CHAPTER III

MATERIALS AND METHODS

3.1 Materials

3.1.1 Plant materials

Dry leaf materials of 32 species of one plant representing each species were taken from the living collection held at the Royal Botanic Garden Edinburgh, UK. Scientific names of the voucher specimens were shown in Table 3. More leaf samples of another species, *Aeschynanthus* were collected in the wild from Doi Pui and San Ku in Chiangmai province (names of seven wild samples collected in Chiangmai province shown in Table 4). Field-work expeditions to collect wild samples were done twice. Photographs of *Aeschynanthus* were taken and their leaf samples were collected and kept in silica gel for further molecular analysis. Herbarium specimens were prepared and collected in Chiangmai herbarium (CMU) in Chiangmai University.

Additionally, six *Aeschynanthus* cultivars (Table 5) were brought from Jatujuk flee market, a plant-selling area in Bangkok. The Thai local name of *Aeschynanthus* (Wan Kai-dang) was used when asking plant sellers whether they sold this plant or whether they could give any other information. All of these six *Aeschynanthus*, one pot each, were brought from the market and kept in a grass house of the Botany Department, Chulalongkorn University, for further study. Species identification of each

wild and cultivated plant collected in Thailand was also perform using the key to species of Thai *Aeschynanthus* of Mary Mendum (RBGE, UK). One representative plant sample per species was used according to the nature of the target gene as they evolve fast enough to show the differences between taxa at the species level. Duplicated samples for analysis of each species were not necessary.

Table 3 The scientific names and RBGE accession numbers of *Aeschynanthus* species used in this study. Asterisks represent *Aeschynanthus* found in Thailand.

Taxon	Section	RBGE accession No
Aeschynanthus acuminatus [Wall. Ex] A. DC*	*Haplotricium*	19991444
Aeschynanthus andersonii C.B. Clarke*	Section *X*	19970465
Aeschynanthus burttii Mendum	N/A	20000562
Aeschynanthus burttii Mendum	N/A	20000536
*Aeschynanthus fecundus*P. Wood. *	*Polytrichium*	19672219
*Aeschynanthus fulgens*Wall. Ex] R. Br *	Section *X*	20002032
Aeschynanthus garrettii Craib*	*Microtrichium*	19750205
Aeschynanthus gracilis C.B. Clark*	Section *X*	19821970
Aeschynanthus hildebrandii Hemsl.*	*Haplotricium*	19991628
Aeschynanthus hookeri C.B. Clark *	*Diplotrichium*	19892128
Aeschynanthus hosseusii Pellegr.*	N/A	19611982
Aeschynanthus humilis Hemsl.*	Section *X*	19850473
Aeschynanthus irigaensis [Merr.] B.L.Burtt & P.Woods	*Microtricium*	19991999
Aeschynanthus aff. *javanicus*	*Aeschynanthus*	20010484
Aeschynanthus lineatus Craib*	*Diplotrichium*	19991622
Aeschynanthus longicaulis Wall. Ex R. Br.*	*Polytrichium*	20001430
Aeschynanthus macranthus [Merr.] Pellegr.*	Section *X*	19801140

Taxon	Section	RBGE accession No
Aeschynanthus malulidii Mendum	N/A	19980282
Aeschynanthus malulidii Mendum	N/A	19980283
Aeschynanthus parviflorus [D. Don] Spreng*	*Diplotricium*	19671067
Aeschynanthus aff. *parviflorus**	N/A	19672220
Aeschynanthus parvifolius R. Br.*	*Aeschynanthus*	19671069
Aeschynanthus radicans Jack*	*Aeschynanthus*	19672224
Aeschynanthus siphonanthus C.B.Clarke	N/A	N/A
Aeschynanthus aff. *siphonanthus*	N/A	N/A
Aeschynanthus sp.	N/A	20000557A
Aeschynanthus sp.	N/A	20002051
Aeschynanthus sp.nov.	N/A	2000 0512
Aeschynanthus speciosus Hook.*	N/A	19762988
Aeschynanthus superbus C.B.Clarke *	N/A	QBG 15899
Aeschynanthus tricolor X *parvifolius* (hybrid)	N/A	19715379
Aeschynanthus viridiflorus Teijsm. & Binn.	*Polytricium*	20021227
Aeschynanthus viridiflorus Teijsm. & Binn.	*Polytricium*	20000332

Table 4 The list of wild *Aeschynanthus* samples which were performed molecular identification analyses

Taxon	Collecting locality
Aeschynanthus sp. CM_007	San-Ku, Chiangmai
Aeschynanthus sp. CM_009	San-Ku, Chiangmai
Aeschynanthus sp. CM_013	San-Ku, Chiangmai
Aeschynanthus sp. CM_022	Doi Pui, Chiangmai
Aeschynanthus sp. CM_026	Doi Pui, Chiangmai
Aeschynanthus sp. CM_030	Doi Pui, Chiangmai
Aeschynanthus sp. CM_034	Doi Pui, Chiangmai

Table 5 The list of *Aeschynanthus* cultivated bought from Jatujak market and performed molecular identification

Taxon	Morphological characteristics
Aeschynanthus sp. JJ_001	It is climber perennials and epiphytes plant. It has flexuous pendulous green stems. The leaves are ovate thick and leathery and lightly green.

Taxon	Morphological characteristics
Aeschynanthus sp. JJ_002	It is creeping perennials and epiphytes plant. It has flexuous pendulous red stems. The leaves are ovate-elliptic, thick and leathery, lightly and dark green.
Aeschynanthus sp. JJ_003	It is creeping perennials and epiphytes plant. It has flexuous pendulous lightly green stems. The leaves are ovate to lance shaped, thick and leathery, lightly green and the smallest than the others.
Aeschynanthus sp. JJ_004	It is creeping perennials and epiphytes plant. It has flexuous pendulous lightly green stems. The leaves are ovate to lance shaped, thick and leathery, lightly green.
Aeschynanthus sp. JJ_005	It is subshrubs and epiphytes plant. It has dark green and warty stem. The leaves are ovate thick and leathery, lightly green and bigger than the others.
Aeschynanthus sp. JJ_006	It is a robust plant. The dark green leaves have hairs below and above. The stem are dark green.

3.1.2 Equipments

- Autoclave: model Conbraco (Conbraco Ind. Inc., USA)

- Automatic micropipette, P10, P20, P200 and P1000 (Gilson, France)

- Microcentrifuge: model Centrifuge 5410 (Eppendorf, Germany)

- Electronic UV transilluminator (Ultra lum Inc., USA)

- Electrophoresis chamber set: model Mupid (Advance Co., Ltd., Japan)

- Maxima ultra pure water: model Maxima UF (ELGA, England)

- Microwave oven: model Sharp carousel R7456 (Sharp, Thailand)

- PCR machine: model GeneAmp® PCR system 9700 (Applied Biosystem, Singapore)

- Polaroid camera: model Direct screen instant camera DS 34 H-34 (Peca products, UK)

- Power supply: model EC 5 70-90 LVD CE (E-C Apparatus corporation, USA)

- pH meter: model Cybersean 500 (Eutech Cybernatics, Singapore)

- Vortex: model MS I Minishaker (IKA-Works, Inc., USA)

- Light microscope (Olympus)

- Stereomicroscope (Olympus)

- Microscope slides

-Cover slip

- Waterbath

3.1.3 Chemicals

- DNeasy® plant minikit (QIAGEN UmbH, (Germany)

- Nucleospin® DNA minikit (Machery-Nagel, Finland)

- QIAquick® PCR purification kit (QIAGEN GmbH, Germany)

- QIAquick® gel extraction kit (QIAGEN GmbH, Germany)

- Bromophenol blue, $C_{19}H_{10}Br_4O_5S$, M.W. = 670 (Research organics,USA)

- EDTA (Ethylene diamine tetra-acetic acid), $C_{10}H_{14}N_2O_8Na_2.H_20$, M.W. = 372.24 (Bio Basic Inc, USA)

- Absolute Ethanol, CH_3CH_2OH, M.W. = 46.07 (Merck, Germany)

- Agarose gel (Research organics,USA)

- DNA Ladder Marker 100 bp (SibEnzyme, Russia)

- Taq DNA polymerase (Finnzyme, Finland)

- 99.5% (v/v) Glycerol ($C_3H_80_3$) M.W. = 92.10 (Research organics,USA)

- Sodium Hydroxide (NaOH) M.W. = 40 (Merck, Germany)

- Boric acid (Research organics, USA)

-Tris-base (Research organics, USA)

- Glacial acetic acid (Merck, Germany)

- Pectinase (Research organics, USA)

- Cellulase (Research organics, USA)

- 8-Quinoline crystalline (Sigma, Germany)

- Activated charcoal (Sigma, Germany)

- p-rosaline (Sigma, Germany)

- Sodium metabisulphite (Sigma, Germany)

- Carmine (Sigma, Germany)

- Hematoxylin

- Iron alum

- 10X TBE (Tris-base 108g, Boric acid 55g, 0.5 M EDTA (pH 8.0) 80 ml, distilled water upto 1 l)

- 6X loading dye (Glycerol 4 ml, Bromophenal blue 25 mg, 1X TBE upto 100)

- 4% pectinase (pectinase powder (prepared from *Rhizopus* sp.))

- 4% cellulose (cellulase powder (prepared from *Tricoderma viridis*.))

- Aceto-carmine (carmine powder 0.5g, Glacial acetic 48 ml, Distilled water 66 ml)

- Famer' solution (Glacial acetic acid 75% ml, 95% ethanol 25 ml)

3.1.4 Oligonucleotide primers

- ITS 1P, 2G, 3P, 4P (Biogenomed, USA)

- ITS 5P, 8P (Biobasic Inc., Canada)

- RAPD primer No. UBC001, No.UBC002 and No.UBC003 (University of Brithish Columbia, Canada)

3.2 Molecular phylogenetic experiments

3.2.1 DNA extraction

The genomic DNA extraction was performed using by QIAGEN Dneasy Plant Mini Kit to give rapid and high quality DNA extraction. The kit uses a spin column to isolate very pure DNA free from inhibitory contaminants. A leaf powder ground under liquid nitrogen was added with 400 μl of AP1 buffer and 4 μl of 100 mg/ml RNase A stock solution. The tube was vortexed and then incubated at 65 ^{o}C for 10 minutes, also mixed several times by inverting the tube during incubation. A 130 μl of buffer AP2 was added to the tube, mixed, and incubated for 5 minutes on ice. The lysate was applied to a QIAshredder spin-column set and centrifuged for 2 minutes. A flow-through fraction was transferred to a new tube and 1.5 volumes of AP3 added. A 650 μl of the mixture was applied to the DNeasy mini spin-column set, centrifuged for one minute, and flow-through discarded. The remaining sample was added to the spin column and centrifuged for another minute. The column was placed in a new tube and 500 μl of

buffer AW added. It was then centrifuged for another minute, another 500 µl of AW buffer added, and centrifuged for 2 minutes. The spin column was transferred to a new tube and 50 µl of 65 °C preheated buffer AE was pipetted onto the DNAeasy membrane. The column was incubated for 2 minutes at room temperature and then centrifuged for one minute. Another 50 µl of preheated buffer was added to elute the DNA. The centrifuged DNA was then stored in -20 °C freezer.

3.2.2 Agarose gel electrophoresis

Each extracted genomic DNA solution was checked by electrophoresis 0.8% (w/v) agarose gel in lx TBE buffer (0.05M Tris-HCl, O.05M Boric acid and 0.65M EDTA) as a running buffer. Electrophoresis was normally operated at 100 V for 30 min. DNA ladder marker 100 bp was used as a standard DNA marker. Loading sample composed of 5 µl of the extracted DNA and 1 µl of a loading dye (6x loading dye buffer: 0.25% bromophenol blue, 40% (v/v) glycerol and diluted in lx TBE running buffer). After that the gel was stained with ethidium bromide solution and destained in ultra pure water. The DNA band was then be visualised and photographed under UV light with a UV transilluminator.

3.2.3 PCR Amplification of ITS regions.

The polymerase chain reaction (PCR) technique described by Mullis and Faloona (1987) was used to make an in vitro amplification of an expected DNA segment. The principle of this method is separated into three steps as follows. The first process is termed "Denaturation" in which the reaction is heated to 94-96 ^{o}C in order to separate the two strands of the double stranded DNA template. Secondly, the reaction is cooled to 40-60 ^{o}C, which lets two oligonucleotide primers anneal to the single stranded DNA. This step is called "Annealing". The reaction is then heated to about 72 ^{o}C which is the optimal temperature for the synthesis of new DNA by the thermostable DNA polymerase. This third process is termed "Extension". The cycle of denaturation, annealing and extension is repeated for the required number of cycles and DNA continues to accumulate exponentially.

Before used, a PCR flow cabinet must be wiped down with 70% ethanol to prevent any cross-contamination. Double-stranded DNA of the ITS region was amplified using two primers (primers ITS 5P (forwards) and ITS 8P (reverse) in the case of complete ITS1-5.8s-ITS2 region; (see Table 6 for the primer sequences). Each PCR reaction contained 5 μl of 10x Taq polymerase buffer, 1 μl of a 10mM dNTP mix, 5 μl each of 10 μM primer 1 and primer 2, about 2 μl of genomic DNA template, 1U of Taq polymerase, and topped up with sterile distilled water to reach a total of 50 μl. When a number of reactions was performed, a master mix comprising all the reagents except the target DNA was prepared and aliquoted to the reaction tubes. DNA was thereafter

added as the last component before thermal cycling.

PCR amplification of the ITS region was carried out in 0.2 ml microcentrifuge tubes in a Perkin Elmer thermal cycler. Each PCR reaction cycle proceeded at follows: initial denaturation at 94 °C for 3 minutes; 30 Cycles of denaturation at 94 °C for 1 minute, annealing at 55 °C for 1 minute, and extension at 72 °C for 1.5 min.; and final extension at 72 °C for 5 min. The PCR products were detected by gel electrophoresis with 1.5% agarose gel with 1x TBE buffer at 60 - 80 Volts for about 2 hours.

Table 6 A list of the primers used in this study and their sequences (from Denduangboripant and Cronk, 2000; Möller and Cronk, 1997).

Primer name	Direction	Sequence (5´ to 3´)
ITS 5P	forwards	GGAAGGAGAAGTCGTAACAAGG
ITS 8P	reverse	CACGCTTCTCCAGACTACA
ITS 1P	forwards	TCCGTAGGTGAACCTGCGG
ITS 2G	reverse	GTGACGCCCAGGCAGACGT
ITS 3P	forwards	GCATCGATGAAGAACGTAGC
ITS 4	reverse	TCCTCCGCTTATTGATATGC

3.2.4 PCR-RAPD amplification

Primary PCR-RAPD amplification experiments were conducted on DNA samples of the three synonymous species (*A. andersonii, A. humilis*, and *A. hildebrandii*) to study their genetic relationship. The optimised reaction conditions for all RAPD experiments in this study are as follows: PCR reaction mixtures of 25 ul contained 20 ng of template

DNA, 2.5 U of Dynazyme thermostable DNA Polymerase and $MgCl_2$ optimised buffer (Finzyme, Finland), 2.5 mM of mix dNTP, and 12 ng of a single 10-mer primer obtained from the University of British Columbia (Canada). Their sequences were shown in Table 7. Amplifications were performed in a DNA Thermo-cycler (Perkin Elmer) programmed for 45 consecutive cycles each consisting of 1 min at 94°C, 2 min at 37°C and 2 min at 72°C. Following amplification, the samples were subjected to electrophoresis in 1.5% agarose gels, stained with 0.5 mg/ml of ethidium bromide and viewed under ultra-violet light.

Table 7 A list of the primers used in RAPD analysis and their sequences

Primer name	Sequence (5´ to 3´)
UBC 001	CCT GGG CTT C
UBC 002	CCT GGG CTT G
UBC 003	CCT GGG CTT A

3.2.5 Purification of the PCR products

Before sequencing the PCR products, any potential contaminants in the PCR mixture must be removed by purification. The purification was performed by using a QIAquick PCR purification kit as specified by the manufacturer. This kit is designed to separate either single- or double-stranded DNA ranging from 100 bp to 10 Kb from any contaminants, i.e. remaining primers, Taq DNA polymerase, unincorporated nucleotides and contaminated compounds introduced with the template DNA. Five volumes of buffer PB were mixed with one volume of the PCP product. The mixture was then

applied to a QIAquick spin column placed in a 2-ml collection tube, and centrifuged for 1 minute at 13000 rpm. The flow-through was discarded. A 0.75 ml of buffer PE was added to the column, which then was centrifuged for 1 minute at 13000 rpm. The flow-through was discarded again and the column was centrifuged for an additional 1 minute at 13000 rpm. The column was transferred to a new eppendorf tube and 50 μl of buffer EB or sterile distilled water was added to the center of the column, which was left standing for 1 minute before centrifuging for 1 min at 13000 rpm.

3.2.6 DNA cycle sequencing

The sequencing reaction was prepared according to the protocol of all PE Applied Biosystems thermal cyclers. About 500 ng of double-stranded DNA were used as template. Eight μl of terminator ready reaction mix was added to the sample. Then, it was followed by adding 3.2 pM of the forward primer or the reverse primer. Deionized water was added to the reaction to quantitate total volume to be 20 μl. The reaction was mixed well and spun briefly. The reaction tube was placed in a thermal cycler. DNA cycle sequencing was performing for 25 cycles as followed: 96°C for 10 sec, 50 °C for 5 sec, and 60 °C for 4 min. Then, the sample was stored at 4°C until the purification of extension products was performed. Purification of the extension product for sequencing was followed by the protocol of Centri-sep columns (Princeton separations, Inc.). Then, the sequencing was performed automatically by ABI prism 377.

3.2.7 Phylogenetic analyses

Before performing phylogenetic analyses, sequences of suitable outgroups must be added to determine the ancestral state (or polarity) of the homologous nucleotide characters. An outgroup taxon (or taxa) are known to lie outside the study group in question and are thus believed to be ancestral to the ingroup. Once a reliable phylogenetic tree has been produced based on a data set of characters properly rooted with an outgroup, one can determine the polarity or direction of evolution provided by the outgroup to analyze the patterns of character evolution in general. In this study, members of two other genera of the family Gesneriaceae were selected and used as outgroups: *Lysionotus* of the tribe Trichosporeae to which *Aeschynanthus* belongs and a member of the tribe Cyrtandreae, *Cyrtandra*, as a check in the unlikely event that *Aeschynanthus* proved to be paraphyletic with respect to *Lysionotus*. ITS sequences of the two outgroups were received from GenBank database. Additionally, *A. hildebrandii* sequences from GenBank was also used in these phylogenetic analysis to compare with our *A. hildebrandii* samples. The outgroups and additional *A. hildebrandii* sequences from GenBank used in the phylogenetic analyses were listed in Table 8.

Table 8 The list of outgroups and additional *Aeschynanthus* sequences retrieved from Genbank to use in these phylogenetic analyses.

Taxon	GenBank accession number	ITS 1 lenghts	ITS 2 lenghts	Reference
Aeschynanthus hildebrandii	AY 047040	227	262	Zimmer *et al.*, 2002
Crytandra baleyi	AF 349151, AF 349232	241	257	Denduangboripant *et al.*, 2001
Lysinotus forrestii	AF 349152, AF 349233	227	262	Denduangboripant *et al.*, 2001

Computational analyses of the obtained DNA sequence data were performed using following computer programs:

- **Chromas**: a program to check and compare the DNA sequence data received from sequencing procedure. The sequence data is then changed into FASTA format file before aligned using ClustalX program.

- **ClustalX**: a program to prepare a DNA data matrix by aligning all DNA sequences with each other (multiple alignment) until the homology of DNA data matrix becomes highest. The program splits each DNA sequence into small parts and compared all sequences by left-right moving alignment with penalty scoring. Then the data matrix was converted to a NEXUS file format before reconstructing a phylogenetic tree.

- **PAUP* (Phylogenetic Analysis Using Parsimony and other methods) version 4.0b10**: a program to reconstruct a phylogenetic tree. This programme works only with Macintosh PowerPC and mainly using maximum parsimony searching approaches to analyse the completely aligned data matrix.

Aligned ITS sequence matrices were analysed by the phylogenetic reconstruction program PAUP* version 4.0b (Swofford, 1998), with character states unordered and initially equally weighted. Polymorphic characters were treated as uncertain. Gaps were treated as missing values. Ambiguous regions in the aligned matrix were excluded from phylogenetic analysis. Indels were scored as a separate presence/absence character (0 was coded for an insertion and 1 for a deletion) and added to the sequence data matrix. Since the ITS analyses contained a large number of taxa (more than 20), the use of exact algorithms (exhaustive and branch-and-bound methods) became limited by the excessive amount of computing time needed. Therefore, heuristic approaches which are considered to be approximate and relatively fast methods were employed. Heuristic searches were used to find the most parsimonious trees. MULPARS (save all equally parsimonious trees), COLLAPSE (collapse any zero-length branches) and steepest descent (not abandon a round of swapping until all input trees from the previous round have been examined by the swapping algorithm) options were selected. ACCTRAN (accelerated transformation) was chosen for character-state optimisation, i.e. to prefer reversals to parallelisms.

When more than one most parsimonious tree was generated, strict and semistrict and 50% majority-rule consensus phylogenetic trees were computed. Bootstrap analysis ("fast" stepwise-addition) (Felsenstein, 1985) and jackknife analysis (Lanyon, 1985) with 50% deletion ("fast" stepwise-addition) were used as indications of statistic-supports for internal branches. Bootstrap and jackknife values were calculated using PAUP* set to heuristic search option with TBR swapping, 10,000 replicates respectively. Descriptive statistics reflecting the amount of phylogenetic signal in the parsimony analyses were given by branch length, the consistency index (CI; CI =*m/s*, where *m* is the minimum possible length of a tree based on the number of variant characters, *s* is the actual tree length; Kluge and Farris, 1969), retention index (RI; RI = *M-s/M-m*, where *M* is the maximum possible tree length; Farris, 1989), and the rescaled consistency index (RC; RC = CI x RI; Farris, 1989). The higher the values of these indices, the more congruent the characters in the data set are with each other and with the tree.

3.3 Cytogenetic experiments

3.3.1 Root preparation and collection

Healthy growing roots of six *Aeschynanthus* species (*A. hildebrandii, A. humilis, A. andersonii, A. obconicus, A. radicans, A.* sp. JJ_001) were collected at about noon of the day by cutting with a sharp scalpel or removing with a pair of fine forceps, then placed into a petri-dish filled with tap water. Adhering soil particles were removed with a

fine brush or by gentle rubbing. The root were transferred to distilled water. Then the root tips were collected and placed on a piece of filter/blotting paper to remove surface water. All roots were transferred to a vial and immersed simultaneously without delay into a pretreatment chemical included 8- hydroxyquinoline and distilled water. Note that the vial should be shaked well a few times and keep at 10°C for 5 hrs. during the pretreatment.

3.3.2 Chromosome staining and counting

- Staning by aceto-carmine using enzyme

First, pretreated root materials were transferred into a Farmer's Fluid fixative solution composed of 3 parts of ethanol to one parts of glacial acetic acid and incubated about 30 minutes at RT. After that, the roots were washed in water for several times. The next step was a hydrolysation which the sample was incubated in 5N HCl for 30-50 min at RT and agitated occasionally. Then, it was washed with water for 1 min to remove the acid. All materials were placed in Feulgen's reagent for 2 hrs, kept reagent corked. The roots were transferred into tap water and rinsed for 5-10 min several times. After that, repeatly rinsed in distilled water and kept in water while squashes were made. Then, the roots were transferred to 4% pectinase and 4% cellulose to be softened. Soft root tips were placed on a clean slide, their very small apical regions were dissected out. Excess water should be blotted out before dissecting. The apical meristem was added with a small drop of acito-carmine and the slide was heat gently by passing over a spirit lamp two or three times before examining under microscope. Finally,

the selected slide had to be sealed with nail varnish for long-term examination.

- Staining with hematoxylin

Small amounts pretreated *Aeschynanthus* roots were washed twice in distilled water for 5 min each. Under the stereomicroscope, regions actively growing or in miotic division, were transferred to a drop of 45% acitic acid on a microscope slide. The material was fragmented into pieces as small as possible using the find needles, then air dry for 30 min or longer. Dried slides were plunged into 5 N HCl at room temperature for 20 min then dried slids containing hydrolyzed material were immediately stained. To staining, a drop of aceto hematoxylin iron alum (4g hematoxylin plus 1g iron alum diluted in 100 ml 45% acitic acid) was added, and the specimen was covered with a coverslip and squashed. Cell analysis and photography were done using freshly stained slides.