# CHAPTER 3

# MATERIALS AND METHODS

## 3.1 Materials

### 3.1.1 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)

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### 3.1.2 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)
- α-bromonaphthaline (Fluka chemika, Switzerland)

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### 3.1.3 Plant materials

Plant materials of 16 from 17 *Cassia* species used in this study were collected from the wild or obtained as cultivated plants from recorded localities in Thailand. The plants were identified by Petchsri (2002) based on the key to species proposed by Larsen *et al.* (1984). Pressed and dried plant specimens were prepared as described in Boonkerd *et al.* (1987) and deposited at BCU. Another species, *Cassia grandis* L., was identified by Phadungrakwitya (2002) after compared with a herbarium specimen at the forest herbarium (the Royal Forest Department, Ministry of Agriculture and Cooperative, Bangkok) and its herbarium specimen was kept at the Department of Phamacognosy, Faculty of Phamacy, Mahidol University. Only one representative sample was sampled per species according to the nature of the target genes in this M.Sc. Thesis that they evolve in fast enough rates suitable for showing the differences between taxa at the species level. Thus, more duplicated samples for each species were not necessary. Scientific names of the voucher specimens were shown in table 3.

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**Table 3** Plant materials used in the study.

| Taxa | Voucher accession number |
|---|---|
| *Cassia alata* L. | *S. Pechsri 55* |
| *Cassia bakeriana* Craib | *S. Pechsri 53* |
| *Cassia fistula* L. | *S. Pechsri 51* |
| *Cassia garrettiana* Craib | *S. Pechsri 59* |
| *Cassia grandis* L. f. | BKF. No. 120511 |
| *Cassia hirsula* L. | *S. Pechsri 60* |
| *Cassia javanica* L. var. *javanica* | *S. Pechsri 54* |
| *Cassia leschenauitiana* DC. | *S. Pechsri 50* |
| *Cassia timoriensis* DC. | *S. Pechsri 77* |
| *Cassia obtusifolia* L. | *S. Pechsri 61* |
| *Cassia occidentalis* L. | *S. Pechsri 67* |
| *Cassia pumila* Lamk. | *S. Pechsri 57* |
| *Cassia siamea* Lamk. | *S. Pechsri 78* |
| *Cassia sophera* L. | *S. Pechsri 56* |
| *Cassia spectabilis* DC. | *S. Pechsri 66* |
| *Cassia surattensis* Burm f. | *S. Pechsri 79* |
| *Cassia tora* L. | *S. Pechsri 77* |

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## 3.2. Molecular phylogenetic experiments

### 3.2.1 DNA extraction

#### - Genomic DNA extraction using DNeasy® Plant Mini Kit

Dry leaf samples were grinded under liquid nitrogen with a motar and pestle. Tissue powder was transferred to a microcentrifuge tube. After that, 400 µl of lysis buffer AP1 and 4 µl of RNase A stock solution (100 mg/ml) were added to the tube mixed them by votexing vigorously, and then incubated at 65°C for 10 min. Buffer AP2 130 µl was added to the lysate mixture, incubated for 5 min on ice and centrifuged at maximum speed for 5 min The mixture was applied into a QIAshredder spin colunm and centrifuged at maximum speed for 2 min. A flow-through was transferred to a new microcentrifuge tube and 600 µl of buffer AP3/E was mixed with the cleared lysate by pipetting. Six hundred and fifty µl of the mixture was applied to a DNeasy mini spin column and centrifuged at a maximum speed for 1 min. The flow-through was discarded and the DNeasy column was placed in a new 2 ml collection tube. The remaining mixture was applied with this step. Five hundred µl of buffer AW was added. Then 500 µl of buffer AW was added to the DNeasy column again and centrifuged for 2 min to dry a membrane in the column. For DNA elution step, the DNeasy column was transferred to a microcentrifuge tube then 100 µl of preheated of buffer AE was added to elute DNA from the column. The column was incubated for 10 min at room temperature, centrifuged for 1 min at maximum speed to elute, and the DNA solution was kept at -20°C before used.

- **Genomic DNA extraction using Nucleospin® DNA mini kit**

There are 2 types of lysis bufier (C0 and C1) provided in each kit. The buffer Cl was chosen to use with these *Cassia* samples because it is suitable for legume plants such as *Pisum sativum*. After grinded 20 mg of dry tissue samples by a pestle and motar, 400 µl of buffer C1 was added to homogenise and lyse the tissue. Ten µl of RNase A solution was added and the mixture was then incubated for 30 min at 60°C. Centrifugation of the mixture was done at maximum speed for 5 min and 300 µl of the clear lysate was transferred to a new microcentrifuge tube. Three hundred µl of buffer C4 and 200 µl of absolute ethanol were added into the tube (C4 buffer and absolute ethanol must be premixed before used). The mixture was loaded into a provided 2 ml Nucleospin® Plant column, centrifuged for 1 min, and the flow-through was discarded. To wash a silica membrane in the column, it was firstly washed by 400 µl of buffer CW. About 700 µl and 200 µl of buffer C5 were then respectively used to wash a silica membrane for the second and third times. To dry the silica membrane completely, the column was centrifuged at maximum speed for 2 min. Finally, a highly pure genomic DNA was eluted from the membrane by adding 50 µl heated buffer CE twice and the eluted DNA solution was kept at -20°C before used.

- **Agarose gel electrophoresis**

Each extracted genomic DNA solution was checked by electrophoresis 0.8% (wlv) agarose gel in lx TBE buffer (0.05M Tris-HCl, O.05M Boric acid and 0.65M EDTA) as a running buffer. An 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

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milliQ water. The DNA band will then be visibled and photographed under UV light with a UV transilluminator.

### 3.2.2 Polymerase Chain Reaction (PCR)

PCR mixture was prepared by adding 2 unit of Taq DNA Polymerase (Finnzyme), lx Taq DNA Polymerase buffer with 1.5 mM $MgCl_2$ included (Finnzyme), 10 mM dNTP and 1 µM of each primer (forward and reverse), and the final volume was quantitated to be 50 µl by adding milliQ water. The PCR cycling profile to amplify a *trnL* intron from chloroplast genome was modified from Bruneau *et al.* (2001) and the profile for ITS regions of nuclear genome was followed White *et al.*. (1991)

The PCR programme to amplify *trn*L intron was: pre-denaturation at 95°C for 5 min; 35 cycles of denaturation at 94°C 1 min; annealing 55°C 1 min and extension 72°C 2 min; the final extension step was at 72°C for 7 min.

The PCR programme to amplify ITS regions was: pre-denaturation at 94°C 3 min; 30 cycles of denaturation 94°C 1 min, annealing 55°C 1 min and extension 72°C 1 min 30 sec; the final extension was at 72°C for 5 min. After PCR amplification completed, an electrophoresis loading sample was prepared by mixing 5 µl of PCR product with 1 µl of loading dye. An electrophoresis was performed on 0.8% agarose gel at 100 V for 30 min One hundred base pair DNA ladder marker was used as DNA standard marker. The gel was then stained with ethidium bromide and visualised on a UV transilluminater.

### 3.2.3 Purification of PCR products

A PCR product was purified following the protocol of QIAquick PCR purification kit. Two hundred and fifty µl of buffer PC were added to a 50 µl PCR reaction. A QIAquick spin column was placed into a provided 2 ml collection tube and

the sample was transferred into the column before centrifuged for 30-60 sec. After that, a flow-through was discarded and the QIAquick column was put back into the same collection tube. The column was washed with 0.75 ml buffer PE and centrifuged for 30-60 sec; then the flow-through was discarded. The column was centrifuged again at maximum speed for 1 min before placed into a clean 1.5 ml microcentrifuge tube. The column was filled with 15 µl milliQ water and spun for 1 min, twice. A purified DNA was stored at -20°C before used. PCR product was checked by 0.8% (w/v) agarose gel before sequenced. Forward and reverse primers of *trnL* intron and ITS used to sequence the products are listed in the table 3.

**Table 4** sequences of primers in this experiment

| primer name | Nucleotide sequence (in 5' to 3' direction) |
|---|---|
| *trn*L-C (Forward) | 5' cga aat cgg tag acg cta cg 3' |
| *trn*L-D (Reverse) | 5' ggg gat aga ggg act tga ac 3' |
| 5p ITS (Forward) | 5' gga agg aga agt cgt aac aag g 3' |
| 8p ITS (Reverse) | 5' cac gct tct cca gac tac a 3' |
| 4p ITS (Reverse) | 5' tcc tcc gct tat tga tat gc 3' |

For some problematic PCR samples which contain a lot of primer dimers generated from overloaded primers in the PCR mixture, the samples should be purified using QIAquick gel extraction method before sequenced. The PCR product was separated from primer-dimers on 0.8% agarose gel electrophoresis with 25 min running. The PCR band was exised out using a clean and sharp scalpel. One ml and 200 µl of buffer QG was added to 400 mg of sliced agarose gel. Then the mixture

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was incubated at 50°C for 10 min or until the sliced gel was completely dissolved. After that, the colour of the mixture was checked wheather still yellow and having high efficiency of DNA absorbed to the QIAquick membrane. The mixture was applied to a provided QIAquick column and spun for 1 min. A flow-through was discarded. All traces of agarose were removed by adding 500 µl of buffer QG and 1 min centrifugation. Seven hundred and fifty µl of buffer PE was used to wash the silica membrane. The flow-through was discarded and the column was centrifuged again to dry the membrane. The column was transfered into a new and clean 1.5 ml microcentrifuge tube. Purified PCR fragment was eluted with 15 µl of milliQ water and centrifuged for 1 min, twice. The eluted PCR product stored at -20°C before sequencing.

**3.2.4 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 *trn*L-C or ITS-5P as the forward primer or *trn*L-D or ITS-8P as 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 perform 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.). Briefly, the column was tapped shortly to allow dry gel to move to the bottom part of the column. Eight hundred µl of milliQ $H_2O$ was added to the column which then was tapped to get off

air bubbles. The column was incubated at RT for 2 hours. Then, a cap and a stopper of the column was removed. The column was transfered to a 2 ml wash tube to drain milliQ $H_20$ by gravity and the flow-through was discarded. Later, the column was spun at 2500 rpm at RT for 2 min. The flow-through was discarded again. Twenty µl of sample from above was loaded onto the centre of the column. Then, the column was placed in a 1.5 ml tube. It was spun at 2500 rpm at RT for 2 min Then, the column was discarded. The elution was dry by speed vacuum for 20-30 min The solution (3 µl) of sequence dry and formamide at the ratio of 1:5 was added to the sample. The mixture was then heat at 95 °C for 2 min and chilled in ice. The mixture tube was wrapped with aluminium foil to avoid the light exposure. Then, the sequencing was performed automatically by ABI prism 377.

#### 3.2.5 Phylogenetic analyses

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

- **Chromas:** It is a PC computer 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:** It is a PC computer 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:** It is a Macintosh computer 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.

These are samples of commands used in PAUP* analyses:

| Command | Meaning |
|---|---|
| #nexus | * data matrix is converted into nexus format |
| begin data | * beginning data to analyses |
| ; | * ending for each command |
| dimension ntax=n nchar=m | * taxa=n taxa and character=m characters |
| format missing=? | *symbol "?" means that such nucleotide position is noavailable to analyse |
| format gap = - | * symbol "-" represents an insertion or a deletion in the data set |
| format matchar = . | * symbol "." represents related sequence data |
| format datatype=dna | * DNA sequence data was used in the analysis |
| end | * ending the procedure |

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**3.3 Cytogenetic experiment** (Jong, 1997)

**3.3.1 Root preparation and collection**

Healthy growing roots 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 distilled water. Then the root tips were collected and transferred to a piece of filter/blotting paper to remove surface water. All roots were transferred to a vial and must be immersed simultaneously without delay into a pretreatment chemical included α- bromonapthaline and distilled water. Note that the vial should be shaked well a few times during the pretreatment.

**3.3.2 Chromosome staining and counting**

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 45% acetic acid 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.

**3.3.3 Meiotic study in Germline cells** (Jaiyasuta, 1989: Chulalaksananukul *et al.*, 2001)

Young flower buds were collected with different sizes. Then the flowers were fixed in a Farmer's fluid overnight. Samples were washes in absolute or 95% ethanol for 3 times, 5 min each time. After that, the fixed flowers were kept in a refrigerator. These process could maintain samples for 6-12 months. For meiotic study, a pollen mother cell (PMC) was put onto a slide, then broken it by a needle to get microsporocytes out of the PMC. Debrises were removed from the slide and propionocarmine was added. The slide was heated briefly to enlarge chromosomes. A cover glass was put on the slide for chromosome study under microscope.

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