Physic nut germplasm genetic diversity identified by internal simple sequence repeats (ISSR) markers

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ABSTRACT

The physic nut has attracted great interest for their socioeconomic promising vegetable oil production capacity that can be used in biodiesel manufacturing. However, the species is still under a domestication process and many studies are still needed to start a breeding program. In this context, this study aimed to evaluate the genetic diversity in 29 physic nut accessions from two germplasm banks using 14 ISSR markers. Genetic characterization revealed that 86.64% of the genetic variation is present within the groups, whereas 13.36% corresponded to the variation between the groups. The total mean number of alleles per locus was 1.99 and the effective number of alleles corresponded to 1.42. The Bayesian analysis allocated the access into four clusters, showing that the clusters formed showed no relation to the origin of access. The Jaccard similarity index indicated that the extent of similarity between accessions ranged from 0.23 to 0.94. The dendrogram formed the same four access groups as those formed by the Bayesian analysis, and all groups were in agreement in both analyses. The study demonstrated the need and importance of a germplasm collection from several regions and countries in order to preserve the genetic diversity of the species and the development of Euphorbiaceae breeding programs.

Key words: *Jatropha curcas*, molecular characterization, germplasm bank, ISSR.

INTRODUCTION

The *Jatropha L.* genus is constituted of 175 tropical and subtropical species (Thomas et al., 2008), being the *J. curcas* L., also known as physic nut, the most popular in Brazil. This species originated probably in Central America, more specifically in Mexico, and is found in other tropical areas of the world (Openshaw 2000; Dias et al., 2012). In Brazil, it grows in almost all regions, is adaptable to variable climate conditions, but is still under a domestication process (Dias et al., 2012).

Most importantly, the physic nut fruit has attracted great socioeconomic interest for its capacity to produce vegetable oil. A viscous oil is extracted from their seeds and used for many purposes such as soap manufacturing, the cosmetic industry, and as diesel or kerosene substitutes with the advantage of burning without emitting smoke or odors (Suresh et al., 2012). Other characteristics that make this euphorbiaceae attractive for the agribusiness are its tolerance to hydric deficit, the fact that it does not serve as food by vertebrate herbivores, its low demand for nutrients and the capacity to recover degraded areas due to its deep roots and to grow in low fertility soils (Teixeira 2005; Thomas et al., 2008). This oleaginous takes three to four years to reach a productive age, which lasts for at least 40 years and produces a minimum of two tons of oil per hectare. It is estimated that the content of oil found in their seeds varies from 40 to 50% (Heller 1996; Teixeira 2005).

The physic nut has already been explored successfully in India, in the African continent and in Central America (Borman et al., 2103; Osório et al., 2014). In Brazil, however, the lack of scientific knowledge about this culture makes dissemination difficult and the need for more studies by research institutions necessary to make safe technical recommendations on their cultivation, harvesting and industrial use.

In order to get physic nut genetic improvement and superior genotypes development, it is essential to introduce the *J. curcas* L. to expand species germplasm active banks. To have a successful program, it is fundamental to gather the greatest genetic variability possible for the development of new genotypes and to explore accessions with desirable traits (Frankel 1984; Marques and Ferrari 2008). To use this pre-existing genetic variability, extension studies on this variability in the available germplasm are necessary with the use of molecular tools together with several other studies.

Based on the need to generate subsidies for future breeding programs with the species and considering its promising economic potential, several germplasm banks from several institutions are being studied in many countries. The main objective of these studies was to characterize the genetic variability of physic nut accessions gathered in germplasm banks (Basha and Sujatha 2007; Ranade et al., 2008; Tatikonda et al., 2009; Wen et al., 2010; Kanchanaketu et al., 2012; Raposo et al., 2014). A survey based on databases shows that, in Brazil, most studies about this species deals with the plant's morphoagronomic characteristics (De Arruda et al., 2004; Reis et al., 2015). They also analyze the physical and chemical characteristics of its oil and pie (FeijãoTeixiera 1987; De Souza et al., 2009) and the

development and transferability to other macrosatellites initiators for the species (Bressan et al., 2012).

Knowledge of the genetic variability gathered in germplasm banks is essential for the rational use of genetic resources in breeding programs. In addition, such characterization can identify duplicates thus cutting banks maintenance costs by reducing the number of accessions to be preserved (Valois 1998). Therefore, the objective of the present work is to evaluate the genetic diversity of physic nut accessions originated from the UNICAMP Multidisciplinary Center for Agricultural, Biological and Chemical Research Germplasm Bank and accesses from the Sergipe Federal University Active Germplasm Bank, using ISSR (*Inter Simple Sequence Repeats*) markers.

MATERIAL AND METHODS

Twenty-nine *J. curcas* accessions originated from several Brazilian regions and other countries were studied (Table 1) and organized into two groups. One group included accessions 1 to 12, originated from CPQBA (Unicamp's Center for Multidisciplinary Chemical, Biological and Agricultural Research) Germoplasm Bank (Figure 1) and the other included accessions 13 to 29, originated from the Germoplasm Active Bank located at the Sergipe Federal University (UFS) Agronomic Engineering Department, in the county of São Cristovão , SE, Brazil.

Table 1. Physic nut germplasm bank accessions identification.

IDENTIFICATION	ACCESSIONS	ORIGIN
1	CPQBA 36	IAC, SP*
2	ORACÍLIA 39	Janaúba, MG
3	GONÇALO 34	Janaúba, MG
4	FILOMENA 38	Janaúba, MG
5	SOCORRO 37	Socorro, SP
6	GLYN 23	CPQBA
7	FORTALEZA 26	Fortaleza, CE
8	BENTO 37	Janaúba, MG
9	PARAGUAÇU 34	Janaúba, MG
10	CAMBOJA	Indochina
11	UFSCAR	São Paulo
12	CANADIA	Canadá
13	JCUFLA001-1	UFLA, MG
14	JCUFLA001-2	UFLA, MG
15	JCUFLA001-3	UFLA, MG
16	JCUFLA001-4	UFLA, MG
17	JCUFLA001-5	UFLA, MG
18	JCUFLA001-6	UFLA, MG
19	JCUFLA001-7	UFLA, MG
20	JCUFLA001-8	UFLA, MG
21	JCUFLA001-9	UFLA, MG
22	JC016URVSHGO	Goiás
23	JC011URVGO	Goiás
24	JC012URVMG	Minas Gerais
25	JC013URVGVGO	Goiás
26	JC014URVES	Espírito Santo
27	JC015URVSHGO	Goiás
28	JC016URLAGSE	Sergipe
29	JC017URBAHBA	Bahia

*IAC = Instituto Agronômico de Campinas; SP = São Paulo; MG = Minas Gerais; CE = Ceará; UFLA = Universidade Federal de Lavras and CPQBA, SP = Center for Multidisciplinary Chemical, Biological and Agricultural Research at the Unicamp.

For the genetic diversity analysis, the DNA of each accession was extracted from the macerated vegetal tissue using the CTBA protocol modified by Doyle and Doyle (1990). After electrophoresis, DNA samples were quantified by the intensity of the fluorescence emitted by the SYBR® Safe Invitrogen, Carlsbad, USA, under an UV light, in agarose gels at 0.8% p/v. This intensity was compared to patterns with molecular weights and specific concentrations and known phage   DNA.

This study tested 42 ISSR (*Inter Simple Sequence Repeats*) initiators (Zietkiewicz et al., 1994; Wolfe et al., 1998) to define the best amplification conditions such as temperature recoupling. This technique involves the use of SSRs (*Simple Sequence Repeats*) as initiators in PCR (*Polymerase Chain Reaction*) reactions to generate multiloci markers.

Based on amplification patterns obtained, 14 initiators with the best band patterns were selected (Table 2) and used during the analyses. PCR reactions for the ISSR initiators were performed at the final volume of 20 µL with around 10 *n*g of the DNA. They also included 0.25 µM of the initiator; 250 µM of each deoxyribonucleate triphosphate (dNTP); Buffer solution 1 X (50 mM of KCl; 10 mM of Tris-HCl, pH 8.9); 1.5 mM of MgCl2; 5 µg of BSA and 1 U of Taq DNA Polimerase (Invitrogen, São Paulo, Brazil). Amplifications total cycle was done by a

MyCycler thermocycler (Bio-Rad Laboratories Inc., USA), programmed to start at one minute and 30 seconds, at 94º C, 45 seconds of pairing up (see re-association temperature for each initiator in Table 2) and one minute and 30 seconds of extension at 72º C, finalizing with five minutes at 72º C, for the final extension. Amplification products were separated by electrophoresis in agarose gel 2% in TBE 0,5X buffer (90 mM Tris-Base, 90 mM boric acid and 2 mM EDTA) at constant tension of 90V. Fragments were visualized by fluorescence emitted by bromophenol blue and blue Green, under the UV light, and photographed. Amplified fragments sizes were estimated through comparison with the molecular marker of 100 pb DNA Ladder.

Figure 1. Physic nut accesses in the experimental field at Unicamp's Center for Multidisciplinary Chemical, Biological and Agricultural Research (CPQBA), Campinas, SP.

Table 2. Selected ISSR initiators, their respective sequences, reassociation temperature and number of produced bands.

| Name of the initiator	Sequence (5´ 3´)	Tm*	Number of bands
17	GTG TGT GTG TGT	47.9	22
6	CAC ACA CAC ACA CAC AG	50.5	21
1	AGA GAG AGA GAG AGA GAG	54.1	20
844	CTC TCT CTC TCT CTC TAC	47.9	18
18	GAG AGA GAG AGA GAG AGA T	45,0	17
CHRIS	CAC ACA CAC ACA CAT G	47.9	16
1	ACA CAC ACA CAC ACA CT	47.9	15
4	GAG AGA GAG AGA GAG AGA	57	14
M2	GGG CGA GAG AGA GAG AGA GA	50.5	12
5	CAG CAG CAG CAG CAG	54.1	12
OMAR	GAG GAG GAG GAG GC	45,0	11
7	CTC TCT CTC TCT CTC TAG	50.5	11
12	GAG AGA GAG AGA GAG AGA C	47.9	10
7	GTC GTC GTC GTC GTC	54.1	10

*Tm = reassociation temperature ºC

Based on the images obtained (Figure 2), the gels were read, genotyping individuals in regards to presence (1) or absence (2) of fragments, generating a binary matrix to calculate the polymorphism percentage and the Jaccard distance matrix. Later, the UPGMA clustering was realized and its reliability analyzed by the cophenetic correlation coefficient (CCC) and bootstrap analysis. The distance matrix analysis, clustering and CCC were realized by the NTSYS program (Rohlf 2000) and the bootstrap analysis by the BOOD program (Coelho 2001).

The STRUCTURE 2.3 program (Pritchard et al., 2000) was used for the diversity structure analysis. The Bayesian approach was adopted with the no admixture model. K values were tested varying from one to eight, being the K real number chosen from ΔK values, according to Evanno et al. (2005).

Molecular variability analysis (AMOVA) was carried out through the decomposition of the total square sum in the between and within groups components, using the Excoffier et al. (1992) metric distances, with the help of the Arlequim program (Schneider et al., 2000).

Two analyses of molecular variance (AMOVA) were realized, the first taking into consideration two groups, each group formed by a germplasm bank and the second considering the clusters obtained through the Bayesian analysis implemented by the Structure program.

The genetic diversity analysis between and within groups was carried out by the POPGENE VERSION 1.32 program (Yeh et al., 2000) to estimate the number of alleles (n_a); effective number of alleles (ne), and the percentage of polymorphic loci and the Shannon Index (Lewontin 1972).
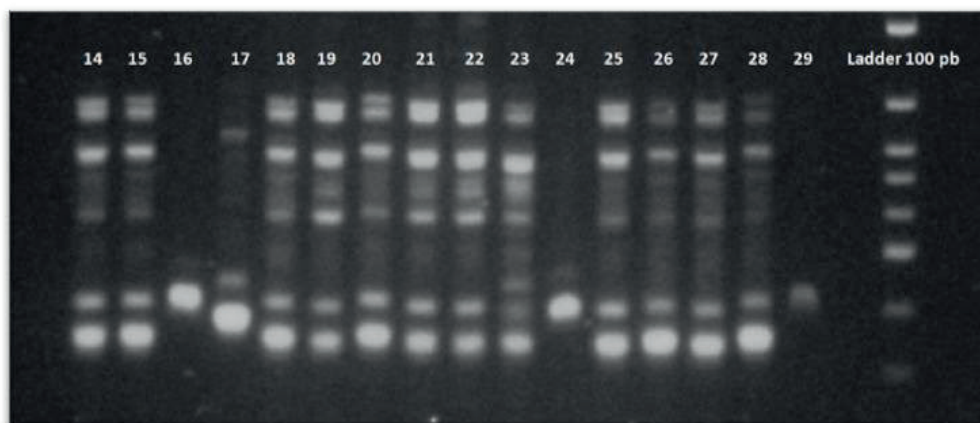
Figure 2. Agarose gel showing polymorphic profile of one of the ISSR primers used (primer M2).

RESULTS AND DISCUSSION

To understand how diversity is structured between and within the groups formed by germplasm banks accesses (CPQBA and UFS), this study used 14 ISSR markers (Table 2) which originated a total of 209 bands, 208 polymorphic, and the variation was of 10 to 12 bands per initiator. Basha and Sujatha (2007) analyzed 42 physic nut accessions from India and one from Mexico, with 48 ISSR markers, and found 29 markers with polymorphism. From the 340 generated bands, 116 were polymorphic, varying from 5 to 19 bands per initiator. These authors report that the polymorphism detected by the ISSR markers was very low (33.5%). On the other hand, results from this work showed amplified loci polymorphism of 99.52%. This can be explained by the different origin of the materials involved in the research and by the use of more polymorphic markers used during the analyses.

The accesses dendrogram was obtained from the similarities measures based on the Jaccard index the UPGMA clustering criterion. Figure 3 shows the dendrogram obtained based on molecular data as well as the correspondence of this analysis with the Structure attribution test. The cophenetic correlation coefficient (CCC) was 0.86, suggesting that the dendrogram explains practically the totality of the original similarity matrix. Jaccard average similarity rate was 0.30, indicating the existence of 30% of an average similarity between the accessions and the similarity extension varied from 0.23 to 0.94. According to this result, it was possible to define four different *J. curcas* clusters (Figure 3).
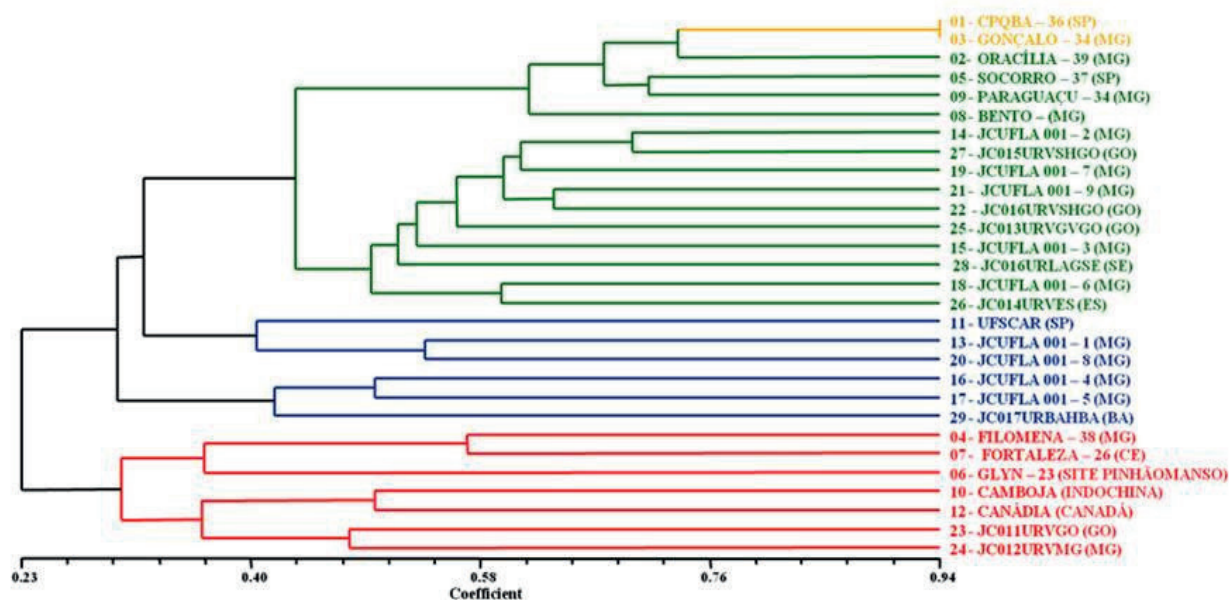
Figure 3. Dendrogram based on the Jaccard similarity and UPGMA clustering criterion in physic nut accesses. Cophenetic correlation coefficient is equal to 0.86 (10.000 bootstrap resampling for the knots consistency percentage). Colors correspond to the main clustering found by the bayesian analysis implemented by the Structure software.

Groups formed by this dendrogram coincided with the four accessions clusters formed by the Bayesian attribution test, confirming and giving more consistency to the results obtained by the present analysis. There was no relationship

among the genotypes of the accessions studied and their origin. One of the reasons for this result may be in the fact that there was a common source of origin among these accessions.

Accessions 1 and 3 were the most similar (94%). Less similar accessions were 12 and 1; 12 and 2; 12 and 3; 12 and 8; 12 and 9; 12 and 14; 12 and 15, 12 and 18; 12 and 19; 12 and 20; 12 and 21; 12 and 22; 12 and 25; 12 and 26; 12 and 27; 12 and 28, with 4% of similarity. With these data it is possible to infer that accessions 1 (CPQBA 36) and 3 (Gonçalo 34) can be considered genetically equal; accession 12 (Canadia), on the other hand, is the least genetically similar to most accessions. However, it is 50% similar to accession 10 (Camboja). In the work developed by Wen et al. (2010), the 45 *J. curcas* accessions formed six groups and showed an average Jaccard similarity rate of 0.76. Based on these results, the authors suggested that the genotypes correlate with their geographical origins, despite the presence of some crossings.

In a similar work using ISSR and RAPD markers, Basha and Sujatha (2007), evaluated the genetic diversity extension of 43 J. curcas accessions originated from different regions in India, including one Mexican no-toxic genotype. The dendrogram based on data from the two markers showed the clustering of 35 of the 43 studied accessions in two main groups. These results were similar to those presented in this study. Researchers concluded that accessions collected in the same region, as well as those collected in different regions in India, formed a group close genetically, showing clearly that the Hindu germplasm geographical difference is not broad, and that there is a great genetic distance between accessions from India and the Mexican genotype.

Tatikonda et al. (2009) observed that the 48 *J. curcas* accessions analyzed with AFLP markers formed four groups in the dendrogram obtained by the UPMGA cluster. With the Jaccard estimates, the similarity coefficient varied from 0.43 to 0.97, suggesting a wide genetic base. The dendrogram helped to understand the analogies among the accessions collected in six states in India. Yadav et al. (2010), with data from the Jaccard similarity matrix generated by 21 EST-SSR markers in 25 *Jatropha curcas* accessions collected in different regions in India obtained a dendrogram with an average similarity coefficient of 0.71, with an amplitude that varied from 0.44 to 0.94. Three main groups were formed and the distribution of accessions in different groups, as in the present study, did not depend on their geographical origins. During the genetic diversity analysis of 78 *J. curcas* accessions with ISSR markers, Mavuso et al. (2016) observed the development of two main clusters that, although unrelated to their cultivation sites, showed close genetic relationship among the populations.

The diversity structure analysis without an *a priori* ranking realized by the STRUCTURE program, showed a K=4 optimum value, according to Evanno et al. (2005). The four clusters developed (Figure 4) corresponded totally to the clusters based on the Jaccard Index by the UPGMA criterion. It is important to emphasize that, for this analysis, the *no admixture model* was adopted. It was possible to observe that accessions 11 (UFSCAR) and 13 (JCUFLA001-1) showed a Q of approximately 95% and 80% for their participation in cluster III, respectively, and only 5% and 20%, respectively, for their participation in cluster IV.
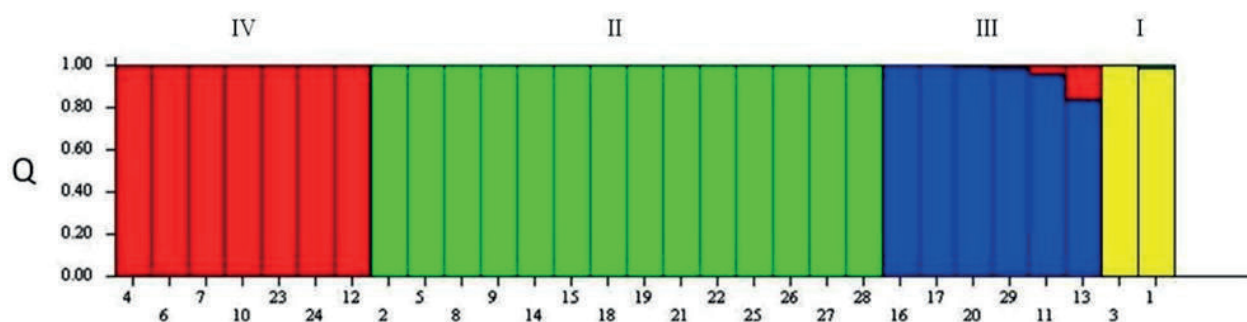
Figure 4. Attribution test for the *J. curcas* (K = 4) accesses studied. Accesses represented by vertical bars. The same color in different accesses indicates that they belong to the same cluster. Different colors in the same individual indicate the genome percentage shared with each cluster. For accesses identification see Table 1. Q = individual participation coefficient in each cluster.

This represents genetic similarity between accessions, i.e., accessions 11 and 13 showed 5% and 20%, respectively, of genetic similarity with accessions 04 (Filomena 38), 07 (Fortaleza 26), 06 (Glyn 23), 10 (Cambodia), 12 (Canádia), 23 (JC011URVGO) and 24 (JC012URVMG). These constituted clusters showed no relation between clusters developed and accessions origins. Studies carried out to visualize genetic diversity structure in the species germplasm bank are relevant, since they introduce researchers to strategies to be adopted by breeding programs.

Bajay et al. (2009), in an analysis similar to this study, using the Structure attribution test, to verify the structure of the genetic diversity of castor bean (*Ricinus communis*) from three germplasm banks and based 41 SSR loci, observed the development of two genetically distinct clusters. This result led the author to infer that the castor bean genetic diversity is well represented in these germplasm banks and the information provided by the analyses

were considered of extreme importance to castor bean breeding programs. By analyzing Brazilian accessions from a turmeric *Curcuma longa* L. germplasm bank together with accessions from other countries, using 19 SSR markers, Sigrist et al. (2010) observed, according to the Structure analysis, the development of three clusters. The first cluster developed by practically all Brazilian accessions, the second developed by Paulista accessions, one by Belém (PA) and by Puerto Rican accessions, and the third group was constituted of accesses from India. The authors were able to conclude that the analyzed Brazilian germplasm bank showed, in general, little genetic variability, stressing once again the importance of including materials from different origins in germplasm banks to increase their genetic variability   and guaranteeing good conditions to develop breeding programs.

Odong et al. (2011) emphasizes the importance of getting information on the genetic structure of the germplasm bank both for preservation as well as for the use of genetic resources gathered and gene banks, being such information also an important aspect in association studies. In this context, the authors highlight the efficiency and reliability of the Structure program for the study of germplasm banks with data generated from molecular markers, compare to traditional techniques such as the UPGMA and Ward.

To evaluate genetic diversity structure, this study carried out two molecular variance analyses (AMOVA) (Table 3), with accessions divided *a priori*. Two groups were considered during the first analysis (Table 3), each one formed by a germplasm bank (CPQBA and UFS). In this case, the analysis detected significant molecular genetic diversity (p < 0.01) between groups as well as within groups. From the total molecular genetic variance verified, 13.36% was due to differences among groups, while 86.64% to differences within groups. In the second analysis (Table 3), accessions were gathered in four clusters according to the Structure attribution test. This analysis also detected significant molecular genetic diversity (p < 0.01) both among clusters and within clusters. From the total molecular genetics verified, 28.85% were due to differences among the four clusters while 71.15% are within clusters. The highest divergence percentage among clusters in this analysis is justified considering that clusters were formed according to the similarity among accessions.

Table 3. Molecular analysis of variance: (a) AMOVA for the two J. curcas access groups (b) AMOVA based on the four clusters of the Structure attribution test.

	Variation source	GL*	SQ	Variance components	Total variation (%)	Statistics Φ	p
(a)	Between group	1	90.89	4.87	13.36	$\Phi GT = 0,13$	p < 0.01
	Within groups	27	757.71	31.56	86.64	$1 - \Phi GT = 0,87$	p < 0.01
	Total	28	848.60	36.43			
(b)	Among clusters	3	231.24	8.70	28.85	$\Phi GT = 0,29$	p < 0.01
	Within the cluster	25	536.55	21.46	71.15	$1 - \Phi GT = 0,71$	p < 0.01
	Total	28	767.79	30.16			

*GL = degrees of freedom; SQ = sum of squares; ΦGT = coefficient of variation among groups.

Mavuso et al. (2016) studied the genetic diversity of 78 *J. curcas* accessions grown in two locations in Taiwan using ISSR markers. They also observed that from the total molecular genetic variability, 31.23% was due to the differences among populations, while 68.77% due to differences within the populations, suggesting low variability for the Jatropha populations grown in Taiwan. These results are in agreement with most tropical species, in which genetic variability is more structured within than between populations (Tatikonda et al., 2009; Mavuso et al., 2016).

Genetic diversity estimates carried out with two groups of physic nut accessions in the present work (Table 4) showed a high polymorphism rate for Group 1 (78.47%) and for Group 2 (89.95%). Total genetic diversity estimates for the 29 *Jatropha curcas* accessions (Table 4) provided the average number of bands (n_a = Group 1 and Group 2), the average effective number of alleles ne and the Shannon index (I). The Shannon Index (I) for the 29 accessions was 0.41. This value is considered high when compared to data presented by Shen et al. (2012) which obtained an average value for I of 0.10. Wen et al. (2010) studied the genetic diversity of 45 *J curcas* accessions using EST-SSR and SSR markers. They estimated an average polymorphism rate of 84% and a Shannon Index average value of 0.56. The authors concluded that the collection showed a restricted and moderate genetic base and that the genetic diversity level was greater between accessions from America and Yunnan (China), confirming the importance of including accessions from different origins to obtain greater genetic variability.

Ranade et al. (2008) evaluated 21 *J. curcas* accessions from different places in India using seven RAPD markers and four DAMD (*Directed Amplification of Minisatellite DNA*) and observed an average value for I of 0.34 and that the percentage of polymorphic loci between theses accessions varied from 3.94 to 39.37%, indicating a low level of variation between accessions. These authors state that the genetic diversity rate, in species habitats in which human intervention is very low or inexistent, may contribute to more substantially to the *J. curcas* germplasm heterogeneity for factors such as adaptability and oil production.

Findings from this study and the lack of correlation between groups formed by the clustering methods used and their geographic origin may have several possible explanations. The first hypothesis to be considered is the possible common origin of the accessions and other possible hypotheses may include the introduction of little physic nut accessions in Brazil and the exchange of genotypes among researchers, institutions and farmers, resulting in the narrowing of the genetic base of the accessions studied in the present work.

Table 4. Diversity estimates: (a) Diversity within each Jatropha curcas group (b) total diversity 29 Jatropha curcas accesses.

		na*	ne	I	P
(a)	Group 1	1.78 (0.41)	1.39 (0.35)	0.36 (0.25)	78.47
	Group 2	1.90 (0.30)	1.40 (0.34)	0.39 (0.22)	89.95
(b)	Total (Group 1 and Group 2)	1.99 (0.07)	1.42 (0.33)	0.41 (0.20)	

*na = mean of the number of observed alleles; ne = mean of the effective number of alleles; I = mean of the Shannon´s index; P = percentage of polymorphic loci; and ( ) = standard deviation.

CONCLUSIONS

The adoption of ISSR markers to characterize accessions in this study revealed that the genetic variability is greater within that among the groups studied and it was impossible to establish a genotypes correlation with their geographic origin. The estimated genetic diversity among group accessions makes evident the need and importance to gather the largest number of accessions possible from different regions and countries to develop a species germplasm bank. The objective is to obtain maximum species variability, guaranteeing their preservation, and last but not least important, to make breeding programs available for this Euphorbiaceae, which has considerable potential for economic exploration.

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