CHAPTER 3

MATERIALS AND METHODS

Sample Sources

A survey and collection of powdery mildew fungi were made in the Northern provinces of Thailand i.e., Chiang Mai, Chiang Rai, Nan, Lampang and Maehongsorn. These collected specimens were observed under microscope for morphological identification. Fungal species, host plants, locations of collection, and voucher number are given in Table 1. Some parts of these specimens were used for sequencing of rDNA ITS region. The last parts were preserved as herbarium specimens and deposited in the Mie University Mycological Herbarium (MUMH), Japan.

Light Microscopy of Fresh Materials

Anamorphic state,

Hyphae, conidiophores, and conidia on fresh materials were stripped off the leaf surfaces with clear adhesive tape, mounted on a microscope slide with the fungal

mycelium uppermost, then examined in water using light microscopy with phase contrast objective lens at 20x, 40x, and 100x magnifications. The following information was noted during the examination of the fresh specimens: Size and shape of conidia, presence or absence of fibrosin bodies, conidiogenesis, conidiophores. (size and shape of foot cell), position of the basal septum, shape and position of hyphal appressoria and the shape of appressoria. Thirty conidia were measured for each examined specimens.

Observations of conidial germ tubes were carried out using the method of Hirata (1942). The inner surface of the onion was cut with a razor to a blade scale of 1 cm^2 and then stripped off by clean forceps. The cell layer was kept in 80% ethanol for more than 2 weeks and rinsed with tap water for 30 minutes before use. The cell layer was put on a microscope slide, then removed the excess water with filter paper, and inoculated with the conidia. The inoculated cell layer was floated on distilled water in a Petri dish and incubated at 20°– 25°C for 24 hours before having microscopic observation.

Teleomorphic state

Chasmothecia on specimens were transferred to a microscope slide using a micro needle. This was conducted under a stereo microscope, and observed in 3% NaOH under a compound microscope. The following informations were noted during the examination: Size and shape of chasmothecia, asci, and ascospores, characteristics of appendages (number, length, color, shape of the apex), number of asci and ascospores.

DNA Extraction

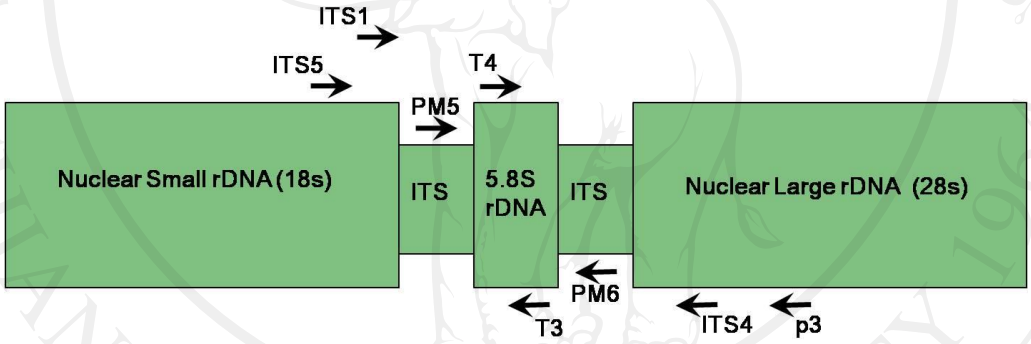
Whole-cell DNA was isolated from fresh fungal specimens or herbarium specimens by the chelex method (Walsh *et al.*, 1991; Hirata and Takamatsu, 1996). Several hundreds of conidia or about 20 chasmothecia were added to 300 µl of 5% Chelex (Bio-Red) in a 1.5-ml microcentrifuge tube and incubated at 56°C for several hours. After stirring or shaking, the extract was incubated in a boiling water bath for 8 min. The extract was mixed vigoriously again and then centrifuged at 15,000 x *g* for 5 min.

Amplification of rDNA and Phylogenetic Analysis

The nuclear rDNA regions were amplified by the polymerase chain reaction (PCR) using nest primers sets. PCR reactions were conducted in a total reaction of 50 µl, including the following reagents: H_2O 28.8 µl; 10X PCR buffer (100mM Tris-HCl, pH 8.3, 500 mM KCl, 15 mM $MgCl_2$) 5 µl; 2.5 mM of each deoxyribonucleotide triphosphate 4 µl; two primer set (20 pmol/µl) 1 µl; Taq DNA polymerase (5 unit/µl) 0.2 µl and DNA 10 µl. A negative control-lacking template DNA was included for each set of reactions. The following thermal cycling conditions were performed in a PCR thermal cycler SP, an initial denaturing step at 95ºC for 1.5 min, thermocycling for 30 cycles, where each cycle took 30 sec at 95ºC followed by 30 sec at 52ºC for annealing and 30 sec at 72ºC for extension and a final extension cycle of 6.5 min at 72ºC. One microliter of the first reaction mixture was used for the second amplification with the partial nested primer set. Components of the reaction mixture and the thermal cycle conditions for the PCR product from the second amplification were the same as for the first PCR.

The rDNA internal transcribed spacer (ITS) region including 5.8S rDNA was amplified using primers ITS5 (White *et al*., 1990) and p3 (Kusaba and Tsuge, 1995) for the first amplification. The ITS5/p3 fragment was subjected to the second amplification using powdery mildew specific primer sets ITS5/PM6 and PM5/p3 according to the procedure of Takamatsu and Kano (2001). The ITS5/PM6 and

PM5/p3 fragments were sent to SolGent Co., Ltd. (Daejeon, South Korea) for sequencing using ITS1 and ITS4 (White *et al.*, 1990) as sequence primers, respectively. Representative sequences determined in this study were deposited in DNA databases (DDBJ, EMBL, GenBank).



ITS1	5´-TCC GTA GGT GAA CCT GCG G-3´
ITS4	5´-TCC TCC GCT TAT TGA TAT GC-3´
ITS5	5´-GGA AGT AAA AGT CGT AAC AAG G-3´
T3	5´-ACG CTC GAA CAG GCA TGC CC-3´
T4	5´-TCA ACA ACG GAT CTC TTG GC-3´
p3	5´-GCC GCT TCA CTC GCC GTT AC-3´
PM5	5´-TTG CTT TGG CGG GCC GGG -3´
PM6	5´-GYC RCY CTG TCG CGA G-3´

Fig. 11 Nucleotide sequences of the PCR primers map and their annealing sites in the rDNA region (Hirata and Takamatsu, 1996).

Data Analysis

The obtained sequences of the rDNA ITS region determination were aligned manually using MS Word ver.5.1 and colour-coded nucleotides with sequences from the genus *Erysiphe*. Phylo including *Typhulochaeta japonica* and *Erysiphe trinae* (*Brasiliomyces trini*) used in genetic trees were obtained from the data using parsimony and distance methods. Maximum parsimony analysis was made, using the parsimony ratchet (Nixon, 1999) in PAUP* 4.0 (Swofford, 2002) and PAUPA at ver. 1 (Sikes and Lewis, 2001) with the heuristic search option of the 'tree-bisection-reconstruction' (TBR) algorithm. All sites were treated as unordered and unweighted, with gaps treated as missing data. The strength of the internal branches of the resulting trees was tested with bootstrap analyses using 1000 replications (Felsenstein, 1985).