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Exploring deeper genetic structures: *Aedes aegypti* in Brazil

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ABSTRACT

Aedes aegypti, being the principal vector of dengue (DENV1 to 4), chikungunya and Zika viruses, is considered as one of the most important mosquito vectors. In Brazil, despite regular vector control programs, *Ae. aegypti* still persists with high urban density in all the states. This study aimed to estimate the intra and inter population genetic diversity and genetic structure among 15 Brazilian populations of *Ae. aegypti* based on 12 microsatellite loci. A total of 510 specimens were analyzed comprising eight locations from northern (Itacoatiara, Manaus, Novo Airão, Boa Vista, Rio Branco, Porto Velho, Guajará-Mirim and Macapá), three from southeastern (Araçatuba, São José de Rio Preto and Taubaté), one from southern (Foz do Iguaçu), one from central west (Cuiabá) and two from northeastern (Campina Grande and Teresina) regions of Brazil. Genetic distances (pairwise values of F_{ST} and *Nm*) and the analysis of molecular variance (AMOVA) were statistically significant, independent of geographic distances among the sites analyzed, indicating that them are under a complex dynamic process that influence the levels of gene flow within and among regions of the country. Bayesian analysis in STRUCTURE revealed the existence of two major genetic clusters, as well as there was genetic substructure within them; these results were confirmed by AMOVA, BAPS and DAPC analyses. This differentiation is the cumulative result of several factors combined as events of multiple introduction, passive dispersal, environmental and climatic conditions, use of insecticides, cycles of extinction and re-colonization followed by microevolutionary processes throughout the country. Isolation by distance also contributed to this differentiation, especially among geographically closer localities. These genetic differences may affect its vector competence to transmit dengue, chikungunya, Zika and the response to vector control programs.

1. Introduction

Aedes aegypti is the most important vector of human arboviruses, including dengue virus (DENV1-4), urban cycle of yellow fever virus (YFV), chikungunya virus (CHKYV) and Zika virus (ZIKV). Consequently, it has been the most studied mosquito in recent times (Brown et al., 2014; Gloria-Soria et al., 2016a). DENV is the most common human arbovirus, which causes approximately 390 million infections every year in more than 125 countries, especially in tropical and subtropical regions (Bhatt et al., 2013). Over the last decade, CHKYV has also emerged as a major cause of concern, causing epidemics in Asia, Indian Ocean islands, southern Europe and the Americas, including Brazil (Fernández-Salas et al., 2015; Madariaga et al., 2016). In Brazil, the CHKYV was recorded for the first time in 2014 (Nunes et al., 2015) and rapidly spread throughout the country. In 2015, ZIKV was reported for the first time in Brazil and in the same year the country experienced a massive ZIKV outbreak, which resulted in several thousand cases of neonatal microcephaly (Kindhauser et al., 2016). To date, there is no vaccine against CHKYV or ZIKV, and even the vaccine against dengue is still in clinical trial phase (Low et al., 2017); therefore, the main tool for the control of these diseases is to combat the main vector, *Ae. aegypti* (Gubler, 1998, 2002).

The high levels of *Ae. aegypti* infestations throughout Brazil, along with favorable conditions in many cities, its adaptive plasticity (Bass and Field, 2011; Linss et al., 2009) and histories of DENV, CHKYV and ZIKV outbreaks, raise concerns regarding the risks of new epidemics

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Fig. 1. Collection sites of *Aedes aegypti*. IT: Itacoatiara, MA: Manaus, NA: Novo Airão, BV: Boa Vista, RB: Rio Branco, PV: Porto Velho, GM: Guajará-Mirim, MP: Macapá, CU: Cuiabá, CG: Campina Grande, TS: Teresina, AR: Araçatuba, RP: São José do Rio Preto, TA: Taubaté, IG: Foz do Iguaçu.

and emphasize the need for effective entomological surveillance and the development of the new control measures for this vector.

Vector competence, as a component of vectoral capacity, is governed by intrinsic (genetic) factors that influence the ability of a vector to transmit a pathogen (Hardy et al., 1983; Woodring et al., 1996). The vector competence of *Ae. aegypti* to the DENV has been observed to vary according to geographical region, as well as between populations from different countries, different states of the same country (Bennett et al., 2002), from a small island (Vazeille-Falcoz and Mousson, 1999) and from the same city (Gonçalves et al., 2014; Chaves, 2018). Differential transmission of Asian and African lineages of ZIKV by *Ae. aegypti* has also been observed in South-Pacific islands (Calvez et al., 2018). Therefore, understanding the dynamics of *Ae. aegypti* populations in a given region, particularly their structure and gene flow patterns, is important for designing more effective entomological surveillance and new control measures for this vector.

In Brazil, earlier studies of genetic structure of *Ae. aegypti* populations involved isozymes (Dinardo-Miranda and Contel, 1996; Fraga et al., 2003) and RAPD (Ayres et al., 2003; Paduan et al., 2006), which demonstrated high polymorphism and genetic differentiation among populations of this vector. In the last decade, analyses with mitochondrial genes indicated the co-existence of two genetic lineages of *Ae. aegypti* in Brazil (Bracco et al., 2007; Scarpassa et al., 2008; Lima-Júnior and Scarpassa, 2009). Linss et al. (2014), using *kdr* (knock-down-resistance) mutant alleles that confer pyrethroid resistance, confirmed two lineages of *Ae. aegypti* in Brazil.

Monteiro et al. (2014) conducted a nationwide study based on microsatellites markers, which reaffirmed the existence of two genetic groups of this vector. These authors also proposed that the *Ae. aegypti* populations from eastern, central and southern Brazil are genetically closer to the populations from Caribbean islands, whereas the populations from northern Brazil are genetically closer to the populations of Venezuela and other North American countries. A recent study conducted by Kotsakiozi et al. (2017), also with microsatellite markers, established the time frame of re-invasion and re-colonization of *Ae. aegypti* in Brazil. The authors suggested that the populations of northern Brazil may have been introduced from neighboring countries, where complete eradication of this vector had never been possible, whereas *Ae. aegypti* populations of southern Brazil may have been actually migrated from the northern areas of Brazil itself.

Microsatellites, due to their high mutation rates and fast evolution, are suitable markers to estimate intra-population genetic diversity, fine-scale population structure, and can detect genetic differentiation even in weakly structured species. In addition, these markers are used to infer contemporary gene flow among populations; therefore, they provide useful information on the dispersion patterns of the vectors (Collins et al., 2000), which is critical for control efforts in the present time. In this study, we analyzed the intrapopulation genetic diversity, population structure and the gene flow patterns among 15 populations of *Ae. aegypti* from different regions of Brazil using 12 microsatellites loci, in order to re-assess the existence of two previously established major genetic groups of this vector in Brazil and to study the underlying genetic connectivity/differentiation within and between these groups.

2. Materials and methods

2.1. Sample collection

Aedes aegypti samples were collected from 15 cities of Brazil, covering all regions of country, as follows: eight from the states of Brazilian Amazon [Itacoatiara (IT), Manaus (MA), and Novo Airão (NA), state of Amazonas; Boa Vista (BV), state of Roraima; Rio Branco (RB), state of

Table 1
Localities sampled for *Ae. aegypti* from different states of Brazil.

State	Locality	Abbreviation	Coordinates (Lat./Long.)	Sample Size	Year of collection
São Paulo	Araçatuba	AR	21°13'42.35"S 50°27'5.15"W	32	2009
São Paulo	São José de Rio Preto	RP	20°52'20.56"S 49°22'25.60"W	31	2005
São Paulo	Taubaté	TA	23° 2'26.05"S 45°33'23.08"W	32	2005
Paraná	Foz de Iguaçu	IG	25°32'24.34"S 54°35'1.29"W	36	2017
Mato Grosso	Cuiabá	CU	15°40'40.56"S 56° 5'42.24"W	36	2007
Paraíba	Campina Grande	CG	7°15'5.88"S 35°52'42.45"W	36	2017
Piauí	Teresina	TS	5° 7'14.19"S 42°48'18.58"W	35	2016
Roraima	Boa Vista	BV	2°46'56.04"N 60°41'2.67"W	36	2005
Amazonas	Itacoatiara	IT	3° 8'42.04"S 58°26'18.20"W	32	2011
Amazonas	Manaus	MA	3°11'34.36"S 60° 1'23.25"W	32	2011
Amazonas	Novo Airão	NA	2°38'12.57"S 60°56'38.95"W	32	2018
Acre	Rio Branco	RB	10° 1'25.00"S 67°49'19.63"W	35	2006
Amapá	Macapá	MP	0° 1'18.79"N 51° 4'11.36"W	36	2017
Rondônia	Porto Velho	PV	8°45'39.68"S 63°54'1.59"W	34	2018
Rondônia	Guajará-mirim	GM	10°47'21.27"S 65°19'48.09"W	35	2018

Acre; Porto Velho (PV) and Guajará-Mirim (GM), state of Rondonia and Macapá (MP), state of Amapá], one from the central west region of the country [Cuiabá (CU), state of Mato Grosso], two from the northeastern region [Campina Grande (CG), state of Paraiba; Teresina (TS), state of Piauí], three from the southeastern region [Araçatuba (AR), São José do Rio Preto (RP) and Taubaté (TA), state of São Paulo] and one from the southern region [Foz do Iguaçu (IG), state of Paraná] (Fig. 1). The details regarding specimen collection including the state, geographical coordinates, year of collection and sample size for each site are shown in Table 1.

The mosquitoes were collected as eggs, larvae or pupae. Larvae and pupae were collected from a variety of artificial recipients near human dwellings, and eggs were collected by using oviposition traps (ovitraps) for 2–7 days, depending on the location. All the samples were collected from multiple breeding sites (25–40 breeding sites) per location to prevent sampling of related individuals. The breeding sites were widespread over different neighborhoods in each city, the geographic distances between breeding sites ranging from 20 m to 5 km (Scarpassa et al., 2008). The specimens collected at each breeding site were transported separately in bottles to the Laboratory of Population Genetics of Malaria and Dengue Vectors at the Instituto Nacional de Pesquisas da Amazônia (INPA), in Manaus, Brazil, and reared to adulthood. In the eggs sampling, each positive ovitrap was immersed in an individual tray with water for hatching eggs and reared to adulthood. The specimens were morphologically identified using the taxonomic key of Forattini (2002), and preserved in 95% ethanol and stored in freezer –20 °C or stored dry in ultra-freezer -80 °C, until DNA extraction. To prevent sampling of related individuals, 2–3 specimens from each breeding site were used in the analyses.

2.2. DNA extraction, PCR and microsatellite genotyping

Genomic DNA was extracted individually from the larva or adult mosquitoes using phenol and chloroform method (Sambrook and Russell, 2001). Twelve previously studied microsatellite loci (Brown et al., 2011; Chambers et al., 2007; Slotman et al., 2007) (Table S1) were genotyped for individual samples, as described by Brown et al. (2011). The *AC1, AC2, AC5, AG2, AG3, AG4, AG5, AG7, B07* and *CT2* loci consist of dinucleotide repeats, whereas *A1* and *B3* loci consist of tri-nucleotide repeats. For the PCR reactions, a total volume of 10 µL of reaction solution was prepared, containing 1 µL of 10–20 ng DNA template, 1 µL 10x buffer, 0.3 µL 50 mM $MgCl_2$, 2.1 µL 1 mM dNTPs, 0.4 µL 4 mM M13-tailed forward primer (Schuelke, 2000), 0.4 µL 4 mM fluorescent M13-labelled primer (FAM, HEX and TAMRA), 0.8 µL 4 mM reverse primer, 0.2 µL of 5U/µL Platinum Taq DNA polymerase (Invitrogen Inc., Carlsbad, CA, USA) and 3.8 µL sterile water to complete the final volume. The microsatellite loci were amplified in 96 well thermocycler Veriti™ Thermal Cycler (Applied Biosystems, Thermo Fisher Scientific, Waltham, MA, USA) according to thermocycle conditions described by Slotman et al. (2007). The PCR products were analyzed in an automated ABI 3130 xl Genetic Analyzer (Applied Biosystems, Thermo Fisher Scientific, Waltham, MA, USA), available at INPA, in Manaus. The allele sizes were scored using GeneScan 500 ROX dye (Applied Biosystems) and genotyped in software GENEMAPPER version 4.0 (Applied Biosystems).

2.3. Microsatellite analyses

The dataset generated was initially analyzed in MICROCHECKER v. 2.23 (Oosterhout et al., 2004) to verify potential genotyping errors, as stuttering and large allele dropout. The same software was used to calculate the probability of occurrence and frequency of null alleles for each locus and in each population. The measures of intrapopulation genetic diversity, such as the number of alleles per locus (*Na*), number of effective alleles per locus (*Ne*), private alleles and their frequencies were estimated using the GENALEX v. 6.41 (Peakall and Smouse, 2012). Allelic richness (*Ar*) and inbreeding coefficient (F_{IS}) were calculated in FSTAT v. 2.9.3 (Goudet, 1995). The measures of observed

(H_O) and expected (H_E) heterozygosity, linkage disequilibrium (*LD*) and the probability tests for Hardy-Weinberg Equilibrium (HWE) were estimated in ARLEQUIN v. 3.1 (Excoffier et al., 2007).

The genetic structure, based on pairwise F_{ST} and *N*m values and Analyses of Molecular Variance (AMOVA), were estimated in ARLEQUIN, v.3.1 (Excoffier et al., 2007), with 10,000 permutations. The partitioning of total molecular variance, AMOVA was evaluated in three different hierarchical levels: (1) all samples (non-grouped) were analyzed as a unique group to test the overall genetic differences among samples; (2) two populations groups as revealed by STRUCTURE analysis (Group 1: AR, RP, CU, CG, TA, IT, MA, NA, BV, RB, PV, GM and Group 2: IG, TS, MP) and (3) four population groups as revealed by STRUCTURE analysis (Group 1: AR, RP, CU, CG ; Group 2: TA, IT, MA, NA, BV ; Group 3: RB, PV, GM; Group 4: IG, TS, MP). The sequential Bonferroni correction was applied for all cases of multiple comparisons (Holm, 1979).

The population structure was evaluated using the Bayesian clustering method calculated in STRUCTURE v. 2.3 (Pritchard et al., 2000). This method identifies clusters of genetically similar individuals from multilocus genotypes, without any prior knowledge about the sampling location. This model assumes K genetic clusters, each having a characteristic set of allele frequencies at each locus. The analysis was performed for ten independent runs for each K (from K = 1 to K = 15, the maximum number of populations used for this study). Consistent results were obtained across runs using a burn-in period of 100,000 permutations, followed by 1,000,000 Markov Chain Monte Carlo (MCMC) repeats. The optimal value of K was estimated by Evanno et al. (2005) method using online version of Structure Harvester v. 0.6.94 (Earl and vonHoldt, 2012). The program CLUMPP v1.1.2 (Jakobsson and Rosenberg, 2007) was used to summarize the results from the 10 independent STRUCTURE runs and the results were plotted using DISTRUCT v.1.1 (Rosenberg, 2003). Depending on the number of clusters (value of K) obtained, we further partitioned our data set for a better understanding of the underlying genetic structure of each population.

A second analysis for test these clusters was accessed by Bayesian Analysis of Population Structure (BAPS) software (Corander et al., 2008; Corander and Tang, 2007) to estimate the number of genetic groups along the area of study. In this analysis, 1–15 clusters were employed (the upper corresponding to the total number of sampled localities), and five independent runs were implemented. The most probable genetic cluster configuration was prepared by comparing the log-likelihood values of the best models.

In order to further explore the genetic structure, we also conducted Discriminant Analysis of Principal Components (DAPC), which submits genetic data to a principal component analysis (PCA) before conducting discriminant analysis (DA) on those principal components (Jombart et al., 2010). In contrast to Bayesian clustering methods, DAPC neither rely on a population genetic model (Hardy-Weinberg or gametic equilibrium, as does STRUCTURE) (Pritchard et al., 2000), nor this multivariate discriminant method is too computationally intense, and it is better at handling hierarchical structure or clinal variation within the populations (Jombart et al., 2010). The discriminant functions are based on linear combinations of alleles harboring the greatest variation between the clusters while minimizing variations between them (Jombart et al., 2010). This method differs from traditional PCA analysis as it minimizes within-group variability. We implemented DAPC in the ADEGENET package (Jombart, 2008) on R version 3.4.1 (R Core Team, 2017). The multivariate analysis defines the groups low levels of support for different numbers of potentially distinct genetic clusters in the absence of a priori population designation (Jombart et al., 2010). The optimal number of Principal Components (PCs) were retained and analyzed using a cross-validation method (and keeping the number of discriminant functions fixed, as proposed by the R's package ADEGENET). The number of PCs retained can have a substantial impact on the results of the analysis. Indeed, retaining too many components with respect to the number of individuals can lead to over-fitting and instability (Jombart and Ahmed, 2011). To explore how the genetic variation was partitioned among sampling sites, DAPC was executed by the function *dapc* using the sampling sites as a prior. Bayesian Inference Criterion (BIC) was used to determine optimal number of population clusters (K) using k values from 1 to 20 with functions *find. clusters* and *k-means,* to provide comparison with STRUCTURE and BAPS. The optimal number of clusters, K was chosen from the lowest value of BIC and subsequent scatterplots and bar plots were prepared. For a detailed cluster-assignment of specimens, we used the function *table(pop(x), grp $grp)* which display a table of the cluster vs sampling site, duly plotted on a comparative graph (function *table.value*).

To assess the significance of correlation between geographic and genetic (F_{ST}) distance matrices among the 15 sampling sites, Isolation by distance (IBD) was estimated using the Mantel test (Mantel, 1967) using the ADEGENET package of R, with the *mantel.randtest* function (999 permutations). As the correlation between genetic and geographic distances can occur under different biological scenarios, like continuous clines or distant patches, we visualized local densities of scatterplot of genetic and geographic distances using a two-dimensional kernel density estimation (function *kde2d*) in the MASS R package (Venables and Ripley, 2002).

Effective population size (*N*e) for each sample, based on the linkage disequilibrium (*LD*) model, was calculated in NeEstimator v. 1.3 (Peel et al., 2004). The dataset was also used to estimate demographic process, such as recent population bottleneck and/or population expansion, and the heterozygosity tests were used to analyze deviations from Mutation-Drift Equilibrium (MDE) for each sample across all loci. At selectively neutral loci, the expected heterozygosity was calculated from allele frequencies data (*H*e) assuming HWE, and from the number of alleles and sample sizes (Heq), assuming a population at MDE, are expected not to be significantly different. Thus, if a significant number of loci show *H*e > Heq, this indicates that the population recently experienced a bottleneck. The estimates of expected heterozygosity were calculated for three mutation models: infinite alleles model (IAM), stepwise mutation model (SMM) and the two-phase model (TPM). In this study, we used TPM with 95% single-step mutations and 5% multiple-step mutations as recommended for microsatellites (Piry et al., 1999). These analyses were performed using BOTTLENECK v. 1.2.02 (Piry et al., 1999).

3. Results

In this study, although the time period of sample collections ranged from 2005 to 2018 (a span of 13 years), temporal genetic studies of *Ae. aegypti,* with microsatellites and SNPs, have shown that allele frequencies and *N*e remain relatively constant over several years and the populations mostly stay in the same genetic grouping over time (Campos et al., 2012; Gloria-Soria et al., 2016b).

A total of 510 specimens of *Ae. aegypti* encompassing 15 Brazilian cities (Fig. 1 and Table 1) was genotyped for 12 microsatellite loci, totaling 6,210 genotypes. All loci were found to be polymorphic (Table S2), with a total of 238 alleles, varying from 3 (locus *AC5* from Itacoatiara and Taubaté) to 14 (locus *AG2* from Boa Vista). Considering all 15 samples, the highest number of alleles were observed for locus *AG2* (28) and the lowest number of alleles for locus *A1* (13). The highest mean allelic richness was observed in Cuiabá (CU) (8.690). Locus *AC1* was found to be in HW disequilibrium in most of the populations (12 out of 15), and also showed highest probability of null alleles in 6 out of 15 samples, followed by *B07* locus with probability of null alleles in 5 out of 15 samples. Eighty of 180 tests (44.44%) deviated significantly from HWE after Bonferroni correction and most of them (56) suggested heterozygote deficiency. The samples of Teresina (TS) and Cuiabá (CU) exhibited highest number of loci deviating from HWE, 11 and 9, respectively, indicating heterozygote deficiencies. The mean observed heterozygosity ranged from 0.526 [Teresina (TS)] to 0.800 [São José de Rio Preto (RP)], whereas mean expected heterozygosity ranged from

0.693 [Porto Velho (PV)] to 0.795 [Foz do Iguaçu (IG)]. The highest mean inbreeding coefficient (F_{IS} = 0.308) was observed in the samples of Teresina (TS), followed by Foz do Iguaçu (F_{IS} = 0.150). The remaining samples showed lower mean F_{IS} (0.004 − 0.085). Populations of Araçatuba (AR), São José de Rio Preto (RP), Itacoatiara (IT), Novo Airão (NA), Boa Vista (BV), Porto Velho (PV) and Guajará-Mirim (GM) showed mean negative values of F_{IS} (-0.001 to -0.095), indicating excess of heterozygotes.

Linkage disequilibrium (*LD*) analysis was carried out to confirm whether the deviations from HWE within samples were due to Wahlund effect, inbreeding, migration, selection or the presence of null alleles. 149 out of 990 (15.05%) locus-by-locus tests for *LD* were found to be significant ($P < 0.0005$), after the Bonferroni correction. The samples of Campina Grande (CG) and Macapá (MP) had the greatest number of significant pair-loci (both with 18), followed by Boa Vista (BV), with 15 (Table S3). In this analysis, no loci pair was consistently significant for *LD* across all 15 populations, indicating that they are not physically linked. This is consistent with the fact that most of the loci used in this study reside on different super contigs of the genome assembly of *Ae. aegypti* (Brown et al., 2011). However, the appearance of possible chromosomal inversions may link physically distant markers in some, but not all populations (Bernhardt et al., 2009).

A total of 51 private alleles were observed in 15 populations (Table S4). The samples of Macapá (MP) had highest number of private alleles (13), but at low frequencies, followed by Teresina (TS) and Foz do Iguaçu (IG) (both with 7), whereas Itacoatiara did not show any private allele (0). The highest frequency of private alleles was observed for Novo Airão (NA) (for one allele at locus *AC5*, with 19.4%), followed by Teresina (TS) (one allele at locus *B07*, with 18.6%; one allele at locus *AG5*, with 15.7%). Considering all 12 loci analyzed, locus *AC2* had the highest number of private alleles (8) followed by locus *B07* (7).

Table S5 presents the estimates of genetic differentiation (F_{ST}) and gene flow (*N*m) among the samples. All pairwise F_{ST} values were highly significant ($P = 0.0000 \pm 0.0000$), before and after the Bonferroni correction. The highest value (F_{ST} = 0.1845) was observed between the samples of Campina Grande (CG) and Macapá (MP), consequently they had lowest level of gene flow (*N*m = 2.2048). On the other hand, the lowest value of genetic distance (F_{ST} = 0.0317) was observed between Manaus (MA) and Itacoatiara (IT), both in state of Amazonas, consequently they showed the highest gene flow (*N*m = 15.2987).

AMOVA analysis including all 15 samples (no grouping) revealed highly significant genetic differentiation among them (F_{ST} = 0.1072; $P = 0.00000 \pm 0.0000$) (Table 2). For hierarchical level with two groups (Group 1: AR, RP, CU, CG, TA, IT, MA, NA, BV, RB, PV, GM; Group 2: IG, TS, MP), the analyses revealed no significant genetic structure between them (F_{CT} = 0.0219); but there was a significant genetic differentiation among samples within groups (F_{SC} = 0.10068; $P = 0.00000 \pm 0.0000$), which can be attributed to the differences within group 1 and group 2. For the hierarchical level with four population groups (Group 1: AR, RP, CU, CG; Group 2: TA, IT, MA, NA, BV; Group 3: RB, PV, GM; Group 4: IG, TS, MP), the percentage of variation was highly significant for all levels (F_{CT} = 0.03448; F_{SC} = 0.08188; $P = 0.00000 \pm 0.0000$). In all three hierarchical levels, the highest percentages of genetic variation were observed within the samples (89.28%; 88.15%; 88.65%, respectively).

In the STRUCTURE analysis (Fig. 2A), Evanno's method identified K = 2 (Figure S1) as the most probable number of genetic clusters and all populations revealed mixed ancestry. The first cluster grouped all samples from southeast [Araçatuba (AR), São José de Rio Preto (RP), Taubaté (TA)] and central west [Cuiabá (CU)], seven populations from north [Boa Vista (BV), Itacoatiara (IT), Manaus (MA), Novo Airão (NA), Rio Branco (RB), Porto Velho (PV), Guajará-Mirim (GM)] and one from northeast [Campina Grande (CG)]. The second cluster grouped one population each from north [Macapá (MP)], northeast [Teresina (TS)] and south [Foz de Iguaçu (IG)] of Brazil. The geographic distribution of these clusters is presented in Fig. 2C.

We also separately analyzed each of the two clusters (Cluster 1 and Cluster 2) generated from the previous STRUCTURE analysis. This analysis revealed the presence of genetic sub-structure within the major clusters (Fig. 2B). Evanno et al. (2005) method subdivided Cluster 1 into three sub-clusters (K = 3) (Figure S2) and Cluster 2 into two sub-clusters (K = 2) (Figure S3). The geographic distribution of these sub-clusters is presented in Fig. 2D.

Thirteen genetic clusters (C1 to C13) were identified by the Bayesian analysis implemented in BAPS [Log (marginal likelihood) of optimal partition = -23513.2655; probabilities for number of clusters = 13 (0.999)]. Out of these, only two clusters (C1 and C5) consisted of two samples. Araçatuba (AR) and São José de Rio Preto (RP), both from state of São Paulo, were grouped in cluster C1, whereas Manaus (MA) and Itacoatiara (IT), both from the state of Amazonas, were grouped in cluster C5. Each of the remaining samples was assigned to a different cluster (Figure S4).

The number of Principal Components (PCs) retained for DAPC analyses were calculated using a cross validation method implemented in *xvalDapc* function from R *ADEGENET* package. In this study, 100 PCs were retained with median and confidence interval for random chance de 97.5% (0.0880) (Figure S5). Based on these values, the first DAPC was implemented to observe the genetic variation among the sampling sites. Also, a bar plot of eigenvalues for the discriminant analysis was used to select 14 discriminant functions to be retained (Fig. 3). This

Table 2
Hierarchical analysis (AMOVA) of the genetic variation in the *Ae. aegypti* samples.

Groups of Samples	Source of variation	Degrees of freedom	Percentage Variation (%)	Fixation index
No Grouping (All)				
AR, RP, CU, CG, TA, IT, MA, NA, BV, RB, PV, GM, IG, TS, MP	Among population	14	10.72	F_{ST} = 0.1072***
	Within populations	1005	89.28	
Two Groups	Among groups	1	1.96	F_{CT} = 0.01958
(1) AR, RP, CU, CG, TA, IT, MA, NA, BV, RB, PV, GM				
(2) IG, TS, MP				
	Among populations within groups	13	9.89	F_{SC} = 0.10068***
	Within populations	1005	88.15	F_{ST} = 0.11846***
Four Groups	Among groups	3	3.45	F_{CT} = 0.03448***
(1) AR, RP, CU, CG				
(2) TA, IT, MA, NA, BV				
(3) RB, PV, GM				
(4) (4) IG, TS, MP				
	Among populations within groups	11	7.91	F_{SC} = 0.08188***
	Within populations	1005	88.65	F_{ST} = 0.11354***

Significance test 10,000 permutations, F_{ST} = fixation index within samples, F_{CT} = fixation index between regions, *Fsc* = fixation index among samples within regions.

*** $P = 0.00000 \pm 0.00000$. See Table 1 for locality abbreviations.

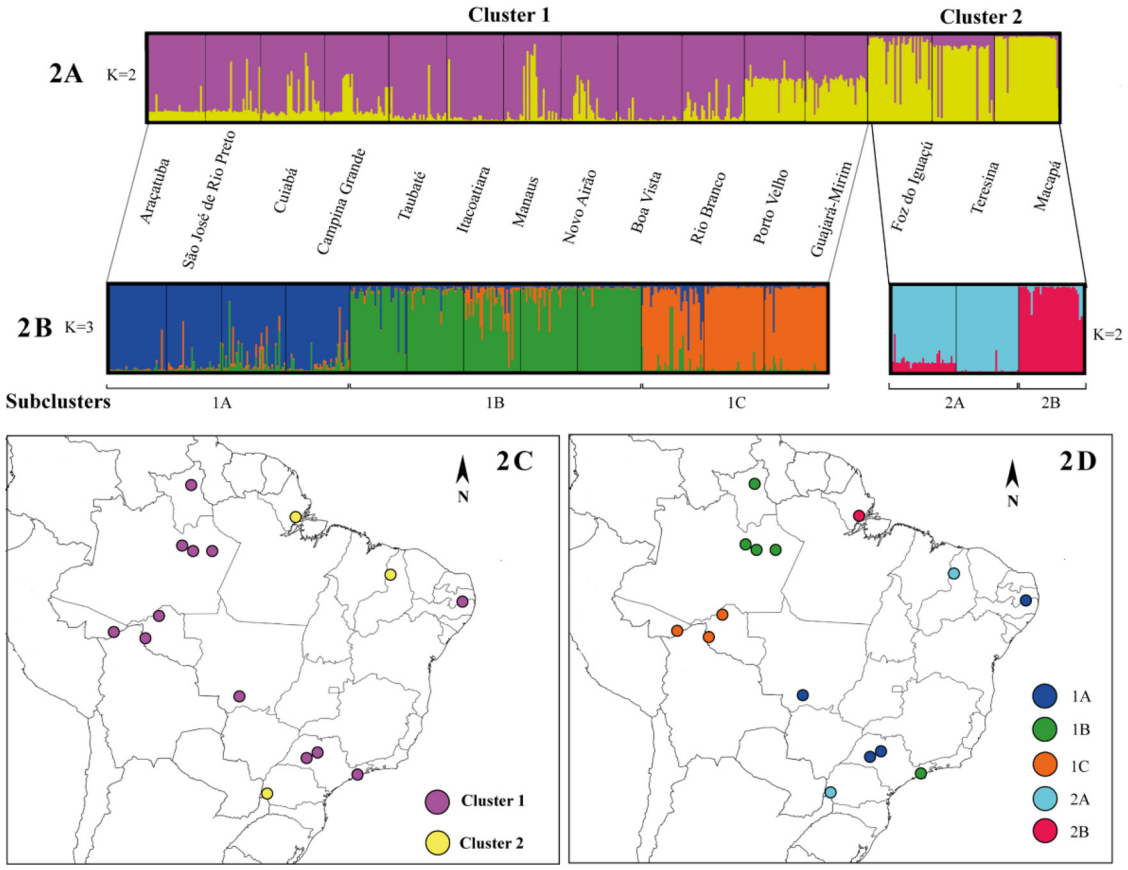

Fig. 2. STRUCTURE bar plot for all *Aedes aegypti* populations used in this study. Each vertical bar along the plot represents an individual. The height of each color represents the probability of assignment to a specific cluster. Black lines within plots indicate population limits. A) Subdivision of all the individuals into K = 2 clusters. B) Sub-cluster 1, K = 3; Sub-cluster 2, K = 2. C) Geographical distribution of two major clusters. D) Geographical distribution of 5 sub- clusters.

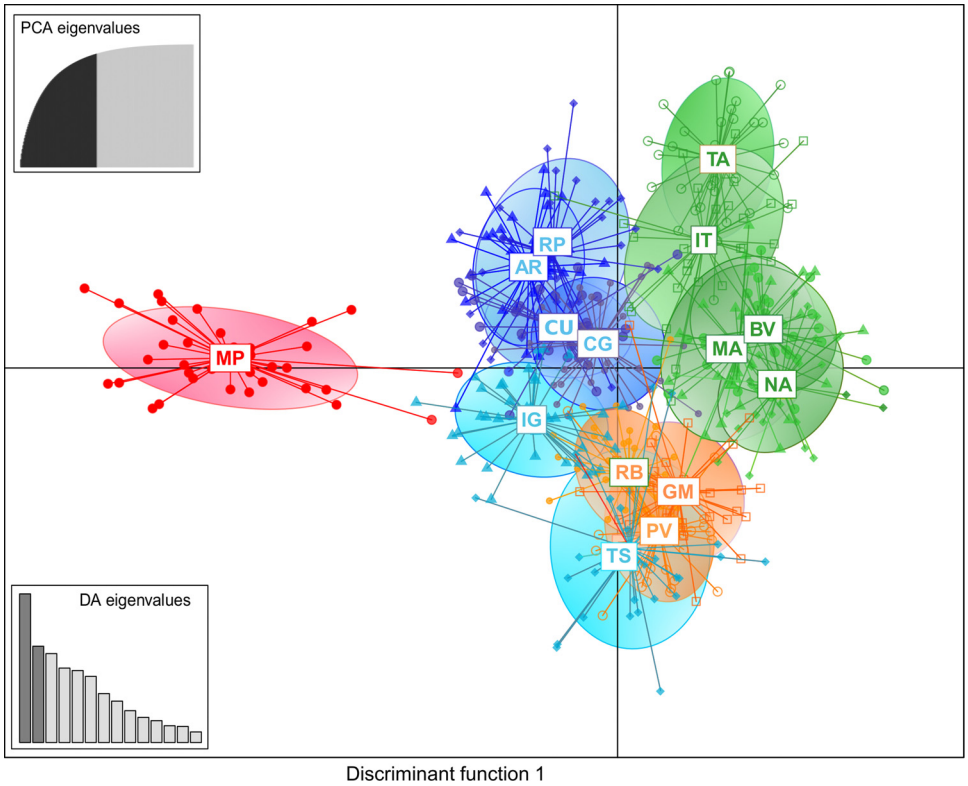

Fig. 3. Discriminant analysis of principal components (DAPC) scatterplot for the 15 sampling sites of *Aedes aegypti* and populations as priors. The optimal number of principal components (PCs = 100) was retained as determined by DAPC cross-validation and 14 discriminant functions. DA and PCA eigenvalues of the analysis are displayed in insets. In this plot, populations were selected a priori based on regional location and the first two principle components served as the axes. The graphs represent the individuals as dots and the groups as inertia ellipses. The colors correspond to the five sub-clusters identified by STRUCTURE analysis (1 A: dark blue; 1B: green; 1C: orange; 2 A: light blue; 2B: red).

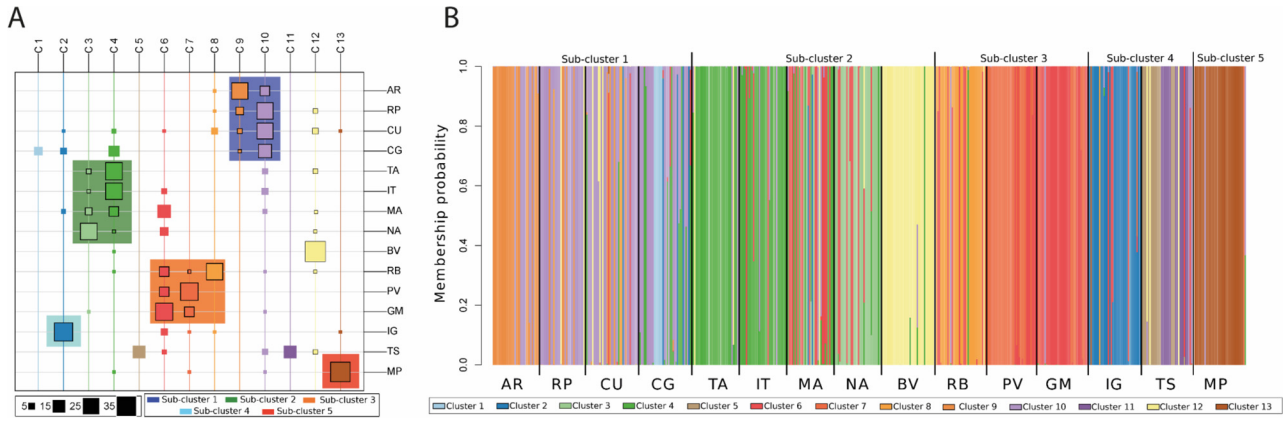

Fig. 4. Population structure of Brazilian populations using DAPC. A) The composition of each of the 13 inferred genetic clusters; larger black box indicates more individuals. The scale of the quantity of specimens for each cluster is presented by the squares from 5 to 35 at the bottom of the figure. For example, inferred cluster 1(C1) contains only few individuals from Campina Grande and inferred cluster 9 (C9) contains a mixture of the individuals from Araçatuba, São José de Rio Preto, Cuiabá and Campina Grande. The colors employed in the clusters are the same as sub-clusters of Structure as in Fig. 2B. B) Membership probabilities assigned to 13 inferred genetic clusters for *Aedes aegypti* individuals. See Table 1 for population abbreviations.

analysis yielded results similar to those retrieved in STRUCTURE (Fig. 2B); therefore, the same colors of sub-clusters were used here to facilitate the comparison. The scatterplot of DAPC based on the sampling sites (*a priori*), revealed that specimens from Macapá (MP) were markedly isolated from other samples (Fig. 3). The remaining samples were distributed in three following groups: 1) TA, IT, MA, NA and BV; 2) RB, PV and GM plus TS; 3) AR, RP, CU and CG. The scatterplot ellipse represented by Foz de Iguaçu (IG) was overlapped partially with the ellipses of the groups 2 and 3.

Based on the Bayesian Inference Criterion (BIC), 13 genetic clusters were identified (BIC = 783.442; K = 13) (Figure S6), which were similar to the analysis of BAPS. Though some of the scatterplot ellipses were overlapped with each other, at least four clearly defined groupings of ellipses were observed (Figure S7): cluster 13 (represented by 33 specimens of Macapá), cluster 5 (represented by 14 specimens of Teresina), cluster 11 (represented by 14 specimens of Teresina) and remaining overlapped ellipses representing one group. Most of the samples were attributed their respective clusters with > 85% of membership probability, as can be observed comparing the composition of each genetic cluster (Fig. 4A), as well as the bar plot (Fig. 4B). Moreover, it was observed that the composition of some genetic clusters can be compared with the sub-clusters retrieved by STUCTURE (Fig. 2B). The clusters C9 and C10 (Fig. 4A) corresponded to the sub-cluster 1A (Fig. 2B), represented by samples from AR, RP, CU and CG; the clusters C3 and C4 corresponded to the sub-cluster 1B (Fig. 2B), represented by a major quantity of samples from TA, MA, IT and NA; the clusters C6, C7 and C8 corresponded to the sub-cluster 1C (Fig. 2B), represented by a major quantity of samples from RB, PV and GM; the cluster C2 corresponded to the sub-clusters 2 A (Fig. 2B), represented by a major quantity of samples from IG and, the cluster 13 corresponded to the sub-cluster 2B (Fig. 2B), represented by major quantity of samples from MP. Only the samples from BV (cluster C12) and TS (clusters C5 and C11), could not be compared with the sub-clusters of STUCTURE.

The Mantel test analysis revealed a weak, but significant correlation between genetic and geographic distances ($r = 0.31$, $P = 0.004$) among 15 sampling sites, covering a range of ˜145 to 3500 km. This result indicates that ˜ 31% genetic differentiation observed can be explained by IBD model (Fig. 5A). The two-dimensional kernel density estimation indicated a patched pattern of genetic differentiation among sampling sites, due to the two clouds observed, a medium density (between 500 and 1500 km) and the other with a high density (between 1800 and 2800 km) (Fig. 5B).

Table S6 presents the effective population size (*Ne*) estimates, based on *LD* model. The lowest *Ne* value was observed in the sample of

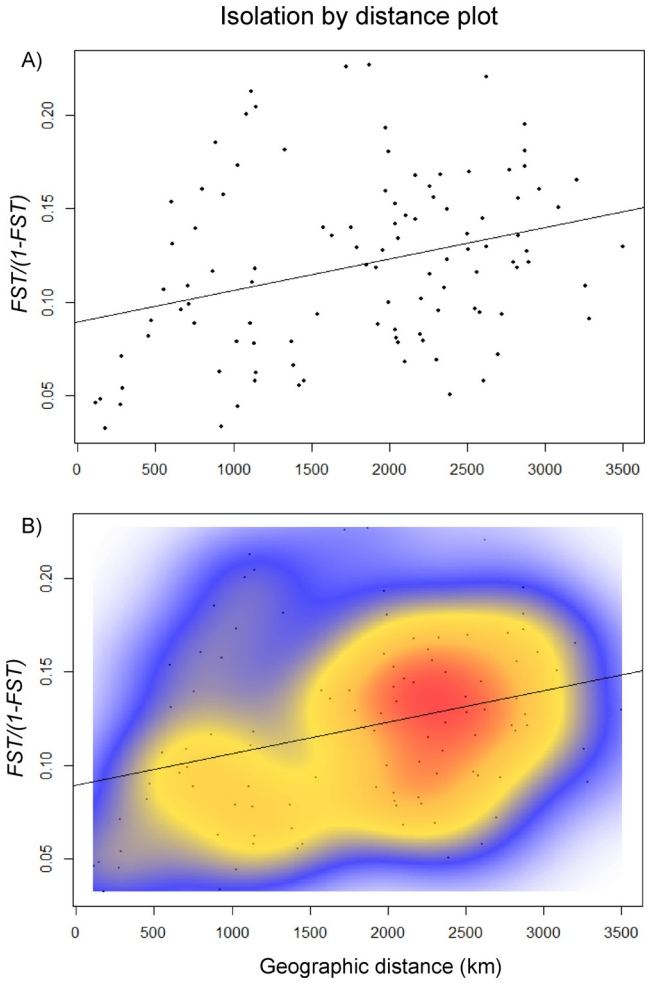

Fig. 5. Isolation by distance (IBD) analysis among 15 samples of *Aedes aegypti* ($r = 0.031$, $p = 0.004$). A) Pairwise $F_{ST}/(1 - F_{ST})$ distances plotted against geographic distances. B) Local density of points plotted using a two-dimensional kernel density estimation. (Line correlation trend is shown; colors represent the relative density of points: blue: low density; yellow: medium density; red: high density).

Campina Grande (CG) (11.8) and the highest value was observed in the sample of Itacoatiara (IT) (∞). The overall average *Ne* value was 75.1, with a 95% CI from 67.8 to 83.2.

The heterozygosity tests were performed under IAM, SMM and TPM models (Table S6). Under IAM, heterozygote excess was detected for all the populations tested, as well as all of them were statistically significant ($P < 0.05$). Under SMM model, two [Araçatuba (AR), and Manaus (MA)] populations showed significant heterozygosity excess. For the TPM (95%), six populations [Araçatuba (AR), Campina Grande (CG), Itacoatiara (IT), Manaus (MA), Porto Velho (PV) and Guajará-Mirim (GM)] showed significant heterozygote excess. The populations of Araçatuba (AR) and Manaus (MA) had significant heterozygosity excess for all three mutation models tested, indicating that these populations might have experienced a recent bottleneck.

4. Discussion

4.1. Genetic clusters and gene flow patterns

The findings of this study, based on genetic distances, AMOVA and cluster analysis, indicated a low to moderate genetic differentiation (Wright, 1978) and the existence of two major genetic clusters of *Ae. aegypti* circulating in Brazil. The Structure analysis (Fig. 2) also indicated that these clusters were sub-structured in five sub clusters. These sub clusters showed higher level of genetic differentiation among them than between the two major clusters, as can be seen in AMOVA and DAPC analyses (Table 2; Figs. 3 and 4). The presence of genetic clusters and sub-clusters in *Ae. aegypti* populations of Brazil has also been reported in studies of Monteiro et al. (2014); Gloria-Soria et al. (2016a) and Kotsakiozi et al. (2017).

This study revealed that most geographically closer populations were genetically similar and were included in the same sub cluster [Araçatuba (AR) and São José de Rio Preto (RP); Rio Branco (RB), Porto Velho (PV) and Guajará-Mirim (GM); Manaus (MA) and Itacoatiara (IT)], suggesting gene exchanges ($Nm > 1$).

Samples of Rio Branco (RB), Porto Velho (PV) and Guajará-Mirim (GM) were included into sub cluster 1C (Figs. 2 and 3) and had low F_{ST} values (0.04 - 0.07). This subcluster was genetically separated from other northern samples, such as BV, MA, IT and NA. Guajará-Mirim (GM), located at the border between Brazil and Bolivia, may have been an entry point of a new genetic group of *Ae. aegypti* from Bolivia, which later spread to PV and RB, due their geographical proximity and well-connected highway network among these cities. Considering the genetic similarity between Manaus and Itacoatiara, Manaus was the first city in the state of Amazonas to be infested with *Ae. aegypti* in 1996 (Figueiredo, 2003), which caused a DENV-1outbreak two years later. Manaus and Itacoatiara are connected by both highway and fluvial routes; thus, we think that the population of Itacoatiara (IT) may have been established by two or more waves of colonization from Manaus (MA). When we (VMS) collected mosquito in Itacoatiara, *Ae. aegypti* was present in high density throughout the city, and all human dwellings were found to be positive, indicating adaptation of this mosquito those environment. These observations are consistent with the large $Ne = \infty$ and lack of private alleles in this sample.

On the other hand, geographically distant populations also showed similarity genetic and were grouped together in a same sub cluster – 1B (Manaus/Itacoatiara/Novo Airão and Taubaté). Considering that *Ae. aegypti* has a low flight range varying from 50 to 800 m (Honório et al., 2003; Harrington et al., 2005), this genetic connectivity between distant localities might be attributed to its passive dispersal mediated by human and commercial traffic by highways, fluvial and air networks (Bosio et al., 2005; Gonçalves da Silva et al., 2012; Kotsakiozi et al., 2017). As Manaus was infested by *Ae. aegypti* in 1996, several years after the infestation have occurred in southeastern Brazil (Figueiredo, 2003); it is possible that the mosquitos which have colonized Manaus may have been introduced from southeastern Brazil. In Manaus, *Ae. aegypti* was collected for the first time in the neighborhood Praça 14 de Janeiro. Later, Scarpassa et al. (2008), analyzing *COI* gene, observed that the mosquitoes collected from this neighborhood shared haplotypes with specimens from Taubaté. Taken together, these findings could explain the genetic connectivity between Manaus/Itacoatiara/Novo Airão and Taubaté. Our data and Scarpassa et al. (2008) suggest that the populations of *Ae. aegypti* from north region (especially Manaus) may have suffered at least two colonization events, one from southeastern Brazil and the other from northern region (Boa Vista). This finding differs from the hypothesis of Kotsakiozi et al. (2017) that the southern Brazilian populations were colonized by the northern populations, and not the other way around.

The sample of Cuiabá (CU) was genetically closer to Araçatuba (AR), São José do Rio Preto (RP) and Campina Grande (CG) and clustered into sub cluster 1 A. The locality of Cuiabá can represent an admixture zone, where it receives migrants of AR, RP and CG and also migrants represented by the sub clusters 1B and 1C (Figures 2B and 4). This hypothesis is consistent with the highest number of alleles, allelic richness and deviations of HWE found in this population (Table S2).

On the other hand, the sample of Foz do Iguaçu (IG) was genetically different from other populations analyzed in this study. Foz do Iguaçu city, located at the border of Brazil, Argentina and Paraguay, is an important tourist attraction site with an intense flux of human and trade, thereby favoring the introduction of new genetic group of this vector. Similarly, the population of Teresina (TS) was genetically isolated. The geographical location of this city and its peculiar climate (hot and dry) may have created favorable conditions (=genotypes more adapted these conditions) to originate a genetically differentiated population from the remaining populations analyzed (Louise et al., 2015; Wilke et al., 2017) or this population may represent a distinct lineage introduced from other region not studied here.

Considering that private alleles are an indirect estimator of gene flow (Slatkin, 1985), the largest number of private alleles (13) verified in the sample of Macapá indicate a reduced gene flow with other populations analyzed in this study, resulting in its highest genetic divergence (Table S5). Kotsakiozi et al. (2017) reported very similar findings, where the highest differences were found between Macapá/Belém/Santarém and remaining samples from Brazil. Monteiro et al. (2014) reported highest differences between Marabá/Tucuruí and remaining samples from Brazil. Lima Júnior and Scarpassa (2009) had also observed highest number of private haplotypes in Belém. Belém is a busy port city with a gateway to Amazon river and situated ˜ 100 km upriver from Atlantic Ocean. Macapá, the capital city of Amapá state (shares its border with Suriname and French Guiana), is located on the northern channel of Amazon river near its mouth on the Atlantic Ocean. These cities and the neighboring areas may be favorable points for the introduction of novel genetic groups of this vector. Taken together, we can propose that the *Ae. aegypti* from Santarém, Belém, Marabá, Tucuruí (state of Pará) and Macapá (state of Amapá) – (ranged from central to north-eastern Brazilian Amazon) – may consist of a separate genetic group from the remaining populations of Brazil.

Thus, the genetic structure observed among samples of *Ae. aegypti* of the present study is in part explained by IBD model, especially among geographically closer localities, and also by passive dispersal mediated by human (commercial roads, highways, river and air routes) and by new introduction events. Additionally, in each locality, the subpopulation of this vector is exposed to distinct environmental and climatic conditions (also socioeconomic), besides the urbanization and the intensity of the vector control measures, which can expand or contract its size. Such populational dynamic is under action of microevolutionary processes (genetic drift, selection-adaptation) that directly acts at the intrapopulation genetic diversity levels. This complex dynamic influence the levels of gene flow causing genetic structure in the populations of this vector within and among regions and localities by throughout country. Brazil is a country of continental extension comprising very diverse environmental and climatic conditions, as well as

various cities with different models of urbanization. In addition, its extensive border sharing with several neighboring countries and sea favors the introduction of new genetic groups of this mosquito, impacting the vector competence and the response to control programs.

4.2. Demographic changes among the populations

The relatively low *N*e values observed in the samples of this study are in accordance with those obtained for four others samples of this vector from Manaus analyzed by Mendonça et al. (2014) and for the two Brazilian samples from Jacobina and Cachoeiro analyzed by Saarman et al. (2017). However, the *N*e values observed in this study were seven-fold lower when compared with *N*e values observed in anopheline malaria vectors (Scarpassa and Conn, 2007). This finding may be related to frequents founder and bottleneck effects that the populations of *Ae. aegypti* are exposed, which reduce the *N*e sizes, and are exactly the low *N*e value intrapopulation that lead to genetic differentiation between populations of this vector by genetic drift. In this study, this was clearly evidenced in the samples of CG and MP; both had the lowest *N*e values and, consequently, the highest genetic distance was observed between them. Interestingly, these samples also exhibited the highest number of significant loci-pairs for *LD* test. The low *N*e may have magnified differentiation between them by genetic drift causing *LD*. A very similar result was found in one population of *Anopheles nuneztovari* that probably consists of two distinct species (Scarpassa et al., 2016) and also in *Anopheles darlingi* (Scarpassa and Conn, 2007). Taken together, these evidences suggest that the high number loci-pairs in *LD* (15.05%) of this study may be in part due to microevolutionary processes (mutation, inbreeding, gene flow, selection, genetic drift or mixture of subpopulations with different allelic frequencies) acting on the populations of *Ae. aegypti* (Templeton, 2011), a consequence to its own populational dynamics.

The genetic bottleneck analysis indicated that none of the samples analyzed in this study appear to be expanding. On the contrary, a significant signal of bottleneck effect was observed in the samples of Araçatuba (AR) and Manaus (MA), under the three mutation models tested, and in the sample of Campina Grande (CG), Itacoatiara (IT), Porto Velho (PV) and Guajará-Mirim (GM) under the two models tested (IAM and TPM). These tests indicate that these populations experienced a recent bottleneck event, which are mostly in agreement with the *N*e sizes observed in these samples (Table S6).

Every population of this study have exhibited different demographic dynamics, such as low *N*e with signs of bottleneck [Campina Grande (CG), and Aracatuba (AR)], high *N*e with no signs of bottleneck [Foz do Iguaçu (IG), São José de Rio Preto (RP), Taubaté (TA), Cuiabá (CU), Rio Branco (RB)], low *N*e with no signs of bottleneck [Macapá (MP) and Teresina (TS)] and high *N*e with signs of bottleneck [Itacoatiara(IT)]. As discussed above, this demographic dynamic may be a consequence (=adaptation) of different environmental and climatic conditions, urbanization and intensity of vector control measures that the populations of *Ae. aegypti* are exposed at local levels, resulting in the genetic differentiation between localities regardless of geographic distance.

This knowledge is important for the control programs - in subpopulations with very low *N*e, the genetic drift effect may be more intense and to cause loss of genetic diversity in few generations. Thus, in genetic modification programs, the *N*e size of the target population should be a parameter of extreme importance to be considered to achieve desired success in the control of this vector.

Additional studies with *Ae. aegypti*, involving the use genotyping by sequencing with nextRAD markers to detect SNPs, which provide higher resolution of genetic structure and analysis of relatedness individuals, are needed to help understand better the populational dynamic, adaptative plasticity, biology and ecology of this vector, with the goal to reduce outbreaks these diseases in Brazil. In addition, the strong commitment of the people is also extremely important in the fight against this powerful vector.

Conflict of interest

The authors declare that they have no conflict of interest.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.actatropica.2019.04.027.

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