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Identification of hybrids of intra and interspecific crosses in Annonaceae by RAPD markers

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ABSTRACT – *The purpose of this work was to identify hybrids in intraspecific crosses between sugar apple accessions and interspecific crosses between sugar apple and atemoya accessions by using RAPD markers. Four sugar apple accessions were selected: Seedless P_1, P_2, P_3 and P_4 and the atemoya cultivar Gefner (G1). In the pre-female phase the flowers were adequately protected and reciprocal crosses were performed. In crosses where the sugar apple accession Seedless P_1 was used as the male parent, the fruits contained seeds, indicating that the pollen grains of Seedless P_1 are viable. The fruits of reciprocal crosses where Seedless P_1 was used as a female parent contained no seeds. The percentage of true hybrids in the crosses P_4 x Seedless P_1, P_3 x Seedless P_1, P_2 x Seedless P_1, and G_1 x Seedless P_1 were, respectively, 100%, 95.55%, 82.86%, and 44.44%. Primer OPF10 was efficient in obtaining polymorphic bands in all Annonaceae hybrid populations.*

Key words: *Annona squamosa, Annona cherimola x Annona squamosa, seedless fruits, molecular markers.*

INTRODUCTION

The Annonaceae encompass a group of economically relevant fruit trees of several countries, e.g., Chile, Mexico, Australia, the USA, and Brazil (Ferreira 2001). In Brazil, these crops are found from the northern region of the country to the state of São Paulo, but are grown mostly in the semiarid region. It is estimated that Annonaceae are planted on 10 thousand ha in Brazil, of which about 1,000 ha are atemoya, partly in the Northeast and partly in the Southeast region of the country (Nogueira et al. 2005). The main national producer is the state of Bahia, followed by Pernambuco, Alagoas and São Paulo (IBGE 2008).

The Annonaceae family is native to tropical and subtropical regions, among which some of the genus *Annona* are fruit plants of commercial interest, such as sugar apple (*Annona squamosa* L.), soursop (*Annona muricata* L.), cherimoya (*Annona cherimola* Mill.) and atemoya (*Annona squamosa* x *Annona cherimola*) (Araujo et al. 1999). Studies with these species indicate the occurrence of the phenomenon known as dichogamy of the protogynous type i.e., maturation of carpels before the stamens, limiting the occurrence of selfing (Pinto et al. 2005).

In Brazil there are no defined sugar apple cultivars, except for the seedless sugar apple, derived from a somatic mutation that produces parthenocarpic fruit (Couceiro 1983). Luna (1988) reports that the most adequate selections for planting in Brazil are Pinha FAO I, Pinha AP and Pinha FAO II. Dantas et al. (1991) also identified the

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selections IPA-2.1, IPA-18.2 and IPA-C-1, with a production of more than 14 kg of fruits per plant per year.

In breeding, molecular markers have been used as a tool to reduce the time required to develop a new cultivar. This technology allows breeders to produce superior genotypes more efficiently, rapidly, accurately and at a lower cost than by conventional breeding, which is usually rather time-consuming (Borém and Caixeta 2009). Among the various uses of molecular markers, the identification of true hybrids is an extremely useful tool in a breeding program (Alzate-Marin et al. 1996). In species with long life cycles, the conventional process of hybrid identification is slow, increasing the time and resources requirements for improvement. Therefore, the use of molecular markers to identify plants from crosses is highly important, so that plants from self or undesirable crosses can be identified and discarded in the F_1 generation (Cordeiro et al. 2006).

The purpose of this study was to identify hybrids in interspecific crosses between accessions of the sugar apple and atemoya cultivar Gefner and in intraspecific crosses between accessions of sugar apple by using RAPD markers.

MATERIAL AND METHODS

The sugar apple accessions Seedless P_1, P_2, P_3 and P_4 and atemoya hybrid G_1, grown in the experimental area of the Universidade Estadual de Montes Claros (Unimontes), Janaúba, Minas Gerais, were used as parents and their eight F_1 hybrid combinations with the reciprocal crosses were evaluated. The accessions were properly identified and artificially pollinated using a number two brush. All flowers of both parents were wrapped in paper bags before reaching the pistila and stamina function stages. When the flowers of the selected accessions used as female parents reached the pistila function stage, pollen grains of the male parent were collected from flowers at the stamina function stage and artificial pollination was carried out from 6:30 to 7:30 am. After hybridization, the crosses were properly identified and the protection of the flowers used as female parents was maintained for another 10 days. Besides the crosses between sugar apple and atemoya accessions, 22 flowers at the pre-female stage of the seedless sugar apple accession Seedless P_1 were covered with bags to evaluate the fruit establishment rate without prior natural or artificial pollination.

Approximately 100 days after artificial pollination, the fruits were harvested and wrapped individually in paper bags, identified and sent to the Laboratório de Fisiologia Pós-Colheita de Frutas. The pulp was removed from the fruits and the seeds packed in labeled paper bags and stored in a refrigerator.

Two seeds of each fruit from each cross were sown in plastic bags, filled with a 3:1:1 (dirt:sand:manure) substrate, properly identified. After germination, the seedlings were thinned to one seedling per bag.

Upon germination, two leaves of each seedling and the parents were collected by hand, the midribs removed and stored in a refrigerator until DNA extraction. The DNA of the parents and plants derived from the crosses was extracted from young leaves by the hexadecyltrimethylammonium bromide (CTAB) buffer method as described by Doyle and Doyle (1990), associated to polysaccharide purification as proposed by Cheung et al. (1993). For the RAPD analysis, the methodology proposed by Williams et al. (1990) was used at the following concentrations: genomic DNA (30 ng), 10X buffer, MgCl2 (2 mM), dNTPs (2.5 mM each base), primer (5 pmol), Taq DNA polymerase (1 unit) and ultra pure water to complete 25 µL. The amplifications were performed under the following conditions: one cycle at 94 ºC for 3 minutes, a cycle of 94 ºC for 10 seconds, 35 °C for 30 seconds and 72 ºC for 1 min, repeated 40 times. A final cycle at 72 ºC for 7 minutes was added. A total of thirty-eight RAPD primers were randomly amplified.

The amplification products were separated by electrophoresis in 1.2% agarose gel at 5 V cm^{-1} in 1x TBE buffer and stained in a 0.05 mg mL^{-1} ethidium bromide solution for three hours. The amplified fragments were photographed under UV light by a UVP Life Science Camera.

The band patterns were interpreted based on the principle that bands generated by a same primer and in the same relative position indicate amplification of the same DNA fragment, in other words, they belong to the same gene locus. On the other hand, bands in different relative positions belong to different loci. The primers were tested first for the parental genotypes. The primers with bands in the male parent and none in the female parent were chosen for the amplification of DNA from plants of the crosses. The accessions with the same bands in the male parents were confirmed as true hybrids.

RESULTS AND DISCUSSION

The fruits of crosses between sugar apple accessions and atemoya, with the female parents P_2, P_3, P_4 and G_1 and

the seedless male parent Seedless P_1 all contained seeds, in variable amounts (Table 1). This result indicates that the pollen grains originating from accession Seedless P_1 are viable. In the fruits of reciprocal crosses however, none contained seeds. The cross between Seedless P_1 x G_1 produced no fruits (Table 1). Of the 22 flowers of accession Seedless P_1 covered at the pre-female stage, no fruit formation was observed either.

Table 1. Crosses, total number of harvested fruits (TNHF), total number of seeds (TNS), mean number of seeds per fruit (MNSF) and number of plants analyzed (NPA) per cross between Annonaceae accessions and atemoya hybrid

Crosses*	TNHF	TNS	MNSF	NPA
P2 x *Seedless* P1	33	2135	70	35
P3 x Seedless P_1	48	2846	92	46
P4 x Seedless P_1	17	667	34	12
G1 x Seedless P_1	10	513	20	9
Seedless P_1 x P2	6	0	0	0
Seedless P_1 x P3	11	0	0	0
Seedless P_1 x P4	3	0	0	0
Seedless P_1 x G1	0	0	0	0

* Seedless P_1, seedless sugar apple accession, P2, P3 and P4 sugar apple accessions with seeds and G1 atemoya cultivar.

According to Varoquaux et al. (2000), a plant can be considered seedless if it is capable of producing seedlesss fruit, fruits with traces of aborted seeds or a very small number of seeds. There are different types of mechanisms that result in seedlessness, among them parthenocarpy and stenospermocarpy. Parthenocarpy may occur in cases where the ovary is able to develop without fertilization of the oosphere. In other situations parthenocarpy can be optional, depending on the plant fertility. If the plant is sterile, parthenocarpy occurs without any external stimulus and will require a method of vegetative propagation, as in the case of banana and pineapple. Besides, parthenocarpy can be associated with environmental factors such as temperature, light or physical and chemical treatments of the flowers, which can inhibit fertilization (Varoquaux et al. 2000). A species may also be seedless due to stenospermocarpy. In this case, the fruits contain partially formed seeds that were aborted after fertilization (Stout 1936).

Couceiro (1983) reports that the seedless sugar apple originated from a somatic mutation and that it produces parthenocarpic fruit. According to the results of this study the absence of seeds in accession Seedless P_1 is not necessarily associated with parthenocarpy. Of the 22 protected flowers, none had the ability to produce fruits without natural pollination. In view of the results and observations made so far, studies on this issue should be conducted to determine the mechanism responsible for the absence of seeds in *Annona squamosa*.

The cross P_2 x Seedless P_1 produced 33 fruits, with an average of 64 seeds per fruit. From the cross P_3 x Seedless P_1 48 fruits were collected, with a total of 2846 seeds and a mean of 59 seeds per fruit. From the cross P_4 x Seedless P_1, 17 fruits were collected, totaling 667 seeds, with an average of 39 seeds per fruit. The lowest number of fruits was harvested from the cross between G_1 x Seedless P_1, with 10 fruits and 513 seeds, and an average 51 seeds per fruit (Table 1).

In reciprocal crosses where the seedless sugar apple accession Seedless P_1 was used as the female parent, fruits were only found in crosses between accessions of the same species, *Annona squamosa*, although without any seeds, which confirmed the seedlessness trait in the accession Seedless P_1 (Table 1).

Twenty-eight primers were used to characterize the sugar apple accessions and the atemoya cultivar Gefner used as parents in the crosses. In nine primers no polymorphic band was found, while in the others it was possible to observe at least one polymorphic band between at least two genotypes (Table 2). These results may be useful at a more advanced stage of the Annonaceae breeding program when aiming to obtain molecular markers linked to the gene(s) that determine(s) the traits seed presence or seedlessness in Annonaceae fruits. To obtain these molecular markers the first step is the search for primers that will generate fragments (bands) that differentiate the parents of the cross (Dirlewanger et al. 2004, Francia et al. 2005, Steele et al. 2006; Collard and Mackill 2008). Polymorphism was detected in 19 primers tested among sugar apple accessions and the atemoya cultivar and may be considered candidates to obtain possible molecular markers. In grapevines, the production of seedless fruits is controlled by three recessive genes, inherited independently and controlled by a dominant regulator gene (*I*). Lahogue et al. (1998) identified a RAPD marker, later converted into a SCAR marker, linked to the gene; they used it to distinguish plants with and without seeds in a progeny. This marker is being used successfully in table grape breeding programs for assisted selection for the seedlessness trait (Revers et al. 2006).

The primers that detected polymorphism between the female parent and male parent, and where the band

Table 2. Primers used to characterize accessions of sugar apple without seeds (Seedless P1), sugar apple with seeds (P2, P3 and P4) and the atemoya cultivar Gefner (G1) with the respective sequences and the number of amplified and polymorphic bands

Primer	Sequence 5'→3'	Number of amplified bands					Number of polymorphic bands
		Seedless P1	P2	P3	P4	G1	
OPB10	CTGCTGGGAC	10	10	7	9	10	3
OPC07	GTCCCGACGA	18	14	17	18	18	4
OPC14*	TGCGTGCTTG	17	17	-**	17	10	7
OPF10*	GGAAGCTTGG	8	10	8	9	8	3
OPG058	CTGAGACGGA	10	10	9	10	9	1
OPH11	CTTCCGCAGT	14	14	16	14	21	7
OPH18	GAATCGGCCA	16	15	17	16	18	3
OPI12	AGAGGGCACA	14	14	-	14	17	3
OPM06*	GCCACACACT	8	10	6	10	11	5
OPN11	TCGCCGCAAA	9	9	12	-	12	3
OPP14	CCAGCCGAAC	10	10	6	10	10	4
OPQ19	CCCCCTATCA	5	5	5	4	10	5
OPR11	GTAGCCGTCT	10	10	9	10	11	2
OPS15	CAGTTCACGG	9	8	10	10	8	2
OPT20	GACCAATGCC	11	12	10	10	12	2
OPU06	ACCTTTGCGG	9	5	6	-	10	7
OPV15	CAGTGCCGGT	12	12	7	-	11	6
OPX07	GAGCGAGGCT	13	11	10	-	13	4
OPZ18*	AGGGTCTGTG	14	14	15	14	16	2

* = primers with a polymorphism level that allowed hybrid identification; ** = no information.

was observed in the male parent were used to verify true hybrids in the populations. Two primers were selected for each cross. The primers Z18 and F10, F10 and G05, M06 and F10, F10 and C14 were selected for the evaluation of the progenies of the following crosses: P_2 x Seedless P_1, P_3 x Seedless P_1, P_4 x Seedless P_1 and G_1 x Seedless P_1, respectively. According to Faleiro et al. (2003) the use of one or two primers or primer combinations with at least one informative band is enough to confirm or refute the occurrence of hybridization.

The percentage of hybrid formation was highest in the crosses P_4 x Seedless P_1 and P_3 x Seedless P_1, with 100% and 95.55% true hybrids, respectively (Table 3). The cross P_2 x Seedless P_1 (Figure 1) produced 82.86% true hybrids. The RAPD marker analysis showed 29 informative bands with both primers in the 35 plants analyzed. Only three plants were not identified as true hybrids by both primers, and the other three showed an informative band in only one of the primers, one with primer OPF10 and two with OPZ18.

The lowest percentage (44.44%) of true hybrids was recorded for the cross between the atemoya cultivar and the seedless sugar apple accession (G_1 x Seedless P_1), (Table 3). Of the nine plants analyzed, four were confirmed as true hybrids by the two primers, three plants were identified with the OPF10 primer and two only with primer OPC14. Atemoya is an interspecific hybrid from a cross between *Annona squamosa* and *Annona cherimola* (Bonaventure 1999), with characteristics of both species. Studies by several authors showed that this species can be artificially pollinated using pollen grains of atemoya or sugar apple (Melo et al. 2002, Pinto et al. 2005). Mansour (1997) reports that in spite of the protogynous dichogamy the species of the Anonaceae family have a period of 2 to 3 hours during which the stigma is still receptive even after anthesis, especially when the humidity is low. This

Table 3. Percentage of intra and interspecific Annonaceae hybrids confirmed by RAPD markers

Crosses	Number of evaluated plants	Number of true hybrids	Percentage of true hybrids
P_2 x Seedless P_1	35	29	82.86
P_3 x Seedless P_1	45	43	95.55
P_4 x Seedless P_1	12	12	100.00
G_1 x Seedless P_1	9	4	44.44
Total	**101**	**88**	**87.13**

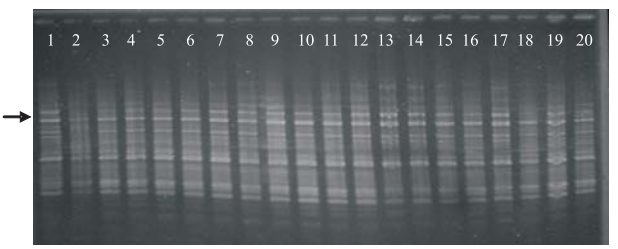

Figure 1. Amplification product with primer OPF10. Columns 1 and 2 contain parent DNA of Seedless P_1 and P_2, respectively, followed by the F_1 plants (3 to 20) derived from the cross between P_2 x Seedless P_1. The arrow indicates the polymorphic band used to verify the true hybrids.

may have been one of the factors that led to the formation of fruits that were not identified as true hybrids, whether in the intra nor interspecific crosses.

Primer OPF10 was shown to be effective in detecting polymorphic bands in all Annonaceae hybrid populations. According to Oliveira et al. (2003), certain hybrids can be considerably different from the parents or very close to them, but hybrids tend to have intermediate characteristics, due to the type of gene inheritance involved.

Samples of the possible parents were included in the analysis. This procedure is important because of the possibility of intra-specific polymorphism. According to Cordeiro et al. (2006) the RAPD marker was efficient and provided information on the genetic similarity of mango varieties, producing specific DNA fragments, which is important in the identification of hybrids. Valente et al. (2005) detected polymorphic loci in the *Arachis* germplasm using RAPD markers and concluded that these are simple and efficient for the characterization of the *Arachis* germplasm and for the characterization of hybrid progenies in crosses between *Arachis hypogaea* L., the cultivated peanut, and synthetic amphidiploids. Furthermore, the RAPD marker was recommended for distinguishing genotypes with the same genetic background in *Coffee arabica* (Teixeira-Cabral et al. 2002, Dias et al. 2005).

Some studies indicated that the hybrid or nuclear nature of morphologically selected tangerine and 'Pêra' sweet orange plants can be confirmed by RAPD markers, selecting the primers OPG8, OPG13 and OPG19. The use of RAPD markers makes an early and efficient selection of tangerine and 'Pêra' sweet orange plants possible with a high probability of being hybrids, which can also be confirmed by morphological traits (Oliveira et al. 2003).

The RAPD marker was found to be an excellent tool for the verification of true hybrids of crosses between Annonaceae genotypes. This technique can also be helpful to identify possible parents of hybrids of unknown origin, or to study the genetic variability to indicate crosses between heterotic groups of interest.

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Identificação de híbridos de cruzamentos intra e interespecíficos em Anonáceas por marcadores RAPD

Resumo - *O objetivo do presente trabalho foi identificar híbridos em cruzamentos intraespecíficos entre acessos de pinheira e interespecíficos entre acessos de pinheira e híbrido de atemoieira por meio do uso dos marcadores RAPD. Foram selecionados quatro acessos de pinheira (*Seedless P_1, P_2, P_3 e P_4*) e um cultivar híbrido de atemoia 'Gefner' (G_1). As flores nos estádios de pré-fêmea foram devidamente protegidas sendo realizados cruzamentos recíprocos. Nos cruzamentos onde os acessos de pinha sem semente (*Seedless P_1*) foi utilizado como genitor masculino foram obtidos frutos com semente, indicando que os grãos de pólen do acesso* Seedless P_1 *são viáveis. Nos cruzamentos recíprocos, em que o acesso* Seedless P_1 *foi utilizado como genitor feminino todos os frutos obtidos não apresentaram sementes. O percentual de híbridos verdadeiros nos cruzamentos $P_4 x$* Seedless P_1, $P_3 x$ Seedless P_1, $P_2 x$ Seedless P_1 *e $G_1 x$* Seedless P_1 *foram 100%, 95.55%, 82.86% e 44.44% respectivamente. O* primer *OPF10 mostrou ser eficiente na obtenção de bandas polimórficas em todas as populações de híbridos de anonáceas.*

Palavras-chave: *Annona squamosa*, *Annona cherimola* x *Annona squamosa*, frutos sem sementes, marcadores moleculares.

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