Comparative Genetics Using Three mtDNA Markers in *Aedes aegypti* (Linnaeus) Populations from Four Municipalities in the State of Mato Grosso, Brazil.

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Research Article

Keywords: Comparative Genetics, Three mtDNA Markers, *Aedes aegypti* (Linnaeus)

Posted Date: August 12th, 2021

DOI: <https://doi.org/10.21203/rs.3.rs-785088/v1>

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Abstract

Aedes aegypti mosquito has spread throughout the tropical and subtropical world and is currently the primary species responsible for transmitting dengue, urban yellow fever, Chikungunya, and Zika virus. This study aimed to investigate the inter- and intrapopulational genetic variability of *Aedes aegypti* through mitochondrial DNA, COI, ND4, and ND5 molecular markers in four municipalities in Mato Grosso. We used the Geneious software to build dendrograms for differentiating populations from each municipality. The interpopulational genetic distance obtained from sequence analysis showed a difference within populations through groups' formation in the ordering. Besides, we identified a difference in the interindividual genetic distance values, notably for the ND5 gene from the populations captured in the four municipalities. We recorded the smallest interindividual genetic distance within populations for populations from Chapada dos Guimarães. Extrinsic factors, including breeding habitat removal, can contribute to decreasing variability. Consequently, the dendrogram showed some similarities. Ovitrap monitoring, vector elimination, and genetic flow investigation stimulate actions to prevent transmitted diseases and support essential effective measures to control and fight *Ae. aegypti*.

Introduction

Aedes (Stegomyia) aegypti mosquito (Linnaeus, 1762), a mosquito native to Africa¹, has spread throughout tropical and subtropical areas due to anthropogenic activities. Today, *Ae. aegypti* is responsible for transmitting dengue, yellow fever, Chikungunya, and Zika virus infection. Dengue is currently one of the most frequent diseases in Brazil². Its annual variations in reporting and expansion is directly related to several factors, such as favorable environment, new serotype circulation, human population movements, and vector local infestation level. For this reason, *Ae. aegypti* adaptation to urban environments favors anthropic long-distance dispersion, supported by the eggs' resistance to desiccation and human host³.

An alternative monitoring method of *Ae. aegypti* is the detection and quantification of eggs deposited in oviposition traps installed in the home environment, which allows the identification of areas with the presence of mosquitoes and the analysis of the spatial and temporal distribution of its population⁴. An essential tool in this vector's study is the capture employing ovitraps to control and monitor populations by obtaining a significant number of specimens. This tool provides information on the presence and population density in different environments that can be estimated using samples, based on genetic methods⁵. Population diagnosis of *Ae. aegypti* through ovitraps allows checking the presence of mosquitoes throughout the year by attracting the pregnant female to oviposition⁶. A study in China, which employed this capture method, showed that vector control is critical and sometimes the only effective way to block or decrease dengue transmission⁷. Infestations can increase according to higher temperatures, humidity⁸, and disordered urbanization.

The genetic diversity of some South American populations have been assessed using a wide range of genetic markers. Mitochondrial DNA (mtDNA) genes are widely used for the identification of genetic variants, dispersal patterns, phylogeny, and population dynamic studies of *Ae. Aegypti*^{9,10}. Besides the populational quantification of *Ae. Aegypti*, the literature reports the use of markers, including mitochondrial DNA^{4,11}, especially the Cytochrome Oxidase I (*COI*) gene, widely used in culicid research, to analyze its genetic variability¹². Molecular techniques, including the polymerase chain reaction (PCR), can contribute to the understanding of vector-human relationships and also enable the genetic study of populations¹³ and their diagnostic investigation in different biological samples¹⁴.

The evaluation of genetic diversity on a smaller scale, such as in cities, allows us to verify viral dispersion. *Ae. aegypti* genetic diversity analysis might be applied to know the structure of the populations to comprehend their dynamics, providing data that might lead to new control measures¹⁵. Several genetic mechanisms are known to generate variability within and between populations, whose differences can arise from random occurrences, including the genetic composition of specimens that disperse and create new populations¹⁶. In addition, there may be changes in allele frequencies resulting from occasional breeding in small¹⁷ or large populations, whose effect can be underestimated in short periods. Differences between populations under different environmental conditions for survival and reproduction (adaptive value¹⁸) can accumulate and result in the development of a new species¹⁹.

Mitochondrial DNA (mtDNA) is an excellent target for molecular analysis due to the absence of introns, haploid inheritance, and because it contains only genes associated with mitochondrial functions. mtDNA is used in population genetic analysis²⁰ and traces a species' evolutionary history²¹. Interestingly, repair mechanisms for this molecule have not yet been described, which likely facilitates the accumulation of mutations and, consequently, the detection of intraspecific variations²¹. Base substitutions, mtDNA deletions, and tRNA-encoding gene translocations cause changes in gene order between phylogenetically related organisms²².

Given the above, studies on the genetic variability of mosquitoes transmitting prominent urban viruses are critical in warmer regions with favorable environments for *Ae. aegypti* proliferation, as in the state of Mato Grosso. Therefore, this research aimed to investigate the inter- and intra-populational genetic variability of this vector in four municipalities in Mato Grosso by using three mitochondrial DNA markers: *COI*, *ND4*, and *ND5*.

Results

Genetic Distance with Dendrograms.

Of the 400 specimens identified as *Ae. aegypti*, 169 underwent PCR using *COI*, *ND4*, and *ND5* primers. However, due to the low annealing capacity, the amplifications occurred in about 30% for *COI* primer,

which had the lowest annealing rate (Table 1). The *ND5* primer presented the best genetic variability results.

Through the Geneious application, we ordered the three primers to identify the different populations in each municipality. The dendrograms ordered the specimens' distance between the municipalities (interpopulational, Fig. 1) and within each municipality (intrapopulational, Fig. 2). We also observed that the obtained sequences had interindividual differences within populations, especially when considering each municipality's sampling location. The most distant specimens indicate that there is a genetically variation, which may arise from a transitory individual into this population.

Table 1
Number of post-sequencing samples from the four municipalities analyzed.

Municipalities	Primers		
	COI (F/R)	ND4 (F/R)	ND5 (F/R)
Cuiabá	7	11	10
Várzea Grande	8	11	10
Santo Antônio do Leverger	5	11	8
Chapada dos Guimarães	7	10	11
Total	27	43	39

In the interpopulation analysis of the trees generated, we verified distances that only one specimen from the municipality of CB was significantly distant from the others. This finding was based on observations from the COI mitochondrial gene (Fig. 1). Similarly, one sample based on this gene in the municipality of VG. Lastly, for CP and SA municipalities, we did not observe any significant genetic distance among the evaluated specimens. However, intrapopulation analysis of these three genes together (Fig. 2) showed a significant distance, especially in VG municipality with COI gene.

Genetic Distance by Analysis of Variance - ANOVA between Factors.

We identified highly significant interpopulational genetic distance values between *Ae. aegypti* specimens in the different municipalities, especially for the *ND5* gene (Fig. 3). The values were: CB ($F_{2,25}=29003$; $p = 0.0000$), VG ($F_{2,26}=24492$; $p = 0.0000$), CP ($F_{2,25}=76,577$; $p = 0.00000$) and SA ($F_{2,21}=50186$; $p = 0.0000$). In CP, the *ND4* gene presented the lowest interpopulational genetic distance among the analyzed specimens (Fig. 4A). Comparatively and significantly ($F_{6,97}=5.4826$, $p = 0.00006$), we considered the sequence of the three primers together between the four municipalities (Fig. 4B), and the municipality with the shortest distance was also CP.

We considered intrapopulation differences in the genetic distance for each gene (Fig. 5). The most significant intrapopulation genetic distance was recorded for the *COI* gene from specimens captured in the municipality of CB ($F_{2,25} = 34562$; $p = 0.0000$). The recording in the VG municipality was significant, with low variability for all primers ($F_{2,26} = 34.069$; $p = 0.000$). In CP ($F_{2,25} = 1169.2$; $p = 0.000$), the *ND5* gene reached the highest genetic distance. We observed a trend of less variability in the *ND5* gene in the intrapopulation genetic distance of specimens from the municipality of SA ($F_{2,21} = 19.911$; $p = 0.000$).

Gene polymorphisms and DNA sequencing.

We sequenced the segments of the mitochondrial DNA *COI*, *ND4*, and *ND5* genes using the Sanger method, whose factorial ANOVA results derived from nitrogenous bases sequencing for each gene. Based on the sequencing results of the three genes combined, the Kruskal-Wallis test demonstrated significant differences ($H_{33,52} = 69.53$; $p = 0.000$) compared to the three genes analyzed and frequency values between genes (Fig. 5; $F_{8,194} = 245.24$; $p = 0.000$). However, when we analyzed specimens from the four municipalities, the frequency values of adenine, thymine, cytosine, and guanine did not present significant differences (Fig. 5; $F_{9,396} = 0.32682$; $p = 0.966$).

Genetic diversity values were high ($h = 0.702$; $p = 0.015$) for the four municipalities. Such analysis allowed us to identify the greatest genetic diversity, which indicated that the variation occurred within populations, with an F_{ST} value of 0.329.

Haplotypic analysis map using Median Joining.

Based on genes *COI*, *ND4*, and *ND5* a network haplotype of *Ae. aegypti* populations from Baixada Cuiabana has complemented this research (Fig. 6). Under this, *COI* was represented with a total number of mutations of S/Eta: 54, the variance of haplotype diversity: 0,01009, and nucleotide diversity, P_i : 0,02972. For *ND4*, the number of polymorphic (segregating) sites was S: 48, the total number of mutations Eta: 61 and, the variance of Haplotype diversity: 0,00358; the Nucleotide diversity had the P_i : 0,03916. In this same analysis for the *ND5* gene, the number of polymorphic (segregating) sites was S: 19, the total number of mutations was Eta: 19 and, the variance of haplotype diversity: 0,00210 with nucleotide diversity, P_i : 0,00396.

Discussion

Our study is the first to investigate the genetic variability of *Ae. aegypti* populations in municipalities of Mato Grosso. We observed a genetic variation in *COI*, *ND4*, and *ND5* mtDNA sequences between specimens per analyzed location, with inter- and intrapopulation genetic distance. These genes accumulate base substitutions in the mitochondrial genome most rapidly³³. In this study, when

populations analyzed together and by gene, the distance found unlikely represents populational fragmentation nor events that could significantly distance a representative sample of individuals.

Birungi and Munstermann²⁶ investigated *Ae. albopictus* in Brazil and the United States and stated that the effect of genetic drift is more pronounced in mtDNA than in nuclear *loci*. The present study results showed variability in the inter- and intrapopulational genetic distance for the *Ae. aegypti* populations analyzed, notably for the *ND5* gene in between municipalities' populations. *Ae. aegypti* specimen analysis confirmed that these markers are essential in population sampling.

The dispersal of mosquitoes over long distances only occurs passively (eggs and adults transported), which may explain the diversity of some haplotypes in different locations. The shortest genetic distance between genes was found for *ND4* in specimens captured in CP, with an exception in one sample only. Ponce et al.¹⁰ compared Ecuadorian populations of *Ae. aegypti* from 17 sites and revealed the presence of only two haplotypes. The variations detected between *ND5* gene sequences in the municipalities may indicate a genetic structure in *Ae. aegypti* resulting from several factors, including extinction and recolonization events, genetic drift, and geographical differentiation.

Interpopulational genetic distance values for the *COI* gene showed low variability in the municipalities. However, population genetic analyzes of *Ae. aegypti* performed by Lv et al.³⁴ identified a relatively high degree of polymorphism in the *COI* and *ND4* sequences in eight populations, which were divided into eleven haplotypes. In studies using *ND4* primer by Scarpassa et al.³⁵, the polymorphism values were higher for the nucleotides, the same as what we found, probably because in *Ae. aegypti* this gene is under most mutations in many samples. In some studies, distance isolation may not be significant, indicating that genetic distance is not always linked to geographic distance ($r=-0.1216$ and $p = 0.755^{36}$).

The genetic differentiation study of 15 populations from Maranhão³⁷, based on the mitochondrial marker *ND4*, found 15 haplotypes among the polymorphic sites. It revealed that most of the variation (58.47%) was found within populations. In our study, fewer haplotypes variations occurred using *ND5* and confirmed the total separation per marker applied. Of the 71 distinct haplotypes, *ND4* represented 41% of the variation, *COI* with 35%, and *ND5* with 24%. Few haplotypes were evidenced with a high mutation rate or variation about the others, within the same gene. The *ND4* gene had the smallest interpopulation genetic distance among the analyzed specimens, especially in the municipality of Cuiabá.

Regarding the four municipalities analyzed in this study, the populations had specimens that were distant because of the *ND5* gene, as shown by the genetic diversity values ($h = 0.702$; $p = 0.015$) and the F_{ST} value of 0.329, which indicated more considerable intrapopulational variation. Highly significant genetic distances can suggest speciation events. We identified a difference in the interindividual genetic distance values, notably for the *ND5* gene from the populations captured in the four municipalities. Thus, our results show a robust hypothesis that new morphospecies is adapted to local environmental conditions, although morphological data is still lacking to support this assumption.

Specimens with the most considerable genetic distance may originate from distinct lineages from areas adjacent to the capture site or reflect the natural selection. They might also result from passive transport by human movement with vehicles and cargo³⁸. Barbosa et al.³⁹ mention that the passive dispersion of vectors found mainly in tires started due to the intense tire trade between the municipalities of the regions where the first outbreaks occurred (in the state of São Paulo), with subsequent dissemination. Thus, tires can become a severe problem for public health.

The species populations from other microhabitats introduced into adjacent areas may share polymorphisms within the same and between different populations. However, the local gene flow can also be stopped due to geographical barriers; thus, no new characteristics are shared⁴⁰. In the present study, we found no significant barriers between populations with the highest genetic distance and intrapopulation variation.

The ordering using dendrograms with the highest genetic distance indicates the dissimilarity between some *loci*, as found for the *ND4* gene in CP in the interpopulational analyses. Population-extrinsic factors, including breeding habitat elimination, may contribute to the low variability at some points, with some dendrogram similarity per analyzed gene. Eleven *ND4* gene and 10 *ND5* gene specimens formed a distinct group in the alignment of 28 individuals in the municipality of CB. Such formation confers distance and genetic variability between the specimens within the populations analyzed. Similarly, this observation was also present in the other investigated cities, and the *ND5* gene presented variability between populations, which may be related to old and repeated introductions of the species into different habitats. This statement is supported by Seixas et al.³ in an article on the colonization and populational diversity of *Ae. aegypti* on Madeira Island, Portugal.

In the municipality of CB, we statistically recorded the highest intra-population variability for the mitochondrial *COI* gene. With analyses of this gene, Van de Vossemberg et al.²¹ identified specimens in different groups formed in dendrograms. These results are in keeping with those from this study, which can imply intraspecific variation between *Ae. aegypti* specimens. According to Seixas et al.³, different specimens analyzed based on *COI* exhibit greater vectorial competence than other populations with a single haplotype. The higher the genetic variability in these mosquitoes, the greater likelihood they will disperse viruses and other disease-causing parasites.

Factorial ANOVA results for the investigated genes of each *Ae. aegypti* specimen showed the differences derived from variations between populations, leading to a genetic distance within each municipality. In general, the populations' genetic profile varies by place throughout the distribution of a species. These differences can arise from random occurrences, including the genetic composition of specimens that disperse and create a new population¹⁴.

Population-intrinsic changes can suggest the influence of genetic distance, with the consequent genetic drift of rare or restricted genes to a single population or geographically close populations, as the municipalities of CB and VG. More considerable genetic distances between specimens for the *ND5* gene

in these municipalities can be associated with multiple introductions linked to different strains, as their habitats have high movement of people, passive dispersion patterns, and infestation control activities.

Based on nucleotide differences and sequencing, given the nitrogenous base proportionality, molecular markers did not show differences between the municipalities through the Kruskal-Wallis Test (analysis performed using the joint gene sequences). The differences were present only in the base frequency between the encoded genes. The polymorphism among *Ae. aegypti* population was greater for the ND4 gene, with a greater number of mutations and nucleotide diversity, according to the results of the haplotype network such as the template proposed by Bandelt et al. (1999) and Rozas et al. (1999). Certain specimens of *Ae. aegypti* may be more similar to each other when using COI and ND5 primers.

Scarpassa et al.³⁵ researched in fourteen locations in Brazil, including the Cuiabá city, using mitochondrial gene Cytochrome Oxidase I (COI) to examine gene flow among 163 mosquitoes from 14 cities. Phylogenetic analysis identified two clades in genetic variability. They recorded two types of haplotypes and found a significant polymorphism among other loci. These authors' analysis revealed two strains separated by 8 fixed mutations, suggesting that *Ae. aegypti* populations likely came from eastern and western Africa, with evidence of multiple introductions.

Our findings complement those results of earlier studies and have significant implications for understanding how these mosquitoes behave under distinct environments and, under human interventions. Therefore, the monitoring of *Ae. aegypti* species is essential for preventing and controlling vector-borne infectious diseases and, governments need to designate effective control measures, how mentioned by Lv et al³⁴. Our principal limitation was, less than 20% of de DNA samples had been amplified using the primer COI of distinct populations (see material and methods) and, the authors suggest another's primers need to be designed for Brazilian populations of *Ae. aegypti*.

In conclusion, this pioneering study conducted in the state of Mato Grosso shows the intrapopulation diversity of *Ae. aegypti* in each municipality investigated. The monitoring with ovitraps and genetic research of *Ae. aegypti* aims to identify genetic variations to boost actions to prevent diseases transmitted by this species. Genetic flow and dispersion estimates can support the government's measures that are essential to effectively control and fight the vector.

Material And Methods

Sampling and data.

This study was carried out in four municipalities in the state of Mato Grosso: Cuiabá (CB), Várzea Grande (VG), Chapada dos Guimarães (CP), and Santo Antônio do Leverger (SA) (Fig. 7). We selected such regions due to their higher human population movement (CB and VG), distinct geographical position and altitude (SA, at about 100 m, and CP, at about 700 meters above sea level). The number of adults resulting from the development of eggs in the laboratory are shown in table 2.

This was a descriptive observational study with a quantitative and qualitative approach. Data were collected with the aid of ovitraps during December/2015 (flooding), February/2016 (flood), June/2016 (drought), and November/2016 (flood) to investigate the number (*n*) of copies of *Ae. aegypti* in the four municipalities (Table 2).

Table 2

Number of adults resulting from the development of eggs in the laboratory. Source: IFBIOTEC Laboratory/IFMT/Cuiabá MT.

Month/Year	<i>Aedes aegypti</i> samples
December/2015	135
February/2016	355
June/2016	243
November/2016	374
Grand Total	1,107

To install the ovitraps, we selected three sampling points with a high movement of people in each municipality, as recommended by Fay and Eliason²³. We counted the eggs in a stereomicroscope and kept them in the laboratory for hatching and maintenance up to adulthood. We relied on relevant literature to identify adult specimens²⁴. Then, we placed specimens in polypropylene tubes containing 100% ethanol and labeled and stored them in a freezer (-20 °C). Individual DNA extraction from 400 *Ae. aegypti* specimens followed the manufacturer's protocol for insect DNA (Invisorb Spin Tissue Mini Kit). DNA quantification analysis was carried out after electrophoresis in 1% agarose gel submerged in Tris-Borate-EDTA buffer (1x) and Nano Spectrophotometer (Denovix D5). Subsequently, we amplified 169 samples with the best DNA result using primers for mitochondrial DNA (mtDNA): Cytochrome Oxidase subunit I (*COI*- F and R: Paduan and Ribolla 2008), Nicotinamide Adenine Dinucleotide Dehydrogenase Subunit 4 (*ND4*- F and R²⁵) and Nicotinamide Adenine Dehydrogenase Subunit 5 (*ND5*- F and R²⁶). After amplification and purification, the genes underwent Sanger sequencing. We deposited the sequences on the National Center for Biotechnology Information (GenBank, www.ncbi.nlm.nih.gov) under access number (available after acceptance of the article).

We analyzed the sequencing data for *COI*, *ND4*, and *ND5* genes using SeqTrace, Mega, and Geneious applications (available online and Trial version, respectively). SeqTrace was used to perform DNA strand consensus; Mega for DNA sequence, distance, and dendrogram analysis; and Geneious was used to perform sequence cutting and alignment. We checked the sequences manually using Bioedit Sequence Alignment Editor 7.0.5.2 in²⁹ Geneious®²⁷ Muscle software²⁸. We checked substitution saturation (transitions and transversions) of nucleotide sequence through DAMBE³⁰. The inter- and intraspecies

genetic distance was determined through Kimura 2-parameter (K2P) using the nucleotide replacement model³¹ in MEGA v6.0³².

We tested the independent variables (variable X - sampling locations and collection months) and dependent variables (variable Y - PCR results) through:

- a) analysis of variance (ANOVA) to verify the significance of data variability;
- b) test for mean comparison, which consists of the smallest significant difference and amplitude of data distribution; and
- c) Kruskal-Wallis test (Trial version) - a multiple comparisons test -, consisting of the average between samples in the gene and base variability between populations from the municipalities. We applied
- d) this test for ANOVA treatments as the results of this analysis were significant.

We applied factorial ANOVA considering the interindividual genetic distance data based on dendrogram results per municipality. We considered gene factors (*COI*, *ND4*, and *ND5*) as independent variables and genetic distance as the dependent variable (factorial). The factors 'genes' and 'municipalities' were analyzed jointly using two-way ANOVA together with nitrogenous base proportionality. We used *Ae. albopictus* as an external group and identified significant differences between each primer for the two vector species ($F_{1,119} = 32,962$; $p = 0.000$). Haplotypic diversity calculation was performed using the DNASp 6.0 program and, haplotic maps were made by Median Joining in the NETWORK 10 program (41, 42).

Declarations

Acknowledgements

This research was funded by FAPEMAT Fundação de Amparo à Pesquisa do Estado do Mato Grosso (Process 0316549/2018) by financial support (Edital PPSUS-2013-MS / CNPq / FAPEMAT / SES).

Authors contributions

Sandra Mariotto: coordinator of project, conducted all sample collections and the research on the genetic laboratory. Responsible accountable for all aspects of the work, and that manuscript in all steps.

Mari Rose de Oliveira Silva: applied primers to obtain sequences on genetic, and wrote a preview of this manuscript.

Cristina Marcia de Menezes Butakka: contributed to writing a preview of this manuscript, too to all statistical analysis, and revising it critically.

Lenicy Lucas de Miranda Cerqueira: Helped to sample collections, contributed to all of this manuscript.

Rosina Djunko Miyazaki: Helped to sample collections, contributed to all versions of this manuscript.

Fabiana Aparecida Caldart Rodrigues: Helped with genetics analysis and, revising this manuscript.

Competing interests

The authors declare no competing interests.

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Figures

Figure 1

Dendrogram for the interpopulational analysis based on A) COI, B) ND4, and C) ND5 genes from *Ae. aegypti* populations recorded in the four municipalities. We used *Ae. albopictus* as an outside group for each primer. Legend 2. CB- Cuiabá; VG- Várzea Grande; CP- Chapada dos Guimarães, SA- Santo Antônio do Leverger.

Figure 2

Dendrogram for intrapopulational analysis based on the results for COI, ND4, and ND5 genes from *Ae. aegypti* populations recorded in the four municipalities. We used *Ae. albopictus* as an outside group, using the results for each primer. Legend 3. A) CB- Cuiabá; B) VG- Várzea Grande; C) SA- Santo Antônio do Leverger; D) CP- Chapada dos Guimarães.

Figure 3

Specimens' interpopulational genetic distance mean values (standard deviation) for each gene (COI, ND4 and ND5), generated based on the dendrogram, municipalities of Cuiabá (CB), Várzea Grande (VG), Chapada dos Guimarães (CP), and Santo Antônio do Leverger/MT (SA).

Figure 4

Specimens' interpopulational genetic distance mean values (standard deviation) for each gene (A) and all primers generated (B) based on the dendrogram between the 4 municipalities analyzed. Legend: Cuiabá (CB), Várzea Grande (VG), Chapada dos Guimarães (CP), and Santo Antônio do Leverger/MT (SA).

Figure 5

Specimens' intrapopulation genetic distance mean values (standard deviation) for each gene (COI, ND4, and ND5), generated based on the dendrogram, municipalities of Cuiabá (CB), Várzea Grande (VG), Chapada dos Guimarães (CP), and Santo Antônio do Leverger/MT (SA).

Figure 6

Haplotypes network map showing the three primers, COI in yellow color, ND4 in blue and, ND5 in green color. The outgroup of *Ae albopictus* is shown in red. Some haplotypes per each gene were significantly segregated.

Figure 7

Graphical representation and location of the study area and collection points in the municipalities of Cuiabá (CB), Várzea Grande (VG), Chapada dos Guimarães (CP), and Santo Antônio do Leverger (SA), Mato Grosso. Legend 1. Cuiabá, triangle (CB-01, 02,03), Várzea Grande, star (VG-01, 02, 03) Chapada dos Guimarães, circle (CP-01, 02, 03), and Santo Antônio do Leverger, square (SA-01, 02, 03).