Hoehnea 44(4): 580-587, 4 tab., 1 fig., 2017 http://dx.doi.org/10.1590/2236-8906-22/2017

Development of microsatellite loci for *Cryptocarya mandioccana* Meisner (Lauraceae) and their genotyping success in different tissues

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Received: 28.04.2017; accepted: 10.08.2017

ABSTRACT - (Development of microsatellite loci for *Cryptocarya mandioccana* Meisner (Lauraceae) and their genotyping success in different tissues). Nine polymorphic microsatellite loci were isolated and characterized for *Cryptocarya mandioccana* Meisner, a tree from the Atlantic Rainforest with seeds dispersed by large animals. The loci were characterized using 48 individuals from two populations and their genotyping success tested in four tissues: leaves from adults and seedlings, and two diaspore maternal tissues. Maternity analyses were also performed on diaspores and leaves from nine adult trees. The number of alleles per locus ranged from nine to 15 and the observed and expected heterozygosities ranged from 0.214 to 0.864 and 0.745 to 0.892, respectively. The loci genotyping success did not significantly differ between tissues and varied from 56 to 96%. The microsatellites showed enough polymorphism to assign the nine adult trees to their diaspores. The successful genotyping in all tissues and identification of mother trees show that the microsatellites are suitable for studies such as spatial genetic structure and maternity analyses.
Keywords: Atlantic forest, Lauraceae, maternity analysis, microsatellite, seed maternal tissue

RESUMO - (Desenvolvimento de locos de microssatélites para *Cryptocarya mandioccana* Meisner (Lauraceae) e o sucesso de genotipagem em diferentes tecidos). Nove locos de microssatélites polimórficos foram isolados e caracterizados para *Cryptocarya mandioccana* Meisner, uma árvore da Mata Atlântica cujas sementes são dispersas por grandes animais. Os locos foram caracterizados usando 48 indivíduos de duas populações e o sucesso de genotipagem testado em quatro tecidos: folhas de adultos e plântulas, e dois tecidos maternos de diásporos. Análises de maternidade foram também realizadas em diásporos e folhas de nove árvores adultas. O número de alelos por loco variou de nove a 15 e as heterozigosidades observada e esperada de 0,214 a 0,864 e 0,745 a 0,892, respectivamente. O sucesso de genotipagem dos locos não diferiu entre os tecidos e variou de 56 a 96%. Os microssatélites mostraram polimorfismo suficiente para atribuir as nove árvores aos seus diásporos. O sucesso de genotipagem em todos os tecidos e a identificação das árvores mães mostraram que os microssatélites são adequados para estudos como estrutura genética espacial e análise de maternidade.
Palavras-chave: análise de maternidade, Lauraceae, Mata Atlântica, microssatélite, tecido materno das sementes

Introduction

Studies on the effects of defaunation on seed dispersal and consequently on gene flow are still scarce in tropical forests, specifically in Atlantic Forest (Carvalho *et al.* 2016). Yet this knowledge is of paramount importance to understand the spatial distribution of genetic variation and how this affects the evolutionary potential of the species (Garcia & Grivet 2011). Direct and indirect methods exist to study seed and gene dispersal such as the determination of seed dispersal distances through parentage analysis (parent tree - seed) or the spatial genetic structure and maternal correlation analysis, respectively (Garcia & Grivet 2011). In this context, microsatellites markers are widely used for studies using both approaches (Hardesty *et al.* 2005; Zucchi *et al.* 2003).

Cryptocarya mandioccana Meisner (Lauraceae) is a tree species from the Brazilian Atlantic Forest.

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It is mainly found in mountain and submountain ombrophilous dense forests, between 10 and 1180 m asl. *C. mandioccana* is likely to be affected by anthropogenic activities such as forest fragmentation and defaunation, mainly due to its dependence to large mammals and birds to disperse its seeds (1.34-3.00 cm length and 1.16-1.92 cm width) (De Moraes 2007). Indeed, *C. mandioccana* is mainly dispersed by two large-sized primate species, the Southern muriqui (Primates, Atelidae: *Brachyteles arachnoides*) and the brown howler monkey (Primates, Atelidae: *Alouatta guariba clamitans*), by the Lowland tapir (Perissodactyla, Tapiridae: *Tapirus terrestris*) (Bueno *et al.* 2013; De Moraes 2007), and the Black-fronted piping guan (Galliformes, cracidae: *Aburria jacutinga*), a large-sized bird (L. Culot, obs. pers). The Southern muriqui (Mendes *et al.* 2008) and the Black-fronted piping guan (BirdLife International 2016) are classified as endangered by the IUCN Red List while the Lowland tapir is classified as "endangered in the Atlantic Forest" (Medici *et al.* 2012).

In the present study, we aimed to: 1) isolate and characterize microsatellite loci designed to *C. mandioccana*, 2) test these markers on four distinct tissues (leaves from adult trees, leaves from seedlings, pericarp and testa from the diaspores), and 3) use the markers to check the maternal origin of both diaspore tissues. The morphology of *C. mandioccana* seeds differ from the morphology of classic fruits since the pulp comes from the augmented floral axis while the diaspore is formed by the seed and the pericarp (De Moraes & Paoli 1996). To check the maternal origin of the tissues, we used the pericarp and the testa, a brownish tissue localized just under the pericarp. The analysis of the success of DNA extraction and amplification protocols in different tissues enabled us to determine the potential of *C. mandioccana* microsatellite markers for future applications such as the determination of the spatial genetic structure of seedlings or the mother-tree of dispersed seeds.

Material and methods

Sampling and DNA extraction - For the isolation and characterization of microsatellite loci, we collected leaves from 48 *C. mandioccana* adult trees from two populations of the Sao Paulo State (SP), Brazil. One population is located in São Miguel Arcanjo, in Carlos Botelho State Park (CBSP), and the other one in Cananéia, in Cardoso Island State Park (CISP). For the optimization of the protocol in other tissues, we used the pericarp of 27 *C. mandioccana* fruits directly collected in nine trees (3 fruits/tree) and the leaves of 27 seedlings randomly selected among the seedlings within a 12-ha plot in CBSP. To confirm the maternal origin of diaspore tissues, we collected leaves from the nine adult trees from which fruits were collected. We stored all samples in silica gel while in the field and then in freezer (-15 °C).

We extracted the genomic DNA using the CTAB/chloroform:IAA protocol for DNA extraction in plant tissues described by Doyle & Doyle (1987), following distinct optimizations according to the tested tissue. We cut around 150 mg of all tissues in 0.5-cm²-fragments and macerated them with 2-mm-beads of zirconium in a Mini-BeadBeater macerator (Biospec Products - USA). The time of maceration varied according to the tissues: 1min 30s for diaspore tissues and 45s for leaves. Higher concentrations of DNA were obtained by adding the extraction buffer after the maceration process. Therefore, we performed dry maceration of the plant tissues.

Construction of a microsatellite-enriched library and primer design

The DNA extracted from leaves of ten *C. mandioccana* adult trees from CISP was used by *Genetic Marker Services* company (Brighton, United Kingdom) to build a microsatellite-enriched library and design primers. Genomic DNA was digested with the Rsa I restriction enzyme. The library was enriched using size-restricted DNA with filter-bonded synthetic repeat motifs, (AG)17, (AC)17, (AAC)10, (CCG)10, (CTG)10, and (AAT)10. A total of 37 positive clones were sequenced from which 17 primer pairs could be designed through the Primer 3 version 3.0 (Rozen & Skaletsky 1999). All primer pairs were synthetized with a M13 tail (5' -TGTAAAACGACGGCCAGT-3') at the end to allow labeling with a tailed fluorescent dye M13 primer and multiplex genotyping procedures (Schuelke 2000).

Amplification conditions and validation of primers - All amplifications were performed by polymerase chain reactions (PCR) in a 96 Well Thermal Cycler (Applied Biosystems) thermocycler. The final volume of the reaction was 11 µl and contained: 1-10 ng of DNA, 1 µl of Nuclease-Free H2O, 5 µl of GoTaq® Colorless Master Mix 2X (Promega), 8 pmol of primer without M13-tail, 2 pmol of primer with M13-tail, 8 pmol of universal M13 primer tagged with fluorochromes (FAM or NED), and 0.42 ng ml^{-1} of BSA. We used the following "Touchdown" program:

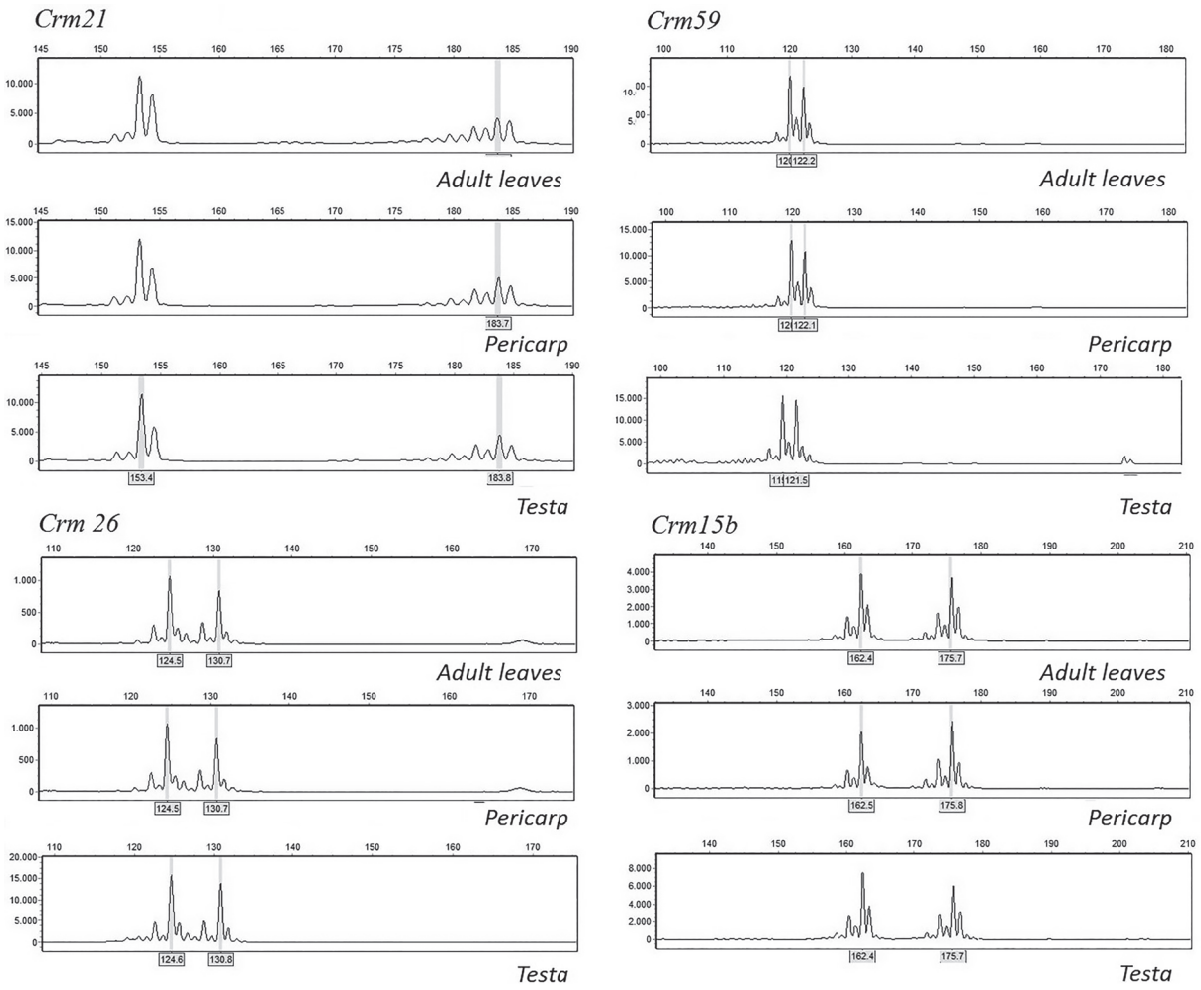

Figure 1. Genotyping results for four loci of the DNA extracted from the adult leaves and the diaspore tissues (pericarp and testa) collected directly on one individual adult tree of *C. mandioccana* (SM 36).

95°C for 3min, followed by 10 cycles of 94 °C for 30s, 58 - 48 °C decreasing 1 °C per cycle during 30s, 72 °C for 30s followed by 30 cycles of 94 °C for 30s, 48 °C for 30s, 72 °C during 30s, followed by a final extension of 10 min at 72 °C according to the method described by Palma-Silva *et al.* (2007). The amplicons were visualized by electrophoresis in agarose gel 1.5% with GelRed (Biotium, Hayward, California, USA). We performed the genotyping of the samples in a DNA automated sequencer (Applied Biosystems 3500 Series Genetic Analyzer) using the GeneScan 500 Liz as dye-labeled size standard. We identified the alleles with the GeneMarker v.4.1 software (Applied Biosystems).

Data Analyses - All genotypes were submitted to the Micro-Checker software (Van Oosterhout *et al.* 2004) where genotyping errors due to stuttering, dropout and null alleles were identified. The levels of genetic diversity within each population were described through allelic richness (*AR*) (El Mousadik & Petit 1996), number of alleles (*A*) per locus and population, allelic size variances, and observed (H_o) and expected (H_e) heterozygosities. These values were estimated with MSA software (Dieringer & Schlötterer 2003), GenAlEx 6.5 (Peakall & Smouse 2006, 2012), and Fstat 1.2 (Goudet 1995). The GENEPOP 3.5 software (Raymond & Rousset 1995) was used to test the principle of Hardy-Weinberg Equilibrium (HWE), the coefficient of inbreeding F_{is} (Weir & Cockerham 1984) within the populations, and the linkage disequilibrium between pairs of loci. To confirm the maternal origin of diaspore tissues, we compared the genotypes of the pericarps, testa, and leaves from adult trees.

Results and Discussion

Microsatellite polymorphism and genetic diversity analysis for *C. mandioccana* - Out of 17 primer pairs

Table 1. Genetic characterization of the microsatellite loci designed for *C. mandioccana*. Locus name, Sequence 5'-3', repeat motif, size range, number of alleles (A), observed heterozygosity (H_o), expected heterozygosity (H_e), and access code to GenBank. Significant departure from HWE (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$).

Locus	Sequence (5' – 3')	Repeat motif	Size range (bp)	A	H_o	H_e	GenBank
Crm2	F: CCTTCTGCTGACCACTTAAAACA	(CA)14	108-129	10	0.795	0.827	MF979811
	R: TCATACAGCCACCAAATCCA						
Cm3	F: GGTAACTAACTCG	(TC)25	153-190	15	0.356	0.870***	MF979812
	R: CACAAGCAAATTCAATCTG						
Crm13	F: TGTGTGTGTGAGATAGTGGTTTTC	(AG)18	154-175	10	0.214	0.858***	MF979813
	R: TGACAATCAAAATGGGAAATTG						
Crm15b	F: AGGGGTGTGCGTGAATAGAA	(GT)13 (GA)12	151-178	14	0.681	0.840	MF979814
	R: TGCACTATATGGAAAAGCATGTG						
Crm21	F: CAGAACCCGTCCCAATTTTT	(GA)20	154-188	13	0.864	0.892	MF979815
	R: CTCCCCGGCTCTAATACCAT						
Crm23	F: TCTCTCTCATGTATCAATTTAAGC	(TC)15	111-146	9	0.310	0.824***	MF979816
	R: TACCATGCCTTAGCTGTGAA						
Crm26	F: CGTAGGCCGAAACGACAAGT	(TG)18	118-134	10	0.756	0.848*	MF979817
	R: TTCCACATGGACATGGCTTG						
Crm27b	F: CCATTTTTTCAATCCAACGG	(AC)12	171-200	10	0.405	0.746***	MF979818
	R: ATGCATCTTAGGGGAGTGCT						
Crm59	F: CATGAACAATAAAATAGTGATAAA	(AC)11	114-134	9	0.667	0.697**	MF979819
	R: TCGTAGGCAACTCATCTCAG						

Table 2. Genetic characterization of *C. mandioccana* populations. Number of sample individuals (N), number of alleles (A), allelic richness (Ar), observed heterozygosity (H_o), expected heterozygosity (H_e), and inbreeding coefficient (Fis). Significant departure from HWE ($P < 0.0001$).

Pop	Latitude S	Longitude W	N	A	Ar	H_o	H_e	Fis
CISP	25°08'01.4"	47°57'42.2"	24	8.444	6.886	0.524	0.785	0.357*
CBSP	24º03'37"	47º59'44"	24	8.778	7.281	0.580	0.807	0.221*

Table 3. Genotyping success (%) for distinct tissues and loci of *C. mandioccana.*

	Loci					
	Crm 21	Crm 59	Crm 26	Cm 3	Crm 15b	Mean ± SD
Pericarp	85	67	82	70	63	73.4 ± 8.6
Testa	78	66	82	59	56	68.2 ± 10.2
Adults	85	78	96	78	82	83.8 ± 6.6
Seedlings	66	82	74	59	81	74.0 ± 9.2

Table 4. Genotypes of the mother tree (adult leaves) and of the tissues (pericarp and testa) of three diaspores (differentiated by a different number) of nine adult individuals (Ind) of *C. mandioccana* collected directly from the mother trees.

Ind	Tissue	Crm 21		Crm 59		Crm26		Cm3		Crm15b	
	Adult leaves	170	172	120	124	122	132	165	165	NA	NA
	Pericarp 1	170	172	120	124	NA	NA	165	165	162	162
	Testa 1	170	172	120	124	NA	NA	165	176	162	162
SM53	Pericarp 2	170	172	120	124	122	132	165	165	162	172
	Testa 2	170	172	120	124	122	132	165	165	162	172
	Pericarp 3	NA	NA	NA	NA	122	132	NA	NA	162	172
	Testa 3	NA	NA	NA	NA	NA	NA	NA	NA	162	172
	Adult leaves	172	172	118	120	126	126	186	188	162	176
	Pericarp 4	NA	NA	118	120	126	126	186	188	162	176
	Testa 4	172	172	118	120	126	126	186	188	162	176
SM33	Pericarp 5	172	180	NA	NA	126	122	186	188	162	176
	Testa 5	172	172	NA	NA	126	126	NA	NA	162	176
	Pericarp 6	172	172	NA	NA	126	122	186	188	162	176
	Testa 6	172	172	NA	NA	126	126	NA	NA	162	176
	Adult leaves	NA	NA	NA	NA	124	130	164	166	162	176
	Pericarp 7	174	176	120	122	124	130	164	166	NA	NA
	Testa 7	174	176	120	122	124	130	164	166	NA	NA
SM6	Pericarp 8	174	176	120	122	NA	NA	164	166	162	176
	Testa 8	174	176	120	122	NA	NA	164	166	162	176
	Pericarp 9	174	176	120	122	NA	NA	164	166	NA	NA
	Testa 9	174	176	120	122	NA	NA	164	166	NA	NA
	Adult leaves	174	180	120	122	128	130	NA	NA	NA	NA
	Pericarp 10	174	180	120	122	128	130	NA	NA	NA	NA
SM10	Testa 10	NA	NA	120	122	128	130	NA	NA	NA	NA
	Pericarp 11	174	180	NA	NA	124	128	164	166	NA	NA
	Testa 11	NA	NA	NA	NA	124	128	164	166	NA	NA

continue

Table 4 (continuation)

Ind	Tissue	Crm 21		Crm 59		Crm26		Cm3		Crm15b	
SM10	Pericarp 12	174	180	NA	NA	124	128	NA	NA	NA	NA
	Testa 12	174	180	NA	NA	124	128	NA	NA	NA	NA
SM12	Adult leaves	NA	NA	NA	NA	124	130	174	174	NA	NA
	Pericarp 13	172	176	120	122	124	130	174	174	178	178
	Testa 13	NA	NA	NA	NA	124	130	NA	NA	NA	NA
	Pericarp 14	172	176	NA	NA	124	130	NA	NA	178	178
	Testa 14	172	176	NA	NA	124	130	NA	NA	NA	NA
	Pericarp 15	176	176	120	120	NA	NA	NA	NA	NA	NA
	Testa 15	172	176	120	120	124	130	NA	NA	NA	NA
SM82	Adult leaves	NA	NA	NA	NA	NA	NA	176	188	NA	NA
	Pericarp 16	NA	NA	122	122	122	128	176	188	NA	NA
	Testa 16	NA	NA	NA	NA	122	128	176	188	NA	NA
	Pericarp 17	168	172	122	122	122	128	176	188	NA	NA
	Testa 17	168	172	122	122	122	128	176	188	NA	NA
	Pericarp 18	NA	NA	NA	NA	122	128	NA	NA	NA	NA
	Testa 18	NA	NA	122	122	122	128	176	188	NA	NA
SM79	Adult leaves	NA	NA	NA	NA	124	128	NA	NA	NA	NA
	Pericarp 19	170	176	118	120	124	128	NA	NA	NA	NA
	Testa 19	170	176	118	120	124	128	NA	NA	NA	NA
	Pericarp 20	170	176	118	120	124	128	NA	NA	154	162
	Testa 20	170	176	118	120	124	128	NA	NA	154	162
	Pericarp 21	170	176	118	120	124	128	170	188	154	162
	Testa 21	170	176	118	120	124	128	188	188	154	162
SM36	Adult leaves	154	184	120	122	122	128	170	188	158	182
	Pericarp 22	154	184	120	122	122	128	170	188	158	182
	Testa 22	154	184	120	122	122	128	170	188	158	182
	Pericarp 23	154	184	120	122	122	128	170	188	158	182
	Testa 23	154	184	120	122	122	128	NA	188	158	182
	Pericarp 24	154	184	120	122	122	128	170	188	158	182
	Testa 24	154	184	120	122	122	128	170	188	158	182
SM42	Adult leaves	170	184	120	120	124	130	186	188	154	176
	Pericarp 25	170	184	120	120	NA	NA	186	188	154	176
	Testa 25	170	184	120	120	NA	NA	186	188	154	176
	Pericarp 26	170	184	NA	NA	124	130	186	188	154	176
	Testa 26	170	184	120	122	124	130	186	188	154	176
	Pericarp 27	170	184	NA	NA	124	130	186	188	154	176
	Testa 27	170	184	NA	NA	124	130	186	188	154	176

designed for *C. mandioccana*, nine loci amplified successfully and were polymorphic for both studied populations (table 1). We detected 78 alleles in this set of polymorphic markers, varying from nine to 15 per locus. Observed and expected heterozygosities varied from 0.214 to 0.864 and from 0.697 to 0.892, respectively (table 1). Only three loci were in HWE: Crm2, Crm15b, and Crm 21 at the 0.05 level (table 1). The loci Crm26 and Crm 59 presented linkage disequilibrium ($P < 0.05$). Although the presence of null alleles cannot be ruled out, we did not detect any genotyping errors due to stuttering or dropout.

The indices of genetic diversity suggest high diversity in both populations (table 2). We detected 73 alleles in the CISP population, varying from five to 12 alleles per locus, and observed and expected heterozygosities of 0.524 and 0.785, respectively, with a significant inbreeding coefficient (Fis = 0.357). In the CBSP population, we detected 77 alleles, varying from five to 11 per locus and observed and expected heterozygosities of 0.580 and 0.807, respectively, with a significant inbreeding coefficient (Fis = 0.221).

Genotyping success in distinct tissues - Although the diaspore tissues and seedlings presented lower genotyping success than leaves from adult trees, the difference was not significant (N = 20, Kruskal-Wallis $\chi^2 = 5.15$, df = 3, P = 0.16) (table 3).

Validation of the maternal origin of diaspore tissues - We used five of the nine validated microsatellites (Crm21, Crm59, Crm26, Cm3, and Crm15b) to compare the genotypes of potential mother trees with those of pericarp and testa tissues. The five microsatellite loci were sufficiently polymorphic to enable the genetic assignment of diaspores to the nine mother trees (table 4, figure 1). Moreover, both tested diaspore tissues presented the same genotypes as the mother trees, indicating that both tissues are of maternal origin (table 4, figure 1). These results show that both tissues can be used in maternity analysis (without the need to separate them for DNA extraction) and therefore in studies aiming to determine seed dispersal distances.

The nine microsatellite loci we isolated and characterized are highly polymorphic and will be useful for future studies about spatial genetic diversity or seed and gene dispersal. In addition, these markers will enable the determination of the genetic diversity of *C. mandioccana* populations as well as the effects of human disturbances on the genetics of this plant species.

Acknowledgements

We would like to thank our field assistants (Danilo Eugenio Ferreira, Sergio Carlos Neves, and Eduardo Pereira) for their valuable help in the field, to Genetic Marker Services for primer designs, and to Sérgio Kakazu for his collaboration during the genotyping process. We are deeply grateful to Dra. Alexandra Sanches who initiated the tests of microsatellite markers and to Prof. Dr. Mauro Galetti for his involvement in the first phases of the project. T.C, G.G., C.C. and L.C. received a fellowship from the Fundação de Amparo à Pesquisa do Estado de São Paulo - FAPESP (2014/20621-2, 2014/20622-9, 2014/01029-5, and 2010/16075-1, respectively) and C.P. received a funding from FAPESP (2009/52725-3). The project was financed by a bilateral funding between Brazil and Belgium - CAPES/WBI program (n°009/10) and by CNPq (Conselho Nacional de Desenvolvimento Científico e Tecnológico - Universal n° 456549/2014-5). A FAPESP funding to L.C (Jovem Pesquisador program, n° 2014/14739-0) provided final support for this publication.

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