Short Communication

Genetic variability in geographical populations of *Aedes aegypti* (Diptera, Culicidae) in Brazil elucidated by molecular markers

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Abstract

The tropical mosquito, *Aedes aegypti* is the most important domestic vector of urban yellow fever and dengue. Genetic population studies on this vector are important because they may lead to new tools for surveillance. An analysis of genetic structure was conducted among populations of *A. aegypti* from 11 localities in four demographic regions within six Brazilian federal states. Markers included 21 random amplified polymorphic DNA (RAPD) loci. RAPD markers were detected among populations and cluster analysis revealed two main groups. We found high genetic polymorphism (H_s = 0.224) and high levels of genetic differentiation between populations from different states (G_{ST} = 0.430), as well as in populations from cities in the same state (G_{ST} = 0.410). These results indicate significant differentiation in *A. aegypti* populations in Brazil. Regression analyses of geographic distances and pairwise F_{ST} values estimated from RAPD markers showed that there is a correlation between genetic structure and geographic localization.

Dengue is an important viral disease transmitted to humans by arthropod vectors. Yearly, an estimated 50-100 million cases of dengue fever (DF) and 250.000-500.000 cases of dengue hemorrhagic fever (DHF) occur worldwide (Gubler, 2002). Four sorotypes of dengue viruses, DEN 1-4, are commonly maintained in urban transmission cycles by *Aedes aegypti*. The primary vector of dengue has a global distribution in tropical and subtropical areas, and exhibits a distinct preference for urban areas.

In the 1940s the Pan American Health Organization (PAHO) initiated an *A. aegypti* eradication program in order to eliminate the vector in the Americas. This program eliminated the mosquito from 19 countries, including Brazil (Schleissman and Calheiros, 1974). Unfortunately, eradication was difficult to sustain, and by the 1970s reinvasion by this mosquito was frequent. The expansion continued during the 1980s and 1990s (Gubler, 2002). In Brazil, dengue cases represent 80% of all cases occurring in the Americas (Schatzmayr, 2000). In 2002, 794.219 dengue cases were registered, with 2.714 cases of the severe form of the disease (HF), which caused 150 deaths (FUNASA 2003).

Overall, mosquito-borne diseases have re-emerged as a significant human health problem due to a number of factors, including the lack of progress in vaccine development, the emergence of drug resistence in pathogens and inseticide resistence in mosquitoes, and the decline in socioeconomic conditions in many countries that limit disease monitoring and mosquito control efforts (Gubler 1998). Mosquito control remains the only viable strategy for preventing dengue and other mosquito borne-disease.

At present, mosquito populations are being investigated at the molecular and behavioral levels, with the goal of developing innovative control strategies. *A. aegypti* has been the subject of numerous studies conducted in laboratories throughout the world. Extensive DNA-based genetic studies have been made using RAPD (Random amplified polymorphic DNA) markers and this technology has been used to study a variety of characteristics in *A. aegypti* populations around the world, including genetic mapping and strain identification (Clark and Lanigan, 1993), the number of families at ovoposition sites (Apostol *et al.*, 1994) and the differentiation of related species (Ballinger-Crabtree *et*

al., 1992). Research regarding vector population genetics can provide new tools to improve vector surveillance elucidating genetic traits of mosquito populations that could be related to vector capacity and/or inseticide resistance.

Recently in Brazil, RAPD markers were used to demonstrate the existence of high levels of genetic differentiation of *A. aegypti* at both macro and micro-geographic levels (Ayres *et al.,*. 2003). In the present study, the genetic diversity in 11 Brazilian populations of *A. aegypti* representing some important commercial routes, in four demographic regions within six states were examined using RAPD. The major objectives were to obtain data on their genetic structure that may contribute to improving vector surveillance.

A total of 12 populations was analyzed. The collection sites are listed in Table 1, and the geographic locations of all sampling sites are shown in Figure 1. Samples were obtained from 11 cities distributed in the Brazilian states of Alagoas, Ceará, Mato Grosso do Sul, Paraná, Rondônia and São Paulo, from January to March in 2002. In Porto Velho, we performed 2 collections at different times (2001 and 2002). Rockefeller is a laboratory strain originally from Puerto Rico that has been reared for at least 25 years. Eggs were collected at different sites by ovitraps (usually 500 - 1000 ovitraps/city) and transported to the laboratory. Eggs from all positive ovitraps were pooled together and a random sample was used in this work in order to minimize relationship between mosquitoes. Mosquitoes were reared until adult stage under standard conditions (temperature 27 to 29 °C, relative humidity 80 to 90% and 12-h light/dark cycle). Pupae were isolated and emerging virgin females were used for analysis.

DNA was extracted from each individual in lysis buffer (Tris-HCl 10mM pH 8.0 containing NaCl 0.2 M, EDTA 25mM, sodium dodecyl sulfate 0.5%) by ethanol

Figure 1 - Map of Brazil showing the *A. aegypti* collections sites.

precipitation following a modification of the procedure of Bender *et al.* (1983). The final DNA pellet was reconstituted in 100 µL TE buffer (Tris-HCl 10mM pH 8.0, 1mM EDTA). DNA concentrations were estimated by comparison with known amounts of electrophoretic standards (*Hind* III digested). DNA working solutions were prepared at a concentration of 10ng/ µL in TE buffer and stored at -70 °C.

Each amplification was performed in a total of 25 µL of reaction mixture consisting of Tris-HCl 10mM pH 9.0, KCl 50mM, $MgCl_2$ 2mM, dNTP 0.2mM, 10 pmoles of primer, 0.75U of *Taq* DNA polymerase (Pharmacia Biotech) and 10ng of genomic DNA. Reactions were performed in a Whatman Biometra (T Gradient) thermal cycler. The PCR program consisted of one step for 4 min at 94 °C, 45 cycles of 1 min at 94 °C, 1 min at 36 °C, 2 min at 72 °C, followed by a final extension step of 5 min at 72 °C (ramp time from 36 °C to 72 °C equal to 2 min). All primers used were 10-base oligomers obtained from Operon Tech-

Table 1 - Locations, coordinates, and sample sizes of *Aedes aegypti* collections in Brazil.

State	Cities	Latitude/Longitude	NR
Ceará	Juazeiro do Norte (JUA)	07°12'47" S/39°18'55" W	5
Alagoas	Maceió (MAC)	09°39'57" S/35°44'07" W	10
Mato Grosso do Sul	Campo Grande (CGR)	09°57'28" S/36°47'30" W	9
Rondônia	Vilhena (VIL)	12°44'26" S/60°08'45" W	10
Rondônia	Porto Velho (PVE)	15°38'42" S/56°05'52" W	20
Paraná	Londrina (LON)	23°18'37" S/51°09'46" W	5
São Paulo	Barretos (BAR)	20°33'26" S/48°34'04" W	10
São Paulo	Bauru (BAU)	22°18'53" S/49°39'38" W	10
São Paulo	Botucatu (BOT)	22°53'09" S/48°26'42" W	10
São Paulo	Campinas (CAM)	28°57'39" S/51°31'55" W	10
São Paulo	Santos (SAN)	23°57'39" S/46°20'01" W	5
Puerto Rico	Rockefeller (RCK)	18°25'22" N/66°02'59" W	10
Total			114

N_R the numbers of individuals from each population used for the RAPD analysis.

nologies (Alameda, CA). Three oligonucleotides were described previously in the literature A02, B03, B13 (Ballinger-Crabtree *et al.*, 1992). We screened eleven other primers (A10, B06, B10, B16, B20, K20, X16, W01, W03, W10, W16) and used two of them in the analyses. Oligonucleotide primers were selected on the basis of their reproducibility and presence of polymorphic products. Primers used in this analysis have the following sequences (5'-3'): A02, TGCCGAGCTG: B03, CATCCCCCTG: B13, TTCCCCCGCT: W01, CTCAGTGTCC: W03, GTCCGGAGTG. All amplification reactions were repeated twice in order to test reproducibility. The RAPD products were analyzed by electrophoresis in 1.5% (w/v) agarose gels run for 1.5h using 1X TAE buffer (Tris-acetate 40 mM pH 7.6, EDTA 1 mM). Gels were stained with ethidium bromide according to Sambrook *et al.* (1989) and photographed under ultraviolet light using a Nikon 750 camera. The sizes of DNA fragments were estimated by comparisons with the standard Ladder 250 bp (Invitrogen).

Amplified fragments, named by the primer used and the molecular mass in base pairs (bp), were scored as the presence (1) or absence (0) of homologous bands, and a matrix of the different RAPD genotypes was assembled. The RAPD markers were analyzed as genetic markers under the following assumptions: (1) RAPD markers segregate in a Mendelian fashion; and (2) genotype frequencies at RAPD loci are in Hardy-Weinberg proportions (Gorrochotegui-Escalante *et al.*, 2000).

RAPDDIST in RAPDPLOT (Black, 1996) softwares were used to compute pairwise distances between samples and populations. We analyzed the data using all the genetic distance measures available in RAPDDIST with the Lynch and Milligan (1994) method correction factor for small sample sizes. F_{ST} values between collections were compiled into a distance matrix. Genetic similarity was summarized in an unweighted pair-group method with an arithmetic average dendogram (UPGMA), for which bootstrap values based on 1,000 replicates were calculated (Felsenstein, 1985).

Comparing distance matrices between matrices of genetic and linear geographic distances tested the possibility of isolation by distance. Geographic coordinates were obtained with GPS and geographic distance matrices were made using GenALEx package (Peakall and Smouse, 2005) to compute the Mantel statistics (Mantel and Valand, 1970). Regression analysis and the Mantel test were performed using Mantel (Rousset, 1997). Resulting *r*-values were interpreted as correlation coefficients. Additionally, the frequencies were used to calculate mean heterozygosity. The population differentiation parameter G_{ST} and unbiased genetic distances (Nei, 1978) were calculated among populations using POPGENE software (Yeh *et al.*, 1997).

Amplifications of *A. aegypti* genomic DNA with 5 primers generated 21 reproducible and polymorphic mark-

ers among natural Brazilian populations. Two markers were present only in the populations of Juazeiro do Norte and Alagoas. Beside these 21 markers, six others were exclusive to Rockefeller (laboratory strain). The number of bands per primer varied from two to six, ranging from 250 to 1500 base pairs.

The dendogram constructed using the UPGMA method (Figure 2) shows that the Rockefeller population represents a distinct group from the Brazilian populations in 100% of the 1000 bootstraps trees. *A. aegypti* Brazilian populations could be separated into two clusters, one group representing two populations from the Northeast (Maceió and Juazeiro do Norte), while the other group (Vilhena, Porto Velho, Campo Grande, Botucatu, Barretos, Bauru, Santos, Campinas and Londrina) consists of 10 populations from the Southeastern, Central and Northern regions. The second cluster was further divided into two smaller clusters that correspond to the geographical origin of the samples. Genetic distance between the two main clusters suggests that these populations are undergoing genetic differentiation, a process that may be driven by ecological, and evolutionary or historical factors.

The differentiation observed in the *A. aegypti* population from Botucatu (when compared to Bauru that is 80 km apart), probably resulted from repeated founder events followed by a reduction of genetic variability due to the city's characteristics. Botucatu is a relative small city, compared with others in this study, with a pronounced cold period. Captures performed in the cold period lead to very few eggs, and during the hot period *Aedes albopictus* is the major mosquito species (Nogueira *et al.*, 2005).

Heterozygosities ranged from 0.209 (±0.019) in the SE (Southeast) to 0.211 (±0.028) in the NE (Northeast). In a similar manner, the total average gene diversity was unusually high ($H_T = 0.388 \pm 0.009$). The overall differentiation among the eleven analyzed wild Brazilian populations was high ($G_{ST} = 0.430$; Nm = 0.65). The intrastate G_{ST} value
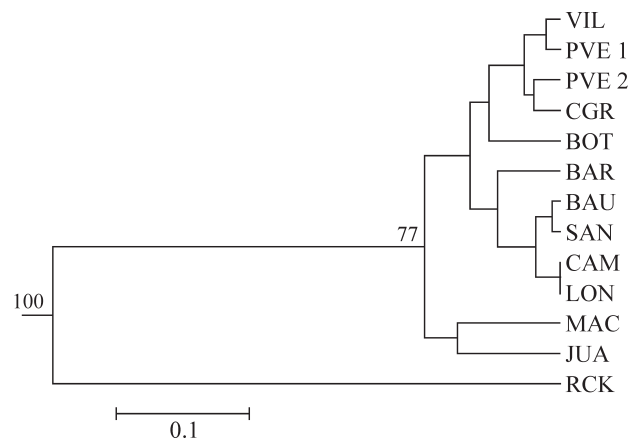

Figure 2 - Dendogram based on Nei's genetic distance (Nei 1978) among *A. aegypti* populations in Brazil. Bootstrap values over 50%, based in 1,000 permutations are indicated on nodes. Analysis was done using the random amplified polymorphic DNA markers.

varied from 0.374 (Nm = 0.835) in Sao Paulo State (BAU, BAR, BTU, CAM, SAN) to 0.208 (Nm = 1.90) in the populations of Central and Northern regions (CGR, PVE, and VIL).

The RAPD method has been reported as an efficient tool to detect differentiation of geographically and genetically isolated populations (Ballinger-Crabtree *et al.*, 1992; Apostol *et al.*, 1994; Gorrochotegui-Escalante *et al.*, 2000; Souza *et al.*, 2001). The results presented in this study demonstrate a significant correlation between genetic and geographic distances of Brazilian *Aedes* populations. The data also showed the presence of two partially isolated groups, one representing mosquitoes from the Northeast and another one with populations from the North, Central and South of Brazil.

The average heterozygosity among the 21 RAPD loci (H_T = 0.388) was similar to that obtained in an analysis in Brazil using 27 RAPD loci (H_T = 0.390) (Ayres *et al.*, 2003) and also to that obtained for 57 loci in Puerto Rico (H_T = 0.354) (Apostol *et al.*, 1996). Genetic diversity among Brazilian *A. aegypti* populations was significant and shows that these populations are highly differentiated (G_{ST} = 0.430; Nm = 0.65) and very polymorphic (Hs = 0.224). These values were similar to those described for other populations of this species in Brazil (G_{ST} = 0.317; Nm = 0.54; H_S = 0.274) (Ayres *et al.*, 2003) and Argentina (G_{ST} = 0.249; Nm = 0.75) (Souza *et al.*, 2001).

Analysis among populations of two geographic regions where commercial traffic is intense, mainly due soybean transportation by road, as seen in the North (PVE and VIL) and Central West (CGR), showed low population differentiation (G_{ST} = 0.208) with a higher degree of gene flow (Nm = 1.90), indicating the importance of human activity to mosquito spread. Genetic distances also tended to be smaller between samples from northeastern Mexico (Gorrochotegui-Escalante *et al.*, 2000), suggesting that in this locality *A. aegypti* dispersal occurs primarily through human commerce; whereas in the Yucatàn, genetic distances were larger, indicating that here *A. aegypti* dispersal may occur mainly through flight).

F_{ST} values were estimated following Lynch and Milligan (1994). F_{ST}/(1 - F_{ST}) values were plotted against log_{10} (geographical distances (km)) estimated from GPS coordinates. This analysis indicated a significant correlation between genetic distances and the geographic distance (r^2 = 0.2089, p < 0.005) of *A. aegypti* in Brazil. *A. aegypti* populations from French Guiana are highly differentiated (F_{ST} = 0.111) even among seven sites that are less than 30 km apart (F_{ST} = 0.137), but show no positive correlation with geographic distance. Ravel *et al.* (2002) studied the genetic variation of *A. aegypti* in Côte d'Ivoire, by microssatellite and detected evidence for a microgeographic genetic differentiation of mosquitoes from Bouaké, with populations being genetically distinct along a north-south transect not related to geographic distances. In another study, Huber *et al.* (2002) detected a significant genetic structuring of the species in the city area of Ho Chi Minh, Vietnam.

Structured populations usually show a dynamic equilibrium between factors that favor differentiation (mutation, drift, and directional or disruptive natural selection, differing in each area) and homogenizing factors (migration, purifying natural selection, and balanced or differential natural selection, uniform in each area) (Solé-Cava, 2001). In our study, analyses of *A. aegypti* in Brazil suggest three geographically differentiated regions (the Northeast, the Southeast and the Central/North). The Northeastern differentiation may be due to a lack of migration between this region and the rest of the country. The Central and Northern differentiation could be result of the connections between cities through the main routes of commercial transit and not the consequence of active migration by mosquito populations. High diversity within and high similarities between groups of populations, as indicated by the RAPD markers, reflect the recent introduction of this species into Brazil, which involved a large number of individuals.

Acknowledgments

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