Molecular markers to assess genetic diversity and mutant identifications in *Jatropha curcas*

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Abstract

Jatropha curcas (Linnaeus) belongs to the Euphorbiaceae family, is a multipurpose use, drought resistant and perennial plant. It is an economically important crop, which generates wide interest in understanding the genetic diversity of the species towards selection and breeding of superior genotypes. *Jatropha* accessions are closely related family species. Thus, better understanding of the effectiveness of the different DNA-based markers is an important step towards plant germplasm characterisation and evaluation. It is becoming a prerequisite for more effective application of marker techniques in breeding programmes. Inter-simple sequence repeats (ISSRs) has shown rapid, simple, reproducible and inexpensive means in molecular taxonomy, conservation breeding and genetic diversity analysis. These markers were used to understand diversity and differentiate amongst accessions of *Jatropha* population and mutant lines generated by acute gamma radiation. The ISSR for marker applications are essential to facilitate management, conservation and genetic improvement programmes towards improvement of biodiesel production and medication substances. A total of 62 ISSR primers were optimised for polymorphism evaluations on five foreign accessions (Africa, India, Myanmar, Indonesia, Thailand), nine local accessions and two mutants of *Jatropha*. Optimisation was resulted 54 ISSR primers affirmative for the polymorphism evaluation study, which encountered 12 ISSR primers, showed significance polymorphism amongst the accessions and mutants. Marker derived from ISSR profiling is a powerful method for identification and molecular classification of *Jatropha* from accession to generated mutant lines.

Key words: ISSR, *Jatropha*, diversity, DNA markers, mutant

Abstrak

Jatropha curcas (Linnaeus) tergolong dalam keluarga Euphorbiaceae yang merupakan tanaman saka pelbagai guna dan tahan kemarau. Ia adalah tanaman ekonomi yang penting dan mendorong minat untuk pemahaman kepelbagaian genetik spesies tersebut untuk tujuan pemilihan dan pembiakan genotip superior. Spesis *Jatropha* adalah berkait rapat di kalangan aksesinya. Pemahaman yang baik tentang penanda molekul berasaskan DNA adalah amat berkesan sebagai langkah penting untuk pencirian germplasma tumbuhan dan penilaiannya. Ia menjadi satu syarat penting sebagai penanda molekul yang berkesan di dalam program-program pembiakan. *Inter-simple sequence repeats* (ISSRs) adalah kaedah yang mudah, mempunyai pengulangan yang tinggi dan menggunakan kos yang rendah bagi diaplikasikan dalam taksonomi molekul, pembiakan pemuliharaan dan analisis kepelbagaian genetik. Penanda molekul ini digunakan dalam memahami kepelbagaian dan membezakan antara populasi spesis *Jatropha* serta mutan yang dihasilkan melalui penyinaran gama secara akut. Aplikasi ISSR adalah penting bagi memudahkan pengurusan, pemuliharaan dan program perbaikan genetik ke arah peningkatan pengeluaran biodiesel dan substrat farmaseutikal. Sejumlah 62 pencetus ISSR telah dioptimumkan untuk menilai polimofik pada lima sampel *Jatropha* berasal dari negara asing (Afrika, India, Myanmar, Indonesia, Thailand), sembilan sampel tempatan dan dua mutan. Sebanyak 54 pencetus telah berjaya dioptimumkan dan 12 pencetus telah memberikan polimorfik yang signifikan di kalangan sampel-sampel tersebut. Penanda molekul dari pencirian ISSR merupakan kaedah berkesan dalam mengenalpasti dan mengklasifikasikan aksesi *Jatropha* untuk penghasilan mutan.

Kata kunci: ISSR, *Jatropha* , kepelbagaian, penanda DNA, mutan

INTRODUCTION

The genus *Jatropha* belonging to Euphorbiaceae family of plant kingdom, is a diploid plant with chromosome number x (2n) = 22. They are closely related family and consist of about 175 species in the world. *Jatropha curcas* is commonly known as physic nut, and is a multipurpose plant valued not only for its medicinal properties and resistance to various stresses i.e. biotic and abiotic stresses but also as a biofuel crop (Divakara *et al.,* 2010). Thus, it has received extensive attention for the use of its seed oil as a commercial source of biofuel and related products (Divakara *et al*, 2010).

The potential economic importance of *J. curcas* has generated wide interest in understanding the genetic diversity of the species for selection and breeding of superior genotypes (Shen *et al.*, 2010). Observations in upcoming plantations indicate that the productivity of the individual *J. curcas* show high variations (Basha *et al.*, 2009). Unfortunately, information regarding the extent and pattern of genetic variation in *J. curcas* population is limited and obstacle by its nature of out-crossing breeding. Global exploration, introduction, germplasm characterisation and evaluation are necessary to provide strong base for development of elite varieties.

The use of DNA markers has been realised a key factor for increasingly accurate genetic diversity assessment of taxonomic relationships and history of gene flow as most of the morphological characteristic are plastic and strongly influenced by environmental factors (Kumar *et al.*, 2009). Amongst all markers available, inter-simple sequence repeats (ISSRs) marker has been shown to provide rapid, simple, reproducible and inexpensive means in molecular taxonomy, conservation, breeding and genetic diversity analysis as no genetic information requires to perform this evaluations. Thus, ISSR is ideal for fingerprinting and characterisation of accessions and inter-specific hybrids (Carvalho *et al.*, 2008; Kumar *et al.*, 2009). The amplification of this microsatellite-based molecular marker depends on the variation, motif and frequency of SSRs that changed due to natural crossing and/or mutation induction (Carvalho *et al.*, 2008).

Among various PCR-based markers, ISSR markers have been revealed to be useful as novel DNA markers in studies on purposing crop improvement such as genomic fingerprinting, phylogenetic analysis and gene tagging (Isshiki *et al.*, 2008). ISSR markers amplify regions between adjacent and inversely oriented microsatellites using di-, tri-, tetra- and pentanucleotide SSR primers with the advantage that the information of the target DNA sequence is not required. Studies have shown that ISSRs provide a powerful, rapid, simple, reproducible and inexpensive means to access genetic diversity among closely related cultivars, characterization of accessions and identification of cultivar and varieties (Kumar *et al.*, 2008).

MATERIALS AND METHOD

Young and healthy leaves of *J. curcas* samples were collected from Nuclear Malaysia greenhouse, LGM and LTKN. Genomic DNA isolation followed the Doyle and Doyle (1990) procedures with small modification. DNA quality was visualised under UV transilluminator after separation by 1% agarose gel electrophoresis at 70 V for 45 min in TBE buffer (Tris boric-EDTA) stained by ethidium bromide (0.5 µg/mL). For DNA purity, the isolated genomic DNA was evaluated by NanoDrop Spectrophotometer at absorbance of 260 nm and 280 nm.

Table 1: Samples and code used for genetic diversity assessments

Representative Location	Collection Site
Africa (AF)	LGM
Borneo Mega Power (BM)	LGM
D1BP	LGM
India (DN)	LGM
Jatroleum (JR)	LGM
Myanmar (MM)	LGM
Melaka (MK)	LGM
Kuala Pilah (KP)	LGM
Indonesia (ND	LGM
Sabah (SB)	LGM
Negeri Sembilan (SEM)	LGM
Semarak (SM)	LTKN
Sungai Buloh (SH)	LGM
Thailand (TH)	LGM

Mutant Plants	Collection Site
JM 308C 1	ANM
JM 308C 2	ANM
JM 308C 3	ANM
JM 308S 1	ANM
JM 308S 2	ANM
JM 308S 3	ANM
JM 308S 4	ANM
JM 352C 1	ANM
JM 352C 2	ANM
JM 352C 3	ANM

Note: LGM (Lembaga Getah Malaysia), LTKN (Lembaga Tembakau dan Kenaf Malaysia), C (cutting), S (seed)

A total of 62 ISSR primers were used for polymorphism evaluations on both foreign and local *Jatropha* accessions (Table 1) including two mutants (propagation by cuttings and seeds) from Nuclear Malaysia germplasm which are previously exposed to gamma radiation. The mutants originated from Semarak.

Table 2: List of ISSR primers used in this study

Primer	Primer Sequence 5' - 3'	Tm (C°)
IS 1	$(CAC)_7T$ (22 mer)	55.3
IS 2	$(GA)_9C$ (19 mer)	46
IS 3	$G(TG)_9$ (19 mer)	46
IS 4	$(CAC)_7G$ (22 mer)	57.2
IS 5	$(CAC)_7GT$ (23 mer)	57.3
IS 6	$(GTG)_7C$ (22 mer)	57.2
IS 7	$(CA)_{10}G$ (21 mer)	49.2
IS 8	$(CT)_9G$ (19 mer)	46
IS 9	$(GA)_9AY$ (20 mer)	45.6
IS 10	$BDBT(CCT)_6$ (18 mer)	49
IS 11	$HVH(TCC)_6$ (20 mer)	48.2
IS 12	$(AG)_8T$ (19 mer)	39.5
IS 13	$(AG)_8G$ (17 mer)	41.9
IS 14	$(GA)_8T$ (17 mer)	39.5
IS 15	$(GA)_8C$ (17 mer)	41.9
IS 16	$(GA)_8A$ (17 mer)	39.5
IS 17	$(CT)_8A$ (17 mer)	39.5
IS 18	$(CT)_8G$ (17 mer)	41.9
IS 19	$(CT)_8T$ (17 mer)	39.5
IS 20	$(CA)_8A$ (17 mer)	39.5
IS 21	$(CA)_8G$ (17 mer)	41.9
IS 22	$(GT)_8A$ (17 mer)	39.5
IS 23	$(GT)_8C$ (17 mer)	41.9
IS 24	$(GT)_8T$ (17 mer)	39.5
IS 25	$(TC)_8A$ (17 mer)	39.5
IS 26	$(GT)_9C$ (19 mer)	46
IS 27	$(GT)_7GGTG$ (17 mer)	45.2
IS 28	$(AC)_8T$ (17 mer)	39.5
IS 29	$(AC)_8C$ (17 mer)	41.9
IS 30	$(AC)_8G$ (17 mer)	41.9
IS 31	$(TG)_8A$ (17 mer)	39.5
IS 32	$(TG)_8G$ (17 mer)	41.9

Primer	Primer Sequence 5' - 3'	Tm (C°)
IS 33	$(AG)_8YT$ (18 mer)	41.8
IS 34	$(GA)_8YT$ (18 mer)	41.8
IS 35	$(CT)_8RA$ (18 mer)	41.8
IS 36	$(CT)_8RC$ (18 mer)	44
IS 37	$(CA)_8RT$ (18 mer)	41.8
IS 38	$(CA)_8RC$ (18 mer)	44
IS 39	$(GT)_8YA$ (18 mer)	41.8
IS 40	$(GT)_8YG$ (18 mer)	44
IS 41	$(TC)_8RT$ (18 mer)	41.8
IS 42	$(AC)_8YG$ (18 mer)	44
IS 43	$(AC)_8YA$ (18 mer)	41.8
IS 44	$(AC)_8YT$ (18 mer)	41.8
IS 45	$(TG)_8RT$ (18 mer)	41.8
IS 46	$(TG)_8RC$ (18 mer)	44
IS 47	$(ACC)_6$ (18 mer)	49.7
IS 48	$(ATG)_8$ (24 mer)	45.4
IS 49	$(CTC)_6$ (18 mer)	49.7
IS 50	$(GAA)_6$ (18 mer)	36.1
IS 51	$(GACA)_6$ (24 mer)	52.3
IS 52	$(TCC)_5RY$ (17 mer)	46.7
IS 53	$(CT)_8C$ (17 mer)	41.9
IS 54	$(AG)_8C$ (17 mer)	41.9
IS 55	$(AG)_8A$ (17 mer)	39.5
IS 56	$(TC)_8C$ (17 mer)	41.9
IS 57	$(GA)_8CT$ (18 mer)	42.9
IS 58	$(GT)_8YC$ (18 mer)	44
IS 59	$(GGAGA)_3$ (15 mer)	39.5
IS 60	$BDB(CA)_7$ (17 mer)	41.1
IS 61	$(GA)_9T$ (19 mer)	43.8
IS 62	$(GA)_9A$ (19 mer)	43.8

PCRs were performed in a 25 µl reaction volume containing 1xPCR buffer (Promega), 2.5 mM $MgCl_2$, 0.23 µM dNTPs (Bioline), 0.4 mM of each degenerate primer, 0.2U *Taq* DNA Polymerase (Promega) and 50-100 ng/µl template genomic DNA. Cycling conditions is 94°C for 3 minutes (initial denaturation) followed by 30 cycles denaturation at 94 °C for 30 s, annealing temperature dependent on primers optimisation for 30-50 s and elongation at 72 °C for 60 s. Final elongation was 72 °C for 1 min. Amplification was performed in a PTC-200 Peltier Thermal Cycler (MJ Research) in 0.2 mL tubes. PCR products were separated in TBE buffer by 1.8 % agarose gel electrophoresis at 70 V for 2 h.

RESULTS AND DISCUSSION

PCR technique is a fundamental tool for genome analysis with widely recognised advantages. The first advantage will be the extraordinary sensitivity of the method of exponential amplification of minute numbers of target sequences to large amounts of yield. Theoretically, even a single copy of a target sequences can be detected and amplified by PCR technique. Despite the wonders of PCR technique, it comes with disadvantages as well. Because of its sensitivity, the PCR results are susceptible to misinterpretation even if a trace amounts of contaminating DNA are present (Birren *et al.*, 2006). In addition, *Taq* DNA polymerase that does not hold proof reading capability results in a relatively high error rate. On average, it incorporates the wrong nucleotide every 9000 nucleotides which can lead to a cumulative error rate of 1 in 300 nucleotides over 30 cycles (Lodge *et al.*, 2007). PCR could also be a technically challenging quantisation method as it often requires substantial pre-experimental planning to design suitable primers and concentration optimisation of each reaction reagent.

Among various PCR-based markers, ISSR markers have been revealed to be useful as novel DNA markers in studies on purposing crop improvement such as genomic fingerprinting, phylogenetic analysis and gene tagging (Isshiki *et al.*, 2008). ISSR markers amplify regions between adjacent and inversely oriented microsatellites using di-, tri-, tetra- and pentanucleotide SSR primers with the advantage that the information of the target DNA sequence is not required (Shen *et al.*, 2010). As ISSR amplification is independent, variation and frequency of the patterns can easily be detected among species. Thus, the system is reliable to mutagenesis study for molecular breeding works. Studies have shown that ISSRs provide a powerful, rapid, simple, reproducible and inexpensive means to access genetic diversity among closely related cultivars, characterisation of accessions and identification of cultivar and varieties (Kumar *et al.*, 2008).

Since no single set of conditions can be applied to all PCR amplifications especially for new primers. The most important step is related to optimise annealing temperature. Optimisation of annealing temperature was achieved by performing PCR gradient. Of 62 ISSR primers optimised, 54 primers (87%) showed amplification. Band patterns were slightly different at different PCR gradient annealing temperatures. Bands that were present at lower annealing temperatures were absent at higher annealing temperature. At lower annealing temperature, non-specific annealing primers occur, resulting in the amplification of unwanted segments of DNA. Conversely, too high annealing temperature resulting in poor amplified DNA yield as a result of oligonucleotide primers anneal poorly to the template DNA (Sambrook and Rusell, 2001). The annealing temperature was determined according to acceptable yields of amplified products and calculated melting temperature provided.

A total of 54 primers were used to assess polymorphism among the 14 accessions of *J. Curcas*; and 12 ISSR primers coded as IS1, IS2, IS6, IS9, IS12, IS19, IS21, IS28, IS42, IS46, IS50 and IS58 produced significant polymorphic bands. The results obtained here indicated that ISSR were appropriate markers to disclose polymorphism in the repeat regions of *J. curcas* accessions from Africa, Myanmar, Negeri Sembilan, Semarak, Sabah and Thailand (Figure 1). Twenty positions at different size in gel electrophoresis shows polymorphism among *Jatropha* accessions. These positions are important and could be used as markers for certain accessions.

In accessing the mutants, three ISSR primers IS1, IS13 and IS15 revealed remarkable results, which showed the evidence of segregation in seedling propagation. In Figure 2, sample Semarak was used as control, in comparing the seedlings derived from mutants 308 and 384. The comparison PCR product obtained from IS1 primers showed a typical elimination bands (related to copy numbers and sequence sizes) at approximately 500 bp and strong bands at position 600 bp. All these might be related the occurrence of out-crossing events. Amplification derived from all three primers i.e. IS1, IS13 and IS15 of genomic DNA from mutant sample 308C3 showed significantly polymorphic as compared to control. Both samples from cutting showed polymorphism in all the three ISSR primers. The results obtained here indicated that ISSR were appropriate markers to disclose polymorphism in the repeat regions of *J. curcas* mutants.

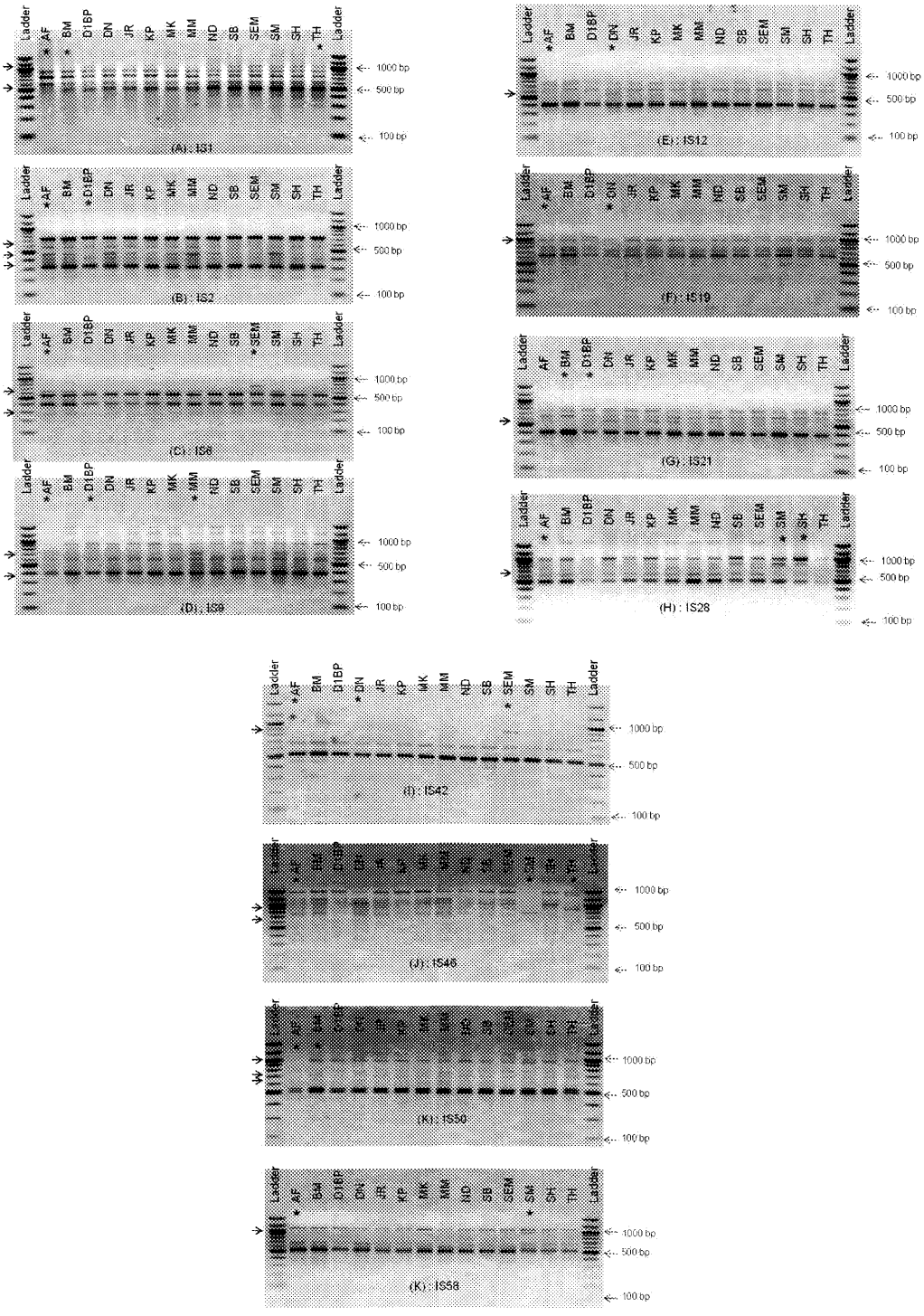

Figure 1: PCR product of different accessions derived from amplification of ISSR primers (A-K) visualised under UV-light on 1.8% agarose gel stained with ethidium bromide. A total of 14 accessions are coded i.e.AF (Africa), BM (Borneo Mega Power), DIBP, DN (India), JR (Jatroleum), MM (Myanmar), MK (Melaka), KP (Kuala Pilah), ND (Indonesia), SB (Sabah), SEM (Negeri Sembilan), (SM (Semarak), SH (Sg Buluh), TH (Thailand).observed under UV lights. 12 ISSR primers shows significant polymorphism in identify the accessions. Arrows on the left gel show additional and/or missing bands which, supported by 100 bp ladder to estimate the size of each polymorphic bands. Asterisks show number of polymorphic in the samples studied.

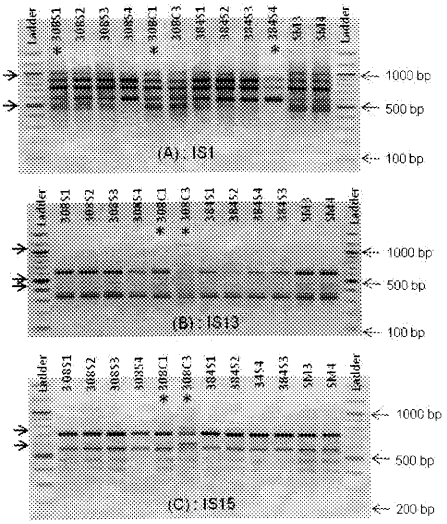

Figure 2: PCR product of two mutants 308 and 384. Genomic DNA were isolated from different seeds and cuttings for polymorphism observation. The amplification of ISSR primers (A-C) were visualised under UV-light on 1.8% agarose gel stained with ethidium bromide. Three ISSR primers (IS1, IS13, and IS15) show significant polymorphism in identify the mutants as compared to control accessions. Arrows on the left gel show additional and/or missing bands which, supported by 100 bp ladder to estimate the size of each polymorphic bands. Asterisks show number of polymorphic in the samples studied.

CONLCUSION

As conclusion, ISSR markers can offer great potential for differentiating polymorphism in the repeat regions of *J. curcas* accessions. In addition, changes at the motifs repeats in mutants also can be identified. However, the changes need to be observed further in field evaluation for certain traits improvement. ISSR markers were capable to discriminate the *J. curcas* from samples 308 (cutting), 308 (seed) and 384 (seed). About 87 % ISSR primers were optimised from this study showed that ISSR primers are capable in assessing genetic diversity and can be further used for identification of different accessions. The genetic diversity pattern based on this ISSR analysis provides guidelines for future *J. curcas* germplasm analysis to facilitate management, conservation and genetic improvement program towards bio-diesel production.

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