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Transferability of microsatellite primers developed for stingless bees to four other species of the genus *Melipona*

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ABSTRACT. Microsatellite markers are a useful tool for ecological monitoring of natural and managed populations. A technical limitation is the necessity for investment in the development of primers. Heterologous primers can provide an alternative to searching for new loci. In bees, these markers have been used in populational and intracolonial genetic analyses. The genus *Melipona* has the largest number of species among bee genera, about 70, occurring throughout the Neotropical region. However, only five species of the genus *Melipona* have specific microsatellite markers. Given the great diversity of this genus, this number is not representative. We analyzed the transferability of 49 microsatellite loci to four other species of the genus *Melipona* (*M. scutellaris*, *M. mondury*, *M. mandacaia*, and *M. quadrifasciata*). Four individuals of each species, from different localities, were used in amplification tests. Primer pairs described for five *Melipona* species and for *Trigona carbonaria* were tested. Among the 49 loci,

22 gave amplification products for all four species, while three gave nonspecific bands and five showed no amplification products. The remaining loci varied in the pattern of amplification, according to the species examined. The number of alleles ranged from 1 to 6. The results demonstrate the possibility of using these heterologous markers in other *Melipona* species, increasing the number of loci that can be analyzed and contributing to further genetic analyses of intra- and intercolonial structure, which is required for conservation measure planning, genetic improvement and resolution of taxonomic problems.

Key words: Microsatellite; Transferability; Bees; *Melipona*; Conservation

INTRODUCTION

Microsatellites are codominant molecular markers based on simple repeated and frequent sequences common in eukaryotic genomes. Each microsatellite *locus* is highly variable, multiallelic and has high information content (Ferreira and Grattapaglia, 1998). These markers are a useful tool for ecological monitoring of natural populations, for developing stock populations and for genetic improvement (Bech et al., 2010). The limitation of the technique is the investment required for the development of primers. The use of primers developed for related species, i.e., transferability, can provide an alternative for the development of new markers (Patel et al., 2010). Various studies have demonstrated the possibility of using heterologous markers in plants (Gidugli et al., 2010, Chu et al., 2010), vertebrates (Bench et al., 2010; Mantellato et al., 2010) and invertebrates (Daly et al., 2002). In bees, these markers have been used in analyses of populations (Carvalho-Zilse et al., 2009) and for examining intracolonial genetic structure (Alves et al., 2009, 2010).

Bees are insects of the order Hymenoptera, whose major ecological role is pollination, which is necessary for the reproduction of plants and/or the reduction of inbreeding depression; in addition, their presence is associated with increased production and conservation of habitats (Michener, 2007). The *Melipona* genus has the largest number of species, about 70 (Moure et al., 2007), and can be found throughout the Neotropical region, from Mexico to Misiones, Argentina, with highest diversity in the Amazon basin (Camargo and Pedro, 1992, Silveira et al., 2002). Until now, microsatellite *primers* have only been described for five species of this genus: *Melipona bicolor* (Peters et al., 1998), *Melipona rufiventris* (Lopes et al., 2009), *Melipona seminigra merrillae* (Francini et al., 2009a), *Melipona interrupta manaosensis* (Francini et al., 2009b) and *Melipona mondury* (Lopes et al., 2010).

In order to increase the number of *Melipona* species to be studied using this methodology, we analyzed the transferability of 49 microsatellite loci to four other species of the genus *Melipona*.

MATERIAL AND METHODS

The species that we tested were *Melipona* (*Michmelia*) *scutellaris*, *M.* (*Michmelia*) *mondury*, *M.* (*Melipona*) *mandacaia*, and *M.* (*Melipona*) *quadrifasciata*. Four individuals of each species, from different locations, were used for amplification tests. Genomic DNA was isolated according to the protocol described by Waldschmidt et al. (1997).

A total of 49 pairs of primers described for the species *Melipona* (*Eomelipona*) *bicolor* (Peters et al., 1998), *Melipona* (*Michmelia*) *rufiventris* (Lopes et al., 2009), *Melipona* (*Michmelia*) *mondury* (Lopes et al., 2010), *Melipona* (*Melikerria*) *interrupta manaosensis* (Franciniet al., 2009b), *Melipona* (*Michmelia*) *seminigra merrillae* (Francini et al., 2009a) and *Trigona carbonaria* (Green et al., 2001) were tested (Table 1).

The standardized 10µL reaction mixture consisted of 0.1 mM of each deoxyribonucleoside triphosphate (dATP, dNTP, dGTP, dTTP), 0.4 mM of each primer, 1.5 mM $MgCl_2$, 0.5 U Taq polymerase in 1X buffer and 20 ng total genomic DNA. The mixture was incubated in an ATC201 model thermal cycler (NyxTechnik). The amplification program included an initial step at 94°C, for 3 min, 40 cycles with a first step at 92°C (denaturation), for 30 s, a second step with annealing temperature specific to each primer, for 1 min, and a third step at 72°C (extension), for 30 s; there was a final extension at 72°C, for 5 min.

The amplification products were subjected to electrophoresis on 8% nondenaturing polyacrylamide gel and visualized by staining with 0.2% silver nitrate. Results of amplifications were classified as amplified, when expected size bands were detected, and as not amplified, when there was no amplified PCR product, or unspecific, when there were bands of unexpected size (Gao et al., 2005).

RESULTS

Mbi28, Mbi32, Mbi33, Mbi215, Mbi218, Mbi232, Mbi233, Mbi254, Mbi259, Mbi305, Mbi522, Mru14, Mmo15, Mim05, Msm03, Msm04, Msm05, Tc1-20, Tc3-155, Tc3-302, Tc4-214, and Tc4-287 loci were amplified in all of the species (45% of all loci). The Mim11 *locus* was amplified along with nonspecific bands, while Mbi221, Mim01, Msm12, Msm13 and Tc3-349 loci gave no amplification products (10% of total). The other loci varied in the pattern of amplification, according to the species analyzed. The number of alleles ranged from 1 to 6.

The success and polymorphism rates in amplifications for each species were: *Melipona mondury,* 59 and 24%; *Melipona mandacaia*, 65 and 37%; *Melipona quadrifasciata*, 69 and 37%.

Table 1 shows the results, including the number of alleles and original and standardized annealing temperatures.

DISCUSSION

Due to the low number of specific primers developed for stingless bees so far, heterologous primers have been used in population studies, and in studies of mating and genetic structure in stingless bees. Carvalho-Zilse and Kerr (2006) tested 10 heterologous microsatellite primers, designed for *Melipona bicolor* and *Apis mellifera*, for population studies in *Melipona scutellaris*. Only the primers designed for *M. bicolor* were successful. With the purpose of studying the genetic variation of *M. scutellaris* colonies that were founded by only two colonies and multiplied for a period of 10 years in an apiary outside the natural area of occurrence of the species, Alves et al. (2010) used three microsatellite markers designed for *Melipona bicolor* and *Scaptotrigona postica*. In the state of Minas Gerais, Tavares et al. (2007) distinguished populations of *Melipona rufiventris*: *Melipona mondury*, which occurs in the Atlantic Forest, and *M. rufiventris*, which occurs in the Cerrado, through the use of microsatellite markers designed for *Melipona bicolor*, isozymes and RAPDs; they suggested

Table 1. Amplification results (number of bands in bold) and PCR parameters in the testing of 49 heterologous microsatellite loci on four *Melipona* species.

Loci	Ta (°C)	*M. scutellaris* (A)	*M. mondury* (A)	*M. mandacaia* (A)	*M. quadrifasciata* (A)
Mbi11	55	1	n.a.	2	2
Mbi28	57.5	2	3	1	1
Mbi32	57.5	1 [60°C*]	1	2	3
Mbi33	60	1 [57.5°C]	1 [57.5°C]	1 [57.5°C]	1
Mbi 88	57.5	1	n.a.	2	3
Mbi103	50	n.a.	n.a.	1	1
Mbi215	57.5	2 [60°C]	1	2 [62.5°C]	2
Mbi218	60	2	4 [63°C**]	3	2
Mbi219	57.5	n.a. [60°C]	n.a. [60°C]	2 [60°C]	1
Mbi221	60	n.a.	n.a.	n.a.	n.a.
Mbi232	50	5 [53°C]	2	2	4
Mbi233	57.5	5 [53°C]	3	5	4
Mbi254	55	4 [53°C]	6 [64°C**]	3	3
Mbi256	57.5	5 [53°C]	in.	2	2
Mbi259	57.5	1 [53°C]	1	1	1
Mbi278	60	1	2	1 [65ºC***]	n.a.
Mbi305	60	1	1	2	1
Mbi522	60	1	1	2	1
MRU 03	63	2 [57°C]	1 [53°C]	in. [53°C]	3
MRU 14	62.5	2 [58°C]	2 [60°C]	3 [60°C]	3
MMO 08	59	1 [58°C]	-	n.a.	n.a.
MMO 15	54	2	-	1	1
MMO 21	57	1 [56°C]	-	in. [56°C]	in.
MMO 22	61,5	4 [59°C]	-	in. [59°C]	in.
MMO 24	52	1	-	n.a.	n.a.
MIM 01	t.d. [62-52]	n.a. [54°C]	n.a. [54°C]	n.a. [54°C]	n.a. [54°C]
MIM 05	t.d. [62-52]	1 [54°C]	1 [54°C]	2 [54°C]	2 [54°C]
MIM 07	t.d. [62-52]	n.a. [54°C]	n.a. [54°C]	n.a. [54°C]	in. [54°C]
MIM 08	t.d. [62-52]	2 [54°C]	1 [54°C]	n.a. [54°C]	n.a. [54°C]
MIM 09	t.d. [62-52]	n.a. [54°C]	n.a. [54°C]	n.a. [54°C]	1 [54°C]
MIM 11	t.d. [62-52]	in. [54°C]	in. [54°C]	in. [54°C]	in. [54°C]
MIM 12	t.d. [62-52]	in. [55°C]	in. [55°C]	in. [55°C]	4 [55°C]
MSM02	t.d. [62-52]	n.a. [60°C]	n.a. [60°C]	n.a. [60°C]	n.a. [60°C]
MSM03	t.d. [62-52]	1 [60°C]	1 [60°C]	1 [60°C]	1 [60°C]
MSM04	t.d. [62-52]	2 [60°C]	2 [60°C]	2 [60°C]	1 [60°C]
MSM05	t.d. [62-52]	1 [60°C]	1 [60°C]	1 [60°C]	1 [60°C]
MSM07	t.d. [62-52]	2 [60°C]	n.a. [60°C]	1 [60°C]	n.a. [60°C]
MSM08	t.d. [62-52]	in. [60°C]	in. [60°C]	in. [60°C]	2 [62°C]
MSM 09	t.d. [62-52]	1 [55°C]	1 [55°C]	4 [55°C]	in. [55°C]
MSM12	t.d. [62-52]	n.a. [59°C]	n.a. [59°C]	n.a. [59°C]	n.a. [59°C]
MSM13	t.d. [62-52]	n.a. [59°C]	n.a. [59°C]	n.a. [59°C]	n.a. [59°C]
Tc1-20	55	1	1	1	2
Tc3-155	58	1	1	1	2
Tc3-302	56	2	2	2	3
Tc4-63	58	1	n.a.	1	1
Tc4-214	55	1	2	1	1
Tc4-287	58	2	1	2	4
Tc4-349	55	n.a.	n.a.	n.a.	n.a.
Tc7-13	58	n.a.	n.a.	1	n.a.

Ta - original annealing temperature, (A) - number of alleles; td - Touchdown; n.a. - not amplified; in. - unspecific bands; [] tested temperatures; *Carvalho-Zilse and Kerr 2006; **Lopes, 2004; ***Werneck, 2008.

that the two populations from the Cerrado - Brasilândia de Minas and Dom Bosco - belong to a new species. For analysis of multiple mating and kinship among worker bees, Takahashi and Nakamura (2003) used primers designed for *Apis mellifera* to amplify four microsatellite loci from two colonies of *Apis laboriosa*. Palmer et al. (2002) used microsatellite markers designed for *A. mellifera*, *Scaptotrigonapostica*, *Melipona bicolor*, and *Trigona carbonaria*

in seven species of the genera *Scaptotrigona, Trigona* and *Austrophlebeia* from Mexico and Australia for evaluation of multiple mating and multiple maternity of male Australian bees. Alves et al. (2009) assessed the maternity of males produced in colonies of *M. scutellaris* by means of three microsatellite *primers* designed for *M. bicolor* and *S. postica*.

As demonstrated by Carvalho-Zilse and Kerr (2006) with markers for *A. mellifera,* in *M. scutellaris*, and Lopes et al. (2009), with markers for *M. rufiventris*, in *Partamona helleri*, the success in the use of heterologous *primers* is lower in phylogenetically more distant species. In this study, results obtained regarding the four species of the genus *Melipona* (*Melipona* and *Michmelia*) using markers developed for other species of the same genus (subgenera *Eomelipona, Michmelia* and *Melikerria*) and for *T. carbonaria* show that, at least for species within the same tribe (Meliponini), evolutionary distance does not always prevent transfer of microsatellite markers (Table 2).

Table 2. Amplification success rate of loci for the bee species.

Loci	*AR*	*M.* (*Michmelia*) *scutellaris*	*M.* (*Michmelia*) *mondury*	*M.* (*Melipona*) *mandacaia*	*M.* (*Melipona*) *uadrifasciata*
M. (*Eomelipona*) *bicolor*	83.3	83.3	66.6	94.4	88.8
M.(*Michmelia*) *rufiventris*	87.5	100	100	50	100
M. (*Michmelia*) *mondury*	46.6	100	-	20	20
M. (*Melikerria*) *interrupta manaoensis*	28.5	28.5	28.5	14.28	42.85
M. (*Michmelia*) *seminigra merrilae*	50	55.5	44.4	55.5	44.4
Trigona carbonaria	75	75.5	62.5	87.5	75.5

AR = amplification rates (in %) of primer pairs designed for each species.

According to Lopes et al. (2010), though heterologous markers can be used, the result must be carefully analyzed due to the presence of null alleles arising from mutations in the annealing sites. Only five species of the genus *Melipona* have specific microsatellite markers. This is not a representative number, considering that around 70 species have been described for this genus (Moure et al., 2007). In order to enable conservation studies, we need more information on the population genetics structure of these species.

Due to the large number of bee species and the high cost required to develop microsatellite markers, heterologous primers are a useful alternative. We were able to use heterologous markers in these *Melipona* species, increasing the number of loci that can be analyzed, allowing analysis of genetic diversity, intra and inter-colonial structure for conservation programs, as well as genetic improvement and problem solving in the classification of species.

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