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Isolation and characterization of ten new microsatellite markers in *Machaerium villosum* Vogel (Fabaceae)[1]

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ABSTRACT - (Isolation and characterization of ten new microsatellite markers in *Machaerium villosum* Vogel (Fabaceae)). *Machaerium villosum* is an important tree species from Southeastern Brazil. We report hereby 10 new microsatellite markers to investigate the structure and genetic diversity of this species. Ninety-seven alleles were detected in 60 specimens from a natural population in Minas Gerais State. High genetic diversity has been found. The mean observed and expected heterozygosities were 0.771 and 0.802, respectively. One locus showed significant Hardy-Weinberg departure and five loci combinations showed significant linkage disequilibrium. These 10 new microsatellite loci will be used to evaluate the genetic diversity of this species in order to understand the fragmentation effects of the Brazilian Atlantic Rain Forest.
Keywords: Atlantic forest, conservation genetics, genetic structure, *Machaerium villosum*, SSR markers

RESUMO - (Isolamento e caracterização de dez marcadores microssatélites inéditos em *Machaerium villosum* Vogel (Fabaceae)). *Machaerium villosum* é uma importante espécie do Sudeste do Brasil. Com a finalidade de investigar a estrutura e a diversidade genética desta espécie, foram desenvolvidos 10 marcadores microssatélites inéditos. Identificaram-se 97 alelos em 60 indivíduos de uma população localizada no Estado de Minas Gerais. Elevados índices de diversidade genética foram detectados. As heterozigosidades médias observadas e esperadas foram 0,771 e 0,802, respectivamente. Apenas um loco demonstrou desvio do equilíbrio de Hardy-Weinberg, enquanto cinco combinações de locos mostraram-se em desequilíbrio de ligação. Estes novos marcadores serão úteis para avaliar a diversidade genética de *Machaerium villosum* e contribuir para o entendimento do efeito da fragmentação da Mata Atlântica.
Palavras-chave conservação genética, estrutura genética, Mata Atlântica, microssatélites, *Machaerium villosum*

Introduction

Machaerium villosum Vogel is a woody species typically found in altitude forests of Southeastern Brazil, in which mature trees can reach height of up to 30 m (Hoehne 1941, Polido & Sartori 2007). The colorful wood is used in the manufacture of luxury furniture, parquets, frames and crossties (Andrade & Vecchi 1916, Nogueira 2010). The species is placed on the IUCN Red List of Threatened Species due to the severe deforestation in its habitat (IUCN 2013). Microsatellite markers are one of the most popular choices for population genetic studies because they provide relevant information for identifying conservation units and investigating the genetic processes which occur in populations (Oliveira *et al*. 2006, Selkoe & Toonen 2006). This study was aimed at developing and characterizing microsatellite markers from *Machaerium villosum* for future studies of population structure and genetic diversity in the remaining populations of this species.

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Material and methods

Microsatellite enriched DNA libraries were constructed following the method of Glenn & Schable (2005) with $(GA)_{10}$, $(TC)_{10}$ and $(CA)_{10}$ probes. Total genomic DNA was extracted from fresh leaf tissue by using CTAB method and digested with *Hae III* restriction enzyme. The fragments were selected by hybridization with biotinylated oligonucleotides and recovered by streptavidin coated magnetic beads. Microsatellite-rich fragments were amplified by Polymerase Chain Reaction (PCR) with SuperSNX24 primer, cloned into TOPO TA® vector (Invitrogen) and transformed into One Shot® TOP 10 Competent Cells (Invitrogen). Enriched fragments were sequenced and primers complementary to flanking regions of simple sequence repeats (SSR) were designed with PRIMER3 (Rozen & Skaletsky 2000). We obtained 26 microsatellites out of 116 clones, however, only 10 were standardized by using the modified tailed primer method (Schuelke 2000). Tail consists in a sequence (GCCAACACTCCTCAAATC) from *Planctomyces limnophilus* DSM 3776 genome. PCR reaction mixture contained 1U *Taq*, 0.2 mM of each dNTP, 0.1334 uM of each primer, 2.5-3.0 mM of $MgCl_2$, PCR buffer [KCl or $(NH_4)_2SO_4$] and DMSO in 15 uL reactions with 15-30 ng of DNA template. Cycling conditions were: 95 ºC, 2min30s, 17x [95 ºC, 30s; T_a (between 51 and 55 ºC), 1min; 72 ºC, 45s], 72 ºC, 10min. The PCR was interrupted to add a solution of 1.5 uL with 0.47 nM of each dNTP, 2 µM fluorescent dye-labeled forward primer (with 6-FAM, NED, VIC or PET) (Arruda *et al*. 2010), 1.33 µM reverse primer and 0.25 U *Taq* DNA Polymerase and resumed under the following conditions: 36x [94 ºC, 1min; 52 ºC, 45s; 72 ºC, 30s], 72 ºC, 10min. The final labeled product was analyzed on a laser detection system in ABI 3130*xl* Genetic Analyzer (Applied Biosystems).

Genetic analyses were carried out on individuals from a natural population located at the Parque Estadual de Nova Baden (21º56'S and 45º18'W), in Lambari region, in Minas Gerais State (N = 60). Null alleles, allele dropout and scoring errors due to stutter were tested with Micro-Checker version 2.2.3 (Van Oosterhout *et al*. 2004) and genotypes were adjusted using Null Allele Estimator. We used GDA version 1.1 (Lewis & Zaykin 2002) in order to estimate the number of alleles per locus ($\hat{A}$), expected ($\hat{H}_e$) and the observed heterozygosity ($\hat{H}_0$). It was also used to calculate the linkage disequilibrium (LD) by chi-square test. Deviations from Hardy-Weinberg equilibrium (HWE) were calculated with pooled

Table 1. Characterization of ten polymorphic microsatellite loci in *Machaerium villosum* Vogel. T_a: annealing temperature.

Locus	Primer sequence (5'-3')	Repeat motif	Allele size range (bp)	T_a (ºC)	GenBank Accession number
MV3	F: CATCCCAATCACCTCATCAAAC R: AGCAAGCTAGTTCACCGCAT	$(GA)_{19}.(GT)_3$	224-246	53	KF147171
MV8	F: CAAGGTACGAGATCCAAGGT R: ATGTGGTTGGTCGCAGTTC	$(GA)_{16}$	130-154	53	KF147172
MV11	F: AACACTGCATCAGCAACAAC R: ATGGACTTCACCACCTCCTG	$(TCT)_4.(GA)_8$	123-168	51	KF147173
MV17	F: ATTACACAGCGGCGTTCA R: TTGTAGCGAGTTTGTTCTGT	$(GT)_9$	144-221	51	KF147174
MV18	F: GCATTGATAAACTGGGTTTTGTG R: AAGTGTTTTGAGTGTGAAGGAAG	$(GT)_{10}$	79-118	51	KF147175
MV24	F: GGGAACTGAGAGAAACGAAACT R: CCCTAACTCCAAACACCAAACT	$(GA)_7.(GT)_3$	275-300	54	KF147176
MV26	F: CTTGGGGCGATGGGTTGT R: TGATGAACGGGCAAGATGAAG	$(GT)_4$	379-404	54	KF147177
MV27	F: CTGTGGGAAAGTGGGAACAA R:ACGAATAAGAAGCAGATGAACTAAC	$(GA)_{11}$	333-368	51	KF147178
MV30	F: TTCTTCCTCATCCCACAACA R: CGCTATGGAATCTCTTGCTC	$(GT)_8(AG)_9$	152-172	55	KF147179
MV31	F: TGAAGTAGCACGAAGCCAAC R: TTTCACCACAAGAGAACGAG	$(GT)_4$	261-288	53	KF147180

Table 2. Genetic diversity estimates in ten polymorphic microsatellite loci from a natural population of *Machaerium villosum* (N = 60). Â: number of alleles per locus; $\hat{\hat{H}}_e$ and $\hat{\hat{H}}_0$: expected and observed heterozygosity, respectively; P_{value}: Hardy-Weinberg Equilibrium probability (bold number indicates significant HWE departure).

Locus	Â	$\hat{\hat{H}}_e$	$\hat{\hat{H}}_0$	P_{value}
MV3	10	0,873	0,767	0,7153
MV8	9	0,853	0,783	0,5436
MV11	10	0,845	0,733	0,2007
MV17	17	0,878	0,817	1,0000
MV18	8	0,825	0,833	0,3789
MV24	9	0,781	0,810	1,0000
MV26	6	0,683	0,650	1,0000
MV27	14	0,892	0,850	1,0000
MV30	5	0,728	0,887	**0,0002**
MV31	9	0,668	0,583	0,4455
Mean	9,7	0,802	0,771	

genotypes using TFPGA version 1.3 (Miller 1997). Probabilities were calculated by Exact Test, according to Haldane (1954).

Results

Primer sequences and diversity estimate indexes are presented in table 1 and 2. Loci produced 97 alleles. The mean number of alleles per locus (Â), $\hat{H}_0$ and $\hat{H}_e$ were 9.7, 0.771 and 0.802, respectively. One locus showed significant deviations from HWE. Five combinations (MV11/MV17, MV17/MV31, MV18/MV30, MV26/MV27 and MV30/MV31) showed significant Linkage Disequilibrium ($P < 0,05$). Using allozyme markers in three populations at South of Minas Gerais State, H_e and H_0 mean estimates were 0.487 and 0.592 (Botrel & Carvalho 2004). As expected, microsatellite markers had a much higher degree of polymorphism as well as diversity than allozyme. However, estimates of genetic structure are expected to be similar (Conte *et al*. 2008). These 10 microsatellite markers will be important tools for studies about structure and diversity in *Machaerium villosum* and therefore, will contribute for its conservation and management practices.

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