

Full Length Research Paper

Genetic diversity and molecular characterization of physic nut genotypes from the active germplasm bank of the Agricultural Research Company of Minas Gerais, Brazil

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The genetic diversity among 46 accessions of physic nut was estimated with Nei and Li's similarity coefficient based on a collection of 69 random amplified polymorphic DNA (RAPD) sequences and 37 inter-simple sequence repeat (ISSR) polymorphic loci. The genetic distance between accessions ranged from 0.13 to 0.76, with an average genetic distance of 0.21. The most divergent genotypes were 86, 71 and 83. A dendrogram (generated by the unweighted pair group method with arithmetic mean, UPGMA) of the joint data matrix was constructed and presented only two phylogenetic groups, one of which contained only three individuals; the remaining group included 95.6% of the analyzed genotypes. The low genetic diversity measured in this study indicates the need to broaden the genetic base and increase the variability of this species. The amplification products generated by amplification of SSR primers were used to characterize toxicity alleles, and none of the accessions presented patterns characteristic of non-toxic accessions.

Key words: *Jatropha curcas*, inter-simple sequence repeat (ISSR), random amplified polymorphic DNA (RAPD), microsatellites, toxicity.

INTRODUCTION

Concerns about the scarcity and high price of oil, as well as global climate change have caused studies of renewable energy sources to be intensified in recent years. Tests of biofuels are ongoing across the world, especially in the European Union, China and India (Fairless, 2007). Brazil is the country with the greatest potential for renewable energy production due to its tropical location with high luminosity and means annual

temperatures, which are associated with water availability and regular rainfall. It is estimated that there are more than 200 species of oilseeds with the potential to produce vegetable oil for renewable energy feed stocks (Teixeira, 2005).

Physic nut (*Jatropha curcas* L.) is an oil crop found in nearly all intertropical areas. The nut occurs naturally throughout Brazilian territory and may be a promising oilseed alternative for biodiesel production (Rosado et al., 2010). The genus *Jatropha* includes over 170 species, including *J. gossypifolia*, *J. mollissima* and *J. curcas* (Saturnino et al., 2005). The oil content has shown a

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phenotypic range from 31 to 41%, with abundant oleic and linoleic acid (Wang et al., 2008). In addition to the physic nut's value as an oilseed, the cake resulting from oil extraction can be used as a fertilizer or a protein-rich supplement for the diets of ruminant and monogastric animals. However, physic nut cake contains a number of toxic compounds, allergens and anti-nutritional factors, the most prevalent of which is phorbol ester (diterpene) (Rakshit et al., 2008). Although there are no reports of non-toxic Brazilian genotypes, several accessions from some provenances in Mexico contain very low or non-detectable levels of phorbol esters (Martinez-Herrera et al., 2006).

Although *J. curcas* has gained recognition as a crop with great potential for biofuel production, it is still considered a semi-wild undomesticated plant with considerable performance variability (Achten et al., 2008). Understanding the degree of genetic diversity in physic nut accessions is essential for the establishment of pre-breeding programs that will contribute to the development of improved cultivars (Achten et al., 2010). The use of molecular markers has allowed for studies on the intra- and inter-specific genetic variability in *Jatropha* spp (Basha and Sujatha, 2007). Molecular markers, such as microsatellites or simple sequence repeats (SSR), inter simple sequence repeat (ISSR) and random amplified polymorphism DNA (RAPD), have been important tools for the analysis of genetic diversity, germplasm characterization and the evaluation and identification of important agronomic genes, permitting considerable progress in the genetic improvement programs of several crops (He et al., 2007).

Several studies have analyzed the genetic diversity of *J. curcas* accessions from different regions of the world. Low levels of genetic diversity have been reported in *Jatropha* accessions from India (Ginwal et al., 2004, 2005; Kaushik et al., 2007; Rao et al., 2008), and from China (Shen et al., 2010; Sun et al., 2008). However, high genetic variability have been related in *J. curcas* accessions from India (Gupta et al., 2008; Gohil and Pandya, 2008), and from Mexico (Pecina-Quintero et al., 2011). Analysis of Brazilian accessions were performed by Abreu et al. (2009), Rosado et al. (2010) and Grativol et al. (2011). The results confirm there are limited genetic variations in the Brazil gene pool. Besides genetic diversity studies, molecular markers have been applied to identify and to select genotypes with remarkable traits. In a study developed by Pamidimarri et al. (2009a), the authors sought to characterize and identify the molecular markers that differentiate toxic and non-toxic *J. curcas* accessions using RAPD, amplified fragment length polymorphism (AFLP) and SSR markers.

The objective of the present study was to analyze the genetic diversity among 46 accessions of physic nut from the Active Germplasm Bank of the Agricultural Research

Company of Minas Gerais/Northern Minas Gerais Regional Unit (Empresa de Pesquisa Agropecuária de Minas Gerais-Unidade Regional do Norte de Minas - EPAMIG/URENM) using RAPD and ISSR markers, as well as to characterize the presence of toxicity alleles using SSR markers.

MATERIALS AND METHODS

Young leaves of 46 accessions of physic nut were obtained from the Active Germplasm Bank (AGB) of the Agricultural Research Company of Minas Gerais-Northern Minas Gerais Regional Unit (EPAMIG/URNM). The origins of the evaluated accessions are presented in Table 1. Genomic DNA was extracted from the leaves using the method proposed by Basha and Sujatha (2007), with a modification for DNA purification proposed by Michaels et al. (1994).

Analysis of genetic diversity

Amplification reactions for the RAPD analysis were performed according to the methodology proposed by Williams et al. (1990) using the following final concentrations: genomic DNA (30 ng/ μ l), Tris-HCl/KI 10x buffer (10 mM/50 mM), $MgCl_2$ (2.8 mM), dNTPs (0.1 mM each), primer (5 pmol), Taq DNA polymerase (1 unit) and ultrapure water to a final volume of 25 μ l. Amplifications were performed under the following conditions: one cycle at 94°C for 3 min; 40 cycles at 94°C for 15 s, 35°C for 30 s and 72°C for 1 min; and a final period at 72°C for 7 min. The temperature of the mixture was then reduced to 4°C. The primers were selected randomly and acquired from the Kit Operon® (OPA06, OPA10, OPB10, OPC07, OPC10, OPC14, OPC18, OPD04, OPD07, OPE05, OPF16, OPF19, OPG17, OPH11, OPH14, OPJ15, OPJ20, OPK01, OPL03, OPM08, OPN11, OPO10, OPP03, OPP09, OPU06, OPU10, OPU19, OPV08, OPV15, OPV17, OPY20, OPW02 and OPW17).

13 ISSR primers were used in the following amplification concentrations for a final volume of 10 μ L: DNA (2.5 ng); Tris-HCl/KCl pH 8.3 (10 mM/50 mM), $MgCl_2$ (25 mM); dNTPs (0.2 μ M); primer (0.4 μ M); Taq DNA polymerase (0.6 units) and ultrapure water to complete the volume. The reactions consisted of an initial denaturation phase at 94°C for 4 min, followed by 35 denaturation cycles (94°C/30 s), annealing (1 min), extension (72°C/2 min) and a final extension phase at 72°C for 7 min. After this step, the temperature was reduced to 10°C until removal of the samples. The primers used for amplification were obtained from the UBC collection (primers developed by the Laboratory of Biotechnology, University British Columbia, collection number 9): UBC 812, UBC 829, UBC 834, UBC 867 with annealing temperature (T_a) = 50°C; UBC 810, UBC 817, UBC 847, UBC 861, UBC 866, UBC 873, UBC 880 and UBC 891 with T_a = 55°C, as well as UBC 889 with T_a = 58°C.

Products resulting from the RAPD and ISSR marker amplifications were separated by 1.2% agarose gel electrophoresis at 100 V in TBE 1x buffer for approximately 2 h and stained with an ethidium bromide solution at 0.2 mg L⁻¹ for 15 min. The amplified fragments were visualized under ultraviolet light and photographed in the UVP® Life Science software digital photo-documentation system. The data obtained by electrophoresis were scored for the presence (1) and absence (0) of bands. The distance between genotypes was calculated using Nei and Li's similarity coefficient. The genotypes were grouped in a dendrogram using the

Table 1. State and country, city/population and collection for the *J. curcas* accessions used in the genetic diversity analysis.

State/Country	City/population	Collection number
Minas Gerais – BR	Araçuaí	43
Minas Gerais – BR	Arinos	63, 65
Minas Gerais – BR	Carbonita	55, 58, 59
Minas Gerais – BR	Diamantina	44
Minas Gerais – BR	Jaíba	71, 83, 86, 87, 88
Minas Gerais – BR	Janaúba	23, 25, 27, 45, 74, 76, 78, 79, 81, 82
Minas Gerais – BR	Januária	50, 52, 54
Minas Gerais – BR	Malacacheta	41
Minas Gerais – BR	Nova Porteirinha	1, 4, 7, 30, 32, 36
Minas Gerais – BR	Porteirinha	10, 12, 15, 16, 17, 21
Bahia – BR	Valente	69
China		CH1, CH2, CH3
Cabo Verde		CB0, CB7, CB11, CB13

unweighted pair group method with arithmetic mean (UPGMA) based on the genetic distance data generated by the two sets of markers. The statistical program Genes was used (Cruz, 2006) for data analysis.

Characterization the presence of toxicity alleles by SSR specific primers

Pamidimarri et al. (2009a) described 12 pairs of SSR primers that have been used to amplified specific bands associated to toxicity/non-toxicity trait in *J. curcas*. In the present study, all accessions from AGB/EPAMIG were characterized by using the same methodology in order to confirm the presence or absence of the toxicity alleles. Amplification was performed in a total volume of 25 mL with the following components: genomic DNA (25 ng), Tris-HCl/KCl (10 mM/50 mM), MgCl₂ (3.0 mM), dNTPs (0.2 mM), primer (0.4 µM each), Taq DNA polymerase (1.0 unit) and ultrapure water to complete a volume of 25 µl. The reactions consisted of an initial denaturation at 94°C for 3 min; 35 cycles at 94°C for 30 s, the specific annealing temperatures of each primer for 30 s and extension at 72°C for 40 s; and a final extension at 72°C for 4 min. The temperature was then decreased to 4°C until removal of the samples. The primers Jc10, Jc24, Jc1, Jc6, Jc9 and Jc21 were subsequently amplified at an annealing temperature of 47°C, and Jc41, Jc58 and Jc66 were amplified at 50°C. The amplification products were separated using electrophoresis on a 30% polyacrylamide gel at 100 V in 1× TAE buffer and stained with a 0.2% silver solution (AgNO₃) according to the methodology of Beidler et al. (1982). The fragments produced by SSR specific primers were compared by size in base pairs (bp) with those reported by Parmidarri et al. (2009a), using molecular weight marker.

RESULTS AND DISCUSSION

Analysis of genetic diversity

A total of 33 RAPD primers were used, of which 24

presented polymorphisms and 9 showed no polymorphic bands. The number of amplified bands per primer ranged from 1 (OPA06, OPC14, OPC18, OPD04, OPD07, OPG17, OPK01 and OPW17) to 10 (OPH11) with an average of 2.8 polymorphic bands per primer. A total of 281 bands were amplified, of which 69 (24.5%) presented polymorphism and 212 (75.5%) were monomorphic. All 13 ISSR primers presented polymorphism in the 46 accessions evaluated. Out of 189 total bands, 37 (19.9%) were polymorphic and 149 (80.1%) were monomorphic. The number of polymorphic bands amplified per primer ranged from 1 (UBC 861, UBC 866, UBC 867 and UBC 891) to 6 (UBC 834) with an average of 2.8 polymorphic bands per primer.

Basha et al. (2009) studied physic nut accessions from different countries and attributed the low genetic diversity between accessions of the same country revealed by RAPD and ISSR markers to the limited number of initial *J. curcas* germplasm introductions in these countries. Conversely, He et al. (2007), also using ISSR markers, reported high levels of genetic diversity among eight *J. curcas* populations. Assessments of intraspecific variations in *J. curcas* involving accessions from different agro-climatic zones of India and China was conducted using a wide variety of molecular markers, and these studies have also shown low to moderate levels of genetic diversity (Tatikonda et al., 2009; Basha et al., 2009).

Basha and Sujatha (2007) applied 400 RAPD primers and 100 ISSR primers to evaluate the extent of genetic diversity in a representative group of 42 *J. curcas* accessions covering different agro-climatic zones of India, along with a Mexican non-toxic accession, and detected modest levels of genetic variation in the Indian germplasm. In support of these results, Reddy et al.

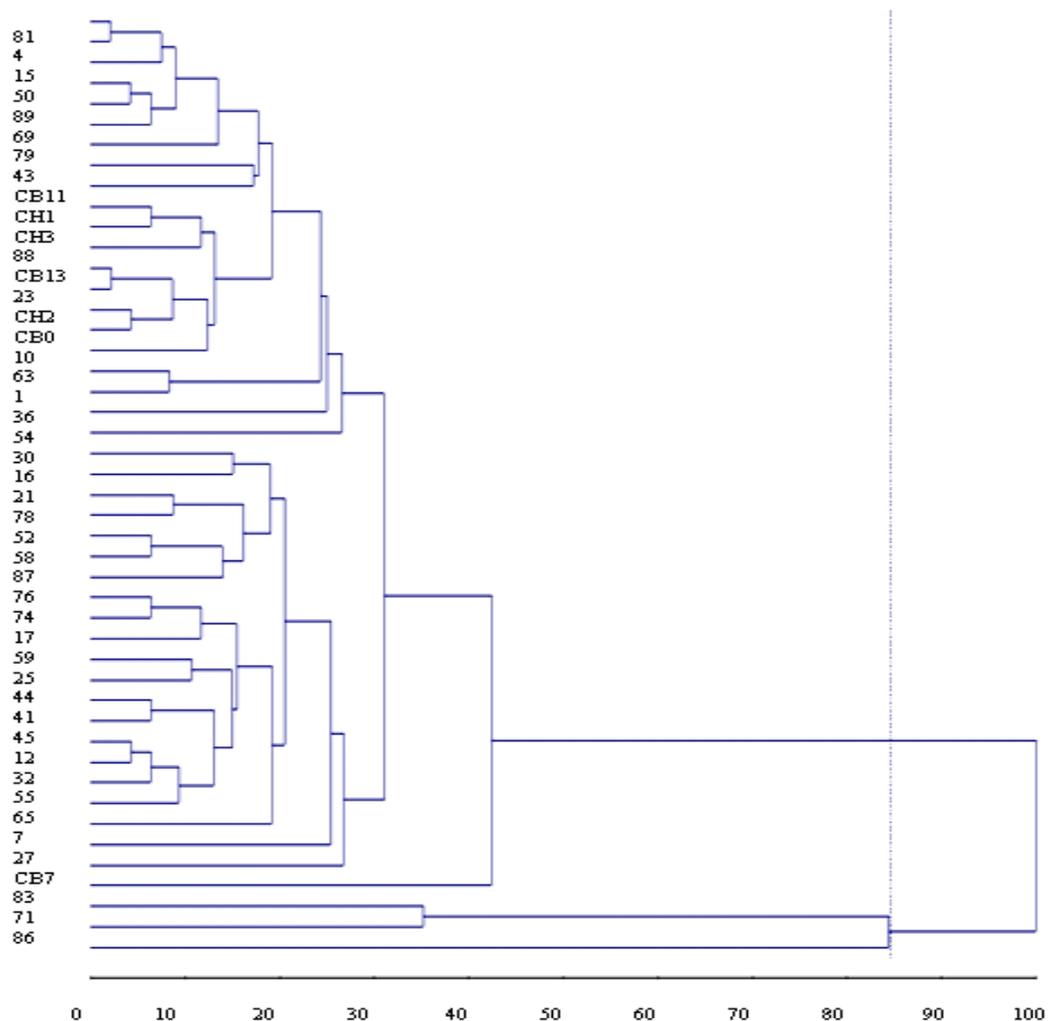


Figure 1. Dendrogram (UPGMA) representing the genetic relationships among the 46 accessions of *J. curcas* based on Nei and Li's similarity coefficient obtained from the group analysis of RAPD and ISSR primers.

(2007) reported relatively low levels of polymorphism in RAPD and AFLP markers among 23 accessions selected from across India.

In Brazil, low levels of genetic diversity have been reported in many studies. Rosado et al. (2010) analyzed 192 accessions of *J. curcas* distributed over a wide geographic area of Brazil and showed that of the 96 RAPD primers used, only 12 showed polymorphism (6.2%). In the analysis of the six SSR primers used in the same study, one to two bands were amplified per primer. A low genetic distance was observed, ranging from 0 to 0.86, with mean value of 0.11.

Grativol et al. (2011) worked with seven ISSR primers in 322 Brazilian accessions of *J. curcas* and encountered a 91% polymorphism rate, identifying 275 rare polymorphic bands present in less than 15% of the

accessions. The average distance between accessions was 0.10 and ranged from 0.05 to 0.17. Despite the elevated number of polymorphisms generated by the ISSR primers, generally low genetic diversity was observed among genotypes. The genetic distance matrix generated by Nei and Li's similarity coefficient showed a minimum genetic distance of 0.13 between accessions 4 (Nova Porteirinha-MG) and 81 (Janaúba-MG) and a maximum distance of 0.76 between accessions CB7 (Cape Verde) and 71 (SADA-Jaíba-MG) with an average distance of 0.21.

Analysis of the dendrogram (Figure 1) revealed the two separate groups of accessions, one consisting of accessions 86, 71 and 83 (all from the SADA Company) and one composed of the remaining 43 accessions. On subdividing group I, it was found that accession 86

remained isolated, while accessions 83 and 71 remained grouped with an approximately 64% degree of similarity. The second group consisted of 93.48% of the population analyzed and was also subdivided into three sub-groups. Sub-group I, consisting of accession CB7, originated from Cape Verde; sub-group II consisted of accessions 27 to 30, grouping materials collected exclusively in Brazil; and the third subgroup was composed of genotypes 54 to 81, characterized by accessions of different origins. Although this third subgroup contained accessions obtained from China, as well as Brazilian lines, the high similarity found in the subgroup (greater than 70%) demonstrates the low genetic diversity among accessions from different origins, confirming many previous studies.

The formation of a main group containing most of the accessions reinforces the hypothesis that physic nut genotypes from across the world share a common ancestry and rejects the idea of genetic diversity by origin. By characterizing 48 genotypes of physic nut from different regions of India and one from Mexico with RAPD markers and microsatellites, Basha and Sujatha (2007) found that 83% of the characterized Indian accessions were placed into two major groups, while one group was formed exclusively of the Mexican accession. Sun et al. (2008), using 17 microsatellite markers, was unable to separate 58 Chinese genotypes. When studying *J. curcas* genotypes from different regions of the world, Basha et al. (2009) encountered only two groups, one contains genotypes from the Central American region and another including genotypes from the other regions of the world.

The low genetic variability observed both in the present study and in previously published work can be attributed to the few initial introductions of the species, both in Brazil and in other parts of the world. This problem may also have been exacerbated by the diffusion of the physic nut, mainly through plant propagation (Rosado et al., 2010). Other factors may contribute to the low genetic variability observed among accessions. Although the physic nut is considered an allogamous plant, both inbreeding and apomixis are present at significant levels. Apomixis rates between 5 and 32% and natural inbreeding rates greater than 20% have been observed in *J. curcas* populations (Bhattacharya et al., 2005; Chang-Wei et al., 2007; Juhász et al., 2009).

To obtain genetic gain in breeding programs, it is essential to introduce material from other locations or countries, primarily from the center of origin and/or diversity of the species (Bueno et al., 2001). Among the objectives improving physic nut are the development of cultivars that have no significant toxicity, increased grain yield and high oil content, synchronous opening of male and female flowers and tolerance to disease, drought and insect pests (King et al., 2009). However, obtaining the genetic improvement to meet these goals is directly

dependent on the presence of genetic variability in the breeding population. Besides, there is an immediate need to study methods for bolstering the genetic base of *J. curcas* by using different strategies like the selection of superior genotypes, mutagenesis or interspecific hybridization and polyploidy.

High levels of intraspecific and inter-specific genetic diversity were found in *Jatropha* spp. by using different molecular markers. The authors recommend the use of inter-specific crosses to increase the genetic diversity and add new alleles to develop new hybrids with higher yield potential (Wen et al., 2010; Pamidimarri et al., 2009b; Tanya et al., 2011).

Characterization of the presence of toxicity alleles by SSR specific primers

Among the 12 pairs of SSR primers tested for the characterization of toxicity alleles in *Jatropha*, nine generated amplification products and three (Jcps20, Jcms21 and Jcms30) were not amplified. The differences between accessions in this study were determined by the presence or absence of specific bands compared with those characterized by Pamidimarri et al. (2009a).

The primers Jcids10, Jcids24, Jcids58, Jcps6 and Jcps9 generated at least one allele with lengths (108, 104, 288 and 140 base pairs, respectively) similar to the toxic accessions observed in the work of Pamidimarri et al. (2009a) (Table 2). Primers Jcids66 and Jcps1 presented two alleles each; for each primer, one allele was of a size similar (228 and 162 base pairs, respectively) present in both the toxic and non-toxic genotypes, while the other allele was of a different size (400 and 80 base pairs, respectively) than that suggested by Pamidimarri et al. (2009a). The primer Jcps21 showed only one allele, with a size (208 base pairs) similar to that of the toxic individuals. None of the 46 accessions evaluated amplified the alleles for non-toxicity identified by Pamidimarri et al. (2009a). Although the origin of the accessions obtained from the SADA company (71, 83, 86, 87 and 88) was unknown, none of the analyzed individuals showed specific allele for non-toxic materials.

According to Heller (1996), toxic genotypes which contain high amounts of phorbol ester, trypsin inhibitors, curcin and phytates, are found in Latin America and Africa; the only non-toxic genotype yet discovered is of Mexican origin. The toxicity in *J. curcas* seeds is caused by the presence of high levels of phorbol esters (high concentration located mainly in the tegument) 1000 times higher than in the non-toxic genotypes, otherwise the variation in curcin levels is non-significant among toxic and non-toxic accessions (He et al., 2011). A total of six RAPD primers, two ISSR primers and one SCAR primer were reported as specific markers for toxic accessions

Table 2. Primers for SSR loci of the *Jatropha curcas* genome used by Pamidimarri et al. (2009a) to differentiate toxic from non-toxic genotypes.

Primer	SNTA ¹ (pb)	STA ² (pb)	SAIS ³ (pb)
Jc ds 10	108/122	108/122	108
Jc ds 24	204/216	204/216	204/216
Jc ds 41	102/114	102/114	102/114
Jc ds 58	104/112	104/112	104
Jc ds 66	216/228	216/228	228/400
Jc ps 1	132/162	132/162	80/162
Jc ps 6	288/305	288/380	288
Jc ps 9	140/132	140/132	140
Jc ps 20	271/260	271/278	-
Jc ps 21	189/200	189/208	208
Jc ms 21	81/89	75	-
Jc ms 30	135/144	144/148	-

SNTA¹, size of non-toxic alleles and STA², size of toxic alleles reported by Pamidimarri et al. (2009a); SAIS³, size of alleles identified in the study; bp, base pairs.

obtained from India, and for Mexican accessions, 17 RAPD primers, four ISSR primers and one SCAR primer have been obtained (Basha and Sujatha, 2007). He et al. (2011) also identified AFLP primers that can be used to characterize toxic and non-toxic accessions. According to same authors, the phorbol ester trait is controlled by only one locus with maternal effect and the use of specific molecular markers must be considered in order to select the accessions. In addition to revealing the level of genetic diversity of the local germplasm, the use of specific primers allows for the differentiation of accessions, facilitating assisted selection and the development of new cultivars.

The development of *J. curcas* cultivars, non-toxic, with high potential of yield, high oil content and pests tolerant would provide the provision of non-toxic cake for animal feed and human consumption (Francis et al., 2013). In the present study, the *J. curcas* accessions from o AGB/Epamig showed low levels of genetic diversity. Priority should be given to the introduction of new genotypes and/or by performing inter-specific cross pollination between *J. curcas* and other *Jatropha* species. In addition, analysis of phenotypic diversity in germplasm collections may facilitate the identification and selection of accessions that can be used as parents in breeding programs.

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