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Characterization of gametophytic self-incompatibility of superior clones of *Coffea canephora*

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Genet. Mol. Res. 17 (1): gmr16039876

Received November 14, 2017

Accepted January 28, 2018

Published February 08, 2018

DOI http://dx.doi.org/10.4238/gmr16039876

ABSTRACT. The ability to avoid self-pollination is a trait that evolved as a manner of reducing the deleterious effects of inbreeding in various allogamous plant species, including *Coffea canephora*. The aim of this study was to perform directed hybridizations to characterize the compatibility groups of superior *C. canephora* clones, since plant selection can reduce variability for this trait. For that purpose, directed hybridizations were carried out in cross arrangements using a balanced diallel without self-fertilization and without reciprocals. The genotypes evaluated were derived from open pollination and from directed hybridizations using Encapa03 as a male parent donor of pollen grains and Robusta640, Robusta 2258, Robusta1675 as female parent receptors of pollen grains. To control the occurrence of type I and type II errors, the likelihood ratio test was used. Compatible crosses were predominant (73.7%). Compatible crosses exhibited a mean rate of fruit set of 44%, with amplitude from 26% to 77%. Of the total of 80 crosses performed, three crosses exhibited unexpected results according to the grouping proposed (P08 x P16, P09 x P15, P09 x P16). From the degree of kinship, the genealogy of the S gene and the segregation of the alleles from one generation to another were estimated. The genotypes P12, P14, and P10 exhibited the highest estimates of LOD score associated with their clustering in groups I, II,

and III. These genotypes may be used as tester plants of their compatibility groups.

KEY WORDS: Plant breeding, selection, gametophytic self-incompatibility.

INTRODUCTION

The ability to avoid self-pollination is an important trait of the reproductive system of various allogamous plant species, including *C. canephora*. It evolved as a way of reducing the deleterious effects of inbreeding (WRIGHT et al., 2013). Self-incompatibility (SI) is a physiological mechanism that impedes a fertile plant from forming viable seeds when fertilized by its own pollen (SILVA et al., 2016). In the *C. canephora*, this barrier to self-pollination is due to arrested development of the pollen tubes, which makes fertilization of the female gametophyte impossible (BERTHAUD, 1980; ASQUINE et al., 2011).

C. canephora exhibits gametophytic self-incompatibility, in which the incompatibility reaction occurs between the pollen tube and the pollen grain, that must not share the same allele of the receptor plant (LASHERMES et al., 1996; NOWAK et al., 2011). In this specie, the expression of self-incompatibility is governed by only one multiallelic gene, identified by the letter S (BERTHAUD, 1980). Interrupted growth of the pollen tube is due to the action of ribonucleases that degrade the ribosomal RNA, impeding the growth of the pollen tube (NOWAK et al., 2011; ZHANG et al., 2009).

In clonal coffee growing, the planting of incompatible clones can compromise yield, due to reduction in the fruit fertilization rate and coffee bean quality, resulting in an increase in the rate of peaberries (FERRAO et al., 2007; ROCHA et al., 2015a). Although evaluations at the center of origin indicate up to five allelic forms of the S gene (OMOLAJA & FAWOLE, 2004), field observations of Brazilian germplasm indicate the predominance of only three allelic forms in expression of this trait (S_1, S_2, and S_3) (FERRAO et al., 2017).

The study of segregation of the S gene is complex, since evaluation of a few dozen plants results in many hybridizations, due to the multiplicative nature of the possible number of hybridizations among “n” parents (CRUZ et al., 2012; RESENDE, 2015). Characterization of tester plants of the compatibility groups has the potential to qualitatively increase the efficiency of field evaluations, reducing the number of hybridizations necessary to characterize the genotypes of the S gene. According to Borém and Miranda (2013), a tester plant can be defined as that which correctly classifies the genotypes of the plants under evaluation.

The aim of this study was to carry out directed hybridizations to characterize the compatibility groups of superior *C. canephora* clones, since selection can reduce the variability for this trait, and to consider the genotypic characterization of plants that may be used as testers of the compatibility groups.

MATERIALS AND METHODS

Directed hybridizations

Directed hybridizations were carried out in the field of Embrapa Rondônia – Porto Velho, RO, Brazil, located on the federal highway BR 364 km 5.5 in the direction of Cuiabá, in the rural area (CEP 76815-800), in the period from June to August 2016. The climate is predominantly tropical of the AM type (Köppen classification): hot and humid, with a well-defined dry period, with the occurrence of water deficit from June to September, mean annual temperature of 25ºC, mean annual rainfall of 2354 mm, and mean annual evapotranspiration of 851 mm.

Eighty hybridizations were made in the period from June to August 2016 in a 5×5 balanced diallel designs without self-fertilization and without reciprocal crosses, between twelve high performance *C. canephora* genotypes. Of this total, four genotypes did not have any degree of relatedness and eight genotypes were derived from full sib progenies (**Table 1)**.

Table 1. Listing of the genotypes (clones) used in the directed hybridization procedures, identified according to the botanical variety, to the maturation cycle and genealogy. The dotted line separates open pollination genotypes from the full sib progenies.

Genotype	Botanical variety	Cycle	Genealogy
P01[1] – 125[2]	Conilon	Intermediate	Open pollination
P02 – 160	Conilon	Intermediate	Open pollination
P04 – 199	Conilon	Intermediate	Open pollination
P08 – 193	Hybrid	Early	Open pollination
P09 – P_1C_1[3]	Hybrid	Intermediate	Encapa03 x Robusta640
P10 – P_2C_1	Hybrid	Intermediate	Encapa03 x Robusta2258
P12 – P_2C_2	Hybrid	Intermediate	Encapa03 x Robusta2258
P13 – P_2C_3	Hybrid	Intermediate	Encapa03 x Robusta2258
P14 – P_1C_2	Hybrid	Intermediate	Encapa03 x Robusta640
P15 – P_2C_4	Hybrid	Intermediate	Encapa03 x Robusta2258
P16 – P_3C_1	Hybrid	Intermediate	Encapa03 x Robusta1675
P20 – P_3C_2	Hybrid	Intermediate	Encapa03 x Robusta1675

[1]Identification of the original plots of the clones in the experiment, [2]Numerical identification of the open pollination genotypes, [3]Numerical identification of the genotypes of known genealogy: Progeny 1, Clone 1: P_1C_1.

In each cross were used 2 branches per plant, with the protection of the entire plagiotropic branch using 5 to 7 rosettes per branch. One day before anthesis, the plagiotropic branches with white flower buds were protected using 55 × 25 cm kraft paper bags. Pollinations were made from 7:00 to 10:00 in the morning, the period of greatest abundance of pollen grains.

Donor and receptor branches were selected among those that had inflorescences at the same phenological stage. To put the donor and receptor branches in contact, an opening at the tip of the paper bag was used. After the contact between the inflorescences, the procedure was completed with the closing of the paper bag and identification of the cross (ROCHA et al., 2015b; SOUSA et al., 2015). The compatibility between two clones was evaluated from the rate of fruit set evaluated weekly over 90 days with the growth of fruit.

Data interpretation

Considering that all the hybridization procedures have probability of error, a probabilistic test was used to determine the odds of the compatibility clustering in relation to the null hypothesis in which all the genotypes are part of the same compatibility group.

A compatibility group is formed by individuals that have the same genotype for the S gene, and for that reason, they are not compatible. The number of combinations (N), defined as the number of different ways that the genotypes can be organized in the compatibility groups may be estimated by the expression:

(i) $N = C^g$

In which N = possible number of combinations that can be obtained between genotypes and C compatibility groups.

Each hybridization procedure carried out has probability of error, due to handling or environmental conditions. Type I error (called false negative) occurs when the H_0 hypothesis, that the individuals are part of the same compatibility group is erroneously rejected, and type II error (called false positive) occurs when fruit develops due to contamination in the hybridization procedure.

Maximum Likelihood Estimation was used to estimate the group of greatest probability of occurrence according to the observed results (Schuster & Cruz, 2008). For this it was considered that the probability of occurrence of the genotypic classes assumes multinomial distribution, in accordance with the number of allelic forms of the S gene, as follows, according to the notation of Schuster & Cruz, 2008:

(ii) $L(p_i / n_i) = \lambda p_1^{n1} p_2^{n2} p_3^{n3} ..... p_n^{nn}$

In which: $\lambda = \frac{N!}{n_1! n_2! ... n_n!}$, $N = \sum_{i=1}^{n} n_i$, N is the number of compatibility groups and n_i is the number of plants of a determined genotypic class that are part of the i-eth compatibility group, with probability p_i.

This method allows estimates of minimum variance to be obtained from the information contained in all the genotypic classes (SCHUSTER & CRUZ, 2008). Considering an error rate of 5%, the likelihood ratio test was interpreted, which consists of comparison between the maximum likelihood estimator (θ_A) and the occurrence of the null hypothesis (θ_N), that all individuals are part of the same compatibility group:

(iii) $Z = Log_{10}\left[L \frac{(\theta_A / x)}{(\theta_N / x)} \right]$

Interpreted using the base 10 logarithm of the likelihood ratio, the estimate indicates that the alternative hypothesis is 10^Z times more probable than the null hypothesis. Thus, the likelihood ratio estimates of the upper limit (Z_{max}), of the grouping (Z_{group}), and of the individual within groups (Z_{ind}) were interpreted:

(iv) $Z_{\max} = Log_{10}\left[L \frac{(\theta_{A1} / x)}{(\theta_N / x)} \right]$; $Z_{group} = Log_{10}\left[L \frac{(\theta_{A2} / x)}{(\theta_N / x)} \right]$; $Z_{ind} = Log_{10}\left[L \frac{(\theta_{A3} / x)}{(\theta_N / x)} \right]$

In which θ_{A1} is the ideal clustering in which all the hybridizations agree with the genealogy, θ_{A2} is the grouping of maximum likelihood obtained from the hybridizations and θ_{A3} is the individual clustering probability within the compatibility group.

RESULTS AND DISCUSSION

The study of segregat ion of the S gene is complex due to the large number of combinations (N) that can be obtained in evaluation of a relatively small number of plants. In an attempt to simplify this issue, Lashermes et al., 1996, developed double haploid populations obtained from duplication of the haploid genome and, for that reason, homozygous for the S gene. Although the double haploid populations allow fixation of a single allele and segregation of only two genotypic classes, their development is a costly and time-consuming process.

In general, predominance of compatible crosses was observed (73.7%). After the artificial hybridizations, a dormancy phase began which lasted for 60-90 days, in which it was not possible to observe fruit growth (CAMARGO & CAMARGO, 2001; CHARRIER, A. & ESKES, 2004; GOMES et al., 2016)). After this period, the fruit began growth, characterized by irreversible increase in the weight and formation of the endosperm, with development of green fruit in a phenological phase that preceded the fruit maturation **(Figure 1**).

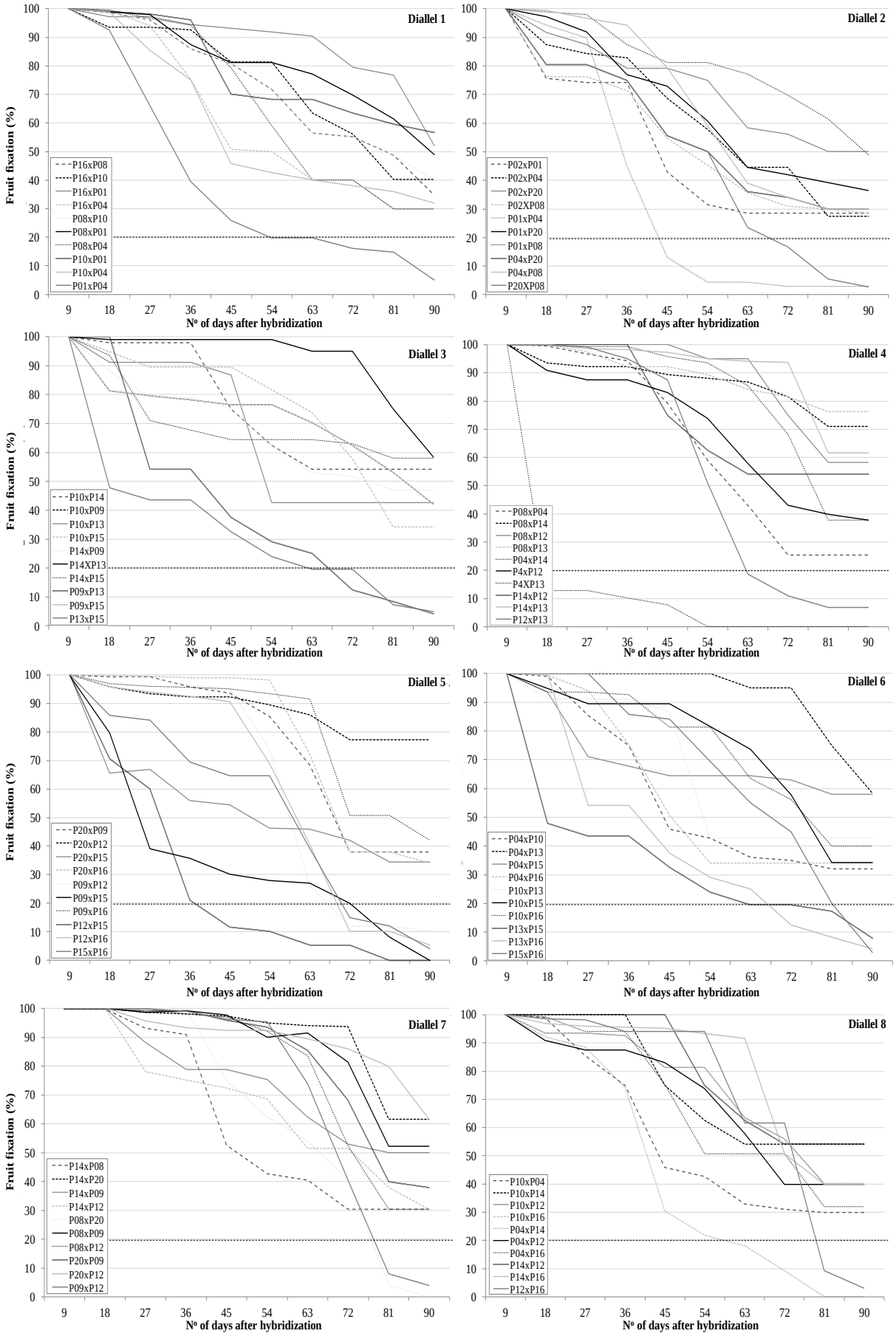


Figure 1. Percentage of fruit set evaluated over 90 days after directed hybridization performed in July and August 2016. The dotted line separates the compatible crosses from the incompatible crosses.

The rate of fruit set after directed hybridization varies according to the species and the environment (CARVALHO et al., 1991). The compatible crosses exhibited a mean rate of fruit set of 44%, with amplitude from 26% to 77% **(Figure 1**). According to Carvalho et al. 1991 in *C. canephora* the artificial hybridization reaches average rate of fruit set inferior to 50%. The literature reports artificial hybridization seed set rates of 66% in *Hemerocallis hybrida*, 65% in *Eucalyptus spp*., 62% in *Ilex paraguariensis*, and 49% in *Coffea arabica* (MENEZES & OLIVEIRA, 2011, ASSIS et al., 1993, CARVALHO et al., 1991). In *C. arabica*, high temperatures of the tropics may provoke abortion of flowers from dehydration of the flower structures (CAMARGO & CAMARGO, 2001).

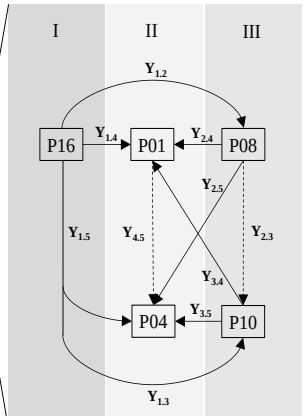
The incompatible crosses exhibited a mean rate of fruit set of 3%, caused by contamination during the hybridization procedures. According to Charrier & Eskes, 2004 and Krug, 1935, contamination is one of the most important sources of error in directed hybridization of *C. canephora*. Care in the action of insects at the time of pollination and afterwards in protection of the inflorescences are important for reducing the percentage of contamination.

In the study of the pattern of inheritance, it is important to consider that each hybridization procedure has a probability of error, whether this is due to handling or environmental conditions.Two types of errors are associated with the hypothesis test. Type I error, called false negative, occurs when the H_0 hypothesis (that all the individuals are part of the same compatibility) is erroneously rejected. Non-synchronized opening of the flowers is a factor which impedes controlled crosses from being made. Type II error, called false positive, occurs when incompatible plants produce fruit, due to contamination, and the true hypothesis is rejected.

In studies of pattern of inheritance unexpected results cannot be discarded, as there is the need for optimization methods that allow inferences regarding the characterization accuracy (PIRES & BRANCO, 2010; NOVITSKI, 2004). Upon considering error rates of 1% and 5%, because of chance, there is a probability of 55% and of 98%, respectively, that at least one hybridization shows an inconsistent result in 80 crosses. Maximum like hood method allows to utilize the information produced in all the genotypic classes to obtain the clustering of maximum probability of occurrence, penalizing the reliability of the groupings that exhibit inconsistent crosses. Among the 80 crosses made, three crosses exhibited unexpected results according to the proposed grouping (P08 x P16, P09 x P15, P09 x P16) (**Figure 2**).

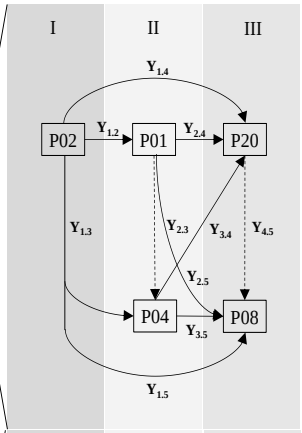
Diallel 1

Clones	P16	P08	P10	P01	P04
P16		C	C	C	C
P08			NC	C	C
P10				C	C
P01					NC
P04					



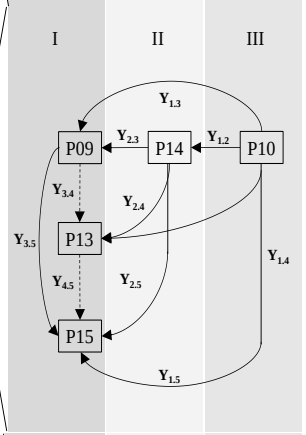
Diallel 2

Clones	P02	P01	P04	P20	P08
P02		C	C	C	C
P01			NC	C	C
P04				C	C
P20					NC
P08					



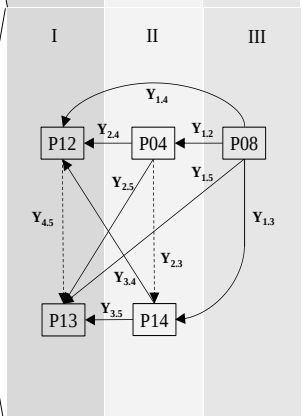
Diallel 3

Clones	P10	P14	P09	P13	P15
P10		C	C	C	C
P14			C	C	C
P09				NC	C*
P13					NC
P15					



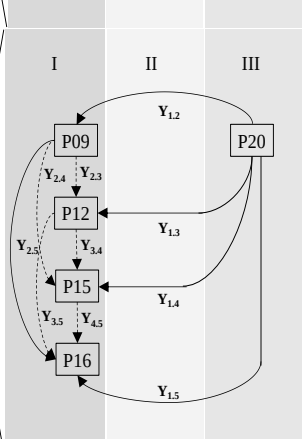
Diallel 4

Clones	P08	P04	P14	P12	P13
P08		C	C	C	C
P04			NC	C	C
P14				C	C
P12					NC
P13					



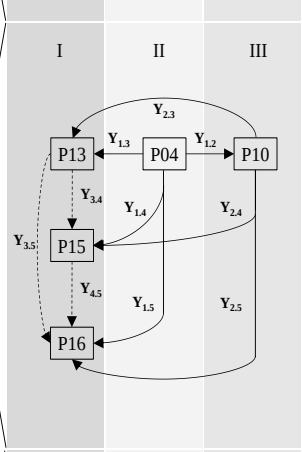
Diallel 5

Clones	P20	P09	P12	P15	P16
P20		C	C	C	C
P09			NC	NC	C*
P12				NC	NC
P15					NC
P16					



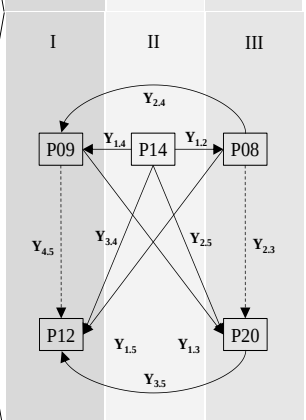
Diallel 6

Clones	P04	P10	P13	P15	P16
P04		C	C	C	C
P10			C	C	C
P13				NC	NC
P15					NC
P16					



Diallel 7

Clones	P14	P08	P20	P09	P12
P14		C	C	C	C
P08			NC	C	C
P20				C	C
P09					NC
P12					



Diallel 8

Clones	P10	P04	P14	P12	P16
P10		C	C	C	C
P04			NC	C	C
P14				C	C
P12					NC
P16					

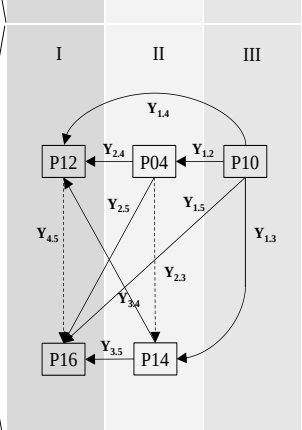


Figure 2. Results of directed hybridizations performed among twelve clones of *Coffea canephora* and the most like hood clustering of the genotypes in their respective compatibility groups, indicated by the Roman numerals I, II, and III. C – compatible cross, NC – incompatible cross, and * indicate three hybridizations with inconsistent results in relation to the groupings.

The maximum likelihood clustering was estimated from a universe of 5.3 x 10^5 combinations that can be obtained considering the distribution of twelve genotypes in three compatibility groups (**Table 2**). The likelihood ratio considers the probability of occurrence of the null hypothesis (H_0) and of the alternative hypothesis (H_a) in accordance with the data obtained. The upper limit of this statistic is obtained when all the crosses indicate the same grouping without inconsistencies (Z_{max} =74.2).

The maximum like hood clustering was 10^{70} times more probable than the H_0 hypothesis that all the genotypes belong to the same compatibility group (Z_{group} =70.3) (**Table 2**)

Table 2. Distribution of accessions characterized according to compatibility groups determined by directed hybridization and accompaniment of fruit development in the field. The compatibility groups are represented by the Roman numerals from I to III.

I	II	III
P02* Zind=2.6	P01* Zind =15.3	P08* Zind =14.1
P09 Zind =5.1	P04* Zind =12.8	P10 Zind =19.2
P12 Zind =12.8	P14 Zind =16.6	P20 Zind =11.5
P13 Zind =9.0		
P15 Zind =5.1		
P16 Zind =9.0		
Z_{max} = 74.2		
Z_{group} = 70.3		

* Genotypes from open pollination, Z_{max} : upper limit of the likehood ratio estimate, Z_{group} : likehhod ratio estimate of the clustering, Z_{ind} : Likehood ratio estimate of individuals within groups.

According to Cruz et al., 2004, LOD score estimates should be higher than 3 to be interpreted, indicating that the H_0 hypothesis is 1000 times more probable than the alternative hypothesis. In this criterion, the genotype P02 exhibited an estimate lower than 3 (Z_{P02} = 2.86), indicating that its position in compatibility group I should be confirmed by performing new crosses. The genotypes evaluated in this study were derived from open pollination and from directed hybridizations using Encapa03 as a male parent donor of pollen grains and Robusta640, Robusta 2258, Robusta1675 as female parents, receptors of the pollen grains. Upon considering the degree of kinship among the genotypes, it was possible to estimate the genealogy of the S gene considering the segregation of the alleles from one generation to the other (**Figure 3**).

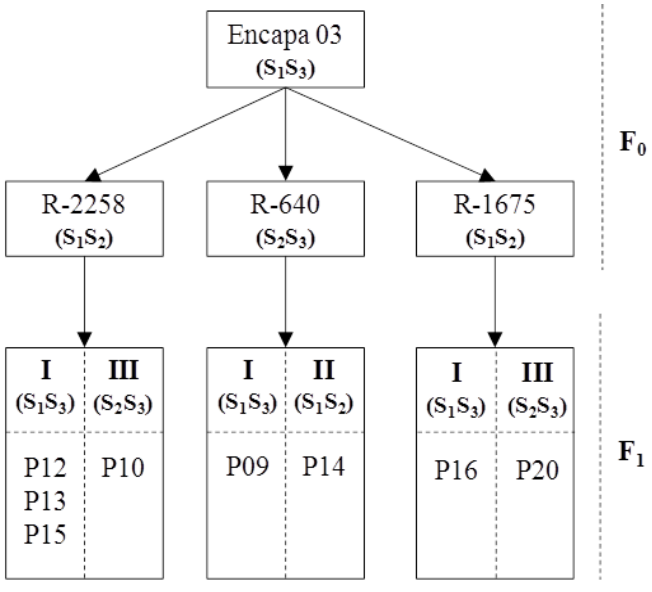


Figure 3. Genealogy of the S gene evaluated in three full sib progenies derived from directed hybridizations using Encapa03 as the male parent and Robusta640, Robusta 2258, and Robusta1675 as female parents. The compatibility groups are identified by the Roman numerals I, II, and III. F_0: parent generation, F_1: progenies of the following generation.

In contrast with the results of Omolaja & Fawole, 2004, who observed the occurrence of 5 different allelic forms, evaluations of Brazilian populations have indicated the segregation of only 3 alleles (CONAGIN & MENDES, 1961, FERRAO et al., 2007). The possibility of identifying new alleles of the S gene in Brazilian germplasm opens the possibility for an expressive reduction in the percentage of peaberries of the Conilon and Robusta botanical varieties, by naturally favouring compatible crosses.

According to Nowak et al., 2011, the low allelic polymorphism of the S gene observed in *C. canephora* suggests that this species passed through a genetic bottleneck in its past. The genetic determination of the C. *canephora* self-incompatibility results in a negative frequency-dependent selection of the S gene, which means that the frequency of an allele is inversely proportional to its adaptive value (RESENDE, 2015). Individuals with the rarest allelic forms of this gene benefit from the higher frequency of potential compatible pollen donor plants, making the individual's fecundity rate inversely proportional to the frequency of the allele in the population. Since the self-incompatibility is a pre-zygotic mechanism that influences the fecundity rate, it contributes to: none of the allelic forms of the S gene may fix in a breeding population, the individuals are heterozygous for this gene and the S gene rate of mutation is higher than a neutral gene.

Unlike the context normally employed in plant breeding of use of tester plants for determination of the specific and general combining ability, the use of tester plants for determination of compatibility groups is based on evaluation of the compatibility response in comparison to a known genotype for the S gene. The characterization of tester plants has potential to significantly reduce the number of hybridizations necessary to characterize the genotypes of the S gene by carrying out test crosses.

Under this same principle, tester plants of *C. canephora* are being used for identification of the races of rust (*Hemileia vastatrix*) since that the resistance response is also governed by few genes of greater effect (ZAMBOLIM, 2016). The genotypes P12, P14, and P10, of higher estimates of LOD score associated with their grouping in groups I, II, and III respectively, can be used as tester plants of their groups (**Table 2**).

Although recent studies of genetic mapping have not provided evidence of a connection between yield traits and the segregation of the S gene (LASHERMES et al., 1996; NOWAK et al., 2011), it should be considered that by chance, selection can drastically reduce the variability of this gene.

Evaluations at the center of origin indicate that this gene is in equilibrium in natural populations (OMOLAJA & FAWOLE, 2004). According to VEKEMAN & SLATKIN, 1994, the S gene has a genealogy like that of neutral genes that do not have their frequency altered by natural selection. In a population in Hardy Weinberg equilibrium (HWE), in which allele frequencies are identical (n=p=…=z), the probability of a cross being compatible is 0% when only two allelic forms are present, 66.7% when three allelic forms are present, and 83.3% when four different allelic forms are present (RESENDE, 2015).

Even though few studies have measured the damaging effect of self-incompatibility to coffee plantations (FERRÃO et al., 2007), the planting of incompatible genotypes can increase the rate of peaberries and reduce the coffee bean yield due to isolation of incompatible plants. Characterization of the genotype of the S gene allows clones to be grouped in the field according to their respective compatibility groups.

The presence of genetic divergence in *Coffea spp.* populations is essential for commercial plantations and contributes to success within breeding programs (MACHADO et al., 2017). In this context, the results of this study should not be used to subsidize the cultivation of few clones, which generates a risk to coffee fields, due to low plant genetic variability. Low genetic variability is associated with lower ability of plants to respond to changes in the environment, which is reflected in greater susceptibility to pests and diseases (SOUZA et al., 2013; RAMALHO et al., 2016), and other defects that are observed from growing a reduced number of genotypes on a large scale, including the lower flower synchronism observed when the genotypes flower on different days.

CONCLUSIONS

In studies of pattern of inheritance unexpected results cannot be discarded, as there is the need for optimization methods that allow inferences of the breeding accuracy. The main advantage of the strategy used is to identify the maximum likehood clustering and the segregation pattern of the S gene, considering the number of evaluated crosses and the probability of errors in the hybridization procedures. The characterization of tester plants has potential to significantly reduce the number of hybridizations necessary to characterize the genotypes of the S gene by carrying out test crosses. Characterization of self-incompatibility systems is important for *C. canephora* breeding considering the composition of commercial varieties.

CONFLICTS OF INTEREST

The authors declare no conflict of interest.

ACKNOWLEDGMENTS

To the CNPq for grant concession and to Brazilian Coffee Research and Development Consortium for financial support.

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