Characterization of 10 New Nuclear Microsatellite Markers in Acca sellowiana (Myrtaceae)

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Characterization of 10 new nuclear microsatellite markers in *Acca sellowiana* (Myrtaceae)[1]

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- *Premise of the study:* Microsatellite primers were identified and characterized in *Acca sellowiana* in order to expand the limited number of pre-existing polymorphic markers for use in population genetic studies for conservation, phylogeography, breeding, and domestication.
- *Methods and Results:* A total of 10 polymorphic microsatellite primers were designed from clones obtained from a simple sequence repeat (SSR)–enriched genomic library. The primers amplified di- and trinucleotide repeats with four to 27 alleles per locus. In all tested populations, the observed heterozygosity ranged from 0.269 to 1.0.
- *Conclusions:* These new polymorphic SSR markers will allow future genetic studies to be denser, either for genetic structure characterization of natural populations or for studies involving genetic breeding and domestication process in *A. sellowiana*.

Key words: *Acca sellowiana*; feijoa; *Plinia cauliflora*; *Plinia jaboticaba*; *Plinia trunciflora*; population genetics.

Acca sellowiana (O. Berg) Burret (Myrtaceae), commonly known as feijoa or pineapple guava, naturally occurs in southern Brazil and Uruguay (Thorp and Bieleski, 2002). Santos et al. (2009) have suggested that there is potential to develop this fruit resource as a food product and as a medicinal plant. In addition to its unique taste, the species has several bioactive properties that make it potentially useful for pharmacological purposes (Weston, 2010). The species is neither well known nor thoroughly exploited in its center of origin; however, commercial cultivars of the species have been cultivated in other parts of the globe (e.g., Colombia and New Zealand). The development of simple sequence repeat (SSR) markers can be very useful for a number of studies, such as cultivar fingerprinting and genetic diversity assessment of germplasm, which are essential for conservation, phylogeography, breeding, and characterization of population genetic structure of *A. sellowiana*.

Some specific molecular markers have already been developed (Santos et al., 2008); however, preliminary studies using those markers show that only nine of the 13 markers are being successfully used (data not published). Therefore, the development of new SSR markers will help to characterize the levels and distribution of genetic diversity; they will also be essential for phylogeographic, species domestication, and genetic mapping studies. Therefore, in this paper, we present 10 new microsatellite markers for *A. sellowiana*.

METHODS AND RESULTS

Total DNA was isolated from leaves of a single feijoa plant according to Doyle and Doyle (1990), and an SSR-enriched library was constructed based on Billotte et al. (1999). Briefly, total DNA was digested using *Afa*I restriction enzyme (Invitrogen, Carlsbad, California, USA) at 37°C for 1 h, followed by enzyme inactivation at 65°C for 20 min. Digested DNA was then ligated to *Afa*I adapters (Afa21: 5′-CTCTTGCTTACGCGTGGACTA-3′ and Afa25: 5′-TAGTCCACGCGTAAGCAAGAGCACA-3′) in a 50-µL reaction containing 500 ng of digested DNA, 10 µM of each *Afa*I adapter, 1× T4 DNA Ligase Buffer (Promega Corporation, Madison, Wisconsin, USA), and 1 U/µL of T4 DNA Ligase (Promega Corporation). The reaction was incubated at 20°C for 2 h, followed by enzyme inactivation at 70°C for 10 min. Fragments were PCR amplified using a Veriti Thermal Cycler (Life Technologies, Carlsbad, California, USA) to yield adequate DNA for the next step. The reaction was performed in a 50-µL reaction containing 5 µL of the ligation reaction, 10 µM of Afa21 adapter, 0.2 mM of each dNTP (Fermentas, Vilnius, Lithuania), 1× *Taq* buffer (Fermentas), 1.5 mM of magnesium chloride (Fermentas), and 1 unit of *Taq* DNA Polymerase (Fermentas). A Veriti Thermal Cycler (Life Technologies) was used to amplify the ligated DNA with the following sequence: 95°C for 4 min; followed by 20 cycles of 94°C for 30 s, 60°C for 1 min, 72°C for 1 min; and a final extension step at 72°C for 8 min. The DNA was enriched for microsatellite repeat regions (CT_n, GT_n, and TTC_n) using Streptavidin MagneSphere Paramagnetic Particles (Promega Corporation) using the protocol suggested by the manufacturer.

Twenty microliters of the enriched DNA was PCR amplified with the same conditions used in the previous reaction, cloned using pGEM-T Easy Vector (Promega Corporation) according to the protocol suggested by the manufacturer, and transformed in chemically competent *Escherichia coli* XL1-Blue cells (Agilent Technologies, Santa Clara, California, USA). A total of 192 positive clones were sequenced with a MegaBACE 1000 automated sequencer (GE Healthcare Biosciences, Pittsburgh, Pennsylvania, USA). Of the sequenced

TABLE 1. Characteristics of 10 microsatellite primers developed in *Acca sellowiana*.

Locus	Primer sequences (5′–3′)	Repeat motif	Allele size (bp)	T_a (°C)	GenBank accession no.
Fse04	F: <6-FAM>TTCGTTTGGCGTTTACCTTC R: CGGTGCTCGTTTGTTGTATG	$(CT)_{12}$	204	59	JX978696
Fse06	F: <6-FAM>CGAATAGGGCACTCCAACTC R: GCCGTCCTTAGGTTCAACAC	$(CT)_9$	232	54	KJ14661
Fse08	F: <6-FAM>TCAGGTGTGGAATCTGCTTCT R: CCCTCTCATTAGGTGGTTGG	$(CT)_{10}$	195	53	KF011978
Fse09	F: <6-FAM>ACCTTGCAAAAGGATGTCGT R: GCTGTTGGAGGAAAAAGCAG	$(TTC)_7$	166	55	JX978698
Fse10	F: <6-FAM>TTTCGTCTCATACAAGCCTACAG R: CGAGGAATTCCCAAAACAAC	$(CT)_6$	249	55	KJ146612
Fse11	F: <6-FAM>AAAAGGAAAGTACGCATCCA R: CCCAGCAACAGGTGTATGA	$(AG)_{13}$	236	55	JX978699
Fse12	F: <6-FAM>GCGATTGAACCATGAAGTCC R: CGGTGGATTCAATGGAGAAG	$(CT)_8$	193	55	KF011979
Fse16	F: <6-FAM>CCATTGTTTTGGAAGGAACA R: TTGCGATTTTGAGAGTGGAG	$(CT)_{20}$	167	55	JX978702
Fse17	F: <6-FAM>CCATGAAATTGCTAAGCTGGA R: CGTGCTCGATGAAGAGGAA	$(CT)_{30}$	204	55	JX978703
Fse21	F: <6-FAM>ATCAGAAAATGCTGCCGAGT R: GCAACATTGCTCAGCAGGTA	$(CT)_{12}$	168	57	JX978700

Note: T_a = annealing temperature.

clones, 36 presented unique SSR regions, and only 26 were suitable for primer design using Primer3 (Rozen and Skaletsky, 2000). Ten SSRs (Table 1) were genotyped in four populations of *A. sellowiana*: Ipê, Urubici, Bituruna, and Guarapuava (Fig. 1). The remaining designed primers were not included in this work due to PCR reaction problems (e.g., non-amplification, several off-target peaks). PCR reactions were performed in 12-µL reactions containing 20 ng of DNA template, 0.2 mM from each dNTP (Fermentas), 1 unit of *Taq* DNA Polymerase (Fermentas), 1× *Taq* buffer (Fermentas), 2 mM of magnesium chloride (Fermentas), and 5 pmol of each primer. A Veriti Thermal Cycler (Life Technologies) was used to amplify the 10 SSR loci with the following sequence: 95°C for 5 min; followed by 32 cycles of 95°C for 30 s, annealing temperature (Table 1) for 30 s, 72°C for 30 s; and a final extension step of 7 min at 72°C. Genotyping was performed using the MegaBACE 1000 automated sequencer, with ET-ROX 400 size standard (GE Healthcare). Sizing was carried out using Fragment Profiler software version 1.2 (GE Healthcare). MICRO-CHECKER version 2.2.4 (van Oosterhout et al., 2004) was used to assess null alleles and scoring errors. Deviations from Hardy–Weinberg equilibrium (HWE) for each of the four populations were calculated using GenAlEx 6.5
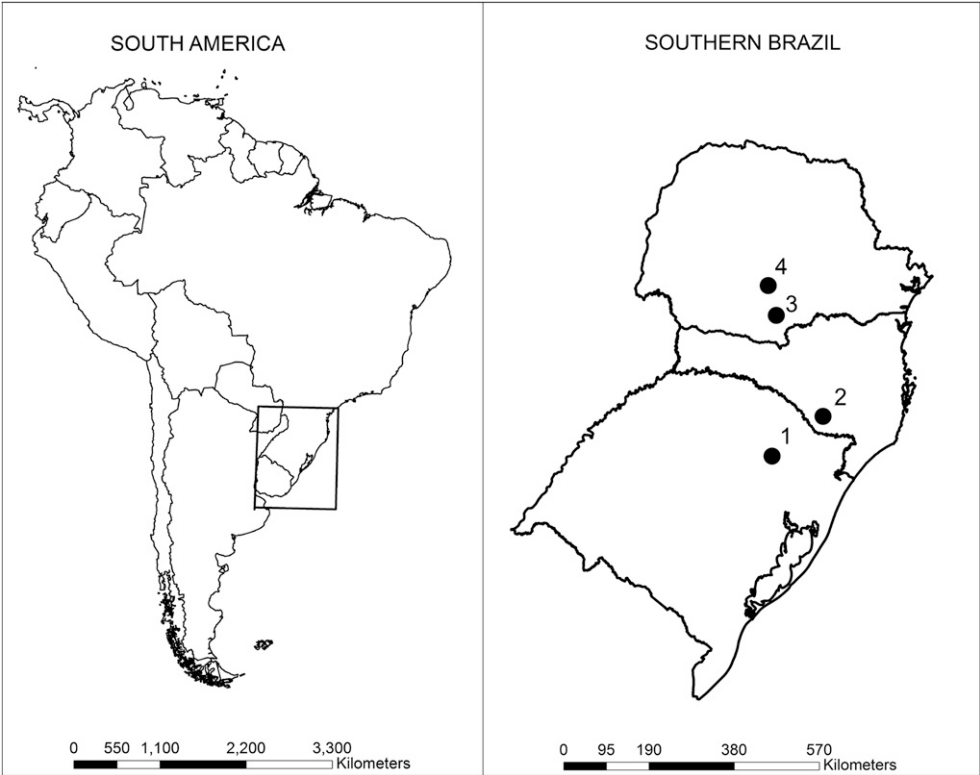

Fig. 1. Geographic localities of the populations sampled in this paper: 1 = Ipê, Rio Grande do Sul (28°75′14″S, 51°35′39″W); 2 = Urubici, Santa Catarina (28°04′18″S, 50°29′70″W); 3 = Bituruna, Paraná (26°02′52″S, 51°70′20″W); and 4 = Guarapuava, Paraná (25°26′29″S, 51°39′17″W).

TABLE 2. Results of initial primer screening in four populations of *Acca sellowiana*.

Locus	Ipê, Rio Grande do Sul ($n = 15$)				Urubici, Santa Catarina ($n = 15$)				Bituruna, Paraná ($n = 15$)				Guarapuava, Paraná ($n = 15$)			
	A	H_e	H_o	PIC	A	H_e	H_o	PIC	A	H_e	H_o	PIC	A	H_e	H_o	PIC
Fse04[ns]	6	0.671	0.667	0.596	9	0.809**	0.733	0.761	7	0.744**	0.813	0.676	9	0.874**	0.667	0.826
Fse06*	10	0.877	0.750	0.822	11	0.932	0.800	0.874	5	0.788	0.667	0.680	8	0.894**	0.714	0.846
Fse08*	5	0.458	0.400	0.421	5	0.743	0.733	0.672	4	0.599	0.500	0.493	5	0.637**	0.400	0.541
Fse09[ns]	4	0.690**	0.667	0.610	5	0.533	0.467	0.479	4	0.651	0.688	0.578	5	0.729**	0.467	0.650
Fse10*	3	0.530	0.333	0.424	2	0.467**	0.200	0.332	3	0.654	0.273	0.553	2	0.536	0.250	0.359
Fse11[ns]	7	0.724	0.800	0.654	8	0.832**	0.867	0.781	7	0.778	0.813	0.720	6	0.699	0.933	0.635
Fse12*	14	0.940	1.000	0.902	13	0.906	0.800	0.866	7	0.740	0.563	0.686	12	0.887**	0.867	0.844
Fse16[ns]	12	0.913	1.000	0.872	15	0.949	1.000	0.912	12	0.911	1.000	0.871	8	0.855	1.000	0.806
Fse17[ns]	12	0.931	0.933	0.891	15	0.915	0.800	0.875	14	0.883	0.875	0.845	11	0.864**	0.733	0.821
Fse21[ns]	7	0.825**	0.867	0.769	6	0.848**	0.867	0.794	7	0.851**	0.875	0.800	5	0.789**	0.533	0.724

Note: A = number of alleles sampled; H_e = expected heterozygosity; H_o = observed heterozygosity; n = number of individuals sampled; ns = nonsignificant values for null alleles analysis ($P > 0.05$); PIC = polymorphism information content.

*Significant values for null alleles analysis ($P < 0.05$).

**Indicates deviation from Hardy–Weinberg equilibrium ($P < 0.05$).

(Peakall and Smouse, 2012). Number of alleles (A), expected (H_e) and observed heterozygosities (H_o), and polymorphic information content (PIC) were analyzed using CERVUS 3.0.3 software (Kalinowski et al., 2007).

All PCR products met the expected sizes based on sequence information from the DNA library. Considering all populations and loci, A ranged from four to 27 alleles per locus (average: 14.10). H_o and H_e, considering all populations and loci, ranged from 0.269 to 1.0 (average: 0.735) and 0.569 to 0.938 (average: 0.805), respectively. The PIC, considering all populations and loci, ranged from 0.495 to 0.923 (mean: 0.773) (Table 2). Out of 10 new SSR markers developed, four showed PIC values greater than the SSR markers developed by Santos et al. (2008), thus expanding the number of highly polymorphic markers for the species. HWE was tested in all populations. All loci were in disequilibrium in at least one population (except the locus Fse16, $P > 0.05$), most likely due to heterozygote deficiency. However, these results should be treated with caution, given the small sample size for the populations ($n = 15$) (Hedrick, 2000).

In addition, for the first time, the transferability of these 10 new SSR markers was tested against DNA from four other Myrtaceae species—*Plinia jaboticaba* (Vell.) Kausel, *P. cauliflora* (DC.) Kausel, *P. trunciflora* (O. Berg) Kausel, and *Eugenia uniflora* L. Six loci successfully amplified products in *P. jaboticaba*, *P. cauliflora*, and *P. trunciflora* (Fse04, Fse09, Fse11, Fse16, Fse17, Fse21), and none of them amplified products in *E. uniflora*. These are the first SSR markers specific for *A. sellowiana* transferred to other Myrtaceae species.

CONCLUSIONS

This is the second study characterizing SSR markers for *A. sellowiana*. The new polymorphic markers double the sampling ability in the feijoa genome as well as the genome of other Myrtaceae species (due to the successful transferability of six markers), and will allow additional studies on breeding and the domestication process. Furthermore, the increase in genome sampling can help to better elaborate the proposition of conservation measures for this important species from southern Brazil.

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APPENDIX 1. Information on voucher specimens for *Acca sellowiana* deposited in the herbarium of Universidade Federal de Santa Catarina, Florianópolis, Brazil (FLOR).

Collection locality	Geographic coordinates	Voucher specimen
Ipê, Rio Grande do Sul	28°75′14″S, 51°35′39″W	FLOR 49843
Urubici, Santa Catarina	28°04′18″S, 50°29′70″W	FLOR 49841
Bituruna, Paraná	26°02′52″S, 51°70′20″W	FLOR 49842
Guarapuava, Paraná	25°26′29″S, 51°39′17″W	FLOR 49839