Population structure of jatropha and its implication for the breeding program

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ABSTRACT. Jatropha (*Jatropha curcas* L.) has potential as an oilseed crop that requires the development of technology for its exploitation. The objective of this study was to assess the population structure and the genetic diversity in jatropha accessions at a global level using simple sequence repeat (SSR) molecular markers. Jatropha accessions (N = 109) from 10 countries were genotyped using 10 SSR markers. The results showed a low level of genetic diversity among 92 accessions originating from India, Mozambique, Ethiopia, Tanzania, Brazil, Honduras, and Indonesia, which were grouped in one cluster. In contrast, accessions from Mexico and Costa Rica showed high level of genetic variability. These accessions may be used to increase the genetic diversity of jatropha in the breeding

populations. The study also showed the need of collecting activity from the center of diversity (Mexico and Costa Rica) to aggregate the genetic diversity in the international collections of jatropha.

Key words: *Jatropha curcas*; Physic nut; Microsatellite markers; Conservation; Genetic diversity

INTRODUCTION

Jatropha (*Jatropha curcas* L.) is a tropical species in the Euphorbiaceae. It is native to Mexico and Central America and distributed in Latin America, Africa, India, and South East Asia (Pandey et al., 2012). It is also classified as a source of high quality oil with biofuel potential, mainly biodiesel and bio jet fuel (Dias et al., 2012). This plant is widely recognized as a potential feedstock for biodiesel production (Pandey et al., 2012; Edrisi et al., 2015).

The cultivation and popularization of jatropha around the word have been mooted by three important ethos: i) achieving energy security, ii) revitalizing marginal and degraded lands (Biswas et al., 2010), and iii) alleviating rural poverty through employment and sustainable biofuel production (Edrisi et al., 2015). Due to these facts, jatropha is considered a plant with ecological and social appeal (Contran et al., 2013).

Among the many topics related to jatropha that have been discussed, the genetics continues to be of major interest. The literature agrees that jatropha shows high phenotypic variation. However, most of the genetic variability in *J. curcas* was shown to be essentially epigenetic, which causes phenotypic variation with no requirement of changing DNA sequences (Achten et al., 2010).

Different studies on various populations of jatropha have shown a low to moderate level of genetic variability (Sun et al., 2008; Gupta et al., 2012; Alves et al., 2013; Maghuly et al., 2015). This low variability is probably due to human impact including intensive selection, or the jatropha mating system (Alves et al., 2013; Bressan et al., 2013; Negussie et al., 2014).

The identification of the cause and absence of genetic variability in the germplasm bank and breeding populations of jatropha is an important step that will provide insight into future breeding programs of the species. In addition, it can help to identify the collections with high genetic variability that can be exploited to increase the genetic variation in the breeding process.

The study of genetic diversity can be done using molecular markers. These markers have an advantage over morphological markers because they are not affected by environmental factors (Basha and Sujatha, 2007) and may provide high quality information. Among the available molecular markers, microsatellites (simple sequence repeats - SSRs) are the most genetically stable, species-specific, and multi-allelic. Microsatellite regions are highly variable and polymorphic. Microsatellite markers are robust in their amplification and are widely used in the characterization of germplasm banks, including soybean (Qiu et al., 2013), olive (Cicatelli et al., 2013), grape (Salmaso et al., 2009), and jatropha. Sanou et al. (2015) used SSR markers to study the genetic diversity among jatropha accessions and concluded a high level similarity among them.

Maghuly et al. (2015) emphasized that one of the limitations in the jatropha breeding materials is the low or incomplete information about the germplasm resources. This highlights the importance of generating more information on the international jatropha collections, for a better exploitation of the species as an energy source that may benefit resource poor farmers. Increasing the genetic variation is a crucial step in any breeding program, which requires incorporating new sources of germplasm especially from the center of origin (Heller, 1996).

Among the international collections of jatropha, the University of Florida gene bank contains various accessions of different origin. Therefore, studying the population structure and genetic diversity of this collection, using molecular markers, can provide insight into which part of the continent contains more variability, and can be exploited in future breeding programs, or can be targeted for further collection.

Therefore, the objective of this research is to study the population structure and genetic diversity of jatropha accessions on a global level, using SSR markers, as well as to identify the countries with most divergent jatropha populations.

MATERIAL AND METHODS

The experiments were conducted in the ornamental horticulture laboratory at the tropical research and education center (TREC), University of Florida, Homestead, FL, USA. The center is located at latitude 25.5°N, longitude 80.4°W and at 1 m in altitude. The germplasm bank is kept at a subtropical climate, with an average annual temperature of 24°C, maximum and minimum temperature of 29° and 19°C, respectively. The region receives 1473 mm rainfall annually.

The jatropha germplasm bank at TREC is comprised of 109 plants, representing 10 different countries. Within each country, different accessions were collected at different times indicated in Table 1 (e.g., Brazil 1, Brazil 2, etc).

DNA extraction

DNA extraction of the 109 jatropha accessions was performed using the DNeasy Plant Mini Kit (Qiagen Inc., Valencia, CA, USA). Samples consisting of 100 mg young leaves were ground in liquid nitrogen and the extracts were poured into 2-mL microfuge tubes. To each microfuge tube, 400 μL AP1 buffer and 4 μL RNAse were added. The mixtures were incubated at 65°C for 10 min and mixed by inverting the tubes three times. Thereafter, 130 μL AP2 buffer was added, the tubes were placed on ice for 5 min and then centrifuged at 18,894 *g* for 5 min. The supernatant was removed and approximately 400 μL was added to the column collector tube, which was centrifuged at 13,000 rpm for 5 min. The supernatant was transferred to a new microfuge tube, to which AP3 buffer was added at 1.5 times its volume and the mixture was centrifuged at 8000 rpm for 1 min. The supernatant was discarded, the DNA retained on the column, which was transferred to a new microfuge tube. Then, 500 μL AW buffer was added and the column was centrifuged at 8000 rpm for 2 min. The column was transferred again to a new microfuge tube and 100 μL AE buffer was added twice, yielding 200 μL DNA solution. The DNA concentration of the samples was measured using a 2000/2000c NanoDrop spectrophotometer (Thermo Scientific, Wilmington, DE, USA) and all measurements were normalized to 15 ng DNA.

Genotyping with microsatellite markers

For the genotyping, 10 markers previously shown to be polymorphic for *J. curcas* (Pamidimarri et al., 2009; Achten et al., 2010; Phumichai et al., 2011) were used. A detailed description of the primers is presented in Table 2. The expected sizes of the fragments were calculated based on the original sequences deposited in GenBank, where the SSR primers were designed and provided a means for consistency of results.

Table 1. Jatropha (*Jatropha curcas* L.) germplasm collection at the Tropical Research and Education Center, University of Florida, Homestead, FL, USA. Accessions are indicated with their laboratory identifications (ID) and geographic origin.

ID	Origin	ID	Origin	ID	Origin
1	India 3	38	India 2	75	Brazil 2
2	India 4	39	India 2	76	Brazil 2
3	India 4	40	Brazil 1	77	Indonesia 2
4	India 4	41	Brazil 1	78	Indonesia 2
5	India 4	42	India 3	79	Indonesia 2
6	India 4	43	India 3	80	Indonesia 2
7	India 4	44	India 3	81	Indonesia 2
8	India 4	45	Costa Rica	82	Indonesia 2
9	India 4	46	Costa Rica	83	Indonesia 2
10	India 4	47	Costa Rica	84	Indonesia 2
11	India 4	48	Costa Rica	85	Indonesia 2
12	Mozambique	49	Costa Rica	86	Indonesia 1
13	Mozambique	50	Costa Rica	87	India 3
14	Mozambique	51	Costa Rica	88	India 3
15	Mozambique	52	Costa Rica	89	Tanzania
16	Ethiopia	53	Costa Rica	90	Tanzania
17	Ethiopia	54	Costa Rica	91	Tanzania
18	Ethiopia	55	Costa Rica	92	Tanzania
19	Ethiopia	56	Honduras	93	Tanzania
20	Tanzania	57	Honduras	94	Tanzania
21	India 3	58	Honduras	95	Tanzania
22	India 3	59	Honduras	96	Tanzania
23	Mexico	60	Brazil 1	97	Tanzania
24	Mexico	61	Brazil 1	98	Tanzania
25	Mexico	62	Brazil 1	99	Brazil 3
26	Mexico	63	Brazil 1	100	Brazil 3
27	Mexico	64	Brazil 1	101	Brazil 4
28	Mexico	65	India 3	102	Brazil 4
29	Mexico	66	India 3	103	Brazil 4
30	Mexico	67	Brazil 2	104	Brazil 4
31	Mexico	68	Brazil 2	105	Brazil 4
32	Mexico	69	Brazil 2	106	Brazil 4
33	Mexico	70	Brazil 2	107	Brazil 4
34	India 1	71	Brazil 2	108	Brazil 4
35	Guatemala	72	Brazil 2	109	India 3
36	Guatemala	73	Brazil 2		
37	Guatemala	74	Brazil 2		

The amplification reactions were performed in a final volume of 20 μL with 30 ng DNA, 200 μM dNTP (Thermo Scientific), 8 pM M13F primer (forward-5'TGTAAAACGACGGCCAGTATGC3'), 32 pM primer R (reverse), 32 pM M13 fluorescent 6-FAM (Eurofins MWG/Operon Huntsville, USA), 0.5 U Taq polymerase (New England BioLabs, Ipswich, MA, USA), and 1.5 mM $MgCl_2$ included in 1X buffer (New England BioLabs, Hitchin, England). The final volume (20 μL) was achieved by addition of MilliQ water (Invitrogen, Life Technologies, Grand Island, NY, USA).

Amplifications followed the methodology described by Hufford et al. (2011), with modifications to the annealing temperature. The program consisted of an initial denaturation at 94°C for 5 min and 30 cycles of the following sequence: denaturation at 94°C for 30 s, annealing at 54°C for 45 s, and extension at 72°C for 45 s. This was followed by eight cycles of the following sequence: denaturation at 94°C for 30 s, annealing at 52°C for 45 s, and extension at 72°C for 45 s. The final extension was done at 72°C for 10 min. Amplifications were performed in a PTC-100 thermocycler (Bio-Rad, Hercules, CA, USA).

The presence of amplified fragments was confirmed on a 1% agarose gel, dyed with 6 μL 10,000X GelRed (Phenix Research, Candler, NC, USA) and visualized in a high UV performance transilluminator (UVP, Upland, CA, USA). After confirmation of amplification efficiency, 1:10 dilutions were made using MilliQ water. The samples were then placed in polystyrene boxes containing dry

ice and shipped overnight to the interdisciplinary center for biotechnology research, University of Florida, Gainesville, FL, USA, for genetic analysis and genotyping. Prior to the genetic analysis, performed on an ABI 3730 sequencer, Hi-Di formamide and the weight marker GeneScan 600 LIZ (ThermoFisher Scientific, Grand Island, NE, USA) were added to the samples.

Table 2. Microsatellite primers for *Jatropha curcas* with their respective sequences, annealing temperatures (Tm), expected sizes (bp), and bibliographic references.

Primer	Sequences	Tm (ºC)	bp	Reference
Jcps21	F: CCTGCTGACAGGCCATGATT R: TTTCACTGCAGAGGTAGCTTGTATA	54.8	190	Achten et al., 2010
Jcds58	F: TCCATGAAGTTTGCTGGCAAT R: AGGTCATCTGGTAAAGCCATACC	54.0	109	Achten et al., 2010
Jcps10	F: CATCAAATGCTAATGAAAGTACA R: CACACCTAGCAAACTACTTGCA	46.5	112	Pamidimarri et al., 2009
Jcps24	F: GGATATGAAGTTTCATGGGACAAG R: TTCATTGAATGGATGGTTGTAAGG	51.0	204	Pamidimarri et al., 2009
Jct27	F: GCCATTAGAATGGACGGCTA R: TGCGTGAAGCTTTGATTTGA	60.0	235	Phumichai et al., 2011
Jcps20	F: ACAGCAAGTGCACAACAATCTCA R: TACTGCAGATGGATGGCATGA	55.0	224	Achten et al., 2010
Jct15	F: AATTCTCTTTCCGCGATCCT R: CGTAGACCTTCCAACAGCAA	60.0	201	Phumichai et al., 2011
Jct16	F: GCCTCCAGCATCTTTCAATC R: AACAATCCCCATTCCTCCTC	60.0	103	Phumichai et al., 2011
Jct68	F: AGCGATAATCGGCCTACCTT R: CAACGTCACTGCCTCCTACC	60.0	212	Phumichai et al., 2011
Jct81	F: CCATTTAGAACCACAACCAT R: GATGTCCAATAAGCCTGAAT	54.0	143	Phumichai et al., 2011

Data analysis

Data were tabulated from the allelic profiles of the 10 SSR markers derived from 109 jatropha accessions. First, a Bayesian model based cluster analysis was performed to determine the possible genetic structure using STRUCTURE v. 2.3.3 (Pritchard et al., 2000), as described in detail by Nunes et al. (2013). The appropriate number of clusters (K) was determined following the method proposed by Evanno et al. (2005) using Structure Harvester (Earl and vonHoldt, 2012). The polymorphic information content (PIC), which measures the ability of the primers to detect differences among the accessions analyzed, was estimated using PowerMarker v. 3.25 (Liu and Muse, 2005).

To understand the existing genetic diversity and population structure of collections from different regions of the world, a clustering analysis was performed using the unweighted pair group method with arithmetic mean (UPGMA) clustering method based on Cavalli-Sforza chord distance (Cavalli-Sforza and Edwards, 1967).

The principal coordinate analysis (PCoA) and molecular analysis of variance (AMOVA) (Excoffier et al., 2005) were performed using GenAlEx v. 6.41 (Peakall and Smouse, 2006).

RESULTS

The number of alleles produced per primer ranged from 1 to 7. The estimated PICs showed high values for primers Jct27, Jct15, Jct16, Jct68, and Jct81. Among the 10 SSR markers, primer Jct68 provided the highest PIC value (0.4161) (data not shown). The cluster analysis grouped the 109 jatropha accessions into two groups (K = 2) (Figure 1). The first group (Group 1; red), with 92 individuals, was formed by all jatropha plants from India, Mozambique, Ethiopia, Tanzania, Brazil, Honduras, and Indonesia, as well as one Mexican (ID 30) and four Costa Rican (ID 45, 50, 51,

and 52) accessions. This indicates a close genetic relationship within the group. The second group (Group 2; green), comprised 17 accessions from Mexico and Costa Rica that showed a high level of genetic diversity. This group showed some level of admixture compared to the first group.

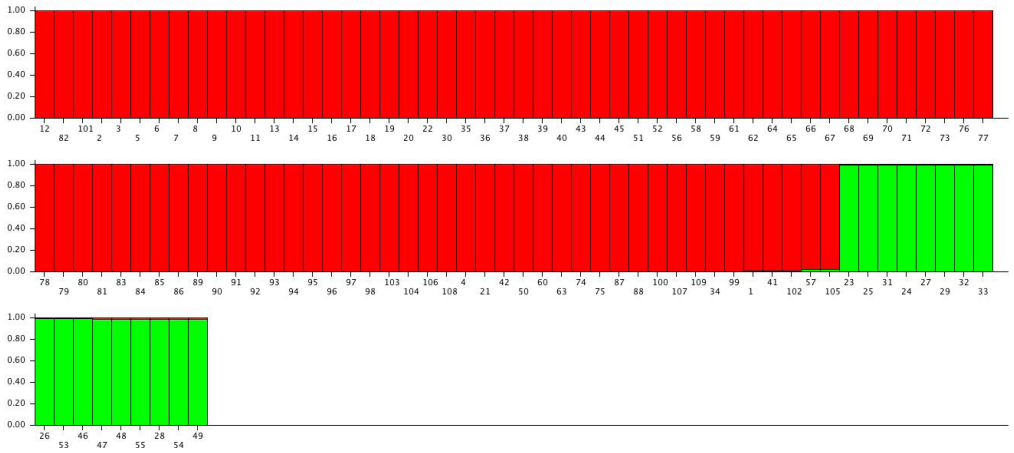

Figure 1. Analysis of the genetic structure of the 109 accessions of *Jatropha curcas* L. produced using Bayesian based clustering analysis using Structure v. 2.3.4. The numbers of the x-axis represent the encoding of each accession following the description in Table 1. The red and green colors represent the group to which each individual belongs.

The PCoA (Figure 2) classified the 109 accessions into four groups, where the Costa Rican and Mexican accessions were relatively closer to each other, while maintaining distance from the other countries. In addition, the accessions from the other countries were classified into two groups where the first group contains the Brazilian, Tanzanian and Indonesian accessions (below the x-axis) and the rest grouped in the second cluster (above the x-axis). The first two principal coordinates explained 52.32% of the total variation, clearly classifying the accessions according to their place of origin (Figure 2).

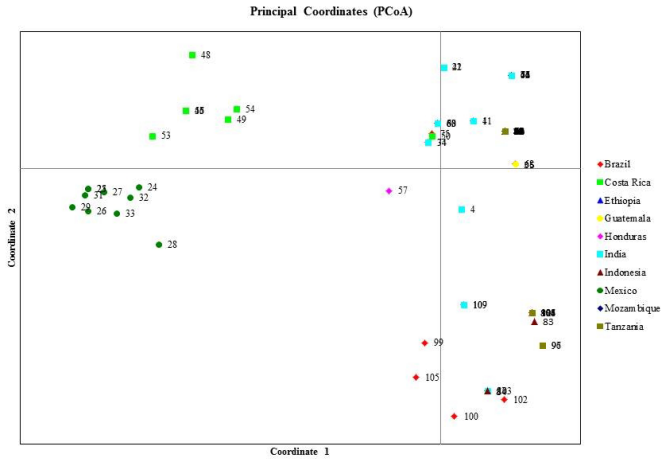

Figure 2. The results from the PCoA analysis for the 109 accessions of jatropha from the germplasm bank of University of Florida, obtained with 10 SSR markers.

The phenetic tree of the 109 accessions based on the UPGMA (Figure 3), confirmed the grouping pattern reported in the PCoA. The dendrogram showed clear separation of the Costa Rican and Mexican accessions from the rest of the accessions, as presented in the PCoA (Figure 2), and showed a higher level of diversity than the accessions from other countries.

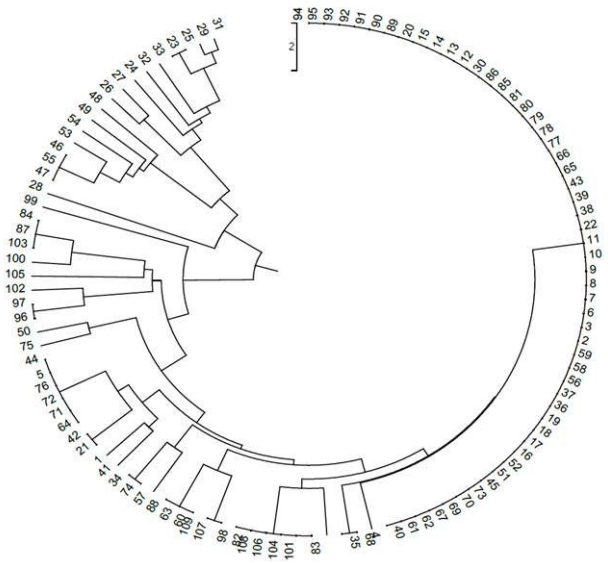

Figure 3. Phenetic UPGMA tree obtained by the 10 SSR markers based on Cavalli-Sforza chord distance (1967), showing the genetic relationships among the 109 accessions of the jatropha germplasm bank at the University of Florida. The edge numbers represent each accession as presented in Table 1.

Based on the place of origin (10 countries), the AMOVA showed that 65% of the total variation was within population and the rest, 35%, among populations (Table 3). This showed some level of genetic variation among the accessions from some countries, especially Costa Rica and Mexico.

Table 3. AMOVA of the 109 jatropha accessions.

Source of variation	d.f.	MSD	Estimated variance	Percentage of molecular variance
Between groups	9	18.149	1.496	35
Within groups	98	2.812	2.812	65
Total	108		4.308	100

DISCUSSION

The Bayesian cluster analysis classified the 109 accessions into two principal groups: the majority of the Mexican and the Costa Rican collections were allocated to the first group, whereas the other accessions clustered in the second group. This grouping indicated a low level of genetic diversity in the collections of jatropha in the world. Maghuly et al. (2015) and Sanou et al. (2015) also reported a low level of genetic diversity among accessions of jatropha from Africa, Asia, Central and South America.

The PCoA and UPGMA classified the accessions into four groups. These analyses classified the accessions in more detail and indicated the existence of some level of variability

among accessions. The information obtained using the PCoA showed some level of variability among the accessions from Mexico and Costa Rica.

The observed variability is supported by the morphological evaluation reported in Nietsche et al. (2013). These authors evaluated 17 accessions of jatropha from the collection at the University of Florida and observed that the Mexican and Costa Rican accessions differed in reproductive characteristics. Costa Rican accessions have a higher number of female flowers and a higher frequency of pollen germination. Due to these superior features, these accessions were suggested as potential parents in crossing programs with the aim of developing cultivars. The clear separation of the Mexican and Costa Rican accessions from all other accessions indicates that these groups may contain unique genes produced through mutation related to their place of origin.

Our results also showed that the Costa Rican and Mexican accessions can be used as potential parents in breeding programs of jatropha because they showed a high level of genetic diversity. The high genetic diversity and distinctness of the Mexican accessions in this study, has also been observed in Pecina-Quintero et al. (2011). Therefore, the inclusion of Mexican and Costa Rican accessions in every country's national collections and breeding programs is recommended, to increase the genetic base of the breeding population.

We found that 65% of the total variation is partitioned within populations and 35% among populations, which indicated some level of differentiation among populations from different regions. The high level of within population variation is frequently observed in allogamous species (Maghuly et al., 2015). In accordance with our results, Bressan et al. (2013) found 68.3% outcrossing in jatropha species using SSR markers confirming that this species is allogamous. In addition, the dominance and efficiency of cross-fertilization in jatropha was reported by Raju and Ezradanam (2002) and Kaur et al. (2011), further supporting our own findings.

The low genetic diversity observed in this and other studies may be the result from the apomixis reproduction system, as reported in phytotechnical studies in jatropha (Bhattacharaya et al., 2005; Kaur et al., 2011), which potentially fixes the alleles at each locus and limits genetic recombination. In addition, using flow cytometry analysis, Ambrosi et al. (2010) reported the occurrence of non-gametophytic apomixis in jatropha.

The germplasm bank used in this study includes accessions from diverse geographical origins that are expected to show medium or high levels of genetic variability. However, this prediction was not supported in the present study. The results also showed that the accessions from continents outside of jatropha center of origin, with different soil and environmental conditions may not be able to create adaptive genes over time. The low genetic diversity observed can also be associated with the low number of alleles per locus obtained in this study (1-7 alleles). However, our results are in accordance with the findings reported in other studies of the same nature (Sanou et al., 2015; Maghuly et al., 2015). A similarly low number of alleles was also documented by Sharma and Chauhan (2011), who found 2-6 alleles by transferring the SSR castor primer to jatropha. Similarly, Na-Ek et al. (2011) observed 2-4 alleles per locus, and Bressan et al. (2012) found 2-8 alleles per locus from new SSR markers developed for jatropha.

The low genetic variability in jatropha may also be due to evolutionary processes. This species may have undergone genetic drift over time, both due to the founder effect and the effect of a bottleneck, leading to an increase in homogeneity. Jatropha suffered a founder effect during its dissemination to Asia and Africa by Portuguese navigators 400 years ago, due to the small sample size that was not representative of the original, natural population (Heller, 1996). The effect of a bottleneck occurs when the size of a population is drastically reduced due to the selection process (agronomic or not) driven by man (Veasey et al., 2011). The existence of human actions that

reduced the population of jatropha is backed by Ambrosi et al. (2010), who claimed that jatropha was initially not selected for characteristics of productivity and/or quality. Thus, the selection process was poorly performed and reduced the size of the population, where the new accessions of jatropha originated from this group of selected plants.

The result of isolated environments colonized by very similar accessions is that, even with cross-fertilization, the formation of new variability will probably not be possible. This may have caused high genetic similarity among accessions of jatropha in the new habitats. This observation is also supported by our study, where the accessions collected from every country outside Mexico and Costa Rica showed low levels of genetic diversity and clustered in the same group as illustrated using different analysis methods. Thus, there was a decrease in the genetic variability of the new populations in relation to the original population. Bressan et al. (2013) also showed that a small number of individuals in the same location can result in a high rate of correlated mating, when the open-pollinated seeds were mainly full-sibs, as observed in this case.

Achten et al. (2010) also suggested that the complex history of introduction and domestication in other continents contributed to the low level of genetic variability observed in this species. The low level of admixture among the accessions of the jatropha observed in this study suggests that most accessions have developed from the same origin and have not produced any considerable change over time in its place of adaptation. Alternatively, jatropha requires more time to create new variability.

Other possible reasons may be the vegetative propagation method used by former farmers. According to Ovando-Medina et al. (2011), a vegetative propagation was used for centuries, to ensure the spread of jatropha in rural areas. Thus, a few selected accessions of jatropha have become widely spread clonally. The high level of similarity observed in this study also supports this conclusion, because most of the accessions clustered in one single group with a membership coefficient of 100% using Bayesian clustering analysis. One might also expect that these individuals remain highly related to each other across generations.

In general, the species' natural environment (in Mexico and Costa Rica) contains high levels of genetic diversity that may be exploited to enrich the germplasm bank and the breeding populations of this species. Therefore, the introduction of accessions from these regions may sustain future production of jatropha. This crop is an important source of income, especially for small-scale farmers that live in the semiarid regions of the tropics. The gain from the productivity of this crop can improve the livelihood of people living in the sub-tropical regions of the world, by providing an additional income source.

Conflicts of interest

The authors declare no conflict of interest.

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