Online journal ISSN - 1676-5680
GMR Genetics and Molecular Research
EVOLUTION AND TECHNOLOGY

# Reduced levels of genetic variation in *Aedes albopictus* (Diptera: Culicidae) from Manaus, Amazonas State, Brazil, based on analysis of the mitochondrial DNA *ND5* gene

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Genet. Mol. Res. 8 (3): 998-1007 (2009)
Received April 7, 2009
Accepted June 7, 2009
Published August 18, 2009

**ABSTRACT.** *Aedes albopictus*, a mosquito originally from Southeast Asia, is considered to be one of the main vectors of dengue fever, yellow fever and other arboviruses. We examined the genetic variability and population structure of 68 individuals of *Ae. albopictus* collected from five neighborhoods of the city of Manaus, based on the mitochondrial gene coding for NADH dehydrogenase subunit 5 (*ND5*). Two haplotypes were found, separated by a single mutational event (T ↔ C), with extremely low levels of genetic variability ($h = 0.187 \pm 0.059$; $\pi = 0.00044 \pm 0.00014$). Based on AMOVA, we concluded that most of the variation (99.08%) occurred within populations, though the levels of variation were not significant. Neutrality tests (Tajima's *D* and Fu's *Fs*) were non-significant, indicating that these populations are in genetic equilibrium. The most frequent haplotype (H1) is restricted to Brazilian populations of *Ae. albopictus*, while the rarer haplotype (H2) is shared with populations from the United States and Asia. We suggest that the reduced variability and low genetic structure identified in our study is a

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consequence of the recent introduction of this species in Manaus, possibly through a founder effect, followed by expansion throughout the city neighborhoods. Genetic similarity would therefore be due to insufficient time to have accumulated genetic differences between the populations of *Ae. albopictus* and not to extensive gene flow among them.

**Key words:** Dengue; Population genetics; Mitochondrial gene; Vector control

## INTRODUCTION

*Aedes albopictus*, commonly known as the "Asian tiger mosquito", originated from Southeast Asia (Forattini, 1986), where it is often found in peri-urban, rural and forested areas (Mousson et al., 2005). This species has spread from its native range to different regions around the globe, largely through the international trade in used tires (Reiter, 1998; Benedict et al., 2007). In the 1980s, *Ae. albopictus* was introduced to the Americas (Mitchell, 1995; Urbanelli et al., 2000), where it has been progressively expanding. Furthermore, in temperate zones *Ae. albopictus* has developed a photoperiodic egg diapause and freezing tolerance (Hawley et al., 1987).

In Brazil, *Ae. albopictus* was registered for the first time in 1986 in the States of Rio de Janeiro, Minas Gerais, and São Paulo, and in 1987, in the State of Espírito Santo. This species was probably introduced in Brazil through the international transport of used tires and, since then, has been largely expanding to other Brazilian States (Consoli and Lourenço-de-Oliveira, 1994). In 2006, *Ae. albopictus* had already been registered in 22 of the 27 Brazilian States (Castro-Gomes et al., 2008). In the State of Amazonas, the first record of *Ae. albopictus* occurred in the municipality of Tabatinga, in June 1996, where there it was temporarily eradicated. In 1997, this species was again collected in that municipality and also in Leticia (Southeast of Colombia), a city situated across the border of the State of Amazonas, Brazil (Vélez et al., 1998). In the same year, *Ae. albopictus* was registered in Terra Nova (periphery of Manaus) (Sá, 2004), and in 2002, it was captured in an urban area in Manaus (Fé et al., 2003). Nowadays, this species is present throughout the city; however, its frequency has been higher in more forested areas.

*Ae. albopictus* is an important dengue vector in Japan, Indonesia, the Seychelles, Thailand, Malaysia, and Hawaii (Hawley et al., 1987; Mitchell, 1995). On the American continents, this species has never been involved in dengue outbreaks; however, it was found naturally infected with dengue virus in Mexico (Ibanez-Bernal et al., 1997). In Brazil, the serotype DENV-1 virus was isolated from larvae of *Ae. albopictus* collected in the field in Campos Altos, State of Minas Gerais (Serufo et al., 1993). Nevertheless, it was never implicated in dengue fever transmission (Forattini, 2002).

Nonetheless, because *Ae. albopictus* can colonize peri-urban, rural and forested areas, health authorities are concerned about the possibility of this species becoming a link between the wild and urban transmission cycles of yellow fever virus (Consoli and Lourenço-de-Oliveira, 1994; Lourenço de Oliveira et al., 2003). Recent studies have shown that in several areas of the States of Goiás and Minas Gerais (Brazil), where wild yellow fever and an epizootic in monkeys have been recorded, adults of *Ae. albopictus* were found (Castro-Gomes et al., 2008).

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Mitochondrial DNA (mtDNA) shows a faster evolution rate compared to nuclear DNA; it has predominantly maternal inherence, and therefore does not undergo genetic recombination (Brown et al., 1979; Avise, 1994). This marker, because of these characteristics, has been widely used in studies of population genetics and evolutionary relationships of many groups of organisms (Avise, 1994; Spanos et al., 2000). *ND5* (coding for subunit 5 of NADH dehydrogenase) is one of the most variable genes of mtDNA and has been studied in several species of the *Anopheles* and *Aedes* genera (Besansky et al., 1997; Birungi and Munstermann, 2002; Molina-Cruz et al., 2004). Polymorphic analyses of the *ND5* gene in American and Asian populations of *Ae. albopictus* showed haplotypes restricted to Brazil, suggesting a single introduction of this species in the country (Birungi and Munstermann, 2002; Mousson et al., 2005).

Thus, the aim of this study was to determine the genetic variability and structure of five wild populations of *Ae. albopictus* from the city of Manaus, using polymorphic analysis of the mtDNA *ND5* gene.

## MATERIAL AND METHODS

### Sampling

The samples of *Ae. albopictus* were collected from five neighborhoods of the city of Manaus, State of Amazonas, Brazil: Coroado, Cidade de Deus, Cidade Nova, Compensa, and Mauazinho (Figure 1). Larvae and pupae were captured through inspection of natural and artificial breeding sites and also using oviposition traps. Subsequently, the specimens of each breeding site were transferred to separate containers and transported to the insectary of the Laboratory of Population Genetics and Evolution of Mosquito Vector, at the National Institute for Research in the Amazon (INPA), in Manaus, State of Amazonas, Brazil. Larvae were fed fish food and wheat germ. Upon reaching the 4th-instar larvae and adults, they were identified according to Forattini (2002), and frozen at -80°C until genomic DNA extraction. The geographic locations, coordinates and sample sizes are described in Table 1.

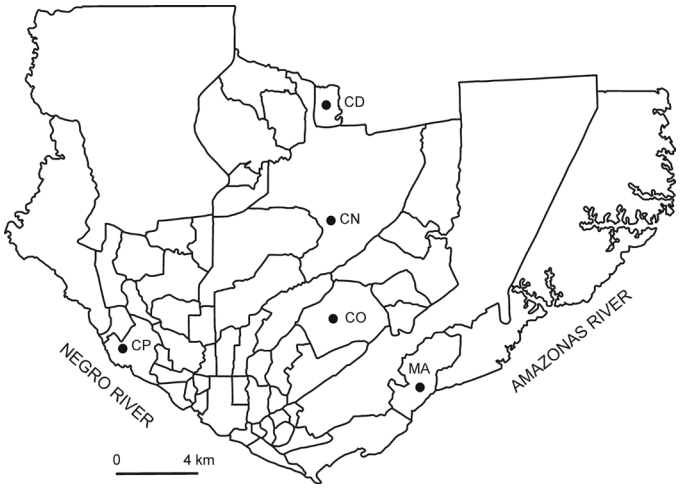


**Figure 1.** Collection sites of *Aedes albopictus*. Abbreviations of localities are in Table 1.

**Table 1.** Geographic localization of the populations of *Aedes albopictus* analyzed in Manaus.

| Locality | Region | Abbreviation | Coordinates | Sample size |
|---|---|---|---|---|
| Coroado | East/Central-Southeast | CO | 03°05'S, 59°59'W | 13 |
| Cidade de Deus | North | CD | 03°00'S, 59°56'W | 16 |
| Cidade Nova | North | CN | 03°02'S, 09°04'W | 13 |
| Mauazinho | East | MA | 03°07'S, 59°55'W | 13 |
| Compensa | West | CP | 03°06'S, 60°03'W | 13 |

## DNA extraction, PCR amplification, and sample sequencing

Genomic DNA was extracted individually from 4th-instar larvae and/or adults, as described by Collins et al. (1987). The primer sequences (forward and reverse) and amplification conditions were according to the method described by Birungi and Munstermann (2002). Subsequently, polymerase chain reaction (PCR) products were checked on 1% agarose gel electrophoresis, stained with ethidium bromide and analyzed under UV light. Following purification, the PCR purified products were then sequenced using the DYEnamic™ ET dye Terminator kit (GE Healthcare, UK), following manufacturer recommendations. Subsequently, they were sequenced in an automated MegaBACE 1000 Analysis System sequencer. DNAs of all individuals were sequenced in both directions.

## Statistical analysis

The sequences were aligned using the BioEdit (Hall, 1999) and Chromas programs (Griffith University, Queensland, Australia). For each population, the haplotype (*h*) and nucleotide ($\pi$) diversities, number of polymorphic sites (*S*), mean number of nucleotide differences (*K*) and Tajima's *D* (Tajima, 1989) and Fu's *Fs* (Fu, 1997) neutrality tests were calculated using the DnaSP program (Rozas et al., 2003). The Arlequin program (Excoffier et al., 2006) was used to calculate the values of $F_{ST}$ and *Nm* (genetic distances and gene flow, respectively), analysis of molecular variance (AMOVA) and the correlation between genetic and geographical distances, by the Mantel test. A haplotype network was generated based on the parsimony method using the TCS program (Clement et al., 2000). To infer the evolutionary relationships of the haplotypes obtained in the present study, we made comparisons with sequence haplotypes available at GenBank [Accession number: AJ971016-AJ971028, Birungi and Munstermann (2002); AY049968-AY049975, Mousson et al. (2005)].

# RESULTS

## Distribution and haplotype frequency

In this study, 68 individuals of *Ae. albopictus* were analyzed using a 430-bp fragment of the *ND5* gene. Two haplotypes were observed, which are separated by a single transition (C ↔ T) at position 209 from our input file. The frequencies of the haplotypes in five studied populations can be seen in Table 2. Haplotype 1, the most frequent (89.71%), was found in all populations. Haplotype 2, was observed in seven individuals (10.29%), which was present in

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four of the five populations analyzed. The haplotypes found in this study were included in the network (Figure 2) together with the haplotypes found by Birungi and Munstermann (2002) in *Ae. albopictus* populations from Brazil, United States, Madagascar, Malaysia, Indonesia, and Japan, and by Mousson et al. (2005) in populations from Brazil, Vietnam, United States, Cambodia, Madagascar, France, Hawaii, Réunion, and Thailand. The origin of each haplotype of this network is shown in Table 3. Figure 2 shows that haplotype 2, the rarer in our study, is the most spread throughout the world (Table 3) and is probably the older.

**Table 2.** Haplotype frequencies in *Aedes albopictus* populations of Manaus.

| Population | H1 | H2 |
|---|---|---|
| CO | 11 | 2 |
| CD | 15 | 1 |
| CN | 12 | 1 |
| MA | 10 | 3 |
| CP | 13 | 0 |
| Total | 61 | 7 |

H1 and H2 = haplotypes 1 and 2. For abbreviation of populations, see Table 1.

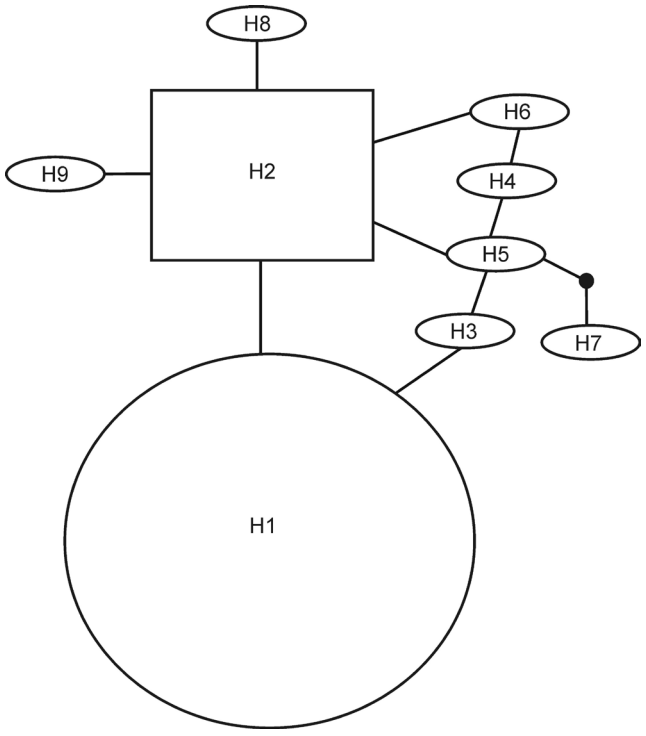


**Figure 2.** Haplotype network of *Aedes albopictus* generated using the haplotypes (H) of this study and those from previous studies. The solid circle represents a mutational event.

**Table 3.** Distribution of the haplotypes shared between the present study and previous studies for *ND5* gene in *Aedes albopictus*.

| Haplotype | Occurrence | References |
|---|---|---|
| **H1** | CO, CN, CD, MA, CP | Present study |
| | Anita Garibaldi, Praia de Fora, Jacarepaguá (Brazil) | **H1** named by Birungi and Munstermann (2002) |
| | Represa do Cigano and São Luís (Brazil) | Mousson et al. (2005) |
| **H2** | CO, CN, CD, MA | Present study |
| | Jacksonville (USA), 3D Salvage (USA) | **H3** named by Birungi and Munstermann (2002) |
| | AAA Salvage (USA), Oslo Mall (USA), Atlanta (USA), Malaysia | |
| | Hanoi (Vietnam), Jacksonville (USA), Chiang Mai (Thailand), Seam Reap (Cambodia), Diego Suarez (Madagascar), MontSecret and Naintré (France), Nha Trang (Vietnam), Oahu (Hawaii), and La Possession and La Providence (Réunion) | Mousson et al. (2005) |
| **H3** | Anita Garibaldi and Praia de Fora (Brazil) | **H2** named by Birungi and Munstermann (2002) |
| **H4** | 3D Salvage and Jacksonville (USA) | Birungi and Munstermann (2002) |
| **H5** | 3D Salvage (USA) | Birungi and Munstermann (2002) |
| **H6** | Oslo Mall (USA) | Birungi and Munstermann (2002) |
| **H7** | 3D Salvage and Jacksonville (USA) | Birungi and Munstermann (2002) |
| **H8** | Madagascar | Birungi and Munstermann (2002) |
| **H9** | Madagascar | Birungi and Munstermann (2002) |

For abbreviations of populations, see Table 1.

## Genetic diversity and neutrality tests

Table 4 shows the values of genetic diversity and neutrality tests (Tajima's *D* and Fu's *Fs*) for each population analyzed. The mean values of haplotype (*h*) and nucleotide ($\pi$) diversities were 0.187 ± 0.059 and 0.00044 ± 0.00014, respectively. The sample from Mauazinho showed the highest level of genetic variability, whereas the sample from Compensa did not display variation. The low levels of genetic variability as well as those of *S* and *K* are due to the presence of two haplotypes, separated by a single mutational event. The two neutrality tests were not statistically significant ($P < 0.05$) for all five populations, despite the negative values for the samples of Cidade de Deus and Cidade Nova.

**Table 4.** Genetic diversity and neutrality tests calculated for the five populations of *Aedes albopictus* from Manaus.

| Population | NH | S | *K* | *h* | $\pi$ | Tajima's *D* test | Fu's *Fs* test |
|---|---|---|---|---|---|---|---|
| CO | 2 | 1 | 0.154 | 0.282 ± 0.0201 | 0.00066 ± 0.00033 | -0.274 | 0.240 |
| CD | 2 | 1 | 0.125 | 0.125 ± 0.106 | 0.0001 ± 0.00025 | -0.491 | 0.035 |
| CN | 2 | 1 | 0.154 | 0.154 ± 0.126 | 0.00036 ± 0.00029 | -1.150 | -0.537 |
| MA | 2 | 1 | 0.385 | 0.385 ± 0.132 | 0.00089 ± 0.00031 | 0.426 | 0.689 |
| CP | 1 | 1 | 0.000 | 0.000 ± 0.000 | 0.00000 ± 0.00000 | ND | ND |
| Total | 2 | 1 | 0.187 | 0.187 ± 0.059 | 0.00044 ± 0.00014 | 0.518 | 0.315 |

NH = haplotype number; *S* = number of polymorphic sites; *K* = mean number of nucleotide differences; *h* = haplotype diversity; $\pi$ = nucleotide diversity; ND = not determined. The level of significance for neutrality tests was $P > 0.05$. For abbreviation of populations, see Table 1.

## Analysis of molecular variance and genetic differentiation

AMOVA was performed to determine the origin of genetic variability in the different hierarchical levels. In this analysis, all populations were considered to be as a single group (not clustered). The results showed that almost all observed variation (99.08%) occurred within populations, whereas less than 1% of variation ($F_{ST}$ = 0.009; P > 0.05) occurred among the populations (data not shown).

The genetic differentiation among the populations is represented by pairwise estimates of $F_{ST}$ and *Nm* values, respectively (Table 5). These results indicated low $F_{ST}$ values and extensive gene flow among the populations. All pairwise $F_{ST}$ values were not statistically significant. Populations from Mauazinho and Compensa had the highest level of genetic differentiation ($F_{ST}$ = 0.167; *Nm* = 2.5) of all populations, which can be attributed to the presence and absence of H2 in Mauazinho and Compensa, respectively.

**Table 5.** Estimates of genetic distance ($F_{ST}$) and gene flow (*Nm*), above and below diagonal, respectively, among the populations of *Aedes albopictus* from Manaus.

| Population | CO | CD | CN | MA | CP |
|---|---|---|---|---|---|
| CO | - | -0.028 | -0.052 | -0.063 | 0.083 |
| CD | Inf. (10.87) | - | -0.073 | 0.046 | -0.014 |
| CN | Inf. (7.69) | Inf. (4.06) | - | 0.011 | 0.000 |
| MA | Inf. (5.73) | 10.4 (14.73) | 45.5 (10.09) | - | 0.167 |
| CP | 5.5 (8.34) | Inf. (14.03) | Inf. (10.9) | 2.5 (13.49) | - |

Inf. = infinity; geographic distances (km) are within parentheses. Number of permutations = 1054. For abbreviations of populations, see Table 1.

## DISCUSSION

The present study revealed only two haplotypes, which was equivalent to the number of haplotypes found for the *ND5* gene in populations of *Ae. albopictus* in other localities in Brazil (Birungi and Munstermann, 2002; Mousson et al., 2005). In this study, genetic variability ($h = 0.187 \pm 0.059$; $\pi = 0.00044 \pm 0.00014$) was extremely low when compared to studies of other species of the family Culicidae, that used the *ND5* gene (Molina-Cruz et al., 2004; Temu and Yan, 2005). According to Nei et al. (1975), low levels of genetic diversity could be related to a long and/or repeated bottleneck effect or founder effect, which both provide the reduction of genetic variation through genetic drift. Therefore, we think that the reduced levels of genetic variability found in populations of *Ae. albopictus* from Manaus may be attributed to a founder effect (i.e., populations founded by few individuals) (Davies et al., 1999; Martocq and Villablanca, 2001), which resulted in the loss of genetic variability through genetic drift, followed by the recent expansion of this species throughout neighborhoods of city (Fé et al., 2003). Furthermore, the recent expansion of this mosquito in Manaus may also explain the high *Nm* values and low levels of genetic variation found in these samples. Consequently, the low genetic structure observed here may be due to an insufficient time for accumulating genetic differences among populations of *Ae. albopictus* in Manaus, and not due to the extensive gene flow presently occurring among them. In *Ae. albopictus* populations from the south and southeast coast of Brazil, with the use of *ND5* gene, genetic diversity ($\pi = 0.0002$) was lower

than that found in the present study (Birungi and Munstermann, 2002). Similarly, Kambhampati and Rai (1991) and Kambhampati et al. (1991) also observed low genetic variation in 17 populations of *Ae. albopictus* from Asia, using RFLP-mtDNA makers. These authors suggested a recent expansion of this species in that region.

Tajima's *D* neutrality test was non-significant for all populations analyzed, indicating that these populations are in genetic equilibrium (Kimura, 1983). Tajima's *D* test performed by Birungi and Munstermann (2002) was also non-significant. Fu's *Fs* neutrality test, proposed to detect population growth, also did not show significant values, not providing, therefore, evidence that *Ae. albopictus* populations in Manaus are expanding.

Haplotype network suggests that H1 is restricted to Brazilian populations, supporting the finding of Birungi and Munstermann (2002). H2, however, the rarer haplotype of this study, is the one more widely distributed throughout the world and is probably the ancient haplotype. This haplotype network also suggests recent expansion of this mosquito across the world, as discussed in previous studies.

In relation to the possible origin of the populations of *Ae. albopictus* in Manaus, H1, found in higher frequency in all populations (89.71%), was also observed in populations from the south and southeast Brazilian coast by Birungi and Munstermann (2002), and in the city of São Luis (State of Maranhão) and in the municipality of Represa do Cigano (State of Rio de Janeiro) (Mousson et al., 2005). H2, the less frequent in the present study, is shared with haplotypes found in populations from the coast of Florida, in the USA (Birungi and Munstermann, 2002), and in populations from Hanoi (Vietnam), Chiang Mai (Thailand), Seam Reap (Cambodia), Deigo Suarez (Madagascar), Montsecret and Naintre (France), Nha Trang (Vietnam), Oahu (Hawaii), and La Possession and La Providence (Réunion) (Mousson et al., 2005). This distribution shows that, although H2 was the less frequent haplotype in Manaus, it is more widely distributed in the world than H1.

The presence of these two haplotypes suggests that there were at least two independent introduction events of *Ae. albopictus* in the city of Manaus. One of these introductions would have probably occurred through populations that originated along the south and southeast Brazilian coast, and the other probably through populations from the coast of Florida. Haplotype 1 was also found in São Luis, which could represent another possible site of introduction. This hypothesis, however, is less probable, since the official record of *Ae. albopictus* in that city is more recent than the one for the State of Amazonas (La Corte dos Santos, 2003). These results contest the hypothesis of Mousson et al. (2005) who suggested that there was a single introduction of *Ae. albopictus* to Brazil and that Brazilian and Asian populations of *Ae. albopictus* are paraphyletic. Birungi and Munstermann (2002) also suggest the occurrence of a single introduction of *Ae. albopictus* in Brazil and in the United States. Nonetheless, the presence of H1 and H2 in this study suggests the occurrence of more than one introduction in the city of Manaus due to the fact that these haplotypes probably originated from distinctive localities.

However, the hypothesis of more than one introduction in Manaus can be better elucidated with use of multiple markers (nuclear and mitochondrial) and a larger range of samples of *Ae. albopictus*.

## ACKNOWLEDGMENTS

The authors thank Henrique Bleise de Carvalho (INPA) for help with the mosquito

collections in the field. The authors also thank the Tematic Laboratory of Molecular Biology team at INPA for MegaBace sequencing and for help in the sequencing of automated electroinjections. Research supported by the Brazilian agency "Fundação de Amparo à Pesquisa do Estado do Amazonas - FAPEAM" and Ministry of Science and Technology - MCT/INPA, Brazil. R.T. Maia obtained a Master's degree through the Graduate Program in Genetics, Conservation and Evolutionary Biology at INPA, and received a fellowship from CNPq, and L.H. Maciel-Litaiff was an undergraduate student, and received a fellowship from FAPEAM.

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