CHAPTER 3

MATERIALS AND METHODS

3.1 Morphology and growth development of plant species

Plant material

Habenaria

1. *H. lindleyana* Steud.
2. *H. myriotricha* Gagnep.
3. *H. rhodocheila* Hance.
    - *H. rhodocheila* Hance. (red flower)
    - *H. rhodocheila* Hance.(pink flower)
    - *H. rhodocheila* Hance. (orange flower)
4. *H. xanthocheila* Ridl.

Pecteilis

1. *Pecteilis hawkesiana* King &Pantl.
    - *P. hawkesiana* King &Pantl. (white lip)
    - *P. hawkesiana* King &Pantl. (yellow lip)
2. *P. susannae* (L.) Rafin

Method

Four plants of each orchid species were collected. Characteristics of each plant, i.e. leaves length and width, leaf color, number of leaf per plant, inflorescences height, number of flowers per plant, flower color, petal length and width, and lip length and width were measured and recorded.

3.2 Analysis of genetic relationship of *Habenaria* and *Pecteilis* by RAPD technique

Plant materials

1. Six orchid species of 2 genera, i.e. *H. lindleyana* Steud., *H. myriotricha*, *H. rhodocheila* Hance (orange, pink and red flowers), *H. xanthocheila*, *P. hawkesiana* (white and yellow lip) and *P. susannae* (L.) Rafin were cultivated at orchid nursery of Horticulture Division, Department of Plant Science and Natural Resources, Faculty of Agriculture, Chiang Mai University, and H.M. the King's Initiative Centre for Flowers and Fruits Propagation at Ban Rai Village, Hang Dong District, Chiang Mai, Thailand.
2. Five samples of each species were collected for RAPD analysis

Materials for RAPD technique

Equipment

1. Electrophoresis apparatus (BIO-RAD, USA)
2. Gel Documentation (SYNBIOSIS, UK)
3. Thermal Cycler (Perkin Elmer Gene Amp PCR System 2400, Perkin-Elmer Cetus Co., Norwalk, Connecticut, USA)
4. Nanodrop 2000 (Thermo Scientific, USA)

Chemical reagents

1. Agarose (Promega, USA)
2. Ammonium acetate (Merck, USA)
3. Boric acid (Merck, USA)
4. Cetyltrimethyl ammonium bromide (USB Cooperation, USA)
5. Chloroform (Merck, USA)
6. Deoxyribonucleoside triphosphates (Invitrogen, USA)
7. 100 to 3,000 bp DNA marker (Invitrogen, USA)
8. Ethidium bromide (AMRESCO. Inc., USA)
9. Ethyl alcohol (Merck, USA)
10. Ethylene diamine tetra-acetic acid (EDTA) (Biobasic. Inc., Canada)
11. EZ Load Precision Molecular Mass Standard(Thermo Scientific, USA)
12. Isopropanol (Merck, USA)

13. Isoamyl alcohol (Merck, USA)
14. Liquid nitrogen
15. Proteinase K (Invitrogen, USA)
16. RNase A (Thermo Scientific, USA)
17. Magnesium chloride (Invitrogen, USA)
18. PCR reaction buffer (Invitrogen, USA)
19. Polyvinyl pyrrolidone-40 (SIGMA CHEMICAL. CO., USA)
20. Primer (Operon Technologies Inc., USA)
21. Sodium chloride (Merck, USA)
22. *Taq* DNA polymerase (Invitrogen, USA)
23. Tris (hydroxyl methyl) aminomethane (Merck, USA)

DNA extraction

Young leaf was cut and individually kept in plastic bag, then placed in plastic box with ice. Individual plant leaf was cleaned and rinsed with distilled water. About 0.1 g of leaf was used. Four extraction buffers were tested, CTAB (cetyltrimethylammonium bromide) (Doyle and Doyle, 1990) and SDS (sodium dodecyl sulfate) in order to compare the extraction materials. After that, DNA was extracted using four different buffers. SDS buffer was followed Ichiro *et al.* (2013). SDS and CTAB buffer protocol was modified by adding 1% PVPP (polyvinyl pyrrolidone).

DNA extraction by CTAB buffer

Leaf tissue was ground in a mortar to give a fine powder form. The powder was mixed with 1 ml 2x CTAB or CTAB + 1% PVPP buffers and transferred into a 1.5 ml centrifuge tube. Next, 10 µL proteinase K, 1 mg/µL was added into the mixture and incubated at 60ºC for 30 min in a water bath, mixed every 10 min. Then, 500 µL 24 chloroform: 1 isoamyl alcohol was added into this mixture 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, mixed and then incubated at 4ºC overnight. Then the mixture was centrifuged at 10,000 rpm for 10 min. The supernatant was discarded. Precipitated was washed with 500 µL of wash buffer, which were included 10 mM ammonium acetate, and 75% of ethanol and centrifuged at 10,000 rpm for 5

min. The liquid was carefully thrown away and the pallet was air dried. After that, it was dissolved by 40 µL of TE buffer which was contained 10 mM Tris-HCl and 0.5 mM EDTA and 10 units of RNase A was added into the mixture, then, put it into incubator at 37ºC for 30 min. This DNA was diluted by 10 ng/µL distilled water (dH_2O). Finally, a total of 1 µL of this dilution was used for polymerase chain reaction (PCR) (Taywiya, 2010).

DNA extraction by SDS buffer

Leaf tissue was ground in a mortar to give a fine powder form. The powder was mixed with 1 ml 1x SDS or SDS + 1% PVPP buffers and transferred into a 1.5 ml centrifuge tube. Then, 500 µL 25 phenols: 24 chloroform: 1 isoamyl alcohol was added into this mixture and then put into the centrifuge at 13,000 rpm for 5 min. The liquid was transferred into a new tube and added with an equal amount of isopropanol, mixed and then incubated at 25 ºC for 1 minute. Then the mixture was centrifuged at 13,000 rpm for 5 min. The supernatant was discarded. Precipitated was added 95% of ethanol and centrifuged at 13,000 rpm for 5 min. After that, Precipitated was added 75% of ethanol and centrifuged at 13,000 rpm for 5 min. The liquid was carefully poured off and the pallet was air dried. After that, it was dissolved by 40 µL of TE buffer which was contained 10 mM Tris-HCl and 0.5 mM EDTA and 10 units of RNase A was added into the mixture, then, put it into incubator at 37ºC for 30 min. This DNA was diluted by 10 ng/µL distilled water (dH_2O). Finally, a total of 1 µL of this dilution was used for polymerase chain reaction (PCR) (Taywiya, 2010).

RAPD analysis

PCR was performedin a 20 µL reaction mixture including 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. One hundred forty primers, OPA01-20, OPC01- 20, OPD01-20, OPF01-20, OPG01-20, OPN01-20 and OPU01-20, were used for PCR amplification. DNA fragments were amplified in the thermal cycler (Perkin Elmer Gene Amp PCR System 2400, Perkin-Elmer Cetus Co., Norwalk, Connecticut, USA). The PCR program was conducted as Taywiya (2010). In the first step, one cycle of denaturation at 94ºC

for 180 sec was applied. The second step employed the following process: 94ºC for 90 sec, 43.6ºC for 45 sec and 72ºC for 60 sec for 40 cycles, with a final extension at 72ºC for 480 sec. The PCR products were stored at 4ºC prior to analysis.

PCR products were separated by 1.5% agarose gel electrophoresis in 1x TBE buffer at 60V. 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). Banding patterns were analyzed by Gene tool and Gene directory (Syngene, UK). Genetic distances were calculated using UPGMA (Unweighted pair group method with arithmetic averages) and PCoA (Principal coordinate analysis) method for clustering and drawing dendrogram.