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Full Length Research Paper

Evaluation of genetic diversity between toxic and non toxic *Jatropha curcas* L. accessions using a set of simple sequence repeat (SSR) markers

Massimo Vischi*, Steluta Raranciuc and Mario Baldini

Dipartimento di Scienze Agrarie e Ambientali, Università di Udine , via delle Scienze 208, 33100 Udine, Italy.

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Scepticism about Jatropha as a competitive biofuel feedstock especially on marginal soils has been growing; in fact, the jatropha-biofuel chain is risky economically and often financially unfeasible without significant government subsidies under these conditions. A valorization of the by-products and in particular of the extruded seed cake (about 70% (w/w) of the processed seed), as animal feed, currently prevented by the presence of phorbol esters (PE) toxins, could contribute to a significant improvement in the economic sustainability of the crop. Strategies for breeding improved varieties could be accelerated by DNA-based molecular marker technology. Wild Mexican accessions and accessions from other parts of the world (South America and Africa) were analyzed by 40 simple sequence repeat (SSR) markers. SSR primers were chosen on the grounds of their Tm, length, degree of polymorphism and specificity for toxic trait. The genetic study pointed out a high degree of similarity both within and among the non Mexican accessions. The Mexican accessions proved to be non toxic and genetically differentiated forming a well separated cluster from out of Mexico accessions. Some polymorphic loci were close correlated with the character toxicity and useful, once validated their association in segregating populations for Marker Assisted Selection (MAS).

Key words: *Jatropha curcas*, genetic variability, molecular markers, non-toxic accession, phorbol esters, simple sequence repeat (SSR) genotyping.

INTRODUCTION

Jatropha curcas L. has received increasing interest in the last years as a new biofuel crop due to its potential use in the so-called “marginal areas”, where there is no competition with food crops, as usually occurs on more fertile land (Tiwari et al., 2007; Prasad et al., 2012). The plant is a shrub/perennial tree producing high quality oil. It is resistant to high temperatures, drought, pests and diseases, with low nutritional input and a well-developed rooting system to protect soil from erosion (Ceasar and Ignacimuthu, 2011). It is widely cultivated in Asia, Latin America and Africa, the International Jatropha Organization (Siddharth and Sharma, 2010), forecast a strong expansion of this crop in the near future up to 12.8 million ha worldwide in 2015. An increase in seed yield and oil content remains the main goal of genetic improvement of *J. curcas,* but a wider acceptability of the whole jatropha-biofuel chain can only be fully achieved with the utilization of the seed cake (about 65-75% w/w of the processed seed) as animal feed. Its use is limited by the presence of phorbol esters (PE), which are highly toxic for animals even at very low concentrations and resistant to high temperature (Makkar and Becker, 2009; Baldini et al., 2012). In the near future, the solution may come from non-toxic *J. curcas* accessions. These genotypes of Mexican origin can be used in breeding

*Corresponding author. E-mail: massimo.vischi@uniud.it. Tel: +390432558609. Fax: +390432558603.

Abbreviations: AFLP, Amplified fragment length polymorphism; **EST,** expressed sequence tag; **ISSR,** inter simple sequence repeat; **Tm,** melting temperature.

programmes to recombine non-toxic traits into elite (toxic) varieties. Strategies for breeding improved varieties could be accelerated by DNA-based molecular marker technology. In the last years different classes of molecular markers have been developed to analyse the germplasm of *J. curcas.*

Different classes of universal molecular markers such as random amplified polymorphic DNA (RAPD), inter simple sequence repeat (ISSR), and amplified fragement length polymorphism (AFLP) were used initially. Sujatha et al. (2005) calculated a similarity index between toxic Indian varieties and a non-toxic variety of Mexican origin by means of about 400 RAPD markers. The similarity index was about 95%, plus their crossing studies pointed out a maternal effect on PE content of seeds. Other studies with RAPD and ISSR markers show high genetic diversity in Mexican germplasm and low genetic variation in accessions from other countries in terms of both PE levels and molecular profiles (Basha et al., 2009). The AFLP analysis indicated that there was very little genetic diversity in the material obtained from Madagascar, Tanzania and Suriname. For a large subset of these samples, no polymorphisms were observed, suggesting that this material is almost clonal. Within the Mexican samples genetic diversity was relatively high, with a total of 85 polymorphic bands being observed (PPB25.2%) (He et al., 2011).

Microsatellites or simple sequence repeats (SSRs) were the second generation of markers used in *J. curcas* because of their co-dominant nature, abundance in genomes, high reproducibility, hyper polymorphism and high rates of transferability across species/genera. SSRs were developed both from genomic DNA libraries and *in silico* as expressed sequence tag (EST)-SSR. Wen et al. (2010) tested the transferability of 419 EST-SSR and 182 G-SSR primer pairs between Cassava and five accessions of *J. curcas*. Among these primers, 234 (55.85%) EST-SSR and 68 (37.36%) Genomic-SSR primer pairs produced amplicons, but only 187 (44.63%) EST-SSRs and 54 (29.67%) Genomic-SSRs were polymorphic among the five accessions. Yadav et al. (2011) assembled 1 227 contigs from 13 201 expressed sequence tags (ESTs) of *J. curcas* from the National Center for Biotechnology Information database. Four hundred and six primer pairs were designed out of the 702 SSR-containing sequences. Fifty randomly selected EST-SSR markers were amplified in 25 accessions collected from different geographical regions of India. Polymorphic information content value ranged between 0.04 and 0.61 with an average of 0.25 ± 0.16, indicating low to moderate level of informativeness within these EST-SSRs.

SSRs were also developed from enriched genomic libraries. Phumichai et al. (2011) isolated 55 microsatellite markers, among these 11 were polymorphic when tested in 26 accessions of *J curcas* from Thailand. One use of molecular markers regards the possibility of discriminating the toxic from the non-toxic varieties, given that no significant morphological, qualitative or quantitative differences are known between these except for the PE content in the toxic varieties (Commission of the European Communities, 2007; Sahoo and Das, 2009). Given that the SSRs are the markers of choice for this analysis, we used them to evaluate the level of polymorphism and the capacity to discriminate between the toxic and non-toxic accessions of a sample of primers chosen from those published, developed both from enriched genomic libraries and from EST databases.

MATERIALS AND METHODS

Plant material

A total of 29 accessions were collected from America (North, Central, South) and Africa starting in 2009. They included 19 populations from different geographical areas in Mexico. The seed of each accession (one to four individuals per accession) was carefully characterized for PE content. The complete list of populations with their provenance and PE content is reported in Table 1.

Phorbol esters extraction and analysis

Sample preparation and PE extraction was done according to the method of Makkar et al. (2007). Briefly, the fruit seeds were pre-dried before de-hulling and drying at 100-105°C for 30 min. Five kernels of each variety were ground and 2 g of kernel powder was subjected to PE extraction by methanol. The methanol extraction step was repeated three times with sonication and centrifugation at 3000 *g* for 8 min at room temperature. PE concentration was obtained by methanol evaporation using a rotary evaporator at 40°C and by flushing with nitrogen. The obtained sample was adjusted to the final volume of 2 ml with methanol, filtered and injected manually (20 µL) into the high performance liquid chromatography (HPLC).

HPLC conditions for quantification of phorbol esters

The analysis was conducted according to the method of Haas and Mittelbach (2000). The HPLC instrument was equipped with a vacuum degasser and a variable UV-VIS wavelength detector (Bio-Rad 1801, Bio-Rad Laboratories, USA). The separation was performed on a 150 × 4.6 mm Poroshell 120 EC-C18 column packed with 2.7 µm particles (Agilent Technologies, USA). 80% acetonitrile solution (HPLC grade, Sigma-Aldrich, Germany) was used as eluent at a flow rate of 2 ml/min at 25°C. The detector wavelength was set at 280 nm. A calibration curve was prepared using phorbol 12-myristate 13-acetate (12-O Tetradecanoylphorbol 13-acetate, TPA, Sigma, Germany) as an external standard (Wink et al., 1997). The detection limit was calculated as: $y = y_B + 3s_B$, where y is the detection limit, y_B is the blank signal and s_B is standard deviation of the blank. The detection limit was 1.0 mg kg^{-1}.

SSR genotyping

Seed coats were removed from seeds after a preliminary imbibition

Table 1. *Jatropha curcas* accessions used for genetic analysis with SSR markers. The table indicates the origin and the seed kernel content of phorbol esters (PE) expressed as equivalent to a standard, phorbol-12-myristate 13-acetate (limit of detection 1 mg kg $^{-1}$).

Accession number	Country	State-region	PE (mg kg $^{-1}$)
1	Mexico	Morelos	30
2			41
3			14
4			6
5			-
6			10
7		Jalisco	26
8			10
9		Quintana Roo	10
10			4
11			7
12			78
13			72
14		Yucatan	60
15		Veracruz	1
16		Hidalgo	3
17			153
18			44
19			41
20	Guinea-Bissau	Nhacra-Oio	3341
21	Senegal	Kaolack	2374
22		Thies	2607
23		Diurbel	2096
24		Kaffrine	2400
25		Saint-Louis	2391
26	Brasil	Bahia	918
27		Piauì	3324
28	Perù	Lima	3115
29	Ghana	Volta	3011

-, Not detected.

in water for 24 h. Separated cotyledons were ground into a powder in the presence of liquid nitrogen: DNA was extracted from 2-4 individuals per accession using the Dneasy 96 Plant Mini Kit (Qiagen GmbH, Hilden, Germany). A total of forty pairs of primers were used. The primers were chosen on the grounds of their Tm, length, degree of polymorphism and specificity for toxic varieties from those published by Pamidimarri et al. (2009), Phumichai et al. (2011), Yadav et al. (2011), Wen et al. (2010) and Basha et al. (2009). The complete list of primers is reported in Table 2.

A tailed polymerase chain reaction (PCR) primer was used for SSR analysis by adding a 19-base M13 oligo sequence (M13 tail) to the 5′ end of each forward SSR primer. Thus, each SSR reaction used three primers: two unlabelled SSR primers with one having an attached M13 sequence tail, and one universal M13 primer labelled with FAM with the same sequence as the tail sequence attached to one of the SSR primers (Schuelke, 2000; Boutin-Ganache et al., 2001; Fukatsu et al., 2005). The PCR reaction was carried out in 10 µL of a solution containing 10 ng genomic DNA, 1x Mg-free PCR buffer solution, 0.25 mM dNTPs, 1.5 mM $MgCl_2$, 0.15 pmol forward primer, 0.3 pmol reverse primer, 0.3 pmolM13-labelled primer, 0.5 U AmpliTaq Gold DNA polymerase (Applied Biosystems) and dH_2O. Amplification was performed in a 9700 Thermal Cycler (Applied Biosystems) as follows: 5 min at 95°C followed by 30 cycles of: 30 s at 94°C, 45 s at 55°C, 45 s at 72°C, 8 cycles of: 30 s at 94°C, 45 s

Table 2. List of 40 microsatellite markers of which seven (bold) shows polymorphic bands, eight (italics) no amplicons, while 25 show monomorphic bands across 29 Jatropha accessions.

Locus	Repeat motif	Primer sequence(5′–3′)	Tm (°C)	Expected size	Reference
jcds24	$(CA)_5(TA)_8(CA)_4…(TA)_3GA(TA)_4$	F:GGATATGAAGTTTCATGGGACAAG	51.0	n.d	Pamidimarri et al.(2009)
		R:TTCATTGAATGGATGGTTGTAAGG			
jcps6	$(AT)_3G(TA)_3…(CT)_3…(GT)_5CT(GT)_3$	F:CCAGAAGTAGAATTATAAATTAAA	44.0	n.d	
		R:AGCGGCTCTGACATTATGTAC			
jcps20	$(TG)_{12}(GA)_{22}$	F:ACAGCAAGTGCACAACAATCTCA	55.0	n.d	
		R:TACTGCAGATGGATGGCATGA			
jcps21	$(CA)_2…(CA)_4$	F:CCTGCTGACAGGCCATGATT	54.8	n.d	
		R:TTTCACTGCAGAGGTAGCTTGTATA			
jcms21	$(CA)_7$	F:TAACCTCTTCCTGACA	43.0	n.d	
		R:ATAGGAAATAAGAGTTCAAA			
jcms30	$(GT)_5T(TG)_2$	F:GGGAAAGAGGCTCTTTGC	48.5	n.d	
		R:ATGAGTTCACATAAAATCATGCA			
JEM023T	TA (5)	F: AGCAGCTCAACTTCCAGGAG	52.0	159	Yadav et al. (2011)
		R: CTGCAAATTTGGGCAGAATA			
JEM013T	AGAGGC (4)	F: TGTTCCACCAATGATCAACC	52.0	224	
		R: CGGGTCAAATCCTCAATCAT			
JEM035NT	TA (8)	F: TCCATTCCGCCTAAAATCTG	52.0	217	
		R: CGCCCGGTGAGCCTAATA			
JEM041T	AT (7)	F: ATTCCAGCATCCCTGCTATG	50.0	221	
		R: GCAATGATCACCACCAAAAA			
JEM081T	CT (8)	F: TGCTGGTGCTAATGCTGCTA	52.0	162	
		R: CAGAAATGCAAAGCCTTCAG			
JEM089T	CTT (5)	F: GCAAACTGAGCAAAAATCGAG	58.0	155	
		R: GAGGGAGGAGGAGCGATACTA			
JEM099T	TA(5)	F: TGACTGAAAAACGATCCATGA	52.0	154	
		R: GGAAGAAAATCCCTTGGATG			
JEM100T	TA(6)	F: CGGCAGATGGAGGATGTAAC	52.0	152	
		R: AATGTAAACGGCATCGGTAT			
JEM105T	CCG(6)	F: GGAGAGCATTCTGTCCCTTG	50.0	242	
		R: GGAATCGGAAAACCCAAAAT			
JMDB04	(AT)7	F: CTTCTTTCCCTGTGGCTTTG	52.0	106	
		R: ACCTCTCCCTTTTGGTCTGC			
JMDB11	(CT)14	F: GCACCACTTCAGGCGAAA	56.0	114	
		R: GAGCGATAGGGCTGAGTAAGAA			
JMDB31	(AGA)7	F: CCATCACCAATTCCTGGTTT	54.0	152	
		R: TGGCGAGAAAGGTAGGGTTA			

Table 2. Continued.

JMDB38	(TC)11	F: GGCCCTTTACAATTTTCTACTGTC R: GCAGACACGATTTTCCGACT	54.0	181	
JMDB57	(AAT)8	F: TGGCAGAGCAACTGCAAATA R: TCTCACACACCCCAAATTCA	56.0	187	
JCT16	(GT)11	F: GCCTCCAGCATCTTTCAATC R: AACAATCCCCATTCCTCCTC	60.0	n.d	Phumichai et al. (2011)
JCT27	(CT)17	F: GCCATTAGAATGGACGGCTA R: TGCGTGAAGCTTTGATTTGA	60.0	n.d	
JCT31	(TC)18	F: TGGAAAACGAATGAGGCTCT R: GGACACTCTGGAAAGGAACG	59.0	n.d	
JCT37	(AG)20	F: ATTCGACAATCTACGGGATA R: CACCTTATACGTCTCTCTCTCTC	54.0	n.d	
JCT81	(CT)18	F: CCATTTAGAACCACAACCAT R: GATGTCCAATAAGCCTGAAT	540.	n.d	
JCT86	(GA)17	F: TATTTCCTCTTCCTGCACAT R: GTTTGGCTAAAAAGGTGATG	56.0	n.d	
JCT89	(CT)16	F: GCCGATAAACCACAGATAAA R: GAAAAATAAAGCCAGCAAGA	54.0	n.d	
JCT103	(AG)17	F: CAACGACTCTTTGAAGAAAAA R: GCCGATAAACCACAGATAAA	54.0	n.d	
JGAA1	(GAA)9	F: AAAGGTCACAGTGTTTCAAAG R: TTCTTTCTCAACTTCCTCCA	56.0	n.d	
JSSR-225	di—	F: CGCAAGGTAAATCGGAGCTA R: ACAATCAAAGGAGTCGTGTAATC	55.0	117	Wen et al. (2010)
JSSR-228	di—	F: TAGAGCAGCTGCAAAGCAAA R: TCGTTTTCCTGTTGAAATCTTG	55.0	169	
JSSR-232	di—	F: CAGGACATGACGCAATTCTG R: GCATGTTAGAAGTTTTTGCAATTT	55.0	247	
JSSR-233	di—	F: CTTTTTGCCAGTCTTCCTGC R: AATGGATCATGTTCAATGTCTTC	55.0	205	
JSSR-236	di—	F: CGACTGCATCAGAACAATGC R: AGCATGTCATTGCACCAAAC	55.0	296	
JSSR-237	tri—	F: CAGTGAGCAGAAACTAAAAACATTG R: GGCACTTTGGAAAGGAAGAG	55.0	212	
JcSSR-19	(AC)21	F: CTTGAAAGTTTTTGTAATTTC R: CGCCAATCATAGATC	50.0	214	Basha et al. (2009)

Table 2. Continued.

JcSSR-20	(AC)10	F: GGCTGAACTTGCGCC R:GCCCTGATTTCTGGTC	60.0	260
JcSSR-21	(C)7(A)5(CA)9	F: CTGAAATGGAGAAATTGG R: ACATATCGAAGATAGGG	50.0	249
JcSSR-22	(TC)16	F: GAATCTCAACAGTGCCC R: GAAGGATGGGAAGTGGG	52.0	152
JcSSR-26	(CA)18	F: CATACAAAGCCTTGTCC R: AACAGCATAATACGACTC	55.0	211

at 53°C, 45 s at 72°C and a final extension stage of 10 min at 72°C. PCR products were separated with an ABI 3730 DNA sequencer (Applied Biosystems) and the fragments were sized by means of a ladder labelled with a fluorochrome VIZ (LIZ500 Applied Biosystems). Data were analysed with GeneMarker software (Version 2.2.0, SoftGenetics).

Statistical analysis

GenAlEx6 software (Peakall and Smouse, 2006) was used to estimate allele number (An), expected heterozygosity (He), number and frequency of genotypes for each accession. Polymorphism information content (PIC) was computed for each SSR locus according to the study of Botstein et al. (1980). Nei's (1983) genetic distance was calculated in the Populations software program (Langella, 1999). A cluster diagram was constructed based on these distances by the unweighted pair-group method using arithmetic averages (UPGMA) (Sneath and Sokal, 1973) with the UPGMA tree searching algorithm of the software. A thousand replicate distance matrices were bootstrapped (Felsenstein, 1985) to evaluate the robustness of the trees. The tree file was visualized and manipulated using the software MEGA version 5 (Tamura et al., 2011).

RESULTS AND DISCUSSION

PE content

The levels of PE in the accessions not coming from Mexican areas (Table 1) varied from a minimum of 919 (just one genotype collected in Bahia, Brazil) up to a maximum of 3341 $mgkg^{-1}$, with an average of 2558 $mgkg^{-1}$, confirming that PE present at levels of 2000 to 4000 $mgkg^{-1}$ are typical of "toxic" accessions and these are prevalent throughout the world (Rakshit et al., 2010; Makkar and Beker, 2009). On the contrary, all accessions collected in Mexico have shown values between 1 and 78 ppm, with several accessions very close to the detection limit (1 $mgkg^{-1}$) and just one (collected in Hidalgo) exceeded 100 $mgkg^{-1}$; this level is considered the maximum threshold for seeds categorized as "edible" or "non-toxic" (Makkar et al., 1998). The above results confirm that the non-toxic genotypes exist only in Mexico and some of these are better than the conventional toxic jatropha varieties in seed yield and seed oil content (Francis et al., 2013). These genotypes could be directly utilized as seed cake, in animal feeding, after mechanical oil extraction.

SSR analysis

The data obtained were classified according to a qualitative scale, with scores ranging from 1 to 5, describing the complexity of the amplification profile for each primer (Stephenson et al., 1998). Of the 40 loci considered, 26 of them generated an electropherogram of a single locus, of easy interpretation as stuttering was absent or very slight (score 1, 2), in 6 loci the presence of irregular stutter bands and/or multilocus peaks hindered reliable interpretation (score 4, 5), the last 8 loci failed to give rise to amplification products. Despite the good quality of 26 loci, only 7 of them revealed polymorphism across the populations considered in this study. The 7 loci revealed a total of 16 alleles in the 29 *J. curcas* accessions. The seven polymorphic microsatellite markers and their properties are reported in Table 3. An overview of SSR markers as detected by DNA fragment analysis with a fully automated capillary electrophoresis system is given in Figure 1. The number of alleles per locus was very limited, ranging from 2 (JMDB04, JCT16, JcSSR26, JCT27, JCT31, JCT81) to 3 (jcms21). The average PIC (Polymorphism Information Content) value was 0.3617 within the range 0.351 (JCT27) to 0.389 (jcms21). The loci were completely or highly homozygotic and consequently the alleles were fixed.

Genetic relationships between accessions

The genetic distance between the accessions examined was calculated using Nei's index (1983). Cluster analysis applied to the matrix

Table 3. List of the **s**even polymorphic loci and their proprieties.

LOCUS	Accession	Na[a]	Ho[b]	He[c]	PIC[d]	Allele size (bp) non-toxic	Allele size (bp) toxic
jcms21	EU586350	3	0.015	0.4912	0.388	90-92	78
JMDB04	JMDB04	2	0.000	0.4632	0.354	125	123
JCT16	AB512288	2	0.000	0.5027	0.374	120	122
JCT27	AB512290	2	0.000	0.4576	0.351	259	253
JcSSR-26	(EU099526)	2	0.000	0.4632	0.354	210	230
JCT31	AB512291	2	0.000	0.4663	0.355	214	208
JCT81	AB512296	2	0.000	0.4632	0.354	157	161

a, number of alleles; b, observed heterozygosity; c, expected heterozygosity; d, polymorphism information content; bp, base pairs.

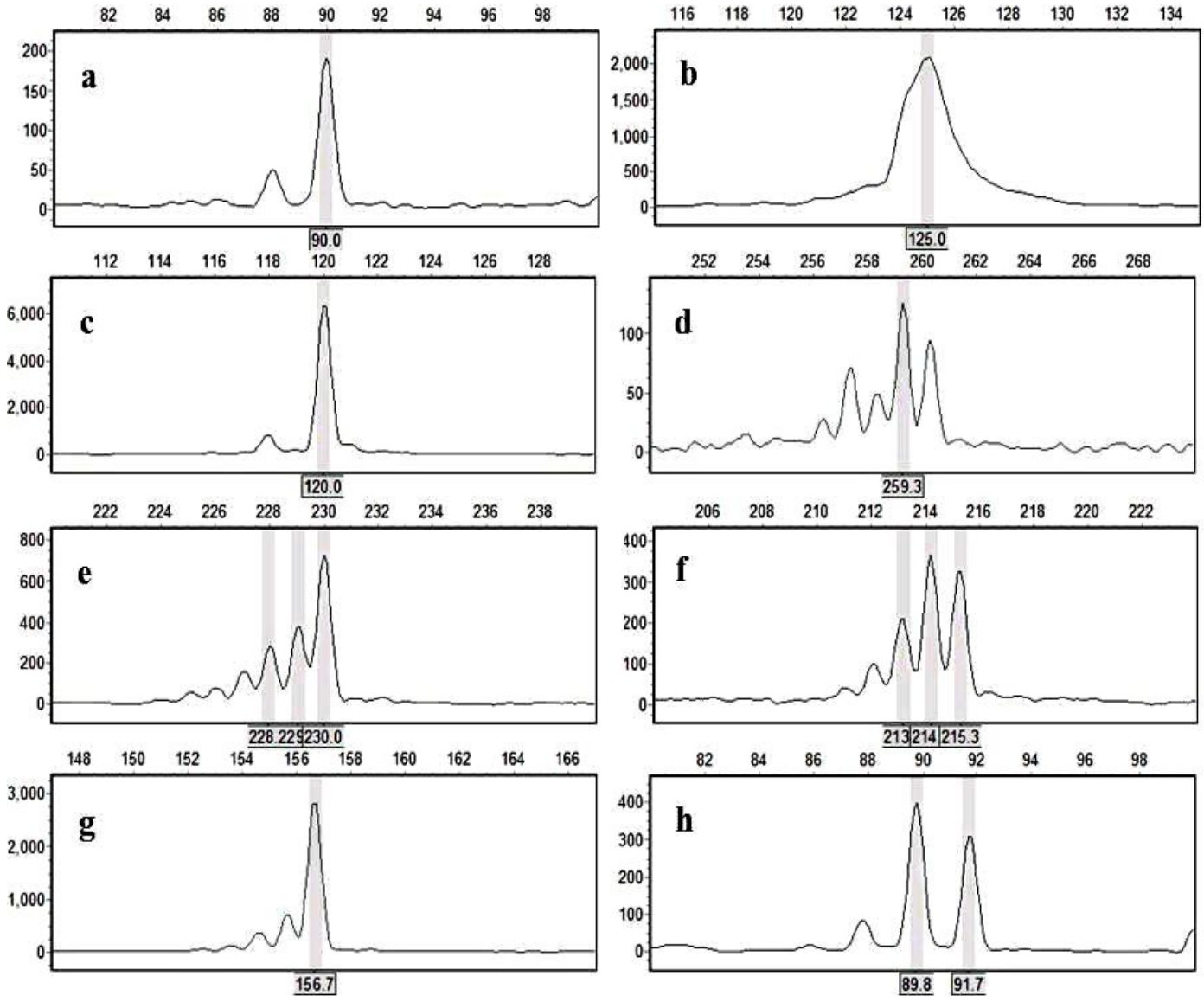

Figure 1. DNA fragment profiles of some alleles displayed by the 7polymorphic loci. The non Mexican accessions proved to be homozygous in all loci. (**a**, Jcms21; **b**, JMBD04; **c,** JcSSR16; **d,** JCT27; **e,** JcSSR26; **f,** JCT31; **g,** JCT81). The Mexican accessions were highly homozygous with some exception (**h**, Jcms21). To note the heterozygous locus for alleles of 90 and 92 bp. **a, b, d, f**, specific alleles (90, 125, 259, 214 bp respectively) for Mexican accessions, **e. g.** specific alleles (230, 160 bp respectively) for non Mexican accessions.

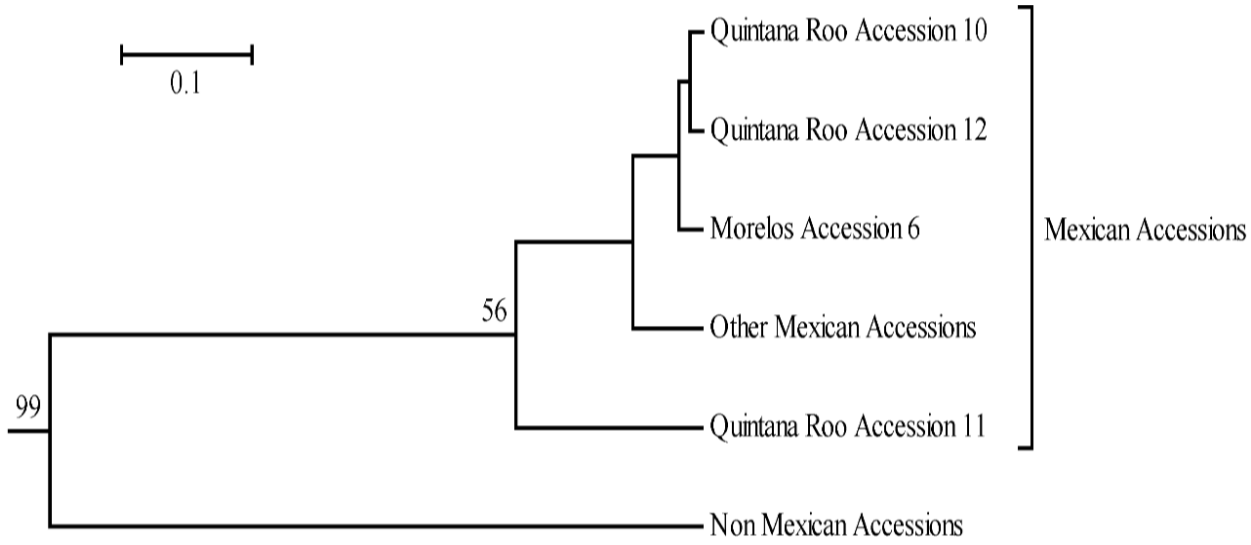

Figure 2. UPGMA cluster analysis of *J. curcas* accessions based on Nei's genetic distances. Only values above 50% of bootstrap node support are shown. Accession numbers are reported as in Table 1.

of genetic distances produced a diagram in which two branches are created that identify two completely different clusters, each of which is composed of very similar populations (Figure 2). The first group includes the non-Mexican toxic populations and the second, the non-toxic Mexican populations. Strong genomic uniformity was found within non-Mexican group of accessions. Some genetic variability was noted in the Mexican group, the accessions 10, 12 from Quintana Roo and accession 6 from Morelos regions were on separated branches from the other Mexican accessions. Moreover accession 11 from Quintana Roo Region formed a distinct sub-group supported by a bootstrap value greater than 50 (Figure 2). These results pointed out Quintana Roo region as the most promising area for genetic variability of *J. curcas*. The high homozygosis is surprising in a monoic and allogamous species but in agreement with previous reports by Ambrosi et al. (2010), Rosado et al. (2010) and Tanya et al. (2011) who found high values of homozygosis estimated on the basis of co-dominant SSR markers, especially in non-Mexican accessions. The species is a native to Central and South America, but is now widely present throughout Central America, Africa and Asia. Limited genetic variation of *J. curcas* populations outside the centre of origin is likely due to infrequent introductions, predominant vegetative propagation or the occurrence of asexual reproduction (that is, apomixis) (Ambrosi et al., 2010). This restricted genetic base generates phenomena of inbreeding that could explain the high homozygosis (Rosado et al., 2010).

Out of 7 polymorphic loci, jcms21 (EU586350), JMDB04, JCT27 (AB512290), JCT31 (AB512291), JCT81 (AB512296) and JcSSR-26 (EU099526) clearly displayed distinct allele patterns between 19 Mexican accessions (nontoxic) versus 10 non-Mexican accessions (toxic) (Table 2), with the only exception being JCT16 (AB512288) that shared 2 alleles in both groups. Interestingly, loci jcms21 (EU586350) and JcSSR-26 (EU099526) displayed specific alleles for each geographical group well differentiated in length (78 vs. 90 bp and 210 vs. 230 bp, respectively). According to a Stepwise Mutation Model this implies a well separated evolutionary history that could strengthen the hypothesis that the current distribution of *J. curcas* is not only a result of recent human activity but is also due to the historical separation and the subsequent spreading of *Jatropha* in Africa and America (Dehgan and Webster, 1979; Dehgan and Schutzman, 1994). The high homozygosis and low genetic variability found also in the Mexican accessions may at least in part be explained by the fact that the samplings were done on isolated plants distant from other possible pollinators and this has led to a high inbreeding over the years with the consequent fixation of the alleles. The low genetic variability found between the Mexican accessions may instead be due to the limited number of markers and that those polymorphic are anyway associated to the toxicity as described in materials and methods. In order to find greater variability, further samplings should be carried out in different Mexican areas. As already reported by Ovando-Medina et al. (2011), a high genetic diversity was found within and among *J. curcas* populations in the State of Chiapas. These markers could be utilized to easily identify the toxic varieties from that non-toxic given that no particular morphological differences exist (Pamidimarri et al., 2009). The association to toxicity should anyhow be validated with the development of segregating populations and the construction of genetic maps to avoid spurious associations on a geographical basis rather than genetic.

These markers could then be utilized for programmes of selection assisted by markers to introduce the non-toxicity character in elite varieties of *J.curcas*.

The sustainability and hence wider acceptance of the jatropha-based biofuel chain will be fully achieved only with the utilization of the *J* seed cake as animal feed. The successful solution could be to obtain a non-toxic *J. curcas* plant, starting from the accessions already present in Mexico improved by suitable breeding programmes or utilized as a genetic source for the introgression of the non-toxic trait into elite toxic varieties. So a primary goal in the genetic improvement of *J. curcas* will be the identification of accessions with high levels of both phenotypic and genetic diversity. Moreover studies should also be done on the maternal inheritance of PE content by direct and reciprocal crosses between toxic and non-toxic genotypes. A further development of the analysis with molecular markers can be obtained with the integration of the results obtained with different classes of markers and developing mapping populations for linkage analysis of toxicity associate markers.

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