GMR Genetics and Molecular Research
Online Journal
ISSN:1676-5680

# Genetic structure of *Aedes aegypti* populations determined using pairwise comparisons

**T. de F. Patarro[1], M.M. Guirado[2], L.M. Ravazzi[1] and H.E.M. de C. Bicudo[1]**

[1]Departamento de Biologia, Letras e Ciências Exatas,
Instituto de Biociências, Universidade Estadual Paulista,
São José do Rio Preto, SP, Brasil
[2]Superintendência de Controle de Endemias, Araçatuba, SP, Brasil

Corresponding author: H.E.M. de C. Bicudo
E-mail: bicudo@ibilce.unesp.br

Genet. Mol. Res. 12 (3): 3775-3787 (2013)
Received January 20, 2013
Accepted August 22, 2013
Published September 19, 2013
DOI http://dx.doi.org/10.4238/2013.September.19.9

**ABSTRACT.** The biological characteristics of *Aedes aegypti* (Diptera, Culicidae), which is a vector of dengue and yellow fever, make this organism a good model for studying population structure and the events that may influence it under the effect of human activity. We assessed the genetic variability of five *A. aegypti* populations using RAPD-PCR technique and six primers. Four populations were from Brazil and one was from the USA. A total of 165 polymorphic DNA loci were generated. Considering the six primers and the five populations, the mean value of inter-population genetic diversity (G*st*) was 0.277, which is considered high according to the Wright classification. However, pairwise comparisons of the populations gave variable G*st* values ranging from 0.044 to 0.289. This variation followed the population's geographic distance to some extent but was also influenced by human activity. The lowest G*st* values were obtained in the comparison of populations from cities with intensive commercial and medical contacts. These mosquito populations were previously classified as insecticide resistant, susceptible, or with decreased susceptibility; this parameter apparently had an effect on

 

the G*st* values obtained in the pairwise comparisons.

**Key words:** Polymorphic loci; Passive migration; Geographic isolation; Insecticide mortality percentage; Genetic markers

## INTRODUCTION

In general, the alleles of a gene do not all exist at an equal frequency within a population and differ among populations throughout a species' distribution. Some alleles are far more common than others and their frequencies may change over time in response to external forces, the basic categories of which are environmental variation, random crosses in small-sized populations (genetic drift) and gene flow. Determining the pattern of how the genetic diversity of species is distributed or organized is important for understanding their biology and history (Falk et al., 2001).

The factors that affect the pattern of genetic variability in populations of the mosquito *Aedes aegypti* (*A. aegypti*) are of special interest because the biological characteristics of this organism cause it to be closely involved with human life. The expansion of the distribution area of this mosquito is highly influenced by human activity, mainly by the transport of eggs and larvae in commerce involving objects such as tires, wells and cans. In addition, their populations are subjected to size variation due to applied control programs, which mainly involve the use of insecticides and the elimination of rearing sites. As a consequence, the factors that normally act on the genetic variability of organisms, especially in *A. aegypti* may be directly and differentially affected by human behavior.

The peculiar biological aspects of *A. aegypti* may affect the organization of the genetic variability of this species because it includes various types of populations, such as populations that were most likely founded with a small passively transported sample, populations that have been continuously or eventually subjected to strong reduction through control means, and populations resulting from multiple introductions of mosquitoes originating from the same or different origins. Thus, investigation of the genetic variability of *A. aegypti* populations may be considered to be of biological interest and ultimately of interest to control programs targeting this species.

In this study, the random amplified polymorphic DNA (RAPD-PCR) technique was used with the main aim of estimating the degree of polymorphism and genetic relatedness between *A. aegypti* populations. RAPD-PCR basically involves the amplification of random regions of genomic DNA using decameric primers. The polymorphism observed using this technique generally results from base substitutions, insertions or deletions which may alter primer annealing sites or change the size of amplified products. The use of RAPD-PCR requires special care in the control of the experiments to preserve the quality and repeatability of the amplified fragments in gels. RAPD-PCR has been used in thousands of studies of different organisms to identify and differentiate populations, verify their genetic relationships and obtain information about their patterns of colonization, among other aims (Sandoval-Castellanos et al., 2007; Ram et al., 2008; Paraguison et al., 2012).

The results of the present study produced new information regarding the genetic characterization of *A. aegypti* populations, showing differential involvement of geographic isolation and passive transport in promoting genetic divergence. The data led to other inferences which are also discussed.

 

## MATERIAL AND METHODS

### Mosquitoes

The origins, coordinates, codes, degrees of insecticide resistance and numbers of mosquitoes analyzed per primer in each of the five *A. aegypti* populations are presented in Table 1. Four of them were from Brazil (São Luís - SL, Bauru - BA, São José do Rio Preto - RP and Araçatuba - AR). The fifth (Rockefeller - RO) was from the USA. The geographic locations of the Brazilian populations are shown on a map in Figure 1. The analyzed mosquitoes were the F1 generation of the collected individuals, except for the RO population, which has been maintained in laboratory for at least 25 years (Paduan et al., 2006). Three of the samples (RO, SL and BA) were furnished by the Superintendence of Endemic Disease Control (SUCEN), located at Marilia in São Paulo State, Brazil, which also performed the tests to classify the five populations as resistant, susceptible or having an intermediate *status* designated "decreased susceptibility". The RO specimens were originally provided to SUCEN by the Center of Disease Control of Puerto Rico (Macoris et al., 2007). RO is a very important population for comparisons because, due to the widespread use of insecticides promoting resistance, susceptible populations are no longer found. The samples from Araçatuba and São José do Rio Preto were furnished by SUCEN and the Health Secretariat located in these cities, respectively. The mosquitoes were received in the egg stage and allowed to develop until the adult stage in the Vector Laboratory of our Institution. The adults were frozen and stored until processed. Only males were used.

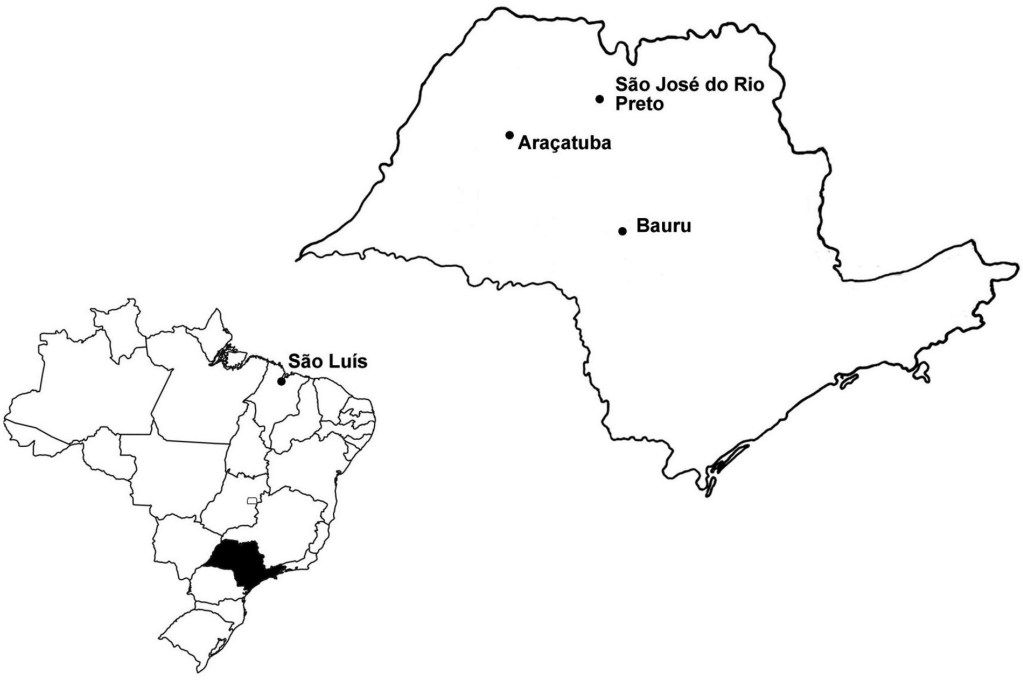


**Figure 1.** Geographic origin of the Brazilian populations analyzed. At the left, the Brazilian map, and at the right, in detail, the State of São Paulo.

**Table 1.** Information for the populations studied: origin, coordinates, code, and classification regarding their insecticide resistance and mortality percentage.

| Population origin | Coordinates | Code | Classification and %mortality | NM/Primer | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | P2 | P9 | P10 | P18 | P20 | P21 |
| Brazil | | | | | | | | | |
| São Luís, MA | 2°31'48"S, 44°18'10"O | SL | R (52.3) | 12 | 13 | 13 | 10 | 12 | 13 |
| Bauru, SP | 22°18'54"S, 49°03'39"O | BA | DS (95.7) | 24 | 20 | 21 | 18 | 17 | 19 |
| São José do Rio Preto, SP | 20°49'12"S, 49°22'44"O | RP | R (88.3) | 16 | 20 | 19 | 7 | 7 | 19 |
| Araçatuba, SP | 21°12'32"S, 50°25'58"O | AR | R (73.0) | 14 | 21 | 15 | 8 | 13 | 20 |
| USA | | | | | | | | | |
| Rockefeller, FL | 25°47'16"N, 80°13'27"W | RO | S (99.5) | 10 | 20 | 23 | 18 | 15 | 17 |

R= resistant, DS= decreased susceptibility and S= susceptible. The number of mosquitoes analyzed per population and primer (NM/primer) are also given.

## Insecticide resistance

The classification of the Brazilian populations regarding their level of insecticide resistance was part of the National Program of Monitoring, coordinated by the Health Ministry and held at SUCEN in Marília and other Brazilian centers. Macoris et al. (2003) described the technique used for classification. The mortality tests used the rules established by the World Health Organization-WHO (1976; 1978). Diagnostic doses of the organophosphorous insecticide temephos and fenitrothion, which are normally applied by health authorities for control, are used in the tests. Mean mortalities greater than 98% indicate susceptibility, while those less than 80% indicate resistance, and mean mortalities between these two values are considered indicative of populations with decreased susceptibility. These criteria were proposed by Davidson and Zahar (1973).

## RAPD-PCR

In the present study, six decameric random primers (from Integrated DNA Technologies, Inc., Coraville, IA USA) were used to generate fragment patterns from the samples analyzed (Table 2). They were chosen from 22 primers previously tested for quality and repeatability of the bands obtained in gels.

**Table 2.** Primers used in the RAPD-PCR analysis: code, base sequence, and C + G content.

| Code | Sequence (5' to 3') | G + C Content (%) |
|---|---|---|
| 2 | CTC CCT GAG C | 70 |
| 9 | GAG CAC CAG T | 60 |
| 10 | GAG CAC TAG C | 60 |
| 18 | CCA TTT ACG C | 50 |
| 20 | ATC GGG TCC G | 70 |
| 21 | ATT CTA TTT C | 20 |

## DNA preparation

DNA was extracted as described by Ayres et al. (2003). Mosquitoes were homogenized individually in microtubes containing 400 µL lysis buffer (10 mM Tris, 0.4 M NaCl and

2 mM EDTA), 72 µL 10% SDS and 7 µL Proteinase K (10 mg/mL). The tubes were mixed by repeated inversions and incubated overnight at 60°C. Subsequently, 420 µL 5 M NaCl were added to each tube, followed by vortexing for 30 sec and centrifugation for 20 min at 13000 rpm. The supernatant was transferred to new microtubes containing 700 µL isopropanol for DNA precipitation, and the mixture was then vortexed, frozen for one hour and centrifuged for 20 min at 13000 rpm. Next, the supernatant was discarded and 500 µL 70% ethanol was added to wash the pellet. The tubes were subsequently vortexed and centrifuged for 10 min at 13000 rpm. The supernatant was discarded again and the tubes were left to dry for 20 min at room temperature. Finally, the pellet was resuspended in 200 µL ultrapure $H_2O$. The DNA content in each sample was quantified using a Nanodrop ND-100. Only samples containing at least 30 ng/µL DNA were analyzed by RAPD-PCR.

## DNA amplification

Extracted DNA was amplified as described by Williams et al. (1990) including modifications indicated by Ayres et al. (2003). PCR was carried out in a 30 µL-reaction mixture containing 1.5 mM $MgCl_2$, 50mM KCl, 0.2 mM each dNTP, 400 pM primers, 10 ng of mosquito DNA and 5U Taq polymerase, and PCR buffer and sterile ultrapure water in a variable volume. Amplifications were performed in a MJ Minicycler™ Research PCR system programmed as follows: 94°C for 1 min, followed by 40 cycles of 94°C for 1 min, annealing at the specific temperature required by each primer for 1 min, and extension at 72°C for 2 min. A final extension was performed at 72°C for 10 min. The amplified products were kept at 4°C until use.

## Electrophoresis

The PCR products obtained (named bands, loci or fragments) were visualized by electrophoresis in 8% polyacrylamide gels (30% Bis-Acrylamide, 5X TBE, glycerol, 10% Ammonium persulfate). A 10 µL aliquot of the amplified products was applied to the gel which was then subjected to vertical electrophoresis at 90 V for 3 h in 1X TBE buffer. The fragments (or bands) generated were visualized after fixation (with 10% ethanol, 0.75% glacial acetic acid) and staining of the gels with silver nitrate, followed by development (1.5% sodium hydroxide, 0.15% formaldehyde). The gels were dried at room temperature and preserved wrapped in cellophane (Ceron et al., 1992).

## DNA analysis

The bands obtained in the gels from DNA amplified from each mosquito were analyzed to determine the presence or absence and size in base pairs (bp) of specific bands. The frequency of bands in every population was also evaluated. Fragment sizes were obtained by comparison with DNA size markers (1kb DNA Ladder, GIBCO BRL). Fragments larger than 7000 bp or with frequencies below 10% were not considered in the analysis.

## Genetic variability of populations

To compare the populations regarding their genetic variability, the bands were

scored to generate a matrix of 1 and 0 (presence and absence, respectively). The analysis of these data to estimate allele frequencies was based on the assumptions that 1) bands with different molecular sizes in the gel are each the product of a dominant allele of a locus; 2) different loci segregate independently; 3) bands that comigrate are homologous; and 4) the genotype frequencies at a RAPD locus are in Hardy-Weinberg equilibrium (Ayres et al., 2003).

POPGENE 1.32 software (Yeh et al., 1999) was used to estimate intra and interpopulation heterozygosity (H*s* and H*t*, respectively), the genetic differentiation of populations (G*st*) and gene flow (N*m*) (Nei, 1987).

## RESULTS

The data on the presence and absence of loci obtained using each primer in every population and their frequencies in terms of the percentage of mosquitoes carrying them are shown in Table 3. For each primer, the bands or loci were designated L1 to Ln, following the increasing order of size in terms of the number of base pairs (bp). Because the primers have different bp sequences, the bands with the same name produced by each of them are different. To illustrate the banding patterns obtained, an image of one gel is shown in Figure 2.

A total of 178 loci were generated from the six primers, in the five *A. aegypti* populations, varying in terms of numbers per primer from 24 (P10) to 38 (P21) and, in size (bp), from 506 (P9) to 6871(P2). Among the 178 loci, 165 were polymorphic (Table 4).

Considering the polymorphism revealed by the primers in the five populations, we calculated the average heterozygosity among individuals within populations (H*s*), the average heterozygosity for the populations (H*t*), the proportion of genetic differentiation (G*st*) between populations and gene flow (N*m*). These parameters were obtained using the statistics described by Nei (1987). The total averages considering all primers and populations are also presented (Table 5).

The H*t* values were higher than the H*s* values for the products of every primer. The H*t* values ranged from 0.155 ± 0.009 (P9) to 0.201 ± 0.021 (P18), while the H*s* values ranged from 0.100 ± 0.004 (P20) to 0.162 ± 0.007 (P10). For the total assembly of the primers and populations, the total average (TA) for *Ht* was 0.181 (±0.016), while that for H*s* was 0.129 (± 0.006).

The proportion of total genetic variation (G*st*), which is a function of how heterozygosity is divided within and between populations, was calculated according to Nei's formula 1 - (H*s* / H*t*). The G*st* per primer for all populations ranged from 0.056 (for P9) to 0.463 (for P18). Gene flow (N*m*), where *m* is the number of migrants per generation, inferred from G*st*, produced inverse values as expected, with the highest value being detected for P9 (8.444) and the lowest for P18 (0.581). The total average (TA) for G*st* and N*m* for the six primers were 0.277 and 2.627, respectively.

A more detailed analysis using the same computer program allowed the detection of different values for G*st* and N*m* in pairwise comparisons of the populations (Table 6). Table 6 also includes the geographic distances in km for each pair. The data indicated a positive correlation between genetic differentiation and geographic distance, which was confirmed by a Pearson correlation coefficient = 0.45. The scatter plot (Figure 3) shows that in addition to the linear effect, there is a quadratic effect.

**Table 3.** Loci and their frequencies (percentages) produced by primers 2, 9, 10, 18, 20, and 21 in the populations SL (São Luís), RO (Rockefeller), BA (Bauru), RP (São José do Rio Preto) and AR (Araçatuba).

| Loci | Primers | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 2 | | | | | 9 | | | | | 10 | | | | | 18 | | | | | 20 | | | | | 21 | | | | |
| | SL | RO | BA | RP | AR | SL | RO | BA | RP | AR | SL | RO | BA | RP | AR | SL | RO | BA | RP | AR | SL | RO | BA | RP | AR | SL | RO | BA | RP | AR |
| L1 | 92 | 40 | 92 | 0 | 0 | 15 | 20 | 30 | 45 | 0 | 77 | 13 | 10 | 0 | 0 | 0 | - | 44 | 0 | 0 | 0 | 0 | 18 | 0 | 0 | 0 | 94 | 0 | 0 | 0 |
| L2 | 92 | 0 | 42 | 0 | 0 | 0 | - | 0 | 30 | 0 | - | 0 | 29 | 26 | 0 | 100 | 94 | 0 | 0 | 0 | 83 | 93 | 65 | 0 | 0 | 0 | 0 | 16 | 0 | 0 |
| L3 | 0 | 30 | 63 | 0 | 0 | 15 | 0 | 0 | 10 | 0 | 0 | 13 | - | 37 | 0 | 0 | 0 | 17 | 0 | 0 | 0 | 0 | 18 | 0 | 0 | 0 | 0 | 47 | 0 | 0 |
| L4 | 100 | 50 | 79 | 0 | 0 | 23 | 0 | 15 | 10 | - | 15 | 13 | 0 | 0 | 0 | 90 | 0 | 0 | 0 | 0 | 100 | 47 | 0 | 0 | 15 | 15 | 0 | 0 | 0 | 0 |
| L5 | 100 | 0 | 0 | 0 | 0 | 31 | 10 | 40 | 60 | 43 | 0 | - | 29 | 37 | 20 | 0 | 33 | 61 | 0 | 13 | 0 | 0 | 35 | 0 | 0 | 0 | 0 | 37 | 0 | 0 |
| L6 | 0 | 40 | 21 | 0 | 0 | - | 10 | - | 10 | - | - | - | - | 0 | 13 | 0 | 83 | 50 | 0 | 0 | 0 | 0 | 35 | 0 | 0 | 0 | 100 | 11 | 0 | 0 |
| L7 | 67 | 0 | 58 | 0 | 0 | - | 0 | 15 | 15 | 10 | 0 | 13 | 10 | 21 | 40 | 100 | 0 | 78 | 29 | 13 | 100 | 80 | 47 | 0 | 0 | 15 | 0 | 63 | 0 | 0 |
| L8 | 33 | 40 | 38 | 0 | 0 | 15 | 25 | 35 | 25 | 0 | - | 0 | 10 | 21 | 20 | 0 | 100 | 0 | 0 | 0 | 0 | 0 | 35 | 0 | 0 | 31 | 0 | 0 | 0 | 0 |
| L9 | 50 | 0 | 54 | 0 | 43 | 0 | 0 | 0 | 10 | - | 23 | 17 | 10 | - | - | 0 | 0 | 0 | 43 | 75 | 42 | 0 | 0 | 0 | 0 | 38 | 0 | 0 | 42 | 25 |
| L10 | 0 | 30 | 17 | 0 | 0 | - | 0 | 15 | 0 | 10 | 0 | 0 | 24 | 16 | 27 | 100 | 0 | 100 | 0 | 0 | 0 | 100 | 41 | 0 | - | 0 | 0 | 21 | 16 | 0 |
| L11 | 0 | 20 | 0 | 0 | 0 | 15 | 10 | - | 0 | 0 | 31 | 39 | 29 | 21 | 27 | 0 | 50 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 46 | 0 | 94 | 0 | 0 | |
| L12 | 0 | 0 | 50 | 0 | 0 | - | 10 | 15 | 15 | 10 | 0 | 30 | 19 | 26 | 40 | 0 | 0 | 22 | 0 | 50 | 33 | 0 | 0 | 0 | 0 | 0 | 0 | 68 | 0 | 30 |
| L13 | 67 | 0 | 0 | 0 | 0 | 23 | 25 | - | 15 | 52 | - | 48 | 38 | 37 | 80 | 90 | 72 | 22 | 0 | 0 | 0 | 0 | 59 | 0 | 0 | 8 | 0 | 47 | 42 | 25 |
| L14 | 0 | 30 | 29 | 0 | 0 | 54 | 35 | 40 | 0 | 52 | 0 | - | - | 26 | 0 | 0 | 0 | 0 | 0 | 13 | 17 | 80 | 0 | 14 | 54 | 0 | 65 | 26 | 0 | 0 |
| L15 | 0 | 0 | 58 | 0 | - | 15 | 15 | 45 | 0 | 24 | 0 | 13 | 10 | 21 | 20 | 0 | 0 | 0 | 29 | 13 | 0 | 0 | 24 | 0 | 0 | 38 | 0 | 0 | 42 | 40 |
| L16 | 58 | 0 | 0 | 13 | 50 | - | 15 | - | 30 | 14 | 0 | 39 | 38 | 47 | 27 | 40 | 0 | 0 | 0 | 0 | 0 | 40 | 18 | 0 | 0 | 0 | 53 | 0 | 0 | - |
| L17 | 0 | 30 | 13 | 0 | 0 | - | 10 | 20 | - | 0 | 23 | 22 | 14 | 0 | 13 | 40 | 0 | 0 | 0 | 0 | 75 | 93 | 0 | 14 | - | 62 | 0 | - | 32 | 15 |
| L18 | 67 | 0 | 0 | 0 | 0 | 77 | 65 | 55 | 20 | 90 | 0 | 35 | 38 | 53 | 67 | 0 | 0 | 0 | 0 | 13 | 0 | 0 | 59 | 0 | 0 | 0 | 0 | 32 | 0 | - |
| L19 | 0 | 60 | 0 | - | - | - | 0 | 0 | 25 | 0 | 0 | 0 | 0 | 0 | 27 | 0 | 0 | 0 | 43 | 13 | 100 | 0 | 0 | 0 | 31 | 0 | 94 | 16 | 16 | 0 |
| L20 | 50 | 10 | 29 | 0 | 0 | 15 | - | 40 | 0 | 0 | - | 52 | 52 | 74 | 100 | 0 | 33 | 61 | 57 | 75 | 0 | 13 | 65 | 0 | 31 | 46 | 0 | 53 | 37 | 25 |
| L21 | 0 | 0 | 0 | 31 | 0 | 38 | 25 | 20 | 20 | 48 | 31 | 13 | 10 | 0 | 0 | 0 | 0 | 0 | 0 | 38 | 0 | 33 | 12 | 0 | - | - | 0 | - | 53 | 25 |
| L22 | 0 | 20 | 50 | 13 | 50 | 54 | 45 | 40 | 10 | 67 | 31 | 22 | 19 | - | 33 | 0 | 39 | 0 | 0 | 0 | 0 | 0 | 29 | 0 | - | 23 | 0 | 53 | 21 | 45 |
| L23 | 0 | 60 | 0 | 38 | 29 | 15 | 15 | 15 | 0 | 19 | 15 | 43 | 24 | 16 | 53 | 0 | 0 | 22 | 14 | 38 | 0 | 0 | 0 | 14 | - | 0 | 0 | 0 | 47 | 45 |
| L24 | 25 | 0 | 0 | 13 | 0 | 15 | - | - | - | 0 | 23 | 13 | - | 11 | - | 0 | 0 | 0 | 43 | 13 | 0 | 0 | 0 | 29 | 23 | 69 | 0 | 0 | - | 15 |
| L25 | 0 | 60 | 33 | 13 | 29 | - | - | 10 | - | 19 | | | | | | 0 | 0 | 17 | 43 | 75 | 0 | 100 | 0 | 29 | 31 | 0 | 0 | 0 | - | 30 |
| L26 | 0 | 0 | 0 | 38 | 57 | 38 | 25 | 20 | 15 | 33 | | | | | | | | | | | 0 | 0 | 0 | 14 | 0 | - | 94 | - | 58 | 60 |
| L27 | 0 | 30 | 0 | 0 | 0 | 38 | 35 | 25 | 20 | 48 | | | | | | | | | | | 0 | 0 | 35 | 0 | 0 | 0 | 0 | 42 | 0 | 40 |
| L28 | 0 | 0 | 0 | 50 | 57 | 31 | 15 | 30 | 20 | 10 | | | | | | | | | | | 0 | 0 | 29 | 0 | 0 | 0 | 0 | - | 32 | 30 |
| L29 | | | | | | 23 | - | - | 0 | 24 | | | | | | | | | | | 0 | 0 | 0 | 43 | 23 | 77 | 0 | 0 | 47 | 15 |
| L30 | | | | | | 46 | 25 | 30 | 0 | 19 | | | | | | | | | | | 0 | 0 | 35 | 14 | 54 | 0 | 41 | 0 | 0 | 0 |
| L31 | | | | | | 0 | - | - | 10 | 0 | | | | | | | | | | | | | | | | 0 | 12 | 0 | 0 | 0 |
| L32 | | | | | | 31 | 30 | 25 | 0 | 10 | | | | | | | | | | | | | | | | 0 | 0 | 0 | 21 | 10 |
| L33 | | | | | | - | 15 | 10 | - | 19 | | | | | | | | | | | | | | | | 0 | 76 | 0 | 63 | 35 |
| L34 | | | | | | | | | | | | | | | | | | | | | | | | | | 0 | 0 | 0 | 0 | 20 |
| L35 | | | | | | | | | | | | | | | | | | | | | | | | | | 8 | 0 | 21 | 37 | 60 |
| L36 | | | | | | | | | | | | | | | | | | | | | | | | | | 0 | 0 | 0 | 0 | 15 |
| L37 | | | | | | | | | | | | | | | | | | | | | | | | | | 31 | 0 | 0 | 0 | 0 |
| L38 | | | | | | | | | | | | | | | | | | | | | | | | | | 0 | 0 | 0 | 79 | 65 |

- = bands not considered due to the frequency below 10%.

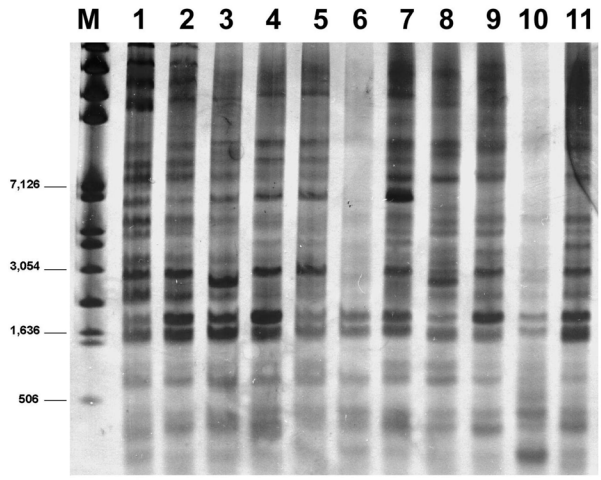


**Figure 2**. Bands in a polyacrylamide gel produced via RAPD-PCR using primer 2. *Lane M* = 1-kb molecular marker; *lanes 1-3* = São Luís; *lanes 4-6* = Rockefeller; *lanes 7-11* = Bauru.

**Table 4.** Intrapopulation variability.

| Primers | No. of loci per primer | Size variation (bp) | No. of polymorphic and monomorphic loci | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | SL | | RO | | BA | | RP | | AR | |
| | | | P | M | P | M | P | M | P | M | P | M |
| P2 | 28 | 634-6871 | 10 | 2 | 15 | 0 | 16 | 0 | 8 | 0 | 7 | 0 |
| P9 | 33 | 506-6108 | 21 | 0 | 21 | 0 | 21 | 0 | 20 | 0 | 20 | 0 |
| P10 | 24 | 559-6617 | 9 | 0 | 17 | 0 | 18 | 0 | 16 | 0 | 16 | 0 |
| P18 | 25 | 546-5792 | 4 | 3 | 7 | 1 | 10 | 1 | 8 | 0 | 13 | 0 |
| P20 | 30 | 553-6532 | 5 | 3 | 8 | 2 | 18 | 0 | 8 | 0 | 9 | 0 |
| P21 | 38 | 543-6560 | 11 | 0 | 9 | 1 | 15 | 0 | 17 | 0 | 21 | 0 |
| T | 178 | NT | 60 | 8 | 77 | 4 | 93 | 1 | 77 | 0 | 86 | 0 |

Number of loci produced per primer, their size variation in base pairs, numbers of polymorphic and monomorphic loci produced per primer in every population, the total number considering the six primers (T), and the total of polymorphic and monomorphic loci per population (NT). For other abbreviation, see Table 1.

**Table 5.** Total heterozygosity (H*t*), mean heterozygosity (H*s*), genetic differentiation (G*st*) and gene flow (N*m*), for every primer considering the five populations. SD= Standard Deviation. GA = general average considering all the primers.

| Primer | Parameters | | | |
|---|---|---|---|---|
| | H*t* (SD) | H*s* (SD) | G*st* | N*m* |
| P2 | 0.197 (0.011) | 0.136 (0.004) | 0.308 | 1.122 |
| P9 | 0.155 (0.009) | 0.146 (0.007) | 0.056 | 8.444 |
| P10 | 0.184 (0.013) | 0.162 (0.007) | 0.119 | 3.700 |
| P18 | 0.201 (0.021) | 0.108 (0.006) | 0.463 | 0.581 |
| P20 | 0.174 (0.021) | 0.100 (0.004) | 0.428 | 0.670 |
| P21 | 0.175 (0.021) | 0.125 (0.004) | 0.286 | 1.246 |
| GA | 0.181 (0.016) | 0.129 (0.006) | 0.277 | 2.627 |

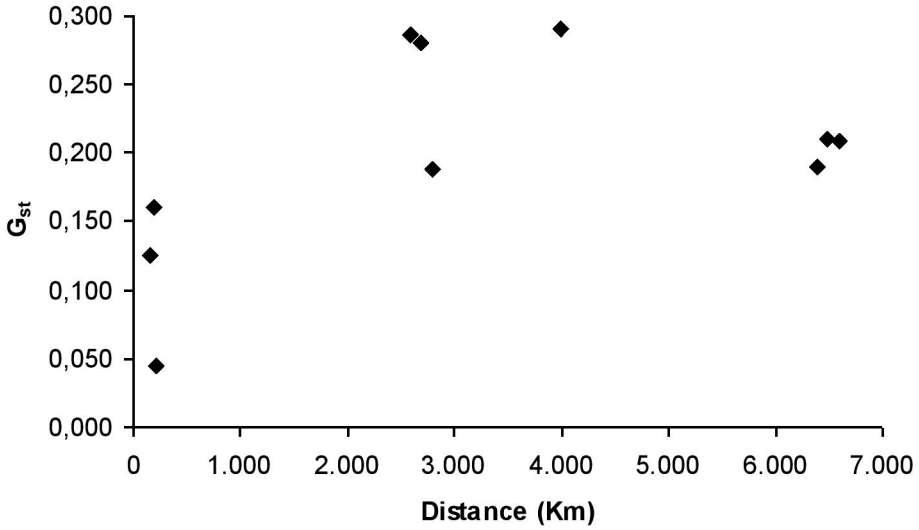


**Figure 3.** Scatter plot of G*st* values versus the geographic distances of the populations analyzed. Pearson's correlation coefficient = 0.45.

**Table 6.** Pairwise comparisons of populations.

| Comparisons of populations | G*st* | N*m* | Geographic distance (km) |
|---|---|---|---|
| Brazilian | | | |
| SL X BA | 0.187 | 6.860 | 2.800 |
| SL X RP | 0.286 | 2.830 | 2.600 |
| SL X AR | 0.279 | 2.562 | 2.700 |
| BA X RP | 0.125 | 8.826 | 170 |
| BA X AR | 0.160 | 4.881 | 200 |
| AR X RP | 0.044 | 341.580 | 230 |
| Average | 0.180 | 61.256 | 1.450 |
| Brazilian *vs* RO | | | |
| SL X RO | 0.289 | 8.232 | 4.000 |
| BA X RO | 0.189 | 13.113 | 6.400 |
| RO X RP | 0.209 | 5.787 | 6.500 |
| RO X AR | 0.207 | 3.727 | 6.600 |
| Average | 0.224 | 7.715 | 5.875 |

Mean genetic diffrentiation (G*st*) and gene flow (N*m*) for each comparison. SL= São Luís, RO= Rockefeller, BA= Bauru, RP= São José do Rio Preto, and AR= Araçatuba. Averages are shown separately for comparisons between Brazilian populations and for Brazilian versus RO populations.

Pairwise comparisons performed to detect bands that were present only in the compared populations showed that the numbers of these bands, considering the six primers were as follows: RO X BA (12) RO X SL (3), RO X RP (2), RO X AR (0), BA X SL (5), BA X RP (2), BA X AR (5), SL X RP (2), SL X AR (3) and RP X AR (13). Considering the numbers, among the 12 bands that were exclusively shared by RO (susceptible population) and BA (population with decreased susceptibility), 9 showed frequencies in BA that were approximately half or even less of those in RO and one did not differ. The comparisons involving the susceptible RO population versus the resistant populations and between resistant populations showed low numbers of shared bands, with the exception of the RP X AR comparison.

## DISCUSSION

It is known that isolated populations may be different from each other, while the individuals within each of them are genetically similar. The opposite situation may occur when populations are closely located geographically and are subjected to a high level of gene flow (Falk et al., 2001). In the present study, considering the six primers and the five *A. aegypti* populations involved in the analysis, the average total heterozygosity (H*t* = 0.181) was higher than the average heterozygosity within each population (H*s* = 0.129), indicating the role of geographic isolation in the production of genetic differentiation among the populations studied.

Additionally, taking into account all of the primers and populations, the average for G*st* values (0.277) showed that, on the basis of Wright (1978) classification, the degree of genetic differentiation of these populations is very high. According to this author, genetic differentiation between populations ranging from 0 to 0.05 is indicative of low differentiation, 0.05 to 0.15 of moderate differentiation, 0.15 to 0.25 of high differentiation and greater than 0.25 of very high differentiation.

A high average genetic differentiation between *A. aegypti* populations has also been obtained in other studies involving mosquitoes from different regions of the world analyzed using RAPD-PCR or other techniques. For example, in Brazilian populations, Ayres et al. (2003) obtained G*st* = 0.317 for populations from different states; Hiragi et al. (2009) reported G*st* = 0.3689, and Paduan et al. (2006) found an even higher G*st* value (0.430). The average G*st* values have been similarly high in studies of *A. aegypti* from other geographic origins, including Mexico (García-Franco et al., 2002), Argentina and Uruguay (de Sousa et al., 2001; Julio et al., 2009; Soliani et al., 2010), and Puerto Rico (Apostol et al., 1996).

Average G*st* values for population groups have been predominantly used in the literature to discuss population structure of *A. aegypti.* However, in the present work, pairwise comparisons of populations showed that different situations are obscured in the average G*st* value estimated for all populations. Using pairwise comparisons, individual analyses produced G*st* values ranging from 0.044 (low differentiation) to 0.289 (very high differentiation). Examination of these individual G*st* values indicated the involvement of geographic distance, but also of other factors affecting the structure of the populations. A positive correlation of the G*st* values with geographic distance was confirmed by application of the Pearson coefficient (0.45). However, the scatter plot showed that, in addition to the linear effect, there is a quadratic effect, meaning that the two parameters are correlated but not in a proportional way.

In analyzing the genetic relationships of these populations, we must remember the short flight distance of *A. aegypti* and the above-mentioned easy transport, mainly of eggs, during the trade of different objects. Regarding the flight distance, Scarpassa et al. (2008) noted that a range of 50 to 800 m has been found in different studies on this mosquito.

Considering the Brazilian populations, the three pairwise comparisons associated with the greatest geographic distances involve SL with BA (2600 km), with RP (2700 km) and with AR (2800 km), whose *Gst* values were 0.187, 0.286 and 0.279, respectively. These were the highest G*st* values obtained among the pairwise comparisons of Brazilian populations. São Luís is located in the state of Maranhão, in northeastern Brazil, while Bauru, São José do Rio Preto and Araçatuba are in the state of São Paulo, in the southeast. The two regions are very different in terms of climate. In addition, due to the great distance and transport difficulties involved, SL is situated in a region where there is scarce commercial activity with the Brazil-

ian south, favoring the isolation from the other populations studied, explaining the low gene flow (N*m*) values obtained.

The Brazilian populations from the state of São Paulo, which are geographically closer to each other (BA X RP =170 km, BA X AR = 200 km and AR X RP = 230 km) showed lower G*st* values (and higher N*m* values) than those observed in the comparisons involving SL, as expected. However, even with the relatively small geographic distance involved in BA X AR comparison, its G*st* value (0.160) is considered indicative of high genetic differentiation according to the Wright classification. The BA X RP comparison showed moderate genetic differentiation.

The AR X RP comparison deserves special consideration. As mentioned above, these populations are separated from each other by 230 km (60 km more than between BA X AR and 30 km more than between BA X RP). However, their G*st* value (0.044) was the lowest obtained in the present study, indicating a low level of genetic differentiation. Conversely, their N*m* value was the highest. The occurrence of gene flow via passive transport may explain the low genetic differentiation observed between populations AR and RP because intensive trade and human traffic between the cities where they are located have continued for a long period. The cities of Araçatuba and São José do Rio Preto are more isolated in the northwestern region of the state, while the third population, BA, from Bauru, is more central and this city is preferentially involved in exchanges with other cities. These different interrelationships have been promoted by the fact that theses sites are served by different highways. The hypothesis of the repeated occurrence of passive transport between Araçatuba and São José do Rio Preto is highly supported by the generally intensive traffic of buses from the former to the latter to transport patients to the Hospital of the Medical School (FAMERP), located at São José do Rio Preto. People also predominantly travel from Araçatuba to São José do Rio Preto for shopping and for purchasing utilities. This coming and going may have propitiated (and perhaps still is) high gene flow through multiple passive migrations between these cities. This hypothesis does not rule out the possibility of a common origin for the two populations with the genetic similarity between them being maintained by the passive gene flow.

The fact that the populations studied were previously classified regarding the degree of mortality they exhibited when subjected to insecticide treatment allowed comparison the data on genetic differentiation from this perspective. The pairwise comparisons of the Brazilian populations with RO are the most informative in this respect because RO is the only population classified as susceptible among the populations studied. As expected on the basis of their large geographic distances (from 4000 to 6800 Km), the G*st* values obtained in comparisons of RO with the Brazilian populations were predominantly higher than those obtained for comparisons between Brazilian populations. The average G*st* for the group of pairwise comparisons involving RO versus Brazilian populations was 0.224, whereas 0.180 for the Brazilian group. However, taking into account whether the populations were insecticide resistant or not, the data appear to suggest that the genetic variation underlying resistance or susceptibility to insecticides may also be involved in the genetic differentiation of the populations compared. Thus, the comparisons involving the susceptible RO population with the resistant SL, RP and AR populations produced G*st* values = 0.289, 0.209 and 0.207, respectively, values which are greater than those detected in the comparison involving the susceptible RO population with BA, which, when this study was performed, was considered to be in an intermediate state between resistance and susceptibility (G*st* = 0.189). A hypothesis to explain these results could be that the genetic constitution of BA is more similar to that of RO because BA has not

yet completed the genetic differentiation required to attain the resistant phenotype. In addition, 9 of the 12 loci that were common to these populations showed frequencies in BA that were half, or even less than half, of those present in RO, suggesting that the ¨susceptible genotype¨ is being substituted with the "resistant one".

According to Sousa-Polezzi and Bicudo (2005) the decrease in the frequency of some esterase bands and the simultaneous increase for others that they observed over time in *A. aegypti* suggest substitution of alleles under the environmental pressure caused by the intensive use of insecticides for control of this species. According to these authors, resistance is due to a group of genes that are simultaneously impacted by this environmental pressure, rather than to a single changed gene. In our study, the low number of bands shared between resistant populations might have been due to the selection of at least partially different genotypes for resistance, in different environments. In contrast, the high number of bands shared between AR and RP can be explained by intensive gene flow via passive migration as discussed above.

Thus, the data presented herein obtained through pairwise comparisons of *A. aegypti* populations, analyzed individually, allowed the detection of variable degrees of genetic differentiation and the analysis of the specific conditions underlying the differentiation of each pair, which are aspects that cannot be revealed by averaged data. The hypothesis that the genetic constitution underlying the degree of resistance of these populations could be used to evaluate the degree of genetic differentiation still requires further study for confirmation.

## ACKNOWLEDGMENTS

Research supported by FAPESP and CAPES (master's fellowship, Thais de França Patarro). We thank Dr. Maria de Lourdes Macoris from SUCEN of Marília for providing the samples of mosquitoes.

## REFERENCES

Apostol BL, Black WC, Reiter P and Miller BR (1996). Population genetics with RAPD-PCR markers: the breeding structure of *Aedes aegypti* in Puerto Rico. *Heredity* 76: 325-334.

Ayres CF, Melo-Santos MA, Solé-Cava AM and Furtado AF (2003). Genetic differentiation of *Aedes aegypti* (Diptera: Culicidae), the major dengue vector in Brazil. *J. Med. Entomol.* 40: 430-435.

Ceron CR, Santos JR and Campos-Bicudo HEM (1992). The use of gelatin to dry cellophane wound slab gels in an embroidering hoop. *Rev Bras Genet.* 15: 201-203.

Davidson G and Zahar AR (1973). The practical implications of resistance of malaria vectors to insecticides. *Bull. World Health Organ.* 49: 475-483.

de Sousa GB, Blanco A and Gardenal CN (2001). Genetic relationships among *Aedes aegypti* (Diptera: Culicidae) populations from Argentina using random amplified polymorphic DNA polymerase chain reaction markers. *J. Med. Entomol.* 38: 371-375.

Falk DA, Knapp E and Guerrant EO (2001). How is Genetic Diversity Distributed in Natural Populations? National Park Service, Available at [http://www.nps.gov./plants/restore/pubs/restgene/2.htm]. Accessed March 30, 2011.

García-Franco F, Muñoz ML, Lozano-Fuentes S, Fernandez-Salas I, et al. (2002). Large genetic distances among *Aedes aegypti* populations along the South Pacific coast of Mexico. *Am. J. Trop. Med. Hyg.* 66: 594-598.

Hiragi C, Simões K, Martins E, Queiroz P, et al. (2009). Variabilidade genética em populações de *Aedes aegypti* (L). (Diptera: Culicidae) utilizando marcadores de RAPD. *Neotrop. Entomol.* 38: 542-547.

Julio NB, Chiappero MB, Rossi HJ, Rondan Dueñas JC, et al. (2009). Genetic structure of *Aedes aegypti* in the city of Cordoba (Argentina), a recently reinfested area. *Mem. Inst. Oswaldo Cruz* 104: 626-631.

Macoris MLG, Andrighetti MTM, Takaku L, Glasser CM, et al. (2003). Resistance of *Aedes aegypti* from the State of São Paulo, Brazil, to organophosphates insecticides. *Mem. Inst. Oswaldo Cruz* 98: 703-708.

Macoris MLG, Andrighetti MT, Otrera VCG, Carvalho LR, et al. (2007). Association of insecticide use and alteration on *Aedes aegypti* susceptibility status. *Mem. Inst. Oswaldo Cruz* 102: 895-900.

Nei M (1987). Genetic Variation Within Species. In: Molecular Evolutionary Genetics. Columbia University Press, New York.

Paduan KS, Araújo-Júnior JP and Ribolla PEM (2006). Genetic variability in geographical populations of *Aedes aegypti* (Diptera, Culicidae) in Brazil elucidated by molecular markers. *Genet. Mol. Biol.* 29: 391-395.

Paraguison RC, Faylon MP, Flores EB and Cruz LC (2012). Improved RAPD-PCR for discriminating breeds of water buffalo. *Biochem. Genet.* 50: 579-584.

Ram SG, Parthiban KT, Kumar RS, Thiruvengadam V, et al. (2008). Genetic diversity among Jatropha species as revealed by RAPD markers. *Genet. Resour. Crop Evol.* 55: 803-809.

Sandoval-Castellanos E, Uribe-Alcocer M and Díaz-Jaimes P (2007). Population genetic structure of jumbo squid (*Dosidicus gigas*) evaluated by RAPD analysis. *Fish. Res.* 83: 113-118.

Scarpassa VM, Cardoza TB and Cardoso Junior RP (2008). Population genetics and phylogeography of *Aedes aegypti* (Diptera: Culicidae) from Brazil. *Am. J. Trop. Med. Hyg.* 78: 895-903.

Soliani C, Rondan-Dueñas J, Chiappero MB, Martinez M, et al. (2010). Genetic relationships among populations of *Aedes aegypti* from Uruguay and northeastern Argentina inferred from ISSR-PCR data. *Med. Vet. Entomol.* 24: 316-323.

Sousa-Polezzi RC and Bicudo HE (2005). Genetic variation along time in a Brazilian population of *Aedes aegypti* (Diptera: Culicidae), detected by changes in the esterase patterns. *Genetica* 125: 43-53.

Williams JG, Kubelik AR, Livak KJ, Rafalski JA, et al. (1990). DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. *Nucleic Acids Res.* 18: 6531-6535.

Wright S (1978). Evolution and the genetics of populations. University of Chicago Press, Chicago.

Yeh FC, Yang R and Boyle T (1999). POPGENE Version 1.32: Microsoft Window-Based Freeware for Population Genetics Analysis. University of Alberta, Edmonton. Available at [http://www.ualberta.ca/~fyeh/popgene_download.html]. Accessed March 30, 2011.