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Evidence of Genetic Diversity Gradients in *Melipona rufiventris* (Hymenoptera: Apidae) within the Brazilian Semiarid Region

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ABSTRACT

Melipona rufiventris Lepeletier, a stingless bee species native to Brazil and referred to as uruçu-amarela, has experienced significant population declines in recent years due to the destruction of native semi-arid ecosystems and the overharvesting of honey. This study investigates the genetic diversity and population structure of *M. rufiventris* in the semiarid region of Brazil using microsatellite markers, laying the groundwork for monitoring the genetic shifts of bee populations across both space and time in Brazil. After testing 37 potential microsatellite markers, only 9 markers (24.3%) showed polymorphism in *M. rufiventris*. When the data were analyzed at three collection sites—Campo Maior, Castelo do Piauí, and Guadalupe—Campo Maior showed the highest mean number of alleles per population (3.0), ranging from 1 to 7, compared to the other sites. Principal coordinate analysis (PCoA) and Bayesian clustering (structure) revealed a clear separation between two genetic groups, with some overlap, confirming significant genetic differentiation. This insight is crucial for conservation efforts, as it suggests that the groups from Campo Maior and Castelo do Piauí + Guadalupe should be considered as separate conservation units. Therefore, conservation strategies should focus on reducing habitat destruction within each area and preventing the movement of colonies, especially considering the role of the species in meliponiculture.

Keywords: Uruçu-amarela, Rufiventris group, Stingless bee, Microsatellites markers

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Introduction

Stingless bees, classified under the Apidae family in the Hymenoptera order, belong to the Meliponini tribe, which includes all living genera of these bees [1, 2]. These bees are essential pollinators of native plants and crops [3-7], highlighting their significant ecological and economic roles. They are versatile and can thrive in various habitats, including different types of forests, savannas, wetlands, protected areas, agricultural lands, and even within human-made structures like wooden houses [8]. However, the intensification of agricultural practices focused on boosting food and forage yields has led to habitat destruction and fragmentation, which are key factors in the decline of stingless bee populations, largely due to the excessive use of pesticides and fertilizers [9-12]. The species *Melipona rufiventris* Lepeletier, 1836, also known as uruçu-amarela, is native to Brazil and has suffered significant population losses in recent years because of the ongoing destruction of native semi-arid vegetation and

the overexploitation of honey. As a result, it is currently listed as endangered on Brazil's red list of threatened species [13].

Beyond the challenges posed by human-induced disturbances to natural habitats, which have led to a significant decline in many populations, research has revealed that *M. rufiventris* virgin queens mate with only one male (monandry) during their nuptial flights [14]. As a result, all female offspring inherit the same paternal chromosomes, leading to limited genetic variability within the colony. Although single mating is an ancestral trait in this species, low genetic diversity at the colony level cannot be assumed, as other mechanisms may develop to enhance intra-colonial genetic variation and overcome biological limitations [15, 16].

Understanding the genetic diversity and population structure of *M. rufiventris* is crucial for interpreting population dynamics. This knowledge also helps predict how disturbances might affect their habitat and inform the development of effective conservation strategies [17]. Molecular markers are valuable tools for assessing genetic diversity and variability within and between populations of a species from different geographic regions [18]. This information is vital for addressing key conservation concerns for this endangered stingless bee species.

This research presents an analysis of the genetic diversity and structure of *M. rufiventris* populations in the Brazilian semiarid region using microsatellite markers, offering a foundation for understanding the spatial and temporal genetic variations of these populations in Brazil.

Materials and Methods

Bee materials and genomic DNA isolation

Worker bees were gathered from natural colonies across three locations: 25 nests in Campo Maior (CAM; 4°49'19" S, 42°09'52" W), 7 nests in Castelo do Piauí (CAP; 5°23'15" S, 41°31'17" W), and 6 nests in Guadalupe (GUA; 6°47'30"S, 43°34'14"W), all within the state of Piauí, Brazil. Once collected, the samples were transported to the laboratory and preserved at -20 °C for future analysis. DNA extraction was performed on the thoraxes of adult worker bees using the HotSHOT technique [19]. The alkaline lysis buffer was heated to 95 °C for an hour, then cooled to 4 °C, and the pH was adjusted to 5 with 40 mM Tris-HCl. DNA concentration was quantified using a NanoDrop 2000 Spectrophotometer (Thermo Scientific), and its quality was assessed by electrophoresis on 0.8 percent agarose gels.

Microsatellite markers testing and genotyping

To identify polymorphic loci in the *M. rufiventris* genome, cross-transferability of microsatellite primers was first assessed **(Table 1)**. A total of nineteen microsatellite primer pairs from *M. subnitida* [20] and eighteen from *M. fasciculata* [8] were used in PCR amplifications.

Each reaction was prepared in a 10 µL mix containing 10-50 ng of DNA, 1× PCR buffer (40 mM Tris-HCl; 100 mM KCl), 0.2-0.25 µM of each primer, 2.5-3.0 mM MgCl2, 0.5-1.0 µM of each dNTP, and 0.25-0.75 U of Invitrogen Taq DNA polymerase. Amplifications were performed using a VERITITM Gradient Thermal Cycler (Life Technologies). The initial PCR protocol (PCR1) involved: 94°C for 5 minutes, followed by 30 cycles of (94 °C for 40 seconds, Ta (50-60) °C for 30 seconds, and 72 °C for 40 seconds), with a final extension step of 72 °C for 7 minutes. For optimization of the Mfsc11 primer, an alternate PCR protocol (PCR2) was applied: 94 °C for 1 minute, followed by 40 cycles of (94 °C for 30 seconds, Ta °C for 30 seconds, 72 °C for 30 seconds), and concluding with 72 °C for 3 minutes.

SSR markers were visualized using silver nitrate staining on 6% denatured polyacrylamide gels. A 10-bp ladder (life technologies) was used as a size marker. The amplification was deemed successful when the gel displayed one or two clear, consistent bands that were close in size to those from the original species.

Table 1. List of markers, primer sequences, and experimental conditions employed for amplifying microsatellite loci in *M. rufiventris*, along with their corresponding GenBank accession numbers.

Loci	**Primers (5' → 3')**	**Repeat motif**	**Ta (°C)**	**Allele size range (bp)**	**PCR profile**	**GenBank accession number**
*Msub*2	F:GCCCAAAGATGGTATGCCG R: ACGAGGCGGATTCAACGAG	$(ACG)_{14}$	60	172-177	PCR_1	KM494946
*Msub*3	F: CTCGGCGCACAATTCGAG R: GGTTATTTCGCCGGCAAGC	$(CGTT)_{11}$	60	132-136	PCR_1	KM494947

*Msub*18	F: TCCCGATTTCCACCGATCC R: GCCGACCTCTTCGACGG	$(ACG)_{18}$	60	142-160	PCR_1	KM494953
*Msub*31	F: TTACCGTCTGTGCTACTGATCC R:TGTCTGTCTGTCTGTCTATCTTTCTG	$(AGAT)_{14}$	60	134-150	PCR_1	KM494956
*Msub*38	F: AATACTCTGTTTCTTCCAGGGG R: CTGAAATTGCTTTCGTGCC	$(AAAG)_{15}$	60	110-135	PCR_1	KM494958
*Msub*46	F: CACTGTTTCTCCAGTTGCTGTC R: GTTTCGTTCGCGTGATTTC	$(AAAG)_{12}$	60	113-132	PCR_1	KM494960
*Msub*48	F: AAAGAGCGTAGGACTTCCACAG R: CATCCATCTATCCGTACATCCA	$(GGAT)_{10}$	58	115-119	PCR_1	KM494961
*Msub*51	F: GGCGTTACAAAGGGGAGAA R: AGTTGACAGCGTTTCCTACCTC	$(AGAA)_9$	60	148-152	PCR_1	KM494962
*Mfsc*11	F: GGAAGGACGAGAGAATTCAAGA R: ATAGTCGTTTGTCGCGAGTGTA	$(CTT)_{13}$	50	142-168	PCR_2	KT730153
*Mfsc*13	F: GCAGTAACGGTAGCAGTGGTG R: ACTCCTTTCTCCTTCTCGGTCT	$(ACG)_{16}$	52	157	PCR_1	KT730154

Ta, Annealing temperature; PCR profiles: (PCR_1 = [94 °C-5 min; 30 cycles × (94 °C-40 seg; Ta-30 seg; 72 °C-40 seg); 72 °C-7 min], PCR_2 = [94 °C-1 min; 40 cycles × (94 °C-30 seg; Ta-30 seg; 72 °C-30 seg); 72 °C-3 min].

Data analysis

Genotypic data were processed with Micro-Checker 2.2.3 [21] to check for null alleles or potential scoring errors. CERVUS 3.0.3 [22] was employed to calculate the number of alleles (A), observed and expected heterozygosities (HO and HE), and polymorphic information content (PIC). Allelic richness (AR) was estimated using FSTAT version 2.9.3.2 [23]. Hardy–Weinberg Equilibrium (HWE) and linkage disequilibrium tests were carried out via the web tool GENEPOP [24], with significance determined using Bonferroni-corrected P-values ($P < 0.05$). Population structure was inferred using a Bayesian admixture model in STRUCTURE v2.3.3 [25], running 1,000,000 Markov chain Monte Carlo iterations after an initial burn-in of 100,000 steps. The optimal number of populations (K) was estimated through ten replications for each K (ranging from one to four), as outlined by [26], using STRUCTURE HARVESTER v.0.6.92 [27]. The program CLUMPP v.1.1.2 [28] was used to align the best K from five repetitions, and DISTRUCT v.1.1 [29] was utilized to visualize these results. Additionally, population structure was examined through principal coordinate analysis (PCoA) in GENALEX v.6.5 [30].

Results and Discussion

Genetic diversity research on bee populations has frequently employed transferred microsatellite markers [31-35]. Out of 37 heterologous microsatellite primer pairs tested via PCR, only nine (24.3%) showed polymorphism in *M. rufiventris*. A primer (Mfsc13) provided clear, repeatable bands but only produced a single band across the studied populations. The remaining 73% of primers yielded unsatisfactory results, either generating non-specific bands or failing to amplify altogether, even though *M. subnitida* and *M. fasciculata* are closely related to *M. rufiventris*. Silva *et al*. [8] suggested that amplification success decreases with increasing genetic distance, emphasizing that phylogenetic closeness is the primary factor influencing the successful transfer of primers. Other elements, such as genome size, complexity, and the microsatellite's location (whether in coding regions or not), might also impact the transferability of microsatellite markers.

In the overall analysis of the dataset, four loci—Mfsc11, Msub31, Msub38, and Msub51—deviated significantly from Hardy-Weinberg Equilibrium ($P < 0.05$), mainly due to null alleles or missing data. These loci exhibited a heterozygote excess, which could be attributed to the mis-scoring of non-specific or stuttering bands [21] and the small effective population size [36, 37]. The allelic richness (AR) ranged from 2 to 6.6, with an average of 3.2. The polymorphic information content (PIC) varied between 0.12 and 0.67, with a mean of 0.37, reflecting moderate informativeness. PIC values exceeding 0.5 are considered highly informative, between 0.25 and 0.5 are moderately informative, and those below 0.25 are less informative [38]. The observed heterozygosity (Ho) varied from 0.00 to 0.85, with a mean of 0.47, while the expected heterozygosity (He) ranged from 0.14 to 0.72, averaging 0.43. The Msub31 locus exhibited the greatest polymorphism, while Msub51 showed some evidence of null alleles, though with a frequency lower than 0.200 **(Table 2)**. Frequencies of null alleles below 0.200 are generally acceptable in microsatellite data analysis [39].

The mean expected heterozygosity (He) observed in this study was similar to that reported by Lopes *et al*. [40] for *M. rufiventris* (He = 0.43), but higher than values found in other meliponine species, including 0.38 for *Melipona mondury* and 0.35 for *M. mandacaia* [41], as well as 0.105 for *M. mondury* and 0.189 for *M. quadrifasciata* [31]. Although the genetic diversity in *M. rufiventris* was greater than in most of these studies, it remains relatively low, which could be linked to genetic, environmental, and biological factors, particularly anthropogenic impacts. These factors can lower genetic variation due to habitat fragmentation and the predatory effects of honey harvesting, which reduce colony numbers in certain areas [40-44].

When the dataset was categorized into three collection sites—Campo Maior, Castelo do Piauí and Guadalupe—it was found that Campo Maior exhibited the highest average number of alleles per population (3.0), ranging from one to seven, compared to the other two sites. After applying Bonferroni correction, departures from Hardy-Weinberg equilibrium were noted at the Mfsc11, Msub18, Msub31, and Msub38 loci in Campo Maior. No significant deviations from HWE were observed in samples from Castelo do Piauí or Guadalupe (P > 0.05) **(Table 2)**.

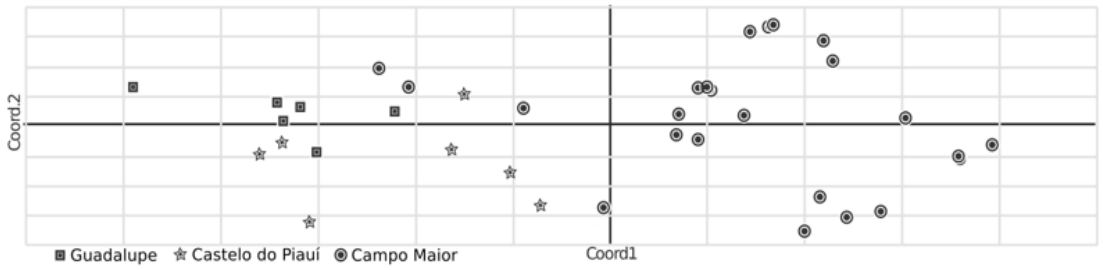
Table 2. Variability across 9 microsatellite loci and the genetic diversity estimates for *M. rufiventris* in the Brazilian semiarid region.

Loci	Campo Maior-PI (n = 25)					Castelo do Piauí-PI (n = 7)					Guadalupe-PI (n = 6)				
	A	**Ho**	**HE**	**PIC**	**pHWE**	**A**	**Ho**	**HE**	**PIC**	**pHWE**	**A**	**Ho**	**HE**	**PIC**	**pHWE**
*Msub*2	2	0.360	0.301	0.252	0.556	2	0.250	0.250	0.195	1.000	1	0.000	0.000	0.000	-
*Msub*3	2	0.640	0.444	0.341	0.057	2	0.333	0.333	0.239	1.000	2	0.333	0.333	0.239	1.000
*Mfsc*11	2	0.818	0.495	0.367	0.0017*	1	0.000	0.000	0.000	-	3	0.667	0.667	0.535	0.309
*Msub*18	4	0.750	0.557	0.466	0.0005*	1	0.000	0.000	0.000	-	1	0.000	0.000	0.000	-
*Msub*31	7	1.000	0.79	0.741	0.000*	2	0.500	0.409	0.305	1.000	2	0.500	0.429	0.239	1.000
*Msub*38	5	0.421	0.679	0.606	0.0003*	2	1.000	0.600	0.375	0.398	2	1.000	0.571	0.535	0.314
*Msub*46	2	0.571	0.455	0.346	0.344	1	0.000	0.000	0.000	-	0	0.000	0.000	0.000	-
*Msub*48	2	0.240	0.216	0.189	1.000	2	0.400	0.356	0.269	1.000	1	0.000	0.000	0.000	-
*Msub*51	1	0.000	0	0	0	2	0.000	0.485	0.346	0.030	1	0.000	0.000	0.000	-
Mean	3	0.533	0.437	0.367	–	1.6	0.275	0.270	0.192	–	1.4	0.277	0.222	0.172	–

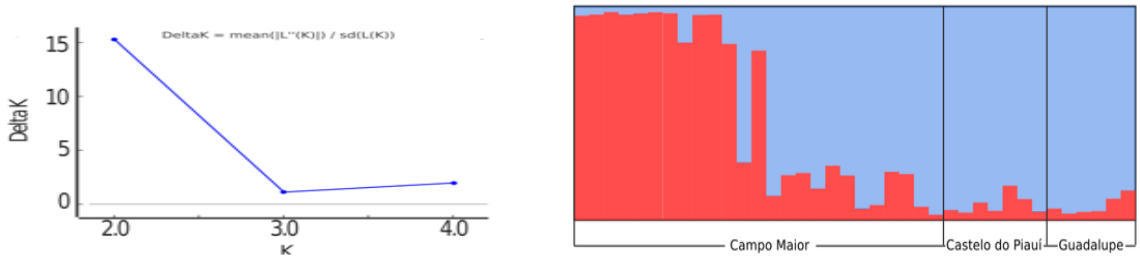
A = number of alleles in the population; Ho = observed heterozygosity; He = expected heterozygosity; PIC = polymorphic information content; pHWE = Hardy-Weinberg equilibrium probability; and * = 5% significance (Bonferroni correction < 0.005).

The analysis of F statistics based on molecular markers revealed a Fis of -0.177, indicating a minimal level of inbreeding. Meanwhile, the Fst and Rst values were 0.151 and 0.288, respectively. The Fst value observed in this study was comparable to that of *Melipona asilvai* populations, where a similar Fst of 0.166 was reported using microsatellite markers [45]. Although the Fst value of 0.151 is not extremely high, it suggests some degree of population differentiation, which is particularly important for a species such as *M. rufiventris*, which is at risk. Following Nei's classification [46], Fst values below 0.05 are low, between 0.05 and 0.15 are moderate, and above 0.15 are considered high, which indicates a considerable population structure in the studied area.

The principal coordinate analysis (PCoA) revealed the separation of the species into 2 main clusters with slight overlap, highlighting significant genetic differences among the three populations **(Figure 1a)**. The Bayesian analysis, with the optimal K-value being 2, confirmed two distinct groups **(Figures 1b, 1c)**.



a)



b) c)

Figure 1. a) principal coordinate analysis (PCoA) scatter plot based on Melipona microsatellite loci, b) identification of the optimal number of clusters from the STRUCTURE analysis, c) bar plot showing the inferred population structure of *M. rufiventris* using the Bayesian admixture model in STRUCTURE (K = 2); each individual is represented by a corresponding bar.

Although the findings from this study suggest a degree of population structure within the semiarid regions, further research is needed to explore the full extent of genetic differentiation within the 'rufiventris group.' Additional sampling from Castelo do Piauí and Guadalupe, as well as more extensive sampling across the broader landscape, would provide valuable insights. While the current sample sizes at these sites were adequate to generate robust data and perform an initial evaluation of the population structure of *M. rufiventris* in this semiarid area, small sample sizes could potentially introduce fluctuations in the results [47]. As such, the findings should be interpreted cautiously. Nevertheless, the information obtained remains crucial for the species conservation efforts. The two groups identified in this study—(1) Campo Maior and (2) Castelo do Piauí + Guadalupe—should be treated as separate conservation units.

Conclusion

Our analysis revealed a clear separation of individuals into 2 distinct groups, with some overlap, indicating notable genetic differentiation between the populations. This finding is crucial for the conservation of the species, suggesting that the two groups identified should be treated as separate units for conservation, potentially qualifying as Evolutionary Significant Units (ESUs). Conservation efforts should prioritize minimizing habitat degradation in each area and prevent the movement of colonies, particularly given the exploitation of the species in meliponiculture.

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