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Molecular markers useful to discriminate *Coffea arabica* cultivars with high genetic similarity

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Abstract New cultivars are released every year to meet market demands. However, in species with a narrow genetic base, such as *Coffea arabica,* the cultivars are closely related and phenotypically similar. This hinders the accurate discrimination of genotypes using morphological descriptors in distinctness, uniformity, and stability (DUS) testing, which is required for the registration and protection of new cultivars. In this sense, molecular markers are an auxiliary tool for accurate and precise discrimination of cultivars. This study aimed to verify the informative capacity and effectiveness of a molecular marker set to discriminate among *C. arabica* varieties, create a database of DNA profiles and allele frequencies, analyze the genetic diversity in this collection, and explore genetic kinships. Thirty-four *C. arabica* cultivars/progenies, which belong to the Brazilian Cultivar Trial, were analyzed using 31 microsatellite markers. Markers with weak bands were removed, and of the remaining, 74.07% were polymorphic and revealed 47 alleles. The obtained molecular profiles revealed segregation between and within cultivars/progenies, and genetic variability was observed between all the cultivars/progenies. Sixteen markers were selected for dendrogram construction and for fingerprinting analysis of the cultivars. The ability of these markers to detect varietal mixture and analyze diversity between and within cultivars was also discussed in detail. The results demonstrated the effectiveness of markers in distinguishing related genotypes from those with similar phenotypic traits. This biotechnological tool will assist breeders in DUS testing of cultivars.

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Introduction

Plant breeding programs aim at more productive and adapted cultivars. However, with the large number of cultivars that have already been released, identification and characterization of these materials, based solely on morphological traits, is not easy (Ercisli et al. 2008; Lanteri et al. 2012; Korir et al. 2013).

For a cultivar to be released, it must be registered, and to ensure the intellectual property rights to the breeder, this cultivar needs to be protected. Registration of cultivars facilitates production, processing, and marketing of seeds and seedlings (Santos et al. 2012). In addition, protection of plant variety enables public and private research companies to be benefited from the royalties received from the rights on the cultivars they develop (Carvalho et al. 2009).

Both registration and protection of plant cultivars require distinctness, uniformity, and stability (DUS). Distinctness, uniformity, and stability testing, which is carried out by the evaluation of morphological descriptors recommended for each species. This approach is subjective, time consuming, and expensive (Korir et al. 2013). As an auxiliary alternative, the use of molecular descriptors has been widely discussed in the Working Group on Biochemical and Molecular Techniques (BMT) and DNA Profiling of the International Union for The Protection of New Varieties of Plants (UPOV 2010) in particular, and it is recommended for the identification, registration, and protection of new cultivars (Wang and Chuang 2013; Chen et al. 2016). Molecular markers aid breeders in DUS testing of cultivars with a narrow genetic base (Ferreira et al. 2016), and in the case of *Coffea arabica*, this strategy is especially useful (Ferrão et al. 2015).

Similar to other species, new *C. arabica* cultivars are released every year to meet the demand of the world market. Cultivars with much higher yield potential than those initially introduced in Brazil were developed by genetic breeding programs (Carvalho 1981). In Brazil alone, to date, 131 *C. arabica* cultivars have been registered on the National Register of Cultivars (NRC) of the Ministry of Agriculture, Livestock and Supply (MAPA) (Brasil 2017a). However, these cultivars were obtained from a few parents, resulting in low variability (Setotaw et al. 2013). This hinders the accurate and precise discrimination of genotypes by morphological descriptors (Lanteri et al. 2012).

Other factors have also contributed to the reduction in genetic variability of coffee plants and have favored the increase in related genotypes. Thus, the spread of *C. arabica* globally and the history of the introduction of this species in Brazil should be mentioned. All coffee plants originated from a limited number of seeds collected from Ethiopia, its center of origin, diversity, and dispersion. It is noteworthy that the genetic base of most coffee cultivars available in the world is from a single progeny cultivated in Europe (Silvestrini et al. 2007). In Brazil, few seedlings and seeds from French Guiana were introduced in Belem, Pará state. These materials are the genetic base of the plants of coffee plantations in Brazil. Furthermore, autogamy is a crucial factor, through which approximately 90% of the flowers are fertilized by the plant's own pollen (Anthony et al. 2002). In addition, selection carried out by the breeder for the best traits can lead to unconscious selection of related genotypes. Several studies have demonstrated the low genetic variability of commercially planted *C. arabica* cultivars (Lashermes et al. 2000; Anthony et al. 2001; Maluf et al. 2005; Cubry et al. 2008; Lashermes et al. 2011; Setotaw et al. 2013; Motta et al. 2014; Pestana et al. 2015).

Thus, the development of a molecular marker set to establish the molecular pattern of each cultivar (fingerprinting) is extremely important to breeding programs. This cultivar discrimination must be fast, accurate, and precise (Collard et al. 2005; Le et al. 2016). Molecular markers have been increasingly used in recent times and is also important for the continuity of breeding programs and for the guarantee of the intellectual property of the final product of genetic selection (Ercisli et al. 2011; Rauscher and Simko 2013; Scarano et al. 2015).

Among the several types of molecular markers, microsatellites (SSR) are most commonly used in genetic diversity and fingerprinting studies. Besides being locus specific, it possess several advantages, including high degree of polymorphism, repeatability, reproducibility, codominance, and multiallelism

(Banerjee et al. 2012; Kaur et al. 2015). The advantages of molecular markers have also been highlighted in various studies on coffee plants (Diola et al. 2011; Missio et al. 2011; Motta et al. 2014; Ferrão et al. 2015; Pestana et al. 2015).

Thus, this study aimed to: (a) verify the informative capacity and effectiveness of molecular markers to discriminate amongst coffee varieties (b) create a database of DNA profiles and allele frequencies (c) analyze the genetic diversity existing in this collection, and (d) explore genetic kinships.

Materials and methods

Genetic material

A total of 34 *C. arabica* genotypes of were used in this study (Table 1); these included cultivars carrying genes that confer resistance to rust, the most important disease in coffee plants. This genetic material is maintained at the experimental area of the Department of Plant Pathology of the Universidade Federal de Viçosa. These plants, which belong to the Brazilian Cultivar Trial, were selected due to the difficulty in their discrimination based on morphological traits and commercial importance. The trial has been carried out in the main coffee-producing regions of Brazil.

The genotypes evaluated were composed of 26 cultivars, five elite progenies resistant to coffee leaf rust, and three susceptible cultivars. From the three susceptible cultivars, two belonged to the group Catuaí and one belonged to the group Bourbon. Catuaí is the most planted group of commercial cultivar in Brazil (Fernandes et al. 2012), while the group Bourbon is recognized for its excellent cup quality worldwide; this quality is a highly valued trait in special coffee markets (Ferreira et al. 2013).

Currently, of the 131 cultivars of *C. arabica* registered in the NRC of MAPA (Brasil 2017a), 14 cultivars are protected by the National Service of Plant Variety Protection (NSPVP) (Brasil 2017b). Out of these cultivars, seven (Araponga MG 1 [28], Catiguá MG 1 [24], Catiguá MG 2 [25], IPR 98 [18], Pau Brasil MG 1 [29], Sacramento MG 1 [27], and IAC 125 RN [14]) were evaluated in this study.

In each cultivar/progeny, six plants were analyzed, constituting a total of 204 genotypes. The occurrence of genetic variability per locus, between and within cultivars, was verified. The genetic variability within cultivars was evaluated by analyzing the six individuals that constituted each genetic material. Genetic variability between cultivars was evaluated in the same locus as follows: first, the alleles observed in the bulk data of individuals were analyzed and then the alleles observed per individual were analyzed.

DNA extraction and SSR marker amplification

In each selected genotype, healthy, fully expanded, young, and light green colored leaves were collected. The leaves were lyophilized and powdered. Genomic DNA was extracted using the method proposed by Diniz et al. (2005). DNA quality and quantity were evaluated using the NanoDrop 2000—Thermo Scientific spectrophotometer. The samples were standardized at 25 ng μL^{-1} and stored at −20 °C.

A total of 31 pairs of microsatellite primers were used (Online Resource 1). PCR amplification was carried out as follows: 50 ηg DNA, 1 U Taq polymerase, 1X enzyme buffer, 1.0 mM $MgCl_2$, 150 μM of each dNTP, and 0.1 μM of each primer, completing the total volume of 20 μL with milli-Q sterile water. The reactions were carried out on PTC-200 (*MJ Research*) and Veriti (*Applied Biosystems*) Thermal Cyclers. After the initial denaturation at 94 °C for 2 min, 10 touchdown PCR cycles were carried out at 94 °C for 30 s; with decreasing annealing temperature of 1 °C for every cycle (66 to 57 °C) for 30 s; and extension at 72 °C for 30 s, followed by 30 cycles of denaturation at 94 °C, annealing at 57 °C, and extension at 72 °C for 30 s at each stage. Final extension was carried out at 72 °C for 8 min. The resulting products of the PCR reaction were separated by 6% polyacrylamide denaturing gel electrophoresis and visualized by silver nitrate staining (Brito et al. 2010).

Statistical analysis

The fragments amplified by SSR markers (codominant) were coded according to the observed genotype and software requirements. Thus, for example, for the locus with four alleles, individuals were assigned 11, 22, 33, and 44 codes for the homozygote genotype, or 12, 13, 14, 23, 24, and 34 for heterozygote genotypes. The distance matrix was generated by the arithmetic complement of the unweighted index (Cruz et al.

Table 1 Coffee trees analyzed with molecular markers

No.	Cultivar or Progeny	Origin	RNC[a] number	Rust resistance reaction	Resistance source[b]	Observations
1	Catuaí Vermelho IAC 144	IAC	02934	S[c]	–	
2	Catuaí Vermelho IAC 15	IAC	02927	S	–	
3	Bourbon Amarelo UFV 535	UFV	–	S	–	Cultivar with cup quality standard
4	Catucaí Amarelo 2SL	MAPA/Fundação Procafé	04915	MR[d]	Icatu	
5	Catucaiam 24137	MAPA/Fundação Procafé	28888	MR	Icatu	
6	Catucaiam 2015479	MAPA/Fundação Procafé	28885	MR	Icatu	
7	Catucaí 785-15	MAPA/Fundação Procafé	04996	MR	Icatu	Resistance to the nematode *M. exigua*
8	Catucaí Vermelho 20/15	MAPA/Fundação Procafé	04910	MR	Icatu	
9	Sabiá tardio	MAPA/Fundação Procafé	04992	MR	CIFC 832/1	
10	IBC-Palma-2	MAPA/Fundação Procafé	04998	MR	CIFC 832/1	
11	Acauã	MAPA/Fundação Procafé	04995	R[e]	CIFC 832/2	
12	Tupi Amarelo IAC 5162	IAC	–	R	CIFC 832/2	Elite progeny of the IAC breeding program
13	Tupi IAC 1669-33	IAC	02957	R	CIFC 832/2	
14	IAC 125 RN	IAC	28587	R	CIFC 832/2	Cultivar protected by the NSPVP[f] and resistance to the nematode *M. exigua*
15	Obatã IAC 1669-20	IAC	02956	R	CIFC 832/2	
16	Obatã Amarelo IAC 4932	IAC	–	MR	CIFC 832/2	Elite progeny of the IAC breeding program
17	Iapar 59	IAPAR	02324	R	CIFC 832/2	
18	IPR 98	IAPAR	09950	R	CIFC 832/2	Cultivar protected by the NSPVP[f]
19	IPR 99	IAPAR	09949	MR	CIFC 832/2	
20	IPR 100	IAPAR	09948	MR	BA-10	
21	IPR 103	IAPAR	09945	MR	Icatu	Resistance to the nematodes *M. paranaensis* and *M. incognita*
22	IPR 104	IAPAR	09944	R	CIFC 832/2	
23	Oeiras MG 6851	EPAMIG/UFV	04755	MR	CIFC 832/1	
24	Catiguá MG1	EPAMIG/UFV	18632	R	UFV440-10	Cultivar protected by the NSPVP[f]
25	Catiguá MG2	EPAMIG/UFV	18633	R	UFV440-10	Cultivar protected by the NSPVP[f]
26	MGS Catiguá 3	EPAMIG/UFV	22098	R	UFV440-10	Resistance to nematode *M. exigua* and *Coffea Berry Disease*

Table 1 continued

No.	Cultivar or Progeny	Origin	RNC[a] number	Rust resistance reaction	Resistance source[b]	Observations
27	Sacramento MG1	EPAMIG/UFV	18631	R	UFV438-52	Cultivar protected by the NSPVP[f]
28	Araponga MG1	EPAMIG/UFV	18635	R	UFV446-08	Cultivar protected by the NSPVP[f]
29	Pau Brasil MG1	EPAMIG/UFV	18634	R	UFV442-34	Cultivar protected by the NSPVP[f]
30	Paraíso MG H 419-1	EPAMIG/UFV	15981	R	UFV445-46	
31	H 419-3-3-7-16-4-1	EPAMIG/UFV	–	R	UFV445-46	Elite progeny of the Epamig/UFV breeding program
32	H 419-10-6-2-5-1	EPAMIG/UFV	–	R	UFV445-46	Elite progeny of the Epamig/UFV breeding program
33	H 419-10-6-2-10-1	EPAMIG/UFV	–	R	UFV445-46	Elite progeny of the Epamig/UFV breeding program
34	H 419-10-6-2-12-1	EPAMIG/UFV	–	R	UFV445-46	Elite progeny of the Epamig/UFV breeding program

[a] Number of National Register of cultivars

[b] Source of rust resistance used for the development of the cultivar

[c] Susceptible

[d] Moderately resistant

[e] Resistante

[f] *NSPVP* National Service of Plant Variety Protection

2011) implemented in the GENES software (Cruz 2013), and the node consistency dendrogram was generated using the MEGA7 software (Kumar et al. 2016).

Genetic distance can be estimated by the following equation

$$D_{ii'} = 1 - \left(\frac{1}{2L} \sum_{j=1}^{L} C_j \right)$$

where Dii′ is the genetic distance between pairs of accessions i and i′; L is the total number of loci studied; and C_j is the number of common alleles between pairs of accessions i and i′.

A dendrogram was constructed, and two fingerprinting analysis were performed. The genotypes were analyzed separately; however, in the construction of the dendrogram and in the first fingerprinting analysis, the bulk of data of the six genotypes for each cultivar/progeny was used. Thus, if the marker appeared in only one individual, it would be considered in the molecular profile of the cultivar. The second fingerprinting analysis was performed with individual data of the genotypes composed of each cultivar/progeny.

The dendrogram was constructed using the UPGMA clustering technique from the values of the distance matrix generated. A set of markers to be used in the discrimination of the cultivars/progenies evaluated were also defined, establishing the unique marker profiles for each cultivar (fingerprinting).

Results

Of the 31 pairs of microsatellite primers analyzed in the cultivars/progenies, four amplified weak bands and were therefore removed from the study. Of the remaining markers, 20 were polymorphic (74.04%) and revealed 47 alleles. The number of alleles per

locus ranged from two to four, with an average of 2.35 alleles.

Molecular profiles obtained using SSR markers presented segregation between and within cultivars/progenies. Genetic variability was observed between all the cultivars/progenies studied. Online Resource 2 shows polymorphism within the cultivars Catiguá MG1 (24) and Sacramento MG1 (27) and polymorphism between the cultivars Catiguá MG2 (25) and Araponga MG1 (28) for CaEST-031 SSR marker. In this example, heterozygote individuals carrying alleles A^1A^2 were encoded as 12; and homozygote individuals carrying only allele A^1 and allele A^2 were encoded as 11 and 22, respectively.

Of the 204 analyzed genotypes, four plants (IBC-Palma-2 [10] plant 07-B2-P2, Tupi IAC 1669-33 [13] plant 16-B3-P1, IAC 125 RN [14] plant 30-B2-P4, and Catuaí Vermelho IAC 15 [2] plant 32-B1-P6) showed different alleles in several loci compared to the molecular profile of its cultivar. These plants were removed and not included for further analyses.

Analysis of the 20 polymorphic markers showed that the number of polymorphic microsatellite loci among individuals within the cultivar/progeny ranged from 0 to 11 (Table 2). Eight cultivars/progenies showed no segregation among the six individuals which constitute them. On the other hand, in Catiguá MG1 (24) cultivar individuals, polymorphism was observed in 11 SSRs markers (55%).

Although the number of polymorphic loci was the same for a few cultivars, variability was also found among them. Polymorphism in one locus (5%) was observed for cultivars IAC 125 RN (14), MGS Catiguá 3 (26), IPR 99 (19), and IPR 100 (20). However, cultivar IAC 125 RN (14) presented one segregating individual, cultivar MGS Catiguá 3 (26) presented two segregating individuals, and cultivars IPR 99 (19) and IPR 100 (20) presented segregation in three of the six individuals which constitute them.

Only the microsatellite polymorphic markers whose loci were diploid and codominant were considered for dendrogram construction and for the establishment of cultivar molecular profiles (fingerprinting). Thus, of the 20 polymorphic markers analyzed, 16 were selected and all subsequent analyses were performed using them. The markers CaEST-006, CaEST-040, CaEST-089, and SSRCa 52 were eliminated, since they amplified three or four alleles per individual.

From the set of 16 SSR markers, two showed a heterozygous molecular profile for most of the cultivars/progenies evaluated, although *C. arabica* is an autogamous species. CaEST030 revealed homozygous pattern only in the individuals of the cultivar IBC-Palma-2 (10), and CaEST-024 revealed only in tree cultivars Tupi Amarelo IAC 5162 (12), Tupi IAC 1669-33 (13), and IAC 125 RN (14).

The dendrogram, with 40% maximum dissimilarity observed at the last fusion level (0.41), showed 14

Table 2 Number of polymorphic loci within the cultivar/progeny analyzed by 20 polymorphic markers

No. of polymorphic loci	Cultivars/progenies
0	Catuaí Vermelho IAC 144, Catuaí Vermelho IAC 15, Bourbon Amarelo UFV535, Catucaí Vermelho 20/15, Sabiá tardio, Obatã IAC 1669-20, IPR 103 e H 419-3-3-7-16-4-1
1	IAC 125 RN, IPR 99, IPR 100 e MGS Catiguá 3
2	Catucaí Amarelo 2SL, Catucaiam 24137, Catucaí 785-15, IBC-Palma-2, Acauã, Tupi IAC 1669-33, Obatã Amarelo 4932, Oeiras MG 6851, Araponga MG1 e H 419-10-6-2-10-1
3	Catucaiam 2015479 e Catiguá MG2
4	IPR 98 e H 419-10-6-2-12-14
5	Paraíso MG H 419-1
6	IPR 104 e Sacramento MG1
7	Tupi Amarelo IAC 5162 e Iapar 59
8	Pau Brasil MG1
9	H 419-10-6-2-5-1
11	Catiguá MG1

groups (Fig. 1). Three groups clustered two cultivars/progenies: the first group comprised cultivars Iapar 59 (17) and IPR 104 (22), the second group consisted of cultivars Tupi IAC 1669-33 (13) and IAC 125 RN (14), and the third group comprised H 419-10-6-2-10-1 (33) and H 419-10-6-2-12-1 (34). One group clustered three cultivars, namely Tupi Amarelo IAC 5162 (12), IPR 98 (18), and Pau Brasil MG1 (29). Nine cultivars/progeny, namely (IBC-Palma-2 [10], Acauã [11], IPR 99 [19], Catiguá MG1 [24], Catiguá MG2 [25], MGS Catiguá 3 [26], Sacramento MG1 [27], Paraíso MG H 419-1 [30], and H 419-10-6-2-5-1[32]) did not cluster with the group containing one cultivar.

The remaining cultivars were allocated to a single cluster. This cluster was divided into two sub-clusters, one consisting of the cultivars/progenies Catuaí Vermelho IAC 144 (1), Catuaí Vermelho IAC 15 (2), Bourbon Amarelo UFV535 (3), Catucaiam 2015479 (6), Sabiá tardio (9), Obatã IAC 1669-20 (15), Obatã Amarelo 4932 (16), Araponga MG1 (28), and H 419-3-3-7-16-4-1 (31); and the other consisting of the cultivars Catucaí Amarelo 2SL (4), Catucaiam 24137 (5), Catucaí 785-15 (7), Catucaí Vermelho 20/15 (8), IPR 100 (20), IPR 103 (21), and Oeiras MG 6851 (23).

Genetic distance between the cultivars Catuaí Vermelho IAC 144 (1), Catuaí Vermelho IAC 15 (2), and the progeny H 419-3-3-7-16-4-1 (31) was 0. This set of 16 selected SSR markers could not be distinguished among these cultivars. Similarly, it was not possible to distinguish between Catucaí Amarelo 2SL (4) and Catucaiam 24137 (5).

The dissimilarity obtained at the last fusion level was 0.41. Maximum dissimilarity (0.6563) was observed between the cultivars Catucaí Amarelo 2SL (4) and MGS Catiguá 3 (26). The same genetic distance was observed between the cultivars Catucaí 785-15 (7) and MGS Catiguá 3 (26).

A total of 31 distinct molecular profiles were obtained through fingerprinting analysis, which considered the genotyping data of the bulk of six

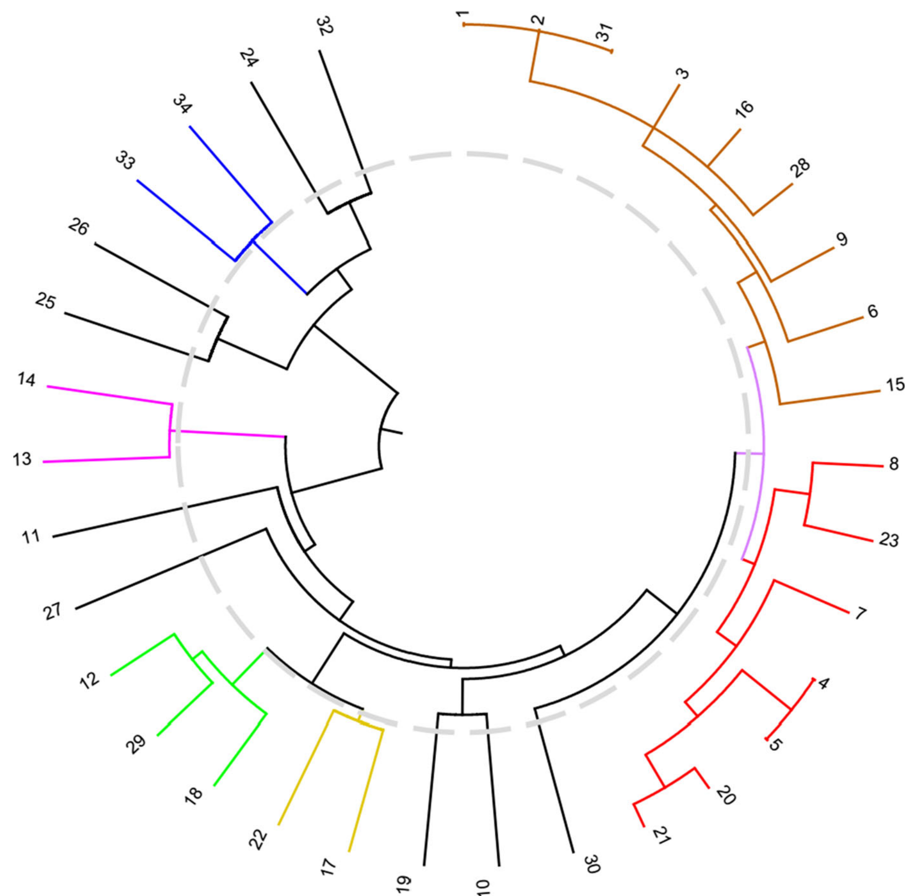

Fig. 1 Dendrogram obtained by the UPGMA technique, based on the dissimilarity matrix of the arithmetic complement of the unweighted index from 34 *C. arabica* cultivars/progenies. The *numbers* in the dendrogram are related to the cultivars described in the Table

individuals of each cultivar/progeny (Table 3). To facilitate visualization and interpretation, each genotype received a distinct color, and genotypes 11, 22, and 33 were recoded as 1, 2 and 3, respectively. A unique molecular profile was obtained for cultivars Catuaí Vermelho IAC 144 (1), Catuaí Vermelho IAC 15 (2), and progeny H 419-3-3-7-16-4-1 (31). Thus, as in the dendrogram, these cultivars/progenies were not discriminated. The same occurred for Catucaí Amarelo 2SL (4) and Catucaiam 24137 (5).. (Color table online)

In the second fingerprinting analysis, molecular profiles of cultivars/progenies were obtained through the individual data of the genotypes that constitute them (Table 4). One to three different genotypes per cultivar/progeny were observed in each locus. All the different genotypes presented by a cultivar/progeny were taken into account for the construction of molecular profile. For instance, evaluation of CaEST-001 locus in Sacramento MG1 (27) cultivar revealed one plant with the allele (A^1A^1), the second with (A^1A^2), and the third with (A^2A^2). Therefore, Sacramento MG1 (27) cultivar profiles for CaEST-001 marker are 1, 12, and 2 (Table 4). This indicates that these three genotypes can be found in this cultivar.

Discussion

In 34 *C. arabica* cultivars, a mean of 2.35 alleles per primer was obtained using 20 polymorphic markers. Similar to this result, a mean of 2.5 alleles per microsatellite primer has been reported in a study on 19 *C. arabica* cultivars (Vieira et al. 2010); indicating a narrow genetic base between the cultivars/progenies. This can be explained by the low number of plants that were initially introduced in Brazil, which constitute the genetic base of the current cultivars (Setotaw et al. 2013). According to these authors, the genetic base of the 121 cultivars released in Brazil between 1939 and 2009 originated only from 13 parents. They also found that out of these parents, seven contributed to 97.55% of the genetic base of *C. arabica* cultivars from Brazil, the world's largest coffee producer.

A high percentage of polymorphic primers (74.04%) was observed. However, most primers used in the analysis were selected for being polymorphic in other studies on this species (Capucho et al. 2009; Ferrão et al. 2015; Pestana et al. 2015), explaining the high polymorphism found in this study. In several studies, approximately 10% polymorphic SSR markers have been observed in *C. arabica* (Capucho et al. 2009; Pestana et al. 2015). In an F_2 population derived from the cross between Catuaí Amarelo IAC 64 (UFV 2148-57) and Híbrido de Timor UFV 443-03, 7.34% polymorphism was observed in 286 SSR primers (Capucho et al. 2009). This same population analyzed by 373 pairs of microsatellite primers presented 15.5% polymorphic primers (Pestana et al. 2015). These results demonstrate the low genetic variability of this species, since the studied population originated from a contrasting cross in the F_2 generation. In several studies, a small number of polymorphic loci was observed for *C. arabica* (Combes et al. 2000; Anthony et al. 2001; Anthony et al. 2002; Sera et al. 2003; Diniz et al. 2005; Vieira et al. 2010).

Another factor that influences the high genetic similarity between *C. arabica* cultivars analyzed in the present study was the parents used as sources of rust-resistant genes. Coffee rust is considered the most important disease in coffee (Zambolim 2016); researchers use the same sources of rust-resistant genes worldwide. These parents are derived primarily from Híbrido de Timor, Icatu, and Indian Selections (Avelino et al. 2015). Cultivars/progenies carrying rust-resistant genes, which have been released and form part of the Cultivars National Trial, are derived from Híbrido de Timor and Icatu. The cultivar IPR 100 (20) is the only exception, whose parent used as a source of rust-resistant genotype was BA-10, an Indian selection.

The high genetic similarity between coffee cultivars highlights the need to identify a set of informative molecular markers that are able to differentiate among them and consequently be used for cultivar fingerprinting. Once identified, this set of markers will facilitate discrimination between cultivars in DUS testing.

The molecular profiles of the cultivars were obtained using 20 polymorphic markers. Four plants were eliminated because they showed different alleles in several loci. This indicated the possibility of varietal mixture. The mixture was confirmed by the phenotypic data in the field. The plant 32-B1-P6 (cultivar Catuaí Vermelho IAC 15 [2]) presented yellow fruits, differing from the fruit color standard of the cultivar. Genotype 16-B3-P1 (cultivar Tupi IAC 1669-33 [13]) was susceptible to rust, differing from the standard of

Table 3 Molecular profile of 34 evaluated cultivars/progenies obtained by bulking of individuals that constitute the cultivars/progenies of *Coffea arabica*. (Colour table online)

Cultivar/progeny	Microsatellite markers															
	CaEST														SSR	
	001	002	022	024	028	029	030	031	034	045	048	058	071	072	16	95
1 Catuaí Vermelho IAC 144	2	3	2	12	2	2	12	1	2	2	1	1	1	2	1	1
2 Catuaí Vermelho IAC 15	2	3	2	12	2	2	12	1	2	2	1	1	1	2	1	1
3 Bourbon Amarelo UFV535	2	2	2	12	2	2	12	1	2	2	1	1	1	2	1	1
4 Catucaí Amarelo 2SL	2	3	2	12	2	2	12	1	2	2	1	1	1	2	2	13
5 Catucaiam 24137	2	3	2	12	2	2	12	1	2	2	1	1	1	2	2	13
6 Catucaiam 2015479	2	3	12	12	2	2	12	1	2	2	1	1	1	2	12	1
7 Catucaí 785-15	2	3	2	12	12	2	12	12	2	2	1	1	1	2	2	3
8 Catucaí Vermelho 20/15	2	3	1	12	2	2	12	1	2	2	1	1	1	2	2	1
9 Sabiá tardio	2	3	2	12	2	2	12	1	2	2	1	1	1	2	1	2
10 IBC-Palma-2	2	2	2	12	12	1	2	1	2	2	1	1	1	2	12	1
11 Acauã	12	1	2	12	2	1	12	3	2	2	1	1	1	2	2	12
12 Tupi Amarelo IAC 5162	2	23	2	12	2	2	12	13	2	2	1	12	1	2	12	1
13 Tupi IAC 1669-33	12	2	2	1	2	2	12	1	2	2	1	2	2	2	2	1
14 IAC 125 RN	2	3	2	1	2	2	12	3	2	2	1	2	2	2	2	1
15 Obatã IAC 1669-20	2	2	2	12	2	2	12	1	2	2	1	1	1	2	2	1
16 Obatã Amarelo 4932	2	23	2	12	2	12	12	1	2	2	1	1	1	2	1	1
17 Iapar 59	12	23	2	12	2	1	12	13	2	2	1	2	12	2	12	13
18 IPR 98	2	23	2	12	2	1	12	13	2	2	1	12	12	2	2	1
19 IPR 99	2	2	2	12	1	2	12	13	2	2	1	1	1	2	2	1
20 IPR 100	2	3	2	12	2	2	12	2	2	2	1	1	1	2	2	13
21 IPR 103	2	3	2	12	2	2	12	2	2	2	1	1	1	2	2	1
22 IPR 104	12	3	2	12	2	1	12	3	2	2	1	12	12	2	1	1
23 Oeiras MG 6851	2	3	2	12	2	12	12	1	2	2	1	1	1	2	12	3
24 Catiguá MG1	2	13	2	12	2	2	12	13	12	2	12	12	12	12	1	1
25 Catiguá MG2	2	3	2	12	2	2	12	3	12	1	12	12	2	1	2	1
26 MGS Catiguá 3	2	3	2	12	2	2	12	3	1	1	2	1	1	1	12	1
27 Sacramento MG1	12	13	2	12	12	2	12	13	1	2	1	1	1	2	12	1
28 Araponga MG1	2	23	2	12	2	2	12	1	2	2	1	1	1	2	1	13
29 Pau Brasil MG1	2	23	2	12	2	12	12	13	12	2	1	12	12	2	12	1
30 Paraíso MG H 419-1	2	3	2	12	2	2	12	1	12	2	1	12	12	2	2	13
31 H 419-3-3-7-16-4-1	2	3	2	12	2	2	12	1	2	2	1	1	1	2	1	1
32 H 419-10-6-2-5-1	1	13	2	12	2	2	12	13	1	12	12	12	12	12	2	1
33 H 419-10-6-2-10-1	1	1	2	12	2	2	12	3	1	1	2	2	2	12	12	1
34 H 419-10-6-2-12-1	2	1	2	12	2	2	12	3	1	12	12	2	2	12	1	1

1 = A^1A^1; 2 = A^2A^2; 3 = A^3A^3; 12 = A^1A^2; 13 = A^1A^3; 23 = A^2A^3

Table 4 Molecular profile of cultivars/progenies obtained from the individual data of genotypes, which constitute the cultivars/progenies of *C. arabica*

Molecular marker	Number of cultivar/progeny[a]																																	
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34
CaEST-001	2	2	2	2	2	2	2	2	2	2	1 2	2	1 2	2	2	2	12 2	2	2	2	2	1 2	2	2	2	2	1 12 2	2	2	2	2	1	1	2
CaEST-002	3	3	2	3	3	3	3	3	3	2	1	2 3	2	3	2	13 3	2 23	2 23 3	2	3	3	3	3	1 13 3	3	3	1 13 3	2 23 3	2 23 3	3	3	1 13 3	1	1
CaEST-022	2	2	2	2	2	1 2	2	1	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2
CaEST-024	12	12	12	12	12	12	12	12	12	12	12	1 12	1	1	12	12	12	12	12	12	12	12	12	12	12	12	12	12	12	12	12	12	12	12
CaEST-028	2	2	2	2	2	2	1 2	2	2	1 2	2	2	2	2	2	2	2	2	1	2	2	2	2	2	2	2	1 2	2	2	2	2	2	2	2
CaEST-029	2	2	2	2	2	2	2	2	2	1	1	2	2	2	2	1 12	1	1	2	2	2	1 12	1 12 2	2	2	2	2	2	1 12 2	2	2	2	2	2
CaEST-030	12	12	12	12	12	12	12	12	12	2	12	12	12	12	12	12	12	12	12	12	12	12	12	12	12	12	12	12	12	12	12	12	12	12
CaEST-031	1	1	1	1	1	1	1 2	1	1	1	3	1 13 3	1	3	1	1	13 3	1 3	1 3	2	2	3	1	1 13 3	3	3	13 3	1	1 3	1	1	13 3	3	3
CaEST-034	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	1 12 2	1 12 2	1	1	2	12 2	1 12	2	1	1	1
CaEST-045	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	1	1	2	2	2	2	2	12 2	1	1 12 2
CaEST-048	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1 12	12	2	1	1	1	1	1	1 12 2	2	1 12 2
CaEST-058	1	1	1	1	1	1	1	1	1	1	1	1 12	2	2	1	1	2	1 12 2	1	1	1	1 2	1	1 12	1 2	1	1	1	1 12	12 2	1	12 2	2	2
CaEST-071	1	1	1	1	1	1	1	1	1	1	1	1	2	2	1	1	12 2	1 12 2	1	1	1	1 2	1	1 12 2	2	1	1	1	1 12	12 2	1	12 2	2	2

Table 4 continued

Molecular marker	Number of cultivar/progeny[a]																																	
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34
CaEST-072	2	1	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	1	1	1	2	2	2	2	2	12	1	1
		2																						12								2	12	12
																								2										2
SSR-016	1	1	1	2	2	1	2	2	1	1	2	1	2	2	2	1	1	2	2	2	2	1	1	1	1	1	1	1	1	2	1	1	1	1
						2				2		2					12						2			2	2		2				2	
																	2																	
SSR-095	1	1	1	1	13	1	3	1	2	1	1	1	1	1	1	1	1	1	1	1	1	1	3	1	1	1	1	1	1	1	1	1	1	1
				13	3						12						3			13								13		13				
				3							2									3														

1 = A^1A^1; 2 = A^2A^2; 3 = A^3A^3; 12 = A^1A^2; 13 = A^1A^3; 23 = A^2A^3

[a] The *numbers* 1–34 represent the cultivars as listed in Tab. 1

the cultivar which harbors resistant genes. The plant 30-B2-P4 (cultivar IAC 125 RN [14]) was more vigorous than other plants of this cultivar. These results demonstrate the ability of the marker to detect mixtures; therefore, in further studies and in the absence of field data, markers should be used to eliminate varietal mixtures.

Segregation between and within cultivars/progenies was observed by molecular analysis of the markers used in this study. The existence of genetic variation between the majority of cultivars demonstrated the effectiveness of SSR markers to differentiate cultivars of species with narrow and phenotypically similar genetic base.

Segregation in *C. arabica* cultivars can be explained by ploidy (tetraploid); although this species is self-pollinated, it presents approximately 10% outcrossing (Lashermes et al. 2000). Thus, greater number of selfing generations is required to increase the level of homozygosity. In addition, this species is perennial with a long juvenile period, a fact that extends the advancement of generations (Sera 2001). However, for the viability of breeding programs and to release new cultivars, plant breeders register *C. arabica* cultivars that can segregate even when they are in F_6 or more advanced generations. The occurrence of late segregations was observed in progenies in which no phenotypic segregations were found in earlier generations.

Analysis of the 20 polymorphic markers revealed that the number of polymorphic loci within each cultivar/progeny ranged from 0 to 11. Eight cultivars/progenies did not segregate among the six individuals which constitute them, suggesting high level of homozygosity. In cultivar Catiguá MG1 (24), polymorphism was observed in 11 loci (55%). Cultivar Catiguá MG1 (24) originated from an artificial cross between cultivar Catuaí Amarelo IAC 86 and Híbrido de Timor UFV 440-10, which were susceptible and resistant to rust, respectively. These parents are genetically divergent, which explains the genetic variation observed in the cultivar. Furthermore, this cultivar was released in the F_5 generation; in this generation, relatively high levels of heterozygosity are commonly observed in polyploid species.

Of the 20 polymorphic SSR markers evaluated, four were eliminated for being tetraploid. This is due to the probable origin of *C. arabica*. It is believed that this species originated from the fusion of unreduced

gametes of the diploid species *C. eugenioides* and *C. canephora* (Lashermes et al. 1999). The genomes of these species have high similarity, and there may be duplicated regions in *C. arabica* genome. The SSR marker located in the duplicated regions, present in the genome of the two-parent species of *C. arabica*, have four alleles per individual (tetraploid). The SSR markers of non-duplicated regions presented two alleles per individual (diploid). The exclusion of tetraploid molecular markers occurred in order to take advantage of the codominant nature of the marker. Therefore, all subsequent analyses were performed with 16 SSR that performed as diploid and codominant molecular markers.

The molecular markers CaEST024 and CaEST030 presented a heterozygous molecular pattern for most cultivars/progenies evaluated. This would not be expected since *C. arabica* is an autogamous species. However, this may be justified by the fact that the molecular marker amplifies the alleles in homeologous chromosomes of the two genomes that comprise this species. Thus, in each homologous chromosome, the alleles are homozygous and, with the self-fertilization cycles, no genetic variation is expected by the meiotic process. In polyploid crops, genome duplication results in a considerable number of duplicated genes or homeologs. Duplicated genes or homeologs may be differentially expressed depending on the organ, developmental stage, and environment, which increase the difficulty in discriminating the function of individual genes. In addition, numerous duplicated genes or homeologs and repetitive sequences hinder the correct sequence assembly and the accurate quantification of gene expression (Fu et al. 2016).

Dendrogram analysis showed 14 groups. Cultivars Iapar 59 (17) and IPR 104 (22) were allocated in the same group. These cultivars were developed by breeding program of the Instituto Agronômico do Paraná (IAPAR), and both are derived from Sarchimor (Villa Sarchi × Híbrido de Timor CIFC 832/2). The other two groups were also allocated materials of the same breeding institution. One group with cultivars Tupi IAC 1669-33 (13) and IAC 125 RN (14) belongs to Instituto Agronômico de Campinas (IAC) and the other group with progenies H 419-10-6-2-10-1 (33) and H 419-10-6-2-12-1 (34) originated from Empresa de Pesquisa Agropecuária de Minas Gerais (EPAMIG) and Universidade Federal de Viçosa (UFV). Cultivars Tupi IAC 1669-33 (13) and IAC 125 RN (14) were selected from seeds of the same hybrid (CIFC H361/4). The same occurred with the progenies H 419-10-6-2-10-1 (33) and H 419-10-6-2-12-1 (34), which were selected from the cross between Catuaí Amarelo IAC 30 and Híbrido de Timor UFV 445-4. This justifies the allotment of the genotypes to the same group.

Cultivars Tupi Amarelo IAC 5162 (12), IPR 98 (18), and Pau Brasil MG1 (29) constitute a single group. The other cultivars were allocated to another group that can be subdivided into two subgroups. In the first subgroup, the cultivars Catuaí Vermelho IAC 144 (1), Catuaí Vermelho IAC 15 (2), and the progeny Bourbon Amarelo UFV 535 (3) are susceptible to coffee rust (Patricio et al. 2010). In addition, the first two cultivars belong to the Catuaí group, justifying the high similarity between them. The other cultivars in this first subgroup carry rust-resistant factors and present the cultivar Catuaí Amarelo in their genetic constitutions.

All cultivars allocated in the second subgroup are moderately resistant to coffee rust. This subgroup allocated the cultivars that have Icatu as source of resistance factors, except for cultivar Catucaiam 2015479 (6), which was allocated to the first subgroup.

Similarity was observed between the cultivars Catuaí Vermelho IAC 144 (1), Catuaí Vermelho IAC 15 (2), and the progeny H 419-3-3-7-16-4-1 (31). Similarity between the cultivars Catuaí Vermelho IAC 144 (1) and Catuaí Vermelho IAC 15 (2) can be explained by their genealogy, since they are derived from the genotype H 2077-2. Phenotypic traits of progeny H 419-3-3-7-16-4-1 (31) demonstrate its genetic proximity to cultivar Catuaí, corroborating the high similarity found between these materials in the present study.

Genetic distance between the cultivars Catucaí Amarelo 2SL (4) and Catucaiam 24137 (5) was also 0. Cultivars of the Catucaí group are derived from natural cross between germplasm Icatu and Catuaí, which is a possible reason for the high similarity observed. Moreover, these cultivars have high phenotypic similarity.

Maximum dissimilarity (0.656) obtained between cultivars Catucaí Amarelo 2SL (4) and MGS Catiguá 3 (26), and between cultivars Catucaí 785-15 (7) and MGS Catiguá 3 (26), may be because of the parents used as a source of rust resistance. In Catucaí cultivars, one of the parents was Icatu; in contrast, in cultivar MGS Catiguá 3 (26), the parent used as source of rust resistance was Híbrido de Timor.

Fingerprinting analysis with the bulk of data of the individuals that compose each cultivar/progeny revealed 31 unique molecular profiles. This result is very important to distinguish these materials. Besides presenting narrow genetic base, these materials are phenotypically similar. Application of SSR molecular markers in fingerprinting analysis, together with genetic diversity, has been reported in several plant species (Barchi et al. 2011; Triwitayakorn et al. 2011; Hajibarat et al. 2015; Chen et al. 2016; Rayda et al. 2016; Maldonado Dos Santos et al. 2016). These authors have highlighted the importance of using molecular markers. In sugarcane, with three SSR markers, 1,205 genotypes (Maccheroni et al. 2009) were differentiated. Seven microsatellite polymorphic markers were used to analyze paternity in seven parent sugarcane polycrosses (Tew and Pan 2010). SSR molecular markers have proved to be accurate in the discrimination of cultivar/progenies (Dutta et al. 2011). With the use of SSR markers, 128 *Camellia sinensis* cultivars were correctly discriminated (Tan et al. 2015).

Once genetic variation is detected between individuals of the same cultivar, analysis of the molecular profile based on bulk data may mislead the detection of some genotypes belonging to the cultivar. Therefore, in the present study, the molecular profile was also made available with all genotypic possibilities, considering allelic variations of the cultivar (Table 4).

The unique molecular profile of a cultivar will facilitate distinguishing kinship genotypes from those with similar phenotypic traits. Thus, fingerprinting is an auxiliary tool to the recommended descriptors, being extremely useful in DUS tests required for the registration process and for plant variety protection.

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Compliance with Ethical Standards

Conflict of interest The authors declare no conflict of interest.

Data archiving statement The authors have not submitted biological data to any of the public databases.

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