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Genetic Diversity of Croton (*Codiaeum variegatum* (L.) Rumph.ex A. Juss) and its Offspring Based on RAPD Markers

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ABSTRACT

Croton (Puring) is a native plant of Indonesia which has varied leaf shapes and colors. The diversity of croton increases through hybridization. The information on genetic diversity and relationship between parent and its offspring of crotons is very limited. This study aimed to analyze the genetic diversity of croton cultivar Mawar (MW) and Walet (W) as parents compared to their offspring, i.e. Black Marlet (BM), Kingkit 1 (KA), Kingkit 2 (KB), and Kamaratih (KM) using RAPD markers. This study used DNA extraction from the fresh leaf of the six cultivars. The next steps were DNA quantification, primary optimization, DNA amplification with PCR, and electrophoresis. Statistical analysis was carried out using Genalex software. A total of 40 primers were screened, out of which 10 were selected for the analysis of genetic diversity. A total of 106 polymorphic bands were generated, ranging from 130 to 1850 bp. The results of RAPD analysis showed that Mawar as female parent had the highest polymorphic bands percentage of 69.01%, while the polymorphic bands percentage of Walet as male parent and the offspring ranged from 31.15 % to 43.94%. The genetic distance between Walet and the offspring ranged from 0.176 to 0.234 and the genetic distance between Mawar and the offspring ranged from 0.314 to 0.372. It indicated that all of offspring were closer to the male parent.

Keywords: Croton, genetic diversity, RAPD, genetic distance.

INTRODUCTION

Croton (*Codiaeum variegatum* (L.) Rumph.ex A. Juss) is one of ornamental plants which has varied leaf shapes and colors. It is a species of plant in the genus Codiaeum, which is a member of the family Euphorbiaceae. It is native plant from Malaccan Islands, and spread widely to some region of Indonesia and other countries such as Philippines, Papua New Guinea, India, Sri Lanka, Thailand, Malaysia and some other Pacific Islands countries (Magdalita *et al*., 2014; Deng *et al*., 2010). According to Mollick and Yamasaki (2012), there are over 300 croton cultivars in the world. Croton can be used for corsages, flower arrangements and wedding bouquets (Stamps and Osborne, 2003), and can be used as a medicinal plant for peptic ulcers (Rahman and Akter, 2013).

According to Brown (1995) *cit*. Mollick *et al*. (2011), the shape of croton leaves is categorized into nine groups, which are: 1). Broad leaf, 2). Oak leaf, 3). Semi oak leaf, 4). Spiral leaf, 5). Recurved leaf, 6). Narrow leaf, 7). Very narrow leaf, 8). Small leaf, and 9). Interrupted leaf. The variation of croton leaf is apparently ascribed to a high frequency of somatic mutation on the leaves (Mollick and Yamasaki, 2012). Essentially, the natural color of croton leaves is green, however, in some cultivated varieties, the leaves have varied colors, streaked, blotched or banded with green, white, red, orange, purple, pink, indigo, violet, yellow, crimson, scarlet, and brown or cream (Ogunwenmo *et al*., 2007). Because the croton has various color pattern, analysis and typing for coloration and color pattern are very difficult (Shimoji *et al*., 2006). Magdalita *et al*. (2014) categorize the color of croton leaf into three group, which are red-leaf group, green-leaf group and yellow-leaf group.

Croton hybridization produces new cultivars which have a variety of leaf shapes and colors. However, information on genetic diversity between parent and its offspring is very limited. Genetic diversity can be analyzed using morphological markers. The advantages

of morphological markers are cheap, easy and efficient. Morphological markers, however, can be influenced by environments (Xu, 2010; López-Caamal and Tovar-Sánchez, 2014) and the age of the plant (Xu, 2010).

Morphological markers are limited to the number observed and some plant growth characteristics expressed late such as flower colors. Morphological markers can also be influenced by other morphological markers or traits of interest due to pleiotropic gene action (Andersen and Lubberstedt, 2003). Therefore, with these limitations, a stable marker is needed. Molecular markers are more stable than morphological markers. Molecular markers can be used to analyze plant genetics, allowing to obtain genes that control target characters quickly and accurately. One of genetic markers is RAPD (Random Amplified Polymorphic DNA).

According to William *et al*. (1990), RAPD markers can be used as genetic mapping, plant and animal breeding application, and DNA fingerprinting, especially in population genetics study. RAPD markers have several advantages, such as simplicity, low cost, and the use of small amount of plant material (Guasmi *et al*., 2012). RAPD markers are also more efficient, able to quickly identify and isolate DNA fragments on specific chromosome (William *et al*., 1990).

Furthermore, RAPD primers are universal, which means that it can be used for genome analysis in many species' varieties (William *et al*., 1990). RAPD markers has been used to estimate the genetic diversity in tetraploid alfalfa population (Naq *et al*., 2011), genetic authentication of *Gardenia jasminoides* Ellis var. grandiflora Nakai (Mei *et al*., 2015), parentage confirmation of cotton (Asif *et al*., 2009) and gossypium (Mehetre *et al*., 2004), genetic analysis of Chrysanthemum (Huang *et al*., 2000; Wolff and Rijn, 1993), and investigation on the partitioning of variation populations of *Medicago sativa* (Crochemore *et al*., 1996). The purpose of this study was to identify the genetic diversity of croton and its offspring using RAPD.

MATERIALS AND METHODS

Molecular analysis was conducted in Plant Genetics and Breeding Laboratory, Faculty of Agriculture of UGM from September 2017 to January 2018. Plant materials used in this study were six croton cultivars, Mawar (MW) and Walet (W), as the parental lines and their offspring, i.e. Black Marlet (BM), Kingkit 1 (KA), Kingkit 2 (KB) and Kamaratih (KM) taken from Keboen Alma Nursery, Pakem, Sleman, Yogyakarta. Three leaf samples in each cultivar were taken from three different plants , except for Walet, in which two leaf samples were taken from one of plant.

The chemicals used in DNA analysis by the RAPD-PCR method were: CTAB (CTAB 2%, 1.4 M NaCl, 0.1 M Tris-HCl, 0.02 M EDTA, 2% PVP, 2% mercaptoethanol and aquabidest), sterile distilled water, 24 chloroform: 1 isoamyl alcohol (CIAA), 70% ethanol, 70% alcohol, buffer PCR mix Go Taq® Green (Promega), nuclease free water, DNA, RAPD primers, DNA staining dyes, agarose, and ladder. The equipments used were PCR tube, 1.5 ml tube, 2 ml tube, white tip, yellow tip, blue tip, centrifuge, vortex, water heater (waterbath), PCR Thermal Cycler BIO RAD machine, electrophoresis machine, UV lamp and digital camera.

The total genomic DNA was extracted from 0.1 g of fresh leaves of croton using CTAB method (Doyle and Doyle 1990). DNA concentration and purity were determined using GeneQuant Spectrophotometer.

Table 1. Primers and their sequences used for RAPD analysis

Primer	Sequence
OPC 2	5'- GTGAGGCGTC -3'
OPC 9	5'- CTCACCGTCC -3'
OPC 11	5'- AAAGCTGCGG -3'
OPC 12	5'- TGTCATCCCC -3'
OPC 14	5'- TGCGTGCTTG -3'
OPC 16	5'- CACACTCCAG -3'
OPC 19	5'- GTTGCCAGCC -3'
OPC 20	5'- ACTTCGCCAC -3'
OPD 8	5'- GTGTGCCCCA -3'
OPD 20	5'- ACCCGGTCAC -3'

Quantification results were used as the basis of DNA dilution. There were 10 RAPD primers based on primer optimization used in this study, which were OPC 2, OPC 9, OPC 11, OPC 12, OPC 14, OPC 16, OPC 19, OPC 20, OPD 8 and OPD 2 (Table 1).

Genomic DNA was used as template for PCR amplification (Williams *et al*., 1990). Amplification was performed in a 10 µl reaction volume containing 2.5 µl template DNA, 5 µl Go Taq® Green (Promega) PCR mix, 0.25 µl of selected RAPD primers, and 2.25 µl of nuclease free water. Amplification conditions were maintained at 94ºC for 5 minutes, followed by 45 cycles of 94ºC for 30 seconds (denaturation), 38ºC for 30 seconds (annealing), 72 ºC for 1 minute 30 seconds (elongation) and final elongation at 72 ºC for 7 minutes, based on modified method from Andreastuti *et al*. (2015). The amplified DNA was loaded on 1.5% agarose and separated in 1x TBE buffer pH 8 at 100 A, 400 volts for 55 minutes, then visualized under UV light and documented using camera.

The bands produced were counted. All bright/ visible fragments were scored as present (1) or absent (0) for each sample. Data were used for genetic distance analysis using GenAlex 6.1 software (Peakall and Smouse, 2007).

RESULTS AND DISCUSSION

Croton hybridization has been carried out to improve the genetic diversity. Hybridization between Mawar (MW) as female parent and Walet (W) as male parent produced four offspring, i.e. Black Marlet (BM), Kingkit 1 (KA), Kingkit 2 (KB) and Kamaratih (KM). The four offspring had different leaf shapes and colors (Figure 1). Mawar leaf was small, multicolor, and varied in shape (linear, oblong and orbicular). The color of Mawar leaf was green with yellow and red dots. Walet leaf was red and linear. Black Marlet was red and varied in shapes (linear, elliptic, and orbicular). Kingkit 1 (KA) and Kingkit 2 (KB) leaf was oblong and green with yellow dots. Kamaratih leaf was multicolor (green with yellow and red dots) and varied in shape (linear, cordate and orbicular).

RAPD analysis using selected ten primers produced 118 bands with a DNA fragment size of 130–1850 bp. Among of the 118 bands, 12 of them were monomorphic bands (10.17%) and 106 of them were polymorphic bands (89.83%). The highest number of polymorphic bands was achieved with primer OPC 11 and OPC 12 (100%), while the lowest number of polymorphic bands was achieved with primer OPC 19 (50%) (Table 2). Monomorphic bands were present in all individuals, while polymorphic bands were present in at least one but not all individuals (Mehetre *et al*., 2004).

A total number of bands owned by the parent and its offspring was ranging from 56 to 71. Mawar produced the highest number of bands, while Kamaratih produced the lowest number (Table 3). The highest percentage of polymorphic bands was found in Mawar (MW) of 69.01%, while the lowest one was found in Walet (W) of 31.15 %. The polymorphic bands of offspring ranged from 35.48 % to 43.94%.

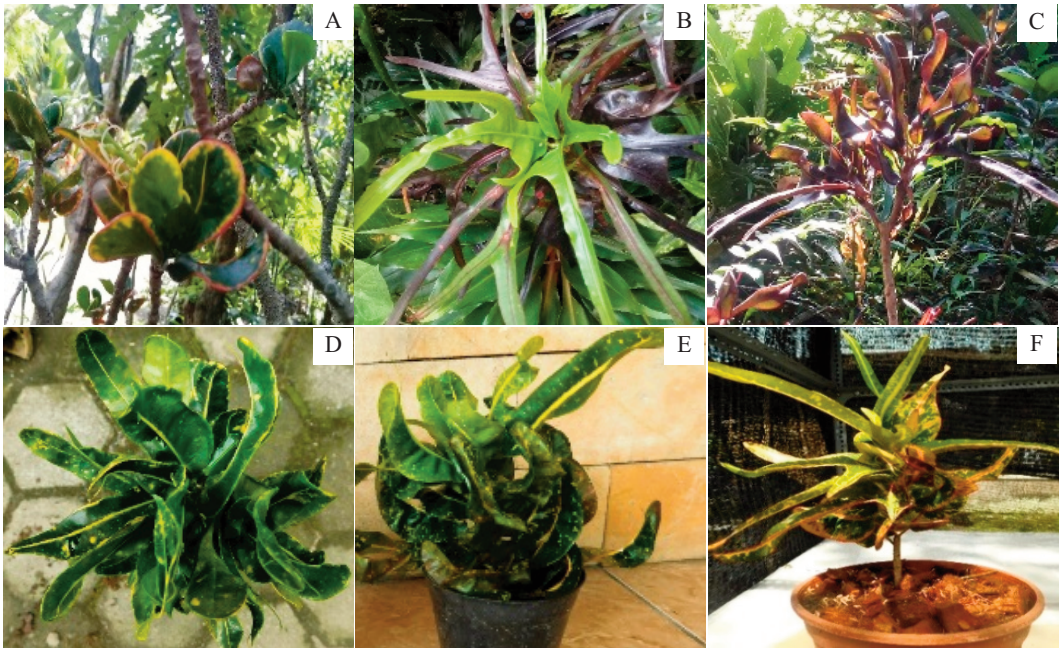

Figure 1. Profile of croton cultivars. A) Mawar parent, B) Walet parent, C) Black Marlet-BM, D) Kingkit 1-KA, E) Kingkit 2-KB and F) Kamaratih-KM

Table 2. Polymorphic and monomorphic bands in each primers used for RAPD analysis

Primer	Total Bands	Total Polymorphic Bands	Total Monomorphic Bands	Polymorphism (%)	Monomorphism (%)
OPC 2	8	6	2	75	25
OPC 9	14	12	2	85.71	14.29
OPC 11	18	18	0	100	0
OPC 12	16	16	0	100	0
OPC 14	5	4	1	80	20
OPC 16	12	11	1	91.67	8.33
OPC 19	6	3	3	50	50
OPC 20	11	10	1	90.91	9.09
OPD 8	14	13	1	92.86	7.14
OPD 20	14	13	1	92.86	7.14
Total	118	106	12	89.83	10.17

Table 3. Percentage of polymorphic and monomorphic bands in the parents croton and their offspring based on RAPD markers

Cultivar	Total Bands	Total Polymorphic Bands	Total Monomorphic Bands	Polymorphism (%)	Monomorphism (%)
Mawar (MW)	71	49	22	69.01	30.99
Walet (W)	61	19	42	31.15	68.85
Black Marlet (BM)	62	22	40	35.48	64.52
Kingkit 1 (KA)	66	29	37	43.94	56.06
Kingkit 2 (KB)	67	26	41	38.81	61.19
Kamaratih (KM)	56	20	36	35.71	64.29
Mean	63.83	27.50	36.33	43.08	56.92

Polymorphic bands are particularly useful in genetic mapping or in segregation pattern of a population. Polymorphism may occur because of the deletions of priming sites, insertion which causes priming too far to support amplification, or insertion which changes the size of the DNA segment without preventing amplification (Williams *et al*., 1990). Mehetre *et al*. (2004) added that the polymorphisms of RAPD markers were observed as different sized DNA fragments from amplification. According to Penner *et al*. (1993), variability in RAPD fragments is manifested in two ways, i.e. variability in the size range amplified and intrinsic differences in the reproducibility of the primers involved.

From this study, we found seven pattern types of molecular markers according to the presence or absence of the bands. Huang *et al*. (2000) classified the RAPD markers into seven types. They were Type I markers shared bands in both parents, and offspring; Type II markers shared bands in male and female parents; Type III markers shared bands in male parent and offspring; Type IV markers shared bands in female parent and offspring; Type V markers were present in the male parent only; Type VI markers were present in the female parent only; Type VII markers were present in offspring only. This study showed type III markers in the primer OPC 19 at 950 bp and in the primer OPD 8 at 475, 700, 1350, 1500bp (Figure 2 and 3). According to Huang *et al*. (2000), Type III markers were suitable for identifying the true male parent. The true hybrid should have the specific bands to the male parent (Ilbi, 2003 and Akhare *et al*., 2008).

Not all of bands found in the parent were descended into its offspring. There were bands not found in either male or female parent, but appeared in the offspring as new bands. This study showed type VII or new bands in the primer OPC 19 at 1100 bp (Figure 3). The new bands were also detected in the alfalfa progenies (Taški-Ajduković *et al*., 2014), diploid banana hybrids (Martanti *et al*., 2017), chrysanthemum hybrids (Huang *et al*., 2000) and Nepenthes hybrids

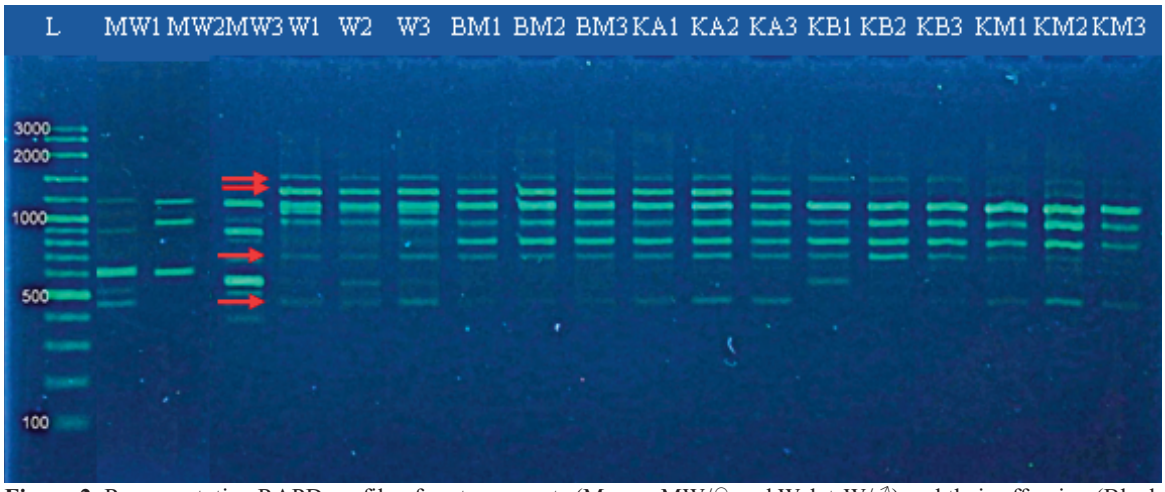

Figure 2. Representative RAPD profile of croton parents (Mawar-MW/♀ and Walet-W/♂) and their offspring (Black Marlet-BM, Kingkit 1-KA, Kingkit 2-KB, and Kamaratih-KM) using OPD 8 primer. The number following the labels indicates replication. L = ladder 100 bp. Arrowhead indicate Type III (bands in male parent and the offspring).

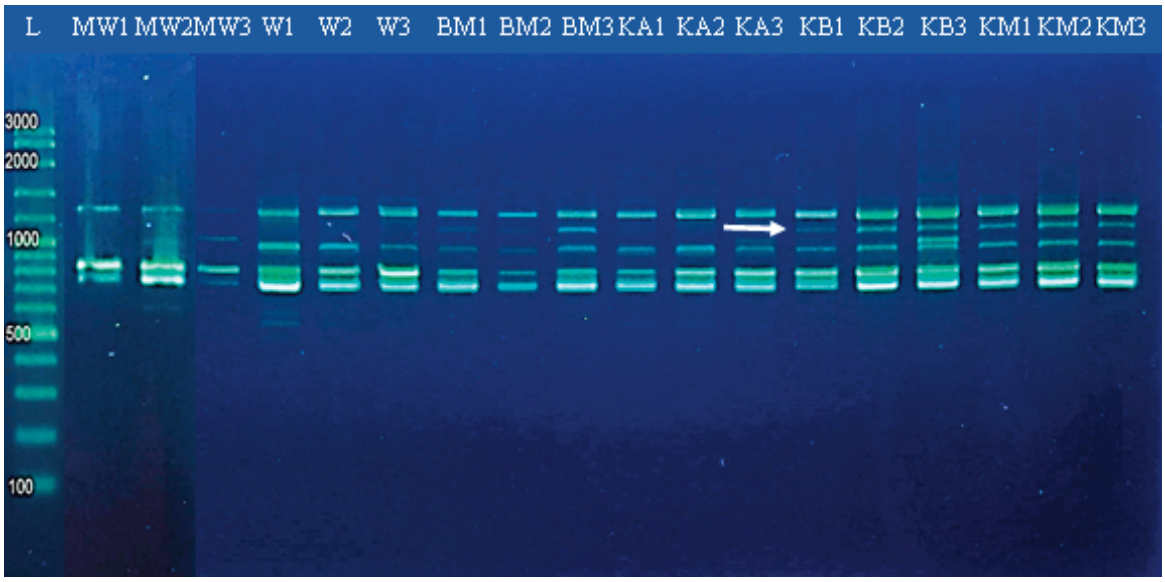

Figure 3. RAPD profile of OPC 19 primer showing type III or bands in male parent and its offspring (red arrowhead) and type VII or new bands in the offspring (white arrowhead). Croton parents (Mawar-MW/♀ and Walet-W/♂) and their offspring (Black Marlet-BM, Kingkit 1-KA, Kingkit 2-KB, and Kamaratih-KM). The number following the labels indicates replication. L = ladder 100 bp.

(Enjelina *et al*., 2018). Appearance or disappearance of the bands may indicate the occurrence of genetic changes in the genome of the hybrids either through the loss or rearrangement of some of their nucleotides (Li and Quiros, 2001; Farzaneh *et al*., 2010).

It was unclear whether the bands appearing in the parents with RAPD markers were dominant homozygote or heterozygote because RAPD markers are dominant markers, where dominant homozygote markers are indistinguishable from heterozygote markers (Lynch and Milligan, 1994). However, by looking at the bands' patterns produced by the offspring, it could be identified whether the bands on the parent were dominant homozygote or recessive (Xu, 2010). According to Williams *et al*. (1990), method of distinguishing homozygote or heterozygote alleles by using RAPD markers can be done on parents that have different genotypes. For example, Walet had heterozygote bands in the primer OPD 8 at 475, 700, 1350, 1500bp (Figure 2). Besides, Walet had dominant homozygote bands in the primer OPC 19 at 950 bp, since those bands were shared to all of offspring (Figure 3). Based on RAPD bands pattern, Walet was dominant to the offspring. Black Marlet was morphologically closed to male parent as shown in the RAPD results.

Based on the genetic distance (Table 4), the male parent was closer to the offspring which ranged from 0.176 to 0.234. The genetic distance between Mawar and Walet was 0.348, and the genetic distance between

Table 4. Croton genetic distance in the parents and their offspring

MW	W	BM	KA	KB	KM	
0.000						MW
0.348	0.000					W
0.314	0.178	0.000				BM
0.322	0.176	0.086	0.000			KA
0.372	0.229	0.148	0.098	0.000		KB
0.321	0.234	0.199	0.117	0.101	0.000	KM

Mawar and its offspring ranged from 0.314 to 0.372. According to the results, it can be suggested that hybridization between the parents having long genetic distance can produce offspring that have long genetic distance from one of the parents or both of them. The genetic distance between Black Marlet and Kingkit A was the lowest reaching 0.086. It indicated that Black Marlet was closer to Kingkit A. Then, Kingkit A and Kingkit B had genetic distance of 0.098. It showed that Kingkit A was closer to Kingkit B, and they had similar leaf shape (Figure 1). All of offspring had short genetic distance from male parent (Walet). According to Kobayashi *et al*. (1996), genetic distance between the parents can account morphological variations among the hybrids. Based on the genetic distance, Mawar and Walet were quite distinct and therefore used for their desirable characteristics in the croton breeding program.

The RAPD technique is simple and rapid, it can be used to identify the offspring of croton hybridization. The problem of this technique is reproducibility and reliability, so it can be improved by the conversion of RAPD into SCAR markers, by developing longer and consequently, more specific primers from RAPD sequences (Rajesh *et al*., 2014).

CONCLUSIONS

This study showed that the RAPD technique was effective for the identification of the offspring of croton hybridization. From this study, Mawar as female parent had the highest polymorphic bands percentage of 69.01%, while the polymorphic bands percentage of Walet as male parent and the offspring ranged from 31.15 % to 43.94%. The values of genetic distance confirmed close relationship between Wallet and the offspring.

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