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Erin LaFon Date

Exploring Genetic Diversity and Focal Hot-Spots of Deer Tick Virus in Wells National Estuarine Research Reserve

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Epidemiology

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By

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MSc
University of Florida
2021

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An abstract of
A thesis submitted to the Faculty of the
Rollins School of Public Health of Emory University
in partial fulfillment of the requirements for the degree of
Master of Public Health
in Epidemiology
2023

# Abstract

Exploring Genetic Diversity and Focal Hot-Spots of Deer Tick Virus in Wells National Estuarine Research Reserve

By Erin LaFon

Powassan virus (POWV) is a tick-borne flavivirus that can cause severe encephalitis and meningitis in humans. It is the sole tick-borne encephalitis serological complex virus found in North America. It has two lineages, with the second lineage, known as deer tick virus (DTV), being more prevalent and associated with a tick vector that also transmits Lyme disease and other tick-borne diseases. This study used phylogenetic analyses to understand the geographic distribution and evolution of DTV lineages and sub-lineages over time. We collected and aligned complete genome sequences of DTV from the National Center for Biotechnology Information (NCBI) GenBank database, filtered them based on quality and relevance, and created a maximum likelihood tree to determine phylogenetic relationships. We included 99 sequences in the final analysis, including samples from ticks collected at hotspot locations in Wells National Estuarine Research Reserve (WNERR) in Maine, USA. The phylogenetic analysis conducted in this study reveals four major clades from WNERR, each showing different levels of genetic diversity. Transects within WNERR also exhibited different levels of viral diversity, with some transects associated with only one clade and others linked to two or more clades. This indicated the potential differences in the composition and distribution of viral variants across different transects. This study uniquely examines the viral diversity of Powassan virus on a fine-scale geographic level, shedding light on the dynamics of DTV populations in a localized area. Our results provide insights into the geographic distribution and evolution of DTV lineages and highlight the importance of understanding the ecology and epidemiology of tick-borne diseases for effective surveillance and control strategies. Further research is needed to investigate the factors driving the persistence and spread of DTV in tick populations and its potential impact on human health.

**Keywords:** tick-borne flavivirus, emerging virus, phylodynamics, Powassan virus, deer tick virus

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# Table of Contents

# Introduction

Powassan virus (POWV) is a tick-borne flavivirus and is the sole tick-borne encephalitis serological complex virus found in North America (Pesko, 2010). It was first identified in 1958 in Powassan, Ontario (McLean & Donohue, 1959). POWV is known to cause fever, headache, vomiting, and weakness; it causes encephalitis and meningitis. Death occurs in 1 in 10 infected patients with severe disease, and about 50% of the survivors continue to have life-long neurological deficits (*Symptoms, Diagnosis, and Treatment | Powassan | CDC*, n.d.). Before 2006, there were only 20 cases reported to the CDC; however, between 2010 and 2020, there were 186 reported cases (*Statistics and Maps | Powassan |* CDC, n.d.).

POWV has two lineages; the first was estimated to have emerged 2000-6000 years ago (Bondaryuk et al., 2021) and circulates in nature among *Ixodes cookei* and *I. marxi* ticks and their hosts: woodchucks, mustelids, and wild canids (McLean & Donohue, 1959). The second lineage emerged in 1995 and was identified in the deer tick *(I. scapularis)* in the northeastern U.S. (Telford et al., 1997) before later being identified in Wisconsin (Ebel et al., 1999). This second lineage is also known as deer tick virus (DTV). *I. scapularis* is a human-biting species; therefore, there is a significant risk to human health where it is present. Until recently, the reservoir of the virus was unknown; however, recent data concludes that the shrews are a likely reservoir host (Goethert, 2021).

POWV is generally considered a minor public health concern due to the relative host-specificity of the arthropod vector. However, DTV could be a more significant threat since it is associated with a tick vector that has driven Lyme disease, human babesiosis, and human granulocytic anaplasmosis (Pesko, 2010).

## Aims

This project aims to use phylogenetic analyses to understand the geographic distribution and evolution of DTV lineages and sub-lineages within the Wells National Estuarine Research

Reserve (WNERR) in Wells, Maine, USA. Our primary hypothesis is that there will be significant genetic differentiation of Powassan virus lineages and sub-lineages between transects within the WNERR. Our null hypothesis is that there will be no significant differentiation of Powassan virus lineages and sub-lineages between transects within the WNERR. By comparing the genetic diversity of DTV in the WNERR with samples from nearby cities and states, we hope to gain insight into the factors that influence the evolution and spread of this emerging pathogen.

## Preliminary Data

The study utilizes 99 sequenced isolates from the northeast region (Connecticut, Maine, Massachusetts, and New York) to represent a range of geographic and temporal data. The majority of samples used in this analysis are from WNERR. Our study builds upon an epidemiological study that investigated the focal distribution of DTV in *I. scapularis* within WNERR. WNERR provides an ideal location for studying the viral diversity of POWV on a small geographic scale. Our collaborators wanted to determine whether natural nidality can explain the continued persistence of DTV at WNERR. Natural nidality, also known as natural foci or natural transmission cycles, refers to the occurrence of infectious diseases in specific geographic areas or ecological settings where the disease-causing agent (such as a pathogen) is endemic and maintained in a cycle of transmission between susceptible hosts and reservoir hosts. WNERR was selected as the study site for several reasons. First, it is a protected reserve known for its diverse habitat types. Second, it has a longstanding data set covering flora, fauna, and environmental conditions, providing a valuable reference for comparison. Last, previously published data showed high infection rates of DTV in *I. scapularis* ticks at this site, making it an ideal location for investigating the virus.

Our collaborators at WNERR conducted a hotspot analysis based on ten 100-meter transects, with 2 transects per habitat type. Each transect consisted of 10 consecutive 10x1 meter plots, with a total of 100 plots (20 plots per habitat type). Latitude and longitude coordinates at plot centers were recorded using a handheld GPS unit. Tick collections were conducted using a dragging technique weekly from

April through November 2018-2022. Ticks were sorted by sex, life stage, transect, and date collected, and only live ticks were tested for DTV infection. RNA was isolated from ticks using a viral RNA extraction kit, and reverse transcription polymerase chain reaction (RT-PCR) was used to test for DTV infection using specific primers. They found two foci of elevated entomological risk index (ERI) posed by nymph and adult ticks during the questing season from April to November (Figure 1). The two foci occurred at transects 3 and 4, which ran through the forest with invasive species in the understory. Transect 3 appeared more extensive and intense (0 - 5.5 DTV-infected ticks per hour) than transect 4 (1.3 - 2.8 DTV-infected ticks per hour).

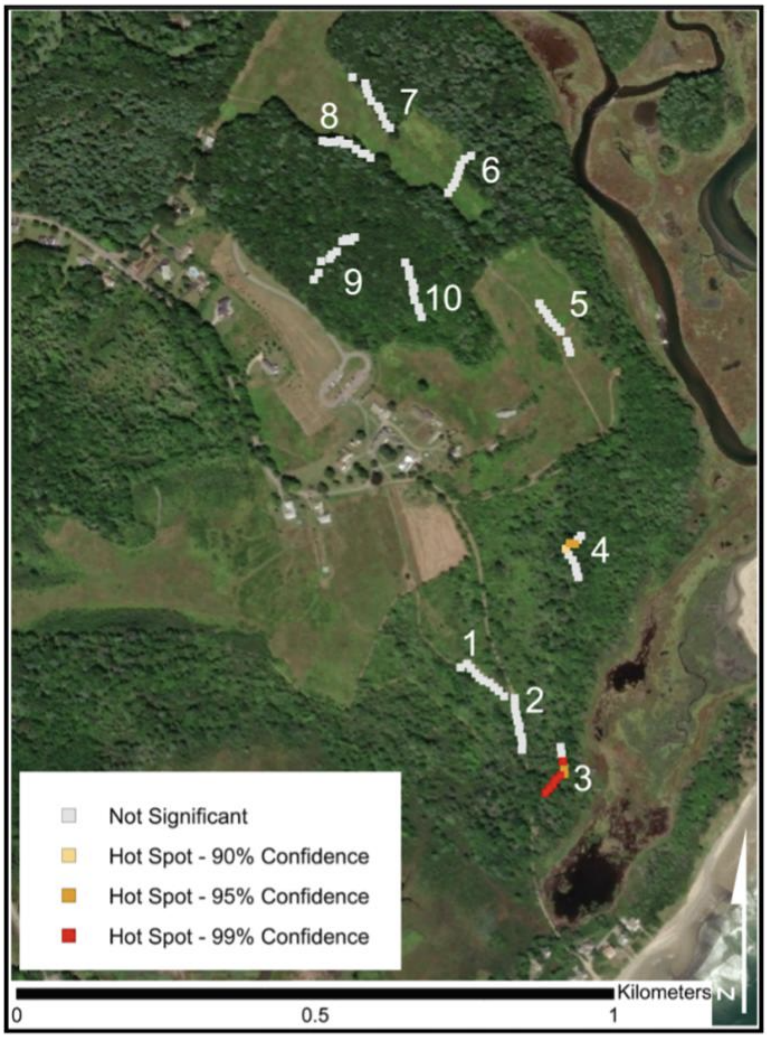


**Figure 1.** Map of WNERR transects and hotspots Squares represent 1m x 10m plots within 100m transects, two transects per habitat: a shrub (Transects 1 and 2), forest w/ invasive shrubs (3 and 4), field (5 and 6), edge (7 and 8), and forest w/ native shrubs (9 and 10).

# Methods

## Collecting and aligning complete genomes

Our group generated 53 DTV genome sequences from samples collected in WNERR between 2018-2021. We downloaded complete genome sequences of DTV and its related strains from the National Center for Biotechnology Information (NCBI) GenBank database. We filtered the sequences based on their quality, completeness, and relevance to the study. We started with 300 genetic samples that are

available from GenBank. The sequences were then uploaded to Geneious Prime and aligned using MAFFT with default settings. After the sequences were aligned, we used IQ-TREE version 1.6.12 with ultrafast bootstrap approximation (1,000 replicates) to determine phylogenetic relationships (Hoang et al., 2018; Nguyen et al., 2015) and visualized the maximum likelihood tree in Interactive Tree of Life (iTOL) (Letunic & Bork, 2021). After visualizing the tree, we selected sequences to be included in the final analysis based on their proximity to the sequences from WNERR. Specifically, we aimed to include samples that demonstrated relationships with locations within other areas of Maine and states outside of Maine. Out of the original 300 sequences, we kept 42 full-length DTV genomes from GenBank (Table 1). Combined with the 53 sequences from ticks collected for the Maine study between 2018-2021 and four sequences our group generated from ticks collected at additional locations in Maine, we ended up with 99 total samples. We estimated a new maximum likelihood tree using IQ-TREE. The best-fit substitution model, TIM+F+G4, was determined using the Bayesian information criterion. We constructed a maximum likelihood tree using this model, with rate heterogeneity modeled using a gamma distribution with four categories. The tree was rooted at the midpoint and had high bootstrap support for all branches (88.9% to 100%). This new tree was also visualized in iTOL.

**Table 1.** Reference sequence metadata

| NAME | Collection Date | Year | City | State | Accession |
|---|---|---|---|---|---|
| CT_G_OL704188_2016 | 2016-11-02 | 2016 | Groton | CT | OL704188 |
| CT_NB_OL704156_2010 | 2010-10-29 | 2010 | North Branford | CT | OL704156 |
| CT_W_OL704194_2019 | 2019-04-09 | 2019 | Westport | CT | OL704194 |
| CT_W_OL704196_2019 | 2019-05-07 | 2019 | Westport | CT | OL704196 |
| MA_HM440559_1996 | NA | 1996 | NA | MA | HM440559 |
| ME_CE_MK104144_2017 | NA | 2017 | Cape Elizabeth | ME | MK104144 |
| ME_CE_OL704211_2016 | 2016-04-08 | 2016 | Cape Elizabeth | ME | OL704211 |
| ME_CE_OL704212_2016 | 2016-06-13 | 2016 | Cape Elizabeth | ME | OL704212 |
| ME_CE_OL704216_2016 | 2016-10-06 | 2016 | Cape Elizabeth | ME | OL704216 |
| ME_CE_OL704221_2016 | 2016-11-18 | 2016 | Cape Elizabeth | ME | OL704221 |
| ME_CE_OL704224_2017 | 2017-11-15 | 2017 | Cape Elizabeth | ME | OL704224 |
| ME_CE_OL704226_2017 | 2017-11-15 | 2017 | Cape Elizabeth | ME | OL704226 |
| ME_CE_OL704227_2017 | 2017-11-15 | 2017 | Cape Elizabeth | ME | OL704227 |
| ME_CE_OL704228_2017 | 2017-11-15 | 2017 | Cape Elizabeth | ME | OL704228 |
| ME_CE_OL704229_2017 | 2017-11-15 | 2017 | Cape Elizabeth | ME | OL704229 |
| ME_CE_OL704230_2017 | 2017-11-15 | 2017 | Cape Elizabeth | ME | OL704230 |
| ME_CE_OL704231_2017 | 2017-11-15 | 2017 | Cape Elizabeth | ME | OL704232 |
| ME_CE_OL704232_2017 | 2017-11-07 | 2017 | Cape Elizabeth | ME | OL704233 |
| ME_CE_OL704233_2017 | 2017-11-07 | 2017 | Cape Elizabeth | ME | OL704234 |
| ME_R_OL704215_2016 | 2016-10-14 | 2016 | Rockland | ME | OL704215 |
| ME_S_OL704222_2016 | 2016-11-17 | 2016 | Standish | ME | OL704222 |
| ME_S_OL704223_2016 | 2016-11-17 | 2016 | Standish | ME | OL704223 |
| ME_T_OL704237_2019 | 2019-05-22 | 2019 | Thomaston | ME | OL704237 |
| ME_W_MK309362_2017 | NA | 2017 | Wells | ME | MK309362 |
| ME_W_OL704213_2016 | 2016-10-07 | 2016 | Wells | ME | OL704213 |
| ME_W_OL704214_2016 | 2016-10-07 | 2016 | Wells | ME | OL704214 |
| ME_W_OL704217_2016 | 2016-11-07 | 2016 | Wells | ME | OL704217 |
| ME_W_OL704218_2016 | 2016-11-07 | 2016 | Wells | ME | OL704218 |
| ME_W_OL704219_2016 | 2016-11-09 | 2016 | Wells | ME | OL704220 |
| ME_W_OL704220_2016 | 2017-11-15 | 2017 | Wells | ME | OL704236 |
| ME_W_OL704236_2017 | 2019-06-18 | 2019 | Wells | ME | OL704238 |
| ME_W_OL704238_2019 | 2016-10-07 | 2016 | Wells | ME | OL704213 |
| NY_AN_OL704287_2015 | 2015-11-21 | 2015 | Ancramdale | NY | OL704287 |
| NY_AR_OL704251_2017 | 2017-06-13 | 2017 | Armonk | NY | OL704251 |
| NY_G_OL704386_2019 | 2019-11-04 | 2019 | Glenville | NY | OL704386 |
| NY_H_OL704318_2018 | 2018-10-14 | 2018 | Hermon | NY | OL704318 |
| NY_I_OL704285_2014 | 2014-11-10 | 2014 | Islip | NY | OL704285 |
| NY_M_OL704343_2014 | 2014-11-04 | 2014 | Montebello | NY | OL704343 |
| NY_M_OL704352_2018 | 2018-06-18 | 2018 | Montebello | NY | OL704352 |
| NY_N_OL704322_2015 | 2015-11-22 | 2015 | Nassau | NY | OL704322 |
| NY_S_OL704354_2018 | 2018-02-27 | 2018 | Smithtown | NY | OL704354 |
| NY_S_OL704356_2018 | 2018-06-06 | 2018 | Smithtown | NY | OL704356 |

### Assessing temporal signal and building a maximum likelihood tree in Interactive Tree of Life (iTOL)

After ensuring the final ML tree contained all relevant sequences, the untranslated regions (UTRs) were removed in Geneious. A new ML tree was generated in IQ-TREE using the GTR with gamma-distributed rate variation and 1,000 bootstrap replicates. To assess the temporal signal in TempEST, we utilized the best-fitting root. Temporal signal refers to the ability of genetic data to reflect the timing of evolutionary events. It is crucial in dating evolutionary processes accurately. Our analysis showed the following results: an estimated date range of 25, a slope (rate) of 2.347E-4, an X-Intercept of 1987.9, a correlation coefficient of 0.62, an R squared value of 0.39, and a residual mean squared of 7.5966E-7. Finally, we constructed a new ML tree and annotated it in iTOL after aligning the sequences and evaluating the temporal signal.

## Results

The final ML tree included samples from four states, 25 different locations within these states, and two samples from a known state but unknown cities (Figure 2). This tree showed that the WNERR sequences did not all cluster together, but had intermingled sequences from other cities in Maine and other states.

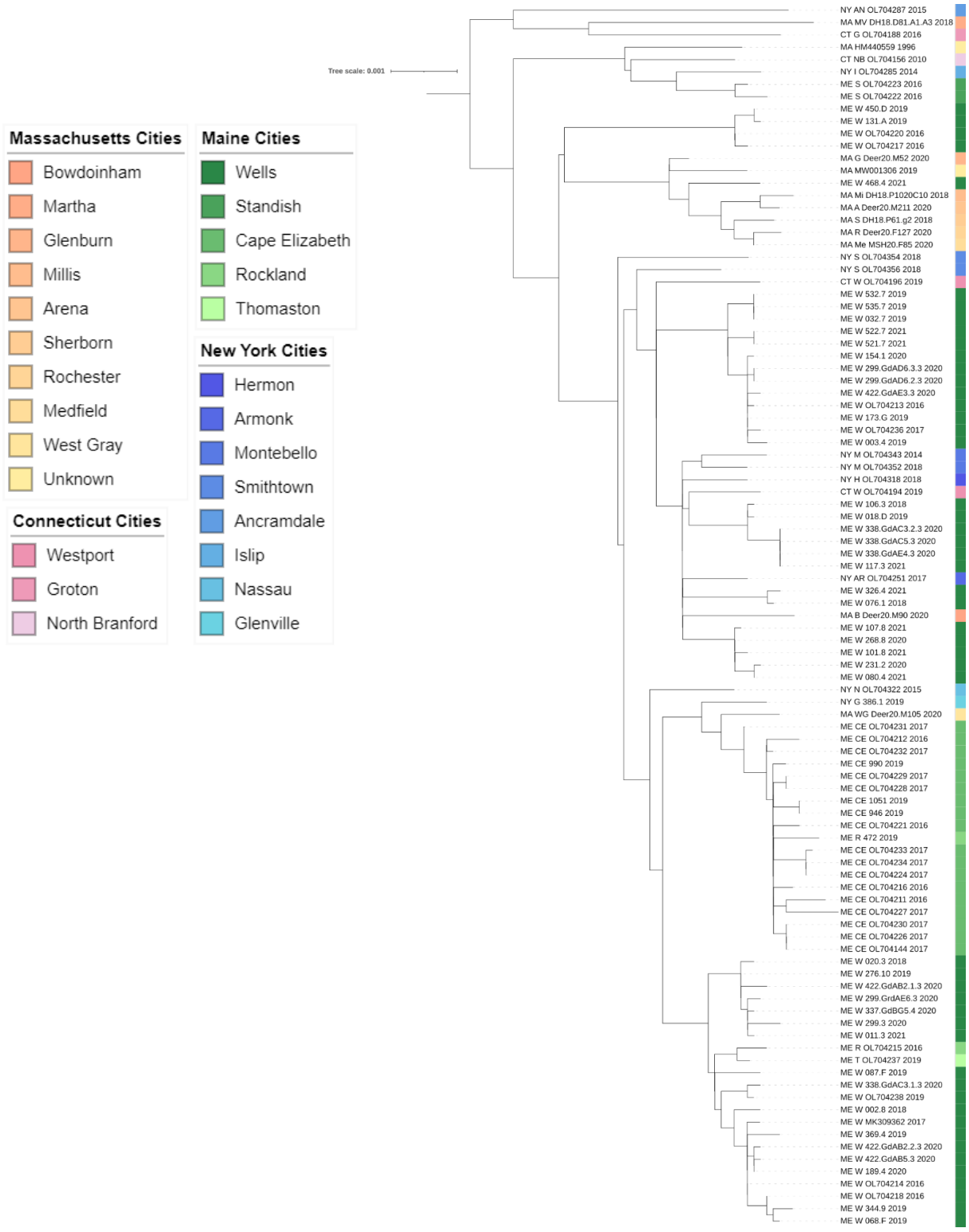


**Figure 2**. Maximum likelihood tree with cities and states annotated
Maximum likelihood tree showing the relationships between cities within the same state and the relationship between states.

Phylogenetic analysis revealed four major clades from WNERR (Figure 3). The pairwise distances calculated for the different clades in this study provide essential information about the genetic diversity and evolutionary relationships among the viral sequences. Clade A, which contained four sequences from WNERR and was most closely related to sequences from Massachusetts, had the lowest average number of pairwise distances between sequences (3.5), indicating that the sequences within this clade were genetically similar with only a small number of differences or substitutions. This degree of conservation was particularly notable since samples in this clade were collected across multiple years, 2016-2019. On the other hand, Clade B, which contained 13 sequences from WNERR, showed a much higher average number of pairwise distances (55.4), suggesting a higher level of genetic diversity among the sequences within this clade. Clade C, which has 19 sequences and included sequences from Connecticut, Massachusetts, and New York, showed an intermediate level of genetic diversity with an average pairwise distance of 32.4. Clade D, which consisted of 22 sequences and included sequences from other parts of Maine (Rockland and Thomaston) also showed an intermediate level of genetic diversity with an average pairwise distance of 28.0. Comparing these pairwise distances, it can be inferred that Clade C has, on average, a higher genetic diversity or evolutionary divergence compared to Clade D. This means that the sequences within Clade C exhibit more genetic variation or differences from each other compared to the sequences within Clade D. The finding that the clades each have a different amount of diversity within them has several potential interpretations. First, it suggests that there may be varying degrees of genetic similarity or differences among the viral sequences within each clade. Clade A, which has the lowest average number of pairwise distances, indicates that the sequences within this clade are genetically similar, with only a small number of differences or substitutions. In contrast, Clade B shows a much higher average number of pairwise distances, indicating a higher level of genetic diversity among the sequences within this clade. Clade C and Clade D, which show intermediate levels of genetic diversity, suggest that there may be varying degrees of genetic variation within different clades. Second, the differences in genetic diversity among the clades may reflect distinct evolutionary relationships among the viral sequences. Clades that contain sequences from other regions, such as Clade C and Clade

D, reflect introductions to or from WNERR, whereas clades that only contain WNERR sequences, such as A and B, reflect isolated foci. Furthermore, the varying levels of genetic diversity within each clade could have implications for the virus's transmission dynamics, evolution, and dispersal patterns in the study area. Clades with higher genetic diversity, such as Clade B, may be experiencing more rapid evolution or higher transmission rates. In comparison, clades with lower genetic diversity, such as Clade A, may be more stable or have more limited transmission dynamics.

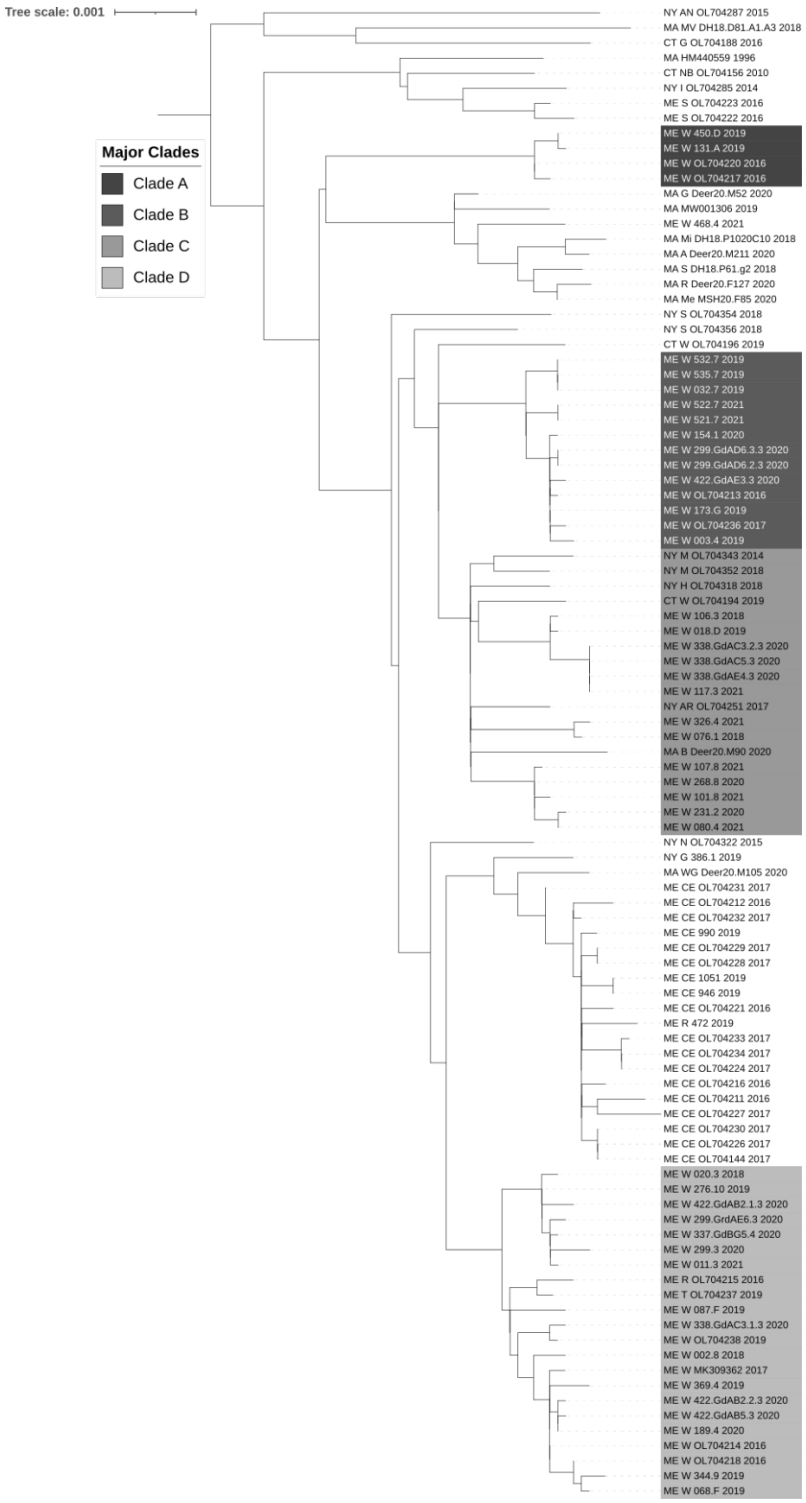


**Figure 3.** Maximum likelihood tree with major clades annotated
Maximum likelihood phylogenetic tree of DTV showing four major WNERR clades highlighted in iTOL.

As previously mentioned, ten 100-meter transects were established, with two transects per habitat type, each consisting of ten consecutive 10 x 1-meter plots. Our analysis revealed the presence of clusters of closely related viruses within WNERR that showed persistence in the same transect across multiple years, as well as clusters distributed across different transects and years (Figure 4). Furthermore, we observed sequences highly similar to these clusters in different states, suggesting the potential dispersal of closely related viruses to and from WNERR. Notably, within the primary hotspot of transect 3, the average number of pairwise distances between sequences was 90.8, similar to the overall average of 78.1 between all sequences in this study. The finding that similar levels of viral diversity were observed within one small transect throughout the entire study is noteworthy. This finding implies that there may be local dynamics of virus transmission and persistence within specific transects, as well as potential dispersal of closely related viruses to and from the study site, potentially to other states. These findings highlight the complex dynamics of viral transmission and dispersal within a localized geographic space and provide insights into the potential mechanisms driving viral diversity patterns observed in the study. Similarly, in transect 4, another hotspot, the average number of pairwise distances was 41.5. Figure 4 demonstrates that each clade was distributed across multiple transects, indicating a wide spatial distribution of viral diversity. Moreover, the number of clades represented within each transect varied. Transects 2, 7, 9, and 10 were associated with only one clade, while transects 1, 3, 4, 8, and L was linked to two or more clades, indicating potential differences in the composition and distribution of viral variants across different transects. These findings indicate that certain areas within WNERR exhibit higher genetic similarity among viruses, possibly indicating localized transmission dynamics and persistence of specific viral strains over time.

The study findings revealed strong bootstrap support for the major clades identified in the phylogenetic analysis, with a cutoff set at 95% or higher. Bootstrap values of 95% or higher indicate a high confidence level in the inferred relationships among viral sequences within each clade. This robust statistical support enhances the reliability of the findings and adds credibility to the conclusions drawn from the analysis of viral diversity. The importance of obtaining strong bootstrap support for the major

clades cannot be overstated. It provides a solid foundation for the observed patterns of viral diversity and lends confidence to the conclusions drawn from the study. The high bootstrap support indicates that the identified clades are likely to be biologically meaningful and not simply the result of random variation or sampling artifacts. Strong bootstrap support also strengthens the validity of the observed patterns of viral diversity across different transects and years.

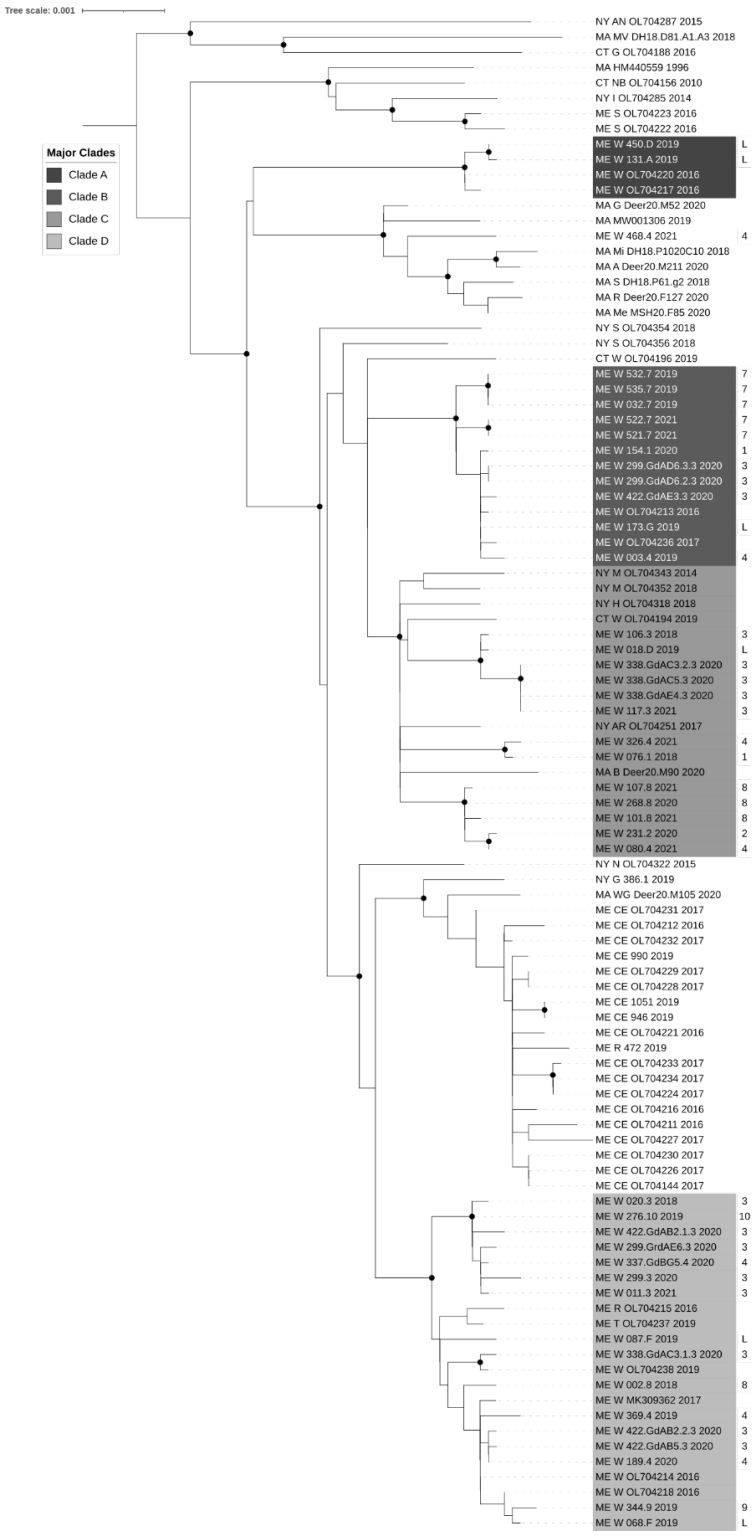


**Figure 4.** Maximum likelihood tree showing the relationship between major clades and transects Transects, major clades, and bootstraps with 95% or greater support are labeled on the maximum likelihood tree to show the relationships between transects and clades, states, and years.

# Discussion

In this study, we investigated the geographic distribution and evolution of DTV lineages and sub-lineages using phylogenetic analyses of 99 sequenced isolates from the northeastern United States. The maximum likelihood tree showed distinct clades and clusters of closely related viruses, providing insights into DTV's genetic diversity and dynamics in the study area.

One of our study's main findings was identifying several major clades of DTV from the Wells National Estuarine Research Reserve (WNERR) in Maine. These clades showed genetic differences of up to 4.42, indicating significant divergence within the virus population in this region. Interestingly, one of the WNERR clades was more closely related to sequences from other locations in Maine, such as Cape Elizabeth and Rockland, than it was to the other WNERR clade. This suggests that DTV may have multiple sources or routes of introduction into the WNERR area, possibly through different reservoir hosts.

Furthermore, we observed a small clade of DTV sequences from WNERR that was most closely related to sequences from New York. This finding raises questions about the potential movement of DTV across state borders and the role of long-distance dispersal in spreading the virus. Recent data has implicated shrews as a potential primary reservoir for DTV. Shrews are known to establish a home range of 370 to 630 $m^2$ (440 to 750 $yd^2$), with males often extending their boundaries during the breeding season in search of females (Common Shrew Articles-Encyclopedia of Life, n.d.). This indicates that shrews have the potential to cover considerable distances in their search for resources and mates, which may contribute to their ability to disperse viruses, including DTV, over varying distances and potentially across different habitats. Future studies could explore the movement patterns of shrews, including their potential for long-distance dispersal, as well as other mechanisms of viral dispersal. Techniques such as radio telemetry or genetic markers could be used to track the movement of shrews and shed light on their potential role in spreading DTV across different habitats and geographical areas. Additionally,

investigating other potential vectors, such as insects or other animals, could provide further insights into the transmission dynamics of DTV.

It is worth noting that the emergence of DTV as a tick-borne virus has raised concerns about its potential impact on human health. While POWV has been considered a minor public health concern due to the relatively host-specificity of its arthropod vector, DTV could pose a more significant threat. Transmission to the mammalian host can occur within 15 minutes of attachment (Ebel & Kramer, 2004). Transmission in human cases has occurred with tick attachment in as little as 3-6 hours (Feder et al., 2021). Quick dissemination from tick to host is likely due to DTV residing in the salivary glands of the ticks before the subsequent blood meal acquisition; this is a stark contrast compared to the *B. burgdorferi* spirochete which is housed in the midgut before migrating to the salivary glands (Ebel & Kramer, 2004; Ribeiro et al., 1987). Since nymphs can also transmit the virus, there is a high potential for causing disease in humans. POWV also transmits transstadially, and some evidence shows potential for vertical transmission (Ebel & Kramer, 2004; Costero & Grayson, 1996). The increasing number of reported cases of POWV and DTV infections in recent years highlights the need for continued surveillance and research to understand better the epidemiology, transmission dynamics, and clinical implications of these viruses.

The analysis of viral diversity at a small geographic scale, specifically within WNERR, has provided valuable insights into the ecology and evolution of DTV lineages. Identifying four major clades within WNERR, each with different levels of genetic diversity and evolutionary relationships, suggests the presence of distinct viral populations within this localized area. The observation of transects associated with different clades further underscores the potential differences in viral composition and distribution, even within a small geographic area. These findings highlight the dynamic nature of DTV populations and the need for comprehensive surveillance and control strategies considering local ecological factors. The results also support the importance of incorporating phylogenetic analysis to better understand the evolution and spread of tick-borne diseases and to inform public health efforts in managing and mitigating their impact on human health. Further research exploring the factors driving the

persistence and spread of DTV within tick populations and its potential impacts on human health would provide valuable insights for addressing this emerging public health concern.

In conclusion, our study provides insights into DTV's geographic distribution, evolution, and sub-lineages in the northeastern United States. Identifying distinct clades and clusters of closely related viruses in the study area suggests complex DTV transmission and evolution dynamics. The emergence of DTV as a tick-borne virus with potential implications for human health shows the importance of continued surveillance and research to understand and mitigate the risks associated with this virus.

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