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Molecular Characterization of Arabica and Conilon Coffee Plants Genotypes by SSR and ISSR Markers

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ABSTRACT

The molecular characterization of ten genotypes of the Coffea arabica *plants and of seven genotypes of* C. canephora *having interesting features for coffee breeding programs was carried to select the parents for breeding. A total of 40 SSR and 29 ISSR primers were used. The primers generated a total of 331 (307 polymorphic and 24 monomorphic) bands. Analysis of genetic diversity presented dissimilarity intervals ranging from 0.22 to 0.44 between the Conilon genotypes, from 0.02 to 0.28 between the Arabica genotypes, and from 0.49 to 0.60 between the genotypes of the two species in the joint analysis. Four groups were formed: I = genotypes of* C. arabica, *II = four progenies of* C. canephora, *Conilon group, and one non defined* C. canephora *(Conilon or Robusta), III = one progeny of un-defined* C. canephora *(Conilon or Robusta) and IV = one progeny of* C. canephora *of Robusta group. The grouping formed was consistent with the origins of each group. High stabilities of the bifurcations were found by bootstrap analysis. The use of molecular markers of the SSR and ISSR types in the diversity study was efficient in distinguishing genotypes between and within* C. arabica *and* C. canephora.

Key words: *Coffea Arabica*, *Coffea canephora*, genetic diversity, molecular markers

INTRODUCTION

The domestication of species, culminating in the successive reduction of genetic diversity, can be mitigated through the use of traditional breeding tools, such as the introduction and selection of germplasm, hybridizations, and backcrosses (Hendre and Aggarwal 2007). The selection of superior genotypes of coffee requires an insightful work. The coffee plant is a perennial crop in which many traits are expressed by mutual actions of multiple genes and by their interaction with the environment. This hinders a more immediate identification of the genetic material traits of interest (Ferrão et al. 2007).

Assessing genetic variability has a great importance in breeding programs. Molecular markers allow direct access to the genome sequences and, thus, isolate the genetic variation of the environmental interferences (Ferrão et al. 2007). The potential use of the Simple Sequence Repeats (SSR) and Inter-Simple Sequence Repeats (ISSR), microsatellite primers, has been demonstrated for *Coffea* related with genetic

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diversity of native species and germplasm bank accessions, geographical origin detection, domestication degree, coffee plant dispersion history, species identification and genealogy (Masumbuko and Bryngelsson 2006; Lopez-Gartner et al. 2009; Tshilenge et al. 2009; Missio et al. 2010; Setotaw et al. 2010; Mishra et al. 2011). The good prospects presented by the application of ISSR and SSR markers to assess the diversity in *Coffea* have led to this study. Thus, the aim of the study was the molecular characterization of *C. arabica* and *C. canephora* genotypes by the molecular markers SSR and ISSR to assist in selecting parents for breeding.

MATERIAL AND METHODS

Seventeen coffee genotypes of the breeding program of the Instituto Capixaba de Pesquisa, Assistência Técnica e Extensão Rural (Incaper) were used in the study. Ten Arabica coffee plant materials were collected from the Experimental Farm of Venda Nova do Imigrante-ES and seven of Conilon coffee plants were collected from the Bananal do Norte Experimental Farms-ES. Some distinctive characteristics of the materials are presented in Table 1.

Young, healthy, and fully-expanded leaves from the 17 genotypes were collected in the field and used for DNA extraction using the protocol proposed by Doyle and Doyle (1990) modified by Abdelnoor et al. (1995) for *C. canephora* and the protocol proposed by Diniz et al. (2005) for *C. arabica* with slight modification in which the leaves were macerated in liquid nitrogen, rather than lyophilized. The quality and quantity of the DNA extracted were tested on 0.8% agarose gel in 1X TBE buffer (pH 8.3) (Tris-Borate-EDTA), stained with ethidium bromide (0.25/mL) and visualized under UV light.

Table 1 - List of the genetic materials of *Coffea arabica* and *Coffea canephora* used in the study of molecular characterization.

Coffea arabica	
Genetic materials	**Short description**
'Bourbon Amarelo'	Progeny selected in a 'Bourbon Vermelho' tillage, susceptible to rust, high.
'Bourbon Amarelo Nanicão'	Progeny selected in a 'Bourbon Vermelho' tillage, susceptible to rust, short.
'Icatu Amarelo'	Progeny originated from *C. arabica* x *C. canephora* natural crossing, moderately resistant to rust, tall.
'Caturra Amarelo'	Progeny selected in a 'Bourbon' tillage, susceptible to rust, short.
'Mundo Novo'	Progeny originated from 'Sumatra' x 'Bourbon Vermelho'natural crossing, susceptible to rust, high.
'Catuaí Vermelho'	Progeny originated from 'Caturra Vermelho' x' Mundo Novo' artificial crossing, susceptible to rust, short.
'Iapar 59'	Progeny originated from 'Villa Sarchi' x 'Híbrido de Timor' artificial crossing, rust resistant, short.
'Bourbon Vermelho I'	Progenies selected in an over 40 years tillage in ES, high adaptability, high.
'Bourbon Vermelho II'	
'Bourbon Vermelho Nanicão'	Progeny selected in an over 40 years tillage in ES, high adaptability, short.
Coffea canephora	
'Progênie 03'	Improved clone, Conilon characteristics, early maturation.
'Progênie 83'	Improved clone, Conilon/Robusta characteristics, intermediate to late maturation.
'Progênie 153'	Improved clone, Conilon characteristics, late maturation.
'Progênie Bukobensis04'	Improved clone, Conilon characteristics, small leaf, thin and light, drought tolerant, early maturation.
'Progênie Robusta IAC 2286-3'	Improved clone, Robusta characteristics, late maturation.
'Progênie Robusta Precoce'	Clone introduced by producer, Conilon/Robusta characteristics, super early maturation.
'Progênie Conilon JP'	Clone selected by producer, Conilon characteristics, early maturation.

To analyze the genetic diversity of the genotypes of *C. arabica* and *C. canephora*, heterologous amplification of 71 SSR primer pairs was developed for *C. arabica* in the *C. canephora* genotypes (data not published). To carry out the analyses in all the genetic materials, primers

showing amplification of well-defined bands were selected. Thus, 40 SSR primers, available in the literature, were used (Combes et al. 2000; Rovelli et al. 2000; Baruah et al. 2003; Coulibaly et al. 2003; Moncada and Mccouch 2004; Poncet et al. 2004; Missio et al. 2009a), as well as 29 ISSR primers of the UBC series in the 17 *Coffea* genotypes (Tables 2 and 3).

PCR reactions for the SSR markers were made in 20 µL containing 2.0 µL of 10x buffer, 150 mM/L of dNTP, 0.1 mM /L of each primer, 50 ng of DNA, 1 mM / L MgCl2, 0.6 U Taq DNA polymerase and the remaining volume was completed with water (MISSIO et al. 2009b). The amplification reactions of the SSR primers were performed using a 96 Veriti Applied Biosystems thermocycler by a touchdown PCR procedure as Missio et al. 2009b modified. This consisted of initial denaturation at 94°C/2 min, followed by 10 cycles of denaturation at 94°C/ 30 s, annealing of 66°C to 57/30 s, decreasing 1°C in each cycle, and extension at 72°C/30 s. The last 30 cycles were 94°C/30 s 57°C/30 s and 72°C/30 s, followed by a final extension at 72°C/8 min. The samples were run on polyacrylamide gel (6%) in the presence of 1X TBE buffer. The electrophoretic separation was done for 2h30 min at 100 volts. At the end of the run, the gels were stained in ethidium bromide solution (0.25 mg/mL).

The amplification reactions of the ISSR primers were performed in a Analítica/Techne/TC-412 thermocycler in a total volume of 20 µL containing 2.0 µL of 10x buffer, 1 mM/L of each dNTP, 0.2 mM/L primer, 30 ng DNA, 5 mM/L MgCl2, 1U of Taq DNA polymerase and the remainder of the volume was completed with water. The program used consisted of initial denaturation at 94°C/4 min, 35 cycles of denaturation at 94°C/1 min, annealing at 52°C/1 min, extension at 72°C/2 min, followed by final extension at 72°C/7 min. The fragments were applied on 2% agarose gel. All the images of the fragments obtained in the gels were photo-documented (Locus Biotechnology) using the LPIx Image program.

To record the molecular data for the markers, a binary values matrix was prepared, where code " 0" referred to absence and "1" to presence of amplification of a fragment (Cruz 2008). This strategy allowed the performance of a statistical analysis of diversity between the species of different ploidy and of dominant and multi-allelic markers (Powell et al. 1996; Souza et al. 2008; Vieira 2010). The genetic similarity values were estimated by the complement of the Jaccard similarity index and clustering analysis by the Unweighted Pair Group Method Arithmetic Mean (UPGMA). The coefficient of variation obtained with the bootstrap analysis was measured to verify the degree of association between the genetic similarity matrix and the dendrogram was generated by this re-sampling method with 1000 simulations. Thus, it was possible to evaluate the stability of bifurcations formed with the aid of the program Genes 2011.9.0 (Cruz 2008).

Table 2 - List of the 29 ISSR primers used for molecular characterization of *Coffea arabica* and *Coffea canephora*.

Primer	Sequência (5'- 3')	Primer	Sequência (5'- 3')
UBC808	AGAGAGAGAGAGAGAGC	UBC857	ACACACACACACACACYG
UBC810	GAGAGAGAGAGAGAGAT	UBC859	TGTGTGTGTGTGTGTGRC
UBC813	CTCTCTCTCTCTCTCTT	UBC862	AGCAGCAGCAGCAGCAGC
UBC818	CACACACACACACACAG	UBC864	ATGATGATGATGATGATG
UBC819	GTGTGTGTGTGTGTGTA	UBC868	GAAGAAGAAGAAGAAGAA
UBC827	ACACACACACACACACG	UBC873	GACAGACAGACAGACA
UBC834	AGAGAGAGAGAGAGAGYT	UBC875	CTAGCTAGCTAGCTAG
UBC841	GAGAGAGAGAGAGAGAYC	UBC879	CTTCACTTCACTTCA
UBC842	GAGAGAGAGAGAGAGAYG	UBC880	GGAGAGGAGAGGAGA
UBC843	CTCTCTCTCTCTCTCTRA	UBC881	GGGGTGGGGTGGGGTG
UBC845	CTCTCTCTCTCTCTCTRG	UBC889	DBDACACACACACACAC
UBC848	CACACACACACACACARG	UBC890	VHVGTGTGTGTGTGTGT
UBC849	GTGTGTGTGTGTGTGTYA	UBC895	AGGTCGCGGCCGCNNNNNNAT
UBC854	TCTCTCTCTCTCTCTCRG	UBC899	CATGGTGTTGGTCATTGTTCCA
UBC855	ACACACACACACACACYT		

Table 3 - List of the 40 pairs of SSR primers used for molecular characterization of *Coffea arabica* e *Coffea canephora*.

Primer	*Forward* primer (5'−3')	*Reverse* primer (5'−3')
M764*	CTGGCATTAGAAAGCACCTTG	GCTTGGCTCACTGTAGGACTG
M793*	CTGAGCGCATGGAAGGAGTA	GGAGACGCAGGTGGTAGAAG
M804*b*	TGGGTTGGTTCTGATTTTGG	CCTCCCTCTCTCCCTGACTC
M747	CCCCAACCTCATGTCTCTGT	GAGTTTTGCGTGTGTGTGCT
CM5*	GTAACCACCACCTCCTCTGC	TGGAGGTAACGGAAGCTCTG
CM11	AATCACCTTCGCAAACCAAC	CCGAACGCAATATCTTATGC
CM17	CCAGCCTTTTCACAATTCTCACCC	TGCCCCCTAGATATGGTACAAGCTTTC
CFGA54*	AGTAATGAACCTGCCGCCTCTTT	TTGTCATTCTTGTGTTTTCCATCC
CFGA99	ATTCGACGACTCCAAAGCATA	CCTTGCTGGCCCTTCCTT
CFGA189	CATCCATCCGAAAACTTGTAACG	CAGCACTGGCAAATAGCAACTCTT
CFGA222	GGGACCCACTAGTGCGAAAAG	CCTTACCTTTCCAACAACTTCACA
CFGA236	TTTCTCGTCTTCCATTCCAGT	TGTACCACGTCTATCACCAATG
E6-3CTG	CTGGGTTGGTTCTGATTTTG	GGTTCCCAGAGATTCTCTCC
SSRCa021*	GCTGAGAGTTTTGAGGGAAA	CCGACGTAGTTGATGATTGA
SSRCa033	GTTTTTACGCGCACGATTA	TTCAAAAGTCAACTCATTCTCC
SSRCa040*	AGGGATGTAGAACCAGCAAA	CCAATAGCTCACAACAAAGG
SSRCa045	GACTTGTTGCATTCCCCTA	GCGCATGTGAAGAGAAAGT
SSRCa052*	GATGGAAACCCAGAAAGTTG	TAGAAGGGCTTTGACTGGAC
SSRCa054*	CCGAACCCAACTAACATCTC	GCAGGTCTTCCATTGTCTGT
SSRCa091*	CGTCTCGTATCACGCTCTC	TGTTCCTCGTTCCTCTCTCT
SSRCa006*	CTTGCTCAGTGAACCATCC	TGCCTCTTATGCCACTACTAAA
SSRCa016	AGCAGATTCCATCCTTATCCT	CCACTAATCCATTCCATTCC
SSRCa055	AAGGAAAACAACACCCAAGA	CGAGACAAGAGAGGGGAAA
SSRCa061	GCAGGTGCAAGTGATAAAAG	CGTCTTGTGATGTGTTAGGG
SSRCa065	ATCTAACAAAATCCCCGTCA	ATCGGTCGCCCTTCTAAT
SSRCa084*	ATCGGAAAGATGTCAACCAT	CAAATTGAAGCCAGTGGTG
SSRCa085*	ATGTGAAAATGGGAAGGATG	CACAGGAAAGTGACACGAAG
SSRCa087*	TCACTCTCGCAGACACACTAC	GCAGAGATGATCACAAGTCC
SSRCa088*	TACCTCTCCTCCTCCTTCCT	ATTTCTATGGACCGGCAAC
SSRCa095*	GAGAGAGCCGAGTGAAGAGA	GAGAGAGAAGCCATGATTTGA
SSRCa096	GAAATGGTGAACTCTCTCTTGG	ATTTGCATGGCTTTGGTG
SSRCa018*	GTCTCGTTTCACGCTCTCTC	ATTTTTGGCACGGTATGTTC
SSRM20*	CTTGTTTGAGTCTGTCGCTG	TTTCCCTCCCAATGTCTGTA
SSRM24*	GGCTCGAGATATCTGTTTAG	TTTAATGGGCATAGGGTCC
SSRM25*	CCCTCCCTGCCAGAAGAAGC	AACCACCGTCCTTTTCCTCG
SSRM32*	AACTCTCCATTCCCGCATTC	CTGGGTTTTCTGTGTTCTCG
SSRM3	ATTCTCTCCCCCTCTCTG	TGTGTGCGCGTTTTCTTG
SSRM11	ACCCGAAAGAAAGAACCAAG	CCACACAACTCTCCTCATTC
SSRM27	AGGAGGGAGGTGTGGGTGAAG	AGGGGAGTGGATAAGAAGG
SSRM47*	TGATGGACAGGAGTTGATGG	TGCCAATCTACCTACCCCTT

*Polymorphic primers

RESULTS

The analysis of polymorphism of the fragments generated resulted in 307 polymorphic and 24 monomorphic bands. The 331 bands were used in the analyses of similarity between the genetic materials. Of the total number of fragments analyzed, 47% were polymorphic only for Conilon, 33% differentiated only the Arabica genotypes and 17% of the bands were efficient in detecting differences between the genotypes of the two species. There were significant differences between *C. canephora* and *C. arabica*. The analysis of the genetic diversity of *C. canephora* and *C. arabica* by the complement of Jaccard similarity index generated dissimilarity intervals ranging from 0.22 to 0.44 between the Conilon coffee genotypes, 0.02 to 0.28 between the arabica coffee genotypes, and 0.49 to 0.6 between the genotypes of the two species (Fig. 1)

The molecular bands obtained in this study were also used to construct a dendrogram based on Unweighed Pair Group Method Arithmetic Mean (UPGMA), using the genetic distances between the genotype pairs (Fig. 2). The dendrogram building by the UPGMA method for the 17 genotypes divided them into groups, where the genotypes of each species, *C. arabica* and *C. canephora*, were maintained separated. Considering the origin of the materials, the value 0.60 was used as a cutoff point in the dendogram.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
1	0	0.2222	0.3522	0.3636	0.3931	0.3721	0.2895	0.5475	0.5526	0.5548	0.5976	0.576	0.5548	0.5329	0.5688	0.5633	0.5461
2		0	0.3416	0.3333	0.4221	0.3256	0.2667	0.5402	0.5395	0.5613	0.575	0.5696	0.5667	0.5333	0.5688	0.5669	0.549
3			0	0.3036	0.3855	0.3592	0.2756	0.5306	0.5305	0.5333	0.5523	0.5361	0.513	0.5183	0.5361	0.5389	0.5217
4				0	0.4011	0.3741	0.3089	0.526	0.5345	0.5367	0.5807	0.5568	0.5394	0.5668	0.558	0.5665	0.5476
5					0	0.3985	0.4436	0.5472	0.573	0.5611	0.5659	0.5682	0.5602	0.5568	0.5556	0.5607	0.5529
6						0	0.3364	0.5339	0.5238	0.5313	0.5839	0.5482	0.5539	0.5231	0.5468	0.5482	0.5338
7							0	0.5192	0.5339	0.5424	0.5593	0.5424	0.5268	0.4907	0.5254	0.5339	0.5089
8								0	0.2289	0.1195	0.1503	0.0993	0.1133	0.1361	0.1307	0.1494	0.1342
9									0	0.1913	0.2732	0.2197	0.2395	0.2686	0.2486	0.2647	0.2814
10										0	0.2011	0.1024	0.1304	0.1754	0.1404	0.1807	0.1625
11											0	0.1751	0.1627	0.2135	0.1864	0.237	0.2108
12												0	0.0255	0.0864	0.0808	0.0932	0.0779
13													0	0.053	0.0932	0.1139	0.0909
14														0	0.0988	0.1358	0.109
15															0	0.052	0.0641
16																0	0.0892
17																	0

Figure 1 - Genetic dissimilarity building for the 17 *Coffea* a*rabica* and *Coffea canephora* genotypes by the Jaccard index.

1- ES03; 2- ES83; 3- ES153; 4- Bukobensi 04; 5- Robusta IAC 2286-3; 6- Robusta Precoce; 7- Conilon JP; 8- Bourbon Amarelo; 9- Bourbon Amarelo Nanicão; 10- Icatu Amarelo; 11- Caturra Amarelo; 12- Mundo novo; 13- Catuaí Vermelho IAC 81; 14- Iapar 59; 15- Bourbon Vermelho I; 16- Bourbon Vermelho II; 17- Bourbon Vermelho Nanicão. The dotted line delimits the genetic dissimilarity values within *C. canephora*, between *C. canephora* and *C. arabica* and within *C. arabica*.

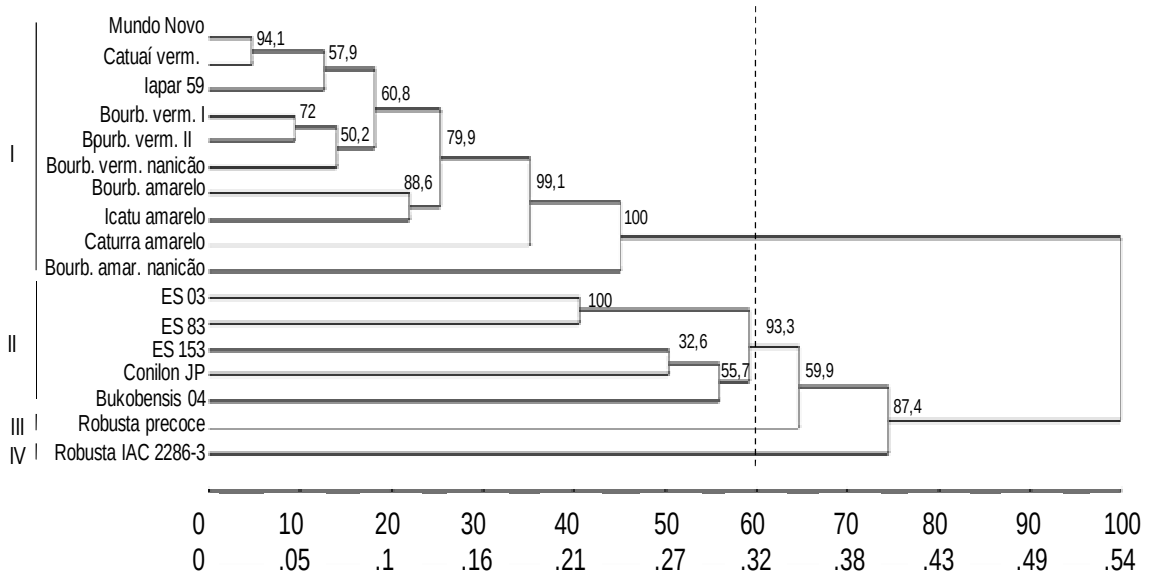

Figure 2 - Dendrogram building by the UPGMA method based on the genetic distances expressed by the Jaccard coefficient index estimated for 17 genotypes of *Coffea*.

To validate the results, the optimal number of binary markers was estimated, using the Genes program (Cruz 2008). The correlation was calculated between the Jaccard of Mi, genetic distance matrix, Mi sampled bands ($2 \leq Mi \leq 307$), and the original distance matrix with 307 bands. The correlation of 0.9 corresponded to 224 alleles. This number of bands was sufficient to generate a dendrogram similar to that obtained by the 307 bands considered in this study (Fig. 3).

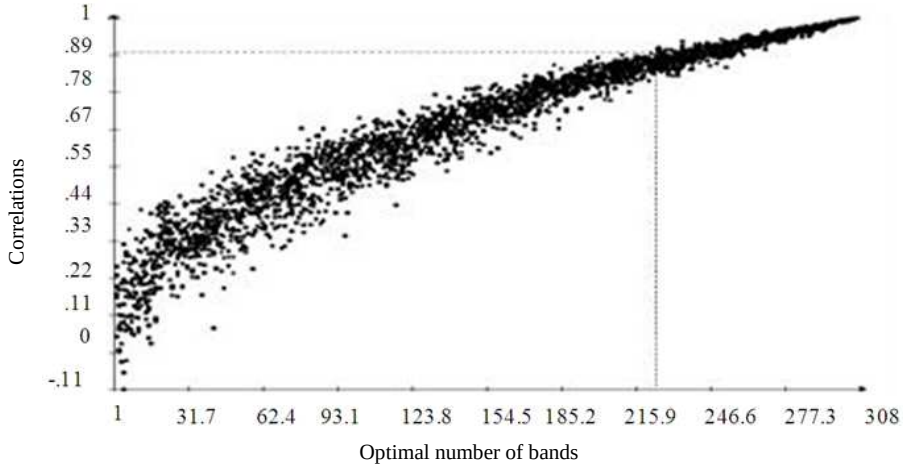

Figure 3 - Optimal number of bands generated by a set of SSR+ISSR primers to assess the genetic diversity of 17 genotypes of *Coffea*. The dots represent the projection of the correlations between the number of M_i bands sampled ($2 \leq M_i \leq 307$) and the original matrix with M bands (M = 307).

DISCUSSION

The low genetic diversity among the *C. arabica* genotypes evaluated was expected. This was due to the process of autogamy and narrow genetic base, resulting from the process of homozygosis and the successive selection cycles (Moncada and Mccouch 2004; Missio et al. 2009b). This was also confirmed by the fact that the arabica genetic materials were probably derived from a few seeds that survived the efforts of expanding the Yemen borders for the cultivation of coffee plants, reaching the present cultivation sites (Vossen 1985).

The *C. canephora* genotypes used in this study showed greater genetic diversity among themselves, with the most dissimilar being 5 & 7, 5 & 2 and 5 & 4, and the most similar being 1 & 2, 2 & 7, 3 &7 and 1 & 7. The genetic dissimilarity obtained with molecular data for the ES Bukobensis 04 progeny, compared to the Conilon group, was also phenotypically observed in the field (data not published). The considerable genetic variability found in *C. canephora* allowed the selection of superior genotypes for different traits, such as broad adaptation, high yield, plant height, resistance to diseases, pests, abiotic stress, quality, etc. (Hendre and Aggarwal 2007). Despite the low dissimilarity found for *C. arabica*, the ISSR and SSR markers were efficient in distinguishing the genotypes studied and the results obtained were in agreement with the genealogy of the materials.

Based on the *cutoff* point, four groups were formed. Group I was composed of 'Mundo Novo', 'Catuaí Vermelho', Iapar 59, 'Bourbon Vermelho I', 'Bourbon Vermelho II', 'Bourbon Vermelho Nanicão', 'Bourbon Amarelo', 'Icatu Amarelo', 'Caturra Amarelo', and 'Bourbon Amarelo Nanicão' ; Group II was composed of ES 03, ES 83, ES 153, Conilon JP, Bukobensi 04; group III was composed of 'Robusta Precoce'; and group IV of Robusta IAC 2286-3. The grouping formed was consistent with the origins of each group. High stability of the bifurcations was found by the means of bootstrap analysis per 1000 re-samplings, with most presenting values above 50%. 'Catuaí Vermelho IAC 81' originated from the 'Caturra' x 'Mundo Novo' cross, shared many bands with the parental 'Mundo Novo' and was approximately 30% distant from 'Caturra Amarelo'. Small genetic differences were

expected between ‘Catuaí’ and ‘Mundo Novo’, as ‘Catuaí’ originated from the cross between ‘Caturra’ and ‘Mundo Novo’ (Carvalho and Monaco 1972). ‘Mundo Novo’ (Sumatra x ‘Bourbon Vermelho’) was also grouped with the parental ‘Bourbon Vermelho’.

Among the Bourbon progenies used in this study, the molecular bands were efficient in separating ‘Bourbon Vermelho’, ‘Bourbon Vermelho Nanicão’, ‘Bourbon Amarelo’ and ‘Bourbon Amarelo Nanicão’. The genetic distance between ‘Bourbon Vermelho’ and ‘Bourbon Amarelo’ was due to the fact that the latter originated from the spontaneous cross between the wild cultivars ‘Bourbon Vermelho’ and ‘Amarelo de Botucatu’ (Carvalho et al. 1957). This greater genetic variability could be associated with the fact that progeny ‘Bourbon Amarelo’ was not submitted to intense crossing cycles and selection in the breeding programs (Maluf et al. 2005).

The *C. canephora* genotypes were analyzed in relation to the groups of origin. The grouping was consistent with the origin of the genotypes (Conilon or Robusta). The progenies ES83 and ‘Robusta Precoce’, which presented phenotypic characteristics intermediary to the two groups (Conilon or Robusta), were more similar to the Conilon genotypes. Among the materials studied, the largest genetic divergence found in the C. *canephora* species was the progenies ‘Robusta IAC 2286-3’ and ‘Conilon JP’, while in the species *C. arabica*, the highest diversity was between ‘Bourbon Vermelho Nanicão’ and ‘Bourbon Amarelo Nanicão’.

CONCLUSIONS

The use of molecular markers of the SSR and ISSR types in the study of diversity was efficient in carrying out the molecular characterization of coffee genotypes between and within *C. arabica* and *C. canephora*. The results showed that microsatellites markers were efficient in estimating the genetic similarity and could be used to increase the efficiency in classifying the materials and selecting the candidates for parental crosses.

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