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Characterizing *Borrelia burgdorferi* in Virginia:

Lyme Disease Prevalence as a Matter of Bacterial Genetic Variation

by

Hannah Cornman

Honors Thesis

Submitted to

Department of Biology
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Advisor: Dr. Jory Brinkerhoff

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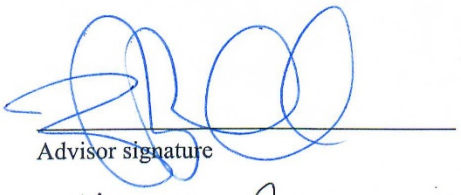

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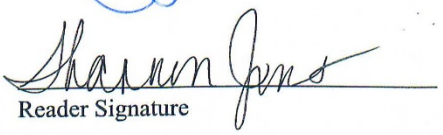

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Abstract

Lyme Disease (LD) incidence and spatial distribution has grown dramatically in the past 20 years. In Virginia, the growth has been especially drastic, primarily in the Western part of the state. This research project investigates how genetic variability of the Lyme Disease causing bacteria, *Borrelia burgdorferi*, might be contributing to this increase in LD incidence in western Virginia. To do this, *B. burgdorferi* samples were isolated from ticks found across the state and were characterized by multilocus sequence typing (MLST). Phylogenetic and minimum spanning tree analyses were also performed. It was hypothesized that the increasing number of Lyme disease infections in western Virginia is partially due to the recent influx of highly pathogenic *B. burgdorferi* sequence types from the Northeast US, the Midwest US, or eastern Virginia. This research has found evidence suggesting there was a recent migration event that affected the western *B. burgdorferi* population. It provided evidence that rejects the hypotheses that *B. burgdorferi* is migrating from the Midwest or eastern Virginia but supports the hypothesis that it is migrating from the Northeast. The results suggest that *B. burgdorferi* sequence types vary significantly across the state and that *B. burgdorferi* strains from eastern Virginia samples exhibit more genetic diversity than those found in western Virginia. None of the identified sequence types from Virginia matched those from the Midwest, but they did match those from the Northeast. Sequence Types 3 and 36 are classified as highly pathogenic, and make up roughly a third of the samples characterized from western Virginia.

Introduction

Lyme borreliosis, also known as Lyme Disease (LD), is an infectious disease caused by the bacteria *Borrelia burgdorferi,* which is spread to humans when they are bitten by infected

ticks of the *Ixodes* genus (Shapiro, 2014). LD is the most prevalent vector-borne disease in North America and its incidence continues to increase (Bacon et al., 2008). There has been a growing National concern about LD in the United States because the yearly number of cases has been increasing steadily for the past two decades (from 9,465 in 1991 to 26,203 in 2016), and in the last 15 years the distribution of LD has greatly expanded (CDC). Some locations have remained relatively stable in LD incidence, while others have had especially dramatic increases in LD cases (CDC). Virginia is one area that has experienced a great increase in LD. In Virginia, LD incidence has increased by 300% since 2007, primarily in the western part of the state. The first cases were noted in the 1980s in northern and eastern Virginia, but since 2006 there has been a significant southwestern expansion of case distribution (Brinkerhoff et al., 2014).

The striking increase and spatial expansion of LD cases in the United States and Virginia has sparked the interest of many public health officials and researchers. Given the complex nature of vector-borne disease transmission, which involves the interplay of vector, host, pathogen, and reservoir species, not to mention the influence of abiotic environmental factors, the causes for LD outbreaks are particularly hard to dissect. An assemblage of studies have shed light on the many factors that may contribute to increasing LD incidence. Spielman (1994) suggested that the spread of Lyme disease and its vectors in the northeastern United States was linked to the recent proliferation of deer, and the abundance of deer was due to environmental changes like the process of reforestation. In 2006, Ostfeld et al. used a model comparison approach to suggest that the abundance of white-footed mice, the primary reservoir species for the LD pathogen *B. burgdorferi,* was a strong predictor of Lyme disease incidence. Most recently, research by Brinkerhoff et al. (2014) in Virginia found that abundance of I. scapularis ticks and prevalence of B. burgdorferi infection in the tick population were consistent with recent

changes in human disease data. One area of LD transmission that has not been extensively explored with regard to its possible contribution to geographic expansion of LD cases is the pathogen, *B. burgdorferi*, itself.

In the United States, *B. burgdorferi* is the only bacterial species that is known to cause LD (Shapiro, 2014). *B. burgdorferi* is a spirochete pathogen that was isolated by William Burgdorfer and his colleagues in 1982 from an *Ixodes* tick, and was subsequently detected in the serum of LD patients (Burgdorfer et al., 1982). An important characteristic of *B. burgdorferi* that may account for differences in LD incidence is the pathogen's extensive genetic variability. It is known that there are many different strains of *B. burgdorferi,* and that the different strains vary in invasiveness and ability to infect humans or other animals (Steere et al., 2008). Small genetic differences in bacterial genotypes can result in significant differences in the particular strain's pathogenicity.

The highly polymorphic outer surface protein C gene (ospC) and the 16 S–23 S (rrs-rrl) rRNA intergenic spacer (IGS) have been the most commonly used genetic markers for *B. burgdorferi* strain identification in the US, and variation in these genes have been associated with variable strain pathogenicity (Jones et al., 2016). It has been observed that strains expressing restriction fragment length polymorphism in the 16 S–23 S rRNA intergenic spacer, which have been classified as RST1 are highly invasive and capable of causing disease in humans. (Hanincova et al., 2008). Additionally strains that possess ospC alleles A, B, H, I and K have been classified as highly pathogenic (Brisson & Dykhuizen, 2004). Multilocus sequence typing (MLST), which is the technique used in this study for strain identification, also has revealed significant variability in the pathogenicity of *B. burgdorferi* strains by examining eight housekeeping genes. MLST characterized sequence types 3, 7, 9, 11, 15, 16, 35, 36, 42, 236 and

410 were found to cause severe and disseminated infection in humans (Hanicova et al., 2013) and are therefore considered highly pathogenic. These differences in pathogenicity could contribute to increasing cases of LD.

When thinking about how *B. burgdorferi* might play into the spread of LD it is also important to consider the origin of this bacteria and therefore where it might be spreading from. The ecological history of *B. burgdorferi* in the United States has greatly shaped the geographic distribution of this bacteria in the country through the following sequence of events. During the period of industrialization between 1600 and 1900, there was very little forest area or deer in the United States, and therefore there were very few ticks and very little LD. After the Civil war, agriculture moved west and forests were able to regrow in the east. As the forests came back, ticks were able to rebound in areas where tiny, fragmented deer populations had survived in Long Island and the North Woods of the upper Midwest. With the growing tick populations came the presence of *B. burgdorferi,* and just as the deer and tick populations were stuck in isolated pockets in the northeast and upper midwest, *B. burgdorferi* became stuck there too. This produced two distinct populations of *B. burgdorferi,* one in the northeast and one in the midwest. Gatewood et al. (2009) showed that these two populations are genetically distinct, and that there is very little gene flow between the two populations. It is around these two pockets of *B. burgdorferi* that LD first emerged.

It is around these two pockets of *B. burgdorferi* that LD first emerged and spread throughout the US. *B. burgdorferi* migrated from the northeastern United States to the eastern shore area of Virginia, and has existed there for at least 30 years (Levine et al., 2001), likely much longer. Surprisingly, the number of LD cases reported from this area is currently much lower than the number of cases being reported in western Virginia areas (CDC). The objective of

this research project was to investigate the genotypes of *B. burgdorferi* strains found across Virginia, and use this information to decipher how genetic characteristics of the pathogen might be contributing to the increase in LD incidence in western Virginia. It was hypothesized that the increasing number of Lyme disease infections in western Virginia is at least partially due to the recent migration of highly pathogenic *B. burgdorferi* genotypes into the area. Based on previous knowledge of the main sources of *B. burgdorferi* nearby, the goal was to specifically investigate whether the new *B. burgdorferi* strains were migrating from the northeast US, the Midwest US, or from eastern Virginia.

In order to carry out this investigation, I planned to genetically characterize samples of *B. burgdorferi* extracted from ticks found in numerous counties across Virginia (Giles, Chesterfield, Goochland, Albemarle, Nelson, New Kent, King William, and King & Queen) using a bacterial typing scheme known as multilocus sequence typing (MLST) (Enright & Spratt, 1999). I then planned to compare eastern and western Virginia genotypes to each other and to those found in the northeast and midwest United States, where genotypes have already been characterized and published in an online database (https://pubmlst.org/borrelia/). I expected that the genotypes of samples from western Virginia would be most closely related to the genotypes commonly found in the region from which they had migrated, either the northeast, the midwest, or eastern Virginia. I also expected that samples from western Virginia would include many genotypes known to be highly pathogenic.

Methods

Collection of Ticks

Ixodes Scapularis ticks were collected from 8 different Virginia counties, which were grouped into four general regions: west (Giles), west-central (Albemarle and Nelson.), east-central (Chesterfield and Goochland), and east (New Kent, King William, King & Queen). A map depicting the site locations and the four regions can be found in **Figure 1**. The ticks were collected via a drag sampling method in which a 1-m^2 piece of corduroy was dragged along the ground and through vegetation at the site and checked periodically for tick removal. This method is described in detail on the official website of the National Park Service https://www.nature.nps.gov/biology/ipm/manual/ticks.cfm. The ticks were then stored in microcentrifuge tubes filled with ethanol until they were needed for further use in the lab.

DNA Isolation

To extract the entirety of the tick's DNA, ticks were flash frozen individually using liquid nitrogen, crushed using a sterilized pestle, and then processed using Machery-Nagel Nucleospin Tissue Kit according to the manufacturer's protocols.

Genetic Analysis: Multilocus Sequence Typing (MLST)

An important aspect of this project was the decision to use multilocus sequence typing (MLST) rather than a single gene sequence analysis. Most methods of characterizing bacteria strains are based on the DNA sequence of one gene, whereas MLST for *B. burgdorferi* is based on the DNA sequence of eight housekeeping genes (Margos et al., 2008). Each gene is represented by an allele, and the combination of all 8 alleles for a particular sample is known as

the allelic profile, which is collectively called a sequence type (Enright & Spratt, 1999). From this point on, the genotype of a sample will be referred to as a sequence type, as is custom in MLST analysis. Because MLST analysis uses eight genes as compared to one, it has the potential to discover eight times as much variation, and to create a much higher resolution picture of the genetic population of *B. burgdorferi* than a single gene analysis would.

MLST has been tremendously successful in similar projects in recent years. For example, MSLT was used to show that North American and European populations of *B. burgdorferi* exhibit significant differences in genotypes. In fact, this MLST study done on 64 samples of *B. burgdorferi* found that North America and Europe did not share a single sequence type. This same study found that *B. burgdorferi* sequence types from California were closely related to the sequence types derived from the midwest United States, which supported the conclusion that the bacteria in California migrated from this area (G. Margos et al., 2008).

A *B. burgdorferi* MLST database was created by Dr. Gabriele Margos, on which every MLST analyzed sample of *Borrelia burgdorferi* has been recorded (http://borrelia.mlst.net/). This database has identified 772 different sequence types of B. burgdorferi and allows users to search the entries for one that matches their sequenced samples by inputting the DNA sequence as a search query. This database will be mentioned later in the methods, as it was used to characterize the collected *B. burgdorferi* samples.

PCR amplification and purification for MLST analysis

The DNA sequences of conserved regions of eight housekeeping genes (clpA, clpX, nifS, pepX, pyrG, rplB, and uvrA) were amplified using nested PCR. The MLST scheme and the primers used for PCR are outlined in Margos et al., (2008).

Confirmation of *B. burgdorferi* presence and gene amplification

After amplification, all samples were subjected to gel electrophoresis on an agarose gel to confirm that *B. burgdorferi* was present and that the genes had amplified, in which case a band would appear downstream from the well into which the sample DNA was placed.

Purification of *B. burgdorferi* genes and nanodrop analysis

After it was confirmed that a particular B. burgdorferi gene was successfully amplified via gel electrophoresis, the DNA was purified using Machery-Nagel Nucleospin PCR Clean Up Kit in order to remove any contaminating macromolecules. Manufacturer protocol was followed. To confirm that the DNA concentration was appropriate for sequencing (10-100 ng/μL), a nanodrop analysis was performed to determine DNA concentration for all samples, and these findings were compared to a positive and a negative control.

DNA Sequencing of *B. burgdorferi* genes and analysis via Sequencher

For all samples with the appropriate DNA concentration, sequence plates were prepared and sent to Operon Eurofins lab in Louisville, KY for sequencing. Bidirectional chromatogram sequence data for each individual gene were aligned and assembled using Sequencher 4.10.1 DNA analysis software. ClustalW (http://www.clustal.org) was used to create a concatenated sequence of all eight MLST genes for each DNA sample.

Determination of alleles and sequence types

The allele of each gene was determined by using the "sequence query" feature on the *B. burgdorferi* MLST database, which searches the database for a known allele that matches the input sequence. Once all eight genes of a sample had been identified as a specific allele, the

sequence type of that sample was determined by using the "Search by combinations of MLST alleles" feature on the *B. burgdorferi* MLST database, which searches the database for a previously determined sequence type that contains the same alleles as the sample being analyzed.

Phylogenetic and minimum spanning tree (MST) analyses

A phylogenetic tree was created by aligning all sequences with ClustalW (http://www.clustal.org) and implemented in MEGA 5.0 (http://www.megasoftware.net/) which selected among models of evolution to reconstruct a probable phylogeny. The DNA sequences used to make this tree included the concatenated sequences determined for the 49 samples I successfully characterized, as well as previously published DNA sequences for common sequence types downloaded from http://borrelia.mlst.net/. Additionally, a minimum spanning tree (MST) was created using the same concatenated sequences file aligned with ClustalW implemented in PhyloViz analytic software which uses the goEburst algorithm to represent the possible evolutionary relationships between strains.

Results

Sequence type identification and distribution by geographic region

DNA was extracted from hundreds of ticks and about 20% were found to be infected with B. burgdorferi. In total, 501 genes were sequenced and were identified as a known allele. A sequence type (defined as successful characterization of all eight genes required for MLST analysis) was determined for 49 samples, including fourteen samples from the west, sixteen from the west-central, ten from the east-central, and nine from the east region. A total of 28 unique sequence types were represented in the results. 30 of the 49 (61.2%) identified samples were characterized as sequence types that had been previously published in the online MLST database,

whereas the other 19 (38.8%) were novel sequence types. **Table 1** shows the characterized alleles and sequence types of the 49 samples for which a sequence type was determined. **Figure 2** shows the sequence types that were identified and the quantities at which they were found in each region. The eastern and western sites did not share a single sequence type. In fact, the east only shared one sequence type (ST 8) with the east-central region. There were four sequence types (37, 36, 3, and 5) that were shared between the west and the two central regions but were not found in the east.

Phylogenetic analysis

The constructed phylogenetic tree **(Figure 3)** allows visualization of the evolutionary relationships between sequence types. It shows that all samples found at western Virginia sites exhibited a clustering pattern in which they were identified as closely related sequence types that are characterized as or are closely related to sequence types 3, 36, or 37. There are no samples found at eastern and western sites that are closely related to one another, but there does not appear to be a clear division of clades by geographic region, as there are a considerable number of samples from different regions that are closely related.

Minimum spanning tree (MST) analysis

The constructed minimum spanning tree **(Figure 4)** allows visualization of how closely the sequence types are related, as each line connecting one sequence type to another represents one mutation that gave rise to a new sequence type. The MST produced by my results was a single cluster, suggesting that all sequence types that I collected and included from the online database have a common ancestor. In this tree, the patterns observed were similar to those seen in the phylogenetic tree. The sequence types identified from the western region are again

clustered together indicating that they are all closely related, whereas the sequence types from the eastern region are spread across the tree, indicating that they are distantly related. Once again, the samples from western Virginia were characterized as or clustered around sequence types 3, 36, and 37. There is still no clear division of sequence types by geographic region because there are sequence types from the two central regions dispersed throughout the tree.

Discussion

Sequence type variability and pathogenicity are consistent with Lyme Disease prevalence

It is immediately obvious that *B. burgdorferi* sequence types vary considerably across the state, which is consistent with the variability in LD prevalence shown by recent reports from the CDC, which indicate low incidence in the southeastern part of the state, and high incidence in the northern and western parts. Roughly 30% of the samples collected from western Virginia were characterized as sequence types 3 or 36, both of which have been identified as highly pathogenic (Hanicova et al., 2013). The recent emergence of these highly pathogenic strains in Western VA could be contributing to the increasing incidence in LD because they are more likely to cause disease if a tick bites a human and transmits the pathogen. Previous research has shown that variability in the abundance of *I. scapularis* nymphs and prevalence of *B. burgdorferi* infection in the ticks are consistent with increases in LD incidence (Brinkerhoff et al., 2014). My findings add an additional layer of complexity by suggesting that not only can the *B. burgdorferi* infection prevalence affect LD risk in an area, but the specific sequence types and consequential pathogenicity of *B. burgdorferi* that are infecting the ticks might also play a role.

Evidence of a recent *B. burgdorferi* migration event in western Virginia

The results of this study provide evidence that suggests there was a recent migration event that affected the western population of *B. burgdorferi*. Looking at the phylogenetic or minimum spanning tree, most of the sequence types identified from western sites are characterized as or clustered closely together around sequence types 37, 3, or 36, indicating that these groups of sequence types are closely related. This is indicative of a recent migration event to the area because the newly arrived species have had little time to accumulate mutations and differentiate from one another. This is similar to the "founder's effect", a well-known phenomenon in evolutionary biology, when a small number of individuals from a larger population establish a new population and a loss of genetic variation occurs (Matute, 2013). This evidence supports our hypothesis that the increasing number of Lyme disease infections in western Virginia is at least partially due to the recent migration of new *B. burgdorferi* strains to that area, because it appears that the western *B. burgdorferi* population is comprised of a few founder strains (STs 3, 36, and 37) which have just begun to differentiate into closely related sequence types (HC_GC042, HC_GC027, HC_GC120, and HC_GC117). A study investigating the diversity of *B. burgdorferi* in Canada, where LD is also emerging, found similar results in which there was evidence for founder events skewing the diversity in emerging tick populations where LD incidence was increasing. The researchers proposed that dispersal of ticks infected with *B. burgdorferi* from the US could be contributing to the emergence of LD in Canada (Ogden et al., 2016).

Eastern *B. burgdorferi* population shows evidence of genetic isolation

While there are a considerable amount of shared sequence types between the west and the two central regions, the east shared only one sequence type with the east central region, suggesting that the eastern *B. burgdorferi* population is isolated from the rest of the state. Further

evidence of the isolated nature of the *B. burgdorferi* population in eastern Virginia can be found by analyzing the phylogenetic and minimum spanning trees. Both trees show that the sequence types identified in eastern Virginia are spread out across the entire tree, indicating that they have become more distantly related through the accumulation of mutations and evolution. In addition, samples from the east had a greater number of mutations and a greater proportion of their samples were characterized as novel sequence types. The accumulation of mutations often occurs during long periods of isolation, and therefore this observation supports the conclusion the eastern *B. burgdorferi* population is genetically isolated (Schluter & Conte, 2009).

I was surprised by the fact that the eastern population was isolated because previous research has found that geographic barriers are generally what separate one population from another (Boenigk et al., 2006), but there is no major geographic barrier that separates the eastern region from the rest of the state. On the other hand, the Blue Ridge Mountains run through the western part of the state, but these did not prevent the sharing of sequence types between the west and the two central regions. Therefore, it appears that there must be another factor that is causing isolation of the eastern *B. burgdorferi* population, and further research should be conducted to determine what it might be.

Migration of *B. burgdorferi* from eastern Virginia to western Virginia is not supported

The apparent isolation of eastern *B. burgdorferi,* along with the fact that the east and west regions did not share a single sequence type, suggests that these pathogen populations are distinct and provides evidence that calls to reject the hypothesis that *B. burgdorferi* is migrating from eastern Virginia to western Virginia. If the migration had occurred from eastern to western Virginia I would expect to see shared sequence types or at least closely related sequences between the samples collected at the eastern and western sites.

Migration of *B. burgdorferi* from the Midwest to western Virginia is not supported

This research also provides evidence that there was no migration of *B. burgdoferi* from the Midwest to western Virginia. This evidence is best displayed in the minimum spanning tree (MST) analysis. **Figure 5** shows the expected minimum spanning trees if B. burgdorferi was migrating from either the Northeast US or the Midwest US based on the following logic. If the *B. burgdorferi* in Western VA had migrated down from the Midwest I would expect to see two separate clusters in my MST. One would have a founder from the Northeast and would be surrounded by sequence types collected in the east, because previous research has shown that *B. burgdorferi* in eastern Virginia migrated decades ago from the Northeast (Levine et al., 2001). The other cluster then, would have a founder from the Midwest, and would be surrounded by sequence types collected in the west, indicating the recent migration of *B. burgdorferi* from the Midwest to western Virginia. There would be no connection between the two clusters because the Northeast and Midwest populations are genetically distinct (Gatewood et al., 2009). On the other hand, if the *B. burgdorferi* in Western VA had migrated down from the Northeast, I would expect to see one single cluster in my MST, because all the sequence types would have originated in the Northeast.

The results of this study produced a single cluster MST, supporting the theory of migration from the Northeast over the Midwest. Additionally, none of the sequence types identified in this study were those commonly found in the Midwest, so it seems that migration of *B. burgdorferi* from the Midwest is unlikely (Gatewood et al., 2009).

Migration of *B. burgdorferi* from the Northeast into western Virginia is supported

On the other hand, there is evidence that supports the hypothesis that *B. burgdorferi* is migrating from the Northeast into western Virginia. As was explained above, a single cluster MST is what would be expected as a result if *B. burgdorferi* in western Virginia has migrated from the Northwest, and that is what our results produced. Further evidence in support of this hypothesis can

be found by looking at the relation of the sequence types found in western Virginia as compared to those found in the Northeast US. On the MST in **Figure 6** I have marked with arrows three sequence types that are commonly found in the Northeast and that were also identified in western Virginia (STs 3, 36, and 37). The presence of these sequence types in the Northeast suggests that the Northeast could be the source of these strains. Furthermore, in **Figure 6** the two red arrows mark sequence types 3 and 36, both of which have been identified as highly pathogenic (Hanicova et al., 2013) and make up roughly 30% of the Western samples. The highly pathogenic nature of these could be responsible for the high incidence of LD in the Northeast and the increasing incidence of LD in western Virginia.

Future Directions

Future research will focus on investigating the genetic populations of *B. burgdorferi* at two new sites: Loudon County in northern Virginia and Grayson County in southwestern Virginia. The plan is to screen ticks from these sites for *B. burgdorferi* and then analyze the bacterial genotypes by the same methods used in this study. These sites are of interest to investigate because if the hypothesis that *B. burgdorferi* is migrating from the Northeast US is correct, then these two counties align with the most likely path of migration of the bacteria.

Loudon County is located north of the western site (Giles County) that was analyzed in this study, and according to the yearly incidence data from the CDC, Loudon experienced increasing cases of Lyme disease years before Giles did. Therefore, in the Loudon *B. burgdorferi* population I expect to find many of the same sequence types as those that were found in Giles, but also some additional sequence types that may have not become established in Giles yet. I would also expect to find somewhat higher diversity in the Loudon *B. burgdorferi* population as well, because the strains have been in the area longer and had more time to accumulate mutations. Adding analysis of Giles *B. burgdorferi* primarily serves as a way to further validate the results of this study if they are consistent, or suggest an alternative hypothesis if they are not.

Grayson County is located south of Giles County and is likely to be one of the next counties hit by the expansion of *B. burgdorferi* and LD. Abundant populations of *I. scapularis* ticks infected with *B. burgdorferi* are present at southwestern Virginia

locations close to the Tennessee Border, which has caused fear about the possibility of a Lyme disease outbreak in these areas (Hickling, 2018). Genetic information about the *B. burgdorferi* found in these ticks would provide further insight about the risk of LD. If the *B. burgdorferi* infected ticks along the Tennessee border are a continuation of the hypothesized migration events from the Northeastern US, then I would expect to find some of the same sequence types that were found in Giles in Grayson, but I would expect fewer sequence types and lower diversity because they would have arrived at this site later and had less time to accumulate mutations. Adding analysis of Grayson *B. burgdorferi* provides an opportunity for early detection of a LD outbreak.

Conclusion

The goal of this research was to investigate the sequence types via MLST of *B. burgdorferi* strains found at various sites in Virginia, and use this information to identify characteristics of the pathogen and sources of pathogen dispersal that might be contributing to the increase in LD incidence in western Virginia. Sequence types were identified for 49 samples from 49 *Ixodes* ticks collected in western, west-central, east-central, and eastern Virginia. The results provided evidence that sequence type variability and pathogenicity are consistent with LD prevalence in Virginia. They also lend support to the hypothesis that there was a recent migration event that affected the *B. burgdorferi* population in western Virginia. Specifically, the results suggest that highly pathogenic strains of *B. burgdorferi* have recently migrated into western Virginia from the Northeast US, and not from the Midwest or eastern Virginia. The fact that many of the strains characterized in western Virginia were identified as highly pathogenic sequence types supports the conclusion that these strains are contributing to the increasing number of LD cases. A peripheral and surprising finding was that the eastern population of *B. burgdorferi* appears to be somewhat isolated genetically. Overall, this study has expanded on the knowledge about the genotypes of *B. burgdorferi* populations in Virginia, specifically by revealing the pathogenicity and possible geographic links of the bacteria strains in western Virginia where LD has become a major health concern. This knowledge will hopefully facilitate speculation about the continued expansion of pathogenic *B. burgdorferi* strains and may help to control the growing infection rate.

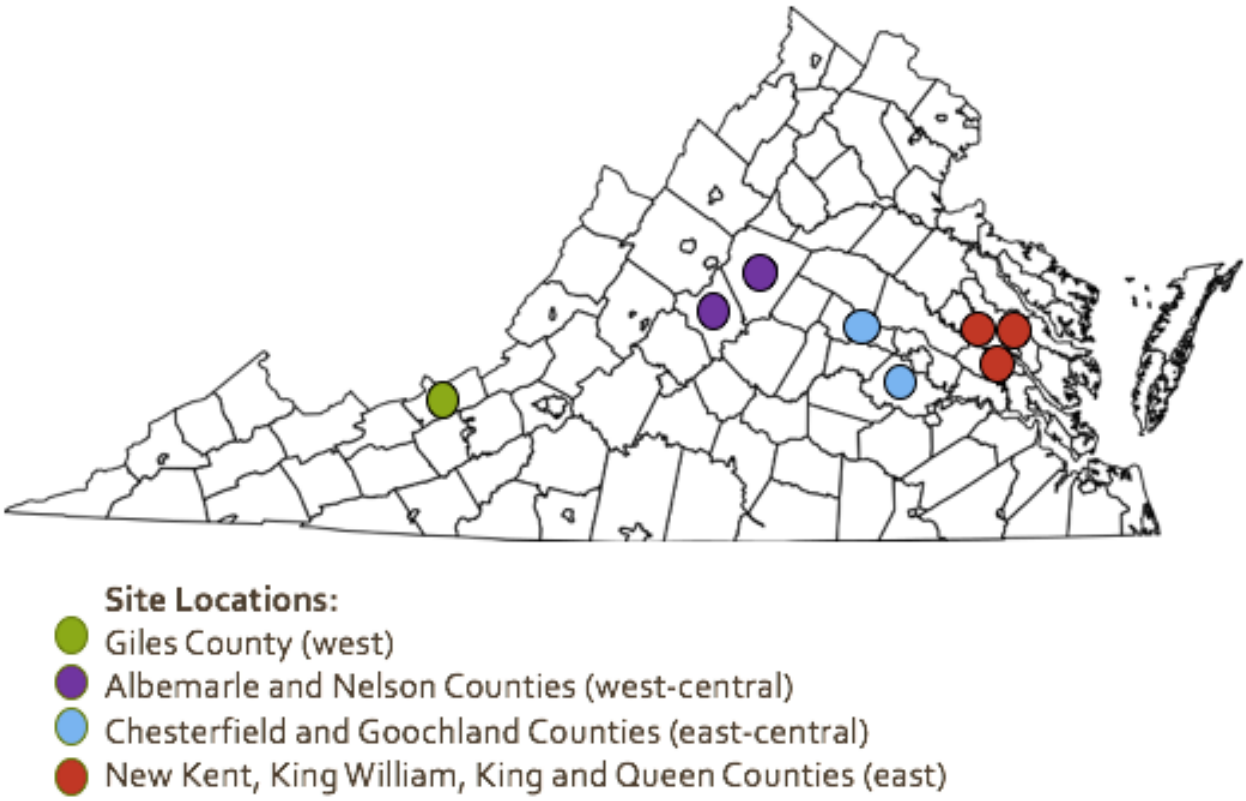

Figure 1. Map of Virginia depicting the sites from which *Ixodes Scapularis* ticks were collected. Ticks were scanned for infection with *B. burgdorferi* and used to obtain *B. burgdorferi* DNA samples for MLST analysis. Individual sites are indicated by circles. The region into which the sites were grouped for analysis is indicated by the color of the circles in the legend.

West Samples	clpA	clpX	nifS	pepX	pyrG	recG	rplB	uvrA	Sequence Type
Giles 027	7	6	12	1	1	5	5	5	HC_GC027
Giles 032	7	6	12	1	1	5	5	5	37
Giles 039	7	6	12	1	1	5	5	5	37
Giles 042	10	5	4	6	168	15	1	6	HC_GC042
Giles 094	7	6	12	1	1	5	5	5	37
Giles 102	4	1	1	1	1	6	1	7	3
Giles 103	10	5	4	6	1	15	1	6	36
Giles 105	10	5	4	6	1	15	1	6	36
Giles 110	4	1	1	1	1	6	1	7	3
Giles 111	7	6	12	1	1 (1*)	5	5	5	HC_GC111
Giles 115	7	6	12	1	1 (1*)	5	5	5	HC_GC111
Giles 117	7	1	6	1	1	136	1	128	HC_GC117
Giles 118	7	6	12	1	1	5	5	5	37
Giles 120	7	9	1	1	1	136	5	1	HC_GC120
West-Central Samples									
Albemarle 3158	4	4	3	3	3	3	3	3	19
Albemarle 2311	21	1	12	8	168	3 (1*)	1	14 (2*)	HC_2311
Albemarle 2312	6	1	5	1	1	7	1	19	59
Albemarle 2315	9	1	1	7	1	1	1	10	307
Albemarle 2318	4	1	1	1	1	6	1	7	3
Albemarle 2388	8	1	1	1 (1*)	4	6	1	7	4
Albemarle 3011	6	1	5	1	1	7	1	8	7
Albemarle 3145	21	1	15	8	1	18	1	7	HC_3145
Albemarle 3190	10	5	4	6	1	6	1	6	9
Albemarle 3193	15	1	1	1	1	6	1	7	HC_3193
Albemarle 3373	6	1	5	1	1	7	1	1	58
Nelson 5207	4	1	1	1	1	6	1	7	3
Nelson 5211	8	1	1	1	1	6	1	7	HC_5211
Nelson 5220	10	5	4	6	1	6	1	6	9
Nelson 5223	10	5	4	6	1	15	1	6	36
Nelson 5238	10	5	4	6	1	15	1	6	36
East-Central Samples									
Chesterfield 5095	5	5	4	5	5	5	1	6	8
Chesterfield 5107	6	1	5	1	1	7	1	19	59
Chesterfield 5116	1	1	1	1	1	1	1	1	1
Chesterfield 5121	10	5	4	6	1	15	1	5	HC_5121
Chesterfield 5126	7	6	12	1	1	5	5	5	37
Chesterfield 5132	10	5	4	6	1	15	1	6	36
Goochland 5153	1	1	4	1	1	136	1	1	HC_5153
Goochland 5160	4	1	1	1	1	6	1	7	3
Goochland 5170	1	1	1	1	1	1	1	11	403
Goochland 5175	4	1	1	1	1	6	1	7	3
East Samples									
King & Queen 66	5	5	4	5	5	5	1	6	8
King & Queen 267	9	1	1	7	1	6	1	10	14
King William 422	21	1	12	8	3 (1*)	3	1	14 (2*)	HC_E422
King William 423	21	1	12	8	3 (1*)	18	1	14 (2*)	HC_E423
King William 652	21	1	12	8	3 (1*)	3	1	14 (2*)	HC_E422
King William 980	21	1	12	8	3 (1*)	5	1	16 (2*)	HC_E422
New Kent 672	11	1	8	1	1	6	4	16 (2*)	6
New Kent 707	11	1	1	1	1	6	1	16 (2*)	HC_E707
New Kent 883	21	1	15	8	1	5	4	7	HC_E883

Table 1. Eight housekeeping gene alleles and sequence type for 49 MLST characterized *B. burgdorferi* samples. (1*) or (2*) indicates one or two nucleotides in the sample differ from the reference sequence

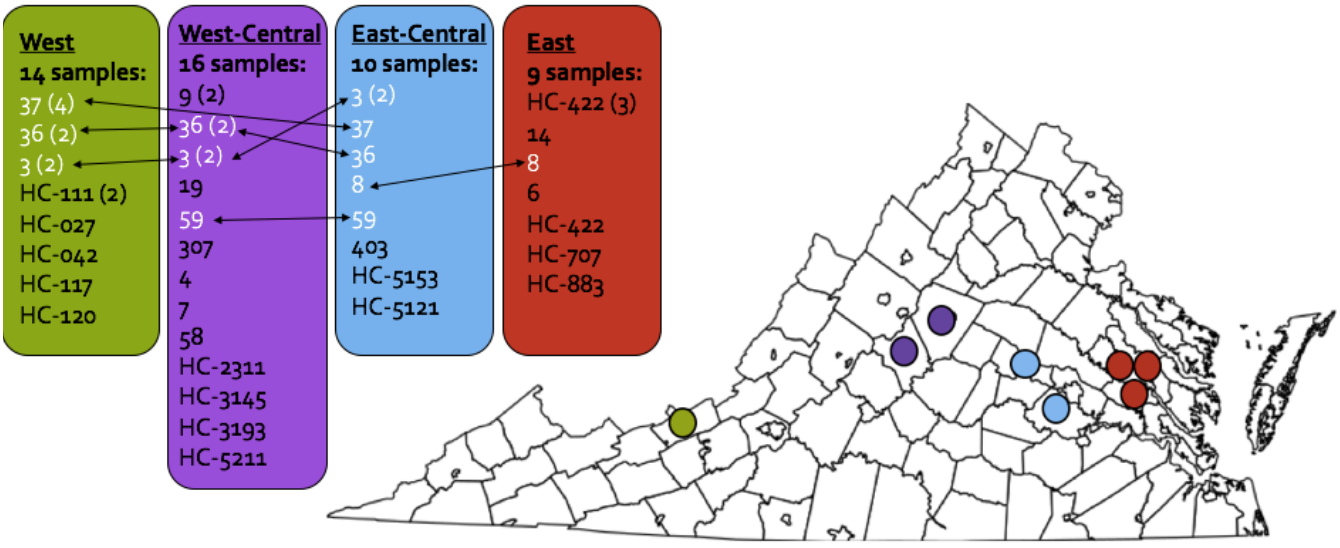

Figure 2. Quantity and geographic distribution of *B. burgdorferi* sequence types in Virginia. The identified sequence types and the regions in which they were found are shown in the color-coded table. The table colors correspond to the regional site colors on the map.

Figure 3. Phylogenetic tree of 49 *B. burgdorferi* samples based on concatenated sequences of eight MLST housekeeping genes. Stars indicate that a sequence type was found in the particular region that matches their color in the legend.

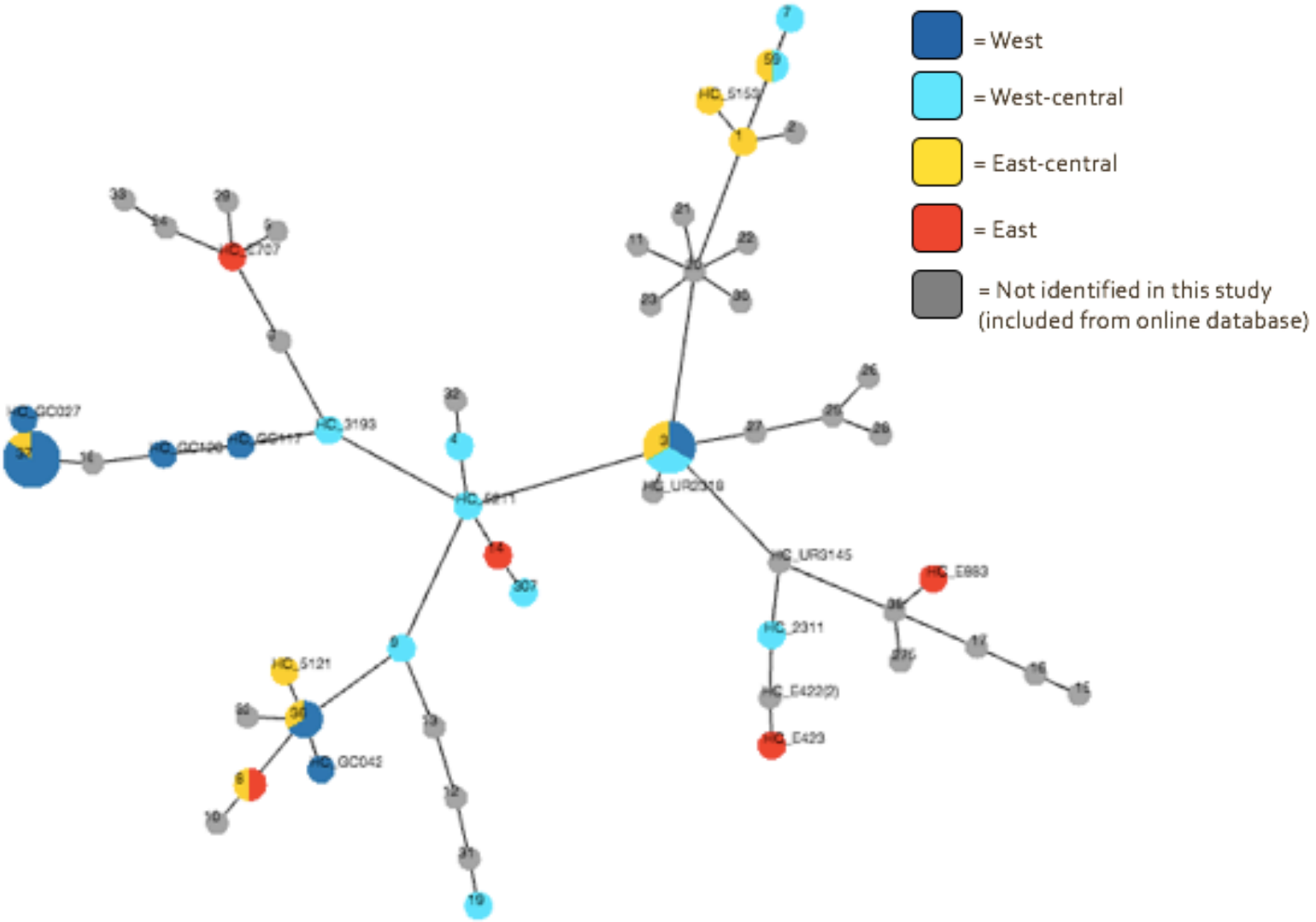

Figure 4. Minimum Spanning Tree (MST) of 49 *B. burgdorferi* samples based on concatenated sequences of eight MLST housekeeping genes. Each circle represents a sequence type and each line represents a polymorphism that gave rise to a new sequence type. The regions in which the sequence types were found are indicated by the color of the circles. If a sequence type was found in more than one region, the proportion of samples from each region is indicated as a division of the circle.

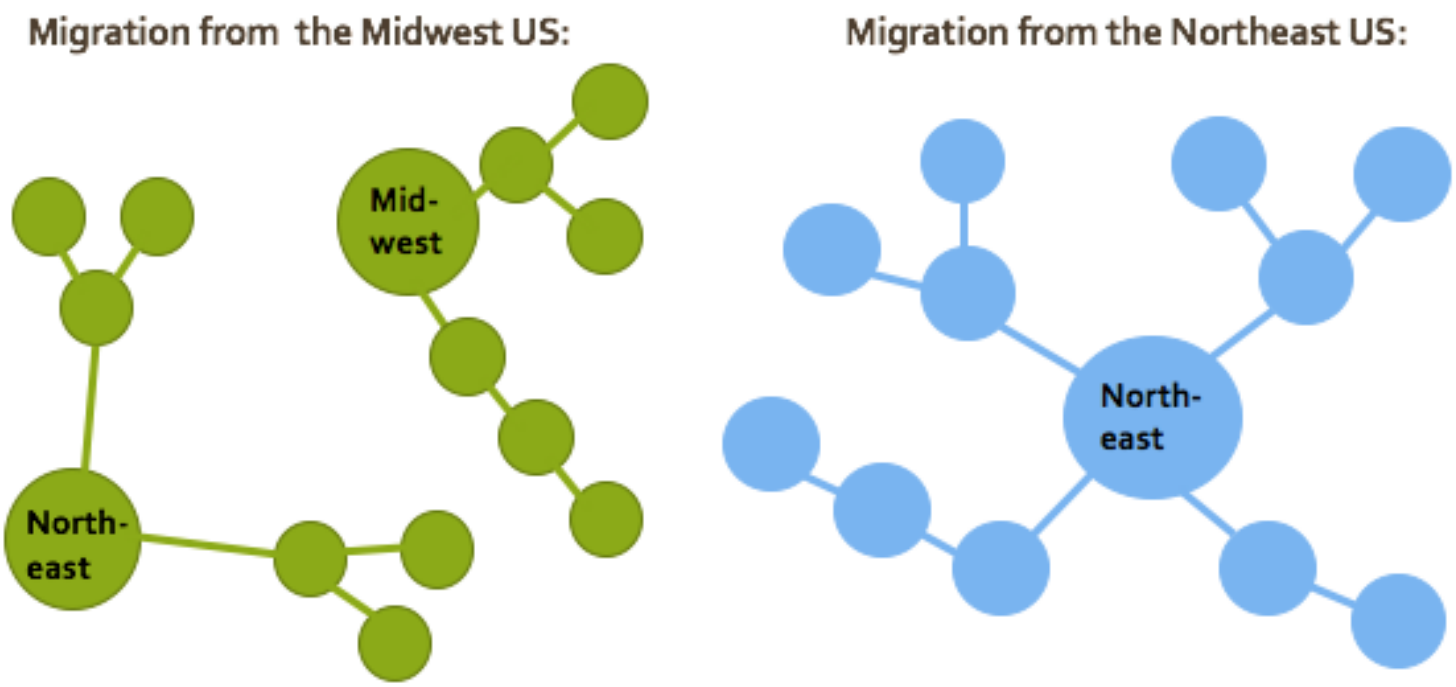

Figure 5. Expected minimum spanning trees if B. burgdorferi had migrated from the Midwest vs. the Northeast US into western Virginia. Migration from the Midwest would be indicated by a MST with two separate clusters. Migration from the Northeast would be indicated by a single cluster.

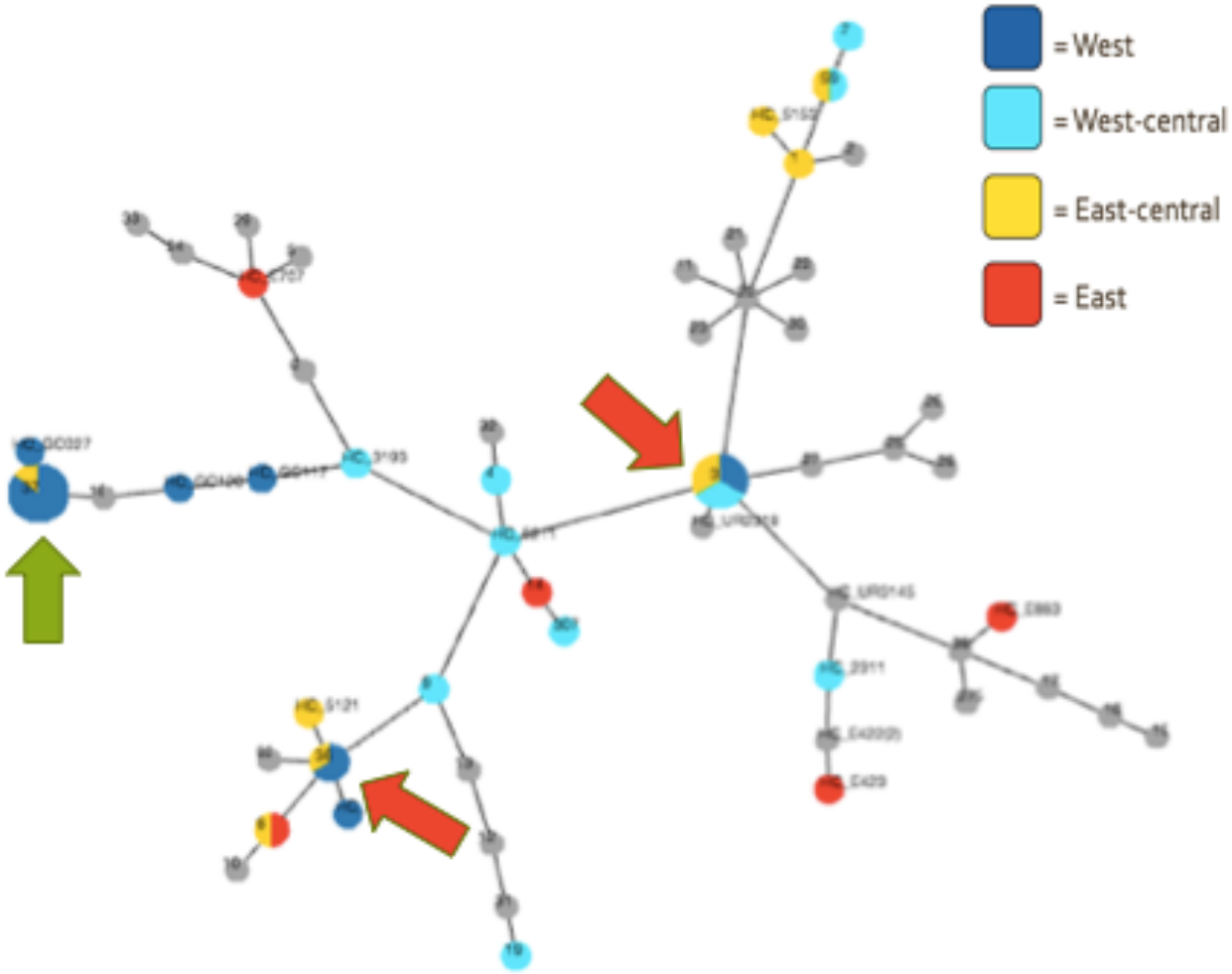

Figure 6. Minimum spanning tree with labeled sequence types.
All sequence types common in the Northeast US and found in western Virginia (STs 3, 36, and 37) labeled with arrows. Highly pathogenic sequence types 3 and 36 are marked with red arrows. Sequence types 3 and 36 make up about 30% of the western *B. burgdorferi* population.

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