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Genetic Diversity of Indonesian Physic Nut (*J. curcas*) Based on Molecular Marker

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ABSTRACT

Various reports of molecular genetic diversity evaluation of physic nut (*J. curcas*) have given inconsistent results. Part of the reasons were because of the used of unrealiable markers. This study was conducted to evaluate genetic diversity of Indonesian physic nut germplasm using four types of molecular markers (RAPD, ISSR, SSR and SCAR markers). Twenty four *J. curcas* accessions planted in Pakuwon, Sukabumi, with various phenotypes were evaluated. Twenty eight SSR marker loci yielded monomorphic allele pattern and indicated that the evaluated accessions probably were all genetically homogeneous for the respective loci. Eight RAPD and 4 ISSR primers out of the total 31 tested primers produced scoreable markers and some (i.e. UBC 873, OPG 17, OPP 03 and OPQ 11 primers) generated polymorphics markers. Genetic similarity coefficiens among evaluated accessions ranged from 0.6 to 1.0 with a population mean of 0.9 indicating low diversity and narrow genetic background among accessions in all populations. Therefore, breeding program utilizing such population would only result in low genetic gain. Based on the evaluated SCAR markers, all accessions belonged to the non-toxic Mexican type of physic nut. This information is important inputs for designing future physic nut breeding strategies in Indonesia.

Keywords: germplasm characterization; molecular analysis; SSR markers

INTRODUCTION

One of the limiting factors in the cultivation and commercialization of *J. curcas* as the biofuel producer was the unavailability of high yielding and high oil content varieties (Surwenshi, Kumar, Shanwad, & Jalageri, 2011). Availability of germplasm with high genetic diversity is the basic requirement for *J. curcas* genetic improvement. Information on genetic diversity is useful for selecting parents for breeding programs and germplasm management. Results of genetic diversity analysis could be used as the decision making tool to avoid duplicate collections. Therefore, availability of reliable methods for assessing *J. curcas* genetic diversity is essential. As in many different plants (Kustanto, Basuki, Sugiharto, & Kasno, 2012; Zulhermana, Sudarsono, Asmono, & Yulismawati, 2010; Saptadi, Hartati, Setiawan, Heliyanto, & Sudarsono, 2011; Ajambang, Sudarsono, Asmono, & Toruan, 2012; Sutanto, Hermanto, Sukma, & Sudarsono, 2013), molecular marker could potentially be used to asses *J. curcas* genetic diversity.

Collection and phenotype diversity analysis of *J. curcas* germplasm have been done in various countries, such as in India, China, Brazil and Indonesia (Ou, Wang, & Li, 2009; Tatikonda et al., 2009; Hartati, Setiawan, Heliyanto, Pranowo, & Sudarsono, 2009). The presence of high variation in morphological characters has also been noted by several researchers (Kaushik, N., Kumar, K., Kumar, S., Kaushik, N, & Roy, 2007; Kumar & Sharma, 2008). Moreover, Makkar, Becker, Sporer, & Wink (1997) reported the existence of high phenotype diversities among *J. curcas* accessions are from West and East Africa, North and Central America and Asia.

Indonesian Center for Estate Crop Research and Development (ICERD) maintains a collection of *J. curcas* germplasm from all regions in Indonesia.

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There were high phenotype diversities among the accessions, such as for plant height, stem diameter, branching type, flowering dates, inflorescence, fruit bunch, fruit, and seed number per plant and seed oil content (Hartati, 2008; Hartati, Setiawan, Heliyanto, Pranowo, & Sudarsono, 2009; Sudarmo, Heliyanto, Suwarso, & Sudarmadji, 2007). However, not all of those phenotype diversities could be attributed to actual genetic diversities since previous studies indicated that there wasa large genotype by environment effects for those characters in *J. curcas* (Heller, 1996; Makkar, Becker, Sporer, & Wink, 1997; Kaushik, N., Kumar, K., Kumar, S., Kaushik, N, & Roy, 2007).

Evaluation of China and Indian collections of *J. curcas* genetic diversity using molecular markers has been done previously (Zhang, Guo, Liu, Tang, & Chen, 2011). Unfortunately, the results were inconsistent. Basha & Sujatha (2007) reported that there were moderate diversities among 42 Indian *J. curcas* accessions. Ranade, Srivastava, A. P., Rana, Srivastava, J., & Tuli (2008) reported that wild, semi-wild and accessions of *J. curcas* have been naturalized and therefore they showed sufficient genetic diversities based on the SPAR marker analysis. Using AFLP markers, Tatikonda et al. (2009) reported that there was a wide genetic diversity among Indian *J. curcas* accessions. On the other hand, evaluation of 38 worldwide accessions of *J. curcas* using RAPD and AFLP markers by Popluechai et al. (2009) resulted in a low genetic diversity. Cai, Sun, Wu, & Peng (2010) reported, there was a high genetic diversity among 219 accessions *J. curcas* from China, while Sun, Li, L. F., Li, Y., Wu, & Ge (2008) reported China's *J. curcas* accessions exhibited a very low genetic diversity. The most comprehensive evaluation was conducted by Montes et al. (2008) on 225 *J. curcas* accessions from 30 countries of Latin America, Africa and Asia using AFLP markers. Result of these studies indicated that *J. curcas* accessions from Africa and India had a low genetic diversity while those from Latin America showed a high diversity.

High phenotypic diversity in Indonesian *J. curcas* (Hartati, 2008; Hartati, Setiawan, Heliyanto, Pranowo, & Sudarsono, 2009) needs to be evaluated further to prove that it is representing the genetic diversity. Unfortunately, there are no available information indicating such association between phenotype and genetic diversity. Therefore, the objectives of this study were to evaluate genetic diversity of ICERD's *J. curcas* germplasm collections from various places in Indonesia using SSR, SCAR, RAPD and ISSR markers.

MATERIALS AND METHODS

The research was done at Bogor Agricultural University from July to December 2011.The plant materials were taken from Pakuwon, Sukabumi East Java owned by ICERD. The molecular marker analysis was done in the Plant Molecular Biology Laboratory (PMB Lab.), Department of Agronomy and Horticulture, Faculty of Agriculture, Bogor Agricultural University (IPB), Bogor, Indonesia.

Plant Materials

Twenty four accessions of *J. curcas* were evaluated. The accessions were the offsprings of the original provenances collected from various regions in Indonesia (Table 1). The samples were chosen using purposive random sampling and the samples representing each area of origin and exhibiting different morphological characters, such as contrasting days of flowering and seed yield. The sampled provenances were grown in the same field and they were about two years old during the sample collection.

DNA Extraction

A total of 0.1 g of young leaves was collected from each sample and total nucleid acid was extracted using modified CTAB procedure as described by Pamidimarri, Meenakshi, Sarkar, Boricha, & Reddy (2009). The isolated DNA was quantified using UV spectrophotometer (Shimadzu UV - 1800) at λ 260 nm and its purity was determined by calculating absorbance ratio at λ 260 and 280 nm (Sambrook, Fritsch, & Maniatis, 1989).

Molecular Marker Analysis

In this research, 28 SSR loci that have been previously evaluated (Saptadi, Hartati, Setiawan, Heliyanto, & Sudarsono, 2011) and the additional 10 SSR loci derived from genomic data bases of *Manihot esculenta* (Wen et al., 2010; Table 2) were evaluated. Moreover, 22 RAPD primers, 9 ISSR primers, and 2 SCAR primers were also evaluated. The SCAR primers were specifically used to identify Indian type (toxic) and the Mexican type (non-toxic) of *J. curcas* accessions as described by Basha & Sujatha (2007). List of the evaluated RAPD, ISSR and SCAR primers are presented in Table 3 and Table 4.

Table 1. Accession numbers, the progenitor origin, days of flowering and seed yield of the evaluated *J. curcas* accessions

No.	Accession No.	Origin	Days to flowering* (days)	Seed yield* (g plant^{-1})
1.	PT7-1	Lampung	200	388
2.	PT33-2	Lampung	97	628
3.	PT13-2	Lampung	86	704
4.	PT14-1	Lampung	86	640
5.	PT-33	Lampung	84	446
6.	PT26-2	Lampung	84	622
7.	PT3-1	Lampung	84	392
8.	IP-1P-3	Lampung	80	422
9.	PT18-1	Lampung	75	274
10.	PT15-1	Lampung	75	780
11.	554-1	West Nusa Tenggara	222	456
12.	575-3	West Nusa Tenggara	145	90
13.	2555-1	West Nusa Tenggara	125	70
14.	3012-3	West Nusa Tenggara	111	244
15.	IP-1A-2	West Nusa Tenggara	99	680
16.	3189-2	West Nusa Tenggara	84	606
17.	3012-1	West Nusa Tenggara	84	748
18.	IP-M-3	East Java	>360	0
19.	IP-1M-2	East Java	180	238
20.	MT7-1	East Java	89	584
21.	SP6-3	South Sulawesi	274	74
22.	SP8-1	South Sulawesi	142	390
23.	SP16-2	South Sulawesi	125	46
24.	HS49-2	East Nusa Tenggara	91	900

Remarks: * = source: Hartati, Setiawan, Heliyanto, Pranowo, & Sudarsono (2009); one-year observation period

Table 2. List of additional primers used to generate markers at 10 SSR loci for genetic diversity analysis of *J. curcas.* These SSR primers were developed from available genomic database of *M. esculenta* as described in Wen et al. (2010)

No.	Accession No.	Primer Sequences	
		Forward	Reverse
1.	JESR-083	ACAGCCTCGTCATTTCACT	TAATGAATGGTTCGTAGCCT
2.	JESR-086	TCCCTCTCCTTCAGATTAAA	ATGATAGCCAAACAGCAACT
3.	JESR-088	CCCTCCCTTTGGTTTCTG	GGAGGAAAGGAGAGGAAATA
4.	JESR-089	AACAACTGGTTGTGGAGTTC	TTGATGCTGTGGATATGAGA
5.	JESR-090	TGACATTTGTCAGTCTTGGA	TCACCATACCACACAATCAC
6.	JESR-097	ACCGCTTCTTCTTTCTCTCT	TAGCCGGCAATATACAGAAT
7.	JESR-104	CCACAGTTCATCCTCAATTT	GATATTCACTCTGGAACCCA
8.	JESR-107	CCTGTGTAGAATCGTCCTTT	AACCAGAACCAATCTCAATG
9.	JESR-108	CTAGTAGAGCAGGTGTTGGG	CATCCCACTCAACAATTCA
10.	JESR-118	CTAAAGGCTGTGAAGAAGGA	TCCGAGCCAATTTCTTATTA

Table 3. List of primers used to generate RAPD markers for genetic diversity analysis of *J. curcas*

No	Primer Name	Primer Sequences	No	Primer Name	Primer Sequences
1.	OPC 10	TGTCTGGGTG	12.	OPP 03	GTGGTCCGCA
2.	OPC 14	TGCGTGCTTG	13.	OPP 09	GGAGTGCCTC
3.	OPC 18	TGAGTGGGTG	14.	OPQ 11	TCTCCGCAAC
4.	OPE 05	TCAGGGAGGT	15.	OPQ 19	CCCCCTATCA
5.	OPF 16	GGAGTACTGG	16.	OPT 14	AATGCCGCAG
6.	OPG 17	ACGACCGACA	17.	OPU 10	ACCTCGGCAC
7.	OPG 18	GGCTCATGTG	18.	OPU 19	GTCAGTGCGG
8.	OPH 14	ACCAGGTTGG	19.	OPV 08	GGACGGCGTT
9.	OPJ 15	TGTAGCAGGG	20.	OPV 14	AGATCCCGCC
10.	OPK 01	CATTCGAGCC	21.	OPV 17	ACCGGCTTGT
11.	OPK 12	TGGCCCTCAC	22.	OPW 17	GTCCTGGGTT

Table 4. List of primers used to generate ISSR and SCAR markers for genetic diversity analysis of *J. curcas*

No.	ISSR Primers	Primer Sequences	No.	SCAR Primer	Primer Sequences
1.	UBC810	GAGAGAGAGAGAGAGAT	1.	ISPJ1-F	GAGAGAGAGAGAGAGGTG
2.	UBC812	GAGAGAGAGAGAGAGAA		ISPJ1-R	GAGAGAGAGAGAGAAAAC
3.	UBC834	AGAGAGAGAGAGAGAGYT			AAT
4.	UBC847	CACACACACACACACARC	2.	ISPJ2-F	GAGAGAGAGAGTTGGGTG
5.	UBC880	GGAGAGGAGAGGAGA		ISPJ2-R	AGAGAGAGAGAGCTAGAG
6.	UBC816	CACACACACACACACAT			AG
7.	UBC891	HVHTGTGTGTGTGTGTG			
8.	UBC866	CTCCTCCTCCTCCTCCTC			
9.	UBC 873	GACAGACAGACAGACA			

Polymerase chain reaction (PCR) was performed in a total volume of 25 µL, consisting of 0.2 µM primers, 1.25 U TaqDNA polymerase (Real Biotech Corporation), 2.5 µL 10x PCR buffer, 0.1 MmdNTPs (10 mM mix), 1 µL DNA template, and ddH2O as needed. The steps for PCR cycles used to amplifly SSR markers were one cycle of denaturation at 95 °C for 5 minutes; 36 cycles consisting of denaturation at 94 °C for 30 seconds, primer annealing at the appropriate temperature for each primer for 30 seconds, and primer extension at 72 °C for 1 minute; and onecycle of final extension at 72 °C for 5 minutes. PCR cycles to generate RAPD markers were one cycle of denaturation at 94 °C for 3 minutes; 45 cycles consisting of denaturation at 94 °C for 45 seconds, primer annealing at 36 °C for 30 seconds, and primer extension at 72 °C for 2 minutes; and one cycle of final extension at 72°C for 7 minutes, whereas the PCR cycles to generate ISSR markers were one cycle of denaturation at 94°C for 4 minutes; 35 cycles consisting of denaturation at 92°C for 30 seconds, primer annealing was set at temperature according to the appropriate Ta for each primer for 1 minute, and primer extension at 72°C for 2 minutes; and one cycle of final extension at 72°C for 7 min. PCR amplification to generate SCAR markers were conducted as follow, one cycle of denaturation at 94°C for 4 minutes; 35 cycles consisting of denaturation at 94°C for 30 seconds, primer annealing at 56°C (ISPJ1 primers) and 54°C (ISPJ2 primers) for 15 seconds, and primer extension at 72°C for 1 minutes; and one cycle of final extension at 72°C for 7 minutes. All PCR amplifications were done using Perkin Elmer DNA thermal cycler model PE2400.

The PCR amplified DNA fragments of RAPD, ISSR and SCAR markers were fractionated using 1 % horizontal agarose gel electrophoresis and visualized using 1 % ethidium bromide staining. The stained DNA fragments were visualized on UV transluminescent and photographed using digital camera for records. Size control of 1 Kb DNA ladder was used to estimate the size of amplified DNA fragments. The PCR amplified DNA fragments of SSR markers were fractionated using vertical 6 % polyacrylamide gel electrophoresis (PAGE) on a Dedicated Height Manual Sequencer (Cole-Parmer) using 1x TBE buffer at a constant voltage of 1,100 V for 3 hours.

The loaded volume of the PCR product for each sample was 1.8 µL and there are 60 loaded samples per gel. The SSR markers were visualized using silver staining and 100 bp DNA ladders were used to estimate the amplified PCR products.

Data Analysis

Allele scoring for each of the generated SSR locus was conducted for the most obvious bands and the alleles were recorded based on their PCR product sizes. Scoring for RAPD and ISSR markers were carried out based on the presence or absence of PCR amplified products. The markers will be scored '1' if certain size of scorable PCR amplified product is presence and '0' if absence.

All of the generated marker data was tabulated for all evaluated individuals and primers and they were used to generate dissimilarity matrixfrom single data. Dissimilarity matric was generated using Dice as the dissimilarity index. Statistical analysis was also performed on the binary data to generate genetic similarity value (Nei & Li, 1979) using the following equation: Sij = 2a / (2a + b + c), in which: Sij is genetic similarity between individual i and j; a is number of generated PCR products for the i and the j individuals, b is number of generated PCR products appearing only in the i individuals, and c is that appearing only in j individuals. Genetic similarity was calculated using NTSys software version 2.0 (Rohlf, 1998). The percentage of polymorphism (PP) was calculated based on the ratio of total number of polymorphic loci to the total number of loci.

Principal Coordinate analysis to produce graphical representations on Euclidean plans and tree construction using weighted Neighbor Joining methods were done using previously generated dissimilarity index as the genetic distance. Boostrap analysis used 10,000 iterations. Dissimilarity index calculation, bootstap analysis, principal coordinate analysis, and tree construction were done using DARWIN Software version 6.0.13 (Perrier & Jacquemoud-Collet, 2006).

RESULTS AND DISCUSSION

SSR Marker Analysis in *J. curcas*

All SSR primers evaluated in this study could yield PCR products from all accessions of *J. curcas* genomic DNA. Nineteen primers evaluated yielded two alleles per locus while the remaining 19 yielded only one band per locus. The evaluated *J. curcas* specific SSR markers (28 loci) generated a total of 47 alleles. However, the 24 accessions of *J. curcas* evaluated in this study produced the same pattern of SSR allele (monomorphic pattern). Similarly, the ten SSR loci developed based on *M. esculenta* genome could also generate amplified product using *J. curcas*, indicating the SSR primers cross-amplify the *J. curcas* genome. Moreover, the generated SSR alleles using *M. esculenta* SSR primers were also monomorphic in all accessions of *J. curcas*. Example of allele patterns generated using two SSR primers in 24 accessionsof *J. curcas* are presented in Fig. 1.

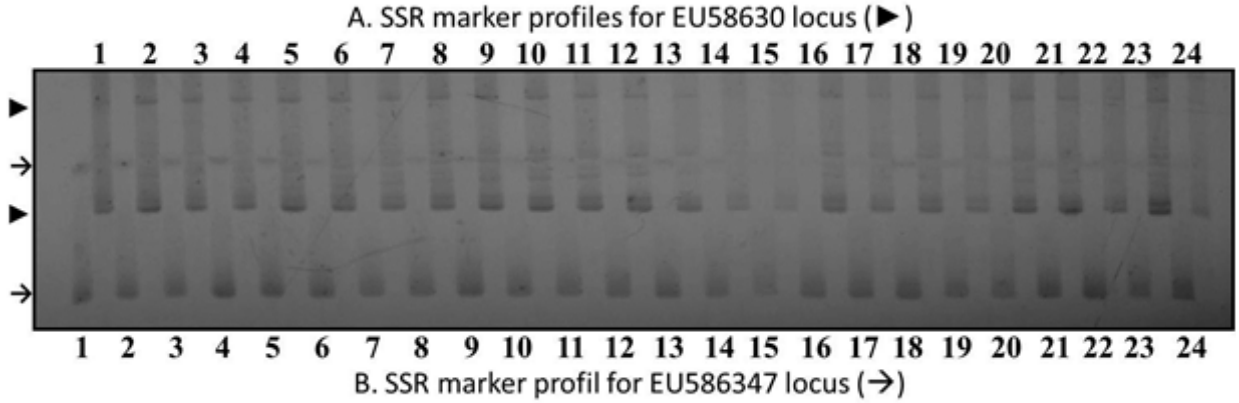

Fig. 1. Examples of allele profiles generated using J. curcas specific SSR primers for 24 accessions (1 – 24). A. The patterns of SSR alleles (►) generated using EU58630 SSR primers and B. The patterns (→) generated using EU586347 SSR primers, respectively

Previous report has indicated SSR marker analysis using accessions of *J. curcas* generated either one or two PCR products and the pattern of amplified products were monomorphics across accessions (Saptadi, Hartati, Setiawan, Heliyanto, & Sudarsono, 2011). Further analysis on the progenies derived from crosses of accessions indicated the two generated PCR products were not allelic and probably from two different locis since there was no segregation between the two bands in the progenies (Saptadi, Hartati, Setiawan, Heliyanto, & Sudarsono, 2011).

Results of the current evaluation using 24 different *J. curcas* accessions from diverse origin in Indonesia also generated the same results as previously described in Saptadi, Hartati, Setiawan, Heliyanto, & Sudarsono (2011). All of the evaluated accessions show monomorphic banding pattern for the evaluated 28 SSR loci (Fig. 1). These results were surprising since *J. curcas* was a cross-pollinated plant. However, this result was similar to those of Rosado et al. (2010) which reported low level of genetic diversity among *J. curcas* germplasm from Brazil.

The low levels of marker polymorphisms among 28 SSR loci might be possible because of two hypotheses; it might indicate the evaluated SSR marker loci are originated from highly conserved regions of the *J. curcas* genome or it indicated actual low level of genetic diversity among the evaluated germplasm. The first hypothesis could not be confirmed since there were no supporting data for the genome of the evaluated *J. curcas* accessions. The second hypothesis is more acceptable since SSR is a hyper variable sequences and it changes frequently in generations. Therefore, unless the low diversity of the accessions, it should detect some degrees of allele diversity among the *J. curcas* specific - 28 SSR loci evaluated. Furthermore, evaluation of additional 10 SSR loci, randomly chosen from the genome of *M. esculenta* (member of the same family as *J. curcas*), resulted in cross amplification using genome of *J. curcas*. However, there was no allele diversity among 24 accessions of *J. curcas* for the cross amplified marker loci. More than 300 SSR primer pairs have been identified to generate SSR loci from the genome of *M. esculenta*. No further evaluation for the SSR loci originated from *M. esculenta* was attempted.

The same 28 SSR loci have been used to evaluate accessions of *J. curcas* in China and they have successfully been used to differentiate genotypes of the accessions (Wen et al., 2010). Therefore, since there is almost no polymorphism among Indonesian accessions of *J. curcas* for the 38 SSR loci evaluated, those results strongly indicated the diversity of *J.curcas* in Indonesia is low. Sun, Li, L. F., Li, Y., Wu, & Ge (2008) has evaluated different sets of *J. curcas* accessions from China and found low diversities among accessions. Furthermore, Rosado et al. (2010) also evaluated 192 accessions of *J. curcas* germplasm from various regions in Brazil using SSR markers. They found only one out of six loci evaluated generating polymorphic alleles.

To validate the researh finding, the DNA from additional six accessions of *J. curcas* from Aceh Besar, Medan, Lombok, Bima, Sumba and Papua were evaluated using the same sets of 38 SSR loci. The results also indicated thatthere was no allele polymorphism among the six accessions (data not presented). These validation results further indicating the possibility of either the low genetic diversity among *J. curcas* accessions from Indonesia or at least a low diversity of the genome regions where the SSR loci resided.

RAPD and ISSR Marker Analysis in *J. curcas*

The evaluated primers have previously been reported to generate polymorphic markers for *J. curcas* (Basha & Sujatha, 2007). Out of the total RAPD and ISSR primers evaluated, 8 RAPD and 4 ISSR primers generated a number of scorable markers. The total numbers of PCR amplified products was 39 fragments and 29 out of 39 fragments (74.36 %) were polymorphic. The number of generated DNA fragments ranged from 1 to 6 (Fig. 2 and Fig. 3). The UBC812 primer generated the least number and OPP33 primer generated the highest number of amplified DNA fragments. DNA fragments amplified using UBC873, OPG17, OPP03 dan OPQ11 primers are all polymorphics (Table 5).

Pair-wise comparison among 24 accessions indicated the highest genetic similarity coefficients (1) were among accession no. 3189-2 and PT13-2; MT7-1 and PT15-1; PT3-1, 2555-1 and SP8-1; 2555-1 and PT3-1 while the lowest (0.60) were between accession no. 554-1 and HS49-2. The average of genetic similarity coefficient was 0.9. The lowest polymorphism percentage (0 %) was among accession no. 3189-2 and PT13-2; MT7-1 and PT15-1; PT3-1, 2555-1 and SP8-1; 2555-1 and PT3-1 while the highest (55.3 %) was between accession

no. 554-1 and HS49-2. The verage of the percentage of polymorphism was 15.9 %. There were two unique PCR amplified DNA fragments in accession no. HS49-2 by using OPV 17 primer and in accession no. PT7-1 by using OPG 17 primers (Fig. 2 and Fig. 3).

Results of the principal coordinate analysis using 12 axis indicated that only five axis contribute a significant amount of variabilities and it accounted for 85 % of variabilities among the evaluated individuals in the population. Distribution of individuals using axis 1 and 2 is presented in Fig. 4.

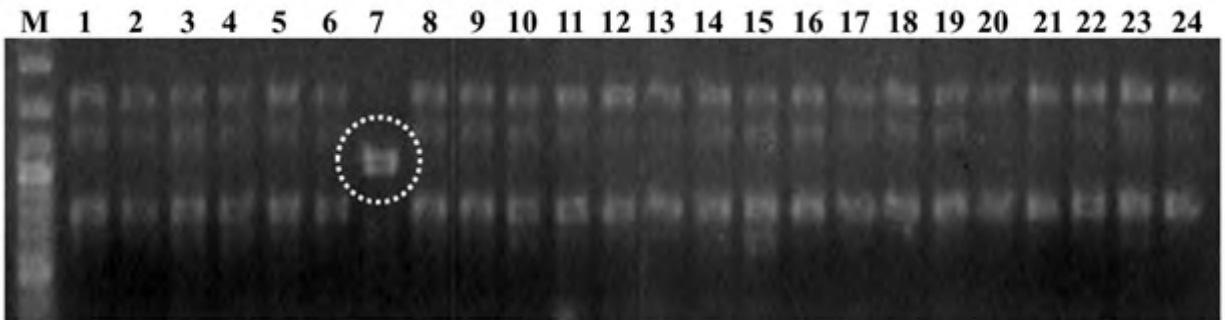

Fig. 2. Electrophoregram of RAPD marker profiles of 24 *J. curcas* accessions (1 – 24) amplified using random primer OPV 17; M = 1 Kb ladder DNA control sizes. Only accession no. 7 (HS49-2) generated unique profiles of DNA fragments while the rest are the same.

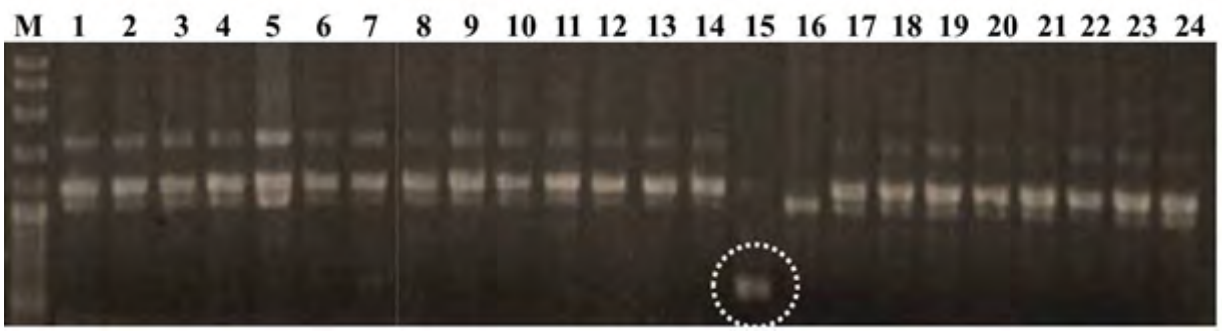

Fig. 3. Electrophoregram of RAPD marker profiles of 24 *J. curcas* accessions (1 – 24) amplified using random primer OPG 17; M = 1 Kb ladder DNA control sizes. Only accession no. 15 (PT7-1) generated unique profiles of DNA fragments while the rest are the same.

Table 5. Number of total amplified DNA fragments, monomorphic and polymorphic fragments, and percentage of polymorphisms generated using the evaluated RAPD and ISSR primers

Primer	Primer Type	Number of generated amplified DNA fragments			Percentage of polymorphism
		Total	Monomorphic	Polymorphic	
OPC 10	RAPD	2	1	1	50
OPC 14	RAPD	3	1	2	66.7
OPG 17	RAPD	4	0	4	100
OPG 18	RAPD	3	2	1	33.3
OPK 01	RAPD	3	1	2	66.7
OPP 03	RAPD	6	0	6	100
OPQ 11	RAPD	4	0	4	100
OPV 17	RAPD	5	1	4	80
UBC 810	ISSR	4	1	3	75
UBC 812	ISSR	1	1	0	0
UBC 834	ISSR	2	2	0	0
UBC 873	ISSR	2	0	2	100
Total		39	10	29	74.4

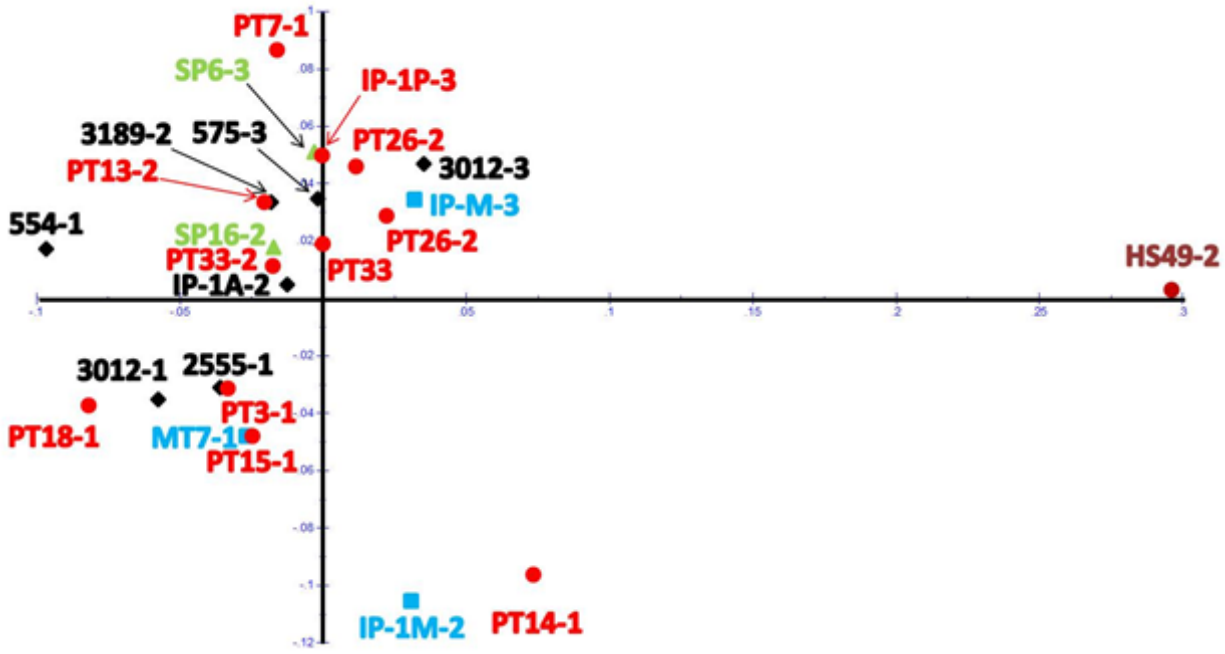

Fig. 4. Principal component analysis for the 24 *J. curcas* accessions from various locations in Indonesia based on the generated RAPD and ISSR marker data. Colors indicate origins of the progenitors: red is from Lampung, black - from West Nusa Tenggara, blue – from East Java, green – from South Sulawesi, and brown – from East Nusa Tenggara, respectively.

Three outlier accessions are presented in the Fig. 4 and they are HS49-2 – an accession from East Nusa Tenggara, IP-1M-2 – from East Java, and PT14-1 – an accession from Lampung. All other accessions are distributed to form a single population mixture (Fig. 4).

In line with the results obtained using SSR markers, evaluation of the *J. curcas* accessions originated from various places in Indonesia using RAPD and ISSR markers also showed a low diversity with polymorphisms percentage of only 15.87 %. Based on the constructed weighted Neighbor Joining (NJ) tree using dissimilarity matric, the *J. curcas* accessions from various places in Indonesia are grouped into three clusters (cluster I.a and I.b, cluster II, and cluster III, Fig. 5). The cluster I.a consisted of three accessions, such as HS49-2, originated from Ende, East Nusa Tenggara, IP-1M-2, from East Java and PT14-1, from Lampung while cluster I.b consisted of nine accessions, PT 13-2, PT26-2, and IP-IP-3, originated from Lampung, SP6-3 – from South Sulawesi, IP-M-3 – from East Java, and IP-1A-2, 575-3, 3012-3, and 3189-2 – from West Nusa Tenggara (Fig. 5, Table 1). The cluster II consisted of four accessions, the 554-1 from West Nusa Tenggara; the PT7-1 and PT33-2 from Lampung and SP16-2 from South Sulawesi while the cluster III consisted of six accessions, the 2555-1 and 3012-1 from West Nusa Tenggara, the MT7-1 from East Java, and PT3-1, PT18-1, and PT15-1 from Lampung (Fig. 5, Table 1). However, the clustering of the *J. curcas* accessions from Indonesia do not follow the geographical patterns, indicating they form a single population with probably a number genetic background.

SCAR Marker Analysis in *J. curcas*

The SCAR marker ISPJ1 specifically developed to identify the toxic Indian type while ISPJ2 identify the non-toxic Mexican type of *J. curcas* (Basha & Sujatha, 2007). These primers were used in this research to identify the type of the evaluated *J. curcas* accessions from various places in Indonesia. PCR amplification of all accessions using ISPJ1 primer pairs did not yield any PCR product. On the other hand, PCR amplification of all accessionsusing ISPJ2 primer pairs yield the expected size of PCR product (Fig. 6), indicates all of the *J. curcas* accessions are non-toxic Mexican type descendant.

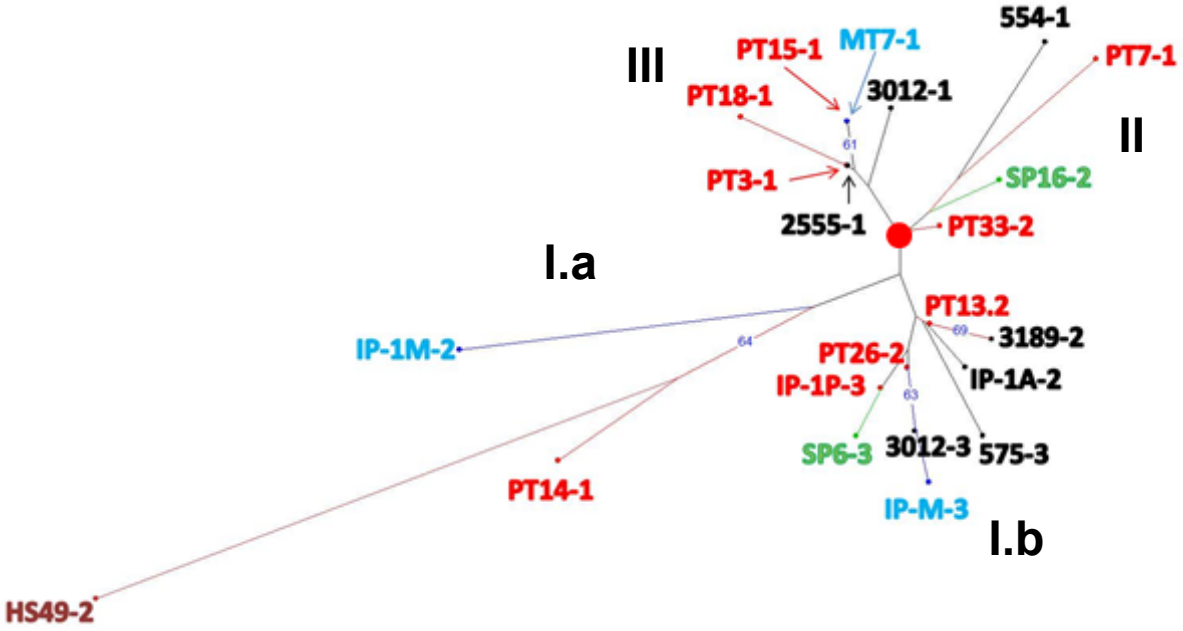

Fig. 5. Constructed Neighbor Joining tree for the 24 *J.curcas* accessions from various locations in Indonesia based on the generated RAPD and ISSR marker data. Red dot in the middle of the tree indicates the root position. Colors indicate origins of the progenitors: red is from Lampung, black – from West Nusa Tenggara, blue – from East Java, green – from South Sulawesi, and brown – from East Nusa Tenggara, respectively. Bootstrap analysis was conducted using 10,000 iterations and branching with bootstrap values higher than 60 are indicated in the figure.

Fig. 6. PCR amplified product of 24 *J. curcas* accessions using ISPJ1 primer (upper) and ISPJ2 primer (lower) generating SCAR marker only from the non-toxic Mexican type *J. curcas* (←).

Previous researcher proposes the spread of *J. curcas* from its center of origin in Mexico and South America was possibly brought by Portuguese explorers through Cape Verde Island and Guinea Bissau towards Africa and Asia (Heller, 1996). Zhang, Guo, Liu, Tang, & Chen (2011) and Sun, Li, L. F., Li, Y., Wu, & Ge (2008) hypothesized *J. curcas* came to China from India as the first area visited by Portuguese explorers through Southeast Asia. Based on these hypotheses, the Indonesian *J. curcas* is most likely to come from India. However, the results obtained from evaluation of the SCAR markers to *J. curcas* accessions from various places in Indonesia indicated all of the evaluated accessions belong to the non-toxic Mexican-type. Therefore, the origin of *J. curcas* in various places in Indonesia is unlikely to come from India.

The narrow of genetic diversity among *J. curcas* accessions in Indonesia is likely caused by the limited number of the original introduced materials from the center of origin and to the vegetative propagation nature of the plants, as previously proposed by Basha & Sujatha (2007), Sun, Li, L. F., Li, Y., Wu, & Ge (2008) and Zhang, Guo, Liu, Tang, & Chen (2011). The introduction of *J. curcas* to the regions by Portuguese explorers was probably happened about 5 centuries ago (Heller, 1996). Therefore, if the original introduced *J. curcas* genotypes are limited, the period between the times this plant was introduced up to now was not long enough to generate genetic diversity from limited genetic background of the initial materials (Zhang, Guo, Liu, Tang, & Chen, 2011). The current findings indicate that there are only limited genetic backgrounds among *J. curcas* accessions from various places in Indonesia, they are not grouped according to geographical distribution, and they most probably form a single population. Such findings supported previous results about the limited variabilities of the *J. curcas* accessions in the regions (Sun, Li, L. F., Li, Y., Wu, & Ge, 2008, Zhang, Guo, Liu, Tang, & Chen, 2011).

Although the evaluated populations tend to indicate low genetic diversity, they exhibit phenotype diversity (Table 1). Therefore, the investigation of breeding value of the selected population through heritability studies (Hartati & Sudarsono, 2013), through inbreeding and outbreeding depression measurement (Hartati & Sudarsono, 2014) and combining ability and heterosis determination (Hartati & Sudarsono, 2015) for various vegetative characters and seed yield of *J. curcas* have previously been conducted. It remains to be seen whether these breeding activities will eventually generate new superior *J. curcas* varieties or not.

CONCLUSION AND SUGGESTION

Evaluation on 24 *J. curcas* accessions having diverse phenotypes originated from various places in Indonesia was conducted. The accessions were genetically evaluated using 28 pairs SSR specific, 22 RAPD, 9 ISSR and 2 SCAR primers. All of the evaluated SSR primers yield monomorphic banding pattern and indicated that they were all genetically homozygous for the respective SSR loci. As many as 8 RAPD and 4 ISSR primers out of the total 31 tested primers produced scoreable markers and some (i.e. UBC 873, OPG 17, OPP 03 and OPQ 11 primers) were polymorphics. Genetic similarity coefficiens among evaluated accessions ranged from 0.6 to 1.0 with a population mean of 0.9 indicating low diversity and narrow genetic background among accessions in all populations. Therefore, breeding program utilizing such population would only result in low genetic gain. Based on the evaluated SCAR markers, all accessions belonged to the non-toxic Mexican type of physic nut. This information is important inputs for designing future physic nut breeding strategies in Indonesia.

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