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Research article

Microsatellite markers based heterozygosity assessment in *Jatropha curcas* L.: A potential bioenergy crop

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Abstract: The tree breeding is more difficult by the changes that occur during the transition from juvenility to maturity. A correlation between individual heterozygosities of parents and their offspring arises from the fact that, at most allelic frequencies, heterozygous parents produce higher proportion of heterozygous progeny than do homozygous parents. Microsatellite markers are an efficient tool for the assessment of heterozygosity and homozygosity. In order to assess the level of heterozygosity of *Jatropha curcas*, 56 SSRs markers were used for genotyping 48 progeny derived from selfed seeds of a single *J. curcas* plant. Out of 56, 7 SSRs could not produce sufficient and significant data as they failed to amplify in more than 70% genotypes and thus not considered for further analysis. Therefore, genotypic data of 49 SSRs were used for heterozygosity assessment. Out of 49 SSRs, 31 SSRs were found to be monomorphic and 18 polymorphic indicating homozygosity and heterozygosity on plants, respectively. The polymorphic SSRs showed allele variation from 2 to 9 with an average of 3.56 alleles per SSRs. The SSR JGM_CD232 showed maximum of 9 alleles followed by SSR JGM_CD348, JGM_CD421, and JGM_CD092 with 5 alleles. The heterozygosity, calculated as proportion of heterozygous individuals in population, varied from 0.00 to 1.00 with an average of 0.37. However, majority of the markers (61%, 11 out of 18) showed heterozygosity variation from 0.00 to 0.22 indicating low level of heterozygosity at these loci. The rest 7 SSRs showed heterozygosity from 0.6 to 1.0 (mean 0.84) indicating higher proportion of heterozygosity at these loci. In the present investigation, the heterozygosity assessment in *J. curcas* indicating low level of heterozygosity showed there is need to create genetic variability in *J. curcas* for genetic improvement.
Keywords: *Jatropha curcas* - SSR marker - Heterozygosity - Bioenergy crop.

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INTRODUCTION

Climate change is one of the biggest threats to the earth's biodiversity loss. Due to high energy demand humans are continuously deployed the natural resources like coal, gas and oil which continuously increase the level of carbon dioxide in the environment and contribute to the global climate change. Over exploitation of fossils fuel reserves and the increasing incidences of environmental pollution demand the search for alternate and renewable sources of biofuels, including biodiesel. In order to fight the climate change biodiesel production from Jatropha oil has potential effect to reduce the hunger of carbon dioxide and to fight against the global climate change. Biofuels are renewable in nature and less polluting than petroleum based fuels (Openshaw 2000, Mandpe *et al.* 2005, Ong *et al.* 2011a). Among the various biodiesel sources, *Jatropha curcas* attracted more attention as a potential source of biofuels due to its non-food nature, oil-rich and widely adaptable properties (Heller 1996, Openshaw 2000, Sujatha *et al.* 2008, Makkar *et al.* 2009, King *et al.* 2009, Devappa *et al.* 2010, Johnson *et al.* 2011). The biodiesel production from *J. curcas* has been considered as an environment friendly renewable fuel alternative to alleviate the energy crisis (Fairless 2007, Ghosh *et al.* 2007). *J. curcas* L. is belonging to the family Euphorbiaceae having chromosome number 2n=22 (Dehgan 1984) with relatively smaller genome size of ~416 Mb (Carvalho *et al.* 2008, Sato *et al*. 2011). *J. curcas* L. is a tropical species native

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to Mexico and Central America, but widely distributed in other tropical and sub-tropical areas of the world, especially in Africa, India and South-East Asia (Heller 1996, Sujatha & Prabhakaran 1997, Openshaw 2000). All part of Jatropha can be used for a wide variety of purposes rising from traditional medicine for common human and animal ailments, protection against land erosion and as a boundary fence or live hedge to newly found high economic potential as a fossil fuel replacement (Openshaw 2000, Sirisomboon *et al.* 2007, Rao *et al.* 2008).

Considering its global importance as promising biofuel plant genetic studies in *J. curcas* have been undertaken towards its genetic improvement through various means including traditional as well as biotechnological methods. A traditional approach of genetic improvement for polygenic traits is time and labor consuming. The molecular markers technique is most widely exploited with conventional breeding program to enhance the more accuracy and save the time. Number of molecular markers have been developed and used for the study of genetic diversity in *J. curcas* such as RAPD (Rosado *et al.* 2010, Machua *et al.* 2011, Rafii *et al.* 2012, Kumar *et al.* 2013, Pamidimarri & Reddy 2014), ISSR (Kumar 2011, Grativol *et al.* 2011, Camellia *et al.* 2012, Soonthornyatara *et al.* 2015), AFLP (Quintero *et al.* 2011, Shen *et al.* 2012, Sinha & Tripathi 2013, Osorio *et al.* 2014, Avendano *et al.* 2015), SSRs (Yadav *et al.* 2011, Ricci *et al.* 2012, Kumari *et al.* 2013, Osorio *et al.* 2014, Maurya *et al.* 2013, 2015) and SNPs markers (Gupta *et al.* 2012, Montes *et al.* 2014, Trebbi *et al.* 2015).

Knowledge of genetic nature of *J. curcas* such as homozygosity and heterozygosity is one of the important aspects for the genetic improvement. Traditionally, biochemical markers were used for the detection of heterozygosity but facing problem for detecting polymorphic loci and heterozygosity (David 1998). Over recent years microsatellite have been applied extensively as a genetic markers for the detection of homozygosity and heterozygosity which replacing other techniques, such as self-pollination with subsequent progeny testing and morphological markers (Murovec *et al.* 2007). The most frequently used molecular markers for homozygosity and heterozygosity testing used to be randomly amplified polymorphic DNAs (Eimert *et al.* 2003, Yahata *et al.* 2005), but microsatellite markers are used much more nowadays because of their co-dominant nature and unambiguous result. Genetic enhancement of this important plant is now a major target for its sustainable productivity. Genetic improvements of *J. curcas* with the implementation of molecular markers with conventional breeding could be increased the productivity. Recently, some efforts have been done by some researcher for the development of improved better yielding genotypes through interspecific hybridization *J. curcas* with other related *Jatropha* species (Basha *et al.* 2009, Dhillon *et al.* 2009, Popluechai *et al.* 2009, Muakrong *et al.* 2013, Laosatit *et al.* 2014, Aruna *et al.* 2015).

The previous literature showed that the number of researchers have worked on the genetic diversity assessment in *J. curcas* using different molecular markers. However, there are no studies conducted in *J. curcas* for the heterozygosity assessment using SSR markers. Therefore, present investigation was undertaken for the assessment of heterozygosity in *J. curacs* which could be utilized in future for the genetic improvement of *J. curcas* to develop high yielding genotype.

MATERIALS AND METHODS

Plant materials and genomic DNA isolation

For the assessment of heterozygosity level in *Jatropha curcas*, one accessions *i.e.* NBJC147 was selfed to check cross pollination. The mature seeds were collected to raise seedling for heterozygosity assessment. Total genomic DNA was extracted from fresh young leaves of 48 seedling of selfed Hansraj and Chhatrapati using a modified CTAB (Cetyl Trimethyl Ammonium Bromide) method. The quality of DNA was checked on 0.8% agarose gel, and DNA concentration was determined using a Nanodrop spectrophotometer ND1000 (Nanodrop Technologies, DE, USA).

Polymerase chain reaction and fragment analysis

PCR amplification was carried out in 10µl reaction mixtures that contained 10 ng genomic DNA, 1X PCR master mix (AmpliTaq Gold®, Applied Biosystems, USA), 0.1 µl (5pmol/µl) of forward primer (tailed with M13 tag), 0.3µl (5pmol/µl) each of both reverse primer and M13 tag (labeled with either 6- FAM, VIC, NED and PET). PCR was performed on Verti Thermal Cycler (Applied Biosystems, USA) using the following cycling condition: initial denaturation at 95ºC for 5 min followed by 36 cycle of 94ºC for 30 s, 50-55ºC (primer specific) for 45 s and 72ºC for 1 min. Subsequently, 10 cycles of denaturation for 30 s at 94ºC, annealing for 45

s at 53ºC, extension for 45 s at 72ºC followed by final extension for 15 min at 72ºC was performed. The amplified PCR products from the parents and hybrids were resolved by TBE agarose gel electrophoresis using 1.5% Agarose (Geni) and then post PCR multiplex sets was prepared based on fluorescence labeled primers. For post PCR multiplex set, 1 µl FAM and 2 µl of each VIC, NED, and PET labeled PCR product were combined with 13 µl of water. 1 µl of this mixture was then added to 10 µl Hi-Di formamide containing 0.25 µl GeneScan ™ 600 LIZ® as internal size standard. This was then denatured for 5 min at 95^0C, quick chilled on ice for 10 min and run on a capillary-based 3730xl DNA analyzer (ABI, USA). Microsatellite loci repeats were assayed on the basis of their observed heterozygosity and number of alleles detected with PCR amplification profile. Fragment analysis was performed using GeneMapper software ver.4.0 and data were scored as allele size (bp).

Data scoring and Statistical analysis

SSR bands were scored as single independent locus. Well-resolved fragments were scored as present (1) or absent (0) for each marker locus. The allelic data of polymorphic SSRs were subjected to statistical analysis using Power Markers (Liu & Muse 2005) to calculate observed heterozysity (Ho), gene diversity or expected heterozygosity (He), major allele number and polymorphic information content (PIC) value. The PIC value was calculated following Botstein et al. (1980) as follows:

$$PIC = 1 - \left[\sum_{i=1}^{n} Pi^2\right] - \left[\sum_{i=1}^{n-1}\sum_{j=i+1}^{n} 2Pi^2Pj^2\right]$$

Where, Pi and Pj are the frequencies of i^{th} and j^{th} allele.

RESULTS

Identification of polymorphic markers

Initially, 56 polymorphic SSR markers were selected from previously developed SSR markers from four SSR enriched genomic libraries (Maurya *et al.* 2013, 2015). These 56 SSR polymorphic markers were selected on the basis of PIC value which polymorphism was detected with a panel of 7 accessions of *Jatropha curcas* and 1 another species of *J. integgerima* (Maurya *et al.* 2013, 2015).

Heterozygosity Assessments

Table1. Details of 56 SSRs for used for the heterozygosity detection.

Total SSRs screened	No. of polymorphic SSRs	No. of monomorphic SSRs	No. of failed SSRs
56	18	31	7

These selected 56 SSR markers were used for the heterozygosity assessment with 48 selfed *J. curcas* genotype. Among these, 49 showed clear PCR products across all the genotype, while only 9 were found non-specific amplifications (Table 1). Out of 49 SSRs, 31 SSRs were found monomorphic indicating that all the plants were homozygous at these loci and the rest, 18 SSRs were found polymorphic producing more than one allele and thus indicating heterozygous condition on these loci (Table 2). The heterozygosity, calculated as proportion of heterozygous individuals in population, varied from 0.00 to 1.00 with an average of 0.37.

Table 2. Polymorphism feature of 18 polymorphic SSRs among 48 progenies of single plants used for heterozygosity assessment.

Marker	Allele No.	Gene Diversity	Heterozygosity	PIC
JGM_A439	3.00	0.06	0.02	0.06
JGM_A464	2.00	0.49	0.63	0.37
JGM_B034	4.00	0.48	0.22	0.40
JGM_B038	4.00	0.12	0.04	0.12
JGM_B041	2.00	0.17	0.06	0.16
JGM_B054	3.00	0.10	0.02	0.10
JGM_B062	3.00	0.25	0.13	0.23
JGM_B190	2.00	0.50	0.96	0.37
JGM_B479	3.00	0.50	0.84	0.39
JGM_B586	3.00	0.54	1.00	0.44
JGM_CD005	3.00	0.06	0.02	0.06
JGM_CD092	5.00	0.29	0.00	0.28
JGM_CD106	3.00	0.52	0.77	0.41
JGM_CD232	9.00	0.25	0.08	0.25

JGM_CD421	5.00	0.53	1.00	0.42
JGM_CD469	3.00	0.50	0.09	0.40
JGM_CD128	2.00	0.04	0.00	0.04
JGM_CD348	5.00	0.55	0.73	0.48
Range	2.00-9.00	0.04-0.55	0.00-1.00	0.04-0.48
Mean±SD	3.56±1.69	0.33±0.20	0.37±0.41	0.28±0.45

However, majority of the markers 11 (61%) showed heterozygosity variation from 0.00 to 0.22 with an average of 0.22 indication low level of heterozygosity at these loci. The rest 7 SSR showed heterozygosity from 0.6 to 1.0 with an average 0.84 indicating higher proportion of heterozygosity at these loci. The JGM_CD232 was showed maximum seven alleles followed by JGM_CD170, JGM_CD348 produced six allele while JGM_CD421, JGM_CD092 and JGM_B034 produced five alleles. The di-nucleotide SSR markers (JGM_B) showed less amplification with 48 genotype. However, tri-nucleotide SSR markers (JGM_CD) showed maximum amplification with 48 genotypes. A representative snap shot from GeneMapper ver. 4.0 showing the polymorphic and monomorphic allele in heterozygosity assessment (Fig. 1).

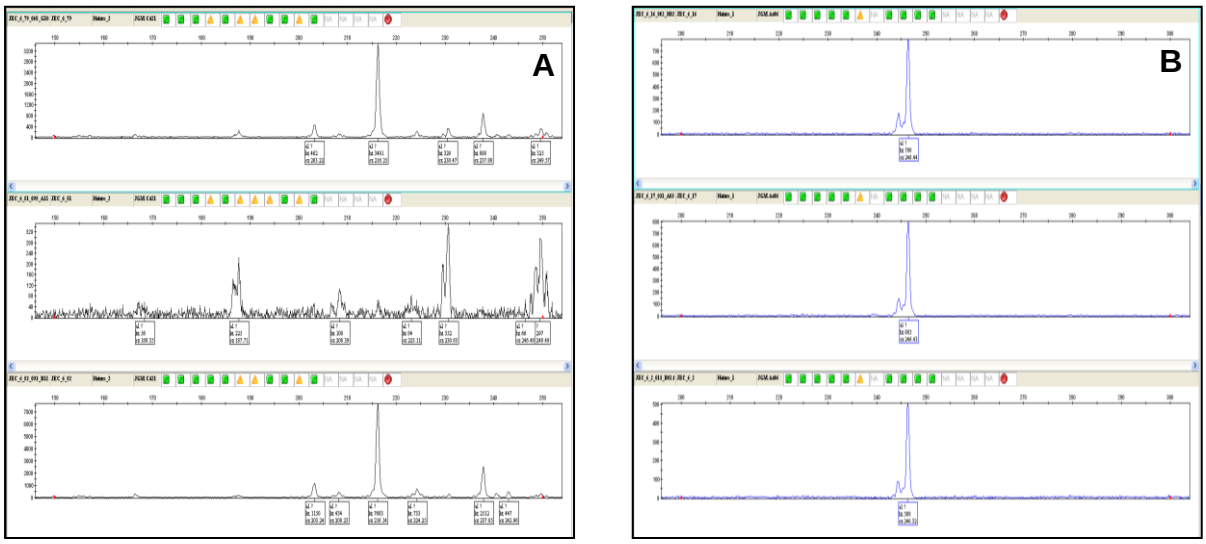

Figure 1. Snap shot showing **A,** Polymorphic (heterozygous) **B,** monomorphic (homozygous) allele.

DISCUSSIONS

Genetic variability is one of the essential requirements for crop improvement through plant breeding. The prime step in this process involves germplasm screening for establishment of genetic diversity. With the increasing knowledge on genome, molecular markers have been assisting the other marker system like morphological and quantitative data trait for genetic characterization studies. There are number of molecular markers have been developed in *J. curcas* and used for the genetic diversity assessment of Jatropha from different growing regions like China (Sun *et al.* 2008), Brazil (Rosado *et al.* 2010, Grativol *et al.* 2011), Mexico (Quintero *et al.* 2011), Thailand (Tanya *et al.* 2011), India (Bhasha *et al.* 2009, Maurya *et al.* 2013, 2015), but there is no any study on the screening of heterozygosity on the basis of molecular markers. The number of molecular markers like RAPD and AFLP were very quickly used for the genetic diversity analysis in *J. curcas* but these markers has its own disadvantages lacking of reproducibility (Karp *et al.* 1997, Hansen *et al.* 1998, Virk *et al.* 2000). However, microsatellite markers are codominant and highly polymorphic which can discriminate the homozygosity and heterozygosity of an individual. The selfed 48 genotypes were assessed with 56 SSR markers showed the 18 polymorphic which indicate the heterozygosity of these markers at different loci. The understanding the genetic basis genotypes will be quite useful to select suitable parental lines for hybridization programmes for the genetic improvement of *J. curcas.* Some preliminary research of homozygosity and heterozygosity have been done in different crops like rice (Liang *et al.* 2011), *Mimulus aurantiacus* (Murovec *et al.* 2007). The tree breeding is more difficult by the changes that occur during the transition from juvenility to maturity. Breeding populations can be characterized by quantifying the levels and organization of genetic variation within and between different breeding groups. Under the appropriate conditions, markers can replace phenotypic selection, thereby removing the need for growing or rearing of individuals (Chen *et al.* 2010). Markers- based systems have been used to study and compare the levels of

random genetic variation throughout the different cycles of a breeding programme, thus allowing much greater flexibility and control over the rate of reduction of genetic variability (Lia & Wua 2007). A correlation between individual heterozygosities of parents and their offspring arises from the fact that, at most allelic frequencies, heterozygous parents produce higher proportion of heterozygous progeny than do homozygous parents (Mitton *et al.* 1993). Microsatellite markers are an efficient tool for the assessment of heterozygosity and homozygosity. In the present investigation, the heterozygosity was assessed in *J. curcas* using SSR markers. Majority of the SSRs (64%) used to assess the heterozygosity were found to be monomorphic and 36% polymorphic. Majority of the markers (61%, 11 out of 18) showed heterozygosity variation from 0.00 to 0.22 indicating low level of heterozygosity at these loci. According to Lerner (1954) the high levels of heterozygosity enhanced the developmental homeostasis among individuals within populations and highly heterozygous individuals have lower level of phenotypic variation than predominantly homozygous individuals.

CONCLUSION

In conclusion, the present study was the heterozygosity assessment using SSR markers of selfed *Jatropha curcas* conducted at juvenility stage. The heterozygosity assessment in *J. curcas* indicating low level of heterozygosity which warrants the urgent need to create genetic variability in *J. curcas* for genetic improvement.

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