A phylogenetic approach to following West Nile virus in Connecticut

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The 1999 outbreak of West Nile (WN) virus in the northeastern United States was the first known natural occurrence of this flavivirus in the Western Hemisphere. In 1999 and 2000, 82 independent Connecticut WN virus isolates were cultured from nine species of birds, five species of mosquitoes, and one striped skunk. Nucleotide sequences obtained from these isolates identified 30 genetic changes, compared with WN-NY99, in a 921-nt region of the viral genome beginning at nucleotide position 205 and ending at 1125. This region encodes portions of the nucleocapsid and envelope proteins and includes the entire coding regions for the premembrane and membrane proteins. Amino acid changes occurred at seven loci in six isolates relative to the WN-NY99 strain. Although 34 of the isolates showed sequences identical to the WN-NY99 isolate, we were able to show geographical-based clusters of mutations. In particular, 26 isolates were characterized by mutation of C to T at position 858. This group apparently originated in Stamford, CT and disseminated to sites located as far as 54 miles from Stamford. Sequences of WN virus isolated from both brain and heart tissues from the same avian host were identical in all 14 tested individual birds, suggesting that the mutations we have documented are real and not caused by culture, RNA extraction, or PCR procedures. We conclude that this portion of the viral genome will enable us to follow the geographical and temporal movement of variant WN virus strains as they adapt to North America.

viral evolution | epidemiology

West Nile (WN) virus is geographically distributed in Africa, the Middle East, western and central Asia, India, Australia (Kunjin virus), and Europe (1–3). Mosquitoes, particularly in the genus *Culex*, transmit this virus among birds and other vertebrates (2–5). The first recorded epidemic occurred in Israel in the early 1950s. More recently, outbreaks of human encephalitis caused by WN virus have been documented in Romania and Russia (6, 7).

WN virus, introduced recently into the northeastern United States, caused the deaths of seven humans among 62 confirmed cases in New York City and nearby counties in late summer 1999. Relatively large numbers of birds, particularly crows, and horses died. Reverse transcriptase–PCR analysis of human brain tissue and isolates from mosquitoes and birds confirmed the cause as WN virus (8–11). Nucleic acid sequence analysis indicated a common origin with a WN virus isolate from a domestic goose that died in Israel in 1998 (8). By September 1999, isolates were identified from nearby Fairfield County in Connecticut, and monitoring programs for WN virus were established in several northeastern states in the United States. Isolations of WN virus from *Culex* mosquitoes and a red-tailed hawk (*Buteo jamaicensis*) during the 1999–2000 winter suggested that this virus had become established in the New World (12, 13). The subsequent recovery of WN virus from mosquitoes and birds in the spring, summer, and fall of 2000 confirmed that the virus had become established in the northeast United States (14).

Extensive genetic variation exists among WN virus isolates in Africa, Australia, Asia, and Europe (15–17). Phylogenetic studies of isolates from Europe and Africa suggest the introduction of WN virus into Europe by birds migrating out of sub-Saharan Africa. For example, the nucleotide sequence of a Romanian WN virus isolate from *Culex pipiens* (RO97–50, GenBank accession no. AF130362) was similar to strains from *Culex neavei* collected in Senegal (SenArD93548, GenBank accession no. AF001570) and from *Culex univittatus* collected in Kenya (KN3829, GenBank accession no. AF146082) (16).

The introduction of WN virus into the United States presents many opportunities for epidemiological study. The ancestral genome sequence of the WN virus strain introduced from abroad into the United States, and possibly directly into the New York City area, is likely to be similar to that isolated from the Chilean flamingo (*Phoenicopterus chilensis*) kept in the New York Zoological Garden (GenBank accession no. AF196835) (8). The potential health effects of this virus on humans will result in an extensive monitoring program in birds, mosquitoes, humans, and mammals in the New World. The study of mutations will enable a phylogenetic analysis to be used to determine evolutionary relationships among isolates. Through nucleotide sequence analysis, we can trace the spread of WN virus mutants over space and time to give a picture of their movement. It is therefore important to document genetic changes arising early in this virus' establishment in the New World. For this study we determined the sequence of a 921-nt region of the West Nile virus genome (9) for 82 isolates and report on a divergent lineage in Connecticut.

Abbreviation: WN, West Nile.

Data deposition: The sequences (corresponding to Fig. 1) reported in this paper have been deposited in the GenBank database [accession nos. 1 (crow) AF385219; 2 (crow) AF385221; 3 (crow) AF385226; 4 (crow) AF385227; 5 (crow) AF385229; 6 (*Cx. pipiens*) AF385235; 7 (crow) AF206519; 8 (crow) AF385239; 9 (crow) AF385240; 10 (crow) AF385241; 11 (crow) AF385243; 12 (Cooper's hawk) AF206520; 13 (crow) AF385246; 14 (*Cx. restuans*) AF385249; 15 (crow) AF385257; 16 (*Cx. salinarius*) AF385252; 17 (*Cx. salinarius*) AF385255; 18 (crow) AF385266; 19 (crow) AF385215; 20 (crow) AF385216; 21 (crow) AF385217; 22 (*Cx. pipiens*) AF385218; 23 (crow) AF385220; 24 (crow) AF385222; 25 (crow) AF385223; 26 (crow) AF385224; 27 (crow) AF385225; 28 (crow) AF385228; 29 (crow) AF385230; 30 (crow) AF385231; 31 (crow) AF385232; 32 (crow) AF385233; 33 (blue jay) AF385234; 34 (*Cx. restuans*) AF385236; 35 (*Cx. pipiens*) AF385237; 36 (*Ae. vexans*) AF206517; 37 (crow) AF385238; 38 (*Cx. pipiens*) AF206518; 39 (cowbird) AF385242; 40 (red-shouldered hawk) AF385244; 41 (crow) AF385245; 42 (crow) AF385247; 43 (crow) AF385248; 44 (crow) AF385250; 45 (crow) AF385251; 46 (*Cx. restuans*) AF385253; 47 (crow) AF385254; 48 (crow) AF385256; 49 (crow) AF385258; 50 (crow) AF385259; 51 (*Cs. melanura*) AF385260; 52 (crow) AF385261; 53 (crow) AF385262; 54 (crow) AF385263; 55 (crow) AF385264; 56 (crow) AF385265; 57 (Canada goose) AF385267; 58 (crow) AF385269; 59 (mourning dove) AF385270; 60 (crow) AF385271; 61 (blue jay) AF385272; 62 (American robin) AF385273; 63 (crow) AF385274; 64 (striped skunk) AF385275; 65 (crow) AF385276; 66 (crow) AF385277; 67 (crow) AF385278; 68 (*Cx. pipiens*) AF385279; 69 (*Cs. melanura*) AF385268; 70 (*Cx. restuans*) AF385280; 71 (crow) AF385291; 72 (*Cx. pipiens*) AF385281; 73 (blue jay) AF385282; 74 (house sparrow) AF385283; 75 (American robin) AF385284; 76 (crow) AF385285; 77 (crow) AF385286; 78 (crow) AF385287; 79 (crow) AF385288; 80 (crow) AF385289; 81 (crow) AF385290; 82 (blue jay) AF385292].

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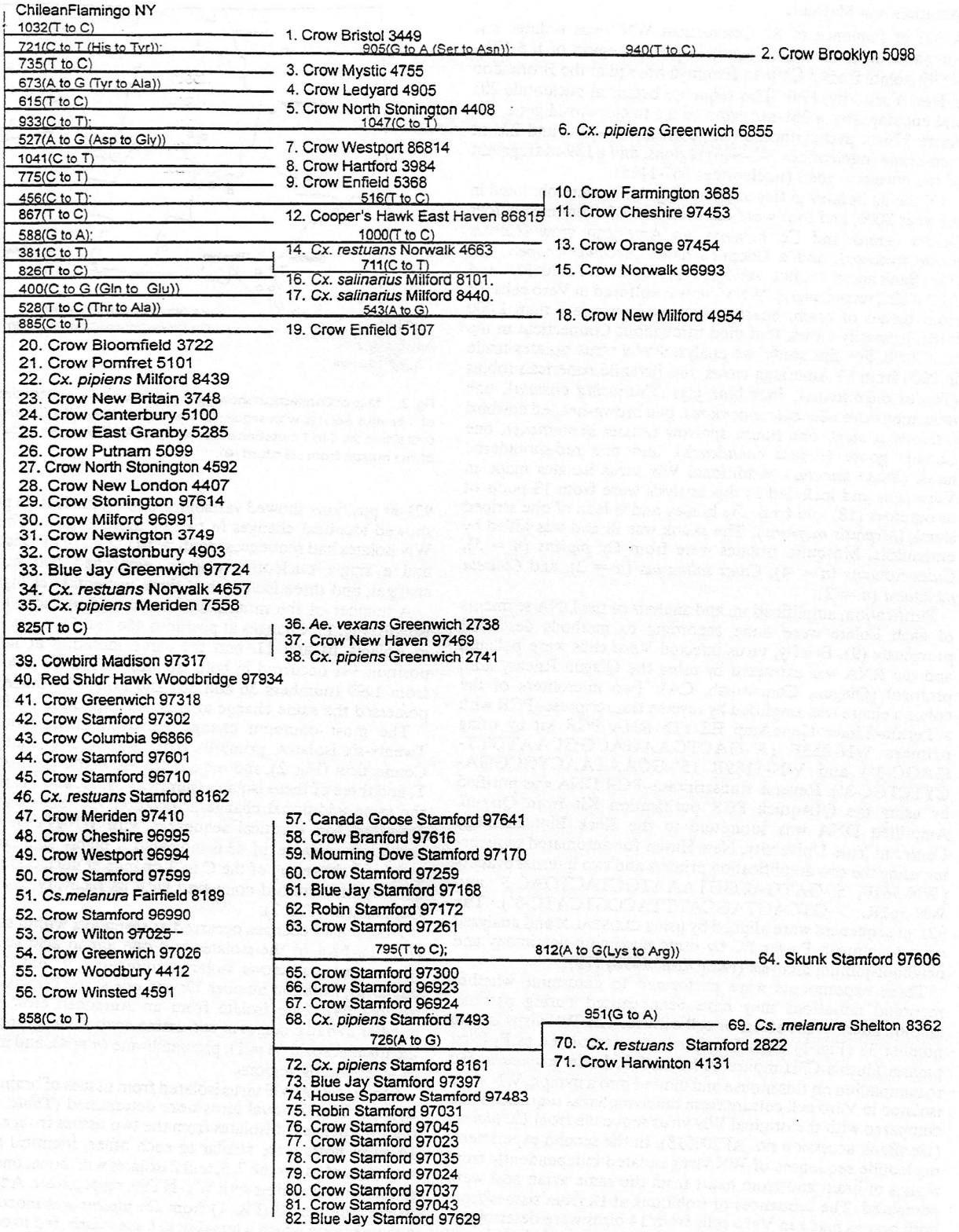

Fig. 1. Unrooted phylogram based on maximum parsimony analysis (heuristic search) comparing a reverse transcriptase–PCR of a 921-nt sequence of the WN-NY99 isolate (GenBank accession no. AF196835) with 82 WN isolates from Connecticut. Branch lengths represent 0, 1, 2, or 3 nucleotide changes. Neighbor-joining analysis gave identical results. Each mutation is identified above the branch as a nucleotide of the WN virus genome. Amino acid changes resulting from nucleotide changes also are identified above the line. Isolation number, host, Connecticut town where host was collected, and WN strain identification number are provided for each isolate. GenBank accession numbers are provided for each isolate in the data deposition footnote.

Materials and Methods

A 921-nt sequence of 82 Connecticut WN virus isolates was compared with the homologous sequence region of the WN-NY99 isolate from a Chilean flamingo housed at the Bronx Zoo in New York City, 1999. This sequence begins at nucleotide 205 and encompasses a 261-nt region of the nucleocapsid gene, the entire 276-nt premembrane (nucleotides 466–741) and 225-nt membrane (nucleotides 742–966) regions, and a 159-nt fragment of the envelope gene (nucleotides 967-1125).

Of the 82 isolates in this analysis (Fig. 1), 78 were obtained in the year 2000, and four were cultured in 1999 from mosquitoes (*Aedes vexans* and *Cx. pipiens*), an American crow (*Corvus brachyrhynchos*), and a Cooper's hawk (*Accipiter cooperi*) (9) (GenBank accession nos. AF206517, AF206518, AF206519, and AF206520, respectively). WN virus was cultured in Vero cells (9) from tissues of brain, heart, or kidney from more than 1,000 birds, primarily crows, that died throughout Connecticut in the year 2000. For this study, we analyzed WN virus isolates made in 2000 from 53 American crows, two juvenile American robins (*Turdus migratorius*), four blue jays (*Cyanocitta cristata*), one mourning dove (*Zenaida macroura*), one brown-headed cowbird (*Molothrus ater*), one house sparrow (*Passer domesticus*), one Canada goose (*Branta canadensis*), and one red-shouldered hawk (*Buteo lineatus*). Additional WN virus isolates made in Vero cells and included in this analysis were from 13 pools of mosquitoes (18) and from the kidney and spleen of one striped skunk (*Mephitis mephitis*). The skunk was ill and was killed by euthanasia. Mosquito isolates were from *Cx. pipiens* (n = 5), *Culex restuans* (n = 4), *Culex salinarius* (n = 2), and *Culiseta melanura* (n = 2).

Purification, amplification, and analysis of the DNA segments of each isolate were done according to methods described previously (9). Briefly, virus-infected Vero cells were pelleted and the RNA was extracted by using the Qiagen Rneasy mini protocol (Qiagen, Chatsworth, CA). Two microliters of the column eluate was amplified by reverse transcriptase–PCR with a Perkin–Elmer GeneAmp EZ rTh RNA PCR kit by using primers WN-233F (5′-GACTGAAGAGGGCAATGTT-GAGC-3′) and WN-1189R (5′-GCAATAACTGCGGA-CYTCTGC-3′). Reverse transcriptase–PCR DNA was purified by using the QIAquick PCR purification Kit from Qiagen. Amplified DNA was submitted to the Keck Biotechnology Center at Yale University, New Haven for automated sequencing using the two amplification primers and two internal primers (WN-561F, 5′-GATGACGGTAAATGCTACTGAC-3′ and WN-561R, 5′-GTCAGTAGCATTTACCGTCATC-3′). The 921-nt sequences were aligned by using CLUSTAL X and analyzed on a MacIntosh Power PC by using maximum parsimony and neighbor-joining analysis (PAUP 4.061; 1998) (19).

Three experiments were performed to determine whether recorded mutations may have been caused during passage, amplification, sequencing, or cell culture. The WN virus isolate number 38 (Fig. 1) from *Cx. pipiens* was inoculated as P_1 (first passage) into a C3H mouse. A larval tick, *Ixodes scapularis*, fed to completion on this mouse and molted into a nymph. WN virus isolated in Vero cell culture from the nymph was sequenced and compared with the original WN virus sequence from *Cx. pipiens* (GenBank accession no. AF206518). In the second experiment, nucleotide sequences of WN virus isolated independently from tissues of brain and from heart from the same avian host were compared. The sequences of isolations at P_0 from tissues from both organs made in Vero cells from 14 birds were determined. In the third experiment, 14 isolates were passed 1–3 times in cell culture and were compared with the original sequence.

Results

The results of the sequence analysis of the 921-nt region of the 82 isolates from Connecticut are presented in Fig. 1. Thirty of the 921-nt positions showed variability, and six nucleotide positions showed identical changes in two or more isolates. Thirty-four WN isolates had sequences identical to the WN-NY99 isolate, 37 had a single nucleotide change, eight had two nucleotide changes, and three isolates had three nucleotide changes.

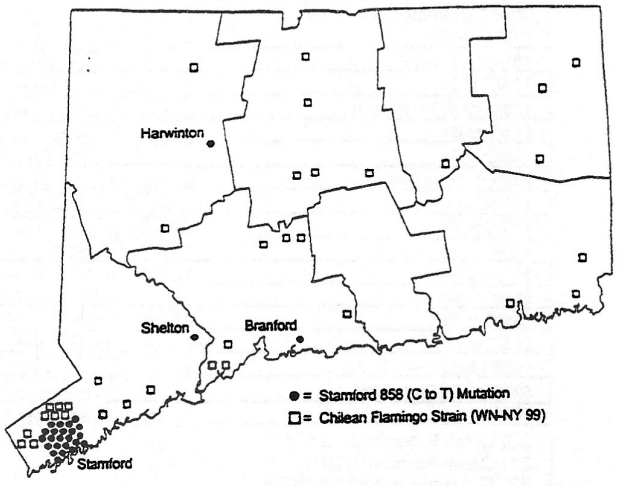

Fig. 2. Map of Connecticut showing county boundaries and the distribution of WN virus isolates with sequences identical to WN-NY99 (□) and isolates containing the C to T mutation at genome position 858 shown in the majority of the isolates from Stamford (●).

A number of the mutations were shared by more than one isolate (Fig. 1). Changes at positions 456 and 516 were identical in isolates 10 and 11, and the same mutation at nucleotide position 381 occurred in both isolates 14 and 15. Two isolates from 1999 (numbers 36 and 38) and isolate 37 made in 2000 possessed the same change at genome nucleotide position 825.

The most common change occurred at nucleotide 858. Twenty-six isolates, primarily from Stamford in southwestern Connecticut (Fig. 2), showed a mutation at this position of C to T, and three of these isolates (numbers 69, 70, and 71) possessed the same additional change at position 726. Six isolates from Stamford had identical sequences with WN-NY99. A Yates corrected χ^2 value of 43.6 indicated a highly significant geographical clustering of the C to T change at nucleotide position 858 within Stamford compared with its recovery elsewhere in Connecticut (Fig. 2).

Amino acid changes occurred at seven loci within six isolates (Fig. 1). Five of the isolates had one amino acid substitution (three American crows, isolation numbers 4, 7, and 18; one *Cx. salinarius*, isolation number 17; and one striped skunk, isolation number 64). One isolate from an American crow (isolation number 2) had changes in two amino acids. Changes occurred in the nucleocapsid (n = 1), premembrane (n = 4), and membrane (n = 2) coding regions.

Sequences of WN virus isolated from tissues of brain and from heart of 14 individual birds were determined (Table 1). Nucleotide sequences of isolates from the two tissues from each of the 14 avian hosts were similar to each other. Identical sequences were recorded for the 7, 5, and 2 isolates with none, one, and two mutations compared with WY-NY99, respectively. Additionally, isolate number 38 (Fig. 1) from *Cx. pipiens* was inoculated into a mouse upon which a larval tick, *I. scapularis*, fed to completion and subsequently molted more than 30 days later into a nymph. The 921-nt sequence from WN virus isolated from this nymphal tick was identical to the original isolate cultured from the mosquito, including the T to C mutation at position 825. Sequences of 14 isolates that had been passed in Vero cells 1–3

Table 1. Comparison of the 921-nt sequence (genome positions 205-1125) of WN-NY99 to Connecticut West Nile virus isolates from tissues of brain (P_0) and heart (P_0) from the same avian host

Isolate no.	Host	Reverse transcriptase–PCR sequences of isolates that differ from WN-NY99	
		Brain	Heart
11	Crow	456, C to T; 516, T to C	456, C to T; 516, T to C
13	Crow	588, G to A; 1000, T to C	588, G to A; 1000, T to C
29	Crow	None	None
33	Blue jay	None	None
37	Crow	825, T to C	825, T to C
41	Crow	None	None
42	Crow	None	None
44	Crow	None	None
47	Crow	None	None
50	Crow	None	None
58	Crow	858 C to T	858 C to T
73	Blue jay	858 C to T	858 C to T
74	House sparrow	858 C to T	858 C to T
82	Blue jay	858 C to T	858 C to T

Isolate numbers are from Fig. 1.

times were compared with sequences of P_0 or P_1 (Table 2). The sequence of each of the 14 isolates in passages 1–3 was identical to the original recorded sequence.

Discussion

Our WN virus sequence data suggest that we are seeing micro-evolutionary events unfold and that we can, by sequence analysis, observe temporal and geographical genetic variation. This 921-nt region beginning at genome position 205 and ending at 1125 appears to change rapidly enough to differentiate a significant percentage of the isolates.

Although the majority of the 30 mutations were silent, seven of the mutations, or 23%, resulted in amino acid substitutions. Similarly, Porter *et al.* (17) reported that most mutations among seven WN virus isolates from six countries were silent. However, Chambers *et al.* (20) reported a single nucleotide substitution of T to A at position 463 of the WN virus envelope gene that specified the substitution of asparagine for tyrosine at amino acid site 155 and produced an N-linked glycosylation site. The amino acid substitution in isolation 2 as described in this study at nucleotide position 905 resulted in a putative glycosylation site. Phentotypic mutations within this 921-nt sequence eventually could produce significant changes in viral antigenicity with concomitant alteration of the immune response to this virus. Rapid dissemination of one strain relative to other strains may indicate a selective advantage as the virus adapts to North American ecosystems. Such a possibility makes continued study of WN virus changes important.

The clustering of isolates from Stamford with the mutation C to T at position 858 likely represents a single event and is not a repeated example of convergence among unrelated isolates. This C to T change was recovered from six species of birds, a striped skunk, and three species of mosquitoes in Stamford, further indicating this variant WN virus is widespread within vertebrate and mosquito populations. Our isolation of this variant from three other Connecticut towns, one of which was 54 miles from Stamford (see Fig. 2), leads us to believe this nucleotide change may be a valuable genetic marker to follow dispersion of a specific WN strain in North America. Berthet *et al.* (15) previously reported that WN subtypes freely moved between regions

Table 2. Comparison of 921-nt sequence (genome positions 205-1125) of WN-NY99 to Connecticut West Nile virus isolates in different passages in Vero cell culture

Isolate no.	Host	Reverse transcriptase–PCR sequences of isolates that differ from WN-NY99	
		Sequence (passage number)	Sequence (passage number)
1	Crow	1032 T to C (P_1)	1032 T to C (P_3)
2	Crow	721 C to T, 905 G to A, 940 T to C (P_1)	721 C to T, 905 G to A, 940 T to C (P_3)
4	Crow	673 A to G (P_1)	673 A to G (P_3)
6	*Cx. pipiens*	933 C to T, 1047 C to T (P_0)	933 C to T, 1047 C to T (P_2)
11	Crow	456 C to T, 516 T to C (P_0)	456 C to T, 516 T to C (P_2)
12	Cooper's hawk	867 T to C (P_0)	867 T to C (P_1)
15	Crow	381 C to T, 711 C to T (P_0)	381 C to T, 711 C to T (P_2)
18	Crow	528 T to C, 543 A to G (P_0)	528 T to C, 543 A to G (P_3)
33	Blue jay	None (P_0)	None (P_1)
46	*Cx. restuans*	None (P_0)	None (P_2)
57	Canada goose	858 C to T (P_0)	858 C to T (P_1)
64	Striped skunk	795 T to C, 812 A to G, 858 C to T (P_0)	795 T to C, 812 A to G, 858 C to T (P_1)
69	*Cs. melanura*	726 A to G, 858 C to T, 951 G to A (P_0)	726 A to G, 858 C to T, 951 G to A (P_2)
71	Crow	726 A to G, 858 C to T (P_0)	726 A to G, 858 C to G (P_3)

Isolate numbers are from Fig. 1.

of Europe and Africa and between organisms without any obvious selection. WN virus isolates identical to the NY99 strain showed a much broader range of distribution in Connecticut, including Stamford (Fig. 2). Birds, such as crows, likely disperse the virus relatively long distances in Connecticut and elsewhere in the United States (21).

In addition to the 858 C to T cluster, there were four other examples of small clusters of mutations. Isolates 69, 70, and 71 (Fig. 1) from *Cs. melanura* from Shelton, *Cx. restuans* from Stamford, and a crow that died 54 miles away from Stamford in Harwinton (Fig. 2), members of the Stamford group (858 C to T), showed an additional common mutation (726 A to G). Isolates 36, 37, and 38 showed a common mutation of T to C at position 825. Two of these isolates were from mosquitoes from Greenwich, CT, which is adjacent to Stamford, in 1999 and one from a crow that died in south central Connecticut in 2000. Isolates 10 and 11, each from a crow that died in adjoining towns in central Connecticut, possessed the changes of C to T at position 456 and T to C at position 516 (Fig. 1). Also, two isolates from Norwalk, 14 and 15 from *Cx. restuans* and from a crow, shared a common mutation at nucleotide 381 of CTOT. Analysis of more isolates at additional geographical sites may identify additional mutations that may be as prevalent as the C to T mutation at position 858.

Sequences of WN virus isolated in Vero cells from both brain and heart tissues from the same avian host were identical whether there were none, one, or two mutations compared with WN-NY99. Host cell type has been reported to influence epitope expression of Kunjin virus (22, 23), a virus that likely is a strain of WN virus (16). The indistinguishable sequences from viral isolates independently cultured from two different organs within the same host suggest that the mutations we have documented are real and not caused by culture, RNA extraction, or PCR procedures. Additionally, sequences of isolates before and after 1–3 passages in Vero cell culture were identical to sequences in the original or first passages, and the recovery of WN virus from a nymphal tick, which had become infected as a larva, with the identical sequence to the isolate inoculated into the larval tick's murine host also suggests stability of recorded mutations.

Although WN virus is changing relatively rapidly, it is not changing too rapidly to obscure the relationships among these isolates. In fact, the data set we have presented shows no homoplasy. That is to say, the tree is described without any need for postulating convergent mutations or nucleotide reversions back to a former state. As a result we have changes at 30 nucleotide positions and our shortest tree is 30 steps. The introduction of WN virus into the United States is clearly a unique opportunity for studying the causes, dissemination, and control of a flavivirus disease in nonimmune host and vector populations. The comparative phylogenetic approach using RNA sequences may be an important method for understanding these processes.

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