Molecular Characterization of Important Biofuel Plant Species Through Rapd Markers

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Abstract- *The current advancement in plant biology research encompassing generation of huge amount of molecular-genetic data and development of impressive methodological skills in molecular markers is applicable in finding solutions to the challenging goals of plant breeding efforts ultimately leading to gainful applications in crop improvement. The present study is aimed to identify suitable molecular marker by screening different RAPD markers for Ricinus communis, Jatropha curcas, Pongamia pinnata and Simarouba glauca and to sequence the markers to map it onto the genome or gene. The non-edible oils from the four biofuel species were extracted by Soxhlet extraction which is a method for phenotyping by measuring the oil content. Due to the significant role of RAPD (random amplified polymorphic DNA) in providing information ranging from diversity at nucleotide level to gene and allele frequencies, the extent and distribution of genetic diversity and population structure, investigation through RAPD markers OPA02, OPA13, OPB10, OPD02, OPA05, OPB19, OPC07 and 970 was undertaken. OPC07 showed a high intensity band of J.curcas which was cloned to get a good copy number of clones of the band and was sequenced by automated cycle sequencing method. The RAPD method has successfully discriminated the plant species, in order to provide information for breeding programs of these important biofuel species. The partial sequence obtained would give a new insight to develop molecular markers directly from parts of genes in Jatropha curcas. Applying the same methodology molecular markers could be developed for all the above biofuel species.*

Keywords- Biofuel Plants, Molecular Markers (RAPD).

I. INTRODUCTION

Non-renewable hydrocarbons are being used as the major energy source the world over. Their fast depletion has forced the scientific community to search for renewable sources, which could be used as fuel. Besides the threat of depleted reserves, their excessive use can aggravate green house gases, which are now held responsible for global warming. Biofuels and bioenergy encompass a wide range of alternative sources of energy of biological origin. These renewable energy sources have the potential of increasing energy supplies in a self reliant way in developing countries. Plant-based fuels create a better balance between the formation and consumption of CO_2; decrease particulate matter, CO_2 unburnt hydrocarbons and SO_2 emission into the atmosphere (Sheehan *et al*. 1998). In this regard, biodiesel derived from the seed-oil of *Jatropha curcas* is fast emerging as an alternative to fossil fuels (Benerji *et al.* 1985) and has the desirable physico-chemical characteristics and performance, even superior to diesel (Mandpe *et al*. 2005)

Ricinus communis is a species that belongs to the Euphorbiaceae family and it is commonly known as castor oil plant and Palma Christi. This plant originates in Africa but it is found in both wild and cultivated states in all the tropical and subtropical countries of the world. However, in Asia and Africa, which are mostly net importers of vegetable oil, *Jatropha curcas* has been recognized as new energy crop for the countries to grow their own renewable energy source with many promising benefits. With the growing interest in biofuels worldwide, there is need for national governments in Asia and Africa to develop mechanisms for harnessing the potential of the fast growing industry and benefit from the growing international trade in biofuels.

Many multinational companies, particularly Scandinavian, Chinese, European and Indian ones are scrambling for African land for Jatropha plantations. It is also reported that wireless communication giants Ericsson, GSMA and MTN are investing in using biofuel from Jatropha and other oils to power cellular network base stations in the developing world for the untapped market of the potential mobile users (Katembo and Gray 2007).

Pongamia pinnata also referred to as Pongam, is a biodiesel tree legume well adapted to arid zones and has many traditional uses (Brijesh *et al.* 2006; Scott *et al*. 2008; Mukta *et al.* 2009). The pongamia tree has the rare property of producing seeds with high oil content (Sarma *et al.* 2005; Kesari *et al.* 2010). Despite the importance of this versatile

plant and the availability of appropriate molecular genetics tools, the full potential of Pongamia is far from being realized. The natural constraints that limit its large-scale production and availability, to meet the demand for biodiesel production, are its long gestation period (4–7 years), plant height, seed storage behaviour, insect pests, and the seed oil yield and quality (NOVOD 2010). Until recently, the identification of elite individuals of *P. pinnata* was mainly described in terms of morphological and agronomic traits which are known to be deeply influenced by environmental factors (Kaushik *et al.* 2007; Kesari *et al.* 2008). The cross-pollinating nature of *P. pinnata* contributes to its wide germplasm biodiversity. Thus, it becomes an important step to examine the genetic variations among naturally growing elite individuals of *P. pinnata* at inter and intra-population levels, and to prepare strategies for its specific exploitation by plant breeders in promoting it as a versatile biodiesel plant.

The presence of polyphenolics and polysaccharide content makes the isolation of high quality intact genomic DNA problematic. Although several successful DNA extraction protocols for plant species containing polyphenolics and polysaccharides compound have been developed, none of these are universally applicable to all plants (Varma *et al*. 2007) and the published protocols are also limited because of degradation of DNA by DNases and other nucleases (Kesari *et al.,* 2009). Therefore, researchers often modify a protocol or blend two or more different procedures to obtain DNA of the desired quality (Varma *et al*. 2007).

RAPD markers have been widely used in plant research for phylogenetic studies, genome mapping and genetic variation analysis both at intra and inter-population levels (Li *et al.* 2008). The technique has several advantages including simplicity, low cost, speed and lack of requirement for DNA sequence information (Williams *et al.* 1990; Lopes *et al*. 2007). However, RAPD technology has several limitations including dominance, uncertain locus homology, sensitivity and reliability. Inter-simple sequence repeat represents the marker of choice for varietal identification studies as they are transferable, hypervariable, highly polymorphic, multi allelic dominant markers, relatively simple to interpret and show high information content (Souframanien and Gopalkrishna 2004).

II. MATERIAL AND METHODS

2.1 Plant material

The germplasm used in this study for genetic diversity studies were young seeds, collected during the month of August 2010 from *Jatropha curcas, Ricinus communis, Pongamia pinnata and Simarouba glauca.* All the seed samples were appropriately stored at -20 ^{0}C for further analysis.

2.2 Soxhlet Extraction

Soxhlet extraction is only required where the desired compound has a limited solubility in a solvent, and the impurity is insoluble in that solvent. The oil content of *Jatropha curcas, Ricinus communis, Pongamia pinnata and Simarouba glauca* seeds were obtained by complete extraction using the Soxhlet extractor (Konte, USA). Extraction of oils from seeds was carried out by Soxhlet extraction method. The 100 gm of each powdered seed sample was put into a porous thimble and placed in a Soxhlet extractor, using 150 cm^3 of n-hexane (with boiling point of 40- 60^o C) as extracting solvent for 6 hours. The oil was obtained after the solvent was removed under reduced temperature and pressure and refluxing at 70^o C to remove the excess solvent from the extracted oil. The oil was then stored in freezer at – 4^o C for subsequent analysis (Warra *et al.* 2011)

2.3 DNA Isolation by CTAB Method

A modified CTAB buffer method (Kim and Hamada, 2005) was employed to post-extraction analyses of DNA from leaves. The DNA quantity of each sample was estimated and DNA concentrations were normalized at 2.5 ng/µl. The DNA quality of each sample was evaluated by running 1 µl of DNA on a 1% agarose gel.

2.3.1 PCR Conditions:

The thermo cycler was programmed for 1 min 30 sec for Initial Denaturation at 94°C, followed by 35 cycles of 15 sec at 94°C (Denaturation), 30 sec at 40°C (Primer Annealing), & 60 sec at 72°C (Elongation).The programme ends with 5 min at 72°C for extra elongation. This process takes 3 hours 30 min to 4 hours to complete the 35 cycles. RAPD-PCR products are stored in thermo cycler at 4°C after the programme ends. The samples were run in a 2% agarose gel with appropriate marker.

2.3.2 RAPD assay

The principle states that, in a single, short oligonucleotide primer, which binds to many different loci, is used to amplify random sequences from a complex DNA template. This means that the amplified fragment generated by PCR depends on the length and size of both the primer & the target genome. The assumption is made that a given DNA sequence (complementary to that of the primer) will occur in

the genome, on opposite DNA strands, in opposite orientation within a distance that is readily amplifiable by PCR.

Table 1: The different RAPD primers and their sequences taken are as tabulated below

Sl. No	Primers	Sequence
1	OPA-02	5′-GGACCCAACC-3′
2	OPA-13	5'-CAGCACCCAC-3'
3	OPB-10	5′-CTGTCGGGAC-3′
4	OPD-02	5′-GGACCCAACC-3′
5	OPA-05	5′-AGGGGTCTTG-3′
6	OPB-19	5′-ACCCCCGAAG-3′
7	OPC-07	5′-GTCCCGACGA-3′
8	970	5′-GTAAGGCCG-3′

2.4 Cloning and Transformation

Cloning is to incorporate a DNA sequence into a vector which can replicate in another organism. The first step in cloning is to fragment the genome into a collection of smaller, more manageable DNA pieces. The pieces of DNA are normally cloned and propagated in bacteria, usually *E.coli*. The most widely used method for cloning uses vectors. Vectors are specialized plasmids which are used to clone pieces of DNA.

Insert used: High intensity band of *Jatropha curcas* obtained after RAPD.

Vector used: Vector is a high copy number with pMB1 origin of replication. In addition, the vector also has f1-origin from filamentous phage f1 for the preparation of SS-DNA. The size of the vector is 3 kb. The vector carries Ampicillin resistant marker. The MCS is within the *lac Zα* fragment that allows blue-white screening.

Host organism: *E. coli* DH5α strain.

2.4.1 Dephosphorylation:

0.5µl alkaline phosphatase was added to the vector vial of restriction digestion & incubated for more than 20 min. The volume was made to 200µl with sterile water. Equal volume of Phenol & Choloroform was added, mixed & spinned. Carefully the aqueous layer was pipette out.

2.4.2 DNA precipitation:

1/10th volume of sodium acetate & equal volumes of isopropanol were added to the DNA sample. It was incubated at room temperature for 15 minutes & centrifuged at 10000 rpm for 10 minutes. The supernatant was decanted off & the pellet was air dried. The DNA was suspended in 30µl of TE buffer.

2.4.2.1 Gel electrophoresis of vector & insert DNA:

1% agarose gel was prepared & the vector sample was loaded completely into the first well. The insert DNA was loaded into the well and suitable marker was used. The electrophoresis was run till the fragments were separated completely.

2.4.2.2 Purification of DNA from Agarose Gel using Silica Method:

- ✓ The gel was weighed along with the vial. 2.5 times of NaCl was added & kept in 55°C till the gel melts completely.
- ✓ 20µl of silica suspension was added. The solution was mixed for 5 min by inverting.
- ✓ The vial was spun at 6000 rpm for 1 min. The supernatant was saved in another fresh vial. The silica pellet was suspended & 200µl of wash buffer consisting of 50% ethanol was added to remove the salt from sample.
- ✓ The vial was mixed for 5-10 times & spun for 6000 rpm for 1 min. The supernatant was saved to the previously saved vial.
- ✓ The pellet was suspended & 200µl of wash buffer was added and the vial was spun. The vial was mixed for 5-10 times & spun at 8000 rpm for 2 min and supernatant was saved.
- ✓ The pellet was air dried. 30µl of pre-warmed elution buffer consisting of 10mM Tris-EDTA buffer was added to the vial containing the air dried pellet & was suspended properly.
- ✓ The vial was incubated for 5 min & was spun at 8000 rpm for 2 min. The supernatant was saved in afresh vial & was labelled as elution I.
- ✓ To the pellet 30µl of elution buffer was added & suspended properly. The vial was spun at 8000 rpm for 2 min& the supernatant was saved & was labelled as elution II.

2.4.2.3 Quantification of DNA:

For cloning it is always advised to visualize the DNA to be used. The sample of 40-50ng was loaded on to the gel, along with 50ng of known DNA. The intensities were compared & the concentrations were determined.

The amount of insert to be added was calculated using the formula:

$$\text{Amount of insert} = \frac{\text{Size of insert x Amount of vector}}{\text{Size of vector}}$$

2.4.2.4 Ligation:

The ligation mix was incubated at 16°C for overnight.

Table 2: Ligation mixture

Components	Stock Conc.	Req. Conc	1:1(µl)	1:2(µl)	1:4(µl)
Distilled Water	-	-	14.5	14	13
Ligase assay buffer	10X	1X	2	2	2
Vector	50 ng/µl	50 ng	1	1	1
Insert DNA	60 ng/ µl	30 ng	0.5	1	2
T4 DNA Ligase	200 CEU/µl	200 CEU	1	1	1
Total Volume			**20**	**20**	**20**

2.5 Transformation

2.5.1 Preparation of competent cells: 3–4 moderately sized host colonies were picked from LB plate and was inoculated into 5ml of LB broth. The shaker was incubated at ~200 rpm at 37°C till the cells are visibly turbid. 1.5 ml culture was aseptically transferred into 3 sterile 1.5ml vials and spun at 4000 rpm for 5 minutes at 4°C. The supernatant was drained completely by quickly tapping on tissue. The pellet was dispersed gently with pipette tip and the suspension was pooled in one of the vials by keeping the tubes on ice. It was re-suspended very gently in 100µl of ice-cold solution A. Immediately 7µl of solution B was added just above the suspension and mixed by finger flicking. It was kept on ice for 10 min. To this 10µl of ligation mix was added into three separate vials, tapped gently & incubated on ice for 15-20 min. After that heat shock has been given by keeping the tubes in water bath adjusted to 42°C for 30 seconds. Immediately the vials were chilled in ice for 10 min. added 0.5 ml of LB broth aseptically to the vials and incubated the culture for 45mins to 1 hr at 37°C.

2.5.2 Plating: Before plating, Samples 1, 2 & 3 were spun for 5 mins and retained 100 µl of supernatant, discard remaining. From vial 1, 2 & 3 completely plated on three different LB ampicillin with *X-gal* plate and spread thoroughly using a spreader. Incubated the plates inverted overnight at 37°C.

2.5.3 Screening of White Colonies: All the white colonies were inoculated separately along with 1 Blue colony as Control into 5ml LB broth + Ampicillin and incubated at 37°C overnight.

2.5.4 Clone Retardation Check: Plasmid was isolated from 1.5ml of overnight grown culture (Alkaline Lysis Method), Suspended in 35µl 1X TE. It was loaded in 5µl of plasmid sample + 10µl of diluted Gel loading Dye on 1% Agarose Gel electrophoresis.

2.5.5 Clone confirmation by restriction digestion: Clones were checked by digesting cloned plasmid with **pvuII enzyme** and vector primer is used for checking the amplification of clones.

2.6 Sequencing: The successful clones were given to Applied Biosciences for sequencing.

III. RESULTS AND DISCUSSION

3.1 SOXHLET EXTRACTION

The % of oil was found by the formula =

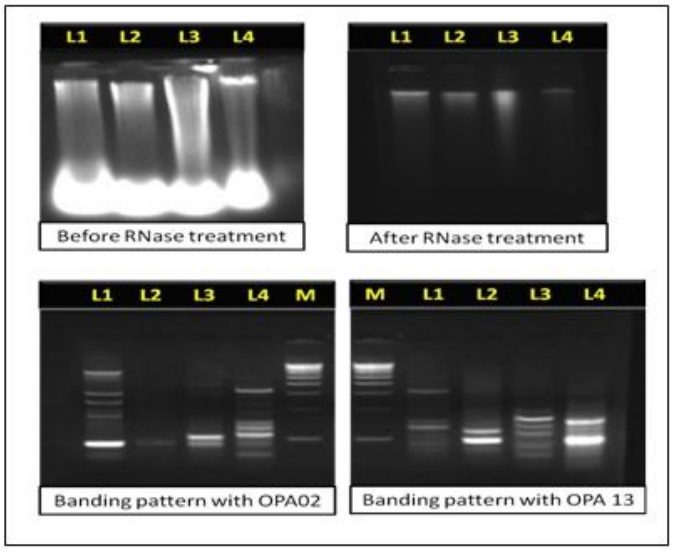
$$\frac{\text{Weight of oil obtained in gms}}{\text{Weight of seeds taken in gms}} \times 100$$

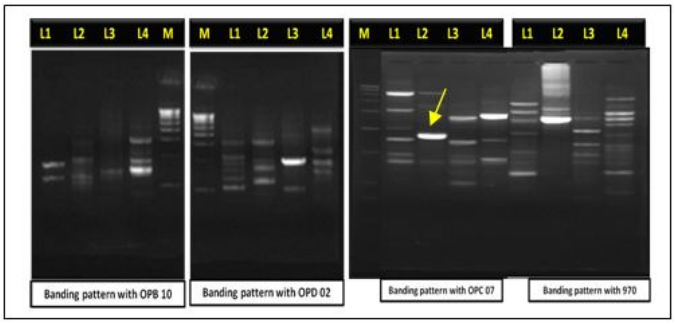
Table 3: The % of oil extracted was tabulated as follows

Species	Weight of oil obtained (gms)	Weight of seeds taken (gms)	Oil %
Jatropha curcas	10	39.19	25.5
Ricinus communis	22.1	39	56.9
Pongamia pinnata	12.61	39.19	32.17
Simarouba glauca	18.25	36	50.6

3.2 DNA isolation:

Plant genomic DNA was isolated by CTAB method from all the four plants and was run on the agarose gel to view the DNA content. In figure the DNA sample contained large amount of RNA which was treated with RNase to get pure DNA samples. Where, L1 is *R. communis*, L2 is *J. curcas*, L3 is *P. pinnata* & L4 is *S. glauca*.





The primer OPC7 showed unique variation in Jatropha. High density band was obtained which was cloned and sequenced.

Table 4: Primers used and the number of bands generated

Primer	*R. communis*	*J. curcas*	*P. pinnata*	*S. glauca*
OPA02	7	1	5	8
OPA13	7	3	6	3
OPB10	2	3	2	5
OPD02	6	5	3	5
OPA05	0	0	0	0
OPB19	0	0	2	0
OPC07	7	4	6	4
970	6	3	5	9

3.3 SEQUENCING

The sequence obtained was:

>0513_072_001_6_M13F-A07.ab1

ACGGTTCTTCTAATAGGGCGATTGGGCCGACGTCGC
ATGCTCCCGGCCGCCATGGTTCCATGGCCGCGGGAT
ATCACTAG
TGCGGCCGCCTGCAGGTCGACCATATGGGAGAGCTC
CCAACGCGTTGGATGCATAGCTCTGATGAATCCCCTA
ATGATTT
TTATCAAAATCATTAAGTTAAGGTAGATACACATCTT
GTCATATGATCAAATGGTTTCGCCAAAAATCAATAAT
CAGACA
ACAAAATGTGCGAACTCGATATTTTACACGACTCTCT
TTACCAATTCTGCCCCGAATTACACTTAAAACGACTC
AACAGC
TTAACGTTGGCTTGCCACGCCTTACTTGACTGTAAAA
CTCTCACTCTTACCGAACTTGGCCGTAACCTGCCAAC
CAAAGC
GAGAACAAAACATAACATCAAACGAATCGACCGATT
GTTAGGTAATCGTCACCTCCACAAAGAGCGACTCGC
TGTATACC
GTTGGCATGCTAGCTTTATCTGTTCGGGCAATACGAT
GCCCATTGTACTTGTTGACTGGTCTGATATCCGTGAG
CAAAAA
CGGCTTATGGTATTGCGAGCTTCAGTCGCACTACACG
GTCGTTCTGTTACTCTTTATGAGAAAGCGTTCCCGCT
TTCAGA
GCAATGTTCAAAGAAAGCTCATGACCAATTTCTAGC
CGACCTTGCGAGCATTCTACCGAGTAACACCACACC
GCTCATTG
TCAGTGATGCTGGCTTTAAAGTGCCATGGTATAAATC
CGTTGAGAAGCTGGGTGTACTGGTAGTCGAGTAAGA
GAAAAGT
ACAATATGCAGACCTAGGGAGCGGAAACTGGAACTA
TCAGCACTTACATGAATATTGTCATCTAGTCCACCTC
AAAGAAC
TTAGGCTATAAGAAGGGCTGACTAAAAGCACCCATC
TCTCATGCGAATTTGATTGTATAATCTTCGCCTCTAA
GCCCGGA
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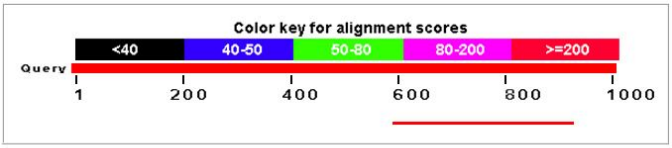
ACATAGC
ATTCCAGACCTATCTACCTGTTGAATGT



Descriptions

Sequences producing significant alignments:

DESCRIPTION

Jatropha curcas DNA, contig: Jcr4S01857_1, strain: Palawan, whole genome shotgun sequence

Alignments

Jatropha curcas DNA, contig: Jcr4S01857_1, strain: Palawan, whole genome shotgun sequence
Sequence ID: dbj|BABX02004926.1|Length: 14754Number of Matches: 1
Related Information
Range 1: 1 to 389GenBankGraphicsNext MatchPrevious Match

Score	Expect	Identities	Gaps	Strand
514 bits(278)	4e-144	366/403(91%)	27/403(6%)	Plus/Plus

Query 619
AGAAAGCGTTCCCGCTTTCAGAGCAATGTTCAAAGA
AAGCTCATGACCAATTTCTAGCCG 678
||||||||||||||||||||||||||||||||||||||||||||||||||||||||

Sbjct 1
AGAAAGCGTTCCCGCTTTCAGAGCAATGTTCAAAGA
AAGCTCATGACCAATTTCTAGCCG 60

Query 679
ACCTTGCGAGCATTCTACCGAGTAACACCACACCGCT
CATTGTCAGTGATGCTGGCTTTA 738
||||||||||||||||||||||||||||||||||||||||||||||||||||||||

Sbjct 61
ACCTTGCGAGCATTCTACCGAGTAACACCACACCGCT
CATTGTCAGTGATGCTGGCTTTA 120

Query 739
AAGTGCCATGGTATAAATCCGTTGAGAAGCTGGGT-
G-TACTGGT-A-GTCGAGTAAGAG 794
|||||||||||||||||||||||||||||||| | ||||||| | ||||||||||||

Sbjct 121
AAGTGCCATGGTATAAATCCGTTGAGAAGCTGGGTT
GGTACTGGTTAAGTCGAGTAAGAG 180

Query 795 -
AAAAGTACAATATGCAGACCTAGGGAGCGGAAA-
CTGGAA-C-TATCAGCA-CTTACAT 849
|||||||||||||||||||||| |||||||| |||||| | ||||||| |||||||

Sbjct 181 GAAAAGTACAATATGCAGACCTAGG-
AGCGGAAAACTGGAAACCTATCAGCAACTTACAT
239

Query 850
GAATATTGTCATCTAGTCCACCTCAAAGAACTTAGGC
TATAAGAAGGGCTGACTAAAAGC 909
|| ||| |||||||||| || |||||| |||||||||||| || ||||||||||||

Sbjct 240 GA-TAT-GTCATCTAGTC-AC-
TCAAAGACTTTAGGCTATAAGA-GG-
CTGACTAAAAGC 293

Query 910 AC-CCATCTCTCATGCGAA-
TTTGATTGTATAA-TCTTCGCCTCTAA-
GCCCGGAAAAAT 965
| ||| |||||||| || || |||||||| ||| ||| ||||| | ||| ||||||

Sbjct 294 AATCCAA-
TCTCATGCCAAATTCTATTGTATAAATCT-CGC-
TCTAAAGGCCGAAAAAAT 350

Query 966
CCAGCGCCTCGACACGGACTCAATTGTCACCACCCG
CTAACCT 1008
| ||||| |||||||||||||| |||||||||||||| | ||||

Sbjct 351 C-AGCGC-TCGACACGGACTCA-
TTGTCACCACCCG-TCACCT 389

In bioinformatics, Basic Local Alignment Search Tool, or BLAST, is an algorithm for comparing primary biological sequence information, such as the amino-acid sequences of different proteins or the nucleotides of DNA sequences. A BLAST search was enabled to compare the query sequence with a library or database of sequences, and identify library sequences that resemble the query sequence above a certain threshold.

Due to advances in molecular biology techniques, large numbers of highly informative DNA markers have been developed for the identification of genetic polymorphism. In the last decade, the random amplified polymorphic DNA (RAPD) technique based on the polymerase chain reaction (PCR) has been one of the most commonly used molecular techniques to develop DNA markers (Fevzi Bardakci, 2001).

For instance, eight random primers were used to identify suitable molecular marker for four biofuel species. As the plants chosen for the present study were the biofuel species the oil yield (wt %) of *Ricinus communis, Jatropha curcas, Pongamia pinnata* and *Simarouba glauca* was found by soxhlet extraction method. Soxhlet extraction is presented as the standard and reference method for fatty acids extraction in most cases. The desired compounds are extracted by an interactive percolation of a fresh solvent (Anne *et al*., 2012). The oil yield (wt %) obtained are 56.9%, 25.5%, 32.17% and 50.6% respectively. This observation was supported by the study that shows the oil yield (wt %) of *R. communis, J. curcas, P. pinnata* and *S. glauca* as 55%, 27-40% ,30-35% and 50-55% respectively by soxhlet extraction (Hemant *et al*., 2011).

A high quality DNA extraction is highly desirable and essential for plant breeding and other molecular screening techniques (Iqbal *et al*., 2013) therefore, CTAB method of DNA isolation was used which yielded a high quality DNA after RNase treatment for RAPD. The random primers OPA02, OPA13, OPB10, OPD02, OPA05, OBP19, OPC07 and 970 primers were used for the characterization. The

primers OPA02, OPA13, OPB10, OPD02, and OPC07, 970 showed good polymorphic bands indicating a wide genetic variance and OPA05, OBP19 showed least number of polymorphic bands.

The findings outlined above had explained the capability of individual primers to amplify the less conserved and highly repeated regions of the genomic DNA and high possibility for the amplified fragments to contain repeated sequences. Out of eight primers OPC07 gave a high intensity band of *J. curcas* of lesser base length compared to other bands and which was absent in other biofuel species obtained in our study.

IV. CONCLUSION

For the effective genetic improvement program of biofuel plants, maintenance of high genetic diversity is one of the most important issues as the species have immense multipurpose importance, yet genetically uncharacterized. A wide range of variability exists in these plants with respect to seed and oil traits. Morphological markers for identifying the individuals are limited in number and they do not often reflect genetic relationships because of interaction with the environment. In the current study, Eight RAPD primer combinations were used to disclose a proper number of all comparisons. RAPD detected polymorphism more efficiently due to greater number of loci detected. One of the most widely used applications of RAPD technique is the identification of markers linked to a trait of interest without the necessity for mapping the entire genome which was exploited in our study.

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