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Genetic Variation Associated with Mammalian Feeding in *Culex pipiens* from a West Nile Virus Epidemic Region in Chicago, Illinois

Shaoming Huang,[1] Gabriel L. Hamer,[2] Goudarz Molaei,[1] Edward D. Walker,[3]
Tony L. Goldberg,[4] Uriel D. Kitron,[5] and Theodore G. Andreadis[1]

Abstract

Mosquitoes of the *Culex pipiens* complex are important vectors of West Nile virus in the United States. We examined the genetic variations of *Cx. pipiens* mosquitoes from Chicago, Illinois that were determined to be principally ornithophilic but exhibited a relatively higher inclination for mammalian hosts including humans. Microsatellite analysis of 10 polymorphic markers was performed on 346 engorged *Cx. pipiens* specimens with identified avian or mammalian blood meals. Our results indicated that there were no significant differences in allelic richness, the pattern of conformity to Hardy-Weinberg equilibrium, and linkage disequilibrium, nor was there overall genetic differentiation between specimens with avian- and mammalian-derived blood meals. However, *Cx. pipiens* form pipiens with mammalian- (including human-) derived blood meals had significantly higher ancestry ($p < 0.001$) and proportion of hybrids ($p < 0.01$) from the *Cx. pipiens* form molestus (population from New York City) than did those with avian-derived blood meals. By contrast, there were no significant differences in the ancestry ($p > 0.05$) and the proportion of hybrids ($p > 0.05$) from *Cx. quinquefasciatus* (population from Harris Country, Texas). No temporal genetic variation was detected in accordance with the observation that there was no shift in blood feeding from birds to mammals. The results of this study in conjunction with regional host-feeding behavior suggest that the probability of genetic ancestry from *Cx. pipiens* f. molestus may predispose mosquitoes to feed more readily on mammals; however, the genetic mechanisms are unknown.

Key Words: Microsatellite analysis; Hybrid ancestry; Host-feeding behavior; *Cx. pipiens* form pipiens; *Cx. pipiens* form molestus

Introduction

West Nile virus (WNV) continues to be the major mosquito-borne arbovirus in the United States since its discovery in New York City in 1999 (Lanciotti et al. 1999). The Chicago suburban area (Cook County, IL) has experienced high levels of virus activity with accompanying human cases, ranking Illinois the first and second in the United States in 2002 and 2005, respectively (Illinois Department of Public Health: http://www.idph.state.il.us/envhealth/wnv.htm). The mosquito *Cx. pipiens* (L.) has been implicated as the principal enzootic and epidemic vector in this region based upon incriminating evidence including: local abundance (Lampman et al. 2006), high prevalence of infection, diverse feeding on avian and mammalian hosts including humans (Hamer et al. 2008a), and vector competence (Sardelis et al. 2001, Turell et al. 2001, 2005).

Host-feeding pattern studies indicate that populations of *Cx. pipiens* from the northeastern United States are principally ornithophilic (Apperson et al. 2002, Molaei et al. 2006), whereas populations from the southeast (Apperson et al. 2004, Savage et al. 2007), mid-Atlantic (Kilpatrick et al. 2006), and upper midwest (Hamer et al. 2008a) are more mammalophilic in nature. The underlying bases for these apparent variations are poorly understood. One hypothesis suggests that varying degrees of introgression between aboveground *Cx. pipiens* form pipiens and the underground *Cx. pipiens* form molestus, which is reported to be highly

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[1] The Connecticut Agricultural Experiment Station, New Haven, Connecticut.
[2] Department of Fisheries and Wildlife, Michigan State University, East Lansing, Michigan.
[3] Department of Microbiology and Molecular Genetics, Michigan State University, East Lansing, Michigan.
[4] Department of Pathobiological Sciences, University of Wisconsin, Madison, Wisconsin.
[5] Department of Environmental Studies, Emory University, Atlanta, Georgia.

mammalophilic and aggressive human biter (Harbach et al. 1984), is an important contributing factor (Spielman 2001, Fonseca et al. 2004). Indeed, microsatellite analysis has demonstrated that the U.S. *Cx. pipiens* populations contain a relatively large number of hybrids with signatures of European forms of pipiens and molestus (Fonseca et al. 2004). However, in an examination of populations from the northeastern United States, the number of hybrids was shown to be considerably lower when a U.S. population of *Cx. pipiens* f. molestus from New York City was used to identify hybrids (Huang et al. 2008).

Microsatellite genotyping and blood meal analyses of engorged mosquitoes from Washington DC and Maryland, suggest that the probability of ancestry from *Cx. pipiens* f. molestus may influence the probability of feeding of *Cx. pipiens* on humans (Kilpatrick et al. 2007). Such studies on the genetic predisposition and its relevance to the hostfeeding behavior of *Cx. pipiens* from the metropolitan Chicago area have not been conducted. The current research initiative was undertaken to examine genetic variations among a relatively large number of blood-fed mosquitoes acquiring blood meals from avian and/or mammalian hosts in order to examine the potential influence of introgressions in the host-feeding behavior of *Cx. pipiens* in this region for the first time.

Materials and Methods

Details on mosquito collection at study sites in metropolitan Chicago and blood-meal identification are described elsewhere (Hamer et al. 2008b). The sample included 346 *Cx. pipiens* f. pipiens mosquitoes, identified to species by polymerase chain reaction (Hamer et al. 2008b) including 254 that had fed on birds and 92 on mammals. Of these, 91 specimens with avian- and 55 with mammal-derived blood meals were collected in 2005 (Hamer et al. 2008a) and the remainder in 2006 (Hamer et al. 2009). Specimens with human-derived blood accounted for 85.9% of the mosquitoes that had fed on mammals. To examine introgressions in the *Cx. pipiens* population in Chicago, an underground population of *Cx. pipiens* f. molestus from New York City (48 specimens) (Huang et al. 2008) and a population of *Cx. quinquefasciatus* Say from Harris County, Texas (48 specimens) (Molaei et al. 2007) were included in the analysis for comparison.

Mosquitoes were genotyped at 10 previously characterized, polymorphic microsatellite loci (Keyghobadi et al. 2004, Smith et al. 2005), including CxpGT9 F2/R, CxpGT12 F2/R2, CxpGT4 F/R, CxpGT40 F/R, CxpGT46 F/R, CxpGT51 F/R, CQ11 F2/R3, CxqGT4 F3/R, CxqGT6b F/R, and CxqTri4 F/R. Microsatellite loci were amplified and resultant allele sizes determined as described previously (Huang et al. 2008).

Allelic richness was estimated per locus per sample and adjusted to account for uneven sample size by a rarefaction procedure implemented in FSTAT 2.9.3.2 (Goudet 2001). Evaluations of Hardy-Weinberg equilibrium, linkage disequilibrium and fixation indices, F_{ST} and R_{ST}, were carried out as described in an earlier study (Huang et al. 2008). Genetic introgressions were analyzed by Bayesian clustering implemented in the software STRUCTURE 2.2 (Pritchard et al. 2000) with the models of admixture and independent allele frequency. The analyses were performed with 100,000 "burn-in" steps followed by 1,000,000 Markov chain Monte Carlo repetitions. Because populations of *Cx. pipiens* f. molestus and *Cx. quinquefasciatus* were genetically distinct from each other and from *Cx. pipiens* f. pipiens here and elsewhere (Fonseca et al. 2004, Kent et al. 2007, Huang et al. 2008), the cluster value was designated as $K = 3$ rather than determined by averaging the log $\Pr(X \mid K)$ (the probability of individual X belong to cluster K) across runs (Pritchard et al. 2000). The analysis was conducted 10 times to ensure consistency. Program Distruct (Rosenberg 2004) was used to graphically portray individual cluster coefficients generated by STRUCTURE 2.2. Individuals with membership coefficient (the fraction of an individual's genome with ancestry in the cluster) equal to or greater than 0.05 from more than one cluster were considered hybrids.

TABLE 1. COMPARISON OF GENETIC DIVERSITY AT THE 10 MICROSATELLITE LOCI BETWEEN *CULEX PIPIENS* MOSQUITOES THAT FED ON BIRDS AND HUMANS

	Cx. pipiens *fed on birds (2N = 508)*				Cx. pipiens *fed on mammals (2N = 184)*			
Locus	*Allele no.*	H_E	H_O	F_{IS}	*Allele No.*	H_E	H_O	F_{IS}
CxqTri4 F/R	5 (4.9)	0.57	0.53	0.06	5 (5.0)	0.54	0.55	−0.02
CxqGT6b F/R	7 (5.3)	0.71	0.67	0.05	6 (5.0)	0.70	0.62	0.10
CxqGT4 F3/R	8 (5.9)	0.25	**0.17**	0.31	5 (4.0)	0.24	**0.21**	0.14
CQ11 F2/R3	13 (10.5)	0.73	**0.25**	0.63	10 (10.0)	0.75	**0.30**	0.57
CxpGT12 F2/R2	9 (8.6)	0.69	**0.58**	0.14	9 (10.0)	0.65	**0.47**	0.28
CxpGT4 F/R	9 (10.7)	0.74	0.69	0.08	8 (12.9)	0.73	0.67	0.08
CxpGT9 F2/R	15 (15.6)	0.85	**0.81**	0.05	12 (13.0)	0.89	**0.77**	0.11
CxpGT40 F/R	24 (24)	0.72	**0.60**	0.16	18 (23.6)	0.777	**0.67**	0.12
CxpGT46 F/R	20 (20.7)	0.88	**0.71**	0.09	17 (17.9)	0.87	**0.61**	0.26
CxpGT51 F/R	28 (29.2)	0.89	0.89	−0.02	22 (24.0)	0.89	0.90	−0.02
Average	13.8 (13.5)	0.70	0.59	0.16	11.2 (12.5)	0.70	0.58	0.16
S. E.	2.5 (2.7)	0.05	0.07	0.06	1.9 (2.3)	0.06	0.06	0.05

Numbers in bold indicate significant heterozygote deficiency after sequential Bonferroni correction ($p < 0.05$). Allele numbers in parentheses represent those adjusted to account for uneven sample size according to the rarefaction method. N, numbers of specimen genotyped; H_E, expected heterozygosity; H_O, observed heterozygosity; F_{IS}, inbreeding coefficient; S. E., standard error.

Table 2. Genetic Distances at 10 Microsatellite Loci

	Cx. quinquefasciatus	Cx. pipiens *f. pipiens fed on birds*	Cx. pipiens *f. pipiens fed on mammals*	Cx. pipiens *f. molestus*
Cx. quinquefasciatus		**0.2726**	**0.2874**	**0.4940**
Cx. pipiens f. pipiens fed on birds	**0.3453**		0.0012	**0.1897**
Cx. pipiens f. pipiens fed on mammals	**0.3522**	0.0001		**0.1843**
Cx. pipiens f. molestus	**0.4970**	**0.1951**	**0.1797**	

Numbers above the diagonal are F_{ST} values and those below the diagonal are R_{ST} values.
Numbers in bold are significant ($p < 0.001$) after sequential Bonferroni correction.

Results

Microsatellite analyses of 346 *Cx. pipiens* using 10 polymorphic markers revealed that there were no significant differences in allelic richness between *Cx. pipiens* with avian- and mammalian-derived blood meals both before and after adjustment for uneven sample size (Table 1). Deviations from Hardy-Weinberg equilibrium were observed in six markers (Table 1); however, the patterns of conformity were not different between *Cx. pipiens* with avian- and mammalian-derived blood meals. No significant tests for linkage disequilibrium were observed in all the locus pairs after sequential Bonferroni correction. Pairwise F_{ST} and R_{ST} values indicated that there was no overall genetic differentiation between the *Cx. pipiens* f. pipiens that had fed on birds and mammals (Table 2).

Results of Bayesian clustering indicated that *Cx. pipiens* f. pipiens, *Cx. pipiens* f. molestus, and *Cx. quinquefasciatus* were

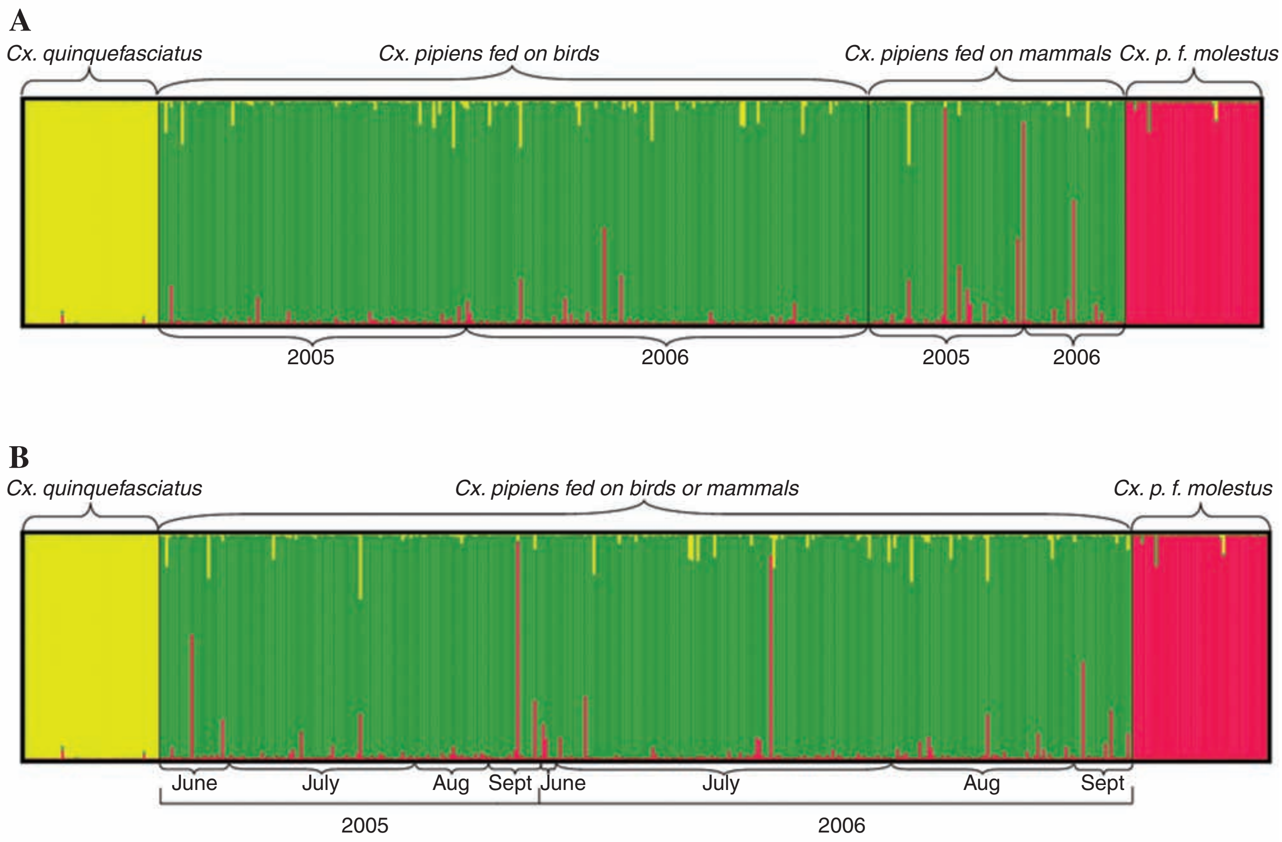

FIG. 1. Bayesian clustering analysis of *Culex pipiens* f. pipiens collected from Chicago suburban area. The yellow, green, and red colors represent *Cx. quinquefasciatus*, *Cx. pipiens* f. pipiens, and *Cx. pipiens* f. molestus cluster, respectively. Each mosquito is represented by a thin vertical line partitioned into three colored segments corresponding to the individual's estimated ancestry in the three clusters. Mosquito populations and groups are separated by vertical black lines. (**A**) Genetic comparison between *Cx. pipiens* mosquitoes fed on avian and mammalian hosts. (**B**) Analysis of seasonal shift in genetic composition in engorged *Cx. pipiens* mosquitoes. *Culex pipiens* f. pipiens mosquitoes collected in each year are arranged in temporal order from June to October.

genetically distinct entities (Fig. 1A), as indicated by the F_{ST} and R_{ST} values as well (Table 2). Furthermore, a pattern of asymmetric gene flow was evident from *Cx. pipiens* f. molestus and *Cx. quinquefasciatus* populations to *Cx. pipiens* f. pipiens population (Fig. 1A). This apparent unidirectional introgression from *Cx. pipiens* f. molestus resulted in significant ancestry variation in the *Cx. pipiens* f. pipiens population. On average, *Culex pipiens* f. pipiens mosquitoes that had fed on mammals had significantly higher ancestry ($p < 0.001$), and there was a greater proportion of hybrids ($p < 0.01$) from *Cx. pipiens* f. molestus than those that fed on birds (Figs. 1A, 2). Three specimens of *Cx. pipiens* f. pipiens with mammalian-derived blood and none with avian-derived blood had molestus ancestry greater than 0.5, a value theoretically denoting half proportion of the genome is derived from the parental population.

No significant differences either in ancestry (Fig. 1A; average ancestry difference $= 0.003 \pm 0.002$, $p > 0.05$) or in proportion of hybrids (proportion test: proportion difference $= 0.06 \pm 0.03$, $p > 0.05$) from *Cx. quinquefasciatus* were observed between mosquitoes that fed on birds and mammals. Additionally, the Bayesian clustering analysis showed no seasonal changes in proportion of *Cx. pipiens* f. molestus and *Cx. quinquefasciatus* ancestries nor in hybrid percentages (Fig. 1B).

Discussion

The present microsatellite analysis of genetic variation in a relatively large sample of blood fed *Cx. pipiens*, the principal vector of WNV in metropolitan Chicago (Hamer et al. 2008a, 2008b, 2009), suggests the importance of population substructure to bird or mammal host selection. Specifically, our results document a significant association between molestus hybrid ancestry in individuals and the presence of mammalian blood, reinforcing an earlier proposition that host selection by *Cx. pipiens* mosquitoes is influenced by genetic predisposition (Kilpatrick et al. 2007).

Considerable geographic variation in host selection patterns of *Cx. pipiens* populations from various regions in the United States has been reported (Apperson et al. 2002, 2004, Molaei et al. 2006, Savage et al. 2007, Hamer et al. 2008a). In the northeastern United States, most (>94%) blood meals were from birds (Apperson et al. 2002, Molaei et al. 2006). However, recent studies in more southerly and midwestern regions showed that a substantial number of individuals had fed on mammals, including humans: Washington DC and Maryland (13%) (Kilpatrick et al. 2006), Chicago (22.4%) (Hamer et al. 2008a), Tennessee (24%) (Apperson et al. 2004), and New Jersey (38%) (Apperson et al. 2004). Although no significant differences in genetic structure and degrees of hybridization were found among populations predominantly feeding on birds in the northeast (Huang et al. 2008), higher fractions of molestus ancestry were detected in the populations with a substantially greater percentage of mammalian-derived blood meals, particularly human, from Washington DC and Maryland (Kilpatrick et al. 2007). In our study, *Cx. pipiens* specimens with mammalian-derived blood meals had a significantly higher proportion of molestus ancestry, suggesting an underlying genetic basis for mammalian versus avian host selection. Although the genetic mechanism is unknown, it would be of value to determine if it is a selectable phenotype, albeit likely a complex one.

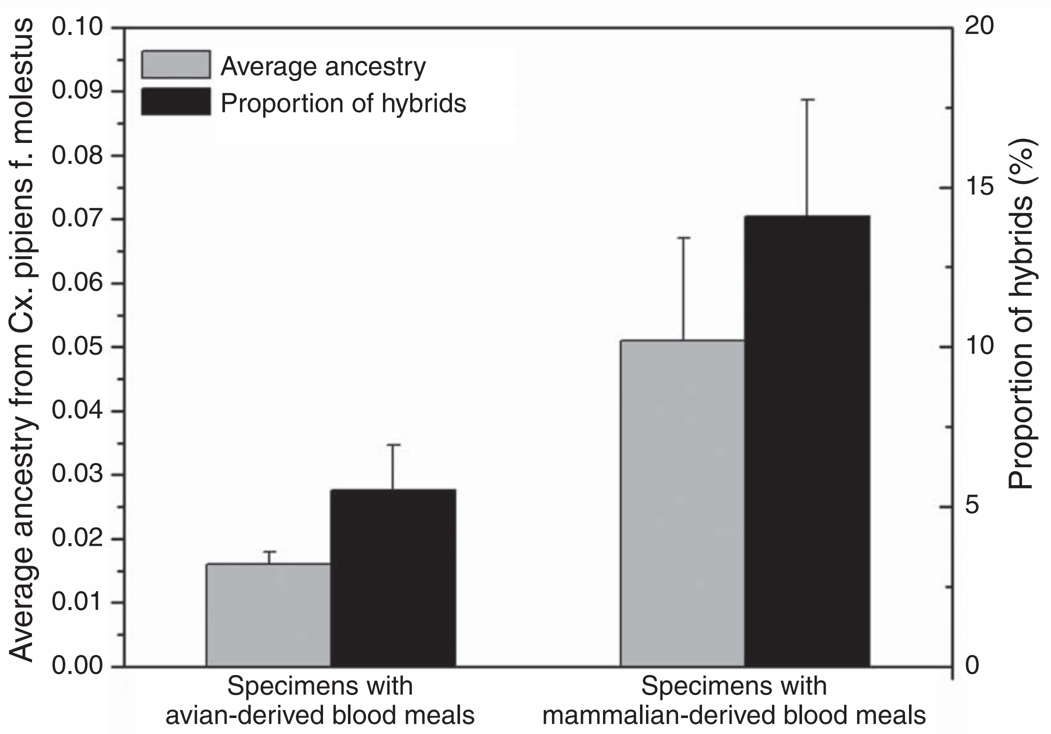

FIG. 2. Analysis of ancestry and hybrid proportion in engorged *Culex pipiens* f. pipiens mosquitoes with avian- and mammalian-derived blood from *Cx. pipiens* f. molestus. Average ancestry and hybrid proportion were plotted on the Y-axes to the left and right, respectively.

Because *Cx. pipiens* has been indicted as the sole (i.e., enzootic, epizootic, and bridge) vector of West Nile virus in the Chicago region (Hamer et al. 2008a), an analysis of the mechanisms influencing feeding on birds (virus amplifying hosts) and mammals (especially, humans) is warranted. Bridging transmission by this species would require flexibility in the phenotype, such that an earlier feeding on a viremic bird was followed by a later feeding on a human. Field evidence for a virus-infected *Cx. pipiens* feeding on a human has recently been published (Hamer et al. 2008a). Our data suggest that such a link is not accompanied by a seasonal shift to *Cx. pipiens* with marked molestus ancestry (Fig. 1B). Earlier reports on the blood-feeding behavior of several mosquito species indicate a temporal shift from avian to mammalian hosts, or from a number of avian species to different birds (Tempelis et al. 1965, 1967, Edman and Taylor 1968). A recent study by Kilpatrick et al. (2007) also made note of genetic determinants for a seasonal change in host-feeding of *Cx. pipiens* from avian to mammalian hosts (particularly humans) as determined by microsatellite analysis of engorged females collected from Washington DC and Maryland. However, the genetic composition showed no correlated seasonal trend. A seasonal shift from mostly American robin, a major host species to other birds, has also been reported in Connecticut (Molaei et al. 2006), but analyses of unengorged populations from the region showed no temporal population differentiation nor changes in molestus ancestry or hybrid percentages (Huang et al. 2008). *Cx. pipiens* from Chicago was shown to exhibit a similar pattern of decline in feeding on American robin followed by an increase in feeding on other bird species, particularly house sparrow, though no seasonal shift from bird-to-mammal feeding was noticed (Hamer et al. 2009). Our genetic analyses on the same populations did not reveal temporal changes in ancestries or hybrid percentages from *Cx. pipiens* f. molestus and *Cx. quinquefasciatus*. Host availability and abundance could potentially play a significant role in seasonal variations in host-feeding behavior, but further investigations are required to more precisely evaluate the contributions of these as well as other (e.g., genetic) factors.

Attempts to associate the pronounced mammalian blood-feeding behavior of *Cx. pipiens* with genetic composition are based on the occurrence of possible hybridization between the ornithophilic *Cx. pipiens* f. pipiens and the mammalophilic *Cx. pipiens* f. molestus. The latter form has been shown to be an aggressive human biter in Europe (Harbach et al. 1984). However, the feeding preference and geographic distribution of molestus populations in the United States have not been thoroughly investigated. The feeding behavior of hybrid populations has only been studied in a mixed population containing both forms in Boston, Massachusetts (Spielman 1964, 2001), where 6 of 353 mosquitoes (1.7%) were reportedly heterozygotes for autogeny (Spielman 1964), consistent with low degrees of hybridization demonstrated in other *Cx. pipiens* populations in northeastern United States (Huang et al. 2008). However, 3 of 9 and 6 of 13 human-biting mosquitoes were identified as heterozygotes in the aforementioned studies (Spielman 1964, 2001). Our results further indicate a positive correlation between genetic ancestry and mammalian blood-feeding behavior in *Cx. pipiens*. Collectively, the limited extent of hybridization between *Cx. pipiens* f. pipiens and *Cx. pipiens* f. molestus can be considered a contributing genetic factor that may influence host-feeding behavior.

We did not identify an association between *Cx. quinquefasciatus* ancestry and mammalian blood-feeding by *Cx. pipiens* f. pipiens. This was most likely due to the locations of our collection sites (41° 42′ N) that were well beyond the recognized sympatric zone (36° N and 39° N latitude), where *Cx. pipiens* f. pipiens and *Cx. quinquefasciatus* extensively hybridize (Barr 1957, Urbanelli et al. 1997). A recent study on mosquitoes collected from Champaign, Illinois (40° 05′ N), which is approximately 160 km south of our sampling sites, detected 7.5% hybrids by DV/D ratio (that is the relative positions of the dorsal [D] and ventral [V] arms of male phallosome), suggesting that the hybridization zone may be wider than previously thought (Sanogo et al. 2008). Our microsatellite analysis detected approximately 5.5% hybrids, which may represent a gradient penetration of *Cx. quinquefasciatus* alleles into the northern edge along the north-south axis. Blood-meal analysis of *Cx. pipiens* complex mosquitoes from Memphis, Tennessee did not reveal greater mammalian blood-feeding among hybrids of *Cx. pipiens* and *Cx. quinquefasciatus* (Savage et al. 2007), and the impact of hybridization was not assessed.

In conclusion, our microsatellite analyses indicate the presence of heterogeneity in the *Cx. pipiens* population. Our results also suggest that the probability of genetic ancestry from *Cx. pipiens* f. molestus may predispose mosquitoes to feed more readily on mammals, although the genetic mechanisms are not known. The impact of extensive gene flow on the population structure of this species and its implications for blood-feeding behavior and vectorial capacity merit further investigation. These studies will help clarify the role that *Cx. pipiens* plays in both enzootic and epidemic transmission of arboviruses including WNV.

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Address reprint requests to:
Dr. Theodore G. Andreadis
The Connecticut Agricultural Experiment Station
123 Huntington Street
PO Box 1106
New Haven, CT 06504

E-mail: theodore.andreadis@ct.gov