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ARTICLE

Molecular diversity in *Coffea canephora* germplasm conserved and cultivated in Brazil

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Abstract – *This work aimed to characterize accessions that represent the* C. canephora *germplasm conserved and cultivated in Brazil. A total of 130 accessions from germplasm banks of IAC (São Paulo), UFV (Minas Gerais) and also collected in plantations of the State of Espírito Santo and Rondônia were evaluated with a set of 20 new microsatellite primers. Multivariate methods were used to estimate the relationship among the accessions. High level of polymorphism and two major diversity clusters were identified. First cluster was composed by the accessions conserved in the IAC and UFV collections and the second was formed by accessions collected in areas under cultivation. Accessions from Espírito Santo and Rondônia were clear separated, composing two subclusters. Despite the great polymorphism found in Brazilian plantations, the diversity may be increased, because a new threshold in the genetic gains is expected on breeding programs with the intensification of the use of conserved germplasm.*

Key words: *Robusta coffee, genetic diversity, microsatellite marker.*

INTRODUCTION

Coffea canephora Pierre ex. Froehner presents a wide genetic variability, with one of the widest geographic natural distribution within the subgenus *Coffea* (Maurin et al. 2007). Likewise most diploid species in genus *Coffea*, *C. canephora* is allogamous and presents a self-incompatibility system.

Brazil is the second largest producer of *C. canephora*, producing about 25% of the world yield (USDA 2012). The States of Espírito Santo and Rondônia are responsible for over 75% of the production (CONAB 2013). In that country, main *C. canephora* germplasm collections are placed in governmental institutions, where breeding programs are developed, i.e.: Instituto Agronômico de Campinas (IAC), in São Paulo; Universidade Federal de Viçosa (UFV), in Minas Gerais; Instituto Capixaba de Pesquisa, Assistência Técnica e Extensão Rural (Incaper), in Espírito Santo and Embrapa (Embrapa Rondônia), in Rondônia. The germplasm conserved in IAC and UFV are mainly composed by accessions introduced from Africa, after FAO expeditions in that continent during the last century (Silvestrini et al. 2008, Fazuoli et al. 2009). On the other hand, Incaper and Embrapa Rondônia have collected a great amount of accessions in plantations from their respective states (Ferrão et al. 2007a, Souza and Santos 2009). As a consequence, those four institutions harbor a representative sample of the germplasm conserved or grown in that country.

Brazilian *C. canephora* accessions were studied using phenotypic traits (Fonseca et al. 2006, Ivoglo et al. 2008, Souza and Santos 2009) and RAPD markers (Ferrão et al. 2007b, Silvestrini et al. 2008). Those studies have confirmed that there is a wide variability within the germplasm maintained in the Brazilian collections. However, despite the advantages of microsatellites - *e.g.*: high reproducibility, multi-allelic locus, co-dominant inheritance, high degree of polymorphism, relative abundance and good coverage of the genome (Powell et al. 1996) - there are a few works using these markers to investigate *C. canephora* diversity. Furthermore, there is no report of comparisons about the diversity among accessions from different institutions, providing a well representative coverage of this germplasm.

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Thus, this study aimed to characterize accessions representing the germplasm cultivated in Brazilian plantations and conserved in research institutions, in order to propose guide lines to management of gene banks and breeding strategies.

MATERIAL AND METHODS

Plant material

A total of 130 accessions of *C. canephora* (Table 1) were genotyped. These accessions comprise a good sample of the germplasm used in the Brazilian breeding programs. Forty three accessions were obtained from IAC (18 belonging to varietal group Kouillou and 25 to varietal group Robusta) and 11 accessions were obtained from UFV. The other accessions were collected by Incaper and Embrapa in traditional coffee producing areas at Espírito Santo (40 accessions) and Rondônia (36 accessions). Accessions of *C. arabica* and Híbrido de Timor (*C. arabica* x *C. canephora*) were included in the analysis as out group species.

DNA extraction

Young and completely extended leaves were collected from each accession, frozen at –80 ºC, lyophilized, ground to become a fine powder and kept at –20 ºC until used. Genomic DNA was extracted using the method described by Diniz et al. (2005) and all DNA samples were prepared to a final concentration of 25 ηg μL^{-1}.

Microsatellite markers

Twenty new microsatellites were used in this study. These DNA markers were developed from non-redundant Express Sequence Tags (EST) of the Brazilian Coffee Genome Project (Table 2), in Coffee Biotechnology Lab (BIOCAFE – UFV).

Each reaction was set to a final volume of 20 µL, containing 50 ηg of genomic DNA, 0.6 unit of *Taq* DNA polymerase, *Taq* buffer 1x, 1mM of $MgCl_2$, 150 µM of each dNTP and 0.1 µM of each primer. PCR amplifications were carried out using touchdown proceeding, which comprises initial denaturation at 94 ºC for 2 min, followed by 10 cycles of denaturation at 94 ºC for 0.5 min, annealing at 67ºC for 0.5 min, decreasing 1 ºC after each cycle, and extension at 72 ºC for 0.5 min. After that, another set of 30 cycles, comprising denaturation at 94 ºC for 0.5 min, annealing at 55 ºC for 0.5 min and extension at 72 ºC for 0.5 min, was accomplished followed by a final 8 min extension time at 72 ºC. Before electrophoresis, PCR products were denatured in 8 µL of denaturing dye (95% formamide) at 94 ºC for 5 min and 7 µL of sample were loaded on a standard 6% polyacrylamide gel at 50 ºC and run at a constant power of 90 W for about 2 h. Post-PCR multiplex, which involved the multi-loading of individual PCR assays (two to four SSRs per running), was performed spacing successive loads by 10 to 30 min during electrophoresis, depending on the prior information about fragment size. At last, the gel was treated with ethanol (10%) + acetic acid (1%), followed by nitric acid (1,5%); stained with silver nitrate (4%), developed with sodium carbonate (3%) and formaldehyde (0.03%); and fixed with acetic acid (5%) and dried for posterior analysis at a transilluminator apparatus.

Data analysis

The evaluation of each locus was performed considering homozygotes and heterozygotes, individuals which showed one or two alleles, respectively. The dissimilarity between the accessions was estimated based on the complement ($1 - S_{ii'}$) of the weighted coincidence index, using the equation: $S_{ii'} = \frac{1}{2}\sum_{j=1}^{L} p_j c_j$, where $S_{ii'}$ is the similarity between the accessions *i* and *i'*; *L* is the total number of loci; c_j is the number of common alleles between *i* and *i'*; and p_j is the weight associated to *j* locus, obtained by a_j/A, being a_j, the number of alleles in locus *j* and A total number of alleles. Principal Coordinate Analysis (PCoA) was performed to view the overall diversity. The dissimilarity matrix was also represented in a dendrogram based on the unweighted pair-group method using arithmetic averages (UPGMA) to establish genetic relations among accessions. Goodness-of-fit of the tree was tested comparing cophenetic value matrix, with the original dissimilarity matrix. Statistical procedures were accomplished using the software packages: Genes (Cruz 2001), Darwin 5.0 (Perrier et al. 2003) and NTSYS-pc (Rohlf 1998).

RESULTS AND DISCUSSION

Genetic relationships among the 130 accessions of *C. canephora* and others species of the genus *Coffea* were evaluated with UPGMA clustering technique (Figure 1). The cophenetic correlation was high (81.5%), and the levels of stress and distortion were low (1.4% and 11.8%, respectively), demonstrating that the dendrogram satisfactorily represents the original matrix of dissimilarities. C. *arabica* and Híbrido de Timor, as expected, composed an out group, confirming the efficacy of the new microsatellites to distinguish different species in genus *Coffea*. Accessions of germplasm collections and those sampled in plantations formed distinct cluster, but no logical subdivisions were observed inside each one. IAC and UFV accessions appeared merged on upper cluster and Rondônia and Espírito Santo accessions were also mixed in lower cluster.

The overall diversity among the 130 accessions of *C. canephora* was also represented in the bi-dimensional

Table 1. List of *Coffea sp* accessions genotyped in this study

Accession	Code	AS[1]	Accession	Code	AS[1]	Accession	Code	AS[1]
Kouillou IAC66-1.1	K66-11	1	Robusta UFV 3587.3	R35873	2	Encapa 07	ES07	4
Kouillou IAC 66-1.2	K66-12	1	Robusta UFV 3751.1	R37511	2	Encapa 14	ES14	4
Kouillou IAC 66-1.3	K66-13	1	Robusta UFV 3751.2	R37512	2	Encapa 16	ES16	4
Kouillou IAC 66-3.1	K66-31	1	Robusta UFV 3754.1	R37541	2	Encapa 19	ES19	4
Kouillou IAC 66-3.2	K66-32	1	Robusta UFV 3754.2	R37542	2	Encapa 28	ES28	4
Kouillou IAC 68-7.1	K68-71	1	Robusta UFV 3755.1	R37551	2	Encapa 104A	ES104A	4
Kouillou IAC 68-7.2	K68-72	1	Robusta UFV 3755.2	R37552	2	Encapa 104B	ES104B	4
Kouillou IAC 68-7.3	K68-73	1	Robusta UFV 3755.3	R37553	2	Encapa 106	ES106	4
Kouillou IAC 69-15	K69-15	1	Cpafro 010	RO010	3	Encapa 110A	ES110A	4
Kouillou IAC 69-5.1	K69-51	1	Cpafro 016	RO016	3	Encapa 110B	ES110B	4
Kouillou IAC 69-5.2	K69-52	1	Cpafro 022	RO022	3	Encapa 112	ES112	4
Kouillou IAC 69-5.3	K69-53	1	Cpafro 024	RO024	3	Encapa 116	ES116	4
Kouillou IAC 70-1.1	K70-11	1	Cpafro 036	RO036	3	Encapa 120	ES120	4
Kouillou IAC 70-1.2	K70-12	1	Cpafro 044	RO044	3	Encapa 132	ES132	4
Kouillou IAC 70-1.3	K70-13	1	Cpafro 045	RO045	3	Encapa 139	ES139	4
Kouillou IAC 70-14.1	K70-141	1	Cpafro 056	RO056	3	Encapa 143	ES143	4
Kouillou IAC 70-14.2	K70-142	1	Cpafro 063	RO063	3	Encapa 148	ES148	4
Kouillou IAC 70-14.3	K70-143	1	Cpafro 077	RO077	3	Encapa 149	ES149	4
Laurenti.1	Laur1	1	Cpafro 085	RO085	3	Encapa 154	ES154	4
Laurenti.2	Laur2	1	Cpafro 086	RO086	3	Encapa 201	ES201	4
Apoatã IAC 2258.1	Apo1	1	Cpafro 089	RO089	3	Encapa 26	ES26	4
Apoatã IAC 2258.2	Apo2	1	Cpafro 098	RO098	3	Encapa 29	ES29	4
Apoatã IAC 2258.3	Apo3	1	Cpafro 100	RO100	3	Encapa 36	ES36	4
Robusta IAC 640.1	R6401	1	Cpafro 103	RO103	3	Encapa 45	ES45	4
Robusta IAC 640.2	R6402	1	Cpafro 119	RO119	3	Encapa 49	ES49	4
Robusta IAC 640.3	R6403	1	Cpafro 127	RO127	3	Encapa 99	ES99	4
Robusta IAC 1641.1	R16411	1	Cpafro 138	RO138	3	Encapa V.1	ESV1	4
Robusta IAC 1641.2	R16412	1	Cpafro 140	RO140	3	Encapa V.2	ESV2	4
Robusta IAC 1655.1	R16551	1	Cpafro 142	RO142	3	Encapa V.3	ESV3	4
Robusta IAC 1655.2	R16552	1	Cpafro 143	RO143	3	Encapa V.4	ESV4	4
Robusta IAC 1675.1	R16751	1	Cpafro 147	RO147	3	Encapa V.5	ESV5	4
Robusta IAC 1675.2	R16752	1	Cpafro 155	RO155	3	Encapa V.6	ESV6	4
Robusta IAC 1675.3	R16753	1	Cpafro 156	RO156	3	Encapa V.7	ESV7	4
Robusta IAC 2257.1	R22571	1	Cpafro 160	RO160	3	Encapa V.9	ESV9	4
Robusta IAC 2257.2	R22572	1	Cpafro 164	RO164	3	Encapa V.10	ESV10	4
Robusta IAC 2259.1	R2259	1	Cpafro 183	RO183	3	Encapa V.11	ESV11	4
Robusta IAC 2286.1	R22861	1	Cpafro 184	RO184	3	Encapa V.12	ESV12	4
Robusta IAC 2286.2	R22862	1	Cpafro 189	RO189	3	Encapa V.13	ESV13	4
Robusta Col - 10.1	Rcol-101	1	Cpafro 190	RO190	3			
Robusta Col - 10.2	Rcol-102	1	Cpafro 193	RO193	3	C. arabica var. Typica UFV 2945	*Carabica*	2
Robusta Col - 10.3	Rcol-103	1	Cpafro 194	RO194	3			
Robusta Col - 5.1	Rcol-51	1	Cpafro 196	RO196	3			
Robusta Col - 5.2	Rcol-52	1	Cpafro 199	RO199	3	Híbrido de Timor CIFC 1343/269	HibTimor	2
Robusta UFV 3580	R3580	2	Cpafro 203	RO203	3			
Robusta UFV 3587.1	R35871	2	Encapa 02	ES02	4			
Robusta UFV 3587.2	R35872	2	Encapa 03	ES03	4			

[1] Accession source: 1) Coffee Germplasm Collection of Instituto Agronômico de Campinas (IAC), São Paulo; 2) Coffee Germplasm Collection of Universidade Federal de Viçosa (UFV), Minas Gerais; 3) Accessions collected in commercial coffee fields in Rondônia State, by Embrapa, and 4) in Espírito Santo State by the Instituto Capixaba de Assistência Técnica, Pesquisa e Extensão Rural (INCAPER).

Table 2. Identification, sequences of forward and reverse primer, temperature of melt and allele size for 28 microsatellites from *Coffea canephora*

Id		Foward Primer	Tm (°C)	Reverse *Primer*	Tm (°C)	Allele size (bp)
EST-SSR 001	AC 01	GAAGACCAAGCACCCTCAAC	59.4	ACACCAACTACGGGCAGACA	59.4	151
EST-SSR 002	AC 02	GAAGGGACAAAGACGCCTAA	57.3	CGACAGATGCAGGAATAAACTG	58.4	184
EST-SSR 003	AC 03	TGAATGGTCATGGCAGGTAAG	57.9	AATCGAATCACAGACCCACTC	57.9	244
EST-SSR 010	AC 13	CTTCTTCATCCAACAACACG	49.6	TGCCATTCCACTGTGTCACT	51.7	152
EST-SSR 014	AC 17	CCTGTTAGAGCTGCTTCTCG	53.7	TCTTCAGATCCGGAGGTTGG	53.7	160
EST-SSR 017	AT 02	TTGAGTGCCAGCATTAGTTG	55.3	TAGAAGGGAGAAGGGCAGGA	59.4	288
EST-SSR 019	AT 04	GGGTCAAATGGCTAATGTTGCT	58.4	CATCGGCTGAAACCTCTCGT	59.4	199
EST-SSR 022	AT 08	TCCAGTCGTCCAATCCAAAC	57.3	CCCACATTTCTTGCCTTCCA	57.3	155
EST-SSR 025	AT 12	AGATACCCACCGCCTAATCCT	54.2	GCAACAACTTCTGCTCATCC	51.7	108
EST-SSR 026	AT 14	TCCGTTCCGGGCTTATGAT	51.0	AAACAGACGCAGATCCCAGA	51.7	224
EST-SSR 027	AT 15	ATGGAAGTGTCCTTGTCGTG	51.7	ATGTCGGTGGGTCGGTCAAA	53.7	259
EST-SSR 033	AT 23	AGTCCTTGGCACTTGCTTT	48.8	CAGACAACGATCAATACCTTCC	52.9	200
EST-SSR 036	AT 26	AGCTGCTGATGGTGTGAAGG	53.7	GCCCAAGTCCAGCTTACATTTC	54.7	271
EST-SSR 039	AAC 01	GCACAATCCTCGATCTCAACA	57.9	TAAAGAACAGAGCCGCCACA	57.3	209
EST-SSR 058	AAT 06	CACACTTGATTCCGCTCACA	51.3	GGATGCTTGCTGCTGCTATT	51.7	201
EST-SSR 067	CCG 03	CGCCCGAAGATCAAACAA	47.9	TTATATCCCGCGGCAAGTCC	53.7	100
EST-SSR 069	CCG 05	TGAGCTAACCAAGACCAGTTCC	54.7	CAACAGGAAATCACCGCCTA	51.7	101
EST-SSR 074	CCG 15	GCATCCTACCGAGTACATACAA	52.9	TCCATCAACAACAACCGAAG	49.6	259
EST-SSR 096	ACGG 01	GTGAACCTCCCTTTCCCTTG	59.4	ACTGGTCTCTCGTCTGTGAA	57.3	152
EST-SSR 097	ACGG 04	TGTTGCACAGGTCGAGAAGA	49.6	TTGGCTGTTTGTACGGTTGA	51.7	256
EST-SSR 108	ACTA 14	GGCTTCTTGGATGTTGTTGT	49.6	CTAGTAAGTGCCTCCATCTTCA	52.9	121
EST-SSR 007	AC 10	AGTGGCTGGGAACAAAGAGA	57.3	TTCTCCTCCCGCAAACAGAG	59.4	155
EST-SSR 021	AT 07	CTTCCCTGATACTGCTGCTC	59.4	TCCCAAATGTCAAGTCCATC	55.3	201
EST-SSR 075	CCG 17	CCCTCCCTCCTACTTGTCCTAA	56.6	ATCCGGCATCATCATCAGAG	51.7	234
EST-SSR 090	AACC 14	GGGCAGTTCTTGTGTTGTGT	51.7	CCGCAGTAGCAATGAATTTGG	52.3	116
EST-SSR 103	ACTA 08	AGACAGCTTTGGTGGTCCTG	53.7	TGAATGTGTGGCCCTTTAGC	51.7	223
EST-SSR 105	ACTA 10	CCTCATTCCACAATCCACTCC	54.2	GTTGACGGGAAGCCTAATCC	53.7	112

graphics based on the principal coordinate analysis (Figure 2). The result corroborated the previous clustering observed by UPGMA, but a better layout of that diversity was found. In the graphic, first, second and third axis exhibited, respectively, 19.1%, 5.8% and 3.3% of the total variability. Accessions were clearly separated in two major groups, in accordance their origin, *i.e.*: 1) accessions preserved in germplasm banks and 2) accessions collected in Brazilian plantations. Cultivated accessions were plotted at the left side of the plan, composed by the 1st and 2nd coordinates. A division by location was also observed in this cluster. Accessions from Rondônia and Espírito Santo occupied upper and lower halves of the plan, respectively. Nevertheless, some misclassifications were observed, i.e.: five accessions of Rondônia (RO098, RO189, RO143, RO100 and RO063) were positioned in the Espírito Santo cluster and two accessions of Espírito Santo were included in Rondônia cluster (ES02 and ES03). Accessions from UFV collection and the Robustas from IAC were positioned in the lower right side of the plan and no remarkable sub-clustering was observed. This similarity among them may indicate that UFV accessions also belong to the Robusta varietal group. Kouillou accessions from IAC occupied a slightly upper position in right side of the plan. In the graphic, composed by 1st and 3rd coordinates, accessions of preserved and cultivated accessions continued apart, reinforcing their genetic distance.

In Brazil, the cultivated plants of *C. canephora* are generically called 'Conilon', what it supposed to be a linguistic derivation of Kouillou. Nevertheless, is necessary to mention that the word 'Kouillou' was historically defined according to solely morphological criteria and may represent different populations in many countries as Ivory Coast, Benin, Gabon (Montagnon et al. 1998). Notably, only six accessions from Rondônia (RO056, RO190, RO193, RO194 and RO199) and one from Espírito Santo (ESV.3) were grouped among

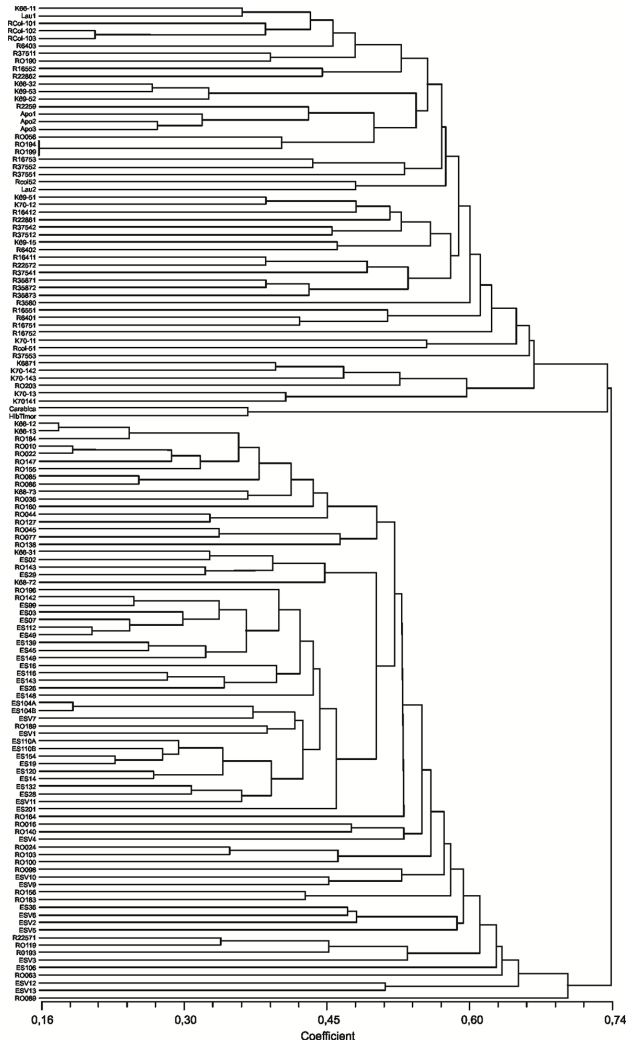

Figure 1. Dendrogram representing the dissimilarity among 130 *Coffea canephora* accessions, obtained by UPGMA method, based on the weighted coincidence index estimated over the polymorphism of 20 microsatellites

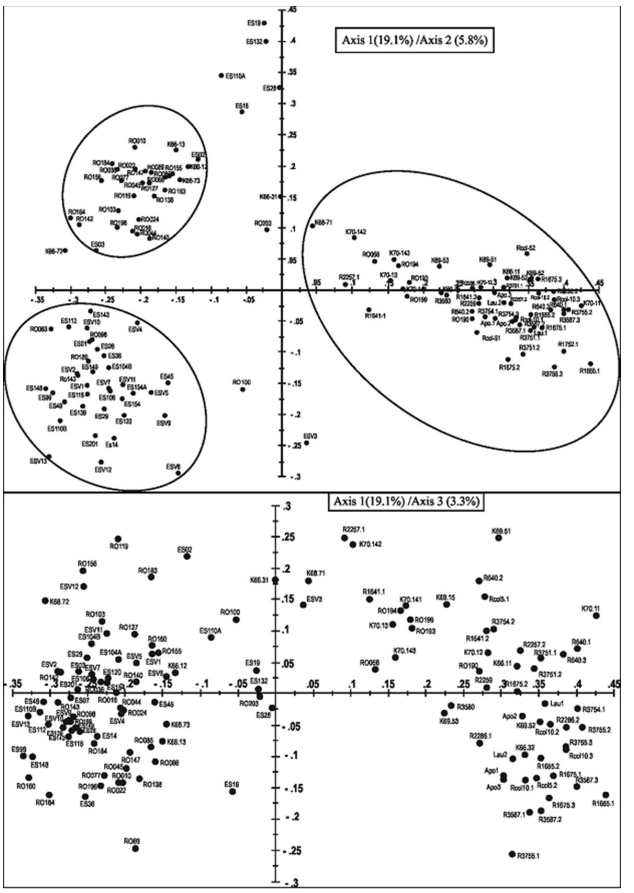

Figure 2. Principal coordinate analysis based on SSR data of 130 *Coffea canephora* accessions.

the accessions of IAC and UFV, which are composed by plants labeled as 'Kouillou' and 'Robusta'. Considering that only six, in a total of 73 accessions collected in plantations, share alleles with those genotypes, it is possible to infer the presence of that varietal group is still small in the most cultivated areas. Consequently, increasing the participation of that germplasm in Brazilian breeding programs would imply a lot of benefits. For instance, the use of Robusta alleles may promote the development of *C. canephora* clones highly resistant to leaf rust (*Hemileia vastatrix* Berk & Br.) and nematodes (*Meloidogyne spp*). Besides, those genotypes could aid to increase yield and improving beverage quality in new cultivars (Fazuoli et al. 2009, Souza and Santos 2009). Another aspect to be explored is the utilization of the heterosis resulting from intergroup crosses. Considering the genetic divergence observed between accessions of the varietal groups (Robusta and Kouillou) and the cultivated germplasm (clone from Rondônia and Espírito Santo), it is expected that their intercrosses may be advantageous. Similar strategy has been used for so long in some breeding programs around the world with remarkable success (Bouharmont et al. 1986, Leroy et al. 1997). Furthermore, 'Robusta' and 'Kouillou' are divergent heterotic groups with complementary characteristics. Robusta plants present high resistance to rust and nematodes, and give good beverage. On the other hand, Kouillou plants are tolerant to drought and they are easier to cultivate due to the smaller size. So, these populations compose a ideal combination to use in a reciprocal recurrent selection program, as it has been already performed in Ivory Cost, since 1984 (Leroy et al. 1993, Leroy et al. 1994, Leroy et al. 1997).

The set of new microsatellites performed a suitable molecular characterization and allowed assessing an im-

portant part of the diversity of *C. canephora* gene pool in Brazil. For the first time, representative samples of accessions from cultivated areas and germplasm collections were examined by microsatellites analysis. These markers revealed a high degree of polymorphism, which provided a satisfactory understanding of the genetic diversity among *Coffea canephora* accessions. Moreover, they allowed the proper grouping of different populations and varietal groups and showed to be able to resolve doubts about the accession classification. This is of great advantage, because the high intra-specific variability and the environmental effects can hinder the differentiation of populations or varietal groups based only on the phenotypic evaluation.

Despite the great polymorphism found in accessions came from areas under cultivation, the diversity may be increased. The present diversity has been enough to support advances of Brazilian breeding programs, but a new threshold of genetic gains is expected with the intensification of the use of Robusta germplasm. Nowadays, Brazil plays a fundamental role in the *C. canephora* world production. Therefore, the establishment of new ways of germplasm interchanging with other collections around the world should be an important initiative to promote introduction of new accessions.

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Diversidade Molecular no germoplasma de Coffea canephora conservado e cultivado no Brasil

Resumo – *Este trabalho objetivou caracterizar acessos de C. canephora oriundos de cultivos comerciais e bancos de germoplasma brasileiros. Um total de 130 acessos das coleções do IAC (São Paulo), UFV (Minas Gerais) e coletados em plantios comerciais no Espírito Santo e Rondônia foram genotipados com 20 novos microssatélites. Métodos multivariados foram utilizados para estimar a relação entre os acessos. Foi observado alto nível de polimorfismo e dois grupos foram identificados: o primeiro foi constituído pelos genótipos conservados nas coleções de germoplasma do IAC e UFV e o segundo foi composto pelos acessos coletados em plantios comerciais. Os acessos do Espírito Santo e de Rondônia formaram dois subgrupos distintos. Apesar do grande polimorfismo encontrado nas lavouras brasileiras de café canéfora, incrementar essa diversidade é necessário, pois um novo limiar de ganhos genéticos é esperado nos programas de melhoramento com a intensificação do uso do germoplasma conservado.*

Palavras-chave: *Café robusta, diversidade genética, microssatélites.*

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