ARTICLE

Molecular marker-assisted selection for seedlessness in atemoya breeding

Bruno Rafael Alves Rodrigues[1], Silvia Nietsche[2*], Samy Pimenta[1] and Marlon Cristian Toledo Pereira[1]

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Abstract: Abstract - *Perennial plant breeding is an expensive and time-consuming process, mainly due to the extended growth time for juveniles. In these cases, the use of molecular marker-assisted selection (MMAS) allows for the selection of a characteristic of interest at the seed or seedling stages. The objective of this work was to characterize the segregation of the INO locus and to use MMAS for the early selection of seedless genotypes of atemoya 'Gefner' [(G; Annona cherimola Mill. × Annona squamosa L.) × Brazilian seedless (Bs; Annona squamosa)]. After primer validation and MMAS, 24 plants of the F_2 population were selected and designated as candidate genotypes for the absence of seeds, as INO alleles were absent in them. For further studies on breeding programs for this species, 53 heterozygous seedlings were considered as genetic resources during selection.*

Keywords: *Annona cherimola × Annona squamosa, INO locus, inheritance study, seedless fruits, breeding of fruit trees*

INTRODUCTION

The use of conventional hybridization methods for the development of new cultivars of perennial plants, such as Annonaceae, is laborious, time-consuming, expensive, and requires a large space (McClure et al. 2014). The breeding of these species involves the generation of segregant populations, and these plants can take years to produce fruits because of extended juvenile stage (Van Nocker 2014).

Brazilian seedless (Bs) is a mutant of *Annona squamosa* with a stenospermocarpy phenotype and was originally identified in northeast Brazil. It was first described in 1940 in the state of São Paulo and was used for intraspecific crossings to develop new seedless sugar apple cultivars with combined desirable quality attributes (Cunha 1953, Nassau et al. 2023). Interspecific hybridizations were also carried out between the mutant Bs and the atemoya 'Gefner' (*A. cherimola* Mill. × *A. squamosa* L.) (Souza et al. 2010, Pereira and Borém 2021).

Molecular marker-assisted selection (MMAS) for perennial plants is an attractive tool, as it allows for selection at the seed or seedling stage, considering the time required to complete one breeding cycle for these species when using conventional breeding methodologies (Collard et al. 2005). MMAS increases the efficiency of incorporating desirable characteristics present in the wild germplasm into domesticated or elite cultivars (Migicovsky and Myles 2017). In addition, MMAS is important at the beginning of the breeding process because

***Corresponding author:**
E-mail: silvia.nietsche@gmail.com
ORCID: 0000-0002-8188-3057

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[1] Universidade Estadual de Montes Claros, Campus Universitário Prof. Darcy Ribeiro, Avenida Prof. Rui Braga, s/n, Vila Mauriceia, 39401-089, Montes Claros - MG, Brazil
[2] Universidade Federal de Minas Gerais - Instituto de Ciências Agrárias, Avenida Universitária, 1000, Universitário, 31270-901, Montes Claros - MG, Brazil

it decreases the number of descendants required for evaluation and accelerates the time to generate a new cultivar.

A related study showed the molecular basis for the absence of seeds in Thai seedless (Ts), an *A. squamosa* cultivar, resulting in the development of a set of LMINO primers (Lora et al. 2011). The use of LMINO primers in the mutant Bs were validated in another study, justifying the molecular basis for *INO* locus deletion (Nassau et al. 2023). The authors also suggested that the inheritance of the presence/absence of seeds in *A. squamosa* is probably controlled by only a single dominant gene, whereas the recessive condition determines the absence of seeds in fruits. These findings provide important information with potential implications for the breeding of other Annonaceae species.

Considering these important results on molecular characterization and the inheritance study carried out with mutant Bs, the objective of the present work was to use MMAS strategies associated with the *INO* locus for the early selection of individuals for the presence/absence of seeds in a segregating population (F_2) of 'Gefner' × Brazilian seedless atemoya progenies.

MATERIAL AND METHODS

Experiment location

Field, greenhouse, and laboratory experiments were conducted at the Department of Agricultural Sciences (lat 15º48'09''S, long 43°18'32''W, alt 516 m)of the State University of Montes Claros (UNIMONTES) in the municipality of Janaúba, Semiarid region of the state of Minas Gerais, Brazil. The region has an Aw-type climate, (dry winter with wet tropical savanna climate) according to the Köppen classification, with a mean temperature of 22 °C in the winter and 24.4 °C in the summer (Sá Júnior et al. 2012).

Genetic material and population segregation

The parents selected for the present study were: atemoya 'Gefner' (G), a genotype characterized by the presence of seeds in their fruits, which was used as the female parent (♀), and the mutant Brazilian seedless (Bs), characterized by the absence of seeds in their fruits, which was used as the male parent (♂).

Hybrids (F_1, G × Bs) were obtained through artificial pollination, as described by Souza et al. (2010). In 2011, 28 F_1 genotypes were transplanted into the experimental area on the UNIMONTES farm. The segregating population (F_2) was obtained during the 2018 crop season (May to December) from hybrids (F_1) selected in the field (Table 1). Species of the genus *Annona* exhibit protogynous dichogamy; hence, seeds of the F_2 population were obtained through geitonogamy of F_1 plants, that is, hand pollination between different flowers of the same plant. Artificial hybridization, seed extraction, and seedling production were performed following the methodology described by Pereira et al. (2003).

Table 1. Number of seeds planted, number of seedlings formed, and percentage of emergence of plants (F_2) from May to December 2018 through geitonogamy of F_1 plants from the crossing between atemoya 'Gefner' × 'Brazilian seedless'

Identification of plants (F_1)	**Seeds** (F_2, n)	**Seedlings** (F_2, n)	**Emergence** (F_2, %)
UNI - PL 01	84	23	27.4
UNI - PL 02	82	3	3.7
UNI - PL 03	160	16	10
UNI - PL 05	91	13	14.3
UNI - PL 11	65	16	24.6
UNI - PL 12	22	2	9.1
UNI - PL 16	70	2	2.9
UNI - PL 20	41	2	4.9
UNI - PL 21	81	10	12.3
UNI - PL 25	7	1	14.3
UNI - PL 30	26	3	11.5
UNI - PL 34	43	5	11.6
Total	772	96	12.2[1]

[1] Mean

Molecular analysis

Samples of young leaves were collected from adult plants of the F_1 population established in the field and from seedlings of the F_2 segregating population in the greenhouse (Table 1). The total number of seedlings obtained for the F_2 population differed from the number of plants analyzed due to the loss of samples during the experimental phases and molecular analyses.

DNA extraction was carried out with hexadecyltrimethylammonium bromide buffer, as described by Doyle and Doyle (1990), combined with the purification of polysaccharides, as proposed by Cheung et al. (1993). DNA quantity was estimated using a

spectrophotometer (UV-1650PC, Shimadzu) at 260 nm. DNA concentrations were estimated using the following equation: [DNA] = 50 µg mL^{-1} × DO260 × dilution factor, where 1 DO = 50 µg mL^{-1} of DNA. Samples were then diluted and standardized to 10 ng of DNA per µL

The assisted selection procedure was carried out by selecting primers of the LMINO dominant type to amplify a region or sequence immediately flanked by the *INO* locus related to the presence of seeds. AsINODel L/R primers were used to amplify a deletion fragment of length 456 bp of the *INO* gene in the mutant Hawaii seedless (*Hs*) (Table 2). The combined use of LMINO and AsINODel L/R primers in a single reaction enabled a codominant test for *A. squamosa* wild and mutant seedless genes. In the present study, these primers were validated for populations obtained from the atemoya cultivar 'Gefner' and the *A. squamosa* Bs and Hs genotypes, which may or may not carry the *INO* locus.

Table 2. List of oligonucleotide primers their sequences and sizes(bp)

Primers	Sequence of the initiator (5' - 3')	bp
F LMINO1 R LMINO2	CCTAAATGAAGGGTTTACATGTGGC GCCCACCTTCATTTGCTCCTTGG	350
AsINODel L AsINODel R	AAACCAAGAGCTGGAAGCAG TGCATGTACGGGAACATCAT	456

LMINO1/2: Lora et al. (2011); AsINODel L/R: Charle S. Gasser (UCLA, Davis, USA; unpublished data, 2022).

Polymerase chain reaction (PCR) was carried out as follows: 25 ng μL^{-1} of DNA was prepared for each sample, and PCR was carried out with molecular markers. The solution volume for each sample was 25 µL, with KCl 50 mM, Tris-HCl 20 mM (pH 8.5), $MgCl_2$ 3.0 mM, dNTPs 0.2 mM, 0.4 mM of each primer, 2.5 µL of genomic DNA, 1.0 unit of DNA Taq Polymerase, and autoclaved ultrapure water.

The amplifications were carried out in a thermocycler (Techne, TC-412) using a program under the following conditions: initial denaturation at 94 °C for 3 min, followed by 35 cycles at 94 °C for 30 s, girdling temperature of 60 °C for 30 s for each primer, and extension at 72 °C for 1 min and 30 s, followed by another cycle at 72 °C for 4 min, followed by a cycle at 4 °C until the removal of the samples from the thermocycler. The resulting product from the amplifications was separated by electrophoresis on a 1.2% agarose gel (m v^{-1}) stained with ethidium bromide solution (0.2 mg L^{-1}) and conducted in TBE 1× buffer (89 mM Tris-based, 89 mM boric acid, 2 mM EDTA, pH 8.0) at 80 V for approximately 2 h. The amplified fragments were analyzed under ultraviolet light and photographed using a digital system (UVP, Life Science Software).

Statistical analysis

The chi-square (χ^2) test was used to confirm the segregation of markers, testing the 1:2:1 hypothesis (dominant homozygote: heterozygote: recessive homozygote) due to the codominant nature of the markers. The tests were carried out at a 5% significance level, with the aid of the statistical software Genes (Cruz 2016).

RESULTS AND DISCUSSION

The phenotype analysis results showed that all F_1 plants from the G × Bs crossing produced fruits with seeds, as did the female parent the atemoya cultivar 'Gefner', whereas the mutant Bs, the male parent, produced seedless fruits. Additionally, molecular analysis confirmed that the LMINO primers amplified only a single sharp fragment of 350 bp, only in the parent G, whereas AsINODel primers allowed for the reproduction of a 456 bp fragment in the mutant Bs and the control genotype (Hs), confirming that the combined use of these primers in the present study showed codominant dynamics and polymorphism for parents G and Bs (Figure 1). The primers tested in F_1 thus showed codominant dynamics, as all F_1 genotypes analyzed were heterozygous, presenting 456 bp and 350 bp fragments (Figure 2).

The results obtained from phenotypic characterization and primer validation in the atemoya 'Gefner' and the F_1 population originated from crossing with the parent Bs confirmed the Mendelian segregation ratio of 1:0 (presence: absence of seeds). First, the inheritance of seedlessness seems to be governed by only a single recessive gene, as all plants of the F_1 population produced fruits with seeds and the genotypic results corresponded perfectly to the phenotypic expectations. Second, the results are consistent with those obtained in the studies on *A. squamosa* by Nassau et al. (2023), who also found that the plants of the three F_1 population (Bs x M_1, Bs x M_2 and Bs x M_3) also produced fruits and they suggested that the presence or absence of seeds is probably governed by only a single gene with an allelic interaction of complete dominance.

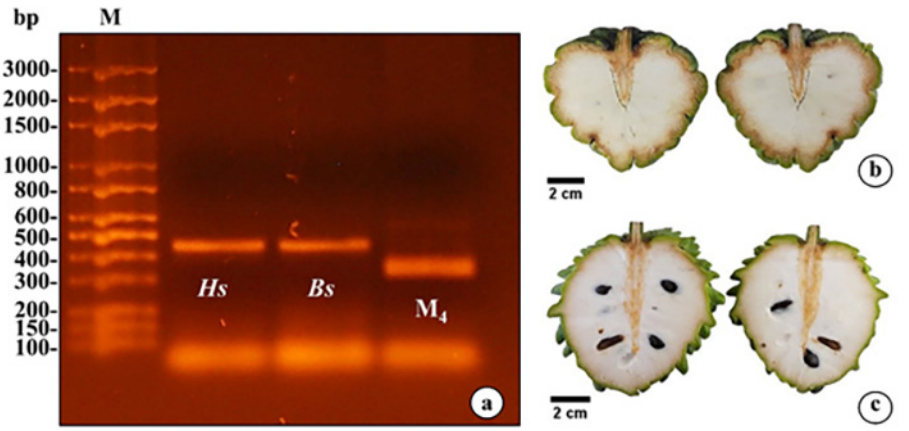

Figure 1. a) Amplification products using the primer pairs LMINO1/2 and AsINODel L/R obtained from DNA samples of different genotypes (Hs: Hawaii seedless, Bs: Brazilian seedless and M4: atemoya Gefner) on agarose gel (1.2%) in TBE 1× buffer. M: molecular weight marker on a linear scale from 100 to 3000 bp; Longitudinal sections of fruits of: b) *Annona squamosa*, 'Brazilian seedless' (Bs) and c) Atemoya 'Gefner' (G). Hs: 'Hawaii seedless'.

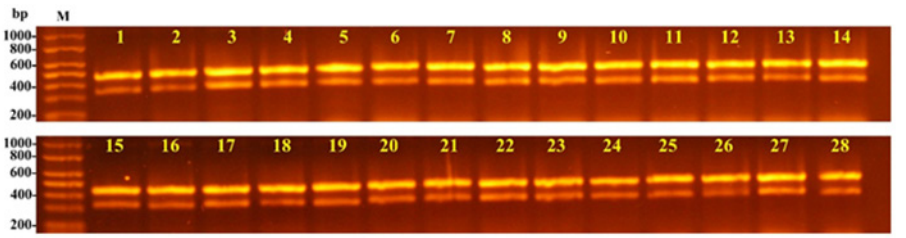

Figure 2. Amplification products using the primer pairs LMINO1/2 and AsINODel L/R obtained from all DNA samples of F_1 hybrid genotypes from G × Bs crossing, identified as 1 to 28 on agarose gel (1.2%) in TBE 1× buffer. M: molecular weight marker on a linear scale from 200 to 1000 bp.

Validation of primers and identification of segregation patterns enabled the application of MMAS to identify genotypes that probably carry the specific traits; however, we focused on the deletion of the *INO* locus to generate a seedless-fruit phenotype at the adult stage for efficient incorporation and conduction of this trait in breeding programs for Annonaceae species (Akkurt et al. 2013, Nassau et al. 2023).

Validated markers in the F_2 population were used to evaluate allelic segregation, with subsequent analysis of assisted selection from the genotype of the seedlings. Ninety-two seedlings were evaluated: 15 were homozygous dominant (16.3%, *INO INO*), with amplification of only the 350 bp band; 24 were homozygous recessive (26.1%, *ino ino*), with amplification of only the 456 bp band; and the other 53 seedlings were heterozygous (57.6%, *INO ino*) and, therefore, presented both bands (350 and 456 bp) for the *INO* locus (Figure 3).

Segregation analysis of molecular markers showed that the observed ratios were consistent with those expected for Mendelian segregation for a single locus 1:2:1 [$\chi^2 = 0.389$ ($P = 0.14$)]; the dominant and the recessive alleles were responsible for the presence and absence of seeds, respectively (Table 3).

The utility of molecular markers to select important agronomic characteristics at the seedling stage has been demonstrated for several perennial crops, including papaya (Dillon et al. 2006, Oliveira et al. 2010), apple (Bassett et al. 2015), cocoa (Royaert et al. 2011), banana (Umber et al. 2016), and coffee (Alkimim et al. 2017). For example, in coconut, genotyping of a single marker can help distinguish tall from dwarf plants at the seedling stage, which is useful for the breeding of this species (Rajesh et al. 2013). Similarly, markers related to disease resistance genes are currently used on a large scale to discard susceptible seedlings at the initial developmental stages in several breeding programs (Di Gaspero and Cattonaro 2010).

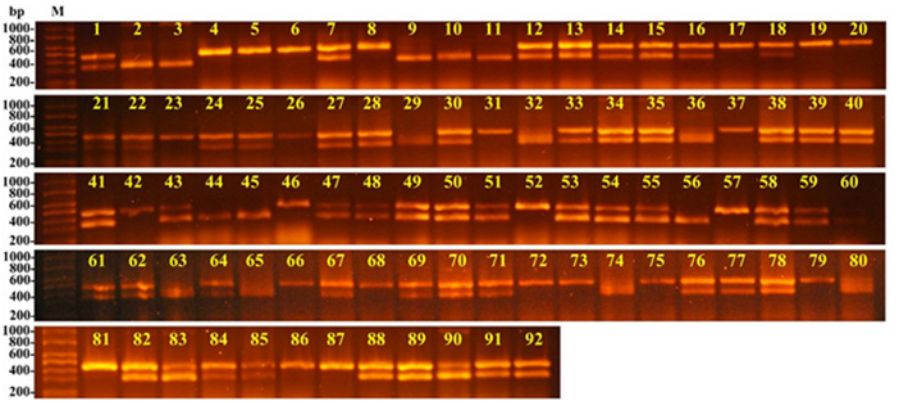

Figure 3. Amplification products using the primer pairs LMINO1/2 and AsINODel L/R obtained from all DNA samples of the F_2 segregating population, identified as 1 to 92 on agarose gel (1.2%) in TBE 1× buffer. M: molecular weight marker on a linear scale from 200 to 1000 bp. *INO INO*: 2, 3, 9, 10, 11, 29, 32, 36, 44, 45, 56, 63, 65, 74, 80. *ino ino*: 4, 5, 6, 8, 17, 19, 20, 23, 26, 31, 37, 42, 46, 52, 57, 66, 68, 72, 73, 75, 79, 81, 86, 87. *INO ino*: 1, 7, 12, 13, 14, 15, 16, 18, 21, 22, 24, 25, 27, 28, 30, 33, 34, 35, 38, 39, 40, 41, 43, 47, 48, 49, 50, 51, 53, 54, 55, 58, 59, 60, 61, 62, 64, 67, 69, 70, 71, 76, 77, 78, 82, 83, 84, 85, 88, 89, 90, 91, 92.

The use of markers also allowed the selection of homozygous recessive seedlings, with a decrease of approximately ¾ of the population (from 92 to 24 plants), representing almost 26% of those originally obtained. This result, obtained through MMAS, is important because 24 plants [PL01 (9, 28, 38); PL2-17; PL3 (4-6, 8); PL5 (28, 32, 45, 78); PL11 (17, 21, 22); PL16-7; PL21 (59, 62, 77); PL25-2; PL30 (10, 11); and PL34 (13.15)] were designated as candidate genotypes for the development of a seedless cultivar and were already selected to be proceeded to the second stage of the breeding program (Table 1 and Figure 3). These genotypes could be vegetatively propagated and grown for field evaluation of agronomic characteristics and production potential, in addition to the characteristics of seminal rudiments.

Table 3. Molecular analysis of codominant segregation of the parents atemoya 'Gefner' (G) and 'Brazilian seedless' (Bs) and the F_1 and F_2 populations

Parent/ generation	Observed ratios			Expected ratio	χ^2	*P*-value
	INO INO	*INO ino*	*ino ino*			
G	1	0	0			
Bs	0	0	1			
F_1	0	28	0	0:1:0	-	-
F_2	15	53	24	1:2:1	3.8913	0.1429

χ^2 estimated using the chi-square test.

Molecular markers related to the absence of seeds were also identified for some species of agronomic interest in studies carried out recently. For example, in grapevines, markers connected to stenospermocarpy, determined mainly by the presence of the dominant allele in the SDI locus (Bouquet and Danglot 1996), have been obtained (Lahogue et al. 1998, Mejía and Hinrichsen 2003, Cabezas et al. 2006) and tested using MMAS (Karaagac et al. 2012, Akkurt et al. 2013). Jinping et al. (2009) identified two SCAR markers connected to the seedless trait in Ponkan tangerines (*Citrus reticulata* Blanco), and Chavez and Chaparro (2011) identified four markers connected to the stenospermocarpy locus in *Citrus kinokuni* through bulk segregant analysis (BSA).

Although the recessive genotypes selected were the most interesting genotypes for fruit tree breeding programs, 53 heterozygous genotypes for the *INO* locus were identified, representing almost 58% of the total plants. This differentiation between heterozygotes and dominant homozygotes is important for selection because this distinction of dominant traits is not possible with phenotypic selection. Thus, determining heterozygosity is important and should be considered during selection, crossing, and advancement of generations, as no self-pollination or test crossing is required to detect traits controlled by recessive alleles (Jiang 2013).

Finally, MMAS for obtaining seedless genotypes using primers for the *INO* locus allowed for screening at the seedling stage, identifying progenies that may be characterized by the absence of seeds, thus reducing the time, space, and resources for growing other plants. This is important because, under specific crop conditions, it takes at least three years after sowing of seeds for flowering to begin in adult plants (Pereira and Borém 2021). Therefore, the results of this study can be considered a satisfactory contribution to breeding programs to generate new seedless cultivars.

CONCLUSIONS

The molecular markers (LMINO and AsINODel primers) allowed for the distinction of homozygous from heterozygous genotypes for seedlessness traits in atemoyas.

Marker-assisted selection identified candidate genotypes for the development of a seedless atemoya cultivar.

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