CHAPTER-5

Study of Genetic Diversity of *J.curcas* by RAPD

5.0 Introduction

The genetic difference between two individuals of a species is the basis of evolution and adaptation. Conservation and sustainable use of biological diversity is important. The International Union for the Conservation of Nature and Natural resources (IUCN) identifies three levels of biological diversity that are equally important to conserve: ecosystem, species and genetic diversity (McNeely et al, 1990). Plant genetic resources comprise the present genetic variation that is potentially useful for the future of mankind. Plant genetic resources should hence be studied and conserved with the ultimate aim of eventually being a source of potentially useful genetic variation. There is virtually no information with regard to the number of introductions and the genetic diversity of *J.curcas* populations grown in India. Several researchers have attempted to define the origin of *J.curcas*, but the source remains controversial (Dehgan and 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 devoid of phorbol esters. There are no named varieties of *J.curcas* in India with the exception of the variety SDAUJ1 (Chatrapathi) that was released during the year 2006 based on selection from local germplasm (Basha and Sujatha, 2007). Hence the need for studies on the varieties of *J.curcas* using molecular markers becomes imperative. In our state of Gujarat too numerous studies on *J.curcas* plantations have been done but molecular studies have not been reported. The conservation and sustainable use of plant genetic resources require accurate identification of their accession (Arif et al, 2010). In this study an attempt has been made to study the genetic diversity existing in *J.curcas*. The study of genetic diversity with the help of molecular markers can be broadly classified into phenotypic markers, biochemical

markers and molecular markers. Of these, molecular markers are more promising as any change in the protein sequence would be brought about by a mutation in its DNA sequence. DNA markers are not typically influenced by environmental conditions and therefore can be used to help describe patterns of genetic variation among plant populations and to identify duplicated accessions within germplasm collections (Ganesh Ram et al, 2008). Molecular markers can be studied by techniques based on polymerase chain reaction (PCR) and non-polymerase chain reaction. RFLP (Restriction Fragment Length Polymorphism) dominates the non-PCR based techniques. This technique is time consuming and highly expensive. Among the PCR based techniques, RAPD is generally a preferred method. This is more so when the genome to be studied is unknown. The advantage it has over other molecular techniques is that it is less time consuming, more cost effective and the starting material (genomic DNA) requirement is low. Reports have shown that RAPD analysis can be used to detect variation within a restricted range, to identify suitable parents for linkage map construction, and for gene tagging for drought resistance (Virk et al, 1995)

The prerequisite for RAPD is good quality DNA. DNA isolation from plant source is at times difficult owing to the large concentration of polysaccharide, protein, pigment or phenolic compounds. Some plant taxa may not permit optimal DNA yield. Closely related species of the same genus also may require different isolation protocols. Thus, an efficient protocol for isolation of DNA as well as the optimization of the PCR conditions is required (Subramanyam et al, 2009).

5.1 Literature Studies

Detection and analysis of genetic variation can help us to understand the molecular basis of various biological phenomena in plants. Since the entire plant kingdom cannot be covered under sequencing projects, molecular markers and their correlation to phenotypes provide us with requisite landmarks for elucidation of genetic variation. Genetic markers can be classified into three types: morphological trait based markers, protein based (biochemical) markers and DNA based (molecular) markers. Traditionally, diversity within and between populations was determined by assessing differences in morphology. Its advantages are being readily available and non requirement of sophisticated equipment. However these attributes are subject to change due to environmental factors and vary at different time points. Biochemical markers also have similar limitation of being influenced by environment. Genetic or DNA based marker techniques such as RFLP (restriction fragment length polymorphism), RAPD (random amplified polymorphic DNA), SSR (simple sequence repeats) and AFLP (amplified fragment length polymorphism) are routinely being used in ecological, evolutionary, taxonomical, phylogenic and genetic studies of plant sciences. These techniques are well established and their advantages as well as limitations have been realized (Agarwal et al, 2008).

An ideal molecular marker technique should have the following criteria: (i) highly polymorphic in nature as it is polymorphism that is investigated in genetic diversity studies (ii) co-dominant in nature as it allows determination of homozygous and heterozygous states of diploid organisms (iii) frequently occurring in the genome (iv) neutral in behavior, easy, cheap and (v) highly reproducible (Kumar et al, 2009). Genomic abundance, level of polymorphism detected, locus specificity, reproducibility, cost etc. are important aspects known to influence various techniques. Table 5.1 enlists these important features regarding the techniques most frequently used (Agarwal et al, 2008).

Table 5.1 Comparision of various aspects of frequently used molecular maker techniques

	Abundance	Reproducibsility	Degree of Polymorphism	Locus specificity	Technical requirements	Quantity of DNA required	Major application
RFLP	High	High	Medium	Yes	High	High	Physical mapping
RAPD	High	Low	Medium	No	Low	Low	Gene tagging
SSR	Medium	Medium	Medium	No	Medium	Low	Genetic diversity
SSCP	Low	Medium	Low	Yes	Medium	Low	SNP mapping
CAPS	Low	High	Low	Yes	High	Low	Allelic diversity
SCAR	Low	High	Medium	Yes	Medium	Low	Gene tagging & Physical mapping
AFLP	High	High	Medium	No	Medium	Medium	Gene tagging
IRAP/ REMAP	High	High	Medium	Yes	High	Low	Genetic diversity
RAMPO	Medium	Medium	Medium	Yes	High	Low	Genetic diversity

RFLP restriction fragment length polymorphism, RAPD random amplified polymorphic DNA, SSR simple sequence repeats, SSCP single strand conformational polymorphism, CAPS cleaved amplified polymorphic sequence, SCAR sequence characterized amplified region, AFLP amplified fragment length polymorphism, IRAP/REMAP inter-retrotransposon amplified polymorphism/retrotransposon-microsatellite amplified polymorphism.

Genetic diversity assessment with molecular markers is important for efficient management and conservation of plant genetic resources in gene banks. Very little information regarding the genetic diversity and number of introductions of *J.curcas* populations grown in India is available. Keeping in view the commercial applications of *J.curcas* it becomes imperative to use high quality planting material for all future plantations. Genetic diversity studies of *J.curcas* in India are limited to the accession available around in here. Exception to this is the study reported by Basha and Sujatha, 2007 where they have incorporated a non-toxic accession from Mexico along with 43 accessions from different regions from India. Montes et al, 2008 reported study of accessions from 30 countries. Senthil Kumar et al, 2009 reported the use of accessions from India and Zimbabwe.

Very little analysis of genetic polymorphism in *J.curcas* has been performed so far. Protein based isozyme markers have been reported to be used to determine the genetic relatedness of the members of the genus *Jatropha* and *Ricinus* sp. (Sujatha et al, 2008). Gupta et al, 2008 used RAPD (Random Amplification of Polymorphic DNA) and ISSR (Inter Simple Sequence Repeat) markers to study different accessions of four geographical locations of India and divided them into four populations. ISSR markers have also been reported to be used to study inter and intra population variability in *J.curcas* (Basha and Sujatha, 2007; Senthil Kumar et al, 2009). Ganesh Ram et al, 2008 assessed the genetic diversity of 12 *Jatropha* species using RAPD. Irrespective of the geographical locations of different accessions and primers, it was observed that all accessions from India clustered together. Diversity analysis with local germplasm showed a narrow genetic base in India (Basha and Sujatha, 2007; Ganesh Ram et al, 2008).This indicates the need of widening the genetic base of *J.curcas* through introduction of accessions with broader geographical background and creation of variation through mutation and hybridization techniques (Mukherjee et al, 2011). Gupta et al, 2008 reported 40%-100% polymorphism using RAPD and approximately similar percentage polymorphism using ISSR markers in 13 *Jatropha* accessions from different geographical locations of India. Senthil Kumar et al, 2009 reported

nearly 100% polymorphism using RAPD and ISSR markers in eight *Jatropha* species and three *Jatropha curcas* accessions. RAPD analysis in *J.curcas* shows a narrow genetic base (Basha and Sujatha 2007; Ganesh Ram et al, 2008).

5.2 Materials and Method

5.2.1 Plant material

Fresh and young plant tissue material from leaves and petioles were used to extract DNA using ~~available~~ protocols. Standardization of protocol was done using plant species grown in Department of Biochemistry, The M.S. University of Baroda, Vadodara. They were rinsed with distilled water and blotted gently on filter paper.

5.2.2 Reagents

DNA Extraction buffer A

Sr. No.	Final concentration	Reagent
1)	2%	CTAB (cetyltrimethylammonium bromide)
2)	100mM	Tris-HCl
3)	20mM	EDTA
4)	1.4M	NaCl
5)	4%	PVP (polyvinyl pyrrolidone)

DNA Extraction buffer B

1)	
2)	
3)	
4)	
5)	

Give Bullets or numbers

DNA Extraction buffer B

100mM	Tris-HCL
50mM	EDTA
100 mM	NaCl.
5M NaCl	

3M sodium acetate (pH 5.2)

5M Potassium acetate

5 M potassium acetate, 60.0 ml

Glacial acetic acid, 11.5 ml

H2O, 28.5 ml

The resulting solution is 3 M with respect to potassium and 5 M with respect to acetate. Store the solution at 4°C and transfer it to an ice bucket just before use.

Solutions and buffers were autoclaved at 121°C at 15 psi pressure and stored at RT (Room Temperature).

5.2.3 Chemicals

Chloroform: isoamyl alcohol (24:1)

80% ethanol

5.2.4 DNA Isolation Protocol (Keb--Llanes et al, 2002)

1. Grind 0.3 g of leaf tissue to a fine power using a mortar, pestle, and liquid nitrogen. Transfer the powder to an Eppendorf tube.

2. Add 300 µL buffer A, 900 µL buffer B, and 100 µL SDS.

3. Vortex the mixture. Incubate in a water bath at 65°C for 10 min.

4. Add 410 µL cold potassium acetate. Mix thoroughly. Centrifuge at 15,300 g for 15 min at 4°C.

5. Transfer 1 mL of the supernatant to a clean Eppendorf tube. Add 540 µL cold isopropanol. Incubate on ice for 20 min.

6. Centrifuge at 9600 g for 10 min. Discard the supernatant. Wash the pellet with 500 µL 70% ethanol and let dry.

7. Resuspend pellet in 600 µL buffer TE. Add 60 µL 3 M sodium acetate (pH 5.2) and 360 µL cold isopropanol. Incubate on ice for 20 min.

8. Centrifuge at 9600 g for 10 min. Repeat steps 5-7 twice.

9. Resuspend the pellet in 50 µL buffer TE.

5.2.5 Agarose Gel Electrophoresis

0.8% agarose gel electrophoresis used to separate, analyze and quantitate nucleic acids and buffer used will be 1x TAE (Tris-Acetate EDTA) buffer.

5.2.6 Spectrophotometric determination of DNA concentration

DNA quantification is a very important step for many downstream applications such as cloning , RAPD etc. DNA yield and purity were checked spectrophotometrically by measuring absorbance at 260 and 280 nm. Nucleic acid concentration was calculated using the following formula:

A260 x 50µg/ml x dilution factor (995/5). This will provide the concentration of the stock DNA (µg/ml).

5.2.7 Restriction Digestion

In existing protocol extracted DNA sample showed higher absorbance at A260/280nm than expected. So in order to ensure good quality DNA, RE (Restriction enzyme) digestion was performed. In this method HindIII was used by varying its concentrations 2, 4, 6 and 8 Units respectively along with control (without adding enzyme) in the corresponding buffer at 37°C for 3 h. Digested DNA along with control was analyzed by running the samples on 1% agarose gel.

5.2.8 DNA Amplification

Thirty Seven IDT

(37) decamer primers from Operon Technologies Inc., (USA) and Integrated DNA Technology () were initially screened for their repeateable amplification with *Jatropha* accessions. Amplification was carried out in 25 µl reaction volumes containing 1X Assay buffer (50 mM KCl, 2.5mM of each dNTP, 0.8 µM primers, 1.5 U of Taq DNA polymerase (Banglore Genei Pvt. Ltd. India) and 20 ng of template DNA. PCR conditions were as shown below. Amplification products were separated on 1.8% agarose gel and stained with ethidium bromide and photographed under UV light.

Keep it separately, it is final extension step

Step I	Step II			Step III			Step IV	Hold
94°C	94°C	38°C	72°C	94°C	45°C	72°C	72°C	4°C
5 min	45 sec	1 min	1.5min	45 sec	1 min	1 min	10 min	store
10 cycles				35 cycles				

Give caption → PCR cycle condition for RAPD marker

5.3 Results and discussion

5.3.1 Standardization of DNA isolation from *J.curcas*

Molecular aspects of biological studies are highly valued and the first approach to such studies is extraction of nucleic acids. Lots of limitations in genetic material extractions are solved by some changes in compound and pH of functional buffers, so that extracted DNA is much more quantified and also better qualified (Alaey et al, 2005). It is also important to use a method which can be done acceptably and economically too. DNA isolation from plant source is at times difficult owing to the large concentration of polysaccharide, protein, pigment or phenolic compounds. As mentioned earlier some plant taxa may not permit optimal DNA yield. Closely related species of the same genus also may require different isolation protocols. Thus, an efficient protocol for isolation of DNA as well as the optimization of the PCR conditions is required (Subramanyam et al, 2009). *Jatropha curcas* like other Euphorbiaceae family members contain exceptionally high amounts of polysaccharides, polyphenols, tannins and other secondary metabolites such as alkaloids, flavanoids, phenols, terpenes etc. which might interfere with successful DNA isolation. Subramanyam et al, 2009 reported certain problems encountered during the isolation and purification of DNA especially from *Jatropha curcas* which included degradation of DNA due to endonucleases, co-isolation of highly viscous polysaccharides, inhibitor compounds like polyphenols and other secondary metabolites that interfere with enzymatic reactions. Many a times, RNA co-precipitates with DNA resulting in many problems including suppression of PCR amplification during RAPD analysis.

Keeping in view the above mentioned problems associated with successful DNA isolation from *J.curcas*, protocol given by Kebb Llanes et al, 2002 was used in the current study. It is generally observed that plant DNA being difficult to isolate due to above mentioned reasons, requires strong treatments with chemicals like liquid nitrogen or cetyltrimethylammonium bromide (CTAB). However, the problem with liquid nitrogen is its toxic nature and non-availability in some remote areas. On the contrary CTAB is not only easily available but also yields good quantity of DNA. In the present study, certain modifications to the existing protocol (Kebb Llanes et al, 2002) were required to be made as lot of polysaccharide co-precipitated with DNA. The first study undertaken was to check the required levels of CTAB (2%-as mentioned in actual protocol, 3% and 4%) and its effect on DNA yield as shown in table 5.2. For all the optimization studies young leaves and petioles of *J.curcas* were used as samples.

Table 5.2 Effect of varying percentage of Cetyl Trimethyl Aammonium Bromide on genomic DNA yield

Samples	2% CTAB	3% CTAB	4%CTAB
$A_{260/280}$ Mean±SEM	2.600±0.4933	2.233±0.2186	2.300±0.2082
DNA (µg/g fresh weight of tissue)	143	182	253

It could be observed from table 5.2 that higher levels of CTAB yields higher DNA, however, fire type bands were seen at higher levels of CTAB (3% and 4%).

Hence, it was desirable to use CTAB at 2% level in the extraction buffer. Incorporation of PVP (polyvinylpyrrolidone), beta-mercaptoethanol and ascorbic acid in extraction buffer A as mentioned in the reported protocol did not help in obtaining good quality DNA. When a buffer devoid of these was used and by introduction of a new Chlorofom:Isoamyalcohol step (24:1) a better yield and quality of DNA was obtained.

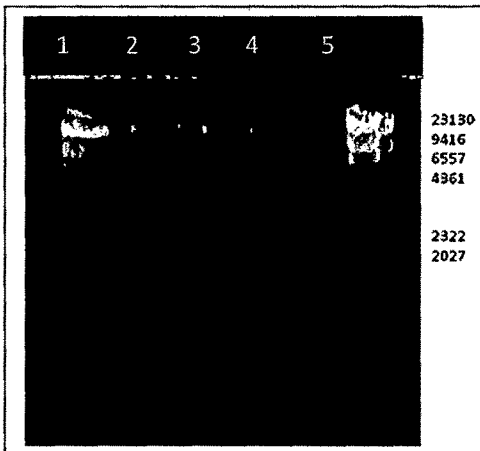

Figure 5.1: Electrophoresis of Genomic DNA of *J.curcas*, Lane1&5-λ *Hin*dIII marker, Lane 2, 3&4-Genomic DNA

Figure 5.1 shows DNA as observed on agarose gel electrophoresis along with λ HindIII marker.

It was observed that when fresh samples were used for DNA isolation no phenolic exudates were seen and hence a transparent pellet of DNA was observed after ethanol wash. However, in stored samples (-80°C) pigmentation was observed and also secondary metabolite contamination was seen. This was overcome by addition of pinch of PVP (44,000 Dalton) and ascorbic acid during crushing of tissue with mortar and pestle. The purity and clean nature of DNA samples could be confirmed through complete digestion by the restriction enzyme HindIII after incubating the reaction tubes at 37 °C for 1.5 h (Figure 5.2).

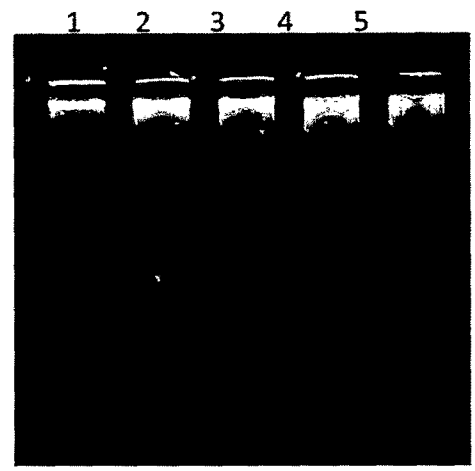

Figure 5.2: Electrophoresis of *J.curcas* DNA digested with *Hin*dIII on 1% agarose gel,Lane1-Undigested DNA, Lane2-5 DNA digested with 2,4,6 and 8 U of *Hin*dIII

This indicated that the isolated DNA was amenable to further processing in cloning experiments as well as DNA fingerprinting. Similar results have been reported by Khanuja et al, 1999 for plants producing large amount of secondary metabolites and essential oils, Pamidimarri et al, 2009 for *Jatropha curcas*, Doulis et al, 2000 for Cupressus sempervirens L.

The second crucial step for effective DNA isolation is the heat shock treatment. In the protocol used heat treatment was at 65°C for 10 minutes. However, this did not yield good results; hence varied time of heat treatment was studied (10, 30, 50, 70, 90, 110, 120 minutes) (figure 5.3). As seen is table 5.3, DNA yields increase even after an hour of treatment and best results were found at 110 minutes of treatment. Though it could be argued that increasing the time duration of heat treatment would lead to a lengthy procedure, during the course of the study, it was observed that heat treatment duration of 60 minutes yielded good quality DNA suitable for many

downstream processes. Pamidimarri et al, 2009 reported 90 minutes of heat treatment gave best results (132.5±7.8 µg/g of tissue). Overall higher DNA yield (from 150 µg/gm of tissue onwards) was obtained in the present study. This far exceeds the reported range (70-120 µg/ gm tissue) in the actual protocol (Kebb Llanes et al, 2002). Yield of DNA in the present study was higher when compared to other reported work (85.95-105.35 µg/ gm tissue by Suramanyam et al, 2009; 57-132 µg/ gm tissue by Pamidimarri et al, 2009). However, Dhakshanamoorthy and Selvaraj, 2009 have reported (2360 ±52 µg/ gm tissue) DNA from *J.curcas* using modified CTAB protocol.

Table 5.3 Variation in duration of heat treatment in leaf samples

Time of incubation at 65°C (minutes)	$A_{260/280}$ Mean±SEM	DNA yield (µg/g of tissue)
10	2.400±0.100	152
30	1.867±0.066	315
50	1.700±0.0577	326
60	1.820 ±0.066	347
70	1.900±0.0577	282
90	1.567±0.133	448
110	1.900±0.100	744
120	1.733±0.333	447

Values are represented as Mean ± SEM (N=9)

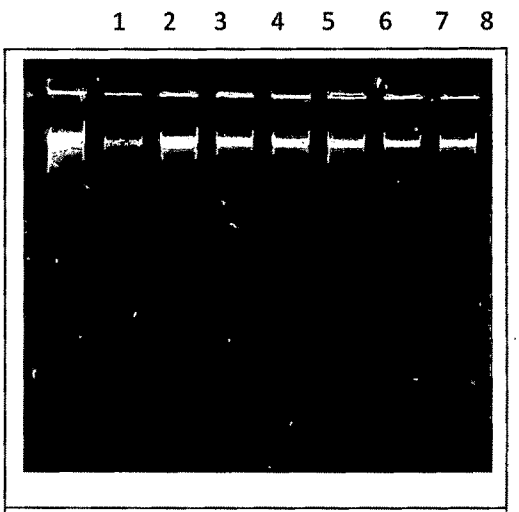

Figure 5.3: Electrophoretic separation of genomic DNA extracted from leaf using different incubation time at 65°C lane no. 1(10'), lane no.2(30'), lane no.3(50'), lane no.4(60'), lane no.5(70'), lane no.6(90'), lane no.7(110'), lane

Table 5.4 Variation in heat treatment attempted in petiole samples

Time of incubation at 65°C (Minutes)	$A_{260/280}$ Mean±SEM	DNA yield (µgDNA/gm tissue
10	1.767±0.1764	207
30	1.800±0.0577	419
50	1.767±0.0333	207
60	1.867±0.066	330
70	2.100±0.4041	215
90	1.767±0.0333	304
110	1.867±0.0333	541
120	1.933±0.0881	207

Values are represented as Mean ± SEM (N=9)

One of the other problems experienced with DNA isolation was increased protein co-precipitation as the reported protocol does not encompass any phenol chloroform treatment. Protein co-precipitation has also been reported by Dhakshanamoorthy and Selvaraj, 2009 where they have stated that photosynthetically active tissue contains phenolic compounds that oxidize during extraction and irreversibly interact with proteins and nucleic acids to form a gelatinous matrix. This matrix might inhibit proper extraction and amplification. Phenol is known to degrade protein present in the sample. However, it is also seen to be a deterrent as it not only is

toxic in nature but residual phenol in the extract also hampers yield of DNA. When phenol was used in the current study, it did not help in eradication of protein that was contaminating the DNA sample. This problem was not overcome even by the use of beta-mercaptoethanol as mentioned in the reported protocol. Hence, the phenol treatment step was dropped and DNA extraction was attempted using chloroform: isoamyl alcohol (24:1). Treatment with chloroform:isoamylalcohol (24:1) was introduced to remove extra proteins present in the sample. Pamidimarri et al, 2009 emphasized on the use of phenol for removal of proteins as it was found to affect the A260/280. However, in the present study A260/280 in the range of 1.7 to 2.0 was obtained even in the absence of phenol in extraction procedure.

The protocol mentions the use of TE along with salt and isopropanol for DNA precipitation. However, residual EDTA of the TE buffer could affect the further series of reactions like PCR amplification and hence, TE was omitted from the treatment and only salt and isopropanol were used for salt–DNA complex precipitation. This also did not affect the yield of isolated DNA. This modified protocol could be used for different tissues like nodes, leaves and petioles. Of all the samples tried it could be concluded that petiole proved to be a better sample for obtaining high quality and quantity of DNA suitable for further downstream processing. Since in this study young leaf samples were incorporated, many a times pigmentation was seen even in the DNA so obtained. On the other hand, nodes being hardy in nature, may at times, lead to lower levels of DNA. The modified protocol for successful DNA isolation from various plant samples is thus outlined below:

1) Chop 0.3 g of leaf tissue using fine blade and grind petiole samples to a fine power using pre-cooled (-20°C) mortar and pestle . Transfer the powder to microfuge tube. Add 300 µl buffer A, 900 µl buffer B, and 100 µl SDS.

2) Vortex the mixture. Incubate in a water bath at 65°C for 60 minutes.

Give numbers or bullets

followed by centrifugation

3) Add 410 µL cold potassium acetate. Mix thoroughly/~~Centrifuge~~ at 12,000 rpm for 25 min at 4°C.

4) Transfer 1 ml of the supernatant to a clean microfuge tube. Add equal amount of chloroform: isoamyl alcohol. Invert it gently and centrifuge at 10,000 rpmfor 10 min at 4°C

5) Transfer 1 ml of the supernatant to a clean Eppendorf tube. Add 540 µL cold isopropanol. Incubate in -20 °C for 1 hour

6) Centrifuge at 10,000rpm for 10 min. Discard the supernatant. Wash the pellet with 500 µl 80% ethanol.

7) Add 60 µl 3 M sodium acetate (pH 5.2) and 360 µl cold isopropanol. Incubate in -20 °C for 1 hr. Centrifuge at 12,000rpm for 10 min. Resuspend the pellet in 20-25 µl TDW. Quantify the DNA spectrophotometrically at 260 nm. 260/280 name of spectrophotometer

either it is nanodrop or picodrop

5.3.2 Optimization of RAPD- PCR parameters

Parameters for random amplification of polymorphic DNA from *J.curcas* were studied and optimized. Parameters studied were variation in annealing temperature; optimal concentration of template DNA, optimal primer concentration, $MgCl_2$ concentration, number of PCR cycles etc. Artifactual non-genetic variation in analysis can be considerable if primer-template concentration and annealing temperature are not carefully optimized (Caetano-Anolles, 1993). RAPD in *J.curcas* has been reported by many others with different goals (Basha and Sujatha, 2007 for development of population (inter and intra population) specific SCAR markers; Basha and Sujatha, 2009 for genetic analysis of *Jatropha* species and interspecific hybrids using nuclear and organelle specific markers; Gupta et al, 2008 for comparative analysis of genetic diversity among *Jatropha curcas* genotypes using both ISSR and RAPD; Ganesh Ram et al, 2008 for studying genetic diversity among *Jatropha* species using RAPD markers; Pamidimarri et al,

2009 for molecular characterization of *Jatropha* resources through ISSR; Dakshanamoorthy and Selvaraj, 2009 for extraction of Genomic DNA from *Jatropha sp.* using modified CTAB method. Each of these studies has a lot of variation in the PCR conditions, DNA concentration, primer levels etc. in their reports. In the present study varied concentrations of DNA (20ng, 40ng, 60ng, 80ng, 100ng, 120ng, 140ng, 160ng, 180ng, and 200ng) were tried to achieve a amplifiable PCR reaction. Out of these 20ng of DNA was suitable for PCR analysis.

In most cases 1.5mM concentration is available along with reaction buffer which is sufficient for Taq DNA polymerase to work (Padmalatha and Prasad, 2006; Basha & Sujatha, 2007). However, in certain instances 3mM of $MgCl_2$ is incorporated (Pamidimarri et al, 2009). In a few instances 2mM of $MgCl_2$ is also reported to be added (Jubera et al, 2009). In the present study, 1mM-3mM of $MgCl_2$ was included in the reaction mix. As shown in figure 2.6, 2mM, 2.5mM and 3mM when included in the reaction mix gave a amplification product on agarose gel electrophoresis.

M – Molecular wt marker ($\lambda_{HindIII}$)

3- 2mM $MgCl_2$

4- 2.5mM $MgCl_2$

5- 3mM $MgCl_2$

1- 1mM $MgCl_2$

2- 1.5mM $MgCl_2$

Figure 5.4 Amplification of *J.curcas* by RAPD in varying $MgCl_2$

Table 5.5 Different *Jatropha* genotypes with their oil content

Sample Code	Sample Name	Seed oil%
A-2	JCP-4, Pantnagar	31.09
A-3	PJA-1, Hyderabad	32.32
A-5	TNAU, Mettupalayam	28.27
A-7	TFRI, Jabalpur	22.94
A-8	JIP-12, Jammu	26.12
A-9	MSU, Vadodara	36.36

Table 5.5 enlists the six different genotypes used in the present study along with their oil content. Out of 37 primers used to study the genetic diversity of 6 *Jatropha curcas* accessions, 7 primers could generate reproducible amplification products. Rest of the primers resulted either in no amplification or smeared products. These primers yielded 95 amplified bands/fragments. The number of amplified fragments ranged from 1 (RAN-10, RFU-10) to 7 (RAN-3) with an average of 9.42 bands per primer (table 5.6). The size of amplified fragments ranged from 190-3000bp (figure 5.5 – 5.11). Similar results have been reported by Ikbal et al, 2010 in *Jatropha curcas* genotypes from different states of India. Ganesh Ram et al, 2008 have reported the size of amplified products in the range of 200-2400bp for different *Jatropha* genotypes. Similar work has been documented by Gupta et al, 2008 in different *Jatropha curcas* genotypes. As shown in table 5.6, in the current study, of the 95 bands scored 66 were polymorphic whereas 28 were monomorphic. Table 5.7 shows eleven unique alleles detected with a total of five primers in five genotypes. The putatively similar bands originating for RAPDs in different individuals may not necessarily be homologous, although they may share the same size in base pairs (Gupta et al, 2008).

The pairwise comparison of the RAPD profiles based on both shared and unique amplification products was made to generate a similarity matrix. As shown in table 5.8 Jaccard's similarity coefficient varied from (0.34 – 0.66). This narrow range of similarity co-efficient value suggests a close genetic population. This could be due to the fact that *Jatropha* is not a cultivated variety and has been propagated randomly throughout India. The highest value of similarity coefficient (0.66) was detected between JIP-12 and JCP-4 and PJA-1 respectively. The lowest value of similarity coefficient (0.345) was detected between accessions from Jammu (JIP-12) and TNAU (Mettupalayam). This also explains the geographical conditions playing a decisive role as the climatic conditions of both the places are different. Cluster analysis based on Jaccard's similarity coefficient generated a dendogram (figure 5.12) which depicts the overall genetic relationship among the genotypes studied. Two distinct clusters could be observed. Genotypes JIP-12 and

TNAU which show a low similarity coefficient are also placed in different clusters. Ecological and geographical differentiation are two important factors which influence breeding and sampling strategies of tree crops which further help in understanding the population structure. Variation in genetic diversity within species is usually related with geographic range, mode of reproduction, mating system, seed dispersal and fecundity (Ikbal et al, 2010). The genetic diversity observed between the genotypes in the present investigation could be due to all the above mentioned factors. Similar conclusions have been drawn by Ikbal et al, 2010 and Gupta et al, 2008.

Table 5.6 Amplified DNA bands and polymorphism generated in *J.curcas* genotypes

Primer (Sequence 5'- 3')	Total Bands (A)	Polymorphic Bands (B)	Monomorphic Bands
RAN-3 GGC ACG TAA C	18	15	3
RAN-10 GTG CCC GAT G	9	8	1
RAN-14 TCG CCG CTT A	9	2	7
RFU-6 CCT GGG CTA C	9	3	6
RFU-10 CCT GGG TGA C	7	4	3
RBA-13 CCG GCC ATA C	10	4	6
RBA-12 CCG GCC TTA A	4	2	2
Total	66	38	28
Mean	9.42	5.42	4

Table 5.7 Primers showing amplification of unique alleles of different genotypes of *J.curcas*

Primer	Total Bands	No. of unique alleles	Allele size (bp)	Genotypes
RAN-3	18	5	190, 210 & 450	JCP-4
RAN-10	9	2	220 & 700	TFRI
RAN-14	9	1	230	JCP-4
RAN-3	18	1	320	MSU
RBA-13	10	1	570	MSU
RAN-13	18	1	580	TFRI
RAN-14	9	1	750	PJA-1
RFU-10	7	1	1000	JIP-12

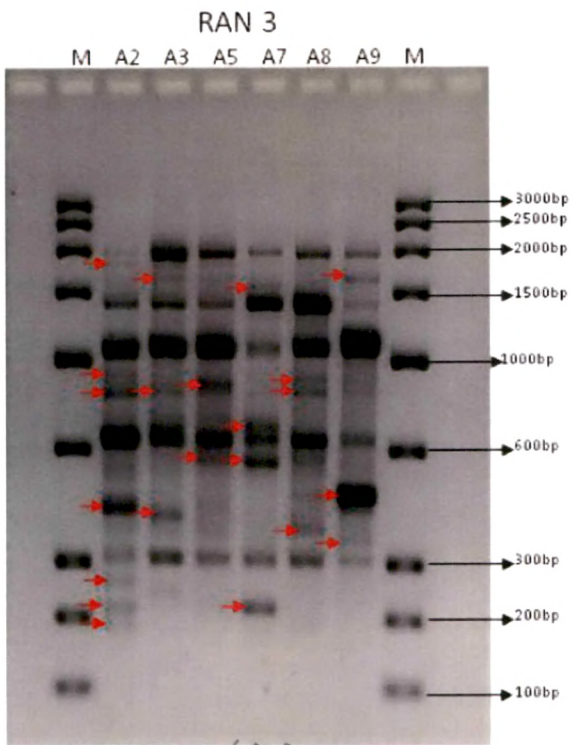

Figure 5.5: RAPD profile generated with RAN3 primer

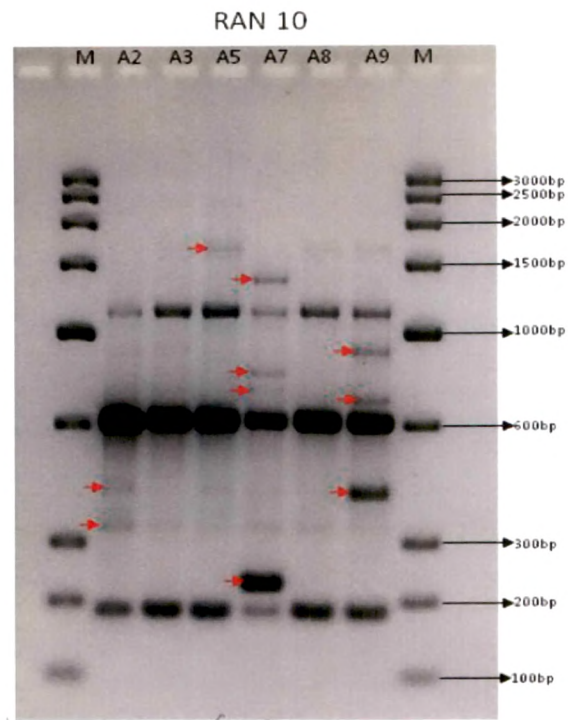

Figure 5.6: RAPD profile generated with RAN10 primer

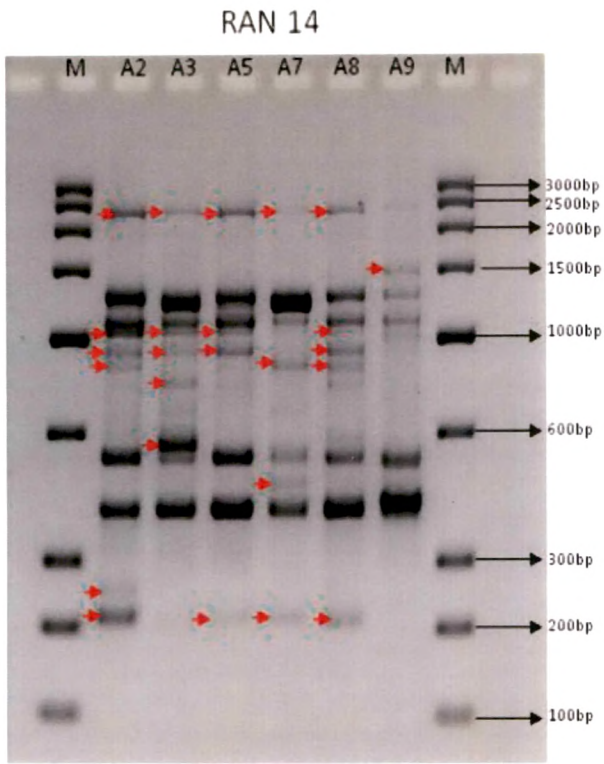

Figure 5.7: RAPD profile with RAN14

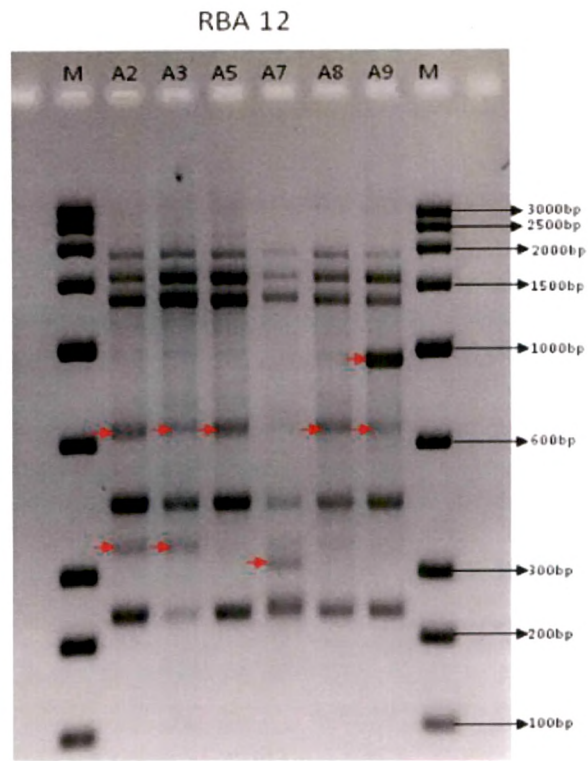

Figure 5.8: RAPD profile with RBA14

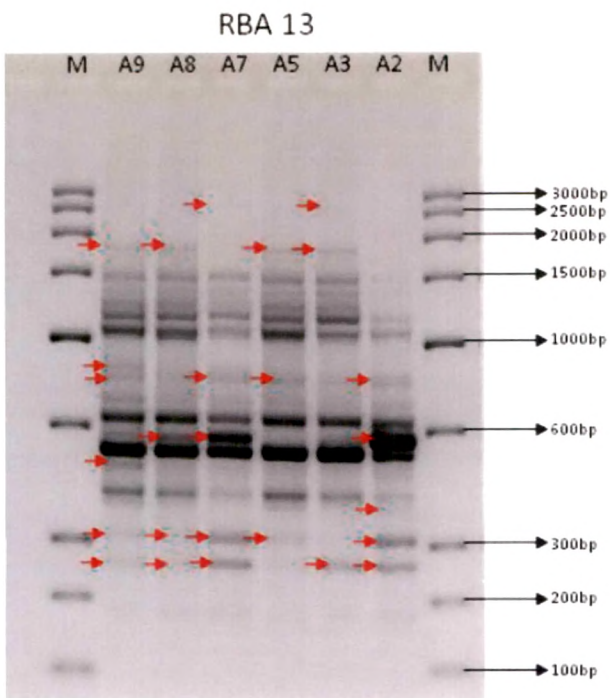

Figure 5.9: RAPD profile with RBA13

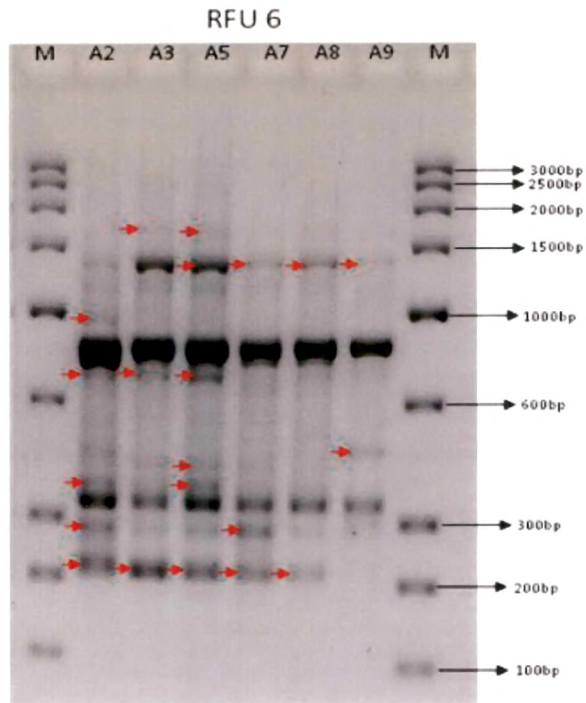

Figure 5.10: RAPD profile with RFU6

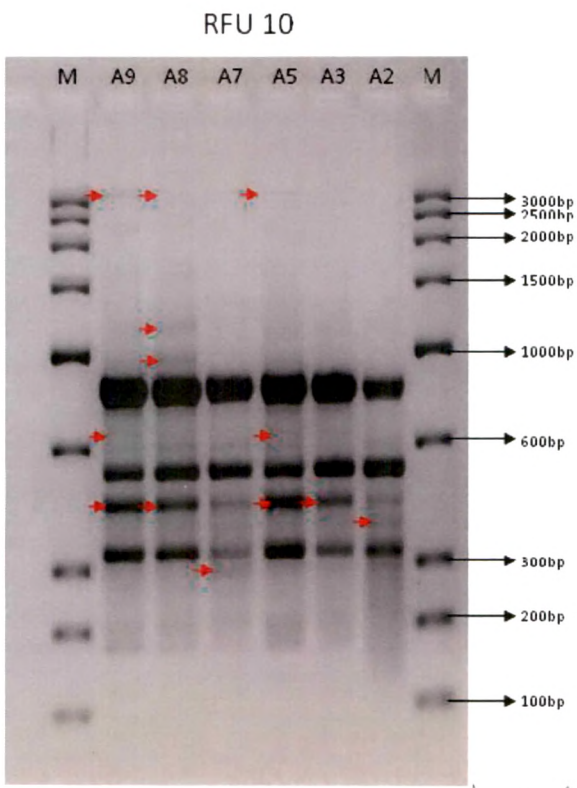

Figure 5.11: RAPD profile with RFU10

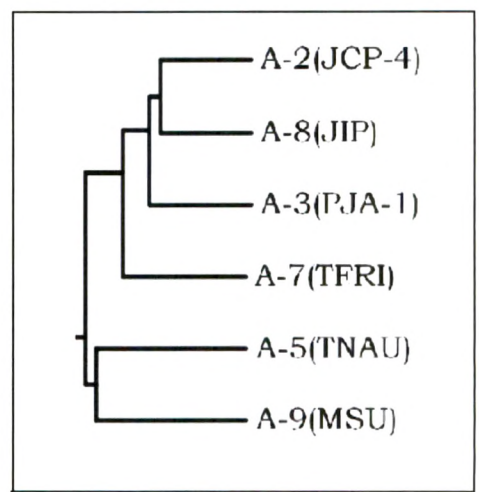

Figure 5.12 Phylogenetic tree analysis

Dendrogram of seven Jatropha accessions based on Jaccard similarity Index revealed by RAPD markers

Table 5.8 Jaccard's similarity matrix of 7 *Jatropha curcas* accessions

	JCP-4	PJA-1	TNAU	TFRI	JIP-12	MSU
JCP-4	1	0.585	0.439	0.566	0.667	0.418
PJA-1		1	0.414	0.509	0.667	0.368
TNAU			1	0.397	0.345	0.434
TTFRI				1	0.549	0.375
JIP-12					1	0.423
MSU						1

Table 5.9 Distance matrix based on Jaccard coefficient

	JCP-4	PJA-1	TNAU	TFRI	JIP-12	MSU
JCP-4	0	0.415	0.561	0.434	0.333	0.582
PJA-1		0	0.586	0.491	0.333	0.632
TNAU			0	0.603	0.655	0.566
TFRI				0	0.451	0.625
JIP-12					0	0.577
MSU						0

Table 5.10 Distance matrix based on RMSD coefficient

	JCP-4	PJA-1	TNAU	TFRI	JIP-12	MSU
JCP-4	0	0.549	0.662	0.561	0.468	0.662
PJA-1		0	0.682	0.608	0.468	0.702
TNAU			0	0.692	0.721	0.641
TFRI				0	0.561	0.692
JIP-12					0	0.641
MSU						0

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