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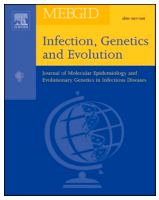

Mansonia spp. population genetics based on mitochondrion whole-genome sequencing alongside the Madeira River near Porto Velho, Rondonia, Brazil

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ABSTRACT

In high abundance, females of the genus *Mansonia* (Blanchard) can be a nuisance to humans and animals because they are voraciously hematophagous and feed on the blood of a myriad of vertebrates. The spatial-temporal distribution pattern of *Mansonia* species is associated with the presence of their host plants, usually *Eichhornia crassipes, E. azurea*, *Ceratopteris pteridoides*, *Limnobium laevigatum*, *Pistia stratiotes*, and *Salvinia* sp. Despite their importance, there is a lack of investigation on the dispersion and population genetics of *Mansonia* species. Such studies are pivotal to evaluating the genetic structuring, which ultimately reflects populational expansion-retraction patterns and dispersal dynamics of the mosquito, particularly in areas with a history of recent introduction and establishment. The knowledge obtained could lead to better understanding of how anthropogenic changes to the environment can modulate the population structure of *Mansonia* species, which in turn impacts mosquito population density, disturbance to humans and domestic animals, and putative vector-borne disease transmission patterns. In this study, we present an Illumina NGS sequencing protocol to obtain whole-mitogenome sequences of *Mansonia* spp. to assess the microgeographic genetic diversity and dispersion of field-collected adults. The specimens were collected in rural environments in the vicinities of the Santo Antônio Energia (SAE) hydroelectric reservoir on the Madeira River.

1. Introduction

Mosquitoes of the genus *Mansonia* (Blanchard) are currently assigned to two different subgenera: *Mansonioides* (Theobald), which comprises species distributed in Asia and Africa (Ronderos and Bachmann, 1963), and the subgenus *Mansonia,* which includes species that are mainly found in the Neotropical Region (Forattini, 2002; Harbach, 2013), including 12 species registered in Brazil. Females of *Mansonia* are voraciously hematophagous and feed on the blood of a myriad of vertebrates. These mosquitoes can be an enormous nuisance to humans and domestic animals when present at high population densities, leading to death among small livestock and stress for large livestock. Moreover, some species of *Mansonia*, such as *Mansonia indubitans* (Dyar and Shannon) and *Mansonia titillans* (Walker), have been implicated in the transmission of the Venezuelan equine encephalitis virus (VEEV) in Peru (Turell et al., 2000) and Venezuela (Mendez et al., 2001). The primary habitats of *Mansonia* larvae are permanent water collections containing floating aquatic vegetation, such as *Eichhornia crassipes* (Mart.), *Limnobium laevigatum* (Humb. & Bonpl. ex Willd.), *Pistia stratiotes* (L.), and *Salvinia molesta* (D. Mitch.). Immature individuals use their spiracular apparatus to attach themselves and pierce submerged plant tissues to obtain oxygen from the aerenchyma (Harbach, 2013). The abundance of aquatic plants is directly related to a reduction in water flow and the presence of organic matter content (eutrophication), which may lead to the spread of these mosquitoes by contributing to the formation of new breeding sites (Forattini, 2002).

There is an overall lack of research on *Mansonia* species' dispersion and population genetics. Such studies are pivotal to evaluate the genetic structuring, which ultimately reflects populational expansion-retraction patterns and dispersal dynamics of the mosquito, particularly in areas

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with a history of recent introduction and establishment. Knowledge obtained from such studies can lead to better understanding of how anthropogenic changes to the environment modulate the population structure of *Mansonia* species, impact mosquito population density, disturb humans and domestic animals, and affect putative vector-borne disease-transmission patterns. Moreover, in order to develop methods for the control of *Mansonia* species with focus on immature population management, it is essential to understand the population structure and dispersion dynamics. Such information can reflect the overall genetic diversity of larval breeding sites that are spatially distributed in a determined area.

Mitochondrial genes are commonly used for molecular-evolution and population-genetics assessments of different mosquito species. Mitochondrial genes have a faster evolution rate than nuclear DNA, a predominantly maternal inheritance, a lack of genetic recombination, a relatively high mutation rate, and high levels of polymorphism and divergence due to their inherent sensitivity. Thus, they are extremely useful as molecular markers (Kang et al., 2015; Opiro et al., 2017). Particularly, there have been many studies using COI and ND5 as markers to determine whether a species has been introduced or to determine the genetic diversity of a population (Kamgang et al., 2013; Žitko et al., 2011).

In this study, we employed a low-density genome Illumina sequencing protocol to obtain whole-mitogenome sequences of *Mansonia* to assess the microgeographic genetic diversity and dispersion of field-collected adult specimens. The specimens were collected in rural environments alongside the Madeira River in rural areas in the vicinities of the Santo Antonio Energia (SAE) reservoir in the municipality of Porto

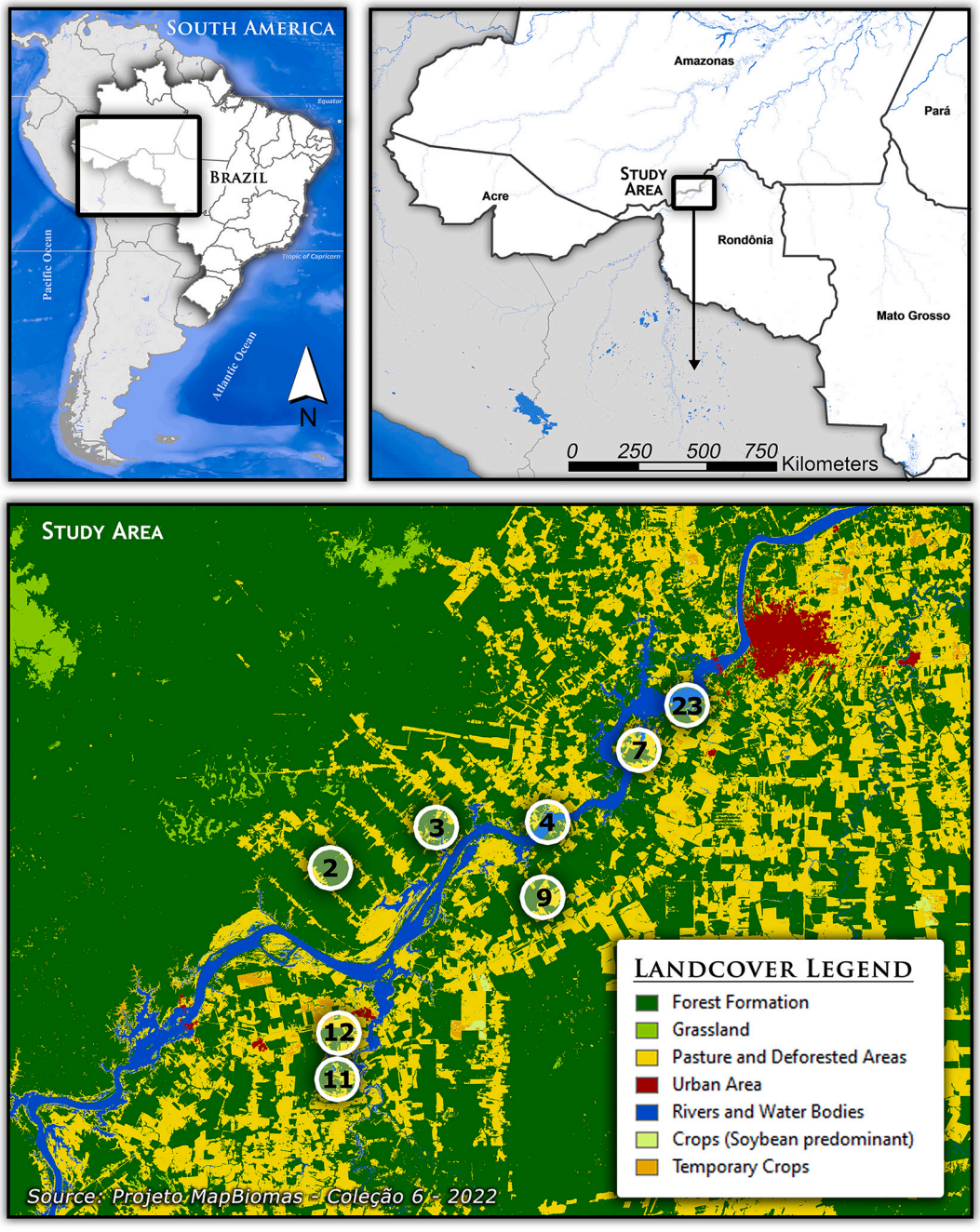

Fig. 1. Specimen collection sites in five municipalities in the Rondônia state, Brazil. The numbers represent the collection sites: 2, 3 and 4 - Joana D'Arc settlement; 7 - Nova Teotônio village; 9 - Santa Rita settlement; 11 - Jaci Paraná River; 12 - Samaúma, Jaci Paraná; 23 - São Domingos settlement.

Velho, Rondônia, Western Brazil.

2. Material and methods

2.1. Study area and sampling points

A total of 81 specimens of *Mansonia* adults were collected at 8 sites (RO-02 ($n = 12$), RO-03 ($n = 10$), RO-04 ($n = 7$), RO-07 ($n = 13$), RO-09 ($n = 11$), RO-11 (n = 10), RO-12 ($n = 9$) and RO-23 (n = 9)). Specimens were collected in the municipality of Porto Velho, Rondônia, Brazil, along a 70-km section of the Madeira River (Fig. 1). The collection area extends from the Jaci-Parana district to approximately 20 km west of the municipality of Porto Velho (Table S1). According to the Köppen classification, the climate is AW – rainy tropical with average temperature varying from 21 to 34 °C and average rainfall varying from 17 to 264 mm monthly. The rainy season is from October to April, and the dry season is from June to August, with transition periods in May and September (Ab'Sáber, 2003).

Field collections were carried out in March 2020 at the end of the rainy season, when the precipitation and the river water levels were highest. The collection sites were surrounded by a mosaic of fragments of primary tropical rain forest and agricultural areas. Two collection methods were employed: CDC light and barrier screen sampling methods. Prior to DNA extraction, all specimens were morphologically identified to the species level using Forattini's (2002) identification key. Only morphotype 1 (classified as *Mansonia titillans*) and morphotype 2 (assigned as "near" *Mansonia titillans*) were used in the study and are described throughout the manuscript as *Mansonia* spp.

2.2. Sample preparation and sequencing

For DNA extraction, heads and thoraces of mosquitoes were separated from the rest of the body using a sterile scalpel. Each specimen was extracted individually using a ReliaPrep™ Blood gDNA kit (Promega, Madison, USA) according to the manufacturer's recommendations. DNA quantification was performed by fluorometric quantitation using a Thermo Fisher Scientific QuBit dsDNA HS Assay Kit according to the manufacturer's recommendations.

DNA libraries were prepared using one-fifth of the total recommended volume for the Nextera XT Library prep kit (Illumina) according to the manufacturer's recommendations. DNA samples were multiplexed from the total of 81 samples, loaded on two mid-output flowcells each, and sequenced using the NextSeq500 platform (Illumina) in a 151-cycle single-read run. Sequence-quality analysis was performed using the FastQC program, and reads were used if results from all analysis modules were approved without errors.

2.3. Variant calling and mitochondrial DNA analysis

All sequencing reads were aligned against the reference mitochondrial genome of *Mansonia amazonensis* (NC_044657.1, available from GenBank) using the program Burrows-Wheeler Aligner (BWA) (Li and Durbin, 2009). Variant calling was performed with the SamTools software package (Li, 2011), and the panel of variants was exported in the VCF version 4.2 format. SNPs were removed from the pre-imputation panel based on a minimum allele frequency (MAF) < 0.1 and missing data (MD) > 0.3 using the LCVCFtools program (Alvarez, 2020). Genotypes with sequencing depth (DP) < 5 or phred genotype-quality score (GQ) < 20 were imputed with BEAGLE 4.1 software (Browning and Browning, 2016) using genotype-normalized probability values (PL). After imputation, genotypes were removed from the panel if the probability of the imputed genotype (GP) was <0.8. Finally, SNPs were filtered using MAF > 0.1 and MD < 0.3.

Statistical analyses were performed with PLINK 1.9 (Purcell et al., 2007), and graphs and figures were generated using the GGPlot2 package in RStudio. Pairwise *Fst* estimates of genetic diversity and neutrality tests were computed using ARLEQUIN 3.5 (Excoffier and Lischer, 2010). The phylogenetic reconstruction was built using MEGA11 (Tamura et al., 2021). The Identical by State (IBS) pairwise genetic distance matrix was calculated by the PLINK program, and the multidimensional scaling procedure (MDS) was applied. Hierarchical clustering analysis was run in RStudio using the pvclust v2.2–0 package.

3. Results

3.1. Sequencing

After sequencing, the average depth for each specimen sequenced was approximately 1,200,000 reads, representing an average coverage of 78×. For subsequent analyses, all reads were aligned with the reference mitochondrial genome of *Ma. amazonensis* (NC_044657). After variant calling and imputation processes, the final dataset consisted of 133 SNPs distributed throughout the mitogenome (Fig. 2).

3.2. MDS analysis and hierarchical clustering

Our first approach to assess the overall genetic differentiation in the collected mosquitoes was to perform an MDS analysis considering 1) the locality where the specimens were collected (Fig. 3a), 2) the Madeira River margin (i.e., left margin or right margin) from which the specimens were collected (Fig. 3b), and 3) the morphotype assigned to each specimen collected (Fig. 3c). MDS analysis revealed a clear division of the analysed samples into two distinct groups, which is clearly supported by the hierarchical clustering analysis (Fig. 4). Interestingly, the observed clustering pattern seems to not be related to the morphotype of the specimens or to the margin of origin since the distribution of individuals proved to be highly heterogeneous when these parameters were applied to assess the clustering trend. On the other hand, when the specimens were analysed from the perspective of the collection locality, there was a discrete grouping tendency of mosquitoes collected at the same place, especially for locations R09 and R03.

3.3. Population genetics analysis

We further investigated the genetic structure using pairwise *Fst* estimates (Table 1) and *Tajima's D* and *Fu's FS* neutrality tests (Table 2). For the *Fst* statistics, significant moderate to high values were found in pairwise population comparisons, especially for the R03 and R09 populations compared to the others (e.g., *Fst* for R02 x R09 was 0.89, and *Fst* for R02 x R03 was 0.77). *Fst* estimates were also used as a distance metric for phylogenetic reconstruction to evaluate the evolutionary relationships between the assigned populations. Once again, the differentiation of R03 and R09 populations compared to the others was supported (Fig. 5). On the other hand, no significant *Fst* statistics were found in the morphotype or margin-origin analysis.

In the neutrality tests, 4 populations (R02, R03, R09, and R11) presented statistically significant negative values of *Tajima's D*. Only the R09 population presented the same pattern for *Fu's FS* test. When the analysis considered the morphotype assignment and margin origin of the sampled populations, statistically significant negative values were obtained for only *Fu's FS* test.

4. Discussion

The results of this study represent a comprehensive analysis of the genetic structure of field-collected *Mansonia titillans* and *Mansonia* near *titillans* at a microgeographic scale. In general, the availability of lentic habitats increases in response to anthropic modifications of the environment, such as the formation of large hydroelectric reservoirs and other minor water collections spread throughout the floodplain (Schiesari et al., 2020). In turn, this may result in an imbalance of native mosquito populations, which could lead to huge interspecific

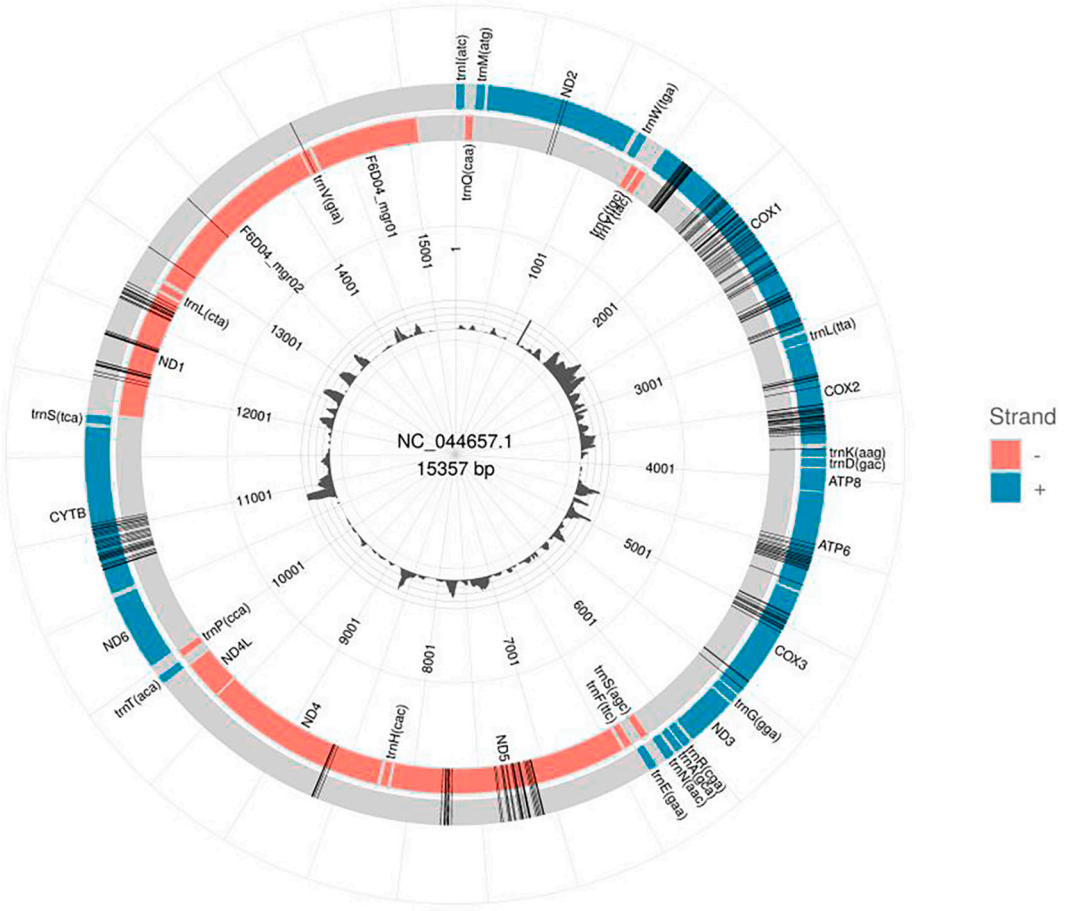

Fig. 2. Map of the reference mitochondrial genome of *Mansonia amazonensis* (NC_044657). After variant calling and imputation processes, the final data set consisted in 133 SNPs distributed throughout the mitogenome. SNPs are represented as black bars and a relative coverage graph is showed in the center of the figure.

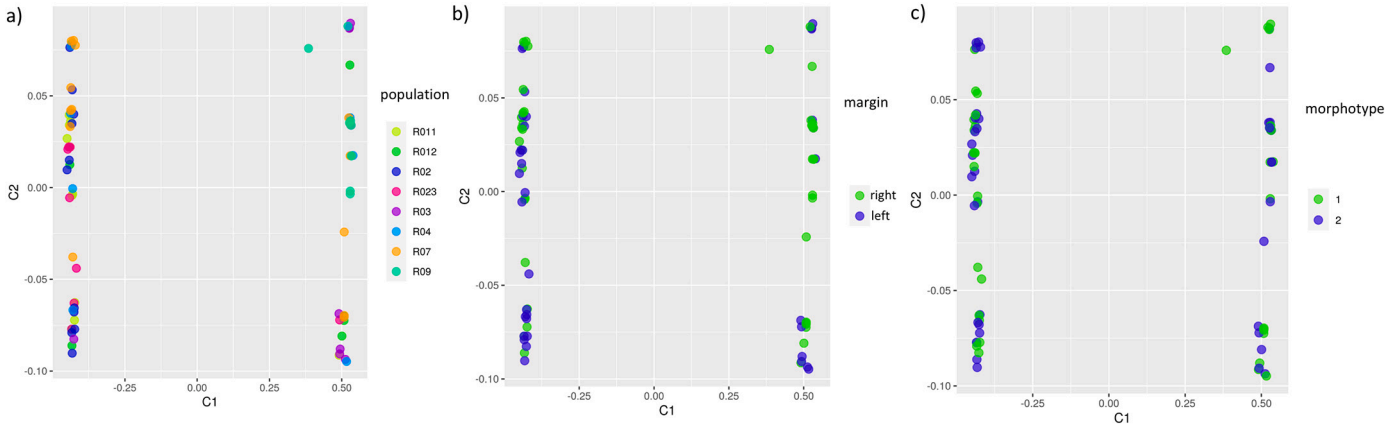

Fig. 3. Multidimensional scaling analysis of *Mansonia* spp. specimens based on: a) the locality where the specimens were collected, b) the Madeira River margin (i.e. left margin or right margin) on which the specimens were collected and c) the assigned morphotype to each specimen collected.

competition for breeding sites and ultimately the thriving of particular species (Amorim et al., 2022; Galardo et al., 2022; Gil et al., 2021). In the case of *Mansonia* spp., the formation of a lentic system tends to favour the establishment of aquatic macrophytes (de Resende et al., 2019), which creates a perfect scenario for oviposition and the development of immature stages of the mosquito. Moreover, the dispersal dynamics of these mosquito populations may be influenced by human activities and the presence of livestock and poultry farming since *Mansonia* spp. females are aggressive biters with both exophilic and endophilic behaviours (Galardo et al., 2022).

The results of the population-genetics analyses provide evidence for population structuring at a microgeographic scale. There are consistent differences in pairwise *Fst* estimates for the different localities where *Mansonia* spp. were sampled. Interestingly, no significant *Fst* statistics were found in the morphotype or margin-origin analyses. This indicates that the Madeira River is not a physical barrier to the dispersal of *Mansonia* spp. because no unique representative genotypes from each margin were found. Likewise, the genetic differentiation found in the

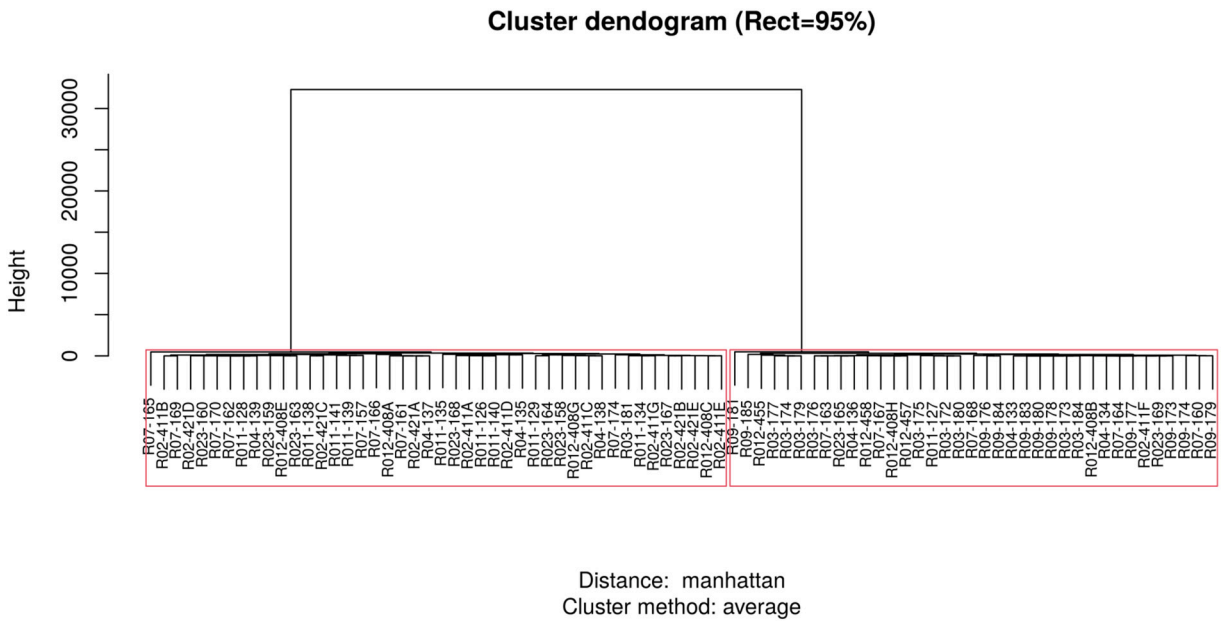

Fig. 4. Hierarchical clustering showing two clearly distinct mitochondrial lineages for the specimens sampled.

Table 1
Fst values of population comparisons (triangular matrix) for a) locality, b) margin origin and c) morphotype classification. Values in bold are statistically significant ($p < 0.05$).

a)

	R02 (n = 12)	R03 (n = 10)	R04 (*n* = 07)	R07 (n = 13)	R09 (n = 11)	R11 (n = 10)	R12 (n = 09)	R23 (n = 09)
R02	–							
R03	**0.77939**	–						
R04	0.19797	0.34196	–					
R07	0.14081	**0.37926**	0.00000	–				
R09	**0.89265**	0.00000	**0.54570**	**0.52522**	–			
R11	0.00000	**0.75465**	0.1519	0.1091	**0.87915**	–		
R12	**0.37360**	0.17853	0.00000	0.00000	**0.36987**	**0.32874**	–	
R23	0.00000	**0.59951**	0.00000	0.00000	**0.75454**	0.00000	0.11965	–

b)

	Left margin (*n* = 38)	Right margin (*n* = 43)
Left margin	–	
Right margin	0.00000	–

c)

	Morphotype 1 (*n* = 41)	Morphotype 2 (*n* = 40)
Left margin		
Right margin	0.00000	

individuals analysed does not seem to be related to the assigned morphotype of the mosquitoes.

Statistically significant negative values of *Tajima's D* and *Fu's FS* were observed, which generally reflect a recent event of population expansion detected as an excess of low-frequency polymorphisms and excess number of alleles, respectively. These results may point towards a scenario of a recent invasion of *Mansonia* spp. alongside the hydroelectric reservoir of the Santo Antonio HPP. Another possibility is the occupation of new niches created in the floodplain as different cycles of flooding occur yearly.

Species of the genus *Mansonia* spp. are known to have a considerable dispersion range and can fly across swamps, ponds, and lakes to find oviposition sites or obtain blood meals (Verdonschot and Besse-Lototskaya, 2014). In fact, despite being capable of covering distances >2 km from the point where adults emerge, the dispersal pattern of *Mansonia* spp. is predominantly characterized by random short flights. Furthermore, they show a tendency to remain near the breeding sites (30 to 100 m) in certain fragments of vegetation (de Mello and Alencar, 2021). This behaviour may explain the observed genetic structuring for these mosquitoes. After the invasion of a new available niche, females take blood meals and lay eggs in a limited area, which favours inbreeding, and after some generations, genetic structuring can be detected.

The groundwater collections and small lakes adjacent to the Madeira River are habitats for mosquito development. In addition, the presence of extensive areas across the river basin that are occupied by cattle farms

Table 2
Tajima's D and Fu's FS neutrality tests values for a) locality, b) margin origin and c) morphotype classification. Values in bold are statistically significant (*p* value is given in the table).

a)

Neutrality tests	Statistics	R02	R03	R04	R07	R09	R011	R012	R023
Tajima's D test	Tajima's D	**−2.34250**	**−2.16534**	2.28909	2.57214	**−1.64123**	**−2.09990**	2.61337	0.29533
	Tajima's D *p*-value	0.00000	0.00000	0.98300	0.98600	0.04200	0.00000	0.98800	0.58200
Fu's FS test	FS	**−1.77569**	−0.71399	1.65034	0.00170	**−12.81346**	−0.71217	0.66695	0.25322
	FS p-value	0.11100	0.22300	0.81600	0.47700	0.00000	0.22600	0.65900	0.42700

b)

Neutrality tests	Statistics	Left margin	Right margin
Tajima's D test	Tajima's D	3.66541	3.88156
	Tajima's D p-value	0.99500	0.99900
Fu's FS test	FS	**−6.62729**	**−7.85640**
	FS p-value	0.01500	0.01000

c)

Neutrality tests	Statistics	Morphotype 1	Morphotype 2
Tajima's D test	Tajima's D	3.77786	3.98584
	Tajima's D p-value	0.99700	0.99900
Fu's FS test	FS	**−7.32965**	**−7.85640**
	FS p-value	0.02200	0.02000

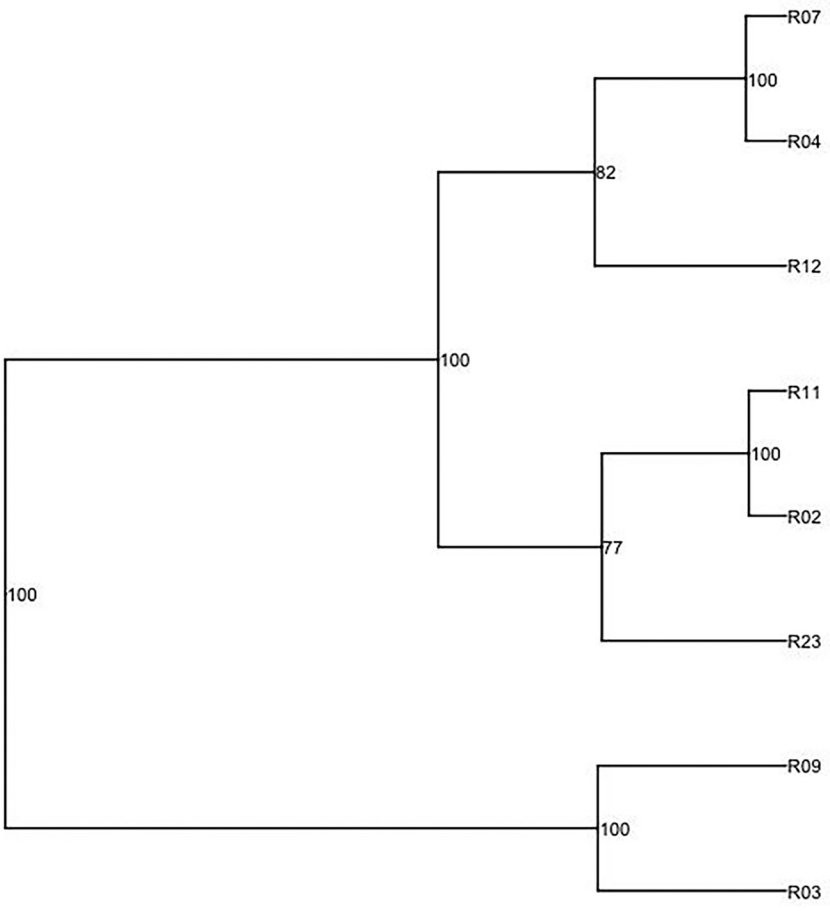

Fig. 5. UPGMA tree calculated from the Fst matrix of population comparisons. Values inside the tree show bootstrap support for the branches.

represent blood sources for a group of mosquitos that is known for haematophagic eclecticism (Luz and Lourenço-de-Oliveira, 1996; Kengne et al., 2003). Furthermore, ecological conditions that favour mosquito occurrence near human settlements arise from so-called visiting behaviour, where females are strongly attracted by artificial light but predominately bite outdoors with exploratory indoor activities (Navarro-Silva et al., 2004; Galardo et al., 2022). This is particularly evident in localities R03 (Joana D'Arc settlement) and R09 (Santa Rita settlement). Both of these localities had statistically significant negative values for both *Tajima's D* and *Fu's FS* and are agricultural areas near the river floodplain.

There is a general scarcity of studies regarding genetic structuring and diversity at both macro and microgeographic scales for *Mansonia* spp. Nevertheless, to implement efficient control programs, it is essential to assess and understand the local mosquito populations' dispersion dynamics, population structure, spatio-temporal structuring, and genetic diversity (Campos et al., 2017; Campos et al., 2012; Endersby et al., 2011). Mitochondrial DNA-based studies on the genetic structure of *Aedes aegypti* (L.) populations in Brazil have examined only broad geographic scales. Such studies have revealed co-occurrence of two divergent lineages (Bracco et al., 2007; Paduan and Ribolla, 2008; Scarpassa et al., 2008) and substantial gene flow among populations from different regions of Brazil (Gonçalves da Silva et al., 2012). Conversely, a mitochondrial DNA-based population-structuring study of *Aedes albopictus* (Skuse) in Manaus showed that populations of this vector had gone through a recent expansion event following a founder effect (Maia et al., 2009). The results demonstrated that mitochondrial markers can be used in such studies at a microgeographic scale.

To our knowledge, this is the first study to use a low-density whole-mitogenome sequencing protocol to assess the microgeographic genetic diversity and dispersion of field-collected *Mansonia* spp. Considering the amount of SNPs obtained and subsequent analyses, this protocol could be applied to any mosquito species when lower cost of NGS library construction cost and lower sequencing effort are needed. Finally, for small-area interventions to be successful, it is key to have knowledge of the peculiarities of the environmental characteristics. Mosquito control planning should identify the genetic differences and structuring of vector populations at a microgeographical scale to delineate strategies that consider genetic, biological, and ecological characteristics of target populations (Li et al., 2020).

5. Conclusions

This study has provided evidence of genetic structuring of *Mansonia* spp. in the vicinities of the SAE reservoir in Porto Velho, Rondônia, Western Brazil. This appears to be the first study to use a low-density whole-mitogenome sequencing protocol to assess the microgeographic genetic diversity and dispersion of field-collected *Mansonia* spp. Moreover, this protocol could be applied to any mosquito species when a lower cost of NGS library construction and lower sequencing effort are needed. This molecular tool could also be suitable for further elucidation of vector populations at a microgeographical scale.

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Authors' contributions

MAMS, DPC and KANR designed the field and laboratory work; MAMS, JAA, ILRS, PEMR, and DPA performed the laboratory research; MVNA, PEMR, and DPA analysed data. DPA wrote the manuscript with contributions from MAMS, PEMR and MVNA. All authors actively contributed to the interpretation of the findings and development of the final manuscript. All authors have read and agreed to the published version of the manuscript.

Data availability

The datasets generated and analysed during the current study are deposited in the Sequence Read Archive (SRA) linked to PRJNA818682 BioProject identifier.

Conflict of interest

The authors declare no conflict of interest.

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