Molecular characterization of genetic and epigenetic divergence in selected *Jatropha curcas* L. germplasm using AFLP and MS-AFLP markers

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Abstract

Jatropha curcas L. (*Euphorbiaceae*) has gained considerable importance as a renewable source of energy. Various molecular markers were employed to explore the genetic diversity in *J. curcas* germplasm. In present study, methylation-sensitive AFLP (MS-AFLP) markers were evaluated to analyze the divergence among five elite germplasm (CSMCRI- 1 to 5) of *J. curcas* and subsequently the results were compared with that of AFLP markers to assess the potential utility of MS-AFLP markers for phylogenetic characterization. Study compared the genetic (obtained through AFLP marker) polymorphism with that of epigenetic (obtained through MS-AFLP marker) polymorphism. Among the germplasms studied, 07 selective combinations of MS-AFLP primers produced 40 epigenetic (methylation) polymorphic markers, while 17 AFLP primers produced 105 genetic polymorphic markers. MS-AFLP analysis showed 44.44% epigenetic polymorphism while AFLP showed 15.20% genetic polymorphism. Epigenetic and genetic polymorphism among the germplasms ranged from 12.50% to 66.67% and 2.27% to 48.57% respectively. Highest mean epigenetic polymorphism was recorded in CSMCRI- 3, while highest mean genetic polymorphism was recorded in CSMCRI- 1. Results showed that the genetic and epigenetic structures of *J. curcas* were not same and epigenetic variations are greater than the genetic variations. Differences in DNA methylation and polymorphism among the studied genotypes under prevailing environment indicate evolution of genetic and epigenetic components in different directions. The study paves idea that genotypes exhibiting less genetic divergence might possess high epigenetic divergence and this along with genetic background could be a base for selection of elite cultivar adapted to particular geo-climatic conditions for further breeding program.

Keywords: AFLP, epigenetic, MS-AFLP, *Jatropha*, methylation, polymorphism.

Introduction

The continuous exhaustion of conventional sources of fuel and ever-increasing demand for energy resources especially for petro-diesel, rekindled the interest in utilization of alternative

non-conventional sources of energy. The use of renewable sources of energy is essential for developing economies worldwide as the availability of energy resources determines the economic feasibility of a nation. Among various renewable sources of energy, plant derived biofuel is one of the most suitable alternatives to the fossil fuel and has gained importance as a substitute of conventional petro-diesel. In recent past, *Jatropha curcas* L. (*Euphorbiaceae*), commonly called Jatropha has drawn interest as a potential biodiesel feedstock. Besides Jatropha; Soybean, Sunflower, Palm, Rapeseed, Canola and Cotton are considered major biofuel crops, however all these produce edible oil while Jatropha produces non-edible oil. Higher prices of edible oil, dilemma for conversion of edible oil into petro-diesel due to food security, short gestation period for Jatropha, superior performance of Jatropha biodiesel over the conventional petro-diesel, use of wasteland for Jatropha cultivation and easy adaptation of Jatropha on different kinds of marginal land thus leaving arable land for agriculture practices, drought endurance of certain limits and avoidance by animals for grazing purpose make *J. curcas* a candidate crop for biodiesel production. Besides all the attractive attributes, the development of Jatropha as candidate biodiesel crop to meet future energy requirements is eagerly awaited [Krishnan and Paramathma, 2009].

Jatropha is characterized by variable and unpredictable yield for various reasons [Ginwal et al., 2005], that warrants the need for genetic improvement of the plant species. For collection, conservation and genetic improvement of a plant species, characterization of both phenotypic and genetic diversity to understand the genetic background of a plant species is the preliminary requirement. To assess morphological variations, very few systematic provenance trials were conducted using various Jatropha collections and clear phenotypic, physiological and biochemical differences among collections were reported [Heller, 1996; Kaushik et al., 2007; Popluechai et al., 2009]. On the other hand, various molecular marker systems namely RAPD, ISSRs, SSRs, AFLP, nrDNA ITS and combinatorial tubulin based polymorphism (cTBP) were employed to study the extent of genetic diversity in *J. curcas* [Populuechai et al., 2009; Xu et al., 2012; Mastan et al., 2012a; Sudeer et al., 2010; Basha and Sujatha, 2007; Basha et al., 2009; Tatikonda et al., 2009]. Also molecular markers were reported for identification of non-toxic from toxic, less phorbol ester containing, genotypes [Basha and Sujatha, 2007; Sudheer et al., 2009; Mastan et al., 2012b]. Wide variations among biochemical (protein, oil and phorbol ester) compositions [Basha et al., 2009] and other phenotypic traits [Yi et al., 2010] have been

established, however molecular marker based studies revealed narrow genetic diversity in *Jatropha* except polymorphism in toxic and non-toxic genotypes.

In present scenario, the yield potential of *Jatropha* accessions is low due to the cultivation of uncharacterized germplasm or non-availability of consistently high yielding genotypes for *Jatropha* plantation. *Jatropha* possess climate-specific adaptations to a varying degree and show easy adaptation on different kinds of marginal land. Thus it is important to characterize the germplasm adapted to particular geo-climatic conditions. Ecologically adaptive epigenetic variations in natural population have been reported independent from genetic variations [Bossdorf et al., 2008] and these are inherited as pre-adaptations by subsequent generations in the form of epigenetic memories. These memories and natural epigenetic variations have been reported responsible for phenotypic variations and capability of an individual to adapt to ecological niches [Pascual et al., 2014]. Epigenetic processes seem to play a crucial role as a linker between the environment and the genome being involved in growth and development under prevailing environment. Epigenetic characterization is a very promising way to obtain genotypes pre-adapted to different environmental conditions. Characterization of *Jatropha* germplasm having higher seed yield and oil content with well adaptation to different environment conditions would help to develop varieties with high potential *Jatropha* crop. *J. curcas* as an undomesticated and potential biodiesel crop, naturally exhibiting narrow genetic base at a global level [Popluechai et al., 2009]. In addition to the diversity analysis, epigenetic characterization would add to the breeding program for genetic improvement through selection of genotypes adapted to a particular environment.

Among various epigenetic changes, methylation of cytosine in DNA strand is an important epigenetic change and has been suggested to be associated with alteration of gene transcription leading morphological changes hence altered phenotypes. The changes in methylation have been shown both temporal [Bossdorf et al., 2008] providing plasticity of gene expression [Zhong et al., 2009] and perpetual/persistent depending up on causes of variations. The adaptive phenotypic modifications have been reported to be heritable by successive generations through meiosis in the form of epialleles [Schulz et al., 2014] and thus have evolutionary consequences. For crop improvement a natural variation or phenotypic trait is used rather than particular molecular difference and the natural variation is outcome of genetic and epigenetic variants. Besides genetic polymorphism, methylation polymorphism is an important factor to be studied

for identification of novel characters for crop improvement [Lukens and Shuhua Zhan, 2007]. Clear potential for heritable epigenetic natural variations to contribute quantitative trait variation and crop improvement has been reported [Springer, 2013]. Thus study of epigenetic variations will add to the information for better understanding on the potentiality of epigenetics for crop improvement. The potentiality of AFLP has been well established in genetic divergence studies. A variant of AFLP, methylation sensitive amplification polymorphisms (MS-AFLP), is employed to study methylation variations (epigenetic divergence) of individuals or population studies which differentiate unmethylated, CG-methylated and CHG-hemimethylated states of genome. The ecological significance of epigenetic variations have been studied using model plants under artificial conditions. In *J. curcas*, the importance of epigenetic differences between individuals as a source of intraspecific diversity in natural plant populations remains to be studied. In present study, attempts were made to assess genetic and epigenetic diversity among selected elite germplasm of *J. curcas* using AFLP as genetic marker and MS-AFLP as epigenetic markers. Subsequently the results obtained with genetic and epigenetic markers were compared for establishment of phylogenetic relationship. The study would provide information on epigenetic polymorphism with respect to genetic polymorphism and about homologous nucleotide sequences in genome that are involved in adaptive responses irrespective of the nucleotides involved in genetic polymorphism.

Material and Methods

Plant sampling and genomic DNA extraction

Five elite germplasm (CSMCRI- 1 to 5) growing in CSIR-CSMCRI Jatropha experimental plot, Chorvadla (Gujarat) and Brahmapur (Odhisra) based on their performance in the field trials *viz.* plant height, canopy circumference, seeds per fruit, 100 seed weight, seeds per gram per plant and oil yield in percentage (supplementary table-1) were selected for present study. The selection was done based on the high yielding potentiality considered in the earlier studied germplasm [Mastan et al., 2012a]. The physio-chemical properties soil from the cultivation sites of these germplasm were given in supplementary table 2. The plantation in experimental plot was maintained since more than last ten years by employing best agronomic practices. From each identified plant 3rd, 4th and 5th leaves from fresh sprouts were harvested as representative sample for study, immediately stored in liquid nitrogen and transported to laboratory for further processing. The total genomic DNA from leaf samples was extracted using cetrimonium bromide

(CTAB) protocol [Doyle and Doyle, 1990] with slight modifications [Mastan et al., 2012c] and quantified using spectrophotometer (BioTek Instruments Inc. USA). The aliquots were diluted to the final concentration as per requirement for AFLP and MS-AFLP profiling. Pure and analytical grade chemicals of Sisco Research Laboratories Pvt. Ltd. (SRL), Mumbai, India and Sigma-Aldrich Chemical Company, USA were used throughout the experiment.

AFLP analysis

AFLP fingerprinting was performed using AFLP analysis system-II kit (Invitrogen Life Science Ltd., USA) according to Vos et al. [1995]. The genomic DNA (300 ng) was digested with *EcoRI* and *MseI* at 37°C for 2 h and digested aliquot was ligated to *EcoRI* and *MseI* specific adaptors at 20°C for 90 min. The ligated DNA was diluted for 1:10 and pre-amplified using *EcoRI* and *MseI* primer with one selective nucleotide at the 5' and 3' ends. The pre-amplified product was diluted 1:10 with sterile Tris–EDTA (TE) buffer. The diluted products were amplified using different combinations of *EcoRI* and *MseI* primer each with three selective nucleotides at the 5' and 3' ends, respectively. Primers for selective amplification were prepared by combining the primers related to the *EcoRI* specific adaptor ligated at 5' and that of *MseI* specific adaptor ligated at 3' for the digested product having respective restriction sites. Selective amplifications were performed using 65°C as the initial annealing temperature for the first cycle and for subsequent 11 cycles the annealing temperature was successively reduced by 0.7°C. Twenty-three cycles were run at 56°C annealing temperature. To the PCR product equal amount of formamide dye was added and subjected to electrophoretic separation on 6% denaturing polyacrylamide gel in 1X TBE buffer in a sequencing gel system (LKB, Sweden). The Gels were stained with silver nitrate using silver staining kit (Sigma, USA) and photographed for further recording.

MS-AFLP analysis

MS-AFLP was performed according to Xu et al. [2000]. The genomic DNA (300 ng) of each sample was digested with *EcoRI/MspI* and *EcoRI/HpaII* restriction enzymes at 37°C for 2 h. Digested aliquots were ligated to *EcoRI* and *MspI* or *HpaII* specific adaptors at 20°C for 90 min to avoid reconstruction of respective restriction sites. The ligated DNA was diluted for 1:10 and pre-amplified using *EcoRI* and *MspI* or *HpaII* primer with one selective nucleotide at the 3' end. The PCR conditions for pre-amplifications consisted 94°C denaturation, 65°C annealing, 72°C extension for one min each up to 34 cycles, followed by final extension at 72°C for 7 min. The pre-amplified product was diluted 1:10 with sterile tris–EDTA (TE) buffer. The diluted products

were amplified with different combinations of *EcoRI* and *MspI* or *HpaII* primers each with three selective nucleotides at the 5' and 3' ends respectively. Selective amplifications were performed using 65°C as the initial annealing temperature for the first cycle and for subsequent 11 cycles the annealing temperature was successively reduced by 0.7°C. Twenty-three cycles were run at 56°C annealing temperature. To the PCR products, equal volume of formamide dye was added and subjected to electrophoretic separation on 6.0% denaturing polyacrylamide gel (PAGE) in 1X TBE (90 mM Tris–borate, 2 mM EDTA, pH 8.0) buffer in a sequencing gel system (LKB, Sweden). The Gels were stained with silver nitrate and scanned for further data recording.

Profiling scoring and data analysis

The AFLP and MS-AFLP reactions were performed thrice and the fingerprints showing reproducible results were scored. The scored bands were transformed into a binary character matrix, using “0” and “1” to indicate the absence and presence, respectively, of particular loci by assuming the fragment size as biallelic locus. Only those loci amplified strongly in each instance with reproducibility were scored and included in the analysis.

For AFLP analysis, genetic similarity (GS) was calculated using Jaccard's coefficient of similarity [Jaccard, 1998] with the help of NTSYS-pc package (version 2.2) [Rohlf, 2001]. Percentage of polymorphism was calculated as total number of polymorphic bands x 100 / total number of bands. Dendrograms were constructed according to unweighted pair-group method with arithmetic mean (UPGMA) method using binary data generated by AFLP followed by bootstrapping analysis across the loci [Felsenstein, 1985] with the help of NTSYS-pc software package.

For MS-AFLP data analysis, a site for a particular accession was classified as “methylated” if a band was present in either the *MspI* or *HpaII* lane but not in the other; “not methylated” if bands were present in both lanes, or “unknown” if bands were absent in both lanes. The bands obtained were classified into four types (Type I to IV) accordingly [Rathore et al., 2015; Mastan et al., 2014]. A site was considered “methylation polymorphic” (MP) if there was at least one accession in which the site was methylated and at least one accession for which the site was not methylated. Percentage methylation was calculated as number of methylated bands x 100 / total number of bands. Percentage methylation polymorphism was calculated as the number of polymorphic methylated bands x 100 / number of methylated bands.

Results

In AFLP fingerprinting out of 21 primer combinations 17 primers were selected for data analysis based on reproducibility of results and consistent pattern of banding (Fig. 1a, b). Seventeen AFLP primers produced a total of 691 markers with an average of 40.65 markers per primer. The numbers of AFLP amplicon ranged from 19 (P-64) to 57 (P-12). Out of 691 markers 586 loci were found monomorphic and 105 loci were polymorphic with an average of 6.18 polymorphic loci per primer combination. Highest (17 nos.) numbers of polymorphic markers were obtained with P-50 primer and least (1 no.) with P-4, 11 and 64. In consonance with numbers of polymorphic markers maximum genetic polymorphism i.e. 48.57% was obtained with P-50 and least i.e. 2.27% with P-11. Overall seventeen primers produced 15.20% genetic polymorphism in the studied genotypes (Table-1). In pair wise comparison among germplasm average polymorphism was found to be 6.8% ranging from 3.62% between CSMCRI- 3 and 4 to 10.27% between CSMCRI- 1 and 5 (Supplementary table 2). CSMCRI- 1 was found to be the most diverged genotype while CSMCRI- 3 was least diverged genotype in AFLP analysis. The genetic similarity in the studied germplasm ranged from 0.94 to 0.98 with an average of 0.96. CSMCRI- 1 and 5 showed least genetic similarity (0.94), while CSMCRI- 4 showed highest genetic similarity (0.98) with CSMCRI- 2 and 3 (Supplementary table 3). In AFLP dendrogram all the genotypes formed a major cluster except CSMCRI- 1 and this showed highest genetic divergence from rest of the genotypes in the present study. CSMCRI- 5 followed CSMCRI- 1 and formed a separate sub-group in major cluster. CSMCRI- 3 and CSMCRI- 4 were observed least diverged (Fig. 2a). The AFLP principal co-ordinate analysis (PCA) analysis showed same trend as that of AFLP dendrogram (Fig. 2b).

In methylation profiling, out of 10 MS-AFLP primer (MSAP) combinations 07 MSAP primers were selected for data analysis based on reproducibility of results (Fig. 1c, d). Seven MSAP primers produced 282 MS-AFLP markers in the studied genotypes. Number of MS-AFLP markers ranged from 28 (MSAP-8) to 51 (MSAP-2) with an average of 40.29 markers per primer. Out of 282 loci, 90 loci were found methylated in the studied genotypes with an average of 12.86 loci per primer. Number of methylated markers and percentage methylation ranged from 11 (MSAP-1, 3 and 5) to 16 (MSAP-2) and 23.91% (MSAP-1) to 46.43% (MSAP-8) respectively. Among 90 methylated loci, 40 loci were found methylation polymorphic in the studied genotypes with an average of 4.56 loci per primer. Number of methylated polymorphic loci and percentage polymorphism ranged from 02 (MSAP-2 and 8) to 10 (MSAP-4) and 12.50%

(MSAP-2) to 66.67% (MSAP-4) respectively. Seven MSAP primers showed 44.44% epigenetic polymorphism among the studied genotypes (Table- 2) with an average of 6.35% per primer and 8.89% per genotype. In pairwise comparisons average numbers of methylated and methylated polymorphic bands ranged from 7.8 (MSAP-1) to 14.8 (MSAP-2) and 0.7 (MSAP-2) to 2 (MSAP-1) respectively. Percentage methylation and methylation polymorphism ranged from 16.96% (MSAP-1) to 42.14 (MSAP-8) and 4.86 (MSAP-2) to 27.45 (MSAP-1) respectively. In pairwise comparisons average percentage methylation and methylation polymorphism observed was 24.68% and 12.44% respectively.

Number of methylated markers among the studied accessions ranged from 67 (CSMCRI- 1) to 76 (CSMCRI- 3). Among the accessions studied, CSMCRI- 3 showed highest methylation (26.95%) while CSMCRI- 4 showed least (23.05%); and CSMCRI- 3 showed highest polymorphism (15.34%) while CSMCRI- 2 showed least (9.12%). In overall analysis CSMCRI- 2 showed minimum epigenetic divergence, while CSMCRI- 1 showed highest epigenetic divergence from rest of the genotypes as compared with other genotypes (Table- 3). CSMCRI- 3 showed highest (19.72%) polymorphism from CSMCRI- 5 while CSMCRI- 2 showed least (9.09%) polymorphism from CSMCRI- 4 among accessions (Supplementary table 4). In dendrogram generated on the basis of MS-AFLP data all the genotypes formed a major cluster except CSMCRI- 1 showing highest epigenetic divergence in the genotypes. CSMCRI- 2 and 5; and CSMCRI- 3 and 4 formed two separate sub-groups in the major cluster. CSMCRI- 3 and CSMCRI- 4 were observed least diverged (Fig. 2c). In MS-AFLP PCA analysis, CSMCRI- 1 showed same trend as that of AFLP dendrogram (Fig. 2d) while rest of the genotypes formed two different clusters.

In dendrogram generated on the basis of pooled genetic and epigenetic data, similar to dendrogram generated separately for genetic/epigenetic data all the genotypes formed a major cluster except CSMCRI- 1 showing highest divergence in the studied genotypes. CSMCRI- 3 and 4 formed one sub-group and this was followed by CSMCRI- 2 and then 5. CSMCRI- 3 and CSMCRI- 4 were observed least diverged (Fig. 2e). In PCA analysis CSMCRI- 1 showed same trend as that of dendrograms (Fig. 2f) obtained in genetic, epigenetic and pooled data analysis.

The AFLP fingerprinting with seventeen primers showed 15.20% genetic polymorphism in the studied genotypes with an average of 3.04% per genotype, while MS-AFLP with seven MSAP primers showed 44.44% epigenetic polymorphism among the studied genotypes. The magnitude

of epigenetic divergence might be higher due to various environmental factors which directly influence epigenetic status as compared to genetic makeup of a plant species. Also the epigenetic makeup of a plant species is governed by interaction of genetic components and prevailing environment thus it is directly influenced by prevailing environment. CSMCRI- 1 and 3 showed highest genetic and epigenetic polymorphism respectively (Fig. 3) among the studied genotypes while rest genotypes showed mix type of relationship.

Discussion

During present course of study, epigenetic divergence was studied among the selected genotypes and a comparison has been made among genetic and epigenetic divergence. In present study, genetic similarity and polymorphism obtained with AFLP were 0.96 and 15.20% respectively and these were found to be correlated with previous reports [Mastan et al., 2012a; Sudheer et al., 2010; Tatikonda et al., 2009]. In AFLP analysis, CSMCRI- 1 was found highly diverged from rest of the genotypes, while rest of the genotypes formed one major cluster. However CSMCRI- 3 and 4 were found closer in the major cluster formed. CSMCRI- 1, 3 and 5 were collected from same geographical location and CSMCRI- 2 from different location, the genetic divergence of CSMCRI- 1 and relatedness of CSMCRI- 2 irrespective of geographical areas might be due to anthropogenic activities [Mastan et al., 2012; Basha and Sujatha, 2007].

Methylation and methylation polymorphism in the studied genotypes varied and MS-AFLP resulted 31.91% methylation and 44.44% methylation polymorphism in methylated genome. Methylation ranged from 23.05% (CSMCRI- 4) to 26.95% (CSMCRI- 3) and methylation polymorphism ranged from 9.12% (CSMCRI- 2) to 15.34% (CSMCRI- 3). In MS-AFLP, highest and least methylation was shown by CSMCRI- 1 and 2 respectively, while CSMCRI- 5 and 2 showed highest and least epigenetic divergence respectively. Occurrence of epigenetic diversity along with genetic diversity is an important factor governing an ecologically balanced population and it has been shown to increase the productivity and stability of plant populations as in *Arabidopsis thaliana* epigenetically diverse populations has been reported to produce up to 40% more biomass than epigenetically uniform population [Latzel et al., 2013].

The variations in the DNA methylation and polymorphism in methylated DNA could occur due to the micro-environmental conditions of the existing geo-ecological conditions. The environmental conditions affect methylation levels and could result in differential methylation patterns in different genotypes. The variation of both DNA methylation and polymorphism was

in the order of CSMCRI- 3 > CSMCRI- 5 > CSMCRI- 2 > CSMCRI- 1 > CSMCRI- 4 and CSMCRI- 3 > CSMCRI- 4 > CSMCRI- 1 > CSMCRI- 5 > CSMCRI- 2 respectively. The variation in both DNA methylation and polymorphism of genotypes indicates the extent of DNA methylation favoring plant adaptation as per prevailing micro-environment resulting in local adaptation of plants [Mastan et al., 2014]. The individual genotype could cope up with the prevailing conditions due to the phenotypic plasticity of the genes of the DNA segments affected by the natural methylation [Lira-Medeiros et al., 2010]. In *Jatropha* collections, moderate level but significant epigenetic diversity has been reported [Yi et al., 2010] and it has also been reported that most of epigenetic differential markers to be inherited as epialleles following Mendelian segregation. This suggests possible involvement of epigenetics in *Jatropha* development under prevailing environment. These epialleles (variations in the degree of methylation of a gene) and adaptive phenotypes are selected by natural forces producing novel and improved ecotypes.

The present results clearly indicate that the genetic and epigenetic structure is not same in *J. curcas* and show greater epigenetic variation (44.44%) than genetic variation (15.20%). These results are in consonance with [Medrano et al., 2014; Kaifeng et al., 2013; Lira-Medeiros et al., 2010]. The genetic variations among the genotypes were found to differ 29.24% lower than epigenetic variation. Higher epigenetic than genetic variance suggest different evolutionary pressures over these two components of the genome leading divergent genetic and epigenetic structure [Lira-Medeiros et al., 2010]. The dendrogram and PCA analysis using pooled data of AFLP and MS-AFLP among the selected germplasms showed less genetic divergence between CSMCRI- 3 and 4 as collected from similar locations, this did not correlate with yield variations. In terms of seed and oil yield the germplasm potentiality is in the order of CSMCR- 5>2>3>4>1 and CSMCRI- 2>1>3>4>5 respectively. In terms of 100 seed weight the potentiality of germplasm is in order of CSMCRI- 2>4>5>1>3. When the genotypes are compared the average genetic and epigenetic polymorphism was in order of CSMCRI- 1>5>2>3>4 and CSMCRI- 5>4>1>2>3 respectively. The geo-ecological conditions for CSMCRI- 1, 3, 4 and 5 were similar (Chorwadla, Gujarat) and CSMCRI- 2 (Brahmapur, Odhissa) was different from the rest of genotypes. This correlates with earlier study showing higher degree of methylation and polymorphism growing under different geo-ecological environments. The environmental conditions have been reported to affect methylation levels, and could result in different

methylation patterns. Also the different accessions exhibited highest and lowest level of both DNA methylation and polymorphism [Mastan et al., 2014]. The epigenetic variations have been reported as an important source of intraspecific functional diversity in plant species [Medrano et al., 2014] and the present results i.e. higher epigenetic divergence than genetic divergence possibly endowing *J. curcas* having capacity to be exploited under broad range of ecological conditions despite its narrow genetic diversity.

In the present study though the overall genetic diversity among the *J. curcas* germplasm was found to be less in comparison with previous studies, however the mean percentage of polymorphism was found to be in accordance with [Sudheer et al., 2010; Basha and Sujatha, 2007]. The study also revealed occurrence of variations both in DNA methylation and polymorphism in methylated DNA among the *J. curcas* germplasm. Different accessions exhibited highest and lowest level of both DNA methylation and polymorphism under prevailing environment indicating different directions of evolution of genetic and epigenetic components. Determining the degree of independence of epigenetic variations in relation to genetic variations; and whether the adaptive changes in a genotype simultaneously involve genotypes and epigenotypes, are two key aspects that remain unexplored in natural populations [Richards, 2006; Richards et al., 2010; Herrera and Bazaga, 2010; Bossdorf et al., 2008]. These aspects could be explored by looking for associations between genotypes and epi-genotypes in the broader context of a causal framework linking genotype and epi-genotype at the individual plant level [Herrera and Bazaga, 2011]. Natural variation in gene/DNA methylation is under genetic control, and establishing the degree to which epigenetic variations are independent from genetic variations is important to evaluate the relevance of the former as an essential, rather than unnecessary inheritance system. The evolutionary significance of epigenetic variations depends on epigenetic and genetic variations being largely independent of each other [Richards, 2006; Bossdorf et al., 2008]. Thus the process of DNA methylation can be a source of random variations in natural populations [Massicotte et al., 2011] and in subsequent generations these changes become permanent as epialleles [Yi et al., 2010] thus leading improved ecotypes under prevailing environment.

The present investigations generate knowledge on methylation pattern of plant genotypes along with their genetic relationship. The present study gives an idea about genetic and epigenetic structures; and the plant species showing less genetic polymorphism would show higher

methylation polymorphism. These findings show that epigenetic variations through DNA methylation are potential source of phenotypic plasticity in plants during evolution [Zhang et al., 2013]. This shows that different plants exhibit various degree of methylation polymorphism for their adaptive response. These findings are relevant to ecologists to understand the mechanism underlying natural phenotypic variation and the responses of plants to prevailing environmental conditions. This gives the status of genetic relatedness among the genotypes and the status of methylation in their genome and methylation polymorphism among them to achieve adaptation to the prevailing environment.

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Author contribution

Conceptualization and design of experiment- MSR; execution of experiment- SGM; data analysis, result interpretation and MS writing- SGM+MSR; maintenance and identification of elite germplasm based on agronomic data, suggestions during the experiments- AG.

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Figure 2: Dendrogram and PCA diagrams generated for *J. curcas* genotypes based on AFLP fingerprint data (a, b), MS-AFLP data (c, d) and pooled data (e, f).

Figure 3: A comparative pictogram of genetic and epigenetic polymorphism in *J. curcas* genotypes.

Table- 1: Details of AFLP primers, number and nature of amplified loci and percentage genetic polymorphism obtained in AFLP based genetic diversity analysis in *J. curcas*.

S. No.	Primer combinations (E/M)	Total number of amplified bands	Number of monomorphic bands	Number of polymorphic bands	% genetic polymorphism
1	P4 (EAAC/MCAT)	43	42	1	2.33
2	P6 (EAAG/MCAG)	38	33	5	13.16
3	P11 (EAAG/MCAG)	44	43	1	2.27
4	P12 (EAAG/MCAT)	57	45	12	21.05
5	P16 (EAAG/MCTT)	41	36	5	12.20
6	P17 (EACA/MCAA)	38	36	2	5.26
7	P 18 (EACA/MCAC)	45	36	9	20.00
8	P20 (EACA/MCAT)	50	43	7	14.00
9	P 27 (EACT/MCAG)	37	34	3	8.11
10	P 29 (EACT/MCTA)	45	37	8	17.78
11	P 31 (EACT/MCTG)	46	37	9	19.57
12	P35 (EACC/MCAG)	32	26	6	18.75
13	P39 (EACC/MCTG)	47	38	9	19.15
14	P40 (EACC/MCTG)	37	29	8	21.62
15	P49 (EAGC/MCAA)	37	35	2	5.41
16	P50 (EAGC/MCAC)	35	18	17	48.57
17	P64 (EAGG/MCTT)	19	18	1	5.26
TOTAL		691	586	105	15.20
Average percentage genetic polymorphism per primer					
E/M- E: <i>Eco</i> RI series primer and M: <i>Mse</i> I series primer					

Table- 2: Details of MS-AFLP primers, number and nature of amplified loci, and percentage methylation and methylation polymorphism obtained in MS-AFLP based epigenetic diversity analysis in *J. curcas*.

Primer	Sequence of primer 5'-3'	Total no. of bands	No. of methylated bands	Overall analysis			Pairwise analysis			
				% Methylation	No. of methylation polymorphic bands	% epigenetic variation	Average No. of methylated bands in CSMCRI 1 - 5	Average % Methylation in CSMCRI 1 - 5	Average No. of methylated polymorphic bands in CSMCRI 1 - 5	Average % epigenetic variation in CSMCRI 1 - 5
MSAP1	E-AAC / HM-CAC	46	11	23.91	7	63.64	7.8	16.96	2	27.45
MSAP2	E-ACA / HM-CAC	51	16	31.37	2	12.50	14.8	29.02	0.7	4.86
MSAP3	E-ACG / HM-CAC	42	11	26.19	4	36.36	8.8	20.95	0.8	8.83
MSAP4	E-AGC / HM-CAC	44	15	34.09	10	66.67	8.2	18.64	1.4	14.67
MSAP5	E-ACT / HM-CAC	30	11	36.67	7	63.64	8.2	27.33	1.1	12.63
MSAP7	E-ACA / HM-CAA	41	13	31.71	8	61.54	10	24.39	1.4	13.18
MSAP8	E-ACG / HM-CAA	28	13	46.43	2	15.38	11.8	42.14	1.3	11.86
Total		282	90	31.91	40	---	69.9	24.68	8.7	---
Average percentage epigenetic polymorphism among germplasm						44.44				12.44

Table- 3: Details of amplified loci, methylated bands and polymorphic bands; and percentage methylation and epigenetic polymorphism in *J. curcas* L.

Parameter/genotype details	CSMCRI 1	CSMCRI 2	CSMCRI 3	CSMCRI 4	CSMCRI 5
Total number of MS-AFLP markers	282				
Number of methylated markers	67	68	76	65	72
Percentage methylation	23.76	24.11	26.95	23.05	25.53
Average No. of methylated polymorphic markers	8.5	7	11	9	7.6
Average % methylation polymorphism	11.20	9.12	15.34	12.68	10.32