Plant Science 176 (2009) 812–823

Contents lists available at ScienceDirect

Plant Science

journal homepage: www.elsevier.com/locate/plantsci

A comparative study of biochemical traits and molecular markers for assessment of genetic relationships between *Jatropha curcas* L. germplasm from different countries

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ARTICLE INFO

Article history:
Received 29 August 2008
Received in revised form 6 March 2009
Accepted 11 March 2009
Available online 24 March 2009

Keywords:
Genetic diversity
Jatropha curcas
Microsatellites
Phorbol esters
SCAR

ABSTRACT

Jatropha curcas has gained popularity as a potential biofuel crop but the major constraint for improvement of the crop for yield and seed quality traits is the narrow genetic base of the germplasm. Genetic background of 72 *J. curcas* accessions representing 13 countries has been elucidated using molecular analysis and biochemical traits. Seed kernel protein, oil content, ash content and phorbol esters revealed variation with accessions from Mexico containing low levels of phorbol esters. Molecular characterization disclosed polymorphism of 61.8 and 35.5% with RAPD and ISSR primers, respectively and Mantel test revealed positive correlation between the two marker systems. Dendrogram based on pairwise genetic similarities and three-dimensional principal coordinate analysis using data from RAPD and ISSR marker systems showed close clustering of accessions from all countries and grouped the Mexican accessions separately in clusters III, IV, V and VI. Presence of the toxic phorbol esters is a major concern and analysis of 28 Mexican accessions resulted in identification of molecular markers associated with high and low phorbol ester content. The identified RAPD and ISSR markers were converted to SCARs for increasing the reliability and use in marker assisted programmes aimed at development of accessions with reduced toxicity. Twelve microsatellite primers differentiated the non-toxic Mexican accessions and disclosed novel alleles in Mexican germplasm. Amplification with primers specific to the curcin coding sequence and promoter region of ribosome-inactivating protein (RIP) revealed polymorphism with one primer specific to RIP promoter region specifically in accessions with low phorbol ester levels. Narrow genetic variation among accessions from different regions of the world and rich diversity among Mexican genotypes in terms of phorbol ester content and distinct molecular profiles indicates the need for exploitation of germplasm from Mexico in *J. curcas* breeding programmes.

1. Introduction

Jatropha curcas, belonging to the family Euphorbiaceae, is a low-growing tree, native to Central and South America and is widely present throughout Central America, Africa and Asia. *Jatropha* is a vigorous, relatively drought- and pest-tolerant plant and is not browsed by animals. It is planted in tropical countries principally as a hedge, protecting cropland from the cattle, sheep and goats [1–3]. *J. curcas* can grow under a wide range of rainfall regimes (200 mm to over 1500 mm per annum) and also survives on marginal lands under harsh climatic conditions.

Traditionally, Jatropha seed and other plant parts have been used for oil, soap and medicinal compounds [4]. However, its recent popularity is due to the potential of its seed oil as a source of biodiesel. Jatropha is unique among renewable energy sources in terms of the number of potential benefits that can be expected from its widespread cultivation. Its cultivation requires simple technology, and comparatively modest capital investment. The seed yield reported for Jatropha varies from 0.5 to 12 tons/(year ha) – depending on soil, nutrient and rainfall conditions – and the tree has a productive life of over 30 years [1,3]. The seeds contain 30–35% oil that can be converted into good quality biodiesel by transesterification [5,6].

The true potential of Jatropha has, however, not yet been realized because of a host of factors. The framework conditions for its exploitation have improved considerably in recent times because of the increase of crude oil prices and policy incentives for the introduction of indigenous and renewable fuels. However, several agro-technological challenges remain for the exploitation of Jatropha as a commercial crop. Even though Jatropha has been

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doi:10.1016/j.plantsci.2009.03.008

scientifically investigated earlier for useful secondary metabolites, the kind of comprehensive research and development efforts necessary to generate profitability and the critical information for the different climatic and edaphic regions have only recently started. Results of such research are trickling in slowly. Yet, the high market demand for biodiesel has resulted in many organisations starting with Jatropha plantations. The success of these ventures rests on the continuous inflow of relevant information from research into practise [7].

One of the most important inputs for successful cultivation of *J. curcas* is the selection of planting material. Observations in upcoming plantations indicate that the productivity of the individual plants show high variations. The major concern is promotion of the crop in its undomesticated condition. Gaining insights into the genetic variability of *J. curcas* provenances and elucidation of correlations between the genetic parameters and biochemical characteristics of seed provenances collected from different regions of the world would be a critical input for the selection of appropriate genotypes for cultivation and breeding purposes.

Recent interest in promotion of *J. curcas* as a biodiesel crop demands genetic improvement of the crop for increased seed yield and oil content. Germplasm characterization is necessary to enhance germplasm management and utilization. Information regarding the extent and pattern of genetic variation in *J. curcas* population is limited. Previous studies on characterization of *J. curcas* germplasm were confined to locally available germplasm using morphological and molecular markers [8]. This study is in continuation to the previous study wherein molecular characterization of germplasm accessions from different regions in India using RAPD and ISSR primers revealed low genetic variability between accessions from same country and maximum divergence between Indian accessions and a non-toxic Mexican accession [9]. Similar variation between six toxic accessions and one non-toxic Mexican accession has been reported by employing RAPD, AFLP and SSR markers [10]. Likewise, genetic diversity assessment of local germplasm revealed low molecular variability among accessions [4,11,12]. In all the above studies genetic variation was mainly due to inclusion of geographically isolated accessions [9,10] or wild species [11,12]. This has necessitated assessment of genetic diversity in *J. curcas* accessions from different parts of the world. The current investigation presents the results of characterization of locally dominant ecotypes collected from 13 *J. curcas* growing countries using biochemical parameters and molecular analysis to understand the genetic richness and geographic structure of the germplasm.

2. Materials and methods

2.1. Plant material

A set of 72 predominantly grown accessions of *J. curcas* from 13 countries was used for the present study. The representative collection includes one each of the Indian and Mexican accessions used in the earlier study [9] as standards for toxic and non-toxic germplasm. In all cases, the predominantly grown accession was used. In case of some countries where variation has been reported different provenances were collected. Accordingly, the accessions from India were from the states of Orissa, Maharashtra, Andhra Pradesh and Kerala; the regions of collection in Mexico include Juan Sarabia, Q-Roo, Veracruz, Puebla and Guerrero and in Madagascar from Amparihi Analavory, Miadanarivo Ampefy, Ambomanarivo Nord, Tsarahonemana Ankazobe, Antafofo Waterfalls Lily, Aniome Ramartine, Axe Majunga, Tulear Sakahara and Fiamaraantsoa. The germplasm was maintained at the research farm of the Directorate of Oilseeds Research, Hyderabad, India and the details of the accessions are presented in Table 1.

2.2. Proximate composition

The analysis for crude protein, lipid, and ash were done in accordance with the standard methods of AOAC [13], i.e. crude protein was measured as macro-Kjeldahl N × 6.25, lipids by extraction with petroleum ether in a Sohxlet apparatus and ash by burning at 550 °C to a constant weight in a Muffle furnace.

2.3. Extraction and estimation of phorbol esters through HPLC

About 1 g of seed kernel was weighed, ground and subsequently extracted with methanol as described by Makkar et al. [14]. The analytical column was reverse phase C18 (LiChrospher 100, endcapped 5 μm) 250 mm × 4 mm I.D. column protected with a guard column containing the same material as the main column. The separation was performed at room temperature (23 °C) and the flow rate was 1.3 ml min^{-1} using a gradient elution [15]. The four phorbol ester compound peaks that appeared between 26 and 31 min were identified and integrated at 280 nm. The results are expressed as equivalent to a standard, phorbol-12-myristate 13-acetate, which appeared between 34 and 36 min.

2.4. DNA extraction

Total genomic DNA was extracted from younger leaves of five plants of 2-year-old plants for each of the *J. curcas* accessions following the standard CTAB method with minor modifications [16]. Five grams of leaves were ground in liquid nitrogen, then homogenized in 20 ml of extraction buffer (2% CTAB, 20 mM EDTA, 2% PVP, 1.4 M NaCl, 100 mM Tris–HCl pH 8.0 and 1% β-mercaptoethanol) and incubated at 65 °C for 1 h. The supernatant was twice extracted with chloroform:isoamylalcohol (24:1, v/v) and treated with RNase A (100 μg/ml), incubated at 37 °C for 30 min. The DNA was precipitated with isopropanol and washed twice with 70% ethanol. The pelleted DNA was air dried and resuspended in 500 μl of sterile MilliQ water and stored at −20 °C. The DNA concentration was determined electrophoretically using known amount of λ DNA as standard.

2.5. RAPD analysis

From the previous study wherein 400 primers were tested, 100 RAPD primers producing robust amplification profiles were chosen for molecular analysis [9]. The PCR amplification reaction (10 μl) consisted of 2.5 ng of DNA, 1× PCR buffer (10 mM Tris pH 9.0, 50 mM KCl, 1.5 mM $MgCl_2$), 100 μM of each of the four dNTPs, 0.4 μM of RAPD primer and 0.3 U of Taq DNA polymerase (Bangalore Genei, India). PCR amplifications were performed in a GeneAmp 9700 Thermal Cycler (PerkinElmer Applied Biosystems) with an initial denaturation at 94 °C for 3 min followed by 45 cycles at 94 °C for 45 s, 36 °C for 30 s and 72 °C for 2 min with a final extension at 72 °C for 7 min. The PCR products were separated on 1.5% agarose gel in 1× TAE buffer by electrophoresis at 100 V for 3 h and visualized with ethidium bromide staining. In general, RAPD markers suffer from a lack of reproducibility, but to check the consistency of the electrophoretic patterns and the polymorphism detected, every PCR reaction disclosing polymorphism among accessions was repeated. All the PCR amplifications included a negative control (no DNA) to avoid erroneous interpretations.

2.6. ISSR-PCR amplification

One hundred ISSR primers (UBC primer set no. 9, University of British Columbia, Canada) were used in the study. The PCR reaction mixture (10 μl) consisted of 2.5 ng of DNA, 200 μM of each of the four dNTPs, 1× PCR buffer (10 mM Tris pH 9.0, 50 mM KCl, 1.5 mM

Table 1
Geographical location of *Jatropha curcas* accessions used in diversity analysis and their biochemical characteristics.

Accession number	Country	Crude protein (%) in kernel	Oil (%) DM	Ash (%) DM	Phorbol ester (mg/g)
WJc-1[a]	India-1	–	–	–	2.21
WJc-2	India-2	30.9	57.2	6.0	2.30
WJc-3	India-3	24.2	56.6	4.0	3.21
WJc-4[b]	India-4	29.9	53.8	5.1	1.054
WJc-5[b]	India-5	33.4	49.7	6.4	1.35
WJc-6[c]	India-6	28.9	55.8	5.6	0.823
WJc-7	India-7	31.2	54.2	5.9	1.74
WJc-8	India-8	–	–	–	4.87
WJc-9	India-9	–	–	–	3.21
WJc-10	India-10	–	–	–	3.97
WJc-11	India-11	–	–	–	2.27
WJc-12	India-12	26.6	–	4.5	1.653
WJc-13	India-13	23.3	60.8	5.1	3.208
WJc-14	India-14	25.1	62.9	4.8	4.058
WJc-15	India-15	29.7	56.4	4.6	2.908
WJc-16	India-16	21.4	63.4	4.4	3.982
WJc-17	India-17	28.7	51.1	6.7	3.259
WJc-18	India-18	25.2	57.3	5.3	3.161
WJc-19	India-19	–	–	–	–
WJc-20	Cape Verde	22.2	57.8	3.2	2.70
WJc-21[a]	Mexico-1	27.2	58.5	4.3	ND
WJc-22	Mexico-2	29.7	61.2	4.3	ND
WJc-23	Mexico-3	27.3	59.2	4.7	ND
WJc-24	Mexico-4	28.4	60.7	4.0	0.01
WJc-25	Mexico-5	29.0	55.3	5.2	0.02
WJc-26	Mexico-6	26.9	61.9	4.7	0.086
WJc-27	Mexico-7	26.9	60.3	3.8	0.057
WJc-28	Mexico-8	27.7	60.5	4.7	0.049
WJc-29	Mexico-9	26.8	60.9	4.7	1.204
WJc-30	Mexico-10	27.3	59.2	4.4	1.36
WJc-31	Mexico-11	27.8	55.9	4.8	2.026
WJc-32	Mexico-12	27.2	58.5	4.3	0.11
WJc-33	Mexico-13	25.9	56.0	5.3	0.018
WJc-34	Mexico-14	27.4	59.8	5.4	0.038
WJc-35	Mexico-15	34.5	57.2	3.8	ND
WJc-36	Mexico-16	33.6	56.3	3.9	3.85
WJc-37	Mexico-17	32.1	55.3	5.1	ND
WJc-38	Mexico-18	31.1	57.8	4.7	ND
WJc-39	Mexico-19	33.6	56.3	3.9	3.85
WJc-40	Mexico-20	31.9	52.7	4.6	ND
WJc-41	Mexico-21	33.3	45.9	4.1	0.60
WJc-42	Mexico-22	28.9	57.5	3.8	ND
WJc-43	Mexico-23	27.6	58.4	5.1	ND
WJc-44	Mexico-24	29.7	58.8	4.7	ND
WJc-45	Mexico-25	29.9	57.1	5.3	ND
WJc-46	Mexico-26	18.8	64.5	5.8	ND
WJc-47	Mexico-27	23.2	57.8	5.5	1.88
WJc-48	Mexico-28	33.6	56.3	3.9	3.85
WJc-49	Madagascar-1	20.0	59.9	5.4	4.503
WJc-50	Madagascar-2	28.9	59.7	3.9	5.213
WJc-51	Madagascar-3	26.8	61.0	4.0	5.718
WJc-52	Madagascar-4	25.0	62.3	3.9	5.166
WJc-53	Madagascar-5	25.8	59.3	4.2	6.167
WJc-54	Madagascar-6	27.5	58.5	4.3	4.71
WJc-55	Madagascar-7	24.8	53.9	4.9	2.04
WJc-56	Madagascar-8	28.4	55.9	4.3	3.76
WJc-57	Madagascar-9	27.9	55.3	4.0	3.57
WJc-58	El Salvador	28.2	57.2	3.9	1.23
WJc-59	Uganda-1	–	–	–	6.44
WJc-60	Africa	27.2	59.1	4.6	1.71
WJc-61	Egypt	31.8	48.3	5.3	4.406
WJc-62	Vietnam	27.7	56.3	4.7	5.60
WJc-63	China-1	31.5	48.6	5.2	3.727
WJc-64	China-2	31.0	56.8	4.0	3.451
WJc-65	China-3	31.8	54.4	4.4	4.984
WJc-66	China-4	32.2	45.4	6.2	2.259
WJc-67	Malaysia	26.5	58.0	3.5	3.018
WJc-68	Philippines-1	32.3	54.9	5.4	–
WJc-69	Philippines-2	28.3	60.5	3.5	–
WJc-70	Thailand	–	–	–	–
WJc-71	Uganda-2	–	–	–	–
WJc-72	Uganda-3	–	–	–	–
Min		18.8	45.4	3.2	0.01
Max		34.5	64.5	6.7	6.44
Average		28.3	57.1	4.7	2.19

Table 1 (*Continued*)

Accession number	Country	Crude protein (%) in kernel	Oil (%) DM	Ash (%) DM	Phorbol ester (mg/g)
Standard deviation		3.41	3.89	0.76	1.88

ND: not at detectable levels, –: indicates not determined due to small sample size, DM: on dry matter basis.
[a] Represents the toxic Indian and non-toxic accessions used in the study of Basha and Sujatha [9].
[b] Seeds from open pollinated flowers of toxic accessions from India that were grown along with the non-toxic Mexican accession, Wjc-6.
[c] Wjc-6 and Wjc-21 are from the same source but Wjc-6 has been raised in Maharashtra along with toxic accessions and seeds are from open pollinated flowers while Wjc-21 is from self-pollinated seeds.

$MgCl_2$), 0.2 μl of 25 mM $MgCl_2$, 0.4 μM ISSR primer and 0.6 U Taq DNA polymerase (Bangalore Genei, India). PCR amplifications were performed with initial denaturation at 94 °C for 4 min followed by 35 cycles of 30 s at 92 °C, 1 min at the annealing temperature (38–64 °C depending on the primer), 2 min elongation at 72 °C and final extension at 72 °C for 7 min. The amplified products were electrophoresed in buffer with 1× TAE at 100 V on a 1.7% agarose gel using EcoRI and HindIII double digest as the molecular weight standard.

2.7. SCAR marker development

For the development of SCAR markers, three RAPD bands specific to toxic genotypes and five RAPD and two ISSR bands specific to non-toxic genotypes were excised and purified from the agarose gel using QIAquick gel extraction kit (Qiagen, Germany). The purified fragments were ligated in to pTZ57R (Insta) T/A cloning vector using cloning kit (MBI Fermentas, USA) and then the recombinant plasmids were transformed in to *E. coli* strain (DH5α) by heat shock method. From the randomly selected white colonies plasmid DNA were isolated using the alkaline-lysis method [17]. Colony PCR and restriction enzyme digestion (EcoRI and HindIII) were performed to confirm the presence of the insert and subsequently the cloned DNA fragments were sequenced at Bioserve Biotechnologies (Hyderabad, India) using M13 vector-specific primers.

Based on the sequence of cloned fragments, two oligonucleotide primers with higher *Tm* were designed as SCAR primers. SCAR amplification was performed in 10 μl of reaction mixture containing 2.5 ng of DNA, 1× PCR buffer (10 mM Tris pH 9.0, 50 mM KCl, 1.5 mM $MgCl_2$), 150 μM of each dNTP, 4 pmol of each primer and 0.3 U of Taq DNA polymerase (Bangalore Genei, India). PCR amplifications were carried out with an initial denaturation at 94 °C for 3 min followed by 35 cycles at 92 °C for 20 s, appropriate annealing temperature for 15 s and 72 °C for 90 s with a final extension at 72 °C for 5 min. The amplified products were resolved on 1.5% agarose gel and stained with ethidium bromide. Of the 10 markers, 7 gave single reproducible bands. The sequences were edited and assembled using the Chromas 1.45 software and deposited in the NCBI GenBank nucleotide sequence database (accession numbers EU748549–EU748555).

2.8. SSR analysis

Primer pairs specific to 17 microsatellite containing sequences of *J. curcas* from NCBI GenBank nucleic acid sequence database (accession numbers EU099518–EU099534) were designed manually considering the parameters like primer length, 16–22 nucleotides; oligomer *Tm*, 50–65 °C; GC content of 30–70% and absence of self-annealing or dimer formation (Table 7). The designed primers were synthesized from Bioserve Biotechnologies (Hyderabad, India).

PCR reactions were set up in a total volume of 10 μl, consisting of 2.5 ng of template DNA, 1× PCR buffer (10 mM Tris pH 9.0, 50 mM KCl, 1.5 mM $MgCl_2$), 200 μM of each of the four dNTPs, 0.2 μM of each of the forward and reverse primers and 0.3 U of Taq DNA polymerase (Bangalore Genei, India). PCR amplifications were done with initial denaturation at 94 °C for 4 min followed by 35 cycles of 30 s at 92 °C, 30 s at annealing temperature (*Ta*), 1 min elongation at 72 °C and final extension at 72 °C for 5 min. All amplified products were resolved on 4% agarose gel using 100 bp ladder (Bangalore Genei, India) as molecular weight standard.

2.9. Curcin-specific primers

Oligonucleotide primers were designed for three sequences each specific to the *J. curcas* curcin gene complete coding sequence and the promoter region of ribosome-inactivating protein gene (Table 2). PCR amplifications were carried out in a final volume of 10 μl, containing 2.5 ng of genomic DNA, 1× PCR buffer (10 mM Tris pH 9.0, 50 mM KCl, 1.5 mM $MgCl_2$), 150 μM of each dNTP, 4 pmol of each primer and 0.3 U of Taq DNA polymerase (Bangalore Genei, India). PCR amplifications were performed with an initial denaturation at 94 °C for 3 min followed by 35 cycles at 92 °C for

Table 2
Curcin oligonucleotide primer sequences, annealing temperature and expected size for each marker.

Primer	GenBank accession no.	Primer sequence for amplification	Amplicon length (bp)	*Tm* (°C)	*Ta* (°C)
Curcin cds1 F	EU395775	GTCATCAAATCAGCGCCC	1518	56	60
Curcin cds1 R		GGATTAAAGCCATGGCAG		54	
Curcin cds2 F	EU195892	GGAATAGAAGACTTTGTTTTC	1819	56	54
Curcin cds2 R		GCTCATACATTGGAAAGATG		56	
Curcin cds3 F	AF469003	GGAATAGAAGACTTTGTTTTC	1702	56	52
Curcin cds3 R		CATACATTGGAAAGACGAGG		58	
Curcin-P1 F	EF612739	GAATAGAAGACTTTGTTTTC	547	52	54
Curcin-P1 R		GAGATAGAATGTTCACAAG		52	
Curcin-P2 F	EF612740	CGTGTCATATTTCTCGTTTG	548	56	54
Curcin-P2 R		GAGATAGAATGGTCACAAGT		56	
Curcin-P3 F	EF612741	CAACGGTCATCAAATTAGC	487	56	50
Curcin-P3 R		CAATATCATTATACGGAATAC		54	

Tm = theoretical annealing temperature [4(G + C) + 2(A + T)].
Ta = optimal annealing temperature.

30 s, appropriate annealing temperature for 30 s and 72 °C for 2 min with a final extension at 72 °C for 5 min. The amplified products were resolved by electrophoresis on 1.5 or 4.0% agarose gel depending on the product size and visualized by ethidium bromide staining. Each PCR reaction was done three times to confirm the amplification pattern.

2.10. Statistical analysis

The gel images were recorded using the Alpha Innotech Fluorchem gel documentation system. For each RAPD and ISSR primer, the presence or absence of bands in each accession was visually scored and set in a binary matrix. The number of polymorphic and monomorphic fragments for each primer pair was scored and the monomorphic markers were excluded from the analysis. The binary matrices were read by NTSYS-pc version 2.02i [18] with Jaccard's similarity coefficients and estimates of genetic distances for all pairwise comparisons between accessions were determined using Similarity for Qualitative Data (SIMQUAL). Dendrograms were constructed independently for both the marker systems and also based on pooled marker data using Unweighted Pair Group Method with Arithmetical Averages (UPGMA). The correlation between matrices was determined using Mantel test. Principal coordinate analysis was performed and the ordination displayed in three dimensions.

3. Results

Morphologically, the accessions did not show gross phenotypic variations during the first 2 years of growth with exceptions. The accessions Wjc-39 and 40 had distinctly large leaves (1.5-fold) as compared to leaves of other accessions. Accession Wjc-3 flowered early while Wjc-41 had small seeds and was also early flowering.

3.1. Biochemical characterization

The proximate composition analysis of the seed samples collected from different sites showed wide variation in crude protein, oil and ash contents (Table 1). The crude protein content varied between 18.8 and 34.5% on dry matter basis in the kernels. The oil content varied between 45.4 and 64.5% and the ash content between 3.2 and 6.7%, also on a dry matter basis in the seed kernels. The standard deviation in all cases was relatively low. Most of the seed kernels had values that were closer to the average with only few divergences from the pattern.

The phorbol ester content also showed significant variation among the genotypes (Table 1). Some genotypes from Mexico contained very low levels of phorbol esters or none at all. In the provenances that contained phorbol esters, their content varied quite considerably. Correlations were negative between protein and oil content (−0.5958), protein and phorbol ester level (−0.058), oil and ash content (−0.3748) and ash and phorbol ester level (−0.231) while the correlations were positive between protein and ash content (+0.03714) and oil and phorbol ester level (+0.063). Genotypes from Madagascar possessed higher oil content and phorbol ester levels.

3.2. RAPD

The 100 tested primers were polymorphic and produced amplification products with all accessions. The number of bands amplified per primer varied between 3 (OPC-7) and 27 (OPD-16) with an average of 12.3 bands per primer. A total of 1233 bands were amplified of which 762 were polymorphic resulting in a polymorphism frequency of 61.8% and an average of 7.6 polymorphic bands per primer. The size of bands varied between 200 and 3500 bp. The extent of polymorphism per primer ranged from 15.3 (OPT-20) to 83.3% (OPE-2). Similarity matrix values using Jaccard's coefficient based on RAPD markers ranged from 0.213 between Wjc-49 (Madagascar) and Wjc-38 (Mexico) to 0.885 between accessions Wjc-15 and Wjc-16 from India. At 41% similarity, the accessions separated out into six clusters. Clustering of accessions based on dendrogram and PCO analysis was similar for most accessions except for the accessions from Malaysia (Wjc-67) and Madagascar (Wjc-49) which were grouped with accessions from Uganda in PCO. Two accessions from Mexico (Wjc-38 and -46) remained as outliers. The three axes of principal coordinate analysis explained 47.1, 8.4 and 3.8% of the total variation (Fig. 1a).

3.3. ISSR

Of the 100 tested primers, 48 primers produced amplification products of which only 36 primers revealed polymorphic loci across the *J. curcas* accessions tested. A total of 586 bands were amplified of which 208 amplicons were polymorphic resulting in polymorphism of 35.5%. The average number of polymorphic amplicons per primer was 5.8. The number of bands amplified per primer ranged between 5 (UBC-816) and 20 (UBC-889) and the amplicon size varied from 100 to 3500 bp. The extent of polymorphism varied between 8.3 (UBC-886) and 69.2% (UBC-866). Of the five categories of ISSR primers tested, mononucleotide primers failed to produce amplification products. Polymorphism was 35.3, 26.0, 39.3 and 38.5% with di-, tri-, tetra- and pentanucleotide primers, respectively with the tetranucleotide primer UBC-866 giving maximum polymorphism. Similarity matrix values ranged from 0.198 between Wjc-52 (Madagascar) and Wjc-38 (Mexico) to 0.949 between Wjc-61 (Egypt) and Wjc-59 (Uganda). Dendrogram analysis separated the accessions into five clusters at 53% similarity. There were minor differences in grouping based on dendrogram and PCO clustering. Accessions from Uganda (Wjc-71 and -72) and Malaysia (Wjc-67) were grouped in cluster I based on UPGMA, while the three accessions were placed distantly in PCO analysis. The main three components of PCO analysis explained 73.3% of the total variation. PCO analysis revealed distinct demarcation between accessions of Central and South America from accessions of other regions (Fig. 1b). The accessions Wjc-38 and Wjc-46 from Mexico had distinct OTUs as in the case of RAPD analysis.

3.4. RAPD–ISSR

The genetic similarity matrix data generated using RAPD and ISSR systems were compared. Mantel test for congruence of RAPD and ISSR data matrices indicated a goodness of fit ($r = 0.84588$) indicating good correlation between the two molecular marker systems. The most divergent accessions were Wjc-49/Wjc-38 (Madagascar/Mexico; similarity matrix 0.218), while the most closely related accessions were Wjc-15/Wjc-16 (Indian accessions; similarity matrix 0.899).

The genetic relatedness of the accessions was determined using Jaccard's similarity coefficients. Dendrogram constructed based on RAPD–ISSR polymorphism separated the accessions into eight distinct clusters at 48% of variation (Fig. 2). Group I was the largest cluster and consisted of 40 accessions which included all the Indian (19), China (4), Philippines (2), Mexico (2), Madagascar (8), and 1 each from Cape Verde, Uganda, Africa, Egypt, Vietnam and Thailand. Group II consisted of two accessions from Uganda. Group III included one accession each from Madagascar and Malaysia. Group IV had the lone accession Wjc-6 that has its origin in Mexico and cultivation in India. Group V included 21 accessions which have their origin in Mexico. In Group VI, three accessions from Mexico and one accession from EI Salvador clustered together. Clusters VII and VIII had one accession each from Mexico.

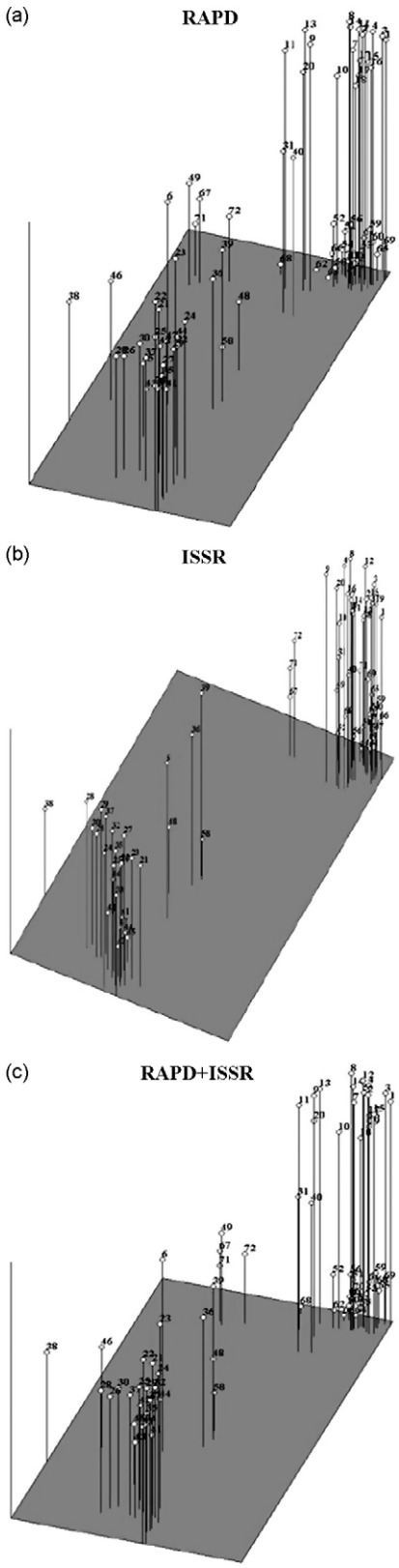

Fig. 1. Three-dimensional plot of 72 accessions of *Jatropha curcas* by principal coordinate analysis using the Jaccard's similarity coefficients. (a) RAPD markers, (b) ISSR markers, and (c) combined markers from RAPD and ISSR analysis. The numbers represent the accession codes as given in Table 1.

The PCO analysis based on RAPD + ISSR polymorphism grouped the accessions in six clusters (Fig. 3). Two genotypes from Mexico (Wjc-38 and -46) failed to cluster with any other accessions. The three axes of PCO analysis explained 49.9, 9.2 and 3.4% variation among accessions (Fig. 1c). A few differences in clustering were observed with UPGMA clustering and PCO analysis. Two accessions from Uganda (Wjc-71 and 72) and one accession each from Malaysia (Wjc-67) and Madagascar (Wjc-49) which formed two distinct clusters in UPGMA grouped together in PCO analysis. The accession from Mexico that was grown in India (Wjc-6) formed a separate OTU in dendrogram while it grouped with the Mexican accessions in PCO analysis.

3.5. Markers specific to toxic and non-toxic genotypes

In the previous study (Basha and Sujatha [9]), markers were identified that could distinguish the 42 toxic Indian accessions and the non-toxic Mexican accession. However, polymorphism between toxic (45) and non-toxic genotypes (27) in this study indicate specificity of 3 RAPD primers to the toxic genotypes and 5 RAPD and 2 ISSR primers to the non-toxic genotypes (Table 3). Similarly, four RAPD markers specific to Mexican accessions are presented in Table 4. Molecular markers specific to 20 accessions were identified (Table 5). The accessions possessing distinct bands were from India (5), Mexico (10), Madagascar (2), Malaysia (1) and Uganda (2). These markers could be converted to SCAR primers for enhancing the reliability of the markers.

3.6. SCAR markers

Previously two diagnostic SCAR markers based on ISSR polymorphism viz., ISPJ-1 and ISPJ-2 were developed [9] and were presumed to be specific either to the Mexican origin or non-toxic character. Validation of these two markers on the 72 accessions used in this study showed association of the markers with toxicity. While ISPJ-1 was specific to toxic genotypes, ISPJ-2 was specific to the non-toxic accessions (Fig. 4a and b). In the present study, an additional 10 markers were identified (Table 3) of which 7 were sequenced and deposited in NCBI Genbank (accession nos. EU748549–EU748555). Blast search of these sequences showed no similarity to any of the known sequences of *J. curcas* or any toxic germplasm nucleotide sequence. Three of the seven SCAR markers could be successfully validated on the representative set of germplasm (Table 6). Amplification of DNA from non-toxic accessions with SCAR marker ISPJ-3 converted from ISSR marker UBC-807 is presented in Fig. 4c. Exceptions were observed with regard to the association of markers with the trait. Accessions Wjc-36, -39 and -48 with high phorbol ester levels failed to show toxicity specific band with ISPJ-1. Likewise, accession Wjc-40 with low phorbol ester content lacked the non-toxic specific amplicon with ISPJ-2 and ISPJ-3 primers.

3.7. SSR analysis

Of the 17 primer pairs tested, 12 gave amplicons in the expected band size. A total of 25 alleles were detected of which 18 were polymorphic (Table 7). The SSR primers produced two to four alleles and maximum polymorphism was obtained with jcSSR-26. Interestingly, microsatellite polymorphism was observed only in the accessions from Mexico and El Salvador while accessions from other countries failed to be distinguished (Fig. 4d and e). The primer jcSSR-26 clearly differentiated toxic accessions from non-toxic accessions with two exceptions. Accession Wjc-39 with high phorbol ester content showed allele specific to non-toxic accessions. Likewise, the accession from El

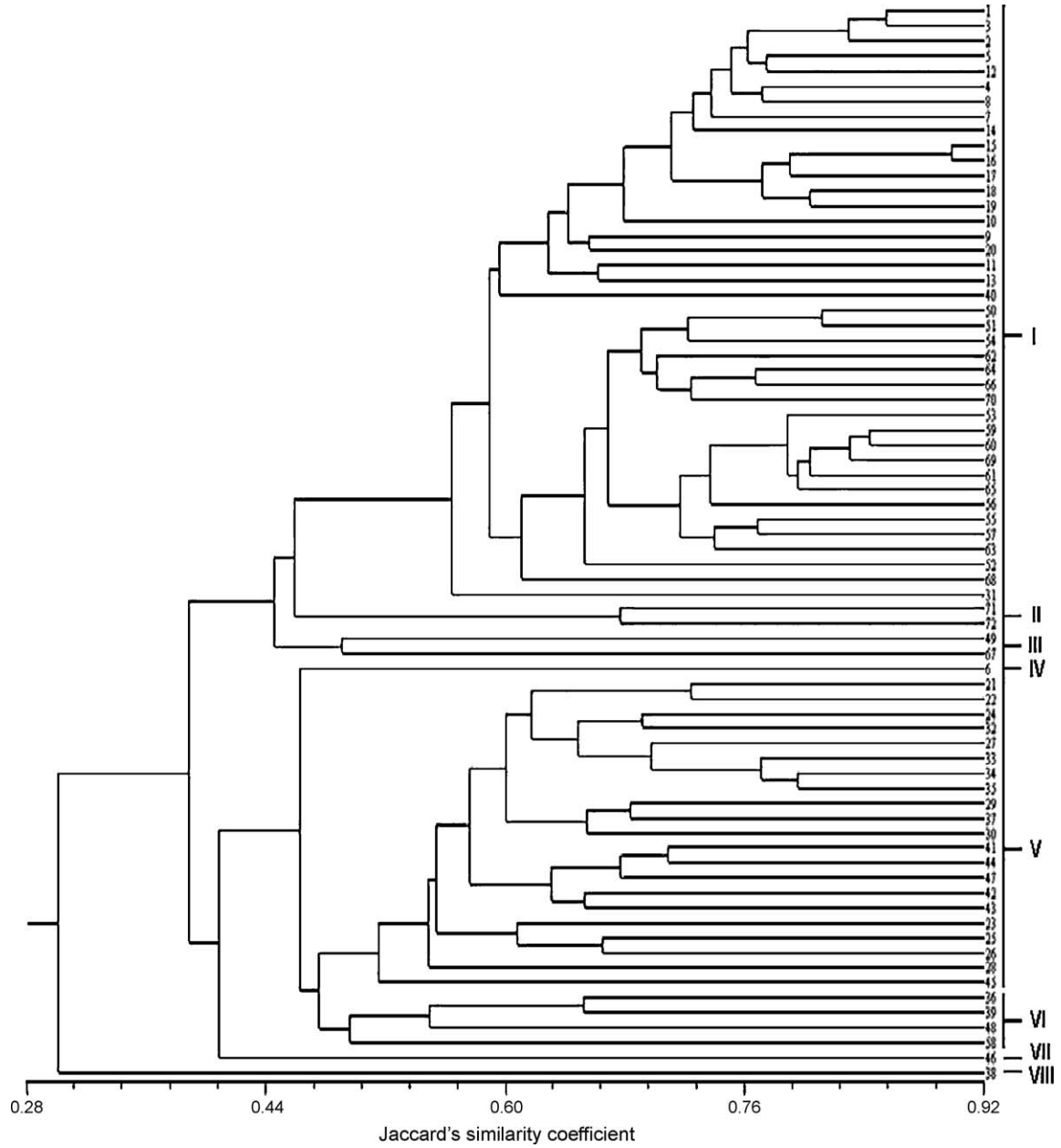

Fig. 2. Dendrogram (UPGMA) representing genetic relationships among 72 accessions of *J. curcas* based the Jaccard's similarity coefficients obtained using the pooled allelic profile of RAPD and ISSR primers.

Salvador (Wjc-58) which showed banding pattern similar to non-toxic accessions with all the tested primers showed allele specific to toxic accessions. The accession from El Salvador could be a hybrid or expressing heterozygosity at jcSSR-26 locus and possessed one allele (211 bp) common to that of other toxic accessions and another allele (234 bp) specific to that accession (Fig. 4d). Use of these microsatellite primers on 42 *J. curcas* accessions from India representing different geographic regions

Table 3
Molecular markers specific to toxic and non-toxic genotypes.

S. no	Toxic genotypes			Non-toxic genotypes		
	Marker type	Primer identity	Amplicon length (bp)	Marker type	Primer identity	Amplicon length (bp)
1.	RAPD	OPC-9	1260	RAPD	OPC-9	1077
2.	RAPD	OPC-18	693	RAPD	OPK-12	1150
3.	RAPD	OPM-12	1083	RAPD	OPP-9	1900
4.				RAPD	OPR-13	856
5.				RAPD	OPW-6	797
6.				ISSR	UBC-807	995
7.				ISSR	UBC-810	1200

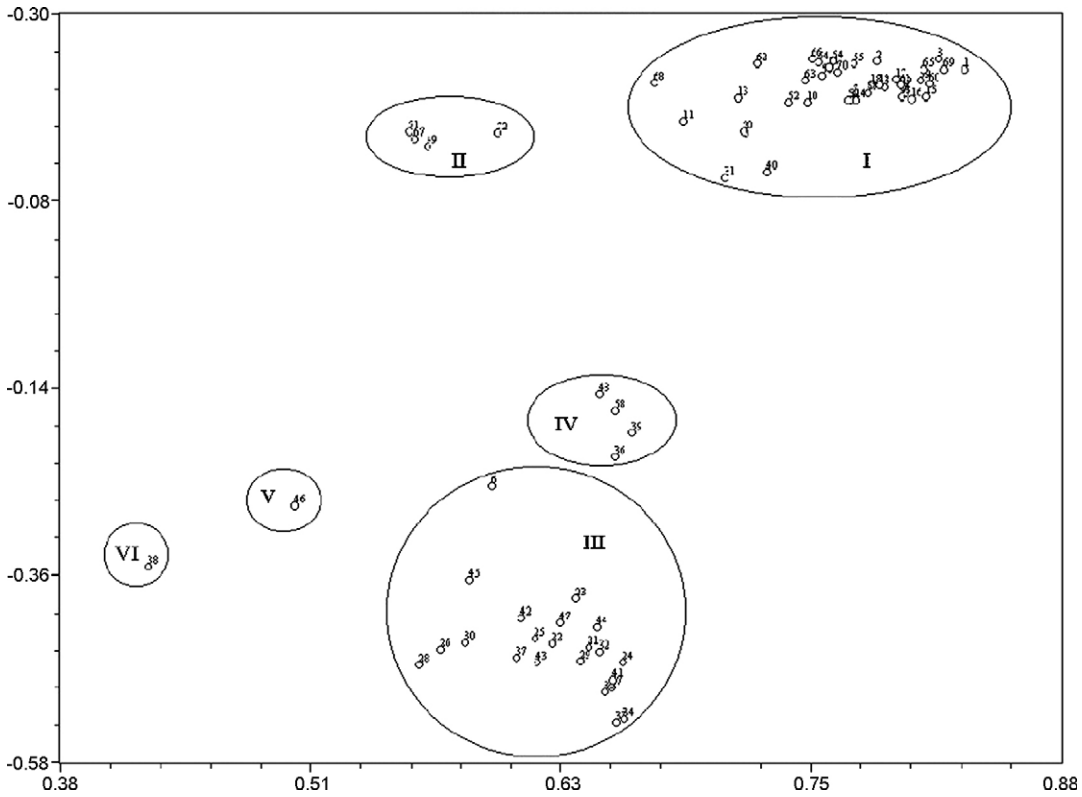

Fig. 3. Two-dimensional scaling of 72 accessions of *J. curcas* by principal coordinate analysis using the Jaccard's similarity coefficients based on the pooled data of RAPD and ISSR primers. The numbers represent the accession codes as given in Table 1.

Table 4
Molecular markers specific for Mexican accessions.

S. no	Marker type	Primer identity	Amplicon length (bp)
1.	RAPD	OPC-14	700
2.	RAPD	OPH-14	1300
3.	RAPD	OPQ-11	1200
4.	RAPD	OPV-17	800

(accessions as in Basha and Sujatha [9]) failed to reveal polymorphism (data not shown).

3.8. Curcin-specific primers

Amplification with the three curcin cds specific primers and curcin-P1 produced single amplicons of specified size. Curcin-P3 amplified bands of specified size in addition to two bands of lower molecular weight. The curcin-P2 primer was informative and revealed polymorphism among the accessions (Fig. 4f). Interestingly, accessions displaying polymorphism were mostly the non-toxic accessions (Wjc-6, -21, -22, -24, -26, -29, -32, -42, -44, -45, -

Table 5
Accession-specific molecular markers.

S. no	Genotype	Marker type	Primer identity	Amplicon length (bp)
1.	WJc-3	RAPD	OPB-11	400
2.	WJc-9	RAPD	OPC-8	750
3.	WJc-11	RAPD	OPB-16	800
4.	WJc-14	ISSR	UBC-828	1200
5.	WJc-18	ISSR	UBC-873	900
6.	WJc-21	RAPD	OPB-20	1000
7.	WJc-22	RAPD	OPD-16	1800
8.	WJc-26	RAPD	OPD-14	450
9.	WJc-28	RAPD	OPB-20	650
10.	WJc-30	ISSR	UBC-840	600
11.	WJc-33	RAPD	OPB-12	1000
12.	WJc-38	RAPD	OPJ-4	450
13.	WJc-42	RAPD	OPB-12	2200
14.	WJc-46	RAPD	OPC-13	1300
15.	WJc-47	RAPD	OPD-1	600
16.	WJc-51	RAPD	OPC-20	1300
17.	WJc-55	RAPD	OPJ-4	550
18.	WJc-67	ISSR	UBC-825	1000
19.	WJc-71	RAPD	OPS-3; OPU-15	1000; 550
20.	WJc-72	RAPD	OPK-12	1000

Table 6
Characteristics of SCAR primers developed to distinguish toxic genotypes from non-toxic genotypes.

S. no	Primer	SCAR primer	GenBank accession no.	Primer sequence for SCAR amplification	Amplicon length (bp)	*Tm* (°C)	*Ta* (°C)
1.	OPM-12	RSPJ1-F RSPJ1-R	EU748555	GGAACAAGAACAGTCGCCG GGGACGTTGGTTAACACTTC	961	60 60	62
2.	OPC-9	RSPJ2-F RSPJ2-R	EU748549	CTCACCGTCCTGAAGCC CTCACCGTCCCTCACTC	1077	56 56	54
3.	UBC-807	ISPJ3-F ISPJ3-R	EU748552	GGTGATGATGGAGAGATGG GAGAGAGAGAGAGTATACTG	964	58 58	56

Tm = theoretical annealing temperature [4(G + C) + 2(A + T)].
Ta = optimal annealing temperature.

Table 7
Oligonucleotide sequences and associated information for the 12 microsatellite primer pairs.

SSR primer	EMBL#	Repeat motif	Primer sequence (5′–3′)	*Ta* (°C)	Expected size (bp)	Number of alleles and size (bp)
JcSSR-18	EU099518	$(TA)_3(GT)_{18}$	GGCGACAGGAAGAGCATG GCAATCTTGGACAGGAAACG	62	394	3 (387, 394, 397)
JcSSR-19	EU099519	$(AC)_{21}$	CTTGAAAGTTTTTGTAATTTC CGCCAATCATAGATC	50	214	2 (214, 218)
JcSSR-20	EU099520	$(AC)_{10}$	GGCTGAACTTGCGCC GCCCTGATTTCTGGTC	60	260	2 (260, 266)
JcSSR-21	EU099521	$(C)_7(A)_5(CA)_9$	CTGAAATGGAGAAATTGG ACATATCGAAGATAGGG	50	249	3 (245, 249, 324)
JcSSR-22	EU099522	$(TC)_{16}$	GAATCTCAACAGTGCCC GAAGGATGGGAAGTGGG	52	152	2 (152, 165)
JcSSR-24	EU099524	$(C)_6G(C)_6(AC)_5$	CACACACAACCAAACTGG GGTTCTCTGAGATCCTC	56	287	1 (287)
JcSSR-26	EU099526	$(CA)_{18}$	CATACAAAGCCTTGTCC AACAGCATAATACGACTC	55	211	4 (193, 198, 211, 234)
JcSSR-28	EU099528	$(CA)_{17}$	GCATTTAGCAGAACCCCA CTAGCTAGTGTTATGTCTC	54	179	Multilocus (3) and monomorphic
JcSSR-29	EU099529	$(CTT)_3CT(CTT)_2TG(T)_5$	GCCATCCAATTATGGG ACAAGTAAGAAGTGAAG	50	156	1 (156)
JcSSR-31	EU099531	$(GT)_5(G)_5C(G)_6$	CTGGTGCTAAAACTATGG ACTGGTCATTCAGCTCC	52	290	2 (290, 294)
JcSSR-32	EU099532	$C(A)_5G(A)_{15}$	TTAGTAGAGAACAAAAAG CGTTACTCTCTACCG	42	298	1 (298)
JcSSR-34	EU099534	$(GAA)_7$	AGAAGAAAGAGGCGAC TCTTGTTGTTCATGAGG	44	147	1 (147)

Ta = optimal annealing temperature.

46 and -58). One accession with high phorbol ester content (Wjc-48) also showed polymorphism.

4. Discussion

In the present investigation, genetic variation has been assessed in *J. curcas* germplasm collected from 13 countries that have initiated research and/or cultivation programmes on Jatropha. Biochemical analysis distinguished *J. curcas* accessions based on differences in phorbol ester content while molecular analysis revealed variation among accessions of Mexican origin. Non-toxic genotypes of *J. curcas* are available only in Mexico and the seeds which were free of or low in phorbol esters were from the edible/non-toxic genotypes [14,15,19]. Drastic poisoning of mice and rats was not found with seeds of Mexican origin [20].

4.1. Biochemical characterization

It is interesting to note wide variation in crude protein (18.8–34.5%) and oil (45.4–64.5%) contents in the tested genotypes. Variation in seed protein (13.7–22.4%), oil content (28.4–42.3%) and ash content (3.6–5.2%) in 13 provenances from different countries in North and Central America, West and East Africa and Asia was lower as compared to that in the present study [2]. This probably could be due to use of fewer accessions or inclusion of only one provenance from Mexico unlike in the present investigation where provenances from 13 countries and with 28 accessions from Mexico are represented. The Jatropha kernel meal has high crude protein content (order of 60%) and with good amino acid composition [14,19] and has the potential for use as animal feed. The removal of phorbol esters from the toxic genotypes is imperative in order to make this by-product of biodiesel industry a valuable livestock feed. While, the kernel meal from the non-toxic genotype would be an excellent animal feed and the oil could possibly be used as edible oil. Phorbol esters have strong insecticidal and molluscidal properties [21] and the oil of the toxic genotypes could be an invaluable source of phorbol esters. Oleic acid content in the oil of the non-toxic genotype was higher than that of the toxic genotype whereas reverse was the case for linoleic acid [22], which could possibly be due to genetic factors.

4.2. RAPD and ISSR analysis

The RAPD primers (400) revealed 42% polymorphism with 482 polymorphic bands/primer in Indian germplasm [9]. In this study, use of 100 primers revealed 61.8% polymorphism with 7.62 polymorphic bands per primer indicating more genetic diversity in the accessions from diverse regions. However, use of ISSR primers failed to reveal higher polymorphism in the world collection (35.5%) when compared with Indian accessions (34.1%). While RAPD scans the entire genome in coding and noncoding regions including repeated or single-copy sequences, ISSR markers disclose polymorphism in the repeat regions. ISSR primers detect more polymorphism than RAPD primers because of variability in microsatellite loci due to DNA slippage. However, SSR analysis using 12 polymorphic primers showed no variation among accessions with the exception of accessions from Mexico. This probably could be the reason for low level of variation with ISSR primers and existence of two major clusters (I and III) in PCO analysis with ISSR markers. Among the ISSR primers, the tri-nucleotide primer UBC-866 was found to be highly polymorphic when tested on Indian accessions [9]. The same primer disclosed maximum polymorphism between accessions from diverse regions.

Both the dominant-marker systems showed similar clustering pattern and disclosed predominantly two groups of accessions. One cluster comprised of accessions from the Central American region while the other cluster encompassed accessions from all other regions. The PCO analysis and dendrogram based on UPGMA

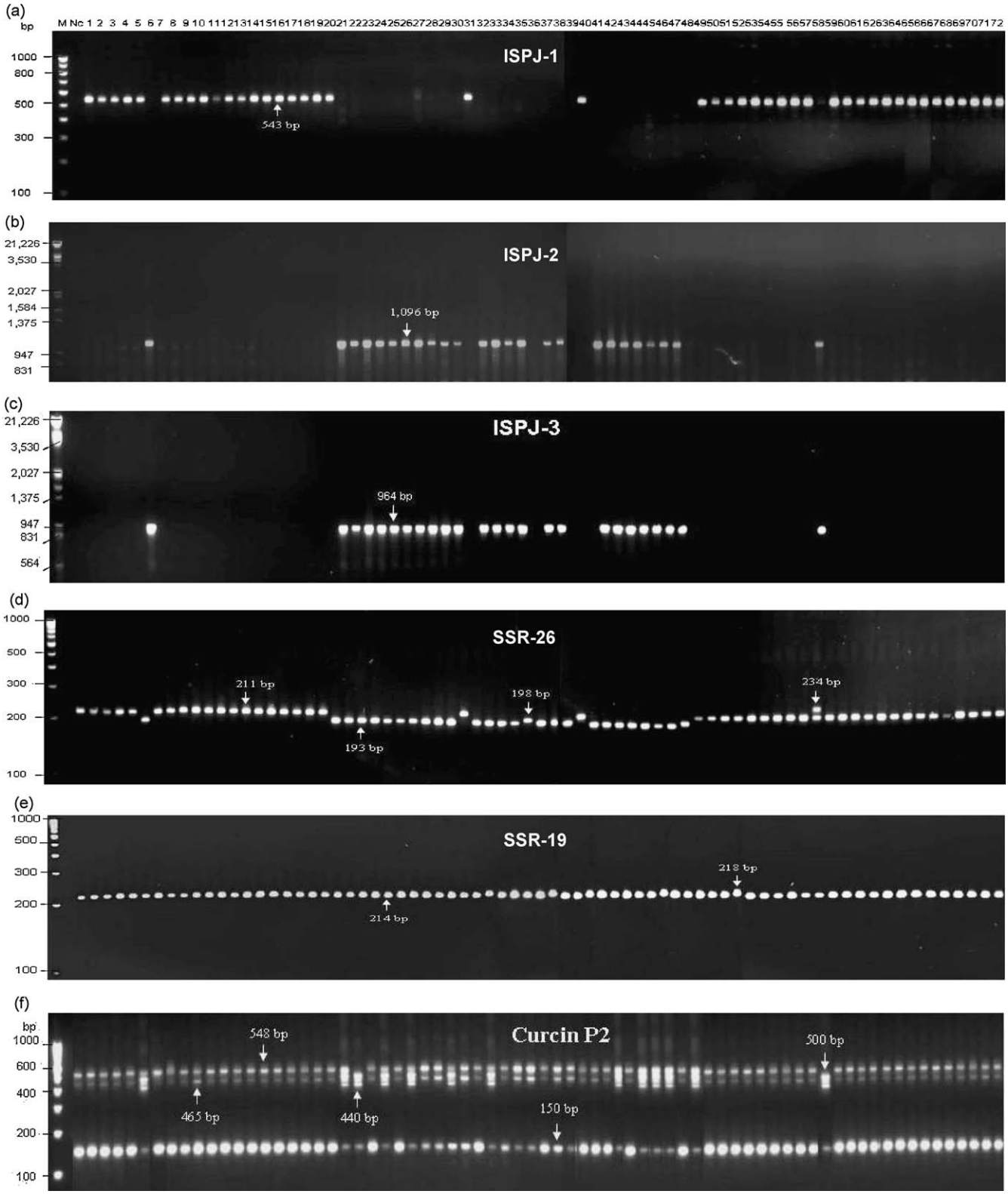

Fig. 4. Molecular analysis of *J. curcas* germplasm using SCAR and SSR primers. (a) Electrophoretic analysis of DNA amplification with SCAR primer ISPJ-1. Lanes designated as M represents 100 bp molecular weight marker, Nc-negative (no DNA) control and 1–72 the samples used in the study. Arrow indicates the amplification of 543 bp band in the toxic genotypes. (b) Electrophoretic analysis of DNA amplification with SCAR primer ISPJ-2. Lanes designated as M represents λ DNA double digest with *Eco*RI and *Hind*III restriction enzymes, Nc-negative (no DNA) control and 1–72 the samples used in the study. Arrow indicates the amplification of 1096 bp band in the non-toxic genotypes. (c) Electrophoretic analysis of DNA amplification with SCAR primer ISPJ-3. Lanes designated as M represents λ DNA double digest with *Eco*RI and *Hind*III restriction enzymes, Nc-negative (no DNA) control and 1–72 the samples used in the study. Arrow indicates the amplification of 964 bp band in the non-toxic genotypes. (d) PCR amplification of genomic DNA using SSR primer pair JcSSR-26 and separated on 4% agarose gel. Lanes designated as M represents 100 bp molecular weight marker, Nc-negative (no DNA) control and 1–72 the samples used in the study. Arrows indicate variation in band sizes of respective genotypes. (e) PCR amplification of genomic DNA using SSR primer pair JcSSR-19 and separated on 4% agarose gel. Lanes designated as M represents 100 bp molecular weight marker, Nc-negative (no DNA) control and 1–72 the samples used in the study. Arrows indicate variation in band sizes of respective genotypes. (f) PCR amplification of genomic DNA using curcin-specific primer pair Curcin-P2 and separated on 4% agarose gel. Lanes designated as M represents 100 bp molecular weight marker, Nc-negative (no DNA) control and 1–72 the samples used in the study. Arrows indicate variation in band sizes of respective genotypes.

revealed low genetic variation in accessions collected from different countries and genetic segregation of accessions from Central America. Narrow genetic diversity in accessions from different countries reiterates the fact that only few introductions constitute the germplasm base in the respective countries. The genetic closeness of accessions in clusters I and II comprising of germplasm from different countries indicate a common source for the material. Reports indicate that the accession from Cape Verde has been transported by Portugese seafarers to different regions in Asia and Africa [2]. Molecular analysis of Indian germplasm along with the exotic collections showed clustering of the Cape Verde accession with the Indian germplasm [9]. Geographically isolated populations accumulate genetic differences as they adapt to different environments. Clustering together of accessions from different regions indicates lack of gene flow between adjacent populations in each region. In outcrossing species, genetic diversity will be generally high but low genetic diversity in *J. curcas* reflects on the mode of propagation, reproductive behavior and the manner in which it has been domesticated. Narrow genetic base of *J. curcas* in African and Asian region could probably be due to limited introductions without further augmentation, predominant propagation of the crop through vegetative cuttings or due to occurrence of apomixis [23].

4.3. Markers specific to toxic and non-toxic genotypes

Molecular profiling based on RAPD, ISSR and SSR markers clearly showed distinction of Mexican accessions from accessions obtained from other countries. A total of 6 RAPD, 2 ISSR and 1 SCAR primers were found specific to toxic Indian (42) and 17 RAPD, 4 ISSR and 1 SCAR primers were specific to non-toxic Mexican accessions [9]. Validation of the primers on the world germplasm collection showed specificity of 3 RAPD primers to toxic genotypes, 5 RAPD and 2 ISSR primers to non-toxic accessions and 4 RAPD primers to Mexican accessions. Thus, molecular markers could be identified for the non-toxic accessions as well as the Mexican population. Of the 21 accession-specific bands, 10 were found specific to the Mexican accessions. The specific markers may simply represent rare alleles and would most likely no longer be specific if a larger germplasm collection is used. Nevertheless, accessions possessing distinct DNA profiles are likely to contain the greatest number of novel alleles and this study confirms presence of rich genetic diversity in the Mexican germplasm. According to Wilbur [24], the genus *Jatropha* must have originated in Mexico because of the occurrence of *J. curcas* along with several closely related taxa. Heller [2] states that it is highly probable that the centre of origin of *J. curcas* is in Mexico and Central America as it is found in different vegetation forms unlike in Africa, Asia and Guatemala where it is found only in cultivated form. The present study confirms these observations as the Mexican accessions were genetically distinct from accessions collected from other parts of the world besides possessing several unique alleles.

4.4. SCAR markers

In the previous study we had developed two SCAR primers that distinguished a non-toxic Mexican accession from the Indian germplasm [9]. Similarly, Pamidimarri et al. [10] identified molecular markers that could differentiate a single non-toxic accession from the toxic accessions. Nonetheless, these markers could be specific to the Mexican origin or to the non-toxic trait and cannot be attributed to non-toxicity reliably. However, the present study in which 72 accessions were subjected to characterization for phorbol ester levels and molecular distinctness clearly showed association of the developed SCAR markers with the non-toxic trait. As SCAR markers are more reliable than other markers owing to their specificity and reproducibility, the markers developed in this study will facilitate marker assisted breeding programmes aimed at development of non-toxic genotypes. Exceptions were observed in the identified markers and trait association which could probably be due to genetic recombinations. Development of appropriate mapping population and determination of recombination frequency along with linkage analysis will disclose the genetic distance of the markers to the target trait.

4.5. SSR analysis

Diversity analysis using microsatellite markers support the dominant-marker data and revealed variation in accessions from Mexico and El Salvador while accessions from other countries failed to be distinguished. In the present investigation, a single allele was observed for all the accessions with all the SSR primers with the exception of Wjc-58 from El Salvador which showed codominant band with jcSSR-26. Conversely, in studies of Pamidimarri et al. [10] SSR analysis showed heterozygosity of the accessions with all SSR primers with the exception of the marker jcms21. Heterozygosity at all SSR loci is unexpected in a crop that is predominantly vegetatively propagated and exhibiting low intra-accessional variability. Use of such heterozygous material offers limited scope in diversity analysis and breeding.

The accession from El Salvador remained distinct and clustered separately. SSR analysis revealed heterozygosity with regard to loci amplified with jcSSR-26. While the 211 bp allele is common with all other Jatropha accessions, the 234 bp allele is specific to the accession. This indicates the existence of genetically divergent and compatible accessions in El Salvador as well. Of the different marker classes, SSRs are more advantageous owing to their multiallelic nature, codominant inheritance, relative abundance, ease for multiplexing and transferability across laboratories. In the present investigation we used 17 microsatellite primers. Pamidimarri et al. [10] used a different set of 12 SSR primers and an additional 293 microsatellite sequences are deposited in NCBI (www.ncbi.nlm.nih.gov/). SSR markers are available for Hevea, Cassava and EST resources are developed for castor [25,26, http://castorbean.tigr.org/] which should provide a critical mass of primers for cross-taxa transferability and in unraveling the genetic diversity in *J. curcas* accessions from Central American region.

4.6. Curcin-specific primers

Use of curcin gene-specific primers and primers for promoter region of RIP revealed information on the curcin gene and promoter. The curcin-P2 primer showed polymorphism in 13 accessions which included 12 of the 25 non-toxic accessions tested and 1 toxic accession. It is interesting to observe variation with regard to promoter region of RIP as well in the non-toxic genotypes. Functional significance of this variation in the promoter region can be ascertained only after determination of RIP levels, transcript profiling and sequence analysis of the gene products.

In conclusion, the present study showed high genetic diversity in Mexican germplasm and low genetic variation in accessions from other countries both in terms of phorbol ester levels and molecular profiles. All the marker types used in the study were informative to differentiate the Mexican germplasm from other accessions and also the toxic from non-toxic accessions. Accession-specific diagnostic SCAR markers were developed for distinguishing the toxic genotypes from non-toxic accessions. Low genetic variation in a crop reveals lack of evolution in the concerned crop species. *J. curcas* has never been bred for productivity and the varieties reported are selections from local germplasm. There is an immediate need for undertaking systematic breeding research in *J. curcas*. This study shows clear geographic structuring and indicates rich genetic diversity of *J. curcas* accessions from Mexico and the

widespread distribution of one or few toxic accessions in all other regions of the world where it is cultivated. The presence of distinct molecular profiles of accessions from Mexico and presence of diverse alleles present it as a valuable source of diversity for exploitation in *J. curcas* breeding programmes.

Acknowledgement

This research is supported by the RSAD project on *Jatropha curcas* sponsored by the Government of Andhra Pradesh, Hyderabad, India.

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