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Polymorphic information content of SSR markers for *Coffea* spp.

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ABSTRACT - *Thirty-three coffee SSR primers from enriched genomic library with $(GT)_{15}$ and $(AGG)_{10}$ repeats were analyzed in 24 coffee tree accessions. Twenty-two primers were polymorphic among accessions; the number of alleles ranged from 2 to 13, with the mean number of 5.1 alleles per primer. PIC values ranged from 0.08 to 0.79. The highest mean PIC values were found for* C. canephora *(0.46), and the lowest values for* C. arabica *(0.22) and triploids (0.22) accessions. The polymorphic SSR markers used in this study were useful for genetic fingerprinting in the coffee tree, especially in the* C. canephora *and the leaf rust resistant arabica cultivars.*

Key words: SSR, arabica coffee, molecular markers, genomic DNA libraries enriched, PIC.

INTRODUCTION

The *Coffea* genus consists of 103 known species, of which *Coffea arabica* L. is the most important commercially and the most cultivated worldwide. Previous studies using different molecular markers showed low levels of polymorphism among *C. arabica* accessions (Poncet et al. 2006, Aggarwal et al. 2007, Missio et al. 2009a), hindering further genetic research in this species. Simple Sequence Repeats (SSRs) or microsatellite markers are potentially useful in this situation, especially for exploring highly variable regions of the genome among individuals or populations of the same species. Furthermore, SSRs have other relevant features, such as high reproducibility, codominant inheritance, and the possibility of automation.

Notwithstanding all the advantages of SSR molecular markers for genetic studies, few SSR primers have been developed for coffee (Combes et al. 2000, Rovelli et al. 2000, Baruah et al. 2003, Moncada and McCouch 2004, Bhat et al. 2005, Poncet et al. 2006, Aggarwal et al. 2007, Hendre et al. 2008, Missio et al. 2009b), compared to other crops (http://www.gramene.org/). In this study, the potential of 33 SSR markers, developed from *C. arabica* were used for assessed the Polymorphism Information Content (PIC) in 24 accessions of the *Coffea* genus, including *C. arabica*. We also show here the potential these SSR markers for genetic fingerprinting in coffee tree.

MATERIAL AND METHODS

The genomic DNA of the *C. arabica* Bourbon Amarelo (UFV 570) genotype was extracted from leaves according to Diniz et al. (2005). The genomic DNA (50mg) was digested using the *Eco*RI, *Nhe*I, *Hae*III and

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*Rsa*I restriction enzymes. Fragments were blunted with the *Mung Bean Nuclease* enzyme, dephosphorylated with *Calf intestinal phosphatase* and linked to the double strand SNX adapters (Hamilton et al. 1999). Enriching was done by DNA hybridization using two biotinylated SSR probes, $(GT)_{15}$ and $(AGG)_{10}$. Enriched fragments where digested with *Nhe*I and linked to the *pBluescript* SK+ plasmid, previously digested with *Xba*I. Competent *Escherichia coli* DH5α cells were transformed with the recombinant plasmids by thermal shock. Selection and diagnosis of white colonies was done by PCR using the T3 and T7 primers (Invitrogen). Colonies containing transformants with insertions over 400bp were selected. Positive clones were sequenced and the DNA fragments were analyzed using the CodonCode Aligner 1.6.3 (CodonCode Corporation) and the SSRIT software.

The PCR reaction were done in a 20μL volume containing 50ηg of DNA, 0.6 U of *Taq* DNA polymerase and a 1 X buffer (Promega), 1mM of $MgCl_2$, 150μM of each dNTP and 0.1mM of each primer. Amplification was performed in a PTC-200 Thermocycler (MJ Research) using a touchdown PCR procedure consisted of initial denaturation at 94 °C/2min, followed by 13 cycles of denaturing at 94 °C/30s, annealing at 67 °C to 55 °C/30s, sinking 1 °C in each cycle, and extension at 72 °C/30s. The last 30 cycles were at 94 °C/30s, 55 °C/30s and 72 °C/30s, followed by final extension at 72 °C/8min. SSR marker polymorphism was verified in 6% silver stained denaturing polyacrylamide gel.

The potential of the 33 primer pairs as molecular markers was assessed in 24 accessions of the *Coffea* genus. The analysis included six arabica (*C. arabica*) accessions, five robusta accessions (*C. canephora*), three Híbrido de Timor (*C. arabica* x *C. canephora*), three Triploids (*C. arabica* x *C. racemosa*) and one racemosa (*C. racemosa*) accession. Six leaf rust resistant arabica were also included in this study (Table 1). The DNA of each of the 24 accessions was extracted from young leaves, according to the protocol described by Diniz et al. (2005).

The allele richness and the PIC (Polymorphism Information Content) were evaluated for each SSR marker. The allele richness was the number of alleles of each microsatellite *locus*. The PIC was calculated using

Table 1. Coffee tree accessions used in this study

Group	Accessions	Species (ploidy)
Arabica	UFV 2144 (Catuaí Vermelho IAC 44)	*Coffea arabica* (2n = 4x = 44)
Arabica	Típica UFV 2945	*Coffea arabica* (2n = 4x = 44)
Arabica	Bourbon UFV 2952	*Coffea arabica* (2n = 4x = 44)
Arabica	Bourbon Amarelo UFV 535-1	*Coffea arabica* (2n = 4x = 44)
Arabica	Arábica UFV 10832	*Coffea arabica* (2n = 4x = 44)
Arabica	Bourbon Amarelo UFV 10745	*Coffea arabica* (2n = 4x = 44)
Robusta	T 3751 (Robusta)	*Coffea canephora* (2n = 2x = 22)
Robusta	T 3580 (Robusta)	*Coffea canephora* (2n = 2x = 22)
Robusta	Conillon UFV 513 (Conillon)	*Coffea canephora* (2n = 2x = 22)
Robusta	Guarini UFV 514 (Robusta)	*Coffea canephora* (2n = 2x = 22)
Robusta	Apoatã IAC 2258 (Robusta)	*Coffea canephora* (2n = 2x = 22)
Híbrido de Timor	Híbrido de Timor CIFC 832/2	*C. arabica* x *C. canephora* (2n = 4x = 44)
Híbrido de Timor	Híbrido de Timor CIFC 4106	*C. arabica* x *C. canephora* (2n = 4x = 44)
Híbrido de Timor	Híbrido de Timor CIFC 1343/269	*C. arabica* x *C. canephora* (2n = 4x = 44)
Triploid	UFV 557-2	Triploid (*C. arabica* x *C. racemosa*) (2n = 3x = 33)
Triploid	UFV 557-3	Triploid (*C. arabica* x *C. racemosa*) (2n = 3x = 33)
Triploid	UFV 557-4	Triploid (*C. arabica* x *C. racemosa*) (2n = 3x = 33)
Racemosa	*Coffea racemosa*	*Coffea racemosa* (2n = 2x = 22)
Resistant arabica	Catiguá MG2	Commercial variety (*C. arabica* x HT) (2n = 4x = 44)
Resistant arabica	IAPAR 59	Commercial variety (*C. arabica* x HT) (2n = 4x = 44)
Resistant arabica	Oeiras MG6851	Commercial variety (*C. arabica* x HT) (2n = 4x = 44)
Resistant arabica	Sacramento MG1	Commercial variety (*C. arabica* x HT) (2n = 4x = 44)
Resistant arabica	Catucai Amarelo 2SL	Commercial variety (*C. arabica* x Icatu Vermelho) (2n = 4x = 44)
Resistant arabica	Obatã Amarelo IAC 4932	Commercial variety (*C. arabica* x HT) (2n = 4x = 44)

the CENES software (http://www.ufv.br/dbg/genes/genes.htm) (Cruz 2007).

RESULTS AND DISCUSSION

From the 33 primer pairs used in this study, named SSRCa, 26 (79%) amplified well-defined bands. Twenty two of them (67%) showed polymorphism among the 24 accessions. These polymorphic loci amplified 112 alleles, a mean 5.1 alleles per primer (Table 2).

PIC values of the polymorphic loci in the 24 *Coffea* accessions ranged from 0.08 to 0.79. The highest mean PIC values were found for *C. canephora* (0.46), while the lowest values were in *C. arabica* (0.22) and triploid (0.22) accessions. The low polymorphism found for *C. arabica* is in agreement with Baruah et al. (2003) work, and may be explained by the autogamous nature and the narrow genetic base of this species. All SSR primers were able to detect genetic differences between *C. arabica* and *C. canephora* populations, as well as among *C. canephora* and the remaining populations. This may be attributed to the *C. canephora* species allogamy, which results in high genetic variability. The SSRCa 018 and 091 had the highest PIC values (0.87 and 0.82) for *C. canephora* genotypes (Table 1). Primers with the highest PIC values for *C. arabica* were the SSRCa 094 (0.59), SSRCa 018 (0.54) and SSRCa 087 (0.54) (Table 2).

The SSRCa 018 and 091 primers were able to differentiate all robusta accessions (T 3751; T 3580; Guarini UFV 514 and Apoatã IAC 2258, of the Robusta group; and UFV 513, of the Conillon group). These primers may be useful for genetic fingerprinting and certification of clonal commercial varieties derived from *C. canephora* (Missio et al. 2009a). The SSRCa 068 and 018 primer differentiated the IAPAR 59 cultivar among the others leaf rust resistant cultivar of arabica (Catiguá MG2, Oeiras MG6851, Sacramento MG1, Catucai Amarelo 2SL and Obatã Amarelo IAC 4932) indicating the potential of this SSR markers in variety identification studies and in genetic fingerprinting (Figure 1).

The SSRCa 003, 016, 019, 020, 023, 026, 062, 078, 079, 082, 083, 091, 094 and 095 amplified not more than two alleles per individual in all accessions. The SSRCa 018, 052, 068, 080, 087, 088 and 092 revealed more tree or four alleles per individual in some triploid and allotetraploid accessions (Figure 1). In the *Coffea* allotetraploid accessions, SSR markers may show diploid and tetraploid segregation. Markers behave as tetraploids when amplifying homologous regions in both ancestral genomes of the species, and as diploids when amplifying only one of the genomes. No primer in this study showed a higher than expected number of alleles per individual for tetraploid species (up to four alleles), triploid species (up to three alleles) and diploid species (up to two alleles), making them adequate for breeding and genetic studies.

Figure 1. Allelic variation revealed by SSRCa 068 (A), SSRCa 092 (B) and SSRCa 018 (C) primers. M: Molecular weight marker; C.a: *Coffea arabica*; C.c: *Coffea canephora*; HT: Híbrido de Timor; T: Triploids; C.r: *Coffea racemosa*; V: Leaf rust resistant arabica varieties

Table 2. The allele richness and Polymorphic Information Content of SSR loci for *Coffea* genus

Primer	Repeats	Primer sequence (5'→ 3')	Size of frag. (bp)	Number of alleles/PIC						Total number of alleles/PIC (n=24)
				C.c (n=5)	C.a (n= 6)	HT (n=3)	T (n=3)	C.r[1]	V (n=6)	
SSRCa 003	$(GT)_{12}$	F: ATG ATTCG TA GG TGGA GTGG R: CTAAG CCGC AAATG A CAG A	196	2/0.31	1/0.00	1/0.00	1/0.00	2	1/0.00	4/0.21
SSRCa 010*	$(CT)_6$	F: G TTG A TTGG T GG AGTG ATTG R: AA GCA TCA AG TA AG GG AG GA	105	2/0.00	2/0.00	2/0.00	2/0.00	2	2/0.00	2/0.00
SSRCa 016	$(GA A)_3$/ $(GG AA AG)_3$	F: AGC AG ATTCC ATCCTTATCCT R: CCACT A ATCC ATTCCATTCC	172	1/0.00	2/0.38	2/0.38	2/0.38	2	2/0.38	3/0.54
SSRCa 017*	$(ATTTT)_3$	F: T ATG A TTGG TTG CTTG GATG R: ATCCT ACA AG GCGG T GTG	205	2/0.00	2/0.00	2/0.00	2/0.00	2	2/0.00	2/0.00
SSRCa 018	$(GT)_{18}(G A)_{10}$	F: G TCT CGTTTCA CGCTCTCTC R: ATTTTT GG CACGG T ATG TTC	115	9/0.87	3/0.54	2/0.38	3/0.54	2	3/0.54	13/0.74
SSRCa 019	$(GA)_{11}$	F: G GGTT AG AT AG AGC AA GA AT GA R: CTGT GA AGG TGT GG AGTTTT	329	4/0.67	1/0.00	2/0.24	2/03.7	1	1/0.00	4/0.44
SSRCa 020	$(AG A)G(AG A)_3$/ $(TG)_4$/$(ATT)_6$	F: G GT AGG CG AAG GA CAG AT AA R: TGG GGCA GA GT GA AG AT AA G	264	1/0.00	1/0.00	1/0.00	1/0.00	1	1/0.00	3/0.08
SSRCa 023	$(AA TG)_3$	F: G ACCC TTG CCTTTTGTTG R: GCC ATTCA TCCA TTCATTC	259	2/0.38	2/0.36	2/0.38	2/0.38	-	2/0.38	2/0.37
SSRCa 026	$(T)_{16}N_{12}(TC)_7$/$(CA C)_4$	F: G AATCTGG TG GG CTTTGA R: AA GG AG AG GGG A AGA AA ATG	289	4/0.60	2/0.38	2/0.38	2/0.38	-	2/0.38	5/0.60
SSRCa 036*	$(CA)_8$	F: ATG TTCGT GA AA CACA CGTC R: GG TTT GCCTT CAT CTTTGTT	128	2/0.00	2/0.00	2/0.00	2/0.00	2	2/0.00	2/0.00
SSRCa 052	$(TTG)_7$	F: G AT GG AAA CCC AG A AAG TTG R: TAG AAG GG CTT TGA CTG GA C	129	3/0.34	2/0.38	3/0.15	2/0.38	1	3/0.36	4/0.46
SSRCa 062	$(CAA)_2G(AG AA)_2$ $(AG)_4N_8(G A)_4$	F: A AGTT ATTA GG GCA AG AG TG GA R: AA GCT CCA AGA CCA AAG ATG	275	2/.038	2/0.38	2/0.38	2/0.38	1	2/0.38	3/0.37
SSRCa 063	$(TG)_3A(GT)_3N(TG)_4$	F: CTCCGCT GA TT TT GTCTTTT R: ACC ACTTTTTCCT CCCTCTC	222	-	-	-	-	-	-	-
SSRCa 064*	$(TTCT)_3$	F: TGCA GT AA GT GA GA CCAA CC R: TGG ACT AT CCCAT ACA TAACC A	242	2/0.00	2/0.00	2/0.00	2/0.00	2	2/0.00	2/0.00
SSRCa 068	$(AG G)_7$ / $(GA A)_4$	F: ATG TTGTT GG AGG CATTTTC R: AG GA GCA GTTG TTG TTTTCC	236	2/0.31	1/0.00	4/0.26	1/0.00	-	2/0.14	4/0.38
SSRCa 078	$(TCC)_5$	F: AG CCT CCCTTAG TTTGTTCTC R: GG AA AG TCG TCA G AT TG GTT	210	2/0.27	1/0.00	1/0.00	1/0.00	-	1/0.00	2/0.10
SSRCa 079	$(CCCT)_2N_5(G AAA A)_3$	F: A AGTG GAG G AGTTTTGTG GA R: CCA AGTGG AT AG GTG TG AG AG	287	2/0.37	1/0.00	1/0.00	1/0.00	2	1/0.00	3/0.19
SSRCa 080	$(CA)_9N_8(CT)_{30}$	F: G TTCTTT CCGCCG TC AAT R: GA GA AG AG AG AGG AA GG GA AA	250	4/0.72	1/0.00	4/0.61	2/0.44	-	4/0.71	10/0.79
SSRCa 081	$(CT)_{38}$	F: A CCGT TGTT G GAT AT CTTTG R: GG TTG AACCT A GAC CTT ATTT	229	3/0.59	1/0.00	2/0.24	1/0.00	-	1/0.00	4/0.28
SSRCa 082	$(CT)_{17}CG(CT)_6$	F: G CTT GT TTCCA TCG CT AAA R: TTA CACG TCA ACCCA CAA AC	178	3/0.50	2/0.24	3/0.54	2/0.35	1	3/0.36	7/0.62
SSRCa 083	$(TC)_{32}$	F: TCCAA CAA CATT AAG CGT ATTC R: GA CAA ACCT GA GG GA AA AG A	223	3/0.59	1/0.00	2/0.35	1/0.00	-	1/0.00	5/0.32
SSRCa 084	$(CCA)_4$ / $(CA C)_5$	F: ATCG GA AAG AT GTCA ACCAT R: CAA AT TGA AGCC AGT G GTG	157	+	+	+	+	+	+	+
SSRCa 085	$(TC)_{24}$	F: ATG TG AA AAT G GG AAG GA TG R: CAC AGG AA AG TG ACA CGA AG	105	-	-	-	-	-	-	-
SSRCa 086	$(AC)_{11}$	F: AG AG AG AA GCCA TG ATT TG A R: TCAGT CCCA GA GA AT AA GG A	105	-	-	-	-	-	-	-
SSRCa 087	$(TC)_{22}$	F: TCACT CTCGCAG A CACACT AC R: GCA GA GA TG AT CAC AAG TCC	143	4/0.57	3/0.54	4/0.67	3/0.13	1	4/0.53	8/0.65
SSRCa 088	$(TTT TCT)_3$	F: T ACCT CT CCTCCT CCT TCCT R: ATT TCT AT GG ACCG GC AAC	180	3/0.49	3/0.25	3/0.13	2/0.38	2	2/0.38	5/0.47
SSRCa 090	$(GA)_{21}$	F: TGA CTCG ATT ACAT CCCT AA TG R: GT ATT TTG GT TCCCCA TG TT	120	-	-	-	-	-	-	-
SSRCa 091	$(GT)_8(GA)_{10}$	F: CGTCT CGT ATCA CGCTCTC R: TGTTCCT CGTT CCTCTCTCT	110	8/0.82	3/0.48	2/0.35	1/0.00	-	3/0.46	10/0.60
SSRCa 092	$(CCA)_7CT(TCCACC)_5$	F: ATA GCCT GA GCCG TA ACCA R: GG GT AA TT AT G ACG AG GG ACA	142	4/0.64	2/0.38	3/0.15	2/0.38	2	2/0.38	6/0.56
SSRCa 093	$(CT)_{37}$	F: TTG CCT ACAA TACCT GTCTCC R: CCCA ATTCCT CTCCATTCT	196	-	-	-	-	-	-	-
SSRCa 094	$(TC)_4(TTCT)_3$/ $(TTTCCT)_3(TTTC)_5$	F: G TG TCCT AG GG AA GG GT AA G R: GA GT GCT AG GA GA GG GA GA G	195	2/0.30	3/0.59	2/0.38	2/0.38	1	2/0.24	4/0.49
SSRCa 095	$(TG)_{11}$	F: G AG AG A GCCG AG TG AA GA GA R: GA GA GA GA AGC CAT GAT TTGA	185	4/0.45	1/0.00	1/0.00	1/0.00	-	1/0.00	4/0.30
SSRCa 096	$(CT)_{18}$	F: G AAA TGGTG AACTCTCTCTTGG R: ATT TG CAT GG CTT TGG TG	183	+	+	+	+	+	+	+
Mean				3.3/0.46	1.8/0.22	2.2/0.27	1.7/0.22	1.5	2.0/0.26	5.1/0.43

C.a: *Coffea arabica*; C.c: *Coffea canepohora*; HT: Hibrido de Timor; T: Triploid (*C. arabica* x *C. racemosa*); C.r: *Coffea racemosa*; V: rust-resistant commercial varieties; [1]only one accession; (-) no amplification product; (+) primers that amplified multiple bands; * monomorphic primers.

Although developed from *C. arabica*, all polymorphic loci obtained in this study amplified fragments in *C. canephora*, Híbrido de Timor and Triploid accessions. Thirteen of these (59%) were also validated for *C. racemosa* (Table 2). These results demonstrate the potential of these SSR markers for genetic studies in related species of *Coffea*. Cross-species transferability primers in *Coffea* has been observed previously for EST-SSR markers (Bhat et al. 2005, Aggarwal et al. 2007).

According to our results the 22 polymorphic SSR loci showed a considerable Polymorphic Information Content among the accessions of coffee, and can enable molecular advances, especially in genetic studies of the *C. arabica* species. In addition, it was showed that it is possible to use these SSRs for coffee variety identification studies.

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Conteúdo de informação polimórfica de marcadores SSR para *Coffea* spp.

RESUMO - *Trinta e três primers SSR para o gênero* Coffea*, oriundos de biblioteca genômica enriquecida com repetições* $(GT)_{15}$ *e* $(AGG)_{10}$*, foram analisados em 24 acessos de cafeeiros. Vinte e dois primers foram polimórficos entre os acessos; o número de alelos variaram de 2 a 13, com um número médio de 5,1 alelos por primer. Os valores de PIC variaram de 0,08 a 0,79. Os maiores valores médios de PIC foram encontrados para* C. canephora *(0,46), e os menores valores para acessos de* C. arabica *(0,22) e Triplóides (0,22). Os marcadores SSR polimórficos utilizados neste estudo foram úteis para fingerprinting genético em acessos de cafeeiros, especialmente em* C. canephora *e cultivares arábicas resistente a ferrugem.*

Palavras chave: SSR, café arabica, marcadores moleculares, biblioteca genômica enriquecida, PIC.

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