HAYATI
Journal of Biosciences

Vol. 26 No. 3, July 2019 139-146
DOI:10.4308/hjb.26.3.139
ISSN: 1978-3019
EISSN: 2086-4094

Androgynomonoecious *Jatropha curcas*: Chromosomes, Isozymes, and Flowers Gender

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ARTICLE INFO

Article history:
Received September 28, 2018
Received in revised form May 21, 2019
Accepted June 30, 2019

KEYWORDS:
flowers inflorescence,
Jatropha curcas,
andromonoecious

ABSTRACT

***Jatropha curcas* (*J. curcas*) is usually monoecious plants, which have male and female flowers on the same inflorescence. However, *J. curcas* can be found as an androgynomonoecious plant (have male, female, and hermaphrodite flowers), even though very rare. Androgynomonoecious *J. curcas* can be identified after six months of planting when it had started flowering. Therefore, it is important to identify the characteristics of androgynomonoecious *J. curcas* that can differentiate between androgynomonoecious and monoecious plants in earlier stages of growth. The objectives of the research were to observe isozymes, chromosome and flowers gender of androgynomonoecious and monoecious *J. curcas* Banten and Lampung accessions. Seeds from five genotypes of *J. curcas* were used in the research. The observation was carried out on the chromosome and isozymes (Peroxidase and Esterase isozymes) could be used as markers to differentiate androgynomonoecious and monoecious plants. Observations about the flower gender from offsprings derived from different seeds were important to know the inheritance of flower gender. The androgynomonoecious and monoecious *J. curcas* were diploid with number of chromosomes 2n = 2x = 22. The chromosomes of androgynomonoecious have longer than that of monoecious *J. curcas*. The isozymes of androgynomonoecious *J. curcas* had four alleles and monoecious *J. curcas* (Banten female monoecious) had three alleles. The flower inflorescence and gender derived from androgynomonoecious plants were unstable, due to androgynomonoecious is intermediate state.**

1. Introduction

Jatropha curcas (*J. curcas*) is an original plant from tropical America belonging to the family of *Euphorbiaceae*. Commonly, *J. curcas* has male and female flowers on the same inflorescence in the plant (Hartati *et al.* 2009), there is currently some *J. curcas* with hermaphrodite flowers, but very rarely found. *J. curcas* with hermaphrodite flowers are classified as andromonoecious type (have hermaphrodite and male flowers in the same plant) and androgynomonoecious type (have male, female, and hermaphrodite flowers in same the plant) (Dellaporta and Urrea 1993; Makkar *et al.* 2008; Andriano-Anaya *et al.* 2016). Hermaphrodite flowers tended to perform self-pollination (Hartati 2009; Dasumiati *et al.* 2015). Self-pollinated plants will produce homogeneous offsprings than cross-pollinated plants (monoecious) which generally produce heterogeneous offsprings (Raju and Ezradanam 2002). Therefore, it is very important to study early detection for sex type of *J. curcas* that produces hermaphrodite flowers as an indicator for *J. curcas* breeding.

The sex type of *J. curcas* cannot be identified when the plant still seedling due to it does not have flowers. Sex types of *J. curcas* can be identified after six months of planting or when it has started flowering. Therefore, the androgynomonoecious *J. curcas* characters need to be observed as a selection criterion in the seedling phase.

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The early detection of sex types in *J. curcas* can be identified through the chromosome analysis, isozymes, and continued by the analysis of reproductive character (flower gender). Chromosome analysis is used to explain the inheritance of genetic materials and the relationship between species through the number and size of chromosomes (Plummer *et al.* 2003). Chromosome analysis also has been used to analyze the diversity of plants, such as those performed by Escudero *et al.* (2010), which evaluates the variation of chromosome number in Cyperaceae. Talukdar (2010) used a chromosome analysis to characterize and compare the three varieties of crops grass pea. To measure the diversity of plant species, also have been carried through isozymes techniques. Isozyme is an enzyme that is a direct product of a gene that has an active molecule and different chemical structures but catalyzes the same chemical reaction. Enzymes are protein biocatalisator for physiological processes that its role controlled genetically (Asante and Laing 2001). Some isozyme known to associated with or related to agronomic characters (Stalker and Mozingo 2001). The isozyme analysis can be used as genetic markers for studying variations of individual in a population (Yunus 2007). Some enzymes were used to differentiate the flower gender i.e, Peroxidase (PER) and Esterase (EST) enzymes (Sharma *et al.* 2010), as in the *Hippophae rhamnoides* L. (Shirkot *et al.* 2009).

Studies on early detection of sex types of plant on *J. curcas* is still limited. The purpose of this research was to identify markers of androgynomonoecious on *J. curcas* by comparison to monoecious *J. curcas* through chromosomes and isozymes characters. Characters of *J. curcas* that can be used to differentiate between androgynomonoecious and monoecious *J. curcas* were chromosomes and isozymes. Furthermore, this study observed the flowers gender inherited from the seeds of androgynomonoecious flowers. The results of this study are expected to characterize the characters of *J. curcas* as selection criteria in *J. curcas* breeding.

2. Materials and Methods

2.1. Plant Materials

The seeds from five genotypes of *J. curcas*, i.e.: seeds from female flowers of androgynomonoecious Lampung accession (LFT), seeds from hermaphrodite flowers of androgynomonoecious Lampung accession (LHT), seeds from female flowers of androgynomonoecious Banten accession (BFT), seed from hermaphrodite flowers of androgynomonoecious Banten accesion (BHT), and seeds from female flowers from monoecious of Banten accession (BFM). Each genotype was represented by 15 seeds for germinated and planted. This study used collection of *J. curcas* genotypes belonging to the first author.

2.2. Chromosomes Analysis

Five germinated seeds from every five genotypes of *J. curcas* (BFT, BHT, LFT, LHT, and BFM) were using for this analysis. The seedling roots from each genotype were used for measurement of chromosomes number and length. Fresh root tips (5-10 mm length) were incubated in 0.2% (w/v) colchicine for 3 hours at 7°C. The root tips were fixed in a 45% (w/v) acetic acid solution for 10 minutes at room temperature. The root tips were hydrolysed in 1 N HCl: 45% acetic acid (3:1, v/v) solution for 3 minutes in a 60°C water bath. The pieces of root tips were stained with 1% (w/v) aceto orcein for 24 hours. Furthermore, root pieces were made into preparation by placed on a glass slide, it was closed with a cover glass and pressed. Preparation was observed under a microscope to count the number of chromosomes and measure the length of chromosomes. The chromosomes' length was measured using the application ImageJ 1.42q (http://rsbweb.nih.gov/ij/).

2.3. Isozymes Analysis

Five seeds of each five genotypes of *J. curcas* (BFT, BHT, LFT, LHT, and BFM) were planted in a polybag. Isozymes analysis using young leaf (second leaf of shoot tip) from two months old-plant. Two grams of a leaf was extracted using 0.5 ml buffer solutions (extraction buffer) and transferred to the starch gel. Extraction buffer consist of 10 mM L-ascorbic acid, 40 mM L-cystein, 20% Triton X-100, 0.25 g PVP-40 (polyvinyl polypyrrolidone), and 0.1 M $Na_2HPO_2{\cdot}H_2O$ pH 7.0 (phosphate buffer). The isozymes were separated using starch gel that was made from 13% (w/v) potato starch in 20 ml gel buffer (5 mM L-histidin monohydrate pH 6.0). Electrophoresis analysis was performed using electrophoresis buffer (50 mM citric acid monohydrate, 150 mM tris hydroxymethyl aminomethane, pH 6.0) during four hours with a constant voltage of 200 V (Soltis and Soltis 1989). Peroxidase (PER) and Esterase (EST) were observed by soaking the gel in substrate solution for PER (50

mM buffer sodium acetate pH 5.0 50 ml, $CaCl_2$ 50 mg, H_2O_2 3% 0.25 ml, 3-amino-9 ethylcarbazole 25 mg, N,N-dimethylformamide 2 ml) and EST (100 mM buffer Na-phosphate pH 6.0 50 ml, α-naphthylacetic acid 25 mg, β-naphthylacetic acid 25 mg, acetone 5 ml, fast blue RR Salt 1 ml) for 60-120 minutes. The position of isozyme bands was measured as relative electrophoretic mobility between band positions to relative electrophoresis mobility (Rf). Only clear isozyme bands were scored and enzymatic scheme diagrams painted according to Rf values.

2.4. Observations of Flower Gender and Plant Sex Types

Each of five genotypes of *J. curcas* (BFT, BHT, LFT, LHT, and BFM) were planted randomly on the field with a planting distance of 2 x 2 m. We observed flower gender and plant sex type from the seedling which had been observed chromosome and isozymes. To analyzed flower gender and plant sex type stabilization, we observed during the first flowering season.

2.5. Data Analysis

Data were analyzed using SPSS version 22. The data analysis of chromosomes and isozyme displayed in the description. The length of chromosomes was conducted using analysis of variance (ANOVA) followed by the *Duncan Multiple Range Test* (DMRT) at 5% level.

3. Results

3.1. Chromosomes Analysis

The characters that observed in chromosome analysis of *J. curcas* were chromosomes number and length. The five genotypes of *J. curcas* have 22 chromosomes (diploid: 2n = 2x = 22) (Figure 1). The range of chromosomes length of five genotypes of *J. curcas* was 1.19 to 2.69 µm. The average chromosomes length were different ($p<0.05$) among the five genotypes were observed. The chromosomes' length of BHT and LHT larger than that of LFT, BFT, and BFM, on the other side, the chromosomes length of BFT and BFM were similar (Table 1).

3.2. Isozymes Analysis

Isozymes analysis was performed using the PER and EST enzymes. Isozymes of EST and PER have a difference in the number of bands on electrophoregram. Band pattern based on Rf value

Table 1. Chromosome length of androgynomonoecious and monoecious *J. curcas*

Chromosome pairs	Chromosome length (µm)				
	Androgynomonoecious plants*				Monoecious plants*
	BFT	BHT	LHT	LFT	
1	1.96	2.57	2.42	2.69	BFM
2	1.87	2.78	2.71	2.45	1.93
3	1.71	2.64	2.47	2.00	1.84
4	1.62	2.16	2.42	2.19	1.67
5	1.61	2.53	2.40	2.15	1.40
6	1.55	2.31	2.34	2.04	1.56
7	1.47	2.28	2.23	1.96	1.50
8	1.42	2.09	2.22	1.88	1.43
9	1.36	1.88	2.16	1.76	1.41
10	1.26	1.66	1.99	1.66	1.39
11	1.19	1.54	1.67	1.68	1.26
Average**	1.55[c]	2.22[a]	2.28[a]	2.04[b]	1.22
					1.51[c]

*BFT: seedling from female flower of androgynomonoecious Banten accession, BHT: seedling from hermaphrodite flower of androgynomonoecious Banten accession, LHT: seedling from hermaphrodite flower of androgynomonoecious Lampung accession, LFT: seedling from female lower of androgynomonoecious Lampung accession, BFM: seedling from female flower of monoecious Banten accession
**Number followed by the same letter in the same row indicate was not significantly different (Duncan test, $p<0.05$)

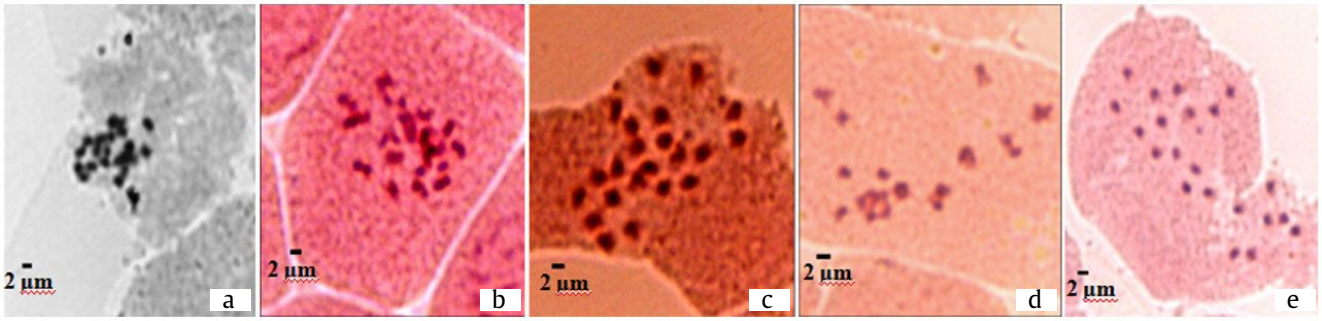

Figure 1. Chromosomes of androgynomonoecious and monoecious *J. cucras*. (a) BFT: seedling from female flower of androgynomonoecious Banten accession, (b) BHT: seedling from hermaphrodite flower of androgynomonoecious Banten accession, (c) LHT: seedling from hermaphrodite flower of androgynomonoecious Lampung accession, (d) LFT: seedling from female flower of androgynomonoecious Lampung accession, (e) BFM: seedling from female flower of monoecious Banten accession

of these isozymes was varied. Esterase isozyme has two band patterns (Figure 2a and c). In the first band pattern consists of four bands (at Rf 0.20, 0.37, 0.50, and 0.77) was formed by LFT, LHT, BHT, and BFT genotype. The second pattern consists of three bands (at Rf 0.37, 0.50, and 0.77) was formed by BFM genotypes. On the other side, Peroxidase isozyme has two band patterns (Figure 2b and d). In the first band pattern consists of four bands (at Rf 0.10, 0.80, 0.83, and 0.96) was formed by LFT, LHT, BHT, and BFT genotypes. The second pattern consists of three bands (at Rf 0.10, 0.80, and 0.96) was formed by BFM genotype.

3.3. The Flower Gender and Plant Sex Type in *J. curcas*

Seeds derived from female flowers (BFT and LFT) and hermaphrodite flowers from androgynomonoecious (BHT and LHT) *J. curcas* produced offsprings of androgynomonoecious plants sex type. On the other side, seeds derived from the female flowers (BFM) of monoecious *J. curcas* produced offsprings of monoecious plants sex type (Table 2).

Plant sex type of androgynomonoecious (from BFT, BHT, LHT, and LFT seeds) produced four flower gender inflorescence, whereas monoecious plants (from BFM seeds) produced monoeciuos flower inflorescence. The androgynomonoecious plants have different percentages of flower gender on the plant. Among androgynomonoecious plants, BFT and LHT genotypes have four flower inflorescence types, that is androgynomonoecious (female, male, and hermaphrodite flowers), andromonoecious (male and hermaphrodite flowers), monoecious (female and male flowers), and male flower inflorescences. On the contrary, androgynomonoecious plants, BHT genotype only has three inflorescence types

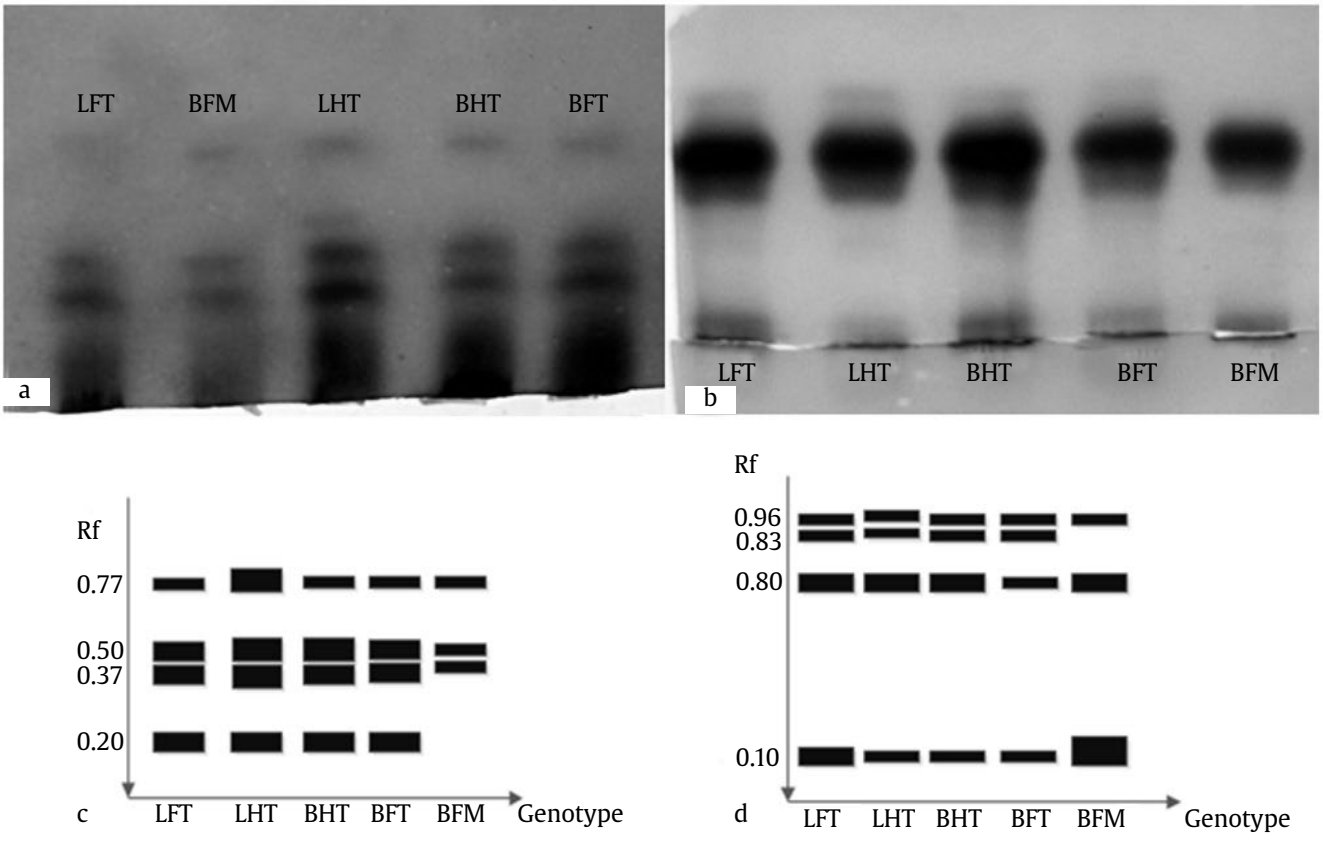

Figure 2. Electrophoregram of Esterase (a and c) and Peroxidase (b and d) enzymes. Electrophoregram of gel electrophoresis (a and b), schematic of electrophoregram (c and d). BFT: seedling from female flower of androgynomonoecious Banten accession, BHT: seedling from hermaphrodite flower of androgynomonoecious Banten accession, LHT: seedling from hermaphrodite flower of androgynomonoecious Lampung accession, LFT: seedling from female flower of androgynomonoecious Lampung accession, BFM: seedling from female flower of monoecious Banten accession

Table 2. Plant, inflorescence sex types, flowers gender, and percentage of flower inflorescence type on the plant produced by genotypes of *J. curcas*

Genotypes	Plant sex type	Flower gender and flower inflorescence type	Percentage of flower inflorescence type on the plant (%)
BFT	Androgynomonoecious	Hermaphrodite, female, Male (Androgynomonoecious)	33.33
		Hermaphrodite, Male (Andromonoecious)	4.17
		Female, Male (Monoecious)	60.41
		Male	2.08
BHT	Androgynomonoecious	Hermaphrodite, Female, Male (Androgynomonoecious)	12.50
		Hermaphrodite, Male (Andromonoecious)	85.00
		Female, Male (Monoecious)	0.00
		Male	2.50
LHT	Androgynomonoecious	Hermaphrodite, Female, Male (Androgynomonoecious)	21.74
		Hermaphrodite, Male (Andromonoecious)	52.17
		Female, Male (Monoecious)	17.39
		Male	8.70
LFT	Androgynomonoecious	Hermaphrodite, Female, Male (Androgynomonoecious)	21.05
		Hermaphrodite, Male (Andromonoecious)	5.26
		Female, Male (Monoecious)	28.95
		Male	44.74
BFM	Monoecius	Female, Male (Monoecious)	85.71
		Male	14.29

BFT: Banten female androgynomonoecious, BFT: female of androgynomonoecious Banten accession, BHT: hermaphrodite of androgynomonoecious Banten accession, LHT: hermaphrodite of androgynomonoecious Lampung accession, LFT: female of androgynomonoecious Lampung accession, BFM: female of monoecious Banten accession. Observation of flowering androgynomonoecious and monoecious *J. curcas* at the same age was five months after planting

that are, androgynomonoecious (female, male, and hermaphrodite flowers), andromonoecious (male and hermaphrodite flowers) and male flowers. The BFT genotype has the highest percentage of androgynomonoecious and monoecious flower inflorescence.

Seedlings derived from the female flowers of Banten accession monoecious *J. curcas* produced two flower inflorescence types that were monoecious inflorescence (male and female flowers on the same inflorescence) and androecious (male flowers). The seedlings of female flowers from androgynomonoecious *J. curcas* produced monoecious inflorescence flowers, while the seedlings of hermaphrodite flowers produced higher andromonoecious inflorescence (male and hermaphrodite flowers in the same inflorescence) than other those flower inflorescence types. The seedlings of female flower from monoecious *J. curcas* produced of monoecious inflorescence flowers (Table 2).

Plants from seed originating from the female flowers on androgynomonoecius *J. curcas* produced offsprings with monoecious inflorescence flowers (male and female flowers on the same inflorescence) higher than other flower inflorescences. Plants from seed originating from hermaphrodite flowers on androgynomonoecius *J. curcas* produced andromonoecious flower inflorescence (hermaphrodite and male flowers on the same inflorescence) higher than other flower inflorescences. Plants from seed originating from hermaphrodite flowers on *J. curcas* Banten accessions were not generated inflorescence which has male and female flowers (Table 2).

4. Discussion

The chromosomes number of *J. curcas* genotypes in this study were 2n = 22 chromosomes. This chromosomes number similar to the study conducted by Carvalho *et al.* (2008) and Sasikala and Paramathma (2010). The chromosomes length of *J. curcas* in this data was in line with the study of Reddy *et al.* (2013) which stated that the length of chromosome of *J. curcas* between 1 to 3.67 µm. The average length of the androgynomonoecious plant's chromosome was longer than the average for monoecious plants. The length of chromosomes can differ in different species in the same family, eventhough the numbers of the chromosomes are the same. The difference length between the androgynomonoecious plant's chromosome and the monoecious plant's indicated that there was a difference in the nitrogenous bases number (Harrison and Schwarzacher 2011). On the other hand, Moliterni *et al.* (2004) stated that chromosomes in plants with hermaphrodite flowers tend to have large variability, making a large difference in chromosomes size among genotypes possible. As in this study, that the androgynomonoecious plants have hermaphrodite flowers, whereas in the monoecious plants have no hermaphrodite flowers.

The variation of band pattern in PER and EST isozyme tends to be classified as qualitative variation, i.e. the presence or the absence of a band in the gel. These isozymes in this study as genetic markers for identifying the androgynomonoecious *J. curcas* genotype. The Peroxidase enzyme also has been used as a marker in the *Hippophae rhamniodes* female reproductive organs by Sharma *et al.* (2010). Band patterns of androgynomonoecious and monoecious plants which produced different variation showed there was a genetic difference between androgynomonoecious and monoecious. This difference was due to more than one gene in each of those plants which control each isozyme (Dewatisari *et al.* 2008).

The Rf value band pattern based on the result of Esterase and Peroxidase isozymes on *J. curcas* can be described as the pattern of alleles between genotypes of *J. curcas* were observed. The EST and PER enzymes from the five *J. curcas* genotypes were differentiated into two band patterns with three to four alleles. The BFT, BHT, LHT, and LFT genotypes which were androgynomonoecious plants had four alleles. Only the BFM genotype was a monoecious plant with three alleles (Figure 2). The difference between monoecious and androgynomonoecious *J. curcas* can also be seen from the types of flower gender owned by each inflorescence and sex type of plant.

Flower inflorescences produced from the androgynomonoecious plants (BFT, BHT, LHT, and LFT) did not have a consistent percentage of flower inflorescence type. Based on the flower gender and sex types of plants produced by each genotype showed that the androgynomonoecious *J. curcas* consistently has the male, female, and hermaphrodite flowers in the same flower inflorescence. Monoecious *J. curcas* consistently produced plant which has flower gender only male and female flowers in the same flower inflorescence. Adriano-Anaya *et al.* (2016) found female, male, and hermaphrodite flowers in the study accessions and based on the proportion of each flower gender, plants were classified as gynoecious, androecious, andromonoecious, androgynomonoecious. Based on evolution of sexuality, androgynomonoecious and andromonoecious *J. curcas* are the intermediate state, whereas monoecious *J. curcas* is modern state (Charlesworth 2016). *J. curcas* sex types in this study similar with studied by Dasumiati *et al.* (2017) that androgynomonoecious and monoecious of *J. curcas* have stable sex type after stem cuttings propagation. The expression of flower gender is controlled by of genetic (Susila *et al.* 2010) and controlled by a pair of sex chromosomes, like in papaya (Ming *et al.* 2007).

The females and hermaphrodites flowers have different resource allocation pattern. This pattern suggests that female flowers may allocate resources to increase their fitness in a less favorable environment more than those hermaphrodite flowers (Delph and Carrol 2001). The hermaphrodites flowers would be stronger facilitators than females because they incur a greater allocational cost of producing pollen (Cranston *et al.* 2012). Another factor that intervenes in sex modification is photoperiod, which has masculinizing or feminizing effects, depending on day duration. The short days decreased temperature to female sex phenotypisation and increase the amount of flower hermaphrodite flowers. This fact is realizable by the regulation of endogenous hormones level (Trutã *et al.* 2007).

In this study, we provided valuable information based on isozyme characters, and chromosomes showed the relationship between the size of the chromosome, the number of alleles, with flower gender in inflorescences type. The genotypes of androgynomonoecius *J. curcas* has four alleles, whereas genotypes offsprings derived from the seeds of hermaphrodite flowers have a size longer than the chromosome derived from the seeds of the female flower. Therefore, isozymes (Peroxidase and Esterase) and chromosome size could be used as markers to differentiate between androgynomonoecious and monoecious plants *J. curcas*. Both androgynomonoecious and monoecious *J. curcas* plants have diploid chromosomes (2n = 22), but chromosomes from androgynomonoecious plants from hermaphrodite flowers have a longer than that of monoecious plants. Characteristics of the androgynomonoecious plants in this study were almost the same as with their mother plant. The flower inflorescence type derived from androgynomonoecious plants were unstable, due to androgynomonoecious is intermediate state (Charlesworth 2016). This information is essential for the establishment of controlled crossing program and relevant for the development of a clone-based breeding program.

Acknowledgements

We greatly thanks to the Research Center for Biological Resources and Biotechnology (PPSHB), and The Indonesian Institute of Sciences Cibinong for the laboratory facilities.

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