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Full Length Research Paper

Analogy of ISSR and RAPD markers for comparative analysis of genetic diversity among different *Jatropha curcas* genotypes

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The phylogenetic relationships of 13 Jatropha genotypes from different parts of the India were analysed using 34 polymerase chain reaction (PCR) markers (20 random amplified polymorphic DNAs (RAPDs) and 14 inter simple sequence repeats (ISSRs)). Amplification of genomic DNA of the 13 genotypes, using RAPD analysis, yielded 107 fragments that could be scored, of which 91 were polymorphic, with an average of 4.55 polymorphic fragments per primer. Number of amplified fragments ranged from one (OPA20, OPB19, OPD13) to nine (OPA18) and which varied in size from 200 to 2,500 bp. Percentage of polymorphism ranged from 40% (OPB18) to a maximum of 100% (14 primers). Resolution power ranged from a minimum of 0.153 (OPA20, OPB19) to a maximum of 11.23 (OPB15). Out of 25 ISSR primers used, 14 were able to amplify. These primers produced 81 bands across 13 genotypes, of which 62 were polymorphic. The number of amplified fragment ranging from two (ISSR 7, ISSR 8, ISSR 16) to nine (ISSR 12) and which varied in size from 200 to 2,500 bp. Of the 81 fragments amplified bands, 62 were polymorphic, with an average of 4.42 polymorphic fragments per primer. Percentage of polymorphism ranged from 37.5% (ISSR 2, ISSR10) to a maximum of 100% (seven primers). The primers based on poly (GA) produced maximum number of bands (nine) while, poly (AT) and many other motifs gave no amplification at all with any of these thirteen genotypes. RAPD markers were more efficient than the ISSR assay with regards to polymorphism detection, as they detected 84.26% as compared to 76.54% for ISSR markers. But, resolving power (Rp), average bands per primer, Nei's genetic diversity (h), Shannon's Information Index (I), total genotype diversity among population (Ht), within population diversity (Hs) and gene flow (Nm) estimates were more for ISSR (7.098, 5.79, 0.245, 0.374, 0.244, 0.137 and 0.635, respectively) as compared to RAPD markers (5.669, 5.35, 0.225, 0.359, 0.225, 0.115 and 0.518, respectively). The regression test between the two Nei's genetic diversity indexes gave r^2 = 0.3318, showing low regression between RAPD and ISSR based similarities. Regression value for ISSR and ISSR + RAPD combined data is moderate (0.6027), while it is maximum for RAPD and ISSR+RAPD based similarities (0.9125). Thus both the markers are equally important for genetic diversity analysis in *Jatropha curcas.* Clustering of genotypes within groups was not similar when RAPD and ISSR derived dendrogram were compared, whereas the pattern of clustering of the genotypes remained more or less the same in RAPD and combined data of RAPD + ISSR. Principal Coordinates Analysis (PCA) analysis was also employed to evaluate the resolving power of the markers to differentiate between the genotypes. These analyses, carried out for both (ISSR and RAPD) markers, allowed us to identify four main groups partially corresponding to the four *J. curcas* collection sites. The results of the present study can be seen as a starting point for future researches on the population and evolutionary genetics of these genotypes.

Key words: *Jatropha curcas*, ISSR, RAPD, genetic diversity.

INTRODUCTION

Jatropha also called Physic Nut, is a genus of approximately 175 succulents, shrubs and trees (some are deciduous, like *Jatropha curcas* L.), from the family Euphorbiaceae. It adapts well to semi-arid marginal sites, its oil can be processed for use as a diesel fuel substitute and it can be used for erosion control. Despite its ecological and economic importance, the taxonomy and genetic structure of the genus *Jatropha curcas* is not entirely clarified, due also to the occurrence of natural hybridization among species (Airy Shaw, 1972). Until now, no study has been carried out on the phylogeny of *J. curcas* for genetic variation among populations using molecular markers. The major constraints in achieving higher quality oil yield of this crop are lack of information about its genetic variability, oil composition, and absence of suitable ideotypes for different cropping systems. Research on this species has lagged behind that of other crop of this family like Ricinus. Therefore, improvement of this crop is needed through utilization of available genetic diversity. The evaluation of genetic diversity and construction of linkage maps would promote the efficient use of genetic variations in the breeding program (Paterson et al., 1991). DNA markers provide an opportunity to characterize genotypes and to measure genetic relationships more precisely than other markers (Soller and Beckmann, 1983). The polymerase chain reaction (PCR) technology has offered new marker systems for diagnosis of genetic diversity in large scale studies (Saiki et al., 1988). Over the last 15 years, polymerase chain reaction technology has led to the development of two simple and quick techniques called random amplified polymorphic DNA (RAPD) and Inter Simple Sequence Repeats (ISSR). The former detects nucleotide sequence polymorphisms, using a single primer of arbitrary nucleotide sequence, and the latter permits detection of polymorphisms in inter-microsatellite loci, using a primer designed from dinucleotide or trinucleotide simple sequence repeats (Wu et al., 1994; Zietkiewicz et al., 1994). Inter simple sequence repeat (ISSR) analysis involves the polymerase chain reaction (PCR) amplification of regions between adjacent, inversely oriented microsatellites, using a single simple sequence repeat (SSR) motifs (di-, tri-, tetra-, or pentanucleotides) containing primers anchored at the 3′ or 5′ end by two to four arbitrary, often degenerate nucleotides (Zietkiewicz et al., 1994). The potential supply of ISSR marker depends on the variety and frequency of microsatellites, which changes with species and the SSR motifs that are targeted (Depeiges, et al., 1995). RAPD and ISSR markers have been used both for DNA fingerprinting (Martín and Sánchez-Yélamo, 2000; Moreno et al., 1998; Blair et al., 1999) and population genetic studies (Wolfe et al., 1998). A large number of polymorphic markers are required to measure genetic relationships and genetic diversity in a reliable manner. This limits the use of morphological characters and isozymes, which are few or lack adequate levels in *J. curcas*. Molecular genetic markers would aid the long-term objective of identifying diverse parental lines to generate segregating popula-tions for tagging important traits, such as gene(s) for high content of specific fatty acids like oleic, linolenic, etc. The objectives of the present investigation were: (1) To study and compare genetic diversity among 13 elite genotypes, using random amplified polymorphic DNA (RAPD) and inter simple sequence (ISSR) markers, and (2) to evaluate of the degree of polymorphism generated from each technique as a pre-requisite for their applicability to population genetics studies in *J. curcas*.

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MATERIALS AND METHODS

Plant material

Thirteen genotypes were collected from four different jatropha growing regions of India i.e. Rajasthan, Uttaranchal, Uttar Pradesh and Orissa (Table 1). These were classified into four different populations for analysis. These genotypes were classified into four different populations for analysis.

DNA isolation

Young leaves were collected from the plants, which were obtained from different states. Total genomic DNA was isolated from the leaves according to Doyle and Doyle (1987). Leaves were ground in liquid nitrogen using mortar and pestle to fine powder. It was then transferred to pre-warmed extraction buffer and incubated at 65°C for 1 h. An equal amount of chloroform: isoamyl alcohol (1:1) was added, mixed well by gentle inversion and centrifuged. The supernatant was transferred to a fresh tube and DNA was precipitated by adding ¾ volume of isopropanol. After centrifugation, the pellet was washed in 70% ethanol, dried and dissolved in TE buffer. RNA was removed by RNase treatment. DNA was quantified by comparing with uncut λ DNA on the agarose gel, diluted to 12.5 ng μl^{-1} and used in PCR.

RAPD analysis

The conditions for RAPD were: 25 ng of template DNA, 200 µM of each dNTPs (Promega, USA), 20 ng of primer (Operon Technologies Inc., USA), 0.5 U of Taq DNA polymerase (Bangalore Genei, India) and 1x reaction buffer (Bangalore Genei, India) in a total volume of 25 µl. Amplification was carried out for 45 cycles of 1 min at 94°C, 1 min at 37°C and 2 min at 72°C in a Applied Biosystems 2720 thermal cycler.

ISSR analysis

The conditions for ISSR were: 25 ng of template DNA, 200 µM of each dNTPs (Promega, USA), 20 ng of primer (Applied Biosciences), 0.5 U of Taq DNA polymerase (Bangalore Genei, India)

Table 1. Details of 13 *J. curcas* genotypes used in present investigation.

S/N	Jatropha cultivars	Accession No./Genotype	State
1	UT41	IC-545441	Uttaranchal
2	UT43	IC-555443	Uttaranchal
3	UT46	IC-545446	Uttaranchal
4	UT49	IC-545449	Uttaranchal
5	UT50	IC-545450	Uttaranchal
6	RJ53	IC-545453	Rajasthan
7	RJ54	IC-545454	Rajasthan
8	RJ62	IC-545462	Rajasthan
9	RJ63	IC-545463	Rajasthan
10	Orissa6	O-06	Orissa
11	Orissa7	O-07	Orissa
12	GKP1	G-01	Uttar Pradesh
13	GKP5	G-05	Uttar Pradesh

and 1x reaction buffer (Banglore Genei, India) in a total volume of 25 µl. Amplification was carried out for 45 cycles of 1 min at 94°C, 1 min at 45 (+,- 5)°C and 2 min at 72°C in a Applied Biosystems 2720 thermal cycler.

Agarose gel electrophoresis

Amplified products were separated in 1.5% agarose gel containing ethidium bromide using 1x TAE buffer. A constant voltage of 55 was provided for 3 - 4 h. DNA fragments were visualized under UV light. The patterns were photographed using Geldoc system (Bio-Rad) and stored as digital pictures. The reproducibility of the amplification was confirmed by repeating each experiment three times.

Data collection and Analysis

The relatedness of DNA samples was assessed by comparing RAPD and ISSR fragments of DNA separated according to their sizes and the presence/absence of shared fragments. The banding patterns obtained from RAPD and ISSR were scored as present (1) or absent (0), each of which was treated as an independent character regardless of its intensity. Jaccard's similarity coefficient (J) was used to calculate similarity between pairs of accessions. Where, $[J = n_{x,y} / (n_t - n_z)]$, $n_{x,y}$ is the number of bands common to accession A and accession B; n_t the total number of bands present in all samples and n_z the number of bands not present in A and B but found in other samples. The similarity matrix was subjected to cluster analysis by unweighted pair group method for arithmetic mean (UPGMA) and a dendrogram was generated using the programme NTSYS-PC (Rohlf, 1992). Support for clusters was evaluated by bootstrapping analysis (Felsenstein, 1985). FreeTree software (Pavlicek et al., 1999) was used for construction of phylogenetic trees on the basis of distance data and for bootstrap analysis of the trees robustness. POPGENE software was used to calculate Nei's unbiased genetic distance among different species with all markers, including monomorphic markers. Nei's unbiased genetic distance is an accurate estimate of the number of gene differences per locus when populations are small. Within species diversity (Hs) and total genetic diversity (Ht) (Nei, 1978) were calculated within the species and within four major groups (as per their collection site) by POPGENE software. The RAPD and ISSR data were subjected to a hierarchical analysis of molecular variance (AMOVA), as described by Excoffier et al. (1992), using three hierarchical levels; individual, population and their regions. The analysis was performed using GenALEx software (Peakall and Smouse, 2001). GenAlEx was also used to calculate a Principal Coordinates Analysis (PCA) that plots the relationship between distance matrix elements based on their first two principal coordinates. Regression between two matrices obtained with two marker types was estimated using Nei's genetic diversity. This yielded the regression (r^2) which is one measure of relatedness between two matrices. In this instance, the matrix regression corresponds to two independently derived dendrograms.

Resolving Power

According to Prevost and Wilkinson (Prevost and Wilkinson, 1999) the Resolving power (Rp) of a primer is: $Rp = \Sigma\ IB$ where *IB* (band informativeness) takes the value of: $1 - [2 \times (0.5 - p)]$, p being the proportion of the 13 genotypes (*Jatropha* cultivars analysed) containing the band.

RESULTS

A set of 20 RAPD and 25 ISSR primers were used for initial screening of 13 genotypes of *J. curcas* for phylogenetic analysis (Table 2).

RAPD band patterns

Thirteen genotypes as obtained from four different regions (Uttaranchal, Rajasthan, Orissa and Uttar Pradesh) of India were amplified using the 20 RAPD oligonucleotides as indicated in Tables 1 and 2. Amplification of genomic DNA yielded 735 fragments that could be scored. All the chosen primers amplified fragments across the 13 genotypes studied, with the number of amplified fragments ranging from one (OPA20, OPB19, and OPD13) to 9 (OPA18) and which varied in size from

Table 2. Details of all the 45 primers (twenty RAPD and twenty five ISSR) used in the present investigation.

Primer	Nucleotide sequence (5'- 3')	G + C content (%)
ISSR		
ISSR 1	ATA TAT ATA TAT ATA TT	0
ISSR2	AGA GAG AGA GAG AGA GT	47.05
ISSR 3	GAG AGA GAG AGA GAG AT	47.05
ISSR 1	ATA TAT ATA TAT ATA TT	0
ISSR 4	CTC TCT CTC TCT CTC TT	47.05
ISSR 5	GTG TGT GTG TGT GTG TA	47.05
ISSR 6	TCT CTC TCT CTC TCT CA	47.05
ISSR 7	ACA CAC ACA CAC ACA CT	47.05
ISSR 8	TGT GTG TGT GTG TGT GA	47.05
ISSR 9	ATA TAT ATA TAT ATA TYA	0
ISSR 10	AGA GAG AGA GAG AGA GYT	44.44
ISSR 11	TAT ATA TAT ATA TAT ART	0
ISSR 12	GAG AGA GAG AGA GAG AYT	44.44
ISSR 13	CTC TCT CTC TCT CTC TRA	44.44
ISSR 14	GTG TGT GTG TGT GTG TYC	50
ISSR 15	ACC ACC ACC ACC ACC ACC	66.66
ISSR 16	CCG CCG CCG CCG CCG CCG	100
ISSR 17	GGCGGCGGCGGCGGC GGC	100
ISSR 18	TGC TGC TGC TGC TGC TGC	66.66
ISSR 19	CTA GCT AGC TAG CTA G	50
ISSR 20	TGC ATG CAT GCA TGC A	50
ISSR 21	CTT CAC TTC ACT TCA	40
ISSR 22	TAG ATC TGA TAT CTG AAT TCC C	36.36
ISSR 23	AGA GTT GGT AGC TCT TGA TC	45
ISSR 24	CAT GGT GTT GGT CAT TGT TCC A	45.45
ISSR 25	ACT TCC CCA CAG GTT AAC ACA	47.61
RAPD		
OPA01	CAG GCC CTT C	70
OPA02	TGC CGA GCT G	70
OPA04	AAT CGG GCT G	60
OPA08	GTG ACG TAG G	60
OPA11	CAA TCG CCG T	60
OPA13	CAG CAC GCA C	70
OPA18	AGG TGA CCG T	60
OPA20	GTT GCG TACC	60
OPB11	GTAGACCCGT	60
OPB15	GGA GGG TGT T	60
OPB18	CCA CAG CAG T	60
OPB19	ACC CCC GAA G	70
OPC08	TGG ACC GGT G	70
OPC12	TGT CAT CCC C	60
OPC15	GAC GGA TCA G	60
OPC16	CAC GCC CTT C	70
OPD05	TGA GCG GAC A	60
OPD08	GTG TGC CCC A	70
OPD11	AGC GCC ATT G	60
OPD13	GGG GTG ACG A	70

Table 3. Combined details of thirty four primers (20 RAPD + 14 ISSR) and amplified bands of all the DNA samples as obtained from thirteen populations of *J. curcas.*

Primer	No. of Total bands	No. of polymorphic bands	No. of monomorphic bands	%of polymorphic bands (P %)	Total No. of bands amplified	Resolution power
ISSR						
ISSR2	8	3	5	37.5	89	13.692
ISSR 3	7	7	0	100	56	8.615
ISSR 7	2	2	0	100	11	1.692
ISSR 8	4	2	2	50	29	4.461
ISSR 10	8	3	5	37.5	76	11.692
ISSR 12	9	9	0	100	62	9.538
ISSR 13	5	4	1	80	25	3.846
ISSR 16	5	2	3	40	41	6.307
ISSR 17	3	3	0	100	21	3.230
ISSR 21	5	5	0	100	34	5.230
ISSR 22	7	5	2	71.42	63	9.692
ISSR 23	4	4	0	100	16	2.461
ISSR 24	7	6	1	85.71	67	10.307
ISSR 25	7	7	0	100	56	8.615
RAPD						
OPA01	2	2	0	100	3	0.462
OPA02	5	5	0	100	39	6.000
OPA04	7	7	0	100	58	8.923
OPA08	7	7	0	100	42	6.462
OPA11	6	4	2	66.66	70	10.770
OPA13	8	5	3	62.50	66	10.153
OPA18	9	9	0	100	70	10.770
OPA20	1	1	0	100	1	0.153
OPB11	5	5	0	100	26	4.307
OPB15	8	6	2	75	73	11.230
OPB18	5	2	3	40	51	7.846
OPB19	1	1	0	100	1	0.153
OPC08	7	4	3	57.14	45	6.923
OPC12	5	5	0	100	30	4.615
OPC15	5	5	0	100	9	1.384
OPC16	3	3	0	100	9	1.384
OPD05	8	5	3	62.50	34	5.230
OPD08	6	6	0	100	49	7.538
OPD11	8	8	0	100	49	7.538
OPD13	1	1	0	100	10	1.538

200 to 2,500 bp. Of the 108 amplified bands, 91 were polymorphic, with an average number of bands per primer and average number of polymorphic bands per primer was 5.35 and 4.55, respectively. Percentage polymorphism ranged from 40% (OPB18) to a maximum of 100% (14 primers), with an average of 84.26% polymorphism. Only 5 out of 20 primers showed less than 75% polymorphism. Total number of bands amplified ranged from one (OPA20 and OPB19) to 73 (OPB15). The resolving power (Rp) of the twenty RAPDs primers ranged from 0.153 (OPA 20 and OPB 19) to 11.23 (OPB15) (Table 3). The total number of bands (TNB), number of polymorphic bands (NPB), percentage of polymorphic bands (P%), average no of bands per primers, average no of polymorphic bands per primer and resolving power (Rp) obtained for each primer are shown in the Table 3 and comparative list is presented in the Table 4.

Genetic variability details across all the populations using RAPD Markers

Data for observed number of alleles, effective number of

Table 4. A comparative list showing different markers details (RAPD, ISSR and RAPD + ISSR) obtained from 13 *J. curcas* populations.

Primer	RAPD	ISSR	RAPD + ISSR
Number of primers used	20	14	34
Total number of polymorphic bands	91	62	153
Total number of monomorphic bands	17	19	36
Total number of bands	108	81	189
Total number of bands amplified	735	646	1381
Percentage polymorphism (%)	84.26	76.54	80.95
Average number of bands/primer	5.35	5.79	5.53
Average number of polymorphic bands/ primer	4.55	4.42	4.52
Resolving power	5.669	7.098	6.257

Table 5. A comparative list of genetic variability across all the 13 populations of *J. curcas* using RAPD, ISSR and RAPD + ISSR primers.

Primers	Observed no. of alleles	Effective no. of alleles	Nei's genetic diversity	Shannon's information Index	Ht	Hs	Gst	Estimate of gene flow
RAPD	1.843 (0.366)	1.350 (0.305)	0.225 (0.155)	0.359 (0.213)	0.225 (0.024)	0.115 (0.012)	0.491	0.518
ISSR	1.765 (0.426)	1.407 (0.355)	0.245 (0.182)	0.374 (0.253)	0.244 (0.033)	0.137 (0.013)	0.441	0.635
RAPD + ISSR	1.810 (0.394)	1.374 (0.328)	0.233 (0.167)	0.365 (0.230)	0.233 (0.028)	0.124 (0.013)	0.468	0.568

The values in the brackets are standard deviation.

Table 6. Summary of analysis of molecular variance (AMOVA) based on RAPD, ISSR and RAPD + ISSR marker data of genotypes of *J. curcas* (levels of significance are based on 1000 iteration steps).

Source of variance	Primers	Variance component	Percentage (P%)	P-value
Among groups	RAPD	5.503	38	< 0.003
	ISSR	3.543	31	< 0.002
	RAPD + ISSR	9.046	35	< 0.001
Among population within groups	RAPD	8.933	62	< 0.003
	ISSR	8.006	69	< 0.002
	RAPD + ISSR	16.939	65	< 0.001

P-value: Probability of null distribution.

alleles, Nei's genetic diversity, Shannon's information index, for all the four populations were analysed using twenty RAPD markers and their respective values were found as 1.843, 1.350, 0.225 and 0.359. Value for total genotype diversity among population (Ht) was 0.225 while within population diversity (Hs) was found to be 0.115. Mean coefficient of gene differentiation (Gst) value 0.491 indicated that 50.9% of the genetic diversity resided within the population. Estimate of gene flow in the population was found as 0.518 (Table 5). AMOVA helps in partitioning of the overall RAPD variations among groups and among populations within the group. 38% is the molecular variance found among population while, within the population this value was found to be 62% (Table 6) indicating that there is more variations within the population. This is helpful in making strategy for germ plasm collection and evaluation.

Dendrogram analysis for *J. curcas* as obtained with RAPD markers

A dendrogram based on UPGMA analysis grouped the 13 genotypes into four main clusters, with Jaccard's simi-

larity coefficient ranging from 0.38 to 0.91 (Figure 1.1). The dendrogram obtained present four main clusters (I, II, III, and IV) with five, four, three and one cultivars, respectively. The cluster I has two sub-clusters (Ia and Ib). Sub-cluster Ia has all the three genotypes from Uttaranchal (UT41, UT49 and UT50). The Ib sub cluster contains two cultivars one each from Uttaranchal and Rajasthan (UT 43 and RJ 53). When all the genotypes were seen together there are four genotypes from Uttaranchal and one from Rajasthan which forms cluster I. The cluster II has again two sub-clusters (IIa and IIb). IIa sub-cluster has two genotypes from Rajas-than (RJ62 and RJ63) while, sub-cluster IIb also contains two genotypes one each from Uttaranchal and Rajasthan (UT46 and RJ54). III cluster is made up of three genotypes which can be again sub-divided into two sub-clusters. Sub-cluster IIIa contains two genotypes from Uttar Pradesh (GKP1 and GKP5) while IIIb has only one genotype from Orissa (Orissa7). Cluster IV is unique as it contains only one genotype from Orissa (Orissa6). Boot-strapping was done using the Tree view (http//www.treeview.net) program to estimate the relative support at clades. The robustness of the cluster was not very strong in *J. curcas.* The results of PCA analysis were comparable to the cluster analysis (Figure 2.1). The first three most informative PC components explained 61.20% of the total variation. Genotypes from Orissa (Orissa 6 and Orissa 7) appear to be distinct from other genotypes in the PCA.

ISSR band patterns

Twenty-five ISSR oligonucleotides (Table 2) were used for amplification of all the thirteen genotypes. These ISSR primers are obtained from Applied Biosciences and out of 25 only 14 primers give rise to reproducible amplification products, while 11 primers did not gave any amplification. The sequences of these 25 primers seem to indicate that microsatellites more frequent in Jatropha contain the repeated dinucleotides (AG)*n*, (GA)n, (TG)n, (CT)n, (AC)*n*, (TG)*n,* and trinucleotides (CCG)n, (GGC)n and the repeated pentanucleotides (CTTCA)*n*. The number of bands produced with different repeat nucleotide was more with the poly (GA) primers (ISSR3 and ISSR9) and with the poly (AG) primers (ISSR2 and ISSR10). The primers that were based on the poly (GA) and poly (AG) motif produced more polymorphism on average (8 bands per primer) than the primers based on any other motifs used in the present investigation. The primer sequences which did not amplified in the present investigation contain the dinucleotides repeat sequences as (AT)n, (GT)n, (TC)n, (TA)n, (GT)n, (CT)n with T at the 5' end, the repeated trinucleotides (ACC)n, (TGC)n and the repeated tetranucleotides (CTAG)n and (TGCA)n (Table 2 and 3).

The 14 primers on an average produced 81 bands across 13 genotypes, of which 62 bands were polymorphic, accounting for 76.54%. Number of bands varied from two (ISSR 7) to nine (ISSR 12), and sizes ranged from 200 to 2,500 bp. Average numbers of bands and polymorphic bands per primer were 5.79 and 4.42 respectively. Percentage of polymorphism ranged from 37.5 (ISSR 2 and 10) to 100% (ISSR 3, 7, 12, 17, 21, 23 and 25), with an average polymorphism of 76.54 % across all the genotypes. Total no of bands amplified ranged from 11 (ISSR 7) to 89 (ISSR 2). The resolving power (Rp) of the fourteen ISSR primers ranged from 1.692 for primer ISSR 7 to 13.692 for primer ISSR 2. Three ISSR primers (ISSR 2, 10 and 24) possess the highest Rp values (13.692, 11.692 and 10.307 respectively). Primer amplification details as obtained for each ISSR primer are shown in Table 3 and comparative list is presented in Table 4.

Genetic variability details across all the populations using ISSR markers

Data for observed number of alleles, effective number of alleles, Nei's genetic diversity, Shannon's information index, for all the four populations were analysed using fourteen ISSR markers and their respective values were found as 1.765, 1.407, 0.245 and 0.374 (Table 5). Value for total genotype diversity among population (Ht) was 0.245 while within population diversity (Hs) was found to be 0.137. Mean coefficient of gene differentiation (Gst) value 0.441 indicated that 55.9% of the genetic diversity resided within the population. Estimate of gene flow in the population was found as 0.635. AMOVA analysis showed that 31% is the molecular variance found among population while, within the population this value was found to be 69% (Table 6) indicating that there is more variations with in the population.

Dendrogram analysis for *J. curcas* as obtained with ISSR markers

The PCR amplification using 14 ISSR primers gave rise to reproducible amplification products. The complete data was based on a total of 646 bands. A dendrogram based on UPGMA analysis with ISSR data is shown in Figure 1.2. Jaccard's similarity coefficient ranged from 0.46 to 0.90. The 13 genotypes were clustered into three clusters (I, II and III) with seven, five and one genotypes respectively. Cluster I comprised of two sub-clusters. Sub-cluster Ia consisted of four genotypes of which three are from Uttaranchal (UT41, UT43 and UT50) and one from Rajasthan (RJ53).While, Ib comprised of three genotypes of which two are from Uttaranchal (UT49 and UT46) and one from Rajasthan (RJ54). Thus, all the five genotypes from Uttaranchal and two genotypes from Rajasthan are grouped together in cluster I. Cluster II again comprised of two sub clusters. Sub-cluster IIa consists of three genotypes of which two are from Rajasthan (RJ62 and RJ63) and one from Uttar Pradesh (GKP1). One geno-

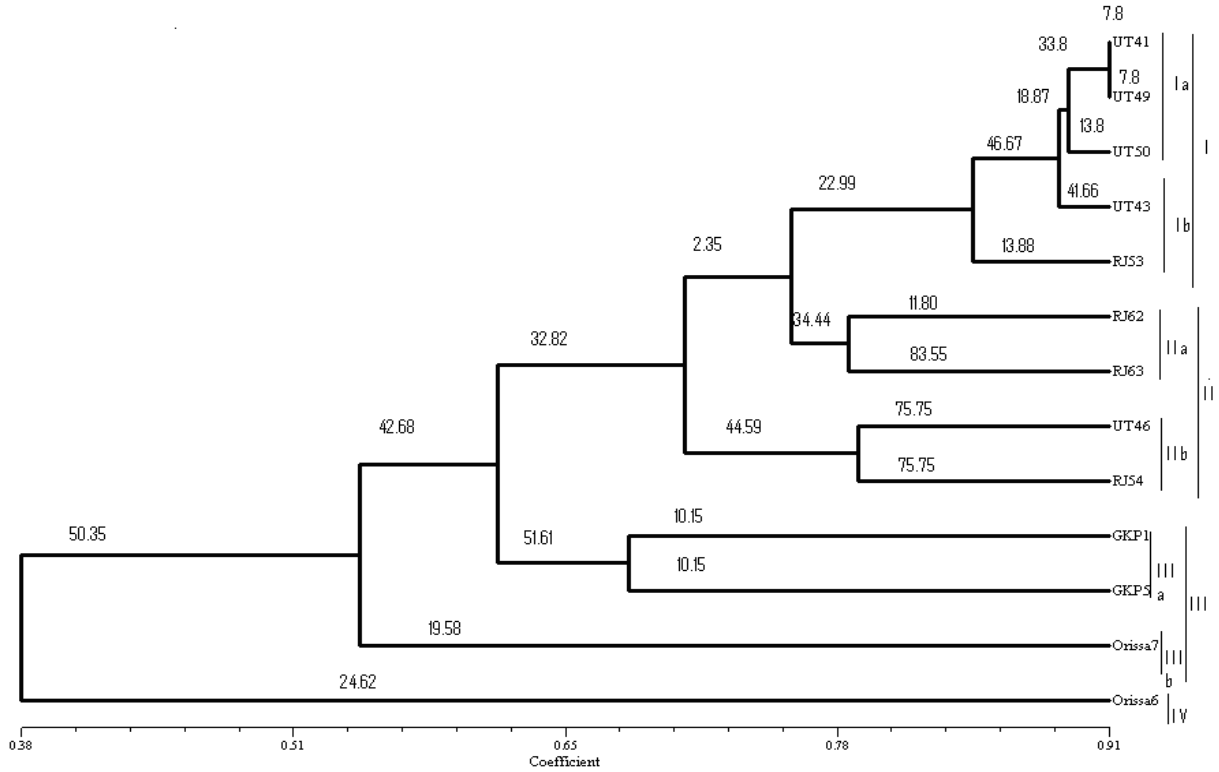

Figure 1.1. RAPD

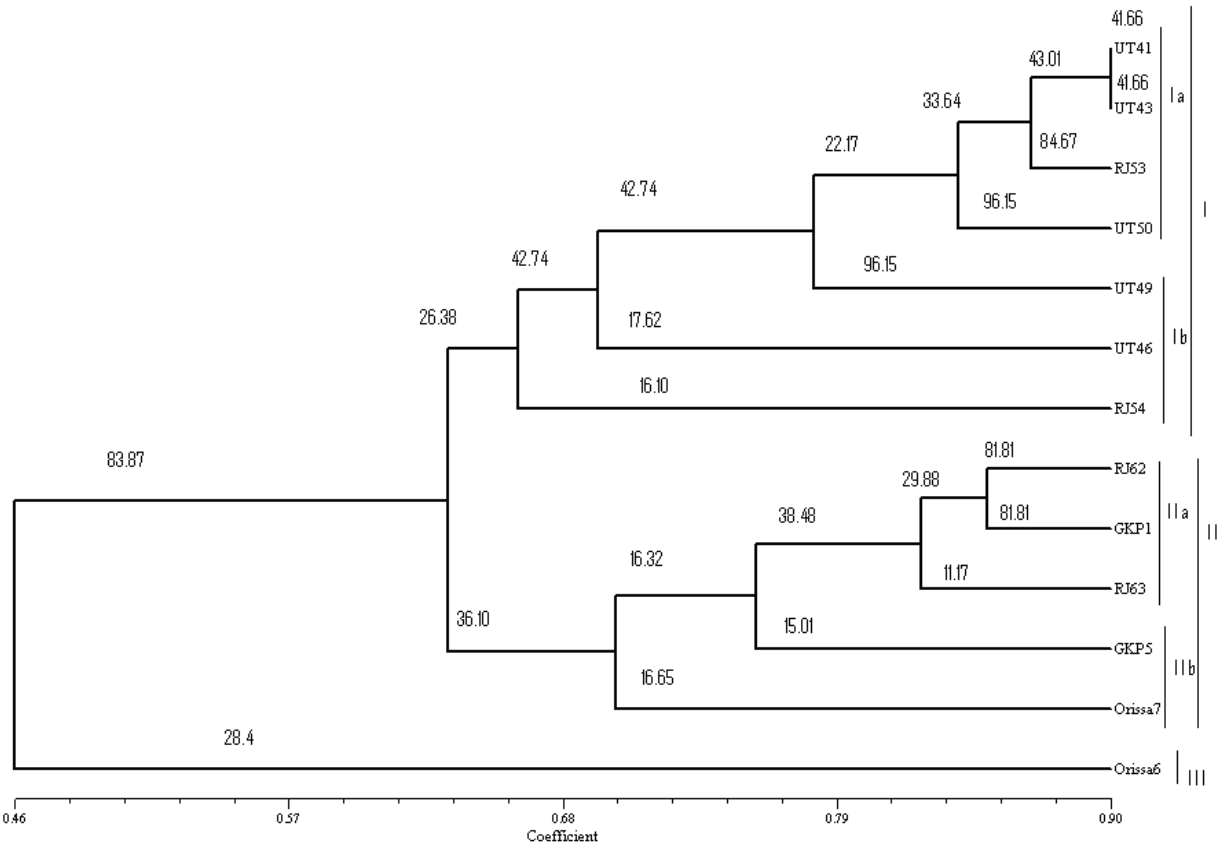

Figure 1.2. ISSR

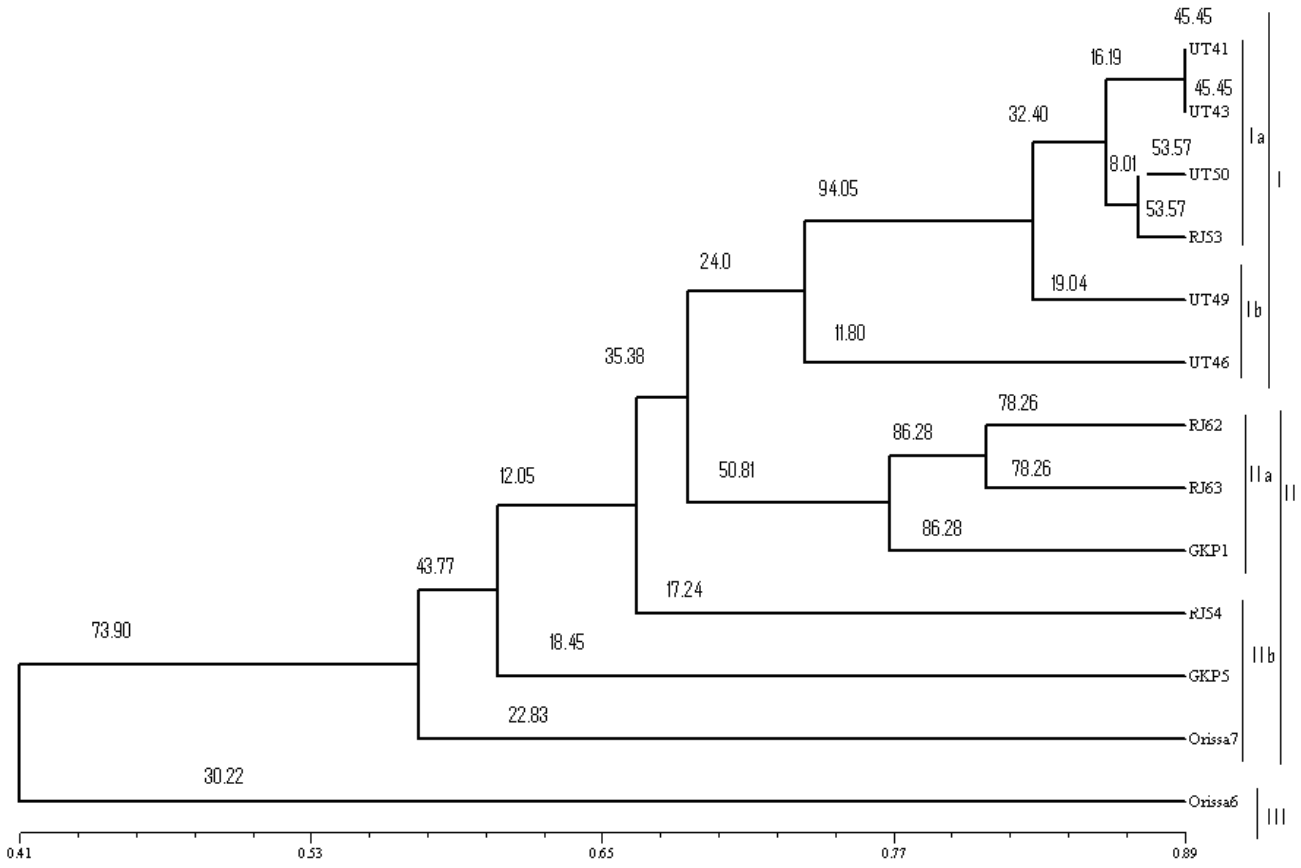

Figure 1.3. ISSR+RAPD

Figure 1. Dendrograms generated using unweighted pair of group method with arithmetic average analysis, showing relationships between Jatropha genotypes, using **(1.1)** RAPD, **(1.2)** ISSR and **(1.3)** ISSR+RAPD data. The numbers at the forks indicate the confidence limits for the grouping of those species in a branch occurred, based on 1,000 cycles in bootstraps analysis, using the free tree program.

type each from Uttar Pradesh and Orissa are the part of sub-cluster IIb (GKP5 and Orissa 7). This cluster consisted of a mixture of genotypes from Rajasthan Uttar Pradesh and Orissa. Cluster III consisted of only one genotype, that is, Orissa 6 which is significantly different from all other genotypes used in the present investigation. The results of PCA analysis were comparable to the cluster analysis (Figure 2.2). The first three most informative PC components explained 60.70% of the total variation. Three genotypes (RJ54, Orissa7 and Orissa6) appear to be distinct from other genotypes in the PCA.

RAPD and ISSR combined data for cluster analysis

The RAPD and ISSR data were combined for UPGMA cluster analysis. The UPGMA dendrogram obtained from the cluster analysis of RAPD and ISSR combined data gave near similar clustering pattern, with Jaccard's similarity coefficient ranging from 0.41 to 0.89. Three major clusters were formed which more close to ISSR cluster are whereas, the dendrogram based on RAPD showed some variation in the clustering of genotypes. Thirteen genotypes were grouped in three clusters in ISSR and ISSR plus RAPD data; whereas a dendrogram based on RAPD data showed four clusters. The dendrograms based on ISSR and RAPD combined markers showed partially similar clustering pattern when used individually. But when used together ISSR based cluster is more similar to the combined cluster than RAPD based cluster. In all the three dendrograms, Orissa 6 is present in different cluster. The dendrogram based on combined data are shown in Figure 1.3. The results of PCA analysis were comparable to the cluster analysis (Figure 2.3). The first three most informative PC components explained 57.90% of the total variation. The two-dimensional ordination confirms the cluster analysis results showing that four genotypes (UT46, RJ54, Orissa7 and Orissa 6) were separated. Besides formation of dendrogram other genetic variation studies were also performed on ISSR and RAPD combined data which are represented in different tables (Tables 4 - 6). As expected all the other para-

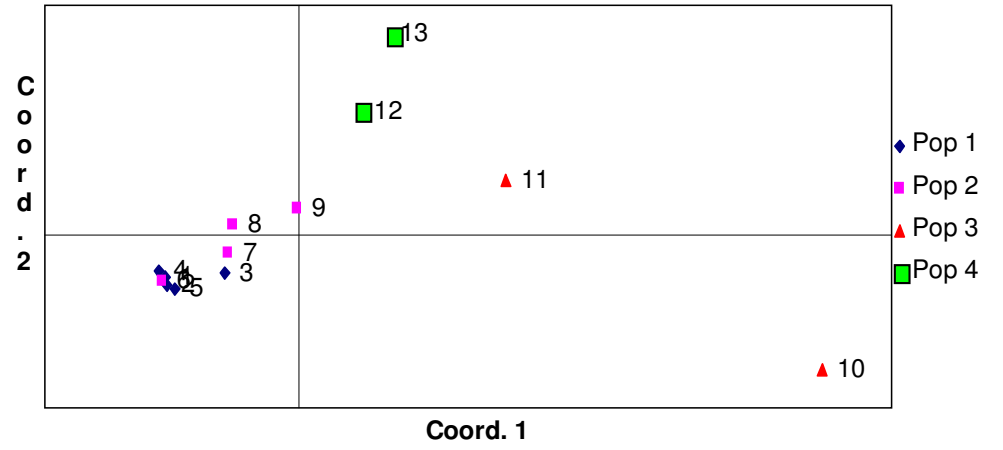

Figure 2.1. RAPD.

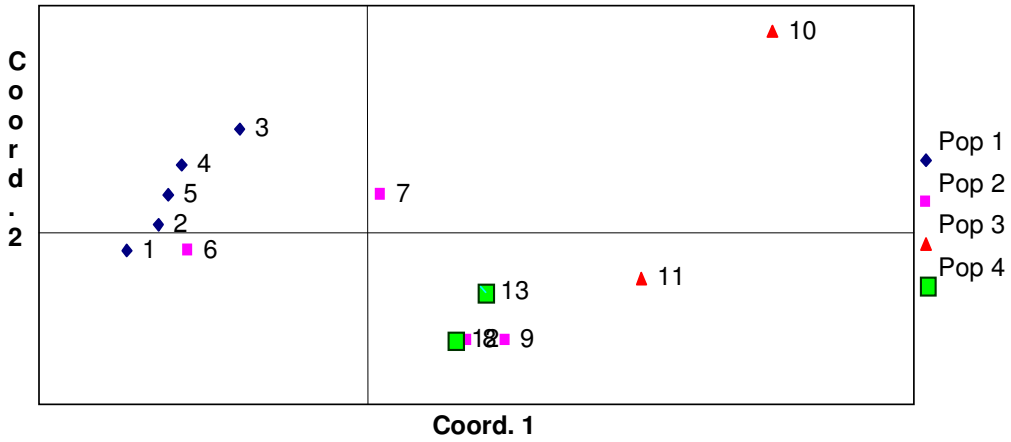

Figure 2.2. ISSR.

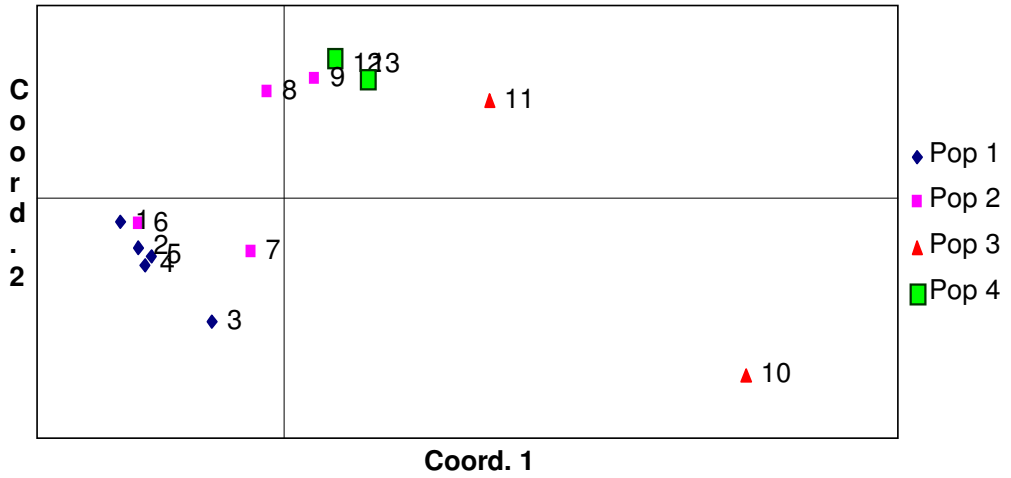

Figure 2.3. RAPD + ISSR

Figure 2. Two-dimensional plot of principal component analysis of thirteen elite *Jatropha* genotypes using **(2.1)** RAPD, **(2.2)** ISSR and **(2.3)** RAPD + ISSR analysis. The numbers plotted represents individual cultivars (where, Pop1 = Uttaranchal, Pop 2 = Rrajasthan, Pop 3 = Orissa, Pop 4 = Uttar Pradesh).

meters are as the mean of both the marker data.

Comparative analysis of RAPD with ISSR markers

RAPD markers were more efficient than the ISSR assay with regards to polymorphism detection, as they detected 84.26% as compared to 76.54% for ISSR markers. Also, the average number of polymorphic bands per primer and total number of polymorphic bands are more for RAPD (4.55 and 91 respectively) than for ISSR (4.42 and 62 respectively) (Table 4). But, resolving power (Rp), average number of bands per primer, Nei's genetic diversity (h), Shannon's Information Index (I), total heterozygosity (Ht), average heterozygosity (Hs) and gene flow (Nm) estimates were more for ISSR (7.098, 5.79, 0.245, 0.374, 0.244, 0.137 and 0.635, respectively) than RAPD markers (5.669, 5.35, 0.225, 0.359, 0.225, 0.115 and 0.518, respectively). The regression test between the two Nei's genetic diversity index was performed. This resulted in r^2 = 0.3318, showing low regression between RAPD and ISSR based similarities. Regression for ISSR and ISSR + RAPD combined data is moderate (0.6027), while it is maximum for RAPD and ISSR + RAPD based similarities (0.9125). This shows that RAPD data is more close to ISSR + RAPD combined data (Table 7).

In *J. curcas*, genetic similarity was calculated from the Nei's similarity index value considering ISSR and RAPD approaches individually as well as together. Based on ISSR marker system, the similarity index values ranged from 0.46 to 0.90 while that on the basis of RAPD markers ranged from 0.38 to 0.91. Similarity indices values based on both the marker systems together ranged from 0.41 to 0.89 indicating more diversity in case of RAPD. Clustering of genotypes within groups was not similar when RAPD and ISSR derived dendrogram were compared, whereas the pattern of clustering of the genotypes remained more or less the same in ISSR and combined data of RAPD + ISSR. But all these values are not significantly different from each other. Therefore, both ISSR and RAPD markers are equally efficient marker system in *J. curcas* because of their capacity to reveal several informative bands in a single amplification.

DISCUSSION

Polymorphism in a given population is often due to the existence of genetic variants represented by the number of alleles at a locus and their frequency of distribution in a population. Heterozygosity corresponds to a probability that two alleles taken at random from a population can be distinguished using the marker in question. Thus a convenient quantitative estimate of marker utility and the polymorphism detected can be given in terms of the Nei's geneitic diversity (h), Shannon's information index (I), total heterozygosity (Ht), average heterozygosity (Hs), coefficient of population differentiation (Gst) and estimate of gene flow (Zhao et al., 2006).

Table 7. Regression analysis between Nei's genetic diversity as obtained from RAPD, ISSR and RAPD + ISSR combined data.

Marker combinations	R^2 (Nei's geneic diversity)
RAPD and ISSR	0.3318
RAPD and RAPD + ISSR	0.9125
ISSR and RAPD + ISSR	0.6027

In this work we compared the applicability of ISSRs and RAPDs as genetic markers to characterize the thirteen *J. curcas* genotypes. However, no such reports on genetic diversity using molecular markers were availa-ble in the genus *Jatropha*. In the present study, an attempt has been made to examine the level of genetic variation within. *curcas*. Accessions from Rajasthan and Uttaranchal were obtained from germplasm collection center at NBPGR (N. Delhi), and local nurseries at Gorakhpur (Uttar Pradesh) and Sambhalpur (Orissa). The *J. curcas* accessions were selected in order to represent different regions of the India where it is grown. The two marker systems, ISSR and RAPD used in the present study have also been used as effective tools to evaluate genetic diversity and to throw light on the phylogenetic relationships in different cultivated and uncultivated plants. The RAPD technique has been widely used both for studies on wild plants (Yeh et al., 1995; Khasa and Dancik, 1996) and for studies on culti-vated plants (Sharma and Dowsons, 1995; Ratnaparkhe et al., 1995). By contrast researches employing the ISSR technique have mainly focused on cultivated species (Moreno et al., 1998; Blair et al., 1999; Wang et al., 1998). In our case, we have observed a greater regression using RAPDs than ISSRs primers, probably due to the low number of cultivars analysed. The evolution of varieties in distinct agro-climatic zones demonstrates significant levels of variation in response to the selection pressure in the zones (Singh et al., 1998). It is, therefore, not surprising to find significant levels of polymorphism among the 13 genotypes of *J. curcas* with RAPD and ISSR markers. RAPD markers were more efficient than ISSR the assay, as they detected 84.26% polymorphism in *J. curcas* as compared with 76.54% for ISSR markers. This is in contrast to the results as obtained for several other plant species like wheat (Nagaoka and Ogihara, 1997) and vigna (Ajibade et al., 2000). The 20 RAPD and 14 ISSR primers in the present study yielded 153 polymorphic markers that unambiguously discriminated 13 genotypes into three clusters. The number of total polymorphic and discriminant fragments is higher for RAPD than ISSRs. In fact, the ISSRs have a high capacity to reveal polymer-phism and offer great potential to determine intra- and inter-genomic diversity as compared to other arbitrary primers like RAPDs (Zietkiewicz et al., 1994). Geographically isolated population accumulates genetic differences as they adapt to different environment. Genetic variation among elite genotypes of *J. curcas* based on RAPD and

ISSR analysis could be useful to select parents to be crossed for generating appropriate populations intended for both genome mapping and breeding purposes.

The regression between RAPD and ISSR Nei's genetic diversity value was low in magnitude. But this value is moderate for ISSR and ISSR+RAPD combined data while, it is very high for RAPD and ISSR+RAPD combined data. A possible explanation for the difference in resolution of RAPDs and ISSRs is that the two-marker techniques target different portions of the genome. The ability to resolve genetic variation among different genotype may be more directly related to the number of polymorphisms detected with each marker technique rather than a function of which technique is employed. Studying the sampling variance of heterozygosity and genetic distance estimates, Nei (1978) reported that a relatively reliable estimate of average heterozygosity can also be obtained from a small number of individuals if a large number of loci are examined. The number of polymorphisms detected among genotypes influences the standard errors of the genetic diversity estimates. There was some consensus between the RAPD and ISSR based grouping of the 13 genotypes.

With this study we can conclude that the molecular analyses of RAPD and ISSR markers were extremely useful for studying the genetic relationships between Jatropha cultivars, providing both ISSR and RAPD markers a powerful tool for the generation of potential fingerprinting diagnostic markers for cultivars. Also the phylogenetic analysis on the basis of RAPD and ISSR-derived dendrogram supports the fact that region specific variations are there, which is because of the multiple generations of selection carried out after their introduction. Both RAPD and ISSR markers might detect non-coding, and therefore, more polymorphic DNA by exploiting the different regions of the genome. The differences found among the dendrograms generated by RAPDs and ISSRs could be partially explained by the different number of PCR products analysed (735 for RAPDs and 646 for ISSRs) reinforcing again the importance of the number of loci and their coverage of the overall genome, in obtaining reliable estimates of genetic relationships among barley cultivars. Similar results have been observed by Loarce et al. (1996) in barley. Another explanation could be the low reproducibility of RAPDs (Karp et al., 1997).

The differences in clustering pattern of genotypes using RAPD and ISSR markers may be attributed to marker sampling error and/or the level of polymorphism detected, reinforcing again the importance of the number of loci and their coverage of the overall genome in obtaining reliable estimates of genetic relationships among cultivars (Loarce et al., 1996). The putatively similar bands originating for RAPDs in different individuals are not necessarily homologous, although they may share the same size in base pairs. This situation may lead to wrong results when calculating genetic relationships (Fernandez et al., 2002). Dendrograms in the present study did not indicate very clear pattern of clustering according to the location in which they were collected. Similar results were obtained in Azukibean (Fernandez et al., 2002) and groundnut (Dwivedi et al., 2001). The genetic closeness among the Uttaranchal cultivars can be explained by the high degree of commonness in their genotype. Six genotypes grouped in cluster I (RAPD + ISSR dendrogram) of which five are from Uttaranchal and one from Rajasthan. Similarly cluster I of ISSR marker too contain all the five genotypes from Uttaranchal and two genotypes from Rajasthan. Cluster derived II derived from ISSR and ISSR + RAPD marker data contain the genotypes from Rajasthan, Uttar Pradesh and Orissa. III cluster is unique as it contains only one genotype from Orissa (Orissa 6). Similar results were observed in blackgram (Gaffor et al., 2001). In all the dendrograms, Orissa-6 was represented as an OTU. The lowest genetic similarity of Orissa-6 with other genotypes is probably associated with their difference in the amplified region. The level of observed polymorphism is very high, and the ability of the ISSR technique to effectively distinguish species in the genus Vigna was reported by Ajibade et al. (2000). The primers with poly (GA)n and poly (AG)n motifs produced more polymorphism than any other motif. While primers with (AT)n, (GT)n and other motifs (Table 2 and 3) did not gave any amplification. Somewhat similar results were also reported by Ajibade et al. (2000) where, they found that the primer containing the CT repeats was one of those, which did not give interpretable phenotype analyzed, while primers with GA and CA repeats revealed polymorphism in the genus Vigna.

Accessions with the most distinct DNA profiles are likely to contain the greatest number of novel alleles. Our results indicate the presence of great genetic variability among elite genotypes of *J. curcas*. ISSR and RAPD markers are useful in the assessment of *J. curcas* diversity, the detection of duplicate sample in germplasm collection, and the selection of a core collection to enhance the efficiency of germplasm management for use in *J. curcas* breeding and conservation. Our results indicate these markers have great potential to discriminate between the thirteen genotypes. In ISSR and ISSR+RAPD combined marker data the UPGMA dendrograms, we obtained the almost same sample distributions while dendrogram from RAPD is little different. Despite the great and similar discriminating power of both markers, some differences between the two could be detected: (1) genetic similarity values lower for ISSRs than for RAPDs, when different genotypes were compared (2) a number of total, polymorphic fragments higher for RAPDs than ISSRs. Although further investigations should be made to verify such a preliminary observation, one possible explanation could be found in the different background on which such differentiation estimates were based. In fact the inter-simple sequence repeats are regions lying within the microsatellite repeats, have a high capacity to reveal polymorphism and offer great potential to determine intra-genomic and inter-genomic

diversity as compared to other arbitrary primers, like RAPDs (Zietkiewicz et al., 1994). Such charac-teristics, which were confirmed in the present study, makes the both the markers as better potential tool to carry out future population genetics studies. Problems of the reliability and repeatability of RAPD markers are well known. Nagaoka and Oigihara (1997) in their studies found that ISSR primers, compared with RAPD primers, produce more reliable and reproducible bands. However, in our experiments, once the PCR conditions were well set up, we obtained a high reproducibility for both RAPD and ISSR markers; only very faint fragments were not reproducible and such fragments were discarded. As previously pointed out, during the ISSR screening we obtained good amplification products from primers based on (GA)*n* and (AG)*n* repeats while (AT)*n* and some other primers gave no amplification products (Table 2 and 3), despite the fact that poly (AT) dinucleotide repeats are thought to be the most abundant motifs in plant species (Martín and Sánchez-Yélamo, 2000; Morgante and Olivieri, 1993). Similar results were obtained in grapevine (Moreno et al., 1998), rice (Blair et al., 1999) and wheat (Nagaoka and Ogihara, 1997). A possible explanation of these results is that ISSR primers based on AT motifs are self-annealing, due to sequence complementarity, and would form dimmers during PCR amplification (Blair et al., 1999). Reason behind non amplification of other repeats may be their absence in the genome.

The results of the present study can be seen as a starting point for future researches aimed at defining the level of intra- and inter-specific genetic diversity and to detect hybrids among these species. For this purpose, a larger number of natural populations of the three species collected from the whole distribution area should be analysed and additional primers tested. Furthermore, in order to design new diagnostic primers more effective in genetic discrimination among genotypes, discriminant bands could be cloned and sequenced. These studies have given important clues in understanding genotype relationship, which may further assist in developing and planning breeding strategies.

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