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Genetic diversity of arabica coffee (*Coffea arabica* L.) using 20 microsatellite markers in the germplasm bank of UNESUM, Ecuador.

Diversidad genética del café arábigo (Coffea arabica L.) aplicando 20 marcadores microsatélite en el banco de germoplasma de la UNESUM, Ecuador

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Summary: With the objective of determining the genetic diversity of arabica coffee (Coffea arabica L.) by applying 20 microsatellite markers in 20 accessions of the germplasm bank of the Universidad Estatal del Sur de Manabí (UNESUM), young leaflets were collected from the upper middle third of each plant of the 20 coffee accessions conserved in vivo at Finca Andil, in small sealed envelopes and placed in a box with 500 g of silica gel for drying and preservation of the leaflet samples, which were then sent to the Molecular Biology laboratory of the Santa Catalina Experimental Station of the National Institute of Agricultural and Forestry Research (INIAF). The QTA - genotyping analysis was performed with M13 Tailing technology for 20 microsatellite markers. The gels were analyzed visually, determining the weight of the fragments in base pairs (bp) amplified for QTA-genotyping in reference to a marker of known fragment weight. The bp of each marker for each of the coffee accessions (absence/presence) were recorded in an Excel spreadsheet for subsequent statistical analysis. For each marker assayed, a Chi-square test was applied to compare the means of presence levels in the accessions belonging to each marker class (absence/presence) determined, with respect to the bp of the alleles reported by other researchers. The results showed that the SSR markers used have a low or limited detection of genetic diversity, and that limitations could be observed in the levels of heterozygosity and homozygosity at specific loci due to the inability of these SSRs to distinguish alleles from homologous chromosomes, as well as the probability of finding null alleles in polyploids. Moreover, the Cam22 marker was found to be monomorphic.

Keywords: Accession, in vivo, DNA, genotyping, gels, markers.

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Resumen: Con el objetivo de determinar la diversidad genética del café arábigo (*Coffea arabica* L.) aplicando 20 marcadores microsatélite en 20 accesiones el banco de germoplasma de la Universidad Estatal del Sur de Manabí (UNESUM), se colectaron foliolos jóvenes del tercio medio superior de cada planta de las 20 accesiones de café conservados *in vivo* en la Finca Andil, en sobres pequeños con cierre y ubicados en una caja con 500 g de sílica gel para el secado y conservación de las muestras de foliolos, para luego enviarlas al laboratorio de Biología Molecular de la Estación Experimental Santa Catalina, del Instituto Nacional de Investigaciones Agropecuarias y forestales (INIAF). El análisis de QTA – genotyping fue realizado con la tecnología M13 Tailing para 20 marcadores microsatélite. Los geles fueron analizados de forma visual, determinando el peso de los fragmentos en pares de bases (pb) amplificado para el QTA-genotyping en referencia a un marcador de peso conocido del fragmento. Los pb de cada marcador para cada una de las accesiones de café (ausencia/presencia), fueron anotados en una hoja de cálculo en Excel para su posterior análisis estadístico. Para cada marcador ensayado se aplicó un test de Chi cuadrada para comparar las medias de niveles de presencia en las accesiones que pertenecen a cada clase de marcador (ausencia/presencia) determinado, respecto de los pb de los alelos reportados por otros investigadores. Los resultados mostraron los marcadores SSR utilizados tienen una baja o limitada detección de la diversidad genética, y que se pudo observar limitaciones en los niveles de heterocigosis y homocigosis en los locus específicos por la incapacidad de estos SSR para distinguir alelos de cromosomas homólogos, así como la probabilidad de hallar alelos nulos en poliploides. Por otra parte, se determinó que el marcador Cam22 fue monomórfico.

Palabras clave: Accesión, in vivo, ADN, genotipado, geles, marcadores.

Introduction

Sánchez Barrantes (2017) mentions that coffee is the most popular non-alcoholic beverage in the world and the second most important product in world trade after petroleum products (Dessalegn et al., 2009). The International Coffee Organization (ICO) for the year 2016, mentions that the average world production was 153 869 million 60 kg bags. Brazil was the main producing country contributing 35.7% of world production. The *Coffea* genus presents great economic importance in the Rubiaceae family (Montoya et al., 2006), it consists of more than 100 known species (Missio et al., 2009a) of which only *Coffea arabica* (coffee arabica) and *C. canephora* (robusta coffee) are commercially cultivated (Tornincasa et al., 2010). Currently, *C.* arabica is the most important species in trade as it produces high

quality coffee compared to *C. canephora*, and contributes around 70% of the world's total coffee production (Anthony et al., 2002, Tornincasa et al., 2010). In Ecuador coffee is of great economic importance and is grown on approximately 199 215 hectares, of which 68% are of the species C. *arabica*, and 32% of the species C. *canephora*, which are distributed in 23 of the 24 provinces of the country, so it has a wide social range (Valverde et al., 2020). Arabica coffee, unlike the other species, possesses conditions such as good fruit yield, bean size and is known for its excellent cup quality (Pérez, 1977). However, it has a major drawback, as it suffers from low genetic diversity due to its origin and domestication process, this low diversity is reflected in a higher susceptibility to diseases and pests (Pereira et al., 2015, Prakash et al., 2002). Therefore, coffee requires adaptation to the different environments in which it is grown to maintain market requirements. To achieve this requires the genetic improvement of coffee, which is mainly carried out through the use of conventional methods, which are generally slow and laborious (Hendre & Aggarwal, 2007). This situation calls for the development of tools that accelerate and provide reliability in the characterization of the gene pool, which will lead to a more efficient utilization of the germplasm available in coffee breeding programs (Hendre & Aggarwal, 2014, Brandão Motta et al., 2014). In this context, genetic markers based on DNA (deoxyribonucleic acid) polymorphism become an important tool that proved to be of great value in the characterization and genetic improvement of plant genetic resources. Among the main molecular markers used in commercially important cultivars are alloenzymes, RFLP (Restriction fragment length polymorphic), RAPD (Random amplified polymorphic DNA), AFLP (Amplified fragment length polymorphism), SNP (Single nucleotide polymorphism) and SSR (Simple sequence repeats) microsatellites (Azofeifa, 2006, Gabriel, 2009, Brandão Motta et al., 2014). Alloenzymes were the first molecular markers used in plant genetics. They are co-dominant markers and have been of great value in breeding studies in both natural populations and plantations, however, it is a very laborious technique, one must know the enzymes very well and it is poorly reproducible between laboratories (Azofeifa, 2006). RFLPs are also codominant markers that can be used with both nuclear DNA and organelle DNA. This technique requires large amounts of good quality DNA for the detection of single copy loci; specific probes are needed, it requires many manipulations and only a

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fraction of the variability of sequences existing in the genome is detected, i.e., its information is limited (Alcántara, 2007). RAPDs amplify both coding and non-coding regions of DNA and reveal higher levels of variation than RFLPs and isoenzymes (Parker et al., 1998). It is a technique that does not require prior knowledge of the DNA sequence and does not require species-specific probes (Azofeifa, 2006), however, it has low reproducibility and cannot differentiate between homozygous and heterozygous individuals as they are dominant markers (Zhivotovsky, 1999). AFLPs are highly polymorphic, do not require any prior sequence information for analysis and can be dominant or codominant markers. This technique is useful for generating genetic fingerprints and mapping; it has also been used for germplasm characterization, phylogenetic studies in plants, bacteria, fungi and in population genetics studies (Alcántara, 2007). However, it requires a high amount of DNA and the technique is more complicated to perform than RAPD (Azofeifa, 2006). SNPs are markers that have been widely used in genetic diversity studies; however, they have a rather low information content, so many have to be used to reach a good level of information (Rischkowsky & Pilling, 2010). Finally, there are simple sequence repeats (SSR) or microsatellite markers that are DNA sequences composed of short tandemly repeated motifs (Cristacho & Gaitán, 2008, Poncet et al., 2004). These markers have a wide site-specific length polymorphism due to different amounts of repeat units; they are also robust, transferable, codominant, chromosomally located at a single locus and can be developed as markers based on the polymerase chain reaction (PCR) technique, with amplification of complementary repetitive regions of the genome. The flanking regions of microsatellite sequences are conserved, this allows high reproducibility of the technique because the primers are designed for these desired regions (Motta et al., 2015). In addition, microsatellites are multiallelic and have numerous advantages compared to other types of markers because they are highly informative, require little genetic sample material and have the possibility of automation (Rovelli et al., 2000). Initially, the identification of microsatellite markers is expensive, as they need to be isolated de novo in most of the species being analyzed for the first time and the development of the primers requires a very long and laborious process (Zane et al., 2002). Currently, there are different strategies for the development of these primers, 4 however the most widely used method is the genomic library enriched with selective hybridization (Zane et al. 2002). Microsatellites are found in coding and non-coding regions of the genome (Missio et al., 2011), so

the strategy of designing universal primers does not work very well for this type of markers. However, some microsatellites have been reported to have highly conserved flanking regions, which allows amplification of these microsatellites in divergent species (Zane et al., 2002). In varieties with a narrow genetic base such as arabicas, the use of molecular markers has been very useful for several studies. Berthou and Trouslot (1977) conducted an isoenzyme study to determine the enzyme polymorphism of *Coffea*, and later Lashermes et al. (1999) conducted an RFLP analysis to characterize the characterization and origin of *Coffea arabica*; however, both studies found that polymorphism is extremely low among *Coffea* accessions and these markers were not sufficiently informative. Another molecular marker that has been important in *Coffea* studies was RAPD, which has been used to construct dendrograms that were found to be consistent with the known history and evolution of *Coffea arabica* (, Orozco et al., 1994, Lashermes et al., 1999). In addition, Orozco et al. (1994) report that RAPD markers have also been good markers for differentiating between *C. arabica* var. *typica* and *C. arabica* var. *bourbon*, as well as for detecting natural and interspecific introgression between diploid *C. canephora* and *C. arabica* (Rume Sudan RS-510 accession). A few years later, Steiger et al. (2002) used AFLP markers to determine the diversity between different cultivars of *Coffea arabica*. They mention that the AFLP technique used in this study is as reliable as RFLP and SSR at a lower cost, and is more reliable than RAPD markers. They found polymorphisms among some cultivars that allowed for cultivar differentiation; however, the genetic variation among arabica cultivars was similar to the variation within cultivars, and no cultivar-specific DNA markers were detected. The main disadvantage they mention with the use of the technique is that erroneous marker data may arise due to partial digestion of genomic DNA. On the other hand, SSR or microsatellite markers are potentially useful, especially for exploring highly variable regions of the genome between individuals or populations of the same species (Hurtado & Herrera, 2013, Missio et al., 2009a). These markers are ideal for the study of genetic diversity, population structure, phylogenetic relationships, construction of linkage maps, QTL interval assignment, among others (Hendre & Aggarwal, 2014). In previous studies of *Coffea arabica* the degree of allelic diversity revealed in SSR loci in most of these organisms indicates that SSRs are ideal for linkage analysis, agronomic trait selection, germplasm evaluation, and cultivar identification (Powell et

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al., 1996). However, despite all the advantages provided by these markers, the availability of polymorphic SSR markers remains a constraint in *C. arabica* (Ferrao et al, 2015, Missio et al., 2011), this due to the low abundance of SSRs present in its genome, which has caused great difficulty in the development of specific markers (Hendre & Aggarwal, 2007). For the reasons mentioned above, the present research aimed to determine the genetic diversity of arabica coffee (*Coffea arabica* L.) by applying 20 microsatellite markers in 20 accessions of the germplasm bank of the Universidad Estatal del Sur de Manabí.

Methodology

Location

The research was carried out at the Andil Farm, belonging to the State University of Southern Manabi (UNESUM), located at kilometer 5 of the road leading to the Noboa Parish of Canton 24 de Mayo. The canton Jipijapa, is bounded to the north by the cantons Montecristi, Portoviejo and Santa Ana, to the south by the province of Santa Elena and Puerto Lopez, to the east by the cantons Paján and 24 de mayo; and, to the west by the Pacific Ocean [Decentralized Autonomous Government (GAD, 2015)]. Jipijapa's predominant climate is hot dry in the western zone and hot humid with dry seasons in the eastern zone, with an average temperature of 24°C and a relative humidity of 85%, affected by the presence of two seasons, dry (between May and October) and rainy (between November and April) (GAD, 2015)

Plant material

The plant material used for this research consisted of 20 accessions from the coffee germplasm bank of the Universidad Estatal del Sur de Manabí, which is conserved *in vivo on* the grounds of the Granja Andíl, in Jipijapa. The characteristics of each of the accessions are described in Table 1.

Table 1. *Accessions of the germplasm bank of Coffea arabica L. of the Universidad Estatal del Sur de Manabí.*

No.	Accession	Origin	Progenitors	Feature	Source
1	Catimor 8666 (4-3).	Portugal	Timor x Caturra	Tolerant to rust and high grain production.	Villacreses (2017). Lucas-Suarez (2018), Parrales Marcillo (2018).
2	Red Catuai UFV	Brazil	(Sumatra x Bourbon	Susceptible to rust	Villacreses (2017). Lucas-Suarez (2018), Parrales Marcillo (2018).
3	Gheisha.	Ethiopia	*C. arabica*	Cup quality	Villacreses (2017). Lucas-Suarez (2018), Parrales Marcillo (2018).
4	Yellow bourbon	Africa	*C. arabica*	Susceptible to coffee rust**.** It has a tall growth habit and is of high quality.	Villacreses (2017). Lucas-Suarez (2018), Parrales Marcillo (2018).
5	Yellow Caturra T-3386	Brazil	Bourbon mutants	High grain production.	Villacreses (2017). Lucas-Suarez (2018), Parrales Marcillo (2018).
6	Catimor CIFC-P2.	Portugal	Caturra x Timor	Resistant to rust. High grain yield.	Villacreses (2017). Lucas-Suarez (2018), Parrales Marcillo (2018).
7	Catimor CIFC-P1	Portugal	Caturra x Timor	Resistant to rust. High grain production.	Villacreses (2017). Lucas-Suarez (2018), Parrales Marcillo (2018).
8	Castle	It is located in Africa	Caturra x de Timor	High grain production.	Villacreses (2017). Lucas-

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				Resistance to rust and cherry disease (*Colletotrichum coffeanum varvirulans*).	Suarez (2018), Parrales Marcillo (2018).
9	Arara	Brazil	Catuaí x Sarchimor	Rust resistance	Villacreses (2017). Lucas-Suarez (2018), Parrales Marcillo (2018).
10	Pache	Guatemala	mutation of the Típica variety	Susceptible to rust	Villacreses (2017). Lucas-Suarez (2018), Parrales Marcillo (2018).
11	Acawa	Brazil	Mundo Novo IAC 388-17 x Sarchimor IAC 1668	Drought and rust resistance. Tolerant to nematodes. Cup quality and late cycle.	Villacreses (2017). Lucas-Suarez (2018), Parrales Marcillo (2018).
12	Catimor CIFC-P3	Portugal	Caturra x de Timor	Low bearing and rust resistant. High yield and more production.	Villacreses (2017). Lucas-Suarez (2018), Parrales Marcillo (2018).
13	Catucai Yellow - SL	Brazil	Icatu and Catuai	Moderate rust resistance	Villacreses (2017). Lucas-Suarez (2018), Parrales Marcillo (2018).
14	Catimor UFV-5607		Timor # 832 x Caturra		Villacreses (2017). Lucas-Suarez (2018), Parrales Marcillo (2018).
15	Red Caturra-Pichilingue	Brazil	Bourbon mutation	Low growth and high productivity. Sun tolerant.	Villacreses (2017). Lucas-Suarez (2018), Parrales Marcillo (2018).
16	Catimor 8664 (2-3)	Portugal	s Timor and Caturra	It is tolerant to rust and has a high grain yield. Cup quality.	Villacreses (2017). Lucas-Suarez (2018), Parrales

					Marcillo (2018).
17	Catucai Yellow	Brazil	Mundo Novo and Caturra		Villacreses (2017). Lucas-Suarez (2018), Parrales Marcillo (2018).
18	Sarchimor 4260	Portugal	Villa Sarchi CIFC 971/10 x Timor hybrid CIFC 832/2	Rust resistance	Villacreses (2017). Lucas-Suarez (2018), Parrales Marcillo (2018).
19	Tipica	Ethiopia	*C. arabica*	Susceptible to coffee rust.	Villacreses (2017). Lucas-Suarez (2018), Parrales Marcillo (2018).
20	Catucai Red 785-15				Villacreses (2017). Lucas-Suarez (2018), Parrales Marcillo (2018).

Source. Gabriel et al. (2023)

Microsatellite marker analysis (SSR) for QTA-genotyping

QTA genotyping was carried out using M13 Tailing technology on 20 coffee accessions with 20 microsatellite markers (SSR) in the Molecular Biology laboratory of the Santa Catalina Experimental Station of the National Institute of Agricultural and Forestry Research (INIAF). The molecular markers used are described in Table 2

Based on the defined model and after analysis of normality and homogeneity of variance for each case, analysis of variance (ANOVA) was performed to test hypotheses of fixed effects, as well as comparisons of treatment means using Tukey's test at $P<0.05$ probability. ANOVA of the data was also used to estimate variance components for random effects. The indicated analyses were performed using Proc GLM of SAS (SAS, 2020).

The economic analysis was performed to determine the benefit/cost of

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each treatment applied. This analysis allowed defining the profitability or not of the treatments (Boardman et al., 2018).

Table 2. *Microsatellite markers (SSR) used for QTA-genotyping of 20 coffee accessions from the UNESUM Germplasm Bank.*

Name	Direct primer	Reverse primer	Repetition	Ta (°C)	Amplicon (bp)	Reference
CaM42	TGGGTCAAGGATCCGTGTGTAAGAAAGA	CCCTCACCACCAGTTCCCGATGTCAG	(CT)8	55	190	Hendre et al. (2008)
CaM41	ATGGGGGGGGGGTGTCGGTCTATGTGA	CGCAATTCGCTGTCACCTCCG	(GA)4(G)4(A)27	50	183	Hendre et al. (2008)
CaM38	GAAGCTGAAGCGGGGGAGGGTAGTAATT	CCCATCCACCCAACCAACCTTCATTTC		55	228	Hendre et al. (2008)
SSRCa091	CGTCTCGTATCACGCTCTCTC	TGTTCCTCGTTCCTCTCTCTCTCTCTCT	(G T) 8(GA)10	56	110	Missio et al. (2011)
CaM17	GGATTCGACAAGGTTGGCAGAGC	TGCCGAGAAGAAGAGGGAGAGATAGTGATG	(CCT)5-87 bp-(CTG)6	57	193	Hendre et al. (2008)
CaM26	CGAGCTAGAATGGATGGATGACTTGGTTGG	GTTGCTCGCACCCCCGCTTCC	(TGGAAG)5	55	203	Hendre et al. (2008)
CaM32	GGGTCAAGGATCCGTGTGTAAGAAAGA	CCCTCACCACCAGTTCCCGATGTCAG	(CT)8	55	191	Hendre et al. (2008)
CaM55	ATGGGGGGGGGGTGTCGGTCTATGTGA	CGCAATTCGCTGTCACCTCC	(GA)4(G)4(A)27	50	183	Hendre et al. (2008)
CaM24	GGATTCGACAAGGTTGGCAGAGC	TGCCGAGAAGAAGAGGGAGAGATAGTGATG	(CCT)5-87 bp-(CTG)6	57	193	Hendre et al. (2008)
CaM22	CCCCTCCTCCTCCTCCTACTACTAGATGGTGGTGG	AACCACCCCCACGCCCACCAATTAAAT	(AT)9 (AC)12	55	222	Hendre et al. (2008)
CaM33	GCGCATTAGGCGTGGGAGAAGCGCATTAGGCGTGGGAGAA	CAGAGAGGTTGTCGGTCGGTCAGGTGGAGAA	(A)13-5 bp-(AG)18	55	240	Hendre et al. (2008)
CaM46	GGTGCGGTGTTTTTTTCAGTTTGGAGA	AACCACCCCCACGCCCACCAATTAAAT	(AT)9 (AC)12	55	222	Hendre et al. (2008)
CaM40	TTGACACACGAAACAGGAAATAAATATAG	CCCTTCCCCTCATAGCCCTTT	(CGA)8	55	238	Hendre et al. (2008)
SSRCa018	GTCTCGTTTCACGCTCTCTCTCTC	ATTTTTTTGGCACGGTATGTTC	(GT)18 (GA)10	57	115	Brandao et al. (2014, 2015), Missio et al. (2009, 2011),
CaM03	CGCGCTTGCTCCCTCTCTCTGTCTCTCT	TGGGGGGGAGGGGGCGGTGTT	(AC)11	57	173	Hendre et al. (2008), Geleta et al. (2012).
CaM18	CCGACTTGGACTGATGCGAAATTGA	AAAGCAAAAAAAACCAGAAAACCAGAAAACGAAGA	(TC)9	57	181	Hendre et al. (2008)
CaM20	AAGAGAGTGTTTGGGATTGCATTTTTAT	CCGCGCGTAGGCTTTGTTTGG	(TA)7(GT)14	55	178	Hendre et al. (2008)
CaM49	CCGGGGTTAATACATTGGTCTTT	ATGACATTGTTGTTGACTTTGCTATAA	(A)33	55	200	Hendre et al. (2008)
CaM36	TGGTTTTTTAGTTTGTTTATTTTTATTTTGATGTGAT	CGAGCCCTCCCCTTGCA	(TTA)7	55	185	Hendre et al. (2008)
CaM02	CGCCAGCCACAGCCACTTGC	GCGGGGGGGTAAGAAAGAGGCGAG	(AGG)7	50	224	Hendre et al. (2008)

SSR: Simple Sequence Repeats

Young, healthy, fully expanded leaves of the 20 genotypes from the germplasm bank of the Southern Manabi State University (Table 1) were collected in the field and used for DNA extraction using the protocol proposed by Doyle and Doyle (1990) as modified by Diniz et

al. (2005) for *C. arabica* with a slight modification in which the leaves were macerated in liquid nitrogen, instead of lyophilized. The quality and quantity of extracted DNA were tested on 0.8% agarose gel in TBE buffer 1X (pH 8.3) (Tris-Borate-EDTA), stained with ethidium bromide (0.25/mL) and visualized under UV light.

To analyze the genetic diversity of *C. arabica* genotypes, heterologous amplification of 20 pairs of SSR primers for *C. arabica* was developed. To perform the analyses on all genetic materials, SSR primers available in the literature were used (Hendre et al., 2008, Missio et al. 2011) (Tables 2).

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

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gels were photodocumented (Locus Biotechnology) using the LPIx Image program.

Allele analysis

The gels were analyzed visually, determining the weight of the fragments in base pairs (bp) amplified for QTA-genotyping in reference to a marker of known fragment weight. The bp of each marker for each of the coffee accessions (absence/presence) were annotated in an Excel spreadsheet for subsequent statistical analysis. For each marker assayed, a Chi-square test (Gabriel et al., 2022) was applied to compare the means of presence levels in the accessions belonging to each marker class (absence/presence) determined, with respect to the bp of the alleles reported by other researchers (Hendre et al. 2008, Missio et.al, 2011).

Results

Allele analysis

Table 3 shows the QTA-genotyping for the 20 SSR molecular markers evaluated, determining that in this set of 20 markers were not effective to differentiate between the accessions of Pache, Acawa, Catimor CIFC-P3, Catucai Amarillo - SL, Catimor UFV-5607, Caturra rojo-Pichilingue, Catimor 8664 (2-3), Catucai Amarillo, Sarchimor 4260, Tipica, Catimor 8666 (4-3), Catucai Rojo 785-15, Catuai rojo UFV, Gheisha, Bourbon amarillo, Caturra amarillo T-3386, Catimor CIFC-P2, Catimor CIFC-P1, Castillo and Arara.

It was also noted that microsatellites CaM42, CaM41, CaM38, SSRCa091, CaM17, CaM26, CaM32, CaM55, CaM24, CaM22, CaM33, CaM46, CaM40, SSRCa018, CaM03, CaM18, CaM20, CaM49 (Table 3 and Figure 1) showed the expected allele fragments in all coffee accessions evaluated. However, two SSRs such as CaM36 and CaM02 did not react with any of the accessions.

Likewise, it is noteworthy to observe that Yellow Caturra T-3386 showed no reaction to the CaM41 marker. Yellow Bourbon did not

react to markers SSRCa091, CaM17, CaM26, CaM55 and CaM49. Catuai red UFV did not react to markers CaM32 and CaM55. Pache did not react to Cam24 markers CaM20 and CaM49. Acawa did not react to marker CaM33. Catucai Amarillo - SL did not react to marker CaM49; and, finally Catucai Amarillo did not react to marker CaM49.

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Table 3. *Height of base pair fragments of each microsatellite marker or SSR in each coffee accession evaluated.*

Accession	CaM42	CaM41	CaM38	SSRCa091	CaM17	CaM26	CaM32	CaM55	CaM24	CaM22	CaM33	CaM46	CaM40	SSRCa018	CaM03	CaM18	CaM20	CaM49	CaM36	CaM02
Pache	193	223	222	104	176	254	207	156	-1	104	240	224	229	119	167	183	-1	-1	-1	-1
Acawa	193	223	222	104	176	254	207	156	199	104	-1	224	229	119	167	183	205	200	-1	-1
Catimor CIFC-P3	193	223	222	104	176	254	207	156	199	104	240	224	229	119	167	183	205	200	-1	-1
Catucai Yellow - SL	193	223	222	104	176	254	207	156	199	104	240	224	229	119	167	183	205	-1	-1	-1
Catimor UFV-5607	193	223	222	104	176	254	207	156	199	104	240	224	229	119	167	183	205	200	-1	-1
Red Caturra-Pichilingue	193	223	222	104	176	254	207	156	199	104	240	224	229	119	167	183	205	200	-1	-1
Catimor 8664 (2-3)	193	223	222	104	176	254	207	156	199	104	240	224	229	119	167	183	205	200	-1	-1
Catucai Yellow	193	223	222	104	176	254	207	156	199	104	240	224	229	119	167	183	205	-1	-1	-1
Sarchimor 4260	193	223	222	104	176	254	207	156	199	104	240	224	229	119	167	183	205	200	-1	-1
Tipica	193	223	222	104	176	254	207	156	199	104	240	224	229	119	167	183	205	200	-1	-1
Catimor 8666 (4-3).	193	223	222	104	176	254	207	156	199	104	240	224	229	119	167	183	205	200	-1	-1
Catucai Red 785-15	193	223	222	104	176	254	207	156	199	104	240	224	229	119	167	183	205	200	-1	-1
Red Catuai UFV	193	223	222	104	176	254	-1	-1	199	104	240	224	229	119	167	183	205	200	-1	-1
Gheisha.	193	223	222	104	176	254	207	156	199	104	240	224	229	119	167	183	205	200	-1	-1
Yellow bourbon	193	223	222	-1	-1	-1	207	-1	199	104	240	224	229	119	167	183	205	-1	-1	-1
Yellow Caturra T-3386	193	-1	222	104	176	254	207	156	199	104	240	224	229	119	167	183	205	200	-1	-1

Catimor CIFC-P2.	193	223	222	104	176	254	207	156	199	104	240	224	229	119	167	183	205	200	-1	-1
Catimor CIFC-P1	193	223	222	104	176	254	207	156	199	104	240	224	229	119	167	183	205	200	-1	-1
Castle	193	223	222	104	176	254	207	156	199	104	240	224	229	119	167	183	205	200	-1	-1
Arara	193	223	222	104	176	254	207	156	199	104	240	224	229	119	167	183	205	200	-1	-1

Figure 1. *Reading frame of the SSR markers CaM42 and CaM41, for the coffee accessions evaluated.*

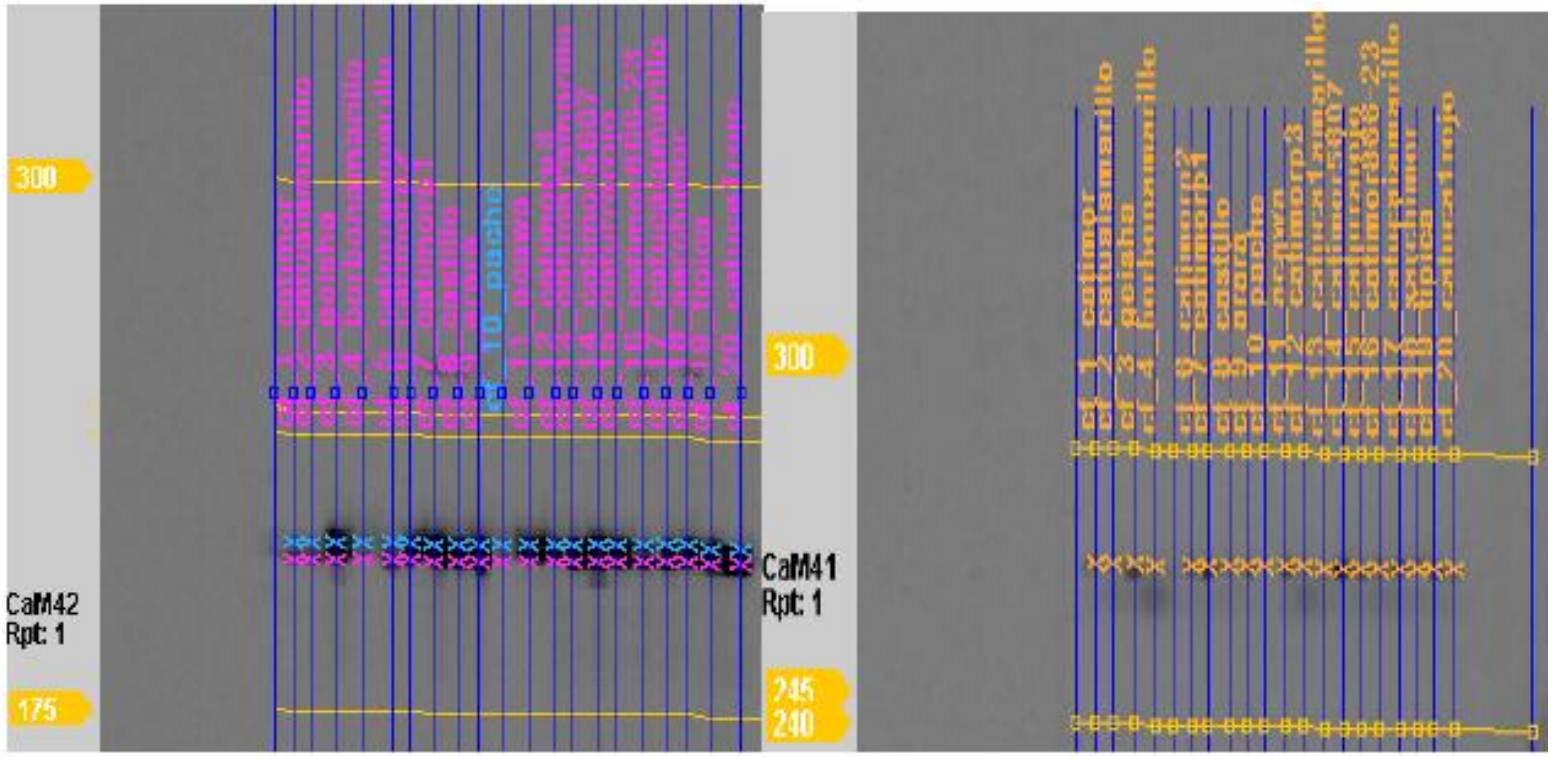
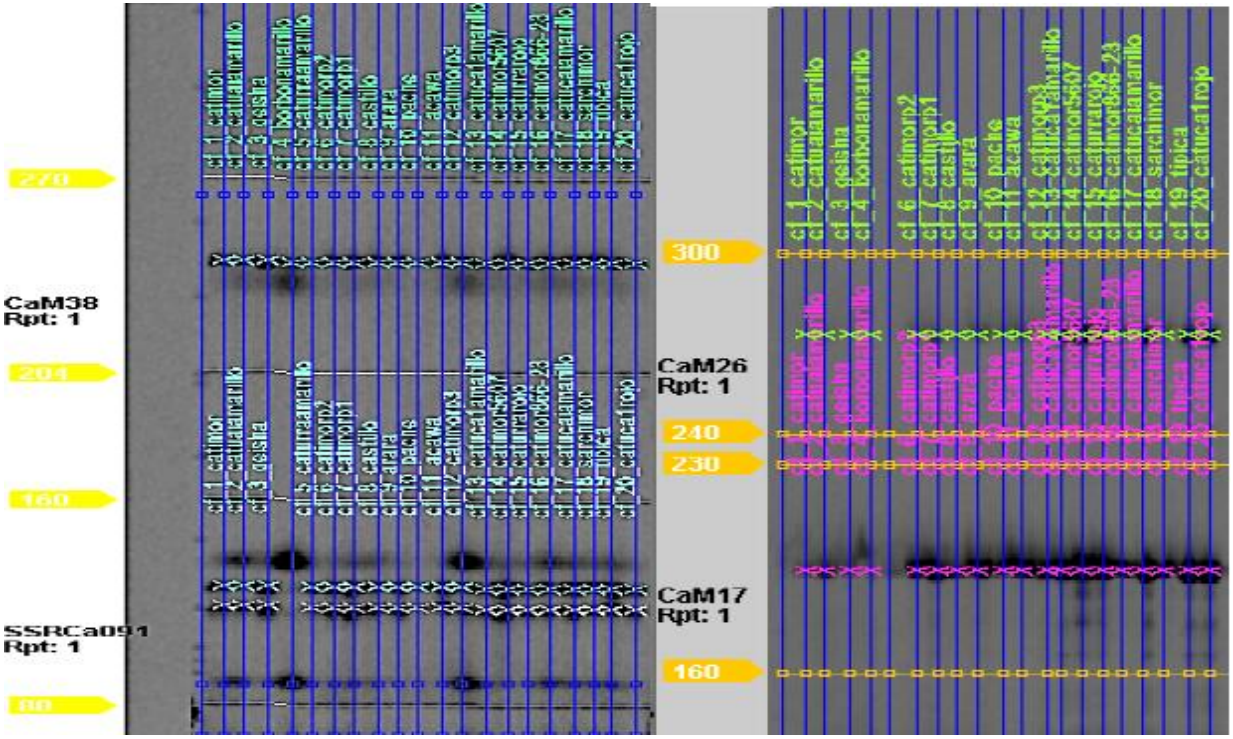


Figure 2. *Reading frame of the SSR markers CaM38, CaM26, SSRCa091 and CaM17, for the coffee accessions evaluated.*



A statistical analysis was performed using the Chi-square test (X^2) in order to check whether the fragments determined in the QTA-genotyping analysis of this research correspond to the expected fragments found by other researchers (Table 4).

Table 4. *Chi-square analysis for QTA-genotyping fragments (bp) determined.*

Microsatellite	0i	ei	oi-ei	(oi-ei)2	(oi-ei)2/ei
CaM42	193	190	3,00	9,00	0.05ns
CaM41	223	183	40,00	1600,00	8,74**
CaM38	222	228	-6,00	36,00	0.16ns
SSRCa091	104	110	-6,00	36,00	0.33ns
CaM17	176	193	-17,00	289,00	1.50ns
CaM26	254	203	51,00	2601,00	12,81**
CaM32	207	191	16,00	256,00	1.34ns
CaM55	159	183	-24,74	704,11	3,84*
CaM24	199	193	6,00	36,00	0.19ns
CaM22	104	222	-118,00	13924,00	62,72
CaM33	240	240	0,00	0,00	0.00ns
CaM46	224	222	2,00	4,00	0.02ns
CaM40	229	238	-9,00	81,00	0.34ns
SSRCa018	119	115	4,00	16,00	0.14ns
CaM03	167	173	-6,00	36,00	0.21ns
CaM18	183	181	2,00	4,00	0.02ns
CaM20	205	178	27,00	729,00	4,10*
CaM49	201	197	4,50	121,50	0.65ns

X^2 analysis (Table 3) showed that overall all fragments (bp) determined were not significant, which would indicate that these fragments are as expected; however, markers CaM41 and CaM26 were highly significant ($P<0.01$) and markers CaM55 and CaM20 were significant ($P<0.05$). This would suggest that these markers had different expression than expected.

The co-dominance characteristic of SSR markers was not considered in this genetic diversity study. There are difficulties in using the SSR marker as codominant in species with a polyploid genome such as *Coffea arabica*, which is an allotetraploid species. This could be explained by limitations in establishing heterozygosity or

homozygosity levels at specific loci, given the inability of SSR markers to distinguish alleles from homologous chromosomes, as well as the possibility of finding null alleles in polyploids (Missio et al., 2009, Cordeiro et al., 2003).

To determine the useful attributes of the genetic markers evaluated, the 20 microsatellite markers were tested in a panel of 20 *C. arabica* genotypes. Allelic amplification was obtained for all markers in all genotypes tested, except CaM36 and CaM02 which did not amplify. Contrary to Hendre et al. (2007), who determined that these markers did amplify and CaM54 did not amplify in arabicas, this marker was not used in our research. In general, the markers revealed low to medium allelic diversity, and in particular 18 of them (CaM42, CaM41, CaM38, SSRCa091, CaM17, CaM26, CaM32, CaM55, CaM24, CaM22, CaM33, CaM46, CaM40, SSRCa018, CaM03, CaM18, CaM20 and CaM49) resulted in double alleles in the case of all *C. arabica* genotypes tested. In contrast, Hendre et al. (2007) found that markers CaM03, CaM15, CaM18, CaM21, CaM31, CaM34, CaM35, CaM39, CaM43, CaM55, CaM57 and CaM58) resulted in low to medium allelic diversity. Comparing both studies, we observed that different markers were validated and only two of the markers used (CaM03 and CaM18) coincided with this characteristic of allelic diversity detection.

We determined that of the 20 validated molecular markers, one (CaM22) was monomorphic. In contrast, Hendre et al. (2007) also determined that seven markers (CaM08, CaM09, CaM11, Cam12, CaM23 and CaM53) were monomorphic.

Regarding the SSRCa091 markers, the fragment at 104 bp was determined. In this regard, this fragment was reported at 110 bp (Missio et al., 2011), with a moderate significant correlation (0.40). In our analysis using the Chi-square test we observed that there was no significant difference, which would indicate that it is the same fragment.

Conclusions

The SSR markers used have low or limited detection of genetic diversity, and limitations in the levels of heterozygosity and homozygosity at the specific locus could be observed due to the inability of these SSRs to distinguish alleles from homologous

chromosomes, as well as the probability of finding null alleles in polyploids. The Cam22 marker was monomorphic.

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