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Self-incompatibility characterization in segregating populations of apple trees with DNA markers for *S*-alleles

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Abstract - The objective of this study was to characterize the parents and respective populations of apple trees regarding *S*-alleles to confirm their genealogy and to evaluate the efficiency of the molecular markers used. Sixteen specific sets of primers were used for identification of apple *S*-alleles by PCR. Two segregating populations of the Epagri Apple Breeding Program resulting from crosses between 'Fred Hough' × 'Monalisa' and 'M-11/00' × 'M-13/91' were evaluated. The expected segregations are 1:1:1:1 for full compatibility and 1:1 for semi-compatibility, which can be confirmed by the X^2 test. The 'Fred Hough' (S_5S_{19}) × 'Monalisa' (S_2S_{10}) cross proved to be fully compatible; and two triploids were identified among the hybrids as well. The 'M-11/00' (S_3S_{19}) × 'M-13/91' (S_3S_5) cross was characterized as semi-compatible based on DNA markers, and the segregation of the *S*-alleles in the hybrids was 1:1, as expected. The segregation of the DNA markers occurred together with their respective *S*-alleles: S_2, S_3, S_5, S_{10}, and S_{19}. Thus, characterization of the *S*-alleles not only allowed identification of compatibility between parents but also identified contaminations in segregating populations.

Index terms: *Malus* × *domestica* Borkh., *S* genotype, *S*-RNase, allele-specific PCR, segregation.

Caracterização da autoincompatibilidade em populações segregantes de macieira via marcadores de DNA para alelos *S*

Resumo - O objetivo deste trabalho foi caracterizar genitores e respectivas populações de macieiras quanto aos alelos *S* para confirmar sua genealogia e para avaliar a eficiência dos marcadores moleculares utilizados. Conjuntos específicos de iniciadores foram utilizados para a identificação dos alelos *S* via PCR. Foram avaliadas duas populações segregantes do Programa de Melhoramento Genético de Macieira da Epagri resultantes dos cruzamentos entre 'Fred Hough' × 'Monalisa' e 'M-11/00' × 'M-13/91'. As segregações esperadas são 1:1:1:1 para compatibilidade total e 1:1 para semi compatibilidade, que podem ser confirmadas pelo teste X^2. O cruzamento 'Fred Hough' (S_5S_{19}) × 'Monalisa' (S_2S_{10}) foi identificado como totalmente compatível, e foram identificados dois triploides entre os híbridos. O cruzamento entre 'M-11/00' (S_3S_{19}) × 'M-13/91' (S_3S_5) mostrou-se semicompatível baseado nos marcadores moleculares, e a segregação dos alelos S nos híbridos foi de 1:1, como esperado. A segregação dos marcadores de DNA para S_2, S_3, S_5, S_{10} e S_{19} ocorreu juntamente com seus respectivos alelos *S*. Dessa forma, a caracterização dos alelos *S*, além de permitir identificar a compatibilidade entre os genitores, serviu para identificar contaminações em populações segregantes.

Termos para indexação: *Malus* × *domestica* Borkh., genótipo *S*, *S*-RNase, PCR alelo-específico, segregação.

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Introduction

Gametophytic self-incompatibility is present in the reproductive process of several *Malus* species, including *Malus* × *domestica* Borkh. (BROOTHAERTS, 2003). Control of self-incompatibility is performed by the multiallelic locus '*S*', located in the terminal portion of chromosome 17 (YAMAMOTO et al., 2002; DE FRANCESCHI et al., 2011, 2016). Each allele is responsible for production of a protein with co-dominance behavior, which acts within the pistil (BATLLE et al. 1995; LI et al., 2012; RAMALHO, 2012). The mechanism of self-incompatibility is as efficient as dioicia in the requirement of cross-fertilization between plants, helping to increase genetic variability (ALLARD, 1971).

Currently, DNA markers are used for identification of *S*-alleles (MA et al., 2016; MIR et al., 2016; DE FRANCESCHI et al., 2018). However, allele-specific markers and microsatellite markers must be linked to the respective *S*-alleles they identify to be considered efficient (FERREIRA; GRATTAPAGLIA, 1995). When the primer is adapted to a region distant from the gene of interest, distortions may occur in the expected frequency of alleles in the segregating populations, resulting in false negative or positive results.

In crosses between fully-compatible plants (all different *S*-alleles), the segregation pattern in the progenies is expected to follow the proportion 1:1:1:1 (RAMALHO, 2012; AGAPITO-TENFEN et al., 2015). In contrast, in crosses of semi-compatible plants, the expected pattern of *S*-allele segregation is expected to follow the ratio 1:1 (CHOI et al. 2002; DE FRANCESCHI et al., 2016). This is because the pollen grain bearing the *S*-allele, common to the diploid pistil tissue, is aborted when in contact with the pistil *S*-RNases (MATSUMOTO, 2014). However, in crosses between incompatible plants, in which the pair of *S*-alleles in each parent are the same, abortion of all pollen grains occurs, not allowing the formation of viable seeds.

Therefore, the objective of this study was to characterize the parents and their respective descendants regarding identification of *S*-alleles by DNA markers to confirm their genealogy and to evaluate the efficiency of the markers used to identify the *S*-alleles.

Materials and methods

For characterization of self-incompatibility by identification of the *S*-alleles, two segregating populations of apple trees belonging to the Apple Breeding Program of the Empresa de Pesquisa Agropecuária e Extensão Rural de Santa Catarina - Epagri (Agricultural Research and Rural Extension Company of Santa Catarina) were evaluated. The experimental orchard is in the Experimental Station in the municipality of Caçador, in the midwestern region of the state of Santa Catarina (26°49'5'' S and 50°59'12'' W at 940 m AMSL).

One population originated from crossing the apple varieties 'Fred Hough' × 'Monalisa' (54 plants) and the other from crossing the selections 'M-11/00' × 'M-13/91' (120 plants). These crosses were made in 2007, following routine crosses of the Apple Breeding Program for the generation of segregating populations.

Young healthy leaves were collected from each of the two segregating populations and respective parents and were kept deep-frozen at -20°C in plastic bags until DNA extraction, which was performed according to Revers et al. (2005) using 0.1 g of ground plant tissue. Each polymerase chain reaction (PCR) was performed in a final volume of 15 µL, containing 1 U of Taq DNA polymerase, 1x enzyme buffer, 2.00 mM $MgCl_2$, 0.2 mM dNTPs, 1 µM of each primer (forward and reverse), and 50 ng of genomic DNA. Primers developed by Kitahara and Matsumoto (2002) and Broothaerts (2003) were used to identify 16 *S*-alleles of apple trees (Table 1). PCR was performed with a T100™ thermocycler (BioRad® California, USA) programmed for 3 min at 94°C, followed by 30 denaturation cycles at 94°C for 1 min, annealing depending on the primer characteristics (see Table 1) for 1 min, and extension at 72°C for 1 min, followed by 7 min at 72°C. For the S_{10} primer, the final extension step was at 72°C for 10 min. For discrimination of the S_4, S_{16}, and S_{22} alleles, part of the PCR-amplified product (10 µL) was digested by the restriction enzyme *TaqI* (for 1 h in a 65°C water bath). Likewise, for discrimination of the S_{20} and S_{10} alleles, 10 µL of the amplification product was digested by the restriction enzyme *NarI* (for 4 h in a 37°C water bath).

Table 1. Primer sequences and temperature conditions for allele-specific PCR to identify the *S*-alleles of apple tree (*Malus* × *domestica* Borkh.) and restriction enzyme digestion.

S-Allele	Primers	Sequence (5' → 3')	Annealing temperature (ºC) / restriction enzymes	Amplified size (bp)
S_1	FTC168	ATATTGTAAGGCACCGCCATATCAT	60	530
	FTC169	GGTTCTGTATTGGGGAAGACGCACAA		
S_2	OWB122	GTTCAAACGTGACTTATGCG	60	449
	OWB123	GGTTTGGTTCCTTACCATGG		
S_3	FTC177	CAAACGATAACAAATCTTAC	55	500
	FTC226	TATATGGAAATCACCATTCG		
S_4	FTC5	TCCCACAATACAGAACGAGA	60 / *TaqI*	274 (194+77)
	OWB249	CAATCTATGAAATGTGCTCTG		
S_5	FTC10	CAAACATGGCACCTGTGGGTCTCC	59	346
	FTC11	TAATAATGGATATCATTGGTAGG		
S_6	FTC141	ATCAGCCGGCTGTCTGCCACTC	58 [1]	850
	FTC142	AGCCGTGCTCTTAATACTGAATAC		
S_7	FTC143	ACTCGAATGGACATGACCCAGT	60	302
	FTC144	TGTCGTTCATTATTGTGGGATGTC		
S_9	OWB154	CAGCCGGCTGTCTGCCACTT	62	343
	OWB155	CGGTTCGATCGAGTACGTTG		
S_{10}	[2]	AACAAATCTTAAAGCCCAGC	60 / *NarI*	282 (185+97)
		GGTTTCTTATAGTCGATACTTTG		
S_{16}	FTC5	TCCCACAATACAGAACGAGA	60 / *TaqI*	274 (243+41)
	OWB249	CAATCTATGAAATGTGCTCTG		
S_{19}	FTC229	TCTGGGAAAGAGAGTGGCTC	60	304
	FTC230	TTTATGAACTTCGTTAAGTCTC		
S_{20}	FTC141	ATCAGCCGGCTGTCTGCCACTC	60 [1] / *NarI*	920 (800+120)
	FTC142	AGCCGTGCTCTTAATACTGAATAC		
S_{22}	FTC5	TCCCACAATACAGAACGAGA	60 / *TaqI*	274 (199+44+31)
	OWB249	CAATCTATGAAATGTGCTCTG		
S_{23}	FTC222	CAATCGAACCAATCATTTGGT	60	237
	FTC224	GGTGTCATATTGTTGGTACTAATG		
S_{24}	FTC231	AAATATTGCAACGCACAGCA	60	580
	FTC232	TTGAGAGGATTTCAGAGATG		
S_{26}	FTC14	GAAGATGCCATACGCAATGG	54	194
	FTC9	TTTAATACCGAATATTGGCG		

Values in parentheses refer to the fragment size generated after digestion with the respective restriction enzymes. [1] Cycle extension time of 45 sec. [2] Primer proposed by Kitahara & Matsumoto (2002). FTC and OWB primers were developed by Broothaerts (2003).

The following cultivars, previously characterized for the respective *S*-allele, were used as positive controls for the presence of each *S*-allele: Fuji (S_1 and S_9; SASSA et al., 1996), Golden Delicious (S_2 and S_3; BROOTHAERTS et al., 1995), Gloster (S_4; VAN NERUM et al., 2001), Gala (S_5; JANSSENS et al., 1995), Marubakaido (S_6 and S_{26}; AGAPITO-TENFEN et al., 2015), Idared (S_7; JANSSENS et al., 1995), McIntosh (S_{10}; RICHMAN et al., 1997), Delicious (S_{19}; MATSUMOTO and KITAHARA, 2000), Alkmene (S_{22}; VAN NERUM et al., 2001), Mutsu (S_{20}; MATSUMOTO et al., 1999), Granny Smith (S_{23}; SCHNEIDER et al., 2001), and Braeburn (S_{24}; KITAHARA et al., 2000). The only exception was allele S_{16}, since no genotype with this pre-identified allele is maintained in Epagri. In addition, the same cultivars were used for primer optimization.

The amplification products were analyzed by 3% agarose gel electrophoresis using a 50 bp DNA marker. The gels were stained with GelRed® fluorescence intercalation. The profiles of the amplified fragments were analyzed by images captured with a Kodak Gel Logic 212 Pro Imaging System. The *S*-allele amplifications whose size coincided with the positive control were identified as present. The segregation of the *S*-alleles was assessed using the X^2 test, considering the *S*-alleles that were identified and the expected segregation (complete compatibility = 1:1:1:1 and semi-compatibility = 1:1). In addition, if there were *S*-alleles common to the parents, field crosses were carried out to obtain the fertilization rate. Self-fertilization of the parents and reciprocal cross-pollination were performed. At 40 days after pollination, the fertilization index (number of fruit with more than 20 mm formed after pollination) of each cross (fruit set) was evaluated.

Results and discussion

The genotypes of the parents 'Fred Hough' (S_5S_{19}), 'Monalisa' (S_2S_{10}), 'M-11/00' (S_3S_{19}), and 'M-13/91' (S_3S_5) identified by Brancher et al. (2020) were confirmed (Figure 1).

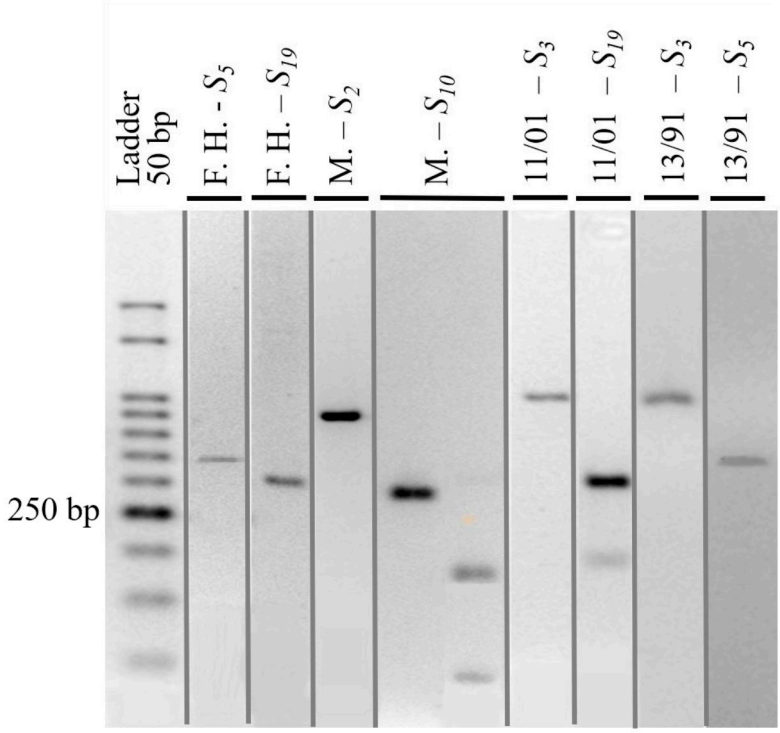

Figure 1. Characterization of the *S*-alleles of the parents of the segregating populations ('F.H.' Fred Hough – S_5S_{19}, 'M.' Monalisa – S_2S_{10}, '11/00' M-11/00' – S_3S_{19}, and '13/91' M-13/91' – S_3S_5) comparing the size of the PCR fragments identified on agarose gel (3% and 50 bp ladder) with the sizes available in the literature (S_2: 449 bp; S_3: 500 bp; S_5: 346 bp; S_{10}: 282 [185 + 97] bp; S_{19}: 304 bp).

In the segregating population resulting from 'Fred Hough' (S_5S_{19}) × 'Monalisa' (S_2S_{10}), 49 apple trees exhibited one of the genotypes expected from this fully-compatible cross (S_2S_5, S_5S_{10}, S_2S_{19}, and $S_{10}S_{19}$). Distribution of the plants among the possible *S*-allele genotypes (Table 2) followed the expected proportion for crossing of fully-compatible genotypes: 1:1:1:1 ($p > 0.05$). Thus, the DNA markers used were effective in identification of the respective alleles, and confirmed that these plants were the result of the 'Fred Hough' × 'Monalisa' cross.

Table 2. *S*-allele genotype and number of plants identified with each genotype in apple tree populations.

'Fred Hough' (S_5S_{19}) × 'Monalisa' (S_2S_{10})			
S-alleles identified in the segregating population	Number of plants observed	Number expected	χ^2
S_5S_2	13	13.5	1.85^{ns}
S_5S_{10}	15	13.5	
S_2S_{19}	9	13.5	
$S_{10}S_{19}$	12	13.5	
$S_2S_5S_{19}$	1	0	
$S_5S_{10}S_{19}$	1	0	
S_5S_9	1	0	
S_9S_{19}	1	0	
$S_{19}S_?$	1	0	
Total	54		
' M-11/00' (S_3S_{19}) × 'M-13/91' (S_3S_5)			
S-alleles identified in the segregating population	Number of plants observed	Number expected	χ^2
S_3S_5	66	60	2.62^{ns}
S_5S_{19}	49	60	
S_5S_{10}	2	0	
$S_5S_?$	3	0	
Total	120		

$S_?$: unidentified *S*-allele.

In addition, two plants were identified as having three alleles each: one with $S_2S_5S_{19}$ and the other with $S_5S_{10}S_{19}$ (Table 2). The occurrence of three *S*-alleles suggests the triploidy of these two plants, since the *S*-alleles identified were common to the *S*-alleles present in the parents. Triploid plants can occur naturally in the *Malus* genus, both in interspecific crosses and among diploid crosses (BROWN, 2012). Two of the three alleles of these plants were inherited from the female parent 'Fred Hough' (S_5 and S_{19}). This result coincides with that found by Janssens et al. (1995) and Sakurai et al. (2000), who identified the maternal parent as the donor of the gamete 2n (gamete not reduced) in different crosses between diploid parents, from which triploid descendants can originate.

Three plants of the first cross had a *S*-allele different from the alleles expected for this population. The plants were genotyped as S_5S_9, S_5S_{19}, and $S_{19}S_?$, according to Table 2. These plants may have resulted from some exchange during the seedling development process or contamination by pollen that did not correspond to the cross-breeding parent.

In the cross 'M-11/00' (S_3S_{19}) × 'M-13/91' (S_3S_5), four different genotypes were identified in the population (Table 2). There is one *S*-allele in common between the parents, characterizing semi-compatibility because of the gametophytic self-incompatibility mechanism (BATLLE et al., 1995; RAMALHO, 2012; MATSUMOTO, 2014; DE FRANCESCHI et al., 2016; PRATAS et al., 2018). The distribution of the plants among the possible *S*-allele genotypes (S_5S_{19} and S_3S_5) followed the expected proportion for the crossing of semi-compatible genotypes: 1:1 ($p > 0.05$). In addition, there are five plants with alleles that likely are a result of contamination during formation of the population (S_5S_{10} and $S_5S_?$).

Because the S_3 allele is in common, the cross 'M-11/00' x 'M-13/91', the reciprocal cross, and self-fertilization of both parents were performed again to determine the fruit set of this cross. The results of the four crosses are shown in Table 3. Self-fertilization of 'M-13/91' did not produce fruit; the cross 'M-13/91' (♀) × 'M-11/00' (♂) exhibited 15.4% fruit set; and the cross 'M-11/00' (♀) × 'M-13/91' (♂) exhibited 35.8% fruit set. It was notable that self-fertilization of 'M-11/00' produces 7.5% fruit set. A hypothesis for this fruit set is that 'M-11/00' has some degree of self-fertility, through which some self-fertilization could naturally occur (LI et al., 2016). The formation of viable seeds in the fruit will indicate if the egg(s) was (were) fertilized or not, and then

parthenocarpy may be dismissed, which is characterized by the formation of fruit without fertilization of the eggs, resulting in the absence of seeds or the presence of sterile seeds (HEGEDÜS, 2006). This fruit set obtained in both crosses diverges from the results of the original cross made in the year 2007, which had 87% fruit set (data not shown), probably because of some environmental effect or the germination capacity of the pollen currently used compared to the past. After harvest, seeds from the fruit from crosses between 'M-11/00' and 'M-13/91' and from the 'M-11/00' self-pollinations (if there are seeds in the self-fertilized fruit) will be genotyped to check for *S*-alleles.

Table 3. Cross between the selections 'M-13/91' and 'M-11/00', the reciprocal cross, and self-fertilization of the parents regarding number of pollinated flowers, number of apples formed, and fruit set (%) at 40 days after pollination.

Parents		Number of pollinated flowers	Number of apples formed	Fruit set (%)
Female (♀)	Male (♂)			
M-11/00 (S_3S_{19})	M-11/00 (S_3S_{19})	133	10	7.5
M-11/00 (S_3S_{19})	M-13/91 (S_3S_5)	123	44	35.8
M-13/91 (S_3S_5)	M-13/91 (S_3S_5)	198	0	0
M-13/91 (S_3S_5)	M-11/00 (S_3S_{19})	156	24	15.4

Therefore, characterization of the *S*-alleles by DNA markers not only allowed identification of compatibility between the parents, but also served to find paternity divergences (or cross-contamination) in segregating populations, as already found by Sakurai et al. (2000) and Choi et al. (2002).

Conclusion

The cross 'Fred Hough' (S_5S_{19}) × 'Monalisa' (S_2S_{10}) is characterized as fully-compatible, with corresponding segregation of the *S*-alleles (1:1:1:1). The results obtained from the segregating population of 'M-11/00' x 'M-13/91' indicate semi-compatibility and a segregation ratio of 1:1.

DNA markers for the S_2, S_3, S_5, S_{10}, and S_{19} alleles co-segregated with the respective *S*-alleles, which was effective for characterization of genotypes.

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