Fine-scale genetic variation and evolution of West Nile virus in a transmission "hot spot" in suburban Chicago, USA.

Available online at www.sciencedirect.com
ScienceDirect
Virology 374 (2008) 381–389
VIROLOGY
www.elsevier.com/locate/yviro

Fine-scale genetic variation and evolution of West Nile Virus in a transmission "hot spot" in suburban Chicago, USA

Luigi Bertolotti [a,*], Uriel D. Kitron [b], Edward D. Walker [c], Marilyn O. Ruiz [b], Jeffrey D. Brawn [d], Scott R. Loss [d], Gabriel L. Hamer [e], Tony L. Goldberg [b,*]

[a] *Università degli Studi di Torino, Dipartimento di Produzioni Animali, Epidemiologia, Ecologia, Via Leonardo da Vinci, 44, 10095 Grugliasco (TO), Italy*
[b] *University of Illinois, Department of Pathobiology, 2001 South Lincoln Avenue, Urbana, IL 61802, USA*
[c] *Michigan State University, Department of Microbiology and Molecular Genetics, East Lansing, MI 48824, USA*
[d] *University of Illinois, Department of Natural Resources and Environmental Sciences, 1102 South Goodwin Avenue, Urbana, IL 61801, USA*
[e] *Michigan State University, Department of Fisheries and Wildlife, East Lansing, MI 48824, USA*

Received 24 October 2007; returned to author for revision 27 November 2007; accepted 28 December 2007
Available online 7 February 2008

Abstract

Mosquitoes and birds were sampled for West Nile virus (WNV) in suburban Chicago, USA, in a "hot spot" of arboviral transmission. Viral genetic diversity within this area was similar to that within Illinois and the United States. Diversity was higher among viruses from mosquitoes than from birds, higher among viruses from birds in urban "green spaces" than from birds in residential areas, but lower among viruses from mosquitoes in green spaces than from mosquitoes in residential areas. Viral transmission was distance-limited, as evidenced by decreasing autocorrelation of WNV sequences with increasing geographic separation. The evolutionary rate of WNV within the study area between 21 July and 4 October 2005 was ten times higher than that for WNV across North America between 2002 and 2005. These results indicate that WNV transmission and evolutionary dynamics can vary seasonally and in response to fine-scale environmental conditions and landscape characteristics related to urbanization.

Keywords: West Nile virus; Genetic diversity; Phylogenetics; Molecular epidemiology; Spatial epidemiology; Molecular evolution

Introduction

West Nile virus (WNV), a mosquito-borne virus in the family *Flaviviridae* and the causative agent of West Nile febrile illness and neurologic disease, has spread rapidly throughout North America since its introduction into the United States in 1999 (Lanciotti et al., 1999). Ecological studies of WNV have demonstrated that viral transmission is maintained through a cycle involving avian hosts and mosquito vectors, and that localized environmental conditions can influence viral persistence and the dynamics of outbreaks (Andreadis et al., 2004; Gu et al., 2006; Kilpatrick et al., 2006; Ezenwa et al., 2007). Molecular studies of WNV evolution during its establishment and spread have shown that the virus evolved rapidly during the early years of the epidemic and that, by 2002, a fitter "WN02" variant had effectively replaced the original "NY99" variant (Moudy et al., 2007; Snapinn et al., 2007). Partial genome sequence analysis provides little evidence for genetic substructure within the North American WNV population, either geographically or with respect to host species (Bertolotti et al., 2007).

To date, molecular epidemiological studies of WNV have yielded valuable insights into broad patterns of viral evolution and emergence, but on coarser spatial and temporal scales (e.g. states, regions, across multiple years) than those on which ecological studies of viral transmission and amplification have tended to focus. The spatial scale of such studies has generally spanned the USA or North America (e.g. Davis et al., 2005; Bertolotti et al., 2007; Snapinn et al., 2007), although some studies have concentrated on particular states (e.g. Connecticut; Anderson et al., 2001) or cities (e.g. Houston; Davis et al.,

* Corresponding authors. L. Bertolotti is to be contacted at Fax: +39 011 6709196. T.L. Goldberg, Fax: +1 217 244 7421.
E-mail addresses: luigi.bertolotti@unito.it (L. Bertolotti), tlgoldbe@uiuc.edu (T.L. Goldberg).

0042-6822/$ - see front matter © 2008 Published by Elsevier Inc.
doi:10.1016/j.virol.2007.12.040

2007). To our knowledge, however, no studies have investigated the genetic structure of WNV on the same spatial scales on which short-term movements of birds and mosquitoes occur (e.g. less than one to several kilometers). As a result, little is known about how WNV may respond genetically to localized environmental conditions.

During the summer of 2005, we began an intensive study of WNV ecology and epidemiology in southwestern Chicago in an area known to be a "hot spot" for human West Nile cases (Ruiz et al., 2004). Interestingly, this area was also a geographic focus of human cases during a 1975 outbreak of St. Louis encephalitis (SLE), another mosquito-borne arboviral disease (Zweighaft et al., 1979). By sampling WNV from live birds and mosquitoes prospectively and in a geographically systematic way throughout the WNV transmission season, we were able to examine viral genetic diversity on a finer spatio-temporal scale than has previously been investigated. Here, we use spatial and molecular epidemiological analytical methods to compare patterns of WNV genetic diversity and evolution within our study area to those on regional and national scales. We use population genetic analyses to compare the diversity of WNV between birds and mosquitoes and to examine how viral genetic diversity varies across urban habitat types in both hosts and vectors. Our analyses yield new insights into how local environmental conditions within an urban setting affect the transmission dynamics and evolution of arboviral pathogens.

Results

We generated 140 WNV envelope (ENV) gene sequences from 12 birds and 128 mosquito pools, from 15 sites (Fig. 1); these samples contained 74 unique WNV ENV sequences (Table 1). Mosquito infection rates varied among sites (Table 1) and were high in some cases, but fewer than 5% of mosquito pool sequences contained secondary peaks on chromatograms that might indicate infection by more than one viral genotype. Phylogenetic analysis yielded a tree similar to that described by Davis et al. (2005) and Bertolotti et al. (2007), in which all newly generated samples are of the "WN02" type and fall within a large, poorly differentiated clade (Fig. 2). The topology of this tree and its degree of phylogenetic resolution did not change when other viral genes (complete polyprotein, 3′ UTR, and 5′ UTR) associated with similar or identical ENV genes were analyzed (data not shown).

Analyses of nucleotide diversity (Nei, 1987) demonstrated that WNV ENV sequences from our study area display a level of genetic variation similar to that of sequences from much broader geographic regions. Mean nucleotide diversity for WNV sequences from the suburban Chicago study area was 0.31%± 0.014% (SEM), which was similar to that of sequences from Illinois (0.27%±0.018%) and the entire United States (0.33%± 0.041%).

Hierarchical analyses of molecular variance showed that, within the suburban Chicago study area, most WNV genetic diversity was contained within individual sampling sites (93% of total variance, $p<0.001$; Table 2), while differences among sampling sites accounted for only 6.61% of WNV genetic diversity. Samples from Cook County were compared to samples from all other Illinois counties in order to evaluate population subdivision at a coarser geographic scale; results showed that the majority of viral diversity (98.2%) was again contained within locations (counties in this case; Table 2).

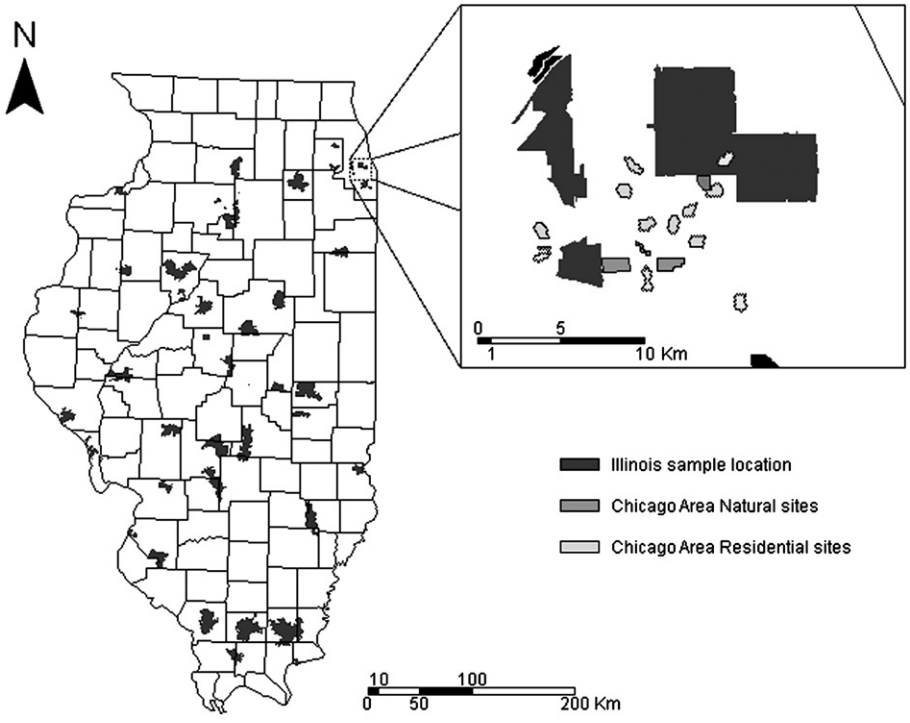

Fig. 1. Map of sample locations. Locations of Illinois samples are indicated as dark gray polygons representing postal code areas. Samples from the Chicago study area are shown in gray (natural sites) and light gray (residential sites).

We compared nucleotide diversity for viruses from birds and mosquitoes from both natural and residential sites within the study area (Fig. 3). We found higher nucleotide diversity among viruses from mosquitoes than from birds (0.32%±0.016% and 0.22%±0.036%, respectively; $p<0.001$). Among viruses from mosquitoes, those from residential sites had higher nucleotide diversity than did those from natural sites (0.36%±0.022% and 0.26%±0.021%, respectively; $p<0.001$). However, among viruses from birds, those from residential sites had significantly lower nucleotide diversity than did those from natural sites (0.15%±0.029% and 0.32%±0.072%, respectively; $p=0.0011$).

Matrix correlation tests showed a positive and significant association between pairwise genetic and spatial distances for sequences from the suburban Chicago study area (Mantel's $r=0.143$, $p=0.003$). However, we observed no such relationship between pairwise genetic distance and spatial distance for statewide Illinois data (Mantel's $r=-0.184$, $p>0.5$). Further examination of the suburban Chicago data using spatial autocorrelation analysis showed a "clinal" pattern of isolation by distance, characterized by significant positive spatial autocorrelation within individual sampling sites and generally decreasing autocorrelation with increasing geographic separation, with statistically significant negative spatial autocorrelation emerging only at distances >4 km (Fig. 4).

Because we knew the exact dates of collection for all viral samples from the Chicago study area, we were able to calculate the evolutionary rate of the WNV ENV gene between 21 July and 4 October, 2005. This rate was $8.20\times10^{-3}\pm2.75\times10^{-3}$ substitutions per site per year, which is about ten times higher than previous evolutionary rate estimates of 0.85×10^{-3} substitutions per site per year for samples collected from across Illinois between 2002 and 2005 (Bertolotti et al., 2007) and 0.30×10^{-3} substitutions per site per year for viruses collected from across North America between 2002 and 2005 (Snapinn et al., 2007). Evolutionary rate estimates did not differ significantly when calculated using sequences from birds and mosquitoes separately ($8.17\times10^{-3}\times0.81\times10^{-3}$ and $7.57\times10^{-3}\times2.49\times10^{-3}$ respectively). Sequences from the Chicago area collected in 2005 and sequences from across Illinois collected between 2002 and 2005 showed ratios of non-synonymous to synonymous substitutions per site that were comparable and well below 1 ($K_a/K_s=0.024\pm0.005$ and 0.044 ± 0.012, respectively).

Discussion

We describe genetic diversity in West Nile virus circulating in birds and mosquitoes in a small geographic area in suburban Chicago, USA, known to be a "hot spot" of arboviral transmission, as evidenced by clustering of human WNV cases in this area in 2002 and clustering of SLE cases in the same area in 1975. Our results demonstrate a degree of sequence-level viral genetic diversity within this limited geographic area that is similar to that within the state of Illinois and to that across the United States as a whole. Within our study area, greater than 95% of viral genetic diversity was contained within sampling sites, which span only one to several km^2 in area.

Although spatial substructure in WNV was not apparent in our phylogenetic analyses, other spatially explicit analyses revealed a significant association between viral genetic and spatial distances. Matrix correlation tests and spatial autocorrelation analyses both indicated isolation by distance among sequences collected from our suburban Chicago study area. Viral nucleic acid sequences collected from the same sampling site were significantly positively autocorrelated, implying that "pockets" of viral diversity may be generated at very local scales (less than 1 km^2). Our finding of negative autocorrelation for viral sequences separated by geographic distances >4 km implies that viral transmission was distance-limited on this approximate spatial scale. Distance-limited movement of mosquitoes or resident (non-migratory) birds within our study area would most parsimoniously explain this trend.

Interestingly, we found no correlation between genetic and spatial distance for WNV sequences from across Illinois. We attribute the absence of a space-genetic relationship for Illinois samples despite the presence of such a relationship within our study area to our sampling strategy. We conducted prospective and geographically systematic sampling and did not rely on opportunistically collected samples from diagnostic laboratories, where avian samples generally predominate and where sampling may be biased towards certain host species or locations (Ward et al., 2006). Anderson et al. (2001) notably studied West Nile virus in Connecticut and found a significant association between WNV genetic and spatial distances, although still on a coarser spatial scale (the State of Connecticut) than ours. Our results extend the generality of these conclusions to a finer geographic scale and to a different geographic region of the USA, providing evidence that WNV genetic diversity is generated as a result of highly localized processes of viral transmission.

Our data also indicate that WNV genetic diversity differed between hosts and vectors within our study area, with diversity being higher in mosquitoes than in birds. Jerzak et al. (2005) previously reported similar results, arguing that tissue-specific selection in hosts may limit the diversity of WNV, and that the mosquito may therefore provide the primary environment for the generation of WNV genetic variation. Our data lend further support to this interpretation. We note that such results could differ for viral genes other than ENV that may be subject to different selective pressures and constraints (Liu et al., 2006; Brault et al., 2007), although our own phylogenetic results did not differ when other regions of the viral genome were considered. We also note that we did not examine within-host or within-vector viral diversity, which could be informative for understanding the genesis of such variation (Jerzak et al., 2005; Ciota et al., 2007).

Our data provide the first evidence that local ecological conditions related to urbanization can influence the genetic diversity and evolution of an arboviral pathogen. We found that viruses from mosquitoes captured in residential sites were more diverse genetically than were viruses from mosquitoes captured in natural "urban green space" sites, but that this relationship was reversed for viruses from birds. In the case of birds, this trend may reflect a difference in the diversity of avian species between natural and residential sites. In our study area, residential sites

Table 1
Numbers of West Nile virus envelope gene sequences from each collection site and from each bird and mosquito species, and site-specific mosquito infection rates

	Residential sites										Natural sites					Total Residential sites	Total Natural sites	Total
	Alsip	Blue Island	Burbank	Chicago - Ashburn East	Chicago - Mt. Greenwood	Evergreen Park - North	Evergreen Park - West	Oak Lawn - Central	Oak Lawn - North	Palos Hills - North	Palos Hills - South	Evergreen Cemetery	Holy Sepulcher Cemetery	Saint Casmir Cemetery	Wolf Wildlife Area			
Agelaius phoeniceus														1			1	1
Carpodacus mexicanus	1								1							2		2
Colaptes auratus								4							2		2	2
Passer domesticus	2							4							1	6	1	7
Total birds	3							4	1					1	3	8	4	12
Culex pipiens pools	5	6	4	2	3	6	10	4	19	14	6	8	13	4	23	79	48	127
Culex restuans pools											1					1		1
Total mosquito pools	5	6	4	2	3	6	10	4	19	14	7	8	13	4	23	80	48	128
Culex infection rate[a]	10.0 (5.6–16.7)	13.3 (7.3–22.7)	9.7 (4.3–19.2)	9.7 (3.2–23.7)	9.8 (4.8–17.9)	13.9 (7.6–23.7)	11.3 (6.4–18.9)	12.5 (7.1–20.9)	18.1 (10.6–29.4)	9.7 (5.4–16.2)	9.1 (5.6–14.1)	9.4 (3.9–19.7)	13.6 (9.5–19.1)	9.8 (5.5–16.4)	24.8 (18.3–33.2)	11.4 (9.5–13.5)	15.7 (12.8–19.1)	12.9 (11.3–14.6)
Total sequences	8	6	4	2	3	6	10	8	20	14	7	8	13	5	26	88	52	140

[a] Maximum likelihood estimates (95% confidence intervals in parentheses) for 2005 *Culex* spp. infection rates (percent) were calculated in Excel (Microsoft, Inc., Redmond, WA) using the PooledInfRate version 3.0 add-in (Biggerstaff, 2006).

contained limited natural habitat (5.1% of total land cover, on average) with the majority of green space found in parks less than 2 ha. Impervious surfaces and buildings dominated the remainder of residential sites. These "backyard" habitats support low avian diversity with dominance by a few species well adapted to urban landscapes, such as the house sparrow (*Passer domesticus*), European starling (*Sturnus vulgarus*), and American robin (*Turdus migratorius*). Natural sites contained significant shrubland and early-successional woodland (76.4% of total land cover, on average) with few impervious surfaces and buildings. These sites supported a comparatively diverse community of breeding birds, including red-winged blackbirds (*Agelaius phoenecius*), song sparrows (*Melospiza melodia*), and gray catbirds (*Dumetella carolinensis*), as well as ducks, herons, and raptors. Greater avian species diversity in natural sites could account for the greater genetic diversity of avian WNV from natural sites, especially if WNV displays different replicative properties in different avian species (Reisen et al., 2005).

Because 99.2% of our WNV-positive mosquito pool samples were *Culex pipiens*, we cannot attribute differences in viral genetic diversity between mosquitoes from residential versus natural sites to differences in vector species diversity. Rather, we suspect that higher viral genetic diversity in mosquitoes from residential sites versus mosquitoes from natural sites may be attributable to local environmental differences between the two types of sites. For example, urban "heat island" effects are known to occur on fine spatial scales, such as between urban areas and the surrounding countryside (Arnfeld, 2003). In a comprehensive study of Chicago, the minimum average summer temperature during the day was about 2.7 °C higher in urban areas than in adjacent rural areas (Ackerman, 1985). Microgeographic temperature differences within urban areas have also been observed, mimicking the phenomenon seen at broader scales (Harlan et al., 2006; Weng et al., 2006). Higher temperatures in our residential sites than in our natural "urban green space" sites could lead to higher viral evolutionary rates, either through direct effects of temperature on viral mutation rates in mosquitoes (body temperatures of birds, although higher than body temperatures of mosquitoes, would be relatively constant among locations) or, more likely, through indirect effects of temperature on the dynamics of host-vector-virus interaction. Weaver et al. (1991, 1994) have suggested such effects as an explanation for regional differences in the evolutionary rate of eastern equine encephalitis virus, but no studies to date have documented similar effects for WNV. Several studies have, however, demonstrated direct effects of temperature on the replication rate of different WNV variants *in vitro* (Kinney et al., 2006) and on the efficiency of transmission of WNV variants in *Culex* mosquitoes (Dohm et al., 2002; Reisen et al., 2006; Richards et al., 2007). Examining the direct and indirect effects of temperature and other environmental variables on the evolution of WNV among regions where these parameters vary naturally would be a fruitful area for future research.

Further support for the importance of fine-scale ecological conditions on WNV evolutionary dynamics comes from our observation of a 10-fold higher evolutionary rate for viruses collected in suburban Chicago between July and October, 2005, than for viruses collected from across Illinois between 2002 and

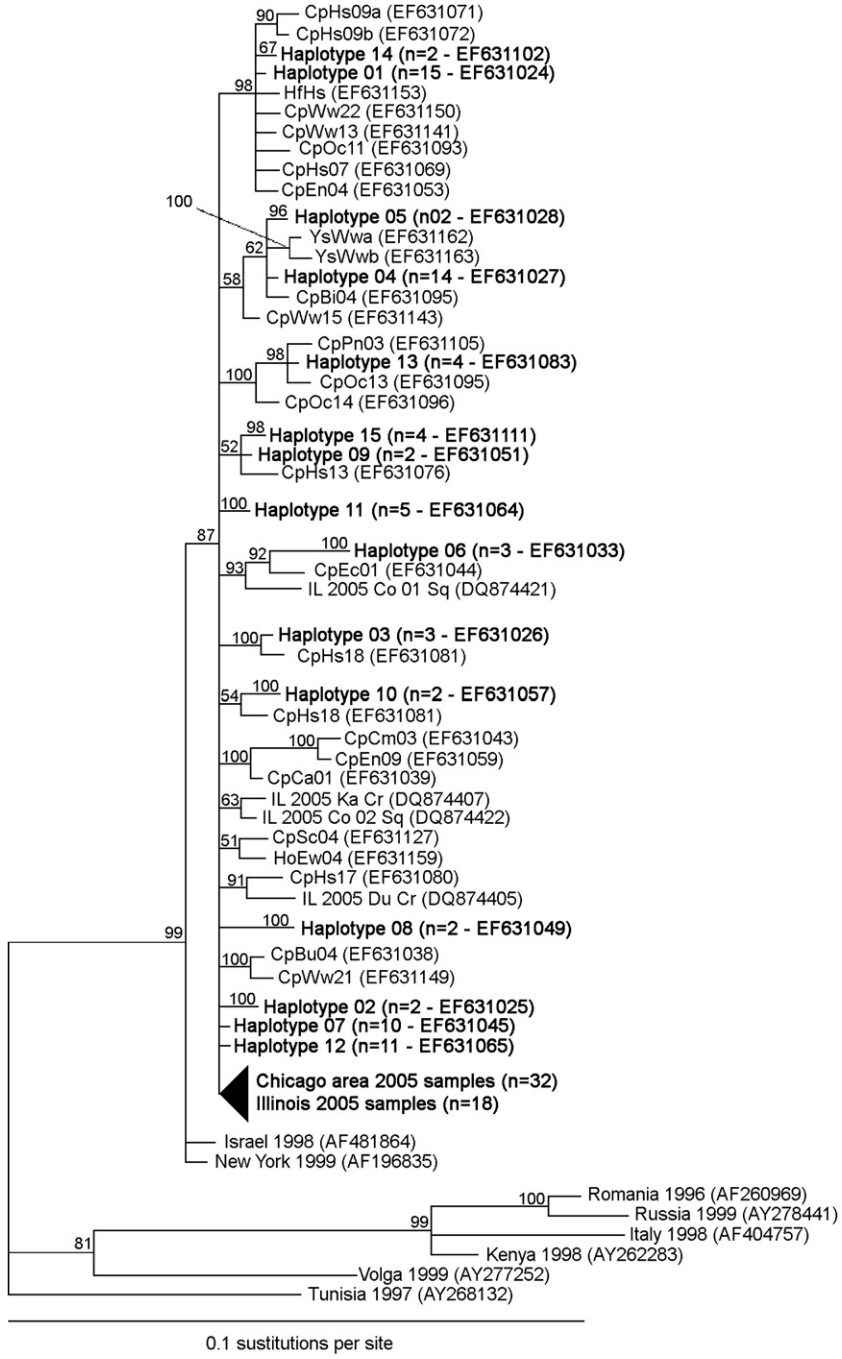

Fig. 2. Phylogenetic tree constructed by Bayesian analysis of 170 WNV envelope gene unique sequences (length 1,575 nucleotides). Taxon names of newly generated sequences from the Chicago area study site indicate species (Cp = *Culex pipiens*; Cr = *Culex resturans*; Hf = *Carpodacus mexicanus*; Ys = *Colaptes auratus*; Ho = *Passer domesticus*), collection site (Pn = Palos Hills — North; Ps = Palos Hills — South; Oc = Oak Lawn — Central; On = Oak Lawn — North; Cm = Chicago — Mt. Greenwood; Ew = Evergreen Park — West; En = Evergreen Park — North; Bi = Blue Island; Ca = Chicago — Ashburn East; Al = Alsip; Bu = Burbank; Ec = Evergreen Cemetery; Hs = Holy Sepulchre Cemetery; Sc = Saint Casmir Cemetery; Ww = Wolf Wildlife Area), serial identification numbers, and GenBank accession numbers. Haplotypes shared by more than one sample are indicated in bold, followed by the number of samples represented and the GenBank accession number in parentheses. Illinois sequence names are followed by the year of collection, county name, and species (Bertolotti et al., 2007). United States sequences names include State name, collection year, and accession number. Posterior probabilities of clades are indicated above branches.

2005. Our suburban Chicago area samples were collected primarily over the summer months, such that evolutionary rate calculations for these viral sequences reflected nucleotide substitutions that had occurred only during the WNV transmission season. Conversely, our WNV samples from across Illinois were collected over multiple years, such that evolutionary rate calculations for these viral sequences reflected nucleotide substitutions that had occurred across seasons, including winters when viral transmission was presumably minimal. Different selective forces operating on the two viral populations cannot parsimoniously account for these evolutionary rate differences, since ratios of non-synonymous to synonymous substitutions per

Table 2
Hierarchical analyses of molecular variance (AMOVA) for WNV envelope gene sequence data

Variance component	*d.f.*	Variance	% Total	P [a]	Φ_{ST} Statistic
Among Chicago area collection sites [b]					
Among sites……σ^2_a	14	0.16431	6.61	<0.0001	0.06613
Within sites……σ^2_c	125	2.32051	93.39		
Among Illinois counties [c]					
Among Counties……σ^2_a	1	0.04446	1.83	0.00425	0.01832
Within Counties……σ^2_c	206	2.38219	98.17		

[a] Probability of obtaining a more extreme variance component and Φ statistic than the observed values by chance alone, calculated from 16,000 random permutations of the data using the computer program ARLEQUIN, version 3.01 (Excoffier et al., 2005).
[b] 15 sampling sites and 140 full-length WNV ENV sequences; see Table 1.
[c] Samples were divided in two groups: samples from Cook county and samples from other Illinois counties.

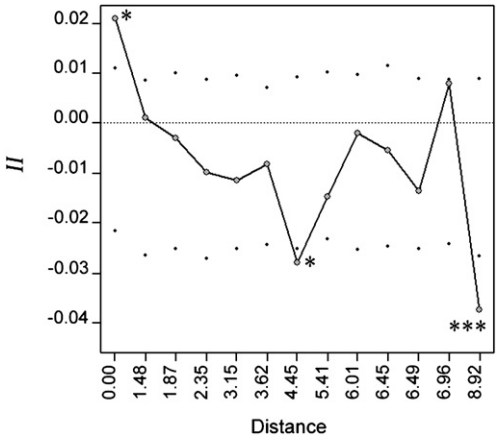

Fig. 4. Spatial autocorrellogram based on an autocorrelation index for DNA analysis (Bertorelle and Barbujani, 1995) for sequences from the Chicago area study site. Y axis (*II*) indicates the degree of sequence-level spatial autocorrelation, or the degree to which sequences separated by a given range of distances (shown as distance classes on the X axis) are genetically similar. The first distance class (0 km) includes comparisons only among sequences collected from the same site. *$p<0.05$; ***$p<0.001$.

site indicated a pattern of genetic drift and strong purifying selection for both viral populations, as has been described previously for WNV (Jerzak et al., 2005; Bertolotti et al., 2007). WNV may therefore evolve at different rates in a cyclic, seasonal pattern, in parallel with the intensity of transmission and "amplification" (*i.e.* population expansion) of the virus in hosts and vectors. Such reasoning has been used to explain differences in arboviral evolutionary rates across broad (intercontinental) scales (Weaver et al., 1991, 1994), but never before to explain fine-scale geographic variation in arboviral genetic diversity or fine-scale temporal variation in arboviral evolutionary rates within an urban environment.

Regardless of the underlying mechanisms, our results clearly demonstrate that WNV varies genetically over geographic and temporal scales that are finer than has previously been appreciated. Our study also demonstrates that fine-scale variation in habitat characteristics within an urban setting contributes to the generation and maintenance of viral diversity. Anthropogenic factors related to urbanization may therefore play a larger role than previously appreciated in arboviral evolution. Further prospective molecular epidemiological studies of WNV across a broad range of spatial and temporal scales should help elucidate how microgeographic and microclimatic processes affect the transmission and evolution of this and other emerging arboviral pathogens in urban settings.

Materials and methods

Study area

We conducted the study in a suburban area of about 11 km by 14 km located 15 km southwest of downtown Chicago (Fig. 1). For field site selection, we first divided the area into 150 m

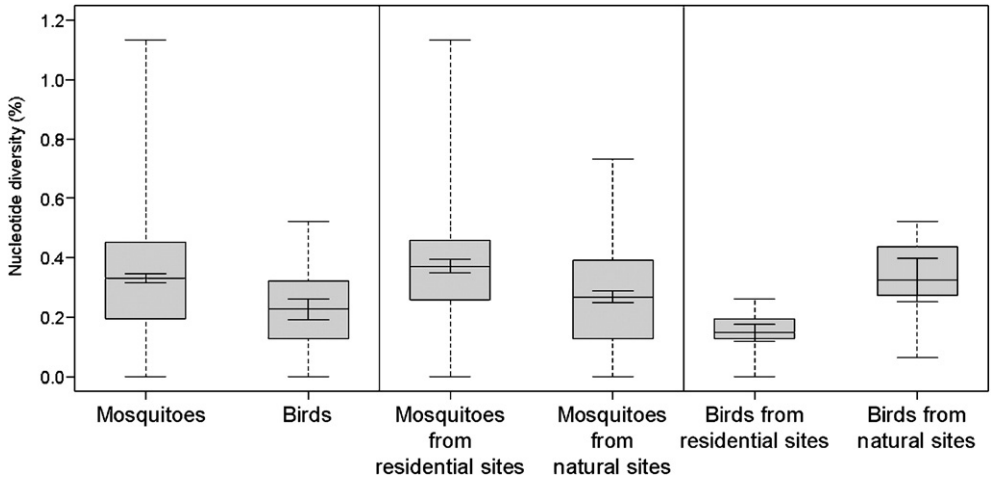

Fig. 3. Nucleotide diversity (Nei, 1987), or mean proportion of nucleotide differences among sequences from the Chicago area study site, corrected for multiple substitutions using a model of molecular evolution derived from a hierarchical likelihood ratio test approach implemented using computer program Modeltest, version 3.7 (Posada and Crandall, 2001). Samples were divided by host/vector species (mosquitoes or birds) and suburban habitat type (natural or residential). Boxplots show means (horizontal lines), standard errors of the mean (internal whiskers), first and third quartiles (lower and upper limits of boxes) and ranges (external whiskers).

hexagonal cells (measured from center to center). We then characterized the hexagons using GIS analysis with ArcMap 9.1 (ESRI, Redlands CA). For each hexagon, we interpolated housing density from census data, determined residential land cover from a published land cover map, and classified urban land cover type by a separate analysis (Ruiz et al., 2007). We further classified residential hexagons in terms of distance from a "natural area" (an "urban green space"). We then selected sites by grouping 20 to 30 adjacent cells that were homogeneous in terms of housing density, urban class type, and distance to natural area to generate a set of 11 residential sites that reflected the social and biological variability of the study area. We selected natural sites ($n=4$) opportunistically, including an urban wildlife refuge and three cemeteries.

Sample collection and testing

During the summer of 2005, we systematically trapped live birds and mosquitoes at all 15 study sites (Hamer et al., in press). We captured birds using mist nets (ATX type, 6 or 12 m length, 36 mm mesh) from each site six times, every 3 to 4 weeks from mid-May to early-October. Birds were identified, aged, sexed, marked with a U.S. Fish and Wildlife Service band (U.S. Department of Interior Bird Banding Laboratory), as authorized by Federal Bird Banding Permit #06507. We collected blood samples by jugular or brachial venipuncture, added 0.8 ml of BA-1 diluent to each blood sample, and stored samples on ice until serum was separated by centrifugation (within 5 h of collection). Serum samples were stored at −20 or −80 °C until tested for viral RNA. We extracted RNA from 100 ml of bird serum in a 1:20 dilution with BA-1 diluent using the ABI 6100 nucleic acid prep station (Applied Biosystems, Foster City, CA). WNV RNA was detected using real-time, reverse transcription-PCR (RT-PCR) (Lanciotti et al., 2000; Hamer et al., in press) on an ABI Prism 9700HT sequence detector at the Research Technology Support Facility at Michigan State University, following the TaqMan One-Step RT-PCR Master Mix Protocol (Applied Biosystems, Foster City, CA).

We collected mosquitoes from each of the field sites once every two weeks from mid-May through early-October, 2005. We used CO_2-baited CDC miniature light traps, CDC gravid traps baited with rabbit pellet infusion, and battery-powered backpack aspirators to collect mosquitoes. Mosquitoes were identified (Andreadis et al., 2005) and pooled into groups of 25 or fewer, grouped by species, sex, collection site, and date. We processed mosquitoes in the laboratory and detected WNV RNA using RT-PCR as described in Hamer et al. (in press).

Molecular analyses

We generated full-length sequences of the WNV envelope (ENV) gene, which is the most variable in the WNV genome and contains a high density of phylogenetically informative nucleotide positions, making it suitable for differentiating among even closely related strains (Davis et al., 2005; Bertolotti et al., 2007). We conducted reverse transcription-PCR on total RNA extracted from positive mosquito pools and bird sera, and we sequenced amplicons directly following previously published protocols (Bertolotti et al., 2007).

To compare WNV diversity and evolution in our study area to that on a wider geographic scale, we included in our analyses 68 published ENV sequences from Illinois and 118 published sequences from across the United States (Bertolotti et al., 2007). To describe evolutionary relationship among these viruses, we constructed phylogenetic trees, selecting models of molecular evolution using a hierarchical likelihood ratio test approach and the Akaike information criterion (Posada and Buckley, 2004) implemented with the computer program Modeltest ver. 3.7 (Posada and Crandall, 2001), combined with Bayesian methods available in the computer program MrBayes ver. 3.1.1 (Ronquist and Huelsenbeck, 2003). To compare our results to those derived from other viral genes, we constructed phylogenetic trees using identical methods and complete polyprotein (10,301 bases), 3′ UTR (631 bases), and 5′ UTR (96 bases) sequences of a selection ($n=61$) of WNV isolates from the literature (Lanciotti et al., 2002; Ebel et al., 2004; Davis et al., 2005), choosing for analysis isolates with identical or similar ENV genes to those from our data set. We calculated tree statistics and performed other phylogenetic manipulations with the computer program PAUP* ver. 4.0b10 (Swofford, 2003).

We quantified viral genetic diversity at the nucleotide level as Nei's nucleotide diversity (Nei, 1987), using the computer programs MEGA4 (Tamura et al., 2007) and PAUP* (Swofford, 2003). We tested the statistical significance of differences between nucleotide diversity estimates for different viral populations using 10,000 bootstrap resamplings of genetic distance matrices. To examine viral population genetic substructuring, we conducted analyses of molecular variance (AMOVA; Excoffier et al., 2005), with viral populations defined both temporally and spatially. We tested the significance of spatiotemporal subdivisions using 16,000 permutations of the data, performed with the computer program Arlequin, version 3.11 (Excoffier et al., 2005). To examine space-time-genetic associations, we conducted tests of matrix correlation (Mantel, 1967) using the computer program R (R. Development Core Team, 2007). Because matrix correlation tests are poor at detecting non-linear associations, we also conducted tests of genetic spatial autocorrelation, using a specifically designed Autocorrelation Index for DNA Analysis (Bertorelle and Barbujani, 1995). To examine and compare the strength and direction of selection in different viral populations, we calculated ratios of non-synonymous to synonymous substitutions per site using the computer program DNAsp (Rozas et al., 2003). Finally, we calculated WNV evolutionary rates using Bayesian methods and relaxed molecular clock assumptions available in the computer program BEAST (Drummond et al., 2006). We performed other statistical tests and procedures with the computer program R (R. Development Core Team, 2007); R codes are available upon request.

Acknowledgments

We thank M. Goshorn, B. Pultorak, M. Neville, T. Thompson, and J. McClain for their assistance in the field, and B. Bullard,

B. Morgan, A. Thelen, M. Bender, L. Abernathy, K. Vashisht, and J. McClain for their assistance in the laboratory. L. Mosher and the Michigan Department of Community Health kindly provided the positive control strain of WNV. G. Ebel and one anonymous reviewer provided helpful comments on the manuscript. Many thanks to J. Fahey and the Archdiocese of Chicago, and to the municipalities of Evergreen Park, Palos Hills, Burbank, Alsip, Blue Island, the City of Chicago, and private landowners in these municipalities for allowing us to conduct this research, as well as to the Village of Oak Lawn for providing field laboratory facilities. This material is based upon work supported by the National Science Foundation/National Institutes of Health Ecology of Infectious Diseases program under Award No. 0429124.

References

Ackerman, B., 1985. Temporal march of the Chicago heat-island. J. Appl. Meteorol. 24, 547–554.

Anderson, J.F., Vossbrinck, C.R., Andreadis, T.G., Iton, A., Beckwith 3rd, W.H., Mayo, D.R., 2001. A phylogenetic approach to following West Nile virus in Connecticut. Proc. Natl. Acad. Sci. U. S. A. 98, 12885–12889.

Andreadis, T.G., Anderson, J.F., Vossbrinck, C.R., Main, A.J., 2004. Epidemiology of West Nile virus in Connecticut: a five-year analysis of mosquito data 1999–2003. Vector Borne Zoonotic Dis. 4, 360–378.

Andreadis, T.G., Thomas, M.C., Shepard, J.J., 2005. Identification Guide to the Mosquitoes of Connecticut. The Connecticut Agricultural Experiment Station, New Haven, CT.

Arnfeld, A.J., 2003. Two decades of urban climate research: a review of turbulence, exchanges of energy and water, and the urban heat island. J. Climatol. 23, 1–26.

Bertolotti, L., Kitron, U., Goldberg, T.L., 2007. Diversity and evolution of West Nile virus in Illinois and The United States, 2002–2005. Virology 360, 143–149.

Bertorelle, G., Barbujani, G., 1995. Analysis of DNA diversity by spatial autocorrelation. Genetics 140, 811–819.

Biggerstaff, B.J., 2006. PooledInfRate, Version 3.0: a MicrosoftR ExcelR add-in to compute prevalence estimates from pooled samples. Centers for Disease Control and Prevention, Fort Collins, CO.

Brault, A.C., Huang, C.Y., Langevin, S.A., Kinney, R.M., Bowen, R.A., Ramey, W.N., Panella, N.A., Holmes, E.C., Powers, A.M., Miller, B.R., 2007. A single positively selected West Nile viral mutation confers increased virogenesis in American crows. Nat. Genet. 39, 1162–1166.

Ciota, A.T., Ngo, K.A., Lovelace, A.O., Payne, A.F., Zhou, Y., Shi, P.Y., Kramer, L.D., 2007. Role of the mutant spectrum in adaptation and replication of West Nile virus. J. Gen. Virol. 88, 865–874.

Davis, C.T., Ebel, G.D., Lanciotti, R.S., Brault, A.C., Guzman, H., Siirin, M., Lambert, A., Parsons, R.E., Beasley, D.W., Novak, R.J., Elizondo-Quiroga, D., Green, E.N., Young, D.S., Stark, L.M., Drebot, M.A., Artsob, H., Tesh, R.B., Kramer, L.D., Barrett, A.D., 2005. Phylogenetic analysis of North American West Nile virus isolates, 2001–2004: evidence for the emergence of a dominant genotype. Virology 342, 252–265.

Davis, C.T., Li, L., May, F.J., Bueno Jr., R., Dennett, J.A., Bala, A.A., Guzman, H., Quiroga-Elizondo, D., Tesh, R.B., Barrett, A.D., 2007. Genetic stasis of dominant West Nile virus genotype, Houston, Texas. Emerg. Infect. Dis. 13, 601–604.

Dohm, D.J., O'Guinn, M.L., Turell, M.J., 2002. Effect of environmental temperature on the ability of *Culex pipiens* (Diptera: Culicidae) to transmit West Nile virus. J. Med. Entomol. 39, 221–225.

Drummond, A.J., Ho, S.Y., Phillips, M.J., Rambaut, A., 2006. Relaxed phylogenetics and dating with confidence. PLoS Biol. 4, e88.

Ebel, G.D., Carricaburu, J., Young, D., Bernard, K.A., Kramer, L.D., 2004. Genetic and phenotypic variation of West Nile virus in New York, 2000–2003. Am. J. Trop. Med. Hyg. 71, 493–500.

Excoffier, L., Laval, G., Schnieder, S., 2005. Arlequin version 3.0: an integrated software package for population genetics data analysis. Evolutionary Bioinformatics Online, vol. 1, pp. 47–50.

Ezenwa, V.O., Milheim, L.E., Coffey, M.F., Godsey, M.S., King, R.J., Guptill, S.C., 2007. Land cover variation and West Nile virus prevalence: patterns, processes, and implications for disease control. Vector Borne Zoonotic Dis. 7, 173–180.

Gu, W., Lampman, R., Krasavin, N., Berry, R., Novak, R., 2006. Spatio-temporal analyses of West Nile virus transmission in *Culex* mosquitoes in northern Illinois, USA, 2004. Vector Borne Zoonotic Dis 6, 91–98.

Hamer, G.L., Walker, E.D., Brawn, J.D., Loss, S.R., Ruiz, M.O., Goldberg, T.L., Schotthoefer, A.M., Brown, W.M., Wheeler, E., and Kitron, U.D., in press. Rapid amplification of West Nile virus: the role of hatch year birds. Vector-Borne and Zoonotic Dis. 8.

Harlan, S.L., Brazel, A.J., Prashad, L., Stefanov, W.L., Larsen, L., 2006. Neighborhood microclimates and vulnerability to heat stress. Soc. Sci. Med. 63, 2847–2863.

Jerzak, G., Bernard, K.A., Kramer, L.D., Ebel, G.D., 2005. Genetic variation in West Nile virus from naturally infected mosquitoes and birds suggests quasispecies structure and strong purifying selection. J. Gen. Virol. 86, 2175–2183.

Kilpatrick, A.M., Daszak, P., Jones, M.J., Marra, P.P., Kramer, L.D., 2006. Host heterogeneity dominates West Nile virus transmission. Proc. Biol. Sci. 273, 2327–2333.

Kinney, R.M., Huang, C.Y., Whiteman, M.C., Bowen, R.A., Langevin, S.A., Miller, B.R., Brault, A.C., 2006. Avian virulence and thermostable replication of the North American strain of West Nile virus. J. Gen. Virol. 87, 3611–3622.

Lanciotti, R.S., Roehrig, J.T., Deubel, V., Smith, J., Parker, M., Steele, K., Crise, B., Volpe, K.E., Crabtree, M.B., Scherret, J.H., Hall, R.A., MacKenzie, J.S., Cropp, C.B., Panigrahy, B., Ostlund, E., Schmitt, B., Malkinson, M., Banet, C., Weissman, J., Komar, N., Savage, H.M., Stone, W., McNamara, T., Gubler, D.J., 1999. Origin of the West Nile virus responsible for an outbreak of encephalitis in the northeastern United States. Science 286, 2333–2337.

Lanciotti, R.S., Kerst, A.J., Nasci, R.S., Godsey, M.S., Mitchell, C.J., Savage, H.M., Komar, N., Panella, N.A., Allen, B.C., Volpe, K.E., Davis, B.S., Roehrig, J.T., 2000. Rapid detection of West Nile virus from human clinical specimens, field-collected mosquitoes, and avian samples by a TaqMan reverse transcriptase-PCR assay. J. Clin. Microbiol. 38, 4066–4071.

Lanciotti, R.S., Ebel, G.D., Deubel, V., Kerst, A.J., Murri, S., Meyer, R., Bowen, M., McKinney, N., Morrill, W.E., Crabtree, M.B., Kramer, L.D., Roehrig, J.T., 2002. Complete genome sequences and phylogenetic analysis of West Nile virus strains isolated from the United States, Europe, and the Middle East. Virology 298, 96–105.

Liu, W.J., Wang, X.J., Clark, D.C., Lobigs, M., Hall, R.A., Khromykh, A.A., 2006. A single amino acid substitution in the West Nile virus nonstructural protein NS2A disables its ability to inhibit alpha/beta interferon induction and attenuates virus virulence in mice. J. Virol. 80, 2396–2404.

Mantel, N., 1967. The detection of disease clustering and a generalized regression approach. Cancer Res. 27, 209–220.

Moudy, R.M., Meola, M.A., Morin, L.L., Ebel, G.D., Kramer, L.D., 2007. A newly emergent genotype of West Nile virus is transmitted earlier and more efficiently by *Culex* mosquitoes. Am. J. Trop. Med. Hyg. 77, 365–370.

Nei, M., 1987. Molecular Evolutionary Genetics. Columbia University Press, New York.

Posada, D., Crandall, K.A., 2001. Selecting the best-fit model of nucleotide substitution. Syst. Biol. 50, 580–601.

Posada, D., Buckley, T.R., 2004. Model selection and model averaging in phylogenetics: advantages of Akaike information criterion and Bayesian approaches over likelihood ratio tests. Syst. Biol. 53, 793–808.

R. Development Core Team, 2007. R: A language and environment for statistical computing 2.5. Vienna, Austria: R Foundation for Statistical Computing.

Reisen, W.K., Fang, Y., Martinez, V.M., 2005. Avian host and mosquito (Diptera: Culicidae) vector competence determine the efficiency of West Nile and St. Louis encephalitis virus transmission. J. Med. Entomol. 42, 367–375.

Reisen, W.K., Fang, Y., Martinez, V.M., 2006. Effects of temperature on the transmission of west nile virus by *Culex tarsalis* (Diptera: Culicidae). J. Med. Entomol. 43, 309–317.

Richards, S.L., Mores, C.N., Lord, C.C., Tabachnick, W.J., 2007. Impact of extrinsic incubation temperature and virus exposure on vector competence of

Culex pipiens quinquefasciatus Say (Diptera: Culicidae) for West Nile virus. Vector Borne Zoonotic Dis. 7 (4), 629–636.

Ronquist, F., Huelsenbeck, J.P., 2003. MrBayes 3: Bayesian phylogenetic inference under mixed models. Bioinformatics 19, 1572–1574.

Rozas, J., Sanchez-DelBarrio, J.C., Messeguer, X., Rozas, R., 2003. DnaSP, DNA polymorphism analyses by the coalescent and other methods. Bioinformatics 19, 2496–2497.

Ruiz, M.O., Tedesco, C., McTighe, T.J., Austin, C., Kitron, U., 2004. Environmental and social determinants of human risk during a West Nile virus outbreak in the greater Chicago area, 2002. Int. J. Health Geogr. 3, 8.

Ruiz, M.O., Walker, E.D., Foster, E.S., Haramis, L.D., Kitron, U.D., 2007. Association of West Nile virus illness and urban landscapes in Chicago and Detroit. Int. J. Health Geogr. 6, 10.

Snapinn, K.W., Holmes, E.C., Young, D.S., Bernard, K.A., Kramer, L.D., Ebel, G.D., 2007. Declining growth rate of West Nile virus in North America. J. Virol. 81, 2531–2534.

Swofford, D., 2003. PAUP*. Phylogenetic Analysis Using Parsimony (*and Other Methods) 4.0. Sunderland, Massachusetts: Sinauer Associates.

Tamura, K., Dudley, J., Nei, M., Kumar, S., 2007. MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) Software Version 4.0. Mol. Biol. Evol. 24, 1596–1599.

Ward, M.R., Stallknecht, D.E., Willis, J., Conroy, M.J., Davidson, W.R., 2006. Wild bird mortality and West Nile virus surveillance: biases associated with detection, reporting, and carcass persistence. J. Wildl. Dis. 42, 92–106.

Weaver, S.C., Hagenbaugh, A., Bellew, L.A., Gousset, L., Mallampalli, V., Holland, J.J., Scott, T.W., 1994. Evolution of alphaviruses in the eastern equine encephalomyelitis complex. J. Virol. 68, 158–169.

Weaver, S.C., Scott, T.W., Rico-Hesse, R., 1991. Molecular evolution of eastern equine encephalomyelitis virus in North America. Virology 182, 774–784.

Weng, Q.H., Lu, D.S., Liang, B.Q., 2006. Urban surface biophysical descriptors and land surface temperature variations. Photogramm. Eng. Remote Sensing 72, 1275–1286.

Zweighaft, R.M., Rasmussen, C., Brolnitsky, O., Lashof, J.C., 1979. St. Louis encephalitis: the Chicago experience. Am. J. Trop. Med. Hyg. 28, 114–118.