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Test of equivalence of PCR-based marker systems in assessing genetic variability and molecular characterization of *Jatropha curcus*: A case study

Narendra Singh Yadav, Arvind Shukla, Yogesh Ruwali, Debjyoti Sen Gupta*[1]

Department of Genetics & Plant Breeding, College of Agriculture, G.B. Pant University of Agriculture & Technology, Pantnagar 263 145; [1]Present Address: North Dakota State University (NDSU), Harris Hall 222, 1250 Bolley Drive, Fargo, ND-58108-6050, USA

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Abstract

PCR-based molecular markers, Random Amplified Polymorphic DNA (RAPD), Inter-Simple Sequence Repeat (ISSR) and Simple Sequence Repeat (SSR) used for genetic characterization in *Jatropha* revealed 100, 98.68 and 97.47 per cent polymorphism, respectively. The highest genetic correlation according to combined RAPD, ISSR, and SSR data was between genotypes PKVJ-DHW-1 and P.J.-03004 (83.7%), while the lowest similarity was found between genotypes TNMC-2 and TNMC-4. The dendrogram of combined RAPD, ISSR and SSR data grouped genotypes into nine major clusters. Each marker grouped TNMC-2 into unique cluster. Cophenetic correlation (r) for RAPD, ISSR, SSR and their combination were 0.9873, 0.9840, 0.7769 and 0.9886, respectively, indicating very good fit of the cluster analysis and showed significant correlation between similarity matrix and cluster analysis. The PCoA for RAPD, ISSR, SSR and combined markers also resulted in similar relationship between the score plots and the pattern of genetic diversity estimated by the UPGMA cluster analysis. Results clearly indicated that the combination of ISSR vs. SSR (r = 0.4835) would be more effective for genetic diversity analysis than other combinations. Thus, bulk analyses of RAPD, ISSR and SSR-PCR markers provides a quick, reliable and highly informative system for DNA fingerprinting in *Jatropha* and also permit to establish genetic relationships which agree with, by other means, known origin of the *Jatropha* cultivars.

Key words: Biofuel, molecular markers, RAPD, ISSR, SSR, *Jatropha curcus*

Introduction

Jatropha genus comprises approximately 200 succulent plants, shrubs and trees from the family Euphorbiaceae [1]. This shrub has originated in Latin America but presently grows throughout the arid, semi-arid tropical and subtropical regions of the world [2-4]. *Jatropha* seeds contain 46-58% of oil kernel weight and 30-40% on seed weight [5, 6]. It has the potential use as bio-diesel [7] to cater the energy needs of the industrial sector without compromising the agricultural field output. *Jatropha* being a cross pollinated plant is expected to have a high level of genetic variation and therefore, selection of superior types is possible. Since selection depends on the existing degree of genetic variation, plant breeders have the *prima facie* to determine the extent of genetic variability in *Jatropha curcus*.

Molecular marker based techniques are reliable to detect extent of genetic variability in *Jatropha* without any influence of environmental variability [1, 12, 13]. However, the most efficient PCR based marker technique has not yet been reported for this species. We compared here three molecular marker techniques *viz*., RAPD (Randomly Amplified Polymorphic DNA), ISSR (Inter-Simple Sequence Repeat) and SSR (Simple Sequence Repeat). Knowing about the success of molecular markers in revealing genetic diversity in other crop plants, a study was conducted to compare molecular markers in *Jatropha curcus* to test transferability of SSR markers from other crops to *Jatropha*, to assess the extent of genetic variability of the test genotypes, to explore the potential of these markers in varietal identification and to compare the different marker systems to select for the best one or combination thereof in case of *Jatropha*.

*Corresponding author's e-mail: debgpb@gmail.com
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Materials and methods

Twenty *Jatropha curcus* L. genotypes, namely, 1. IGAU Raipur, 2. IGAU Bilaspur, 3. IGAU Surzuja from Madhya Pradesh (M.P.), 4. TNMC-2, 5. TNMC-3, 6. TNMC-4, 7. TNMC-5, 8. TNMC-7 and 9. TNMC-22 from Coimbatore, Tamilnadu, 10. Sagar (SFRI), 11. Indore (SFRI), 12. TFRI-1, 13. TFRI-2 from Jabalpur, (M.P.), 14. Pant J. Sel-1, 15. Pant J. Sel-2 from Pantnagar, Uttarakhand, 16. RJ-117 from Rahuri, Maharastra, 17. PKVJ-MKV - 1 and 18. PKVJ-DHW-1, from Akola, Maharastra, 19. PJ-03004 (LC-I) and 20. PJ-03031 (LC-II) from, Pantnagar, Uttarakhand belonging to different locations were used in the present study. Each genotype was reduced to a pool of 20 plants, and 100 mg of young leaves from each plant were used to create the pool. The method described by Dellaporta et al. [15] was followed for extraction of genomic DNA. Further purification by RNase and quantification was done by taking the absorbance on Genesys UV spectrophotometer.

Primers used in the PCRs

A total of 35 decamer RAPD primers were tested for polymorphism and among these 20 polymorphic RAPDs (Table 1) were used for diversity analysis. For ISSR, 24 primers based on dinucleotide, tetranucleotide or pentanucleotide repeats were tested and out of which 19 polymorphic ones (Table 2) were used to genotype test cultivars. Out of 40 rice SSR markers, 17 SSR primer pairs (Table 3) amplified and were polymorphic which subsequently used for diversity analysis.

PCR conditions

The protocol for RAPD analysis was adapted from Williams *et al*. [16]. The reactions were carried out in a 25-µl volume PCR tube. 20-µl master mix was prepared individually for each marker type and DNA amplifications were performed in 96 well plates PTC-100 thermocycler (MJ Research Thermocycler) with 35 cycles of 60 s at 94^{o}C, 60 s at 36^{o}C and 2 min at 72^{o}C and for ISSRs and SSR the cycles were 40 and 35 with TA at 51^{o}C and 54^{o}C respectively. Samples of 10µl PCR products were analysed on 1.4% agarose gel in 0.5X TBE buffer running at 50 V for 4 hrs. The gels were stained using ethidium bromide solution. For ISSR and SSR agarose concentration was kept 1.8 and 2.0%, respectively, whereas rest of the procedure was common.

Molecular marker data analysis

RAPD and ISSR bands were scored for the presence (1) or absence (0) of homologous bands for all cultivars. SIMQUAL program was used to calculate Jaccard's similarity coefficients. A common estimator of genetic identity was calculated as Nab/ (NAB + NA + NB). The dendrograms were constructed by UPGMA cluster analysis using NTSYS-pc V 2.11 [17].

Results and discussion

RAPD amplification

Twenty genotypes were genotyped with a total of thirty five primers, out of which 20 were found polymorphic. The average expected gene diversity, PIC, unique band, per cent polymorphism, polymorphic bands, monomorphic bands, total bands and amplified product range obtained by each primer are shown in Table 1. These 20 primers showed a total of 158 reproducible bands. Each of the primer varied greatly in their ability to resolve variability among the genotypes. The individual primer produced bands in a range of 4 (LC-77, LC-84, and LC-96) to 22 (LC-89) with an average of 7.9 bands per primer. All the bands were found polymorphic. Out of 20 primers, five namely, LC-72 for genotypes IGAU-Raipur, LC-76 for TNMC-4 and TNMC-22, LC-87 for genotype TNMC-7, LC-89 for PJ-03031 and LC-95 for genotype PKVJ-MKV-1 gave a total of six unique bands, which can be converted into STS markers with a potential application for identification of cultivars in varietal seed mixtures.

Average expected gene diversity

The average expected gene diversity ranged from 0.20 for primer LC-89 followed by 0.28 for primers LC-76 and LC-87 to a maximum of 0.47 for primer LC-93 (Table 1). The unweighted pair group method with arithmetic average (UPGMA) cluster analysis method was followed for the construction of the phylogenetic tree (Fig. 1). The results showed clear distinction among all 20 genotypes by grouping the genotypes into seven clusters at 75% similarity. Clustering pattern indicated that the genotypes in any cluster are genetically diverse from the genotypes in other clusters. Also principal coordinate analysis was performed in order to highlight the resolving power of ordination. The high level of polymorphism detected by the tested RAPD primers in this study is similar with the other reports in *Jatropha* as well as other perennial species [1, 18-20].

ISSR amplification

Out of 24 primers, 19 were found polymorphic to differentiate all the genotypes. A total of 152 bands were detected using 19 ISSR primers out of which 150 were

Table 1. Details of RAPD analysis

S.No.	Primer code	Primer sequence	Ampified product range (bp)	Unique band	PIC	Average expected gene diversity (H_i)
1	LC-71	TGCCGAGCTG	350-3050	-	0.19	0.46
2	LC-72	AGTCAGCCAG	350-3000	1	0.39	0.39
3	LC-73	AATCGGGCTG	275-3050	-	0.31	0.37
4	LC-75	GAAACGGGTG	350-3000	-	0.49	0.32
5	LC-76	GTGACGTAGG	250-2350	2	0.55	0.28
6	LC-77	GGGTAACGCC	800-1255	-	0.05	0.34
7	LC-78	GTGATCGCAG	450-3100	-	0.36	0.41
8	LC-80	CAGCACCCAC	400-3050	-	0.08	0.38
9	LC-84	AGGTGACCTG	850-2800	-	0.05	0.34
10	LC-87	AGGTGACCGT	450-2550	1	0.65	0.28
11	LC-89	AGTCAGCCAC	100-4000	1	0.60	0.20
12	LC-90	GTGAGGCGTC	650-2850	-	0.32	0.33
13	LC-91	TGGACCGGTG	250-1903	-	0.26	0.39
14	LC-93	GGACCCAACC	1185-1658	-	0.20	0.47
15	LC-94	GTCGCCGTCA	220-3000	-	0.20	0.41
16	LC-95	TGAGCGGACA	230-3500	1	0.52	0.30
17	LC-96	TTGGCACGGG	1090-2850	-	0.23	0.30
18	LC-97	GTGTGCCCCA	330-1903	-	0.29	0.42
19	LC-99	AGCGCCATTG	380-3570	-	0.33	0.38
20	LC-101	GGGGTGACGA	385-2250	-	0.14	0.44

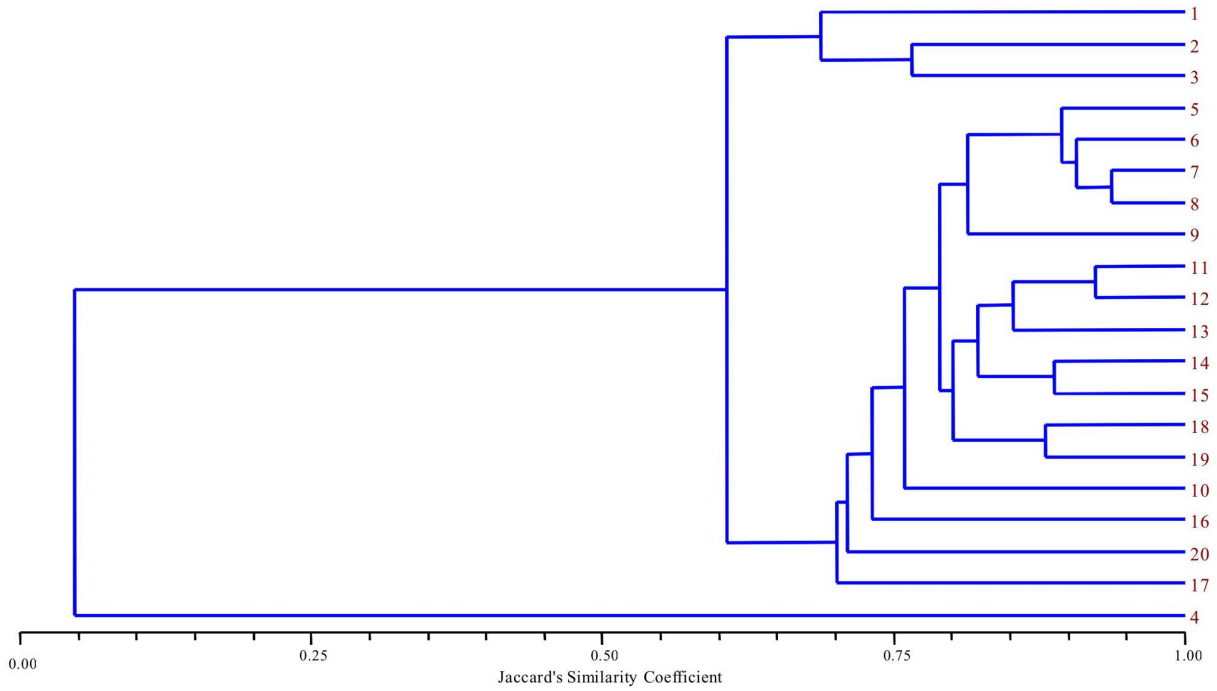

Fig 1. UPGMA dendrogram showing clustering of 20 genotypes of *Jatropha curcus* based on 20 RAPD primers

polymorphic and only two were monomorphic (Table 2). The number of amplified bands varied from one in primers LC-57 and LC-58 to a maximum of 13 in primers, LC-46, LC-48, LC-62 and LC-63 with an average of 8.0 bands per primer. The PIC values ranged from 0.08 for primer LC-55 to as high as 0.75 for primer LC-57, with an average of 0.33 for all the 19 primers (Table 2). One primer, LC-62, produced unique amplification for PJ-03031.

Average expected gene diversity

The lowest average expected gene diversity was recorded for primer LC-57(0.23) followed by primer LC-58 (0.27) and the highest for primer LC-65 (0.46). The mean value for all the ISSR primers was 0.36 over all the genotypes showing high level of average expected gene diversity among the genotypes (Table 2). Jaccard's similarity coefficients were estimated using nineteen ISSR primers. Genotype TNMC-2 showed least similarity coefficient with other genotypes. All 20 genotypes were clearly separated by dendrogram. The genotypes were grouped into six clusters based on similarity coefficients at 75% (Fig. 2).

SSR amplification

Though in case of Euphorbiaceous plants like castor, manihot and rubber tree, SSRs have already been reported but to check the transferability of rice SSR primers with the *Jatropha*, these SSR primers were used in the experiment as today huge number of rice SSR primers are available compared to mentioned euphorbiaceous genera. Out of 40 rice SSR primers screened, 17 were polymorphic revealing 119 bands in 20 genotypes of which 116 bands were found to be polymorphic in nature with 97.47% polymorphism. All the primers gave 100% polymorphism except, LC304 and LC321, which geve 87.5 and 70 % polymorphism. PIC values of most of ISSR and RAPD primers are poor, but that of SSR primers from rice are high revealing the success of rice SSR used in the present study.

The number of bands per primer varied widely among these primers, ranging from one (LC-298) to 16

Table 2. Details of ISSR analysis

S.No.	Primer code	Primer sequence	Ampified product	PIC	Average expected range (bp) gene diversity (H_i)
1	LC-46	AGAGAGAGAGAGAGAGT	400-3500	0.18	0.34
2	LC-48	AGAGAGAGAGAGAGAGG	100-3500	0.15	0.37
3	LC-49	GAGAGAGAGAGAGAGAT	300-3600	0.20	0.33
4	LC-51	GAGAGAGAGAGAGAGAA	325-2950	0.23	0.31
5	LC-52	CTCTCTCTCTCTCTCTG	425-2800	0.51	0.30
6	LC-53	TCTCTCTCTCTCTCTCC	420-1150	0.10	0.38
7	LC-54	TCTCTCTCTCTCTCTCG	750-3000	0.37	0.44
8	LC-55	ACACACACACACACACT	500-2800	0.08	0.39
9	LC-56	ACACACACACACACACG	350-2400	0.17	0.42
10	LC-57	TGTGTGTGTGTGTCTGA	1185	0.75	0.23
11	LC-58	AGAGAGACAGACAGAGYA	800-1400	0.70	0.27
12	LC-59	GAGAGAGAGAGAGAGAYT	275-1500	0.32	0.42
13	LC-60	TCTCTCTCTCTCTCTCRT	200-3000	0.60	0.29
14	LC-62	HBHAGAGAGAGAGAGAG	280-3000	0.20	0.42
15	LC-63	BHBGAGAGAGAGAGAGA	200-2500	0.16	0.32
16	LC-64	VHVGTGTGTGTGTGTGT	375-1600	0.38	0.30
17	LC-65	HVHTGTGTGTGTGTG	375-2500	0.26	0.46
18	LC-67	ATGATGATGATGATGATG	450-2900	0.44	0.40
19	LC-68	CTCCTCCTCCTCCTCCTC	450-3200	0.48	0.41

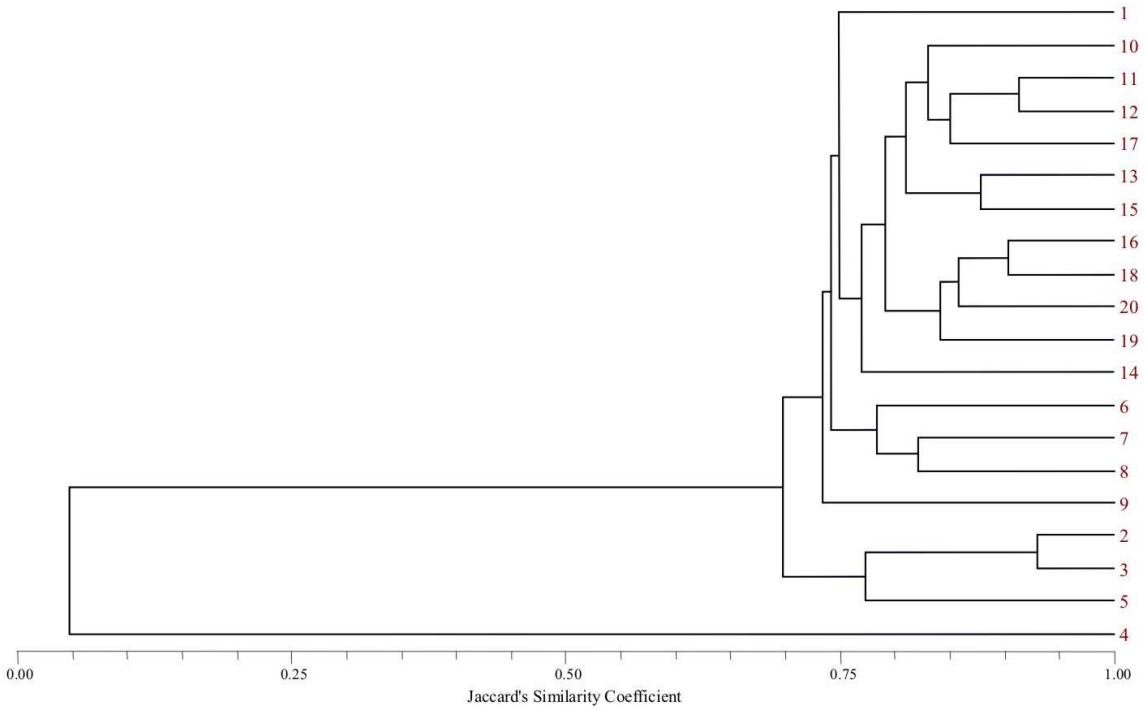

Fig. 2. UPGMA dendrogram showing clustering of 20 genotypes of *Jatropha curcus* based on 19 ISSR primers

(LC-304). The average number of bands per primer was 7.0 per primer. PIC values ranged from 0.05 for primer LC-298 to 0.95 for primer LC-313 with an average of 0.66 for all the primers. Table 3 gives detail of SSRs used in the study. Twelve primer pairs produced 25 unique amplifications for one or the other test cultivars.

Average expected gene diversity

The expected gene diversity ranged from 0.04 for primer LC-297 followed by 0.05 for primer LC-313 to a maximum of 0.38 for primer LC-310 followed by 0.35 for primer LC-298 with an average of 0.19 for all the primers used in the study among all the genotypes at all loci (Table 3). A dendrogram was constructed using SAHN module NTSYS-pc following UPGMA method, and a total of five clusters were constructed at 50% similarity coefficient (Fig. 5). The value of Jaccard's similarity coefficient ranged from 23.2% to a maximum of 71.7% revealing sufficient variability among the genotypes. Genotype TNMC-2 can be separated from other cluster even at 32.5% similarity coefficient value. Principal coordinate analysis was also performed in order to highlight the resolving power of ordination.

Combined analysis of RAPD, ISSR and SSR markers

The combined allelic diversity data set using three different PCR based molecular markers (RAPD, ISSR, and SSR) resulted in 429 bands from 56 primers with an average of 7.66 bands per primer. Out of all primers used in the study, 18 resulted in 32 unique bands (six by RAPD, one by ISSR and 25 by SSR primers) by amplifying single genotype.

Similarity coefficient

The Jaccard's similarity coefficients were estimated using pooled markers diversity data. The range of coefficient varied from 8.5% to a maximum of 83.7%. Genotype TNMC-2 showed lower similarity coefficient value with rest of the genotypes (Fig. 4). The phylogenetic tree constructed through SAHN module of NTSYS-pc using UPGMA method revealed nine major clusters at 75 % similarity. Genotype TNMC-2 was clustered into separate group even at 10% similarity. Thus combined analysis of markers again grouped genotypes into different clusters on the basis of variability present among the genotypes and showed that sufficient variability is present among the genotypes. Principal coordinate analysis was also performed in order to highlight the resolving power of ordination (Fig. 5).

Matrix comparison and Mantel test

Matrix comparison was performed using Mx COMP programme of NT SYS-pc v 2.11 to calculate the

Table 3. Details of SSR analysis

S.No.	Primer code		Primer sequence	Ampified product range (bp)	Unique band	PIC	Average expected gene diversity (H_i)
1	LC-291	RM11-F R	TCTCCTCTTCCCCCGATC ATAGCGGGCGAGGCTTAG	80-1000	1	0.71	0.24
2	LC-292	RM13-F R	TCCAACATGGCAAGAGAGAG GGTGGCATTCGATTCCAG	90-500	-	0.55	0.28
3	LC-295	RM17-F R	TGCCCTGTTATTTTCTTCTCT GGTGATCCTTTCCATTTCA	140-800	1	0.84	0.15
4	LC-297	RM19-F R	CAAAAACAGAGCAGATGAC CTCAAGATGGACGCCAAGA	100-500	1	0.46	0.04
5	LC-298	RM20-F R	ATCTTGTCCCTGCAGGTCAT GAAACAGAGGCACATTTCATT	200	-	0.05	0.35
6	LC-304	RM26-F R	GAGTCGACGAGCGGCAGA CTGCGAGCGACGGTAACA	90-2500	-	0.42	0.28
7	LC-306	RM29-F R	CAGGGACCCACCTGTCATAC AACGTTGGTCATATCGGTGG	350-1700	3	0.93	0.07
8	LC-310	RM34-F R	GAAATGGCAATGTGTGCG GCCGGAGAACCCTAGCTC	100-2300	-	0.45	0.38
9	LC-312	RM36-R F	CAACTATGCACCATTGTCGC GTACTCCACAAGACCGTACC	80-600	1	0.84	0.15
10	LC-313	RM38-F R	ACGAGCTCTCGATCAGCCTA TCGGTCTCCATGTCCCAC	75-200	2	0.95	0.05
11	LC-314	RM39-F R	GCCTCTCTCGTCTCCTTCCT AATTCAAACTGCGGTGGC	90-1875	3	0.85	0.13
12	LC-315	RM41-F R	AAGTCTAGTTTGCCTCCC AATTTCTACGTCGTCGGGC	90-3000	-	0.75	0.23
13	LC-318	RM47-F R	ACTCCACTCCACTCCCCAC GTCAGCAGGTCGGACGTC	150-2500	2	0.63	0.29
14	LC-319	RM48-F R	TGTCCCACTGCTTTCAAGC CGAGAATGAGGGACAAATAACC	150-900	6	0.87	0.12
15	LC-321	RM50-F R	ACTGTACCGGTCGAAGACG AAATTCCACGTCAGCCTCG	80-1400	1	0.39	0.18
16	LC-325	RM103F	CTTCCAATTCAGGCCGGCTGGC CGCCACAGCTGACCATGCATGC	900-1500	3	0.93	0.06
17	LC-326	RM104-F R	GGAAGAGCAGAGAAAGATGTG TCAACAGACACACCGCCACCGC	80-2500	1	0.53	0.23

cophenetic correlation between the similarity matrix and the cophenetic value matrix. The cophenetic correlation (r) was also used to measure the goodness of fit for different cluster analysis. Significance of cophenetic correlation (r) was tested using t-test. The estimated cophenetic correlation (r) for RAPD was 0.9873, for ISSR it was 0.9840 while for SSR its value was 0.7769 indicating good fit of the cluster analysis and showed significant correlation between similarity matrix and cluster analysis. The relationship between three matrices is depicted in Table 4. For combined molecular markers the cophenetic correlation, r, was equal to 0.9886 and thus indicated very good fit of the cluster generated using SAHN UPGMA method with the similarity matrix. The relationship between two matrices is depicted in Table 4.

Comparison of tested marker systems

The relative efficiency of a marker was assessed by correlating the genetic similarity measures of different

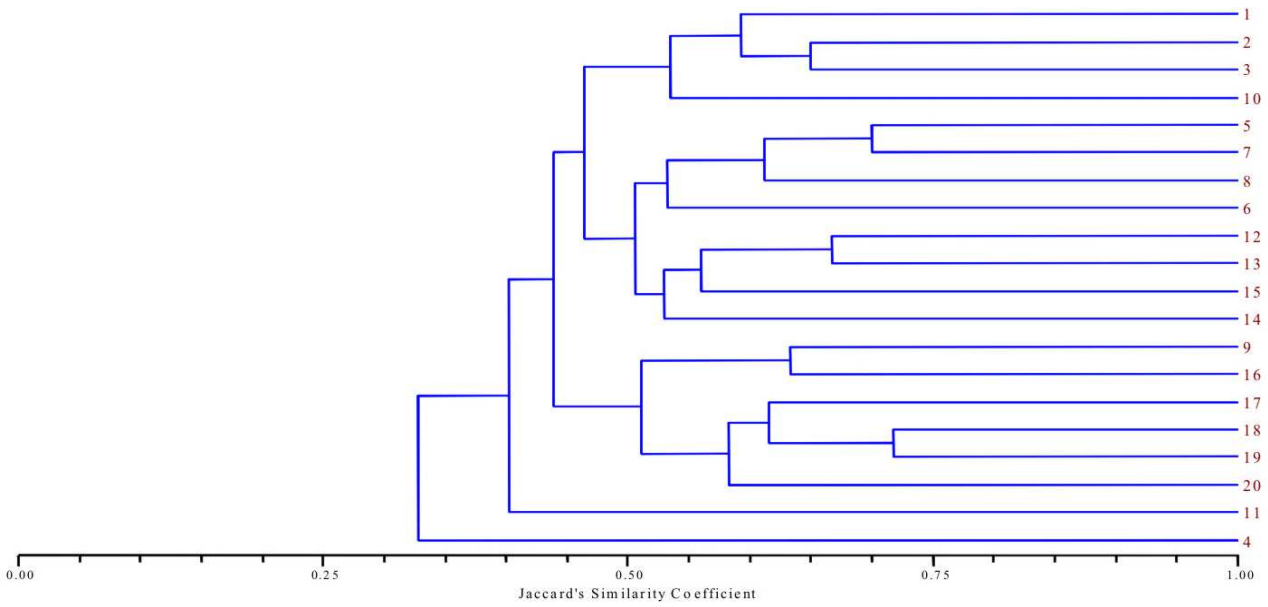

Fig. 3. UPGMA dendrogram showing clustering of twenty genotypes of *Jatropha curcus* based on seventeen SSR primers

markers. The similarity coefficient of twenty genotypes measured through different molecular markers data (RAPD, ISSR and SSR) were similar in other studies involving a few other perennial species [13, 21, 22], were subjected to calculate the Pearson's correlation coefficient (Table 4). Among the molecular markers highest Pearson's correlation value (r = 0.9259) was recorded between RAPD and ISSR, which suggested that these two markers are concordant to each other in terms of the genetic diversity produced. It means that these two markers are giving polymorphism at same loci for the same genotypes i.e. genetic diversity revealed using these two markers is more or less similar. Thus, either of these two markers can be used for studying the genetic diversity among *Jatropha curcas* genotypes. The RAPD vs. SSR markers produced the second highest correlation value (r = 0.5136) followed by ISSR vs. SSR (r = 0.4835). But both of these values are not very high, thus, it can be concluded that for studying genetic variability among *Jatropha curcas* genotypes, these markers should be used in combination to arrive at any conclusion on genetic variability. It means that RAPD should be used with SSR and ISSR. The low value of Pearson's correlation between these markers indicates that they are giving polymorphism at different loci in the same genotype. It was observed that the involvement of RAPD markers increases the Pearson's correlation coefficient with either of the two markers (ISSR and SSR). The close association between RAPD and ISSR in comparison to RAPD and SSR suggested that the combination of RAPD and SSR markers would be more effective for genetic diversity analysis. It was revealed in the markers amplification that at any level of similarity (e.g. at 75%

Table 4. Pearson's correlation coefficient (r) and Mantel t-test

S. No.	Marker system	r	t	Probability
1.	RAPD vs. SSR	0.5136	3.4554	0.9997
2.	RAPD vs. ISSR	0.9259	4.2318	1.0000
3.	SSR vs. ISSR	0.4835	3.2420	0.9994
4.	Combined molecular markers vs. RAPD	0.9751	4.4779	1.0000
5.	Combined molecular markers vs. SSR	0.5888	3.9642	1.0000
6.	Combined molecular markers vs. ISSR	0.9766	4.4673	1.0000

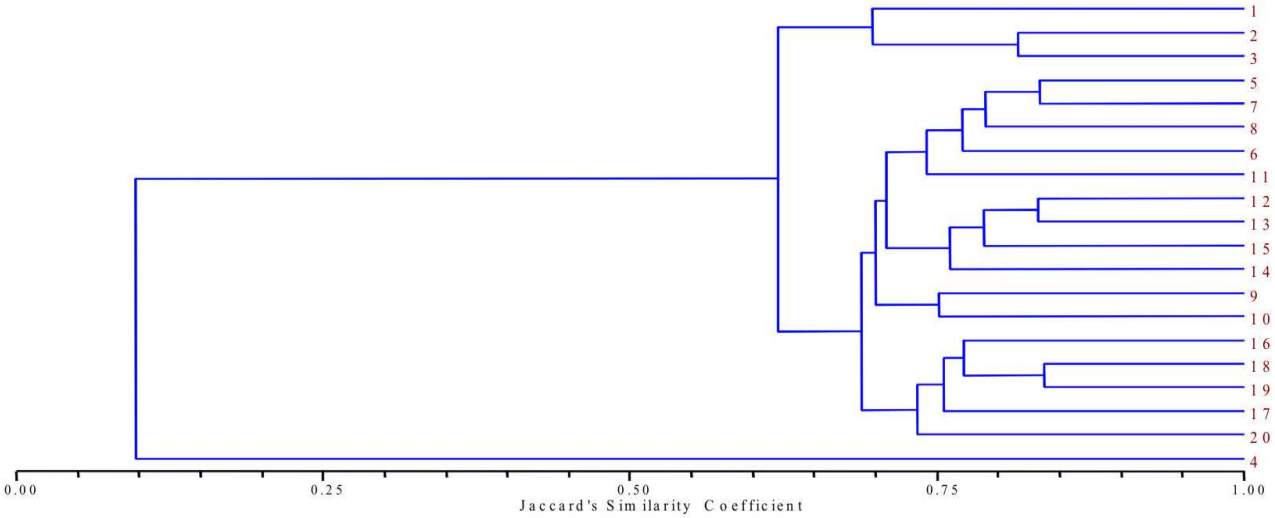

Fig. 4. UPGMA dendrogram showing clustering of twenty genotypes of *Jatropha curcus* based on combined molecular marker data

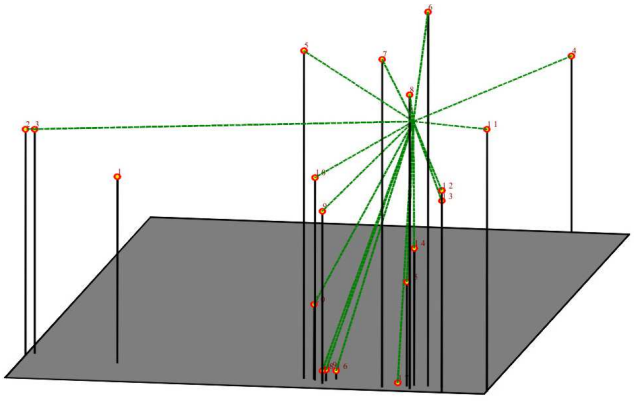

Fig. 5. Relationship among twenty genotypes of *Jatropha curcus* visualized by PCoA of combined molecular marker based genetic similarities

similarity level) RAPD resulted in seven clusters, ISSR resulted in six clusters and SSR resulted into 20 clusters among the 20 genotypes.

The pattern of cluster analysis was further confirmed by principal coordinate analysis (PCoA) for molecular markers. The results for different markers are presented in two and three-dimensional score plots. The PCoA for RAPD, ISSR, SSR and combined molecular markers also resulted in similar relationship between the score plots and the pattern of genetic diversity estimated by the UPGMA cluster analysis.

The total variation of first five principal components for RAPD, ISSR, SSR and combined molecular marker data was estimated as 64.54%, 65.17%, 48.02% and 55.68%, respectively. These results indicated variations between the markers for assessing the degree of genetic relationship between the clusters and the score plots. The clusters generated through the combination of three molecular markers (RAPD, ISSR, and SSR) were compared with the score plots generated by PCoA analysis. The first five components of combined molecular markers resulted in a cumulative variance of 55.68%, which showed relatively similar results with individual molecular markers.

Though technical simplicity of RAPD and ability to scan the whole genome has resulted in its use in the analysis of genetic relationships in several genera [22-24] it was found that SSR produced most specific clustering pattern followed by RAPD and ISSR. The clustering pattern obtained through combined molecular markers find its place in between SSR and RAPD. The results revealed that SSR markers came out with best discrimination among all the genotypes studied (highest number of unique bands and grouped the test cultivars into maximum number of clusters) for genetic diversity analysis, hence may be considered more efficient markers technique for *Jatropha curcas* and in this study seventeen SSR markers have been transferred from *Oryza sativa* to *Jatropha*.

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