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Correlating the phenotypic and molecular diversity in *Jatropha curcas* L.

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ABSTRACT

Thirty four *Jatropha* germplasm accessions, selected based on unique phenotypic traits from 180 accessions collected from diverse geographical regions were subjected to field evaluation and molecular analysis. The field evaluation using eight quantitative traits showed significant variation among the germplasm. The molecular analysis using 56 RAPD and 40 ISSR primers resulted in 7 and 8 clusters, respectively. The accession IC541633 from Bastar (Chattisgarh) emerged as the most diverse accession. An attempt has been made to correlate the clustering based on molecular data with the quantitative traits. There was partial correlation between the quantitative traits and molecular data. Interestingly, the diverse accessions according to molecular diversity were characterized by unique phenotypes. Time of flowering, inflorescence type and number, leaf colour and texture were the traits contributing to variation. These traits may be used in identification of diverse accessions during germplasm exploration surveys or short listing of accessions for crossing in *Jatropha* improvement programmes.

1. Introduction

Among the plant sources identified for biodiesel, *Jatropha* (*Jatropha curcas* L.) has gained a prominent place because of the quality of the biodiesel produced from the crop [1–3]. Despite its naturalization in all the tropics and sub-tropics, morphological variability was reported to be low [4]. Characterization of *J. curcas* germplasm using morphological and molecular markers revealed modest levels of genetic diversity in the germplasm available in India [5], China [6] and also around the world [7]. Most of the earlier studies on germplasm characterization using either morphological or molecular markers are based on random access to the material. However, there is a need to assess for trait based diversity based on phenotypic and genotypic variation. Information on the number of introductions being made and the genetic diversity of *J. curcas* germplasm available in India is rather limited [5] and [8]. With an objective to effectively tap the potential of this biofuel crop, efforts were made to collect the diverse germplasm accessions available in the country.

Molecular markers have been used to study the genetic diversity among the different species of *Jatropha*, using RAPD markers [9], to confirm hybridity of interspecific hybrids using RAPD and ISSR markers [10] to assess inter and intra-population variability using arbitrary primers [5], [8] and [11]. *In-situ* assessment of diversity using phenotypic traits has been reported [12]. Since lack of morphological variation is a major challenge in the genetic improvement of the crop an effort has been made to understand the genetic diversity through molecular markers and correlate it with the

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phenotypic features (both unique and quantitative traits) for facilitating the collection of diverse germplasm accessions. Assessment of genetic diversity among accessions was unsuccessful due to strong influence of environment on highly heritable traits like 100-seed weight, seed protein and oil content in *J. curcas* [13]. However, significant differences in the seed traits and growth characters, female: male ratio and seed yield have been reported by [14]. In this present study, germplasm accessions with unique phenotypic traits were identified. These accessions were subjected to field evaluation (quantitative traits) and molecular analysis using RAPD and ISSR markers to study the relatedness of phenotypic traits (both unique and quantitative traits studied) and genetic variation based on molecular markers.

2. Experimental

2.1. Plant material

A set of 34 accessions of *J. curcas* from diverse geographical regions, which exhibited unique phenotypic traits in the experimental site was selected from germplasm block of 180 accessions established in 2005 and 2006 at the National Bureau of Plant Genetic Resources, Regional Station, Hyderabad, India. The passport information and associated unique trait of each accession is presented in Table 1. The crop was cultivated as rainfed under North Telangana Zone of Andhra Pradesh. One-month-old rooted stem cuttings of 34 selected accessions were raised in polythene bags were field transferred into the pits (60 × 60 × 60 cm) containing 2 kg Farm Yard Manure along with N:P:K at the rate of 2:10:8 g, with a spacing of 2 × 2 m in rainy 2007. The stem cuttings were raised in a Randomized Block Design with three replications with three cuttings per replication. The latitude, longitude and altitude of the experimental site were 17°.37′.53″, 78°.47′.44″and 542 m MSL (Mean Sea Level) respectively. The soils were virgin containing red sandy loam type with pH of 7.2.The intercultural operations such as weeding, basin making and earthing up were carried out as per the requirement. The minimum mean air temperature during December to January (14 °C) and maximum temperature during the month of April to May fluctuated between 40 and 45 °C, while weather during the rest of the period was moderate enabling optimum growth and development of plants. Two peaks of rains were received, one during June to August through South-West monsoon which is assured and other peak during mid-October to December that was scanty. Overall, the mean annual precipitation ranged between 750 and 800 cm. The variation observed in eight quantitative traits *viz.*, plant height, crown spread, collar length, number of primary branches, petiole length, pedicel length, number of fruit clusters per plant and number of fruits per plant was recorded on two-year-old plants (Table 2). The seed oil content was estimated using Soxhlet method. The data was analyzed using free downloadable WASP2 statistical software [15].

2.2. DNA extraction

The total genomic DNA was extracted from younger leaves of three plants for each accession following the standard CTAB method with minor modifications [16]. 5 g of leaf tissue was ground in liquid nitrogen, then homogenized in 20 ml of extraction buffer (2% CTAB, 20 mmol dm^{-3} EDTA, 2% PVP, 1.4 mmol dm^{-3} NaCl, 100 mmol dm^{-3} Tris–HCl pH 8.0 and 1% β-mercaptoethanol) and incubated at 65 °C for 1 h. The supernatant was treated with RNase A (10 mmol dm^{-3}), incubated at 37 °C for 30 min and twice extracted with chloroform:isoamyl alcohol (24:1 vv^{-1}). The DNA was precipitated with isopropanol and washed twice with 70% ethanol. The pelleted DNA was air dried and re-suspended in 50 μl of sterile Millipore water and stored at −20 °C.

2.3. RAPD PCR-amplification

A total of 56 decamer primers from Operon kits—OPA to OPX (Operon technologies, Alameda, USA) were used for DNA amplification [17]. The PCR amplification reaction (10 μl) consisted of 2.5 ng of DNA, 1× PCR buffer (10 mmol dm^{-3} Tris pH 9.0, 50 mmol dm^{-3} KCl, 1.5 mmol dm^{-3} $MgCl_2$), 100 mmol dm^{-3} each of the four dNTPs, 0.4 mmol dm^{-3} 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. Regardless of the marker system used, all the PCR amplifications included a negative control (no DNA) to avoid erroneous interpretations.

2.4. ISSR PCR-amplification

Forty ISSR primers (UBC primer set No. 9, University of British Columbia, Canada) were used for the analysis. The PCR reaction mixture (10 μl) consisted of 2.5 ng of DNA, 200 mmol dm^{-3} of each of the four dNTPs, 1× PCR buffer (10 mmol dm^{-3} Tris pH 9.0, 50 mmol dm^{-3} KCl, 1.5 mmol dm^{-3} $MgCl_2$), 0.2 μl of 25 mmol dm^{-3} $MgCl_2$, 0.4 mmol dm^{-3} ISSR primer and 0.6 U *Taq* DNA polymerase (Bangalore Genei, India). PCR amplifications were performed in the 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 (Ta), 2 min elongation at 72 °C and final extension at 72 °C for 7 min. The amplified products were resolved on 1.7% gel and documented in a gel documentation system (Alpha Innotech Fluorchem).

2.5. Data analysis

Marker index for RAPD and ISSR markers was calculated in order to characterize each primer for its ability to detect polymorphic loci among the genotypes. It is the sum total of the polymorphism information content (PIC) values of all the markers produced by a particular primer. PIC value was calculated using the formula $PIC = 1 - \sum pi^2$, where *pi* is the frequency of the ith allele [18]. Genetic similarities were calculated using Jaccard's similarity coefficient for RAPD and ISSR polymorphisms individually and combined as well. The Mantel test of significance was determined to measure the goodness of fit between the

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

S. No	Accession No.	Village	Mandal	District	State	Latitude	Longitude	Altitude (m)	Unique trait
1	IC565048	Ishwarnagar	Indervelli	Adilabad	Andhra Pradesh (A.P)	19°28′40"	78°41′12"	440	Early yield
2	IC565044	Machapur	Gudihatnur	Adilabad	A.P	19°32′23"	78°29′37"	442	Loose inflorescence
3	IC537863	Reddipalli	Chegunta	Adilabad	A.P	17°58′40"	78°27′37"	550	Leaf size (small)
4	IC537862	Chegunta	Chegunta	Adilabad	A.P	17°58′40"	78°27′37"	550	Leaf size (small)
5	IC537874	Pipri	Hutnoor	Adilabad	A.P	19°24′20"	78°15′25"	441	Many Primary branches
6	IC537914	Rangapur	Parigi	Ranga Reddy	A.P	17°1′29"	77°15′17"	627	Good plant structure ©
7	IC537916	Rangapur Aamabal	Parigi	Ranga Reddy	A.P	17°11′29	77°15′17"	627	Leaf colour (light green)
8	IC541651	Sulphipadhar	Bastar	Bastar	Chattisgarh	19°18′91"	81°47′01"	566	Identified as promising ®
9	IC541654	Narayanpal	Bastar	Bastar	Chattisgarh	19°12′06"	81°44′39	563	Good foliage
10	IC541633	Sonarpal Munjlapara	Bastar	Bastar	Chattisgarh	19°17′56"	81°55′81"	569	Late flowering
11	IC544693	Chinndarpalli	Hanwada	M'nagar	A.P	16° 45′54"	77°58′01"	545	Leaf no. (less)
12	IC541664	Arandi	Keskal	Bastar	Chattisgarh	19°59′29"	81°33′96"	653	Good plant structure ©
13	IC471314	Loharcha Banyagaon	Sirohi (Mt. Abu)	Sirohi	Rajasthan	–	–	–	Typical leaf (papaya leaf like)
14	IC541634	Nayapara	Kondgaon	Bastar	Chattisgarh	19°30′40"	81°41′72"	568	Identified as promising ®
15	IC541660	Pharasgaon	Pharasgaon	Bastar	Chattisgarh	19°52′01"	81°37′86"	616	No primary branches
16	IC537938	Kulkurti	Manoor	Medak	AP	17°56′05"	77°42′45"	540	High oil content
17	IC544674	Lingampet	Lingampet	Nizambad	A.P	18°14′12"	78°07′24"	474	Leaf no. (less)
18	IC471318	Khamnor	Udaipur	Udaipur	Rajasthan	–	–	–	Bushy
19	IC544678	Rajupalayam	Kothapatnam	Prakasam	A.P	15°23′48"	80°07′38"	10	Hardy plant (thick stems)
20	IC471333	Saira Patha	Udaipur	Udaipur	Rajasthan	–	–	–	Many primary branches
21	IC544659	Kothagudem	Kothagudem	Khammam	A.P	17°32′37"	80°58′22"	105	Prolific vegetative growth
22	IC538055	Chopla	Vardakhan	Champawat	Uttarakhand	–	–	–	Geographic location β
23	IC471313	Loharcha	Sirohi(Mt. Abu)	Sirohi	Rajasthan	–	–	–	Good plant structure © & geographic location ∞.
24	IC544676	Karedu	Ulvapadu	Prakasam	A.P	15°11′08"	80°00′55"	12	Leaf colour (light green)
25	IC544654	Kuravi	Kuravi	Warangal	A.P	17°31′16"	80°00′20"	203	Good foliage
26	IC537940	Budmetpalli	Tekmal	Medak	A.P	17°56′21"	77°58′49"	507	More primary branches and ideotype©
27	IC565041	Pasorapalle		Medak	A.P	18°05′04"	78°17′46"	497	Bushy plant type
28	IC537877	Chinncholi	Neredigonda	Adilabad	A.P	19°19′43"	78°24′02"	435	Leaf size (small)
29	IC544660	Kondaigudem	Chundrugonda	Khammam	A.P	17°19′06"	80°42′32"	181	Good yield and early bearing
30	IC544685	ChinnaKalavala	Sultanbad	Karimnagar	A.P	18°33′23"	79°21′15"	267	Many inflorescences
31	IC537933	Gollapalle	Kondapur	Ranga Reddy	A.P	17°31′06"	78°02′32"	580	Early yield
32	IC550849	Peddabanthupally	Gorla	Vizianagarm	A.P	18°19′10"	83°29′59"	119	Leathery leaf
33	IC565039	Akkanapet	Ramayampet	Medak	A.P	18°06′48"	78°23′47"	556	Good foliage
34	IC550847	Gurjavalasa	Dattirajeru	Vizianagarm	A.P	18°26′42"	83°19′44"	138	Leathery leaf texture

© – Good plant type with optimum leaf and stem growth.
® – Ref. [12].
β – Collected from temperate climate.
∞ – Collected from arid climate.

similarity matrices produced with the two marker systems. Phenotypic inter-relations, using the quantitative data, among the accessions were assessed using Euclidean distance. Dendrograms were constructed using the unweighted 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).

3. Results and discussion

3.1. Phenotypic variation

The data recorded on all the eight quantitative traits and oil content are presented in Table 2. There was significant variation in all the phenotypic traits recorded ($p < 0.05$). Phenotypic variation indicated the existence of diversity for quantitative traits *viz.*, plant height (90–225 cm), canopy spread (40–170 cm), collar length (12–32 cm), primary branches (2–16), petiole length (8.5–25.5 cm), pedicel length (1.7–7.0 cm), number of fruit clusters (4–23) and number of fruits per plant (4–55) and oil content (17.5–36.6%). A dendrogram based on the quantitative traits separated the accessions into seven clusters (Fig. 1). The cluster I emerged as largest, as it recorded maximum of 19 accessions (IC565048, IC537874, IC541651, IC544676, IC565041, IC537863, IC565044, IC538055, IC537877, IC537914, IC471318, IC544685, IC550847, IC544693, IC537916, IC537938, IC541634, IC537933 and IC549678). The Cluster-II had accommodated 6 accessions (IC541664, IC471313, IC541660, IC550849, IC565039 and

Table 2 – Variation in the phenotypic traits of the *J. curcas* accessions used in diversity analysis.

S. No.	Accession No.	Plant height (cm)	Canopy spread (cm)	Collar length (cm)	Primary branches (no.)	Petiole length (cm)	Pedicel length (cm)	Fruit clusters (no.)	Fruits per plant (no.)	Oil content (%)
1	IC565048	148.3	128.7	23.7	4.3	10.1	2.4	8.7	21.3	34.6
2	IC565044	143.3	71.3	23.3	3.3	10.5	2.1	8.7	11.0	23.3
3	IC537863	139.7	92.0	23.7	7.7	12.2	1.9	16.3	12.3	35.3
4	IC537862	182.0	138.7	29.3	3.7	15.8	2.1	3.3	8.3	36.5
5	IC537874	113.7	107.7	20.3	3.7	9.5	2.8	4 .3	11.3	30.3
6	IC537914	94.0	85.0	15.5	5.3	15.2	2.5	5.3	12.3	24.1
7	IC537916	161.3	81.3	22.0	4.3	17.1	2.2	5.3	5.3	35.7
8	IC541651	105.0	71.0	14.0	4.3	11.8	1.8	10.7	21.7	36.3
9	IC541654	112.0	95.7	22.3	2.3	15.4	6.0	2.3	6.0	35.2
10	IC541633	141.3	80.0	20.3	2.7	20.0	5.7	3.3	8.7	21.8
11	IC544693	97.0	50.7	11.7	2.3	12.0	2.2	4.3	8.3	22.9
12	IC541664	141.3	161.7	24.0	4.7	15.7	2.3	17.0	28.3	31.6
13	IC471314	123.0	133.3	20.3	5.3	14.7	2.2	19.0	16.0	22.3
14	IC541634	114.0	52.0	21.8	2.3	16.5	2.4	4.0	15.0	36.2
15	IC541660	136.0	42.0	18.0	3.3	14.5	5.3	3.3	7.0	35.0
16	IC537938	171.3	93.0	25.2	4.3	16.0	2.4	3.7	5.3	36.7
17	IC544674	225.3	140.3	33.7	3.7	13.1	1.9	8.3	5.3	34.5
18	IC471318	116.7	92.0	16.1	6.7	14.3	2.3	3.7	4.7	27.7
19	IC544678	204.3	94.3	22.3	2.3	13.4	3.2	7.3	9.3	32.5
20	IC471333	159.0	139.0	27.3	9.0	12.3	3.0	7.0	6.3	25.5
21	IC544659	220.0	169.7	33.3	4.3	25.0	2.4	21.0	50.0	32.5
22	IC538055	103.3	66.0	20.7	5.3	10.0	2.5	2.0	5.3	23.2
23	IC471313	180.3	148.3	26.0	4.7	17.6	2.0	21.7	20.3	28.0
24	IC544676	113.0	92.7	17.7	2.3	8.7	2.1	17.0	22.7	33.5
25	IC544654	165.0	130.3	18.0	15.0	12.7	2.6	16.3	25.7	28.5
26	IC537940	91.0	80.3	12.0	3.7	11.7	5.3	3.0	4.7	21.2
27	IC565041	106.7	99.0	23.7	4.3	12.6	3.7	16.0	29.3	34.4
28	IC537877	127.0	73.0	18.0	4.3	11.3	3.7	5.3	20.3	21.2
29	IC544660	188.0	123.0	16.2	3.3	12.8	2.5	8.3	35.3	17.5
30	IC544685	147.0	89.0	16.0	4.3	15.2	2.5	8.3	5.3	24.2
31	IC537933	141.0	72.3	22.0	2.3	14.2	2.3	4.3	7.3	33.5
32	IC550849	152.0	108.0	26.5	5.1	14.3	3.4	10.3	16.3	22.2
33	IC565039	146.3	112.3	25.0	5.7	10.4	2.1	13.7	20.3	25.1
34	IC550847	133.0	92.0	16.0	4.0	13.3	2.2	7.4	13.3	30.3
	Mean	142.5	100.2	21.4	4.5	13.8	2.8	8.8	14.7	29.2
	CV%	7.4	3 .5	6 .4	13.3	3.2	9.8	10.3	8.0	1.6
	CD (.05)	17.2	5.7	2.2	1.0	0.7	0.5	1.5	1.9	0.8

IC471333). Cluster III had one accession – IC544660, cluster IV included 2 accessions (IC537862 and IC544674), cluster V had 4 accessions (IC541654, IC541633, IC537940 and IC471314) and cluster VI and VII had one accession each *viz.*, IC544654 and IC544659, respectively, which emerged as diverse. The dendrogram (Fig. 1) based on quantitative phenotypic traits studied revealed that the diverse accessions cannot be identified using these traits as similar values were recorded across diverse clusters for all the traits studied namely, plant height, canopy spread, collar length, primary branches, petiole length, number of fruit clusters, number of fruits per plant and oil content with the exception of pedicel length, which recorded distinguishing variation across some clusters which could help in the identification of diverse accessions.

3.2. *Molecular variation*

A set of 56 RAPD and 40 ISSR primers were used for screening of 34 genotypes of *J. curcas* for their diversity.

3.2.1. *RAPD analysis*

The total number of markers observed among the *J. curcas* accessions based on RAPD analysis with 56 primer pairs was 632 (Table 3). All the tested primers amplified fragments across the 34 genotypes under study. The number of amplified fragments ranged from 3 (OPD 19 and OPG 17) to 24 (OPJ 5) and the size of the products ranged from 200 to 3000 bp. The total number of polymorphic markers was 543 with a mean of 9.69 per primer and the percentage of polymorphism was 87.3. The percentage of polymorphism ranged from 50 (OPH 14) to 100% (19 primers) and only 12 of the 56 primers showed less than 75% polymorphism. RAPD marker profile produced by the primer OPF 16 is presented in Fig. 2. The similarity coefficients based on 543 RAPD markers ranged from 0.33 to 0.80 with a mean similarity index of 0.57. The accessions IC541690 and IC471333 were almost similar with similarity index of 0.80 although they are from geographically distant locations of Andhra Pradesh and Rajasthan, respectively. The accession IC541633 from Bastar (Chattisgarh) and accessions IC537863 and IC565048 from

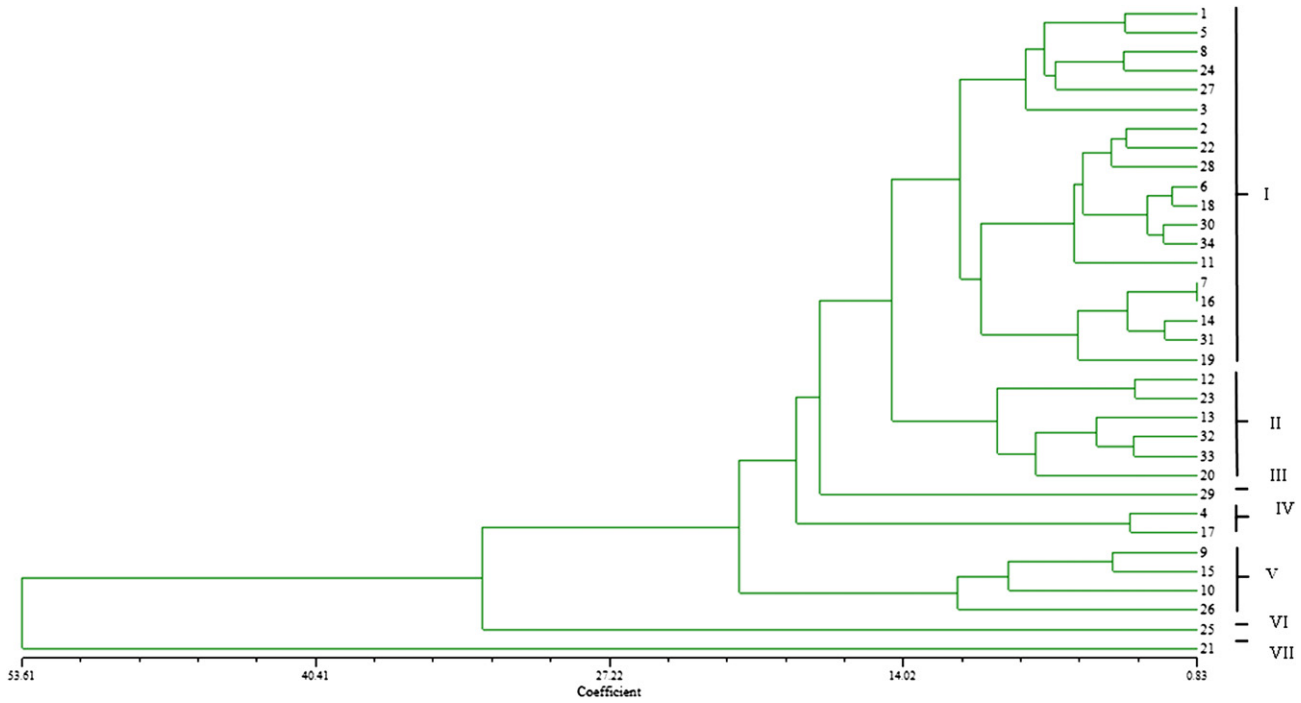

Fig. 1 – Dendrogram generated using UPGMA analysis, showing relationships between *Jatropha* genotypes using quantitative phenotypic traits data (Table 2).

Adilabad (Andhra Pradesh) showed the lowest similarity index (.33). The PIC values, a reflection of allele diversity and frequency among the accessions, were not uniformly higher for all the RAPD loci tested. The PIC values ranged from 0.07 (OPL 6) to 0.92 (OPO 9) with a mean of 0.58.

The dendrogram based on UPGMA analysis grouped the 34 genotypes into seven clusters, with Jaccards' similarity coefficient ranging from 0.80 to 0.33 at a mean of 0.57 (Fig. 3). The first cluster had 17 accessions *viz.*, IC471333, IC537862, IC537863, IC537916, IC537933, IC537940, IC538055, IC541634, IC541660, IC544654, IC544659, IC544660, IC544674, IC544678, IC550847, IC565039 and IC565048 comprising of accessions from Andhra Pradesh, Chattisgarh and Uttarakhand. Within A.P too, the accessions were from diverse agro-climatic regions and include the districts of Adilabad, Medak, Prakasam and Khammam. The second cluster includes 11 accessions, *viz.*, IC471313, IC471314, IC537862, IC537874, IC537914, IC541651, IC541654, IC541664, IC544693, IC565041 and IC565044 from the states of Andhra Pradesh, Chattisgarh and Rajasthan. The third cluster had 2 accessions IC471318 and IC537938 originating from Rajasthan and Andhra Pradesh, respectively. The fourth, fifth, sixth and seventh clusters were characterized by one accession each *viz.*, IC544685, IC550849, IC544676 and IC541633 collected from Karimangar, Vizianagaram, Prakasam districts of A.P and Bastar district of Chattisgarh, respectively.

3.2.2. *ISSR analysis*

Forty primers produced a total of 494 scorable markers among the genotypes. The size of amplified products ranged from 250 to 3000 bp. The number of markers per primer ranged from 5 (UBC 813 and UBC 840) to 21 (UBC 812). The total number of polymorphic markers and the percentage of polymorphism were 325 and 68.5, respectively (Table 4). The percentage polymorphism ranged from 23.5 (UBC 885) to 100% (7 primers). The amplification profiles produced by the ISSR primers UBC 827 is shown in Fig. 4. The PIC values ranged from .04 (UBC 881) to 0.97 (UBC 857) with a mean PIC value of 0.4.

ISSR marker analysis separated the 34 accessions into 8 major clusters at similarity of 33% (Fig. 5). The first cluster had 15 accessions *viz.*, IC471333, IC537862, IC537863, IC537916, IC537933, IC537940, IC541634, IC541660, IC544654, IC544660, IC544674, IC544678, IC550849, IC565039 and IC565048 from diverse agro-climatic zones of the states of Rajasthan, A.P and Chattisgarh. The 4 accessions in cluster II *viz.*, IC471314, IC537862, IC541664, IC544693 were from Rajasthan, A.P and Chhattisgarh. The third cluster had 10 accessions *viz.*, IC471313, IC471318, IC537874, IC537914, IC538055, IC541651, IC541654, IC544659, IC544676 and IC565041. These accessions were from diverse regions of A.P, Chhattisgarh, Rajasthan and Uttarakhand. The fourth, fifth, sixth, seventh and eight clusters had one accession each *viz.*, IC537938, IC550847, IC544685, IC565044 and IC541633 from Medak, Vizianagaram, Karimnagar and Adilabad districts of A.P and Bastar district of Chhattisgarh, respectively.

3.2.3. *RAPD + ISSR analysis*

The dendrogram based on both marker systems separated the genotypes into seven clusters, with similarity coefficient ranging from 0.50 to 0.92 (Fig. 6). Cluster I included 12 accessions *viz.*, IC471333, IC537862, IC537916, IC537933, IC537940, IC544654, IC544674, IC544678, IC550847, IC565039, IC565044

Table 3 – Polymorphism and number of bands generated with RAPD primer for 34 accessions of *J. curcas*.

S.No.	RAPD Primer	Sequence (5′–3′)	Number of bands	Number of polymorphic markers	Percentage polymorphism	PIC value
1	OPB 1	GTTTCGCTCC	7	6	85.7	0.74
2	OPB 2	TGATCCCTGG	8	8	100.0	0.68
3	OPB 3	CATCCCCCTG	11	11	100.0	0.84
4	OPB 7	GGTGACGCAG	13	7	53.8	0.39
5	OPB 10	CTGCTGGGAC	11	6	54.5	0.28
6	OPB 19	ACCCCCGAAG	7	7	100.0	0.83
7	OPC 1	TTCGAGCCAG	12	9	75.0	0.45
8	OPC 6	GAACGGACTC	6	5	83.3	0.66
9	OPC 11	AAAGCTGCGG	10	9	90.0	0.38
10	OPC 16	CACACTCCAG	12	12	100.0	0.91
11	OPC 18	TGAGTGGGTG	7	7	100.0	0.73
12	OPD 1	ACCGCGAAGG	13	11	84.6	0.86
13	OPD 3	GTCGCCGTCA	23	20	87.0	0.69
14	OPD 11	AGCGCCATTG	11	11	100.0	0.67
15	OPD 15	CATCCGTGCT	9	8	88.9	0.6
16	OPD 16	AGGGCGTAAG	11	11	100.0	0.76
17	OPD 19	GGGGTGACGA	3	2	66.7	0.9
18	OPE 2	GGTGCGGGAA	23	23	100.0	0.65
19	OPE 6	AAGACCCCTC	12	10	83.3	0.73
20	OPE 7	AGATGCAGCC	12	9	75.0	0.45
21	OPE 11	GAGTCTCAGG	7	7	100.0	0.36
22	OPE 14	TGCGGCTGAG	12	12	100.0	0.7
23	OPE 20	AACGGTGACC	9	9	100.0	0.55
24	OPF1	ACGGATCCTG	12	8	66.7	0.29
25	OPF7	CCGATATCCC	20	18	90.0	0.78
26	OPF 16	GGAGTACTGG	12	9	75.0	0.51
27	OPG 8	TCACGTCCAC	11	11	100.0	0.61
28	OPG 11	TGCCCGTCGT	10	7	70.0	0.62
29	OPG 17	ACGACCGACA	3	3	100.0	0.08
30	OPG 18	GGCTCATGTG	7	4	57.1	0.41
31	OPG 19	GTCAGGGCAA	9	5	55.6	0.39
32	OPH 9	TGTAGCTGGG	12	12	100.0	0.73
33	OPH 12	ACGCGCATGT	13	10	76.9	0.44
34	OPH 13	GACGCCACAC	10	10	100.0	0.63
35	OPH 14	ACCAGGTTGG	4	2	50.0	0.03
36	OPI 2	GGAGGAGAGG	18	11	61.1	0.36
37	OPI 19	AATGCGGGAG	14	10	71.4	0.64
38	OPI 20	AAAGTGCGGG	20	19	95.0	0.84
39	OPJ 4	CCGAACACGG	12	11	91.7	0.89
40	OPJ 5	CTCCATGGGG	24	21	87.5	0.81
41	OPJ 6	TCGTTCCGCA	11	9	81.8	0.71
42	OPJ 9	TGAGCCTCAC	11	6	54.5	0.35
43	OPJ 13	CCACACTACC	10	8	80.0	0.59
44	OPK 1	CATTCGAGCC	9	9	100.0	0.55
45	OPL 6	GAGGGAAGAG	4	4	100.0	0.07
46	OPL 9	TGCGAGAGTC	8	7	87.5	0.89
47	OPM12	CACAGACACC	12	11	91.7	0.46
48	OPM13	GGTGGTCAAG	6	5	83.3	0.17
49	OPO 2	ACGTAGCGTC	8	6	75.0	0.45
50	OPO 6	CCACGGGAAG	6	6	100.0	0.48
51	OPO 7	CAGCACTGAC	12	10	83.3	0.19
52	OPO 9	TCCCACGCAA	20	20	100.0	0.92
53	OPO 20	ACACACGCTG	12	11	91.7	0.76
54	OPP 2	TCGGCACGCA	12	9	75.0	0.82
55	OPP 3	CTGATACGCC	17	16	94.1	0.78
56	OPP 9	GTG GTC CGC A	14	9	64.3	0.52
		Total	632	543	87.28	0.58

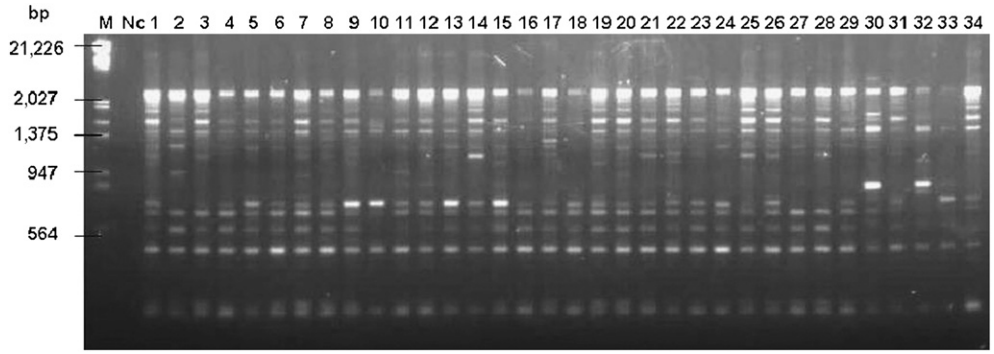

Fig. 2 – RAPD marker profiles of 34 genotypes of *J. curcas* generated with OPF 16 primer, M-marker, *EcoR* 1–*Hind* III double digest of λ DNA; NC-negative control (no DNA), 1–34 represent the genotypes according to Table 1.

and IC565048; cluster II had seventeen accessions *viz.*, IC471313, IC471314, IC471318, IC537862, IC537874, IC537914, IC537938, IC538055, IC541634, IC541651, IC541654, IC541660, IC541664, IC544659, IC544660, IC544693 and IC565041; cluster III, IV, V, VI and VII had one accession each *viz.*, IC550849, IC544676, IC565044, IC544685 and IC541633. The grouping from the combined analysis was similar in composition to that obtained from RAPD analysis. The correlation between the similarity matrices generated by RAPD and ISSR polymorphism based on Mantel statistic had reasonable fit ($r = 0.67$). Similarity values based on both the marker systems ranged from 0.49 to 0.80.

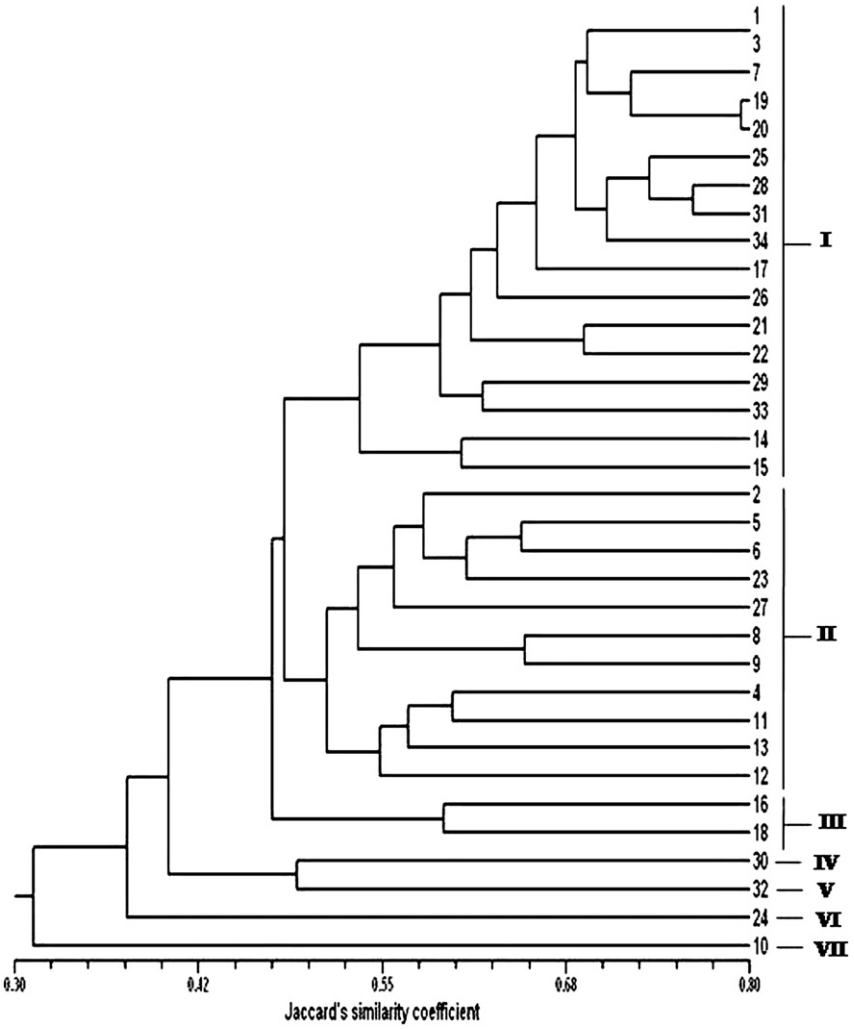

Fig. 3 – Dendrogram generated using UPGMA analysis showing relationships between *Jatropha* genotypes using RAPD data.

Table 4 – Polymorphism and number of bands generated with ISSR primer for 34 accessions of *J. curcas*.

S.No	Primer	Sequence (5′–3′)	Number of bands	Number of polymorphic markers	Percentage polymorphism	PIC Value
1.	UBC 807	AGAGAGAGAGAGAGAGT	20	9	45.0	0.31
2.	UBC 808	AGAGAGAGAGAGAGAGC	12	6	50.0	0.09
3.	UBC 809	AGAGAGAGAGAGAGAGG	14	5	35.7	0.16
4.	UBC 811	GAGAGAGAGAGAGAGAC	15	7	46.7	0.23
5.	UBC 812	GAGAGAGAGAGAGAGAA	21	16	76.2	0.35
6.	UBC 813	CTCTCTCTCTCTCTCTT	5	4	80.0	0.26
7.	UBC 814	CTCTCTCTCTCTCTCTA	19	13	68.4	0.54
8.	UBC 815	CTCTCTCTCTCTCTCTG	6	6	100.0	0.77
9.	UBC 817	CACACACACACACACAA	11	8	72.7	0.36
10.	UBC 818	CACACACACACACACAG	10	7	70.0	0.43
11.	UBC 823	TCTCTCTCTCTCTCTCC	8	7	87.5	0.41
12.	UBC 825	ACACACACACACACACT	18	14	77.8	0.36
13.	UBC 826	ACACACACACACACACC	9	3	33.3	0.14
14.	UBC 827	ACACACACACACACACG	10	6	60.0	0.49
15.	UBC 828	TGTGTGTGTGTGTGTGA	8	7	87 .5	0.57
16.	UBC 834	AGAGAGAGAGAGAGAGYT	18	11	61.1	0.29
17.	UBC 835	AGAGAGAGAGAGAGAGYC	16	14	87.5	0.24
18.	UBC 836	AGAGAGAGAGAGAGAGYA	17	10	58.8	0.38
19.	UBC 840	GAGAGAGAGAGAGAGAYT	5	2	40.0	0.1
20.	UBC 841	GAAGGAGAGAGAGAGAYC	15	11	73.3	0.58
21.	UBC 842	GAGAGAGAGAGAGAGAYG	9	9	100.0	0.88
22.	UBC 844	CTCTCTCTCTCTCTCTRC	12	10	83.3	0.52
23.	UBC 846	CACACACACACACACART	15	12	80.0	0.56
24.	UBC 847	CACACACACACACACARC	13	9	69.2	0.3
25.	UBC 848	CACACACACACACACARG	12	12	100.0	0.78
26.	UBC 856	ACACACACACACACACYA	14	8	57.1	0.34
27.	UBC 857	ACACACACACACACACYG	11	11	100.0	0.97
28.	UBC 860	TGTGTGTGTGTGTGTGRA	11	6	54.5	0.74
29.	UBC 862	AGCAGCAGCAGCAGCAGC	15	12	80.0	0.41
30.	UBC 865	CCGCCGCCGCCGCCGCCG	15	10	66.7	0.44
31.	UBC 866	CTCCTCCTCCTCCTCCTC	9	8	88.9	0.49
32.	UBC 867	GGCGGCGGCGGCGGCGGC	8	8	100.0	0.48
33.	UBC 868	GAAGAAGAAGAAGAAGAA	10	8	80.0	0.37
34.	UBC 876	GATAGATAGACAGACA	7	7	100.0	0.88
35.	UBC 878	GGATGGATGGATGGAT	6	6	100.0	0.6
36.	UBC 880	GGAGAGGAGAGGAGA	13	4	30.8	0.28
37.	UBC 881	GGGTGGGGTGGGGTG	15	5	33.3	0.04
38.	UBC 885	BHBGAGAGAGAGAGAGA	17	4	23.5	0.09
39.	UBC 887	DVDTCTCTCTCTCTCTC	12	6	50.0	0.1
40.	UBC 888	CGTAGTCGTCACACACACACACA	13	4	30.8	0.26
		Total	494	325	68.5	0.4

Based on dendrograms of RAPD, ISSR and RAPD + ISSR seven genotypes *viz.*, IC537938, IC541633, IC544676, IC544685, IC550847, IC550849 and IC565044 were observed to be diverse. IC541633 emerged as distinctly diverse among all the accessions. Principal Coordinate analysis (PCoA) based on RAPD + ISSR polymorphism grouped the accessions into six clusters (Fig. 7). There was no similarity between the dendrogram based on phenotypic traits and the RAPD ($r = 0.06$) and ISSR ($r = 0.09$) clustering. However, the similarity between the phenotypic traits and the RAPD + ISSR was good ($r = 0.66$).

3.3. *Comprehending the variation*

Determination of genetic variation among genotypes of *J. curcas* using phenotypic traits and molecular analysis is essential to select parents to be crossed for generating appropriate populations intended for creating variation, genome mapping and breeding purposes. The objective of the present study was to assess the molecular variation that could be corroborated with phenotypic variation so that distinct genotypes, if any, identified phenotypically could be utilized in the breeding programmes. Previous studies [5], [9], [19] and [8] were mostly based on random collections of *J. curcas* and other *Jatropha* species, and correlations were drawn based on geographical isolation. Geographically isolated populations accumulate genetic differences as they adapt to different environments. However, dendrograms in the present study do not indicate a very clear pattern of clustering according to geographical location. The genotypes from different locations were clustered in one cluster and *vice versa*, which clearly shows that geographic differentiation of Indian *J. curcas* is not extensive.

The germplasm, characterized by significant variation in phenotypic quantitative traits and some unique traits, which formed the basis of the study, was subjected for molecular confirmation to identify plus trees and diverse accessions. The

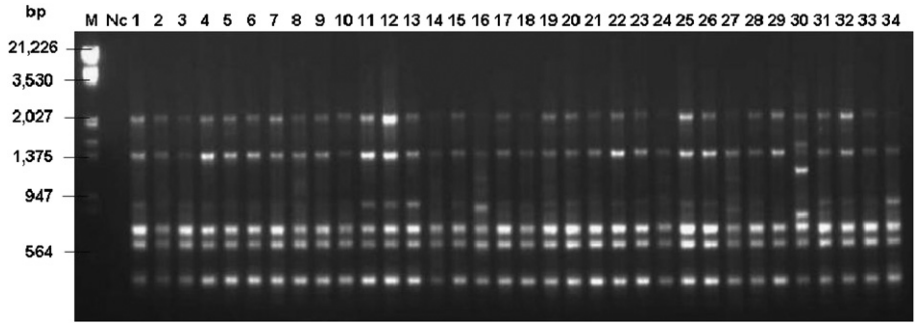

Fig. 4 – ISSR marker profile of 34 genotypes of *J. curcas* generated with UBC 827 primer, M-marker, *EcoR* 1–*Hind* III double digest of λ DNA; NC-negative control (no DNA), 1–34 represent the genotypes according to Table 1.

guiding traits for plus trees in *Jatropha* have been reported [12]. Applying the guiding traits to the present study, one accession, IC544659, was found promising for three traits *viz.*, plant height (220 cm), petiole length (25 cm) and fruits per cluster (21). Further, there were two accessions IC537863 and IC541651 that were found promising for two traits of fruits per cluster (>10) and oil content (>35%).

Molecular characterization using 56 RAPD and 40 ISSR primers, to identify the diverse accessions, revealed high polymorphism of 87.5% and 68.5%, respectively. The molecular markers (RAPD and ISSR) yielded 868 polymorphic markers that discriminated the 34 genotypes into 7 and 8 clusters, respectively. The reproducibility factor for RAPD and ISSR is 84.4% and 87%, respectively [20]. High capacity of ISSR primers to reveal polymorphism offers great potential to determine intra- and inter genomic diversity as compared to other arbitrary primers like RAPD as has been reported by [21]. However, the number of total polymorphic and discriminate fragments recorded in the present study was higher for RAPD than ISSR. The differences found among the dendrograms generated by RAPD and ISSR could be partially explained by different number of PCR products analyzed (632 for RAPD and

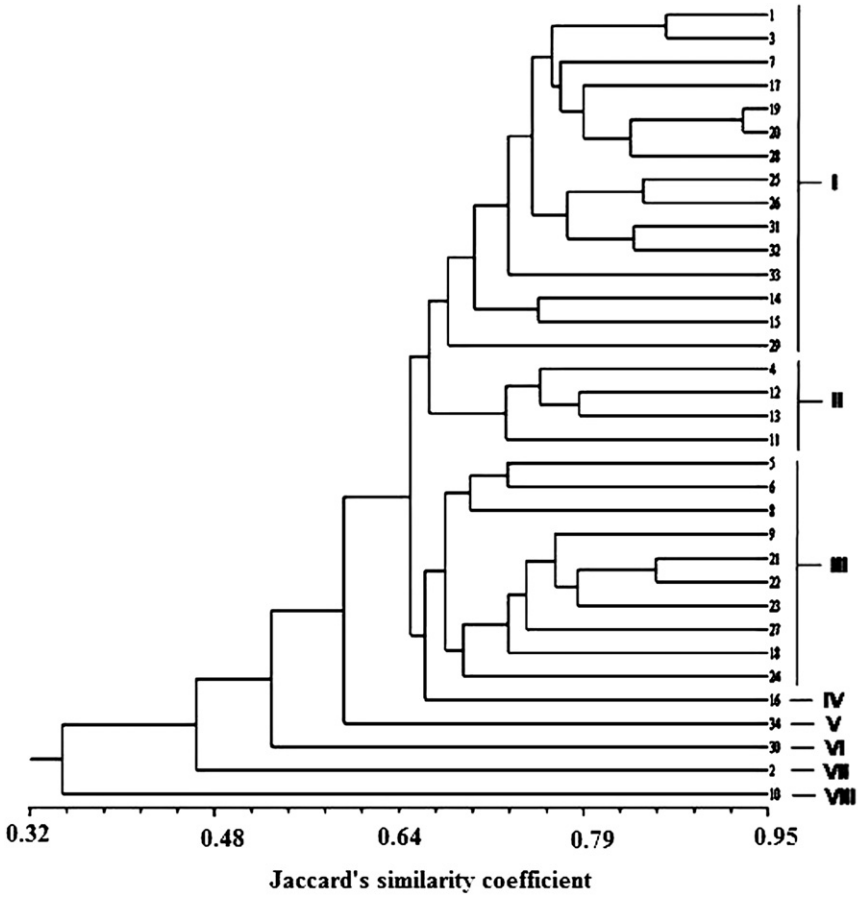

Fig. 5 – Dendrogram generated using UPGMA analysis showing relationships between *Jatropha* genotypes using ISSR data.

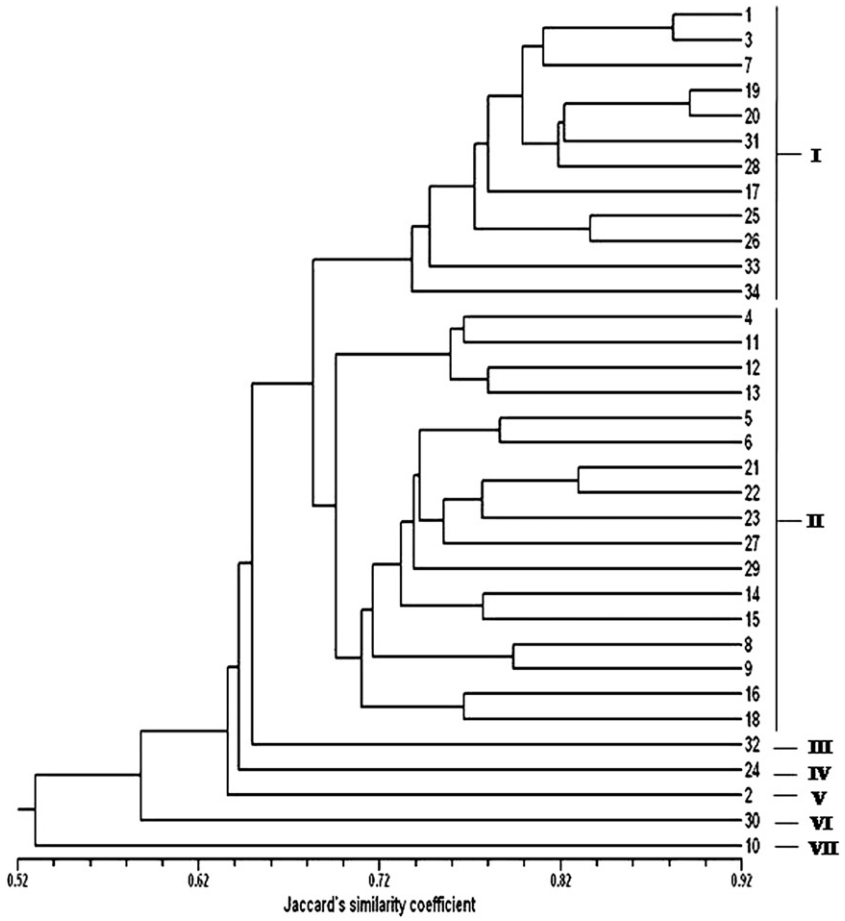

Fig. 6 – Dendrogram generated using UPGMA analysis, showing relationships between *Jatropha* genotypes using RAPD + ISSR data.

494 for ISSR) and marker sampling error and or the level of polymorphism detected, reinforcing the importance of the number of loci and their coverage of the overall genome in obtaining reliable estimates of genetic relationships among the cultivars [22]. ISSR markers are directed at multiple microsatellite loci distributed across the genome while RAPD markers scrutinize the whole genome facilitating the detection of genome wide genetic variation. Both the markers resulted in polymorphic loci but of the two marker systems RAPD was superior to ISSR; similar observation has been made by [5] where the number of total polymorphic fragments was higher for RAPDs than ISSRs.

The dendrogram based on pair-wise genetic similarity coefficients with RAPD and ISSR markers showed grouping of the 34 accessions into 7 and 8 clusters, respectively. Partial corroboration was found between the molecular and phenotypic (quantitative) data. For example, cluster III formed by the RAPD marker, with two accessions *viz.*, IC537938 and IC471318 within the cluster, had showed non-significant variation with respect to traits of canopy spread, pedicel length, number of fruit clusters and fruits per plant and Cluster IV for plant height. Similarly, Cluster II of the ISSR had accessions which were similar with respect to pedicel length. Hence, pedicel length, among the quantitative traits could guide in identification of diverse accessions. However, a more detailed study with more molecular markers and a larger set of germplasm may provide further understanding regarding the quantitative traits influencing the grouping of accessions and the cause of diversity. Focused study on such traits, which aid in identification of diverse accessions during germplasm collection and evaluation, expedite results in the crop improvement programme of this potential biodiesel crop.

Furthermore, in the analysis with RAPD markers, accessions IC541633 of cluster VII, IC544676 of cluster VI and IC550849 of cluster V emerged as being the most diverse when compared with cluster I comprising of 17 accessions. With regard to ISSR markers, the accession IC541633 of cluster VIII, IC565044 of cluster VII and IC544685 of cluster VI were found to be diverse when compared to cluster I with 15 accessions. In RAPD + ISSR analysis, as well the accession IC541633 was found to be diverse and was included in cluster VII. Despite the distinctness of the accession IC541633, in terms of its molecular profile, analysis of quantitative traits *viz.*, plant height, canopy spread, collar length, petiole length, pedicel length, clusters per plant and fruits per plant showed no significant variation. However, it was found to possess a unique phenotypic trait of late flowering. Similarly, the accessions, IC544676 and IC550849 which emerged as diverse

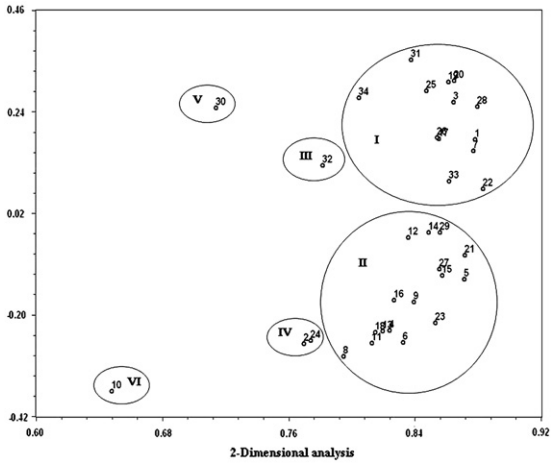

Fig. 7 – Two dimensional scaling of 34 accessions of *J. curcas* by principal coordinate analysis using the Jaccard's similarity coefficients based on RAPD + ISSR primers. The numbers represent the accession codes (S. no) as given in Table 1.

from RAPD and RAPD + ISSR analysis were characterized by unique traits of light green leaves and leathery leaves, respectively. And the accessions, IC565044 and IC544685, which were diverse in ISSR and RAPD + ISSR analysis, had exhibited unique traits – lax (loose) type of inflorescence and more number of inflorescences, respectively. The PCoA complimented the RAPD + ISSR dendrogram well both in the number of clusters and the accessions comprising the clusters except for two variations *viz.*, IC565044 and IC544676 which were in separate clusters in dendrogram grouped together in PCoA and IC538055, which was in group II in PCoA was in cluster I in dendrogram analysis. The three axes of Eigen values captured 75.9% of the total variation. Based on dendrograms and PCoA of RAPD + ISSR analysis, the accessions IC541633, IC544676, IC544685, IC550849 and IC565044 have been identified as diverse. The accessions, IC541633, IC544676, IC544685 and IC550849 are common outliers in RAPD and RAPD + ISSR analysis and accessions IC541633, IC544685, IC565044 are outliers in ISSR and RAPD + ISSR analysis. This indicates that, among the phenotypic quantitative traits studied, pedicel length partially and unique traits, such as, time of flowering, inflorescence number, leaf colour and leaf type can be used to identify diverse germplasm in *Jatropha*.

4. Conclusions

Accessions, IC544654 and IC54459 which emerged as diverse from analysis of eight phenotypic traits (quantitative traits) were not corroborated as diverse by molecular analysis. Among the phenotypic (quantitative) traits, pedicel length could guide in the identification of diverse accessions. Accessions IC541633, IC544676, IC550849 were outliers in the RAPD analysis and were characterized by unique phenotypic traits of late flowering, light green leaves and leathery leaves respectively. Accessions IC565044, IC544685, outliers in the ISSR analysis, apart from IC541633, were characterized by unique phenotypic traits of loose (lax) inflorescence type and many inflorescences, respectively. In RAPD + ISSR analysis, IC550849, IC544676, IC565044, IC544685 and IC541633 were categorized as diverse. Hence, unique phenotypic traits like, leaf colour (light green), leaf type (leathery), time of flowering, inflorescence type (lax inflorescence) and inflorescence number and pedicel length (partially) among the quantitative traits can guide in the identification of diverse accessions.

Thus, such a study including more unique traits (qualitative or quantitative) of the *Jatropha* germplasm accessions and corroborating the variation in the traits with the molecular traits would result in several such traits which could serve as morphological indicators for the study of diversity in *Jatropha*. The identified traits could be effectively harnessed as a handy tool for selection of diverse germplasm accessions at the time of germplasm collection or short listing of accessions for the crossing programmes.

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