

## Genetic variability in *Jatropha curcas* L. from diallel crossing

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**ABSTRACT.** Physic nut (*Jatropha curcas* L.) presents high oilseed yield and low production cost. However, technical-scientific knowledge on this crop is still limited. This study aimed to evaluate and estimate the genetic variability of hybrids obtained from diallel crossing. Genetic variability was carried out using ISSR molecular markers. For genetic variability, nine primers were used, and six were selected with 80.7% polymorphism. Genetic similarity was obtained using the NTSYS pc. 2.1 software, and cluster analysis was obtained by the UPGMA method. Mean genetic similarity was 58.4% among hybrids; the most divergent pair was H1 and H10 and the most similar pair was H9 and H10. ISSR PCR markers provided a quick and highly informative system for DNA

fingerprinting, and also allowed establishing genetic relationships of *Jatropha* hybrids.

**Key words:** Physic nuts; ISSR; Hybrids

## INTRODUCTION

Physic nut is of Mexican and Central American origin, and is cultivated in many other Latin American, Asian, and African countries as hedge. It was also an important export product from the Cape Verde Islands during the first half of this century. These species had been used for medicinal purposes and widely cultivated in the tropics long before recorded history. Physic nut has been considered as one of the oilseed plants species with good yield characteristics and with good yield traits of biodiesel production, with about 38% oil content. These competitive advantages qualify physic nut as promising oilseed crop for commercial use (Brasileiro et al., 2013).

When comparing with other oilseed species, physic nut stands out for its economic and social viability for biodiesel production; and despite being a perennial species it can reach the productive stage from the second year after growing. However, Brazil does not have definitive system for commercial production yet (Dias et al., 2012).

Many studies on the botanical classification (Pessoa, 2011; Pimenta et al., 2014) and multiple uses of *Jatropha* have been reported. From its seeds, it is possible to produce insecticides, larvicides (Sakthivadivel and Daniel, 2008), fungicides (Cordova-Albores et al., 2014), soap, fertilizers (cake resulting from pressing), and biodiesel (Kazembe and Chaibva, 2012). The leaves, stem, and root have biocide activities (Ribeiro et al., 2012; Rahmam et al., 2014). Several types of active substances present in different parts of the plant and their mechanisms of action have also been associated with a number of uses (Prasad et al., 2012), such as reduction of *in vitro* tumor cell growth.

Due to the demand for propagation material aiming at the establishment of commercial cultivation of physic nut, no breeding program has resulted in at least a cultivar, nor a production system minimally validated for propagation of physic nut cultivars that has been certified by the National Register of Cultivars (Brasil, 2008).

Physic nut is a non-domesticated plant that stands out for its use as biofuel and medicine. Knowledge on the genetic diversity associated with molecular markers is necessary for the planning of selection and breeding strategies (Kaushik et al., 2007; Montes et al., 2014). Molecular marker technology for germplasm characterization is a relatively simple, rapid, and very important tool used in many different ways, such as genome mapping, genetic tagging, phylogenetic analysis, forensic investigations, and genetic diversity (Bered et al., 1997; Grover and Sharma, 2016).

Inter-simple sequence repeats (ISSR) is a class of markers that amplifies genome regions and does not need any previous genomic information on the target species. It is a relatively inexpensive, simple, accurate technique, and requires small amounts of DNA for PCR amplifications; it can be performed quickly, and is effective in detecting genetic diversity within a short period of time (Jingura and Kamusoko, 2015). ISSR has been widely used in plants to identify the genetic diversity of physic nut (Semagn et al., 2006; Kumar et al., 2011; Pecina-Quintero et al., 2014), promoting useful information for the plant breeding of this species (Soonthornyatara et al., 2015).

In breeding programs, hybridization and crossings between hybrids are resources that increase the genetic variability and gather desirable traits in different genotypes, aiming at genetic gains and selection efficiency. The identification of heterotic groups by molecular markers is important, as well as the evaluation of the hybrid produced from crossings representative of each group (Hausmann et al., 2004).

Few researches on *Jatropha* hybrids are available in the literature. However, many studies have been accomplished seeking the phenotypic selection of individuals for selective breeding program aiming at hybrid seeds (Mat et al., 2015). Studies on the development of interspecific and intergeneric hybrids (*J. integerrima* and *J. multifida*, *J. podagrica* and *Ricinus communis*) present hybridization as suitable tool for elite hybrid programs, evidencing its success in the production of hybrids between *J. curcas* and closely related species (Laosatit et al., 2014).

Although few studies have been carried out using physic nut hybrids, further studies are required to really understand the genetic gains that may exist with hybridizations. Santana et al. (2013) tested the general and specific combining abilities, genetic parameters, and the correlation between morphological traits. General combining ability was predominant for most of the evaluated traits. Estimated heritability was higher than 70% for number of secondary branches, number of female flowers, stem diameter, and branch height. The number of female flowers accounted for more than 50% of the variation of *Jatropha* hybrids. However, the genetic diversity of hybrids obtained from these diallel crossings was not evaluated. Thereby, this study aimed to evaluate the genetic variability of ten hybrids obtained by Santana et al. (2013) using ISSR markers of *J. curcas*.

## MATERIAL AND METHODS

### Area and plant material

For field tests, the experiment consisted of completely randomized block design with three replications. Each experimental plot comprised six plants, spaced 2 x 2 m apart, and totaling 24 m<sup>2</sup>. The ten hybrids are derived from crossings involving seven physic nut genotypes (JCUFS-01, JCUFS-08, JCUFS-13, JCUFS-05, JCUFS-03, JCUFS-04, and JCUFS-15) (Table 1). Hybrids were grown in a Rural Campus Experimental Farm of the Federal University of Sergipe (UFS), São Cristóvão, SE, Brazil (lat. 10°55'26''S, long. 37°11'57''W), under rainfed conditions. The soil of the experimental area is classified as Red Yellow Argisol, characterized by landscape units (Empresa Brasileira de Pesquisa Agropecuária, 2013). According to the Köppen classification, the climate is rainy tropical type, with average annual temperature of 25.5°C, and average annual rainfall of 1300 mm.

### PCR

DNA samples were isolated from young leaves, as described by Nienhuis et al. (1995). The initial primer selection resulted in nine informative ISSR primers (UBC 808, UBC 809, UBC 811, UBC 813, UBC 834, GOOFY, OMAR, MAO, and JOHN) from IDT (Integrated DNA Technologies). PCRs were carried out at 94°C for 5 min, followed by 35 cycles at 94°C for 45 s, annealing at temperature indicated for each specific primer, and 72°C for 90 s, with a final extension at 72°C, for 5 min, using the TPersonal Thermocycler. Then, the amplified

products were separated by 1.5% agarose gel horizontal electrophoresis (1X TBE - 89 mM Tris, 89 mM boric acid, 2.5 mM EDTA, pH 8.3) (Loccus Biotecnologia LCH 20X25), and constant voltage of 120 V for 2 h and 30 min. The gel was stained by GelRed-Biotium® (Biotium Inc., Hayward, CA, USA). The amplified products were visualized under UV light.

**Table 1.** Hybrids of *Jatropha curcas* used in the analysis of genetic diversity and quality of seeds collected in the Rural Campus of the Federal University of Sergipe (UFS).

Denomination	Hybrid
H1	JCUFS-01 x JCUFS-08
H2	JCUFS-01 x JCUFS-13
H3	JCUFS-04 x JCUFS-08
H4	JCUFS-03 x JCUFS-05
H5	JCUFS-04 x JCUFS-05
H6	JCUFS-04 x JCUFS-13
H7	JCUFS-03 x JCUFS-08
H8	JCUFS-04 x JCUFS-15
H9	JCUFS-03 x JCUFS-13
H10	JCUFS-01 x JCUFS-05

## Data analysis

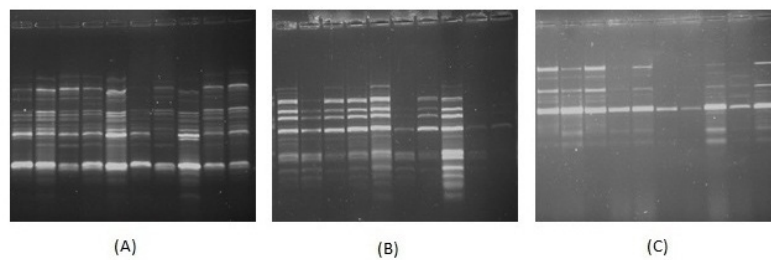
Intense amplified fragments were used for analysis. The electrophoretic profile of each gel was visually examined and set as absence (0) or presence (1) of fragment in order to build a binary matrix.

To determine the ideal number of amplified fragments, correlation ( $r$ ) and stress value ( $E$ ) estimates among similarities were obtained. Similarities were estimated by the Jaccard coefficient (Jaccard, 1908). The optimum number of fragments was estimated by the Genes software (Cruz, 2006). The number of different alleles observed, the number of effective alleles, the percentage of polymorphic loci, the Shannon diversity index, and the Nei differentiation were estimated using the POPGENE 1.32 software (Peakall and Smouse, 2006).

Hybrid genetic similarity clustering was carried out using the NTSYSpc 2.1 software (Rohlf, 2001).

## RESULTS

Sequences of nine primers used in ISSR analysis and their amplification products were tested, resulting in six informative primers (Figure 1). Table 2 shows the sequences of the primers, the annealing temperature, the total number of polymorphic loci, and polymorphism for each ISSR primer used in physic nut hybrids.



**Figure 1.** Fragments produced by standart primers OMAR (A), UBC 809 (B) and UBC 811 (C).

**Table 2.** Sequences of the primers, annealing temperature (Ta), total number of loci (N), number of polymorphic loci, and percent for each ISSR primer performed in hybrids of *Jatropha curcas* (UFS).

Primer	Sequences	Ta (°C)	N	Polymorphic loci	(%)
UBC 808	(AG) 8-C	47	27	25	92.59
UBC 809	(AG) 8-G	48	19	18	94.74
UBC 811	(GA) 8-C	45	12	11	91.67
UBC 834	(AG) 8-YT	47	12	7	58.33
GOOFY	(GT) 7-YG	48	10	8	80.0
OMAR	(GAG) 4-RC	47	24	18	66.67
Mean	-	-	17.3	14.5	80.7
Total	-	-	104	87	-

D, Y, and V mean degenerate oligonucleotides: D = (A, G, T); Y = (C, T); and V = (A, C, G).

Of the 104 loci, 87 were polymorphic, corresponding to 80.7%, with mean polymorphism of 17.3 loci per primer. The maximum and minimum percentages of polymorphic loci were 94.74% (UBC 809) and 58.33% (UBC 834). Among the six primers used, only the UBC 834 presented less than 60% polymorphism. Physic nut hybrids presented genetic similarity of 58.4% among them (Table 3).

**Table 3.** Genetic similarity among 10 hybrids of *Jatropha curcas* L. (UFS).

Hybrids	H1	H2	H3	H4	H5	H6	H7	H8	H9	H10
H1	1.00									
H2	0.66	1.00								
H3	0.63	0.64	1.00							
H4	0.60	0.67	0.65	1.00						
H5	0.62	0.59	0.68	0.60	1.00					
H6	0.59	0.61	0.64	0.56	0.65	1.00				
H7	0.63	0.59	0.58	0.60	0.49	0.48	1.00			
H8	0.59	0.65	0.56	0.60	0.65	0.55	0.57	1.00		
H9	0.57	0.61	0.54	0.52	0.49	0.57	0.68	0.51	1.00	
H10	0.47	0.51	0.52	0.50	0.51	0.55	0.55	0.55	0.69	1.00

Similar results found in this study using UBC 834 primer were obtained in a study on estimative genetic diversity among 16 physic nut accessions with ISSR markers, with percentage of polymorphism equal to 57% (Camellia et al., 2012). However, in a study on the correlation of phenotypic and genetic diversity, 35.7% polymorphism was obtained using the primer UBC 809 (Sunil et al., 2011), which is much lower than that observed in this study.

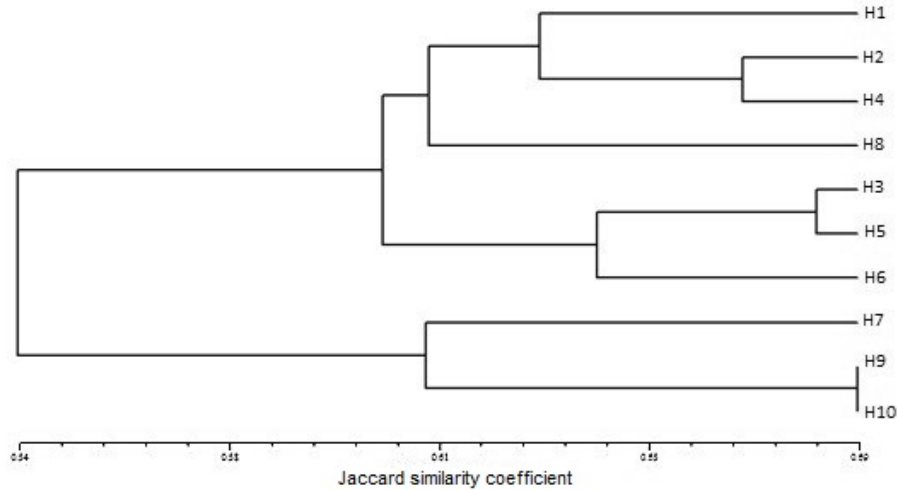
The total number of genotype specific marker loci is 104, and it varies from 10 (GOOFY) to 27 (UBC 808). A study with 224 physic nut genotypes from different regions of China, evaluated by ISSR markers, selected 15 primers with good reliability and reproducibility. The primers UBC 808, 809, and 834 were set as more informative. A total of 169 amplified fragments were obtained, ranging from 160 bp to 2.4 kb (Cai et al., 2010).

The most divergent physic nut genotype pair was H1 and H10 (Figure 2). The highest similarity (68.7%) was observed between hybrids H9 and H10 (Table 3).

The branching patterns in the dendrogram resulted in three major groups. Group I, formed by the hybrids H1, H2, H4, and H8; group II, formed by the hybrids H3, H5, and H6; and group III, formed by the hybrids H7, H9, and H10.

In Malaysia, molecular characterization of 48 physic nut accessions from three different regions was carried out using 10 ISSR primers. The percentage of polymorphism observed between populations ranged from 90.75 to 100%, and 94% of the total variation was

observed within populations, with 11 distinct groups. The estimate of the Shannon index, the effective number of alleles, and the number of different alleles are 0.556, 1.690, and 1.926, respectively (Arolu et al., 2012).



**Figure 2.** Dendrogram of genetic similarity estimated by Jaccard coefficient by UPGMA for 10 hybrids of *Jatropha curcas*. H1. JCUFS-01 x JCUFS-08; H2. JCUFS-01 x JCUFS-13; H3. JCUFS-04 x JCUFS-08; H4. JCUFS-03 x JCUFS-05; H5. JCUFS-04 x JCUFS-05; H6. JCUFS-04 x JCUFS-13; H7. JCUFS-03 x JCUFS-08; H8. JCUFS-04 x JCUFS-15; H9. JCUFS-03 x JCUFS-13; H10. JCUFS-01 x JCUFS-05.

In this study, the mean number of effective alleles is 1.542; whereas the number of different alleles is 1.817, and the Shannon index is 0.450.

Hybrids were analyzed based on 104 loci obtained with six primers. ISSR seems to be suitable and efficient to detect polymorphism in physic nut. Lower percentage of polymorphism was obtained by He et al. (2007) and Cai et al. (2010) using ISSR; however, it was higher than that reported by Machua et al. (2011), of 55.33%, using RAPD. Additional information was reported by Mastan et al. (2012), who obtained polymorphism of 56.43, 57.9, and 36.84, using RAPD, AFLP, and SSR, respectively. The optimum number of loci for the obtainment of genetic estimation accuracy is 103 polymorphic fragments for a stress of 0.0097, and the optimum correlation ( $r$ ) is 0.9962.

## DISCUSSION

Low genetic variability has been observed for physic nut by molecular markers, such as AFLP, SSR, RAPD, and ISSR in several studies in Brazil and in the world (Sun et al., 2008; Rosado et al., 2010; Salvador-Figueirôa et al., 2015). High genetic diversity is essential to obtain genetic gains by hybridization methods. Results obtained in this study, such as high polymorphism rate between the studied hybrids, may contribute to future breeding programs of this species.

Results showed high percentage of polymorphism (80.7%), with exception of the UBC 834 primer. High polymorphism rates among physic nut accessions were also found

in studies carried out by Arolu et al. (2012) and Senthil Kumar et al. (2009), highlighting the wide range of intrapopulation polymorphism.

High polymorphism rates (81.1%) and high genetic diversity were reported with 134 physic nut accessions collected in Chiapas (Mexico) using AFLP markers. Number of effective alleles, Shannon index, and Nei diversity was 1.303, 0.306, and 0.192, respectively (Ovando-Medina et al., 2011).

Results of this study are similar to those estimated for physic nut accessions collected in the State of São Paulo (Brazil), using AFLP, in which the mean number of different alleles was of 1.809, and the Shannon index was 0.386 (Pioto et al., 2015).

These results corroborate the mean values found in this study, which found mean number of effective alleles of 1.542, mean number of different alleles of 1.817, and Shannon index of 0.450. Studies with ISSR markers in 158 individuals from different populations of Yunnan (China) obtained mean values for the effective number of alleles of 1.382, Shannon index of 0.317, and polymorphism rate of 55.05% (Xiang et al., 2007).

Genetic distance provides wide genetic base (0.47 to 0.69), which can be used for breeding to obtain elite hybrids. However, considering that the lower the Shannon index, the lower is the degree of uncertainty and diversity of the individuals, and that the higher the Shannon index, the higher is the diversity, results show low to moderate levels of genetic diversity among hybrids. In general, suggesting low variability within the groups.

Similar values were found for the total number of loci. The primers UBC 808, UBC 809, and UBC 811 confirm the results obtained by Tanya et al. (2011), who also reported higher polymorphism rates with these primers.

In contrast to the results of this study, low percentage of polymorphism (19.9%) is observed using 13 primers, with 2.8 polymorphic bands per primer. Primer UBC 834 generated the highest number of polymorphic fragments (Alkimin et al., 2013), which is similar to the value obtained in this study.

Low percentage of polymorphism (35.5%) was also observed for physic nut from different countries by ISSR markers. Results are related to the limited number of germplasm in those countries (Basha et al., 2009). Results found by these authors corroborate those from Basha and Sujatha (2007), who detected low variation (33.5%) among the genotypes from a group of 42 genotypes from India. However, their results differ from those of Grativol et al. (2011), who tested 322 accessions from different regions of Brazil. Results indicate high polymorphism (91%) between accessions, and are similar to the values obtained in the present study.

Studies on markers have been reported for several species, such as *Ricinus communis* L., *Manihot esculenta* (Vidal et al., 2015), *Hevea brasiliensis* (Mantello et al., 2012), and *J. curcas* (Cammellia et al., 2012). In these studies, the minimum number of locus markers was determined, which results in good accuracy and optimizes time and resources for genetic characterization. During the analysis of genetic diversity among physic nut genotypes, previous selection of the most responsive primers for amplification and polymorphism detection should be carried out.

In this study, ISSR markers showed several informative bands in a single amplification. Similar results were obtained in genotypes of the genus *Jatropha*, revealing 100% polymorphism (Senthil Kumar et al., 2009). Mavuso et al. (2015) observed 85.19% polymorphism. These results highlight the effectiveness of ISSR markers in polymorphism detection in physic nut, when compared with other molecular markers. Hybrids tested in this study are clustered in three groups (Figure 2).

Proportionally, this study better discriminates the genetic diversity among hybrids when compared with results reported by Soonthornyatara et al. (2015), who assessed 138 physic nut accessions from different countries. These authors used ISSR and AFLP markers and obtained two groups, while Kole et al. (2015) (with 182 genotypes) and Verma et al. (2013) (with 30 accessions) used RAPD markers and found two and seven groups, respectively.

Based on the dendrogram, maximum homogeneity and heterogeneity of hybrids within and between groups, respectively, were evaluated. Information on the genetic diversity observed among hybrids could help obtain genetic gains associated with the productive potential of future hybrids.

Group I enclose the hybrids obtained from parents JCUFS-01, JCUFS-03, JCUFS-04, JCUFS-05, JCUFS-08, JCUFS-13, and JCUFS-15; group II presents hybrids with the parents JCUFS-04, JCUFS-05, JCUFS-08, and JCUFS-13. The hybrids H3 (JCUFS-04 x JCUFS-08), H5 (JCUFS-04 x JCUFS-05), and H6 (JCUFS-04 x JCUFS-13) showed high similarity (65.7%) and belong to the same group (Group II), and present JCUFS-04 as common parent.

Hybrid H8 (JCUFS-04 x JCUFS-15), despite belonging to group I, also showed moderate similarity level (58.7%) with hybrids H3, H5, and H6.

The parent JCUFS-04 is present only in groups I and II. This common parent concentrates favorable alleles for seed production, mass of one hundred seeds, stem diameter, branch number, height branch, and crown diameter. JCUFS-05 presents favorable alleles for seed production, stem diameter, and plant and branches height. Besides JCUFS-04 and JCUFS-05, the parent JCUFS-13 also stood out due to favorable alleles for mass of one hundred seeds, number of secondary branches, and canopy diameter (Santana et al., 2013). Groups I and II comprise hybrids derived from crossings between these parents, evidencing the potential of these groups to improve the genetic gains in physic nut.

Group III consists of hybrids H7 (JCUFS-03 x JCUFS-08), H9 (JCUFS-03 x JCUFS-13), and H10 (JCUFS-01 x JCUFS-05). JCUFS-01 parent is present in crossings of groups I and III. JCUFS-13 parent can be distinguished by higher seed oil content; it has short vegetative stage, low proportion of staminate to pistillate flowers in an inflorescence, and high seed production performance (Pessoa, 2011).

Hybrids H1 and H10 were the most divergent; however, they showed moderate level of genetic diversity (47.0%). Hybrid H9 (JCUFS-03 x JCUFS-13) and H10 (JCUFS-01 x JCUFS-05) presented high genetic similarity (69.0%) (Figure 1 and Table 3). These hybrids are distinguished by the existence of positive interaction between parents, i.e., specific combining ability (Santana et al., 2013).

Heterotic groups are fundamental in breeding programs due to the possibilities of obtaining elite hybrids. The information generated by ISSR markers in this study, could be useful to plan crossings aiming at heterosis in future hybrid combinations, in order to obtain genetic material with consistent superiority.

Studies show the high genetic similarity among physic nut accessions in several countries, obtained by different molecular markers (Sudheer Pamidimarri et al., 2009; Singh et al., 2010; Khurana-Kaul et al., 2012). Physic nut germplasm from islands of the Pacific Ocean and mainlands of Brazil, Mozambique, and Senegal shows high level of genetic similarity within populations (Ricci et al., 2012), reinforcing the hypothesis that physic nut populations may present high homozygosity (Basha et al. 2009).

As a monoecious species, heterozygous locus is expected, with predisposition for outcrossing. However, physic nut is able to produce fruits by both self-pollination and cross-



pollination (Divakara et al., 2009; Wang and Ding, 2012). This species does not present self-incompatibility problems, resulting in high fertilization rates in geitonogamy processes (above 80%), whether or not the pollen-donor flower belongs to the same inflorescence of the same plant (Paiva Neto et al., 2010).

Low genetic diversity detected among physic nut accessions in different regions of the world may result from the introduction of seeds of a small number of plants in countries with low diversity; or seeds from geographical sources that do not have high genetic diversity of this species, or both. Few events might have occurred in the past for the introduction of the genetic material of physic nut, which increased the inbreeding level due to the narrow genetic diversity, resulting in increased homozygosity in subsequent generations (Pratima et al., 2013).

Physic nut accessions from different regions of Brazil present narrow genetic base, probably due to a common ancestral and to the reproduction of these genetic materials, preferably by geitonogamy. These phenomena contributed to isolated occurrence of plants in the country. Despite the narrow genetic base, a high phenotypic plasticity enables wide adaptation in different environments (Rosado et al., 2010).

This information corroborates with moderate to high levels of genetic similarity obtained in this study. This is probably due to the hybrids of crossings between parents from the same region of Brazil (Minas Gerais), with the exception of parents JCUFS-13 and JCUFS-15 (Goiás). Thus, the genetic variability among accessions is genetically dependent on their origin (Santos et al., 2010). The genetic and geographic isolation affects the level of genetic diversity between species (Tripathi et al., 2007).

Human activities are crucial for genetic diversity of physic nut, and the low level of variability can be explained by these activities, resulting in high frequency of homozygotes. However, most of the genetic variability can be essentially epigenetic, which could explain the phenotypic variation without the need for changes in DNA sequences (Maghuly et al., 2015).

In order to obtain genetic and phenotypic gains for the success of physic nut-breeding program, the introduction of accessions of the centers of origin is crucial, due to the high diversity found in these sites. High homozygosity and genetic uniformity were observed among physic nut accessions cultivated in several countries in South America, Africa, and Asia, and supports the general idea that the region of the Mexican Central America and Guatemala is the center of origin and presents wide genetic variability and levels of heterozygosity (Trebbi et al., 2015).

Despite the several information on the genetic variability among physic nut accessions from different regions of the world, few studies (Basha and Sujatha, 2009; Parthiban et al., 2009) have been carried out on hybrids, and thus, studies on the association based on the co-segregation between potential genotypes for the traits of interest, with the use of molecular markers, such as SSR, AFLP, and ISSR, are necessary (Achten et al., 2010).

Therefore, the development of new hybrids and cultivars is an essential requirement to increase yield. The exploitation of genetic diversity and increased characterization of genetic resources is paramount criterion for physic nut breeding. Besides the use of information on molecular parameters and genetic similarity, phenotypic traits are also important for the obtainment of hybrids with superior agronomic performance, such as oil yield, content, and quality. Hybridization is a method used in genetic plant-breeding programs, as well as gathering the desirable traits of different parents in a single individual. Studies on intraspecific hybridization revealed high associations between agronomic traits and yield components in six physic nut hybrids (Tar et al., 2011).

The generation of hybrid aims to gradually increase the frequency of favorable alleles and to obtain superior individuals to their populations. When evaluating the yield of the seeds obtained, superior hybrids may be obtained based on information regarding heterosis (Islam et al., 2011) and genetic variability. Thus, the commercialization of physic nut hybrids may be considered based on heterosis for several production traits.

ISSR primers used in this study are important for the detection of polymorphism. They are also efficient in studies of genetic variability among physic nut hybrids from diallel crossings. In addition, good discrimination was found for the clustering when compared with several studies on genetic variability between individuals grown in different regions of the world.

Therefore, more achievements are important for the constitution of core collections aiming at genetic diversity for the obtainment of superior genotypes, since the success of these hybrids will result from the heterotic effect obtained by crossing genotypes with specific and general combining ability.

### Conflicts of interest

The authors declare no conflict of interest.

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