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AFLP-based molecular characterization of an elite germplasm collection of *Jatropha curcas* L., a biofuel plant

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ABSTRACT

Amplified fragment length polymorphism (AFLP) was employed to assess the diversity in the elite germplasm collection of *Jatropha curcas*, which has gained tremendous significance as a biofuel plant in India and many other countries recently. Forty-eight accessions, collected from six different states of India, were used with seven AFLP primer combinations that generated a total of 770 fragments with an average of 110 fragments per primer combination. A total of 680 (88%) fragments showed polymorphism in the germplasm analyzed, of which 59 (8.7%) fragments were unique (accession specific) and 108 (15.9%) fragments were rare (present in less than 10% accessions). In order to assess the discriminatory power of seven primer combinations used, a variety of marker attributes like polymorphism information content (PIC), marker index (MI) and resolving power (RP) values were calculated. Although the PIC values ranged from 0.20 (E-ACA/M-CAA) to 0.34 (E-ACT/M-CTT) with an average of 0.26 per primer combination and the MI values were observed in the range of 17.60 (E-ACA/M-CAA) to 32.30 (E-ACT/M-CTT) with an average of 25.13 per primer combination, the RP was recognized the real attribute for AFLP to determine the discriminatory power of the primer combination. The RP values for different primer combinations varied from 23.11 (E-ACA/M-CAA) to 46.82 (E-ACT/M-CTT) with an average of 35.21. Genotyping data obtained for all 680 polymorphic fragments were used to group the accessions analyzed using the UPGMA-phenogram and principal component analysis (PCA). Majority of groups obtained in phenogram and PCA contained accessions as per geographical locations. In general, accessions coming from Andhra Pradesh were found diverse as these were scattered in different groups, whereas accessions coming from Chhattisgarh showed occurrence of higher number of unique/rare fragments. Molecular diversity estimated in the present study combined with the datasets on other morphological/agronomic traits will be very useful for selecting the appropriate accessions for plant improvement through conventional as well as molecular breeding approaches.

1. Introduction

Jatropha (*Jatropha curcas* L.) commonly known as purging nut or physic nut, is a perennial deciduous, multipurpose shrub belonging to the family Euphorbiaceae. Primarily Jatropha plant was used for its medicinal values however, in the recent years the seed-oil has gained tremendous significance as biofuel plant. Jatropha is a native to Mexico and Central American region and later naturalized in many parts of the tropic and subtropic regions of the world, where it is grown as a hedge crop to protect gardens and fields from animals and for traditional use [1].

The seed-oil of Jatropha has been reported to have insecticidal [2], molluscicidal [3] and fungicidal [4] properties. The decorticated seeds yield 40–50% oil, which is transesterified, blended with the diesel and used as biodiesel. Among the non-edible oil yielding crops, Jatropha has gained paramount significance as its suitability for the application as a biofuel plant for its short gestation period, drought endurance, low cost of seeds, high oil content, easy adaptation on marginal and semi marginal lands, suitability as fuel substitute with out any alteration to the existing engines [5].

Although Jatropha is known for its insecticidal and toxicity properties, when grown as a monocrop there are reports of insect pests like *Scutellera nobilis* Fabr. (scutellarid bug) in India and *Pachycoros klugii* Burmeister in Nicaragua (South America), affecting flower fall, fruit abortion and malformation of seeds causing huge economic losses [6]. In order to develop insect, pest resistant varieties and also fill up the gap between the demand and the supply

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of elite planting material, there is a need for improving the species. However, not much work has been carried out on this species in terms of genetic improvement especially regarding the number of introductions, usage of the wild or locally available germplasm.

In India, Jatropha is grown in several states as a part of promoting biofuel program, several germplasm lines from different locations of India have been collected at ICRISAT and Osmania University. An understanding of germplasm diversity and genetic relationships in a germplasm collection is an invaluable aid for crop improvement strategies [7]. Diversity studies, based on their morphological traits, are not reliable as morphological traits are heavily influenced by environment. Molecular diversity assessed by using molecular markers is independent of the influence of environment and estimated by using DNA from any growth stage. Among the various molecular markers, employed to assess diversity studies, PCR-based markers such as RAPD (random amplified polymorphic DNA [8]), ISSR (inter simple sequence repeat [9]) and AFLP (amplified fragment length polymorphism [10]) have become popular, as their application does not need any prior sequence information. On the other hand, microsatellite or simple sequence repeat (SSR) are the markers of choice for breeding applications, but their development is an expensive process [11].

Among different marker systems available at present, AFLP represents dominant marker system and provides multilocus and genome wide marker profiles. These features make the AFLP technology suitable for molecular characterization and DNA fingerprinting of any germplasm collection [7]. AFLP was widely used to assess phylogenic and genetic diversity in different tree species like almond [12], cashew [13] and Euphorbiaceae members like *Hevea* [14]. In the direction of analyzing the diversity of Jatropha germplasm, a few studies have been carried out recently using, RAPD, ISSR, SSR, SPAR (single-primer amplification reaction) and AFLP markers [5,15–17].

The present study surveys the molecular diversity in forty-eight accessions of Jatropha collected from six states from a representative collection of 200 accessions from India, by using seven AFLP primer combinations. The genotyping data were used to understand the relationships among germplasm lines and identify genetically diverse lines for using them in genetic improvement of Jatropha.

2. Material and methods

2.1. Plant materials

A set of 48 accessions including 13 (OJC series) from Osmania University and the remaining 35 from ICRISAT was analyzed for assessing molecular diversity. These accessions represent different geographical regions of India [Uttar Pradesh (UP), Gujarat (GJ), Rajasthan (RJ), Madhya Pradesh (MP), Chhattisgarh (CG) and Andhra Pradesh (AP)] (Table 1).

2.2. DNA extraction

About 5 g of tender, fresh leaf material was harvested from 21-day-old seedlings from ten individual plants and bulked for each accession. The quality and quantity of genomic DNA isolated using traditional CTAB (3%) protocol was poor, as Jatropha is a latex yielding plant, rich in polyphenols and polysaccharides. Hence a modified protocol was followed, as described here. The leaf material was ground in liquid nitrogen, along with 3% polyvinylpyrrolidone (PVP; Sigma, USA), and then homogenized in 20 ml CTAB (4%) extraction buffer with 2% β-mercaptoethanol incubated at 65 °C for 1 h followed by two extractions with chloroform and isoamylalchol (24:1, v/v). Pelleted DNA was air dried and suspended in high salt TE and RNase A (10 mg/ml).

Purification steps were carried out twice with phenol, phenol: chloroform (1:1) and with chloroform: isoamyl alcohol (24:1). Finally, DNA was pelleted with 0.5 volume of sodium acetate (3 M) and 2 volumes of absolute ethanol, followed by ethanol wash (70%), air dried and resuspended in appropriate volume of low salt TE. The quantity of the DNA was estimated spectrophotometrically and quality was checked on 0.8% agarose electrophoretically.

2.3. AFLP analysis

AFLP fingerprinting was carried out as described by [10] with some modifications as described below. Genomic DNA (350 ng/μl) was digested with 5 U each of EcoRI and MseI (New England Biolabs, USA) at 37 °C for 16 h. Digested samples were incubated at 70 °C for 15 min to deactivate the restriction enzymes.

Adapters [(EcoRI (5 pmol) and MseI (25 pmol)] were ligated to the restricted DNA fragments in ligation buffer [(1× T4 DNA ligase buffer, 0.2 U of T4 DNA ligase (New England Biolabs, USA)] and incubated at 20 °C for 3 h. Preamplification of the diluted (10-fold), ligated DNA was carried out with primers (complimentary to the EcoRI and MseI adapters, with one selective nucleotide adenine and cytosine respectively) in the thermal cycler (MJ Research, USA) using the following cycling parameters: 20 cycles set at 94 °C for 30 s, 56 °C for 60 s and 72 °C for 60 s.

The diluted (10-fold), amplified products were used as the template for selective amplification. The second amplification was carried out with seven selective primer combinations of EcoRI (labeled) and MseI with three selective nucleotides (Table 2) in a total volume of 10 μl. The PCR program consisted of two segments. The first segment comprised of 12 cycles with one cycle at 94 °C for 30 s, 65 °C for 30 s, and 72 °C for 60 s. The annealing temperature was lowered by 0.7 °C per cycle during the first 12 cycles. The second segment comprised of 20 cycles at 94 °C for 30 s, 56 °C for 60 s and 72 °C at 60 s. The PCR products were resolved on 6% (w/v) denaturing polyacrylamide gels by loading 4 μl of the sample per lane for 2 h at constant power (50–55 W). The gels were dried in a gel dryer (BioRad, USA) for 45 min and autoradiographs were obtained by exposing Kodak Biomax MR film (Eastman Kodak Co., USA) to the dried gel in an exposure cassette (General Electrics, UK) for 14–20 h depending on the signal intensity and the film was developed.

2.4. Statistical analysis

Genotyping data obtained for the AFLP primer combinations was used for assessing the discriminatory power of AFLP primer combinations by evaluating three parameters [18]: (i) polymorphism information content (PIC), (ii) marker index (MI) and (iii) resolving power (RP).

The PIC value for each AFLP primer combination was calculated as proposed by Roldan-Ruiz et al. [19]:

$$PIC_i = 2 f_i(1 - f_i),$$

where PIC_i is the polymorphism information content of marker i, f_i the frequency of the marker fragments, which were present and $1–f_i$ the frequency of marker fragments, which were absent. PIC was averaged over the fragments for each primer combination.

The marker index was calculated as given in Varshney et al. [20]:

$$MI = PIC \times EMR$$

where, EMR is "The effective multiplex ratio (E) is defined as the product of the total number of loci/fragments per primer (n) and the fraction of polymorphic loci/fragments (β) ($E = n \cdot \beta$)".

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Table 1
Details on Jatropha accessions used in the study.

Accession number[a]	Collection site	State	Two letter state code	Percentage oil content	1000-seed weight (g)
UT1	Durgapur	Uttar Pradesh	UP	30.6	660
UT2	Godhra	Gujarat	GJ	29.7	590
UT3	Indore	Madhya Pradesh	MP	30.6	700
UT4	Jhabua	Madhya Pradesh	MP	29.5	610
UT5	Maymbhaje	Uttar Pradesh	UP	28.9	640
UT6	Raipur	Chhattisgarh	CG	30.4	660
UT7	Ratnapur	Uttar Pradesh	UP	29.5	660
UT8	Udaipur	Rajasthan	RJ	30.0	620
UT10	NBRI Lucknow	Uttar Pradesh	UP	25.0	600
UT11	Biotech Park Lucknow	Uttar Pradesh	UP	25.0	580
SMHP	Cuddapah	Andhra Pradesh	AP	32.3	670
SDP	Sudepally, Kurnool	Andhra Pradesh	AP	25.7	530
CSM1	Bhavnagar	Gujarat	GJ	33.1	635
CSM2	Bhavnagar	Gujarat	GJ	32.8	620
CSM3	Bhavnagar	Gujarat	GJ	28.5	600
CSM5	Bhavnagar	Gujarat	GJ	30.8	660
CSM9	Bhavnagar	Gujarat	GJ	31.4	650
CSM11	Bhavnagar	Gujarat	GJ	30.2	625
CSM12	Bhavnagar	Gujarat	GJ	32.2	630
SKNJ2	Bhavnagar	Gujarat	GJ	32.1	615
SKNJ7	Bhavnagar	Gujarat	GJ	34.9	660
IJC7	Churu	Rajasthan	RJ	29.3	510
IJC8	Jabalpur	Madhya Pradesh	MP	34.7	726
IJC9	Jabalpur	Madhya Pradesh	MP	32.8	673
IJC11	Jabalpur	Madhya Pradesh	MP	34.4	694
OJC1	Rain shadow area department	Andhra Pradesh	AP	27.4	620
OJC2	Chhattisgarh	Chhattisgarh	CG	28.1	600
OJC3	Karimnagar	Andhra Pradesh	AP	30.6	640
OJC4	Mahaboobnagar	Andhra Pradesh	AP	19.0	600
OJC5	Mahaboobnagar	Andhra Pradesh	AP	37.9	540
OJC6	Chandanagar	Andhra Pradesh	AP	31.2	563
OJC7	Chintapally	Andhra Pradesh	AP	29.3	590
OJC8	Jodhpur	Rajasthan	RJ	28.7	540
OJC9	Adilabad	Andhra Pradesh	AP	32.7	610
OJC10	Sumerpur	Rajasthan	RJ	32.0	605
OJC11	Sumerpur	Rajasthan	RJ	33.8	545
OJC12	Sumerpur	Rajasthan	RJ	33.4	620
OJC13	Sumerpur	Rajasthan	RJ	31.5	530
SNES25	Velchal	Andhra Pradesh	AP	39.4	860
SNES34	Chevella	Andhra Pradesh	AP	34.4	820
SNES37	Chevella	Andhra Pradesh	AP	38.9	780
BAAS30	Cheevurugaon Pujaripara	Chhattisgarh	CG	34.5	700
BAAS35	Banyagaon Nayapara	Chhattisgarh	CG	36.2	750
BAAS38	Sonabat Chepdapara	Chhattisgarh	CG	35.3	590
BAAS48	Karli Choakpara	Chhattisgarh	CG	34.5	585
BAAS53	Aamabal Gudipara	Chhattisgarh	CG	32.7	765
BAAS57	Devda	Chhattisgarh	CG	36.9	715
BAAS61	Pharasgaon	Chhattisgarh	CG	35.5	700

[a] Same accession numbers have been presented in dendrogram (Fig. 3) and Principal component analysis (Fig. 4).

Table 2
Sequences of oligonucleotide adapters and primers used in AFLP.

Name	Code	Sequence
EcoRI adapter		5′-AAT TGG TAC GCA GTC TAC-3′ 3′-CC ATG CGT CAG ATG CTC-5′
MseI adapter		5′-TAC TCA GGA CTC AT-3′ 3′-G AGT CCT GAG TAG GAG-5′
EcoRI primer	E-A00	5′-GTA GAC TGC GTA CCA ATT C A-3′
MseI Primer	M-C00	5′-GAT GAG TCC TGA GTA A C-3′
EcoRI + 3-ACT	E-ACT	5′-GTA GAC TGC GTA CCA ATT C ACT-3′
EcoRI + 3-ACA	E-ACA	5′-GTA GAC TGC GTA CCA ATT C ACA-3′
EcoRI + 3-AGG	E-AGG	5′-GTA GAC TGC GTA CCA ATT C AGG-3′
EcoRI + 3-ACG	E-ACG	5′-GTA GAC TGC GTA CCA ATT C ACG-3′
EcoRI + 3-AGC	E-AGC	5′-GTA GAC TGC GTA CCA ATT C AGC-3′
MseI + 3-CTT	M-CTT	5′-GAT GAG TCC TGA GTA A CTT-3′
MseI + 3-CAA	M-CAA	5′-GAT GAG TCC TGA GTA A CAA-3′
MseI + 3-CAT	M-CAT	5′-GAT GAG TCC TGA GTA A CAT-3′
MseI + 3-CAG	M-CAG	5′-GAT GAG TCC TGA GTA A CAG-3′
MseI + 3-CTA	M-CTA	5′-GAT GAG TCC TGA GTA A CTA-3′

Resolving power of each primer was calculated according to Prevost and Wilkinson [21]:

$$RP = \Sigma I_b,$$

where, I_b represents fragment informativeness. The I_b can be can be represented into a 0–1 scale by the following formula:

$$I_b = 1 - [2 \times |0.5 - p|],$$

where, p is the proportion of the 48 accessions containing the fragment.

2.5. Construction of phenogram

Prominent AFLP fragments for each primer combination were scored visually as present (1) or absent (0). Genetic similarity (GS) was estimated between pairs of accessions according to Jaccard's similarity coefficient [22] using the NTSYS-pc 2.02 software package [23]. Genetic similarity matrix obtained based on Jaccard's similarity

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coefficient was used to prepare the phenogram following unweighted pair group method with arithmetic average (UPGMA).

To find the robustness of the phenogram, bootstrapping analysis was carried out for these 48 accessions (hundred replicates) with PAUP Win 32 software (4.0 beta version) [24]. The data were also analyzed using multivariate methods for conducting principal component analysis (PCA).

3. Results

For understanding the molecular diversity in Jatropha accessions available at ICRISAT, in the first instance, a set of 48 genotypes were selected in such a way that all the six states from where Jatropha accessions have been collected are represented. For instance, if four accessions have been collected from one town from a particular state of India, only one accession out of four accessions was selected. AFLP markers were employed for molecular profiling as AFLP provide genomewide fingerprints.

3.1. Marker polymorphism

A total of seven primer combinations (primer combinations) from five EcoRI and five MseI primers were used to generate AFLP profiles on 48 Jatropha accessions (Table 2). While analyzing the genome wide AFLP fingerprint profiles, only prominent fragments (well resolved on gel) were considered. In total, all seven primer combinations generated a total 770 fragments, of which 680 (88%) were polymorphic and 59 (8.7%) of the polymorphic fragments were unique and accession specific (Table 3). The total number of fragments (monomorphic and polymorphic) for each assay ranged from 90 to 135 with an average of 110 per primer combination. The percentage of polymorphic fragments varied from 82.8% to 95.1% with an average of 88.2% per primer combination.

3.2. AFLP features

Marker informativeness for the seven AFLP primer combinations was analyzed using several parameters (Table 3). A total number of fragments (NTF) generated were 770, of which 680 (88.3%) were polymorphic fragments (NPF) and the remaining 90 (11.7%) were monomorphic fragments (NMF). Thus, percentage of polymorphic fragments varied from 82.8% (E-ACA/M-CAT) to 95.1% (E-ACG/M-CAA) with an average of 88.2% per primer combination. While analyzing frequency distribution of the polymorphic fragments across the AFLP primer combinations, higher number of polymorphic fragments were obtained in the frequency classes

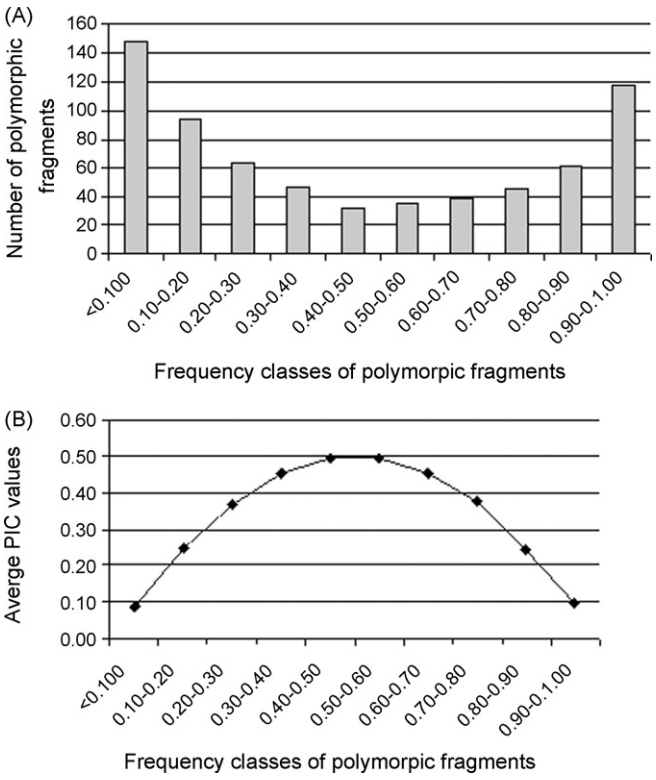

Fig. 1. (A) Frequency distribution for polymorphic AFLP fragments in Jatropha germplasm collection. (B) Relationships between PIC value and frequency of polymorphic fragments.

0.0–0.10 and 0.90–1.0 (Fig. 1A). However, when these data were correlated with the PIC value data for individual fragments, it was found that higher number of polymorphic fragments had lower PIC values (Fig. 1A). Whereas the fragments falling under 0.50–0.60 are highly informative (average PIC value 0.50) following by those present in classes 0.40–0.50 (average PIC value 0.49) and 0.60–0.70 (average PIC value 0.45) (Fig. 1A and B).

The polymorphic fragments were categorized further as unique fragments, rare fragments, shared fragments and similar fragments as given below:

3.2.1. Unique fragments

These are the specific fragments, present in only one accession for the given primer combination. A total of 59 (8.7% of NPF) unique

Table 3
Degree of polymorphism and information content for 7 AFLP primer combinations used.

S. No.	Primer combinations	NTF[a]	NMF[b]	NPF[c]	% Polymorphism	NUF[d]	NRF[e]	NSF[f]	NSIF[g]
1	E-ACT/M-CTT	107	12	95	88.8	2	12	63	18
2	E-ACA/M-CAA	102	14	88	86.3	11	22	30	25
3	E-ACA/M-CAT	93	16	77	82.8	7	18	37	15
4	E-AGG/M-CAG	120	19	101	84.2	8	13	36	44
5	E-AGG/M-CTA	135	16	119	88.2	14	16	47	42
6	E-ACG/M-CAA	123	6	117	95.1	13	16	47	41
7	E-AGC/M-CTA	90	7	83	92.2	4	11	40	28
8	Total	770	90	680	88.3	59	108	300	213
9	Minimum	90	6	77	82.8	2	11	30	15
10	Maximum	135	19	119	95.1	14	22	63	44
11	Average	110.0	12.9	97.1	88.2	8.4	15.4	42.9	30.4

[a] Total number of fragments generated (NTF).
[b] Number of monomorphic fragments (NMF).
[c] Number of polymorphic fragments (NPF).
[d] Total number of unique fragments (NUF).
[e] Total number of rare fragments (NRF).
[f] Total number of shared fragments (NSF).
[g] Total number of similar fragments (NSIF).

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fragments (NUF), specific to only one accession, were observed with an average of 8.4 unique fragments per primer combination (Table 3). Highest number of unique fragments (14) were detected by the primer combination E-AGG/M-CTA in four accessions (BAAS53, IJC11, OJC1 and OJC2) followed by 13 unique fragments in two accessions (BAAS53 and BAAS57) by the primer combination E-ACG/M-CAA. Lowest number of unique fragments (2) were observed by the primer combination E-ACT/M-CTT in BAAS53 accession. While checking the unique fragments across the accessions, BAAS53, BAAS57, OJC1, OJC2 and IJC11 showed highest number of unique fragments. In general, higher number of unique fragments were recorded in accessions coming from Chhattisgarh (BAAS53 and BAAS57).

3.2.2. Rare fragments

AFLP fragments observed in less than 10% of the accessions with a given primer combination were considered as rare fragments (NRF). A total of 108 rare fragments were observed with an average of 15.4 rare fragments per primer combination (Table 3). Highest number of rare fragments (22) were detected by the primer combination E-ACA/M-CAA, followed by 18 rare fragments by the primer combination E-ACA/M-CAT. Lowest number of rare fragments (11) were observed by the primer combination E-AGC/M-CTA. Among the 48 accessions BAAS53, IJC11, OJC1 and OJC2 showed higher number of rare fragments than the other accessions.

3.2.3. Shared fragments

These are the fragments that are scored in 70% accessions for a particular locus. A total of 300 shared fragments (NSF) were observed with an average of 42.9 shared fragments per primer combination (Table 3). Highest number of shared fragments (63) were detected by the primer combination E-ACT/M-CTT, followed by 47 shared fragments by the primer combination (E-AGG/M-CTA and E-ACG/M-CAA). These fragments were recorded in accessions from different collections of Uttar Pradesh (UT), Gujarat (CSM), Chhattisgarh (BAAS) and Andhra Pradesh (SNES) accessions. Lowest number of shared fragments (30) were observed by the primer combination E-ACA/M-CAA.

3.2.4. Similar fragments

These are the fragments that are present in more than 70% accessions for a particular locus. A total of 213 similar fragments (NSIF) were observed with an average of 30.4 similar fragments per primer combination (Table 3). Highest number of similar fragments (44) were detected by the primer combination E-AGG/M-CAG, followed by 42 with the primer combination E-AGG/M-CTA. Lowest number of similar fragments (15) were observed by the primer combination E-ACA/M-CAT. Majority of the similar fragments were recorded among UT accessions (e.g., UT1 (Uttar Pradesh) and UT2 (Gujarat), UT3 (Madhya Pradesh) and UT4 (Madhya Pradesh), UT5 (Uttar Pradesh), UT6 (Chhattisgarh) and UT7 (Uttar Pradesh), CSM accessions (e.g., CSM1 and CSM3 from Gujarat), BAAS accessions (e.g., BAAS30, BAAS35 and BAAS38 from Chhattisgarh) and OJC accessions (e.g., OJC6 and OJC7 from Andhra Pradesh).

3.3. Marker attributes

3.3.1. Polymorphism information content (PIC)

The PIC value for 680 polymorphic fragments ranged between 0.05 and 0.50 with an average of 0.25 per fragment. Majority of the fragments (114) showed the PIC value between 0.45 and 0.50, while 89 fragments showed lower (<0.05) PIC value (Fig. 2). The remaining 566 fragments showed moderate PIC value between 0.05 and 0.45. In order to distinguish different primer combinations, the PIC values for all the polymorphic fragments generated by a primer combination were averaged to get an average PIC value for the corresponding primer combination. As a result, the highest PIC value (0.34) was observed for the primer combination E-ACT/M-CTT and the lowest PIC value (0.20) was recorded for the primer combination E-ACA/M-CAA (Table 4). Average PIC value per the primer combination was 0.26.

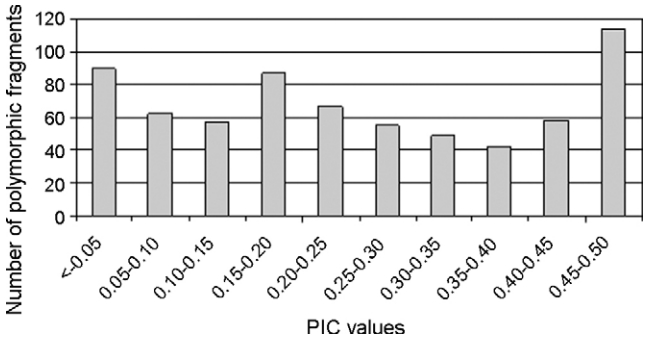

Fig. 2. PIC distribution for polymorphic fragments generated by AFLP primer combinations use.

3.3.2. Marker index (MI)

MI is a feature of a marker and therefore the MI values were calculated for all primer combinations. The MI values ranged from 17.60 to 32.30 with an average of 25.13 per primer combination. Highest value (32.30) was scored with the primer pair for E-ACT/M-CTT and the lowest value (17.60) for the primer pair E-ACA/M-CAA (Table 4). A positive correlation was observed between MI and PIC value ($r^2 = 0.66$, $p < 0.005$).

3.3.3. Resolving power (RP)

The RP is a feature of the primer combination that indicates the discriminatory potential of the primer combination. RP ranged from 23.11 to 46.82 with an average of 35.21 per primer combination. Highest value (46.82) was scored with the primer combination for E-ACT/M-CTT and the lowest value (23.11) for the primer pair E-ACA/M-CAA (Table 4). The RP values were found in positive correlation with MI ($r^2 = 0.99$, $p < 0.005$).

3.4. Genetic diversity and phylogenetic analysis

Genotyping data obtained for all 680 polymorphic fragments from seven primer combinations were used to estimate pairwise similarity comparisons among the accessions and a genetic similarity matrix was calculated using Jaccard's similarity coefficient by using NTSYS-pc programme. Subsequently, the genetic

Table 4
Marker attributes for AFLP combinations used.

Primer combinations	PIC[a]	EMR[b]	MI[c]	RP[d]
E-ACT/M-CTT	0.34	95	32.30	46.82
E-ACA/M-CAA	0.20	88	17.60	23.11
E-ACA/M-CAT	0.25	77	19.25	26.21
E-AGG/M-CAG	0.25	101	25.25	34.81
E-AGG/M-CTA	0.24	119	28.56	39.17
E-ACG/M-CAA	0.24	117	28.08	38.84
E-AGC/M-CTA	0.30	83	24.90	37.53
Minimum	0.20	77	17.60	23.11
Maximum	0.34	119	32.30	46.82
Average	0.26	97	25.13	35.21

[a] Polymorphism information content (PIC).
[b] Effective multiplex ratio (EMR).
[c] Marker index (MI).
[d] Resolving power (RP).

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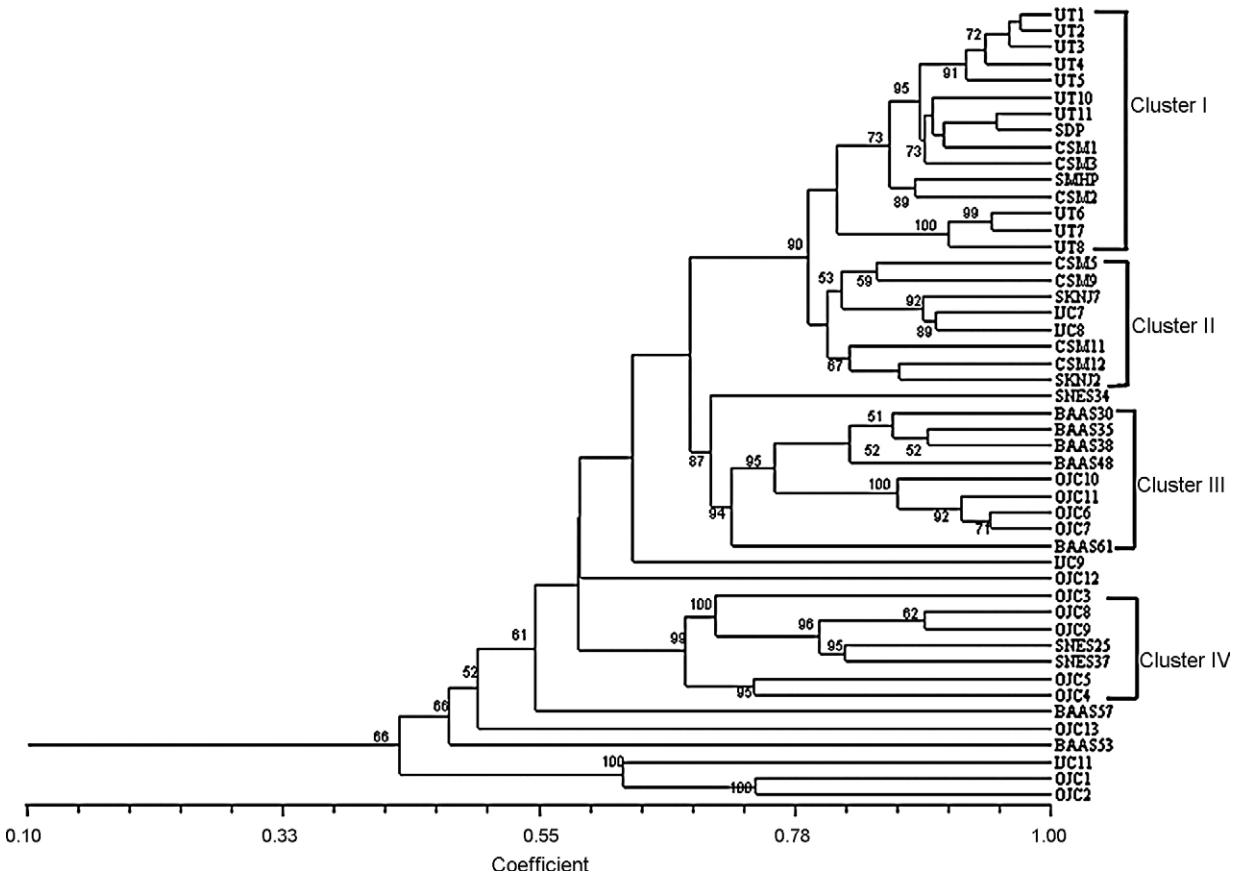

Fig. 3. UPGMA phenogram for of 48 Jatropha accessions based on genotyping data for 680 polymorphic AFLP fragments obtained by 7 primer combinations. The values on the nodes of the cluster indicate the bootstrap values higher than 50%. The scale represents Jaccard's similarity coefficient values.

similarity matrix was used for constructing the unweighted pair group method with arithmetic average phenogram with help of NTSYS-pc programme. The similarity coefficient values of the phenogram ranged from 0.43 to 0.97 suggesting a broad genetic base (Fig. 3).

In order to prepare a reliable phenogram, initially the individual data matrix for each of seven primer combination was used for preparing the phenogram. Majority of the major nodes generated in all the seven individual phenograms were in congruent except for some minor subgroups with in the subclusters (data not shown). Finally, the combined data matrix containing genotyping data for all seven primer combinations were used to generate the phenogram to understand the relationships among accessions examined.

This phenogram (Fig. 3) could be classified into four major clusters (Cluster "I", "II", "III" and "IV"). Cluster "I" contained a total of 15 accessions, 5 accessions from Uttar Pradesh (UT1, UT5, UT10, UT11 and UT7), 4 accessions from Gujarat (UT2, CSM1, CSM2 and CSM3), one each from Rajasthan (UT8) and Chhattisgarh (UT6) and similarly 2 each from Madhya Pradesh (UT3 and UT4) and Andhra Pradesh (SDP and SMHP). At least 7 accessions (UT1, UT2, UT3, UT4, UT5, UT10 and UT11) in this cluster showed high degree of homology (90% similarity). The Cluster "II" contained a total of 8 accessions that includes 6 from Gujarat (CSM5, CSM9, CSM11, CSM12, SKNJ2 and SKNJ7) and 1 each from Rajasthan (IJC7) and Madhya Pradesh (IJC8)). Cluster "III" grouped 9 accessions including 5 from Chhattisgarh (BAAS30, BAAS35, BAAS38, BAAS48 and BAAS61) and 2 each from Andhra Pradesh (OJC6 and OJC7) and Rajasthan (OJC10 and OJC11). The Cluster "IV" containing 7 accessions basically includes 6 accessions from Andhra Pradesh (OJC3, OJC4, OJC5, OJC9, SNES25 and SNES37) except the accession OJC8 that has come from Rajasthan. However, 3 accessions from Chhattisgarh (BAAS57, BAAS53 and OJC2) 2 each from Andhra Pradesh (SNES34 and OJC1), Madhya Pradesh (IJC9 and IJC11) and Rajasthan (OJC12 and OJC13) were not grouped in any clusters.

Genetic similarity matrix obtained based on Jaccard's similarity coefficient was also subjected for principal component analysis. Two-dimensional graph generated so grouped the accessions into five groups (Fig. 4). Group "I" contained 26 accessions from six states namely Gujarat (10), Uttar Pradesh (5), Madhya Pradesh (4),

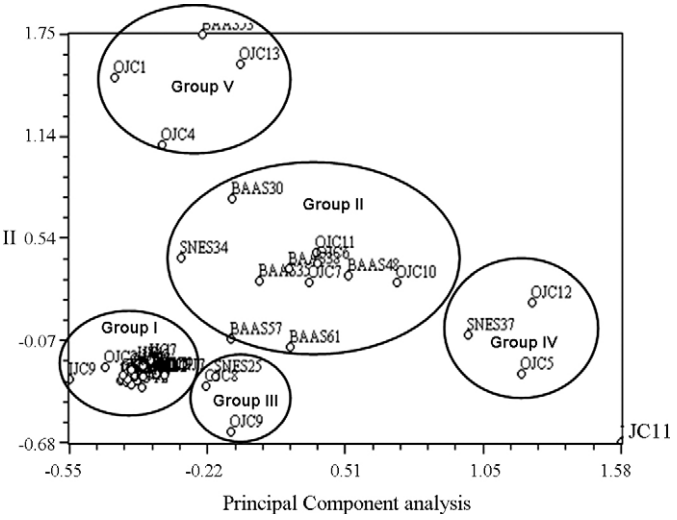

Fig. 4. Principal component analysis (PCA) of Jatropha accessions based on 680 polymorphic AFLP fragments.

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Andhra Pradesh (3), Rajasthan (2) and Chhattisgarh (2). The Group "II" contained 11 accessions from Chhattisgarh (6), Andhra Pradesh (3) and only 2 accessions from Rajasthan. The Group "III" and "IV" contained 3 accessions each (including 2 accessions from Andhra Pradesh) and 1 accession from Rajasthan. Group "V" contained 4 accessions coming from Andhra Pradesh (2), Chhattisgarh (1) and Rajasthan (1).

4. Discussion

AFLP is an information rich marker system due to its ability to generate a large number of polymorphic/informative loci simultaneously in a single lane with a single-primer combination as compared to RAPDs, RFLPs and microsatellites [25–27]. AFLP has been successfully used to investigate the diversity in several plant species including Euphorbiaceae members like *Hevea* [14], cassava [28].

In case of Jatropha, a few recent studies have been conducted to assess molecular diversity in the Jatropha germplasm collection. For instance Sujatha et al. [29] utilized RAPD markers to assess the genetic similarity between toxic Indian and non-toxic Mexican accessions. This study was extended further by converting RAPD markers into SCAR markers that differentiates Indian and Mexican accessions [5]. Similarly two other studies [15,16] involving molecular characterization of Jatropha identified markers for toxic and non-toxic varieties.

4.1. Optimization of DNA isolation protocol

High quality genomic DNA is a primary prerequisite for molecular genetic analysis of crops. Extraction of intact, high-molecular-weight DNA used for PCR, and fingerprinting is challenging when the plant tissue is rich in polysaccharides, secondary metabolites, or polyphenolics as these contaminants results in co-precipitation with genomic DNA yielding poor quality and quantity [30]. Therefore, significant changes were made in traditional CTAB protocol in the present study to isolate the good quality of genomic DNA of Jatropha. For instance, use of young leaf tissues with 4% CTAB solution and addition of PVP (3%) and repeating the purification steps twice, yielded intact and good quality DNA for AFLP assays. The protocol, available in Section 2, should be very useful for isolation of high quality DNA for not only Jatropha but also for other members of Euphorbiaceae family.

4.2. AFLP profiling and marker polymorphism

Efficiency of a molecular marker technique depends upon the amount of polymorphism it can detect among the set of accessions under investigation. High quality marker profiling was obtained with 7 AFLP primer combinations on 48 accessions collected from 6 states of India. All the primer combinations used were found polymorphic and yielded about 110 total and 97.1 (88.2%) polymorphic fragments per primer combination. The level of polymorphism detected by AFLP in the present study is comparable to the other AFLP diversity study (97.2%) to understand genetic divergence and phylogenetic analysis of genus Jatropha [15]. While analyzing frequency distribution of the polymorphic fragments across the AFLP primer combinations, higher number of polymorphic fragments were obtained in the frequency classes 0.0–0.10 and 0.90–1.0. However, when these data were analyzed together with PIC value data, it was found that higher number of polymorphic fragments generally have lower PIC values and therefore these fragments are not informative. Our analysis suggests that fragments falling under 0.50–0.60 are highly informative (average PIC value 0.50) following by those present in classes 0.40–0.50 (average PIC value 0.49) and 0.60–0.70 (average PIC value 0.45).

The present study also surveyed the unique, rare, shared and similar fragments detected by different AFLP primer combinations, although such features, to the best of our knowledge, have not been reported in other AFLP studies. Detection of 59 unique fragments that are specific to only one accession and 108 rare fragments that are present in 10% of the accessions examined will be very useful to select the diverse accessions for plant improvement. Two primer combinations namely E-AGG/M-CTA and E-ACG/M-CAA were found most effective to detect higher number of unique fragments and a good number of rare fragments (Table 3). Therefore, these primer combinations should be useful for detecting such informative fragments at least in Jatropha germplasm, if not in general across the other plant species. It is also important to note that the accessions containing higher number of unique/rare fragments have come from Chhattisgarh (e.g. BAAS53 and BAAS57) and Andhra Pradesh (e.g. OJC1) Unique/rare fragments detected in particular accessions can be used to develop sequence tagged site markers to identify particular accession [31].

Occurrence of shared fragments/similar fragments in accessions are useful for understanding the relationships/similarity in accessions. For instance, accessions CSM1, CSM2 and CSM3 having the similar/shared fragments have come from Gujarat, and similarly the accessions BAAS30, BAAS35 and BAAS38 have come from Chhattisgarh. Such kind of information will be useful in molecular profiling of accessions from different geographical locations and/or deducing the probable geographical origin of unknown accessions.

4.3. Discriminatory power of AFLP primer combination

A number of marker attributes like PIC, MI and RP have been used in several studies to assess the informativeness or discriminatory power of the primer combinations for genetic diversity studies. Although PIC has been used most extensively in majority of diversity/marker studies [11,20]. Prevost and Wilkinson [21] introduced the concept of RP (resolving power) to assess the discriminatory power of AFLP primer combinations.

In terms of calculating the PIC value of different fragments obtained by a particular primer combination, the fragments showed an average PIC value 0.25. The maximum PIC value for biallelic markers like AFLP can be expected as 0.50. As mentioned earlier, we found higher PIC value (0.49) for the fragments which were detected in 50–60% of accessions. Based on our analysis (Fig. 1A and B), we recommend targeting the fragments occurring in 40–70% accessions, that showed PIC value in the range of 0.45–0.50, for diversity analysis in case a larger number of fragments (>200) are detected by a particular primer combination. Based on the overall PIC value (0.34) for different primer combinations, E-ACT/M-CTT primer combination is recommended for germplasm analysis of Jatropha.

Another parameter, MI (marker index) together with PIC value has been used to assess the informativeness of the AFLP primer combination in several crop species e.g. soybean (PIC = 0.32, MI = 6.14 [25]), wheat (PIC = 0.32, MI = 3.41 [32]), cornsalad (PIC = 0.25, MI = 4.47 [33]), etc. Our study has reported the MI in the range of 17.60–32.30 (average 25.13) and PIC in the range of 0.20–0.34 (average 0.26) which are comparable to the earlier studies. Although PIC and MI value have been useful to compare the informativeness of AFLP and other marker systems (e.g. [25]), discrimination of maximum number of accessions would be the most important feature of the given primer combination when the purpose of study is to distinguish accessions analyzed [18]. In order to assess the discriminatory power of AFLP primer combinations, Prevost and Wilkinson [21] used the new attribute-resolving power. The primer combinations used in the present study showed the RP values in the range of 23.11–46.82 (average 35.21). As

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Prevost and Wilkinson [21] and Fernandez et al. [31] observed a strong and linear relationship between the ability of a primer combination to distinguish accessions and RP, the primer combination E-ACT/M-CTT with the highest RP value (46.82) should be most informative primer combination for distinguishing the accessions. Indeed, the phenogram prepared based on genotyping data for this primer combination alone, is very much comparable to the reported phenogram, prepared based on genotyping data for all seven primer combinations, in the present study (data not shown). A very strong and positive correlation observed between RP value and MI ($r^2 = 0.99$, $p < 0.005$) in our study indicates the use of any of two parameters to select the informative primer combination.

4.4. Germplasm diversity

To understand genetic diversity in the germplasm analyzed, genotyping data obtained for all the seven primer combinations were used to generate the UPGMA-based phenogram and principal component analysis. Both UPGMA-phenogram as well as PCA displayed similar grouping of accessions with some minor deviation. For instance, UPGMA-phenogram classified the accessions into four clusters while PCA grouped them into five groups (with one accession IJC11 as outlier) as shown in several other studies (e.g. [5,34]). Majority of the accessions clustered/grouped in the phenogram/PCA were in accordance with their geographical location. To find the robustness and stability of the phenogram to group accessions in different clusters, the data were analyzed for bootstrap analysis with hundred replicates. Higher bootstrap values (>50) obtained for all the major nodes in phenogram indicate the stability of grouping of accessions in different clusters [34].

As percentage oil content data and 1000-seed weight were available for all accessions analyzed, an attempt was made to understand the correlation between different groups/clusters with percentage oil content and/or 1000-seed weight. However, any specific trend was not observed between groups/clusters with percentage oil content and 1000-seed weight. For instance, as per PCA, the group "I" contained accessions with 25–34.9% oil content, the group II contained accessions with 29.3–36.9% oil content, the group III had accessions with 28.7–39.4% percentage oil content while the groups "IV" and "V" included the accessions with 33.4–38.9% and 19.0–32.7% percentage oil content respectively. Similar was the case for 1000-seed weight.

The phenogram showed highest genetic similarity (Jaccard's similarity coefficient 0.97) between UT1 and UT2 that have come from Uttar Pradesh and Gujarat, respectively followed by UT3 and UT4 that have come from Madhya Pradesh. As the pedigree data are not available on the accessions analyzed, the higher genetic similarity indicates the higher probability of origin of all these accessions (UT1, UT2, UT3 and UT4) from the same source and eventually distribution to three different states (Uttar Pradesh, Gujarat and Madhya Pradesh). The PCA grouped all the accessions coming from Gujarat together in group "I" that indicates no introduction of any diversity in these accessions from outside or through breeding.

It is interesting to note that three accessions namely IJC11 (Madhya Pradesh), OJC1 (Andhra Pradesh) and OJC2 (Chhattisgarh) showed high genetic dissimilarity with other accessions in both phenogram as well as PCA. Indeed, the IJC11 was identified quite diverse accession in the study of [5] based on RAPD and ISSR marker data. The PCA showed wide scattering of all 12 accessions of Andhra Pradesh in all five groups: "I" (3), "II" (3), "III" (2), "IV" (2) and "V" (2). Furthermore, majority of the accessions from Andhra Pradesh and Chhattisgarh also showed higher amount in oil percentage content. These facts indicate the presence of higher amount of genetic diversity in the accessions of Andhra Pradesh.

In summary, the present study provided a larger number of reliable and reproducible fingerprint profiles for an elite collection of 48 accessions of Jatropha collected from six states of India. On one hand analysis of different marker parameters established the fact that probably RP is the best parameter for assessing the discriminatory power of AFLP primer combination, on the other hand, a higher genetic diversity was documented in the accessions of Andhra Pradesh and Chhattisgarh. Availability of unique or rare fragments present in different accessions together with genetic dissimilarly data would be very useful for improvement of the species through conventional breeding methodologies as well as molecular breeding approaches such as marker assisted selection (MAS).

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