Studies on Molecular Diversity in *Jatropha* and Development of Molecular Markers

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by

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Under Supervision of

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Discipline of Wasteland Research

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Bhavnagar - 364 021, Gujarat

October -2008

Dedicated to my Family

&

Friends

CSMCRI

केन्द्रीय नमक व समुद्री रसायन अनुसंधान संस्थान
गिजुभाई बधेका मार्ग, भावनगर- ३६४ ००२

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CERTIFICATE BY GUIDE

This is to certify that the contents of this thesis entitled **"Studies on Molecular Diversity in *Jatropha* and Development of Molecular Markers"** is the original research work of **Mr. D V N Sudheer Pamidimarri** carried out under my supervision. I further certify that this work has not been submitted either partly or fully to any other university/institution for the award of any diploma/degree.

Place: Bhavnagar

Date:

M. P. Reddy
(Supervisor)

CANDIDATE'S STATEMENT

I hereby declare that the work incorporated in the present thesis is original and has not been submitted to any university/institution for the award of any diploma/degree. I further declare that the results presented in the thesis and conclusions made therein, contribute in general to the advancement of knowledge in **Botany** and in particular to **"Molecular Diversity in *Jatropha* and Development of Molecular Markers".**

Place: Bhavnagar

Date: **(D V N Sudheer Pamidimarri)**

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CONTENTS

ABBREVIATIONS AND SYMBOLS

And others	*et al.,*
base pair	bp
Degree centigrade	°C
deoxynucleotides	dNTPs
Enzyme units	U
For example	e.g.
Genetic similarity	GS
Genetic distance	GDS
Gram	g
Hour	h
Liters	L
Meter	m
Micro	µ
Milli	m
Minute	min
Molar	M
Nano	n
Optical density	O.D.
Per	/
Percentage	%
Percentage of polymorphism	PP
Pico	p
Precipitate	ppt
Room temperature (25 ºC)	RT
Seconds	sec
That is	i.e.
Ultraviolet	UV
Versus	*vs*
Volume by volume	v/v
Weight	Wt
Weight by volume	w/v

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CHAPTER 1

INTRODUCTION

Global warming which is due to aggravated green house gases is now responsible for changes occurring in the environment. These changes are showing inevitable consequences, also deterring the human life and development. In pursuit of better life, renewable energy resources are being extracted for alternative to the depleting resource in particular to "petroleum products". Bio-energy also has the ability to minimize aggravation of green house gases. Bio-fuels and bio-energy encompass wide range sources of renewable energy based on biological origin. These renewable energy sources offer prospects of increasing energy supplies in a self reliant way in developing countries like India and also work as checkpoint for aggravating green house gases [Fairless, 2007]. Plant based fuels are best renewable sources, and its use can lead to a better balance of carbon dioxide and other green house gases formation responsible for global warming. Biodiesel, a methyl ester of fatty acids, made out of edible or non-edible vegetable oils, is an appropriate alternative to petroleum based diesel. Use of bio-diesel will not only reduce pollution levels dramatically but also helps in meeting growing demand of fuel and reduce the burden on imports of crude oil. Biodiesel derived out of *Jatropha curcas* seed oil is fast emerging as an alternative to fossil fuel as it has desired physiochemical characteristics and performance even superior to conventional petro-diesel, albeit after suitable modification [Heller, 1996; Openshaw, 2000; Mandpe *et al.,* 2005].

Genus *Jatropha* with 172 species having significant economic importance is native to Central America and distributed in Africa and Asia [Fairless, 2007]. *J. curcas, J. glandulifera, J. gossypifolia, J. integerrima, J. multifida, J. podagrica,* and *J. tanjorensis* are widely distributed in India. *J. curcas, J. integerrima* and *J. glandulifera* are native to America [Sunita *et al.,* 2005]. *J podagrica* is a multipurpose shrub

commonly found in Africa, Asia and Latin America [Olapeju *et al.,* 2007]. *J. tanjorensis* Ellis & Saroja, reported to be native to India distributed in only few districts of Tamil Nadu, generally grown as a hedge plant and reported as natural interspecific hybrid between *J. curcas* L. and *J. gossypifolia* [Prabakaran & Sujatha, 1999]. *J. gossypifolia* L. also called as bellyache bush and is a major weed in Australia [Taofeeq *et al.,* 2005]. *J. multifida* found naturally in Mexico is a popular landscape plant in South Florida [Yotam *et al.*, 2000]. *J. integerrima, J. multifida*, and *J. podagrica* are important ornamental plants, whereas, *J. curcas* and *J. glandulifera* have medicinal value [Ramchandani & Jolly, 1988]. Besides having medicinal value *J. curcas* has emerged as a potential biodiesel crop alternative to petro-diesel [Banerji *et al.,* 1985; Gubitz *et al.,* 1999; Mandpe *et al.,* 2005; Martin & Mayeux, 1984; Staubmann *et al.,* 1999; Takeda 1982]. In addition the plant has characteristics such as tolerance to stress and is not grazed by animals. The by-products obtained while, preparing biodiesel has industrial applications [Yotam et al., 2005].

J. curcas can be cultivated on lands, which are largely unproductive and are located in poverty-stricken, watershed areas and degraded forests. Added to above, this crop does not compete with other important food crops and in turn will not have a major impact on cropping pattern. The cultivation of this species on degraded soils will not only help in their re-vegetation but also help to reduce dependency on oil imports. However, large scale cultivation of *J. curcas* remains the most critically important issue that will ultimately decide success. It is reported that the crop is characterized by variable and unpredictable yield for reasons that have not been identified [Ginwal *et al.,* 2004] which limits the large scale cultivation and warrants the need for genetic improvement of the species. Therefore, measures need to be taken to improve the species genetically for higher yield by exploiting the available plant genetic resources. By establishing genetic distances through DNA fingerprinting, and other efficient tools like nrDNA ITS sequence will generating huge amount of genetic information regarding diversity which will not only useful for phylogenetic and evolutionary studies of *J. curcas* and its sister taxa, but also for genetic improvement of the species using marker based breeding techniques.

Molecular marker analysis in genome wide studies greatly enhanced the speed and efficiency of crop improvement. Molecular markers closely linked to traits of economic importance have been developed in several crops [Stein & Keller, 2001]. These have allowed the selection of desirable traits in a genotype. DNA fingerprinting techniques like Restriction Fragment Length Polymorphism (RFLP), Amplified Fragment Length Polymorphism (AFLP), Randomly Amplified Polymorphic DNA (RAPD), micro-satellites markers/Simple Sequence Repeats, Sequence Characterized Amplified Regions (SCARs), Sequence Tagged Sites (STS), nrDNA ITS have been used for the generation of molecular markers and were efficiently used in breeding programs [Farooq & Azam, 2002; Spada *et al.,* 2004; Gupta *et al.,* 1999; Waycott *et al.,* 1999].

RAPD and AFLP markers have been proved to be valuable tools in the characterization and evaluation of genetic diversity and phylogenetic analysis of any species. RAPD [Williams *et al.,* 1990] technique acquired importance because of its technical simplicity, cost efficiency and possibility of generating a large number of markers. AFLP [Vos *et al.,* 1995] is very efficient and reproducible method which generates hundreds of informative genetic markers in a short time provides high quality fingerprints with high marker reproducibility. The major advantage of RAPD and AFLP fingerprinting techniques is no need of previous knowledge of the genome sequence for its application and require very small amount of DNA per analysis.

ITS region belongs to the nrDNA family which has the property of under going rapid concerted evolution. The small size, ease of amplification and rapid concerted evolution made nrDNA ITS region, one of the most important tool from phylogenetic standpoint which will promote accurate reconstruction of taxonomic relationships. Many studies were undertaken to deduce the phylogetic relation among species of many genus [Wen *et al.,* 1996; Alice & Campbell, 1999; Sang *et al.,* 1995] including those belonging to family Euphorbiaceae [Berry *et al.,* 2005] but till date there are no reports in and among the species of genus *Jatropha*.

In recent times microsatellites/SSRs have become one of the most popular molecular markers used extensively for various molecular studies. High polymorphism

and the relative ease of scoring are the two major features that made the microsatellites suitable choice for many genetic studies. Microsatellites are tandemly repeating motifs of 1-6 bases found in all prokaryotic and eukaryotic genome characterized by a high degree of length polymorphism. Soon after their first description [Litt & Luty, 1989; Tautz, 1989; Weber & May, 1989] microsatellites have proven to be an extremely valuable tool for genome mapping in many organisms [Schuler *et al.,* 1996; Knapik *et al.,* 1998]. The major drawback of microsatellites is that they need to be isolated *de novo* from species being examined for the first time.

There are very few empirical reports on the molecular characterization of the *Jatropha* species whose economic importance span over widely in industrial, medicinal and energy resource management. So, there is a need to study the genetic diversity and deduce the phylogenetic relationships in and among the species of genus *Jatropha* and also complete characterization of the species *J. curcas* to improve the species for better utilization by exploiting the available plant genetic resources. Therefore, the present investigation was undertaken employing the most efficient DNA fingerprinting techniques (RAPD and AFLP), single locus nuclear ribosomal DNA sequence (nrDNA ITS) and microsatellites (SSR) as tools to study the molecular diversity and development of molecular markers in *Jatropha* with the following objectives:

- Molecular diversity analysis by RAPD.
- Molecular diversity analysis by AFLP.
- Comparative analysis of both RAPD and AFLP molecular diversity studies.
- Development and characterization of molecular markers.

CHAPTER 2

REVIEW OF LITERATURE

Genus *Jatropha* is derived from the Greek word 'Jatros' meaning 'doctor' and 'trophe' meaning 'nutrition'. Genus *Jatropha* Linn., comprising of 172 species, is distributed in tropical and sub-tropical parts of the world, of which nine species namely *J. curcas, J. gossypifolia, J. multifida, J. glandulifera, J. integerrima, J. podagrica, J. tanjorensis, J. maheshwaria and J. villosa* occur in India [Openshaw, 2000]. Out of nine, seven species (*J. curcas, J. gossypifolia, J. multifida, J. glandulfera, J. integerrima, J. podagrica, J. tanjorensis*) are economically important and distributed in India.

Table 1: Classification of the Genus *Jatropha*.

Kingdom	**Plantae**
Subkingdom	**Tracheobionta**
Super division	**Spermatophyte**
Division	**Magnoliophyta**
Class	**Magnoliopsida**
Subclass	**Rosidae**
Order	**Euphorbiales**
Family	**Euphorbiaceae**
Genus	***Jatropha***

J. curcas, J. integerrima and *J. glandulifera* are native to America [Sujatha *et al.,* 2005]. *J podagrica* is a multipurpose shrub commonly found in Africa, Asia and Latin America [Olapeju *et al.,* 2007]. *J. tanjorensis* Ellis & Saroja, reported to be native to India is found only in few districts of Tamil Nadu, generally grown as a hedge plant and reported as natural interspecific hybrid between *J. curcas* and *J. gossypifolia* [Prabakaran & Sujatha, 1999]. *J. multifida* occurs as natural population in Mexico and is a popular

landscape plant in South Florida [Yotam *et al.,* 2000]. *J. gossypifolia* also called "bellyache bush", is a major weed in Australia [Taofeeq *et al.,* 2005].

2.1. BOTANICAL DESCRIPTION OF DIFFERENT SPECIES OF *JATROPHA*

2.1.1 *Jatropha curcas*:

J. curcas is a 3–5 m tall deciduous shrub. The leaves are cordate, 3-5 lobed and 10-15 cm long. It flowers twice a year once during May-June and other in September-November. Seeds usually mature within a month. The flowers are unisexual, monoecious yellowish green in glabrous or pubescent cymes at the end of the branches. Fruits are trilocular capsules 1.5–3.0 cm long.

The seeds contain 30-35% viscous, non-edible oil that has attracted the attention of world as alternate fuel [Takeda, 1982; Banerji *et al.,* 1985; Martin and Mayeux, 1985; Openshaw, 2000]. Seed cake obtained after expelling the oil is rich in protein (19.0%), carbohydrates (17.0%), fiber (16.0%), Nitrogen (6%), Phosphorous (2.75%) and Potassium (0.94%). The deoiled cake is similar to chicken manure and can be used as excellent organic fertilizer [Makkar & Becker K, 1997]

J. curcas grows under wide range of arid and semi-arid climatic conditions. Hot and humid climate favours seed germination. It can be cultivated successfully in the regions having scanty to moderate rainfall and can be used to control soil erosion. Oil extracted from seeds can be processed for obtaining biodiesel whose exhausts have proved to be safe; making it as a good alternative for conventional petrodiesel.

2.1.2. *Jatropha gossypifolia*:

J. gossypifolia is a medium sized shrub with thick stout stem, profuse branching, alternate, dark brown leaves with 3-5 lobes, serrate margin with dense pigmentation and glands. The flowers are small purple red, monoecious, unisexual arranged in cymose pattern. Each flowers has six to eight yellow stamens arranged in two whorls, the pollen are highly fertile.

2.1.3. *Jatropha integerrima*:

J. integerrima is slender-stemmed, multi-trunked tropical evergreen tree or large shrub with long leaves varying in shape from oblong to fiddle or even-lobed. One inch wide red flowers are produced round the year in beautiful clusters which are held upright above the foliage. The seed capsules are brown which hold smooth, speckled, and toxic seeds.

2.1.4. *Jatropha multifida*:

J. multifida is a small shrub with loose spreading crown and reaches upto 20 feet (~ 6 m). Large leaves, growing upto 12 inch (30.5 cm) wide and cut deeply into 7-11 narrow lobes. A margin of each lobe is dissected into narrow pointed segments. Sun facing part of leaf is dark green and lower side of leaf is light greenish. The flowers are bright coral red and borne in flat-topped clusters on long stalks held high above the foliage. Coral plant looks more like cloudy water.

2.1.5. *Jatropha podagrica*:

J. podagrica is a shrub and can grow up to 2-3 feet high. It has knobby, grey-skinned, swollen stem, a large bottle-like caudex, and huge three-lobed smooth waxy leaves upto 10-12 inches in diameter. Pretty, brilliant scarlet coral-like flowers are arranged in large terminal clusters.

2.1.6. *Jatropha glandulifera*:

J. glandulifera is a medium sized shrub with thick stout stem, profuse branching, green leaves alternately arranged with 3-5 lobes, margins are serrate with dense pigmentation and glands. The flowers are small purple red, monoecious, unisexual arranged in cymose pattern. Each flowers has six to eight yellow stamens arranged in two whorls, the pollen are highly fertile.

2.1.7. *Jatropha tanjorensis*:

J. tanjorensis is a medium sized shrub with thick stout stem. Leaves are light to dark green coloured, alternately arranged, five lobed, have distinct serrate margins, long petiole with dense pigmentation, cymose inflorescence with co-inflorescence,

monoecious, unisexual and bisexual, medium sized green calyx with pale pink tinged flowers, eight stamens are arranged in a single layer with sterile pollen.

2.2. STUDIES CARRIED OUT IN *JATROPHA*

2.2.1. Biochemical analysis of *Jatropha* spcecies:

Many studies have been carried out to evaluate the biochemical and immunological aspects of *Jatropha species*. Lin *et al.,* [2003]; studied the antidiarrhea, antitumor effect of curcin extracted from *J. curcas.* Mujumdar *et al*., [2000] and Lin *et al*. [2003] studied antidiarrhea activity of *J. curcas* root extract on albino mice. The results indicated that action of methanol extracted from *J. curcas* root elevated the prostaglandin biosynthesis and reduced propulsive movement of the small intestine. Liu *et al.* [1997] studied the phorbolester in *J. curcas* and reported its mollusicidal activity against the schistosome vector snails. Aregheore *et al*. [2003] had detoxified the level of lactin and phorbolesters extracted from *J. curcas* using heat and chemical treatment. They have studied the detoxification effects of 14 different chemical treatments and observed that the level of lactin and phorbolester tolerance reduced to 0.09 mg/g. Augustusa *et al*. [2002] studied the gross heat value (20.85 MJ/kg) and fatty acid composition of oil extracted from the seeds of *J. curcas*. They also studied influence of growth regulators on the yield of extractable chemicals from the seeds of *J. curcas*. The oil fraction consisted of saturated fatty acids, palmitic acid (14.1%), stearic acid (6.7%) and unsaturated fatty acids oleic acid (47.0%) and linoleic acid (31.6%) [Mandpe *et al.,* 2005]. Shweta *et al.* [2005] have developed a suitable procedure for extraction of oil from seeds of *J. curcas* by ultrasonication and aqueous enzymatic method. Maximum yield of oil which was obtained from the seed kernels by ultrasonication for 5 min followed by aqueous enzymatic oil extraction using an alkaline protease at pH 9.0 was 74%. Kaushik *et al*. [2007] observed the variability in seed traits and oil content of 24 germplasm of *J. curcas*. A significant difference was found in seed size, weight and oil content between germplasm and it was concluded that phenotypic coefficient of variation was higher than the genotypic coefficient of variation indicating the predominant role of environment. Martinez-Herrera *et al.* [2006] studied the chemical composition of toxic or anti-

metabolic constituents of seed kernels and oil of *J. curcas* and found that the seed kernel contain proteins, lipids, fibres, starch, soluble sugars etc. oil mainly contains oleic, linoleic, palmitic, stearic acids and occasionally cis-11-eicosenoic acid (C20:1) and cis-11, 14-eicosadienoic acid (C20). They also reported that trypsin inhibitors, phytates, saponins and lectins were the other major antinutrients present in all the seed meals. Modi *et al*. [2006] have used propan-2-ol as an acyl acceptor for immobilized lipase-catalyzed preparation of biodiesel and gave the optimum conditions for transesterification of crude *J. curcas* oil, based on alcohol to oil as 4:1 (molar ratio) at 50 ºC for 8 h and the maximum conversion achieved was 92.8%.

Biswanath *et al.* [2003] isolated a coumarino-lignoid, cleomiscosin A, from the stems of different species of *Jatropha* that was originally isolated from *Cleome viscosa*. These compounds were suggested to be useful taxonomic markers within the genus. Kosasi *et al*., [1989] and Albert *et al.* [1995] isolated Labaditin and Cyanoglucoside named as 'multifidin' from the latex of *J. multifida*. Labaditin is an immunologically active novel cyclic decapeptide, consisting of 1 Alanine, 2 Glycine, 1 Isoleucine, 2 Threonine, 2 Trypsin and 2 Valine and cyanoglucoside, consisting 1-cyano-3-fl-D-glucopyranosyloxy-(Z)-1-methyl-1-propene. Albert *et al.* [1996] isolated two novel cyclic peptides podacycline A and B from the latex of *J. podagrica*. Podacycline A is a cyclic nonapeptide with the sequence Glyl-Leu2-Leu3-Gly4-Ala5-Val6-Trp7-Ala8-Gly9-Glyl and podacycline B, a cyclic heptapeptide, was determined to be Phel-Ala2-Gly-3-Thr4-Ile5-Phe6-Gly7-Phel. Somyote *et al.* [2003] isolated a macrocyclic diterpene integerrimene with a novel 8, 9-*seco*-rhamnofolane skeleton and a new rhamnofolane endoperoxide 2-epicaniojane together with caniojane and 1,11-bisepicaniojane from the roots of *J. integerrima*. Ee *et al.* [2005] isolated a new ferulic ester (n-heptyl ferulate together with 8-hydroxy-6, 7- dimethoxycoumarin, acetylaleuritolic acid) and γ-sitosterol was isolated from the stem and roots of *J. podagrica*. Compounds dimethoxycoumarin**,** acetylaleuritolic and γ-sitosterol were found to be cytotoxic towards the HeLa (cervical carcinoma) cell line. Dror Koltin *et al*. [2006] first reported the case of *J. multifida* poisoning that differed from organophosphate intoxication and observed that irrespective of the symptoms of vomiting and diarrhea, the cholinesterase activity level was normal

after *Jatropha* ingestion and decreased in case of organophosphate poisoning. Biswanath and Anjani [1999] isolated a new lignan gossypidien from the stems of *J. gossypifolia*. Iracelle *et al.* [2003] have shown that ethanolic extract of *J. gossypifolia* elicited hypotension in conscious normotensive rats. Omolaja *et al.* [2003] studied the coagulant activity of the latex of *J. curcas* and showed that whole latex significantly reduced the clotting time (3.83 ± 1.01 min) of human blood. Oduola *et al.* [2005] determined the suitability of the leaf extract of *J. gossypifolia* as an anticoagulant for biochemical and hematological analyses. Sarin *et al.* [2007]; examined the blend of *Jatropha* and Palm biodiesel and studied physico-chemical properties to get an optimum mix of them to achieve better low temperature properties, with improved oxidation stability.

2.2.2. Molecular studies in *Jatropha* species:

Information regarding genetic background, intraspecies, non toxic and toxic variation in genus *Jatropha* is limited. Puangpaka and Thaya, [2003] studied the karyology of five *Jatropha* species by staining chromosomes of the microsporocyte with propionocarmine. The study showed most of the species were paired as bivalents at first metaphase and separated to 11:11 at first anaphase. Chromosomes of the taxa studied were of very small size. Bivalent length ranged from 1–3.67 μm. Most of the species had chromosome numbers of 2n = 22 and a base number of x = 11. The study reported that it was *J. curcas* and *J. multifida* were chromosomally similar. Both of these taxa had a meiotic configuration of 7 ring II + 4 rod II, *J. integerrima*'s 'Red' and 'Pink' flower had the same meiotic configuration of 6 ring II + 5 rod II. *J. podagrica* meiotic configuration was 8 ring II + 3 rod II. The karyology of *J. gossypifolia* was determined from first anaphase cells and the chromosomes separated to 11:11. According to them, *J. curcas* and *J. multifida, perhaps*, *J. gossypifolia* appeared closely related to each other based on their meiotic configuration and morphological similarity. Carvalho *et al.* [2008] studied the genome size, base composition and karyotype of *J. curcas*. The results shows 2C value of genome of *J. curcas* was 0.85 pg and an average base composition of 38.7% GC. The karyotype of *J. curcas* is made up of 22 relatively small metacentric and submetacentric chromosomes whose size range from 1.71 to 1.24 μm.

Prabakaran and Sujatha [1999] reported *J. tanjorensis* as a natural interspecific hybrid of *J. curcas* and *J. gossypifolia* based on cytological and peroxidase isozyme studies. The species has widespread distribution in Tanjore, Pudukottai, Trichirapalli and Ramnad districts of Tamil Nadu, India.

Also Sujatha and Prabakaran [2003] reported successful interspecific hybrids between *J. curcas* and *J. integerrima* stating that interspecific hybrid, exhibited intermediate morphological and vegetative characteristics. Further backcrossing of the hybrids resulted in a number of flower colours varying from dark pink through green to white enhancing the ornamental value of the genus. Hybrids were confirmed through PCR amplification using RAPD primers.

2.3. ECONOMIC IMPORTANCE OF *J. CURCAS*:

2.3.1. *J. curcas* as a source of bio-fuel:

Among various renewable energy choices, oil seed crops have potential for meeting the ever increasing demand of petroleum products [Bhasbutra & Sutiponpeibun, 1982]. *Jatropha* is a potential oil crop; the seeds contain 30 to 40% of oil, 19.0% protein, 17.0% carbohydrates and 16.0% fiber. The oil contains 21% saturated fatty acids and 79% unsaturated fatty acids. Central and state planning commissions are encouraging the cultivation of *Jatropha* on different lands for production of raw material for biodiesel production. Economic analysis has demonstrated that *J. curcas* fuel can compete with conventional petro-diesel and it can be used with out any modification in general diesel engines [Demant & Gajo, 1992; Henning & von Mitzlaff, 1995; Mandpe *et al.,* 2005]. Among the different bio-diesel derived fromdifferent vegetable oils including physic nut oil based bio-diesel and it was found that performance of *Jatropha* seed oil was superior to other oils [Lutz, 1992; Lutz, 1992; Pak & Allexi, 1994].

2.3.2. *J. curcas* as folk medicine:

Besides the huge importance as a source of renewable energy it has very good medicinal properties. Extracts of *J. curcas* are used in folk remedies. It has been reportedly used as abortifacient, anodyne, antiseptic, cicatrizant, depurative, diuretic,

emetic, hemostat, lactagogue, narcotic, purgative, rubefacient, styptic, vermifuge and vulnerary. Physic nut is a folk remedy for alopecia, anasorca, ascites, burns, carbuncles, convulsions, cough, dermatitis, diarrhea, dropsy, dysentery, dyspepsia, eczema, erysipelas, fever, gonorrhea, hernia, incontinence, inflammation, jaundice, neuralgia, paralysis, parturition, pleurisy, pneumonia, rash, rheumatism, scabies, sciatica, sores, stomach ache, syphilis, tetanus, thrush, tumors, ulcers, uterosis, whitlows, yaws and yellow fever [Duke & Wain, 1981].

The leaves of *Jatropha* contain apigenin, vitexin and isovitexin. The amyrin, stigmasterol and stigmastenins along with two new flavonoid glycosides present in leaves and twigs are found every effective against seabies, paralysis, rheumatism and hard tumors. The leaf juice is used as external application for piles, the leaf decoction in arthritis, venereal disease. Heated leaves are used on the breast as a lactagogue, leaf tea is used for marasmus and for jaundice [Watt & Breyer-Brandwijk, 1962; Morton, 1981; Perry, 1980].

The latex of *J. curcas* contains alkaloids such as Jatrophine, Jatropham, Jatrophone and curcain which are believed to have anti-cancerous properties. Latex is also used for dress sore, ulcer and inflamed tongue, people drink the decoction for heartburn. Colombians and Costa Ricans apply the latex to burns, hemorrhoids, ringworm and ulcers. Seeds are used for dropsy, gout, paralysis, and skin ailments [Watt & Breyer-Brandwijk, 1962]. Roots are used in decoction as a mouthwash for bleeding gums, tooth ache and Venezuelans take for dysentery [Morton, 1981]. Three deoxypreussomerins, palmarumycins, CP1, JC1 and JC2 have been isolated from the stems of *J. curcas* and are found to be antibacterial in nature [Ravindranath *et al.,* 2004].

2.3.3. *J. curcas* other uses:

There are many other uses of *J. curcas* besides, biodiesel and medicinal value. The oil has been used for illumination, for manufacturing soap and candles. Nuts collected from a non toxic Mexican variety are roasted and eaten [Watt & Breyer-Brandwijk, 1962]. The latex is strong inhibitor of watermelon mosaic virus [Tewari & Shukla, 1982]. Bark is used as a fish poison [Watt & Breyer-Brandwijk, 1962]. In South

Sudan, the seeds as well as the fruits are used as contraceptive List and Horhammer, 1969–1979]. Pressed cake of *Jatropha* is used as organic fertilizers and as soil improver [Jones & Miller, 1991].

2.4. GENETIC VARIATION IN *J. CURCAS*

So far, few records of systematic provenance trials exist, where an attempt was made to examine the genetic variation of the physic nut. Adaptive trials on *J. curcas* and *J. gossypiifolia* were undertaken at Hisar, Bangalore and Sardar Krushinagar in India by agricultural universities. The evaluation of five cultivars revealed a good degree of variation in plant height, branches per plant and seed yield per plot at Hisar. In Northern Nicaragua in two different places, Nicaraguan and Cape Verde provenances were planted on 1200 ha and the two types looked different in the field. The Nicaraguan type has fewer branches, larger and paler leaves and bigger seeds, whereas, the Cape Verde provenance produced high seed yield. In the Nicaraguan material, a male sterile plant was observed which produces more fruits than the hermaphrodite types.

Heller [1992] studied a collection of 13 provenances in multiple locations field trials in two countries of the Sahel region: Senegal and Cape Verde. In Cape Verde the climate is semi-arid with a short rainy season (approximately 4 months) and longer dry season (approximately 8 months) with a wide variation in rainfall (200-800 mm). The trials were conducted on the island of Santiago at Sao Jorge and Tarrafal (Chao Bom) and vegetative development was evaluated at each location and significant differences were reported in the vegetative development among the various provenances.

Molecular diversity through RAPD was studied in 42 germplasm of *J. curcas* collected from different regions in India along with a non toxic genotype from Mexico to identify genetically divergent materials for use in breeding programme. The report clearly indicated the existence of polymorphism is very low. The study also indicates an immediate need for widening the genetic base of *J. curcas* germplasm [Basha & Sujatha, 2007].

In India extensive experimental field trials are being conducted by CSMCRI (Central Salt and Marine Chemical Research Institute), Bhavnagar to assess the possibility of developing *J. curcas* as a bio-diesel crop. Many field experiments are being conducted with national and international network projects to assess the performance and identification of best performing germplasm in the field. In the studies germplasm were collected from different geographical locations including those from the centre of species origin (Mexico/Central America). Significant variation in growth and yield attributing characters were recorded. The major limitation observed is low and inconsistent yield which limits the large scale cultivation. So there is a need to identify and improve the species for higher yield and oil production. For needful improvement in the species there is essential to understand the extent of genetic diversity and identification of molecular markers which in turn can be utilized for breeding and improving the species using the naturally available plant genetic resources.

Plant genetic resources are one of the most valuable assets available to mankind. An important component for effective and efficient management of plant genetic resources is characterization of the germplasm, their phylogenetics and evolution which is essential not only for identification of various species but also to determine genetic relatedness within and among the species The information generated could be used in breeding programs and molecular mapping. Assessment of diversity and their phylogenetic relationships has traditionally been through morphological characters, biochemical markers like isozymes. However, these analysis have their inherent disadvantages like limited number of markers and often found to be rather less effective due to its inconsistency to short term environmental fluctuations [Crawford *et al.,* 1994; Essilman *et al.,* 1997; Francisco *et al.,* 1996; Lesica *et al.,* 1998; Lowrey *et al.,* 1985; Soltiset *al.,* 1992]. DNA based molecular analysis tools like DNA fingerprinting became ideal for germplasm characterization and phylogenetic studies due to their inconsistency to environmental fluctuations

2.5. A BRIEF HISTORY ON APPLICATIONS OF DNA FINGERPRINTING IN PLANT SCIENCE

DNA fingerprinting techniques like, Restriction Fragment Length polymorphism (RFLP), Amplified Fragment Length Polymorphism (AFLP), Randomly Amplified Polymorphic DNA (RAPD), micro-satellites markers/SSR, Sequence Characterized Amplified Regions (SCAR), Sequence Tagged Sites (STS), nrDNA-ITS have been used for generation of molecular markers for efficient use in breeding and genetic resource management. In 1988 the first attempt on application of DNA fingerprinting in plants was reported, which was based on southern blot analysis and restriction fragment polymorphism (RFLP) technique. The initial experiments used either M13 repeat probe discovered by Vassart *et al.* [1987] or the human minisatellite probes developed by Jeffreys *et al.* [1985]. In the very first DNA fingerprint report dealing with plants, Ryskov *et al.* [1988] demonstrated DNA fingerprint pattern differences between two varieties of barley (*Hordeum vulgare*), following southern blot hybridization of *Hae*III-digested DNA samples with M13 probe. Same probe was also used by Rogstad *et al.* [1988] to generate DNA fingerprints from a panel of gymnosperms and angiosperms. Dallas [1988] applied the human minisatellite probe to distinguish rice cultivars.

In 1989, synthetic oligonucleotides that recognize simple repetitive DNA sequences were introduced in plant DNA fingerprinting. Weising *et al.* [1991] showed that polymorphic DNA fragment patterns were produced when restriction-digested barley and chickpea DNA was separated by agarose gel electrophoresis, and the dried gels were hybridized with radiolabeled $(GACA)_4$ or $(GATA)_4$ probe. Numerous articles were published on so called oligonucleotide fingerprinting approach in plants, showing that the levels of detected variations was highly dependent on the chosen probe [Beyeramann *et al.,* 1992; Depeiges *et al.,* 1995; Sharma *et al.,* 1995; Weising *et al.,* 1991; 1992].

The introduction of PCR-based methods constituted a new milestone in the field of DNA fingerprinting. Two methods using primers with arbitrary sequence were published in 1990 [Welsh & McClelland, 1990; Williams *et al.,* 1990] and third one was published in 1991[Caetano-Anolles *et al.*]. The random amplified polymorphic DNA

(RAPD) approach using arbitary primers developed by Williams *et al.* [1990] has become the best known variant of this prototype of PCR-based DNA profiling

In 1995 Vas *et al.* [1995] reported a new method called AFLP, incorporating the elements of both RFLP and RAPD. A third group of PCR-based DNA profiling technique which guides the PCR amplification to certain types of (mostly repetitive) DNA, was also developed which does not require species-specific primers. This type of approach is best exemplified by the inter-simple sequence repeat (ISSR)-PCR developed by Zietkiewicz *et al.* [1994].

The rapidity, with which large number of samples can be processed, made PCR based methods increasingly popular. AFLP, RAPD, and ISSR are broadly used techniques, although RAPD in particular have some demerits with reproducibility and competitive priming. These problems are less pronounced in AFLP, which is currently regarded as the method of choice when high numbers of markers are desired. All the three methods usually arrive at very similar estimates of genetic diversity and genetic distances, when applied to the same plant material. However, the detectable loci are mostly biallelic (a band is present or absent), and initial attempts to distinguish hetero from homozygotes by band intensity have largely abandoned. Consequently, the bands generated by these multilocus techniques were dominant markers, which reduced their potential for use in population genetics and in-depth genetic analyses.

The best proved markers for population genetics and in-depth genetic analyses were microsatellites/SSR. PCR with primers complementary to the DNA sequences flanking hypervariable microsatellites were introduced [Litt & Luty, 1989; Smeets *et al.,* 1989 Tautz, 1989; Weber & May, 1989] and became increasingly attractive in animal genetics in the early 1990s. The availability of locus specific co-dominantly inherited bands with high levels of polymorphism soon prompted botanists to explore the potential of this approach for the genetic analysis of plants. The feasibility of PCR amplification of microsatellites in plants was first demonstrated in soybean [Akkaya *et al.,* 1992]. Microsatellites soon proved to be excellent tools for discriminating between genotypes, for population studies, gene tagging, and linkage mapping. The major limitations of

microsatellite markers are the time and cost involved in developing specific primer pairs [Akagi *et al.,* 2001; Aljanabi & Martinez, 1997]. Fortunately, several studies have demonstrated the possibility of microsatellite marker transfer to congeneric specific, or occasionally, even to other genera [Arnold *et al.,* 2002; Peakall *et al.,* 1998].

Molecular characterization, analysis of genetic divergence and phylogenetic analysis of the germplasm, is not only essential for identification of various species but also to determine genetic relatedness within and among the species and for management of plant genetic resources which are the most valuable natural assets available to the mankind. DNA based molecular analysis tools like RFLP, AFLP, RAPD, SSR, SCAR, STS and nrDNA-ITS became ideal for germplasm characterization and have been used for the generation of molecular markers for efficient use in breeding. Out of these, most efficient multilocus marker techniques such as RAPD, AFLP, and single locus nrDNA ITS sequences were used in the present study for the genetic divergence and phylogetic analysis. Further efforts were made to isolate and characterize highly variable single locus markers microsatellites (SSRs) from *J. curcas*.

2.5.1. Random Amplified Polymorphic DNA (RAPD):

Random amplified polymorphic DNA (RAPD) markers [Williams *et al.,* 1990] have been increasingly employed for population studies and for analyzing the molecular diversity [Hogbin *et al.,* 1998; Fischer *et al.,* 2000]. RAPD technique has the advantage of assessing a greater number of potential polymorphic loci distributed randomly in the genome than allozymes. In addition, when compared to other DNA-based markers, the procedure is technically simple, economic and does not require any prior knowledge of the target DNA sequence in the genome. However, most RAPD loci show dominant segregation and possess only two alleles per locus, which may bias some population genetic parameters.

The standard RAPD technique utilizes short synthetic oligonucleotides (10 bases long) of random sequences as a primer to amplify nanogram amounts of total genomic DNA under low annealing temperature by PCR (Figure 1a). During annealing at appropriate temperature, oligonucleotide primers of random sequence bind several

priming sites on the complementary sequences in the template genomic DNA and produce discrete DNA products. The profile of amplified DNA primarily depends on nucleotide sequence homology between the template DNA and oligonucleotide primer at the end of each amplified product. Welsh and McClelland [1990] independently developed a similar methodology using primers, 15 nucleotides long and called it as Arbitrarily Primed Polymerase Chain Reaction (AP-PCR) technique.

2.5.2. Amplified Fragment Length Polymorphism (AFLP):

AFLP analysis [Vos *et al.,* 1995] belongs to the category of selective restriction fragment amplification technique, which is based on the ligation of adapters (i.e., linkers and indexers), to genomic restriction fragments followed by a PCR based amplification with adapter specific primers having the extension with selective nucleotides (Figure 1b). For AFLP analysis, only a small amount of purified genomic DNA is needed and is digested with two restriction enzymes, one with an average cutting frequency (like *Eco*RI) and a second one with a higher cutting frequency (like *Mse*I or *Taq*I). Double-stranded oligonucleotide adapters are designed in such a way that the initial restriction site is not restored after ligation, which allows simultaneous restriction and ligation, while religated fragments are cleaved again. An aliquot is then subjected to two subsequent PCR amplifications under highly stringent conditions with adapter-specific primers that have at their ends an extension of one to three nucleotides running into the unknown chromosomal restriction fragment. An extension of one selective nucleotide amplifies 1 of 4 of the ligated fragments, whereas, three selective nucleotides in both primers amplify 1 of 4,096 fragments. The PCR primer which spans the average-frequency restriction site is labeled and after polyacrylamide gel electrophoresis a highly informative pattern of 40 to 200 bands can be obtained. The patterns obtained from different strains are polymorphic due to (i) mutations in the restriction sites, (ii) mutations in the sequences adjacent to the restriction sites and complementary to the selective primer extensions, and (iii) insertions or deletions within the amplified fragments. AFLP markers offer the following advantages:

1. The quantity of information generated, using AFLP markers are superior or equivalent to standard molecular markers.
2. AFLP markers can be generated for any organism with no prior knowledge about the genomic makeup of the organism. Therefore, AFLP has broad taxonomic applicability and has been used effectively in a variety of taxa, including bacteria [Keim *et al.,* 1997; Huys *et al.,* 1996], fungi [Majer *et al.,* 1998; Rosendahl & Taylor 1997], animals, vertebrates [Otsen *et al.,* 1996; Liu et al. 1998; Ajmone-Marsan *et al.,* 1997] and plants [Triantaphyllidis *et al.,* 1997].
3. AFLP amplifications are performed under conditions of high selectivity (high stringency), thus eliminating the pseudo variation that observed routinely in RAPD–PCR. Repeated AFLP amplifications show near perfect reproducibility [Vos *et al.,* 1995, Jones *et al.,* 1997] and overall errors generally amount to less than 2% [Huys *et al.,*1996; Tohme *et al.,* 1996; Arens *et al.,* 1998; Winfield *et al.,* 1998].
4. AFLP analysis requires minimal amounts of DNA and partially degraded samples can be used. Therefore, extremely small samples and even very small organisms can be examined with AFLP fingerprinting.
5. AFLP markers can be generated at great speed, as illustrated by the high ratio of polymorphism generated per PCR experiment (multiplex ratio) and by the high percentage of polymorphism in all markers generated. Studies routinely report the screening of hundreds or even thousands of markers [Maughan *et al.,* 1996; Liu *et al.,* 1998; Ajmone-Marsan *et al.,* 1997].
6. AFLP markers segregate in a Mendelian fashion [Vos *et al.,* 1995; Maughan *et al.,* 1996] and can be used for population genetics and QTL analyses.
7. And because of the nearly unlimited number of markers that can be generated with AFLP–PCR, using a series of different primer combinations, at least some AFLP markers will be located in variable regions [Vos *et al.,* 1995] and thus reveal even minor genetic differences within any given group of organisms. Single nucleotide differences between AFLP fragments can be resolved with either manual

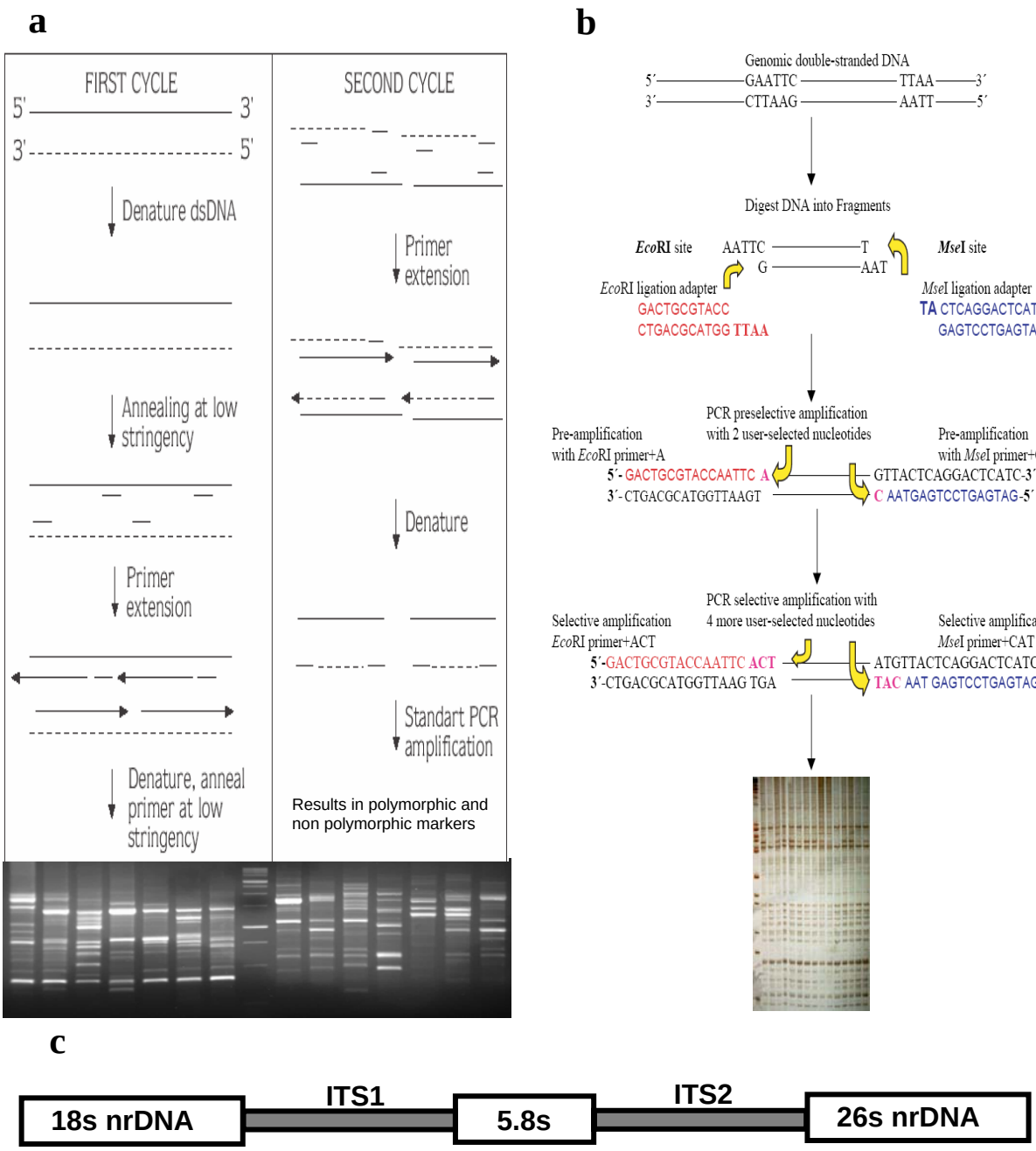
polyacrylamide gel electrophoresis or with the help of automated genotyping sequencers.

2.5.3. Nuclear Ribosomal DNA ITS (nrDNA ITS) region for genetic diversity and phylogenetic analysis:

Most of the biologists such as parasitologists, geneticists, and taxonomists have preferred to use RFLP, RAPD, or allozyme data rather than sequence based molecular markers to resolve phylogenetic problems, because the former methods are economical and rapid in obtaining the final result. Furthermore, acquisition of DNA sequence data is more difficult, expensive and needs much more time. Nevertheless, DNA sequencing is the best way to estimate genetic variations of specific genes among taxa examined directly. Due to the arbitrariness of taxonomic categories and the differences in the evolutionary rates of analyzed genes or molecular markers among the taxa examined, no sweeping generalizations could be made about the taxonomic rank at which particular markers or gene regions are useful. For instance the evolutionary rate (the degree of sequence variation) of the selected molecular markers or gene regions is much faster than that of independent organisms [Hwang *et al.,* 1998].

Nuclear ribosomal DNA, which encodes rRNAs, has been most commonly applied in phylogenetic approaches. Eukaryotic nuclear rDNA is tandemly organized with high copy numbers up to ca. 5,000. Each repeat unit consists of genes coding for the nuclear small subunit (SSU), large subunit (LSU), and 5.8S rDNAs. These coding regions are separated from each other by spacers. The SSU and LSU rDNAs are separated by the two external transcribed spacers (ETS) and a non-transcribed spacer (NTS). Both spacers are called as intergenic spacer (IGS). The 5.8S rDNA is embedded in the two internal transcribed spacers (ITS1 and ITS2) (Figure 1c). The nrDNA spacer regions evolve much faster than the nrRNA coding regions because the substitutions occurring in spacer regions do not show lethal effects on the organisms. This results in a relatively fast evolutionary rate of spacer regions.

Figure 1: Multilocus DNA fingerprinting (a) RAPD, (b) AFLP; (c) Single locus nrDNA ITS



Due to the high variability, nrDNA spacer regions, IGS and ITS have been employed to resolve phylogenetic problems in lower categorical levels among genera, species, or populations [Morgen and Blair, 1998; Navajas *et al.,* 1998; Perera *et al.,* 1998]. The size of IGS (ca. 4-5 kb) is far larger than those of ITS regions (ca. 1 kb). Due to the large size of the IGS, ITS regions have been preferred over IGS in phylogenetic approaches.Molecular studies using the internal transcribed spacer (ITS) region in 18S-26S nuclear ribosomal DNA (nrDNA) have been applied widely to phylogenetic questions in flowering plants [Baldwin 1995]. These investigations have concentrated on lower level taxonomic problems, but subfamily (e.g. *Apioideae*, Downie and Downie, 1996) and family level [e.g. *Winteraceae,* Suh *et al.,* 1993] studies also have been conducted. Furthermore, at least in ITS-2, there are regions conserved across flowering plants; Hershkovitz and Zimmer [1996] presented an alignment in which one-third to one-half of the ITS-2 sequence is alignable above the family level in angiosperms. Hence, the ITS region has the potential for contributing phylogenetic signal at higher taxonomic levels than was earlier anticipated. For some angiosperm groups more than one molecular region has now been investigated phylogenetically. Especially interesting are studies based on both chloroplast (cp) and nuclear (n) regions, e.g. Wendel *et al.* [1991], Baldwin [1992], Downie and Downie [1996], Gielly *et al.* [1996], Soltis *et al.* [1996] and Oxelman *et al.* [1997].

2.5.4. Microsatellites or Simple Sequence Repeats (SSRs):

Microsatellites or simple sequence repeats (SSRs) are tandem repeating motifs of 1-6 bases found in all prokaryotic and eukaryotic genomes. They are present in both coding and non-coding regions and are usually characterized by a high degree of length polymorphism. The origin of such polymorphism is still under debate though it appears that it is due to slippage events during DNA replication [Schlotterer & Tautz, 1992]. Despite the fact that the mechanism of microsatellite evolution is still unclear; soon after their first description [Litt & Luty, 1989; Tautz, 1989; Weber & May, 1989] SSRs are being widely employed in many fields because of their high variability, which makes them very powerful genetic markers. Microsatellites have proven to be an extremely

valuable tool for genome mapping in many organisms [Schuler *et al.,* 1996; Knapik *et al.,* 1998], their applications span over different areas ranging from ancient and forensic DNA studies, to population genetics and conservation management of biological resources [Jarne & Lagoda, 1996]. A microsatellite locus typically varies in length from 5 to 40 repeats; dinucleotide, trinucleotide and tetranucleotide repeats are the most common choices for molecular genetic studies. Dinucleotide repeats account for the majority of microsatellites for many species [Li *et al.,* 2002]. Trinucleotide and hexanucleotide repeats are the repeat classes that appear mostly in coding regions because they do not cause frameshift [Toth *et al.,* 2000]. Mononucleotide repeats are less reliable because of problems with amplification, less common longer repeat types, and limited data exists to examine their evolution [Li *et al.,* 2002].

The DNA surrounding the microsatellite locus is termed as 'flanking region'. As the sequences of flanking regions are generally conserved (i.e. identical) across the individuals of the same species and sometimes in different species, a particular microsatellite locus can often be identified by its flanking sequences. Short stretches of oligonucleotides or primers, can be designed to bind to the flanking region and guide the amplification of the microsatellite locus with polymerase chain reaction (PCR). Due to their exceptional variability and relative ease of scoring, microsatellites are now considered to be the most powerful genetic markers. It is possible to observe the loci with more than 10 alleles or above in relatively small samples [Bowcock *et al.,* 1994; Deka *et al.,* 1995; Primmer *et al.,* 1996]. In addition to being highly variable, microsatellites are also densely distributed throughout eukaryotic genomes, making them as preferred marker for very-high-resolution genetic mapping [Dib *et al.,* 1996; Dietrich *et al.,* 1996]. Advantages of microsatellites like-high heterozygosity, ubiquity through the genome (often adjacent to coding sequences, though rarely transcribed), and PCR typability, combined with ease of multiplexing-make them obvious candidates for markers of choice in genome maps. However, since they are underrepresented in chromosome centromeres and telomeres, minisatellite data are complementary for uniform genome coverage by markers [Weissenbach *et al*., 1992].

From identifying relatives to inferring demographic parameters; microsatellites are rapidly replacing RFLPs and RAPDs in most applications in population biology [Blouin *et al.,* 1996; Bowcock *et al.,* 1994; Goldstein *et al.,* 1996; Jarne & Lagoda, 1996]. Part of the appeal of microsatellites over RFLPs and RAPDs is that the genetic basis of microsatellite variability is readily apparent: unique primers amplify a genomic region including a well-defined repeat structure that is responsible for the observed variation. This allows the development of inferential methods based on explicit models of microsatellite evolution [Feldman *et al.,* 1996; Goldstein *et al.,* 1996; Pollock *et al.,* 1998; Slatkin 1995a, b]. Above advantages suggest that microsatellites are the most efficient tools in population genetics studies. One perceived difficulty with microsatellites is the long lead time in identifying and characterizing microsatellites in new taxonomic groups. However, this problem is partially alleviated by the continuing popularity of microsatellites in genetic mapping. Microsatellite maps are now available in nearly all organisms of genetic and/or economic interest including humans, mice, fruit flies, cows, sheep, chickens, pigs, tomatoes, soybeans, and rice etc [Akkaya *et al.,* 1995; Broun and Tanksley, 1996; Causse *et al.,* 1994; Crawford *et al.,* 1995; Crooijmans *et al.,* 1996; Dib *et al.,* 1996; Dietrich *et al.,* 1996; Goldstein & Clark, 1995; Ma *et al.,* 1996; Postlethwait *et al.,* 1994; Rohrer *et al.,* 1996; Su & Willems, 1996; Taramino & Tingey, 1996].

A variety of *in vivo* and *in vitro* studies indicate that microsatellite loci are highly unstable, having some of the highest mutation rates observed at molecular loci. Microsatellite mutation processes have been inferred by direct observations both on artificial constructs in yeast [Henderson & Petes, 1992] and in human pedigrees [Weber & Wong, 1993]. The majority of observed mutations are of a single step (one repeat unit); a significant minority of mutations may be of larger size. Out of 22 observed germ line mutations, Weber and Wong, [1993]; observed no mutation larger than two repeats. Twenty of these mutations involved a change of a single step for a ratio of 0.91 single-step to two-step mutations. A subsequent study by Amos *et al.* [1996]; confirmed only a single mutation of larger than one repeat unit out of 15 observed mutations. Engineered repeat tracks in yeast also show a great preponderance of single- and two-step mutations

[Henderson & Petes, 1992]. The general conclusion from these studies is that the majority of mutations are of one or two steps. It remains possible that mutations of much larger sizes occur, but too infrequently to be routinely picked up in such studies. Indirect evidence for such mutations comes from the study of distances among human populations in which the calibrated mutation rate is some what higher than that observed in pedigrees [Goldstein *et al.,* 1995].

Microsatellites can be used to determine genetic diversity within a species, to distinguish varieties and individuals even, as well as for parentage analysis [Edwards *et al.,* 1996]. The feasibility of PCR amplification of microsatellites in plants was first demonstrated in soybean in 1992 [Akkaya *et al.,* 1995] and their use as a tool for genome mapping of organisms was first demonstrated in 1996 [Schuler *et al.,* 1996; Knapik *et al.,* 1998].

2.5.4.1. Isolation of microsatellites:

The major drawback of microsatellites is that they need to be isolated *de novo* from most species being examined for the first time. Consequently, the strategy of designing universal primers matching conserved sequences, which was very effective for mitochondrial DNA [Kocher *et al.,* 1989], is problematic for microsatellites. However, the presence of highly conserved flanking regions has been reported for some microsatellite loci in cetaceans [Schlotterer *et al.,* 1991], turtles [FitzSimmons *et al.,* 1995] and fish [Rico *et al.,* 1996], allowing cross-amplification from species that diverged as long as 470 million years ago. The task of microsatellite isolation can be quite involving in terms of effort and time because it traditionally consists of screening genomic libraries with appropriate probes [Rassmann *et al.,* 1991] and sequencing the positive clones. The number of positive clones (containing microsatellites) that can be obtained by means of this traditional method usually ranges from 12% to less than 0.04%. Such an isolation strategy can be effective only in taxa with a high frequency of microsatellites as in some fish or other vertebrates and whenever only a relatively low number of microsatellites are needed. Traditional strategies are less useful while dealing with taxa with a very low frequency of microsatellites such as birds or plants or when

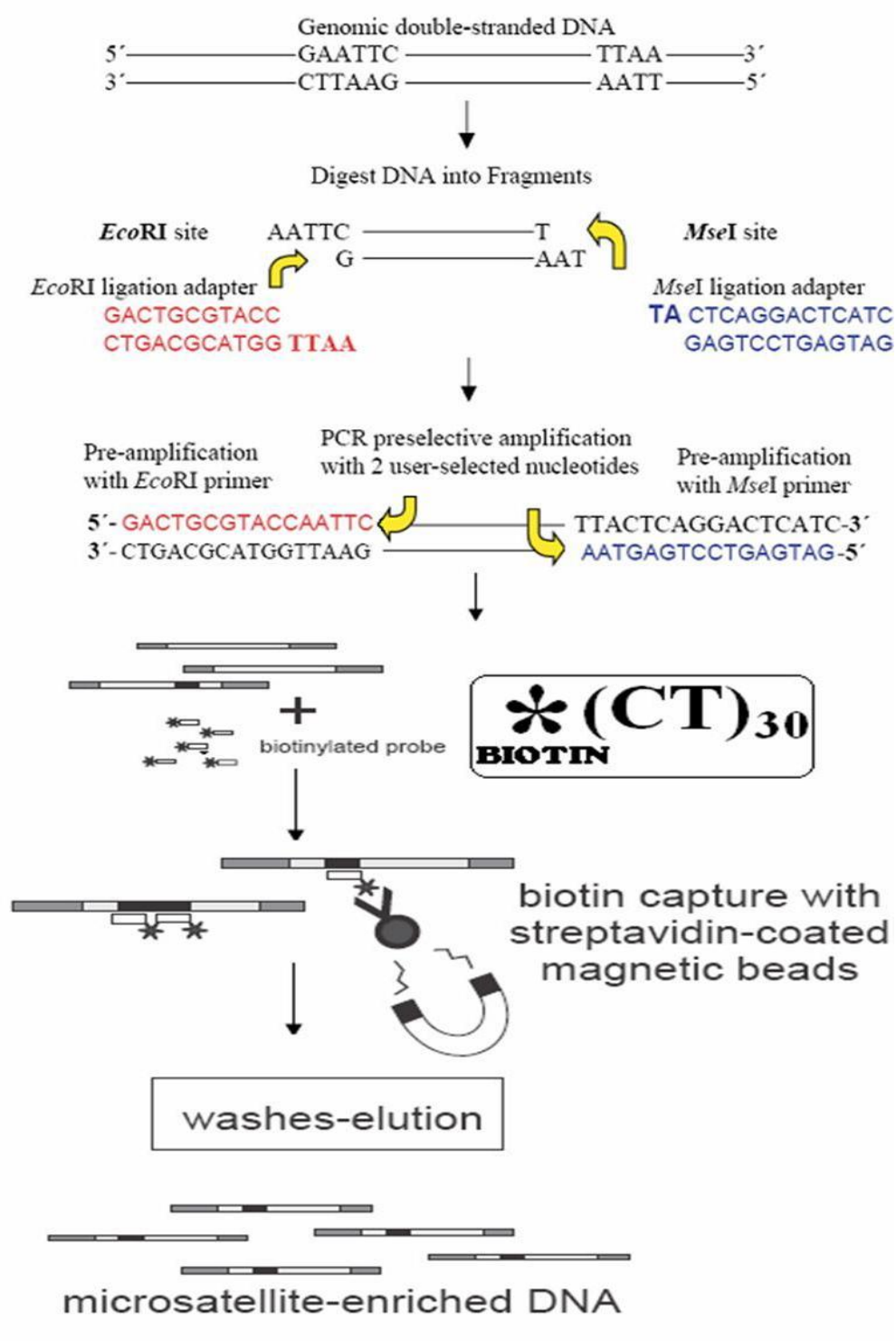
large number of microsatellites are required as in the case of studies on genetic distances between populations [Zhivotovsky & Feldman, 1995; Cooper *et al.,* 1999] or while constructing a genetic map [Liu, 1997].

A number of new protocols overcoming these limitations have appeared in literature in the last few years. There are many approaches for isolation of the microsatellites like isolation through genomic library construction, clone identification by repeat probe hybridization, RARP (repeat-anchored random primers), PIMA (PCR isolation of microsatellite arrays) [Lunt *et al*., 1999] and many enrichment protocols based on biotin-streptavidin-magnitic complex separation. These methods differ only slightly from one another. Out of these enrichment techniques, FIASCO (Fast Isolation by AFLP of Sequences COntaining repeats) which involves restriction digestion, adapter ligation and selective separation of repeat sequences through biotinylated repeat probe streptavidin coated magnetic complexes, is the most suitable and cost effective method for labs where the AFLP technique is already established. FIASCO protocol was successfully applied for isolation of microsatellites from birds (*Passera lagia*), fish (*Sparus aurata* and *Lophius americanus*), crustacean (*Meganyctiphanes norvegica*) and red coral (*Corallium rubrum*). Its application takes limited time isolating SSRs for the organism under study for the first time and was taken as a tool for isolating the microsatellite makers of *J. curcas* in the present study.

2.5.4.1.1 FIASCO (Fast Isolation by AFLP of Sequences COntaining repeats):

FIASCO is a fast, simple and effective technique as many unnecessary steps are eliminated and proved to be efficient in isolation of SSR in different organisms. The percentage of clones containing dinucleotide repeats varied from a minimum of 50% (*Passera lagia*) to a maximum of 95% (*Sparus aurata*). The results are indicative of high microsatellite isolation efficiency though the information concerning the frequency of polymorphic loci among the positive ones is not available completely [Zane *et al.,* 2002]. The technique relies on the extremely efficient digestion-ligation reaction of the AFLP procedure.

Figure 2: FIASCO (Fast Isolation by AFLP of Sequences COntaining repeats)



The FIASCO procedure offers an important advantage that is, the cost of isolating microsatellites will be minimum, since restriction enzyme digested and adapter ligated product which is obtained in the 2nd step of the AFLP will serve as the starting material for the isolation procedure which minimizes the cost of initial requirements needed in the labs having established AFLP technique (Figure 2). As over-amplification will change the average representative amplified fragments; so, care should be taken to optimize the number of cycles in the PCR amplification of pre-amplified product. The bands can be over-represented in the final PCR product, and they tend to be carried over during enrichment, especially if they cross-hybridize with the biotinylated probe, accounting for repetition of a significant fraction of the obtained recombinant clones. PCR conditions producing a visible product on agarose gel in the form of a smear are considered optimal and are selected for further use. PCR products, from the organisms tested to date, are always larger than 200 bp, thus eliminating the need for size selection. PCR amplification under optimal conditions is replicated to obtain several hundred nanograms of amplified DNA. Unless the yield of the DNA amplification is very poor, sample concentration is not recommended because it can result in loss or low recovery of DNA.

DNA obtained is then hybridized with a biotinylated (nucleotide repeat)$_{15}$ probe. DNA denaturation and annealing is performed at room temperature. DNA molecules are hybridized to biotinylated probes and are selectively captured by streptavidin coated beads. The beads-probe-DNA complex is separated by a magnetic field. Nonspecific DNA is removed by non-stringency and stringency washes. DNA is separated from the beads-probe complex by alkaline denaturation. The last non-stringency wash, the last stringency wash and the two elutions obtained from the denaturation steps should harbor an increasing proportion of DNA fragments containing the selected repeat and should carry the *Mse*I-N primer target site at each end. The PCR products of the two elution steps are used for producing a highly enriched microsatellite library, because they are likely to contain the largest proportion of repeat-containing fragments.Enrichment protocols appear preferable because they are fast and efficient, only basic skills in molecular biology is required and limited laboratory equipment is needed in addition to what is required for subsequent microsatellite screening. Starting from DNA that has

already been extracted, cloned products ready to be sequenced can be obtained in about three days.

2.6. A BRIEF HISTORY OF MOLECULAR PHYLOGENETICS AND EVOLUTION

Molecular phylogenetics is component of molecular evolution study. Fundamentally the subject of molecular evolution encompasses two areas of study (i) the evolution of macromolecules and (ii) the reconstruction of the evolutionary history of genes and organisms. The first area includes the rates and pattern of changes in the genetic material (eg. DNA sequences) or/and its encoded products (eg. proteins) during evolutionary time and the mechanisms responsible for such changes. The second area, also known as "molecular phylogeny" or "molecular phylogenetics" deals with the evolutionary history of organisms and macromolecules, as inferred from molecular data.

It might appear that the two areas of study constitute independent fields of inquiry, for the object of the first is to elucidate the causes and detects the evolutionary changes in molecules, while the second uses molecules merely as a tool to reconstruct the evolutionary history of organisms and their genetic constituents. In practice, however, the two disciplines are intimately interrelated, and progress in one facilitates studies in the other. For instance, phylogenetic knowledge is essential for determining the order of changes in the molecular characters under study, and knowing the order of such changes is usually the first step in inferring their cause. Conversely the knowledge of the pattern and rate of change of a given molecule is crucial for attempts to reconstruct the evolutionary history of a group of organisms.

The study of molecular evolution has its tools in two disparate disciplines: population genetics and molecular biology. Population genetics provides the theoretical foundation for the study of evolutionary process, while molecular biology provides the empirical data. Thus, to understand molecular evolution it is essential to acquire some basic knowledge of both molecular biology and theory of population genetics. Although as a discipline molecular evolution is still at a young stage of development, the pursuit of this subject actually began at the turn of the century. Studies in immunochemistry at the end of the last century and the beginning of this showed that serological cross-reaction

were stronger for more closely related organism than for less related ones. Seeing the evolutionary implications of this finding, Nuttall [1904] conducted precipitin tests of serum proteins to understand the phylogenetic relationships among various groups of animals. He determined, for example, that man's closest relative was ape, followed, in order of relatedness, by Old World monkeys, New World monkeys, and prosimians. These techniques stimulated much interest in the molecular phylogeny of humans, apes, and other primates [Goodman *et al.,* 1962; Zuckerkandl *et al.,* 1963] and made a great impact on the study of molecular phylogeny in general.

The 1960s was a period of rapid progress and intense controversies in molecular evolution. Considerable sequence data for hemoglobins and cytochromes had become available in the early 1960s and comparative studies of the data suggested that the rate of amino acid substitution in each of these proteins was approximately the same among different mammalian lineages [Zuckerkandl & Pauling, 1965; Margoliash, 1963]. Zuckerkandl and Pauling [1965] therefore proposed that for any given protein the rate of molecular evolution is approximately constant in all evolutionary lineages or, in other words, there is a molecular clock. This proposal immediately stimulated a great deal of interest in the use of macromolecules for evolutionary studies. Indeed, the proteins evolve at constant rates; they can be used to estimate the dates of species divergence and to reconstruct phylogenetic relationships among organisms. This proposal had a tremendous impact on the development of molecular evolution.

The 1960s and 1970s also were years of tremendous progress in molecular phylogenetics. The accumulation of protein sequences, which were more informative and easier to analyze than other types of molecular data then available, provided for the first time adequate data for studying long-term evolution such as evolutionary relationships among orders or beyond. These data stimulated many studies on reconstruction of phylogenetic trees and on the development of tree-making methods [Eck & Dayhoff 1966; Fitch & Margoliash 1967; Dayhoff 1972]. In addition to protein sequence analysis, protein electrophoresis and analysis; immunological techniques such as methods of microcomplement fixation were also extensively used in phylogenetic studies [Sarich &

Wilson 1966; 1967l; Maxon & Wilson 1975]. The advent of various recombinant DNA techniques has led to a rapid accumulation of DNA sequence data, thus stimulating even greater interest in molecular systematics.

Molecular Phylogenetics is the study of evolutionary relationships among organisms or genes by a combination of molecular biology and statistical techniques. It is also commonly called as molecular systematics, if the relationships of organisms are the concern. The molecular approach to systematics was initiated at the turn of the century by Nuttall [1904] as discussed above who used serological cross-reaction for phylogenetic analysis. Yet, the development of the polymerase chain reaction (PCR) method [Saiki *et al.,* 1985] has made systematic studies even easier, resulting in an unprecedented high level of activities in phylogenetic reconstruction.

There are several reasons why molecular data are much more powerful for evolutionary studies than morphological and physiological data. First, DNA and proteins generally evolve in a more regular manner than do morphological and physiological characters and therefore can provide a clear picture of relationships of organisms. Second, molecular data are often much more amenable to quantitative treatments than morphological data. In fact, sophisticated mathematical and statistical theories have already been developed based on molecular data [Nei 1987; Felsenstein 1988; Swofford and Olsen 1990; Miyamoto and Cracraft 1991]. Molecular data will be useful for studying the polygenetic relationships among closely related populations of species [eg. Cann *et al.,* 1987; Vifilant *et al.,* 1991; Hedges *et al.,* 1992]. In the future, most phylogenetic issues are likely to be resolved by molecular data and we may eventually be able to fulfill Darwin's dream of having ***"a fairly true genealogical tree of each great kingdom of nature"***.

The rapid progress in the study of molecular evolution has been greatly facilitated by the development of high-speed computers. Most of the procedures involved in molecular evolution and population genetic analysis were computation intensive and could not have been routinely done with out computers. Their ever-increasing speed and accessibility, analysis methods became more sophisticated and made them tremendously

incredible. However, only recently has it been possible to use some to of the more rigorous methods eg.maximum likelihood/parsimonious methods for the construction of trees and phylogenetic analysis through computer simulations.

Although RAPD, AFLP and microsatellite techniques which are proven to be the best molecular analysis tools to study diversity and phylogenetic releationships between many genera and populations of the species, there are very few attempts made in *Jatropha*. Survey work conducted by CSMCRI, Bhavnagar, India and other organizations involved in genetic improvement of *Jatropha* have observed significant variability in morphology, seed yield, and oil content, tolerance to biotic and abiotic stress in natural population. Therefore efforts were made to study releating to molecular diversity and phylogenetic analysis of *Jatropha* with the main objectives as (i) Molecular diversity analysis by RAPD, (ii) Molecular diversity analysis by AFLP, (iii) Comparative analysis of both RAPD and AFLP molecular diversity studies and (iv) To develop and characterization of molecular markers.

These studies will be highly useful in molecular breeding, MAS (Marker Assisted Selection), QTL (Quantitative Tract Loci) mapping and generation of physical maps which would ultimately in genetic improvement of *J. curcas*.

CHAPTER 3

MATERIALS AND METHODS

3.1. CHEMICALS

Acrylamide, bis-acrylamide, bind saline, repel saline (Amersham Bioscience, USA). bromophenol blue, RNase, Taq DNA polymerase, xylene cyanol (Biogene, USA), AFLP analysis system I (Invitrogen, USA), PolyATtract® System 1000 (Promega, USA), ammonia, ammonium acetate, acetic acid, formaldehyde, methanol, sodium carbonate (Na_2CO3), sodium chloride (NaCl) (Qualigens, India), ß-mercaptoethanol, agarose, decyltrimethyl-ammonium bromide (CTAB), ethylenediaminetetra-aceticacid (EDTA), polyvinyl pyrophosphate (PVP), silver nitrate (AgNO3), sodium hyroxide (NaOH), sodiumdodecyl sulphate (SDS), sucrose, tetramethylethylenediamine (TEMED), tris-base (trishydoxy-methylaminomethane), urea; (Sigma-Aldrich, USA), ammonium persulphate (APS) (SRL Pvt. Ltd, India).

3.2. INSTRUMENTATION

Agarose gel electrophoretic unit (Sigma, USA), autoclave (Tomy, Japan); bio-clean bench (Thompson, India), fume hood (Labexcel, England), gel documentation system (G-Box chemiUV trans, Syngene, UK), refrigerated orbital shaker (Syngenics, India), lyophilizer (Heto Pvt. Ltd, UK), microcentrifuge (Tarson, India), spectrophotometer (CARY 500 scan UV visible spectrophotometer, USA), thermal cycler (Eppendorf ep gradientS, Germany), UV transilluminator (Fisher biotech Ltd, USA), vertical sequenceing gel eletrophoretic unit, voltage powerpack system (LKB Pvt. Ltd., USA) and water bath system (Remi equipments, India).

3.3. MEDIA AND THEIR COMPONENTS

3.3.1. 100 mg/mL ampicillin (stored at -20 ºC).

3.3.2. 40 mg/mL isopropylthio- ß -D-galactoside(IPTG)

3.3.3. 20 mg/mL X-gal (5-bromo-4-chloro-3-indolyl- ß -D-galactopyranoside) in dimethylsulfoxide(DMSO)

3.3.4. Luria Bertani (LB) agar:

Tryptone	-	10 g
Yeast extract	-	5 g
NaCl	-	10 g
Agar	-	15 g
Distilied water	-	1000 mL
pH	-	7.5 $\pm$ 0.2

3.3.5. LB broth:

Tryptone	-	10 g
Yeast extract	-	5 g
NaCl	-	10 g
Distilled water	-	1000 mL
pH	-	7.5±0.2

3.3.6. SOC medium:

Tryptone	-	2 g
$MgCl_2$	-	0.5 g
NaCl	-	0.05 g
KCl	-	0.18 g
Yeast extract	-	0.5 g
Distilled water	-	100 mL

pH	-	7.2

3.3.7. Resuspension solution:

Glycerol	-	15 mL (15%)
$CaCl_2$	-	10 mM
Distilled water	-	85 mL

3.4. BUFFERS

3.4.1. Extraction buffer:

Tris-HCl	-	100 mM
EDTA	-	50 mM
NaCl	-	3.5 M
CTAB	-	2%
PVP	-	1%
SDS	-	5%
ß-mercaptoethanol	-	1%
pH	-	8.0.

3.4.2. TE buffer:

Tris-HCl	-	10 mM (pH - 8.0)
EDTA	-	1 mM (pH - 8.0)

3.4.3. Urea Polyacrylamide gel loading buffer:

Sucrose	-	10% w/v
Bromophenol blue	-	0.02% w/v
Xylene cyanol	-	0.02% w/v
Formamide	-	90% v/v

3.4.4. Agarose gel loading buffer:

Sucrose	-	10% w/v
Bromophenol blue	-	0.02% w/v
Xylene cyanol	-	0.02% w/v
Distiled water	-	10 mL

3.4.5. 10X TBE buffer:

Tris-base	-	0.9 M
Boric acid	-	0.9 M
EDTA	-	20 mM
pH	-	8.0

3.4.6. 20X SSC buffer:

NaCl	-	3 M
Trisodium citrate dihydrate	-	44.1 g
Distilled water	-	500 mL
pH	-	7.2

3.5. GENOMIC DNA EXTRACTION

Genomic DNA was extracted from fresh leaves collected from plants established on genetic garden/experimental field station of CSMCRI, Chorvadala, Gujarat and extraction was carried out in two phases as described below:

3.5.1. Solutions for exraction:

Tris saturated phenol, phenol:chloroform:isoamyl alcohol (25:24:1), chloroform:isoamyl alcohol (24:1), 70% and 80% ethanol, 5 M NaCl, 3 M sodium acetate (pH 5.2) and TE buffer (10mM Tris-HCl, 1 mM EDTA, pH 8.0). Solutions and buffers prepared were autoclaved at 121 ºC temperature and 15 psi pressure (Tommy autoclave,

Japan). The stock solution of RNase 10 mg/ml was prepared freshly as per the user manual (Sigma, USA).

3.5.2. Extraction phase:

- Fresh young leave collected were rinsed with distilled water and blotted gently with soft tissue paper.
- 0.1 g of leaf tissue was ground in a pre-cooled mortar with pestle to a fine powder using liquid nitrogen along with 10 mg (2% of extraction buffer) of PVP (Sigma, USA). The powdered tissue was scraped into a 2.0 mL microcentrifuge tube containing pre-heated (65 ºC) extraction buffer in 1:5 ratio (0.5 mL). β-mercaptoethanol was added to the final concentration of 0.2 M and mixed well. The mixture was incubated in water bath at 65 ºC for 90 min and cooled for 5 min.
- An equal volume of chloroform:isoamyl alcohol mixture (24:1) was added to the extract and mixed by gentle inversion for 5 to 10 min to form a uniform emulsion. The mixture was centrifuged at 8000 rpm for 8 min at RT. Chloroform:isoamyl alcohol extraction step was done twice. The aqueous phase was pipetted out gently, avoiding the interface.
- To the above solution, 5 M NaCl (to final concentration 2 M) and 0.6 volumes (V/V) isopropanol was added and incubated at RT for 1 h.
- Two volumes of 80% ethanol was added to the above solution and incubated again for 10 min at RT for DNA precipitation. After incubation the mixture was centrifuged at 10,000 rpm for 15 min at RT. The white/translucent pellet was washed with 70% ethanol, dried and resuspended in 200 µL of TE buffer.

3.5.3. Purification phase:

- The sample was treated with RNase (10 µL of 10 mg/mL of RNase) and incubated at 37 ºC for 60 min.

- After incubation with RNase, one volume of Tris saturated phenol (pH 8.0) was added and mixed gently by inverting the micro-centrifuge tube till it formed a milky white emulsion. The emulsion was then centrifuged at 10,000 rpm for 5 min at RT. The supernatant was pipetted out into a fresh tube.
- The sample was then extracted with equal volume of chloroform:isoamyl alcohol (24:1) twice. The DNA was reprecipitated with 0.6 volumes of isopropanol, 2.0 M NaCl (final concentration) and incubated for 10 min.
- To the above solution, 20 µL of sodium acetate and 1 volume of 80% ethanol was added, incubated at RT for 30 min, and centrifuged at 10,000 rpm for 15 min to pelletout the DNA. The pellet was then washed with 70% ethanol twice; air dried and finally suspended in 40-50 µL of TE buffer.

3.6. QUANTIFICATION OF GENOMIC DNA

After extraction of genomic DNA, quantification was done according to Sambrook *et al.,* 1982. 15 µL of extracted DNA was dissolved in 735 µL of TE buffer and O.D. was taken at 260 and 280 nm (CARY 500 scan UV visible spectrophotometer). Quantity of DNA was calculated by using following formula:

Amount of DNA (ng/µL) = O.D. at 260 X dilution factor X 50 (extension coefficient)

Quality was assessed by taking the (O.D. at 260)/(O.D. at 280). Samples which gave the O.D. between 1.6-1.8 were used in further work.

3.7. RESTRICTION DIGESTION

The extracted genomic DNA was digested by incubating with *Eco*RI, *Mse*I restriction endonucleases along with control (without adding enzyme) in the corresponding buffers at 37 ºC for 3 h according to the users manual. Digested DNA

along with control was analyzed by running the samples in 1.2% agarose gel at 50 V and stained with ethidium bromide.

3.8. POLYMERASE CHAIN REACTION (PCR)

PCR was carried out as per the required volume with stock concentrations of reaction buffer (10X), $MgCl_2$ (25 mM), dNTPs, Taq DNA Polymerase (5 U/µL) (Bioenzyme, USA) and, template DNA (250 ng/µL). The reaction was carried out in Thermal cycler (Eppendorf ep gradientS)

Final concentration of PCR reagents in reaction mixture (100 µL)

Taq polymerase 5 U

1X reaction buffer

3.5 mM $MgCl_2$

800 µM dNTPs

0.4-1 µM of each Primer

50-100 ng Template DNA

Reaction was carried out as per the program given bellow:

Step	**Temperature**	**Time**
Initial denaturation	94 ºC	3 min
Denaturation	94 ºC	30 sec
Annealing	----	30 sec
Extension	72 ºC	----
Final extension	72 ºC	5 min

3.9. AGAROSE GEL ELECTROPHORESIS

3.9.1. Plate preparation and casting the gels: Cleaned agarose gel casting cassette and comb were wiped with methanol. The open sides of the tray were sealed with gel sealing

tape. The comb was placed in the given slits of the plate. Calculated amount of agarose in TBE buffer was mixed to prepare 1.5% solution. The agarose was dissolved completely in the buffer by heating the mixture at 80-85 ºC in microwave oven and was cooled to 50 ºC. Liquid was gently poured into the casting tray before it gets solidified. The combs and sealed tape were removed slowly after complete solidification of the agarose gel.

3.10. PREPARATION OF SAMPLES AND SCANNING OF GELS

The amplified DNA samples having approximately 15 µL volume were mixed with 4 µL gel loading dye and were carefully loaded in the wells using gel loading tips. Electrophoresis was carried out at 100-50V. The gel was stained by ethidium bromide solution having of 0.1mg/ml concentration for 15-20 min. The gel images were recorded in JPEG or TIF formats using gel documentation system (Syngene, USA). The gels were analyzed by using the software Gene Tool (Syngene, USA).

3.11. RANDOM AMPLIFIED POLYMORPHIC DNA (RAPD) ANALYSIS

Amplification of RAPD fragments was performed according to Williams *et al.* [1990] using decamer arbitrary primers (Operon technologies Inc, USA; IDT, USA). The reaction was carried out in a volume of 25 µL of reaction mixture containing final concentration of 10 mM Tris-HCl (pH 9.0), 50mM KCl, 0.1% Triton X-100, 0.2 mM dNTPs, 3.0 mM $MgCl_2$, 0.4µM primer, 25 ng template, 1unit Taq DNA polymerase (Sigma, USA). Amplification was performed in programmed thermal cycler with a program of initial denaturation at 94 ºC for 3 min, 42 cycles of denaturation at 94 ºC for 30 sec, primer annealing at 32 ºC for 1min, extension at 72 ºC for 2.5 min, and final extension at 72 ºC for 4 min. amplification products were electrophoresed in 1.5% TBE. The gels were stained with ethidium bromide and documented using gel documentation system (Syngene, UK). Experiment with each primer was done three times those primers gave reproducible fingerprints were considered for data analysis.

3.12. AMPLIFIED FRAGMENT LENGTH POLYMORPHISM (AFLP) ANALSIS

3.12.1. Reagents for AFLP:

- *Eco*RI/*Mse*I [1.25 units/µL each in 10 mM Tris-HCl (pH 7.5), 50 mM NaCl, 0.1 mM EDTA, 1 mM DTT, 0.1 mg/mL BSA, 50% (v/v) glycerol, 0.1% Triton X-100]. 100 µL
- 5X reaction buffer [50 mM Tris-HCl (pH 7.5), 50 mM Mg-acetate, 250 mM K-acetate] 250 µL
- Distilled water 1.25 mL
- Adapter/ligation solution [*Eco*RI/*Mse*I adapters, 0.4 mM ATP,10 mM Tris-HCl (pH 7.5), 10 mM Mg-acetate, and 50 mM K-acetate] 1.2 mL
- T4 DNA ligase [1 unit/µL in 10 mM Tris-HCl (pH 7.5), 1 mM DTT,50 mM KCl, 50% glycerol (v/v)] 50 µL
- TE buffer [10 mM Tris-HCl (pH 8.0), 0.1 mM EDTA] 4.5 mL
- Pre-amp primer mix 2 mL
- Selective primers (6.7 ng/µL, dNTPs)

  EcoR I primers (27.8 ng/µL):

Primer E-AAC 46 µL

Primer E-AAG 46 µL

Primer E-ACA 46 µL

Primer E-ACT 46 µL

Primer E-ACC 46 µL

Primer E-ACG 46 µL

Primer E-AGC 46 µL

Primer E-AGG 46 µL

Mse I primers (6.7 ng/µL, dNTPs):

Primer M-CAA 900 µL

Primer M-CAC	900 µL
Primer M-CAG	900 µL
Primer M-CAT	900 µL
Primer M-CTA	900 µL
Primer M-CTC	900 µL
Primer M-CTG	900 µL
Primer M-CTT	900 µL

3.12.2. Steps involved in AFLP technique:

(1) Restriction endonuclease digestion of genomic DNA

(2) Ligation of adapters

(3) Pre-amplification reaction

(4) Selective amplification reaction

3.12.2.1. Endonuclease digestion:

The purified DNA sample showing sutable OD of 260/280 was taken and digested with *Eco*RI and *Mse*I which generated the template for next step. *Eco*RI has 6 bps recognition sites and *Mse*I has 4 bps recognition sites. Using these two enzymes together, small DNA fragments of optimal size range (<1 kb) are generated to obtain good fingerprints. The reaction was carried out as follows:

Component	**Control**	**Sample**
5X reaction buffer	5 µL	5 µL
Sample DNA (250 ng in ≤18 µL)	——	≤18 µL
EcoR I/Mse I	2 µL	2 µL
Distilled water	18 µL	5.5 µL-25 µL

Tubes containing reaction mixture were mixed gently and total reaction mixture was collected to bottom of the tube by centrifugation. Tubes were incubated at 37 ºC for 120 min followed by heat inactivation of endonuclease at 70 ºC for 15 min. The reaction mixture was stored at -20 ºC till further step.

3.12.2.2. Ligation of adapters:

Following the heat inactivation of the restriction endonuclease, the genomic DNA fragments were ligated to *Eco*RI and *Mse*I adapters to generate template DNA for pre-amplification. The common adapter sequences flanking variable genomic DNA sequences serves as primer binding sites on these DNA fragments. Using this strategy, it was possible to amplify many DNA fragments without having prior sequence knowledge. Ligation reaction was carried out as follows:

Component	**Volume**
Adapter ligation solution	24 µL
T_4 DNA Ligase	1 µL

Reaction mixture was gently mixed at room temperature, centrifuged briefly to collect contents, and incubated at 20 °C ± 2 °C for 120 min.

1:10 dilution of the ligation mixture was done (10 µL of reaction mixture was taken and transferred to a 1.5 mL microcentrifuge tube. 90 µL of TE buffer was added to it and mixed well).

The unused portion of the reaction mixture was stored at –20 °C.

3.12.2.3. Pre-amplification:

In this step genomic DNA template obtained from step 3.12.2.1 was amplified with AFLP primers each having one selective nucleotide. The PCR products of the pre-amplification reaction were diluted and used as a template for the selective amplification. The pre-amplification was carried out as follows:

Component	**Volume**

Diluted template DNA (from Ligation mix)	5 µL
Pre-amp. primer mix	40 µL
10X PCR buffer plus Mg^{+}	5 µL
Taq DNA polymerase (5 unit/µL)	1 µL
Total volume	51 µL

Gentle mixing of contents was done and mixture was centrifuged briefly to collect the reaction. 20 cycles were performed at:

94 ºC for 30 sec

56 ºC for 60 sec

72 ºC for 60 sec

Final incubation temperature was kept at 4 ºC.

15 µL of pre-amplified product was transferred to a 1.5 mL microcentrifuge tube containing 135 µL TE buffer. This was sufficient to carryout 30 selective AFLP amplifications. If necessary, new dilutions can be made from the pre-amplification reactions to give additional template for the selective AFLP amplifications. Both unused diluted and undiluted reactions were stored at -20 ºC.

3.12.2.4. Selective amplification:

In the selective amplification reaction, genomic DNA was amplified with two AFLP selective primers, each containing three selective nucleotides. For each primer pair, following components were added to a 1.5 mL microcentrifuge tube and labelled as "Mix 1".

Component	**Volume**
Diluted *Eco* RI primer	5 µL

(For non radioactive detection, primers were diluted as follows: 18 µL of *Eco*RI primer with 32 µL of distilled water for use with the AFLP Non-Radioactive Probe)

*Mse*I primer (contains dNTPs)	45 µL
Total volume (sufficient for 10 reactions)	50 µL

Following components were added to another 1.5 mL microcentrifuge tube and were labelled as "Mix2".

Component	**Volume**
Distilled water	79 µL
10X PCR buffer plus Mg^{+}	20 µL
Taq DNA polymerase (5 units/µL)	1 µL
Total volume (sufficient for 10 reactions)	100 µL

Each AFLP amplification was conducted by combining the following in a 0.2- or 0.5-mL thin-walled PCR tube:

Component	**Volume**
Diluted template DNA (from preamplification)	5 µL
Mix 1 Primers/dNTPs	5 µL
Mix 2 (Taq DNA polymerase/buffer)	10 µL

- One cycle was performed at 94 ºC for 30 sec; 65 ºC for 30 sec; and 72 ºC for 60 sec.
- Annealing temperature was lowered at each cycle by 0.7 ºC for 12 cycles. This gives a touchdown phase of 13 cycles.
- 23 cycles were performed at:

  94 ºC for 30 sec,

  56 ºC for 30 sec, and

  72 ºC for 60 sec.

Total Time: 82 min.

3.13. RAPD AND AFLP DATA ANALYSIS

Acquired RAPD finger prints were statistically analyzed with following the assumptions.

1) The populations are in hardy Weinberg equilibrium.

2) Each band represents the phenotype at a single biallelic distinct locus.

3) Comigrating band represent homologous loci.

4) Polymorphic loci are inherited in a Mendelian fashion.

Fragment sizes were designated as loci, and were considered as Biallelic (present = 1, absent = 0) and made the binary matrix. Only those loci amplified strongly in each instance with reproducibility were scored and included in the analyses ignoring the intensity of the bands.

Genetic similarity was calculated according to the following formula:

$$F = \frac{2Nxy}{(Nx+Ny)}$$

Nxy = the number of bands shared by two species.

Nx and Ny = are the number of fragments in each sample.

Genetic disparity was calculated by formula.

$$P=1-F$$

Percentage of polymorphism was calculated by using following formula:

$$\text{Percentage of Polymorphism} = \frac{\text{No. of Polymorphic Bands}}{\text{Total number of Bands}} \text{ X } 100$$

Phylogenetic trees were constructed according to Jaccard [1908] using binary data generated by RAPD and AFLP excluding the intraspecific polymorphic markers, followed by bootstrapping analysis across the loci [Felsenstein, 1985] with the help of statistical analysis software SYSTAT version 12.

3.14. FIASCO (FAST ISOLATION BY AFLP OF SEQUENCES CONTAINING REPEATS):

3.14.1 Reagents for FIASCO:

3.14.1.1. Pre-amplified product:

Pre-amplification product of *J. curcas* genomic DNA from step 3.12.2.1 was taken with the following modifications; 17 cycles instead of 20 cycles showing proper amplification (uniform smear) obtained by primer having no selective nucleotide were taken as the starting material for isolating microsatellite enriched library.

3.14.1.2. Preparation of paramagnetic particles of streptavidin (SA-PMP's):

SA-PMPs were resuspended until the particles are completely dispersed by gently flicking the bottom of the stock bottle and one mL of the mixture was taken in 1.5 mL tube provided in the kit (Promega, USA).The SA-PMPs were captured by placing the tube in the magnetic stand until they were collected at the side of the tube (approximately 30 seconds) and the supernatant was decanted. Then SA-PMPs were then washed three times with 0.5X SSC (300 µL per wash). After each wash, SA-PMPs were captured using the magnetic stand and the wash solution was carefully removed. After washing SA-PMPs were resuspended in 100 µL of 0.5X SSC.

3.14.2. Preparation of DNA probe hybrid:

- 500 ng of pre-amplification product was added to 80 pM of 5' biotin-labelled oligonucleotide probes [5'biotin $(CT)_{15}$3'].
- Volume was made up to 100 µL so that final concentration of the solution will be 4.2X SSC, 0.07% SDS.

- Mixture was incubated at 95 ºC for 5 min and then shifted to room temperature (24 ºC) for 15 min.

3.14.3. SA-PMP-PA-SSR-Probe hybrid complex preparation:

- 100 µL of PA-Probe hybrid was added in to 50 µL of SA-PMP's.
- 300 µL of Milli Q water was added and incubated for 30 min at RT with constant gentle mixing.

3.14.4. Capture and washing of annealed SA-PMP-PA-SSR-Probe hybrid complex.

- SA-PMP-PA-SSR-Probe hybrid complex were separated from hybridization buffer by magnetic field and the buffer was discarded.
- Non-specific DNA fragments were removed by three non-stringency (with 400 µL TEM_{1000}) washes and three stringency (with 400 µL of 0.2X SSC, 0.1% SDS) washes.

NOTE: Each wash is carried out for five min at RT with gentle mixing.

- Last non-stringent and stringent washes were stored for final analysis.

3.14.5. Enriched microsatellites library elution (alkaline denaturation):

- The washed beads were treated with 12 µL of 0.15 M NaOH and the supernatant was recovered by separating the SA-PMP's with magnetic field.
- The recovered supernatant was immediately neutralized by addition of appropriate volume of pre-titrated acetic acid.
- To the above neutralized mixture equal volume of 2X TE was added and stored at -20 ºC till further use.

3.14.6. PCR amplification of enriched microsatellites library:

The enriched product microsatellites isolated in the step 3.14.5 was diluted 5 times with TE buffer and amplified for 12 cycles using Pre-amp primers without selective nucleotide mix. The pre-amplification was carried out as follows:

Component	Volume
Diluted template DNA (from enriched product)	5 µL
Pre-amp primers without selective nucleotide	40 µL
10X PCR buffer plus Mg^{+}	5 µL
Taq DNA polymerase (5 unit/µL)	1 µL
Total volume	51 µL

Reaction was collected by mixing gently and centrifuging briefly and 12 cycles were performed at:

94 °C for 30 sec

56 °C for 60 sec

72 °C for 60 sec

3.14.7. Ligation of PCR amplified enriched microsatellites in to T-Vector:

Ligation reaction was carried out as per manufacturer's instruction (Fermentas, USA) as follows:

Component	Volume
pTZ57T/R	3 µL
PCR product	7 µL
5X reaction buffer	6 µL
T_4 DNA Ligase	1 µL
Distilled water	13 µL

Reaction mixture was incubated at 22 ºC for 6-8 hours.

3.14.8. Preparation of competent cells of *E. coli* (DH_5 α):

Competent cells for *E. coli* were prepared as described by Sambrook *et al*., [1989]; with minor modifications. Fresh overnight cultures were made in Luria-Bertani

medium (LB) at 37 ºC for 6 hrs so that mid-log phase is reached and then sub-cultured (1%) in 10 mL of SOC medium and the culture was incubated at 37 ºC for 2.5 h till it reaches early log phase. 4 mL of culture was centrifuged at 6000 rpm for 2 min at 4 ºC. The pellet obtained was then gently resuspended in 1 mL of resuspension solution and centrifuged at 6000 rpm for 2 min at 4 ºC. The supernatant was discarded and the pellet was suspended in 100 µL of resuspension solution and incubated in ice for 6-8 h.

NOTE : Chilled resuspension solution is used to avoid heat-shock to cells.

3.14.9. Transformation into *E. coli*:

Transformation was carried out according to Sambrook *et al.*, [1989]; with minor modifications. 7 µL of ligated sample was added to 100 µL of competent cells. The microcentrifuge tube was then kept in ice for 10-15 min and heat shock was given to cells by exposing the tube to 42 ºC (maintained in water bath) for 90 sec. The tube was then immediately transferred to ice and incubated for 15 min. The mixture was diluted in 900 µL of LB broth and the tube was incubated at 37 ºC for 45 min on incubator shaker (125 rpm) for the expression of antibiotic marker (Amp^r). After incubation the microcentifuge tubes were centrifuged at 8000 rpm for 2 min and 600 µL of supernatant was discarded and the pellet was suspended in the leftover 400 µL of supernatant. The culture was then plated onto LB plates containing ampicillin (50 µg/mL), X-gal (40 µg/mL) and IPTG (20 µg/mL). The plates were incubated at 37 ºC for 12 h. After incubation, plates were checked for white and blue colonies.

3.14.10. Colony PCR and clone selection:

100 µL aliquots of sterile water were dispensed into 1.5 mL microcentrifuge tubes. Aliquots were prepared as per the number of bacterial colonies to be screened. A single white colony was picked with a sterile wire loop and suspended into the tube. After picking all the colonies of interest the cells were lysed by keeping the bacterial suspension at 95 ºC for 5 min in water bath. The cells were then immediately transferred to ice for 15 min and centrifuged at 10,000 rpm for 3 min to separate cell debris and 5

µL supernatant was taken as the template for total 12 µL PCR . PCR was conducted as described the section 3.8. using the following program:

94 ºC for 2 min

94 ºC for 30 sec (30 cycles)

56 ºC for 30 sec

72 ºC for 60 sec

Final extension at 72 ºC for 3 min

The amplified products were then run in 1.6% agarose gel with template prepared with *E. coli* (DH5 α) strain as negative control and *E. coli* (DH5 α) transformed with non ligated T-vector as positive control. Clones were selected by comparing the DNA bands size with the size of positive control (165 bp).

3.14.11. Amplification of selected clones:

Template for the amplification of the clones and PCR was performed as per section 3.14.10 in a volume of 60 µL reaction mixture containing 20 µL of template.

3.14.12. Sample purification:

The sample DNA (55 µL) was loaded with 10 µL of loading buffer in 1.6% agarose gel. After running the samples in electrophoretic unit, DNA bands of interest were identified using UV tranilluminator. Bands were than excised from the gel with a clean sharp blade. Gel pieces containing DNA bands were collected in individual, previously weighed microcentrifuge tubes of 2 mL and the pieces were weighed. Three volumes (w/v) of solubilization buffer was added to one volume of gel piece. The tubes were incubated at 50 ºC until the gel piece got dissolved (10 to 15 min). One gel volume of iso-propanol was added to the tube and mixed properly. Sample was then applied to spin column of collection tube provided with the kit (Qiagen gel purification kit, USA). Collection tube was centrifuged at 10,000 rpm for 1 min. Supernatant was discarded and 500 µL of wash-buffer was added to spin column. Tubes were centrifuged again at 10,000 rpm for 1 min. the columns were then proceed with wash solution at 10,000 rpm

for 1 min twice and empty spin was performed at 10,000 rpm for 2 min. Spin column was placed in 1.5 mL microcentrifuge tube. 40 µL of elution buffer was added to spin column and tube was kept for 1 min incubation at RT. Tube was then centrifuged at 10,000 rpm for 2 min. Spin column was removed from the microcentrifuge tube. 2 µL of purified sample was checked with 2 µL of loading buffer in the 1.2% agarose gel and sample was stored in -20 ºC for further use.

3.14.13. Lyophilisation and sequencing of purified samples:

Amplified DNA quantification was done as described in the section 3.6. Approximate 750 ng of DNA was lyophilized in 1.5 mL microcentrifuge tube using HETO VR-1 lyophilizer (HETOVAC, Denmark). The lyophilized samples were packed and sent to Macrogen INC, Seoul, Korea for sequencing analysis.

3.14.14. Sequence editing and primer designing:

The raw sequence data was retrieved from Macrogen INC in Seoul, Korea. From the sequence, the vector and adapters sequences were edited using BioEdit software. The primer to the flanking regions of the microsatellite was designed using Net Primer software. The optimal length of PCR primers was kept 18-22 bp. This length is long enough for adequate specificity and short enough for primers to bind easily to the template at the annealing temperature. With the help of the software, T_m of the primers, internal looping at 3' and 5' ends as well as undesirable pairings of primers were analyzed and the care was taken to have the forward and reverse primers base composition 50-60% (G+C) and ended with (3') in a G or C, or CG or GC which prevents "breathing" of ends and increase efficiency of priming. T_m of both primers was taken between 50-65 ºC. The primer pairs having the optimum T_m with minimum number of internal loops and primer-dimers were designed. The designed oligos were procured from IDT (Integrated DNA Technology) USA.

3.15. PRIMER RECONSTITUTION

Primer vials were centrifuged at 6000 rpm for 1 min. Calculated amount of milli Q water was added to vials. One min vortexing was done to dissolve the transparent

lyophilized oligo pellet. The vial was then heated at 60-65 ºC for 10 min and then transferred to ice. The primers were diluted to 10 pM/µL in 1.5 mL microcentifuge and the tubes were stored at -20 ºC.

3.16. MICROSATELLITE AMPLIFICATION

Microsatellite markers were amplified from in a volume of 25 µL containing 10 mM Tris-HCl (pH 9.0), 50mM KCl, 0.1% Triton X-100, 0.2 mM each dNTPs, 30 mM $MgCl_2$, 0.4 µM primer, 25 ng template, 1 unit of Taq DNA polymerase (Biogene,USA). Amplification was performed in a thermal cycler (master cycle epgradient S eppendorf, Germany) with following program:

94 ºC for 2 min

94 ºC for 30 sec (30 cycles)

--- ºC for 30 sec (Table 19)

72 ºC for 40 sec

Final extension was at 72 ºC for 3 min. The amplified products were resolved in denaturing Polyacrylamide (12%) gel in 1X TBE buffer. The size of the allele was determined using GeneTool analysis software (Syngene, UK).

3.17. DENATURING POLY ACRYLAMIDE GEL ELECTROPHORESIS (PAGE)

Polyacrylamide gel electrophoresis was carried out in a vertical matrix of acrylamide gel containing urea with TBE Buffer as running buffer.

3.17.1. Plate preparation:

Plates were wiped four times with ethanol. Little amount of bind or repel saline was spread to corresponding plates uniformly and wiped again with ethanol for four times. Plates were assembled having 0.3 mm spacer gap and plates were placed appropriately in the casting base.

3.17.2. Preparation of 12% denaturing polyacrylamide gel: (35 mL): The required percentage of gel was prepared by adding the reagents as given bellow:

Component		Volume
Urea	:	15.75 g
Distilled water	:	17.5 mL
Acrylamide stock solution (30%)	:	14.0 mL
TBE Buffer (10X)	:	3.5 mL
TEMED [N, N, N, N'- tetramethylethylenediamine]	:	40 µL
Ammonium persulphate (10%)	:	350 µL

3.17.3. Casting of the denaturing PAGE gel:

Solution was drawn into syringe immediately after addition of APS. Syringe was filled without liftingout of the acrylamide solution to avoid bubbles. Syringe was placed in the tube and then turned upward. Gel solution was injected with moderate pressure and combs were inserted carefully in the plates. Plates were left undisturbed for polymerization. Combs were removed carefully and wells were cleaned.

3.17.4. Loading and electrophoretic separation of the amplified product:

The amplified AFLP/microsatellite PCR product was mixed with 4 µL of formamide loading buffer. The sample mixture was denatured by boiling at 95 ºC for 5 min in a water bath. 3 µL of sample was loaded into corresponding wells in pre-run gel and the samples were resolved by electrophoresis by running the gel at 410 V in 1 X TBE Buffer. The plates were separated gently and carefully to make sure that gel is stuck to the plate applied with bind saline.

3.18. SILVER STAINING OF DENATURING PAGE GELS

3.18.1. Reagent preparation:

3.18.1.1. Fix solution: 5% CTAB solution w/v [0.5 g of CTAB was added in 500 mL of double distilled water and mixed well till it forms clear solution]

3.18.1.2. Ammonia Solution: 1.5% Ammonia solution v/v [6.5 mL of Ammonia was added in 493.5 mL of double distilled water and mixed well]

3.18.1.3. Staining solution: Ammoniacal silver nitrate [0.5 g of silver nitrate was dissolved in 560 mL of double distilled water, 2.5 mL of 1 N NaOH was added and solution was mixed till it forms uniform brown colour. Ammonia solution was added drop wise till it turns colorless and was kept in dark].

3.18.1.4. Developing solution: 2.5% Na_2CO_3 with 450 µL of Formaldehyde [450 µL of Formaldehyde was added to 10 g of Na_2CO_3 in 400 mL of double distilled water]

3.18.1.5. Stop Solution: 7.5% Acetic acid solution [35 mL of acetic acid in 465 mL of double distilled water].

3.18.2. Procedure:

- Gels were rinsed twice with double distilled water and placed in tray containing distilled water and incubated for 5 min with gentle shaking.
- Distilled water was removed and the gels were incubated in CTAB solution for 30 min.
- Gels were rinsed twice with double distilled water and incubated in ammonia solution for 15 min.
- The gels were rinsed twice with double distilled water and incubated in staining solution for 20 min.
- Gels were rinsed again with distilled water and then placed in the tray containing developing solution till the required contrast was obtained in bands.
- After getting required contrast development of band intensity was stopped with stop solution.

- Gels were scanned and saved as compressed TIF or JPEG format using gel A4 pro-gel scanning system (Syngene, UK).

3.19. MICROSATELLITE DATA ANALYSIS

The size of each microsatellite allele was calculated by taking standard 20 bps marker (Fermentas, USA) using the GeneTool analysis software (Syngene, USA). The allele data was scored in microsoft excel as number of the repeats according to the calculated size. The data in excel was converted to Arliquin data input file format using CRATE software [Coombs, 2007]. Parameters such as expected heterozygosity, observed heterozygosity, Hardy-Weinberg equilibrium and P-value were calculated using using Arlequin analysis software version 3.0 [Excoffier *et al.,* 2005] and dendrogram of analyzed sample based on SSR markers diversity was generated using Gene Directory analysis software (Syngene, USA).

3.20. ISOLATION AND SEQUENCEING OF nrDNA ITS SEQUENCE

3.20.1. Amplification and sequencing of nrDNA ITS sequence:

Candidate primers JCITS-1-F (5'ACCTGCGGAAGGATCATTGTCGAAA3') and JCITS-2-R (5'CCTGGGGTCGCGATGTGAGCGT3') were designed from conserved 5' and 3' ends of the reported species belongs to Euphorbiaceae family primers were designed by taking in to consideration (i) melting temperature (Tm), (ii) G+Ccontent and (iii) self-complementarity using the NETPRIMER computer program. The PCR amplification was carried out in a volume of 50 µL reaction mixture containing final concentration of 10 mM Tris-HCl (pH 9.0), 50mM KCl, 0.1 Triton X-100, 0.2 mM each dNTP, 1.5 mM $MgCl_2$, 0.2 µM primes JCITS-1-F and JCITS-2-R, 50 ng template, 2 units Taq DNA polymerase (Sigma, USA). Amplification was performed in programmed thermal cycler (Master cycle, Eppendorf, Germany). The amplified PCR products were purified using PCR cleanup kit (Sigma, USA) and sequencing was done with the same primers using the service provided by Macrogen Pvt Ltd, Korea.

3.20.2. nrDNA ITS sequence analysis:

Sequences were aliened and edited with the help of BIOEDIT sequence analysis software. In the resulted data files all positions containing gaps and missing data were eliminated from the dataset (Complete-deletion option). The files were converted saved in fasta format. With the resulted data file, maximum composite likelihood estimate of the pattern of nucleotide substitution was done and rate variation among sites was modeled with a gamma distribution (shape parameter = 1). The transition/transversion (ti/tv) rate ratio (R) was calculated based on the formula $R = [A*G*k_1 + T*C*k_2]/[(A+G)*(T+C)]$ using the MEGA software. Genetic distance was obtained with the help of the same software and mean genetic distance of inter and intraspeciefic distance was calculated by sum of individual genetic distance divide by number of samples. Phylogenetic tree based on nrDNA ITS sequence data was obtained based on maximum parsimony method along with bootstaping with 1000 replicates using MEGA anlaysis software [Kumar *et al.,* 1993; Felsenstein, 1985]. Since the earlier reports indicate that *Jatropheae* and *Crotoneae* together had a new world origin and more phylogenetically close [Berry *et al.,* 2005]; so, phylogram was rooted by taking one of the species of genus croton (*Croton argyranthemus*) as an out group.

CHAPTER 4

RESULTS

Part A:

4. A. STANDARDIZATION OF METHOD FOR HIGH QUALITY GENOMIC DNA EXTRACTION FROM *J. CURCAS* FOR GENETIC DIVERSITY AND MOLECULAR MARKER STUDIES.

Several experiments based on available protocols were performed using fresh plant material collected from one year old plants growing in the genetic garden, CSMCRI, Bhavnagar. In initial experiments, the major problem associated with DNA extraction was co-precipitation of large amount of polysaccharides along with genomic DNA. Using 3.5 M NaCl in extraction buffer followed by the use of 80% ethanol along with 2.0 M NaCl (final concentration) during precipitation has significantly improved the quality of DNA avoiding the co-precipitation of polysaccharides. The above high salt included extraction buffer was further used to

(i) determine the type of plant material to be used for the extraction, (ii) determine incubation time of buffer and tissue mixture at 65 ºC, (iii) buffer to tissue ratio and (iv) extraction with phenol-chloroform-isoamyl alcohol vs. Tris saturated phenol followed by chloroform-isoamyl alcohol extraction in extraction and purification phases. All the experiments were repeated 3-4 times to check reproducibility.

The next stage of optimization was to deduce the optimum buffer to tissue ratio for extraction. The best yield with good quality of genomic DNA was obtained when the tissue to buffer concentration was 1:5. The resulted DNA concentration was 142.00 ± 15.8 µg/g of tissue with O.D.1.81 ± 0.069 $A_{260/280}$ (Table 2). Optimization of incubation

period at 65 ºC was found by conducting the experiment in gradient of incubation times.The best yield (132.50 ± 7.8 ng/µL with O.D. 1.80 ± 0.059 $A_{260/280}$) was obtained following incubation at 65ºC for 90 min (Table 3). With the above optimized conditions the extraction procedure was applied to different sources of the plant like root, petiole, stem, germinated seedlings and also from callus generated from leaf (Table 4). The best yield was obtained from leaves (126.40 ± 8.9) with good $A_{260/280}$ O.D. (1.81 ± 0.063) followed by callus (88.30 ± 3.4) and no change in the yield was observed by conducting all the extraction steps at RT.

The key steps in the optimized protocol are as follows:

- Use of 3.5 M NaCl in extraction buffer,
- Use of 2.0 M NaCl (final concentration) during precipitation.
- Tris saturated phenol in place of phenol-chloroform-isoamyl alcohol at purification phase.
- 80% ethanol for DNA precipitation.

The DNA thus extracted had 1.81±0.063 (from the leaves), OD at $A_{260/280}$ and the yield was 120 to 140 µg per gram of material.The optimized extraction method was found to be suitable for the extraction of DNA from all parts of the plant and also from callus. The suitability of the extracted DNA to restriction digestion, ligation and to PCR was carried out following the procedure given in the materials and methods section. The results showed that the extracted DNA with optimized protocol in this study (section 3.5) was found to be useful for all the subsequent procedures in molecular analysis and DNA fingerprinting techniques like RAPD, AFLP and SSR analysis (Figure 7, 8 & 9).

Table 2: Effect of tissue: buffer ratio on quality and quantity of genomic DNA extracted from *J. curcas* leaf (mean ± SD of 4 independent experiments).

Tissue – Buffer Ratio	$A_{260/280}$	DNA concentration obtained (µg/g of tissue)
1:3	1.67 ± 0.063	66.00 ±12.3
1:4	1.72 ± 0.11	72.50 ± 8.6
1:5	1.81 ± 0.069	142.00 ± 15.8
1:6	1.84 ± 0.12	112.50 ± 11.3
1:7	1.77 ± 0.059	95.50 ± 6.9

Table 3: Effect of incubation time on quality and quantity of genomic DNA extracted from *J. curcas* leaf (mean ± SD of 4 independent experiments).

Time of Incubation at 65 ºC(min)	$A_{260/280}$	DNA concentration obtained (µg/g of tissue)
30	1.74 ± 0.062	57.50 ± 5.3
60	1.75 ± 0.073	77.50 ± 5.8
90	1.80 ± 0.059	132.50 ± 7.8
120	1.95 ± 0.063	122.50 ± 11.3
150	1.82 ± 0.071	56.25 ± 8.6
180	1.84 ± 0.079	59.75 ± 8.2

Table 4: Qualitative and quantitative differences in genomic DNA extracted from different tissues of *J.curcas*. (Mean ± SD of 4 independent experiments).

Plant part	$A_{260/280}$	DNA concentration obtained (µg/g of tissue)
Germinated seedling	1.64 ± 0.061	78.60 ± 6.8
Petiole	1.84 ± 0.042	43.75 ± 7.2
Root	1.29 ± 0.23	45.00 ± 5.3
Stem	1.75 ± 0.093	46.25 ± 4.1
Callus (leaaf)	1.82 ± 0.079	88.30 ± 3.4
Leaf	1.81 ± 0.063	126.40 ± 8.9

Figure 3: Electrophoretic separation of genomic DNA extracted from *J. curcas* leaf using different protocols. Lane 1-1kb marker; lane 2-5 DNA extracted using published protocols.

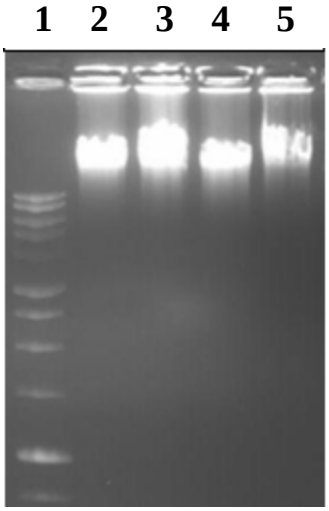


Figure 4: Electrophoretic separation of genomic DNA extracted from *J. curcas* leaf using phenol, chloroform, isoamyl alcohol combinations. Lane1-DNA extracted with Tris saturated phenol; lane 2-DNA extracted with phenol, chloroform, isoamyl alcohol (25:24:1); lane 3-1kb marker; lane 4-DNA extracted with chloroform, isoamyl alcohol (24:1).

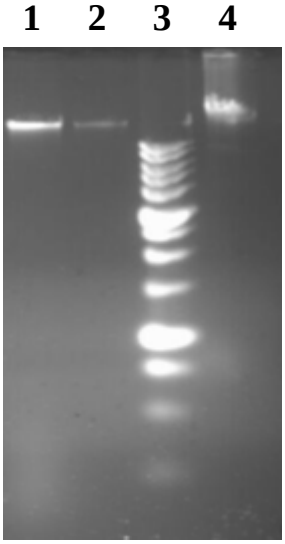


Figure 5: Restriction digestion of genomic DNA. Lane1, digested with *EcoR*I; Lane2, digested with *EcoR*I and *Mse*I; Lane 3, control reaction (DNA without endonuclease).

Figure 6: Electrophoretic separation of genomic DNA extracted from different tissue of *J. curcas*. lane 1-1 Kb DNA marker; lane 2- germinated seedlings; lane 3- roots, lane 4- stem of 2 weeks plant, lane 5- stem of matured plant, lane 6- petiole, lane 7- leaf and lane 8- callus.

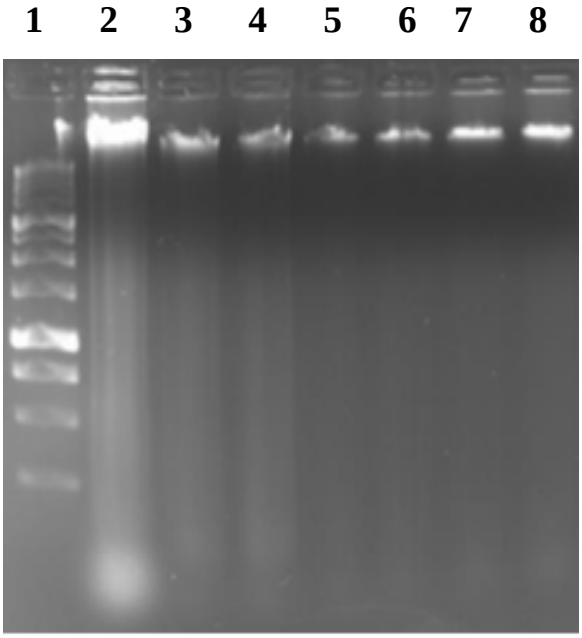


Figure 7: RAPD fingerprint analysis of *J. curcas* genomic DNA. lane 1, 1 Kb marker; lane2-15, RAPD profile of *J. curcas* with primer OPL5.

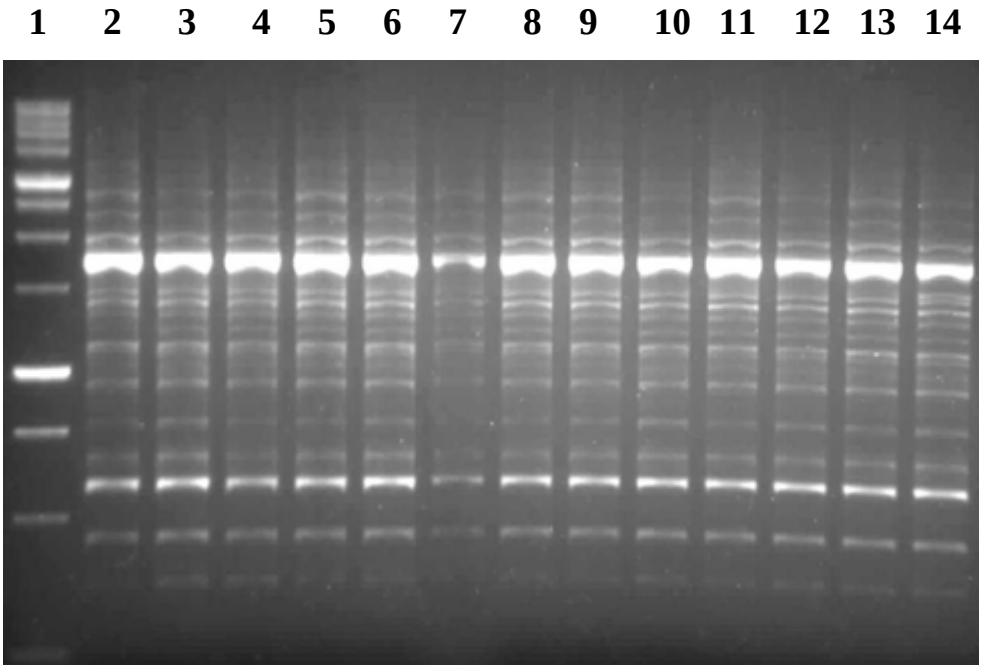


Figure 8: Lane 1-17, SSR marker (jcds10) amplified from 17 germplasm of *J. curcas*; Lane M, 10 bp marker.

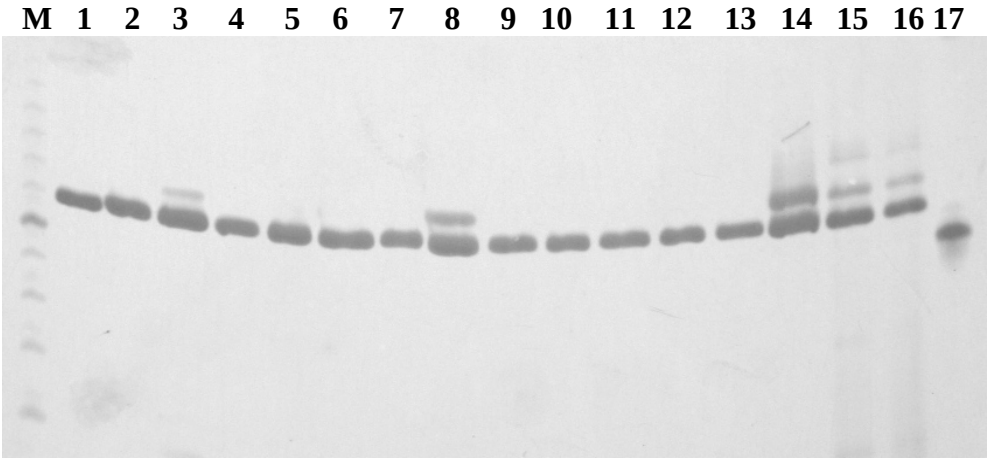
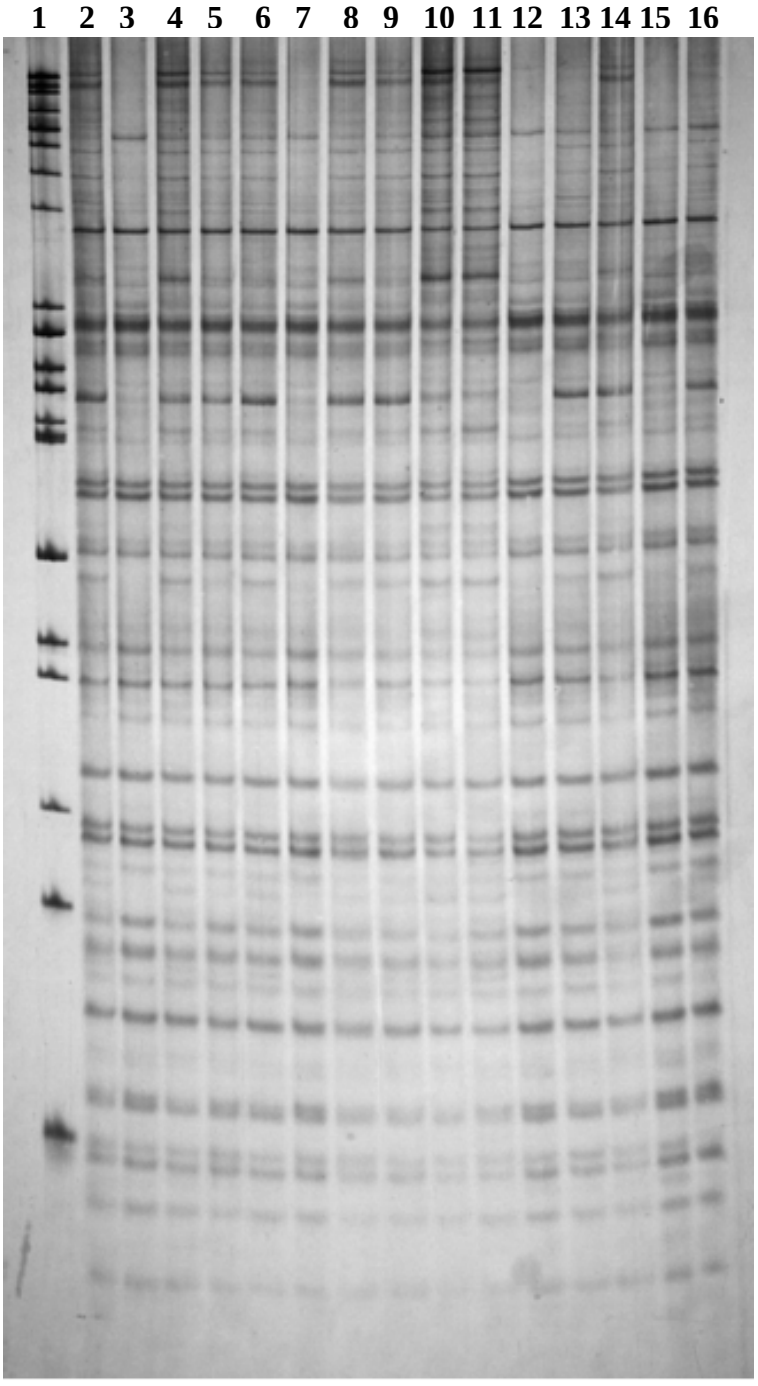


Figure 9: AFLP fingerprint of *J. curcas*, genomic DNA isolated using the standardized potocol. Lane 1-1kb+100bp mixture; lane 2 to16- selective amplified with primer set E-ACC/M-CAC.



Part B:

4.B. COMPARATIVE STUDIES ON INTERSPECIFIC GENETIC DIVERGENCE AND PHYLOGENIC ANALYSIS OF GENUS *JATROPHA* BY RAPD, AFLP AND nrDNA ITS SEQUENCE ANALYSIS.

Initially 180 RAPD primers (20 primers Kit E, IDT USA; 160 primers, kit- J, K, L, N, O, P, Q, R, Operon technologies Inc., USA) were screened and out of which 52 primers responded with more than 6 markers were included in the study. In the further screening 33 primers which gave fingerprints with good resolution and band reproducibility were used in the final analysis to characterize *J. curcas, J. glandulifera, J. gossypifolia, J. integerrima, J. multifida, J. podagrica* and *J. tanjorensis*. For AFLP DNA fingerprinting initially 64 combinations of *Eco*RI and *Mse*I with three nucleotide selective primers were screened and out of which 27 primer combinations that gave sharp fingerprints were selected for molecular analysis of above described *Jatropha* species. In total 619 RAPD markers and 1853 AFLP markers were generated and used to study molecular divergence and to deduce phylogenetic relation among the species.

4.B.1. RAPD analysis:

Use of thirty three RAPD primers has produced totally 619 markers out of which 605 markers were found to be polymorphic. Markers obtained for each primer varied from 7 (OPP10) to 44 (IDT E-5). On an average each primer produced 18.76 markers out of which 18.33 were polymorphic markers. Primer IDT E-4 has produced 2 common markers in all the species studied which is the highest number with any primer used in this study. 100 percent polymorphism was observed when primers IDT E5, OPJ13, OPN10, OPN12, OPN16, OPN19, OPO20, OPP15, OPP6, OPQ9, OPJ9, OPL14, OPO7, OPO15, OPP1, OPP5, OPP10, OPQ16, OPQ20 and OPR2 were used. Whereas, use of IDT E7, OPJ20, OPL5, OPN20, OPO5, OPQ2, IDT E20, OPL7, OPN3, OPQ11, OPQ12 and OPR8 primers resulted one marker each common to all the species studied. Use of primer OPP10 has resulted in lowest number of markers (7) without any common marker and OPQ12 has given lowest number of markers (8) with one marker common to all the

species studied and showed lowest PP (87.50). The PP within the species was found to be 1.49 for *J. curcas*, 2.03 for *J. glandulifera*, 3.10 for *J. gossypifolia*, 2.60 for *J. integerrima*, 2.95 for *J. multifida*, 2.18 for *J. podagrica*, 2.18 for *J. tanjorensis* whereas, GS was found to be 0.992 for *J. curcas*, 0.989 for *J. glandulifera*, 0.984 for *J. gossypifolia*, 0.986 for *J. integerrima*, 0.985 for *J. multifida*, 0.988 for *J. podagrica*, for 0.988 *J. tanjorensis*. Intraspecific diversity was found to be maximum in *J. gossypifolia* and minimum in *J. curcas.* On the contrary intraspecific GS was found to be maximum in *J. curcas* and minimum in *J. gossypifolia*. Over all PP among seven species through RAPD was found to be 97.74 with 14 markers common in all the species studied. In the pair wise comparison the mean PP was 68.48 and GS was 0.48. The highest PP (76.53) and lowest GS (0.38) was found between *J. glandulifera* and *J. tanjorensis*. Lowest PP (54.49) and highest GS (0.63) was found between *J. curcas* and *J. integerrima* (Table 5 & 6).

4.B.2. AFLP analysis:

Using 27 combinations of AFLP selective primers 1853 markers were generated out of which 1802 makers were found to be polymorphic. On an average each primer has produced 68.63 markers with 66.74 polymorphic markers. Markers obtained for each primer varied from 83 (M-CAT/E-ACA) to 53 (M-CAG/E-ACT). Over all PP among seven species was found to be 97.25. The PP within the species was 2.95 for *J. curcas*, 3.60 for *J. glandulifera*, 5.49 for *J. gossypifolia*, 3.10 for *J. integerrima*, 5.47 for *J. multifida*, 2.32 for *J. podagrica*, 4.28 for *J. tanjorensis*. Whereas, the GS was found to be 0.985 for *J. curcas*, 0.981 for *J. glandulifera*, 0.971 for *J. gossypifolia*, 0.984 for *J. integerrima*, 0.971 for *J. multifida*, 0.988 for *J. podagrica*, 0.978 for *J. tanjorensis*. Similar to RAPD, AFLP also showed the maximum diversity in *J. gossypifolia* and minimum in *J. curcas*. The GS was also highest in case of AFLP as observed with RAPD in *J. curcas* and minimum in *J. gossypifolia*. Use of primer set M-CAT/E-ACA has resulted maximum number of markers (83) with no common marker to all the species indicating 100% polymorphism among species studied. Use of primers combination E-AAC/M-CAA, E-AAC/M-CTG, E-ACC/M-CTG, M-CTG/E-ACTP, E-ACC/M-CTT and

E-AGC/M-CAC also resulted in 100% polymorphic markers. When primers combination M-CAT/E-AAG was used highest numbers of non polymorphic markers (7) were obtained and resulted in lowest PP (90.91). Maximum GS and minimum PP was observed between *J. multifida* and *J. podagrica* followed by *J. podagrica* and *J. integerrima*. Maximum PP and minimum GS was found between *J. glandulifera* and *J. podagrica*. Mean PP and GS between any two species was found to be 71.33 and 0.44 respectively (Table 7 & 8).

The phylogenetic trees were generated according to Jaccard [1908] from the binary data of RAPD and AFLP following bootstrapping across the loci. Both the phylogenetic trees showed similar grouping of the species with minor changes (Figure 13 & 14). The bootstrapping values obtained supported the phylograms constructed in the present study. The phylogram of RAPD showed one major clade with *J. curcas*, *J. integerrima* and *J. gossypifolia* with *J. tanjorensis* as a sister clade. In RAPD phylogram *J. curcas*, *J. integerrima* grouped with least distance followed by *J. multifida* and *J. podagrica*. The phylogram of AFLP showed one major clade with five species of *Jatropha* (*J. curcas, J. glandulifera, J. gossypifolia, J. integerrima* and *J. tanjorensis*) and one minor clade with *J. multifida* and *J. podagrica*. In the AFLP phylogram *J. multifida* and *J. podagrica* grouped with least branch distance, followed *by J. curcas, J. integerrima* and then followed by *J. glandulifera* and *J. tanjorensis*.

4.B.3. nrDNA ITS Analysis:

nrDNA ITS sequences amplified with primers JCITS-1-F and JCITS-2-R isolated from 7 species, 3 germplasm of each species (*J. curcas, J. glandulifera*, *J. gossypifolia*, *J. integerrima*, *J. multifida, J. podagrica,* and *J. tanjorensis*) showed very minor size variations ranging in length from 647 to 654 bp (Figure 15, Table12). In case of intraspecific ITS region size variations were seen in the species, *J. glandulifera, J. integerrima* and *J. tanjorensisis* of only one basepair difference; however, the size variations were shown only by ITS1 but not either by 5.8s or ITS2. Over all size variations observed was found to be same (8 bp) in ITS1 (ranging from 647 to 654) and ITS2 (ranging from 202 to 209) whereas 5.8s nrDNA sequence showed only one base

pair size difference. Inter species G+C content of the sequences found to be broad and it was in the range of minimum 53.82 by *J. multifida* and maximum 63.43 by *J. gossypifolia*. The intraspecies G+C content variation was found to be very narrow. There appeared to be no variation in case of *J. multifida* on the contrary *J. podagrica* showed high variations (Table12).

The overall mean genetic distance of genus *Jatropha* based on nrDNA ITS was found to be 0.385. Highest interspecific genetic distance (GDS) (0.419) was found between the species *J. glandulifera* and *J. multifida*. The second highest interspecific genetic distance (0.405) was found to in between *J. multifida* and *J. tanjorensis*. The least interspecific genetic distance (0.085) was found in between *J. gossypifolia* and *J. tanjorensis* and next least genetic distance was found between *J. curcas* and *J. integerrima*. The highest intraspeciefic genetic distance was observed in *J. podagrica* (0.011) followed by *J. tanjorensis* (0.009). The least intraspecific genetic distance was found in *J. gossypifolia* (0.002) followed by *J. multifida* (0.004). The intraspecies mean genetic distance was found to be 0.006 (Table 10 & 11).

Majority of nucleotide variations observed were transitions/transversions and very minor variations are deletions or insertions. The levels of variations varied significantly in three regions and major variations were observed in regions of ITS 1 and 2 than in 5.8s nrDNA. The R value for 21 sequences together was found to be 11.292. The k_1, k_2 was found to be 0.585 and 36.511 respectively. transitions/transversions ratio (ti/tv ratio) between the pair of species was summarized in the (Table 13). ti/tv ratio was found to be maximum in between *J. integerrima* and *J. multifida* (42.78) followed by *J. gossypifolia* and *J. multifida* (37.52). The least mean of ti/tv ratio was found in case of *J. curcas* with other species of the genus. In comparison with other species *J. curcas* showed high ti/tv rario (2.62) with *J. integerrima* which shows the recent divergence of these species. Over all mean ti/tv ratio between any pair of the species was found to be 10.03.

The phylogram was generated based on maximum parsimonious method from the 21 sequences obtained with the bootstrap support by taking 1000 replicates and the resulted tree showed two major clades (Figure 16). The bootstrapping values obtained

strongly supported the phylogram generated with nrDNA ITS sequence (Figure 16). The major clade was formed with *J. curcas, J. gossypifolia, J. integerrima* and *J. podagrica*. The minor clade was formed with *J. glandulifera, J. multifida* and *J. tanjorensis*. In the major clade though the least branch distance was observed between *J. curcas* and *J. podagrica* but notable observation was that one of the germplasm sequence of *J. integerrima* (J.INT3) has shown high similarity with *J. curcas* than with any other species and clustered with *J. curcas* clade, a feature supported by a high bootstrap value (99) which shows the genetic closeness between these two species. In the minor clade of phylogram *J. glandulifera* and *J. multifida* clustered with least distance followed by *J. tanjorensis*. Over all the phylogram based on ITS region showed *J. curcas* and *J. integerrima, J. glandulifera* and *J. multifida* were phylogenetically close followed by *J. gossypifolia, J. integerrima.*

Table 5. Percentage of polymorphism calculated from RAPD data in different species of *Jatropha*.

	J. tanjorensis	*J. glandulifera*	*J. gossypifolia*	*J. multifida*	*J. podagrica*	*J. integerrima*
J. curcas	62.80	71.43	69.95	69.80	69.35	54.49
J. integerrima	71.57	68.73	61.56	69.35	70.08	
J. podagrica	75.12	68.91	69.90	57.02		
J. multifida	76.38	67.99	67.26			
J. gossypifolia	71.57	68.39				
J. glandulifera	76.53					

Table 6. Genetic similarity calculated from RAPD data in different species of *Jatropha*.

	J. tanjorensis	*J. glandulifera*	*J. gossypifolia*	*J. multifida*	*J. podagrica*	*J. integerrima*
J. curcas	0.54	0.45	0.46	0.46	0.49	0.63
J. integerrima	0.44	0.48	0.56	0.47	0.46	
J. podagrica	0.40	0.48	0.47	0.60		
J. multifida	0.38	0.49	0.50			
J. gossypifolia	0.44	0.48				
J. glandulifera	0.38					

Table 7: Percentage of polymorphism calculated from AFLP data in different species of *Jatropha*.

	J. tanjorensis	*J. glandulifera*	*J. gossypifolia*	*J. multifida*	*J. podagrica*	*J. integerrima*
J. curcas	68.19	71.75	70.90	71.57	73.47	62.08
J. integerrima	66.02	71.20	63.95	76.01	76.65	
J. podagrica	73.11	78.41	75.50	55.90		
J. multifida	74.45	75.62	74.37			
J. gossypifolia	71.97	74.75				
J. glandulifera	72.05					

Table 8: Genetic similarity calculated from AFLP data in different species of *Jatropha*.

	J. tanjorensis	*J. glandulifera*	*J. gossypifolia*	*J. multifida*	*J. podagrica*	*J. integerrima*
J. curcas	0.48	0.44	0.45	0.44	0.42	0.55
J. integerrima	0.51	0.45	0.53	0.39	0.38	
J. podagrica	0.42	0.36	0.39	0.61		
J. multifida	0.41	0.39	0.40			
J. gossypifolia	0.44	0.40				
J. glandulifera	0.44					

Table 9: ti/tv ratio in different species of *Jatropha.*

	J. podagrica	*J. integerrima*	*J. glandulifera*	*J. gossypifolia*	*J. tanjorensis*	*J. multifida*
J. curcas	0.12	2.62	0.07	0.57	0.10	0.65
J. multifida	30.50	42.78	4.73	37.51	7.36	
J. tanjorensis	3.20	2.57	3.82	7.48		
J. gossypifolia	29.59	18.10	7.88			
J. glandulifera	3.69	3.03				
J. integerrima	4.33					

Table 10: Intraspecific mean genetic distance obtained by nrDNA ITS sequence analysis in different species of *Jatropha*.

Name of the species	Genetic distance
J. podagrica	0.011
J. curcas	0.005
J. multifeda	0.004
J. tanjorensis	0.009
J. gossypifolia	0.002
J. glandulifera	0.010
J. integerrima	0.004
Mean genetic distance	0.0064

Table 11: Pairwise inter and intraspcific genetic distance (GDS) in different species of *Jatropha* calculated based on nrDNA ITS sequence data.

	J.POD2	J.POD3	J.CUR1	J.CUR2	J.CUR3	J.MUL1	J.MUL2	J.MUL3	J.TAN1	J.TAN2	J.TAN3	J.GOS1	J.GOS2	J.GOS3	J.GLN1	J.GLN2	J.GLN3	J.INT1	J.INT2	J.INT3
J.POD1	0.011																			
J.POD2	0.011	0.011																		
J.POD3	0.215	0.215	0.210																	
J.CUR1	0.214	0.214	0.209	0.002																
J.CUR2	0.218	0.218	0.212	0.003	0.002															
J.CUR3	0.343	0.343	0.342	0.366	0.361	0.366														
J.MUL1	0.337	0.337	0.337	0.360	0.355	0.360	0.003													
J.MUL2	0.343	0.343	0.342	0.366	0.361	0.366	0.000	0.003												
J.MUL3	0.265	0.265	0.262	0.161	0.158	0.161	0.395	0.389	0.395											
J.TAN1	0.265	0.265	0.262	0.161	0.158	0.161	0.395	0.389	0.395	0.000										
J.TAN2	0.279	0.279	0.275	0.172	0.169	0.172	0.410	0.403	0.410	0.007	0.007									
J.TAN3	0.257	0.257	0.255	0.192	0.189	0.192	0.370	0.364	0.370	0.085	0.085	0.092								
J.GOS1	0.257	0.257	0.255	0.192	0.189	0.192	0.370	0.364	0.370	0.085	0.085	0.092	0.000							
J.GOS2	0.254	0.254	0.253	0.195	0.192	0.195	0.367	0.361	0.367	0.087	0.087	0.094	0.002	0.002						
J.GOS3	0.241	0.241	0.243	0.165	0.163	0.165	0.405	0.399	0.405	0.163	0.163	0.171	0.168	0.168	0.170					
J.GLN1	0.250	0.250	0.253	0.174	0.171	0.174	0.419	0.412	0.419	0.159	0.159	0.168	0.165	0.165	0.167	0.005				
J.GLN2	0.250	0.250	0.253	0.174	0.171	0.174	0.419	0.412	0.419	0.159	0.159	0.168	0.165	0.165	0.167	0.005	0.000			
J.GLN3	0.237	0.237	0.232	0.155	0.152	0.155	0.365	0.359	0.365	0.184	0.184	0.190	0.213	0.213	0.216	0.158	0.166	0.166		
J.INT1	0.237	0.237	0.232	0.155	0.152	0.155	0.365	0.359	0.365	0.184	0.184	0.190	0.213	0.213	0.216	0.158	0.166	0.166	0.000	
J.INT2	0.242	0.242	0.237	0.160	0.157	0.160	0.372	0.365	0.372	0.187	0.187	0.192	0.216	0.216	0.219	0.163	0.171	0.171	0.003	0.003

Table 12: nrDNA ITS size and their G+C content in different species of *Jatropha*.

Species Name(germplasm code) [Genbank accession number]	ITS region	ITS1	5.8s	ITS2	G+C content
J. podagrica (J.POD1)[EU881714]	647	278	163	206	58.58
J. podagrica (J.POD2) [EU881715]	647	278	163	206	58.58
J. podagrica (J.POD3) [EU881716]	647	278	163	206	59.04
J. curcas (J.CUR1) [EU700418]	649	278	164	207	60.4
J. curcas (J.CUR2) [EU700419]	649	278	164	207	60.55
J. curcas (J.CUR3) [EU700420]	649	278	164	207	60.55
J. multifeda (J.MUL1) [EU881717]	654	289	163	202	53.82
J. multifeda (J.MUL2) [EU881718]	654	289	163	202	53.82
J. multifeda (J.MUL3) [EU881719]	654	289	163	202	53.82
J. tanjorensis (J.TAN1) [EU881720]	654	282	164	208	62.84
J. tanjorensis (J.TAN2) [EU881721]	653	281	164	208	62.79
J. tanjorensis (J.TAN3) [EU881722]	653	281	164	208	62.63
J. gossypifoila (J.GOS1) [EU881723]	648	280	164	204	63.27
J. gossypifoila (J.GOS2) [EU881724]	648	280	164	204	63.27
J. gossypifoila (J.GOS3) [EU881725]	648	280	164	204	63.43
J. glandulifera (J.GLN1) [EU881726]	646	274	163	209	60.99
J. glandulifera (J.GLN2) [EU881727]	647	275	163	209	60.59
J. glandulifera (J.GLN3) [EU881728]	647	275	163	209	60.59
J. integerrima (J.INT1) [EU881729]	648	278	164	206	62.35
J. integerrima (J.INT2) [EU881730]	649	279	164	206	62.4
J. integerrima (J.INT3) [EU881731]	649	279	164	206	62.71

Table 13: Intraspecific mean genetic distance (GD) obtained by nrDNA ITS sequence analysis of genus *Jatropha*.

Name of the species	Genetic distance
J. podagrica	0.011
J. curcas	0.005
J. multifeda	0.004
J. tanjorensis	0.009
J. gossypifolia	0.002
J. glandulifera	0.010
J. integerrima	0.004
Mean genetic distance	0.0064

Figure 10: RAPD profiles of different species of *Jatropha* with primer OPL 5 (1-7) and IDT E 4. (8-14). Lane 1&8, *J. tanjorensis*; 2&9, *J. curcas*; 3&10, *J. glandulifera*; 4&11, *J. gossypifolia*; 5&12, *J. multifida*; 6&13, *J. podagrica*; 7&14, *J. integerrima*; M- 1kb DNA ladder.

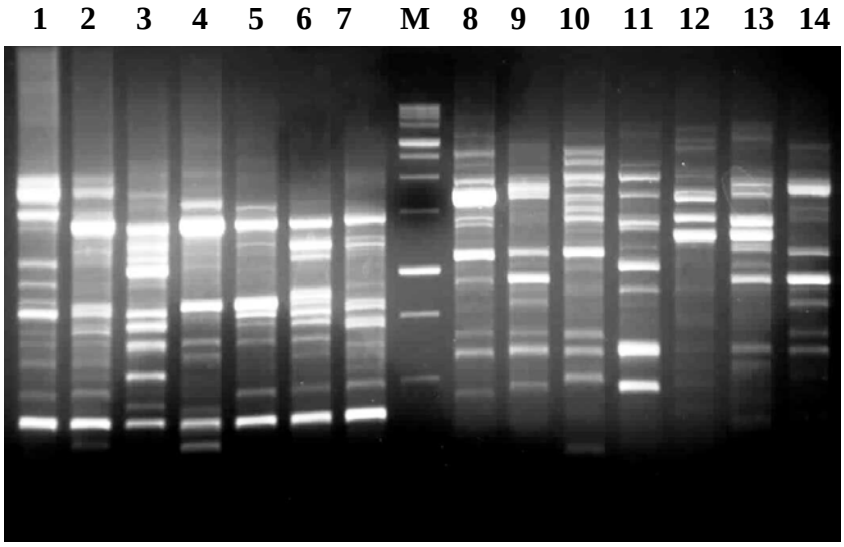


Figure 11: RAPD profiles of different species of *Jatropha* with primer OPL16 (1-7) and OPJ13 (8-14). Lane 1&8, *J. tanjorensis*; 2&9, *J. curcas*; 3&10, *J. glandulifera*; 4&11, *J. gossypifolia*; 5&12, *J. multifida*; 6&13, *J. podagrica*; 7&14, *J. integerrima*; M- 1kb.

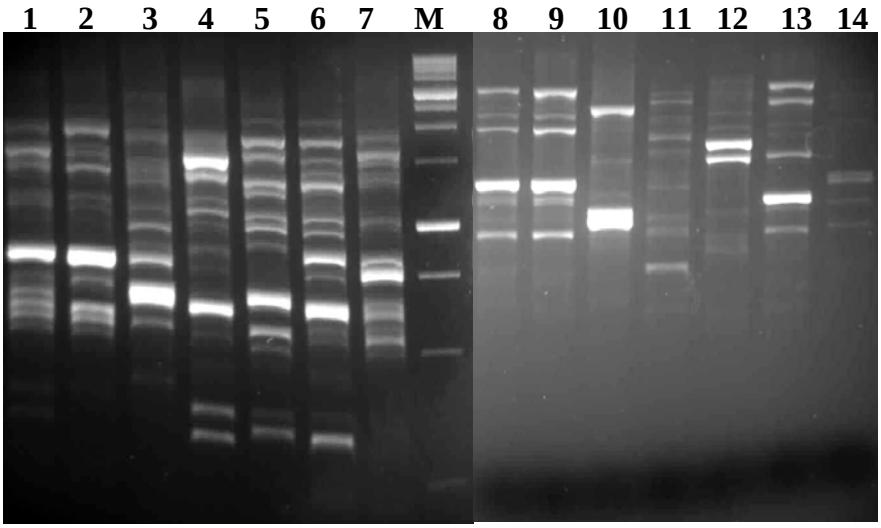


Figure 12: AFLP profiles of different species of *Jatropha* with selective amplification with primers CAT/E-AAG, (1-7); M-CAA/E-ACA (8-14) and M-CAA/E-ACT (8-14). Lane 1,8&15 - *J. curcas*; 2,9&16 - *J. tanjorensis*; 3,10&17, *J. glandulifera*; 4,11&18 - *J. gossypifolia*; 5, 12&19 - *J. multifida*; 6,13&20 - *J. podagrica*; 7,14&21 - *J. integerrima*; M- 1Kb+100bp DNA ladder mix.

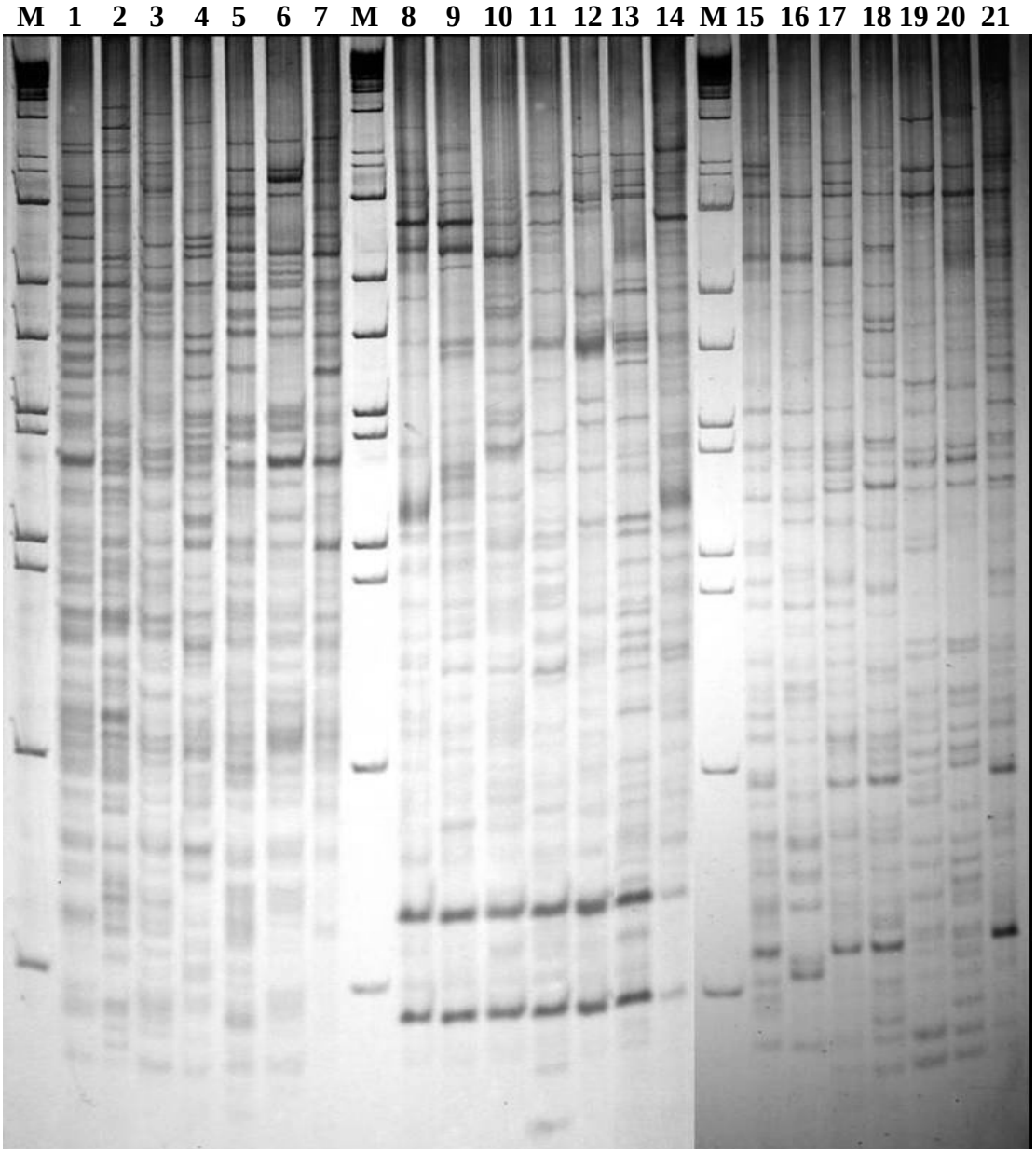


Figure 13: RAPD-based phylogenetic tree for seven species of *Jatropha* constructed according to Jaccard and tree supported with bootstrap analysis values.

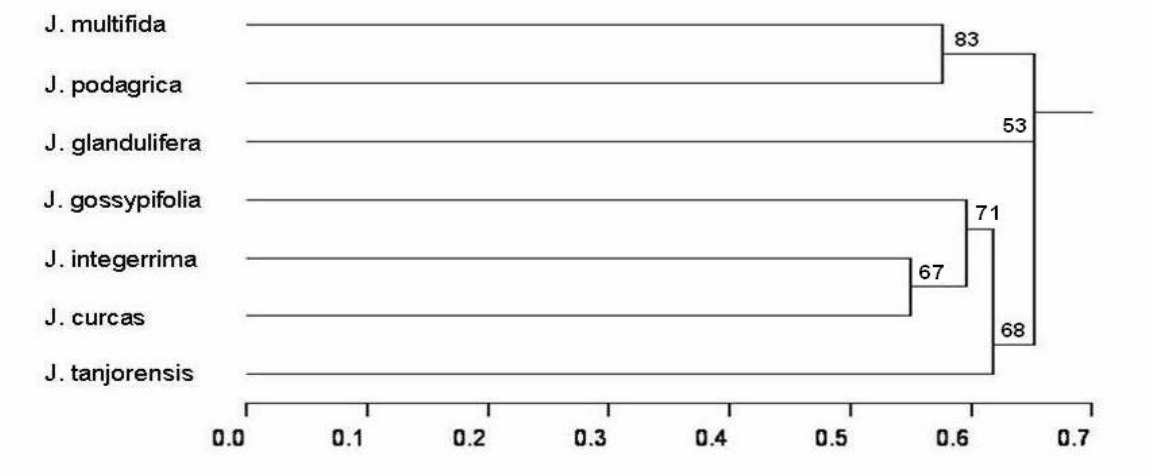


Figure 14: AFLP- based phylogenetic tree for seven species of *Jatropha* constructed according to Jaccard and tree supported with bootstrap analysis values.

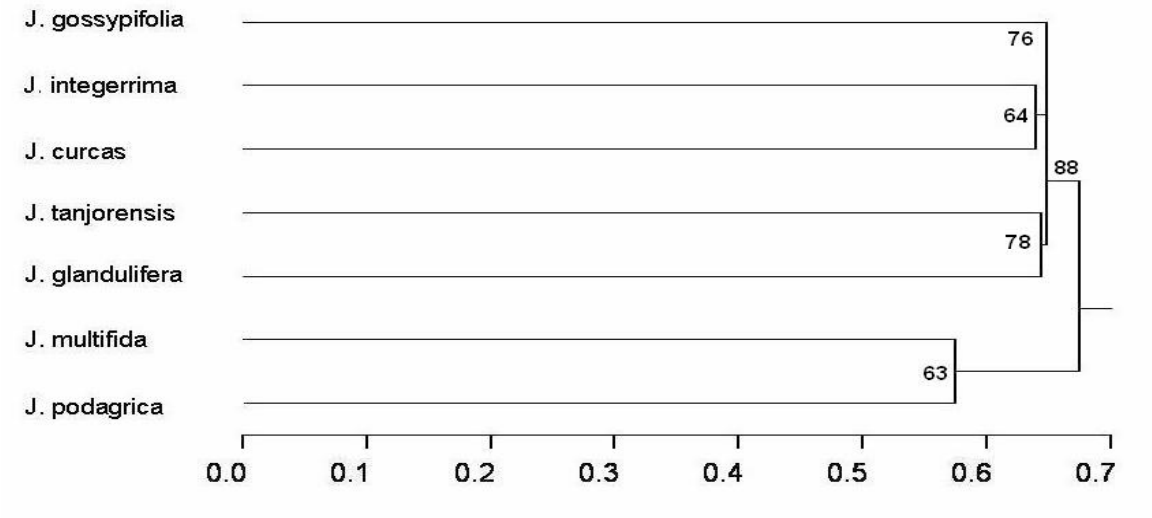


Figure 15: nrDNA ITS region amplified from seven species of *Jatropha* using JCITS-1-F and JCITS-2-R primers; 1-*J. integerrima*, 2- *J. gossypifolia*,3- *J. multifida*, 4- *J. podagrica*, 5- *J. curcas*, 6- *J. glandulifera*, 7- *J. tanjorensis* and M- 1kb DNA ladder.

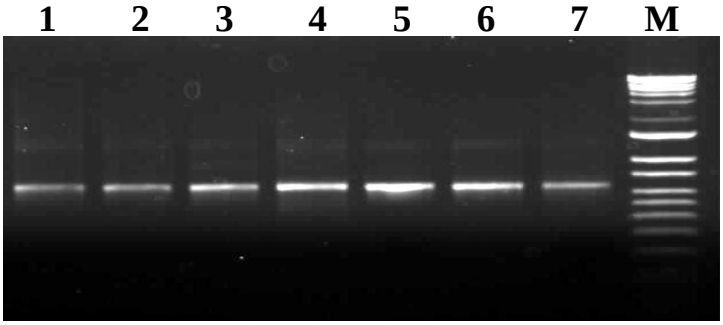
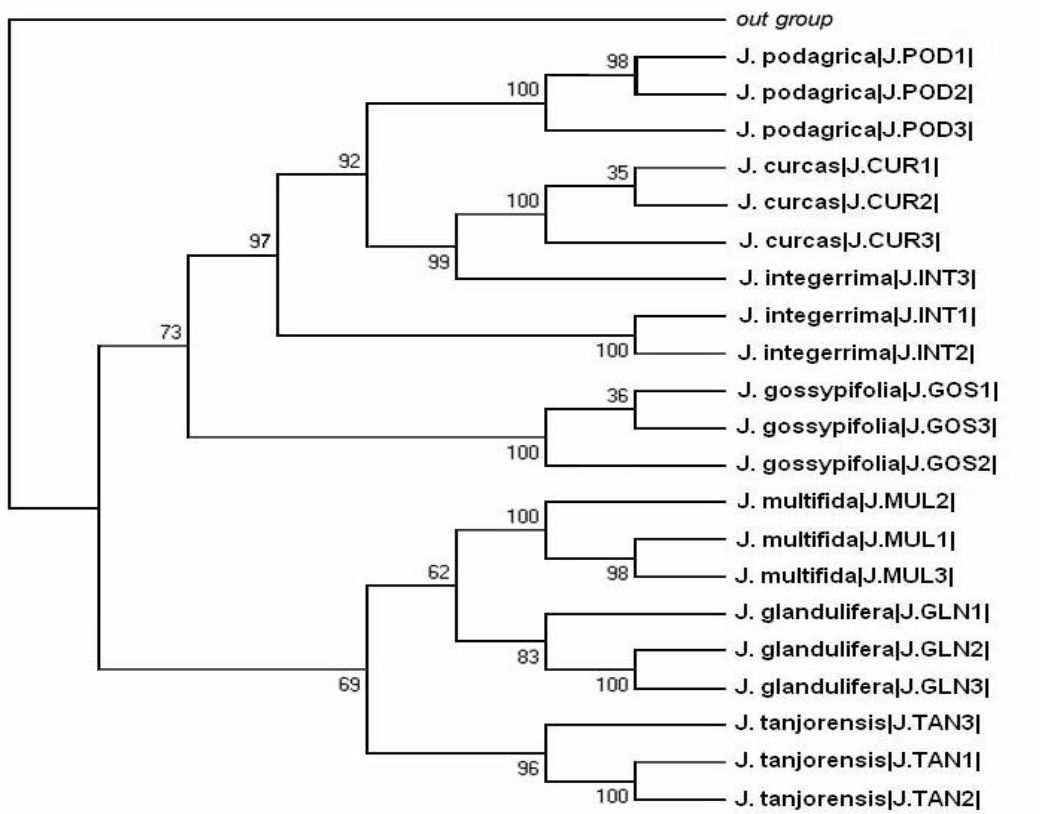


Figure 16: Phylogenetic tree of seven species of *Jatropha* generated with the help of nrDNA ITS sequence using maximum parsimonious method supported with bootstrapping analysis.



Part C:

4.C. MOLECULAR CHARACTERIZATION AND IDENTIFICATION OF MARKERS FOR TOXIC AND NON-TOXIC VARIETIES OF *J. CURCAS* USING RAPD, AFLP AND SSR MARKERS.

In this study a non-toxic Mexican variety was compared with five germplasm of *J. curcas* collected from different geographical regions of India along with a Mexican toxic variety using RAPD and AFLP techniques with an aim to develop markers specific to non-toxic variety. For identification of specific markers and to calculate the percentage of polymorphism and genetic similarity between toxic and non-toxic varieties, the markers present only in non-toxic but not in toxic germplasm and the markers present in all the *J. curcas* toxic germplasm but not in non-toxic variety were taken. In case of RAPD, the primers selected in the previous section (4.C.) were taken for this part of investigation. Out of 52 RAPD primer screened 39 primers resulted with polymorphic markers. In AFLP analysis 64 selective primer combinations were screened and out of them 56 combinations resulted with polymorphic bands between the toxic and non-toxic varieties. Out of total 371 RAPD and 1442 AFLP markers analyzed 56 (15.09%) RAPD and 238 (16.49%) AFLP markers were found to be polymorphic. The percentage of similarity was 84.91 by RAPD and 83.51 by AFLP fingerprinting. Genetic similarity between toxic and non-toxic variety was found to be 0.92 by RAPD and 0.90 by AFLP fingerprinting techniques. The results obtained by both these techniques are comparable and showed the competitive validity of their application in molecular characterization of *J. curcas* varieties.

Out of 52 RAPD primers screened for identification of selective markers between toxic and non-toxic varieties, no polymorphic markers were observed between these two varieties with 13 primers and remaining 39 primers resulted in total 56 polymorphic markers. Primer OPO19 has given highest number (5) of polymorphic markers specific to toxic variety. The lowest number of polymorphic markers (1) was observed with 13

primers IDT E-12, 18, OPJ20, OPL1, OPN3, 8, 12, OPP1, 2, 15, OPQ7, 15 and 20. Use of primer OPQ15 has resulted in one specific marker each to toxic (approximately 810 bp) and non-toxic (approximately 425 bp) variety. Primers IDT E-18, OPL14 resulted in one marker each of approximately 900 and 2100 bp respectively whereas, OPR8 resulted in two specific markers of approximately 1450 and 700 bp (Figure 17 & 18). In case of AFLP the primers E-ACC/M-CAC combination resulted in maximum number of polymorphic markers (3) each for toxic and non-toxic and minimum marker (1) for non-toxic variety was recorded when primers combination E-AAG/M-CTG was used. Prominently amplified AFLP markers specific to toxic and non-toxic variety and their molecular weights was recorded (table 14, Figure 19). In this study 12 SSR markers were analyzed (Table 2) to find out their size polymorphism among non-toxic and toxic varieties. Minor miss-amplifications were minimized by gradient PCR and optimizing the annealing temperature (Table 15). The polymorphic nature of SSR markers were characterized for toxic and non-toxic varieties. The results showed that out of 12 makers tested, 7 (jcms21, jcds24, jcms30, jcps20, jcps21, jcps6, jcps3) found to be size polymorphic. Among the 7 markers, jcms21 showed homozygous allele in the toxic variety. The markers, jcms30, jcps21, jcps3 and jcps20 showed at least one allele of same size repeats common in both nontoxic and toxic varieties. The highest size allele polymorphism was found with jcds24, followed by jcms30 and jcps6 (Figure 20, Table15).

Table 14: AFLP molecular markers specific to non-toxic and toxic varieties of *J. curcas*.

AFLP specific primer set combination	Number of specific markers	Molecular weight of specific marker to non-toxic variety(bp)	Molecular weight of specific marker to toxic variety(bp)
E-AAC/M-CAA	3	882,446	957
E-AAC/M-CAC	1	1054	Nil
E-AAC/M-CAG	2	Nil	559,85
E-AAC/M-CAT	1	Nil	575
E-AAC/M-CTC	2	Nil	584, 259
E-AAC/M-CTG	1	59	Nil
E-AAC/M-CTT	3	678, 98	484
E-ACC/M-CAA	5	529, 275	916, 373, 279
E-ACC/M-CAC	6	1533, 553, 521	1067, 593, 323
E-ACC/M-CAG	5	98	1435, 1247, 911, 567
E-ACC/M-CAT	1	1427	Nil
E-ACC/M-CTA	1	602	Nil
EACC/M-CTC	4	624, 529, 394	516
E-ACC/M-CTG	1	1612	Nil
E-ACC/M-CTT	5	1341, 1047, 623, 496, 172	Nil
E-AGC/M-CAA	2	926, 441,	Nil
E-AGC/M-CAC	5	665, 624, 150, 251	251
E-AGC/M-CTA	2	64	133
E-AGC/M-CTC	3	293, 91	228
E-AGC/M-CTG	2	633	590
E-AGC/M-CTT	3	1110	502, 213

Table 15: Characteristics of 12 microsatellite loci in non-toxic and toxic varieties of *J. curcas*.

Marker GenBack No.	Primer sequence(5'-3')	T_a(ºC)	Repeat motif	Allele size in rangen non-toxic(bp)	Allele size in Toxic (bp)
jcds10 (EU586340)	F:CATCAAATGCTAATGAAAGTACA R:CACACCTAGCAAACTACTTGCA	46.5	$(TG)_6CACGCA(TG)_4$	108/122	108/122
jcds24 (EU586341)	F:GGATATGAAGTTTCATGGGACAAG R:TTCATTGAATGGATGGTTGTAAGG	51.0	$(CA)_5(TA)_8(CA)_4$.....$(TA)_3GA(TA)_4$	204/210	204/216
jcds41 (EU586342)	F: AACACACCATGGGCCACAGGT R:TGCATGTGTGCGGGTTTGATTAC	56.5	$(CA)_6(TA)_2$	102/114	102/114
jcds58 (EU586343)	F:TCCATGAAGTTTGCTGGCAAT R:AGGTCATCTGGTAAAGCCATACC	54.0	$(GT)_4(GA)_5$	104/112	104/112
jcds66 (EU586344)	F:CCTACGAGTGATTGGATAGTTTCTCA R:TCTTCCATCAAGAGTCGTTGGGCA	54.0	$(CT)_2(GT)_3ATTGCA(AT)_4$	216/228	216/228
jcps1 (EU586345)	F:GAGGATATTACAGCATGAATGTG R:AATCAATCAATCTTTGGCAAA	47.5	$(TG)_4$…$(GT)_3$…$(GT)_4$	132/162	132/162
jcps6 (EU586346)	F:CCAGAAGTAGAATTATAAATTAAA R:AGCGGCTCTGACATTATGTAC	44.0	$(AT)_3G(TA)_3$...$(CT)_3$...$(GT)_5CT(GT)_3$	288/305	288/380
jcps9 (EU586347)	F:GTACTTAGATCTCTTGTAACTAACAG R:TATCTCTTGTTCAGAAATGGAT	48.0	$(GT)_3GC(TG)_2A(GT)_3$	140/132	140/132
jcps20 (EU586348)	F:ACAGCAAGTGCACAACAATCTCA R:TACTGCAGATGGATGGCATGA	55.0	$(TG)_{12}(GA)_{22}$	271/260	260/278
jcps21 (EU586349)	F:CCTGCTGACAGGCCATGATT R:TTTCACTGCAGAGGTAGCTTGTATA	54.8	$(CA)_2$...$(CA)_4$	189/200	189/208
jcms21 (EU586350)	F:TAACCTCTTCCTGACA R: ATAGGAAATAAGAGTTCAAA	43.0	$(CA)_7$	81/89	75
jcms30 (EU586351)	F:GGGAAAGAGGCTCTTTGC R:ATGAGTTCACATAAAATCATGCA	48.5	$(GT)_5T(TG)_2$	135/144	144/148

Figure 17: Toxic and non-toxic RAPD markers; a- RAPD profile with primer IDT E-18, b- RAPD profile with primer OPQ15, c- RAPD profile with primer OPO19;1-6:Toxic and 7-non-toxic varieties of *J. curcas;* M: 1kb marker.

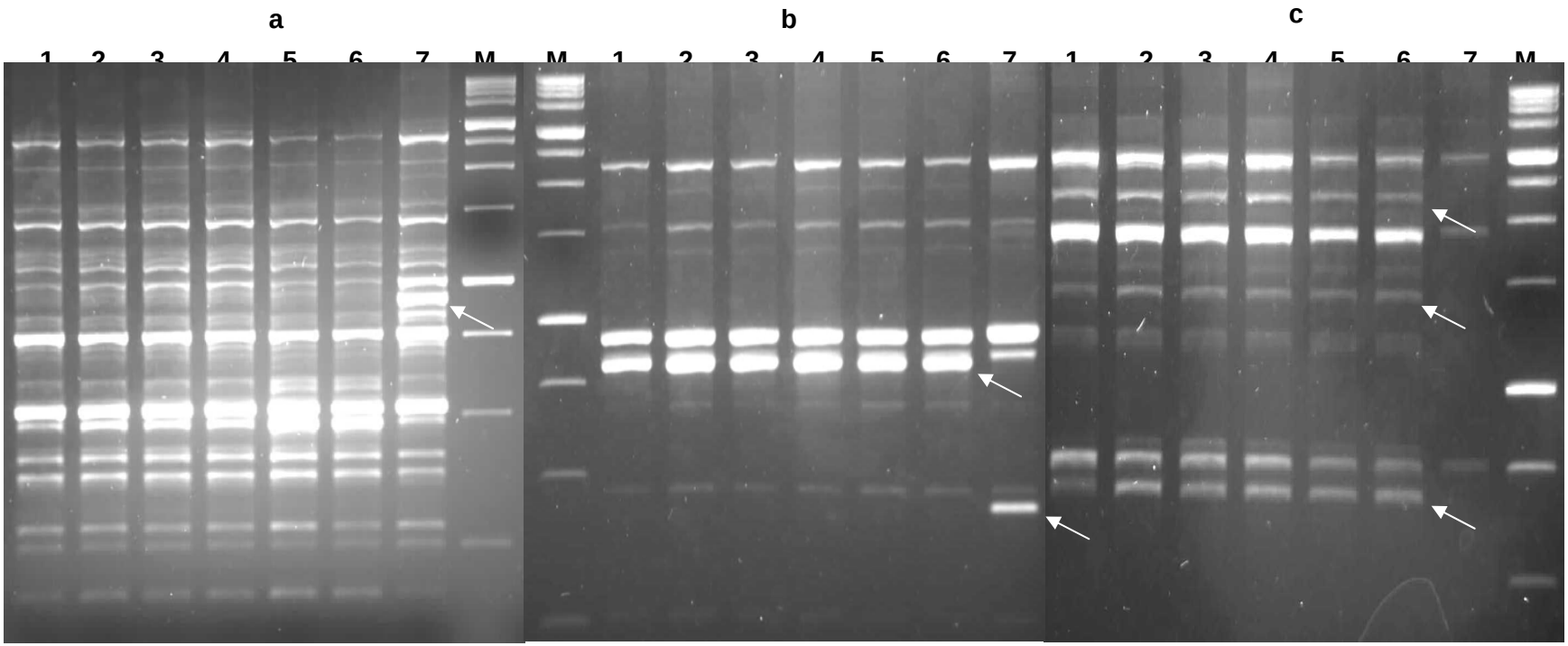


Figure 18: Toxic and non-toxic RAPD markers; a- RAPD profile with primer OPL14, b- RAPD profile with primer OPR8; 1-6: Toxic and 7-non toxic varieties of *J. curcas*; M: 1kb Marker.

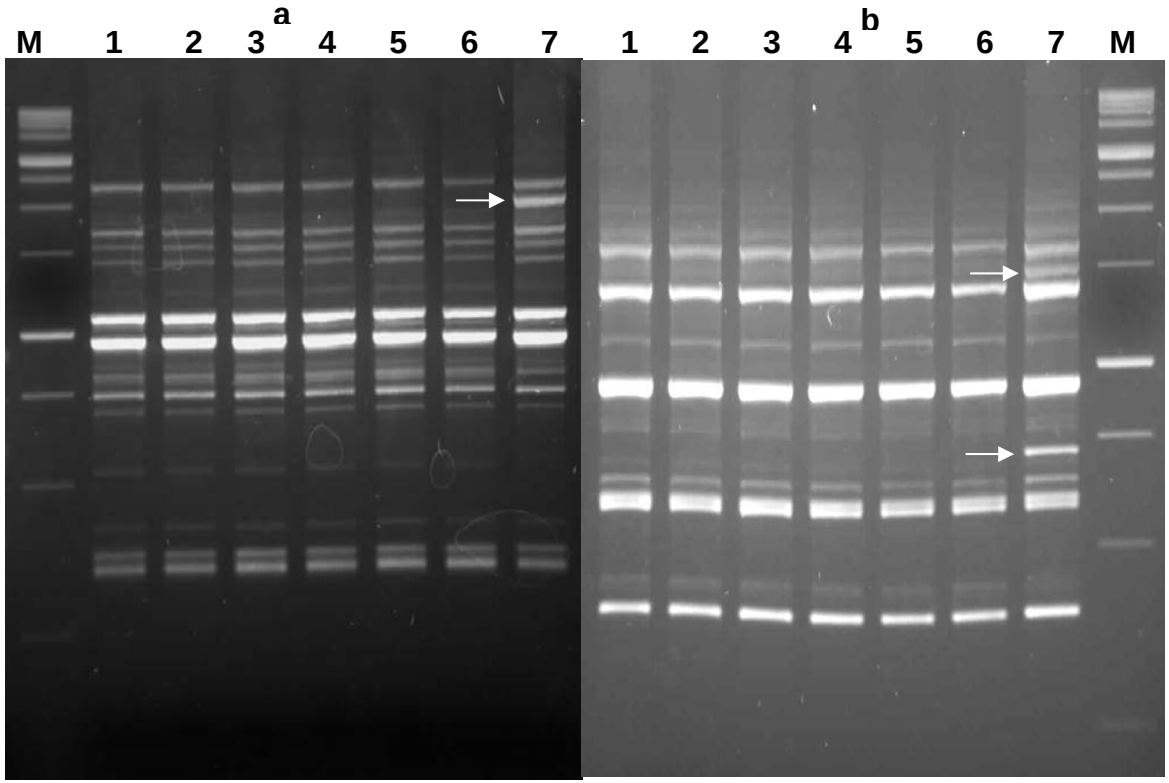


Figure 19:

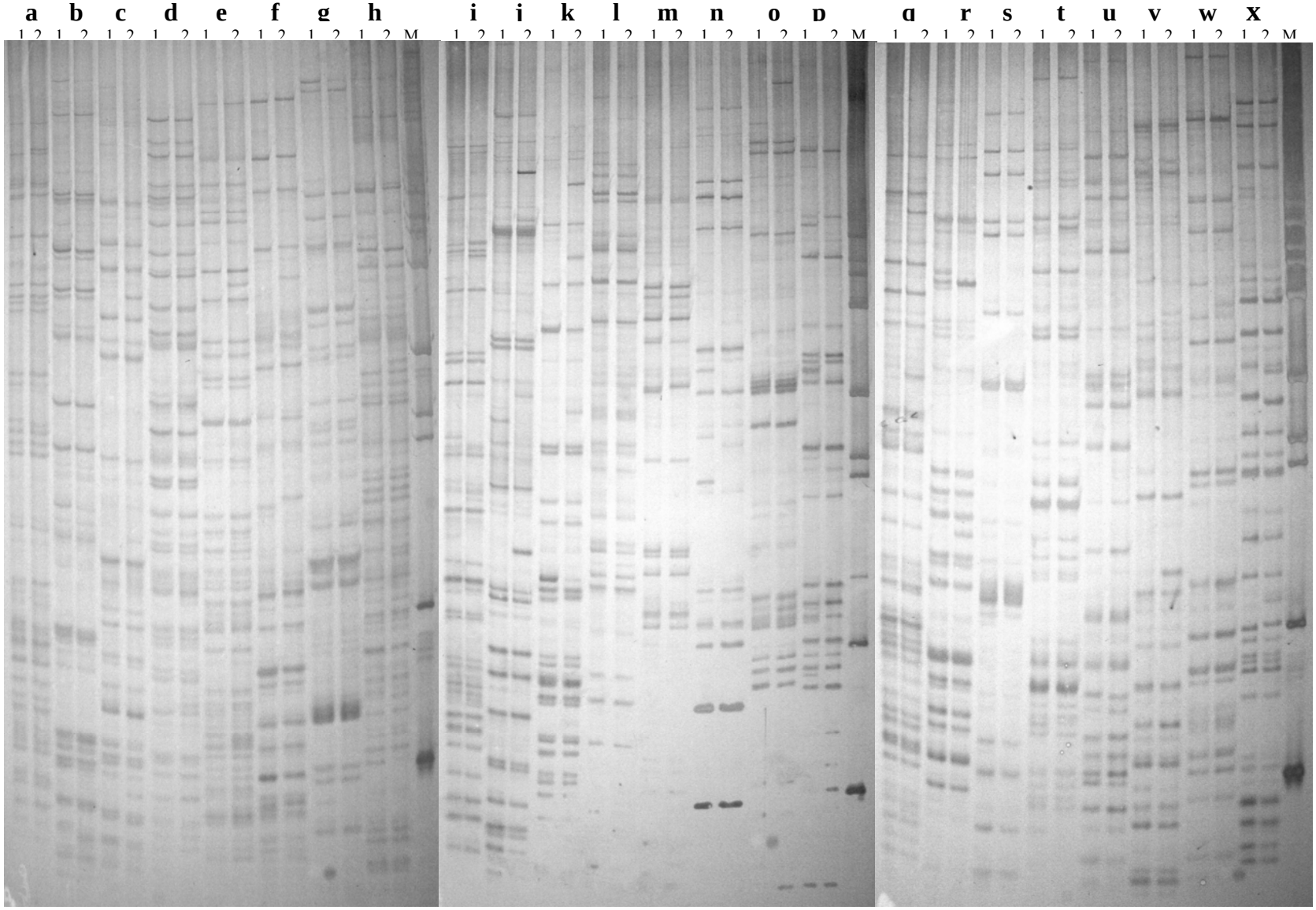
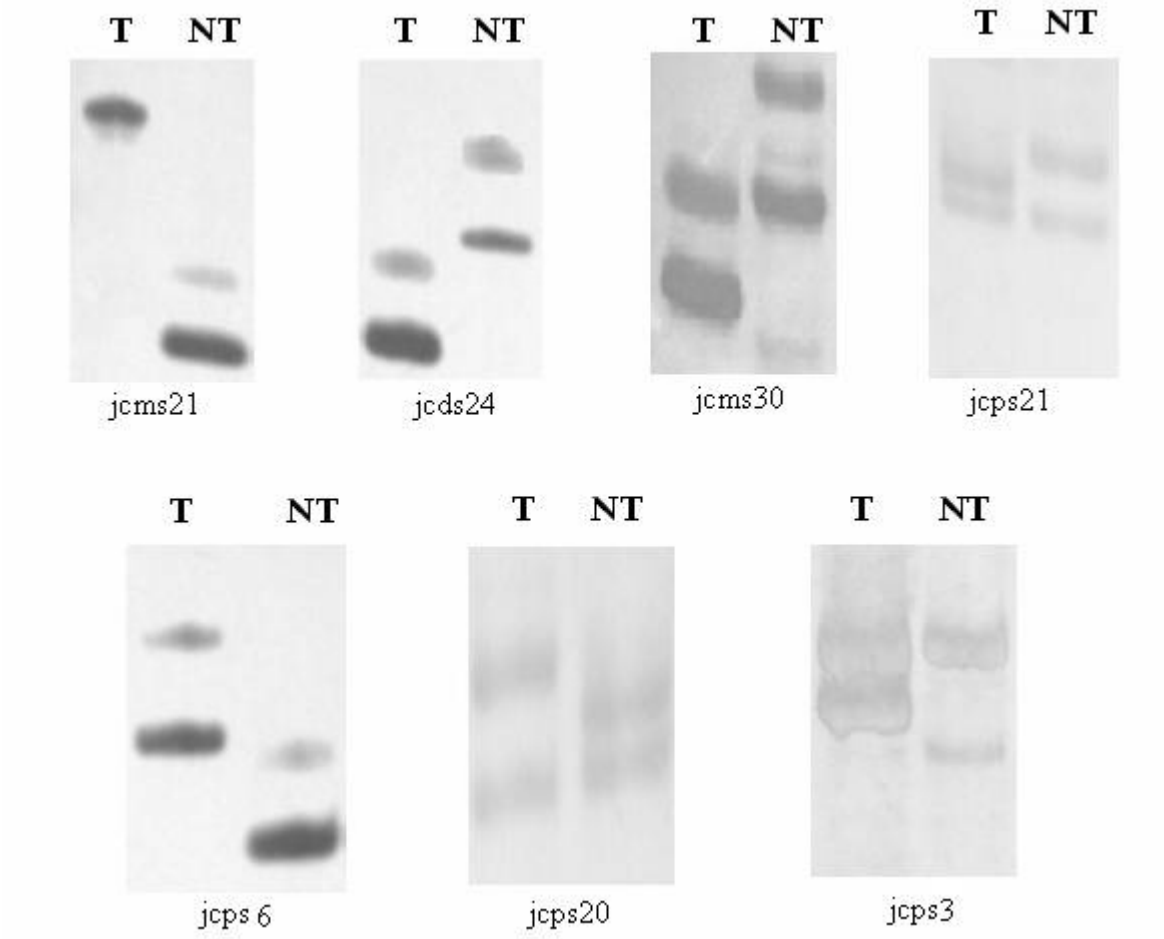

a b c d e f g h i i k l m n o p q r s t u v w x
1 2 1 2 1 2 1 2 1 2 1 2 1 2 1 2 M 1 2 1 2 1 2 1 2 1 2 1 2 1 2 1 2 M 1 2 1 2 1 2 1 2 1 2 1 2 1 2 1 2 M

Figure 19: Toxic and non-toxic AFLP markers; 1-non-toxic variety of *J. curcas*, 2-toxic varieties of *J. curcas*. **a-x** Selective amplification with AFLP primers (**a**-E-AAC/M-CAA; **b**- E-AAC/M-CAC; **c**- E- AAC/M-CAG; **d**- E- AAC/M-CAT; **e**- E- AAC/M-CTA; **f**- E- AAC/M-CTC; **g**- E- AAC/M-CTG; **h**- E- AAC/M-CTT; **i**- E-ACC/M-CAA; **j**- E- ACC/M-CAC; **k**- E ACC/M-CAG; **l**- E- ACC/M-CAT; **m**- E- ACC/M-CTA; **n**- E- ACC/M-CTC; **o**- E- ACC/M-CTG; **p**- E- ACC/M-CTT; **q**- E-AGC/M-CAA; **r**- E- AGC/M-CAC; **s**- E- AGC/M-CAG; **t**- E- AGC/M-CAT; **u**- E- AGC/M-CTA; **v**- E- AGC/M-CTC; **w**- E- AGC/M-CTG; **x**- E- AGC/M-CTT).

Figure 20: Size polymorphism SSRs in toxic (T) and non-toxic (NT) varieties of *J. curcas* (jcms21, jcds24, jdms30, jcps21, jcps6, jcps20 and jcps3).



Part D:

4.D. PHYLOGEOGRAPHY AND MOLECULAR DIVERSITY ANALYSIS OF *J. CURCAS* AND DISPERSION ROUTE REVELED FROM RAPD, AFLP AND nrDNA ITS ANALYSIS.

4.D.1 RAPD analysis:

The primers selected in the section (4.C.) were also used for this part of investigation. Out of 52 RAPD primer screened 27 primers responded with band clarity and reproducibility at each instant of repetition were taken for the final analysis. Totally 395 markers were obtained and 304 markers were found to be polymorphic. Overall PP was found to be 76.96. The maximum PP (55.92) was found between the germplasm JCE51 and JCE56. The minimum PP (0.27) was found between the germplasm JCI01 and JCI02. Among the germplasm collected in India showed the overall PP to be 55.30 and the PP of Mexican germplasm showed 64.49. The PP of the germplasm excluding the Indian germplasm was found to be 72.78. The mean PP among 42 germplasm was found to be 26.47. The mean PP with the Indian germplasm was found to be 14.66. The mean PP between the germplasm excluding Indian germplasm was found to be 35.86. The mean PP of Mexican germplasm observed was 33.18. Among all maximum genetic similarity was found between the Indian germplasm (1.00). Within the Mexican germplasm the highest GS was found to be 0.91 between JCE51 and JCE58. Mean genetic similarity was found to be 0.80 among Mexican germplasm. The highest genetic similarity between the germplasm excluding Indian germplasm was found to be 0.88 between JCE59 and JCE52. The mean genetic similarity among the germplasm excluding the Indian germplasm found to be 0.78. Highest pair wise GS (0.99) was observed in between JCI01 and JCI02. Lowest pair wise GS 0.61 found between JCE51 and JCE56.

4.D.2. AFLP analysis:

Using 18 combinations of AFLP selective primers which resulted in superior quality fingerprints with above 30 markers were taken for the present analysis. Out of 911 markers generated, 667 makers were found to be polymorphic. On an average each primer

has given 50.6 markers with 31.6 polymorphic markers. Overall PP among 42 germplasm was found to be 73.22. The maximum PP (50.18) was found between the germplasm JCI26 and JCE50. The minimum PP (1.28) was found between the germplasm JCI02 and JCE52. Among the germplasm collected in India showed overall PP 60.95 and the PP of Central America/Mexico germplasm was 55.89. The PP of the germplasm excluding the Indian germplasm was found to be 66.42. The mean PP among 42 germplasm was found to be 31.46. The mean PP within the Indian germplasm observed was 20.57. The mean PP between the germplasm excluding Indian germplasm was 38.87. The mean PP of Mexican germplasm was found to be 39.95. Among all maximum GS (1.00) was observed between JCI02 and JCE52. Within the Mexican germplasm the highest GS was observed between 0.92 between JCE57 and JCE58, and lowest 0.37 observed between JCE61 and JCE62. Mean GS obtained among Central American/Mexican germplasm was 0.74. The highest GS among the germplasm excluding Indian germplasm was 0.92, between JCE58 and JCE59. The mean GS among the germplasm excluding the Indian germplasm was 0.75. Lowest GS (0.67) in among 42 germplasm is in between JCE26 and JCI50. Highest GS (0.99) found between JCI02 and JCI52.

4.D.3. ITS sequence analysis:

The germplasm of *J. curcas* taken for the RAPD and AFLP analysis were also taken for nrDNA ITS sequence analysis. The nrDNA ITS region sequences ranged in length from 646 to 657 bp in germplasm *J. curcas*. The size variation was observed nearly same in both ITS1 (range 278-280, mean 278.31±0.71) and ITS2 (range 204-208, mean 206.71±0.74). 5.8S nrDNA sequence has shown standard length 164 except the germplasm JCI01 and JCI26 (163) with a single bp deletion. A relatively broad range of G+C content was observed and the range was found to be 59.20% to 64.45%. The G+C content in Indian germplasm except JCI19 (64.14%), JCI28 (64.25%), JCI01 (59.20%) and JCI26 (59.20%) all other germplasm showed almost similar G+C content (60. 48±0.14). The sequence alignment shows the minimum value of 91 maximum of 100. Overall deletions/insertions are lower than the transitions/tranversions. Only one deletion was observed in the 5.8S nrDNA (position 383 bp) in the germplasm JCI01 and JCI26. Altogether 50 mutations were observed in ITS1, 9 in 5.8S nrDNA, 15 in ITS2. Maximum

diversity was observed in ITS1 followed by ITS2 and least was observed in 5.8S nrDNA. Highest genetic distance (0.096) calculated based on nrDNA ITS region was found between JCI01 and JCE52, JCI26 and JCE52; and followed by JCI01and JCE55, JCI25 and JCE55(0.093). The mean genetic distance was found to be 0.03.

4.D.4. Phylogenetic analysis:

Phylogenetic trees were constructed using binary data generated by RAPD and AFLP according to maximum parsimonious method followed by bootstrapping across the loci. The resulted RAPD and AFLP showed major correlation in clustering of the germplasm, but the relationships were best resolved in AFLP than RAPD. Both the phylograms showed three distinct clusters where most of the samples collected near by geographical areas were grouped however the intermixing was observed. The Indian germplasm did not cluster according to the geographical region of collection. In both the phylograms the exotic germplasm collected out of India clustered according to the geographical region and the major correlations were observed (Cluster 1, in RAPD; cluster 3, in AFLP) (Table 16, Figure 21 & 22). Cluster 2 in RAPD was formed by JCE51, 56, 63, 50, 53, 20, 59, 60, 62, 61, 55, 57, 58, JCI20 and AFLP these exotic germplasm grouped in cluster 3 and cluster includes JCE30, 63, 50, 56, 58, 57, 55, 62, 59, 60, 61, 54, JCI20. In both the clusters the Indian germplasm JCI20 grouped along with the exotic germplasm. In the RAPD and AFLP the cluster2 in both the phylograms was formed with almost similar germplasm JCI1, 2, 3, 4, 5, 6, 7, 8, 9, 11, 12, 13, 14 except JCI10 where it was grouped along with them in RAPD, was grouped in cluster 1 in AFLP which is with the group JCE 52, JCI26, 24, 25, 27, 28, 22, 23, 21, 18, 19, 17, 16, 10. In case of RAPD cluster 3 was formed with JCI16, 21, 18, 19, 20, 23, 24, 25, 27, 28, 26, 17, and JCE31. In comparision phylogenetic trees of both multilocus DNA fingerprinting anlaysis (RAPD and AFLP) showed reasonable corulation.

The phylogram obtained with the nrDNA ITS sequence showed a total of 3 clusters two major and one minor cluster (Figure 23). Large cluster was formed with 22 Indian germplasm (JCI01, 02, 03, 04, 05, 06, 07, 08, 09, 10, 13, 14, 15, 16, 17, 18, 20, 21, 25, 25, 26 and 27) and the second largest major cluster was formed by group of 16 germplasm, 14 of exotic (JCE50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62 and 63) and

2 Indian (JCI19, 28). A single minor cluster was formed with the 4 Indian germplasm (JCI11, 22, 23, 24). In comparison with phylograms generated based on of multilocus DNA finger printing the phylogram of nrDNA ITS sequence was atypical with Indian germplasm. Whereas, in fingerprinting data phylogram showed similar corulation with exotic germplasm. The Phylograms of RAPD and AFLP showed two major clusters in Indian germplasm but the phylogram of nrDNA ITS showed only one major cluster and a single minor cluster.

Figure 21: Phylogenetic tree generated based on maximum parsimonious method using RAPD fingerprinting analysis data from 42 germplasm of *J. curcas* collected from different geographical areas combined with bootstrapping analysis across loci..

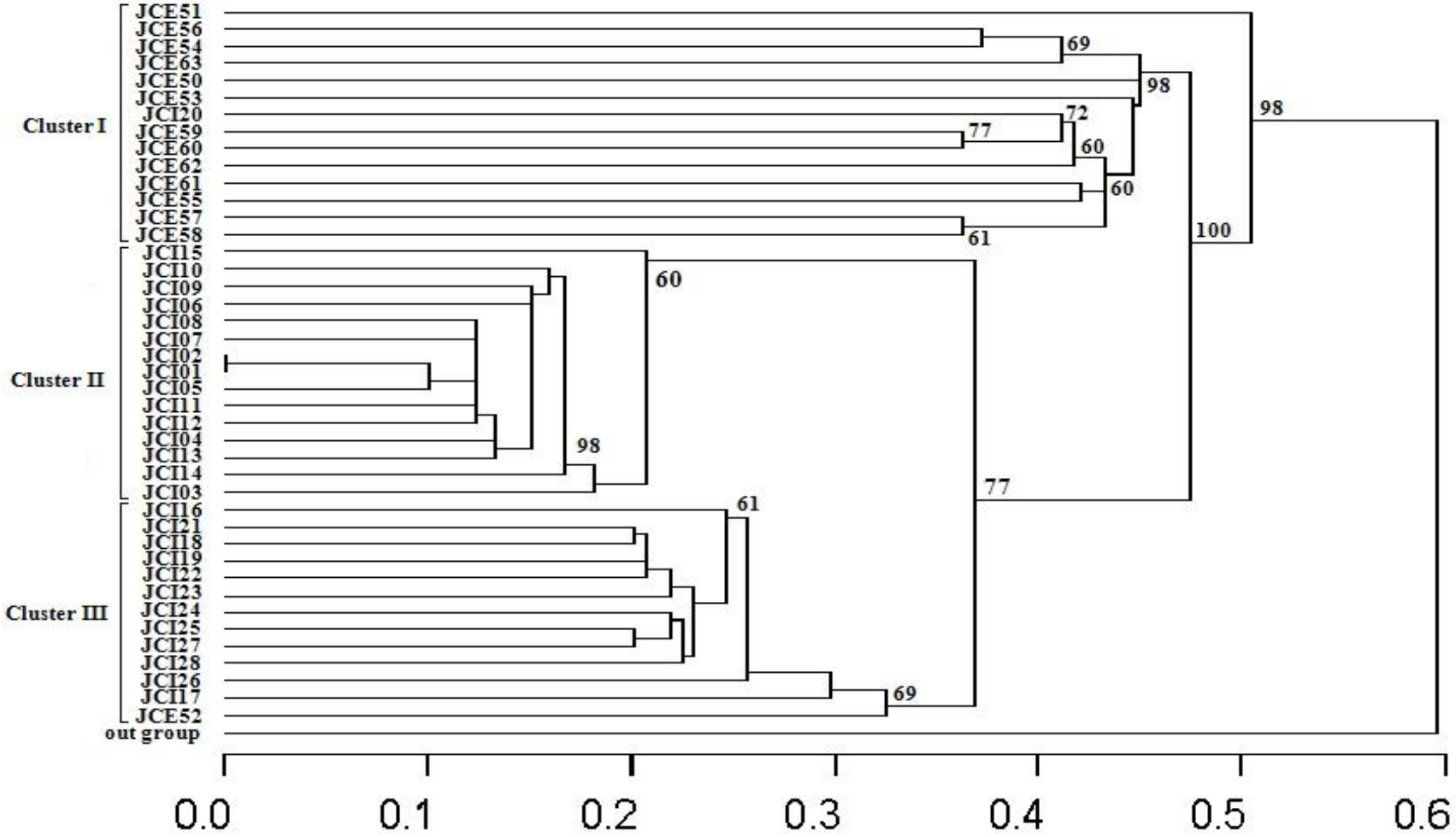


Figure 22: Phylogenetic tree generated based on maximum parsimonious method using AFLP fingerprinting analysis data from 42 germplasm of *J. curcas* collected from different geographical areas combined with bootstrapping analysis across loci.

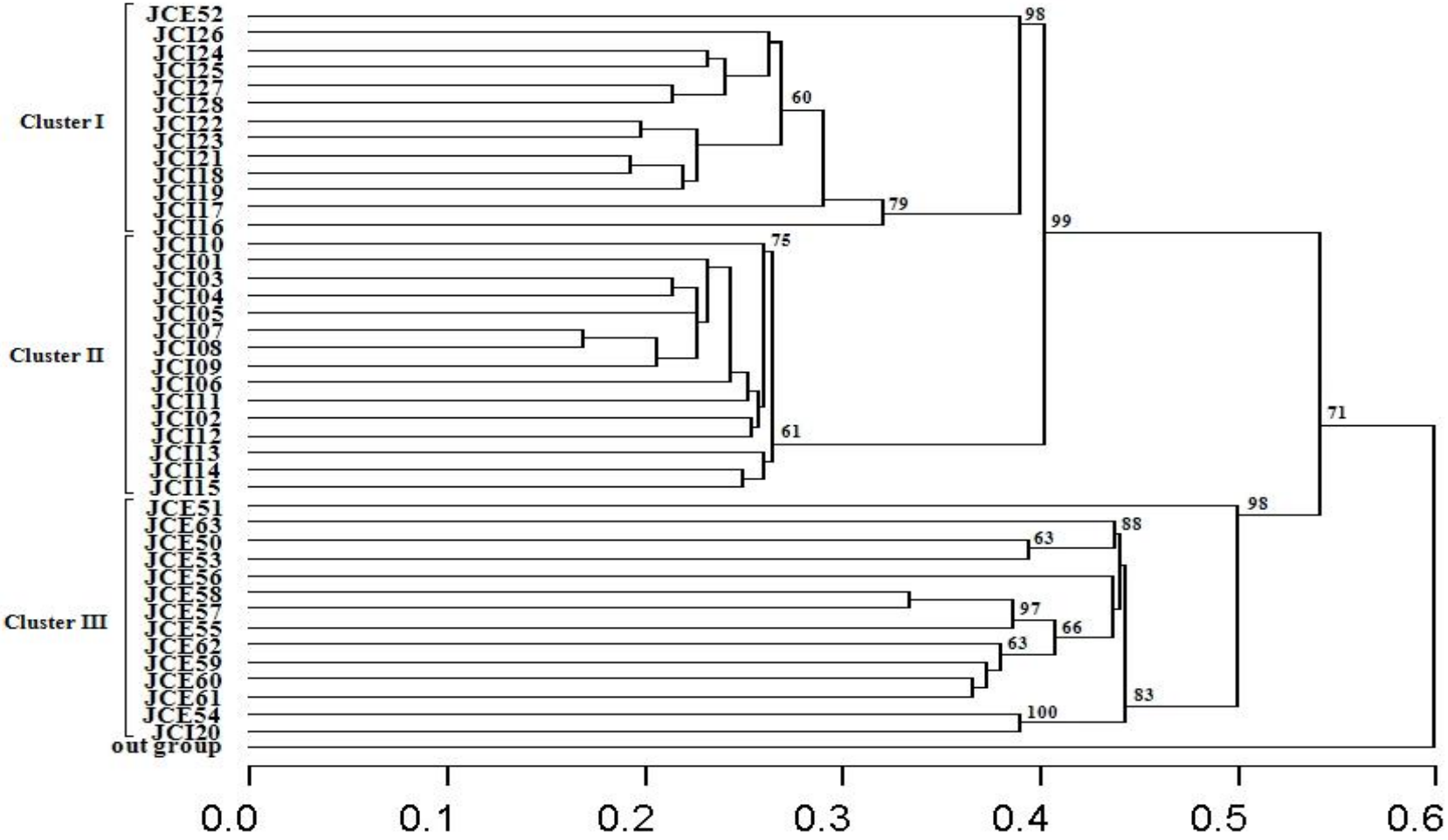


Figure 23: Phylogenetic tree generated based on maximum parsimonious method using nrDNA ITS sequence analysis from fingerprinting analysis data from 42 germplasm of *J. curcas* collected from different geographical areas combined with bootstrapping analysis across loci.

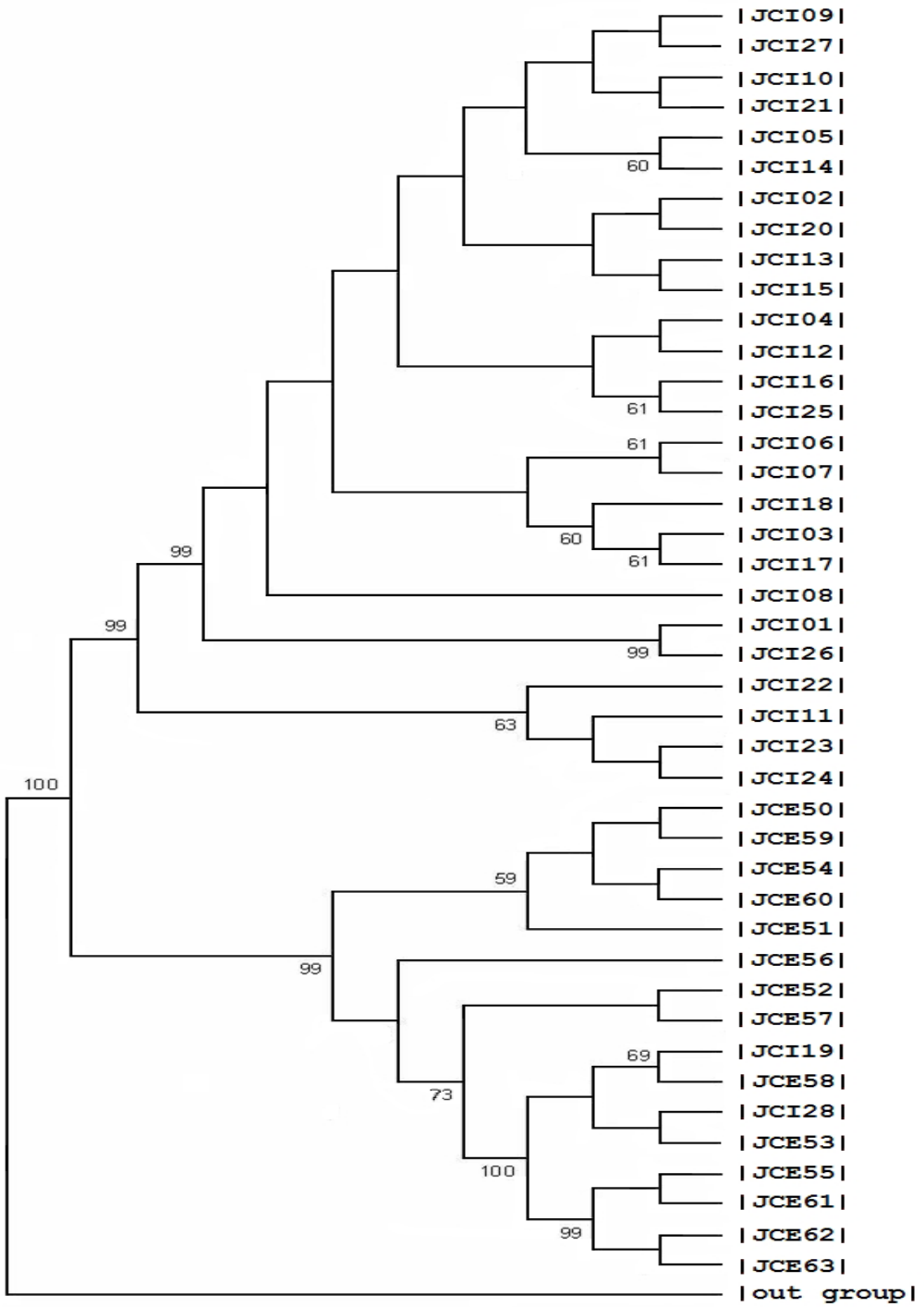


Table 16: Germplasm and its geographical place of collection, genbank accession number of nrDNA ITS region.

Germplasm code	GenBank Acession No.	Country of sample belongs
JCI01	EU700417	India
JCI02	EU700418	India
JCI03	EU700419	India
JCI04	EU700420	India
JCI05	EU700421	India
JCI06	EU700422	India
JCI07	EU700423	India
JCI08	EU700424	India
JCI09	EU700425	India
JCI10	EU700426	India
JCI11	EU700427	India
JCI12	EU700428	India
JCI13	EU700429	India
JCI14	EU700430	India
JCI15	EU700431	India
JCI16	EU700432	India
JCI17	EU700433	India
JCI18	EU700434	India
JCI19	EU700435	India
JCI20	EU700436	India
JCI21	EU700437	India
JCI22	EU700438	India
JCI23	EU700439	India
JCI24	EU700440	India
JCI25	EU700441	India
JCI26	EU700442	India
JCI27	EU700443	India
JCI28	EU700444	India
JCE50	EU700445	Madagascar
JCE51	EU700446	Africa
JCE52	EU700447	Cape Verde
JCE53	EU700448	Madagascar
JCE54	EU700449	Spain
JCE55	EU700450	Mexico
JCE56	EU700451	Mexico
JCE57	EU700452	Mexico
JCE58	EU700453	Cape Verde
JCE59	EU700454	Cape Verde
JCE60	EU700455	Cape Verde
JCE61	EU700456	Mexico
JCE62	EU700457	Mexico
JCE63	EU700458	Mexico

Part E:

4.E. ISOLATION AND CHARACTERIZATION OF NOVEL MICROSATELLITES FROM *J. CURCAS* AND THEIR CROSS SPECIES AMPLIFICATION.

Out of 38 sequenced clones obtained by optimized FIASCO technique as described in materials and methods (3.14) (Figure 24, 25, 26, 27, 28 &29), 21 (62%) clones were found to have microsatellite repeats. However, all the sequences that have microsatellite markers were found not suitable for the primer designing due to lack of sufficient flanking region either in 3' end or 5' end needed for primer designing. Only 14 sequences having proper flanking regions were taken for primer designing and out of 14, 12 sequences amplified properly with expected size of amplification. Among these 12 microsatellite markers, one was found to be perfect repeat and rest were imperfect repeats (Table 18). With the designed forward and reverse primers miss-amplification was found to be high and this may be due to the conserved nature of the flanking regions of the microsatellite distributed in the genome. The miss-amplifications was minimized by conducting the gradient PCR experiments for each microsatellite by taking ±8 °C and the optimum annealing temperature without miss-amplification for each marker was determined (Table 19).

Cross species amplification in 6 species (*J. tanjorensis, J. gossypifolia, J. multifida, J. integerrima, J. glandulifera,* and *J. podagrica*) was checked for all the 12 microsatellite and all the markers showed cross species amplification. Markers jcds10, jcds58, jcps1, jcps21 and jcms21 showed amplification in all the species checked (Figure 30). The minimum number of cross species amplification (i.e. with 3 species) was found with the markers jcds24 and jcds66. Out of all the species *J. tanjorensis* gave amplification with all the markers (i.e. 12 markers) which were isolated from the *J. curcas*. *J. integerrima* amplified next highest to *J. tanjorensis*. *J. gossypifolia, J. multifida, J. glandulifera, and J. podagrica* amplified with the least number of markers (i.e. 9 markers) (Table 17).

The isolated markers were characterized in *J. curcas* with 32 germplasm collected from a natural population in Junagarh Gir forest region and 21 representative diverged germplasm collected randomly in India. Among the populations collected

from Junagarh Gir forest region showed highest number of alleles (11) by marker jcds24 and lowest (2) by jcps1 and jcms30. The observed and expected heterozygosities ranged from 0.94 to 0.54 and from 0.95 to 0.56, respectively. Tests for Hardy–Weinberg equilibrium (HWE) showed that loci jcds58, jcds66, jcps1, jcps6 and jcms30 were not in HWE equilibrium. Whereas, the representative of 21 Indian germplasm were shown highest number of alleles (16) were found with marker jcds24 and lowest (2) with jcms30 and mean observed and expected heterozygosity were found to be 0.78 ± 0.10 and 0.72 ± 0.13, respectively. Tests for Hardy–Weinberg equilibrium (HWE) and pairwise linkage disequilibrium test showed that, except jcds24 all other loci deviated significantly from HWE (Table 19, Figure 31).

Table 17: Cross species amplification of isolated markers in different species of genus *Jatropha*.

Markers	*J. curcas*	*J. tanjorensis*	*J. glandulifera*	*J. gossypifolia*	*J. multifida*	*J. podagrica*	*J. integerrima*
Jcds10	+	+	+	+	+	+	+
Jcds24	+	+	+	-	-	-	-
Jcds41	+	+	+	+	-	-	+
Jcds58	+	+	+	+	+	+	+
Jcds66	+	+	-	-	-	+	-
Jcps1	+	+	+	+	+	+	+
Jcps6	+	-	+	+	+	+	+
Jcps9	+	+	-	+	+	+	+
Jcps20	+	+	-	+	+	+	+
Jcps21	+	+	+	+	+	+	+
jcms21	+	+	+	+	+	+	+
jcms30	+	+	-	-	+	-	+

Table18: Characteristics of 12 microsatellite loci isolated from *J. curcas*.

Marker (GenBack Acc. No.)	Primer sequence (5'-3')	Repeat motif	Allele size range(bps)
jcds10 (EU586340)	F:CATCAAATGCTAATGAAAGTACA R:CACACCTAGCAAACTACTTGCA	$(TG)_6CACGCA(TG)_4$	108-122
jcds24 (EU586341)	F:GGATATGAAGTTTCATGGGACAAG R:TTCATTGAATGGATGGTTGTAAGG	$(CA)_5(TA)_8(CA)_4.....(TA)_3GA(TA)_4$	204-246
jcds41 (EU586342)	F: AACACACCATGGGCCACAGGT R:TGCATGTGTGCGGGTTTGATTAC	$(CA)_6(TA)_2$	102-114
jcds58 (EU586343)	F:TCCATGAAGTTTGCTGGCAAT R:AGGTCATCTGGTAAAGCCATACC	$(GT)_4(GA)_5$	104-112
jcds66 (EU586344)	F:CCTACGAGTGATTGGATAGTTTCTCA R:TCTTCCATCAAGAGTCGTTGGGCA	$(CT)_2(GT)_3ATTGCA(AT)_4$	216-228
jcps1 (EU586345)	F:GAGGATATTACAGCATGAATGTG R:AATCAATCAATCTTTGGCAAA	$(TG)_4…(GT)_3…(GT)_4$	132-162
jcps6 (EU586346)	F:CCAGAAGTAGAATTATAAATTAAA R:AGCGGCTCTGACATTATGTAC	$(AT)_3G(TA)_3...(CT)_3...(GT)_5CT(GT)_3$	288-305
jcps9 (EU586347)	F:GTACTTAGATCTCTTGTAACTAACAG R:TATCTCTTGTTCAGAAATGGAT	$(GT)_3GC(TG)_2A(GT)_3$	140-165
jcps20 (EU586348)	F:ACAGCAAGTGCACAACAATCTCA R:TACTGCAGATGGATGGCATGA	$(TG)_{12}(GA)_{22}$	224-260
jcps21 (EU586349)	F:CCTGCTGACAGGCCATGATT R:TTTCACTGCAGAGGTAGCTTGTATA	$(CA)_2...(CA)_4$	189-200
jcms21 (EU586350)	F:TAACCTCTTCCTGACA R: ATAGGAAATAAGAGTTCAAA	$(CA)_7$	75-88
jcms30 (EU586351)	F:GGGAAAGAGGCTCTTTGC R:ATGAGTTCACATAAAATCATGCA	$(GT)_5T(TG)_2$	135-144

Table 19: Characterization of 12 microsatellites in a local population and representatives of total Indian population of *J. curcas*.

Marker GenBack No.	T_a (°C)	H_O	H_e	P-value	A	T_a (°C)	H_O	H_e	P-value	A
	Indian representative germplasm					Germplasm from Gir forest region				
jcds10 (EU586340)	46.5	0.86	0.96	< 0.0001	6	46.5	0.86	0.81	0.63	4
jcds24 (EU586341)	51.0	0.95	0.95	0.899	16	51.0	0.94	0.95	0.899	11
jcds41 (EU586342)	56.5	0.81	0.75	0.009	5	56.5	0.81	0.75	0.096	5
jcds58 (EU586343)	54.0	0.86	0.67	0.004	4	54.0	0.86	0.67	< 0.0001	3
jcds66 (EU586344)	54.0	0.81	0.75	0.002	5	54.0	0.81	0.75	0.031	3
jcps1 (EU586345)	47.5	0.80	0.75	0.003	6	47.5	0.84	0.75	< 0.0001	2
jcps6 (EU586346)	44.0	0.80	0.76	< 0.0001	4	44.0	0.80	0.76	0.016	4
jcps9 (EU586347)	48.0	0.74	0.56	0.196	4	48.0	0.54	0.56	0.196	4
jcps20 (EU586348)	55.0	0.90	0.89	0.000	15	55.0	0.89	0.92	0.223	9
jcps21 (EU586349)	54.8	0.52	0.61	0.018	3	54.8	0.57	0.61	0.180	3
jcms21 (EU586350)	43.0	0.53	0.61	0.017	3	43.0	0.77	0.71	0.091	3
jcms30 (EU586351)	48.5	0.85	0.76	< 0.0001	2	48.5	0.75	0.87	< 0.0001	2

T_a (ºC), Annealing temperature; A, number of alleles; H_O, observed heterozygosity; H_e, expected heterozygosity.

Figure 24: Restriction digestion of genomic DNA. Lane1, digested with *EcoR*I; Lane2, digested with *EcoR*I and *Mse*I; Lane 3, control reaction (genomic DNA without endonuclease).

Figure 25: Pre-amplification. Lane 1, Pre-amplification with 12 cycles; Lane 2, Pre-amplification 17 cycles; Lane 3, Pre-amplification with 20; Lane 4, 100 bp marker.

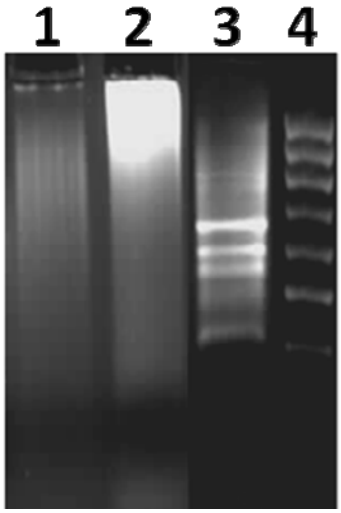


Figure 26: SSR enrichment confirmation by selective amplification. Lane 1, selective amplification with enriched DNA; Lane 2, selective amplification with direct pre-amplification product; Lane 3, 1kbs marker.

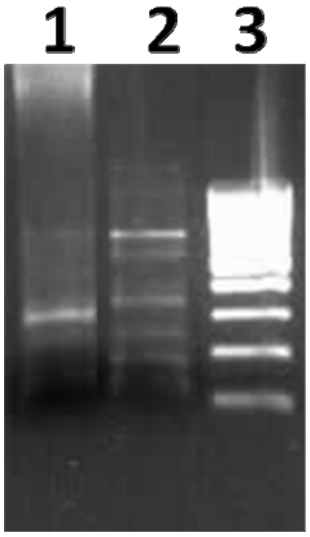


Figure 27: Pre-amplification of enriched microsatellite DNA. Lane 1, 1kbs marker; Lane 2, Pre-amplification with 8 cycles ; Lane 3, Pre-amplification with 12 cycles; Lane 4, Pre-amplification with 16 cycles.

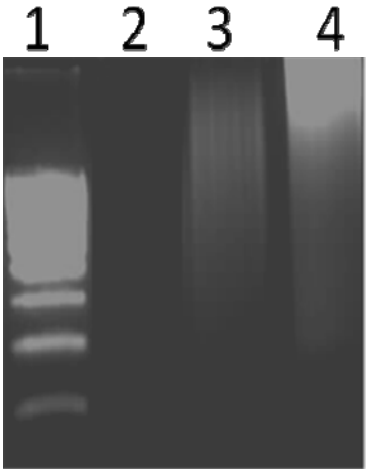


Figure 28: Colony PCR for selection of obtained white colonies. Lane 1, 100 bps marker.

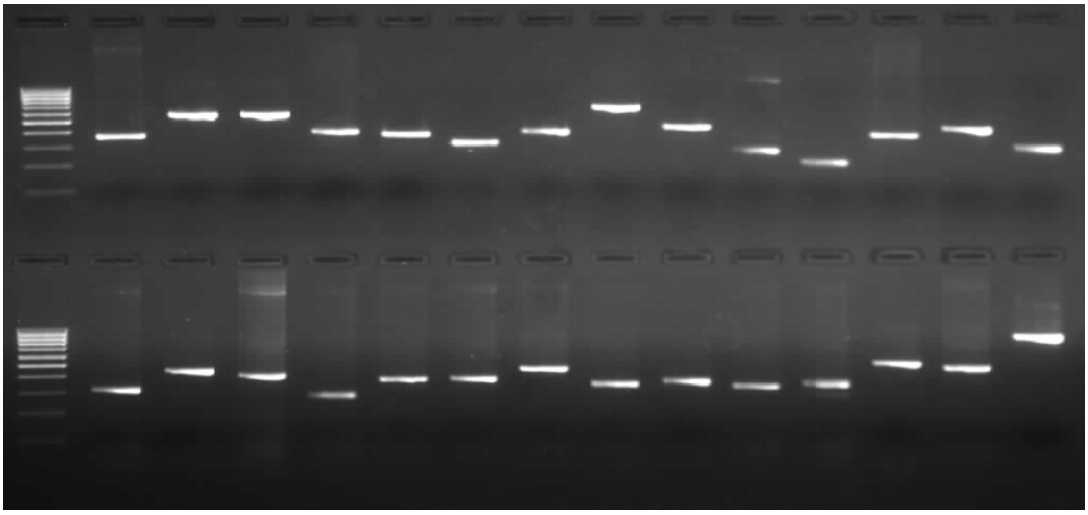


Figure 29: Sequenced microsatellite.

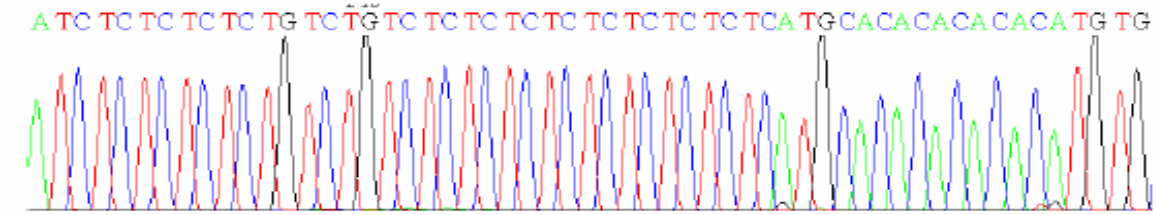


Figure 30: Cross species amplification of microsatellite marker jcps21, Lane 1. 100 bps marker; Lane 2. *J. curcas*; Lane 3. *J. tanjorensis*; Lane 4. *J. glandulifera*; Lane 5. *J. gossypifolia*; Lane 6. *J. multifida*; Lane 7. *J. podagrica*; Lane 8. *J. integerrima*.

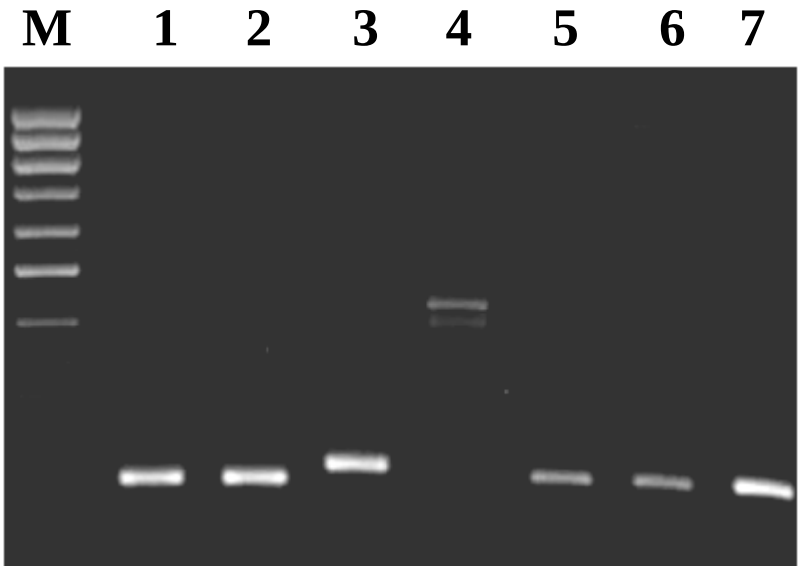
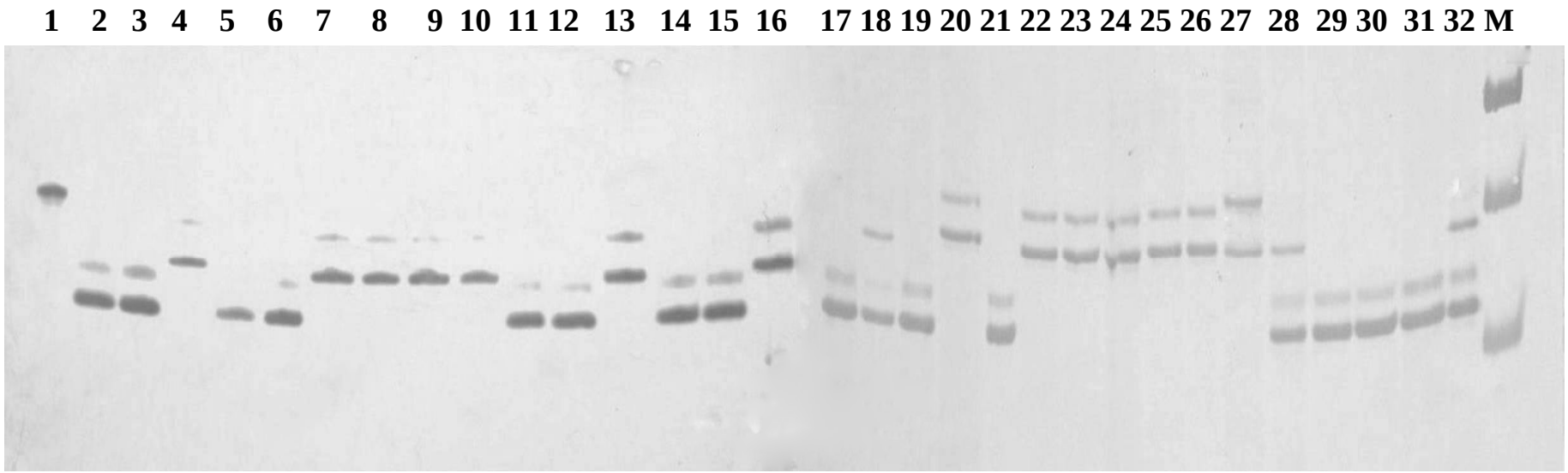


Figure 31: Microsatellite marker (jcms21) allele polymorphism in 32 germplasm of *J. curcas.*



CHAPTER 5

DISCUSSION

As early as in 1911, Rudolf Diesel, who invented the diesel engine, made the statement "***It is generally forgotten, that vegetable and animal oils can be used directly in diesel engines***". A small diesel engine ran with peanut oil during the world exhibition, Paris in 1900, which worked exceptionally well, however, the change of fuel was realized by only a few [Kiefer, 1986]. In experiments carried out until 1950, vegetable oils were used without any problem in common engines with prechamber injection. Henning and Kone reported activities involving the use of physic nut oil in engines in Segou, Mali during World War II.

Since the oil crisis of the 1970s and recognition of the limitations of world oil resources, Bio-fuel has received special attention. Most of the research was carried out in temperate regions with the aim of making oil crops available to farmers, opening new avenues for diversifying in view of the increasing subsidy-driven surpluses in traditional commodities. Another benefit of the cultivation of oil crops for energy purposes is; it can act as check point for the increasing global warming/greenhouse effect. In the current era of energy crisis due to the depletion of natural resources and the global warming problems *J. curcas* has acquired notable importance as an alternative renewable energy source. Special interest has been shown in the cultivation of *J. curcas* for this purpose, especially since it is drought resistant and can potentially be used to produce oil from marginal semi-arid lands, without competing food crops. Short gestation period, easy adaptation to different kinds of marginal and semi marginal lands, drought endurance and antiherbivory made the species more attractive for cultivation [Jones & Miller, 1991; Heller, 1996; Gubitz *et al.*, 1999; Francis *et al.*, 2005]. In addition, these fuels can be used partly to substitute costly oil imports for landlocked countries.

Biodiesel derived from *J. curcas* seed oil has the desirable physiochemical characteristics, performance was shown to be superior to conventional petro-diesel [Heller, 1996; Openshaw, 2000] and more advent in prospect of environmental concern [Mandpe *et al.,* 2005]. The use of physic nut seed oil in car engines is reported in the literature [Mensier & Loury, 1950; Cabral, 1964; Takeda, 1982; Ishii & Takeuchi, 1987]. Environmental benefits of its use in place of conventional diesel include lower exhaust emissions of particulate matter, greenhouse gases such as CO, CO_2 and SO_2 [Mandpe *et al.,* 2005].

In India extensive experimental field trials are being conducted at CSMCRI (Central Salt and Merine Chemical Research Institute), Bhavnagar to assess the possibility of developing *J. curcas* for bio-diesel. Many field experiments were being conducted on various factors and the major limitation observed is low and inconsistent yield which limits the large-scale cultivation. So, there is a need to identify and improve the species for higher yield, which can be easily achieved by exploiting the natural plant genetic resources like available natural genetic diversity through DNA based molecular analysis tools and utilize the information in breeding programs for the genetic improvement of the species.

Assessment of diversity has traditionally been through morphological characters, biochemical markers like isozymes. However, these analyses have its inherent disadvantages like limited number of markers, and often found to be less effective due to its inconsistency to short term environmental fluctuations [Les *et al.,* 1991]. DNA based molecular analysis tools like Restriction Fragment Length polymorphism (RFLP), Amplified Fragment Length Polymorphism (AFLP), Random Amplified Polymorphic DNA (RAPD), micro-satellites markers (SSR), Sequence Characterized Amplified Regions (SCAR), Sequence Tagged Sites (STS) and nrDNA-ITS became ideal for germplasm characterization and have been used for the molecular diversity assessment, generation of molecular markers for efficient use in breeding.

Although these molecular analysis tools have been used to create extensive linkage maps and study diversity in many plants, very few attempts were made in case of

Jatropha. DNA fingerprinting techniques like RAPD, AFLP and generation of microsatellite markes, and their analysis offers efficient characterization of *Jatropha* at molecular level. The generated markers can be efficiently employ in breeding and linkage mapping to improve the species for better attributes like yield and adaptability. Hence, the present study was undertaken by taking *Jatropha* as experimental system with the following objectives (i) To analyze molecular diversity by RAPD, (ii) To analyze molecular diversity AFLP, (iii) To compare both RAPD and AFLP molecular diversity studies (iv) To develop and characterize molecular markers.

Since their was no published information available, the present investigation was started with standardization of the protocol for isolation of high quality genomic DNA extraction for molecular marker analysis, studies carried on analysis of inter and intra generic diversity using RAPD and AFLP fingerprinting, the data was substantially compared with the single locus based (nrDNA ITS sequence) sequence analysis system. The most efficient polymorphic markers like SSRs were isolated by standardized FIASCO enrichment technique and were characterized to analyze allele polymorphism between toxin and non-toxic varieties, the genetic structure of *J. curcas* in representative germplasm of overall India and a small population belongs to Gujarat Gir forest region.

Part A:

5.A. STANDARDIZATION OF METHOD FOR GENOMIC DNA EXTRACTION FROM *J. CURCAS* FOR GENETIC DIVERSITY AND MOLECULAR MARKER STUDIES.

The unsuccessful attempts for extraction of good quality of DNA from *J. curcas* for the molecular marker analysis with the available protocols [Doyle & Doyle, 1990 Stewart & Via, 1993; Stange *et al.*, 1998] made us to search for extraction protocols and developed a simple and efficient method of genomic DNA extraction from different tissues of *J. curcas* including callus by modifying CTAB protocol [Doyle & Doyle, 1990]. Different CTAB extraction methods reported in the literatures [Khanuja, 1999; Michiels, 2003] gave very less quantity of DNA with loads of polysaccharide and protein contamination, samples were very viscous and took long time to get dissolved in TE buffer. The $A_{260/280}$ O.D. ratio of 1.34 to 1.65 indicated heavy contamination of the DNA with proteins. The extracted sample, upon electrophoresis gave fire-type bands, uneven migration, and often remained in the wells during electrophoresis, as reported in the literature [Do & Adams, 1991; Sharma *et al.*, 2002] confirmed the presence of polysaccharides and protein contamination (Figure 3; Lane 2-5). Polysaccharides can co-precipitate with DNA after addition of alcohol during DNA precipitation to form highly viscous solutions. By using 3.5 M NaCl in extraction buffer and 80% ethanol with 2.0 M NaCl (final concentration) during precipitation and further purification with Tris saturated phenol during purification phase the quality (A260/280 1.81±0.063, Table3) and quantity (120 to 140 µg per gram) of DNA was improved significantly without protein and polysaccharide contamination. On agarose gel electrophoresis DNA gave sharp bands (Figure II; Lane-1). Use of 100% ethanol along with 2.0 M NaCl (final concentration) in extraction and purification phases not only decreased the quantity of DNA but also precipitated salts along with DNA. On electrophoresis the DNA gave fire type bands. Use of high concentration of NaCl in the extraction buffer decreased polysaccharides contamination [Danshwar *et al.*, 2004]. In the present protocol use of 3.5

M NaCl in the extraction buffer reduced 90% of polysaccharides contamination and very little or no jelly like precipitate was found during precipitation of DNA. One of the most significant steps of our protocol was use of only Tris saturated phenol (pH 8.0), followed by chloroform-isoamyl alcohol extraction. Most of the protocols in the literature used phenol, chloroform, isoamyl alcohol (25:24:1) or chloroform, isoamyl alcohol (24:1) for protein removal (Doyle and Doyle, 1990; Dellaporta 1983); whereas, in our experiments use of either phenol:chloroform:isoamyl alcohol (25:24:1) or chloroform:isoamyl alcohol (24:1) led to the formation of yellowish pellet with O.D. 1.60±0.75 at $A_{260/280}$ which confirmed the presence of contamination, especially proteins. By using Tris saturated phenol (pH 8.0) followed by chloroform:isoamyl alcohol (24:1) extraction, protein impurities were successfully removed without affecting DNA yield (Figure 4, Lane-1; Figure 6, Lane 7). It was also observed that buffer to tissue ratio, and incubation time were also important factors for obtaining higher yields of DNA and in case of *J. curcas* 1:5 tissue to buffer ratio (Table 2) and 90 min incubation at 65 ºC (Table 3) gave the best result. Compared to precipitation at -20 ºC, precipitation at RT could archive the same without compromising the quality and quantity of DNA. The extracted DNA was suitable for restriction digestion, ligation, PCR amplification and other downstream processes necessary for DNA fingerprinting (Figure 3, 4 & 5). This protocol was also found suitable for extraction of genomic DNA from other parts of *J. curcas* i.e. root, petiole, stem, germinated seedlings and also from callus (Table 4, Figure 6). The optimized technique was simple, efficient and can be carried out at room temperature with out compromising the quality and quantity. This protocol for isolating genomic DNA was used further molecular diversity and marker analysis studies in the present investigation

Part B:

5.B. COMPARATIVE STUDIES ON INTERSPECIFIC GENETIC DIVERGENCE AND PHYLOGENIC ANALYSIS OF GENUS *JATROPHA* BY RAPD AND AFLP AND nrDNA ITS SEQUENCE ANALYSIS.

In pre DNA era many studies were carried out to analyze the diversity within and among the population of plant species using allozymes [Francisco *et al.,* 1996; Hamrick & Godt, 1989]. The limitations with these techniques were low number of markers and pseudo variations [Crawford *et al.,* 1994; Essilman *et al.,* 1997; Lesica *et al.,* 1998; Lowrey & Crawroford, 1985; Soltis *et al.,* 1992]. Advances in the field of molecular biology have provided many tools for studying the diversity in genome level to get phylogenetic relationship among different species. Out of many PCR based fingerprinting techniques RAPD and AFLP emerged as very useful and efficient methods for analyzing the molecular diversity due to ease of working and efficiently utilized for studying the divergence between different plant species [Adams & Demeke, 1993; Campos *et al.,* 1994, Catalan *et al.,* 1995; Essilman *et al.,* 1997; Ranade *et al.,* 1997; Spooner *et al.,* 1997], within the species [Brucci & Menozzi, 1993; Brunell & Whitkus, 1997; Volis *et al.,* 2001], varieties and also in QTL mapping of interested characters [Brunell & Whitkus, 1997; Hadrys *et al.,* 1992].

In the present study RAPD and AFLP fingerprinting techniques were used to analyze the molecular relatedness of seven species of genus *Jatropha* (*J. curcas, J. glandulifera, J. gossypifolia, J. integerrima, J. multifida, J. podagrica* and *J. tanjorensis*) and the results were compared with the results of single locus nrDNA ITS sequence analysis.

Maximum intraspecies percentage of polymorphism (PP) and minimum genetic similarity (GS) was found in *J. gossypifolia* followed by *J. multifida* in both RAPD and AFLP analysis. Highest GS and minimum PP was found in *J. curcas* by RAPD analysis. Basha and sujatha [2007] and Ganesh *et al.* [2008] also observed similar results. In AFLP analysis lowest PP was found in *J. podagrica* followed by *J. curcas*. In the present study

RAPD analysis showed that *J. glandulifera* is the most diverged species among the *Jatropha* species studied and the same results were also observed Ganesh *et al.* [2008]. The maximum GS was found between *J. curcas* and *J. integerrima*. Whereas, Ganesh *et al.* [2008] reported that the genetic similarly between *J. curcas* and *J. integerrima* was second highest however the highest GS reported was with in the *J. curcas* germplasm only.

Earlier interspecific hybrids between *J. curcas* and *J. integerrima* [Sujatha & Prabakaran, 2003] and genetic variation between toxic and non-toxic Mexican variety of *J. curcas* [Sujatha *et al.*, 2005] were confirmed using RAPD technique. The extents of genetic diversity at inter and intra population level in *J. curcas* was also studied using RAPD along with ISSR analysis [Basha & sujatha, 2007]. In this present investigation, the phylogenetic relationship among different species of *Jatropha* (*J. curcas, J. glandulifera, J. gossypifolia, J. integerrima, J. multifida, J. podagrica* and *J. tanjorensis*) which are distributed prominently in India was deduced using RAPD and AFLP techniques. The PP among the seven species of *Jatropha* in this study was found to be 97.74 by RAPD and 97.25 by AFLP.

Prabakaran and Sujatha [1999] reported *J. tanjorensis* as a natural hybrid between *J. curcas* and *J. gossypifolia* using morphological and biochemical markers as indicators. However, the present result of RAPD and AFLP are not in agreement with the findings since the PP observed between *J. curcas* and *J. tanjorensis* was 62.80 by RAPD and 68.19 with AFLP and between *J. tanjorensis* and *J. gossypifolia* the PP was 71.57 by RAPD and 71.97 by AFLP which are far from the expected PP for any hybrid. Similar results were observed in phylogram analysis (Figure 13 & 14).

In the karyotyping analysis (*J. curcas, J. gossypifolia, J. integerrima, J. multifida* and *J. podagrica*) *J. curcas* and *J. multifida* have similar meiotic configuration and *J. curcas, J. podagrica* has meiotic configuration different to other species studied [Soontornchinaksang & Jenjittikul, 2005]. The present study also showed more or less similar result. The GS was high between *J. curcas* and *J. integerrima* (0.63 by RAPD, 0.55 by AFLP) whose genetic similarities were higher than the mean genetic similarities

(0.48 by RAPD and 0.44 by AFLP) of the species studied. The PP was high between *J. curcas, J. podagrica* (69.35 by RAPD, 73.47 by AFLP) and the values were higher than the mean PP (68.48 by RAPD and 71.33 by AFLP). Our molecular analysis results support the reason for the successful interspecific hybridization reported between *J. curcas* X *J. integerrima* [Sujatha & Prabakaran, 2003; Rupert *et al.,* 1970]. From the results of RAPD primers IDT E4, OPL 5, OPL16 and OPJ13 primers (Figure 10 & 11); and AFLP selective primer combinations M-CAT/E-AAG, M-CAA/E-ACA and M-CAA/E-ACT (Figure 12), can be efficiently used in differentiating all the seven species and to identify the interspecies hybrids, since these primers resulted in maximum number of polymorphic markers which are specific to species and also non polymorphic markers common to all the species studied. PP loci between most pairs of species for the phylogenetic analysis were showing minor deviation according to the Bussell *et al.*, [2005]. The results with RAPD and AFLP were similar, and supported by bootstrap analysis, shows that the genetic relationships reported among different species are reliable.

The phylogenetic trees generated using the RAPD and AFLP data showed relatively good correspondence with each other and were also supported by bootstrapping analysis (Figure 13 & 14). In case of RAPD *J. curcas* and *J. integerrima* showed maximum GS and were grouped together with least branch distance. Similar grouping was also observed in case of AFLP. *J. glandulifera* whose geographical distribution was stated to be wider and has morphological distinctiveness, been separated from major and minor clades, the results are in agreement with Ganash *et al.* [2008]. The highest GS (0.60) shown by *J. multifida* and *J. podagrica* with AFLP analysis were grouped together and showed the least branch distance in AFLP phylogram and same were also grouped together in the RAPD phylogram. *J. tanjorensis* considered to be the natural hybrid [Prabakaran & Sujatha, 1999] was grouped neither with *J. curcas* nor with *J. gossypifolia* in both the phyolograms. This study demonstrated the usefulness of both RAPD and AFLP techniques for molecular characterization and to study the phylogenetic relations between the species of the genus *Jatropha*. This is the first attempt in the phylogenetic analysis of species of *Jatropha* and the study has given a background for genetic

relatedness among the seven species studied. The consistency of the result was proved with comparative analysis of the both RAPD and AFLP.

The internal transcribed spacer (ITS) has been used in numerous systematic studies at genus and species levels of a wide array of plant taxa [Baldwin *et al.,* 1995]. Its small size, ease of amplification and rapid concerted evolution made nrDNA ITS region as most important tool from a phylogenetic standpoint and promotes accurate reconstruction of species relationships. Many studies were undertaken to deduce the phylogetic relation among species of many genus [Wen-Hsiung, 1997; Morgen & Blair, 1995; 1998] including those genera belongs to family Euphorbiaceae [Berry et al., 2005]. So, in this study phylogenetic relationship of seven species of genus *Jatropha* was also assessed using nrDNA ITS sequence and the results obtained were compared with our previous analysis, which were based on multilocus marker systems. From the results it was evident that overall mean genetic distance (GDS) of genus *Jatropha* was found to be 0.385 which is far less from the values obtained with multilocus marker systems (RAPD and AFLP). The intraspecies GDS obtained in the present study using nrDNA ITS sequence has shown contrary result with earlier analysis based on multilocus marker system. The reason may due to the fact that the data based on multilocus marker system is representative of total genome variations; whereas, nrDNA ITS analysis is based on small region which is not the representative of the total genome. Based on the GDS the most genetic related species pair was found to be *J. gossypifolia*/*J. tanjorensis* and *J. curcas*/*J. integerrima*. And the most distant species pair was *J. multifida* and *J. tanjorensis*. The interspecies genetic relatedness based on ITS sequence data showed correlation with multilocus marker based analysis. This study also supports the fact that *J. curcas* and *J. integerrima* are most genetically related, which may be the reason for successful hybridization between these two species [Alice & Campbell, 1999].

The reports on mutational analysis revealed that transitions are more probable than transversions [Li & Graur, 1991] therefore; transversions are considered as more reliable mutations in assessing GDS [Quicke, 1993]. Estimation of the ti/tv rate bias is important not only to understand the patterns of DNA sequence evolution, but also to

reliably estimate sequence distance and phylogeny reconstruction. However, estimated ti/tv ratio varies with species included in the analysis [Yang & Yoder, 1999]. In the present study, the estimated ti/tv ratio supported the previous RAPD and AFLP result for example, the pair of *J. curcas* / *J. integerrima* which with a high ti/tv ratio was found to be least diverged. Molecular divergence data of nrDNA ITS sequence also supported the above conclusion (Table 9).

The phylogram obtained using nrDNA ITS sequence showed moderate congruence with the phylogenetic trees obtained using multilocus marker system. The phylogenetic tree based on ITS sequence also supports the relatedness observed for the species pair *J. curcas*/*J. integerrima* which was clustered together on basis of maximum parsimony. Though the least genetic branch distance in the phylogram was found between *J. curcas* and *J. podagrica*, one of the nrDNA ITS sequences of germplasm (J.INT3) of *J. integerrima* has clustered with *J. curcas* cladode which indicates the phylogenetic relatedness among them. *J. tanjorensis* which was reported to be a natural hybrid [Prabakaran & Sujatha M, 1999] between *J. curcas* and *J. gossypifolia* clustered neither with *J. curcas* nor with *J. gossypifolia*. *J. glandulifera* and *J. tanjorensis* which formed a single clade in phylogram generated by AFLP, also clustered together in the ITS sequence based phylogram. The same was observed in case of *J. gossypifolia*/*J. integerrima*. The major exception observed in the phylogram generated with nrDNA ITS sequence was *J. podagrica*, which was clustered with *J. multifida* in the phylograms of RAPD and AFLP, grouped with *J. curcas* and formed a major clade. Overall good correlation was able to found among the phylograms obtained with nrDNA ITS sequence data and multilocus marker system with very minor differences.

The present study elucidates the usefulness of the nrDNA ITS sequence in phylogenetic analysis of genus *Jatropha* and would pave way for future phylogetic and/or evolutionary studies among the taxa belonging to the family Euphorbiaceae. The sequence data generated would serve as a platform for further studies in intraspecies population, their phylogenetic origins, biogeographical and molecular evolutionary studies. The generated molecular markers can be utilized in species differentiation,

molecular identification and characterization of interspecific hybrids. The study concludes that both RAPD and AFLP are efficient in diversity studies of *Jatropha*. However, AFLP gives better resolution and marker consistency. The markers generated by RAPD and AFLP can be employed efficiently in interspecific hybrids identification, marker assisted selection and genetic resource management.

Part C:

5.C. MOLECULAR CHARACTERIZATION AND IDENTIFICATION OF MARKERS FOR TOXIC AND NON-TOXIC VARIETIES OF *J. CURCAS* USING RAPD, AFLP AND SSR MARKERS.

J. curcas, a multipurpose shrub, has acquired high agro-industrial significance globally because of its seed oil which is a potential source of biodiesel and also for its beneficial by-products [Mandpe *et al.*, 2005; Jones *et al.*, 1991; Heller, 1996; Gubitz *et al.*, 1999]. The seed cake remaining after oil extraction is toxic owing to the presence of different toxic substances in seed. So, oil and deoiled cake is not suitable for animal consumption [Makkar *et al.*, 1998], despite of having best protein composition and favorable amino acid profile [Makkar *et al.*, 1997]. Though various processing techniques have been attempted to detoxify, no treatment has been successful in completely eliminating the toxic principles of defatted kernel meal [Martinez-Herrera *et al.*, 2006]. In addition to common toxic cultivar, a non-toxic *J. curcas* has been reported from Mexico whose innocuous nature was established [Francis *et al.*, 2005; Makkar & Becker, 1997]. Cultivation of non-toxic *J. curcas* may assume to acquire prime importance as it can provide oil for biodiesel and de-oiled seed cake as a live stock feed [Makkar *et al.*, 1998]. However, no qualitative and quantitative differences were reported between these two cultivars. Development of any simple marker will enable to identify non-toxic variety from toxic variety which will not only add the quality control for selective cultivation of non-toxic variety, but also avoid any toxic adulteration in the animal feeds.

In our study a non-toxic Mexican variety was compared with five germplasm of *J. curcas* collected from different geographical regions of India and a Mexican toxic variety using RAPD and AFLP technique with an aim to develop markers specific to either non-toxic or toxic variety. Sujatha *et al.* [2005] reported 94.6 percentage of similarity between toxic and non-toxic varieties using RAPD fingerprinting whereas, in the present study percentage of similarity was 83.59 by RAPD and 84.91 by AFLP fingerprinting. Genetic

similarity between toxic and non-toxic variety was found to be 0.90 by RAPD and 0.91 by AFLP fingerprinting techniques.

Use of primer OPQ15 has resulted in one specific marker each to toxic (approximately 810 bp) and non-toxic (approximately 425 bp) variety. Primers IDT E-18, OPL14, resulted in one marker each of approximately 900 and 2100bp respectively whereas, OPR8 resulted in two specific markers of approximately 1450 and 700bp (Figure 17 & 18) and the AFLP the primers E-ACC/M-CAC combination which resulted in maximum number of polymorphic markers three markers each for toxic and non-toxic varieties can be used for the identification of either of the varieties. Prominently amplified AFLP markers specific to toxic and non-toxic variety and their molecular weights was recorded (Table 14, Figure 19). Though the marker related studies for toxic *J. curcas* has been reported using ISSR [Basha & Sujatha, 2006] in the present study markers were identified specific to both toxic and non-toxic varieties using RAPD and AFLP fingerprinting which have better application in molecular breeding studies.

In this study 12 SSR markers were analyzed among nontoxic and toxic varieties. Minor miss-amplifications were minimized by gradient PCR and optimized the annealing temperature (Table 15). The average length of the microsatellites in the present study was between 75 bps to 250 bps. Similar results were reported in other species also [Zane *et al.*, 2002]. The polymorphic nature of SSR markers were characterized for toxic and non-toxic varieties. The results showed that out of 12 markers tested, 7 were found to be size polymorphic. Among the analyzed microsatellite markers, presence of high polymorphism (7) implies the high availability of polymorphism of microsatellites between two varieties and their applicability in MAS and QTL analysis studies.

This is the first report on molecular characterization of non-toxic and toxic *J. curcas* using both multilocus and single locus marker systems. The specific markers generated using RAPD and AFLP fingerprinting will help to distinguish non-toxic from toxic varieties of *J. curcas* or vice versa and markers of RAPD and AFLP together with the polymorphic SSR markers can be exploited in Marker Assisted Selection (MAS), QTL analysis and for other molecular breeding studies.

Part D:

5.D. PHYLOGEOGRAPHY AND MOLECULAR DIVERSITY ANALYSIS OF *J. CURCAS* AND DISPERSION ROUTE AS REVEALED FROM RAPD, AFLP AND nrDNA ITS ANALYSIS.

Distribution of species may consist of geographically structured population because of their natural evolution, distribution, and dispersal mechanisms. The genetic structure depends on historical, biogeographical, demographical, and domestication process. Yet the genetic structure, apparent geographical distribution and dispersion of the plant species is poorly understood due to their poor mechanisms for long dispersal of the race, interference by forces like domestication and distribution thorough anthropogenic activity. In the recent past, due to availability of sophisticated molecular analysis tools, there was a paradigm shift of population and phylogeographical structuring from morphological based analysis to genetic level diagnosis and historical process of the species [Avise, 2000].

The knowledge of the phylogeography and the genetic distribution of a plant species are extremely important for the optimum utilization of the naturally available plant generic resources for the breeding programs and inturn to improve the species for their economically important characters. In case of *J. curcas* only inter and intra population diversity in India was reported [Sujatha & Basha 2007] and till date there are no reports regarding the diversity distribution, number of introductions and the genetic diversity of *J. curcas* populations grown globally. Several researchers have attempted to define the origin of *J. curcas*, but the source remains controversial [Dehgan & Webster 1979; Heller, 1996]. Three distinct varieties are reported viz., the Cape Verde variety that has spread all over the world, the Nicaraguan variety with few but larger fruits and a non-toxic Mexican variety with the reduced phorbol esters content [Henning, 2006; Heller, 1996].

J. curcas reported to be the most primitive species of genus *Jatropha* [Dehgan & Webster, 1979] is an ecologically important species and has the potential to rehabilitate

the waste and degraded land [Sudheer *et al.,* 2008b]. For optimum utilization of any plant species, it is very necessary to characterize the total germplasm and deduce its biogeography through efficient molecular techniques. An understanding of the extent of genetic diversity and knowing the biogeography of the plant species is critical and very much needed for the success in breeding programme to improve the species for better exploitation of its benefits to mankind.

RAPD, AFLP data allow an assessment of population structure based on multiple loci, i.e. representative of the entire genome and nrDNA estimates the diversity and evolution based on significant DNA region. nrDNA ITS and fingerprinting data together can provide complementary information about evolutionary patterns [Davies & Bermingham, 2002].

5.D.1. Genetic diversity of *J. curcas*:

The diversity distribution of *J. curcas* existing globally was analyzed by RAPD and AFLP fingerprinting techniques. Correlative data was obtained from both the techniques and the genetic diversity was found to be equitable. Our RAPD and AFLP data corroborate the fact that overall genetic diversity within population component is low in comparison with the diversity among the population as reported earlier [Basha & Sujatha, 2007]. Overall GD was high in the germplasm collected from the Mexico as compared to any other group of collections (Table 16). The GD was narrow among the Indian germplasm with both RAPD and AFLP analysis and conforms to earlier findings [Basha & Sujatha, 2007; Sudheer *et al.,* 2008]. Though, overall diversity given by both the techniques are almost in congruence but RAPD could not resolve some of the germplasm as AFLP could, which implies the advantage of AFLP in genetic diversity studies of *J. curcas*. The mean genetic diversity of population from Mexico, previously reported as center of origin based on morphological data and natural distribution, was found to be the highest [Heller, 1996]. However, overall genetic diversity found was limited in comparison with diversity of many taxa and also of many endemic species reported till date [Thompson & Nelson 1998; Colombo *et al.,* 2000 Chengxin *et al.,* 2003; Torres *et al.,* 2003; Jain *et al.,* 2003; Ram *et al.,* 2004]. The same conclusions can be

drawn in case of nrDNA ITS sequence analysis. The mean genetic distance was however, far less than the mean diversity found with DNA fingerprinting data. The genetic distance reported among the Indian population was less than the genetic distance observed among of the population of Mexico.

5.D.2. Phylogeography:

In phylogram analysis, total germplasm were grouped in three clusters in both RAPD and AFLP. Indian germplasm formed two clusters except JCI20. Only one germplasm belonging to Cape Verde (JCE52) clustered with Indian germplasm in both RAPD and AFLP phylograms. The phylogram generated with the help of nrDNA ITS sequence was not much correlative with RAPD and AFLP in case of Indian germplasm. The reason may be that RAPD and AFLP fingerprinting data was based on the variations in whole genome but the nrDNA sequence is based on the single locus sequence variations. nrDNA ITS sequence data showed narrow diversity among the Indian germplasm but formed a single major cluster with Indian germplasm except JCI26. In comparison with RAPD, AFLP, the nrDNA ITS sequence data showed narrow molecular diversity among Indian germplasm. As like multilocus markers nrDNA ITS sequence also showed maximum diversity among Mexican germplasm.

Phylogenetic trees obtained by RAPD and AFLP showed good correlation in compression to nrDNA ITS sequence. The low bootstrap values may be due to the low number of parsimony-informative characters available. The conclusions with poise can be drawn by comparative analysis of phylograms generated by all the three analysis. The phylogenetic tree generated by RAPD and AFLP suggests that two distinct germplasms of *J. curcas* were introduced simultaneously in India and were distributed throughout the country. A strong genetic relationship was found between the Mexican and Cape Verde germplasm. In the phylograms based on RAPD, AFLP indicate that African germplasm has separated from main group and formed a separate branch. However, based on nrDNA ITS sequence analysis Cape Verde germplasm showed more relation to African germplasm. The Indian germplasm showed nearest relationship with Cape Verde and Mexican germplasm. nrDNA ITS sequence phylogram showed equal relationship with

both Cape Verde and Madagascar but the node of Indian with Madagascar germplasm was poorly supported with bootstrap value. Taken as a whole analysis conclusions may be drown as, two distinct germplasms were introduced in to India one probably from Cape Verde via Spain and other an alternate route that could not be revealed by the present investigation as the study requires more diverse collections of germplasm. The present results also indicate that introduced germplasm in India were distributed throughout the country through human activity [Basha & Sujatha, 2008].

5.D.3. Origin and centre of diversity:

A number of attempts were made to define the origin of physic nut, but the source remained controversial. Dehgan and Webster [1979] Cite-Wilbur [1954] for the first time stated that Central America might be the most likely source of origin of *J. curcas.* According to other sources also, the physic nut seems to be native to Central America as well as to Mexico where it occurs naturally in the forests of coastal regions [Aponte, 1978]. Martin and Mayeux [1984] identified the Ceara state in Brazil as a centre of origin without giving any proof. Herbarium specimens of the Americas were usually collected from hedges along roads and paths, live fence posts or disturbed sites ("disturbed forest") [1949] confirm this, and also the information provided by many collectors in the herbariums seems [Dehgan's Horticultural Systematics Laboratory, Standley and Steyermark] to support the argument that the species collected in America were from natural vegetation. Whereas, the collections in other places like Africa and Asia not found to be natural but only in cultivated form. So, it was stated that highly probable centre of origin of the physic nut is Mexico/Central America. The "true" centre of origin, however, still has to been confirmed [Heller, 1996].

In the present study, the original collecting sites in Mexico and other parts of the world have provided the extent of genetic diversity of *J. curcas* existing globally. The nucleotide diversity data of RAPD and AFLP showed that the maximum diversity was found among the Mexican germplasm than any other group of population. This concludes *J. curcas* has its centre of diversity in Mexico, since origin of species will have maximum time of evolution process which will provide the diversity to the species. The nrDNA ITS

sequences of germplasm belonging to Central America showed minimum sequence similarity scores than any other group of populations, which inturn supports the conclusions obtained by the multilocus based fingerprinting (RAPD and AFLP) analysis data. The genetic diversity found in the Indian germplasm was very narrow as reported earlier [Basha & Sujatha, 2007], However, in the present study the analysis gave better polymorphism than the earlier report, though the samples analyzed were less (28 germplasm). The phylograms showed that unlike exotic germplasm the clustering of Indian germplasm was random and not in correspondence to the geographical region of collection. This random clustering clearly indicates the human interference in distribution of *J. curcas* as said earlier [Basha & Sujatha, 2007]. The low genetic diversity may be due to the recent introduction of the species in India and its fast distribution, since it is an economically important plant.

5.D.4. Dispersal route revealed by RAPD, AFLP and nrDNA ITS sequence analysis:

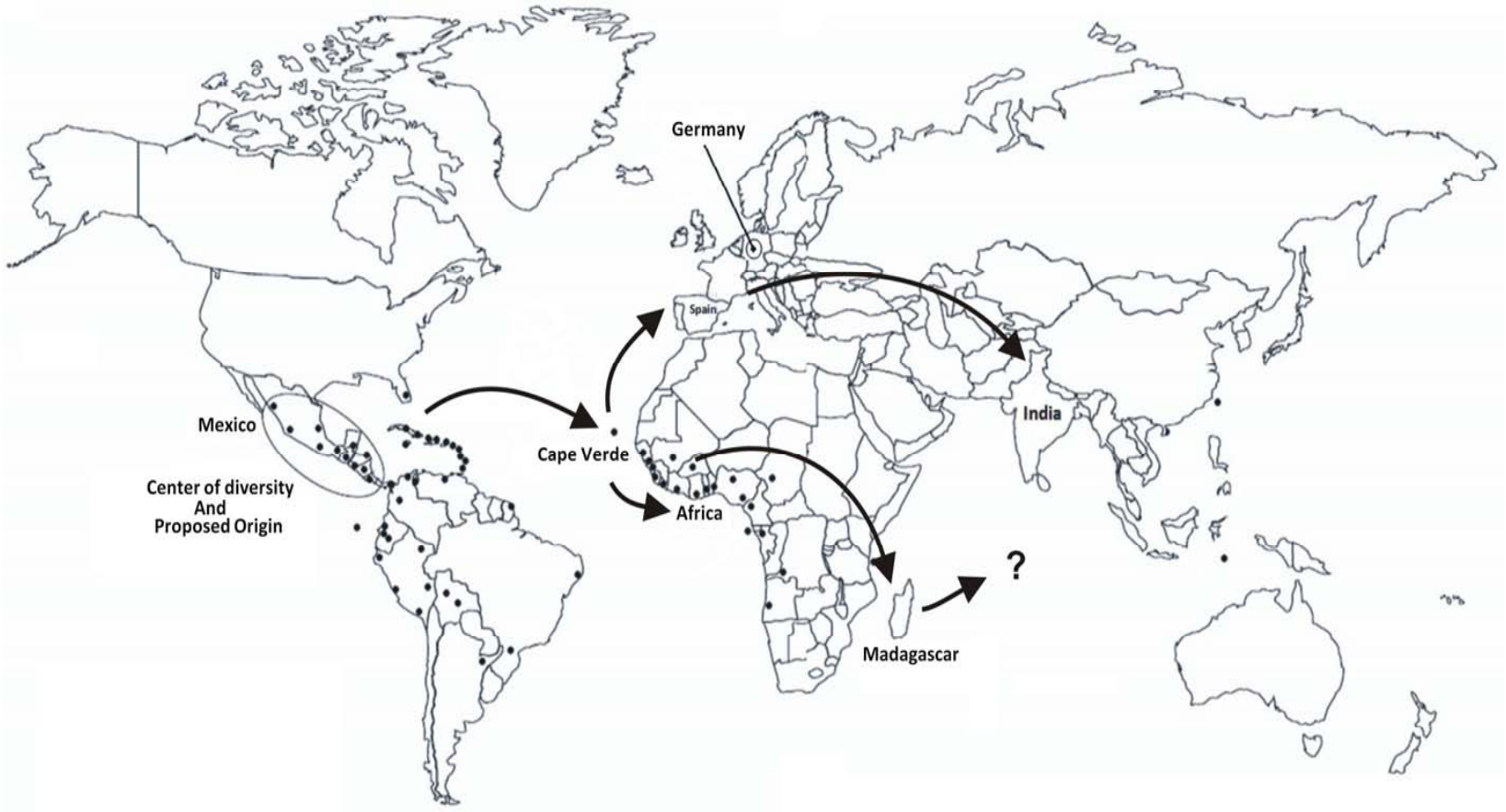
The migratory route of the *J. curcas* was not yet completely described but some attempts were made based on preliminary data available. Heller [1996] mentioned that this species was probably distributed by Portuguese seafarers via the Cape Verde Islands and former Portuguese Guinea (now Guinea Bissau) to other countries in Africa and Asia. No facts are available in the literature before 1800 as to when the physic nut was introduced into Cape Verde [Serra, 1950]. Freitas [1906] says that the physic nut was already known several years prior to 1810, as he mentioned it in his book “Memoria ou descripção physico-politica das ilhas de Cabo Verde”. Burkill [1966] assumes that the Portuguese brought the physic nut to Asia. Nevertheless, the reports say Portuguese transported it to the Old World. Though the information was available, the proof for the proposed migration till the date does not exist. The molecular data analysis in the present study can provide the means to deduce the putative migratory route of *J. curcas* from center of origin to India.

The phylogram analysis based on RAPD data showed that Indian germplasm were very much close to Cape Verde and followed by Mexico. The same can be inferred from phylogram of AFLP fingerprinting data where in JCI20, Cape Verde germplasm,

Spainish germplasm showed maximum genetic similarity and were placed together in the cluster. In either of the cases the African germplasm and Madagascar samples did not showed near phylogenetic relationships with any Indian germplasm. Phylograms based on nrDNA ITS sequence also support the above observations. On the larger scenario it is clear that African samples showed near relation with Madagascar. So, with the above data analysis the putative distribution of *J. curcas* was as follows: *J. curcas* from the center of the origin was introduced to Cape Verde, than might have spread to Spain Portugal, simultaneously spreading to other neighboring countries and to Africa. As said Burkill [1996] Portuguese might have introduced *J. curcas* in to India. The subsequent spread to Philippines was not deduced in the present study due to lack of samples but most probable route would have been from Asia rather than Africa or Madagascar.

The present study based on RAPD, AFLP and nrDNA ITS analysis concludes that the existing molecular diversity is moderate but still less than many species reported. The study supports that the most probable origin of *J. curcas* is Mexico since the diversity was high among the Mexican germplasm studied. As discussed above the putative migratory route deduced from the data was from Mexico/Central America to Cape Verde to Spain and Portugal, from where it was introduced to Asia and in India most probably by Portuguese people. This study not only supports the view that *J. curcas* was distributed through human activity in India [Sujatha, 2007] but also from origin to other places of the world.

Figure 33: Migratory route of *J. curcas* reveled by RAPD, AFLP and nrDNA ITS sequence data.



Part E:

5.E. ISOLATION, CHARACTERIZATION OF NOVEL MICROSATELLITES FROM *J. CURCAS* AND THEIR CROSS SPECIES AMPLIFICATION.

Microsatellites or simple sequence repeats (SSR) are tandemly repeating motifs of 1-6 bases present in both coding and non-coding regions and are usually characterized by a high degree of length polymorphism. Due to their exceptional variability and relative ease of scoring, microsatellites are now considered to be the most powerful genetic markers. It is observed that loci with more than 10 alleles and heterozygosities above exists even in relatively small samples [Bowcock *et al.,* 1994; Deka *et al.,* 1995], while certain loci can be considerably more variable [Primmer *et al.,* 1996]. In addition to being highly variable, microsatellites are also densely distributed throughout eukaryotic genome, making them the preferred marker for very-high-resolution genetic mapping [Dib *et al.,* 1996; Dietrich *et al.,* 1996]. Advantages of microsatellites like-high heterozygosity, ubiquity through the genome (often adjacent to coding sequences, though rarely transcribed), and PCR typability, combined with ease of multiplexing render them suitable candidates for markers of choice in genome maps. However, since they are underpresented in chromosome centromeres and telomeres, minisatellite data are complementary for uniform genome coverage by markers. [Weissenbach *et al.,*1992].

From identifying relatives to inferring demographic parameters, microsatellites are rapidly replacing RFLPs and RAPDs in most applications in population biology [Blouin *et al.,* 1996; Bowcock *et al.,* 1994; Goldstein *et al.,* 1996; Jame & Lagoda, 1996] but one perceived difficulty with microsatellites is the long lead time in identifying and characterizing microsatellites in new taxonomic groups. However, this problem is partially alleviated by the continuing popularity of microsatellites in genetic mapping. There are many approaches for isolation of the microsatellites but in the present study FIASCO enrichment technique which is proven to be the most suitable, efficient method for the labs where the AFLP system is already established was used. The present part of

study 12 microsatellites isolated from *J. curcas,* to characterize them in available diverse germplasm and check the marker cross amplification ability in different species of genus *Jatropha.*

In the present study the most efficient technique FIASCO was used and found to be well suitable for the isolation of microsatellite markers for biodiesel plant *J. curcas*. This procedure has been used to produce the enriched library of GT repeats using the probe Biotin $(GT)_{15}$. The principle of this method is readily applicable for the labs where the AFLP is already standardized and the same sample from third step (pre-amplification) act as the starting DNA material for the enrichment. Intial screening results demonstrated that the enrichment is very proficient, easy and quick generating a large number of clones containing desired microsatellite sequences. The screening result shows that the efficiency of enrichment is 62% but some of the sequences were without flanking regions which is essential for designing primers. On the other hand, the efficiency of random clone sequencing without enrichment was reported to be 0.2-0.5% [Zane *et al.*, 2002]. This enrichment technique used in the present investigation, with minor modifications, can be applied very efficiently to other species of genus *Jatropha* as well as to other related members of family Euphorbiaceae. The reported efficiency (62%) in the present enrichment represents that FIASCO it is the better choice, more resourceful method, comparable to those of pre-existing protocols for the microsatellite enrichment.

The present study is the first report on isolation and use of microsatellite markers within and between populations of *J. curcas*. The 12 microsatellite markers isolated showed reasonable variations and moderate levels of allelic variations which suggest that these markers are highly valuable assets for the population genetic studies and other genetic improvement programs. The genetic studies carried out in 32 germplasm collected from natural populations showed better balance of HWE equilibrium. Seven markers (58%) out of 12 showed the HWE equilibrium. Only one marker out of 12 markers was in HWE equilibrium among the germplasm collected randomly from distinct geographical areas within India. The information given by each SSR marker showed significant deviation of observed heterozygosity from expected heterozygosity. It

concludes the fact that there is external interference in natural distribution of the species by anthropogenic activity. And it warns us to conserve the available natural genetic resource of *J. curcas*. This data represents the first example of use of SSR markers to investigate the genetic variations and to deduce the population genetic structure of *J. curcas*.

There are many reports on cross species amplification of SSR markers among the different species belonging to the same genus and rarely across genera [Kurt *et al.*, 2005]. The isolated markers in the present study were also checked for their cross amplification ability in other prominent species of genus *Jatropha* and the results obtained were appreciable. The results showed 100% cross species amplification of markers isolated. Each marker was able to amplify more than 4 species revealing the conserved nature of the markers among the species of *Jatropha*.

The optimized enrichment technique FIASCO can be further utilized for the high throughput isolation of microsatellite marker. These SSRs can be utilized in studies of population genetics, genetic variations, gene flow among the natural population, molecular mapping, marker assisted selection, molecular breeding and various crop improvement programs. The application of these markers not restricted only to *J. curcas*, the cross species amplification ability of these markers can be exploited in other prominent species of *Jatropha*.

The present investigation will pave way for further intraspecies molecular characterization in other species of *Jatropha*. The generated molecular markers can be utilized in species differentiation, molecular identification, population genetic structure within and among populations, molecular breeding, identification, characterization of interspecific and intraspecific hybrids, MAS (Marker assisted selection), QTL (quantitative Trait loci) mapping and to generate the physical maps.

CHAPTER 6

SUMMARY AND CONCLUSIONS

In the current era of energy crisis due to the depletion of natural resources and global warming, *J. curcas* has acquired notable importance as an alternative renewable energy source since it has the ability to provide the oil yielding seeds which can be converted to bio-diesel that has shown better performance than the conventional petro-diesel. The major limitation in large scale cultivation of *Jatropha* as an energy crop observed is low and inconsistent yield. Therefore, there is a need to improve the species for higher yield by exploiting the naturally available plant genetic resources.

Plant genetic resources are the most valuable natural assets available to the mankind. An important component for effective and efficient management of plant genetic resources is molecular characterization and analysis of genetic divergence of germplasm, which is essential not only for identification of various species but also to determine genetic relatedness within and among the species. RAPD and AFLP markers have proved to be valuable tools in the characterization and evaluation of genetic diversity, phylogenetic analysis intern help in species improvement through breeding. The comparision of the data obtained using the multilocus techniques (RAPD and AFLP) with phylogenetically significant single locus DNA sequence like nrDNA ITS will provide substantial comparative analysis and will provide comprehensive conclusions.

Hence, efforts were made to study molecular diversity in *Jatropha* with the objectives; (i) To analyze molecular diversity by RAPD, (ii) To analyze molecular diversity by AFLP, (iii) To compare both RAPD and AFLP molecular diversity studies and (iv) To develop and characterize molecular markers. Since, there is no published literature available, the present investigation was started with standardization of protocol for isolation of quality genomic DNA for marker analysis. Studies were carried on

analysis of inter and intra generic diversity using RAPD and AFLP fingerprinting, the data was substantially compared with the single locus based (nrDNA ITS sequence) sequence analysis. The most efficient polymorphic markers like SSRs were isolated by FIASCO enrichment technique and were characterized to analyze the genetic structure of *J. curcas* in representative germplasm of India and a small population belongs to Gujarat, Gir forest region.

The unsuccessful attempts for extraction of good quality DNA from *J. curcas* with the available protocols made us to search for a new extraction protocol which in turn led to the stardardization of a simple and efficient method for genomic DNA isolation from different tissues of *J. curcas* by modifying CTAB protocol. By using 3.5 M NaCl in extraction buffer and 80% ethanol with 2.0 M NaCl (final concentration) during precipitation and further purification with Tris saturated phenol during purification phase the quality (A260/280 1.81±0.063) and quantity (120 to 140 µg per gram) of DNA was improved significantly without protein and polysaccharide contamination. 5:1 buffer to tissue ratio and 90 min incubation at 65 ºC gave the best result. Compared to precipitation at -20 ºC, we could archive the same result without compromising the quality and quantity of DNA at RT. The extracted DNA was found suitable for restriction digestion, ligation, PCR amplification and other downstream processes necessary for DNA fingerprinting.

Using RAPD, AFLP fingerprinting techniques seven species of genus *Jatropha* (*J.curcas, J. glandulifera, J. gossypifolia, J. integerrima, J. multifida, J. podagrica* and *J. tanjorensis*) were studied for their molecular and phylogenetic relatedness. Maximum intraspecies percentage of polymorphism (PP) and minimum genetic similarity (GS) was found in *J. gossypifolia* followed by *J. multifida* by both RAPD and AFLP analysis. Highest GS and minimum PP was found in *J. curcas* by RAPD analysis. In AFLP analysis lowest PP was found in *J. podagrica* followed by *J. curcas*. RAPD analysis showed that *J. glandulifera* is the most diverged species among the *Jatropha* species studied. The present study ruled out *J. tanjorensis* as a natural hybrid between the *J. curcas* X *J. gossypifolia* as reported earliar in the literature.

The phylogenetic trees generated using the RAPD and AFLP data showed relatively good correspondence with each other which also supported by bootstrapping analysis. The phylogenetic trees constructed using RAPD, AFLP and nrDNA ITS sequence data suppoted the highest genetic simlatiy of the *J. curcas* and *J. integerrima. J. glandulifera* whose geographical distribution was stated to be wider and has morphological distinctiveness been separated from major and minor clades.

Globally *J. curcas* is promoted for large acreage cultivation in a big way for biodiesel production. Cultivation of non-toxic *J. curcas* may assume to acquire prime importance as it can provide oil for biodiesel and deoiled seed cake as a live stock feed. In the absence of significant qualitative and quantitative differences between toxic non-toxic varieties except for the phorbol esters content, development of any simple marker will enable to identify non-toxic variety from toxic variety which will not only add the quality control but also avoid any toxic adulteration in the animal feed. A non-toxic Mexican variety was compared with six germplasm (five collected from different geographical regions of India and one Mexican toxic variety) using RAPD, AFLP and successfully identified the varietal specific makers. Out of 12 SSR markers tested, 7 (jcms21, jcds24, jcms30, jcps20, jcps21, jcps6, jcps3) found to be size polymorphic. The highest size allele polymorphism was found with jcds24, followed by jcms30 and jcps6.

In this study, 42 germplasm on *J. curcas* were analyzed and their genetic structure and phylogeography was deduced using RAPD, AFLP and nrDNA ITS analysis. On an average each primer has given 50.6 markers with 31.6 polymorphic markers. Over all PP among 42 germplasm was found to be 73.22. The maximum PP (50.18) was found between the germplasm JCI26 and JCE50. The minimum percentage of polymorphism (1.28) was found between the germplasm JCI02 and JCE52. Among the germplasm collected in India, the over all PP was 60.95 and the PP of Mexico/Central America germplasm was 55.89. The PP of the germplasm excluding the Indian germplasm was found to be 66.42. The ITS region sequences ranged in length from 646 to 657 bp. 5.8S nrDNA sequence has shown standard length 164 except the sample JCI01 (163) a single

base pair deletion. In comparision with DNA fingerprinting data, nrDNA ITS sequence showed narrow genetic diversity.

This study based on RAPD, AFLP and nrDNA ITS analysis in *Jatropha* concludes that the existing molecular diversity is moderate but still less than the diversity reported for many species. The study supports the most probable origin of *J. curcas* to be Mexico/Central America. Results shows that the putative dispersion route deduced from the data was from center of the origin (Mexico/Central America) was introduced to Cape Verde, than might have spread to Spain Portugal, simultaneously spreading to other neighboring countries Asia and to Africa. Portuguese might have introduced *J. curcas* in to India. This study not only supports the view that *J. curcas* was distributed through anthropogenic/human intervention in India but also species was distributed from its place of origin to other places of the world.

In this study efforts were made to isolate the most efficient co-dominant markers like microsatellites. The efficiency of the FIASCO technique for isolation of the microsatellites from *J. curcas* was found to be 62%. With the data obtained the imperfect repeat markers were more abundant in *J. curcas* than prefect repeats.The average length of the microsatellites isolated from *J. curcas* was between 75 bp to 250 bp which is in accordance with the previous reports in other species. 12 markers were isolated and characterized in 32 germplasm within a single population and 21 germplasm of geographically diverse representatives in India. The population genetic studies done in 32 germplasm collected from natural population showed better balance of HWE equilibrium. Seven markers out of 12 showed the HWE equilibrium. Whereas, 21 germplasm collected randomly, from distinct geographical areas on India showed only one marker is in HWE equilibrium. Markers isolated showed 100% cross species amplification.

Conclusions

- Standaridized the protocol for extraction of high quality genomic DNA for molecular marker studies in *J. curcas*.

- Inter and intrageneric molecular diversity studied indicated that genetic diversity among the species is reasonably high for the genus *Jatropha*.
- *J. curcas* and *J. integerrima* are genetically closer than any other pair of species studied in genus *Jatropha*.
- Molecular analysis indicated that *J. tanjorensis* is not a natural hybrid as reported in the literature.
- Present molecular studies indicated that RAPD and AFLP are equally compitative in resolving molecular diversity and phylogenetic releations ships of genus *Jatropha.*
- Markers specific to toxic and non-toxic varieties of *J. curcas* were identified using RAPD, AFLP and SSRs.
- Phylogentic anlaysis carried out with geographically diverised germplasm and for the first time provided the molecular evidence as; Mexico/Central America is the center of origin of *J. curcas*.
- The phylogenetic clustering was not in accordance with the geographical place of collection in Indian germplasm. This may be result of anthropogenic activities due to the economic importance of the species.
- This study also concludes that *J. curcas* species was introduced in two major germplasm and later got distributed throughout India simultaneosly.
- For the first time SSR markers of *J. curcas* showed reasonably good allelic polymorphism within and among the populations, varieties and showed 100% cross species amplification among the other species of genus *Jatropha*.

In the present investigation entitled "***Molecular Diversity in Jatropha and Development of Molecular Markers"*** the phylogenetic relationship among the species of genus *Jatropha* was deduced. Specific markers for toxic and non-toxic varieties of *J. curcas* were generated. The genetic structure and phylogeography of *J. curcas* was analyzed with efficient markers. Highly polymorphic co-dominant molecular markers (SSR) were isolated.

The markers identified and isolated in the present study can be utilized in species differentiation, molecular identification, molecular breeding, characterization of interspecific hybrids, MAS (Marker assisted selection), QTL (Quantitative Trait Loci) mapping, to generate molecular physical maps.

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1. **D. V. N. Sudheer Pamidiamarri**, Nirali Pandya, Muppala P. Reddy, T. Radhakrishnan (2008) Comparative study of interspecific genetic divergence and phylogenic analysis of genus *Jatropha* by RAPD and AFLP. *Mol Biol Rep.* DOI 10.1007/s11033-008-9261-0

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4. **D. V. N. Sudheer Pamidimarri**, Ramini Sinha, Pragya Kothari, Muppala P. Reddy (2008) Isolation of novel microsatellites from *Jatropha curcas* L. and their cross species amplification. *Mol ecol resour.*

5. Nitish Kumar, **Sudheer D.V.N. Pamidimarri**, Meenakshi Kaur, Girish Boricha, & Muppala P. Reddy (2008) Effects of NaCl on growth, ion accumulation, protein, proline contents and antioxidant enzymes activity in callus cultures of *Jatropha curcas*. *Biologia.* **63/3:**378—382.

6. Nitish Kumar, Meenakshi Kaur, **Sudheer D.V.N. Pamidimarri**, Girish Boricha, & Muppala P. Reddy, Differtial response of calli and seedlings of *Jatropha curcas L.* to the Salinity stress. *J. Forset Science.* **24/2:**69-77.

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8. **D. V. N. Sudheer Pamidimarri** and M P Reddy, Phylogeography and molecular diversity analysis of *Jatropha curcas* and Migration route reveled from RAPD, AFLP and nrDNA ITS analysis (Under communication).

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10. **D. V. N. Sudheer Pamidimarri** and M. P. Reddy, Published ITS region sequences of 51 *J. curcas* strains in NCBI (Accessions no. EU700417 to EU700458 & EU340796 to EU340804).

11. **D. V. N. Sudheer Pamidimarri** and M. P. Reddy, Published ITS region sequences of 7 *Jatropha* species in NCBI (Accessions no. EU340789 to EU340795).

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Comparative study of interspecific genetic divergence and phylogenic analysis of genus *Jatropha* by RAPD and AFLP

Genetic divergence and phylogenic analysis of genus *Jatropha*

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Abstract Genus *Jatropha* with 172 species having significant economic importance belongs to the family Euphorbiaceae. There are no reports on molecular characterization and phylogenetic relationship among the species of *Jatropha*. Hence, the present study was undertaken to assess the extent of genetic variability that exist and also to establish phylogenetic relationship among *Jatropha curcas*, *J. glandulifera*, *J. gossypifolia*, *J. integerrima*, *J. multifida*, *J. podagrica* and *J. tanjorensis* using RAPD and AFLP. The percentage of loci that are polymorphic among the species studied was found to be 97.74% by RAPD and 97.25% by AFLP. The mean percentage of polymorphism (PP) was found to be 68.48 by RAPD and 71.33 by AFLP. The phylogram generated with RAPD and AFLP data showed maximum similarity. With the generated data maximum relatedness was found between *J. curcas* and *J. integerrima* this may be the reason for the success of inter hybrid crosses between these two species. Neither RAPD nor AFLP data generated in this study supports the view of *J. tanjorensis*, a natural interspecific hybrid between *J. curcas* and *J. gossypifolia*. The present study concludes that both RAPD and AFLP techniques are comparable in divergence studies of *Jatropha* species. The markers generated by RAPD and AFLP can be employed efficiently for interspecific hybrids identification, marker assisted selection and genetic resource management.

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Introduction

Genus *Jatropha* with 172 species having significant economic importance is native to Central America and distributed in Africa and Asia [1]. *Jatropha curcas, J. glandulifera*, *J. gossypifolia*, *J. integerrima*, *J. multifida*, *J. podagrica*, and *J. tanjorensis* are widely distributed in India. *J. curcas*, *J. integerrima*, and *J. glandulifera* are native to America [2]. *J podagrica* is a multipurpose shrub commonly found in Africa, Asia and Latin America [3]. *J. tanjorensis* Ellis & Saroja, reported to be native to India appears in only few districts of Tamil Nadu, generally grown as a hedge plant and reported as natural interspeciefic hybrid between *J. curcas* L. and *J. gossypifolia* L. [4]. *J. multifida* found naturally in Mexico is a popular landscape plant in South Florida [5]. *J. gossypifolia* also called as bellyache bush and is a major weed in Australia [6]. *J. integerrima*, *J. multifida*, *and J. podagrica* are important ornamental plants, whereas, *J. curcas*, *J. glandulifera* have medicinal value [7]. Besides, medicinal value, in the recent past *J. curcas* has emerged as a potential biodiesel crop alternative to petro-diesel [8–13]. However, the crop is characterized by variable and unpredictable yield for reasons, that have not been identified [14] which limit the large-scale cultivation and warrant the need for genetic improvement of the species. Establishing genetic distances through DNA fingerprinting methods and the information generated can be used for genetic improvement of the species.

From the literature it is evident that there are no reports on phylogenetic analysis of *Jatropha*. Therefore, the present investigation was undertaken with the objective to characterize the seven prominently distributed species of *Jatropha* (*J. curcas*, *J. glandulifera*, *J. gossypifolia*, *J. integerrima*, *J. multifida*, *J. podagrica*, and *J. tanjorensis*) in India using RAPD and AFLP DNA fingerprinting techniques, to deduce the phylogenic relationship among these species and identify specific markers that could be useful in identification of interspecific hybrids, genetic improvement of the species and genetic resources management.

Materials and methods

Genomic DNA extraction

Genomic DNA was extracted from three diverged accessions from each species (*J. curcas*, *J. glandulifera*, *J. gossypifolia*, *J. integerrima*, *J. multifida*, *J. podagrica*, and *J. tanjorensis*) established in the genetic garden of Central Salt and Marine Chemicals Research Institute, Bhavnagar, Gujarat, India, using CTAB protocol [15]. 0.1 g of leaf tissue was ground in liquid nitrogen and taken in to a 2 ml microcentrifuge tube. To the ground sample 0.5 ml of extraction buffer (2% CTAB, 100 mM Tris–HCl, 3.5 M NaCl, 20 mM EDTA, 0.2 M β-Mercaptoethanol, 2% PVP, pH 8.0) was added and incubated at 65°C for 90 min. The above sample was extracted with equal volume of Chloroform: Isoamyl alcohol (24:1) and supernatant was transferred in to a new tube. The sample was treated with RNase and extracted with Tris saturated phenol. The supernatant after extraction with Tris saturated phenol was taken and extracted further with chloroform: Isoamyl alcohol (24:1) twice, and precipitated with 80% of ethanol. The pellet was air dried and was dissolved in known volume of Milli Q water.

RAPD analysis

Amplification of RAPD fragments was performed according to Williams et al. [16] using decamer arbitrary primers (Operon technologies Inc, USA; IDT, USA). The reaction was carried out in a volume of 25 µl of reaction mixture containing final concentration of 10 mM Tris–HCl (pH 9.0), 50 mM KCI, 0.1 Triton X-100, 0.2 mM each dNTP, 3.0 mM $MgCl_2$, 0.4 µM primer, 25 ng template, 1 U *Taq* DNA polymerase (Promega, USA). Amplification was performed in programmed thermal cycler (Master cycle epgradient S, eppendorf, Germany) with program of initial denaturation at 94°C for 3 min, 42 cycles of denaturation at 94°C for 30 s, primer annealing at 32°C for 1 min, extension at 72°C for 2.5 min, and final extension at 72°C for 4 min. amplification products were electrophoresed in 1.5% TBE. The gels were stained with ethidium bromide and documented using gel documentation system (Syngene, UK). Experiment with each primer was done three times and those primers gave reproducible fingerprints were considered for data analysis.

AFLP analysis

AFLP analysis system-II kit (Invitrogen life science Ltd., USA) was used for AFLP fingerprinting [17]. The genomic DNA (300 ng) was digested with *Eco*RI and *Mse*I at 37°C for 2 h and digested aliquot was ligated to *Eco*RI and *Mse*I specific adopters at 20°C for 90 min. The ligated DNA was preamplified using *Eco*RI and *Mse*I with one selective nucleotide at the 3′ end primer each. The preamplified product was diluted 1:20 with sterile TE buffer. The diluted product was amplified using primers with three selective nucleotides for *Eco*RI primer and three selective nucleotides for *Mse*I primer at the 3′. PCR was 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 1× TBE buffer in a sequencing gel system (LKB, Sweden). The Gels were stained with silver nitrate using silver staining kit (Sigma, USA). Experiment with each primer combination of *Eco*RI, *Mse*I was done three times and those combinations gave reproducible fingerprints were considered for data analysis.

Data analysis

Acquired RAPD and AFLP fingerprints were individually scored and statistically analyzed by following the assumption that fragment size as a locus was considered as biallelic (present = 1, absent = 0) and made the binary matrix. Only those loci amplified strongly in each instance with reproducibility were scored and included in the analyses ignoring the intensity of the band. Genetic similarity (GS) was calculated according to $GS = 2Nxy/(Nx + Ny)$, where, Nxy was the number of bands shared by two species and Nx and Ny are the number for fragments in each sample. The percentage of polymorphism (PP) was calculated by using formula PP = total number of polymorphic bands/total number of bands multiplied with 100. Phylogenetic trees were constructed according to Jaccard [18] using binary data generated by RAPD and AFLP excluding the intraspecific polymorphic markers, followed by bootstrapping analysis across the loci [19] with the help of statistical analysis software SYSTAT version 12.

Results

Initially 180 RAPD primers (20 primers Kit E, IDT USA; 160 primers, kit-J, K, L, N, O, P, Q, R, Operon technologies Inc., USA) were screened and out of which 52 primers responded with more than six markers were included in the study. In the further screening 33 primers which gave fingerprints with good resolution and band reproducibility were used in the final analysis to characterize *J. curcas*, *J. glandulifera*, *J. gossypifolia*, *J. integerrima*, *J. multifida*, *J. podagrica*, and *J. tanjorensis*. For AFLP DNA fingerprinting initially 64 combinations of *Eco*RI and *Mse*I with three nucleotide selective primers were screened and out of which 27 primer combinations, which gave sharp fingerprints were selected for molecular analysis of above described *Jatropha* species. In total 619 RAPD markers and 1853 AFLP markers were generated and used to study molecular divergence and to deduce phylogenic relation among the species.

RAPD analysis

Use of 33 RAPD primers has produced totally 619 markers out of which 605 markers were found to be polymorphic. Markers obtained for each primer varied form 7 (OPP10) to 44 (IDT E-5). On an average each primer has produced 18.76 markers out of which 18.33 are polymorphic markers. Primer IDT E-4 has produced two common markers in all the species studied, which is the highest number with any primer used in this study. 100% polymorphism was observed when primers IDT5, OPJ13, OPN10, OPN12, OPN16, OPN19, OPO20, OPP15, OPP6, OPQ9, OPJ9, OPL14, OPO7, OPO15, OPP1, OPP5, OPP10, OPQ16, OPQ20, and OPR2 were used. Whereas, use of IDT7, OPJ20, OPL5, OPN20, OPO5, OPQ2, IDT20, OPL7, OPN3, OPQ11, OPQ12, and OPR8 primers resulted one marker each common to all the species studied. Use of primer OPP10 has resulted in lowest number of markers (7) without any common marker and OPQ12 has given lowest number of markers (8) with one marker common to all the species studied and showed lowest PP (87.50). The PP within the species was found to be 1.49 for *J. curcas*, 2.03 for *J. glandulifera*, 3.10 for *J. gossypifolia*, 2.60 for *J. integerrima*, 2.95 for *J. multifida*, 2.18 for *J. podagrica*, 2.18 for *J. tanjorensis* whereas, GS was found to be 0.992 for *J. curcas*, 0.989 for *J. glandulifera*, 0.984 for *J. gossypifolia*, 0.986 for *J. integerrima*, 0.985 for *J. multifida*, 0.988 for *J. podagrica*, for 0.988 *J. tanjorensis.* Intraspecific diversity was found maximum in *J. gossypifolia* and minimum in *J. curcas* on the contrary intraspecific GS was found maximum in *J. curcas* and minimum in *J. gossypifolia*. Over all PP among seven species through RAPD was found to be 97.74 with 14 markers common in all the species studied. In the pair wise comparison the mean PP was 68.48 and GS was 0.48. The highest PP (76.53) and lowest GS (0.38) was found between *J. glandulifera* and *J. tanjorensis*. Lowest PP (54.49) and highest GS (0.63) was found between *J. curcas* and *J. integerrima* (Tables 1, 2).

AFLP analysis

Using 27 combinations of AFLP selective primers 1853 markers were generated out of which 1802 makers were found to be polymorphic. On an average each primer has given 68.63 markers with 66.74 polymorphic markers. Markers obtained for each primer varied form 83 (M-CAT/E-ACA) to 53 (M-CAG/E-ACT). Over all PP among seven species was found to be 97.25. The PP within the species was 2.95 for *J. curcas*, 3.60 for *J. glandulifera*, 5.49 for *J. gossypifolia*, 3.10 for *J. integerrima*, 5.47 for *J. multifida*, 2.32 for *J. podagrica*, 4.28 for *J. tanjorensis.* Whereas the GS was found to be 0.985 for *J. curcas*, 0.981 for *J. glandulifera*, 0.971 for *J. gossypifolia*, 0.984 for *J. integerrima*, 0.971 for *J. multifida*, 0.988 for *J. podagrica*, 0.978 for *J. tanjorensis.* Similar to RAPD, AFLP also showed the maximum diversity in *J. gossypifolia* and minimum in *J. curcas.* The GS was also highest, same as observed with RAPD in *J. curcas* and minimum in *J. gossypifolia*. Use of primer set M-CAT/E-ACA has resulted maximum number of markers (83) with no common marker to all the species indicating 100% polymorphism among species studied. Use of primers combination E-AAC/M-CAA, E-AAC/M-CTG, E-ACC/M-CTG, M-CTG/E-ACTP, E-ACC/M-CTT and E-AGC/M-CAC were also resulted in 100% polymorphic markers. When primers combination M-CAT/E-AAG was used highest numbers of non polymorphic markers (7) were obtained and resulted in lowest PP (90.91). Maximum GS and minimum PP was observed between *J. multifida* and *J. podagrica* followed by *J. podagrica* and *J. integerrima*. Maximum PP and

Table 1 Percentage of polymorphism calculated from RAPD data in different species of *Jatropha*

	J. tanjorensis	*J. glandulifera*	*J. gossypifolia*	*J. multifida*	*J. podagrica*	*J. integerrima*
J. curcas	62.80	71.43	69.95	69.80	69.35	54.49
J. integerrima	71.57	68.73	61.56	69.35	70.08	
J. podagrica	75.12	68.91	69.90	57.02		
J. multifida	76.38	67.99	67.26			
J. gossypifolia	71.57	68.39				
J. glandulifera	76.53					

Table 2 Genetic similarity calculated from RAPD data in different species of *Jatropha*

	J. tanjorensis	*J. glandulifera*	*J. gossypifolia*	*J. multifida*	*J. podagrica*	*J. integerrima*
J. curcas	0.54	0.45	0.46	0.46	0.49	0.63
J. integerrima	0.44	0.48	0.56	0.47	0.46	
J. podagrica	0.40	0.48	0.47	0.60		
J. multifida	0.38	0.49	0.50			
J. gossypifolia	0.44	0.48				
J. glandulifera	0.38					

minimum GS was found between *J. glandulifera* and *J. podagrica*. Mean PP and GS between any two species was found to be 71.33 and 0.44, respectively (Tables 3, 4).

The phylogenetic trees were generated according to Jaccard from the binary data of RAPD and AFLP and followed by bootstrapping across the loci. Both the phylogenetic trees showed similar grouping of the species with minor changes (Figs. 3, 4). The bootstrapping values obtained supported the phylogram obtained in the present study. The phylogram of RAPD showed one major clade with *J. curcas*, *J. integerrima*, and *J. gossypifolia*, with *J. tanjorensis* as a sister clade. In RAPD phylogram *J. curcas*, *J. integerrima* grouped with least distance followed by *J. multifida* and *J. podagrica*. The phylogram of AFLP showed one major clade with five species of *Jatropha* (*J. curcas*, *J. glandulifera*, *J. gossypifolia*, *J. integerrima*, and *J. tanjorensis*) and one minor clade with *J. multifida* and *J. podagrica*. In the AFLP phylogram *J. multifida*, *J. podagrica* grouped with least branch distance, followed by *J. curcas*, *J. integerrima* and then followed by *J. glandulifera* and *J. tanjorensis*.

Discussion

Many studies were carried out to analyze the diversity within and among the population of plant species using allozymes [20, 21]. The limitations with these techniques are low number of markers and pseudo variations [22–27]. Advances in the field of molecular biology have provided many tools for studying the diversity in genome level to get phylogenetic relationship among different species. Out of many PCR based fingerprinting techniques RAPD and AFLP emerged as very useful and efficient methods for analyzing the molecular diversity due to ease of working and efficiently utilized for studying the divergence between different plant species [28–33], within the species [34–36], varieties and also in QTL mapping of interested characters [37, 38].

In the present study RAPD and AFLP fingerprinting techniques were used to analyze the molecular relatedness of seven species of genus *Jatropha* (*J. curcas*, *J. glandulifera*, *J. gossypifolia*, *J. integerrima*, *J. multifida*, *J. podagrica*, and *J. tanjorensis*). Maximum intraspecies PP and minimum GS was found in *J. gossypifolia* followed by *J. multifida* in both RAPD and AFLP analysis. Highest GS and minimum PP was found in *J. curcas* by RAPD analysis. Basha and Sujatha [39] and Ganesh et al. [40] also observed similar results. In AFLP analysis lowest PP was found in *J. podagrica* followed by *J. curcas*. In the present study RAPD analysis showed that *J. glandulifera* is the most diverged species among the *Jatropha* species studied. Ganesh et al. [40] also observed similar results. The maximum GS was found between *J. curcas* and *J. integerrima.* Whereas, Ganesh et al. [40]

Table 3 Percentage of polymorphism calculated from AFLP data in different species of *Jatropha*

	J. tanjorensis	*J. glandulifera*	*J. gossypifolia*	*J. multifida*	*J. podagrica*	*J. integerrima*
J. curcas	68.19	71.75	70.90	71.57	73.47	62.08
J. integerrima	66.02	71.20	63.95	76.01	76.65	
J. podagrica	73.11	78.41	75.50	55.90		
J. multifida	74.45	75.62	74.37			
J. gossypifolia	71.97	74.75				
J. glandulifera	72.05					

Table 4 Genetic similarity calculated from AFLP data in different species of *Jatropha*

	J. tanjorensis	*J. glandulifera*	*J. gossypifolia*	*J. multifida*	*J. podagrica*	*J. integerrima*
J. curcas	0.48	0.44	0.45	0.44	0.42	0.55
J. integerrima	0.51	0.45	0.53	0.39	0.38	
J. podagrica	0.42	0.36	0.39	0.61		
J. multifida	0.41	0.39	0.40			
J. gossypifolia	0.44	0.40				
J. glandulifera	0.44					

reported that the GS between *J. curcas* and *J. integerrima* was second highest however the highest GS reported was within the *J. curcas* germplasm only.

Interspecific hybrids between *J. curcas* and *J. integerrima* [41] and genetic variation between toxic and nontoxic Mexican variety of *J. curcas* [42] were confirmed by using RAPD technique. The extents of genetic diversity at inter and intra population level in *J. curcas* was also studied using RAPD along with ISSR analysis [39]. Our study was to analyze the phylogenetic relationship among different species of *Jatropha* (*J. curcas*, *J. glandulifera*, *J. gossypifolia*, *J. integerrima*, *J. multifida*, *J. podagrica*, and *J. tanjorensis*), which are distributed prominently in India using RAPD and AFLP techniques. The PP among the seven species of *Jatropha* in this study was found to be 97.74 by RAPD and 97.25 by AFLP.

Prabakaran and Sujatha [4] reported *J. tanjorensis* as a natural hybrid between *J. curcas* and *J. gossypifolia* using morphological and biochemical markers as indicators. However, the present result of RAPD and AFLP are not in agreement with the findings since the PP observed between *J. curcas* and *J. tanjorensis* was 62.80 by RAPD and 68.19 with AFLP and between *J. tanjorensis* and *J. gossypifolia* the PP was 71.57 by RAPD and 71.97 by AFLP which are far from the expected PP for any hybrid. Similar results were observed in phylogram analysis also (Figs. 3, 4).

In the karyotyping analysis (*J. curcas*, *J. gossypifolia*, *J. integerrima*, *J. multifeda*, and *J. podagrica*) *Jatropha curcas* and *J. multifida* have similar meiotic configuration and *J. curcas*, *J. podagrica* has meiotic configuration different to other species studied [43]. The present study also showed more or less similar result. The GS was high between *J. curcas* and *J. integerrima* (0.63 by RAPD, 0.55 by AFLP) whose genetic similarities were higher than the mean genetic similarities (0.48 by RAPD and 0.44 by AFLP) of the species studied. The PP was high between *J. curcas*, *J. podagrica* (69.35 by RAPD, 73.47 by AFLP) and the values were higher than the mean PP (68.48 by RAPD and 71.33 by AFLP). Our molecular analysis results support the reason for the successful interspecific hybridization reported between *J. curcas* × *J. integerrima* [42, 44]. From the result RAPD primers IDT E 4 and OPL 5 primers (Fig. 1); and AFLP selective primer combinations M-CAT/E-AAG, M-CAA/E-ACA and M-CAA/E-ACT (Fig. 2), can be efficiently used in differentiating all the seven species and to identify the interspecies hybrids, since these primers resulted in maximum number of polymorphic markers which are specific to species and also non polymorphic markers common to all the species studied. Though PP loci between the most pairs of species for the phylogenetic analysis were showing minor deviation according to the Bussell et al. [45]. However, the results with RAPD and AFLP were similar and supported by bootstrap analysis shows that genetic relationships reported among different species are reliable.

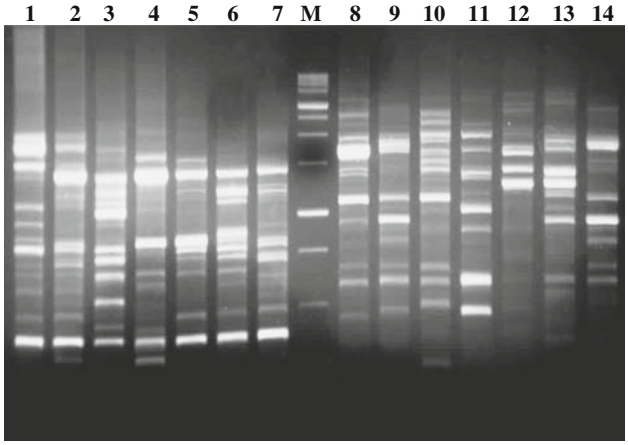

Fig. 1 RAPD profiles of different species of *Jatropha* L. with primer OPL 5 (1–7) and IDT E 4. (8–14). Lane 1 & 8—*J. tanjorensis*; 2 & 9—*J. curcas*; 3 & 10—*J. glandulifera*; 4 & 11—*J. gossypifolia*; 5 & 12—*J. multifida*; 6 & 13—*J. podagrica*; 7 & 14—*J. integerrima*; M—1 Kb DNA ladder

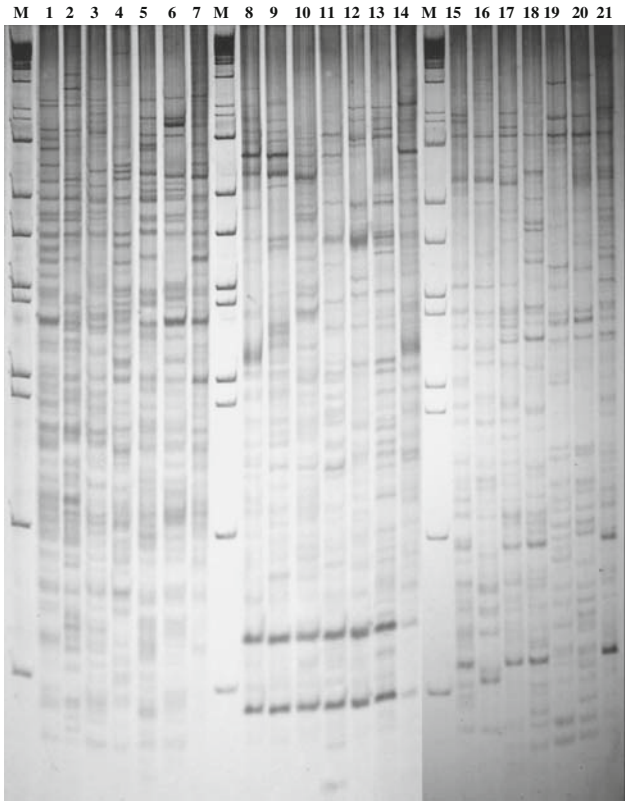

Fig. 2 AFLP profiles of different species of *Jatropha* L. with selective amplification with primers CAT/E-AAG, (1–7); M-CAA/E-ACA (8–14) and M-CAA/E-ACT (8–14). Lane 1, 8 & 15—*J. curcas*; 2, 9 & 16—*J. tanjorensis*; 3, 10 & 17—*J. glandulifera*; 4, 11 & 18—*J. gossypifolia*; 5, 12 & 19—*J. multifida*; 6, 13 & 20—*J. podagrica*; 7, 14 & 21—*J. integerrima*; M—1 Kb + 100 bp DNA ladder mix

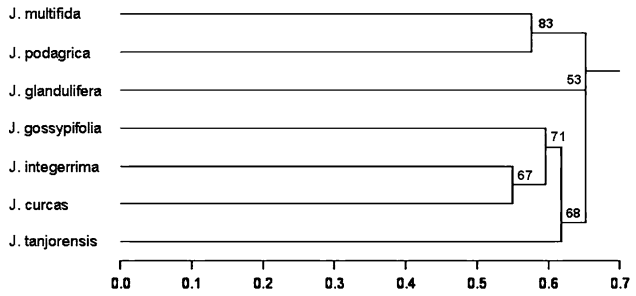

Fig. 3 RAPD-based phylogenetic tree for seven species of *Jatropha* constructed according to Jaccard and tree the supported with bootstrap analysis values

The phylogenetic trees generated using the RAPD and AFLP data showed relatively good correspondence with each other and also were supported by bootstrapping analysis (Figs. 3, 4). In case of RAPD, *J. curcas* and *J. integerrima* showed maximum GS were grouped together with least branch distance were also grouped together in case of AFLP. *J. glandulifera* whose geographical distribution was stated to be wider and has morphological distinctiveness been separated from major and minor clades and the results are in agreement with Ganesh et al. [40]. The highest GS (0.60) shown by *J. multifida* and *J. podagrica* with AFLP analysis were grouped together and showed the least branch distance in AFLP phylogram and same were also grouped together in the RAPD phylogram. *J. tanjorensis* considered to be the natural hybrid by Prabakaran and Sujatha was grouped neither with *J. curcas* nor with *J. gossypifolia* in both the phylograms. This study demonstrated the usefulness of both RAPD and AFLP techniques for molecular characterization and to study the phylogenetic relations between the species of the genus *Jatropha*. This is the first attempt in the phylogenetic analysis of species of *Jatropha* and the study has given a background for genetic relatedness among the seven species studied. The consistency of the result was proved with comparative analysis of both RAPD and AFLP.

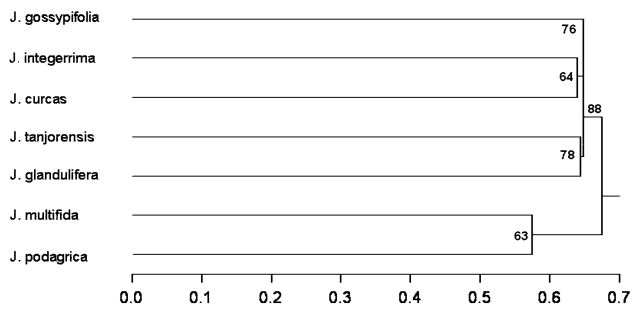

Fig. 4 AFLP-based phylogenetic tree for seven species of *Jatropha* constructed according to Jaccard and tree the supported with bootstrap analysis values

The present investigation will pave way for further intraspecies molecular characterization. The generated molecular markers can be utilized in species differentiation, molecular identification and characterization of interspecific hybrids. The study concludes that both RAPD and AFLP are efficient in diversity studies of *Jatropha*. However, AFLP gives better resolution and marker consistency. The markers generated by RAPD and AFLP can be employed efficiently in interspecific hybrids identification, marker assisted selection and genetic resource management.

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Molecular characterization and identification of markers for toxic and non-toxic varieties of *Jatropha curcas* L. using RAPD, AFLP and SSR markers

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Abstract *Jatropha curcas* L., a multipurpose shrub has acquired significant economic importance for its seed oil which can be converted to biodiesel, is emerging as an alternative to petro-diesel. The deoiled seed cake remains after oil extraction is toxic and cannot be used as a feed despite having best nutritional contents. No quantitative and qualitative differences were observed between toxic and non-toxic varieties of *J. curcas* except for phorbol esters content. Development of molecular marker will enable to differentiate non-toxic from toxic variety in a mixed population and also help in improvement of the species through marker assisted breeding programs. The present investigation was undertaken to characterize the toxic and non-toxic varieties at molecular level and to develop PCR based molecular markers for distinguishing non-toxic from toxic or vice versa. The polymorphic markers were successfully identified specific to non-toxic and toxic variety using RAPD and AFLP techniques. Totally 371 RAPD, 1,442 AFLP markers were analyzed and 56 (15.09%) RAPD, 238 (16.49%) AFLP markers were found specific to either of the varieties. Genetic similarity between non-toxic and toxic verity was found to be 0.92 by RAPD and 0.90 by AFLP fingerprinting. In the present study out of 12 microsatellite markers analyzed, seven markers were found polymorphic. Among these seven, jcms21 showed homozygous allele in the toxic variety. The study demonstrated that both RAPD and AFLP techniques were equally competitive in identifying polymorphic markers and differentiating both the varieties of *J. curcas*. Polymorphism of SSR markers prevailed between the varieties of *J. curcas*. These RAPD and AFLP identified markers will help in selective cultivation of specific variety and along with SSRs these markers can be exploited for further improvement of the species through breeding and Marker Assisted Selection (MAS).

Keywords *Jatropha curcas* L. · Non-toxic · Toxic · RAPD (Random Amplified Polymorphic DNA) · AFLP (Amplified Fragment Length Polymorphism) and Microsatellites

Introduction

Jatropha curcas L., belonging to the family Euphorbeaceae, is native to South America and widely distributed in South and Central Americas, Africa and Asia. *Jatropha curcas* is a multipurpose shrub with significant economic importance and having the capabilities to rehabilitate the degraded lands [1]. Since its seed oil can be converted to biodiesel, it is emerging as a renewable energy source, alternative to petro-diesel. Several reports have demonstrated better performance of the *Jatropha* biodiesel compared with the conventional petro-diesel [1–3]. The short gestation period, easy adaptation to different kinds of marginal and semi marginal lands, drought endurance and avoidance by animals, make this plant species more attractive for cultivation [4–7]. Inspite of best nutritional composition, seed cake obtained from the toxic *J. curcas* remains unutilized as an animal feed due to its toxic nature [8, 9] and no successful attempts are made till now for completely eliminating toxic principle [10]. Globally *J. curcas* is promoted for large acreage cultivation in a big way for biodiesel production

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[11, 12]. Selective cultivation of non-toxic variety reported from Mexico, whose innocuous nature was established [7, 8, 13], will add value to the crop through utilization of deoiled seed cake as a safe animal feed.

Cultivation of non-toxic variety of *J. curcas* could provide oil for biodiesel and deoiled seed cake as a live stock feed [9]. No significant morphological, qualitative and quantitative differences are known between toxic and non-toxic varieties except for the phorbol esters content in the toxic variety [1, 8]. Development of any simple marker will enable identification of non-toxic variety from toxic variety, which will not only add to the quality control for selective cultivation of non-toxic variety but also avoid any toxic adulteration in the animal feeds. In our previous study with multilocus marker systems RAPD and AFLP, we found both the marker systems equally competent [14]. The same marker systems have been selected for molecular characterization, estimation of genetic diversity between toxic and non-toxic varieties, possibility of distinguishing non-toxic from toxic variety or vice versa and identifying polymorphic microsatellite markers for both the varieties. The identified polymorphic markers can be exploit for genetic improvement of the species through breeding and Marker Assisted Selection (MAS).

Materials and methods

Genomic DNA was extracted from six diverged toxic *J. curcas* accessions including one toxic variety collected from Mexico and a Mexican non-toxic variety. The extraction was carried from fresh leaves as described by Pamidiamarri et al. [14]. About 0.1 g of leaf tissue was ground in liquid nitrogen and put in a 2 ml eppendorf tube. To the ground sample 0.5 ml of extraction buffer (2% CTAB, 100 mM Tris–HCl, 3.5 M NaCl, 20 mM EDTA, 0.2 M β-Mercaptoethanol, 2% PVP, pH 8.0.) was added and incubated at 65°C for 90 min. The above sample was extracted with equal volume of Chloroform: Isoamyl alcohol (24:1) and supernatant was transferred to a new tube. The sample was treated with RNase and was extracted with Tris saturated phenol. The supernatant was further extracted with chloroform: Isoamyl alcohol (24:1) twice, and precipitated with 80% ethanol. The pellet was air dried and was dissolved in 100 µl of Milli Q water.

Amplification of RAPD fragments was performed according to Williams et al. [15] using decamer arbitrary primer (Operon Technologies Inc, USA; IDT, USA). The reaction was carried out in a volume of 25 µl containing 10 mM Tris-HCl (pH 9.0), 50 mM KCl, 0.1% Triton X-100, 0.2 mM each dNTPs, 3.0 mM $MgCl_2$, 0.4 µM primer, 25 ng template, 1 unit Taq DNA polymerase (Biogene, USA). Amplification was performed in a thermal cycler (master cycle, eppendorf, Germany) with program of initial denaturation at 94°C for 3 min, 42 cycles of denaturation at 94°C for 30 s, primer annealing at 32°C for 1 min, extension at 72°C for 2.5 min, and final extension at 72°C for 4 min. Amplified products were separated in 1.5% TBE agarose gel. The gels were stained with ethidium bromide and documented using gel documentation system (Syngene, UK). Experiment with each primer was done three times and those primers gave reproducible fingerprints were considered for data analysis.

AFLP analysis system-II kit (Invitrogen Life Science Ltd, USA) was used for AFLP fingerprinting [16]. The genomic DNA (300 ng) was digested with *Eco*RI and *Mse*I at 37°C for 2 h and digested aliquot was ligated to *Eco*RI and *Mse*I specific adapters at 20°C for 3 h. The ligated DNA was preamplified using *Eco*RI and *Mse*I with one selective nucleotide at 3′ of primer each. The pre-amplified product was diluted 1:20 with sterile TE buffer. The diluted product was amplified using primers with three selective nucleotides for *Eco*RI primer and three selective nucleotides for *Mse*I primer at the 3′ end. PCR was 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 appropriate formamide dye was added and subjected to electrophoretic separation on 6% denaturing polyacrylamide gel in 1× TBE buffer in a sequencing gel system (LKB, Sweden). The gels were stained with silver nitrate using silver staining kit (Sigma, USA). Experiment with each primer combination of *Eco*RI and *Mse*I was done three times and those primers which gave reproducible fingerprints were considered for data analysis.

Microsatellite markers were amplified from toxic and non-toxic *J. curcas* varieties in a volume of 25 µl containing 10 mM Tris-HCl (pH 9.0), 50 mM KCl, 0.1% Triton X-100, 0.2 mM each dNTP, 3.0 mM $MgCl_2$, 0.4 µM primer, 25 ng template, 1 unit Taq DNA polymerase (Biogene,USA). Amplification was performed in a thermal cycler (master cycle, eppendorf, Germany) with program of initial denaturation at 94°C for 3 min, 35 cycles of denaturation at 94°C for 30 s, primer annealing (Table 2) for 30 s, extension at 72°C for 40 s, and final extension at 72°C for 4 min. To the PCR product appropriate formamide dye was added and subjected to electrophoretic separation on 10% denaturing polyacrylamide gel in 1× TBE buffer in a sequencing gel system (LKB, Sweden). The gels were stained with silver nitrate using silver staining kit (Sigma, USA).

Acquired RAPD and AFLP finger prints were individually scored and statistically analyzed by assuming that fragment size as a locus was considered as biallelic (present = 1, absent = 0) and made the binary matrix.

Only those loci amplified strongly in each instance with reproducibility were scored and included in the analyses. Genetic similarity was calculated according to $F = 2Nxy/(Nx + Ny)$, where Nxy was the number of bands shared by two species and Nx and Ny are the number for fragments in each sample. Genetic distance was calculated by formula $P = 1 - F$. The percentage of polymorphism was calculated by using formula PP = total number of polymorphic bands/total number of bands multiplied with 100. Percentage of Similarity was obtained by formula PS = 100 − PP. The allele size of the each SSR markers were determined by GeneTool analysis software (Syngene, UK).

Results

Totally 180 RAPD primers were screened and 52 primers those resulted more than 6 scorable bands were selected for further study. In AFLP analysis 64 selective primer combinations were tried and out of them 56 combinations resulted with polymorphic bands between the toxic and non-toxic varieties. For identification of specific markers and to calculate the percentage of polymorphism and genetic similarity between toxic and non-toxic varieties the marker present only in non-toxic but not in toxic accessions and the marker present in all the *J. curcas* toxic accessions but not in non-toxic variety were taken. Out of total 371 RAPD and 1,442 AFLP markers analyzed 56 (15.09%) RAPD and 238 (16.49%) AFLP markers were found to be polymorphic. The percentage of similarity was 84.91 by RAPD and 83.51 by AFLP fingerprinting. Genetic similarity between toxic and non-toxic variety was found to be 0.92 by RAPD and 0.90 by AFLP fingerprinting techniques. The results obtained by both these techniques are comparable and showed the competitive validity of their application in molecular characterization of *J. curcas* varieties.

Out of 52 RAPD primers screened for identification of selective markers between toxic and non-toxic varieties, no polymorphic markers were observed between these two varieties with 13 primers and remaining 39 primers resulted in total 66 polymorphic markers. Primer OPO19 has given highest (5) number of polymorphic markers specific to toxic variety. The lowest number (1) of polymorphic markers was observed with 13 primers IDT E-12, 18, OPJ20, OPL1, OPN3, 8, 12, OPP1, 2, 15, OPQ7, 15 and 20. Use of primer OPQ15 has resulted in one specific marker each to toxic (approximately 810 bp) and non-toxic (approximately 425 bp) variety. Primers IDT E-18, OPL14 resulted in one marker each of approximately 900 and 2,100 bp, respectively; whereas, OPR8 resulted in two specific markers of approximately 1,450 and 700 bp (Figs. 1, 2). In case of AFLP the primers E-ACC/M-CAC combination resulted in maximum number of polymorphic markers (3) each for toxic and non-toxic and minimum marker (1) for non-toxic variety was recorded when primers combination E-AAG/M-CTG was used. Prominently amplified AFLP markers specific to toxic and non-toxic variety and their molecular weights were recorded (Table 1, Fig. 3). In this study 12 SSR markers (personal

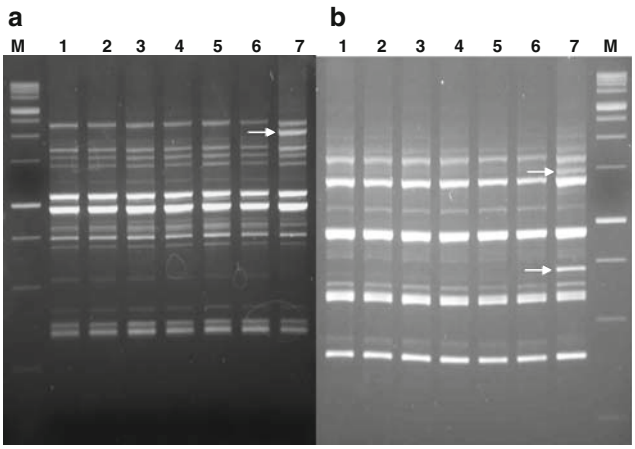

Fig. 2 (**a**) RAPD profile with primer OPL14, (**b**) RAPD profile with primer OPR8; 1–6: Toxic and 7: Non-toxic variety of *J. curcas*; M: 1 kb Marker(Biogene, USA)

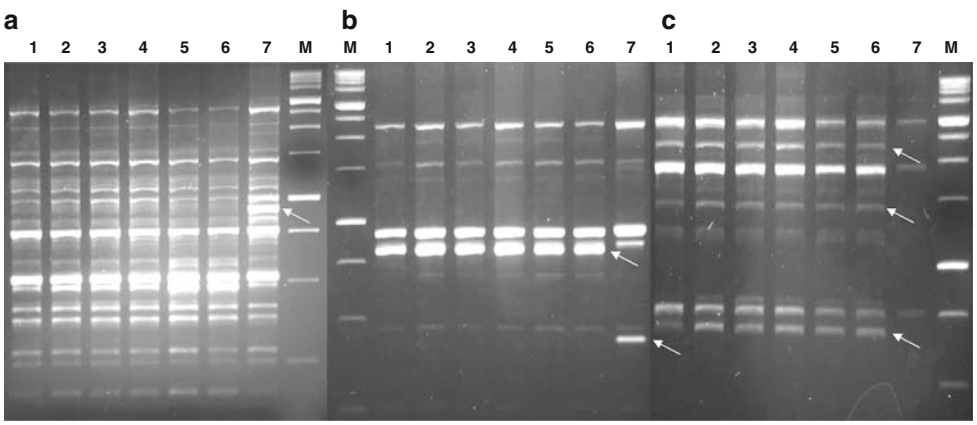

Fig. 1 (**a**) RAPD profile with primer IDT E-18, (**b**) RAPD profile with primer OPQ15, (**c**) RAPD profile with primer OPO19;1–6: Toxic and 7: Non-toxic variety of *J. curcas;* M: 1 kb marker(Biogene, USA)

Table 1 AFLP molecular markers specific to non-toxic and toxic variety of *J. curcas*

AFLP specific primer set combination	Number of specific markers	Molecular weight of specific marker to non-toxic variety (bp)	Molecular weight of specific marker to toxic variety (bp)
E-AAC/M-CAA	3	882, 446	957
E-AAC/M-CAC	1	1054	Nil
E-AAC/M-CAG	2	Nil	559, 85
E-AAC/M-CAT	1	Nil	575
E-AAC/M-CTC	2	Nil	584, 259
E-AAC/M-CTG	1	59	Nil
E-AAC/M-CTT	3	678, 98	484
E-ACC/M-CAA	5	529, 275	916, 373, 279
E-ACC/M-CAC	6	1533, 553, 521	1067, 593, 323
E-ACC/M-CAG	5	98	1435, 1247, 911, 567
E-ACC/M-CAT	1	1427	Nil
E-ACC/M-CTA	1	602	Nil
EACC/M-CTC	4	624, 529, 394	516
E-ACC/M-CTG	1	1612	Nil
E-ACC/M-CTT	5	1341, 1047, 623, 496, 172	Nil
E-AGC/M-CAA	2	926, 441,	Nil
E-AGC/M-CAC	5	665, 624, 150, 251	251
E-AGC/M-CTA	2	64	133
E-AGC/M-CTC	3	293, 91	228
E-AGC/M-CTG	2	633	590
E-AGC/M-CTT	3	1110	502, 213

communication) were analyzed (Table 2) to find out their size polymorphism among non-toxic and toxic varieties. Miss-amplifications were minimized by gradient PCR and optimized the annealing temperature (Table 2). The polymorphic nature of SSR markers were characterized for toxic and non-toxic varieties. The results showed that out of 12 makers tested, 7 (jcms21, jcds24, jcms30, jcps20, jcps21, jcps6, jcps3) found to be size polymorphic. Among the seven markers, jcms21 showed homozygous allele in the toxic variety. The markers, jcms30, jcps21, jcps3 and jcps20 showed at least one allele of same size repeats common in both non-toxic and toxic varieties. The highest size allele polymorphism was found with jcds24, followed by jcms30 and jcps6 (Fig. 4, Table 2).

Discussion

Jatropha curcas, a multipurpose shrub, has acquired high agro-industrial significance globally because of its seed oil which is a potential source of biodiesel and also for its beneficial by-products [3–6]. The seed cake remaining after oil extraction is toxic and owing to the presence of different toxic substances in seeds, oil and deoiled cake are not suitable for animal consumption [9] and cannot be used as fodder/feed despite having best protein composition and favorable amino acid profile [17]. Short gestation period, easy adaptation to different kinds of marginal and semi marginal lands, drought endurance and avoidance by animals, made the species more attractive for cultivation [4–7]. Though various processing techniques have been attempted to detoxify, no treatment has been successful in completely eliminating the toxic principles of defatted kernel meal [10]. In addition to common toxic cultivar, a non-toxic *J. curcas* has been reported from Mexico whose innocuous nature was established [7, 8, 13]. However, no qualitative and quantitative differences were reported between these two cultivars. Development of any simple marker will enable identification of non-toxic variety from toxic variety which will not only add the quality control for selective cultivation of non-toxic variety, but also avoid any toxic adulteration in the animal feeds.

In the present study a non-toxic Mexican variety was compared with five accessions of *J. curcas* collected from different geographical regions of India and one Mexican toxic variety using RAPD and AFLP technique and identified markers specific to non-toxic and toxic varieties. Sujatha et al. [12] reported 94.6% of similarity between toxic and non-toxic varieties using RAPD fingerprinting whereas, in the present study the percentage of similarity was 84.91 by RAPD and 83.59 by AFLP fingerprinting. Using the RAPD primers IDT E-18, OPL14 and AFLP

selective primer combination E-ACC/M-CAC resulted in polymorphic markers for both toxic and non-toxic varieties can be used for identification. Though the marker related studies for toxic *J. curcas* has been reported using ISSR [18]; in the present study markers were identified specific to both toxic and non-toxic varieties using RAPD and AFLP fingerprinting which have better application in molecular breeding studies.

From the last one decade microsatellites have become one of the most popular molecular markers utilized in different fields. High polymorphism and the relative ease of scoring represent the two major features that make microsatellites of large interest for many genetic studies. Soon after their first description [19–21] SSRs were being widely employed in many fields because of their high variability which made them very powerful genetic markers. Microsatellites have proven to be an extremely valuable tool for genome mapping in many organisms [22, 23]. In the present study, 12 markers isolated [personal communication] (Table 2) showed average length of the microsatellites between 75 and 250 bps which is similar to results reported in other species [24]. Among 12 markers analyzed, seven markers found polymorphic, which implies the high polymorphism of microsatellites between two varieties and their applicability in MAS and QTL analysis studies.

This is the first report on molecular characterization of non-toxic and toxic *J. curcas* using both multilocus and single locus maker systems. The specific markers generated using RAPD and AFLP fingerprinting will help to distinguish non-toxic from toxic varieties of *J. curcas* or vice versa and markers of RAPD and AFLP together with the

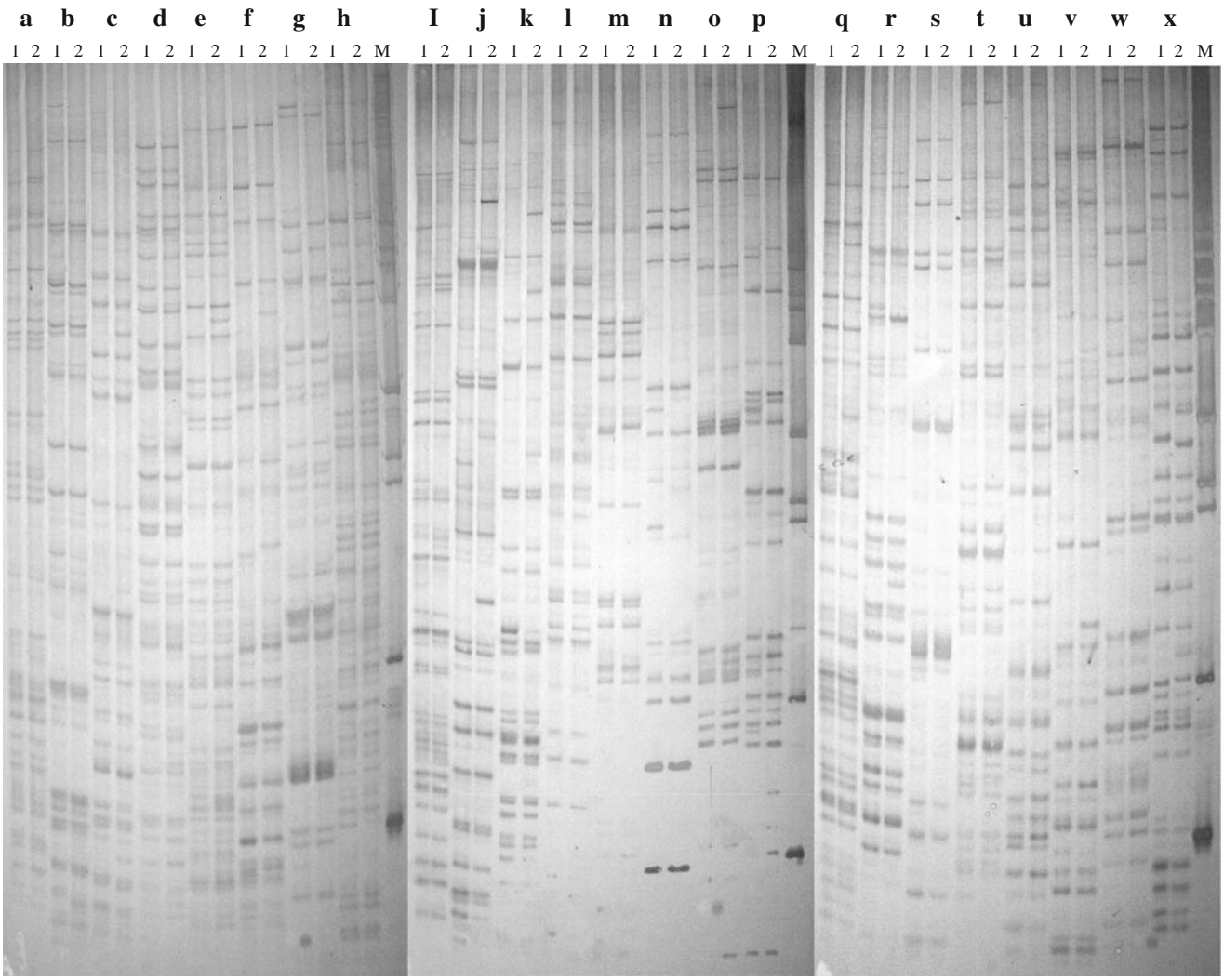

Fig. 3 1-Non-toxic variety of *J. curcas*, 2-Toxic variety of *J. curcas*. (**a**–**x**) Selective amplification with AFLP primers. (**a**) E-AAC/M-CAA; (**b**) E-AAC/M-CAC; (**c**) E-AAC/M-CAG; (**d**) E-AAC/M-CAT; (**e**) E-AAC/M-CTA; (**f**) E-AAC/M-CTC; (**g**) E-AAC/M-CTG; (**h**) E-AAC/M-CTT; (**i**) E-ACC/M-CAA; (**j**) E-ACC/M-CAC; (**k**) E-ACC/M-CAG; (**l**) E-ACC/M-CAT; (**m**) E-ACC/M-CTA; (**n**) E-ACC/M-CTC; (**o**) E-ACC/M-CTG; (**p**) E-ACC/M-CTT; (**q**) E-AGC/M-CAA; (**r**) E-AGC/M-CAC; (**s**) E-AGC/M-CAG; (**t**) E-AGC/M-CAT; (**u**) E-AGC/M-CTA; (**v**) E-AGC/M-CTC; (**w**) E-AGC/M-CTG; (**x**) E-AGC/M-CTT)

Table 2 Characteristics of 12 microsatellite loci in non-toxic and toxic variety of *J. curcas*

Marker GenBank No.	Primer sequence(5′–3′)	T_a (°C)	Repeat motif	Allele size in range non-toxic (bp)	Allele size in Toxic (bp)
jcds10 (EU586340)	F:CATCAAATGCTAATGAAAGTACA R:CACACCTAGCAAACTACTTGCA	46.5	$(TG)_6CACGCA(TG)_4$	108/122	108/122
jcds24 (EU586341)	F:GGATATGAAGTTTCATGGGACAAG R:TTCATTGAATGGATGGTTGTAAGG	51.0	$(CA)_5(TA)_8(CA)_4…(TA)_3GA(TA)_4$	204/210	204/216
jcds41 (EU586342)	F: AACACACCATGGGCCACAGGT R:TGCATGTGTGCGGGTTTGATTAC	56.5	$(CA)_6(TA)_2$	102/114	102/114
jcds58 (EU586343)	F:TCCATGAAGTTTGCTGGCAAT R:AGGTCATCTGGTAAAGCCATACC	54.0	$(GT)_4(GA)_5$	104/112	104/112
jcds66 (EU586344)	F:CCTACGAGTGATTGGATAGTTTCTCA R:TCTTCCATCAAGAGTCGTTGGGCA	54.0	$(CT)_2(GT)_3ATTGCA(AT)_4$	216/228	216/228
jcps1 (EU586345)	F:GAGGATATTACAGCATGAATGTG R:5′AATCAATCAATCTTTGGCAAA	47.5	$(TG)_4…(GT)_3…(GT)_4$	132/162	132/162
jcps6 (EU586346)	F:CCAGAAGTAGAATTATAAATTAAA R:AGCGGCTCTGACATTATGTAC	44.0	$(AT)_3G(TA)_3…(CT)_3…(GT)_5CT(GT)_3$	288/305	288/380
jcps9 (EU586347)	F:GTACTTAGATCTCTTGTAACTAACAG R:TATCTCTTGTTCAGAAATGGAT	48.0	$(GT)_3GC(TG)_2A(GT)_3$	140/132	140/132
jcps20 (EU586348)	F:ACAGCAAGTGCACAACAATCTCA R:TACTGCAGATGGATGGCATGA	55.0	$(TG)_{12}(GA)_{22}$	271/260	260/278
jcps21 (EU586349)	F:CCTGCTGACAGGCCATGATT R:TTTCACTGCAGAGGTAGCTTGTATA	54.8	$(CA)_2…(CA)_4$	189/200	189/208
jcms21 (EU586350)	F:TAACCTCTTCCTGACA R:ATAGGAAATAAGAGTTCAAA	43.0	$(CA)_7$	81/89	75
jcms30 (EU586351)	F:GGGAAAGAGGCTCTTTGC R:ATGAGTTCACATAAAATCATGCA	48.5	$(GT)_5T(TG)_2$	135/144	144/148

T_a, annealing temperature; bp, basepairs

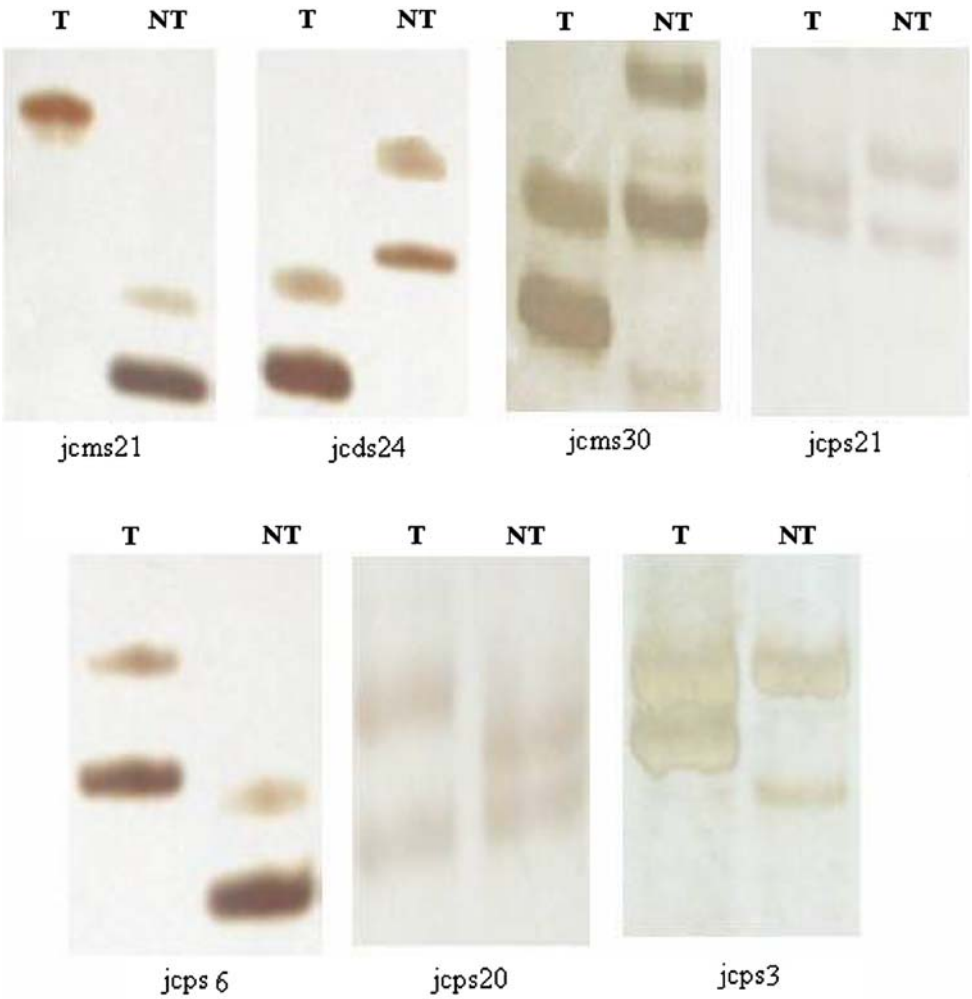

Fig. 4 Size polymorphism SSRs in toxic (T) and non-toxic (NT) variety of *J. curcas* (jcms21, jcds24, jdms30, jcps21, jcps6, jcps20 and jcps3)

polymorphic SSR markers can be exploited in Marker Assisted Selection (MAS) QTL analysis and for other molecular breeding studies.

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Effects of NaCl on growth, ion accumulation, protein, proline contents and antioxidant enzymes activity in callus cultures of *Jatropha curcas*

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Abstract: *Jatropha curcas* is an oil bearing species with multiple uses and considerable economic potential as a biofuel crop. The effect of NaCl stress on growth, ion accumulation, contents of protein, proline, and antioxidant enzymes activity in callus cultures of *J. curcas* was investigated. Exposure of callus to NaCl decreased growth in a concentration dependent manner. NaCl treated callus accumulated Na and declined in K, Ca and Mg contents. Na/K ratio increased steadily as a function of external NaCl treatment. NaCl induced significant differences in quality and quantity of proteins, whereas, proline accumulation remained more or less constant with treatment. NaCl stress enhanced the activity of superoxide dismutase (SOD; E.C. 1.15.1.1) and peroxidase (POX; E.C. 1.11.1.7). Further in the isoenzyme studies, four SOD isoenzymes (SOD 1, 2, 3, and 4) and two POX isoenzymes (POX 1 and 2) were detected with the treatment. NaCl strongly induced activity of SOD 4 isoenzyme in 40, 60, 80 mM and POX 2 isoenzyme in 40 and 80 mM NaCl concentrations. Increase in antioxidant enzymes activity could be a response to cellular damage induced by NaCl. This increase could not stop the deleterious effects of NaCl, but it reduced stress severity and thus allowed cell growth to occur.

Key words: *Jatropha curcas*; antioxidant enzymes; ion accumulation; NaCl; proline; proteins

Introduction

Salinity is a major impediment in irrigated agriculture especially in the arid and semiarid environment. Today, ∼20% of the world's cultivated land and nearly half of the irrigated land is affected by salinity (Zhu 2001). Growing demand for food and plant products to feed the expanding world population with ever decreasing soil resources and dwindling fresh water supplies warrants the need for biological and technical solutions to overcome the physiological limitations, which restrict crop productivity. This demands the extension of cultivation on uncultivable degraded/saline soils which are not suitable for conventional agriculture (Reddy & Iyengar 1999).

High salinity levels in the external medium are known to affect many physiological and metabolic processes leading to growth reduction. High concentration of NaCl causes ion imbalance and osmotic stress in many plant species (Maggio et al. 2000). As a consequence of these primary effects, secondary stresses such as oxidative damage occur (Zhu 2001). Plants with high levels of antioxidant activity was reported to have a greater resistance to oxidative damage (Hernandez et al. 2000; Cherian & Reddy 2003; Elkahoui et al. 2005; Niknam et al. 2006). Cell destruction factors like superoxide anion (O_2^-), hydroxyl radical ($^{\bullet}OH$), singlet oxygen ($^1O_2$) and hydrogen peroxide (H_2O_2) raised due to NaCl stress are controlled mainly by antioxidant enzymes (Elkahoui et al. 2005).

In vitro culture technique serves as a useful tool to study the salt stress response of undifferentiated callus to salinity in controlled and uniform conditions, thus, avoiding complications arising from physiological and structural variability of the whole plant (Piqueras et al. 1996; Bajji et al. 1998; Elkahoui et al. 2005) and means of rapid selection and improvement for salinity tolerance. The plant cell culture studies also allow isolation and selection of salt tolerant lines to elucidate mechanism of tolerance operating at cellular level (McCoy 1987; Piqueras et al. 1996; Bajji et al. 1998; Cherian & Reddy 2003; Elkahoui et al. 2005; Niknam et al. 2006) and the possibility of developing salt tolerant lines (Petrusa & Winicov 1997).

Jatropha curcas (purge nut/physic nut) belongs to the family Euphorbiaceae and is distributed in arid and semi arid areas of South America and all tropical regions. *J. curcas* recently received tremendous attention for its seed oil that can be converted into biodiesel and is considered to be a universally acceptable energy crop

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(Takeda 1982). It is reported that the species grows in areas with extreme climates and soil conditions that could not be inhabited by most of the agriculturally important plant species (Francis et al. 2005). However, there are no reports on the salt tolerance of *J. curcas* neither at cell nor at whole plant level. Therefore, the present study was undertaken to evaluate the changes in the activity of enzymes involved in oxidative metabolism (SOD and POX), accumulation of ions and proline involved in osmoregulation in *J. curcas* callus exposed to different concentrations of NaCl with a view to assess the possibility of improving salt tolerance in *J. curcas*.

Materials and methods

Callus culture and growth determination

Callus of *J. curcas* was produced from in vitro grown leaf on MS (Murashige & Skooge 1962) medium (pH 5.8) containing 0.70% agar, 5 mg/L 6-benzyl amino purine (BAP) and 1 mg/L naphthalene acetic acid (NAA) and sterilized by autoclaving at 1.05 kg /cm^2 pressure and 121℃ for 20 min. Friable callus was sub cultured for every 4 weeks after initiation and grown for another 4 weeks before evaluated for salinity tolerance. At the beginning of experiment, callus (100 mg) was transferred to fresh medium without or with the different NaCl concentrations (20, 40, 60, 80, 100 mM) and allowed to grow at 25 ± 2℃ in dark. After four weeks, the callus was washed thoroughly with distilled water to remove the adhering ions, the moisture was removed by blotting and fresh mass was taken. Dry mass was determined after drying the callus at 60℃ for 48 h to a constant mass. Water content as a percentage of fresh mass was calculated.

Determination of ions content

Oven-dried callus was digested in 10 mL of HNO_3:$HClO_4$ (10:4) at 60℃. After complete digestion of the sample, the final volume was adjusted to 50 mL with distilled water and contents of Na, K, Ca and Mg were determined by inductively coupled plasma emission mass spectroscopy (ICP, Perkin-Elmer).

Proline extraction and estimation

Free proline content was determined according to Bates et al. (1973). 200 mg of fresh callus was homogenized in 4 mL of 3% aqueous sulphosalicylic acid and centrifuged at 10 000 *g* for 10 min to remove debris. 2 mL of the supernatant was mixed with 2 mL of acid ninhydrin (625 mg ninhydrin in 15 mL glacial acetic acid and 10 mL of 6 M orthophosphoric acid) and 2 mL glacial acetic acid in a test tube and boiled at 100℃ for 1 h. The reaction was stopped by cooling the tubes in an ice bath. The chromophore formed was extracted with 6 mL of toluene and the absorbance of the resulting organic layer was measured at 520 nm (Shimadzu UV-160A, Japan). The concentration of proline was estimated by referring to a standard curve prepared using L-proline.

Enzyme extraction and assays

Callus (100 mg) was homogenized at 4℃ with 2 mL of extraction buffer [(200 mM Tris-HCl, 10 mM ethylenediaminetetraaceticacid (EDTA), 0.5 M sucrose, 2 mM phenylmethylsulfonyl fluoride (PMSF) and 1.0% insoluble polyvinylpyrrolidone (PVP) (pH 8.5)]. The homogenate was sonicated for 5 min and centrifuged at 20 000 g for 15 min. The supernatant obtained was used for estimation of protein content (Bradford 1976) and enzymes activity.

The activity of superoxide dismutase (SOD; E.C. 1.15.1.1) was assayed by measuring its ability to inhibit the photochemical reduction of nitroblue tetrazolium (NBT) according to the method of Beauchamp & Fridovich (1971). The reaction mixture (3 mL) contained 50 mM of phosphate buffer (pH 7.8), 13 mM of methionine, 75 μM of NBT, 0.1 mM of EDTA, 2 μM of riboflavin and 0.1 mL of enzyme extract. Samples were illuminated using two 15 W fluorescent lamps for 10 min. The absorbance of reaction mixture was read at 560 nm. A non-irradiated reaction mixture served as control. Log A_{560} was plotted as function of the volume of enzyme extract used in the reaction mixture (Giannopolitis & Ries 1977). In the resultant graph the volume of enzyme extract corresponding to 50% inhibition of the reaction was considered as one enzyme unit.

Peroxidase (POX; E.C. 1.11.1.7) activity was measured according to Shannon et al. (1966) by following the change in absorbance at 460 nm due to o-dianisidine oxidation in the presence of H_2O_2 and enzyme. The assay mixture consisted of 2.8 mL of O-dianisidine-buffer (16 mL of 0.5% (w/v) o-dianisidine, 48 mL of 0.6 M sodium acetate, pH 5.5, and 416 mL of H_2O), 0.1 mL of H_2O_2 (1% w/v) and 0.1 mL of enzyme extract. The amount of enzyme required to change the absorption (Δ OD) by 0.01 min^{-1} mg^{-1} protein was taken as unit enzyme activity.

Separation of isoenzymes of SOD and POX were performed by Native PAGE (7.5% polyacrylamide) by loading 50 μg of protein for POX and 100 μg for SOD. The gels were run in an electrode buffer composed of 0.025 M Tris and 0.192 M glycine (pH 8.8) for 3 h at 4℃ at a constant current of 30 mA. After electrophoresis, the gels were stained for SOD (Misra & Fridovich 1977) by incubating in a solution containing 2.5 mM NBT for 25 min followed by incubation in 50 mM potassium phosphate buffer (28 μM riboflavin and 28 mM EDTA, pH 7.8) for 20 min in dark and then exposed to light for 15 min. The gels were stained for POX (Gulati 1989) by incubating in 0.02 M acetate buffer (pH 5.5) for 10 min and then transferred to fresh 0.02 M acetate buffer (pH 5.5) containing 0.66% guaiacol. After 5 min of incubation 3% H_2O_2 was added drop-wise till desired contrast was obtained and the reaction was stopped by replacing the solution with distilled water.

The total protein equivalent to 25 μg was subjected to SDS-PAGE (12.5% polyacrylamide) at 24℃ for 3 h at a constant current of 30 mA (Laemmli et al. 1970). The protein gels were stained with silver nitrate (Damerval et al. 1987) and relative molecular weights were determined by running standard molecular weight marker mix (Sigma, USA)

The data are based on mean of minimum five independent experiments. All the data were subjected to two-way analysis of variance and significance was determined at 95% confidence level.

Results

Effect of NaCl on callus growth

NaCl inhibited the growth of callus at all concentration and the inhibition was proportional to NaCl concentration used in the medium. When callus was cultured in the presence of 20 mM NaCl, a reduction of 18% fresh mass and 43% dry mass was observed as compared to control. Further reduction of 56, 71, 73, and 74% fresh

Table 1. Effect of different NaCl concentration on growth, protein and proline content in callus cultures of *J. curcas*. Mean ± SE, $n = 5$.

NaCl (mM)	Fresh mass (FM) [g]	Dry mass (DM) [g]	Protein [mg g^{-1}FM]	Proline [μg g^{-1}FM]	Water [%]
0	6.43 ± 0.33	0.67 ± 0.06	12.63 ± 0.21	5.14 ± 0.06	89.60 ± 0.45
20	5.25 ± 0.33	0.38 ± 0.02	15.31 ± 0.29	6.05 ± 0.05	92.83 ± 0.35
40	2.82 ± 0.20	0.15 ± 0.015	18.38 ± 0.52	6.05 ± 0.05	94.46 ± 0.92
60	1.90 ± 0.18	0.10 ± 0.015	20.59 ± 0.72	5.75 ± 0.07	94.33 ± 0.70
80	1.76 ± 0.09	0.11 ± 0.011	15.71± 0.31	5.14 ± 0.06	93.43 ± 0.94
100	1.70 ± 0.05	0.11 ± 0.011	8.17 ± 0.10	5.09 ± 0.04	93.10 ± 0.80

Table 2. Effect of different NaCl concentration on accumulation of Na, K, Ca and Mg (mg g^{-1} DM) in callus cultures of *J. curcas*. Mean ± SE, $n = 5$.

NaCl (mM)	Na	K	Ca	Mg	Na/ K
0	19.2 ± 2.33	29.85 ± 3.33	9.8 ± 1.01	5.00 ± 2.11	0.64
20	24.96 ± 3.21	23.85 ± 3.99	7.8 ± 1.11	4.77 ± 1.01	1.05
40	32.00 ± 3.56	18.20 ± 2.77	6.99 ± 0.99	5.21 ± 1.11	1.75
60	36.00 ± 4.01	15.85 ± 2.11	5.01 ± 0.88	5.11 ± 1.55	2.27
80	47.50 ± 5.78	14.43 ± 1.88	4.09 ± 1.00	4.78 ± 0.99	3.29
100	57.17 ± 6.11	13.01 ± 1.09	3.11 ± 0.56	4.88 ± 0.88	4.39

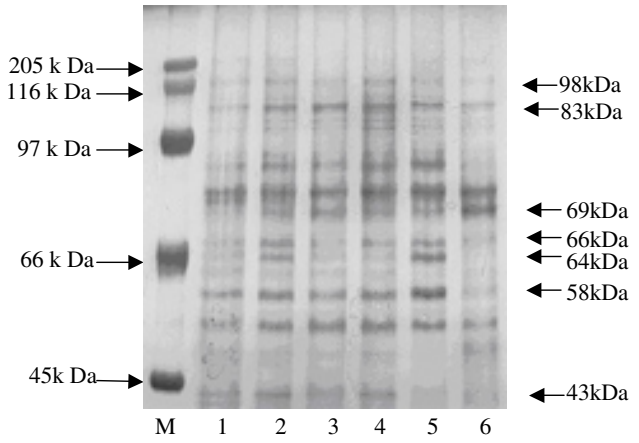

Fig. 1. SDS-PAGE profile of proteins in callus cultures of *J. curcas* under different concentration of NaCl: lane 1, 2, 3, 4, 5 and 6; 0, 20, 40, 60, 80 and 100 mM NaCl treated callus respectively and M represent molecular marker.

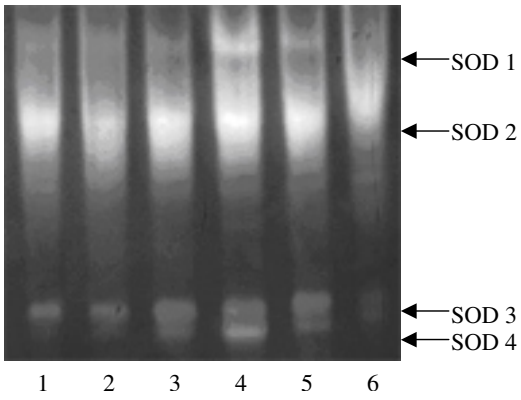

Fig. 2. Activity staining of SOD in callus cultures of *J. curcas* under different concentration of NaCl: lane 1, 2, 3, 4, 5 and 6; 0, 20, 40, 60, 80 and 100 mM NaCl treated callus respectively.

mass and 63, 85, 83 and 83% dry mass occurred at 40, 60, 80, and 100 mM NaCl concentrations respectively. However, no significant differences were recorded for water content with NaCl treatment (Table 1).

Effect of NaCl on ions accumulation

Na accumulation increased and K content declined as a function of external NaCl treatment. In parallel with Na accumulation and a decline in K content, the Na/K ratio increased at all concentrations of NaCl, while Ca and Mg contents decreased with the increase in NaCl concentration (Table 2).

Effect of NaCl on quantitative and qualitative differences in proteins and proline accumulation

NaCl stress resulted in accumulation of soluble protein. An increase of 21, 45, 63, and 24% was observed at 20, 40, 60, and 80 mM NaCl respectively over control. At the presence of NaCl 100 mM, the protein content declined to 35% as compared to control (Table 1). Qualitative differences in expression of proteins were also observed with increase in the NaCl concentration (Fig. 1). The intensity of the 98, 69, 66 and 64 kDa proteins increased in NaCl treated callus as compared to control, whereas, the intensity of the 83, 58 and 43 kDa proteins decreased. In contrast to proteins, no significant change was observed in proline content at all NaCl concentrations studied (Table 1).

Effect of NaCl on the activity and isoenzyme patterns of antioxidant enzymes

An increase in the activity of both SOD and POX was observed as a function of external NaCl concentration (Table 3). According to PAGE analysis three SOD isoenzymes were observed in control cells (SOD 1, SOD 2, and SOD 3). NaCl induced one more isoenzyme

Table 3. Effect of different NaCl concentration on the activity of superoxide dismutase (SOD) and peroxidase (POX) in callus cultures of *J. curcas*. Mean ± SE, $n = 5$.

NaCl (mM)	Superoxide dismutase [U mg^{-1}(protein) min^{-1}]	Peroxidase [U mg^{-1}(protein) min^{-1}]
0	1.47 ± 0.03	8.49 ± 0.35
20	1.72 ± 0.04	15.78 ± 1.07
40	2.33 ± 0.05	14.40 ± 0.79
60	2.11 ± 0.03	14.99 ± 0.99
80	1.85 ± 0.02	14.61 ± 0.57
100	1.41 ± 0.01	27.61 ± 2.43

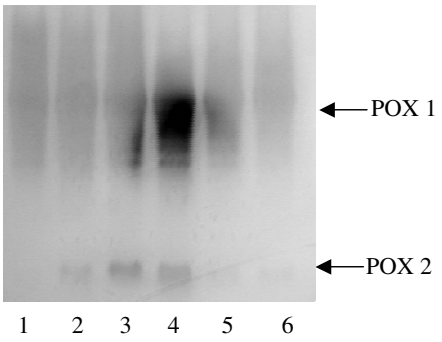

Fig. 3. Activity staining of POX in callus cultures of *J. curcas* under different concentration of NaCl: lane 1, 2, 3, 4, 5 and 6; 0, 20, 40, 60, 80 and 100 mM NaCl treated callus respectively.

(SOD 4) and all the isoenzymes were more prominent, which was in agreement with the spectrophotometric assay (Fig. 2). As compared to one isoenzyme (POX 1) in control two isoenzymes (POX 1 and POX2) were observed in NaCl treated callus. At 40, 60 and 80 mM NaCl treatment the isoenzyme bands appeared more prominent as it was observed for SOD (Fig. 3).

Discussion

Plant cell culture is a useful technique for the study of responses to environmental stress at cell level. In the present study it was observed that NaCl provoked an inhibition of cell growth, which may be due to nutritional imbalance, osmotic and metabolic disturbances. Elkahoui et al. (2005) in *Catharanthus roseus*, Cherian & Reddy (2003) in *Suaeda nudiflora*, Niknam et al. (2006) in *Trigonella* species, and Shibli et al. (2007) in *Lycopersicon* species observed similar results. Na accumulation in tissues under salinity stress is generally considered as a major factor behind the adverse effect of salinity on nutrient uptake and growth (Shibli et al. 2001). The increase in Na content of cells was accompanied by a decrease in K accumulation and differences in Na/K ratio under saline conditions (Cherian & Reddy 2003). In the present study Na/K ratio increased as a function of external NaCl concentration. Potassium takes part in many enzymatic activities in plant cell and maintaining cystosolic Na/K ratio is a key requirement for growth under high saline conditions (Apse et al. 1999). The decrease in Ca accumulation observed in our study may be due to displacement of Ca from cell membrane by Na (Cramer et al. 1985; Shibli et al. 2007). Decreased Mg accumulation under saline condition is in agreement with earlier results (Shibli et al. 2007). The decrease in protein content observed at the higher NaCl concentration (100 mM) in nutrient medium may be due to decrease in the synthesis of protein (Hall & Flowers 1973) or stimulation of protein hydrolysis (Uprety & Sarin 1976) whereas, the increase in protein content at 20, 40, 60 and 80 mM may be attributed to stress induced synthesis of proteins (Cherian & Reddy 2003). NaCl treatment resulted in qualitative changes in proteins and increased the expression of 98, 69, 66 and 64 kDa proteins whereas, 83, 58 and 43 kDa proteins decreased with increase in NaCl concentration. These results are in accordance with Niknam et al. (2006). Accumulation of proline as a compatible solute has been reported although the increase of the proline pool upon salt treatment does not seem to be a general phenomenon (Stewart & Lee, 1974). The significance of proline accumulation in osmotic adjustment is still debated and varies according to the species (Meloni et al. 2004). No significant differences in proline accumulation due to NaCl treatment observed in the present study are in agreement with the results of Feitosa de Lacerda et al. (2001). NaCl provoked a dose dependent increase in SOD activity in *J. curcas* callus which could represent a defense mechanism against NaCl induced generation of superoxide anion (O_2^-), hydroxyl radical ($^{\bullet}OH$), singlet oxygen ($^1O_2$). Increased activity of SOD under saline conditions was also reported (Elkahoui et al. 2005; Cherian & Reddy 2003). The NaCl induced increases in POX activity in callus culture of *J. curcas* indicate that these cells have a higher capacity for the decomposition of H_2O_2 generated by SOD (Hernandez et al. 2000; Cherian & Reddy 2003; Niknam et al. 2006), which may be required for preventing the peroxidation of membrane lipids generated by salt stress. Induction of SOD and POX isoenzyme in native PAGE, increase in their intensities and activity under saline conditions might be a useful adaptation in the defense mechanism of *J. curcas* against oxidative damage (Piqueras et al. 1996).

This study has revealed that NaCl induced an oxidative stress in *J. curcas* callus, the increase in the activity of SOD and POX may not be enough to eliminate the deleterious effects provoked by NaCl and only alleviated the impact of stress for survival of callus.

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Comparative Response of Callus and Seedling of *Jatropha curcas* L. to Salinity Stress

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ABSTRACT : *Jatropha curcas* L. is an oil bearing species with many uses and considerable economic potential as a biofuel crop. Salt stress effect on growth, ion accumulation, contents of protein, proline and antioxidant enzymes activity was determined in callus and seedling to understand the salt tolerance of the species. Exposure of callus and seedling to salt stress reduced growth in a concentration dependent manner. Under salt stress Na content increased significantly in both callus and seedling whereas, differential accumulation in the contents of K, Ca, and Mg was observed in callus and seedling. Soluble protein content differed significantly in callus as compared to seedling; however proline accumulation remained more or less constant with treatments, however, proline concentration was ~2 to 3 times more in callus than in seedling. Salt stress induced qualitative and quantitative differences in superoxide dismutase (SOD; E.C. 1.15.1.1) and peroxidase (POX; E.C. 1.11.1.7) in callus and seedling. Salt induced changes of the recorded parameters were discussed in relation to salinity tolerance.

Keywords : Antioxidant enzymes, *Jatropha curcas*, Seedling, Callus, Salt

INTRODUCTION

Soil salinity is an inevitable impediment for agriculture world wide. Excess salinity in the soil has devastating effects on plant growth, crop yield and even leading to complete crop failure in the worst affected areas (Owens, 2001). It is estimated that salinity limits the production of nearly 40% of agricultural lands world over (Serrano and Gaxiola, 1994). Growing demand for food and plant products to feed the expanding world population, with ever decreasing soil resources and dwindling fresh water supplies warrants the need for biological and technical solutions to overcome the physiological limitations, which restrict crop productivity (Reddy and Iyengar, 1999). This demands the extension of cultivation on uncultivable degraded/ saline soils, which are not suitable for conventional agriculture, identification of salt tolerant crops or increase salt tolerance in existing crops. Despite advances in increasing plant productivity and resistance to number of pests and diseases, improving salt tolerance in crop plants remain elusive, mainly because salinity simultaneously affects several aspects of plant physiology. To achieve salt tolerance three interconnected aspects of plant activity are important; (1) damage must be prevented, (2) homeostatic conditions must be re-established and (3) growth must resume (Zhu, 2001). Salt stress causes ionic imbalance (Zhu et al., 1997), with excess sodium and chloride ions having a deleterious effect on many cellular systems (Serrano et al., 1999) therefore, plant survival and growth depends on adaptations to re-establish homeostasis, osmoregulation and oxidative damage (Cherian and Reddy, 2003; Elkahoui et al., 2005; Niknam et al., 2006). To cope up with salt stress, plant responds with physiological and biochemical changes (Hernandez et al., 1993; Lutts et al., 1996; Cherian and Reddy, 2003; Elkahoui et al., 2005; Niknam et al., 2006). These changes aim at retention of water in spite of high external osmoticum and maintenance of metabolic activities (Hasegawa et al., 2000).

In vitro culture technique avoids physiological and structural complexities of the plant (Bajji et al., 1998) and provides an important tool for studying salt effects and its mechanisms in plants (McCoy, 1987; Chen et al., 1998).

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It is also reported that both cellular and whole plant mechanisms are equally important in elucidating salt tolerance mechanisms (Dracup, 1991; Bajji et al., 1998; Niknam et al., 2006). However, there were relatively few studies comparing responses of intact higher plants and their tissues (Dracup, 1991; Naik and Widholm, 1993; Gulati and Jaiwal, 1994), even fewer addressed physiological changes during salt stress (Cherian and Reddy 2003; Elkahoui et al. 2005; Niknam et al., 2006). Plant cell and tissue culture studies are also relevant to crop improvement because they offer a means of rapid selection and development of salinity resistant crops. (Cherian and Reddy, 2003; Elkahoui et al., 2005).

Jatropha curcas (physic nut) is a multipurpose species belongs to the family Euphorbiaceae and is distributed in arid and semi arid areas of South America and all tropical regions. *J. curcas* recently received tremendous attention for its seed oil that can be converted into biodiesel and is considered to be a universally acceptable as an energy crop and alternative to conventional fossil fuels (Takeda, 1982; Mandpe et al., 2005). The species grows in areas with extreme climate and soil conditions that could not be inhabited by most of the agriculturally important plant species (Francis et al., 2005). However there are no reports on existence of *J. curcas* on saline soils or studies related to salt tolerance either at whole plant or at cell level. Therefore the present study was undertaken to evaluate the responses of *J. curcas* to salt stress both at cellular and whole plant level by studying the changes in the activity of antioxidative enzymes, accumulation of ions and proline to assess the possibility of improving salt tolerance in the species.

Materials and methods

Callus culture and growth determination

Callus of *J. curcas* were produced from in vitro grown leaf on MS (Murashige and Skooge, 1962) medium (pH 5.8) containing 0.70% agar, 5 mg/L 6-benzyl amino purine (BAP) and 1 mg/L naphthalene acetic acid (NAA) and sterilized by autoclaving at 1.05 kg/cm^2 pressure and 121℃ for 20 min. Friable callus were sub cultured for every 4 weeks after initiation and grown for further 4 weeks before being used for salinity tolerance studies. NaCl was used as salt in all experiments. At the beginning of experiment, callus (100 mg) was transferred to fresh medium amended with different concentrations of salt (0 (control), 20, 40, 60, 80, 100 mM). The callus was grown at 25± 2℃ in dark and was harvested after four weeks of growth. Callus was washed thoroughly with distilled water to remove the adhering ions and fresh mass (fm) was taken after removing the moisture by blotting. Dry mass (dm) was determined after drying the callus at 60℃ for 48 h to a constant mass.

Germination percentage and seedling growth determination

100 uniform seeds of *J. curcas* collected from a single plant, were surface sterilized with 0.1% mercuric chloride solution and then thoroughly washed with distilled water and germinated in Petri dishes lined with double layer of Whatman No. 1 filter paper. The petri dishes were moistened with 20 ml of different concentrations (0, 20 40, 60, 80, and 100 mM) of salt and maintained in dark at 28±2℃. Seeds were considered germinated when the radicle was at least 5 mm long. The experiment was continued for 10 days. Germination percentage was calculated 10 days after the beginning of experiment using the equation: final germination percentage = number of germinated seeds/ total number of seeds planted × 100. Full grown seedling of 10 cm long, were transferred to 30 plastic containers, each with five seedling, after a week the containers were randomly arranged and treated with 300 ml of Hoagland nutrient solution supplemented with above said concentrations of salt (Haogland and Arnon, 1950) and allowed to grow in controlled condition (25±2℃ temperature, 16 h photoperiod at a photon flux intensity of 46 μmol m^{-2} s^{-1} and 85% relative humidity) and the plants were continuously aerated. The nutrient solution was changed after every week. From each treatment fresh mass and increased shoot length were measured after 6 weeks. For

dry mass determination, the seedling were dried at 60℃ for 48 h to a constant mass and weighted.

Determination of ion content

Oven dried callus and seedling were digested in 10 ml of HNO_3:$HClO_4$ (10:4) on hot plate in a fume hood. After digestion the solution was filtered and final volume was adjusted to 50 ml and analyzed for Na, K, Ca and Mg using inductively coupled plasma emission mass spectroscopy (ICP, Perkin-Elmer).

Proline extraction and estimation

Proline was extracted and determined following the method described by Bates et al. (1973) using L-proline as standard. 200 mg each of fresh callus and seedling were homogenized in 4 ml of 3% aqueous sulphosalicylic acid and centrifuged at 10,000 rpm for 10 min to remove debris. 2 ml of the supernatant was mixed with 2 ml of acid ninhydrin (625 mg ninhydrin in 15 ml glacial acetic acid and 10 ml of 6 M orthophosphoric acid) and 2 ml glacial acetic acid in a test tube and boiled at 100℃ for 1 h. The reaction was stopped by cooling the tubes in ice bath. The chromophore formed was extracted with 6 ml of toluene and the absorbance of resulting organic layer was measured at 520 nm (Shimadzu UV-160A, Japan). The concentration of proline was estimated by referring to a standard curve for L-proline.

Enzyme extraction and assays

Callus and seedling (100 mg) were homogenized at 4℃ with 2 ml of extraction buffer [(200 mM Tris-HCl, 10 mM ethylenediaminetetraaceticacid (EDTA), 0.5 M sucrose, 2 mM phenylmethylsulfonyl fluoride (PMSF) and 1.0% insoluble polyvinylpyrrolidone (PVP) (pH 8.5)]. The homogenate was sonicated for 5 min and centrifuged at 15,000 rpm for 15 min. The supernatant obtained was used for estimation of protein content (Bradford, 1976) and enzymes activity.

The activity of superoxide dismutase (SOD; E.C. 1.15. 1.1) was assayed by measuring its ability to inhibit the photochemical reduction of nitroblue tetrazolium (NBT) according to the method of Beauchamp and Fridovich (1971). The reaction mixture (3 ml) contained 50 mM phosphate buffer (pH 7.8), 13 mM methionine, 75 μM NBT, 0.1 mM EDTA, 2 μM riboflavin and 0.1 ml of enzyme extract. Samples were illuminated by two 15 W fluorescent lamps for 10 min. The absorbance of reaction mixture was read at 560 nm. A non-irradiated reaction mixture served as control. Log A_{560} was plotted as function of the volume of enzyme extract used in the reaction mixture (Giannopolitis and Ries, 1977). From resultant graph the volume of enzyme extract corresponding to 50% inhibition of the reaction was considered as one enzyme unit.

Peroxidase (POX; E.C. 1.11.1.7) activity was measured according to Shannon et al. (1966) by following the change in absorbance at 460 nm due to o-dianisidine oxidation in the presence of H_2O_2 and enzyme. The assay mixture consisted of 2.8 ml of O-dianisidine-buffer (16 ml of 0.5% (w/v) o-dianisidine, 48 ml of 0.6 M sodium acetate, pH 5.5, and 416 ml of H_2O), 0.1 ml H_2O_2 (1% w/v) and 0.1 ml enzyme extract. The amount of enzyme required to change the absorption (Δ OD) by 0.01 min^{-1} mg^{-1} protein was taken as unit enzyme activity.

Separation of isoenzymes of SOD and POX was performed by Native PAGE (7.5% polyacrylamide) by loading of 50 μg of protein for POX and 100 μg for SOD. The gels were run in electrode buffer composed of 0.025 M Tris and 0.192 M glycine (pH 8.8) for 3 h at 4℃ at a constant current of 30 mA. After electrophoresis gels were stained for SOD (Misra and Fridovich, 1977) by incubating in a solution containing 2.5 mM NBT for 25 min, followed by incubation in 50 mM potassium phosphate buffer (28 μM riboflavin and 28 mM EDTA, pH 7.8) for 20 min in dark and then exposed to light for 15 min.The gels were stained for POX (Gulati 1989) by incubating in 0.02 M acetate buffer (pH 5.5) for 10 min, then transferred to fresh 0.02 M acetate buffer (pH 5.5) containing 0.66% guaiacol. After 5 min of incubation 3%

H_2O_2 is added drop wise till desired contrast was obtained and reaction was stopped by replacing the solution with distilled water.

The data are based on a mean of minimum five independent experiments. All the data were subjected to two way analysis of variance and significance was determined at 95% confidence level.

Results

Effect of salt on germination percentage and growth of callus and seedling

Salt inhibited seed germination and seedling growth at all concentration and the inhibition was proportional to salt concentration used. At lower concentration (20 mM) seed germination was not effected, however, at 40, 60, 80, and 100 mM concentrations seed germination inhibited by 15, 25, 41, and 46% respectively as compared to control (Table 1). Fresh mass and dry mass of both callus and seedling also decreased gradually at all salinity levels and inhibition was proportional to salt concentration in the medium. The decrease in fresh mass was 18, 56, 70, 72, and 74%, and for dry mass it was 43, 77, 85, 83, and 84% respectively as compared to control in callus. Whereas, in seedling the decrease was 32, 49, 52, 76, and 85% for fresh mass and 44, 61, 50, 72, and 75% for dry mass respectively at 0, 20, 40, 60, and 100 mM salt concentration as compared to control (Fig. 1 a&b). Shoot growth of seedling also showed a similar trend as shown for fresh and dry mass (Table 1).

Effect of salt on ion accumulation

Salt provoked a dose dependent increase in Na ion accumulation in both callus and seedling and accumulation was ~1 to 1.5 times higher in seedling as compared to callus (Fig. 2a). K content decreased in callus, where as, in seedling it increased however, concentration was 2 to 3 times higher in callus than seedling at low salt concentration (Fig. 2b). The Na/K ratio increased gradually with increasing concentration of salt both in callus and seedling and ratio was ~2 times higher in seedling (Fig. 2e). Ca accumulation decreased gradually in callus whereas, in seedling it increased up to 60 mM then decreased however, accumulation was more in seedling than callus (Fig. 2c). Mg content also decreased with increasing salt concentration in both callus and seedling; however, Mg concentration was ~2 to 3 times higher in seedling than callus (Fig. 2d).

Effect of salt on protein and proline accumulation

Salt stress resulted in accumulation of soluble proteins in callus up to 80 mM and decreased at 100 mM, where as in seedling protein content remained more or less constant at all the salinity levels. The increase in protein content in callus was 21, 45, 63, and 24% at 20, 40, 60, and 80 mM salt concentrations respectively (Fig. 3a). No significant change was observed in proline content at all salinity

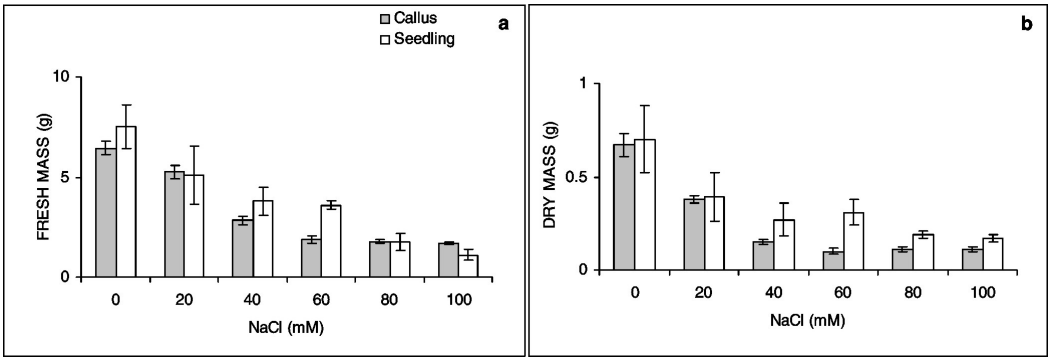

Fig. 1. Effect of salt stress on fresh mass (a) and dry mass (b) of *J. curcas* callus and seedling. Vertical bars indicate SE of five independent experiments.

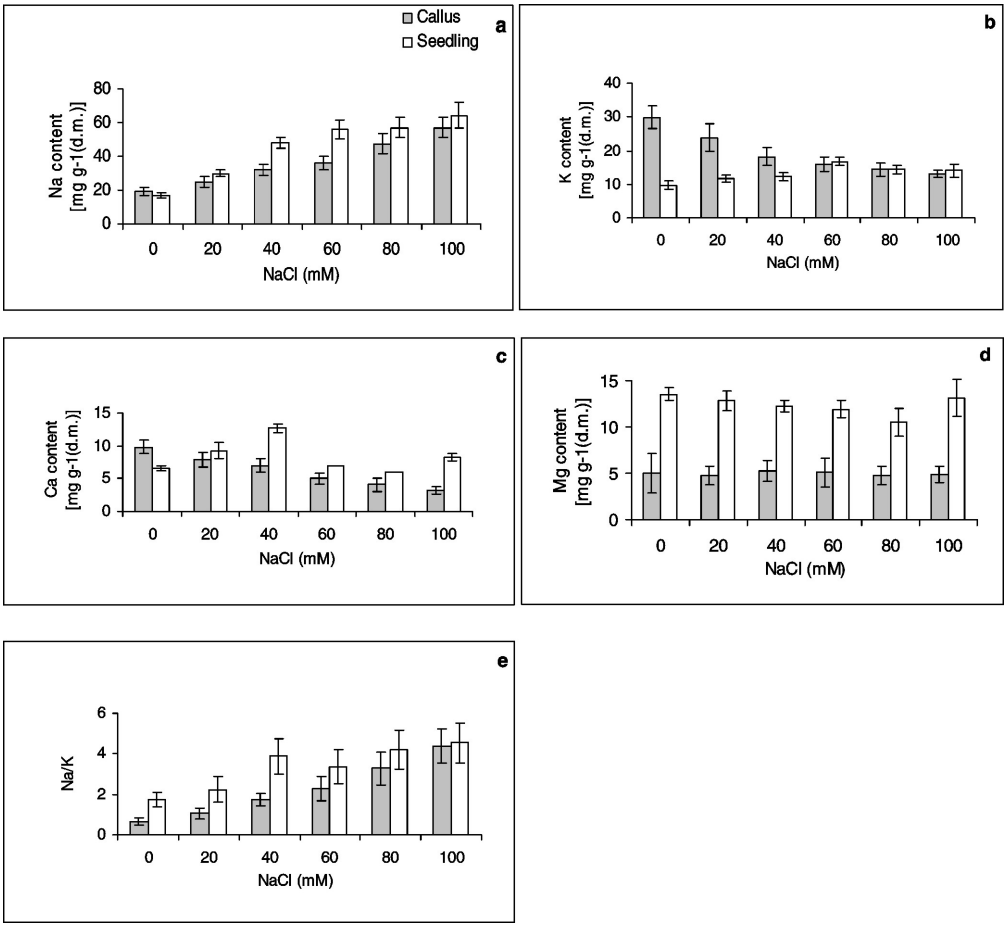

Fig. 2. Effect of salt stress on Na (a), K (b), Ca (c), Mg (d) and Na/K (e) accumulation in *J. curcas* callus and seedling. Vertical bars indicate SE of five independent experiments.

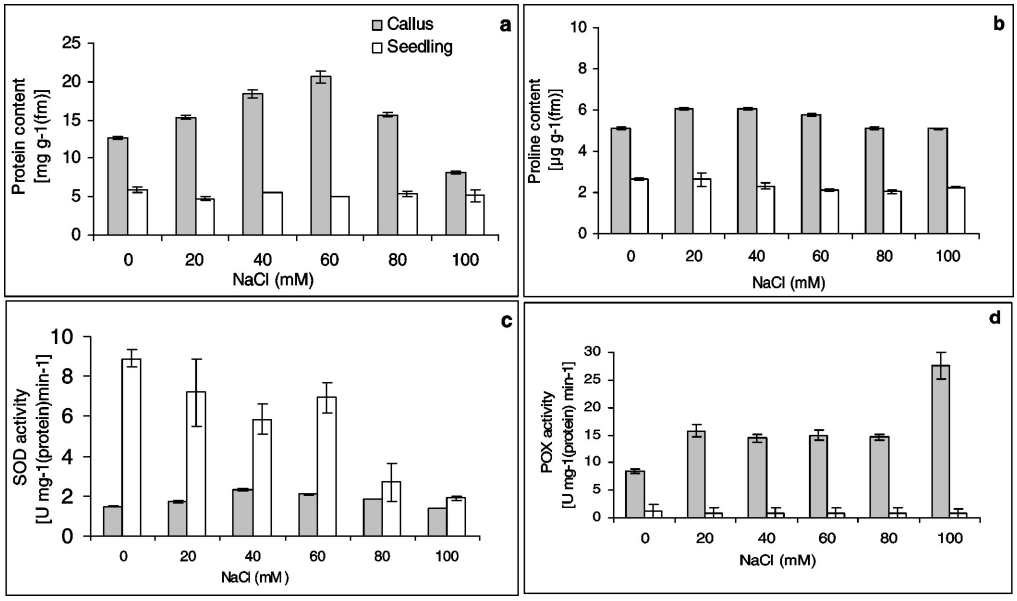

Fig. 3. Effect of salt stress on protein (a) and proline content (b), SOD (c) and POX (d) activity in *J. curcas* callus and seedling. Vertical bars indicate SE of five independent experiments.

level in both callus and seedling. Proline and protein accumulation was ~2 to 3 times more in callus as compared to seedling (Fig. 3b).

Effect of salt on the activity of antioxidant enzymes

The activity of both SOD and POX was determined by both spectrophotometric assay and gel staining following native PAGE. SOD and POX activity increased as function of external salinity in callus whereas, in seedling the activity gradually decreased at all salt concentration. SOD activity increased ~1.5 to 2 fold at 40, 60, and 80 mM salinity level and decreased slightly at 100 mM as compared to control in callus whereas, in seedling the decrease was 1.2, 1.5, 1.2, 3.2, and 4.7 fold at 20, 40, 60, 80, and 100 mM concentration respectively and the activity was ~2 to 4 times higher in seedling as compared to callus (Fig. 3c). In native PAGE separation, four SOD isoenzymes were observed with salt treatment as compared to three isoenzymes in control callus (Fig. 4a). In case of seedling eight isoenzymes were observed in both control and salt treatments (Fig. 4b). The POX activity increased 2 to 3 fold due to salinity stress in callus in contrast to ~1.5 to 2 times decrease in seedling (Fig. 3d). In callus native PAGE two isoenzymes were observed due to salt treatment as compared to one isoenzyme in control cells (Fig. 5a), whereas, five isoenzymes were recorded in seedling irrespective of salt treatment (Fig. 5b). In callus both SOD and POX isoenzyme were in agreement with spectrophotometric assay but in seedling

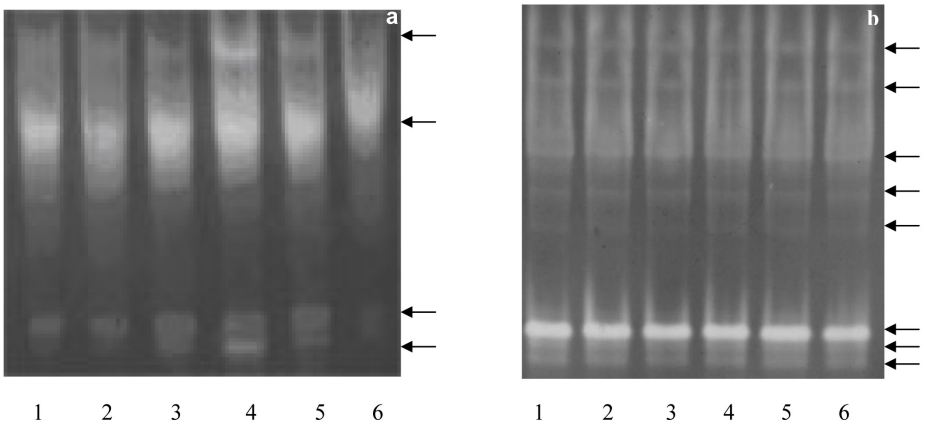

Fig. 4. Effect of salt stress on SOD isoenzymes separation in callus (a) and Seedling (b) of *J. curcas*: lane 1, 2, 3, 4, 5 and 6; 0, 20, 40, 60, 80 and100 mM NaCl treated callus and seedling, respectively.

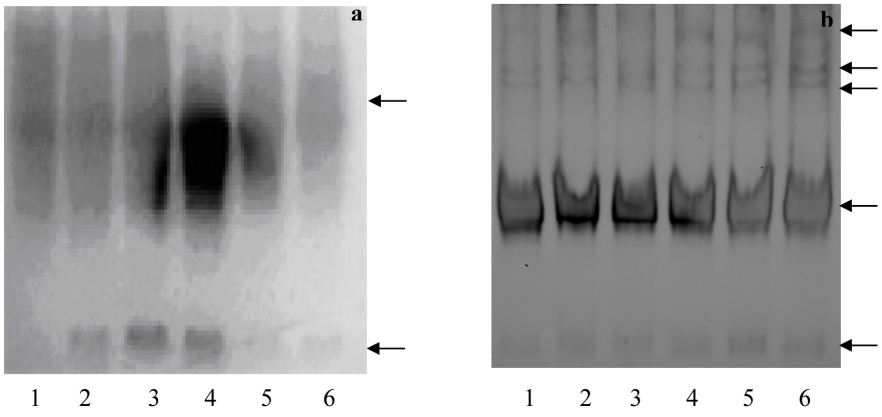

Fig. 5. Effect of salt stress on POX isoenzymes separation in callus (a) and seedling (b) of *J. curcas*: lane 1, 2, 3, 4, 5 and 6; 0, 20, 40, 60, 80 and100 mM NaCl treated callus and seedling, respectively.

no relation between activity and intensity of band was observed.

Discussion

In the present study salt stress provoked an inhibition of seed germination, growth, fresh and dry mass accumulation and both callus and seedling behaved in a similar manner to salt stress. Decrease in seed germination, fresh mass and dry mass due to salt stress was reported in different species (Niknam et al., 2004, 2006). Comparison of salt tolerance of whole plant with that of cell cultures derived from indicated that for certain species salt tolerance was similar for whole plant and cell lines indicating salt tolerance is based on intrinsically cellular process (Tal et al., 1978). Cherian and Reddy (2000, 2003) have shown that salt tolerance at whole plant is substantially greater than cell lines. According to Flowers et al. (1985) and McCoy (1987) cellular tolerance did not always correlate with whole plant tolerance. A negative correlation where, the whole plant is salt tolerant and isolated cells are salt sensitive was regarded as indicator for the operation of the mechanism depending upon organization of cells in whole plant. Hence the salt tolerance trait selected at cell level may not express in whole plant and subsequent sexual progenies, where the whole plant and cultured cells both are salt sensitive or tolerant is interpreted as indicator for the operation or lake of operation of similar mechanisms on both levels (Gulati and Jaiwal, 1994). More or less similar response observed in whole plant and cell cultures in the present study indicates the salt tolerance mechanism operating in whole plant and cell are similar. The decrease in growth was associated with an increase in Na concentration both in callus and seedling, demonstrate that response of this species to salt stress is linked to Na accumulation. Excessive accumulation of Na causes ion imbalance and metabolic disturbances (Cherian and Reddy, 2003; Elkahoui et al., 2005). K content was higher in callus as compared to seedling at all salinity level. The decrease in Ca accumulation observed in our study may be due to displacement of Ca from cell membrane by Na (Cramer et al., 1985; Shibli et al., 2007). Decreased Mg accumulation under saline condition is in agreement with earlier results (Shibli et al., 2007) and the higher concentration of Mg observed in seedling than the callus indicated the requirement of Mg for chlorophyll formation. The differences observed in various ions content in callus and seedling can be attributed to their growing conditions and nature of the plant. The imbalance in ion uptake and Na/K ratio in callus and seedling might have disturbed the growth of callus and seedling (Cherian and Reddy, 2003; Elkahoui et al., 2005). Protein content in callus and seedling showed dissimilar trend. In seedling no significant change was observed whereas, in callus it increased up to 80 mM and then decrease at 100 mM salt concentration. Cherian and Reddy (2003) and Nikman et al. (2004, 2006) also reported similar trend in callus cultures of *Suaeda nudiflora*, *Nicotiana tabacum* and *Tigonella species*. The decreased protein content at high salinity observed in callus cultures may be due to the decrease in the synthesis of protein (Hall and Flowers, 1973), whereas, the increase in protein content at 20, 40, 60 and 80 mM may be due to stress induced synthesis of proteins (Cherian and Reddy, 2003; Niknam et al., 2006). Although accumulation of proline as a compatible solute has been reported for several plant species (Cherian and Reddy, 2000; Maggio et al., 2000; Cherian and Reddy, 2003; Niknam et al., 2006), the increase in proline pool upon salt treatment does not seem to be a general phenomenon (Stewart and Lee, 1974) and the significance of proline accumulation in osmotic adjustment is still debated and varies according to the species (Meloni et al., 2004). No significant differences observed in both callus and seedling due to salt stress in the present study is in agreement with the results of Lutts et al. (1996) and Lacerda et al. (2001). Since *J. curcas* is very succulent material with approx 90% water, the proline content estimated may not be actual, due to dilution effect of xylem sap the real concentration of cellular proline might have been under estimated (Maggio et al., 2000). Salt stress provoked a dose dependent increase in SOD activity in callus but decreased in seedling however the activity was higher in seedling than callus at all salinity

level. Cherian and Reddy (2003) also reported increased SOD activity in seedling and callus cultures of *S. nudiflora* under saline conditions. Higher POX activity observed in callus than seedling indicates that cells have a higher capacity for the decomposition of H_2O_2 generated by SOD in callus than whole plant (Cherian and Reddy, 2003; Niknam et al., 2006). Salt stress induced synthesis of new isoenzymes in both callus and seedling under salt stress are in conformity with the results of Elkahoui et al. (2005) and Niknam et al. (2006). The differences in the isoenzymes pattern of SOD and POX observed between seedling and callus suggest tissue specific isoforms. The qualitative and quantitative differences observed in the present study might be a useful adaptation in the defense mechanism of *J. curcas* against active oxygen species. However salt induced changes observed in antioxidant enzymes activity may not be strong enough to eliminate all the deleterious effects provoked by salt, only alleviated the impact of stress, thus allowing cell/plant growth to occur. Many similarities observed between callus and whole plant suggests the existence of cellular based salt induced response in *J. curcas*. However, the differences in the isoenzymes pattern of SOD and POX observed between callus and seedling suggest tissue specific expression of isoenzymes which needs further studies.

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