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Mitochondrial DNA Polymorphism and Heteroplasmy in Populations of *Aedes aegypti* in Brazil

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ABSTRACT The tropical mosquito *Aedes aegypti* (Diptera: Culicidae) is the most important domestic vector of urban yellow fever and dengue viruses. *Ae. aegypti* originated from Africa and was probably introduced into Brazil during the colonial period through embarkations, and dengue epidemics soon followed. Genetic analysis of 12 *Ae. aegypti* populations from five states in Brazil was conducted based on two mitochondrial DNA fragments: cytochrome oxidase I and NADH dehydrogenase subunit 4. Analyses comparing individual haplotypes indicated the existence of two well-defined clades, probably representing two mitochondrial lineages. Analysis of molecular variance showed significant variability in genetic structure among collections within groups. Mantel regression analysis showed a correlation between genetic and geographic distances, mainly because of northern and northeastern populations, in comparison with those in the southeast. The population from Santos, the largest port in Brazil, showed the greatest diversity, with 10 unique haplotypes, an indication of recent introductions that have not yet spread to other Brazilian cities. Different mitochondrial DNA sequences were found in three specimens, indicating the presence of heteroplasmy.

KEY WORDS *Aedes aegypti*, population genetics, mitochondrial DNA, genetic variability, heteroplasmy

Aedes (Stegomyia) *aegypti* (L.) is a geographically widely distributed mosquito of great epidemiological importance, because it is the major vector involved in the transmission of several debilitating or lethal human arboviruses, including yellow fever and dengue viruses (Chow et al. 1998). Dengue is a mosquito-borne endemoepidemic viral disease, caused by any one of the four dengue serotypes. It is a major health problem in many tropical regions of the world. More than 50 million cases of dengue fever and dengue hemorrhagic fever are reported annually worldwide, although the majority of infections may be asymptomatic (Holmes and Twiddy 2003).

Efforts to control *Ae. aegypti* in the Americas began before the 1940s, when the Pan American Health Organization (PAHO) initiated an eradication program to prevent urban epidemics of yellow fever. The vector was eliminated from 19 countries, including Brazil (Schleissman and Calheiros 1974). Unfortunately, eradication was difficult to sustain, and the reintroduction of this vector insect in Brazil occurred in 1967, with frequent reinvasions since (Monath 1994, Gubler 1997). The direct causes of the present dengue emergency are social and demographic. Population growth and unplanned urbanization have increased the densities of both vector and host (Gubler 1997). Its wide distribution nowadays, infesting up to 3,592 municipalities nationwide (Honório and Lourenço-de-Oliveira 2001), has made dengue infection a major public health problem, with ~40 million cases of dengue fever and several hundred thousand cases of dengue hemorrhagic fever each year. Dengue cases in Brazil represent 80% of those occurring in the Americas (Schatzmayr 2000).

There are many components associated with the epidemiological and transmission cycles of this and other arboviruses transmitted by *Ae. aegypti*, and genetic factors are responsible for most of the characteristics. Studies have shown variation in vector competence between different populations, and consequently, a significant amount of research has focused on genetic diversity within the species (Bennett et al. 2002, Lourenço-de-Oliveira et al. 2002, 2004). Studies of the genetic structure of Brazilian *Ae. aegypti* populations may lead to novel ways of understanding and control of disease transmission.

Mitochondrial DNA (mtDNA) is commonly used for molecular evolution studies in insects (Kambhampati 1995, Tang et al. 1996) and in population genetics studies (Conn et al. 1993, Besansky et al. 1997, Fonseca et al. 2000), because, as a molecular marker, mtDNA has many advantages (Avise 1994, Howell et al. 1996, Jenuth et al. 1996). The *COI* gene is the largest of the

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Table 1. Locations, coordinates, sample sizes, and mitochondrial haplotypes of *Ae. aegypti* collections in Brazil

State	Cities	Latitude/longitude	*N*	Haplotype frequencies
Alagoas	Maceio	09°39′57″ S/35°44′07″ W	10	H1(10)
Ceará	Juazeiro do Norte	07°12′47″ S/39°18′55″ W	10	H1(5), H2(1),[a] H5(3),[a] H6(1)[a]
Mato Grosso Sul	Campo Grande	09°57′28″ S/36°47′30″ W	10	H1(10)
Paraná	Londrina	23°18′37″ S/51°09′46″ W	10	H21(1), H22(1),[a] **H27(8)**
Rondônia	Vilhena	12°44′26″ S/60°08′45″ W	10	H1(2), H25(1), **H27(7)**
Rondônia	Porto Velho	15°38′42″ S/56°05′52″ W	15	H26(4), H27(2), H28(8),[a] **H29(1)**[a]
São Paulo	Barretos	20°33′26″ S/48°34′04″ W	10	H1(1), H19(1),[a] H20(1),[a] H21(1),[a] **H24(6)**[a]
São Paulo	Bauru	22°18′53″ S/49°39′38″ W	10	H7(2), H10(1),[a] **H27(7)**
São Paulo	Botucatu	22°53′09″ S/48°26′42″ W	10	H1(10)
São Paulo	Campinas	28°57′39″ S/51°31′55″ W	10	H1(7), H3(1), H7(1), **H27(1)**
São Paulo	Marlia	22°12′50″ S/49°56′45″ W	10	H1(4), H5(3), H7(1),[a] H8(1),[a] H9(1)[a]
São Paulo	Santos	23°57′39″ S/46°20′01″ W	10	H4(1),[a] H11(1),[a] H12(1),[a] H13(1),[a] H14(1),[a] H15(1),[a] H16(1),[a] H17(1),[a] H18(1),[a] **H23(1)**[a]

Haplotypes in bold represent clade 2 and the others represent clade 1.
[a] Rare haplotypes.
N, sample size used for the COI and ND4 analysis; Hx (*N*), no. of individuals in each haplotype.

three mitochondrial-encoded cytochrome oxidase subunits and the most conserved among the three cytochrome oxidase coding genes in mtDNA (Clary and Wolstenholme 1985, Beard et al. 1993). The NADH dehydrogenase complex (or mitochondrial respiratory complex I) is composed of a large number of subunits, several of which are encoded by mtDNA. Genetic analysis of *Aedes* and *Anopheles* species has been conducted in particular with ND4 (NADH dehydrogenase subunit 4) and ND5 (NADH dehydrogenase subunit 5) mtDNA loci, and these loci have been shown to be highly polymorphic (de Merida et al. 1999, Gorrochoteghi-Escalante et al. 2000, 2002).

mtDNA does not recombine (Hayashi et al. 1985), although some evidence of recombination events has recently been reported (Eyre-Walker et al. 1999, Hagelberg et al. 1999). Individuals are usually homoplasmic for one mitochondrial haplotype, although heteroplasmic conditions have been reported in many species (bats, Wilkinson and Chapman 1991; *Drosophila*, Volz-Lingenhöhl et al. 1992; perch, Nesbø et al. 1998). In *Drosophila*, a small (0.1% or less) but frequent inheritance of sperm mtDNA has been found (Kondo et al. 1992). Studies using sequence analysis of two types of mtDNA in *D. simulans* revealed that two sequences differed by ≈2%, which clearly rules out heteroplasmy produced by a single mutation event and strongly suggests paternal transmission of mtDNA in *Drosophila* (Solignac et al. 1986, Satta and Chigusa 1991).

In Brazil, an early population genetic study using random amplified polymorphic DNA (RAPD) (Paduan et al. 2006) investigated *Ae. aegypti* populations representing certain important commercial routes in five demographic regions spanning six states. The results indicated that the populations were clustered by geographic region and showed isolation by distance. However, this molecular marker showed few genetic variations between cities within the regions. In this study, mtDNA COI and ND4 sequences were used to characterize variation among these samples in Brazil.

Materials and Methods

Mosquito Collection and DNA Extraction. Populations were obtained from 12 cities in the Brazilian states of Alagoas, Ceará, Mato Grosso do Sul, Paraná, Rondônia, and São Paulo from January to March 2002 as part of work for the Brazilian *Aedes* survey Agencies (SUCEN and FNS). A total of 125 individuals were analyzed. The collection sites are listed in Table 1, and the geographic locations of all sampling sites are shown in Fig. 1. Specimens were collected as eggs by ovitraps (usually 500–1,000 ovitraps/city) and were reared to adult stage in insectaries under standardized conditions (25 ± 1°C, 80 ± 10% RH, and 12-h light/dark cycle). The pupae were isolated, and emerging female adult mosquitoes were frozen and stored at −20°C.

Individual female mosquitoes were placed in 1.5-ml microcentrifuge tubes and homogenized with 100 μl lysis buffer (10 mM Tris-HCl, pH 8.0, containing 0.2 M NaCl, 25 mM EDTA, and 0.5% [vol:vol] sodium dodecyl sulfate). DNA was extracted by ethanol precipitation following a modification of the procedure described by Bender et al. (1983). The final DNA pellet was reconstituted in 100 μl of TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0). DNA concentration was estimated by comparison with known amounts of electrophoretic standards (λ *Hind*III digested). DNA working solutions were prepared at a concentration of 10 ng/μl in TE buffer and stored at −70°C.

Polymerase Chain Reaction and Sequencing. DNA samples were used as templates for the amplification of specific fragments of mtDNA: a 450-bp fragment for COI and a 390-bp fragment for ND4. Two sets of primers were used: for COI, Fly5IP (5′-GGATTATTAGGATTTATTGT-3′) and Fly10IP (5′-GCAAATAATGAAATTGTTCT-3′) (Sallum et al. 2002); and for ND4, ND4 F (5′-TGATTGCCTAAGGCTCATGT-3′) and ND4R (5′-TTCGGCTTCCTAGTCGTTCAT-3′), modified by Gorrochoteghi-Escalante et al. (2000). The *COI* gene was amplified with Fly5 primer (representing nucleotides 842–861 in *Anopheles quadrimaculatus* [NC 000875]) and Fly10 primer (repre-

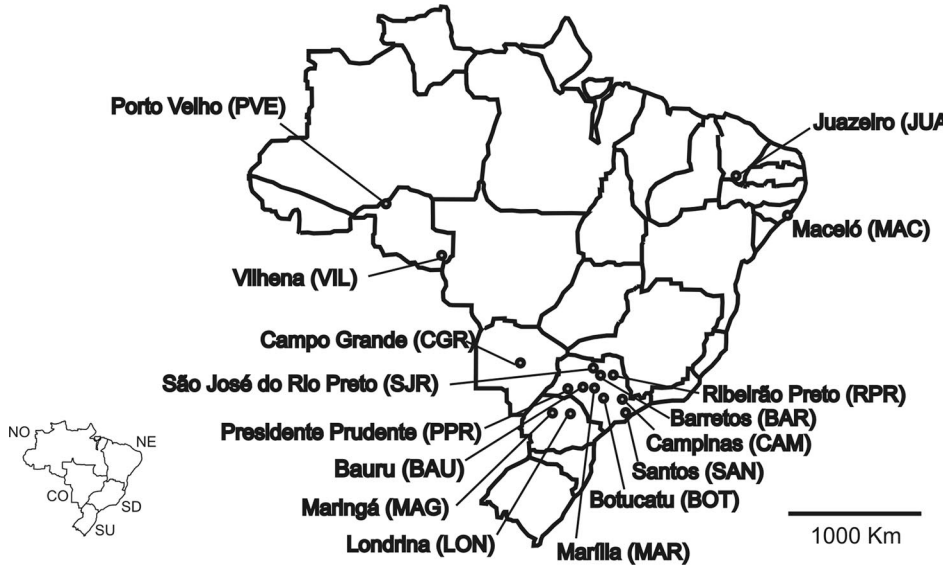

Fig. 1. Map of Brazil showing the *Ae. aegypti* collection sites. A smaller map shows Brazillian Geographic regions (NO, north; NE, northeast; CO, central west; SD, southeast; SU, south).

senting positions 1,371–1,392 in *Drosophila yakuba*). For the *ND4* gene, the amplified region corresponds to nucleotides 8,519–8,880 in *Ae. albopictus* (GenBank no. AY072044).

Each reaction was performed in a Biometra thermal cycler, in a final volume of 25 μl. For the COI and ND4, the polymerase chain reaction (PCR) mixture contained ≈100 ng genomic DNA, 1× buffer, 2 mM $MgCl_2$, 2.5 mM dNTP (GE Healthcare, Bucks, United Kingdom), 20 μM of each primer, and 1.5 U *Taq* polymerase (GE Healthcare). The PCR program involved three cycles of 94°C for 2 min, 37°C for 2 min, 72°C for 1 min, and 35 cycles of 94°C for 30 s, 50°C for 30 s, 72°C for 1 min, and a final extension step of 72°C for 5 min. PCR products were separated by agarose-gel electrophoresis and purified using an Eppendorf DNA Kit. Purified DNA fragments were directly sequenced in an automated DNA sequencer (ABI Prism 377), using the Big Dye Terminator Cycle Sequencing Ready Reaction Kit (PE Applied Biosystems, Foster City, CA). Consensus sequences were obtained through the analysis of the sense and antisense sequences using the MERGER program (http://bioweb.pasteur.fr/seqanal/alignment/intro-uk.html). The CLUSTAL W program (Thompson et al. 1994) with default parameters was used to align.

Statistical Analysis of Mitochondrial Haplotype Frequencies. Variations in haplotype frequencies within and among cities were examined by analysis of molecular variance (AMOVA) (Excoffier et al. 1992) using Arlequin 2.0 (Scheneider et al. 2000). The significance of the variance components associated with each level of genetic structure was computed using a nonparametric permutation test with 1,000 permutations.

For each collection, the nucleotide sequence and the frequency of haplotypes for each collection were entered into DnaSP software, version 3.5 (Rozas and Rozas 1999). The number of polymorphic sites, the average number of nucleotide differences (κ), the nucleotide diversity (π_1), the nucleotide diversity with Jukes and Cantor correction (π_2), the synonymous and nonsynonymous sites, and haplotype diversity (*Hd*) were estimated. The neutrality tests Tajima's D (Tajima 1989) and F* and D* (Fu and Li 1993) were applied to study forces that may be involved in alterations of DNA sequences such as mutations, natural selection, and the combination of both, because the polymorphism pattern in populations is affected by selection (Tajima 1989, Fernandes-Matioli 2001).

Pairwise genetic differences among populations were estimated as N_{ST}, which is similar to F_{ST} but incorporates sequence divergence among haplotypes into the distance estimate. This is equivalent to the infinite-sites model of F_{ST} based on DNA sequence data with the Jukes and Cantor correction applied. Pairwise effective migration rates (Nm, expressed as the number of reproductive migrants per generation) were calculated from N_{ST}. Pairwise N_{ST} values were transformed to $N_{ST}/(1 - N_{ST})$ and regressed on pairwise geographic distances. Geographic coordinates were obtained with a GPS. The geographic distance matrices were constructed using the GenALEx package (Peakall and Smouse 2006) and transformed ($\log_{10}$) geographic distances (km) among populations to compute the Mantel statistics (Mantel and Valand 1970) and to test for isolation by distance. Regression analysis and the Mantel test were performed using Mantel (Rousset 1997).

The relationships between *A. aegypti* haplotypes were inferred in Network Software version 4.2.0.1 (Fluxus Technology, Suffolk, England) to create an unrooted haplotype network, using star contraction.

Table 2. The no. of polymorphic sites and mutation positions of each haplotype in the *COI* and *ND4* genes

	0 0 3	2 2 5	2 4 6	3 9 0	4 0 5	4 4 4	4 5 0	5 0 1	5 4 0	5 6 1	5 7 0	5 7 9	6 1 8	6 4 5	6 6 3	6 9 6	6 9 9	7 0 5	7 1 1	7 4 1	N^a
H01	C	C	C	T	G	C	T	C	A	T	C	G	C	G	C	G	A	T	G	T	49
H02	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	1
H03	.	T	T	C	A	T	.	T	G	C	T	A	T	A	T	A	T	.	A	C	1
H04	T	.	.	.	.	.	C	.	.	.	.	A	.	.	.	.	.	C	A	.	1
H05	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	A	.	6
H06	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	1
H07	.	.	T	C	A	.	.	T	.	C	T	A	T	.	T	A	.	.	A	.	4
H08	.	T	T	C	A	T	.	T	G	C	T	A	T	.	T	A	T	.	A	C	1
H09	.	T	T	C	A	.	C	T	.	C	T	A	T	A	T	A	T	.	A	C	1
H10	.	T	T	C	A	T	.	T	.	C	T	A	T	.	T	A	T	.	A	C	1
H11	T	.	.	.	.	T	.	T	.	.	.	A	.	.	.	.	.	.	A	.	1
H12	.	.	.	C	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	1
H13	.	.	.	.	.	.	C	.	.	.	.	A	.	.	.	.	.	.	.	.	1
H14	T	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	A	.	1
H15	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	A	C	1
H16	T	.	.	.	.	.	C	.	.	.	.	A	.	.	.	.	.	.	A	.	1
H17	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	1
H18	.	.	.	.	.	.	C	.	.	.	.	A	.	.	.	.	.	C	A	.	1
H19	.	T	T	C	A	T	.	T	G	C	T	A	T	.	T	A	.	.	A	C	1
H20	.	.	T	C	A	T	.	T	G	C	T	A	T	.	T	A	.	.	A	C	1
H21	.	T	T	C	A	T	C	.	.	C	.	A	T	A	T	A	T	.	A	C	2
H22	.	T	T	C	A	.	C	.	.	.	.	A	.	.	.	.	.	.	A	.	1
H23	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	C	1
H24	.	T	T	C	A	.	.	T	G	C	T	A	T	.	T	A	T	.	A	C	6
H25	.	T	T	C	A	.	C	T	G	C	T	A	T	.	T	A	T	.	A	C	1
H26	.	T	T	C	A	T	.	T	.	C	T	A	T	.	T	A	.	.	A	C	4
H27	.	.	.	.	A	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	25
H28	.	T	T	C	A	T	.	T	.	C	T	A	T	.	T	A	T	.	A	.	8
H29	.	T	T	C	A	T	.	T	.	C	T	A	T	A	T	A	T	.	A	C	1

The first five polymorphic sites (003, 225, 246, 390, and 405) are for the *COI* gene, and all others are for the *ND4* gene.
[a] No. of individuals in each haplotype.

Results

Sequence Variation. Mitochondrial fragments of the *COI* and *ND4* genes from 125 specimens of *Ae. aegypti* were sequenced. The COI fragment contained 450 nucleotides; however, a sequence made up of 417 sites was used. Five sites were polymorphic, and all were phylogenetically informative. The analyses revealed the existence of seven different haplotypes (GenBank accession numbers AY851649–AY851654), resulting from five transitions. The haplotypes were distinguished by single C ⇔ T transitions at positions 003, 225, 246, and 390 and by a single G ⇔ A transition at position 405. The collections showed an average number of nucleotide differences among individuals (k = 2.042) with the nucleotide diversity (π = 0.00485) and haplotype (gene) diversity (Hd = 0.596).

The ND4 fragment contained 390 nucleotides; however, a sequence made up of 376 sites was used. The alignment of 125 sequences showed that 15 sites were polymorphic, and all were informative. The analyses of gene fragments revealed the existence of 24 different haplotypes (GenBank accession numbers AY906835–AY906853). Fifteen synonymous polymorphic sites were recognized. Haplotypes were distinguished by 14 single C ⇔ T or G ⇔ A transitions and by 1 single A ⇔ T transversion at position 282. The collections showed an average number of nucleotide differences among individuals (k = 6.542) with the nucleotide diversity (π = 0.01740) and haplotype (gene) diversity (Hd = 0.787).

Combined Data and Genetic Analysis. When the combined analysis for the two genes was performed, 20 informative characters were found in 793 bp, and 29 distinct haplotypes were identified among the *Ae. aegypti* populations. The predominance of two haplotypes (H1 and H27) was observed in almost all of the populations in the different geographical areas. Beside these, we found rare or exclusive haplotypes in populations such as Barretos, Juazeiro do Norte, Marília, and Santos. All haplotypes of the Santos population were exclusive. The distributions of haplotypes in populations are listed in Table 1. The genetic parameters obtained for the populations of this species indicate good levels of genetic variability. The collections showed an average number of nucleotide differences among individuals (k = 8.553), with moderate nucleotide diversity (π = 0.0107) and high haplotype (gene) diversity (Hd = 0.800). The number of variable loci and the observed frequencies for each collection and for all mosquitoes are shown in Table 2.

Neutrality tests were applied in all Brazilian populations. The results were statistically significant, Tajima's D (3.692; $P < 0.001$), Fu and Li's D* (1.745; $P < 0.02$), and Fu and Li's F* (2.980; $P < 0.02$), indicating that the populations are in balance in relation to the haplotypes of mtDNA. However, when the tests were repeated only in populations with polymorphic se-

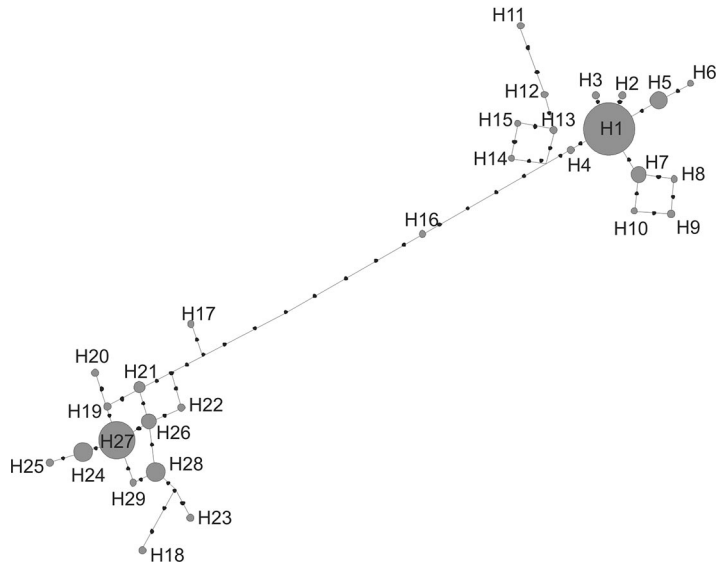

Fig. 2. Statistical parsimony network of 29 *Ae. aegypti* haplotypes of Brazilian populations for COI and ND4 mitochondrial fragments. Black dots along the lines indicate mutation steps between haplotypes (theoretically extinct or unrepresented in the sample). The size of the spheres is proportional to the number of individuals carrying the haplotype.

quences, they deviated from neutrality, which as a whole was caused by the heterogeneity of sequences.

The analysis using a haplotype network based on statistical parsimony resulted in an unrooted cladogram where each mutational step is represented (Fig. 2). Twenty-seven different haplotypes were represented in two main groups, which also supported two clades. These showed high levels of variation: within the first clade, there were 38 polymorphic sites, and within the second clade, there were 33 polymorphic sites. There were eight polymorphic sites between the two clades. The two clades represent two main maternal lineages in Brazil. The first clade accounted for ≈57% of the samples; the most prevalent haplotype was H1, with ≈39% of the individuals. Although high, the level of variation of the clades was much lower than the level of variation of all of the samples. This is reflected in κ, π_1, and π_2 values for the individual clades (Table 3). The Santos population showed the most mutations among individuals: this can be seen in Fig. 2, where the haplotypes are the most distant of the main haplotypes (e.g., H16, H11, H17, and H18) in each respective clade. When the Santos population was removed from the analyses, the indexes decreased mainly in clade 1 compared with those of the entire data set (Table 3).

Gene flow estimates varied widely among populations. Pairwise N_{ST} values ranged from 0 to 0.686, corresponding to an Nm of infinity (identical haplotype frequencies) to 0.23 reprodutive migrants per generation. All of the highest N_{ST} values (>0.900) were contributed by the Londrina and Porto Velho populations. The dendogram based on linearized N_{ST} values identified two distinct, very well-defined clades among the 29 haplotypes detected in Brazilian *Ae. aegypti* populations, with indication of a paraphyletic origin (data not shown).

Pairwise linearized N_{ST} values were regressed against the natural logarithm of the pairwise geographic distances among populations to test for isolation by distance. This analysis indicated a nonsignificant correlation between genetic and geographic distances among *Ae. aegypti* in Brazil ($r^2 = 0.018$, $P = 0.17$). However, when the Mantel test was performed excluding the cities of Botucatu and Londrina, where winter temperatures are lower, there was a positive correlation between geographic localities and genetic diversity ($r^2 = 0.069$, Mantel $P = 0.03$).

Variation in mtDNA sequences was partitioned by AMOVA. When grouped by cities at or near where the groups originated, most (53.09%) of the variation was found among populations within groups ($P < 0.0001$). Within groups, a smaller (25.26%), but highly significant proportion of the variation was found ($P < 0.0001$). This indicates that substantial population structuring occurs within populations at this geographic scale. Among groups, 21.64% of the variation was found, showing significant genetic structuring at this level. The high fixation indices ($F_{ST} = 0.74738$) also showed the significance of genetic structuring when different urban populations were compared.

Analysis of the 125 chromatograms revealed the existence of at least three individuals containing two different ND4 sequences. Sequences in both directions showed the existence of a polymorphism at the same site. All three cases of heteroplasmy occurred at the polymorphic sites described in this study.

Table 3. Variability estimates in the COI and NADH mitochondrial fragments among *Ae. aegypti* populations in Brazil

Locality	Polymorphic sites	k[a]	π_1[a]	π_2[a]
Northeast/north				
Juazeiro do Norte	3	0.933	0.00118	0.00118
Maceio	0	0	0	0
Vilhena	17	6.266	0.00790	0.00801
Porto Velho	2	0.857	0.00108	0.00108
West central				
Campo Grande	0	0	0	0
Southeast				
Londrina	3	0.755	0.00095	0.00095
Barretos	17	4.311	0.00544	0.00549
Bauru	16	7.355	0.00928	0.00940
Botucatu	0	0	0	0
Campinas	17	3.711	0.00468	0.00474
Marlia	4	1.488	0.00188	0.00188
Santos	18	7.733	0.00975	0.00984
Clade 1				
Haplotypes H1–H16	12	1.235	0.00156	0.00156
Clade 1 without Santos	6	0.602	0.00076	0.00076
Clade 2				
Haplotypes H17–H29	13	1.586	0.00200	0.00201
Clade 2 without Santos	7	1.246	0.00157	0.00157
All samples	20	8.553	0.01079	0.01092

[a] κ, avg. no. of differences; π_1, nucleotide diversity; π_2, nucleotide diversity with Jukes and Cantor correction.

Discussion

Different studies of *Ae. aegypti* populations throughout the world have shown that breeding structure varies geographically. The goal of this study was to use two mitochondrial loci to characterize the pattern of genetic differentiation and population structure of this species in Brazil. The results reported here suggest several hypotheses regarding the colonization history and current population dynamics of *Ae. aegypti* in Brazil.

Nucleotide diversity (π) in the *ND4* mitochondrial gene in *Ae. aegypti* was significantly greater than π in the mitochondrial *COI* gene. This difference may arise because of higher constraints on the mutation rate (i.e., balancing selection) in the *COI* gene. Alternatively, the higher diversity rate in the *ND4* gene may be caused by the existence of two well-supported clades in *Ae. aegypti*, which were detected in Mexican populations (Gorrochotegui-Escalante et al. 2002). Compared with studies of ND4 from Mexico, the nucleotide diversity was similar to the Brazilian northeastern region (0.01434) (Gorrochotegui-Escalante et al. 2000), the Yucatan Peninsula (0.2161), and the Mexican northeastern region (0.1636) (Gorrochotegui-Escalante et al. 2002).

Analysis of ND4 sequences from *Ae. aegypti* in Brazil revealed the presence of 24 different haplotypes, whereas 25 haplotypes were detected in Mexico (Gorrochotegui-Escalante et al. 2002). There was a similar number of polymorphic sites (16–24), although with two well-supported clades, differing from the structure observed in Mexico. Members of clade 1 were present in the basal lineage identified in the Mexico study, and clade 2 members were grouped into the derived lineage. The presence of two major clades may support the hypothesis presented by the Mexico and Thailand studies, in that two lineages present in both regions evolved before the introduction of this mosquito from its ancestral source, presumably in North Africa (Tabachnick 1991). However, combined analysis of the *COI* and *ND4* genes indicated two distinct lineages without a common origin. In our case, the combined analysis provided a larger number of informative sites and explanatory power for the sequences. The existence of these distinct mitochondrial lineages in *Ae. aegypti* may support the existence of distinct, historic, matriarchal lineages within the Brazilian population. This suggests that different mitochondrial lineages were introduced into Brazil or represent a recent introgression of a new maternal lineage. These results are also consistent with the analysis of the collections from Mexico (Gorrochotegui-Escalante et al. 2000).

Northeastern Brazil is more arid than other regions, with extended periods of drought. In this study, these populations did not seem to be strongly genetically isolated by distance from any other. However, a previous study by our group using RAPD markers showed that *Ae. aegypti* populations from northeastern Brazil seem to be strongly genetically isolated by distance from all the others, but are genetically homogeneous within the region. Similar results were obtained in northeastern Mexico, when analyses were performed using RAPD and mitochondrial markers (Gorrochotegui-Escalante et al. 2002). The patterns of diversity identified in this study suggest that haplotypes among *Ae. aegypti* in Brazil vary by region. Mosquitoes in northeastern Brazil contained only 4 of the 29 haplotypes that have been detected thus far. Furthermore, in this study, genetic diversity was low among northeastern populations.

Data from RAPDs of the same samples (Paduan et al. 2006) showed no indication of recent introductions of *Ae. aegypti*, because there was good correlation between geographic and genetic data. Populations from the northeast seem to be partially isolated from the rest of the country. In contrast to what was observed with RAPD analysis, this study showed indications of different introductions in Santos, which contains 10 exclusive haplotypes (H07, H12–H19, and H22), suggesting multiple introductions that have not yet spread to the rest of the Brazilian territory.

When the haplotype diagram was analyzed, two major haplotypes (H1 and H27) were observed. Most of the variations from these two haplotypes, apart from H11–H18, were seen principally in Santos, where certain haplotypes showed four or five mutation steps from the two main groups (H1 and H27), indicating different introductions. Because Santos is the largest port in Latin America, these variants may represent recent introductions that have not yet spread to the rest of the country.

Unique haplotypes were also found in Barretos (H19, H20, H21, H24), Marilia (H08, H09), Bauru (H10), Campinas (H03), Londrina (H22), Porto Velho (H26, H28, H29), and Vilhena (H25). Haplotype H01 was the most common haplotype, found in eight populations, and was most frequent in southeastern, northeastern, and central-western populations. Haplotype H27 was the second most common haplotype, found in four populations, and was most frequent in southeastern, southern, and northern populations.

Genetic diversity among southeastern *Ae. aegypti* populations also varied greatly, showing high diversity between cities. Gene flow was low among southeastern populations ($Nm = 0.96$), and some populations situated <250 km apart shared no common haplotypes. The results showed significant population structuring within these populations; however, the Mantel analysis showed no isolation by distance in populations in the state of São Paulo. Human movements can transport mosquitoes between cities, thus increasing genetic similarity among geographically distant populations in São Paulo state. In Mexico, over distances of 100–300 km, the populations remained genetically uniform, and at distances >300 km, the populations seemed to be isolated. In that case, collections within 130 km of each other in northeastern Mexico and within 180 km in the Yucatan showed no barrier to gene flow (Gorrochotegui-Escalante et al. 2002). This pattern suggests that gene flow among populations decreases with increasing geographic distance.

Analysis of populations also revealed the existence of several genetically distinct populations. These included CGR, BTU, and MAC, all of which showed low genetic variability, with the presence of a unique haplotype (H1). This is consistent with the hypothesis that these populations were established by few mosquitoes and that a substantial bottleneck occurred during or after the founding of these populations. Genetic bottlenecks in local populations could be generated by vector-control efforts, thus affecting their genetic similarity with other populations. Mantel analysis only showed significant correlation between genetic and geographic distances when the Botucatu and Londrina populations were excluded. These two cities have the coldest winters of all the localities analyzed, with temperatures as low as 0°C, which may also lead to bottlenecks in *Ae. aegypti* populations. No correlation between southeastern populations occurred. This result is consistent with results for the *Ae. aegypti* populations in Mexico, where populations from the Yucatan and the Pacific coastal region showed genetic isolation by distance. The present AMOVA results were highly consistent, with 53% of the variation found among populations within groups. More importantly, variation among populations within cities showed a high degree of genetic differentiation at this spatial scale. Genetic structure at this level was highly significant ($P < 0.001$).

Taken together, the analysis of mtDNA in Brazilian populations of *Ae. aegypti* showed that dispersion of the vector seems not to be correlated with human activity, and the island model could be used to explain population structure. New mosquito introductions are not significant in the cities analyzed, except for Santos, where the only exclusive haplotypes were found. These introductions in Santos have not penetrated Brazil by land, another indication of low rates of mosquito dispersal.

In some specimens (3 of 125), the presence of more than one sequence of mtDNA was found within cells, a phenomenon called heteroplasmy. Most of the heteroplasmy observed in insects consist of length variation of the AT-rich control region of the mtDNA (Solignac et al. 1986, Satta and Chigusa 1991). This region is characterized to be rich in tandem repeats that can vary in length by slipped-strand mispairing during replication (Solignac et al. 1986, Buroker et al. 1990, Hayasaka et al. 1991, Lewis et al. 1994). In this case, the occurrence of mutation within the individual could be the mechanism for the heteroplasmy.

The occurrence of two distinct mitochondrial gene sequences in one individual could be explained by three factors: sequencing of a nuclear pseudo-gene, mutation within the mtDNA within the cells, or paternal leakage of mitochondria. The sequence of a pseudo-gene would produce polymorphism in different sites that we observed in mtDNA gene. Because the polymorphism that we found occurs at the same point where we observed polymorphism in Brazilian populations, we believe that paternal leakage is the most parsimonious explanation for the observed heteroplasmy. It is possible that this event was underestimated, because sequencing is not the best way to reveal this kind of heteroplasmy in the mitochondrial genome.

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