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Detection of Mitochondrial DNA Restriction Site Differences Between the Subspecies of *Melipona quadrifasciata* Lepeletier (Hymenoptera: Apidae: Meliponini)

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Detecção de Diferenças no Padrão de Restrição do DNA Mitocondrial Entre Duas Subespécies de *Melipona quadrifasciata* Lepeletier (Hymenoptera: Apidae: Meliponini)

RESUMO - A distribuição geográfica da abelha sem ferrão *Melipona quadrifasciata* Lepeletier, no Brasil, atinge do Rio Grande do Sul até a Paraíba, habitando originalmente regiões de Mata Atlântica. A espécie compreende duas subespécies, *M. q. quadrifasciata* e *M. q. anthidioides*, que são reconhecidas morfologicamente pelo padrão das bandas tergais (listras amarelas no abdome). O presente trabalho teve por objetivo detectar diferenças no padrão de restrição do DNA mitocondrial entre as duas subespécies. Foram coletadas amostras de quatro colônias de cada subespécie e uma operária por colônia foi utilizada nas análises moleculares. Nove regiões do genoma mitocondrial foram amplificadas via PCR e posteriormente digeridas com 13 enzimas de restrição (PCR+RFLP). Três enzimas apresentaram padrão de restrição diferente entre as duas subespécies. Diferenças no padrão de restrição do genoma mitocondrial poderão contribuir na identificação da origem materna de indivíduos híbridos de *M. q. quadrifasciata* e *M. q. anthidioides*, pois estes ocorrem naturalmente ou por influência humana.

PALAVRAS-CHAVE: Enzima de restrição, PCR+RFLP, polimorfismo

ABSTRACT - The endemic range of the stingless bee *Melipona quadrifasciata* Lepeletier extends from the southern most state in Brazil, Rio Grande do Sul, up to the state of Paraíba, where it originally inhabited the Atlantic Rain Forest. Two subspecies have been morphologically recognized based on the tergal band pattern (yellow stripes across the abdomen), *M. q. quadrifasciata* and *M. q. anthidioides*. The objective of the present study was to characterize restriction site variation in the mitochondrial DNA of each subspecies. Four colonies of each subspecies were sampled. One individual per colony was used for DNA extraction and further analysis through amplification of nine fragments of the mitochondrial genome and digestion with 13 restriction enzymes (PCR+RFLP). Three enzymes showed restriction pattern differences between the subspecies. Those differences will be useful to determine the maternal origin of hybrids colonies, which may occur naturally or due to colony transportation among breeders.

KEY WORDS: Restriction enzyme, polymorphism, PCR+RLFP, mtDNA

The tribe Meliponini (Michener 2000) consists of stingless bees that exhibit highly advanced social organization. In Brazil, more than 300 species of this tribe have been described and are widely distributed in the country (Kerr *et al*. 1999).

The stingless bees play very important ecological and economic roles. Several species are commercially exploited for honey production. *Melipona quadrifasciata* Lepeletier ("mandaçaia") is one of such species and comprises two subspecies, *M. quadrifasciata quadrifasciata* and *M. quadrifasciata anthidioides* (Schwarz 1932). The subspecies can be easily discriminated by the yellow stripe pattern from the third to the sixth tergite, with *M. q. quadrifasciata* exhibiting continuous yellow bands and *M. q. anthidioides* exhibiting stripes that are interrupted in the central part (Aidar 1996).

The endemic range of this species goes across the state of Paraíba (northeastern Brazil) down to the state of Rio Grande do Sul (southern Brazil) (Moure & Kerr 1950). However, the geographic distribution seems to be very distinct

for each subspecies. *M. q. quadrifasciata* is found in regions with colder climates, being more abundant in the states of Paraná and Santa Catarina (Monteiro 2000), but it is also found in altitudes above 1500 m in São Paulo, Rio de Janeiro and Minas Gerais (Moure 1975). In contrast, *M. q. anthidioides* is distributed in warmer regions, being frequent in the states of Rio de Janeiro and Minas Gerais (Melo & Campos 1987 in Aidar 1996). Nonetheless, natural hybrid zones have been described in regions of the state of São Paulo and in southern Minas Gerais, where hybrid individuals showing a mixture of both tergal band patterns have been registered (Moure & Kerr 1950, Kerr 1951 in Aidar 1996, Moure 1975).

Few molecular studies have been conducted on this species. Using RAPD methods, Waldschimidt *et al*. (2000) identified a DNA marker that seemed to be present in *M. q. quadrifasciata,* but absent in *M. q. anthidiodes*. However, the authors stated that more data were needed to develop a consistent marker to identify the subspecies. RFLP has also been used to characterize mitochondrial DNA variation in the two subspecies (Weinlich *et al*. 2004). Among 18 restriction sites mapped with 15 six-base cutter restriction enzymes, only one site was variable (*Bgl* II). The total genome size was estimated at approximately 18,500 base pairs for both subspecies (Weinlich *et al*. 2004).

As mentioned above, hybridization between the two subspecies occurs and although they can be easily identified by morphology, hybrid individuals present intermediate tergal band pattern. Thus the possibility of having reliable molecular markers would certainly help to identify the maternal origin of hybrids and also to understand other biological aspects of the species, like migration and gene flow. Therefore the present study is a current effort directed toward detecting genetic markers differences between these subspecies. Here is reported a further mtDNA characterization of *M. q. quadrifasciata* and *M. q. anthidiodes* with PCR+RFLP methods.

Material and Methods

Four colonies of each subspecies were sampled. *M. quadrifasciata quadrifasciata* workers were obtained from colonies located in three different regions of the state of Santa Catarina (Blumenau, Leoberto Leal and Mafra) and one of the state of Paraná (Prudentópolis). Workers of *M. q. anthidioides* were collected from colonies located in three different states: Paraná (Curitiba), Rio de Janeiro (Cunha) and São Paulo (Bauru and Ribeirão Preto). Total DNA was extracted as described by Sheppard & McPheron (1991) using one thorax per extraction. One individual per colony was sampled. The mtDNA was analyzed by PCR+RFLP, a technique that consists of amplification of specific regions of the mitochondrial genome by PCR and subsequent digestion of the fragments with restriction enzymes. 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 µM), 5 µl of 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 initial denaturation at 94ºC/5 min, followed by 35 cycles of denaturation at 94ºC/ 1 min, annealing for 1 min and 20 s at the specific temperature for each pair of primers (Table 1) and elongation at 64ºC/2 min. A final elongation step at 64ºC for 10 min was

Table 1. Pairs of *primers* used to amplify mitochondrial DNA regions of *M. quadrifasciata*, the genes amplified, and respective annealing temperatures.

Pair	Name	Sequence (5'→3')	Reference	Main genes	Temp (ºC)
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 & Smith 1991)		
3	COI-IIF	TCTATACCACGACGTTATTC	(Hall & Smith 1991)	COII	44
	mtD18	CCACAAATTTCTGAACATTGACCA	(Simon *et al.* 1994)		
4	mtD19	GAAATTTGTGGAGCAAATCATAG	(Simon *et al.* 1994)	ATPases (8, 6),	42
	mtD22	TCAACAAAGTGTCAGTATCA	(Simon *et al*. 1994)	COIII	
5	5612R	GAAATTAATATAACATGACCACC	(Francisco *et al.* 2001)	COIII, ND3	42
	tPheF	GCGTAATATTGAAAATATTAATGA	(Francisco *et al.* 2001)		
6	mtD24	GGAGCTTCAACATGAGCTTT	(Simon *et al.* 1994)	ND4, ND6,	42
	mtD28	ATTACACCTCCTAATTTATTAGGAAT	(Simon *et al.* 1994)	CytB	
7	mtD26	TATGTACTACCATGAGGACAAATATC	(Simon *et al.* 1994)	CytB, ND1	42
	mtD30	GTAGCATTTTTAACTTTATTAGAACG	(Simon *et al.* 1994)		
8	Mel 3	TAAAGTTAAAAAAGCAACTC	(Francisco *et al.* 2001)	16S	42
	16SF	CACCTGTTTATCAAAAACATGTCC	(Hall & Smith 1991)		
9	16SR	CGTCGATTTGAACTCAAATCATG	(Hall & Smith 1991)	16S, 12S	42
	mtD36	AAACTAGGATTAGATACCCTATTAT	(Simon *et al.* 1994)		

Table 2. Restriction fragment sizes and number of restriction sites (in parenthesis) generated after enzymatic digestion. Polymorphic sites are indicated in bold.

MtDNA regions	Fragment total size (pb)	Restriction enzymes															
		Bcl I		*Cla* I		*Eco* R I		*Hae* III		*Hind* III		*Hinf* I		*Nde* I		*Pst* I	
		A	B	A	B	A	B	A	B	A	B	A	B	A	B	A	B
ND2, COI	2200	1500 500 200 (2)	1500 500 200 (2)	2200 (0)	2200 (0)	2200 (0)	2200 (0)	2200 (0)	2200 (0)	2200 (0)	2200 (0)	2200 (0)	2200 (0)	1850 350 (1)	1850 350 (1)	2200 (0)	2200 (0)
COI	1700	1050 550 (1)	1050 550 (1)	1700 (0)	1700 (0)	1700 (0)	1700 (0)	1700 (0)	1700 (0)	1700 (0)	1700 (0)	**930 770 (1)**	**930 470 300 (2)**	**1150 550 (1)**	**1700 (0)**	1700 (0)	1700 (0)
COII	950	950 (0)	950 (0)	850 100 (1)	850 100 (1)	950 (0)	950 (0)	950 (0)	950 (0)	950 (0)	950 (0)	950 (0)	950 (0)	950 (0)	950 (0)	950 (0)	950 (0)
ATPases (6,8), COIII	1800	1800 (0)	1800 (0)	1800 (0)	1800 (0)	940 860 (1)	940 860 (1)	960 840 (1)	960 840 (1)	1000 800 (1)	1000 800 (1)	1050 750 (1)	1050 750 (1)	1800 (0)	1800 (0)	1800 (0)	1800 (0)
COIII, ND3	1300	1300 (0)	1300 (0)	1300 (0)	1300 (0)	1000 250 (1)	1000 250 (1)	1300 (0)	1300 (0)	1100 200 (1)	1100 200 (1)	950 350 (1)	950 350 (1)	1300 (0)	1300 (0)	1300 (0)	1300 (0)
ND4, ND6, CytB	2560	2560 (0)	2560 (0)	2000 560 (1)	2000 560 (1)	2560 (0)	2560 (0)	2560 (0)	2560 (0)	2560 (0)	2560 (0)	1960 600 (1)	1960 600 (1)	2560 (0)	2560 (0)	2560 (0)	2560 (0)
CytB, NDI	1800	1800 (0)	1800 (0)	1650 150 (1)	1650 150 (1)	950 850 (1)	950 850 (1)	1800 (0)	1800 (0)	1800 (0)	1800 (0)	870 600 330 (2)	870 600 330 (2)	1800 (0)	1800 (0)	1800 (0)	1800 (0)
16S	790	790 (0)	790 (0)	790 (0)	790 (0)	790 (0)	790 (0)	790 (0)	790 (0)	790 (0)	790 (0)	790 (0)	790 (0)	790 (0)	790 (0)	560 230 (1)	560 230 (1)
16S, 12S	1850	1850 (0)	1850 (0)	1850 (0)	1850 (0)	1850 (0)	1850 (0)	1850 (0)	1850 (0)	1850 (0)	1850 (0)	1850 (0)	1850 (0)	1850 (0)	1850 (0)	1400 450 (1)	1400 450 (1)

A: *M. quadrifasciata quadrifasciata;* B: *M. q. anthidioides*

performed. Nine pairs of primers (Table 1) were used to amplify specific mtDNA regions of *M. q. quadrifasciata* and *M. q. anthidioides*. The PCR products were separated by electrophoresis in 0.8% agarose gels, stained with ethidium bromide, visualized under a UV light and photographed.

To determine the presence of restriction sites, the PCR-amplified mtDNA fragments were digested for a minimum period of 6h with the following restriction enzymes: *Bam* HI, *Bcl* I, *Bgl* II, *Cfo* I, *Cla* I, *Dra* I, *Eco* RI, *Eco* RV, *Hae* III, *Hind* III, *Hinf* I, *Nde* I and *Pst* I. The digested products were analyzed in 1.5% agarose (agarose 1000 Gibco) gels, which were stained and visualized as above.

Results and Discussion

The PCR+RFLP technique applied in this study enabled us to determine the number of restriction sites and size of the fragments (Table 2), except for the enzymes *Bgl* II, *Bam* HI, *Cfo* I and *Eco* RV which did not produced detectable digestion products for either of the two subspecies studied.

Weinlich *et al*. (2004) determined a mtDNA restriction map for seven species of *Melipona* including *M. quadrifasciata quadrifasciata* and *M. q. anthidioides*. Among a total of 18 restriction sites mapped for these two subspecies, a variable site (*Bgl* II mapped at the cytochrome B gene) was present in *M. q. quadrifasciata* but absent in *M. q. anthidioides*. However, in the present study we did not find any restriction sites for the enzyme *Bgl* II in the mtDNA regions amplified. Compared to the published map, another difference was a previously undetected *Hae* III site in the fragment containing the genes ATPase 6, 8 and COIII (Table 2) in both subspecies.

In addition to the enzymes used by Weinlich *et al*. (2004), we analyzed mtDNA fragments with three other enzymes (*Dra* I, *Hinf* I and *Nde* I). These enzymes presented some restriction pattern differences between the two subspecies. For the fragment comprising COI as the main gene, the enzymes *Hinf* I and *Nde* I cut once versus twice and once versus uncut in *M. q. quadrifasciata* and *M. q. anthidioides*, respectively (Table 2). The enzyme *Dra* I recognized one or more cleavage sites in each PCR-amplified mtDNA region, generating various small fragments whose sizes could not be precisely estimated. Thus, these data are not included in Table 2. However a distinct restriction pattern could be clearly visualized for the cytochrome B region. As shown in Fig. 1, the largest band has about 390 and 423 bp in *quadrifasciata* and *anthidioides* subspecies, respectively.

The enzyme *Dra* I has been widely used in *Apis mellifera* L. to investigate subspecies diversity. It recognizes the sequence TTTAAA, a very likely restriction site in *A. mellifera* A+T rich mitochondrial genome (Crozier & Crozier 1993). High variability has been detected in the COI-COII intergenic region with this enzyme, generating distinct restriction patterns for several honey bee subspecies (Garnery *et al*. 1993, 1995; Franck *et al*. 1998). However, the COI-COII intergenic region is absent in Meliponini (Silvestre *et al*. 1999, Francisco *et al*. 2001, Fernandes-Salomão *et al*. 2002, Weinlich *et al*. 2004) but the mtDNA genome of *Melipona bicolor* Lepeletier has a higher A+T content than that of *A. mellifera* (Silvestre 2002). This latter evidence, although found in a different species of the same genus, suggests that enzymes recognizing A+T cleavage sequence sites may still be useful to detect polymorphism in *M. quadrifasciata*.

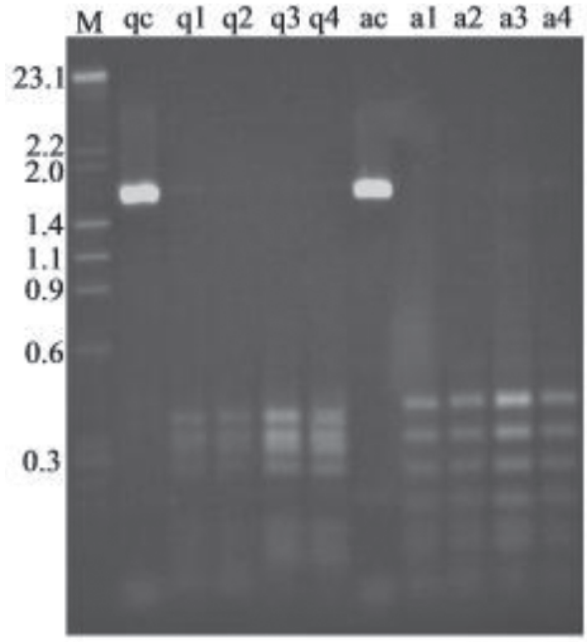

Figure 1. Agarose gel (1.5%) showing the different banding patterns observed between *M. q. quadrifasciata* (q_1, q_2, q_3 and q_4) and *M. q. anthidioides* (a_1, a_2, a_3 and a_4) for the cytochrome B region amplified with mtD26/mtD30 after digestion with *Dra* I. M: molecular weight markers λ/*Hin*d III and ϕx174/*Hae* III (in Kb). qc and ac: control of no digested PCR fragment.

The data obtained in this present work combined with those from Weinlich *et al*. (2004), indicate five restriction enzymes (*Bgl* II, *Hae* III, *Dra* I, *Hinf* I and *Nde* I) that can be used to evaluate variation in mtDNA of *M. q. quadrifasciata* and *M. q. anthidioides* and serve as markers for population analyses. Traditionally, the identification of *M. quadrifasciata* subspecies was carried out by analyzing the pattern of stripes on the abdomen (Moure & Kerr 1950). Hybrid colonies that originate through contact between reproductive individuals from the two subspecies can lead to changes in the tergal band pattern and misidentification. This is a relevant issue nowadays as the economic value of *M. quadrifasciata* has encouraged the practice of exchanging queens among commercial breeders. This practice can promote contact between the two subspecies and lead to gene flow and development of secondary hybrid populations. The development of reliable genetic markers will be of significant benefit in studies where the identification of maternal origin in commercial populations or of subspecies in natural popations is required.

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