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Molecular characterization of *Jatropha curcas* germplasm using inter simple sequence repeat (ISSR) markers in Peninsular Malaysia

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Abstract

Molecular characterization and evaluation of germplasm was carried out using 10 Inter simple sequences repeat (ISSR) on 48 accessions of *Jatropha curcas* (L) collected from three states (Kelantan, Selangor and Terengganu) in Peninsular Malaysia. The stem cuttings of these *J. curcas* accessions were collected, raised in the nursery and then transferred to the experimental site at University Agricultural Park. The 48 *J. curcas* accessions were grouped into three different populations based on the state from where they were collected. Percentage polymorphism in these three populations ranged from 90.75% (Terengganu) to 100% (Kelantan). Analysis of molecular variance (AMOVA) showed that 94 % of the total variation was observed within the populations while variation among the populations accounted for the remaining 6%. A dendrogram produced by Unweighted Pair Group Method with Arithmetic Mean (UPGMA) based on Nei's genetic distance grouped the whole germplasm into 11 distinct clusters. Based on the information from this dendrogram, accessions that are far from each other by virtue of genetic origin and diversity index are strongly recommended to be use as parent for crossing. This will bring about greater genetic diversity, thus resulting into increase in selection gain. This will also lead to high productive index in terms of increase in fruit yield per hectare, oil yield, seed weight and other yield components. Therefore, accessions, B-01-03, T-01-09, B-04-01 and T-01-01 could be crossed with accessions D-04-02, B-05-05, B-01-04, and D-01-06 for the improvement of *J. curcas* in future breeding program.

Keywords: ISSR; *Jatropha curcas* L; Genetic diversity; Molecular breeding; AMOVA; Selection gain.
Abbreviations: ISSR - Inter simple sequences repeat; AMOVA- Analysis of molecular variance; PCA - Principal component analysis.

Introduction

Global increase in demand for renewable energy to combat the greenhouse effect and rapid depletion of ozone layer as a result of discharge of harmful gases into the environment, couple with the depletion of reserved fossil fuel has mandated the use of biomass energy feedstock for sustainable production of biofuel. Biofuel has been known to be a good alternative to fossil fuel due to its cheap, sustainable and environmental friendly properties (Biabani et al., 2012; Srivastava et al., 2011; Divakara et al., 2010). Feedstocks for this renewable energy are sourced from canes such as in sugarcane and fruits of different plant materials (Srivastava et al., 2011). *Jatropha curcas* L family has been ranked first among all other feed stocks. Other uses of this crop include greening of wasteland and control of desert encroachment. (Tanya et al., 2011; Reddy et al., 2008). *J.curcas*, a tree crop of euphorbiaceae family was believed to have originated from Central America with productive life of about 30-40 years. It is a multipurpose tree of ethnomedicinal and industrial importance (Rafii et al., 2012a). This plant has been given several names due to its importance in production of drugs, lubricants, colouring, dyes, abortifacients (Batar and Sardana 2000). It is widely found across wide vegetation due to its low agronomical input requirement and it ability to withstand environmental stress. *J. curcas* was initially used as a live fence around the farmland to prevent animal from browsing on the food crop because of its toxic nature. However, it can be cultivated successfully under wide range of rainfall regimes (200mm to more than 1500 mm per year) (Divakara et al., 2010). Jatropha as a biodiesel plant is unique due to its inherent attributes compare to other renewable green energy sources. It production requires little management, simple technology and comparative low capital investment coupled with its, ability to grow on marginal land, low gestation period, continuous fruiting throughout the year (Ranade et al., 2008). Despite the great prospect of this crop, its genetics and agronomical make up have not been fully understood and these are necessary for the improvement in the growth and yield of this crop. Efforts to cultivate *J. curcas* have been going on all over the world with India as a leading country in its cultivation, and domestication follow by China, Brazil and many others (Sorrell et al., 2010). However, the profit margin realize from this crop is still very small compare to the effort invested in its cultivation , this due to a number of reasons among which are its low fruits, less number of female compare to male, lack of improved or hybrid varieties and uneven ripening. All these are caused by low understanding of it optimum agronomical requirements and genetic makeup (Divakara et al., 2010). In-depth research in the field of genetics and breeding is essential to overcome the problems facing Jatropha cultivation. The success of any genetics and breeding program depends on the collection of planting material (germplasm) from different agro-vegetational

Table1. Information of 48 Jatropha accessions collected in Malaysia.

Code	I.C/ Number	Origin	Area	State	Latitude	Longitude
A1	B-01-01	Seri Serdang	Serdang	Selangor	3°0'1.24"	101° 43' 1.1994"
A2	B-01-02	Seri Serdang	Serdang	Selangor	3°0'38.88"	101° 42' 35.9994"
A3	B-01-03	Seri Serdang	Serdang	Selangor	3°0'38.16"	101° 42' 21.6"
A4	B-01-04	Taman Serdang Raya	Serdang	Selangor	3°0'38.52"	101° 42' 25.1994"
A5	B-01-05	UPM-Cemetery	Serdang	Selangor	2°59' 52.44"	101° 43' 4.8"
A6	B-01-06	UPM- Kolej 17	Serdang	Selangor	2°58'45.48"	101° 42' 39.5994"
A7	B-01-07	UPM- Kolej 17	Serdang	Selangor	2°58'45.479"	101° 42' 39.5994"
A8	B-01-08	UPM-Kolej 17	Serdang	Selangor	2°58'46.199"	101° 42' 43.2"
A9	B-02-01	Ladang Raja Musa	Kuala Selangor	Selangor	2°24'29.519"	101° 16' 55.1994"
A10	B-02-02	Bukit Belimbing	Kuala Selangor	Selangor	2°24' 29.88"	101° 16' 51.6"
A11	B-02-03	Sri Angala Aman	Kuala Selangor	Selangor	2°23'48.479"	101° 16' 30"
A12	B-02-04	Kota Hulu Moram	Kuala Selangor	Selangor	3°23'33.72"	101° 17' 27.5994"
A13	B-02-05	Taman Sri Blimbing	Kuala Selangor	Selangor	3°23'23.639"	101° 16' 19.2"
A14	B-02-06	Lorong Intan A	Kuala Selangor	Selangor	3°25'10.92"	101° 13' 15.6"
A15	B-03-01	Sungai Choh, Rawang	Hulu Selangor	Selangor	3°20' 45.6"	101° 35' 24"
A16	B-03-02	Batu 16, Kampong Melayu	Hulu Selangor	Selangor	3°18'15.839"	101° 35' 45.6"
A17	B-04-01	Kampong Sungai Buloh	Kuala Selangor	Selangor	3°14' 44.16"	101° 28' 22.7994"
A18	B-04-02	Jalan Rahidin	Kuala Selangor	Selangor	3°11' 6.3994"	101° 32' 56.4"
A19	B-05-01	Bangi Lama	Hulu Langat	Selangor	2°54' 5.04"	101° 46' 40.8"
A20	B-05-02	Bangi Lama	Hulu Langat	Selangor	2°54' 2.8794"	101° 46' 37.2"
A21	B-05-05	Pekan Beromang	Hulu Langat	Selangor	2°52'35.759"	101° 52' 22.8"
A22	B-05-06	Kampong Sungai Jai	Hulu Langat	Selangor	2°52' 15.96"	101° 52' 55.2"
A23	B-05-11	Near Hulu Langat river	Hulu Langat	Selangor	3°9'52.9194"	101° 50' 59.9994"
A24	B-06-01	Batu Laut, Banting	Kuala langat	Selangor	2° 40' 23.52"	101° 31' 19.2"
A25	B-06-02	Banting	Kuala langat	Selangor	2°40'22.439"	101° 31' 19.2"
A26	B-06-03	Taman Changang	Kuala langat	Selagor	2°49'45.479"	101° 37' 8.3994"
A27	D-01-01	PLT. Pasir Puteh	Pasir Puteh	Kelantan	5°49'38.639"	102° 22' 15.5994"
A28	D-01-02	PLT. Pasir Puteh	Pasir Puteh	Kelantan	5°49'38.999"	102° 22' 15.5994"
A29	D-01-03	PLT. Pasir Puteh	Pasir Puteh	Kelantan	5°49'38.639"	102° 22' 15.5994"
A30	D-01-04	PLT. Pasir Puteh	Pasir Puteh	Kelantan	5°49'38.639"	102° 22' 15.5994"
A31	D-01-05	PLT. Pasir Puteh	Pasir Puteh	Kelantan	5°49'38.28"	102° 22' 15.5994"
A32	D-01-06	PLT. Pasir Puteh	Pasir Puteh	Kelantan	5°49'37.92"	102° 22' 15.5994"
A33	D-01-07	Kampong Gong Tinggi	Pasir Puteh	Kelantan	5°48'12.96"	102° 28' 11.9994"
A34	D-01-08	Kampong Tebing Tinggi	Pasir Puteh	Kelantan	5°49'33.599"	102° 26' 16.8"
A35	D-01-09	Kampong Tok Bali	Pasir Puteh	Kelantan	5°54'28.8"	102° 27' 50.3994"
A36	D-01-10	Kampong Tok Bali	Pasir Puteh	Kelantan	5°53'56.04"	102° 28' 29.9994"
A37	D-02-01	Jabatan Pertanian, Kota Bharu	Kota Bharu	Kelantan	6°6'6.8394"	102° 16' 1.1994"
A38	D-02-02	Jabatan Pertanian, Kota Bharu	Kota Bharu	Kelantan	6°6' 6.8394"	102° 16' 1.1994"
A39	D-03-01	Jambu Tawar	Machang	Kelantan	5°42'48.599"	102° 12' 39.5994"
A40	T-01-01	Merang	Setiu	Terengganu	5°30'24.48"	102° 56' 16.8"
A41	T-01-03	Merang	Setiu	Terengganu	5°30'24.48"	102° 56' 9.6"
A42	T-01-04	Merang	Setiu	Terengganu	5°30'24.48"	102° 56' 6"
A43	T-01-05	Merang	Setiu	Terengganu	5°30'25.199"	102° 56' 9.6"
A44	T-01-06	Penarik	Setiu	Terengganu	5°28'14.519"	102° 48' 57.6"
A45	T-01-08	Merang	Setiu	Terengganu	5°32'13.199"	102° 57' 39.5994"
A46	T-01-09	Batu Rakit	Setiu	Terengganu	5°26'53.16"	103° 2' 59.9994"
A47	T-01-10	Batu Rakit	Setiu	Terengganu	5°26'35.879"	103° 3' 21.5994"
A48	T-01-11	Kampong Sungai Bari	Setiu	Terengganu	5°23'31.919"	102° 51' 46.7994"

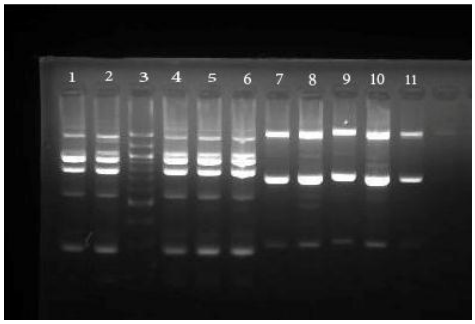

Fig 1. The polymorphic band of primer UBC990505 on the ethidium bromide Gel of *J. curcas* accessions.

zones and the presence of genetic diversity in the population. These will enable the selection and breeding of plant with desired traits (Surwenshi et al., 2011; Ranade et al., 2008). Morphological and yields traits have previously been used as a descriptors for genetic relationship however, they have failed to reveal the accurate and exact taxonomical relationship existing among populations due to the high influence of environmental factors. For effective and thorough exploitation of genetic diversity and similarity, molecular breeding as conventional breeding tools has gained ground and proofed to be efficient in doing this. (Kumar et al., 2009; Rafii et al., 2012b). Molecular breeding involves the use of molecular markers based on polymerase chain reaction of DNA finger printing. Various molecular markers such as AFLP (Mastan et al., 2012), RAPD (Gupta et al., 2010; Ikbal and Dhillon 2010), SSR (Sudheer Pamidimarri et al., 2009) and ISSR (Tanya et al., 2011; Senthil Kumar et al., 2009; Grativol et al., 2010) have been used in studying the diversity of *J. curcas* in various part of the world. Of all the markers, ISSR has been selected because of it rapid, simple, reproducible and high polymorphic means of assessing the diversity and identification of close related accessions. ISSR has been used extensively in many areas such as conservation, molecular genotyping and breeding of many tree crops such as casuarina, cassava, tea, mango in the plant. (Balasaravanan et al., 2005; Blair et al., 1999; Kumar Mondal, 2002; Xin-Hua et al., 2007) and food crop such as dock weed (Xue et al., 2012). Therefore, this present work examines the diversity of *J. curcas* accessions collected over the Peninsular Malaysia, representing the various agro-ecologies existing in the country. This is done as a part of breeding strategies for identification of superior accessions, which will be use in hybridization to aid the development of new varieties. These varieties will be capable of producing high number of fruits with high oil percentage, and this eventually lead to increase in profit margin of individuals, public and private companies.

Results

Banding patterns

From this research, a total of 10 ISSR primers were used to study the diversity among 48 accessions. The number of bands ranged from 10 to 21 for each of the DNA sample. Primer two showed the highest number of band while the least number of band was recorded for the primer six. Ten (10) primers yielded a total of about 156 scorable loci (Table 2).

Genetic diversity in Jatropha curcas population

The 48 accessions were divided into three populations (group) based on their geographical location. The percentage of polymorphic loci for each group ranged from 90.75% (Terengganu) to 100% (Kelantan) with average of 96.3% (Tables 3 and 4). Shannon's information index and Nei genetic index were estimated to be 0.556 and 0.015, respectively. Genetic variability among the groups as revealed by expected heterozygosity (He) showed that Kelantan population possessed greater level of variability with value of 0.403 as compare to Selangor and Terengganu populations with of 0.382 and 0.361, respectively. Variation within and among populations was partitioned by Analysis of Molecular Variance (AMOVA). Variation among the populations accounted for 6% while variation within populations was 94% (Table 5).

Cluster analysis

Genetic similarity was calculated using Jaccard's similarity coefficient and a UPGMA dendrogram was constructed. Eleven major clusters were formed at coefficient of 0.60 (Fig. 6 and Table 6) They were cluster I (B-01-03, D-03-01, B-03-02, D-02-01, B-02-01, D-01-02, D-01-04, B-05-06, D-01-07, B-01-06, T-01-01, B-02-06, B-04-01, B-06-03, B-05-02, T-01-09, B-06-01,B-02-03, T-01-05, B-01-07,T-01-10, T-01-04, B-01-08, D-01-03, D-01-08, D-01-05, B-05-01, D-02-02, B-02-04, B-01-02, D-01-01, B-02-05, B-01-05, T-01-08, B-02-02, T-01-03), cluster II (B-05-11), cluster III (D-01-09), Cluster IV (B-01-01), cluster V (T-01-06) and cluster VI (B-06-02), cluster VII (B-03-01), cluster VIII (D-01-10, B-04-02), cluster IX (B-05-05), cluster X (T-01-11, B-01-04) and cluster XI (D-01-06).

Principal component analysis

In principal component analysis, 48 accessions were also grouped into 11 groups as shown in two dimensional graph (Fig. 3). The first three principal components resulted into 75.45% of total variation and this accounted for more than ¾ of the total variation observed in the populations. The first three principal components (PC1, PC2, and PC3) are 61.69%, 8.68% and 5.07% respectively (Table 7). From PC 1, highest value was 0.93 followed by 0.92 and 0.91 while the least were 0.19, 0.22, and 0.26, respectively. The highest (0.93) was found in accession D-02-01, D-01-02, and B-05-06 while the least was found in accession B-05-05. In PCA 1, all the accessions contributed positively toward the diversity of one group than another, while in PC 2 and PC 3 had 19 and 22 members, respectively which contributed positively.

Discussion

Efficient and reliable use of molecular markers such as ISSR for study of genetic diversity in any food crop or tree crop requires selection and application of primers which will give clear, distinct, reliable and sufficient information required to study the divergence that occur within the crop. In this research the number of polymorphic loci detected per primer combination varies according to the primer. The number of polymorphic loci ranged from 8 to 21 with the average of about 14.7 for each of the primer (Table 2). Similar results were observed by several authors using ISSR (Tanya et al., 2011; Blair et al., 1999; Gupta et al., 2010). Also reported (Shafie et al., 2011), that more than 283 fragments were generated by ten ISSR primers when a study on genetic diversity of worm wood capillary (Artemisia capillaries) from Negeri Sembilan state of Malaysia was carried out. These findings have demonstrated the ability of ISSR primers to generate large amount of polymorphic loci. In addition to that Rosado et al. (2010) also observed distinct polymorphism in germplasm comprising of 192 accessions of *J. curcas* from all over the Brazilian's state, using 96 RAPD primers. Similar thing was observed in the diversity study of 26 Mexican, 3 Chinese, 3 Thai and 4 Vietnamese *J. curcas* using ISSR (Tanya et al., 2011). Furthermore, cluster analysis grouped the 48 accessions into 11 distinct clusters, with cluster I having 36 accessions while other clusters are having one accession each, except for cluster VIII and cluster XX with two accessions in each of them. It was observed that most of the accessions from Selangor and Kelantan populations are found to have majority in cluster I, this shows

Table 2. Polymorphic primers showing synthesis ID, length and annealing temperature, number of bands, percentage polymorphism.

No.	Synthesis ID	Sequence	Len	Tm (◦C)	No. of bands	No. of polymorphic marker	Polymorphism (%)
1	UBC990505	5'-AGA GAG AGA GAG AGA GT-3'	17	54.8	20	20	100.0
2	UBC990508	5'-GAG AGA GAG AGA GAG AGA T-3'	19	58	21	21	100.0
3	UBC990509	5'-AGA GAG AGA GAG AGA GC-3'	17	57.2	14	13	92.9
4	UBC990511	5'-GAG AGA GAG AGA GAG AT-3'	17	54.8	21	19	90.5
5	UBC990512	5'-GAG AGA GAG AGA GAG AC-3'	17	57.2	16	16	100.0
6	UBC990513	5'-DBD ACA CAC ACA CAC AC-3'	17	55.6	10	8	80.0
7	UBC990514	5'-HVH GTG TGT GTG TGT GT-3'	17	55.6	10	9	90.0
8	UBC990517	5'-ACA CAC ACA CAC ACA CYA-3'	18	56.5	15	13	86.7
9	UBC990518	5'-GAC AGA CAG ACA GAC A-3'	16	54.2	17	16	94.1
10	UBC990519	5'-DBD ACA CAC ACA CAC AC-3'	17	55.6	12	12	100.0
Total					156	147	94.2

Note: Len= Length, Tm= Melting temperature, DBD and CYA= the sequence code.

Table 3. Genetic diversity in *J.curcas* accession Gemplasm as detected by ISSR primers.

Population		N	Na	Ne	I	He	%P
Selangor		26.000	1.963	1.684	0.558	0.382	98.150
Terengganu		9.000	1.815	1.651	0.526	0.361	90.750
Kelantan		13.000	2.000	1.736	0.583	0.403	100.000
Grand mean Total	Mean	16.000	1.926	1.690	0.556	0.382	96.300
	SE	0.572	0.030	0.025	0.014	0.011	2.830

Na = No. of Different Alleles; Ne = No. of Effective Alleles; I = Shannon's Information . He = Expected Heterozygosity P= Percentage of Polymorphic Loci; SE= Standard Error.

the out crossing nature of *J.curcas*. This crop undergoes of cross pollination and this allows for variability in the population. Geographically these two states are said to have been place at the same longitude, and this favour the cross pollination among different crop. This shows that accessions having the same state are said to have greater similarity in genetic composition and make up as evident from previous study of Shen et al. (2012). The authors reported that 17 accessions from India and five accessions from Mexico respectively are found in similar cluster. Clustering of genotypes of *J. curcas* having similar state has also been reported (He et al., 2011; Leela et al., 2011). Jatropha has been a wild crop which was recently domesticated because of its inherent potentials as a biofuel feedstock, dermatological creams and soap production ingredient. This might be one of the reasons for multiple clustering as reported by the previous researcher (Balasaravanan et al., 2005). Looking at the coefficient at which these clusters are constructed, it showed that the diversity among the accessions of this *J. curcas* is generally low. This has been the trends of occurrence recorded by many authors (Basha and Sujatha 2009; Rao et al., 2009). The center of origin for this crop was traced back to Mexico in South America, it was transported through vegetative part to other part of the world by Portuguese traders where it got acclimatized. Previous studies done (Owusu-Danquah et al., 2012) also raised the fact that the vegetative plant materials of this crop are been shared by farmers among themselves through informal distribution (borrowing of plant vegetative parts from friends) and consequently resulted into narrow genetic base. Presently different types of genotypes and varieties of this crop are found in Mexico including the toxic and non- toxic. Furthermore, three dimensional principal coordinate analysis (figure 5), accessions from Selangor which includes B-01-03 (3), B-01-07 (7), B-02-06 (14), B-03-02 (16), B-04-02 (18), are positioned very close to the centroid. This implies that, accessions close to the centroid are having similar genetic divergence as reported by (Latif et al., 2011). While others mostly accessions from Kelantan D-01-02(28), D-02-02(38)

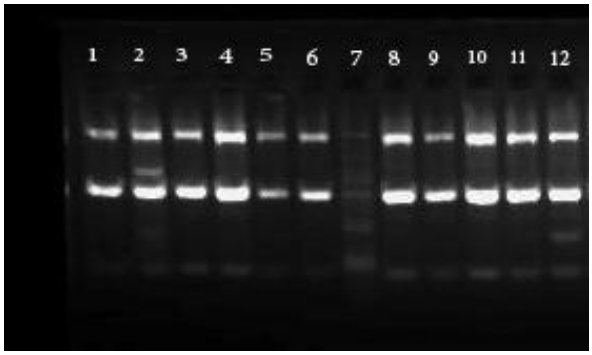

Fig 2. The polymorphic bands of primer UBC990511 on the ethidium bromide Gel of *J. curcas* accessions.

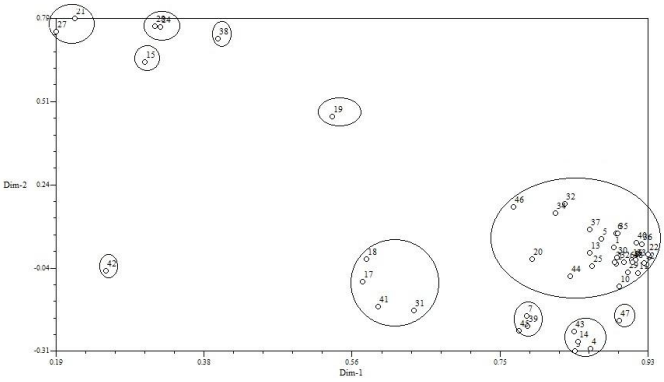

Fig 3. Two dimensional principal component analysis. This shows the 11 grouped formed by 48 different accessions across Peninsular Malaysia.

Table 4. Pair wise population matrix of Nei genetic identity.

States	Selangor	Terengganu	Kelantan
Selangor	1.000		
Terenganu	0.985	1.000	
Kelantan	0.982	0.973	1.000

Table 5. Analysis of molecular variance (AMOVA) within and between the populations of *J. curcas.*

Source of variation	d.f	SS	MS	Est. Var.	% variation	F_{ST}	P value
Among Populations	2	0.924	0.462	0.016	6%		<0.001
Within Populations	45	10.889	0.242	0.242	94%	0.016	<0.001
Total	47	11.813		0.258	100%		

Table 6. Accessions comprising in various clusters as shown in the dengrogram based on UPGMA.

Cluster	Accession
Cluster I	B-01-03, D-03-01, B-03-02, D-02-01, B-02-01, D-01-02, D-01-04, B-05-06, D-01-07, B-01-06, T-01-01, B-02-06, B-04-01, B-06-03, B-05-02, T-01-09, B-06-01, B-02-03, T-01-05, B-01-07, T-01-10,T-01-04, B-01-08, D-01-03, D-01-08, D-01-05, B-05-01, D-02-02, B-02-04, B-01-02, D-01-01, B-02-05, B-01-05 T-01-08, B-02-02, T-01-03
Cluster II	B-05-11
Cluster III	D-01-09
Cluster IV	B-01-01
Cluster V	T-01-06
Cluster VI	B-06-02
Cluster VII	B-03-01
Cluster VIII	D-01-10, B-04-02
Cluster IX	B-05-05
Cluster XX	T-01-11, B-01-04
Cluster XI	D-01-06

and Terengganu T-01-10(47), positioned far from the centroid are more genetically diverse in terms of their origin. This result was also supported by Ashkani et al. (2012) while studying the genetic diversity in rice genotype from various part of south East Asia resistance to blast disease. Variation among the populations accounted for 6% while variation within population was 94%. Similar result (87.8% within the population) was observed when AFLP maker was used to study the genetic diversity between Mexico and Chiapas varieties of *J.curcas* (Ovando-Medina et al., 2011). In addition to that, a study was carried out on 36 varieties of *J.curcas*, 68.88% of total variation was observed with the varieties of two populations from Assam and Meghalaya in the Northeast India (Kumar et al., 2011).

More of these results following similar trend have been observed in jatropha population from Brazil, China, Thailand, Vietnam, when AMOVA was used to partition the variation into within and among the populations (Tanya et al., 2011; Santos et al., 2010; Na-ek et al., 2011) and these enable one to conclude that higher variation are present within the populations of any germplasm. Furthermore, determination of genetic variability among the accession of *J. curcas*, using molecular marker is essential in selection of parental material to be used for crossing in order to develop an elite population needed for hybridization and improvement program as reported by (Ricci et al., 2012; Mastan et al., 2012). Therefore, crossing should be made between two distant clusters. Accessions B-01-03, T-01-09, B-04-01 and T-01-01 of clusters I could be hybridized with accessions D-04-02, B-05-05, B-01-04, and D-01-06 of clusters VIII, IX, X and XI as this will help in improving the diversity within and among the populations of *J.curcas* in the germplasm. Generally speaking, this study has showed that the genetic resource of *J. curcas* in Malaysia is low, with this there is immediate need for further research toward widening the plant genetic resource base for a substantial improvement in the yield of the present germplasm. This could be attained by introduction of the plant material from other countries which are recognized for their advancement in the improvement of this crop (Xin-Hua et al., 2007; Na-ek et al., 2011) and compare with germplasm collected from peninsular Malaysia to further aid in understanding of variation in this crop.

Materials and methods

Collection of plant materials

A total of 48 accessions of *Jatropha curcas* (Table: 1) were collected over different agro ecological zones in Malaysia. The collection areas, three states of Malaysia include Terengganu, Selangor and Kelantan. All the collections were assembled at the University Agricultural Park, Universiti Putra Malaysia before they were taking to the plant breeding experimental site for field evaluation. The stem cuttings of each of the accessions were raised in the nursery and seedlings were transplanted to the field.

DNA extraction and quantification.

Total genomic DNA was extracted from 21-days young leaves of 48 accessions using CTAB method (Tanya et al. 2011). Young leaves sample were ground in mortal with pestle using liquid nitrogen. DNA was extracted in a 2 ml micro centrifuge tube by adding 0.5g ground leaf tissue and 900 µl CTAB extraction buffer [2g PVP, 10 mM Tris HCl (pH (8.0), 4ml 20 mM EDTA (pH 8.0), 2% CTAB powder, 1.4M NaCl) and 2ml of 2% B mercapoethanol]. The

extracted DNA was precipitated from the aqueous phase by adding equal volume of 4^{o}C isopropanol, incubated at -25^{o} C for 30 min and centrifuged at 13000 rpm for 10 min. The DNA was precipitated and washed twice by adding 500µl ice-cold 70% ethanol.

DNA was later dissolved in 50 ul of TE buffer and with 10 ul of RNase to kill the RNA and later incubated at 37^{0}c for 1 hr. The DNA Purity and concentration was measured with spectrophotometer NANODROP Spectrophotometer (Alpha. USA). The DNA were later stored at -20^{0}c for further use.

Polymerase chain reaction (PCR) protocols.

A total of 15 ISSR primers were tested with *J. curcas* DNA for reproducible amplification with 48 accessions and 10 were chosen based on their ability to detect distinct polymorphic band across all the 48 accessions and the remaining primers were discarded (Table 2). The reaction mixture was prepared using Qiagen master mix pack (1x Taq buffer with $(NH_4)_2SO4$, 2mM $MgCl_2$, 10 mM dNTPs of each nucleotide, 10 uM primer, and 1 U Taq polymerase). The total reaction mixture was 25 ul and the PCR tubes together with the mixture were put in BIORAD-My cycle thermocycler (Bio-Rad Lab. Inc., California, USA). The initial denaturation was set for 5 minutes at 94^{0}c with each of the cycle having 1 minute denaturation at 94^{0}c, 1 minute annealing at 60 ^{0}C, 2 minute extension at 72^{0}c and 10 minutes for final extension. Annealing temperature was varied based on each primer. The separation of amplified PCR product was done by electrophoresis with ethidium bromide stain, 2 % agarose gel, 1x TBE buffer of p^{H} 8 at room temperature. The gel documentation and visualization was done using Alpha Gel imaging system (Alpha inc. USA).

Band scoring and data analysis

The acquired image were individually scored and analyzed in a binary fashion by representing present "1" and absent "0". The scorings were done using UVIDoc version 99.02 to analyze the image. The only bands that were scored are those that are > 50bp in length and reproducible. Band that were similar are assumed to be homologous.

Genetic similarity (GS) was calculated using Jaccard's similarity coefficient, and the GS was subjected to unweighted pair group method analysis (UPGMA) in order to construct dendrogram with NTSYS-pc. version 2.1. The principal component analysis (PCA) was calculated to confirm the similarity and diversity among the accessions. Analysis of Molecular variance (AMOVA) on Euclidean pair-wise genetic distances was calculated as described by (Excoffier et al. 1992) using GenAlEx 6.41 by (Peakall and Smouse 2006) to partition the total genetic variation into hierarchical system, among the population(AP) and within the population (WP). The Fst analog for Fst was calculated based on Euclidian genetic distances, and test of significance was carried out using the per-mutational procedures made available in GenAlEx. POPGEN was used in analyzing the ISSR Diploid data for each population for the calculation Nei's gene diversity and Shannon information index were calculated respectively.

Conclusion

Since the motive of diversity and evaluation study of this crop is to identify the group of accession that is worthy of

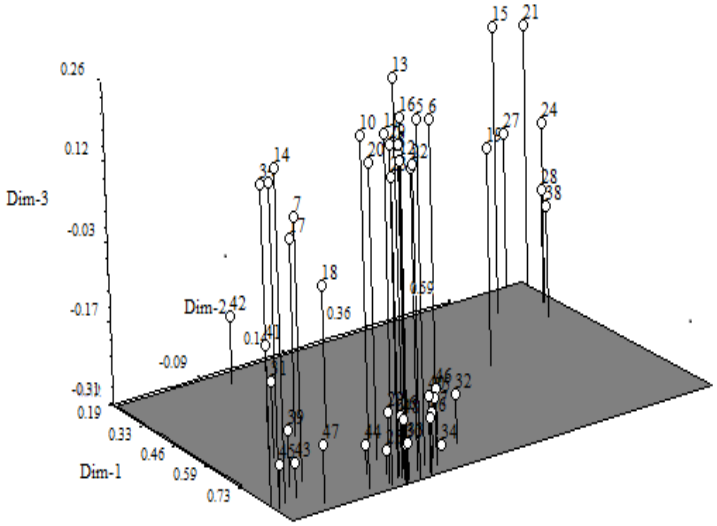

Fig 4. Three dimensional principal component analysis. The figure shows the position of various accessions to the centroid. It depicts how genetically similar or diverse these *J.curcas* are to each other.

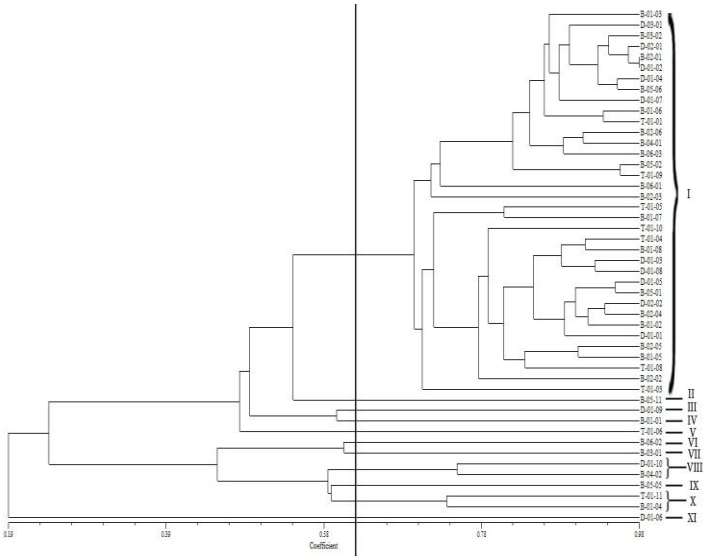

Fig 5. Dendrogram based on Unweighted pair group method with arithmetic average (UPGMA), depicting the genetic relationship among the *J.curcas* accessions from Peninsular Malaysia.

been use as a material for future improvement and breeding program, in order to bring heterosis into the populations. Accessions that are far from each other by virtue of genetic origin and diversity index are strongly advised to be use asparent for crossing. This will bring about greater diversity which will lead to high productive index in terms of increase in fruit yield per hectare, oil yield, seed weight and others. Therefore, accessions, (B-01-03, T-01-09, B-04-01 and T-01-01 could be crossed with accessions (D-04-02, B-05-05, B-01-04, and D-01-06) for the improvement of *J. curcas* for future breeding program.

Table 7. Principal component analysis and percentage variation contributed by each of the component.

Accessions	PC1	PC2	PC3
Variation (%)	61.6914	8.6833	5.0718
B-01-03	0.89	0.03	0.2
D-03-01	0.89	-0.02	0.26
B-05-02	0.84	-0.31	0.23
T-01-09	0.86	-0.3	0.24
B-01-06	0.87	0.06	0.28
T-01-01	0.89	0.08	0.28
B-02-03	0.78	-0.19	0.14
B-03-02	0.91	-0.01	0.24
D-02-01	0.93	-0.02	0.26
B-02-06	0.89	-0.1	0.29
B-02-01	0.92	-0.05	0.29
D-01-02	0.93	-0.02	0.23
B-06-03	0.86	0.01	0.36
B-04-01	0.84	-0.28	0.25
B-06-02	0.31	0.64	0.21
D-01-04	0.91	-0.01	0.31
T-01-06	0.58	-0.08	0.03
D-01-09	0.58	-0.01	-0.07
B-03-01	0.54	0.46	0.07
B-06-01	0.79	-0.01	0.2
D-01-10	0.22	0.79	0.16
B-05-06	0.93	0.01	0.23
D-01-07	0.89	-0.02	0.2
B-04-02	0.33	0.76	0.01
T-01-04	0.86	-0.03	-0.28
B-01-08	0.9	-0.02	-0.21
B-05-05	0.19	0.74	-0.04
T-01-11	0.32	0.76	-0.11
D-01-05	0.9	-0.05	-0.2
B-05-01	0.89	0	-0.27
B-05-11	0.64	-0.18	-0.19
B-02-05	0.83	0.18	-0.24
D-02-02	0.91	-0.01	-0.26
B-01-05	0.82	0.14	-0.32
B-02-04	0.89	0.08	-0.2
B-01-02	0.92	0.04	-0.22
T-01-08	0.86	0.09	-0.24
B-01-04	0.4	0.72	-0.12
T-01-05	0.78	-0.23	-0.22
D-01-01	0.92	0.05	-0.19
B-01-01	0.6	-0.16	-0.14
D-01-06	0.26	-0.04	-0.2
T-01-10	0.84	-0.24	-0.26
B-02-02	0.83	-0.06	-0.27
B-01-07	0.77	-0.24	-0.28
T-01-03	0.76	0.17	-0.24
D-01-03	0.89	-0.21	-0.22
D-01-08	0.91	-0.02	-0.22

PC1= Principal component 1; PC2= Principal component 2; PC3= Principal component 3.

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