (Fig. 3A). The two clades showed evidence of star radiations from AFF4 and AFF17, with an intermediate grouping of AFF9 to AFF11.

3.1.3 Population structure—Sampled sites with ≥ 5 individuals were used for AMOVA and pairwise Φ_{ST} analyses. Global structure within (accounting for 55.7% of variation) and among groups (44.3% of variation) was detected among the 12 resulting sites (AMOVA Φ_{ST} = 0.44, $p<0.001$). Haplotype frequency as measured by Φ_{ST} was significantly different between many of our sampling sites (Table 1). Haplotype frequencies were most often significantly different between sites in the north (North Carolina northward) and in the south (South Carolina southward), while sites within each region showed little difference in haplotype frequency.

AFF17 was the most frequently encountered haplotype (115 individuals), followed by AFF4 (50 individuals), the latter of which had been detected in previous studies in Georgia (Genbank accession numbers IAU95879 and AF549834). A Mantel test comparing matrices for spatial and genetic distance between the 12 sites with ≥ 5 ticks showed evidence for isolation by distance ($r = 0.58$, $p = 0.003$, 2000 permutations). Partial Mantel tests indicated that this initial result was due to spatial clustering of *I. affinis* populations, with no significant isolation by distance observed when using a matrix accounting for clustering as a covariate ($r = 0.00$, $p = 0.51$), and significant isolation by distance when using the geographic distance matrix as a covariate ($r = 0.41$, $p = 0.006$). Haplotypes from Clade A

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were predominant in southern sites, while haplotypes from Clade B were predominant in northern sites (Fig. 4A). SAMOVA analysis also supported that the southern sites represented a separate population from the northern sites, with the model containing two groups yielding the best-supported F_{CT} value (Supplemental Table 2, $F_{CT} = 0.63$).

Mismatch distributions revealed no evidence of spatial ($\tau = 7.79$, $p = 0.19$) or demographic expansion ($\tau = 9.4$, $p = 0.08$) for the population when analyzed as a whole. Neutrality tests ($D = 0.50$, $p = 0.72$; $F_S = -0.12$, $p = 0.55$) also revealed no significant evidence for departures from neutrality when all samples were included. There were also no significant indicators for spatial expansion or departures from neutrality when we analyzed separately the northern ($\tau = 8.22$, $p = 0.35$; $F_S = 0.23$, $p = 0.57$; $D = 0.23$, $p = 0.64$) and southern ($\tau = 2.04$, $p = 0.42$; $F_S = -1.47$ $p = 0.19$ $D = -1.37$, $p = 0.06$) populations that were supported by SAMOVA analysis. Both northern and southern populations, however, showed evidence of recent demographic expansion ($\tau = 0$, $p < 0.001$).

3.2 Amblyomma maculatum

3.2.1 Sample size and haplotype variation—*Amblyomma maculatum* were collected from 18 sites across the eastern US. The number of *A. maculatum* collected and sequenced from each site varied from one to 90 across the four years, totaling 370 individuals (Supplemental Table 1). Rarefaction curves were generated for all sites, treating all years as independent samples, and curves started approaching asymptote at around 10 individuals (Fig 1B). The haplotype accumulation curve failed to reach an asymptote, indicating that haplotype sampling was not nearing completeness when all sites were included, which was supported by ACE and Chao 1 richness estimators (Supplemental Figure 1).

3.2.2 Heteroplasmy—Of the *A. maculatum* analyzed, 13 out of 370 ticks exhibited heteroplasmy (3.5% of individuals) at one or more sites in the mitochondrial 16S rRNA gene. Most heteroplasmic sites were within a short stretch of base pairs on a hairpin loop region of the rRNA molecule. Heteroplasmic sites were confirmed using TA cloning on a representative sample of individuals exhibiting multiple peaks at a given base pair site. Cloned plasmids containing rRNA from one individual exhibited one phase when sequenced. The ticks that exhibited heteroplasmy were from six sites that were geographically distant from one another, and included five ticks from CHS, two ticks from VB1, two from NCA, two from FX2, one from KY2 and one from MS5. The ticks that exhibited heteroplasmy were not included in our analyses, due to the inability to resolve the phase of the heteroplasmic sites.

3.2.3 Phylogeny—We identified 36 unique haplotypes that were not heteroplasmic (Genbank accession numbers TBD), several of which had been partially sequenced by other researchers (Black and Piesman 1994, Ketchum et al. 2009, Ferrari et al. 2013). To construct our phylogenic trees, we used only our complete 416 bp sequences, thus conserving all variable sites within our analyses (Fig. 2B). Although there was abundant haplotype diversity in the *A. maculatum* samples sequenced, no evidence of clades emerged from our trees. By visualizing our 36 haplotypes using an MST, it was evident that the lack of clades was due to very few base pair changes between haplotypes (Fig. 3B). There was evidence

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for star radiations from haplotypes MAC16, MAC8, and MAC9, and haplotypes MAC1 and MAC2 were distinct from the rest of the haplotypes by seven base pair changes.

3.2.4 Population structure—Sites with ≥ 10 and 5 individual *A. maculatum* were used for AMOVA and pairwise Φ_{ST} analyses, respectively. Global structure within (85.44% of variation) and among groups (14.56% of variation) was detected among the 9 resulting populations with ≥ 10 individuals (Φ_{ST} = 0.15, df = 318, p < 0.001). Haplotype frequency as measured by Φ_{ST} was significantly different between almost all of the sampling sites, indicating little connectivity between sites with ≥ 5 individuals (Table 2). Sites VB1 and FX2 (Φ_{ST} = 0.04, q = 0.12) and MS2 and TNS (Φ_{ST} = 0.05, q = 0.08), had haplotype frequencies that were not significantly different. Those sites with the smallest sample sizes (MS3, MS4, and MS7, all from Mississippi) exhibited more statistically similar haplotype frequencies to other sites in our pairwise Φ_{ST} analysis (Table 2).

A Mantel test comparing matrices for physical and genetic distance among the 12 sites with ≥ 5 ticks showed no evidence for isolation by distance (r = 10.01, p = 0.44, 2000 permutations). Although there were significant differences in haplotype frequencies between most sites, no geographic pattern was evident (Fig. 4B). Sites in geographic proximity to each other were not more likely to have similar haplotype frequencies. MAC8 was the most frequently encountered haplotype (60 individuals), followed closely by MAC16 (52 individuals), and both of these were centers of star radiations (Fig. 3B) and found at multiple sites (Fig. 4B). SAMOVA analysis supported that each of our sampling sites represented a genetically isolated populations (Supplemental Table 3).

Mismatch distributions revealed no evidence of spatial (τ = 2.26, p = 0.20) or demographic expansion (τ = 3.09, p = 0.27) when all individuals were analyzed as one population. Tajima's *D* statistic revealed no evidence for departures from neutrality for the whole population (*D* = -0.45, p = 0.38), but Fu's F_S statistic suggested evidence of recent expansion (F_S = −13.88, p = 0.001). There were few significant indicators for departures from neutrality or expansion when examining individual sites, with only populations YRK (F_S = −1.41, p = 0.03) and MS3 (F_S = −2.95, p = 0.02) departing from neutrality by Fu's *F* statistic, and only MS3 indicating spatial expansion (τ = 1.16, p = 0.04).

4. Discussion

Because of the recent reports documenting the range expansions of *I. affinis* (Harrison et al. 2010, Nadolny et al. 2011) and *A. maculatum* (reviewed by Paddock and Goddard 2015), we expected to see evidence of northward population expansion in both species. Instead, we found the genetic population structures of each species to be unique, and neither pattern supported our original simple hypothesis of populations spreading north from closely related southern populations. Populations of both species from sites in northern and southeastern Virginia are <10 years old, and are unlikely to contain significant on-site mutation.

4.1 Ixodes affinis

This is the first study of the population genetics of *I. affinis*. We found *I. affinis* was characterized by two genetic clades with a clear geographic break, with Clade A dominant in

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South Carolina and Georgia and Clade B dominating in North Carolina and Virginia (Fig. 4A, Table 1, Supplemental Table 2). Clade A was more diverse than Clade B, indicating that *I. affinis* has been established longer at the southern sites than the northern sites. This pattern was expected, but was especially striking since 82% of individual *I. affinis* in this study were collected from northern sites, but 66% of haplotypes were from southern sites. The star radiations evident in each clade, however, indicate that both clades are expanding independently of each other (Fig. 3A). Our mismatch analyses further support that these clades are both undergoing significant demographic expansion. Results from SAMOVA and partial Mantel tests suggest that each of these populations is independent. These results indicate that *I. affinis* are newly arrived in the north (North Carolina and Virginia) and that this population is expanding. The source of northern populations is, however, not southern populations, but rather neighboring northern populations.

So how did the northern population become established? There are a number of possible explanations for the establishment of and subsequent genetic barrier between the two *I. affinis* populations. One possibility is that this population is the result of a founder event, facilitated by the long-distance dispersal of an engorged *I. affinis* female north of South Carolina. Those initial offspring from a female of haplotype AFF17 may have since colonized the rest of the northern portion of the *I. affinis* range, radiating into closely related haplotypes in Clade B (Fig. 3A, Fig. 4A). *Ixodes affinis* feed on a variety of mammalian and avian hosts, but are most commonly found on cervids, canids, and felids as adults (Harrison et al. 2010). These large mammals could transport adult *I. affinis* over long distances; however, such long-distance dispersal events are relatively uncommon for hosts of *I. affinis*, which would account for the lack of genetic exchange between the two populations after the initial establishment. Anthropogenic movement of *I. affinis* on white-tailed deer (*Odocoileus virginianus*) is unlikely, as adult *I. affinis* are not active during the fall and winter seasons when deer are killed and transported by hunters (Oliver et al. 1987).

Other possible explanations for the genetic segregation of the two *I. affinis* populations include haplotype-correlated behavioral or physiological differences that allow increased survival in their respective regions. As *I. affinis* has moved northward along a latitudinal and climatic gradient, adaptations corresponding with haplotype clade may have resulted in differential survival. For example, the Northern and American clades of *I. scapularis* exhibit different host-seeking behaviors and survival ability dependent upon their respective regional clades (Ginsberg et al. 2014, Goddard and Piesman 2006). A third explanation could be the presence of a geographic break in the range of a critical host that dictates the presence or absence of particular haplotypes. For example, the ranges of the northern short-tailed shrew (*Blarina brevicauda*) and the southern short-tailed shrew (*B. carolinensis*) overlap only in the area where northern clade *I. affinis* are found (George et al. 1986, McCay 2001). Field studies on shrews and the genetic makeup of their associated ticks would be needed to test this hypothesis.

We found *I. affinis* haplotype diversity was generally low with few haplotypes present at each site, indicating that only a few individual ticks would be needed to initiate a population. Adult *I. affinis* likely play an important role in forming these populations via short-distance founder events, where one or two engorged adult females might drop off a deer or other

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large mammal. Few of our populations sampled were monohaplotypic, and we documented gene flow between nearby sampling sites, indicating that multiple short-distance dispersal events are needed to establish and maintain populations. *Ixodes affinis* are predominantly dispersed by mammals at all stages of their lives (Harrison et al. 2010). The small mammals that play an important role as hosts for juvenile *I. affinis* have limited vagility, thereby limiting the dispersal potential of immature *I. affinis*. Long distance dispersal events as discussed above would also be relatively uncommon for most hosts of adult *I. affinis*; studies of the role of white-tailed deer in seed dispersal found that most dispersal events were only within hundreds or thousands of meters (Myers et al. 2004). Each of the two *I. affinis* clades is likely to be undergoing demographic expansion through these short-distance dispersal events, assisted by mammals and perhaps non-migratory birds. These short-distance dispersal events would be facilitated by the riparian woodland habitats that *I. affinis* favor, which are abundant in the eastern US and have relatively high connectivity between habitat patches (Griffith et al. 2003).

At present, all populations of *I. affinis* in the US are in Atlantic coastal plain ecosystems (Oliver et al. 2003, Harrison et al. 2010, Nadolny et al. 2011). *Ixodes affinis* are found in secondary successional woodland habitats, and the adults are relatively easy to collect using sampling flags where they are found (Nadolny et al. 2011). It is possible that coastal plains ecosystems provide all the essential resources *I. affinis* need to survive, including common flooding events and abundant woodland habitat, but these ticks are unable to establish outside of these environmental constraints. We suggest that *I. affinis* may yet establish in areas north of Virginia considered part of the Atlantic coastal plain (Auch, 2014).

4.2 Amblyomma maculatum

In contrast to *I. affinis*, the genetic structure of *A. maculatum* indicated that there is low gene flow among sites, including those that are geographically proximate (Fig. 4B, Table 2, Supplemental Table 3). Little evidence of genetic clades was uncovered, and most haplotypes were separated by only 1–3 base pairs (Fig. 3B). Fu's F_S statistic indicates recent population growth of the population as a whole, with star radiations (Fig. 3B) supporting recent expansion of *A. maculatum* throughout the eastern US. Populations at individual sample sites, however, were generally not undergoing expansion, with only a few exceptions. This was contrary to our expectations, as *A. maculatum* has been documented spreading into the mid-Atlantic, presumably from established populations further south (Fornadel et al. 2011, Wright et al. 2011), so we expected to see evidence of northward population expansion.

Previous studies of *A. maculatum* population genetics have generally documented low genetic diversity and high levels of gene flow (Araya-Anchetta et al. 2015). Ferrari et al. (2013) described four 16S mitochondrial rRNA haplotypes in Mississippi and three in North Carolina *A. maculatum*, with little genetic variation between all sampled populations (F_{ST} = 0.02). However, they found strong support for genetic differences between individual sites within regions. A separate study on *A. maculatum* ticks from Texas, Oklahoma and Kansas (Ketchum et al. 2009) reported seven 16S mitochondrial rRNA haplotypes, with low genetic variation among populations that had been established for 20 or more years. Our study was

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performed on a larger scale on younger populations, which could explain why we found moderate genetic variation across the eastern US (Φ_{ST} = 0.15), no isolation by distance, and significant genetic differences between sites.

Each population of *A. maculatum* comprised multiple haplotypes, indicating that multiple females were involved in their formation. Interestingly, we found the same haplotypes widely dispersed throughout the eastern US, indicating that there must be some limited gene flow between even widely separated populations (Fig 4B). In contrast, populations geographically close to one another were not more likely to share haplotypes (Table 2). Only sites with very few individuals sampled, all from Mississippi, exhibited evidence of significant gene flow among populations (Table 2), which may be a statistical artifact of small sample size. Previous studies of *A. maculatum* populations have shown polyphyletic haplotypes and implicated multiple introductions in the genetic diversity observed (Ferrari et al. 2013, Ketchum et al. 2009). Ferrari et al. (2013) have suggested that observed *A. maculatum* inter-population gene flow could be facilitated by long-distance anthropogenic movements of cattle or by migratory birds.

In order for gene flow to occur between geographically distant populations, long distance dispersal must be taking place. Immature *A. maculatum* parasitize many species of birds, which could provide opportunities for long-distance dispersal (Teel et al. 2010). In one study, up to 2.4% of ticks collected from mist-netted songbirds over 2 years were *A. maculatum* larvae and nymphs (Florin et al. 2014). Like *I. affinis*, the adults of *A. maculatum* largely parasitize deer and other large mammals – none of which regularly migrate between northern Virginia and Mississippi. Unlike *I. affinis*, *A. maculatum* nymphs and larvae have been reported from more than 35 species of birds, including migratory species that regularly travel long distances (Florin et al. 2014, Ogden et al. 2008, Teel et al. 2010). Immature *A. maculatum* have been collected from migratory birds in Canada, well outside this species' range (Ogden et al. 2008). Larvae or nymphs arriving at a new area after feeding on birds are more likely to initiate populations with multiple haplotypes than a single adult. This is especially likely considering mixed flocks of birds can support nymphs or larvae that are unlikely to represent identical haplotypes, possibly founding a population with more than one *A. maculatum* haplotype during a migration stop. We found that even new populations in northern Virginia (<2 years old) generally exhibited multiple haplotypes in the adult ticks collected, indicating population establishment by more than one female.

Migratory birds are important agents of long-distance dispersal of other tick species, especially *I. scapularis* as it expands its range northward into Canada (Ogden et al. 2008, Smith et al. 1996). However, models of *I. scapularis* range expansion into Canada suggest that migratory birds are not the sole source of new ticks, with mammalian dispersal resulting in faster population establishment (Leighton et al. 2012). One challenge in understanding how immature bird-borne ticks could initiate new populations is the low likelihood of survival to adulthood and successful reproduction. Ticks spend up to 90% of their lives off-host, surviving environmental stressors and predation (Needham and Teel 1991). Risk of desiccation is a particularly important consideration for ticks of all life stages, and *A. maculatum* is sensitive to low humidity levels (Fleetwood 1985). As such, the range of *A. maculatum* is restricted to warm, moist microhabitats in coastal environments with

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temperate climates and high humidity (Cooley and Kohls 1944, Goddard and Norment 1983, Yoder et al. 1998).

Amblyomma maculatum adults mate on-host with males using pheromones to attract females, which eliminates some reproductive challenges when ticks are at low densities (Gladney 1971, Gladney et al. 1974). Adults are generally considered more likely to establish populations, as one female *A. maculatum* can lay 15,000 eggs (Drummond and Whetstone 1970, Wright 1971). One or more deer arriving at a successional site carrying engorged *A. maculatum* females of several haplotypes could be responsible for founding a genetically diverse new population. However, deer do not disperse far enough to account for the presence of identical haplotypes in geographically distance populations, such as our sampling sites in southern Mississippi and Northern Virginia. We also found no evidence of isolation by distance, which should be evident if there was significant migration between sites that are close to one another. It is likely that some combination of avian and mammalian hosts is responsible for the patterns observed, and additional research will be required to conclusively answer questions of what hosts are important in *A. maculatum* range expansion.

A preference for grassy, often xeric habitat helps to explain why *A. maculatum* populations are so genetically isolated from nearby populations. *Amblyomma maculatum* are often found in grassy, successional habitats (Paddock and Goddard 2015, Wright et al. 2011), which are common but often ephemeral in the southeastern US. Due to a history of fire suppression in the eastern US, anthropogenic actions such as clear-cutting and prescribed burns are the major mechanism by which open habitats are created and preserved. In particular, prescribed burns to recreate lost grassland habitat have been increasing in recent decades, with the unintended result of creating ideal patches of *A. maculatum* habitat (Paddock and Goddard 2015). Burned areas are recolonized quickly by *A. maculatum*, and have even resulted in increased tick populations within a year of a prescribed burn (Scifres et al. 1988). Human manipulation may create patches of ideal habitat in which a particular haplotype may establish and thrive for a sufficient amount of time to be picked up by a traveling host and redistributed across the landscape to another suitable spot.

Populations of *A. maculatum* are patchy, and are likely mediated by local extirpations as these open habitats complete the successional process and become unsuitable for *A. maculatum* and its preferred host community. Small mammals are likely to be important for *A. maculatum* population maintenance in successional habitats, but are not likely to contribute to dispersal due to canopy closure extirpating local rodent populations (Langley and Shure 1980). To facilitate formation of genetically diverse populations, multiple founders must arrive at an appropriate site at the correct time in the successional process when hosts are abundant. However, appropriate mixes of hosts and successional habitat are only available until succession completes, which might not provide sufficient time for subsequent generations of ticks to disperse locally like *I. affinis*. Unless open habitat is maintained by prescribed burning or other intervention, the successional process may swiftly extirpate populations of *A. maculatum*, so the nearest neighbor populations remain genetically and geographically isolated, seeded only by long-distance dispersers. As a result

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of the ephemeral and geographically isolated nature of these populations, one limitation of our study is that we have likely missed many existing populations in our sampling.

Heteroplasmy was discovered in the 16S rRNA gene of 3.5% of sampled *A. maculatum* ticks. As we saw with the resolved haplotypes, heteroplasmy was distributed across the eastern US and was not limited to any site or geographic region. Heteroplasmy has been reported previously in *Amblyomma* ticks; Xiong et al. (2013) described 166 heteroplasmic sites in *A. cajennense*, including 22 sites in the rRNA genes. Heteroplasmy may therefore be common in ticks of the genus *Amblyomma*; recognizing the possibility of heteroplasmy in these ticks is important for accurate population genetics studies.

5. Conclusions

The present study is unique because it compares patterns of genetic structure across two tick species that share many of the same vertebrate hosts, but inhabit different habitats across the eastern US. We found marked differences in population structure, connectivity and isolation, as well as differing levels of genetic diversity. Our evidence suggests that diverse populations of *A. maculatum* are founded via multiple founder events, including long-distance dispersal events, and are maintained in relative isolation by ephemeral assemblages of hosts in successional habitats. We also suggest that the northern *I. affinis* population may have been founded by a rare long-distance founder event, but has since been maintained and expanded via short-distance dispersals by mammalian hosts with small home ranges. Short-distance haplotype differentiation due to external pressures influencing tick survival provides an alternative theory for the separation of the northern and southern populations of *I. affinis*. While host choice is a key ecological factor in tick range expansions, equally important are habitat stability, habitat connectivity and the life stages that are responsible for dispersal. Although there is considerable host overlap at all life stages for *I. affinis* and *A. maculatum*, these other ecological differences result in strikingly different geographic and genetic patterns.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- We compare the population genetics of two invading ixodid tick species
- Despite shared hosts, each tick exhibited unique genetic patterns of connectivity
- *Ixodes affinis* exhibited two genetically and geographically isolated populations
- Each *Amblyomma maculatum* population sampled was genetically distinct
- Habitat connectivity, stability, and host choice are key in tick range expansions

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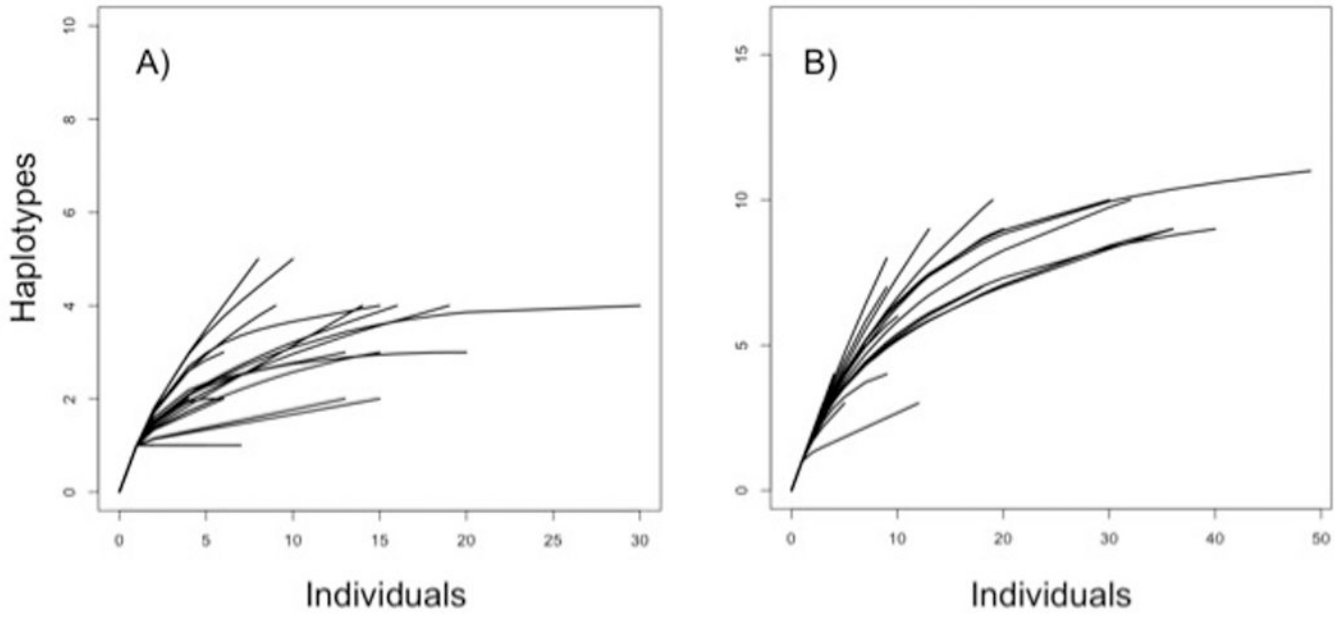

Figure 1.

Haplotype rarefaction curves for A) *Ixodes affinis* and B) *Amblyomma maculatum* using haplotypes instead of species. Each line is representative of a single geographic population from one year, and indicates the number of individuals sampled and the number of haplotypes found.

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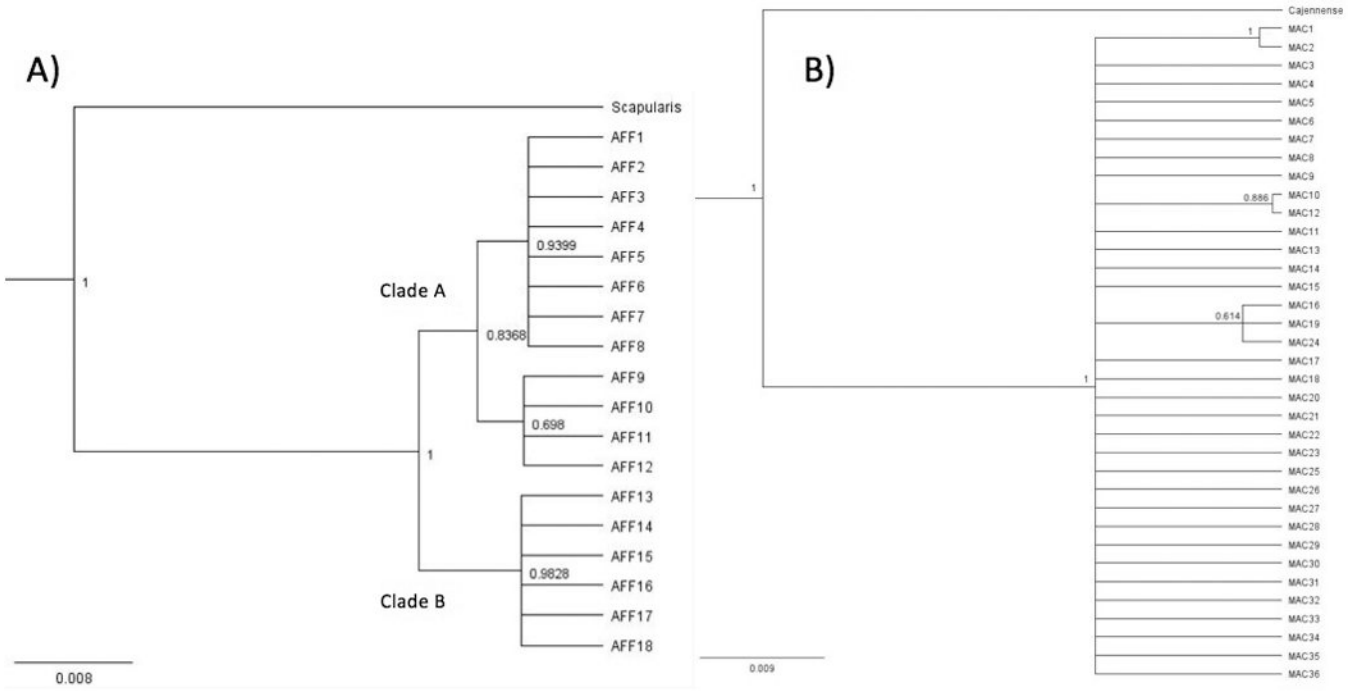

Figure 2.

Phylogenetic relationships among 16S rRNA mitochondrial haplotypes based on Bayesian (BA) inference for *I. affinis* (A) and *A. maculatum* (B). Topology was consistent between neighbor-joining (NJ), maximum-likelihood (ML) and Bayesian (BA) inferences. Branch lengths are according to the indicated scale of nucleotide substitutions per base pair. Node values indicate branch support, based on 1000 replicates. (A) *Ixodes affinis* specimens (n = 236) collected across the southeastern United States resolved in 18 haplotypes (454 base pairs). The evolutionary model that best fit our data was the Tamura 3-parameter model with a discrete gamma distribution (T93+G). A single *Ixodes scapularis* sample (Genbank accession number KF146631) was used to root the tree. (B) *Amblyomma maculatum* specimens (n = 357) collected across the eastern United States resolved in 36 nonheteroplasmic haplotypes (416 base pairs). The Tamura 3-parameter evolutionary model best fit our data. A single *Amblyomma cajennense* sample (Genbank accession number L34317) was used to root the tree.

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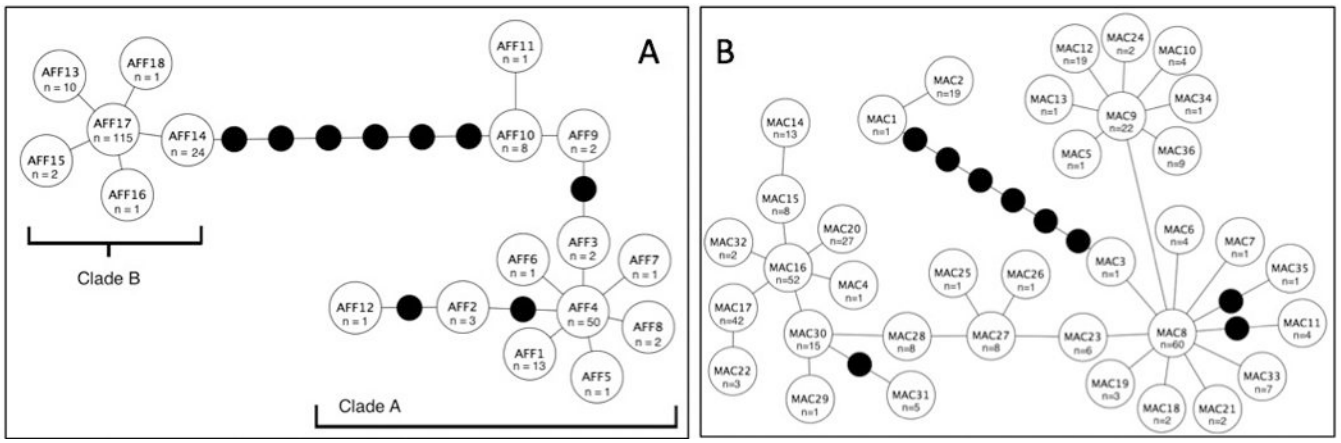

Figure 3.

Minimum spanning trees of (A) 18 *I. affinis* haplotypes (AFF1 – AFF18) and (B) 36 non-heteroplasmic *A. maculatum* haplotypes detected in this study (MAC1 – MAC36). Each labeled circle represents a haplotype that differs by one base pair from a connecting haplotype; black circles indicate the number of additional base pair changes needed to connect two haplotypes. Each haplotype is labeled with the number of individuals with that haplotype. Each haplotype is labeled with the number of individuals with that haplotype. *Ixodes affinis* Clade A haplotypes were mostly found in the south, while Clade B haplotypes were mostly found in the north. We found no strong evidence of clades in our *A. maculatum* samples.

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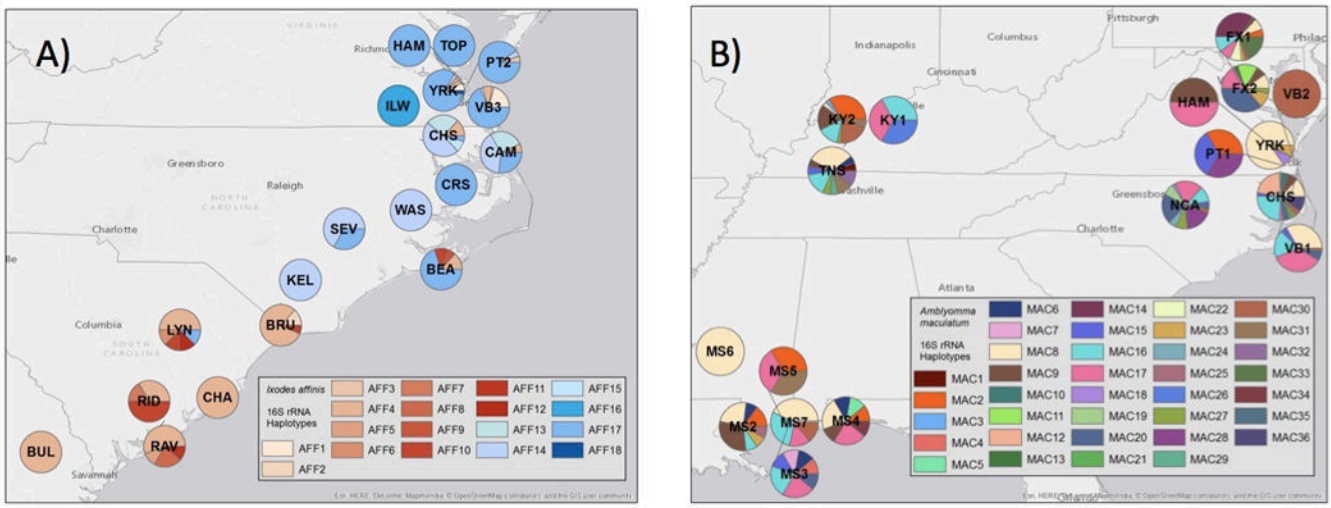

Figure 4.

(A) Map of *I. affinis* haplotypes collected across 19 sites. Red haplotypes are from Clade A (AFF1 – AFF12) and blue haplotypes are from Clade B (AFF13 – AFF18). (B) Map of haplotypes of *A. maculatum* ticks collected across 19 sites. Although there were significant differences in haplotype frequencies between some sites, no geographic pattern was evident.

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Table 1

Pairwise Φ_{ST} and false discovery rate (FDR) adjusted q values for *I. affinis*. Columns and rows are arranged from northernmost site to southernmost site.

	YRK (n=31)	PT2 (n=21)	VB3 (n=54)	CHS (n=23)	CAM (n=15)	BEA (n=30)	BRU (n=15)	LYN (n=8)	CHA (n=7)	RID (n=6)	RAV (n=9)	BUL (n=5)
YRK		0.6554	0.1471	0*	0*	0.1642	0*	0*	0*	0*	0*	0*
HOC	−0.0146		0.0084*	0*	0*	0.0499*	0*	0*	0*	0*	0*	0*
VB3	0.0216	0.0774		0*	0*	0.0386*	0*	0*	0*	0*	0*	0*
STP	0.4684	0.5164	0.3944		0.5341	0*	0*	0*	0*	0*	0*	0*
CAM	0.3412	0.4035	0.2666	−0.0083		0*	0*	0*	0*	0*	0*	0*
BEA	0.0231	0.0622	0.0333	0.3754	0.2484		0*	0*	0*	0*	0*	0*
BRU	0.6477	0.7381	0.5270	0.3837	0.4165	0.5029		0.0653	0.5139	0.0084*	0.0326*	0.6107
WOB	0.4542	0.5548	0.3316	0.2039	0.1837	0.2732	0.0577		0.0318*	0.3569	0.5732	0.2278
TIB	0.7363	0.8628	0.5994	0.4631	0.5058	0.5909	0.0177	0.1905		0.0184*	0.0791	0.9879
GIF	0.5557	0.6605	0.4319	0.2503	0.2442	0.3679	0.2722	0.0131	0.4815		0.1525	0.0326*
CAW	0.5442	0.6353	0.4254	0.2431	0.2511	0.3872	0.0678	−0.0239	0.1771	0.1258		0.2634
BUL	0.7232	0.8522	0.5859	0.4336	0.4687	0.5706	−0.0233	0.1304	0	0.4128	0.1211	

Above the grey cells with stars are FDR adjusted q values, those that are significantly different are marked with *. Below are pairwise Φ_{ST} values for all sites with ≥ 5 ticks.

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Table 2

Pairwise Φ_{ST} and false discovery rate (FDR) adjusted q values for *A. maculatum*.

Site	VB1 (n=48)	YRK (n=13)	TNS (n=19)	FX1 (n=38)	FX2 (n=30)	MS2 (n=13)	MS3 (n=9)	MS4 (n=9)	MS7 (n=7)	NCA (n=38)	CHS (n=85)	KY2 (n=35)
VB1		0*	0.0234*	0.0234*	0.1167	0*	0.4836	0.0234*	0.4675	0*	0*	0*
YRK	0.4369		0*	0*	0*	0*	0*	0*	0.0118*	0*	0*	0*
TNS	0.0705	0.2293		0.0118*	0.0438*	0.0840	0.1285	0.1277	0.8918	0*	0.0118*	0.0334*
FX1	0.0432	0.3497	0.0854		0*	0*	0.1277	0.0626	0.2557	0*	0*	0*
FX2	0.0374	0.3563	0.0540	0.0954		0*	0.3134	0.0334*	0.4675	0.0371*	0*	0*
MS2	0.2294	0.1085	0.0500	0.1526	0.1714		0*	0.7781	0.0969	0*	0.0334*	0*
MS3	−0.0235	0.5769	0.0360	0.0298	0.0150	0.2092		0.0118*	0.6166	0.2779	0.0334*	0.0371*
MS4	0.1496	0.2251	0.0497	0.0739	0.1206	−0.0600	0.1436		0.2140	0*	0.3960	0.0277*
MS7	−0.0407	0.4707	−0.0719	0.0165	−0.0233	0.0722	−0.0513	0.0331		0.3576	0.2291	0.1703
NCA	0.0648	0.4334	0.0798	0.1059	0.0355	0.2498	0.0194	0.2096	0.0048		0*	0*
STP	0.0894	0.2020	0.0772	0.0870	0.0960	0.0632	0.1280	−0.0024	0.0215	0.1644		0*
KY2	0.2085	0.3360	0.0990	0.1715	0.1846	0.1166	0.1363	0.1186	0.0936	0.2033	0.2251	

Above the grey cells with stars are FDR adjusted q values, those that are significantly different are marked with *. Below are pairwise Φ_{ST} values for all sites with ≥ 5 ticks.