Identification of Culex pipiens Complex Mosquitoes in a Hybrid Zone of West Nile Virus Transmission in Fresno County, California

Rory D. McAbee,* Emily N. Green, Jodie Holeman, Julie Christiansen, Niki Frye, Katherine Dealey, F. Steve Mulligan III, Aaron C. Brault, and Anthony J. Cornel

Department of Entomology, University of California at Davis, Mosquito Control Research Laboratory, Parlier, California; Center for Vector-Borne Diseases, University of California at Davis, California; Department of Pathology, Microbiology and Immunology, School of Veterinary Medicine, University of California at Davis, California; Consolidated Mosquito Abatement District, Selma, California

Abstract. Culex pipiens sensu lato mosquitoes were collected from 24 gravid traps (mid-June to mid-October, 2005) in Fresno County, CA. Captured gravid females were allowed to oviposit before sibling species identification by Ace.2 PCR and detection of West Nile virus (WNV) RNA by RT-PCR were performed on the mother and her offspring. Of the 442 Cx. pipiens s.l. female mosquitoes collected, 88 were positive for WNV viral RNA (peaked in August) with no significant differences among complex members or habitat. Vertical transmission was detected in 4 out of 20 families originating from WNV-positive mothers, however, in only a small number of offspring from each family. Out of 101 families that had PCR-based maternal and offspring identifications, the offspring from 15 families produced inexplicable amplicon patterns, suggesting ambiguities in the PCR assay identifications. Male genitalia (DV/D ratio) and Ace.2 PCR identifications revealed numerous discrepancies in our ability to accurately determine the identity of Cx. pipiens complex members in the hybrid zone of Fresno County.

INTRODUCTION

In summer 2004, West Nile virus (family Flaviviridae, genus Flavivirus, WNV) activity was detected in most of California, including Fresno County. This activity was expected to continue and perhaps become more extensive in 2005. Given this scenario, we considered the summer of 2005 to be the optimal time to examine infection rates of WNV in a hybrid zone of Culex pipiens complex mosquitoes. Cx. pipiens s.l. mosquitoes have consistently been identified as major WNV vectors from numerous field isolations1–3 and from laboratory vector competence assays.4–7 In this paper no assumptions have been made about their taxonomic status, but herein after the terms Cx. pipiens and Cx. quinquefasciatus will be used to distinguish complex members.

Fresno County is located within the 36°N and 39°N latitude introgression zone in the United States where Cx. pipiens and Cx. quinquefasciatus and their hybrid populations occur in sympathy.8–10 Fresno County, therefore, represents a good locality to compare WNV infection rates among the two nominal taxa and hybrids of the Cx. pipiens complex. If spatial and temporal differences in infection rates were identified among the members of the complex, this information would be useful for mosquito abatement. For example, control and surveillance efforts could focus on sites that have a higher propensity to breed and maintain populations of Cx. quinquefasciatus versus Cx. pipiens and their hybrids in rural, peri-urban, or urban situations. Alternatively, if vertical WNV transmission is identified, control efforts could also be warranted in the winter to reduce over-wintering populations.

The justification for examining and comparing infection rates among Cx. pipiens s.l. stems from an ongoing debate about genetic differentiation and associated behavioral differences among the complex members.11,12 which was rekindled due to the invasion of WNV into the United States. Members of this complex display a variety of behavioral adaptations8,13–17 and show some morphologic differences8,16–22 and variation in WNV vector competency.4,23–25 It is not clear if the observed variation is associated with genetically discrete populations or taxa or if it represents a high degree of polymorphism within a single panmictic unit.

There are currently two methods to distinguish between Cx. pipiens and Cx. quinquefasciatus. One is based on relative overlap and measurements of the dorsal and ventral arms in male genitalia (DV/D ratio).26 Cx. pipiens have DV/D ratios of less than 0.2, Cx. quinquefasciatus has DV/D ratios greater than 0.4, and hybrids have intermediate ratios of 0.2–0.4. The other method that distinguishes the two nominal taxa target nucleotide differences in the Ace.2 gene that when amplified by polymerase chain reaction (PCR) produces a different size fragment for each of the two taxa.27,28 The Ace.2 gene is not associated with insecticide resistance.29,30

In this study we used a combination of male DV/D ratio measurements and the diagnostic PCR assay of Smith and Fonseca28 to identify wild females and their offspring. Identiﬁcations based on Smith and Fonseca28 were favored over the Aspen and Savage27 methods because of the simplicity of conducting one PCR procedure as opposed to two. Furthermore, Aspen and Savage27 PCR often did not amplify Cx. pipiens-speciﬁc product on specimens from California and elsewhere, such as in Africa, despite altering reaction conditions. After a wild female laid eggs, her DNA was extracted from a portion of the body and the rest of the carcass was used for WNV RNA detection. Wild female families were reared separately so that the DV/D ratios and PCR identifications of her male offspring could be matched to her identity

* Address correspondence to Rory D. McAbee, Mosquito Control Research Laboratory, 9240 S. Riverbend Avenue, Parlier, CA 93648. E-mail: rdmcabee@uckac.edu
to determine whether she mated with a conspecific or hybrid. Knowing the identity of the mother, in some instances PCR identification and $DV/D$ ratios of the male offspring produced identifications that could not be possible genetically. This led us to consider evaluating the accuracies of both identification methods by setting up crosses and back-crosses between California Cx. pipiens north of 39°N and Cx. quinquefasciatus south of 36°N and comparing the hybrid and back-cross PCR and $DV/D$ ratio identifications. These results indicate that both identification methods are unreliable, which has profound implications for systematic and ecological studies and resolving public health impacts of sibling species in the hybrid zone.

**MATERIALS AND METHODS**

**Mosquito collection, rearing, and identification.** Gravid female members of Cx. pipiens s.l. were captured in 8 rural, 8 urban (high-density housing, commercial areas), and 8 periurban (low-density housing on outskirts of cities) locations using modified gravid traps baited with 7-day-old Bermuda grass infusion. Previous evaluations found that gravid traps consistently collected more WNV positive Cx. pipiens complex mosquitoes than did CDC-style CO2 traps. We collected from a total of 24 trap sites, sampling 8 sites per week on a 3-wk rotation starting in June and ending in October.

Gravid females were placed into individual holding vials and provided with Bermuda grass-infused water to oviposit. After oviposition, the head and legs of the female were separated from the rest of the body for DNA extraction and the abdomen and thorax were stored in RNA later (Ambion, Inc., Austin, TX) for RNA extraction and subsequent virus detection. In addition, all nongravid and nonovipositing mosquitoes were dissected and processed similarly.

Each egg raft (family) was reared separately in 2 L of Bermuda grass-infused water and larvae fed a diet of ground rodent diet 5001 (PMI Nutrition International LCC, Brentwood, MO). Ten adult males (8 days old) from each family were separated into 3 parts. The head and legs were stored in 70% ethanol for DNA extraction, the thorax and abdomen were preserved in RNA later for WNV RNA detection, and the genitalia were stored dry for $DV/D$ ratio determination. PCR identification of the mother and her male offspring allowed for inferences to be made as to which complex member the mother mated with in the wild in the hybrid zone in Fresno County. Furthermore, congruence between PCR identification and $DV/D$ ratio of the male offspring was evaluated. For the Smith and Fonseca PCR assay, the ACEpips, ACEquin, and B1246s primers at the concentration recommended by manufacturer specifications. Real-time PCR analysis used 10 μL of eluted RNA and the One-Step RT-PCR kit (Applied Biosystems, Foster City, CA) using an ABI 7500 Sequence Detection System. Primers spanning a portion of the envelope—WNVenv-forward, WNVenv-reverse, and WNV-probe—were used for the initial screening, whereas primers spanning a section of the NS1 region—3111V, 3239C, with probe 3136V—were used for confirmatory purposes. Only samples that had a logarithmic increase in fluorescent signal above the threshold for both sets of primers and probes were considered positive.

**Laboratory crosses.** To evaluate the correlation of $DV/D$ ratio to PCR identification we crossed males with females from laboratory colonies of Cx. quinquefasciatus (LA, originating in downtown Los Angeles, CA) and Cx. pipiens (SH, originating from Redding in Shasta County, CA). The LA mosquitoes have been in colony since 2000 and have consistently produced Cx. quinquefasciatus-specific Ace2 PCR products and $DV/D$ ratios while in colony. The SH mosquitoes have been in colony since 2003 and have consistently produced Cx. pipiens-specific Ace2 PCR products and $DV/D$ ratios while in colony. Thirty individuals of each colony were first subjected to both $DV/D$ ratio determination and PCR to ensure that the current generation still consistently produced identifications as either Cx. pipiens or Cx. quinquefasciatus. Virgin male and females were obtained by separating the sexes based on pupal morphology. All crosses were conducted in screened 28 cm × 28 cm × 28 cm cages in an insectary maintained at 26°C and 85–95% RH. The larvae were reared in 2 L of tap water and fed ground rodent diet 5001 (PMI Nutrition International LCC). Mice were used as a blood source for egg production 3 days after the virgin females and males were combined. The crossing schemes and the labels used are provided in Table 1. The crossing scheme was initiated by placing 20 virgin LA ♀ and 20 SH ♂. The LA × SH hybrid egg rafts were reared, and the pupae were then sexed and reared to adults separately. Then, 20 hybrid ♀ were back-crossed to 20 LA ♀, 20 hybrid ♂ were back-crossed to 20 LA ♀, and 20 hybrid ♂ were back-crossed to 20 SH ♀.

**WNV RNA detection from mosquitoes.** Individual mosqui-

| Designations for laboratory crosses between Cx. pipiens pipiens (Shasta) and Cx. pipiens quinquefasciatus (LA) colonies |
|---|---|---|
| Mother, ♀ | Father, ♂ | Offspring |
| LA | LA | LA |
| Shasta | Shasta | Shasta |
| LA | Shasta | AF1 |
| AF1 | AF1 | AF2 |
| AF1 | LA | AF2 |
| AF1 | AF1 | A2F2 |
| AF1 | Shasta | A2F2 |
| Shasta | AF1 | A4F2 |
| Shasta | LA | BF1 |
| BF1 | BF1 | BF2 |
| BF1 | LA | BF2 |
| BF1 | BF1 | B2F2 |
| BF1 | Shasta | B3F2 |
| Shasta | BF1 | B4F2 |
crossed to 20 SH ♀, and the reciprocal back-cross of 20 hybrid ♀ to males of each parental type was also done. The genitalia of 30 males not used in the inbreeding crosses or back-crosses were dissected for DV/D ratio measurements, and the corresponding remaining body was stored in 70% ethanol for DNA extraction and diagnostic PCR. This entire scheme was repeated except for initiating it with the reciprocal cross of 20 virgin LA ♀ with 20 SH ♀. Each crossing series is designated by a letter A or B (Table 1). The A series consists of hybrids generated by a male SH and a female LA (AF1). These hybrids were then crossed with their siblings (AF2), and females were back-crossed to LA males (A2F2) and SH males (A3F2). Male AF1 mosquitoes were also back-crossed to LA females (A2F2) and SH females (A4F2). The B series of crosses is in the exact same pattern except the hybrids (BF1) are produced by mating between a male LA and a female SH.

RESULTS

WNV infection in wild females and offspring. From mid-June until mid-October, 442 Cx. pipiens complex mosquitoes were collected from gravid traps in Fresno County and tested for presence of WNV RNA. The numbers of mosquitoes collected at each site varied considerably throughout this period. Overall we collected more mosquitoes in gravid traps in urban environments; however, there was much variation between collection sites. As an example, Figure 1 depicts numbers collected in urban trap sites showing this variation for the duration of the study. Because of limited resources, we were unable to sample mosquitoes from all 24 traps every week; that would have enabled us to determine if trap counts showed significant differences between urban, rural, and peri-urban locations. The mosquitoes collected were identified by PCR of which 271 were Cx. quinquefasciatus, 7 Cx. pipiens, and 98 were hybrids. Of these 442 mosquitoes collected, 88 (19.9%) tested positive for WNV RNA based on the two RT-PCR primer-pair reactions. West Nile viral RNA-positive mosquitoes were collected in rural, urban, and peri-urban sites throughout Fresno County, with hot spots occurring in Navelencia and Selma (Figure 2).

Of the 88 mosquitoes that were positive for WNV RNA, 58 were Cx. quinquefasciatus, 10 were Cx. pipiens, and 20 were hybrids as determined by Ace.2 PCR. This overall infection rate of 19.9% was not significantly different among members of the Cx. pipiens complex at 95% confidence ($\chi^2 = 0.85$, Figure 3). There was also not a significant difference ($\chi^2 = 0.93$, 95% confidence) between the overall infection rate and mosquitoes collected in different local habitats (Figure 4). The vector index for each urban (0.73), peri-urban (0.50), and rural (0.61) site also indicated similar infection rates between habitats. There were significant differences between habitats. There were significant differences ($\chi^2 > 9.49$) between the overall infection rate and the rates of infection determined for each month, with a peak of infection occurring in August (Figure 3).

Two hundred eight of the 442 females collected, oviposited, from which iso-female families were reared. Twenty of the females that oviposited were WNV RNA positive. From these 20 families, 5 individual offspring out of 47 tested (10.6%), representing 4 different families, were positive for WNV RNA, confirming that transovarial transmission occurred.

Identification of Cx. pipiens complex members in Fresno. Because we had offspring reared from a mother of known PCR identity, we attempted, on the basis of offspring PCR identity, to predict which member of the complex the mother mated with in the wild. After the first round of PCR, 15 families produced combinations of offspring and mother identifications that could not be possible, such as producing offspring homozygous for the Cx. pipiens-specific allele from mothers homozygous for the Cx. quinquefasciatus-specific allele. The opposite was also observed. A second round of PCR performed on the 15 un-interpretable families produced similar results.

DV/D measurements of the male offspring for which we had PCR identifications also revealed lack of congruence between the male genitalia morphology and the PCR identification (Figure 5). Both PCR and DV/D ratios were obtained for 475 male offspring. Ten males were selected from each of the 20 families originating from WNV RNA-positive mothers,

FIGURE 1. Numbers of Cx. pipiens s.l. collected in gravid traps from mid-June through to mid-October 2005 located in urban areas in Fresno County, California.
and 10 males were chosen from 48 families originating from WNV RNA-negative mothers for male genitalia dissections. From each family selected, not all 10 of the males chosen were used to produce data as some of the genitalia could not be measured for $DV/D$ ratios because of broken appendages. For males of $DV/D$ ratios of less than 0.2 (diagnostic for Cx. pipiens), the PCR identified 26/55 as Cx. pipiens, 12/55 as hybrids, and 17/55 as Cx. quinquefasciatus. Males with $DV/D$ ratios between 0.2 and less than 0.4 (hybrid category) produced PCR identifications of 42/293 as Cx. pipiens, 73/293 as hybrids, and 178/293 as Cx. quinquefasciatus. Males with $DV/D$ ratios above 0.4 (Cx. quinquefasciatus category) produced PCR identifications 9/127 as Cx. pipiens, 21/127 as hybrids, and 98/127 as Cx. quinquefasciatus. Most lack of congruence occurred due to the PCR identifying many Cx. pipiens and hybrid $DV/D$-categorized specimens as Cx. quinquefasciatus. Although less frequent, identification of Cx. pipiens by PCR was found in $DV/D$ hybrid and Cx. quinquefasciatus categories and PCR hybrid identifications were found in $DV/D$ Cx. pipiens and Cx. quinquefasciatus categories. As we do not know the parents of these field-collected mosquitoes, it is impossible to tell which identification method is more flawed. These data indicate that either one or both methods of identification do not work for properly identifying Cx. pipiens complex mosquitoes of Fresno County.

Inconsistent results were also found in hybrids originating from laboratory-generated crosses of mosquito colonies of known identity. Colony mosquitoes consistently produced congruent PCR and $DV/D$ ratio identifications as either Cx. pipiens (SH colonies) or Cx. quinquefasciatus (LA colony) based on at least 100 individuals throughout their time in colony. This indicates that there is no polymorphism at the primer site of the Ace.2 locus, which would incorrectly identify them. Out of 30 hybrid males picked from the AF1 cross (LA $\delta \times$ SH $\delta$) the PCR identified 27 as hybrids (2 bands of equal intensity) and 3 as Cx. pipiens (Figure 6). In the recipi-
local BF1 cross (LA $\delta \times$ SH $\varphi$), 16 males produced 2 bands, and in 14 only the Cx. quinquefasciatus diagnostic fragment was amplified (Figure 6). In all 16 cases, according to the naked eye, the Cx. quinquefasciatus fragment was more than twice the intensity of the Cx. pipiens diagnostic fragment.

A summary of DV/D ratios of 30 males each from the parents, hybrids, and back-crosses are given in Figures 7 and 8. Figure 7 represents the DV/D results from the cross originating from SH $\delta \times$ LA $\delta$. DV/D ratios of hybrids from both the SH $\varphi \times$ LA $\delta$ and SH $\varphi \times$ LA $\delta$ were all smaller than 0.2, which is in fact within the Cx. pipiens range and not within the expected hybrid range between 0.2 and 0.4. Inbreeding the hybrids produced variable results where in the case of the SH $\varphi \times$ LA $\delta$ hybrids (AF2) the DV/D ratios were similar to their hybrids and in the case of the SH $\varphi \times$ LA $\delta$ hybrids (BF2) the DV/D values were mostly within the expected hybrid range of 0.2-0.4. Back-crossing to Cx. quinquefasciatus parents caused a general shift toward Cx. quinquefasciatus DV/D values but were mostly still within the hybrid range of 0.2-0.4. Because the SH $\varphi \times$ LA $\delta$ hybrids (BF2) had DV/D values typical for Cx. pipiens, back-crossing them to Cx. pipiens parents caused no change in values.

**DISCUSSION**

From mid-June to mid-October 2005, considerable WN virus activity was detected in Fresno County. While this study focused only on WNV infections in gravid Cx. pipiens s.l., at the same time, mosquito pool (from CDC-style carbon dioxide traps) and sentinel chicken seroconversion surveillance for WNV was carried out by the Consolidated Mosquito Abatement District (CMAD) in collaboration with the California Vector-Borne Disease Surveillance System (http://www.calsurv.org/arbo.html). The CMAD submitted and received positive RT-PCR results from 22/87 Cx. tarsalis, 33/147 Cx. pipiens s.l., and 3/4 Cx. stigmatosoma for an overall minimum infection rate of 8/1000. Mosquito pool sizes ranged from 6 to 50, and pools that were WNV RNA positive were evenly spread throughout the CMAD (Figure 2). Cx. tarsalis and Cx. quinquefasciatus are competent bridge vectors that permit the virus to move from a strictly enzootic pattern to humans. This was confirmed by 43 human cases in people who live within the CMAD district boundaries. The CMAD set up 6 sentinel surveillance flocks of 10 chickens in each flock for a total of 60 chickens. The first chickens seroconverted in the 3rd week of July, about 5 weeks after the first Cx. pipiens member was positive. The next week (first week of Aug), a further 22 seroconverted, and by the middle of October 51/60 chickens had seroconverted, confirming once again intense WN transmission in Fresno County.

**FIGURE 5.** Proportions of male progeny identified by Ace.2 PCR and categorized according to DV/D ratio showing the lack of congruency between the 2 identification methods.

**FIGURE 6.** Example of 1.5% agarose gel showing the Ace.2 PCR bands characterizing Cx. pipiens s.l. hybrids produced from crosses of parent colonies from Northern (Shasta colony, Cx. pipiens) and Southern California (Los Angeles, Cx. quinquefasciatus). Lanes 1 and 18, 100-bp ladder; lanes 2–9, hybrids from LA $\delta \times$ SH $\varphi$ (AF1); lanes 10–17, hybrids from SH $\varphi \times$ LA $\delta$ (BF1). Three of the individuals produced the expected bands diagnostic for hybrids (lanes 5, 7, 9). Lanes 2 and 10–14 showed a weak Cx. pipiens-specific band (610 bp) and an intense Cx. quinquefasciatus-specific band (274 bp), while the rest produced only the Cx. quinquefasciatus-specific band.
The infection rate based on testing of individual gravid trap \textit{Cx. pipiens} s.l. females conducted was double that of the MIR of CO$_2$ \textit{Cx. pipiens} s.l. pooled trap collections. The higher infection rates in gravid trap and individually sampled females can be explained. Firstly, MIRs were calculated assuming that each positive pool contained a single infected mosquito,\textsuperscript{30} which gives an underestimate of actual infection rates. Conversely, they can also give an inflated value if you have a positive in a pool with a small number of mosquitoes. Secondly, CO$_2$-baited traps collect mostly young, unfed, nuliparous females (> 65%) of \textit{Cx. tarsalis} and \textit{Cx. quinquefasciatus}.\textsuperscript{40-42} Hence, the majority of the mosquitoes collected in CO$_2$-baited traps have never come into contact with a viremic host, unlike that of gravid-female–based collections consisting of females who would have at least had one opportunity to come in contact with an infective host. From our experience, no members of the \textit{Cx. pipiens} complex are autogenous in Fresno County (unpublished data). Many of the feral \textit{Cx. pipiens} s.l. positive for WNV RNA will probably not transmit virus in nature. Several will succumb to predation, lethal insecticide exposure, or natural death before completion of the extrinsic incubation period. Furthermore, vector competence of different \textit{Cx. pipiens} s.l. populations varies greatly depending on dose of infection and the genetic determinants of barriers to infection. For example, transmission rates can vary from 52% (Bakersfield \textit{Cx. quinquefasciatus}) to as low as 6% (Coachella Valley \textit{Cx. quinquefasciatus}) when infected with the same dose of WNV.\textsuperscript{4}

WNV infection rates in \textit{Cx. quinquefasciatus} rose steadily from early June, reaching a peak in August, which is the hottest time of the year, and then declining as the season progressed through to October (Figure 4). Total numbers of gravid \textit{Cx. pipiens} s.l. collected stayed fairly constant throughout the season, peaking in July at 130 and declining to 100 in September (only 2 trapping rotations occurred in October, collecting 39 mosquitoes). This trend of peak WNV infection in mosquitoes coinciding with the warmest temperatures also occurred in 2004 in Coachella Valley, Los Angeles, and Kern County.\textsuperscript{38} According to Reisen and others,\textsuperscript{38} the invading NY99 WNV strain requires warm temperatures for efficient virus growth and transmission.

In this study, vertical transmission of WNV was confirmed among members of the \textit{Cx. pipiens} complex by testing 10 male offspring from positive feral mothers. In all instances, only 1 or 2 out of the 10 male offspring tested were positive. This means that only a small proportion of the offspring become infected via transovarial transmission. If males become infected via transovarial transmission, then this raises the possibility of venereal transmission. Venereal transmission of WNV was demonstrated by force mating experiments; however, titers of virus in females infected via this route remained too low for this infection to be passed vertically or horizontally to her progeny or vertebrate host, respectively.\textsuperscript{43}

It is unfortunate that we cannot comment on differences in WNV infection rates among members of the \textit{Cx. pipiens} complex in Fresno County because of inconsistency of the identification methods that distinguish complex members. Problems arose when assessments were made to ascertain, based on the mothers and her progeny PCR identifications, the degree of conspecific matings between \textit{Cx. pipiens} and \textit{Cx. quinquefasciatus} in the Fresno County hybrid zone. Identifications of offspring homozygous for the \textit{Cx. pipiens}-specific Aec.2 allele were obtained from mothers homozygous for the \textit{Cx. quinquefasciatus}-specific allele and vice versa. These offspring and mother identifications cannot be possible as no combinations of \textit{Cx. pipiens} s.l. mating pair would give these results. This ambiguity of diagnostic PCR reaction results based on 15 families even raises concerns about the accuracy of the other 193 families with respect to with whom the mother mated and makes assessments of degree of conspecific mating, hybrid inbreeding, and back-crossing among complex members in Fresno County unjustified. There was also very poor congruence between DV/D ratio and PCR identifications of each of the males. No particular trend was evident in which direction the 2 diagnostic methods mismatched.

These incongruencies between DV/D ratio and PCR identifications required taking a step back to reconsider the validity of DV/D that is ubiquitously used as the \textit{a priori} method to distinguish \textit{Cx. pipiens} from \textit{Cx. quinquefasciatus} and their hybrids. Crosses of northern California \textit{Cx. pipiens} to southern California \textit{Cx. quinquefasciatus} revealed several inconsis-
tencies in the general understanding of the validity of DV/D. All the hybrids had ratios typical for Cx. pipiens, and only the back-crosses to the Cx. quinquefasciatus parents had intermediate ratios that, in fact, consisted of a continuous range of values between hybrids and the parental form. This means that, at the very least, among California Cx. pipiens s.l. categorizing DV/D into finite values corresponding to taxa and hybrids is a futile exercise. This suggests that the DV/D phenotype and expression occurs as a result of a complex interaction of underlying genetic and environmental (epigenetic) factors and cannot be considered a neutral genetic marker. The maintenance of the DV/D Cx. pipiens and Cx. quinquefasciatus phenotypes north of 39°N and south of 36°N latitudes, respectively, is most likely a result of environmental factors and perhaps, more specifically, temperature. The influence of temperature on DV/D ratio outcome has previously been shown by Wilton and Jakob.44 While differences in the clinal change in temperature can explain the DV/D ratio value clines in North America, it still remains to be explained why only the 2 discrete Cx. pipiens and Cx. quinquefasciatus DV/D ratio categories occur in sympathy in South Africa.10

Misidentification of Cx. pipiens/Cx. quinquefasciatus hybrids from known California parental origin by Acc2 PCR identification warrants further discussion and investigation. The outcome and interpretation of the PCR are critically affected by the primer concentrations, as there is only one nucleotide that differentiates Cx. pipiens from the other complex members.28 In this study, we often obtained amplification of 1 band in hybrids, and when we did obtain 2 bands we observed a continuous range from that of equal intensities to faint bands (just visible to the eye) of fragment size diagnostic for one or other of the taxa. Separating hybrids from Cx. pipiens and Cx. quinquefasciatus remains an issue as there is still no appropriate diagnostic character.

An over-riding issue that still needs to be resolved in California is to assess gene flow among Cx. pipiens s.l. The wide-spread spatial distribution of similar insecticide resistance mechanisms and genes in Californian Cx. pipiens s.l. populations suggests that extensive gene flow is not just restricted to the hybrid zone in the Central Valley but extends further north and south. The organophosphate resistance-causing A2B2 esterase complex and the pyrethroid knock-down resistance (kdr-type) allele in the voltage-gated sodium channel gene occur in both northern Cx. pipiens (Shasta County) and the southern Cx. quinquefasciatus (San Diego County) populations (unpublished data). It is possible that these resistance mechanisms arose independently in the California Cx. pipiens and Cx. quinquefasciatus populations, but the spread of these mechanisms via gene flow between the 2 taxa seems a more plausible explanation. This extensive gene flow provides credence to Tabachnick and Powell’s conclusion that individuals classified as Cx. pipiens in northern California possess large amounts of the southern Cx. quinquefasciatus genotype and vice versa. This means that the current classification of Cx. pipiens and Cx. quinquefasciatus and hybrid populations/individuals used in systematics studies and comparative ecological, behavioral, vectorial capacity, and epidemiologic investigations may produce misleading results.

Received May 9, 2007. Accepted for publication November 25, 2007.


