ZOOLOGIA 32 (1): 14–22, February 2015
http://dx.doi.org/10.1590/S1984-46702015000100003

Insecticide resistance and genetic variability in natural populations of *Aedes* (*Stegomyia*) *aegypti* (Diptera: Culicidae) from Colombia

Oscar A. Aguirre-Obando[1], Ana C. Dalla Bona[1], Jonny E. Duque L.[2] & Mário A. Navarro-Silva[1,3]

[1]*Laboratório de Entomologia Médica e Veterinária, Departamento de Zoologia, Setor de Ciências Biológicas, Universidade Federal do Paraná. Caixa Postal 19020, 81531-980 Curitiba, Paraná, Brazil.*
[2]*Centro de Investigaciones en Enfermedades Tropicales y Grupo de Investigación en Enfermedades Infecciosas y Metabólicas, Escuela de Medicina, Universidad Industrial de Santander. Bucaramanga A.A. 678, Colombia.*
[3]*Corresponding author. E-mail: mnavarro@ufpr.br*

ABSTRACT. Mosquito control prevails as the most efficient method to protect humans from the dengue virus, despite recent efforts to find a vaccine for this disease. We evaluated insecticide resistance and genetic variability in natural populations of *Aedes aegypti* (Linnaeus, 1762) from Colombia. This is the first Colombian study examining kdr mutations and population structure. Bioassays with larvae of three mosquito populations (Armenia, Calarcá and Montenegro) were performed according to the World Health Organization (WHO) guidelines, using Temephos. For the analysis of the Val1016Ile mutation and genetic diversity, we sampled recently-emerged adults from four mosquito populations (Armenia, Calarcá, Montenegro and Barcelona). Following the WHO protocol, bioassays implemented with larvae showed resistance to Temephos in mosquito populations from Armenia (77% ± 2) and Calarcá (62% ± 14), and an incipient altered susceptibility at Montenegro (88% ± 8). The RR_{95} of mosquito populations ranged from 3.7 (Montenegro) to 6.0 (Calarca). The Val1016Ile mutation analysis of 107 genotyped samples indicates that 94% of the specimens were homozygous for the wild allele (1016Val) and 6% were heterozygous (Val1016Ile). The 1016Ile allele was not found in Barcelona. Genetic variability analysis found three mitochondrial lineages with low genetic diversity and gene flow. In comparison with haplotypes from the American continent, those from this study suggest connections with Mexican and North American populations. These results confirm that a continuous monitoring and managing program of *A. aegypti* resistance in the state of Quindío is required.

KEY WORDS. Bioassays; gene flow; knockdown resistance; mitochondrial DNA; ND4 gene.

Dengue, a viral disease transmitted by a mosquito, has the greatest epidemic potential in the world (Who 2013). Given that there is no effective vaccine, in order to control dengue outbreaks it is necessary to control the vector, *Aedes* (*Stegomyia*) *aegypti* (Linnaeus, 1762) (Urdaneta-Marquez & Failloux 2011). Vector control strategies in Colombia are mostly dependent on community participation through education campaigns to reduce potential larval breeding sites. However, in epidemic situations, insecticides are applied on a large scale (Ministerio de la Protección Social 2011). In Colombia, two main classes of insecticides are used: the organophosphates (OP) Temephos since 1970 (Motta-Sanchez et al. 1976) and Malathion, since 1980 (Ocampo et al. 2011), and pyrethroids (PY), used since 1990 (Maestre 2012).

Currently, resistance to OP and PY is present on a large scale and has been reported to occur in most regions where *A. aegypti* is established (Who 2013). Despite the existence of a national network for the evaluation of insecticide resistance to malaria and dengue vectors in Colombia (Ministerio de la Protección Social 2011), the insecticide resistance status of *A. aegypti* has not been systematically monitored. The first report of resistance was to DDT, an insecticide that is no longer used in Colombia for dengue control, in a mosquito population from Cucuta, near the border with Venezuela (Gast-Galvin 1961). Resistance to OP Temephos was first documented in Cali, Valle del Cauca (Suárez et al. 1996), followed by the states of Norte de Santander, Sucre, Antioquia, Huila, Nariño, Cundinamarca, Santander, Caquetá, Meta, Guaviare and Atlántico (Maestre et al. 2009, Ocampo 2011, Santacoloma et al. 2012, Grisales et al. 2013). Regarding the evaluations of Malathion, the populations are not yet resistant to adulticide (Ocampo et al. 2011, Santacoloma et al. 2012).

PY resistance in Colombia was first documented in 2006 in mosquito populations from Santander, Cundinamarca, Meta, Caqueta and Guaviare (Santacoloma et al. 2010). Metabolic resistance and target site insensitivity represent the two major forms of PY resistance (Soderlund & Knipple 1999, 2003). Studies have suggested that mutations in the voltage-gated sodium channel (Na_V), the target site for PY and DDT, may play a role in PY resistance (Irac 2011). Na_V is a transmembrane protein present in the neuronal axons and is composed of four ho-

mologous domains (I-IV), each with six hydrophobic segments (S1-S6) (Catterall 2000). 'Knockdown resistance' (kdr) is a generic term applied to insects that fail to lose coordinated activity immediately following PY exposure.

Kdr mutations in the Na_V (associated or not with PY resistance) have been observed in a range of insects, including *A. aegypti* (Saavedra-Rodriguez et al. 2007). In populations of *A. aegypti* from Latin America and Southeast Asia, the mutations Val1016Ile, Val1016Gly, Phe1534Cys and Asp1794Tyr, all in the IIS6 and IIIS6 segment, are correlated with insecticide resistance (Brengues et al. 2003, Saavedra-Rodriguez et al. 2007, Chang et al. 2009, Harris et al. 2010, Linss et al. 2014). However, only one of these, a valine to isoleucine substitution at codon 1016, has been clearly linked to insecticide resistance; selection pressure under laboratory conditions, bioassays with adults, biochemical assays and molecular screening have confirmed this finding (Rodpradit et al. 2005, Saavedra-Rodriguez et al. 2007, Strode et al. 2008, García et al. 2009, Martins et al. 2009, Lumjuan et al. 2011, Marcombe et al. 2012).

Understanding the patterns of genetic structure and gene flow among *A. aegypti* populations is pivotal for the development of rational dengue control programs (Urdaneta-Marquez & Anna-Bella 2011). The current trend is to use microsatellites (Monteiro et al. 2014) and/or SNPs (single nucleotide polymorphism) (Rasic et al. 2014). Nevertheless, mitochondrial DNA (mtDNA) has been widely used in population genetics studies of *A. aegypti* from different geographic points and dengue endemic regions (Gonçalves et al. 2012). The ND4 mitochondrial gene, which codifies the subunit 4 of the NADH dehydrogenase enzyme, is an effective tool to analyze genetic population structure and colonization events in *A. aegypti*. Such analyses were carried out in mosquito populations from Brazil (Twerdochlib et al. 2012), Bolivia (Paupy et al. 2012), Peru (Yáñez et al. 2013), Venezuela (Urdaneta-Marquez et al. 2008) and Mexico (Gorrochotegui-Escalante et al. 2002). So far, only RAPDs (Random Amplified Polymorphic DNA) (Ocampo & Wesson 2004, Mejía et al. 2011) and more recently the mtDNA (Caldera et al. 2013) have been used to analyze the genetic structure of *A. aegypti* populations in Colombia.

Here, we evaluated the insecticide resistance and genetic variability in natural populations of *A. aegypti* from Colombia. This is the first Colombian study to look at insecticide resistance (OP and PY (kdr mutation)) and population structure. Our findings have shown that insecticide resistance is spreading in the country owing to the $1016Ile^{kdr}$ allele (in low frequency), and that OP resistance is found in most populations of *A. aegypti* studied by us. Genetic variability analysis shows that the vector population has low genetic diversity and limited gene flow.

MATERIAL AND METHODS

We collected *A. aegypti* larvae in 2011, from three municipalities in the state of Quindío: Armenia (4°32'0"N, 75°40'0"W, 1,483 m), Montenegro (4°34'23"N, 75°45'20"W, 1,292 m), Calarcá (4°31'55"N, 75°39'1"W, 1,573 m) and Barcelona (4°25'53", 75°43'26", 1,573 m), a district of Calarcá. We followed the standard methods of the Pan-American Health Organization (PAHO) for determining the infestation rate of *A. aegypti* (Ops 1995). At each municipality, we randomly collected immatures from at least 25 different containers located in selected residences in the urban area. This included domestic breeding sites such as water storage vessels, plastic pails, tires, and cans. Each container was located at least 100 m away from the others. Larvae from the same municipality were pooled in the laboratory and stored until adults emerged under controlled conditions (25 ± 1°C, humidity 80 ± 10% and photoperiod 12:12 hours) in the medical entomology laboratory of the Center for the Study of Tropical Diseases (Centro de Investigaciones en Enfermedades Tropicales – CINTROP), Industrial University of Santander (UIS), Santander, Colombia. We collected recently-emerged adults from each population for the analysis of the Val1016Ile mutation and genetic diversity. The mosquitoes were individually placed in absolute ethanol (99.5%) and stored in a freezer at -20°C. The remaining F_0 adults were used to produce the F_1 generation. *Aedes aegypti* F_1 larvae were used as the source in bioassays to determine Temephos susceptibility. Adults were fed a 10% honey solution and blood meals that were provided by rats, *Rattus norvegicus* (Berkenhout, 1769), twice a week to induce oviposition.

Bioassays were carried out with *A. aegypti* natural populations from Armenia, Montenegro and Calarcá. We were unable to run the bioassay with the population from Barcelona, since it was not possible to establish the colony base because there were few immatures on the field. Bioassays included F1 generation larvae and the insecticide Temephos pestanal 250 mg 97.5% (Sigma-Aldrich) following the World Health Organization (WHO) guidelines (Who 1998). Bioassays were calibrated with Rockefeller, a susceptible strain of *A. aegypti* (Centers for Disease Control, CDC), using a diagnostic concentration of 0.0162 ppm Temephos. This is twice the LC_{99} (lethal concentration that kills 99% of the larvae) of the susceptible strain. The results from larvae were expressed as mortality rates 24h after exposure to Temephos. The following criteria proposed by the Who (1998) guidelines were adopted to classify population susceptibility status: susceptible (percentage of mortality > 98%), susceptibility incipiently altered (80-98%), or resistant (< 80%).

Dose-response bioassays followed the WHO guidelines to determine larval susceptibility to Temephos (Who 1981). In these experiments, third instar or initial fourth instar larvae were exposed to 10 concentrations of the insecticide to determine larval mortality between 5 and 95%. At each concentration and in the control, four replicates containing 20 larvae each were tested. Larval mortality was checked 24 h after exposure. All tests were repeated three times on different days.

Mortality data (expressed as a number of dead specimens per dose) was applied to calculate lethal concentrations to 50

and 95% (LC_{50} and LC_{95}) of exposed individuals, and were analyzed by the log-probit method of Finney (1971) using the Probit software by Raymond (1993). Resistance ratios (RR_{50} and RR_{95}) were obtained by dividing the lethal concentration of the population by the equivalent lethal concentration of the Rockefeller population.

DNA extraction followed Bona et al. (2012). Individual mosquitoes from each locality were genotyped at position1016 of the genomic DNA using allele-specific PCR (AS-PCR). We used three primers to determine the presence of the Val1016Ile mutation, one for the 1016Val allele: 5'-GCG GGC AGG GCG GCG GGG GCG GGG CCA CAA ATTGTT TCC CAC CCG CAC CGG -3', one for the 1016Ile allele: 5'-GCG GGC ACA AAT TGT TTC CCA CCC GCA CTG A -3', and a third common to both alleles: 5'-GGA TGA ACC GAA ATT GGA CAA AAG C -3'. PCR reactions followed the protocol described by Saavedra-Rodriguez et al. (2007) and Martins et al. (2009). Amplified PCR products were checked in 10% polyacrylamide gel. Using the gel results, we calculated genotypic and allelic frequencies, and Hardy-Weinberg equilibrium (HW) (Salman 2007, Hartl 2008). The Rockefeller strain of *A. aegypti*, a standard for insecticide susceptibility reared in the laboratory, and life-history trait parameters, were used as reference for the wild-type alleles (1016Val) of the Nav gene.

After DNA extraction, we used two primers to amplify a segment of the ND4 gene, a universal ND4R primer: 5'-ATT GCC TAA GGC TCA TGT AG-3' and a reverse NDAR primer: 5'-TCG GCT TCC TAG TCG TTC AT-3 (Costa-da-Silva et al. 2005). PCR reactions and sequencing followed Twerdochlib et al. (2012).

Consensus sequences were obtained using the Staden software version 1.5, and were aligned with the program BioEdit version 7.0 (Hall 2004), using the ClustalW tool (Thompson et al. 1994). The sequences were compared with others available on GenBank using Tblastx to verify the amplified fragment. Genetic diversity and neutrality tests were calculated using the program DnaSP, version 5.0 (Librado & Rozas 2009). Molecular variation analysis (AMOVA) was performed with the program Arlequin version 3.5 (Excoffier & Lischer 2010). Population structure was determined using the Wright fixation index (F_{ST}, Wright 1921) and gene flow (Nm) was obtained by the program Arlequin 3.5 (Excoffier & Lischer 2010) followed by Bonferroni correction.

The Mantel test was used to estimate the correlation between genetic (F_{ST}) and geographic (km) distances. The GenAlEx6 software was used to test isolation by distance (Peakall & Smouse 2012). Geographical distances for this analysis were obtained using Google Earth 6.0. The software Mega ver. 5.05 (Tamura et al. 2007) was used to create a tree with the Neighbor-Joining method, following the Jukes-Cantor genetic distance model. Bootstrap support was estimated with 1,000 replicates. *Aedes* (*Stegomyia*) *albopictus* (Skuse, 1894) (GenBank#EF153761) was used as the external group.

The haplotypes of this study were deposited in the GenBank under accession KF241755 – KF241757. In order to estimate gene flow among the populations analyzed, they were compared with the haplotypes available from America, published by Gonçalves et al. (2012); these haplotypes are free of nuclear mitochondrial pseudogenes (NUMTs). Samples containing mixtures of mtDNA and NUMT sequences are expected to significantly affect the outcome of genealogy- and frequency-based analyses. This is because mtDNA and NUMTs have separate genealogies and thus different evolutionary histories (Gonçalves et al. 2012, Ribeiro 2012). To reduce the error caused by NUMTs in the samples two analyses were carried out: 1. We searched for heterozygous sites in the chromatogram and additional termination codons (Ribeiro 2012), and 2. We compared the haplotypes with a list of NUMTs verified by Black & Bernhardt (2009) and Hlaing et al. (2009). If any NUMT was found, it was removed from the analysis.

RESULTS

We collected 1976 immatures (NI = Number of immatures) from 77 domestic breeding sites (DB = Domestic breeding sites) of four mosquito populations, Armenia (NI = 739, DB = 25), Calarcá (NI = 628, DB = 25), Montenegro (NI = 531, DB = 19) and Bacelona (NI = 78, DB = 8).

Diagnostic concentration (% mortality)

The results of bioassays with larvae following the WHO protocol showed that *A. aegypti* populations (Mortality rate ± SD) from Armenia (77% ± 2) and Calarcá (62% ± 14) are resistant to OP Temephos and that the Montenegro population has incipient, altered susceptibility to the insecticide (88% ± 8).

Multiple concentrations (RR)

Dose-response bioassays following the WHO protocol resulted in resistance ratios (RR_{95}) greater than three, and were the highest in the *A. aegypti* populations from Calarcá. In general, the slope values of the *A. aegypti* populations studied were lower than those obtained from the Rockefeller strain, confirming their heterogeneity in comparison to the reference strain and the differences in their response to OP Temephos. The LC_{50} and LC_{95} of all population studied are presented for comparison in Table I.

Table I. Temephos susceptibility profile from Colombian populations of *Aedes aegypti*, showing means (standard deviations) for slopes, LC and RR.

Location	G	Slope	LC ppm		RR	
			50	95	50	95
Rockefeller	Fn	5.0(0.2)	0.0027(0.0002)	0.0049(0.0004)	–	–
Armenia	F1	3.9(0.2)	0.0116(0.0011)	0.0308(0.0048)	3.4	4.9
Calarcá	F1	3.3(0.1)	0.0121(0.0011)	0.0379(0.0070)	3.5	6.0
Montenegro	F1	4.9(0.2)	0.0109(0.0010)	0.0235(0.0028)	3.2	3.7

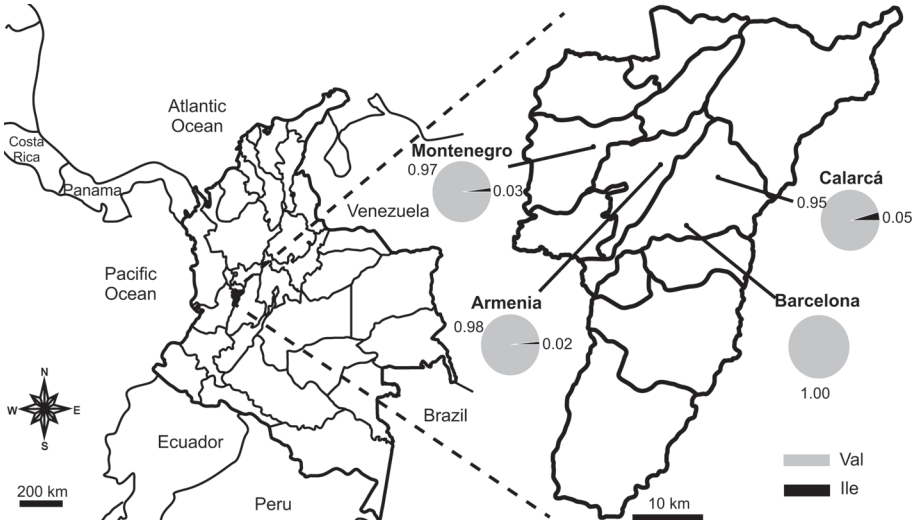


Figure 1. Allelic frequencies of 1016Val and 1016Ile in the Na_v of four *A. aegypti* populations from Colombia.

Genotyping the 1016 fragment of Na_v

A total of 107 *A. aegypti* individuals were genotyped for the Val1016Ile mutation. Of these individuals, 94% were homozygous dominant (Val/Val); 6% were heterozygous (Val/Ile), and homozygous recessive genotypes (Ile/Ile) were not found. In all populations, the genotypic frequencies of Val/Val were greater than the frequencies of Val/Ile and Ile/Ile (Table II). The frequencies of the allele 1016Val were greater than the frequencies of the 1016Ile allele in all populations (Fig. 1). The populations from Armenia, Calarcá and Montenegro are in Hardy-Weinberg equilibrium. Nevertheless, bioassays with adults should be carried out to confirm the susceptibility status in these populations.

Table II. Genotypic frequency of the mutation Val1016Ile in the NaV of four *Aedes aegypti* populations from Quindío, Colombia.

Location	n	Genotypic Frequency			HW equilibrium test
		Val/Val	Val/Ile	Ile/Ile	χ^2, (df)*
Armenia	30	0.97	0.03	0.00	0.009, (1)
Calarcá	30	0.90	0.10	0.00	0.083, (1)
Montenegro	30	0.93	0.07	0.00	0.036, (1)
Barcelona	17	1.00	0.00	0.00	–

* Not significant difference ($p > 0.05$).

Genetic diversity – fragment of mitochondrial gene ND4

The amplified product of the ND4 gene was 311bp. There were four polymorphic sites and 307 monomorphic sites. The analysis of the amplified fragment of 42 individuals resulted in three haplotypes without NUMTs: H1-Col (GenBank# KF241755), H2-Col (GenBank# KF241756) and H3-Col (GenBank# KF241757). The most frequent of these haplotypes was H2-Col (48%), followed by H3-Col (28%) and H1-Col (24%). Only H3-Col occurred in Montenegro; H1, H2 and H3-Col occurred in Barcelona; and H1 and H2-Col occurred in Armenia and Calarcá (Table III). Haplotypes were determined by three transitions: G↔A (site 48); T↔C (sites 144, 249) and one transversion: A↔T (site 90) (Table IV). The average nucleotide composition was 20% Cytosine, 29% Thymine, 43% Adenine and 8% Guanine. Haplotypes found in this study were similar to the H2 haplotype, which is found in Mexico and North America (Fig. 2).

Table III. Number of individuals observed for each haplotype in samples of four populations of *Aedes aegypti* from Colombia.

Location	H1-COL	H2-COL	H3-COL	Total
Armenia	2	10	0	12
Calarcá	1	9	0	10
Montenegro	0	0	10	10
Barcelona	7	1	2	10
Total	10	20	12	42
%	24	48	28	100

Table IV. Variable sites in three haplotypes of ND4 mitochondrial gene in *Aedes aegypti* from Colombia.

Haplotypes	Position of nucleotide change				N
	48	90	144	249	
H1-COL	G	A	T	T	10
H2-COL	A	T	C	T	20
H3-COL	A	T	C	C	12

N = Number of individuals that share each haplotype.

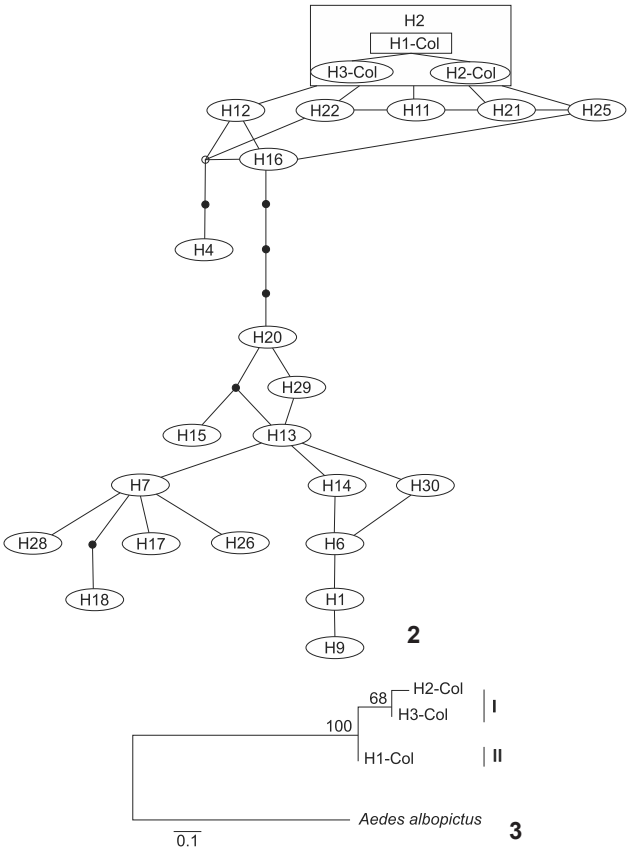
Haplotype diversity was 0.67 ± 0.036 (mean ± SD, n = 42), nucleotide diversity was 0.0058 ± 0.0002 and there were 1.81 nucleotide differences on average. The neutral selectivity test results ($p > 0.05$) were in agreement with the assumptions of the neutral mutation model ($p > 0.05$, Tajima's D Test 2.24, Fu Fs Test 2.58).

Analysis of molecular variance (AMOVA, Fst = 0.58, $p < 0.05$) indicated genetic structure. Most of the variation occurred among populations (58%), while 42% occurred within populations. Significant Fst values after Bonferroni correction indicated that mosquito populations from Armenia-Montenegro, Barcelona-Montenegro, Barcelona-Calarcá and Calarcá-Montenegro are genetically structured, and that they are about 11 km apart from one another (Table V). Genetic distance (F_{ST}) and geographic distance (km) were correlated (Mantel, $R^2 = 0.77$; $p < 0.05$) as expected. Two groups were resolved with Neighbor-Joining: Group I comprised of the most frequent haplotypes, H2-Col (48%) and H3-Col (28%), and group II of the least frequent haplotype, H1-Col (24%, Fig. 3).

Table V. Genetic distances (Fst values, above the diagonal) and effective number of migrants (Nm, below the diagonal) of four natural *Aedes aegypti* populations from Quindío, Colombia.

Population	Armenia	Barcelona	Calarcá	Montenegro
Armenia	–	0.3	-0.1	0.8 *
Barcelona	1.1 (10.4)	–	0.5 *	0.5 *
Calarcá	Inf. (6.1)	0.6 (11.1)	–	0.9 *
Montenegro	0.1 (11.1)	0.4 (11.3)	0.1 (17.0)	–

Inf. = infinity; geographic distances (km) are in parentheses; * significant difference ($p < 0.0083$ after Bonferroni test).



Figures 2-3. (2) Haplotype network among mitochondrial ND4 haplotypes. The rectangle represents the ancestral haplotype. Each black dot indicates a single substitution. The size of the circle is not proportional to the haplotype frequency. The numbers inside the circle indicate the Haplotype number. H1-H3-Col present study; H1-H4, H7, H9, H18, H21-25, H27 (Haplotype present at Mexico and North America), H6 (Brazilian Amazon, Southeastern Brazil, Peru, Mexico and North America), H11, H17, H26, H28 (Brazilian Amazon), H10, H15 (Brazilian Amazon, Southeastern Brazil, Venezuela, Mexico and North America); H12, 14 (Brazilian Amazon, Venezuela), H13 (Brazilian Amazon, Southeastern Brazil), H16 (Brazilian Amazon, Southeast Brazil, Mexico and North America), H19 (Southeastern Brazil, Mexico and North America), H20 (Venezuela); H29, 30 (Southeastern Brazil). (3) Dendrogram for three *A. aegypti* haplotypes from Colombian populations using the Neighbor-Joining method, according to the Jukes-Cantor model. Bootstrap values are at the nodes of each branch.

DISCUSSION

We found that insecticide resistance is present in most studied populations of *A. aegypti* and is determined by the1016Ilekdr allele (showing low frequency), which confers OP resistance. Even though resistance to Temephos had been previously documented in Colombia (Grisales et al. 2013),our findings on Temephos resistance are important because it has been the main insecticide used to control the immature stages of natural *A. aegypti* populations in the country (Ocampo et al. 2011). Additionally, Temephos pressure on larvae may generate cross-resistance to PY or other OP used in the control of the adult stages (Rodríguez et al. 2002, Tikar et al. 2009).

The application of the OP Temephos on breeding sites is essential to control *A. aegypti* immatures worldwide (Who 2013). However, long-term use of this insecticide has caused the emergence of resistance in several Latin American countries (Rodríguez et al. 2007, Lima et al. 2011, Bisset et al. 2013). Colombia's participation in the continental vector control campaign led by OPS (Santacoloma et al. 2010), and several dengue outbreaks in 1970 and in 1980 in most Colombian regions (Boshell et al. 1986) has been the cause of intensive insecticide use to reduce dengue cases, selecting resistant vector populations.

Regarding the weak presence of the $1016Ile^{kdr}$ allele associated with PY resistance, the detection of the Val1016Ile mutation in *A. aegypti* natural populations could have dire consequences for the continued use of PY, since studies on se-

lection pressure using PY insecticides under laboratory conditions have documented fixation of the 1016Ile^{kdr} allele after only five generations (Irac 2011, Saavedra-Rodriguez et al. 2012). In the last decade, the 1016Ile^{kdr} allele has rapidly spread in *A. aegypti* populations from Mexico and Brazil, simultaneously with the intensification of PY usage due to the emergence of dengue outbreaks (García et al. 2009, Linss et al. 2014). Therefore, enhanced surveillance for resistance should be a priority in localities where the 1016Ile^{kdr} allele is found, before new adaptive alleles can be selected for decreasing the deleterious effects of kdr (Brito et al. 2013). Consequently, bioassays with adults should be performed to confirm the susceptibility status of these populations.

Our results suggest that alternative control strategies need to be found before Temephos resistance compromises operational control. An alternative to reduce selection pressure for resistance is to devise an insecticide swapping program implementing the criteria proposed by the Brazilian Ministry of Health. According to these guidelines, Temephos needs to be replaced with another insecticide with a different action mechanism in populations with $RR_{95} \geqslant 3$ (Ministério da Saúde 2006). In studies conducted on populations of *A. aegypti* from Brazil, it was observed that when the application of Temephos is interrupted in locations where RR_{95} is greater than 10, resistance declines only gradually, and several years are needed for Temephos to be effective again (Montella et al. 2007). On the other hand, despite the fact that Colombia has its own vector control program, the susceptibility status of the populations we evaluated should be considered as "populations with susceptibility loss to OP Temephos"; hence continuous monitoring of susceptibility status is recommended in order to determine the accurate moment to change the active substance (Ministerio de la Protección Social 2011). Nevertheless, the RR_{95} values in all populations were greater than three.

Chemical measures used in vector control programs could affect the genetic diversity of *A. aegypti* populations, and as a result, induce genetic changes through bottleneck and genetic drift effects (Urdaneta-Marquez & Anna-Bella 2011). Low genetic diversity is most likely a result of a decline in population size caused by insecticide use, as it was observed in vector populations from Trinidad and Tobago, and Venezuela (Yan et al. 1998, Herrera et al. 2006). However, some studies have revealed the presence of greater genetic diversity in areas that are frequently treated with insecticides, as shown for *A. aegypti* populations from French Polynesia and Brazil (Paupy et al. 2000, Ayres et al. 2004). In our results, genetic diversity (Hd), number of haplotypes (N) and nucleotide diversity (π) were lower (N = 3, Hd = 0.67 and π = 0.006) than in other studies on the ND4 gene of *A. aegypti*. For example, 36 locations in the Americas, Asia and Africa (N = 20, Hd = 0.82 and π = 0.020) (Bracco et al. 2007), five states in Brazil (N = 24, Hd = 0.80 and π = 0.017) (Paduan & Ribolla 2008) and two populations from Colombia (N = 10, Hd = 0.068 and π = 0.009) (Caldera et al. 2013).

The studied Colombian *A. aegypti* populations were genetically structured, a trend also found in other populations from South America and Central America (Gorrochotegui-Escalante et al. 2002, Costa-da-Silva et al. 2005, Urdaneta-Marquez et al. 2008, Paupy et al. 2012, Twerdochlib et al. 2012, Caldera et al. 2013). The genetic structure of the studied Colombian populations occurred between regions separated by less than 17 km, similar to the patterns observed in Venezuela (distance less than 15 km between them), which suggest that gene flow is restricted (Herrera et al. 2006). Geographic barriers associated with Andean mountains in the state of Quindío probably obstruct gene flow. However, in Quindio state, where tourism is intense, there is more road traffic between the studied populations (Gobernación del Quindío 2013, Invias 2013), which ties the gene flow in populations of *A. aegypti* to human transport (Huber et al. 2004, Costa-da-Silva et al. 2005).

Overall, two mitochondrial lineages were present in the studied Colombian populations, and the most frequent haplotypes came from group I. This pattern is in agreement with previous studies (Gorrochotegui-Escalante 2002, Bosio et al. 2005, Herrera et al. 2006, Bracco et al. 2007, Paduan & Ribola 2008). The haplotypes found in this study indicate a relationship between the Mexican and North American populations. These connections result from passive dispersal of *A. aegypti* among different countries, and passive vector dispersal is likely to be the most common pattern world-wide (Gorrochotegui-Escalante et al. 2002, Huber et al. 2004, Bosio et al. 2005, Gonçalves et al. 2012). Further studies are necessary to ascertain how the vector entered Colombia, since the connection among the populations in this study is clear. We conclude that continuous monitoring and managing programs are needed to control *A. aegypti* populations in Colombia. Given that insecticide resistance could potentially compromise vector control programs, a threshold of $RR_{95} \geqslant 3.0$ should be established for swapping among insecticides with different modes of action.

ACKNOWLEDGEMENT

We thank Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq, process 140224/2013-0).

LITERATURE CITED

Ayres C, Melo-Santos M, Prota J, Solé-Cava A, Furtado A (2004) Genetic structure of natural populations of *Aedes aegypti* at the micro- and macro geographic levels in Brazil. **Journal of the American Mosquito Control Association 20**(4): 350-356.

Bisset J, MArin R, Rodríguez M, Severson D, Ricardo Y, French L, Días M, Perez O (2013) Insecticide resistance in two *Aedes aegypti* (Diptera: Culicidae) strains from Costa Rica. **Journal of Medical Entomology 50**(2): 352-361.

Black WC, Bernhardt SA (2009) Abundant nuclear copies of mitochondrial origin (NUMTs) in the *Aedes aegypti* genome.

Insect Molecular Biology 18(6): 705-713. doi: 10.1111/j.1365-2583.2009.00925.x

Bona A, Piccoli C, Leandro A, Kafka R, Twerdochilib A, Navarro-Silva M (2012) Genetic profile and molecular resistance of *Aedes* (*Stegomyia*) *aegypti* (Diptera: Culicidae) in Foz do Iguaçu (Brazil), at the border with Argentina and Paraguay. **Zoologia 29**(6): 540-548. doi: 10.1590/S1984-46702012000600005

Boshell J, Groot H, Gacharná M, Márquez G, González M, Gaitán M, Berlie C (1986) Dengue en Colombia. **Biomédica 6**(3-4): 101-106.

Bosio C, Harrington L, Jones J, Sithiprasasna R, Norris D, Scott T (2005) Genetic structure of *Aedes aegypti* populations in Thailand using mitochondrial DNA. **American Journal of Tropical Medicine and Hygiene 72**(4): 434-442.

Bracco J, Capurro M, Lourenço-de-Oliveira R, Sallum M (2007) Genetic variability of *Aedes aegypti* in the Americas using a mitochondrial gene: evidence of multiple introductions. **Memórias Instituto Oswaldo Cruz 102**(5): 573-580.

Brengues C, Hawkes N, Chandre F, McCarroll L, Duchon S, Guillet P, Hemingway J (2003) Pyrethroid and DDT cross-resistance in *Aedes aegypti* is correlated with novel mutations in the voltage-gated sodium channel gene. **Medical and Veterinary Entomology 17**(1): 87-94.

Brito L, Linss J, Lima-Camara T, Belinato T, Peixoto A, Lima J, Valle D, Martins A (2013) Assessing the Effects of *Aedes aegypti* kdr Mutations on Pyrethroid Resistance and Its Fitness Cost. **PLOS One 8**(4): e60878. doi: 10.1371/journal.pone.0060878

Caldera S, Jaramillo S, Cochero S, Pérez-Doria A, Bejarano E (2013) Diferencias genéticas entre poblaciones de *Aedes aegypti* de municipios del norte de Colombia, con baja y alta incidencia de dengue. **Biomedica 33**(1): 89-98. doi: 10.7705/biomedica.v33i0.1573

Catterall W (2000) From ionic currents to molecular mechanisms: the structure and function of voltage-gated sodium channels. **Neuron 26**(1): 13-25.

Costa-da-Silva A, Capurro M, Bracco J (2005) Genetic lineages in the yellow fever mosquito *Aedes* (*Stegomyia*) *aegypti* (Diptera: Culicidae) from Peru. **Memórias do Instituto Oswaldo Cruz 100**(6): 539-544.

Chang C, Shen W, Wang T, Lin Y, Hsu E, Dai S (2009) A novel amino acid substitution in a voltage-gated sodium channel is associated with knockdown resistance to permethrin in *Aedes aegypti*. **Insect Biochemistry and Molecular Biology 39**(4): 272-278. doi: 10.1016/j.ibmb.2009.01.001

Excoffier L, Lischer H (2010) Arlequin suite ver 3.5: A new series of programs to perform population genetics analyses under Linux and Windows. **Molecular Ecology Resources 10**(3): 564-567. doi: 10.1111/j.1755-0998.2010.02847.x

Finney D (1971) **Probit Analysis.** Cambridge, University Press, 3rd ed.

García G, Flores A, Fernández-Salas I, Saavedra-Rodríguez K, Reyes-Solis G, Lozano-Fuentes S, Bond J, Casas-Martínez M, Ramsey J, García-Rejón J, Domínguez-Galera M, Ranson H, Hemingway J, Eisen L, Black W (2009) Recent rapid rise of a perrmethrin knock down resistance allele in *Aedes aegypti* in México. **PLOS Neglected Tropical Diseases 3**(10): 531-541. doi: 10.1371/journal.pntd.0000531

Gast-Galvis A (1961) Una década de labor del Instituto Carlos Finlay de Colombia. **Boletín de la Oficina Sanitaria Panamericana 50**(1): 44-58.

Gobernacion Del Quindío (2013) **Página oficial del departamento del Quindío.** Available on line at: http://www.quindio.gov.co [Accessed: 8 April 2013]

Gonçalves A, Cunha I, Santos W, Luz S, Ribolla P, Abad-Franch F (2012) Gene flow networks among American *Aedes aegypti* populations. **Evolutionary Applications 5**(7): 664-676. doi: 10.1111/j.1752-4571.2012.00244.x

Gorrochotegui-Escalante N, Gomez-Machorro C, Lozano-Fuentes S, Fernandez-Salas I, Muñoz M, Farfan-Alej J, Garcia-Rejon J, Beaty B, Black W (2002) Breeding structure of *Aedes aegypti* populations in Mexico varies by region. **American Journal of Tropical Medicine and Hygiene 66**(2): 213-222.

Grisales N, Poupardin R, Gomez S, Fonseca-Gonzalez I, Ranson H, Lenhart A (2013) Temephos resistance in *Aedes aegypti* in Colombia compromisos dengue vector control. **PLOS Neglected Tropical Diseases 7**(9): 1-10. doi: 10.1371/journal.pntd.0002438

Hall T (2004) **Bioedit.** Carlsbad, Ibis Therapeutics, v. 7.0.

Harris A, Rajatileka S, Ranson H (2010) Pyrethroid resistance in *Aedes aegypti* from Grand Cayman. **American Journal of Tropical Medicine and Hygiene 83**(2): 277-284. doi: 10.4269/ajtmh.2010.09-0623

Hartl D (2008) **Princípios de genética de populações.** Ribeirão Preto, FUNPEC, 3rd ed.

Hemme R, Thomas C, Chadee D, Severson D (2010) Influence of urban landscapes on population dynamics in a short-distance migrant mosquito: Evidence for the dengue vector *Aedes aegypti*. **PLoS Neglected Tropical Diseases 4**(3): 634-643. doi: 10.1371/journal.pntd.0000634.g001

Herrera F, Urdaneta L, Rivero J, Zoghbi N, Ruiz J, Carrasquel G, Martínez J, Pernalete M, Villegas P, Montoya A, Rubio-Palis Y, Rojas E (2006) Population genetic structure of the dengue mosquito *Aedes aegypti* in Venezuela. **Memórias Instituto Oswaldo Cruz 101**(6): 625-633.

Hlaing T, Tun-Lin W, Somboon P, Socheat D, Setha T, Min S, Chang M, Walton C (2009) Mitochondrial pseudogenes in the nuclear genome of *Aedes aegypti* mosquitoes: implications for past and future population genetic studies. **BMC Genetics 10**: 11. doi: 10.1186/1471-2156-10-11

Huber K, Loan L, Chantha N, Failloux A (2004) Human transportation influences *Aedes aegypti* gene flow in Southeast Asia. **Acta Tropica 90**(1): 23-29. doi: 10.1016/j.actatropica.2003.09.012

Invias (2013) **Resumen del estado de la red vial con criterio técnico 2013.** Available online at: www.invias.gov.co/index.php/hechos-de-transparencia/informacion-financiera-y-contable/doc_download/916-resumen-del-estado-de-la-red-vial-con-criterio-tecnico-2013 [Accessed: 8 April 2013]

Irac (2011) **Prevention and Management of Insecticide Resistance in Vectors of Public Health Importance.** Atlanta, Insecticide Resistance Action Committee (IRAC), 70p.

Librado P, Rozas J (2009) DnaSP v5: A software for comprehensive analysis of DNA polymorphism data. **Bioinformatics 25**(11): 1451-1452. doi: 10.1093/bioinformatics/btp187

Lima E, Paiva M, Araújo A de, Silva E da, Silva U da, Oliveira L de, Santana A, Barbosa C, Paiva C de, Goulart M, Wilding C, Ayres C, Melo M (2011) Insecticide resistance in *Aedes aegypti* population from Ceará, Brazil. **Parasites & Vectors 4**(5): 1-12. doi: 10.1186/1756-3305-4-5

Linss J, Brito L, Garcia G, Saori A, Bruno R, Lima J, Valle D, Martins A (2014) Distribution and dissemination of the Val1016Ile and Phe1534Cys Kdr mutations in *Aedes aegypti* Brazilian natural populations. **Parasites & Vectors 7**(25): 1-12. doi: 10.1186/1756-3305-7-25

Lumjuan N, Rajatileka S, Changsom D, Wicheer J, Leelapat P, Prapanthadara L, Ranson H (2011) The role of the *Aedes aegypti* Epsilon glutathione transferases in conferring resistance to DDT and pyrethroid insecticides. **Insect Biochemistry Molecular Biology 41**(3): 203-209. doi: 10.1016/j.pt.2010.08.004

Maestre R (2012) Susceptibility Status of *Aedes aegypti* to Insecticides in Colombia, p. 203-209. In: Farzana P (Ed.) **Insecticides – Pest Engineering.** Croatia, InTech Press.

Maestre R, Rey G, Salas J, Vergara C, Santacoloma L, Goenaga O (2009) Susceptibilidad de *Aedes aegypti* (Diptera: Culicidae) a temephos en Atlántico – Colombia. **Revista Colombiana de Entomologia 35**(2): 54-57.

Marcombe, S, Blanc R, Pocquet N, Muhammad-Asam R, Poupardin R, Sélior S, Darriet F, Reynaud S, Yébakima A, Corbel V, Jean-Philippe D, Chandre F (2012) Insecticide Resistance in the Dengue Vector *Aedes aegypti* from Martinique: Distribution, Mechanisms and Relations with Environmental Factors. **Plos ONE 7** (2): e30989. doi: 10.1371/journal.pone.0030989

Marcombe S, Paris M, Paupy C, Bringuier C, Yebakima A, Chandre F, Despres L (2013) Insecticide-Driven Patterns of Genetic Variation in the Dengue Vector *Aedes aegypti* in Martinique Island. **PLoS ONE 8** (10): e77857. doi: 10.1371/journal.pone.0077857

Martins A, Lima J, Peixoto A, Valle D (2009) Frequency of Val1016Ile mutations in the voltage-gated sodium channel gene of *Aedes aegypti* Brazilian populations. **Tropical Medicine & International Health 14**(11): 1351-1355. doi: 10.1111/j.1365-3156.2009.02378.x

Mejia G, Mora G, Ramos E, Maestre R, Mazenett E, Malambo D, Gómez D (2011) Identificación genética de subpoblaciones de *Aedes aegypti* en Cartagena de Indias – Colombia. **Revista Ciencias biomédicas 2**(1): 5.

Ministério da Saúde (2006) **Reunião técnica para discutir status de resistência de *Aedes aegyptia* inseticidas.** Brasília, Ministério da Saúde, Secretaria de Vigilância em Saúde, Coordenação Geral do Programa Nacional de Controle da Dengue.

Ministerio de la Protección Social (2011) **Guía de Vigilancia Entomológica y Control de Dengue.** Colombia, Ministerio de la Protección Social, Instituto Nacional de Salud, Organización Panamericana de la Salud (OPS/OMS). Available online at: http://new.paho.org/col/index.php?option = com_docman&task = doc_download&gid = 1215&Itemid [Accessed: 8 April 2013]

Monteiro F, Shama R, Martins A, Gloria-Soria A, Brown J, Powell J (2014) Genetic diversity of Brazilian *Aedes aegypti*: Patterns following an eradication program. **PLos Neglected Tropical Diseases 8**(9): e3167. doi: 10.1371/journal.pntd.0003167

Montella I, Martins A, Viana-Medeiros P, Lima J, Braga I, Valle D (2007) Insecticide Resistance Mechanisms of Brazilian *Aedes aegypti* Populations from 2001 to 2004. **American Journal of Tropical Medicine and Hygiene 77**(3): 467-477.

Motta-Sanchez A, Tonn R, Uribe L, Calheiros L (1976) A comparison of methods of application of several insecticides for the control of *Aedes aegypti* in villages in Colombia. **WHO/VBC 76** (23): 1-33.

Ocampo C, Salazar-Terreros M, Mina N, McAllister J, Brogdon W (2011) Insecticide resistance status of *Aedes aegypti* in 10 localities in Colombia. **Acta Tropica 118**(1): 37-44. doi: 10.1016/j.actatropica.2011.01.007

Ocampo C, Wesson D (2004) Population dynamics of *Aedes aegypti* from a dengue hyper endemic urban setting in Colombia. **American Journal of Tropical Medicine and Hygiene 71**(6): 506-513.

OPS (1995) **Dengue y dengue hemorrágico en las Américas: guías para su prevención y control.** Washington, DC, Organización Panamericana de la Salud.

Paduan K, Ribolla P (2008) Mitochondrial DNA Polymorphism and Heteroplasmy in Populations of *Aedes aegypti* in Brazil. **Journal of Medical Entomology 45**(1): 59-67. doi: 10.1603/0022-2585(2008)45[59:MDPAHI]2.0.CO;2

Paupy C, Goff G Le, Brengues C, Guerra M, Revollo J, Barja Z, Jean-Pierre H, Fontenille D (2012) Genetic structure and phylogeography of *Aedes aegypti*, the dengue and yellow-fever mosquito vector in Bolivia. **Infection, Genetics and Evolution 12**(6): 1260-1269. doi: 10.1016/j.meegid.2012.04.012

Paupy C, Vazeille-Falcoz M, Mousson L, Rodhain F, Failloux A (2000) *Aedes aegypti* in Tahiti and Moorea (FrenchPolynesia): isoenzyme differentiation in the mosquito population according to human population density. **American Journal of Tropical Medicine and Hygiene 62**(2): 217-224.

Peakall R, Smouse P (2012) GenAlEx 6.5: genetic analysis in Excel. Population genetic software for teaching and research-an update. **Bioinformatics 28**(19): 2537-2539. doi: 10.1093/bioinformatics/bts460

Raymond, M. 1995. **PROBIT.** France, software.

Rasic G, Filipoviæ I, Weeks A, Hoffmann A (2014) Genome-wide SNPs lead to strong signals of geographic structure and relatedness patterns in the major arbovirus vector, *Aedes aegypti*. **BMC Genomics 15**(275): 1-12. doi: 10.1186/1471-2164-15-275

Ribeiro L (2012) Mitochondrial pseudogenes in insect DNA barcoding: differing points of view on the same issue. **Biota Neotropica 12**

(3): 301-308. doi: 10.1590/S1676-06032012000300029

Rodpradit P, Boonsuepsakul S, Chareonviriyaphap T, Bangs M, Rongnoparut P (2005) Cytochrome P450 genes: molecular cloning and overexpression in a pyrethroid-resistant strain of Anopheles minimus mosquito. **Journal of American Mosquito Control Association 21**(1): 71-79. doi: 10.2987/8756-971X(2005)21[71:CPGMCA]2.0.CO;2

Rodríguez M, Bisset J, Ruiz M, Soca A (2002) Cross-resistance to pyrethroid and organophosphorus insecticides induced by selection with temephos in *Aedes aegypti* (Diptera: Culicidae) from Cuba. **Journal of Medical Entomology 39**(6): 882-888. doi: 10.1603/0022-2585-39.6.882

Rodríguez M, Bisset J, Fernández D (2007) Levels of insecticide resistance and resistance mechanisms in *Aedes aegypti* (Diptera: Culicidae) from some Latin American countries. **Journal of the American Mosquito Control Association 23**(4): 420-429. doi: 10.2987/5588.1

Saavedra-Rodriguez K, Urdaneta-Marquez L, Rajatileka S, Moulton M, Flores A, Fernandez-Salas I, Bisset J, Rodriguez M, McCall P, Donnelly M, Ranson H, Hemingway J, Black W (2007) A mutation in the voltage-gated sodium channel gene associated with pyrethroid resistance in Latin American *Aedes aegypti*. **Insect Molecular Biology 16**(6): 785-798.

Saavedra-Rodriguez K, Salas I, Strode C, Ranson H, Hemingway J, Black W (2012) Transcription of detoxification genes after permethrin selection in the mosquito *Aedes aegypti*. **Insect Molecular Biology 21**(1): 61-77. doi: 10.1111/j.1365-2583.2011.01113.x

Salman A (2007) **Conceitos básicos de genética de populações.** Porto Velho, Embrapa, 118p.

Santacoloma L, Chaves B, Brochero H (2010) Susceptibilidad de *Aedes aegypti* a DDT, deltametrina y lambdacialotrina en Colombia. **Revista Panamericana de Salud Publica 27**(1): 66-73.

Santacoloma L, Chaves B, Brochero H (2012) Estado de la susceptibilidad de poblaciones naturales del vector del dengue a insecticidas en trece localidades de Colombia. **Biomédica 32**(3): 333-343. doi: 10.7705/biomedica.v32i3.680

Soderlund D, Knipple D (1999) Knockdown resistance to DDT and pyrethroids in the house fly (Diptera: Muscidae): from genetic trait to molecular mechanism. **Annals of the Entomological Society of America 92**(6): 909-915.

Soderlund D, Knipple D (2003) The molecular biology of knockdown resistance to pyrethroid insecticides. **Insect Biochemistry and Molecular Biology 33** (6): 563-577.

Strode C, Wondji C, David J, Hawkes N, Lumjuan N, Nelson D, Drane D, Karunaratne S, Hemingway J, Black W, Ranson H (2008) Genomic analysis of detoxification genes in the mosquito *Aedes aegypti*. **Insect Biochemistry and Molecular Biology 38**(1): 113-123. doi: 10.1016/j.ibmb.2007.09.007

Suárez M, González R, Morales C (1996) Temefos resistance to *Aedes aegypti* in Cali, Colombia. **American Journal of Tropical Medicine and Hygiene Supplements 55**(2): 257.

Tamura K, Dudley J, Nei M, Kumar S (2007) MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. **Molecular Biology and Evolution 24**(8): 1596-1599. doi: 10.1093/molbev/msm092

Tikar S, Kumar A, Prasad G, Prakash S (2009) Temephos-induced resistance in *Aedes aegypti* and its cross-resistance studies to certain insecticides from India. **Parasitology Research 105**: 57-63. doi: 10.1007/s00436-009-1362-8

Thompson J, Higgins D, Gibson T (1994) CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. **Nucleic Acids Research 22** (22): 4673-4680.

Twerdochlib A, Dalla A, Leite S, Chitolina R, Westphal B, Navarro-Silva M (2012) Genetic variability of a population of *Aedes aegypti* from Paraná, Brazil, using the mitochondrial ND4 gene. **Revista Brasileira de Entomologia 56**(2): 249-256. doi: 10.1590/S0085-56262012005000030

Urdaneta-Marquez L, Bosio C, Herrera F, Rubio-Palis Y, Salasek M, Black W (2008) Genetic relationships among *Aedes aegypti* collections in Venezuela as determined by mitochondrial DNA variation and nuclear single nucleotide polymorphisms. **American Journal of Tropical Medicine and Hygiene 78**(3): 479-491.

Urdaneta-Marquez L, Anna-Bella F (2011) Population genetic structure of *Aedes aegypti*, the principal vector of dengue viruses. **Infection, Genetics and Evolution 11** (2): 253-261. doi: 10.1016/j.meegid.2010.11.020

WHO (1981) **Instructions for determining the susceptibility resistance of mosquito larvae to insecticides.** Geneva, World Health Organization, VBC, 81.806.

WHO (1998) **Test procedures for insecticide resistance monitoring in malaria vectors, bioefficacy and persistence of insecticides on treated surfaces.** Geneva, World Health Organization, WHO/CDS/CPC/MAL/98.12.

WHO (2013) **Sustaining the drive to overcome the global impact of neglected tropical diseases.** Available online at: http://apps.who.int/iris/bitstream/10665/77950/1/9789241564540_eng.pdf [Accessed: 2 April 2013]

Yan G, Chadee D, Severson D (1998) Evidence for genetic hitchhiking effect associated with insecticide resistance in *Aedes aegypti*. **Genetics 148**(2): 793-800.

Yáñez P, Manami E, Valle J, Garcia M, León W, Villaseca P, Torres D, Cabezas C (2013) Variabilidad genética del *Aedes aegypti* determinada mediante el análisis del gen mitocondrial ND4 en once áreas endémicas para dengue en el Perú. **Revista Peruana de Medicina Experimental y Salud Pública 30**(2): 246-250.

Submitted: 15 July 2014
Received in revised form: 24 October 2014
Accepted: 23 December 2014
Editorial responsibility: Maria Wilhelmina Pil