SYSTEMATICS, MORPHOLOGY AND PHYSIOLOGY

Mitochondrial DNA Polymorphism among Populations of *Melipona quadrifasciata quadrifasciata* Lepeletier (Apidae: Meliponini) from Southern Brazil

Rogelio R Torres[1], Maria C Arias[2], Geraldo Moretto[3]

[1]Depto. de Producion Animal, Univ. Nacional de Colombia, Bogotá D.C.; rogeroto15@yahoo.com; [2]Depto. de Genética e Biologia Evolutiva, Instituto de Biociências, Univ. de São Paulo 05.508-090, São Paulo, SP; mcarias@ib.usp.br; [3]Depto. de Ciências Naturais, Univ. Regional de Blumenau, 89.010-971 Blumenau, SC; gmoretto@furb.br

Edited by Fernando Noll – UNESP

Neotropical Entomology 38(2):208-212 (2009)

Polimorfismo do DNA Mitocondrial entre Populações de *Melipona quadrifasciata quadrifasciata* Lepeletier (Apidae: Meliponini) do Sul do Brasil

RESUMO - A distribuição geográfica da abelha sem ferrão *Melipona quadrifasciata quadrifasciata* Lepeletier compreende desde o Rio Grande do Sul até Minas Gerais. O objetivo do presente estudo foi verificar a variabilidade genética em amostras de *M. q. quadrifasciata* coletadas na Região Sul do Brasil. Para tanto, 29 colônias de três localidades (Blumenau e Mafra/SC e Prudentópolis/PR) foram amostradas e a técnica de PCR-RFLP para o DNA mitocondrial foi utilizada. Sete regiões do genoma mitocondrial foram amplificadas e digeridas com 15 enzimas de restrição. Cinco haplótipos foram identificados: dois exclusivos das amostras de Prudentópolis e os outros três registrados nas amostras de Mafra e/ou de Blumenau.

PALAVRAS-CHAVE: mtDNA, PCR-RLFP, abelha sem ferrão, haplótipo

ABSTRACT - The geographical distribution of the Brazilian endemic stingless bee *Melipona quadrifasciata quadrifasciata* Lepeletier ranges from Rio Grande do Sul to Minas Gerais states. The objective of the present study was to verify mtDNA polymorphisms among samples of *M. q. quadrifasciata* collected in southern Brazil. Twenty nine colonies from three localities (Blumenau and Mafra/SC and Prudentópolis/PR) were sampled. Seven mtDNA regions were amplified and further digested with 15 restriction enzymes (PCR-RFLP). Five composite haplotypes were identified, with two unique to samples from Prudentópolis and the remaining three to samples from Mafra and/or Blumenau.

KEY WORDS: mtDNA, PCR-RLFP, stingless bee, haplotype

The tribe Meliponini exhibits Pantropical distribution. The vast majority of species occurs in the Neotropical region (Michener 2000). In Brazil, this tribe is widely distributed throughout the country and is well represented, with more than 300 described species (Camargo & Pedro 2007). Their ecological and economic importance is unquestionable, since they are responsible for 40% to 90% of the native flora pollination (Kerr *et al* 1999). Also, the importance of several species for honey production and crop pollination has increased the beekeeping practice and queen exchange among breeders.

Melipona quadrifasciata Lep. ("mandaçaia") comprises two subspecies, *M. quadrifasciata quadrifasciata* and *M. quadrifasciata anthidioides* (Schwarz 1932). The geographic distribution of each subspecies seems to be very distinct. *Melipona q. quadrifasciata* is found in regions with colder climates, being more abundant in the states of Paraná and Santa Catarina (Monteiro 2000), and altitudes higher than 1500 m, in São Paulo, Rio de Janeiro and Minas Gerais states (Moure 1975). Conversely, *M. q. anthidioides* is naturally distributed in warmer regions, being frequent in Rio de Janeiro and Minas Gerais states (Aidar 1996). The two subspecies are morphologically discriminated by the yellow stripe pattern presented from the third to the sixth tergite (*M. q. quadrifasciata* presents continuous bands and *M. q. anthidioides* presents interrupted bands in the dorsal midline) (Aidar 1996). Molecular differences between both subspecies have been recently reported, as a putative RAPD marker of *M. q. quadrifasciata* was absent from *M. q. anthidiodes* (Waldsmchmidt *et al* 2000). Mitochondrial DNA (mtDNA) variation was also described between subspecies through RFLP analyses (Weinlich *et al* 2004, Moretto & Arias 2005). Souza *et al* (2008) reported a reliable and simple method

to discriminate between both subspecies based on mtDNA RFLP, reinforcing the importance of describing new molecular markers for subspecies identification and maternal origin, since hybrids can be originated through natural contact or mediated by beekeepers as a consequence of queen or brood combs exchange among different geographic regions.

In this paper, we detailed the methodology and discuss the results which were briefly reported as unpublished data in a review article (Arias *et al* 2006), focusing in detecting mtDNA polymorphisms among samples of *M. q. quadrifasciata* from three localities (here called as populations) of southern Brazil in an attempt to identify haplotypes closely associated to geographic sites. Moreover, as an effort to improve the data described by Souza *et al* (2008), seven mtDNA regions were analyzed and a large number of restriction enzymes were utilized.

Material and Methods

A total of twenty nine feral *M. quadrifasciata quadrifasciata* colonies were sampled. Workers were obtained from twelve, eight and nine colonies located in Blumenau, Mafra (Santa Catarina state) and Prudentópolis (Paraná state), respectively. Blumenau (26º 55'26'' S) is located at the Itajaí Valley, at 21 m altitude and exhibits an average temperature of 21.6ºC; Mafra (26º 06'55''S) is located at the north of Santa Catarina state at 809 m altitude, with an average temperature of 18ºC; and Prudentópolis (25º 12'40''S) is at 730 m altitude, with an average temperature of 19ºC. According to Koppen (1948), these three regions have a humid mesothermic climate, with no distinct dry period.

Total DNA was extracted as described by Sheppard & McPheron (1991) using one thorax per extraction. The mtDNA was analyzed by PCR-RFLP, a technique that consists in amplifying mitochondrial genome regions by PCR and subsequent digestion of the fragments by 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 an 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 performed. Seven primer pairs (Table 1) were used to amplify the specific mtDNA regions of *M. q. quadrifasciata*. The PCR-amplified fragments 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 fragments were digested for a minimum period of 6h with the following restriction enzymes: *Ase* I, *Bam* HI, *Bcl* I, *Bgl* II, *Cfo* I, *Cla* I, *Dra* I, *Eco* RI, *Eco* RV, *Hae* III, *Hind* III, *Hinf* I, *Nde* I, *Pst* I and *Ssp* I. The digested products were analyzed in 1.0% agarose (Agarose 1000 Gibco) or 12 % acrylamide gels (non-denaturing conditions). The latter were silver stained, dried and scanned.

Results

The fragment size of the seven amplified mtDNA regions ranged from 950 bp to 2200 bp (Table 1) according to the expected (Francisco *et al* 2001, Moretto & Arias 2005). The restriction enzymes *Bgl* II, *Bam* HI, *Cfo* I and *Eco* RV did not cleave any fragment. The enzymes *Bcl* I, *Cla* I, *Dra* I, *Eco* RI, *Hae* III, *Hind* III, *Nde* I and *Pst* I presented one or more restriction sites in each mtDNA region, but they did not yield polymorphic restriction patterns. In contrast, the enzymes *Ase* I, *Hinf* I and *Ssp* I detected polymorphic sites at intra and inter population levels (Figs 1, 2, Table 2).

Table 1 Primer pairs used to amplify mitochondrial DNA regions of *Melipona quadrifasciata quadrifasciata*, their main genes constituents, respective annealing temperatures and expected fragment size.

Pair	Name	Sequence (5'→3')	Reference	Main genes	T (ºC)	Fragment size (bp)
1	mtD2	GCTAAATAAGCTAACAGGTTCAT	(Simon *et al* 1994)	ND2, COI	42	2200
	mtD9	CCCGGTAAAATTAAAATATAAACTTC	(Simon *et al* 1994)			
2	mtD7	GGATCACCTGATATAGCATTCCC	(Simon *et al* 1994)	COI, COII	44	1700
	COI-IIR	GATCAATATCATTGATGACC	(Hall & Smith 1991)			
3	COI-IIF	TCTATACCACGACGTTATTC	(Hall & Smith 1991)	COI, COII	44	950
	mtD18	CCACAAATTTCTGAACATTGACCA	(Simon *et al* 1994)			
4	mtD19	GAAATTTGTGGAGCAAATCATAG	(Simon *et al* 1994)	ATPases (8, 6), COIII	42	1800
	mtD22	TCAACAAAGTGTCAGTATCA	(Simon *et al* 1994)			
5	5612R	GAAATTAATATAACATGACCACC	(Francisco *et al* 2001)	COIII, ND3	42	1300
	tPheF	GCGTAATATTGAAAATATTAATGA	(Francisco *et al* 2001)			
6	mtD26	TATGTACTACCATGAGGACAAATATC	(Simon *et al* 1994)	CytB, ND1	42	1700
	mtD30	GTAGCATTTTTAACTTTATTAGAACG	(Simon *et al* 1994)			
7	16SR	CGTCGATTTGAACTCAAATCATG	(Hall & Smith 1991)	16S, 12S	42	1850
	mtD36	AAACTAGGATTAGATACCCTATTAT	(Simon *et al* 1994)			

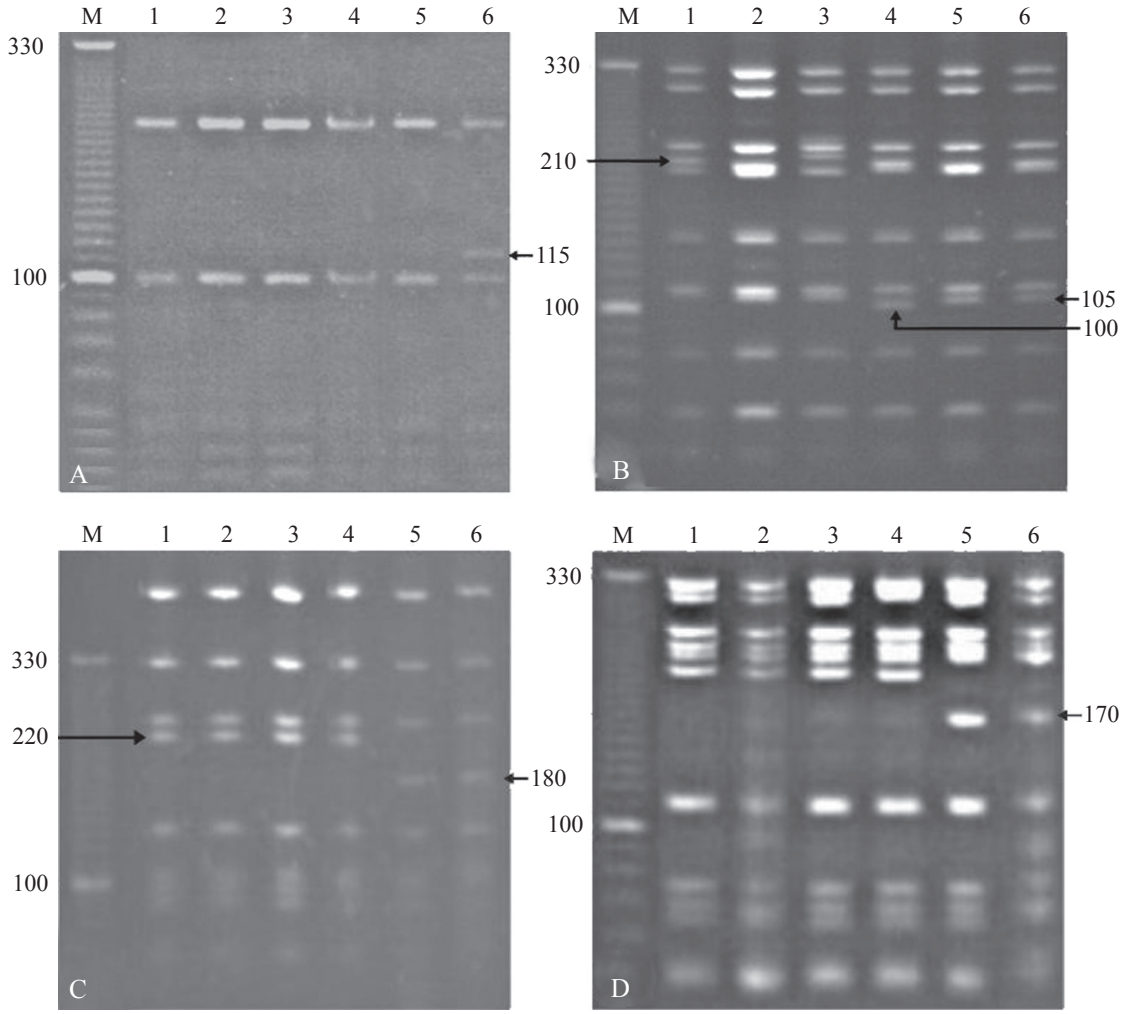

Fig 1 Acrylamide gels (12%) showing the banding patterns observed in *Melipona quadrifasciata quadrifasciata* for the 16S-12S region after digestion with enzymes *Ssp* I (A), *Ase* I (B), the COI-COII and ND2/COI regions digested with *Ase* I (C and D, respectively). Lanes 1 and 2 = Blumenau; 3 and 4 = Mafra; and 5, 6 = Prudentópolis. M: 10 bp DNA Ladder.

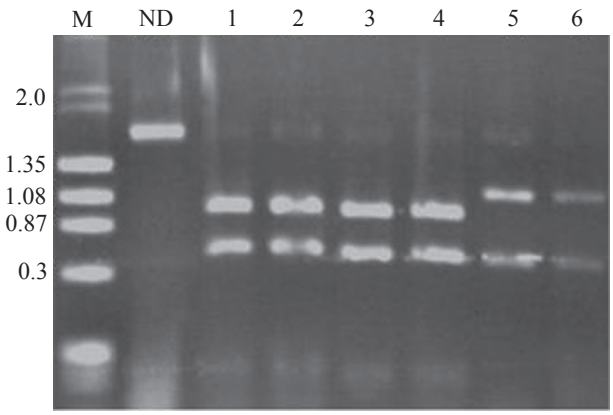

Fig 2 Agarose gel (1.0%) showing the different banding patterns observed in *Melipona quadrifasciata quadrifasciata* for the COI-COII region after digestion with *Hinf* I (ND = no digested fragment; 1, 2 = Blumenau; 3, 4 = Mafra and 5, 6 = Prudentópolis). M: molecular weight markers λ/*Hin*d III and ϕx174/*Hae* III (in Kb).

The variation considered to compose the haplotypes was the presence/absence of restriction fragments and shift in fragment gel mobility, which led to the identification of five composite haplotypes identified, which were distributed according to the collecting sites (Table 3). Two haplotypes were found per population. Haplotype 5 (H5) was the only one shared between populations (Blumenau and Mafra). Differences among H5, H4 and H3 found only in Santa Catarina reside in gel mobility polymorphism, not in restriction pattern. Haplotypes H1 and H2 were exclusive of Prudentópolis and differed in four out of six RFLP markers when compared to H3, H4 and H5. Haplotype differences within population were restricted to a single or two RFLP markers (Table 3).

Discussion

Few molecular studies on stingless bees have been conducted (Arias *et al* 2006), so the data available are

Table 2 Restriction fragments showing polymorphism after enzyme digestion verified in three mitochondrial regions for *Melipona quadrifasciata quadrifasciata* populations. The percentage of each pattern is presented. RE: restriction enzyme; dash: fragment absent; Prud: Prudentópolis; Blumen: Blumenau; Sample size is shown in parenthesis. * indicates fragments showing gel mobility polymorphism.

mtDNA region+RE		16S/12S-*Ssp* I		16S/12S-*Ase* I		16S/12S-*Ase* I			COI/COII-*Ase* I		COI/COII-*Hinf* I		ND2/COI–*Ase* I	
Haplotype		A	B	A	B	A	B	C	A	B	A	B	A	B
Fragm. size (bp)		120	-	210*	-	105*	100*	-	180	220	1,100	1,000	170	210
Collecting sites and haplotype frequency (%)	Prud. (9)	22	78	-	100	100	-	-	100	-	100	-	100	-
	Mafra (8)	-	100	-	100	-	37	63	-	100	-	100	-	100
	Blum. (12)	-	100	8	92	-	-	100	-	100	-	100	-	100

still limited and are poorly representative, considering the hundreds of Meliponini species and their wide distribution (Michener 2000, Camargo & Pedro 2007). Weinlich *et al* (2004) determined the mtDNA restriction map for seven species of *Melipona* including *M. quadrifasciata*. Eighteen restriction sites were mapped for this species, however comparing the published map with the present study some differences should be pointed out. We did not find the *Bgl* II site mapped at the cytochrome B gene, and moreover this enzyme did not cut any mtDNA region here studied. Nonetheless, a previous undetected *Hae* III site in the fragment containing the ATPase 6, 8 and COIII genes was detected in our study. These results were consistent among all samples, thus not representing inter population variation.

Recently, Moretto & Arias (2005) published a comparative study between the two subspecies of *M. quadrifasciata* by analyzing mtDNA restriction patterns. Polymorphisms between *M. quadrifasciata quadrifasciata* and *M. quadrifasciata anthidioides* were detected for COI region digested with *Hinf* I and *Nde* I, and CytB region digested with *Dra* I. Souza *et al* (2008) analyzed a large sample of *M. quadrifasciata* and reported RFLP patterns generated by CytB region after *Vsp* I digestion closely associated to each subspecies. Although the latter study presented a strong correlation between molecular markers and subspecies, authors could not identify molecular markers associated to the nest geographic origin.

The present study aimed at the molecular characterization of *M. quadrifasciata quadrifasciata* from three distinct geographic areas from Southern Brazil by mtDNA PCR-RFLP analysis. Most of the restriction enzymes were not informative; however, polymorphism was detected by the enzymes *Ssp* I, *Hinf* I and *Ase* I. Polymorphisms verified at the 16S/12S region were not related to any population exclusively, representing intra-specific variability. The restriction patterns generated by *Ase* I digestion are worthwhile as they differ only by differences in gel mobility of two fragments (210 and 105 bp). This mobility shift was reported as a consequence of double-strand conformation polymorphism (DSCP) due to base substitutions (Hagerman 1990). The same phenomenon has been verified in other studies on bees for the 16S and CytB regions (Collet *et al* 2007, Souza *et al* 2008). Nonetheless, the COI/COII region digested with *Ase* I and *Hinf* I, and the ND2 region digested with *Ase* I presented distinct restriction patterns between samples from Prudentópolis (Paraná state) and Santa Catarina state, and exclusive haplotypes were identified. It is interesting that the COI/COII region of *Apis mellifera* L. contains an intergenic non-coding region (Crozier *et al* 1989), which is highly informative to differentiate among *A. mellifera* geographic races and evolutionary lineages (Garnery *et al* 1992, 1995, Franck *et al* 1998). Although this intergenic region was reported as absent in Meliponini (Arias *et al* 2006), the surrounding genes (COI and COII) revealed polymorphic and exclusive restriction sites between *M. quadrifasciata quadrifasciata* samples from Paraná and Santa Catarina.

Table 3 *Melipona quadrifasciata quadrifasciata* composite haplotypes and their occurrence per collecting sites.

Haplotypes	Prudentópolis	Mafra	Blumenau
H1 (BBAAAA)	7	0	0
H2 (ABAAAA)	2	0	0
H3 (BBBBBB)	0	3	0
H4 (BACBBB)	0	0	1
H5 (BBCBBB)	0	5	11

The colonies sampled in Santa Catarina had three haplotypes: H3 was present only in Mafra, H4 only in Blumenau and H5 in both localities, while samples from Prudentópolis had two haplotypes (H1 and H2). Geographic characteristics, as altitude, indicate similarities between Mafra and Prudentópolis, both at around 700 m high, whereas Blumenau is located at 23 m above sea level. The high similarity among Mafra and Blumenau haplotypes and the high incidence of haplotype H5 in both regions could suggest a current gene flow mediated by females between these two geographic regions. In fact these two localities are connected by Atlantic Rain Forest remnants (Moretto, personal information), which may function as a natural corridor to the dispersion of reproductive individuals. The two haplotypes identified in Prudentópolis were exclusive, indicating a possible isolation of this population in relation to the other in here studied. Although Prudentópolis and Mafra are located in areas of Araucaria Forest, the former seems to be isolated as no ecological corridor exists due to Araucaria

and Atlantic forest fragmentation. Arias *et al* (2006) studied populations of the stingless bee *Plebeia remota* Holmberg and through mtDNA RFLP verified that samples collected from Prudentópolis did not share haplotypes with populations from São Paulo, Santa Catarina and eastern Paraná. Moreover, a multidisciplinary study provided extra evidences based on wing morphology, cuticular hydrocarbons and mtDNA RFLP that *P. remota* from Prudentópolis should be considered as a distinct species (Francisco *et al* 2008). Whether this is indicative of an ancient event where populations of several species were kept isolated in southern Paraná refuge or is related to natural selection due to climatic or biotic factors, it should be a subject for further studies, including a broad sampling of *M. quadrifasciata* and other species for comparative studies from this area and nearby .

References

Aidar D (1996) A mandaçaia: biologia de abelhas, manejo e multiplicação de colônias *Melipona quadrifasciata* Lep (Hymenoptera, Apidae, Meliponinae). Sociedade Brasileira de Genética, Ribeirão Preto, 103p.

Arias M C, Brito R M, Francisco F O, Moretto G, Oliveira F F, Silvestre D, Sheppard W S (2006) Molecular markers as a tool for population and evolutionary studies of stingless bees. Apidologie 37: 259-274.

Camargo J M F, Pedro S R M (2007) Meliponini Lepeletier, 1836. In Moure J S, Urban D, Melo G A R (Orgs) Catalogue of bees (Hymenoptera, Apoidea) in the Neotropical Region, Curitiba, Brazil, 1058p.

Collet T, Arias M C, Del Lama M A (2007) 16S mtDNA variation in *Apis mellifera* detected by PCR-RFLP. Apidologie 38:47-54.

Crozier R H, Crozier Y C, Mackinlay A G (1989) The CO-I and CO-II region of honeybee mitochondrial DNA: evidence for variation in insect mitochondrial evolutionary rates. Mol Biol Evol 6: 399-411.

Francisco F O, Nunes-Silva P, Francoy T M, Witmann D, Imperatriz-Fonseca V L, Arias M C, Morgan E D (2008) Morphometrical, biochemical and molecular tools for assessing biodiversity. An example in *Plebeia remota* (Holmberg, 1903) (Apidae, Meliponini). Insect Soc 55: 231-237.

Francisco F O, Silvestre D, Arias M C (2001) Mitochondrial DNA characterization of five species of *Plebeia* (Apidae: Meliponini): RFLP and restriction map. Apidologie 32: 323-332.

Franck P, Garnery L, Solignac M, Cornuet J-M (1998) The origin of West European subspecies of honeybees (*Apis mellifera*): new insights from microsatellite and mitochondrial data. Evolution 52: 1119-1134.

Garnery L, Cornuet J-M, Solignac M (1992) Evolutionary history of the honey bee *Apis mellifera* inferred from mitochondrial DNA analysis. Mol Ecol 1: 145-154.

Garnery L, Mosshine E H, Oldroyd B P, Cornuet J-M (1995) Mitochondrial DNA variation in Moroccan and Spanish honey bee populations. Mol Ecol 4: 465-471.

Hall H G, Smith D R (1991) Distinguishing African and European honeybee matrilines using amplified mitochondrial DNA. Proc. Natl Acad Sci USA 88:4548-4552.

Hagerman P J (1990) Sequence-directed curvature of DNA. Annu Rev Biochem 59:755- 781.

Kerr W E, Nascimento V A, Carvalho G A (1999) Preservation of native Brazilian bees: a question of historical and ecological conscience. Environ Biodiv 51: 390-393.

Koppen L W (1948) Climatologia con un estudio de los climas de la Tierra. Fondo de Cultura Econômica, Panuco, 63, Mexico, DF.

Michener C D (2000) The bees of the world. The Johns Hopkins University Press, Baltimore, MD, USA, 913p.

Monteiro W R (2000) Meliponicultura (criação de abelhas sem ferrão): a mandaçaia. Mensagem Doce 57:15-17.

Moretto G, Arias M C (2005) Detection of mitochondrial DNA restriction site differences between the subspecies of *Melipona quadrifasciata* Lepeletier (Hymenoptera:Apidae: Meliponini). Neotrop Entomol 34: 381-385.

Moure J S (1975) Notas sobre as espécies de Melipona descritas por Lepeletier em 1836 (Hymenoptera, Apidae). Rev Bras Biol 3: 15-17.

Schwarz H F (1932) The genus *Melipona*. Bull Amer Mus Natur Hist 63: 231-460.

Sheppard W S, McPheron B (1991) Ribosomal DNA diversity in Apidae, p.89-102. In Smith D R (ed) Diversity in the genus *Apis*. Westview Press, Oxford, 265p.

Simon C, Frati F, Beckenbach A, Crespi B, Liu H, Flook P (1994) Evolution, weighting and phylogenetic utility of mitochondrial gene sequences and a compilation of conserved polymerase chain reaction primers. Ann Entomol Soc Am 87: 651-701.

Souza R O, Moretto G, Arias M C, Del Lama M A (2008) Differentiation of *Melipona quadrifasciata* L. (Hymenoptera, Apidae, Meliponini) subspecies using cytochrome b PCR-RFLP patterns. Genet Mol Biol 30 : 445-450.

Waldschmidt A M, Barros E G, Campos L A O (2000) A molecular marker distinguishes the subspecies *Melipona quadrifasciata quadrifasciata* and *Melipona quadrifasciata anthidioides* (Hymenoptera: Apidae, Meliponinae). Genet Mol Biol 23 : 609-611.

Weinlich R, Francisco F O, Arias M C (2004) Mitochondrial DNA restriction and genomic maps of seven species of *Melipona* (Apidae: Meliponini). Apidologie 35: 365-370.

Received 28/IX/07. Accepted 29/I/09.