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Molecular Characterization of Orchid Variants *Spathoglottis plicata* Blume Based on RAPD Analysis

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ABSTRACT
The development of *S. plicata* orchid variants is important to increase their genetic diversity. This study aims to determine the genetic diversity of *S. plicata* orchid variants based on RAPD analysis. The research sample was 15 plant variants consisting of wild type and mutant orchid variants. Molecular characterization was used to identify the genetic diversity of orchid variants based on the PCR-RAPD using eight universal primers from the screening results. The DNA amplicon electropherogram from RAPD results was converted to binary data and analyzed with GenAlex 6.1 to obtain the genetic distance, scatter diagram, and the percentage of DNA polymorphism. The data were also analyzed using NTSYS ver.2.2 to obtain a dendrogram cluster using the Unweighted Pair-Group Method using Arithmetic Average (UPGMA). The results showed that DNA polymorphism was found in the wild-type and mutant orchid variants. The percentage of DNA polymorphism of mutant orchid groups was greater than that of wild type orchids. The results of the Principal Coordinate Analysis based on the genetic distance showed that the *S. plicata* mutant orchid variant was in a different quadrant from the wild type group. Based on the dendrogram cluster, the mutant orchid variants were located away from the wild orchid, which confirmed that the mutant orchid variants have undergone genetic changes.

Keywords: *Spathoglottis plicata variants, genetic diversity, RAPD.*

1. INTRODUCTION

Spathoglottis plicata orchid is a significant commodity with a high economic value, especially for orchids with unique characters and beautiful flowers. Spathoglottis orchids with purple flowers are the most common species found. This happens because most orchid propagation is carried out by splitting pseudobulb or tillers. Morphologically, the orchid variation can be observed from the characteristics of the leaf length, plant height, number of tillers, flower stalk length, and flower color. Another type of Spathoglottis orchid found is Spathoglottis with yellow and white flowers. In addition to conservation, the development of this orchid variant also needs to be improved. For this reason, morphological and molecular characterization is important for all variants found to determine their conservation and development strategies.

Spathoglottis orchid is one of the best-selling ornamental orchid plants. Many orchid fans and growers have collected this orchid. This plant is easily cultivated only by splitting the pseudobulb, but such reproduction will only produce clones and not produce variants. In some countries, this plant is reportedly vulnerable to extinction. The status of this orchid is not stated in the red list plant at IUCN (International Union for Conservation of Nature), but it is declared as a vulnerable plant in Australia [1] and India [2]. There are three challenges associated with the reproduction of orchid plants, including the preservation of orchid species to avoid extinction, the development of clones with certain superior characters, and the creation of variants with new characters. The development of diversity will increase the economic value of these orchids as commodity plants. Variants with superior morphological characters are found on the habitus profile, color and number of flowers, and the length of the stems, which is proportional to the plant height. Orchids that are not too short or too long have interesting characters [3].

Based on previous research [4], it was reported that one of the 118 wild type orchids found in the Yogyakarta Region was *S. plicata*. From the results of in vitro culture, the orchid variant obtained was *S. plicata* with purple, purplish, or white flowers [5]. The

question is whether morphological variation in orchid variants is related to the genetic variation, which is an interesting problem to observe. Molecular variations can be detected by DNA, RNA, or protein analysis [6]. One of the most widely used molecular characterization methods is a DNA analysis using RAPD (Random Amplification of Polymorphic DNA) markers. The research aims were to identify the genetic variability of *S. plicata* variant plants based on the RAPD analysis.

2. RESEARCH METHOD

2.1. Research material

The research material is about 30 months old variants of *S. plicata* Blume orchid, consisting of wild type and mutant variants derived from in vitro seed culture. Cultured seeds come from the normal seeds and seeds irradiated with X-rays.

2.2. DNA isolation

The DNA analyzed was genome DNA from the wild type orchids and S. plicata variant taken from small pieces of leaves. The DNA isolation was carried out by the CTAB method, according to Murray and Tompson [7], with modification. The DNA quantification was performed by spectrophotometry at λ 260 and 280 nm to determine the concentration and purity of the isolated DNA.

2.3. DNA analysis with RAPD

Molecular analysis (DNA) was performed by PCR-RAPD method (Polymerase Chain Reaction - Random Amplified Polymorphism DNA) [8]. It was used to detect DNA polymorphisms to determine the level of genetic diversity (DNA) in the wild-type orchid and mutant variants. The molecular markers used for PCR-RAPD were determined based on the results of screening 22 universal primers using three DNA genome samples. The screening results obtained nine primers (Table 1)which showed the presence of DNA polymorphism

Table 1. Selected universal primers of DNA analysis with PCR-RAPD

N0	Primer	Nucleotide sequence 5'— 3'
1	OPA-02	TGCCGAGCTG
2	OPA-11	CAATCGCCGT
3	OPA-12	TCGGCGATAG
4	OPA-14	TCTGTGCTGG
5	OPB-1	GTTTCGCTCC
6	OPB-4	GGACTGGAGT
7	OPD-12	CACCGTATCC
8	OPD-14	CTTCCCCAAG

The PCR reaction was carried out using a 200 µL PCR microtube with a final volume of 10 µL, containing 5 µL of Go Taq Green (Promega), 0.25 µL (10 µM) primer (decamer), 2.5 µL DNA template (diluted 25x), and 2.25 µL nuclease free water. PCR was performed in stages: pre-denaturation (94o C, 1 minute), denaturation (94o C, 30 seconds), annealing (37o C, 30 seconds), elongation (72o C, 1 minute 30 sec), expansion (72o C, 7 minutes), and hold (4o C, 5 minutes). In addition, PCR is run in 45 cycles.

The DNA amplicon from the PCR was then visualized by electrophoresis on 1.5% agarose gel with 1 µL of DNA staining (FloroSafe). The DNA size marker used is 1kb DNA Ladder Vivantis, which provides variations in the size of DNA fragments between 100-10,000 bp. Electrophoresis was carried out in a 1x TBE solution with 100 volts in 55 minutes. The electrophoresis gel was then observed under UV and photographed with a digital camera

2.4. Data processing

The DNA amplicon electropherogram from RAPD results is converted into binary data by giving a score of 1 if there is a DNA band, and a zero score if there is no DNA band. The data were then analyzed using the GenAlex 6.1 program [9] to obtain genetic distance, scatter diagram, and percentage of DNA polymorphism. The data were then analyzed using the NTSYS version 2.2 program to obtain dendrogram clusters [10], using the UPGMA (Unweighted Pair Group Methods with Arithmetic Averages) method.

3. RESULT AND DISCISSION

3.1. DNA analysis with RAPD

The collection of *S. plicata* orchid variants is derived from the results of in vitro culture of normal and irradiated seeds. The *S. plicata* orchid has a variety of morphological characters, especially in terms of flower size and color, including its labellum character (Figure 1). Other morphological variations appear in the leaf length, flower stalk length, plant height, clump size, or the number of tillers (Figure 2). This morphological variation is likely related to genetic variation.

Figure 1: Flower color variations on several *S. plicata* orchid variants

Figure 2: Differences in morphology of several *S. plicata* orchid variants; Ruler size = 40 cm

Variations of flowers in *S. plicata* were found in both wild type and mutants Variations in the wild type orchid are due to genetic recombination. According to Soraka & Lyakh [11], plant mutations can occur in various organs, including mutations of leaves, stems, roots, flowers, fruits, and seeds. The results of X-ray irradiation on *S. plicata* seeds also produce phenotypic variations in leaves, roots, and stems; and induce premature flowering of mutant seedlings in bottles [12]. Romeida et al. [13] also found albino mutants, purple stem mutants, variegated mutants, and flower color mutants in *S. plicata* derived from orchid pods radiated with gamma rays.

3.2. DNA analysis with RAPD

The DNA analysis results using RAPD markers on 15 *S. plicata* variant samples showed variations or differences in DNA band patterns (Figure 3). The number of DNA bands and DNA polymorphisms produced from RAPD using 8 primers showed a wide variation. Most primers produced polymorphisms of 60-100%, except for the OPB1 primer.

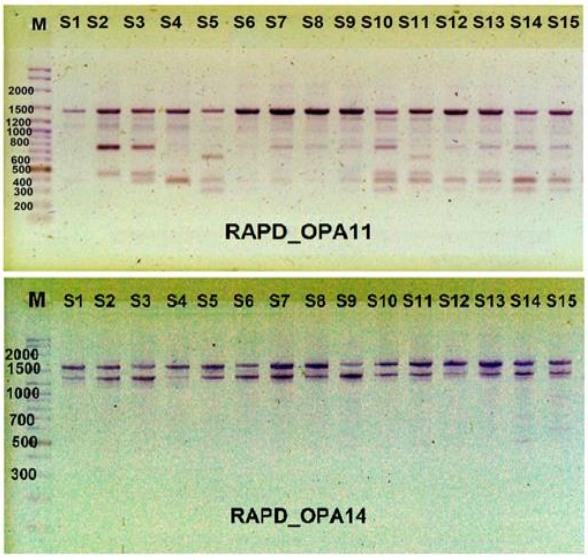

Figure 3: DNA electropherogram of *S. plicata* orchid variants from PCR RAPD with OPA-11 & OPA14 primers

Based on the results of the RAPD analysis, genomic DNA samples from the *S. plicata* orchid variant produced 3-12 DNA bands with a percentage of polymorphism reaching 90-100% (Table 2). This indicates that the primers are able to strongly demonstrate DNA polymorphism in the *S.plicata* orchid variants. Variations or differences between the variants can also be seen from the genetic dsitance (Table 3).

Table 2: Number of DNA bands and DNA polymorphism percentage produced by the primer

Primer	Nucleotide sequence	Total band	Number of polymorphism	Number of monomorphism	Polymor-phisme (%)
OPA-02	TGCCGAGCTG	9	9	0	100
OPA-11	CAATCGCCGT	10	9	1	90
OPA-12	TCGGCGATAG	7	7	0	100
OPA-14	TCTGTGCTGG	5	3	2	60
OPB-1	GTTTCGCTCC	4	4	0	100
OPB-4	GGACTGGAGT	1	0	1	0
OPD-12	CACCGTATCC	12	12	0	100
OPD-14	CTTCCCCAAG	8	8	0	100

Table 3. Genetic Distance Matrix of *15 S. plicata* Variants

S1	S2	S3	S4	S5	S6	S7	S8	S9	S10	S11	S12	S13	S14	S15	
0															**S1**
6	**0**														**S2**
9	**7**	**0**													**S3**
12	**16**	**17**	**0**												**S4**
15	**17**	**20**	**7**	**0**											**S5**
14	**16**	**19**	**4**	**3**	**0**										**S6**
13	**15**	**18**	**13**	**12**	**11**	**0**									**S7**
17	**19**	**20**	**13**	**12**	**11**	**8**	**0**								**S8**
17	**19**	**22**	**13**	**10**	**9**	**6**	**8**	**0**							**S9**
22	**24**	**27**	**16**	**15**	**14**	**9**	**13**	**9**	**0**						**S10**
22	**24**	**23**	**14**	**13**	**12**	**15**	**13**	**9**	**14**	**0**					**S11**
24	**26**	**29**	**16**	**17**	**14**	**15**	**19**	**15**	**14**	**18**	**0**				**S12**
19	**21**	**24**	**15**	**10**	**11**	**8**	**10**	**8**	**9**	**13**	**13**	**0**			**S13**
23	**25**	**26**	**19**	**16**	**17**	**14**	**14**	**12**	**13**	**15**	**21**	**14**	**0**		**S14**
22	**24**	**25**	**14**	**13**	**14**	**15**	**13**	**15**	**16**	**14**	**18**	**13**	**15**	**0**	**S15**

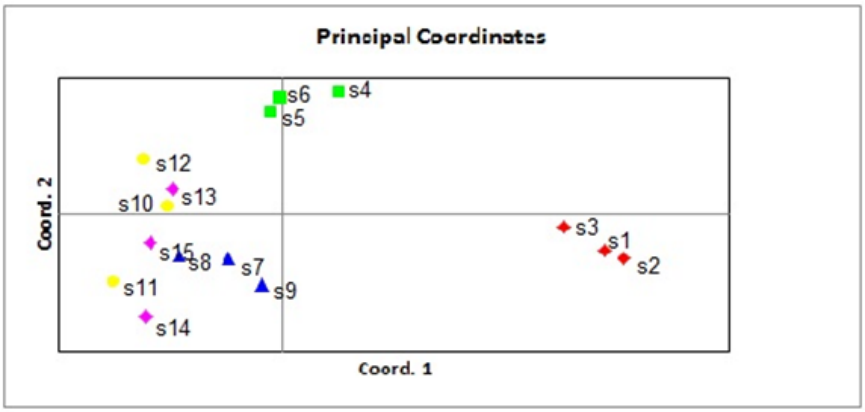

Figure 4. Scattered Diagram Based on Principal Coordinat Analysis of Genetic Distance of *S. plicata* Orchid Variants

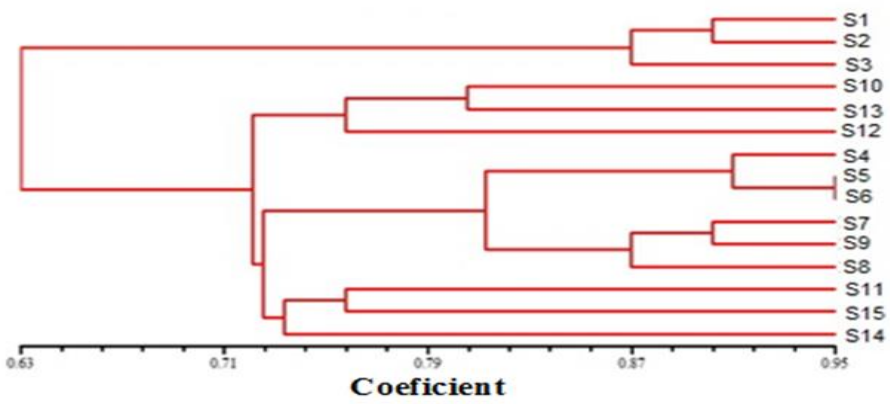

Figure 5. Dendrogram Cluster Based on Genetic Distance of 15 *S. plicata* Orchid Variants

Regarding the Principal Coordinate Analysis (PCA) of the GenAlex ver 6.1 program (Figure 4), the wild-type *S. plicata* variants are clustered in the same quadrant, whereas groups of the mutant variants are scattered in the opposite quadrants.

The molecular character of *S. plicata* orchid consisting of wild type variants S1, S2, and S3 had a very high similarity index that reached 0.87 or had a very high level of similarity of 87% (Figure 5). The PCA analysis results showed that these wild type orchid variants cluster in the same quadrant (Figure 4). In contrast, the mutant variants had a lower similarity index (<0.64) than that of the wild type variants. Mutant plant variants were scattered in different quadrants. Among the 12 mutant *S. plicata* orchid variants, some of them have a large genetic distance (Figure 5). This gave an indication that several plants had undergone significant genetic changes, as indicated by a higher percentage of DNA polymorphisms. Nonetheless, the differences due to DNA changes in the mutant group did not show any effects on the morphology of the plants.

The appearance of morphological variations due to gamma-ray radiation was also found in roses [14]. Morphological changes due to radiation also occur in Gypsophila paniculata [15] and Moluccella laevis [16]. Based on the results of the DNA analysis of homologous POH1 [5], X-ray irradiation resulted in insertion, deletion, and substitution of DNA nucleotides in *S. plicata* orchids that experience shoot mutations. Rick [17] reported the occurrence of chromosomal aberrations, namely ring deletion in the Tradescantia pollen due to X-ray irradiation. Murata et al. [18] found mutation deletion and insertion of one or several nucleotides in the medicinal plant group of Angelica acutiloba in Japan. X-ray irradiation on *S. plicata* Blume seeds has been proven able to induce the prominent changes in morphology and genetics in *S. plicata* seeds so that it makes sense that the mutated orchids exhibit greater genetic variability than that of wild orchids.

4. CONCLUSION

Genetic variability was found in *S. plicata* orchid variants, both in wild type and in mutant variants. It was shown by the higher average percentage of DNA polymorphism; the more diffuse the distribution of variants in the scatter diagram, the greater the molecular similarity index in the dendrogram cluster. A higher percentage of DNA polymorphisms in the mutant variant group than in the wild type group indicates an increase in the genetic variability due to DNA changes. The similarity index of the mutant group is lower (<0.64) than that of the wild type group (> 87%), indicating that some variants of the mutant are potential to become new cultivars.

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