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Genetic Differences Between *Culex pipiens* f. molestus and *Culex pipiens pipiens* (Diptera: Culicidae) in New York

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Abstract

The definition and phylogenetic placement of the autogenous molestus form of *Culex pipiens* has puzzled entomologists for decades. We identified genetic differences between *Cx. p. pipiens* (L.) and *Cx. pipiens* f. molestus Forskål in the SH60 fragment described previously. Single-strand conformation polymorphism analysis, cloning, and sequencing of this fragment demonstrated high polymorphism within and among individual *Cx. p. pipiens*, with common SH60 variants shared among individuals from distant locations. In contrast, *Cx. pipiens* f. molestus from New York City each contained a single SH60 variant, which was not identified in any other *Cx. p. pipiens* specimens analyzed. Supporting microsatellite analysis demonstrated significant but reduced gene flow between *Cx. p. pipiens* and *Cx. pipiens* f. molestus in New York relative to *Cx. p. pipiens* populations in New York and California. Results are discussed in the context of two contrasting hypotheses regarding the origin of *Cx. pipiens* f. molestus populations.

Keywords

Culex pipiens pipiens; *Culex pipiens* f. molestus; genetics; microsatellites; West Nile virus

Culex pipiens f. molestus Forskål is a morphologically identical ecological biotype of *Culex pipiens pipiens* (L.) defined by a host of behavioral and physiological characteristics. In contrast to *Cx. p. pipiens, Culex pipiens* f. molestus has the ability to produce eggs without a vertebrate bloodmeal (autogeny); can mate in confined spaces (stenogamy); foregoes winter diapause; occupies subterranean environments with limited surface access; and feeds readily on mammals, including humans (Mattingly 1952, Vinogradova 2000). The potential public health importance of the mammal-feeding *molestus* biotype of *Cx. p. pipiens* as a human disease vector has led to a search for markers to readily identify these two forms.

Despite the numerous biological differences that distinguish *Cx. pipiens* f. molestus from *Cx. p. pipiens*, reliable identification of *Cx. pipiens* f. molestus by using morphology and/or molecular tools and phylogenetic placement of this form within the *Cx. pipiens* complex

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remains unresolved. Morphological and biochemical studies using larval chaetotaxy, variation in length of dorsal and ventral arms of the phallus in adult males (DV/D ratios), and chromatography have all failed to reliably separate these two forms (Jobling 1938, Mattingly 1952, Micks 1954, Harbach et al. 1984). Micks and Scrollini (1954) identified biochemical differences among *Cx. p. pipiens, Cx. p. quinquefasciatus* Say, and *Cx. pipiens* f. molestus by using infrared spectrometry. Recently Fonseca et al. (2004) reported unique microsatellite signatures among worldwide populations of *Cx. p. pipiens* and *Cx. pipiens* f. molestus. This report was followed by Bahnck and Fonseca (2006) who developed a rapid molecular assay to differentiate between *Cx. p. pipiens, Cx. pipiens* f. molestus, and putative hybrid populations based on sequence differences in the genomic regions flanking the CQ11 microsatellite locus. These advances provide evidence of a molecular and genetic basis for the observable phenotypes that distinguish *Cx. p. pipiens* and *Cx. pipiens* f. molestus, but they also demonstrate that progress in identifying genetic differences between them has been challenging despite decades of work. Identification of additional markers and a more thorough characterization of genetic differences between *Cx. p. pipiens* and *Cx. pipiens* f. molestus are needed as well as further evidence supporting the evolutionary origin of *Cx. pipiens* f. molestus.

Two hypotheses predominate regarding the origin of *Cx. pipiens* f. molestus populations. Byrne and Nichols (1999) concluded with allozyme analysis that underground, autogenous *Cx. pipiens* f. molestus in London were most likely founded from a single colonization event from local aboveground *Cx. p. pipiens* populations. This hypothesis was also proposed by Dobrotworsky (1967), who through comparative studies of DV/D ratios of *Cx. p. pipiens, Cx. pipiens* f. molestus, and *Cx. p. fatigans* Weidemann in the South Pacific postulated that *Cx. pipiens* f. molestus originated from *Cx. p. pipiens*. Alternatively, microsatellite data generated by Fonseca et al. (2004) supports a hypothesis that *Cx. p. pipiens* and *Cx. pipiens* f. molestus are genetically distinct forms and that northern European underground populations were founded instead by a southern autogenous, mammal-feeding form that moved northward and colonized underground habitats. This conclusion was reached because the genetic signature of the underground, autogenous specimens from London and northern Europe was more similar to African and Middle Eastern autogenous populations than with sympatric, aboveground *Cx. p. pipiens*. It was further postulated that North American *Cx. p. pipiens* had a hybrid ancestry between Old World *Cx. p. pipiens* and *Cx. pipiens* f. molestus (Fonseca et al. 2004).

Our aims were therefore to identify genetic differences between *Cx. p. pipiens* and *Cx. pipiens* f. molestus in New York and to address the question of whether *Cx. pipiens* f. molestus populations in New York City were derived from sympatric, aboveground *Cx. p. pipiens* or existed as a separate or hybrid genetic entities. We applied polymerase chain reaction (PCR); single-strand conformation polymorphism (SSCP) analysis; restriction fragment length polymorphism analysis; and sequencing to mitochondrial, ribosomal, and genomic DNA markers in colony and field-collected *Cx. p. pipiens* and *Cx. pipiens* f. molestus from New York. We also analyzed 12 microsatellite loci in field-collected specimens of *Cx. p. pipiens, Cx. pipiens* f. molestus, and *Cx. p. quinquefasciatus* to compare gene flow between *Cx. pipiens* complex mosquitoes from New York and California. The

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collective results presented provide a comprehensive picture of the genetic makeup of New York *Cx. p. pipiens* and *Cx. pipiens* f. molestus through the integration of novel and existing genetic markers. Results are discussed in the context of both existing hypotheses regarding the origin of *Cx. pipiens* f. molestus populations.

Materials and Methods

Sample Sources and DNA Isolation

Colony and field-collected *Cx. p. pipiens, Cx. pipiens* f. molestus, and *Cx. p. quinquefasciatus* were obtained from sources listed in Table 1. *Cx. pipiens* f. molestus from New York City (NYC) were defined at the time of collection by subterranean habitat, autogenous reproduction, stenogamy, and mammalian blood-feeding behavior. *Cx. p. pipiens* from NYC and Syracuse were collected aboveground by resting collections and larval collections from storm drains and containers. Specimens from Baltimore, Co., MD, were aspirated in mid-March from a known overwintering site for this species. Specimens from Pennsylvania also were collected aboveground as part of mosquito surveillance and control programs. All *Cx. p. pipiens* and *Cx. pipiens* f. molestus samples listed in Table 1 were evaluated by SSCP, described below. Microsatellite analysis was performed only on field-collected samples of *Cx. p. pipiens*, and *Cx. pipiens* f. molestus from NYC and Syracuse as well as Shasta, San Bernadino County, and Coachella Valley populations from California. Due to geographic proximity, *Cx. p. quinquefasciatus* populations from San Bernadino and Coachella Valley were treated as one population for the microsatellite analysis. DNA was extracted from whole mosquitoes by a modified salt procedure as described previously (Norris et al. 2001, Kent and Norris 2005).

Polymerase Chain Reaction

The relative quality of all DNA extractions was evaluated by PCR amplification of a fragment of the mitochondrial NADH dehy-drogenase subunit four (ND4) by using arthropod-specific primers (Simon et al. 1994, Kent and Norris 2005). Molecular identification for all samples was obtained using the Crabtree et al. (1995) PCR diagnostic to confirm placement of each specimen in the *Cx. pipiens* complex. The Crabtree et al. (1997), Aspen and Savage (2003), and Smith and Fonseca (2004) PCR diagnostics also were used to differentially identify *Cx. p. pipiens* and *Cx. p. quinquefasciatus* as well as potential *Cx. p. pipiens/quinquefasciatus* hybrids. The Bahnck and Fonseca (2006) diagnostic was used to characterize the genetic background of specimens originating from NYC or Syracuse.

To further analyze the SH60 fragment amplified by the Crabtree et al. (1997) PCR assay, alternative forward and reverse primers were designed manually from the original SH60 sequence (GenBank accession no. U90782). The F4 forward primer was shifted five bases to the right, and the R4 reverse primer was similarly shifted both to the right 20 bases and to the left five bases. The 5′ to 3′ sequence of the alternative, left-shifted reverse primer used successfully with the reported F4 forward primer (Crabtree et al. 1997) was ACTGCCCACCCACTCCATAG. DNA amplifications were completed on a MJ Research PTC-200 thermal cycler (Bio-Rad Laboratories, Hercules, CA) and visualized on ethidium

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bromide-stained 2% agarose gels. All gels were run with GeneRuler 100-bp molecular mass marker (MBI Fermentas, Hanover, MD).

SSCP Analysis

To identify nucleotide substitutions, insertions, and deletions within and between mosquito populations, *Cx. p. pipiens* and *Cx. pipiens* f. molestus SH60 PCR products derived from the Crabtree et al. (1997) PCR diagnostic were compared by SSCP following the methods of Hiss et al. (1994) with some modification. Four microliters of PCR product was mixed with 8 μl of denaturing loading mix (DLM stock solution: 0.005 g of xylene cyanol blue + 0.005 g of bromophenol blue + 9.0 ml of deionized formamide + 0.2 ml of 1 M NaOH + 0.8 ml of double distilled H_2O), heated at 95°C for 5 min, and then immediately plunged into ice. Samples were electrophoresed at 4°C at constant power on medium-format 5% native polyacrylamide gels and stained with SYBR Green (Cambrex Bio-Science Rockland Inc., Rockland, ME) for band visualization. Variation in banding patterns was assessed manually by comparing the relative mobility of both upper and lower bands among samples run on the same gel (Black and DuTeau 1997). To ensure accurate scoring, samples producing similar patterns on different plates were rerun on the same gel. After samples were condensed in this manner and unique banding patterns recognized, representative samples of each unique banding pattern were then selected and polymorphisms confirmed by sequencing. When trying to identify identical SH60 variants between *Cx. p. pipiens* and *Cx. pipiens* f. molestus, the same *Cx. pipiens* f. molestus SH60 samples were electrophoresed with each plate of *Cx. p. pipiens* samples as an internal standard.

Cloning and Sequencing

Individual *Cx. p. pipiens* contained multiple variants at the SH60 locus. Therefore, SH60 PCR products from 13 NYC *Cx. p. pipiens*, 25 Syracuse *Cx. p. pipiens*, three NYC *Cx. pipiens* f. molestus, one Cornell colony *Cx. pipiens* f. *molestus*, and one Cornell colony *Cx. p. pipiens* were purified with the QIAquick PCR Purification or Gel Extraction kits (QIAGEN, Valencia, CA) and cloned using chemically competent TOP10 One Shot *Escherichia coli* (TOPO TA Cloning kit with pCR 2.1-TOPO vector catalog K4500-01, Invitrogen, Carlsbad, CA) to evaluate differences between SH60 variants. Transformed *E. coli* were grown at 37°C overnight on LB agar plates containing 50 μg/ml carbenicillin, and transformed colonies were identified by blue-white selection. The SH60 insert was recovered from individual colonies by the Crabtree et al. (1997) PCR, and amplicons from 10 to 15 clones from each mosquito in a series of individual mosquitoes representing each taxon were evaluated for inter- and intra-individual variability by SSCP. Clones representative of unique SH60 mobility classes on SSCP were selected for sequencing. Sequencing was performed using the universal M13 forward and reverse primers to ensure sequence information for the entire fragment was obtained. Multiple sequence alignments were performed using Multalin (Corpet 1988). All SH60 sequences from four New York City *Cx. p. pipiens*, three New York City *Cx. pipiens* f. molestus, three Syracuse *Cx. p. pipiens*, one colony *Cx. p. pipiens* from Cornell University, and one colony *Cx. pipiens* f. molestus from Cornell University are available from GenBank (accession nos. AY923229–AY923239, EF015564–EF015568, and DQ421381–DQ421386) (Table 2).

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Microsatellites

To corroborate the SSCP results, 12 microsatellite loci were selected for analysis (Table 3). Primers designed by Fonseca et al. (1998) and Keyghobadi et al. (2004) had previously demonstrated to be polymorphic within the *Cx. pipiens* complex and among related species (Fonseca et al. 2000, 2004; Smith et al. 2005). An alternate reverse primer for locus CQ11 was used due to nulls and misamplification by the original primer set (Smith et al. 2005). The *Cx. tarsalis* Coquillett microsatellite loci used for this study represent a subset of loci that were experimentally determined to amplify from *Cx. pipiens* complex mosquitoes. All PCR reactions were performed in 20-μl reaction volumes containing 10 mM Tris, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% gelatin; 1.0 mM dNTPs; 1.0 U of *Taq* polymerase, and 25 pmol (0.25 μM) each of forward and reverse primer. An initial denaturation of 5 min at 96°C was followed by 30 cycles of 94°C for 40 s, 54°C for 45 s, and 72°C for 45 s. The final 72°C extension step was 45 min (Norris et al. 2001). The forward primer in each reaction was labeled with a fluorescent marker (FAM, TET, or HEX) compatible with ABI PRISM (PerkinElmer Life and Analytical Sciences, Boston, MA) electrophoresis. Single locus PCR products were mixed for multiplexed analysis (CxpGT4 + CxpGT46 + CQ26; CxpGT9 + CQ29 + CQ11; CUTA1+CUTD107+CUTD4; CUTD120+CUTB1+CUTD113). Multiplexed products were evaluated on an ABI Prism 3100-Avant Genetic Analyzer (Applied Biosystems, Foster City, CA), and data were analyzed using GeneScan and Geno-Typer Fragment Analysis software packages to derive microsatellite genotypes and allele sizes (Applied Biosystems). Arlequin version 1.1 (Schneider et al. 1997) was used to calculate allele frequencies, evaluate for compliance to Hardy-Weinberg equilibrium, and estimate FST and number of migrants per generation (Nm) values for NYC *Cx. p. pipiens*, NYC *Cx. pipiens* f. molestus, Syracuse *Cx. p. pipiens*, Shasta *Cx. p. pipiens*, and San Bernadino *Cx. p. quinquefasciatus* populations.

Results

PCR and SSCP Analysis of the SH60 Fragment

The identity of all specimens as *Cx. p. pipiens, Cx. p. quinquefasciatus*, or *Cx. p. pipiens/ quinquefasciatus* hybrids was confirmed by PCR. Molecular results corroborated with original species designations listed in Table 1, except for the *Cx. p. pipiens* colony from San Joaquin County, CA. This colony seemed to be of hybrid origin between *Cx. p. pipiens* and *Cx. p. quin-quefasciatus* according to specific primer annealing of the ACE2 locus (Aspen and Savage 2003, Smith and Fonseca 2004). We identified consistent differences in genetic diversity in the *Cx. p. pipiens*-specific SH60 fragment described by Crabtree et al. (1997) between *Cx. p. pipiens* and *Cx. pipiens* f. molestus populations in New York. Because this fragment was *Cx. p. pipiens*-specific, no amplification could be obtained from *Cx. p. quinquefasciatus* for comparison, even when forward and primers were shifted in either direction. The identity and genomic location of the SH60 fragment remains unknown; however, the serendipitous observation of differential SSCP banding patterns between *Cx. p. pipiens* and *Cx. pipiens* f. molestus prompted further investigation into SH60 diversity between biotypes and across geographic locations. Both colony and field-collected *Cx. p. pipiens* are highly polymorphic at this locus, whereas *Cx. pipiens* f. molestus have greatly reduced diversity (Fig. 1). SSCP analysis of additional *Cx. p. pipiens* populations listed in

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Table 1 revealed similar patterns of inter- and intra-individual diversity as illustrated in Figs. 1 and 2 (data not shown). The complex banding patterns observed in individual *Cx. p. pipiens* specimens were the result of multiple SH60 variants amplified simultaneously during PCR (Fig. 2A, B). Variants were identified through SSCP analysis of PCR products from genomic DNA and clones from the same individual mosquito elec-trophoresed side by side. When evaluating all *Cx. p. pipiens* and *Cx. pipiens* f. molestus populations sampled, some SH60 variants were shared among *Cx. p. pipiens* individuals from widely separated locations, for example Cpp24F (Syracuse, NY) and Cpp10P (New York City, NY) (Table 2).

To address the possibility that *Cx. pipiens* f. molestus contained additional SH60 variants that were not amplified due to mutations in the priming region, one additional forward and two reverse primers were manually selected by shifting the existing primers in either direction. Primer combinations consisting of a novel forward primer shifted inward five bases coupled with the either the original reverse or a novel reverse shifted to the right 20 bases, did not amplify the target sequence from either *Cx. p. pipiens* or *Cx. pipiens* f. molestus templates. The primer combination that used the existing F4 forward primer together with a novel reverse primer shifted to the left five bases did produce the expected product in both *Cx. p. pipiens* and *Cx. pipiens* f. molestus. Using the latter alternative primer set, still only one SH60 variant was amplified from *Cx. pipiens* f. molestus, whereas multiple variants were amplified from *Cx. p. pipiens*.

Finally, a multiple sequence alignment of SH60 clones from *Cx. p. pipiens* and *Cx. pipiens* f. molestus revealed that the observed diversity of SH60 banding patterns was generated by nucleotide polymorphism at a limited number of variable sites. Notably, all four *Cx. pipiens* f. molestus SH60 clones contained an adenine residue at position 187, whereas each of the 14 clones from *Cx. p. pipiens* contained cytosine (Table 2) at this position. Despite this finding, attempts to develop a reliable restriction fragment length polymorphism diagnostic to differentiate between *Cx. p. pipiens* and *Cx. pipiens* f. molestus based on this single mutation were unsuccessful. In addition, extreme sequence diversity was observed at the remaining variable positions among the *Cx. p. pipiens* SH60 variants (Table 2).

Microsatellites

Of the 12 microsatellite loci evaluated, only CUTB1, CUTD113, CxpGT4, CxpGT46, and CQ11 complied with Hardy-Weinberg equilibrium in at least three of five populations (Tables 3 and 4). These results could be due to small sample sizes in some populations or the existence of null alleles. Therefore, only these five loci were included in population analyses. Diversity indices for these loci are reported in Table 4 for New York *Cx. p. pipiens* and *Cx. pipiens* f. molestus. *Cx. pipiens* f. molestus in NYC were monomorphic at locus CQ11 for a 283 bp allele. Table 5 compares the FST values and corresponding estimated Nm among five populations of *Cx. p. pipiens, Cx. pipiens* f. molestus, and *Cx. p. quinquefasciatus*. Microsatellite CQ11 allele sizes, Bahnck and Fonseca (2006) PCR results and SH60 variant data for field-collected New York *Cx. p. pipiens* and *Cx. pipiens* f. molestus are integrated in Table 6.

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Discussion

Numerous attempts were made to identify genetic differences between *Cx. p. pipiens* and *Cx. pipiens* f. molestus. There were no consistent differences between these forms in the small subunit ribosomal 12S (EF028702, EF028703), CO1 (DQ072277, DQ072278, DQ072279), or ND4 (EF028084, EF030092). Additionally, screening of *Cx. p. pipiens* and *Cx. pipiens* f. molestus from New York with 10 different random amplification of polymorphic DNA-PCR primers (Sigma-Genosys, GEN1-10, 50% G+C) did not reveal any consistently unique amplicons for either form. However, the serendipitous analysis of the *Cx. p. pipiens*-specific SH60 fragment identified by Crabtree et al. (1997) resulted in some interesting discoveries.

Our analysis of the SH60 fragment can be interpreted in two ways. First, taking into account the diversity of the SH60 amplicon in *Cx. p. pipiens* populations versus *Cx. pipiens* f. molestus, these data may suggest that this population of *Cx. pipiens* f. molestus in NYC was locally founded from aboveground populations of *Cx. p. pipiens* through a geographically independent colonization event associated with a dramatic genetic bottleneck. This colonization event resulted in a genetically similar sympatric population but with greatly reduced diversity. Alternatively, these PCR, SH60, and microsatellite data may support the idea that these observed differences distinguish *Cx. pipiens* f. molestus from *Cx. p. pipiens* as genetic entities. Consistent genetic differences were identified between *Cx. p. pipiens* and *Cx. pipiens* f. molestus at the SH60 locus, and these results are easily interpreted in the context of the rapid assay published by Bahnck and Fonseca (2006). Supporting data for these two hypotheses are presented below.

We have demonstrated that New York *Cx. p. pipiens* populations as well as additional *Cx. p. pipiens* populations from around the United States, are highly polymorphic at the SH60 locus, whereas NYC *Cx. pipiens* f. molestus are genetically consistent. Two SH60 variants differing by a single nucleotide substitution at position 239 were identified in *Cx. pipiens* f. molestus from NYC, with individual mosquitoes each containing only a single variant. In contrast, individual *Cx. p. pipiens* invariably contained multiple SH60 variants, with the number of variants existing in each collection of mosquitoes too large to be completely catalogued in this study. For example, 18 NYC *Cx. p. pipiens* comprised 11 unique SSCP banding patterns. From cloning, each banding pattern represented a composite of between two and five different SH60 variants that were amplified simultaneously during PCR. The 30 *Cx. p. pipiens* specimens from Syracuse, NY contributed an additional 20 SSCP banding patterns unique from those observed in NYC *Cx. p. pipiens* (data not shown). Some of the SH60 variants were shared between individuals in these two locations (Table 2). Cloning, SSCP, and sequencing analysis of amplicons generated by alternative primers flanking the SH60 fragment confirmed that *Cx. pipiens* f. molestus individuals contained only a single SH60 variant, whereas *Cx. p. pipiens* individuals contained multiple variants. Thus, diversity in *Cx. pipiens* f. molestus at the SH60 locus was not underestimated due to polymorphisms in the PCR priming region. Finally, because the SH60 fragment was *Cx. p. pipiens*-specific, it could only be amplified from *Cx. p. pipiens* and *Cx. pipiens* f. molestus, nullifying the use of a phylogenetic analysis due to absence of the outgroups necessary to provide context.

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Contrary to what would be expected if the reduced diversity in *Cx. pipiens* f. molestus is indeed due to a founder's effect, the diversity at this locus seems to be maintained in colony *Cx. p. pipiens* populations. Because the identity and genomic location of the SH60 fragment is unknown, it is difficult to interpret these results. One possible explanation is that the *Cx. p. pipiens* colonies evaluated did not experience a severe bottleneck due to recent colonization, founding from a larger number of individuals, reproduction of many individuals each generation, and/or subsequent introductions into the colony to maintain genetic diversity. A more plausible explanation is that the SH60 fragment is genetically linked to a *Cx. pipiens* f. molestus-associated trait, leading to its fixation in those populations and apparent absence of genetic selection or reduction in *Cx. p. pipiens* colonies. Linkage of phenotypes with genetic elements controlling autogeny has been investigated previously (Spielman 1957).

All SH60 clones from 38 *Cx. p. pipiens*, representing both NYC and Syracuse collections, were analyzed by SSCP simultaneously with SH60 amplicons from *Cx. pipiens* f. molestus. No shared SH60 variants were identified between either of these *Cx. p. pipiens* collections and *Cx. pipiens* f. molestus from NYC. However, due to the enormous variability in SH60 variants among *Cx. p. pipiens*, exhaustive sequencing of all SH60 variants was not completed, and the presence of shared variants between these two biotypes cannot be ruled out. Should shared SH60 variants be discovered, this finding would argue in favor of a founder's effect and local origination of this *Cx. pipiens* f. molestus population from surface-dwelling *Cx. p. pipiens*. Because the analysis stands however, NYC *Cx. pipiens* f. molestus embodied a unique SSCP banding pattern characterized in part by an adenosine residue at position 187 that was not shared by any other sequenced *Cx. p. pipiens* clones.

These PCR and SSCP experiments were supplemented with a population analysis at five microsatel-lite loci, including *Cx. p. pipiens* and *Cx. p. quinque-fasciatus* collections from California as geographic outgroups. The *Cx. pipiens* f. molestus population had less microsatellite allelic diversity at each locus as compared with sympatric populations of *Cx. p. pipiens* from NYC (Table 4). Additionally, there was at least one shared allele between *Cx. pipiens* f. molestus and *Cx. p. pipiens* from both New York locations at all five loci. The shared alleles present in the *Cx. pipiens* f. molestus population were likely derived from contemporary or ancestral genetic exchange with surface-dwelling *Cx. p. pipiens*. The reduced allelic diversity across all microsatellite loci in general in *Cx. pipiens* f. molestus relative to *Cx. p. pipiens* mirrors the results of the SH60 fragment analysis.

At microsatellite locus CQ11, which is purportedly diagnostic in size for *Cx. pipiens* f. molestus (Bahnck and Fonseca 2006), all 16 NYC *Cx. pipiens* f. molestus were monomorphic for a 283-bp allele (Tables 4 and 6). This finding is similar with that reported by Fon-seca et al. (2004) in which the majority of *Cx. pipiens* f. molestus contained a 285-bp allele, and Bahnck and Fonseca (2006), in which CQ11 for *Cx. pipiens* f. molestus was 284 bp. Slight differences in allele size between this study and previous publications could be due to differences in the settings used to designate alleles.

Microsatellite analysis also revealed largely unrestricted gene flow among populations of *Cx. p. pipiens* collected from East and West coasts of the continental United States,

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indicating that this species exists as a panmictic population in much of North America. In contrast, genetic structuring was confirmed among the different members of the species complex. There was relatively more gene flow estimated between New York *Cx. pipiens* f. molestus and *Cx. p. pipiens* populations than between either *Cx. pipiens* f. molestus and *Cx. p. quinquefasciatus* or *Cx. p. pipiens* and *Cx. p. quinquefasciatus* (Table 5). Because this latter cross is known to occur in the United States throughout the hybrid zone between the 36th and 39th parallels (Barr 1957), the estimated gene flow between *Cx. p. pipiens* and *Cx. pipiens* f. molestus is also likely to be biologically significant. The finding of relatively greater gene flow between *Cx. pipiens* f. molestus and *Cx. p. pipiens* could be the result of a common ancestry between these two biotypes, the founding or derivation of *Cx. pipiens* f. molestus from *Cx. p. pipiens*, or the existence of some degree of ongoing but restricted gene flow between sympatric *Cx. p. pipiens* and *Cx. pipiens* f. molestus populations.

The key data from all aspects of this study are summarized in Table 6, from which we posit the following hypotheses. Our collection of *Cx. pipiens* f. molestus collected from the 91st St. sewer in NYC represents a population of true molestus and is characterized by one SH60 variant per individual mosquito and fixation of allele 283 at the CQ11 microsatellite locus. In contrast, *Cx. p. pipiens* populations from New York and elsewhere exhibit a much greater diversity of CQ11 alleles as well as SH60 variants. From these data, either of the following interpretations could be argued. (1) NYC *Cx. pipiens* f. molestus were locally founded from surface *Cx. p. pipiens* populations. Evidence supporting this theory is the greatly reduced genetic diversity in *Cx. pipiens* f. molestus relative to *Cx. p. pipiens* in the SH60 amplicon as well as the five microsatellite loci statistically analyzed. Microsatellite alleles, including CQ11 283, were shared between NYC *Cx. p. pipiens* and *Cx. pipiens* f. molestus. Although no common SH60 variant was identified between biotypes, it likely exists given the extreme polymorphism seen among *Cx. p. pipiens* at this locus. Furthermore, there was relatively more gene flow between *Cx. p. pipiens* and *Cx. pipiens* f. molestus than between any other combination of members of the species complex. (2) Alternatively, the patterns observed between *Cx. p. pipiens* and *Cx. pipiens* f. molestus in New York at the CQ11 microsatellite locus and SH60 locus could represent true genetic differences that characterize these biotypes, which also readily hybridize. For populations where hybridization is occurring between *Cx. p. pipiens* and *Cx. pipiens* f. molestus, CQ11 alleles and SH60 variants once present in *Cx. p. pipiens* or *Cx. pipiens* f. molestus are introduced and dispersed throughout the larger geographic population through introgression and recombination. As a result, we also observe “M” types that have multiple SH60 variants (Cpp10), and “P” types that have only one SH60 variant (Cpp20), and individuals such as Cpp01 which have the P/M molecular type and multiple SH60 variants but not the CQ11 283 allele (Table 6). Bahnck and Fonseca (2006) cautioned that their PCR results must be interpreted on the population level, because after the F1 generation the assay is not accurate in identifying individual mosquitoes of hybrid ancestry due to independent assortment and recombination among microsatellite alleles. This phenomenon is evident through the presence of “P,” “M,” and “P/M” types among individual mosquitoes collected from the same geographic location (i.e., St. Albans, NYC, and Prospect Park, NYC, Table 6). This latter hypothesis is also supported by the micro-satellite analysis which demonstrates the presence of reduced but biologically significant degree of gene flow between *Cx. p. pipiens* and *Cx. pipiens* f. molestus (Table 5).

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Despite an extensive genetic analysis of these populations, we are unable to indisputably side with one hypothesis over the other. Evidence of gene flow and "hybridization" between *Cx. p. pipiens* and *Cx. pipiens* f. molestus could be interpreted as two genetically distinct forms that are converging and hybridizing in ecological zones of sympatry. Alternatively, *Cx. pipiens* f. molestus represents an autogenous biotype derived from surface dwelling *Cx. p. pipiens* with the residual appearance of hybridization as the two forms are behaviorally and physiologically divergent but are not yet reproductively isolated. A more thorough evaluation of sympatric populations of *Cx. P pipiens* and *Cx. pipiens* f. molestus biotypes in the United States and globally is needed before these results can be validated and placed in a broader phylogenetic context.

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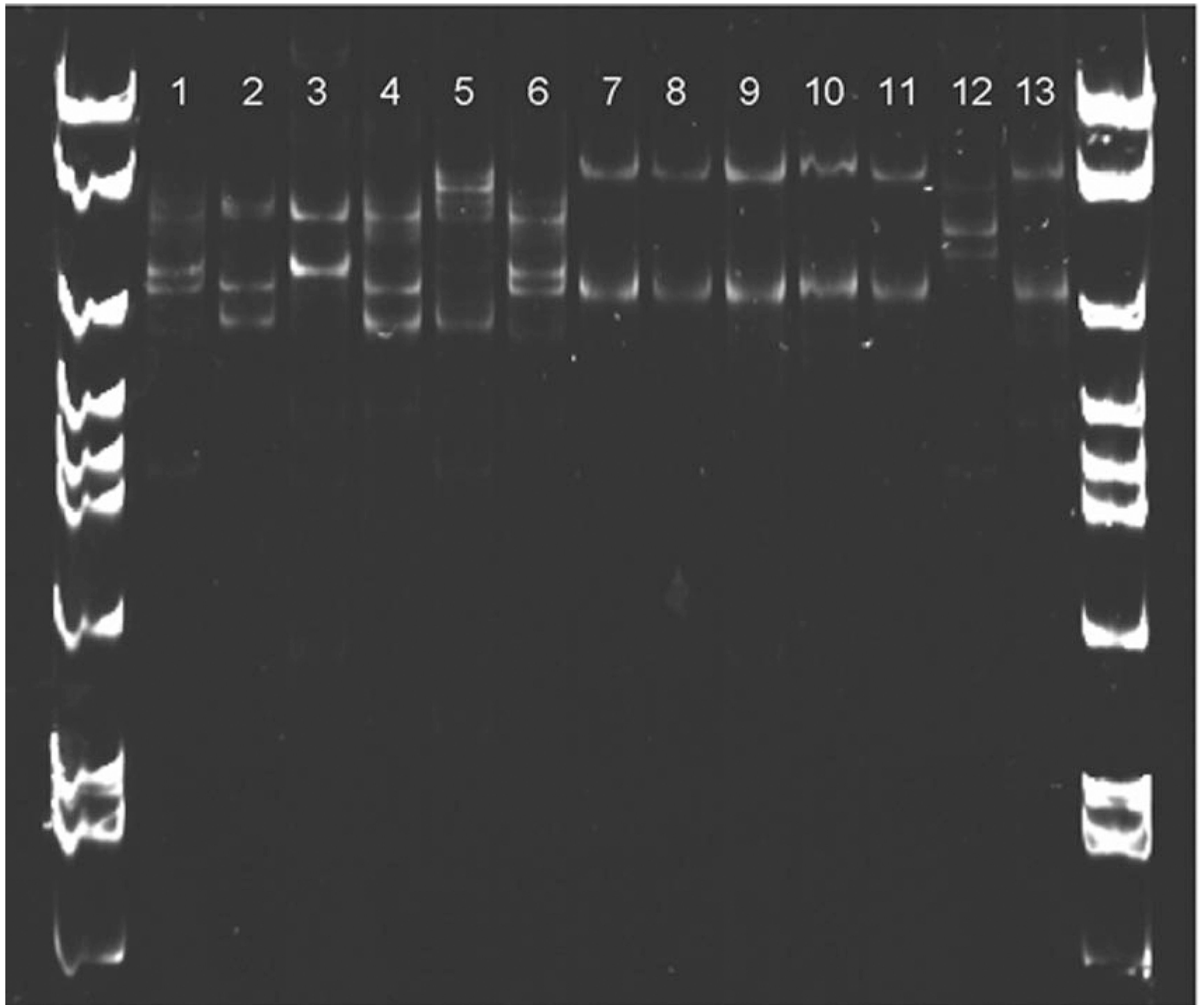

Fig. 1.

Genetic variability between *Cx. p. pipiens* and *Cx. pipiens* f. molestus in the SH60 fragment by SSCP analysis. Lanes 1–6, colony *Cx. p. pipiens;* lanes 7–11, colony *Cx. pipiens* f. molestus; lane 12, field-collected *Cx. p. pipiens;* and lane 13, field-collected *Cx. pipiens* f. molestus.

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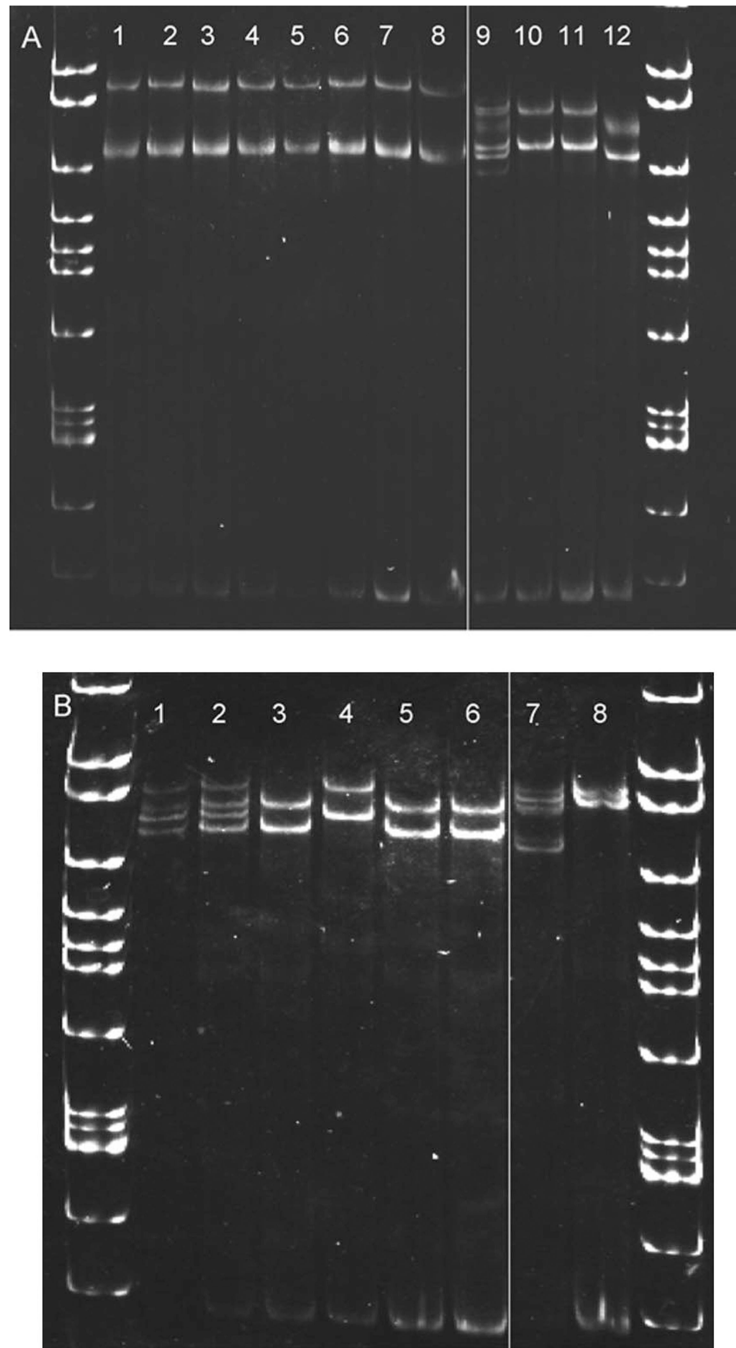

Fig. 2.

Genomic DNA and corresponding clones of the SH60 fragment illustrating that *Cx. pipiens* f. molestus contains a single SH60 variant, whereas *Cx. p. pipiens* specimens contain multiple variants that are additive to form the complex banding patterns seen in Fig. 1. (A) NYC *Cx. p. pipiens* and *Cx. pipiens* f. molestus. Lane 1, *Cx. pipiens* f. molestus genomic DNA; lanes 2–5, corresponding clones from the same specimen; lane 6, *Cx. pipiens* f. molestus genomic DNA; lanes 7–8, SH60 clones; lane 9, *Cx. p. pipiens* genomic DNA; and lanes 10–12, SH60 clones. (B) NYC and Syracuse *Cx. p. pipiens*. Lane 1, Syracuse *Cx. p.*

pipiens genomic DNA; lanes 2–6, SH60 clones; lane 7, NYC *Cx. p. pipiens* genomic DNA; and lane 8, one corresponding clone.

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Table 1

Sources of *Cx. pipiens* complex mosquitoes used in genetic analyses

	Strain	Source	*n*
Cx. p. pipiens	Colony: Cornell	Cornell University, Tompkins Co., Ithaca, NY	20
	Colony: Shasta F3	UC Davis, Shasta County, Davis, CA	8
	Colony: LIN	Sierra Pacific log decks, Placer Co., Lincoln, CA	10
	Colony: Iowa State University	AIDL, Colorado State University, Larimer Co., Ft. Collins, CO	15
	Colony: San Francisco, CA	AIDL, Colorado State University, Larimer Co., Ft. Collins, CO	18
	Colony: SJCMVC	San Joaquin Co., Rancho Cordova, CA	18
	Field-collected	St. Albans, Prospect Park, North Central Park, Clove Lake Park Staten Island, Split Rock Golf Course, New York Co., NYC, NY	20
	Field-collected	Onondaga Co., Syracuse, NY	30
	Field-collected	Philadelphia Co, Philadelphia; Union Co., Buffalo, Lewis, White Deer, Mifflinburg Boro and Kelly Townships; Chester Co., Wallace Township; Bucks Co., Falls Township; Montgomery Co., Norristown, and Erie Co., PA	19
	Field-collected	Fort Howard bunkers, Baltimore Co., Ft. Howard, MD	11
C.x pipiens f. molestus	Colony: Cornell	New York Co., NYC, NY	12
	Field-collected	91st St. sewer, New York Co., NYC, NY	16
Cx. pipiens quinquefasciatus	Field-collected	San Mateo Co., West Valley, CA	20
	Field-collected	Orange, Riverside, and San Diego Counties, Coachella Valley, CA	20

AIDL, Arthropod-borne Infectious Disease Laboratory; SJCMVC, San Joaquin County Mosquito and Vector Control District.

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Table 2

Nucleotide substitutions present among selected *Cx. p. pipiens* and *Cx. pipiens* f. molestus SH60 clones from New York

Biotype	Source	Accession no.	Clone name	Nucleotide position												
				25	40	44	49	69	87	102	187	207	209–217	239	246	249
Cx. p. pipiens	Cornell Univ. colony	AY923231	P16B	T	C	T	C	C	T	G	C	C	GGAAGGGCC	T	A	C
Cx. p. pipiens	Syracuse, NY	AY923233	Cpp50J	.	.	.	.	.	.	.	.	.	.	.	.	T
Cx. p. pipiens	Syracuse, NY	AY923234	Cpp50D	G	.	.	.	.	.	A	.	.	———	.	G	T
Cx. p. pipiens	Syracuse, NY	AY923232	Cpp50C	G	.	.	.	.	C	A	.	.	———	.	G	T
Cx. p. pipiens	Syracuse, NY	AY923236	Cpp24G	G	.	C	.	.	.	.	.	T	———	.	G	T
Cx. p. pipiens	Syracuse, NY	AY923235	Cpp24F	G	.	C	.	.	.	.	.	.	———	.	G	T
Cx. p. pipiens	Syracuse, NY	EF015565	Cpp32A	G	.	C	.	T	.	.	.	.	———	.	G	T
Cx. p. pipiens	Syracuse, NY	EF015566	Cpp42B	G	.	C	.	T	.	.	.	.	———	.	G	T
Cx. p. pipiens	Syracuse, NY	EF015567	Cpp45H	G	T	C	.	.	.	.	.	.	———	.	G	T
Cx. p. pipiens	Syracuse, NY	EF015568	Cpp45Q	G	.	C	.	.	.	.	.	.	———	.	G	T
Cx. p. pipiens	St. Albans, NYC	DQ421382	Cpp01G	G	.	C	T	.	.	.	.	.	———	.	G	T
Cx. p. pipiens	St. Albans, NYC	DQ421381	Cpp01H	G	.	C	T	.	.	.	.	T	———	.	G	T
Cx. p. pipiens	St. Albans, NYC	DQ421383	Cpp01I	G	.	C	T	.	.	.	.	T	———	.	G	T
Cx. p. pipiens	Staten Island, NYC	DQ421385	Cpp10A	G	.	C	.	T	.	.	.	.	———	.	G	T
Cx. p. pipiens	Staten Island, NYC	DQ421384	Cpp10B	G	.	C	T	.	.	.	.	.	———	.	G	T
Cx. p. pipiens	Staten Island, NYC	AY923239	Cpp10P	G	.	C	.	.	.	.	.	.	———	.	G	T
Cx. p. pipiens	Central Park, NYC	AY923230	Cpp17A	G	.	C	T	.	.	.	.	.	———	.	G	T
Cx. p. pipiens	Prospect Park, NYC	EF015564	Cpp19F	G	.	C	.	.	.	.	.	T	———	.	G	T
Cx. p. pipiens	Crabtree et al. (1997)	U90782	U90782	G	.	C	.	T	.	.	.	.	———	.	G	T
Cx. pipiens f. molestus	91st St. sewer, NYC	DQ421386	Cpm4A	G	.	C	T	.	.	.	A	T	———	.	G	T
Cx. pipiens f. molestus	91st St. sewer, NYC	AY923229	Cpm7B	G	.	C	T	.	.	.	A	T	———	.	G	T
Cx. pipiens f. molestus	91st St. sewer, NYC	AY923238	Cpm3C	G	.	C	T	.	.	.	A	T	———	C	G	T
Cx. pipiens f. molestus	Cornell Univ. colony	AY923237	M4	G	.	C	T	.	.	.	A	T	———	.	G	T

Dots indicate nucleotides shared with the reference sequence (AY923231, P16B), whereas dashes indicate gaps.

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Table 3

Microsatellite loci examined in North American populations of *Cx. p. pipiens, Cx. pipiens* f. molestus, and *Cx. p. quinquefasciatus*

Locus	Designed for	Repeat motif	Reference
CUTA1	*Cx. tarsalis*	$(AC)_n$	Rasgon et al. (2006)
CUTD107	*Cx. tarsalis*	$(CAG)_n CAA(CAG)_n$	Rasgon et al. (2006)
CUTD4	*Cx. tarsalis*	$(CAG)_n$	Rasgon et al. (2006)
CUTD120	*Cx. tarsalis*	$(CAG)_n$	Rasgon et al. (2006)
CUTB1	*Cx. tarsalis*	(GA)	Rasgon et al. (2006)
CUTD113	*Cx. tarsalis*	(CAG)	Rasgon et al. (2006)
CQ26	*Cx. p. quinquefasciatus*	$(GTGTGTAT)_2 + (GT)_{10} + (GT)_5$	Fonseca et al. (1998)
CQ29	*Cx. p. quinquefasciatus*	(GT) GA(GT)	Fonseca et al. (1998)
CQ11	*Cx. p. quinquefasciatus*	$(GT)_2 ACTTC(GT)_9$	Fonseca et al. (1998), Smith et al. (2005)
CxpGT4	*Cx. p. pipiens*	$(GT)_5(GTTT)_2 GC(GT)_2 CT(GT)_5$	Keyghobadi et al. (2004)
CxpGT9	*Cx. p. pipiens*	$(GT)_{13}$	Keyghobadi et al. (2004)
CxpGT46	*Cx. p. pipiens*	$(TG)_{15}$	Keyghobadi et al. (2004)

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Table 4

Genetic diversity of two populations of *Cx. p. pipiens* and one population of *Cx. pipiens* f. molestus from New York

Locus	New York City *Cx. p. pipiens* Allele frequency							New York City *Cx. pipiens* f. molestus allele frequency								Syracuse *Cx. p. pipiens* allele frequency							
	2N	A	MC	LC	H_o	H_e	P	Locus	2N	A	MC	LC	H_o	H_e	P	Locus	2N	A	MC	LC	H_o	H_e	P
CUTBl	32	5	0.41	0.09	0.625	0.786	0.632	CUTBl	32	3	0.59	0.19	0.438	0.605	0.17	CUTBl	20	5	0.35	0.05	1	0.77	0.619
CUTD113	32	4	0.66	0.03	0.333	0.537	0.223	CUTD113	32	2	0.56	0.44	0.625	0.508	0.609	CUTD113	20	2	0.55	0.45	0.5	0.57	1
CxpGT4	32	8	0.34	0.03	0.625	0.825	0.2	CxpGT4	32	6	0.41	0.03	0.688	0.754	0.158	CxpGT4	20	5	0.40	0.05	0.8	0.76	0.381
CxpGT46	32	12	0.22	0.03	0.933	0.892	0.813	CxpGT46	32	7	0.38	0.03	0.75	0.78	0.016*	CxpGT46	20	7	0.35	0.05	0.9	0.82	0.844
CQ11	32	5	0.34	0.03	0.333	0.777	0.002*	CQ11	32	1	1.00	0.00	Monomorphic			CQ11	20	2	0.80	0.20	0.2	0.41	0.301

2N is the number gene copies, A is the no. of alleles per locus, MC represents the frequency of the most common allele, LC is the frequency of the least common allele, H_O represents observed heterozygosity, and H_e represents expected heterozygosity.

*Denotes significant deviation from Hardy-Weinberg equilibrium.

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Table 5

Relative gene flow among populations of *Cx. p. pipiens, Cx. pipiens* f. molestus, and *Cx. p. quinquefasciatus* in the United States

	NYC *Cx. p. molestus*	**NYC *Cx. p. pipiens***	**Syracuse *Cx. p. pipiens***	**Shasta *Cx. p. pipiens***
NYC *Cx. p. molestus*				
NYC *Cx. p. pipiens*	61.5 (0.00806)*			
Syracuse *Cx. p. pipiens*	121.88 (0.004009)	Infinity (−0.01002)		
Shasta *Cx. p. pipiens*	65.47 (0.00758)	Infinity (−0.00205)	Infinity (0.00000)	
San Bernadino *Cx. p. quinquefasciatus*	28.39 (0.01731)*	49.01 (0.01010)*	46.85 (0.01110)*	44.53 (0.00000)

Values presented are the estimated number of migrants per generation (Nm) followed by the FST in parentheses.

*Denotes significant FST.

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Table 6

Combined CQ11 and SH60 data for field-collected New York *Cx. p. pipiens* and *Cx. pipiens* f. molestus. The no. of SH60 variants was determined by cloning and SSCP. “Cpp” denotes specimens identified as *Cx. p. pipiens* and “Cpm” denotes specimens identified behaviorally and physiologically as *Cx. pipiens* f. molestus. Column (P/M) refers to Bahnck and Fonseca (2006) PCR results, where P = “pipiens” and M = “molestus”. “+” indicates instances where not all SH60 variants present were isolated by cloning.

Mosquito	Collection location	CQ11 alleles	P/M	# SH60 variants
Cpp01	St. Albans, NYC	251,279	P/M	3+
Cpp02	St. Albans, NYC	283,283	M	2+
Cpp06	St. Albans, NYC	251,251	P	4
Cpp09	St. Albans, NYC	251,251	P	2
Cpp10	Clove Lake Park, Staten Is.	283,283	M	2
Cpp12	North Central Park, NYC	283,283	M	1
Cpp14	North Central Park, NYC	251,251	P	4
Cpp17	North Central Park, NYC	265,265	P	3
Cpp18	Silver Lake Golf Course, NYC	259,283	P/M	5
Cpp19	Prospect Park, NYC	259,283	P/M	4
Cpp20	Prospect Park, NYC	259,265	P	1
Cpp21	Syracuse, NY	259,259	P	2
Cpp22	Syracuse, NY	283,283	M	3
Cpp23	Syracuse, NY	259,283	P	2
Cpp24	Syracuse, NY	259,283	P/M	2
Cpp26	Syracuse, NY	259,283	P	2+
Cpp27	Syracuse, NY	259,259	P	2+
Cpp31	Syracuse, NY	259,259	P	2
Cpp32	Syracuse, NY	251,251	P	2
Cpp33	Syracuse, NY	259,259	P	4
Cpp45	Syracuse, NY	259,283	P	2
Cpp48	Syracuse, NY	259,259	P	4
Cpp49	Syracuse, NY	283,283	M	3
Cpp50	Syracuse, NY	259,259	P	3
Cpm01	91st St. sewer, NYC	283,283	M	1
Cpm02	91st St. sewer, NYC	283,283	M	1
Cpm03	91st St. sewer, NYC	283,283	M	1
Cpm04	91st St. sewer, NYC	283,283	M	1
Cpm05	91st St. sewer, NYC	283,283	M	1
Cpm06	91st St. sewer, NYC	283,283	M	1
Cpm07	91st St. sewer, NYC	283,283	M	1
Cpm08	91st St. sewer, NYC	283,283	M	1
Cpm09	91st St. sewer, NYC	283,283	M	1
Cpm10	91st St. sewer, NYC	283,283	M	1

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Mosquito	Collection location	CQ11 alleles	P/M	# SH60 variants
Cpm11	91st St. sewer, NYC	283,283	M	1
Cpm12	91st St. sewer, NYC	283,283	M	1
Cpm13	91st St. sewer, NYC	283,283	M	1
Cpm14	91st St. sewer, NYC	283,283	M	1
Cpm15	91st St. sewer, NYC	283,283	M	1
Cpm16	91st St. sewer, NYC	283,283	M	1

$^{+}$indicates that not all SH60 variants present in that individual were identified by cloning, so the true no. is unknown.

Pure molestus are fixed at the CQ11 locus for allele 283, and contain one SH60 variant which in this case has the one point mutation unique from all pipiens analyzed.

Populations that have mixed gene flow between P and M have additional microsatellite alleles present and at the population level, show both P and M and P/M types on the rapid assay, and have more than one SH60 variant per individual. The rapid assay is only accurate for the F1 generation in showing true hybrids, after which recombination and backcrossing will skew results if viewed only on an individual basis. Therefore, hybrid populations may have individuals that are homozygous for either P or M CQ11 alleles or have one or more SH60 variant. In Cpp01, the 283 allele has been lost altogether.

There is or was at one point, gene flow between surface and subterranean populations.