International Journal of ChemTech Research
CODEN(USA): IJCRGG ISSN : 0974-4290
Vol.5, No.2, pp 735-740, April-June 2013

ICGSEE-2013[14th – 16th March 2013]
International Conference on Global Scenario in Environment and Energy

Molecular Characterization Of Jatropha Genetic Resources Through Amplified Fragment Length Polymorphism (AFLP) Markers

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Abstract: *Jatropha curcas* was emerged as one of the major biodiesel crop in the last decade. In this study 6 Jatropha species namely *J. curcas, J. integerrima, J. glandulifera, J. podagrica, J. dioca,* and *J. gossypifolia* were analysed for genetic diversity using four AFLP primer combinations. Four AFLP primer combinations produced a total of 178 fragments with an average of 44.5 fragments per primer combination. A total of 167 (93.80%) fragments showed polymorphism in the germplasm analyzed. In order to access the discriminatory power of four primer combinations used, a variety of marker attributes like polymorphism information content (PIC), percentage polymorphism and effective multiplex ratio were calculated. The polymorphic information content (PIC) (0.38), effective multiplex ratio (EMR) (41.75) showed the efficiency of used primer combinations. The Jaccard's similarity coefficient ranged from 0.27 to 1. Dendrogram generated by unweighted pair group method for arithmetical averages (UPGMA) cluster analysis revealed the level of similarity among 6 Jatropha species and maximum similarity was observed between *J. curcas* and *J. dioca* which was 59 %, *J.` integerimma* (53%). was found next to it in similarity matrix generated. *J. glandulifera* showed highest dissimilarity with other Jatropha species. For this study AFLP was found to be a reliable molecular marker technique and provides one of the most informative approaches to establish genetic relationships relatedness in Jatropha, which may also be true for other related species.
Keywords: *Jatropha curcas*; Biodiesel; AFLP.

Introduction

Jatropha was emerged as a renewable energy source, alternative to petro diesel. Due to an increasing demand for energy and declining fossil fuel resources[1] biofuels were worldwide recognized as an alternative source of energy. The genus Jatropha was very diverse, belongs to tribe Joannesieae in the Euphorbiaceae family and contains approximately 175 known species[2]. Different species of Jatropha are widely spread all over the world. *J. curcas, J. integerrima, J. glandulifera, J. podagrica, J. dioca,* and *J. gossypifolia* were collected from different locations in India. Seeds of *J. curcas* contain 20-40% oil in it[3]. It was found that energy value of *J. multifida* oil was highest (13.647 kcal/g) and the oil samples of *J. curcas, J. glandulifera, J. gossypifolia* and *J. multifida* were rich in oleic and linoleic acids (72.2-84%). The oil content in all the four species of Jatropha ranged from 27.2-48.5%, being highest in

J. curcas[4]. Jatropha oil was non edible, it was suggested that that the factor responsible for toxicity in *Jatropha curcas* was phorbol esters present in high quantity in its toxic varieties[5]. Curcin is one of the major toxins present in Jatropha which deteriorates the quality of its oil and make it non edible for use. Curcin cleaved the N- glycosidic bond of adenine A4234 of 28s r RNA. As a result of this ribosomes were unable to bind to the elongation factors 1 or 2; hence protein synthesis was stopped[6-8].

Molecular markers were based on DNA polymorphism that was readily detected and whose inheritance can be monitored easily. Random amplified polymorphic DNA (RAPD), inter simple sequence repeats (ISSR), restriction fragment length polymorphism (RFLP), simple sequence repeats (SSR) and amplified fragment length polymorphism (AFLP) markers were some of the commonly used molecular markers. AFLP is a firmly established molecular marker technology with broad applications in linkage mapping, parentage analysis, population genetics and genetic diversity analysis. It is a combination of RAPD and RFLP technique which involves both restriction digestion and PCR amplification. Compared to RAPD, ISSR and DAMD markers, amplified fragment length polymorphism (AFLP) and simple sequence repeat (SSR) markers were more suitable for diversity analysis and fingerprinting because of their consistent results and cost effectiveness.

Materials And Methods

Genomic Dna Extraction

Genomic DNA was extracted from 06 species of *Jatropha* (*J. curcas, J. integerrima, J. gossypifolia, J. glandulifera, J. Podagrica* and *J. dioca*) collected from different locations of India (Table.1) and established in experimental farm of "The Energy and Resources Institute", New Delhi, India. Fresh leaves were lyophilized for 24 hours in a Freezemobile G lyophiliser (VirTis, New York, USA). DNA was isolated from lyophilized tissue using CTAB based method with few modifications earlier standardized in this laboratory[9,10].

Table 1. List of Jatropha species and details of their collection site

S.No.	Scientific name	Source/Collection site	State	Latitude and longitude
1	*J. curcas*	Eluru	Andhra Pradesh	16.7000°N, 81.1000° E
2	*J. integerrima*	South Delhi	New Delhi	29.0167°N, 77.3833° E
3	*J. gossypifolia*	Bhilwada	Rajasthan	25.3500°N, 74.6300° E
4	*J. glandulifera*	Ghaziabad	Uttar Pradesh	28.6618°N, 77.4242° E
5	*J. podagrica*	Chandrapur	Maharashtra	19.9500°N, 79.3000° E
6	*J. dioca*	Gurgaon	Haryana	28.4700°N, 77.0300° E

AFLP Method

Amplified fragment length polymorphism (AFLP) method was (11) used for the assessment of genetic diversity between the species as well as within the species of several plant crops. AFLP approach has been preferred over other molecular marker techniques such as RAPD and ISSR due to their high multiplex ratio and reproducibility. AFLP analysis was carried out using the standard AFLP protocol[11]. In brief, 250 ng of DNA was digested in 30 µl volumes using 5 µl of 5X restriction ligation buffer containing ATP, 2.0 µl of 0.5 M NaCl, 1.50 µl of BSA (1 mg/ml), 5 units of *Mse* I and 5 units of *Eco* RI at 37ºC for 2 hours followed by enzyme inactivation at 65ºC for 10 minutes. Adapter ligation was done at 18ºC for 1 hour 45 minutes by adding 10 pmol of *Eco* RI adaptor, 50 pmol of *Mse* I adaptor and 1 unit of T_4 DNA ligase to the restriction mix. The ligation mix was diluted to 1:10 in TE buffer (10 mMTris, 0.1 mM EDTA) and 5 µl of diluted ligation reaction was used as template for amplification with adapter specific primers *Eco* RI + A and *Mse* I + C in a total of 20 µl volume. The PCR reaction was performed in a Gene Amp PCR 9700 Thermal cycler (Applied biosystems) using following cycling parameters: 20 cycles of 30 s at 94ºC, 60 s at 56ºC and 60 s at 72ºC. The pre-amplification mix was diluted 50 fold for selective amplification. The selective amplification was carried out using *Eco* RI and *Mse* I primers with 2 selective nucleotides respectively in a total of 10 µl reaction volumes employing following PCR parameters: 1 cycle of 30 s at 94ºC, 30 s at 65ºC and 60 s at 72ºC. The annealing temperature was reduced by 0.7ºC per cycle during the first 11 cycles. The subsequent 23 cycles were performed at 94ºC for 30 s, 56ºC for 30 s and 72ºC for 70 s. The

samples were size-fractionated on 6% polyacrylamide gels and next day AFLP fragments were detected by autoradiography.

Data Analysis

The AFLP loci (bands) were scored manually for their presence (denoted as `1') or absence (denoted as '0') for each primer combination. The amplicons scored were in the size range of 75-450 bp. Only distinct bands were taken up for the analysis. The binary matrix was used to estimate genetic similarity coefficient using Jaccard's coefficient [GS_1= a / (a+b+c)],[12].Where GS is the measure of genetic similarity between individuals 'i' and 'j', '*a*' is the number of bands present in both 'i' and 'j' while '*b*' is the number of bands present in 'I' and absent in 'j', and '*c*' is the number of bands present in j and absent in 'i'. Clustering was done which was based on UPGMA (Unweighted Pair Group Method of Arithmetic Averages)[13]. It was based on Euclidean distance and squared for pair-wise comparisons among all species evaluated to construct phenetic dendrograms. All the above-mentioned statistical analysis was performed using NTSYS pc software (version 2.1)[14]. The total numbers of bands within each primer combinations were scored to arrive at the proportion of polymorphic loci. Genotypic data from AFLP analysis were used to assess the inequitable of the respective assays by evaluating, (a) polymorphic information content (PIC) (b) effective multiplex ratio (EMR) (c) percentage polymorphism (PP) etc.

Results

AFLP data was analysed in the form of 0-1 binary data. Six different species of Jatropha viz., *J. curcas, J. integerrima, J. gossypifolia, J. glandulifera, J. podagrica* and *J. dioca* were studied for assessment of genetic diversity. Four primer combinations generated a total of 178 bands, out of which 167 were polymorphic (Table 3). Maximum polymorphic bands were obtained by primer combination E-AA x M-CTA while minimum where observed for primer combination E-AG x M-CAG. Percentage polymorphism was calculated for each primer combination individually.

Table 2. Sequence of *Eco* RI and *Mse* I adaptors

Adapters	Sequence
Eco RI adapter	5'-CTCGTAGACTGCGTACC-3' 3'-CATCTGACGCATGGTTAA-5'
Mse I adapter	5'-GACGATGAGTCCTGAG-3' 3'-TACTCAGGACTCAT-5'

Table 3. Different marker attributes calculated from AFLP data

Name of Primer	Number of bands	Polymorphic bands	Monomorphic bands	% polymorphism	PIC
E-AGxM-CAG	37	31	6	83.78	0.32
E-AGxM-CTA	48	47	1	97.91	0.40
E-AAxM-CAG	38	35	3	92.1	0.40
E-AAxM-CTA	55	54	1	98.18	0.39
Total	178	167	11		
Average	44.5	41.75	2.75	92.99	0.38

Table 4. Similarity matrix generated by UPGMA cluster analysis

Species	*J. curcas*	*J. integerimma*	*J. gossypifolia*	*I. glandulifera*	*J. podagrica*	*J. dioca*
J. curcas	1.00					
J. integerimma	0.53	1.00				
J. gossypifolia	0.40	0.34	1.00			
J. glandulifera	0.24	0.31	0.30	1.00		
J. podagrica	0.36	0.39	0.29	0.23	1.00	
J. dioca	0.59	0.51	0.39	0.28	0.37	1.00

Primer combination E-AG x M-CAG (83.78%) was least polymorphic while E-AA x M-CTA (98.18%) showed highest polymorphism among all four primer combinations. Very high (92.99 %) average percentage polymorphism obtained in this study, as all accessions belongs to different species of Jatropha. On the basis of marker frequency polymorphic information content (PIC) was calculated which was highest for the primer combination E-AG x M-CTA and E-AA x M-CAG (0.40) and lowest for primer combination E-AA x M-CAG. The average PIC value for all markers was 0.38. Effective multiplex ratio (EMR) was 41.75 for all the four primer combinations. As a result of UPGMA cluster analysis, maximum similarity of *J. curcas* was observed with *J. dioca* (59%) then with *J. integerrima* (53%), *J. gossypifolia* (40%), *J. podagrica* (36%) and *J. glandulifera* (24%). Dendrogram (Figure 1) was generated by unweighted pair group method for arithmetical averages (UPGMA) using the Jaccard's similarity coefficient. In dendrogram similar result was depicted as with similarity matrix (Table 4). Two major clusters formed, in which *J. curcas, J. dioca, J. integerrima, J. gossypifolia, J. podagrica* grouped together under same cluster at different similarity levels while *J. glandulifera* grouped separately in another cluster. Jaccard's similarity coefficient lies between 0.27 -1.00. Average genetic diversity obtained was 63.13 %.

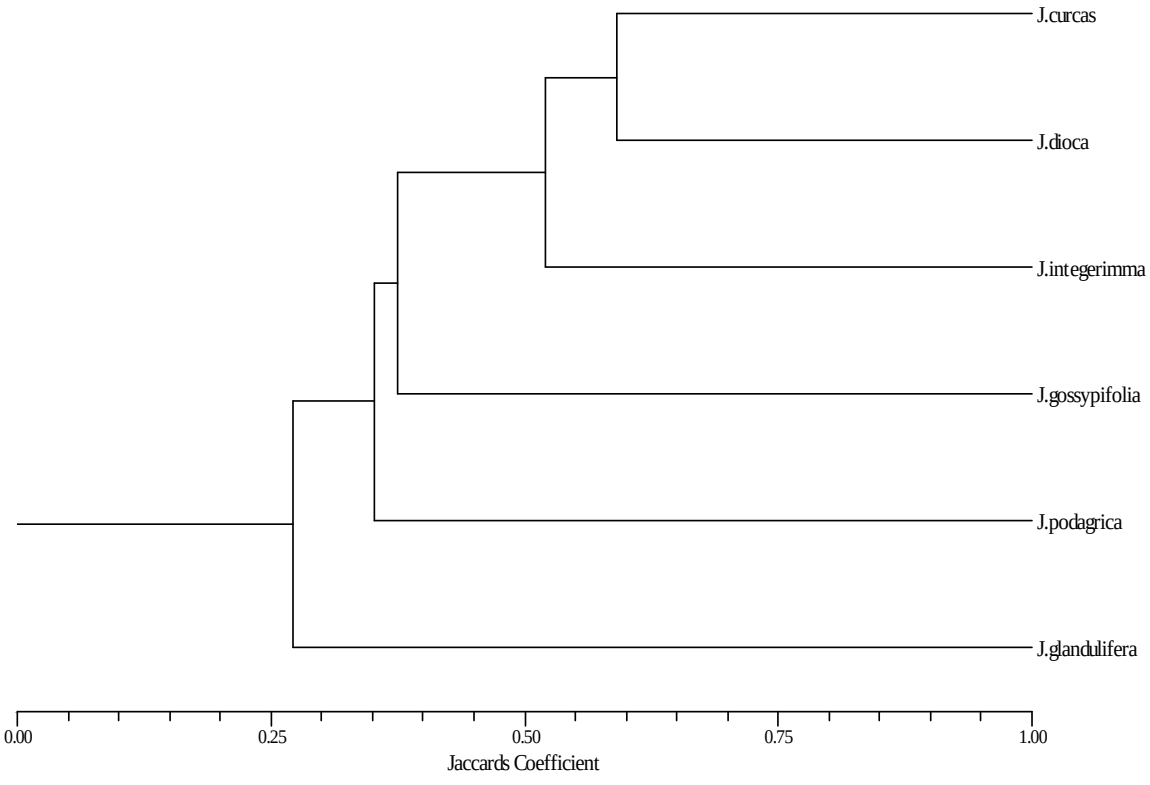

Fig 1. Dendrogram generated by NTSYS pc software version 2.1

Discussion

Level of genetic diversity was very low in Indian *Jatropha curcas*. RAPD and ISSR markers were used to analyze genetic diversity and found 42% polymorphism with 400 RAPD markers while, 33.5% polymorphism with 100 ISSR primers[15]. Similarly AFLP markers were used to analyze 58 *J. curcas* accessions from China and found that only 14.3% bands were polymorphic[16]. Genetic diversity of different Jatropha species were also tested by various molecular markers like RAPD, ISSR, AFLP and microsatellite markers. The genus Jatropha is native of Tropical America with more than 200 species widely distributed in tropics. RAPD and AFLP markers were used for the comparative study of interspecific genetic divergence and phylogenic analysis of genus Jatropha[17]. RAPD analysis showed that *J. glandulifera* was the most diverged species among the Jatropha species studied. They found maximum similarity between *J. curcas* and *J. integerimma* and supported the view that these were the most compatible species and can be used for the development of interspecific hybrids in Jatropha. Similar results were[18] observed and illustrated the distinctness of *J. glandulifera* from other species of Jatropha. They reported that the GS among *J. curcas* accessions were highest and GS between *J. curcas* and *J. integerrima* was next to it. The PP (percentage polymorphism) among the seven species of Jatropha was found to be 97.74 by RAPD and 97.25 by AFLP. As the considerable level of similarity exhibited between *J. curcas* and *J. integerrima*[19], inter specific hybrids successfully developed in them. In our study, 92.99 % polymorphism was obtained among different species of Jatropha. Of which *J. curcas* and *J. dioca* were clustered together and showed maximum similarity of 59%, while *J. integerrima* was next to it and showed 53% similarity to *J. curcas.* Study of diversity among different species of Jatropha will help in proper understanding of compatability behavior among them and use them as parents for further breeding purpose for the genetic improvement of *J. curcas*. *Jatropha curcas* contains 30-40 % oil in its seed[20] that can provide a sustainable and economical substitute for diesel. Palmitic, stearic, oleic and linoleic acids were commonly found fatty acids in Jatropha. Other species of Jatropha like *J. gossypifolia, J. integerrima, J. glandulifera* and *J. multifida* were found to be rich in linoleic acids (51.3-73.9%) whereas *J. curcas* contained high oleic acid (49%)[21]. Palmitoleic acid was reported to be found in *J. curcas*[22]. Whereas it was reported that *J. gossypifolia* oil was rich in palmitic acid (31.4%) which was a saturated fatty acid[23]. By incorporating all these findings interspecific hybrids will be developed in Jatropha which contained high levels of useful fatty acids as per the requirement. We can improve oil characteristics; yield performance etc and the generating germplasm can be released as variety which will be further used in diesel industry for the production of biodiesel of high quality to fulfill the increasing demand of fuel against present energy crisis. Knowledge and proper understanding of breeding behavior, existing genetic diversity, and floral mechanism will help the researchers for developing better strategies for developing Jatropha as a future biodiesel crop. These results were helpful for providing valid guidelines for collection and conservation of germplasm and for further characterization of Jatropha genetic resources.

Conclusion

Genetic diversity analysis in six species of Jatropha using four AFLP primer combinations proved very useful for the further genetic improvement programs in it. It paved way for the use of other species of Jatropha in molecular breeding so that their useful characters can also be exploited for genetic enhancement of oil trait and yield performance in *Jatropha curcas*. Different species of Jatropha have different useful traits which can be utilized for eg. stem hardiness, frost tolerance in *J. integerrima* and *J. gossypifolia*. AFLP markers also proved to be very useful for such type of interspecific genetic diversity studies.

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