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Genetic differentiation in populations of *Aedes aegypti* (Diptera, Culicidae) dengue vector from the Brazilian state of Maranhão

Andrelina Alves de Sousa[a,b], Elmary Fraga[a], Iracilda Sampaio[c], Horacio Schneider[c], Maria Claudene Barros[a,*]

[a] *Universidade Estadual do Maranhão, Centro de Estudos Superiores de Caxias (CESC), Caxias, MA, Brazil*
[b] *Universidade Estadual do Maranhão, Centro de Ciências Agrárias, São Luís, MA, Brazil*
[c] *Universidade Federal do Pará (UFPA), Instituto de Estudos Costeiros/IECOS, Bragança, PA, Brazil*

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ABSTRACT

Aedes (*Stegomyia*) *aegypti* is the vector responsible for the transmission of the viruses that cause zika, yellow and chikungunya fevers, the four dengue fever serotypes (DENV – 1, 2, 3, 4), and hemorrhagic dengue fever in tropical and subtropical regions around the world. The present study investigated the genetic differentiation of the 15 populations of this vector in the Brazilian state of Maranhão, based on the mitochondrial ND4 marker. A total of 177 sequences were obtained for *Aedes aegypti*, with a fragment of 337 bps, 15 haplotypes, 15 polymorphics sites, haplotype diversity of $h = 0.6938$, and nucleotide diversity of $\pi = 0.01486$. The neutrality tests (*D* and *Fs*) were not significant. The AMOVA revealed that most of the variation (58.47%) was found within populations, with $F_{ST} = 0.41533$ ($p < 0.05$). Possible isolation by distance was tested and a significant correlation coefficient ($r = 0.3486$; $p = 0.0040$) was found using the Mantel test. The phylogenetic relationships among the 15 haplotypes indicated the existence of two distinct clades. This finding, together with the population parameters, was consistent with a pattern of genetic structuring that underpinned the genetic differentiation of the study populations in Maranhão, and was characterized by the presence of distinct lineages of *Aedes aegypti*.

Introduction

Aedes (*Stegomyia*) *aegypti* is a mosquito native to Africa, which is now found throughout the tropical and subtropical regions of the world (Chow et al., 1998). This species is the vector responsible for the transmission of the viruses that cause zika, yellow and chikungunya fevers, the four dengue fever serotypes (DENV – 1, 2, 3, and 4), and hemorrhagic dengue fever. This mosquito is mainly urban in distribution, and transmits viruses through the bite of infected females. The intimate relationship of the species with humans is an important factor determining outbreaks and epidemics (Lima Júnior and Scarpassa, 2009; Twerdochlib et al., 2012; Yáñez et al., 2013). Given this relationship, eradication of dengue fever, in particular, is one of the principal challenges faced by public health authorities worldwide, one that has increased in recent years, with the resurgence and dispersal of the vector over a wide geographic area. Economic and social factors, such as the ongoing expansion of unplanned urban development, population movements, and the resistance of the vector to insecticides, have all contributed to the propagation of dengue fever during recent years (Urdaneta-Marquez et al., 2008). *A. aegypti* was considered to have been eradicated from Brazil in 1955, according to the Pan-American Health Organization (PAHO), based on its program for the eradication of urban yellow fever in the Americas. This program was interrupted in 1960, however, and in 1976, the vector was recorded in the state of Bahia, followed by Rio de Janeiro, in 1977 (Scarpassa et al., 2008). In the first half of 2016, the Brazilian Health Ministry recorded 1117 high risk cases of dengue fever in Brazil, of which, 91 were critical, and 51, fatal (Brasil, 2016). In the state of Maranhão, ten high risk cases were reported, with one critical case, and one death. A total of 3748 suspected cases of Chikungunya fever were also reported, of which, 284 were confirmed, 48 by laboratory testing, and 236 by clinical-epidemiological diagnosis, while 3281 are still under investigation. Laboratory tests have also confirmed the autochthonous transmission of the Zika virus by *A. aegypti* in 22 Brazilian states since April 2015. While this disease is generally considered to be benign, more severe symptoms have recently been recorded in French Polynesia and Brazil, including the debilitation of the Central Nervous System (Guillain-Barré syndrome, transverse myelitis, and meningitis), highlighting how little

* Corresponding author.
E-mail: mbdene@yahoo.com.br (M.C. Barros).

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is still known about the Zika virus (Oehler et al., 2014; Campos et al., 2015).

Given the epidemiological importance of the species, molecular studies can provide important insights into the dynamics of *A. aegypti* populations, in particular for the understanding of their differences in their efficiency as vectors, resistance to insecticides, and the ecological adaptations of the species (Bracco et al., 2007; Paupy et al., 2012; Fraga et al., 2013). The analysis of the genetic differentiation of *A. aegypti* populations will thus be essential for the identification of new lineages and the dispersal mechanism that may have a significant effect on the spatial distribution of outbreaks of dengue fever (Silva et al., 2012).

Considering the continuing presence of the vector in the Brazilian state of Maranhão, the present study investigated the genetic characteristics of the local *Ae aegypti* populations and analyzed population dynamics in the context of the circulation of a number of dengue serotypes which represent a latent potential for the occurrence of major epidemics.

Material and methods

Origin and collection of samples

Specimens (eggs) were obtained using egg traps, which were set in areas adjacent to domestic residences and retrieved after five days. The eggs were taken to the Genetics and Molecular Biology Laboratory at the Caxias Center for Higher Studies (CESC/UEMA), where they hatched and the larvae were raised in artificial containers (separated by municipality) until the emergence of the adults for identification using Consoli and Lourenço-de-Oliveira's classification key (1994). The adults were then transferred to entomological cages (one for each municipality) for mating.

The adults were fed with a 10% sucrose solution, maintained in the cage. After two meals, 20 females were isolated in plastic cups to lay their eggs, and 20 of these clutches were separated in individual artificial hatcheries for the hatching of the eggs. The larvae were then fed until the fourth stage of development, based on the protocol of Santos et al. (1981). The larvae were then frozen (−20 °C) for subsequent extraction of the genomic DNA, for which the larvae were selected randomly from different clutches to ensure a lack of relatedness between specimens (Table 1).

Extraction of the DNA, and the amplification and sequencing of the ND4 gene

The total DNA was extracted using the protocol developed by Wilkerson et al. (1995). Following extraction, the DNA was visualized in a 1% agarose minigel. The amplification of the ND4 gene from the total DNA was conducted using the PCR technique, with the conditions and primers described by Costa-da-Silva et al. (2005), in which the forward primer was ND4L: 5′-ATTGCCTAAGGCTCATGTAG-3′ and the reverse primer was ND4H: 5′-TCGGCTTCCTAGTCGTTCAT-3′. The PCR products were purified with ExoSAP-IT, following the manufacturer's recommendations. The DNA of the purified products was sequenced using the dideoxyterminal method (Sanger et al., 1977) with a Big Dye Terminator v.3.1 Cycle Sequencing Ready Reaction kit (Applied Biosystems). The product was precipitated and the unincorporated coloring was removed by washing before the samples were sequenced in a DNA ABI 3500 automatic sequencer. All samples were sequenced in both directions.

Table 1
Municipalities sampled in the state of Maranhão, Brazil.

Population	Coordinates	Sample size (N = F1)
Balsas	7°31′58.50″ S, 46°02′14.84″ W	10
Caxias	4°53′14.15″ S, 43°20′23.36″ W	13
Fortuna	5°43′23.43″ S, 44°09′29.55″ W	10
Imperatriz	5°31′53.9″ S, 47°29′10.19″ W	17
Mirador	6°21′42.93″ S, 44°20′56.31″ W	06
Parnarama	5°40′08.83″ S, 43°05′58.21″ W	08
Pedreiras	4°34′29.41″ S, 44°35′56.72″ W	10
Rosário	2°56′24.00″ S, 44°14′26.88″ W	09
São Bernardo	3°21′40.96″ S, 42°25′08.10″ W	12
São Mateus	4°02′25.94″ S, 44°28′05.72″ W	11
Timon	5°05′59.03″ S, 42°50′15.15″ W	13
Paço do Lumiar[a]	2°32′37.87″ S, 44°05′31.85″ W	10
Raposa[a]	2°25′57.38″ S, 44°05′07.39″ W	16
S.J. de Ribamar[a]	2°33′47.45″ S, 44°03′45.23″ W	09
São Luís[a]	2°33′28.84″ S, 44°14′43.92″ W	23
Total		177

[a] Fraga et al. (2013).

Population and phylogenetic analyses

The sequences were edited using the BIOEDIT program, version 7.0.5.2, (Hall, 1999) and aligned in CLUSTAL W (Thompson et al., 1994), using Ribeiro et al.'s (2007) complete sequence of the *A. aegytpi* ND4 gene as a reference (1–1344 pb, Genbank # DQ440274), as well as Behura et al.'s (2011) sequence of the complete mitochondrial genome (Genbank # NC_010241).

The number of haplotypes and polymorphics sites, haplotype (h) and nucleotide diversity (π), Tajima's (1989) neutrality test (D) and Fu's (1997) *Fs*, which provide information on selective neutrality in natural populations, were all run in DNAsp version 5.0 (Librado and Rozas, 2009). The ARLEQUIN program, version 3.01 (Excoffier et al., 2006) was used to estimate the genetic distance and gene flow, based on the F_{ST} values, as well as a hierarchical analysis to estimate inter- and intra-population genetic differentiation, based on an Analysis of Molecular Variance (AMOVA), with different hierarchical levels. For this, the specimens were arranged in predefined groups based on non-genetic criteria, such as geographic, ecological or environmental variables.

The F_{ST} was used to estimate the genetic structure of the populations. Genetic isolation by distance was estimated by Mantel's (1967) method, which provides the significance of the correlation between a genetic matrix based on the F_{ST} values and a matrix of geographic distance (km) using the IBDWS in the site IBDWS (http://ibdws.sdsu.edu/~ibdws/distances.html) (Jensen et al., 2005).

The origin of the different haplotypes was inferred through the construction of a haplotype network in the HAPLOVIEWER program (Salzburger et al., 2011). The genetic divergence within and between populations was determined by the p distance, obtained for the corrected Tamura and Nei (1993) parameters in MEGA version 6.0 (Tamura et al., 2013). In the specific case of the ND4 gene, Corander et al.'s (2013) Bayesian grouping was also run in BAPS 6.0, to identify possible population clusters.

The phylogenetic relationships among the haplotypes were inferred. The most adequate evolutionary model was determined by the maximum likelihood ratio test, run in MEGA version 6.0 (Tamura et al., 2013). The significance of the clusters was estimated by a bootstrap analysis, with 1000 replicates (Felsenstein, 1985). Two haplotype sequences of the ND4 gene – from *Aedes albopictus* (Costa et al. (2006): Genbank # EF153761) and *Anopheles marajoara* (Wilkerson et al. (2005): Genbank # AY846347) – were included as outgroups.

The haplotypes identified in the present study were also compared with those available in GenBank. These sequences included those derived from a study the other regions of Brazil, such as Amazonia (Lima Júnior and Scarpassa, 2009: #EU650405–EU650417) and Paraná (Twerdochlib et al., 2012: #JN089748–JN089755), as well as the study of Paduan and Ribolla (2008) – #AY906835–AY906853. Further afield, sequences

were obtained from Mexico (Gorrochotegui-Escalante et al., 2002: #AF334842–AF334865) and Peru (Costa-da-Silva et al., 2005: #DQ177153–DQ177155), as well as Bracco et al.'s (2007) research in the Americas (#DQ176828–DQ176831), Africa (#DQ176833–DQ176843), and Asia (#DQ176845–DQ176849).

Analysis of the quality of the ND4 sequences

The quality of the chromatograms was evaluated using the DNA Sequencing Analysis Software, version 5.1 (Applied Biosystems/www.appliedbiosystems.com), and a BlastN survey of the Vector Base (http://www.vectobase.org) was conducted to verify the possible correlation between the haplotype sequences and the regions of the nuclear mitochondrial DNA (NUMTs). Over the past few years, the presence of insertions of the nuclear genome in mitochondrial sequences (NUMTs) has been reported in an increasing number of studies (Black and Bernhardt, 2009; Behura et al., 2011; Leite, 2012; Moore et al., 2013; Fraga et al., 2013). The sequences containing NUMTs may be amplified by PCR together with those of the target mtDNA, thus generating misleading results in population studies based on molecular markers (Paupy et al., 2012).

In the present study, however, no NUMTs were identified, so the sequences analyzed represent real mitochondrial DNA lineages. This conclusion is reinforced by the ample occurrence of haplotypes H2 and H3, which correspond to H1 and H20 from Mexico (Gorrochotegui-Escalante et al., 2002), and were validated as true haplotypes rather than NUMTs (Black and Bernhardt, 2009). Haplotypes H1 and H2 from São Luís Island (Fraga et al., 2013) also corresponded to H1 and H2 from Mexico (Gorrochotegui-Escalante et al., 2002).

Results

Frequency of haplotypes and polymorphism of the ND4 gene

Samples were obtained from 11 municipalities in the Brazilian state of Maranhão, which were complemented with 58 sequences taken from GenBank (Fraga et al., 2013: access numbers #KF922333–KF922342), derived from four populations (São Luís, Paço do Lumiar, Raposa, and São José do Ribamar) located on São Luís Island in Maranhão (Fig. 1).

A total of 177 sequences were analyzed from the *A. aegypti* specimens from Maranhão. The fragments sequenced had a total of

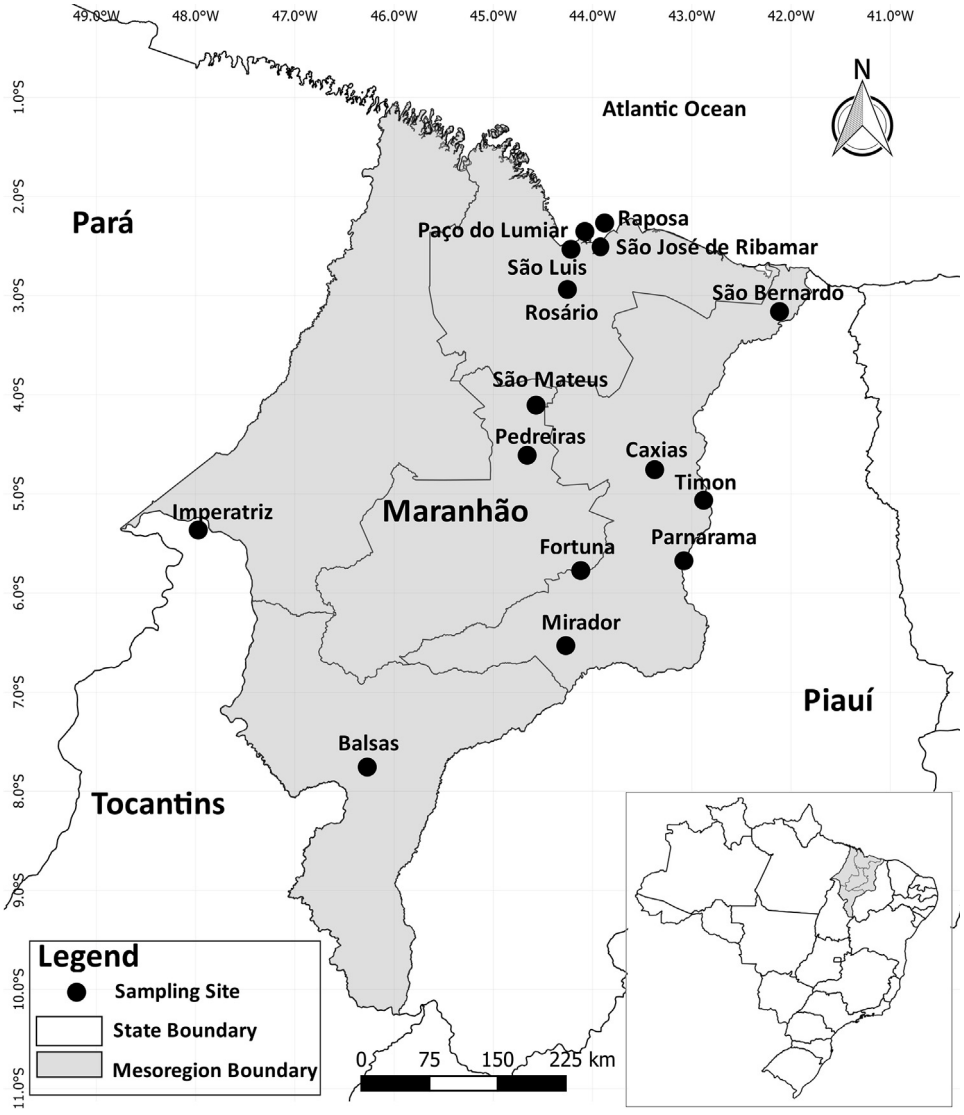

Fig. 1. Map of the Brazilian state of Maranhão, showing the municipalities in which the *Aedes aegypti* specimens were collected.

Table 2
Number of haplotypes, polymorphic sites, and frequency of haplotypes of the ND4 mitochondrial marker identified in the *Aedes aegypti* populations from the Brazilian state of Maranhão.

Haplotypes	Polymorphic sites 111111 22222 2558011266 14469 3049309847 25800	Frequency	Populations[a]
H1	TTTGTCTAAT TATAC	46	Bal, Cax, Imp, Mir, Par, Ped, SBer, SMat, Tim, Rap.
H2	.C.A.TCG.C CGAGT	85	Bal, Cax, Fort, Imp, Mir, Ped, Ros, SBer, SMat, Tim, PLu, Rap, SJR, SLu.
H3	.C.A.TCG.C CGAG.	14	Cax, Fort, Par, Ros, SBer, SJR, SLu
H4	.C...T.G.C CGAGT	1	Fort
H5	....A..	2	Fort, Par
H6	.....G.	1	Imp
H7	...A..... ..A.T	7	Ped
H8	.C.A.TCG.C CGA..	3	Ros, SLu
H9	.C...TCG.C CGAG.	1	SMat
H10	.C.A..C..C C.AGT	10	PLu, Rap
H11	...A.TCG.C CGA..	1	SJR
H12	.C.A.TCG.. CGA..	1	SJR
H13	.CAACTCG.. CGA..	1	SJR
H14	.CAACTCG.. CGAG.	1	SJR
H15	AC.A.TCG.C CGAGT	3	SLu

[a] Populations: Bal, Balsas; Cax, Caxias; Fort, Fortuna; Imp, Imperatriz; Mir, Mirador; Par, Parnarama; Ped, Pedreiras; Ros, Rosário; SBer, São Barnardo; SMat, São Mateus; Tim, Timon (PLu, Paço do Lumiar; Rap, Raposa; SJR, São José de Ribamar; SLu, São Luís; Fraga et al., 2013).

337 base pairs (bps), corresponding to sites 8374 through 8711 of the mitochondrial genome of *A. aegypti*, see GenBank #NC_010241 (Behura et al., 2011). A total of 15 haplotypes and 15 polymorphic sites were identified in the study populations (Table 2).

The H2 haplotype was the most common (f = 85), representing 48.02% of the specimens analyzed, and occurring in all populations except that from Parnarama. The second most common haplotype was H1 (f = 46, 25.98%), which was found in all populations except Fortuna, Rosário, Paço do Lumiar, Raposa and São Luís. All the other haplotypes were far less frequent. The third most common, H3 (f = 14, 7.90%) was recorded only in the Caxias, Fortuna, Parnarama, Rosário, São Bernardo, São José de Ribamar and São Luís populations, while H7 (f = 7, 3.95%) was exclusive to Pedreiras. Haplotype H8 (f = 3, 1.69%), occurred in Rosário and São Luís, while H15, with the same frequency (f = 3) was exclusive to São Luís. Haplotype H5 (f = 2, 1.12%) occurred in Fortuna and Parnarama, while H10, with the same frequency (f = 2) occurred in Paço do Lumiar and Raposa. haplotypes H4, H6, H9, H11, H12, H13, H14 and H15 were all unique, being recorded in Fortuna, Imperatriz, São Mateus, and São José de Ribamar, respectively (Table 2).

Haplotype diversity for the present study population as a whole was h = 0.6938, while nucleotide diversity was π = 0.01486. When each population was considered separately, haplotype diversity (h) ranged from 0.318 in the São Bernardo population to 0.889 in São José de Ribamar, while nucleotide diversity (π) varied between 0.00167 in São Luís to 0.01741 in Mirador. Neither of the neutrality tests (D and Fs) produced significant results ($p > 0.05$), whether for the population as a whole or each local population individually, indicating that the observed polymorphism was consistent with the neutral mutation model (Table 3).

Haplotypes shared with other studies

While it was the second most common haplotype, H1 was not recorded in the populations from Fortuna, Rosário, Paço do Lumiar, Raposa or São Luís. Even so, this haplotype was recorded in Amazonia, as H6 (Lima Júnior and Scarpassa, 2009) and in Paraná, as H3 (Twerdochlib et al., 2012).

Haplotype H2 is shared with other populations in Maranhão and other regions of Brazil (Paduan and Ribolla, 2008), corresponding to haplotype H10 in Amazonia (Lima Júnior and Scarpassa, 2009), H4 in Paraná (Twerdochlib et al., 2012), as well as other parts of the world, such as Mexico (H1) (Gorrochotegui-Escalante et al., 2002), Peru (H2) (Costa-da-Silva et al., 2005), and H5 in the Americas, Africa, and Asia (Bracco et al., 2007).

Haplotype H3 was identified as H2 on other parts of Brazil (Paduan and Ribolla, 2008), as well as in the Paraná (Twerdochlib et al., 2012) and in Mexico as H20 (Gorrochotegui-Escalante et al., 2002).

Haplotype H7 was recorded as H1 in Paraná (Twerdochlib et al., 2012) and Amazonia (Lima Júnior and Scarpassa, 2009), and H11 in other regions of Brazil (Paduan and Ribolla, 2008), H15 in the Americas, Africa and Asia (Bracco et al., 2007), and H3 in Peru (Costa-da-Silva et al., 2005). Haplotype H8 corresponds to H4 from São Luís Island (Fraga et al., 2013). The haplotypes H4, H5, H6, H8, H9, H10, H11, H12, H13, H14 and H15 have not been observed in previous studies (Table 4).

Analysis of Molecular Variance (AMOVA) and phylogenetic relationships

The genetic differentiation of the populations was investigated using AMOVA. When all the populations were analyzed

Table 3
Genetic diversity and the results of the neutrality tests for the 15 *Aedes aegypti* populations from Maranhão, Brazil, analyzed in the present study.

Population	Genetic diversity		Tajima's D	Fu's Fs
	h	π		
Balsas	0.356	0.01161	0.02622	6.473
Caxias	0.564	0.01248	1.98116	6.194
Fortuna	0.644	0.00765	−1.20010	1.176
Imperatriz	0.522	0.01475	1.50245	7.000
Mirador	0.533	0.01741	1.31709	6.057
Parnarama	0.607	0.01304	0.68813	3.614
Pedreiras	0.511	0.01022	−0.51119	3.536
Rosário	0.556	0.00264	0.71533	0.134
São Bernardo	0.318	0.00584	−1.91083	2.269
São Mateus	0.345	0.00680	−1.67815	2.524
Timon	0.513	0.01674	2.39502	9.447
Paço do Lumiar[a]	0.533	0.00475	1.83053	3.338
Raposa[a]	0.567	0.00999	0.05886	4.792
S. J. de Ribamar[a]	0.889	0.00742	0.57782	1.603
São Luís[a]	0.447	0.00167	−0.78905	−1.176
Total	0.695	0.01486	2.37285	1.877

[a] Fraga et al. (2013), h, haplotype diversity; π, nucleotide diversity.

Table 4
Distribution of haplotypes of the ND4 mitochondrial marker identified in the *Aedes aegypti* populations from the Brazilian state of Maranhão and haplotypes shared with other studies.

H[a]	Populations															Other studies					
	Bal[b]	Cax	Fort	Imp	Mir	Par	Ped	Ros	SBer	SMat	Tim	PLu*	Rap*	SJR*	SLu*	PR	AM	BR	AAA	Peru	Mex
H1	8	8	–	11	4	5	1	–	1	1	5	–	2	–	–	H3	H6	–	–	–	–
H2	2	1	6	5	2	–	2	6	10	9	8	4	10	3	17	H4	H10	–	H5	H2	H1
H3	–	4	2	–	–	2	–	1	1	–	–	–	–	2	2	H2	–	H2	–	–	H20
H4	–	–	1	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
H5	–	–	1	–	–	1	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
H6	–	–	–	1	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
H7	–	–	–	–	–	–	7	–	–	–	–	–	–	–	–	H1	H1	H11	H15	H3	–
H8	–	–	–	–	–	–	–	2	–	–	–	–	–	–	1	–	–	–	–	–	–
H9	–	–	–	–	–	–	–	–	–	1	–	–	–	–	–	–	–	–	–	–	–
H10	–	–	–	–	–	–	–	–	–	–	–	6	4	–	–	–	–	–	–	–	–
H11	–	–	–	–	–	–	–	–	–	–	–	–	–	1	–	–	–	–	–	–	–
H12	–	–	–	–	–	–	–	–	–	–	–	–	–	1	–	–	–	–	–	–	–
H13	–	–	–	–	–	–	–	–	–	–	–	–	–	1	–	–	–	–	–	–	–
H14	–	–	–	–	–	–	–	–	–	–	–	–	–	1	–	–	–	–	–	–	–
H15	–	–	–	–	–	–	–	–	–	–	–	–	–	3	–	–	–	–	–	–	–

[a] H, haplotypes.
[b] The codes are those used in the respective studies for the same haplotype.
Bal, Balsas; Cax, Caxias; Fort, Fortuna; Imp, Imperatriz; Mir, Mirador; Par, Parnarama; Ped, Pedreiras; Ros, Rosário; SBer, São Barnardo; SMat, São Mateus; Tim, Timon; (PLu*, Paço do Lumiar; Rap*, Raposa; SJR*, São José de Ribamar; SLu*, São Luís; Fraga et al., 2013); PR, Paraná (Twerdochlib et al., 2012); AM, Amazonia (Lima Júnior and Scarpassa, 2009); BR, Brazil (Paduan and Ribolla, 2008); AAA, Americas, Africa, and Asia (Bracco et al., 2007); Peru (Costa-da-Silva et al., 2005); Mex, Mexico (Gorrochotegui-Escalante et al., 2002).

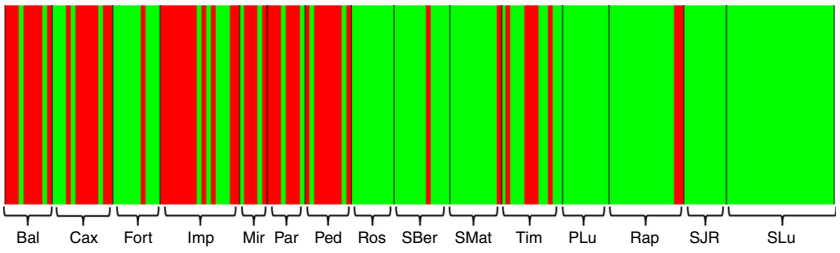

Fig. 2. A *priori* estimate of the probable groups of populations produced by the BAPS (Bayesian Analysis of Population Structure v 6.0) program, indicating a total of two groups.

as a single group (Maranhão state), an F_{ST} value of 0.38939 was recorded ($p < 0.05$), with 38.94% of the variation being found among populations, and 61.06% within populations (Table 5), although this situation shifted when the populations were classified by geographic mesoregion, i.e., North (Rosário, Paço do Lumia, São José de Ribamar, Raposa, and São Luís), Center (Fortuna, Pedreiras, and São Mateus), West (Imperatriz), East (Caxias, Parnarama, São Bernardo, and Timon), and South (Balsas and Mirador), with an F_{ST} value of 0.41533 ($p < 0.05$), and 21.34% of the variation among populations, 20.20% among populations within each of the five groups (North, Center, West, East, and South), and 58.47% within populations (Table 6).

Based on pairwise F_{ST} indices, genetic differentiation ranged from −0.012 (Raposa vs. São Luís) to 0.813 (Balsas vs. São Luís), with $p < 0.05$, with the lowest mean inter-population divergence being found between the Rosário and São Luís populations (0.002), and the highest mean being observed between the populations from Balsas and São Luís (0.027). The lowest mean intra-population genetic divergence was recorded in São Luís (0.0016) and the highest (0.018) in the Mirador population (Table 7). The Bayesian inference (BAPS) indicated the existence of two ($K = 2$) population clusters (Fig. 2). Even so, the correlation coefficient for the Mantel test ($r = 0.3486$, $p = 0.0040$) was significant, indicating a isolation by distance (Fig. 3).

The analysis of the haplotypes produced a non-rooted haplotype network, with 15 well-defined clusters, with the size of the circle and the number within each circle corresponding to the frequency of occurrence of each haplotype in the state of Maranhão (Fig. 4).

The phylogenetic relationships among the 15 haplotypes were analyzed in the context of other haplotypes obtained from studies of populations from Brazil and other parts of the world. The resulting tree has two distinct clades, with clade I encompassing four haplotypes (H1, H5, H6, and H7) from the present study, including the second most common type (H1) and one (H6) that was unique to the Imperatriz population. Clade II includes the remaining 11 haplotypes (H2, H3, H4, H8, H9, H10, H11, H12, H13, H14 and

Table 5
Analysis of Molecular Variance (AMOVA) of the *Aedes aegypti* populations from the Brazilian state of Maranhão.

Type of variation	Component of the variation	Variation (%)	F_{ST}	P^a
Among populations	1.00187	38.94	0.38939	<0.05
Within populations	1.57106	61.06		

[a] Value calculated based on 1023 random permutations.

Table 6
Analysis of Molecular Variance (AMOVA) of the five groups (North, South, Center, East, West) of the *Aedes aegypti* populations from the Brazilian state of Maranhão.

Type of variation	Component of the variation	Variation (%)	F_{ST}	P^a
Among populations	0.57333	21.34	0.41533	<0.05
Among populations within groups	0.54269	20.20		
Within populations	1.57106	58.47		

[a] Value calculated based on 1023 random permutations.

Table 7
Pairwise F_{ST} values (above the diagonal) and the media genetic divergence interpopulation (below the diagonal) and intrapopulation (diagonal bold) in *Aedes aegypti* populations from the Brazilian state of Maranhão.

Populations	Balsas	Caxias	Fortuna	Imperatriz	Mirador	Parnarama	Pedreiras	Rosário	S. Bernardo	S. Mateus	Timon	P. Lumiar	Raposa	S. J. Ribamar	S. Luís
Balsas	**0.012**	0.328	0.209	0.549	0.058	0.085	0.040	0.781	0.680	0.370	0.016	0.802	0.655	0.745	0.813
Caxias	0.014	**0.016**	0.185	0.448	0.085	0.070	0.109	0.585	0.482	0.167	0.123	0.561	0.404	0.458	0.613
Fortuna	0.025	0.020	**0.007**	0.020	0.122	0.012	0.010	0.428	0.398	0.050	0.023	0.481	0.342	0.349	0.521
Impertriz	0.013	0.015	0.023	**0.015**	0.466	0.264	0.264	0.551	0.497	0.199	0.283	0.615	0.461	0.502	0.643
Mirador	0.014	0.015	0.021	0.015	**0.018**	0.018	−0.017	0.674	0.557	0.212	−0.025	0.678	0.503	0.582	0.708
Parnarama	0.012	0.014	0.023	0.014	0.014	**0.013**	−0.088	0.540	0.454	0.086	−0.072	0.546	0.387	0.423	0.592
Pedreiras	0.015	0.017	0.020	0.016	0.016	0.015	**0.010**	0.570	0.418	0.110	−0.089	0.582	0.423	0.463	0.620
Rosário	0.026	0.021	0.005	0.024	0.022	0.025	0.021	**0.002**	0.157	0.204	0.595	0.220	0.194	0.109	0.230
S. Bernardo	0.026	0.021	0.007	0.023	0.022	0.024	0.020	0.004	**0.006**	0.129	0.510	−0.073	−0.043	−0.122	−0.060
S. Mateus	0.025	0.021	0.007	0.023	0.022	0.024	0.021	0.005	0.006	**0.007**	0.142	0.177	0.068	0.030	0.230
Timon	0.020	0.018	0.014	0.019	0.018	0.019	0.018	0.014	0.014	0.014	**0.017**	0.616	0.456	0.505	0.649
P. Lumiar	0.024	0.021	0.009	0.022	0.021	0.023	0.017	0.007	0.008	0.008	0.014	**0.004**	−0.071	−0.124	−0.098
Raposa	0.023	0.020	0.009	0.022	0.021	0.023	0.018	0.008	0.008	0.009	0.015	0.008	**0.010**	−0.177	−0.012
S. J. Ribamar	0.026	0.021	0.008	0.024	0.023	0.024	0.022	0.006	0.008	0.008	0.015	0.011	0.011	**0.007**	−0.100
S. Luís	0.027	0.022	0.005	0.025	0.023	0.026	0.021	0.002	0.004	0.004	0.014	0.006	0.007	0.006	**0.001**

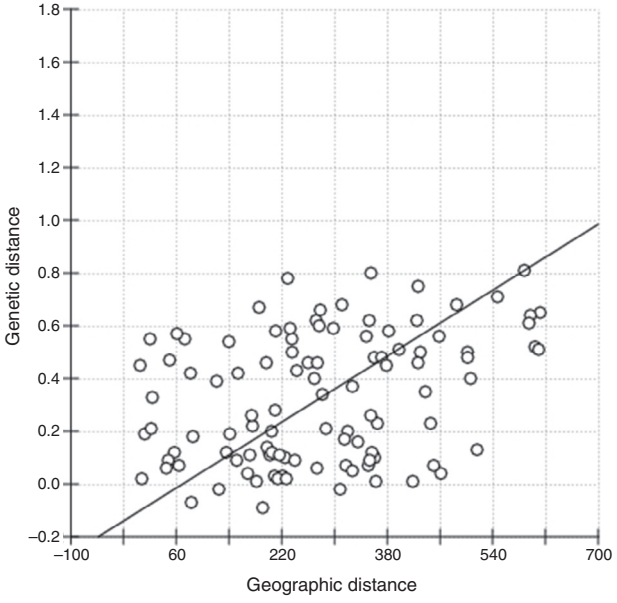

Fig. 3. Mantel test correlation of genetic distance and geographic distance across all sampled cities in the *Aedes aegypti* populations of the Brazilian state of Maranhão.

H15), one of which (H2) was the most common, and seven (H4, H9, H11, H12, H13, H14 and H15) that were exclusive to the Fortuna, São Mateus and São José de Ribamar populations, respectively (Fig. 5).

Discussion

Polymorphism of the ND4 gene and the distribution of haplotypes

The results of the present study of the ND4 mitochondrial marker indicate the presence of a relatively large number of haplotypes (15) in comparison with studies in Peru (Costa-da-Silva et al., 2005), Bolivia (Paupy et al., 2012) and the Brazilian state of Paraná (Twerdochlib et al., 2012). However, a larger total number of haplotypes was recorded Amazonia by Lima Júnior and Scarpassa (2009), as well as in Mexico (Gorrochotegui-Escalante et al., 2002) and the Americas (Bracco et al., 2007).

The H2 haplotype is widespread in the Maranhão sample, and those from other regions, including Amazonia, where it corresponds to haplotype H10, as well as Mexico, where it is H1, Peru (H2), identified as H5 in the study of Bracco et al. (2007), covering the Americas, Africa, and Asia and H4 in Paraná. The ample distribution of this haplotype indicates that it represents one of the vector's oldest lineages, which may have dispersed following the bottleneck created by the programs established for the control of the vector in the 1950s and 1960s. This distribution pattern would thus be accounted for either by the resistance of this lineage to the pesticides used by these programs or through its introduction from the countries or regions where there was no control.

The H1 haplotype was absent from six populations in Maranhão (Fortuna, Rosário, Paço do Lumiar, São José de Ribamar and São Luís), although it was found in Amazonia, as H6, H3 in Paraná. Lima Júnior and Scarpassa (2009) suggested that this haplotype may have either originated in Brazil or been introduced into the country from a region not yet surveyed. Our results further reinforce this

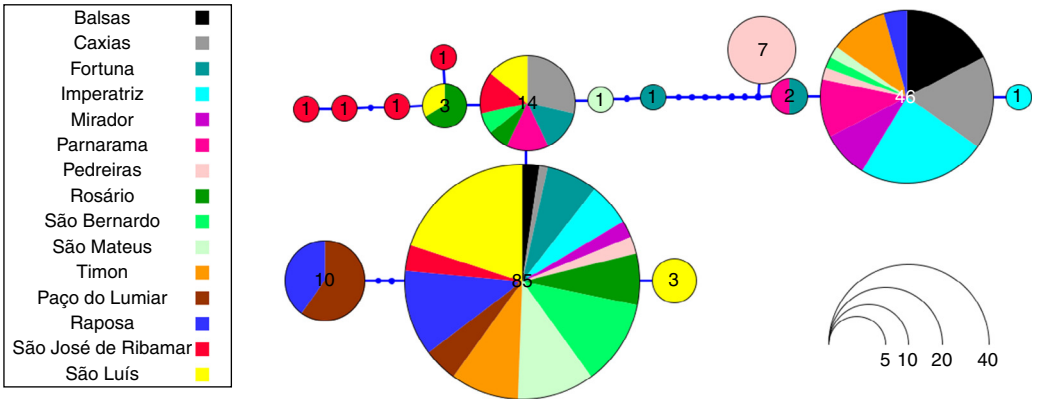

Fig. 4. Haplotype network found in the present study for the *Aedes aegypti* populations of the Brazilian state of Maranhão.

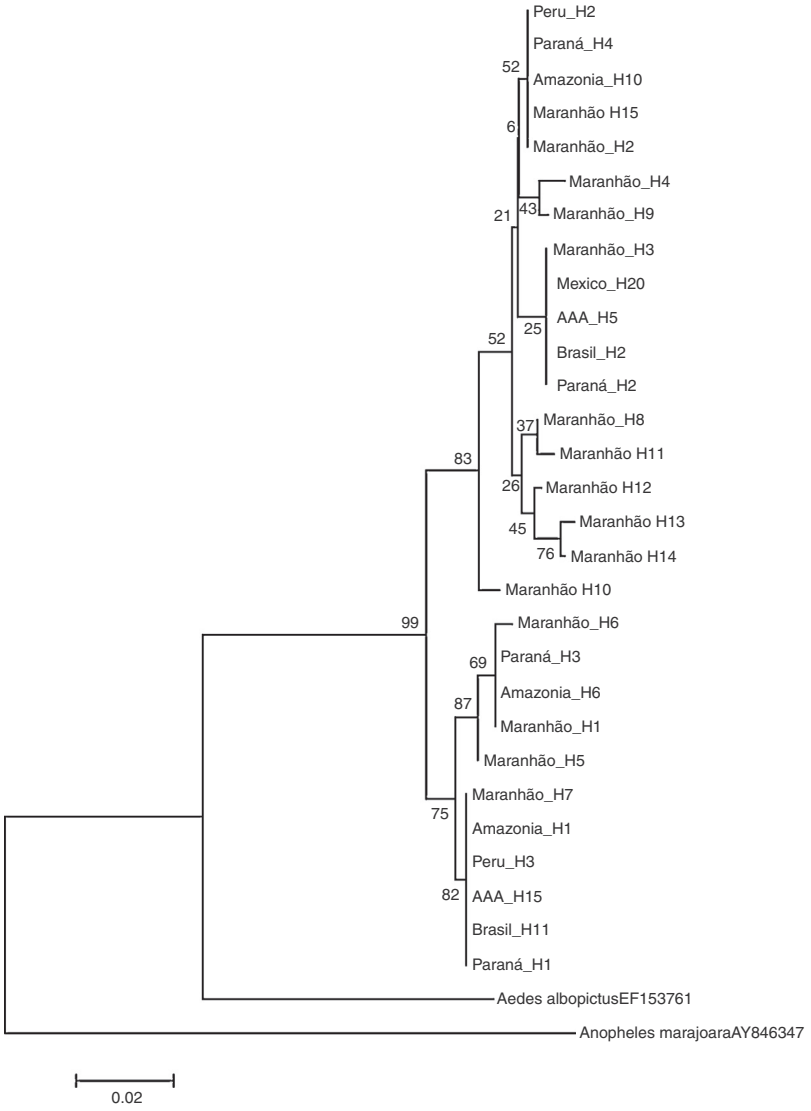

Fig. 5. Phylogenetic relationships among the haplotypes of the *Aedes aegypti* populations of the Brazilian state of Maranhão and those recorded in other studies – Americas (Bracco et al., 2007), Amazonia (Lima Júnior and Scarpassa, 2009), Brazil (Paduan and Ribolla, 2008), Mexico (Gorrochotegui-Escalante et al., 2002), Paraná (Twerdochlib et al., 2012) and Peru (Costa-da-Silva et al., 2005). The analysis was based on the Neighbor-Joining algorithm using the Tamura-Nei genetic distance model, with bootstrap support estimated from 1000 repetitions.

conclusion, given that this haplotype was only observed in Brazilian populations (Amazonia, Paraná, and Maranhão). Haplotype H3 was found in the populations from Caxias, Fortuna, Parnarama, Rosário, São Bernardo, São José de Ribamar, and São Luís, and was recorded as H20 in Mexico. The ample distribution of the H3 haplotype in different parts of the world indicates that it has been fixed in the regions where it was recorded.

Haplotype H7 was found only in the population from Pedreiras in the present study, but despite its reduced frequency in Maranhão, it has also been recorded in Paraná and Amazonia (as H1) and other regions of Brazil (as H11), as well as H3 in Peru, and H15 in the Americas, Africa, and Asia. The H8 haplotype found in Rosário and São Luís in the present study. While this haplotype was only observed in the populations from Maranhão, a more detailed analysis of the other studies of *A. aegypti* would be necessary to confirm that it occurs only in this state.

The other haplotypes recorded in the present study – H4 in Fortuna, H5 in Fortuna and Parnarama, H6 in Imperatriz, H8 in Rosário and São Luís, H9 in São Mateus, H10 in Paço do Lumiar and Raposa and H11, H12, H13, H14, H15 in São José de Ribamar – have not been recorded in any other studies of this molecular marker, which suggests that they may represent either new mutations that have yet to disperse or haplotypes introduced from regions that have yet to be surveyed.

The indices of genetic diversity recorded in the present study ($h = 0.6938$ and $\pi = 0.01486$) were relatively high, in contrast with the findings of previous studies (Gorrochotegui-Escalante et al., 2002; Bosio et al., 2005; Costa-da-Silva et al., 2005; Herrera et al., 2006; Bracco et al., 2007; Lima Júnior and Scarpassa, 2009; Paupy et al., 2012; Twerdochlib et al., 2012).

The diversity indices recorded in the present study indicate reduced levels of gene flow between the *A. aegypti* populations found in Maranhão, which has resulted in marked genetic differentiation among populations. This low level of gene flow may be related to the reduction in the effective size of the populations resulting from bottlenecks caused by the intense use of insecticides during control campaigns. The findings of the present study are consistent with those of Ayres et al. (2004), who found that populations of *A. aegypti* in areas where control campaigns were frequent present high levels of genetic differentiation.

The results of the *Fs* (Fu, 1997) and *D* (Tajima, 1989) tests were not significant ($p > 0.05$), which indicates that the populations of *A. aegypti* in the state of Maranhão are not undergoing expansion, despite the presence of unique haplotypes. These findings are consistent with those of Lima Júnior and Scarpassa, (2009) for Amazonian populations, Twerdochlib et al. (2012) for populations from the state of Paraná and Bracco et al. (2007) for those from the Americas.

Analysis of molecular variance and phylogenetic relationships

In the present study, the AMOVA was run based on two different hierarchical arrangements – a single group encompassing all the populations of the state of Maranhão, and five groups corresponding to the different geographic mesoregions of the state. Irrespective of the arrangement, however, most of the variation was found within the populations, that is, 61.06% for the single population ($F_{ST} = 0.38939$; $p < 0.05$), and 58.47% for the five geographic groups ($F_{ST} = 0.41533$; $p < 0.05$). When compared with the populations analyzed in Amazonia (Lima Júnior and Scarpassa, 2009: 72.69%; $F_{ST} = 0.273$; $p \leq 0.05$), Paraná (Twerdochlib et al., 2012: 67%; $F_{ST} = 0.32996$; $p \leq 0.05$), and Venezuela (Herrera et al., 2006: 77.60%; $F_{ST} = 0.224$; $p \leq 0.05$), these results indicate that the Maranhão populations are undergoing a process of genetic differentiation.

The capacity of individuals and populations to adapt to alterations in the environment depends on their intraspecific genetic diversity (Rieger et al., 2006), which is reinforced by new mutations and recombinations of existing genetic material. As in previous research, such as that of Gorrochotegui-Escalante et al. (2002), Bosio et al. (2005), Costa-da-Silva et al. (2005), Lima Júnior and Scarpassa (2009), Twerdochlib et al. (2012), the results of the present study indicated relatively high intra-population variation, which may reflect the evolutionary success of the species, given that the results of the AMOVA indicate the presence of population structuring consistent with the presence of a number of different lineages of *A. aegypti* in Maranhão.

Mean genetic divergence ranged from 0.2% to 2.7%, with the lowest values being observed between geographically proximate populations (i.e., Raposa and São Luís), whereas higher means were found between more distant populations, such as Balsas and São Luís (see Fig. 1), as confirmed by the Mantel test, which indicated a significant correlation between genetic (F_{ST}) and geographic (Km) distances for the *A. aegypti* populations of Maranhão (Fig. 3). In their global analysis of Brazilian *A. aegypti* populations, Monteiro et al. (2014) also found a significant correlation between the genetic and geographic distances between populations.

Despite the significant correlation indicated by the Mantel test, the pairwise F_{ST} matrix (Table 7) and the population clusters generated by Bayesian inference (Fig. 2) both indicate that, while the Maranhão populations are well structured genetically, there is still considerable gene flow, reflected in the relatively ample distribution of haplotype H2. In their analysis of the NAHD4 marker in Amazonian populations of *A. aegypti*, Lima Júnior and Scarpassa (2009) found high levels of differentiation and gene flow, even between widely-displaced populations, such as Santarém and Boa Vista (880 km), similar to that observed in the present study.

The phylogenetic tree presented two well-supported clades supported by a 99% bootstrap value (Fig. 5). The presence of the two lineages in ours results are consistent with other studies of the ND4 gene in *A. aegypti* populations (Bracco et al., 2007; Lima Júnior and Scarpassa, 2009; Paupy et al., 2012; Twerdochlib et al., 2012), as well as with studies of the microsatellite loci (Monteiro et al., 2014) support the conclusion that the populations in Maranhão have originated from multiple introductions.

Conflicts of interest

The authors declare no conflicts of interest.

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References

Ayres, C.E.J., Melo-Santos, M.A.V., Prota, J.R.M., Solé-Cava, A.M., Regis, L., Furtado, A.F., 2004. Genetic structure of natural populations of *Aedes aegypti* at the micro- and macrogeographic levels in Brazil. J. Am. Mosq. Control Assoc. 20, 350–356.

Behura, S.K., Lobo, N.F., Haas, B., deBruyn, B., Lovin, D.D., Shumway, M.F., Puiu, D., Romero-Severson, J., Nene, V., Severson, D.W., 2011. Complete sequences of mitochondria genomes of *Aedes aegypti* and *Culex quinquefasciatus* and comparative analysis of mitochondrial DNA fragments inserted in the nuclear genomes. Insect Biochem. Mol. Biol. 41, 770–777.

Black Iv, W.C., Bernhardt, S.A., 2009. Abundant nuclear copies of mitochondrial origin (NUMTs) in the *Aedes aegypti* genome. Insect Mol. Biol. 18, 705–713.

Bosio, C.F., Harrington, L.C., Jones, J.W., Sithiprasasna, R., Norris, D.E., Scott, T.W., 2005. Genetic structure of *Aedes aegypti* population in Thailand using mitochondrial DNA. Am. J. Trop. Med. Hyg. 72, 434–442.

Bracco, J.E., Capurro, M.L., Lourenço-de-Oliveira, R., Sallum, M.A.M., 2007. Genetic variability of *Aedes aegypti* in the Americas using a mitochondrial gene: evidence of multiple introductions. Mem. Inst. Oswaldo Cruz 102, 573–580.

Brasil, 2016. MS – Ministério da Saúde. SVS – Secretaria de Vigilância em Saúde, Boletim Epidemiológico da Dengue; Chikungunya, Zika vírus, Available at: http://www.portaldasaude.gov.br/ (accessed: 24.02.16).

Campos, G.S., Bandeira, A.C., Sardi, S.I., 2015. Zika vírus out break, Bahia, Brazil. Emerg. Infect. Dis. 21, 5.

Chow, V.T.K., Chan, Y.C., Yong, R., Lee, K.M., Lim, L.K., Chung, Y.K., Lam-Phua, S.G., Tan, B.T., 1998. Monitoring of dengue viruses in field-caught *Aedes aegypti* and *Aedes albopictus* mosquitoes by a type-specific polymerase chain reaction and cycle sequencing. Am. J. Trop. Med. Hyg. 58, 578–586.

Consoli, R.A.G.B., Lourenço-de-Oliveira, R., 1994. Principais Mosquitos de Importância Sanitária no Brasil. Fiocruz, Rio de Janeiro.

Corander, J., Cheng, L., Marttinen, P., Sirén, J., Tang, J., 2013. BAPS: Bayesian Analysis of Population Structure. Version 6.0.

Costa, M.C.V., Paduan, K.S., Ribolla, P.E., Lourença-de-Oliveira, R., 2006. Temporal analysis of mitochrondrial gene (NDH4) in *Aedes aegypti* populations fron endemic and non-endemic areas in Brazil. Departamento de Entomologia, Fiocruz, Rio de Janeiro, Brasil.

Costa-da-Silva, A.L., Capurro, M.L., Bracco, J.E., 2005. Genetic lineages in the yellow fever mosquito *Aedes* (*Stegomyia*) *aegypti* (Diptera: Culicidae) from Peru. Mem. Inst. Oswaldo Cruz 100, 539–544.

Excoffier, L., Laval, G., Schneider, S., 2006. Arlequin ver. 3.1: an integrated software package for population genetics data analysis. Evol. Bioinf. Online, 47–50.

Felsenstein, J., 1985. Confidence limits on phylogenies: an approach using the bootstrap. Evolution 39, 783–791.

Fraga, E.C., Oliveira, D.R.S., Aragão, D.G., Schneider, H., Sampaio, I., Barros, M.C., 2013. Genetic variability and evidence of two distinct lineages of *Aedes aegypti* (Diptera, Culicidae) on São Luís Island in Maranhão, Brazil. Open Trop. Med. J. 6, 11–18.

Fu, Y.X., 1997. Statistical tests of neutrality of mutations against population growth, hitchhiking and background selection. Genetics 147, 915–925.

Gorrochotegui-Escalante, N., Gomez-Machorro, C., Lozano-Fuentes, S., Fernandez-Salas, L., De Lourdes Munoz, M., Farfan-Ale, J.A., Garcia-Rejon, J., Beaty, B.J., Black, W.C., 2002. Breeding structure of *Aedes aegypti* populations in Mexico varies by region. Am. J. Trop. Med. Hyg. 66, 213–222.

Hall, T.A., 1999. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucl. Acids Symp. Ser. 41, 95–98.

Herrera, F., Urdaneta, L., Rivero, J., Zoghbi, N., Ruiz, J., Carrasquel, G., Martinez, J.A., Pernalete, M., Villegas, P., Montoya, A., Rubio-Palis, Y., Rojas, E., 2006. Population genetic structure of the dengue mosquito *Aedes aegypti* in Venezuela. Mem. Inst. Oswaldo Cruz 101, 625–633.

Jensen, J.L., Bohonak, A.J., Kelley, S.T., 2005. Isolation by distance, web service. BMC Genet. 6, 13.

Leite, L.A.R., 2012. Mitochrondrial pseudogenes in insect DNA barcoding: differing points of view on the same issue. Biota Neotrop. 12, 3, Available at: http://www.biotaneotropica.org.br/v12n3/en/abstract?thematic-review+bn02412032012 (accessed 18.10.16).

Librado, P., Rozas, J., 2009. DnaSP v5: a software for comprehensive analysis of DNA polymorphism data. Bioinformatics 25, 1451–1452.

Lima Júnior, R.S., Scarpassa, V.M., 2009. Evidence of two lineages of the dengue vector *Aedes aegypti* in the Brazilian Amazon, based on mitochondrial DNA ND4 gene sequences. Gen. Mol. Biol. 32, 414–422.

Mantel, N., 1967. The detection of disease clustering and a generalized regression approach. Cancer Res. 27, 209–220.7.

Monteiro, F.A., Shama, R., Martins, A.J., Gloria-Soria, A., Brown, J.E., et al., 2014. Genetic Diversity of Brazilian Aedes aegypti: Patterns following

an Eradication Program. PLoS Negl Trop Dis 8, e3167, http://dx.doi.org/10.1371/journal.pntd.0003167.
Moore, M., Massamba, S., Goss, L., Burugu, M.W., Sang, R., Kamau, L.W., Kenya, E.U., Bosio, C., Munoz, M.L., Sharakova, M., Black, W.C., 2013. Dual African origins of global *Aedes aegypti* s.l. populations revealed by mitochrondrial DNA. PLOS Negl. Trop. Dis. 7, 2175.
Oehler, E., Watrin, L., Larre, P., Leparc-Golfrt, I., Lestère, S., Valour, F., 2014. Zika virus infection complicated by Guillain-Barré syndrome: case report, French Polynesia, December 2013. Euro Surveill. Mar 19, 9.
Paduan, K.S., Ribolla, P.E.M., 2008. Mitochondrial DNA polymorphism and heteroplasmy in populations of Aedes aegypti in Brazil. J. Med. Entomol. 45, 59–67.
Paupy, C., Goff, G.L., Brengues, C., Guerra, M., Revollo, J., Simon, Z.B., Hervé, J.-P., Fontenille, D., 2012. Genetic structure and phylogeografic of *Aedes aegypti*, the dengue and yellow-fever mosquito vector in Bolivia. Infect. Genet. Evol. 12, 1260–1269.
Ribeiro, J.M.C., Arcà, B., Lombardo, F., Calvo, E., Phan, V.M., Chandra, P.K., Wikel, S.K., 2007. An annotated catalogue of salivary gland transcripts in the adult female mosquito, *Aedes aegypti*. BMC Genomics 8, 6.
Rieger, T.T., Campos, S.R.C., Santos, J.F., 2006. A biologia molecular como ferramenta no estudo da biodiversidade. Floresta e Ambiente 13, 11–24.
Salzburger, W., Ewing, G.B., Von Haeseler, A., 2011. The performance of phylogenetic algorithms in estimating haplotype genealogies with migration. Mol. Ecol. 20, 1952–1963.
Sanger, F., Nichlen, S., Coulson, A.R., 1977. DNA sequencing with chain termination inhibitors. Proc. Natl. Acad. Sci. U. S. A. 74, 5463–5468.
Santos, J.M.M., Contel, E.P.B., Keer, W.E., 1981. Biologia de anofelinos amazônicos. I – Ciclo biológico, postura e estádios de Anopheles darlingi, Root, 1926 (Diptera: Culicidae) da Rodovia Manaus/Boa Vista. Acta Amaz. 11, 789–797.
Scarpassa, V.M., Cardoza, T.B., Cardoso Junior, R.P., 2008. Population genetics and phylogeography of *Aedes aegypti* (Diptera: Culicidae) from Brazil. Am. J. Trop. Med. Hyg. 78, 895–903.
Silva, A.G., Cunha, I.C.L., Santos, W.S., Luz, S.L.B., Ribolla, P.E.M., Abad-Franch, F., 2012. Gene flow networks among American *Aedes aegypti* populations. Evol. Appl., 1752–4571.
Tajima, F., 1989. The effect of change in population size on DNA polymorphism. Genetics 123, 597–601.
Tamura, K., Nei, M., 1993. Estimation of the number of nucleotide substitutions in the control region of mitochondrial DNA in humans and chimpanzees. Mol. Biol. Evol. 10, 512–526.
Tamura, K., Stecher, G., Peterson, D., Filipski, A., Kumar, S., 2013. MEGA 6: molecular evolutionary genetics analysis, version 6.0. Mol. Biol. Evol. 30, 2725–2729.
Thompson, J.D., Higgins, D.G., Gibson, T.J., 1994. Clustal W, improving the sensitivity of progressive multiple sequence alignment through sequence weighting position specific gap penalties and weight matrix choice. Nucleic Acids Research hitchhiking and background selection. Genetics 147, 915–925.
Twerdochlib, A.L., Bona, A.C.D., Leite, S.S., Chitolina, R.F., Betina Westphal, B., Navarro-Silva, M.A., 2012. Genetic variability of a population of *Aedes aegypti* from Paraná, Brazil, using the mitochondrial ND4 gene. Revista Brasileira de Entomologia 56, 249–256.
Urdaneta-Marquez, L., Bosio, C., Herrera, F., Rubio-Palis, Y., Salasek, M., Black, W.C., 2008. Genetic relationships among *Aedes aegypti* collections in Venezuela as determined by mitochondrial DNA variation and nuclear single nucleotide polymorphisms. Am. J. Trop. Med. Hyg. 78, 479–491.
Wilkerson, R.C., Foster, P.G., Li, C., Sallum, M.A., 2005. Molecular phylogeny of neotropical *Anopheles* (Nyssorhynchus) *albitarsis* species complex (Diptera: Culicidae). Ann. Entomol. Soc. Am. 98, 918–925.
Wilkerson, R.C., Parsons, T.J., Klein, T.A., Gaffigan, T.V., Bergo, E., Consolim, J., 1995. Diagnostic by random amplified polymorphic DNA polymerase chain reaction of four cryptic species related to *Anopheles* (*Nyssorhynchus*) *albitarsis* (Diptera: Culicidae) from Paraguay, Argentina and Brazil. J. Med. Entomol. 32, 697–704.
Yáñez, P., Mamani, E., Valle, J., Paquita García, M., León, W., Villaseca, P., Torres, D., Cabezas, C., 2013. Variabilidad genética de *Aedes aegypti* determinada mediante el análisis del gen mitocondrial ND4 en once áreas endémicas para dengue en el Perú. Rev. Peru Med. Exp. Salud Publica 30, 246–250.