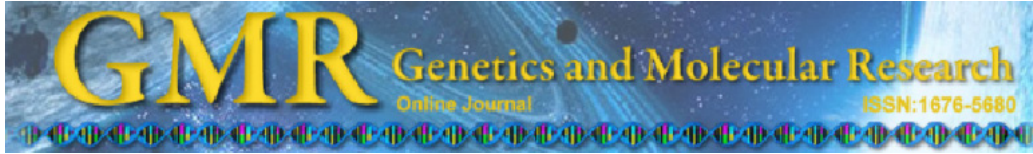

Genetic diversity analysis of *Jatropha curcas* L. (Euphorbiaceae) based on methylation-sensitive amplification polymorphism

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ABSTRACT. Genetic analysis of 56 samples of *Jatropha curcas* L. collected from Thailand and other countries was performed using the methylation-sensitive amplification polymorphism (MSAP) technique. Nine primer combinations were used to generate MSAP fingerprints. When the data were interpreted as amplified fragment length polymorphism (AFLP) markers, 471 markers were scored. All 56 samples were classified into three major groups: γ-irradiated, non-toxic and toxic accessions. Genetic similarity among the samples was extremely high, ranging from 0.95 to 1.00, which indicated very low genetic diversity in this species. The MSAP fingerprint was further analyzed for DNA methylation polymorphisms. The results revealed differences in the DNA methylation level among the samples. However, the samples collected from saline

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areas and some species hybrids showed specific DNA methylation patterns. AFLP data were used, together with methylation-sensitive AFLP (MS-AFLP) data, to construct a phylogenetic tree, resulting in higher efficiency to distinguish the samples. This combined analysis separated samples previously grouped in the AFLP analysis. This analysis also distinguished some hybrids. Principal component analysis was also performed; the results confirmed the separation in the phylogenetic tree. Some polymorphic bands, involving both nucleotide and DNA methylation polymorphism, that differed between toxic and non-toxic samples were identified, cloned and sequenced. BLAST analysis of these fragments revealed differences in DNA methylation in some known genes and nucleotide polymorphism in chloroplast DNA. We conclude that MSAP is a powerful technique for the study of genetic diversity for organisms that have a narrow genetic base.

Key words: *Jatropha curcas* L.; Genetic diversity;
Methylation-sensitive amplification polymorphism

INTRODUCTION

Jatropha curcas L. (JCL) or physic nut is a drought-resistant plant that belongs to the family Euphorbiaceae. All parts of JCL can be used for a wide variety of purposes ranging from traditional medicine for common human and animal ailments, protection against land erosion and as a boundary fence or live hedge to newly found high economic potential as a fossil fuel replacement (Openshaw, 2000; Sirisomboon et al., 2007; Rao et al., 2008). However, the most attractive feature of JCL nowadays is its potential as an alternative source of energy, as biodiesel, to alleviate the energy crisis (Carvalho et al., 2008; Ranade et al., 2008). Seeds of JCL contain non-edible oil in the range 4-40% (Jha et al., 2007). JCL has now been promoted in many countries but importantly, before any commercial exploration of a plant in any application, it is imperative to have enough information about the plant, such as its biology or the nature of the plant, so that the potential of the plant can be utilized maximally (Kumar and Sharma, 2008). Although nowadays, there is much research on JCL, genetic diversity is one of the most important studies because understanding the extent of genetic diversity not only helps identify existing species, but also contributes to the critical knowledge required for the success of a breeding program (Basha and Sujatha, 2007). Many DNA markers have been used to study genetic diversity in JCL, such as random amplified polymorphic DNA (RAPD; Subramanyam et al., 2009), RAPD and inter-simple sequence repeat (ISSR; Gupta et al., 2008), single-primer amplification reaction (Ranade et al., 2008), and amplified fragment length polymorphism (AFLP; Tatikonda et al., 2009). The studies also included related species such as *J. intergerima* Jacq., *J. podagrica* Hook., *J. gossypifolia* L. in their analysis (Basha and Sujatha, 2007; Ram et al., 2008). Molecular markers linked to toxic and non-toxic varieties were also identified (Pamidimarri et al., 2009).

Although the previously mentioned reports revealed some genetic diversity in

JCL, genetic diversity study using the AFLP technique on the physic nut grown in Thailand resulted in very low genetic diversity (Soonthornyart et al., 2007). Methylation-sensitive amplification polymorphism (MSAP) or methylation-sensitive AFLP (MS-AFLP) is a modification of the AFLP technique used to evaluate genetic diversity. This technique provides not only standard AFLP data, but also detection of differences in DNA methylation. DNA methylation is the process by which the methyl group (-CH_3) is transferred to a nucleotide, especially in CpG or CpNG sequences by the activity of S-adenosyl methyltransferases (Hafiz et al., 2006). It is one of the most well-known epigenetic regulations, causing changes of gene expression without any changes in DNA sequences. DNA methylation has been implicated in a number of cellular processes in higher plants, including regulation of gene expression during development, silencing of transposable elements and genes, and chromatin organization (Pavlopoulou and Kossida, 2007; Gehring and Henikoff, 2007).

The MSAP technique is used to detect changes at the DNA methylation level. This technique is based on the use of the two isochizomers, *Hpa*II and *Msp*I, which are different in their sensitivity to methylation of the recognition sequences. Both enzymes recognize the tetra-nucleotide sequence 5'-CCGG-3', but the action is determined by the methylation status of the external or internal cytosine residues. MSAP has been used to detect cytosine methylation changes in DNA methylation between rice and wild rice strains (Takata et al., 2005), and infraspecific DNA methylation polymorphism in cotton (Keyte et al., 2006). MSAP markers have been used to study genetic diversity in many plants such as *Arabidopsis thaliana* (Cervera et al., 2002), detection of DNA methylation polymorphism and the epigenetic population structure in a wild barley species (Li et al., 2008). Moreover, polymorphisms from the MSAP technique could also identify the narrow genetic basis of *Musa* whereas SSR and standard AFLP marker showed lower polymorphisms (Baurens et al., 2003; Noyer et al., 2005).

MSAP could also indicate the role of the environment, causing alteration of DNA methylation that could affect the phenotype. Kaushik et al. (2007) studied seed traits and the oil content of JCL accessions and the results showed that general phenotypic variation was higher than the genotypic variation, which indicated the predominant role of the environment. However, DNA methylation patterns are faithfully inherited. Changes in DNA methylation that arise somatically during the plant life cycle have the possibility of being propagated. Therefore, epimutations might be an important source of variation during plant evolution (Gehring and Henikoff, 2007). The objective of the present study was to investigate the genetic diversity of the physic nut using the MSAP technique in order to estimate the DNA methylation level.

MATERIAL AND METHODS

Plant materials and DNA extraction

Fifty-six accessions of JCL samples from Thailand and other countries were collected, including samples with high-yield production, γ-irradiation and non-toxic varieties. The samples used in the experiment are listed in Table 1. DNA was extracted from young leaves by the modified CTAB method (Doyle and Doyle, 1990).

Table 1. *Jatropha curcas* L. (JCL) used in this study.

No.	Name of sample	No.	Name of sample
1	JCL Anna 16	29	JCL 300 rad3
2	JCL Anna 20	30	JCL USA 8.45 Krad
3	JCL Anna 33	31	JCL USA
4	JCL Anna 34	32	JCL Hawaii
5	JCL Anna 78	33	JCL Suriname (South America)
6	JCL Anna 80	34	JCL India 1
7	JCL Anna 27	35	JCL India 2
8	JCL Nakornratchasima 1	36	JCL Chennai (India)
9	JCL Anna 18	37	JCL India 3
10	JCL Anna 83	38	JCL Rajahstan (India)
11	JCL Anna 69	39	JCL Dee1 (India)
12	JCL Anna 6	40	JCL India 4
13	JCL FF25B-14	41	JCL Lao
14	JCL FF20-Sbr-3	42	JCL Sri Lanka 1
15	JCL E-L-23	43	JCL Sri Lanka 2
16	JCL Chantaburi	44	JCL China 1
17	JCL Supanburi	45	JCL China 2
18	JCL 108-21-50	46	JCL Cambodia
19	JCL 101-32-115	47	JCL Myanmar1
20	JCL Nakornratchasima 2	48	JCL Myanmar2
21	JCL India × Satul	49	JCL Indonesia
22	JCL US Anna × India	50	JCL Pichai-Piset (non-toxic)
23	JCL Anna 21 × E-L-23	51	JCL SS 1 (non-toxic, Mexico)
24	JCL JD	52	JCL SS 2 (Peru)
25	JCL Mukdahan 10 Krad	53	JCL SS 3 (Peru)
26	JCL Suksan 10 Krad	54	JCL SS 4 (non-toxic, Mexico)
27	JCL 300 rad1	55	JCL SS 5 (non-toxic, Mexico)
28	JCL 300 rad2	56	JCL SS 6 (non-toxic, Mexico)

MSAP procedure

Two sets of DNA digestion/ligation reactions were carried out simultaneously. In the first reaction, 100 ng genomic DNA was digested with 10 U *Eco*RI (Fermentas, Canada) plus 10 U *Msp*I (Fermentas) in a final volume of 50 μL containing digestion reaction buffer (33 mM Tris-acetate, pH 7.9, 10 mM magnesium acetate, 66 mM potassium acetate, 0.1 mg/mL BSA), 5 pmol *Eco*RI adapter, 25 pmol *Hpa*II/*Msp*I adapter, 5 U T_4 DNA ligase and ligation reaction buffer (33 mM Tris-acetate, pH 7.9, 10 mM magnesium acetate, 66 mM potassium acetate, 0.1 mg/mL BSA). The mixture was then incubated at 37°C for 5 h. The reaction was stopped by incubating at 65°C for 10 min and diluting 10 times in 0.1X TE (1 mM Tris-HCl, 0.1 mM EDTA, pH 8) for polymerase chain reaction (PCR) amplification. The second reaction was carried out as above, except that *Hpa*II (Fermentas) was used instead of *Msp*I. The pre-selective amplification reaction was performed using 2 μL diluted digestion/ligation reactions, in 25 μL PCR mixture containing 200 mM Tris-HCl, pH 8.4, 500 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM of each dNTP, 0.2 pmol *Eco*RI and *Hpa*II/*Msp*I adapter-directed primers (each possessing a single-selective base, E+1; HM+1) and 1 U *Taq* DNA polymerase (Invitrogen, Norway). PCR was performed with the following profile: 94°C for 60 s, 25 cycles of 30 s denaturing at 94°C, 30 s annealing at 55°C and 60 s extension at 72°C, and finally 10 min at 72°C to complete extension. After confirming the presence of a smear of fragments (100-1000 bp in length) by agarose electrophoresis, the amplification product was diluted 40 times in 0.1X TE.

Selective amplification (second PCR) of the diluted pre-amplification product was carried out using a total of nine selected primer combinations (E1+HM1, E1+HM6, E2+HM5, E3+HM1,

E3+HM6, E4+HM3, E5+HM4, E5+HM6, and E6+HM6; Table 2). A selective PCR was performed with the following profile: 94°C for 60 s, 36 cycles of 30 s denaturing at 94°C, 30 s annealing and 60 s extension at 72°C, ending with 10 min at 72°C to complete extension. Annealing was initiated at 65°C, which was then reduced by 0.7°C for the next 12 cycles and maintained at 56°C for the subsequent 23 cycles. The second PCR product was mixed with 10 µL loading dye (98% formamide, 10 mM EDTA, 0.01% (w/v) bromophenol blue and 0.01% (w/v) xylene cyanol), denatured at 95°C for 5 min and separated by electrophoresis on 6% denaturing polyacrylamide sequencing gels (6% polyacrylamide 29:1, 7 M urea) in 1X TBE buffer. The gel was pre-run at 300 V for about 30 min before 10 µL of the mixture was loaded. Gel was run at 400 V for about 2 h, and stained with silver nitrate using the modification method from Benboza et al. (2006).

Table 2. Sequences of primers and adapters used.

Adapter/Primer	Sequence
*Eco*RI adapter	5'-CTCGTAGACTGCGTACC-3'
	3'-CATCTGACGCATGGTTAA-5'
*Hpa*II/*Msp*I adapter	5'-GATCATGAGTCCTGAT-3'
	3'-AGTACTCAGGACGAGC-5'
E+A	5'-GACTGCGTACCAATTCA-3'
HM+T	5'-ATCATGAGTCCTGCTCGGT-3'
E+AAC (E1)	5'-GACTGCGTACCAATTCAAC-3'
E+ACT (E2)	5'-GACTGCGTACCAATTCACT-3'
E+AGT (E3)	5'-GACTGCGTACCAATTCAGT-3'
E+AAG (E4)	5'-GACTGCGTACCAATTCAAG-3'
E+AGC (E5)	5'-GACTGCGTACCAATTCAGC-3'
E+AGG (E6)	5'-GACTGCGTACCAATTCAGG-3'
HM+TAA (HM1)	5'-ATCATGAGTCCTGCTCGGTAA-3'
HM+TCC (HM2)	5'-ATCATGAGTCCTGCTCGGTCC-3'
HM+TTC (HM3)	5'-ATCATGAGTCCTGCTCGGTTC-3'
HM+TCG (HM4)	5'-ATCATGAGTCCTGCTCGGTCG-3'
HM+TAC (HM5)	5'-ATCATGAGTCCTGCTCGGTAC-3'
HM+TAG (HM6)	5'-ATCATGAGTCCTGCTCGGTAG-3'

Data analysis

Cleared MSAP bands were scored using two methods modified from Noyer et al. (2005) and Keyte et al. (2006). Firstly, only *Eco*RI-*Msp*I bands were score as "1" for the presence of bands and "0" for the absence of bands for standard AFLP analysis. Secondly, bands from both *Eco*RI-*Msp*I and *Eco*RI-*Hpa*II of individuals were compared to interpret the MS-AFLP data. If the band was present in both digestion sets, it was scored as "1" and if the band was present in only one set, it was scored as "0". The scored bands from both methods were analyzed separately and in combination. Genetic similarity was estimated using a simple-matching coefficient (Sneath and Sokal, 1973). Phylogenetic tree construction and principal component analysis (PCA) were performed using the NTSYS-Pc computer software, version 2.2k (Rohlf, 2005). The cophenetic values and Maxcomp command were used to measure the goodness of fit for a cluster analysis. The polymorphic information content (PIC) of each marker was also calculated using PIC = $1 - \Sigma Pi^2$, where *Pi* is the frequency of the present and absent alleles (Ott, 1991).

Cloning and sequencing of polymorphic bands between toxic and non-toxic JCL samples

Some nucleotide and methylation polymorphic bands from the MSAP fingerprint were

selected and repeated to confirm polymorphisms. Bands were excised from polyacrylamide gel and re-amplified using the following conditions: 94°C for 60 s, 35 cycles of 30 s denaturing at 94°C, 30 s annealing at 55°C and 60 s extension at 72°C, and finally 10 min at 72°C to complete extension. The PCR products were purified using a HiYield Gel PCR DNA Fragment Extraction Kit (RBC, Taiwan) and ligated to pGEM-T easy vector (Promega, USA). Ligated plasmids were transformed with *Escherichia coli* XL1-blue competent cells. Recombinant clones were selected and plasmid extraction performed using a High-Speed Plasmid Mini Kit (Geneaid, Taiwan), after which, the plasmid was sent for sequencing at Bio-Basic (Canada). Sequence data were then edited and searched for homology using BLAST (available at http://blast.ncbi.nlm.nih.gov/Blast.cgi).

RESULTS AND DISCUSSION

Five categories of JCL samples were selected and used in this study. The first category was JCL accessions collected from various parts of Thailand and some were reported to have high-yield production. The second category was JCL accessions from other countries in Asia, Central and South America. The third and fourth categories were hybrids and mutation-induced JCL. The last category was non-toxic JCL from Mexico. Sixty-four *Eco*RI-*Hpa*II/*Msp*I or E-HM primer combinations were used to conduct a preliminary screening of the genome of JCL and nine combinations producing clear bands were selected for further analysis. In AFLP analysis, nine primer combinations provided 471 clear and scorable bands with an average of 52.3 markers per primer pair. PIC values of these markers ranged from 0.00 to 0.49 and the mean PIC score was 0.015. Genetic similarity estimated by Sneath and Sokal (1973) among samples studied was extremely high, ranging from 0.95 to 1.00. Cluster analysis using UPGMA was consistent with the PCA. The principal components 1, 2 and 3 accounted for 89.6, 4.65 and 2.53% of the total variation, respectively. The results from AFLP analysis classified the 56 samples into three distinct groups at a genetic similarity of 0.97 (Figure 1A). The cophenetic correlation coefficient revealed a very good fit of cluster analysis, with a value of r = 0.993 for the dendrogram. The first group was composed of 49 JCL samples with a similarity index greater than 0.99. The second and the third groups were irradiated JCL and non-toxic JCL, respectively. However, from the total 471 markers used in the AFLP analysis, only 51 markers (10.8%) were polymorphic markers. This indicated the very low genetic variation among the JCL samples studied. For radiation-induced mutation JCL accessions, only JCL25 and JCL26 were separated in another group, whereas irradiated JCL27, JCL28 and JCL29 showed no polymorphism at nucleotide or DNA methylation and were clustered along with JCL in the first group. Irradiation by X-rays and γ-rays can induce nucleotide changes in many plants such as mungbean (Sangsiri et al., 2005) and soybean (Atak et al., 2004) and these changes could be detected using DNA markers (Danylchenko and Sorochinsky, 2005). The results of the present study indicated that mutation induced by irradiation caused some changes in DNA sequences in samples JCL25 and JCL26 as well as DNA methylation alterations, but possibly not in irradiated JCL27, JCL28 and JCL29, which were induced mutations at low levels of radiation (300 rad). Interestingly, JCL25 and JCL26 shared some specific bands with non-toxic JCL, while the bands were absent in all toxic accessions. These results agreed with Pamidimarri et al. (2009) that DNA markers could be used to distinguish non-toxic and toxic JCL. The genetic similarity between toxic and non-toxic varieties from our AFLP analysis was about 0.95, whereas results from Pamidimarri et al. (2009) found that genetic similarity

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between toxic and non-toxic varieties was 0.92 from RAPD and 0.90 from AFLP analysis. The results suggested that non-toxic JCL not only had some distinct morphology compared to the toxic JCL, but also had some polymorphism at the DNA level.

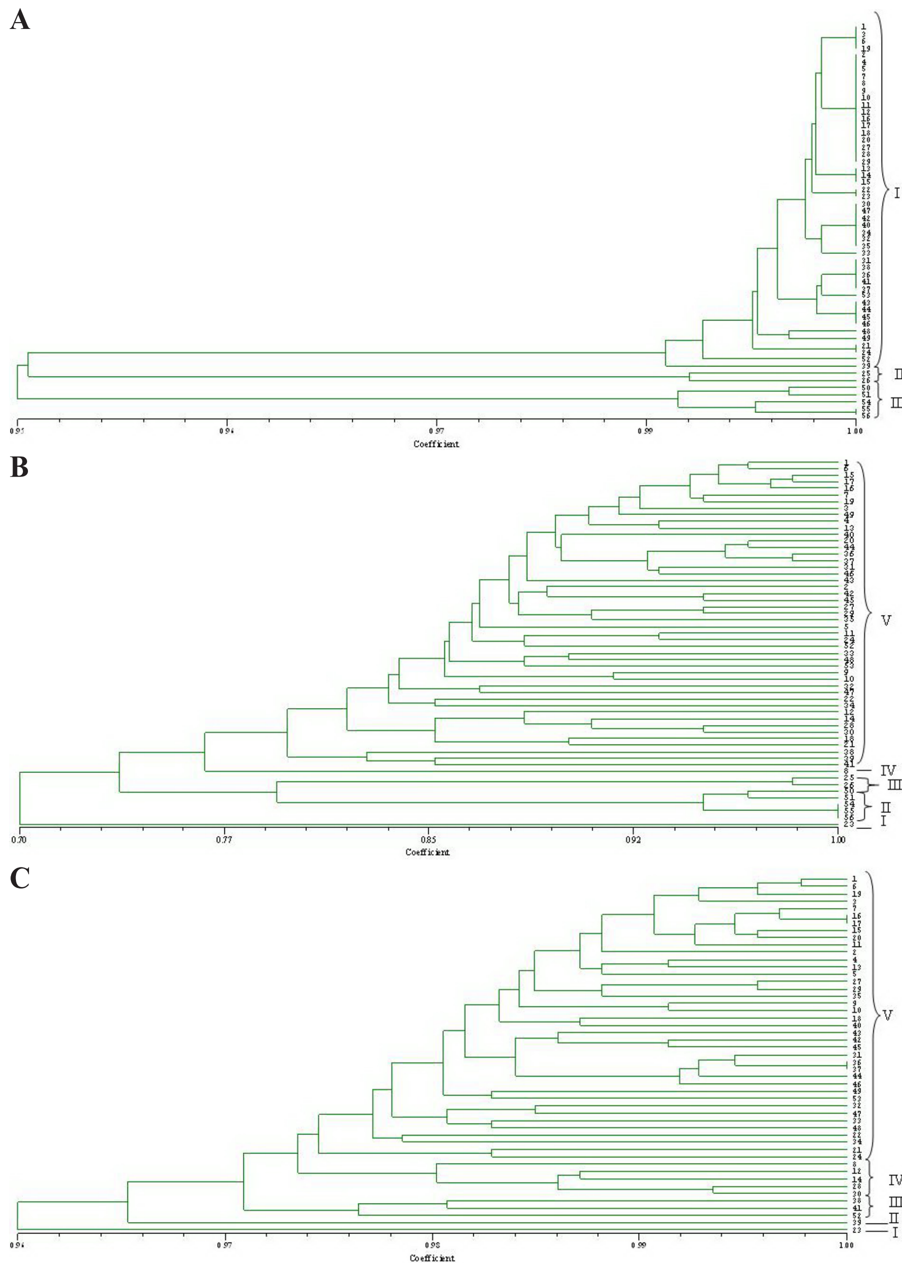

Figure 1. A. Phylogenetic tree of 56 *Jatropha curcas* L. accessions from AFLP data. **B.** Phylogenetic tree of 56 *J. curcas* L. accessions from MS-AFLP data. **C.** Phylogenetic tree of 49 *J. curcas* L. accessions in Group I of AFLP analysis constructed from combination data.

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In the MS-AFLP analysis, polymorphisms were analyzed by comparing the presence or absence of bands between two sets of digestion/ligation reactions. Band presence in only one digestion set and absence in the other set were recorded as methylation polymorphism in order to eliminate nucleotide polymorphism. The polymorphic bands were scored as binary data and analyzed using the same procedure as for standard AFLP. From nine primer combinations, 60 methylation polymorphisms were detected. Estimates of the PIC value had a similar range as the AFLP data ranging from 0.03 to 0.46 with an average of 0.179. Genetic similarity estimated by Sneath and Sokal (1973) among samples studied was moderately high, ranging from 0.70 to 1.00. Results from cluster analysis using UPGMA separated 56 samples into five groups (Figure 1B). The cophenetic correlation coefficient revealed a good fit of cluster analysis, with a value of r = 0.84 for the dendrogram. The first group comprised only one sample, the hybrid from Anna21 and E-L23 (JCL23). The second group was all non-toxic JCL. The third group was mutation-induced JCL (JCL25 and JCL26). The fourth group was the JCL sample from an area with saline soil (JCL8). The fifth group consisted of the rest of the samples including mostly Thai and foreign accessions, as well as some mutants and hybrids. PCA showed the same pattern as the UPGMA tree. Principal components 1, 2 and 3 accounted for 14.5, 9.06 and 8.40% of the total variation, respectively. MS-AFLP analysis revealed the alteration in the DNA methylation level among the samples, which possibly was caused by different environmental effects. The results showed that although there was very low polymorphism at the nucleotide level, the DNA methylation level was much altered. Moreover, JCL8, which had been grown in a saline soil area, was separated in a particular group. Salinity is a well-known stressor in many plants and possibly caused an alteration in DNA methylation (Mahajan and Tuteja, 2005; Zhong et al., 2009). DNA methylation might be altered in the same variety, caused by different factors associated with the environment, stress, pathogen infection, or even epimutation. Although data from DNA methylation alone cannot be used to study genetic diversity, methylation-sensitive markers were very useful to identify evidence of epigenetic regulation involving sex determination, somaclonal variation, pathogen resistance or environmental changes. The MS-AFLP in the current study could separate all non-toxic JCL accessions, mutation-induced JCL25 and JCL26, saline soil grown JCL (JCL8), and hybrids from JCL Anna21 and E-L23 (JCL23).

In order to separate samples in Group I from AFLP analysis, data from AFLP and MS-AFLP analyses were combined and analyzed together to elucidate the effects of methylation-sensitive data to tree topology. After excluding the samples of JCL25, JCL26 and non-toxic accessions, which were clearly separated according to AFLP and MS-AFLP phylogenetic tree, the combination data were analyzed for genetic similarity based on Sneath and Sokal (1973) and a phylogenetic tree was constructed using the UPGMA method. All 49 JCL samples in Group I of the AFLP analysis were clearly separated into 5 subgroups at a genetic similarity of 0.972 (Figure 1C). The cophenetic correlation coefficient revealed a good fit of cluster analysis, with a value of r = 0.81 for the dendrogram. Interestingly, the hybrid from Anna21 and E-L23 (JCL23) was separated from the major group independently. Genetic similarity estimated by Sneath and Sokal (1973) among samples studied was extremely high, ranging from 0.96 to 1.00. Principal components 1, 2 and 3 accounted for 80.98, 4.70 and 2.94% of total variation, respectively.

From MSAP fingerprinting, some nucleotide and methylation-sensitive polymorphisms between toxic and non-toxic JCL were detected. Six polymorphic bands (P1-P6) were

cloned and sequenced. Homology search results and sequence identification are shown in Table 3. The selected polymorphic bands were three methylation-sensitive polymorphic bands and three nucleotide polymorphic bands. Fragments P2 and P5 revealed nucleotide polymorphism between toxic and non-toxic JCL, whereas fragment P1 showed hypermethylation in non-toxic JCL, but these three fragments showed no reasonable similarity match with BLAST analysis. However, another three polymorphic bands did match some entries in the NCBI database. Fragments P3 and P6, revealed hypermethylation in non-toxic JCL, matched pheophorbide A oxygenase (PAO) and the gene encoded for the SEYI protein in *Ricinus communis*, respectively, while fragment P4 showed nucleotide polymorphism in the RNA polymerase β-subunit protein (*rpo*C1) gene in the JCL chloroplast genome. The results from sequencing of polymorphic bands revealed some nucleotide differences in chloroplast genes of toxic and non-toxic JCL. Genes in the chloroplast genome have been widely used to study phylogeny in many plant species (Wen et al., 2009; Kumagai et al., 2010). The present study also identified different DNA methylation in two regions, namely, the *PAO* gene and the gene encoded for the SEYI protein. PAO is located at the inner envelope of senescing chloroplasts and is involved in chlorophyll breakdown and senescence (Hörtensteiner et al., 1998; Pruzinská et al., 2003). The SEYI protein is similar to the root hair defective 3 (RHD3) GTP-binding protein encoded by the *RHD3* gene. This gene is essential for plant cell expansion and is also required for cell wall biosynthesis and actin organization (Wang et al., 2002; Hu et al., 2003). Moreover, another three sequences also showed polymorphism in nucleotide sequences and DNA methylation in some unknown regions in the genome. These data could provide differences between toxic and non-toxic JCL at the molecular level.

Table 3. Sequence details and results from homology search using BLAST.

Name	Primer combination	Size (bp)	Polymorphic type	Homology
P1	E+AGG/HM+TCC	290	MET/hypermethylated in non-toxic JCL	Unknown match
P2	E+AGG/HM+TCC	250	SEQ/nucleotide polymorphism	Unknown match
P3	E+AAC/HM+TAA	500	MET/hypermethylated in non-toxic JCL	*Ricinus communis* pheophorbide A oxygenase, putative, mRNA (XM002523689.1)
P4	E+AGT/HM+TAG	400	SEQ/nucleotide polymorphism	*Jatropha curcas* chloroplast, complete genome (FJ695500.1)
P5	E+ACT/HM+TAC	250	SEQ/nucleotide polymorphism	Unknown match
P6	E+ACT/HM+TAC	250	MET/hypermethylated in non-toxic JCL	*Ricinus communis* protein in SEY1, putative, mRNA (XM002527359)

JCL = *Jatropha curcas* L.; MET = methylated polymorphism; SEQ = nucleotide sequence polymorphism.

A previous study on the genetic diversity of JCL from Thailand and other countries using the AFLP technique revealed very low genetic variation among the samples (Soonthornyart et al., 2007). In the present study, the genetic variation of JCL accessions from Thailand and other countries was evaluated, including mutation-induced JCL, hybrids and non-toxic JCL. The MSAP technique was selected in order to confirm the information that low genetic diversity may exist in JCL. MSAP detected not only the nucleotide polymorphisms but also the differences in the DNA methylation level as it was used to identify low genetic diversity in *Musa* (Baurens et al., 2003; Noyer et al., 2005).

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From experience in growing JCL for many years, the authors have found obvious differences in yield production and flowering date but no polymorphism at the DNA level. The substantially different performance was also found even in the plants propagated from the same clones. The differences of DNA methylation level among JCL could be one reasonable explanation for the phenotypic differences without nucleotide polymorphisms. Moreover, DNA methylation has been proposed to be one of the possible factors involved in heterosis or hybrid vigor (Zhao et al., 2008). The results from the analysis of AFLP, MSAP and combination data are summarized in Table 4.

Table 4. Comparison of results from amplified fragment length polymorphism (AFLP), methylation-sensitive amplification polymorphism (MSAP) and combination analysis.

	AFLP	MSAP	Combination (AFLP+MSAP)
No. of markers	471	60*	531
No. of polymorphic markers	51	60	
No. of monomorphic markers	420		
Similarity	0.95-1.00	0.70 - 1.00	0.96 - 1.00
Average PIC value	0.015	0.179	
PCA 1-3	96.78%	31.96%	88.62%

*No. of markers with methylation polymorphism. PIC = polymorphic information content; PCA 1-3 = principal component analysis 1, 2 and 3.

Forty-nine accessions of JCL with almost identical genetic background based on AFLP analysis were clustered in Group I, but they were clearly separated into 5 subgroups in combination analysis. When methylation-sensitive data were analyzed together with AFLP data, higher resolution of the classification in cluster analysis was obtained. Combination analysis also separated JCL23 (the hybrid from JCL Anna21 and E-L23) into another subgroup. The results also provided evidence that environmental factors could have some major effects on the traits of JCL. This could explain the phenotypic variation observed in JCL without nucleotide polymorphism. From the present study, high genetic similarity (more than 0.99) among cultivated JCL samples was observed. The results of the present study were in agreement with Basha and Sujatha (2007) that JCL has low genetic diversity and requires an immediate widening of its genetic base. Mutation induced by irradiation was able to cause some changes in nucleotide sequences and the level of DNA methylation in some samples. However, high polymorphism was still detected among wild and cultivated JCL samples (Tatikonda et al., 2009; Subramanyam et al., 2009).

In conclusion, the genetic base among cultivated JCL was low, but the DNA methylation level was fairly diverse, suggesting that environmental factors may have a major impact on the performance of cultivated JCL. Wide cross and induced mutation should be considered in a JCL breeding program. It is suggested that MSAP is a powerful technique to study genetic diversity, especially for JCL with a narrow genetic base.

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