markers in *Eucalyptus grandis* and *E. urophylla*. *Theoretical and Applied Genetics*, **97**, 816–827.
Chakravarat I, Li CC (1983) The effect of linkage on paternity calculations. In: *Inclusion Probabilities in Parentage Testing* (ed. Walkera RH), pp. 411–420. American Association of Blood Banks, Arlington, Virginia.
Collevatti RG, Grattapaglia D, Hay JD (2001) Population genetic structure of the endangered tropical tree species *Caryocar brasiliense*, based on variability at microsatellite loci. *Molecular Ecology*, **10**, 349–356.
Creste S, Tulmann-Neto A, Figueira A (2001) Detection of single sequence repeat polymorphisms in denaturing polyacrylamide sequencing gels by silver staining. *Plant Molecular Biology Report*, **19**, 299–306.
Doyle JJ, Doyle JL (1987) Isolation of plant DNA from fresh tissue. *Focus*, **12**, 13–15.
Goudet J (2002) *FSTAT (Version 2.9.3.2): A Program to Estimate and Test Gene Diversities and Fixation Indices*. Available from http://www.unil.ch/izea/softwares/fstat.html.
Kanashiro M, Harris SA, Simons A (1997) RAPD diversity in Brazil nut (*Bertholletia excelsa* Hunb. & Bonpl.: Lecythidaceae). *Silvae Genetica*, **46**, 219–223.
Lemes MR, Grattapaglia D, Grogan J, Proctor J, Gribel R (2007) Flexible mating system in a logged population of *Swietenia macrophylla* King (Meliaceae): implications for the management of a threatened Neotropical tree species. *Plant Ecology*, **192**, 169–179.
Mori SA, Prance GT (1990) Taxonomy, ecology, and economic botany of the Brazil nut (*Bertholletia excelsa* Humb. & Bunpl.: Lecythidaceae). *Advances in Economic Botany*, **8**, 130 –150.
Nei M (1978) Estimation of average heterozygosity and genetic distance from a small number of individual. *Genetics*, **89**, 583–590.
O'Malley DM, Buckley DP, Prance GT, Bawa KS (1988) Genetics of Brazil nut (*Bertholletia excelsa* Humb. & Bonpl.: Lecythidaceae). II. Mating system. *Theoretical Applied Genetics*, **76**, 929–932.
Peres CA, Baider C, Zuidema PA *et al*. (2003) Demographic threats to the sustainability of Brazil nut exploitation. *Science*, **302**, 2112–2114.
Rafalski JA, Morgante M, Powell W, Vogel JM, Tingey SV (1996) Generating and using DNA markers in plants. In: *Analysis of Non-mammalian Genomes: a Practical Guide* (eds Birren B, Lai E), pp. 75–134. Academic Press, New York.
Weir BS (1996) *Genetic Data Analysis II*. Sinauer Associates, Sunderland, Massachusetts.
Weir BS, Cockerham CC (1984) Estimating *F*-statistics for the analysis of population structure. *Evolution*, **38**, 1358–1370.

doi: 10.1111/j.1755-0998.2008.02481.x

Microsatellite loci for the stingless bee *Melipona rufiventris* (Hymenoptera: Apidae)

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Abstract

Eight microsatellite primers were developed from ISSR (intersimple sequence repeats) markers for the stingless bee *Melipona rufiventris*. These primers were tested in 20 *M. rufiventris* workers, representing a single population from Minas Gerais state. The number of alleles per locus ranged from 2 to 5 (mean = 2.63) and the observed and expected heterozygosity values ranged from 0.00 to 0.44 (mean = 0.20) and from 0.05 to 0.68 (mean = 0.31), respectively. Several loci were also polymorphic in *M. quadrifasciata*, *M. bicolor*, *M. mandacaia* and *Partamona helleri* and should prove useful in population studies of other stingless bees.

Keywords: ISSR, *Melipona*, microsatellite, population genetics, stingless bees

Received 13 May 2008; revision accepted 15 October 2008

Melipona rufiventris is part of a group of bees native to Brazil, known as stingless bees, which are important pollinators in native forests and for several cultivated plants. Stingless bee species construct their nests inside holes in the trunks of trees and have low dispersal abilities. The joint effects of beekeepers, fire and the destruction of natural habitat have drastically reduced many populations of this species, and a number are threatened with extinction.

Generally, *M. rufiventris* queens mate with a single male during the nuptial flight and the genetic variability among its offspring is therefore low. There is thus a growing interest

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Table 1 Characterization of the microsatellite loci developed for *Melipona rufiventris*. GenBank Accession numbers are given in brackets below locus names

Locus	Repeat	Size (bp)	T_a (°C)	Primer sequence (5′–3′)	*A*	H_O	H_E
Mru03 (EU678247)	$(AG)_6(CGTT)_6$	120	63	F: AGAGAGAGAGAGAGACGC R: GGTGAAAGGAGAACGAAC	5	0.44	0.68
Mru04 (EU678248)	$(AG)_4GG(AG)_2G(GA)_5$	115	59	F: TTATCCTTGGCTCGACTC R: GATGTTCTCCACCTTCGT	2	0.35	0.30
Mru05 (EU678249)	$(TA)_8$	103	52	F: CAGAGATGGAAAGAACGG R: AAGAGATTTGCTGGAACG	2	0.05	0.05
Mru06 (EU678250)	$(AC)_5$	109	57	F: GGCGAAATTAGCGATACT R: TAACAGCGTAGAGAAAGG	2	0.05	0.05
Mru08 (EU678251)	$(CGAG)_6$	143	62	F: ATAAATCACGCACACTTG R: TCGAAAATTGCACTCTCG	3	0.22	0.21
Mru09 (EU678253)	$(GA)_7AA(GA)_3$	139	64	F: GTGATACCTCCGTGGAAC R: CTTCGCCAGAGACATCGC	3	0.30	0.60
Mru12 (EU678252)	$(GA)_8GG(GA)_4$	121	57.5	F: GATAGATAGACAGACAGG R: GTGTAATCCGTTTAAGGG	2	0.20	0.43
Mru14 (EU678254)	$(TCG)_8TT(GTC)_4$	136	62.5	F: GCTGTTTCCCTGTTCGAG R: CCCCTATCTTTTATGCCG	2	0.00	0.18

F and R: forward and reverse primers, respectively; T_a: annealing temperature; *A*: number of alleles; H_O: observed heterozygosity; H_E: expected heterozygosity.

in developing genetic markers that will allow the quantification of genetic diversity in these bees and hence assist with conservation efforts.

To date, however, microsatellite primers have been developed for only three stingless bee species: *M. bicolor* (Peters *et al*. 1998), *Scaptotrigona postica* (Paxton *et al*. 1999) and *Trigona carbonaria* (Green *et al*. 2001). The objective of this study was to identify and characterize microsatellite primers for *M. rufiventris* from intersimple sequence repeats (ISSR). This technique is based on the amplification of sequences by a single primer that is anchored in the microsatellite region and has been applied in population studies of various organisms (Kanchanaprayudh *et al*. 2002; Francisco-Candeira *et al*. 2007; Provan & Wilson 2007).

The amplifications were carried out in a PTC-100 thermocycler (MJ Research) using the DNA extracted from an *M. rufiventris* individual and the following ISSR primers: $(CA)_8G$, $(GA)_8YG$, $(TG)_8RC$, $(GATA)_2(GACA)_2$ and $DVD(TC)_7$. The amplification programme consisted of an initial denaturation step at 94 °C (3 min), followed by 40 cycles at 92 °C (1 min), 55 °C (2 min) and 72 °C (2 min) with a final extension of 72 °C (7 min). The polymerase chain reaction (PCR) amplifications were carried out in volumes of 25 μL consisting of 0.2 μM primer, 8 μM dNTP, 0.5 U *Taq* DNA polymerase (Phoneutria), 1× PCR buffer and 20 ng genomic DNA. The ISSR fragments were purified and linked in the pGEM-T Easy (Promega) vector following the manufacturer's instructions. After transformation in *Escherichia coli* DH5α, inserts from 32 positive clones were sequenced.

Results showed that the sequenced fragments presented microsatellites corresponding to the ISSR primers at either the 5′ or the 3′ end. Furthermore, 12 clones presented internal sequences corresponding to one or more microsatellites. The analysis of these sequences permitted the design of eight independent primer pairs. These primers were tested in 20 *M. rufiventris* workers, representing a single population from Minas Gerais state. Cross-species amplification was tested in *M. quadrifasciata*, *M. bicolor*, *M. mandacaia* and *Partamona helleri*. The PCR amplifications were carried out in 10 μL reactions containing 12.5 ng genomic DNA, 1× Promega *Taq* PCR buffer (10 mM Tris-HCl, pH 8.3; 50 mM KCl), 0.5 or 0.25 μM of each forward and reverse primer, 0.1 mM dNTP, 1.5 or 1.0 mM $MgCl_2$, and 1 U *Taq* DNA polymerase (Promega). The conditions for the PCR were: 94 °C (3 min), followed by 40 cycles at 92 °C (30 s), specific annealing temperature for each primer (Table 1) (1 min) and 72 °C (30 s) with a final extension step at 72 °C (5 min). The products were separated in 8% nondenatured polyacrylamide gel and the alleles were scored using a molecular size marker (10 bp). Values for the observed and expected heterozygosities, tests for the Hardy–Weinberg (HW) equilibrium and linkage disequilibria were calculated using the PopGene software (Yeh *et al*. 1999). The eight loci tested were polymorphic (Table 1). The number of alleles ranged from 2 to 5 (mean = 2.63) and the observed and expected heterozygosity values ranged from 0.00 to 0.44 (mean = 0.20) and 0.05 to 0.68 (mean = 0.31), respectively. No significant linkage disequilibrium was detected. Significant heterozygote deficiencies and deviation from HWE ($P < 0.05$) were detected at loci Mru03, Mru09, Mru12 and Mru14, even after sequential Bonferroni correction (Rice 1989).

Several of these loci were also polymorphic in *M. quadrifasciata*, *M. bicolor*, *M. mandacaia* and *P. helleri* (Table 2),

Table 2 Size range and number of microsatellite alleles observed in four stingless bee species, using microsatellite primers designed for *Melipona rufiventris*

	Species			
Locus	*M. quadrifasciata* (*N* = 8)	*M. bicolor* (*N* = 8)	*M. mandacaia* (*N* = 8)	*P. helleri* (*N* = 8)
Mru03	112–128 (5)	100–110 (2)	110–122 (4)	100 (1)
Mru04	—	—	—	—
Mru05	100–104 (3)	100–112 (3)	98–104 (2)	—
Mru06	110 (1)	106–108 (2)	110 (1)	90–106 (3)
Mru08	125 (1)	130–134 (2)	—	—
Mru09	130–150 (4)	120–136 (3)	120–140 (3)	132 (1)
Mru12	108 (1)	100–120 (5)	110 (1)	—
Mru14	122–136 (3)	122–136 (2)	120–132 (3)	120–130 (3)

N, number of individuals analysed.

and should prove useful in population studies of other stingless bees.

Acknowledgements

The authors are grateful to the Brazilian agencies FAPEMIG (EDT 245/05); CNPq and UFV for financial support and to CAPES for supporting D. M. Lopes.

References

Francisco-Candeira M, González-Tizón A, Varela MA, Martínez-Lage A (2007) Development of microsatellite markers in the razor clam *Solen marginatus* (Bivalvia: Solenidae). *Journal of the Marine Biological Association of the United Kingdom*, **87**, 977–978.

Green CL, Franck P, Oldroyd P (2001) Characterization of microsatellite loci for *Trigona carbonaria*, a stingless bee endemic to Australia. *Molecular Ecology*, **1**, 89–92.

Kanchanaprayudh J, Lian C, Zhou Z, Hogetsu T, Sihanonth P (2002) Polymorphic microsatellite markers of a *Pisolithus* sp. from a *Eucalyptus* plantation. *Molecular Ecology Notes*, **2**, 263–264.

Paxton RJ, Weißschuh N, Quezada-Euăn JJG (1999) Characterization of dinucleotide microsatellite for stingless bees. *Molecular Ecology*, **8**, 685–702.

Peters JM, Queller DC, Imperatriz-Fonseca VL, Strassmann JE (1998) Microsatellite loci for stingless bees. *Molecular Ecology*, **7**, 783–792.

Provan J, Wilson PJ (2007) Development of microsatellites for the peat moss S*phagnum capillifolium* using ISSR cloning. *Molecular Ecology Notes*, **7**, 254–256.

Rice WR (1989) Analyzing tables of statistical tests. *Evolution*, **43**, 223–225.

Yeh FC, Yang R, Boyle T (1999) *PopGene Version 1.31: Microsoft Windows-based Freeware for Population Genetic Analysis. Quick User Guide*. University of Alberta and Centre for International Forestry Research, Canada.

doi: 10.1111/j.1755-0998.2008.02486.x

Isolation and characterization of microsatellite loci for the Asian longhorned beetle, *Anoplophora glabripennis*

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Abstract

We describe the first isolation of SSR (simple sequence repeat) markers for *Anoplophora glabripennis*, a pest of forests in China and urban trees in the USA. Ten markers were developed using FIASCO (fast isolation by AFLP of sequences containing repeats) with an $(AC)_{17}$ probe, and five markers were identified in expressed sequence tags found on the