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Identification of interspecific hybrid between *Jatropha curcas* × *J. integerrima* using morphological and molecular markers

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Abstract. *Saptadi D, Asbani N, Heliyanto B, Setiawan A, Sudarsono. 2020. Identification of interspecific hybrid between Jatropha curcas x J. integerrima using morphological and molecular markers. Biodiversitas 21: 814-823.* Eight F_1 progenies derived from *Jatropha curcas* × *J. integerrima* hybridizations were evaluated for their morphological characters and using RAPD, ISSR and SSR markers. Morphological variations among the hybrids were limited and they were intermediate between the *Jatropha* parents. The eight F_1 progenies derived from *J. curcas* × *J. integerrima* hybridizations were most probably the interspecific F_1 hybrids. The confirmed identity of the progenies as interspecific hybrids between *J. curcas* × *J. integerrima* was based on the presence of several phenotypic characters from both parents in the F1 progenies and by similarity of the molecular marker banding patterns among the parents and the F_1 progenies. Among the evaluated molecular markers, the ISSR primers and the majority of either RAPD and SSR primers were not able to generate marker for confirming the identity of F_1 progenies as interspecific hybrids between *J. curcas* × *J. integerrima*. However, the RAPD primer OPC 10 and the SSR primers AF469003, EU099522 and EU586348 were able to generate polymorphic markers in the *Jatropha* parents and their F_1 progenies. Therefore, these four primers were able to generate usable markers for confirming the identity of F_1 progenies as interspecific hybrids between *J. curcas* × *J. integerrima*. The evaluated interspecific F_1 progenies are potentially useful to increase genetic diversity of *J. curcas* and support its breeding program.

Keywords: F_1 interspecific, microsatellite, molecular marker, morphological variation

INTRODUCTION

Breeding activities require the presence of high genetic diversity for the targetted characters (Acquaah 2007) and for other supporting characters. Physic nut (*Jatropha curcas*) is a perennial crop (Raju and Ezradanam 2002). Compare to annual crops, new cultivar development for *J. curcas* requires more time (Divakara et al. 2010). On the other hand, new varieties having high oil content, yield and other desirable characters are still a priority for *J. curcas* breeding. Increased in *J. curcas* yield may be achieved through improving agronomic traits, resistance to biotic and abiotic stresses and developing better seed oil quality (Heller 1996). Although it needs some improvement, the yield potential of Indonesian *J. curcas* genotypes is relatively high (Hartati et al. 2009; Yi et al. 2010; Hartati et al. 2012).

Unfortunately, a report has also indicated that Indonesian *J. curcas* genetic diversity was low (Saptadi et al. 2017). Moreover, *J. curcas* breeding activities using such a narrow genetic base will not be efficient since the genetic gain for each breeding cycle will be low (Acquaah 2007). Therefore, activities to increase genetic diversity of Indonesian *J. curcas* collections should be beneficial. One such endeavor can be done by using interspecific hybrid among *Jatropha* species (Dhillon et al. 2009). Interspecific hybrids are used to increase genetic variation in many crops Gomes et al. 2014; Subroto et al. 2018; Banjanac et al. 2019). Hence, increase genetic diversity of *J. curcas* may be done by introducing desirable characters from other *Jatropha* species (Dhillon et al. 2009).

Jatropha curcas has a soft stem which makes the crop sensitive to waterlogging and root rot disease (Dhillon et al. 2009). *J. curcas* is also susceptible to both shoots infecting mites (*Eriophyid* and *Polyphagotarsonemus latus*) and thrips (*Selenothrips rubrocinctus* and *Rhipiphorothrips cruentatus*) (Asbani 2008). On the other hand, *J. integerrima* (the *Jatropha* species grown as an ornamental plant) has the tolerance to low temperature, resistance to stem rot disease, very resistance to leaf-eating caterpillar, and sturdy inflorescence characters (Lakshminarayana and Sujatha 2001). Asbani (2008) also reported that *J. integerrima* was probably resistant to shoot mites and thrips. The *J. integerrima* seeds also contain a high level of linoleic acid which affects oil quality (Rao and Lakshminarayana 1987). Therefore, *J. integerrima* may be used as the source of desirable characters for an interspecific hybridization with *J. curcas* (Dhillon et al. 2009).

Interspecific hybrid production is not an easy task since many constrain hamper the production of the hybrids such as sexual incompatibilities which limit the hybridization success, embryo abortion, low progeny quantity, and sterile hybrid plants (Van Tuyl 1997; Kaneko and Bang 2014).

Moreover, identification of the true-to-type interspecific hybrid plants may not be an easy task since the hybrid seedlings may have similar morphologies than those of the self progenies (Thomasset et al. 2011). Grow out test (GOT) has been widely used to identify the interspecific hybrid progenies (Alam et al. 2015; Pattanaik et al. 2018) and it includes planting of the putative hybrid seed progenies, observing their morphological characters, and selecting progenies having the combined characters of both male and female parents. Such approach is time-consuming, expensive and requires extensive resources (Wu et al. 2010), especially for such perennial crops as the *Jatropha* species. The GOT approach is also sensitive to environmental bias which reduces its effectiveness (Moose and Mumm 2008). Other studies proposed isoenzyme analysis as an effective alternative method (Ronis et al. 1990; Hirose et al. 1993). However, the presence of polymorphic isozyme banding patterns in closely related species is limited (Wu et al. 2010). Therefore, the evaluation of other more sensitive methods to distinguish progenies of *Jatropha* interspecific hybrids is necessary and DNA-based markers may be the desired alternative methods (Spooner et al. 2005). Moreover, the DNA based markers may be used to select out undesirable progenies as early as at the seedling stage (Dhillon et al. 2009). Therefore, early identification of the interspecific hybrid progenies is possible.

Dhillon et al. (2009) used RAPD markers to identify progenies of the inter-specific hybrid between *J. curcas* and *J. integerrima*. The RAPD markers have also been used to identify progenies derived from hybridization of *Passiflora* (Conceição et al. 2011), *Chrysanthemum* (Huang et al. 2000), and *Mentha* (Shasany et al. 2005). Another alternative DNA marker such as simple sequence repeat (SSR) markers has also been used to identify hybrid progenies of *Helianthus* (Iqbal et al. 2011), corn (Wu et al. 2010), walnut (Pollegioni et al. 2009), and peanut (Gomez et al. 2008).

In earlier research (Asbani 2008), an array of putative interspecific hybrid progenies has been produced by intercrossing Indonesian *J. curcas* accessions to *J. integerrima*. The limited number of progenies formed from interspecific crossing can be due to incompatibility between the two species (Pershina and Trubacheeva 2017). Moreover, the putative interspecific hybrid progenies were morphologically closely related to the female *J. curcas* parent (Asbani 2008). Therefore, the DNA based markers may be used to confirm true identities of the putative *Jatropha* interspecific hybrids.

The aims of this research were to evaluate morphological variations among putative interspecific hybrid progenies between *J. curcas* × *J. integerrima* and establish methods for identifying putative hybrids progenies using a combination of the SSR, RAPD, and ISSR markers. The identified interspecific hybrid may then be used to widen the genetic diversity of the existing *J. curcas* in Indonesia. Moreover, the availability of effective DNA based markers for interspecific hybrid identification may be beneficial for supporting future *J. curcas* breeding program using the interspecific hybrid progenies.

MATERIALS AND METHODS

Plant materials and morphological characterization

All plant materials used in this research were from Indonesian Sweetener and Fiber Crops Research Institute (ISFRI), Malang, Indonesia. Molecular analysis was done at the Plant Molecular Biology Laboratory (PMB Lab), Department of Agronomy and Horticulture, Faculty of Agriculture, Bogor Agricultural University, Bogor, Indonesia. The only surviving putative interspecific hybrids progenies (eight plants) between *J. curcas* as the female parent and red-flowered *J. integerrima* as the male counterpart (Asbani 2008) were used.

Observation of the morphological characters was performed to the parents and their interspecific hybrids to determine the inheritance of the characters from the parents to the progenies. The evaluated characters include the leaf shapes and colors, flowers, fruits, mature seeds, and the branching shape. Variations of morphological characters are photographed and documented to provide detail information.

Primers for molecular characterization

The putative interspecific hybrid progenies and their respective parents were used for identifying informative SSR, RAPD, and ISSR markers. The DNA samples were isolated from young leaves of the evaluated progenies and their respective parents. DNA samples were used as templates for PCR amplification using RAPD (5 primers), ISSR (3 primers) (Table 1) and SSR (9 loci) markers (Table 2). We have previously evaluated the effectiveness of these DNA markers for *J. curcas* (Saptadi et al. 2011).

DNA extraction

The DNA was extracted from all of the *Jatropha* leaf samples using the standard CTAB protocols (Doyle 1990). A total of 0.1 g fresh *Jatropha* leaf was homogenized in 500 µL of extraction buffer (CTAB 2%, 100 mM Tris HCl pH 8, 3.5 M NaCl) and 1% polyvinylpolypyrrolidone (PVP). Leaf extract was then transferred into a 2.000 µL microtube, 1.5% β-mercaptoethanol was added, and after thoroughly mixing, the mixture was incubated at 65 °C for 90 minutes. Chloroform: isoamyl alcohol (24: 1) of the equal volume was added to the heated mixtures, and the mixture was shaken gently for 10 min. The mixture was centrifuged at 8,000 rpm for 8 min at room temperature, and the upper liquid phase was transferred to a new tube. After adding an equal volume of 2 M NaCl and a 0.6 time of isopropanol to the final volume, the mixtures were incubated at room temperature for 60 minutes. Subsequently, 80% cold ethanol was added to the mixture for as much as twice of the final volume. Subsequently, the final mixtures were incubated for 10 minutes and centrifuged at 10,000 rpm at room temperature for 15 min. The DNA pellet was washed with 70% ethanol, dried and dissolved in 200 µL TE buffer. Quantification of the DNA was done using a spectrophotometer and validated by running the DNA on an agarose gel electrophoresis.

Table 1. List of primers used to generate RAPD and ISSR markers in the studies

Primers	Sequences	Primers	Sequences
RAPD		**ISSR**	
OPC 10	TGTCTGGGTG	UBC 810	GAG AGA GAG AGA GAG AT
OPG 17	ACGACCGACA	UBC 812	GAG AGA GAG AGA GAG AA
OPG 18	GGCTCATGTG	UBC 834	AGA GAG AGA GAG AGA GYT
OPQ 11	TCTCCGCAAC		
OPV 17	ACCGGCTTGT		

Table 2. List of primer sets used to generate simple sequence repeat (SSR) markers in the studies

Accessions		Primer sequences	PCR product (bp)	Ta (ºC)	Repeat pattern
EU586348	F	GGGCTGGGATTTTGTCTCTT	246	55	(GT)12(AG) 23
	R	GGCATGACCCTTGTGACTCT			
EU586343	F	CATGAAGTTTGCTGGCAATG	129	54	GT(4)..(GA)5
	R	AAAGGTCATCTGGTAAAGCCATA			
EF612741	F	GGCATTTCCTTGCATTTTCA	489	55	(TAA)10..(A)8
	R	CTGAGCAAACGGGGAAGTAA			
EF612739	F	GGCATTTCCTTGCATTTTCA	620	54	(TAA)10..(A)8
	R	GAAGGGCAGAGGCTTCACTA			
EU099518	F	CTCATGAACAACAAGAATTT	137	55	(TA)3(TG)18.. (TA)6
	R	CAGATTCTAATGAAGGTACG			
EU099522	F	CAAATAGATTCCTCAATCC	122	44	(TC)16
	R	GGGACCCAAAGAAACAAT			
EU099524	F	ATTCATGTACCAGTCAAGTC	109	44	(C)6..(C)5(AC)5
	R	TGCTAAAACTCTGGTTCTCT			
AF469003	F	CATCTTATGAAACTGTCGTT	145	45	(TAA)8
	R	TACTTACAAAGAAAGCGAGA			
EU586349	F	CAAAATAAGTCGAAACAAAC	143	44	(A)6..(A)8..(CA)4
	R	TATAGGCTCTTGCATAAATC			

Note: F = forward primer, R = reverse primer, Ta = annealing temperature

Amplification and separation of amplicon

PCR amplification for the target sequences was performed in a total volume of 25 µL containing 0.2 µM of the evaluated primers, 1.25 U Taq polymerase (Real Biotech Corporation), 1x PCR buffer, 0.1 µl of 10 mM dNTP mix, and 1 µl of DNA template. The cycle used to amplify the SSR markers include one cycle of denaturation at 95°C for 5 mins; 36 cycles of denaturation at 94° C for 30 s, annealing at the suitable temperatures for each primer for 30 s, elongation at 72°C for 1 min. One cycle of primer extension at 72°C for 5 mins was added at the end of the PCR steps. The steps for RAPD markers amplification include one denaturation cycle at 94°C for 3 mins followed by 45 cycles, each at 94°C for 45 s, 36 °C for 30 s and 72°C for 2 min. A final primer extension at 72°C for 7 mins was added at the end of PCR cycles. The DNA amplification conditions for ISSR is as follow: one denaturation cycle at 94°C for 4 min followed by 35 cycles, each at 92°C for 30 s, Ta of each primer for 1 min, 72°C for 2 mins and a final extension at 72°C for 7 mins.

The PCR amplified DNA of either the RAPD or the ISSR markers were fractionated by agarose gel electrophoresis (1%), visualized by ethidium bromide staining, and observed under UV transluminescent. One kb DNA ladder was used as the size markers of the PCR amplicon. SSR markers were fractionated in a 6% polyacrylamide gel electrophoresis (PAGE) using standard procedures (Saptadi et al. 2011). PAGE separation was performed in a Dedicated Height Sequencer (Cole-Palmer) using 1X TBE buffer at a constant voltage of 1100 V for 3 hours. The 100 bp DNA ladders were used to estimate the size of the SSR amplicon, and the PAGE results were visualized using silver staining.

Data analysis

The SSR, RAPD and ISSR markers were scored based on the present or absent of the amplicons for the evaluated individual using the electropherograms. For each of the amplicon sizes in the SSR, RAPD and ISSR analysis, score "1" was given if the DNA band present and score "0" if the DNA band absent. A scoring table was created to facilitate the comparison of DNA banding patterns between parent and interspecific hybrids; binary data was created from the marker scores and used to calculate the genetic distances among individuals. We calculated dissimilarity matrix based on the score data for the combined SSR, RAPD, and ISSR markers and used simple matching dissimilarity index to estimate genetic distance. The iterations for bootstrap analysis were set at 10,000 and the weighted Neighbour Joining approach was used to do the tree construction. Dissimilarity Analysis and Representation for WINDOWS (DARwin) software version 6.05 (Perrier and

Jacquemoud-Collet 2006) was used to calculate the dissimilarity matric, bootstrap, and tree construction for the evaluated *Jatropha* accessions.

RESULTS AND DISCUSSION

Morphological characterizations

All individuals of the putative interspecific hybrids grow vigorously, able to produce flowers and show intermediate morphological characteristics between the two *Jatropha* parents. Some of the putative interspecific hybrids follow the male (*J. integerrima*) parental characteristics for the branch, stem, inflorescence and leaf pigmentation, petiole and flower stalk characters. The seed formation in the putative interspecific hybrids also similar to the flowering and fruiting characters of the male parent. The stem in the putative interspecific hybrids resembles the male parent which was sturdier than the female *J. curcas*. Leaf shapes resemble the female parent, but leaf sizes were intermediate between the two parents. The fruit shape and size were also midway between those of the two parents (Table 3; Figure 1).

Characterization using RAPD and ISSR markers

Five RAPD and three ISSR primers were used to generate either RAPD or ISSR markers (Table 1, Table 4). Using total DNA isolated from P_1 and P_2 genomic DNA, the random primers OPQ 11, OPC 10, OPG 17 and OPG18 generated either one or two RAPD marker loci (Table 4). On the other hand, random primer OPV17 only generated one RAPD marker for P_1 parent and absence for P_2 (Table 4). Although capable of amplifying two RAPD marker bands (2 loci) from P_1 and a single band (1 locus) from P_2 (Table 4), the putative interspecific hybrids only have marker bands from the P_1 (OPG 17-1 and OPG 17-3 loci) and absence for the P_2 origin (OPG 17-2 locus), indicating the genotype of the P_2 for OPG 17-2 locus was probably (+/-). The OPG 18 primer was capable of amplifying one RAPD marker band (1 locus, OPG 18-3) from P_1 and two bands (2 loci, OPG 18-2 and OPG 18-3) from P_2 (Table 4). However, all the progenies yielded RAPD marker band (1 locus, RAPD 18-1) which was absent in the P_1 and P_2 parents. Hence, OPG 17 and OPG 18 primers were not usable for detecting the putative interspecific hybrids of *J. curcas* x *J. integerrima*. Meanwhile, OPQ 11 primer generated two DNA marker bands in either P_1 (OPQ 11-2 and OPQ 11-4) or the P_2 parents (OPQ 11-1 and OPQ 11-3). Moreover, the OPC 10 primer generated a single RAPD marker for P_1 (OPC 10-1) and two RAPD markers for P_2 (OPC 10-2 and OPC 10-3). The putative interspecific hybrids of *J. curcas* x *J. integerrima* also carry allele combinations for the loci derived from both parents (Table 4). Therefore, only two out of five RAPD primers evaluated were useful for detecting the putative interspecific hybrid progenies.

Two of the evaluated ISSR primers (UBC 810 and UBC 834) yielded monomorphic markers in all the tested individuals, and one ISSR primer (UBC 812) did not produce amplified DNA (Table 4). Therefore, the evaluated ISSR primers in this study were useless for finding the *Jatropha* interspecific hybrids.

Characterization using SSR markers

PCR amplification using eight pairs of the evaluated SSR primers yielded amplicons in the putative interspecific hybrids and their parents while one primer pairs (EU099524) resulted in no amplicon for the male parent DNA template. The detected number of loci were 1 to 3 loci for the parents and 1 to 5 loci for the putative interspecific hybrid populations. Most of the alleles associated with the putative interspecific hybrids were originated from either the parents, except for two alleles. The irregular allele was generated by the SSR primer of AF469003. These irregular SSR alleles were found in the putative interspecific hybrid (IH) #6. Most of the generated SSR were either polymorphic against the two parents or among parents and their interspecific hybrids.

Table 3. Evaluation results of various morphological characters among the *Jatropha curcas* (P_1), *J. integerrima* (P_2) and eight putative interspecific hybrid progenies (F_1)

Characters	P_1 (*J. curcas*)	Interspecific hybrids	P_2 (*J. integerrima*)
Leaf	Spiral, cordate, palmately with 5 deep lobes, dark green	Spiral, cordate with 5 shallow lobes, dark green,	Spiral, obovate with 3 shallow lobes, dark green
Petiole	Long, non-pigmented	Medium, light purple pigment	Short, strong purple pigment
Stem	Stout, thick bark, frangible	Slim, thin bark, strong, woodier	Slim, thin bark, strong, woodier
Branching	Many, pointing up	Many leads to side	Many leads to side
Flower	Small size, greenish-yellow petals rolled on end, non-pigmented stalk, light yellow pollen	Medium size, pink petals with light roll on end, light purple pigmented stalk and non-pigmented, yellow pollen	Big size, flat dark pink petals, purple stalk, yellow pollen
Inflorescence	Cymose, unisexual, monoecious, short non-pigmented stalk	Cymose, unisexual, monoecious, long light pigmented stalk	Cymose, unisexual, monoecious, long dark pigmented stalk
Fruit	Green, big, drupaceous	Green, medium, some pigmented, shallow lobes	Green with purple pigment, small with deep lobes
Seed	Big, black	Medium, black to brownish-black	Small, brown with a black dot

The EU099518 SSR primer pairs amplified the same allele (allele 2) for the P_1, the P_2, and in the seven out of eight putative F_1 interspecific hybrids (Table 5). Meanwhile, the EF612741 and EF612739 SSR primer pairs amplified allele 2 in the P_1 and allele 1 in the P_2 parents. However, the putative F_1 interspecific hybrids mostly carry the allele 1 from P_2 parent. Moreover, the EU099524 SSR primer pairs amplified allele 1 and 2 in the P_1 and no amplicon in the P_2. PCR using the EU099524 SSR primer pairs from all the putative F_1 interspecific hybrids amplified the same allele 1 and 2 as in the P_1 parent (Table 5).

The EU586343 and the EU586349 SSR primer pairs amplified allele 1 and 2 (EU586343) and allele 1,2, and 3 (EU586349) in the P_1 (Table 5). On the other hand, the EU586343 and EU586349 SSR primer pairs amplified alleles 2 and 3 respectively in the P_2. The putative F_1 interspecific hybrids mostly carry the allele 1 and 2 (EU586343) and allele 1,2, and 3 (EU586349). Therefore, the generated SSR alleles of the EU586343 and the EU586349 primer pairs in the P_1 and P_2 parents segregated in the F_1 interspecific hybrid progenies (Table 5).

The following SSR primer pairs (Table 5) amplified allele 2 in the P_1 and allele 1 in the P_2 parents (AF469003), allele 2,3,5 in the P_1 and allele 1,4 in the P_2 (EU099522) and allele 1,3 in the P_1 and allele 2,4,5 in the P_2 (EU586348). Moreover, the generated SSR alleles of the AF469003, the EU099522 and the EU586348 SSR primer pairs in the P_1 and P_2 parents were also segregated in the F_1 interspecific hybrid progenies (Table 5). Representative samples of the polymorphic SSR markers are presented in Figure 2.

Genetic diversity of the evaluated *Jatropha*

The value of genetic dissimilarity between the P_1 (*J. curcas*) and P_2 (*J. integerrima*) based on all molecular markers was 66% (Table 6). The highest genetic dissimilarity score (45%) resulted between P_1 and IH#1, and the lowest genetic similarity (7%) resulted between IH#3 and IH#5 (Table 6). The mean values of genetic dissimilarity among IH, among IH and P_1, and among IH and P_2 were 18%, 62%, and 78%, respectively.

Table 4. Scoring of the molecular markers generated by five RAPD and three ISSR primers for the parental (P_1 and P_2) and the putative interspecific hybrids (8 individuals) of *Jatropha curcas* x *J. integerrima.*

Primer pairs	Parents:		Putative interspecific hybrids (IH #):							
	P_1	**P_2**	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**
RAPD markers										
OPQ 11	2,4	1,3	2,4	1,4	1	1	1,3	1,3	1,3	1,3
OPC 10	1	2,3	1,2,3	-	1,2,3	-	-	1,2,3	1,2,3	1,2,3
OPG 17	1,3	2	1,3	1,3	1,3	1,3	1,3	1,3	1,3	1,3
OPG 18	3	2,3	-	1,3	-	1,2,3	1,3	1,2,3	1,2,3	1,3
OPV 17	1	-	-	-	-	-	1	-	-	-
ISSR markers										
UBC 810	1,2	1,2	1,2	1,2	1,2	1,2	1,2	1,2	1,,2	1,2
UBC 834	1	1	1	1	1	1	1	1	1	1
UBC 812	-	-	-	-	-	-	-	-	-	-

Note: Parental line P_1 = *J. curcas*; P_2 = *J. integerrima* and 1-8 = the putative interspecific hybrid progenies. -- = No DNA band was detected. *Numbers showed the identity of the yielded PCR amplified products using the respective primers and the individual DNA template.

Table 5. Scoring of the molecular markers generated by nine SSR primer pairs for the parental (P_1 and P_2) and the putative interspecific hybrids (8 individuals) of *Jatropha curcas* x *J. integerrima*.

SSR primer pairs	Individuals									
	P_1	**P_2**	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**
EU586348	1,3*	2,4,5	1,2,3,4	1,3,5	1,3,5	2,4	3,5	1,3,5	3,5	3,5
EU586343	1,2	2	1,2	1,2	1,2	1,2	1,2	1,2	1,2	1,2
EF612741	2	1	1	1	1	1	1	1	1	1
EF612739	2	1	2	1	1	1	1	1	1	2
EU099518	2	2	2	2	2	2	2	1	2	2
EU099522	2,3,5	1,4	1,2,3,4,5	1,2,3,4,5	1,2,3,4,5	1,2,3,4,5	1,2,3,4,5	1,2,3,4,5	1,2,3,4,5	1,2,3,4,5
EU099524	1,2	--	1,2	1,2	1,2	1,2	1,2	1,2	1,2	1,2
AF469003	2	1	1,2	1,2	2	1,2	1,2	3	1,2	2
EU586349	1,2,3	3	1,2,3	1,2,3	1,2,3	1,2,3	1,2,3	1,2,3	--	3

Note: Parental line P_1 = *J. curcas*; P_2 = *J. integerrima* and 1-8 = the putative interspecific hybrid progenies. -- = No DNA band was detected. *Numbers indicated the identity of the PCR amplified products yielded by PCR using the respective primers and the individual DNA template.

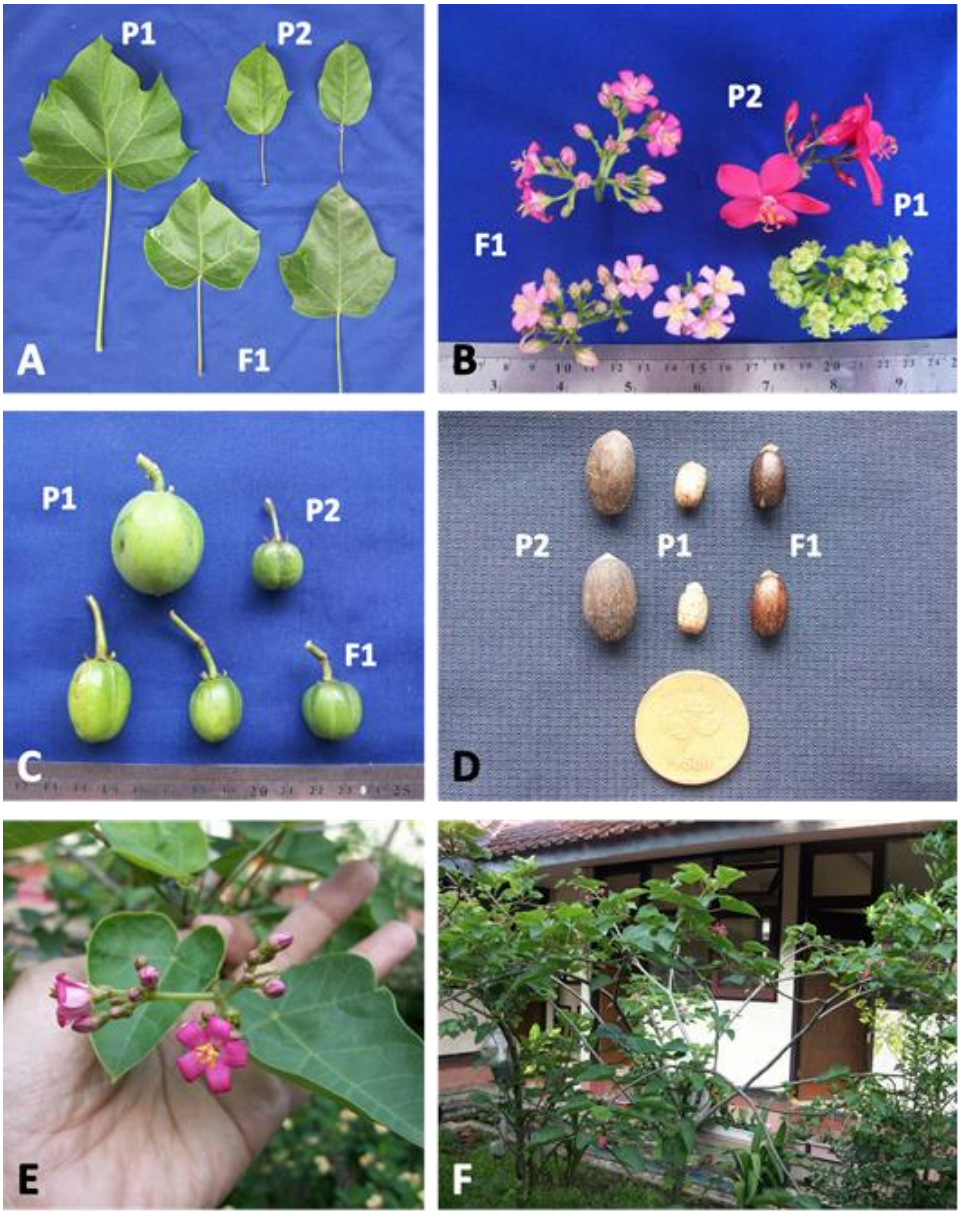

Figure 1. Result of morphological observation: (A) leaves, (B) flowers, (C) fruits, and (D) seeds of *Jatropha curcas* (P1), *J. integerrima* (P2), and their interspecific hybrids. (E) F1 inflorescence pigmentation, and (F) F1 branching

Table 6. The coefficient of genetic dissimilarity among *Jatropha curcas*, *J. integerrima*, and eight of their interspecific hybrid (IH) progenies. The dissimilarity coefficient was determined based on the result of genotyping using 9 SSR, 5 RAPD, and 3 ISSR marker loci. Parental line P_1 = *J. curcas*; P_2 = *J. integerrima* and IH#1-IH#8 = the interspecific hybrid progenies.

	P_1	**P_2**	**IH#1**	**IH#2**	**IH#3**	**IH#4**	**IH#5**	**IH#6**	**IH#7**	**IH#8**
P_1	-									
P_2	0.66	-								
IH1	0.45	0.18	-							
IH2	0.39	0.25	0.21	-						
IH3	0.38	0.28	0.15	0.13	-					
IH4	0.39	0.26	0.21	0.11	0.2	-				
IH5	0.39	0.26	0.28	0.07	0.16	0.11	-			
IH6	0.38	0.37	0.28	0.19	0,14	0.22	0.19	-		
IH7	0.31	0.37	0.24	0.21	0.2	0.14	0.18	0.19	-	
IH8	0.38	0.28	0.19	0.24	0.19	0.2	0.2	0.21	0.09	-

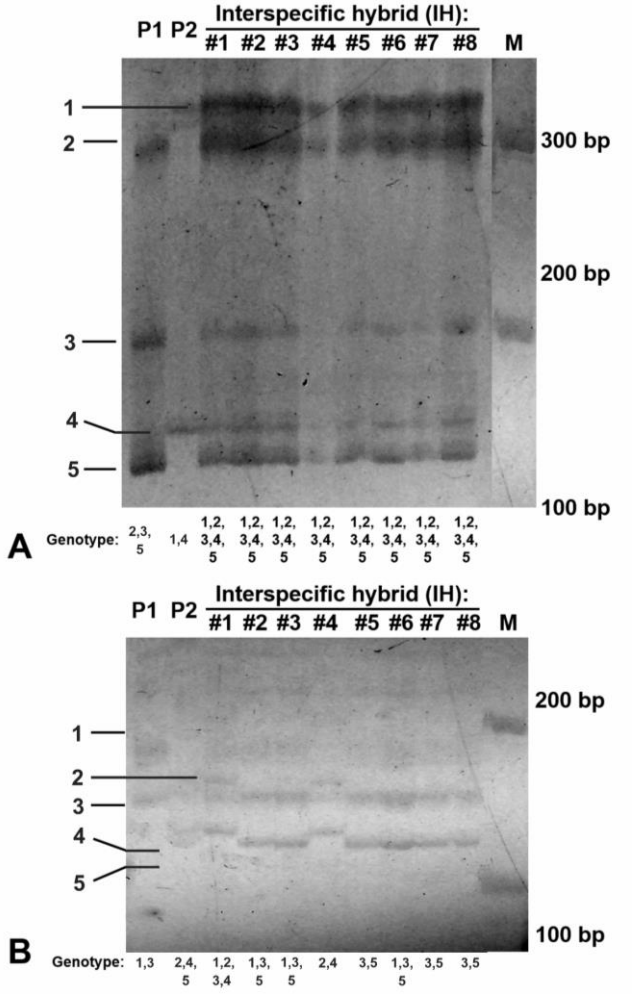

Figure 2. Electropherogram of DNA amplification result of eight F_1 individuals (#1-#8) as results of crossing between *Jatropha curcas* (P_1) and *J. integerrima* (P_2) by using 2 SSR markers EU099522 (A) and EU586348 (B). M = 100 bp DNA markers.

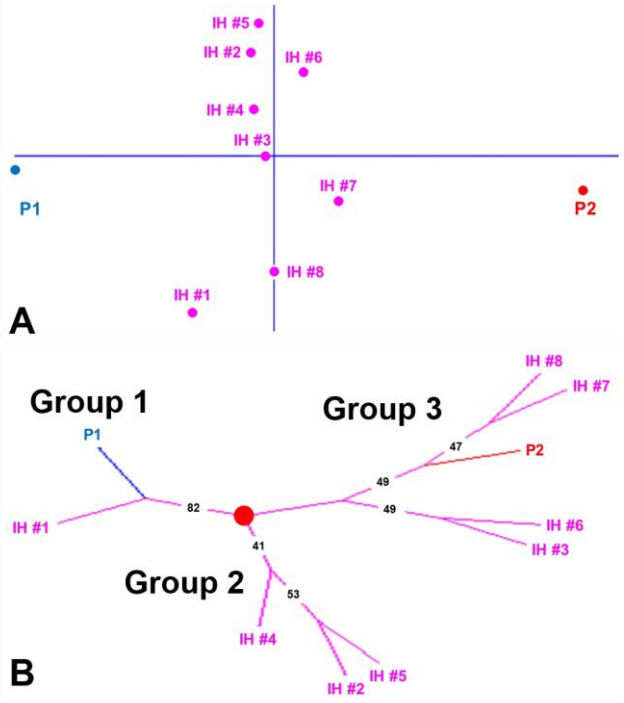

Figure 3. Results of the factorial and cluster analysis of P_1 (*Jatropha curcas*), P_2 (*J. integerrima*), and eight F_1 interspecific hybrids (IH #1 - IH#8) between P_1 and P_2 constructed using the dissimilarity coefficient. The dissimilarity coefficient was estimated based on the genotyping results of the *Jatropha* using RAPD, ISSR and SSR markers.

Factorial and cluster analysis results among *J. curcas* (P_1), *J. integerrima* (P_2), and their interspecific hybrid (IH) progenies constructed based on the dissimilarity coefficient values were presented in Figure 3.A and 3.B. Results of the factorial analysis showed that *J. curcas* (P_1) and *J. integerrima* (P_2) were indeed genetically distance-related (Figure 3.A). Moreover, the interspecific hybrid (IH) progenies were genetically in between the *J. curcas* (P_1) and *J. integerrima* (P_2), and there was less genetic diversity among the interspecific hybrids (Figure 3.A).

Figure 3.B. presented the result of cluster analysis among the evaluated *Jatropha*. Results of the cluster analysis showed that the *Jatropha* accessions were divided into three groups. The first group only has two members (P_1 and IH#1), the second group consisted of IH#2, IH #4, and IH#5, and the third group consisted of P_2 and the rest of the interspecific hybrid (IH) progenies (Figure 3.B).

Discussion

Although many scientists are able to generate interspecific hybrid progenies between *J. curcas* and *J. integerrima* and yielded hybrid progenies, the genetic materials were difficult to access by Indonesian scientists interested in working with the interspecific hybrids. Therefore, developing the interspecific hybrids locally in Indonesia is necessary. Sujatha and Prabakaran (2003) have successfully obtained ca. 9.3% seed formation out of the total interspecific hybridization between *J. curcas* and *J. integerrima*. Meanwhile, Dhillon et al. (2009) reported a 7.3% success rate for the same scheme of interspecific hybridizations. Hence, Asbani, (2008) has initiated the interspecific hybrid progenies between *J. curcas* and *J. integerrima* using locally available *Jatropha* sp. genetic materials from Indonesia.

As in previously reported interspecific *Jatropha* hybridizations (Sujatha and Prabakaran 2003; Dhillon et al. 2009), some degrees of sexual incompatibilities also exist in the interspecific hybridizations between *J. curcas* and *J. integerrima* from Indonesia (Asbani 2008). Fortunately, a limited number of fertile F_1 interspecific hybrids between the two *Jatropha* sp. were identified and grown to maturity. Those fertile interspecific progenies were kept as *Jatropha* collections at Indonesian Sweetener and Fiber Crops Research Institute (ISFRI), Malang, Indonesia.

Although the remaining accessions of interspecific hybrids maintained at ISFRI showed many intermediate phenotypes between the two *Jatropha* parents, genetic analysis has not been done among the interspecific hybrids. Therefore, some of the putative hybrids could have been served progenies of the *J. curcas.* Therefore, before the putative interspecific hybrids be used for further breeding activities to improve Indonesian *J. curcas*, molecular characterizations are needed to confirm the identity of the putative interspecific hybrids.

Polymorphic markers (RAPD, ISSR, and SSR) for Indonesian *J. curcas* accessions have previously been identified (Saptadi et al. 2011). Therefore, these markers may potentially be used to validate the identity of putative interspecific hybrids. The RAPD and ISSR markers are dominant and multi-loci markers. On the other hand, the SSR marker is a co-dominant one. Therefore, the mode of inheritance for the RAPD, ISSR and SSR markers among putative interspecific F_1 hybrids are different. In the RAPD and ISSR markers, a single primer was used to amplify various loci in the genome. For each target locus, there would be either (+) or (-) DNA band as the allele. Therefore, the genotype of a certain locus in a diploid individual can either be homozygous (+/+) or (-/-) or a heterozygous (+/-) and the RAPD marker scores for both the (+/+) or (+/-) genotypes were (+) while for the (-/-) genotype was (-).

Even though in previous report (Saptadi et al. 2011), the three ISSR primers were capable of generating polymorphic markers among *J. curcas* from Indonesia, they were not informative for identifying putative interspecific hybrids between *J. curcas* and *J integerrima*. The first ISSR primer (UBC 812) did not produce any PCR amplicon in all tested *Jatropha*. The second ISSR primer (UBC 834) yielded one amplicon (1) from P_1 (*J. curcas*), P_2 (*J. integerrima*), and from the eight putative interspecific hybrids. The third ISSR primer (UBC 810) yielded two amplicons (1,2) from P_1 (*J. curcas*), P_2 (*J. integerrima*), and from the eight putative interspecific hybrids. Therefore, the three ISSR primers cannot be used to confirm the identity of the putative interspecific hybrids.

Out of five RAPD primers previously reported capable of yielding polymorphic markers (OPQ 11, OPC 10, OPG 17, OPG 18 and OPV 17) (Saptadi et al. 2011), only OPC 10 was effective for confirming identity of the eight putative interspecific hybrids. The OPC 10 primers were (+) for amplicon 1 from P_1 (*J. curcas*) and (+) for both amplicon 2 and 3 from P_2 (*J. integerrima*) parents. The amplicons from putative interspecific hybrids were either (+) for amplicon 1,2,3 (IH #1, #3, #6, #7 and #8) or (-) for all amplicons (IH #2, #4, and #5). Based on the banding pattern of the interspecific hybrid progenies, the genotype of P_1 parent was probably (+/-) for amplicon 1, while the genotype of P2 parent was probably (+/-) for both amplicon 2 and 3. Therefore, the expected RAPD banding patterns of the interspecific hybrids would either be (+) for amplicon 1,2,3; for amplicon 1,2; 1,3; 2,3 or (-) for all amplicons (no amplicon). As expected, two out of the five banding patterns which are (+) for amplicon 1,2,3 and (-) for all amplicon (no amplicon) were present in the eight progenies.

The number of identified loci by RAPD marker in this study was limited. However, we can use RAPD marker generated using OPC 10 to identify the interspecific hybrid progenies between *J. curcas* and *J. integerrima*. RAPD marker was a dominant marker (Williams et al. 1990) and the primer can be used to identify hybrid profiles by evaluating the co-inherited markers. Using RAPD markers, screening for putative hybrid genotypes can be faster and more cost-effective than evaluation using morphological characters. In some cases, there are some RAPD marker bands that appeared in the F_1 progenies but they were not found in their parents. Chromosome recombinations during meiosis could also lead to the loss of the RAPD priming sites, resulted in such anomaly in the RAPD markers among parents and their disappearance in the F_1 progenies and vice versa (Tyagi et al. 1992).

The primers to generate SSR markers used in this study were developed using *J. curcas* genome, and they were validated and informative against Indonesian accessions of *J. curcas* (Saptadi et al. 2011). The SSR markers were also tested for their cross-species amplification potential to other *Jatropha* species. The results those studies confirmed that the evaluated SSR markers were also informative for *Jatropha* sp. (Saptadi et al. 2011; Sudheer et al. 2011).

To generate SSR marker, a pair of primers were used to amplify a target locus in the genome. For each target locus, there could be many sizes of amplified DNA bands as the alleles from different individuals. The genotype of a diploid individual can either be homozygous (1/1) or (2/2) or a heterozygous (1/2) and the SSR marker scores for the 1/1 genotype was 1, for the 2/2 genotype was 2, for the 1/2 genotype was 1,2.

Out of nine SSR primer pairs used, three were effective for confirming identity of the eight putative interspecific hybrids (EU586348, EU099522, and AF469003). The EU586348 primers generated amplicon 1,3 from P_1 (*J. curcas*) and amplicon 2,4,5 from P_2 (*J. integerrima*) parents. The amplicons from putative interspecific hybrids were either 1,2,3,4,5 (IH #1), 1,3,5 (IH #2, #3, #6), 2,3,4 (#4), or 3,5 (#5, #7, #8). The EU099522 primers generated amplicon 2,3,5 from P_1 (*J. curcas*) and amplicon 1,4 from P_2 (*J. integerrima*) parents. The amplicons from putative interspecific hybrids were all 1,2,3,4,5 which are the combine alleles of the P_1 and P_2 parents. The AF469003 primers generated amplicon 2 form P_1 (*J. curcas*) and amplicon 1 from P_2 (*J. integerrima*) parents. The amplicons from putative interspecific hybrids were either 1,2 (IH #1, #2, #4, #5 and #7) which are the combine alleles of the P_1 and P_2 parents; 2 (IH #3, #8) or 3 (IH #6). Hence, out of nine SSR loci previously reported capable of yielding polymorphic markers (Saptadi et al. 2011), only three primer pairs were effective for confirming identity of the eight putative interspecific hybrids.

Among the 9 SSR primers used, the primers EU099522 and EU586348 were most informative primers because they produce most amplification bands, polymorphic for the parent and co-inherited in F1 individuals. The generated alleles from those primers segregated following

normal Mendelian genetics in the F_1 generation. If the alleles for the SSR marker are polymorphic for the parent genome and they were co-inherited in the interspecific F_1 hybrid progenies, then the progenies can be used for genetic inheritance analysis (Kang et al. 2011). The SSR marker amplified using EU586343 was not informative because the allele was monomorphic for *J. curcas* and *J. integerrima*.

The genetic distance between *J. curcas* and *J. integerrima* was closer than that of other *Jatropha* species. Finding in this research confirmed the previous report about the existence of a correlation between the rate of success in interspecific hybridization and genetic distances among the parents. The previous report indicated that the hybridization success among plant species of the same genus depended on their genetic distance (Sudheer et al. 2009; Kumar Yadav et al. 2011). The previous report also proposed the success of interspecific hybrid depends on the use of *J. curcas* as the female parents (Sujatha and Prabakaran 2003; Dhillon et al. 2009).

Genetic diversity between F_1 ranged from 7 to 28% with an average of 18% indicated the low variation among crossbreeds. The hybrid plants grouped closer to the *J. curcas* parent (P_1) are individual #1, #2, #3, #4, #5 and #8, while hybrid individual #6 is closer to *J. integerrima* parent (P_2) as presented in Figure 3.B. Those groupings were also supported with results of the SSR marker analysis. The SSR markers used in this study were developed using *J. curcas* genome. However, our results were in contrast to those of Dhillon et al. (2009), who reported *J. curcas* was more distantly related to the interspecific progenies. Our further evaluation indicated the grouping based on the molecular marker analysis was not complementary to those based on morphological characters.

The RAPD, ISSR, and SSR marker evaluation in this experiment aimed at obtaining effective molecular markers for the interspecific hybrid identification among interspecific progenies of *J. curcas* × *J. integerrima*. Validation of the progeny identity using the selected molecular markers could be done in a more accurate way and a shorter period compare to the use of phenotypes. Moreover, screening for the interspecific hybrid progeny could also be done at the seedling level.

Several desirable characters are found among the interspecific hybrid progenies between *J. curcas* and *J. integerrima* parents. The desirable characters include all year flowering, resistance to major insects (thrips and mites) probably because of differences in trichome densities, leaf thickness, and leaf antixenosis or antibiosis compounds (Asbani 2008). Moreover, the hybrid progenies of *J. curcas* × *J. integerrima* evaluated in this research showed vigorous stature and capable of producing normal flower (fertile). Phenotypic characters of the interspecific hybrids were intermediate between those of *J. curcas* and *J. integerrima* parents. However, for the stem characters, most of the interspecific hybrid progenies exhibited the characters of *J. integerrima*. Such observation validated the identity of progenies as interspecific hybrids. The intermediate character in the F_1 individuals further confirmed that they were the results of hybridization between *J. curcas* and *J. integerrima*.

This study was the first using SSR markers for the identification of interspecific F_1 hybrids between *J. curcas* and *J. integerrima* from Indonesia. The selected markers found in this study can be used to support *Jatropha* breeding program in Indonesia. However, more evaluations are necessary for studying linkage among the markers and the desired traits or phenotypes. Moreover, we have proven that interspecific hybrids between *J. curcas* x *J. integerrima* accessions from Indonesia can be regenerated and fertile interspecific hybrids found. Therefore, generating F_2 progenies and subsequent advance generation from the interspecific F_1 hybrids should be possible. Our preliminary observation among the open-pollinated F_2 progenies derived from the hybrids showed the flowers and leaf morphological characteristics of *J. curcas* and the partial pigmentation character of *J. integerrima*. Subsequently, the confirmed interspecific F_1 hybrids may also be used to introgress genes controlling desired characters from *J. integerrima* into the *J. curcas* genetic background through backcross breeding. The introgression of such genes into *J. curcas* genomes will widen their genetic diversity.

Based on the overall evaluation results, the eight F1 progenies derived from *J. curcas* × *J. integerrima* hybridizations were most probably the interspecific F1 hybrids between the two *Jatropha* parents. The confirmed identity of the eight F_1 progenies as interspecific hybrids between *J. curcas* × *J. integerrima* was based on the presence of a number of phenotypic characters from both parents in the F_1 progenies and by similarity of the molecular marker banding patterns among the parents and the F_1 progenies. Among the evaluated molecular markers, the ISSR primers and the majority of either RAPD and SSR primers were not able to generate marker for confirming the identity of F_1 progenies as interspecific hybrids between *J. curcas* × *J. integerrima*. However, the RAPD primer OPC 10 and the SSR primers AF469003, EU099522 and EU586348 were able to generate polymorphic markers in the *Jatropha* parents and their F1 progenies. Therefore, these four primers were able to generate usable markers for confirming the identity of F1 progenies as interspecific hybrids between *J. curcas* × *J. integerrima*. The evaluated interspecific F_1 progenies of *J. curcas* × *J. integerrima* hybridizations are potentially useful to increase genetic diversity of *J. curcas* and support its breeding program in Indonesia.

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