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Genetic Relationships of Jamestown Canyon Virus Strains Infecting Mosquitoes Collected in Connecticut

Philip M. Armstrong* and Theodore G. Andreadis
The Connecticut Agricultural Experiment Station, New Haven, Connecticut

Abstract. Jamestown Canyon virus (JCV) (family *Bunyaviridae*, genus *Orthobunyavirus*) is maintained in a mosquito-deer cycle and has been implicated in the etiology of meningitis and encephalitis with human cases reported from Ontario, Canada, Michigan, Connecticut, and New York. Despite the recognition of symptomatic cases in the northeastern United States, little is known about the genetic relationships of JCV variants circulating in this region. Accordingly, we compared the phylogenetic relationships of 56 JCV isolates from mosquitoes collected in Connecticut over a 40-year period to evaluate their evolutionary history and characterize patterns of genetic diversity in the state. We distinguished at least two major lineages in Connecticut on the basis of phylogenetic reconstruction of small (S), medium (M), and large (L) segment nucleotide sequences. Viruses representing each lineage infected a diverse group of mosquito species over multiple years of sampling and appeared to be geographically structured along an east-west axis. One of these lineages was detected in Connecticut from 1966 through 2006 with few mutational changes accumulating over time. Phylogenetic trees generated from portions of the M and L segments yielded different topologies from S segment sequences as three clades became consolidated into two. Although direct evidence for genetic exchange by reassortment was lacking among cocirculating strains in Connecticut, molecular trees from S, M, and L segments were incongruent, which suggests a distinct evolutionary history or process for each genomic segment. These results suggest that JCV variants are stably maintained in Connecticut where they infect a wide diversity of mosquito species.

INTRODUCTION

Jamestown Canyon virus (JCV) belongs to the California serogroup within the genus *Orthobunyavirus*, family *Bunyaviridae* and is broadly distributed throughout the United States and Canada. The virus is amplified by transmission between mosquito vectors (mainly *Aedes* and *Ochlerotatus* species) and deer hosts, and may persist over winter in mosquito eggs by vertical transmission.[1] (The separation of *Ochlerotatus* and *Aedes* into different genera is contested[2,3] but we adopt this designation on the basis of morphologic characters and molecular evidence.[4,5]) Infection in humans appears to be most prevalent in regions where deer are abundant[6] and may occasionally cause neurologic illness including meningitis and encephalitis, particularly in adults.[7,8] In Connecticut, serologic evidence of JCV infection was found in 4–10% of blood donors,[9] and the first statewide case was diagnosed in a hospitalized teenage patient during 2001.[10] Other human cases of symptomatic illness have been reported in New York, Michigan, and Ontario, Canada.[11]

Despite the recognition of JCV as a human pathogen, little is known about the genetic relationships of variants circulating in nature. It was originally isolated from *Culiseta inornata* mosquitoes collected in Colorado in 1961 and identified as a subtype of Melao virus (MELV) based on serologic classification.[12,13] Jamestown Canyon virus may be further subdivided into regional strains: Jerry Slough virus (JSV) and South River virus (SRV) isolated from California and New Jersey, which are considered synonyms of JCV by the International Catalogue of Arboviruses.[13] Molecular evidence supports this classification scheme and includes Inkoo virus (INKV) from Finland as another possible variant of JCV.[14,15] These findings indicate that JCV is comprised of several evolutionary lineages that circulate in North America and its geographic range may extend into northern Europe if INKV proves to be antigenically indistinguishable from JCV upon further analysis.

As a member of the family *Bunyaviridae*, the genome of JCV is comprised of three single-strands of negative-sense RNA designated as the small (S), medium (M), and large (L) segments that encode the nucleocapsid, surface glycoprotein precursor, and RNA polymerase, respectively.[16] The segmented nature of JCV allows it to rapidly diversify by reassortment in addition to mutation and possibly recombination.[17,18] Segmented viruses undergo reassortment during mixed infections of the same cell by exchanging whole viral segments among heterologous viruses. Evidence of reassortment has been documented for several members of the family *Bunyavirdae* by comparing phylogenetic trees derived from each genomic segment and identifying inconsistent relationships among taxa.[19–22] The extent of genetic exchange among natural isolates of JCV has not been evaluated; however, reassortant viruses have been generated experimentally by coinfecting mosquitoes with JCV and La Crosse virus.[23]

Statewide mosquito trapping and testing to monitor arbovirus activity has been continuously conducted in Connecticut since 1997. Isolates of JCV obtained during this effort were sequenced and compared by phylogenetic analysis to evaluate the evolutionary history and transmission patterns of JCV variants in this region.[24] In addition, we compared tree topologies generated from the S, M, and L segments to determine whether they are congruent or reflect distinct phylogenetic histories.

MATERIALS AND METHODS

Viruses. Jamestown Canyon virus was isolated from 19 different species of mosquitoes that were collected with CO_2-baited Centers for Disease Control (Atlanta, GA) miniature light traps at 31 different locales in 7 Connecticut counties over a 10-year period from 1997 through 2006 (Figure 1). Viruses were isolated by inoculating mosquito homogenates onto Vero cell cultures as previously described.[24] Connecticut

* Address correspondence to Philip M. Armstrong, The Connecticut Agricultural Experiment Station, 123 Huntington Street, PO Box 1106, New Haven, CT 06504. E-mail: philip.armstrong@po.state.ct.us

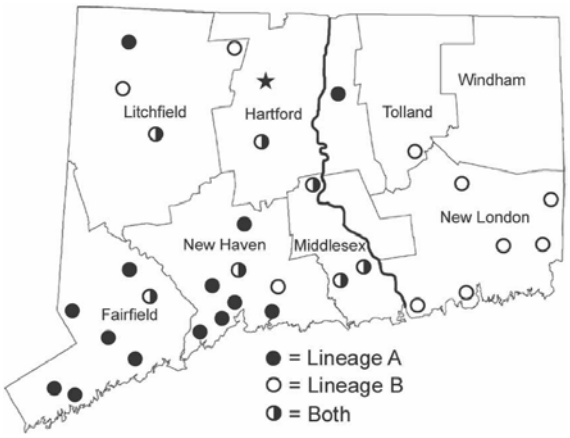

FIGURE 1. County-based map of Connecticut showing distribution of 31 trapping locations where mosquitoes infected with Jamestown Canyon virus were collected from 1997 through 2006. The Connecticut River is depicted by bold line. ★ = 1966 Simsbury collection site.

isolates from 1966 and 1992 were also included in this study,[25,26] in addition to prototype strains of JCV, JSV, INKV, SRV, and MELV.

Reverse transcription–polymerase chain reaction (RT-PCR) and nucleotide sequencing. RNA was extracted from primary viral isolates using the viral RNA Kit (Qiagen, Valencia, CA). RT-PCR was performed using the Titan One-Tube RT-PCR System (Roche Diagnostics, Indianapolis, IN) and three primer pairs targeting genomic segments: S (BUNS+new: 5′-TGACCAGTAGTGTACTCCAC-3′ and BUNS-new: 5′-CAAGCAGTAGTGTGCTCCAC-3′),[27] M (M14C: 5′-CGGAATTCAGTAGTGTACTACC-3′ and M619Rnew: 5′-GACATATGCTGATTGAAGCAAGCATG-3′),[19] and L (CAL-L64f: 5′-GGATAACGCAGAATATCAACAATTC-3′ and CAL-L609r: 5′-GCGACTTTAAGTAAGAATTCTTCATC-3′). These primers were selected because they reliably amplify cDNA from a variety of California and Bunyamwera group viruses and were shown to flank regions with sufficient information for phylogenetic comparisons.[19,28,29] For each RT-PCR, 2 uL of extracted RNA was added to Master mix I containing 500 μM ATP, 500 μM GTP, 500 μM CTP, 500 μM TTP, 12.5 μM dithiothreitol DTT, and 1 μM of each primer in a final volume of 20 μL. This mixture was heated to 85°C for 5 minutes and then quick chilled on ice. Master mix I was added to a second master mix containing the Titan RT-PCR buffer and enzyme mixture to give a final volume of 50 μL. Amplification of the full-length S segment (~950 basepairs) was performed as follows: 1 cycle at 50°C for 30 minutes 94°C for 2 minutes, 10 cycles at 94°C for 15 seconds, 55°C for 30 seconds, and 68°C for 1 minute, followed by 25 cycles at 94°C for 15 seconds, 55°C for 30 seconds, and 68°C for 1 minute plus 5 seconds per cycle, and 1 cycle at 68°C for 7 minutes. Portions of the M segment (~620 basepairs) and L segment (~550 basepairs) were amplified as follows: 1 cycle at 45°C for 30 minutes and 94°C for 2 minutes, 10 cycles at 94°C for 15 seconds, 48°C for 30 seconds, and 68°C for 1 minute, followed by 25 cycles at 94°C for 15 seconds, 55°C for 30 seconds, and 68°C for 1 minute plus 5 seconds per cycle, and 1 cycle at 68°C for 7 minutes. Amplification products of the appropriate size were purified using the PCR purification kit (Qiagen) and sequenced at the Keck Sequencing Facility (New Haven, CT) using the 3730xl DNA Analyzer (Applied Biosystems, Foster City, CA).

Genetic analyses. Overlapping sequence chromatograms were aligned and edited with a minimum of two-fold redundancy using ChromasPro (Technelysium Ltd., Tewantin, Queensland, Australia). Edited nucleotide sequences were deposited in Genbank (accession numbers EF681804–EF681859 and EF687021–EF687137). Multiple sequence alignments were generated by the ClustalW algorithm using Mega 3.0.[30] Nucleotide alignments were visually inspected for errors and coding regions were translated into protein to check the integrity of the open reading frame in the alignment. The S segment alignment consisted of 827 characters and spanned the entire nucleocapsid gene and portions of the 5′ and 3′ non-coding regions. The M segment alignment comprised 471 characters encoding part of the G2 glycoprotein, and the L segment included 408 characters encoding a portion of the RNA-dependent RNA polymerase. There were no gaps in sequence alignments except in the 3′ non-coding region of the S segment alignment and in the M segment alignment, which corresponded to a two-amino acid deletion of the outgroup sequence. Phylogenetic relationships were evaluated by three independent methods: neighbor joining (NJ), maximum likelihood (ML), and maximum parsimony (MP) using PAUP 4.0.[31] The NJ trees were generated using the Jukes-Cantor model of sequence evolution. The ML analyses were performed by implementing the heuristic search method and the HKY85 model with base frequencies and transition/transversion ratios estimated empirically. The MP trees were estimated by the heuristic search method and treating characters as equally weighted and unordered. Melao virus served as the outgroup to root phylogenetic trees. Support for individual nodes was evaluated by performing 200 bootstrap replicates using the maximum likelihood criteria described above. Incongruence length difference (ILD) tests were performed in PAUP 4.0 by parsimony analysis using 1,000 random partition replicates. Patristic distances were calculated from maximum likelihood trees using PATRISTIC 1.0.[32]

RESULTS

Phylogenetic reconstruction of the S segment showed that isolates from Connecticut belonged to three distinct clades designated as lineages A, B1, and B2 (Figure 2). These groupings were well supported by bootstrap analysis and consistent regardless of the criteria used for estimating phylogenetic trees. Viruses representing each lineage infected a wide diversity of mosquito species and persisted over many years of sampling. Lineage A comprised 60.7% of viruses isolated from Connecticut and included the original 1966 isolate obtained from *Ochlerotatus abserratus* in Simsbury, Connecticut. Lineage B1 was the second most prevalent group, representing 33.9% of viruses sampled; viruses of lineage B2 were infrequently detected in mosquitoes and infected 5.4% of the cohort. Mean nucleotide distances between lineages A, B1, and B2 ranged from 5.7% to 8.5%, which was at least 20-fold higher than mean distances within lineages. Lineages B1 and B2 consistently grouped together as sister clades in NJ, MP, and ML analyses; however, this node was weakly supported with 56% bootstrap support. Reference strains JCV and JSV

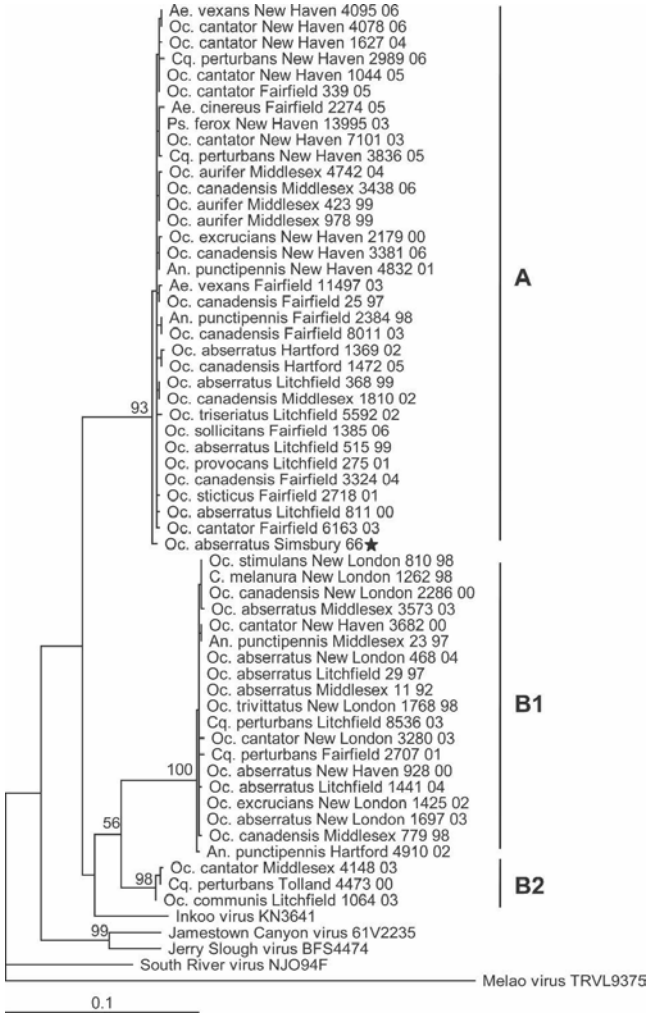

FIGURE 2. Phylogenetic tree depicting relationships of Jamestown Canyon virus isolates on the basis of maximum likelihood analysis of small segment nucleotide sequences. Numbers at nodes indicate bootstrap support for values greater than 50%; support for nodes within lineages are omitted for clarity. Taxon names for Connecticut isolates specify the mosquito species and county where they were collected, in addition to the isolate number and year of isolation (last two digits). Branch lengths are proportional to the number of nucleotide substitutions/site.

grouped together with 99% bootstrap support, yet other major nodes were poorly supported with less than 50% bootstrap support. These findings indicate that distinct lineages of JCV persist in Connecticut and infect a wide variety of mosquito species.

The distribution of lineages A and B (B1 + B2) appeared to be geographically structured along an east-west axis (Figure 1). Using the Connecticut River as a dividing line, lineage A viruses represented 72% of isolates sampled from the western portion of the state versus only 10% of those collected east of the river ($P = 0.0005$, by Fisher's exact test). Nevertheless, the distribution of these lineages clearly overlapped because both were detected in seven sites west of the Connecticut River.

We found that distinct lineages of JCV may cocirculate in the same sites, which is a necessary condition for heterologous reassortment. To evaluate the potential for genetic exchange among JCV strains, we compared the topologies of molecular trees resulting from M and L segment nucleotide sequences. Trees estimated from a portion of the M segment yielded topologies that were essentially identical to each other by NJ, MP, and ML methods (Figure 3), yet differed in many details from the S segment topology (Figure 2). Connecticut isolates that had segregated into lineages B1 and B2 became consolidated into a single group designated as lineage B; otherwise, membership within each group was consistent for both segments. Each lineage was genetically homogeneous; mean nucleotide distances were 0.6 and 0.5% within lineages A and B versus 12.4% between these groups. The branching order of the Connecticut lineages and reference taxa JCV (61V2235), JSV (BFS4474), and INKV (KN3641) also differed in many aspects from S segment trees, yet none of these differences were indisputable because bootstrap support was weak at conflicting nodes in one or both trees.

Sequences from the L segment generated trees that were similar to each other by all three phylogenetic methods (Figure 4) but differed from S and M segment trees. The S and M

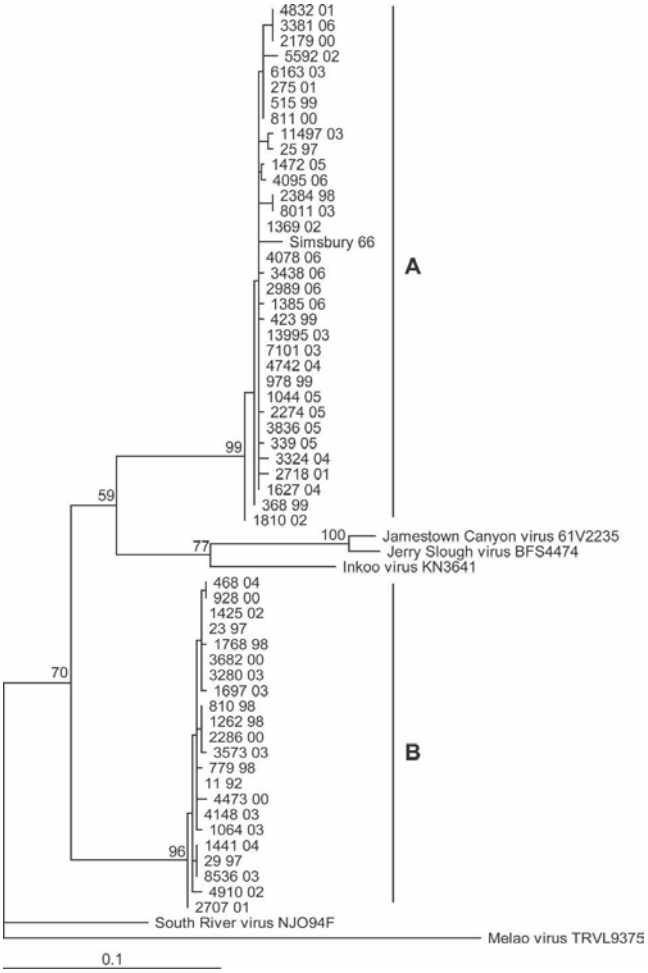

FIGURE 3. Maximum likelihood tree depicting relationships of Jamestown Canyon virus isolates on the basis of analysis of medium segment nucleotide sequences. Numbers at nodes indicate bootstrap support for values greater than 50%; support for nodes within lineages are omitted for clarity. Taxon names for Connecticut isolates are represented by their isolation number and year of isolation (last two digits). Branch lengths are proportional to the number of nucleotide substitutions/site.

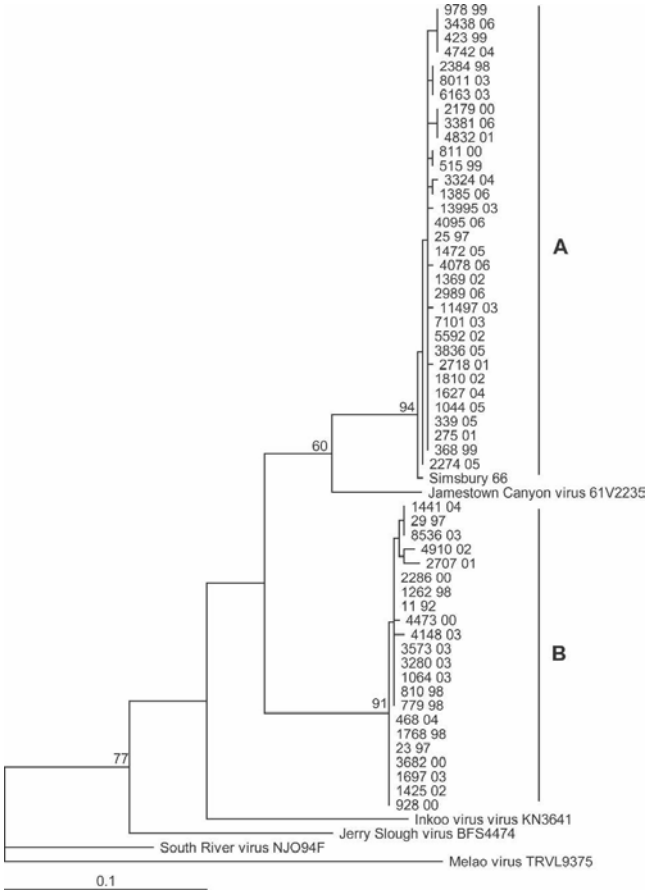

FIGURE 4. Maximum likelihood tree depicting relationships of Jamestown Canyon virus isolates on the basis of analysis of large segment nucleotide sequences. Numbers at nodes indicate bootstrap support for values greater than 50%; support for nodes within lineages are omitted for clarity. Taxon names for Connecticut isolates are represented by their isolation number and year of isolation (last two digits). Branch lengths are proportional to the number of nucleotide substitutions/site.

segment trees paired the prototype strain of JCV (61V2235) with JSV (99–100% bootstrap support), yet the L segment tree placed this virus adjacent to lineage A (60% bootstrap support). Isolates from Connecticut separated into two genetically distinct groups: lineages A and B as defined by the M segment tree. Genetic variation within each of these lineages was limited.

The observed conflicts in branching patterns of the S, M, and L trees may arise from real differences in the evolutionary history or may be explained by errors in phylogenetic inference on the basis of limited sampling of phylogenetically informative characters in this study. To assess whether molecular datasets from each RNA segment differ significantly, we performed ILD tests.[33] These tests assess conflict by comparing the optimal score of trees for the original datasets, or segments for this study, to a null distribution of random partitions of the combined dataset. If there is significant conflict, the original, predefined datasets will out-perform the combined, randomly-partitioned datasets. The ILD tests detected conflicting phylogenetic signal in pairwise comparisons between segments: S and M ($P = 0.03$), S and L ($P = 0.005$), in addition to M and L ($P = 0.004$). On the basis of these results, we suggest that the observed conflicts among RNA segments reflect a separate evolutionary history or process.

Conflicting relationships were identified by comparing patristic distances from each RNA segment. Patristic distances were calculated from the branch lengths of ML trees to estimate the amount of genetic change separating pairs of taxa and the relationship of these distances was compared between segments in two-way scatter plots (Figure 5). A strong correlation was observed when comparing distances between segment trees with a few notable outliers. Patristic distances separating viruses representing lineages B1 and B2 in the S segment tree were 9-fold and 23-fold greater than corresponding distances from the M and L segment trees. Inconsistencies were also observed for the prototype strain of JCV (61V2235). Sequence from the L segment of this strain was more similar to lineage A viruses versus M and S sequences, which were more closely related to JSV. These findings suggest acquisition of RNA segments from different ancestors.

DISCUSSION

This study presents the first molecular phylogeny of JCV by focusing on isolates obtained over a 40-year period from a diverse array of mosquito species collected over a broad geographic range in Connecticut. We found at least two major lineages of JCV cocirculate statewide. Lineages A and B are distinct from reference strains of JCV, JSV, SRV, and INKV isolated from Colorado, California, New Jersey, and Finland, respectively, and both reappeared over multiple years of sampling, which indicates stable transmission of these variants in this region consistent with field observations. Jamestown Canyon virus is the only mosquito-borne virus that has been consistently detected each year during 10 years of continuous surveillance in Connecticut from 1997 through 2006.[24] The virus may persist over winter during periods of mosquito inactivity in mosquito eggs infected by transovarial transmission,[34,35] thereby providing a plausible mechanism for the stable maintenance of JCV from year to year. Alternatively but less likely, the virus could be reintroduced each year from southern sites where mosquitoes are active throughout the year and presumably transmission of JCV is continuous. If annual importation of the virus is a more important mechanism for reinitiating transmission each season in northern, temperate sites, then our phylogenetic trees would show a different pattern. Given this scenario, we would expect periodic replacement of genetic clades each year and the absence of any geographic structure, maintained over multiple years of sampling, which was not observed in Connecticut. Another possibility is that the two JCV lineages are annually introduced into different regions of Connecticut via distinct migratory pathways; however, this is highly unlikely given that the main vertebrate hosts (white-tailed deer) are not long-distance migrants.

The extent of genetic divergence between the initial 1966 Simsbury isolate and extant strains of lineage A suggests that JCV is evolving slowly and provides context for interpreting evolutionary distances among lineages. The different groupings identified in this study probably have been evolving independently in North America for a long time, although precise estimates cannot be ascertained given the lack of earlier isolates needed to accurately calibrate the molecular clock. Rapid changes in viral genotype, therefore, are more likely to

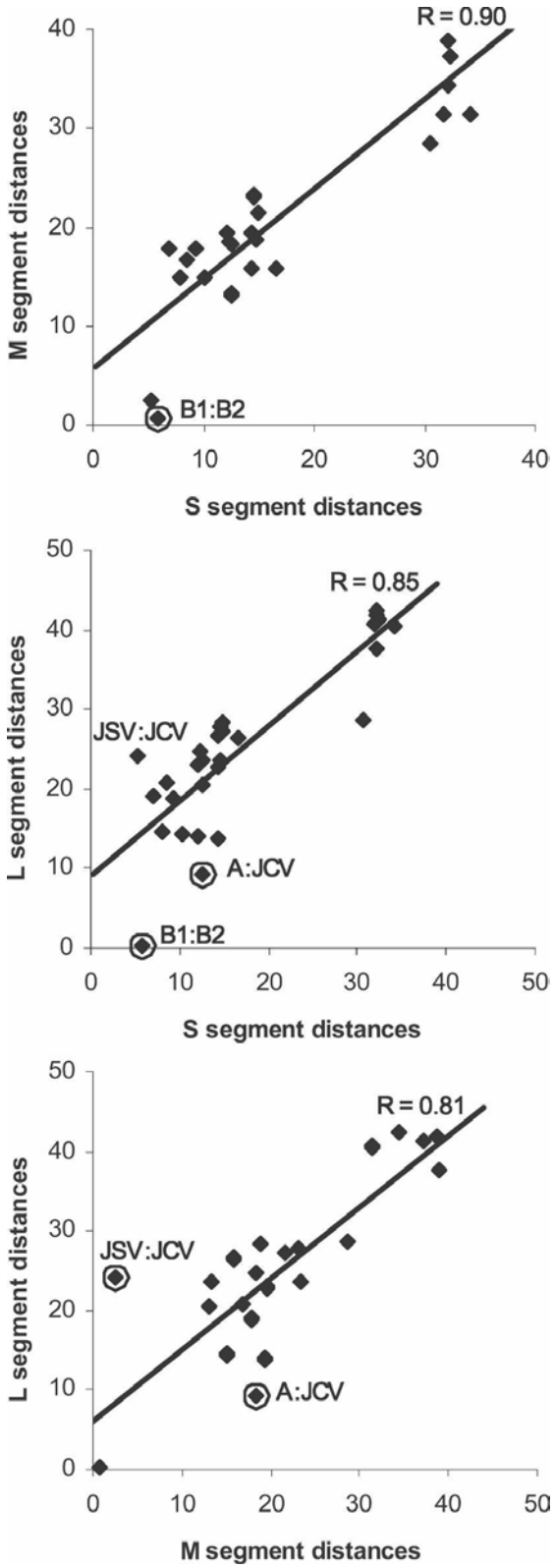

FIGURE 5. Comparisons of patristic distances (percent nucleotide difference) calculated for pairs of taxa from small, medium, and large segment maximum likelihood trees. Regression lines are calculated from paired distances and circled points represent paired distances that are more than two standard deviations from the mean difference. Lineage A, B1, and B2 viruses are represented by isolates 5592, 468, and 1064, respectively. JCV (Jamestown Canyon virus) and JSV (Jerry Slough virus) refer to prototype strains 61V2235 and BFS4474.

be accomplished by genetic exchange of RNA segments between heterologous viruses rather than by the random accumulation of mutations. The prototype strain of JCV carries the genetic signature of hybridization between two different ancestors that later gave rise to lineage A and JSV. These results, however, should be interpreted with caution given that reassortment would have occurred in the distant past.

In this study, we found no evidence for genetic exchange between major lineages A and B, despite the absence of obvious ecologic or geographic barriers between them in this region. Lineage A viruses were more prevalent in the western portion of Connecticut, whereas lineage B predominated in the east, which suggests restricted movement between the two portions of the state. Nevertheless, their distributions overlapped in many of the same sites, both lineages were detected during the same years of sampling, and both infected many of the same mosquito species without any obvious pattern, thus providing ample opportunity for genetic exchange to occur within the same vector or vertebrate host. Viruses designated as lineages B1 and B2 by analysis of the S segment became consolidated into a single lineage when comparing sequence from the M and L segments. This could be a result of reassortment with another unidentified lineage, creating a third distinct clade in the S segment tree, or perhaps the different segments evolved at unequal rates. We cannot differentiate between these two possibilities based on our current sample of viruses.

Our findings indicate that JCV strains in Connecticut are structured more by geography than by year of isolation, which suggests that migration is restricted among populations occupying eastern and western parts of the state. This pattern is not observed in Connecticut and other regions of the northeastern United States for mosquito-borne viruses that perpetuate in avian hosts such as West Nile or eastern equine encephalitis viruses.[36–39] These findings may reflect the host range of the main amplification hosts: white-tailed deer versus birds. Recent studies have shown mean annual home-range sizes for white-tailed deer in suburban landscapes in Connecticut typically range from 40 to 60 hectares.[40,41] Additional sampling of JCV on a broader scale may show that genetic variation is geographically structured across its range in North America and could identify variants that map to the distribution of symptomatic cases of JCV infection. This study provides a foundation for future studies on the molecular evolution and epidemiology of JCV.

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Authors' address: Philip M. Armstrong and Theodore G. Andreadis, The Connecticut Agricultural Experiment Station, 123 Huntington Street, PO Box 1106, New Haven, CT 06504, Telephone: 203-974-8461, Fax: 203-974-8502. E-mail: philip.armstrong@po.state.ct.us.

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