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Mitochondrial genome differences between the stingless bees *Melipona rufiventris* and *Melipona mondury* (Apidae: Meliponini)

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ABSTRACT. Within the Meliponini, a widely distributed group of stingless bees, *Melipona rufiventris* has been considered as a single, cohesive species. Recently, analysis of morphological characters led to the splitting of this species into two species, *M. mondury* and *M. rufiventris*. The former occurs in the Atlantic Rain Forest ranging from Santa Catarina to Bahia States, while the latter is found in other parts of Brazil. We used PCR + RFLP to identify genetic marker patterns of the mtDNA between these species. Nine mtDNA regions were amplified and digested with four restriction enzymes (*Eco*RI, *Eco*RV, *Hin*dIII, and *Hin*fI). Six species-specific restriction sites were identified for *M. mondury* and *M. rufiventris* with all enzymes, except for *Hin*dIII. The molecular data agree with the morphological classification.

Key words: Meliponini, *Melipona rufiventris*, *Melipona mondury*, RFLP, Mitochondrial DNA, Polymorphic site

INTRODUCTION

The stingless bee Meliponini tribe comprises several hundred (400 to 500) species that are restricted to the Neotropical region (Velthuis, 1997; Costa et al., 2005). According to Michener (2000), the total number of species is still not well established because of the many cryptic species. In Brazil more than 300 stingless species have been described from several regions (Kerr et al., 1996). Besides playing a very important ecological role, some species are commercially exploited for honey production and pollination of various cultivated crops (Del Sarto et al., 2005).

Melipona rufiventris Lep. was considered to be a stingless bee species widely distributed in several regions of the Brazil (Vasconcelos, 1998). However, Moure (1975) and Camargo and Pedro (1992) suggested the separation of some *M. rufiventris* populations as different species. Melo (2003) found some morphological differences in populations considered to be *M. rufiventris* and separated these populations into two species, *M. mondury* and *M. rufiventris*. The former is currently located in the Atlantic Rain Forest, ranging from Santa Catarina to Bahia States, whereas the latter is found in other parts of Brazil (Melo, 2003).

Mitochondrial DNA (mtDNA) has been widely used as a molecular genetic marker in vertebrates and invertebrates due to its characteristics, such as high-mutation rates, maternal inheritance, absence of recombination, and small molecular size (Brown et al., 1979; Moritz et al., 1987; Harrison, 1989). Analysis of mtDNA polymorphism has been used in several animal studies, such as population dynamics, species and subspecies characterization, systematics, and phylogeny (Moritz et al., 1987; Patarnello et al., 1994; Weinlich et al., 2004).

Biodiversity of bees was first assessed using morphometric analyses. Ruttner et al. (1978) recognized 24 *Apis mellifera* subspecies and suggested that they could be grouped into three or four evolutionary "branch groups". Later mtDNA analyses confirmed the existence of these four evolutionary branches, with slight modifications to subspecies composition (Estoup et al., 1995; Franck et al., 2000). Since mtDNA is inherited maternally, it has been used in biogeographic studies of *A. mellifera* to investigate the ancestry of individual colonies, and for studies of gene flow patterns among hybridizing populations, such as the spread of African-derived honey bees from Brazil to other parts of South, Central and North America (Sheppard and Smith, 2000).

Among non-*Apis* bees, mtDNA has been used to study genetic variability in the Bombini and Meliponini tribes. In bumble bees (Bombinae), analyses of sequences from the cytochrome *b* and COI regions of the mtDNA were used to determine the genetic structure of several *Bombus* species (Estoup et al., 1996; Widmer et al., 1998; Widmer and Schmid-Hempel, 1999).

mtDNA has also been used for the characterization of species and subspecies within the Meliponini. Restriction maps have been made for species belonging to the genera *Plebeia* and *Melipona* (Francisco et al., 2001; Weinlich et al., 2004), while Moretto and Arias (2005), through RFLP analyses, identified restriction size patterns for *M. quadrifasciata quadrifasciata* and *M. quadrifasciata anthidioides*.

There have been very few molecular studies of *M. rufiventris* and *M. mondury*. Recently, Costa et al. (2005) noted low genetic variability when they analyzed isoenzyme data of some *M. rufiventris* populations. We used PCR + RLFP methods to identify genetic marker differences in the mtDNA between *M. rufiventris* and *M. mondury*.

MATERIAL AND METHODS

The mitochondrial genome analyses were carried out with individuals from eight *M. mondury* colonies sampled in Blumenau, Santa Catarina State and five colonies of *M. rufiventris*, four collected in São Simão, São Paulo State and one in Brasília, the Brazilian capital.

Total DNA was extracted as described by Sheppard and McPheron (1991), using one thorax per extraction. One individual per colony was sampled. The mtDNA was analyzed by PCR + RFLP. PCR was carried out using 1 µL of the total DNA extraction, 5 µL of PCR buffer (Boehringer Mannheim), 1.5 µL of each primer (20 mM), 5 µL of the dNTPs (2 mM each), and 2.5 U of *Taq* DNA polymerase (Boehringer Mannheim) in a total volume of 50 µL. Each PCR reaction was submitted to an initial denaturation at 94°C for 5 min, followed by 35 cycles of 94°C for 1 min, annealing for 1 min and 20 s at the specific temperature for each pair of primers (Table 1), with elongation at 64°C for 2 min. A final elongation step at 64°C for 10 min was performed. Seven pairs of primers (Table 1) were used to amplify specific mtDNA regions of *M. q. quadrifasciata*. The PCR products were separated by electrophoresis on 0.8% agarose gels, stained with ethidium bromide, visualized under a UV light and photographed.

To look for restriction sites, the PCR-amplified mtDNA fragments were digested for a minimum of 6 h with the following restriction enzymes: *Eco*RI, *Eco*RV, *Hin*dIII, and *Hin*fI. The digested products were analyzed on 1.0% agarose (Agarose 1000 Gibco) and were stained and visualized as above.

RESULTS

Most *primer* pairs (Table 1) gave amplification products of nine mtDNA regions in *M. mondury* and *M. rufiventris*. Only the $tRNA^{Leu}$/COII region was not amplified in any of the individuals. The sample from the single *M. rufiventris* colony collected in Brasília did not amplify the ND2/COI region. The sizes in base pairs of the amplified mtDNA regions and digestion fragments are shown in Table 2.

The four restriction enzymes recognized one or more restriction sites in the two bee species. Only one restriction site was detected with *Hin*dIII in the ATPases (6,8) - COIII region, which was not polymorphic between *M. mondury* and *M. rufiventris*. With each of the other enzymes, at least one restriction site polymorphic between the two bee species was detected. *Hin*fI produced polymorphic restriction sites in three mtDNA regions. Fragments with 1200 and 700 bp were observed in the COI region in *M. mondury*, while in *M. rufiventris* a single 950-bp fragment and various small fragments for which the size could not be precisely estimated were detected. Another *Hin*fI polymorphic site was observed in the ND4-ND5 region, where two fragments with 1800 and 650 bp were detected in *M. mondury* and in *M. rufiventris* from Brasília and another with 1900 and 550 bp in *M. rufiventris* from São Simão. In the ND1-16S region *Hin*fI gave fragments with 630 and 120 bp in *M. mondury* and with 650 and 100 bp in *M. rufiventris*.

The enzyme *Eco*RI cut four mtDNA regions, but polymorphic sites were detected only in the CytB-ND1 region, with fragments of 1000 and 700 bp in the five *M. rufiventris* samples and no restriction site in *M. mondury*. Another polymorphic site between the two bee species was found with *Eco*RV. In the CytB-ND1 region, this enzyme gave a restriction site that was

detected only in *M. mondury*; two fragments with 1400 and 300 bp in this species were not visualized in *M. rufiventris*.

Table 1. Pairs of *primers* used to amplify mitochondrial DNA regions of *Melipona mondury* and *M. rufiventris*, their main gene constituents, and respective annealing temperatures.

Pair	Name	Sequence (5'→3')	Reference	Main genes	Temperature (°C)
1	mtD2	GCTAAATAAGCTAACAGGTTCAT	(Simon et al., 1994)	ND2, COI	42
	mtD9	CCCGGTAAAATTAAAATATAAACTTC	(Simon et al., 1994)		
2	mtD7	GGATCACCTGATATAGCATTCCC	(Simon et al., 1994)	COI	44
	COI-IIR	GATCAATATCATTGATGACC	(Hall and Smith, 1991)		
3	mtD19	GAAATTTGTGGAGCAAATCATAG	(Simon et al., 1994)	ATPases (8,6),	42
	mtD22	TCAACAAAGTGTCAGTATCA	(Simon et al., 1994)	COIII	
4	5612R	GAAATTAATATAACATGACCACC	(Francisco et al., 2001)	COIII, ND3	42
	tPheF	GCGTAATATTGAAAATATTAATGA	(Francisco et al., 2001)		
5	mtD24	GGAGCTTCAACATGAGCTTT	(Simon et al., 1994)	ND4, ND6,	42
	mtD28	ATTACACCTCCTAATTTATTAGGAAT	(Simon et al., 1994)	CytB	
6	mtD26	TATGTACTACCATGAGGACAAATATC	(Simon et al., 1994)	CytB, ND1	42
	mtD30	GTAGCATTTTTAACTTTATTAGAACG	(Simon et al., 1994)		
7	Mel 3	TAAAGTTAAAAAAGCAACTC	(Francisco et al., 2001)	16S	42
	16SF	CACCTGTTTATCAAAAACATGTCC	(Hall and Smith, 1991)		
8	16SR	CGTCGATTTGAACTCAAATCATG	(Hall and Smith, 1991)	16S, 12S	42
	mtD36	AAACTAGGATTAGATACCCTATTAT	(Simon et al., 1994)		
9	MtD18	CCACAAATTTCTGAACATTGACCA	(Simon et al., 1994)	COII	44
	COI-IIF	TCTATACCACGACGTTATTC	(Hall and Smith, 1991)		
10	Seq 18	GAACTATCAATTTGATATTG	(Francisco et al., 2001)	ND4 & ND5	42
	8467F	GGAATTTTTTTTTGAATGAAA			

DISCUSSION

The PCR + RFLP technique enabled us to determine some pairs of *primers* that can be used to amplify the mtDNA in *M. mondury* and *M. rufiventris* and to estimate the number of restriction sites and size of the fragments generated by the four restriction enzymes. The mtDNA amplification of *M. rufiventris* and *M. mondury* was conducted with 10 pairs of *primers* previously used to amplify specific mtDNA regions in other Meliponini (Table 1). These *primers* amplified nine mtDNA regions, which encompass approximately 80% of the mitochondrial genome of *M. mondury* and *M. rufiventris*. No amplification products were obtained with the mtD18 + COI-IIF *primers*, which amplified the region that contained the COII gene (approximately 950 bp), in *Plebeia remota* and *M. quadrifasciata* (Francisco et al., 2001; Moretto and Arias, 2005). Therefore, the COII gene and its control region were the only mtDNA regions not amplified in *M. mondury* and *M. rufiventris*.

Table 2. Restriction fragment sizes and number of restriction sites (in parentheses) generated after enzymatic digestion. Polymorphic sites are indicated in bold. A: *Melipona mondury* and B: *Melipona rufiventris*.

MtDNA regions	Fragment total size (bp)	*Eco*RI		*Eco*RV		*Hin*dIII		*Hin*fI	
		A	B	A	B	A	B	A	B
ND2, COI	2200	1500 700 (1)	1500 700 (1)	2200 (0)	2200 (0)	2200 (0)	2200 (0)	**1600** **600** **(1)**	**1850** **350** **(1)**
COI	1900	1900 (0)	1900 (0)	1900 (0)	1900 (0)	1900 (0)	1900 (0)	**1200** **700** **(1)**	**950** **(?)**
ATPases (6,8), COIII	1800	960 840 (1)	960 840 (1)	1000 800 (1)	1000 800 (1)	1050 750 (1)	1050 750 (1)	1800 (0)	1800 (0)
COIII, ND3	1250	900 350 (1)	900 350 (1)	1100 200 (1)	1100 200 (1)	1250 (0)	1250 (0)	1250 (0)	1250 (0)
ND4, ND5	2450	2450 (0)	2450 (0)	2450 (0)	2450 (0)	2450 (0)	2450 (0)	**1800** **650** **(1)**	**1900** **550** **(1)**
ND4, ND6, CytB	2560	2560 (0)	2560 (0)	2560 (0)	2560 (0)	2560 (0)	2560 (0)	2560 (0)	2560 (0)
CytB, NDI	1700	**1700** **(0)**	**1000** **700** **(1)**	**1400** **300** **(1)**	**1700** **(0)**	1700 (0)	1700 (0)	1700 (0)	1700 (0)
ND1, 16S	750	750 (0)	750 (0)	750 (0)	750 (0)	750 (0)	750 (0)	**630** **120** **(1)**	**650** **100** **(1)**
16S, 12S	1850	1850 (0)	1850 (0)	1850 (0)	1850 (0)	1850 (0)	1850 (0)	1850 (0)	1850 (0)

? = small fragments for which the size could not be estimated.

Recent studies of mtDNA of Meliponini have provided additional insight into the *Melipona*. Restriction site patterns have been used to characterize subspecies and populations at a molecular level in the two *M. quadrifasciata* subspecies and among *M. q. quadrifasciata* populations (Moretto and Arias, 2005; Torres RR, Arias MC and Moretto G, unpublished results).

Weinlich et al. (2004) determined the mtDNA maps of *M. rufiventris* and six other *Melipona* species. Twelve restriction sites on the *M. rufiventris* map were determined by eight restriction enzymes. The *Hae*III site mapped at the ND5 gene was exclusive for *M. rufiventris*, while the other sites were shared by at least one other species. Although we did not use the enzyme *Hae*III, some different restriction sites were identified when our results were

compared with this map. The *Eco*RI restriction site at the ND2-COI region, present in the two bee species that we studied, was absent in all species in Weinlich's map. The *Eco*RI site detected in the CytB-ND1 region, absent in *M. mondury* and present in *M. rufiventris* in our study, was undetected in the *M. rufiventris* analyzed by Weinlich et al. (2004). While no restriction site was detected with *Eco*RV in the *M. rufiventris* map, we found three non-polymorphic restriction sites in both bee species (Table 2) and a restriction site in the CtyB-ND1 region that was present in *M. mondury* and absent in *M. rufiventris*, as well as in the seven species of the map determined by Weinlich et al. (2004).

The restriction enzyme *Hin*fI, which has a 4-base recognition site, has been used to discriminate subspecies of *A. mellifera* and *M. quadrifasciata* (Sheppard et al., 1996; Moretto and Arias, 2005). We detected four restriction sites for *Hin*fI polymorphic between *M. rufiventris* and *M. mondury*; in contrast only one polymorphic site was detected among *M. quadrifasciata* subspecies (Moretto and Arias, 2005). The enzymes *Eco*RI and *Eco*RV, both with six-base recognition sites, also revealed a polymorphic restriction site that was not detected in *M. quadrifasciata*.

The stingless bees *M. mondury* and *M. rufiventris* were previously considered as a single species (*M. rufiventris*), with a large geographic distribution across Brazil. Recently, Melo (2003), based on morphometric analyses, concluded that a part of this group of populations is a new species, *M. mondury*, which is found in the Atlantic Rain Forest from Santa Catarina to Bahia. Although our mtDNA analysis was limited to four restriction enzymes, the genetic variability detected between *M. mondury* and *M. rufiventris* supports Melo's conclusions.

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