Studies on genetic diversity of *Jatropha curcas* L. genotypes by RAPD and ISSR markers

A

THESIS REPORT

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CANDIDATE'S DECLARATION

I, hereby declare that the work presented in the thesis entitled, **"Studies on Genetic diversity of *Jatropha curcas* L. genotypes by RAPD and ISSR molecular markers"** in partial fulfilment of the requirement for the award of the degree of Master of science in Biotechnology, to Department of Biotechnology and Environmental Sciences, Thapar University, Patiala, is an authentic record of my own work during the period of six months from January 2013 to July 2013, under the guidance of Dr. N. Das, Associate Professor, Thapar University, Patiala. I have not submitted the matter embodied in this thesis for the award of any other degree or diploma.

Date:

Place: PATIALA ASHA KIRAN

CERTIFICATE

This is certified that the thesis entitled **"Studies on Genetic diversity of *Jatropha curcas* L. genotypes by RAPD and ISSR molecular markers"** submitted by Asha Kiran in partial fulfilment of the requirement for the award of the degree of Master of Science in Biotechnology, to Thapar University, Patiala is a record of student's own work carried out by her under my supervision and guidance. The thesis has not been submitted for the award of any other degree or certificate in this or any other University or Institute.

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ABSTRACT

Jatropha curcas had gained popularity as a potential biodiesel producing crop but the major constraint for improvement of the crop for yield and seed quality traits (like seed oil content) is the narrow genetic base of the germplasm. In the present study, genetic backgrounds of 16 Jatropha curcas accessions from different locations of Punjab have been elucidated using molecular markers. For this work, total genomic DNA was isolated from *Jatropha* using a simple and efficient method. Quality and quantity of the genomic DNA preparations were checked by using agarose gel electrophoresis and nanodrop spectrophotometric analysis. Restriction analysis clearly reveals that the genomic DNA preparations are essentially devoid of inhibitory substances. Molecular analysis was carried out through PCR-based Random Amplified Polymorphic DNA (RAPD) and Inter Simple Sequence Repeat (ISSR) techniques. RAPD and ISSR are inexpensive and rapid methods, they do not require any information regarding the details of the plant genome and have been widely used to ascertain the genetic diversity in *Jatropha curcas* L. This is only a preliminary study with regard to genetic diversity analysis of a few select *Jatropha* germplasm. In this study, only a total of 5 RAPD and 5 ISSR markers were used to investigate polymorphism in 16 *Jatropha curcas*. Most of them refers to elite accessions in terms of seed yield and oil content. Banding patterns of the amplified DNA for a particular primer were found to be nearly identical for all the accessions, but the difference could be noticed with regard to the range of band sizes. For example, in case of RAPD markers the size range was found to be 200- 1500 bp; whereas, for ISSR markers it was found to be in the range of 400-2000 bp. Interestingly, we found some degree of polymorphism in a few *Jatropha* accessions, namely TJS 04 #42, TJS 50 #01, TJS 19 #04, TJS 07 #05, TJS 01 #01.

CONTENTS

ABBREVIATIONS

❖ DNA	Deoxyribonucleic acid
❖ ISSR	Inter Simple Sequence Repeats
❖ PCR	Polymerase Chain Reaction
❖ RAPD	Random Amplified Polymorphic
❖ TBE	Tris borate EDTA
❖ TE	Tris EDTA

1. INTRODUCTION

1.1 Energy crisis

Rapid depletion of non renewable energy resources and increase in the demand of energy due to rise in population having changing lifestyles, compel to develop an alternative renewable source of energy globally. We know that petroleum and diesel fuels are finite on earth; therefore dependence on renewable energy resources has been increasing day by day. By the year 2050, it is estimated that 90% of the world population will live in developing countries, so there is higher need of renewable energy resources to fulfil the energy requirements. Biomass accounts for almost all renewable energy resources. It is estimated that the world production of biomass is 146 billion metric tons per year, mostly from wild plant growth. Biomass as an energy source ranks fourth worldwide, which means it provides 14% of the world's energy needs. In addition, biomass is a clean renewable energy source so; there is no net gain of carbon dioxide. Burning biomass is not the only way to release its energy, but biomass can be converted to other usable forms of energy like bio-fuels. Two important sources of biomass based bio-fuels are ethanol and bio-diesel. Global bio-fuel use is expected to increase twofold by 2015.

1.2 Bio-diesel as renewable energy resource

Bio-diesel, derived from the oils and fats of plants like sunflower, rape seeds, Canola, Soybean, *Jatropha* can be used as a substitute or an additive to conventional diesel. Bio-diesel refers to a short chain alkyl esters made by transesterification of vegetable oils. Bio-diesel can be used alone or mixed in any ratio (B-20 to B-100) with mineral oil diesel fuel. Bio-diesel substantially reduces unburned hydrocarbons like carbon monoxide, sulphur dioxide and particulate matter in exhaust fumes. Biodiesel is an environment friendly, biodegradable, cheaper and clean burning fuel due to low flash point and it can be directly used in powered combustion engines without much engine modifications. It is a renewable liquid fuel that can be produced locally thus helping reduce the country's dependence on imported crude petroleum diesel.

1.3 Biodiesel status in India

The period from 2005 until now has experienced unprecedented growth in global biodiesel demand, production and production capacity. Recently, Government of India launched national mission on biodiesel with a view to find cheap and renewable liquid fuel based on vegetable oils. However, shortage of raw material to produce biodiesel is a major constraint. Many developed countries are using edible oil-seed crops such as sunflower and rapeseed in Europe, soybean in the USA, palm oil in Malaysia and coconut in Philippines for the production of biodiesel. However, developing countries like India having shortage of edible oil for consumption cannot afford to use edible oils for production of biodiesel. The option therefore left with the country is to go for non-edible oilseeds which can be grown in degraded forestlands, un-utilised public lands, field boundaries, and fallow lands of farmers (Biswas *et al*. 2010). According to the National Oilseeds and Vegetable Oils Development Board of the Indian Ministry of Agriculture (NOVOD), there are about 10 species with economic potential for biodiesel production. Out of which, *Jatropha curcas* have received much attention.

In India, biodiesel from *Jatropha curcas* can be produced on a large scale basis by proper cultivation and further seed's processing. According to the Ministry of Rural Development, out of 306 million hectares only 173 million of lands can be used for cultivation, while the remaining land is either eroded farm land or non-arable wasteland. Such previously unused land can be used for *Jatropha* cultivation. India's Planning Commission recommended a national mission on bio fuel, specifically on *Jatropha* curcas with following plans:

Plan A: 500,000 hectares of *Jatropha* would be grown on government land across the country. The fuel would be produced at the village level by local governing bodies.

Plan B: Central government would carry out plantation of *Jatropha* on 12 million hectares of land and privatize the production of biodiesel from this species. Department of Biotechnology (DBT) has undertaken a Mission to develop quality planting material of *Jatropha curcas* and develop agro technologies for its cultivation. DBT has also undertaken some research and development projects related to genetic variability studies on *Jatropha curcas.*

1.4 Botanical features of *Jatropha curcas* L.

Jatropha curcas belonging to family *Euphorbiaceae* comprises 160-175 species of shrubs, rhizomatous shrubs, herbs and small trees which can attain height up-to 5-8 m. It is commonly named as ratanjyot, chandrajyot, Jamal gota, Jangli arandi, Kala aranda and physic nut etc. in the different states of India. It is a native species of Central America and has been later introduced in Africa and Asia by Portuguese as an oil yielding plant (Figure: 1A). About nine species of *Jatropha* have been recorded in India. Out of these important ones are *Jatropha curcas*, *Jatropha gossypifolia*, *Jatropha glandulifera*, *Jatropha multifida*, and Jatropha *podagrica*. Among all the species, *Jatropha curcas* is the most important bio-diesel yielding crop. It is diploid species with 2n = 22 chromosomes.

Jatropha is a perennial, deciduous and diploid (2n = 22) shrub which can attain a height of 3 to 5 m, but can reach a height of 8-10 m under favourable conditions. The trees are deciduous, shedding the leaves in the dry season. The latex containing branches bear alternately arranged leaves with terminal inflorescence. The plant is monoecious and flowers are unisexual; occasionally hermaphrodite flowers occur. Male and female flowers occur separately in same inflorescence in the ratio 29:1 which subsequently effect mature seed production. Each inflorescence yields a bunch of approximately 10 or more ovoid fruits. Fruiting starts from the first year onwards. However, sufficient yield of fruits is obtained after the plant reach at the age of about 3-5 years. *Jatropha* seeds contain 46–58% of oil on kernel weight and 30–40% on seed weight. Fruiting occurs in winter when the shrub is leafless but in the permanently humid region flowering and fruiting occurs throughout the year. (Figure:1B).

Fig: 1A *Jatropha curcas* plant

Fig: 1B *Jatropha curcas* plant bearing fruits

Apart from bio-fuel production, *Jatropha curcas* has many medicinal advantages. Plant parts like seeds, leaves and bark are used as traditional medicines. The latex produced by *Jatropha* known as "Jatprophine", has some quinine like alkaloid properties. Jatprophine is an anti-cancerous agent and can be applied on skin to cure skin diseases. Wound healing properties of latex is due to the presence of proteolytic enzyme called curcain. It latex also has blood coagulating effect on blood plasma and is also being useful in dropsy, sciatica and paralysis. Its leaves can be used against cough and act as antiseptic after birth. The oil is non- edible due to the presence of a toxic substance named as 'curcin'. It forms symbiotic association with arbuscular mycorrhizal (AM) fungi in soil when phosphate deficiency occurs in the soil. The AM provides carbon and energy rich photosynthetic products to plant and which in turn provides essential nutrients especially P, Cu, Zn to AM. *Jatropha curcas* sheds its leaves in winter which leads to organic matter production and increase in earthworm population around the root zone hence, increase in soil fertility. Soap manufacturing can be carried out from *Jatropha* oil because of high saponofication value.

Worldwide use of *Jatropha curcas* for bio-diesel production had met with low seed set as well as oil yield which made its business risky. So, *Jatropha curcas* needs large scale cultivation and breeding of superior elite genotypes with higher seed yield. *Jatropha curcas* needs genetic improvements so that we can change its wild perennial status to high yielding and elite cultivated crop. Crop improvement work in this species is very limited. It is necessary to detect and document the extent of variation within and between populations, in this particular species with the help of genetic marker techniques. These techniques can be classified as morphological, biochemical and molecular markers. Knowledge of genetic variations and relationship in this species will allow organization of germplasm and selection of elite accessions for further breeding programmes. Hence, assessment of genetic diversity and characterization of *Jatropha curcas* L. genotype with the help of marker techniques become must. These techniques can be classified as morphological, biochemical and molecular markers.

1.5 Approaches for genetic diversity assessments

1.5.1 Traditional approaches for genetic diversity assessments

Traditional approaches are mainly based on morphological and biochemical techniques. These approaches are mainly used to study forest tree relationships and variability. Morphological markers are mainly corresponds to a particular trait which can be scored

visually. They may be dominant or recessive. Since genetic markers used to develop maps in plants are those which affect morphological characters including genes for dwarfism, albinism, and altered leave morphology etc. So, we can say that morphological markers can be used to check seed and leaves morphology, seed yield as well as growth characteristics. Unfortunately, a major constraint in the use of morphological markers is that of environment which has predominant role in the morphological variation. Biochemical markers are mainly concerned with the protein molecules like isozymes and seed oil content. Variability in seed oil content is the basis of biochemical marker studies in *Jatropha curcas as* there is limited number of studies on *this species* regarding isozymes.

1.5.2 Molecular marker approaches for genetic diversity assessments

Molecular marker technique helps to define genetic relationships more frequently and precisely than the other techniques. It is the most powerful technique used for genetic characterization. Molecular marker is a DNA sequence which is easily detected and whose inheritance can be monitored easily. The Polymerase Chain Reaction (PCR) has develops new tools for determining genetic variability and to characterise the species. Molecular or DNA based marker fingerprinting technique can detect genetic variation easily and distinguishes species with reliable information. These markers are not influenced by environmental conditions, hence can be used to describe genetic variation among plant population and identify different accessions of same species. Different approaches are available for DNA fingerprinting such as amplified fragment length polymorphism (AFLP), restriction fragment length polymorphism (RFLP), Inter simple sequence repeats (ISSRs) and randomly amplified polymorphic DNAs (RAPD). Among these, RAPD and ISSR are inexpensive and rapid methods, they do not require any information regarding the genome of the plant, and have been widely used to ascertain the genetic diversity in several plants.

1.5.2.1. RAPD Genotyping

This technique is based on arbitrary primed PCR. RAPD technique is based on PCR using genomic DNA from species of interest and amplifies those arbitrary DNA regions which are flanked by primer sequences (usually of 10 bp length). Although the sequences of RAPD primers are arbitrarily chosen, two basic criteria must be met: a minimum of 40% GC content (50 - 80% GC content is generally used) and the absence of palindromic sequence.

1.5.2.2. ISSR Genotyping

This technique is based on site targeted PCR. ISSR involves amplification of DNA segments present at an amplifiable distance in between two identical microsatellite repeat regions oriented in opposite direction. ISSRs use longer primers (15–30 mers) as compared to RAPD primers (10 mers). The amplified products are usually 200–2000 bp long and amenable to detection by both agarose and polyacrylamide gel electrophoresis. The use of ISSRs as genetic markers in crop plants has been demonstrated in several agricultural crop species, including wheat.

RAPD and ISSR marker analysis requires only small amount of DNA and produce higher level of polymorphism and gives diversity analysis in plants. Moreover, these markers have been extensively applied in cultivar and species identification, characterization of inter- or intra-population relationships, population genetic analysis and diversity/polymorphism evaluation of germplasm. Use of such techniques for germplasm characterization may facilitate the conservation and utilization of plant genetic resources, permitting the identification of unique genotypes or sources of genetically diverse genotypes.

Without sufficient genetic variability of the desired types of traits that are of economic interest, an attempt to improve forest tree will be unsuccessful or a failure. Therefore, the first and foremost thing to do for starting any tree improvement programme is to determine the amount, cause and nature of variation that is present in the species of interest. The real status of genetic diversity studies on *Jatropha curcas* germplasm is still very limited in India and nil in the state of Punjab. Keeping above aspects in view, our aim is to validate these molecular markers on *Jatropha curcas* genotype collected from different locations in the state of Punjab.

2. REVIEW OF LITERATURE

Clonal forestry becomes an important source for both capturing genetic information and achieving higher rate of multiplication of elite varieties of species in the field. Studies of clones on the basis of morphological and biochemical markers is the simplest, quickest, and easier methods for identification of clones in the field. But when we talk about species identifications on the basis of above approaches then these approaches are not enough. So, more advance and accurate methods are required for these types of identification and characterization. This could be done by molecular marker techniques which are widely used to study genetic diversity among the plant species and helps to find genetic relationships. For assessment of genetic diversity and characterization of *Jatropha curcas* germplasm it has become essential to apply molecular markers tools. A brief review related to traditional approaches as well as molecular marker techniques on *Jatropha* is given below in this context.

2.1. Traditional approaches

This approach is used in forest tree relationship studies and variation. Morphological markers are mainly related to taxonomy of plant which can be visually detected. Their characterization power is strong and usually preferred for quick identification directly in the field level. The morphological characterization in *Jatropha curcas* is mainly concerned with phenotypic characters like seed yield, shape of the leaves, flowering patterns etc. and biochemical characterization is mainly concerned with seed oil content and toxicity level. Studies have been conducted to identify genetic variation in populations, provenances and clones of *Jatropha curcas* through traditional morphological and biochemical marker techniques.

2.1.1. Morphological markers

J. curcas is classified into three classes on the bases of toxic contents present in its seeds (phorbol esters and curcin). The Cape Verde variety of *Jatropha curcas* has spread all over the world. The Nicaraguan variety has few but large fruit size and non-toxic Mexican variety that has limited phorbol ester content in the seeds (Henning 2006). SDAUJ1 is an Indian *Jatropha curcas* variety that was released during the year 2006 through local selection of germplasm (www.icar.org).

Initially candidate plus tree variation was found to be insufficient. Sakaguchi and Somabhi (1987) did not detect any morphological distinct marker in forty clonal lines of *Jatropha*

curcas which were collected from different locations of Thailand. Sukarin *et al.* (1987); Heller (1992) have found limited variability by using morphological markers. Morphological studies like seed oil content correlation with seed weight stem diameter and leave area etc. were carried out from central India for ten *Jatropha* accessions (Ginwal *et al*.2004). Better result was obtained in various oil yield components, number of branches and number of seeds in individual trees but significant result was obtained from kernel oil content in the *J. curcas* species Pant *et al.* (2006). Kausik *et al.* (2006) found significant variability in seed size, oil content and seed weight of 24 accessions of *J. curcas* species which were collected from Haryana, India. *Jatropha curcas* plants selected from 4 eco-geographical areas have shown prominent results in the nine characters. From a total of 162 *Jatropha* accessions taken from 4 zones, highest variability was found in zones. Similarly other morphological traits were also varied in different zones (Sunil *et al.* 2008). Traditional method using morphological traits for establishment of genetic diversity and relationships among accessions were mostly unsuccessful due to involvement of environmental factors which leads to variably in accessions most of the time.

2.1.2. Biochemical markers

A study based on biochemical markers is mainly focused on seed oil content. There are limited studies on *Jatropha curcas* by biochemical markers. There are some reports which define some genetic variability on the basis of isozymes. Isozyme analysis was carried out to determine phylogenetic relationships of *Jatropha curcas* with other species. Isozymes like peroxidise and superoxide dismutase were used under study Prabakaran and Sujatha (1999). Makkar *et al.* (1998) determined variability in oil content, crude protein content, fibre and ash content in 18 accessions of *Jatropha* collected from countries like West and East Africa, America and Asia. Ginwal *et al.* in 2004 determined association between oil content and rainfall. Similarly, Yunus in 2007 able to differentiate *Jatropha curcas* species collected from different regions in Central java and Indonesia on the basis of enzyme profiles. They have used Isozymes like sorbitol dehydrogenase, shikimate dehydrogenase, alcohol dehydrogenase and isocitrate dehydrogenase for their research work.

2.2. Molecular markers

In the last 20 years, genetic variation of tree species has been understood through an assessment of survival and growth performance parameters. However, above exercises are time consuming, labour-intensive and costly. Gradual advances in technologies have taken good shape for assessing genetic diversity at the molecular level. Recent developments in molecular biology and biotechnology offer quick and easy detection of genetic variation and characterization of genotypes through the use molecular markers. Most of the molecular characterization studies on *Jatropha curcas* were carried out in India, China and other countries but relatively less work has been done in Central America and Africa. Mostly, the molecular marker analysis was carried out in three ways – Inter and intra specific genetic diversity, by phylogenetic relationships and distinguishing of toxic and non-toxic varieties of *Jatropha curcas*. A brief review related to molecular marker analysis is given below.

2.2.1. Inter specific genetic diversity

This type of study showed the comparison of *Jatropha curcas* with other species of *Jatropha*. RAPD markers were used to compare *Jatropha curcas* with *Jatropha intengerrima.* Primer OPA-04 and OPA-08 identified five fragments which were specific to *J. curcas* Sujatha and Prabakaran (2003). Similarly, Sudheer-Pamidiamarri *et al*. (2009) described the relationship between *Jatropha curcas* and *Jatropha intengerrima* with the help of two molecular markers, RAPD and AFLP. Some significant results showed relationship between both of the *Jatropha* species. Similarly, Sudheer *et al.* (2010a) carried out molecular marker analysis by using SSR, RAPD and AFLP on several species of *Jatropha*.

2.2.2. Intra specific genetic diversity

Intra specific genetic diversity studies are based on variability due to geographical location of the same species or variability occurred between the accessions and population of the same species. 42 accessions of *Jatropha curcas* were selected from different crop growing regions in India along with a non-toxic genotype from Mexico as a prelude for utilization of promising and genetically divergent materials in the breeding programmes for molecular characterization. Molecular polymorphism was 42.0% with 400 RAPD primers and 33.5% with 100 ISSR primers between collected accessions indicating modest levels of genetic variation in the Indian germplasm. Within population variation based on RAPD polymorphism was 64.0%. Population-specific bands have been identified for the accession from Kerala (2 RAPD markers), Rajasthan (1 RAPD and 1 ISSR marker) and the non-toxic

genotype from Mexico (17 RAPD and 4 ISSR markers), which serve as diagnostic markers in genotyping (S. D. Basha, M. Sujatha 2007).

Reddy *et al.* (2007) found low polymorphism, 14-16% by RAPD and 8-10% by AFLP markers in 23 selected provenances from the total of 300 collected provenances in India. Ranade *et al.* (2008) were assessed the genetic diversity studies among the accessions by SPAR (Single Primer Amplification Reaction) method. This study showed that wild and newly introduced accessions have genetic variations and the accessions from North East India were the most distant from other accessions in UPGMA analysis.

Analysis among 13 accessions of *Jatropha curcas* (collected from different location of India) was carried out through 20 RAPD and 14 ISSRs markers (Gupta *et al.* 2008). In RAPD analysis, out of 107 fragments, 91 fragments were found polymorphic. In an average 4.45 fragments per primer was found to be polymorphic. Amplified fragments were 1 to 9 in number. One fragment was found in those PCR products in which primer used were OPA20, OPB19, and OPD13 and nine bands were analysed by using OPA18 primer. The sizes of the bands varied from 200 to 2,500 bp. The percentage of polymorphism ranges from 40% (OPB18) to 100% (14 primers). Resolution power ranges from 0.153 (OPA20, OPB19) to 11.23 (OPD15). Few genotype of Orissa (Orissa 6 and Orissa 7) showed distinctness from other Indian genotypes. In ISSR analysis 14 out of 25 were able to give reproducible amplified products. 81 bands were able to amplify with these primers, but only 62 were polymorphic. In an average 4.42 fragments per primer was found to be polymorphic. Amplified fragments were 2 (ISSR 7, ISSR 8, ISSR 16) to 9 (ISSR 12) in number and varied in size from 200 to 2,500 bp. The percentage of polymorphism ranges from 37.5% (ISSR 2, ISSR 10) to 100% (7 primers). Resolution power ranges from 0.153 (OPA20, OPB19) to 11.23 (OPD15). The primers containing poly (GA) able to produced maximum number of 9 bands. While poly (AT) and some other motifs did not give any type of amplification, means no band were found by using primers. For polymorphism detection RAPD markers were more efficient than ISSR markers, as 84.26% polymorphism was detected by using RAPD marker and 76.54% detected by ISSR markers (Gupta *et al*. 2008).

Sun *et al.* (2008) were collected 58 accessions of *Jatropha curcas* from different geographical locations in South China for SSR and AFLP marker analysis. Only one out of 17 SSR markers was found polymorphic. In AFLP analysis, out of 70 polymorphic loci only 14.3% were polymorphic. Through AFLP marker analysis it was found that *Jatropha*

accessions in Guizhou region in China were genetically diverse from the other collected accessions. In India, Tatikonda *et al.* (2009) also accessed AFLP technique on 48 elite accessions of *Jatropha curcas* which were collected from six states for plant genetic improvement and molecular breeding approaches. In total, 770 fragments were generated, but 680 fragments showed polymorphism. Out of 680 fragments which were showing polymorphism, 59 fragments were found to be unique and 108 fragments were rare (only present in 10% of the total accessions). Montes *et al.* (2009) studied 255 accessions of *Jatropha curcas* which were collected from over 30 countries in Latin America, Africa and Asia. The accessions were analysed for AFLP technique. They found that genetic variability was low in African and Indian accessions and high in Guatemalan and other Latin America accessions. Analysis of molecular diversity was carried out among eight populations in Kenya using RAPD markers. Ten random primers generated 251 loci that were scored for diversity (Machua *et. al.* 2011).

2.2.3. Phylogenetic relationship of *J. curcas*

Phylogenetic study describes the origin of particular species and helps to find relationships between their accessions. Phylogenetic study in case of *J. curcas* deals with genetic relationship of different accessions of *J. curcas* by molecular marker techniques like RAPD, AFLP, and ISSR. These studies may also reveal the closeness of one particular accession with other selected accessions.

12 *Jatropha* species were selected and RAPD investigation was carry out to find percentage of polymorphism in *Jatropha curcas* species. From 26 random primers used, 18 primers gave reproducible amplification banding patterns of 112 polymorphic bands from total 134 bands, which showed 80.2% polymorphism across the genotypes. Out of all these primers, three primers (OPA 4, OPF 11, and OPD 14) showed 100% polymorphic pattern. Polymorphic information content was found to be high in OPD 14 (0.50). Jaccard's coefficient varied from 0.00 to 0.85, which reveals higher amount of genetic variability. Phylogenetic tree analysis of these 12 accessions was also carried out by UPGMA clustering. UPGMA cluster analysis indicated three distinct clusters, one comprising all accessions of *J. curcas L.,* while second included six species viz*., J. ramanadensis Ramam., J. gossypiifolia* L*., J. podagrica, J. tanjorensis J.* L. Ellis et *Saroja, J. villosa* and *J. integerrima*, *J. glandulifera* Roxb. Remained distinct and formed third cluster indicating its higher genetic distinctness from other species.The grouping pattern of clustering corresponds well with principal component analysis confirming patterns of genetic diversity observed among the species. The results

were helpful for collection, conservation and characterization of *Jatropha* genetic resources (Ganesh Ram *et al*. 2008).

Pamidiamarri *et al.* (2009a) did phylogenetic analysis with the help of RAPD and AFLP markers to found phylogenetic relationships among seven species of Jatropha: *Jatropha curcas, Jatropha gandulifera, J. gassoypifolia L., J. multifida, J. integerrima, J. tenzorensis* and *J. podagrica.* The percentage of polymorphic loci among the species was found to be 97.74% by RAPD and 97.25% by AFLP. The phylogram made from both the studies were found to be similar. The mean percentage of polymorphism was calculated as 68.48% by RAPD and 71.33% by AFLP markers. These molecular markers study reveals the maximum relatedness between *Jatropha curcas* and *Jatropha integerrima,* which may result due to hybrid crossing between these two species. By using RAPD and AFLP techniques, researcher were able to detect *J. tenzorensis,* a natural interspecific hybrid between *Jatropha curcas* and *Jatropha integerrima* (Prabhakaran and Sujatha 1999). The above study depicted that RAPD and AFLP techniques were helpful for providing genetic divergence data as in case of *Jatropha* species.

In another study Pamidiamarri *et al.* (2009b) did phylogenetic analysis among seven species of *Jatropha* by using specific marker called nuclear ribosomal DNA internal transcribed spacers (ITS) sequences (nrDNA ITS). The sizes of nrDNA ITS regions resulted were found to be in the range of 647 to 654 bp. The overall genetic distance (GD) in *Jatropha* genus was found to be 0.385. This present study also reveals the same result that the *Jatropha curcas* and *Jatropha integerrima* were found very close to each other genetically but now one more species clustered with them that was *J. podagrica.*

Eight *Jatropha* species and three *Jatropha curcas* accessions were selected for ISSR analysis. Nine primers were showing reproducible pattern by amplified 61 polymorphic bands out of 64 and 98.14% polymorphism was analysed. Some of the primers which were showed 100% polymorphism these were named as I1, I2, I3, I4, I5, I6, I7 AND I10. Jaccard's coefficient ranges from 0.346 to 0.807 which indicated higher level of genetic variation. The UPGMA cluster analysis indicated three distinct clusters, one comprising of all accessions of *Jatropha curcas* L., while second cluster included four species: *J. tanjorensis, J. gossipifolia L., J. podogrica* and *J. maheshwarii* and M.P.Nayer and third cluster included J. *villosa, J. multifiga, Jatropha integerrima* and *Jatropha gandulifera* (Senthil Kumar *et al.* 2009).

Some peoples also analysed their result by using nuclear and organelle specific primers. In India, 34 accessions of *Jatropha curcas* comprising 8 agronomical important species (*J. curcas, J. gossipifolia L., J. podogrica J.gandulifera, J.multifida, J. villosa, J. villosa* var. ramnadensis and *J. maheshwarii)* were selected for RAPD, ISSR and microsatellite analysis. 200 RAPD, 100 ISSR and 50 microsetellte primers were used. The nuclear specific markers showed high interspecific genetic variation (98.5% polymorphism). 10 organelle specific primers were showed single, discrete band of which three were functionally disclosing polymorphism among Jatropha species. Artificial hybrids were produced between *Jatropha curcas* and other *Jatropha* species except *Jatropha podogica.* The polymorphic primers showed F1 hybrid specificity to ward parent species and confirmed the hybridity of the interspecific hybrid. The ISSR and RAPD markers revealed that *Jatropha tenjorensis* was the natural hybrid of *Jatropha curcas* and *Jatropha gossypifolia* (Basha and Sujatha 2009).

Microsatellite markers are used to analyze specific regions of oligonucleotides in the *Jatropha* genome. Microsatellite has advantages like locus specificity, co-dominant nature, high reproducibility and substantial size polymorphism (Powell *et al.* 1996). Pamidiamarri *et al.* (2009c) selected 32 accessions of *J. curcas* from Junagadh Gir forest region, India and the library was constructed for characterization with the help of microsatellite markers. In total 12 identifiable microsatellite markers were found and their cross amplification was also checked in six common species of *Jatropha*. Popluechai *et al.* in 2009 assessed genetic variation with the help of RAPD, AFLP and cTAB (combinatorial tubulin based polymorphism) in 38 accessions of *Jatropha curcas* from 13 countries. 6 accessions of India were showed appropriate genetic diversity out of all the accessions. In RAPD analysis, all the samples were examined with 10 RAPD markers. Phylogenetically two clusters were formed, one cluster contained all 17 accessions of *Jatropha curcas* and the second cluster was made up of out group *Jatropha podagrica* which was showing 52% similarity with *Jatropha curcas.* Similarity coefficient among accessions of *Jatropha curcas* was found very narrow (0.78). 14 Thai Nigerian accessions clustered together and separated from 2 Indian accessions. cTBP analysis deals with length variation of first and second intron of the member of beta tubulin gene family. Result concluded that the four accessions from Costa Rica were clearly varied from other accessions and also showed intra-specific polymorphism in both the introns.

Yu Cai in 2010 investigated genetic diversity of Chinese *Jatropha* germplasm by using ISSR Markers. Out of 100 ISSR primers, 15 that had good reproducibility were selected.127 bands

were found to be polymorphic which indicated that *Jatropha curcas in* China showed high genetic variability. Dendrogram was prepared and accessions were clustered in to two sub-groups. Level of polymorphism was found 75.12% among the germplasm of *Jatropha.* Population genetic analysis was also done which showed that distinct genetic differentiation occurred in Chinese *Jatropha*. Slight correlation of oil content variation and genetic diversity was observed. Tropical populations are the most important genetic resources. Above research reviews reveals that in India, 60-80% *Jatropha curcas* population was found to be genetically similar.

2.2.4 Distinguishing toxic and non toxic varieties

Molecular marker is become important tool for distinguishing toxic variety from non-toxic in case of different accessions of *Jatropha curcas.* Various studies were attempted on this. Few of them are mentioned below.

Genetic similarity between toxic and non-toxic variety was analysed by using RAPD and AFLP techniques. Both techniques were found to be equally important. Genetic similarity between toxic and non-toxic variety was concluded by RAPD (0.92) and also by AFLP (0.90). 371 RAPD, 1442 AFLP were analysed in total and 56 (15.09%) RAPD and 238 (16.49%) AFLP markers were specific to the varieties (Pamidiamarri *et al.* 2008). Popluechai *et al.* in 2009 did clustering of non-toxic Mexican accession (NMTA) and other *J. curcas* accessions.

Biochemical and molecular analysis was done on 72 accessions of *Jatropha curcas* representing 13 countries. In biochemical level of analysis various factors were included like seed kernel protein, oil content, ash content and phorbol ester content variation was checked with accessions from Mexico which were containing low level of phorbol content. Level of polymorphism was found 61.8% by RAPD and 35.5% by ISSR primers. With the help of RAPD and ISSR data, close clustering of accessions from all countries were showed by dendrogram based on pairwise genetic similarities. Mexican accessions were clustered separately in the cluster of III, IV, V and VI. 28 Mexican accessions resulted in identification of molecular markers associated with high and low phorbol ester content. SCARs technique was made by the conversion of RAPD and ISSR markers, used for increasing their reliability and marker assisted program was used to identify the accession which was less toxic. 12 microsatellite primers were used to identify novel alleles in Mexican germplasm and to differentiate non-toxic Mexican from other accessions. High diversity was found in Mexican genotype in terms of phorbol ester content than the other accessions of *Jatropha curcas* (Basha *et al.* 2009).

3. Origin of the problem

The presence of genetic diversity is utmost important for any plant species. A complete knowledge of the magnitude and pattern of genetic diversity in crop plant species has significant implications in breeding programmes and for the conservation of genetic resources. Classical technology using morphological markers for checking genetic diversity and relationships among accessions was not very successful due to the strong effect of environment on highly heritable seed traits like 100-seed weight, seed protein and seed oil content. Therefore, selection based on genetic assessment with the help of molecular markers is very essential due to its more reliable and consistent nature. In the present study, the collected germplasm from the different areas of the state of Punjab will be evaluated through RAPD and ISSR molecular markers to detect and document the amount of variation existing within and between the *Jatropha* populations. The understanding of the genetic structure of *Jatropha* populations collected from the state of Punjab will allow us to have genetic material available for future improvement of this biofuel crop.

4. Objectives of the present study

Keeping the above points in view, the following objectives are framed to carry out the thesis work:

- Survey of elite clones of *Jatropha curcas* from the different locations in the state of Punjab
- Extraction of genomic DNA from some field grown *Jatropha* germplasm
- Studies on genetic diversity analyses of the above *Jatropha* accessions with the help of RAPD and ISSR molecular markers

5. MATERIALS & METHODS

5.1. MATERIALS

5.1.1. Survey and selection of elite *Jatropha* germplasm

A thorough and extensive survey of *Jatropha curcas* germplasm was carried out to identify high yielding candidate plus trees (CPTs) from different locations in the state of Punjab. The Individual candidate Plus Plants (CPPs) were selected on the basis of phenotypic assessment of character of economic interests like: Seed yield (kg/plant) number of capsules per cluster, branching pattern, height, crown spread, collar diameter of the tree, disease resistance etc. Care will be taken to avoid trees infested with pests and diseases. Collection of seeds/cuttings from selected plus trees were carried out during the months from January to March.

5.1.2. Procurement of *Jatropha* plant material and other materials

- Leaves from a set of 16 different candidate plus trees (CPTs) were collected from different regions in the state of Punjab (Table: 1)
- RAPD and ISSRs primers were purchased from Xcelris Lab Ltd.
- Mini submarine electrophoresis apparatus was used.
- Various enzymes and fine chemicals used for molecular biology techniques were purchased from Bangalore Genei Pvt. Ltd., Bangalore; Amersham Biosciences, Hong Kong and MBI Fermentas.
- The routinely used chemicals were bought from Himedia Laboratories Pvt. Ltd., Mumbai and Sisco Research Laboratories Pvt. Ltd., Mumbai.
- Glass wares and Plastic wares were bought from Borosil and Tarsons Products Pvt. Ltd.

Table 1:

S.NO.	*Jatropha* Accessions	OIL CONTENT (%)
1.	TJS- 04 #42	54.49
2.	TJS- 61 #33	34.02
3.	TJS- 29 #31	32.47
4.	TJS-15 #11	35.29
5.	TJS-73 #59	28.63
6.	TJS-27 #108	33.19
7.	TJS-01 #01	35.52
8.	TJS-48 #01	36.65

9.	TJS-50 #01	33.40
10.	TJS-19 #04	38.63
11.	TJS-07 #05	29.75
12.	TJS-25 #19	34.64
13.	TJS-23 #17	35.07
14.	TJS-35 #02	35.60
15.	TJS-49 #01	32.31
16.	TJS-30 #06	35.70

5.1.3 Buffer used

a) DNA Extraction buffer

Tris Cl(pH=8.0) : 50.0 mM

Na_2EDTA : 50.0 mM

NaCl : 250.0 mM

Sucrose : 15%

b) TE Buffer (1X)

Tris-HCl : 10.0 mM (pH= 8.0)

EDTA : 1.0 mM (pH= 8.0)

c) Gel Loading Buffer (5X)

Sucrose : 30% (w/v)

EDTA : 25.0 mM (pH=8.0)

Tris HCI : 25.0 mM

Bromophenol Blue : 0.2% (w/v)

d) TBE Buffer (5X)

Tris Base : 54.0 g

Boric Acid : 28.0 g

EDTA : 3.8 g

The pH of the buffer was set at 8.0 and the volume was made up to 1000 ml with sterile double distilled water.

5.1.4. Enzymes used in the study

5.1.4.1. Ribonuclease (RNase)

Stock Solution : 10.0 mg/ml

Working Solution : 10.0-15.0 µg/ml

DNase-free RNase A was prepared as follows:

RNase was prepared in a buffer containing 10.0 mM Tris-HCl (pH= 8.0) and 15.0 mM NaCl. The solution was boiled for 10 minutes, followed by slow cooling, after which, it was dispensed into aliquots and stored at -20 °C for subsequent use.

5.1.4.2. *Taq* DNA polymerase for PCR

Stock Solution : 3 U/µl

Working Solution : 1 U/µl

5.1.4.3. *Sau*3A restriction enzyme

Tetra cutter restriction enzymes *Sau*3A was used in this study. Restriction digestion was carried out in buffer supplied by the manufacturer. Depending upon specific enzyme, reaction was carried out at appropriate temperature and BSA was added as per requirement.

5.1.5. Primer used for molecular marker analysis:

5.1.5.1. Primer description for RAPD (Table 2)

Table 2:

S.NO.	Oligo name	Sequence	Length	GC%	Tm value
1.	RNR-01	5'CCTGGCGAGC	10	80.0	36.0
2.	RNR-02	5'TCCCGACCTC	10	70.0	34.0
3.	RNR-03	5'CCAGGCGCAA	10	70.0	34.0
4-	RNR-04	5'CGGAGAGTAC	10	60.0	32.0
5.	RNR-05	5'CCTGGCGAGC	10	80.0	36.0

5.1.5.2. Primer description for ISSR (Table 3)

Table 3:

S.NO.	Oligo name	Sequence	Length	GC%	Tm value
1.	RNI-01	5'AGAGAGAGAGAGAGAGT	17	47.1	44.6
2.	RNI-02	5'AGAGAGAGAGAGAGAGC	17	52.9	47.1
3.	RNI-03	5'AGAGAGAGAGAGAGAGG	17	52.9	47.1
4.	RNI-04	5'AGAGAGAGAGAGAGAGA	17	47.1	44.6
5.	RNI-05	5'ACACACACACACACACG	17	52.9	47.1

5.1.6. Others

For DNA isolation and purification:

- Liquid nitrogen
- 5 M potassium acetate
- Isopropanol
- Phenol and chloroform
- 3 M sodium acetate
- Dehydrated alcohol
- Normal and cold Centrifuge
- water bath
- -20°C refrigerator
- Nanodrop spectrophotometer
- Agarose gel electrophoretic apparatus.

5.2 METHODS

5.2.1. Extraction of total genomic DNA from different accessions of *Jatropha curcas*

A total 16 elite accessions of field grown *Jatropha curcas* were selected for total plant DNA isolation. The plant material (usually soft leaves) was washed under running tap water followed by sterile distilled water wash. The water was removed from the plant material by blotting it with filter paper. Weighed out 150-200 mg of above plant material and transferred to a clean and dry pestle and motor. The material was grinded in the presence of liquid nitrogen into a fine powder. Extraction buffer 1-1.2 ml was added in the frozen powder and transferred the mixture into eppendorf tubes. The tubes were incubated at 65°c for 15min with intermittent shaking. $1/3^{rd}$ volume of (500 µl) 5M potassium acetate was added to the solution. Mixed vigorously and incubated in ice for 20 minutes. Centrifuged the content at 5500 rpm for 25 minutes in cold centrifuge. Took supernatant in fresh eppendorf tubes and $2/3^{rd}$ volume of iso-propanol was added into it. Shake slowly and the tubes were incubated at -20°C overnight. The DNA was palleted out by centrifugation at 12000 rpm for 15 minutes. The pallet was washed with ice cold 70% ethanol. Centrifuged the content at 11000 rpm for 8 minutes. Suspended the pellet into100 µl TE buffer after complete drying of pellet for about 30-40 min.

Initially, volume make for the DNA preparation was carried out by adding 400 to 500 µl of sterile double distilled water as per requirement. Then, extracted DNA was purified with the addition of 4.0 µl of RNase in the eppendorf tubes. Mixed well and incubate it at 37°C for 30-45 minutes. Phenol - chloroform extraction was done by adding equal volume of phenol: chloroform and Mixed properly again for 5-8 minutes by inversion method. Centrifuged the content at 8000 rpm for 10 minutes. Took supernatant in to fresh eppendorf tubes and added equal volume of chloroform. Mixed properly for 5-8 minutes. Centrifuged at 8000 rpm for 10 minutes. Upper aqueous layer was collected into fresh eppendorf tubes and $1/10^{th}$ volume of 3 M sodium acetate was added into it. Mixed properly for 2-3 min and double amount of dehydrated alcohol was added into each eppendorf tube to precipitate the genomic DNA. Mixing was done for 2-3 minutes. After centrifugation at 12000 rpm for 15 minutes, pellet was air dried and dissolved in 50 µl of TE buffer. The purified DNA Samples were shifted to -20°C for overnight incubation or for long term storage.

5.2.2. Determination of quality and quantity of purified genomic DNA samples from 16 *Jatropha curcas* accessions

- The quality of total purified genomic DNA samples was checked out by running 0.7% agarose gel electrophoresis, taking lamda DNA as molecular marker in one of the well.

- Restriction Digestion of genomic DNA: Restriction digestion of *Jatropha curcas* total genomic DNA was carried out to check the purity of DNA samples. The cellular proteins if present along with the genomic DNA may cause reduction in the activity of restriction enzymes. Proteins masks the binding sites of restriction enzymes present on genomic DNA and thus cause incomplete digestion of DNA. Thus, in order to check protein contamination in the isolated DNA samples, its restriction digestion was performed with *Sau*3A endonuclease enzyme. Restriction digestion was performed according to manufacturer's instructions, making the reaction volume to 15 µl. Efficient working temperature for *Sau*3A restriction digestion was set at 37°C for 15 min. After the reaction, the digested DNA samples were checked out by 1.0% agarose gel electrophoresis.
- The quantity of 16 *Jatropha* DNA sample was checked out by taking ratio of absorbance at 260nm and 280nm (A260/280) by nanodrop spectrophotometer. The concentration of each DNA sample was determined in µg/ml (1µg/ml =1ng/µl).
- Master *Jatropha* DNA stock was diluted to make a working solution of 10ng/µl for PCR analysis to check genetic variation collected accessions through molecular markers.

5.2.3. Molecular characterization of *Jatropha curcas* genotypes

Molecular characterization was carried out using PCR based random amplified polymorphic DNA (RAPD) and Inter simple sequence repeats (ISSRs) technique. DNA of 16 elite *Jatropha* genotypes were isolated and working solution of 10ng/µl were made from each of these 16 samples. PCR reaction was set for RAPD and ISSRs technique. PCR was carried out in 30 µl reaction volume and various components were used as follows:

Double distilled water	19µl
10x PCR buffer	3µl
dNTPs mix	2µl
Primer used	3µl
Template DNA	3µl
Taq DNA polymerase	1 µl

The addition of enzyme was done after giving the reaction mixture a hot start at 94^{o}C for 3.0 minutes.

5.2.3.1. Reaction Mixture for PCR

In our study, we used 5 primers for RAPD and 5 primers for ISSRs analysis. One primer at a time was used to study the polymorphism among 16 elite *Jatropha* genotypes by PCR based RAPD and ISSRs assay. Reaction with double distilled water, 10x PCR buffer, dNTPs and particular primer was prepared to avoid handling errors. The reaction mix was then distributed to 16 tubes (25µl per tube) followed by addition of 3µl of respective template DNA to make the total reaction volume to 30µl.

5.2.3.2. PCR-amplification

The thermal cycling parameters were set as follows (Table 4):

Table 4:

Stages	Steps	Temperature(°C)	Duration (min)	No. of Cycles
1.	Initial denaturation	94.0	3.0	1
2.	a. Denaturation	94.0	1.0	35
	b. Annealing	42.0	2.0	35
	c. Extension	72.0	2.0	35
3.	a. Final Extension (Post PCR)	72.0	5.0	1
	b. Soak	4.0	∞	-

The reaction was carried out for 35 cycles.

After the completion of required cycles of PCR amplification the samples were stored at -20°C in a refrigerator. To check banding pattern the PCR samples were loaded on 1.5% agarose gel. The size of bands can be determined by running 500 bp and 100 bp ladders along with the PCR products in agarose gel. The gel was run in 0.5X electrophoretic TBE buffer for 2-3 hours and photographed using gel documentation system.

6. RESULTS AND DISCUSSION

6.1. Isolation of total DNA from *Jatropha curcas:*

Depending on the nature and complexity of plant material, proper method needs to be employed for extraction of total genomic DNA, along with its performance evaluation by different molecular techniques. Moreover, isolation of good quality genomic DNA from different plant materials is an important prerequisite for many molecular techniques related to both basic and applied research in the area of plant molecular biology, crop improvement, biodiversity studies and conservation of genetic resources (Kumari *et al*. 2012). Application of molecular techniques is essential for enhancing the seed oil content of this crop and to improve the fuel characteristics of biodiesel by altering the fatty acid composition. So, in order to start with the basic molecular studies on *Jatropha*, total genomic DNA was isolated from freshly collected leaves of 16 (sixteen) elite *Jatropha* accessions viz. TJS- 04 #42, TJS-61 #33, TJS- 29 #31, TJS-15 #11, TJS-73 #59, TJS-27 #108, TJS-01 #01, TJS-48 #01, TJS-50 #01, TJS-19 #04, TJS-07 #05, TJS-25 #19, TJS-23 #17, TJS-35 #02, TJS-49 #01, TJS-30 #06 by method given by Kumari *et al*. 2012. The protocol was found to be very efficient, cost effective and yields good quality genomic DNA. The quality of DNA was checked by agarose gel electrophoresis along with bacteriophage lambda DNA (Fig 3). The DNA bands appeared to be compact with negligible smearing indicating little degradation during isolation.

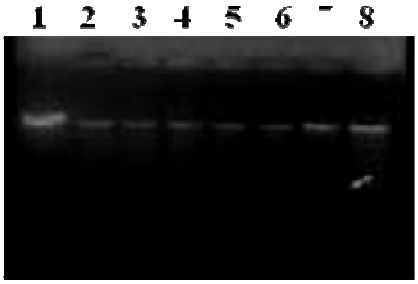


Fig 3. Agarose Gel Electrophoresis of *Jatopha curcas* total genomic DNA
Lane 1: Bacteriophage λ DNA
Lane 2. TJS-04 #42 total DNA
Lane 3: TJS- 61 #33 total DNA
Lane 4: TJS- 29 #31 totalDNA
Lane 5: TJS-15 #11 total DNA
Lane: 6: TJS-73 #59 total DNA
Lane: 7: TJS-27 #108 total DNA
Lane: 8: TJS-01 #01 total DNA

6.2. Spectro-photometric Analysis of DNA samples

The yield of *Jatropha curcas* DNA samples was determined nanao-drop spectro-photometer. The yield of DNA for all the sixteen *Jatropha* accessions was ranging from 40-110 µg/g of the plant tissue. The A_{260}/A_{280} ratio (Table 5) of the DNA samples was approaching 1.8, which clearly indicated that the isolated DNA is free of impurities such as proteins and phenolic compounds and thus of good quality.

Table 5:

S.No.	*Jatropha* accessions	A_{260}/A_{280} ratio
1	TJS 04#42	1.65
2	TJS 61#33	1.92
3	TJS 29#31	1.72
4	TJS 15#11	1.69
5	TJS 73#59	1.74
6	TJS 27#108	1.66
7	TJS 01#01	1.90
8	TJS 48#01	1.73
9	TJS 50#01	1.65
10	TJS 19#04	1.69
11	TJS 07#05	1.34
12	TJS 25#19	1.75
13	TJS 23#17	1.68
14	TJS 35#02	1.80
15	TJS 49#01	1.71
16	TJS 30#06	1.66

6.3. Restriction analysis

The quality of the *Jatropha* DNA preparations were further characterized by restriction analyses using tetracutter restriction enzyme namely *Sau*3A1. Agarose gel electrophoresis of digested samples revealed varying smearing pattern (Fig 4). An extensive smearing was obtained with tetracutter *Sau*3A1 enzyme because of huge number of tetra restriction sites present in the genome of the plant. The digestion pattern clearly indicated that the DNA samples were essentially devoid of inhibitory substances.

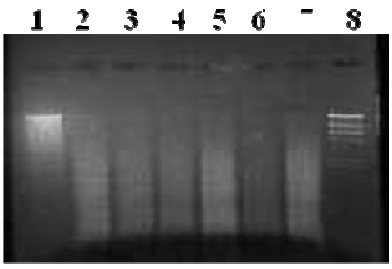


Fig 4: Restriction enzyme digestion of *Jatropha* total genomic DNA
Lane 1: Total DNA TJS- 04 #42 (uncut)
Lane 2: Total DNA TJS- 04 #42 digested with Sau3A1
Lane 3: Total DNA TJS -29#31 digested with Sau3A1
Lane 4: Total DNA TJS-15#11 digested with Sau3A1
Lane 5: Total DNA TJS-73#59 digested with Sau3A1
Lane 6. Total DNA TJS-27#108 digested with Sau3A1
Lane 7: Total DNA TJS-01#01 digested with Sau3A1
Lane 8: λ DNA digested with Hind III

In addition to present work undertaken, isolation of total DNA will serve as foundation step in carrying out further genetic studies on *Jatropha* in future.

6.4. Genetic diversity based on ISSR and RAPD Profiling

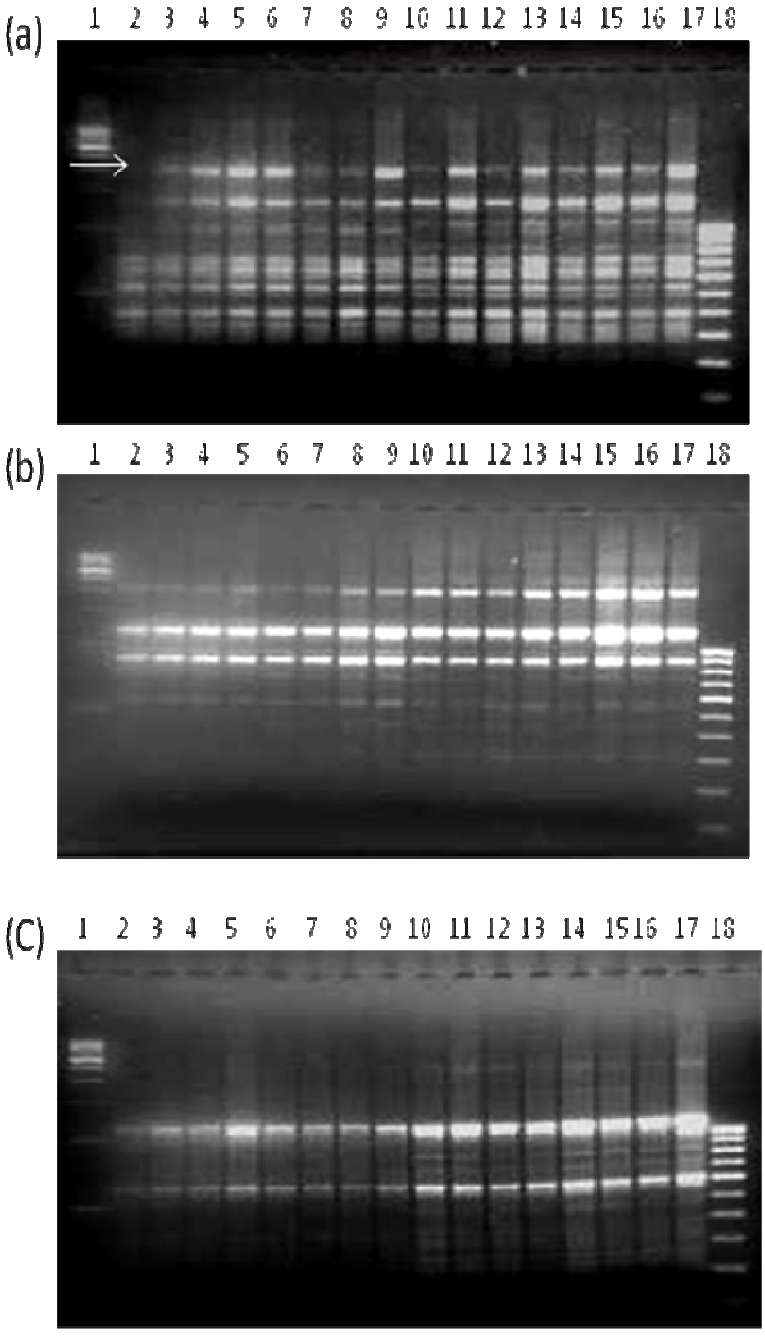
In order to analyze diversity amongst accessions of the same species, it is of huge significance to study wild as well as maintained at local collections and holdings. In the present study we have used two independent RAPD and ISSR methods on different accessions of *Jatropha curcas* collected from the different locations in the state of Punjab. The RAPD profiles usually represent widely distributed portions of the genome and may reveal general similarities, while the ISSR profiles are generated from microsatellite rich regions and reveal similarities among the repetitive sequence families. Thus the two methods

involve regions having quite different evolutionary histories and have different coverage capacity (Powell *et al*. 1996).

6.4.1. ISSR profiling

In the study, five ISSR primers were used for DNA amplification of sixteen elite *Jatropha curcas* accessions. Two primers showed polymorphism amongst *Jatropha* accessions. The results indicated amplification of alleles of different sizes for each primer tested (Fig 5). Different primers produced varied amplification patterns (Table 6). Primer RNI-01 amplified nine alleles of 400-2000 bp size. Out of nine alleles amplified, an allele of 2 kb showed polymorphism amongst different accessions tested. Primer RNI-01 amplified 2.0 kb allele in all accessions except in accession TJS-04 #42. The absence of 2.0 kb allele in TJS-04 #42 but its presence in other 15 *Jatropha* accessions indicated diverse nature of accession TJS-04 #42 when amplification reaction was carried out with RNI-01 primer. Similarly, primer RNI-04 amplified 1.2 kb allele in accessions TJS 50#01, TJS 19#04, TJS 07#05 which was absent in other 13 *Jatropha* accessions. The absence or presence of allelic amplification indicated the

presence of genetic diversity in the *Jatropha* accessions collected from the different locations of Punjab.



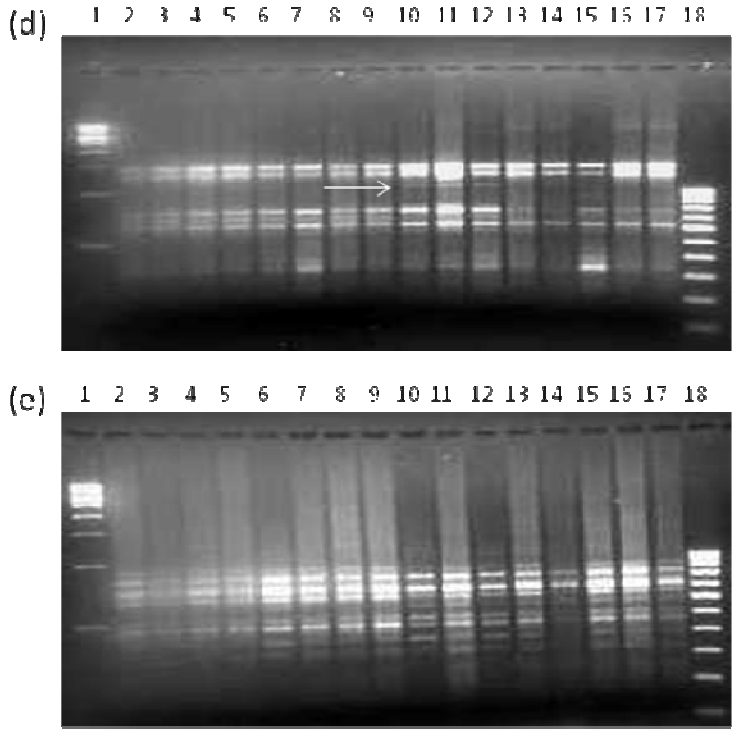


Fig. 5. Molecular analysis of *Jatropha curcas* germplasm using ISSR primers
(a) Electrophoretic analysis of DNA amplification with RNI-01 primer. Lane 1: represents 500 bp molecular weight marker, Lane 2-17: represents samples used in the study, Lane 18: represents 100 bp molecular weight marker. Arrow indicates absence of 2 kb band in TJS-04 #42. (b) Electrophoretic analysis of DNA amplification with RNI-C2 primer. Lane 1: represents 500 bp molecular weight marker, Lane 2-17: represents samples used in the study, Lane 18: represents 100 bp molecular weight marker. (c) Electrophoretic analysis of DNA amplification with RNI 03 primer Lane 1: represents 500 bp molecular weight marker, Lane 2 17: represents samples used in the study, Lane 18 represents 100 bp molecular weight marker. (d) Electrophoretic analysis of DNA amplification with RNI-04 primer. Lane 1: represents 500 bp molecular weight marker, Lane 2-17: represents samples used in the study, Lane 18 represents 100 bp molecular weight marker. Arrow indicates presence of ~ 1.2 kb band in TJS-19 #04, TJS-07 #05 and TJS-25 #19 (e) Electrophoretic analysis of DNA amplification with RN -C5 primer. Lane 1: represents 500 bp molecular weight marker, Lane 2-17: represents samples used in the study, Lane 18: represents 100 bp molecular weight marker.

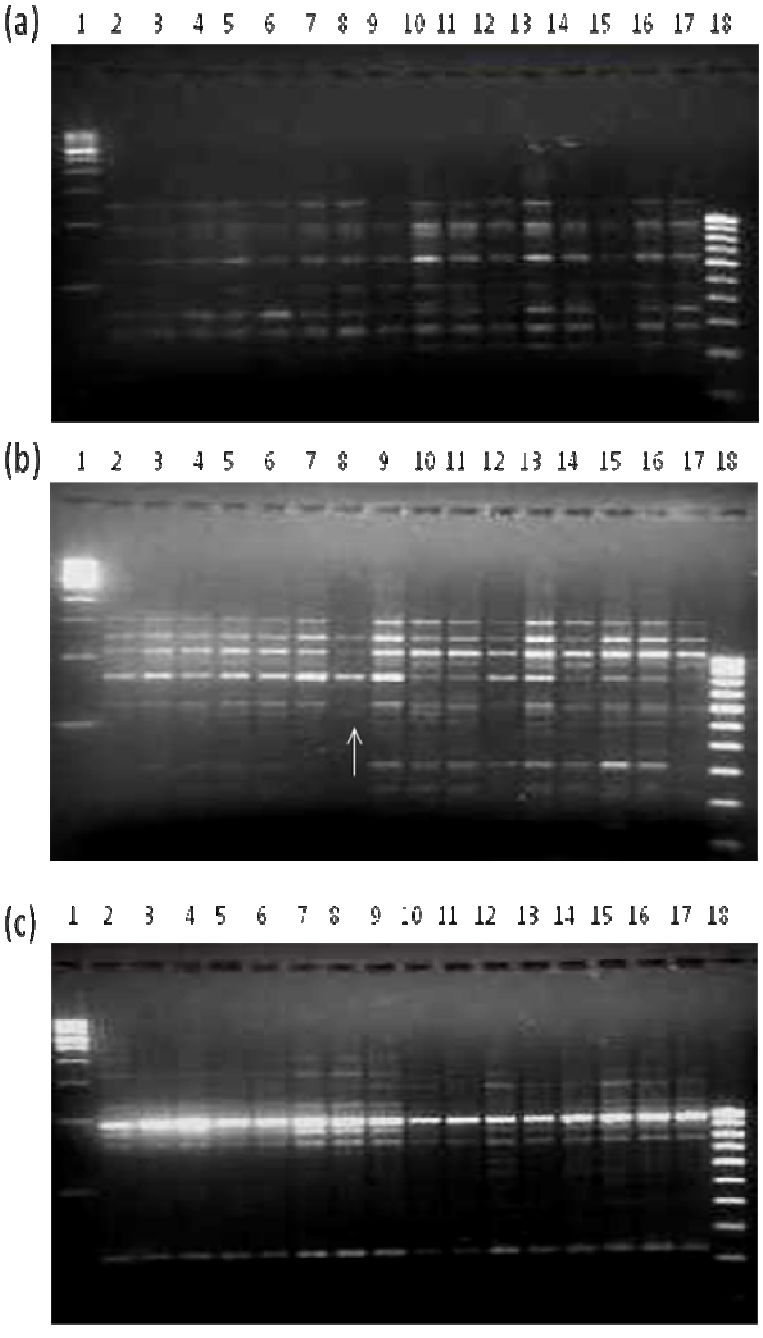
Table 6:

S. No.	Primer	ISSR Sequence	Total no. of amplified alleles	Allele size (bp)
1	RNI-01	AGAGAGAGAGAGAGAGT	9	400-2000
2	RNI-02	AGAGAGAGAGAGAGAGC	4	600-2000
3	RNI-03	AGAGAGAGAGAGAGAGG	2	600-1200
4	RNI-04	AGAGAGAGAGAGAGAGA	4	600-1500
5	RNI-05	ACACACACACACACACG	5	500-850

6.4.2. RAPD Profiling

In the present study, five RAPD primers were tested on sixteen elite *Jatropha curcas* accessions for intraspecific genetic diversity studies. The results indicated amplification of

alleles of different sizes for each primer tested. RAPD primers amplified number of alleles ranging from minimum of one to maximum of six alleles. These amplified alleles were of different size range (220-1500 bp). Results showed the absence of 600 bp allele in accession TJS 01#01 which was present in 15 *Jatropha* accessions when amplification reaction was carried out with RNR-02 primer. The varied allelic amplification indicated the presence of genetic diversity in the *Jatropha* accessions collected from the different locations of Punjab.



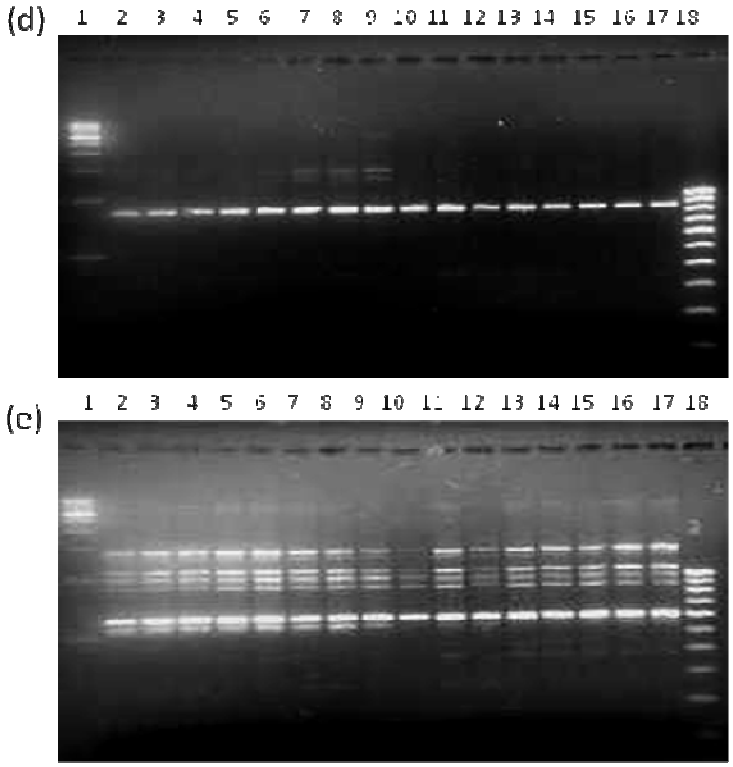


Fig. 6. Molecular analysis of *Jatropha curces* germplasm using RAPD primers
(a) Electrophoretic analysis of DNA amplification with RNR-01 primer. Lane 1: represents 500 bp molecular weight marker, Lane 2-17: represents samples used in the study, Lane 18: represents 100 bp molecular weight marker. (b) Electrophoretic analysis of DNA amplification with RNR-02 primer. Lane 1: represents 500 bp molecular weight marker, Lane 2-17: represents samples used in the study, Lane 18: represents 100 bp molecular weight marker. Arrow indicates absence of ~800 bp band in TJS-01 #01 *Jatropha* accession (C) Electrophoretic analysis of DNA amplification with RNR-03 primer. Lane 1: represents 500 bp molecular weight marker, Lane 2-17: represents samples used in the study, Lane 18: represents 100 bp molecular weight marker. (d) Electrophoretic analysis of DNA amplification with RNR-04 primer. Lane 1: represents 500 bp molecular weight marker, Lane 2-17: represents samples used in the study, Lane 18: represents 100 bp molecular weight marker. (e) Electrophoretic analysis of DNA amplification with RNR-05 primer. Lane 1: represents 500 bp molecular weight marker, Lane 2-17: represents samples used in the study, Lane 18: represents 100 bp molecular weight marker.

Table7:

S. No.	Primer Code	RAPD Sequence	No. fragments per accession	~ Size of the fragments (bp)
1	RNR-01	CCTGGCGAGC	5	300-1400
2	RNR-02	TCCCGACCTC	6	300-1500
3	RNR-03	CCAGGCGCAA	4	220-1100
4	RNR-04	CGGAGAGTAC	1	850
5	RNR-05	CCTGGCGAGC	6	650-1400

Concluding Remarks: The present investigation was a preliminary study to identify genetic diversity among the different accessions of *Jatropha curcas* L. from the different locations in the state of Punjab. We concluded significant polymorphism in the *Jatropha curcas* L. accessions on the bases of ISSR and RAPD genotyping. Different band pattern with different sizes were obtained when all these primers were used respectively. The polymorphism was found when RNI-01, RNI-04 and RNR-02 primers were used for genotyping. In ISSR profiling, the absence of 2 kb allele in TJS 04#42, when their genomic DNA was amplified with RNI-01 primer. The presence of 1.2 kb allele in TJS 50#01, TJS 19#04 and TJS 07#05 accessions when RNI-04 primer was used to amplify their genomic DNA. These bands are unique and absent in other accessions, resulted polymorphism. Similarly, in RAPD profiling, the absence 600bp allele in TJS 01#01 when RNR-02 primer were used for amplification. We also concluded the difference in the size range when 5 ISSR AND 5 RAPD primers were used. The allele size range resulted by ISSR primers was found from 400 bp to 2000 bp and the allele size range resulted by RAPD primer was found from 200 bp to 1500 bp. The understanding of the genetic structure of populations, selection of superior/elite genotypes will further allow us to have genetic material available for future improvement of this important bio fuel crop through biotechnological interventions. These approaches are further used for UPGMA and statistical analysis and some other molecular approaches like AFLP may also applied on *Jatropha curcas* accessions for genetic diversity studies.

7. SUMMARY

Various experimental steps as adopted in the study are briefly discussed below:

- Survey and selection of *Jatropha curcas* germplasm was carried out to identify high yielding candidate plus trees (CPTs) from different locations in the state of Punjab.
- The total genomic DNA was isolated from 16 selected accessions of *Jatropha curcas* by taking fresh and soft leaves as extracted material.
- Quality and quantity of each DNA sample was also determined by agarose gel running and nanodrop spectrophotometer, respectively. By nanodrop spectrophotometer yield was also determined. Restriction analysis was carried out which revealed that the isolated DNA samples were essentially free of inhibitory materials.
- After DNA isolation, molecular characterization was carried out using PCR based random amplified polymorphic DNA (RAPD) and Inter simple sequence repeats (ISSRs) technique. We used 5 primers for RAPD and 5 primers for ISSRs analysis.
- We found polymorphism in banding patterns of amplified genomic DNA of few accessions like TJS 04#42, TJS 50#01, TJS 19#04 and TJS 07#05 by ISSR genotyping and accessions like TJS 01#01 by RAPD genotyping. Variability in band size range was also concluded.
- The absence or presence of few different bands indicates the presence of DNA polymorphism in the *Jatropha* accessions collected from the different locations of Punjab.

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