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Analysis of Genome-Wide Homozygosity in *Jatropha curcas* Accessions Using AFLP Markers

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ABSTRACT

Genetic diversity and level of heterozygosity at amplifed fragment length polymorphism (AFLP) loci was analyzed in the bioenergy species, *Jatropha curcas*, using seven candidate plus tree accessions and their progeny individuals. Seven AFLP primer combinations resulted in scoring of 435 bands out of which 180 (41.3%) were polymorphic across the seven accessions. The majority of polymorphisms were due to inclusion of the two exotic accessions, JIP74 and JIP75. The proportion of segregating bands in these progeny arrays varied from 0 to 31% of the total bands present in the respective parental accession. All the five Indian accessions were found to be homozygous for majority of the AFLP loci detected in this study. The results reveal extremely narrow genetic diversity and high level of genome-wide homozygosity in the Indian accessions but a considerably high level of heterozygosity in the exotic accessions. The probable causes and potential implications of these findings are discussed.

KEYWORDS: AFLP, Biofuels, Genetic diversity, *Jatropha curcas*, Outcrossing rates

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INTRODUCTION

Jatropha curcas (family- Euphorbiaceae) has received great attention as a source of seed oil for biodiesel[1].It is supposedly a native of Mexico and Central America from where it was brought to other countries including India[2].During past decade, efforts have been initiated towards genetic improvement of Jatropha through different breeding and selection approaches.

The currently used approach in Jatropha involves selection of accessions from different geographical regions and their field evaluation through performance trials[2].This is a widely used strategy in majority of forestry species such as eucalyptus and teak[3].In this strategy, the individual tree(s) (also called accessions) are selected, based on a set of predefined parameters, for example, seed and fruit characters, branching habit etc. These accessions (now called candidate plus trees) are then evaluated under field trials for their agronomic value. This strategy is based on the presumption that there is a high rate of outcrossing in these trees and the individual trees are more likely to be heterozygous at majority of loci. It is also assumed that an out breeding population would have higher levels of genetic diversity. The genetic diversity in a population depends on several factors such as the genetic diversity in the founder population, mating system, migration and mutation rates. *J. curcas* bears unisexual flowers where pollination is mediated by insects. So a priory, the mating system of *J. curcas* seems to favour outcrossing. The individual trees are, therefore, expected to be highly heterozygous and genetically distinct. However, no systematic study has been reported so far to prove or disprove these assumptions.

AFLP technique[4] has been widely used for characterization of genetic resources including Jatropha due to its high reproducibility and high multiplex ratio[5,6,7].Further, AFLP markers can be generated in large numbers without any prior nucleotide sequence information in the species under investigation. However, AFLP loci exhibit dominance so that a homozygote for band 'presence' cannot be distinguished from a heterozygote. The AFLP genotype of an individual can only be inferred from the AFLP genotype of its selfed progeny which would display segregation at all heterozygous loci present in the parent individual.

In this study, we used progeny individuals of selected accessions of Indian and exotic accessions to estimate the level of heterozygosity in these accessions using AFLP markers. We also looked for possible outcrossing events in these progenies. We reasoned that presence of any non-maternal band in the progeny should be indicative of outcrossing events during the formation of the new generation while segregation of any maternal band in the progeny should be indicative of its heterozygous state in

the mother plant which might have resulted from outcrossing events in preceding generations. Finally, we discuss the possible reasons and implications of these findings in genetic improvement of Jatropha.

MATERIALS AND METHODS

Two datasets were used in this study. The first dataset was designed to detect the levels of heterozygosity in the mother plants based on genotypes of their progeny arrays. This included a total of seven mother plants and 32 progenies individuals.

Table 1: Details of the plant material used

Sl No	Sample ID	Place/ origin
1	JM01	Madurai, TN
2	JM01/A	Progeny of JM01
3	JM01/B	Progeny of JM01
4	JM01/E	Progeny of JM01
5	JM01/G	Progeny of JM01
6	JM-15	Guwahati, Assam
7	JM-15/A	Progeny of JM-15
8	JM-15/B	Progeny of JM-15
9	JM-15/C	Progeny of JM-15
10	JM-33	Udaipur, Rajasthan
11	JM-33/A	Progeny of JM-33
12	JM-33/B	Progeny of JM-33
13	JM-33/C	Progeny of JM-33
14	TERI-J21	Gurgaon, Haryana
15	TERI-J21/A5	Progeny of TERI-J21
16	TERI-J21/A7	Progeny of TERI-J21
17	TERI-J21/A8	Progeny of TERI-J21
18	TERI-J21/A9	Progeny of TERI-J21
19	TERI-J21/A10	Progeny of TERI-J21
20	TERI-J21/A11	Progeny of TERI-J21
21	TERI-J21/A12	Progeny of TERI-J21
22	TERI-J21/A13	Progeny of TERI-J21
23	TERI-J21/A14	Progeny of TERI-J21
24	TERI-J21/A15	Progeny of TERI-J21
25	BL-3	Bhilwara, Rajasthan
26	BL-3/7	Progeny of BL-3
27	BL-3/8	Progeny of BL-3
28	BL-3/9	Progeny of BL-3
29	BL-3/10	Progeny of BL-3
30	J74	Mexico
31	J74-1	Progeny of J74
32	J74-2	Progeny of J74
33	J74-3	Progeny of J74
34	J75	Mexico
35	J75-1	Progeny of J75
36	J75-2	Progeny of J75
37	J75-3	Progeny of J75
38	J75-4	Progeny of J75
39	J75-5	Progeny of J75

For developing these progeny arrays, selfed seeds of the seven selected accessions were obtained by bagging the whole inflorescences (containing male and female flowers) and manually dusting the stigma with self-pollen. The collected seeds were germinated in greenhouse trays filled with a mixture of garden soil and sand (1:1). Each accession used in this study was selected from the germplasm bank of TERI. Five of these accessions were originally from distant geographical locations of India and two were exotic accessions from Mexico (table 1). Three to 10 individual seedlings from each progeny array were selected randomly for analysis.

The second dataset was used to detect the level of outcrossing in field conditions. This included a progeny array generated from open pollinated seeds from one of the parental accession, TERI-J21, from a field plot where the highly distinct accessions JIP74 and JIP75 were planted around its vicinity within an aerial distance of three to five meters. A total of 20 progenies were used in this dataset.

Young apical leaves were collected from mother trees and 3-month old seedlings and lyophilized for 24 hours in a freeze drier (Freezemobile G, VirTis, New York, USA). DNA isolation was done following CTAB based method with few modifications earlier standardized in this laboratory[8,9].

AFLP REACTION

AFLP analysis was carried out using the standard AFLP protocol [Vos et al. [4]]. In brief, 250 ng DNA was digested in 25 µl volumes using 5 µl of 5X restriction ligation buffer containing ATP, 2.5 µl of 0.5 M NaCl, 1.25 µl of BSA (1 mg/ml), 5 units of *Mse* I and 5 units of *Eco* RI at 37°C for two and half hours followed by enzyme inactivation at 70°C for 10 minutes. Adapter ligation was done at 20°C for 2 hours by adding 6 pmol of *Eco* RI adaptor, 55 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 legation 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 Inc.) using following cycling parameters: 20 cycles of 35 s at 94°C, 65 s at 56.5°C and 65 s at 72°C. 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 and 3 selective nucleotides respectively in a total of 10 µl reaction volumes employing following PCR parameters: 1 cycle of 30 s at 94°C, 35 s at 65.5°C and 65 s at 72°C. The annealing temperature was reduced by 0.7°C per cycle during the first 11 cycles. The subsequent 23 cycles were performed at 94°C for 35 s, 56°C for 35 s and 72°C for 65 s. The samples were size-fractionated on 6% polyacrylamide gels and the 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 80-400 bp. Only distinct bands were taken up for the analysis. The binary matrix was used to estimate genetic similarity coefficient using Jaccard coefficients. Clustering was done based on UPGMA (Unweighted Pair Group Method of Arithmetic Averages) to construct phonetic dendrograms. All the above-mentioned

Table 2: AFLP primer combinations and their banding attribute across the seven progeny arrays

Sl No.	Name of primer combination	Total bands	Polymorphic bands	%
1	E-AG x M-CTG	65	21	32.31
2	E-AG x M-CTA	62	37	59.68
3	E-AG x M-CAG	72	31	43.06
4	E-AG x M-CAA	71	35	49.30
5	E-AG x M-CTG	55	18	32.73
6	E-AG x M-CAT	67	21	31.34
7	E-AG x M-CTC	43	17	39.53
	Total	435	180	41.38

statistical analysis was performed using NTSYSpc software (Exeter Software, Setauket, NY, version 2.02). The number of polymorphic bands within each progeny array was scored to arrive at the proportion of heterozygous loci or to detect the outcrossing events based on whether the band was present or absent in the mother plant.

Table 3: Distribution of segregating AFLP loci in different mother accessions

Mother plant	Number of progenies used	Number of bands in mother plant	Number of segregating bands	% of segregating bands
JM01	4	329	0	0%
JM15	3	329	1	0%
JM33	3	329	0	0%
TERI-J21	10	329	0	0%
BL-3	4	331	14	4%
J74	3	359	62	17%
J75	5	415	130	31%

RESULTS AND DISCUSSIONS

In the first dataset, seven AFLP primer combinations were used to generate banding profiles in seven progeny arrays along with their respective maternal parents. A total of 435 bands were scored out of which 180 (41.3%) were polymorphic across all the 39 samples (Table 2). Across the two exotic accessions, JIP74 and JIP75, 166 out of 430 bands (38.6%) were polymorphic whereas among the Indian accessions, only 24 bands out of 333 bands (7.2%) were found to be polymorphic across the five accessions. The majority of the polymorphic bands were due to variations among the two exotic accessions, JIP74 and JIP75. The Jaccard's similarity coefficient ranged from 1 among Indian accessions to 0.767 between JIP74 and Indian accessions. All five Indian accessions clustered together showing almost no divergence among themselves (Fig. 1). The exotic accessions separated out from this cluster as well as from each other showing high divergence from each other as well as from Indian accessions.

The proportion of segregating bands within each progeny array varied from 0% to 31% (Table 3). Four out the five Indian accessions (JM01, JM15, TERI J-21 and JM33), were found to be homozygous for all AFLP loci scored in this study. The accession BL-3 had about 4% of the loci in heterozygous condition. The exotic accessions, JIP74 and JIP75 had 17% and 31% of the loci respectively in heterozygous state.

The results of cluster analysis showed a surprisingly high level of genetic similarity between the five mother accessions from India. As these accessions were originally sourced from distant geographical locations of India, they were expected to be genetically distinct. However, the AFLP markers could not clearly distinguish all the five accessions (Fig. 1).

A number of research papers have been published during past few years on genetic diversity analysis of Jatropha[10,11,12,13]. Most of these studies have reported low genetic diversity within the accessions of *Jatropha curcas*. In fact, a major proportion of the polymorphism reported in these papers can be attributed to the use of other species of Jatropha or other genera of Euphorbiaceae as outliers [10,11,12.] Low genetic diversity has also been reported in *J. curcas* accessions from China[14]. Some of the studies have used *J. curcas* accessions from other countries (especially from Mexico) which explains the genetic diversity detected in these studies[13]. An exception in this regard is the recent study carried out at ICRISAT, India[7] where an extremely high level of genetic diversity (Jaccard's similarity coefficient up to 0.43) was revealed in accessions from India.

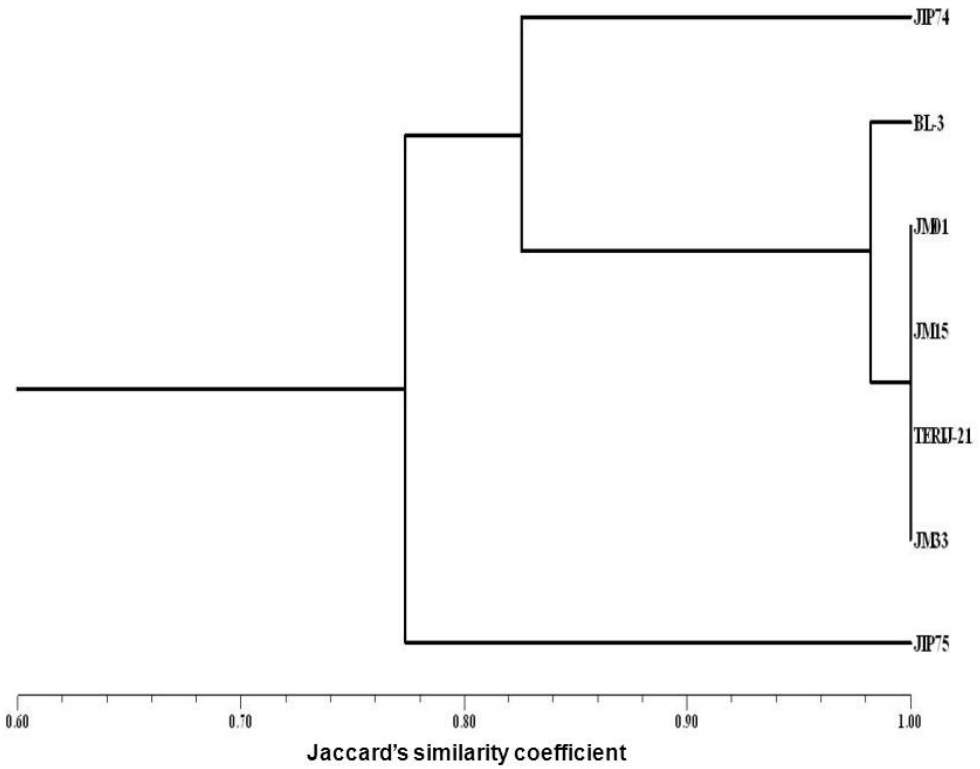

Fig. 1. UPGMA dendrogram of the mother plant accessions based on AFLP data using Jaccard's coefficient. A total of 435 AFLP loci containing 180 polymorphic loci were used to construct the dendrogram.

Our current study corroborates the fact that the overall genetic diversity among Indian accessions is extremely low. Indeed, studies carried out in this laboratory using AFLP markers on over 600 *J. curcas* accessions from different regions of India also revealed an extremely narrow genetic diversity and no clustering based on geographical affiliations of accessions was observed (unpublished data).

The AFLP loci are inherited in dominant fashion. Therefore, the homozygous genotype for the dominant-allele "band presence" (+/+) and heterozygous genotype (+/-) cannot be distinguished from each other[15]. However, any band present in the mother and polymorphic in the progeny array should be in heterozygous (+/-) state in the mother. On the other hand, any band absent in the mother plant but present in any of the progeny is possible only through cross pollination with an individual containing the band present allele (+) either in homozygous or heterozygous state. In the current analysis, none out of 329 loci scored in JM01, TERI-J21 and JM33 were found to segregate in their respective progenies. Only one out of 329 bands was found to segregate in the progeny array of JM15. In BL-3, 14 out of 331 bands were found to segregate in the progeny. Thus, overall percentage of segregating

loci in all five Indian accessions was very low indicating a genome wide homozygosity in these accessions. On the contrary, in JIP74 and JIP75, the number of segregating bands was 62/359 and 130/415 respectively showing that a large proportion of their genomes are in heterozygous state. No non-parental bands were observed in any progeny individuals used in this study showing complete absence of open pollination which was expected in this dataset due to controlled selfing.

In the second dataset, none of the 329 bands generated using seven primers were found to be polymorphic. This indicates that none of 20 progeny individuals was a product of outcrossing. The field information showed that none of the two accessions, JIP74 and JIP75 flowered simultaneously with TERI-J21 which might have led to monomorphism in the progeny individuals.

In the absence of any significant level of polymorphism detectable in other accessions planted in the vicinity of TERI-J21, it was not possible to estimate the outcrossing rates in this case. This is because many of the cross pollination events would become undetectable due to presence of identical alleles in the maternal plant and its surrounding individuals that could be potential pollen donors. Markers with high multiplex ratio such as AFLP and RAPD should be more informative in this condition because it is expected that some of the loci would be polymorphic between the pollen donor and the maternal parent. However, due to extremely high level of genetic similarity among the Indian accessions planted around TERI J-21, no outcrossing event was detectable. These results also show that all AFLP loci scored in TERI J-21 were in homozygous state which is similar to the findings of the first dataset where no segregating bands were found (Table 3). In a recent study, all the 245 microsatellite markers were found to be in homozygous state in two accessions of *J. curcas* from Singapore[16]. Absence of non-maternal bands in the progeny may have two possible explanations which are not mutually exclusive. First, the surrounding potential pollen donors had identical alleles as that of the maternal accession at all the surveyed loci so that any cross pollination between these genetically similar individuals is in fact a case of “biparental inbreeding” which is not distinguishable from selfing. Second, self-pollination could be common in Jatropha just because there is no self-incompatibility reported so far. A close observation of its flowering behavior shows that some of the male and female flowers of an inflorescence frequently open on same day which is one way that can lead to selfing. Further, it is not uncommon to see both male and female flowers on different branches of an individual plant opening on same days. Both of these conditions provide ample opportunities for self-pollination.

For India, *J. curcas* is an exotic species that was supposedly introduced in India by Portuguese travelers about 400 years ago. The extremely low level of genetic diversity reported in Indian

accessions of *J. curcas* could have resulted due to introduction of seeds from few related plants or from geographical sources which did not harbor high genetic diversity or both. It is also probable that very few number of introduction events of Jatropha germplasm took place in the past. High level of inbreeding due to narrow genetic diversity within the population is expected to increase homozygosity in subsequent generations. The results of the current study are in complete agreement with the results of most of the studies carried out so far on Indian Jatropha accessions.

The findings have serious implications in research activities related to genetic improvement of Jatropha. Low genetic diversity of Jatropha germplasm is apparently a major problem in countries where it has been introduced (and these are the countries where it is pursued the most as a biodiesel crop). A large number of field trials using Jatropha accessions from India are in progress. In absence of any significant variability not much genetic gain is expected through these trials. A common practice in Jatropha breeding is to select candidate plus trees based on morphological descriptions and oil content data of single individual trees as is common in case of eucalyptus or other forestry species. The oil content of Jatropha accessions from India has been reported to vary from 18% to 42%[17]. However, it is important to accurately dissect the genetic and environmental components of this variation before using this information and germplasm for genetic improvement through selection. Due to high genetic similarity among these accessions it is likely that a large proportion of these variations are not heritable. Such variations are possible due to the growing conditions of the plants and lot to lot variations in seed quality due to uneven procedures of seed handling. The strategy of candidate plus tree selection is expected to be most efficient when the source genepool contains high levels of genetic diversity. Indeed, some biofuel companies have already collected large number of diverse accessions of Jatropha from its centre of diversity such as Central America and surrounding areas. These accessions have also been evaluated for their productivity leading to significant improvement[18].

Several groups are currently working on genetic improvement of Jatropha through hybridization and selection. Under the current scenario, intra-specific hybridization between these “near-identical pure lines” is unlikely to provide any significant advances due to presence of identical alleles in the parental lines. Genetically, such crosses are equal to selfing. For the same reason, linkage mapping efforts based on pseudo testcross strategy[19] which has been widely used in tree species, is also unlikely to work due to highly homozygous status of a large proportion of loci in these accessions. However, the combination of exotic accessions, JIP74 and JIP75 used in this study is highly appropriate for generating the mapping population for linkage mapping using pseudo test cross strategy because a

large proportion of loci in these accessions are heterozygous which are expected to segregate in the progeny.

There is an urgent need to increase the genetic diversity of *J. curcas* germplasm in India and several other countries where it has been introduced. This can be done through world-wide exchange of accessions from diverse sources. Collections should be made from regions such as Mexico and Central America which are the centers of diversity for this species. Genetic improvement of Jatropha could then proceed through several approaches such as intra- and inter-specific hybridization, mutation breeding and genetic transformation.

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