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Research Article

Intraspecific SSR Marker Screening for Detection of *Dendrobium crumenatum* Mutants Generated from *In Vitro* Gamma Irradiation

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ABSTRACT

Determination of *D. crumenatum* mutant obtained from *in vitro* mutation breeding needs a long time due to its long-life cycle. SSR molecular markers can be used for early mutant detection. Specific SSR markers developed in *D. crumenatum* are not yet available. Alternative published SSR markers were developed from *D. catenatum*. The aims of this study are to screen the most informative SSR markers generated from *D. catenatum* tested in irradiated *D. crumenatum* population and to determine the gamma irradiation dose resulting the most mutants. Ten SSR markers were randomly selected and tested in 25 individuals of *D. crumenatum* plantlets irradiated with several doses (0, 5, 10, 15, and 20 Gy; 5 plantlets each dose). The result showed 7 of 10 primers were polymorphic and the other three were monomorphic. All seven polymorphic primers can be used to identified intraspecific variation in the *D. crumenatum* mutant population. Markers *dnsr28* and *dnsr98* were the most informative, with the highest polymorphic information content (PIC) value of 0.5. Irradiation *D. crumenatum* protocorms using 10-15 gray doses were detected as the highest mutant percentage obtained up to 100% in the sample tested. This resulting marker information can be used to screen wider mutant population to decrease the non-mutant individuals in the population for maintenance and cost efficiency. The 10-15 Gy can be used as reference doses for gamma irradiation in 3 months old *D. crumenatum* protocorm materials.

INTRODUCTION

Mutation breeding through several doses of gamma irradiation has been conducted to increase *Dendrobium crumenatum* diversity. Gamma Irradiation is one of many mutagens in mutation breeding techniques that can randomly induce variability of plant materials (Udage 2021). Many reports showed that gamma irradiation treatment has successfully changed the vegetative, generative, and metabolite performance of the plant species (Simanjuntak et al. 2020; Fathin et al. 2021; Aisyah et al. 2022a). The goal was to obtain novel *D. crumenatum* mutants with longer inflorescence bloom periods and/or novel flower morphology phenotypes. However, flower phenotypes require a long time to be observed due to

long-life cycle especially in the orchid genus. In general, it takes 3-4 years for orchid to complete one life cycle from seed to bloom (Hsu et al. 2018). Early screening of putative mutants will greatly help researchers to reduce the number of plants maintained, costs, and labor. Early screening based on visual or phenotypic plants is difficult to do because not many variances can be observed in vegetative *in vitro* stage and plantlets that do not differ much between controls and those that have been irradiated (except for the plantlet sizes).

Early putative mutant selection can be done molecularly to determine individuals whose genetics have changed post mutation treatment. Among many molecular markers, SSR marker is a codominant marker based on specific simple sequence repeat (SSR) motifs that highly polymorphic and spread in the whole genome possible to differentiate inter- or intraspecific variations (Zhao et al. 2019; Li et al. 2021) that are the best option for diversity analysis in mutant population.

The SSR markers are mainly generated from whole genome (Bhattarai et al. 2021) or RNAseq (Tsai et al. 2015) data. Despite the fact that next-generation sequencing (NGS) has been well developed, the research in the field is relatively pricy. The data analysis can only be performed by bioinformatic specialist supported with super computer. Luckily, some of the SSR motives are conserved in cross species (Zhao et al. 2019) that mean the SSR generated from one species genome can also be used in other species. The SSR markers of *Dendrobium* species have been published and generated from *D. catenatum* genome scaffolds and have not been tested in *D. crumenatum*. The *D. crumenatum* intraspecific diversity, specifically in the mutant population study using informative primer, has not been reported. This study aims to screen the most informative SSR primer generated from *D. catenatum* tested in irradiated *D. crumenatum* population and determine the gamma irradiation dose resulting in the most mutants.

MATERIALS AND METHODS

Materials

Plant material, *in vitro* gamma irradiation mutation, and protocorm maintenances

Plant material used in this experiment are 3 months old *Dendrobium crumenatum* protocorm that have been gamma irradiated in several doses, the main different phenotypic between the materials is the plantlet sizes (Figure 1). The seeds were extracted from seedpod resulting from self-pollinated wild *D. crumenatum* obtained in Bogor, West Java. The extracted seeds then cultured in *in vitro* culture following Sanjaya et al. (2022) germination medium. Briefly half-strength Murashige and Skoog medium supplemented with 15% coconut water, 30 g L^{-1} sugar, 7 g L^{-1} agar. The pH of the medium was adjusted to 5.6-5.8. The protocorms were subcultured to germination medium in the disposable petri dish (50 protocroms/petri) for 1 week and then irradiated in Gamma cell 220 with several doses consisting of 0, 5, 10, 15, and 20 gray with 3 replicates. The protocorms were then directly transferred to grown in fresh growing

Figure 1. *D. crumenatum* gamma irradiated plantlet materials used in this experiment.

orchid medium (Lubis et al. 2020). Briefly 2 gr L^{-1} hyponex 20:20:20, 50 gr L^{-1} ripe banana, 50 gr L^{-1} potato, 2 gr L^{-1} active charcoal, MS vitamins, 20 g L^{-1} sugar, and 7 gr L^{-1} agar, and the pH of the medium was adjusted to 5.6-5.8. The protocroms were maintain in the media for 6 months.

Methods

DNA isolation

The DNA were isolated from five individuals from each treatment with the total of 25 samples following modified Putri et al. (2021). Each plantlet (±0.2 g) was taken and ground in 1.5 mL microtubes manually using mini pestle then 700 µL of CTAB buffer was added and vortexed until homogeneous. The samples were then incubated in a water bath at 65^{O}C for 30 minutes (vortexed every 10 minutes). The samples were then centrifuged at a speed of 8500 rpm for 15 minutes. The upper phase (supernatant) was transferred to a 1.5 mL microtube, taken carefully using a micropipette. CIA (chloroform : isoamyl alcohol, 24 : 1) was added in 1x volume of supernatant and inverted for 10 minutes then centrifuged again at 8500 rpm for 15 minutes. The upper phase was carefully taken using a micropipette and transferred to a new 1.5 mL microtube. A total of 1x the volume of cold absolute ethanol was added to the sample and homogenised by turning it back and forth manually. The sample was then precipitated by incubating in a freezer (-40^{O}C) overnight (16-18 hours). After precipitation, the sample was centrifuged at 8500 rpm for 30 minutes. The supernatant was discarded and the pellet was washed by adding 400 µL of cold 70% ethanol, homogenised by vortexing and centrifuged at 8500 rpm for 5 minutes. Samples were washed two times. The supernatant was discarded and the resulting DNA pellet was air-dried until no visible liquid trace or alcohol odor can be smelled. The DNA pellet was diluted by adding 100 µL ddH_2O. The resulting stock DNA is then stored in a -20^{O}C freezer until use.

Primer screening

Ten SSR Primers were selected from Zhao et al. (2019), Table 1. From 72 of the published primers, the primer selection criteria in this research was the primer that can amplify and resulting PCR product in other species (*D. catenatum*, *D. denneanum*, and *D. nobile*) as the indicator that primers are universal for species in *Dendrobium* genus. The primers were tested by PCR amplifying in 25 *D. crumenatum* irradiated plantlets (5 plantlets each dose; 0, 5, 10, 15, and 20 gy) with the setting of pre-

Table 1. Selected primers from SSR mining from *Dendrobium catenatum* genomic scaffolds generated by Zhao et al. (2019) tested in gamma-irradiated *Dendrobium crumenatum* population.

Primer ID	Forward Sequence (5′-3′)	Reverse Sequence (5′-3′)	Target Size (bp)	Repeat Unit
dnsr9	CCATTTATGTTGCTTGCGAGTT	GCTCCGACGTAAGATTCACAA	181	$AG_{(51)}$
dnsr17	GCCGCCTTTCTTATGTTAGTTG	GCGTAATGCTGTGACACCAA	285	$TAA_{(39)}$
dnsr24	TTCGAGGCAACGGAGTCAG	TCCACCAGCAAAGCACACT	451	$TTA_{(57)}$
dnsr25	GCATCATAAGCAGTAGGTAAAC	CCACTAGACTTGTTGATAGCAT	329	$AC_{(50)}$
dnsr28	TGGAGCAAGACTTGTCTAAGC	ACTTGAGATTAGCAAACAGCAC	217	$TACA_{(11)}$
dnsr55	GTCCTAAGATTCTACCGCATCA	AAGGTGAAGCCTAAGGTCTACT	473	$TG_{(91)}$
dnsr58	GGTAGGTTGAGTAGCTGAGAC	TCCCTAACAACAAACAGACATG	159	$TTTC_{(9)}$
dnsr92	CCAACAGAACTTGCAGGACTAG	CGACTCCACGGGACTACTTT	319	$AT_{(46)}$
dnsr95	CTTCTTCTCCTGAGCCTGTGA	TGCTGCTGCCCTTACTAAGT	344	$ATT_{(73)}$
dnsr98	TTTGTGCTCAGTTTGTGTTTCC	GAATCTCACGCCATCTCTGC	408	$TTA_{(77)}$

denaturation in 94°C for 5 minutes, denaturation in 94°C for 30 seconds, annealing in 55°C for 30 seconds, extension in 72°C for 25 seconds, and final extension in 72°C for 5 minutes. The PCR products were then separated in 1% agarose gel, electrophoresis setting of 100 volts for 30 minutes, and visualised under UV-transilluminator.

Data analysis

The agarose gel visualisations were scored manually. The scoring data were used to calculate the allele number, main allele frequency, and PIC. The PIC formula:

$$PIC=1-\sum_{i=1}^{l} P_i^2 - \sum_{i=1}^{l-1}\sum_{j=i+1}^{l} 2P_i^2 P_j^2$$

P*i* and P*j* are the population frequency of the *i*th and *j*th allele.

The dendrogram was constructed using NTSYS 2.1 software with the command of Sequential Agglomerative Hierarchical and Nested-Unweighted Pair-Group Method with Arithmetic (SAHN-UPGMA) (Rohlf 2000).

RESULTS AND DISCUSSION

Results

The PCR using 10 SSR-selected primers successfully amplified all the *D. crumenatum* sample tested. The primers detected as many as 45 alleles, ranging from 1 to 9 alleles with an average of 4.5 alleles (Table 2). Primer *dnsr95* and *dnsr98* showed the highest number of alleles (9 alleles) and highest number of polymorphic alleles (9 alleles; 100% polymorphic) (Figure 2), while primer *dnsr24*, *dnsr25*, and *dnsr55* were the lowest containing only one allele (0% polymorphic) and monomorphic. The average allele frequency was 0.67, the lowest was 0.51 in *dnsr28* and the highest were 1.00 in *dnsr24*, *dnsr25*, and *dnsr55*. The SSR used are dominant markers because the obtained alleles are more than 2, which is perfect for diversity analysis but may be inappropriate in progeny-parentage analysis.

The alleles from polymorphic SSR primers were used as a base for manual genotyping and phylogenetic tree construction using NTSYS 2.1 software. Based on the phylogenetic tree, the seventh primers can differentiate the samples into two big group (A and B) and totals of 4 sub-

Table 2. Polymorphism of 10 SSR primer pairs in gamma-irradiated *Dendrobium crumenatum* population.

Primer ID	No. of allele	No. of polymorphic allele	% allele polymorphic	Allele Freq.	PIC
dnsr9	7.00	7.00	100.00	0.79	0.33
dnsr17	6.00	6.00	100.00	0.59	0.48
dnsr24	1.00	0.00	0.00	1.00	0.00
dnsr25	1.00	0.00	0.00	1.00	0.00
dnsr28	3.00	3.00	100.00	0.51	0.50
dnsr55	1.00	0.00	0.00	1.00	0.00
dnsr58	3.00	3.00	100.00	0.96	0.08
dnsr92	5.00	5.00	100.00	0.57	0.49
dnsr95	9.00	9.00	100.00	0.76	0.36
dnsr98	9.00	9.00	100.00	0.55	0.50
Total	45.00	42.00			
Average	4.50	4.20		0.67	0.39

Note: PIC= polymorphic information content.

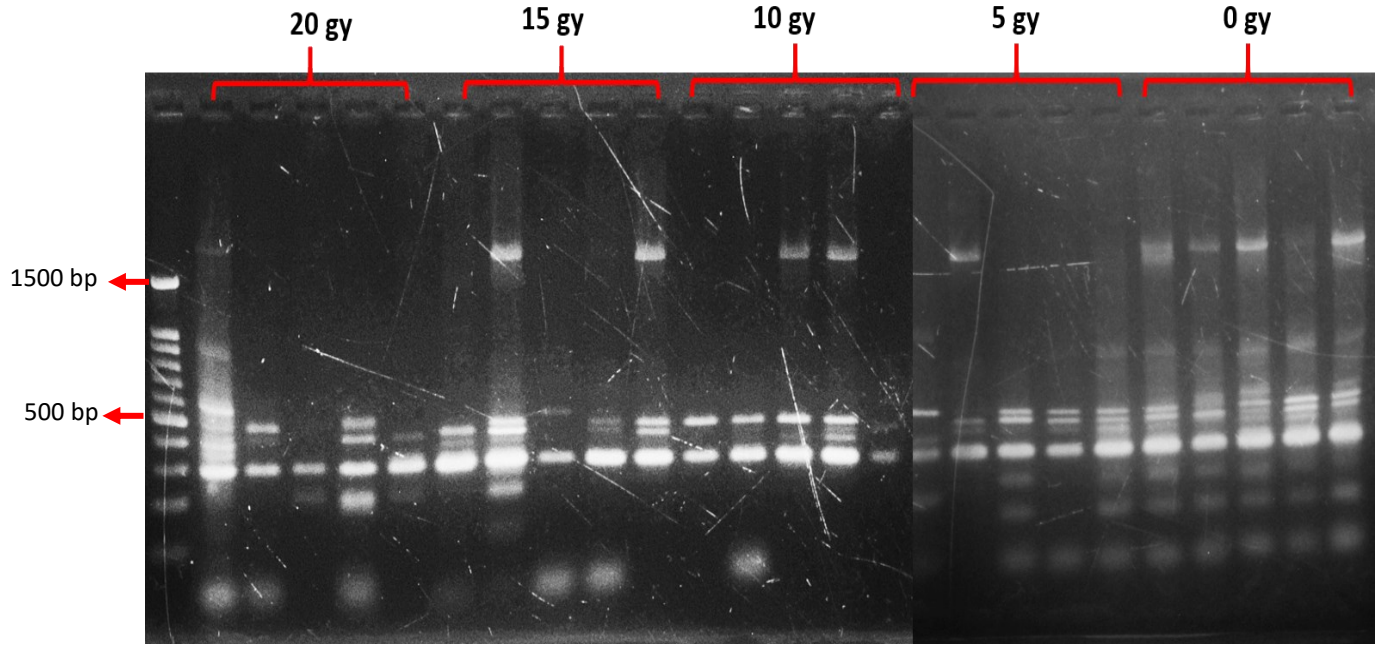

Figure 2. Gel visualisation of amplified PCR products from 25 gamma-irradiated *D. crumenatum* samples using primer *dnsr98* showed the highest polymorphism.

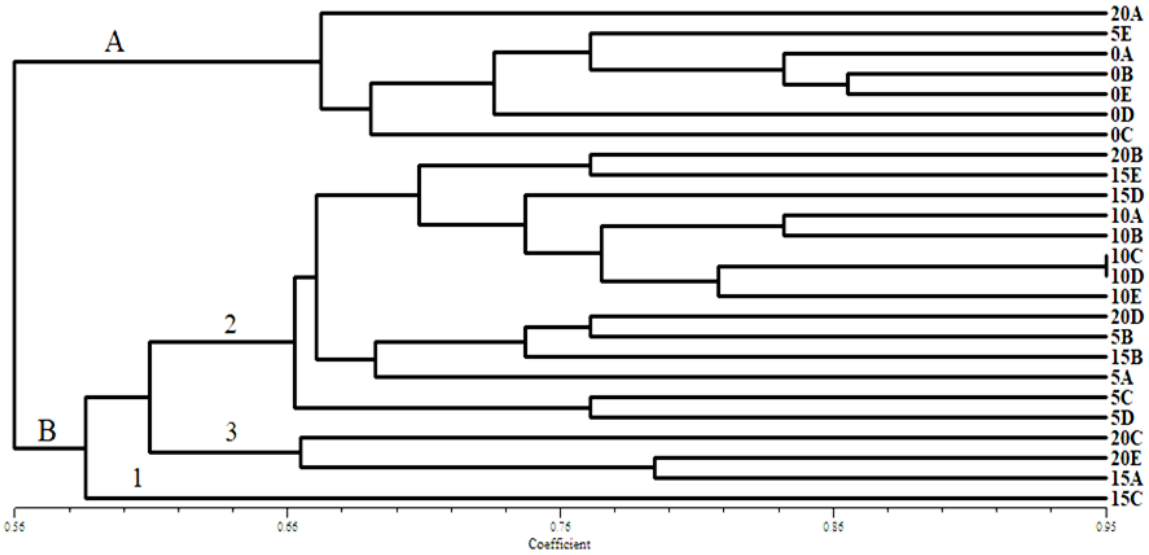

Figure 3. Phylogenetic from 25 individuals *D. crumenatum* Irradiated with five different doses differentiated with 7 SSR primers generated from *D. catenatum* genome sequence.

groups (A1, B1, B2, and B3) (Figure 3). The A1 subgroup consist of all control samples (0Gy; 0A-E), and two irradiated samples (5Gy; 5E and 20Gy; 20A), while the rest samples are scattered into group B1-3. The result indicates that the SSR motives of the samples in subgroup A1 having high similarity means that irradiated samples 5E and 20A are not putative mutants, while the other subgroups have more different SSR motives that resulting from the gamma irradiation means the other samples in subgroup B1-3 are mutants.

We also found that 18 of the 25 samples (72%) are mutants from the result of seven SSR primers screening in the gamma-irradiated samples. The highest mutant percentage, up to 100% were obtained in 10 and 15 Gy samples, while the 5 and 20 Gy samples showed 80% of the materials are mutants (Table 3). The data indicates that 10-15 Gy are effective in generating mutants from 3 months-old *D. crumenatum* protocroms.

Table 3. Mutant percentage determined using SSR primers.

Gama irradiation dose	Mutant with 7 primers
0 gray	0%
5 gray	80%
10 gray	100%
15 gray	100%
20 gray	80%

Discussion

D. crumenatum is an orchid species that is commonly found in Sumatra (Melinda et al. 2022), Yogyakarta (Semiarti et al. 2020), Bali (Darmawati et al. 2018) and spreads in many other areas in Indonesia. The characteristics of *D. crumenatum* are very small (< 20cm) plant height (from base to the tip of the flowering pseudobulb) and small flower (width < 3 cm) (De et al. 2015). Despite the species having potential traits like strong fragrance, multiple flowers, and ease of propagate, the flower has low or no economic value due to the small flower size and short blooming time (overnight) (Figure 4). Some traits modifications of the species may able to improve the species phenotype that may increase the economic value of the species. The long flowering time of the species is the main target to be improved.

Figure 4. *D. crumenatum* plant (left) and flower (right).

Breeding can improve the traits of the species. Mutation breeding through gamma irradiation considered as faster and efficient breeding method compared to cross-breeding or genetic engineering. Mutation breeding is considered more effective and time efficient (Udage 2021). Mutation using gamma irradiation was able to increase the flower phenotype variability in *Portulaca grandiflora* (Aisyah et al. 2022a), *Celosia cristata* (Simanjuntak et al. 2020), *Celosia argentea* var. *plumosa* (Aisyah et al. 2022b) *Echinacea purpurea* (Cahyaningsih et al. 2022), *Chrysanthemum morifolium* 'Donglinruixue' (Wang et al. 2020) and many plant species. Mutation breeding is known as a random induced mutation that can change one or several plant traits. Due to random mutation, it is also possible to obtain other potential mutant of *D. crumenatum* that may attract market interest.

Despite several advantages of mutation breeding, there is a limitation: mutation breeding always works with a vast population that needs to be maintained and selected. Early selection will narrow up the population number. In our chase, the only phenotypic change that we can observed 6 six months post-mutation was only the plant size. The plant size

may be caused by the treatment successfully changed the material genetics or just the epigenetic effect. The most effective method for early mutant determination is molecular screening. SSR is the common molecular marker used for mutant detection (Mansyur et al. 2019; Asadi et al. 2020; Arrufitasari et al. 2022; Vighneswaran et al. 2022). SSR markers are generated from repeat sequences that are primarily found in microsatellites of the chromosome that are relatively more conserved compared to p or q chromosome arms (Mason 2015) and can differentiate plants at inter-and intraspecific level (Zhao et al. 2019; Li et al. 2021; Guerrero et al. 2022; Pauldasan et al. 2022).

Published SSR markers generated from *D. catenatum* genome (Zhao et al. 2019) were used in this study but further optimisation is needed to be applied in the different species population. The screening result showed as many as seven markers are polymorphic and clustering the control material in one subgroup A1 (Figure 2) showed high sensitivity of the marker in mutant detection. Interestingly, two gamma-irradiated materials in dose of 5 (5E) and 20 Gy (20A) also grouped in subgroup A1 indicating both samples may not mutant. The SSR primers developed from *D. catenatum* genome scaffold has been published and tested in *D. catenatum*, *D. denneanum*, and *D. nobile* intraspecific population and showed highest PIC of 0.751, 0.681, and 0.626, respectively (Zhao et al. 2019). But lower PIC (the highest obtained in this research were only 0.5) obtained in this research possibly due to different sequence repeat between *D. crumenatum* and *D. catenatum* from Zhao et al. (2019) report. The material possibly has low diversity even after mutated.

Not many morphological characters can be observed in *in vitro* plantlets even six months post-mutation (nine months old *in vitro* culture from seed). No phenotypic trait was measured due to no distinct phenotype can be observed visually. In this stage, we focused on SSR genotyping result to determine the best primers in differentiating putative mutant. But, the most striking difference is the size of the plantlets which looks more dwarf when treated with higher gamma irradiation doses at 15-20 Gy (Figure 1). The most putative mutants from phenotypic observations based on plantlet size were obtained at 15-20 Gy. However, this is slightly different from what is obtained from SSR-genotyping data. That most mutants were obtained in 10-15 Gy.

Information from this research is important to us to verify that our gamma irradiation treatments were able to increase the variability of the *D. crumenatum* to molecular level and possibly to important commercial traits. The phenotypic of the obtained mutant materials needs to be further evaluated to determine the potential use of the materials. Besides the ornamental values, *D. crumenatum* was reported to have pharmacological effect in dermatological problems like boils and pimples (Wang 2021) also antimicrobial properties and several potential metabolites like saponin, terpenoid, and alkaloid compounds (Sandrasagaran et al. 2014). The strong flower fragrance can also be developed as raw material in perfumery.

CONCLUSION

The SSR marker generated from different species (*D. catenatum*) can differentiate intraspecific *D. crumenatum* population generated from gamma irradiation. Out of the 10 markers tested, three were monomorphic while the rest were polymorphic. All polymorphic markers were able in differentiating individual in the population but the *dnsr28* and *dnsr98* were more informative with the highest PIC value of 0.5. Diversity analysis by the similarity using the polymorphic markers

showed that the tested population is grouped into 2 big groups (A and B) and 4 subgroups (A1, B1, B2, and B3). All of the control samples (0 Gy) were clustered in A1 together with 5E and 20A, while the rest of the samples were scattered in group B. The polymorphic markers were informative to differentiate mutants and non-mutant material. The highest mutant percentage was obtained in 10-15 Gy gamma irradiation doses.

AUTHORS CONTRIBUTION

R.D. conducted the research and wrote the manuscript., I.P.W.S. supervised the manuscript., H.Y. and I.A.D.P., conducted data analysis and provided photographs., I.K.S. and P.K.M. recapitulated data, created tables, and formatted the manuscript., Y.F. conducted the research.

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CONFLICT OF INTEREST

The authors declare there is no conflict of interest in any part of this research.

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