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ORIGINAL ARTICLE

Comparative study of different molecular markers for classifying and establishing genetic relationships in *Coffea canephora*

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Abstract The genetic variability characterization of the accessions of the germplasm collection, using molecular markers, is being applied as a complementary strategy to the traditional approaches to redefine the plant genetic resources. In this study, we compared the informativeness and efficiency of the molecular markers RAPD, AFLP and SSR in the analysis of 94 accessions of *Coffea canephora* germplasm held by the breeding program of the Brazilian Agricultural Research Corporation (Embrapa), Rondônia State, Brazil. For this, we considered the marker's discriminatory power and level of polymorphism detected and also the genetic relationships and clustering (dendrogram) analysis. The RAPD marker yielded low-quality data and problems in the discrimination of some accessions, being less recommended for genetic studies of *C. canephora*. The SSRs had a higher level of information content and yielded high-quality data, while AFLP was the most efficient marker system because of the simultaneous detection of abundant polymorphism markers per few reactions. Our results indicate that AFLP and SSR, allies to the intrinsic characteristics of each technique, are the most suitable molecular markers for genetic studies of *C. canephora*. However, the choice of AFLP or SSR in the species characterization should be made in agreement with some characteristics that are discussed in this work.

Keywords SSR · AFLP · RAPD · Genetic relationships · Robusta coffee

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Introduction

The coffee tree belongs to the botanical family Rubiaceae, which has 500 genera and over 6,000 species. Of these genera, particular attention has been given to the *Coffea* that comprises two cultivated species of economic importance: *Coffea arabica* L. ($2n = 4x = 44$) and *C. canephora* Pierre ex Froehner ($2n = 2x = 22$).

Coffea canephora species, commonly known as "Robusta coffee," can be divided into two distinct genetic groups separated by their centers of diversity. The "Guinean Group" is phenotypically characterized by its long leaves, small growth habit, small internode length, drought tolerance and susceptibility to leaf rust caused by the fungus *Hemileia vastatrix*. The second group, known as the "Congolese Group," is divided into four subgroups, SG1, SG2, B and C. Genotypes from SG1 present morphological characteristics similar to the "Guinean Group."

On the other hand, genotypes from SG2, B and C present large broad leaves and large beans, are highly resistant to leaf rust and are not able to endure long droughts (Cubry et al. 2008; Musoli et al. 2009). The "Guinean Group" has remained restricted to its area of origin (Guinea and the Ivory Coast), and there are no records of its being introduced into other countries. However, the "Congolese Group" is reported to have been introduced into Brazil on several occasions, the first time in approximately 1920, when it was introduced to occupy marginal *C. arabica* areas (Ferrão et al. 2007). Additional introduction events occurred during the 1970s, when accessions from the African continent were sent to several countries (Fazuoli et al. 2009). Subgroups SG1 are represented by varieties locally known as "Conilon coffee," while SG2, B and C are known as "Robusta coffee."

Advances in the coffee crop have been made by genetic breeding programs, which have developed new cultivars to meet specific consumer demands as well as to promote the economic and environmental sustainability of coffee. However, the success of these programs depends primarily on the genetic diversity within the base population. For this, germplasm collections have the important function of maintaining genetic resources, which have significant impact on the plant breeding.

For genetic coffee breeding, some traits of interest are expressed late because coffee is a perennial crop with a long juvenile period (Ferrão et al. 2009). Thus, techniques that allow quick assessment of germplasm variability are especially important. For instance, the use of molecular markers allows faster, easier and more accurate assessment of the genetic variation, providing higher accuracy in the measurement of genetic diversity and offering great contribution to the breeding programs. In recent years, the number of molecular assays available for assessing genetic diversity has increased significantly. The molecular methods differ in principle, in application, in the type and amount of polymorphisms detected and in the cost and time requirements (Russell et al. 1997).

The three most common markers used for genetics studies in the *Coffea* genus are RAPD (Random Amplified Polymorphic DNA) (Lashermes et al. 1993; Orozco-Castillo et al. 1994; Sera et al. 2003; Oliveira et al. 2007; Silvestrini et al. 2008; Ferrão et al. 2009; Tshilenge et al. 2009), amplified fragment length polymorphism (AFLP) (Lashermes et al. 2000; Steiger et al. 2002; Anthony et al. 2002; Brito et al. 2010; Diola et al. 2011) and simple sequence repeat (SSR) (Baruah et al. 2003; Moncada and McCouch 2004; Poncet et al. 2004; Prakash et al. 2005; Silvestrini et al. 2007; Missio et al. 2009, 2010, 2011). Nevertheless, the choice of which marker type to use is often unclear. A better understanding of the effectiveness of these methods is a powerful tool for germplasm management, as well as for genetic studies and breeding programs. Comparative studies of different molecular techniques for measuring genetic variability have already been performed in cereals (Powell et al. 1996), barley (Russell et al. 1997; Varshney et al. 2007), melon (Garcia-Mas et al. 2000), potato (Milbourne et al. 1997), maize (Pejic et al. 1998), rice (Virk et al. 2000), olives (Belaj et al. 2003), apricot (Lamia et al. 2010) and fig (Baraket et al. 2010). Similar studies with the *C. canephora* species have not been reported in the literature.

In this study, we compare the efficiency and discriminating capacity of the RAPD, AFLP and SSR (microsatellite) molecular markers in establishing genetic relationships in *C. canephora* accessions.

Materials and methods

Plant material

A total of 94 accessions of *C. canephora* from different Brazilian research institutions currently maintained at the Coffee Germplasm Collection of the Brazilian Agricultural Research Corporation (Embrapa), in Rondônia State were genotyped (Table 1). These accessions comprise a representative sample of the germplasm used in the Brazilian breeding programs. They were introduced in the Embrapa gene bank over the last 3 decades by means of interchange with the Agronomic Institute of Campinas (Instituto Agronômico de Campinas, IAC), São Paulo State, and the Capixaba Research Institute Technical Assistance and Rural Extension (Instituto Capixaba de Pesquisa, Assistência Técnica e Extensão Rural, Incaper), Espírito Santo State (Souza et al. 2003). In addition, different accessions were collected in traditional coffee-producing areas at Rondônia State and included in this study. According to phenotypic analyses performed previously, the Incaper accessions belong to the Conilon varietal group (Table 1, group 1 accessions), whereas the IAC samples belong to the Robusta varietal group (Table 1, group 2 accessions). The varietal group of the accessions collected in traditional coffee-producing areas at Rondônia State is unknown, as they had not been previously phenotyped (Table 1, group 3 accessions).

Young and completely extended leaves were collected from each accession, frozen at −80 °C, lyophilized, ground and stored at −20 °C. Genomic DNA was extracted using the protocol described by Diniz et al. (2005), and the DNA samples were prepared to a final concentration of 25 ng μl^{-1}.

Molecular analyses

RAPD

Accessions were genotyped using 17 primers from Operon Technology® (OPA-10, OPC-07, OPC-10, OPI-20, OPN-05,

Table 1 *Coffea canephora* accessions used in this study

Acessions	Code	Group[a]
Conilon Incaper 03	ES03	1
Conilon Incaper 16	ES16	1
Conilon Incaper 28	ES28	1
Conilon Incaper 45	ES45	1
Conilon Incaper 110	ES110A	1
Conilon Incaper V.1	ESV1	1
Conilon Incaper V.2	ESV2	1
Conilon Incaper V.3	ESV4	1
Kouillou 661	K661	1
Robusta IAC 640.1	R6401	2
Robusta IAC 640.2	R6402	2
Robusta IAC 1675	R1675	2
Robusta IAC 2259	R2259	2
Robusta IAC 2257.1	R22571	2
Robusta IAC 2257.2	R22572	2
Robusta IAC 2258.1	R22581	2
Robusta IAC 2258.2	R22582	2
Robusta IAC 2258.3	R22583	2
Cpafro 001	RO001	3
Cpafro 003	RO003	3
Cpafro 004	RO004	3
Cpafro 006	RO006	3
Cpafro 010	RO010	3
Cpafro 015	RO015	3
Cpafro 016	RO016	3
Cpafro 017	RO017	3
Cpafro 018	RO018	3
Cpafro 022	RO022	3
Cpafro 024	RO024	3
Cpafro 025	RO025	3
Cpafro 026	RO026	3
Cpafro 030	RO030	3
Cpafro 032	RO032	3
Cpafro 035	RO035	3
Cpafro 036	RO036	3
Cpafro 038	RO038	3
Cpafro 042	RO042	3
Cpafro 043	RO043	3
Cpafro 044	RO044	3
Cpafro 045A	RO045A	3
Cpafro 045B	RO045B	3
Cpafro 047	RO047	3
Cpafro 049	RO049	3
Cpafro 052	RO052	3
Cpafro 056	RO056	3
Cpafro 058	RO058	3
Cpafro 059	RO059	3
Cpafro 064	RO064	3
Cpafro 072	RO072	3
Cpafro 073	RO073	3
Cpafro 075	RO075	3
Cpafro 076	RO076	3
Cpafro 077	RO077	3
Cpafro 085	RO085	3
Cpafro 086	RO086	3
Cpafro 088	RO088	3
Cpafro 089	RO089	3
Cpafro 096	RO096	3
Cpafro 098	RO098	3
Cpafro 100	RO100	3
Cpafro 101	RO101	3
Cpafro 103	RO103	3
Cpafro 115	RO115	3
Cpafro 119	RO119	3
Cpafro 120	RO120	3
Cpafro 125	RO125	3
Cpafro 127	RO127	3
Cpafro 138	RO138	3
Cpafro 139	RO139	3
Cpafro 140	RO140	3
Cpafro 141	RO141	3
Cpafro 142	RO142	3
Cpafro 146	RO146	3
Cpafro 147	RO147	3
Cpafro 149	RO149	3
Cpafro 151	RO151	3
Cpafro 155	RO155	3
Cpafro 156	RO156	3
Cpafro 160	RO160	3
Cpafro 161	RO161	3
Cpafro 164	RO164	3
Cpafro 167	RO167	3
Cpafro 171	RO171	3
Cpafro 172	RO172	3
Cpafro 183	RO183	3
Cpafro 184	RO184	3
Cpafro 189	RO189	3
Cpafro 190	RO190	3
Cpafro 193	RO193	3
Cpafro 194	RO194	3
Cpafro 196	RO196	3
Cpafro 197	RO197	3
Cpafro 199	RO199	3
Cpafro 203	RO203	3

[a] Group 1: Accessions from germplasm of Incaper, belonging to the Conilon varietal group; group 2: germplasm of IAC, belonging to the Robusta varietal group; group 3: accessions collected in traditional coffee-producing areas at Rondônia State, Brazil

OPN-07, OPN-09, OPR-01, OPX-05, OPX-11, OPAB-04, OPAL-08, OPAM-07, OPAN-19, OPAQ-03, OPAS-09 and OPAU-02). DNA fragments were amplified in 25-µl reactions containing 25 ng DNA, 1 U of *Taq* DNA polymerase, 50 mM KCl, 2 mM $MgCl_2$, 10 mM Tris-HCl, 100 mM dNTP and 0.2 mM primers. The PCR reaction consisted of 40 cycles with denaturation at 94 °C for 15 s, primer annealing at 35 °C for 30 s and amplification at 72 °C for 1 min. A final extension step at 72 °C for 7 min was performed after the last cycle. The amplified products were run on 1.2 % agarose gel, and fragments were visualized by ethidium bromide staining.

AFLP

AFLP marker analysis was performed using the method described by Brito et al. (2010). Four selective primer combinations were used (E-CTC/M-AGC, E-CTC/M-AGT, E-CAT/M-AGT and E-CGC/M-ATA).

SSR

SSR primers obtained from ESTs of the Brazilian Coffee Genome Project and from the literature (Combes et al. 2000; Coulibaly et al. 2003; Baruah et al. 2003; Poncet et al. 2004; Bhat et al. 2005) were used for genotyping (Table 2). SSR marker analysis was performed using the method described by Missio et al. (2009).

Data analysis

The dominant markers, RAPD and AFLP, were scored in a binary matrix, with 1 representing the presence and 0 (zero) the absence of bands. SSR is a codominant marker, and the alleles were scored according to molecular weight markers. To compare the efficiency of the three markers (RAPD, SSR and AFLP) in varietal identification, diversity and differentiation, we follow the parameters: polymorphism level and informativeness of the markers, cluster analysis and correlation between markers matrices.

Polymorphism level and informativeness of the markers

1. Number of assay units or the product of PCR amplification obtained with one set of primers (U).
2. Number of polymorphic bands (N_p).
3. Number of non-polymorphic bands (N_{np}).
4. Average number of polymorphic bands per assay unit (N_p/U).
5. Number of loci (L): in the case of RAPD and AFLP markers, the theoretical maximum number of loci is equal to total the number of bands ($N_p + N_{np}$) obtained for each marker. For SSR, this value corresponds to the number of assays (i.e., the number of primer pairs) because this type of marker uses primers that amplify specific genomic sequences.
6. Number of loci per assay unit: $n_u = \frac{L}{U}$.
7. Average number of alleles per locus (N_{av}). For SSR, N_{av} was calculated using the formula: $N_{av} = \frac{N_p}{L}$. RAPD and AFLP markers were assumed to have two loci per assay ($N_{av} = 2$).
8. Expectation of heterozygosity (H_e) was calculated using the formula $H_e = 1 - \Sigma p_i^2$, where p_i is the allelic frequency of the ith allele.
9. Average polymorphic information content (PIC), also known as discriminatory power, was calculated according to the formula: $1\left(\sum_i^a p_i^2 - \sum_{i,j=1}^a \sum_{(i\neq j)}^a p_i^2 p_j^2\right)$, where p is the allelic frequency of the ith allele (Botstein et al. 1980).
10. Fraction of polymorphic loci (β) was calculated according to the formula reported by Powell et al. (1996): $\beta = \frac{N_p}{N_p + N_{np}}$
11. Effective number of alleles per locus (n_e) was calculated according to the formula: $n_e = \frac{1}{\sum p_i^2}$ where p is the allelic frequency of the ith allele (Morgante et al. 1994).
12. Total number of effective alleles (N_e) was calculated according to the formula reported by Pejic et al. (1998): $N_e = \Sigma n_e$.
13. Assay efficiency index (A_i) was calculated according to the formula reported by Pejic et al. (1998): $A_i = \frac{N_e}{U}$.
14. The number of polymorphic markers simultaneously analyzed in one gel was defined by Powell et al. (1996) as the effective multiplex ratio (E) and was calculated using the formula: $E = n_u\beta$.
15. Efficiency of polymorphism detection, defined by Powell et al. (1996) as the Marker index (MI), was calculated using the formula: MI = $E \times$ PIC.
16. The qualitative nature of data (QND) was calculated according to formula from Varshney et al. (2007): QND = DC × QM × PR, where DC is the documentation capacity, QM is the quality of the marker, and PR is the percent reproducibility of the fragment(s)/band(s) of the given marker system across the laboratories. DC and PR values for the AFLP and SSR markers are constant and were defined by Varshney et al. (2007). For the RAPD analyses, it was assumed that the value of the two parameters was 0.25. The QM values are variable and depend on the primers used for each marker.
17. The effective marker index (EMI) was calculated according to the formula reported by Varshney et al. (2007): EMI = MI × QND.

Table 2 Description of the SSR used to amplify the *Coffea canephora* accessions

Primers	Foward sequence (5′–3′)	Reverse sequence (3′′–5′)	T_m (°C)	Size (pb)
EST-SSR11[a]	GTCCGATTCCCATTGCTTC	CTGCTACTTGGACGTTCTCTTT	51	140
EST-SSR48[a]	TCCTCCTCGTGCTTCTCAAC	GGCAGCATTCTCCTGATCCT	53.7	126
EST-SSR67[a]	CGCCCGAAGATCAAACAA	TTATATCCCGCGGCAAGTCC	53.7	100
SSR 029[b]	GGCTTCTTGGGTGTCTGTGT	CCATTGGCTTTGTATTTCTGG	55	110
SSR 030[b]	ATGGGGCCAACTTGAATATG	CAGGGCATCTATCTACTTCTCTTT	55	220
SSR 034[b]	GGAGACGCAGGTGGTAGAAG	TCGAGAAGTCTTGGGGTGTT	58	294
SSR 039[b]	TCCCCCATCTTTTTCTTTCC	GGGAGTGTTTTTGTGTTGCTT	55	116
SSR 043[b]	TTTTCTGGGTTTTCTGTGTTCTC	TAACTCTCCATTCCCGCATT	55	134
SSR 049[b]	TGGAGAAGGCTGTTGAAACC	GGCGTGAAGCAAAAAGGTAT	55	192
SSR 057[b]	CTCGCTTTCACGCTCTCTCT	CGGTATGTTCCTCGTTCCTC	55	102
SSR 059[b]	CCAGCTCTCCTCACTCTTTTCA	GGTGGTGGAGGGGTAATAGG	58	272
SSR 071[b]	GCTAAGTTCAATTGCCCCTGT	GGGTTAATTTGATTGCGTGA	59	232
SSR 074[b]	TGGGGAAAAGAAGGATATAGACAAGAG	GAGGGGGGCTAAGGGAATAACATA	55	129
SSR 076[b]	GGTCCCACTCTCAAGCTGAA	GGCAATTGATTCTGGAACCT	59	157
SSR 106[b]	CCCTCCCTCTTTCTCCTCTC	TCTGGGTTTTCTGTGTTCTCG	60	184
SSR 119[b]	TTGCCATCATCGTTCATTCT	GCATAGTGTCGGTTGTGTTGTT	58	190
SSR 121[b]	CGACACTTTCTTTGGCACTC	AGACACCCACCCATCCAC	50	177
SSR 122[b]	CGTCTCGTTTCACGCTCTCT	GATCTGCATGTACTGGTGCTTC	55	237
SSR 151[b]	GGCCGAGGGGAAAAAGAAGC	GGAAACCTCACGAGAAGATTACACAA	57	100

[a] EST-SSR were developed in Coffee Biotechnology Lab—BIOCAFÉ, Universidade Federal de Viçosa

[b] SSR primers were obtained from the literature (Combes et al. 2000; Coulibaly et al. 2003; Baruah et al. 2003; Poncet et al. 2004; Bhat et al. 2005)

Cluster analysis and correlation between marker matrices

To analyze genetic relationships, we first evaluated the diversity between groups of accessions and then among pairs of accessions. Nei's Genetic Distance (1972) was used to measure the distance between groups. Subsequently, pairs of accessions were compared. For the dominant markers (i.e., RAPD and AFLP), genetic dissimilarity was estimated using Jaccard's Complement Coefficient (1908). The distances for codominant markers (SSR) were estimated as a function of the number of common alleles per locus using a weighted index, calculated according to the formula: $S_{ii'} = \frac{1}{2L}\sum_{j=1}^{l} c_j$, where $S_{ii'}$ is the similarity between the accessions i and i', L is the total number of loci, and c_j is the number of common alleles between i and i' (Cruz et al. 2011). For the combined analysis, the distance matrices for the RAPD, AFLP and SSR molecular markers were combined to obtain a single distance matrix. A weighted sum was used in which the matrix of each marker was multiplied by a weighting factor obtained using the formula $\mathrm{WF} = \frac{L}{N} \times i \times \mathrm{QND}$, where WF is the matrix's weighting factor, L is the number of loci measured by each marker individually, N is the total number of loci measured by all of the markers, i is a constant from the informativeness index (scored as 2 for dominant markers and 3 for codominant markers), and QND is the qualitative nature of data, as defined by Varshney et al. (2007). The neighbor-joining (NJ) algorithm (Saitou and Nei 1987) was used for cluster analyses from the estimated distance matrices for all markers.

The cophenetic correlation coefficient (r) was calculated, and Mantel's test (1967) was performed to check the goodness of fit of a cluster analysis to the matrix on which it was based; 1,000 random permutations were used to test the significance between the matrix correlations. According to Rohlf and Fisher (1968), r values >0.8 indicate goodness of fit. Group discrimination analysis using the k-nearest neighbor algorithm was performed to verify the consistency of the clusters in the dendrograms. All statistical procedures were performed using the GENES Software package (Cruz 2006) and the Fig Tree v1.3.1 (Rambaut 2006).

Results

Polymorphism levels and marker informativeness

All three molecular markers used in this study proved to be highly effective in discriminating the 94 accessions

Table 3 Levels of polymorphism and comparison of the discriminating capacity and informativeness obtained with RAPD, AFLP and SSR markers in 94 *Coffea canephora* accessions

Indexes with their abbreviations		Markers		
		RAPD	AFLP	SSR
Number of assays units or primers	U	17	4	19
Number of polymorphic bands	N_p	65	93	92
Number of non-polymorphic bands	N_{np}	17	24	0
Average number of polymorphic bands per assay unit	N_p/U	3.82	23.25	4.84
Number of loci	L	82	117	19
Number of loci per assay unit	N_u	4.82	29.25	1.00
Average number of alleles per locus	N_{av}	2.00	2.00	4.84
Expectation of heterozygosity	H_e	0.24	0.20	0.43
Average polymorphic information content	PIC	0.19	0.18	0.39
Effective number of alleles per locus	n_e	1.31	1.25	1.75
Total number of effective alleles	N_e	107.42	146.25	33.34
Fraction of polymorphic loci	β	0.79	0.79	1.00
Assay efficiency index	A_i	6.31	36.57	1.75
Effective multiplex ratio	E	3.8	23.10	1.00
Marker index	MI	0.60	4.15	0.39
Qualitative nature of data	QND	0.046	0.093	0.75
Effective marker index	EMI	0.027	0.385	0.292

analyzed (Table 3). The total number of polymorphic bands ranged from 65 to 93 for the RAPD and AFLP markers, respectively. However, the percentage of polymorphism did not correlate to the total number of amplified fragments. The SSR markers had the highest percentage of polymorphic bands (100 %) followed by the AFLP and RAPD markers (84.62 and 79.27 %, respectively).

The AFLP markers were able to detect higher average numbers of polymorphic bands per assay unit ($N_p/U = 23.25$), number of loci per assay unit ($N_u = 29.25$) and effective alleles ($N_e = 146.25$). These estimates resulted in high values of assay efficiency index ($A_i = 36.57$), effective multiplex ratio ($E = 23.10$), marker index (MI = 4.15) and effective marker index (EMI = 0.385). The other dominant marker, RAPD, was able to detect polymorphisms at a satisfactory level, although this was a lower value compared with the AFLP markers. The only exception was the H_e value, for which the RAPD markers (0.24) yielded slightly higher values than did the AFLP markers (0.20).

The SSR, a codominant and locus-specific marker, presented the highest value of the average number of polymorphic bands per assay unit ($N_p/U = 4.84$) and effective number of alleles per locus ($n_e = 1.75$). Out of a total of 19 SSR primers, the number of alleles ranged from two (SSR 151) to eight (SSR 23 and SSR 43) with an average of 4.84 alleles per locus. In addition, the SSR markers had the highest values of expectation of heterozygosity ($H_e = 0.43$), average polymorphic information content (PIC = 0.39) and qualitative nature of data (QND = 0.75). However, these markers had the lowest values on the assay efficiency index ($A_i = 1.75$), effective multiplex ratio ($E = 1.00$) and marker index (MI = 0.39).

Table 4 Minimum and maximum values of the genetic distance (RAPDs, AFLPs and SSRs) among groups and pairs of *Coffea canephora* accessions

	Between groups of accessions			Between pairs of accessions		
	RAPD	AFLP	SSR	RAPD	AFLP	SSR
Minimum	0.204	0.223	0.579	0.454	0.666	0.945
Maximum	0.050	0.053	0.075	0	0.070	0.119

Cluster analysis and correlation between markers matrices

The estimated genetic distances between groups and pairs of accessions, calculated for each marker system, are shown in Table 4. The SSR markers gave the highest distance between groups (0.579) and pairs of accessions (0.945). Null values for genetic dissimilarity were only found for the RAPD markers.

The genetic distances obtained for each marker were represented as dendrograms, based on the genetic distance matrices and the NJ clustering (Figs. 1, 2, 3). The general dendrogram is shown in Fig. 4 and summarizes the genetic relationships obtained from the combined data of the three sets of molecular markers.

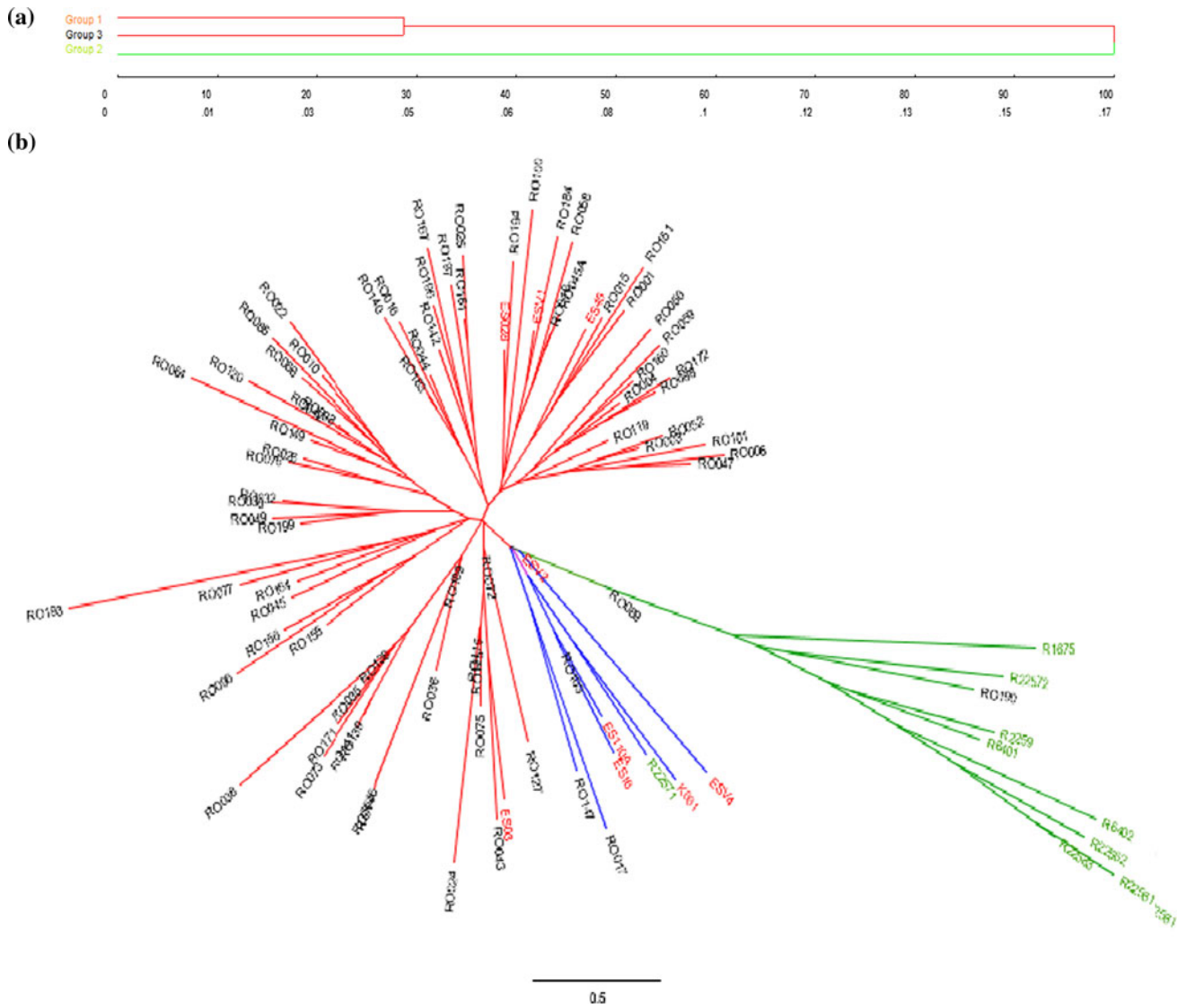

Fig. 1 **a** Dendrogram of the *Coffea canephora* groups obtained by Nei's Genetic Distance (1972); **b** radial tree of the *Coffea canephora* acessions obtained by Jaccard's Complement Coefficient (1908). *Labels* in *red*, *green* and *black* represent, respectively, groups 1, 2 and 3 of Table 1. *Clades* in *red*, *green* and *blue* represent clusters of Conilons, Robust and natural hybrids, respectively. Molecular marker: RAPD

The molecular analyses between groups of accessions (Figs. 1a, 2a, 3a) were consistent with the morphological data. All of the marker systems were able to discriminate the varietal groups Robusta (green clade) and Conilon (red clade). The dendogram generated from comparison data between accessions (Figs. 1b, 2b, 3b) were also consistent with the morphological classifications with two exceptions. The first involved the R22571 genotype, which was morphologically considered as Robusta, but was classified as Conilons by all of the molecular analyses. The other exception involved the ES03 accession, which was classified as Robusta using AFLP markers, but with the other two-marker assay was clustered together with the Conilons. Therefore, evaluation of the cluster analysis suggested that most of the accessions in the Coffee Germplasm Collection of Embrapa belong to the Conilon varietal group, including the random samples collected in Rondônia State (Table 1, group 3). The genotypes located between the two groups (blue clade) can be hybrids resulting from natural crossing among Robust and Conilon.

The results obtained from the dendrograms were confirmed by discriminant analysis, which showed the efficiency of the markers to separate the Conilon and Robusta varietal groups.

The Mantel matrix correspondence test was used to compare the original distance matrix and the dendrogram; *r* was significant for all three genetic markers (Table 5, diagonal). The correlation coefficients between the original

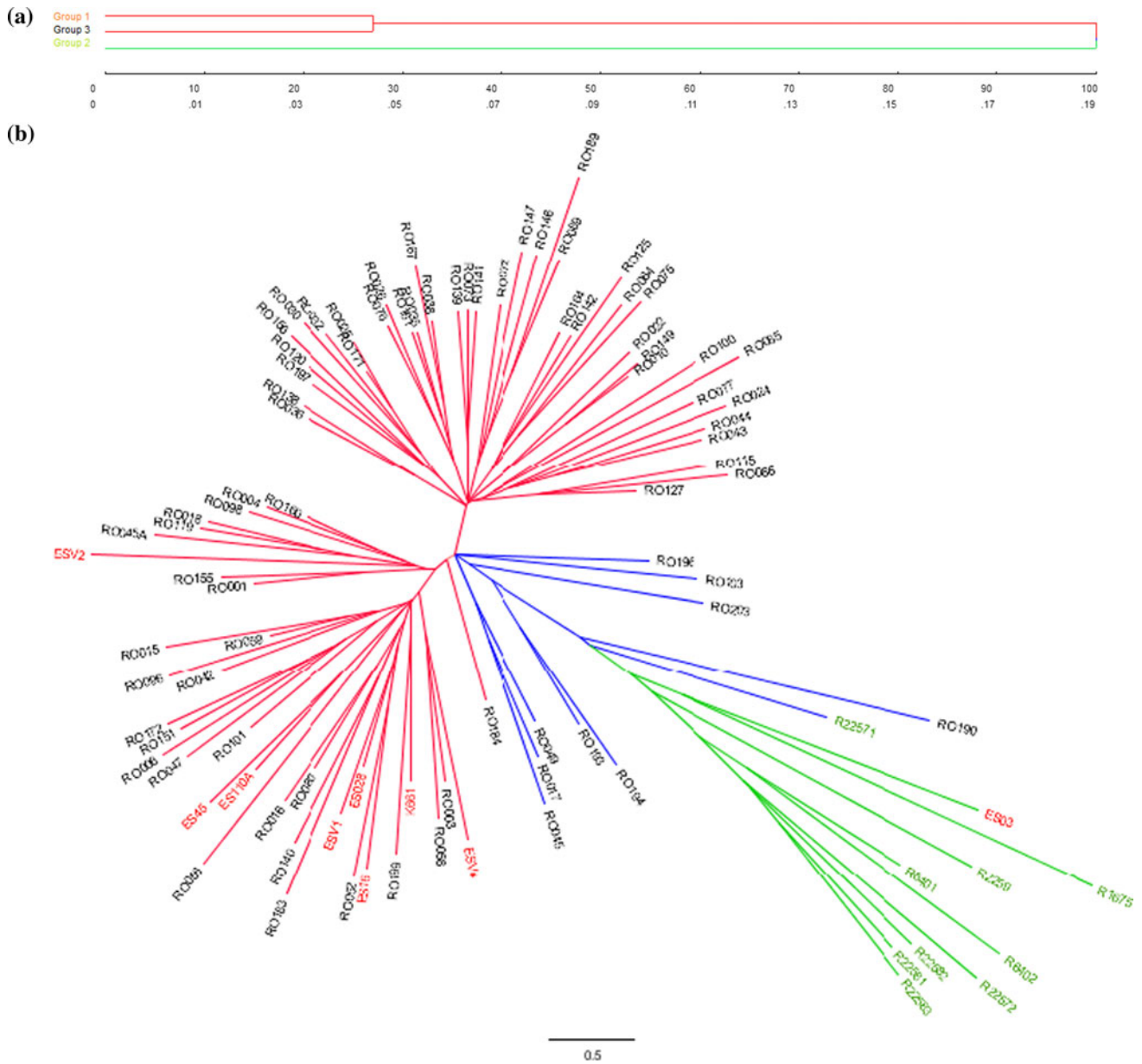

Fig. 2 **a** Dendrogram of the *Coffea canephora* groups obtained by Nei's Genetic Distance (1972); **b** radial tree of the *Coffea canephora* acessions obtained by Jaccard's Complement Coefficient (1908). *Labels* in *red*, *green* and *black* represent, respectively, the groups 1, 2 and 3 of Table 1. *Clades* in *red*, *green* and *blue* represent clusters of Conilons, Robust and natural hybrids, respectively. Molecular marker: AFLP

matrices (Table 5, above the diagonal) and between the dendograms (Table 5, below the diagonal) were also significant for all of the molecular markers.

Discussion

Compared with previous studies in *C. arabica* (Maluf et al. 2005), another economically important species in *Coffea*, the levels of polymorphism detected by the three molecular markers in this study were higher. The differences may be due to the origin and reproduction method of these species and how they were disseminated throughout the world.

Endemic to high altitudes in southeast Ethiopia, Sudan (Thomas 1942) and Kenya (Berthaud and Charrier 1988), *C. arabica* was first cultivated in Yemen 500 years ago. At the beginning of the eighteenth century, the progeny from a single plant were taken from Indonesia to Europe and then to the South American continent, where it became the genetic basis for the major cultivars grown in Brazil and other countries. The consequence of this introduction, in combination with the autogamous reproduction, is a narrow

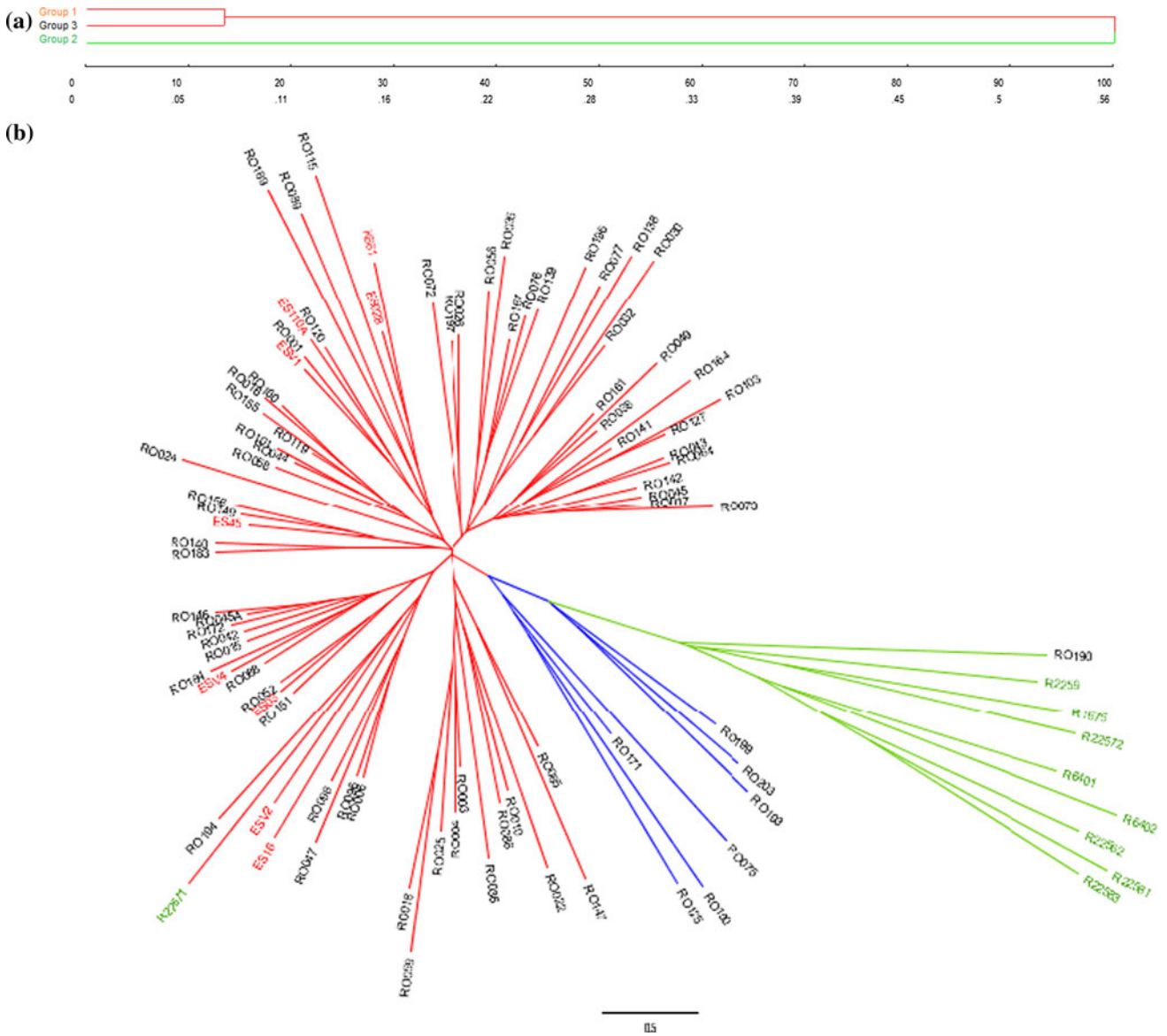

Fig. 3 **a** Dendrogram of the *Coffea canephora* groups obtained by Nei's Genetic Distance (1972); **b** radial tree of the *Coffea canephora* acessions obtained by the weighted index. *Labels* in *red*, *green* and *black* represent, respectively, the groups 1, 2 and 3 of Table 1. *Clades* in *red*, *green* and *blue* represent clusters of Conilons, Robust and natural hybrids, respectively. Molecular marker: SSR

gene pool and low levels of polymorphism. In contrast, *C. canephora* is endemic to western, tropical and subtropical regions of Africa, including large areas of the Republic of Guinea, the Ivory Coast, Sudan, Uganda and other countries. Historical records show that cultivation of *C. canephora* began in Congo in 1870 and subsequently expanded to cover the central part of Africa and later reached Indonesia, Java and the South American continent through trade. The result of these introductions in combination with cross-pollination was the formation of *C. canephora* populations with high levels of genetic variability, high heterozygosity and, consequently, high levels of polymorphism (Lashermes et al. 1993; Ferrão et al. 2007).

In the present study, SSR markers showed the highest polymorphism levels, which was concordant with several other studies (Powell et al. 1996; Russell et al. 1997; Pejic et al. 1998; Gallego et al. 2005; Varshney et al. 2007). According to these authors, the hypervariability is caused by replication slippage and the codominant nature of this marker, which permits the detection of a high number of alleles per locus. However, the marker efficiency also depends on the species under study (Belaj et al. 2003). In potato (McGregor et al. 2000) and yam (Mignouna et al. 2003), AFLP data were more appropriate for genetic studies than SSR or RAPD markers. These results suggest that the choice of method may depend on the genetic

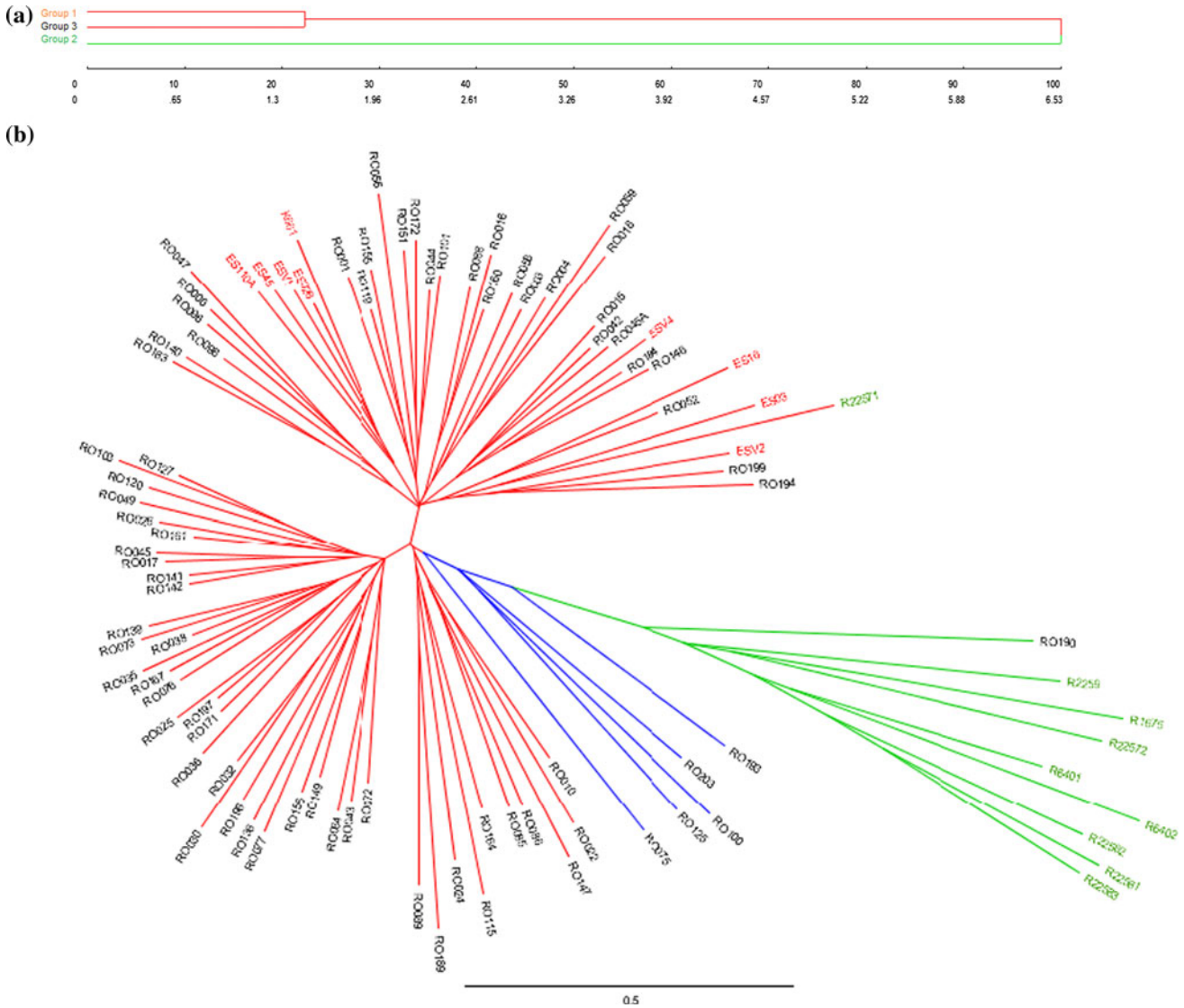

Fig. 4 **a** Dendrogram of the *Coffea canephora* groups obtained by Nei's Genetic Distance (1972); **b** radial tree of the *Coffea canephora* acessions obtained by the matrix's weighting factor (WF). *Labels* in *red*, *green* and *black* represent, respectively, the groups 1, 2 and 3 of Table 1. Clades in *red*, *green* and *blue* represent clusters of Conilons, Robust and natural hybrids, respectively. Molecular marker: combined data set (RAPD/AFLP/SSR)

background of the crop being investigated and the aims of the study (McGregor et al. 2000).

Our data showed that AFLP markers are efficient for detecting polymorphisms using a few assay units, resulting in high values of A_i, N_u, E and MI. In contrast, the SSR markers, which are multi-allelic, presented high values of N_{av}, H_e and PIC. According to Powell et al. (1996), the efficiency of a molecular marker is a balance between the level of polymorphism it can detect (information content) and its capacity to identify multiple polymorphisms. These two parameters are represented, respectively, by the PIC and E values. The product of these parameters provides a global metric for marker efficiency, referred to here as MI. The SSR markers presented high H_e (0.43) and PIC (0.39) values, but a low MI value (0.39). On the other hand, AFLP markers had a low PIC (0.18) and the highest MI (4.15) values. According to Belaj et al. (2003), this discrepancy between MI scores is caused by the large influence of the number of bands used for calculating the final MI. Molecular markers such as AFLP, which represent various genomic regions simultaneously, tend to have higher MI values than locus-specific markers, such as SSR (Baraket et al. 2010). However, Pejic et al. (1998) and Gallego et al. (2005) reported that SSR markers were more informative because of their high H_e, PIC and N_{av} values. In contrast, AFLP markers are more efficient for detecting large levels of polymorphisms with a few assays. To increase the efficiency of detecting high polymorphism levels using a

Table 5 Cophenetic correlations among matrices

	RAPD	AFLP	SSR	RAPD/AFLP/SSR
RAPD	**0.75***	0.59*	0.60*	0.71*
AFLP	0.73*	**0.88***	0.67*	0.85*
SSR	0.79*	0.77*	**0.90***	0.95*
RAPD/AFLP/SSR	0.82*	0.80*	0.95*	**0.94***

Below diagonal, original similarity matrix comparison; diagonal (in bold), goodness of fit of a cluster analysis to the similarity matrix on which it was based; above diagonal, cophenetic value matrix (matrix of ultrametric values) comparison (after clustering procedure)

* Significant at $P < 0.01$

few assay units in SSR analysis, the strategy of multiplex PCR, which allows the simultaneous amplification of more than one SSR per assay, has been used (Guichoux et al. 2011). The significant growth in detecting the level of polymorphisms will permit increases in the effective multiplex ratio (*E*) and consequently the marker index (MI).

In addition to considering the informativeness and efficiency of polymorphism detection, Varshney et al. (2007) introduced the concept of data quality (QND) for comparing molecular markers. The parameter was created to help researchers choose the most appropriate genotyping method. This value depends on the characteristics intrinsic to each technique, such as DC, QM and PR. In the present study, the lowest QND values were obtained for the RAPD (0.046) and AFLP (0.093) because of the difficulty in interpreting multi-locus gels and the low reproducibility of these techniques. The highest QND value was obtained for SSR markers (0.75) because of the robustness and ease of evaluation, making this method the most recommended.

Another parameter that was used was the EMI, which combines the data quality (QND) with all the other possible attributes such as information content, fraction of polymorphic fragments and multiplex ratio. The highest EMI values were found for AFLP (0.385) followed by the SSR (0.292) and RAPD (0.027).

The lowest QND and EMI values for RAPD indicate that this marker is less reliable. For the other analysis, RAPD markers yielded intermediate scores for most of the evaluated parameters. RAPD was not informative as SSR and was not as efficient for detecting polymorphisms as AFLP. Similar results with details on polymorphism levels and informativeness of different molecular markers were reported in barley (Russell et al. 1997), maize (Pejic et al. 1998), olives (Belaj et al. 2003), apricots (Lamia et al. 2010) and figs (Baraket et al. 2010).

The three molecular markers were also used to analyze the genetic relationships between the *C. canephora* accessions. Despite the morphological and adaptive differences that separate the *C. canephora* varietal groups, to classify the access is not an easy task, since these plants are strictly allogamous, and the populations are constituted of plants with high levels of heterozygosity (Conagin and Mendes 1961) and with extensive phenotype variability. These facts reinforce the importance of molecular markers for breeding programs, as well as for conservation of genetic resources in germplasm collection. We observed that all of the markers analyzed were efficient for discriminating the varietal group. Analyses of SSR data revealed higher genetic distances between groups of accessions and dissimilarity between accessions (0.579 and 0.945, respectively), demonstrating a greater discriminatory capacity compared with the other two techniques. Furthermore, the RAPD marker assays yielded null values for dissimilarity between some accession pairs. Taken alone, this would suggest the existence of duplicates within the germplasm collection. However, as this finding was not corroborated with the data of the other marker, we conclude that RAPD markers are inefficient at discriminating some genotypes.

The genetic relationships, shown as dendrograms (Figs. 1, 2, 3, 4), were consistent with existing morphological data (Souza and Santos 2009). Most of the accessions in the Coffee Germplasm Collection of Embrapa belong to the Conilon varietal group, including the accessions collected within the Rondônia State that had not been previously classified (Table 1, group 3). The only genotype collected in the State that belonged to the Robusta varietal group was RO190. The possibility that this genotype represents a natural hybrid of Robusta and Conilon is currently being investigated using morphological and molecular markers. Other possible hybrids, grouped between the two groups (blue taxa in the dendrograms), are also being evaluated. These data were confirmed using discriminant analyses, which demonstrated that these techniques are efficient to separate the Conilon and Robusta varietal groups. According to Cruz et al. (2011), this technique can be used to evaluate the consistency of clustering patterns in dendrograms because the method estimates discriminant functions for genotype classification.

The highest *r* value (0.90) was obtained for SSR followed by RAPD and AFLP markers (0.75 and 0.88, respectively), suggesting that SSR markers provide the best goodness of fit. Using the data from combined analysis (RAPD/AFLP/SSR), a high cophenetic correlation coefficient value was obtained (0.93). The *r* value is an important parameter for assessing the reliability of the technique, as it provides an estimate of the goodness of fit between the distance matrix and the dendrogram. Rohlf and Fisher (1968) showed that the goodness of fit between the original distance and dendrogram is considered to be satisfactory when the *r* value >0.8.

The correlation coefficients between markers in the original distance matrices (Table 5, above the diagonal) and the dendograms (Table 5, below the diagonal) were significant. High values were found for the combined data set (RAPD/AFLP/SSR), showing a good representativeness. According to Belaj et al. (2003), the correlation among different markers may be affected by the type of genetic polymorphism detected by each of the markers, as well as the number of primers used in the analyses. However, Lamia et al. (2010) and Gallego et al. (2005) found low correlation values between different molecular markers and reported the importance of using different methods for estimating genetic diversity. According to these authors, the complementarity of the information that is generated by different markers is valuable for genetic studies. Therefore, we conclude that the diversity measurements obtained by the combined analysis offer accurate and reliable results, because it uses information provided from distinct and complementary methods. However, when analyzing the combined data, it is important to consider the marker factor as informativeness, genomic coverage and the qualitative nature of data. Thus, the weighting procedures used in this study aim to favor the more robust markers (in this case the SSR and AFLP markers) by assigning them higher scores.

The RAPD data showed intermediate values of polymorphism and informativeness; furthermore, this technique had problems in discriminating some accessions and obtained low QND values. Therefore, this method is the least reliable of the three markers; consequently, it is the least recommended for *C. canephora* genetic studies.

The utility of AFLP and SSR markers for genetic diversity studies in *C. canephora* was demonstrated. However, deciding which technique is the most appropriate for any given investigation is not easy and depends on a number of factors, including the purpose of the research, the genetic structure of the population and the resources available (Belaj et al. 2003). Each technique has unique characteristics, but the robustness, the informativeness and the polymorphism level should be the primary criteria for choosing a method (Powell et al. 1996).

For studies of closely related plants with a narrow genetic base and low polymorphism levels, we recommend the AFLP markers. This molecular marker presents reproducibility and is able to detect high polymorphism levels using few assay units. In *C. canephora* studies, this characteristic is shown by high values of A_i, N_p/U, E and MI, because this crop is naturally polymorphic, and the polymorphism level detected using AFLP markers will be significantly higher when compared with the other two-marker system. However, the low QND values demonstrate that it is very difficult to interpret and document the AFLP genotyping, and in addition, this molecular marker is labor-intensive, requiring more resources.

The SSR marker, because of its codominant and multiallelic nature, was considered the most informative marker system. Analyses of SSR data allow detailed studies about the population structure, genetic mapping, phylogenies and germplasm characterization. Other important properties of this marker system are the random distribution in the genome, high informativeness, robustness and reproducibility. Additionally, the SSR marker showed the highest QND value, which, according to Varshney et al. (2007), is the most important parameter for genebank managers and curators, who want genotyping data that can be documented and handled easily in their database. The drawback of this technique is the hard work needed for marker development. However, this problem has been greatly simplified by the complete sequencing of the coffee genome, and new primer sequences are frequently being added to the hundreds already available in the literature.

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