Genetic Variability and Evidence of Two Distinct Lineages of *Aedes aegypti* (Diptera, Culicidae) on São Luís Island in Maranhão, Brazil

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Abstract: *Aedes aegypti* is the principal vector of the yellow fever virus and the four dengue serotypes and its hemorrhagic fever viruses. The genetic variability and differentiation of four *Aedes aegypti* populations from São Luis Island in the Brazilian state of Maranhão was analyzed based on the sequences of a fragment of the mitochondrial ND4 gene. A total of 58 sequences of 337 bps were analyzed, revealing the existence of 10 haplotypes, of which five were considered to be unique. Haplotype diversity for the total population was 0.6273 and nucleotide diversity 0.00748. The haplotype tree produced from the data indicated the presence of two mitochondrial lineages of the dengue vector, one of which was characterized by the H6 haplotype, found only in the population from one site (Raposa), and may represent the recent introduction of this lineage to the island. The results of the AMOVA indicated that the majority of the genetic variation (74.38%) was found within populations. However, the significant *Fst* value of 0.2572 indicates a certain inter-population differentiation which may result in differences in the vectorial capacity of the insect, its susceptibility to the virus or even resistance to insecticides or other ecological adaptations, all of which may limit the effectiveness of programs for the control of *Ae. aegypti*.

Keywords: Dengue Fever, dengue vector, mitochondrial DNA, ND4, population genetics, polymorphism.

INTRODUCTION

Aedes (*Stegomyia*) *aegypti* (L.) (Diptera: Culicidae) is the principal vector of the Yellow Fever virus and of the four dengue virus serotypes (DEN-1, DEN-2, DEN-3, and DEN-4) [1]. This household mosquito, which probably originated in Ethiopia, is active during the day and lays its eggs preferentially in artificial deposits of clean water [2]. The behavior of the species is strictly synanthropic and anthropophilic, and it is the culicid most closely associated with human populations. When the female feeds more than once between successive ovipositions, the possibility of ingesting and transmitting the dengue virus, in particular, increases considerably [3].

While *Ae. aegypti* was considered to have been eradicated from Brazil in 1955 [4], it was introduced into the city of São Luís in 1969, although it only began to attract the attention of the local authorities in 1995, when the first cases of classic dengue fever were detected in the Cohab-Anil neighborhood. In 1996, the first epidemic occurred on the island of São Luís, with 4641 reported cases and approximately 41.40% of the population being sensitized by DEN-1. In 2001, the DEN-2 serotype was isolated for the first time [5].

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Dengue is one of the most common viral diseases in Brazil by number of cases. This arbovirus represents a serious public health problem in most tropical countries, where climatic conditions favor the proliferation of *Ae. aegypti*, a species found in 3970 municipalities in Brazil. In the state of Maranhão, this mosquito is found 73.3% of the municipalities, where three dengue serotypes – DEN-1, DEN-2, and DEN-3 – can be found [6].

In 2009, a total of 3841 cases were reported in Maranhão, a rate of 60.3 cases per 100,000 inhabitants. Of these cases, 14 were confirmed as DHF (Dengue Hemorrhagic Fever) and 34 as DWC (Dengue with complications), with two deaths. Overall, 322 cases (8.4% of the total) were recorded in the state capital, São Luís, followed by Imperatriz, with 268 cases (7.0%), Caxias with 247 (6.4%), and Açailândia, with 244 (6.4%). These numbers represent rates of 32.3, 113.2, 166.8, and 241.2 cases per 100,000 inhabitants, respectively [7]. While there is some oscillation in the number of cases reported each year – 6811 in 2010, 11,427 in 2011, and 5463 in 2012 – the figures are preoccupying, with numerous grave cases, emphasizing the seriousness of the situation in the state [8].

A number of studies have emphasized the genetic variability of *Ae. aegypti* populations, which may reflect both ecological and anthropogenic factors, such as the increasing use of insecticides [9-11], which may favor the adaptation of the vector and increase its potential for the transmission of yellow and dengue fevers [12,13]. The analysis of DNA sequences has provided important insights into the genetic

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structure of populations and helped to identify cryptic species, which are very common in some insect groups. In particular, the mitochondrial DNA has been widely-used, given its easy isolation, large number of copies per cell, small size, and simple organization [14]. In the specific case of *Ae. aegypti*, subunit 4 (ND4) of the mitochondrial NADH gene has been shown to be an excellent tool for the analysis of the genetic differentiation of the populations of this vector [15-20].

The present study is the first to analyze the *Ae. aegypti* populations of the island of São Luís in the Brazilian state of Maranhão based on the sequences of the mitochondrial ND4 gene. The results demonstrated the genetic variability and differentiation in the populations of this dengue virus vector.

MATERIAL AND METHODS

Origin and Collection of Samples

The samples were collected in the municipalities of São Luís, São José de Ribamar, Raposa, and Paço do Lumiar, which make up São Luís Island in the state of Maranhão (Table **1** and Fig. **1**). Ovitraps were set in areas adjacent to domestic residences and were retrieved after five days. The traps were removed to the UEMA Genetics and Molecular Biology Laboratory at Caxias, where the eggs were left to hatch, following which the larvae were raised until their emergence as adults, when they were bred. The males were fed a 10% sucrose solution, while a hamster (*Mesocricetus auratus*) was used to provide the females with a blood meal. After mating, females were isolated for oviposition and later identified by means of the Forattini's taxonomic key [21]. The hatched larvae were raised until the fourth developmental stage, using a protocol modified from Santos *et al.* [22]. The samples – 4th stage larvae – were stored in a freezer at – 20ºC until the extraction of the total DNA.

Extraction of DNA, Amplification and Sequencing of the ND4 Gene

The DNA was obtained using the procedure described by Williams *et al*. [23]. Larvae (one individual from each clutch) with distinct parents were used to represent the four populations of *Ae*. *aegypti*, with samples from around 30 clutches being obtained for each of the four populations. The amplification of the ND4 gene fragment from the total DNA was based on the primers (ND4H: 5' - TCG GCT TCC TAG TCG TTC AT – 3' and ND4L: 5' – ATT GCC TAA CGC TCA TGT AG – 3') and PCR conditions described in the literature [15]. Following the PCR, the samples were visualized in 1% agarose gel. The PCR products were sequenced

Table 1. Location, Geographic Coordinates, and Sizes of the *Aedes Aegypti* Samples from São Luís Island in the Brazilian State of Maranhão

Municipality	Coordinates	Number of Individuals
São Luis	2°33'28.84"S / 44°14'43.92"W	23
São José de Ribamar	2°33'47.45"S / 44°03'45.23" W	09
Paço do Lumiar	2°32'37.87"S / 44°05'31.85" W	10
Raposa	2°25'57.38"S / 44°05'07.39" W	16

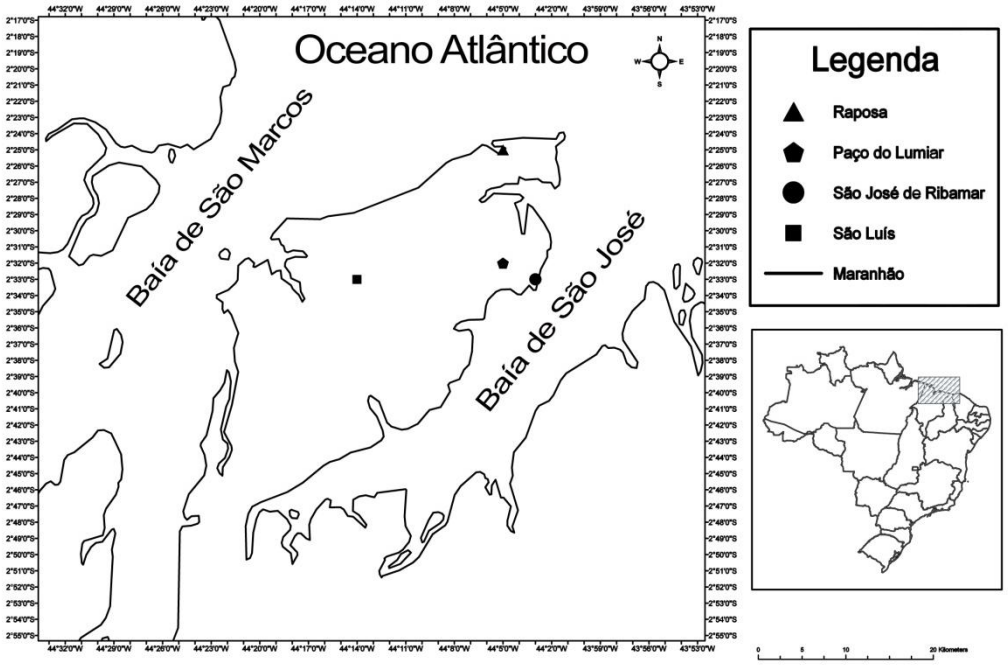

Fig. (1). Map of Brazil showing São Luis Island in the state of Maranhão and the *Aedes aegypti* specimen collection sites.

using the dideoxyterminal method, with reactions being carried out directly in both strands of DNA using [24], with ABI Prism TM kit reagents, followed by electrophoresis in an ABI 377 automatic sequencer. The amplified ND4 region corresponds to 337 bps at positions 8392 to 8729 of the *Ae. aegypti* mitochondrial genome (GenBank accession number NC_010241). Chromatogram quality was assessed using DNA Sequencing Analysis Software, version 5.1 (Applied Biosystems/www.appliedbiosystems.com).

Phylogenetic and Population Analyses

The sequences were aligned using CLUSTAL W [25] and edited in the Bioedit 7.1.9 program [26]. The consensus sequences were obtained through the analysis of the sense and antisense sequences of each PCR fragment in the Bioedit. Sequence similarity of the ND4 sequences generated in this study with those available in GenBank was assessed using a BLASTN search [27].The number of polymorphic sites (*s*), and haplotype (*h*) and nucleotide (π) diversity, as well as the *D* [28] and *F* [29] neutrality tests were obtained using DnaSP 5.10.1 [30]. The relationships among the *Ae. aegypti* haplotypes were inferred from an unrooted haplotype network produced by NETWORK 4.5.1.0 [31] using the median-joining method [32]. The matrix of genetic distances among haplotypes and the tree representing the relationships among them were based on the Neighbor-Joining approach, following the Tamura-Nei genetic distance model, based on 1000 replicates in MEGA version 5.10 [33]. Two sequences obtained from GenBank – *Ae. albopictus*/accession number: EF153761 and *An. marajoara*/accession number: AY846347 – were used as the outgroup.

Population differentiation and the significance of the inter- and intra-population genetic variability were analyzed using an Analysis of Molecular Variance, AMOVA, run in ARLEQUIN, version 3.01 [34], which is based on the evaluation of different hierarchical levels. The haplotypes identified in the present study were compared with those from other regions of Brazil deposited in GenBank, as well as other countries on the American, African, and Asian continents. These included sequences AF334841-AF334865 from Mexico [35], DQ177153-DQ177155 from Peru [15], and DQ176828-DQ176831, DQ176833-DQ176843, and DQ176-845-DQ176849 from around the world [17]. Brazilian sequences included AY906835-AY906853 [18], as well as haplotypes – EU650405-EU650417 – from the Amazon region [19] and JN089748-JN089755 from the state of Paraná [20]. The sequences of the haplotypes identified in the present study were deposited in GenBank under access numbers KF922333–KF922342.

RESULTS

Distribution and Frequency of the Haplotypes

A total of 58 sequences of a 337 base pair (bp) fragment of the ND4 gene were obtained from the *Ae. aegypti* specimens collected on São Luís Island. The analysis of the DNA revealed a total of ten haplotypes and 15 polymorphic sites in the four study populations. The H1 haplotype was the most common, being observed in all the study populations, and in 58.8% of the specimens. The second most common haplotype was H5 (17.3%), but was recorded only in the specimens from Paço do Lumiar and Raposa. Haplotype H6 was found only in Raposa, and was differentiated from all the other haplotypes by 11 mutational steps. Six haplotypes were recorded in São José de Ribamar, of which four – H7, H8, H9, and H10 – were exclusive to this municipality (Table **2** and Fig. **2**).

Table 2. Haplotype Frequencies and Polymorphic Sites Found in the Four *Aedes aegypti* Samples from São Luis Island in the Brazilian State of Maranhão

Haplotype	Polymorphic Sites															Number of Specimens in Population				
	0	0	0	0	1	1	1	1	1	2	2	2	2	2	2					
	2	5	5	9	0	1	2	3	7	1	2	4	5	6	9					
	6	3	7	2	6	3	2	1	0	5	0	8	1	3	3	SL	PL	RAP	SJR	Total
H1	T	C	T	A	T	T	C	G	C	C	T	G	A	G	T	17	4	10	3	34
H2	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	2	-	-	2	4
H3	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	3	-	-	-	3
H4	.	.	.	.	.	.	.	.	.	.	A	.	.	A	C	1	-	-	-	1
H5	.	.	.	.	.	C	.	A	.	.	.	A	.	.	.	-	6	4	-	10
H6	.	T	.	G	.	C	T	A	T	T	.	A	T	A	C	-	-	2	-	2
H7	.	T	.	.	.	.	.	.	.	.	.	.	.	A	C	-	-	-	1	1
H8	.	.	A	.	C	.	.	.	T	.	.	.	.	A	C	-	-	-	1	1
H9	.	.	.	.	.	.	.	.	T	.	A	.	.	A	C	-	-	-	1	1
H10	.	.	A	.	C	.	.	.	T	.	A	.	.	.	C	-	-	-	1	1
Total																23	10	16	9	58

SL = São Luís; PL = Paço do Lumiar; RAP = Raposa; SJR = São José de Ribamar. Dots represent the same nucleotide found in the first sequence (H1).

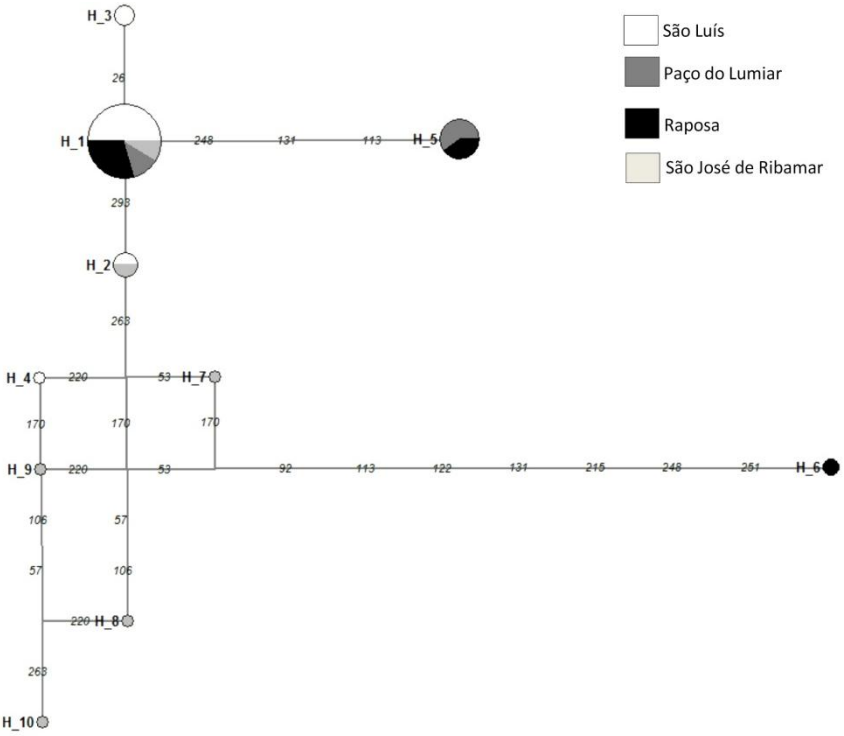

Fig. (2). Haplotype network of a 337 bp sequence of the NADH dehydrogenase subunit 4 region of mtDNA of four populations of *Aedes aegypti* from São Luis Island. The size of the circles is proportional to the number of individuals carrying the haplotype. Numbers represent the positions of mutations on the 337-bp fragment.

The unrooted haplotype network (Fig. **2**) represents the distribution and relative frequency of the different haplotypes found on São Luís Island. Haplotype 1 was recorded in all four populations, and was separated from haplotypes 2 and 3 by only a single mutation, and from haplotypes 4 and 5 by three mutational steps. It is also separated from haplotype 9 by four mutations, and from haplotypes 8 and 10 by five. The most differentiated haplotype was H6, however, which was separated by 11 mutations. Only two cases were found in the Raposa population (Fig. **2**), and it may represent a recent introduction.

Polymorphism of the ND4 Gene and Analysis of Molecular Variance (AMOVA)

The genetic diversity and results of the neutrality tests for each population on São Luís Island are presented in Table **3**. The general haplotype diversity for the four populations was 0.6273, while nucleotide diversity was 0.00748. The haplotype diversity of individual populations varied from 0.4466 in São Luís to 0.8889 in São José de Ribamar, with nucleotide diversity ranging from 0.00196 (São Luís) to 0.01026 (Raposa). Neither of the neutrality tests (*D* or *Fs*) returned significant results for any of the populations (individual or total), indicating that the characteristics of the observed polymorphisms were as expected according to the neutral mutation model.

The AMOVA was applied to the whole data set (population of São Luís Island), with a result of F_{ST} = 0.257 (Table **4**), which is highly significant ($p < 10^{-5}$). Much greater variation was observed within (74.38%) than among populations (25.72%).

Genetic Distances and Phylogenetic Relationships among Haplotypes

The genetic distances observed among the different populations (Table **5**) indicate that the greatest distances were related to the exclusive presence of haplotype H6 in the Raposa population (distances of 2–4%), a pattern observed in other regions of Brazil. The phylogenetic relationships found in the neighbor-joining tree based on the Tamura-Nei algorithm (Fig. **3**) revealed the relationships among the ten haplotypes found on São Luís Island, as well as the others available in GenBank. All the haplotypes were supported conclusively (99% bootstrap support).

Haplotype H6, observed in the Raposa population, grouped strongly (99% bootstrap support) with haplotypes recorded in other regions of Brazil, forming a clade that represents an *Ae. aegypti* lineage with an ample distribution in this country, the presence of which was confirmed on São Luís Island in this study. A second clade was identified with 77% bootstrap support, which grouped nine haplotypes from the present study with those described previously from Brazil, Peru, and Mexico, representing the other mitochondrial lineage of this vector. This clade includes haplotype 5, the second most common in the present study, which was identified as the basal form, with genetic distances of between 1% and 2% (Fig. **3** and Table **5**).

DISCUSSION

Distribution of Haplotypes

Of the studies that have used the ND4 gene as a molecular marker for the analysis of populations of *Ae. aegypti*, the

Table 3. Genetic Variability and the Results of the Neutrality Tests on Four Populations of *Aedes Aegypti* from São Luis Island, Maranhão, Brazil, Based on Sequences of the NADH Dehydrogenase Subunit 4 (ND4) Mitochondrial Gene

Population	N	S	K	NH	Molecular diversity		Tajima's *D*	Fu's *F*s
					h	π		
São Luís	23	4	0.64	04	0.4466	0.00196	-1.11328	-0.85272
São José de Ribamar	09	7	2.88	06	0.8889	0.00852	0.54268	-1.25159
Paço do Lumiar	10	3	1.60	02	0.5333	0.00497	1.83053	3.33815
Raposa	16	11	3.37	03	0.5667	0.01026	0.05886	4.79154
Grouped Populations	58	15	2.37	10	0.6273	0.00748	-0.80462	-0.97127

N = Sample size, S = polymorphic sites, K = average number of nucleotide differences, NH = number of haplotypes, *h* = haplotype diversity; π = nucleotide diversity. Significance level for neutrality tests: $p > 0.05$.

Table 4. Analysis of Molecular Variance in the Frequency of the NADH Dehydrogenase Subunit 4 (ND4) Mitochondrial Haplotypes Among the *Aedes Aegypti* Populations from São Luis Island, Maranhão, Brazil

Source of variation	Variance component	Percentage of variation	Fixation Index F_{ST}	*P**
Among population	0.32792	25.72	0.25721	P < 0.0001
Within population	0.94696	74.38		

*Probability of the test results (1023 permutations).

Table 5. Matrix of Genetic Distances Between the Haplotypes of the *Aedes Aegypti* Populations Based on the Neighbor-Joining (NJ) Algorithm of the Tamura-Nei genetic Distance Model

Haplotype	% Genetic Distance									
	1	2	3	4	5	6	7	8	9	10
1 - H1	-									
2 - H2	0	-								
3 - H3	0	1	-							
4 - H4	1	1	1	-						
5 - H5	1	1	1	2	-					
6 - H6	3	3	4	3	2	-				
7 - H7	1	1	1	1	2	2	-			
8 - H8	2	1	2	1	2	3	1	-		
9 - H9	1	1	1	0	2	3	1	1	-	
10 - H10	2	1	2	1	2	4	2	1	1	-

ten haplotypes recorded in the present study is a higher number than the eight found in the state of Paraná [20], but lower than the 20 detected in the Americas, Africa, and Asia [17], the 24 found in the northern, northeastern, midwestern, southern, and southeastern regions of Brazil [18] or the 13 recorded in the Amazon region [19]. Three of the haplotypes identified in the present study – 1, 2, and 6 – were identical to those reported from other regions of Brazil and the world.

Haplotype H1 was by far the most common (n = 34), representing 58.8% of the specimens analyzed, and was the only haplotype found in all four populations. This haplotype was identical to haplotype 1 in the Mexican study [35] and haplotype 2 in Piura, Peru [15]. This haplotype is also widely distributed in Brazil [17], including the municipality of São Luís, and the states of Alagoas, Ceará, Mato Grosso do Sul, Rondônia, and São Paulo [18]. It was also detected as haplotype 10 in the city of Belém [19] and haplotype 4 in the state of Paraná [20].

Haplotype H2, present only in the populations from São Luís and São Jose de Ribamar, was identical to haplotype 20 in Mexico [35], haplotype 5 in Belém [17], and haplotype 2 in Paraná [20] and the municipality of Juazeiro do Norte, in the Brazilian state of Ceará [18]. Haplotype H6, found only in the Raposa population, was identified with haplotype 17

in Brazilian populations studied by Bracco *et al*. [17], haplotype 14 in southeastern Brazil [18], and haplotype 6 in the states of Acre, Amazonas, and Pará [19]. This haplotype was also recorded as haplotype 3 in Paraná, where it was relatively common [20].

Overall, then, the results of the present study indicate the presence of two distinct mitochondrial lineages of the dengue vector on São Luís Island, with a genetic constitution similar to those recorded in other Brazilian states and other regions around the world (Fig. **3**). However, haplotype 5, which was the second most common in this study (17.3%), was recorded in only two municipalities, Paço do Lumiar and Raposa. The haplotypes H3, H4, H7, H8, H9, and H10 were also recorded only on São Luís Island, and may represent recent introductions or new mutations that have yet to be dispersed to other localities, as observed in other regions of Brazil [18, 19].

Genetic Variability of the Populations

The indices of haplotype (h = 0.627) and nucleotide diversity (π = 0.00748) recorded in the present study were similar to those recorded in a number of previous studies of *Ae. aegypti*, including populations from the Amazon region: h = 0.676, π = 0.0113 [19], and Paraná state: h = 0.702, π = 0.01556 [20]. The analyses of three Peruvian populations [15], and 19 populations from Thailand [13] also produced virtually identical values of nucleotide diversity (π = 0.0079).

Moderate indices of nucleotide diversity (π) were detected in the Raposa population (π = 0.01026). Similar values were also recorded for this vector in Amazonia (π = 0.0115) [19], Mexico (π = 0.0143) [35], and the Brazilian state of Paraná [20] (π = 0.01556). However, much higher values were recorded in other populations, such as Venezuela (π = 0.01877) [16], the Americas, Africa, and Asia [17] with

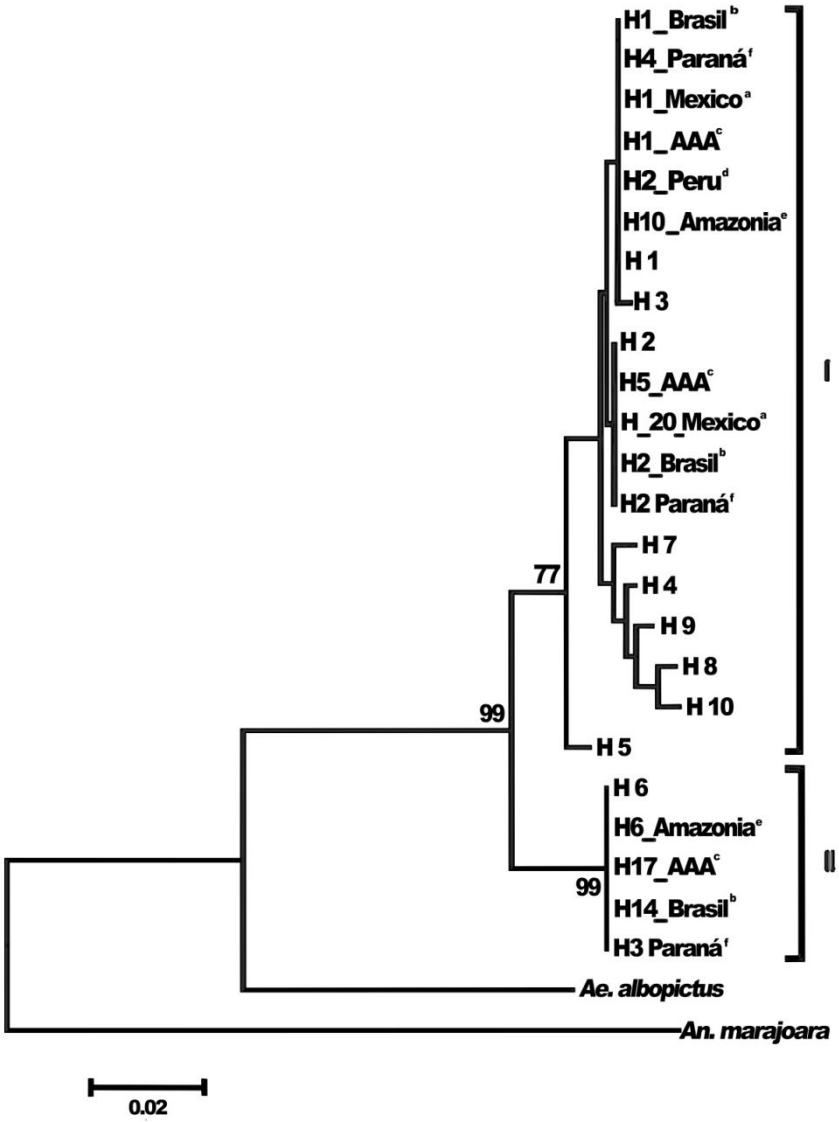

Fig. (3). Phylogenetic relationships among *Aedes aegypti* haplotypes based on the neighbor-joining (NJ) algorithm under the Tamura-Nei genetic distance model. Bootstrap values are marked on the branches. Haplotypes from the present study are shown in bold type. [a]Gorrochotegui-Escalante *et al.* (2002); [b]Paduan & Ribolla (2008); [c]Bracco *et al.* (2007); [d]Costa-da-Silva *et al.* (2005); [e]Lima Júnior & Scarpassa (2009); [f]Twerdochlib *et al*. (2012).

values of $\pi = 0.01997$, and other Brazilian populations, with $\pi = 0.01740$ [18]. These differences may be related to programs implemented for the control of the vector, which may provoke the loss of intermediate lineages and result in increased divergence between haplotypes [19]. A similar process is likely to have occurred on São Luis Island, where considerable efforts have been made to control the spread of the vector.

The results of the neutrality tests were not significant, which indicates that the detected polymorphism was consistent with the neutral model, and that the *Ae. aegypti* populations of São Luís Island are not expanding, despite the presence of four unique haplotypes, such as those detected in the population from São José de Ribamar (Fs = -1.25159, $p > 0.05$). These results are consistent with those obtained from populations in the Amazon basin [19] and worldwide [17].

Genetic Differentiation and Distances Among Populations

The results of the AMOVA for the data collected in the present study indicated greater variability within populations (74.38%; $F_{ST} = 0.25721$; $p < 0.0001$), a pattern also observed in the populations from Paraná (67%; $F_{ST} = 0.32996$; $p < 0.005$) [20], the Amazon region (72.69%; $F_{ST} = 0.273$; $p < 0.005$) [19], and Venezuela (77.60%; $F_{ST} = 0.224$; $p < 0.005$) [16]. The F_{ST} value of 0.2572, with a highly significant p value, indicates that the study population is highly differentiated, which may result in considerable differences in the vectorial capacity of the species, as observed in the Paraná population [20]. The marked intrapopulation genetic variation found in both the present study and other regions [13, 15, 19, 20, 35] may reflect the evolutionary success of the species.

Evidence of the Existence of Two Distinct Lineage of the Dengue Vector on São Luís Island

The analysis of the four *Ae. aegypti* populations from São Luís Island indicates the presence of two distinct lineages or haplotype groups, as found in most previous studies of the ND4 gene in this species [16-20]. The presence of the two lineages is reinforced by the strong tendency (99% bootstrap support) for haplotype H6 to group with those observed in other regions of Brazil [17-20] (Fig. **3**).

The occurrence of haplotype H6 exclusively in the Raposa population, albeit at a low frequency, indicates that this mitochondrial lineage may have been introduced relatively recently. This haplotype was the most common one in Paraná [20], and the second most common in the Amazon basin [19], and is relatively widely distributed in the rest of the country, as haplotype 17 [17]. Given this, the fact that it occurred in only one population on São Luís Island, where it was relatively rare, reinforces that hypothesis that it is a recent introduction.

Over the past few years, a number of studies have highlighted the prevalence of pseudogenes in the nuclear genome of *Ae. aegypti* [40-43]. These Numts (mtDNA) may be amplified by PCR together with the mitochondiral DNA, and when these paralagous sequences are amplified, they may lead to erroneous conclusions in phylogenetic and population-level analyses, given that they end up being analyzed as orthological sequences [44]. However in our analyzes the haplotypes 1 and 20 in Mexico (35) which correspond to lineage 1 (Fig. **3**) of our study (haplotypes 1 and 2 of this study) were validated as true mitochondrial haplotypes and not as Numts [45]. Similarly, the widely-distributed lineage 2, which is represented in the present study by haplotype H6 – also found in the Amazon basin as part of the clade which includes haplotypes 3 and 5 [19] – which correspond to haplotypes 5 and 3 from Mexico [35], has also been validated as a true mitochondrial haplotypes [45]. The results of the present study were also consistent with those of a number of previous analyses from different parts of the world [17-20, 42], reinforcing the conclusion that they represent true mitochondrial lineages.

The results of the present study contribute to the development of strategies for the control of the vector of dengue fever on São Luís Island, given that the identification of two genetic lineages of *Ae. aegypti* is consistent with the situation found in most other urban centers in Brazil. The observed genetic differentiation may be related to the ongoing use of insecticides, which may reduce populations drastically, resulting in the fixation of mutant alleles through bottlenecks or recolonization. These genetic modifications may affect the vectorial capacity of the mosquito, susceptibility to the parasite, resistance to the insecticide or ecological adaptation [12, 36-39], which in turn may limit the success of campaigns designed to control the proliferation of the disease or the vector.

CONFLICT OF INTEREST

The authors confirm that this article content has no conflict of interest.

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