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A mutation in the voltage-gated sodium channel gene associated with pyrethroid resistance in Latin American *Aedes aegypti*

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Abstract

Pyrethroids are commonly used as mosquito adulticides and evolution of resistance to these compounds is a major threat to public health. 'Knockdown resistance' to pyrethroids (kdr) is frequently caused by nonsynonymous mutations in the voltage-gated sodium channel transmembrane protein (para) that reduce pyrethroid binding. Early detection of kdr is critical to the development of resistance management strategies in mosquitoes including *Aedes aegypti*, the most prevalent vector of dengue and yellow fever viruses. Brengues *et al.* described seven novel mutations in hydrophobic segment 6 of domain II of para in *Ae. aegypti*. Assays on larvae from strains bearing these mutations indicated reduced nerve sensitivity to permethrin inhibition. Two of these occurred in codons Iso1011 and Val1016 in exons 20 and 21 respectively. A transition in the third position of Iso1011 encoded a Met1011 replacement and a transversion in the second position of Val1016 encoded a Gly1016 replacement. We have screened this same region in 1318 mosquitoes in 32 additional strains; 30 from throughout Latin America. While the Gly1016 allele was never detected in Latin America, we found two new mutations in these same codons. A transition in the first position of codon 1011 encodes a Val replacement while a transition in the first position of codon 1016 encodes an Iso replacement. We developed PCR assays for these four mutations that can be read either on an agarose gel or as a melting curve. Selection experiments, one with deltamethrin on a field strain from Santiago de Cuba and another with permethrin on a strain from Isla Mujeres, Mexico rapidly increased the frequency of the Iso1016 allele. Bioassays of F_3 offspring arising from permethrin susceptible Val1016 homozygous parents and permethrin resistant *Iso*1016 homozygous parents show that Iso1016 segregates as a recessive allele in conferring kdr. Analysis of segregation between alleles at the 1011 and 1016 codons in the F_3 showed a high rate of recombination even though the two codons are only separated by a ~250 bp intron. The tools and information presented provide a means for early detection and characterization of kdr that is critical to the development of strategies for resistance management.

Keywords: pyrethroids, knockdown resistance, *Aedes aegypti*, para, mutation detection.

Introduction

Synthetic pyrethroids such as permethrin, deltamethrin, resmethrin and sumithrin are commonly applied mosquito adulticides. In addition, bednets, curtains and other household items treated with pyrethroids for personal protection are seeing increased use (Zaim *et al.*, 2000). With the deployment of pyrethroid-treated materials for dengue vector control likely to become more widespread in the future (Kroeger *et al.*, 2006), the occurrence of resistance may become common in many populations worldwide, and monitoring will be crucial to ensuring vector control. Metabolic resistance and target site insensitivity are both major forms of pyrethroid resistance (Soderlund & Bloomquist, 1990; Soderlund, 1997; Soderlund & Knipple,

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1999). 'Knockdown resistance' (kdr) is a generic term applied to insects that fail to lose coordinated activity immediately following pyrethroid exposure. Typically kdr is unaffected by synergists that inhibit esterases and monooxygenases. Instead kdr arises through nonsynonymous mutations in the voltage-gated sodium channel transmembrane gene (paralysis locus in *Drosophila melanogaster*) that reduce pyrethroid binding to the sodium channel passing through the nerve cell membrane. Kdr usually limits the effectiveness of pyrethroids to varying degrees depending on whether the insecticide contains a descyano-3-phenoxybenzyl alcohol (type I pyrethroid) or an α-cyano-3-phenoxybenzyl alcohol (type II). Thus detection of kdr in the field has severe consequences for sustained use of pyrethroids in mosquito control. The early detection and characterization of kdr is critical to the development of strategies for resistance management.

The mosquito *Aedes aegypti* is the main vector of the four serotypes of dengue virus (DENV1–4) (Monath, 1994; Gubler, 2002a). There are 50–100 million DENV infections each year and while most of these are mild or asymptomatic, the numbers of severe infections with shock and haemorrhaging have been increasing and 20% of these cases are fatal (Pan American Health Organization, 1994; Gubler, 2002b). Brengues *et al.* (2003) examined DDT and pyrethroid resistance in *Ae. aegypti* from 13 localities and found evidence for DDT and pyrethroid resistance in strains from Semarang in Central Java, Belem in Brazil and Long Hoa in Vietnam. They obtained sequences of the gene region that encodes hydrophobic segment 6 of domain II of the voltage-gated sodium channel gene from 10 mosquitoes in all 13 strains. They analysed exons 19–21 that encode amino acid residues 908–1036. Exon 19 contains the amino acids associated with 'super kdr' resistance including methionine at position 918, leucine at 925, threonine at 929 and leucine at 932 (Soderlund & Knipple, 2003). Exon 20 contains the leucine to phenylalanine substitution at 1014 that confers nerve insensitivity to pyrethroids in a wide range of insect species including *Anopheles gambiae* (Soderlund & Knipple, 2003).

Brengues *et al.* (2003) discovered seven novel mutations in *Ae. aegypti* relative to *An. gambiae*. The Belem strain had a valine at position 923 (exon 19) while three *Ae. aegypti* strains and *An. gambiae* had a glycine at the same site (hereafter referred to as Val923). All *Ae. aegypti* strains contained an isoleucine at position 952 (exon 20) and a lysine at position 961 (exon 20), whereas *An. gambiae* contained a valine and histidine, respectively, at the same sites. The Long Hoa strain contained a tryptophan at position 982 (exon 20) while the other three *Ae. aegypti* strains and *An. gambiae* had a leucine at the same site (Trp982). The leucine to phenylalanine substitution at 1014 was absent in all *Ae. aegypti* strains. However, the Belem strain had a methionine at position 1011 (exon 20) while three *Ae. aegypti* strains and *An. gambiae* contained a isoleucine at the same site (Met1011). Furthermore, the Thailand strain had a glycine at position 1016 (exon 21) while three *Ae. aegypti* strains and *An. gambiae* encoded a valine at the same site (Gly1016). Direct neurophysiological assays on individual larvae from strains with these mutations demonstrated reduced nerve sensitivity to permethrin or lambda cyhalothrin inhibition when compared to the susceptible strains.

Table 1. Mutations in codons 1011 and 1016 of para in *Aedes aegypti*

	Codons			
Source	1011		1016	
Wild type	Iso	ATA	Val	GTA
Brengues *et al.*, 2003	Met	ATG	Gly	GGA
Current study	Val	GTA	Iso	ATA

In an initial sequencing screen for these nonsynonymous mutations in exons 20 and 21 in 87 mosquitoes from 30 *Ae. aegypti* strains from throughout Latin America, we discovered two new mutations in codons 1011 and 1016. An A/G transition in the first position of 1011 encodes a valine replacement (Val1011) and an A/G transition in the first position of 1016 encodes a isoleucine replacement (Iso1016). The relationships of all six alleles at codons 1011 and 1016 of the voltage-gated sodium channel transmembrane protein (para) in *Ae. aegypti* are listed in Table 1. Herein, we report on the development of a PCR assay for each of these six alleles, the results of which can be read either on an agarose gel or as a melting curve on a real-time PCR machine. In these 30 strains from throughout Latin America and in the Rockefeller and New Orleans susceptible strains we estimated the frequencies of Iso1011, Met1011, Val1011, Val1016, Iso1016 and Gly1016. In addition, we show in two selection experiments, one with deltamethrin on a field strain from Santiago de Cuba (Rodriguez *et al.*, 2005) and another with permethrin on a strain from Isla Mujeres, Mexico that there is a rapid increase in the frequency of the Iso1016 allele but decreases in the frequencies of Met1011 and Val1011 alleles. An F_1 intercross was made between the standard susceptible New Orleans strain and a F_{5} Isla Mujeres strain that had been selected for permethrin resistance. Bioassays of the F_3 generation indicate that Iso1016 acts as a recessive allele in conferring kdr. Analysis of segregation between alleles at the 1011 and 1016 codons in the F_3 offspring also suggested a high rate of recombination even though the two codons are only separated by a ~270 bp intron. Finally, a phylogenetic analysis was performed on the intron spanning exons 20 and 21 in 87 mosquitoes to assess the evolutionary history of these four alleles.

Table 2. Sequences of oligonucleotides used in this study for allele-specific PCR and amplification of the intron spanning exons 20 and 21. The sequences of the short and long tails are provided for the first sequence only. The 3′ allele-specific nucleotide is shaded in grey and the mismatch and the third nucleotide from the 3′ end is underlined.

Primers for allele-specific PCR		Product size (bp)
Val1011		
Val1011f	5′-ATTGTATGCTTGTGGGTGACG-3′	
Iso1011r	5′-GCGGGCTACTTACTACTAGATTTCCAAT-3′	84
Val1011r	5′-GCGGGCAGGGCGGCGGGGGCGGGGCCTACTTACTACTAGATTTCCGAC-3′	104
78+6		
78+26		
Met1011		
Met1011f	5′-GTCCTGTATTCCGTTCTTTTT-3′	
Iso1,011r	5′-[long tail] TACTTACTACTAGATTTGCC-3′	62
Met1011r	5′-[short tail]TACTTACTACTAGATTTACT-3′	62
Iso1016		
Val1016f	5′-[long tail] ACAAATTGTTTCCCACCCGCACCGG-3′	102
Iso1016f	5′-[short tail]ACAAATTGTTTCCCACCCGCACTGA-3′	82
Iso1011r	5′-GGATGAACCSAAATTGGACAAAAGC-3′	
Gly1016		
Gly1016f	5′-ACCGACAAATTGTTTCCC-3′	
Val1016r	5′-[short tail]AGCAAGGCTAAGAAAAGGTTAATTA-3′	80
Gly1016r	5′-[long tail] AGCAAGGCTAAGAAAAGGTTAACTC-3′	80
Primers for intron amplification		
kdr20f	5′-ATGTGGGATTGTATGCTTG-3′	Avg. = 365(357–377)
kdr21r	5′-GATGAACCGAAATTGGAC-3′	

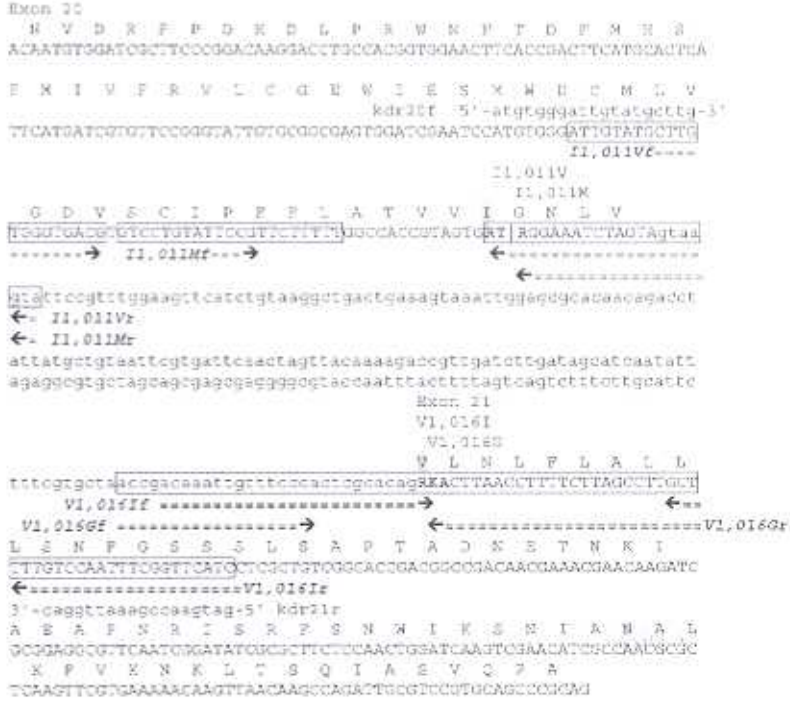

Figure 1. Exon 20, intron (lower-case letters) and exon 21 from the *Aedes aegypti* voltage-gated sodium channel gene. This transcript is a product of VectorBase Transcript ID AAEL006019 and appears in supercont1.186 from nucleotides 18 685–163 945. Exon 20 corresponds to nucleotides 115 258–115 445 while exon 21 corresponds to nucleotides 115 680–115 887. The locations of the oligonucleotides listed in Table 3 appear in boxes. Codons 1011 and 1016 appear in bold letters.

Results

Allele-specific PCR

Table 2 lists all oligonucleotides used in this study and the predicted lengths of the PCR products. Figure 1 shows the locations of these oligonucleotides with respect to exons 20 and 21 and the intervening intron. Melting curve profiles are shown for each genotype at each locus in Fig. 2. Figure 2A displays the melting curves for the Iso1011Val single nucleotide polymorphism (SNP). A single peak at 80 °C indicates an Iso1011/Iso1011 homozygote, while two peaks at 80 °C and 85 °C indicate a Val1011/Iso1011 heterozygote.

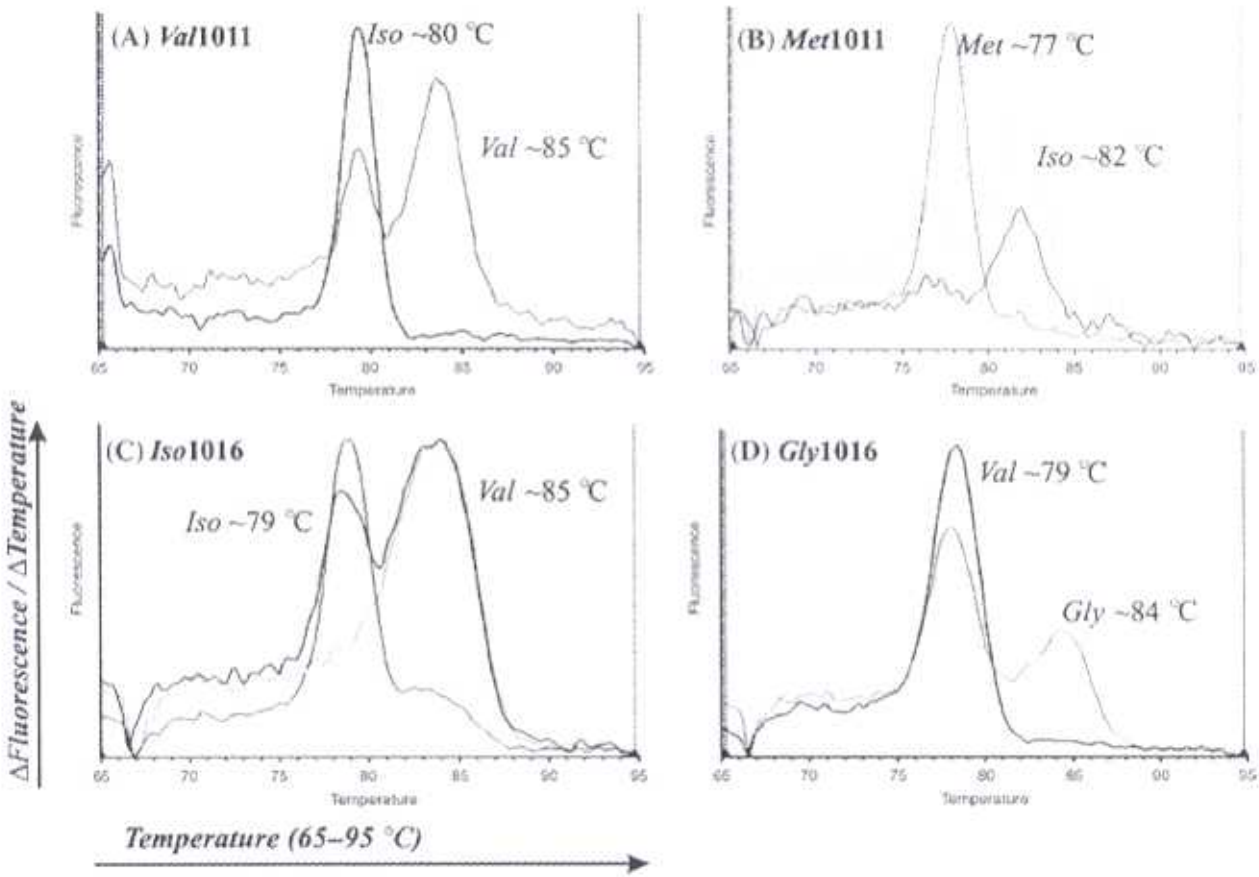

Figure 2. Melting curve patterns for genotypes at the four single nucleotide polymorphism (SNP) loci. (A) Iso1011Val SNP. The melting curve patterns for an Iso1011/Iso1011 homozygote and a Val1011/Iso1011 heterozygote are shown. (B) Iso1011Val SNP. Melting curves for all three genotypes Met1011/Met1011, Met1011/Iso1011 and Iso1011/Iso1011 are shown. (C) Iso1016Val SNP. Melting curves for Iso1016/Iso1016, Iso1016/Val1016 and Val1016/Val1016 are shown. (D) Iso1016Gly SNP. Melting curves are shown for a Val1016/Val1016 homozygote and a Val1016/Gly1016 heterozygote.

Figure 2B displays the melting curves for the Iso1011Val SNP and melting curves for all three genotypes are shown. A Met1011/Met1011 homozygote has a single peak at 72 °C while a Met1011/Iso1011 heterozygote has peaks at 77 and 82 °C and the Iso1011/Iso1011 homozygote displays a single peak at 82 °C. Figure 2C displays the melting curves for the Iso1016Val SNP and the melting curves correspond to Iso1016/Iso1016 (single peak at 79 °C), Iso1016/Val1016 (peaks at 79 and 85 °C) and Val1016/Val1016 (single peak at 85 °C). Figure 2D displays the melting curves for the Iso1016Gly SNP. Melting curves are shown for a Val1016/Val1016 homozygote (single peak at 79 °C) and a Val1016/Gly1016 heterozygote (double peaks at 79 and 84 °C). All three genotype profiles are only shown for Met1011 and Iso1016 SNP loci because we did not find homozygotes for the less common alleles at the other two loci among the 1318 mosquitoes analysed. The Gly1016 allele was obtained from a laboratory strain from Thailand because this substitution was never detected in any of the Latin American collections or in the New Orleans or Rockefeller laboratory strains. The accuracy of the melting curve profiles was assessed by comparing them with the sequences of the original 87 mosquitoes from which we also collected intron sequence data. There were no disagreements between the sequences and the melting curve profiles.

Realizing that real-time PCR machines are not always available, we also fractionated the PCR products on 4.0% Tris-Borate EDTA (TBE) agarose gels by electrophoresis for 90 min at 80 V alongside a 25 bp DNA ladder. Figure 3 shows that the different tail lengths added to the allele-specific primers for the purpose of producing different melting curves could also be detected via agarose gel electrophoresis. These results have been confirmed for the other three loci and among numerous mosquitoes.

Allele and genotype frequencies

Table 3 lists the names of the *Ae. aegypti* strains analysed, the names, collection locations, country and coordinates of the collection sites (or, in the case of the Cuban strains, the

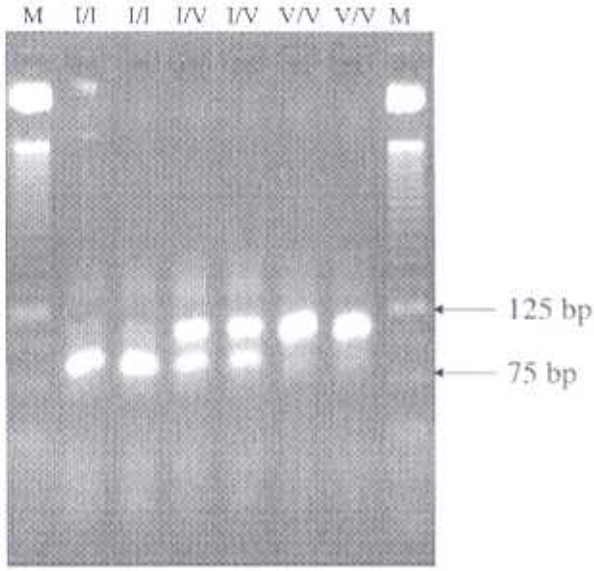

Figure 3. Agarose gel electrophoresis of Iso1016 and Val1016 PCR products in 4% GenePure HiRes agarose gel. Genotype Iso1016/Iso1016 appear in lanes 1 and 2, genotype Val1016/Iso1016 in lanes 3 and 4, Val1016/Val1016 genotypes are in lanes 5 and 6. DNA size markers (25 bp DNA ladder) are shown in lanes M.

publications describing the collection sites). Also listed are the numbers of mosquitoes analysed in each strain.

The four insecticide selected strains from Cuba (F_{12} and F_{13} deltamethrin, F_{14} propoxur and F_6 temephos) all originated from the Santiago de Cuba collection originally described by Rodriguez *et al.*, (1999). *Aedes aegypti* were also collected from Isla Mujeres, an tourist resort island just northeast of Cancun, Mexico where temephos and pyrethroids are regularly used for larval and adult control, respectively (Flores *et al.*, 2006). F_5 adults arose from five generations of permethrin selection.

Numbers of genotypes at each of the three loci in all 32 strains are listed in Table 4. Frequencies of the Iso1016, Met1011 and Val1011 alleles were estimated along with the 95% confidence intervals around those estimates. Wright's F_{IS} was estimated as a way of testing for Hardy–Weinberg equilibrium among genotypes where: $F_{IS} = 1 -$ (heterozygotes observed/heterozygotes expected). If an excess of heterozygotes are observed then $F_{IS} < 0$ and if an excess of homozygotes are observed then $F_{IS} > 0$. A χ^2 goodness-of-fit test was used to test the null hypothesis that $F_{IS} = 0$. Note that $F_{IS} = 0$ at the 1016 locus in all but one case (Iso1016 in F_{14} propoxur) and in all analyses of Val1011. However, with Met1011, genotype frequencies were not in equilibrium in 12 strains. Note that there was no consistent pattern in F_{IS}. In six strains $F_{IS} < 0$ (excess of heterozygotes) in all significant tests but $F_{IS} > 0$ (excess of homozygotes) in the other six strains.

At codon 1016 in field collections that had not been subject to laboratory selection, Iso1016 ranged in frequency from 0.00 in four collections from Tapachula, Mexico and in five collections from Venezuela to a frequency of 0.293 in Ciudad Habana. At locus 1011 in field collections without laboratory selection, Val1011 ranged in frequency from 0.00 in four collections from Tapachula, Mexico, two collections in Cuba and one collection from Venezuela to 0.188 in Huehuetan, Mexico. At the same codon, Met1011 was polymorphic in all strains, ranging in frequency from 0.193 from Huixtla, Mexico to 0.583 in Ciudad Habana.

The F_{12} and F_{13} deltamethrin, F_{14} propoxur and F_6 temephos strains arose through selection with the corresponding insecticide from the Santiago de Cuba collection. The Isla Mujeres strain gave rise through permethrin selection to the F_5 generation. The frequencies of the Met1011, Val1011 and Iso1016 in these strains before and after insecticide selection are listed in Table 4 and shown in Fig. 4. The frequency of Iso1016 increased significantly from 0.033 in the original Santiago de Cuba collection to 0.567 (Fisher's exact test, $P = 6.6 \times 10^{-10}$) in the F_{12} generation and to 0.883 ($P = 2.20 \times 10^{-16}$) following one additional generation of deltamethrin selection. Similarly, the frequency of Iso1016 increased from 0.259 to fixation following five generations of selection in the Isla Mujeres strain with permethrin ($P = 2.20 \times 10^{-16}$). In contrast, Iso1016 decreased insignificantly from 0.033 to 0.018 ($P = 1.000$) in the temephos selected line but increased to 0.400 ($P = 8.78 \times 10^{-7}$) in the propoxur selected line.

An opposite response to selection was noted in the frequency of Met1011. This allele decreased from 0.517 in the original Santiago de Cuba collection to 0.533 and 0.283 in the F_{12} and F_{13} generations respectively following deltamethrin selection (Fisher's exact test, $P = 1.000$, 0.015 respectively). Met1011 also decreased from 0.317 in the Isla Mujeres strain to 0.125 after five generations of permethrin selection ($P = 0.0224$). The frequency of Met1011 increased slightly from 0.517 to 0.621 ($P = 0.3517$) in the temephos selected line but increased to 0.950 ($P = 6.03 \times 10^{-8}$) in the propoxur selected line. None of the changes in the frequency of *Val*1011 was significant.

A total of 420 F_3 offspring were generated in a cross between a Isla Mujeres P. female (Iso1011/Met1011, Iso1016/Iso1016) and a susceptible New Orleans strain male (Iso1011/Iso1011, Val1016/Val1016). F_3 offspring were exposed to 1.2 µg of permethrin in a bottle bioassay (Brogdon & McAllister, 1998). After 1 h, adults were scored as alive (flying or walking). The remainder were placed in a cage and returned to the insectary. After 4 h these adults were scored as active or dead. DNA was collected from all adults and tested for codon 1016 genotypes. Figure 5 shows that all Iso1016/Iso1016 survived, while only 9.4% of Val1016/Iso1016 and none of the Val1016/Val1016 survived. After 4 h 57.5% of Val1016/Iso1016 were active while only 15% of the Val1016/Val1016 were active. A 3×3 contingency χ^2 analysis was highly significant (Fisher's

Table 3. Names of the *Aedes aegypti* strains analysed. The city, country and coordinates of the collection sites are listed for each strain along with the numbers of mosquitoes analysed. For the Cuban strains, the publications describing the collection sites are provided in lieu of coordinates.

Strain name	Location	Country	Latitude or published description of location	Longitude	Sample size
Rockefeller	Puerto Rico	USA	Rodriguez *et al.*, 2000		30
New Orleans	New Orleans	USA	Flores *et al.*, 2006		30
PCHI	Puerto Chiapas	Mexico	14.7142	-92.4152	48
CHID	Ciudad Hidalgo	Mexico	14.6799	-92.0229	48
RIOF	Rio Florido	Mexico	14.2611	-93.3361	48
PIJI	Pijijiapan	Mexico	15.6811	-93.2069	48
HUET	Huehuatan	Mexico	15.0155	-92.3860	48
HUIX	Huixtla	Mexico	15.1376	-92.4698	48
MAZA	Mazatan	Mexico	14.8676	-92.4496	48
MOTZ	Motozintla	Mexico	15.3638	-92.2431	48
ESQI	Esquintla	Mexico	15.3268	-92.6587	48
MAPA	Mapastepec	Mexico	15.4366	-92.9028	48
IM	Isla Mujeres	Mexico	21.2345	-86.7316	30
IM F_5	Isla Mujeres				30
Santiago de Cuba	Santiago de Cuba	Cuba	Rodriguez *et al.*, 2000		30
F_{12} Deltamethrin	Santiago de Cuba	Cuba	Rodriguez *et al.*, 2005		30
F_{13} Deltamethrin	Santiago de Cuba	Cuba	Rodriguez *et al.*, 2005		30
F_{14} Propoxur	Santiago de Cuba	Cuba	Bisset *et al.*, 2006		30
F_6 Temephos	Santiago de Cuba	Cuba	Rodriguez *et al.*, 2002		30
Ciudad Habana	Ciudad de la Habana	Cuba	Rodriguez *et al.*, 2000		30
Nicaragua	Managua	Nicaragua	?	?	30
Costa Rica	Guanacaster	Costa Rica	Rodriguez *et al.*, 2000		30
Panama	Rio Abajo	Panama	?	?	30
Cumana	Sucre	Venezuela	10.4332	-64.1825	58
Barcelona	Anzoategui	Venezuela	8.9093	-64.6768	50
Barquisimeto	Lara	Venezuela	10.0781	-69.4010	50
Guanare	Portuguesa	Venezuela	9.5781	-69.2523	55
Caja Seca	Zulia	Venezuela	9.1944	-71.0033	51
La Fria	Tachira	Venezuela	8.1974	-72.2360	54
Los Teques	Miranda	Venezuela	10.1735	-65.8065	50
Cd. Bolivar	Bolivar	Venezuela	8.0688	-63.5565	55
San Fernando	Apure	Venezuela	7.8422	-67.5283	55

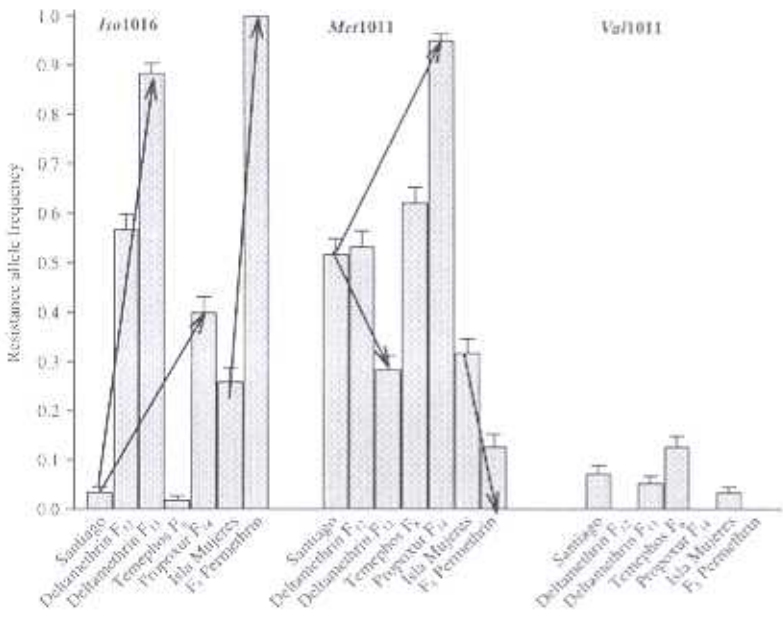

Figure 4. The frequencies of the Met1011, Val1011 and Iso1016 alleles in the original Santiago de Cuba and Isla Mujeres collections and in the strains selected with the respective insecticides.

Table 4. Frequencies of Iso1016, Met1011 and Val1011 alleles and genotypes among 32 strains of *Aedes aegypti*. Also provided are the sample sizes, 95% confidence intervals (CI) around allele frequency estimates and the F_{IS} in each strain

Iso1016 Strain	A/A	A/G	G/G	Total	Frequency	95% CI	F_{IS}
Rockefeller	0	2	27	29	0.034	0.012	−0.071
New Orleans	0	0	30	30	0.000	–	–
Puerto Chiapas	0	0	48	48	0.000	–	–
Ciudad Hidalgo	0	16	29	45	0.178	0.017	−0.216
Rio Florido	0	4	43	47	0.043	0.008	−0.044
Pijijapan	0	0	48	48	0.000	–	–
Huehuetan	0	1	47	48	0.010	0.004	−0.011
Huixtla	0	0	47	47	0.000	–	–
Mazatan	0	9	39	48	0.094	0.012	−0.103
Metozintla	0	0	48	48	0.000	–	–
Esquintla	0	7	39	46	0.076	0.011	−0.082
Mapastepec	0	0	48	48	0.000	–	–
Isla Mujeres (IM)	0	15	14	29	0.259	0.030	−0.349
IM F_5 Permethrin	30	0	0	30	1.000	–	1.000
Santiago de Cuba	0	2	28	30	0.033	0.012	−0.077
F_{12} deltamethrin	8	18	4	30	0.567	0.032	–
F_{13} deltamethrin	24	5	1	30	0.883	0.021	0.191
F_{14} propoxur	2	20	8	30	0.400	0.032	−0.389*
F_6 temephos	0	1	27	28	0.018	0.009	−0.018
Ciudad Habana	3	11	15	29	0.293	0.031	0.085
Nicaragua	1	4	25	30	0.100	0.020	0.259
Costa Rica	0	5	24	29	0.086	0.019	−0.094
Panama	2	7	20	29	0.190	0.026	0.215
Sucre	0	0	58	58	0.000	–	–
Anzoategui	0	14	36	50	0.140	0.014	0.163
Lara	0	1	49	50	0.010	0.004	−0.010
Portuguesa	0	0	55	55	0.000	–	–
Zulia	0	0	51	51	0.000	–	–
Tachira	0	0	54	54	0.000	–	–
Miranda	0	0	50	50	0.000	–	–
Bolivar	0	2	53	55	0.018	0.005	−0.019
Apure	0	2	53	55	0.018	0.005	−0.019
Rockefeller	16	6	8	30	0.633	0.031	0.569**
New Orleans	3	20	6	29	0.448	0.034	−0.394*
Puerto Chiapas	15	15	17	47	0.479	0.021	0.361*
Ciudad Hidalgo	11	22	12	45	0.489	0.022	0.022
Rio Florido	7	24	15	46	0.413	0.021	−0.076
Pijijapan	3	30	14	47	0.383	0.020	−0.351*
Huehuetan	9	11	28	48	0.302	0.019	0.457**
Huxtla	1	15	28	44	0.193	0.018	−0.094
Mazatan	3	19	26	48	0.260	0.018	−0.028
Metozintla	17	14	17	48	0.500	0.020	0.417**
Esquintla	14	16	16	46	0.478	0.021	0.303*
Mapastepec	2	19	20	41	0.280	0.021	−0.146
Isla Mujeres (IM)	0	19	11	30	0.317	0.030	−0.463*
IM F_5 Permethrin	1	4	19	24	0.125	0.027	0.235
Santiago de Cuba	10	11	9	30	0.517	0.033	0.256
F_{12} deltamethrin	11	10	9	30	0.533	0.033	0.330
F_{13} deltamethrin	6	5	19	30	0.283	0.029	0.590***
F_{14} propoxur	27	3	0	30	0.950	0.014	−0.053
F_6 temephos	11	14	4	29	0.62*	0.033	−0.025
Ciudad Habana	11	13	6	30	0.583	0.032	0.109
Nicaragua	1	14	14	29	0.276	0.030	−0.205
Costa Rica	5	10	10	25	0.400	0.038	0.167
Panama	7	13	8	28	0.482	0.035	0.070
Sucre	0	31	24	55	0.282	0.016	−0.392**
Anzoategui	3	28	20	51	0.333	0.018	−0.235
Lara	3	27	19	49	0.337	0.019	−0.234
Portuguesa	1	19	29	49	0.214	0.016	−0.152
Zulia	2	31	18	51	0.343	0.018	−0.348*
Tachira	13	18	12	43	0.512	0.023	0.162
Miranda	0	15	22	37	0.203	0.021	−0.254
Bolivar	3	21	24	48	0.281	0.018	−0.082
Apure	2	33	19	54	0.343	0.017	−0.357**

Table 4. (*Continued*)

Iso1016 Strain	A/A	A/G	G/G	Total	Frequency	95% CI	F_{IS}
Rockefeller	0	4	26	30	0.067	0.016	−0.071
New Orleans	0	0	30	30	0.000	–	–
Puerto Chiapas	0	2	43	45	0.022	0.006	−0.023
Ciudad Hidalgo	0	0	46	46	0.000	–	–
Rio Florido	0	0	47	47	0.000	–	–
Pijijapan	0	1	47	48	0.010	0.004	−0.011
Huehuetan	0	18	30	48	0.188	0.016	−0.231
Huixtla	0	2	46	48	0.021	0.006	−0.021
Mazatan	0	0	47	47	0.000	–	–
Motozintla	0	2	46	48	0.021	0.006	−0.021
Esquintla	0	1	45	46	0.011	0.004	−0.011
Mapastepec	0	0	48	48	0.000	–	–
Isla Mujeres (IM)	0	2	28	30	0.033	0.012	−0.034
IM F_9 Permethrin	0	0	30	30	0.000	–	–
Santiago de Cuba	0	4	24	28	0.071	0.018	−0.077
F_{12} deltamethrin	0	0	28	28	0.000	–	–
F_{13} deltamethrin	0	3	26	29	0.052	0.015	−0.055
F_{14} propoxur	0	0	30	30	0.000	–	–
F_8 temephos	0	7	21	28	0.125	0.023	−0.143
Ciudad Habana	0	5	25	30	0.083	0.018	−0.091
Nicaragua	0	1	29	30	0.017	0.008	−0.017
Costa Rica	0	5	25	30	0.083	0.018	−0.091
Panama	0	3	27	30	0.050	0.014	−0.053
Sucre	0	4	53	57	0.035	0.006	−0.036
Anzoategui	0	9	43	52	0.087	0.011	−0.095
Lara	0	2	46	48	0.021	0.006	−0.021
Portuguesa	0	2	53	55	0.018	0.005	−0.019
Zulia	0	5	45	50	0.050	0.009	−0.053
Tachira	0	2	52	54	0.019	0.005	−0.019
Miranda	0	0	49	49	0.000	–	–
Bolivar	0	9	46	55	0.082	0.010	−0.089
Apure	0	2	52	54	0.019	0.005	−0.019

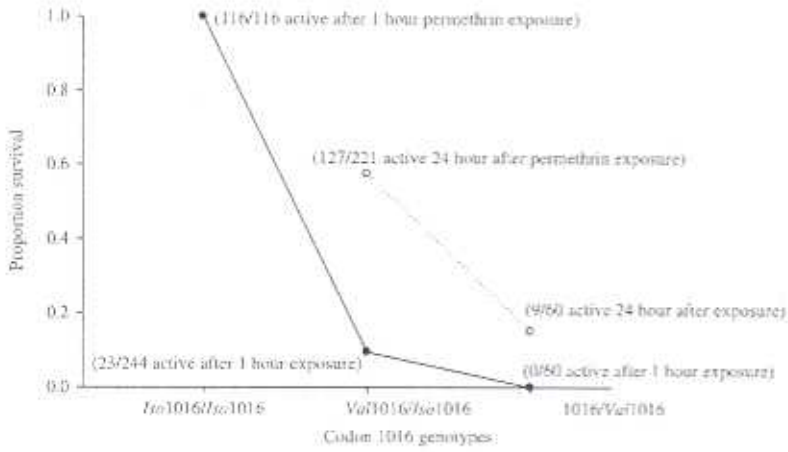

Figure 5. Survival rates following 1 h permethrin exposure (solid lines) and after 4 h (dotted line) among 420 F_2 offspring arising from a cross between an Isla Mujeres P_1 female and a susceptible New Orleans strain male. The numbers of mosquitoes in each genotype (denominator) and numbers of mosquitoes surviving (numerator) appear in parentheses.

exact test, $P = 9.14 \times 10^{-89}$). Iso1016 acts as a mostly recessive allele in conditioning survival of *Ae. aegypti* exposed to permethrin.

This analysis was repeated among 400 of the F_2 offspring for the Met1011 allele but the 3 × 3 contingency χ^2 analysis was not significant ($P = 0.411$), suggesting that this allele may not confer permethrin resistance. This result was not expected given that the two codons are only separated by a ~270 bp intron and that no recombination should have occurred between them in only three generations. Furthermore, the genotypes of the F_1 parents were Iso1011/Met1011 and Iso1016/Val1016 and consequently we expected only three di-locus genotypes in the F_2 generation. Instead we detected nine. A 3 × 3 contingency χ^2

Table 5. Observed and expected numbers of the 1011 and 1016 di-locus genotypes in the F_2 *Aedes aegypti* offspring. Expected frequencies were calculated assuming our a priori hypothesis of complete linkage ($r = 0$ cM), independent segregation ($r = 50$ cM). The optimal fit between observed and expected numbers was obtained assuming $r = 17.8$ cM. In each of these three cases a χ^2 goodness-of-fit test was performed

		Expected		
F_2 Zygotes	Observed	$r = 0$	$r = 50$	$r = 17.8$
Iso1011 Iso1016/Iso1011 Iso1016	11	0	24.6	5.8
Iso1011 Iso1016/Iso1011 Val1016	13	0	49.4	36.8
Iso1011 Iso1016/Met1011 Iso1016	80	0	50.8	36.3
Iso1011 Iso1016/Met1011 Val1016	0	0	49.0	11.7
Iso1011 Val1016/Iso1011 Val1016	3	100	24.8	58.2
Iso1011 Val1016/Met1011 Iso1016	171	200	51.0	115.0
Iso1011 Val1016/Met1011 Val1016	37	0	49.2	37.0
Met1011 Iso1016/Met1011 Iso1016	20	100	26.2	56.8
Met1011 Iso1016/Met1011 Val1016	49	0	50.6	36.5
Met1011 Val1016/Met1011 Val1016	16	0	24.4	5.9
$\chi^2_{(d.f.=3)}$ goodness-of-fit		21183.3	409.5	209.4
Probability		0.0	1.3×10^{-82}	3.8×10^{-42}

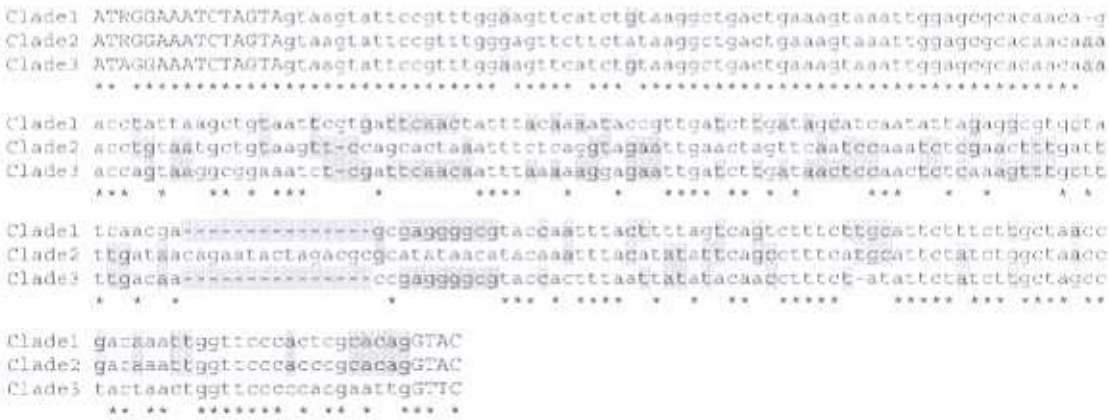

Figure 6. Representative sequences of the intron in clades 1, 2, and 3. Constant sites are indicated with '*'. Single nucleotide polymorphism (SNP) alleles shared between two clades are shaded in grey.

analysis was performed on 1011 and 1016 genotypes and this was also not significant ($P = 0.347$), suggesting independent segregation. Table 5 lists the observed numbers of the 1011 and 1016 di-locus genotypes in the F_2 offspring. Expected frequencies were calculated assuming complete linkage ($r = 0$ cM) or independent segregation ($r = 50$ cM). The optimal fit between observed and expected numbers was obtained assuming $r = 17.8$ cM. In each of these three cases a χ^2 goodness of fit test was performed and in each case there was a poor fit to expectations. In any case, it is clear that we recovered di-locus genotypes that should not have been present in the F_2 offspring without some amount of recombination.

Evolution of kdr mutations

There were 274 sites in the 88 aligned intron sequences (*An. gambiae* used as an outgroup). Among *Ae. aegypti* sequences, there were 114 segregating sites and 31 of these sites contained an insertion/deletion. There were 31 haplotypes, a nucleotide diversity of $\pi = 0.146$ and the average number of nucleotide differences were $k = 35.58$. However, this diversity was not uniformly distributed throughout the intron. The first 100 nucleotides are relatively conserved as are the last 75 (Figs 6 and 7). The minimum number of recombination events was $R_m = 33$, indicating abundant recombination among segregating sites within the intron. However, there was also a large amount of linkage disequilibrium among segregating sites. Of 5356 pairwise comparisons among segregating sites, 2409 (45%) were significant by Fisher's exact test and 1803 (34%) were significant when using Bonferroni corrections.

Linkage disequilibrium was not uniformly distributed among all segregating sites. Figure 8 shows the disequilibrium between all segregating sites and alleles at codon 1011 positions 1 and 3, and codon 1016 at all three positions. Alleles at codon 1011 positions 1 and 3 were in significant disequilibrium with four of 103 sites and the magnitude of the disequilibrium was low. In contrast, positions 1 and 3 at codon 1016 were in significant disequilibrium with 59 and 36 of the 103 sites while position 2 was in significant disequilibrium with three of 103 sites.

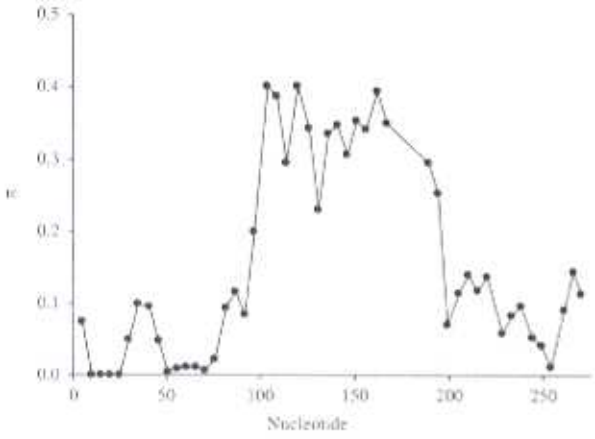

Figure 7. Plot of π (Nei & Miller 1990) among the 87 intron sequences. The sliding window length in DNAsp was set to 10 with a step size of 5.

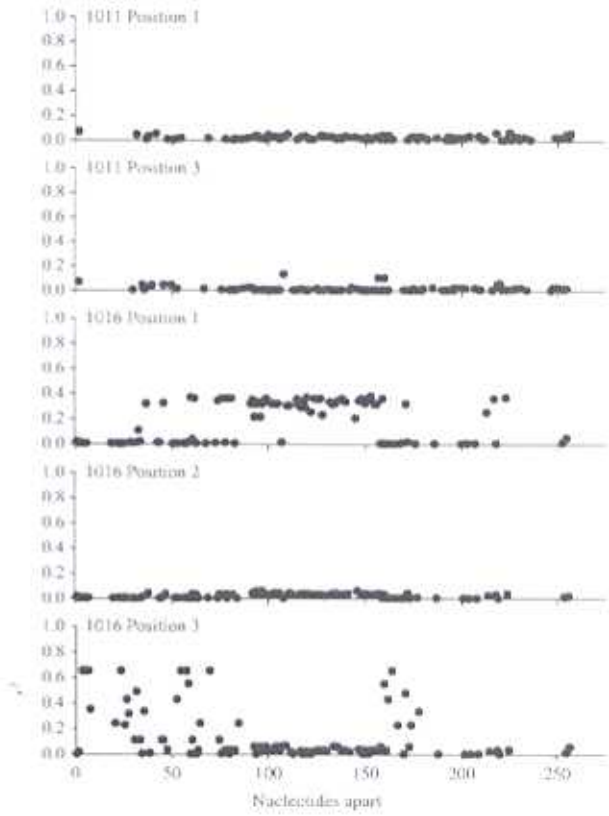

Figure 8. Plot of Hill & Robertson's (1968) linkage disequilibrium coefficient r^2 as a function of the distance (in nucleotides) between pairs of segregating sites for the first and third codons in exon 1011 and the first, second and third codons in exon 1016.

Figure 9 is the maximum parsimony phylogeny of the intron spanning exons 20 and 21 estimated among 88 mosquitoes with different genotypes in exons 1011 and 1016. Bootstrap analysis indicated the presence of three clades with support > 80%. These were arbitrarily labelled clades 1–3. The frequencies of Iso1011, Met1011, Val1011, Val1016, Iso1016 and Gly1016 (from Thailand only) were then compared among the three clades. The frequency of Iso1011 were distributed independently among the three clades (Fisher's exact test, $P = 0.1654$), as was Val1011 ($P = 0.0874$) and Met1011 ($P = 0.7465$). However, there was a very evident excess of Val1016 alleles in clade 1 ($P = 3.38 \times 10^{-8}$), an excess of Iso1016 alleles in clade 2 ($P = 7.39 \times 10^{-10}$), but Gly1016 was distributed independently among the three clades ($P = 0.4629$).

Discussion

We screened segment 6 of domain II of para in 1318 *Ae. aegypti* in 32 strains from throughout Latin America for the six alleles (Table 1) in codons 1011 and 1016 in exons 20 and 21, respectively. Brengues *et al.* (2003) described two additional alleles. The Belem strain had a Val923 allele but this was located in exon 19 and was not screened in the present study. The Long Hoa strain contained a Trp982 allele that was not found in any of our initial sequencing screen of 87 individuals. We also never detected the Gly1016 allele and we found no additional replacement substitutions in segment 6 of domain II of para.

Selection experiments, one with deltamethrin on a field strain from Santiago de Cuba and another with permethrin on a strain from Isla Mujeres, Mexico rapidly increased the frequency of the Iso1016 allele but did not change or decrease the frequencies of exon 1011 alleles. Bioassays of F_3 offspring arising from permethrin susceptible Val1016 homozygous parents and permethrin resistant Iso1016 homozygous parents showed that Iso1016 segregates as a recessive allele in conferring kdr.

We also observed that both the Met1011 and Iso1016 alleles increased to 0.950 and 0.400 in the propoxur selected line. As there is no evidence that kdr confers carbamate resistance, the most parsimonious explanation is that Met1011 and Iso1016 are linked to loci conditioning propoxur resistance and that propoxur selection leads to a rapid increase in these and the linked kdr alleles.

Brengues *et al.* (2003) found pyrethroid resistance in *Ae. aegypti* strains from Semarang in Central Java, Belem in Brazil and Long Hoa in Vietnam. The highest level of resistance (296-fold at the Lethal Concentration $(LC)_{95}$) occurred in the Semarang strain. Significantly, this was the strain in which they also found the Gly1016 allele, suggesting that substitutions in this codon may confer kdr. As in our study, they also found a number of individuals with the Met1011 mutation but this did not appear to be associated with elevated pyrethroid or DDT resistance.

The results of this initial survey are disturbing if Iso1016 does in fact confer kdr. Iso1016 was found in frequencies

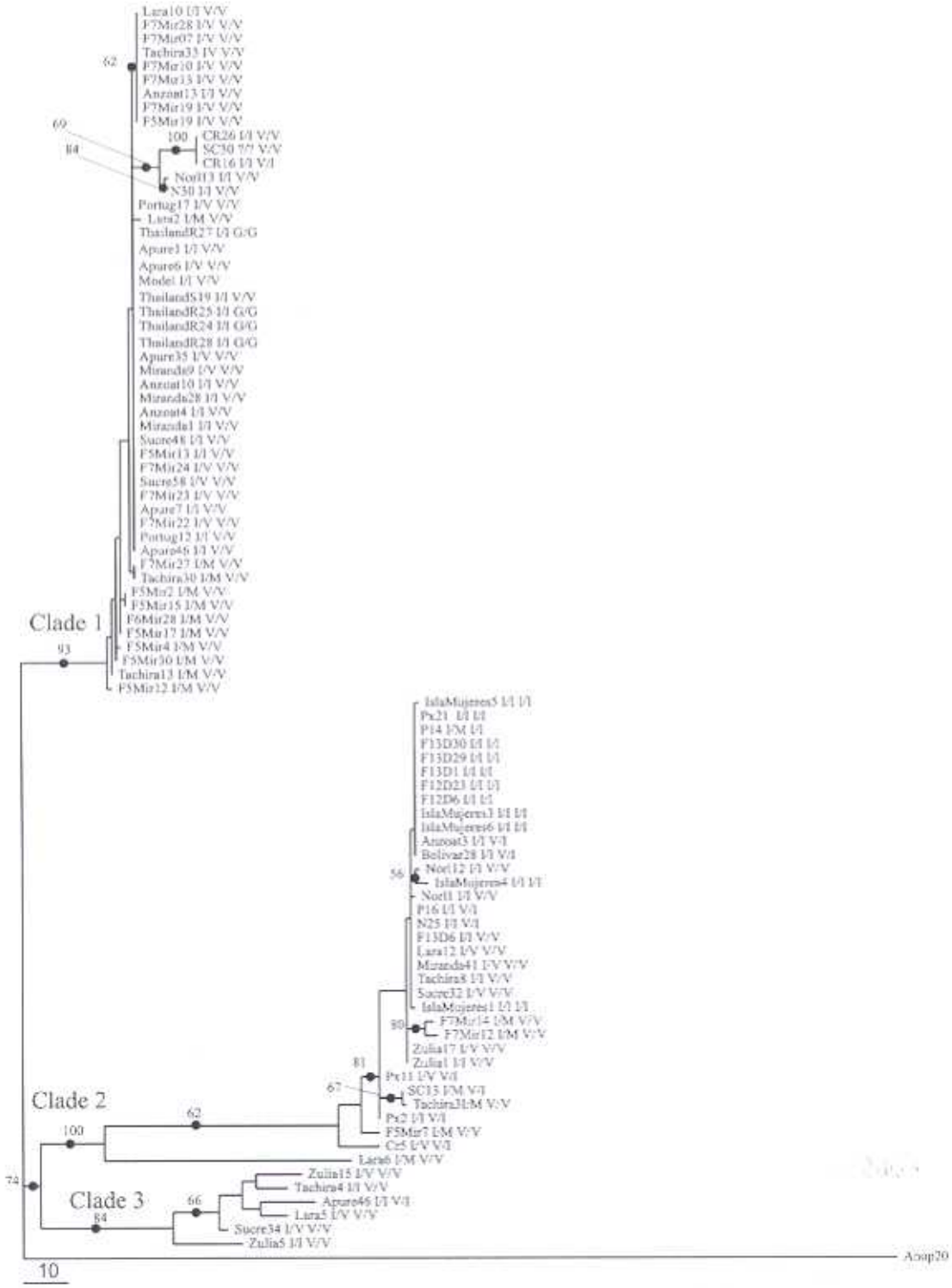

Figure 9. Maximum parsimony phylogeny of the intron spanning exons 20 and 21 estimated among 87 mosquitoes with different genotypes in exons 1011 and 1016. For each mosquito, the strain, number ID is listed as is the amino acid encoded respectively by exons 1011 and 1016. I, Iso; V, Val; M, Met; G, Gly.

ranging from 0.010–0.293 in 15 of the 25 unselected field collections (Table 4). Many of the reports of pyrethroid resistance predate the use of pyrethroids for *Ae. aegypti* control in the field (Rongsriyam & Busvine, 1975). This observation, and the fact that cross resistance to DDT existed in the pyrethroid resistant strains of Brengues *et al.* (2003), suggests that the widespread levels of Iso1016 detected in our survey may be a result of earlier or current uses of DDT.

Results of both the linkage disequilibrium and phylogenetic analyses (Figs 8 and 9) suggest linkage disequilibrium between segregating sequences and the Iso1016 allele. Very little disequilibrium has been detected in the few other *Ae. aegypti* genes that have been analysed. Linkage disequilibrium analyses were completed among 57 segregating sites in the *Abundant Trypsin* gene among 1661 female *Ae. aegypti* from throughout Mexico (Gorrochotegui-Escalante *et al.*, unpubl. data). In four different geographical regions of Mexico, the majority of segregating sites were in linkage equilibrium over a distance of only 777 nucleotides. The rate of recombinations/generation (r, (Hudson *et al.*, 1987)) was estimated as 88×10^{-3}. This same pattern was observed in the *Early Trypsin* and r was estimated as 5.2×10^{-3} recombinations/generation (Gorrochotegui-Escalante *et al.*, 2005). Black *et al.* (2007) estimated that in the *Abundant Trypsin* gene there are ~380–3640 recombination events/generation, whereas in *Early Trypsin* there are ~20–80 recombination events/generation. Thus, in *Ae. aegypti* populations, recombinations/generation is sufficiently large to maintain most segregating sites in linkage equilibrium. The minimum number of recombination events in the intron (R_m = 33) is also relatively large and suggests abundant recombination among segregating sites.

Usually, mutations in the exons encoding residues 954–1084 of para occur singly in species. It is interesting to consider the outcome of cumulative mutations at both 1011 and 1016. Double mutations in the first and third positions would yield a GTG in codon 1011 that encodes a valine but in 1016 double mutations would produce a AGA mutation encoding an arginine. Iso1011, Met1011 and Val1011 alleles occur together in the Anzoategui, Apure, Bolivar and Lara Venezuelan collections, in the Esquintla collection in Tapachula Mexico, and in the Ciudad Habana, Costa Rica, Nicaragua and Panama collections (Table 4), but we did not find any mosquitoes with double mutations in codon 1011. At this time we have no evidence that Val1016, Iso1016 and Gly1016 occur sympatrically.

The observation of more than three 1011 and 1016 di-locus genotypes among the 400 F_2 offspring was not expected given that the two codons are only separated by a ~270 bp intron. It is tempting to suggest that region represents a recombination hotspot in the *Ae. aegypti* genome. However, this hypothesis is probably too simple given that no estimates of r gave a satisfactory fit to the observed numbers of di-locus genotypes.

Despite evidence of recombination in only three generations and the general prediction of linkage equilibrium from studies of other genes in *Ae. aegypti*, Iso1016 alleles occurred in disequilibrium with a large number of segregating sites in the intron and a large excess of Iso1016 alleles were found to be associated with clade 2 in the phylogenetic analysis. Neither pattern was found with any of the three codon 1011 alleles. This pattern is consistent with a hypothesis that a genetic sweep of the Iso1016 allele and proximate intron sequences has occurred through DDT and subsequently pyrethroid selection. Furthermore, the genetic sweep was recent enough that there has been insufficient time for recombination to disrupt the disequilibrium between the Iso1016 allele and proximate intron sequences.

We developed PCR assays for these six alleles that could be read either on an agarose gel or as a melting curve. The allele-specific PCR system presented here has proven to be a rapid and relatively inexpensive means for surveying genotypes at segregating sites in *Ae. aegypti* field collections. The cost per genotype determination using melting curve analysis on a real time machine (Fig. 2) is $0.73 and the cost when using standard PCR conditions and agarose gel electrophoresis (Fig. 3) is $0.35. Approximately $0.15 of this cost is associated with the use of low melting temperature agarose (GenePure HiRes, ISC, BioExpress, Kaysville, UT or Metaphor, BioWhittaker Molecular Applications, Rockland, ME).

Ultimately, validation of Iso1016 as a marker for kdr in Latin American *Ae. aegypti* will require testing for the association between Iso1016 and pyrethroid susceptibility in field-collected material. In addition, Iso1016 has not been tested to confirm reduced sodium channel sensitivity to pyrethroids *in vivo* or in an *in vitro* expression system. Further functional validation is required before it can be used as a molecular tool for monitoring pyrethroid resistance. Therefore, it remains a putative kdr mutation. Because Iso1016 acts as a recessive allele in conditioning survival and the frequency of Iso1016 homozygotes is low in unselected lines, this effort may require applying pyrethroid selection for several generations to generate Iso1016 homozygotes prior to testing the association between Iso1016 and resistance. Alternatively, there was a ~42% difference in survival rate 4 h after permethrin exposure (Fig. 5) between Val1016/Iso1016 heterozygotes and Val1016/Val1016 homozygotes. This suggests that survival tests 4 h following exposure may be a more sensitive test of whether Iso1016 confers kdr. The tools and information presented here should provide a means for early detection and characterization of kdr that is affordable and practical for many laboratories in developing countries. This information will be critical in the development of information and strategies for resistance management.

Experimental procedures

Mosquito strains and DNA isolation

Table 3 lists the names of the *Ae. aegypti* strains analysed, the names, collection locations, country and coordinates of the collection sites (or, in the case of the Cuban strains, the publications describing the collection sites). Also listed are the numbers of mosquitoes analysed in each strain.

The four insecticide selected strains from Cuba (F_{12} and F_{13} deltamethrin, F_{14} propoxur and F_6 temephos) all originated from the Santiago de Cuba collection originally described by Rodriguez *et al.* (1999). Selection conditions for F_{12} and F_{13} deltamethrin are described in Rodriguez *et al.* (2005). Selection conditions for F_6 temephos are described in (Rodriguez *et al.*, 2002) and selection of F_{14} propoxur appears in Bisset *et al.* (2006).

Aedes aegypti were collected from Isla Mujeres, an island just northeast of Cancun, Mexico (Flores *et al.*, 2006). This is a tourist resort and both temephos and pyrethroids are regularly used for larval and adult control, respectively. After establishment of an F_1 population in the laboratory, F_2 adults were exposed to 1.2 µg of permethrin in a bottle bioassay (Brogdon & McAllister, 1998). After a 1 h exposure, mosquitoes were returned to a cage. After 4 h, 14% of inactive mosquitoes survived, were blood fed and laid F_3 eggs. Adult F_3 were again exposed to 1.2 µg of permethrin for 1 h and returned to a cage. After 4 h, 90% of the mosquitoes had survived, these were blood fed and laid F_4 eggs. This was repeated one more time and 98% of F_4 adults survived.

The F_5 generation of the Isla Mujeres strain was crossed with the standard susceptible New Orleans strain for quantitative trait locus mapping (K. Saavedra-Rodriguez, unpubl. data). The F_5 generation was then exposed to 1.2 µg of permethrin in the bottle bioassay for 1 h. Active adults were scored as alive. The remainder of adults were checked after 4 h and scored as active or dead. Only data on the segregation of alleles at the para locus are provided here.

The remainder of mosquito strains analysed arose from F_1 eggs laid by adults that had been raised from field-collected larvae. DNA was isolated from individual mosquitoes by salt extraction (Black & DuTeau, 1997) and suspended in 500 µl TE buffer (10 mM Tris-HCl, 1 mM EDTA pH 8.0). The DNA was divided into 5–100 µl aliquots and stored at –70 °C.

Allele-specific PCR

SNPs were detected using allele-specific PCR. Optimal PCR primers were identified using Primer Premier® software (Premier Biosoft International, Palo Alto, CA). Allele-specific primers were designed first. The 3′ end of an initial 25-nucleotide (nt) oligonucleotide was placed on the SNP in the antisense strand and the primer was then examined for stable ($\Delta G < -6.0$ kcal/mol) self-annealing or secondary structures. If either structure appeared on the 5′ end, we trimmed the sequence down to ≥ 18 nt in an attempt to remove the problem nucleotides. Otherwise, we repeated the process but with the 3′ end of the 25 nt oligonucleotide located on the sense strand. With the four mutations in this study, we were fortunate in being able to identify allele-specific primers without stable self-annealing or secondary structures in at least one direction. Once primers were identified we intentionally replaced the third nucleotide from the 3′ end with a mismatch. This step was originally recommended by Okimoto & Dodgson, (1996) and greatly improved the specificity of the assay. If a purine naturally occurred at this site we replaced it with a pyrimidine and vice versa. In addition, we used different mismatched purines or pyrimidines in the two allele-specific primers (see Table 2). Next, we used Primer Premier® software to identify a reverse primer to the allele-specific primers. Criteria were set to identify reverse primers that would amplify a product < 90 bp as recommended by Wang *et al.* (2005). The reverse primer was also examined for stable self-annealing or secondary structures both with itself and with the allele-specific primers.

Genotypes were determined in a single-tube PCR using the two different 'allele-specific' primers and the reverse primer. Allele-specific primers were manufactured (Operon Inc., Huntsville, AL, USA) with the 5′ tails originally described by Germer & Higuchi (1999) and Wang *et al.* (2005) to discriminate amongst the amplification products either based on size or melting temperatures. These were (5′-GCGGGCAGGGCGGCGGGGGCGGGGCC [allele-specific primer 1]-3′ or 5′-GCGGGC [allele-specific primer 2]-3′).

PCR was performed in a 25 µl volume in 96-well Hard Shell™ plates with white wells (Bio-Rad Laboratories, Hercules, CA, USA). Each reaction contained 12.5 µl of 2× iQ™ SYBR® Green Supermix (Bio-Rad Laboratories) (final concentrations = 50 mM KCl, 20 mM Tris-HCl, pH 8.4, 0.2 mM of each dNTP, 0.625 units iTaq® DNA polymerase, 3 mM $MgCl_2$, 1× SYBR Green I, 10 nM fluorescein), 25 pm of each primer, ~100 ng of template DNA, and sterile filtered ddH_2O water added to make a final 25 µl volume. PCR wells were covered with Flat Cap Strips (Bio-Rad Laboratories) and placed into the Opticon 2 DNA Engine (MJ Research, Waltham MA, USA). Thermal cycling conditions were: (1) 95 °C for 12 min (first denature); (2) 95 °C for 20 s (denature in cycle); (3) 60 °C for 1 min (anneal); (4) 72 °C for 30 s (extension); (5) cycle to step (2) 39 times; (6) 72 °C for 5 min (final extension); and (7) ramp from 65 °C to 95 °C at a rate of 0.2 °C/s (melting curve).

For agarose gel electrophoresis detection, a 4.0% (w/v) GenePure HiRes agarose (ISC BioExpress, Kaysville, UT, USA) gel was poured with 1× Tris-Borate-EDTA (89 mM Tris-borate and 2 mM EDTA, pH 8.3). DNA fragments were fractionated by electrophoresis for 90 min at 80 V alongside a 25 bp DNA ladder (TrackIt, Invitrogen, Carlsbad, CA, USA).

Evolution of kdr mutations

We amplified and directly sequenced the intron spanning exons 20 and 21 to examine the evolution of the four para mutations. Primers kdr20f and kdr21r (Table 2, Fig. 1) were used to amplify the 3′ end of exon 20, the entire intron and the 5′end of exon 21. Sequences were obtained from 87 mosquitoes chosen based upon their genotypes in exons 1011 and 1016. Sequences were aligned using CLUSTALW (Thompson *et al.*, 1994). Trace files contained double peaks when mosquitoes were heterozygous for length polymorphisms in the intron. In these mosquitoes haplotype sequences were ascertained by cloning and sequencing PCR products.

The numbers of segregating sites, insertion/deletions, haplotypes, a nucleotide diversity (π, Nei & Miller, 1990), the average number of nucleotide differences (k, Tajima, 1993), the minimum number of recombination events (R_m, Hudson & Kaplan, 1985) and linkage disequilibrium coefficients (Hill & Robertson, 1968) were estimated using DNASP 4.10.9 (Rozas *et al.*, 2003). DNASP does not analyse aligned positions containing insertions/deletions.

Phylogenetic reconstruction was carried out using maximum parsimony analysis in PAUP 4.0 (Swofford, 1993) in which insertions/deletions were treated as a fifth character and a bootstrap analysis with 1000 replications was performed to test support for the derived phylogeny.

Acknowledgement

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