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Genetic variation of doubled haploids derived from anther culture of M_1 red rice plants

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Abstract. *Nurmansyah, Setyadi AH, Fatumi NC, Fatmawati Y, Wulandari RA, Purwantoro A. 2021. Genetic variation of doubled haploids derived from anther culture of M_1 red rice plants. Biodiversitas 22: 4923-4929.* The doubled haploid (DH) technology when integrated with induced mutation could accelerate development of local rice cultivars with several desirable traits. Anthers from the first generation of mutant (M_1) plants were utilized for DH production. However, the effectiveness of inducing and fixing mutation requires detailed evaluation with the help of molecular techniques for its accuracy and reliability than that of morphological or biochemical assessments. The objective of this research was to develop and detect genetic variation of DH plants derived from anther culture of M_1 plants. Seeds of local red rice cultivar, Cempo Abang, were treated with four gamma-ray doses (0, 100, 200, 300 Gy) and planted to produce the M_1 plants. Panicles at the booting stage of the M_1 plants were collected and used as donor anthers. The present study suggested that gamma irradiation treatments indirectly could increase callus formation. However, it also reduced the number of callus producing plantlets due to higher frequency of brown calli. Eleven spontaneous DH plants obtained in the study along with parental plants were assessed to determine the occurrence of genetic variation using six RAPD primers. The RAPD primers generated 51 bands, of which 34 alleles or 66.7% were polymorphic with an average of 5.6 polymorphic alleles per primer. The genetic similarity among parent and 11 DH lines based on Jaccard's similarity index ranged from 0.622 to 0.902. The DNA polymorphism among the DH plants demonstrated the effect of gamma irradiation to create genetic variation. Therefore, this method could be used as an alternative for rice breeding programs especially to develop preferred traits in the local rice cultivars.

Keywords: Cempo Abang, anther culture, double haploids, M_1 plants, RAPD markers

INTRODUCTION

Pigmented rice is considered a superfood for its antioxidant, anticancer and other nutritional properties (Limbongan et al. 2021). Indonesia is known for its local pigmented rice cultivars due to their high adaptability and diversified genome. However, the local cultivars have a lower yield as compared to improved varieties. In order to overcome various undesirable characteristics and further utilize the local cultivar genome, rice breeders constantly strive to improve the yield potential by several breeding methods, including induced mutation. This technique is an alternative approach utilized in plant breeding for rapidly enriching the genetic variability. Induced mutant alleles act as a genetic variation source for crop improvement and candidate gene discovery through functional genomics in many crops, including rice (Kumawat et al. 2019). Sobrizal (2016) also reviewed how induced mutation benefited in improving the characteristics of the local varieties such as reducing harvest period, plant stature, as well as increasing yield potential. Moreover, mutation breeding is frequently used to discover new genetic sources for abiotic and biotic stress tolerance, herbicide resistance, nutritional quality traits, and climate resilience (Chaudhary et al. 2019).

Although induced mutation demonstrated its ability to improve local varieties, it is equally time consuming as the conventional cross breeding program which requires 6-8 breeding cycles to get the homozygous line. On the other hand, doubled haploid (DH) technology only requires one or two generations to obtain homozygous line which helps in accelerating the breeding program. In rice, DH plants could be generated by in vitro spontaneous diploidization without chromosome doubling treatments through anther culture (Lee at al. 2003). A rather different technique was experimented in which both induced, and DH methods were incorporated and created by mutagens in one generation. The method utilized first generation of mutant (M_1) plants as donor plants for DH production (Szarejko and Forster 2007). Although several studies have focused on this technique in rice (Lee et al. 2003; Myint et al. 2005), to the best of our knowledge, there is no genetic diversity assessment based on molecular markers of DHs derived from mutant plants. The detection of variation using molecular markers is more accurate and reliable than that of morphological or physiological characteristics (Zakiyah et al. 2019). Furthermore, the information will help breeders determines the efficiency of this technique in generating variation.

The objective of this study was to develop and evaluate the genetic variation based on molecular markers of DHs derived from anther culture of the M_1 plants. The M_1 plants were generated from the local red rice cultivar, Cempo Abang, that were subjected to four doses of gamma radiation (0, 100, 200, and 300 Gray). Cempo Abang is

preferred by consumers due to its intermediate amylose content (21.42%) which makes rice softer and stickier. Moreover, it has a high protein content (9.04%), beta carotene (153.15 µg/100g), and iron (16.09 ppm). However, its yield is low due to lower number of grains per panicle and lower number of productive tillers (Kristamtini and Purwaningsih 2009). Random Amplified Polymorphic DNA (RAPD) markers were used in this study which was efficient in assessing the genetic variability and diversity in many rice cultivars (Sholikhah et al. 2019; Zakiyah et al. 2019; Mazumder et al. 2020).

MATERIALS AND METHODS

Gamma radiation treatment

Seeds of Cempo Abang were treated using four different gamma radiation doses (0, 100, 200, and 300 Gray) to generate M_1 seeds at the Center for the Application Isotope and Radiation Technology, National Nuclear Energy Agency (BATAN), Indonesia. The M_1 seeds were then planted in the glasshouse. Five healthy M_1 plants per treatment were selected as donor plants for the anther culture.

Development of DH plants

Explant collection and preparation

Ten panicles per treatment were collected from the M_1 plants, which produced red pericarp color, at the booting stage, when the length between the flag leaf and the penultimate leaf was 8 to 12 cm (Figure 1a). The selected panicles were wrapped in an aluminum foil and stored at 4ºC for 8 days. After cold pretreatment, the leaf sheaths were removed from the panicles. Appropriate spikelets containing anthers at uninucleate microspore stage, in which the length of anthers was less than half the length of the spikelet (Dewi et al. 2017), were surface sterilized with 20% (v/v) of a 5.2% sodium hypochlorite solution for 15 minutes followed by washing five times using sterile water.

Callus induction and plant regeneration

Callus induction medium (CIM) consisted of N6 basal medium supplemented with 2 mg L^{-1} NAA, 0.5 mg L^{-1} kinetin, putrescine 20 µM, 3% (w/v) sucrose, and 0.3% (w/v) phytagel. A total of 300 anthers per treatment were cultured in a petri dish (90×15 mm) containing 25 ml CIM and placed in the dark at 25ºC. Calli with 1-2 mm diameter were transferred to regeneration medium containing MS basal medium supplemented with 2 mg L^{-1} kinetin, 0.5 mg L^{-1} NAA, putrescine 20 µM, 3% (w/v) sucrose, and 0.3% (w/v) phytagel. The transferred calli were incubated in continuous light at 25ºC. Green plantlets (3-5 cm long) were transferred to a glass tube containing 15 ml rooting medium (MS basal medium + 0.5 mg L^{-1} IBA).

Acclimatization

The acclimatization of green plantlets, which had 3 to 4 leaves and roots, consisted of two phases. In the first phase, the green plantlets were transferred into test tubes containing sterile water for 1 to 2 weeks in a room where sunlight can pass through a window. This stage aimed to induce more functional roots and to adjust plants into higher light intensity from sunlight. The result of the first phase is shown in Figure 1h when the plant had healthy root development. After that, the plants were transferred into plastic boxes containing mud in a greenhouse under 25% shading for 2 weeks. The survival plants were then placed into 10-liter plastic pots under full sunlight.

Observation and data analysis

The percentage of callus induction and plant regeneration were investigated from cultured anthers. The onset of callus initiation and total number of browning calli, calli producing plants, green plants, and albino plants were also calculated. The data analysis was carried out using Microsoft Excel.

Genetic diversity assessment using RAPD

DNA extraction

Total genomic DNA was extracted from leaf tissue of the DH plants which were evaluated by normal growth using DNA mini kit from Geneaid Biotech Ltd. The normal growth of DH plants was identified by normal morphological appearance similar to their parental diploid plant with more than 90% grain fertile. Quantification of the genomic DNA was determined by NanoDrop™ spectrophotometer and DNA was then diluted with TE buffer to final concentration of 100 ng L^{-1}.

PCR amplification

Each PCR reaction was conducted on 12.5 µL total volume containing 6.25 µL GoTaq® Green Master Mix (Promega), 3.25 µL nuclease-free water, 2.5 µL of DNA template, and 1 µL of each RAPD primer. PCR amplification was set as follows: pre denaturation at 94°C for 3 minutes, followed by 40 cycles of denaturation at 94°C for 1 minute, annealing at 37°C for 1 minute, and elongation at 72°C for 1 minute 30 seconds, with a final elongation at 72°C for 10 minutes. Amplified products were electrophoresed on 1.5 % (w/v) agarose gel.

Observation and data analysis

Observation was done on the profile of DNA fragments in agarose gels. The amplified products were scored based on the presence of the DNA band i.e., 1 for presence and 0 for absence using Gel Analyzer 19.1 software (www.GelAnalyzer.com). Polymorphism information content (PIC) for each RAPD primer was calculated using Gene Diversity Software (GDdom) that can be accessed online through http://plantmolgen.iyte.edu.tr/GDdom/ (Abuzayed et al. 2017). The PIC value calculates the informativity of each primer which indicates the capability of a primer to generate polymorphic bands. The PIC value of RAPD primer as a dominant marker ranges from 0 to 0.5 (Nagy et al. 2012). Jaccard similarity coefficient was used to estimate genetic similarity among DH plants as well as Principal Coordinates Analysis (PCoA). In addition, cluster analysis was carried out to determine the clustering pattern of DH plants based on the unweighted pair group method, with arithmetic averaging (UPGMA), using Jaccard

similarity index, in the PAST (Paleontological Statistics) v. 4.03 program (Hammer et al. 2001).

RESULTS AND DISCUSSION

Development of DHs derived from M_1 plants

Anthers from all treatments were able to generate callus with relatively similar on the onset of callus initiation, ranging from 24 to 26 days. The percentage of anthers producing callus increased with increasing irradiation dose which was 3.33%, 4.33%, 5.00%, and 5.67% at doses of 0, 100, 200, and 300 Gy, respectively (Table 1). In other studies, the percentage of callus induction of Indica rice cultivars was relatively in a similar range of 2.30%-8.45% (Ali et al. 2021; Rahman et al. 2021). Cempo Abang is classified as the Indica subspecies, which is known as more recalcitrant to callus induction in-vitro than the Japonica subspecies. Grewal et al. (2011) found that the Indica group had lower anther culturability with an average of 1.2% than the Japonica group which had anther culturability of 28.1%. They also discovered that genes that are responsible for anther culturability are considered incomplete dominant.

The onset and frequency of callus initiation is widely known as genotype dependent. It can be improved by adjusting culture media with different combination ratio of auxin and cytokinin (Rahman et al. 2021), modified basal media (Ali et al. 2021), or supplementing with polyamines (Maharani et al. 2020). The present study suggested that gamma irradiation treatments indirectly could increase callus formation. However, it does not affect the onset of callus induction. The increasing number of callus formations on higher doses might be related to gamma rays as stress treatment. Datta (2005) stated that callus induction from anther culture is highly influenced by several types of stress pretreatments. Moreover, Mkuya et al. (2005) found that direct application of gamma rays on panicles improved the callus induction of Indica rice line.

The transferred callus produced the brown callus, rooty callus, and plantlet with different frequencies. The brown and rooty calli were unable to generate plantlets. The brown calli were frequently found in the higher dosages of gamma radiation. Consequently, the frequency of callus produced plantlets decreased with the increasing dose of gamma irradiation which was 60.00%, 53.85%, 33.33%, and 23.35% for 0, 100, 200 and 300 Gy, respectively (Table 2). Myint et al. (2005) also observed that plants that were subjected to the highest gamma irradiation dose (450 Gy) generated the lowest percentage of plant regeneration. The higher number of brown calli could be caused by stress effect of gamma irradiation. Choi et al. (2021) reported that gamma irradiation is not only damaging the plant DNA but also inducing oxidative stress through excessive reactive oxygen species production, leading to indirect cell damage.

Each transferred calli generated albino, green, or mixed shoots which produced both green and albino plantlets (Figure 1e-1g). All the transferred calli derived from 0 and 300 Gy formed albino plants while the remaining treatments generated calli producing albino, green, and mixed shoot. Calli that were generated both albino and green plantlets were also found in anther culture of temperate Japonica rice varieties (Hooghvorst et al. 2018). Albinism or chlorophyll-deficient regeneration is one of the most important challenges in anther cultures of many cereal crops like barley (Makowska and Oleszczuk 2014), triticale (Krzewska et al. 2015), rice (Mishra and Rao 2016), and wheat (Weigt et al. 2019). In rice, the frequency of albino plant varies from 5% to 100%, and it is affected by several factors such as the length of cold pre-treatment exposure, genotype, culture medium, and gelling agent (Mishra and Rao 2016; Tripathy et al. 2019). The study showed a high frequency of albino plant ranging from 67% in 200 Gy treatment to 100% in 0 Gy and 300 Gy treatments. The various responses among the treatments indicated that gamma irradiation treatment did not affect the frequency of albino plant. On the other hand, the average number of shoots per transferred calli reduced with the increase in irradiation dose, from 6.2 shoots in control treatment to 3.5 shoots in 300 Gy treatment (Table 3).

Table 1. Callus induction frequency and the onset of callus initiation at different gamma irradiation treatment

Treatment	No. of anthers cultured	The onset of callus initiation (days after anther inoculation)	The number of callus obtained	
			No.	%
0 Gy	300	26	10	3.33
100 Gy	300	26	13	4.33
200 Gy	300	24	15	5.00
300 Gy	300	25	17	5.67

Table 2. Plant regeneration frequency at different gamma irradiation treatment

Treatment	No. of callus transferred	Brown callus		Rooty callus		Plantlet	
		No.	%	No.	%	No.	%
0 Gy	10	2	20.00	2	20.00	6	60.00
100 Gy	13	6	46.15	0	0.00	7	53.85
200 Gy	15	10	66.67	0	0.00	5	33.33
300 Gy	17	11	64.71	2	11.76	4	23.53

Table 3. Plant regeneration efficiency at different gamma irradiation treatment

Treatment	No. of callus	No. of callus producing shoot			No. of shoot		Total shoots	Percentage of albino plant (%)	Average shoot/ transferred calli
		Albino	Mixed	Green	Albino	Green			
0 Gy	6	6	0	0	37	0	37	100	6.2
100 Gy	7	5	1	1	27	9	36	75	5.1
200 Gy	5	3	1	1	12	6	18	67	3.6
300 Gy	4	4	0	0	14	0	14	100	3.5

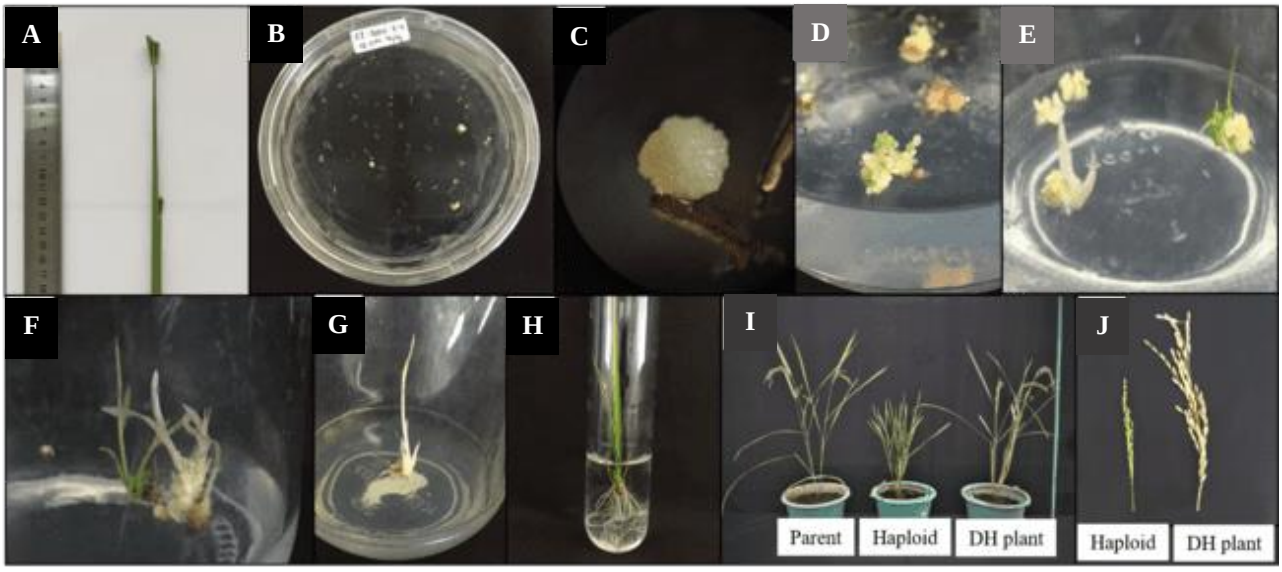

Figure 1. Double haploids development from M_1 red rice plants through anther culture. A. Selected boot length, B-C. Callus initiation, D. Callus generated green spot in regeneration medium, E. Callus regenerated into single type of plantlet (green or albino), F. Callus produced multiple mixed plantlets (green and albino), G. Single albino plantlet, H. Well-formed roots in the first phase of acclimatization, I. Comparison among parent, haploid, and DH plants, J. Comparison between haploid and DH panicles

A total of 15 green plants were regenerated from induced calli derived from plants that were subjected to 100 and 200 Gy. Twelve out of them survived and grew with morphological appearance similar to diploid parental plants, 11 of which were spontaneous DHs, and one was a haploid plant. The 11 DH plants have red pericarp as their parental line. The successful rate of acclimatization was 80 % which is lower than that of the other studies such as 91.7% in F1 hybrid of Indica rice (Dewi et al. 2017), 89% in F2 generation of Mediterranean Japonica rice (López-Cristofanini et al. 2018), and 100% in F1 generation of temperate Japonica rice (Sakina et al. 2020). The spontaneous DH plants were identified by their normal morphological appearance with more than 90% grain fertility whereas haploid plant had smaller appearance with sterile panicle (Figure 1i-1j). The percentage of spontaneous DH was higher (91.7%) than that obtained in different donor plants, such as 11.5%-47.1% in plants generated by crosses among Japonica rice (López-Cristofanini et al. 2018); 30.6%-60.0% in plants generated by crosses among Indica rice (Dewi et al. 2019).

Genetic diversity assessment of double haploids using RAPD

Eleven spontaneous DH plants obtained in this study and parental plant were assessed for genetic diversity using six RAPD primers. The number of amplified bands is shown in Table 4. The RAPD primers generated 51 bands, of which 34 alleles or 66.7% were polymorphic with an average of 5.6 polymorphic alleles per primer. The band size of the six RAPD amplified products ranged from 317 to 2133 bp. The PIC value ranged from 0.100 to 0.318 with an average of 0.206. The RAPD polymorphism among 11 DH plants and parental line using OPA 12 primer is shown in Figure 2.

The polymorphic alleles indicated variability among DH plants when compared with parental plant. The percentage of RAPD polymorphism obtained in this study was lower than that obtained by Zakiyah et al. (2019) which illustrated 21 aromatic rice genotypes with 84.4% polymorphism; Hanum et al. (2020) when analyzed four red rice genotypes with 93.5% polymorphism; and Epe et al. (2021) when characterized 15 rice cultivars with 88.9% polymorphic alleles. The polymorphic difference was due to different uses of rice genotypes as well as RAPD primers. Moreover, the polymorphism among the DH plants is not expected to be high because of the individual plant derived from the same parental line. The DNA polymorphism among the DH plants demonstrated the effect of gamma irradiation to generate genetic variation.

Table 4. Characteristics of six RAPD primers

Table 4. Characteristics of six RAPD primers

Primer	Primer sequence (5′-3′)	Size range (bp)	Total number of bands	Polymorphic bands	Polymorphism (%)	PIC value
OPA 12	TCGGCGATAG	339-1755	11	10	90.9	0.318
OPA 16	AGCCAGCGAA	408-2018	9	7	77.8	0.284
OPA 18	AGGTGACCGT	317-1490	9	7	77.8	0.165
OPD 02	GGACCCAACC	297-1570	9	3	33.3	0.111
OPD 03	GTCGCCGTCA	493-2133	8	6	75.0	0.260
OPD 05	TGAGCGGACA	388-1096	5	1	20.0	0.100
Total			51	34		
Mean			8.5	5.6	66.7	0.206

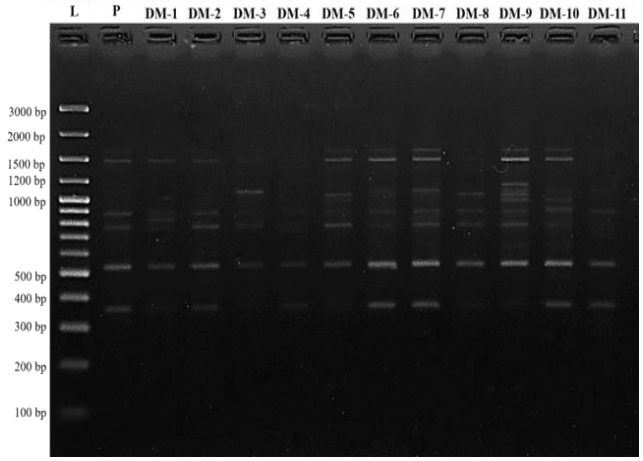

Figure 2. RAPD banding pattern of 11 DH plants and parental line using OPA 12 primer. L: DNA ladder, P: parental line, DM: Double haploid mutants

Gamma-rays induced mutation is one of the most frequently used method to generate valuable mutation in rice mutation breeding. Among the 853 rice mutant varieties registered in the mutant variety database of IAEA, 66% or 564 varieties were generated by gamma-rays, most of them were subjected to irradiation treatment ranging from 100 to 300 Gy. At the genome level, Zhang et al. (2020) analyzed the M_1 plants of rice cv. Gaogengnuo that were previously treated with 300 Gy gamma radiation using whole-genome resequencing. They found a total of 356,314 single nucleotide polymorphisms (SNPs), 9075 structural variations (SVs), 73,495 insertion/deletion polymorphisms (InDels), and 5100 copy number variations. These huge DNA polymorphisms are valuable resources in order to create variation by fixing it into DH plants through anther culture.

The genetic similarity between parental and 11 DH lines based on Jaccard's similarity index ranged from 0.622 to 0.854 while among the DH lines, it ranged from 0.524 to 0.902 (Table 5). The highest similarity value was between double haploid mutant no. 5 (DM-5) and DM-10 while the lowest similarity value was between DM-4 and DM-9. The wide range of variation among parental line and the DH plants was also shown in the scatter biplot of PCoA (Figure 3a). The two principal coordinates that accounted for 68.3% of total variation distributed 11 DH plants and parental lines into four quarters. Although there is no study to assess genetic variation among DH plants derived from anther culture, Kim et al. (2003) studied genetic variation among plants originating from irradiated calli of rice embryos. They detected genetic variation between parent and plant from irradiated calli ranging from 62.7% to 94.1% which is relatively similar to the present study.

The UPGMA dendrogram showed that the DH plants and parental line were divided into three clusters based on the average genetic similarity value of 0.70 (Figure 3b). The first cluster consists of parental plant and six DH plants induced by 200 Gy while the second and third clusters consist of respective three DH plants induced by 100 Gy. This result revealed that the DH plants were separated based on the level of the treatment. Nurmansyah et al. (2021) had also a similar result when comparing genetic similarities among faba bean M_2 mutant plants using AFLP markers. They found that mutant plants from the same gamma irradiation treatment were gathered in the same cluster and separated from the other groups.

The average genetic similarities among DH plants from the same dose of irradiation treatment compared to the parental plant were also calculated. The 200 Gy-gamma irradiation-induced DH plants showed higher genetic similarities to the parental plant (0.810) than that of the 100 Gy-gamma irradiation-induced DH plants (0.655). It indicated that the DH plants generated from 200 Gy are relatively similar to the parent. Moreover, it explained that the higher irradiation dose does not mean a higher mutation rate. It was explained by Li et al. (2016) when evaluating the mutation rate of six M_2 rice plants that were previously treated by three different doses of gamma irradiation rays (150, 250, and 350 Gy) using whole-genome sequencing. They found that plants that were exposed to the same dose had a relatively similar mutation rate. Although there was an increase of mutation rate in the 150 Gy-induced mutant plants from 9.49×10^{-6} to 9.70×10^{-6} in the 250 Gy-induced mutant plant, the 350 Gy-induced mutant plants showed a lower mutation rate with 7.52×10^{-6}.

Table 5. Pairwise Jaccard genetic similarity index of 11 DH plants and parental line

	Parent	**DM-1**	**DM-2**	**DM-3**	**DM-4**	**DM-5**	**DM-6**	**DM-7**	**DM-8**	**DM-9**	**DM-10**	**DM-11**
Parent	1											
DM-1 (100)	0.684	1										
DM-2 (100)	0.658	0.839	1									
DM-3 (100)	0.625	0.639	0.758	1								
DM-4 (100)	0.622	0.800	0.828	0.774	1							
DM-5 (200)	0.805	0.659	0.634	0.683	0.600	1						
DM-6 (200)	0.805	0.619	0.595	0.643	0.561	0.857	1					
DM-7 (200)	0.854	0.707	0.643	0.690	0.610	0.86	0.860	1				
DM-8 (100)	0.711	0.735	0.706	0.714	0.719	0.683	0.683	0.690	1			
DM-9 (200)	0.762	0.545	0.558	0.568	0.524	0.814	0.773	0.778	0.605	1		
DM-10 (200)	0.825	0.659	0.675	0.683	0.641	0.902	0.814	0.86	0.683	0.814	1	
DM-11 (100)	0.628	0.641	0.658	0.757	0.667	0.609	0.644	0.652	0.757	0.542	0.644	1

DM-1-DM-11: Double haploids originated from M_1 plants number 1 to 11. Values in the bracket indicate irradiation doses of each DH plant

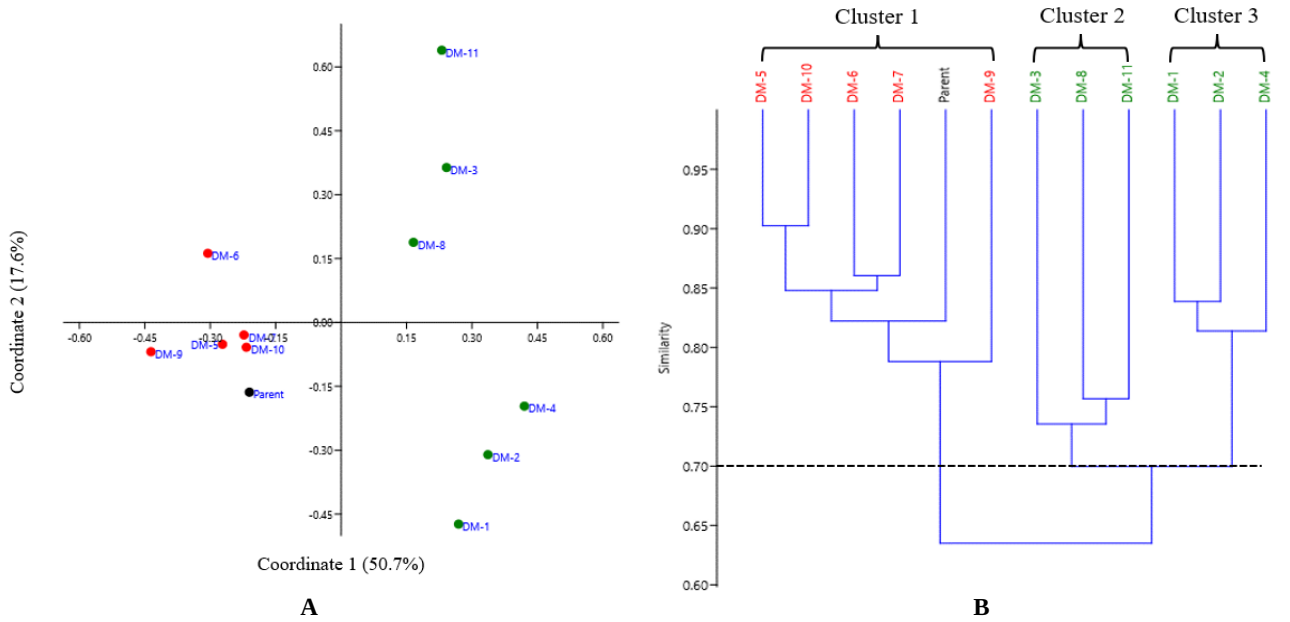

Figure 3. Relatedness among DH plants and parental plant. A. Principal Coordinates Analysis (PCoA) based on Jaccard's similarity index of 11 DH plants and parental plant. B. Cluster analysis of 11 DH plants and parental plant using the UPGMA method. DM-1-DM-11: Double haploids originated from M_1 plants number 1 to 11. Green font/dot indicates 100Gy-gamma radiation-induced DH plants, Red font/dot indicates 200Gy-gamma radiation-induced DH plants

In conclusion, gamma irradiation treatments could increase anther culturability. However, it reduces the number of calli producing plantlets due to higher frequency of brown calli. It also decreases the average number of shoots produced per transferred calli. The DNA polymorphism was detected among the DH plants and parental lines which demonstrated the effect of gamma irradiation to generate genetic variation. Moreover, cluster analysis shows that DH plants from the same dose of irradiation treatment were gathered within the same cluster which indicates plants from the same dose of treatment have a similar mutation rate. Therefore, the DH method integrated with induced mutation could be used as an alternative for rice breeding program especially to improve local rice cultivars.

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