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SHORT COMMUNICATION

Genetic Variation of the First Generation of Rodent Tuber (*Typhonium flagelliforme* Lodd.) Mutants Based on RAPD Molecular Markers

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Rodent tuber (*Typhonium flagelliforme* Lodd.) is a herbal plant from the Araceae family. The plant has high medical potential and is effective to cure cancer. However, the low level of its genetic variation limits its exploration for desirable traits. The low level of genetic variation in Rodent tuber is mainly due to its asexual reproduction system. It usually reproduces vegetatively via tuber separation. Therefore, gamma irradiation had been applied to rodent tuber *in vitro* to increase its genetic diversity. The objective of this study was to analyze the genetic diversity of the first generation (MV1) of gamma irradiated rodent tuber mutant using random amplified polymorphic *DNA* (RAPD) markers. A total of 14 mutant DNA samples were analyzed with 14 RAPD primers. The result showed that 67 out of 123 DNA bands were polymorphic among mutant lines. Based on cluster analysis these mutants showed 0.78-0.97 genetic similarity. Cutting of dendogram at genetic distance of 0.89 produced four main clusters. Mutants with high genetic variation are now available. This increases the opportunity of obtaining mutant lines with high anti-cancer activity.

Keywords: Gamma irradiation, mutant, RAPD, *Typhonium flagelliforme*, variation

INTRODUCTION

Rodent tuber (*Typhonium flagelliforme* Lodd.) is a herbal plant from the Araceae family (Surachman 2009). It has high medical potential and can be used to cure cancer, such as breast and ovarian cancer (Syahid & Kristina 2007; Nurrochmad *et al*. 2011), leukemia (Mohan *et al*. 2011), and lung cancer (Chan *et al*. 2005). The phytochemical constituents of rodent tuber are alkaloid, steroid, flavonoid, and glycoside (Surachman 2009).

Rodent tuber has low genetic diversity. It usually reproduces vegetatively through tuber separation. The pollination of rodent tuber rarely happens. Vegetative propagation of rodent tuber reduces the formation of new genotypes (Syahid & Kristina 2007). One of the methods to increase genetic variation is mutation through gamma irradiation. This technique can be used in genetic variation induction, selection and propagation of plants in a short time in comparison to conventional methods (Afrasiab & Iqbal 2010).

Sianipar *et al*. (2013b) had initiated and propagated rodent tuber from Bogor indirectly through callus formation. Sianipar *et al*. (2013b) had induced the mutation in rodent tuber shoots through gamma irradiation and regenerated into plantlets. Sianipar *et al.* (2013a) showed the toxicity of rodent tuber extract and hexane fraction to *Artemia salina*. Further studies had been done in Universitas Pelita Harapan to detect the genetic changes of *in vitro* rodent tuber mutants.

A molecular marker is a type of genetic markers which is based on DNA analysis. Molecular markers usually do not have biological function. Several types of molecular techniques using genetic markers are restriction fragment length polymorphism (RFLP), random amplified polymorphic DNA (RAPD), amplified fragment length polymorphism (AFLP),

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inter-simple sequence repeats (ISSRs), sequence characterized regions (SCARs) (Semagn *et al*. 2006). RAPD markers use the polymerase chain reaction (PCR) amplification technology (Fauza *et al.* 2007). RAPD markers can be used without prior knowledge of the genome that is being analyzed (Agarwal *et al*. 2008). The RAPD analysis does not need high DNA purity and quantity (Zainudin & Maftuchah 2006). RAPD markers serve as dominant markers in segregating population. It cannot differentiate between homozigotic and heterozigotic individuals (Fauza *et al*. 2007). RAPD markers had been widely used in plant breeding. RAPD markers can be used in detecting genetic variation of mutant plants, such as in *Vigna mungo* (L.) Hepper (Arulbalachandran *et al*. 2010) and *Arachis hypogaea* L. (Bhagwat *et al*. 1997). Several other applications of RAPD molecular markers include the analysis of population structure, detection of somaclonal variation, cultivar identification, and the development of specific genetic markers for certain characters (Kumar & Gurusubramanian 2011).

This research aimed to analyze the genetic variation of the first generation (MV1) rodent tuber mutant lines based on RAPD markers.

MATERIALS AND METHODS

Plant Materials. The first generation of rodent tuber mutants used in this research were obtained from previous research done by Sianipar *et al*. (2013b). Sianipar *et al*. (2013b) irradiated *in vitro* rodent tuber at 0, 10, 20, 30, 40, and 50 Gy of gamma ray. The plantlets survived 20 and 25 Gy gamma-irradiation.

DNA Isolation of Mutant Rodent Tuber. DNA isolation was done to 14 out of 37 selected mutant lines by using modified CTAB procedure (Doyle & Doyle 1987). Young first generation of mutant rodent tuber leaves were homogenized in eppendorf tube in liquid nitrogen. One mL of CTAB buffer solution (2% CTAB, 100 mM Tris-HCl pH 8.0, 20 mM Na_2EDTA pH 8.0, 1.4 M NaCl) and 0.2% 2-Mercaptoethanol was added into the homogenized leaf samples. Samples were then incubated in waterbath at 65 ^oC for 45 min. 500 µL of chloroform: isoamyl alcohol solution (24:1) were added into the samples. The samples were then mixed by invertion for 12 times. Samples were centrifuged for 1 min at 13000 rpm. The supernatant obtained were separated into another tube. One tenth volume of natrium acetate solution (NaOAc) pH 5.2 and one volume of cold isopropanol were added into the supernatants. Samples were incubated at -20 ^oC for 30 min and centrifuged at 13000 rpm for 10 min. Pellets obtained were washed with 200 µL of cold ethanol 70% and were room dryed for 30 min. Pellets were resuspended in 200 µL of TE buffer pH 8.0. Three µL of RNAse A (10 mg/mL) were added to the resuspended DNA samples and were incubated at 37 ^oC for an hour. DNA samples were kept in -20 ^oC and were ready to use.

PCR Analysis. PCR RAPD reaction was done with a total volume of 25 µL which consisted of 5 µL of GoTaq Flexi Buffer (5X), 2.5 µL of $MgCl_2$ (25 mM), 0.5 µL of PCR Nucleotide Mix (10 mM), 1 µL of primer (10 µM), 0.2 µL of GoTaq DNA Polymerase (5U/µL), 2 µL of DNA *template* (100 ng/µL), and 13.8 µL of ddH_2O. PCR cycles consisted of a cycle of predenaturation (95 ^oC for 2 min), followed by 7 cycles of denaturation (95 ^oC for 1 min), annealing (37-34 ^oC for 1 min), and extension (72 ^oC for 3 min), and 38 cycles of denaturation (95 ^oC for 1 min), annealing (34 ^oC for 1 min), and extension (72 ^oC for 3 min), followed by a final extension cycle (72 ^oC for 7 min), and storage at 4 ^oC.

PCR RAPD Visualization. PCR RAPD products were visualized with electrophoresis using 1.2% agarose gels with 110 volt for 90 min. Agarose gels were then soaked at ethidium bromide solution for 10 min and washed with water. Visualization and documentation of PCR RAPD were done with Chemidoc XRS System. The size of DNA bands were determined by comparing them with 1 kb DNA ladder.

Data Analysis. DNA bands obtained were scored into binary format of 0 and 1. Binary matrix obtained were analyzed by using NTsys software to determine the similarity matrix with DICE coefficient. Clustering analysis was done with UPGMA method.

RESULTS

RAPD molecular markers successfully showed the genetic variation between control and selected rodent tuber mutant lines. Mutant lines were analyzed by using 14 RAPD primers (Table 1). All of the primers used in the study produced polymorphic DNA bands. Total DNA bands obtained in the study were 67 polymorphic bands out of 123 bands. Primer OPA-14 produced the most polymorphic DNA bands with 12 bands. The least polymorphic bands were obtained by using primer OPC-14.

Analysis using OPE-03 produced 5 polymorphic bands out of 8 bands, with the size of 800-2500 bp (Figure 1). The 1200 bp DNA band distinguished control and 6-2-5-2, 6-3-2-1, 6-3-3-10, 6-3-3-6, and 6-9-4 mutant lines. The 800 bp DNA band

distinguished control and 6-6-1-9, 6-1-3-3, 6-6-3-6, and 6-9-4 mutant lines. RAPD analysis using OPA-03 primer (Figure 2) produced 5 polymorphic bands out of 11 total bands with the size of 500-10000 bp. The

Table1. Total DNA bands obtained from PCR RAPD

Primer	Sequence	Total DNA bands	Total polymorphic DNA bands	DNA band size (bp)
OPA-02	TGCCGAGCTG	7	2	250- 3000
OPA-03	AGTCAGCCAC	11	5	500-10000
OPA-09	GGGTAACGCC	8	4	600- 4000
OPA-14	TCTGTGCTGG	11	11	500- 3700
OPB-18	CCACAGCAGT	9	4	300- 2800
OPC-08	TGGACCGGTG	6	6	1300- 6000
OPC-05	GATGACCGCC	13	6	400- 3500
OPC-11	AAAGCTGCGG	10	2	600- 2600
OPC-14	TGCGTGCTTG	7	1	600- 2300
OPD-08	GTGTGCCCCA	10	6	600- 3000
OPD-20	ACCCGGTCAC	9	9	600- 3000
OPD-10	GGTCTACACC	6	5	600- 5500
OPE-03	CCAGATGCAC	8	5	800- 2500
OPE-07	AGATGCAGCC	8	1	500- 3000
Total DNA bands		123	67	

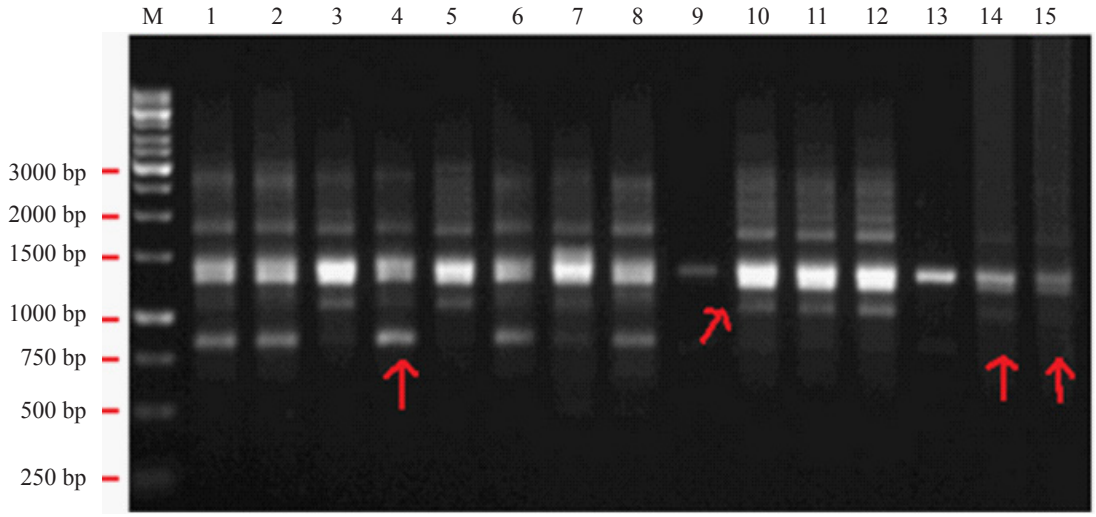

Figure 1. DNA Bands Profile of Mutant Rodent Tuber based on OPE-03. (M) 1kb ladder, (1) Control, (2-15) Mutants: (2) 6-9-3, (3) 6-6-1-9, (4) 6-2-5-2, (5) 6-1-3-3, (6) 6-3-2-1, (7) 6-6-7-8, (8) 6-1-6-1, (9) 6-3-3-10, (10) 6-9-5, (11) 6-1-1-1, (12) 6-2-1-2, (13) 6-3-3-6, (14) 6-6-3-6, (15) 6-9-4.

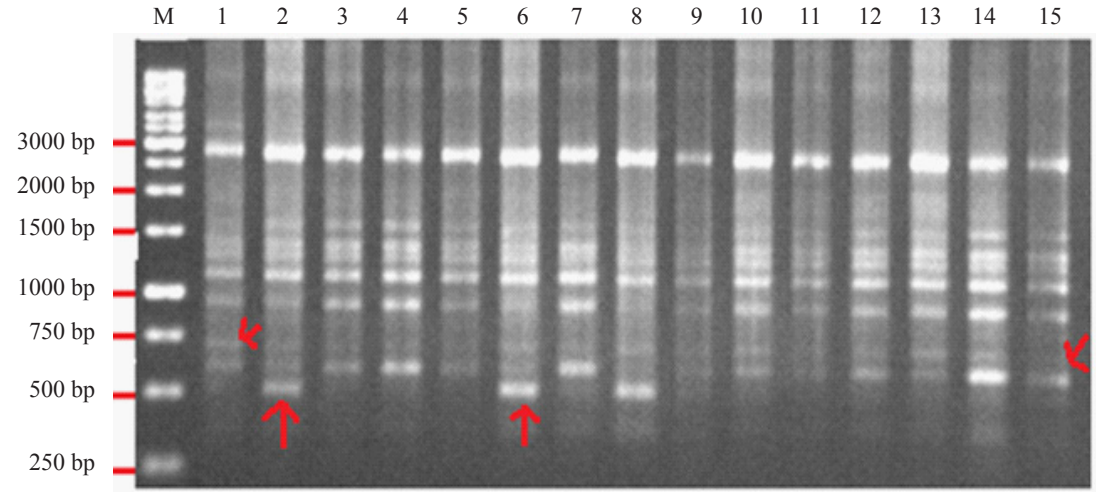

Figure 2. DNA Bands Profile of Mutant Rodent Tuber based on OPA-03. (M) 1kb ladder, (1) Control, (2-15) Mutants: (2) 6-9-3, (3) 6-6-1-9, (4) 6-2-5-2, (5) 6-1-3-3, (6) 6-3-2-1, (7) 6-6-7-8, (8) 6-1-6-1, (9) 6-3-3-10, (10) 6-9-5, (11) 6-1-1-1, (12) 6-2-1-2, (13) 6-3-3-6, (14) 6-6-3-6, (15) 6-9-4.

500 bp DNA band distinguished control and 6-9-3, 6-3-2-1, and 6-1-6-1 mutant lines. The 700 bp DNA band distinguished control and 6-1-3-3, 6-1-1-1, and 6-9-4 mutant lines.

Genetic similarity matrix (Table 3) were obtained from the analysis of DNA bands with NTsys software. Genetic similarity obtained based on RAPD analysis was 0.78-0.97. The lowest genetic similarity was 78% obtained between 6-9-4 and 6-1-6-1 mutant lines. The highest genetic similarity was obtained between 6-1-1-1 and 6-2-1-2 mutant lines, and 6-9-5 and 6-2-1-2 mutant lines. The lowest genetic similarity between mutant line and control was obtained between mutant 6-9-4 and control. Mutant 6-9-3 also had better observed morphological characters compared to control plant (Figure 3). However, RAPD molecular analysis showed higher similarity between 6-9-3 with control plant (0.88) than with 6-9-4 (0.80) (Table 3).

Clustering analysis was done from the genetic similarity data and was visualized in the dendogram (Figure 4). Cutting of dendogram at genetic distance of 0.89 produced four main clusters (Table 4). Cluster 1 with genetic similarity of 0.91 consisted of control and 6-1-6-1 mutant line. Cluster 2 with genetic similarity of 0.89 consisted of 6-9-3, 6-3-2-1, 6-3-3-10, and 6-3-3-6 mutant lines. Cluster 3A with genetic similarity of 0.92 consisted of 6-6-1-9, 6-2-5-2, 6-9-5, 6-1-1-1, and 6-2-1-2 mutant lines. Cluster 3B with genetic similarity of 0.95 consisted of 6-6-3-6 and 6-9-4 mutant lines. Cluster 4 with genetic similarity of 0.90 consisted of 6-1-3-3 dan 6-6-7-8 mutant lines. The phenotype of mutants and control were different. The observation at six weeks after irradiation showed significant differences between control (normal mother plant), 20 and 25 Gy mutants with average plant height of 9.57, 3.41, and 2.43 cm, respectively, and the average number of shoots produced were a 7.85, 6.03, and 5.00 shoots, respectively (Sianipar *et al*. 2013b).

Table 3. Genetic similarity matrix of mutant rodent tuber based on RAPD analysis

	Control	6-9-3	6-6-1-9	6-2-5-2	6-1-3-3	6-3-2-1	6-6-7-8	6-1-6-1	6-3-3-10	6-9-5	6-1-1-1	6-2-1-2	6-3-3-6	6-6-3-6	6-9-4
Control	1.00														
6-9-3	0.88	1.00													
6-6-1-9	0.90	0.86	1.00												
6-2-5-2	0.90	0.88	0.95	1.00											
6-1-3-3	0.85	0.82	0.90	0.88	1.00										
6-3-2-1	0.87	0.92	0.87	0.89	0.84	1.00									
6-6-7-8	0.86	0.82	0.87	0.87	0.90	0.86	1.00								
6-1-6-1	0.91	0.87	0.83	0.85	0.82	0.89	0.84	1.00							
6-3-3-10	0.90	0.90	0.88	0.91	0.87	0.95	0.87	0.90	1.00						
6-9-5	0.90	0.87	0.94	0.94	0.92	0.88	0.87	0.86	0.91	1.00					
6-1-1-1	0.87	0.84	0.92	0.92	0.91	0.86	0.86	0.85	0.89	0.97	1.00				
6-2-1-2	0.88	0.86	0.94	0.92	0.91	0.88	0.86	0.85	0.90	0.97	0.97	1.00			
6-3-3-6	0.87	0.86	0.87	0.89	0.87	0.90	0.85	0.87	0.93	0.90	0.91	0.91	1.00		
6-6-3-6	0.86	0.81	0.93	0.90	0.86	0.84	0.85	0.79	0.85	0.88	0.89	0.89	0.89	1.00	
6-9-4	0.84	0.80	0.91	0.90	0.87	0.84	0.86	0.78	0.85	0.87	0.88	0.87	0.88	0.9	1.00

Figure 3. Mutant Rodent Tubers Plant Vigor at two months old (A) Control plant (B) mutant 6-9-3. Mutant with better plant vigor (6-9-3) was obtained as opposed to control plant.

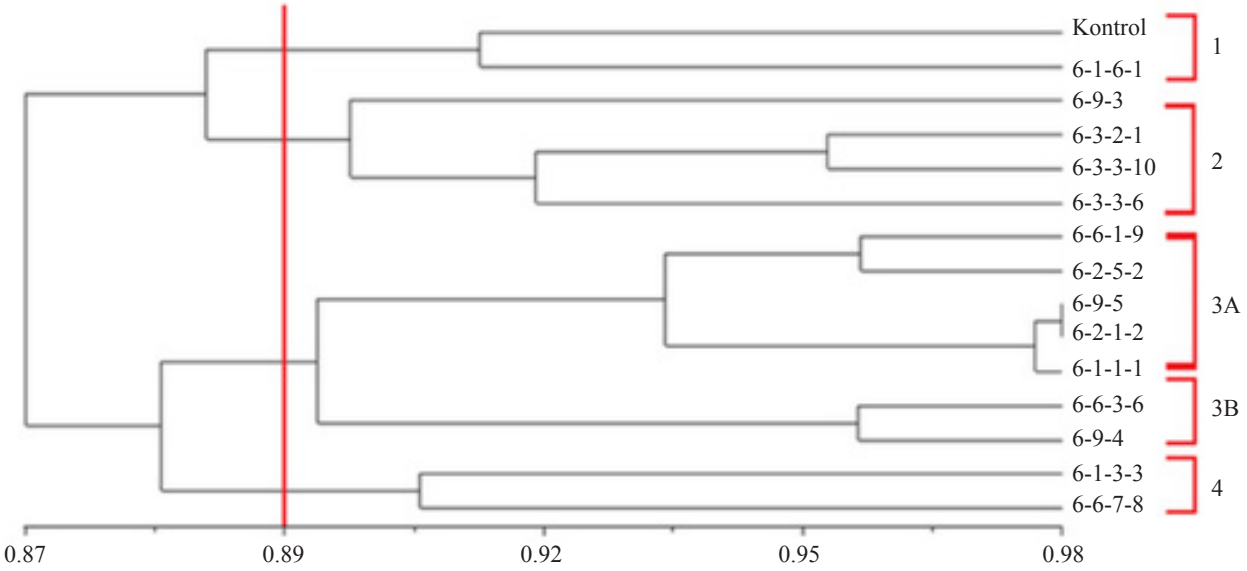

Figure 4. Dendogram of Mutant Rodent Tuber based on RAPD Analysis. Red vertical line showed cut off position at genetic distance 0.89. (1) Control, mutant 6-1-6-1; (2) mutants 6-9-3, 6-3-2-1, 6-3-3-10,6-3-3-6; (3A) mutants 6-6-1-9,6-2-5-2, 6-9-5, 6-2-1-2, 6-1-1-1; (3B) mutants 6-6-3-6,6-9-4; (4) mutants 6-1-3-3, 6-6-7-8.

Table 4. Clustering analysis of mutant rodent tuber based on RAPD analysis

Main groups	Sub group	Similarity coefficient	Group members
1		0.91	control, 6-1-6-1
2		0.89	6-9-3, 6-3-2-1, 6-3-3-10, 6-3-3-6
3	3A	0.92	6-6-1-9, 6-2-5-2, 6-9-5, 6-1-1-1, 6-2-1-2
	3B	0.95	6-6-3-6, 6-9-4
4		0.90	6-1-3-3, 6-6-7-8

DISCUSSIONS

A random fashion of genetic changes, which were most likely due to gamma irradiation from the previous research by Sianipar *et al.* (2013b), were shown in this study. The random changes in the genome because of gamma irradiation had also been shown in coconut mutants (Rohani *et al*. 2012). Several other studies had shown changes in morphological characters due to gamma irradiation, for example in mutant sugarcane (Khan *et al*. 2009) and *Artemisia* (Purnamaningsih *et al*. 2011).

Morphological characters observed in this research were important parameters in agronomy. Leaf area is important for photosynthesis (Stampar *et al*. 1999), plant height is important for light exposure (Aarsen 1995), and number of leaves and shoots are linked to biomass of plant. Morphologically selected mutants should have good agronomy traits, like high biomass. Mutant lines with good agronomy characters will be advantageous for production of anti-cancer compound in the future.

The polymorphism of DNA bands obtained in this study varied between 250 to 6000 bp. This difference in DNA band size was obtained from gamma irradiation to rodent tuber calli. Gamma irradiation was the most effective physical mutagen in changing DNA structure. Gamma irradiation caused dissociation of water molecule and produced hydroxil free radicals. Hydroxil free radicals were the most reactive free radicals and could react with biomolecules in cells, including DNA (Girija *et al*. 2013).

RAPD molecular markers analysis were used in detecting changes in DNA. Changes in DNA were shown by the presence and absence of DNA bands (Atak *et al*. 2011). The intensity of DNA bands might varies. This difference in DNA bands intensity might results from gene copy number which showed the abundance of certain sequence in the genome (Semagn *et al*. 2006). Primer OPA-02, OPA-03, OPA-09, OPA-14, OPB-18, OPC-05, OPC-08, OPC-11, OPC-14, OPD-10, and OPD-20 had previously been used in the analysis of the genus *Typhonium* (Acharya *et al*. 2005; Rout 2006).

Genetic similarity analysis had been done previously in the *Typhonium* genus with RAPD molecular markers by Rout (2006), with genetic similarity of 0.43-0.63. Research about genetic variation of gamma irradiated mutant lines had been

done previously in sugarcane. Sugarcane which was irradiated with 10-50 Gy gamma rays had genetic similarity of 0.70-0.98 based on RAPD molecular markers (Khan *et al.* 2009).

Every mutant lines analyzed in this study had different genetic similarity with control based on genetic similarity matrix. Genetic similarity between control and mutant lines varied between 0.84 to 0.91. Smaller genetic similarity showed bigger effects of gamma irradiation on mutant plant genotype. The smallest genetic similarity obtained between control and mutant lines was obained at 6-9-4, 6-1-3-3, 6-6-7-8, 6-6-3-6, 6-1-1-1, 6-3-3-5, and 6-3-2-1 mutant lines. The genetic similarity between mutant lines varied between 0.78 to 0.97. Smaller genetic similarity means more difference in the genotype of the mutant lines. The phenotype mutants are different with the normal mother plantlets (control). An increase in plant height occurred until the sixth week in control, while the mutant had a decrease in plant height (Sianipar *et al*. 2013b).

RAPD molecular markers were useful for the selection and plant breeding. The analysis of genetic similarity and its clustering were useful to create different genotypes with different genetic backgrounds. The application of genetics background in plants can help the development of top variety with high productivity (Bibi *et al*. 2009).

RAPD molecular markers had been successfully used in this study to show the genetic variation of control and the first generation (MV1) of gamma irradiated rodent tuber mutant lines. RAPD molecular markers had also been applied in detecting the genetic variations of different plants, such as *Vigna mungo* (L.) Hepper (Arulbarachandran *et al*. 2010), *Arachis hypogaea* L. (Bhagwat *et al*. 1997), and *Butea monosperma* (Vashishtha *et al*. 2013). Aside from detection of genetic variation, RAPD markers can also be used to identify new plant cultivars, such as in *Ribes nigrum* (Huo *et al*. 2013). Rout (2006) showed the closest genetic distance was obtained with population of different *Typhonium* species.

Variable mutant lines were observed in this study based on molecular marker analysis. These results indicated that the previous research by Sianipar *et al*. (2013b) has successfully induced the genetic variability of rodent tuber. These results were also supported by the morphological characters results in the previous study. Mutant lines with lower genetic similarity to control and better agronomy characteristics were obtained. One of them was mutant line 6-9-4. Mutant line 6-9-4 has the lowest genetic similarity to control plant. Mutant 6-9-4 also had better observed morphological characters than control plant. Mutant 6-9-3 also had better observed morphological characters compared to control plant (Figure 3). However, RAPD molecular analysis showed higher similarity between 6-9-3 with control plant (0.88) than with 6-9-4 (0.80) (Table 3). This example showed that despite of the good plant vigor observed in 6-9-3 and 6-9-4, the genetic similarity might be different with control plant. Morphological characters observed were influenced by both genetic and environmental factors. This result also indicated that DNA bands obtained by RAPD analysis were not linked with morphological characters. Morphological characters did not contribute to the genetic similarity shown in the dendogram (Figure 4), as it was only constructed based on RAPD molecular analysis.

These mutant lines which have lower genetic similarity and better agronomy characteristics would be important for future applications, such as breeding, conservation, and production of anti-cancer compound. In the case of production of anti-cancer, compound from rodent tuber has to be identified and characterised first. Quantitative analysis of anti-cancer bioactive compounds in mutant lines should be done following the identification and characterisation of anti-cancer bioactive compound to select potential mutant lines for production.

In conclusion, RAPD molecular markers analysis had successfully shown the genetic variation of 14 first generation rodent tuber mutant lines. Total DNA bands obtained were 67 polymorphic DNA bands out of 123 DNA bands. Genetic distance obtained was 0.78-0.97. Dendrogram cutting at the 0.89 genetic similarity showed four main clusters.

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