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Inter and intra-population variability of *Jatropha curcas* (L.) characterized by RAPD and ISSR markers and development of population-specific SCAR markers

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Abstract *Jatropha curcas* (Euphorbiaceae) is an oil-bearing species with multiple uses and considerable potential as a bioenergy crop. The present investigation has been undertaken to assess the extent of genetic diversity in a representative set of 42 accessions of *J. curcas* encompassing different crop growing regions in India along with a non-toxic genotype from Mexico as a prelude for utilization of promising and genetically divergent materials in the breeding programmes. Molecular polymorphism was 42.0% with 400 RAPD primers and 33.5% with 100 ISSR primers between accessions indicating modest levels of genetic variation in the Indian germplasm. The within-population variation based on RAPD polymorphism was 64.0% and was on par with the inter-population variation. Polymorphic ISSR markers have been identified that could differentiate the Indian accessions from the Mexican genotype and two of them were converted to SCAR markers. The SCAR primer pair ISPJ1 amplified a 543 bp fragment in all the Indian populations, while ISPJ2 with a specific amplicon of 1,096 bp was specific to the Mexican genotype. Population-specific bands have been identified for the accession from Kerala (2 RAPD markers), Neemuch-1 from Rajasthan (1 each of RAPD and ISSR markers) and the non-toxic genotype from Mexico (17 RAPD and 4 ISSR markers), which serve as diagnostic markers in genotyping. The study indicates an immediate need for widening the genetic base of *J. curcas* germplasm through introduction of accessions with broader geographical background.

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Introduction

Jatropha curcas L. (Family Euphorbiaceae), also known as Sabudam, purging nut is a multipurpose plant with several attributes and considerable potential and has evoked interest all over the tropics as a potential biofuel crop (Takeda 1982; Martin and Mayeux 1985; Jones and Miller 1991; Openshaw 2000). *J. curcas* is a native of Mexico and Central American region and was later introduced in many parts of the tropics and subtropics where it is grown as a hedge crop and for traditional use (Heller 1996). In the recent past, the oil crisis and depleting fossil fuel reserves has rekindled interest in promotion of tree borne oil species in several African, Asian and Latin American countries. Among the potential oil

bearing tree species, *J. curcas* has assumed importance due to its short gestation period, drought endurance, low cost of seeds, high oil content, easy adaptation on marginal and semi-marginal lands, suitability as fuel substitute without any alteration to the existing engines and above all the plant size that makes seed collection more convenient.

The programmes sponsored by various agencies in different countries have a common mandate of survey of *Jatropha* plantations, selection of candidate plus phenotypes, establishment of seed production areas, evaluation, establishment of high-tech nurseries and vegetative multiplication gardens and progeny trials of high yielding plantations. The success of these programmes lies in the identification of genetically divergent material and development of genetically superior stocks. However, the crop has never been bred for productivity and the major bottleneck in *Jatropha* promotion programmes is the use of material available ''wild'' locally. The initial variations in fruit and seed yield of the candidate plus trees were found insignificant when the plants are grown on common site, indicating low genetic variability. Forty *J. curcas* clonal lines from different locations in Thailand investigated for intra-specific variability revealed no morphological differences (Sakaguchi and Somabi 1987).

There is virtually no information with regard to the number of introductions and the genetic diversity of *J. curcas* populations grown in India. Several researchers have attempted to define the origin of *J. curcas*, but the source remains controversial (Dehgan and Webster 1979; Heller 1996). Three distinct varieties are reported viz., the Cape Verde variety that has spread all over the world, the Nicaraguan variety with few but larger fruits and a non-toxic Mexican variety devoid of phorbol esters (Henning 2006). There are no named varieties of *J. curcas* in India with the exception of the variety SDAUJ1 (Chatrapathi) that was released during the year 2006 based on selection from local germplasm (www.icar.org).

An understanding of the extent of genetic diversity is critical for the success of a breeding programme. Traditional methods using morphological characteristics for establishment of genetic diversity and relationships among accessions were largely unsuccessful due to the strong influence of environment on highly heritable seed traits like 100-seed weight, seed protein and oil content in *J. curcas* (Heller 1996). Hence, selection based on genetic information using neutral molecular markers is essential as it is more reliable and consistent. In Euphorbiaceae, molecular markers such as, RAPD, RFLP and SSRs have been employed for determining the extent of genetic diversity in elite rubber (*Hevea brasiliensis*) clones (Besse et al. 1994; Varghese et al. 1997; Lakawipat et al. 2003) and cassava (Asante and Offei 2003; Fregene et al. 2003). In *Jatropha*, isozyme markers were used to determine the genetic relatedness of the members of the genus *Jatropha* and *Ricinus* (Sathaiah and Reddy 1985). RAPD markers were employed to confirm hybridity of inter-specific hybrids (Sujatha and Prabakaran 2003) and to determine the similarity index between one accession each of the toxic Indian and non-toxic Mexican genotypes (Sujatha et al. 2005). In this present investigation, we evaluated genetic diversity in *J. curcas* germplasm from India along with a non-toxic genotype from Mexico using specifically the RAPD and ISSR primers keeping in view the low development and running costs per data point besides detection of genome-wide variation.

Materials and methods

Plant material

A representative set of 43 accessions of *J. curcas*, that included 42 accessions from different geographical regions of India and one accession from Mexico were used for diversity analysis. The accessions were selected from 340 accessions of *J. curcas* plantations established at three co-operating centres (Acharya N.G. Ranga, Agricultural University, National Bureau of Plant Genetic Resources and International Crops Research Institute for the Semi-Arid Tropics, Hyderabad, India) of the Rain Shadow Area Development project (RSAD) on *J. curcas.* As the germplasm included several accessions from the same region and also the same accession at different centres,

selection was based on the geographical distribution and variations (if any) in qualitative and quantitative characters (Table 1). The representative collection included one sample from Cape Verde grown in Tamilnadu (IJC-3), a sample from Kerala with slightly smaller dark black seeds (Kerala) and a non-toxic variety from Mexico. For assessment of intra-population variability, 65 plants derived from seeds from a single plant were subjected to molecular analysis using polymorphic primers. For confirmation of bands specific to a population, 20 individual plants from the most divergent lines were analysed using polymorphic primers revealing polymorphism with the respective population.

DNA extraction

Total genomic DNA was extracted from younger leaves of ten plants for each accession

Table 1 Geographical location of the *J. curcas* accessions used in diversity analysis

S. No.	Accession identity	Collection site	State/Country	Specific character
1.	AF-1	Coimbatore	Tamilnadu/India	Toxic
2.	AF-2	Coimbatore	Tamilnadu/India	Toxic
3.	AF-3	Coimbatore	Tamilnadu/India	Toxic
4.	AF- 4	Coimbatore	Tamilnadu/India	Toxic
5.	AF-5	Coimbatore	Tamilnadu/India	Toxic
6.	AF-6	Coimbatore	Tamilnadu/India	Toxic
7.	AF-7	Coimbatore	Tamilnadu/India	Toxic
8.	S.J.H	Rajasthan	Rajasthan/India	Toxic
9.	Neemuch-1	Rajasthan	Rajasthan/India	Toxic
10.	VHA	Rajasthan	Rajasthan/India	Toxic
11.	Avinash	Rajasthan	Rajasthan/India	Toxic
12.	Kerala	Kerala	Kerala/India	Small black seeds
13.	TNMC-7	Chinthapalli	Andhra Pradesh/India	Toxic
14.	Kolapari	Chinthapalli	Andhra Pradesh/India	Toxic
15.	Rivyarintaram	Chinthapalli	Andhra Pradesh/India	Toxic
16.	Bayalukunchangi	Chinthapalli	Andhra Pradesh/India	Toxic
17.	N-11	Nellore	Andhra Pradesh/India	Toxic
18.	N-12	Nellore	Andhra Pradesh/India	Toxic
19.	N-13	Nellore	Andhra Pradesh/India	Toxic
20.	Rajasthan	Rajasthan	Rajasthan/India	Toxic
21.	Haryana	Haryana	Haryana/India	Toxic
22.	DOR-Jc	Hyderabad	Andhra Pradesh/India	Toxic
23.	Non-toxic Jc	Papantla region	Veracruz/Mexico	Non-toxic
24.	SNS-2	Rangareddy	Andhra Pradesh/India	Toxic
25.	SNS-6	Rangareddy	Andhra Pradesh/India	Toxic
26.	SNS-7	Rangareddy	Andhra Pradesh/India	Toxic
27.	SNS-8	Rangareddy	Andhra Pradesh/India	Toxic
28.	SNS-9	Rangareddy	Andhra Pradesh/India	Toxic
29.	IJC-1	Rajgarh	Madhya Pradesh/India	Toxic
30.	IJC-2	Chinthapalli	Andhra Pradesh/India	Toxic
31.	IJC-3	Erode	Tamilnadu/India	Toxic (Cape Verde type)
32.	IJC-4	Erode	Tamilnadu/India	Toxic (Erode local)
33.	IJC-5	Erode	Tamilnadu/India	Toxic
34.	IJC-6	Churu	Rajasthan/India	Toxic
35.	IJC-7	Churu	Rajasthan/India	Toxic
36.	IJC-8	JNKVV, Jabalpur	Madhya Pradesh/India	Toxic
37.	IJC-9	CHRK-VSP, Jabalpur	Madhya Pradesh/India	Toxic
38.	IJC-10	MONDC, Jabalpur	Madhya Pradesh/India	Toxic
39.	IJC-11	CHRK-GBR, Jabalpur	Madhya Pradesh/India	Toxic
40.	IJC-12	TFRI, Jabalpur	Madhya Pradesh/India	Toxic
41.	IJC-13	Churu	Rajasthan/India	Toxic
42.	IJC-14	Churu	Rajasthan/India	Toxic
43.	IJC-15	Churu	Rajasthan/India	Toxic

following the standard CTAB method with minor modifications (Doyle and Doyle 1990). 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 treated with RNase A (100 µg/ml), incubated at 37°C for 30 min and twice extracted with chloroform:isoamylalcohol (24:1 v/v). 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 Millipore water and stored overnight at –20°C.

RAPD analysis

A total of 400 decamer primers from Operon kits—OPA to OPX (Operon technologies, Alameda, USA) were used for DNA amplification according to the method of Williams et al. (1990). 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 an GeneAmp 9700 Thermal Cycler (Perkin Elmer 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 was repeated twice and run in an Eppendorf and GeneAmp PCR system 9700 thermal cyclers. Regardless of the marker system used, all the PCR amplifications included a negative control (no DNA) to avoid erroneous interpretations.

ISSR-PCR amplification

One hundred ISSR primers (UBC primer set No. 9, University of British Columbia, Canada) were used for the analysis. A preliminary screening was carried out using gradient annealing temperatures (T_a) in order to select primers that could give good amplification and suitable annealing temperature. 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 $MgCl_2$), 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 using a GeneAmp 9700 thermal cycler (Perkin Elmer Applied Biosystems) 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 (T_a), 2 min elongation at 72°C and final extension at 72°C for 7 min. The amplified products were electrophoresed at 100 V on a 1.7% agarose gel using *Eco*RI and *Hin*dIII double digest as the molecular weight standard.

SCAR marker development

For the development of SCAR markers, polymorphic ISSR bands one each specific to Indian genotypes (UBC 812) and the non-toxic Mexican genotype (UBC 834) were excised and purified from the agarose gel using QIAquick gel extraction kit (Qiagen, Germany). The selected fragments were ligated into pTZ57R (Insta) T/A cloning vector using cloning kit (MBI Fermentas, USA) and then the recombinant plasmids were transformed into *E. coli* strain (DH5α) by heat shock method. The cells were then spread on Luria-Bertani (LB) selection medium containing ampicillin (50 µg/ml), IPTG, and X-gal and incubated at 37°C for 16 h. The purification of plasmid DNA from the positive colonies was carried out using the alkaline-lysis method (Sambrook et al. 1989). Colony PCR and restriction enzyme digestion (*Eco*RI and *Hin*dIII) were performed to confirm the presence of the insert and subsequently the cloned DNA fragments were sequenced (Bioserve Biotechnologies, India).

Based on the sequence of the cloned ISSR fragments, two sequences with higher T_m were designed as SCARs and deposited in the NCBI Genbank (Table 3). SCAR amplifications were performed in 10 μl of reaction mixture containing 2.5 ng of DNA, 1 μl of 10× assay buffer (10 mM Tris pH 9.0, 50 mM KCl, 1.5 mM $MgCl_2$), 0.5 μl of 2.5 mM dNTP, 2 pmol of each primer and 0.6 U *Taq* DNA polymerase (Bangalore Genei, India). The PCR amplification included an initial denaturation step at 94°C for 4 min, followed by 35 cycles of 94°C for 30 s, 56°C for 15 s for SCAR primer ISPJI (Genbank No EF012272) and 54°C for 15 s for ISPJ2 (Genbank No EF012273); 72°C for 1 min followed by extension at 72°C for 7 min. The amplification products were resolved on 1.7% agarose gel stained with ethidium bromide. Following confirmation of the SCAR product amplification profile, the specificity and reproducibility of SCAR markers was verified using DNA from all the 43 accessions as well as from 20 individual plants of the non-toxic *J. curcas* genotype.

Statistical analysis

For each primer, the presence or absence of bands in each accession was visually scored and set in a binary matrix. Genetic similarities were calculated using Dice similarity coefficient for RAPD and ISSR polymorphisms individually as well as together. The Mantel test of significance was determined to measure the goodness of fit between the similarity matrices produced with the two marker systems. Dendrograms were constructed using the un-weighted pair-group method with an arithmetic average (UPGMA) method and principal coordinate analysis was done using the NTSYS PC version 2.11 (Applied Biostatistics Inc, Setauket, USA).

Results

Both the marker systems being employed to assess the genetic diversity in *J. curcas* germplasm were quite informative and were able to generate adequate polymorphism and unique DNA fingerprints for identification of few genotypes (Figs. 1, 2a).

Out of 400 RAPD primers tested, 339 primers produced amplification products of which 267 revealed polymorphic fingerprint patterns. Out of a total of 3,057 bands, 1,287 (42%) were polymorphic with an average of 4.82 polymorphic bands per primer. The total number of bands per amplification varied from 1 to 20 in the molecular size range of 100–3,500 bp. With single letter Operon primer sets from OPA to OPX, the extent of polymorphism varied between 55.5% (OPS) and 95% (OPL). Six RAPD markers were specific to the Indian accessions while 17 markers were specific to the Mexican genotype (Table 2).

Of the 100 ISSR primers screened, 48 primers gave amplification products of which 29 primers generated polymorphic banding patterns. Out of 340 bands, 116 were polymorphic with an average

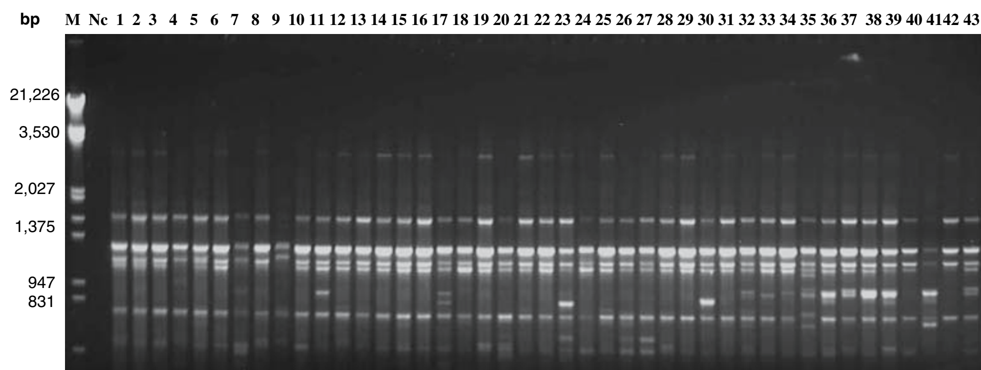

Fig. 1 Electrophoretic analysis of DNA amplification produced using RAPD primer OPV17. Lanes designated as M represents λ DNA double digest with *Eco*RI and *Hin*dIII restriction enzymes, Nc—negative (no DNA) control, and 1–43 the samples used in the study

Table 2 Molecular markers specific to Indian and non-toxic Mexican genotypes

S. No	Indian genotypes			Non-toxic Mexican genotype		
	Marker type	Primer identity	Amplicon length (bp)	Marker type	Primer identity	Amplicon length (bp)
1.	RAPD	OPC 18	650	RAPD	OPC 10	2,100
2.	RAPD	OPG 17	1,050	RAPD	OPC 14	700
3.	RAPD	OPK 1	950	RAPD	OPE 5	2,600
4.	RAPD	OPP 3	400	RAPD	OPF 16	1,200
5.	RAPD	OPV 8	750	RAPD	OPG 18	1,600
6.	RAPD	OPW 17	700	RAPD	OPH 14	1,350
7.	ISSR	UBC 812	550	RAPD	OPJ 15	1,300
8.	ISSR	UBC 880	800	RAPD	OPK 12	1,150
9.				RAPD	OPP 3	550
10.				RAPD	OPP 9	1,900
11.				RAPD	OPQ 11	1,200
12.				RAPD	OPQ 19	1,800
13.				RAPD	OPT 14	800
14.				RAPD	OPU 10	2,500
15.				RAPD	OPU 19	800
16.				RAPD	OPV 14	600
17.				RAPD	OPV 17	800
18.				ISSR	UBC810	1,200
19.				ISSR	UBC812	1,500
20.				ISSR	UBC834	1,100
21.				ISSR	UBC847	1,350

of 11.7 bands per polymorphic primer. The number of bands amplified per primer varied between 5 (UBC primer # 816) and 19 (UBC primer # 891) with band size between 100 bp and 3.5 kb. Frequency of polymorphism ranged from 11.1% to 66.6% with an average polymorphism of 33.5% across the accessions studied. The trinucleotide primer UBC 866 was found to be highly polymorphic (66.6%).

In the present investigation, genetic structuring in terms of accession specific bands was defined for few genotypes. The five divergent accessions (Kerala, non-toxic Jc, Neemuch-1, TNMC-7 and IJC-11) with several unique bands were subjected to individual plant analysis (20 plants) with both types of markers that produced polymorphic bands. Population-specific markers have been identified for only three (Kerala, non-toxic Jc and Neemuch-1) of the five genotypes analysed. From a total of 27 markers (22 RAPD, 5 ISSR), only two RAPD markers viz., $OPJ15_{700}$ and $OPD7_{750}$ were specific to the population from Kerala. For Neemuch-1, 19 RAPD and 12 ISSR markers were found to be polymorphic of which only one marker each of the two types tested viz., $OPC14_{1200}$ and $UBC873_{1300}$ were found unique to the accession. Seventeen RAPD and 4 ISSR primers from a total of 47 polymorphic markers

Table 3 Characteristics of the SCAR primers developed to distinguish Indian accessions from the Mexican genotype

S. No	ISSR primer	SCAR primer	Genbank accession No.	Primer sequence for SCAR amplification	Amplicon length (bp)	T_m (°C)	T_a (°C)
1.	(GA)8 A	ISPJ1-F	EF012272	GAGAGAGAGAGAGAGGTG	543	56	56
		ISPJ1-R		GAGAGAGAGAGAGAAAACAAT		58	
2.	(AG)8 YT	ISPJ2-F	EF012273	GAGAGAGAGAGTTGGGTG	1,096	56	54
		ISPJ2-R		AGAGAGAGAGAGCTAGAGAG		60	

T_m = Theoretical annealing temperature [4(G+C) + 2(A+T)]

T_a = Optimal annealing temperature

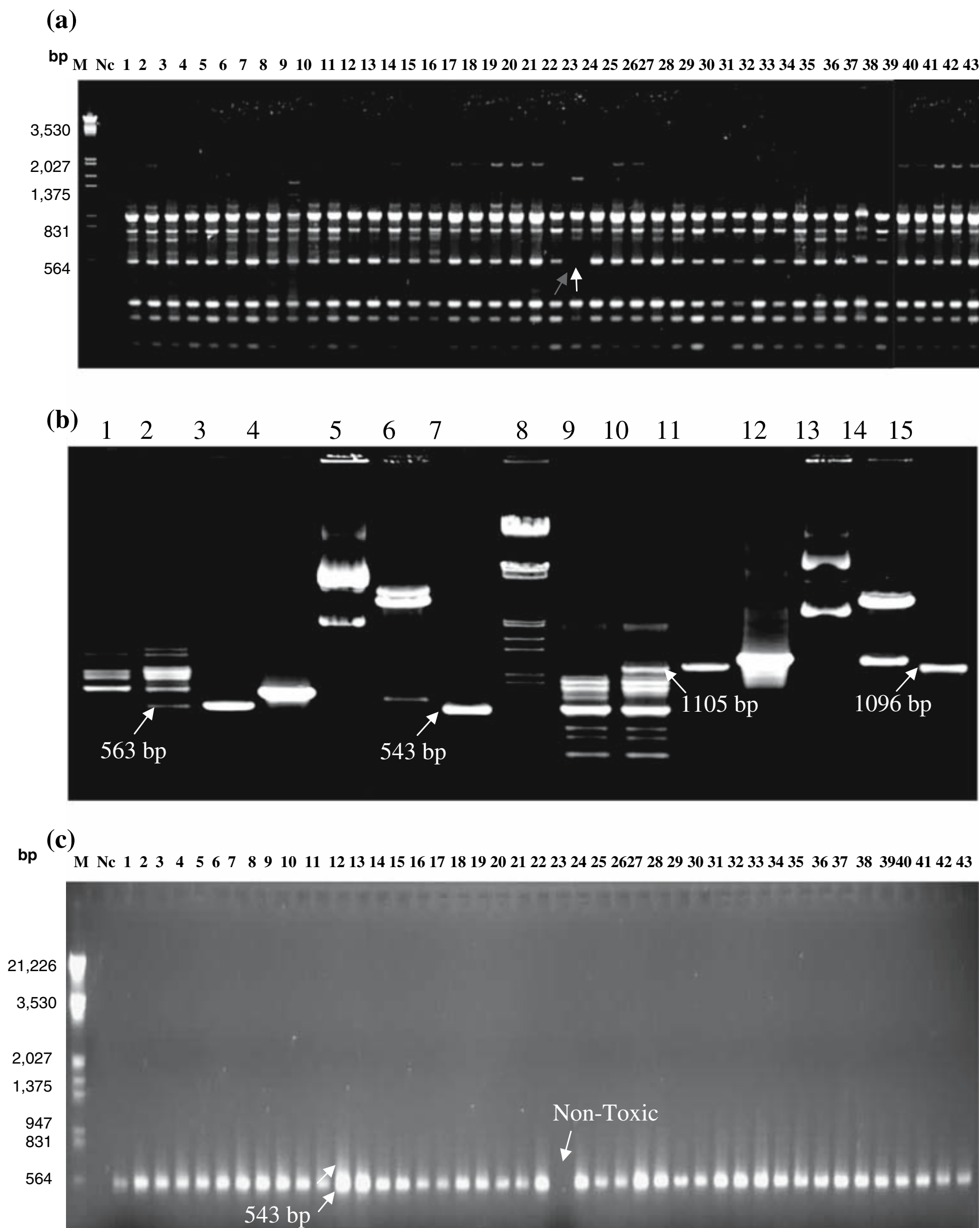

Fig. 2 Amplification profile of *J. curcas* with ISSR primers. (**a**) Electrophoretic analysis of DNA amplification with ISSR primer UBC812. Lanes designated as M represents λ DNA double digest with *Eco*RI and *Hin*dIII restriction enzymes, Nc—negative (no DNA) control, and 1–43 the samples used in the study. Arrows indicate the absence of 563-bp band in the Mexican genotype. (**b**) Conversion of polymorphic ISSR markers (UBC812 and UBC834) into SCAR markers. Lanes designated as 1—Mexican genotype amplified with UBC812, 2—Indian genotype amplified with UBC812, 3—Gel eluted fragment, 4—PCR confirmation of cloning, 5—Undigested plasmid DNA, 6—plasmid DNA restriction (*Eco*RI, *Hin*dIII), 7—Indian genotype amplification with SCAR primer (ISPJ1), 8—λ DNA double digest with *Eco*RI and *Hin*dIII, 9—Mexican genotype amplified with UBC834, 10—Indian genotype amplified with UBC834, 11—Gel eluted fragment, 12—PCR confirmation of cloning, 13—Undigested plasmid DNA, 14—plasmid DNA restriction (*Eco*RI, *Hin*dIII), 15—Mexican genotype amplification with SCAR primer (ISPJ2). (**c**) Electrophoretic analysis of DNA amplification with SCAR primer ISPJ1. Lanes designated as M represents λ DNA double digest with *Eco*RI and *Hin*dIII restriction enzymes, Nc—negative (no DNA) control, and 1–43 the samples used in the study. Arrows indicate the absence of 543-bp band in the Mexican genotype

(35 RAPD and 12 ISSR) were specific to the non-toxic Mexican genotype and distinguished it from the Indian accessions (Table 2). Six RAPD and 2 ISSR markers found in all the 42 Indian populations were absent in the Mexican genotype.

Two ISSR fragments, one each specific to the Indian and Mexican genotypes were selected from the ISSR fingerprints. The SCAR primers designed from UBC 812 (ISPJ1) amplified fragment resulted in an amplicon of 543 bp and from UBC 834 (ISPJ2) in a fragment of 1,096 bp (Table 3). The conversion of ISSR fragments into SCAR marker was confirmed through PCR amplification and restriction digestion pattern (Fig. 2b). The specificity and reproducibility of SCAR markers in discriminating genotypes of the two diverse geographical regions was verified by testing individual plants. The ISPJ1 specific to the Indian genotypes produced the amplicon of specific size in all the Indian accessions but not in the non-toxic genotype (Fig. 2c). The ISPJ2 specific to the non-toxic genotype amplified the fragment of desired length in the non-toxic genotype, but gave an additional band of 100 bp in all the genotypes.

Although the two marker systems sampled different segments of the genome, the clustering pattern of the genotypes was almost similar with both the marker systems and most of the accessions were placed in their respective clusters with minor changes. Based on RAPD polymorphism, the accessions diverged at 57% of variation. The maximum divergence with RAPD markers was observed between Neemuch-1 from Rajasthan and DOR-Jc (0.488), IJC-4 (0.476), IJC-1 (0.512) and IJC-3—the Cape Verde type (0.516). Similarity matrix values based on RAPD analysis ranged from 0.484 to 0.952 and at a threshold of 70% disclosed 6 clusters. Cluster I included 34 accessions while the other 5 clusters had 1–3 accessions. The maximum genetic distance between accessions based on ISSR polymorphism was 0.65. Similarity matrix values based on ISSR analysis ranged from 0.573 to 0.970 and at a threshold of 80% separated the accessions into 7 clusters. Maximum divergence based on ISSR polymorphism was recorded between the Mexican genotype and the accessions Neemuch-1 (0.427) and IJC-11 (0.418). The correlation between the similarity matrices generated by RAPD and ISSR polymorphism based on Mantel statistic was significant with good fit (r = 0.79481). Similarity values based on both the marker systems ranged from 0.498 to 0.952. Dendrograms based on RAPD, ISSR and ISSR-RAPD polymorphism revealed five divergent genotypes from five different locations viz., Kerala from Kerala, TNMC-7 from Chintapalli, non-toxic Jc from Mexico, IJC-11 from Jabalpur and Neemuch-1 from Rajasthan. The accessions from Mexico (non-toxic Jc), Jabalpur (IJC-11) and Rajasthan (Neemuch-1) did not cluster with any of the other genotypes while the accessions from Kerala (Kerala) and Chintapalli (TNMC-7) grouped together and formed a separate cluster (Fig. 3).

Principal coordinate analysis based on RAPD + ISSR polymorphism grouped the accessions into 7 clusters (Fig. 4). Thirty-five Indian accessions were grouped in two clusters indicating the presence of low genetic diversity among the accessions used in the study. Cluster IV comprised of two accessions from Kerala (Kerala) and Chintapalli (TNMC-7), Clusters V to VII included one accession each from Jabalpur (IJC-11), Mexico (non-toxic Jc) and Rajasthan (Neemuch-1). The genotypes from Mexico and Rajasthan failed to cluster with any other accession, either in the dendrogram or on the basis of the principal coordinate analysis. Most of the genotypes clustered together according to their geographical location except for accessions from Rajasthan and Chintapalli, which were distributed across the clusters.

To assess the extent of intra-population variation, 65 seeds derived from a single plant in a population were raised and the DNA isolated from seedlings was subjected to molecular marker analysis with 25 RAPD primers. The individuals diverged at 0.64 indicating high level of heterogeneity in the base population (data not shown).

Discussion

The study constitutes the first successful attempt at assessment of genetic diversity in *J. curcas* using molecular markers and the development of SCAR markers for differentiation of Indian

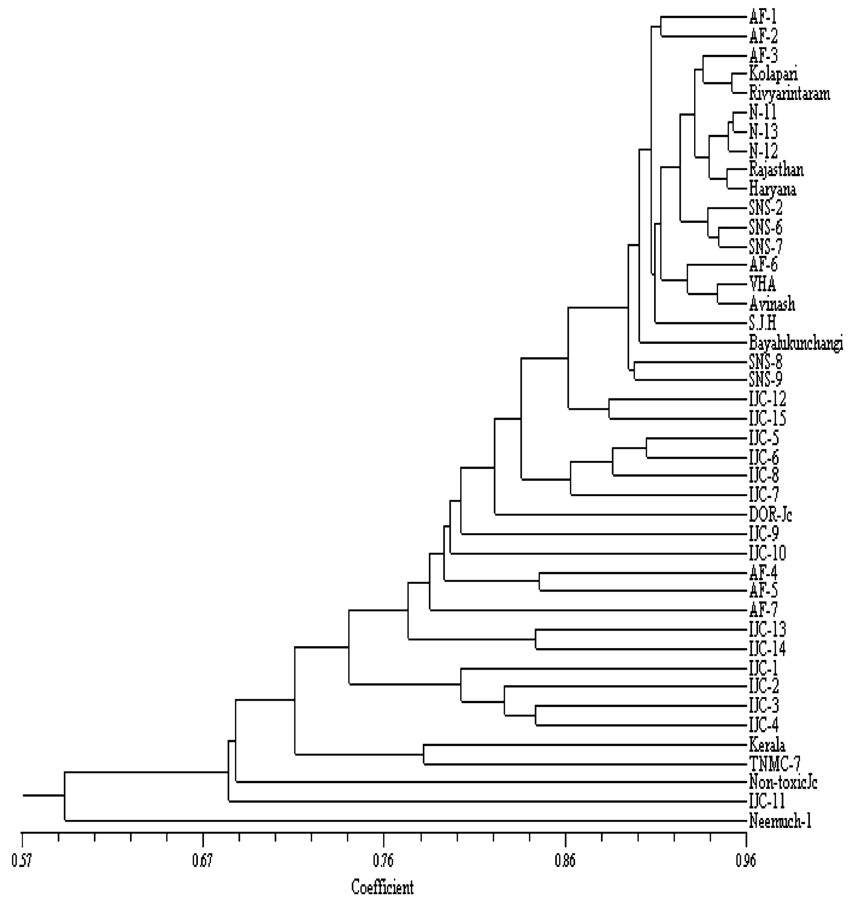

Fig. 3 Dendrogram (UPGMA) representing genetic relationships among 43 accessions of *J. curcas* based on genetic similarity matrix obtained using the pooled allelic profile of RAPD + ISSR primers

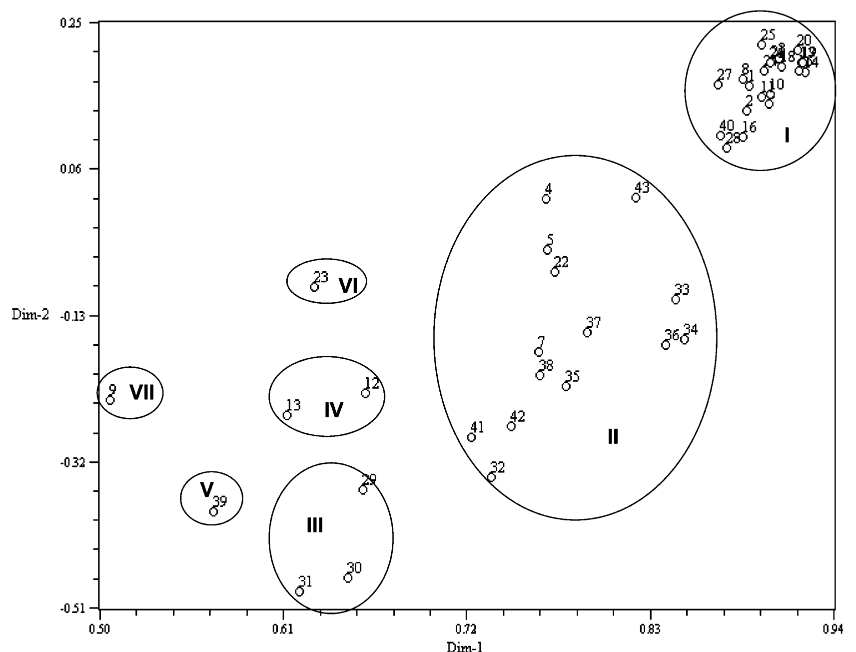

Fig. 4 Two-dimensional scaling of 43 accessions of *J. curcas* by principal component analysis using the Dice similarity coefficients based on the pooled data of RAPD + ISSR primers. The numbers represent the accession codes as given in Table 1

accessions from the Mexican genotype. The earlier study on molecular analysis in *J. curcas* was confined to the assessment of genetic similarity between one accession each of the toxic Indian and the non-toxic Mexican genotypes using 100 RAPD markers (Sujatha et al. 2005). The study could identify polymorphic RAPD markers that could distinguish these two geographically isolated genotypes but could not provide information on the extent of genetic variability available in the *J. curcas* germplasm that is essential for the breeding programmes. In the present investigation, both RAPD and ISSR primers have been used on a representative set of 43 accessions from different locations to describe the genetic structure of *J. curcas* germplasm in India. ISSR markers target multiple microsatellite loci distributed across the genome while RAPD markers scan the entire genome and hence, genome wide genetic variation could be detected with the use of these two marker systems. Discriminative ISSR markers were converted to SCAR markers to improve the specificity and reproducibility.

In the present study, the polymorphism detected with 400 RAPD and 100 ISSR primers was too low (42.0% and 33.5%, respectively) and many of the Indian accessions did not possess distinct molecular profiles and failed to be discriminated indicating a narrow genetic base of the material being investigated. Further, the intra-population variation as determined by RAPD primers was 36.0% and was on par with the genetic variation detected between populations. Such low genetic variation in an allogamous species like *J. curcas* could be due to the few introductions that have spread across the country primarily, through vegetative propagation. Programmes launched in Brazil, Nicaragua and India for introduction of *J. curcas* for varied purposes met with limited success due to the wide gap in potential and actual yields. The species has not been improved for productivity and most of the projects relied on naturally occurring unadapted populations, which are a result of the few initial introductions. Hence, there is an immediate need to systematize research for widening the genetic base of *J. curcas* through selection of superior genetic stocks, mutagenesis and inter-specific hybridization.

The reproducibility factor is 87.0% for ISSR markers and 84.4% for RAPD markers (Mc Gregor et al. 2000). In general, SCAR markers have been developed from RAPD fingerprints to improve the reliability and reproducibility of PCR assays (Paran and Michelmore 1993). In the present investigation, ISSR polymorphic bands have been selected for SCAR marker development as they are known to be highly reproducible, abundant, polymorphic and because of the specific targeted regions of the genomes (Zietkiewicz et al. 1994; Bornet et al. 2002). There was 69% similarity between ISPJ1 and sequences from the NCBI database for *H brasiliensis* microsatellites (AY 486851.1, AY 486747.1, AY 486717.1), while ISPJ2 had 50% sequence similarity to microsatellite sequences of *Arachis hypogea* (clone PM 488) and *Fragaria vesca* (clone CFVCT034). ISSR primers are locus specific and fragments of the same molecular weight are considered as the same locus. ISSR fingerprints could be used for development of microsatellite markers in *J. curcas* as demonstrated for *Eucalyptus* (vander Nest et al. 2000) because development of simple sequence repeat primers is expensive and typically involves screening of enriched or non-enriched genomic libraries for microsatellite sequences (Rafalski et al. 1996).

Two diagnostic SCAR markers based on ISSR polymorphism have been developed for the Indian and Mexican genotypes, which will be useful in further characterization of the samples from the two geographical regions. There were no significant variations in qualitative and quantitative characters between accessions from these two geographically isolated regions except for the phorbol ester content (Makkar et al. 1998). Owing to the presence of different toxic substances in *J. curcas* seeds; the seeds, press cake and the oil are not used for human or animal nutrition (Makkar et al. 1998). The innocuous nature of the non-toxic *J. curcas* variety found in the Papantla region of Veracruz State in Mexico was established using fish and rats as experimental models (Becker and Makkar 1997). Enormous potential lies if this variety is bred for both biodiesel and edible purpose. The association of the SCAR markers to the non-toxic trait can only

be confirmed following validation of the markers on the appropriate breeding population.

We have identified unique bands specific to three populations viz., non-toxic Jc, Kerala and Neemuch-1. Dehgan and Webster (1979) postulated *J. curcas* as the most primitive form of the genus and that species in other sections evolved from *J. curcas* or other ancestral form with changes in growth habit and flower structures. *J. curcas* crosses readily with most of the species and is also known to form natural hybrid complexes (*J. curcas-canascens*; *J. curcas-gossypifolia*), which are associated with high degree of sterility (Dehgan and Webster 1979; Dehgan 1984; Prabakaran and Sujatha 1999). Hence, one must be cautious about the divergent material. However, the accessions being used in the study were highly fertile and produced normal seed. All the putative population-specific markers being identified could be converted to robust SCAR markers to improve the reproducibility.

The dendrogram based on pair-wise genetic similarity coefficients showed grouping of 35 out of 43 accessions studied (83.0%) into two major clusters (clusters I and II). Accessions collected from the same region as well as different regions in India were in closely formed groups, which, clearly indicates that the geographic differentiation of Indian *J. curcas* germplasm is not extensive. The principal component analysis results corresponded well with the grouping of accessions based on cluster analysis with the exception of two rearrangements. The accession IJC-15 from Rajasthan placed in cluster I according to the dendrogram was grouped in cluster II according to PCA. Likewise the accession IJC-4 from Tamilnadu placed in cluster III based on cluster analysis was grouped in cluster II based on PCA. Based on dendrogram and PCA, five divergent accessions viz., Kerala, non-toxic Jc, Neemuch-1, TNMC-7 and IJC-11 have been identified. However, accession specific bands could be established only for three accessions viz., Kerala, non-toxic Jc and Neemuch-1. The accession Kerala grouped with TNMC-7 while the non-toxic Jc and Neemuch-1 accessions were more dispersed on the PCA plot, which is a reflection of their wider genetic variation. However, development of only 2 population specific markers for Neemuch-1 as against 21 markers for the Mexican genotype indicates the greater genetic distance of the Indian and Mexican genotypes.

Till date the 'true' centre of origin of *J. curcas* has not been established and this study will pave way for investigation of the genetic distinctness of this crop in the centre of origin and other regions where it is being introduced. Modest levels of genetic variation in *J. curcas* accessions available in India and wide variation between Indian and Mexican genotypes necessitates characterization of accessions with broader geographical background and comparison of genetic relationships with morphological characteristics in order to develop a greater understanding of this prospective biofuel crop.

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