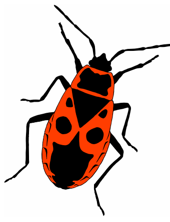

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NOTE

Genome-wide discovery and characterization of microsatellite markers from *Melipona fasciculata* (Hymenoptera: Apidae), cross-amplification and a snapshot assessment of the genetic diversity in two stingless bee populations

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Abstract. Brazilian native meliponines are currently threatened by increased human impacts. The assessment of their genetic variation by microsatellite DNA markers can assist in the conservation of populations and help in the planning and establishment of efficient management strategies. The purpose of this study was to develop the first set of microsatellite markers for *Melipona fasciculata*, selected from partial genome assembly of Illumina paired-end reads. Primer pairs were designed for each detected locus at their flanking regions. Bee samples were genotyped from two different populations of Northeastern Brazil for marker characterization and validation. A total of 17 microsatellite loci displayed polymorphism. Mean H_E and H_O heterozygosities were 0.453 and 0.536, respectively. *PIC* across all loci ranged from 0.108 to 0.714. A genetic diversity analysis revealed high values for population differentiation estimates (F_{ST} = 0.194, R_{ST} = 0.230, and D_{est} = 0.162) within the investigated region. PCoA and Bayesian clustering showed a separation of the species into two distinct clusters. These microsatellite markers have demonstrated strong potential for population-level genetic studies. Moreover, the preliminary analysis of the genetic diversity in *M. fasciculata* provides provisional evidence of significant population differentiation between the two studied populations.

INTRODUCTION

Stingless bees (Hymenoptera: Apidae: Meliponini) are a diverse group of bees regarded for their great economic and ecological importance. For instance, beekeeping provides a sustainable source of income under a low-cost investment for smallholder farming communities, and these native bees provide an efficient pollination service in both natural and agricultural systems (Garibaldi et al., 2013).

Currently, native meliponines are threatened by increased human impacts such as destruction of native vegetation and consequent landscape transformation, and indiscriminate use of pesticides for agriculture (Winfree et al., 2009; Potts et al., 2010; Silva et al., 2015). Anthropogenic disturbances or intervention may negatively affect the existence of small populations of native stingless bees, leading to the risk of local extinction (Klein et al., 2007; Silva et al., 2014). Therefore, a clear understanding of the genetic variation and population structure of meliponine bees can contribute to the development of effective conservation strategies to secure the continued survival of these original populations and the species itself.

The *Melipona* (*Melikerria*) *fasciculata* Smith, 1854 (Hymenoptera: Apidae), popularly known as "uruçu-cinzenta" or "tiúba", is a native stingless bee species that can be found in the neotropical region of Brazil, within the states of Pará, Tocantins, Maranhão, Piauí and Mato Grosso (Silveira et al., 2002). Apart from its role as a pollinator in most ecosystems and crops (Nunes-Silva et al., 2013), a great interest in the species has emerged because (i) stingless beekeeping is relatively easy, as long as

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flowering plants are available, and (ii) its production of honey and geopropolis with antioxidant potential (Dutra et al., 2014) and anti-inflammatory effect (Liberio et al., 2011). The species has also an important place in a rapidly growing market with wide possibilities for economic exploitation, mainly by family farmers (Holanda et al., 2012; Venturieri et al., 2012; Alves, 2013; Gostinski, 2018). As such, beekeepers have been continuously trying to improve honey production by nest transportation and trading among themselves (Kerr et al., 1996; Jaffé et al., 2015). In fact, this unregulated and uncontrolled practice of transporting and trading colonies from one region to another affects the genetic structure of stingless bee populations by altering factors such as geographic distance between populations or physical barriers to gene flow (Nogueira et al., 2014; Byatt et al., 2016; Jaffé et al., 2016).

Microsatellites, stretches of short DNA sequences tandemly repeated, have become the markers of choice for high-resolution assessment of genetic variation and population structure studies, most importantly, due to their abundance throughout the eukaryote genome and their hypervariability (Goldstein & Schlötterer, 1999). Emerging technologies in DNA sequencing (i.e. next generation sequencing – NGS) have proven to be useful for identifying microsatellite loci from the large amounts of sequence data they generate with much less effort and low cost, therefore, challenging traditional approaches for their development (Zalapa et al., 2012; Souza et al., 2015). Microsatellite markers developed for a studied species can also be transferred among related species, of the same genus or different genera in the same family, which considerably reduces their development costs and shortens development times in a non-source species (Selkoe & Toonen, 2006).

In this paper, we describe the first set of microsatellite markers developed for *Melipona fasciculata*, selected from partial genome assembly of Illumina paired-end reads, and test the cross-amplification of all microsatellite loci in four non-source species. A preliminary analysis of its genetic variation within a relatively short geographical range is also performed to characterize and validate these polymorphic markers.

MATERIALS AND METHODS

Bee materials and genomic DNA isolation

Genomic DNA from *Melipona fasciculata* was extracted from each adult worker thorax (n = 50) using the Wizard Genomic DNA Purification Kit (Promega, Madison, WI, USA) according to the manufacturer's instruction. Bees were collected from hives originally from the Northeast region of Brazil, in the states of Piauí (Elesbão Veloso city; 6°11´56.2˝S and 42°11´43.8˝W) and Maranhão (São Bento city; 2°42´30.6˝S and 44°50´18.9˝W). DNA was also isolated from four additional species, *M. marginata*, *M. subnitida*, *Nannotrigona testaceicornis* and *Frieseomelitta varia* (Table 1) for cross-species amplification testing. Three worker bee samples from each species were collected from hives located at Embrapa Meio-Norte in Teresina, Piauí (5°02´22.8˝S and 42°48´12.9˝W). The extracted DNA samples were electrophoresed on 0.8% agarose gel to test for overall quantity and quality of the DNA yield.

Library preparation and next generation sequencing

A single individual with the highest quality DNA yield was selected for sequencing. DNA was quantified using a PicoGreen protocol and was run using a Perkin Elmer Fusion DNA Quantifier (Perkin Elmer, Waltham, MA, USA). An Illumina paired-end library was created using 1 ng of genomic DNA, following the standard protocol of the Illumina Nextera XT Library Preparation kit (Illumina, San Diego, CA, USA). DNA was tagged and fragmented by the Nextera XT transposome, followed by limited-cycle PCR amplification, AMPure XP magnetic-bead purification (Agencourt Bioscience Corporation, Beverly, MA, USA) and the Illumina Nextera XT bead-based normalization protocol. The DNA library was sequenced using a MiSeq Benchtop Sequencer (Illumina). Contigs were created from the resulting paired-end sequence data (reads) using CLC Genomics Workbench 7.0.4 (Qiagen, Carlsbad, CA, USA).

SSR loci search and primer design

All these contigs were subsequently added directly into Msatcommander 0.8.2 (Faircloth, 2008) for detection of possible microsatellite loci with at least four repeats, except for dinucleotides (six repeats), and designing of primer pairs for each detected locus at their flanking regions. Long mononucleotide repeats were ignored for marker development. Primer design was performed with the Primer3 (Rozen & Skaletsky, 2000).

SSR-PCR amplification for primer validation and genotyping

Genomic DNA from five individuals, each from different colonies, were initially used to validate all designed primer pairs using polymerase chain reactions (PCRs). Reactions were performed in a 10-µL total volume containing at least 10 ng of genomic DNA, with 1.25 to 1.5 × buffer, 2 to 2.5 mM $MgCl_2$, 10 mM dNTP mix, 0.25 mM of each primer and 0.25 units of *Taq* DNA polymerase (Thermo Fisher Scientific, Waltham, MA, USA) or HotStar *Taq* DNA Polymerase (Qiagen). All amplifications were run in a Veriti 96-well Thermal Cycler (Applied Biosystems, Foster City, CA, USA) using the PCR temperature profile indicated in Table 1. We also tested the cross-species amplification success of these loci with three samples from each of four additional species, *Melipona marginata*, *M. subnitida*, *Nannotrigona testaceicornis*, and *Frieseomelitta varia*, as the DNA extraction described above. All amplification products, from source and non-source bee species, were screened by silver nitrate detection on denatured 6% polyacrylamide gels. Subsequently, additional *M. fasciculata* samples were genotyped from two different locations, Piauí (20 inds) and Maranhão (20 inds), each from different colonies, to obtain baseline allele frequency information.

Data analysis

The genotyped data were initially analyzed using Micro-checker ver. 2.2.3 (van Oosterhout et al., 2004) to test for the presence of null alleles, large allele dropout and scoring errors by stuttering. Observed and expected heterozygosities (H_O and H_E), the number of alleles (*Na*), and the polymorphic information content (*PIC*) were determined using Cervus ver. 3.0 (Kalinowski et al., 2007). Allelic richness (A_R) as a measure of the number of alleles per locus independent of sample size was calculated by Fstat ver. 2.9.3.2 (Goudet, 1995). Deviations from Hardy-Weinberg equilibrium (*HWE*) and tests for linkage disequilibrium were conducted using Genepop software (Raymond & Rousset, 1995). The Bonferroni correction was applied when multiple pairwise tests were performed to assess the significance ($P < 0.05$).

The genetic diversity for each locus was evaluated by Arlequin ver. 3.5 (Excoffier & Lischer, 2010), which determined the value of θ (F_{ST}) for the whole sample set.

A Bayesian grouping admixture model was used to infer possible population structuring using the software Structure ver. 2.3.3 (Pritchard et al., 2000). The program was set up for 1,000,000 Markov chain Monte Carlo repetitions after an initial burn-in of 500,000 steps. The estimate of the best *K* was calculated based on five replications for each *K* (from 1 to 6) using Structure harvester ver. 0.6.92 (Earl & vonHoldt, 2012). The program Clumpp

Table 1. Characteristics and cross-species amplification of 18 microsatellite markers developed for *Melipona fasciculata*.

Locus	Primer sequence (5'–3')	Motifs repeats	Ta (°C)	*Na*	Size range (bp)	Reaction profile	*Melipona marginata*	*Melipona subnitida*	*Nannotrigona testaceicornis*	*Frieseomelitta varia*	GenBank access. no.
*Mfsc*3	F: GAGCGAGAGGGAGCAAGATA R: TAGTAACGTAATTCTGCGCGAT	$(AGAT)_{14}$	50	2	090–094	PCR_{STD}	+	–	–	+	KT730150
*Mfsc*7	F: TCTACCCATCTCTGTTTCTCTCC R: TCGCAGTTTCGTTGATTTTG	$(ATCT)_{10}$	50	2	232–236	PCR_{STD}	–	–	–	–	KT730151
*Mfsc*10	F: AGTAGAACGATTTCGAGAAC R: ACGAAGCCGTATGCTAA	$(CTTT)_{10}$	43	2	148–160	$PCR_{STD+HOT1}$	–	–	–	–	KT730152
*Mfsc*11	F: GGAAGGACGAGAGAATTCAAGA R: ATAGTCGTTTGTCGCGAGTGTA	$(CTTT)_{10}$	50	5	170–186	PCR_{STD}	+	+	–	–	KT730153
*Mfsc*13	F: GCAGTAACGGTAGCAGTGGTG R: ACTCCTTTCTCCTTCTCGGTCT	$(ACG)_{16}$	52	2	189–207	PCR_{STD}	+	–	–	–	KT730154
*Mfsc*14	F: AGTTGCAGCGTTTGTGAAATC R: GTGGGTTCGGAGATGTGTATAAG	$(AGT)_{17}$	47–57	2	116–122	$PCR_{TCD+HOT1}$	+	–	–	–	KT730155
*Mfsc*17	F: ATTTTCTCAGTAAGCGAGTCCG R: CGACCTTGTTCGTATAATAGCA	$(ATT)_{17}$	50	10	142–187	$PCR_{STD+HOT1}$	–	–	–	–	KT730156
*Mfsc*22	F: GTGACAATAATAGGAGGGAAATCG R: GAAGCTGGTACAGGTATCGGAG	$(GAT)_{14}$	58–48	2	231–234	$PCR_{TCD+HOT1}$	+	+	+	+	KT730157
*Mfsc*23	F: ATTCGGCATCGGCGTTAT R: TTAGAGAAAGTTGTTGGACCCG	$(CGT)_{14}$	48	3	243–261	PCR_{STD}	+	–	–	–	KT730158
*Mfsc*24	F: GTAGAGGAGTAGTAACAGCAA R: CGAGTCCCGGTTAGC	$(AGC)_{14}$	48	4	165–189	PCR_{STD}	+	+	+	+	KT730159
*Mfsc*27	F: CGTCTCCACCGTCTTCTATTTC R: GCGTGTCCTCTCTTTCTCTCTC	$(AGC)_{13}$	50	6	205–227	PCR_{STD}	+	+	–	–	KT730160
*Mfsc*28	F: ATGATTCTCGCTTTCGTCGT R: GTGAGGAGACGCTGGATTTC	$(AGC)_{13}$	52–62	5	160–184	$PCR_{TCD+HOT2}$	+	+	–	+	KT730161
*Mfsc*30	F: TCTATAAGCGCCAGAGAGGAAG R: TTTCAGGGATGCGCC	$(ACG)_{12}$	50	3	186–192	PCR_{STD}	+	+	–	–	KT730162
*Mfsc*31	F: TGTGGTCGCGGTTGC R: TCGCCGCTCGGAACT	$(AAG)_{12}$	50	2	260–263	PCR_{STD}	–	–	–	–	KT730163
*Mfsc*32	F: GTTATCGTTATCGTCATCGTCGT R: CCGTGAGCGAACTCGAAC	$(CGT)_{12}$	47–57	3	105–108	$PCR_{TCD+HOT1}$	–	–	–	–	KT730164
*Mfsc*34	F: AACTTTGAGGACGCACGAG R: CACTTCTTGTTCGACTTGGTTG	$(ACGA)_{12}$	53	1	109	$PCR_{STD+HOT1}$	–	–	–	–	KT730165
*Mfsc*36	F: CGCCTACACCTAGAACCAAAA R: ACGTACACCGATGGCGTT	$(AAAG)_{13}$	55	9	097–109	PCR_{STD}	–	–	–	–	KT730166
*Mfsc*37	F: GAAGGAAGGAAAGAGGCCG R: CCATTGCTACCCGTACCTCC	$(AAAG)_{10}$	55	8	103–119	PCR_{STD}	–	–	–	–	KT730167

Ta – annealing temperature; *Na* – number of alleles; PCR_{STD} – standard PCR [94°C – 1 min; 40 cycles (94°C – 30 s; Ta – 30 s; 72°C – 30 s); 72°C – 3 min]; $PCR_{TCD+HOT1}$: Touchdown PCR with HotStart *Taq* DNA polymerase {95°C – 15 min; 10 cycles [94°C – 30 s; Ta_1 – 30 s; 72°C – 30 s]; 10 cycles [94°C – 30 s; Ta_2(–1°C/cycles) – 30 s; 72°C – 30 s]; 25 cycles [94°C – 30 s; Ta_1 – 30 s; 72°C – 30 s); 72° – 10 min]}; $PCR_{TCD+HOT2}$: Touchdown PCR with HotStart *Taq* DNA polymerase = {95°C – 15 min; 10 cycles [94°C – 30 s; Ta_1(–1,0°C/cycle) – 30 s; 72°C – 30 s]; 25 cycles [94°C – 30 s; Ta2 – 30 s; 72°C – 30 s]; 72°C – 10 min.

ver.1.12 (Jakobsson & Rosenberg, 2007) was used to align the five repetitions of the best *K*. The program Distruct ver. 1.1 (Noah, 2004) was used to graphically display the results produced by Clumpp. Population structure was also analyzed using principal coordinate analysis (PCoA), R_{ST}, a measure of genetic differentiation analogous to F_{ST} but incorporating allele size information, and the D_{est} estimator of actual differentiation as implemented in Genalex v. 6.5 (Peakall & Smouse, 2012).

RESULTS AND DISCUSSION

Sequence assembly, SSR identification and primer design

The genomic library, which was previously loaded as 16% of a MiSeq Reagent Kit v2 300 cycle sequencing run, produced 2,669,884 reads, which were assembled into 47,087 contigs. The program msatcommander 0.8.2 (Faircloth, 2008) identified 9,954 contigs (11.3% of total contigs) containing 11,869 microsatellite loci, being in the majority mononucleotide (6,444) and dinucleotide (3,225) repeats. For ease of imaging and scoring, we chose to examine only tri- (734) and tetranucleotide (574) loci. From these, 37 loci were chosen for primer designing and validation in *M. fasciculata*, based on the presence of long, uninterrupted repeat units (≥ 10 repeats), flanking regions of at least 50 bp in length containing no more than four monobase repeats [$(A)_n$, $(G)_n$, $(C)_n$, $(T)_n$], and amplicon length between 100 and 270 bp.

Polymorphism and validation of *Melipona fasciculata* SSR markers

The Micro-Checker analysis of the entire dataset revealed null alleles for loci *Mfsc*3 and *Mfsc*11, at lower frequencies than 0.2 (Table 2). Null allele frequencies below 0.2 are acceptable in most microsatellite data sets (Dakin & Avise, 2004). When the dataset was divided into two populations (Piauí and Maranhão) only locus *Mfsc*3 indicated the presence of nulls, which may be a possible cause of its deviation from *HWE* in the Piauí population, even after Bonferroni correction for multiple comparisons at the 5% significance level (critical value for $P > 0.0029$). No loci showed significant linkage disequilibrium after Bonferroni correction.

PCR products of expected size with clear and consistent bands were obtained from 18 primer pairs, out of 37 sets tested on 50 individual bees from the two surveyed populations in Northeastern Brazil (Table 2). The proportion of markers that generated consistent amplicons within their expected sizes was 48.6%. Expected product sizes for each microsatellite locus were based on sequence data from the partial genome assembly process. Seventeen microsatellite loci were polymorphic across the entire

Table 2. Variability of 17 microsatellite loci and genetic diversity estimates in *Melipona fasciculata*.

Locus	All individuals (*n* = 50)						Piauí (n = 25)						Maranhão (n = 25)						F_{ST}	R_{ST}	D_{est}
	A_R	H_O	H_E	*PIC*	p*HWE*	Null¶	A_R	H_O	H_E	*PIC*	p*HWE*	Null¶	A_R	H_O	H_E	*PIC*	p*HWE*	Null¶			
*Mfsc*3	2.0	0.000	0.186	0.167	0.000*	0.156	2	0.000	0.423	0.325	0.000*	0.290	1	0.000	0.000	0.000	Mono	0.000	0.321	0.424	0.089
*Mfsc*7	2.0	0.353	0.295	0.248	0.556	–0.048	2	0.522	0.394	0.311	0.267	–0.098	1	0.000	0.000	0.000	Mono	0.000	0.189	0.396	0.079
*Mfsc*10	2.0	0.617	0.431	0.336	0.002*	–0.133	2	0.500	0.384	0.305	0.272	–0.091	2	0.720	0.470	0.355	0.008	–0.177	0.016	0.067	0.012
*Mfsc*11	4.9	0.326	0.646	0.570	0.000*	0.191	2	0.381	0.316	0.261	1.000	–0.056	4	0.280	0.409	0.376	0.01	0.086	0.601	0.158	0.873
*Mfsc*13	2.0	0.306	0.262	0.226	0.575	–0.037	2	0.625	0.439	0.337	0.055	–0.137	1	0.000	0.000	0.000	Mono	0.000	0.309	0.084	0.122
*Mfsc*14	2.0	0.378	0.504	0.373	0.184	0.079	2	0.357	0.516	0.374	0.318	0.094	2	0.391	0.507	0.730	0.403	0.070	–0.038	0.258	–0.039
*Mfsc*17	9.1	0.732	0.610	0.550	0.013	–0.081	5	0.611	0.571	0.501	0.038	–0.036	7	0.826	0.642	0.572	0.047	–0.122	0.003	0.103	0.004
*Mfsc*22	2.0	1.000	0.505	0.375	0.000*	–0.333	2	1.000	0.512	0.375	0.000*	–0.333	2	1.000	0.510	0.375	0.000*	–0.333	0.000	0.083	0.000
*Mfsc*23	3.0	0.864	0.574	0.494	0.000*	–0.189	3	0.762	0.547	0.469	0.056	–0.149	2	0.957	0.510	0.375	0.000*	–0.305	0.161	–0.019	0.207
*Mfsc*24	3.9	0.370	0.335	0.304	0.395	–0.029	1	0.000	0.000	0.000	Mono	0.000	4	0.680	0.528	0.457	0.281	–0.107	0.253	0.094	0.132
*Mfsc*27	5.9	0.979	0.762	0.714	0.000*	–0.129	3	1.000	0.532	0.406	0.000*	–0.315	5	0.960	0.731	0.668	0.008	–0.142	0.290	0.017	0.684
*Mfsc*28	4.8	0.732	0.556	0.509	0.142	–0.118	2	0.375	0.315	0.258	1.000	–0.054	4	0.960	0.626	0.546	0.000*	–0.215	0.182	0.351	0.209
*Mfsc*30	2.9	0.156	0.186	0.173	0.017	0.024	1	0.000	0.000	0.000	Mono	0.000	3	0.292	0.324	0.286	0.031	0.019	0.125	0.035	0.030
*Mfsc*31	2.0	0.073	0.116	0.108	0.120	0.037	2	0.188	0.272	0.229	0.302	0.060	1	0.000	0.000	0.000	Mono	0.000	0.164	0.333	0.023
*Mfsc*32	2.9	0.362	0.334	0.288	0.004*	–0.024	3	0.773	0.538	0.427	0.000*	–0.162	1	0.000	0.000	0.000	Mono	0.000	0.399	0.062	0.226
*Mfsc*36	8.3	1.000	0.746	0.700	0.000*	–0.151	3	1.000	0.613	0.516	0.000*	–0.252	9	1.000	0.817	0.775	0.000*	–0.111	0.051	0.209	0.137
*Mfsc*37	7.4	0.872	0.664	0.601	0.000*	–0.130	2	0.727	0.474	0.356	0.017	–0.181	8	1.000	0.783	0.734	0.000*	–0.132	0.082	0.131	0.151
Mean	3.9	0.536	0.453	0.396	–	–	2.3	0.519	0.403	0.321	–	–	3.4	0.533	0.403	0.367	–	–	0.194	0.230	0.162

A_R – allelic richness; H_O – observed heterozygosity; H_E – expected heterozygosity; *PIC* – polymorphic information content; *pHWE* – probabilities of departure from Hardy-Weinberg equilibrium; Null – null alleles frequency. *Locus that deviated significantly from *HWE* after Bonferroni correction (adjusted critical $P < 0.0029$). ¶Negative null-allele frequencies are a software artefact and can be interpreted as zero.

data set. However, a few revealed a monomorphic banding pattern within a population (*Mfsc*24 and *Mfsc*30 in Piauí and *Mfsc*3, *Mfsc*7, *Mfsc*13, *Mfsc*31 and *Mfsc*32 in Maranhão). Nevertheless, it is expected that these loci may become polymorphic once again when additional individuals are sampled.

Ten out of the 18 *M. fasciculata*-specific microsatellite markers tested were transferable to other stingless bees (*M. marginata*, *M. subnitida*, *Nannotrigona testaceicornis*, and *Frieseomelitta varia*). However, heterologous amplification proved more successful with closely related species, with 10 loci (out of 18 tested loci, i.e. 55.6%) in *M. marginata* and 6 loci (33.3%) in *M. subnitida*. Cross-species amplification tests showed lower success rate for *Frieseomelitta varia* (22.2%) and *Nannotrigona testaceicornis* (11.1%), presumably due to the larger evolutionary distance between the target and the source species (Barbara et al., 2007).

The genotyping of the entire dataset revealed 70 alleles, ranging from one, for locus *Mfsc*34 to 10, for locus *Mfsc*17, with an average of 3.9 ± 2.7 alleles per locus (Table 2). This result was of similar magnitude to that found in other species within the same genus such as *M. rufiventris* (Lopes et al., 2009), *M. seminigra merrillae* (Francini et al., 2009), *M. interrupta manaosensis* (Francini et al., 2010), *M. mondury* (Lopes et al., 2010) and *M. yucatanica* (May-Itzá et al., 2010). The size of alleles in the least polymorphic locus (H_E and *PIC*), *Mfsc*31, ranged from 260 to 263 bp, while for the most polymorphic locus, *Mfsc*27, alleles varied from 205 to 227. These two loci (*Mfsc*31 and *Mfsc*27) were composed of trinucleotide motifs. As shown in Table 2, the level of polymorphism of each locus was also evaluated by the allelic richness (A_R) and the polymorphic information content (*PIC*). The values of allelic richness varied from 2 to 9.1 (average of 3.9 ± 2.4), while *PIC* values ranged between 0.108 and 0.714. Mean *PIC* (0.372 ± 0.198) characterizes all microsatellite loci as reasonably informative markers (Botstein et al., 1980). Overall mean observed and expected heterozygosity was estimated to be 0.536 and 0.453, respectively. These estimates were higher when compared to most heterozygosities found for *Melipona* species, exception made for *M. subnitida* (Souza et al., 2015). It is noteworthy that low levels of heterozygosity are known to occur in social Hymenoptera compared to other insects (Graur, 1985). Nine microsatellite loci exhibited significant probabilities ($P < 0.05$) of departure from Hardy-Weinberg equilibrium, likely due to the mixing of individuals from populations of different allelic frequencies (Templeton, 2006). However, when populations were considered separately, the number of loci that deviated significantly from *HWE* were five in each population (Table 2), mostly caused by excess of heterozygotes. F_{IS} *estimates* for most loci were negative, also indicating a trend towards an excess of observed heterozygosity. In a population this feature could be a signature of population bottleneck events (Cornuet & Luikart, 1996), which might be associated to destruction of native vegetation and consequent landscape transformation by human activities (Winfree et al., 2009; Potts et al., 2010).

Assessment of the genetic diversity in *Melipona fasciculata*

Genetic diversity between *M. fasciculata* populations, as measured by the mean number of alleles per microsatellite locus, mean allelic richness, heterozygosity and *PIC*, was characterized by a slightly higher level of genetic variability from samples collected in Maranhão when compared to those sampled in Piauí (Table 2).

The high F_{ST} (0.194) and R_{ST} (0.230) estimates found in *M. fasciculata* suggest the existence of genetic differentiation. However, additional genetic surveys should be carried out to confirm this observation. Similarly high F_{ST} estimates were reported in wild populations of *M. rufiventris* (Tavares et al., 2007) and *M. beecheii* (Quezada-Euan et al., 2007), with values of 0.250 and 0.280, respectively. D_{est}, which is a measure based on the proportion of alleles that are unique to a subpopulation, provided further evidence of population differentiation ($D_{est} = 0.162$). Low rates of dispersion and short flight distance, less than 2000 m, might have contributed to the levels of population differentiation (Silveira et al., 2002; Araújo et al., 2004; Duarte et al., 2014).

The scatter-plot of the principal coordinates analysis (PCoA) showed a clear separation of the species in two distinct clusters of stingless bees ultimately defined by the origin of each individual population, therefore, confirming a significant molecular genetic difference between the two populations (Fig. 1A). The analysis of microsatellite variation using the admixture model of STRUCTURE, at the first level of sub-population separation ($K = 2$), have also revealed two distinct clusters (Fig. 1B). These clusters rep-

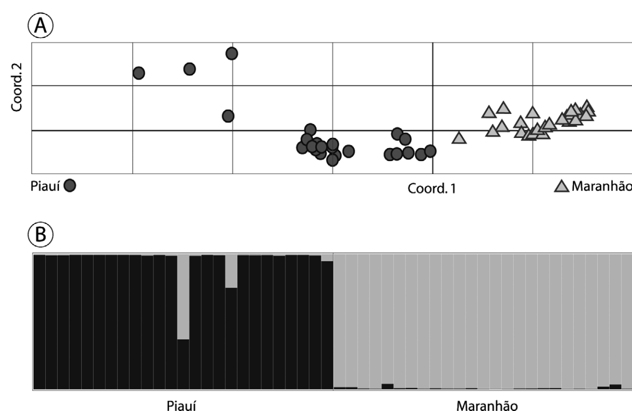

Fig. 1. A – Principal coordinates analysis of microsatellite variation in the stingless bee *Melipona fasciculata*; B – Genetic structure inferred using Bayesian analysis in the program STRUCTURE. Each individual is represented by a vertical bar.

resent each sampling population separately, except for very few individuals mostly located in Piauí that appears to be admixed.

All these estimates indicate that there is little contemporary gene flow between these two *M. fasciculata* populations. This suggests questions about whether the destruction of native semi-arid vegetation is increasing genetic drift by reducing genetic connectivity among *M. fasciculata* populations currently restricted to the remaining fragments of native forests.

Overall, analyses provide provisional evidence of significant population differentiation between Maranhão and Piauí, in *M. fasciculata*. However, the data generated by this study should be further investigated using the same microsatellites markers, but larger sample size and more widespread sampling throughout the distribution of the species. Given all these considerations, the 18 isolated microsatellite loci have demonstrated strong potential for population-level genetic studies and can be used effectively as molecular tools to aid in the conservation of the species. Cross-species amplification further indicates that most of these loci can be useful across a wider range of species. Moreover, the results obtained from this snapshot assessment of the genetic diversity in *M. fasciculata* support their use for conducting population genetics and landscape genetics studies.

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