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Analysis of Genetic Structure and Pathogen Dynamics of Ixodes scapularis in Southwestern Virginia

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Abstract

Ixodes scapularis, or the blacklegged tick, is the primary vector of *Borrelia burgdorferi*. This pathogen is the causative agent of Lyme disease, the most common vector-transmitted disease in the United States. Although *I. scapularis* is distributed throughout the eastern U.S., Lyme disease is only considered endemic in the northeastern region of the country. Prior to 2007, Lyme disease was uncommon in Virginia, but since then cases of Lyme disease have increased dramatically with a hotspot forming in the Roanoke-Blacksburg area. The purpose of the current study was to determine whether *B. burgdorferi* prevalence and the genetic structure of *I. scapularis* populations in Southwest Virginia are unique from other regions of the state. By testing for *Borrelia* spp., we also determined whether *B. miyamotoi*, a newly-emerging pathogen, is present in the region. A phylogenetic analysis using the 16S mitochondrial rRNA gene was completed with *I. scapularis* nymphs and adults (n=48) from four sites in southwestern Virginia. *Borrelia* spp. testing of *I. scapularis* nymphs (n=291) was also done via nested polymerase chain reaction (PCR). Genetic analysis demonstrated that all *I. scapularis* fell within the American clade, primarily associated with northern populations of *I. scapularis*, and yielded 16 different haplotypes, 9 of which were unique within this analysis. These findings suggest expansion of American clade ticks from the northeastern United States into Virginia. Meanwhile, there was an overall *B. burgdorferi* prevalence of 4.5%, which is lower than what has been previously reported in Lyme endemic areas. Interestingly, one nymph tested positive for *B. miyamotoi*, which is the first published report of *B. miyamotoi* in *I. scapularis* in southwestern Virginia. Overall, this study provided evidence indicating that the southern front of Lyme disease is expanding, and gave valuable insights into Lyme disease ecology in an emerging hotspot in southwestern Virginia.

Introduction

All human-biting ticks experience three life stages: larvae, nymph and adult. Between each life stage, ticks must obtain a blood meal from a human or animal host in order to continue their life cycle (Kocan et al. 2015). This behavior makes ticks vectors of numerous pathogens capable of infecting humans and/or animals. One such tick species is *Ixodes scapularis*, or the blacklegged tick, which has major public health significance in the eastern and upper midwestern United States. Pathogens transmitted by *I. scapularis* include, but are not limited to, *Borrelia burgdorferi* (the causative agent of Lyme disease), *Anaplasma phagocytophilum* (the causative agent of anaplasmosis), and *Babesia microti* (the causative agent of babesiosis) (Kocan et al. 2015).

A newly-emerging pathogen transmitted by *I. scapularis* here in the United States is *Borrelia miyamotoi*. It was discovered in Japan in 1994 from *Ixodes persulcatus* (Krause et al. 2015). However, it was not until 2000 that *B. miyamotoi* was identified in *I. scapularis* ticks in the United States (Scoles et al. 2001, Bunikus et al. 2004). Meanwhile, the first case of human infection with *B. miyamotoi* was reported in Russia in 2011, and the first case in the U.S. was reported in 2013. Two studies have been published on *B. miyamotoi* found in residents of southern New England. The studies found a 1% (n=584) and a 3.9% (n=639) prevalence of *B. miyamotoi* respectively (Krause et al. 2013, Krause et al. 2014). The identification of *B. miyamotoi* infection-antibodies in study patients suggests that *B. miyamotoi* may be more widespread than was initially thought (Krause et al. 2013, Krause et al. 2014). The symptoms of *B. miyamotoi* infection include fever, fatigue, headache, chills, myalgia, and arthralgia (Krause et al. 2015), which are very similar to the symptoms of Lyme disease. This has led to speculation that many *B. miyamotoi* cases may have been misdiagnosed as Lyme disease.

Peromyscus leucopus (white-footed mouse) the primary reservoir of *B. burgdorferi* has also been found to be a competent reservoir of *B. miyamotoi* in the northeastern United States (Scoles et al. 2003, Barbour et al. 2009). Other species, including birds, may serve as reservoirs for *B. miyamotoi* as well (Hamer et al. 2012). These findings suggest that the reservoir hosts of *B. miyamotoi* may be similar to the reservoirs for *B. burgdorferi* (Wagemakers et al. 2016). Collectively, the high seroprevalence of *B. miyamotoi* in residents in New England, in conjunction with the initial understanding of reservoir hosts, has led to speculation that *B. miyamotoi* may have a similar distribution in the U.S. to *B. burgdorferi*. However, more studies are needed to explore this hypothesis.

Unlike *B. miyamotoi,* of which little is definitively known in the United States, Lyme disease is the most common vector-transmitted disease in the U.S. (Bacon et al. 2008). It was first recognized in the United States in 1975, and since its discovery, the amount of Lyme disease cases in the country consistently increased. From 1992 to 2006, a total of 248,074 cases were reported to the Centers for Disease Control and Prevention (Bacon et al. 2008). Subsequently, from 2008 to 2015, there were a total of 275,589 cases reported. Most of the cases continued to be reported from states with high incidence in the northeastern United States, and case counts have remained stable or decreased in these areas (Schwartz et al. 2017). Conversely, during this same time period, case counts have increased in states that neighbor those with high incidence (Schwartz et al. 2017).

In acute Lyme disease infection, patients experience flu-like symptoms including fever, cough, sore throat, runny nose, muscle aches, and fatigue. In 70-80% of cases, these symptoms may be preceded by an erythema migrans rash. In later stages patients may experience cranial neuropathies, meningitis, carditis, lymphadenopathy, and arthralgia (Krause et al. 2015). In terms

of Lyme disease ecology, *B. burgdorferi* is not transovarially transmitted (from adult to larvae), so larval ticks do not emerge from eggs infected with the pathogen (Nelder et al. 2016). Thus, *B. burgdorferi* is transmitted to humans by the bite of an *I. scapularis* nymph or adult that has acquired the infection during a blood meal on an infected host as a larva or nymph. As previously mentioned, the white-footed mouse is the principal reservoir for the pathogen. However, *I. scapularis* nymphs also feed on other rodent species, mesomammals (such as raccoons (*Procyon lotor)* and Virginia opossums *(Didelphis virginiana)*), white-tailed deer (*Odocoileus virginianus* and humans (Nelder et al. 2016).

Despite *I. scapularis* and its principal reservoir being distributed throughout the United States, Lyme disease is most common in the Northeast and Upper Midwest (Nelder et al. 2016) and is rare in the Southeast. Accordingly, the northeastern and upper midwestern regions of the U.S. tend to have a higher prevalence of *B. burgdorferi* in *I. scapularis* and in reservoir hosts and higher *I. scapularis* abundance (Sonenshine et al. 1995). One hypothesis for the difference in Lyme disease dynamics between the northeastern and southeastern United States is variation in *I. scapularis* host selection (Arsnoe et al. 2015). Specifically, populations of *I. scapularis* in the Southeast usually feed on a variety of lizard species which are not competent reservoirs for *B. burgdorferi* (Apperson 1993, Kollars et al. 1999, Durden et al. 2002). Conversely, northern populations of *I. scapularis* larvae and nymphs tend to feed on small mammals like the white-footed mouse, which is a competent reservoir for *B. burgdorferi* (Arsnoe et al. 2015). Thus, variation in host selection of *I. scapularis* ticks in the northeastern U.S. and southeastern U.S. could explain the higher incidence of Lyme disease in the Northeast.

Another hypothesis for the greater incidence of Lyme disease in the northeastern versus southeastern United States, which is not mutually exclusive of the first hypothesis, is variation in

vector behavior. *I. scapularis* nymphs from northern populations tend to quest more aggressively, meaning that they more frequently quest higher on vegetation. Southern populations, however, tend to remain on or below the leaf litter (Arsnoe et al. 2015, Sakamoto et al. 2014, Duik-Wasser et al. 2010). Consequently, nymphs from northern populations are more likely to come in contact with humans.

In terms of genetics, there are two distinct lineages of *I. scapularis*, the American clade and the Southern clade. Genetic analyses have indicated that the American clade originates from the Southern clade. Specifically, these analyses demonstrate that *I. scapularis* ticks in the South recolonized northern America after the most recent glaciation event (Norris et al. 1996, Van Zee et al. 2012, Humphrey et al. 2012). Therefore, the American clade, although less genetically diverse due to founder effect, is now widely distributed throughout the northern United States, but has also continued to have individuals in the South. The Southern clade, however, is more genetically diverse, but only exists in the South (Norris et al. 1996).

Importantly, there is some question as to whether questing behavior is genetic or the result of differences in the environment. Findings of Arsnoe et al. (2015), suggest that *I. scapularis* nymphs' questing behavior is genetically driven. However, while aggressive questing behavior is associated with *I. scapularis* populations in the Northeast and Upper Midwest, it was not associated with all *I. scapularis* in the American clade, indicating that some other unknown gene or genes are driving this behavior (Arsnoe et al. 2015).

As stated earlier in this paper, Lyme disease case counts have increased in states that neighbor those with high incidence (Schwartz et al. 2017), and Virginia is one of those neighboring states. Prior to 2007, Lyme disease was uncommon in Virginia. However, the number of confirmed cases nearly tripled from 2006 to 2007 (Bacon and Kugeler 2008), and has

continued to increase (Brinkerhoff et al. 2014). This suggests that the southern front of Lyme disease infection may be expanding. Specifically, Lantos et al. (2015) reported that between 2000 and 2006, Virginia had registered fewer than 400 cases per year. However, by 2014 the annual case count had increased to 1,346. Furthermore, the largest annual increase was seen in southwestern Virginia with a distinct hotspot in the Roanoke-Blacksburg area (Lantos et al. 2015).

To better understand *I. scapularis* dynamics in Virginia, Brinkerhoff et al. (2014) conducted a study examining *I. scapularis* genetic structure and *B. burgdorferi* prevalence across an east-west gradient in Virginia. They detected 17 different haplotypes of the 16S mitochondrial rRNA gene from 85 different nymphs, all falling within the American clade (Brinkerhoff et al. 2014). Their findings suggest that there has been recent spatial and/or demographic expansion of *I. scapularis* in Virginia, thus supporting the hypothesis of a north-to-south migration of American clade ticks into Virginia from the Northeast. This would potentially provide an explanation for the increased human exposure to *B. burgdorferi* in Virginia, but the possibility that the distribution of endemic American clade ticks has expanded in Virginia could not be completely excluded (Brinkerhoff et al. 2014). Notably, Brinkerhoff et al. (2014) obtained a significantly higher prevalence of *B. burgdorferi* from their most western site, leading to speculation that *B. burgdorferi* prevalence may be higher in the western portion of the state. However, their study only had a single site in western Virginia, so more sites in western Virginia need to be tested, particularly in southwestern Virginia which was not tested in their study.

Despite there being a Lyme disease hotspot in southwestern Virginia, few studies on tick ecology have been done within the region (Morris 2019, Herrin et al. 2014). Initial findings from Morris (2019) suggest that *I. scapularis* nymphs exhibit more aggressive questing behaviors

associated with northern populations (Morris 2019), and this aggressive questing behavior may be contributing to the high Lyme disease incidence in the region. The only other study in the southwestern Virginia region (Herrin et al. 2014) tested adult *I. scapularis* for *B. burgdorferi*, but studies on nymphs need to be conducted as well. Specifically, nymphs are more epidemiologically important for the transmission of Lyme disease to humans due to their small size, which makes them less likely to be seen and removed before they pass on the pathogen. Furthermore, additional research is needed to investigate the hypotheses from Brinkerhoff et al. (2014) that 1) *I. scapularis* are migrating southwards into Virginia from the northeast and 2) high *B. burgdorferi* prevalence may also be contributing to the high Lyme incidence in southwestern Virginia.

Thus, to gain better insight into *I. scapularis* dynamics and the drivers behind high Lyme disease incidence in southwestern Virginia, the current study utilized *I. scapularis* ticks collected by Morris (2019) to determine whether *Borrelia* spp. prevalence and the genetic structure of *I. scapularis* populations in southwestern Virginia are unique from other regions of the state. By testing for *Borrelia* spp., we also determined whether *B. miyamotoi* is present in the region. To do this work, nested polymerase chain reaction (PCR) testing for *Borrelia* spp. was conducted along with genetic analyses of the *I. scapularis* 16S mitochondrial rRNA gene. These 16S sequences were then compared to sequences of *I. scapularis* ticks elsewhere in Virginia (Brinkerhoff et al. 2014, Brinkerhoff unpublished data) and in the eastern United States. Ultimately, these data will help provide a better understanding of the rapidly changing Lyme disease dynamics in Southwest Virginia.

Methodology

Tick Collection and Species Identification. Morris (2019) previously collected and identified the ticks for this study. Ticks were collected on a monthly basis between February 2018 and January 2019 at twelve different sites in deciduous forests in the Roanoke Valley (Figure 1) region of Virginia via flagging. Prior to identification and extraction, ticks were stored in 70% ethanol. *I. scapularis* adults and nymphs were identified morphologically to species and genus using dissection keys. 16S mitochondrial rRNA gene sequencing was used to confirm the species of all nymphs. DNA extractions were performed on the nymphs using the Qiagen DNeasy Blood and Tissue Extraction kit (Qiagen, Valencia, CA, USA) per the manufacturer's protocols. A 280 bp region of the *I. scapularis* 16S mitochondrial rRNA gene was amplified by PCR and verified on agarose gels. Successfully amplified PCR products were excised from the agarose gel and extracted using a Qiagen QIAquick Gel Extraction kit (Qiagen, Valencia, CA, USA) and sent for sequencing at Eurofin Genomics (Eurofins Genomics LLC, Louisville, KY, USA).

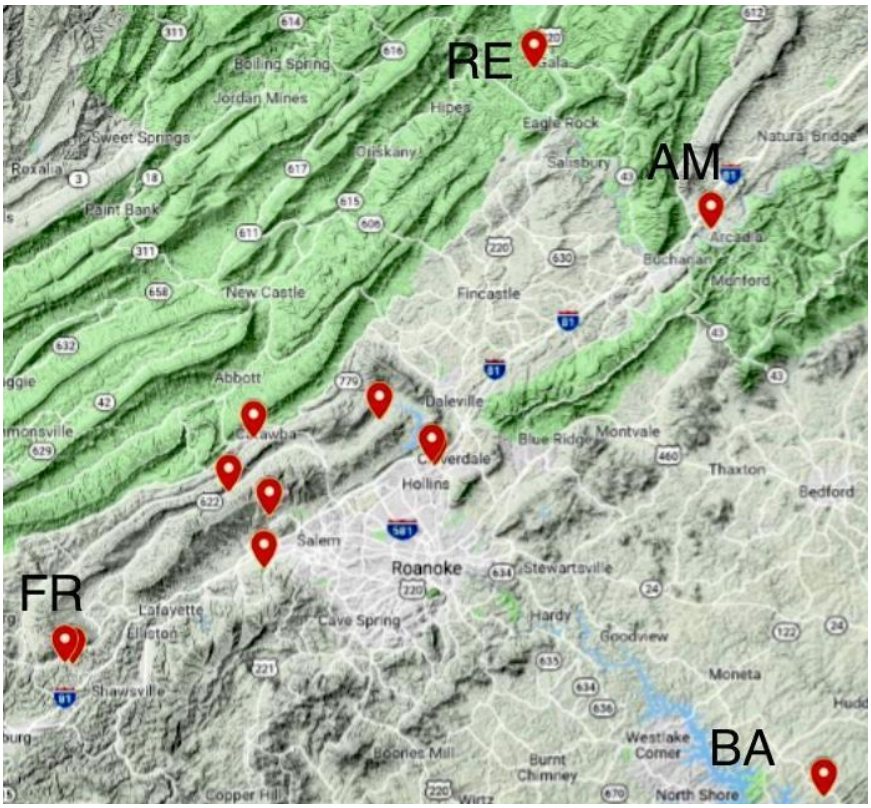

Figure 1. Map image of the 12 collection sites in the Roanoke Valley. The four sites in which ticks were used for genetic analysis were Falls Ridge Preserve, Bourassa State Forest, Private Property 1, and Private Property 2, (FR, BA, AM and RE respectively) thus representing the outermost sites of our study region. Note that all I. scapularis nymphs collected from the 12 sites were used in the pathogen testing.

Genetic Analysis. The ticks from the four sites determined to represent the outermost sites in our study region were used for the genetic analysis: Falls Ridge Preserve, Bourassa State Forest, Private Property 1, and Private Property 2, (FR, BA, AM and RE respectively) (Figure 1). This decision was made because the farther the sites were from each other, the more genetic differences we expected to find, hopefully capturing a representative snapshot of the genetic diversity within our region. Twenty ticks (10 nymph, 5 male adults, and 5 female adults) per site were tested when possible. At sites with fewer than 20 *I. scapularis*, all *I. scapularis* adults and nymphs collected from the site were tested.

Because DNA was not previously extracted from adult ticks, DNA extractions were performed on them as previously outlined above and stored at -20°C until PCR was performed.

For all ticks, a PCR targeting the mitochondrial 16S rRNA gene using 16S +1 and 16S-1 primers (Black and Piesman 1994) was used. Note that the 16S protocol used in the current study is different from the protocol used by Morris (2019) in species identification. Whereas a 280 bp region of the *I. scapularis* 16S ribosomal RNA gene was amplified by Morris (2019), the primers used in the current study amplified a larger, 411 bp region. The 280 bp region used by Morris (2019) was sufficient for confirming species identity, but a larger region was desirable for better detecting haplotype differences within the *I. scapularis* species. Additionally, this particular region of the 16S rRNA gene sequence was used so that our results would be comparable to Brinkerhoff et al.'s (2014) findings. Successfully amplified PCR products were excised from the agarose gel and stored at -20°C until they were extracted using a Qiagen QIAquick Gel Extraction kit (Qiagen, Valencia, CA, USA) and sent for sequencing at Eurofin Genomics (Eurofins Genomics LLC, Louisville, KY, USA). Using Sequencer 5.4 (Sequencher ®, Ann Arbor, MI, USA), sequences were trimmed, identity of ambiguous bases were confirmed, and bidirectional sequences were used to create a continuous sequence. Sequences were then aligned to determine unique haplotypes at our sites. Shannon Diversity Index (SDI) values were calculated using haplotypes in lieu of species as a measure of genetic diversity at each site. Bidirectional sequences were also aligned with reference sequences from *I. scapularis* collected elsewhere in Virginia (Brinkerhoff et al. 2014, Brinkerhoff unpublished data) along with other sequences from throughout the United States which were downloaded from GenBank (GenBank accession numbers KF146631-47). MEGA-X 64 (http://www.megasoftware.net) was used to select among various evolutionary models, which were then used to guide the creation of two maximum-likelihood phylogenetic trees: one tree containing sequences exclusively from the

current study and the other, containing both sequences from this study and sequences from throughout the United States, including elsewhere in Virginia.

Pathogen Testing. Pathogen testing was performed on all 291 *I. scapularis* nymphs collected from all 12 sites by Morris (2019). Due to time constraints, we chose to focus solely on nymphs since they are more likely to successfully transmit *B. burgdorferi* to humans. A nested PCR protocol was used (Barbour et al. 1996). The primers used for the primary reaction were the FLALL and FLARL primers, and primers used for the secondary reaction were the FLALS and FLARLS primers. These primers were used to target the *B. burgdorferi* flagellin and 16S rRNA genes of *Borrelia* spp. Negative PCR controls were used as described above. Positive controls consisting of *Borrelia turicatae* DNA were also used in each series of PCR reactions. Also, different designated rooms were used for primary and secondary PCR reactions. All positive samples were excised from the agarose gel and stored at -20°C until they could be extracted and sequenced as outlined above.

Results

From the 291 nymphs collected, *Borrelia* spp. was detected in 13 nymphs (n=291), demonstrating an overall prevalence of 4.5% (Table 1). Of the 13 nymphs that were *Borrelia* spp. positive, 11 were *B. burgdorferi* (3.8%), and one was *B. miyamotoi* (0.3%). One sequence tested positive for *Borrelia* spp., but was not sequenceable. For the GH site at which the single *B. miyamotoi*-positive tick was detected, there was a 5.6% prevalence of *B. miyamotoi*. The site that had the highest *B. burgdorferi* prevalence was BN (12.5%), while BA, PA, HA, CC, GY, and RE sites had no positives.

Table 1: Borrelia spp. prevalence among I. scapularis nymphs collected at eleven southwestern Virginia sites.

Site	# Nymphs tested	# *Borrelia* Positives (% positive)	# *B. burgdorferi* Positive (% positive)	# *B. miyamotoi* Positives (% Positive)
AM	143	6 (4.2)	5 (3.5)	0 (0)
BA	7	0 (0)	0 (0)	0 (0)
CR	39	2 (5.1)	2 (5.1)	0 (0)
FR	16	1 (6.3)	1 (6.3)	0 (0)
BN	16	2 (12.5)	2 (12.5)	0 (0)
GH	18	2 (11.1)	1 (5.6)	1 (5.6)
PA	20	0 (0)	0 (0)	0 (0)
HA	21	0 (0)	0 (0)	0 (0)
CC	5	0 (0)	0 (0)	0 (0)
GY	2	0 (0)	0 (0)	0 (0)
RE	4	0 (0)	0 (0)	0 (0)
Total	291	13 (4.5)	11 (3.8)	1(0.3)

For the phylogenetic analyses, 52 *I. scapularis* nymphs and adults were initially sent out for sequencing and 4 were left out of the analysis due to sequence ambiguities. Thus, 48 ticks were used for the phylogenetic analyses. A maximum-likelihood phylogenetic tree was constructed using the Hasegawa-Kishino-Yano method with discrete Gamma distribution (+G) and assuming that a fraction of the sites are evolutionarily invariable (+I) (Figure 2). This phylogenetic tree included only *I. scapularis* tick sequences obtained in the current study. Analysis of *I. scapularis* mitochondrial 16S rRNA gene sequences yielded 16 haplotypes from 48 individuals (Figure 2). *I. scapularis* ticks from the AM site exhibited 8 haplotypes, the FR site had 5 haplotypes, RE site had 4 haplotypes, and the BA site had 4 haplotypes. Sample 14-23 represents a haplotype displayed by 27 individual ticks (10 from AM, 13 from FR, 3 from BA, and 1 from RE), 12-18 represents a haplotype displayed by 3 ticks (1 from RE, 1 from AM, and 1 from FR), 28-1 represents a haplotype displayed by 2 ticks (1 from RE, and 1 from AM), 10-19 represents a haplotype displayed by 2 ticks (2 from AM), and 6-2 represents a haplotype

displayed by 2 ticks (2 from BA). All other haplotypes were represented by an individual tick. Finally, the AM site displayed the most haplotype diversity with a Shannon Diversity Index (SDI) of 1.475. Of note, this site also had the highest number of ticks positive for *B. burgdorferi* and highest *I. scapularis* abundance, representing 49.1% of the ticks tested, but did not have the highest *B. burgdorferi* prevalence (3.5%) A second maximum-likelihood phylogenetic tree was constructed using the Tamura Nei method with discrete Gamma distribution (+G) (Figure 3). This phylogenetic tree included all of our sequences as well as *I. scapularis* sequences obtained from elsewhere in the United States and Virginia. All *I. scapularis* haplotypes from our study sites fell within the American clade. There were eight haplotypes (10-12, 24-17, 14-23, 10-19, 28-1, 24-17a, 12-18, 24-13a) that had already been found in eastern Virginia (Brinkerhoff et al. 2014). The rest of the haplotypes were different from all other sequences included in the analysis.

Table 2. Number of haplotypes obtained from each of the four sites included in the phylogenetic analysis and Shannon Diversity Index (SDI) values by site. Note that higher SDI values indicate more haplotype diversity.

Site	# Ticks Tested	# Haplotypes present (# Haplotypes / # Ticks Tested	Shannon Diversity Index Value
AM	20	8 (0.4)	1.474
BA	8	4 (0.5)	1.234
FR	20	6 (0.3)	0.879
RE	4	4 (1.0)	1.386

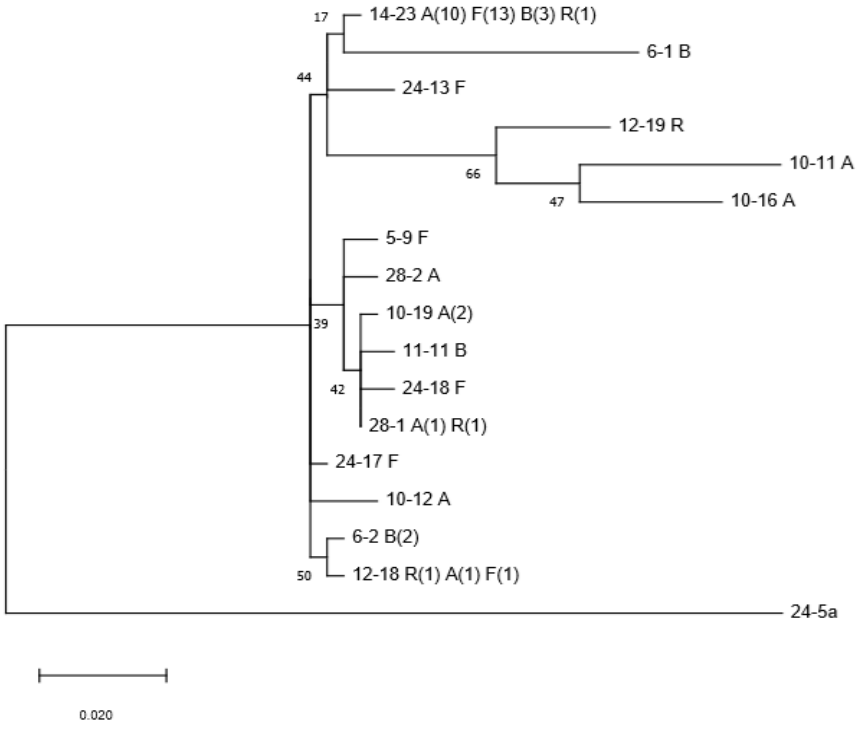

Figure 2. Maximum-likelihood phylogenetic reconstruction of I. scapularis lineages based on the 16S mitochondrial rRNA sequences from the four sites included in this portion of the study (AM [A], BA [B], FR [F], RE [R]) using Tamura Nei +G+I model. 24-5a is an Ixodes brunneus adult serving as an outgroup sequence on this tree. Numbers in parentheses indicate the number of individual ticks with the haplotype at that site. Bootstrap values are based off of 1000 replicates.

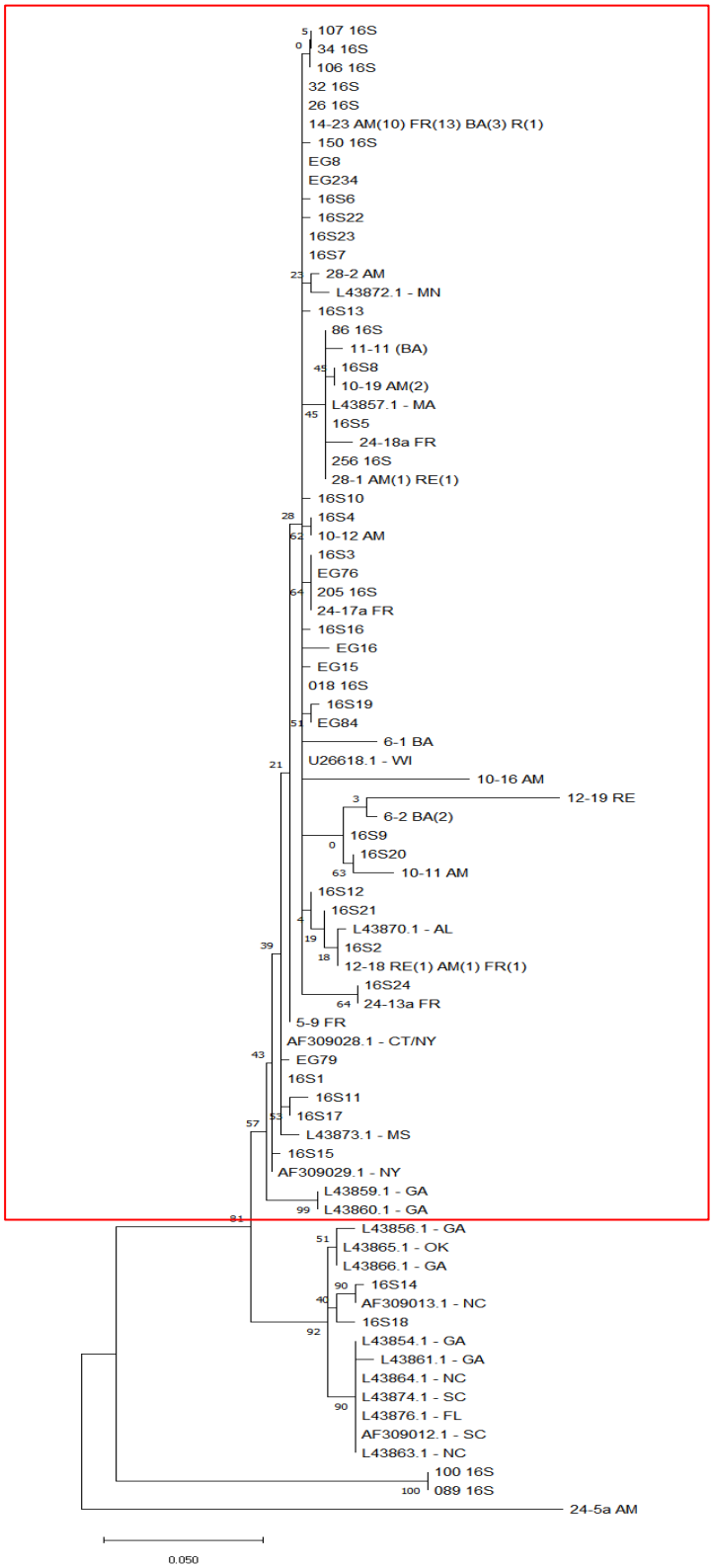

Figure 3. Maximum-likelihood phylogenetic reconstruction of I. scapularis lineage based on 16S mitochondrial rRNA gene sequences using Tamura Nei + G model. Reference sequence GenBank accession numbers are indicated. Samples from Virginia are indicated by the inclusion of 16S or EG in the name, representing samples originating from the American Clade. Sequences following the template 14-23 (a number - another number) represent sequences obtained in the current study. Numbers in parentheses next to these sequences indicate the number of individual ticks with that haplotype per site. The American clade is indicated by the red box. All other sequences (except 24-5a which is an Ixodes brunneus tick serving as an outgroup) represents ticks originating from the Southern clade. Bootstrap values are based off of 1000 replicates.

Discussion

This is the first known study of *Ixodes scapularis* genetics in southwestern Virginia and only the second study in the region of *Borrelia burgdorferi* prevalence. There was a 4.5% prevalence of *B. burgdorferi* in all *I. scapularis* nymphs collected in the current study. The only other study conducted in southwestern Virginia on *B. burgdorferi* (Herrin et al. 2014) detected a prevalence of 32.9% (minimum 31.7%; maximum 35.7%) in *I. scapularis* adults. Because of the high prevalence of *B. burgdorferi* outside the area traditionally considered endemic for Lyme disease, Herrin et al. (2014) suggested that this indicated a recent expansion of Lyme into the southern Appalachian region. Importantly, Herrin et al. (2014) exclusively tested adult *I. scapularis* ticks, which generally have higher prevalence of pathogens when compared to nymphs because they have had one additional blood meal than nymphs and therefore one more opportunity to acquire the pathogen. Thus, the results of Herrin et al.'s (2014) study are not directly comparable to this one.

The current study focused on nymphal *Ixodes scapularis* due to the fact that they are more often associated with human Lyme disease cases because of their small size, which makes them difficult to detect and remove in a timely manner. Also, nymphs emerge in the spring and early summer, which coincides with typical human outdoor activity, as opposed to adult *I. scapularis* which are active in the late fall through the winter when fewer people are outdoors (Reynolds and Brown 2017). Importantly, Brinkerhoff et al. (2014) did test *I. scapularis* nymphs from throughout Virginia and obtained a higher overall *B. burgdorferi* prevalence of 15% as compared to our findings. Furthermore, the most western site from Brinkerhoff et al. (2014) had a significantly higher prevalence of *B. burgdorferi* (20%) than their other sites (0%, 0%, and 6%) located in central and eastern Virginia. However, there were no sites in Southwest Virginia

where the Lyme disease hotspot is found. Thus, although Brinkerhoff et al. (2014) provides useful information regarding Lyme ecology in Virginia, our data offer additional insight into Lyme ecology in southwestern Virginia.

Though Lyme disease is reported in almost every state, *B. burgdorferi* and *I. scapularis* are endemic to the northeastern United States (Barbour et al. 1993, Peppin et al. 2012, Centers for Disease Control and Prevention 2013). Therefore, areas located in the northeastern United States tend to have higher case numbers of Lyme disease and higher prevalence of *B. burgdorferi* in *I. scapularis* ticks. Specifically, Nelder et al. (2016) reported that on average, *B. burgdorferi* was most prevalent in *I. scapularis* ticks in the Northeast. New Hampshire (52%), Maine (49.1%), Minnesota (47.2%), Indiana (45.3%), Michigan (39.5), Illinois (33.9%) and New York (31.6%) displayed the highest *B. burgdorferi* prevalence in the study. These results are reinforced by several other studies conducted throughout the northeastern United States. For example, a study located in Pennsylvania found that adult *I. scapularis* ticks had a 61% prevalence of *B. burgdorferi* at their most northern site (Courtney et al. 2004), and another study conducted in New Jersey found a 33.6% prevalence of *B. burgdorferi* infection in *I. scapularis* ticks (adults and nymphs, Martin et al. 2004), which is similar to the 32.9% prevalence found by Herrin et al. (2014) in *I. scapularis* adults in southwestern Virginia. In New York, Aliota et al. (2014) found that in all of the *I. scapularis* nymphs they collected (n=67), *B. burgdorferi* had the highest prevalence of infection at 67% (Aliota et al. 2014). Similarly, in a study by Gatewood et al. (2009) which used several locations across the northeastern United States, a relatively high prevalence of *B. burgdorferi* was found in their adult *I. scapularis* (33.2%), and these results are also similar to the prevalence found by Herrin et al. (2014). However, the prevalence of *B. burgdorferi* in nymphs was significantly lower (12.5%) (Gatewood et al. 2009). While this is still

higher than the prevalence found in the current study, it is consistent with what was found by Brinkerhoff et al. (2014) in their most western site.

Conversely, in the southeastern United States, *B. burgdorferi,* along with some other pathogens vectored by *I. scapularis* such as *Babesia* spp., are not considered endemic (May et al. 2014). Therefore, *B. burgdorferi* prevalence is much lower and sometimes non-existent in this region of the country. For example, a study conducted in Tennessee found that no *I. scapularis* ticks collected were positive for *Borrelia* spp. (Mays et al. 2014). Similarly, a study conducted by Gleim et al. (2019) in Georgia detected no *B. burgdorferi* infection in the *I. scapularis* collected. In studies conducted in the Southeast where *B. burgdorferi* was detected, the prevalence was still much lower than the prevalence of *B. burgdorferi* infection that have been found in the Northeast. In the Outer Banks of North Carolina, *B. burgdorferi* infection in ticks ranged from 7- 14% in questing adult *I. scapularis* (Levine et al. 2017). From these data, it is obvious that there is a trend of high *B. burgdorferi* prevalence in the northeastern United States where *B. burgdorferi* is endemic, and a low prevalence of *B. burgdorferi* in nonendemic areas like the southeastern United States. Studies of the northeastern and the southeastern U.S. show that Virginia is serving as the frontline of southern expansion of Lyme disease. Thus, we have regions of the state that are in transition and may be exhibiting prevalence somewhere between what we see in the Northeast and Southeast, southwestern Virginia being an example of this.

The high *B. burgdorferi* prevalence found in western Virginia in previous studies (more in line with the prevalence seen in the northeastern U.S.) in conjunction with the increase in Lyme cases indicates that *B. burgdorferi* may be becoming more endemic in the western region of the state. However, our lower prevalence of *B. burgdorferi* in nymphs in the current study indicates that the Lyme disease hotspot in the Roanoke-Blacksburg area may not be due to

increased *B. burgdorferi* prevalence in nymphs. Reasons for this low prevalence are not yet clear but potential causes may be, 1) Greater host diversity and/ or larval *I. scapularis* using other hosts besides the white-footed mouse. Interestingly, LoGiudice et al. (2003) determined that species-poor communities tend to have high numbers of white-footed mice but few other hosts, while species-rich communities have additional host species that are poor reservoirs (LoGiudice et al. 2003). This leads to a "dilution effect" in which species-rich communities have a lower prevalence of *B. burgdorferi*. Indeed, Sonenshine et al. (1995) suggest that other small mammals like *Mus musculus* (feral house mice) and *Oryzomys palustris* (rice rats) are more abundant in Virginia and thus perhaps more commonly used by *I. scapularis* here in Virginia and/or 2) If *I. scapularis* has been migrating from the North, they would have immigrated to southwestern Virginia more recently than northern Virginia. Therefore, there would have been less time for *B. burgdorferi* to become pervasive throughout the sylvatic cycle resulting in the lower prevalence of the pathogen in this region. Ultimately, more studies are needed to explore the drivers of this trend. Furthermore, studies examining other areas within southwestern Virginia are needed.

Thus, there is a discrepancy that exists in the Roanoke Valley whereby there is low *B. burgdorferi* prevalence in *I. scapularis* populations and yet a high case incidence of Lyme disease. Therefore, our findings seem to indicate that high *B. burgdorferi* prevalence is likely not contributing to the high number of Lyme cases in this region. Alternatively, there are several phenomena that may be contributing to this high Lyme disease incidence. Initial testing of a very small number of adult *I. scapularis* (n=19) (data not shown) indicate a notably higher *Borrelia* spp. prevalence (47.4%). Although more adult *I. scapularis* need to be tested to confirm these findings, it indicates the possibility that 1) nymphs may be feeding on a competent reservoir that larvae are not, and 2) adult ticks may be a more epidemiologically important life stage for Lyme

cases in southwest Virginia. Specifically, people in Southwest Virginia may spend more time outdoors in the late fall and winter (when adult *I. scapularis* are active) than people residing in the Northeast due to the milder winters experienced here in Virginia. This would provide more opportunities for adult *I. scapularis* to feed on people here in Virginia as compared to the Northeast. It is also possible that other factors, unrelated to pathogen prevalence, are driving the high Lyme incidence in southwestern Virginia. For example, Morris (2019) found that *I. scapularis* nymphs are exhibiting more aggressive questing behavior, which is more associated with northern *I. scapularis* populations. Importantly, this aggressive questing behavior makes it more likely that they will feed on humans (Morris 2019). Other factors such as more abundant *I. scapularis* also need to be investigated.

Of the *I. scapularis* tested in this study, there was one that tested positive for *B. miyamotoi*, demonstrating 0.3% prevalence. Interestingly, only one other study has examined *B. miyamotoi* in Virginia (Nelder et al. 2016) and they found a prevalence of 3.5% (n=173). More studies need to be done to better understand *B. miyamotoi* dynamics on a regional scale. But, if southwestern Virginia is serving as the southern expansion front of Lyme disease, the presence of *B. miyamotoi* makes sense because *B. miyamotoi* and *B. burgdorferi* are thought to have similar distributions.

All of the *I. scapularis* ticks included in the genetic analysis fell within the American clade, with none being a part of the Southern clade. Interestingly, the ticks in our study appear to have greater genetic diversity (16 haplotypes were obtained from 47 ticks) than the ticks from throughout Virginia that were tested by Brinkerhoff et al. (2014) (17 haplotypes from 85 nymphs). Of the individual ticks included in the analysis, 43.8% belonged to haplotypes previously found in Virginia (Brinkerhoff et al 2014), likely indicating haplotypes that have been

in Virginia for a long time. However, there are 9 haplotypes that were unique. It's possible that these haplotypes do exist elsewhere in Virginia and that our analysis just simply didn't sample them. Alternatively, these unique haplotypes in conjunction with the high haplotype diversity detected in ticks in southwestern Virginia may support the conclusions of Brinkerhoff et al. (2014) which suggested that the population of *I. scapularis* in southwestern Virginia (and western Virginia, more broadly) is genetically distinctive from populations elsewhere in the state due to demographic expansion of *I. scapularis* ticks from the North.

Notably, *I. scapularis* ticks from the FR site displayed the lowest haplotype diversity of the ticks in the genetic analysis with an SDI value of 0.8788. Though no conclusions can definitively be drawn from the current research, this finding may provide support for the expansion of *I. scapularis* from the North migrating southwards. If western Virginia is serving as the southward expansion front of *I. scapularis*, then populations that are further south would not be as diverse. Interestingly, the FR site is the most southwestern site, perhaps supporting this theory.

Conclusion

Prevalence of *Borrelia burgdorferi* in *I. scapularis* nymphs in southwest Virginia is much lower than what has been found in previous studies both in western Virginia and in regions where Lyme disease is endemic. These findings indicate that high *B. burgdorferi* prevalence in *I. scapularis* nymphs is likely not a primary driving factor of the emerging Lyme disease hotspot in the Roanoke-Blacksburg area, and that other factors such as more aggressive nymphal questing behavior, increased outdoor human activity, and/or increased *I. scapularis* abundance are more important drivers. Furthermore, to our knowledge, this is the first published report of *B. miyamotoi* infection in *I. scapularis* in western Virginia. The increase of *B. miyamotoi* may be

related to the southward expansion of Lyme disease. Regardless, more testing of ticks from other locations in the state for this pathogen are needed to better understand its distribution.

All ticks in the current study were part of the American clade. Furthermore, there was relatively high haplotype diversity and a number of haplotypes detected that were unique as compared to other sequences in this study. Comparisons to sequences from other *I. scapularis* ticks in Virginia and elsewhere are needed to better understand their origins. Collectively, this, in conjunction with the low *B. burgdorferi* prevalence and low haplotype diversity at our most southwestern site possibly suggests demographic expansion of American clade ticks from the northeastern United States into Virginia. Overall, this study provides valuable insights into Lyme ecology in an emerging hotspot with evidence potentially indicating that the southern front of Lyme disease is moving southwards. Thus, there is a need for expanded and continued surveillance of *I. scapularis* and wildlife populations in the southern United States. Additionally, given the aggressive questing behavior of nymphs in conjunction with the presence of *B. burgdorferi* in western Virginia, testing for other pathogens vectored by *I. scapularis*, including *Anaplasma phagocytophilum* and *Babesia microti*, is needed.

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Appendix A

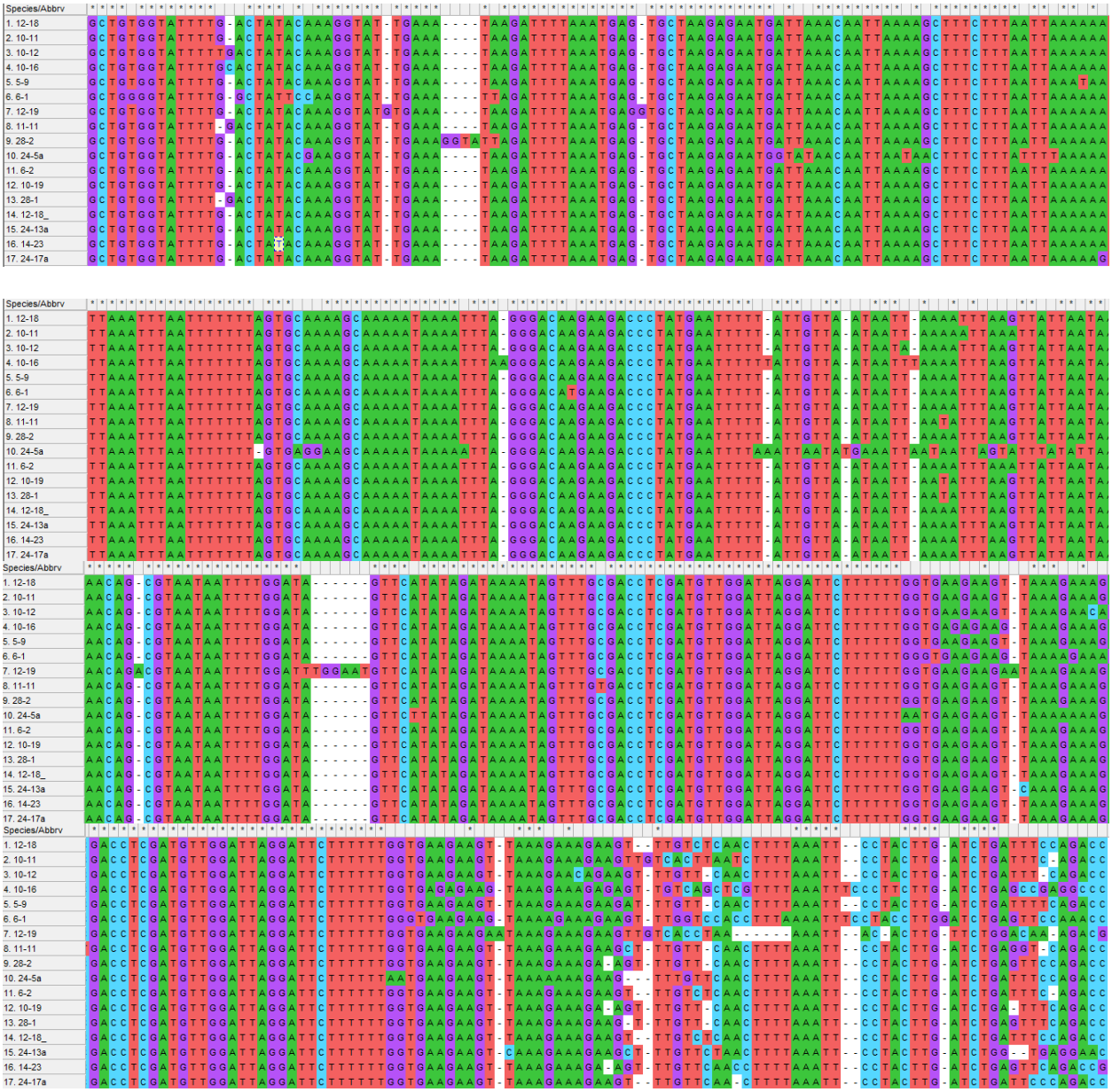

Figure A1. This figure displays the alignment of all 16 different haplotypes described in the paper. Note that due to the length of the sequences, the image has been wrapped in order to show each nucleotide of each sequence in order. 24-5a, which serves as an outgroup in the phylogenetic tree (figure 1 and figure 2) is also included in this alignment.

Table A1. This table displays the number of duplicate haplotypes obtained at each site from which ticks were used to complete the genetic testing.

Haplotype	**Site AM**	**Site FR**	**Site BA**	**Site RE**
14-23	10	13	3	1
24-13	0	1	0	0
12- 18	1	1	0	1
28-1	1	0	0	1
10- 19	2	0	0	0
6- 2	0	0	2	0
28- 2	1	0	0	0
11- 11	0	0	1	0
12- 19	0	0	0	1
6- 1	0	0	1	0
24- 17	0	1	0	0
5- 9	0	1	0	0
10- 16	1	0	0	0
10- 12	1	0	0	0
10- 11	1	0	0	0
24- 18	0	1	0	0
24-5a (Outgroup)	1	0	0	0