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Genetic Insights into the Population Structure of *Culex pipiens* (Diptera: Culicidae) in the Northeastern United States by Using Microsatellite Analysis

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Abstract. Members of the *Culex pipiens* complex are considered as biting nuisance and vectors of important arboviruses including West Nile virus (WNV). To analyze the genetic structure of urban and rural populations of *Cx. pipiens* form pipiens and gain insights into behavioral implications, mosquitoes were collected from established WNV transmission foci in Connecticut from October 2006 through October 2007, examined by using microsatellite markers, and compared with other populations from neighboring states in the northeastern United States. The mean numbers of alleles per locus for the aboveground *Cx. pipiens* form pipiens populations ranged from 11.5 ± 2.3 to 13.2 ± 2.4 and were not significantly different. In contrast, *Cx. pipiens* form molestus had greatly reduced allelic diversities with an average of 4.4 ± 1.2 alleles per locus, which was significantly lower than that of any of the *Cx. pipiens* form pipiens populations analyzed. We did not detect significant genetic differences between urban and rural populations of *Cx. pipiens* form pipiens from Connecticut nor did we observe temporal genetic changes. However, in a comparative analysis with populations of neighboring states, New Jersey, New York, and Massachusetts, genetic variations associated with geographic distance were identified. In the analyses of Bayesian clustering and principal component analysis, we identified two clusters separating *Cx. pipiens* form molestus from *Cx. pipiens* form pipiens populations, indicating that *Cx. pipiens* form molestus was genetically distinct from any of the *Cx. pipiens* form pipiens populations examined during this study.

INTRODUCTION

In the northeastern United States, *Culex pipiens* has been implicated as the primary vector of West Nile virus (WNV).[1–4] The *Cx. pipiens* complex exists in two forms that exhibit substantially different behavioral and physiologic characteristics, but are morphologically indistinguishable. *Culex pipiens* form pipiens generally develop in aboveground environments, mate while swarming in open areas (eurygamous), undergo obligatory winter diapause, and require a blood meal to develop eggs (anautogeny).[5] *Culex pipiens* form molestus, in contrast, inhabit subterranean environments especially in urban areas, mate in confined spaces (stenogamous), remain active throughout the winter, and produce their first batch of eggs without a blood meal (autogeny).[5] Local studies on the host-feeding preferences of aboveground populations presumed to be *Cx. pipiens* form pipiens, have shown that this form has a very strong preference for avian hosts with occasional feeding on mammals including humans.[6–8] Studies in Europe, however, have demonstrated that the molestus form feeds readily on mammals and is an aggressive human biter.[9] Definitive knowledge of the biting behavior of North American populations of *Cx. pipiens* form molestus is lacking. The two forms generally are reproductively isolated in nature, but have been reported to occasionally hybridize in urban areas during the late summer producing hybrid females that feed indiscriminately on avian or mammalian hosts.[10–12]

Populations of *Cx. pipiens* form pipiens and form molestus from northern Europe have been examined by using microsatellite markers, and have shown to be genetically distinct and do not interbreed.[13] However, in an analysis of aboveground populations of *Cx. pipiens* from the northeastern United States, many individuals with hybrid genetic signatures (pipiens versus molestus) alongside individuals with a pipiens signature were noted. This suggests that the high percentage of hybrids of the two behavioral forms contributed to the higher rate and unique feature of human infection in North America.[13] Definitive evidence demonstrating that these hybrid forms feed on mammalian more readily than avian hosts is lacking. However, the genetic composition of different *Cx. pipiens* populations may have important implications for the transmission of WNV in various locales. The extent and distribution of hybrid populations of *Cx. pipiens* in the northeastern United States is unclear, and there is a need to more fully characterize the genetic structure of natural populations of this mosquito vector both spatially and temporally to better interpret epidemiologic studies.

The current study was designed to examine the genetic structure of urban and rural populations of *Cx. pipiens* form pipiens and compare them with *Cx. pipiens* form molestus in the northeastern United States, and to analyze temporal changes in *Cx. pipiens* populations collected from established WNV transmission foci in Connecticut (CT) by using microsatellite markers. These markers are useful in population genetic studies. They are codominant, polymorphic, and assist in estimating relatedness and differentiating individuals.[14] A set of twelve existing microsatellite markers were used to analyze populations of *Cx. pipiens* from five urban/suburban and three rural locations in CT, and urban locales from Trenton, New Jersey, New York City, New York, and Cambridge, Massachusetts.

MATERIALS AND METHODS

Mosquito collection and identification. Mosquitoes were collected either as adults by using gravid traps baited with hay infusion,[15] as larvae by dipper sampling in the open water bodies, or as multiple egg rafts by using oviposition traps from eight sites representing rural and urban localities in CT during June to October 2007 (Figure 1). Egg rafts were hatched separately and only one female from each raft was included in the analyses. Additional aboveground populations of *Cx. pipiens* form pipiens were collected from neighboring states, New Jersey (NJ), New York (NY), and Massachusetts (MA) for comparison purposes (Figure 1). Populations of *Cx. pipiens*

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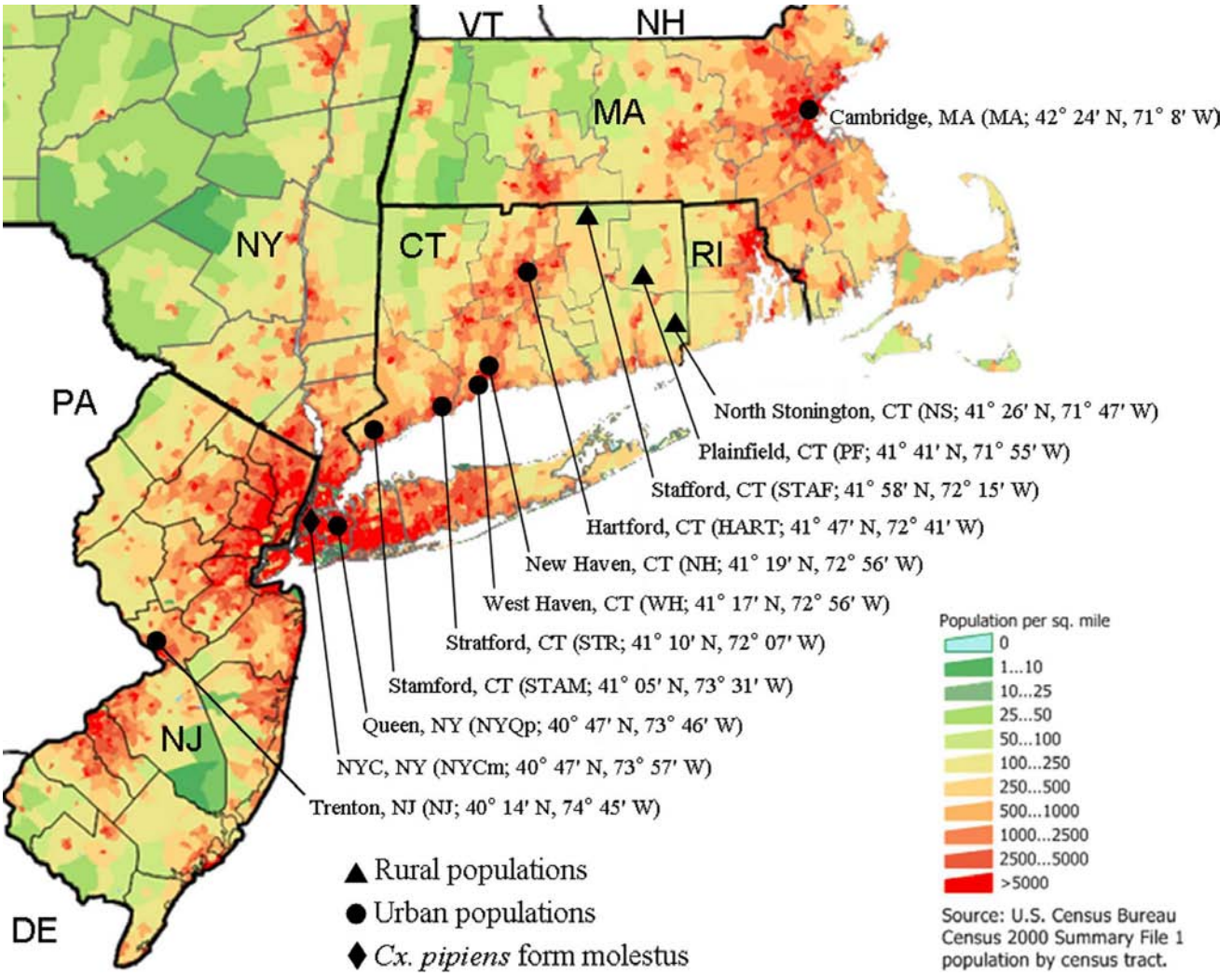

FIGURE 1. Mosquito sampling sites in Connecticut, New Jersey, New York, and Massachusetts. Locations of rural sites are shown in closed triangles, locations of urban sites are depicted by closed circles, and collection site of *Cx. pipiens* form molestus is shown in closed diamond. Population abbreviations followed by the latitude and longitude parameters of the collection sites are included in the parentheses. The map was adopted and modified from http://en.wikipedia.org/wiki/List_of_U.S._states_by_population_density. This figure appears in color at www.ajtmh.org.

form pipiens from New York City (NYC) were collected by hand-held aspirators from aboveground hibernacula located at Fort Totten in the borough of Queens in January 2007. Underground population of *Cx. pipiens* form molestus was collected by using a battery-powered modified CDC backpack aspirator (John W. Hock Co., Gainesville, FL) from several sewer catch basins located on 91st Street in the borough of Manhattan, NYC in January 2007. This population of *Cx. pipiens* form molestus has been examined to be pure molestus population in a previous study.[16] We have confirmed their finding by observations that this population was active when collected in winter from underground habitat, and a colony established in our laboratory is autogenous. Analysis of mosquitoes for potential temporal variations was additionally performed with populations collected monthly on six occasions: October 2006, and June through October 2007 from an active WNV transmission site in New Haven, CT.[2] For space consideration, abbreviations for the collection sites have been used throughout this publication as described in Figure 1. Adult mosquitoes were transported to the laboratory either alive in cages or on dry ice. Larvae were carried alive to the laboratory where they were reared to adults for analysis. Specimens were promptly identified on chill tables with the aid of a stereomicroscope by using descriptive keys.[17,18] *Culex pipiens* specimens were further subjected to a species–specific polymerase chain reaction (PCR) test based on ribosomal DNA[19] to confirm the results of morphologic identifications. Identified specimens were either processed immediately for genomic DNA extraction or stored at –80°C.

Genomic DNA extraction. Before genomic DNA extraction from individual female mosquitoes, abdomens were removed to avoid cross-contamination from sperms in the spermatheca. Each mosquito was homogenized with the aid of a microtube pestle (USA Scientific, Enfield, CT) in a 1.5 mL tube containing 180 μL phosphate buffered saline (PBS) buffer (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, 1.47 mM KH_2PO_4) and subjected to DNA extraction by using the DNeasy Blood & Tissue Kit (QIAGEN, Valencia, CA) according to the manufacturer's recommended protocol. Isolated DNA from each mosquito was reconstituted in 50 μL AE buffer (Qiagen, 10 mM Tris-Cl, 0.5 mM EDTA, pH 9.0), and stored at –20°C for PCR experiments.

Polymerase chain reaction and microsatellite data collection. A total of 12 existing polymorphic microsatellite markers for members of *Cx. pipiens* complex were used in the analyses (Table 1). The forward primer for each pair was labeled at the 5′-end with a fluorescent dye (VIC, NED, 6-FAM, or PET; Applied Biosystems, Foster City, CA). All PCR reactions were performed in 20 μL reaction volume containing 0.4 μL genomic DNA, 2 μL 10 × PCR buffer II (Applied Biosystems, 100 mM Tris-HCl, 500 mM KCl, pH 8.3), 2 μL $MgCl_2$ (25 mM), 0.4 μL dNTP mix (10 mM), 0.4 μL bovine serum albumin (10 mg/μL), 0.4 μL each primer (0.2 μM), and 0.5 units AmpliTaq Gold DNA polymerase (Applied Biosystems). The PCR reactions were initially denatured at 95°C for 10 minutes, followed by 35 cycles of amplification at 95°C for 15 seconds, 54°C for 20 seconds and 72°C for 20 seconds, and finally extended at 72°C for 7 minutes. Allele–specific amplification PCR reactions were performed on a 96-well GeneAmp PCR System 9700 (Applied Biosystems). Compatible primer pairs were multiplexed to increase the overall assay throughput. The PCR products were pooled and mixed with Hi-Di formamide (Applied Biosystems) followed by adding GeneScan 600 LIZ Size Standard (Applied Biosystems) for the reproducible sizing of the fragments, and analyzed by using 3730 Genetic Analyzer (Applied Biosystems). Data were analyzed by using GeneMapper software version 3.7 for fragment analysis (Applied Biosystems) to derive microsatellite allele sizes and genotypes. If the locus had stutter and/or plus A issues resulting in split peaks and making it difficult to designate the correct alleles, a final PCR extension time of 45 minutes was added to facilitate the plus A formation, and the last high peak was called consistently throughout the genotyping procedure. To ensure the consistency of allele amplification throughout this study, a positive control obtained from sequencing each locus was included in every genotyping analyses, and approximately one-fourth of the specimens for each population were genotyped in duplicate. A known population of *Culex quinquefasciatus* was compared with the populations of *Cx. pipiens* analyzed in this study to examine the possibility that results obtained for *Cx. pipiens* was not influenced by introgression. Only 10 of 12 markers were used in this comparison because 2 of them did not amplify with *Cx. quinquefasciatus*. No significant hybridization was found between these two mosquitoes in the study region, nor was a gradient of *Cx. quinquefasciatus* ancestry found. Therefore, *Cx. quinquefasciatus* was excluded from further analyses to use all the 12 markers.

Microsatellite data analysis. The program Micro-Checker was used to identify genotyping errors, and to estimate the frequencies of null alleles prior to statistical analyses.[20] GENEPOP 4.0[21] was used to determine allele frequencies, conformity to Hardy-Weinberg equilibrium (HWE), and Linkage (Gametic) disequilibrium (LD). Allele frequencies were estimated per locus per population. The Statistical Package for the Social Sciences (SPSS) version 15.0 (SPSS, Inc., Chicago IL) was used to examine whether the differences of mean allele frequencies among various populations were significant. Each locus was tested separately for departures from HWE by using the Markov chain algorithm of Guo and Thompson (1992)[22] with 1,000 batches and 100,000 iterations per batch.[23] Pairwise LD was estimated for each population by using Fisher's exact test. Significance levels were adjusted according to the sequential Bonferroni method to account for multiple comparisons in tests of HWE and LD.[24]

Two fixation indices, F_{ST} and R_{ST}, were calculated to measure the population genetic differentiation. F_{ST}, assuming the infinite alleles model (IAM), was calculated based on the absolute frequencies of alleles,[25] whereas R_{ST}, an analogue of F_{ST}, assuming the stepwise mutation model (SMM), was estimated from the sum of squared number of repeat differences.[26] Pairwise F_{ST} and R_{ST} values were calculated in ARLEQUIN[27] and R_{ST} Calc,[28] respectively. The unbiased *P* values of F_{ST} and R_{ST} values were determined by nonparametric permutation procedure with 10,000 replicates. Isolation by distance was tested according to Rousset (1997).[29] Mantel test[30] with 10,000 randomization iterations was used to test the significance of the correlation in the software FSTAT.[31]

To examine the population structure and estimate hybridization between *Cx. pipiens* form pipiens and *Cx. pipiens* form molestus, both Bayesian clustering and principal component analysis (PCA) were performed. Bayesian clustering was performed in the software STRUCTURE[32] with the "admixture" model, which does not use prior information on sampling localities so that individuals are allowed to have ancestry from multiple populations. We coupled admixture model with correlated allele frequencies with 100,000 "burn-in" steps and 1,000,000 follow-on runs. Analyses were performed for K = 1 through K = 10 with 10 runs for each K. The most likely number of clusters, K, was determined by averaging the log Pr(X|K) (the probability of individual X belong to cluster K) across runs.[32] Program Distruct[33] was used to graphically display the results produced by the genetic clustering program STRUCTURE. The PCA was performed in the program

TABLE 1

Primer sequences and repeat motifs of the 12 microsatellite loci used in the genetic analysis of *Cx. pipiens* populations in the northeastern United States

Locus	Origin	Repeat motif	Reference
CxpGT9 F2/R	*Culex pipiens*	$(GT)_{13}$	Smith and others 2005; Keyghobadi and others 2004
CxpGT12F2/R2	*Cx. pipiens*	$(TG)_{14}$	Smith and others 2005; Keyghobadi and others 2004
CxpGT4 F/R	*Cx. pipiens*	$(GT)_5(GTTT)_2GC(GT)_2CT(GT)_5$	Keyghobadi and others 2004
CxpGT20 F/R	*Cx. pipiens*	$(TG)_{15}$	Keyghobadi and others 2004
CxpGT40 F/R	*Cx. pipiens*	$(GT)_{15}$	Keyghobadi and others 2004
CxpGT46 F/R	*Cx. pipiens*	$(TG)_{15}$	Keyghobadi and others 2004
CxpGT51 F/R	*Cx. pipiens*	$(TG)_4CG(TG)_{15}$	Keyghobadi and others 2004
CxpGT53 F/R	*Cx. pipiens*	$(TG)_{22}$	Keyghobadi and others 2004
CQ11 F2/R3	*Cx. quinquefasciatus*	$(GT)_2(ACTTC)(GT)_9$	Fonseca and others 1998; Smith and others 2005
CxqGT4 F3/R	*Cx. quinquefasciatus*	$(GT)_{12}$	Smith and others 2005
CxqGT6b F/R	*Cx. quinquefasciatus*	$(CA)_8$	Smith and others 2005
CxqTri4 F/R	*Cx. quinquefasciatus*	$(TGC)_7$	Smith and others 2005

PCA-GEN version 1.2 (Goudet J, unpublished) with 10,000 randomizations for significance test.

Statistics of ancestry and hybrid percentage were performed from the Bayesian clustering result. For the purpose of comparison, individuals were considered as hybrids if the ancestry coefficient was equal or greater than 0.06 as defined elsewhere.[13]

RESULTS

Allele frequencies. All microsatellite loci amplified in this study were polymorphic. The average number of alleles per locus ranged from 4.7 ± 1.0 to 20.2 ± 2.1. Locus CxqTri4 was the least and CxpGT53 the most polymorphic (Table A1, available at www.ajtmh.org). The mean numbers of alleles per locus per population for the aboveground *Cx. pipiens* form pipiens populations ranged from 11.5 ± 2.3 to 13.2 ± 2.4 and were not significantly different. In contrast, *Cx. pipiens* form molestus had greatly reduced allelic diversity with an average of 4.4 ± 1.2 alleles per locus per population, which was significantly lower than that of any of the *Cx. pipiens* form pipiens populations ($P < 0.05$). Additionally, *Cx. pipiens* form molestus had four loci, CQ11, CxqGT4, CxqTri4, and CxpGT12, which were fixed at alleles 282, 149, 116, and 140, respectively. The *Cx. pipiens* form molestus population was collected from the same location reported in an earlier study.[16] The fixed allele sizes were slightly different from this report most likely because of differences in the amplification conditions and program settings in allele designation. Locus CxpGT12 was also similar to that reported earlier,[13] but in that report it was not fixed, and the major allele size was 144 with a frequency of 0.82. The major alleles in all loci were the same for all *Cx. pipiens* form pipiens populations. However, *Cx. pipiens* form molestus had different distribution of major alleles in loci CQ11, CxpGT9, CxpGT20, CxpGT40, CxpGT51, and CxpGT53.

Conformity to Hardy-Weinberg equilibrium. Exact tests showed significant departures ($P < 0.05$) from HWE after sequential Bonferroni corrections in loci CQ11, CxpGT12, CxpGT20, CxpGT40, and CxpGT53 (Table A1). Locus CQ11 had significant departures in all the *Cx. pipiens* form pipiens populations, whereas CxpGT40 had significant departure only in three populations. All these departures were associated with positive F_{IS} values, reflecting heterozygosity deficits. Heterozygosity deficits are usually caused by inbreeding, selection, Wahlund effect, and null alleles. The first three were unlikely the reasons as they affect all loci not just one or a few. Instead, null alleles caused by mutations in the primer-binding sites was the most likely reason, as suggested by the program Micro-Checker.[20] Locus CQ11 is known for mutations in primer biding sites forcing the authors to redesign the primers,[34,35] and CxpGT12 was reported to have null alleles as well.[36] In PCR amplification, a few individuals repeatedly failed to amplify at one locus, although they amplified successfully at other loci, strongly suggesting the presence of null alleles. Preferential amplification of small alleles (i.e., large allele dropout or short allele dominance),[37] where the larger allele specifically fails to amplify may be another contributing factor. For example, locus CxpGT53 was highly polymorphic with allele sizes ranging from 225 to 335 bp. In some individuals, we hardly observed alleles larger than 325 bp. In this study, we either used all the loci or excluded the aforementioned 5 loci in further analysis for the purpose of comparison.

Gametic (linkage) disequilibrium analysis. Pairwise exact tests of the 12 loci for LD across all populations revealed that locus pairs, CxpGT9 & CxpGT40, CxpGT4 & CxpGT51, CxpGT9 & CxpGT51, and CxpGT40 & CxpGT51, were significant. Upon removal of population NH0610, only one significant test involving locus pair CxpGT4 & CxpGT51 in population NH0706 was found and no global significant LD was present. Therefore, except for the temporal analysis, NH0610 was excluded from any other analysis. When populations of *Cx. pipiens* form pipiens from New Haven were analyzed for temporal genetic changes separately, loci CxpGT4, CxpGT9, CxpGT20, CxpGT40, and CxpGT53 all showed significant LD with locus CxpGT51, but these linkages to locus CxGT51 were not observed when only the New Haven population NH0708 was included in the analyses. Considering results of both HWE and LD, we either used all loci or excluded the five loci showing significant departures from HWE. Locus CxpGT51 in temporal analysis of New Haven populations was also excluded.

Genetic comparison of urban and rural populations of *Cx. pipiens* form pipiens in Connecticut. We examined the population structure of *Cx. pipiens* form pipiens mosquitoes collected from urban and rural localities by analyzing either 12 or 7 loci, which excluded the 5 loci exhibiting departures from HWE. Pairwise F_{ST} and R_{ST} values for all urban and rural populations were lower than 0.02 (Table 2 and 3), a value empirically considered to indicate negligible genetic differentiation. All these F_{ST} and R_{ST} values calculated over either 12 or 7 loci were not significant after sequential Bonferroni correction ($\alpha = 0.05$, $K = 28$), and the overall F_{ST} and R_{ST} means were also not significant between urban and rural populations, suggesting *Cx. pipiens* form pipiens populations in CT were genetically homogenous. In the Bayesian clustering analysis, we identified two clusters ($K = 2$), separating *Cx. pipiens* form molestus from *Cx. pipiens* form pipiens populations (Figure 2A). No population structuring was detected in urban and rural *Cx. pipiens* form pipiens populations, further suggesting lack of major genetic differentiations among these populations. Ancestry and hybrid percentage in rural populations were not significantly different from that of urban populations when all 12 markers were used in the analysis (Table A2, available at www.ajtmh.org). However, when 7 markers were used, ancestry and hybrid percentage in any rural population were significantly lower than that of urban populations (Table A2). Although differences of ancestry and hybrid percentage were evident, no population differentiation was detected. Either other genetic variations offset the ancestry and hybrid differences resulting in insignificant population differentiations, or the ancestry and hybrid estimates were influenced by the markers used.

We further analyzed the effect of the number of markers on the estimates of *Cx. pipiens* form molestus ancestry and hybrid percentage. Because there were many different combinations to choose in a given number of markers, we simply carried out the analysis in a stepwise procedure by removing the least or most polymorphic marker, the second least or most polymorphic marker, and so on until there was only one marker remained. When we removed the least polymorphic marker one at a time, the population structure was well maintained until there were only three markers left, but the ancestry and hybrid estimates were altered (Figure 2B, Table

TABLE 2
Genetic distance at 12 microsatellite loci between sampling localities*

	Urban populations (CT)					Rural populations (CT)			Other urban populations			Molestus
	NH0708	HART	STAM	WH	STR	STAF	PF	NS	MA	NJ	NYQp	NYCm
NH0708		0.0028	0.0075	0.0029	0.0024	0.0003	–0.0006	0.0118	0.0146	0.0115	0.0059	**0.2438**
HART	0.0008		0.0052	–0.0028	–0.0024	0.0069	0.0037	0.0078	0.0022	0.0170	0.0049	**0.2209**
STAM	0.0039	0.0064		–0.0020	–0.0030	0.0021	0.0106	0.0081	0.0114	**0.0220**	–0.0016	**0.2213**
WH	0.0011	0.0049	0.0044		–0.0014	0.0053	0.0088	0.0083	0.0143	0.0215	–0.0035	**0.2505**
STR	0.0007	0.0035	0.0016	0.0040		–0.0024	–0.0020	–0.0013	0.0066	**0.0245**	0.0048	**0.2341**
STAF	–0.0003	0.0017	0.0057	0.0048	0.0028		–0.0030	0.0027	0.0094	**0.0219**	0.0082	**0.1966**
PF	0.0044	0.0042	0.0072	0.0092	0.0047	0.0007		–0.0013	0.0026	**0.0256**	0.0142	**0.2452**
NS	0.0044	0.0047	0.0054	0.0057	0.0023	0.0011	–0.0002		0.0075	**0.0393**	0.0106	**0.2367**
MA	0.0071	0.0047	0.0098	0.0098	0.0076	0.0042	0.0059	0.0026		**0.0278**	0.0136	**0.2169**
NJ	0.0065	**0.0120**	0.0090	**0.0174**	**0.0117**	0.0099	**0.0108**	**0.0142**	**0.0167**		**0.0214**	**0.2287**
NYQp	0.0015	0.0046	0.0031	0.0030	0.0020	0.0033	0.0059	0.0045	**0.0101**	0.0085		**0.2080**
NYCm	**0.1911**	**0.1920**	**0.1915**	**0.2021**	**0.1857**	**0.1831**	**0.2100**	**0.2020**	**0.1919**	**0.1811**	**0.1831**	

* Values below the diagonal are F_{ST} and those above diagonal are R_{ST}. Numbers in bold are significant at $P < 0.05$ after sequential Bonferroni correction. Numbers in bold and underlined are significant at $P < 0.001$ after sequential Bonferroni correction.

A2). Ancestry and hybrid estimates of one of the rural populations of *Cx. pipiens* form pipiens, STAF, were no longer significantly different from that of urban populations even though the hybrid percentage was still significantly different as were the overall average estimates (Table A2). Population PF also exhibited the same pattern when only three markers were used in the analysis. When we removed the most polymorphic marker one at a time, expected simulations of population structure were not achieved (Figure 2C). Although *Cx. pipiens* form molestus was still well separated from *Cx. pipiens* form pipiens populations when the first and second most polymorphic markers were removed, the ancestry and hybrid estimates were no longer applicable. Compared with the earlier study[13] in which 40% of individuals in *Cx. pipiens* form pipiens populations in the United States have been reported as hybrids, we identified fewer hybrids even when only 3 markers were used. Hybrid percentage on average was as low as 11.9% when 7 markers were used. The results clearly demonstrated that the estimates of ancestry and hybrid percentage were not identical when different combinations of markers were used. As expected and as a general consensus in most population genetic studies, results also indicate that the more polymorphic markers are genetically more informative.

Temporal genetic variabilities. Temporal genetic changes were investigated and, over all loci, genetic differentiation was not significant (Table A3 and A4, available at www.ajtmh.org). All pairwise F_{ST} and R_{ST} values (< 0.01) calculated over 12 and 6 loci were not significant after sequential Bonferroni correction ($K = 15, \alpha = 0.05$), suggesting no temporal genetic changes occurred in the New Haven populations. Bayesian clustering analysis in program STRUCTURE also did not detect population structure changes except *Cx. pipiens* form molestus was distinct (Figure 3A). When calculated over 12 loci, only NH0709 had a significantly higher average *Cx. pipiens* form molestus ancestry than NH0708 (0.034 ± 0.011 versus 0.012 ± 0.002, $P < 0.05$), but it was no longer significant at $P = 0.01$ level and was also not significant when calculated over 6 loci. Correspondingly, NH0709 had 16% of hybrids, significantly higher than other populations when 12 loci were used, but it was also no longer significant when 6 loci were used (Table A5, available at www.ajtmh.org). Overall, no temporal genetic changes were observed.

Geographic structure. When *Cx. pipiens* form pipiens populations from NJ, NY, and MA were included in the analysis, the F_{ST} and R_{ST} values ranged from 0.0101 to 0.0393. Several F_{ST} and R_{ST} values were significant after sequential Bonferroni correction ($K = 66, \alpha = 0.05$) (Tables 2 and 3). In the analysis with the 12 markers, pairwise F_{ST} values were significant when NJ population was compared with other populations (HART, WH, STR, PF, NS, MA), and MA to NYQp. The R_{ST} values were significant when NJ population was compared with other populations (STAM, STR, STAF, PF, NS), and MA to NYQp (Table 2). In the analysis with 7 markers, pairwise F_{ST} values were significant involving MA with other populations (STAM, WH, STR, NJ, NYQp), and

TABLE 3
Genetic distance at seven microsatellite loci between sampling localities*

	Urban populations (CT)					Rural populations (CT)			Other urban populations			Molestus
	NH0708	HART	STAM	WH	STR	STAF	PF	NS	MA	NJ	NYQp	NYCm
NH0708		0.0057	0.0143	0.0046	0.0101	0.0016	0.0046	0.0194	**0.0273**	0.0033	0.0102	**0.1655**
HART	0.0033		0.0048	0.0001	–0.0043	–0.0013	0.0009	0.0058	0.0007	0.0100	0.0115	**0.1285**
STAM	0.0076	0.0105		–0.0024	–0.0030	0.0015	0.0171	0.0033	0.0179	0.0115	–0.0023	**0.1228**
WH	0.0003	0.0079	0.0095		–0.0041	–0.0062	0.0083	0.0016	0.0233	0.0109	–0.0015	**0.1439**
STR	–0.0002	0.0037	0.0008	0.0022		–0.0055	0.0015	–0.0022	0.0105	0.0154	0.0089	**0.1455**
STAF	0.0002	0.0012	0.0116	0.0013	0.0042		–0.0045	–0.0066	0.0128	0.0115	0.0060	**0.1365**
PF	0.0079	0.0063	0.0124	0.0107	0.0059	0.0017		0.0022	0.0082	0.0138	0.0232	**0.1593**
NS	0.0045	0.0056	0.0074	0.0047	0.0037	0.0000	0.0003		0.0125	**0.0245**	0.0134	**0.1298**
MA	0.0085	0.0042	**0.0137**	**0.0132**	**0.0126**	0.0058	0.0080	0.0065		0.0164	**0.0264**	**0.1147**
NJ	0.0082	0.0114	0.0043	**0.0126**	0.0082	0.0110	0.0086	0.0056	**0.0122**		0.0008	**0.1285**
NYQp	0.0020	0.0105	0.0041	0.0010	0.0035	0.0061	0.0100	0.0057	**0.0174**	0.0033		**0.1171**
NYCm	**0.1491**	**0.1481**	**0.1372**	**0.1563**	**0.1494**	**0.1536**	**0.1680**	**0.1523**	**0.1484**	**0.1404**	**0.1461**	

* Values below the diagonal are F_{ST} and those above diagonal are R_{ST}. Numbers in bold are significant at $P < 0.05$ after sequential Bonferroni correction. Numbers in bold and underlined are significant at $P < 0.001$ after sequential Bonferroni correction.

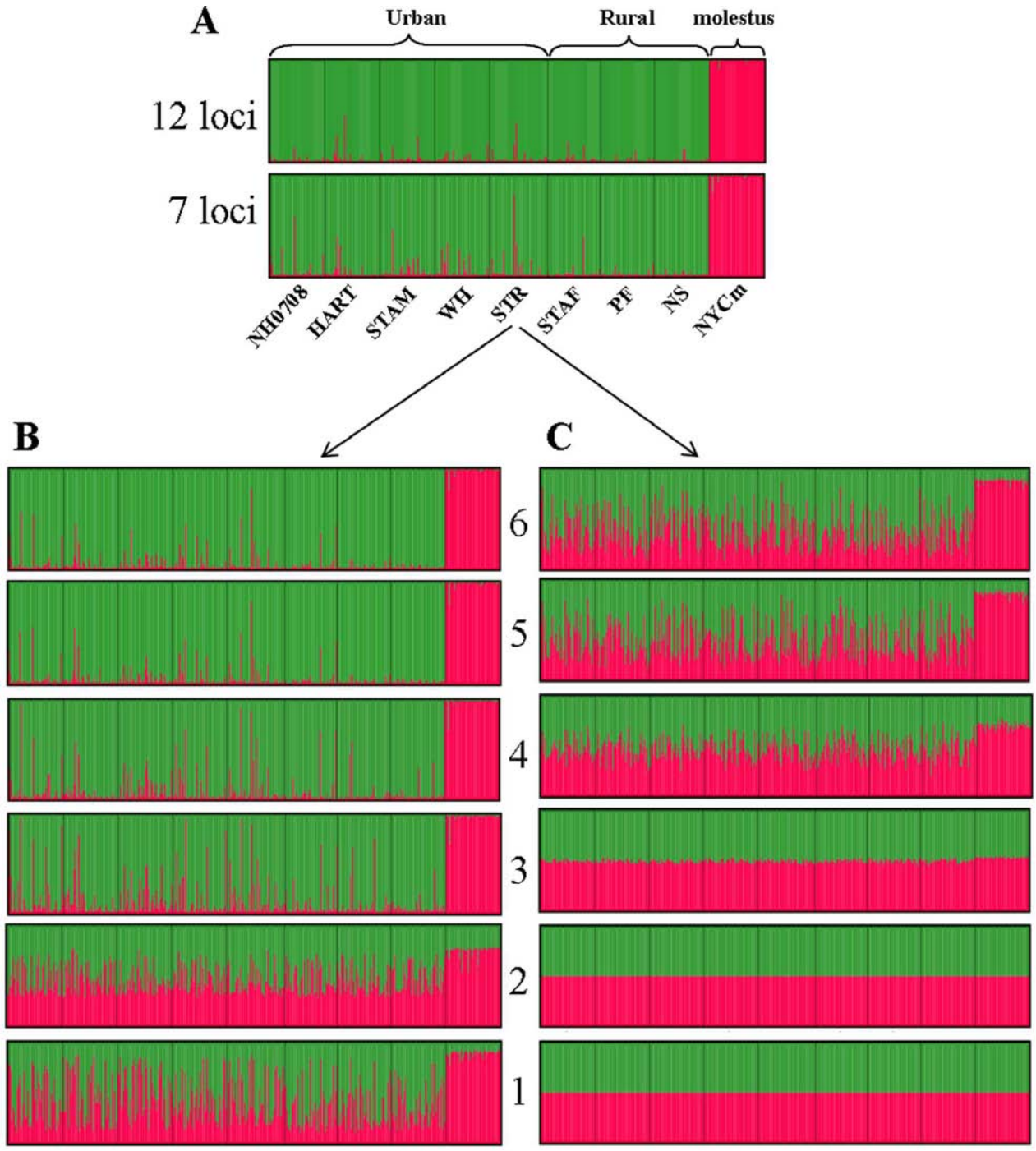

FIGURE 2. Bayesian clustering analysis of urban and rural *Cx. pipiens* form pipiens populations from Connecticut. Each thin vertical line represents each of the 384 individuals. The vertical line is partitioned into two colored segments representing the individual's estimated ancestry in the two clusters. Green and red colors represent *Cx. pipiens* form pipiens and *Cx. pipiens* form molestus cluster, respectively. Black lines separate individuals of different populations. Grouped urban and rural populations, and *Cx. pipiens* form molestus are labeled above the panel **A**, and populations from various sites are labeled below. Values between panels **B** and **C** represent the numbers of loci remaining in the analysis after certain loci were removed. **A**, Clustering results when 12 and 7 loci were used. **B**, Clustering result when the least polymorphic loci were removed in a stepwise procedure. **C**, Clustering result when the most polymorphic loci were removed in a stepwise procedure. This figure appears in color at www.ajtmh.org.

NJ with WH, while R_{ST} values were significant involving MA with other populations (NH0708 and NYQp), and NJ with NS (Table 3). Results suggested that NJ and MA populations were genetically different from some of the other populations. In contrast, Bayesian clustering analysis with models of population admixture and allele frequency correlated or independent did not detect any population structuring in *Cx. pipiens* form pipiens populations with the exception of *Cx. pipiens* form molestus that was always distinct when included in analysis (Figure 3B). As indicated,[32] program STRUCTURE is less powerful to test the population structure when the predefined populations correspond closely to genetic popula-

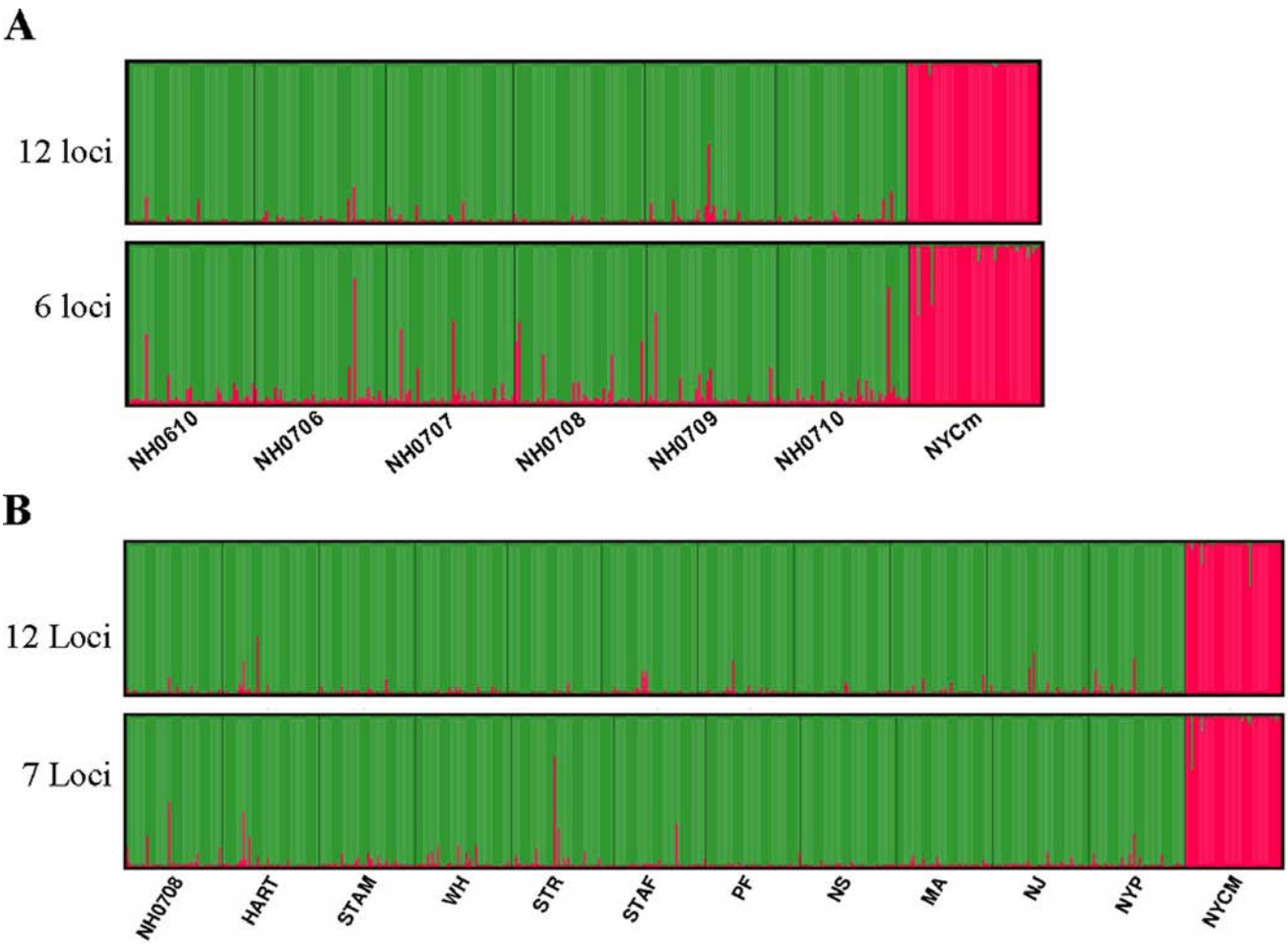

FIGURE 3. **A**, Temporal analysis of *Cx. pipiens* form pipiens populations in New Haven, Connecticut by using Bayesian clustering. **B**, Geographic analysis of *Cx. pipiens* form pipiens populations from Connecticut, New Jersey, New York, and Massachusetts. Green and red colors represent *Cx. pipiens* form pipiens and *Cx. pipiens* form molestus cluster, respectively. This figure appears in color at www.ajtmh.org.

tions, as was the case in this study. Instead, testing for frequency differences, from which F_{ST} derives, is more powerful and appropriate. In the principal component analysis, the first principal component accounted for 69.87% and 63.30% of the total variation calculated over 12 and 7 loci, respectively, and separated *Cx. pipiens* form molestus from *Cx. pipiens* form pipiens populations (Figure A1, available at www.ajtmh.org). The second principal component accounted for 6.06% and 9.10% of the total variations calculated over 12 and 7 loci, respectively. It appeared that NJ and MA populations were separated from the rest in the 12 loci analysis, and MA population was separated from the rest in the 7 loci analysis. The first principal component yielded the same result as Bayesian clustering, but the second principal component yielded better resolution on *Cx. pipiens* form pipiens populations.

Tests of isolation by distance based on F_{ST} and R_{ST} were both highly significant (Figure 4), suggesting differentiation in *Cx. pipiens* form pipiens populations was associated with geographic distance. We further tested whether the significant result was because of one or a few populations. We conducted this analysis by removing populations in a stepwise procedure. New Jersey and MA populations were the most distant geographically. When these two populations were removed from the analyses, the Mantel tests were no longer significant, confirming the population differentiation was because of isolation by distance. Furthermore, we found that ancestry and hybrid percentages in NJ, NYQp, and MA populations were not significantly different from other *Cx. pipiens* form pipiens populations (Table A6, available at www.ajtmh.org). In addition, when NJ, NY, and MA populations were included in the analysis, estimates of ancestry and hybrid percentages for CT populations did not remain the same (Table A2 and A6), suggesting a different number of populations can result in varying estimates.

***Cx. pipiens* form molestus as a distinct population.** *Culex pipiens* form molestus was always genetically distinct from any of the *Cx. pipiens* form pipiens populations examined during the present study. *Culex pipiens* form molestus had F_{ST} and R_{ST} values ranging from a moderate 0.1147 to a high 0.2505 (Table 2 and Table 3). All these F_{ST} and R_{ST} values

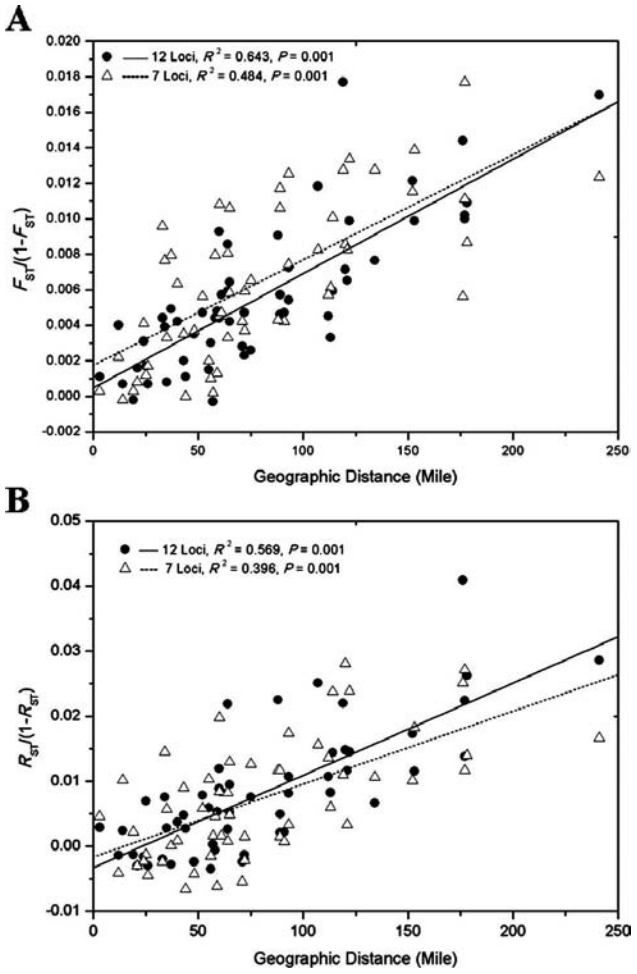

FIGURE 4. Geographic distance versus genetic distance among *Cx. pipiens* form pipiens populations from Connecticut, New Jersey, New York, and Massachusetts. Correlations and probabilities were estimated by using the Mantel test with 10,000 iterations. **A**, Correlation based on F_{ST}. **B**, Correlation based on R_{ST}.

were significant after sequential Bonferroni correction (α = 0.001, K = 66). Both Bayesian clustering and PCA analyses invariably separated *Cx. pipiens* form molestus from all the *Cx. pipiens* form pipiens populations.

DISCUSSION

Our comparative microsatellite analysis of populations of the *Cx. pipiens* complex in CT with other populations from neighboring states provides insights into the genetic structure of the major vector of WNV in the northeastern United States. Some behavioral differences such as host-feeding preference important in evaluating vectorial capacity in this mosquito species complex, have been attributed to genetic diversity and degrees of hybridization between *Cx. pipiens* form pipiens and *Cx. pipiens* form molestus. Scarcity of information on temporal and spatial variations and inadequacy of comprehensive knowledge have led to confusion over the relative contributions of these mosquitoes to the transmission, and in cases to broad generalizations of the transmission dynamics. Thus, population genetic studies will prove vital for evaluating the respective role members of this mosquito species complex play in enzootic and/or epidemic transmission of WNV and presumably other arboviruses in various regions in the US.

Members of *Cx. pipiens* complex display a variety of behavioral adaptations.[5] Whether the observed differences are associated with genetic variation and degrees of polymorphism within the populations is not entirely understood. Although our knowledge of the genetic structure of populations of *Cx. pipiens* complex in various regions is relatively limited, host-vector interactions and feeding behavior of *Cx. pipiens* form pipiens in some localities in the US have been examined. Studies in NY and CT have shown a principally ornithophilic blood-feeding behavior with little inclination for mammalian hosts.[6–8] Emerging evidence, however, indicates that populations of *Cx. pipiens* form pipiens acquire relatively greater portions of blood meals from mammalian hosts in other regions including New Jersey,[7] Delaware,[38] Maryland and Washington DC,[39] Tennessee,[40] and Illinois,[41] There have been attempts to examine the genetic structure of some regional populations and explain variations in host-feeding behavior. Such genetic examinations have suggested that hybridizations between mainly ornithophilic *Cx. pipiens* form pipiens and mammalophilic *Cx. pipiens* form molestus,[13,42] and in some regions between *Cx. pipiens* and its southern counterpart *Cx. quinquefasciatus* within the 36°N and 39°N latitude introgression zone in the United States,[42] may be pivotal factors in determining host preferences. It is not clear whether differences in host-feeding behavior of *Cx. pipiens* form pipiens in northeast and other regions in the United States is solely the result of relative variations in genetic structure, host availability, or a combination of factors.

Results of this study showed that there was no population structuring among aboveground *Cx. pipiens* form pipiens populations collected from urban or rural locales in CT. Although rural populations had lower hybrid ancestry than urban populations in the analysis based on seven markers, hybrid ancestry estimates were not consistent when varying numbers of markers were used, and the ancestry differences did not lead to population structuring. Detailed studies are in progress to examine the host-feeding patterns of rural and urban populations of *Cx. pipiens* form pipiens in CT and to determine possible associations with population differentiation.

The overall genetic differentiation in populations of *Cx. pipiens* form pipiens examined at twelve localities in CT, NJ, NY, and MA was not significant. Although, several significant pairwise genetic distances based on analysis of F_{ST}/R_{ST} and PCA were detected when either NJ or MA populations (These two populations are located outermost north and south, respectively, in collection range.) were included in the analysis. Test of isolation by distance suggested the observed genetic variations were indeed distance associated. Because the number of localities analyzed in the present study represents a relatively small portion of the distribution range, *Cx. pipiens* form pipiens populations are likely to exhibit an even greater degree of heterogeneity and may not exist as a panmictic unit along the south-north axis. Microsatellite analysis of *Cx. pipiens* form pipiens collected from the east and west coasts of continental US indicated a largely unrestricted gene flow among populations.[16] However, in that analysis a limited number of mosquitoes from a colony population from the west coast was included and results may not reflect an entirely clear overview of the population structure.

Temporal changes in genetic structure and hybrid ancestry of *Cx. pipiens* form pipiens populations have not been studied

in detail. The relationship between these changes and behavioral adaptations such as shifts in host feedings from birds to mammals, is poorly understood. Brief episodes of feeding shifts by heterozygote forms of *Cx. pipiens* in Boston, Massachusetts during periods of interbreeding between anautogenous males and autogenous females in September and December have been documented.[10–12] Recently, a shift in feeding preference of *Cx. pipiens* form pipiens from birds to humans during late summer and early fall has been reported in Maryland and Washington DC.[39] However, in a subsequent examination of this population, no temporal changes in hybrid ancestry were detected.[42] In contrast, temporal analysis of the feeding patterns of this mosquito species in Memphis and surrounding areas of Shelby County, Tennessee did not support a shift in feeding behavior away from avian (mostly American robin) to mammalian hosts late in the summer, but rather, a significant degree of temporal variation was noticed in the proportion of robin-derived blood meals throughout the summer.[40] Similarly, blood meal analysis of *Cx. pipiens* form pipiens populations in CT revealed a seasonal shift from American robins to other avian species, but not mammalian hosts.[8] Analyses of microsatellite results consistently showed no seasonal genetic variation and hybrid ancestry change in the *Cx. pipiens* populations in CT during the present study. Because of the aforementioned contrasting findings and the lack of established assumption that hybrid mosquitoes feed on mammals more readily than they do on avian hosts, caution should be exercised in using hybrid ancestry as a genetic basis to interpret the differences in host-feeding patterns of *Cx. pipiens* populations.

Two major hypotheses have been proposed on the origin of *Cx. pipiens* form molestus populations. 1) On the basis of microsatellite analyses, *Cx. pipiens* form pipiens and *Cx. pipiens* form molestus are genetically distinct forms and underground populations in northern Europe were introduced from southern Europe or north Africa[13]; and 2) based on allozyme analysis, these underground, autogenous populations were most likely derived from local aboveground populations of *Cx. pipiens* form pipiens as the result of a single colonization event.[43] Our genetic analyses of F_{ST} and R_{ST}, Bayesian clustering and PCA support the first proposition that *Cx. pipiens* form molestus is genetically distinct from *Cx. pipiens* form pipiens. This was most likely the result of greatly reduced allelic diversity and fixation at a single allele in four loci in *Cx. pipiens* form molestus population. Earlier study reports that northern Europe underground populations have all the major alleles also found in African and Middle Eastern populations, but not in northern Europe aboveground populations.[13] In the present study *Cx. pipiens* form molestus population from NYC did not contain unique alleles, but a subset of alleles also found in *Cx. pipiens* form pipiens populations. Therefore, the distinction of *Cx. pipiens* form molestus from *Cx. pipiens* form pipiens does not necessarily exclude the possibility that US *Cx. pipiens* form molestus populations were derived from local aboveground *Cx. pipiens* form pipiens populations. It is noteworthy that genotyping of a *Cx. pipiens* form pipiens colony maintained in our laboratory revealed a similar pattern of reduced allelic diversity and locus fixation; it appeared this population was as distinct as *Cx. pipiens* form molestus (microsatellite data not shown).

The occurrence of any possible hybridization between stenogamous and epigeous *Cx. pipiens* form pipiens and eurygamous and hypogeous *Cx. pipiens* form molestus is expected to be restricted to localities where the two populations exist in close proximity. Populations of *Cx. pipiens* form molestus in the United States, to the best of our knowledge, almost exclusively occur in the subterranean habitats located in urban areas, and consequently higher degrees of hybridization events are expected in such settings. In the analysis of rural populations of *Cx. pipiens* form pipiens from CT based on seven markers, a relatively lower hybrid ancestry was identified in comparison to urban populations. However, the hybrid ancestry estimates were not always consistent when varying numbers of markers were used. Analysis of *Cx. pipiens* form pipiens populations collected from urban localities in New Jersey, New York, and Massachusetts, where *Cx. pipiens* form molestus populations are known to be present, did not reveal that these populations contain more hybrids than other *Cx. pipiens* form pipiens populations found elsewhere in this study. As a plausible explanation either hybrid alleles were rapidly dispersed throughout the *Cx. pipiens* form pipiens populations, or *Cx. pipiens* form molestus were derived from the aboveground populations as indicated earlier and the hybrid signature is actually a preexisting genetic composition in *Cx. pipiens* form pipiens populations.

It has been reported that US *Cx. pipiens* form pipiens populations contain more than 40% hybrids with ancestry from European *Cx. molestus* based on eight microsatellite markers,[13] but only 11.9% hybrids with ancestry from US *Cx. pipiens* form molestus populations was identified in the present study by using seven microsatellite markers. The differences in hybrid percentages may be because of the number and combination of the markers and populations of *Cx. pipiens* form molestus used in the two studies. *Culex pipiens* form molestus population in the US is not identical to either southern Europe or north African *Cx. pipiens* form molestus populations. Loci CxpGT9 and CxpGT46 are very different in allele frequencies and the distributions of major alleles. Furthermore, several alleles in these two loci are not shared between US *Cx. pipiens* form molestus and the other two populations.

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