CHAPTER 3

MATERIALS AND METHODS

3.1 Analysis of genetic relationship of genus *Phalaenopsis* and related genera, *Doritis* and *Kingidium*, by RAPD technique

Plant materials

Thirty-six samples of 30 representative species were collected from 8 sections of *Phalaenopsis* and 2 related genera, *Doritis* and *Kingidium* (Table 1 and Table 2). Twenty-five species of *Phalaenopsis* were obtained from 8 sections, i.e. section *Phalaenopsis*: *P. amabilis*, *P. aphrodite*, *P. philippinensis* and *P. schilleriana* (Figure 1), section *Proboscidioides*: *P. lowii* (Figure 2), section *Parishianae*: *P. gibbosa* 1, *P. gibbosa* 2, *P. lobbii* and *P. parishii* (Figure 3), section *Polychilos*: *P. cornu-cervi* and *P. mannii* (Figure 4), section *Stauroglottis*: *P. equestris*, *P. lindenii* 1 and *P. lindenii* 2 (Figure 5), section *Fuscatae*: *P. fuscata* and *P. viridis* (Figure 6), section *Amboinenses*: *P. amboinensis* 1, *P. amboinensis* 2, *P. javanica*, *P. micholitzii* and *P. venosa* (Figure 7), and section *Zebrinae*: *P. bellina*, *P. corningiana*, *P. hieroglyphica*, *P. mariae*, *P. pulchra*, *P. sumatrana*, *P. violacea* and *P. violacea* var. *sumatra* (Figure 8). One species of *Doritis*: *D. pulcherrima*, *D. pulcherrima* 'dwarf' (miniature plant) and *D. pulcherrima* var. *buyssoniana* (tetraploid plant) (Figure 9), and four species of *Kingidium*: *K. braceana*, *K. deliciosa*, *K. philippinensis* and *K. minus* (Figure 10). They were cultivated at orchid nursery of Horticulture Division, Department of Plant Science and Natural Resources, Faculty of Agriculture, Chiang Mai University, Chiang Mai, Thailand.

Table 1 Geographical distributions of plant materials used in this study.

No.	Code	Species	Section/genus	Distribution
1	PH01	*P. amabilis*	*Phalaenopsis*	Indonesia, Philippines and Australia
2	PH02	*P. aphrodite*	*Phalaenopsis*	Northern Philippines and southeastern Taiwan
3	PH03	*P. schilleriana*	*Phalaenopsis*	Philippines
4	PH04	*P. philippinensis*	*Phalaenopsis*	Philippines
5	PH05	*P. lowii*	*Proboscidioides*	Myanmar and adjacent western Thailand
6	PH06	*P. gibbosa* 1	*Parishianae*	Vietnam, Laos and Thailand
7	PH07	*P. gibbosa* 2	*Parishianae*	Vietnam, Laos and Thailand
8	PH08	*P. lobbii*	*Parishianae*	India, Bhutan, Myanmar, Vietnam and Thailand
9	PH09	*P. parishii*	*Parishianae*	India, Myanmar and Thailand
10	PH10	*P. cornu-cervi*	*Polychilos*	Northeast India, Indonesia and Thailand
11	PH11	*P. mannii*	*Polychilos*	Northeast India, Nepal, and China to Vietnam
12	PH12	*P. equestris*	*Stauroglottis*	Philippines and Taiwan
13	PH13	*P. lindenii* 1	*Stauroglottis*	Philippines
14	PH14	*P. lindenii* 2	*Stauroglottis*	Philippines
15	PH15	*P. viridis*	*Fuscatae*	Indonesia (Sumatra)
16	PH16	*P. fuscata*	*Fuscatae*	Indonesia, Malaysia and Philippines
17	PH17	*P. amboinensis* 1	*Amboinenses*	Indonesia
18	PH18	*P. amboinensis* 2	*Amboinenses*	Indonesia
19	PH19	*P. javanica*	*Amboinenses*	Indonesia (Java)
20	PH20	*P. micholitzii*	*Amboinenses*	Philippines
21	PH21	*P. venosa*	*Amboinenses*	Indonesia (Sulawesi)
22	PH22	*P. sumatrana*	*Zebrinae*	Myanmar, Thailand, Vietnam, Indonesia, Malaysia and Philippines
23	PH23	*P. corningiana*	*Zebrinae*	Indonesia (Borneo)
24	PH24	*P. hieroglyphica*	*Zebrinae*	Philippines
25	PH25	*P. violacea*	*Zebrinae*	Indonesia and Malaysia
26	PH26	*P. violacea* var. *sumatra*	*Zebrinae*	Indonesia (Sumatra)
27	PH27	*P. bellina*	*Zebrinae*	Malaysia
28	PH28	*P. pulchra*	*Zebrinae*	Philippines
29	PH29	*P. mariae*	*Zebrinae*	Philippines and Indonesia
30	K01	*K. braceana*	*Kingidium*	Bhutan and China
31	K02	*K. deliciosa*	*Kingidium*	Sri Lanka, India, Philippines and Indonesia
32	K03	*K. minus*	*Kingidium*	Thailand
33	K04	*K. philippinensis*	*Kingidium*	Philippines
34	D01	*D. pulcherrima*	*Doritis*	Northeast India, Southern China, Thailand, Indonesia and Malaysia
35	D02	*D. pulcherrima* 'dwarf'	*Doritis*	Thailand
36	D03	*D. pulcherrima* var. *buyssoniana*	*Doritis*	Thailand and Indonesia

Table 2 Flower descriptions of plant materials used in this study.

No.	Code	Species	Section/genus	Flower description		
				Shape	Color	No. of pollinia
1	PH01	*P. amabilis*	*Phalaenopsis*	Round	White	2
2	PH02	*P. aphrodite*	*Phalaenopsis*	Round	White	2
3	PH03	*P. schilleriana*	*Phalaenopsis*	Round	Pink	2
4	PH04	*P. philippinensis*	*Phalaenopsis*	Round	White	2
5	PH05	*P. lowii*	*Proboscidioides*	Round	White and Pink	4
6	PH06	*P. gibbosa* 1	*Parishianae*	Round	White	4
7	PH07	*P. gibbosa* 2	*Parishianae*	Round	White	4
8	PH08	*P. lobbii*	*Parishianae*	Round	White	4
9	PH09	*P. parishii*	*Parishianae*	Round	White	4
10	PH10	*P. cornu-cervi*	*Polychilos*	Star	Yellow with reddish brown bar and spot	2
11	PH11	*P. mannii*	*Polychilos*	Star	Yellow with reddish brown bar and spot	2
12	PH12	*P. equestris*	*Stauroglottis*	Star	Light pink	2
13	PH13	*P. lindenii* 1	*Stauroglottis*	Star	Light pink	2
14	PH14	*P. lindenii* 2	*Stauroglottis*	Star	Light pink	2
15	PH15	*P. viridis*	*Fuscatae*	Star	Yellow with brown spot	2
16	PH16	*P. fuscata*	*Fuscatae*	Star	Yellow with brown spot	2
17	PH17	*P. amboinensis* 1	*Amboinenses*	Star	Yellow with reddish brown bar	2
18	PH18	*P. amboinensis* 2	*Amboinenses*	Star	Yellow with reddish brown bar	2
19	PH19	*P. javanica*	*Amboinenses*	Star	Creamy white with reddish brown bar and spot	2
20	PH20	*P. micholitzii*	*Amboinenses*	Star	White	2
21	PH21	*P. venosa*	*Amboinenses*	Star	Greenish yellow with brown bar	2
22	PH22	*P. sumatrana*	*Zebrinae*	Star	Creamy white with brown bar	2
23	PH23	*P. corningiana*	*Zebrinae*	Star	Creamy white with brown bar and spot	2
24	PH24	*P. hieroglyphica*	*Zebrinae*	Star	Creamy white with brown bar and spot	2
25	PH25	*P. violacea*	*Zebrinae*	Star	Greenish white	2
26	PH26	*P. violacea* var. *sumatra*	*Zebrinae*	Star	Pink	2
27	PH27	*P. bellina*	*Zebrinae*	Star	Greenish white with purple base	2
28	PH28	*P. pulchra*	*Zebrinae*	Star	Dark purple	2
29	PH29	*P. mariae*	*Zebrinae*	Star	Creamy white with reddish brown bar	2
30	K01	*K. braceana*	*Kingidium*	Round	Green	4
31	K02	*K. deliciosa*	*Kingidium*	Round	White	4
32	K03	*K. minus*	*Kingidium*	Round	White with purple bar and spot	4
33	K04	*K. philippinensis*	*Kingidium*	Round	White	4
34	D01	*D. pulcherrima*	*Doritis*	Round	Pink	4
35	D02	*D. pulcherrima* 'dwarf'	*Doritis*	Round	Dark pink	4
36	D03	*D. pulcherrima* var. *buyssoniana*	*Doritis*	Round	Dark pink	4

P. amabilis *P. aphrodite*

P. philippinensis *P. schilleriana*

Figure 1 Flower of *Phalaenopsis* species in section *Phalaenopsis*.

P. lowii

Figure 2 Flower of *Phalaenopsis* species in section *Proboscidioides*.

P. gibbosa *P. parishii*

Figure 3 Flower of *Phalaenopsis* species in section *Parishianae*.

P. cornu-cervi *P. mannii*

Figure 4 Flower of *Phalaenopsis* species in section *Polychilos*.

P. equestris *P. lindenii*

Figure 5 Flower of *Phalaenopsis* species in section *Stauroglottis*.

P. viridis

Figure 6 Flower of *Phalaenopsis* species in section *Fuscatae*.

P. amboinensis *P. javanica*

Figure 7 Flower of *Phalaenopsis* species in section *Amboinenses*.

P. violacea *P. violacea* var. *sumatra*

Figure 8 Flower of *Phalaenopsis* species in section *Zebrinae*.

D. pulcherrima

D. pulcherrima ‘dwarf’

D. pulcherrima var. *buyssoniana*

Figure 9 Flower of species from genus *Doritis*.

K. deliciosa

K. minus

Figure 10 Flower of species from genus *Kingidium*.

Materials for RAPD technique

Equipments

1. Autoclave
2. Automatic pipette P2, P20, P100 (Gilson Medical Electronics S.A., France)
3. Electrophoresis apparatus (BIO-RAD)
4. -20 °C freezer
5. -80 °C freezer
6. Gel Documentation (Lab Focus Co., Ltd.)
7. High speed microcentrifuge
8. Microwave oven
9. Mortar
10. Power supplies (BIO-RAD)
11. Spectrophotometer
12. Temperature controlled microcentrifuge
13. Thermal Cycler (Perkin Elmer Gene Amp PCR System 2400, Perkin-Elmer Cetus Co., Norwalk, Connecticut, USA)
14. UV transilluminator (Syngene)
15. Vortex mixer
16. Water bath

Chemical reagents

1. Agarose (Promega)
2. Ammonium acetate
3. Boric acid

4. Bromophenol blue
5. Cetyltrimethyl ammonium bromide
6. Chloroform
7. Deoxyribonucleoside triphosphates (Invitrogen)
8. 50 - 2,500 bp DNA Marker (Invitrogen)
9. Ethidium bromide
10. Ethyl alcohol
11. Ethylene diamine tetra-acetic acid (EDTA)
12. EZ Load Precision Molecular Mass Standard
13. Isopropanol
14. Isoamyl alcohol
15. Liquid nitrogen
16. Magnesium chloride (Invitrogen)
17. 2- mercaptoethanol
18. PCR reaction buffer (Invitrogen)
19. Polyvinyl pyrrolidone-40
20. Primer (Operon Technologies Inc., Alameda, California, USA)
21. Sodium chloride
22. Sodium dodecyl sulfate (SDS)
23. *Taq* DNA polymerase (Invitrogen)
24. Tris (hydroxyl methyl) aminomethane
25. Xylene cyanol FF

DNA extraction

Young leaf was cut and individually put in a plastic bag, and then placed in an icebox and brought over to the laboratory. It was cleaned and rinsed with distilled water and 70 % ethanol. About 0.1 g of leaf was employed for DNA extraction using the CTAB (cetyltrimethyl ammonium bromide) method (Doyle and Doyle, 1990). Leaf tissue was ground in a mortar to give a fine powder form. After grinding, the powder was mixed with 1 ml 2x CTAB buffer and transferred into a 1.5 ml microcentrifuge tube. Next, 10 µl proteinase K, 1 mg/µl, was added into the mixture and incubated at 60 ^{o}C for 30 min in a water bath, gently mixed every 10 min. Then, 500 µl 24 chloroform : 1 isoamyl alcohol was added into this mixture, strongly mixed, and then put into the centrifuge at 10,000 rpm for 10 min. The liquid was transferred into a new tube and added with an equal amount of isopropanol, gently mixed and then incubated at 4 ^{o}C overnight. After that, the mixture was centrifuged at 10,000 rpm for 10 min. The supernatant was discarded. Precipitate was washed with 500 µl of wash buffer (10 mM ammonium acetate and 75 % ethanol) and centrifuged at 10,000 rpm for 5 min. Then, it was washed with 500 µl of 75 % ethanol and centrifuged at 10,000 rpm for 5 min. This liquid was carefully discarded. The precipitate was air-dried. After that, it was resuspended by 100 µl of TE buffer (10 mM Tris-HCl and 0.5 mM EDTA), and 10 units of RNase A was added into this mixture and incubated at 37 ^{o}C for 30 min. This DNA solution was diluted to 10 ng/µl using distilled water (dH_2O). A total of 1µl of this dilution was used for polymerase chain reaction (PCR).

RAPD analysis

PCR was carried out in a 20 µl reaction mixture containing 10 ng of DNA template, 1x PCR buffer (20 mM tris-HCl pH 8.0, 0.1 mM EDTA, 1 mM DTT, 50 % glycerol), 1.5 mM $MgCl_2$, 100 µM dNTPs, 100 ng primer, 0.8 unit *Taq* DNA polymerase and dH_2O. Twenty decamer primers, OPAK01, OPAK10, OPAK11, OPD03, OPD10 and OPF01 - OPF15 (Operon Technologies Inc.) (Table 3) were used for PCR amplification. The DNA was amplified in the thermal cycler (Perkin Elmer Gene Amp PCR System 2400, Perkin-Elmer Cetus Co., Norwalk, Connecticut, USA). The PCR program was modified from Chen *et al*. (1998) with two-step thermal cycles. In the first step, two cycles of denaturation at 94 °C for 60 sec, annealing at 36 °C for 10 sec and extension at 72 °C for 70 sec were used. The second step was carried out by the following process: 30 cycles of 94 °C for 60 sec, 42 °C for 45 sec and 72 °C for 70 sec, with a final extension at 72 °C for 240 sec. The PCR products were stored at 4 °C prior to analysis.

Table 3 List of 20 decamer primers and their sequences used for RAPD technique.

No.	Primer name	Sequence 5' ⟶ 3'
1	OPAK01	TCTGCTACGG
2	OPAK10	CAAGCGTCAC
3	OPAK11	CAGTGTGCTC
4	OPD03	GTCGCCGTCA
5	OPD10	GGTCTACACC
6	OPF01	ACGGATCCTG
7	OPF02	GAGGATCCCT
8	OPF03	CCTGATCACC
9	OPF04	GGTGATCAGG
10	OPF05	CCGAATTCCC
11	OPF06	GGGAATTCGG
12	OPF07	CCGATATCCC
13	OPF08	GGGATATCGG
14	OPF09	CCAAGCTTCC
15	OPF10	GGAAGCTTGG
16	OPF11	TTGGTACCCC
17	OPF12	ACGGTACCAG
18	OPF13	GGCTGCAGAA
19	OPF14	TGCTGCAGGT
20	OPF15	CCAGTACTCC

The PCR products were separated by 1.8 % agarose gel electrophoresis in 1x TBE buffer at 50 V. The gel was stained with 0.1 μg/mL ethidium bromide and photographed under UV light using Gel Documentation (Lab Focus Co., Ltd.). The RAPD bands were scored as 0 (absent) and 1 (present). Standard measures of genetic diversity were calculated for the estimate of Nei' s (1972) gene diversity (h), polymorphic band and genetic distance values using the POPGENE version 1.32 program (Yeh *et al*., 1999). Genetic distances were determined using the UPGMA (unweighted pair group method with arithmetic averages) method for clustering and drawing dendrogram with the MEGA version 4 program (Tamura *et al*., 2007). Bootstrap analysis using 1,000 replications was performed using WinBoot (Yap and Nelson, 1996) to determine confidence limits of clusters in the UPGMA-based dendrogram.

3.2 Studies on crossability of genus *Phalaenopsis* and related genera, *Doritis* and *Kingidium*.

Plant materials

Seventeen samples from 14 representative species of *Phalaenopsis* and 2 related genera, *Doritis* and *Kingidium*, in the previous experiment were used in this study. Eleven species of *Phalaenopsis* were obtained from 7 sections, 1) Section *Phalaenopsis*: *P. amabilis*, *P. aphrodite* and *P. schilleriana*, 2) Section *Proboscidioides*: *P. lowii*, 3) Section *Parishianae*: *P. gibbosa* 1, and *P. parishii*, 4) Section *Polychilos*: *P. cornu-cervi*, 5) Section *Stauroglottis*: *P. equestris*, 6) Section *Amboinenses*: *P. amboinensis* 2 and *P. javanica*, and 7) Section *Zebrinae*: *P. violacea* and *P. violacea* var. *sumatra*. Three samples from one species of *Doritis*: *D. pulcherrima*, *D. pulcherrima* 'dwarf' and *D. pulcherrima* var. *buyssoniana*, and two species of *Kingidium*: *K. deliciosa* and *K. minus*.

Testing for crossability

Pollinia from male parent were collected and placed on the stigma of female parent. Pollination was done in during 8.00 - 9.00 am morning. Since flowers of each species were not blooming at the same time, pollinia of each species were collected and placed in sealed plastic tube and stored at 8 °C. Each species was used for both male and female parents. The total of 24 interspecific crosses i.e. 21 intersectional (Table 4) and 3 intrasectional (Table 5), and 20 intergeneric (Table 6) hybridizations were made using 1 - 10 flowers for each cross. The result of crossability and number of fruit setting were recorded. Fruits were harvested at four months after pollinations, their seeds were sown under aseptic condition. The sown seeds germinated within

a month, they developed into protocorms and then plantlets. They were subcultured for two times at four months intervals until the plantlets grew up to the size that could be transplanted in the greenhouse. The whole process took about a year. When plantlets were ready to be transplanted, they were deflasked and kept in the nursery for three weeks before they were planted in 1-inch pot using sphagnum moss as growing medium, thereafter, they were kept under a plastic roof. After 3 months, they were individually transplanted into a 3-inch pot using the same growing medium and kept under a shade-house at about 70 % shading. Six months after transplanting, number of plantlets and survival rate were recorded. One year after deflasked, the plant produced flowers. Phenotypic characteristics, i.e. leaf size, leaf color, flower width, flower color and pollinia number of studied plants were recorded.

Table 4 Intersectional hybridization of *Phalaenopsis* species.

No.	Parent 1 (P1)	Parent 2 (P2)	No. of pollinated flower	
			P1xP2	P2xP1
Section *Phalaenopsis* x Section *Polychilos*				
1	*P. amabilis*	*P. cornu-cervi*	5	3
2	*P. schilleriana*	*P. cornu-cervi*	6	5
Section *Phalaenopsis* x Section *Parishianae*				
3	*P. aphrodite*	*P. parishii*	3	3
4	*P. schilleriana*	*P. gibbosa* 1	3	2
Section *Phalaenopsis* x Section *Proboscidioides*				
5	*P. schilleriana*	*P. lowii*	3	3
Section *Phalaenopsis* x Section *Zebrinae*				
6	*P. schilleriana*	*P. violacea*	3	2
Section *Proboscidioides* x Section *Parishianae*				
7	*P. lowii*	*P. gibbosa* 1	5	3
8	*P. lowii*	*P. parishii*	3	2
Section *Proboscidioides* x Section *Polychilos*				
9	*P. lowii*	*P. cornu-cervi*	10	5
Section *Parishianae* x Section *Polychilos*				
10	*P. parishii*	*P. cornu-cervi*	3	2
Section *Parishianae* x Section *Stauroglottis*				
11	*P. parishii*	*P. equestris*	3	2
Section *Parishianae* x Section *Zebrinae*				
12	*P. parishii*	*P. violacea*	3	3
Section *Polychilos* x Section *Stauroglottis*				
13	*P. cornu-cervi*	*P. equestris*	5	5
Section *Polychilos* x Section *Amboinenses*				
14	*P. cornu-cervi*	*P. amboinensis* 2	3	3
15	*P. cornu-cervi*	*P. javanica*	3	2
Section *Polychilos* x Section *Zebrinae*				
16	*P. cornu-cervi*	*P. violacea*	5	5
Section *Stauroglottis* x Section *Amboinenses*				
17	*P. equestris*	*P. amboinensis* 2	2	1
18	*P. equestris*	*P. javanica*	2	1
Section *Stauroglottis* x Section *Zebrinae*				
19	*P. equestris*	*P. violacea*	3	2
Section *Amboinenses* x Section *Zebrinae*				
20	*P. amboinensis* 2	*P. violacea*	2	2
Section *Amboinenses* x Section *Zebrinae*				
21	*P. javanica*	*P. violacea*	2	2

Table 5 Intrasectional hybridization of *Phalaenopsis* species.

No.	Parent 1 (P1)	Parent 2 (P2)	No. of pollinated flower	
			P1xP2	P2xP1
Section *Phalaenopsis*				
1	*P. aphrodite*	*P. schilleriana*	4	3
Section *Parishianae*				
2	*P. gibbosa* 1	*P. parishii*	3	3
Section *Amboinenses*				
3	*P. amboinensis* 2	*P. javanica*	2	2

Table 6 Intergeneric hybridization of genus *Phalaenopsis* and related genera, *Doritis* and *Kingidium*.

No.	Parent 1 (P1)	Parent 2 (P2)	No. of pollinated flower	
			P1xP2	P2xP1
Genus *Phalaenopsis* x Genus *Doritis*				
1	*P. amabilis*	*D. pulcherrima* 'dwarf'	5	5
2	*P. lowii*	*K. deliciosa*	3	3
3	*P. cornu-cervi*	*D. pulcherrima*	2	3
4	*P. cornu-cervi*	*D. pulcherrima* 'dwarf'	5	3
5	*P. cornu-cervi*	*K. deliciosa*	3	2
6	*P. cornu-cervi*	*K. minus*	3	2
7	*P. equestris*	*D. pulcherrima* 'dwarf'	2	2
8	*P. equestris*	*K. deliciosa*	5	2
9	*P. equestris*	*K. minus*	5	4
10	*P. violacea*	*D. pulcherrima*	3	2
11	*P. violacea*	*D. pulcherrima* 'dwarf'	2	2
12	*P. violacea*	*D. pulcherrima* var. *buyssoniana*	3	2
13	*P. violacea*	*K. deliciosa*	2	3
14	*P. violacea*	*K. minus*	3	3
Genus *Doritis* x Genus *Kingidium*				
15	*D. pulcherrima*	*K. deliciosa*	2	2
16	*D. pulcherrima*	*K. minus*	2	2
17	*D. pulcherrima* 'dwarf'	*K. deliciosa*	2	2
18	*D. pulcherrima* 'dwarf'	*K. minus*	4	3
19	*D. pulcherrima* var. *buyssoniana*	*K. deliciosa*	2	3
20	*D. pulcherrima* var. *buyssoniana*	*K. minus*	2	2

3.3 Characterizations of F_1 progenies derived from intersectional and intergeneric hybrids of *Phalaenopsis* and related genera, *Doritis* and *Kingidium*, by RAPD technique

Plant materials

Three *Phalaenopsis* species, *P. cornu-cervi*, *P. equestris* and *P. schilleriana*, two related genera, *D. pulcherrima* 'dwarf' and *K. minus*, and three sets of F_1 progenies derived from the crosses *P. schilleriana* x *P. cornu-cervi*, *D. pulcherrima* 'dwarf' x *P. equestris* and *D. pulcherrima* 'dwarf' x *K. minus* were used in this study. Phenotypic characteristics, i.e. leaf size, leaf color, flower width, flower color and pollinia number of studied plants were recorded.

DNA extraction

DNA extraction was performed as described in 3.1. Quality and quantity of DNA were checked by 1 % agarose gel electrophoresis and then determined by a spectrophotometer.

RAPD analysis

Parental lines and their F_1 progenies were evaluated using the RAPD technique with PCR condition as described in 3.1. The 20 decamer primers were screened for DNA amplification of 3 compatible crosses and their progenies. The RAPD bands were scored as 0 (absent) and 1 (present). Genetic similarity of parental lines and their progenies using principle component analysis (PCA) was performed with the NTSYS-pc version 2.01 program (Rohlf, 2000).

3.4 Specific marker for flower color pattern of *Phalaenopsis cornu-cervi* by AFLP technique

Plant materials

Three types of *P. cornu-cervi* were used in this study: 1) three plants of reddish brown flower, 2) six plants of yellow flower with reddish brown bar and spot, and 3) three plants of pure yellow flower (no spot or bar) (Figure 11).

Materials for AFLP technique

Equipments

1. Autoclave
2. Automatic pipette P2, P20, P100 (Gilson Medical Electronics S.A., France)
3. Electrophoresis apparatus (BIO-RAD)
4. -20 °C freezer
5. -80 °C freezer
6. Gel Documentation (Lab Focus Co., Ltd.)
7. Gel dryer
8. High speed microcentrifuge
9. Microwave oven
10. Mortar
11. Power supplies (BIO-RAD)
12. Spectrophotometer
13. Temperature controlled microcentrifuge

14. Thermal Cycler: (Perkin Elmer Gene Amp PCR System 2400, Perkin-Elmer Cetus Co., Norwalk, Connecticut, USA)
15. UV transilluminator (Syngene)
16. Vortex mixer
17. Water bath

Chemical reagents

1. Acrylamide (Sigma)
2. Acetic acid
3. AFLP Core Reagent Kit (Invitrogen)
4. AFLP Starter Primer Kit (Invitrogen)
5. Agarose (Promega)
6. Bind silane (Promega)
7. Bis-acrylamide (Sigma)
8. Boric acid
9. Bromophenol blue
10. Cetyltrimethyl ammonium bromide
11. Chloroform
12. Deoxyribonucleoside triphosphates (Invitrogen)
13. 100 - 2,000 bp DNA marker (Fermentas)
14. Ethidium bromide
15. Ethyl alcohol
16. Ethylene diamine tetra-acetic acid (EDTA)
17. Formaldehyde (37%)
18. Formamide (98%)

19. Isoamyl alcohol
20. Isopropanol
21. Liquid nitrogen
22. Magnesium chloride (Invitrogen)
23. 2- mercaptoethanol
24. Nitric acid
25. PCR reaction buffer (Invitrogen)
26. Phenol
27. Polyvinyl pyrrolidone-40
28. Proteinase K (Invitrogen)
29. Repel silane
30. RNase ONE^{TM} Ribonuclease (Promega)
31. Silver nitrate
32. Sodium carbonate
33. Sodium chloride
34. Sodium dodecyl sulfate (SDS)
35. Sodium thiosulfate
36. *Taq* DNA Polymerase (Invitrogen)
37. Tris (hydroxymethyl) aminomethane
38. Urea
39. Xylene cyanol FF

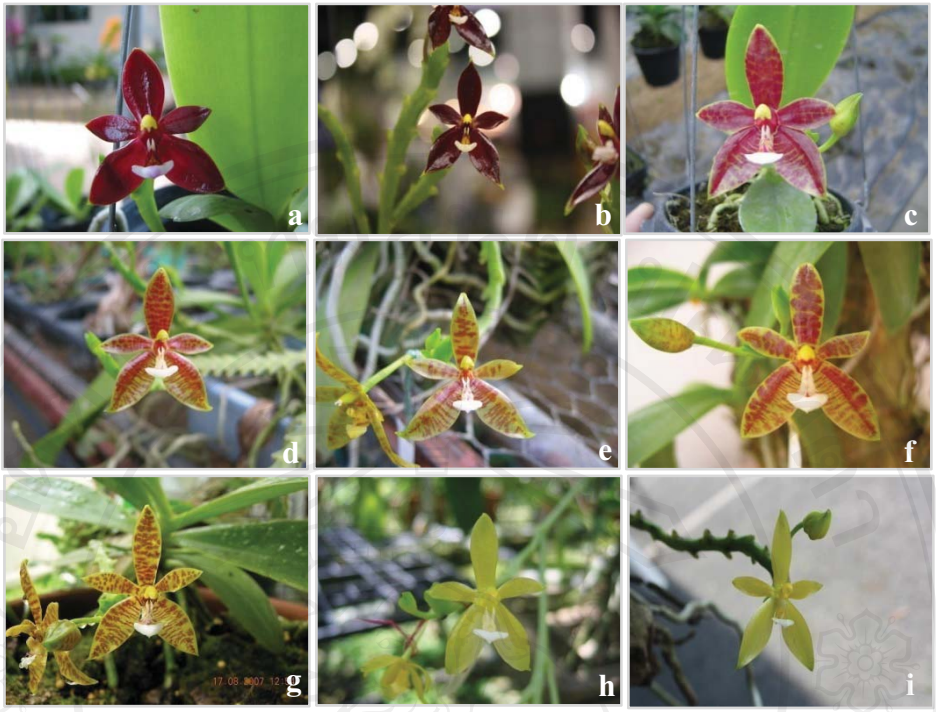

Figure 11 Three groups of *Phalaenopsis cornu-cervi* used in the AFLP analysis, reddish brown flower (a - c), yellow flower with reddish brown bar and spot (d - g), and pure yellow flower (no spot or bar) (h - i).

DNA extraction

DNA extraction was performed as described in 3.1. Quality and quantity of DNA were checked by 1 % agarose gel electrophoresis and then determined by a spectrophotometer.

AFLP analysis

AFLP analysis was carried out based on the protocol described by Vos *et al*. (1995). Genomic DNA was digested with restriction enzymes, *Eco*RI and *Mse*I at 37 °C for 3 hr, and ligated with *Eco*RI and *Mse*I adapter overnight at 4 °C. The product was amplified in the preselective amplification step using primers with one selective base (*Eco*RI+A and *Mse*I+C primers) in a total volume of 20 µl, and the

PCR product was diluted in a ratio of 1:5 with TE buffer, and then used as a template for selective amplification. The selective amplification step was conducted with 2 - 3 selective bases at the 3' end of each primer. Primers were 64 combinations of 8 *Eco*RI and 8 *Mse*I primers: *Eco*RI+AC, *Eco*RI+AG, *Eco*RI+AAC, *Eco*RI+AAG, *Eco*RI+AGA, *Eco*RI+ATC, *Eco*RI+ATG, *Eco*RI+ATT, *Mse*I+CAA, *Mse*I+CAG, *Mse*I+CAT, *Mse*I+CCA, *Mse*I+CTA, *Mse*I+CTC, *Mse*I+CTG and *Mse*I+CTT (Table 7 and Table 8). The conditions for preselective amplification were 25 cycles of denaturation at 94 °C for 30 sec, annealing at 56 °C for 60 sec and extension at 72 °C for 60 sec, followed by 5 min extension at 72 °C. For selective amplification step, touch down PCR was carried out by denaturation at 94 °C for 30 sec, annealing at 65 °C for 30 sec and extension for 60 sec at 72 °C for the first cycle, followed by lowering the annealing temperature by 1 °C for next 24 cycles, then annealing at 56 °C for the remaining 20 cycles; extension at 72 °C for 60 sec. To analyze the DNA pattern, the PCR products were denatured at 95 °C for 5 min and quickly cooled on ice. After selective amplification step, 1.8 % agarose gel electrophoresis of plant No. 1 of *P. cornu-cervi* in reddish brown flower group was used to screen for suitable primer combinations, with polymorphic DNA bands and high resolution. The PCR products of suitable primer combinations were separated on 6 % denaturing polyacrylamide gels in 1x TBE buffer, and electrophoresis was performed at constant power (55 W) and temperature (50 °C) for 5 hr. After electrophoresis, bands were visualized by silver staining by adding 10 % acetic acid for 20 min and then adding 1 % nitric acid for 20 min. The gels were washed 3 times with double distilled water (ddH_2O) and stained with 0.2 % silver nitrate solution for 30 min. After washing with ddH_2O, the gels were developed with 3 % sodium carbonate which was supplemented with

0.02 % formaldehyde, until the DNA bands appeared. The reactions were stopped with 10 % acetic acid for 2 min and washed again with ddH_2O. The gel was dried on filter paper at 55 °C for 2 h under vacuum on the gel dryer. Selective amplification using suitable primer combinations were repeated at least twice.

The polymorphic DNA bands that were specific for *P. cornu-cervi* flower color pattern were identified. The specific DNA bands were retrieved from the gel and used for reamplification by the same primer combination using selective amplification condition. The fragments were then cloned with Clone JET^{TM} PCR Cloning Kit (Fermentas) and automated sequenced (Ward Medic, Ltd.). The DNA sequences were compared with database in National Center for Biotechnology Information (NCBI) GenBank.

Table 7 List of primers and their sequences used for AFLP technique.

Reaction	Primer	Sequence 5'⟶3'
Preselective amplification	E-A	GACTGCGTACCAATTCA
	M-C	GATGAGTCCTGAGTAAC
Selective amplification - *Eco*RI	E-AC	GACTGCGTACCAATTCAC
	E-AG	GACTGCGTACCAATTCAG
	E-AAC	GACTGCGTACCAATTCAAC
	E-AAG	GACTGCGTACCAATTCAAG
	E-AGA	GACTGCGTACCAATTCAGA
	E-ATC	GACTGCGTACCAATTCATC
	E-ATG	GACTGCGTACCAATTCATG
	E-ATT	GACTGCGTACCAATTCATT
Selective amplification - *Mse*I	M-CAA	GATGAGTCCTGAGTAACAA
	M-CAC	GATGAGTCCTGAGTAACAC
	M-CAG	GATGAGTCCTGAGTAACAG
	M-CAT	GATGAGTCCTGAGTAACAT
	M-CTA	GATGAGTCCTGAGTAACTA
	M-CTC	GATGAGTCCTGAGTAACTC
	M-CTG	GATGAGTCCTGAGTAACTG
	M-CTT	GATGAGTCCTGAGTAACTT

Table 8 Sixty-four primer combinations used for AFLP technique.

Primer	M-CAA	M-CAC	M-CAG	M-CAT	M-CTA	M-CTC	M-CTG	M-CTT
E-AC	1 [a]	2	3	4	5	6	7	8
E-AG	9	10	11	12	13	14	15	16
E-AAC	17	18	19	20	21	22	23	24
E-AAG	25	26	27	28	29	30	31	32
E-AGA	33	34	35	36	37	38	39	40
E-ATC	41	42	43	44	45	46	47	48
E-ATG	49	50	51	52	53	54	55	56
E-ATT	57	58	59	60	61	62	63	64

[a]The 1 - 64 numbers correspond to the numbers labeled in Figure 47.