GENETIC VARIABILITY OF BLACK PEPPER (*Piper nigrum* L.) PUTATIVE MUTANTS BASED ON MORPHOLOGY AND SSR MARKERS

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ABSTRACT

Genetic distance or genetic relationship among putative mutants resulting from irradiated black pepper need to be understood to assist in the selection of superior variety. This study aims to analyze and evaluate genetic variability of mutants resulting from gamma irradiation on Ciinten variety based on morphological and SSR markers. The 27 putative mutants and its original variety were used in this study. Morphological characters observed were plant height, leaf size, number of leaves, nodes and branches. Nine SSR primers were screened to analyze genetic variability.The result on morphological characters showed there are changes on mutant leaf characters from the original/unirradiated Ciinten variety on leaf shape, leaf base and leaf margin. At 18.15% similarity coefficient, 27 mutants and the original variety were clusterd into one group (Group I) and separated from mutant number 15 (Group II). Further clustering of group I, at 70 % similarity coefficient, the mutant plants wereclustered into two subgroups, i.e. subgroup I with three mutants (3, 9 and 17) and subgr oup II consist of the Ciinten and 23 mutants. SSR markers could distinguish the original Ciinten variety from the putative mutants. At 63% similarity coefficient, the original Ciinten variety (Group I) is clearly separated from all of its 27 putative mutants (Group II). Molecular (SSR) markers have proven to be more accurate in identifying genetic variability than morphological characters.

Keywords: Black pepper, enhance variability, gamma irradiation, genetic distance, putative mutant, SSR markers

INTRODUCTION

Black pepper (*Piper nigrum* L.) belongs to the family Piperaceae, is a perennial climbing vine grown for its berries and it is extensively used as spices. It is one of the oldest and most popular spices in the world, with total world consumption reaching more than 350 000 ton annually (IPC 2013). Black pepper was introduced to Indonesia during ancient time, probably from India. The species is a predominantly self-pollinated (geitonogamy) perennial vine, propagated by cuttings (Krishnamoorthy & Parthasarathy, 2009), so that gene interchange under natural condition is presumably rare, thereforeit possesses narrow genetic base.

Genetic variability is an important factor in a breeding program. The success of breeding is determined by the availability of genetic material with broad genetic variability (Frankel & Brown, 1984). Genetic variability can be enhanced by mutation using gamma irradiation (Alhoowalia & Maluszynski, 2000). To measure the success of mutation induction, it is necessary to analyze genetic variability.

Studies on genetic variability can be performed using morphological, biochemical or molecular properties. In perennial crops, such as black pepper, the analysis using morphological characters is less favourable, since the morphological characters in the vegetative phase are generally few, whereas for generative characters would take long time to appear. In addition, the appearance of morphological characters are highly influenced by environment, plant development, also by the dominant-recessive and the interaction of epistasis and pleiotropic effects (Idrees & Irshad, 2017). Moreover, genetic analysis based on morphological characters is also a time-consuming and labor-intensive task (Cooke, 1984). However, the advantage of using morphological characters is that the characters are visually visible and can use simple tools.

Biochemical marker, such as isozyme is an effective marker, co dominat inheritance so that can be used to distinguish homozygote and heterozygote (McDonald & McDermont, 1993) can can be apply easily with relatively low cost (Bermawiedan Pool, 1991; Mondini *et al*., 2009). But, the number of markers are limited, the expression is influenced by environment and plant development stage (McDonald & McDermont, 1993; Mangolin *et al*., 1997; Garkava *et al*., 2000) and low genetic diversity at the species or subspecies level (Rafinski & Babik, 2000).

Meanwhile, there have been many reports describing the benefits of molecular markers, such as the expression of such markers are independent of environmental factors, dominant-recessive relationship and epistatic-pleiotropic interaction, highly polymorphic, and the result from the analyses are more reliable and repeatable. Various molecular techniques have been developed to analyze genetic diversity, which can be classified into two groups, based on their abilities to show homozygosity (dominant marker) or heterozygosity (co-dominant marker) (Hartl, 1988). Some of the thedominant markers commonly used are Random Amplified Polymorphic DNA (RAPD) (Williams *et al*., 1990), Inter-simple sequence repeat (ISSR) (Zietkiewicz *et al*., 1994) and Amplified Fragment Length Polymorphisms (AFLP) (Vos *et al*., 1995), whereas some of the co-dominant markers are: Restriction Fragment Length Polymorphisms (RFLP) (Botstein *et al*., 1980), Microsatellites/Simple sequence repeat (SSR) (Akkaya *et al.,* 1992); Expressed sequence tag (EST) (Adams *et al*., 1991) and Single Nucleotide Polymorphism (SNP) (Jordan & Humphries, 1994). These markers can be used for various purposes, such as genetic mapping, detection of mutation, cultivar identification and marker assisted selection (Hartl & Jones, 2005).

One of the molecular markers that has been widely used in genetic studies is the SSR markers. SSR is a recurrent sequence of DNA with high polymorphism, and inherited according to Mendelian segregation law (Powell *et al.*, 1996; Hancock, 1999). Other advantages of the SSR markers areneed little amount of DNA, has forward and reverse primers, easier to work and lower cost. Therefore SSR markers are used to analyze the genetic diversity of the Ciintenputative mutants resulting from mutation induction. This study aims to analyze and evaluate the genetic relationship between individual black pepper plants resulting from gamma irradiation based on morphological and SSR markers.

MATERIALS AND METHODS

Plant Materials

Plant materials used in this study were 27 putative mutants derived from mutation using Co^{60} gamma rays on Ciinten variety, at seed and seed with radicle phases. Ciinten is one of the superior varieties with a number of superior agronomic characters such as long spike, hight 1000 fruits and seed weights, high percentage of fruit sets, big size of fruit and seeds, but lower in number of spike per vine and rather susceptible to foot rot (*Phytophthoracapsici*) disease (Bermawie *et al.*, 2013). The putative mutants were selected from irradiation treatments around LD 50 (25 and 50 Gy) (Meilawati *et al.*, 2016).

M**orphological Observation**

All the putative mutants derived from M1 were planted in polybag and put in a shade house. Observation was carried out on seedlings, eight months after planting on several morphological characters such as plant height, leaf size and number, number of nodes, branch and leaf shape. Methods of observation followed descriptor for black pepper (IPGRI, 1995).

Molecular Analyses using SSR Markers

DNA extraction

Genomic DNA was isolated from freshly collected young leaves of 3-4 months old putative mutant seedlings using the CTAB (cetyltrimethyl ammonium bromide) (Doyle & Doyle, 1990) with a few modifications. 0.5 g of young leaf tissue was ground in 5% Polyvinylpyrrolidine to fine powder using sterile pestle and mortar and suspended in 700 µl of preheated 4% CTAB buffer (2% CTAB, 0.1 M Tris pH 8.0, 20 mM EDTA; 1.4 M NaCl, and 0.5% β-mercapto ethanol). The suspension was incubated at 65°C for 30 min with occasional inversion, then incubated at room temperature for 10 min. An equal volume of chloroform: isoamyl alcohol (24:1 v/v) mixture was added to the suspension and centrifuged at 12,000 rpm for 20 min. The aqueous phase was transferred to a new microfuge tube and added with equal volume of chloroform: isoamyl alcohol (24:1) mixture at 10,000 rpm for 15 min. The Chloroform: IAA extraction step was repeated twice. The aqueous phase was transferred to a fresh tube containing 0.6 volumes of ice-cold isopropanol. After centrifugation, the pellet was washed in 70% ethanol and air dried and dissolved in TE buffer (10 mMTris; 1 mM EDTA, pH 8.0). DNA was quantified using UV-spectrophotometer and diluted to 50 ng/µl and used in PCR.

Primer Screening

NineSSR (psol) primers (Yoshida *et al.*, 2014) were initially used for primer screening. Five primers were selected for SSR analysis based on their ability to generate clear and distinct polymorphic bands. Three primers which generate smeared or monomophicbands were not used for further analyses.

DNA Amplification

PCR reaction was performed on five selected primers. Each PCR reaction contains 2 µL DNA, 6 µL PCR Mix, 0.5 µL SSR, 4 µL deionized water. PCR was runnedusing Thermal Cycler (Thermo, USA), with pre-denaturation at 94 ºC for 4 min followed by 35 cycles each of denaturation at 94 ºC for 4 min, annealing at 37 ºC for 1 min, amplification at 72 ºC for 1 min, extention 72 ºC for 5 min.

Electrophoresis of Amplified Products

Electrophoresis was performed using vertical polyacrylamide gel electrophoresis withTBE buffer at 80 V, for 60 min. Gel was stained ethidium bromide for 10 second, then washed with distilled water for 15 min, and visualized under the UV transilluminatorin gel documentation system (Biorad, USA).

Data Analyses

For morphological characters, data was analyzed using minitab. For SSR markers, the bands were conversed to matrix data. In the matrix data, the presence of band was scored 1 and null (0) for absence. The data was analyzed with SIMQUAL (*Similarity for Qualitative Data*) of NTSYS-pc version 2.01 program and similarities between putative mutant was estimated using DICE (Nei & Li, 1979). Dendrogram was created from the resultant similarity matrices using the UPGMA method following (*Sequencial, Agglomerative, Hierarchical and Nested*) (SAHN) function of NTSYS-pc (Version 2.01).

RESULTS

Morphological Observation

The observation of the leaf character indicates that there are variation of some leaf characters in the putative mutants compared to the unirradiated Ciinten variety (the origin). The variation observedonplant height, leaf length, leaf width, number of leaf, number of node, number of branch, leaf shape, leaf base and leaf margin (Table 1).

The variation on leaf shape on the putative mutants are shown in Table 2. Nine putative mutants (33.33%) derived from treatment at 25 Gy on seeds with radicles; and 18 individuals (66.67%) consisted of mutants derived from treatment at 25 and 50 Gy. Four out of nine mutants (44.4%) derived from seeds with radicles, differ on leaf shape from the origin.While from seed phases, eleven out of 18 mutants (55.6%) showing variation on leaf shape. This indicated the percentage of variation on leaf shape of mutants form seed phase is slightly higher than that from seeds with radicles. This may be related inability of majority seeds with radicles could not grow (Meilawati*et al*. 2015), especially at dose 50 Gy. Leaf shape of original Ciinten variety is ovate, while five of the putative mutants are ovate elliptic, eight of the putative mutants are ovate lanceolate and, and two putative mutants are cordate (Table2, Figure 1).

Changes are also observed on leaf base. Leaf base of the Ciinten variety is mostly rounded, while five of the putative mutants are ovate elliptic, eight mutants are ovate lanceolate, and two mutants are cordate (Table 3; Figure 2). Not much variation on leaf margin. Leaf margin of the orginal Ciinten variety is entire/even and while on some of the mutants are wavy (Table 3; Figure 3).

Table 1. Morphological caharcteristics of Ciinten variety and its putative mutants

No	Putative mutant	Plant height (cm)	Leaf length (cm)	Leaf width (cm)	No. of leaf	No. of node	Leaf thichness	Stem diameter	No. branch
1	MP1(I.D1.3)	8.5	7.5	4.5	13	6	0.24	2.48	1
2	MP2(I.D1.4)	14.4	8.9	6.6	8	7	0.28	2.98	0
3	MP3(I.D1.5)	23.5	8.5	5.2	9	9	0.26	2.99	0
4	MP4(I.D1.13)	8.9	8.7	6.1	4	4	0.25	2.56	0
5	MP5(II.D1.3)	17.5	10.8	6.3	13	10	0.26	0.23	3
6	MP6(II.D1.5)	15.1	9.6	6.7	11	7	0.22	3.15	1
7	MP7(II.D1.11)	7.3	6.0	3.8	12	6	0.20	1.99	0
8	MP8(III.D1.8)	14.2	7.5	4.7	10	7	0.20	3.27	0
9	MP9(III.D1.12)	24.8	8.1	7.8	11	10	0.27	2.72	0
10	MP10(I.25.14)	12.5	8.7	6.5	11	7	0.28	2.98	1
11	MP11(I.25.16)	16.5	11.6	7.2	6	8	0.26	3.43	0
12	MP12(I.50.1)	9.1	5.7	4.7	14	6	0.24	2.19	2
13	MP13(I.50.2)	14.2	8.5	5.3	10	9	0.24	4.12	0
14	MP14(I.50.7)	16.5	11.3	7.5	10	8	0.25	3.53	0
15	MP15(I.50.10)	45.2	12.5	7.3	12	13	0.28	3.34	0
16	MP16(I.50.13)	12.2	9.5	6.9	8	7	0.26	3.21	0
17	MP17(I.50.16)	22.2	9.5	5.7	19	9	0.24	3.10	4
18	MP18(I.50.17)	10.0	6.2	4.8	9	6	0.25	3.72	1
19	MP19(I.50.18)	11.5	6.5	3.8	10	6	0.21	2.92	1
20	MP20(II.25.1)	15.0	9.5	7.7	10	7	0.29	3.82	0
21	MP21(II.25.2)	17.8	9.4	6.9	10	8	0.29	3.45	0
22	MP22(II.25.6)	10.5	8.5	5.8	6	6	0.27	3.29	0
23	MP23(II.25.26)	16.9	10.5	7.3	11	11	0.27	3.13	0
24	MP24(III.25.6)	14.7	9.0	6.5	9	6	0.25	2.82	0
25	MP25(III.25.9)	19.8	11.2	7.6	10	8	0.28	3.49	0
26	MP26(III.25.17)	8.2	6.0	4.8	7	6	0.27	3.49	0
27	MP27(III.25.28)	10.5	7.0	5.8	12	5	0.28	4.21	1
28	Control	9.5	6.4	5.3	11	8	0.23	3.38	0

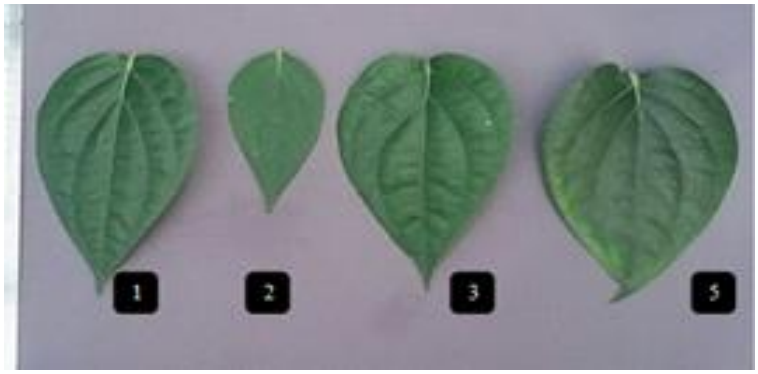

Figure 1. Variation on leaf shape1. Ovate, 2. Ovate lanceolate, 3 Ovate elliptic, 4. Cordate (Meilawati *et al.* 2016)

Table 2. Variation in leaf shape of Ciinten variety putative mutants

No	Putative mutants	Leaf shape			
		Ovate	Ovate elliptic	Ovate lanceolate	Cordate
1	R.I.25.3			√	
2	R.I.25.4		√		
3	R.I.25.5	√			
4	R.I.25.13	√			
5	R.II.25.3	√			
6	R.II.25.5	√			
7	R.II.25.11			√	
8	R.III.25.8	√			
9	R.III.25.12			√	
10	B.I.25.14		√		
11	B.I.25.16	√			
12	B.I.50.1		√		
13	B.I.50.2			√	
14	B.I.50.7			√	
15	B.I.50.10			√	
16	B.I.50.13	√			
17	B.I.50.16			√	
18	B.I.50.17	√			
19	B.I.50.18		√		
20	B.II.25.1				√
21	B.II.25.2			√	
22	B.II.25.6	√			
23	B.II.25.26	√			
24	B.III.25.6		√		
25	B.III.25.9	√			
26	B.III.25.17	√			
27	B.III.25.28				√
28	Origin (control)	√			

Note: R (Seed with radicle phase), B (Seed phase), I-III (replication), 25 Gydan 50 Gy (irradiation dose), 1-28 (plant number)

Figure 2. Variation on leaf base. Left: Rounded, Centre: Oblique (mutant), Right: Oblique (mutant) (Meilawati *et al.* 2016)

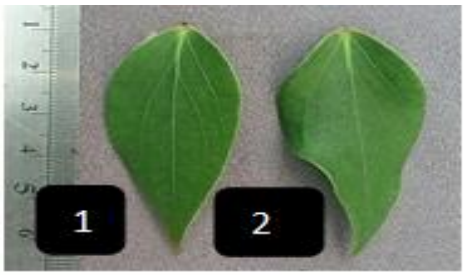

Figure 3.Variation on leaf margin, 1.Entire/even, 2.Wavy (Meilawati *et al.* 2016)

Table 3. Variation on leaf base and leaf margin of the putative mutants and the original Ciinten variety

No	Putative mutants	Leaf base				Leaf margin	
		Rounded	Cordate	Acute	Oblique	Even	Wavy
1	R.I.25.3		√				√
2	R.I.25.4		√			√	
3	R.I.25.5		√				√
4	R.I.25.13		√			√	
5	R.II.25.3			√		√	
6	R.II.25.5		√			√	
7	R.II.25.11	√				√	
8	R.III.25.8	√				√	
9	R.III.25.12		√			√	
10	B.I.25.14		√			√	
11	B.I.25.16		√			√	
12	B.I.50.1		√			√	
13	B.I.50.2	√				√	
14	B.I.50.7	√				√	
15	B.I.50.10	√					√
16	B.I.50.13	√				√	
17	B.I.50.16		√			√	
18	B.I.50.17	√					√
19	B.I.50.18		√			√	
20	B.II.25.1		√				√
21	B.II.25.2	√				√	
22	B.II.25.6		√			√	
23	B.II.25.26		√			√	
24	B.III.25.6		√			√	
25	B.III.25.9		√			√	
26	B.III.25.17		√				√
27	B.III.25.28		√			√	
28	Origin (control)	√				√	

Note: R (Seed with radicle phase), B (Seed phase), I-III (repeat), 25 Gy and 50 Gy (irradiation dose), 1-28 (plant number)

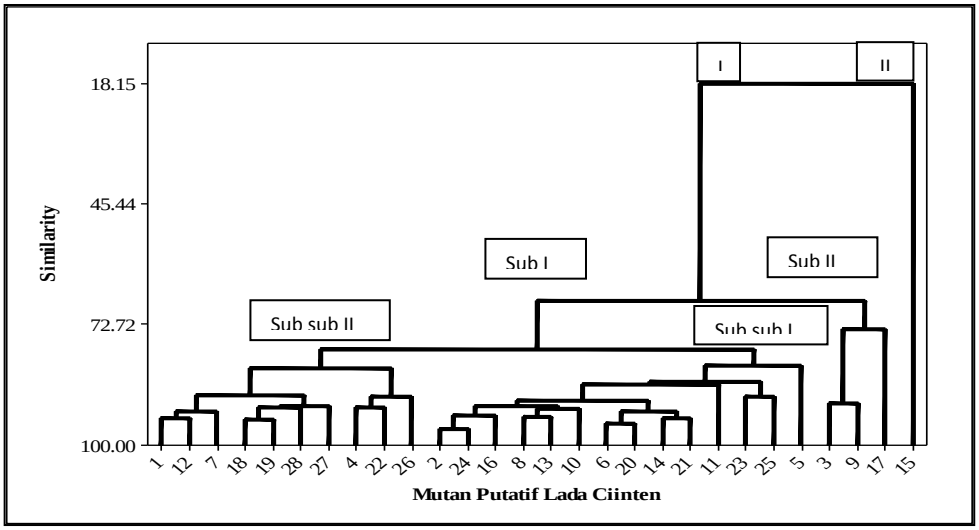

Figure 4. Dendogram of Ciinten variety putative mutants of resulting from mutation using gamma irradiation based on morphological character

Cluster analyses based on morphological characters, grouped the 27 putative mutants into two clusters (Figure 4), with the majority of mutants and the original varietywas clustered into group I and separated from mutant number 15 (group II) at 18.15% similarity coefficient.

Table 4. Grouping characters of Ciinten variety putative mutants

Group		Sub group		Sub sub group		Grouping character
II	15					Plant height
I	1, 12, 7, 18, 19, 28, 27,4, 22, 26, 2, 24, 16, 8, 13, 10, 6, 20, 14, 21, 11, 23, 25, 5, 3, 9, 17	Sub I	3, 9, 17			Leaf length
		Sub II	1, 12, 7, 18, 19, 28, 27,4, 22, 26, 2, 24, 16, 8, 13, 10, 6, 20, 14, 21, 11, 23, 25, 5	Sub sub I	2, 24, 16, 8, 13, 10, 6, 20, 14, 21, 11, 23, 25, 5	Number of node
				Sub sub II	1, 12, 7, 18, 19, 28, 27, 4, 22, 26	

Mutant number 15 formed its own group based on its highest plant height compared to the control and the original Ciinten variety. At the 70% similarity coefficient, the putative mutant plants are further clustered into two subgroups with leaf length as separator, i.e. subgroup I with three mutants and subgroup II with 24 mutants. The unirradiated Ciinten variety was clustered in subgroup II. This indicates that majority of the putative mutants have similar leaf sizewith the original variety.

At the level of similarity 75%, the mutant is further divided into two sub-subgroups with the character of the number of nodes as separators, namely sub sub I with 14 mutants, and sub subgroup II with 10 mutants. The unirradiated Ciinten variety (number 28) has similar morphological characters with mutant number 18, 19, and 27, at 87% similarity. Similar result was also reported in soybean that mutants with similar morphological traits tend to be grouped in the same cluster (Malek *et al.*, 2014).

SSR Analyses

PCR analysis showed that of the nine SSR primers, five primers produced polymorphic bands, while the three primers had monomorphic bands. SSR analyses using five primers Psol10, Psol15, Psol16, Psol17, Psol18amplified 16 polymophic bands, size ranging from 140-430 bp (Table 5).

Page visualization using primer Psol 10, the original Ciinten variety (No.28) differ from several of the putative mutants in band number. The control variety produced one band (200 bp), whereas the band was absence from three mutants (number 2, 7, 26). The absence of band from the mutants may be attributed to deletion of certain sequence.On the contrary there is an insertion or addition of a band on mutants number 19, 15 and 3 at around 100 bp, but missing from the original Ciinten variety.Primer Psol16 produces one band at the control variety (28), while in seven mutants (numbers 9, 10, 11, 12, 13, 14, 15) produce two bands. Changes in the number of bands, allegedly related to the treatment of gamma irradiation.

Table 5. Sequence of SSR primers showing polymorphic bands

Primer	Primer sequence (5'-3')	No. band	Size (bp)
Psol 10	F:CACGACGTTGTAAAACGACCAGACGGATTCCCACTGAT R: GGACTTGTAACCCATCGAGA	3	220-330
Psol 15	F:CACGACGTTGTAAAACGACCGCGGACTAACCAGAGTTAC R: GCCACAAAAACCCACTCA	3	140-150
Psol 16	F:CACGACGTTGTAAAACGACGAAGTCCTAACCAGACCTGTG R: GAGGTGTTGTTGATGTGAGC	2	160-210
Psol 17	F: CACGACGTTGTAAAACGACTATTCCCATGCGAGATGC R: CGGCATAACCACTAAACCAC	3	390-430
Psol 18	F: CACGACGTTGTAAAACGACACTGTTGTGGACCTTGTTGC R: TGTATTAGGCCCCATCGAC	5	150-160
Total number of bands		16	

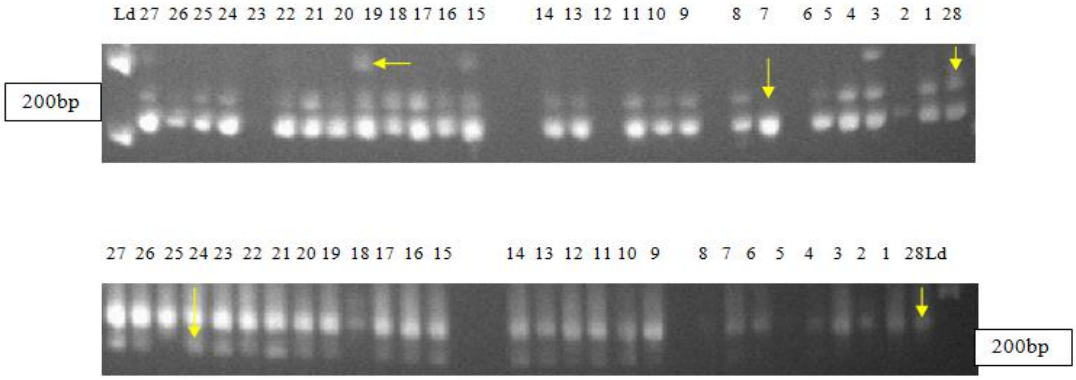

Figure 5. PAGE using (a) Psol 10 and (b) Psol 16 primers on 27 putative mutants derived from gamma irradiation of Ciinten variety

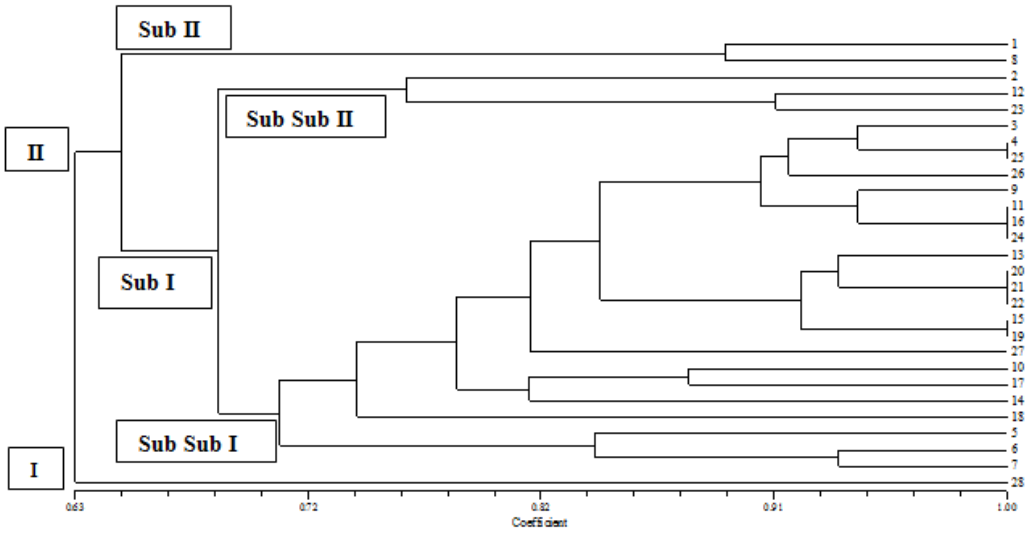

Figure 6. Dendogramof Ciinten variety putative mutants derived from gamma irradiation based on five SSR primers

The genetic relationship among the mutants can be seen from the coefficient of similarity and cluster analysis (clustering analysis) using DNA band patterns (SSR markers) (Figure 6). The dendogram resulting in two groups, and

at 63% similarity coefficient clearly separates the original Ciinten variety (number 28) (group I) with the putative mutants (group II). Group II is divided into two sub-groups namely sub I with 25 mutants and sub II with only two mutants (number 1 and 8), at a similarity level of 65%. Sub group I is further divided into sub sub I consisted with three mutants (number 2, 12, 23), and sub sub II with 22 putative mutants, of which were putative mutants having a similarity level of 100% i.e. number 4 with 25; numbers 11, 16 and 24; numbers 20, 21 and 22 and numbers 15 and 19.

DISCUSSION

A number of mutants showed differences in morphological characters from the unirradiated Ciinten variety, especially on leaf characters. Although this experiment was carried out at seedling stage, but differences in the morphological characters between the unirradiated Ciinten variety with the mutants can be observed. This study was in accordance with Surest (2015) that variation occurred on leaf characters on sugarcane mutants compared to control.

The lowest similarity coefficient based on morphological charaters was 18.15%, and the unirradiated Ciinten variety was clustered the 26 out of 27 mutants. Levels of similarity among other mutants ranged between 70-75%. There are still much similarity between the unirradiated Ciinten variety with the mutant based on morphological characters. This result differ from the SSR markers, in which the unirradiated Ciinten variety clearly separated from the mutants at similarity coefficient 63%.The number of band identified using SSE markers null–three bands. The number of bands generated depends on how many pieces of DNA are produced from PCR.

The banding pattern shows the diversity of the control and the putative mutant at the DNA level, in which deletion and insertion of bands were observed on a number mutants compare to the unirradiated Ciinten variety as shown by primer Psol 10 and Psol 16.Accoding to Muhammad and Othman (2005), the polymorphism of DNA bands based on the emergence and absence of bands may be due to deletion or insertion. Mohr &Schopfer (1995) states hat ionizing radiation (gamma irradiation) will produce ions and radicals in the form of hydroxyl (OH^-). If the hydroxyl radical is attached to the nucleotide chain in the DNA, then single or multiple DNA strands will break, thus undergoing a gene change. Thus, it is probable it occurred in this study.

Qosim (2006) studied the induction of gamma irradiation on the mangosteen nodular callus and resulted in genetic diversity between 60-91% based on RAPD. whereas Harahap (2005) obtained genetic diversity 62-100% from gamma irradiated mangosteen in vitro. On the other hand, Sobir and Poerwanto (2007) based on RAPD analysis on mangosteen seeds from gamma irradiation using five random primers, produce greater genetic variability of irradiated manggosteen (62%) than variation on Java mangosteen accession (27%). In this study, genetic diversity obtained from gamma irradiation of 77-95%.

The DNA marker is a small part of the DNA sequence that can show the diversity due to deletions, duplicates, insertions or substitutions between different mutants, which can be identified by PCR products in the form of band size or band migration distance. Based on molecular character with SSR, band change was observed in this study in gamma irradiated Ciinten variety mutants.

This indicates that irradiation carried out in this study produces mutants that are genetically quite diverse.

CONCLUSION

There are differences among Ciinten putative mutants in morphological characters such as plant height, leaf length, leaf width, number of leaves, number of branches, number of branches, leaf shape, leaf base and leaf margin. One mutant clearly distinct from the other mutants and the original variety, at similarity coefficient 18.15%. Levels of similarity among other mutants ranged between 70-75%. Molecular analysis using SSR showed polymorphisms on five primers namely Psol10, Psol15, Psol16, Psol17, Psol18. Band deletion and insertion were observed in a number of the putative mutants. Cluster analyses based on SSR markers clearly separate the original Ciinten variety from its mutants at similarity coefficient 63%.

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