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Contributed Paper

Assessment of Genetic Diversity of *Jatropha curcas* L. Using AFLP and ISSR Markers

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ABSTRACT

Jatropha curcas L. (family Euphobiaceae) is widely distributed in the tropics as a potential biofuel crop. Two DNA marker techniques, AFLP and ISSR, were used to assess the genetic diversity of *J. curcas* germplasm collected throughout Thailand, introduced accessions from six countries and other four *Jatropha* species. The AFLP and ISSR fingerprint data were combined and used to evaluate genetic relationships among all samples. Pairwise similarity estimated between *J. curcas* ranged from 0.8428 to 1.00 with an average of 0.9955. The UPGMA cluster analysis result showed that almost all *J. curcas* samples were grouped into cluster 1, while a sample from USA formed separate cluster 2. The germplasms from other countries, preferably from the center of origin of this species, should be introduced to broaden genetic diversity of *J. curcas* in Thailand. The genetic relationship among *J. curcas*, *J. integerrima*, *J. gossypifolia*, *J. multifida* and *J. podagrica* was also observed. The result showed that *J. integerrima* was genetically more close to *J. curcas* than the others, while the highest value of genetic distance was found between *J. curcas* and *J. gossypifolia*. This genetic information will be useful for germplasm management in *Jatropha* breeding program.

Keywords: genetic diversity, *Jatropha curcas* L., DNA marker, biodiesel, biofuel plant

1. INTRODUCTION

Jatropha curcas L., also known as physic nut or purging nut, is a bush or small tree belonging to the family Euphobiaceae. *J. curcas* is a native plant of Mexico and Central American region. It was later introduced into many parts of the tropical and subtropical regions of the world [1]. Recently, *J. curcas* has gained a lot of interest as a potential biofuel crop for many logical reasons [2-3]. It has a very high oil content of approximately 45-58% on kernel weight basis and 30-40% on seed weight basis [4].

Biodiesel derived from *J. curcas* has the desirable physiochemical characteristics and it is not competing with food grade oils [1, 5]. Among all the potential biofuel plants, *J. curcas* is assumed importance due to its easy adaptation to different kinds of marginal lands, drought endurance, avoidance by animals and its short interval time to give first yield [6]. Furthermore, *J. curcas* is a multipurpose plant with several utilities, for example, the extracted oil can be used in skin care products, pesticide, cosmetics, lighting, while the seed cake is used as animal feeds and organic fertilizer. Its wood and leaves can be used as fuel and green manure [3]. For those reasons, *J. curcas* is being introduced rapidly in several countries, including Thailand. Breeding of high-yielding *J. curcas* has therefore become a focus of attention. However, the knowledge involving a level of genetic diversity of this plant in Thailand is still limited. Germplasm collection and diversity analysis of *J. curcas* are prerequisites for breeding program.

Nowadays, molecular markers are powerful tools for the evaluation of genetic diversity of plant species. Unlike the morphological markers, the utilization of molecular markers has been effective in assessment of genetic diversity independent of environmental influences and stage of plant growth. Different kinds of molecular markers, such as Random Amplified Polymorphic DNA (RAPD), Amplified Fragment Length Polymorphism (AFLP), Inter Simple Sequence Repeat (ISSR) and Simple Sequence Repeat (SSR), have been used to assess the genetic diversity of *J. curcas*. Almost all reports revealed low to moderate genetic diversity among *J. curcas* accessions from the same country. Some works also included its closely relative species, such as *J. intergerima* Jacq., *J. podagrica* Hook. and *J. gossypifolia* L. [6-11]. In other countries, many works have been conducted to study genetic variation of this biofuel plant, but in Thailand, the experiment on *J. curcas* genetic diversity assessment is still limited. Tanya *et al.* (2010) used ISSR markers to determine genetic relationship and diversity among 30 accessions of *J. curcas* from China, Mexico, Thailand and Vietnam and the outgroups including *J. gossypifolia*, *J. integerrima*, *J. podagrica*, and *Ricinus communis*. The result revealed a narrow genetic diversity within *J. curcas* species [9]. Another research used 10 SSR markers to study the genetic diversity of 32 *J. curcas* accessions, including six accessions from Thailand, nine non-toxic accessions from Mexico, 12 gamma radiation-treated seeds (300 Gy) from Mukdahan province and one accession each from Myanmar, Cambodia, India, Laos, and China. The result of this experiment showed low level of genetic diversity and only two to four alleles were observed [12]. The research that aims to collect Thai *J. curcas* germplasms and then thoroughly measure its genetic relationship has not been reported yet.

AFLP and ISSR techniques are effective methods for revealing DNA polymorphisms. The AFLP technique is appropriate for the DNA fingerprint analysis because AFLP profiles do not alter with minor variations in experimental conditions. ISSR markers are arbitrary multiloci marker produced by PCR amplification with microsatellite primer. The ISSR primers have been shown to provide a rapid, reproducible and cost effective method to assess the genetic diversity and identify closely related cultivars in many species [13]. The AFLP and ISSR techniques are advantageous because no prior genomic information is required for their use.

The current study used AFLP and ISSR markers to elucidate the genetic diversity of *J. curcas* germplasm collected throughout

Thailand, introduced accessions from six different countries and other four *Jatropha* species. The information from this study will be useful for future genetic improvement of this potential biofuel crop.

2. MATERIALS AND METHODS

2.1 Plant Materials and Genomic DNA Extraction

A total of 138 accessions, including *J. curcas* and four other *Jatropha* species; *J. gossypifolia, J. integerrima, J. multifida* and *J. podagrica*; were collected. The accession numbers and location sites are shown in Table 1. The plants were collected in the field and maintained at the National Corn and Sorghum Research Center, Pakchong, Nakhon-Ratchasima province.

Total genomic DNA was extracted from leaf tissue according to the CTAB method, following the procedures of Doyle and Doyle (1990) [14]. The concentration of DNA was quantified by measuring the absorbance of UV light (260 nm) by spectrophotometer.

Table 1. List of samples used in this study.

Samples	No. of accession	Accession no.	Collection site
J. curcas	121	J2, J3, J4, J5, J8, J9, J10, J11, J12, J13, J15, J16, J17, J18, J19 , J20, J21, J22, J23, J24, J25, J26, J27, J29, J30, J31, J32, J33, J34, J35, J37, J40, J41, J42, J44, J45, J46, J47, J49, J52, J53, J54, J56, J57, J58, J59, J60, J63, J64, J65, J66, J67, J68, J69, J70, J71, J72, J73, J74, J75, J76, J77, J78, J79, J80, J81, J82, J83, J84, J85, J86, J87, J88, J89, J90, J91, J92, J93, J94, J95, J96, J97, J98, J99, J100, J101, J102, J103, J104, J105, J106, J107, J108, J109, J110, J111, J112, J113, J114, J115, J116, J117, J118, J119, J120, J121, J122, J123, J124, J125, J126, J127, J128, J129, J132, J133, J134, J135, J136, J137, J138	Thailand
J. curcas	2	J7, J38	USA
J. curcas	1	J55	China
J. curcas	6	J6, J28, J36, J39, J48, J51	India
J. curcas	1	J130	Suriname
J. curcas	2	J50, J131	Sri Lanka
J. curcas	1	J14	Laos
J. integerrima	1	J62	Thailand
J. gossypifolia	1	J1	Thailand
J. multifida	1	J43	Thailand
J. podagrica	1	J61	Thailand

2.2 AFLP Analysis

Genomic DNA (0.25 μg) was digested with 2.5 units of *Eco*RI and *Mse*I (Biolabs, Australia) in a final volume of 25 μL containing digestion reaction solution (50 mM potassium acetate, 20 mM Tris-acetate pH 7.9, 10 mM magnesium acetate, 1mM dithiothreitol, 0.1 mg/mL BSA). After mixing, the DNA samples were incubated for 3 h at 37 °C. Ligation of *Eco*RI and *Mse*I adapters was performed by mixing 25 μL of double digested DNA and 25 μL of ligation solution (1 unit of T_4 DNA ligase, 50 mM Tris-HCl pH 7.5, 10 mM $MgCl_2$, 10 mM dithiothreitol, 1 mM ATP). The mixture was then incubated at 25 °C for 2 h.

The pre-selective amplification reaction was performed using 2 μL of digestion/ligation reactions, in 25 μL of PCR reaction containing 200 mM Tris-HCl pH 8.4, 500 mM KCl, 1.5mM $MgCl_2$, 0.2 mM of each dNTP, 0.2 pmol of *Eco*RI and *Mse*I adapter-directed primers (each possessing a single selective base, E+1; M+1) and 1 U of *Taq* DNA polymerase (Invitrogen, Brazil). PCR reactions were performed with the following profile: 94 °C for 3 min, 30 cycles of 30 s denaturing at 94 °C, 30 s annealing at 56 °C and 60 s extension at 72 °C, ending with 5 min at 72 °C to complete extension. After checking for the presence of a smear of fragments (100-1000 bp in length) by agarose gel electrophoresis, the amplification product was diluted 20 times in 0.1 × TE. Selective amplification (second PCR) of the diluted pre-amplification products was carried out using ten primer combinations (Table 1), screened from 64 combinations commonly used in plants. Selective PCR reactions were 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 a temperature of 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 products were mixed with 10 μL of loading dye (98% formamide, 10mM EDTA, 0.01% w/v bromophenol blue and 0.01% w/v xylene cyanol), denatured at 95 °C for 5 min and separated on 6% denaturing polyacrylamide gels (6% polyacrylamide 29:1, 7 M urea) in 1× TBE buffer. The gels were pre-run at 1,500 V for about 30 min before 10 μL of the mix was loaded. Gels were run at 1,500 V for about 3 h. The AFLP fragments were visualized by silver staining [15].

2.3 ISSR Analysis

All fifteen primers were screened. The PCR reactions were carried out in 12.5 μl volumes containing 20 ng of genomic DNA, 20 mM Tris-HCl pH 8.4, 50 mM KCl, 2.0 mM $MgCl_2$, 0.1 mM of each dNTP, 5 pmol primer and 1 U of *Taq* DNA polymerase (Invitrogen, Brazil). Amplification was performed in a PTC-100 thermocycler (Marshall Scientific, USA) programmed for 5 min at 94 °C, followed by 35 cycles of 30 sec at 94 °C, 1 min at 45 °C and 1.30 min at 72 °C, ending with 10 min at 72 °C to complete extension. Amplification products were analyzed by gel electrophoresis on 1.5% agarose in 1× TBE buffer and visualized by ethidium bromide staining. Finally, five primers, namely $(AC)_8G$, $(GA)_8C$, $(GA)_8T$, $(AG)_8T$ and $(AG)_8YT$, generated clear polymorphic bands were selected to estimate *Jatropha* genetic diversity.

2.4 Data Analysis

For the diversity analysis, each PCR product was assumed to represent a single locus and only polymorphic bands were scored as present (1) or absent (0). A binary matrix was imported into NTSYS-pc version 2.20k for cluster analysis [16].

Genetic similarity among all accessions was calculated according to Jaccard's Similarity Index (JSI) [17] by the SIMQUAL subprogram, and the SAHN subprogram was used for cluster analysis by the UPGMA (unweighted pair-group method with arithmetic means) method [18]. The polymorphic information content (PIC), which is an index for the analysis of the polymorphism of each amplified DNA fragment, was calculated by the equation [19] as following:

$$PIC = 1 - \sum_{i}^{k} Pi^2$$

where: Pi = allele frequency. This parameter is sometimes called heterozygosity. PIC value for the dominant marker means the probability of a polymorphism between two randomly drawn samples. Pi can only be P_A and P_P, where P_A is the frequency of samples in which the DNA fragment was absent (frequency of null allele) and P_P is the frequency of samples in which the DNA fragment was present (frequency of amplified allele). Analysis of molecular variance (AMOVA) was performed using POPGENE version 1.32 [20].

3. RESULTS

Two marker systems, AFLP and ISSR were employed to analyze the genetic diversity of *J. curcas* germplasm collection and four other related species. Genetic diversity was assessed by ten AFLP primer combinations and a total of 680 DNA bands were generated with an average of 68 bands per primer pair (Table 2). In total, 604 polymorphic bands (88.82% of the total amplified bands), ranging from 100 to 1,500 bp, were scored. The average number of polymorphic bands per primer pair was 60.4, while the range for ten primer combinations were 53 to 69. Interestingly, six unique bands of *J. curcas* accession no. J7 (from USA) and a unique band of J41 (from Nan province, Thailand) were found. The AFLP pattern generated by E-AAC/M-CAT primer pair was highly polymorphic containing 98.28% polymorphic bands. PIC values ranged from 0.0144-0.1092 with an average of 0.0313. When considering only *J. curcas* samples, the highest number of polymorphic bands was detected by the E-AGG/M-CTT primer combination with 21 bands or 30.43% of all bands generated by this primer pair were found to be polymorphic.

Five ISSR markers were also performed and a total of 50 DNA bands were produced, of which, 43 bands or 86.00% were found to be polymorphic (Table 3). Four unique bands of *J. curcas* accession no. J7 and a unique band of J8 (from Prae province, Thailand) were found. The ISSR pattern generated by $(AG)_8$YT primer was highly polymorphic containing 100% polymorphic bands. PIC values ranged from 0.0144-0.2050 with an average of 0.0244. On exclusion of other *Jatropha* species, $(AG)_8$YT primer also generated the highest percent of polymorphic bands (83.33%) among the *J. curcas* samples.

The polymorphic bands generated from AFLP and ISSR markers were combined into one binary matrix and then used to estimate the genetic similarity among all samples. On inclusion of outlier species, the result obtained by Jaccard's coefficient showed that the genetic similarity varied from 0.2495 (between *J. gossypifolia* and *J. podagrica*) to 1.00 (between several *J. curcas* samples). The lowest genetic similarity among accessions of *J. curcas* was 0.8428 (between accessions J7 from USA and J105 from Nan province, Thailand) and the average genetic similarity was 0.9955.

The genetic relationship between *J. curcas* and the related *Jatropha* species was also

evaluated. The species specific DNA fragments were found. Of the four related species used in this study, *J. integerrima* was genetically more similar to *J. curcas* than the others, while the highest value of genetic distance was found between *J. curcas* and *J. gossypifolia*. The average genetic similarity value between *J. curcas* and *J. integerrima* was 0.4513, whereas the average genetic similarity between *J. curcas* and *J. gossypifolia* was 0.3116. The average genetic similarity between *J. curcas* and *J. multifida* was 0.3412 and between *J. curcas* and *J. podagrica* was 0.3501.

Table 2. Number of bands, number of polymorphic bands and percentage of polymorphic bands obtained for the 138 accessions from ten AFLP selective primer combinations.

Primer combinations	No. of bands	No. of polymorphic bands	% polymorphic bands
E[a]-AAC/M[b]-CAT	58	57	98.28
E-AAC/M-CTT	61	53	86.88
E-AAG/M-CAT	61	57	93.44
E-AAG/M-CTA	69	62	69.85
E-AGG/M-CAG	66	58	87.88
E-AGG/M-CAT	74	69	93.24
E-AGG/M-CTT	69	62	89.85
E-CAA/M-ATA	78	67	85.90
E-CAA/M-AAG	70	60	85.71
E-CAG/M-ATA	74	59	79.73
Total	680	604	-
Average	68.0	60.4	88.82

E [a] = pre-amplification primer (GACTGCGTACCAATTC) of *Eco*RI
M [b] = pre-amplification primer (GATGAGTCCTGAGTAA) of *Mse*I.

Table 3. Number of bands, number of polymorphic bands and percentage of polymorphic bands obtained for the 138 accessions from five ISSR primers.

ISSR primers	No. of bands	No. of polymorphic bands	% polymorphic bands
$(AC)_8G$	9	8	88.89
$(AG)_8T$	12	9	75.00
$(AG)_8YT$	6	6	100.00
$(GA)_8C$	13	12	92.31
$(GA)_8T$	10	8	80.00
Total	50	43	-
Average	10.0	8.6	86.00

The genetic similarity among all *J. curcas* samples was estimated using NTSYS version 2.20k and the results are shown in Figure 1. The Jaccard's similarity coefficient values ranged from 0.8428 to 1.00 with an average of 0.9955, suggesting a narrow genetic base among all collected *J. curcas* samples. There were a large number of identical samples. The redundant samples were cut off and represent as Jc sample (Figure 1.). Most of the genetically similar samples were from Thailand, compared to several accessions from different countries such as India, China, Suriname, Lao, Sri Lanka and USA. Among all *J. curcas* samples. J7 from USA showed minimum genetic similarity with the rest and found to be most diverged germplasm in this study.

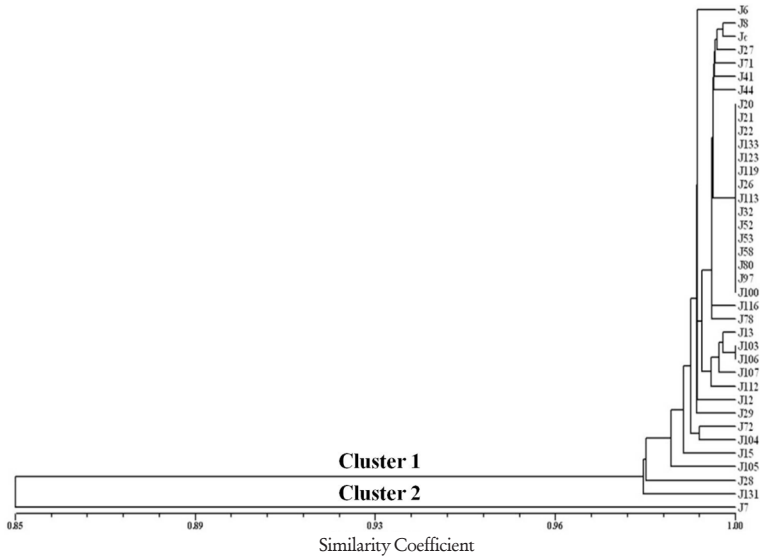

Figure 1. UPGMA clustering of *Jatropha curcas* samples based on AFLP and ISSR markers.

In addition, the AMOVA procedure was used to estimate the partitioning of genetic variance among regions, among and within populations. The result based on both AFLP and ISSR data showed that the genetic variation was found mainly within *J. curcas* population (77%), but variance among populations of Thailand and other countries was only 23%. A measure of population differentiation (F_{st}) among populations of Thailand and other countries was found at moderate level (0.231), while F_{st} among *J. curcas* populations collected from different regions of Thailand was very low (0.014), confirming the low level of genetic differentiation among *J. curcas* germplasm throughout Thailand.

4. DISCUSSION

The genetic diversity of *J. curcas* germplasm collected throughout Thailand and some introduced from other six countries was assessed using two molecular marker systems, AFLP and ISSR. A high level of genetic similarity among *J. curcas* accessions collected from different regions of Thailand was found based on the genetic similarity coefficient and AMOVA analysis. The same results were reported in several studies which were carried out to understand genetic diversity of *J. curcas* using various marker systems. Pamidimarri et al. (2010) [6] used AFLP and RAPD markers to evaluate the genetic diversity of 28 diverse germplasm of *J. curcas* collected from distinct

geographical areas in India and a high similarity ranging from 0.866 to 1.00 was reported. Pamidimarri *et al*. (2010) [6] also mentioned that there were 18 AFLP primer combinations effective for *J. curcas* and three of them matched the combinations identified in this study. When compared the results generated from the same primer pair, the genetic diversity of *J. curcas* germplasm in India reported by Pamidimarri *et al*. (2010) [6] was higher than that of germplasm in the present study.

Shen *et al*. (2010) [11] reported the used of AFLP marker to evaluate genetic diversity of *J. curcas* in Hainan, China. The result showed a high similarity ranged from 0.866 to 0.977, suggesting a low genetic diversity among *J. curcas*. Tanya *et al.* (2010) [9] used ISSR markers to determine genetic relationship and diversity among 30 accessions of *J. curcas* from China, Mexico, Thailand, and Vietnam and the result also revealed a narrow genetic base. In contrary to the above studies, AFLP based molecular characterization of *J. curcas* accessions from Andhra Pradesh was found diverse, showing occurrence of high number of unique/rare fragments and having great variation in percentage of oil content [10]. However, careful understanding of the phylogeny and use of adequate number of molecular markers are essential prerequisites for drawing valid inferences about the genetic affinities.

J. curcas is probably native to Mesoamerica. This plant was dispersed in Thailand by the Portuguese more than two centuries ago [21]. A few introduced plants and their vegetative propagation activity were reported to cause the low genetic variation of *J. curcas* in India and China [7, 22]. Similar situation to those countries, the genetic diversity in our *J. curcas* germplasm is far less when compared to other plant species. Another possible reason for low genetic diversity observed in the invasive plant species is the time in which it was introduced into Asia was not long enough to give rise to the limited genetic variation, so *J. curcas* in Asia has a low genetic diversity [22].

Despite the several reports on limited genetic diversity in *J. curcas*, the results from morphological and physiological diversity assessment showed otherwise. The great variation in important phenotypic, physiological and biological traits such as plant height, seed size, water use efficiency and seed oil content were previously reported [23]. Those phenotypic variances may be caused by epigenetic regulatory mechanisms [24]. Yi *et al*. (2010) [25] designed the experiment to study the phenotypic, genetic and epigenetic diversity of *J. curcas* collections at the same time. The results indicated that the differences in agronomic performance among *J. curcas* were independent from climate and practice. Such agronomic trait variations, however, were matched by very low genetic diversity but significant epigenetic diversity. The further study on epigenetic polymorphisms and epigenetic regulatory mechanisms of this biofuel plant are considerable.

The success of *J. curcas* genetic improvement program requires germplasm collection that has broad genetic base. In this study, a low level of genetic diversity of *J. curcas* germplasm in Thailand was found. The genetic relationships among all *J. curcas* samples (Figure 1) revealed that *J. curcas* samples could be divided into two clusters. Almost all samples were grouped into cluster 1, while a sample from USA, accession no. J7, was found to be the most diverged germplasm and formed separate cluster 2. The close relationships between the samples from Thailand, India and Sri Lanka were observed. The *J. curcas* germplasm from Sri Lanka (accession no.131) and India

(accession no. 6) were clustered in the same group as samples from Thailand (Figure 1). Moreover, we found that some samples from India, China, Suriname, Lao and Sri Lanka were genetically identical. The result in this study indicated that it is necessary to import new germplasm preferably from the center of origin of this species such as Mexico or Central America. Basha *et al*. (2009) [8] reported that genetic variation of *J. curcas* in Mexico was higher than that of the germplasm from other parts of the world. Montes *et al*. (2009) [26] studied 225 accessions of *J. curcas* collected from over 30 countries in Latin America, Africa and Asia. The genetic diversity of this germplasm was assessed using AFLP technique. The result revealed that genetic variability was low in African and Indian *J. curcas* but high genetic variability was found in Guatemalan and other Latin American accessions. The same result was also reported by Ovando-Medina *et al*. (2011) [27], who used AFLP markers to evaluate the genetic diversity of *J. curcas* germplasm collected from Chiapas, Mexico. Cluster analysis result of five populations of *J. curcas* in Chiapas showed a Jaccard's dissimilarity coefficient of 0.893, indicating very high genetic diversity. In addition, there was a report indicating that Brazilian cultivated *J. curcas* germplasms were closely related but had a high level of genetic diversity than accessions from other countries in African, Asian and Central America [28]. Those promising germplasms should be introduced to broaden genetic diversity of *J. curcas* in Thailand.

Another way to increase the genetic diversity of *J. curcas* is the use of chemical and physical mutagens to induce mutation. Mutation breeding is one of the available options for genetic improvement of *J. curcas* with modest levels of variability. In India, mutation breeding has been initiated to create genetic variation for various traits and developed mutants are being characterized using DNA markers [29]. Positive correlation between data generated by SSR and ISSR markers and morpho-physiological traits has been reported in Greek sweet cherry [30]. Those markers could be useful in marker-assisted breeding programs.

Assessment of genetic relationships among species is required for the management of genetic resources and it can predict the success probability of interspecific hybridization. In this study, the genetic relationship among *J. curcas* and four related species; *J. integerrima*, *J. gossypifolia*, *J. multifida* and *J. podagrica*, was also observed. The maximum relatedness was found between *J. curcas* and *J. integerrima*. Our molecular analysis results supported the reason for the success in interspecific hybridization between those two species. The same results were reported by Tanya *et al*. (2010) [9]. Interspecific hybrids have been developed between *J. curcas* and *J. integerrima* and reciprocal crosses are possible [31]. One to two backcrosses of the F_1 hybrids to *J. curcas* resulted in transgressive segregants exhibiting variation for fruit and seed characters [31]. Therefore, the interspecific hybridization is one of the approaches to improve new variety of *J. curcas*. In this study, several polymorphic DNA fragments between different species were found. Those DNA markers can be utilized in species differentiation, molecular identification and characterization of interspecific hybrid.

CONCLUSIONS

AFLP and ISSR markers were used for a study of the genetic diversity in *J. curcas* and four related species, as a first step towards its genetic improvement in

Thailand. The similarity indices, 0.8428 to 1.00, revealed the highly close relationships among *J. curcas* accessions in the germplasm collection. Of the four related species used in this study, *J. integerrima* was genetically more close to *J. curcas* than the others. Our results indicated that the germplasms from other countries, preferably from the center of origin of this species, should be introduced to broaden genetic diversity of *J. curcas* in Thailand.

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