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PERMANENT GENETIC RESOURCES

Development of microsatellite markers in *Annona crassiflora* Mart., a Brazilian Cerrado fruit tree species

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Abstract

***Annona crassiflora* Mart. (Annonaceae) is a native fruit species of the region of Brazilian Cerrado with a high agronomic potential, although without any traces of domestication. A set of 10 microsatellite primer pairs was developed from an enriched genome library (TC13). An average of 19.3 alleles per locus was detected. Observed heterozygosity estimates were consistently lower than those obtained for gene diversity, evidencing a departure from Hardy–Weinberg expected proportions. The reported set of markers showed to be highly informative and constitutes a powerful tool for the development of genetic characterization studies in *A. crassiflora*.**

Keywords: araticum, Brazilian savannah, Cerrado, genetic diversity, molecular marker, SSR

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Annona crassiflora Mart. belongs to the family Annonaceae and is known as araticum in Brazil. It is one of the most typical native fruit tree species of the Cerrado region (Brazilian woodland-savannah) that has received great attention from research in the Brazilian Center-West region in face of the potential for becoming a commercial crop. Its fruits are very much appreciated by the local population for consumption, either fresh or processed (ice cream, popsicles, jellies and pulp) (Almeida *et al*. 1998). The species is also known to possess pharmaceutical properties detected in treatment of tumours, aside from its bactericidal and antifungal effects (Ribeiro *et al*. 2000). The ripe fruit pulp is used as astringent and tonic, and the infusion of leaves and seeds is used against syphilis, diarrhea and rheumatism (Almeida *et al*. 1987). As most native Cerrado fruit species, *A. crassiflora* is still in the wild state and all commercial exploration occurs on an entirely predatory basis, without any degree of domestication.

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It is widely known that simple sequence repeat (SSR) markers are excellent tools for the development of genetic characterization studies of wild plant species. In this study, we describe a set of 10 SSR markers that showed to be highly useful for the development of genetic studies in *A. crassiflora*.

An enriched genomic library for *A. crassiflora* was prepared according to the protocol of Rafalski *et al*. (1996). The genomic DNA extracted from young leaves (50 μg) was digested with enzyme *Sau3A1* (QIAGEN) and separated in 2% agarose gel. The fragments of 200–800 bp were recovered using the Quiaquick Gel Extraction kit (QIAGEN). Individual fragments were ligated to adaptors and hybridized with biotin-labelled probes (TC13) and recovered using magnetic beads (streptavidin-coated magnetic beads). The DNA fragments were then cloned into the plasmid pGEM T-Easy Vector (Promega) and transformed into competent *Escherichia coli* XL1-Blue cells (Sambrook *et al*. 1989). The transformed cells were plated and incubated at 37 °C for 12 h in 1× Luria-Bertani (LB) culture medium containing Ampicillin, Xgal and IPTG (50 μg/mL ampicilin, 40 μg/μL X-gal and 0.5 M IPTG). Positive clones were screened using blue/white selection. The recombinant bacterial clones were lysed in water and an aliquot was used for an amplification reaction

Table 1 Primer sequence, repeat motif, range of fragment size, GenBank Accession number, and genetic characterization of 10 microsatellite markers in *Annona crassiflora* Mart., screened in 32 individuals

Locus	Primers (5′–3′)	$(TC)_n$	Size (bp)	Accession no.	T_a	n	A	H_O	H_E	f	P value
Acr01	F 6FAM-CGGCCTTCAAAAGGGAGATA	20	200–300	EU487524	58	29	31	0.793	0.966	0.182	0.000
	R CATGATTCTTCTGCTTCTGTGG										
Acr10	F 6FAM-TGACGAAAACGAGAAAAGCA	9	150–180	EU487525	58	29	9	0.586	0.803	0.273	0.000
	R ATGTCCCCAACCCAATACAT										
Acr19	F HEX-GAGAGCTGGGAGAAGAGCAA	11	145–175	EU487526	58	29	12	0.690	0.837	0.178	0.080
	R AAAGCTGGGAGAGACGACAC										
Acr20	F 6FAM-AGAGCCAGAGCCAGTGAGAC	21	170–210	EU487527	60	30	17	0.933	0.896	–0.042	0.035
	R TTGCCTCCATCTCTCAATCC										
Acr22	F 6FAM-CTGACTCGCTGGCTCTCTCT	18	180–240	EU487528	58	32	19	0.875	0.924	0.054	0.153
	R CTACAGCCCACATGTGCAAC										
Acr26	F HEX-CACGACCAAGGAGAGAGGAG	15	150–180	EU487529	58	32	10	0.719	0.849	0.156	0.017
	R GGCAACAATCCTGACTCACA										
Acr33	F HEX-CAAACAGGCGATGAGACAGA	32	100–230	EU487530	58	32	22	0.750	0.927	0.193	0.000
	R TGGTTGGCTTTTCTCTTTCAA										
Acr34	F NED-GGAACAGAAGCTGTGGCATT	28	130–190	EU487531	58	31	21	0.903	0.941	0.041	0.274
	R CGCGCAATTCCACAATAAC										
Acr37	F HEX-GGCAACTTCTCCCCTTTACC	24	250–360	EU487532	60	31	28	0.839	0.969	0.137	0.010
	R CCGGTGCCTGCTGTATATG										
Acr44	F NED-CAATTGCAATGGGTAGAGAGAG	26	100–160	EU487533	58	32	24	0.969	0.955	–0.015	0.651
	R CATCACCGCACAAAGAGAA					30	19.3	0.806	0.907	0.113	—

T^a, annealing temperature (°C); n, number of individuals successfully genotyped; A, number of alleles; H_O, observed heterozygosity; H_E, gene diversity; f, intrapopulation fixation index; P value, significance levels associated to the deviates from HWE proportions.

according to the Invitrogen polymerase chain reaction (PCR) protocol. The forward and reverse M13 primers, complementary to the vector region, were used in this reaction. The PCR products were purified using ethanol precipitation with potassium acetate. Cloned fragments were sequenced from both ends using BigDye Terminator version 3.1 kit (Applied Biosystems). Appropriate microsatellite and flanking regions for primer design were found in 44 clones. Primers were designed using the program Primer 3 (Rozen & Skaletsky 2000). Microsatellite loci were initially tested using DNA of 12 plants of natural populations. For the PCR amplification, a final volume of 13 μL was used containing: 4.5 ng of DNA, 0.25 μM of each forward and reverse primers, 0.25 mM dNTPs, buffer (10 mM Tris-HCl pH 8.3, 50 mM KCl), 1.5 mM $MgCl_2$, 0.25 mg/mL Bovine Serum Albumin, 1 U *Taq* polymerase (Invitrogen) and ultrapure water. A thermocycler PTC-100 MJ Research was used with the programme: 94 °C for 5 min, 30 cycles at 94 °C for 1 min, 1 min at primer-pair-specific annealing temperature, 72 °C for 1 min and a final extension at 72 °C for 7 min.

Among the 44 initially synthesized primer pairs, 10 primers pairs (Table 1) were selected, based on consistency and level of polymorphism, for a preliminary genetic characterization of natural *A. crassiflora* populations collected in the state of Goiás, Brazil. Forward primers of each pair were labelled with different fluorochromes (6-FAM, HEX or NED) and arranged in multiplex sets for analyses in an automated fragment analyser (ABI-3100, Applied Biosystems). A mix of fluorescent (ROX) labelled fragments synthesized as described in Brondani & Grattapaglia (2001) was used as internal size standard. The software GeneMapper version 3.5 (Applied Biosystems) was used for allele calling. Genotypes for each loci in 32 adult plants from one natural population (17°19′25″S, 51°33′47″) were evaluated. Genetic data were analysed using the program GDA (Lewis & Zaykin 2001). The results suggest that there is a high level of genetic diversity in natural populations of *A. crassiflora* (Table 1). An average of 19.3 alleles per locus was estimated. The estimated observed heterozygosities (mean = 0.805) was consistently lower than gene diversities estimates (mean = 0.906), evidencing an increase of homozygosity in relation to the expected levels under Hardy–Weinberg equilibrium. Significant departures from Hardy–Weinberg proportions were detected for seven of the 10 described loci, suggesting that the species possibly adopts a mixed self and random mating system for reproduction. The presence of null alleles could not be discarded, however. Significant levels of linkage disequilibrium were not detected between any pair of loci (Table 2). The reported set of markers showed to be highly informative and constitutes a powerful tool for the development of genetic characterization studies in *A. crassiflora*.

	Acr10	Acr19	Acr20	Acr22	Acr26	Acr33	Acr34	Acr37	Acr44
Acr01	—								
Acr10	1.0000								
Acr19	0.0726	0.6947							
Acr20	1.0000	0.6970	1.0000						
Acr22	1.0000	1.0000	1.0000	0.1995					
Acr26	1.0000	0.8044	1.0000	1.0000	1.0000				
Acr33	1.0000	1.0000	0.3309	0.1962	0.0355	1.0000			
Acr34	1.0000	0.0128	1.0000	1.0000	1.0000	0.1072	1.0000		
Acr37	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	
Acr44	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000

Table 2 Significance levels (*P* values) obtained for the linkage disequilibrium tests for all pairs of loci. All values were declared nonsignificant at the 5% level, under the false discovery rate criterion (Benjamin & Hocheberg 1995)

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