Genetics and Molecular Biology, 30, 2, 445-450 (2008)

www.sbg.org.br

Research Article

Differentiation of *Melipona quadrifasciata* L. (Hymenoptera, Apidae, Meliponini) subspecies using cytochrome b PCR-RFLP patterns

Rogério O. Souza[1], Geraldo Moretto[2], Maria C. Arias[3] and Marco A. Del Lama[1]

[1]*Departamento de Genética e Evolução, Universidade Federal de São Carlos, São Carlos, SP, Brazil.*
[2]*Departamento de Ciências Naturais, Universidade Regional de Blumenau, Blumenau, SC, Brazil.*
[3]*Departamento de Genética e Biologia Evolutiva, Instituto de Biociências, Universidade de São Paulo, São Paulo, SP, Brazil.*

Abstract

Melipona quadrifasciata quadrifasciata and *M. quadrifasciata anthidioides* are subspecies of *M. quadrifasciata*, a stingless bee species common in coastal Brazil. These subspecies are discriminated by the yellow stripe pattern of the abdominal tergites. We found *Vsp* I restriction patterns in the cytochrome b region closely associated to each subspecies in 155 *M. quadrifasciata* colonies of different geographical origin. This mitochondrial DNA molecular marker facilitates diagnosis of *M. quadrifasciata* subspecies matrilines and can be used to establish their natural distribution and identify hybrid colonies.

Key words: subspecies differentiation, *Melipona quadrifasciata*, cytochrome b, mtDNA, PCR-RFLP patterns.

Received: April 12, 2007; Accepted: August 28, 2007.

Introduction

The stingless bee *Melipona quadrifasciata* Lepeletier 1936 (popularly known as *mandaçaia*) is a member of the Meliponini tribe, a group with highly eusocial organization and pantropical distribution (Michener, 2000). The exclusively Neotropical genus *Melipona* is the most derived group (Camargo and Pedro, 1992) and is most diverse in the Amazon basin (Silveira *et al.*, 2002). Approximately 40 *Melipona* species are distributed from Mexico to Argentina (Camargo, 1979; Michener, 2000), although the biology and systematics of the described species is still poorly understood (Camargo and Pedro, 1992).

Some *Melipona* species are essential to pollination of Brazilian Atlantic forest (Kerr *et al.*, 1996) and open savannah ("cerrado") flora (Silberbauer-Gottsberger and Gottsberger, 1988), and agricultural crops (Heard, 1999; Klein *et al.*, 2003; Kremen *et al.*, 2004) and are important for the conservation of world flora and fauna (Melendez-Ramirez *et al.*, 2002; Corlett, 2004; VillaNueva *et al.*, 2005). Stingless bees are also increasingly used to improve productivity in commercial crops because they require less specialized handling and maintenance procedures than bees with stings, with some stingless species having been domesticated for honey production.

Send correspondence to Marco Antonio Del Lama. Departamento de Genética e Evolução, Universidade Federal de São Carlos, Rodovia Washington Luis km 235, 13.565-905 São Carlos, SP, Brazil. E-mail: dmdl@power.ufscar.br.

Melipona quadrifasciata quadrifasciata and *Melipona quadrifasciata anthidioides* (Schwarz, 1932) are subspecies commonly observed in coastal Brazil, ranging from the northeastern state of Paraíba southward to the southernmost Brazilian state of Rio Grande do Sul (Moure and Kerr, 1950). These subspecies can be easily discriminated by the yellow stripe pattern from the third to sixth abdominal tergites, the pattern consisting of 3 to 5 continuous bands in *M. q. quadrifasciata* and 2 to 5 discontinuous bands in *M. q. anthidioides* (Schwarz, 1932).

The geographic distribution of the two subspecies is very distinct, with *M. q. anthidioides* being distributed in warmer areas and is hence more frequent in the states of Rio de Janeiro and Minas Gerais (Melo and Campos, 1987), while *M. q. quadrifasciata* is found in colder regions and is hence more abundant in the states of Paraná and Santa Catarina but is also found in the states of São Paulo, Rio de Janeiro and Minas Gerais at altitudes above 1500 m (Moure, 1975). Natural hybrid zones are described in elevated areas (500 to 700 meters) in the states of São Paulo and southern Minas Gerais. Hybrid colonies originating from males and queens of the two subspecies usually consist of bees with intermediate phenotypes in the tergal banding pattern, which can lead to misidentification (Moure and Kerr, 1950; Moure, 1975; Melo and Campos, 1987).

Identification of hybrid colonies is a relevant issue because the economic value of *M. quadrifasciata* has encouraged the exchange of queens among commercial

breeders. This practice has promoted contact between the two subspecies, leading to gene flow and development of secondary hybrid populations. Data on genetic structure of bee species are useful for conservation purposes because population genetic purity is greatly affected by beekeeping and commercial breeding practices. Genetic markers that provide information on the degree of gene flow between these two subspecies can confirm specific subspecies kept by beekeepers and provide a more realistic picture of the natural distribution of *M. quadrifasciata* before drastic anthropic alterations.

In this paper we report a simple method to differentiate the *M. q. quadrifasciata* and *M. q. anthidioides* matrilines using cytochrome b (cyt-b) polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) patterns. This study presents the first population analysis from a large number of colonies and focuses on the identification of a mitochondrial DNA genetic marker at the subspecies level and the detection of hybrid colonies occurring naturally or induced by beekeeping. We also present a comparison between the data herein and previous findings.

Material and Methods

Samples

Adult workers from 155 *Melipona quadrifasciata* colonies were analyzed. Their geographical origin and subspecies identification (based on tergal stripe pattern) are showed in Table 1. All samples from Rio de Janeiro and Curitiba were unambiguously identified as *M. q. anthidioides*, while all colonies from Prudentópolis, Blumenau and Vale do Itajaí were identified as *M. q. quadrifasciata*. Samples from the Universidade Federal de Viçosa and from Lins as well as from two meliponaries in Ribeirão Preto were composed of colonies with *quadrifasciata*, *anthidioides* or intermediate tergal stripe patterns (samples identified as mixture). The samples from the states of Minas Gerais and São Paulo (Table 1) do not reflect the natural occurrence area of these subspecies because the bees at these sites were maintained for a long time in hives where colonies from different areas were introduced; consequently these colonies are not considered "pure" genetic pools of the *M. quadrifasciata* subspecies.

Extraction, PCR- RFLP and electrophoresis and DNA sequencing

The DNA from the thorax of one adult worker per colony was extracted using a phenol-chloroform protocol (Sheppard and McPheron, 1991) and the extracted DNA used for PCR amplification of the mitochondrial cytochrome b region, using the primers 5'-TATGTACTA CCATGAGGACAAATATC-3' (forward) and 5'-ATTAC ACCTCCTAATTTATTAGGAAT - 3' (reverse) described by Crozier *et al.* (1991). Reactions were carried out in a 25 µL final volume containing 1 µM of each primer, 250 µM of each dNTP, 2.5 mM $MgCl_2$, 1x buffer (Biotools), 1 U of Taq DNA Polymerase (Biotools) and 1 µL of the extracted DNA (50-100 ng). Amplification cycles were repeated 30 times following a basic schedule of 30 s at 94 °C for denaturing, 20 s at 48 °C for annealing, and 1 min at 72 °C for extension. Digestion of the cyt-b fragment was subsequently performed in a 20 µL reaction mixture using 3 µL of the PCR product and 2 units of the enzymes in an appropriate buffer. Digestions were allowed to proceed for four hours with different restriction enzymes (*Bgl* II, *Dra* I, *Mbo* I, *Taq* I and *Vsp* I; all from Fermentas). Digested fragments were submitted to electrophoresis in non-denaturing polyacrylamide gels (8% (w/v) for *Bgl* II and 12% (w/v) for *Dra* I, *Mbo* I, *Taq* I and *Vsp* I) and silver stained.

For DNA sequencing each cyt-b haplotype found was cloned and two clones of each haplotype were forward and reverse sequenced on an ABI 3100 (Applied Biosystems) sequencer. Sequences were analyzed using Codon Code

Table 1 - Number of colonies of *Melipona quadrifasciata* sampled, their geographical origin, subspecies identification based on tergal stripe patterns and the cytochrome b polymerase chain reaction restriction fragment length polymorphism (RFLP-PCR) composite haplotypes found. N = number of colonies for each site.

State, city and altitude	Geographical coordinates	Type of tergal band pattern*	A1	B1	B2	C2	N
Minas Gerais, Viçosa, 648 m	20°45'14" S 42°52'55" W	Mixture	9	22	9		40
São Paulo, Ribeirão Preto, 589 m	21°12'42" S 47°48'24" W	Mixture	25	3	11		39
São Paulo, Lins, 459 m	21°40'43" S 49°44'33" W	Mixture	3			1	4
Rio de Janeiro, Rio de Janeiro, 10 m	22°54'23" S 43°10'21" W	*anthidioides*	18	12			30
Paraná, Prudentópolis, 664 m	25°12'47" S 50°58'40" W	*quadrifasciata*				5	5
Paraná, Curitiba, 934 m	25°24'54.5" S 49°17'36.7" W	*anthidioides*	8				8
Santa Catarina, Vale do Itajaí, 0 m	26°55' S 49°03' W	*quadrifasciata*				6	6
Santa Catarina, Blumenau, 150 m	26°55'10" S 49°03'58" W	*quadrifasciata*			1	22	23
Total number of colonies			63	37	21	34	155

*3 to 5 continuous bands for *quadrifasciata* and 2 to 5 discontinuous bands for *anthidioides*.

Aligner V 1.5.2 (Codon Code, Dedham, Massachusetts, United States), Gene Runner version 3.05 (Hastings Software Inc.) and Multalin (Corpet, 1988). The cyt-b nucleotide sequence variation between *M. quadrifasciata* subspecies were compared with GenBank entries for *Melipona bicolor* (AF 466146), *Melipona compressipes* (AF 181615) and *Apis mellifera* (L06178).

Results

The *Melipona quadrifasciata* cyt-b amplicon was 485 bp in length. Two *Vsp* I restriction patterns were observed (Figure 1), haplotype 1 with two fragments (119 bp and 366 bp) and haplotype 2 with three fragments (119 bp, 150 bp and 216 bp). All samples with *M. q. anthidioides* phenotype were associated with haplotype 1, while all samples possessing the *M. q. quadrifasciata* tergal stripe pattern were associated with haplotype 2 (Table 1).

Digestion of the cyt-b amplicon with *Mbo* I generated three patterns (Figure 1), haplotype A with three fragments (122 bp, 143 bp and 220 bp) and haplotypes B and C with four fragments (31 bp, 91 bp, 143 bp and 220 bp), the difference between B and C being the lower mobility of the 220 bp fragment in pattern C. No complete association was observed between tergal stripe phenotype and *Mbo* I restriction patterns, but a very close association was found between *Mbo* I patterns A and B with *M. q. anthidioides* samples and *Mbo* I pattern C with *M. q. quadrifasciata* samples (Table 1).

Digestions using *Dra* I generated three fragments (107 bp, 134 bp and 244 bp) while *Taq* I produced two fragments (138 bp and 347 bp), with no differences being detected between samples. None of the colonies analyzed showed *Bgl* II restriction sites.

Four composite haplotypes were obtained based on the *Mbo* l and *Vsp* I cyt-b patterns, A1, B1, B2, and C2 (Table 1). Each haplotype was submitted to sequencing reactions, with Figure 2 showing the *Mbo* l and *Vsp* I restriction sites and the differences between the haplotypes.

A cyt-b restriction map was created from the sequencing results (Figure 3), with restriction sites potentially useful for differentiating patterns A1, B1, B2 and C2 being represented on this map. For the construction of this map we considered only restriction sites with less than ten cuts per enzyme and, with the exception of the *Vsp* I and *Mbo* I sites, restriction sites shared among haplotypes are not shown. The number of enzymes restriction sites generated in the different haplotypes was six each for A1 and B1, seven for B2 and eight for C2. The two most difficult patterns to discriminate through *Mbo* I and *Vsp* I digestion (B2 and C2) could be easily differentiated using the *Pac* I endonuclease (Figure 3).

Relationships between *M. quadrifasciata* haplotypes and their similarities with *M. bicolor*, *M. compressipes* and *Apis mellifera* sequences are shown in Figure 4. Among *M. quadrifasciata* haplotypes, most substitutions were observed between B1/C2 (15) and A1/C2 (14). Fewer differences were seen between B2/C2 (4) and A1/B1 (5). It is worth to noting that the *Vsp* I patterns (1 *vs.* 2) are associated with a higher number of nucleotide substitutions throughout the cyt-b fragment than the *Mbo* I haplotypes (A, B and C). There were fewer substitutions between *M. bicolor* and *M. compressipes* (n = 36) than the number of substitutions between either of these species and *M. quadrifasciata* subspecies (ranging from 52 to 56 substitutions, respectively). This resulted in two clusters, one comprising *M. quadrifasciata* samples and the second encompassing *M. bicolor* and *M. compressipes*.

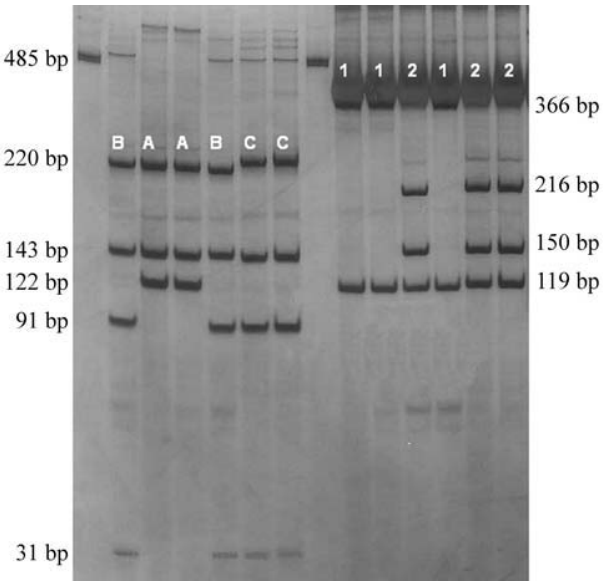

Figure 1 - Non-denaturing silver stained 12% polyacrylamide gel showing *Melipona quadrifasciata* cytochrome b PCR-RFLP patterns for *Vsp* I (haplotypes 1 and 2) and *Mbo* I (haplotypes A, B and C) endonucleases. Fragment profiles pattern (fragment sizes) were based on sequencing results.

Discussion

The complete association found between specific tergal stripe phenotypes and *Vsp* I cyt-b restriction patterns (*Vsp* I pattern 1 in *anthidioides* and pattern 2 in *quadrifasciata*) allows for simple subspecies identification. Although *Mbo* I patterns could not be wholly assigned to a particular subspecies, a close link between *Vsp* I 1 - *Mbo* I pattern A with *M. q. anthidioides* and *Vsp* I 2 - *Mbo* I pattern C with *M. q. quadrifasciata* was observed. The *Vsp* I patterns are associated with higher nucleotide substitutions along the cyt-b fragment. As the dendrogram in Figure 4 shows, this association explains the clustering of *Vsp* I patterns into different clades and highlights the importance of *Vsp* I haplotypes for *M. quadrifasciata* subspecies identification.

The *Mbo* I restriction patterns B and C showed no fragment size differences, although they could be differen-

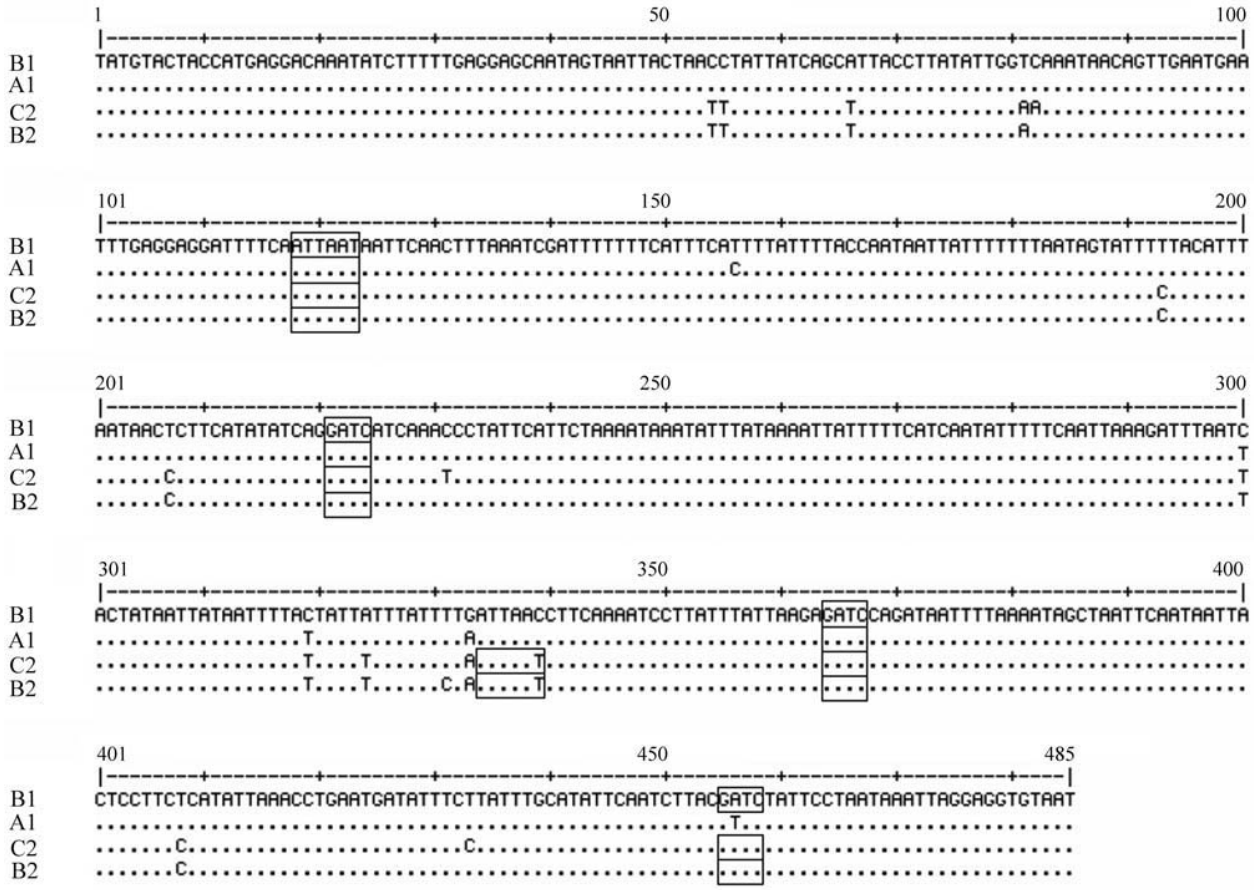

Figure 2 - DNA sequence of cytochrome b haplotypes observed in *Melipona quadrifasciata quadrifasciata* and *Melipona quadrifasciata anthidioides* samples (Accession n. EF 529490 - EF 529493). Recognition sites for endonucleases *Mbo* I (positions 220, 363 and 454) and *Vsp* I (positions 119 and 335) are marked within boxes.

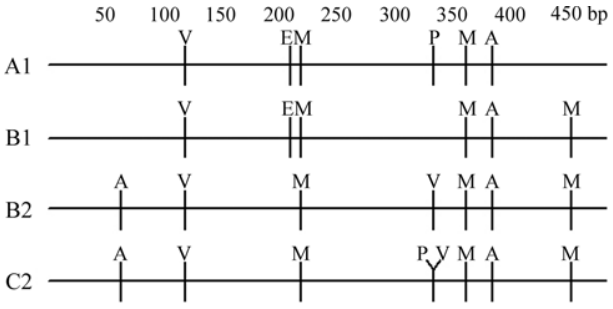

Figure 3 - Linear restriction map for *Melipona quadrifasciata* cytochrome b haplotypes A1, B1, B2 and C2. The restriction sites are represented by letters (A, *Alu* I; E, *Ear* I; M, *Mbo* I; P, *Pac* I; V, *Vsp* I) and their relative positions are indicated.

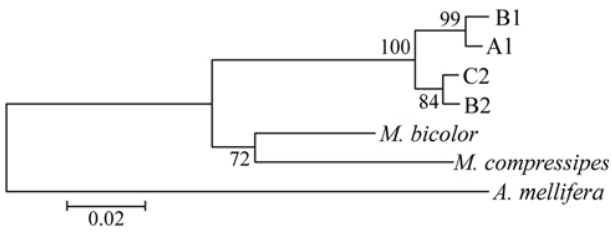

Figure 4 - Neighbor-joining dendrogram illustrating relationships among *Melipona quadrifasciata* haplotypes (A1, B1, B2 and C2), *Melipona bicolor*, *Melipona compressipes* and *Apis mellifera* (as outgroup), based on cytochrome b sequences. Number on branches are bootstrap values over 5000 replicates.

tiated by slower electrophoretic mobility in the 220 bp fragment of pattern C. The difference in electrophoretic mobility may have resulted from base substitutions, leading to double-strand conformation polymorphism (DSCP). This has been described as a potential source of variable genetic markers for termite populations (Atkinson and Adams, 1997). In DSCP, polymorphisms occur when mutations affect the curvature of double-stranded DNA molecules visualized following electrophoresis in non-denaturing acrylamide gels (reviewed in Hagerman, 1990). This is apparently common in AT-rich DNA. Thus, the difference observed in the 220 bp cyt-b fragment between haplotypes B and C is a single base substitution (C ↔ A) at position 82 (Figure 2). A similar pattern of differential fragment mobility without size difference has been observed in the same

mtDNA region of *Apis mellifera* subspecies (KM Ferreira, personal communication).

The cyt-b restriction patterns for *Bgl* II have been proposed by Weinlich *et al.* (2004) as diagnostic markers for *M. quadrifasciata* subspecies. According to these authors, a *Bgl* II restriction site within the cyt-b region was only detected in *M. q. quadrifasciata*. However, the 155 *M. quadrifasciata* colonies analyzed by us showed no *Bgl* II restriction site, a fact confirmed by sequencing analyses. The absence of a *Bgl* II restriction site in *M. quadrifasciata* cyt-b has also been reported by Moretto and Arias (2005).

Waldschmidt *et al.* (2000) identified a random amplified polymorphic DNA (RAPD) marker in *quadrifasciata* absent from *anthidioides* colonies of Brazilian *M. quadrifasciata* populations sampled from the core distribution of each subspecies. In the warmer northern sampling area of Minas Gerais state, where *M. q. anthidioides* colonies are frequent, specimens with *M. q. quadrifasciata* abdominal band patterns were found without this RAPD marker. This finding was interpreted by Waldschmidt *et al.* (2000) as the occurrence of natural hybrid colonies. In this sense, the cyt-b haplotypes described in our current paper provides a better method to identify hybrid colonies because to a specific tergal pattern must correspond the differential cyt-b pattern expected to that subspecies.

Moretto and Arias (2005) recently reported a mitochondrial variant in the cytochrome oxidase I locus as a diagnostic marker for *Melipona quadrifasciata* subspecies, although population analyses are needed to confirm the broader application of this genetic marker. In addition, Moretto and Arias (2005) also reported a *Dra* I polymorphism in the cyt-b+ND1 region enabling the differentiation of the two subspecies. This variation was not observed in our present study, probably due to the different size of the fragment analyzed (1800 *vs.* 485 bp).

Stingless bees are suffering population declines in many areas of the world due to habitat destruction and deforestation (Melendez-Ramirez *et al.*, 2002; Kremen *et al.*, 2004; Samejima *et al.*, 2004; VillaNueva *et al.*, 2005), with some species probably facing extinction. The high degree of environmental alteration due to human activities has led to the current crisis for pollinators of wild and agricultural crops (Kearns *et al.*, 1998; but see Cane, 2001). Loss of genetic variability due to environmental perturbation and the implication of such loss in respect to the conservation of stingless bees needs to be understood, and for this to occur the level of gene variation and genetic structure of stingless bee populations must be quantified.

The PCR-RFLP method outlined here appears well-suited as a molecular tool for the identification of *Melipona quadrifasciata* subspecies, allowing easy diagnosis of matrilines. This molecular analysis, when associated with morphological and behavioral data, will be useful in future studies that will improve our understanding of the biology, biogeography and evolution of *Melipona quadrifasciata*.

Acknowledgments

We would like to thank Davi S. Aidar, Miguel A. Quaggio, Joelson Rodrigues Souza and Lúcio A.O. Campos for providing samples of *Melipona quadrifasciata* subspecies, Margarita M. López-Uribe for a careful reading of the manuscript, Otávio Lino e Silva for computational help and Susy Coelho for technical assistance.

References

Atkinson L and Adams ES (1997) Double-strand conformation polymorphism (DSCP) analysis of the mitochondrial control region generates highly variable markers for population studies in a social insect. Insect Mol Biol 6:369-375.

Camargo CA (1979) Sex determination in bees. XI. Production of diploid males and sex determination in *Melipona quadrifasciata*. J Apic Res 18:77-84.

Camargo JM and Pedro SR (1992) Systematics, phylogeny and biogeography of the Meliponinae (Hymenoptera, Apidae): A mini-review. Apidologie 23:509-522.

Cane JH (2001) Habitat fragmentation and native bees: A premature veredict? Conserv Ecol 5:1-9.

Corlett RT (2004) Flower visitors and pollination in the Oriental (Indomalayan) Region. Biol Rev 79:497-532.

Corpet F (1988) Multiple sequence alignment with hierarchical clustering. Nucleic Acids Res 16:10881-10890.

Crozier YC, Koulianos S and Crozier RH (1991) An improved test for Africanized honeybee mitochondrial DNA. Experientia 47:968-969.

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

Heard TA (1999) The role of stingless bees in crop pollination. Annu Rev Entomol 44:183-206.

Kearns CA, Inouye DW and Waser NM (1998) Endangered mutualism: The conservation of plant-pollinator interactions. Annu Rev Ecol Syst 29:83-112.

Kerr WE, Carvalho GA and Nascimento VA (1996) Abelha Uruçu - Biologia, Manejo e Conservação. Fundação Acangaú, Belo Horizonte, 141 pp.

Klein AM, Steffan-Dewenter I and Tscharntke T (2003) Pollination of *Coffea canephora* in relation to local and regional agroforestry management. J Appl Ecol 40:837-845.

Kremen C, Williams NM, Bugg RL, Fay JP and Thorp RW (2004) The area requirements of an ecosystem service: Crop pollination by native bee communities in California. Ecol Let 7:1109-1119.

Meléndez-Ramirez V, Maganã-Rueda S, Parra-Tabla V, Ayala R and Navarro J (2002) Diversity of native bee visitors of cucurbit crops (Cucurbitaceae) in Yucatán, México. J Insect Conserv 6:135-147.

Melo GAR and Campos LAO (1987) Variações dos padrões de faixas nas populações de *Melipona quadrifasciata* Lepeletier, 1936 no Estado de Minas Gerais (Hymenoptera, Apidae, Meliponinae). Anais do XIV Congresso Brasileiro de Zoologia, Juiz de Fora, pp 76.

Michener CD (2000) The Bees of the World. The Johns Hopkins University Press, Baltimore, 952 pp.

Moretto G and Arias MC (2005) Detection of mitochondrial DNA restriction site differences between the subspecies of

Melipona quadrifasciata Lepeletier (Hymenoptera, Apidae, Meliponini). Neotrop Entomol 34:381-385.

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

Moure JS and Kerr WE (1950) Sugestões para a modificação da sistemática do gênero *Melipona* (Hymen.-Apoidea). Dusenia 1:105-129.

Samejima H, Marzuki M, Nagamitsu T and Nakasizuka T (2004) The effects of human disturbance on a stingless bee community in a tropical rainforest. Biol Conserv 4:577-587.

Schwarz HF (1932) The genus *Melipona*: The type genus of the Meliponidae or stingless bees. Bull Am Mus Nat Hist 63:231-460.

Sheppard WS and McPheron BA (1991) Ribosomal DNA diversity in Apidae. In: Smith DR (ed) Diversity in the Genus *Apis*. Westview Press, Boulder, pp 89-102.

Silberbauer-Gottsberger I and Gottsberger G (1988) A polinização de plantas de cerrado. Rev Bras Biol 48:651-663.

Silveira FA, Melo GAR and Almeida EAB (2002) Abelhas Brasileiras: Sistemática e Identificação. Editora IDM Composição e Arte, Belo Horizonte, 253 pp.

VillaNueva GR, Roubik DW and Colli-Ucán W (2005) Extinction of *Melipona beecheii* and traditional beekeeping in the Yucatán peninsula. Bee World 86:35-41.

Waldschmidt AM, Barros EG and Campos LAO (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 FO and Arias MC (2004) Mitochondrial DNA restriction and genomic maps of seven species of *Melipona* (Apidae, Meliponini). Apidologie 35:365-370.

Assistant Editor: Klaus Hartfelder