# CHAPTER 3

# MATERIALS AND METHODS

## 3.1 Materials

### 3.1.1 Fungal isolates

3.1.1.1 *H. scutata* strains used in this study were isolated from fresh stromatal specimens collected from Sirindhorn Peat Swamp Forest.

3.1.1.2 *H. schizostachyi* NHJ 4547 was kindly provided by Dr. Nigel L. Hywel-Jones.

3.1.1.3 *H. discoidea* NHJ 5004 used was also provided by Dr. Nigel L. Hywel-Jones.

### 3.1.2 Chemicals

Media (Appendix 1 and 2)

- Agarose gel
- Potato dextrose agar (Difco)
- Malt extract agar (Difco)
- Corn meal agar (Difco)
- Glucose yeast extract agar (Difco)
- Potato dextrose broth (Difco)
- Malt extract broth (Difco)

Antibiotics

streptomycin, penicillin G and chloramphenicol (Sigma)

Other chemicals

- 95% ethanol
- glacial acetic acid (Merck)
- NaCl (Meark)

- Tris (Sigma)
- agarose gel
- EDTA (Sigma)

### 3.1.3 Equipments

| Equipments | Company |
|---|---|
| Autoclave machine | Tomy SS-320 |
| Microtome | Triangle Biomedial Science |
| Scanning electron microscope | JSM-5800 LV |
| Compound microscope | Olympus |
| Stereo microscope | Olympus |
| Hot air oven | Memmert |
| Incubators | Memmert, Precision scientific |
| Laminar air flow cabinet | Nuaire |
| Refrigerators | National, Sanyo |
| Rotary shaker | Forma Scientific |
| Water bath | Memmert |
| Balance | Sartorius |
| Centrifuge | Eppendorf |
| Microwave ovens | Sanyo, LG |
| pH meter | Sartorius |
| Gel doc | BIO-RAD |
| UV-transilluminator | Herolab |
| Gel electrophoresis machine | Wealtec |
| Micropipettes | Rainin, Eppendorf |
| Vortex | Genie |
| PCR machine | GeneAmp |
| Spectophotometer | Eppendorf |

### 3.1.4 Other apparatus

- fine insect pins
- glass slides and cover slips
- plastic boxes
- marker pens
- Vernier caliper
- Plastic bags
- rubber gloves
- ice box
- plastic tips
- 1.5 ml microcentrifuge tubes
- PCR tubes

## 3.2 Collection of specimens

### 3.2.1 Survey sites:

Sirindhorn Peat Swamp Forest, Narathiwat
Bala-Hala Wildlife Sanctuary, Narathiwat
Khao Luang National Park, Nakhon Srithammarat
Nam Noa National Park, Petchabun
Ton Nga Chang Wildlife Sanctuary, Songkhla
Khao Yai National Park, Saraburi
Doi Inthanon National Park, Chiang Mai

### 3.2.2 Sample collection:

Collections of *H. scutata* and *H. schizostachyi* were randomly made from *Syzygium* leaves and bamboo twigs and placed in plastic boxes and the

date of collection was recorded. Samples were returned to the Mycology Laboratory, BIOTEC and isolated into pure culture.

### 3.2.3 Isolation of fungi

Potato dextrose agar (PDA) with added antibiotics (streptomycin, penicillin G and chloramphenicol) was used as a standard medium for isolation (Appendix 1). Isolations were made by ejection of ascospores from the fresh mature stroma on PDA plus antibiotics. Isolations from water releasing ascospores were performed when the first isolation method failed.

#### A. Ejection of ascospores from stroma

The mature stromata were stuck on the lid using petroleum jelly or vaseline and covered on the PDA. The plates were incubated at room temperature overnight for shooting of ascospores from fresh mature stroma. The lid with stromata was removed and a new sterile lid replaced. The plates were then incubated at 25°C in a moisture chamber until ascospore germination was observed. Fungal isolation was made by hyphal tipping and transferred to new PDA.

#### B. Isolation from water releasing ascospores

The mature stromata were dipped into sterile water to release ascospores for 15-30 minutes. The ascospore suspension was streaked on PDA. The plates were incubated at 25°C in a moisture chamber until ascospore germination was observed. Fungal isolation was made by hyphal tipping and transferred to new PDA.

## 3.3 Morphological studies

### 3.3.1 Macroscopic structures

The fungal stromata were observed with the naked eye and under stereomicroscope and photographed. The stroma size was measured using Vernier caliper (±0.1 cm).

### 3.3.2 Microscopic structures

Mature fungal stromata were sectioned by using a Microtome. The morphological structures were observed and photographed using light and scanning electron microscopes (SEM).

**Preparation of fungal specimens for SEM**

The fungal stromata and fungal colonies were cut vertically from the edge of the stroma or colony to the central part into 3 parts in triplicate. The fungal samples were treated before observing under SEM.

(1) Primary fixation

The fungal samples were soaked in 2.5% glutaraldehyde for 2 hours. Samples were washed with 0.1 M phosphate buffer, pH 7.3 for 5 minutes, 3 times.

(2) Post fixation

The fungal samples were soaked in 1% $OsO_4$ for 1 hour and washed with 0.1 M phosphate buffer, pH 7.3 for 10 minutes, 2 times.

(3) Dehydration

The fungal samples were dehydrated in 50%, 70%, 80% and 90% ethanol each 2 times for 15 minutes and finally in absolute ethanol for 30 minutes, twice. The samples were dried thoroughly by using SAMDRAI (Polaron CPD7501 Critical Point Drier). After that the samples were stuck on the brass stub and coated with gold by the ion sputter method using a SPI-MODULE Sputter Coater. The treated fungal samples were observed under the JSM-5800 LV scanning electron microscope at Science Equipment Center, Prince of Songkla University.

### 3.4 Distribution of *H. scutata* in experimental *Syzygium* plantation

The distribution of *H. scutata* was studied at an experimental *Syzygium* plantation in front of Sirindhorn Peat Swamp Research Center, Narathiwat by sampling the *Sygygium* leaves (*S. oblatum* and *S. tumida*) from three height levels (high, middle, low) of branches. The number of leaves and observed stromata in each branch were recorded. The data were analysed by statistical method ($\chi^2$ test) to determine the relationship between plant species and the level of branch to the *H. scutata* stromata occurrence.

### 3.5 Effect of media and temperature on the growth of fungi

*H. scutata* and *H. schizostachyi* isolates were determined the growth conditions in the laboratory. The fungal isolates were subcultured onto PDA and incubated at room temperature. Fungal plugs (3 mm diameter) were removed from the peripheral edge of the colonies and inoculated onto four medium plates, PDA (Difco), CMA (Difco), GYA (Difco) and MA (Difco) (Appendix 1). The incubation temperatures were 20°C, 25°C, 30°C and 35°C.

Each set of experiments was performed in triplicate. The diameter of colonies was measured every week up to 2 months for growth study.

### 3.6 Cultivation and harvesting of fungi for molecular studies

Five pieces of fungal mycelia were inoculated into 50 ml of potato dextrose broth, PDB (Difco) and malt extract broth, MEB (Difco) and incubated on a rotary shaker (200 rpm) at 25°C for 3-4 weeks. Contamination of cultures was checked by microscopic examination of growth on PDA. The list of fungi used in this study was shown in Appendix 2.

The fungal biomass was harvested by filtration using sterile gauze. The fresh mycelium was washed thoroughly with sterile warm water and ground in liquid nitrogen using a mortar and pestle.

### 3.7 DNA extraction

#### 3.7.1 CTAB DNA extraction method

The method used was a modified protocol from O' Donnell *et al.* (1997). The fresh 3-4 week old mycelium was ground with liquid nitrogen for 10-15 minutes in a sterilised mortar. The ground mycelium was transferred to 1.5 ml micro-centrifuge tube and resuspended in 400 µl CTAB extraction buffer (Appendix 3) and mixed. This was incubated for 30 minutes at 70°C. The suspension was centrifuged at 12,000 rpm for 5 minutes. The supernatant was transferred to the new tube, with an equal volume of phenol: chloroform: isoamyl alcohol (25:24:1) added and mixed gently. The emulsified solution was centrifuged at 12,000 rpm for 5 minutes. The supernatant was transferred to the new tube, with an equal volume of chloroform: isoamyl alcohol (24:1)

added and mixed gently. The upper aqueous layer was transferred to the new tube. The 0.1 volume of sodium acetate (3M) and 2.5 volume of absolute ethanol were added. The suspension was mixed gently and kept at -20°C for 30 minutes or overnight. The sample solution was then centrifuged at 4°C, 10,000 rpm for 10 minutes. The absolute ethanol was removed. The DNA pellet was washed by 70% ethanol for 2-3 times. After removal of ethanol, the DNA pellet was air-dried with the cap-open. Finally, the DNA was suspended in 30 µl nanopure water, checked for quality and quantity in a 1% agarose gel. The DNA was stored at 4°C.

### 3.7.2 Microwave DNA extraction method

The method used was a modified protocol from White *et al.* (1997). The fungal stroma was cut using a sterile blade to open the ascomata. The ascomata were taken using fine forceps and a needle. The ascomata were placed into a 1.5 micro-centrifuge tube containing 400 µl CTAB extraction buffer. The tube was sealed with plastic wrap without cap. The suspension was microwaved for a total of 30 seconds (15s-10s-5s) on full power (800 Watt). The suspension was incubated at 70°C for 30 minutes or overnight. This was centrifuged at 12,000 rpm for 5 minutes.

DNA extraction was further performed as described in 3.6.1.

### 3.7.3 Quick and dirty method

This method was modified from Wang *et al.* (1993). Fungal aerial mycelium was scraped off the agar surface with fine forceps with care to avoid inclusion of agar in the DNA extraction procedure. A few milligrams of fungal

mycelium was placed into a 1.5 micro-centrifuge tube. Ten µl of 0.5 N NaOH was added to every mg of mycelium. The fungal mycelium was ground until no large mycelia were left. Two µl of the suspension was transferred quickly to a new tube containing 198 µl of 100 mM Tris pH 8.0 and mixed well. One µl of suspension was used directly for polymerase chain reaction.

### 3.7.4 QIAGEN$^{R}$ Dneasy Plant DNA Extraction Kit

The fungal DNA was extracted using this kit following the manufacturer's instructions. The ground mycelium was resuspended in 400 µl extraction buffer plus 4 µl of RNase A stock solution (100 mg/ml) and vortexed vigorously. The suspension was incubated at 65°C for 10 minutes. The lysate was added to the purification buffer and incubated on ice for 5 minutes. The lysate was loaded into QIAshredder spin column sitting in a 2 ml collecting tube and centrifuged at 10,000 rpm for 2 minutes. The flow-through fraction was transferred to a new tube. The 1.5 volume of precipitation buffer was added into the clear lysate and mixed gently. The mixture was applied to Dneasy mini spin column sitting in a 2 ml collecting tube and centrifuged at 10,000 rpm for 1 minute. The flow-through was discarded. Dneasy column was added by the washing buffer and centrifuged at 10,000 rpm for 2 minutes. This step was performed in duplicate. The column was added with the elution and incubated for 5 minutes at room temperature. Finally the Dneasy column sitting in a new 2 ml collecting tube was centrifuged at 10,000 rpm for 1 minute to elute the DNA from the column The DNA was stored at 4°C.

### 3.7.5 Neucleospin$^{R}$ plant DNA extraction kit

The fungal DNA was extracted using this kit following the manufacturer's instructions. The ground mycelium was resuspended in 400 µl

extraction buffer and incubated at 70°C for 30 minutes. The suspension was centrifuged at 10,000 rpm for 5 minutes. The clear lysate was transferred to the new tube. The precipitation buffer was added and mixed well. The mixture was loaded into the Neucleospin column and centrifuged at 10,000 rpm for 1 minute. The flow-through was discarded. The washing buffer was added to the column. The column was centrifuged at 10,000 rpm for 2 minutes. This step was performed in duplicate. The column was added with the elution buffer and incubated at room temperature for 5 minutes. The column sitting in a new 2 ml collecting tube was centrifuged to elute the DNA. The DNA was stored at 4°C.

### 3.7.6 Neucleospin$^{R}$ tissue DNA extraction kit

The fungal DNA was extracted using this kit following the manufacturer's instructions. The fresh mycelium was resuspended in 400 µl extraction buffer and incubated at 70°C for 30 minutes. The suspension was centrifuged at 10,000 rpm for 5 minutes. The clear lysate was transferred to the new tube. The precipitation buffer was added and mixed well. The mixture was loaded into the Neucleospin column and centrifuged at 10,000 rpm for 1 minute. The flow-through was discarded. The washing buffer was added to the column. The column was centrifuged at 10,000 rpm for 2 minutes. This step was performed in duplicates. The column was added with the elution buffer and incubated at room temperature for 5 minutes. The column was centrifuged to elute the DNA. The DNA was stored at 4°C.

## 3.8 Measurement of DNA concentration

The concentration of the extracted genomic DNA was measured by ultraviolet absorbance spectrophotometer at 260 nm. An absorbance of 1.0 is equivalent to 50 µg/ml for double-stranded DNA or 35 µg/ml for single-

stranded DNA. Purity of DNA solution was also estimated by the ratio of the absorbance at 260 nm (absorbance for DNA) and 280 nm (absorbance for protein). Non-contaminated DNA should have a ratio of 1.8-1.9. If the ratio is less than 1.8-1.9, it is contaminated with protein or phenol (Brown, 1993 & 1994)

### 3.9 DNA purification

Fungal DNA was purified using a Neucleospin $^{R}$ Extract DNA purification kit (Macherey-Nagel) following the manufacturer's instructions. The PCR product sample was mixed with the binding buffer and transferred into the Neucleospin column sitting in a 2 ml collecting tube and centrifuged at 10,000 rpm for 1 minute. The flow-through was discarded. The washing buffer was added to the column. The column was centrifuged at 10,000 rpm for 1 minute. This step was performed in duplicate. The flow-through was discarded. The column was added with the elution buffer and incubated at room temperature for 1 minute. The column sitting in a new 2 ml collecting tube was centrifuged to elute the DNA. The purified DNA was directly used for PCR.

### 3.10 Fungal rDNA amplification

#### 3.10.1 Reagent for PCR

DyNazyme II DNA Polymerase PCR kit (Finnzymes)

Figure 3.1 shows the positions of primers on the fungal nuclear ribosomal DNA. Primers for PCR amplification and rDNA sequencing are listed in Table 3.1.

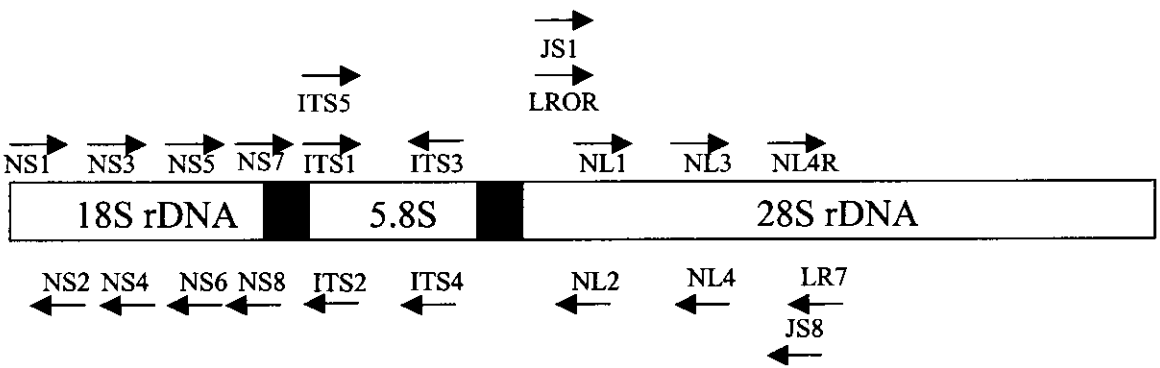


**Figure 3.1** The positions of primers on the fungal nuclear ribosomal DNA

**Table 3.1** Lists of the primers for fungal rDNA amplification (sequence from 5' to 3')

| Primer name | Gene sequence (5' to 3') | Length (base) |
|---|---|---|
| **Nuclear small rDNA (18S)** | | |
| **NS1** | **GTA GTC ATA TGC TTG TCT C** | **19** |
| NS2 | **GGC TGC TGG CAC CAG ACT TGC** | 21 |
| NS3 | **GCA AGT CTG GTG CCA GCA GCC** | 21 |
| NS4 | **CCT CCG TCA ATT CCT TTA AG** | 20 |
| NS5 | AAC TTA AAG GAA TTG ACG GAA G | 22 |
| **NS6** | **GCA TCA CAG ACC TGT TAT TGC CTC** | **24** |
| NS7 | **GAG GCA ATA ACA GGT CTG TGA TGC** | 24 |
| **NS8** | **TCC GCA GGT TCA CCT ACG GA** | **20** |
| **Nuclear ITS** | | |
| **ITS1** | **TCC GTA GGT GAA CCT GCG G** | **19** |
| ITS2 | GCT GCG TTC TTC ATC GAT GC | 20 |
| ITS3 | GCA TCG ATG AAG AAC GCA GC | 20 |
| **ITS4** | **TCC TCC GCT TAT TGA TAT GC** | **20** |
| ITS5 | **GGA AGT AAA AGT CGT AAC AAG G** | 22 |
| **Nuclear large rDNA (28S)** | | |
| **JS1** | **CGC TGA ACT TAA GCA TAT** | **18** |
| **JS8** | **CAT CCA TTT TCA GGG CTA** | **18** |
| NL1 | GCA TAT CAA TAA GCG GAG GAA AAG | 24 |
| NL2 | CTC TCT TTT CAA AGT TCT TTT CAT CT | 26 |
| NL3 | AGA TGA AAA GAA CTT TGA AAA GAG AG | 26 |
| NL4 | GGT CCG TGT TTC AAG ACG G | 19 |
| NL4R | CCG TCT TGA AAC ACG GAC C | 19 |
| **LR7** | **TAC TAC CAC CAA GAT CT** | **17** |
| **LROR** | **ACC CGC TGA ACT TAA GC** | **17** |

### 3.10.2 Amplification of fungal rDNA by PCR

Ribosomal DNA (rDNA) was amplified using the polymerase chain reaction (PCR) in a GeneAmp$^R$ PCR System 9700. Primers NS1, NS6 and NS8 were used to amplify the small subunit rRNA (18S rDNA). The large subunit rRNA Primers (28S rDNA) was amplified by primers LROR, LR7, JS1, JS8, ITS5. As well as the Internal Transcribed Spacers was amplified with ITS1, ITS4 and ITS5. The amplification cycles were performed.

#### A. Preparation of PCR mixture

A master mix was prepared at each PCR reaction, each 0.2 ml sterile reaction tubes contained (total 50 µl):

| **Reagents** | **Volume added (µl)** |
|---|---|
| 10X PCR buffer with 15 mM $MgCl_2$ | 5.0 |
| 10 mM dNTP mix | 1.0 |
| 10 mM upstream primer | 1.0 |
| 10 mM downstream primer | 1.0 |
| *Taq* DNA polymerase | 0.5 |
| Sterile MilliQ $H_2O$ | 39.5 |
| Template DNA | 2.0 |

#### B. PCR procedure

The ingredients were mixed as described. The mixture was put into a 0.2 ml PCR tube and placed on GeneAmp$^R$ PCR System 9700 and run following the PCR conditions.

### C. PCR conditions

The following PCR cycling parameters were employed for the amplification of 5.8S rDNA and flanking ITS and 28S DNA.
The PCR cycle was as follow:

| | | |
|---|---|---|
| Step 1: | 94 °C | 2 minutes |
| Step 2: | 94 °C | 1 minute |
| Step 3: | 58 °C | 1 minute |
| Step 4: | 72 °C | 2 minutes |
| Step 5: Go to Step 2 for additional 34 cycles | | |
| Step 6: | 72 °C | 10 minutes |

## 3.11 Detection of DNA and PCR products by agarose gel electrophoresis

### 3.11.1 Running condition

The 10 µl DNA sample including 1 µl loading buffer were loaded into the wells of the 1 % agarose gel in the gel tank. The gel was run at 85-100 volts and maximum current (400 mA). The electrophoresis process was stopped until the loading dye had migrated to two third of the agarose gel.

### 3.11.2 Visualisation of DNA

After gel electrophoresis, the agarose gel was stained by soaking in 1xTAE containg ethidium bromide (0.5µg/ml). The DNA fragments were observed by placing the agrose gel into a Bio-Rad Gel Doc 1000 UV illuminator connected to a computer with the software molecular analyst. The intensity of the bands was noted and photographs were taken.

### 3.12 DNA sequencing

The PCR products were purified as described in section 3.8 and sent to BIOTEC Service Unit (BSU) for DNA sequencing.

### 3.13 Alignment

The nucleotide sequences of all selected species were aligned using CLUSTAL W software (Thompson *et al.*, 1994) and the BioEdit programme. The sequences were then manually aligned for the phylogenetic tree construction.

### 3.14 Phylogenetic tree construction

After manual adjustment, maximum-parsimony (MP) phylogenetic trees were constructed using the PAUP* 4.6.2 program (Swofford, 1998). Heuristic search was used initially with the consistency index (CI), the retention index (RI) and the rescaled consistency index (RC) calculated. The characters were reweighted and the heuristic search was re-run until the RC value remained unchanged. Finally, bootstrap tests (Felsenstein, 1981, 1985) from 100 replicates were constructed on each supported branch. Group receiving more than 70% bootstrap support are reasonably well supported by the data (Berbee, 1995).