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New molecular evidence for fragmentation between two distant populations of the threatened stingless bee *Melipona subnitida* Ducke (Hymenoptera, Apidae, Meliponini)

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Abstract

For a snapshot assessment of the genetic diversity present within *Melipona subnitida*, an endemic stingless bee distributed in the semi-arid region of northeastern Brazil, populations separated by over 1,000 km distance were analyzed by ISSR genotyping. This is a prerequisite for the establishment of efficient management and conservation practices. From 21 ISSR primers tested, only nine revealed consistent and polymorphic bands (loci). PCR reactions resulted in 165 loci, of which 92 were polymorphic (57.5%). Both Φ_{ST} (ARLEQUIN) and θ^B (HICKORY) presented high values of similar magnitude (0.34, $p<0.0001$ and 0.33, $p<0.0001$, respectively), showing that these two groups were highly structured. The dendrogram obtained by the cluster analysis and the scatter-plot of the PCoA corroborate with the data presented by the AMOVA and θ^B tests. Clear evidence of subdivision among sampling sites was also observed by the Bayesian grouping model analysis (STRUCTURE) of the ISSR data. It is clear from this study that conservation strategies should take into account the heterogeneity of these two separate populations, and address actions towards their sustainability by integrating our findings with ecological tools.

Keywords

Population differentiation, Hymenoptera, Jandaíra, genetic diversity, ISSR markers

Introduction

Melipona subnitida Ducke (Hymenoptera, Apidae, Meliponini) is an endemic stingless bee distributed in the Caatinga, the semi-arid region of northeastern Brazil (Michener 2007, Camargo and Pedro 2013). The species, called Jandaíra by natives, has great ecological significance as a pollinator of the local native flora and cultivated crops and is of economic importance in honey production, which is valued for its alleged medicinal properties and antibacterial activity (Cruz et al. 2004; Alves et al. 2008; Silva and Paz 2012).

The stingless bees are currently threatened by the increasing destruction of native semi-arid vegetation and by the intensification of agriculture in the Caatinga (Roulston and Goodell 2011; Pereira et al. 2011). In response, small populations of stingless bees may gradually decline, resulting in local extinction. The assessment of the genetic diversity present within *Melipona subnitida* populations is therefore a prerequisite for the establishment of efficient management and conservation practices.

Molecular markers have proven to be decisive in elucidating diversity and differences at the DNA level in microorganisms, plants and animals (Panwar et al. 2010; Sebastien et al. 2012; Rana et al. 2012; Bonatti et al. 2014). Among several markers, Inter Simple Sequence Repeats (ISSR) have been used due to their low cost and high level of polymorphism, and as an alternative to overcome reproducibility problems commonly found in other PCR-based markers (Abbot 2001; Lima-Brito et al. 2011). ISSRs are semiarbitrary markers based on DNA amplification by PCR in the presence of single 15- to 20-bp long primer complementary to a target short sequence repeat (Zietkiewicz et al. 1994). Despite the existence of few genetic studies related to stingless bees, it has recently been shown that ISSR markers can be useful in the analysis of natural bee populations, contributing to the development of management strategies of these important genetic resources (Nascimento et al. 2010; Tavares et al. 2013).

The present study uses ISSR analysis to investigate the degree of genetic differentiation between two *Melipona subnitida* populations separated by over 1,000 km in the Brazilian Caatinga.

Worker bees were randomly collected from natural colonies (one bee from each of 30 colonies) distributed in 2 locations only: (1) 15 nests in *Natal* (*NAT*; coordinates: 5°48'04"S, 35°11'08"W; State of Rio Grande do Norte) and (2) 15 nests in *Ilha das Canárias*, Parnaíba River Delta (*PAR*; coordinates: 2°46'39"S, 41°51'59"W; on the border of the states of Piauí and Maranhão) in Brazil (Figure 1A). All the samples were taken to the laboratory and stored at –20°C until further use. Total genomic DNA was extracted from each adult worker thorax using a phenol/chloroformalcohol isoamyl (25:24:1, v:v:v) extraction of SDS/proteinase-K digested tissue of each individual (Sambrook et al. 1989). High molecular weight DNA was isolated by ethanol precipitation and visualized by agarose gel electrophoresis.

The extracted DNA was then amplified by PCR using twenty-one primers developed by the University of British Columbia (primer set #9). PCRs were carried out in 20 μL-reaction volumes containing approximately 10–50 ng of DNA, 1× PCR buffer

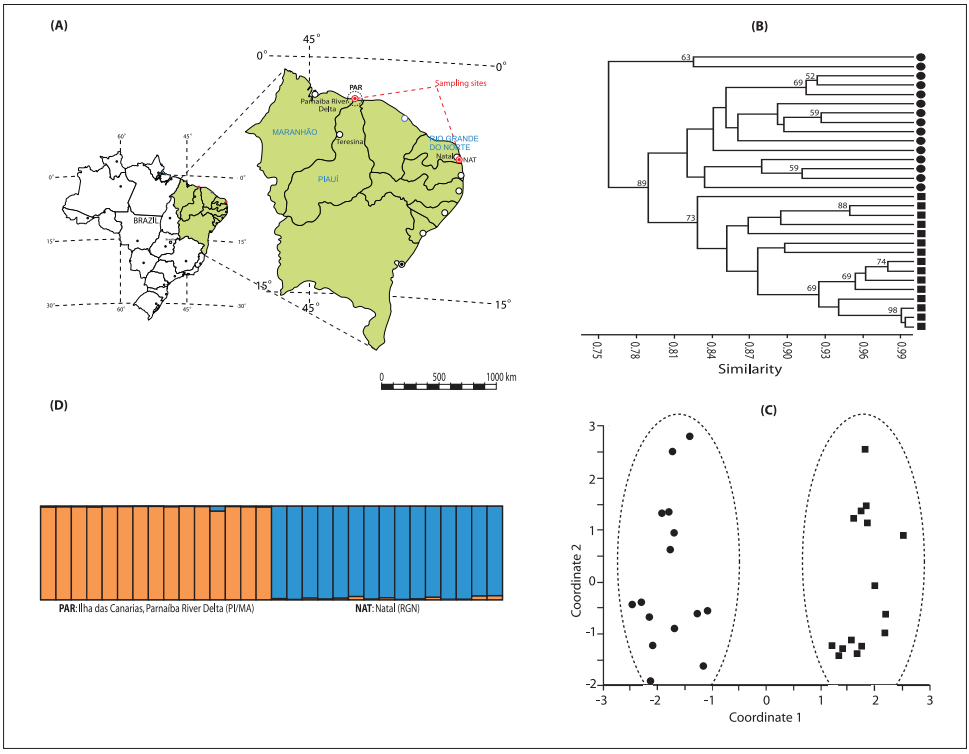

Figure 1. A Sampling sites of *Melipona subnitida*: *NAT* (coordinates: 5°48'04"S, 35°11'08"W; State of Rio Grande do Norte) and *PAR* (coordinates: 2°46'39"S, 41°51'59"W; on the border of the States of Piauí and Maranhão) **B** Clustering analysis using UPGMA for *M. subnitida* genotypes included in this study based on DICE similarity coefficient values. Numbers indicate bootstrap values for nodes retained by more than 50% of bootstrap replicates (1000 replications) **C** Scatter-plot of the principal coordinate analysis (PCoA) using ISSR loci. ■ *PAR* genotypes; ● *NAT* genotypes **D** Bar plot from Inferred population structure of using the Bayesian grouping admixture model-based program STRUCTURE (K = 2).

(40 mM Tris-HCl; 100 mM KCl), 1.5 µM of primer, 6.25 mM $MgCl_2$, 1.5 µM of each dNTP, 1.25 U of Invitrogen *Taq* DNA polymerase. All amplifications were carried out on a VERITI™ Gradient Thermalcycler (APPLIED BIOSYSTEMS). The following PCR conditions were used: an initial denaturation at 94°C for 1.5 min, followed by 45 cycles of 94°C/40 s, 36°C/1 min. and 72°C/2 min., and a final extension of 72°C/5 min. ISSR markers were screened by silver nitrate detection on denatured 6% polyacrylamide gels, which were scored for absence (0) and presence (1) of bands across genotypes and entered into a binary matrix.

Sample polymorphism was estimated as the percentage of polymorphic bands (PPB) in the total number of bands. The program HICKORY v.1.1, which implements the Bayesian method (Holsinger et al. 2002), was used for estimating θ^B (F_{ST} analogue), heterozygosity (H_S), and the inbreeding coefficient (*f*), an F_{IS} analogue for dominant markers. Analysis of Molecular Variance (AMOVA) was conducted using

all the amplified loci to check the occurrence of population structure among sampling localities using ARLEQUIN v.3.11 (Excoffier et al. 2006). The use of different algorithms for the calculation of F_{ST} analogues was an additional effort to check for the reliability of the data presented by ISSR markers.

Further, a Bayesian grouping admixture model (burn-in length 100,000 iteractions; run length 100,000; *K*= 1 to 8 subpopulations tested) was used to infer the number of subpopulations (software STRUCTURE 2.3.3; Pritchard et al. 2000). These results were analyzed using STRUCTURE HARVESTERWeb v.0.6.9 (Evanno et al. 2005).

The similarity among samples within populations was estimated using PAST v1.34 (Hammer et al. 2001) according to Dice's coefficient. Cluster analysis using the unweighted pair-group method arithmetic average (UPGMA) and multidimensional principal coordinate analysis (PCoA) were performed on the data set to reveal the degree of genetic differentiation between sites.

From 21 primers initially screened for their ability to generate ISSR loci, only nine revealed consistent and polymorphic bands (loci) with 30 Jandaíra worker bees. The other 12 ISSR markers were monomorphic or had unreliable amplification and therefore are not included in the genetic diversity analysis. Polymorphic ISSR primers were also considered reproducible after repeated PCRs, under the same reaction conditions and, therefore, selected for genotyping (Table 1). PCR reactions involving these nine primers resulted in 165 loci (bands) or 18.3 bands/primer, of which 92 were polymorphic (10.2 polymorphic bands/primer) ranging in size from 250 to 1636 bp, corresponding to an average polymorphism of 57.5%. Genotyping showed that most of the detected loci were polymorphic. Overall ISSR polymorphism in *Melipona subnitida* was similar to that of *M. quadrifasciata* Lepeletier (67%) (Nascimento et al. 2010). The number of polymorphic bands per primer ranged from 5 (UBC-884 and UBC-888) to 23 (UBC-899).

ISSR genotyping revealed differences in genetic diversity based on the percentage of polymorphic bands (% PPB), which was also estimated separately for each population. Result suggests that the *NAT* population (80.7%) is characterized by a higher genetic diversity than the *PAR* population (64.9%), which in theory might give the *NAT* population an increased ability to adapt to selective pressures.

The θ^B, f and H_S values obtained from four different models of population structure using the Bayesian analysis are shown in Table 2. Of the models applied to the ISSR dataset, the full model, in which θ and f are estimated simultaneously, was preferred primarily because of its smaller deviant information criterion (DIC) value (657.47), with a difference of more than six units required to indicate strong support over all the other models (Holsinger et al. 2002). In the Bayesian approach θ^B (analogue to Wright's F_{ST}), f (analogue to F_{IS}), and H_S (average panmictic heterozygosity across populations) were estimated to be 0.33, 0.31 and 0.29, respectively, indicating a pronounced genetic differentiation between populations, possibly caused by restricted gene flow and random genetic drift (Epperson 1995).

The analysis of molecular variance also provided additional support to the evidence of population differentiation in *Melipona subnitida*. The AMOVA analysis indicated

Table 1. Primer names and sequences used in the ISSR analysis, number of polymorphic bands per primer and range of molecular weight in base pairs (bp) amplified by PCR-ISSR for 30 *Melipona subnitida* worker bees.

Primer code	Primer sequence (5'–3')*	Total number of bands/loci	Number of polymorphic loci						Size range of bands (bp)
			Total	Overall PPL	*PAR*	Polymorphism (%)	*NAT*	Polymorphism (%)	
UBC-827	ACA ACA ACA ACA ACA CG	15	6	0.400	3	50.0	6	100.0	500-1010
UBC-834	AGA GAG AGA GAG AGA GYT	25	8	0.320	7	87.5	2	25.0	300-1000
UBC-841	GAG AGA GAG AGA GAG AYC	21	16	0.762	11	68.8	13	81.3	500-1010
UBC-845	CTC TCT CTC TCT CTC TRG	9	9	1.000	8	88.9	8	88.9	506-1636
UBC-884	HBH AGA GAG AGA GAG AG	18	5	0.278	4	80.0	3	60.0	500-1010
UBC-886	VDV CTC TCT CTC TCT CT	16	11	0.688	6	54.5	11	100.0	500-1600
UBC-888	BDB CAC ACA CAC ACA CA	14	5	0.357	3	60.0	4	80.0	500-1010
UBC-899	CAT GGT GTT GGT CAT TGT TCC A	34	23	0.676	14	60.9	21	91.3	250-1636
UBC-852	TCT CTC TCT CTC TCT CRA	13	9	0.692	3	33.3	9	100.0	950-1636
Total		165	92	-	59	-	77	-	-
Average		-	-	0.575	-	64.9	-	80.7	-

PPL: Proportion of polymorphic loci; *The following designations were used for degenerate sites: Y (C or T), R (A or G), H (A, T, or C), B (G, T, or C), V (C, G, or A) and D (G, A, or T). *PAR*: *Ilha das Canarias*, Parnaiba River Delta; *NAT*: *Natal*.

Table 2. Summary of genetic variability, partitioning of diversity and limits for credible interval by Bayesian statistical procedures.

Models	*f*			θ^B			H_S			DIC
	Mean	2.5 %	97.5 %	Mean	2.5 %	97.5 %	Mean	2.5 %	97.5 %	
Full	0.3096	0.0455	0.67	0.3275	0.2425	0.4188	0.2925	0.2565	0.3254	657.465
f = 0	0	-	-	0.2737	0.2099	0.3451	0.3272	0.3122	0.3418	665.837
θ^B	0.3733	0.1702	0.6037	0	-	-	0.3546	0.3319	0.3761	1107.250
f-free	0.4997	0.0224	0.9742	0.3578	0.2807	0.4389	0.2771	0.2467	0.3098	700.287

θ^B is analogous to Wright's F_{ST}, *f* is analogous to F_{IS}, H_S is the average panmictic heterozygosity across populations, and DIC is deviance information criterion.

Table 3. Hierarchical analysis of molecular variance (AMOVA) detected by the ISSR genotyping.

Source of variation	df	Sum of squares	Variance components	Percentage of total variance (%)	P-value*
Among populations	1	96.333	5.697	34.35	< 0.0001
Among individuals within populations	28	304.800	10.886	65.65	0.014
TOTAL	29	401.133	16.583		
Fixation index			F_{ST} index = 0.3435		

*p-values calculated from a random permutation test (10,000 permutations).

that most of the genetic variation found in *M. subnitida* samples could be attributed to differences among individuals within populations (approximately 66% of the variance), but also a large part of the variation (34.35%) was due to differences among localities. The inbreeding coefficient (*f* = 0.31) provided by HICKORY agrees with the results obtained by the AMOVA, as moderate endogamy might be expected for strongly structured populations.

Both θ^B (HICKORY) and Φ_{ST} (ARLEQUIN) presented high values, but of similar magnitude and significance (0.33, p<0.0001 and 0.34, p<0.0001, respectively), showing that these two populations are highly structured (Tables 2–3). These two different approaches showed a general agreement among the results.

A high degree of population differentiation has also been observed for *Melipona quadrifasciata* populations based on ISSR patterns (Nascimento et al. 2010) and *M. rufiventris* Lepeletier (Tavares et al. 2007) based on allozyme, microsatellite and random amplified polymorphic DNA (RAPD) molecular markers. Both studies were conducted within the State of Minas Gerais (Brazil). However, no correlation was found between the first internal transcribed spacer (ITS1) sequence divergence of *M. subnitida* populations and geographical distances in northeastern Brazil, which might be explained by the extremely high mutation rates of the ITS region in *M. subnitida* (Cruz et al. 2006).

Genetic differentiation within *Melipona subnitida* populations was probably because of low gene flow, caused by limited dispersal ability (Engels and Imperatriz-Fonseca 1998; Tavares et al. 2007), the large distance separating the *NAT* and *PAR*

populations, and extensive disturbances of population dynamics due to anthropogenic habitat degradation and fragmentation (Quezada-Euan et al. 2007). More recently, mtDNA data has also pointed to genetic differentiation between these *M. subnitida* populations from Rio Grande do Norte (Mossoró city) and Piauí (Parnaíba city, on the border of Maranhão state) (Bonatti et al. 2014).

Furthermore, the dendrogram obtained by the cluster analysis (Figure 1B) and the scatter-plot of the PCoA (Figure 1C) revealed a clear separation of the species in two main clusters confirming a significant molecular genetic difference between the two populations. This topology corroborates the data presented by the Bayesian θ^B and AMOVA. A clear evidence of subdivision among sampling sites was also observed by the Bayesian grouping model analysis of the ISSR data (Figure 1D).

This study provides additional evidence that ISSR markers can be useful tools in defining population genetic substructuring in *Melipona* species. More importantly, the distinctiveness of populations in these two regions suggests that the *NAT* and *PAR* populations of *M. subnitida* have separate evolutionary histories. It is clear from this study that conservation strategies should take into account the heterogeneity of these two separate populations, and that actions should be addressed towards their sustainability by integrating our findings with ecological tools. Failing to do so would risk decimating the entire bee population by uncontrolled human activities in the region.

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