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Local Evolution of Pyrethroid Resistance Offsets Gene Flow among *Aedes aegypti* Collections in Yucatan State, Mexico

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Abstract. The mosquito *Aedes aegypti* is the major vector of the four serotypes of dengue virus (DENV1–4). Previous studies have shown that *Ae. aegypti* in Mexico have a high effective migration rate and that gene flow occurs among populations that are up to 150 km apart. Since 2000, pyrethroids have been widely used for suppression of *Ae. aegypti* in cities in Mexico. In Yucatan State in particular, pyrethroids have been applied in and around dengue case households creating an opportunity for local selection and evolution of resistance. Herein, we test for evidence of local adaptation by comparing patterns of variation among 27 *Ae. aegypti* collections at 13 single nucleotide polymorphisms (SNPs): two in the voltage-gated sodium channel gene *para* known to confer knockdown resistance, three in detoxification genes previously associated with pyrethroid resistance, and eight in putatively neutral loci. The SNPs in *para* varied greatly in frequency among collections, whereas SNPs at the remaining 11 loci showed little variation supporting previous evidence for extensive local gene flow. Among *Ae. aegypti* in Yucatan State, Mexico, local adaptation to pyrethroids appears to offset the homogenizing effects of gene flow.

INTRODUCTION

Aedes aegypti is the principal urban vector of dengue, chikungunya, and yellow fever viruses.[1] The mosquito is closely associated with human habitation: the immature stages are found in water-holding containers in the peridomestic environment and adult females commonly feed and rest indoors. In Yucatan State in Southern Mexico, *Ae. aegypti* is ubiquitous in large urban centers and in smaller communities[2] and dengue is hyperendemic.[3,4] The mosquito is controlled through a combination of physical source reduction (removal or alteration of potential water-holding containers) and use of insecticides including the organophosphate temephos to eliminate larvae from water-holding containers and since 2000 pyrethroids have been used to control adults in and around homes.[5,6]

Although insecticide pressure is high in large urban centers in Yucatan State, fine-scale refugia from insecticides are present. Refugia from temephos include atypical development sites (e.g., vacant lots or storm water drains) for larvae that may not be included in the control effort and residential premises that go untreated as a result of a lack of access by mosquito control teams. Indoor environments provide refugia against vehicle-based spraying of pyrethroids caused by limited spray penetration of the relatively closed concrete homes typical of Yucatan State. Moreover, the intensity of insecticide-based mosquito control to a large extent follows the occurrence of dengue disease cases, and therefore is variable both in space and over time.

This complex and dynamic spatiotemporal mosaic of insecticide pressure, together with mosquito migration through flight or human transport of containers infested with eggs or larvae, shape the rate and patterns of gene flow among *Ae. aegypti* populations in Yucatan State. Earlier reports on the genetic structure of *Ae. aegypti* collections based on determination of mitochondrial ND4 haplotypes, indicated that among and within three regions of Mexico—Northeastern, Pacific, and Yucatan Peninsula—collections can be expected to remain genetically uniform within distances up to 150 km.[7,8] Moreover, recent data have been gathered from the Yucatan Peninsula on the emergence and rapid rise to near fixation in large urban centers of a knockdown resistance (kdr)-conferring allele (I1,016) in the voltage-gated sodium channel gene (*para*) of *Ae. aegypti*.[4,9] Another *para* mutation that is also strongly implicated in pyrethroid resistance involves a cysteine replacement (C1,534).[10,11]

Our goal in this study is to test two alternative hypotheses concerning the evolution and spread of pyrethroid resistance in Yucatan State, Mexico. One hypothesis is that pyrethroid resistance is uniformly spread throughout Yucatan State by high rates of gene flow. If this hypothesis is valid then we would expect genes that confer pyrethroid resistance to follow the same patterns of variation as neutral genes distributed throughout the genome. An alternative hypothesis is that, despite high rates of gene flow, pyrethroid evolution occurs locally. Specifically, we would expect the frequencies of I1,016 and C1,534 to increase in the presence of pyrethroids and, possibly when pyrethroids are removed, to decline in frequency if these alleles have a low fitness in the absence of insecticides. If this hypothesis is correct we would then expect genes that confer pyrethroid resistance to exhibit greater spatial variation than detected among neutral genes.

Herein, we examine variation at 13 single nucleotide polymorphisms (SNPs). These include I1,016 and C1,534 in *para* and two cytochrome P_{450} genes, CYP9J32 and CYP9J29 previously associated with permethrin resistance in a QTL mapping study[12] and in a microarray analysis.[13] Moreover, CYP9J32 has exhibited pyrethroid metabolizing activity[14] and was also overexpressed in Mexican mosquito lines selected with temephos alongside a carboxyl/esterase gene CCE1C.[15] The other eight SNPs are putatively insecticide-neutral loci that have been examined in previous population

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genetic and mapping studies.[12,16–19] Alleles at these 13 loci were compared among 27 collections of *Ae. aegypti* originating from eight different communities in Yucatan State. This allowed us to quantify gene flow and genetic structuring among and within communities and collections, and to determine whether patterns of genetic differentiation between putatively neutral genes are similar to patterns observed among insecticide resistance-associated genes.

MATERIALS AND METHODS

Mosquito collections and rearing. *Aedes aegypti* mosquitoes used in this study originated from previously described collections of larvae in Yucatan State over a 12-month period from March 2010 to February 2011.[2] Fourth-instar larvae and pupae were retrieved from a variety of containers found on residential premises or in cemeteries. We typically sampled ≥ 20 containers per collection site. After eclosion, adults were identified to species and *Ae. aegypti* females were artificially blood fed to generate F_1 eggs. Only egg papers that subsequently resulted in > 500 adults were used for the study. We randomly selected from 29 to 60 F_1 adults per collection to minimize the likelihood of oversampling families in single containers. This was particularly important in the current study because family biased sampling could generate genetic clusters that could be misinterpreted as local adaptation.

This study includes 27 collections from eight different communities in Yucatan State, totaling 1,301 mosquitoes (Table 1, Figure 1). Yucatan has a subtropical climate with seasonal heavy rainfall from June to October and sporadic rain during the remaining year. Mosquito collections were made in eight communities with different levels of urbanization. Seven communities surround the main urban city Merida (population = 777,615), which is centrally located and connected by roads among all communities by distances ranging from 5 to 150 km (Figure 1). Altitude ranges from 0 to 20 meters above sea level and human population size in our collection communities varied from 2,111 to 39,611.[2] For small-scale analysis, we obtained nested collections within Merida, Uman, and Caucel (Supplemental Figure 1). Five of these collections (comprising 232 mosquitoes) originated from the city of Merida (the largest urban center in the state), and were located within 3 km of each other (Supplemental Figure 1). Other local collections were made in the satellite communities of Caucel (549 mosquitoes from 11 collections located within 3 km of each other—Supplemental Figure 2) and Uman (285 mosquitoes in five collections located within 5 km of each other—Supplemental Figure 3) or from other single locations in communities > 25 km distant from Merida (234 mosquitoes in five collections Table 1, Figure 1). Supplemental figures are included to show the sizes of the collection areas in Merida, Uman, and Caucel. The largest collection site was Centro in the center of Merida, whereas the smallest was Siglo XXI in Uman. Because > 20 containers were sampled from each collection site, Centro would have had the lowest density of samples, whereas Siglo XXI would have had the greatest sampling density.

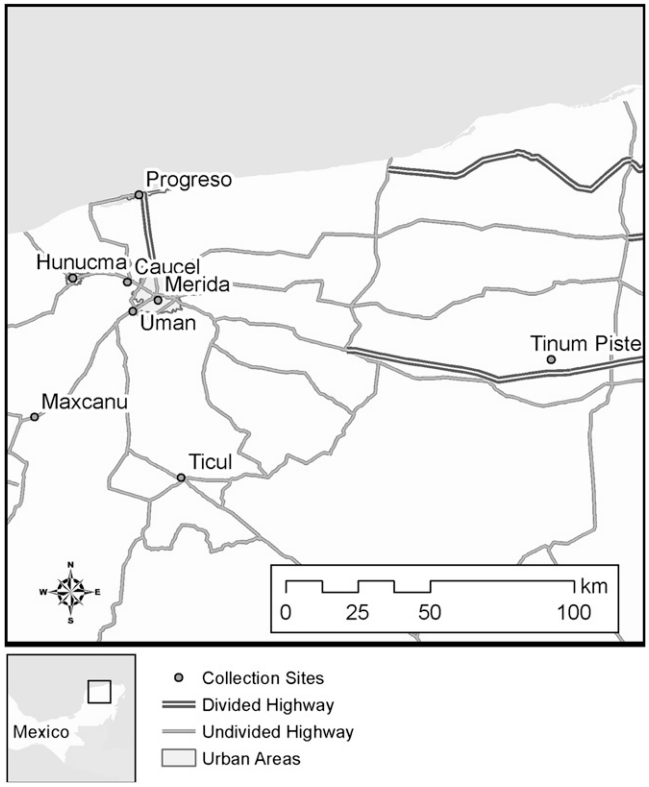

FIGURE 1. Map of collection sites across the Yucatan State.

TABLE 1

Collection sites, geographical coordinates, and number of mosquitoes used for analysis*

Community	Collections	n	Longitude	Latitude
Merida	Centro	46	–89.61745	20.95163
	San Jose Vergel	46	–89.58848	20.95532
	Xoclan Canto	47	–89.66492	20.96700
	Vergel I	47	–89.57865	20.94833
	Chuburna	46	–89.63282	21.01425
Caucel	Balcones I	29	–89.70270	21.00083
	Arboleda	34	–89.70645	21.00285
	Herradura	31	–89.68935	20.99722
	Sol Caucel IV	50	–89.71352	21.00682
	Pedregales Caucel	45	–89.69068	20.99987
	Los Almendros II	60	–89.70195	20.99543
	Los Almendros Caucel	59	–89.70033	20.99758
	Hacienda Caucel	61	–89.71257	21.00577
	Los Cocos Caucel	60	–89.71300	20.99593
	Torres I	60	–89.69425	21.00487
	Viva Caucel	60	–89.71827	20.99827
Uman	Itzincab Palomeque	48	–89.69432	20.91413
	Paseos de Itzincab	48	–89.69603	20.91733
	San Lorenzo	47	–89.73140	20.89287
	Acim 1	47	–89.70578	20.91895
	Arcos I	48	–89.73885	20.88988
	Siglos XX1	47	–89.73452	20.88532
Progreso		47	–89.67783	21.28067
Ticul		47	–89.54522	20.39727
Hunucma		46	–89.88343	21.02115
Maxcanu		47	–90.00250	20.58583
Tinum-Piste		47	–88.39200	20.76650

*Cities Merida, Uman, and Caucel are composed of several nested collections. The remaining sites are composed of a single collection.

DNA isolation and SNP genotyping. The DNA was isolated from individual mosquitoes by the salt extraction method[20] and suspended in 150 μL of TE buffer (10 mM Tris-HCl, 1 mM EDTA pH 8.0). The SNP identification, allele-specific polymerase chain reaction (PCR), melting

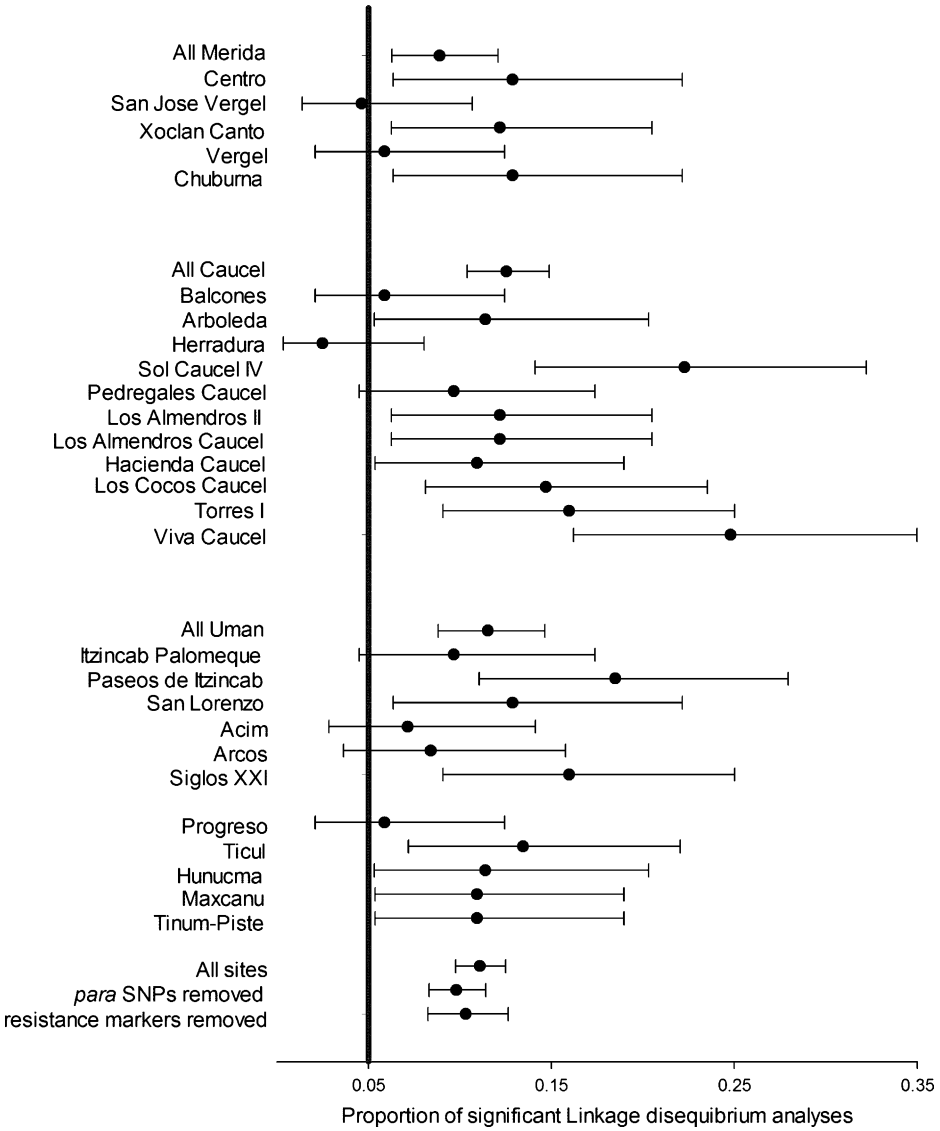

FIGURE 2. Analysis of the proportion of significant linkage disequilibrium ($N = 78$ pairwise comparisons) for each community and collection site. Bars represent Bayesian 95% highest density intervals (95% HDI). Proportions with HDI that overlap 0.05 were not considered to have a credible excess of linkage disequilibrium.

curve conditions, and genotype readings followed published procedures.[11,12,21,22] Table 2 lists the names of the SNP markers, their VectorBase identification numbers, physical or genetic location in the genome (if known) and the primer sequences used for allele-specific PCR. We obtained SNP genotypes at 13 SNP loci in each mosquito. Table 3 describes the salient features of each of the 13 SNPs including: chromosome and linkage location, gene name, the position of the SNP in the gene and in the codon, whether the SNP codes for a replacement substitution, and the alternate nucleotides at the site.

Statistical analysis of allele frequencies. Individual diploid genotypic data at the 13 SNP loci was reformatted with the program CONVERT[23] for subsequent use in the programs GDA[24] and ARLEQUINv.3.5.[25] Pairwise linkage disequilibrium analyses were also conducted in ARLEQUINv.3.5 and tested for significance using the expectation-maximization (EM) algorithm with 10,000 permutations. Proportions of significant linkage disequilibrium among 78 pairwise comparisons were compared by Bayesian 95% Highest Density Intervals (95% HDI) using WinBUGS.[26] All additional analyses were performed using three different sets of data that included: 1) all 13 SNP loci, 2) only the eight neutral SNP loci and, 3) only the five insecticide resistance SNP loci (Table 2). Variation in genotype frequencies among communities and within collections was determined by analysis of molecular variance (AMOVA) using ARLEQUINv.3.5. This program also estimated fixation indexes within (F_{IS}) and among (F_{ST}) collections and computed the significance of the variance components associated with each level of genetic structure by a nonparametric permutation test with 10,000 pseudoreplicates. Population genetic analyses were carried out using the Wright's FST estimate assuming the island model. We performed a Mantel analysis to test the relationship between geographic and genetic distances using the software PASSaGE.[27] A normalized Mantel correlation coefficient (r)

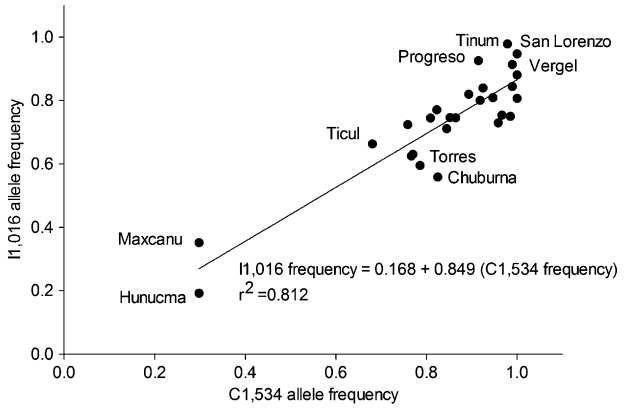

FIGURE 3. Regression analysis of allele frequencies of two *para* mutations: I1,016 and C1,534 in 27 collections from Yucatan.

was obtained from matrices of pairwise Slatkin's linearized F_{ST} $[F_{ST}/(1\text{-}F_{ST})]$[28] and pairwise geographic distances. Significance was tested by randomly permuting the order of the elements within the distance matrices.

RESULTS

SNP allele and genotype frequencies within collections. A total of 1,301 *Ae. aegypti* individuals, from 27 collections in eight different communities, were genotyped at 13 SNP marker loci. Allele frequencies are shown in Supplemental Table 1. Genotype proportions at each of the 13 loci were tested for Hardy Weinberg (HW) equilibrium. Table 4 lists the fixation index (F_{IS} = 1-[H_{obs}/H_{exp}]) where H_{obs} is the observed number of heterozygotes and H_{exp} is the expected number of heterozygotes obtained for each locus in each collection. Seven out of 351 collection-by-locus tests could not be performed as one of the alleles was fixed. From the remaining 344 collection-by-locus tests, 31 (9.0%) did not fit HW proportions at an α = 0.05. Lack of fit to HW proportions occurred eight times in 26 tests at locus CCE1C and this was significantly more than expected ($\chi^2_{[1\ d.f]}$, P = 0.024). Lack of fit to HW proportions occurred at 4 of 12 tests in the Tinum-Piste collection but this was not significantly more than expected ($\chi^2_{[1\ d.f],}$ P = 0.316). Overall, there was an excess of heterozygotes at locus CCE1C but otherwise genotypes conformed to HW expectations. The consistent heterozygote

TABLE 2

Single nucleotide polymorphic markers, gene vector base ID, genetic or physical location, and allele-specific oligonucleotide sequences*

SNP markers	Gene vector base ID	Physical/genetic location	Allele-specific oligonucleotide sequence (5′–3′)
Putative neutral loci			
Amy 447	Amylase II (AAEL013421)	1-32 cM[16]	5LT-ACCGAACGACTTCAATGCG 5ST-ACCGAACGACTTCAATACT CCAGCAGTTACGCACCTGATAG
Amy 450	Amylase II (AAEL013421)	1-32 cM[16]	5LT-TAGTCGTAGATTTCAGAA 5ST-TAGTCGTAGATTTCAAAC AACTTCCCTGCAGTCCCC
TrypE	Early Trypsin (AAEL007818)	2-19 cM[16]	5LT-GGCTACCGCATAACCCTGAACCACA 5ST-CTACCGCATAACCATGAACC TGGCTGAGTCCCAGAAGG
VCP	Vitellogenic carboxypeptidase precursor (AAEL15312)	2-23 cM[17]	5LT-GTTGACACCCGAKCGGTAACCTTCC 5ST-GTTGACACCCGAKCGGTAACCTCCT CAGCCACTTGACGAGAACAT
Chymo	Chymotrypsin (AAEL014188)	2q4.4[18]	5LT-CGTCGTTTGGTTGCGGARTTCAGCG 5ST-CGTCGTTTGGTTGCGGARTTCAACA CGGGACTGACTCCYCCTTGATAG
Malt	Maltase (AAEL009524)	3p3.1[18]	5LT-ACCGTCCARATCCCCGATAGCG 5ST-ACCGTCCARATCCCCGATAACA GAAAYTTCTACCAAGTTTACCCAA
GPI	Glucose-6-phosphate isomerase (AAEL012994)	3q	5LT-GCTGATTGCCATGTACGAACACCAG 5ST-GCTGATTGCCATGTACGAACACTAA CGTCCCAGATGACACCCT
Apyr	Apyrase 2 (AAEL006347)	3-68 cM[12]	5LT-ATTTCCAGTTTGAATCTGA 5ST-ATTTCCAGTTTGAATCAGT GCTTTTAAGTCTCGTTTTCG
Insecticide resistance loci			
CCE1C	Carboxyl/cholinesterase 1C (AEL003201)	1q2.1[19]	5LT-TACTCCTATTGCAGATTTTATTCAC 5ST-TACTCCTATTGCAGATTTTATTTAT TCCACGAGGAATCGGKAT
CYP9J32	Cytochrome P_{450} oxidase 9J32 (AAEL008846)	2q1.1[19]	5LT-ACTGCTTCCTTGATGATTGTG 5ST-ACTGCTTCCTTGATGATTATT AAGTTTGATGATTAAGATGGG
I1,016	Voltage gated sodium channel (AAEL006019)	3q2.5[18]	5LT-ACAAATTGTTTCCCACCCGCACCGG 5ST-ACAAATTGTTTCCCACCCGCACTGA TGATGAACCSGAATTGGACAAAAGC
C1,534	Voltage gated sodium channel (AAEL006019)	3q2.5[18]	5LT-TCTACTTTGTGTTCTTCATCATGTG 5ST-TCTACTTTGTGTTCTTCATCATATT TCTGCTCGTTGAAGTTGTCGAT
CYP9J29	Cytochrome P_{450} oxidase 9J29 (AAEL014610)	3-50cM[12]	5LT-CATCGGGTCACGGTTTCCG 5ST-CATCGGGTCACGGTTTTCA GAACGAAAATCTACGCAGCAT

*Locations were previously determined.[12,16–19] 5LT corresponds to the sequence 5′-GCGGGCAGGGCGGCG GGGGCGGGGCC-3′ and 5ST to the sequence 5′-GCGGGC-3′.

TABLE 3
Physical position of single nucleotide polymorphisms (SNPs)*

Marker	Annotation	Amino acid/SNP	Codon	Silent/ replace	Nucleotide	Nucleotides from ATG
Chrom 1 Amy447	α-amylase	Pro149	CCT	Silent	T/G	447
Amy450	α-amylase	Pro150	CCT	Silent	T/G	450
CCE1C	α-esterase	Asp276	GAT	Silent	T/C	828
Chrom 2						
TrypE	trypsin 3A1 Precursor	5′UTR	–	Indel	CACAGCC/–	–4
VCP	cathepsin B	Pro95	CCA	Silent	A/G	285
Chymo	serine-type endopeptidase	Val110	GTC	Ile	G/A	328
CYP9J32	cytochrome P450	Ser260	TCA	Silent	A/C	780
Chrom 3						
Malt	maltase precursor	Asp40	GAT	Silent	T/C	123
I1,016	*para*	Val776	GTA	Ile	A/G	2328
C1,534	*para*	Phe1269	TTC	Cys	T/G	3807
GPI	glucose-6-phosphate isomerase	Lys500	AAG	Silent	G/A	1500
CYP9J29	cytochrome P450	Ala487	GCA	Silent	A/G	1461
Apyr	apyrase precursor	5′UTR	–		A/T	–260

*Gene annotation, residue position if SNP belongs to coding region; type of replacement and SNP position in coding sequence starting from ATG.

excess at locus CCE1C suggests the genotype assay for this SNP may be inaccurate or that there is a heterozygote advantage conferred by the SNP. The fact that it encodes a transition and is synonymous makes heterozygote advantage unlikely. The excess of heterozygotes at CCE1C might also be associated with duplication. However, in earlier studies we found that the segregation of alleles at the *para* locus[12] and three different CCE-C genes fit a single gene model.[19]

Linkage disequilibrium among loci. An analysis of linkage disequilibrium was performed to determine whether alleles at the 13 SNP loci segregated independently from one another. There were 2,106 ([13 × 12/2] × 27) potentially pairwise linkage disequilibrium comparisons except that alleles were fixed at certain loci in specific collections. From a total of 2,034 dilocus-by collection tests, 225 (11.1%) exhibited significant linkage disequilibrium. The Bayesian HDI around this rate extended from 9.8% to 12.5% for all sites and these proportions changed only slightly when removing the *para* SNPs or the other putative resistance markers (Figure 2). Thus, there were an excess proportion of loci in disequilibrium.

TABLE 4
Fixation index (F_{IS}) for each collection site at each SNP marker*

Community	F_{IS} coefficient and Hardy Weinberg equilibrium												
	Putative neutral loci								Insecticide resistance loci				
Collection	Amy447	Amy450	TrypE	VCP	Chymo	Malt	GPI	Apyr	CCE1C	CYP9J32	I1,016	C1,534	CYP9J29
Merida													
Centro	0.06	–0.08	0.23	0.04	0.11	0.20	0.12	–0.14	–	–0.02	–0.24	–0.08	0.09
San Jose Vergel	–0.07	0.03	–0.12	–0.17	0.15	–0.05	**0.33**	–0.18	–0.03	–0.30	–0.13	–	0.15
Xoclan Canto	–0.10	0.00	–0.11	–0.16	–0.02	–0.11	–0.01	–0.11	–0.01	0.20	–0.23	–0.05	–0.05
Vergel I	0.00	0.01	–0.08	–0.05	–0.33	0.02	–0.16	–0.11	–0.06	–0.28	0.24	0.19	0.11
Chuburna	0.48	**-0.38**	0.32	–0.17	0.24	–0.07	0.23	–0.10	–0.05	0.31	0.02	0.16	0.26
Caucel													
Balcones	–0.10	0.35	0.04	–0.08	–0.17	0.27	0.10	–0.06	**-0.44**	0.05	–0.19	0.26	0.38
Arboleda	–0.02	–0.06	–0.16	–0.29	–0.10	**0.50**	–0.16	–	–0.26	0.01	–0.32	0.00	0.36
Herradura	–0.13	0.28	–0.05	–0.13	–0.20	–0.13	–0.25	–0.18	–0.18	0.01	–0.22	–	0.21
Sol Caucel IV	–0.05	0.07	**-0.33**	–0.09	–0.05	–0.11	–0.13	–0.02	**-0.29**	0.11	0.11	**0.39**	**0.31**
Pedregales Caucel	–0.14	**0.37**	–0.09	–0.07	0.13	0.17	0.30	–0.08	–0.24	–0.04	0.25	**0.67**	–0.04
Los Almendros II	–0.03	0.00	0.17	–0.17	–0.05	0.04	–0.06	–0.18	**-0.34**	0.10	0.19	–0.07	0.21
Los Almendros Caucel	–0.10	0.02	0.07	–0.20	–0.02	0.15	–0.15	–0.05	–0.14	0.11	–0.06	0.00	**0.67**
Hacienda Caucel	–0.06	0.20	–0.09	–0.14	–0.19	–0.13	–0.08	–0.12	–0.24	0.16	0.10	0.10	**0.45**
Los Cocos Caucel	–0.07	**0.32**	**-0.28**	–0.24	0.06	–0.09	0.17	–0.04	**-0.36**	0.08	0.14	0.49	0.06
Torres I	–0.11	–0.04	–0.07	–0.07	–0.08	–0.08	–0.09	–0.11	–0.20	0.20	0.23	**0.37**	0.23
Viva Caucel	–0.06	0.11	0.03	–0.18	**–0.27**	–0.05	–0.10	–0.05	–0.26	–0.02	0.12	0.17	0.12
Uman													
Itzincab Palomeque	–0.15	**0.78**	–0.08	–0.29	–0.02	–0.06	–0.12	–0.07	–0.08	–0.10	0.14	0.00	–0.18
Paseos de Itzincab	–0.15	**0.37**	0.12	–0.21	–0.13	0.17	0.01	–0.13	**-0.31**	–0.02	–0.15	–0.03	–0.05
San Lorenzo	0.08	0.34	–0.19	–0.12	–0.06	0.12	**0.65**	–0.06	–0.14	–0.23	–0.05	–	**0.60**
Acim 1	–0.07	–0.07	–0.20	–0.19	–0.11	–0.15	–0.03	–0.12	**-0.33**	–0.19	–0.11	–0.09	0.11
Arcos I	–0.12	–0.09	–0.24	–0.19	–0.02	–0.19	–0.15	–0.11	**-0.34**	–0.24	–0.17	–0.21	0.09
Siglos XXI	–0.26	–0.24	–0.24	–0.19	0.26	0.26	–0.08	–0.16	–0.23	0.20	–0.21	–0.11	0.02
Progreso	–0.17	0.11	0.18	0.30	**0.03**	–0.39	0.08	–0.02	–0.18	–0.02	–0.01	–0.16	0.27
Ticul	–0.11	–0.07	–0.11	–0.07	–0.08	**-0.12**	–	–0.07	–0.04	–0.04	0.05	–0.21	–0.17
Hunucma	–0.11	0.15	–0.08	–0.07	–0.14	0.24	0.15	–0.16	–0.20	–0.19	–0.08	0.00	0.27
Maxcanu	–0.03	0.17	–0.21	0.00	0.24	0.12	–0.08	–0.04	–0.23	–0.04	0.12	–0.21	0.23
Tinum-Piste	–	–0.01	**0.40**	–0.12	–0.26	0.12	0.15	–0.12	**-0.33**	**0.32**	1.00	**1.00**	0.28

*Bold numbers show markers that did not fit Hardy-Weinberg equilibrium ($P < 0.05$). Positive values show an excess of homozygotes. Negative value is caused by an excess of heterozygotes. (–) indicates fixation.

TABLE 5
Number of collection sites that showed significant linkage disequilibrium at a pairwise di-locus comparison

Chromosome	Loci	Number of collections with significant disequilibrium at each dilocus comparison												
		Amy447	Amy450	CCE1C	TrypE	VCP	Chymo	CYP9J32	Malt	I1,016	C1,534	GPI	CYP9J29	Apyr
1	Amy447	–	5	2	1	3	1	3	1	0	4	2	1	0
1	Amy450		–	5	7*	1	6	3	3	2	2	4	1	5
1	CCE1c			–	1	1	2	0	3	3	2	0	4	2
2	TrypE				–	1	3	4	4	6	3	2	1	3
2	VCP					–	4	1	4	4	4	2	3	3
2	Chymo						–	6	3	3	3	1	2	2
2	CYP9J32							–	5	5	3	2	4	0
3	Malt								–	3	1	1	2	2
3	I1,016									–	20†	2	5	1
3	C1,534										–	2	5	3
3	GPI											–	3	2
3	CYP9J29												–	2
3	Apyr													–

*$P \leq 0.05$.
†$P \leq 0.001$.

Figure 2 indicates that this was not associated with one community or collection site in a community. In Merida, the HDI did not cross the $\alpha = 0.05$ threshold in three of five collection sites. In Caucel, the HDI did not contain the 0.05 threshold in eight of eleven collection sites. In Uman, HDI did not contain the 0.05 threshold in three of six collection sites and only crossed the threshold in one of five remaining sites. Table 5 lists for each of 78 di-locus pairs the number of collections exhibiting disequilibrium. Only two of these, (TrypE/Amy450 and I1,016/C1,534) exceed the $\alpha = 0.05$ threshold. Of possible di-locus comparisons, I1,016 and C1,534 were in disequilibrium in 20 of 27 collections. Figure 3 indicates that frequencies of resistant alleles I1,016 and C1,534 co-occur in 81.2% of the individuals within collections.

SNP allele frequencies among collections. We performed an AMOVA assuming a hierarchical structure of eight communities. Three of these communities—Merida, Uman, and Caucel—contained five, six, and 11 collections, respectively, whereas the remaining five communities (Hunucma, Maxcanu, Ticul, Progreso, and Tinum-Piste) were represented by single collections. An AMOVA including all 13 loci indicated that 3.2% of the total variation was attributable to difference among communities, whereas 93.8% arose within collections ($P < 0.05$) Table 6. However, AMOVAs performed for each locus separately (Table 7) revealed that the percentage of total variation among communities ranged widely from 0% to 23.5%. Putatively neutral loci showed < 1.2% variation among communities but C1,534, I1,016, and CCE1C varied significantly among communities accounting for 23.5%, 13.2%, and 3.2% of the overall variance, respectively.

Two additional AMOVAs were performed to compare variation among collections in the frequency of putatively neutral markers with variation in the frequency of the five resistance markers (Table 6). For the putatively neutral markers, variation among communities was non-significant and accounted for only 2.7% of the variation among collections within communities. For insecticide resistance loci, variation among communities accounted for 6.8% and 3.3% among collections within communities.

Isolation by distance and genetic structure. A Mantel analysis was performed to test for isolation by distance. Figure 4

TABLE 6
Analysis of molecular variance (AMOVA) for three sets of data*

Data set	Source of variation	Sum of squares	F	% Variance
All loci				
Among communities		169.9	0.032*	3.2
Among collections within communities		139.8	0.030*	3.9
Among mosquitoes within collections		4650.4	0.062*	93.8
Total		4960.2		
Putative neutral loci				
Among communities		24.2	0.000	–0.03
Among collections within communities		68.5	0.027*	2.7
Among mosquitoes within collections		2513.4	0.027*	97.3
Total		2606		
Insecticide resistance loci				
Among communities		145.8	0.068*	6.8
Among collections within communities		71.3	0.035*	3.3
Among mosquitoes within collections		2136.9	0.100*	90
Total		2354.1		

TABLE 7
Analysis of molecular variance (AMOVA) by locus*

Loci	Among communities			Among collections within communities		
	SSD	d.f.	%	SSD	d.f.	%
Putative neutral						
Amy447	3	7	1.2	3	19	0.9
Amy450	6	7	0.6	12	19	3.2
TrypE	4	7	–0.1	10	19	2.6
VCP	2	7	–0.1	6	19	1.4
Chymo	2	7	–0.9	14	19	4.1
Malt	3	7	–0.3	10	19	2.9
GPI	3	7	–0.6	10	19	4.3
Apyr	2	7	0.2	3	19	1.0
Insecticide resistance						
CCE1C	12	7	3.3	7	19	1.7
CYP9J32	5	7	–0.6	23	19	4.0
I1,016	57	7	13.2	16	19	3.4
C1,534	67	7	23.5	13	19	4.2
CYP9J29	5	7	0.0	13	19	2.8

*Sum of squares (SSD), degrees of freedom (d.f.), and % of variation obtained among communities (8) and among collection within communities.

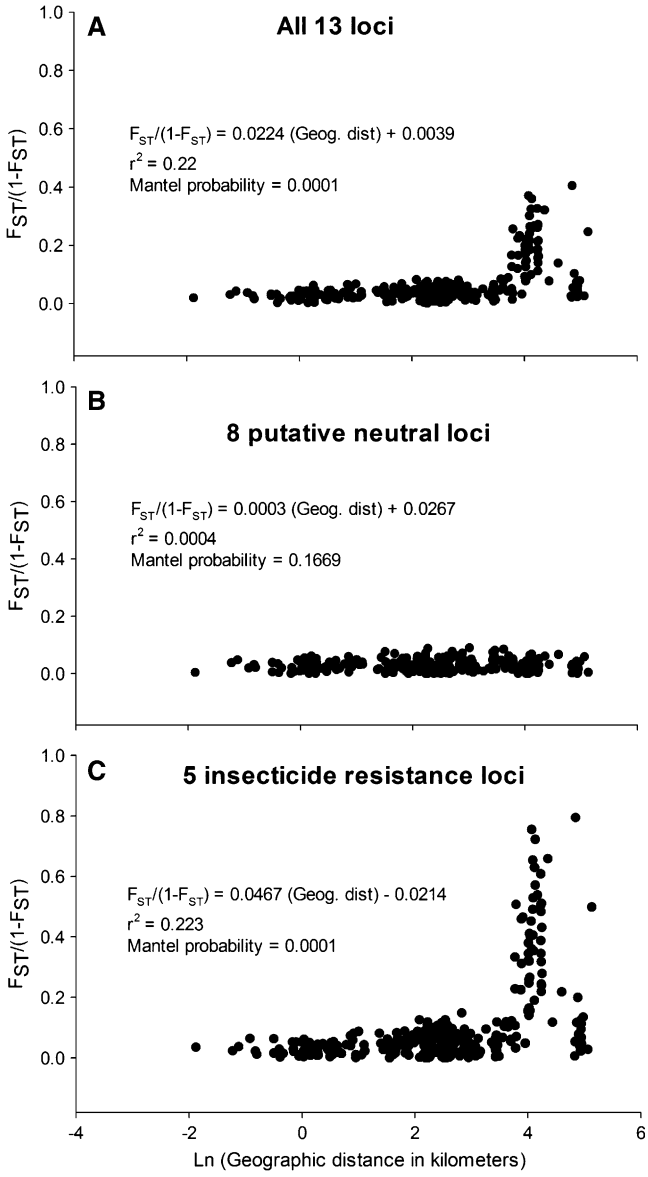

FIGURE 4. (**A**) Regression analysis of pairwise FST/(1-FST) for all 13 loci against ln(geographic distances[km]). (**B**) pairwise FST/(1-FST) for the putatively neutral loci against ln(geographic distances [km]), and (**C**) pairwise FST/(1-FST) for insecticide resistance loci against ln(geographic distances [km]). Regression analysis equation and correlation coefficient are also shown.

shows the relationship between Slatkin's linear pairwise F_{ST} ($F_{ST}/[1-F_{ST}]$) and the natural logarithm of the pairwise geographic distances for the three groups of markers. Populations were isolated by distance when analyzing all 13 markers (r^2 = 0.22, Mantel Prob = 0.0001). However, this pattern was inconsistent when considering only neutral markers (r^2 = 0.0004, Mantel Prob = 0.1669) for which no isolation by distance was evident and insecticide resistance markers, which exhibited isolation by distance (r^2 = 0.22, Mantel Prob = 0.0001), but only among collections that were farther apart. Collections on the left side of the graph were obtained in the same city, whereas collections on the right side came from small towns (Maxcanu, Progreso, Ticul) located further away from the larger cities.

DISCUSSION

The AMOVA and Mantel analyses of putatively neutral SNPs indicated high rates of gene flow among all collections in Yucatan State. However, allele frequencies at putative pyrethroid resistance loci were highly heterogeneous. In particular, C1,534 and I1,016 varied greatly in frequency among communities. These results suggest that these mutations are under strong selection pressure in Yucatan *Ae. aegypti* populations. We propose that this spatial variability is driven by local selection pressure generated by the use of pyrethroids in and around houses of dengue patients. Our indirect estimation of the intensity of insecticide use for vector control based in the reported dengue cases for each community from 2006 to 2010 (Supplemental Figure 4) suggests that insecticide is spatially and temporally heterogeneous across Yucatan communities. For example, a high number of dengue cases was reported for Merida from 2006 to 2010, suggesting high use of insecticides during this time span. Meanwhile, low to moderate intensity was estimated for the remaining examined communities, except for high use in Ticul and Progreso in 2007, and in Uman and Ticul in 2009, where dengue outbreaks occurred. As a common trend the use of insecticides increased in 2010, when most of the communities experienced dengue outbreaks resulting in moderate to high insecticide use by vector control campaigns.

This scenario is supported by our Mantel analysis for insecticide resistance loci, which identified a significant positive correlation between genetic and geographic distances. Our mosquito collections are connected to Merida through single roads. Although some collections are separated by large geographic distances (> 25 km) from Merida, we did not obtained significant variability at putative neutral loci, suggesting that there is abundant mosquito migration through eggs, larvae, and adults among collections that maintains high rates of gene flow. This was not surprising given our earlier studies with mitochondrial ND4 haplotypes that showed free gene flow among Yucatan collections within 150 km of distance.[7,8] Previous studies in Asia using either ND4 or microsatellite markers showed that *Ae. aegypti* populations were genetically different with a pattern of panmictic populations occurring in and near urban centers, but more genetically differentiated with increasing geographical distance from the urban centers.[29,30]

We expected to detect excess linkage disequilibrium among our SNP markers because *Ae. aegypti* in these communities are frequently subject to population suppression that may generate strong population bottlenecks that can, in turn, generate disequilibrium. Furthermore, a selective sweep might cause alleles linked to the *para* locus on chromosome 3 to be in linkage disequilibrium. As shown in Figure 2, there was an overall excess of analyses in which significant disequilibrium was detected. Furthermore, we found strong linkage disequilibrium between the C1,534 and I1,016 loci in the *para* gene. This suggests that either there has been a local selective sweep of both mutations or that the mutations arose independently and have been selected for in parallel. To test genetic sweep we would have to include SNPs present at introns in the *para* gene or surrounding genes in chromosome 3. However, this analysis was done in a prior study[21] and only very low levels of disequilibrium were discovered.

We wished to test two alternative hypotheses concerning the evolution and spread of pyrethroid resistance in Yucatan

State, Mexico. Our first hypothesis was that pyrethroid resistance will be uniformly spread throughout Yucatan State by high rates of gene flow. If so, then we expected genes that confer pyrethroid resistance to follow the same patterns of variation as neutral genes distributed throughout the genome. This pattern was not seen. Even though the frequencies of all neutral markers were uniformly spread throughout all of our 27 collection sites, markers C1,534, I1,016, and to a lesser extent CCE1C varied greatly in frequency among sites.

The alternative hypothesis was that, despite high rates of gene flow, pyrethroid evolution occurs locally. We expected the frequencies of I1,016, C1,534, CCE1C, CYP9J32, and CYP9J29 to increase in the presence of pyrethroids and, possibly when pyrethroids are removed, to decline in frequency if these alleles have low fitness in the absence of insecticides. If so, we would then expect I1,016, C1,534, CCE1C, CYP9J32, and CYP9J29 to exhibit greater spatial variation than detected among neutral genes. This is the pattern observed with I1,016, C1,534, and CCE1C.

These results suggest a general model for the movement of insecticide-resistant genes in the Yucatan. High rates of gene flow occur among *Ae. aegypti* populations within 150 km of one another. This gene flow spreads alleles at all loci, including resistance loci, across the State. In areas where insecticides are applied, the frequencies of resistance alleles increase. Conversely, in areas where insecticides are no longer applied, and assuming negative fitness associated with resistance alleles in the absence of insecticides, the frequencies of resistance alleles will decline. In an operational mosquito control context, our findings indicate that pyrethroid susceptibility can persist locally even with high gene flow and high widespread frequencies of pyrethroid resistance alleles. This situation is beneficial for insecticide resistance management; providing critical "islands" of pyrethroid susceptibility that provide a source for susceptibility alleles. Because wide cross resistance to pyrethroids has been reported among mosquito populations across Mexico,[31,32] the use of non-pyrethroid molecules listed in the new Official Mexican policy for the surveillance and control of vectors[6] will help to maintain the sink of pyrethroid-susceptible *Ae. aegypti* in Yucatan State.

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