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Genetic Differentiation of *Aedes aegypti* (Diptera: Culicidae), the Major Dengue Vector in Brazil

C.F.J. AYRES,[1] M.A.V. MELO-SANTOS, A. M. SOLÉ-CAVA,[2] AND A. F. FURTADO

Departamento de Entomologia, Centro de Pesquisas Aggeu Magalhães-Fiocruz, Recife-PE, Brazil

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ABSTRACT In 2000, Brazil reported 180,137 cases of dengue, ≈80% of the total in the Americas. However, little is known about gene flow among the vector populations in Brazil. Random amplified polymorphic DNA (RAPD) was used to study the genetic structure of *Aedes aegypti* in 15 populations from five states, with a range extending 2,800 km. An analysis was performed of 47 polymorphic RAPD loci quantified gene flow at the macro- (different states) and micro- (different cities) geographical levels. Genetic polymorphism was high ($H_S = 0.274$), and high levels of genetic differentiation existed both between different states ($G_{ST} = 0.317$) and between cities or neighborhoods in each state ($G_{ST} = 0.085–0.265$). These values are higher than those described for any other populations of *A. aegypti*.

KEY WORDS mosquitoes, genetic variability, random amplified polymorphic DNA, population structure

OVER THE LAST FEW years, dengue has become one of the most important public health problems among vector-borne diseases in the Americas. Dengue cases in Brazil represent 80% of the cases occurring in the Americas (Schatzmayr 2000). In the period of January–July 1996, ≈115,000 cases, caused by serotypes 1 and 2, were reported (Da Costa and Natal 1996). Of these, 671 were of the severe form of disease (dengue hemorrhagic fever [DHF]), which caused 26 deaths. Since 1998, reported cases of dengue have increased to 570,148 (Schatzmayr 2000). The increase in the number of autochthonous cases, in addition to the large proportion of states that contained the vector, led to the implementation of the *Aedes aegypti* Eradication Program (PEAa) by the Brazilian Government in 1996.

No vaccines have proven effective against dengue viruses, and no specific chemotherapeutic drugs are available for dengue treatment. Consequently, the susceptible population has little protection against the disease. The only viable way to decrease the incidence of dengue is with integrated vector control measures (Gubler 1989, Rose 2001).

A. aegypti, a mosquito highly adapted to the urban environment, is the major dengue vector in the Americas. It is able to develop and thrive in almost any human settlement, where uncovered containers filled with rainwater or drinking water, provide excellent habitats for the mosquito larvae (Consoli and Oliveira 1994).

Genetic factors are responsible for most of the characteristics that contribute to the success of the insect vectors, such as susceptibility, vector competence, and insecticide resistance. *A. aegypti* has been one of the best-studied species in this respect. The *A. aegypti* dispersal patterns have been extensively investigated in many parts of the world, through estimates of levels of gene flow (Reiter et al. 1995, Edman et al. 1998, Gorrochotegui-Escalante et al. 2000). Knowledge of the mosquito population structure may lead to novel ways of controlling disease transmission (James 1992). However, despite its epidemiological importance, no studies of the genetic population structure of Brazilian *A. aegypti* have been published.

The DNA polymorphism detected by random amplified polymorphic DNA (RAPD) has been used successfully to characterize the genetic structure in several mosquito species (West and Black 1998, Scarpassa et al. 1999) and as a tool to build linkage maps (Mutebi et al. 1997). RAPDs have also been used to estimate the number of families at oviposition sites (Apostol et al. 1993, 1994) and to differentiate related species (Ballinger-Crabtree et al. 1992, Kambhampati et al. 1992). In the current paper, we used RAPD markers to demonstrate the existence of high levels of population structuring of *A. aegypti* in Brazil, both at the macro- (up to 2,600 km) and micro-geographic (within several kilometers) levels.

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Materials and Methods

Sampling. *A. aegypti* samples were collected from 16 sites, from January 1999 to May 2000. The collection sites were selected in a way that facilitated the analysis of gene flow at the macro- and micro-geographic levels (Fig. 1; Table 1). At the macro level, we collected samples in five States of Brazil: Amazonas (northern region of Brazil); Pernambuco, Alagoas, and Sergipe

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Fig. 1. Map of Brazil showing the *A. aegypti* collection sites.

(northeast Brazil); and São Paulo (southeast Brazil). Collections from three adjacent cities in the state of Pernambuco—Olinda, Jaboatão dos Guararapes, and Recife—provided data for micro-geographic comparisons. Three districts were sampled within the city of Recife, in addition to a fourth, a laboratory strain (Graças-CPqAM). An *A. aegypti* population from Orlando, FL, was compared with the Brazilian *A. aegypti* populations. Field mosquitoes were collected from several different natural breeding sites as larvae and pupae to avoid sampling individuals from the same cohort. The larvae and pupae from each site were then pooled and reared, and the adult females stored in liquid nitrogen.

Isolation of Mosquito Genomic DNA. Each mosquito was homogenized in 500 μl of lysis buffer containing 0.4 M NaCl, 2 mM EDTA, 10 mM Tris-HCl (pH 8.0), proteinase K (150 mg/μl), and 1.5% sodium dodecyl sulfate (SDS). The homogenates were incubated at 60°C overnight, and 420 μl of 5 M NaCl were added to the suspension after the incubation. The mixture was gently vortexed for at least 30 s and then centrifuged at 14,000 rpm for 20 min. One volume of isopropanol was added to the supernatant, and the mixture was held at −20°C for 1 h for precipitation of the DNA. The DNA pellet was recovered by centrifugation at 14,000 rpm for 20 min, washed with 70% ethanol, vacuum-dried, and resuspended in 300 μl of sterile TE buffer (10 mM Tris, 1 mM EDTA). Comparison against electrophoresis standards (λ *Hind*III digest) established the DNA concentration in each sample.

Table 1. Origin and number of wild and laboratory samples of *A. aegypti*

State	City	Latitude/longitude	Neighborhood	Sample size
Pernambuco (PE)	Recife	8.05S, 34.88W	Derby	15
			Boa Viagem	15
			Várzea	15
			Graças[a] (CPqAM)	15
	Olinda	8.01S, 34.85W	—	15
	Jaboatão	8.11S, 35.01W	—	15
São Paulo (SP)	Ribeirão Preto	21.17S, 47.81W	—	20
	Baurú	22.31S, 49.06W	—	20
	Araçatuba	21.21S, 50.43W	—	20
Amazonas (AM)	Manaus	3.10S, 60.02W	Cidade Nova	15
			Pq. Laranjeiras	15
Alagoas (AL)	Maceió	9.66S, 35.73W	—	25
	Arapiraca	9.75S, 36.66W	—	20
Sergipe (SE)	Aracaju	10.91S, 37.07W	—	20
	Itabaiana	10.68S, 37.42W	—	20
Florida (FL, USA)[a]	Orlando	28.55N, 81.33W	—	15
Total				280

[a] Laboratory population.

Table 2. Primers used in RAPD analyses, showing number and size of fragments obtained after amplification

Primer	Nucleotide sequence	Number of amplified fragments	Fragment length (base pairs)
G06	5′-GCG GAA ATA G-3′	13	130–1,440
G08	5′-GTC AAC GAA G-3′	11	250–1,800
G09	5′-GAG GAC AAA C-3′	12	290–2,000
G10	5′-GGT ACT CCC A-3′	11	120–2,200

RAPD Analyses. Of the 10 arbitrary primers (Gibco-BRL) tested, four were selected (G06, G08, G09, and G10) based on their reproducibility and efficiency in the polymerase chain reaction (PCR) amplification. We tested the reproducibility of the amplifications using the same DNA sample in three different PCR reactions. The sequences of the oligonucleotide primers are shown in Table 2. Each PCR was carried out in a final volume of 30 μl of 10 mM Tris-HCl (pH 9.0), containing 10 ng of mosquito genomic DNA, 50 mM KCl, 1.5 mM $MgCl_2$, 400 pmol of each primer, 2.0 U of *Taq*DNA polymerase, and 0.2 mM of each dNTP. All amplification reactions were performed in a GENIUS thermocycler (Techne Limited, Cambridge, UK). Amplification proceeded through 40 cycles at 94°C for 1 min, 35°C for 1 min, and 72°C for 2 min, followed by one final extension step at 72°C for 10 min. The PCR products were analyzed by electrophoresis on a 1.5% agarose gel in Tris-borate-EDTA (TBE) buffer (0.089 M Tris; 0.0089 M boric acid, 0.002 M EDTA, pH 8.3) with 5 mg/ml of ethidium bromide, and visualized on an ultraviolet (UV) transilluminator. Negative controls on each set of reactions provided a check for contamination.

Statistical Analyses. Analyses of the RAPD markers depended on the following assumptions: (1) RAPD alleles segregate in a Mendelian fashion; (2) bands that comigrate are homologous; (3) different loci segregate independently; and (4) populations are in Hardy-Weinberg equilibrium. Based on these assumptions, gene frequencies were estimated using the corrections suggested by Lynch and Milligan (1994). The frequencies were used to calculate mean heterozygosity, the population differentiation parameters G_{ST} and θ, and unbiased genetic distances (Nei 1978), using the TFPGA (Miller 1997) and POPGENE (Yeh and Boyle 1997) population genetics programs. Genetic distances between the populations were summarized in an unweighted pair-group method with arithmetic average dendrogram (Sokal and Sneath 1963) for which bootstrap values, based on 1,000 replicates, were added (Felsenstein 1985). Effective migration rates (N_m) were calculated from inbreeding indices (G_{ST}) as $N_m = 0.25\ (1 - G_{ST})/G_{ST}$ (Wright 1978). This method of estimating N_m has been criticized because of its dependence on assumptions that are inappropriate for most natural populations (Whitlock and McCauley 1999). However, it has proven to be fairly robust to violations of those assumptions, covarying positively with direct estimates of migration (Neigel 1997). Therefore, it was used here in a comparative measure with similar estimates obtained for mosquitoes by other authors (Neigel 2002).

Results

Forty-seven markers were distinguished, ranging in size from 120 to 2,200 bp. (Fig. 2; Table 2). Heterozygosities ranged from 0.243 in the state of Pernambuco to 0.300 in the state of Sergipe. Pairwise genetic distances were usually high, varying between 0.047 and

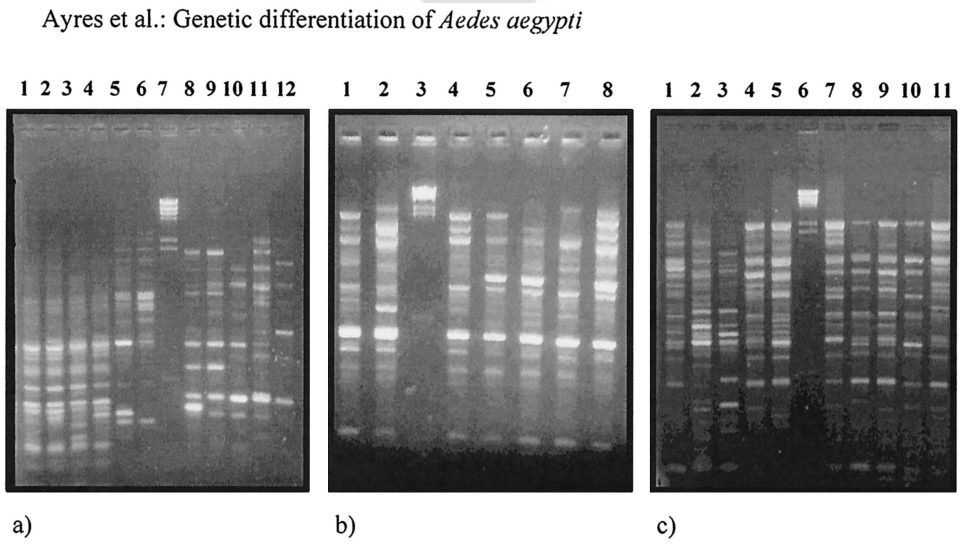

Fig. 2. Amplification products from RAPD. (A) Primer G06 (lanes 1–6) and primer G08 (lanes 8–12), lane 7 = molecular weight marker (λ *Hind*III). (B) Primer G09 (lanes 1, 2, and 4–8), lane 3 = molecular weight marker (λ *Hind*III). (C) Primer G10 (lanes 1–5 and 7–11), lane 6 = molecular weight marker (λ *Hind*III).

Table 3. Diversity, genetic differentiation, and geographic distance among localities in each state of Brazil from where mosquitoes were collected

State	Geographic distance range among samples (K_m)	Genetic differentiation (G_{ST})	Gene flow (Nm)	θ^a	H_s
Pernambuco	1.1–17.0	0.265	0.69	0.315	0.243
São Paulo	189.5–275.0	0.128	1.70	0.155	0.255
Sergipe	55.0	0.085	2.69	0.098	0.300
Amazonas	<10.0	0.144	1.49	0.196	0.285
Alagoas	100.0	0.113	1.63	0.177	0.288
Total		0.317	0.54	0.303	0.274

[a] Lynch and Milligan (1994).

0.381 (data not shown). Similarly, the overall differentiation among the 16 populations was extremely high (θ = 0.303; G_{ST} = 0.317; N_m = 0.54). Intrastate G_{ST} values varied from 0.085 (N_m = 2.69) in the state of Sergipe to 0.265 (N_m = 0.69) in the state of Pernambuco (Table 3). The unweighted pair-group method with arithmetic average dendrogram (Fig. 3) demonstrated two separate clusters. The first represented the populations from the state of Pernambuco (excepting Várzea). The second cluster, representing the remaining populations, was divided further into two smaller clusters. The first consisted of the populations from Orlando, FL and the state of Amazonas. The second cluster was divided into two subgroups, one containing Arapiraca and the remaining populations from São Paulo, and a second sub-group containing the populations from Sergipe and Maceió. The matrices of raw data are available on demand from the authors.

Discussion

The populations of Brazilian *A. aegypti* are highly differentiated (G_{ST} = 0.317; N_m = 0.54) and very polymorphic (H_S = 0.274), indicating that mosquito populations are recruiting locally. At least on a short-term basis, independent eradication programs can operate on a regional basis.

Although the sample sizes per site were relatively small (15–25 mosquitoes per site), unexpectedly high levels of genetic structure were found, even at small geographical scales. High G_{ST} values (0.249; N_m = 0.75) have already been noted among six *A. aegypti* widely separated populations (≈1,000 km apart) in Argentina, using RAPD markers (De Souza et al. 2001). Populations of *A. aegypti* from islands of the French Polynesia also are significantly differentiated (F_{ST} = 0.150; N_m = 1.42), independent of geographical distance (Failloux et al. 1995). Recently, Huber et al. (2002) studied the genetic variation of *A. aegypti* in Ho Chi Minh City, Vietnam, by microsatellites and detected a significant genetic structuring of the species in the city area. Other studies, however, indicate that levels of gene flow between *A. aegypti* populations can be high. In Puerto Rico, for example, allozyme (Wallis et al. 1984) and RAPD (N_m = 11.7; Apostol et al. 1996) studies demonstrated a continuous level of gene flow among *A. aegypti* populations from sites covering ≈100 km. Populations of this species spanning 700 km in Mexico also seem to be genetically very homogeneous (N_m ranging from 5.9 to 19.0 individuals per generation; Gorrochotegui-Escalante et al. 2000). However, it must be cautioned that these rates of gene flow were inferred on the basis of differentiation rates that assumed the island-model population structure

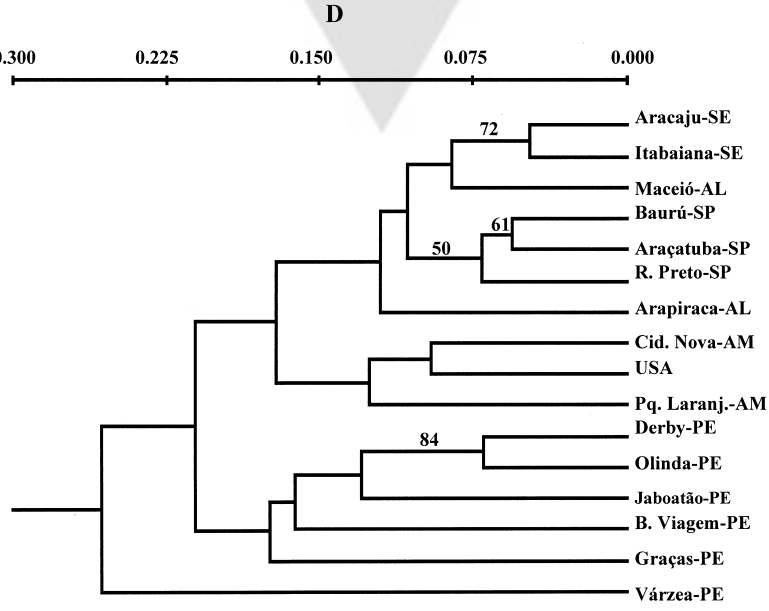

Fig. 3. Dendrogram based on Nei's genetic distances (Nei 1978) among *A. aegypti* populations in Brazil. Bootstrap values over 50%, based on 1,000 permutations are indicated on the nodes. Locations are listed as in Table 1.

and migration-drift equilibrium, a situation that is probably unrealistic for many mosquito populations (Donelly et al. 1999, Fonseca et al. 2001). For example, populations of *Anopheles gambiae* and *Anopheles arabiensis* (Donelly et al. 2001) and of *A. japonicus* (Fonseca et al. 2001) are probably not in equilibrium because of recent population expansions. The main reason for disequilibrium is that populations have not had enough evolutionary time to diverge, so that their similarity will be the result of the inertia of gene frequencies over time, instead of the result of a steady level of gene flow (Avise 2000). In this case, N_m values will usually be overestimated. This study concludes that Brazilian populations of *A. aegypti* are genetically highly differentiated, and this is therefore unlikely to be affected negatively by the possible bias introduced by the lack of migration-drift equilibrium in the populations analyzed.

The average gene diversity within the subpopulations analyzed (H_S) was 0.266, and in the total population (H_T), it was 0.390. These values are similar to those found in *A. aegypti* populations from Puerto Rico ($H = 0.354$; Apostol et al. 1996), Trinidad ($H = 0.390$; Yan et al. 1999), Mexico ($H = 0.339$; Gorrochoteguí-Escalante et al. 2000), and Argentina ($H = 0.350$; De Souza et al. 2001). The *A. aegypti* population from Graças-PE, reared in the CPqAM laboratory for 4 yr, had similarly high levels of heterozygosity ($H = 0.238$) to those observed in wild Brazilian populations (range, $H = 0.193$ in Jaboatão to $H = 0.342$ in Maceió; data not shown). Therefore, RAPD markers can retain much of their variation in laboratory populations, indicating that they may be suitable for the identification of the geographical origin of samples even in laboratory cultures. Indeed, in the RAPD analysis performed here, the laboratory (Graças-CPqAM) population clustered together with the other populations from the state of Pernambuco. Similar results have been obtained in populations of fruit fly (*Ceratitis capitata*), for which the RAPD technique correctly identified the geographical origin of the laboratory population used (Haymer and McInnis 1994, Reyes and Ochando 1998).

The *A. aegypti* populations from the state of Pernambuco were genetically very differentiated ($G_{ST} = 0.265$), despite the small geographical distance between them. The observed differences may result from the frequent and heavy use of insecticides in the area. This treatment may establish a cycle of extinction and recolonization that can, as a consequence of genetic drift, increase the genetic differences between the populations. Similar patterns have been reported for other populations of this species (Tien et al. 1999, Paupy et al. 2000). Note that the population from Várzea, where Temephos treatments occur twice as often as in the other areas (because of the existence of a cemetery with many breeding sites), is also the most differentiated of the Pernambuco populations (Fig. 3).

The high level of genetic differentiation found among the Brazilian samples reveals that the *A. aegypti* population is highly structured compared with populations of the same species in other places of the world. The observed genetic differentiation may reflect important differences of vector competence, parasite susceptibility, or insecticide resistance, so that populations from different areas in Brazil must be treated as independent epidemiological units.

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