



Studies on genetic variability of *Capsicum frutescens* var. Cakra Hijau induced by ethyl methane sulphonate (EMS) using SSR marker

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ABSTRACT

Genetic variability of wild type and EMS induced mutant cayenne peppers (*Capsicum frutescens*) var. Cakra Hijau is studied morphological traits and simple sequence repeat (SSR) markers. Phenotypic characters were investigated using standard pepper descriptors. The polymorphic SSR marker Ca19, Ca26, Ca52, Ca56, and Ca96 were analyzed using unweighted pair group methods with arithmetic means (UPGMA) with Jaccard's similarity index. The Polymorphic information content (PIC) value ranging from 0-0.228 with the highest index on Ca96. The Morphological traits showed of clusters (6 clades) with a lower cut-off value (0.2568) than SSR-based dendrogram did (4 clades; 0.5108). Therefore, genetic variability induced by EMS mutant were differentiated using morphological and SSR genotyping

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INTRODUCTION

Pepper (*Capsicum* sp.) is an economically important crop belonging to the Solanaceae family. The genus *Capsicum* has five domesticated species (*C. annuum*, *C. frutescens*, *C. chinense*, *C. pubescence*, and *C. baccatum*) of which *C. annuum* and *C. frutescens* are the two most widely cultivated species worldwide [1,2]. Besides, *C. frutescens* consists of many cultivars but Cakra Hijau is one of which has been popular to be planted in Indonesia by the high resistance of several pests, as well as air and insect-borne diseases [3]. However, the systematic position of that cultivar remains in doubt, and information available based on the previous statement is confined only in morphological and agronomic traits which leads to the *C. frutescens* belonging cultivar. In aftermath of lack of database, it may enable the plant breeders who intend to produce a distinct breed from two cultivars in one species yet prevention of gene flow among species may happen to offspring sterility [4].

Phenotypic characters from the pepper, either vegetatively and generatively, have been used widely to distinguish between pepper genotypes and then classify them into groups [5&6]. Describing and clustering germplasm by using

phenotypic characters is fundamental in any characterization programme [7]. The presence of environmental factors may lead to many difficulties for distinguishing between individuals that are closely related through phenotypic data [8]. It has, therefore, become inevitable to do molecular characterization which has been proven to be very objective and independent of environmental factors [9].

There are many methods to assess molecular genetic variability. One of which stands out as exceptional in genetic diversity studies is the simple sequence repeat (SSR) analysis. It has been widely applied for genetic diversity assessment for germplasm because of their ability to detect multi-allelic forms of variation and are reproducible thus this analysis is more effective for inbred lines and breeding materials with special attributes [10]. Moreover, the SSR as molecular markers offers profound advances in cost and efficiencies [11].

Following the probability of mutagenic effect of Ethyl Methane Sulphonate (EMS) in previous research [12] in Cakra Hijau designated Genotype 1 (G1), this research also investigated the effect on microsatellite polymorphisms since insertion and deletion may occur [13].

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Despite the popularity of the species in all agro-ecological zones, very little has been achieved in the improvement of the indigenous cultivars probably due to the limited database of genetic diversity within the cultivars. The more the extent of genetic variability within the species, it is better for the breeder in developing elite cultivars through careful selection of superior parents. An understanding of the genetic variability of a population, through the use of both morphological and molecular markers, is of critical importance in developing effective strategies for germplasm conservation and breeding purpose. Therefore, this study aimed to evaluate genetic variability between wild type and mutant peppers which were induced by a mutagenic agent such as EMS, as well as each individual using morphological and molecular analysis (SSR markers).

MATERIAL AND METHODS

The popular chili variety Cakra Hijau grown in greenhouse at Plant Genetics Laboratory, Department of Biology, the University of Brawijaya during Jan 2019. A total of 17 mutants progenies were evaluated which consists of three wild-type and 14 mutant individuals of *Capsicum frutescens* var. Cakra Hijau (wild types seeds were obtained by PT BISI International Tbk).

EMS-mediated Mutation Treatment

The wild chilli seeds were soaked in distilled water for 8 hours and treated with EMS concentration of 0% (control) and 0.01% (mutant). The mutants came from 0.01 % ethyl methane sulphonate (EMS) treatment. The seeds were removed from remaining EMS by 1 % sodium thiosulphate immersion for 5 minutes, running water for 15 minutes, and air-dried. Subsequently, the seeds were immediately germinated and 3 control and 14 mutant plants. The healthy plants were selected randomly and transplanted on the standard potting mixers which consist of soil, compost, and husk media.

Morphology Descriptors Assessment

Morphology of wild and all mutant offspring were measured using quantitative characters at various growth stages like stalk, vegetative, flowering, and fruiting stage according to IPGRI, AVDRC, CATIE Capsicum descriptors [14]. The stalk descriptions include plant height (cm), diameter (cm), total dichotomous branches, total nodes, primary and secondary branch's length. The leaves include leaf length and width (9th node; cm), cotyledon leaves number, and axillary bud number. First flowering periods, as well as fruit set periods and numbers, were also measured.

Capsaicin Estimation

The total capsaicin content from old-green fruits (30 DAP) was extracted by absolute ethanol (0.5 gram per 5 ml), transferred into a microtitre plate, and subjected to microplate reader spectrophotometry at 280 nm. The standard capsaicin was commercially obtained from Sigma Co and a stock of 1 mg/ml

was prepared. Working standards were made from the stock ranging from 20 ppm/ml to 100 ppm/ml in concentration. Total capsaicin was analyzed statistically by descriptive qualitative analysis to compare the pungency level among the samples.

DNA Extraction

DNA extracted using Cetyl trimethyl ammonium bromide (CTAB) method [15] with some modifications. The pre-chilled 50mg leaf is crushed by mortar and pestle using liquid nitrogen. The powder was homogenized by 700 µl CTAB buffer (100 mM Tris-HCl pH 8.0; 1.4 mM NaCl; 20 mM EDTA pH 8.0; 2 % PVP; and 2 % CTAB) supplemented by 2 % -mercaptoethanol and transferred to a 1.5 ml sterile microtube. The homogenate was incubated (65 °C; 30 minutes) and cooled down (room temperature; 5 minutes). Afterward, the homogenate was centrifuged (13000 rpm; 25 °C; 15 minutes) and the supernatant was transferred to a new tube. The supernatant was added by a volume of PCI (phenol: chloroform: isoamyl alcohol; 25: 24: 1; v/v/v), mixed by vortexing, and centrifuged for 5 minutes. The aqueous phase was removed to a new tube, added by a volume of CI (chloroform: isoamyl alcohol; 24: 1; v/v), vortexed, and centrifuged for 10 minutes. The upper layer was transferred to a new tube and added by 1000 µl cold isopropanol, mixed by flicking, incubated (-20 °C; 60 minutes), and centrifuged directly for 15 minutes. The supernatant was discarded and the pellet was washed with 70 % alcohol then centrifuged. The supernatant was discarded and the pellet was air-dried for 12 hours before resuspending the pellet in 40-50 µl sterile ddH₂O.

The quality of DNA verified using a nano spectrophotometer and the quality was also done using 1 % agarose gel along with lambda DNA. The DNA was then diluted with 500-800 µl concentration and used for PCR analysis.

Screening the Mutant Population using SSR Markers

The SSR primer pairs Ca19, Ca26, Ca52, Ca56, and Ca96 were synthesized first Base, Malaysia, and reported [16] were used for PCR optimization (Table 1). The Amplification of microsatellite primers was carried out in 10 µl aliquot by following PCR running program: pre-denaturation (94 °C; 2 minutes); 30 cycles of denaturation (94 °C; 1 minute), annealing (temperature refers to Table 1; 1 minute), extension (72 °C; 1 minute); and post-extension (72 °C; 7 minutes). The amplicon performance was assessed by a simple 1.5 % agarose gel electrophoresis for initial screening and 2.5 % for obtaining clear and distinct PCR pattern.

Construction of Phylogenetic Trees

The morphological data and capsaicin content were classified into 1, 2, 3, and 4 scores and used for the dendrogram construction. The scores were converted into binary numbers (0, 1). The PCR amplicon obtained in SSR analysis was assigned as alleles of SSR loci and scored into binary format (0, 1). The dendrogram is constructed using phenotype and SSR markers data using a simple clustering Unweighted Pair Group Method

with Arithmetic Mean (UPGMA) based on Jaccard's similarity (0-100%). The Clusters from the dendrogram were determined from the cut-off value of an average similarity.

All the amplicon scored from gel electrophoresis were analyzed individually to determine the reproducibility of each primer towards all samples tested. The PIC value of each marker was determined by converting binary data through a formula expressed in Equation 1. Clustering analysis from highly reproducible primers and the highest PIC value was performed by Unweighted Pair Group Method with Arithmetic Mean (UPGMA) and Jaccard's Similarity, ranging from 0 to 1 [17]. Clustering analysis was conducted using PAST 3.14 software.

$$\text{Polymorphic Information Content (PIC)} = 2 \times f_i \times (1 - f_i) \quad (\text{Equation 1})$$

Where f_i = (sum of alleles or bands)/no. samples.

RESULTS

Clustering Results Based on Morphological Data of the Control and Mutant Peppers

The unweighted pair group method with arithmetic mean (UPGMA) algorithm was carried out for clustering wild type and mutant individuals based on Bray-Curtis' similarity index. The dendrogram tree (Figure 2) showed six major clusters which were set by a 0.2568 cut off value, as well as the plant photos

Table 1: Details of microsatellite (SSR) primers used in genetic variability analysis

Primer name	Primer sequence (5'-3')		Annealing temperature (°C)
	Forward	Reverse	
Ca19	CCGCAATGGCA GTATGATCT	CGGCTCTATCT ACAACGGTG	55.1
Ca26*	CGCATATAGG CAGATCAAAT	TGACTCAAAT GCTCTCTGAA	50.6
Ca52	TAGCAGAGG ACCAGTTAGCA	ATGTTCTGAG TCCACGATGC	55.1
Ca56*	<u>CTTCGCATAT</u> <u>GGCAGATCA</u>	TCTCTGTGGC TGACTCAAAT	52.7
Ca96*	CGCATATAGG CAGATCAAAT	AATCTCTGTG GCTGACTCAA	51.8

*underlined some nucleotide sequence (forward and reverse primers, respectively) means a primer has the same template site

representing each cluster. Group I only contains single mutant 8 which was separate from the cluster II.

The mutant phenotypes (Figure 1, Table 2) were generating diverse groups when compared to control. The control group having high similarity which was concentrated on a cluster III (CH1 and CH2) though only CH3 forming cluster IV from a minor cut-off threshold. Cluster III, consisting of the biggest number of the pepper, was predominantly branched into two sub-cluster, III. A consisting mutant 2, 7, 4, and two first controls, as well as mutant 3, 5, and 14 grouped in III. B. Therefore, six out of 14 mutant plants pertained to a small difference of morphological traits from control plants. The shortest primary branch's length was the trait derived which formed synapomorphy with the range of 0-1.5 cm, as well as no secondary branch observed (0 cm; data not shown).

Capsaicin content of Control and Mutant Cakra Hijau Peppers

Out of 14 mutants, only 11 mutants were able to enter the reproducing phase and pungency level were reported to vary among mutant peppers compare to wild type. However, no total capsaicin had been successfully confirmed from mutant 3, 5, and 14, the mutant 3 and 14 were failed to enter the generative phase. The mutant 14 showed a late flowering (77 days after plantation/DAP) and followed by fruit set failures (data not shown). The total capsaicin of the mutants was relatively lower than the control groups. The most pungent pepper was grouped in CH1 which shared a minor margin with mutant 11 at the second position, accounting for 113,000 and 112,000 Schoville Heat Unit (SHU). The capsaicin content was approximately twice as high as mutant 7 which was reported as the lowest pungent pepper among all (Figure 3). Moreover, other mutants, such as 2, 8, and 12, also showed significantly lower pungency level with a concentration below 80,000 SHU.

The SSR Profile of Wild and Mutant Progenies

A total of five SSR primers were finally used to differentiated wild and mutant genotypes and subsequently used for clustering analysis. More than 90% of wild and mutant samples are amplified using SSR primers and the monomorphic alleles/bands were eliminated for genetic distance analysis. The mutant Ca56 and Ca96 which have shown a polymorphic pattern and showing a unique SSR profile. The SSR marker Ca96 exhibited

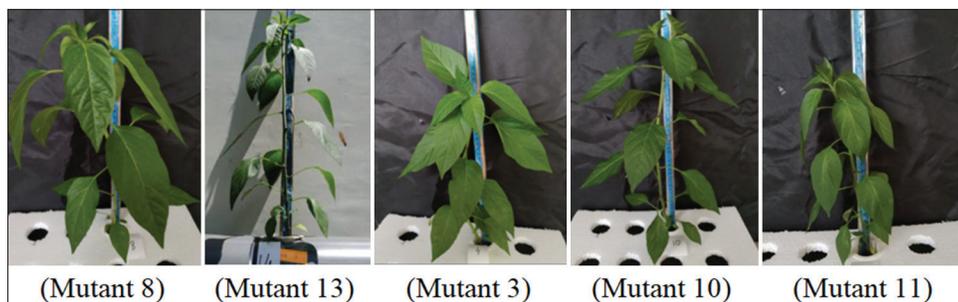


Figure1: Mutants of *Capsicum frutescens* var. Cakra Hijau induced by ethyl methane sulphonate (EMS)

Table 2: Morphological Characteristics of Mutant Plants G1/01

Character	Observation Variable	Hot Pepper Chili G1 / 01														
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
Stem	Plant Height (cm)	37	38	39	34	36	35	44	42	41	41	39.5	27.5	22.5	36	38
	Rod diameter (cm)	0.80	0.80	0.64	0.64	0.73	0.70	0.76	0.70	0.64	0.67	0.67	0.48	0.48	0.64	0.64
	Number of branches	1	1	1	0	1	0	2	1	1	2	0	0	0	2	0
	Number of Nodes / segments	17	17	16	15	17	17	17	15	11	15	18	14	13	15	15
	Length of the First Branch (cm)	0.5	0.5	1	0	1.5	0	4	1	3	1.5	0	0	0	1.5	0
	Length of the Second Branch (cm)	0	0	0	0	0	0	1.5	0	0	1	0	0	0	2	0
Leaf	Leaf Length (9th node) (cm)	13	12.8	11.5	12	11.5	11.5	13.3	12	8.8	11.5	11	8.7	8.5	10	13.5
	Leaf Width (9th node) (cm)	6	6.3	5.5	5.5	5.5	5.3	6.4	5.7	4.5	5.5	5.5	4.5	4	4.5	5.5
	Number of cotyledon leaves	1	2	2	2	2	2	2	2	1	1	0	2	0	1	3
	Axillary shoot	0	0	0	0	0	0	2	3	1	2	3	3	0	3	0
Flower	Flowering (HST)	58	77	70	-	51	77	51	55	51	51	65	58	69	51	-
Fruit	Amount of fruit	9	-	-	-	2	-	4	2	11	9	10	6	4	9	-
	Fruit ripening time	43	-	-	-	55	-	48	65	49	48	46	43	46	45	-

(1-15) – Mutants induced by Ethyl Methane Sulphonate (EMS)

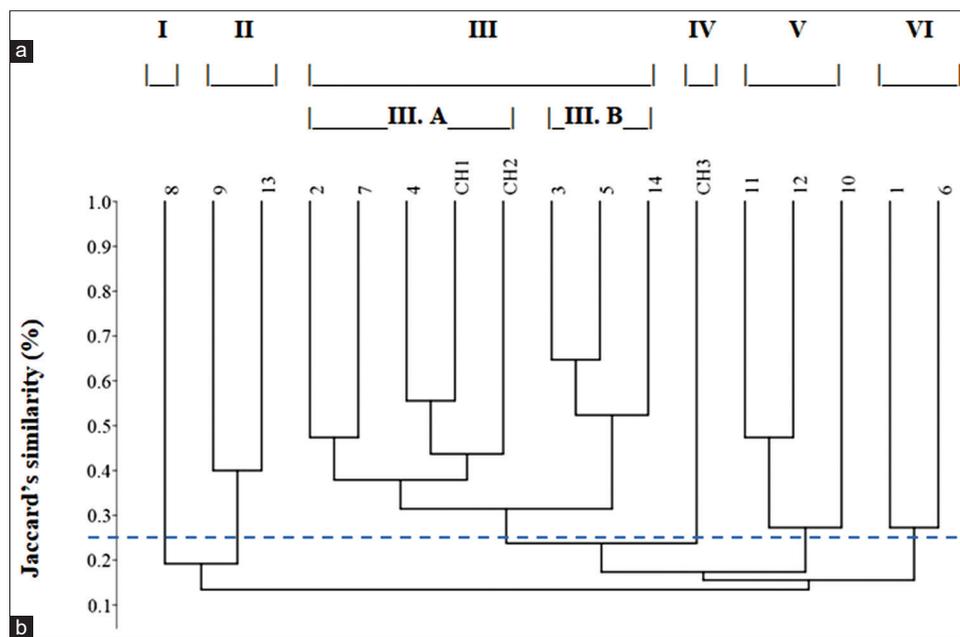


Figure 2: Dendrogram based on Bray-Curtis similarity describing the morphological relationship between control groups (CH1-CH3) and mutant groups (1-14). A dashed line indicates a cutoff similarity value of 25.68 %

the most reproducible among the SSR markers with alleles numbers ranged from 1 to 4 and the highest number of the polymorphic band (2 alleles), as well as the highest polymorphic information content (PIC), accounted for 0.228, slightly higher than that of Ca56 (Table 3).

The primers Ca56, and Ca96 mostly producing polymorphic allele when compared to Ca 26 (Figure 4). The two alleles 1178 and 92-bp band were clustered as a polymorphic class while a unique allele noticed in the SSR primer Ca96. Therefore, Ca56 and Ca96 SSR primers are contributing more for the phylogenetic construction.

Figure 5 shows a dendrogram tree based on five sets of SSR markers using UPGMA analysis and Jaccard's similarity. By comparing to the morphology-based dendrogram tree, the SSR-based dendrogram produced fewer clusters with Jaccard cutoff

value above 50 %. Cluster I which only consists of mutant 14 were distinctly unrelated from the other three clusters (II, III, and IV). Then, most of the plants were classified in Cluster II. Surprisingly, only two mutants consistently showed a genetic relation with two controls, namely mutant 4 and 7 towards CH1 and CH2. Meanwhile, by looking at Figure 2, the presence of a polymorphic 1178-bp band observed corresponds to several lanes (CH3, 2, 3, 5, 6, and 11) grouped into cluster IV.

DISCUSSION

The DNA markers are routinely used to perform genetic variability assessment and hence present study SSR markers were employed for characterization of mutants. The SSR makers showed effective discriminating power is much appreciable in previous studies [18&19]. The SSR markers reveal a wide range of variability compared to other markers

such as random amplified polymorphic DNA (RAPD) [20]. The Ethyl methane sulphonate (EMS) has been widely used mutagen in vegetable crops because of its high effectiveness and rate of induction of point mutation [21&22]. The EMS mutant caused both formations new alleles and loss of alleles of several genotypes in *Capsicum frutescens* L. [16]. The EMS mutant lines in the popular chili variety Cakra Hijau recorded significant morphological variability when compared to wild

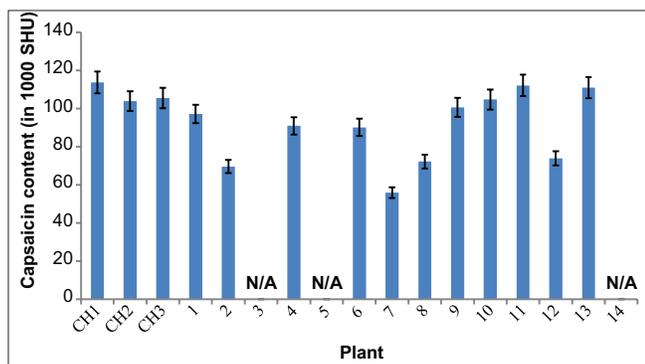


Figure 3: Capsaicin content of Cakra Hijau pepper between mutant and control groups. N/A labels indicate the plant failed to produce fruits while flowering terminated. The cap of each bar shows error standard threshold at 5 %

type while testing with SSR marker. The primer Ca 96 helped to differentiate the mutant type from the wild type.

The reliability of SSR markers detecting molecular diversity depends on the length of the primer itself. [23] showed 20 bp or more primers tend to have a better degree of polymorphism than those of 12-20 bp and less than 12 bp. Besides, the primers used are also able to amplify alleles with a high transferability which can be used in many species as well as a high reproducibility [24]. In the present study, five sets of primers were used specifically for *Capsicum* genera and 20 bp in length, named as Ca19, Ca26, Ca52, Ca56, and Ca96. The SSR primers amplified successfully with different reproducibility levels. The SSR markers which generated single band each sample or/and showed monomorphic alleles were eliminated for constructing dendrogram trees, such as Ca19, Ca26, and Ca52 (Table 3). Meanwhile, two latest primer sets viz., Ca56 and Ca96 were yielding polymorphic bands and hence produced well-defined dendrogram which helped for the differentiation of mutant from wild type

This SSR profile generated in this study revealed different allele sizes, ranging from 56 bp to more than 1000 bp. The SSR primer Ca96 generated up to 4 alleles where a unique 211-bp band was only seen in mutant No.14. Hence the

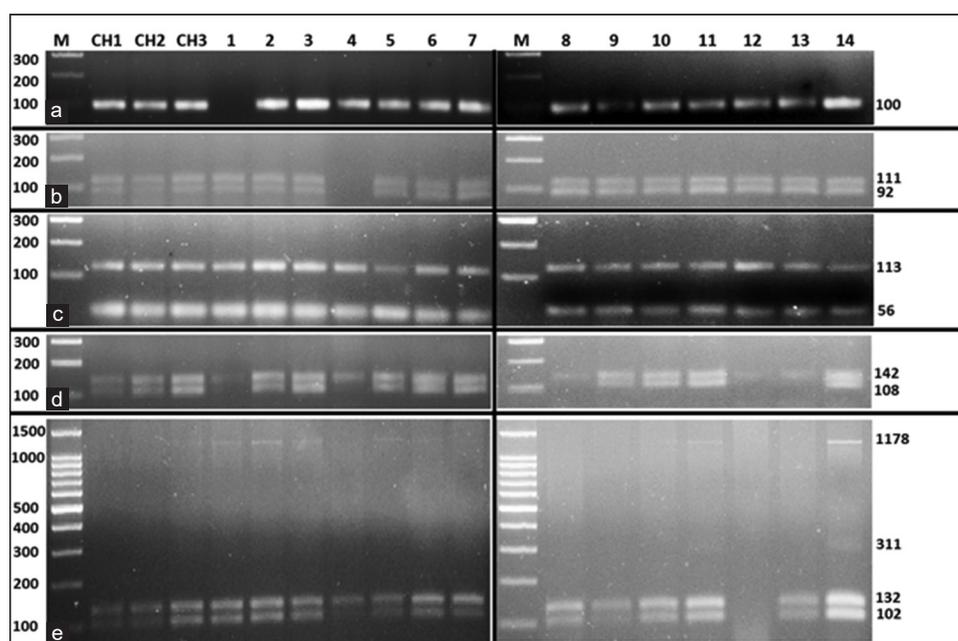


Figure 4: Multi-locus SSR profiles of *Capsicum frutescens* var. Cakra Hijau using different SSR marker primers, namely (a) Ca19; (b) Ca26; (c) Ca52; (d) Ca56; and (e) Ca96 from control groups (CH1-CH3) and mutant (1-14), as well as band ladders (M lane; DNA mark 100 bp; Jena Bioscience)

Table 3: Reproducibility of SSR primers towards *Capsicum frutescens* L. in all samples (Wild types and mutants)

SSR primer	Estimated band size (bp)	Number of monomorphic bands	Number of polymorphic bands	Unique bands	Successful amplification (%)	PIC*
Ca19	100	1	0	0	92.85	0.111
Ca26	92; 111	2	0	0	92.85	0.111
Ca52	56; 113	2	0	0	100	0
Ca56	108; 142	1	1	0	100	0.208
Ca96	102; 132; 211; 1178	1	2	1	92.85	0.228

*PIC: Polymorphic Information Content (PIC)

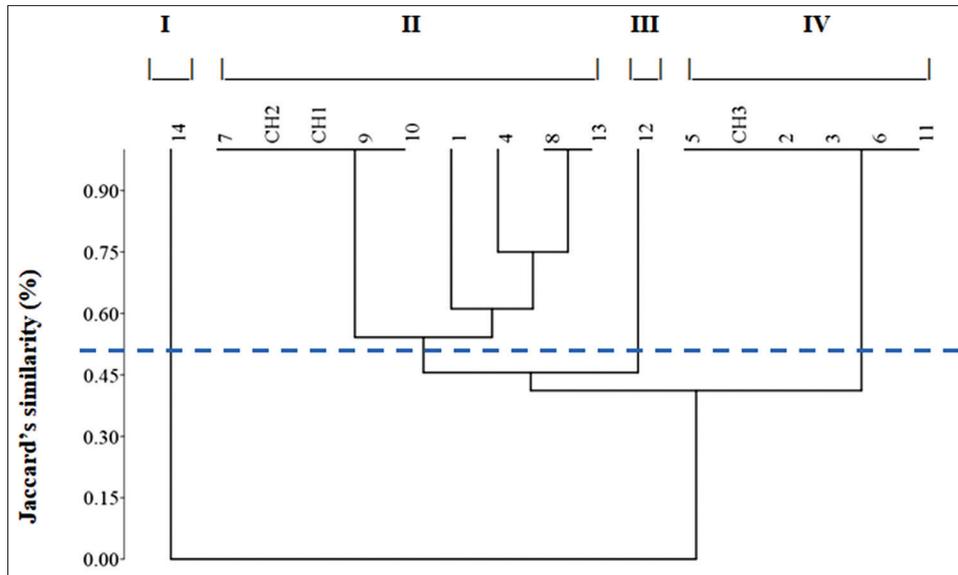


Figure 5: Dendrogram based on Jaccard's similarity describing the genetic relationship between control groups (CH1-CH3) and mutant groups (1-14) using SSR marker results. A dashed line indicates a cutoff similarity value of 51,08 %

mutant No 14 placed as an outgroup separately from all other wild plants investigated. The EMS brings about extension and shrinkage microsatellite alleles length by inserting or deleting nucleotides [25]. Designing primers' strategies for SSR have been well described by several previous studies. [26] mentioned two alternatives proposed for designing markers, firstly, selection of primers for their physical position on the genome if complete whole genome sequence is available in a web-based database, and secondly, prediction can be performed from genome sequencing analysis and mapping exercise in advance.

The polymorphic information content (PIC) calculated is used to measure the usefulness of SSR primers/markers for certain molecular studies [27]. There are three classifications of PIC quality based on the index range, namely highly informative (above 0.5), reasonably informative (between 0.25 and 0.5), and slightly informative (less than 0.25) [28]. The PIC value in this study ranged from 0-0.228 which means all markers used were not considered as a powerful marker even though using the same primers as a previous study [16] has done successfully for evaluating genetic variability of other genotypes. However, two out of five primers (Ca56 and Ca96) tested have been continued for constructing phylogenetic due to the highest PIC score.

Mutation induced by EMS is thought to cause notable genetic variation in some mutant peppers (Figure 6). With five SSR markers used to evaluate genetic variability Surprisingly, in Ca96 dendrogram, one line (mutant 12) tended to be unrelated from the second sub-major cluster consisted of the remaining plants while mutant 14 had a far genetic relationship from the cluster. Walker and Walker (2014) [29] argued that though mutagens show the deleterious impact on plant treated in the first place of plant breeding, genetic variation by thorough screening and selection enables



Figure 6: Morphological observation of the wild (A) and mutant type (B) of Cakra Hijau.

some plants to survive better in the scathe and undesirable environment.

CONTRIBUTION OF AUTHORS

ELA and KS conceived the idea and designed the work, NR executed the work, RK carried out SSR analysis, ELA, KS and NR wrote the paper.

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