

## Geographic distribution and spatial differentiation in the color pattern of abdominal stripes of the Neotropical stingless bee *Melipona quadrifasciata* (Hymenoptera: Apidae)

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ABSTRACT. *Melipona quadrifasciata* Lepeletier, 1836, regionally known as “mandaçaia”, has been traditionally divided in two distinct subspecies: *M. quadrifasciata anthidioides* and *M. quadrifasciata quadrifasciata*. The main difference between the subspecies refers to the yellow metasomal stripes which are continuous in *M. q. quadrifasciata* and discontinuous in *M. q. anthidioides*. This study investigated the geographic differentiation in the metasomal stripes and characterized the restriction sites in the mtDNA of both chromatic types. Specimens from 198 localities were examined, and the variation observed in the pattern of stripes was grouped into distinct classes. The distribution pattern found in the present work agrees with the previously reported pattern: *M. q. quadrifasciata* inhabits the southern portion of the distribution, from Misiones, Argentina, southeastern Paraguay and Rio Grande do Sul to southern São Paulo, and *M. q. anthidioides* ranges from northeastern São Paulo to the northern Diamantina Plateau, Bahia, and westwards to the central portion of the Goiás state. It is documented for the first time the occurrence of two populations with continuous stripes inhabiting disjunct areas in relation to *M. q. quadrifasciata* – one in northern Minas Gerais and another in northeastern Bahia and Sergipe. The data of RFLP showed two restriction patterns, one present in *M. q. quadrifasciata*, and another in *M. q. anthidioides* and in populations with continuous metasomal stripes from northern Minas Gerais and northeastern Bahia and Sergipe. The observed patterns of geographic differentiation of *M. quadrifasciata* suggests the occurrence of repeated events of geographical isolation, followed by range expansion, that occurred probably during the cycles of climatic changes in the Pleistocene.

KEY WORDS. Atlantic forest; biogeography; mtDNA COI.

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The stingless bee *Melipona quadrifasciata* Lepeletier, 1836 regionally known as “mandaçaia” in Brazil, is widespread throughout eastern Brazil, from Rio Grande do Sul to Paraíba states and extending westward to Misiones, in Argentina, and southeastern Paraguay (MOURE & KERR 1950). Traditionally, this species has been divided into two distinct subspecies: *M. quadrifasciata anthidioides* Lepeletier and *M. quadrifasciata quadrifasciata*. The main difference between the subspecies refers to the yellow tergal stripes from the 2<sup>nd</sup> to 5<sup>th</sup> tergum in workers and males, which are continuous in *M. q. quadrifasciata* and discontinuous in *M. q. anthidioides* (DUCKE 1916, SCHWARZ 1932).

The subspecies *M. q. quadrifasciata* is found in colder regions, comprising the southern portion of São Paulo, through Paraná, Santa Catarina and Rio Grande do Sul in the south (KERR 1951). The subspecies *M. q. anthidioides* is found from northern

and northeastern São Paulo state, eastward up to Paraíba (KERR 1951). In the state of São Paulo and southern Minas Gerais there is a hybridization zone between the two subspecies, where intermediary patterns of yellow tergal stripes are detected (MOURE & KERR 1950, KERR 1951).

The occurrence of populations bearing continuous abdominal stripes, similar to the color pattern exhibited by *M. q. quadrifasciata*, in areas outside the distribution range of this subspecies, is known for more than 30 years. NOGUEIRA-NETO (1970) stated that he had received specimens of *M. quadrifasciata* from Carira, in Sergipe, displaying a pattern of tergal stripes similar to that of *M. q. quadrifasciata*. Also, colonies with continuous tergal stripes, similar to the subspecies from southern Brazil, from areas under semi-arid climate in northern Minas Gerais are known since the 1980's (G.A.R. Melo & L.A.O. Campos, unpublished data).

Some studies have showed a molecular differentiation between the subspecies of *M. quadrifasciata*. WALDSCHMIDT *et al.* (2000) detected a RAPD-PCR marker, present in *M. q. quadrifasciata* and absent in *M. q. anthidioides*. WALDSCHMIDT *et al.* (2002), in a further population study using RAPD markers, observed two genetically distinct groups, one comprising *M. q. quadrifasciata* and another composed of *M. q. anthidioides*. In both studies, the samples from Januária, northern Minas Gerais, and bearing continuous tergal stripes, clustered with *M. q. anthidioides*.

In stingless bees, analyses of mitochondrial DNA (mtDNA) by RFLP have showed differences at both inter- and intra-specific levels. FRANCISCO *et al.* (2001) characterized the mtDNA of five species of *Plebeia* using RFLP and detected differences in the restriction sites among them. FERNANDES-SALOMÃO *et al.* (2002) analyzed, via RFLP, the mtDNA of 16 species of *Melipona*, revealing differences among each species. MORETTO & ARIAS (2005) observed distinct RFLP profiles in the mtDNA between the two subspecies of *M. quadrifasciata*. DE LA RÚA *et al.* (2007) analyzed the differences in the RFLP pattern and sequences comprising the ITS2 ribosomal region in the nuclear DNA of *Melipona beecheii* and *M. yucatanica* and they also found intra-specific variation in both species.

The goal of the present work is to investigate the geographic differentiation in the metasomal coloration and characterize the restriction sites in the mtDNA of both morphotypes of *M. quadrifasciata*, as determined by the tergal stripe patterns, in order to achieve a better understanding of the evolution of their biogeographic pattern.

## MATERIAL AND METHODS

Data regarding collecting localities and the pattern of tergal stripes in *Melipona quadrifasciata* were gathered from material deposited in the entomological collections of the Museu de Zoologia at Universidade de São Paulo (in January 21, 1987, by GARM), Coleção Entomológica J.S. Moure at Universidade Federal do Paraná (in February 4, 1988, by GARM and January 15, 2008, by GARM and HBF), Coleção P. Nogueira-Neto at the Instituto de Biociências, Universidade de São Paulo (in July 18, 1988, by GARM), Coleção Camargo at Faculdade de Filosofia, Ciências e Letras of Ribeirão Preto, Universidade de São Paulo (in November 15, 1991, by GARM), and Museu de Entomologia at Universidade Federal de Viçosa (examined by GARM and HBF in several occasions). Data from recently collected samples in northern Minas Gerais, Bahia and Sergipe, and deposited in the entomological collection of the apiary at Universidade Federal de Viçosa, were also included. Data on Argentinean and Paraguayan samples were taken from HOLMBERG (1903), BERTONI (1911) and COCKERELL (1919). Considering that *Melipona mandacaia* Smith, 1863 is the sister species of *M. quadrifasciata* (G.A.R. Melo, unpublished data; see below), its geographic records were compiled from specimens deposited in the collection at Universidade Federal do Paraná and plotted in the distribution map.

The variation observed in the pattern of tergal stripes was grouped into four distinct classes (Fig. 1). Class A represents the pattern in which each yellow stripe is largely interrupted (Fig. 1), corresponding to the so-called *M. q. anthidioides*; class B also presents an interrupted pattern, but the distance between each half band is smaller than that in class A (Fig. 2); class C, represents the hybrid pattern between the extreme forms (Fig. 3); and class D comprises the pattern where the yellow stripes are continuous, corresponding to the pattern found in *M. q. quadrifasciata* (Fig. 4). A few specimens bearing a variant pattern, intermediate between classes A and B, were observed; for practical purposes, they were included in class A. Also, specimens with continuous stripes but displaying a small medial interruption in tergum II were included in class D. The recognition of the hybrid pattern and intermediate forms were based on several controlled crosses between the extreme morphotypes (G. A. R. Melo & L. A. O. Campos, unpublished data).

Representative specimens of the four classes of tergal stripe patterns were photographed using a Leica DFC 500 digital camera attached to a stereomicroscope Leica MZ 16. Images were captured using the software IM 50 (Image Manager) and combined using the software Auto-Montage Pro 5.03.0040.

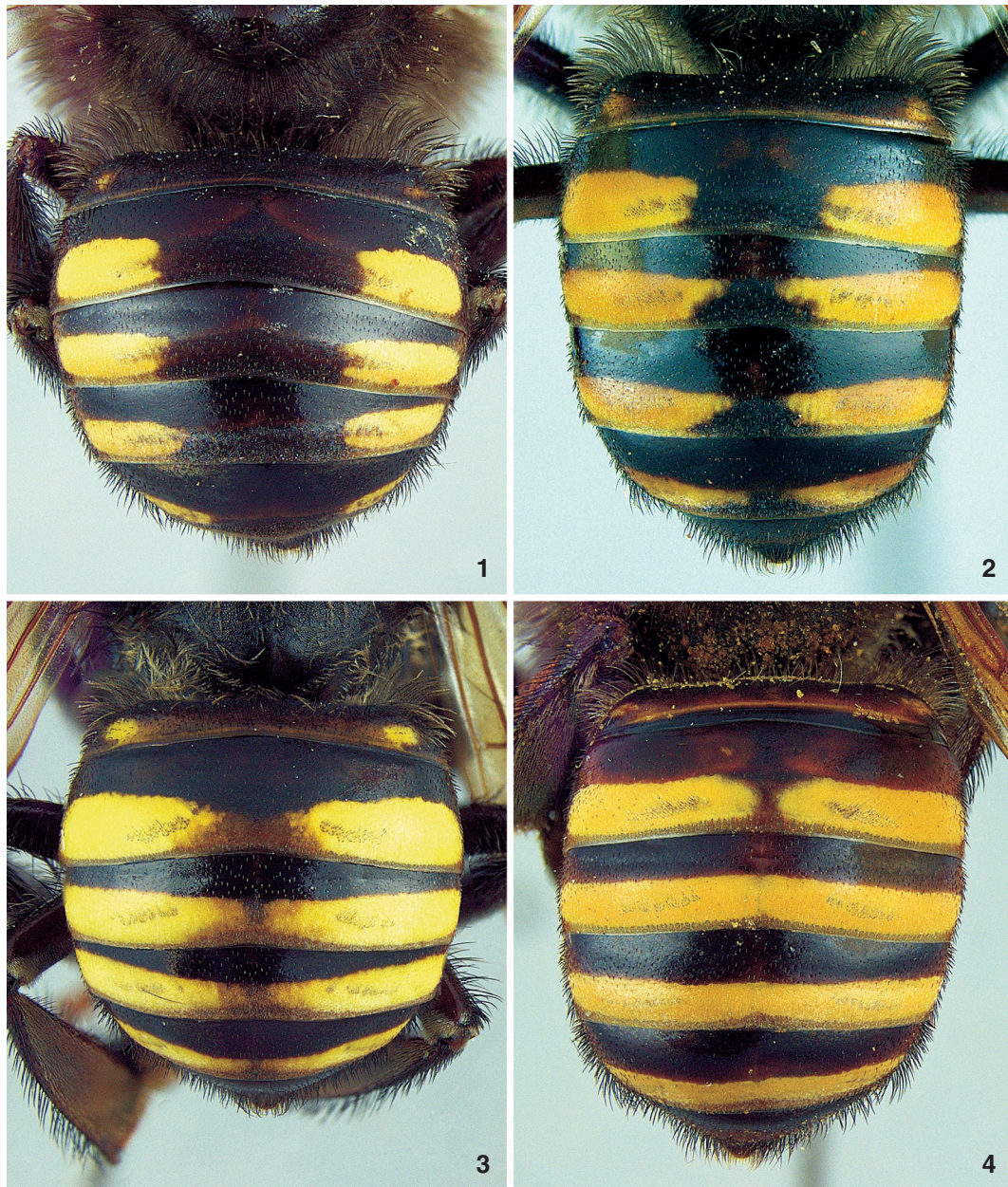
Geographic coordinates for the localities cited in the collecting labels were obtained using the geo-referencing tool *geoLoc* provided by the Centro de Referência em Informação Ambiental – CRIA, available at <http://splink.cria.org.br/geoloc>. Preference was given to coordinates provided by Instituto Brasileiro de Geografia e Estatística – IBGE. Locality coordinates for recent samples, mainly in northern Minas Gerais, Bahia and Sergipe, were obtained in the field using a GPS device. A map of the geographic distribution was generated using the software Arcview Gis 3.2a.

The samples of *M. quadrifasciata* used in the present work were collected and stored in absolute ethanol and kept frozen at -80°C up to their utilization. A worker bee from each of the following localities was analyzed: Itaiópolis (Santa Catarina) and Porto Alegre (Rio Grande do Sul), representing *M. q. quadrifasciata*; Caeté (Minas Gerais), Viçosa (Minas Gerais) and Jequié (Bahia), representing *M. q. anthidioides*; Uruçua and Januária, comprising the population with continuous stripes from northern Minas Gerais; Brejão and São Cristóvão, in Sergipe, representing the population with continuous stripes from northeastern Bahia and Sergipe.

The DNA extraction followed the procedure reported by FERNANDES-SALOMÃO *et al.* (2005) and the samples were quantified in 0.8% agarose gel, in order to check the amount and the quality of DNA samples. A region encompassing the mtDNA COI gene was amplified, using the primers mtD7 (5' GGATCACCTGATATAGCATTCCC 3') and COI-IIR (5' GATCAATATCATTGATGACC 3') described by SIMON *et al.* (1994) and HALL & SMITH (1991), respectively.

The PCR reactions comprised 50ng of genome DNA, 5 µl of buffer (Promega), 3 µl of MgCl<sub>2</sub> (25 mM), 1.5 µl of each primer





Figures 1-4. Patterns of tergal yellow stripes exhibited by workers and males of *M. quadrifasciata*. (1) class A, *M. q. anthidioides*, worker from Santa Tereza, Espírito Santo; (2) class B, worker from Três Marias, Minas Gerais; (3) class C, hybrid pattern, worker from Ourolândia, Bahia; (4) class D, *M. q. quadrifasciata*, worker from Campina Grande do Sul, Paraná.

(20  $\mu$ M), 5  $\mu$ l of dNTPs (2mM each) and 2 U of *GoTaq* DNA polymerase (Promega) and water to a final volume of 50  $\mu$ l. Each reaction was carried out in a PTC-100 thermocycler (MJ Research, Inc.), programmed to an initial denaturation step at 94°C for five minutes, followed by 35 cycles at 94°C for one minute, 45°C for one minute and 64°C for two minutes, plus a final extension

step at 64°C for 10 minutes.

The amplified DNA fragment was digested with the enzymes *Dra*I, *Eco*RI, *Hin*fI, *Mbo*I, *Pst*I, *Sca*I, *Xba*I and *Xho*I. The digestion reaction was performed at 37°C for lasted six hours, and the digested products were run in 2% agarose gel, stained with ethidium bromide and photodocumented under UV light.

## RESULTS

Specimens from 198 localities in Brazil were examined, comprising the entire area of occurrence of *M. quadrifasciata*. The distribution records are shown in figure 5, each one represented by the symbol corresponding to the pattern of tergal stripes herein recognized. The distribution records for *M. mandacaia* are also shown in figure 5.

In general, the distribution pattern of *M. quadrifasciata* found in the present work agrees with the previously reported pattern for this species, i.e., *M. q. quadrifasciata*, a form displaying continuous tergal stripes, inhabits the southern portion of the distribution, from Rio Grande do Sul to southern São Paulo, including Misiones, in Argentina, and southeastern Paraguay to

the west, whereas *M. q. anthidioides*, a form with interrupted stripes, ranges from northeastern São Paulo to the northern portion of Diamantina Plateau, in Bahia, and westwards to the western tip of Minas Gerais and central portion of the Goiás state.

Regarding the distribution pattern reported by previous authors, a few main differences should be pointed out: (1) Samples bearing interrupted stripes were not observed in the states of Alagoas and Sergipe, as shown in KERR (1951); (2) No specimens of *M. quadrifasciata* were recorded from Pernambuco and Paraíba, as reported by KERR (1951) and CAMARGO & PEDRO (2007); (3) It is consistently documented for the first time the distribution pattern of populations with continuous stripes and inhabiting clearly disjunct areas in relation to *M. q. quadrifasciata* – one in northern Minas Gerais and another in northeastern Bahia and Sergipe (Fig. 5).

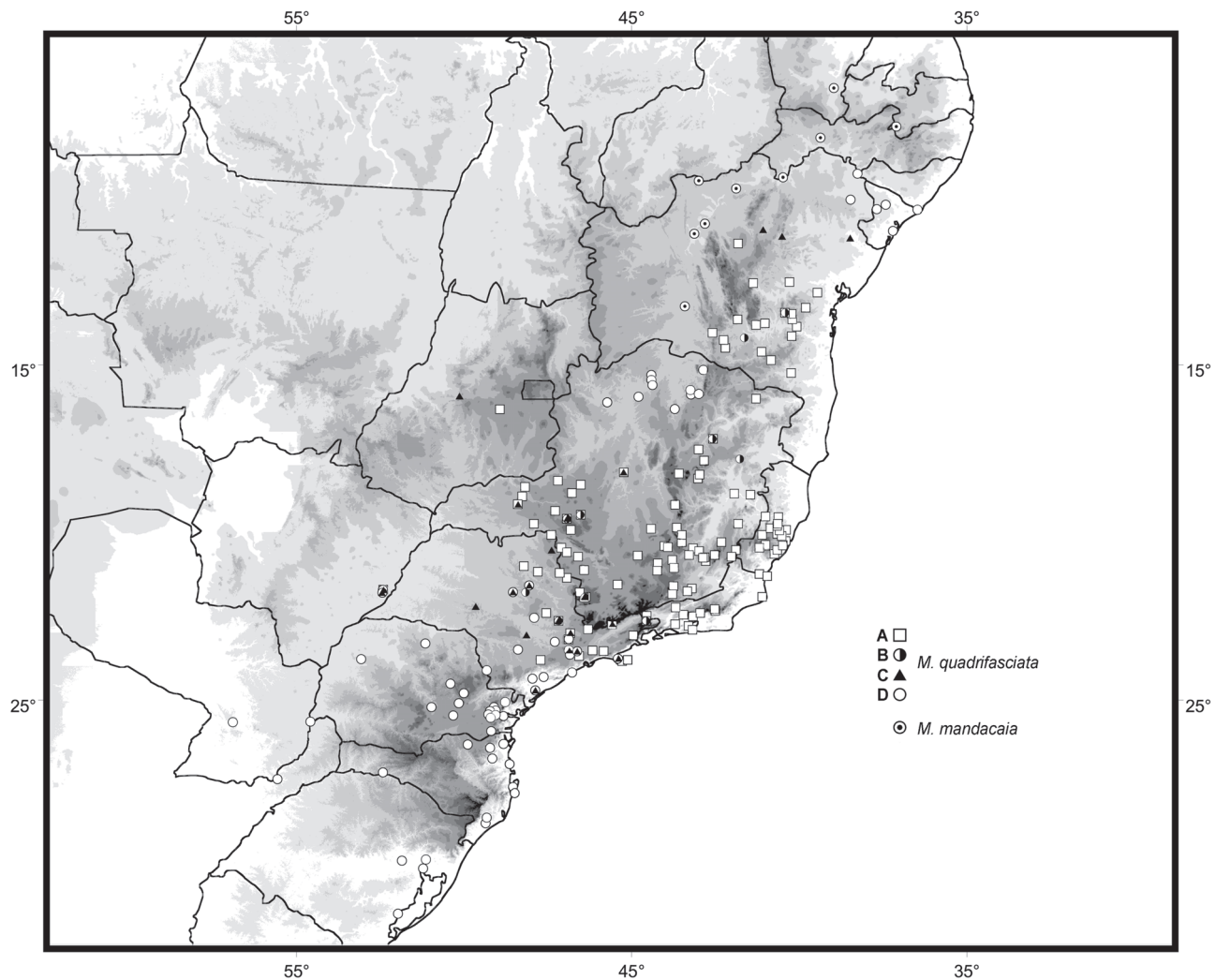


Figure 5. Distribution map for *M. quadrifasciata* and *M. mandacaia*. The records of the different patterns of tergal stripes (class A-D) in *M. quadrifasciata* are discriminated in the map (see text for characterization of the pattern classes). The different gray tones in the map are based on digital reconstruction of the relief, where the darkest tones indicate higher altitude.



Both northern populations exhibiting a pattern of continuous stripes are associated with the basin of the São Francisco River. The former population is apparently restricted to areas of arboreal Caatinga along the middle São Francisco and some of its tributaries in Minas Gerais, such as the Verde River, on the right margin, and Urucuia River, on the left margin, while the latter population occupies Caatinga forests near the mouth of the São Francisco River. It is noteworthy that both populations inhabit areas contiguous with the southern and northern limits of the distribution of *M. mandacaia*, a species occurring along the middle to the upper São Francisco (Fig. 5).

The contact zones between populations of *M. quadrifasciata* bearing contrasting patterns of tergal stripes were recognized through the presence of hybrid forms (black triangles in figure 5). As previously shown by KERR (1951), the hybridization zone between *M. q. quadrifasciata* and *M. q. anthidioides* occupies the central region of the state of São Paulo, along the main channel of the river Tietê (MOURE 1975), and apparently extending to the west up to eastern Mato Grosso do Sul. The hybridization zone between *M. q. anthidioides* and the morphotype with continuous stripes found in the northern portion of the distribution extends along a relatively narrow area in northeastern Bahia.

In relation to the population with continuous stripes in northern Minas Gerais, no specimens that could indicate hybridization with *M. q. anthidioides* were identified, although the occurrence of specimens with a hybrid phenotype in Três Marias, in central Minas Gerais, might be regarded as an evidence of contact between these populations. Individuals displaying a hybrid phenotype were also found in populations from the western tip of Minas Gerais and Goiás, in areas relatively far from the hybridization zone in São Paulo. In populations of *M. q. anthidioides* from northeastern Minas Gerais and southwestern Bahia, specimens exhibiting the class B phenotype were found in a few dispersed localities (Fig. 5).

Based on the distribution pattern shown in figure 5, it seems that *M. q. anthidioides* is associated with higher altitude areas along the mountain ranges in Minas Gerais, Espírito Santo and Bahia, and absent from the lowlands in northern Espírito Santo and southern Bahia, as well as from lower altitude areas in the upper São Francisco river.

A fragment of about 1700bp, encompassing the mtDNA COI gene of each specimen was amplified and digested. From the eight enzymes tested, *EcoRI*, *PstI*, *ScaI*, *XbaI* and *XhoI* failed in digesting the amplified DNA fragment, while the other provided restriction fragments. The restriction pattern after digestion with *DraI* was identical in all samples, while *HinfI* and *MboI* generated polymorphic profiles.

Five restriction fragments (970, 240, 180, 190, and 120 bp) were produced by the enzyme *DraI*, corresponding to four restriction sites, similar in all analyzed samples. The enzyme *HinfI* generated two restriction patterns: (1) one restricted to samples of *M. q. quadrifasciata* (fragments of 950, 320, 280, and 150 bp), corresponding to the localities Itaiópolis and Porto Alegre; and

(2) another present in the remaining studied localities (Caeté, Viçosa, Jequié, Urucuia, Januária, Brejão and São Cristóvão) with fragments of 950, 600 and 150 bp (Fig. 6). Polymorphic profiles, comprising fragments of 640, 340, 320, 230, and 170 bp, were obtained by the enzyme *MboI*, following the pattern seen with *HinfI*: one pattern was found in samples of *M. q. quadrifasciata*, and the second pattern was present in the remaining localities (fragments of 600, 380, 320, 230 and 170 bp).

The RFLP data are only partially congruent with the geographic distribution of the tergal color patterns. The samples with continuous stripes belonging to *M. q. quadrifasciata* presented distinct restriction patterns in relation to those from the other analyzed samples. The samples bearing continuous stripes from northern Minas Gerais and northeastern Bahia and Sergipe, however, exhibited the same restriction pattern observed in samples of *M. q. anthidioides*.

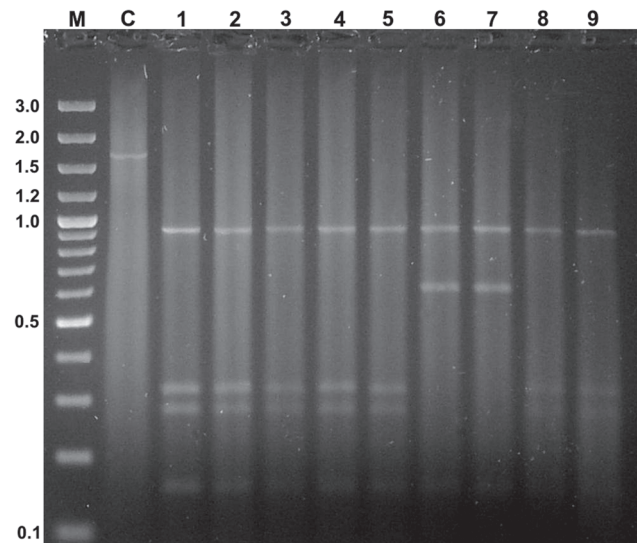


Figure 6. *HinfI* RFLP patterns of a fragment of the gene COI in *M. quadrifasciata*. Locality data for the samples are: 1) Viçosa, Minas Gerais; 2) Caeté, Minas Gerais; 3) Jequié, Bahia; 4) Urucuia, Minas Gerais; 5) Januária, Minas Gerais; 6) Itaiópolis, Santa Catarina; 7) Porto Alegre, Rio Grande do Sul; 8) Brejão, Sergipe; and 9) São Cristóvão, Sergipe. M is the GeneRuler of 100 pb DNA ladder plus (Fermentas). C is the undigested control of the fragment.

## DISCUSSION

*Melipona quadrifasciata* has one of the largest geographic distributions among species of *Melipona*, inhabiting a large area along eastern Brazil. This species is mainly associated with the Atlantic Forest along its southern distribution range and with Cerrado (Brazilian savannah) and seasonal forests in the northern portion of its distribution. Its occurrence in such distinct

biomes reveals a successful history of survival and reproduction under diverse climatic conditions and vegetation when compared to other species of *Melipona*. The occupation of mesic environments, covered by humid forests, seems to represent a derived feature exhibited by this species. The species of the subgenus *Melipona* (*Melipona*), group to which *M. quadrifasciata* belongs to, inhabit mainly dry and seasonal forests, such as for instance *M. mandacaia* in northeastern Brazil (G.A.R. Melo, unpublished data).

The geographic differentiation of *M. quadrifasciata*, bearing forms with distinct tergal stripe patterns at different regions, reflects a complex evolutionary history. The maintenance of two distinguishable patterns of metasomal coloration between *M. q. quadrifasciata* and *M. q. anthidioides* has been related to recent ecological features, such as adaptation to distinct climatic conditions (KERR 1951, MOURE 1975, CAMARGO & PEDRO 2007). However, the occurrence of additional populations bearing continuous tergal stripes in two separate regions suggests that vicariant historical events might have taken place. Based on the present scenario, these disjunct populations bearing continuous stripes would represent remaining descendants of a larger population that became fragmented.

This hypothesis is supported by comparisons between *M. quadrifasciata* and *M. mandacaia*. Within *M. (Melipona)*, *M. mandacaia* is the closest species to *M. quadrifasciata* and, based on several morphological similarities found in workers and males and nest architecture, they can be considered sister-species (G.A.R. Melo, unpublished data). Similarly to other species of *M. (Melipona)* that possess tergal stripes, *M. mandacaia* displays continuous yellow stripes, indicating that the occurrence of such condition in *M. quadrifasciata* should be considered a plesiomorphy, thereby justifying giving those forms with continuous metasomal stripes the status of remaining populations. The presence of a single form possessing interrupted stripes, *M. q. anthidioides*, would be a result of a more recent differentiation within this group. The continuous geographic distribution of *M. q. anthidioides* within the central portion of the species range provides additional support to this hypothesis.

The separation between *M. q. quadrifasciata* and the other populations, as indicated by RFLP profiles using the enzymes *HinfI* and *MboI*, is in agreement with the results of WALDSCHMIDT *et al.* (2000, 2002), who found a RAPD marker present in *M. q. quadrifasciata* and absent in *M. q. anthidioides*, as well as with MORETTO & ARIAS (2005) who also distinguished both subspecies based on distinct RFLP patterns.

As observed by WALDSCHMIDT *et al.* (2000, 2002), the samples with continuous stripes from northern Minas Gerais, and, in the present study, also from northeastern Bahia and Sergipe, were not genetically different from samples of *M. q. anthidioides*. The molecular data suggest that the differentiation process first involved a split between the southern populations, giving rise to *M. q. quadrifasciata*, and those found in the northern portion of the species range. The differentiation of the form with inter-

rupted stripes, corresponding to the subspecies *M. q. anthidioides*, in relation to populations bearing continuous stripes in northern Minas Gerais and northeastern Bahia and Sergipe, would represent a more recent event.

The available evidence does not allow for a more precise estimate of when the differentiation process in *M. quadrifasciata* has started. The complexity in the observed patterns suggests the occurrence of independent events of geographical isolation, followed by range expansion. Other species of *Melipona* Illiger, 1806 from the Atlantic forest, such as *M. bicolor* and *M. marginata*, also present morphological differences over their ranges, in the north-south direction, and these distinct forms have been regarded as subspecies (CAMARGO & PEDRO 2007). A similar differentiation has been reported in other animal groups, as in birds of the genus *Xiphorhynchus* (CABANNE *et al.* 2007). Probably, these events took place relatively recently in the evolutionary history of these species of *Melipona*, most likely during the cycles of climatic changes in the Pleistocene, as revealed in *Xiphorhynchus fuscus* by CABANNE *et al.* (2007).

The identification of populations bearing continuous yellow stripes and isolated in the northern portion of the species range could indicate that additional subspecies of *M. quadrifasciata* should be recognized in order to give them a taxonomical status congruent with the differentiation pattern observed for the species. Alternatively, considering the relatively low degree of differentiation within the species, the use of subspecies within *M. quadrifasciata* could be abandoned. Further studies using mtDNA haplotypes, however, should be conducted in order to elucidate the phylogeographic pattern of populations and define whether phylogroups are present or not within this species.

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