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Genetic Variations and Relationships of Papua's Endemic Orchids Based on RAPD Markers

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ABSTRACT

Orchidaceae has known as an attractive flower and immense species. We have found a large species of Orchidaceae grow naturally in Papua's jungle, Indonesia territorial. This study aims to reveal genetic variation and genetic relationships among endemic orchids in Papua based on RAPD markers. The study included 26 accessions of Papua's endemic orchids used for genomic DNA extraction. Genomic DNAs were extracted by using DNA extraction kit from Qiagen and genomic DNA amplification by using 10 decamer RAPD primers. DNA fragments that were amplified by Polymerase Change Reaction (PCR) were visualized and documented by using UV illumination apparatus. Genetically, endemic Orchids in Papua were described high variation. Fragments amplification by using ten RAPD primers and performed in the PCR tools resulted in 54 numbers of polymorphic fragments and no monomorphic band. The number of polymorphic bands per primer ranged from 4 to 7 with averaged 5.4 bands per assay unit. The genetic dissimilarities (GDs) among examined orchids ranged from 0.10 to 0.94 based on Nei's unbiased coefficients. Dendrogram construction showed that Papua's endemic orchid (PEO) samples different from another and separated to form group by their own at the 0.40 coefficient value and at the 0.6 coefficient value indicate that PEO sample is divided into nine groups *i.e.* samples at the genera level were separated into their own groups.

1. INTRODUCTION

The Orchidaceae is reported a huge species growing naturally and cultivated around the world, especially in the tropical countries. Orchidaceae was reported to consist of 35,000 species under 800 genera [1]

and family of Orchidaceae between 25,000 and 30,000 different species around the world [2]. It has been reported that 5000 species of Orchidaceae existed in Indonesian and approximately a half of them (2770 species) existed in Papua islands, Indonesian territorial [3]. Several of those species which have attractive performance, either flower or vegetative growth were domesticated and cultivated as well as propagated by *in vitro* technique [4].

Papua and West Papua, Indonesia territorial have a unique and interesting variety of orchids, some of which are still found and grow wild in the forest and several species have been domesticated. Several species of Papua's orchid have been used as a parent for breeding program [5]. Orchid growing and developing in the world's wilderness are estimated there are 20,000 - 35,000 species [6] of 900 genera in the world and about 5000 species found in Indonesia [3, 5]. Estimated 2770 species are found in Papua and only 300 species have been identified according to the Chairman of the Indonesian Orchid Association (IOA) of Papua Province [7].

Information of plant genetic variation is very important for sustainable use, breeding program and germplasm conservation. A popular DNA markers used for revealing genetic differentiation and genetic relationships is Random Amplified Polymorphism DNA (RAPD) marker. The RAPD marker is one of many techniques used for molecular research. The advantages of RAPD markers are simpler in their preparation than other molecular markers. The other advantages of RAPD markers are easy to employ for examining the differentiation of organism [8-10], because it is not using radioactive and relative chief [8]. The RAPD analyses were reported to be used as marker for many aims of organism evaluation either alone or in tandem with another molecular marker, such as: genetic relationships among *Brassica napus* [11], genetic analyses of plant progenies [12], molecular characterization of reciprocal crosses of Orchidaceae [13], genetic variation in *Vanillaplanifolia* [14], molecular characterization and phylogenetic revealing of Orchidaceae [15], genetic linkage map [16], identification of grapevine [17], and genetic differentiation [18]. This study is carried out for revealing genetic diversity and genetic relationship among Papua's endemic orchids based on RAPD markers which would help for understanding of genetic profile that can be used to improve strategies for sustainable utilization, breeding program, and germplasm conservation in the future.

2. MATERIALS AND METHODS

2.1. Plant Samples

A total 26 accessions of endemic orchids were collected by local people from the jungle of Papua, Indonesia territorial. Phenotypic of Papua's endemic orchid (PEO) were used as plant materials in this study it sown in the **Figure 1**. The young leaf samples were preserved by using silica gel granules in zip lock plastic according to previous reported procedures [19]. The dried young leafs were grinded directly without using liquid nitrogen by using mortar and pestle until the tissue become fine powder. Isolation and extraction of genomic DNA from the dried leaf samples were performed by using procedures as described in Qiagen DNA extraction kit. The genomic DNA was achieved its keep in −20˚C in the freezer until ready to use.

2.2. PCR Amplfication

Ten decamer RAPD primers were employed in this research as follows: OPA1 (5'-CAGGCCC TTC-3'), OPA4 (5'-AATCGGGCTG-3'), OPA12 (5'-TCTGTGCTGG-3'), OPU3 (5'-CTATGCCGAC-3'), OPU8 (5'-GGCGAAGGTT-3'), OPAW-05 (5'-CTGCTTCGAG-3'), OPAE-16 (5'-TCCGTGCTGA-3'), OPD08 (5'-GTGTGCCCCA-3'), OPD11 (5'-AGCGCCATTG-3'), and OPU8 (5'-AGCGCCATTG-3'). The decamer random primers were provided original sequences by Operon Technologies, Alameda, USA and synthesized by Integrated DNA Technologies. The PCR reagents were mixed in a 25 µl volume containing 10 ng genomic DNA, 2.0 µl of 50 pM primer, 2.5 µl of 10× polymerase buffer, 2.5 µl of 2.5 mM dNTPs, 1.0 U AmpliTaq Gold, 2.0 µl of 3 mM $MgCl_2$, and Milli Q Water.

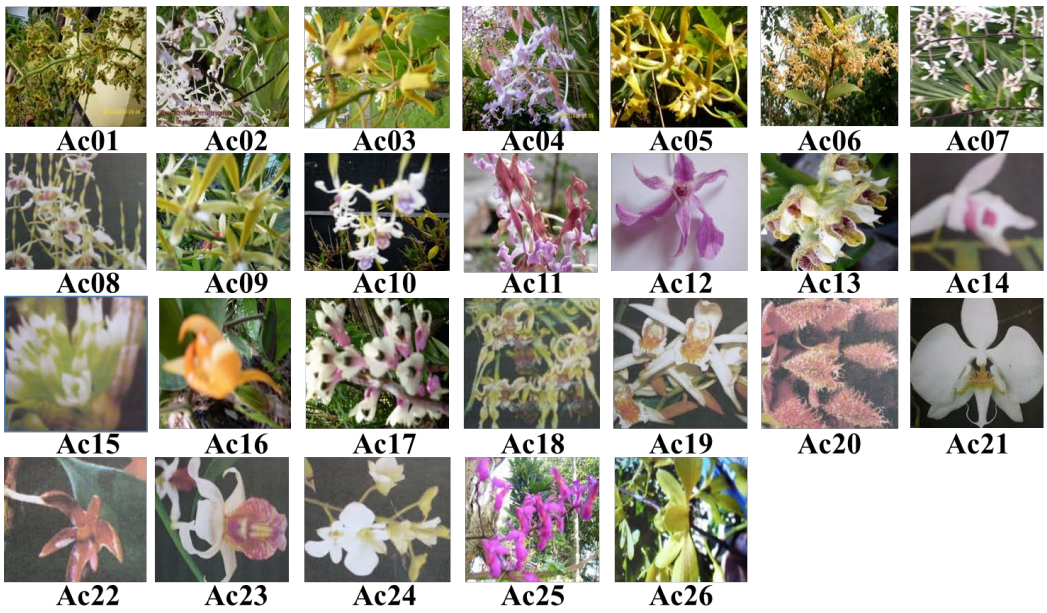

Figure 1. **Flower performances of PEO used in the study.** ***Grammatophyllum scriptum*** **(Ac01).** ***Dendrobium lineale*** **type 1 (Ac02),** ***Dendrobium*** **sp1 (Ac03).** ***Dendrobium gouldii*** **(Ac04),** ***Dendrobium mirbelianum*** **type 1 (Ac05),** ***Dendrobium discolor*** **(Ac06),** ***Dendrobium lineale*** **type 2 (Ac07),** ***Dendrobium antenatum*** **type 1 (Ac08),** ***Dendrobium mirbelianum*** **type 2 (Ac09),** ***Dendrobium antenatum*** **(Ac10),** ***Dendrobium aries*** **(Ac11),** ***Dendrobium*** **sp2 (Ac12),** ***Dendrobium macrophyllum*** **(Ac13),** ***Dendrobium anosnum*** **(Ac14),** ***Dendrobium capituliflorum*** **(Ac15),** ***Dendrobium digibum*** **(Ac16),** ***Dendrobium simillieae*** **(Ac17),** ***Dendrobiums spectabile*** **(Ac18),** ***Collagen asperata*** **(Ac19),** ***Bulbophylum phalaenopsis*** **(Ac20),** ***Phalaenopsis amibilis*** **(Ac21),** ***Bulbophylum patens*** **(Ac22),** ***Dendrobium nindii*** **(Ac23),** ***Dendrobium affine*** **(Ac24),** ***Ascoglossum calepsum*** **(Ac25), and** ***Dendrobium sculery*** **(Ac26).**

Amplification was performed in a programmable Thermal Cycler (Takara) for an initial denaturation at 94˚C for 2 min, 40 cycles denaturation at 94 for 1 min, annealing at 37˚C, extension at 72˚C for 2 min, final extension at 72˚C for 5 min, and holding at 4˚C. The Amplified DNA fragments were resolved in 2.0% agarose gel, electrophoresis at 100 V using 1× TAE buffer, standard molecular weight marker using a 100 bp DNA ladder (Takara), staining with Ethidium bromide (1 µl/l) for 30 min, visualization and documentation by using UV illuminator equipment and digital camera. The PCR reaction was repeated more than one times per sample for each primer to ensure the reproducibility of the amplified bands. Faint bands were not considered for scoring and calculation.

2.3. Data Analysis

RAPD fragments were scored as present (1) and absent (0). The scorer band in the formed of a binary data were analyzed the genetic variation, and genetic relationships among Papua's endemic orchids. The dissimilarity matrix was calculated by using distance coefficient. The dissimilarity was employed for clustering of the Papua's endemic orchids by the Unweighted Pair-Group Method Arithmetic Average (UPGMA), using the Sequential Agglomerative Hierarchical Nested Cluster Analysis (SAHN-clustering) [20] and TREE program from NTSYS-pc, version 2.02 packages [21]. Bootstrap analysis by 1000 permutation times were performed by using software Tools for Genetic Analysis (TFPGA 1.3); [22]. The average polymorphic information contents (PIC) of RAPD primers were calculated by using [23] formula ($PIC_i = 1 - \sum pi^2$, pi is the frequency of the *i* bands).

3. RESULTS AND DISCUSSIONS

3.1. Polymorphism of RAPD Markers

Amplification fragments by using ten RAPD primers and performed in the PCR tools were resulted 54 numbers of polymorphic fragments and no monomorphic band. The number of polymorphic bands per primer ranged from 4 (OPU8) to 7 (OPU3) with averaged 5.4 bands per assay unit. The number of polymorphic band that were observed in this work is lower than the number of bands were reported in the species of *Phalaenopsis* [15] and in the genus of *Catasetum* [24]. The fragment size range among 100 and 1400 bp, and the number of genotypes range from 9 (OPA12) to 17 (OPAW-05) with average 13.5 genotypes (Table 1).

The PIC value was performed to estimate the power of discriminatory the markers. A large proportion of PIC value has a high discrimination power. The PIC values were calculated range from 0.71 to 0.92. In the previous studies were reported that the PIC of within species of *Phalaenopsis* reached 0.99 [15]. Primer OPA1 and OPD11 have the highest PIC value (0.92), therefore both these primers have the highest discrimination power comparing overall primer used in this research. Otherwise, the lowest PIC value was observed at primer OPA12 with 0.71 PIC values (Table 1). An example of amplification pattern for molecular characterization generated by RAPD markers is shown in Figure 2.

Table 1. Amplification results of 10-mer RAPD primers used for discrimination of 26 samples Papu's endemic orchid.

Primer name	Primer Sequences (5' – 3')	Number of polymorphic fragments	Fragment size range (bp)	Number of genotypes	PIC
OPA1	CAGGCCCTTC	6	100 - 1000	15	0.92
OPA4	AATCGGGCTG	6	150 - 1300	15	0.90
OPA12	TCTGTGCTGG	4	250 - 1100	09	0.71
OPU3	CTATGCCGAC	7	150 - 1000	14	0.90
OPU8	GGCGAAGGTT	5	150 - 1000	12	0.90
OPAW-05	CTGCTTCGAG	6	150 - 1100	17	0.91
OPAE-16	TCCGTGCTGA	5	200 - 1400	14	0.90
OPD08	GTGTGCCCCA	6	200 - 800	11	0.83
OPD11	AGCGCCATTG	5	200 - 700	16	0.92
OPU8	AGCGCCATTG	4	150 - 1300	12	0.86

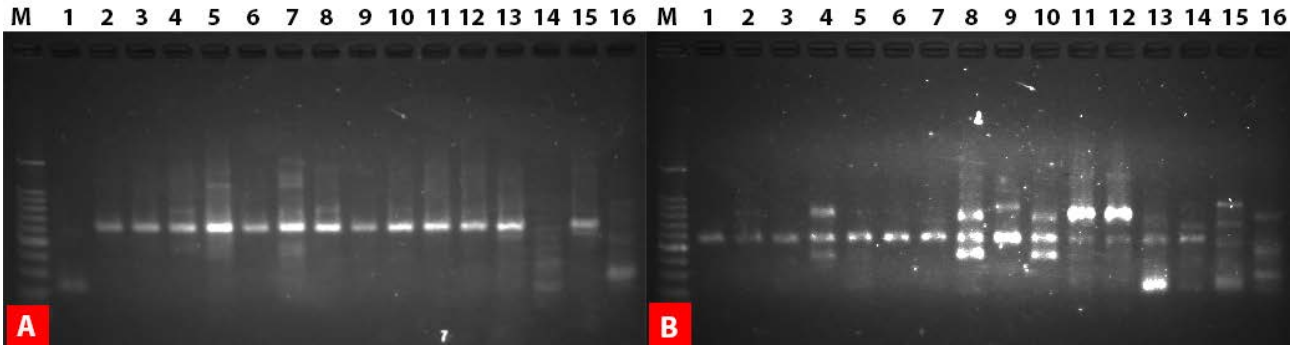

Figure 2. Performance of RAPD fragment by using OPA12 (A) and OPAE-16 (B) on 2.0% agarose gel. Marker (M) and the number of well indicated number of orchid samples.

3.2. Genetic Variations

The genetic dissimilarities (GDs) among examined orchids were range 0.10 to 0.94 based on [25] unbiased coefficients. The highest GDs values were found between *A. calepsum* (Ac25) and *P. amibilis* (Ac21) with 0.94 GDs value then followed by pairwise differences of *A. Calepsum* and *C. asperata* with 0.90 GDs value and the smallest GDs values among genera were found between *D. aries* (Ac11) and *A. Calepsum* (Ac25) with 0.23 GDs value (Table 2). Genetic distances of PEO were found in this study, it showed a

Table 2. Genetic distances of 26 PEO based on fragment amplified of 10 RAPD primers used and calculated by using Nei's unbiased distance.

No	Accession numbers	Accession Numbers																									
		Ac01	Ac02	Ac03	Ac04	Ac05	Ac06	Ac07	Ac08	Ac09	Ac10	Ac11	Ac12	Ac13	Ac14	Ac15	Ac16	Ac17	Ac18	Ac19	Ac20	Ac21	Ac22	Ac23	Ac24	Ac25	Ac26
1	Ac01	0.00																									
2	Ac02	0.35	0.00																								
3	Ac03	0.32	0.10	0.00																							
4	Ac04	0.50	0.23	0.20	0.00																						
5	Ac05	0.49	0.33	0.20	0.25	0.00																					
6	Ac06	0.46	0.43	0.27	0.27	0.18	0.00																				
7	Ac07	0.52	0.35	0.23	0.27	0.23	0.30	0.00																			
8	Ac08	0.77	0.43	0.40	0.25	0.52	0.49	0.38	0.00																		
9	Ac09	0.23	0.27	0.25	0.52	0.40	0.38	0.43	0.73	0.00																	
10	Ac10	0.49	0.27	0.40	0.35	0.66	0.62	0.55	0.30	0.52	0.00																
11	Ac11	0.59	0.30	0.43	0.43	0.49	0.46	0.68	0.43	0.55	0.32	0.00															
12	Ac12	0.66	0.35	0.90	0.43	0.49	0.52	0.59	0.49	0.49	0.43	0.12	0.00														
13	Ac13	0.55	0.49	0.40	0.66	0.46	0.55	0.62	0.81	0.40	0.73	0.77	0.69	0.00													
14	Ac14	0.62	0.62	0.59	0.66	0.81	0.62	0.49	0.90	0.46	0.59	0.77	0.69	0.52	0.00												
15	Ac15	0.38	0.38	0.46	0.66	0.73	0.85	0.55	0.73	0.40	0.46	0.62	0.62	0.66	0.52	0.00											
16	Ac16	0.66	0.52	0.43	0.69	0.55	0.66	0.46	0.77	0.49	0.62	0.59	0.66	0.55	0.49	0.55	0.00										
17	Ac17	0.49	0.69	0.73	0.59	0.81	0.77	0.77	0.66	0.73	0.73	0.62	0.69	0.66	0.66	0.59	0.69	0.00									
18	Ac18	0.52	0.59	0.55	0.49	0.62	0.59	0.46	0.43	0.49	0.32	0.40	0.40	0.69	0.62	0.43	0.66	0.62	0.00								
19	Ac19	0.55	0.49	0.59	0.59	0.73	0.77	0.55	0.66	0.52	0.59	0.55	0.62	0.66	0.40	0.35	0.55	0.59	0.62	0.00							
20	Ac20	0.44	0.49	0.62	0.62	0.77	0.30	0.81	0.62	0.49	0.43	0.59	0.52	0.62	0.55	0.55	0.81	0.62	0.40	0.69	0.00						
21	Ac21	0.46	0.52	0.49	0.62	0.62	0.59	0.52	0.85	0.55	0.55	0.73	0.73	0.38	0.49	0.55	0.40	0.55	0.59	0.49	0.66	0.00					
22	Ac22	0.44	0.52	0.43	0.69	0.55	0.52	0.46	0.69	0.32	0.49	0.66	0.73	0.55	0.49	0.49	0.52	0.69	0.35	0.49	0.40	0.30	0.00				
23	Ac23	0.46	0.52	0.49	0.62	0.55	0.52	0.58	0.55	0.49	0.49	0.52	0.52	0.49	0.62	0.55	0.59	0.77	0.52	0.49	0.52	0.46	0.52	0.00			
24	Ac24	0.49	0.43	0.40	0.59	0.59	0.62	0.55	0.59	0.40	0.46	0.27	0.43	0.46	0.52	0.46	0.38	0.52	0.38	0.52	0.62	0.49	0.43	0.43	0.00		
25	Ac25	0.69	0.55	0.52	0.59	0.66	0.55	0.77	0.52	0.59	0.46	0.23	0.38	0.73	0.73	0.81	0.49	0.66	0.49	0.90	0.62	0.94	0.69	0.55	0.25	0.00	
26	Ac26	0.73	0.35	0.43	0.49	0.49	0.66	0.67	0.62	0.62	0.49	0.46	0.52	0.43	0.77	0.69	0.66	0.62	0.66	0.49	0.59	0.52	0.66	0.46	0.49	0.55	0.00

wide range of genetic distances. In the previous studies were reported that the genetic variation of orchid species also shows high variation [26-28]. The range of high genetic distance values based on the results of Nei's unbiased distance analysis is reflected from the highly variable phenotype of orchid flowers (**Figure 2**).

The GDs among species of *Dendrobium* were range 0.10 to 0.90 based on Nei's unbiased coefficient. The highest GDs values were found between *D. Stratiotes* (Ac08) and *D. anosnum* (Ac14) with 0.90 GDs value then followed by pairwise differences of *D. Stratiotes* (Ac08) and *Dendrobium* sp3 with 0.77 GDs value and the smallest GDs value were found between *D. Lineale* type1 (2) and *Dendrobium* sp1 (Ac03) with 0.10 GDs value. The calculation results of genetic variation among orchid species in this investigation resemble those of *Cajanus cajan*, *Cajanus albicans*, and *Cajanus liniatus* which have a wide range of genetic differences based on RAPD markers [29].

3.3. Genetic Relationships

Dendrogram construction of PEOs is based on 54 polymorphic fragments of 10 RAPD primers as markers by using UPGMA method of Nei's unbiased distance [24]. Dendrogram construction showed that PEO samples different from another and separated to form group by their self at the 0.40 coefficient value. This phenomenon correlated with different morphological phenotypes of flower. In the previous studies were reported that a lot of species of plant showed us their phenotypic traits correlated with molecular assessment [30-33]. Conformities of morphological traits and genetic characteristics by using molecular marker were reported also at sago palm species [34, 35]. At the 0.6 coefficient value indicates that PEO sample is divided into nine groups (**Figure 3**) *i.e.* each sample at the genera level was separated into their own groups. Group 1 consists of two individuals namely *Ascoglossum calepsum* and D*endrobium* sp2; Group 2 consists of four individuals namely *Bulbophylum phalaenopsis, Dendrobium anosnum, Dendrobium Aaries*, and *Dendrobium digibum*; Group 3 consists of seven individuals namely *Dendrobium nindii, Dendrobium capituliflorum, Bulbophylum patens, Dendrobium mirbelianum* type 2, *Dendrobium antenatum* type 1, *Dendrobiums spectabile*, and *Dendrobium mirbelianum* type 1; Group 4 consist *Collagen asperata* only; Group 5 consist *Dendrobium simillieae* only; Group 6 consists of two individuals

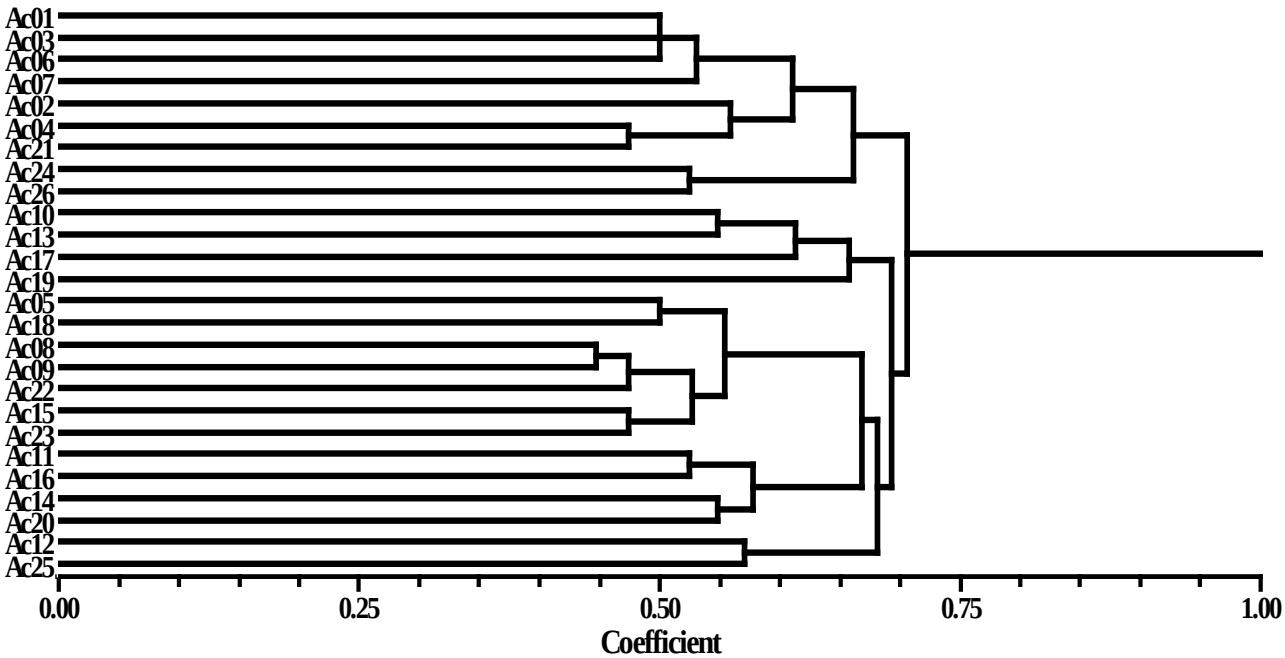

Figure 3. Dendrogram construction based on UPGMA methods by using Nei's unbiased distance of 26 accessions of PEO and determined by 54 polymorphic fragments of 10 RAPD primers as marker.

namely *Dendrobium antenatum* and *Dendrobium macrophyllum*; Group 7 consists of *Dendrobium affine* and *Dendrobium sculery*; Group 8 consists of *Dendrobium lineale* type 2, *Dendrobium gouldii*, and *Phalaenopsis amibilis*; and Group 9 consists of *Grammatophyllum scriptum*, D*endrobium* sp1, *Dendrobium discolor*, and *Dendrobium lineale* type 1.

4. CONCLUSION

Genetically, endemic Orchids in Papua were described high variation. Amplification fragments by using ten RAPD primers and performed in the PCR tools resulted in 54 numbers of polymorphic fragments and no monomorphic band. The number of polymorphic bands per primer ranged from 4 to 7 with an averaged 5.4 bands per assay unit. The genetic dissimilarities (GDs) among examined orchids ranged from 0.10 to 0.94 based on Nei's unbiased coefficients. Dendrogram construction showed that Papua's endemic orchid (PEO) samples different from another and separated to form group by their own at the 0.40 coefficient value and at the 0.6 coefficient value indicate that PEO sample is divided into nine groups *i.e.* each sample at the genera level was separated into their own groups. All samples of PEO used in the study are advised to be conserved prior to extinction.

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