CHAPTER 3
MATERIALS AND METHODS

1. Sample sources

Powdery mildew fungi specimens were collected in the northern Thailand from 2009 to 2011. These specimens were used for morphological observation and ribosomal DNA (rDNA) sequencing. Host plants and their family name, collection localities, and voucher numbers are listed and shown in Table 3. All herbarium specimens were deposited at the mycological herbarium in Department of Entomology and Plant Pathology, Faculty of Agriculture, Chiang Mai University, Thailand and Mie University Mycological Herbarium (MUMH), Japan.

2. Morphological observation

Diseased leaves were examined by using a light microscope with 20X and 40X objective phase contrast lenses. Occasionally, examinations of herbarium specimens were carried out by using lactic acid method (Shin and La, 1993 and Shin, 2000). Before observation, herbarium materials were rehydrated by boiling a small piece of the specimen, with fungal mycelium downwards, in a drop of lactic acid on a slide. After boiling, the rehydrated leaf was scrapped the mycelium off and mounted either lactic acid or cotton blue lactic acid for light microscopic observation.

2.1 Anamorphic state

Morphological observation on fungal colonies of anamorphic state was stripped off from the leaf surfaces with clear adhesive tape mounted on a microscope slide with distilled water or 3%NaOH. Morphological characteristics were measured in 30 replicates for each structure on anamorph: size and shape of conidia, conidiophore, foot cells; position of the basal septum; shape and position of hyphal appressoria and presence
or absence of fibrosin bodies (To-anun *et al*., 2005). Conidial germ tubes observation was carried out by using the method of Hirata (1942).

2.2 Teleomorphic state

Teleomorphic feature (chasmothecia) were examined by transferred them onto a microscope slide with a clean needle and mounted with distilled water or 3%NaOH. The following informations were noted: size and shape ascomata, asci, ascospores; characeristics of appendages; number of asci, ascospores.

3. Molecular phylogenetic analyses

3.1 DNA extraction

Whole-cell DNA was extracted from mycelia or conidia using the Chelex method (Walsh *et al*., 1991; Hirata and Takamatsu, 1996). DNA extraction of powdery mildews were carried out using a clean needle to take mycelium (including conidia) off under stereo microscope or using clear adhesive tape (in size: 0.3 mm × 0.3 mm) stripped mycelium off from the leaf surfaces to 300 µl 5% Chelex solution in 1.5 ml eppendorf tube and incubated at 56˚C for 15 min. Then, the extract was incubated in boiling water for 8 min. The extract was mixed vigoriously using vortex for 40 s. This process was repeated twice and stored the DNA extract at –20˚C used as DNA template in Polymerase Chain Reaction (PCR). Before using, the DNA extract is centrifuged at 15,000 rpm for 5 min.

3.2 DNA Amplification and sequencing

The nuclear rDNA region; ITS including 5.8S rDNA and 28S rDNA including the domains D1 and D2 were amplified by the polymerase chain reaction (PCR) using nested primer sets (Fig. 20 and Table. 3). PCR reactions were performed in a total of 50 µl, including the following reagents: H_2O 33.8 µl, 10X PCR buffer (100mM Tris-HCI (pH 8.3), 15 mM $MgCl_2$, 500 mM KCI) 5 µl, 2.5 mM of each deoxyribonucleotide triphosphate 4 µl, two primer sets (20 pmol/µl) 1 µl, Taq DNA polymerase (5 unit/µl) 0.2 µl and DNA template 5 µl. A negative control without template DNA was included in each set of PCR reactions.

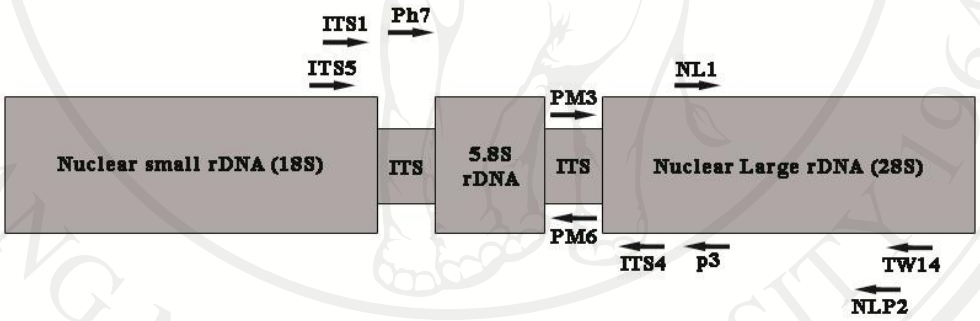

Figure 20 Primer sets were used in this study to amplify nuclear ribosomal DNA (rDNA).

Table 3 A primer nest of ribosomal DNA regions used for Polymerase Chain Reaction (PCR) on powdery mildew fungi in Tribe Phyllactinieae.

Primers	Nucleotide sequences
ITS rDNA	
(including 5.8s rDNA)	
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′
p3	5′-GCC GCT TCA CTC GCC GTT AC-3′
PM6*	5′-GYC RCY CTG TCG CGA G-3′
Ph7**	5′-TG TTG CTT TGG YAG GCC G-3′
28s rDNA	
PM3*	5′-GKG CTY TMC GCG TAG T-3′
NL1	5′-AGT AAC GGC GAG TGA AGC GG-3′
NLP2*	5′-GGT CCC AAC AGC TAT GCT CT-3′
TW14	5′-GCT ATC CTG AGG GAA ACT TC-3′

*Powdery Mildew specific
**_Phyllactinia_ specific

PCR reactions were conducted with TaKaRa Taq DNA polymerase (TaKaRa, Tokyo) under the following thermal cycling conditions in a PCR thermal cycler SP (Takara, Kyoto, Japan): an initial step for denaturing at 95˚C for 2 min; thermocycling for 30 cycles that each cycle consisted of 30s at 95˚C followed by 30s at 52˚C for annealing, and 30s at 72˚C for extension; and a final extension cycle at 72˚C for 7 min.

The following primer sets were used for amplification of 28S rDNA (large subunit): PM3 (Takamatsu and Kano, 2001), TW14, NL1 and NLP2 (Mori *et al*., 2000a). Primers PM3 and TW14 were used for the first PCR. Nested primer sets NL1 and TW14 were used for the second amplification using the first PCR product as a template.

For amplification of the ITS regions, primer sets of ITS1, ITS4, ITS5, p3, PM6 and Ph7 were used. A *Phyllactinia* and *Leveillula* specific primer Ph7 was designed in this study. Primers ITS5 (White *et al*., 1990) and p3 (Kusaba and Tsuge, 1995) were used for the first amplification. Nested primer sets ITS5/PM6 and Ph7/ITS4 were used for the second amplification.

The PCR product was subjected to preparative electrophoresis in 1.5% agarose gel in TAE buffer. The nucleotide fragments of the second PCR products were sent to SolGent Co., Ltd. (Daejeon, South Korea) for sequencing by using NL1 and NLP2 as sequence primers of 28S rDNA, and using ITS1 and ITS4 (White *et al*., 1990) as sequence primers of ITS regions. Sequences were used in BLAST searches against the EMBL database (http://www.ebi.ac.uk/Tools/sss/fasta/nucleotide.html) to identify the most similar sequences available in the databases.

3.3 Phylogenetic analysis

The sequences were initially aligned using MEGA5 (Tamura *et al*., 2011) with MUSCLE program (Edgar and Robert, 2004). The sequences in this study were analyzed with the sequences data that registered in DDBJ (The DNA Databank of Japan) (Table. 4). Maximum parsimony trees were constructed from the alignment data matrix using parsimony ratchet method (Nixon, 1999) in PAUP 4.0b8 (Swofford, 2001) and PAUPRat ver. 1 (Sikes and Lewis, 2001) with the heuristic search option using the tree bisection-reconstruction (TBR) algorithm. All sites were treated as unordered and weighted. All positions containing gaps and missing data were eliminated. The strength of internal branches of the resulting trees were conducted to test with bootstrap (BP) analyses (Felsenstein, 1985) using 1 K replication with the stepwise addition option set as sample. Tree score including tree length, the consistency index (CI), the retention index (RI), the rescaled consistency index (RC) and the homoplasy index (HI) were also calculated. PAUP 4.0b8 (Swofford, 2001) was used for finding a tree which obtained the highest likelihood value among the equally parsimonious trees using the Kishino-Hasegawa (KH) (Kishino and Hasegawa, 1989) and Shimodaira-Hasegawa (SH) (Shimodaira and Hasegawa, 1999).

The strength of the internal branches of the resulting trees was tested by bootstrap analysis (Felsenstein, 1985) using 1,000 replications. Lack of bootstrap value indicates less than 60% support at that node.

Table 4 Isolates of Phyllactinieae included for sequence analysis.

Fungal species	Host plant	Location of collection and voucher material	GenBank accession no.	
			ITS	28S
Phyllactinia				
Ph. broussonetiae-kaempferi	*Broussonetia kazinoki*	Japan; MUMH163	AB080492	AB080382
Phyllactinia sp.	*Castanopsis* sp.	China; HMAS41420	AB080546	AB080443
Ph. roboris	*Castanea crenata*	Japan; MUMH591	AB080516	AB080407
Ph. guttata	*Cornus officinalis*	Japan; MUMH153	AB080496	AB080386
Ph. mali	*Crataegus* sp.	Switzerland; MUMH619	AB080523	AB080414
Ph. broussonetiae-kaempferi	*Ficus tikoua*	China; HMAS37126	AB080547	AB080445
Ph. chubutiana	*Lycium chilense*	Argentina; BCRU4634	AB243690	AB243690
Ph. mali	*Mespilus* sp.	Iran; MUMH918	AB080556	AB080454
Ph. guttata	*Morus alba*	Korea; SMK17215	AB080540	AB080432
Ph. guttata	*Morus australis*	Japan; MUMH79	D84384	AB080372
Ph. guttata	*Morus cathayana*	Japan; MUMHn36	AB080518	AB080409
Ph. guttata	*Paliurus spina-christi*	Iran; MUMH922	AB080560	AB080458

Table 4 Isolates of Phyllactinieae included for sequence analysis. (continued)

Fungal species	Host plant	Location of collection and voucher material	GenBank accession no.	
			ITS	28S
Phyllactinia				
Ph. mali	*Pyrus pyrifolia*	Japan; MUMH435	AB080521	AB080412
Ph. angulata	*Quercus rubra*	USA; MUMH928	AB080566	AB080464
Leveillula				
L. contractirostris	*Alcea* sp.	Iran; IRAN11113	AB045105	AB080467
L. lanuginosa	*Daucus carota*	Iran; IRAN11119	AB042641	AB042641
L. elaeagni	*Elaeagnus angustifolia*	Iran; IRAN11138	AB048350	AB042642
L. taurica	*Glaucium oxylobum*	Iran; IRAN11139	AB045108	AB080474
L. saxaouli	*Haloxylon* sp.	Iran; IRAN11121	AB044382	AB080469
L. taurica	*Helianthus annuus*	Iran; IRAN11134	AB044378	AB080472
L. lactukae-serriolae	*Lactuca serriola*	Iran; IRAN11144	AB044375	AB080476

Table 4 Isolates of Phyllactinieae included for sequence analysis. (continued)

Fungal species	Host plant	Location of collection and voucher material	GenBank accession no.	
			ITS	28S
Leveillula				
L. mindii	*Mindium laevigatum*	Iran; IRAN10909	AB045106	AB080466
L. cylindrospora	*Noaea mucronata*	Iran; IRAN11117	AB044352	AB080468
L. rubiae	*Rubia tinctorum*	Iran; IRAN10575	AB044381	AB080465
L. duriaei	*Salvia nemorosa*	Iran; IRAN11142	AB044373	AB080475
Pleochaeta				
Pl. shiraiana	*Celtis sinensis*	Japan; MUMH36	D84381	
Pl. indica	*Celtis australis*	Japan; MUMH3208	AB243757	AB243757