Seedless fruit in *Annona squamosa* L. is monogenic and conferred by *INO* locus deletion in multiple accessions

Bruno Rafael Alves Rodrigues[1], Charles S. Gasser[2], Samy Pimenta[1], Marlon Cristian Toledo Pereira[1] and Silvia Nietsche[3]*

[1] State University of Montes Claros, Department of Agricultural Sciences, CEP 39401-369, Janaúba, MG, Brazil
[2] Dept. of Molecular and Cellular Biology, University of California, Davis, Davis, CA 95616 USA
[3] Federal University of Minas Gerais, Institute of Agricultural Science, CEP 39404-547, Montes Claros, Minas Gerais, Brazil
*Author for correspondence: silvia.nietsche@gmail.com

Running head: Inheritance of seedless fruit in *A. squamosa*

Summary. Understanding the genetic basis and inheritance of a trait facilitates the planning of breeding and development programs of new cultivars. In the sugar apple tree (*Annona squamosa* L.), the mechanism of the desirable seedless trait in the Thai seedless (Ts) and Brazilian seedless (Bs) accessions was associated with a deletion of the *INNER NO OUTER* (*INO*) locus. Genetic analysis of F_1, F_2 and backcross descendants of crosses of Bs to fertile wild-type varieties showed that seedlessness was recessive and monogenic. Whole genome sequencing of a third accession, Hawaiian seedless (Hs), identified a 16 kilobase deletion including *INO*. The finding of an identical deletion in Ts and Bs indicated a common origin among genotypes, from a single deletion event. Analysis of microsatellite markers could not preclude the possibility that all three accessions are vegetatively propagated clones. The sequence of the deletion site enabled formulation of a codominant assay for the wild-type and mutant genes that validated the *INO* gene deletion as the cause of seedless trait, and can be used in the selection of new seedless varieties. The study findings and obtained progenies should be useful in breeding and introgression programs of the trait into elite sugar apple lines and into other *Annonas* by means of interspecific crossings.

Introduction

The global fruit market has grown appreciably in the past decade. The absence of seeds from fruit that is consumed has become one of the most appreciated traits by consumers. Reducing seed content without changing the size of the fruit is one of the main objectives of the cultivation of many fruit trees. The goal is to enhance the experience of fruit consumption by consumers and improve the quality of fruits for food processing (Varoquaux et al., 2000).

Sugar apple (*Annona squamosa* L.) (2n = 14) is a tropical fruit species in the family Annonaceae. Seedless fruits have been described for some spontaneous mutants of *A. squamosa* whose origin is still unknown (Pinto et al., 2005; Wongs-Aree and Noichinda, 2011; Lora et al., 2018; Pereira and Borém, 2021). They include the Cuban cultivar Cuban seedless (Cs), with good fruit characteristics but lower productivity than fertile cultivars (Araújo et al., 1999); Brazilian seedless (Bs) originally identified in northeast Brazil, which produces small, asymmetric fruits that frequently perish (Santos, 2014); the Thai seedless (Ts) mutant that produces normal size fruits (Lora et al. 2011) among other types with apparently similar fruits, such as in the Philippines and Hawaii (Pinto et al., 2005; this work).

Lora et al. (2011) were among the first researchers to examine details of the seedless trait in *A. squamosa* in Ts. This variety produces fruit following pollination and fertilization. The authors demonstrated that seedlessness results from a defect in ovule development where the outer of the two integuments sheathing the nucellus fails to form. This defect directly mirrors the effect of *INNER NO OUTER* (*INO*) loss of function mutants in *Arabidopsis thaliana* (Villanueva et al., 1999). *INO* encodes a putative transcription factor belonging to the *YABBY* family. Members of this family are involved in the determination of abaxial identity in a variety of plant organs (Sawa et al., 1999; Siegfried et al., 1999; Bowman, 2000). Lora et al. (2011) isolated an *A. squamosa INO* ortholog and demonstrated the association of

the Ts mutant with an apparent deletion of the *INO* gene, indicating a candidate gene for the seedless trait.

The Bs variety was also evaluated in a breeding program undertaken at the State University of Montes Claros. Results showed that the absence of seeds was also associated with failure in the development of the external integument of the ovule and in the development of seeds, similar to that observed in Ts (Mendes et al., 2012; Santos et al., 2014). In addition, preliminary studies of inheritance of the stenospermocarpic absence of seeds involving F_1 progenies in Bs (*A. squamosa* wild type x Bs) also indicated the same expected recessive nature for the mutation as in Ts (Nassau et al., 2021). However, it was not known whether the molecular basis of the *INO* deletion described is widespread in *A. squamosa* and may be responsible for the case of aspermia described in Bs.

The present study addressed the following questions: (i) Is the inheritance of the presence/absence of seeds in *A. squamosa* monogenic?; (ii) What are the molecular details of the *INO* gene deletion?; (iii) Is there a complete correspondence of homozygosity for the deletion with the seedless phenotype; (iv) What is the relationship between the known seedless accessions, and (v) Can codominant molecular markers specific to *INO* be designed for use in assisted selection?

Materials and Methods

Plant material

Wild-types (fertile) M_1, M_2 and M_3 and seedless Bs lines were previously described by de Souza et al. (2010), and Ts was described by Lora et al. (2011). For whole genome sequencing a wild-type sugar apple fruit was purchased from a retail source in the United States, seeds from the fruit were planted, and one plant grown in the UC Davis Conservatory was sampled for sequencing with voucher herbarium samples stored as DAV225058 and

107 DAV225059. The Hawaiian seedless (Hs) line was obtained as budwood from Frankie's
108 Nursery (Waimanalo, HI), grafted to a wild-type *A. squamosa* rootstock and grown in the UC
109 Davis Conservatory with a voucher sample stored as DAV225060.

110 For genetic inheritance studies, plants (M_1, M_2, M_3 and Bs) were grown at the
111 Experimental Farm and molecular analysis were performed at Molecular Biology laboratory
112 of the State University of Montes Claros, latitude 15º48'09''S, longitude 43°18'32''W and
113 altitude 516 m. For phenotypic characterization of seedless versus seeded (fertile) two
114 strategies were applied: (a) fruits were harvested, pulped, and examined for the presence or
115 absence of seeds (Supplemental Figure S1); or (b) flowers either fresh or fixed in formalin
116 acetic acid-alcohol (FAA) were dissected to separate the ovules from the carpel tissue.
117 Isolated ovules were observed with a stereomicroscope (Nikon, SMZ800) and photographed
118 by a digital camera (Sony, Dsc-W350). The wild-type ovules present a domed shape opposite
119 the funiculs, while the mutant ovules come to a point at this position (Supplemental Figure
120 S2).

121 Filial generations (F_1), self-fertilization (F_2), backcrosses with wild-types parents M_1,
122 M_2, M_3 (BC_M), and backcrosses with mutant parent Bs (BC_{Bs}) were obtained. Segregations
123 were evaluated for conformity to predicted ratios with the Chi-square test (χ^2) using the Genes
124 statistical software (Cruz, 2016).

125
126
127 **Polymerase chain reaction (PCR) analysis**
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129 DNA was extracted from young leaf samples with hexadecyltrimethylammonium
130 bromide buffer (CTAB) as described by Doyle and Doyle (1990) and separated from
131 polysaccharides as described by Cheung et al., (1993). Primers used in PCR are listed in
132 Supplemental Table S1. PCR was performed with DreamTaq (ThermoFisher, Waltham, MA)
133 and the included reagents with an initial denaturation at 94ºC for 3 min; 35 cycles with

134 denaturation at 94ºC for 30 sec, annealing at 56°C for 30 sec, and extension at 72ºC for 1.5
135 min; and a final extension of 72ºC for 4 min. For reactions using the AsINODel primers a 60°
136 annealing temperature was used.

137 PCR products were electrophoresed on 1.2 % (w/v) agarose buffered with 1× TBE (89
138 mM Tris-base, 89 mM boric acid, 2 mM EDTA, pH 8.0) or SB (10 mM Sodium Borate pH
139 8.0) and DNA visualized by staining with ethidium bromide (2 µg/ml) an illumination with
140 ultraviolet light.

141 For sequencing, PCR products were processed with ExoSAP-IT (ThermoFisher,
142 Waltham, MA) or Quiapure (Quiagen, Germantown, MD) and sequenced using amplification
143 primers on an ABI 3500 or 3730 genetic analyzer (ThermoFisher, Waltham, MA) at Análises
144 Moleculares Ltda. (Centro de Biotecnologia, UFRGS, Porto Alegre, RS) or the University of
145 California Davis CBS DNA Sequencing Facility (Davis, CA).

146
147
148 **Whole genome shotgun sequencing**
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150 DNA for whole genome sequencing was isolated from young leaves by grinding in
151 100 mM TRIS-Cl, 20 mM EDTA, 1.4 M NaCl, 2% (w/v) CTAB, 1% each
152 polyvinylpyrrolidone and sodium metabisulfite pH 8.0. Samples were treated with 70 µg/ml
153 RNAaseA (ThermoFisher, Waltham, MA), extracted with 1:24 (v/v) mixture of isoamyl
154 alcohol and chloroform, and precipitated with isopropanol. Samples were dissolved in 10 mM
155 TRIS pH 8.0, 1 mM EDTA, adjusted to 0.3 M Na Acetate, pH 4.8, precipitated with 2
156 volumes of ethanol and dissolved in 10 mM TRIS pH 8. Wild-type *A. squamosa* DNA was
157 processed and sequenced at the University of California, Davis Genome Center (Davis, CA).
158 For PacBio (Menlo Park, CA) sequencing, DNA fragments greater than 10 kb were selected
159 by BluePippin (Sage Sciences, Beverly, MA) electrophoresis and were sequenced on a
160 PacBio RSII or Sequel Single Molecule, Real-time device. This resulted in 2.46 million reads

with an average read length of 8 kb comprising more than 29 Gbases, or approximately 37 X genome representation. For Illumina (San Diego, CA) sequencing the DNA was sheared and fragments of an average size of 400 bp were selected and sequenced on a HiSeq 4000 apparatus by the paired-end 150 bp method (PE150) resulting in approximately 390 million sequences. The sequences were trimmed of poor quality regions and primer sequences with Sickle (Joshi and Foss, 2011) and Scythe (Buffalo, 2011), respectively resulting in 229 Gbases or approximately 124 X genome representation. Hs DNA was similarly processed and sequenced by QuickBiology (Pasadena, CA) resulting in 408 million sequences and approximately 130 X genome representation.

Genome sequence assembly and analysis

The PacBio reads of wild-type DNA were assembled using Canu (Koren et al., 2017) with default settings, producing 3519 contigs. The wild-type Illumina reads were aligned with the assembly using BWA MEM (Li, 2013) with default settings, and Pilon (Walker et al., 2014) used the alignment to correct the contigs, changing 148k single nucleotide errors and adding a net of more than 1.8 Mbases of insertions for a final assembly of 707.7 Mbases with an average contig length of 201 kb and an N90 of 93.9 kb. A BLAST (Altschul et al., 1990) search with the known *A. squamosa INO* gene sequence (Lora et al., 2011) identified a 587 kb contig (tig00001115) containing the *INO* gene (Supplemental Figure S3). BWA MEM aligned the Hs Illumina reads with the assembly and Tablet (Milne et al., 2013) was used to examine the alignment with the 587 kb contig containing INO. BLAST was used to search one half of the set of Hs Illumina sequence reads for those extending across a detected deletion and the resulting sequences were aligned and assembled using Sequencher 5.4.1 (Gene Codes, Ann Arbor, MI).

Genetic diversity using SSR markers

Genetic diversity was assessed among varieties of seedless sugar apple Bs, Ts, and Hs, with the fertile parent M_2 as a contrasting control. Sixty-seven pairs of SSR microsatellite markers, described for *A. cherimola* were used, with fifteen having been described by Escribano et al. (2004) and fifty-two by Escribano et al. (2008).

DNA extraction was performed as described above for the markers association of seedless trait with INO deletion. Amplification utilized an initial denaturation at 94ºC for 1 min; 35 cycles at 94ºC denaturation for 30 sec, annealing at 48 to 57°C depending on the primer; and extension at 72ºC for 1 min; and a final extension of 72ºC for 7 min. The amplification products were separated by 3.0% agarose gel electrophoresis buffered, stained and visualized as above.

To calculate diversity, the amplification data of the SSR primers were converted into numerical code per locus for each allele. The presence of a band was designated by 1 and the absence by 0. Although the microsatellite markers can be codominant, molecular analyses of the locus were performed based on the presence/absence of each amplified fragment. The established binary matrix was used to obtain estimates of genetic similarities between genotype pairs, based on the Jaccard coefficient. The Genes statistical program (Cruz, 2016) was used for data processing.

Procedures adopted chronologically

The present study was developed according the following chronological events: (1) obtaining the segregating generations (F_2, BC_M and BC_{Bs}) from the crossing of the fertile parents M_1, M_2 and M_3 with the mutant Bs (2018-2019); (2) application of the dominant marker LMINO primer-set in order to associate the seedless trait with *INO* deletion in the segregating generations (F_1, F_2, BC_M and BC_{Bs}) (2019-2020); (3) sequencing and

identification of the *INO* gene deletion region and design the codominant primer AsINODel (2021); (4) AsINODel marker validation and phenotypic characterization of plants (F_2, BC_M and BC_{Bs}) in the field conditions (2021-2022).

Results

Seedless trait inheritance

The results of the phenotypic analysis of the parents M_2 and Bs, F_1, F_2, backcrosses with the wild-type parent M_2 (BC_M) and with mutant parent Bs (BC_{Bs}) are displayed in Table 1. In generation F_1, all individuals presented fruits with seeds. In the F_2 population, among the plants in reproductive stage during the evaluation period, 48 formed fruits with fully developed seeds and ten presented only seed rudiments, characterized by the absence of seeds (Supplemental Figure S1). Considering segregation hypotheses expected for one, two and three genes (Supplemental Table S2), the Chi-square test revealed that the trait under study segregated at a 3:1 ratio (presence/absence), consistent with a monogenic inheritance. These results were corroborated by data from backcrosses with the parent M_2 (BC_M), where all plants that produced fruits had seeds, consistent with the 1:0 ratio, while the plants evaluated through backcrossing with the mutant parent Bs (BC_{Bs}), showed segregation of 1:1 for presence and absence of seeds. Taken together, these results corroborate the monogenic inheritance found in the analyses of F_2 generations, indicating that a single recessive locus controls the seedless trait in Bs *A. squamosa*.

Table 1. Phenotypic analysis of the segregation of the seed presence/absence in fruits of F_1 and F_2 generations, BC_M and BC_{Bs} backcrosses of the M_2 family in *A. squamosa*. Janaúba-MG, Brazil.

Parents and Generations	Observed proportion		Expected ratio	χ^2	*p*-value
	Presence	Absence			
Parents					
M_2	1				
Bs		1			
Generation F_1					
$M_2 \times Bs$	17	0	1:0		
Generation F_2					
$F_1(M_2 \times Bs) \times F_1(M_2 \times Bs)$	48	10	3:1	1.86	0.172^{ns}
Backcrossing					
BC_M					
$F_1(M_2 \times Bs) \times M_2$	12	0	1:0		
BC_{Bs}					
$F_1(M_2 \times Bs) \times Bs$	8	6	1:1	0.286	0.592^{ns}

^{ns} Non-significant values at the 5% significance level, χ^2 Chi-square test estimate.

Association of seedless trait with *INO* deletion

Previously described molecular markers for the presence of the *INO* gene (Lora et al., 2011) were tested on parents M_1, M_2, M_3, and Bs and displayed the expected band patterns. These markers generated amplification products only in the three wild-type parents, with no amplification of any fragment in Bs for any of the primer pairs used (Supplemental Figure S4A).

The dominant marker LMINO primer-set were also used to amplify DNA from F_1 plants obtained from crosses between genotypes of *A. squamosa* (M_1, M_2, and M_3) with the mutant Bs (Table 1). All evaluated F_1 individuals produced fruits with seeds in the field and amplified the products with all primer pairs, as shown in the Supplemental Figure S4B.

The same procedure was applied in order to genotype segregating generations (F_2, BC_M and BC_{Bs}) in seedling stage in the nursery (Table 2). Figure 1 shows a sample of individuals amplified with the LMINO1/2 primers and the results confirm the discriminatory capacity of those genetic markers (Figure 1A, B and C). The field confirmation of presence/

266 absence of seeds in the fruits in these generations F_2, BC_M and BC_{Bs} were obtained later
267 (Table 1).
268 In the F_2 generations of the three crosses ($M_1 \times Bs$, $M_2 \times Bs$ and $M_3 \times Bs$), there was a
269 segregation of the products of the amplification of the LMINO markers that correlated exactly
270 with the presence/absence of seeds. Fertile plants in this generation uniformly produced an
271 amplification product with the LMINO1/2 primer set, while plants producing no product
272 (indicating homozygosity for the *INO* gene deletion) produced only seedless fruit (Figure
273 1A). The same complete cosegregation pattern seen in F_2 individuals for the presence/absence
274 of seeds and PCR product was also observed in backcross populations of BC_{Bs} (Figure 1B).
275 For BC_M backcross plants, the formation of *INO* amplification products was observed in all
276 DNA samples tested (Figure 1C) for these uniformly fertile/seed bearing plants.
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278

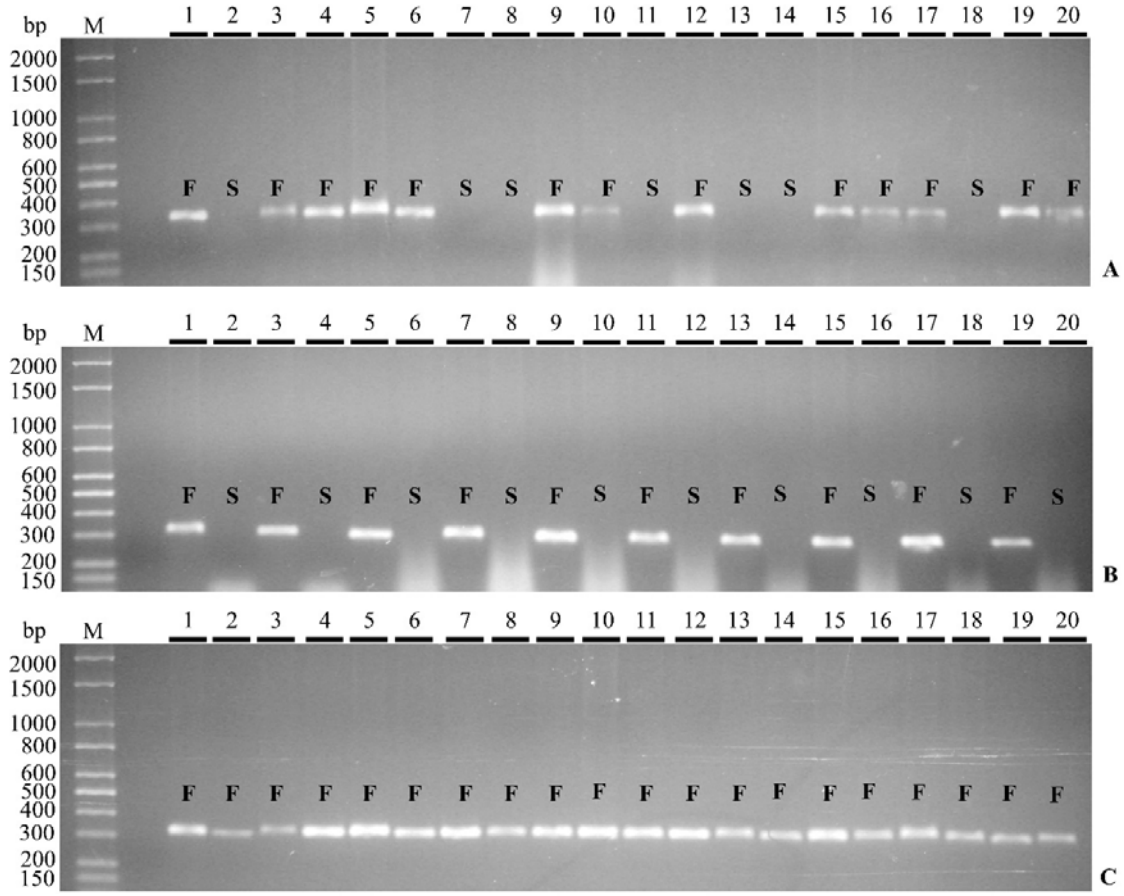

Figure 1. Amplification products for primer pair LMINO1/2 obtained from DNA samples from segregating generations: A) F_2, B) BC_{Bs} C) BC_M in 1.2% agarose gels in 1× TBE buffer. M bp: molecular weight marker in base pairs. Lanes 1 to 20 contain the respective amplified fragments from the 20 genotypes. S: seedless; F: fertile (S and F are phenotypic determinations).

The χ^2 test was performed with the data generated in the molecular analysis to confirm the segregation of the dominant amplification (Table 2). F_1 plants displayed the expected genotypic ratio of 1:0 (presence/absence of amplified band) that had been linked to the trait of seeded fruits. In F_2 generations, six segregation hypotheses expected for one, two and three genes were tested (Supplemental Table S3). Considering a significance of 5% probability, the frequencies of genotypes fit a ratio of 3:1, but allowed rejection of the other predicted ratios, confirming the hypothesis that a single locus confers the phenotype for the trait under study, with the dominant allele responsible for the presence and the recessive allele for the absence of the amplification product.

To identify the homogeneity between the F_2 crossings ($M_1 \times Bs$, $M_2 \times Bs$, and $M_3 \times Bs$), statistical techniques were applied to verify whether the differences observed in the results could be explained by chance or not. The heterogeneity test was not significant and indicated, with a 55% likelihood, that the results of the χ^2 were consistent for the populations of the three families studied, confirming the expected segregation (Table 2).

To further support the hypothesis of segregation in F_2 generations, BC_M and BC_{Bs} backcrosses were used. Similarly, the heterogeneity of segregation between the families of the BC_{Bs} backcrossing was not significant (Table 2). BC_M and BC_{Bs} progenies analysed separately displayed segregation in a manner consistent with the hypothesis of a single gene. In BC_{Bs} backcrossing, carried out between generations F_1 and the parent Bs, the proportion was close to 1:1 presence/absence of seeds in the fruits. χ^2 test was applied and the deviations between the observed and expected frequencies were not significant. In the BC_M backcrossing between generations F_1 and parents (M_1, M_2, and M_3), the proportion was 1:0 presence/absence of seeds in the fruits. These results confirmed the monogenic inheritance found in the analyses of F_2 generations consistent with a single recessive allele being responsible for the seedless trait in *A. squamosa* considering the 3:1 segregation hypothesis.

Table 2. Molecular analysis of segregation of dominant primers in parents (M_1, M_2, M_3 and Bs), generations F_1, F_2, BC_M and BC_{Bs} in *A. squamosa*, Janaúba-MG, Brazil.

Parents and Generations	Observed proportion		Expected ratio	χ^2	*p*-value
	Presence	Absence			
Parents					
M_1	1				
M_2	1				
M_3	1				
Bs		1			
Generation F_1					
$M_1 \times$ Bs	9	0	1:0		
$M_2 \times$ Bs	17	0	1:0		
$M_3 \times$ Bs	6	0	1:0		
Generation F_2					
$F_1(M_1 \times Bs) \times F_1(M_1 \times Bs)$	99	43	3:1	2.113	0.146^{ns}
$F_1(M_2 \times Bs) \times F_1(M_2 \times Bs)$	190	71	3:1	0.676	0.411^{ns}
$F_1(M_3 \times Bs) \times F_1(M_3 \times Bs)$	69	33	3:1	2.941	0.086^{ns}
Heterogeneity				1.182	0.553^{ns}
Backcrosses					
BC_M					
$F_1(M_1 \times Bs) \times M_1$	63	0	1:0		
$F_1(M_2 \times Bs) \times M_2$	67	0	1:0		
$F_1(M_3 \times Bs) \times M_3$	61	0	1:0		
BC_{Bs}					
$F_1(M_1 \times Bs) \times$ Bs	48	61	1:1	1.550	0.213^{ns}
$F_1(M_2 \times Bs) \times$ Bs	57	59	1:1	0.034	0.852^{ns}
$F_1(M_3 \times Bs) \times$ Bs	26	30	1:1	0.286	0.593^{ns}
Heterogeneity				0.586	0.746^{ns}

^{ns} Non-significant values at the 5% significance level, χ^2 Chi-square test estimate.

The nature of the *INO* gene deletion event

Whole genome shotgun sequencing was used to determine the characteristics of the *INO* gene deletion event. A draft wild-type *A. squamosa* genome was assembled through sequencing of total DNA isolated from a plant grown from seed derived from commercially available *A. squamosa* fruit. Genomic DNA was sequenced by both long-read (PacBio (Menlo Park, CA) Single Molecule, Real-Time sequencing) and short read (Illumina (San Diego, CA)) paired-end 150 base (PE150) methods. The long reads were assembled into a draft

sequence that was corrected with the higher coverage short read sequences. The resulting assembly comprised 707 Mbases of DNA in 3,519 contigs, with average contig length of 201 kb. A BLAST (Altschul et al., 1990) search with a previously published *A. squamosa INO* gene sequence (GenBank GU828033.1) was used to identify a 587 kb contig that included the *INO* gene (Supplemental Figure S3). Total Hs *A. squamosa* DNA was used to produce a second short-read sequence set (~140-fold redundant) and this was aligned with the assembled wild-type sequence. Visualization of the alignment of the Hs sequences with the 587 kb contig including *INO* revealed a clear absence of reads over a region of 16,020 bp indicating a 16 kb deletion that included the *INO* gene (Figure 2A). The alignment program truncates read sequences where they do not align with the reference sequence, so a deletion or a deletion with a heterologous insertion would appear similar in this visualization. Truncated sequence reads at the immediate borders of the putative deletion region were used to perform a BLAST search of the Hs sequence reads to identify untruncated read sequences. Identified sequence reads from the two sides of the deletion region overlapped allowing us to assemble the Hs sequence in this region (Figure 3). This showed that the lesion was in fact a clean deletion of the region deficient in read sequences with no inserted DNA and no sequence duplication at the deletion borders (Figure 2B). The deleted region included the entire *INO* gene and part of the first coding region of another putative gene encoding a possible UDP-N-acetylglucosamine-N-acetylmuramyl-pyrophosphoryl-undecaprenol N-acetylglucosamine protein. The genomic region containing *INO* in wild-type and the corresponding region from Hs were deposited in GenBank as accessions ON248606 and ON248607, respectively.

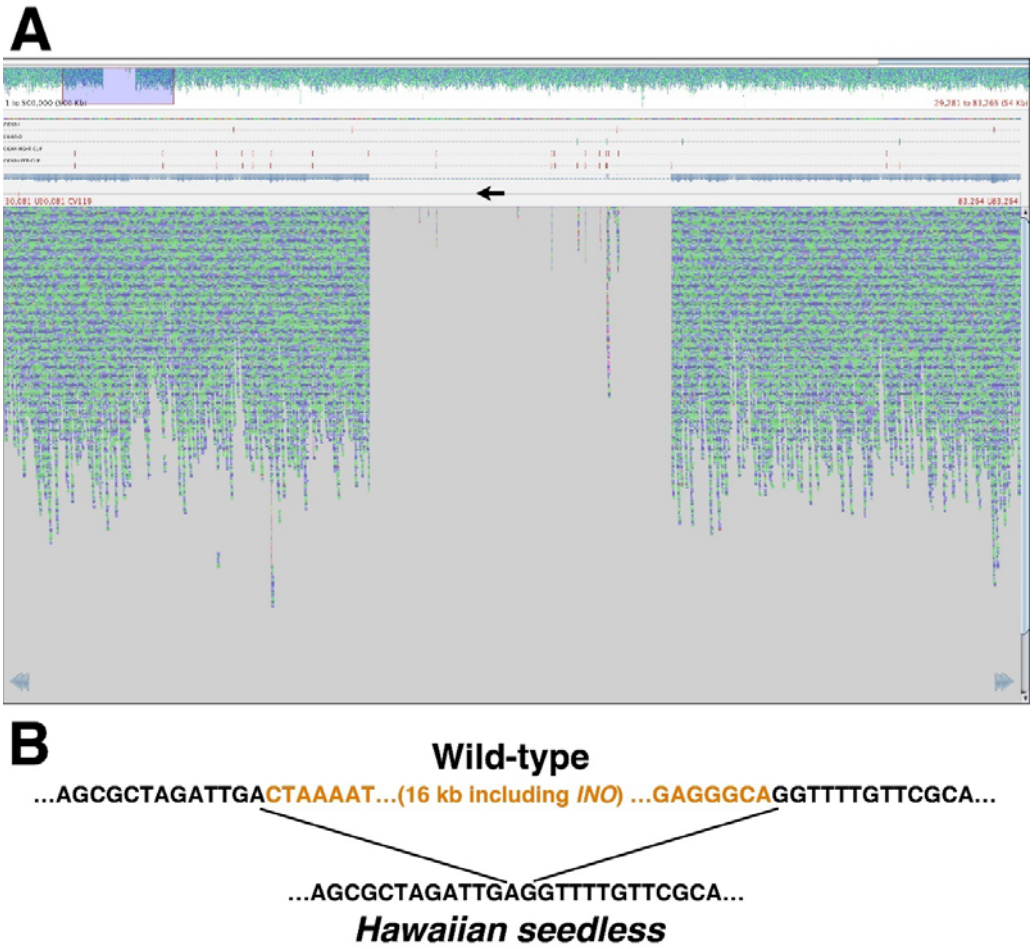

Figure 2. Identification of the *INO* deletion region. A) Screen capture of Tablet (Milne et al., 2013) visualization of the alignment of Hs Illumina sequence reads with the 587 kb contig of *A. squamosa* wild-type sequence that contains the *INO* gene. At top is a view of 500 kb of the alignment with each of the aqua or blue colored dots representing one 150 base read of the Hs sequence. A region including a deficiency of aligning reads is visible and is highlighted by a translucent purple box. The lower portion of the panel is an expanded view of the highlighted region where each small colored line again depicts a 150 base Hs read aligning with the wild-type reference sequence. The near complete absence of aligning reads in the center of this region (barring a small number of reads aligning with repeated sequences within this region) shows a clear deletion in the Hs sequence relative to the wild-type reference sequence. The black arrow edited onto the figure depicts the location of the *INO* gene transcribed region in the wild-type reference sequence. B) Depiction of the wild-type sequence compared to the sequence of the corresponding region in Hs as determined by assembling Hs Illumina reads that overlapped the deletion junction. A deletion of 16,020 bp was observed with no duplication of sequence at the junction site and no additional inserted sequence.

Primers were designed that would amplify the region spanning the deletion in mutant plant DNA (Figure 3). These primers (AsINODel F and AsINODel R) were used for PCR on DNA from Hs and Ts lines. Both lines produced a fragment with migration consistent with a length consistent with the 456 bp expected for the deletion (Figure 4A). Further, because the region between the primers in wild type is too large to be amplified in standard procedures, this procedure provides a positive assay for the presence of the deletion. In combination with primers that will amplify a region of the wild-type *INO* gene, this would enable a codominant test for the wild-type and seedless alleles of the gene in a single reaction. To test this, we modeled amplification from a "heterozygote" by mixing DNA from Hs and wild-type *A. squamosa*. We found that the amplification with the deletion-specific primers and LMINO1/2 primers in a single reaction could clearly differentiate the homozygous seedless, homozygous wild-type and heterozygous lines (Figure 4B).

Subsequent work showed that AsINODel primer PCR on all three seedless isolates produced comigrating fragments (Figure 4A and Figure 5A). The sequences of the PCR products were determined for all three seedless lines and were aligned with the assembled deletion region sequence (Figure 3). The sequence of the deletion junction was confirmed in the sequence from Hs, and an identical deletion was present in the same position in the sequences from mutants Bs and Ts (Figure 3), consistent with all lines producing PCR products of the same size. Further, the presence of identical deletions in all three lines indicates a single origin for the *INO* deletion among the isolates.

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AssembledDelRegion      GCTGTTTCCTTACCTGTTTAGTTGTCTATGTTCCACATGATCAATGATCGACGCCCAAGC
HsDelPCR                ------------------------------------------------------------
TsDelPCR                ------------------------------------------------------------
BsDelPCR                ------------------------------------------------------------

                                 AsINODelF      >
AssembledDelRegion      TATGCATGCATGTACGGGAACATCATTCTTTAATTGCTGAACCTTTTTTTTAAAAAAAAA
HsDelPCR                ----------------------------TTTAATTGCTGAACCTTTTTTTTAAAAAAAAA
TsDelPCR                ----------------------------TTTAATTGCTGAACCTTTTTTTTAAAAAAAAA
BsDelPCR                --------------------------------------GAACCTTTTTTTTAAAAAAAAA

AssembledDelRegion      AAAATTCACACGTGTGATGAATTTAATATCTAATTGTGGAAGTGGGCCCCAATTCAACGG
HsDelPCR                AAAATTCACACGTGTGATGAATTTAATATCTAATTGTGGAAGTGGGCCCCAATTCAACGG
TsDelPCR                AAAATTCACACGTGTGATGAATTTAATATCTAATTGTGGAAGTGGGCCCCAATTCAACGG
BsDelPCR                AAAATTCACACGTGTGATGAATTTAATATCTAATTGTGGAAGTGGGCCCCAATTCAACGG

AssembledDelRegion      ACTTATGCATGTTTTGACTTTTGACTCTATATATTCGTTGTTGTTTTTTCAGACAGAACA
HsDelPCR                ACTTATGCATGTTTTGACTTTTGACTCTATATATTCGTTGTTGTTTTTTCAGACAGAACA
TsDelPCR                ACTTATGCATGTTTTGACTTTTGACTCTATATATTCGTTGTTGTTTTTTCAGACAGAACA
BsDelPCR                ACTTATGCATGTTTTGACTTTTGACTCTATATATTCGTTGTTGTTTTTTCAGACAGAACA

AssembledDelRegion      GGTCAAAAGGCTTCGAAGTTTGGAAGAAGGAAGCCATGGAAATAATGTCTCATTTATTCA
HsDelPCR                GGTCAAAAGGCTTCGAAGTTTGGAAGAAGGAAGCCATGGAAATAATGTCTCATTTATTCA
TsDelPCR                GGTCAAAAGGCTTCGAAGTTTGGAAGAAGGAAGCCATGGAAATAATGTCTCATTTATTCA
BsDelPCR                GGTCAAAAGGCTTCGAAGTTTGGAAGAAGGAAGCCATGGAAATAATGTCTCATTTATTCA

AssembledDelRegion      TATTCATGTGATAATTTAGAAAGCATAGCGCTAGATTGagGTTTTGTTCGCAATGAAGAG
HsDelPCR                TATTCATGTGATAATTTAGAAAGCATAGCGCTAGATTGagGTTTTGTTCGCAATGAAGAG
TsDelPCR                TATTCATGTGATAATTTAGAAAGCATAGCGCTAGATTGagGTTTTGTTCGCAATGAAGAG
BsDelPCR                TATTCATGTGATAATTTAGAAAGCATAGCGCTAGATTGagGTTTTGTTCGCAATGAAGAG

AssembledDelRegion      TGTTGGAGGCCTTGTTTGAAAGAAATCCATCCTGCCTGGCTTCTGATTTTTCGCATTGTG
HsDelPCR                TGTTGGAGGCCTTGTTTGAAAGAAATCCATCCTGCCTGGCTTCTGATTTTTCGCATTGTG
TsDelPCR                TGTTGGAGGCCTTGTTTGAAAGAAATCCATCCTGCCTGGCTTCTGATTTTTCGCATTGTG
BsDelPCR                TGTTGGAGGCCTTGTTTGAAAGAAATCCATCCTGCCTGGCTTCTGATTTTTCGCATTGTG

AssembledDelRegion      GCCTTTTGTGTGCTTGTGCCATTGCTTGTTGTCTATGCAGTTGTTGATGGAAGCAGCATA
HsDelPCR                GCCTTTTGTGTGCTTGTGCCATTGCTTGTTGTCTATGCAGTTGTTGA-------------
TsDelPCR                GCCTTTTGTGTGCTTGTGCCATTGCTTGTTGTCTATGCAGTTGTTGA-------------
BsDelPCR                GCCTTTTGTGTGCTTGTGCCATTGCTTGTTGTCTATG-----------------------

                                          <    AsINODelR
AssembledDelRegion      TTTCACTACTATACTCAGTAAGCTGCTTCCAGCTCTTGGTTTGATTGAACAAAATACTCA
HsDelPCR                ------------------------------------------------------------
TsDelPCR                ------------------------------------------------------------
BsDelPCR                ------------------------------------------------------------

AssembledDelRegion      TGATTGATGAAGTTTAGAAAAGTAACTTTAGAAACTATATGTTTGAAACTCAGTCGAGTT
HsDelPCR                ------------------------------------------------------------
TsDelPCR                ------------------------------------------------------------
BsDelPCR                ------------------------------------------------------------

AssembledDelRegion      CTTGAAATATGCTAGCTTCAATGTTCCATATCCATCTTCATGAAGTGTCGAATGAACATG
HsDelPCR                ------------------------------------------------------------
TsDelPCR                ------------------------------------------------------------
BsDelPCR                ------------------------------------------------------------

AssembledDelRegion      TGGAACATAACTGGCA
HsDelPCR                ----------------
TsDelPCR                ----------------
BsDelPCR                ----------------
```

Figure 3. Aligned sequences of the deletion region junction. “AssembledDelRegion” is the sequence assembled from the Illumina reads selected from among all reads by BLAST searches with the reads flanking the deletion. The deletion falls between the “ag” nucleotides shown in lowercase light blue. The other sequences are those of PCR products produced using the AsINODel primers (indicated by arrows above the sequence) on DNA from Hs, Ts, or Bs as indicated, with sequence determined using the AsINODelR primer. All sequences show an identical deletion.

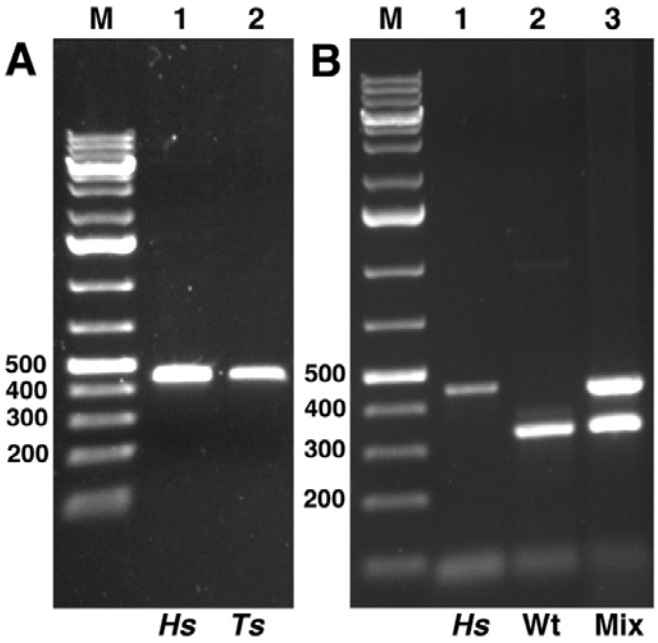

Figure 4. PCR of *INO* deletion region. A) AsINODel primers designed to amplify a fragment spanning the deletion junction were used on Hs and Ts DNA and the products were electrophoresed on an agarose gel. The Hs DNA produced the expected 457 bp fragment, and a comigrating fragment was produced from Ts. B) A combination of the AsINODel and LMINO1/2 primer pairs was used in single reactions to amplify DNA from Ts, wild-type and a mixture of wild-type and Ts ("Mix"). Only the expected 457 and 350 bp fragments were amplified from the Ts and wild-type DNA, respectively. Both bands were amplified from the mixture demonstrating effective detection of a heterozygous state.

Codominant marker analysis

To evaluate use of the AsINODel primers together with the LMINO1/2 pair as codominant markers for plant breeding, lines M_1, M_2, and M_3 were used as wild-type for *INO* gene and the presence of seeds, and the mutant Bs for the *INO* deletion and the absence of seeds. The genotype Hs was used as a positive control for the marker AsINODel. After PCR reactions, the combined primer pairs amplified a single fragment of 350 bp from the wild-type parents, representing the presence of the *INO* gene, and a 456 bp fragment from the mutants Bs and Hs, corresponding to the deletion junction fragment (Figure 5A).

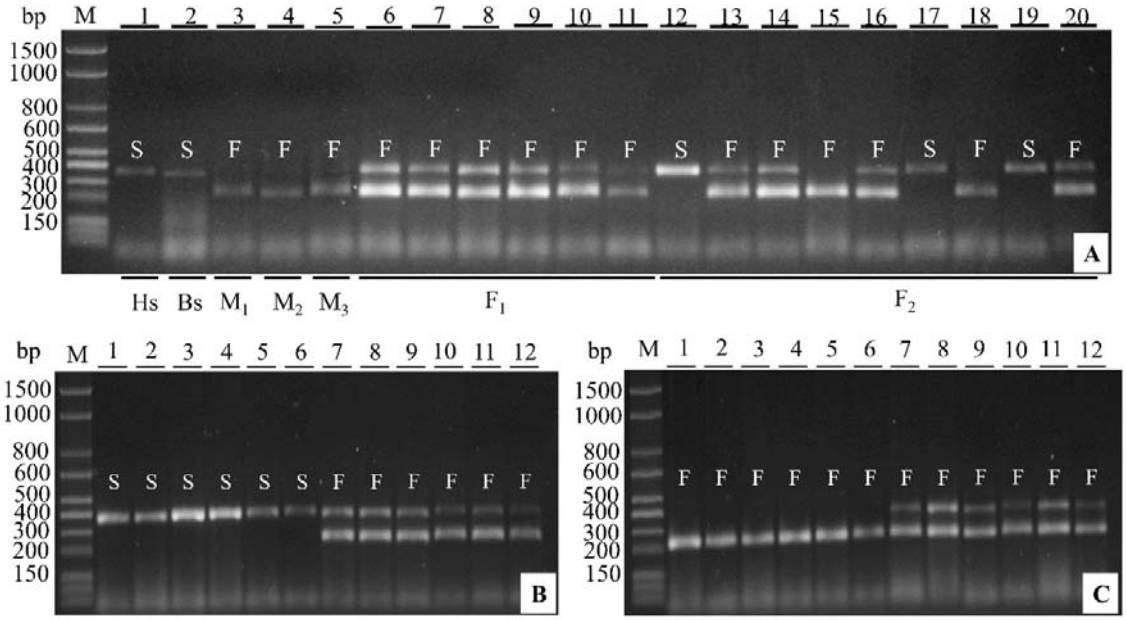

Figure 5. Amplifications products for combined AsINODel and LMINO1/2 primer pairs obtained from DNA samples of populations indicated below the agarose gel lanes (A). M bp: molecular weight marker in base pairs; Hs: Hawaiian seedless; Bs: Brazilian seedless; M_1, M_2, M_3: wild-types; F_1: first filial generation (lanes 6 to 11); F_2: self-fertilization (lanes 12 to 20). Generations BC_{Bs} (B) and BC_M (C) lanes 1 to 12 contain the amplified fragments from the 12 individuals each. S: seedless; F: fertile (phenotypic determinations of the source plants).

As expected, the wild-type fertile parents (M_1, M_2 and M_3) were homozygous for the presence, and the mutant seedless parent Bs was homozygous for the deletion of the *INO* gene. In generation F_1 both *INO* gene and deletion region bands were present in all 32 genotypes evaluated, consistent with the expected heterozygous state (Table 3 and Figure 5A).

The codominant markers were also used in genotyping of individuals of the F_2 and backcross generations (BC_M and BC_{Bs} from M_2×Bs) (Table 3) with some individuals selected to illustrate the discriminatory capacity of markers in Figure 5 A, B and C. In generation F_2, analysis of 145 plants gave results consistent with the expected ratio of 1:2:1 for homozygous *INO*: heterozygous: homozygous *ino* deletion (Table 3). The expected ratios of genotypes were also observed for the sixty-one BC_{Bs} plants and the fifty-six BC_M plants, where ratios close to 1:1:0 and 0:1:1 were observed respectively (Figure 5B, 5C and Table 3). The χ^2 test

449 on each of these generations showed probabilities consistent with a monogenic segregation of

450 the wild-type and mutant alleles.

451

452 **Table 3.** Molecular analysis of codominant segregation of parents (M_1, M_2, M_3 and Bs), F_1
453 generations and generation F_2, BC_M and BC_{Bs} backcrosses of the M_2 family in *A. squamosa*,
454 Janaúba-MG, Brazil.

Parents and Generations	**Observed proportion**			**Expected ratio**	χ^2	***p*-value**
	INO INO	***INO ino***	***ino ino***			
Parents						
M_1	1					
M_2	1					
M_3	1					
Bs			1			
Generation F_1						
$M_1 \times$ Bs	0	9	0	0:1:0		
$M_2 \times$ Bs	0	17	0	0:1:0		
$M_3 \times$ Bs	0	6	0	0:1:0		
Generation F_2						
$(M_2 \times Bs) \times (M_2 \times Bs)$	34	70	41	1:2:1	0.848	0.654^{ns}
Backcrosses						
BC_M						
$F_1(M_2 \times Bs) \times M_2$	30	26	0	1:1	0.286	0.593^{ns}
BC_{Bs}						
$F_1(M_2 \times Bs) \times$ Bs	0	30	31	1:1	0.016	0.898^{ns}

455 ^{ns} Non-significant values at the 5% significance level, χ^2 Chi-square test estimate.

456

457 Fruit was obtained from sixty-three of the genotyped segregating plants. In every case

458 where a band corresponding to the wild-type *INO* gene was present (11 homozygous *INO* and

459 37 heterozygous plants) the seeded phenotype was observed. Similarly, all of the fifteen

460 plants homozygous for the deletion allele exhibited the Bs mutant seedless phenotype. Thus,

461 there was 100% correlation of the seeded and seedless phenotypes with the wild-type

462 containing and homozygous *ino* deletion genotypes, respectively. To obtain more information

463 to further characterize the linkage between the molecular and visible phenotypes we examined

464 the ovules of plants that were flowering but not yet producing fruit where the *ino* mutant

465 ovules could be differentiated from wild-type (Supplemental Figure S2). This determination

466 was done on ninety-five plants and once again there was a complete correspondence between

467 the molecular genotype and the presence of wild-type ovules or aberrant ovules incapable of
468 forming seeds (Supplemental data file 1).

469 If the *ino* mutant gene is only linked to the seedless mutant allele, and not causally
470 responsible for it, then recombination between the molecular and phenotypic markers would
471 be possible. Among segregating F_2 plants, a single recombination event could be observed in
472 the wild-type *INO* containing chromosome of heterozygous progeny, or in either chromosome
473 of homozygous *ino* progeny. The same would be true of chromosomes deriving from the F_1
474 plants in the BC Bs progeny. In these cases, recombination would lead to a switch of the
475 seedless/seeded phenotype and breakage of the 100% cosegregation (Supplemental Methods).
476 Seed or ovule phenotypic data were produced for 57 informative heterozygous plants and 36
477 homozygous mutant plants (allowing examination of a total of 114 chromosomes)
478 (Supplemental data file 1). Thus, no recombination events were observed between the *INO*
479 locus and the Bs gene in 114 chromosomes. This allows an initial limit on the maximum
480 genetic distance between *ino* and Bs. A genetic distance of 3.5 cM would lead to a prediction
481 of 3.5% recombination, where 0% recombination was observed. The comparison of these
482 observed and expected result using the χ^2 test produces $\chi^2 = 3.99$ and a corresponding P-value
483 of 0.046. Thus, a genetic distance of 3 cM (or greater) can be rejected at the 5% probability
484 level (Supplemental data file 1). In addition, within the 587 kb of sequence surrounding the
485 *INO* gene, we find that the 16 kb deletion is the only significant difference between wild-type
486 and Hs in this region (the only other being a single base change in an intron of a predicted
487 gene). Together these observations are consistent with the *INO* gene deletion being the lesion
488 responsible for the seedless phenotype.

489

490 **Genetic diversity using SSR markers**

491

492 Of the 67 SSR primers used, 41 amplified under the tested conditions, 12 presented a
493 null amplification pattern with no bands evident, and the remaining 14 did not present a clear

494 band pattern in the tested samples (Supplemental Table S4). Among the primers that
495 amplified, 38 generated a total of 63 monomorphic alleles; only three primers (LMCH 3, 39,
496 and 137) generated specific polymorphisms among the genotypes evaluated.

497 The LMCH 3 primer generated two alleles between 200 and 300 bp for the accession
498 M_2 genotype and the seedless genotype, respectively. The primer LMCH 39 amplified five
499 alleles in total. Three generated a band only in genotype M_2, distinguishing it from seedless
500 genotypes. Similarly, the primer LMCH 137 amplified five alleles. However, in this marker
501 the seedless genotypes presented two specific bands differing from genotype M_2, displaying
502 amplification of two monomorphic bands common to all individuals. The genetic distance
503 between genotypes, estimated by the complement of the similarity matrix generated by the
504 Jaccard index, ranged from 0 among genotypes Bs, Ts, and Hs, and 0.0933 between the M_2
505 with Bs, Ts, and Hs. Thus, these markers differentiated between the mutant lines and the
506 wild-type line, but could not demonstrate any divergence between the three mutant
507 accessions.

508
509
510 **Discussion**
511
512
513 Three seedless stenospermocarpous sugar apple varieties, Bs, Ts, and Hs, from
514 differing global locales have been described [Lora et al. (2011), Santos et al. (2014) and this
515 work]. The seedless phenotype results from an ovule defect (Lora et al., 2011, Santos et al.,
516 2014) indistinguishable from the ovule effects of the *inner no outer* mutation in *Arabidopsis*
517 (Villanueva et al., 1999), and was associated with a deletion of this locus in the Ts accession
518 (Lora et al., 2011). On this basis, Lora et al. (2011) hypothesized that the deletion was the
519 cause of the seedless trait, but segregation data were not available. We show that the three
520 seedless accessions all carry identical 16 kb deletions of the region including *INO*. The
521 identical sequence of the deletion region in the three accessions indicates that the mutant

522 allele arose as a single deletion event in a common ancestor and propagated globally. The
523 deletion was not associated with any apparent repeated sequence at the deletion junction, but
524 was a precise excision of the deleted region. We tested the causal relationship between the
525 deletion and seedlessness by examining segregation of the seedless trait and the deletion. We
526 demonstrated that seedlessness is a single locus recessive trait and that the fertile/seedless
527 phenotypes cosegregated 100% with the presence/absence of the *INO* gene among progeny
528 plants (114 evaluated chromosomes). Our data demonstrate that the *ino* deletion and the
529 seedless trait are separated by less than 3.5 cM. We have determined the sequence of 587 kb
530 surrounding the deletion region, and based on prior measures of the relationship between
531 genetic and physical mapping distances (Puchta and Hohn, 1996) this would constitute from
532 three to thirty centimorgans and so would include the seedless mutation. Outside of the
533 deletion that includes the *INO* gene, we found no other significant differences from wild type
534 in the sequenced region surrounding the *INO* locus. Together with the clear expectation of an
535 *ino* gene deletion causing the observed ovule phenotype, these data support the *ino* deletion
536 being the sole cause of the seedless trait.

537 It is possible that the deletion and seedless trait was retained in multiple lines by
538 selection despite significant outcrossing, or alternatively that all lines with this deletion are
539 vegetatively propagated clones. Genotyping through the use of SSR markers failed to detect
540 polymorphism among our available seedless accessions (Bs, Hs, Ts) but did show a low level
541 of polymorphism (4.7%) when compared to the wild-type M_2 (indicating low genetic
542 diversity among genotypes). These data therefore do not rule out a possible spread by
543 vegetative propagation. The utilized SSR markers were developed for *A. cherimolia* and no
544 specific SSR primers were available for *A. squamosa* and some outbreeding could have been
545 missed in this analysis. But with the current data the simplest explanation is that a single
546 mutational event was dispersed to different continents through vegetative propagation.

547 Tracing a temporal and geographical route of this dispersal is difficult since the species has
548 been widely developed and naturalized.

549 It is believed that the primary center of origin of *A. squamosa* is in the lowlands of
550 Central America. Historical data indicate accessions to Mexico by the natives of the region
551 (Pinto et al., 2005). According to these authors, after their arrival, the Spaniards were
552 responsible for spreading the seeds to the Philippines. By 1590, the species had been
553 introduced into India. In Brazil, 1626 is the first record of introduction of the species. By the
554 early 17th century the species was already widespread in Indonesia, China, Australia,
555 Polynesia and Hawaii. In 1955, the Cuban seedless variety had been introduced in the state of
556 Florida in the United States. The mutant Brazilian accession Bs was first described in 1940 in
557 the state of São Paulo (Cunha, 1953). We obtained Hs from a commercial nursery in Hawaii.
558 The nursery reports obtaining the line from the Philippines. Their source in the Philippines
559 reports that the line was brought to the Philippines from an unknown location in the 1920s,
560 but documentary evidence of this was not found. Information on the origin of Ts was not
561 available.

562 Lora et al. (2011) designed primers within, or closely flanking the transcribed region
563 of *INO* for detection of the wild-type locus, and demonstrated amplification from wild-type *A.*
564 *squamosa, A. cherimola*, and four other species that cover the phylogenetic range of the genus
565 *Annona*. These primers were confirmed to function as expected in our *A. squamosa* varieties
566 (Nassau et al., 2021 and this work). Notably the primer pairs only allow detection of the wild-
567 type locus and a homozygous mutant could only be detected by the absence of amplification.
568 According to Singh and Singh (2015), markers that can directly identify the traits of interests
569 should be more efficient in incorporating and monitoring genes in breeding programs. Our
570 determination of the sequence of the region from which *INO* has been deleted enabled
571 formulation of primers (AsINODel) for direct detection of the mutant allele allowing

572 codominant detection of both alleles in wild-type and seedless mutant parents and to
573 differentiate fertile heterozygotes from homozygous fertile plants in a single reaction.
574 The improvement of perennial plants, especially fruit species, is a challenging task due
575 to a long juvenile period and seed dormancy, among other factors, which translates into a
576 relatively long generation period (McClure et al., 2014). In *A. squamosa*, for example, the
577 beginning of flowering of adult plants, originated from seeds, under specific cultivation
578 conditions, would take at least three years (Pereira and Borém, 2021) until the
579 characterization of the fruits, limiting the number of genotypes that can be studied, which
580 makes the breeding process slow and expensive. The PCR assay using a combination of the
581 primers detecting the wild-type and mutant alleles allows plants to be genotyped in the
582 seedling stage, accelerating the breeding program. Given the generality of the *INO* gene
583 detecting primers for use in multiple species of *Annona* (Lora et al., 2011), and the fertility of
584 interspecific crosses in this genus, our codominant genotyping is applicable to introgression
585 of the seedless trait into elite sugar apple varieties and into other cultivated *Annona* species.
586
587
588 **Conclusions**
589
590
591 The presence/absence of seeds in *A. squamosa* displays a monogenic inheritance. The
592 *INO* allele, whether homozygous or heterozygosis (*INO__*), conditions the presence of seeds
593 and has complete dominance over the recessive seedless condition (*ino ino*). The Bs, Ts and
594 Hs mutant accessions all display the same deletion sequence for the *INO* locus, demonstrating
595 a common origin of genotypes. The deletion is simple, not resulting in insertion or duplication
596 of any sequence at the deletion junction. Determination of the deletion sequence allowed
597 formulation of primers (AsINODel) that specifically detect the deletion. The AsINODel
598 primers together with the marker LMINO1/2 display co-dominance and can distinguish
599 dominant homozygotes from heterozygotes. The validated LMINO and AsINODel primers

can facilitate introgression of the seedless trait in *A. squamosa* and other members of this genus.

Acknowledgments

We thank Trang Dang for technical support and Debra Skinner for help with planning and analysis for sequence determination.

Funding

We are grateful for the support of the Coordination for the Improvement of Higher Education Personnel - Brazil (*Coordenação de Aperfeiçoamento de Pessoal de Nível Superior* - CAPES) - Financing Code 001, the National Council for Scientific and Technological Development (*Conselho Nacional de Desenvolvimento Científico e Tecnológico* - CNPq), the U. S. National Science Foundation grant IOS1354014 (to CSG) and the Fundação de Amparo à Pesquisa do Estado de Minas Gerais - FAPEMIG) for granting scholarships.

Author Contributions

Experiments were conceived by S.N., S.P and C.S.G. Crossing and genetic analyses were performed by B.R.A.R. with help from M.C.T.P. Whole genome sequence analysis was performed by C.S.G. and B.R.A.R. S.N., B.R.A.R and C.S.G. wrote the manuscript.
The author responsible for distribution of materials integral to the findings presented in this article in accordance with the policy described in the Instructions for Authors (https://academic.oup.com/plphys/pages/general-instructions) is: Silvia Nietsche (silvia.nietsche@gmail.com)

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