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RESEARCH ARTICLE

GENETIC VARIATION IN A JATROPHA CURCAS × JATROPHA INTEGERRIMA HYBRID.

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Abstract

A viable hybrid was produced by an interspecific Jatropha curcas x Jatropha integerrima cross. Artificial cross pollination was used, with J. integerrima being the pollen donor for J. curcas. Hybrid fruit resulted. Seeds from this fruit were germinated in vitro to produce seedlings as a source of leaf explants for genomic DNA extraction and purification. Molecular markers (ISSR and DAMD) were used to confirm hybridization success and characterize the combination of parental characteristics in the hybrid. Based on the band pattern, this interspecific cross resulted in genetic variation in the hybrid's DNA, since 15% of its DNA content exhibited no similarity to either parent genome. Of the remaining DNA content, 35% was similar to the female parent (J. curcas), 25% to the male parent (J. integerrima), and 25% was a combination of both parents.

Introduction:-

Genetic improvement is effective in creating new varieties of plant species and emphasizing or developing traits of interest. Interspecific crossing is a common tool in genetic improvement. In the conventional method two species of the same genus but different species are crossed to produce a hybrid.

The genus Jatropha includes commercially interesting species. The species J. curcas is probably the best known of the species in this genus due to its fruit's very high fatty acids content, which makes it a promising raw material for biofuel production. Other species, such as *J. integerrima*, can be used as ornamentals due to their medium plant size, large crimson red inflorescences and numerous branches and inflorescences.

Despite the well-known incompatibility of many Jatropha interspecific crosses, there are also exceptional cases such as *Jatropha tanjorensis*, a natural cross between *J. curcas* × *J. gossypifolia* (Sahai et al., 2009). The first reported

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natural Jatropha hybrid, it is sterile due to the shape of the anther lobes, which are flat, fibrous and in some cases have callus-like structures; it is these unusual formations, which prevent reproduction.

Various attempts have been made to produce interspecific crosses within the Jatropha genus. In a study of pollen-pistil incompatibility, a common result of interspecific crosses, three interspecific crosses were made with *J. curcas*: *J. gossypifolia*; *J. podagrica*; and *J. villosa* (Kumar et al., 2009). In the *J. curcas* × *J. gossypifolia* the pollen tubes reached the ovaries after pollination but could not produce seeds, while the *J. curcas* × *J. podagrica* showed signs of incompatibility such as bulging pollen tubes and inverse growth of the tube. The *J. curcas* x *J. villosa* exhibited barriers to crossability in its growth pattern: the pollen tube was wrinkled and twisted and did not reach the ovary, preventing reproduction.

A *J. curcas* × *J. integerrima* cross was produced with the aim of increasing seed and fatty acids production per plant in the BC4F1 population, and the specific selection characteristics indicated the possibility of simultaneous improvement of fruit and oil yield per plant (Subashini et al., 2015). In another study of *J. curcas* × *J. integerrima* cross the objective was to produce changes in canopy height and size and thus improve seed harvest rate (One et al., 2014). Through generations F 1, F 2, BC 1 F 1 and BC 1 F 2, six traits were documented: high (TL), intermediate (ID) and dwarf (DW), expansion (SP), upright (UP) and erect canopy angles (ER). Independent segregation identified nine phenotypes: TL-ER, TL-UP, TL-SP, ID-ER, ID-UP, ID-SP, DW-ER, DW-UP and DW-SP, at a 1: 2 : 1: 2: 4: 2: 1: 2: 1 ratio.

Interspecific crosses within Jatropha are useful for obtaining cultivars with desirable agricultural characteristics. However, interspecific crossing does not generally guarantee hybrid production because environmental and physiological factors frequently prevent its success. This is why specialized techniques and tools are needed to verify the success of a hybrid.

The present study objective was to produce a cross between *J. curcas* var. Sikilte and *J. integerrima* to create a new variety. Jatropha curcas var. Sikilte is a Mexican variety produced and registered in Mexico, and has a semi-rigid habit, an average height of 1.91 m, and good tolerance for drought and low temperatures. This variety only produces female inflorescences. Jatropha integerrima has an average height of 2 m, large inflorescences of crimson red, and year-round foliage.

Materials and Methods:-

Selection of J. curcas parents and female inflorescences

Five *J. curcas* var. Sikilte plants were randomly selected from a plantation at the Center for Research and Technology and Design Assistance of the State of Jalisco, Southeast Campus in Merida, Yucatan, Mexico. Cross-pollination by wind and pollinating insects was prevented by covering the inflorescences with paper bags. These have a square plastic-covered window that allows monitoring of inflorescence development to identify the most adequate time for pollination.

Establishing J. integerrima parents and selection of male inflorescences

The *J. integerrima* male parental culture was established using cuttings collected from a mother plant located in the city of Mérida, Yucatan (21°01’34.9” N; 89°38’35.9” W). A total of twelve cuttings were planted in black plastic bags filled with sterile substrate. When these parents produced flowers, male flowers were collected 24 hours before cross-pollination and stored in magenta boxes with moistened cotton at 5 °C.

Pollination of J. curcas with J. Integerrima:

Direct pollination of female J. curcas inflorescences was done using male *J. integerrima* inflorescences. The *J. curcas* flowers were open and had been previously covered with paper bags. Pollination was done between eight and nine h by rubbing pollen directly from a male *J. integerrima* inflorescence onto a female *J. curcas* inflorescence until the pistils were thoroughly covered with pollen. The inflorescences were then covered with paper bags.

Establishing hybrid seeds

Seeds were extracted from the fruit produced by the *J. curcas* x *J. integerrima* hybrid. These seeds were disinfected in a laminar flow hood by immersion in a 5% (v/v) solution of Extran® for 5 min, followed by immersion in 70% (v/v) ethanol for 1 min, and stirring in 30% (v/v) sodium hypochlorite solution for 15 min. They were rinsed three

times with sterile distilled water. The disinfected seeds were planted in MS culture medium at 100% ionic strength (Murashige and Skoog, 1962) supplemented with 30% sucrose (w/v) and 2.2% Gelrite (w/v).

Molecular analysis of parents and hybrids

DNA extraction from J. curcas and J. integerrima explants

Nuclear DNA was extracted from leaf explants of *J. curcas* and *J. integerrima*. Extraction was done using the GenElute™ plant Genomic DNA Miniprep Kit (Sigma-Aldrich, Missouri, United States). Extraction of DNA from some tissues required modification of the protocol with Plant DNAzol™ Reagen (Invitrogen™) with added polyvinylpyrrolidone (PVP) (Sigma-Aldrich), and maceration of tissue samples until producing an aqueous solution. From this point on the manufacturer-suggested methodology was followed.

DNA extraction from hybrid

A sample of leaf (100 mg fresh weight) was pulverized with Plant DNAzol™ Reagen (Invitrogen™) with added polyvinylpyrrolidone (PVP) (Sigma-Aldrich) following the methodology of the GenElute™ plant Genomic DNA Miniprep Kit (Sigma-Aldrich).

PCR amplification of parental and hybrid DNA

Amplification was done using PCR with 2.5 µL 10X $MgCl_2$ PCR buffer; 1 µL 50 mM $MgCl_2$; 0.2 µL Taq polymerase at 5 units per µL (Invitrogen™, California, United States); 2 µL 0.2 mM dNTPs; and 1 µL 10 µM initiator (Integrated DNA technologies). The procedure was done in a 96-well thermal cycler (Applied Biosystems™ Verity™).

DAMD markers

The alignment temperature (T_a) used with the HVA, HBV, HVR and JJ33.6 markers was 55 °C (Zhou et al., 1997; Aguilera-Cauich et al., 2015). For the M13 marker, the alignment phase at 42 °C was programmed as described by Aguilera-Cauich (2015). The cycle began with a denaturation stage (95 °C for 5 min); followed by 40 cycles of 1 min at 92 °C; 2 min at the corresponding T_a, according to marker type; 2 min at 72 °C; and an extension stage of 7 min at 72 °C.

ISSR markers

Three UBC markers (set No. 9 UBC 807, UBC 810, UBC 815; University of British Columbia, Canada) were used with a 35 °C T_a and an IMPN marker (IMPN 28) with 47 °C T_a (Tanya et al., 2011). The cycle for all ISSR markers was initial denaturation at 94 °C for 2 minutes; 40 cycles of 15 seconds at 94 °C; 30 seconds at T_a for each marker; 1 minute at 72 °C; and a final extension of 7 minutes at 72 °C. Samples were stored at -8 °C until use.

Band profile interpretation

The electrophoresis gel was viewed in a high-performance UV transilluminator (UVP Ultraviolet Products, Jena, Germany). The PCR-amplified fragments were separated and observed following the above protocol in 1.5% (w/v) ultrapure agarose gel (Invitrogen™, California, United States) dissolved in 1X TAE. Corroboration of DNA and PCR amplification was done by analyzing all the images with the Gel Analyzer 2010 software (http://www.gelanalyzer.com/). A data matrix using values of 0 and 1 to indicate the presence or absence of bands was generated to calculate the percentages of genetic polymorphism with each primer. These were analyzed with the NTSYSpc ver. 2.10e software.

Results and Discussion:-

Cross-pollination between the *J. curcas* and *J. integerrima* parents resulted in fruit on the female (*J. curcas*) parent. Seeds and embryos were extracted from the fruit for germination, and were the source of the plant material used in DNA extraction.

Integrity of the resulting genomic DNA was confirmed with electrophoresis. Specific sequences were later amplified using DAMD and ISSR molecular markers to identify any differences and similarities between the parents and the hybrid. These markers allow differentiation between individuals of the same genus but different species and between varieties of the same species because these sequences repeat in the same individuals. For example, DAMD markers were reported to be highly efficient in identifying differences and similarities in genetic variation among seventeen Oryza species, and in identifying polymorphisms between accessions of one Oryza species and between individual plants of a wild Oryza species (Zhou et al., 1997). Another study used DAMD markers to identify reliable and

unambiguous specific markers in eleven Capsicum species (Ince et al., 2009). In a comparison of DAMD and RAPD markers using nineteen cucumber accessions DAMD markers were found to have greater potential for detecting polymorphisms (Hu et al., 2011).

Like DAMDs, ISSR molecular markers can identify polymorphisms among individuals of different species of the same genus. For example, ISSR was used to identify polymorphism and similarity between 39 different J. curcas accessions from regions as varied as Taiwan, China, Vietnam and Mexico, as well as between different Jatropha species (*J. integerrima, J. gossypifolia* and *J. podagrica*) (Tanya et al., 2011). In the same study a DICE similarity analysis effectively grouped each accession with its counterparts from each region and each species. Use of ISSR can also assist in cultivar identification and phylogenetic studies as shown in a study using ISSR markers with eight eggplant (*Solanum melongena*) cultivars and twelve accessions of eight related Solanum species in which each accession was grouped with its respective species and these were classified into seven different groups (Isshiki et al., 2008).

Using the ISSR marker matrices reported by Tanya et al. (2011) (HVA, HBV, HVR and 33.6) and the matrix generated in the present study a comparison was made between *J. curcas* and *J. integerrima* accessions (Figure 2A), and the *J. curcas* var. Sikilte and *J. integerrima* parents in this study to identify any relationship between *J. curcas* var. Sikilte and the reported accessions (Tanya et al. 2011). No relation was found between J. curcas var. Sikilte and other *J. curcas* accessions, most probably because the Sikilte variety is an F1. Illustrated as a dendrogram, the Sikilte variety is placed in the same clade as J. integerrima, highlighting its distance from other *J. curcas* rather than its affinity to *J. integerrima*.

To date, which traits are transferred to the F1 progeny of an interspecific cross between a wild species (*J. integerrima*) and an improved variety (*J. curcas* var. Sikilte) cannot be specified. The traits resulting from this cross have been documented (One et al., 2014; Kumar et al., 2009; Subashini et al., 2015), though not using an improved variety with FOF characteristics. In the present study, DAMD and ISSR markers, and the DICE similarity index, were applied to determine what similarities or differences the resulting F1 exhibited compared to its parents. A binary matrix was then generated with the GelAnalyzer 2010 software and this analyzed with the NTSYSpc software (Table 1). Using the DICE similarity index, values were assigned ranging from 0 (zero) (total dissimilarity between hybrid and parents) to 1 (one) (total similarity of hybrid with parents). Shown in a dendogram (Figure 2 B), the F1 is clearly closer to the female parent (*J. curcas*), with a 0.545 similarity coefficient (Table 2); this value exceeds 0.5, is therefore closer to one and indicates greater similarity to that parent. The similarity coefficient value between the F1 and the male parent (*J. integerrima*) was 0.456, which is closer to zero and thus indicates less similarity.

Conclusions:-

The interspecific cross between *J. curcas* and *J. integerrima* was viable, producing fruit with seeds that germinated without difficulty. This is the first report of an interspecific cross between a *J. curcas* variety generated and registered in Mexico and a *J. integerrima* accession. Thirty-five percent (35%) of the F1's DNA corresponded to its female parent (*J. curcas*), 25% to its male parent (*J. integerrima*), 25% was a combination of both parents and the remaining 15% had no relationship to either parent, that is, it was unique to the F1. This is to be expected in part since the female parent in a hybrid exerts a greater genetic load, meaning the hybrid will always exhibit greater similarity to the female parent than to the male parent.

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Table 1:- General binary matrix of DAMD and ISSR used in analyzing *J. curcas, J. integerrima* and the hybrid.

Markers	*J. curcas*	Hybrid	*J. integerrima*	Markers	*J. curcas*	Hybrid	*J. integerrima*
IMPN 28.1	1	0	0	HVA.4	0	0	1
IMPN 28.2	1	0	1	HVA.5	0	0	1
IMPN 28.3	0	1	0	HVA.6	0	1	1
IMPN 28.4	1	0	0	HVA.7	1	1	1
IMPN 28.5	0	1	1	HVA.8	0	0	1

IMPN 28.6	0	0	1	HVA.9	1	1	0
IMPN 28.7	1	1	0	HVA.10	0	1	0
IMPN 28.8	0	0	1	HBV.1	0	1	0
IMPN 28.9	1	1	1	HBV.2	0	0	1
IMPN28.10	1	1	1	HBV.3	0	0	1
IMPN28.11	0	0	1	HBV.4	0	0	1
IMPN28.12	1	0	0	HBV.5	1	1	0
IMPN28.13	1	0	0	HBV.6	0	0	1
UBC 807.1	1	1	0	HBV.7	1	1	0
UBC 807.2	0	1	1	33.6.1	0	0	1
UBC 807.3	1	1	1	33.6.2	1	0	0
UBC 807.4	1	1	1	33.6.3	0	1	1
UBC 807.5	1	1	0	33.6.4	1	0	0
UBC 807.6	0	0	1	33.6.5	0	1	1
UBC 810.1	0	0	1	33.6.6	0	1	1
UBC 810.2	0	0	1	33.6.7	1	1	0
UBC 810.3	0	0	1	33.6.8	0	0	1
UBC 810.4	1	1	1	33.6.9	1	0	0
UBC 815.1	0	1	0	33.6.10	0	1	0
UBC 815.2	0	1	0	33.6.11	1	0	1
UBC 815.3	0	0	1	33.6.12	1	1	0
UBC 815.4	0	1	0	33.6.13	0	0	1
UBC 815.5	0	0	1	33.6.14	0	0	1
UBC 815.6	0	1	0	HVR.1	0	0	1
UBC 815.7	0	1	1	HVR.2	1	0	0
UBC 815.8	0	0	1	HVR.3	0	1	0
UBC 815.9	1	1	0	HVR.4	1	0	0
HVA.1	1	1	1	HVR.5	0	1	1
HVA.2	0	1	1	HVR.6	1	1	1
HVA.3	1	1	1	HVR.7	0	1	0

Note: Includes both ISSR and DAMD markers.

Table 21:- Analysis of DICE similarity coefficient.

Species	J. curcas	Hybrid	J. integerrima
J. curcas	1		
Hybrid	0.545	1	
J. integerrima	0.31	0.456	1

Note: Illustrates similarity of J. curcas with hybrid (0.545), and dissimilarity of hybrid with J. integerrima (0.456).

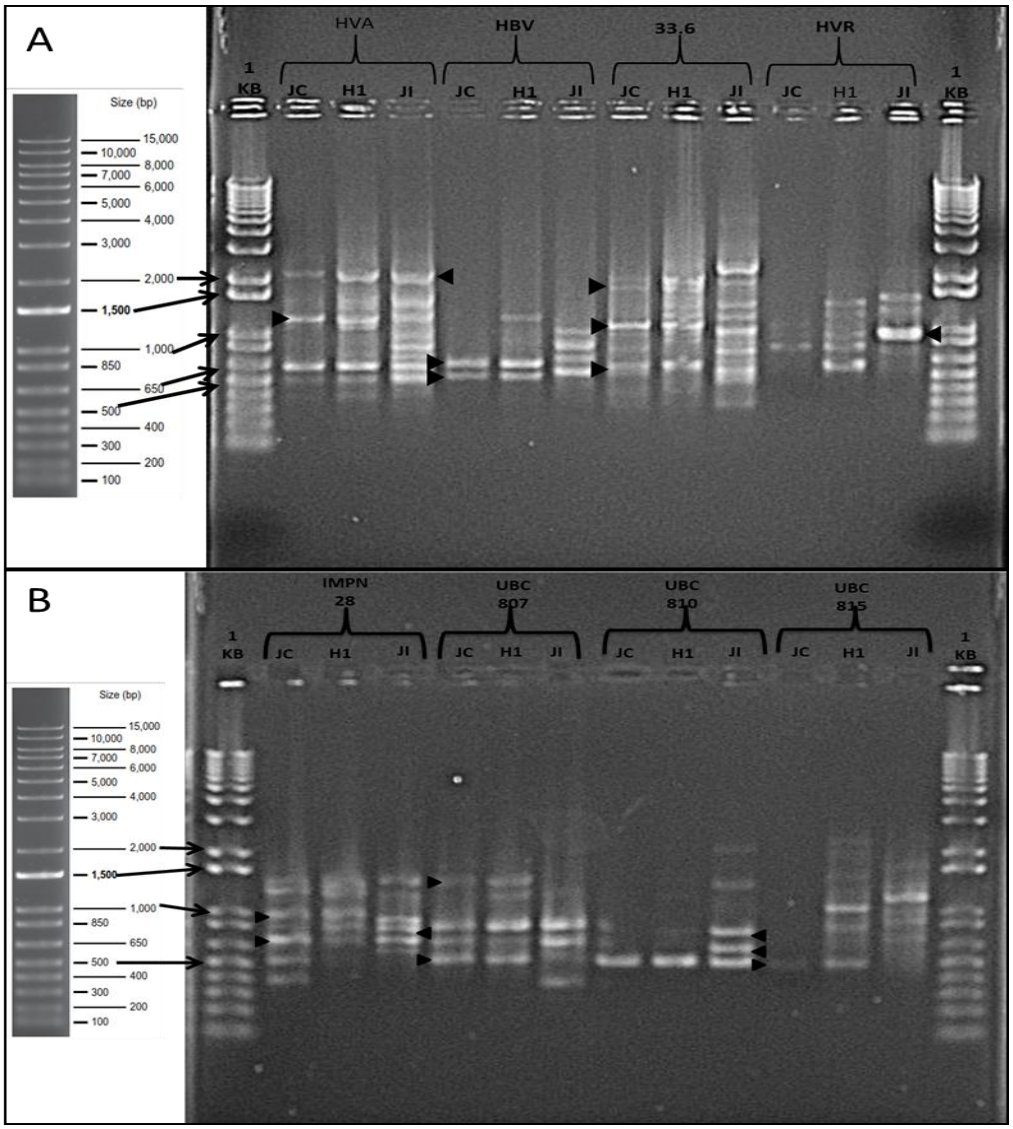

Figure 1:- Electrophoresis of DNA from parents and hybrid using DAMD and ISSR molecular markers. A) electrophoresis of genomic DNA from parents and hybrid using DAMD markers (HVR, HBV, 33.6 and HVA). B) electrophoresis of genomic DNA from parents and hybrid using ISSR markers (IMPN 28, UBC 807, UBC 810 and UBC 815). Triangular arrows pointing to the right indicate band similarity between the female parent (*J. curcas*) and the hybrid. Triangular arrows pointing to the left indicate band similarity between the male parent (*J. integerrima*) and the hybrid.

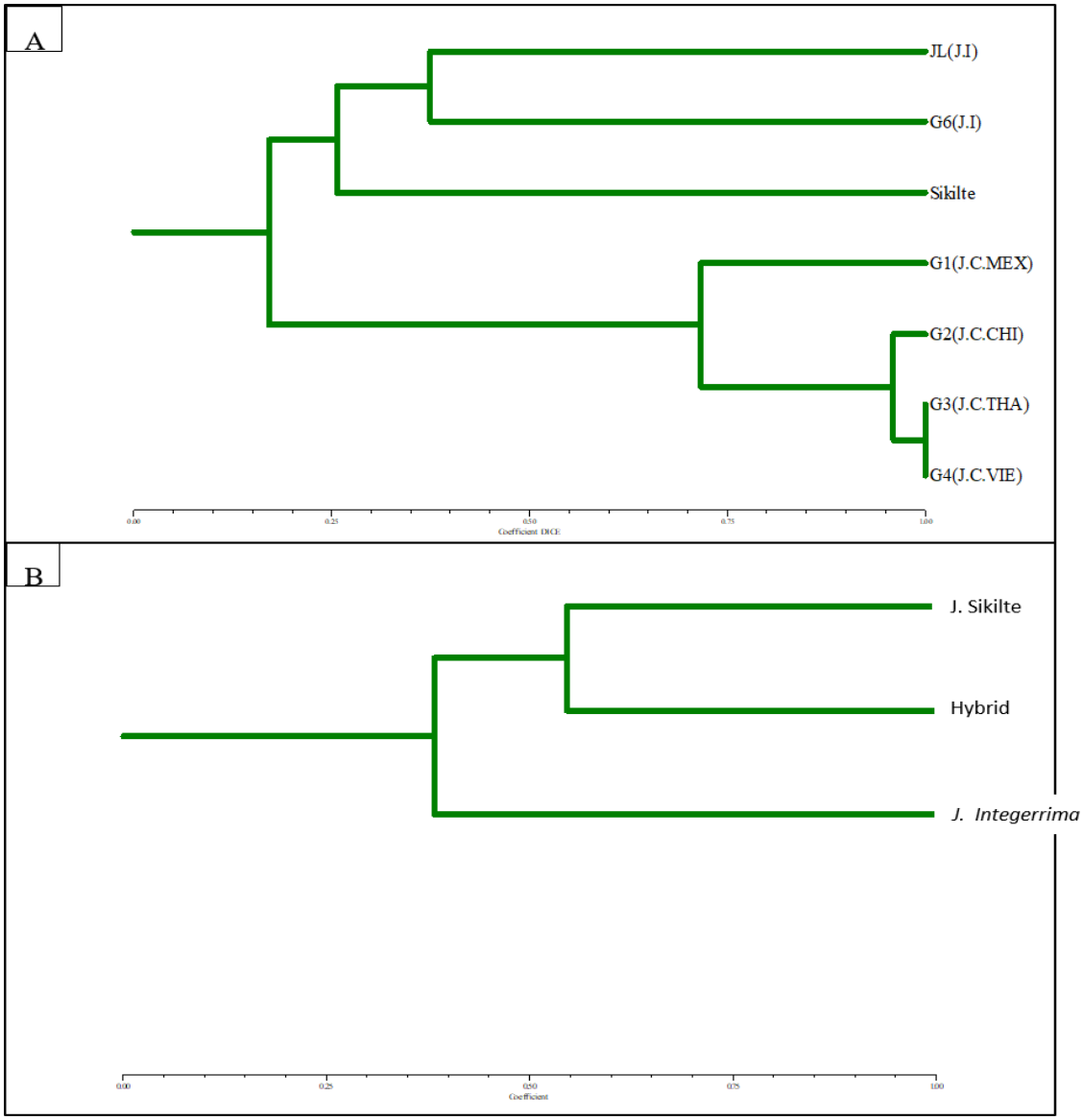

Figure 2:-Dendogram of *J. curcas*, *J. integerrima* and the hybrid using 4 DAMD and 4 ISSR molecular markers. A) Dendrogram comparing results of Tanya et al. (2011) and the present results: *J. curcas*: G1 (Mexico), G2 (China), G3 (Thailand), G4 (Vietnam); G6 *J. integerrima* x *J. curcas* var. Sikilte; and *J. integerrima*. B) DICE similarity dendogram between parents and hybrids of the Jatropha genus with DAMD markers.

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