

## **Segregation analysis of molecular markers in a population derived from *Coffea liberica* Hiern x *C. eugenoides* L.**

**Análisis de segregación de marcadores moleculares en una población derivada de *Coffea liberica* Hiern x *C. eugenoides* L.**

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### **Abstract**

Coffee is a very important crop for the world economy. The commercial coffee production is based on two species, *Coffea arabica* L. (70%) and *C. canephora* Pierre (30%). *C. arabica* is the only allotetraploid ( $2n=4x=44$ ) species, which has its primary center of diversity in the southwestern Ethiopia highlands. *C. arabica* is the only species cultivated in Colombia and represents a very important economic and social value. However, *C. arabica* has a very narrow genetic diversity and therefore, the use of diploid species on coffee breeding is very important because they allow to broad its diversity. In this work, the genetic segregation patterns were evaluated on a population of 101 F1 hybrid plants from a cross between the diploid species *C. liberica* and *C. eugenoides* using 618 molecular markers, of which 168 SSRs and 2 ESTs exhibited polymorphic patterns that allowed segregation analysis. Approximately 24% of the loci were null alleles, and the segregation distortion reached 23.5% at  $\alpha < 0.01$ . A total of four segregant types were observed, from up to seven possibilities. The origins of null alleles, possible causes of segregation distortion, different segregation patterns obtained and recombination frequencies are discussed. The knowledge derived from this study allowed to better understand the genetic segregation behavior of these markers with the aim of developing genetic maps that have important applications for coffee breeding programs as well as other commercial crops.

**Key words:** Coffee, microsatellites, segregation distortion.

### **Resumen**

El café es un cultivo importante para la economía mundial. La producción comercial de café se basa en dos especies, *Coffea arabica* L. (70%) y *C. canephora* Pierre (30%). La primera es la única alotetraploide ( $2n = 4x = 44$ ) que tiene su centro primario de diversidad en las tierras altas del suroeste de Etiopía; la segunda es la única cultivada en Colombia y representa un importante valor económico y social. Sin embargo, *C. arabica* tiene una base genética muy estrecha y por ello es importante el uso de especies diploides en el mejoramiento del café para ampliar su diversidad. En este trabajo fueron evaluados los patrones de segregación genética en una población consistente en 101 plantas híbridas F1 de un cruce entre la especie diploide *C. liberica* y *C. eugenoides* utilizando 618 marcadores moleculares, de las cuales 168 SSR y 2 EST exhibieron patrones polimórficos que permitieron el análisis de segregación. Aproximadamente 24% de los loci fueron alelos nulos y la distorsión de la segregación alcanzó 23.5% ( $\alpha < 0.01$ ). Se observaron un total de cuatro tipos segregantes de entre siete posibilidades diferentes.

Se discuten los orígenes de los alelos nulos, posibles causas de distorsión de la segregación, diferentes patrones de segregación y frecuencias de recombinación. El conocimiento derivado de este estudio permite entender mejor el comportamiento de la segregación genética de estos marcadores, con el fin de desarrollar mapas genéticos que tienen aplicaciones importantes para los programas de mejoramiento de café y de otras especies comerciales.

**Palabras clave:** Café, microsatélites, distorsión de segregación.

## Introduction

According to the International Coffee Organization (ICO), currently there are some 70 coffee producer countries around the world, of which the exporting members of the ICO are responsible for over 97 percent of world output. In 2010 total coffee sector employment was estimated at about 26 million people in 52 producing countries (ICO, 2014).

Coffee belongs to the genus *Coffea*, subgenus *Coffea* (Rubiaceae). The genus includes approximately 80 taxa, most of which are diploids with  $2n = 2x = 22$ . The only tetraploid is *C. arabica* L., with  $2n = 2x = 44$  (Bridson and Verdcourt, 1988). The two main cultivated species are *C. arabica* and *C. canephora* Pierre. An additional species of coffee that is cultivated less extensively is *C. liberica* (Van der Vossen, 1985).

For breeding purposes one of the major obstacles is the lack of genetic diversity in the gene pool of *C. arabica*, making it necessary to use diploid species to introgress genes of agronomic importance (Lahermes *et al.*, 2011). Besides, given the perennial condition of coffee, the implementation of marker-assisted selection (MAS), allows the reduction of time required during selection. The use of co-dominant markers, such as SSR (Simple Sequence Repeats), improve the efficiency and accuracy of linkage analysis in diploid species, particularly in those with a high degree of heterozygosity (Jiang and Zeng, 1997) and therefore helps in the implementation of MAS for quantitative traits.

In order to exploit the genome of coffee and bypass the problem of low genetic variability in cultivated species, it is feasible to use its diploid relatives in the construction of a molecular genetic linkage map. *Coffea eugenioides* is a good candidate to generate a mapping population because of its close evolutionary relationship with the cultivated coffee *C. arabica*, which is derived from

hybridization between *C. eugenioides* and *C. canephora* (Lahermes *et al.*, 1999). An additional diploid species of interest is *C. liberica*, which is known to harbor coffee rust resistance (Prakash *et al.*, 2004) and other traits of economic importance.

However, segregation distortion is a phenomenon of frequent occurrence and wide distribution in plants that has been detected particularly in diverse classes of mapping populations derived from different species (McCouch *et al.*, 1988; Barret *et al.*, 2004). Genetic mapping projects that report segregation distortion propose several different mechanisms to explain this phenomenon. Among the mechanisms that are mentioned with more frequency is the rejection or elimination of male or female gametes during fertilization, the selective fertilization of particular gametic phenotypes and the abortion of zygotes (Xu *et al.*, 1997).

Also it has been suggested that certain loci of the gametophyte can be responsible for a partial or total elimination of gametes carrying certain parental alleles, in which case the distortion of allele segregation is derived from the linkage between the marker alleles and the gametophyte alleles that confer a diminution of the pollination capacity, also known as pollen killer genes (Harushima *et al.*, 1996). This phenomenon has substantial importance for the recovery of specific recombinant genotypes that must be obtained from breeding populations, especially when the populations have been obtained from interspecific crosses, in which the recovery of desirable recombinants is limited due to the nonrandom survival of the descendants (Lahermes *et al.*, 2001).

The main aim of the present study was to analyze the differences in segregation patterns, the genetic distortion of segregant molecular loci in a segregating population derived from the interspecific cross between *Coffea*

*liberica* x *C. eugenoides*, as well as to verify the presence of null alleles and the differences in genetic recombination in the two species coffee studied. This segregation analysis was then used to construct a genetic linkage map in the same population (Lopez *et al.*, 2013).

## Materials and methods

### Plant material

Plant material is maintained in a germplasm bank of the agricultural station of Naranjal at the National Center for Coffee Research (Cenicafé, Chinchiná, Colombia). The segregating population consisted of 101 F1 interspecific hybrid plants developed by cross pollination (CP) between one plant of *C. liberica* ( $2n = 2x = 22$ ) as the female parent and one plant of *C. eugenoides* ( $2n = 2x = 22$ ) as the male parent. Because these two species are heterozygous, this kind of population is known as a pseudotestcross (Grattapaglia and Sederoff, 1994).

Pollinations were performed by hand, using plastic bags to protect emasculated flowers. Young coffee leaves were packed in wet paper in an isothermic box and brought to the laboratory for immediate DNA extraction. Parental plants were chosen based on gametic compatibility and regular meiotic behavior in interspecific hybrids in order to guarantee a reasonable population size for segregation and linkage analysis. *Coffe eugenoides* was selected because it was a progenitor species of the cultivated tetraploid *C. arabica* (Lashermes *et al.*, 1999). Both *C. liberica* and *C. eugenoides* share approximately 50% genetic similarity with *C. arabica* and, between them, share 45% genetic similarity (Moncada and McCouch, 2004). *Coffe liberica* originated in East Africa while *C. eugenoides* originated in Central Africa. Both grow mainly in wild habitats, but *C. liberica* is also commercially cultivated in some countries and used for coffee mixtures. In addition, compared with *C. eugenoides*, *C. liberica* has a high content of chlorogenic acids, implicated in the species' organoleptic properties and defense response mechanisms (Ky *et al.*, 1999). *Coffe eugenoides* has very low caffeine content, 0.2%, when compared with levels in *C. liberica* that reach 1% (Barre *et al.*, 1997). Additionally, there is

evidence that *C. liberica* has genetic resistance to several biotic and abiotic stresses, including resistance to the coffee Berry borer (CBB) *Hypothenemus hampei* Ferrari (Romero and Cortina-Guerrero, 2004) and to the coffee leaf rust caused by *Hemileia vastatrix* Ferrari (1867) (Prakash *et al.*, 2004).

### DNA extraction

Genomic DNA extraction for each plant was done starting with 10 g of young apical leaf tissue, according to a protocol of Ky *et al.* (2000). DNA content and integrity was evaluated by spectrophotometer and by 1% agarose gel electrophoresis, comparing each sample to 2DNA/*EcoRI* + *HindIII* standard (Promega). The DNA samples were re-suspended in TE buffer and kept at -20 °C until use.

### Molecular markers and polymorphism detection

A total of 618 co-dominant PCR-based molecular markers were evaluated. These included 232 microsatellites (Simple Sequence Repeats or SSRs) developed by Cenicafé from a short insert genomic DNA library of *C. arabica* 'Caturra' using hybridization-based screening with 26-mer oligonucleotides, (GA)<sub>13</sub> and (CA)<sub>13</sub>, according to the laboratory protocols of McCouch at Cornell University, Ithaca, N.Y. U.S.A. (Unpublished data). Additionally, the rest of the markers were developed for other groups, but intellectual property rights still prevent from publication of the sequences: 315 SSRs by Giorgio Graziosi, University of Trieste, Italy; 20 ESTs (Expressed Sequence Tags) and 51 COS (Conserved Orthologous Sequences) from *Solanum lycopersicum* (SOL Genomics Network, Cornell University, Ithaca, N.Y. U.S.A.).

The amplification was performed using optimized parameters for each primer pair (Montoya *et al.*, 2006). The amplifications were carried in a total reaction volume of 25 µL. The optimum mix of reagents was 2 µL of DNA (50 ng/µL), 0.75 µL of each primer (diluted to 10 µmol/L), 2.5 µL of 10x buffer, including 15 mmol of MgCl<sub>2</sub>, 0.2 mmol/L each of the dNTPs, and 1 U of *Taq* polymerase. The optimum PCR profile for all markers was an initial denaturation at 95 °C for 5 min, followed by 30 cycles of denaturation at 94 °C

for 1 min, annealing at 55 °C for 1 min and extension at 72 °C for 1 min. After 30 cycles, a final incubation at 72 °C for 5 min was allowed. All amplicons were further evaluated on silver-stained 4% polyacrylamide gels.

### Data analysis

Electrophoretically visible alleles were designated with lower-case italic letters, while failure to detect alleles after several attempts of PCR was interpreted as evidence of null alleles and designated as 0. For the segregation analysis all possible genotype combinations for co-dominant markers with mendelian segregation in a diploid organism were considered, as shown in Table 1 (Ritter and Salamini, 1996; Maliepaard *et al.*, 1997).

Segregation analysis was performed after all the individual genotypes were defined for each marker and a database was created. The database was the object of a verification process that included triple gel reading. The segregation ratios of genotypes were tested against expected ratios using the chi-square test. Segregation distortion calculations were estimated by chi-square tests for pairs of linked loci at LOD  $\geq 10$  taking into the account linkage phase determined by the JoinMap 3.0 software (Van-Ooijen and Voorrips, 2001).

### Detection of null alleles

The presence of null alleles was tested in two ways. First, status was based on the phenotype observed among the progeny and their phenotypic and genotypic proportions. Secondly, the presence of null alleles may be due to the lack of correct annealing of the

marker primers, even after several attempts at PCR optimization. Nulls were verified nulls by redesigning new primers in such a way as to not overlap with the first set of primers in the original sequences adjacent to each microsatellite locus. This strategy was applied to markers CFGA1254, CFGA205, CFGA55, and CFGA99.

## Results and Discussion

A total of 618 molecular markers were evaluated in CP population. It was found that 252 markers were polymorphic; the rest were monomorphic or not scorable for different reasons, including technical difficulties during amplification. From the polymorphic markers, 82 were homozygous in both parents, although they were polymorphic between parents they were not useful for the analysis; the remaining 170 (168 SSRs and 2 ESTs) were used for the segregation analysis.

### Segregant genotypic classes.

Configurations found in the segregation analysis of the molecular markers are graphically represented in Figure 1. There are some configurations that do not segregate in the descendants including type *vvxvv*, monomorphic and monozygotic in both parents; type *vvxzz*, two alleles but homozygous in both parents and type *vvxv0*, two alleles but one of them null in the heterozygous parent.

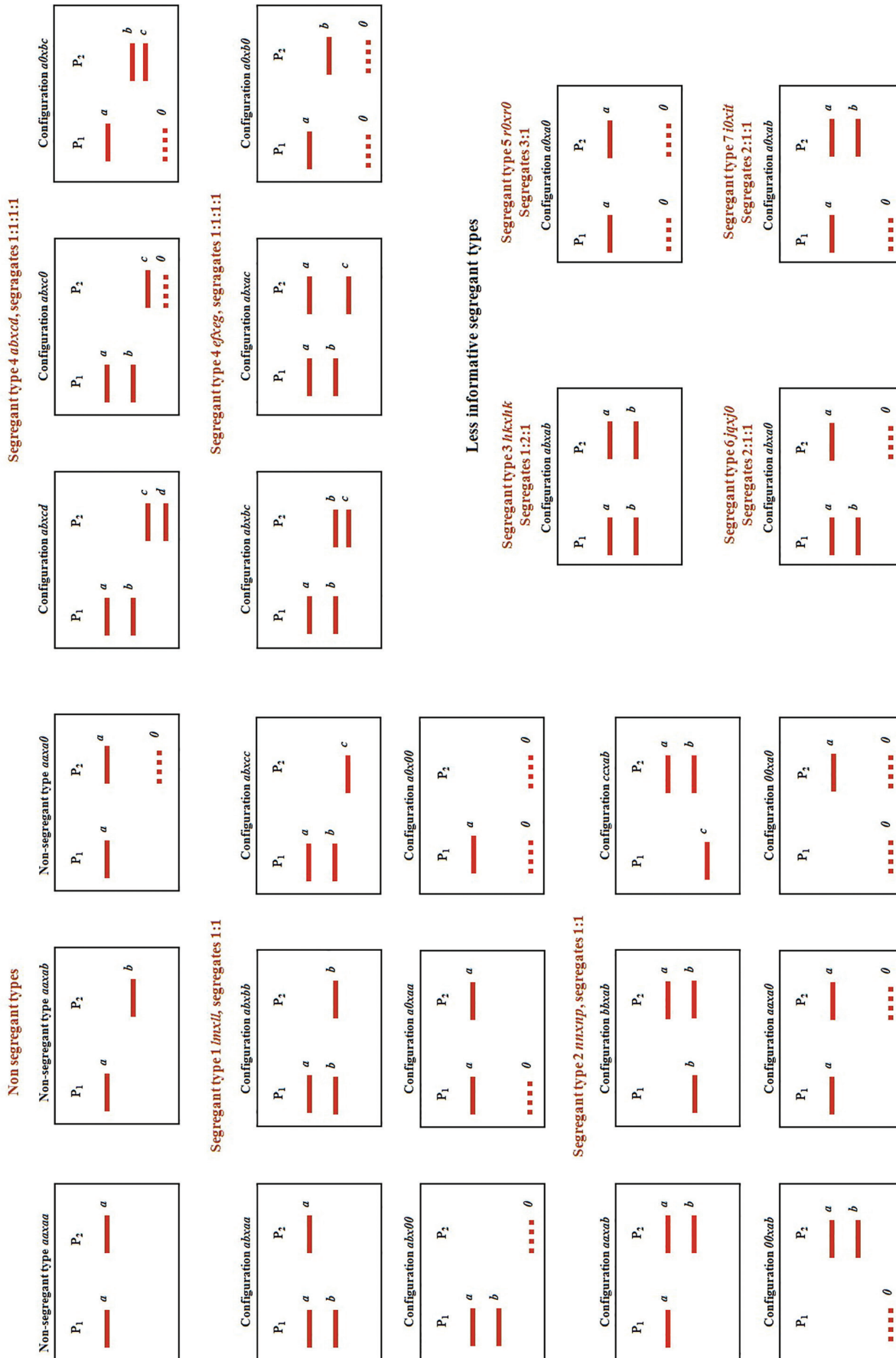
The segregant types considered here are described in the Table 2. Most of the markers analyzed segregate from *C. liberica*, segregant type 2, followed by those of bi-parental origin, segregant type 4.

**Table 1.** Seventeen possible genotype combinations for seven segregant types.

Locus 1†	Locus 2‡						
	<i>ab x aa</i>	<i>aa x ab</i>	<i>ab x ab</i>	<i>ab x cd</i>	<i>a0 x a0</i>	<i>ab x a0</i>	<i>a0 x ab</i>
<i>ab x aa</i>	‡	–	2	3	4	5	6
<i>aa x ab</i>		(1)	(2)	(3)	(4)	(5)	(6)
<i>ab x ab</i>			7	8	9		(10)
<i>ab x cd</i>				11	12	13	(13)
<i>a0 x a0</i>					14	15	(15)
<i>ab x a0</i>						16	17
<i>a0 x ab</i>							(16)

† Crosses between locus 1 and 2 are represented for diploid organisms using co-dominant markers. Left pair of alleles represents the genotype for parent 1 and the right pair for parent 2. Cero means null allele.

‡ Each genotype combination is designated with numbers from 1 to 17. Numbers in parenthesis represent reciprocal crosses, and – means non informative cross.



**Figure 1.** Schematic representations of configurations found in the segregation analysis of markers. Each panel includes the segregant type, genotypic ratios and several examples of possible configurations. Parents are indicated as P<sub>1</sub> and P<sub>2</sub>, solid lines represent visible bands and dotted lines represent null alleles

Indeed, it is important to note that almost a third of the segregant loci (49 loci or 28.8%) are of the type that provides us with the most genetic information. These types of loci can be used to make genetic inferences for both parents because their segregant types of *abxcd* or *efxeg* represent important support for the robustness of the genetic map in these diploid species. In contrast, the segregant type *hkxhk* had the lowest representation with only 3 loci (1.8%), an observation that is advantageous, considering that this segregant class provides the least amount of genetic information because it implies dominance.

The superior number of segregant markers for *C. liberica* compared to *C. eugenioides* could be due to difficulties that arose during the DNA extraction for the latter species, which may have resulted in a decrease in the quality of the template for PCR. The resulting bands associated with the alleles of the male parent were generally less intense, making its visualization difficult and allowing the predominance of informative markers for the female parent.

## Segregation distortion

The magnitude of segregation distortion was analyzed for 170 loci with respect to the expected mendelian proportions of segregation following six different statistical criteria; the three most common and practical criteria are discussed here. Using exact significant levels, less markers tend to be distorted at  $\alpha = 0.01$  and  $\alpha = 0.005$ ; 5 and 8 markers, respectively (Table 3). With the criterion of  $\alpha < 0.10$ , segregation distortions were observed for 77 loci, corresponding to 45.3% (Table 3). Segregation distortion at the significant level of  $\alpha < 0.05$  grouped 62 markers or the 36.5% and with  $\alpha < 0.01$  40 markers were grouped, representing 23.5% (Table 3).

No direct relation between segregation distortion and segregant type was observed. Distorted loci were found for all segregant classes in proportion to the number of loci observed for each segregant type. Nevertheless, some tendency towards preferential distortion can be identified for one of the parents in certain regions of a linkage map when it was calculated (López and Moncada, 2005;

**Table 2.** Amounts of loci in each segregant type considered in this study and their genetic characteristics.

Segregant	Cros	N	%†	Genetic characteristics of each locus‡
Type 1	<i>lmxll</i>	2 8	16 5	Heterozygous in the first parent <i>C. eugenioides</i> (P <sub>1</sub> ). It allows detection of two or three alleles. Possible genotypes are <i>ll</i> and <i>lm</i> in proportions 1:1. Informative of parent P <sub>1</sub> .
Type 2	<i>nnxnp</i>	9 0	52 9	Heterozygous in the second parent <i>C. liberica</i> (P <sub>2</sub> ). It allows detection of two or three alleles (inverse to Type 1). Possible genotypes are <i>nn</i> and <i>np</i> in proportions 1:1. Informative of parent P <sub>2</sub> .
Type 3	<i>hkxhk</i>	3	1.8	Heterozygous in both parents; it allows detection of two alleles. Possible genotypes are <i>hh</i> , <i>hk</i> and <i>kk</i> , in proportions 1:2:1. When null alleles are included ( <i>h0</i> and <i>k0</i> are dominant genotypes of <i>h</i> or <i>k</i> over the null allele), genotypes are <i>h0</i> and <i>kk</i> or <i>hh</i> and <i>k0</i> in proportions 3:1 or 1:3, respectively. In any case the segregation information is partial.
Type 4 (a)	<i>abxcd</i>	4 0	23 5	Heterozygous in both parents allows detection of four no-dominant alleles; possible genotypes are <i>ac</i> , <i>ad</i> , <i>bc</i> and <i>bd</i> in proportions 1:1:1:1. Informative of both parents.
Type 4 (b)	<i>efxeg</i>	9	5.3	Heterozygous in both parents. It allows detection of three no-dominant alleles. Possible genotypes are <i>ee</i> , <i>ef</i> , <i>eg</i> and <i>fg</i> in proportions 1:1:1:1. Informative of both parents.
Type 5	<i>roxro</i>	0	0	Heterozygous in both parents. It allows detection of two alleles, one of them null in each parent. Possible genotypes are <i>r0</i> and <i>00</i> in proportions 3:1. It can be analyzed in JoinMap as type 3, <i>h0xkk</i> or <i>hhxk0</i> with proportions 3:1 or 1:3, respectively, ignoring the null allele in the descendant; however, this will introduce some bias in the analysis.
Type 6	<i>jqxjo</i>	0	0	Heterozygous in both parents. It allows detection of three alleles, one of them null in one parent. Possible genotypes are <i>j</i> -, <i>jq</i> or <i>q0</i> in proportions 2:1:1, where <i>j</i> - represents dominance (genotypes <i>jj</i> or <i>j0</i> ). It can be analyzed in JoinMap by making use of the detectable heterozygous and translating the data into Type 1, <i>lmxll</i> with proportions 1:1 by ignoring the null allele. However, this will introduce some bias.
Type 7	<i>ioxit</i>	0	0	Heterozygous in both parents. It allows detection of three alleles, one of them null in one parent, (inverse to Type 6). Possible genotypes are <i>i</i> -, <i>it</i> , <i>t0</i> in proportions 2:1:1, where <i>i</i> - represents dominance (genotypes <i>ii</i> or <i>i0</i> ). It can be analyzed in JoinMap by making use of the detectable heterozygous and translating the data into Type 2, <i>nnxnp</i> with proportions 1:1 ignoring the null allele. However, this will introduce some bias.

† Percentage of each segregant type with respect to the total of 170 mapped loci.

‡ Each allele is represented by different letter in italics. For null alleles a cero (0) is used.

**Table 3.** Summary data showing number of loci according to the segregant types and segregation distortions.

Parental origin	Segregant Types (and ratios)	Total number of loci (and %)	Percent		Significance level <sup>§</sup>								
			A <sup>†</sup>	B <sup>‡</sup>	0.1	0.05	0.01	0.005	0.0005	0.0001	$\alpha \leq 0.1$	$\alpha \leq 0.05$	$\alpha \leq 0.01$
<i>C. eugenioides</i>	<i>lmxll</i> (1:1)	28 (16.5%)	50.0%	8.2%	1	3	1	0	3	6	14	13	10
<i>C. liberica</i>	<i>nrxnp</i> (1:1)	90 (52.9%)	32.2%	17.1%	3	7	2	5	6	6	29	26	19
Undetermined	<i>hkxhk</i> (1:2:1)	3 (1.8%)	100%	1.8%	0	1	0	1	0	1	3	3	2
Both parents	<i>abxcd</i> or <i>efxeg</i> (1:1:1:1)	49 (28.8%)	63.3%	18.2%	11	11	2	2	3	2	31	20	9
Total		170			15	22	5	8	12	15	77	62	40
Percent		100%									45.3%	36.5%	23.5%

<sup>†</sup> Column A: percentage of distorted loci ( $\alpha \leq 0.1$ ) in each segregant type with respect to each segregant type.

<sup>‡</sup> Column B: percentage of distorted loci ( $\alpha \leq 0.1$ ) in each segregant type with respect to 170 loci analyzed.

<sup>§</sup> Number of distorted loci for each segregant type at the given exact significant level (exact  $\alpha$ ). Shaded columns contain accumulated number of markers under the given value of alpha.

López *et al.*, 2013). These results support the hypothesis that there are genomic regions that are genetically determined by factors of segregation distortion, and that these control the direction of segregation deviation in favor of a specific progenitor within the linked groups. The evidence suggests that *C. eugenioides* is the parental species with a greater tendency to present segregation distortion with respect to each segregant type (Table 3, column A). This finding is consistent with other studies that demonstrated that a deviation in segregation arises most commonly by paternal gametophytic selection through selective processes influenced by the gynoecium (Luo *et al.*, 2001).

The distribution patterns as well as the rank of values of the segregation distortion detected here are similar to those obtained in other experiments of segregation analysis and genetic mapping with populations from different species of the *Coffea* genera. For example, it has been verified in two tetraploids, var. Catimor and var. Mokka, that segregation distortion values ranking from 22% of distorted loci in Catimor to 45% in Mokka are distributed widely in the genomes of these two cultivars (Pearl *et al.*, 2004). In the interspecific map that was constructed in species *C. pseudozanguebariae* x *C. liberica* var. Dewevrei, the segregation distortion reached 30% of loci evaluated (Ky *et al.*, 1999). In another population derived from the cross between *C. canephora* and a double haploid of the same species, proportions of segregation distortion ranking between 13% and 44% ( $\alpha < 0.05$ ) were found in association with a wide distribution of the distorted loci on the genome of this spe-

cies (Lashermes *et al.*, 2001). In contrast, in a map constructed from a population of double haploids of the diploid coffee *C. canephora*, a more moderate proportion of segregation distortion was found, varying between 12% for RFLP markers and 20% for RAPD markers, with  $\alpha < 0.01$  for both kinds of markers (Paillard *et al.*, 1996).

Segregation distortion is a common phenomenon and broadly distributed in plants. It has been detected in different mapping population types of different species and by using different types of genetic markers. Several mechanisms have been argued to explain the existence of this phenomenon, including the elimination of some gametes during some stages of the fecundation process, selective fertilization of gametic genotypes and zygotic abortion (Xu *et al.*, 1997). It has also been suggested that some particular loci in the gametophyte could be responsible for the partial or total elimination of male or female gametes carrying incompatibility alleles, in which case, segregation distortions are due to genetic linkage between incompatibility loci and marker loci (Harushima *et al.*, 1996). Segregation distortions should be considered during construction of genetic linkage maps because in some instances it may seriously affect the calculations of the distances among loci.

#### Null alleles analysis.

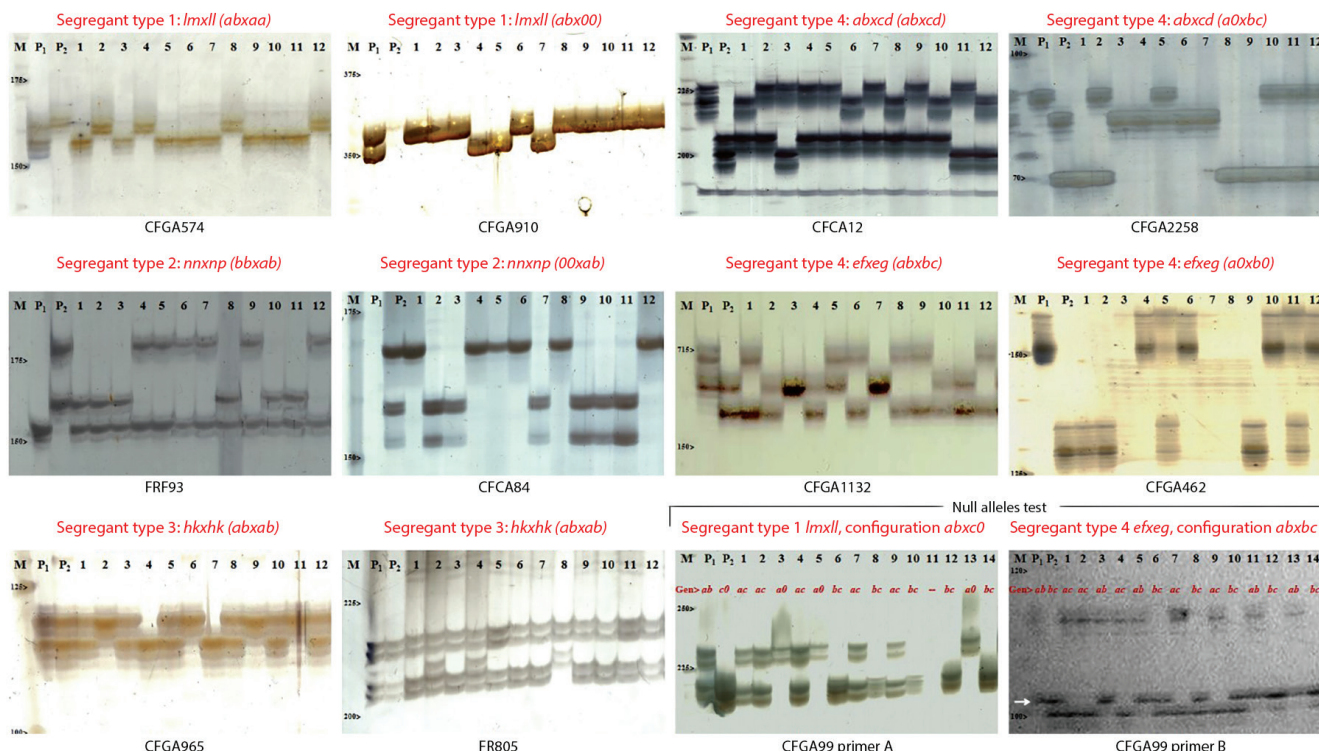
Of 170 segregant loci, 41 (24.1%) exhibited the presence of 1 to 3 null alleles per locus that were undetectable using the original PCR primers. Considering each segregant type, of the 41 loci with null alleles, 15 (36.6% from

nulls, 53.6% from male parent segregants) exclusively presented the null allele in *C. eugenioides*, 18 (43.9% from nulls, 20% from the female parent segregants) only in *C. liberica* and 8 (19.5% from nulls, 4.7% from total segregants) presented the null in both parents. When considering both, the number of null alleles per locus and the segregant type, 22 loci presented 1 null per locus, 6 of them type *nnxnp*, 4 of type *lmxll* and 12 of type *abxcd* or *efxeg*. Fifteen loci presented 2 nulls per locus, 6 of type *nnxnp*, 5 of type *lmxll* and 4 of type *abxcd* or *efxeg*. Finally, 4 loci presented 3 nulls per locus, 3 of type *nnxnp* and 1 of type *lmxll*. Clearly, proportional to the number of segregant loci for each parent, *C. eugenioides* exhibits a larger tendency towards the presence of null alleles, an aspect that is perhaps related to the fact that its DNA was recalcitrant to PCR amplification in many cases.

To verify whether, at least in some cases, the null alleles are due to failure by primer

sequences to recognize the target DNA during PCR, an additional experiment was conducted in which some primers were redesigned for loci suggesting the existence of null alleles. From the null allele assays for the marker loci CFGA1254, CFGA205, CFGA55 and CFGA99, only the last locus gave some evidence of annealing failure as an explanation for the observation of a null allele (Figure 2, lower right).

Given that the markers used in this study were transferred from the tetraploid *C. arabica* var. Caturra, this finding suggests that, at least for some marker loci, it is probable that alterations in the sequence of the flanking regions of the microsatellites have occurred during the course of the evolution of this species. Similar observations have been found in other species where detailed sequence analyses demonstrate that base substitutions may explain the presence of null alleles. In rice it has been shown that up to 62% of null



**Figure 2.** Electrophoretic gels of single sequence repeats (SSRs) showing segregant types and the null alleles test. From the left to the right and from the upper to the lower edge, the first 10 gels correspond to examples of all segregant types studied; at the lower right, the 2 gels of locus CFGA99 show results of the null alleles test; primer A using original set of primers, primer B using redesigned primers. The segregant types are indicated on top of each gel and particular allelic configurations in parenthesis. Locus names are indicated on the bottom of each gel and the lanes are labeled from the left to the right as: M, molecular weight; P<sub>1</sub>, parental *Coffea eugenioides*; P<sub>2</sub>, parental *C. liberica*; then twelve individuals from the F<sub>1</sub> descendants (14 individuals in the null alleles test). For the null alleles test, inferred genotypes are indicated for each individual and the white arrow shows the new allele detected with redesigned primers B.



alleles are the result of sequence alterations in the flanking regions of the SSRs, caused mainly by point mutations (Chen *et al.*, 2002), a mechanism that can affect important segments of polyploid genomes and the process of differentiation of homologous chromosomes (Eckard, 2001).

#### Differences in genetic recombination.

Differences between parents with regard to recombination frequencies were studied for 18 map intervals (13.5% from total intervals and 13.5% of genome coverage in cM). These intervals were chosen using markers that provided the most genetic information, corresponding to segregant types *abxcd* or *efxeg*, heterozygotes in both the parental species. Only pairs of linked loci having LOD scores higher than 10 were selected for this test. The results of the analysis showed that no interval in particular reflects significant inequalities in the frequency of recombination between the parents. Nevertheless, when all the intervals are included in the analysis by chi-square as a unit, the differences between parents become significant and in favor of the male *C. eugenioides* with 137 events of recombination compared with 100 events in the female *C. liberica* ( $X^2 = 5.78$ , significant at  $\alpha < 0.05$ , d.f. = 1).

Differential recombination between sexes has been studied in several species as a common phenomenon in nature, including species of agricultural interest (Burt *et al.*, 1991; Plomion and O'Malley, 1996). In the apple tree, *Malus pumila* Mill., significant differences in recombination frequency have been detected between the parents of a mapping population, and when the difference in frequency are very high, it can have considerable effects on the determination of the correct order of loci (Maliepaard *et al.*, 1998). It is possible that the probability of finding differences in recombination frequency between parents is greater in a cross between two individuals from different species, as it is found in this study. Nevertheless, in this work, the magnitude of those differences does not seem to considerably affect the individual intervals of the map distances. In this case the level of saturation of the map seems to

play a very important role in decreasing the effects of differential recombination between the parents of the mapping population.

### Conclusions

- The segregation analysis in the descendants of the interspecific cross between *C. liberica* and *C. eugenioides* in this study, and specially based on the results presented with respect to the amounts of each segregant type, it is possible to conclude that SSRs markers are a very informative approach for genetic analysis in coffee and appears that the majority of this type of loci are genetically very informative.
- The phenomenon of segregation distortion detected in this study is comparable with that found in other studies. Using a criterion of a  $\sim 0.01$ , 40 loci were distorted, corresponding to 23.5%. These values are considered to be average for plants and, given the fact that the population under investigation was derived from an interspecific cross; it is very probable that this aspect may have played an important role in the segregation distortions found here.
- In addition, based on the proportions of segregant and distorted loci for each of the parents, it was evident that *C. liberica* is the parental species that presented the major proportion of segregant loci, and that *C. eugenioides* is the parental species with greater tendency to cause segregation distortions.
- In this study, a clear tendency to exhibit a major proportion of null alleles was detected in the paternal *C. eugenioides*. In some cases it was possible to verify the presence of null alleles by changing the PCR conditions or the sequence of primers. This allows the validation of the segregant types, thereby avoiding the ambiguity of genotype scoring for plant segregating analysis.
- Considering only the more informative loci (segregant types *abxcd* or *efxeg*), differences between parents with regard to recombination frequencies when all the intervals are included allows to suggest that, in this cross, *C. eugenioides* was the parental with major tendency to chromosomal recombination during meiosis.

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