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Genetic Diversity Analysis of Dendrobium sp Orchid from Indonesia based on Random Amplified Polymorphic DNA (RAPD)

Ahmad Yunus*, Sri Hartati , Fitria Roviqowati

Department of Agrotechnology, Faculty of Agriculture, Universitas Sebelas Maret. Jl. Ir. Sutami 36A, Surakarta 57126, Central Java, Indonesia.
Center for Research and Development of Biotechnology and Biodiversity, Universitas Sebelas Maret. Jl. Ir. Sutami 36 A Surakarta 57126, Central Java, Indonesia.

*Corresponding author: tatik_oc@yahoo.com

Abstract
The orchid genus Dendrobium is a commodity from plants that are sold in pots, and Cut flowers are very popular in the world because they have flowers of various sizes, shapes, and colors. Orchid flowers of the Dendrobium genus flower throughout the year, so they are included in the promising cut flower industry. Information on genetic diversity and genetic relationships between orchids of the genus *Dendrobium* through molecular analysis techniques is needed in plant breeding programs and genetic resources. Orchids *Dendrobium* that have various phenotypes will increase the selling value of the commodity. The purpose of this study was to determine the genetic diversity of the orchid *Dendrobium* using RAPD markers. The scoring data were analyzed using NTSYS-pc (software *Numeral Taxonomic System*) version 2.02, which produced data in the form of cluster dendrograms. Genetic diversity of *Dendrobium spp., analyzed with 5 primers RAPD,* ranges from 43-76% and divides the dendrogram into 2 main clusters based on genetic similarity. The polymorphism of the 5 RAPD primers was very high, namely 91.6-100%.

Keywords: Genetic diversity, *Dendrobium*, RAPD markers, Similarity index, Dendrogram

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Introduction

The genus of plants with the largest flowers in the Orchidaceae family is the genus Dendrobium, which isspread mostly in Oceania and Asia (1). The orchid genus Dendrobium is a commodity from plants that are sold in pots and cut flowers that are very popular in the world because they have flowers of various sizes, shapes, and colors. Orchid flowers of the Dendrobium genus flower throughout the year, so they are included in the promising cut flower industry. The regions of California, Florida, and Hawaii are centers of the cultivation of orchids of the genus *Dendrobium* in the United States. The economic value of selling orchids has been significant in Hawaii for decades, with sales increasing from US$2.4 million in 1991 to US$5.6 million in 2000. Orchid production of *Dendrobium* in pots in the Netherlands reaches 40 to 50 million units, which shows enthusiasm for the commodity. Thailand is the country with the largest exporter of orchids Dendrobium, highestcutat 22% (2).

The orchid genus Dendrobium can also be used as a medicinal plant. The enthusiasm for orchids is motivated by the economic importance of using *Dendrobium* as a traditional medicine. For more than 2300 years, most of the genus *Dendrobium* (*Orchidaceae*) has been used as traditional medicine in China. The fresh or dried stems of this species are a *superior tonic* in traditional Chinese medicine. The efficacy of the plant Dendrobium is that it can nourish the kidneys, produce lost body fluids, and reduce stomach disease (3). Mass production of orchids Dendrobium in biotechnology with tissue culture techniques, flowering in vitro, and genetic transformation is needed for the biomedical, pharmaceutical, and ornamental plant industries, because the genus Dendrobium is one of the commodities with high demand, resulting in profits that are higher

than those of other commodities (4).

Crosses of orchids Dendrobium, which have potential as ornamental and medical flowers, are needed to create superior hybrids that have the desired properties. Orchids have the opportunity to cross with different genera relatively easily because they have a weak reproductive barrier. As many as 250,000 cultivars of orchids and more than 110,000 hybrids with interesting phenotypes in 2015 were registered with the Royal Horticultural Society. As many as 3000 new hybrid orchids are added every year and become the most diverse commodity among other plants. Most of the orchid hybrids are derived from intergeneric crosses, which involve crosses with different genera. In contrast, Dendrobium hybrids have mostly intrageneric crosses, which are produced by species differences and similarities in the genus (3). *Dendrobium* is a promising industry, so the expansion of the cultivation area and the increase in genetic diversity have encouraged the formation of a high-tech agricultural industry that is efficient in planting, processing, and marketing (5).

Information on genetic diversity and genetic relationships between orchids of the genus *Dendrobium* through molecular analysis techniques is needed in plant breeding programs and genetic resources. Orchids *Dendrobium, which have various phenotypes,* will increase the selling value of the commodity (6). The population of orchids Dendrobium, which has a wide genetic diversity due to the plant breeding program, can lead to increased adaptability in new areas. Breeders of the orchid commodity Dendrobium have the challenge of increasing the genetic diversity in order to increase profits in terms of productivity or economic growth. The combination of increasing demand and increasing genetic diversity will increase income while maintaining the sustainability of the orchid *Dendrobium* (7).

Periodic reduction of genetic diversity in germplasm will lead to unintended consequences, such as reduced production potential of crops, plants becoming susceptible to pests and diseases, reduced adaptation to climate and weather changes, and loss of genetic resources. This is avoided so that agricultural production in a commodity becomes stable and does not experience fluctuations that can have an impact on the economy. The contribution of plant breeding to production yields increased from 50 to 88% through genetic analysis. The increase in agricultural productivity from genetic acquisition during 1930-2011 was 79%. Achieving a high percentage of productivity depends on information on genetic diversity, because genetic diversity contributes to the quality and quantity of agricultural products. Increasing agricultural production through the management of genetic diversity is very necessary (8).

Plant breeding is the act of increasing the agronomic production of plants using the scientific principle of plant genetic diversity. Integration between genetic diversity, statistics, and knowledge of phenotypes and traits that exist in a plant can be used to increase functional germplasm diversity (9). Changes in diversity occur due to crossing with other parents, segregation, recombination, genetic drift, and selection by breeders (10).

Molecular analysis is needed to obtain more accurate information about genetic diversity than morphological analysis. Morphological analysis of orchids of the genus *Dendrobium* has been carried out by (11). Still, it is not very accurate because environmental conditions and plant growth influence the morphological analysis. The study of genetic diversity is an important step in the improvement of hybrid crosses. Selection of suitable and efficient molecular markers is needed to produce high polymorphisms (12). The molecular marker used in this study is RAPD (*Random Amplified Polymorphic DNA*). RAPD (*Random Amplified Polymorphic DNA*) uses a primer with a short sequence of 10 nucleotides, which is used to amplify the target DNA sequence randomly, so that RAPD produces high polymorphism (13).

Genetic analysis of orchids is used to determine the genetic diversity of a species and to determine the parentage of the cross. Genetic similarity between species can be analyzed using RAPD molecular markers. The method of analysis in RAPD using random primers is used to see the relationship betweenplant species. The advantage of the RAPD molecular analysis technique is that it has a fast implementation, a small amount of DNA is needed, which is around 0.5-50 ng, and no radioisotope analysis is needed (14).

RAPD has been used for DNA fingerprint identification, cultivar identification, and plant genetic diversity analysis. One of the weaknesses of RAPD is that the resulting reproducibility is lower, so that the sample DNA bands cannot be fully amplified.

Accurate and useful basic information about various plantspecies is obtained through genetic diversity analysis using molecular markers. Data results from analytical methods play an important role in the effect of the results of genetic diversity studies on a species or population. The genetic structure of germplasm is grouped according to the UPGMA method (15). RAPD has been used as an analysis method for the orchid Vanda cuerolea, which produces a high polymorphic band of 64.28%, higher than the polymorphic yield of the ISSR marker, which is only 37.71%. The results show that the RAPD marker is superior to the ISSR marker (16). The purpose of this study was to examine the genetic diversity of the orchid Dendrobium, which was used as basic information on plant breeding.

Materials and Methods

This research was conducted at the Center for Plant Conservation of the Botanical Gardens of LIPI (Indonesian Institute of Sciences), located on Jl. Ir. H. Juanda No. 13, Bogor West Java, Indonesia. Thesamples used in this study were 4 species of the genus *Dendrobium spp,* namely *Dendrobium mirbelianum*, *Dendrobium lamellatum*, *Dendrobium sucundum*, *Dendrobium bracteosum*, and *Dendrobium purpureum* (Table 1). The RAPD primers used for DNA amplification, including their nucleotide sequences, are listed in Table 2.

Table 1. Orchid *Dendrobium spp* and Origin Area

Code	Species Name	Origin
F2	*Dendrobium mirbelianum*	Jawa
F3	*Dendrobium lamellatum*	kalimantan
F8	*Dendrobium sucundum*	Sumatera
F9	*Dendrobium bracteosum*	Papua
F10	*Dendrobium purpureum*	Sulawesi

Table 2. RAPD markers used for DNA Amplification of

Primer	Sekuens Nukleotida (5'- 3')
OPA 2	TGCCGAGCTG
OPA 7	GAAACGGGTG
OPB 12	CCTTGACGCA
OPA 13	CAGCACCCAC
OPB 18	CCACAGCAGT

The chemicals used for DNA analysis using the RAPD method were: CTAB, EDTA, Tris-HCL, PVPP, sterile distilled water, mercaptoethanol, NaCl, quartz sands, chloroform, isoamyl alcohol, absolute ethanol, 70% alcohol, PCR buffer reaction, dNTP mix, Taq DNA polymerase, MgCl2, and primer. The tools used for DNA isolation are an analytical balance, water baths, a minibeadbeater, a vortex, a centrifuge, and a spectrophotometer.The tool used for DNA amplification is PCR. The tools used for electrophoresis are an electrophoresis and a trans-UV illuminator.

Sample DNA was *Dendrobium spp* extracted from young leaves following a modified protocol using PVPP.The purity and quantity of isolated DNA were analyzed using a UV Spectrophotometer with absorbance ratios A260 and A280 compared to standard blanks. According to (17), the analysis of DNA purity and concentration was used to see whether the isolated DNA was good enough to be used for amplification. PVPP is used to reduce contaminants in the sample so that the concentration and purity of isolated DNA have a highvalue.

PCR program with preheating to 94 ^{o}C for 2 minutes, then 45 cycles consisting of denaturation to untie the double helix of the target DNA at a temperature of 94 ^{o}C for 1 minute, the attachment of the primer to thetarget DNA occurs at the annealing stage which takes place at 37 ^{o}C for 1 minute, the elongation of the resultsof amplification by polymerase enzyme elongation takes place on stage with a temperature of 72 $^{\circ}$C for 5 minutes. The last cycle was cooling to 25 ^{o}C.

The PCR results were put into the well (1 well for 1 sample) using a micropipette, and the SMOBIO AccuBand™ ladder was added to the far left well. 1.5% agarose gel was inserted into the electrophoresis, which already contained 1x TBE immersion. The electrophoresis power was set with a voltage of 100 V anda current of 400 mA, with a time of 70 minutes. Visualization using a trans UV illuminator and photographed witha camera.

Genetic diversity is observed by scanning DNA bands. DNA bands are translated into binary data. A valueof 1 means the band exists, and a value of 0 means the band is empty. The analysis was carried out in clusters using NTSYSpc (*Numerical Taxonomy System*) with UPGMA (Unweighted Pair Group Method Arithmetic). The clustering method uses the DICE SIMQUAL (Similarity for coefficients Qualitative Data) and SAHN (Sequential Agglomerative Hierarchical and Nested). Genetic diversity is shown using a dendrogram.

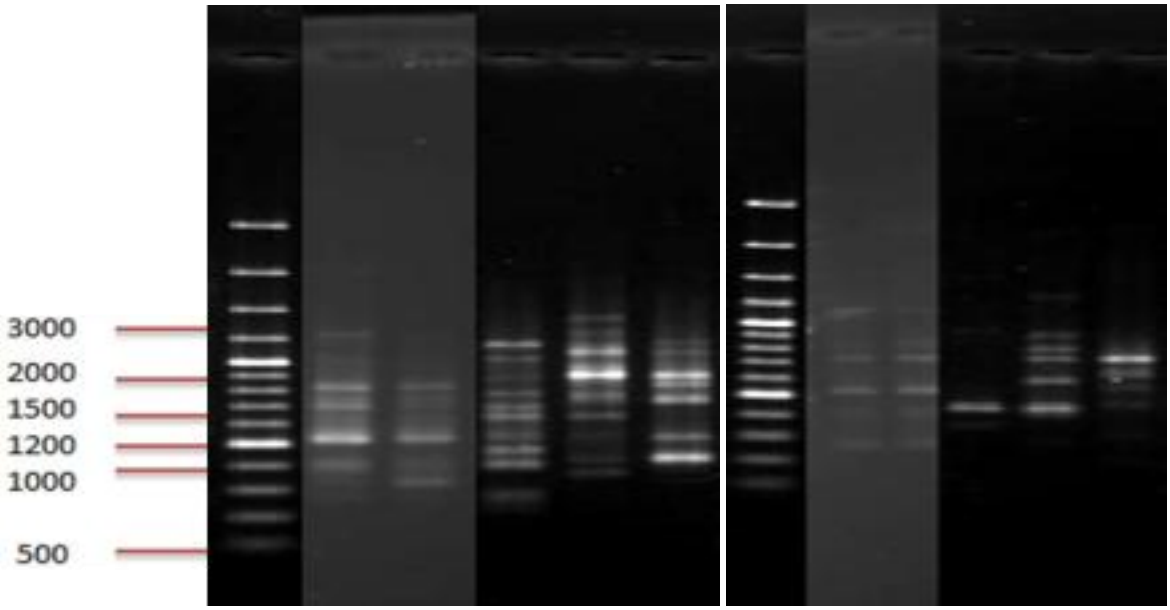
Results and Discussion

DNA Amplification

Genetic diversity is a variation within a species in a population. Genetic variation in a

population to be crossed is the basis for the selection of plant improvements. This genetic diversity must be maintained because it is important for the survival of the population in the future. There has been increased awareness of the importance of biodiversity, including agricultural biodiversity, conservation for sustainable use, and development. The release of hybrids from low genetic diversity will cause the hybrids to become more uniform. Low genetic diversity usually stems from the limited number of lines used in the production of hybrids, so increasing genetic diversity must be done (18).

Plant breeding and the use of germplasm require knowledge of genetic variation and relationships between species. This can be analyzed using molecular markers by determining genetic distance, analyzing genetic diversity, performing genome analysis, and developing molecular maps. Identification of molecular markersis needed to achieve the desired plant breeding results. This study used RAPD (Random Amplified Polymorphic DNA) markers because RAPD is the most cost-effective and simple molecular marker and can be performed in most modern laboratories. RAPD can amplify most of the genome and does not require information about the genome of the sample under study. The results of the amplification were electrophoresed and visualized as shown in Figure 1.



OPA 13 OPB 12

Figure 1 Results of DNA Amplification with Primers OPA 13 and OPB 12

5 RAPD primers were used for amplification, namely OPA 2, OPA 7, OPA 13, OPB 12, and OPB 18. The results of the amplification were then electrophoresed to separate DNA bands based on band size. Assessing genetic variation through morphological characters is very difficult, due to the variability caused by plant growth conditions. Amplification using RAPD OPA 13 primers showed that at sizes 400,650, and 1150 bp, the orchids *Dendrobium sucundum, Dendrobium bracteosum,* and *Dendrobium purpureum* had DNA bands at the same size. DNA bands at sizes 450, 750, 900, and 1100 bp in orchids *Dendrobium bracteosum* and *Dendrobium purpureum* have the same band size. The DNA bands measuring 800 bp in the orchids *Dendrobium mirbelianum* and *Dendrobium lamellatum* have the same bands. Monomorphic bands are found at a size of 500 bp. Monomorphic bands showed that the species *Dendrobium sucundum, Dendrobium bracteosum, Dendrobium purpureum, Dendrobium mirbelianum,* and *Dendrobium lamellatum* amplified DNA bands of the same size and locus because they have genetic similarities. There are specific alleles at sizes 475, 550, and 1000 bp, which only amplify 1 genotype at a certain size.

The presence of the same DNA band at a certain size in *Dendrobium sucundum, Dendrobium bracteosum,* and *Dendrobium purpureum* orchids indicates that these orchids have higher genetic similarity than other orchids and tend to be in the same cluster based on their similarity. According to (19), cluster analysis is based on the similarity of DNA bands amplified by primers. The similarity of size and loci in the amplified DNA bands in the samples showed high similarityof each species. These similarities indicate that the genetic diversity between these species is low.

All species in the RAPD OPA 13 primer can be amplified. According to (20), the high number of amplified bands will increase the percentage of polymorphism, which indicates that the markerused can amplify each genotype well. The similarity in the size of the DNA bands or the presence of DNA bands at the same locus indicates the similarity between the genotypes being analyzed.

Amplification using RAPD OPB 12 primer showed that at sizes 250, 550, and 750 bp, the *Dendrobiummirbelianum* and *Dendrobium mirbelianum orchids* had the same size. DNA bands with a size of 700 bp in orchids *Dendrobium bracteosum* and *Dendrobium purpureum* have the same band size. DNA bands measuring 450 bp in *Dendrobium sucundum* and *Dendrobium purpureum* have the same band size. There are specific alleles at sizes 250, 400, 600, 650, 800, 900, and 1100. According to (21), the number of polymorphic bands and the number of amplified bands are used to identify andshow differences between the genotypes analyzed. Molecular markers can show the identity pattern ofan organism, so that information about genetic variation is important for the development and maintenance of an organism's potential, which is realized through a hybridization program.

There are DNA bands with different thicknesses in each primer; this is due to differences in the concentration and purity of the sample DNA. RAPD primers bind to complementary DNA base sequences so that DNA can be amplified. The results of DNA band amplification were visualized using gel electrophoresis. According to (22), the annealing temperature during the amplification process in PCR affects the quality of the resulting band. Annealing temperature that is toolow or too high can cause the primers to fail to attach to the target DNA sequence. The DNA bands produced in each sample are different in number because each primer has a different sequence and will amplify the sample DNA in different amounts. Primers that have various annealing temperatures will increase the production of the resulting DNA bands. In addition to polymorphism, the quality of the amplified bands is also an important factor in primer selection. Incorrect primers will result in less clear DNA bands and will reduce the accuracy of the data when analyzed.

The results of the electrophoresis that have been visualized were analyzed by scoring, namely a score of 1 for the visible band and a score of 0 for the invisible band. Scoring must be done carefully sothat the results obtained are in line with expectations. A smear under the DNA band can reduce the accuracy of the data used for analysis. Smears occur because the DNA produced from isolation has a low concentration and purity. According to (23), the requirement for a good amplificationresult is the high quality and quantity of isolated DNA. The leaf extraction method has a drawback, namely the high accumulation of phenolics and polysaccharides. Polysaccharides combined with genomic DNA will make the DNA sample thick like glue and difficult to amplify. According to (24), DNA samples that are yellow or brown in color indicate that the sample is contaminated with polyphenols. The extraction method using CTAB as a lysis buffer and PVPP will reduce the polyphenol content in the sample.

The scoring is entered into an Excel table and then analyzed with NTSYS 202 NTSYS-pc (Numerical Taxonomic System) version 2.02 using the SIMQUAL (Similarity Qualitative) UPGMA (Unweighted Pair Group Method Arithmetic) method. Table RAPD polymorphism to 5 is presented in Table 3.

Table 3. Percent Polymorphism 5 Primer on *Dendrobium spp* in RAPD PCR reactions

No	Primer	Sequence5'3 to 3'	Size (bp)	Amplified Band	Polymorphic band	Monomorphic band	% Polymorphic	% Monomorphic
1	OPA2	TGCCGAG CTG	300-1600	12	11	1	91.6	8.3
2	OPA7	GAAACGG GTG	250-1900	15	15	0	100	0
3	OPB12	CCTTGAC GCA	250-1200	13	13	0	100	0
4	OPA13	CAGCACC CAC	300-1200	16	15	1	93.75	6.25
5	OPB18	CCACAGC AGT	250-1750	12	12	0	100	0

Description: % of Polymorphic = (Number of DNA Bands / Polymorphic Bands) x 100%.
% Monomorphic = (Number of DNA Bands/ Monomorphic Bands) x 100%

DNA bands that can be amplified by 5 primers measuring 250-1900 bp with a polymorphism rate of 91.6-100%. OPA 2 primers have a band size of 300-1600 bp with a band count of amplified as manyas 12 bands, the resulting polymorphism is 91%, with monomorphism as much as 8.3%. OPA 7 primerhas a band size of 250-1900 bp with the number of amplified bands as many as 15 bands and 100% polymorphism percentage. OPB 12 primer has a band size of 250-1200 bp with the number of amplifiedbands as many as 13 bands, and the percentage of polymorphism as much as 100%. OPA 13 primerhas a band size of 300-1200 bp with 16 bands amplified. with the percentage of polymorphism as muchas 93.75% and the percentage of monomorphism as much as 6.25%%. OPB 18 primer has a band size of 250-1750 bp with a total of 12 bands amplified. and the percentage of polymorphic bands reached 100%. OPA 13 primer is a primer with the largest number of bands amplified, because the primer is complementary to the target DNA.

RAPD PCR uses 2 types of primers, namely forward and reverse primers, as a specific sequence template that will attach to the target DNA. According to (25), each primer will amplify a different part of DNA, so the length of the resulting nucleotides will be different. The difference in the size of the DNA bands produced will be different due to differences in the primers used. The longer the ribbon, the bigger the size. According to (26), intact DNA bands tend tohave high molecular weights so that they show good DNA quality. DNA with a small size is usually DNAthat is truncated due to inappropriate annealing temperature, which causes DNA degradation.

Setting the annealing temperature is an important step in PCR amplification. According to (27), the annealing temperature is the temperature at which the primer will stick to the target DNA sequence. The temperature is calculated based on the melting temperature value of each primer. Inappropriate annealing causes the primer to be unable to attach to the target DNA, and amplification will not occur. RAPD has been used in polymorphism analysis of the orchid *Coelogyn pandurata*, according to research (28), polymorphism is the difference in DNA bands observed in agarose gel. RAPD primers produced 95.83% polymorphic bands in the amplified orchid Coelogyn pandurata, with 43 bands amplified, measuring 200-2100 bp.

Cluster Analysis Clusters

They are grouped based on the similarity of the similarity matrix; the greater the genetic similarity betweenspecies, the more they will be grouped into the same cluster. Genetic diversity is the inverse of genetic similarity, which is calculated by the formula 1-value of similarity.

Table 4. Genetic Similarity Matrix of *Dendrobium spp* based on 5 Primers RAPD

	F2	F3	F8	F9	F10
F2	1				
F3	0.571	1			
F8	0.229	0.313	1		
F9	0.154	0.122	0.245	1	
F10	0.327	0.304	0.304	0.349	1

Description:
F2. *Dendrobium mirbelianum*; F3. *Dendrobium lamellatum*; F8. *Dendrobium sucundum*; F9. *Dendrobium bracteosum*; F10 *Dendrobium purpureum*

The value of the similarity matrix describes genetic similarity. The closer the value to 1, thehigher the similarity between species and the lower the genetic diversity. Based on Table 4, the lowest genetic similarity was observed between *Dendrobium lamellatum* and *Dendrobium bracteosum*, with a similarity coefficient of 0.122, indicating the highest genetic diversity of 88%. In contrast, the highest genetic similarity was found between *Dendrobium mirbelianum* and *Dendrobium lamellatum*, with a similarity coefficient of 0.57, corresponding to the lowest genetic diversity of 43%. The species *Dendrobium mirbelianum* and *Dendrobium lamellatum* have a coefficient of genetic similarity high, marked by a straight line on the dendrogram (figure 2) so that they are grouped into thesame cluster as in Figure 2. Species in the same cluster tend to have a high similarity of phenotypes orcome from the same geography. But on research, these *Dendrobium mirbelianum* and *Dendrobium lamellatum* are in the same cluster, but are in different geographical areas of Java and Kalimantan. According to (29) research, geographical areas have no impact on the genetic diversity of a species. Different environmental conditions (differences in soil, temperature, and rainfall) cause different clusters of genotypes, but usually populations in the same geographic area tend to cluster into the same cluster.

Figure 2 is a dendrogram compiled by UPGMA analysis, which is a description of the genetic similarity coefficient grouped by cluster.

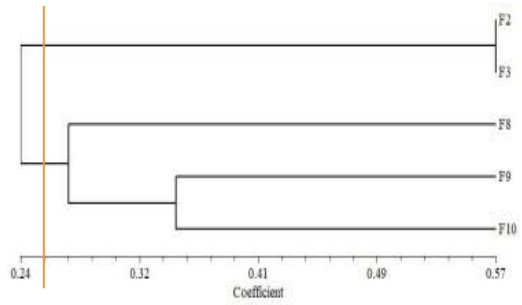


Figure 2 Grouping of 5 species *Dendrobium spp* based on 5 RAPD primers Description: F2. *Dendrobium mirbelianum*, F3. *Dendrobium lamellatum*, F8. *Dendrobium sucundum*, F9. *Dendrobium bracteosum*, F10 *Dendrobium purpureum*

Dendrobium spp has genetic similarity with a value of 0.24-0.57, with a genetic diversity of 43- 76%. The cluster on the dendrogram is divided into 2 main clusters based on the similarity coefficient. value0.24, namely cluster A containing *Dendrobium mirbelianum* and *Dendrobium lamellatum* with a genetic similarity coefficient of 0.57 and a genetic diversity of 43%. Cluster B contains 2 sub-clusters, with*Dendrobium sucundum* in sub-cluster I. Species *Dendrobium bracteosum and Dendrobium purpureum* have a genetic coefficient of 0.35 and a genetic diversity of 65%. The selection of parents with different clusters will increase genetic diversity in hybrid yields.

It can be seen that the highest similarity coefficient is in *Dendrobium lamellatum* and *Dendrobium bracteosum, with a value of 0.122;* the highest genetic diversity is 88%, so that if crossed, it will have a high potential for success. According to (30), basic information about the genetic diversity of the parents to be crossed is very important to know, especially the identification of combinations of parents that have a high heterotic value. Elders with high diversity and value High heteroticity will increase hybrid yield. mother plant with high genetic diversity low will complicate the selection and reduce the variability of the resulting hybrid. According to (31), genetic diversity is the key to theagricultural industry. Sustainable agriculture requires genetic diversity so that the sustainability of a population is maintained and does not decline. Plant breeding programs require populations with high genetic diversity as the basis for parental warfare. Monitoring the genetic diversity of each species in apopulation is needed as an agricultural industry strategy.

Conclusion

The genetic diversity of *Dendrobium spp., analyzed with 5 RAPD primers,* ranged from 43- 76% and divided the dendrogram into 2 main clusters based on genetic similarity. Polymorphism The yield of the5 RAPD primers was very high, namely 91.6-100%. The highest genetic diversity exists in the species *Dendrobium lamellatum* and *Dendrobium bracteosum, with a value of 0.122; the highest genetic diversity is 88%, so that if crossed,* it will have a high potential for success.

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