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Production of F1 *Papilionanthe hookeriana* (Rchb.f.) Schltr. homozygous and heterozygous: Amplified fragment length polymorphism (AFLP) analysis of flower colors and self and cross-pollination ability between different flower colors

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Abstract Results showed that seed germination of *Papilionanthe hookeriana* (Rchb.f.) Schltr. was not significantly different between self-pollination treatments, while the percentage of self-pollination ability and seedling numbers were significantly different at 0.01 and 0.05 levels, respectively. Pink and purple flowers had the highest self-pollination ability at 100% and the highest numbers of seedlings (1,295) for self-pollination of pink flowers. Percentages of cross-pollination ability, pod size and weight, and seed weight were not significantly different between treatments, while percentages of seed germination and seedling numbers were significantly different at the 0.01 level. The PixPu hybrid showed 100% seed germination, whereas the PixWh hybrid yielded the highest number of seedlings (403.2). Amplified fragment length polymorphism (AFLP) technique was used to study the genetic relations between various flower colors of *P. hookeriana* (Rchb.f.) Schltr. and the F1 hybrids of self- and cross-pollinated plants were classified into two clusters by UPGMA methods.

Keywords: *Papilionanthe hookeriana,* DNA fingerprint, AFLP, Breeding

Introduction

Orchids are monocotyledonous flowering plants and members of the Orchidaceae family (Thaithong, 2006). They are the most diverse family of angiosperms (Christenhusz and Byng, 2016) and represent the largest family of

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plant species encompassing 763 genera and exceeding 28,000 species (Zhang *et al*., 2018). Within Thailand, Orchidaceae demonstrate remarkable diversity, comprising 168 genera and over 1,170 species that thrive in various habitats (Sittisatchatum, 2007), including 60 terrestrial orchid genera with more than 200 species (Kitidee *et al*., 2023). In the last two decades, orchid conservation efforts have mushroomed. *Papilionathe hookeriana* (Rchb.f.) Schltr. or pencil orchid is a perennial epiphytic orchid with monopodial plant growth (Romeida *et al*., 2016). The plants grow in swampy deciduous forests of Vietnam, Thailand, peninsular Malaya, Sumatra, and Borneo at elevations of 0 to 700 m (Wood and Cribb, 1994). Pooma (2005) reported finding *P. hookeriana* (Rchb.f.) Schltr. in peat swamp forests in Chumphon Province, Thailand. In its natural habitat, the orchid grows up to 2.5 m high. The stem is cylindrical-rounded, segmented, and covered with a thin sheath with a diameter of 0.5-1.2 cm. The leaf is cylindrical with an acute tip and stands up like a pencil (Romeida *et al*., 2016). Sustainable conservation and utilization of the endemic orchid species *P. hookeriana* (Rchb.f.) Schltr. has been followed since 2005 through *in vitro* seed propagation (Montri *et al*., 2023). However, the natural production of fruits or pods is insufficient to meet the demand. Hence, the practice of self and cross-pollination has become crucial for large-scale sexual propagation and selective breeding for commercial applications. Pollination is the vital process of transferring pollen grains from the stamens to the stigma as a crucial aspect of sexual reproduction. Flowering plants have evolved an array of traits that impact their pollination success including mechanisms aimed at optimization of self-pollination (Fottorini and Glover, 2020). Cross-pollination increases genetic diversity and is favored by selection and selection acts on key floral traits in taxonomically diverse species. Understanding orchid pollination systems is essential for effective conservation if plant-pollinator interaction is disrupted. Lehnebach *et al*. (2005) studied the self and cross-pollination ecology of terrestrial orchids in New Zealand. They determined that reproduction of *Lomanda longifolia* is predominantly by self-pollination, whereas both *Pterostylis alobula* and *P. patens* are cross-pollinated. The breeding system and reproductive success of *Epidendrum secundum* in Southeastern Brazil is based on flower variation and pollen transference between plants of *E. secundum* and another related species (Pansarin and Amaral, 2008). The reproductive success of *Stanhopea lietzei* and *S. insignis* was low as a consequence of deficient pollen transference, while pollinator scarcity was the main factor. The stigmatic cavity is too narrow to admit pollinia when the bee first visits a flower because before drying, the pollinia are too thick to be inserted (Pansarin and Amaral, 2009). *Acianthera aphthosa*, a representative species with self-pollinated flowers, exhibits a lower incidence of embryos compared to cross-pollinated flowers (Pansarin *et al*.,

2016). Conversely, *Dendrobium* displaying both self-compatibility and self-incompatibility traits, constitutes nearly half of all self-compatibility orchids (Niu *et al*., 2018). Hybridization, both natural and artificial, has the effect of integrating the excellent characteristics of the two parents within the hybrid offspring. The F1 progenies derived from two parents with contrasting target traits usually exhibit large phenotypic differences. Various obstacles that impede the process of hybridization, including parent incompatibility and postfertilization embryo abortion, result in the failure of distant hybridization (Li *et al*., 2021). Evidence suggests that recurrent breeding system transitions between cross and self-pollination are an important mechanism for diversification in the *Epipactis youngiana* (Hollingsworth *et al*., 2006). Population genetic diversity is closely related to species fitness (Reed and Frankham, 2003). Populations and species with low genetic diversity may have reduced survival in changing environments (Markert *et al*., 2010). In the natural environment, *P. hookeriana* (Rchb.f.) Schltr. orchid exhibits flowers of three distinct colors. This study ascertained whether genetic variances existed among these color variations.

The various techniques studied for differentiating species are based on differences in genes or DNA. These techniques include RFLP (Restriction Fragment Length Polymorphism), RAPD (Random Amplified Polymorphic DNA), and AFLP (Amplified Fragment Length Polymorphism DNA). These techniques produce different levels of DNA that are used to identify and classify organisms. For example, Tores *et al*. (1993) conducted a study on five rose cultivars using the RAPD marker. Results showed that all cultivars could be differentiated using eight RAPD primers, which produced distinct DNA patterns. Benner *et al*. (1995) studied 8 species of Cattleya orchids and the hybrid F1 using 10 RAPD primers. Results showed that 55% of the DNA fingerprints were different, indicating a hybrid relationship with *Cattleya harrisoniana*. Lu *et al*. (1996) conducted a study on 18 varieties of peach using the RAPD marker, and 80 types of RAPD primers were tested. Among these, 20 RAPD primer types exhibited 40 DNA bands, while 6 types of RAPD primers were suitable for the identification of 18 peach varieties. The constructed dendrogram depicted the genetic relationships among the peach varieties. Lim *et al.* (1999) conducted a study on orchids belonging to the genus Vanda, specifically *V. sanderiana* and *A. miniatum*. The RAPD technique was used along with 8 primers to analyze the genetic characteristics of these orchids. Findings revealed that the orchids could be distinguished from one another. It was established that *A. miniatum* shared a close genetic relationship with *V. sanderiana*, while *V. teres* and *V. hokkeriana* formed a new genus, *Papilionanthe*.

DNA fingerprinting technique called amplified fragment length polymorphism (AFLP) is based on selective PCR amplification of restriction fragments from a total digest of genomic DNA. This technique involves three steps as (1) restriction of the DNA and ligation of oligonucleotide adapters, (2) selective amplification of sets of restriction fragments, and (3) gel analysis of the amplification fragments (Vos *et al.*, 1995). The AFLP and random amplified polymorphic DNA (RAPD) marker techniques were applied to study the diversity and phylogeny of lentils. AFLP detected a much higher level of DNA polymorphism than RAPD analysis (Sharma *et al.*, 1996). A study of a hybrid from an inter-specific, controlled cross between a resistant *Populus* female and a susceptible *Populus* male using the AFLP technique identified three markers tightly linked to the *Mer* locus (Cervera *et al.,* 1996). Three different DNA mapping techniques, restriction fragment length polymorphism (RFLP), RAPD, and AFLP, were used on identical soybean analysis to compare their ability to identify markers in the development of a genetic linkage map. Results showed that AFLP data enhanced the efficiency and ease of the DNA mapping technique (Lin *et al.*, 1996). *Glycine max* and *Glycine soja* were studied using the AFLP technique. The AFLP band results demonstrated differential patterns both between and within the groups of *Glycine max* and *Glycine soja*. Maughan *et al*. (1996) conducted a comparative study of RFLP, RAPD, AFLP, and SSR techniques to differentiate soybean varieties of *Glycine max* and *Glycine soja*. They used the Marker Index (MI) value to compare the efficiency of identifying plant varieties using each technique. Results showed that AFLP had a higher MI value compared to the other techniques. Consequently, AFLP was determined as a more effective technique for grouping soybeans compared to the other methods (Powell *et al*., 1996). Previous studies showed that the AFLP technique had high efficiency and DNA polymorphism for studies of genetic diversity, hybrid progeny, and DNA mapping. AFLP technique was used to determine self and cross-pollination ability between different flower colors of *P. hookeriana* (Rchb.f.) Schltr.

Materials and methods

Plant materials

The plant species *P. hookeriana* (Rchb.f.) Schltr was collected from Chumphon Province, Thailand. Fifty plants of each color were grown in the shade house for one year. Plants in the flowering stage were used as plant material. Measurements including the size of the flower, flower stalk, pollinia (width x length), and pollinia weight were recorded from these 50 plants before

the pollination process, with data shown in Table 1. Healthy plants and uniformity in flower morphology were key criteria when selecting plants for self and cross-pollination experiments.

Table 1. Size of flower and flower stalk, size and weight of pollinia, and diameter of stigma gravity of various flower colors of *P. hookeriana* (Rchb.f.) Schltr.

Flower color[1/]	Flower size [2/] (cm)		Flower stalk [2/] (cm)		Pollinia size [2/] (cm)		Pollinia weight [2/] (mg)	Stigma gravity[2/] (cm)
	Width	Length	Width	Length	Width	Length		
Pi	4.25	5.67	0.21	1.5	0.29	0.30	0.25	0.40
Pu	4.58	5.70	0.20	1.6	0.27	0.28	0.28	0.37
Wh	4.75	5.48	0.19	1.1	0.43	0.25	0.28	0.32
F-test	ns	ns	ns	ns	ns	ns	ns	ns
C.V. (%)	7.23	9.86	6.42	10.25	9.17	8.91	14.58	24.89

[1/] Pi = Pink, Pu = Purple and Wh = White
[2/] ns is not significant at the 0.05 level

Self and cross-pollination

Self and cross-pollination experiments were conducted using 30 plants of each color in the study. The purple (Pu), pink (Pi), and white (Wh) flower colors of *P. hookeriana* (Rchb.f.) Schltr. were self and cross-pollinated. After pollination, the characteristics of flower change were observed at various stages of fruit or pod development until reaching the mature stage and ready for propagation. The self-pollination flowers were purple (Pu), pink (Pi), and white (Wh). Percentages of self-pollination ability, pod size and weight, and seed weight were recorded 90 days after pollination. The seed was then cultured in Vacin and Went (VW) 1949 medium and percentages of germination and seedling numbers were recorded. The characteristics of seedlings were also observed for the color. Cross-pollination combinations of different flower colors were performed as follows: PixPu, PixWh, PuxPi, PuxWh, WhxPi, and WhxPu. Percentages of cross-pollination ability, pod size, pod weight, and seed weight were recorded 90 days after pollination. The seed was then cultured in Vacin and Went (VW) 1949 medium and percentages of germination and seedling numbers were recorded. The characteristics of seedlings were also observed for the color.

DNA extraction

Total DNA was extracted from leaves of *P. hookeriana* (Rchb.f.) Schltr. according to the method of Doyle and Doyle (1987). Methods of total DNA extraction were as follows: The plant sample was ground thoroughly with liquid

nitrogen and 100-200 mg per sample was put in a tube. CTAB buffer of 600 µl and 20 µl of 20% SDS were added and incubated at 60 °C for 60 min. The mixture of phenol: chloroform: isoamyl alcohol of 600 µl was added and centrifuged at 14,000 rpm for 15 min and the supernatant was transferred into a new tube. Then, 1/10X 5 M potassium acetate and 2X absolute ethanol were added to the sample tube and incubated overnight at -20 °C. The next day, the sample was centrifuged at 14,000 rpm for 20 min at 4 °C and the supernatant was discarded. Next, 600 µl of 70% ethanol was added and the sample was centrifuged at 14,000 rpm for 20 minutes at 4 °C. The supernatant was discarded. Then, 50 µl of TE buffer were added to the total DNA and stored at 4 °C. The result of DNA extraction was checked by the electrophoresis technique using 1% agarose gel. For each orchid extract sample, 5 µl of DNA was mixed with 2 µl of loading dye. The electrophoresis was conducted at a voltage of 100 V for 30 min. Subsequently, the agarose gel was stained with ethidium bromide for 1 hour, and the results were examined using a gel document. Finally, the concentration of the total DNA was calculated and adjusted to approximately 50 ng/µl.

AFLP analysis

The methods of the AFLP fingerprint technique were as follows: 50 µg of total DNA of *P. hookeriana* (Rchb.f.) Schltr. from 25 samples of volume 40 µl was digested with 5 units of a mixture of *Eco*RI/*Mse*I in buffer A (33 mM Tris-HCl pH 7.5, 10 mM KCl, and 0.5 mM DTT) at 37 ºC for 1 hour and ligated to *Eco*RI adapter and *Mse*I adapter by 10 µl of buffer containing 5 pmol *Eco*RI adapter, 50 pmol *Mse* I adapter, 30 mM Tris-HCl pH 7.8, 10 mM DTT, 1 mM ATP, 10X ligase buffer, and 1 unit of T4 DNA ligase (Promega) at 37 °C for 3 hours. The sample was diluted 10 times by dH_20 and stored at -20 °C. The pre-amplification step was carried out for each DNA sample ligated with adapters by adding 70 ng of *Eco*RI adapter primer and 70 ng of *Mse*I adapter primer in a PCR reaction containing 50 mM $MgCl_2$, 10X PCR buffer, 2.5 mM dNTPs, and 1 unit of Taq DNA polymerase. Total volume of the PCR solution was 50 µl. The mixture was amplified on a thermal cycler using the following cycling parameters: 20 cycles at 94 °C for 30 sec, 56 °C for 1 min and 72 °C for 1 min. The PCR product (pre-amplification) was diluted 10 times by dH_20 and stored at -20 °C. Selective amplification was then performed using a reaction mixture composed of 3 µl of PCR product from pre-amplification, 30 ng of *Eco*RI and *Mse*I primer, 10x PCR buffer, 50 mM $MgCl_2$, 2.5 mM $dNTP_s$ and 0.4 units of Tag DNA polymerase. Selective amplification PCR was performed as follows: 34 cycles at 94 °C for 30 sec and 65 °C for 30 sec. The temperature was reduced at 1 °C intervals until reaching 56 ºC (annealing only) and 72 ºC for 1 min. The

loading dye (95% formamide, 10 mM NaOH, 0.05% Bromophenol Blue, and 0.05% xylene cyanol) of 10 µl was added to the PCR product. Both pre and selective amplification conditions were modified according to Vos *et al*. (1995). Products from the selective amplification (adding 10 µl loading dye) were checked by 1% agarose in 0.5x TBE. The PCR products from the selective amplification were then separated on 6% denatured polyacrylamide gel electrophoresis in 1xTBE buffer for 30 min at 1600 V, 100 mA, and 70 W. The gel was transferred to a fixer for 5 min followed by silver staining for 7 min. Fingerprint patterns of the AFLP bands were recorded.

Table 2. Nucleotide sequences and primer names for the AFLP technique

No.	Primer name	Sequence (5'⟶3')
1	MseI-adaptor-F	GACGATGAGTCCTGAG
2	MseI-adaptor-R	TACTCAGGACTCAT
3	EcoRI-adaptor-F	CTCGTAGACTGCGTACC
4	EcoRI-adaptor-R	AATTGGTACGCAGTCTAC
5	EcoRI primer	GACTGCGTACCAATTCA
6	MseI primer	GATGAGTCCTGAGTAAC

Data analysis

Data were analyzed using SAS version 9.0 software with a completely randomized design. Each treatment consisted of 10 replications and each replication had 3 flowers for both self and cross-pollination and 3 pods for *in vitro* seed culture. The average data were calculated from all replications. The data was evaluated through an analysis of variance (ANOVA). After the ANOVA, the mean differences among treatments were assessed using Duncan's Multiple-Range Test (DMRT) at significance levels of 0.05 and 0.01 levels. For genetic similarity analysis, AFLP fragments were visually scored as present (1) or absent (0) to create a binary data set that was used to calculate the genetic similarity matrix and dendrogram constructs using NTSYS Version 1.2 software (Rohlf, 2000). The similarity matrix was subjected to an unweighted pair group method analysis (UPGMA) clustering and the dendrogram was then constructed.

Results

Senescence occurred after pollination of *P. hookeriana* (Rchb.f.) Schltr. For 2 days. At 10 days, the flowers turn brown and dry up. At 25 days, the flower stalks change color from white to green and continue to grow until they develop into pods. At 90 days, the pods are green, and when they mature, they turn a dark

brown color. They have elongated shapes and contain numerous seeds that resemble light yellow flour or dust (Figure 1).

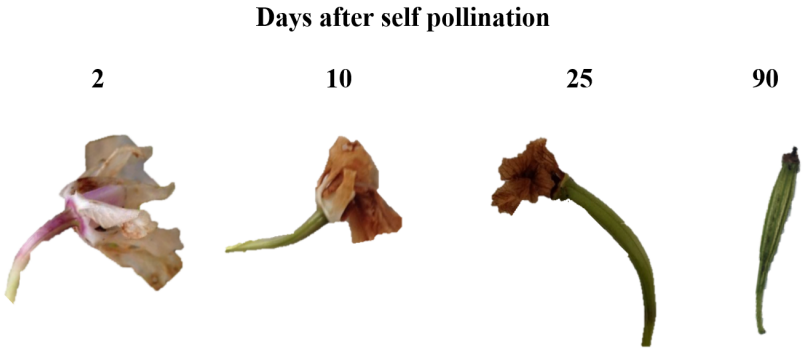

Figure 1. Changes after self-pollination of different flower colors of *P. hookeriana* (Rchb.f.) Schltr. at 2, 10, 25 and 9 days

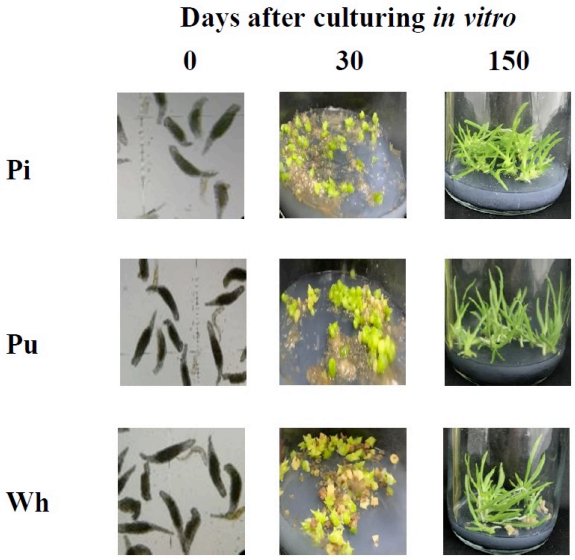

Figure 2. Characteristics of white (Wh), pink (Pi), and purple (Pu) flowers of self-pollinated seeds, protocorms and seedlings of *P. hookeriana* (Rchb.f.) Schltr. after culture on VW medium for 0, 30, and 150 days

The seeds were taken from mature pods (90 days old). These seeds are then cultured on VW medium. The characteristics of seeds are elongated shape and have numerous seeds inside each pod, a flour or dust-like appearance, and a light-yellow color. Under a microscope, the seed coats are thin and closely packed together, with the black color of the endosperm. The size and shape of the seeds

vary among individuals. At 30 days the seeds start developing into the protocorm stage, characterized by round, green structures. When they reach 150 days of age, the young plants begin to grow leaves and roots. The seedlings have different colors. Results from self-pollination of white color flowers showed a light green hue, whereas seedlings derived from self-pollination of pink and purple flowers showed a dark green shade (Figure 2). Seedlings resulting from cross-pollination between PixPu, PixWh, PuxPi, PuxWh, WhxPi, and WhxPu showed a variety of colors ranging from light green to green. However, their size was not sufficient for transplanting to the outside environment (Figure 3).

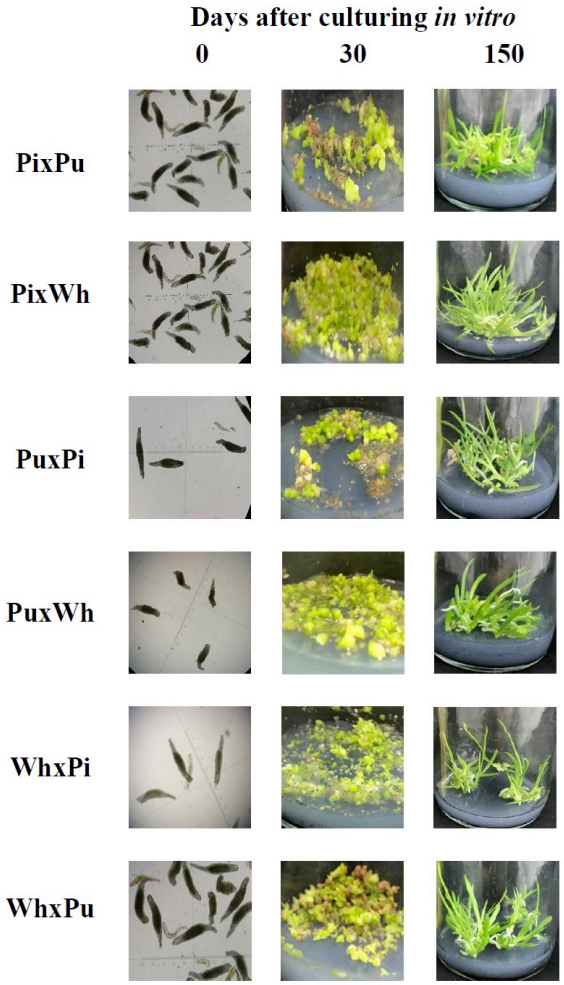

Figure 3. Characteristics of white (Wh), pink (Pi), and purple (Pu) flowers of cross-pollinated seeds, protocorms and seedlings of *P. hookeriana* (Rchb.f.) Schltr. after culture on VW medium for 0, 30 and 150 days

The self-pollination experiment conducted on *P. hookeriana* (Rchb.f.) Schltr. Revealed that fruit (pod) set and seed germination percentages did not show statistically significant among the self-pollination treatments. Nonetheless, the fruit set percentage reached its maximum of 100% in self-pollination of white flower color. A statistically significant was observed in self-pollination ability percentages and the seedlings number at a significant level of 0.01 and 0.05, respectively. The self-pollination ability percentages were 100% for pink and purple colors, whereas white flowers had only 40% self-pollination ability (Table 3). The pod weight and seed weight were significantly different among different flower color self-pollination (Figure 4). The purple color had the highest pod and seed weight compared to the white flower color but pod weight data were not significantly different from the pink color (Figure 5). The highest seedling number at 1,295 was found in self-pollinated pink color flowers, while 382 and 93 seedlings were gained from the self-pollination of purple and white flower colors, respectively (Table 3).

Table 3. Percentage of self-pollination ability, fruit set and seed germination, and seedling number of *P. hookeriana.* (Rchb.f.) Schltr.

Self-hybrid[1/]	**Self-pollination ability (%)** [2/]	**Fruit set (%)** [2/]	**Seed germination (%)** [2/]	**Number of seedlings**[2/]
Pi	100 a	70	71.43	1,295 a
Pu	100 a	80	87.50	382 b
Wh	40 b	100	50.00	93 b
F-test	**	ns	ns	*
C.V. (%)	37.26	15.23	61.66	53.21

[1/] Pi = Pink, Pu = Purple and Wh = White
[2/] ns is not significant, * and ** are significant at 0.05, and 0.01 levels

Results of cross-pollination of PixPu, PixWh, PuxPi, PuxWh, WhxPi, and WhxPu showed that percentages of cross-pollination ability and fruit were not significantly different between cross-pollination treatments (Table 4). The pod weight and seed weight were varied among the cross-pollination treatments. The PixPu, PixWh, PuxPi, WhxPi, and WhxPu had higher pod weight and seed weight when compared with PuxWh. However, for the seed weight, WhxPu was not significantly different from PuxWh (Figure 5). The percentages of seed germination and seedling numbers were significantly different at the 0.01 level. The higher rate of seed germination, reaching 100% was observed in cross-pollination of PixPu but it was not significantly different in statistics with cross-pollination of PixWh and PuxPi. The percentages of seed germination of PuxWh, WhxPu were 50% and the lowest seed germination percentage at 25% was found in WhxPu. The highest seedling numbers were found in the cross-pollination of

PixWh but not significant from cross-pollination of PixPu (304.7 seedlings). Seedling numbers of WhxPi, PuxPi, PuxWh, and WhxPu were 62.4, 46.9, 25.0 and 24.8 seedlings, respectively (Table 4).

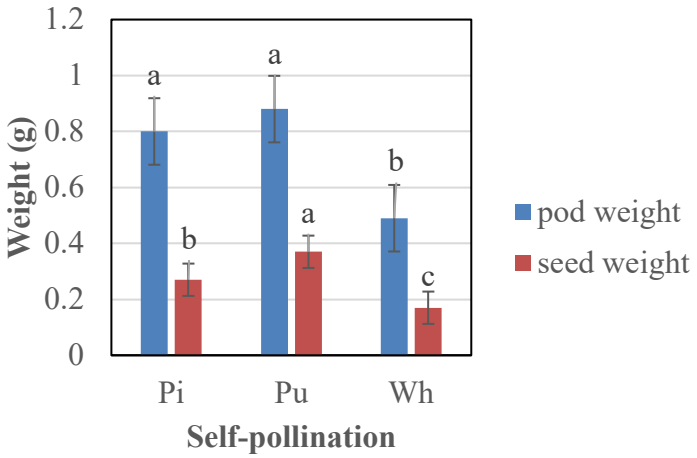

Figure 4. Pod and seed weight of self-pollination of various flower colors (Pi = Pink, Pu = Purple and Wh = White) of *P. hookeriana.* (Rchb.f.) Schltr.

Table 4. Percentages of cross-pollination ability, fruit set percentage, germination, and seedling number in *P. hookeriana.* (Rchb.f.) Schltr.

Cross-hybrid[1/]	Cross-pollination ability (%)[2/]	Fruit set percentage (%)[2/]	Germination (%)[2/]	Seedling number[2/]
PixPu	90.0	88.89	100.00 a	304.7 a
PixWh	80	100	87.50 a	403.2 a
PuxPi	90	100	77.78 a	46.9 b
PuxWh	60	100	50.00 b	25.0 b
WhxPi	80	100	25.00 c	62.4 b
WhxPu	60	100	50.00 b	24.8 b
F-test	ns	ns	**	**
C.V. (%)	18.65	15.23	23.27	20.92

[1/] Pi = Pink, Pu = Purple and Wh = White
[2/] ns is not significant, * and ** are significant at 0.05 and 0.01 levels

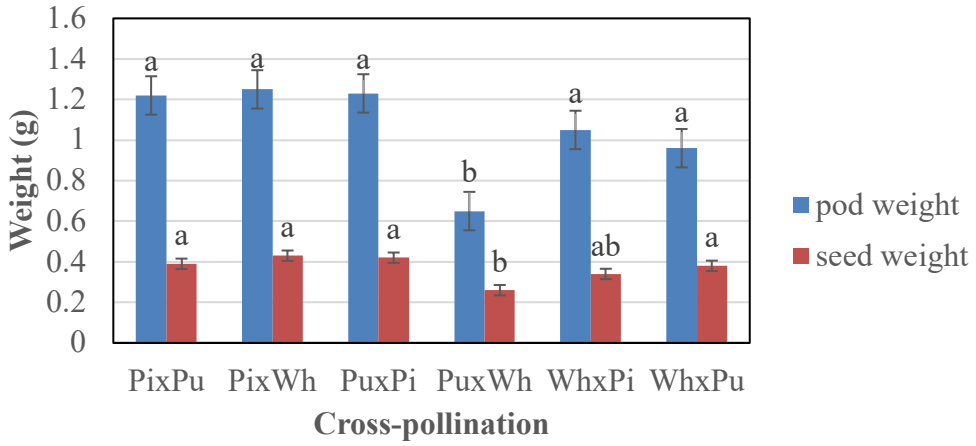

Figure 5. Pod and seed weight of cross-pollination of various flower colors (Pi = Pink, Pu = Purple and Wh = White) of *P. hookeriana.* (Rchb.f.) Schltr.

The AFLP technique was employed to analyze 25 samples using EcoRI/MseI primer and 650 bands were detected. The DNA bands were different sizes, as shown in Figure 7. The DNA bands of the AFLP technique translate to the similarity coefficient, as shown in Figure 6. The DNA band data and similarity coefficient from all the DNA fingerprints were analyzed using the NTSYSpc program version 1.2 and grouped using the UPGMA method. Dendrograms were constructed using UPGMA analysis for self and cross-pollinations, as shown in Figure 8. Results showed that *P. hookeriana.* (Rchb.f.) Schltr. was grouped into two clusters. Cluster I comprised 14 samples including Pi 05-2 (S1), Pu 02 (S2), WrxPu 03-3 (S5), PuxWr 03-1 (S6), PixWr 01-2 (S7), Pi 05-R3 (S9), Pu 02-R1(S10), Wr 10-R1 (S13), WrxPi 01-R1 (S14), WrxPi 01-R2 (S15), WrxPi 01-R3 (S16), WrxPu 03-R1 (S17), WrxPu 03-R2 (S18), and PuxWr 03-R3 (S21) that were divided into two subclusters IA and IB. Subcluster IA consisted of 13 samples including Pi 05-2 (S1), Pu 02 (S2), WrxPu 03-3 (S5), PuxWr 03-1 (S6), PixWr 01-2 (S7), Pi 05-R3 (S9), Pu 02-R1 (S10), Wr 10-R1 (S13), WrxPi 01-R1 (S14), WrxPi 01-R2 (S15), WrxPi 01-R3 (S16), WrxPu 03-R1 (S17), and WrxPu 03-R2 (S18). Subcluster IB contained PuxWr 03-R3 (S21) only. Cluster II comprised 11 samples that were divided into two subclusters IIA and IIB. Subcluster IIA consisted of 5 samples including WrxPi 01-1 (S4), Pu 02-R2 (S11), Pu 02-R3 (S12), WrxPu 03-R3 (S19), and PuxWr 03-R1 (S20). Subcluster IIB comprised 6 samples including PixWr 01-R2 (S22), PuxPi 03-R1-1 (S23), PuxPi 03-R2-1 (S24), PixPu 07-R1 (S25), PixPu 07-R2 (S26), and PixPu 07-R3 (S27). The UPGMA dendrogram of 25 *P. hookeriana.* (Rchb.f.) Schltr. samples is shown in Figure 8.

S1																								
S2	0.000																							
S4	0.654	0.654																						
S5	0.000	0.000	0.654																					
S6	0.083	0.083	0.654	0.083																				
S7	0.128	0.128	0.580	0.128	0.128																			
S9	0.083	0.083	0.654	0.083	0.000	0.128																		
S10	0.174	0.174	1.022	0.174	0.274	0.223	0.274																	
S11	0.916	0.916	0.128	0.916	0.916	0.654	0.916	0.734																
S12	0.916	0.916	0.128	0.916	0.916	0.654	0.916	0.734	0.000															
S13	0.274	0.274	1.022	0.274	0.174	0.223	0.174	0.274	0.916	0.916														
S14	0.223	0.223	0.734	0.223	0.223	0.083	0.223	0.128	0.511	0.511	0.223													
S15	0.223	0.223	0.734	0.223	0.223	0.083	0.223	0.223	0.654	0.654	0.128	0.083												
S16	0.274	0.274	1.022	0.274	0.174	0.223	0.174	0.174	0.734	0.734	0.083	0.128	0.128											
S17	0.223	0.223	0.916	0.223	0.223	0.174	0.223	0.223	0.821	0.821	0.223	0.174	0.174	0.223										
S18	0.223	0.223	0.734	0.223	0.223	0.083	0.223	0.128	0.511	0.511	0.223	0.000	0.083	0.128	0.174									
S19	0.821	0.821	0.274	0.821	0.821	0.580	0.821	0.821	0.223	0.223	1.022	0.580	0.734	0.821	0.580	0.580								
S20	0.821	0.821	0.274	0.821	0.821	0.580	0.821	0.821	0.223	0.223	1.022	0.580	0.734	0.821	0.580	0.580	0.000							
S21	0.274	0.274	0.274	0.274	0.274	0.223	0.274	0.511	0.446	0.446	0.511	0.329	0.329	0.511	0.446	0.329	0.511	0.511						
S22	0.580	0.580	0.329	0.580	0.580	0.511	0.580	0.734	0.386	0.386	0.916	0.511	0.654	0.734	0.511	0.511	0.329	0.329	0.223					
S23	0.821	0.821	0.386	0.821	1.022	0.734	1.022	0.821	0.329	0.329	1.273	0.734	0.916	1.022	0.734	0.734	0.174	0.174	0.654	0.329				
S24	0.654	0.654	0.386	0.654	0.821	0.580	0.821	0.821	0.446	0.446	1.022	0.734	0.734	1.022	0.580	0.734	0.274	0.274	0.511	0.329	0.083			
S25	0.511	0.511	0.386	0.511	0.654	0.446	0.654	0.654	0.446	0.446	0.821	0.580	0.580	0.821	0.446	0.580	0.274	0.274	0.386	0.223	0.174	0.083		
S26	0.734	0.734	0.223	0.734	0.734	0.511	0.734	0.916	0.274	0.274	0.916	0.654	0.654	0.916	0.511	0.654	0.223	0.223	0.329	0.174	0.223	0.223	0.128	
S27	0.734	0.734	0.223	0.734	0.734	0.511	0.734	0.916	0.274	0.274	0.916	0.654	0.654	0.916	0.511	0.654	0.223	0.223	0.329	0.174	0.223	0.223	0.128	0.000

Figure 6. The similarity coefficient of *P. hookeriana.* (Rchb.f.) Schltr. from the AFLP technique

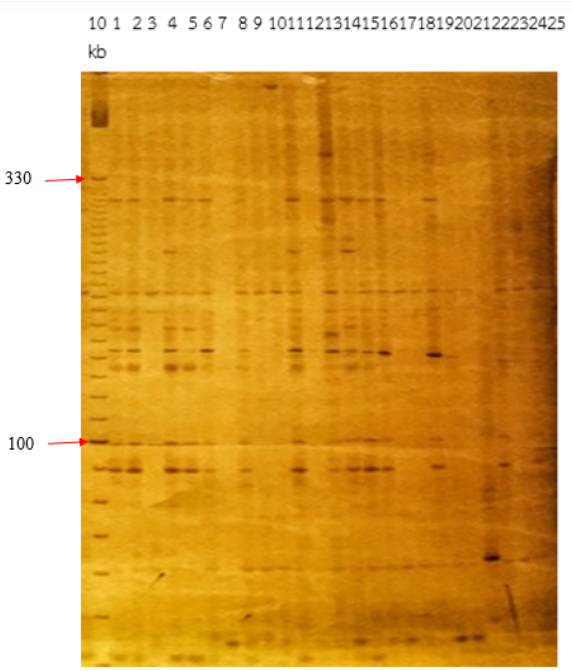

Figure 7. DNA bands from the AFLP technique (left to right include 10 bp DNA: Ladder, Pi 05-2 (1), Pu 02 (2), WrxPi 01-1 (3), WrxPu 03-3 (4), PuxWr 03-1 (5), PixWr 01-2 (6), Pi 05-R3 (7), Pu 02-R1(8), Pu 02-R2 (9), Pu 02-R3 (10), Wr 10-R1 (11), WrxPi 01-R1 (12), WrxPi 01-R2 (13), WrxPi 01-R3 (14), WrxPu 03-R1 (15), WrxPu 03-R2 (16), WrxPu 03-R3 (17), PuxWr 03-R1 (18), PuxWr 03-R3 (19), PixWr 01-R2 (20), PuxPi 03-R1-1 (21), PuxPi 03-R2-1 (22), PixPu 07-R1 (23), PixPu 07-R2 (24) and PixPu 07-R3(25), where Pi = Pink, Pu = Purple and Wh = White)

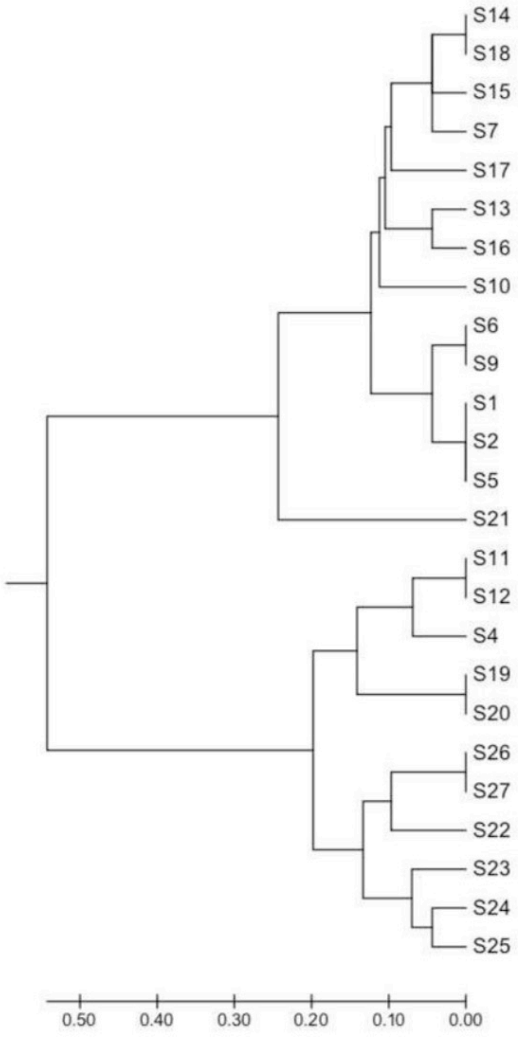

Figure 8. UPGMA dendrogram of 25 *P. hookeriana.* (Rchb.f.) Schltr. samples analyzed by NTSYSpc version 1.2. (Meaning of S number, Pi 05-2 (S1), Pu 02 (S2),Wr 10-1 (S3), WrxPi 01-1 (S4), WrxPu 03-3 (S5), PuxWr 03-1 (S6), PixWr 01-2 (S7), PuxPi 03-3 (S8), Pi 05-R3 (S9), Pu 02-R1(S10), Pu 02-R2 (S11), Pu 02-R3 (S12), Wr 10-R1 (S13), WrxPi 01-R1 (S14), WrxPi 01-R2 (S15), WrxPi 01-R3 (S16), WrxPu 03-R1 (S17), WrxPu 03-R2 (S18), WrxPu 03-R3 (S19), PuxWr 03-R1 (S20), PuxWr 03-R3 (S21), PixWr 01-R2 (S22), PuxPi 03-R1-1 (S23), PuxPi 03-R2-1 (S24), PixPu 07-R1 (S25), PixPu 07-R2 (S26) and PixPu 07-R3 (S27), where Pi = Pink, Pu = Purple and Wh = White)

Discussion

Self and cross-pollination ability are impacted by several factors. Studies in the genus *Bulbophyllum* showed that a smaller diameter of the stigmatic cavity in *B. involutum* reduced the chance of interspecific pollination with *B. weddellii* by 50% (Borba and Semir, 1999). Zhang and Gao (2017) studied *H. davidii* and *H. fordii.* They reported that floral morphology played an important role in floral isolation and the two species shared the same hawkmoth pollinator while in *chiloglottis* orchids, pollination involved highly specific interaction between a pool of nonpollinating 'minor responder' wasps. Six novel compounds, all 2,5-dialkylcyclohexan-1,3-diones were involved in pollinator attraction (Peakall *et al*., 2010). Previous studies confirmed that other factors affected self and cross-pollination ability in orchids including the size of the stigmatic cavity, morphology of the flower (Li *et al*., 2021), and chemical pollination compounds (Gutiérrez-Rodríguez, *et al*., 2022; Lui *et al*., 2006). Fruit production levels are commonly documented in orchid species, and fruit set production serves as the most utilized metric for assessing female reproductive success (Proctor and Harder, 1994). Gonzalez-Diaz and Ackerman (1988) found that self-pollination had a higher percentage of fertile seed compared with cross-pollination but there were no significant differences in fruit set, seed fertility, or seed weight between self and cross-pollinated flowers. Pollen load can affect fruit and seed production in some plants (Cruzan, 1986). The reproductive success of *Stanhopea lietzei* and *S. insignis* also depends on the stigmatic cavity (Pansarin and Amaral, 2009). Our results showed that the size of the pollinia and stigma cavity of *P. hookeriana* (Rchb.f.) Schltr. affected self-pollination ability. Purple and pink flowers of *P. hookeriana.* (Rchb.f.) Schltr. had larger pollinia and stigma gravity than white flowers (Table 1). Self-pollination abilities in purple and pink flowers were recorded at 100%, while only 40% white flowers. Cross-pollination abilities of cross-pollination between PixPu and PuxPi, PixWh and WhxPi, and PuxWh and WhxPu were 90, 80 and 60% and correlated with self-pollination abilities. Purple and pink flower cross-hybrids PixPu, PuxPi had the highest cross-pollination ability of 90%, similar to self-pollination ability at 100%. Purple and white color cross-hybrids PuxWh and WhxPu had the lowest cross-pollination ability of 60% similar to self-pollination ability in white flowers at 40%. A similar result was found in *S. insignis*, with low reproductive success because the stigmatic cavity was too narrow to admit pollinia, and the pollinia were too thick to be inserted (Pansarin and Amaral, 2009). Pollen size also affected pot size and seed weight in *P. hookeriana.* (Rchb.f.) Schltr. Pollen sizes of PixPu were greater than PuxWh flowers, and this probably increased cross-pollination ability. Our results showed that the pollination ability of pink flowers was the most effective

followed by purple flowers, with white flowers as the least productive. Comparable findings were observed in research conducted by Gonzalez-Diaz and Ackerman (1988), who noted that the two pollinia in *O. maculate* orchid resulted in double seed weight per fruit.

As a key part of traditional breeding, seed germination is directly related to the efficiency and success of crossbreeding. (Li *et al*., 2021). The effectiveness of *in vitro* propagation is significantly influenced by factors such as seed maturity, culture conditions, and culture media. *In vitro* propagation research has been conducted on a wide range of orchid species (Gao *et al*., 2020; Montri *et al*., 2023) for large scale propagation and conservation of orchids. Pod size and seed weight also impacted seed germination and seedling number. Higher pod size and seed weight gave a higher rate of seed germination and seedling number. The highest rate of seed germination, reaching 100% in *P. hookeriana.* (Rchb.f.) Schltr. was observed in cross-pollination of PixPu but it was not significant different in statistics with cross-pollination of PixWh and PuxPi. While the percentages of seed germination of PuxWh, WhxPu were 50% and the lowest seed germination percentage at 25% was found in WhxPu. The highest seedling numbers were found in the cross-pollination of PixWh but not significant from the cross-pollination of PixPu (304.7 seedlings). Seedling numbers of WhxPi, PuxPi, PuxWh, and WhxPu were 62.4, 46.9, 25.0 and 24.8 seedlings, respectively.

Various techniques have been studied for polymorphism in organisms such as RFLP, RAPD, ISSR, SCAR and AFLP. These techniques produce different levels of DNA that are used to identify and classify organisms. The technique used in this research was AFLP. This PCR-based assay for plant DNA fingerprinting revealed significant levels of DNA polymorphism in plants (Vos *et al*., 1995). This technique has been applied to study genetic variation and diversity in many plant species including orchids. Studies of *Dendrobium* hybrids using AFLP analysis suggested that AFLP fingerprint profiling gave accurate genetic relationships between tested *Dendrobium* hybrids (Xiang *et al*., 2003). A morphological study and molecular analysis of *Dendrobium* showed a correlation between AFLP data and combined data, indicating the usefulness of AFLP data for discrimination and hybrid identification in the absence of floral morphological characters (Wahba *et al*., 2014). The AFLP technique was also used to study endangered orchids. *L. japonica* is an endangered orchid in Northeast China. Using AFLP techniques suggested that habitat protection and maintaining a stable environment were critical for the conservation of *L. japonica* (Chen *et al*., 2013). *P. longifolium* is an endangered orchid in Costa Rica. In situ conservation, avoiding fragmentation, and mycorrhizal fungi and pollinators were shown to be important factors to protect habitat (Munoz *et al*., 2010). The

AFLP technique had high efficiency and DNA polymorphism for studies in *Dendrobium* species, and endangered orchids are useful tools for studies of genetic diversity, floral morphology, and conservation. Here, the AFLP technique was used to determine self and cross-pollination ability. In this study, the AFLP technique was used to analyze 25 samples for self and cross-pollination ability between different flower colors of *P. hookeriana*. (Rchb.f.) Schltr. Results showed genetic relations between various flower colors of self and cross-pollinated *P. hookeriana.* (Rchb.f.) Schltr. classified into two main clusters including cluster I (subclusters IA and subclusters IB) and cluster II (subclusters IIA and subclusters IIB), as shown in Figure 8. An analysis of the UPGMA dendrogram and flower colors of *P. hookeriana* (Rchb.f.) Schltr. found that cluster IIB had cross-pollination of *P. hookeriana* (Rchb.f.) Schltr. only. All samples in cluster IIB were cross-pollination of PixPu, PuxPi and PixWh, and especially PixPu and PuxPi. Results indicated that the AFLP technique successfully detected DNA polymorphism of cross-pollination in *P. hookeriana* (Rchb.f.) Schltr. Similar findings were recorded by Sandaro *et al*. (2012) for the *Serapias* genus of Italian orchids using the AFLP technique. Thus, evolutionary and taxonomic relationships are indicators of hybrid individuals in the genus.

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