Jurnal Agro*Biogen* 7(1):47-55

Genetic Diversity Analysis of *Jatropha Curcas* Provenances Using Randomly Amplified Polymorphic DNA Markers

Dani Satyawan* and I Made Tasma

Indonesian Center for Agricultural Biotechnology and Genetic Resources Research and Development, Jl. Tentara Pelajar 3A, Bogor 16111
Phone (62-251) 8337975; Facs. (62-251) 8338820; *E-mail: d.satyawan@gmail.com

Submitted: 22 Oktober 2010; Accepted: 9 Maret 2011

ABSTRAK

Analisis Keragaman Genetik Jarak Pagar (*Jatropha Curcas*) Menggunakan Marka RAPD. *Dani Satyawan dan I Made Tasma.* Jarak pagar (*Jatropha curcas* L.) adalah tanaman non pangan yang bijinya dapat menghasilkan minyak untuk digunakan dalam produksi biodiesel atau langsung dipakai sebagai bahan bakar bagi mesin diesel yang telah dimodifikasi. Tujuan penelitian ini adalah untuk mengetahui keragaman genetik koleksi tanaman jarak pagar Badan Penelitian dan Pengembangan Pertanian yang ditanam di Kebun Percobaan Pakuwon (Sukabumi, Jawa Barat) dengan menggunakan marka RAPD. Sebanyak 50 aksesi jarak pagar dan satu aksesi *Jatopha integerrima* dianalisis keragaman genetiknya menggunakan 14 primer RAPD dengan kandungan G+C antara 60-80%. Dari 14 primer tersebut diperoleh 64 pita DNA dengan tingkat polimorfisme sebanyak 95,7%. Beberapa pita DNA yang dihasilkan tidak konsisten dan *reproducible* sehingga dilakukan reaksi ulangan untuk setiap primer yang digunakan demi meminimalisir kesalahan skor marka. Data pita DNA yang dihasilkan selanjutnya digunakan untuk analisis keragaman genetik dengan menggunakan *Unweighted Pair Group Method Arithmetic* (UPGMA) dan koefisien Dice, dan hasil yang didapat menunjukkan bahwa aksesi yang dianalisis memiliki koefisien kesamaan antara 0,2 dan 0,98, dengan rerata sebesar 0,75. Analisis dendrogram membagi aksesi-aksesi ke dalam dua kelompok besar, dengan satu *outlier* dari Lampung Selatan. Pembagian kelompok tidak berkorelasi dengan daerah asal masing-masing aksesi, sehingga konsisten dengan hipotesis bahwa tanaman jarak pagar baru masuk ke Indonesia sekitar 4 abad lalu dan penyebarannya lebih banyak difasilitasi oleh manusia. Tingginya rerata koefisien kesamaan menunjukkan bahwa keragaman genetik koleksi ini cukup rendah, sehingga di masa depan diperlukan penambahan aksesi dengan latar belakang genetik yang bervariasi untuk semakin meningkatkan produktivitas tanaman hasil pemuliaan jarak pagar.

Kata kunci: *Jatropha curcas*, marka RAPD, keragaman genetik.

ABSTRACT

Genetic Diversity Analysis of *Jatropha Curcas* Provenances Using Randomly Amplified Polymorphic DNA Markers. *Dani Satyawan and I Made Tasma.* *Jatropha curcas* nuts are rich in oil that is higly suitable for the production of bio-diesel or to be used directly in modified diesel engines. The objective of this study was to assess the extent of genetic diversity among 50 *J. curcas* provenances and one accession of *J. integerrima* using RAPD markers. The fifty *J. curcas* provenances were collected from ecologically diverse regions of Indonesia, and planted in the Pakuwon Experimental Station (Sukabumi, West Java). Fourteen RAPD primers with 60-80% G+C content were used in this genetic diversity analysis and produced 64 bands with 95.7% polymorphism level. The Polymerase Chain Reactions used to generate the RAPD bands sometimes produced inconsistent and non-reproducible results, necessitating the duplication of each reaction to prevent scoring errors. Sixty one validated bands were subsequently used for genetic diversity analysis using Unweighted Pair Group Method Arithmetic (UPGMA) method and Dice coefficients. It was shown that the similarity coefficients among the provenances ranged from 0.2 to 0.98 with an average similarity of 0.75. Dendrogram analysis produced two major groups of provenances, with one outlier from South Lampung. There was no tendency for provenances originated from nearby regions to cluster together in each group, and several provenances showed more similarities with provenances originated from distant regions. This pattern lent credence to reports that *Jatropha* was introduced to Indonesia around four centuries ago and was mainly spread by humans. Based on the mean similarities among the accessions and their clustering pattern, the genetic diversity of the *Jatropha* collection appeared to be fairly low. Future additions of genetic materials from more diverse genetic background will be necessary to maintain the current progress of *Jatropha* improvement program.

Keywords: Genetic diversity, RAPD markers, *Jatropha curcas*.

INTRODUCTION

Indonesian population and economic developments have increased each year, resulting in a similar increase in national consumption for fossil-based fuels even when the national fuel production is lower than the national demands. As a consequence, the country position has shifted from a net exporter to become a net importer. The world fuel price has also shown extreme volatility, at one time even sky-rocketing close to US$ 150 per barrel, making the

government's expenses from the energy sector increased significantly to cover the national fuel subsidy. This trend is expected to continue as long as the population growth and economic improvements in highly populated countries like Brazil, India, and China progresses at their current rates. A new national energy policy is thus required to mitigate this trend, including exploring alternative energy sources to substitute fossil fuels with renewable ones. One of the alternatives is by using plant-based energy sources such as those derived from palm oil, sugar cane, maize, soybean, sweet sorghum, and *Jatropha curcas* L.

J. curcas has several advantages compared to other bio-energy sources as this crop can be grown quite easily under diverse environmental growth conditions. *J. curcas* oil is not only a renewable fuel, it is also non-edible and consequently it does not compete with the production of plant oils for human consumption. Another attractive feature of *J. curcas* oil is that it produces less carbon dioxide and nitric oxide emission compared to regular diesel oil and therefore it is considered as environmentally cleaner since it creates less green house gasses.

The genus *Jatropha* belongs to the Euphorbiaceae family. This genus consists of 175 species and among those, five can currently be found in Indonesia (Hasnam, 2006). These include *J. curcas* and *J. gossypiifolia* that have generally been used as medicinal crops and *J. integerrima* Jacq, *J. multifida* and *J. podagrica* Hook that have been used as ornamental crops. *J. curcas* mainly attracted scientists around the world due to its unique oil characteristics for diesel replacement (Achten *et al.,* 2008).

Most members of the genus *Jatropha* have chromosome numbers 2n = 44 (Dehgan and Schutzman, 1994). *J. curcas* itself was reported to be consisted of two groups, i.e., 2n = 22 and 2n = 44 for diploid and tetrapoid genomes, respectively. Based on their chromosome configurations during meiosis, *J. curcas*, *J. multifida,* and *J. gossypiifolia* seem to be closely related to each other. However, Dehgan and Schutzman (1994) classified these three species into different groups within genus *Jatropha* and subgenus *curcas*.

J. curcas genetic diversity was thought to be relatively low based on the limited collection currently available in the world (Hasnam, 2006). *J. curcas* is a native of Mexico and Central American region and was later introduced into many parts of the tropics and subtropics where it is grown as a hedge crop and for traditional use (Dehgan and Schutzman, 1994; Heller, 1996). This plant was reported to spread to South East Asia, including Indonesia, around the 17th to 18th centuries, carried by Portuguese and Spanish sailors (Heller, 1996; Hasnam, 2006). Genetic variations that can be found in Indonesia therefore is most likely due to differences in growth environments and geographical isolation within each island that resulted in the emergence of different ecotypes found around the nation. The presence of insect pollinators and differences in maturing time between male and female flowers can also promote outcrossing and increase diversity (Heller, 1996). These two factors can contribute to the genetic variations among Indonesian accessions.

An understanding of the extent of genetic diversity is critical for the success of a breeding program. Traditional methods using morphological characteristics for establishment of genetic diversity and relationship among provenances were largely unsuccessful due the strong influence of the environments on highly heritable seed traits like 100-seed weight, seed protein, and oil content in *J. curcas*. Hence, selection based on genetic information using neutral molecular markers is essential as it is more reliable and more consistent. In the Euphorbiaceae family, molecular markers such as RAPD, AFLP, RFLP, and SSR have been employed in determining the extent of genetic diversity in elite rubber clones (Lakawipat *et al.,* 2003) and cassava (Asante and Offei, 2003).

As a preliminary *J. curcas* phylogenetic study we explored the use of RAPD markers. The RAPD marker development uses a single, arbitrary short oligonucleotide primer (10 nucleotides in length) to amplify *J. curcas* genome segments flanked by two complementary binding sites in inverted orientation (Williams *et al*., 1990). Any primers that bind close enough to each other in the genome will create PCR products. The RAPD markers are observed as the presence and absence of PCR bands in a particular genotype and therefore are classified as dominant markers. RAPD markers are routinely used for genetic diversity analyses on many crop plants especially the ones where no SSR markers have been developed.

In 2005, the Indonesian Center for Estate Crop Research and Development (ICERD) conducted germplasm exploration throughout the Indonesian regions. They collected germplasms from provinces of South Sumatera, Lampung, Banten, West Java, Central Java, East Java, West Nusa Tenggara, East Nusa Tenggara and South Sulawesi (Hasnam, 2007). The collected plant materials were planted in three Indonesian regions representing three different climatic zones: ICERD West Java Pakuwon Experimental Station (representing the wet area), ICERD East Java

Asembagus Experimental Station (representing the very dry area), and ICERD Muktiharjo Experimental Station (representing the dry area). Fifty three *J. curcas* provenances were planted at the ICERD West Java Pakuwon Experimental Station representing all the collected provenances. A provenance is defined as plant materials originated from regions with different agro-ecosystems (Hasnam, 2007). The regions from where the plant materials were collected can be a district or a sub-district as long as the collection area demonstrated variations in their environmental conditions. The genetic diversity level of the collected germplasm would then be determined using randomly amplified polymorphic DNA (RAPD) markers. The data presented in this study will provide important information for future *J. curcas* breeding programs.

The objective of this study was to analyze the genetic diversity levels of ICERD *J. curcas* collection planted in Pakuwon Experimental Station (Sukabumi, West Java). Genetic diversity of the provenances will be evaluated using RAPD markers.

MATERIALS AND METHODS

Plant Materials

The plant genetic materials used in this study consisted of 52 provenances of *J. curcas* and one provenance of *J. integerrima*. These provenances were collected from Indonesian regions from the western, central, and eastern parts of the country covering the wet, medium, and dry areas. The list of the provenances used in this study is shown in Table 1. An individual plant was selected randomly to represent a provenance, so that DNA per provenance was represented by a single plant from the group. The chosen plants were propagated using stem cuttings and planted in ICABIOGRAD green house. Young leaves with approximately 9 cm^2 leaf area were then collected from each plant, except for Bgr-3 and Lam-4 provenances, which were not responsive to propagation by cuttings. The young leaves from the 51 plant samples were collected in small plastic bags that were precooled in ice and were later stored in ice during transport to the laboratory. Upon arrival, the samples were frozen in liquid nitrogen and ground to fine powders using a mortar and pestle. The leaf powders were subsequently used for DNA isolation.

DNA Isolation

DNA was isolated from young leaf powder from the respective plant samples. DNA extraction was conducted using a modified protocol of Michiels *et al*. (2003). *J. curcas* leaf powders were transferred into a 2-ml ependorf tube to fill a third of the tube volume (approximately 667 ul). Six hundreds µl 60°C-prewarmed CTAB extraction buffer (100 mM Tris pH 8, 1,4 M Na Cl, 20 mM EDTA pH 8, 0.2 % (v/v) beta mercaptoethanol, 2% Polyvinylpirrolidone (PVP), 2% CTAB) were added into the tube and mixed well. The solution was then incubated at 60°C for 1 h and the tubes were regularly inverted once in 15 minutes. Next, 600 µl chloroform : isoamyl alkohol (24 : 1) were added into tubes and mixed well by vortexing The samples were then cetrifuged for 5 min at 2,500 rpm and the upper phase of the solution was transferred into a new 2-ml eppendorf tube. The chloroform : isoamyl alkohol (24 : 1) extraction of the samples was repeated two more times untill the upper phase of the solution is clear. The upper phase of the solution was then transferred into a 1.5 mL microtube before adding 400 µl of isopropanol. The tube was later incubated at room temperature overnight. The next day the DNA was collected by centrifugation at 5,000 rpm for 10 min. The collected DNA was then washed using washing solution (10 mM ammonium acetate dan 70% ethanol). The DNA was collected by centrifugation as before. The washing step was repeated one more time, and the collected clean DNA was then dried overnight before diluting it using 100 µl TE buffer (10 mM Tris pH 8, 1 mM EDTA). DNA concentation was mesured using a spectrophotometer (Bio Rad, California, USA). DNA was then diluted to a concentration of 10 ng/µL.

RAPD Primers

Fourteen primers were selected from a list of primer used by Wong *et al.* (1997) that showed excellent polymorphism in cassava (*Manihot esculenta*). The selected RAPD primers were expected to be highly polymorphic in *Jatropha* as well, since cassava is closely related to *J. curcas*. The RAPD primers used in this study and their sequences are shown in Table 2. Primers were synthesized by Invitrogen (California, USA).

PCR and RAPD Genotyping

The 51 purified DNA samples from the *Jatropha* provenances and its wild relative (*J. integerrima*) were used as templates in the PCR reaction. A 10-µl PCR reaction was used. The PCR reaction consisted of 20 ng DNA, 0.4 mM dNTP, 4.0 mM $MgCl_2$, 1.0 µM primer, and one unit of *Taq* DNA polymerase Core System from (Promega, Wiaconsin, USA). PCR reaction was conducted in an MJ Research 96-well PCR machine (MJ Research, New Jersey, USA) as follows: Preheating at 95°C for 5 min, followed by 30 cycles of denaturation

Table 1. Provenances of *J. curcas* along with their wild relative, their origin and number of individual plants available at field collection of the Pakuwon Experimental Station.

No.	Provenance designation	Origin				No. of plants[a]
		Village	Sub-district	District	Province/country	
1.	JT-1	Rawalu	Banyumas	Banyumas	Central Java	30
2.	JT-2	Banjarsari	Nasaung	Cilacap	Central Java	30
3.	JT-3	Palih Raja	Palih Raja	Banyumas	Central Java	27
4.	JT-4	Sidobau	Palih Raja	Banyumas	Central Java	27
5.	JT-5	Sidourip	Binangun	Banyumas	Central Java	24
6.	JT-6	Tegal Kemulyan	Cilacap	Cilacap	Central Java	21
7.	JT-7	Sedayu	Binangun	Cilacap	Central Java	18
8.	JT-8	Gemilir	Cilacap Utara	Cilacap	Central Java	18
9.	JB-1	Cigelam	Babakan Cikao	Purwakarta	West Java	24
10.	JB-2	Ciwaneng	Babakan Cikao	Purwakarta	West Java	24
11.	JB-3	Tonjong	Pelabuhan Ratu	Sukabumi	West Java	24
12.	JB-4	Cacaban	Congeang	Sumedang	West Java	24
13.	JB-5	Pandanaan	Paseh	Sumedang	West Java	24
14.	Btn-1	Sukawaris	Cikeusik	Pandeglang	Banten	24
15.	Btn-2	Cikeruh Wetan	Cikeusik	Pandeglang	Banten	24
16.	Btn-3	Malangsari	Cikeusik	Pandeglang	Banten	24
17.	Skb	Cikadu	Pel. Ratu	Sukabumi	West Java	24
18.	Lam-1	Kahuripan	Panengahan	South Lampung	Lampung	24
19.	Lam-2	Merak Blantung	Panengahan	South Lampung	Lampung	24
20.	Lam-3	Suak	Sidomulyo	South Lampung	Lampung	24
21.	Lam-4	Kr BelalauBatu	Brak	West Lampung	Lampung	9
22.	Tsk-1	Kelapa Genep	Cikalong	Tasikmalaya	West Java	24
23.	Tsk-2	Sindang Kerta	Cikalong	Tasikmalaya	West Java	24
24.	Tsk-3	Sindang Kerta	Cipatujak	Tasikmalaya	West Java	24
25.	Krw-1	Mulya Sejati	Cikampek	Karawang	West Java	24
26.	Krw-2	Curug	Klari	Karawang	West Java	24
27.	Bgr-1	Ciapus	Cikaret	Bogor	West java	24
28.	Bgr-2	Pamayanan	Ciomas	Bogor	West java	24
29.	Bgr-3	Cimahpar	Ciomas	Bogor	West java	24
30.	Tbn-1	Mander	Tambak Boyo	Tuban	East Java	24
31.	Tbn-2	Mander	Tambak Boyo	Tuban	East Java	24
32.	SBr-1	Surantih	Koto Taruhan	South Surantih	West Sumatera	12
33.	SBr-2	Marunggi	Nan Subaris	P. Pariaman	West Sumatera	12
34.	SBr-3	Ombilin	Rambatan	Tanah Datar	West Sumatera	12
35.	Jpr-1	St Portugis	Benteng	Jepara	Central Java	10
36.	Pati-1	Pati	Pati	Pati	Central Java	147
37.	Pati-2	Pati	Pati	Pati	Central Java	123
38.	Smp-1	Bt Belah Barat	Dasuk	Sumenep	East Java	24
39.	Mkr-1[b]	?				2
40.	Mkr-2[b]	?				24
41.	Mkr-3[b]	?				24
42.	Mkr-4[b]	?				24
43.	Bnwg	Banyuwangi	Banyuwangi	Banyuwangi	East Java	346
44.	NTB				West Nusa Tenggara	286
45.	Pak-1	Plant Intro[c]			Malaysia	2
46.	Pak-2	Plant Intro			India	42
47.	Pak-3	Plant Intro			India	500
48.	Pak-4	Plant Intro			India	11
49.	Pak-5	Plant Intro			Malaysia	14
50.	Pak-6	Plant Intro			Malaysia	5
51.	Pak-7	Plant Intro			Malaysia	32
52.	Integ[b]	?				2
53.	RedLeaf[b]	?				1

[a]Number of plants of each physic nut provenance currently available at the Pakuwon Experimental Station, Sukabumi, West Java; [b]No complete information is available on these provenances; Plant Intro = plant introduction; ? = no information available.

at 95°C 1 min, primer annealing at 32°C 3 min, and primer extension at 72°C 2 min. The reaction was closed by DNA extesion at 72°C for 10 min. The PCR products were then electrophoresed using 1.5% agarose at 70 volt for 2 h. The gel was stained with ethidium bromide and visualized under UV light using a Bio Rad Chemidoc (Bio Rad, California, USA). Each RAPD primer reaction was repeated twice to obtain consistent RAPD bands.

Table 2. Amplification results of the 14 RAPD primers assayed in 51 *Jatropha* provenances.

RAPD Primer	Primer Sequence (5' → 3')	Number of amplified RAPD bands[a]	Number of polymorphic bands[b]	Percentage of polymorphic bands
RAPD 1	GGCCACAGCG	6	6	100
RAPD 2	ACCCGTCCCC	4	4	100
RAPD 3	ACCGCCTCCC	5	4	80
RAPD 4	CGACGCGTGC	9	9	100
RAPD 5	CGAGACGGGC	8	8	100
RAPD 6	GCAGCTCCGG	3	3	100
RAPD 7	ACGCCCTGGC	2	2	100
RAPD 8	GGCCTTCAGG	1	1	100
RAPD 9	TGCACGGACG	3	3	100
RAPD 10	GGCCTACTCG	7	7	100
RAPD 11	GTCTCG TCGG	4	4	100
RAPD 12	GTGTAGGGCG	5	5	100
RAPD 13	CGCAGTACTC	2	2	100
RAPD 14	TGGCCGAGGG	5	3	60
Average	-	4.57	4.36	95.71

[a]The number of amplified DNA bands include both thick and medium sizes DNA bands; [b]Polymorphic bands were counted for both medium and bold RAPD fragments.

Data Analysis

The RAPD bands were scored as follows: each DNA band in a gel represents DNA fragment from a locus in a plant genotype. DNA bands having the same migration rate were assumed to be from the same locus and were given a value of 1 when the band exists and 0 when no band appears. Genetic similarity was calculated using the method of Rohlf (2000). A dendrogram then was constructed using a cluster analysis of the Unweighted Pair Group Method Arithmetic (UPGMA) method through the Numerical Taxonomy and Multivariate System (NTSYS) program version 2.1-pc (Rohlf, 2000).

RESULTS AND DISCUSSION

Marker Polymorphism, Scorability, and Consistency

All 14 RAPD markers produced observable and scorable bands from purified *Jatropha* DNA. Since very few genetic studies have been performed on *Jatropha* during the commencement of this study (early, 2007), the RAPD markers were selected based on a study by Wong *et al.* (1997), which concluded that RAPD primers with high G+C content tend to produce better banding patterns in cassava (*Manihot esculenta*). Since *Jatropha* and cassava are both members of the Euphorbiaceae, it was assumed that their DNA composition would be fairly similar and produce prolific RAPD bands from primers with 70-80% G+C content. This assumption appeared to be correct, since the 14 RAPD primers could produce 64 scorable bands, which means that on average each primer was capable of generating 4.57 scorable bands. Among the 64 bands, 61 were polymorphic across the 51 *Jatropha* provenances (Table 2). The level of polymorphism was very high (95.7%), but this was probably due to the incorporation of outliers from other species (jarak zitun/*J. inteerrima*), which tend to produce different banding patterns on loci that are monomorphic among *J. curcas* accessions.

A typical banding pattern observed on a 1.5% agarose gel can be seen in Figure 1. The DNA bands typically consisted of 3-4 intense bands that were easy to score and reproducible when PCR was repeated, along with several faint bands that could potentially introduce false negative results since they were easily lost in wells that were not loaded properly due to pipetting error or diffusion into the electrophoresis buffer. The fainter bands tended to arise from spurious amplifications, as illustrated by the lower sized bands produced by accession number 44 in Figure 1, which only appeared in the second PCR but completely absent in the first PCR. The fainter bands also often failed to appear in PCR products where one band predominated and consumed all available PCR primers in the reaction. However, the fainter bands can actually be thick bands that showed reduced intensity on gels due to PCR error or gel loading error, as illustrated by the single intense band from accession 49 that appeared very faint in the first PCR. The inclusion of the fainter bands in the scoring process, therefore, should be done cautiously after confirming that the bands consistently appear in the duplicated reactions using the same primers and DNA templates.

Duplicated PCR is a necessity for accurate genotyping using RAPD since the short primers by nature are not very specific. This lack of specificity was

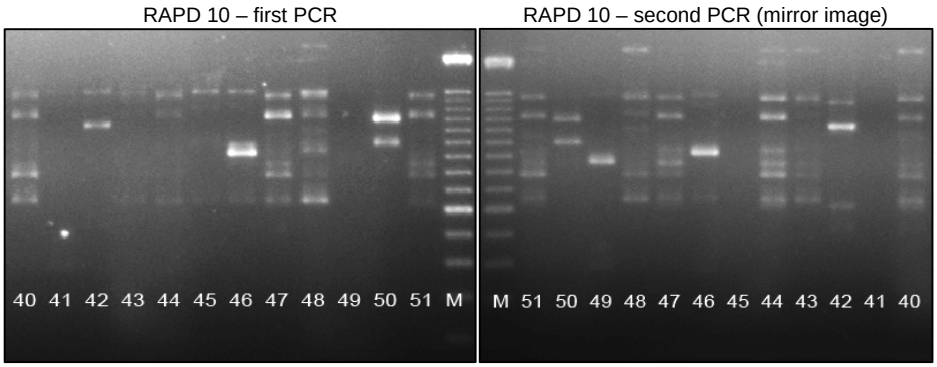

Figure 1. Banding patterns obtained in repeated reactions using the same primer and DNA templates. The gel picture from the second PCR was digitally flipped to its mirror image to aid comparison. M = 100 bp DNA ladder, 40-51 = *Jatropha* accessions used in the RAPD analysis.

further exacerbated by the low stringency during PCR that promotes the amplification of the non-specific products. Several PCR variables were known to improve RAPD consistency, including template DNA purity, consistent primer concentration, a single source of DNA polymerase between reactions, and the use of a single thermocycler set at a certain reaction profile (Tommerup *et al.,* 1995). All those factors were observed during this study, but these optimizations by no means guarantee that the DNA bands, which were visualized on gels, would be free from false positives and negatives. Repetitions are still necessary to improve accuracy of the test. The repeated PCR will add to the cost of genotyping, but overall RAPD is still the cheapest and easiest genotyping methods compared to the more consistent procedures like AFLP and SSR.

Genetic Diversity of the *Jatropha* Collection

The 61 polymorphic bands were used to calculate genetic diversity and genetic relationship between provenances across the 51 *Jatropha* accessions using NTSYSpc software (Rohlf, 2000). Similarity coefficients between provenances were calculated using DICE coefficients (Dice, 1945), and the result showed that genetic similarities between the sampled accessions ranged between 0.2 (between *J. integerrima* and NTB) and 0.98 (between Pati-2 and Tuban-1) with an overall mean genetic similarity of 0.75. The DICE coefficient was chosen to generate the similarity matrix, because it emphasizes positive co-occurences rather than negative co-occurences (Duarte *et al.,* 1999). Thus, it reduces biases and errors created by false negatives. False negatives can occur easily in RAPD scoring, since many DNA bands fail to appear in sufficient intensity due to PCR error, inadequate primer concentration, and gel loading errors. On the other hand, false positives are easier to control by duplicating the PCR for each primer. Consequently, similarity calculations that emphasizes positive scoring are usually more accurate for error-prone methods that produce dominant markers like RAPD (Duarte *et al.,* 1999), especially when closely-related accessions are used in the assay (Dalirsefat *et al.,* 2009).

A dendrogram was constructed from the similarity data using Unweighted Pair Group Method with Arithmetic Mean (UPGMA) clustering method in NTSYSpc (Figure 2A). As expected, *J. integerrima,* a different species used as an outlier in this study, appeared as a single separate branch that shared only 0.36 coefficient of similarity with the other 50 accessions from the *J. curcas* group. Another accession with red-colored young foliage (Jarak Pucuk Merah) that was included as another outlier (Redleaf, Figure 2) was found to group closely with other *J. curcas* accessions. This accession was originally thought to be another species within the genus *Jatropha*. However, the high genetic similarity to other *J. curcas* accessions exhibited by this red-leafed accession most likely means that the accession belongs to the species of *J. curcas*, albeit with an interesting morphological variation.

Another interesting provenance was Lam-3 from South Lampung. It appeared to be sufficiently distinct from other *J. curcas* accessions, and shared only around 0.74 similarity with other *J. curcas* provenances. This is interesting because the other two provenances from Lampung Province were very similar to each other with a significant bootstrap score of 56 (Figure 2B). The fact that Lam-3 provenance is more distinct to other *J. curcas* than provenances introduced from other countries like Malaysia and India makes it an appealing subject for a study on its origin and geographical growth condition.

Aside from the Lam-3 provenance, the dendrogram shows that in general the provenances can be classified into two major groups (Figure 2). However, there appears to be no correlation between the clustering of provenances with their geographic origin. For example, the eight provenances collected from Central Java Province were not clustered together within a group but they were distributed in both major groups and sometimes found to be more similar to provenances collected from other provinces. One of the highest genetic similarity coefficients was found between JT-6 and Btn-2. JT-6 was collected

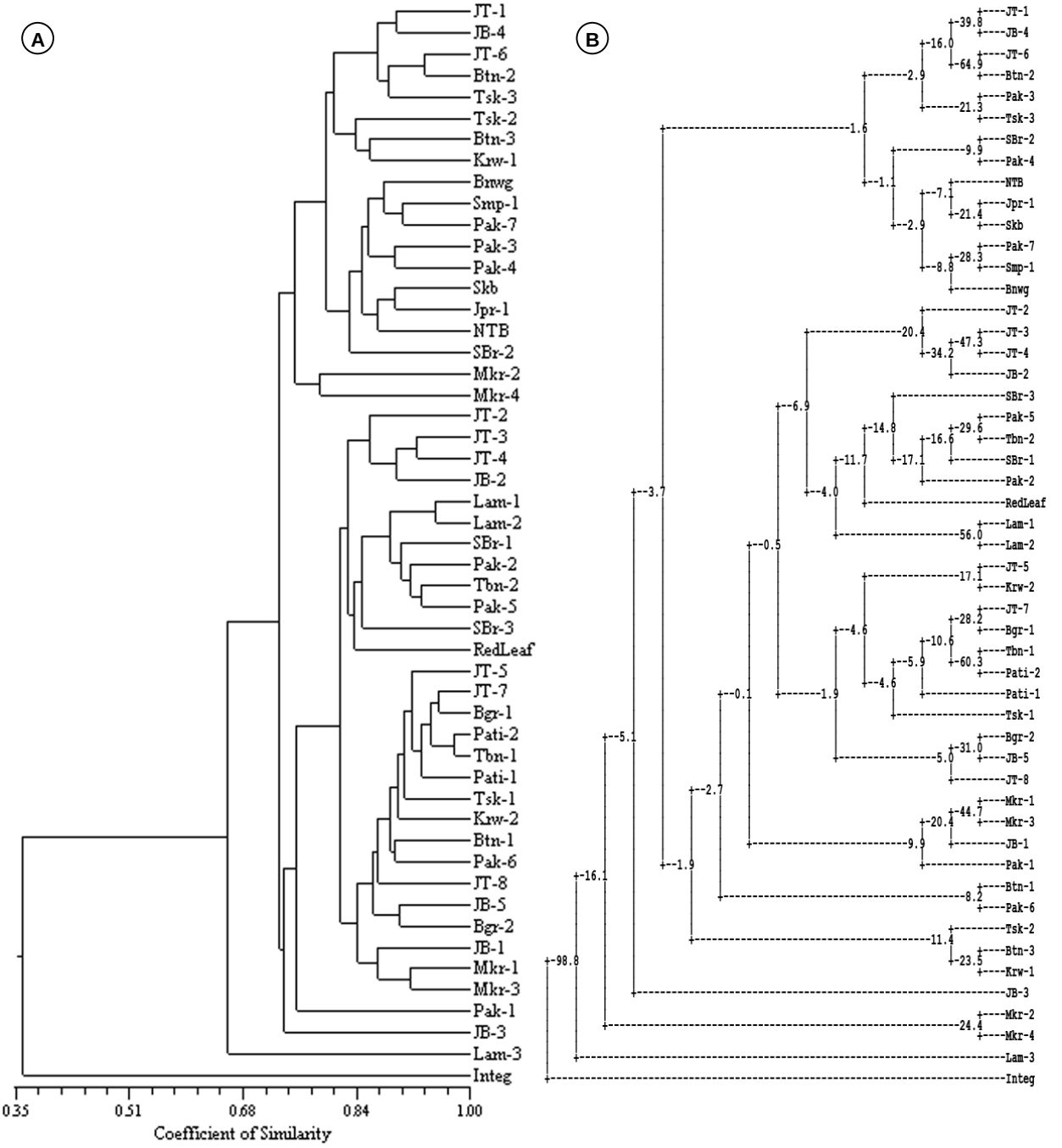

Figure 2. Dendrograms showing genetic relationships among the *Jatropha* provenances originated from different geographical regions of Indonesia. These provenances at the present time are maintained at Pakuwon Experiment Station. A = dendrogram constructed in NTSYSpc using UPGMA clustering method, B = consensus tree from a bootstrapping analysis using Winboot software. The numbers at the branches show the percentage of times the group consisting of the accessions to the right of that branch occurred.

from Cilacap while Btn-2 was from Pandeglang (Table 1), which are around 400 km apart from each other. On the other hand, JT-8 provenance, which was collected from the same regency less than 10 km away from where the JT-6 was collected, shared much lower genetic similarity with JT-6 and was much more similar to an accession collected from Malaysia.

The low correlation between genetic similarity and geographic origin supports the statement by Heller (1996) and Hasnam (2006) that *Jatropha* was introduced to Indonesia around four centuries ago. It is likely that due to such a short introduction time, not enough genetic distinctiveness has developed in the *Jatropha* populations from different locations in Indonesia. Most of the *Jatropha* diversities probably reflect more of the actual origin of the introduced parents where those provenances arose from. The phylogenetic relationship could become even more complicated if humans played a major role in the dispersion of *Jatropha* in Indonesia, since genetic materials could be transported further by humans in modern times than by other natural dispersal mechanisms. Hence, the fact that one provenance from Central Java was more similar to a Malaysian provenance than a provenance collected from an adjacent region is hardly surprising if we consider human mobility pattern during the last four centuries.

To test validity of the UPGMA dendrogram (Figure 2A), a bootstrapping analysis was done using Winboot software (Yap and Nelson, 1996). This analysis was performed to test the confidence level of each tree branch in the constructed phylogenetic tree in Figure 2A by calculating the frequency of a group of provenances to appear together on a particular branch. The output of the bootstrapping analysis can be seen in Figure 2B. The numbers at the branches show the percentage of times the group consisting of the accessions to the right of that branch occurred. The higher the values (maximum of 100) that are observerd on the tree branches, the higher the confidence levels of the cluster statistically. Some people suggest that a bootstrap value of over 50 can be considered as significant (Reddy *et al.*, 2009). The bootstrap analysis produced relatively low confidence level values, with the exception of the branch that differentiate *J. integerrima* from *J. curcas* accessions (bootstrap value of 98.8). The next highest value (64.9) was observed at the branch between JT-6 and Btn-2. Other branches that scored higher than 50 was the branch between Lam-1 and Lam 2, and the branch between Tuban-1 and Pati-2, which has the highest genetic similarity coefficient as well.

The bootstrap analysis also produced a dendrogram that was slightly different from the dendrogram obtained from NTSYSpc. This difference, along with the fact that most of the bootstrap values at each branch were very low, indicate that the clustering of the provenances based on RAPD data obtained in this study was not statistically robust. The addition of more marker data can sometimes increase the bootstrap values, as demonstrated by Jaunet and Wang (1999). However, the effect of each marker data addition was somewhat mixed in this study, where each additional marker data sometimes increase the bootstrap value of some branches but decrease the others.

Nevertheless, the calculated genetic diversity obtained in this study is fairly similar to that obtained in India by Basha and Sujatha (2007). In a study of 42 Indian *J. curcas* accessions using 400 RAPD and 100 iSSR markers, they obtained similar diversity values among their collections. They concluded that the genetic diversity of the Indian *Jatropha* collection was not high enough and a widening of germplasm diversity is required by introducing genetic materials from more diverse backgrounds to increase the gain of future breeding program. Similar program is probably needed in Indonesia since the genetic diversity level is also similar here, even though current efforts in *Jatropha* improvement by the Indonesian Agency for Agricultural Research and Development (IAARD) has resulted in three improved populations (IP3-P, IP-3A, and IP-3M, which are acronyms for the 3rd improved populations from Pakuwon, Asembagus, and Muktiharjo Experimental Stations respectively). The yield improvement of the three populations was significant in each generation of mass selection. This indicates that there was enough genetic variability available among individual plant within a provenance to support the significant annual yield increase. Nevertheless, more work and genetic materials are still needed to create more commercially viable *Jatropha* cultivars in the future.

CONCLUSION

Fourteen RAPD primers with more than 60-80% G+C content could produce 61 polymorphic bands in 50 *J. curcas* and one *J. integerrima* provenances, which confirms RAPD as an economical and easy method to generate DNA markers for this species. However, DNA bands generated by RAPD were sometimes inconsistent, necessitating duplicated reactions for a more consistent scoring and accompanied with algorithms that cancel the disadvantages

of false negatives in diversity analysis. Genetic diversity study using the RAPD marker data generated a phylogenetic tree that divided the provenances into two major groups with an outlier from South Lampung (Lam-3). The grouping did not correlate well with geographical origin, as provenances originated from neighboring regions were not always clustered together and often showed more similiarities to provenances from distant regions than to each other. This grouping pattern lent credence to reports that *Jatropha* was introduced to Indonesia around four centuries ago and was mainly spread by humans. The phylogenetic clustering also showed that all of the *J. curcas* provenances shared more than 70% similarity (with the exception of Lam-3 provenance). This observation, along with the high value of the mean similiarity coefficient (0.75), indicated that the *Jatropha* collection did not have sufficiently high genetic diversity. Further addition of accessions from a more diverse genetic background will be needed to sustain the rapid gain of productivity currently obtained using mass selection system.

ACKNOWLEDGEMENTS

The authors wish to thank Dr. Hasnam and Dibyo Pranowo, MS for the plant materials used in this study, and Mr. Fajar Suryawan and Mr. Ahmad Dadang for laboratory assistance. This study was funded by the 2007 Fiscal Year National Budget for the Indonesian Center for Agricultural Biotechnology and Genetic Resources Research and Development, Project Number: SP 3209.0/018-09.0/XII/2007.

REFERENCES

Achten, W.M.J., L. Verchot, Y.J. Franken, E. Mathijs, V.P. Singh, R. Aerts, and B. Muys. 2008. Jatropha bio-diesel production and use. Biomass and Bioenergy 32(12):1063-1084.

Asante, I.K. and S.K. Offei. 2003. RAPD based genetic diversity study in fifty cassava (*Manihot esculenta* Crantz) genotypes. Euphytica 131:113-119.

Basha, S.D. and M. Sujatha. 2007. Inter and intra-population variability of *Jatropha curcas* (L.) characterized by RAPD and ISSR markers and development of population-specific SCAR markers. Euphytica 156(3):375-386.

Dalirsefat, S.B., A.S. Meyer, and S.Z. Mirhoseini. 2009. Comparison of similarity coefficients used for cluster analysis with amplified fragment length polymorphism markers in the silkworm, *Bombyx mori*. J. Insect Science 9: Article 71.

Dehgan and Schutman. 1994. Contributions toward a monograph of neotropical Jatropha: Phenetic and phylogenetic analyses. Ann. Missouri Bot. Gard. 81:349-367.

Dice, L.R. 1945. Measures of the amount of ecologic association between species. Ecology 26:297-302.

Duarte, J.M., J.B. dos Santos, and L.C. Melo. 1999. Comparison of similarity coefficients based on RAPD markers in the common bean. Genet. Mol. Biol. 22(3):427-432.

Hasnam. 2006. Variasi *Jatropha* L. Info Tek Jarak Pagar (*Jatropha curcas*) 1(2):5.

Hasnam. 2007. Status perbaikan dan penyediaan bahan tanaman jarak pagar (*Jatropha curcas* L.). Prosiding Lokakarya-II Status Teknologi Tanaman Jarak Pagar (*Jatropha curcas* L.) hlm. 7-16.

Heller, J. 1996. Physic nut (*Jatropha curcas* L.). *In* Promoting the Conservation and Use of Underutilized and Neglected Crops 1:1-66.

Jaunet, T.X. and J.F. Wang. 1999. Variation in genotype and aggressiveness of *Ralstonia solanacearum* race 1 isolated from tomato in Taiwan. Ecology and Population Biology 89(4):320-327.

Lakawipat, N., K. Teerawatanasuk, M. Rodier-Goud, M. Sequin, A. Vanavichit, T. Toojinda, and S. Tragoonrung. 2003. Genetic diversity analysis of wild germplasm and cultivated clones of Hevea brasiliensis Muell. Arg by using microsattelite markers. J. Rubber Res. 6:36-47.

Michiels, A., W. Van den Ende, M. Tucker, L. Van Riet, and A. Van Laere. 2003. Extraction of high-quality genomic DNA from latex-containing plants. Analytical Biochemistry 315:85-89.

Reddy, C.S., A.P. Babu, B.P.M. Swamy, K. Kaladhar, and N. Sarla. 2009. ISSR markers based on GA and AG repeats reveal genetic relationship among rice varieties tolerant to drought, flood, or salinity. J. Zhejiang Univ. Sci. B. 10(2):133-141.

Rohlf, F.J. 2000. NTSYSpc. Numerical Taxonomy and Multivariate Analysis System. Version 2.1. Applied Biostatistics Inc.

Tommerup, I.C., J.E. Barton, and P.A. O'Brien. 1995. Reliability of RAPD fingerprinting of three basidiomycete fungi, Laccaria, Hydnangium and Rhizoctonia. Mycological Research 99(2):179-186.

Williams, J.G.K., A.R. Kubelik, K.J. Livak, J.A. Rafalski, and S.V. Tingey. 1990. DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. Nucl. Acid Res. 18:6531-35.

Wong, H.L., H.H. Yeoh, S.H. Lim, and K.C.L. Looi. 1997. Design of primers for rapd analyses of cassava, Manihot esculenta. Phytochemistry 46(5):805-810.

Yap, I. and R.J. Nelson. 1996. WinBoot: A program for performing bootstrap analysis of binary data to determine the confidence limits of UPGMA-based dendrograms. IRRI Discussion Paper Series 14:1-20.