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POLYMERASE CHAIN REACTION ASSAY IDENTIFIES NORTH AMERICAN MEMBERS OF THE *CULEX PIPIENS* COMPLEX BASED ON NUCLEOTIDE SEQUENCE DIFFERENCES IN THE ACETYLCHOLINESTERASE GENE *ACE.2*

STEPHEN ASPEN AND HARRY M. SAVAGE

Division of Vector-Borne Infectious Disease, Centers for Disease Control and Prevention, PO Box 2087, Fort Collins, CO 80522

ABSTRACT. Nucleotide sequence differences in the acetylcholinesterase gene *Ace.2* were used to develop an assay to distinguish among North American mosquitoes of the *Culex pipiens* complex. Taxon-specific polymerase chain reaction primers based on sequence differences within intron 2 of *Ace.2* distinguish among the sibling species *Cx. pipiens* Linneaus and *Cx. quinquefasciatus* Say and their F_1 hybrids. This assay may be used to confirm the species composition of mosquito pools, identify individual specimens collected in arbovirus surveillance programs and other mosquito studies, and define zones of hybridization.

KEY WORDS Acetylcholinesterase gene, *Ace.2, Culex pipiens* complex, *Culex pipiens, Culex quinquefasciatus,* molecular species identification

INTRODUCTION

North American mosquitoes of the *Culex pipiens* complex include *Cx. pipiens* Linnaeus, *Cx. quinquefasciatus* Say, and their hybrids. Members of the *Cx. pipiens* complex are the primary enzootic vectors of West Nile virus in North America (Nasci et al. 2001b) and the primary enzootic and epidemic vectors of St. Louis encephalitis virus (Mitchell et al. 1980). Adult females of the *Cx. pipiens* complex are frequently collected in gravid traps (Reiter 1983, Savage et al. 1993), or with aspirators as part of arbovirus surveillance programs (Nasci et al. 2001a), blood-meal host studies (Apperson et al. 2002), and other mosquito investigations. Mosquitoes of the *Cx. pipiens* complex in North America can be distinguished from other common *Culex* (*Culex*) mosquitoes by careful examination of morphological characters (Apperson et al. 2002) and by taxon-specific polymerase chain reaction (PCR) primers (Crabtree et al. 1995, Aspen et al. 2003).

Identification of mosquitoes to species is essential to assess vectorial status and to develop effective control and prevention strategies. However, progress in our understanding of the relative roles of taxa within the *Cx. pipiens* complex in virus transmission have been stymied because reliable morphological characters to distinguish female adults, larvae, and pupae within this complex are unknown. Adult males of the *Cx. pipiens* complex are morphologically distinguishable only through careful comparison of the male genitalia and use of the DV/D ratio, where DV is the extension of the ventral arm of the phallosome laterally of its intersection with the dorsal arm, and D is the distance between the two intersections of the dorsal and ventral arms (Sundararaman 1949, Barr and Kartman 1951). Based on previous analysis of male specimens from the USA (Barr 1957), *Cx. pipiens* is assumed to be present in areas north of 39°N and *Cx. quinquefasciatus* is assumed to be present in areas south of 36°N. In the middle latitudes of the USA between 36°N and 39°N, both nominal taxa and hybrids (Barr 1957), and introgressed specimens (Tabachnick and Powell 1983, Miller et al. 1996) may be present. This simple "latitude" concept of species distributions is known to be complicated by orographic and other environmental variables in California (Tabachnick and Powell 1983, Urbanelli et al. 1997).

Immunoelectrophoresis (Cupp and Ibrahim 1973) and enzyme electrophoresis techniques (Cheng et al. 1982, Urbanelli et al. 1985, 1995) have been employed with some success to separate members of the *Cx. pipiens* complex. Species-specific PCR primers based on ribosomal DNA (rDNA) internal transcribed spacers have been designed that used subtractive hybridization (Crabtree et al. 1997); however, only *Cx. pipiens*–specific primers could be designed, meaning that *Cx. quinquefasciatus* could only be identified by a lack of PCR amplification, instead of by a positive marker. A restriction digest of an amplified region of rDNA has been reported (Severini et al. 1996) to distinguish *Cx. pipiens* from *Cx. quinquefasciatus* when the specimens are from different continents (*Cx. pipiens* from Italy compared with *Cx. quinquefasciatus* from Africa and North America); however, our laboratory has been unable to reproduce these results when using mosquitoes collected within North America (Aspen and Savage, unpublished data).

Bourguet et al. (1998) reported nucleotide sequence differences between *Cx. pipiens* and *Cx. quinquefasciatus* in an acetylcholinesterase gene, designated *Ace.2* by Malcolm et al. (1998). The *Ace.2* gene is 1 of 2 acetylcholinesterase genes reported to exist in *Cx. pipiens* (Bourguet et al. 1996). The *Ace.2* gene is not associated with insecticide resistance (Malcolm et al. 1998), and the function

Table 1. *Culex* mosquito populations used in this study.

Taxa	Geographic origin	Source
Cx. pipiens	Ft. Collins, CO	H. Savage
	Queens, NY	C. Apperson
Cx. quinquefasciatus	Monroe, LA	H. Savage
	East Baton Rouge Parish, LA	R. Nasci
	Gainesville, FL	J. Reinert
	Jefferson Co., FL	M. Godsey
Cx. pipiens–Cx. quinquefasciatus hybrids	Laboratory hybridization between *Cx. pipiens* from Colorado and *Cx. quinquefasciatus* from Louisiana	
Cx. nigripalpus	Jefferson Co., FL	M. Godsey
	Vero Beach, FL	R. Rutledge
Cx. restuans	Burlington, VT	H. Savage
	Alleghany Co., NC	B. Harrison
Cx. salinarius	Monroe, LA	H. Savage
	Jefferson Co., FL	M. Godsey

of *Ace.2* remains unknown. The *Ace.2* gene is located on chromosome 1 and is tightly linked to the sex locus (Malcolm et al. 1998). A 700-base pair (bp) amplicon of *Ace.2* (encompassing part of exon 2, intron 2, and part of exon 3) can be digested with the restriction enzyme (RE) *Sca*I to reveal different restriction profiles for *Cx. pipiens* and *Cx. quinquefasciatus* (Bourguet et al. 1998). In North America, this assay has limited utility because *Cx. restuans* Theobald and *Cx. salinarius* Coquillett share the same *Sca*I restriction profile as *Cx. quinquefasciatus* (Aspen and Savage, unpublished data). Therefore, the RE assay (Bourguet et al. 1998) cannot be used to evaluate mosquito DNA for the presence of *Cx. pipiens* and *Cx. quinquefasciatus* in mosquito pools from the eastern USA, where *Cx. restuans* and *Cx. salinarius* might be represented.

The goal of this study was to obtain nucleotide sequence data on the *Ace.2* gene for 5 common *Culex* species from the eastern USA and to develop species-specific PCR primers to distinguish *Cx. pipiens* and *Cx. quinquefasciatus* that would not produce amplicons in the presence of DNA from other common *Culex* species from North America.

MATERIALS AND METHODS

Mosquitoes: The origin and source of each mosquito population used for this study are given in Table 1.

A colony of *Cx. pipiens* was established from overwintering adult female mosquitoes collected in a concrete storm sewer in Ft. Collins, CO, by H. Savage. A colony of *Cx. quinquefasciatus* was established by H. Savage from egg rafts collected from East Baton Rouge, LA, by R. Nasci. Pure strains were passed through 1 generation to assure colonization of parent strains and crosses were initiated with 2nd-generation adults. *Culex pipiens–Cx. quinquefasciatus* 1st filial generation (F_1) hybrids, 2nd filial generation (F_2) specimens, and various backcrosses were produced in the insectary.

Mosquito genomic DNA preparation: Mosquito genomic DNA was extracted from individual specimens or mixed pools by using the Qiagen DNeasy Tissue Kit (Qiagen Inc., St. Clarita, CA) with the following modifications. Mosquitoes were ground in BA-1 medium (1X M199-H, 0.05 M Tris, pH 7.6, 1% bovine serum albumin, 0.35 g/liter of sodium bicarbonate, 100 units/ml of penicillin, 100 μg/ml of streptomycin, and 1 μg/ml of fungizone). The DNA was eluted twice in 200 μl of warm (40°C) nuclease-free water (Amresco, Solon, OH) for a total eluate volume of 400 μl. The DNA concentration was determined by using the absorbance of ultraviolet radiation at 260-nm wavelength on a spectrophotometer.

Cloning and sequencing: A region of the acetylcholinesterase gene *Ace.2* (including part of exon 2, intron 2, and part of exon 3) was amplified from mosquito genomic DNA as described by Bourguet et al. (1998). The amplicon was purified by using the Qiaquick PCR Purification Kit (Qiagen) and ligated into the plasmid pGEM-T Easy (Stratagene, La Jolla, CA) before transforming the *Escherichia coli* strain XL-1 Blue (Stratagene) by heat shock. Recombinant clones were selected on agar plates with tetracycline and ampicillin. A single clone from each specimen was sequenced with two different primers in each direction in an ABI 377 sequencer (Applied Biosystems, Foster City, CA) with the BigDye Terminator version 3 reaction mix (Applied Biosystems).

Species-specific PCR primer design: The *Ace.2* sequences from *Cx. pipiens, Cx. quinquefasciatus, Cx. restuans, Cx. salinarius,* and *Cx. nigripalpus* were aligned by using Lasergene's Megalign program, version 5.03 (DNAStar, Inc., Madison, WI). Lasergene's Primerselect program, version 5.03 (DNAStar, Inc.) was used to design one forward-sense PCR primer (PACEF290) based on a *Cx. pi-*

Table 2. Polymerase chain reaction primers used in this study.

Primer	Sequence (5′ to 3′)
B1246	TGGAGCCTCCTCTTCACGGC
PACEF290	TTATAGTGATATGGTGGAAA
QACEF290	TTATAGTAAAATGGTTGAGA

piens–specific sequence and a different forward-sense primer (QACEF290) based on a *Cx. quinquefasciatus*–specific sequence. These primers were designed to anneal within intron 2 and to work with a reverse-sense primer (B1246 [Bourguet et al. 1998]) that complements a consensus sequence within exon 3. The nucleotide sequences of these primers are provided in Table 2.

Species-diagnostic PCR: Each 25-μl reaction mixture contained 1× of GeneAmp PCR Buffer I (10 mM Tris-HCl pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% w/v gelatin, Applied Biosystems), 0.2 mM of each deoxynucleotide triphosphate (Roche Diagnostics Corp., Indianapolis, IN), 80 nM of the reverse-sense primer B1246, 20 ng of template DNA, and 0.5 U of Amplitaq DNA Polymerase (Applied Biosystems). Each reaction also contained either 80 nM of PACEF290 or 320 nM of QACEF290. The reaction mixtures were placed in a PTC-100 thermal cycler (MJ Research, Inc., Incline Village, NV) programmed for 1 cycle at 93°C for 5 min, followed by 27 cycles of 93°C for 1 min, 52°C for 1 min, and 72°C for 90 sec, and completed by 1 cycle at 72°C for 10 min. The PCR product was visualized with ethidium bromide on a 1.8% agarose gel.

RESULTS

Nucleotide sequencing

A region of the *Ace.2* gene for *Cx. pipiens* (Ft. Collins, CO), *Cx. quinquefasciatus* (Jefferson Co., FL), *Cx. restuans* (Alleghany Co., NC), *Cx. salinarius* (Jefferson Co., FL), and *Cx. nigripalpus* (Vero Beach, FL) was amplified, cloned, and sequenced (GenBank acquisition numbers AY196910–AY196914).

Species-diagnostic PCR to identify *Cx. pipiens* or *Cx. quinquefasciatus*

Primers PACEF290 and QACEF290, included in Table 2, were tested on different DNA templates extracted from the mosquito populations listed in Table 1. Reactions containing PACEF290 produced a 487-bp amplicon when *Cx. pipiens* template DNA was used, and reactions containing QACEF290 produced a 487-bp amplicon when *Cx. quinquefasciatus* template DNA was used (Fig. 1). No amplification was observed in reactions containing either PACEF290 and *Cx. quinquefasciatus* template DNA or QACEF290 and *Cx. pipiens* template DNA. When template DNA from *Cx. restuans, Cx. salinarius,* or *Cx. nigripalpus* was used, no amplification occurred. When DNA from mixed species pools composed of up to 40 specimens of various *Cx.* (*Culex*) spp. mosquitoes and including 1 or more specimens of either *Cx. pipiens* or *Cx. quinquefasciatus* was used as template, the presence of the correct species was detected. When template DNA from both *Cx. pipiens* and *Cx. quinquefasciatus* were present in mixed species pools, PCR reactions with either species-specific primers resulted in 487-bp amplicons.

PCR on *Cx. pipiens–Cx. quinquefasciatus* hybrids

When template DNA from individual specimens of both types of laboratory-reared F_1 hybrids between *Cx. pipiens* and *Cx. quinquefasciatus* was used, amplification resulted from reactions containing PACEF290 and from reactions containing QACEF290 (Fig. 1). All F_1 hybrids were detected by the presence of both a *Cx. pipiens* and a *Cx. quinquefasciatus* amplicon.

When individual specimens were tested from selected backcrosses between F_1 hybrids and both *Cx. pipiens* and *Cx. quinquefasciatus,* results corresponded exactly (see Appendix 1) to predictions of a sex-linked model as reviewed by Clements (1992). The only departure from predictions was the apparent overabundance of *Cx. pipiens–Cx. quinquefasciatus* male hybrids and the corresponding deficiency of *Cx. pipiens* males among F_2 specimens in cross 2 (exact binomial test, $P = 0.04$; Appendix 1).

DISCUSSION

Accurate identification of field-collected adult mosquitoes is a critical step in arbovirus surveillance, blood-meal host preference investigations, and other studies on mosquito biology. However, it is impossible to morphologically identify individual adult females of *Cx. pipiens, Cx. quinquefasciatus,* and their hybrids. Our inability to distinguish these taxa morphologically has stymied research on the biology of these nominal taxa and hybrids, and studies on the interaction of these taxa and hybrids as arbovirus vectors in the hybrid zone, which covers much of the middle latitudes of the USA.

We have developed a PCR assay for the identification of individual specimens of *Cx. pipiens, Cx. quinquefasciatus,* and their F_1 hybrids. This assay makes use of PCR primers that are complementary to taxon-specific nucleotide sequences within intron 2 of the *Ace.2* gene. The DNA from the specimen of interest is run in 2 separate PCR reactions, 1 with the *Cx. pipiens* primer and a 2nd with the *Cx. quinquefasciatus* species-specific primer. The reactions can be run simultaneously, because both use the same thermal cycling program. Specimens of *Cx. pipiens* produce a 487-bp amplicon only from the reaction with the *Cx. pipiens* primers, whereas

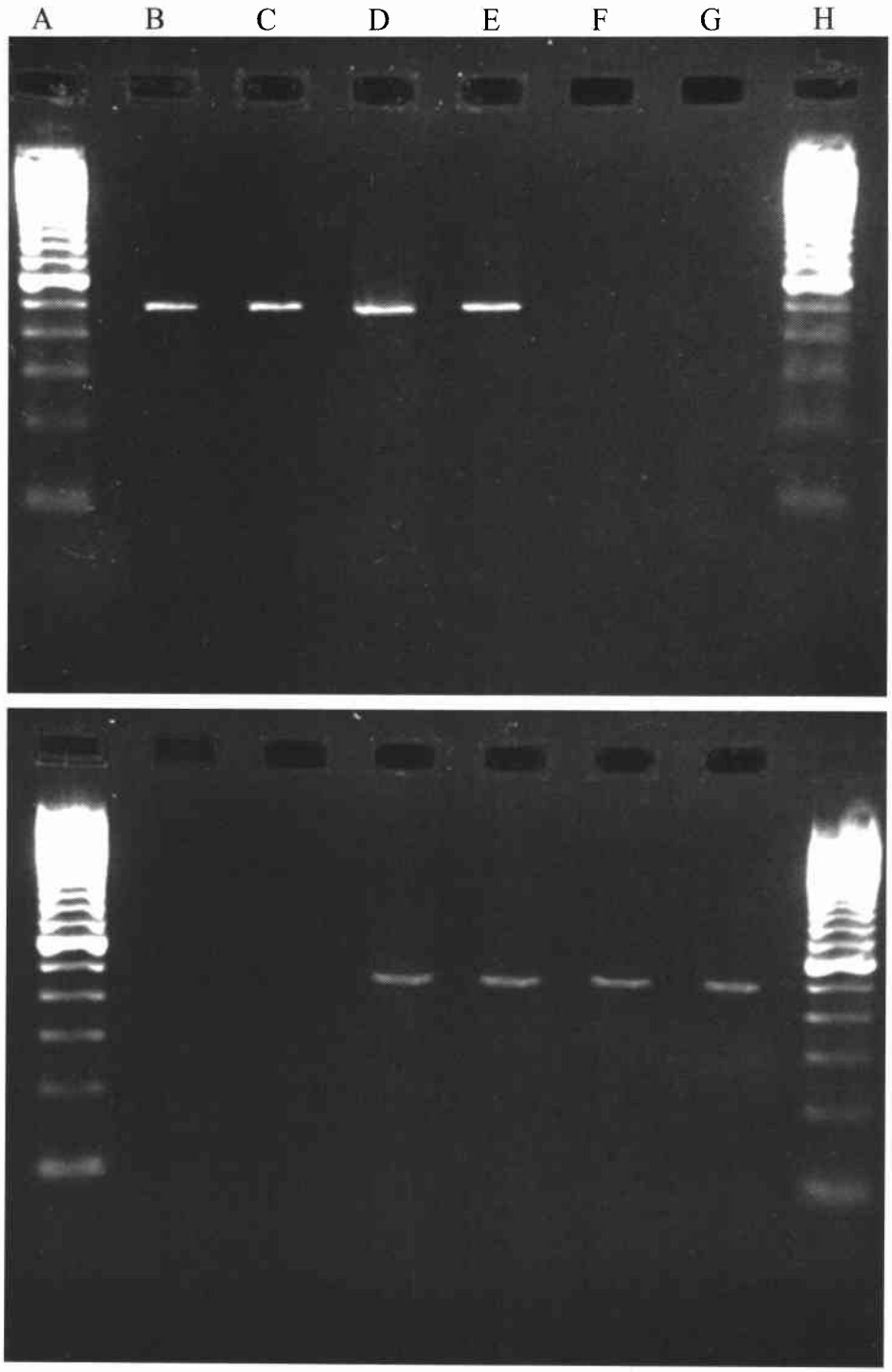

Fig. 1. Polymerase chain reaction amplicons resulting when *Culex pipiens*– or *Cx. quinquefasciatus*–specific forward-sense primers are used with the universal reverse-sense primer B1246. (Top) Polymerase chain reaction amplicons resulting when *Cx. pipiens*–specific primer, PACEF290, is used. (Bottom) Polymerase chain reaction amplicons resulting when *Cx. quinquefasciatus*–specific primer, QACEF290, is used. Both top and bottom reactions contain the following template DNA or markers in lanes A–H: (A and H) 100-bp DNA marker; (B) *Cx. pipiens* (Ft. Collins, CO); (C) *Cx. pipiens* (Queens, NY); (D) and (E) *Cx. pipiens*–*Cx. quinquefasciatus* F_1 hybrids; (F) *Cx. quinquefasciatus* (Monroe, LA); (G) *Cx. quinquefasciatus* (Jefferson Co., FL).

specimens of *Cx. quinquefasciatus* produce a 487-bp amplicon only from the reaction with the *Cx. quinquefasciatus* primers. *Cx. pipiens–Cx. quinquefasciatus* F_1 hybrids are easily detected by the production of both a *Cx. pipiens* amplicon and a *Cx. quinquefasciatus* amplicon.

The interpretation of results from mixed species pools, for example, virus-positive pools processed during arbovirus surveillance, is less straightforward. Pure pools of *Cx. pipiens* or *Cx. quinquefasciatus* would be easily recognizable by the presence of 1 amplicon and the absence of the other. However, if the testing of a mosquito pool resulted in the production of both amplicons, it is possible that the pool includes representatives of both species, or only hybrids, or 1 or both nominal species and 1 or more hybrids. In such an instance, one can only state that the pool contains DNA from both nominal species, and that both nominal taxa and hybrids might be present.

The *Ace.2* locus is located on chromosome 1, or the sex chromosome, and is tightly linked to the sex locus in *Culex* mosquitoes. A PCR assay with the *Cx. pipiens* and *Cx. quinquefasciatus* species-specific primers on laboratory-generated F_1 backcrosses with *Cx. pipiens* and *Cx. quinquefasciatus* confirms the sex-linked inheritance of the *Ace.2* gene (Appendix 1). The composition of genotypes in the F_2 insects indicates that male hybrids may have a selective advantage over *Cx. pipiens* males under laboratory conditions (Appendix 1, cross 2). This is consistent with observations that colony initiation of *Cx. pipiens* is more difficult than with *Cx. quinquefasciatus* and populations of hybrid origin (H. Savage, unpublished data).

Assuming that genotypes of field-collected F_2 specimens correspond to predictions of the Hardy–Weinberg principle, F_2 specimens would display the typical 1PP:2QP:1QQ distribution for the *Ace.2* alleles. Therefore, 50% of F_2 specimens would produce both a *Cx. quinquefasciatus* and a *Cx. pipiens* amplicon and be identified as true hybrids, 25% of F_2 specimens would produce only a *Cx. pipiens* amplicon and be identified as *Cx. pipiens,* and 25% of F_2 specimens would produce only a *Cx. quinquefasciatus* amplicon and be identified as *Cx. quinquefasciatus.* Although all specimens are correctly identified based on the composition of their *Ace.2* alleles, because of recombination, the F_2 specimens of *Cx. pipiens* and *Cx. quinquefasciatus* may indeed possess alleles for other genes that are typical of its sister species. Similar arguments can be presented for the backcrosses. Therefore, when working with specimens in the hybrid zone, an identification system based on a single gene will underestimate the degree of possible gene mixing. The identification of an additional diagnostic gene for these taxa, particularly a mitochondrial gene, would increase our ability to recognize true parental specimens and lead to a better understanding of hybridization between *Cx. pipiens* and *Cx. quinquefasciatus.*

The assay described herein offers new opportunities to investigate questions regarding introgression and hybridization within the *Cx. pipiens* complex as well as a tool for mosquito species identification in virus-positive mosquito pools detected during routine arbovirus surveillance programs. However, this assay requires 2 PCR reactions per sample to identify nominal taxa and hybrids. Future efforts to design species-specific primers that produce different-sized amplicons and allow their use in a single PCR reaction, the development of a real-time PCR assay with allelic discrimination, or both, would be beneficial.

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Appendix 1.

Summary of testing with *Culex pipiens* and *Cx. quinquefasciatus* species-specific *Ace.2* primers of individual specimens from the 2nd filial generation (F_2), and the 1st filial generation (F_1) backcrossed (BC) with parent strains. Inheritance of sex in *Culex* mosquitoes with the M/m system, and inheritance of sex-linked characters are reviewed in Clements (1992). Crosses were made in the laboratory with a *Cx. pipiens* strain from Ft. Collins, CO, and a *Cx. quinquefasciatus* strain from East Baton Rouge Parish, LA. Abbreviations: ♂, male; ♀, female; M, male sex allele, dominant; m, female sex allele, recessive; Q, *Cx. quinquefasciatus Ace.2* amplicon (allele); P, *Cx. pipiens ACE.2* amplicon (allele).

Cross 1. F_2 specimens, ♂ parents were F_1 hybrids MQ/mP, ♀ parents were F_1 hybrids mQ/mP, $n = 29$ (n♂ = 17, n♀ = 12).

	♂ QQ	♂ QP	♀ PP	♀ QP
Expected	8.5	8.5	6	6
Observed	7	10	5	7

Cross 2. F_2 specimens, ♂ parents were F_1 hybrids MP/mQ, ♀ parents were F_1 hybrids mQ/mP, $n = 31$ (n♂ = 15, n♀ = 16).

	♂ PP	♂ QP	♀ QQ	♀ QP
Expected	7.5	7.5	8	8
Observed	3	12	9	7

Cross 3. BC specimens, ♂ parents were F_1 hybrids MQ/mP, ♀ parents were *Cx. quinquefasciatus* mQ/mQ, n = 10.

	♂ QQ	♂ QP	♀ QQ	♀ QP
Expected	5	0	0	5
Observed	5	0	0	5

Cross 4. BC specimens, ♂ parents were F_1 hybrids MQ/mP, ♀ parents were *Cx. pipiens* mP/mP, n = 10.

	♂ PP	♂ QP	♀ PP	♀ QP
Expected	0	5	5	0
Observed	0	5	5	0

Cross 5. BC specimens, ♂ parents were F_1 hybrids MP/mQ, ♀ parents were *Cx. pipiens* mP/mP, n = 10.

	♂ PP	♂ QP	♀ PP	♀ QP
Expected	5	0	0	5
Observed	5	0	0	5

Cross 6. BC specimens, ♂ parents were F_1 hybrids MP/mQ, ♀ parents were *Cx. quinquefasciatus* mQ/mQ, n = 10.

	♂ QQ	♂ QP	♀ QQ	♀ QP
Expected	0	5	5	0
Observed	0	5	5	0

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ERRATA

On page 298 in our paper "Has *Aedes albopictus* established in California?", *Journal of the American Mosquito Control Association* 19(4):297–300, by Minoo B. Madon, Jack E. Hazelrigg, Michael W. Shaw, Susanne Kluh and Mir S. Mulla, the legends for Figures 1 and 2 were reversed. Fig. 1 should read "Ovitrap" and Fig. 2 should read "Modified Encephalitis Virus Surveillance (EVS)/CO_2-baited trap."

Minoo B. Madon

On page 325, in the section subtitled "Species-diagnostic PCR to identify *Cx. pipiens* or *Cx. quinquefasciatus,*" in our paper "Polymerase chain reaction assay identifies North American members of the *Culex pipiens* complex based on nucleotide sequence differences in the acetylcholinesterase gene *ACE.2*," *Journal of the American Mosquito Control Association* 19(4):323–328 by Stephen Aspen and Harry M. Savage, we state that 20 ng of template DNA was used. In the current protocol that we use on a routine basis in our laboratory, the amount of template DNA is typically 300 ng.

Harry M. Savage
Stephen Aspen