



Genetic divergence between *Melipona quadrifasciata* Lepeletier (Hymenoptera, Apidae) populations

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Abstract

Melipona quadrifasciata is a stingless bee widely found throughout the Brazilian territory, with two recognized subspecies, *M. quadrifasciata anthidioides*, that exhibits interrupted metasomal stripes, and *M. quadrifasciata quadrifasciata*, with continuous metasomal stripes. This study aimed to estimate the genetic variability of these subspecies. For this purpose, 127 colonies from 15 Brazilian localities were analyzed, using nine species-specific microsatellite primers. At these loci, the number of alleles ranged from three to 15 (mean: 7.2), and the observed heterozygosity (H_o) ranged from 0.03-0.21, while the expected heterozygosity (H_e) ranged from 0.23-0.47. The genetic distances among populations ranged from 0.03-0.45. The F_{ST} multilocus value (0.23) indicated that the populations sampled were structured, and the clustering analysis showed the formation of two subgroups and two more distant populations. The first group contained the subspecies *M. quadrifasciata quadrifasciata*, and the other, the subspecies *M. quadrifasciata anthidioides* and the two *M. quadrifasciata* populations with continuous metasomal stripes from northern Minas Gerais. These results confirmed that the yellow metasomal stripes alone are not a good means for correctly identifying the different subspecies of *M. quadrifasciata*.

Keywords: genetic differentiation, microsatellites, population genetics, stingless bees.

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Introduction

Despite their ecological importance as pollinators of native trees in Brazil (Kerr *et al.*, 2001; Imperatriz-Fonseca *et al.*, 2006), stingless bee populations have decreased in the Neotropics, similarly to other bee populations, due to habitat degradation and fragmentation, agricultural intensification, indiscriminate use of agrochemicals and pesticides, and introduction/spread of exotic species (Freitas *et al.*, 2009).

Melipona quadrifasciata, in particular, is a stingless bee species with a wide geographic distribution in Brazil (Silveira *et al.*, 2002; revised in Camargo and Pedro, 2007). Few molecular studies, however, have been conducted on this species to analyze its genetic variability and structuring of populations (Waldschmidt *et al.*, 2000, 2002; Moreto and Arias, 2005; Souza *et al.*, 2008; Batalha-Filho *et al.*, 2009, 2010; Nascimento *et al.*, 2010).

In general, these studies have provided evidence that it is possible to recognize molecular differences between the two *M. quadrifasciata* subspecies, confirming the usefulness of the yellow metasomal stripes, which are continuous in *M. quadrifasciata quadrifasciata* and interrupted in

M. quadrifasciata anthidioides, to distinguish them (Schwarz, 1932).

However, according to Moure and Kerr (1950), although *M. quadrifasciata quadrifasciata* is found in the colder regions located from Rio Grande do Sul to southern São Paulo, and *M. quadrifasciata anthidioides* occurs from northeastern São Paulo along eastern Brazil up to Paraíba, a hybridization zone exists in the states of São Paulo and Minas Gerais, with specimens presenting an intermediary pattern of yellow metasomal stripes.

More recently, *M. quadrifasciata* individuals from Januária and Urucuia (northern Minas Gerais) have been observed exhibiting continuous metasomal stripes identical to those of *M. quadrifasciata quadrifasciata* instead of the interrupted pattern characteristic to *M. quadrifasciata anthidioides*, as was expected based on their geographical localization (review in Batalha-Filho *et al.*, 2009). The specimens from Januária, however, did not exhibit the RAPD marker present in individuals of the subspecies *M. quadrifasciata quadrifasciata*, being therefore genetically more similar to *M. quadrifasciata anthidioides* (Waldschmidt *et al.*, 2000).

Another population bearing a continuous pattern of metasomal stripes was later on described in Sergipe and northeastern Bahia (Batalha-Filho *et al.*, 2009). RFLP analysis and sequencing of the mtDNA COI gene again demon-

strated that this population grouped together with populations from northern Minas Gerais and populations of *M. quadrifasciata anthidioides* (Batalha-Filho *et al.*, 2010).

These data demonstrate that a distinction between *M. quadrifasciata anthidioides* and *M. quadrifasciata quadrifasciata* based exclusively on morphological characteristics is not reliable.

Thus, the main objective of this study was to investigate the genetic variability of some *M. quadrifasciata* populations from different locations in Brazil, in order to improve the knowledge about their taxonomic status.

Materials and Methods

Bee samples

Melipona quadrifasciata adult workers from 127 colonies were collected in eight Brazilian states. *M. quadrifasciata quadrifasciata* samples were collected in the states of Rio Grande do Sul (Porto Alegre), Santa Catarina (Içara) and Paraná (Curitiba), while *M. quadrifasciata anthidioides* samples were obtained in Rio de Janeiro (Miguel Pereira), Minas Gerais (Cristiano Otoni, Piranga, Caeté, Rio Vermelho and Poté), Espírito Santo (Domingos Martins) and Bahia (Caitité). We also included samples from Urucua/Januária and São Cristovão in the analyses, representing *M. quadrifasciata* populations with continuous metasomal stripes from northern Minas Gerais and Sergipe, respectively. It is important to emphasize that these populations were located completely outside the distribution area of *M. quadrifasciata quadrifasciata* which typically exhibits continuous yellow metasomal stripes.

Samples from Ourolândia (Bahia) presenting a hybrid pattern of metasomal stripes were also analyzed.

Samples from Cristiano Otoni, Caeté, Urucua and Januária were collected from feral colonies, directly from nature. Samples from Piranga, Rio Vermelho, Poté, São Cristovão, Ourolândia and Caitité were provided by beekeepers that maintain a small number of colonies in the original trunks or in rational boxes. These colonies were collected in their propriety wood or in the neighborhood, approximately 500-1000 m from their present-day localization. Samples from Domingos Martins, Miguel Pereira, Içara, Porto Alegre and Curitiba were obtained from colonies kept in local meliponaries. These colonies, however, were also obtained from the respective regions and did not represent translocated colonies.

The geographic distribution and the number of colonies sampled in each locality are shown in Table 1. Samples were stored in absolute ethanol at -80 °C until DNA extraction.

DNA extraction and amplification

Genomic DNA from 127 individuals (one per colony) was extracted according to the protocol of Waldschmidt *et al.* (1997) and amplified using nine microsatellite primers specifically designed for *M. quadrifasciata* in our laboratory (Table 2).

To perform the amplification, the forward (F) sequence of each primer was modified at the 5' end with the addition of a sequence of the M13 universal primer (5'-TTTCCAGTCACGA-3') (Schuelke, 2000). Additionally, the M13 universal primer was labeled with a fluo-

Table 1 - Sampled localities, geographic coordinates and number of *Melipona quadrifasciata* colonies (N) analyzed.

Subspecies	Locality/State (code)	Coordinates	N
<i>M. q. quadrifasciata</i>	Porto Alegre/RS (PA)	30°01' S; 51°13' W	5
	Içara/SC (IÇ)	28°42' S; 49°17' W	5
	Curitiba/PR (CT)	25°25' S; 49°16' W	18
<i>M. q. anthidioides</i>	Miguel Pereira/RJ (MP)	22°27' S; 43°28' W	4
	Cristiano Otoni/MG (CO)	20°51' S; 43°45' W	8
	Piranga/MG (PI)	20°36' S; 43°11' W	9
	Domingos Martins/ES (DM)	20°23' S; 41°01' W	6
	Caeté/MG (CE)	19°48' S; 43°32' W	15
	Rio Vermelho/MG (RV)	18°18' S; 43°00' W	7
	Poté/MG (PO)	17°46' S; 41°54' W	3
	Caitité/BA (CA)	14°03' S; 42°29' W	7
<i>M. q. anthidioides</i> *	Urucua/MG (UR)	16°04' S; 45°47' W	15
	Januária/MG (JA)	15°25' S; 44°19' W	13
	São Cristovão/SE (SC)	11°01' S; 37°12' W	4
<i>M. quadrifasciata</i> with a hybrid pattern of tergal stripes	Ourolândia/BA (OU)	10°58' S; 41°04' W	8

**M. quadrifasciata anthidioides* with continuous tergal stripes.

Table 2 - Characteristics of the nine microsatellite loci of *Melipona quadrifasciata* used in the present study.

Locus	Repeat motif	Primer sequences (5' -3')	Ta (°C)	Allele size range (bp)	k
Mquad2	(TGC) ₄ (CGC) ₄	F: GTGCTCTTGCTCTTGCTC R: CCGCAGTTTTTGTTTCGCT	55	142-151	4
Mquad4	(GC) ₅	F: CGCTGACTTTAAAATCGTTC R: TCGCTTCTGTTTCGTTCTC	55	121-153	6
Mquad5	(ATT) ₆	F: TGAAATCCAGAAATCTTATG R: TCGTTCGGTTTGGAATG	55	124-130	5
Mquad6	(TAT) ₇	F: TCAGAGAACAGACCCAATC R: GGACCTAATACCTGCACT	55	112-148	8
Mquad7	(AT) ₂₁	F: CGCACACGCTAACGGAACG R: CAGGACGAGGCGTAACCG	65	131-185	15
Mquad13	(AATCGC) ₂	F: GGAGGATCCTCTCGACGAGG R: GGTTCCCGAAACGTGCACCC	65	182-248	8
Mquad19	(TC) ₈	F: GGGACGCACGATCTCGGACG R: GGACACGCCCGTGGGAAGAG	65	120-194	11
Mquad20	(GGACG) ₅	F: CGTGACGGGATAATCCTTGC R: TCACCGAGCCTGTTTCAGG	60	189-234	5
Mquad24	(GA) ₈	F: ACGCGCACGCGCACACATAC R: CGCGCGTCGCTGACTCATGC	67	144-146	3

F and R: forward and reverse primers, respectively; Ta: annealing temperature; k: number of alleles.

rescent dye (FAM-6-carboxy-fluorescein or HEX hexachloro-6-carboxy-fluorescein).

The amplification reaction solutions consisted of 10 ng DNA, 200 µM of each dNTP, 1.0 µM of each primer (F, R and M13), 1.5 mM MgCl₂, and 0.5 unit of *Platinum Taq* polymerase (Invitrogen) in 10x buffer. Amplifications were performed by an initial denaturation step of 94 °C for 3 min, followed by 10 cycles of 94 °C for 15 s, with a specific annealing temperature for each primer for 20 s and 72 °C for 30 s. Subsequently, 25 cycles at 89 °C for 15 s, 53 °C for 20 s, 72 °C for 30 s, and a final extension at 72 °C for 30 min were performed. The polymerase chain reaction (PCR) products were identified using a MegaBACE automated capillary sequencer. Allele sizes were scored against the size standard ET 550R (GE Healthcare Life Sciences, Pittsburgh, PA, USA), and the peaks were analyzed using the MegaBACE *Fragment Profiler* program.

Statistical analysis

The Popgene version 1.32 program (Yeah *et al.*, 1999) was used to calculate the following estimators of genetic diversity: allele frequency, expected (H_e) and observed (H_o) heterozygosity, genetic distance between populations (Nei, 1978), and F-statistics (Wright, 1978). For these statistical analyses, colonies were grouped according to locality, and each of these groups was considered to be a population.

Grouping analysis based on the UPGMA algorithm and the analysis of molecular variance (AMOVA; Excoffier *et al.*, 1992) were carried out using the Genes program (Cruz, 2012). The cophenetic correlation coefficient that measures to which extent the clustering result corresponds

to the original dissimilarity matrix was used to test the efficiency of the clustering analysis for the data (Rohlf and Fisher, 1968), and AMOVA was calculated considering two hierarchic levels and the groups formed in the clustering analysis.

Results

The nine microsatellite loci analyzed presented 65 alleles, ranging from three (Mquad24) to 15 (Mquad7) alleles (mean: 7.2 alleles/locus). All loci showed deviations from the Hardy-Weinberg equilibrium ($p < 0.05$) at one or more sites, but no single locus showed deviations in all cases. Table S1 shows the allele frequencies observed for these loci, considering all the analyzed populations.

The most frequent alleles of the different loci were present in both populations of *M. quadrifasciata quadrifasciata* and *M. quadrifasciata anthidioides*, as well as in the populations with continuous metasomal stripes from northern Minas Gerais and Sergipe and in the hybrid morphotype from Bahia. In general, however, the populations exhibited different allelic constitutions, with some fixed and other private alleles (Table S2).

In the different analyzed populations, the observed heterozygosity (H_o) ranged from 0.03-0.21, while the expected heterozygosity (H_e) ranged from 0.23-0.47.

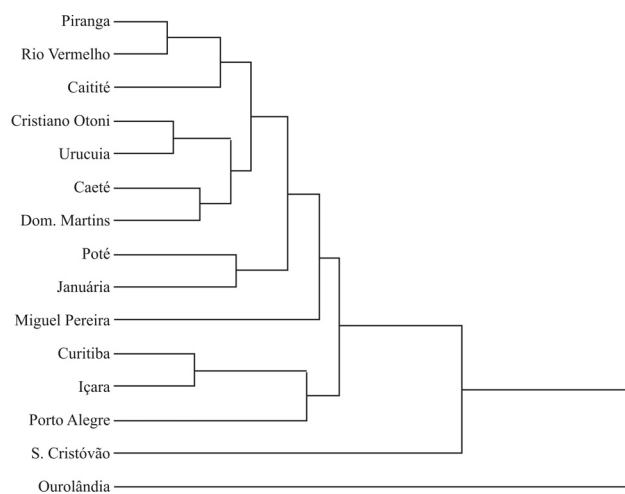
The genetic distances among populations ranged from 0.05 (Piranga and Rio Vermelho, Cristiano Otoni and Caeté, Cristiano Otoni and Urucuia) to 0.45 (Januária and São Cristóvão) (Table 3).

Clustering analysis demonstrated that populations of both subspecies, although clustering together in a single

Table 3 - Genetic distances among the fifteen *Melipona quadrifasciata* populations analyzed (codes are the same as in Table 1).

Pop	PA	IÇ	CT	MP	CO	PI	DM	CE	RV	PO	CA	UR	JA	SC	OU
PA															
IÇ	0.16														
CT	0.13	0.06													
MP	0.18	0.17	0.15												
CO	0.14	0.18	0.11	0.14											
PI	0.18	0.15	0.09	0.09	0.06										
DM	0.25	0.24	0.18	0.18	0.09	0.09									
CE	0.17	0.20	0.14	0.21	0.05	0.07	0.07								
RV	0.19	0.09	0.07	0.14	0.13	0.05	0.18	0.13							
PO	0.21	0.14	0.16	0.13	0.11	0.10	0.11	0.13	0.14						
CA	0.11	0.12	0.09	0.10	0.06	0.07	0.13	0.07	0.09	0.14					
UR	0.15	0.22	0.16	0.18	0.05	0.06	0.13	0.09	0.12	0.11	0.13				
JA	0.24	0.13	0.16	0.15	0.13	0.09	0.15	0.19	0.11	0.10	0.18	0.11			
SC	0.44	0.43	0.35	0.36	0.29	0.28	0.40	0.28	0.33	0.42	0.22	0.37	0.45		
OU	0.19	0.31	0.27	0.37	0.19	0.23	0.23	0.18	0.28	0.21	0.22	0.15	0.27	0.35	

major group, can be divided into two subgroups, one containing the populations of the subspecies *M. quadrifasciata quadrifasciata* and the other the populations of the subspecies *M. quadrifasciata anthidioides* and the two *M. quadrifasciata* populations with continuous metasomal stripes from northern Minas Gerais (Figure 1). Only populations from São Cristovão and Ourolândia, which showed higher genetic distance values than the other populations (Table 3), were isolated in this analysis. The cophenetic correlation of this clustering was high (0.87), further showing a good adjustment between the genetic distances and the graphically represented distances.

**Figure 1** - Dendrogram based on the UPGMA method, showing the Nei genetic distances (Nei, 1978) among the *Melipona quadrifasciata* populations analyzed. Cophenetic correlation = 0.87.

The F_{ST} multilocus value obtained when populations of both subspecies were considered together (F_{ST} : 0.23) also showed that they were structured, and the AMOVA test demonstrated that the interpopulation variation (52.5%) was higher than the intrapopulation variation (47.5%) of the subgroups obtained in the clustering analysis. Differences in the allele frequencies among populations as well as their private alleles probably contributed to these results.

Discussion

Our results showed a relatively high level of multi-allelism in the nine microsatellite loci analyzed, which is characteristic when species-specific microsatellite primers are used (Peters *et al.*, 1998; Paxton *et al.*, 2003; Brito *et al.*, 2009; Kapheim *et al.*, 2009; Lopes *et al.*, 2009a,b; Oliveira *et al.*, 2009; Francisco *et al.*, 2011).

This high number of alleles, however, was evidenced only when all populations were considered together. When each population was considered separately, only few alleles were found in each of the loci analyzed, always with one of them at a higher frequency. Consequently, a low frequency of heterozygotes was detected, which may explain the differences between the expected and observed heterozygosity values found. Additionally, despite having sampled 13 to 18 colonies in some localities, this sample size can be considered relatively small, which might also explain this difference, as well as the observed deviations from the Hardy-Weinberg equilibrium.

Notwithstanding, the lower level of genetic variability detected in the *M. quadrifasciata* populations (that gen-

erally mate once) compared to the values reported for *M. bicolor* (H_0 : 0.40, Peters *et al.*, 1998) and *Apis mellifera* (H_0 : 0.35–0.59, De La Rúa *et al.*, 2003), for example, may be due to biological differences between the species, such as the facultative polygyny of *M. bicolor* (Cepeda, 2006; Velthuis *et al.*, 2006), the multiple mating of honeybee queens (Estoup *et al.*, 1994) or the limited flight dispersion distance of stingless bees (Araújo *et al.*, 2004).

Although *M. quadrifasciata* presents a widespread geographic distribution, occupying distinct biomes such as the Atlantic rainforest, gallery forests (seasonal forests) and arborous caatinga, environment degradation may also be contributing to their reduced genetic variability. This hypothesis should be considered, as it was already observed in *M. rufiventris* and *M. mondury* populations (Tavares *et al.*, 2007) that environmental degradation may isolate populations in small forest fragments, leading to inbreeding and local extinction. An indication of this local extinction is provided by Batalha-Filho *et al.* (2009) who, in contrast to Kerr (1951), found no *M. quadrifasciata* specimens in Pernambuco and Paraíba, despite intensive searching.

It is worthy of note that many of the private alleles detected in the present study were found in samples from Urucuia/Januária (northern Minas Gerais). Similarly, allele A of locus Mquad24 was found only in *M. quadrifasciata* population from São Cristóvão (Sergipe) and in the hybrid population from Ouro-lândia (Bahia). As mentioned above, although the samples from Urucuia, Januária and São Cristóvão contain *M. quadrifasciata* populations with continuous metasomal stripes, they were located completely outside the distribution area of *M. quadrifasciata quadrifasciata*.

Therefore, the presence of these private alleles is evidence of restricted genetic flow between these and the other populations of *M. quadrifasciata anthidioides* and could explain the genetic differentiation detected for the populations of São Cristóvão and Ouro-lândia. Genetic differentiation among some *M. quadrifasciata* populations has also been detected using ISSR markers (Nascimento *et al.*, 2010), PCR-RFLP assays of mtDNA (Batalha-Filho *et al.*, 2009), and mtDNA COI gene sequencing (Batalha-Filho *et al.*, 2010). According to these authors, geographic barriers separating close neighbors of this species can explain this differentiation.

The clustering analyses showed that *M. quadrifasciata* populations from Curitiba, Içara and Porto Alegre, which correspond to the subspecies *M. quadrifasciata quadrifasciata*, form a separate subgroup from the *M. quadrifasciata* populations of Urucuia and Januária, which exhibit a continuous metasomal stripe pattern similar to this subspecies, but are genetically more similar to *M. quadrifasciata anthidioides* populations.

The two other morphologically atypical *M. quadrifasciata* populations (São Cristóvão and Ouro-lândia), however, were found to be isolated in the clustering

analyses performed in this study. These same populations were clustered with *M. quadrifasciata anthidioides* in the phylogeographic study of Batalha-Filho *et al.* (2010), although the presence of exclusive haplotypes was detected in populations with continuous metasomal stripes from Sergipe. This result may represent differences between the two molecular markers used (mitochondrial vs. nuclear). In the present case, as microsatellites represent nuclear markers with a high mutation rate, the presence of two or three exclusive alleles was sufficient to differentiate populations. Further studies are necessary to elucidate the taxonomic status of these populations.

Overall, the results obtained in the present study corroborate those of previous studies demonstrating genetic differences between the two *M. quadrifasciata* subspecies (Waldschmidt *et al.*, 2000; Moreto and Arias, 2005; Souza *et al.*, 2008; Batalha-Filho *et al.*, 2009, 2010). Additionally, as already observed by Batalha-Filho *et al.* (2009, 2010), our results also indicated that the *M. quadrifasciata* populations from northeastern Bahia and Sergipe are genetically different from the *M. quadrifasciata quadrifasciata* samples, even though they show the characteristic continuous metasomal stripes pattern.

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Supplementary Material

The following material concerning this article is available online:

Table S1 - Allele frequencies of the microsatellite loci.

Table S2 - Distribution of the alleles present in the nine loci analyzed.

This material is available as part of the online article at <http://www.scielo.br/gmb>.

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