Advances in Social Science, Education and Humanities Research, volume 528
Proceedings of the 7th International Conference on Research, Implementation, and Education of Mathematics and Sciences (ICRIEMS 2020)

Genetic Diversity of *Spathoglottis plicata* Blume Orchid Variant Based on Inter-Simple Sequence Repeat (ISSR) Molecular Marker

Desri Rofita Sari[1*], Suyitno Aloysius[2]

[1]*Bachelor of Biology Study Program, Faculty of Mathemathic and Natural Science, Yogyakarta State University*
[2]*Academic Staff in Biology Education Department, Faculty of Mathemathic and Natural Science, Yogyakarta State University*
**Corresponding author e-mail: desrih6@gmail.com*

ABSTRACT

The aim of this research is to determine the level of genetic diversity of in vitro cultur *Spathoglottis plicata* variants derived irradiated (mutants) and without irradiation (wild type) seeds. This research is an observation study to detect of genetic diversity of plant variants based on ISSR molecular markers. DNA isolation and quantification was carried out and DNA amplification was then done by Polymerase Chain Reaction (PCR) method with seven ISSR primers. The data were analyzed by the GenAlex program to analyse the genetic distance, percentage of DNA polymorphism and scattered diagram. The NTSYSpc2.0.2 program was used to calculate similarity index and cluster dendrogram by Unweighted Pair-Group Method Using Arithmetic Average (UPGMA). The results showed that the genetic diversity level of *Spathoglottis plicata* variants based on the percentage of plant polymorphism of variants derived from irradiated seed culture results was 92.57%, while wild type variant group was 44.78%, with an average total of 68, 66%. The largest source of genetic was contributed by inter-population diversity with a percentage of 69%. Genetic variation in the mutant plant variant group was higher than the wildtype plant group. The result of dendogram shows that *S. plicata* orchid variants were divided into 3 clusters where the second cluster consisting of a group of mutant plants has a similarity level score <60% which shows that this variant has the potential to develop into new varieties.

Keywords: *Variants of Spathoglottis plicata, genetic diversity, ISSR.*

1. INTRODUCTION

In Indonesia there are about 5,000 species of orchids from 25,000 species of orchids in the world [7]. Orchid is an ornamental plant whose flowers are beautiful and long lasting. Many natural orchids are cultivated for use in the flower industry for decoration and as collections of home garden flowers [11], among them are *Spathoglottis plicata.* Spathoglottis orchids consist of about 40 species, spreading from the Himalayas, China, Indochina, India, Philippines, Indonesia, Papua New Guinea, Australia, Caledonia to the Fiji Islands. There are four cultivars in *Spathoglottis plicata* orchids, including purple, red, violet and white flower cultivars [9].

In some countries, these plants receive less attention and are reported to be vulnerable to extinction. The status of Spathoglottis orchids is not stated in the International Union for Conservation of Nature and Natural Resources (UICN), but is declared extinct in Australia and India [2]. The development of

genetic diversity will increase the economic value of these orchids as commodity crops. Variants with superior morphological character, mainly from the habitus profile,

Plants that have superior character are much in demand by the market. This has led to many developments in the propagation of plant variants such as by induction of mutagenesis. The easiest mutant induction is by mutation induction technique with ionizing rays (X-rays and gamma rays). Aside from being easy, the ionizing ray induction technique is also relatively safe and has been widely applied to horticular plants such as barley and wheat [2].

The genetic diversity of *S. plicata* orchids as seen from the morphological variation needs to be clarified by molecular analysis to see whether these variations are accompanied by genetic variations. molecular research on the Spathoglottis soil orchid is still very limited, such as genetic variability testing with Restriction Fragment Length Polymorphism (RFLP) analysis. In addition, genetic variability analysis has also been carried out based on DNA polymorphisms with markers of Random Amplified Polymorphic DNA (RAPD) [1] and nucleotide polymorphisms of homologous POH1 genes [2] and *S. plicata* orchid RbcL (plastidom) genes. [2].

The Inter Simple Sequence Repeats (ISSR) molecular marker is a marker with a repetitive sequence motif. ISSR markers is amore recent than RAPD and RFLP. ISSR markers have high reproducibility but are also faster, easier, and with a small amount of DNA are able to detect genetic polymorphisms [5]. Identification of the genetic diversity of ISSR markers is widely used because the technique is simple, and fast. All ISSR markers are distributed randomly throughout the genome and usually exhibit high polymorphisms even though the level of polymorphism has been shown to vary with the detection method used [5].

Based on this background, further research needs to be done to see the genetic diversity of the existing *S. plicata* orchid plant variants. In this case molecular analysis will be carried out using Inter-Simple Sequence Repeat (ISSR) molecular markers. Variants of mature *S. plicata* orchids (Suyitno, 2017), from color and number of flowers, the length of the stems that are proportional to the height of the canopy, neither too short nor too long [8].

the *in vitro* culture of natural seeds and from irradiated seeds that have been growing in the FMIPA Biology Education garden, UNY will be investigated.

2. METHODS

This research is an observation study with the object of research in the form of fifteen variants of the *Spathoglottis plicata* orchid plant. The variables studied included the types of orchid plant variants and the level of genetic diversity (DNA) as seen from the percentage of DNA polymorphisms, variant plant scatter diagrams based on genetic distances and number of clusters based on the level of similarity of the dendrogram produced.

2.1. DNA isolation

Data obtained with several stages including DNA isolation and quantification, DNA amplification and visualization and data processing. DNA isolation was carried out using TIANGEN plant genomic DNA kit. A sample of 0.2 grams was washed and dried. Samples were cut into small pieces and added 700 µL of GP1 buffer + 0.1% β-*Mercapto* then grinded using sterile mortar. After that, 700 µL of GP1 buffer + 0.1% β-*Mercapto* was added again and mashed. The mashed sample was transferred to a 1.5 ml microtube and then incubated in a water bath for 20 minutes at 65 ° C (every 5 minutes mixed up). Samples were added with 700 µL chloroform (mixed up) then centrifuged for 5 minutes, 12000 rpm. 600 µL supernatant was transferred to a new 1.5 ml microtube and added 600 µL of GP2 buffer. After that the mixture was transferred to spin column as much as 600 µL and centrifuged for 1 minute, 12000 rpm. The supernatant in the column tube is removed and then put the 600 µL mixture back into the spin column and centrifuged for 1 minute, 12000 rpm. The supernatant is removed and then added 500 µL GD buffer and centrifuged for 1 minute, 12000 rpm. The supernatant was removed and then added 600 µL PW buffer and centrifuged for 1 minute, 1200 rpm. The supernatant is removed

and then added another 600 µL PW buffer and centrifuged for 1 minute, 12000 rpm. Supernatant removed and then centrifuged again for 2 minutes, 12000 rpm. after that, leave it at room temperature for 5-10 minutes. results visualized by electrophoresis method, with agarose concentration of 1%. Agarose gel is made by dissolving agarose with 0.5x TBE (pH 8). Electrophoresis was carried out using a 50volt for 60 minutes. Electrophoresis visualization was carried out using the 1012 series Gel Documentation System. Quantification of DNA from isolation was analyzed using TECAN Nano Quant Nucleic Acid Quantitation to determine the level of purity and concentration of DNA from the isolation.

Spin column was transferred to a 1.5 ml microtube and 100 µL of TE was added and then allowed to stand again and centrifuged for 2 minutes, 12000 rpm. Visualization of DNA isolation

2.2. DNA Amplification

Molecular analysis was done by Polymerase chain reaction (PCR) using Inter-Simple Sequence Repeat (ISSR). The primers used for PCR-ISSR were determined using four genom DNA samples. From 25 types of decamer which were being screened, seven types of oligonucleotide primers were found that showed the presence of polymorphic DNAs (Table 1).

Table 1. Screening results of decamer types oligonucleotides for ISSR

No.	Primer	Nucleotide order 5'—3'
1	UBC 807	AGAGAGAGAGAGAGAGT
2	UBC 818	CACACACACACACACAG
3	UBC 834	AGAGAGAGAGAGAGAGYT
4	UBC 835	AGAGAGAGAGAGAGAGYC
5	UBC 868	GAAGAAGAAGAAGAAGAA
6	I4	ACACACACACACACACAG
7	I34	AGAGAGAGAGAGAGAGAA

DNA samples were amplified using ISSR primers. The final reaction volume for the PCR mix is 15 µL, with a composition: 7.5 µL PCR mix (Promega Go Taq Green Master Mixes), 1µL primers, 1 µL DNA samples, and 5.5 µL Nuclease Free Water (NFW). The PCR reaction started from pre-denaturation step (94ºC, 3 minutes). Followed by further steps consist of denaturation (94ºC, 30 seconds), annealing (42 - 48,9ºC, 30 seconds), and Extension (72ºC, 2 minutes), run in 40 cycles, then final extension (72ºC, 5 minutes) and finally terminated with hold it at 4ºC.

The results of DNA amplification were visualized with electrophoresis in 1% agarose gel, Florosafe DNA staining, 1 kb of Vivantis DNA ladder, and run at 50 volts, 45 minutes. Documentation of the results of electrophoresis visualization was carried out using the 1012 series Gel Documentation System

2.3. Data analysis

The data results from ISSR were converted into binary data by giving scores. Score 1 if there was a DNA fragment and score 0 if there was no DNA fragment. Binary data were analyzed with the Gen Alex 6.1 program to obtain genetic distance, scatter plot and percentage of DNA polymorphism. Data were also analyzed with NTSYSpc2.0.2 to calculate the equation coefficient and dendrogram creation using the Unweighted Pair-Group Method Using Arithmetic Average (UPGMA).

3. RESULTS AND DISCUSSION

DNA amplification of *S. plicata* orchid variants from 7 primers yielded varying lengths and numbers of DNA fragments, between 330 bp-2400 bp with 7-11 loci (Table 2). The total amplification results were able to display 67 loci, consisting of 64 polymorphic loci and 3 monomorphic loci. The number of loci is calculated based on the number of bands that appear at the distance of the DNA fragment migration, according to the size or the size of the DNA fragment. From the results of data analysis, the percentage of DNA polymorphism of *S. plicata* mutant variants reached 68.66% (Table 3).

Based on the analysis of loci polymorphisms, of wild type variant groups (V1-V5) the percentage of DNA polymorphism was 44.78% smaller than the percentage of DNA polymorphism of mutant variant groups (V6-V15) of 92.54% (Table 3) . The percentage of locus mutant DNA polymorphisms higher than wild type variants which indicates an increase in genetic diversity of *S. plicata* orchids. This is thought to be a result of the irradiation treatment which caused mutations by previous studies. Irradiation using X-rays on seeds has been shown to cause gene mutations in *S. plicata* sprouts produced with changes or impaired prominent morphological development [3].

Table 2. The number of DNA fragments as a result of PCR-ISSR

Primer	**∑ of DNA fragments**	**∑ DNA fragments polymorphism**	**∑ DNA fragments Monomorphism**
UBC 807	10	9	1
UBC 818	9	9	0
UBC 834	11	11	0
UBC 835	10	9	1
UBC 868	10	10	0
I4	10	9	1
I34	7	7	0
∑	67	64	3

Table 3. Percentage of DNA polymorphism in *S. plicata* variants

Population	**% P**
V1-V5 (Variant wild type)	44.78%
V6-V15 (Variant mutan)	92.54%
Mean	68.66%
SE	23.88%

The level of polymorphism in mutant variants is greater than wild type variants, this is due to the irradiation given to cause mutations at the DNA level. Mutations in DNA result in the genetic diversity of a variety. Mutations can lead to the emergence of new variations in species resulting in an increase in genetic diversity [12].

Mutations can be divided into small mutations (gene mutations) and large mutations (chromosome mutations). Small mutations are changes that occur in the genes, usually a change in the nucleotide sequence of the DNA, whereas large mutations are changes that occur in the structure and arrangement of chromosomes. Chromosome mutations are changes in the structure of the molecular length consisting of DNA molecules that connect genes.

The most influence occurs by mutagens, especially ionizing rays is the breakage of chromosome threads (chromosome breakage or chromosome aberration) [14]. Besides producing greater DNA polymorphism, the variant of the mutant *S. plicata* orchid also has a greater molecular diversity value than the wild type plant variant. AMOVA can analyze genetic differences between individuals in subpopulations as well as genetic differences between subpopulations. The results of analysis of molecular variance (AMOVA) showed that the highest source of diversity was found among populations with a percentage of 69%, while the percentage of diversity within the population was 31% smaller than the percentage between populations (Table 4).

Genetic distance values in 15 *S. plicata* orchid variant plants based on 7 ISSR molecular markers range between 9 and 40 (Table 5). Fifteen *S. plicata* orchid variant plants that have a low genetic distance value (9) on varieties V4 and V5. While variants that have a high genetic distance value (40) in the V2 and V7 variants. Genetic distance is the level of gene differences (genome differences) between species and species. If the genetic similarity coefficient gets smaller, the farther the kinship is, so that it will increase the high genetic diversity or vice versa. Genetic distance and kinship analysis are basic things that can determine the crossing elders in conservation and plant breeding efforts [5].

Table 4. Analysis Variant Diversity (AMOVA) of *S. plicata* Variant based on ISSR Markers

Source	**Df**	**SS**	**MS**	**Est.ar.**	**%**
Among					
Pops	1	44.400	44.400	4.966	31%
Within Pops	13	146.800	11.292	11.292	69%
Total	14	191.200		16.258	100%
Stat	**Value**	**P(rand >= data)**			

Table 5. The genetic distance of *S. plicata*

	V1	V2	V3	V4	V5	V6	V7	V8	V9	V10	V11	V12	V13	V14	V15	
V1	0															V1
V2	14	0														V2
V3	16	14	0													V3
V4	17	19	15	0												V4
V5	20	20	14	9	0											V5
V6	29	35	33	32	27	0										V6
V7	30	40	30	31	26	21	0									V7
V8	36	34	34	33	34	23	26	0								V8
V9	27	35	25	30	27	26	19	21	0							V9
V10	31	35	27	32	35	24	21	23	18	0						V10
V11	31	29	27	34	35	24	21	25	26	18	0					V11
V12	29	35	33	32	35	32	37	33	32	34	36	0				V12
V13	32	36	30	23	28	29	24	28	29	27	33	19	0			V13
V14	33	37	29	32	31	26	29	31	26	26	34	14	17	0		V14
V15	28	34	26	25	26	25	26	32	27	25	35	19	16	15	0	V15

Principal Coordinate Analysis (PCoA) / Scatter Plot shows the results of the analysis derived from binary data with ISSR molecular markers. The PCoA results can map individuals within their subpopulations in all four quadrants. Variants VI-V5 (Wildtype) tend to group in quadrants I and IV while variants V6-V15 (mutants) are divided into 2 quadrants. Variants V12-V15 are grouped in quadrant II, while V6-V11 are grouped in quadrant III (Figure 1).

Based on the binary data analysis of DNA band scores using the NTSYS program, 3 plant clusters were obtained at a similarity coefficient of 0.63 (Figure 2). Cluster 1 consisting of V1-V5, cluster 2 variant V6 - V11, cluster 3 variant V12 - V15. Based on the similarity index, the second cluster which is part of the mutant variant group has a low similarity index (<64%). This shows the diversity at the genetic level, so it can be said that the mutant variant plants that have a similarity index far from the potential wild type variants as new varieties.

Figure 1. *Scatter Plot* of *S. plicata variant*

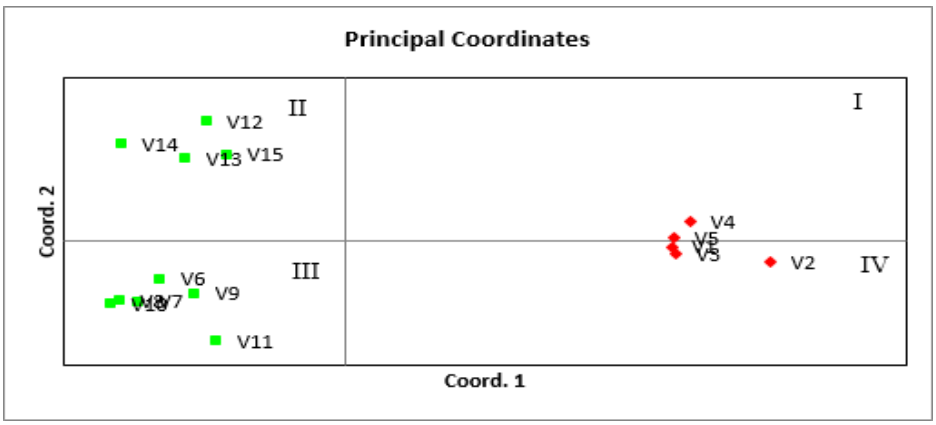
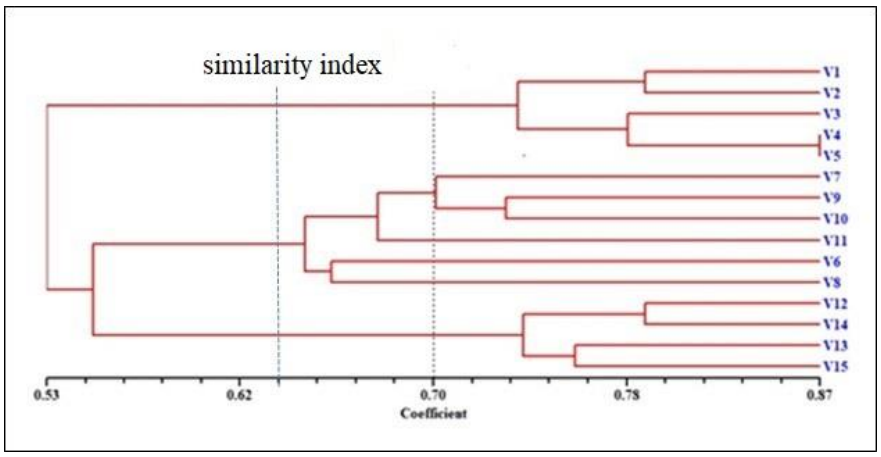


Figure 2. Dendrogram of *S. plicata* variant



ISSR markers have been widely used to identify species, cultivars or populations of a species that are similar to a low level of genetic variation, and are very useful as a means of detecting genetic diversity of a plant species that has very wide genetic variation. ISSR markers has succeeded in detecting the genetic diversity of 31 species of Dendrobium orchid [13], genetic diversity of *Scutellaria baicalensis* [6], genetic diversity among populations of *Rynchostylis retusa* epiphytic orchids from Goa [10], and produced molecular fingerprints of *Dendrobium officinale* orchid population markers [4].

Based on the description above, it can be concluded that the genetic diversity level of *S. plicata* variant based on the percentage of plant polymorphism of variants derived from irradiated seed culture results was 92.57%, while wild type variant group was 44.78%, with an average total of 68, 66%. The largest source of genetic was contributed by inter-population diversity with a percentage of 69%. Genetic variation in the mutant plant variant group was higher than the wildtype plant group. The result of dendogram shows that *S. plicata* orchid variants were divided into 3 clusters where the second cluster consisting of a group of mutant plants has a similarity level score <60% which shows that this variant has the potential to develop into new varieties.

ACKNOWLEDGMENTS

The research was supported by Daftar Isian Pelaksanaan Anggaran (DIPA) Faculty of Mathematics and Natural Science, Universitas Negeri Yogyakarta.

REFERENCES

[1] Aloysius S, Aziz Purwantoro, Kumala Dewi, Endang Semiarti. Improvement of genetic variability in seedlings of *Spathoglottis plicata* orchids through X-ray irradiation. *Biodiversitas*, 2017,Vol 18 (1): 20-27

[2] Aloysius S, Aziz Purwantoro, Kumala Dewi, Endang Semiarti. Phenotypic variation and genetic alteration of *Spathoglottis plicata* resulted from in vitro cultured seed irradiated with X-Ray. *Biodiversitas,* 2018,19 (5) : 1642-1648.

[3] Aloysius S. Variasi morfologis, fisiologis, dan molekular tanaman anggrek *spathoglottis plicata* blume hasil iradiasi sinar-x [*Disertasi*]. Program Studi Biologi Pascasarjana, Fakultas Biologi, Universitas Gadjah Mada.2017.

[4] Chen, Y.P., P.D. Rekha., A.B. Arun., F.T.Shen., W.A. Lai., C.C. Young.,

Phosphate solubilizing bacteria from subtropical soil and their tricalcium phosphate solubilising activities. Applied Soil Ecology , 2006, 34: 33-41.

[5] Erwinda, Zosi. Analisis Keragaman Genetik Anggrek *R. rerusa* Berdasarkan Marka Molekular *Inter- Simple Sequence Repeat* (ISSR). [*Skripsi*, tidak diterbitkan],Universitas Negeri Yogyakarta, Yogyakarta. 2019.

[6] Guo, H.B., Huang, K.Y., Zhou, T.S., Wu Q.H., Zhang, Y.J., and Liang, Z.S. DNA Isolation, Optimization of ISSR-PCR system and Primers Screening of *Scutellaria baicalensis. Journal Medicine Plant Research,*2009. 3(11): 898-901.

[7] Irawati.*Pelestarian jenis anggrek Indonesia*. Buku panduan Seminar Anggrek Indonesia 2002. 34- 45.

[8] Kartikaningrum, S., Sulyo, Y., Hayati, N.Q., Suryanah dan Nety, Y.A. Keragaman karakter kualitatif hasil persilangan anggrek Spathoglottis. *J. Hort. Ed. Khusus*, 2007.5(2): 138-147.

[9] Murthy, K.S.R., Ramulu, D.R. Rao, J.C., Emmanuel, S. and Pullaiah T. *In* vitro flowering of *Spathoglottis plicata* Bl. (Orchidaceae). *Phytomorp.: An Int. J. Plant Morphol.*2012. 56(3-4): 117-120.

[10] Parab, G.V and Krishnan S. Assessment Of Genetic Variation Among Populations of *Rhynchostylis Retusa* an Epiphytic Orchid From Goa, India Using ISSR and RAPD Marker. *Journal of Biotechnology*, 2008, 7(17): 313-319.

[11] Semiarti E., Purwanto A., and Indrianto, A. In Vitro Culture Of orchids: Theroles of class-1 knox gene in shoot development. *J Biol Res*., 2015, 20: 18-27

[12] Sengupta M, Chakraborty A, Raychauduri SS.Ionizing Radiation Induced Changes in Phenotype, Photosynthetic Pigments and Free Polyamine Levels in Vigna radiata (L.) Wilczek. Applied Radiation and Isotop., 2013,75: 44-49.

[13] Wolff, K., E. Zietkiewicz, H. Hofstra. Identifcation of Chrysanthemum Cultivars and Stability of DNA Fingerprint Patterns. Theoretical Applied Genetics.1995, 91:439-447.

[14] Yuwono, Triwibowo. Bioteknologi Pertanian. Yogyakarta: UGM Press.2019.