APPENDIX

Preparing Chelex for DNA extraction

Chelex 100 Resin (Bio-Rad) was adjusted to 5% Chelex solution and suspended in sterile water, and 300 µl aliquots transfer to Eppendorf tubes 1.5ml for DNA extraction.

Conidial germ tubes observation by Hirata's method

Initially, getting the onion skin to peel off and using a razor cut the inner surface of onion in size: 1×1 cm^2. The cell layers (epidermis cells) were stripped off by forceps, then soaked in 80% ethanol alcohol for at least 2 weeks. Before using the cell layers of onion, cell cleaning has been done by rinsed with tap water for 1 hr. The cell layer with cuticle side upwards was put on a microscopic slide and inoculated with the vegetative growth in fungus. The inoculated cell layer was floated on distilled water in a Petri dish at room temperature. Microscopic observation will be operated after 24 hr.

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Poster and oral presentation

Poster section:

1. **Monkhung, S.**, Divarangkoon, R. and To-anun, C. (2008). First report of *Phyllactinia dalbergiae* on *Dalbergia lanceolaria* (Fabaceae) in Thailand. The 3rd Annual Meeting of Thai Mycological Association (TMA) and Mycology conference in Thailand. October 10-11, 2008. Faculty of Agriculture. Khonkhan University, Khonkhan, Thailand.

2. **Monkhung, S.**, Divarangkoon, R. and To-anun, C. (2009). Morphology and Phylogeny of *Oidiopsis* (Erysiphaceae) found on *Capsicum* spp. in Thailand. The 4th Annual Meeting of Thai Mycological Association and Mycology Conference (TMA). October 24, 2009. Maejo University, Chiang Mai, Thailand.

3. Divarangkoon, R., **Monkhung, S.** and To-anun, C. (2009). First report of two *Brasiliomyces (Erysiphaceae)* on *Lithocarpus* spp. (Fagaceae) from Thailand. The Annual Meeting of Thai Mycological Association and Mycology conference in Thailand. (TMA) October 24, 2009. Maejo University, Chiang Mai, Thailand.

4. **Monkhung, S.**, Divarangkoon, R. and To-anun, C. (2010). Two Powdery Mildew Fungi on *Morus alba* in Thailand. RGJ-Ph.D. Congress XI. April 1-3, 2010. Chonburi, Thailand.

5. Meeboon, J., To-anun, C., **Monkhung, S**., Divarangkoon, R. and Takamatsu, S. (2011). A Tropical Powdery Mildew Genus *Brasiliomyces* Occurred at Least Twice During The Evolution of The Erysiphaceae. international Union of Microbiological Societies Congress (IUMS). September 6-10, 2011. Sapporo Convention Center, Higashi-Sapporo, Japan.

6. **Monkhung, S.**, To-anun, C. and Takamatsu, S. (2012). First report of *Phyllactinia cassiae-fistulae* (Erysiphaceae; Ascomycota) from Thailand: molecular and morphological characterization. The 6th Thai Mycological Conference. March 6, 2012. Rama Gardens Hotel, Bangkok, Thailand.

Oral section:

1. **Monkhung, S.**, Divarangkoon, R. and To-anun, C. (2010). Morphology and Phylogeny of *Oidiopsis* (Erysiphaceae) found on *Capsicum* spp. in Thailand. The Thai Phytopathological Society. May 15, 2010. Kasetsart University, Bangkok, Thailand.

2. **Monkhung, S.** and To-anun, C. (2010). First Report of Powdery Mildew on *Cassia fistula* (Leguminosae) in Thailand. RGJ Seminar Series 77th. Fungal Biodiversity and Biotechnology. November 13, 2010. Chiang Rai, Thailand.

PUBLICATIONS

1. **Monkhung, S.**, To-anun, C. and Takamatsu, S. (2011). Molecular approach to clarify taxonomy of powdery mildew on Chilli plants caused by *Oidiopsis sicula* in Thailand. Journal of Agricultural Technology 7(6): 1801–1808.

2. Divarangkoon R., Meeboon J., **Monkhung S.**, To-anun C. and Takamatsu S. (2011). Two new species of *Erysiphe* (*Erysiphales*, *Ascomycota*) from Thailand. Mycosphere 2(3): 231–238.

3. **Monkhung, S.**, To-anun, C. and Takamatsu, S. (2012). First report of *Phyllactinia cassiae-fistulae* (Erysiphaceae; Ascomycota) from Thailand: molecular and morphological characterization. African Journal of Biotechnology. 12(2): 109–114.

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Molecular approach to clarify taxonomy of powdery mildew on Chilli plants caused by *Oidiopsis sicula* in Thailand

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Monkhung, S., To-anun, C. and Takamatsu, S. (2011) Molecular approach to clarify taxonomy of powdery mildew on Chilli plants caused by *Oidiopsis sicula* in Thailand. Journal of Agricultural Technology 7(6): 1801-1808.

Causal agent of powdery mildew on five chilli plants in Thailand viz.: *Capsicum frutescens*, *C. annuum* var. *grossum*, *C. frutescens* × *C. chinense* (Bhut Jolokia), *Capsicum* sp. (maxican chilli) and *Capsicum* sp. (darby chilli) has been identified as *Oidiopsis* sp. based on morphological data in Thailand. Molecular phylogenetic analysis indicated that the powdery mildew on *Capsicum* spp. is *Oidiopsis sicula* which supports the morphological data. This result confirmed that *Oidiopsis sicula* is linked to *Leveillula taurica* in teleomorph state. Maximum Parsimony tree showed that all sequence data are located in a clade consisted of *Leveillula taurica*, a fungal agent causing powdery mildew of *Capsicum* sp.

Key words: morphology, phylogeny, *Leveillula taurica*, *Capsicum* spp.

Introduction

The first systematic taxonomy of powdery mildews were studied based on morphological characteristics (Boesewinkel, 1980; Salmon, 1900; Ferraris, 1910). Some powdery mildews have similar morphological characteristics which cause confusing identification of the fungal group. In addition, sufficient information on morphological characteristics of sexual state (teleomorph) is essential to identify powdery mildews at species level. Unfortunately, most powdery mildews do not produce sexual state in tropical or sub-tropical areas. This is a problem for taxonomy of powdery mildew. Hirata and Takamatsu (1996) has been reported to use molecular analyze associate with anamorphic morphology in taxonomy of powdery mildew.

Nowadays, molecular technique is a useful tool for precise taxonomy for the Erysiphaceae. Khodaparast *et al*. (2001) determined the nucleotide

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sequences of the rDNA ITS regions for 13 *Leveillula* species on 50 host plant species and reported that the morphology of primary conidia mostly provides a good criterion to identify *Leveillula* species. This study also demonstrated that *L. taurica s. lat.* is a species complex composed of several biological species. Glawe *et al*. (2005) determined that powdery mildew on *Triglochin maritime* is caused by *L. taurica*. This result was confirmed by morphological and ITS sequence data. The ITS sequence of this fungus was identical with those reported for *L. taurica* hosted by *Capsicum annuum* in Australia and *Elaeagnus augustifolia* in Iran.

Chilli (Solanaceae) is an economic spice crop and cultivated commercially in all parts of Thailand (Poonpolgul & Kumphai, 2007). Powdery mildew on chilli is an important disease that causes yield losses in growing chilli area. And also, this disease is distributed in the other parts of the world (Palti, 1988). Sontirat *et al.* (1994) reported that *Oidiopsis* sp. is a causal agent of powdery mildew disease in Thailand. However, identification at species level was not shown because its perfect state has never been found on *Capsicum* species. The molecular analysis combined with morphological characteristics is a useful tool to approach for precise taxonomy of powdery mildews.

The present study was conducted to clarify the taxonomy of the fungal pathogen causing powdery mildew on chilli plants (*Capsicum* spp.) at species level on the basis of morphological data associate with molecular approach.

Materials and methods

Morphological observation

Specimens were collected in the northern Thailand since 2007. Fungal colonies on fresh specimens were stripped off by adhesive tape, mounted in distilled water and examined by standard light microscopy with 20X and 40X objective phase contrast lenses. Herbarium specimens were mounted in lactic acid, gently heated, but without any staining (Shin and La, 1993). Morphological characteristics were measured in 30 replicates for each structure: size and shape of conidia, conidiophore; position of the basal septum; shape and position of hyphal appressoria and presence or absence of fibrosin bodies (To-anun *et al*., 2005). Observation of conidial germ tube was carried out using the method of Hirata (1942). Specimens were deposited in the mycological herbarium in Department of Entomology and Plant Pathology, Faculty of Agriculture, Chiang Mai University, Thailand and Mie University Mycological Herbarium (MUMH), Japan.

Phylogenetic analysis

Whole-cell DNA was extracted from mycelia or conidia using the chelex method (Walsh *et al.,* 1991; Hirata and Takamatsu, 1996). The nuclear rDNA ITS region including 5.8S rDNA was amplified by the polymerase chain reaction (PCR) using nested primer sets. The following thermal cycling conditions were performed 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 oligonucleotide primers were used in this study as follows: ITS1, ITS4, ITS5, p3, PM6 and Ph7. A *Phyllactinia* and *Leveillula* specific primer Ph7 (TGTTGCTTTGGYAGGCCG) 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 nucleotide fragments of PCR products were sent to SolGent Co. (Daejeon, South Korea) for sequencing by using ITS1 and ITS4 (White *et al.,* 1990) as sequence primers, respectively.

The nucleotide sequences of rDNA on ITS region were aligned with MUSCLE program (Edgar & Robert, 2004). Phylogenetic trees were constructed from data using maximum-parsimony (MP) analysis in MEGA5 (Tamura *et al*., 2011) with a heuristic search using close-neighbor-interchange algorithm (CNI). All positions containing gaps and missing data were eliminated. 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 50% support at that node.

Result

Morphological observation

Powdery mildew was found on 5 chilli species, viz.: *Capsicum frutescens*, *C. annuum* var. *grossum*, *C. frutescens* × *C. chinense* (Bhut Jolokia), *Capsicum* sp. (maxican chilli) and *Capsicum* sp. (darby chilli). Diseased chilli plants appear symptom on leaves, but other parts of plant did not show symptoms. The lower side of leaves exhibited white-grayish colonies of fungi (hypophyllous) (Fig 1). The upper side of leaves showed a symptom as only yellow spot and then became to necrotic brown spot.

Mycelium hypophyllous; hyphae substraight to wavy, mostly branching near the septum; conidiophores erect, long and slender, arising from the upper part of mother cell, positions not central; foot-cells straight, with a basal septum

near branching point of mycelium up to away from it; appressoria slightly lobed to elongated; conidia formed singly, dimorphic conidia, apically pointed in primary conidia and ellipsoid to cylindric in secondary conidia without conspicuous fibrosin bodies and conidial germination formed pseudoidium type (Fig 2). Chasmothecia can not be found. Table 1 showed size of morphological features of powdery mildew on each chilli species.

Phylogenetic analysis

The five rDNA sequences data on ITS region were aligned with 21 *Leveillula* sequences retrieved from GenBank. The alignment data matrix consisting of 26 taxa and 621 characters were used in the analysis. A total of 1,012 most parsimonious trees (CI = 0.708, RI = 0.832) were constructed by the MP analysis. One of MP tree is shown in Fig 3. The powdery mildew found on five chilli plants were located in the group of *L. taurica* causing powdery mildew on chilli under the accession numbers of AB000940 and MUMH3830.

Fig 1. *Capsicum annuum* var. *grossum* Bail. (sweet chilli) leaves showing a symptom of powdery mildew

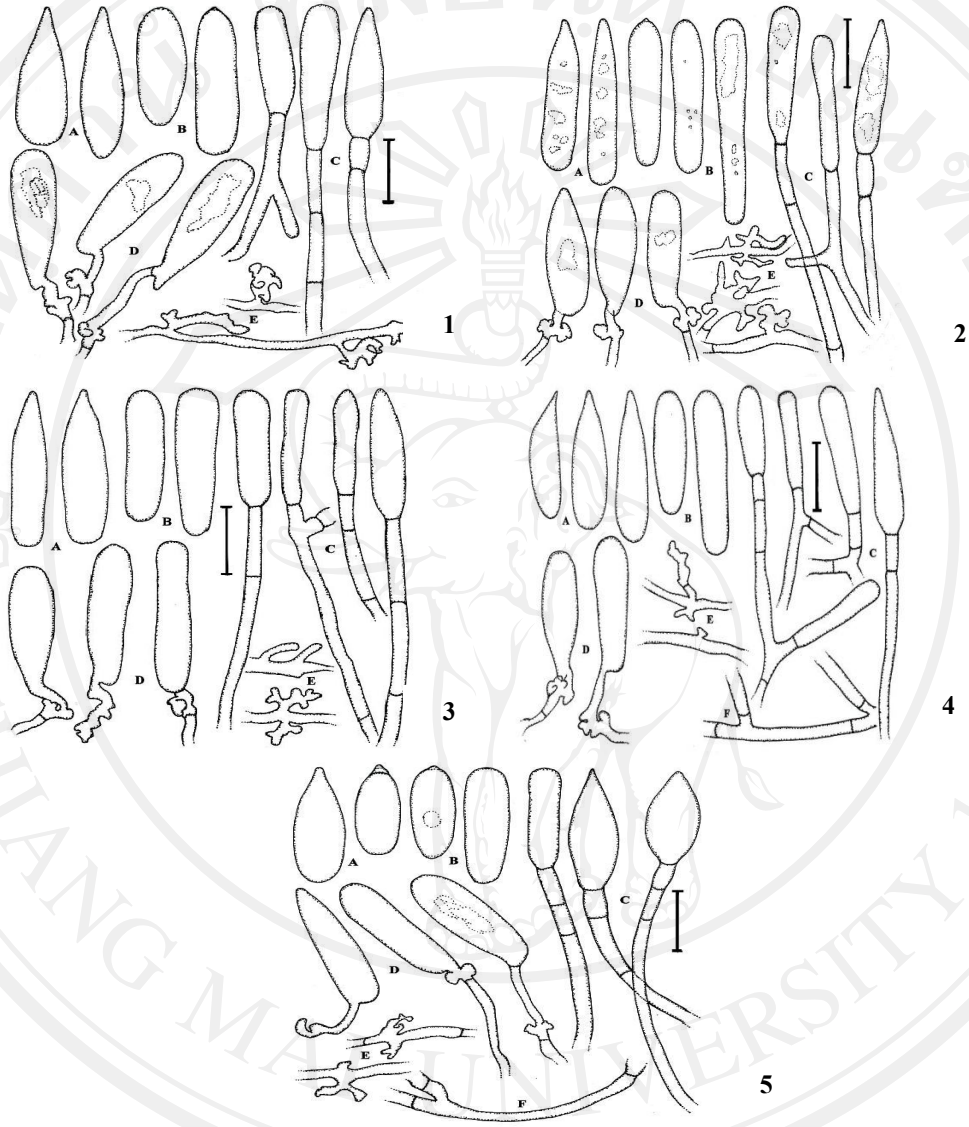

Fig 2. Morphological characteristics of *Oidiopsis* found on chilli plants illustrated using a line drawing under a light microscope (400X); (1) *Capsicum frutescens* (2) *C. annuum* var. *grossum* (3) *C. frutescens* × *C. chinense* (Bhut Jolokia) (4) *Capsicum* sp. (maxican chilli) (5) *Capsicum* sp. (darby chilli). Alphabet in figures described as follows; (A) primary conidia (B) secondary conidia (C) conidiophores (D) conidia with germ tubes of the pseudoidium pattern (E) mycelia with appressorium and (F) mother cells that originate of conidiophore (Bar = 30 µm.)

Table 1. Morphological characteristics of powdery mildew on chilli species.

Host	Size of morphological characteristics (µm)				
	conidiophore	mother cell	foot cell	primary conidia	secondary conidia
Capsicum annuum var. *grossumum* (MUMH5096)	(51-)100-276 (-338)×(5-)8-12(-16)	(28-)30-122 (-134)×5-6(-7)	(41-)49-140 (-202)×5-6(-7)	60-76(-83)× 13-17(-21)	(46-)52-69 (-78)×(12-)13-17
Capsicum spp. (maxican chilli) (MUMH5104)	(66-)133-266(-281)×(10-)12-17	(24-)34-84(-107) ×5-6	(17-)48-137(-234) ×(4-)5-6(-7)	(61-)63-76(-78) ×(13-)15-19(-21)	(46-)58-73(-78) ×(13-)15-17(-19)
Capsicum frutescens (MUMH5083)	(105-)178-273(-310)×(6-)8-15	(46-)49-83(-151)×5-6	(56-)90-149(-174)×5-7	(58-)63-80(-88)×(15-)16-20	(49-)54-71(-76)×(13-)15-19
Capsicum spp. (darby chilli) (MUMH5119)	(37-)110-251(-301)×(7-)8-12.2	(30-)35-89(-100)×5-7	(44-)76-124(-177)×5-7	(47-)58-73(-74)×14-19	(41-)51-68(-76)×(11-)13-18(-19)
C. frutescens×*C. chinense* (Bhut Jologia) (MUMH5106)	(119-)144-195(-278)×(7-)8-15(-17)	(22-)88-105(-115)×(5-)6-7	(39-)71-107(-149)×(5-)6-7	(54-)58-71(-73)×(12-)15-18	(46-)5-72(-73)×(13-)15-17(-18)

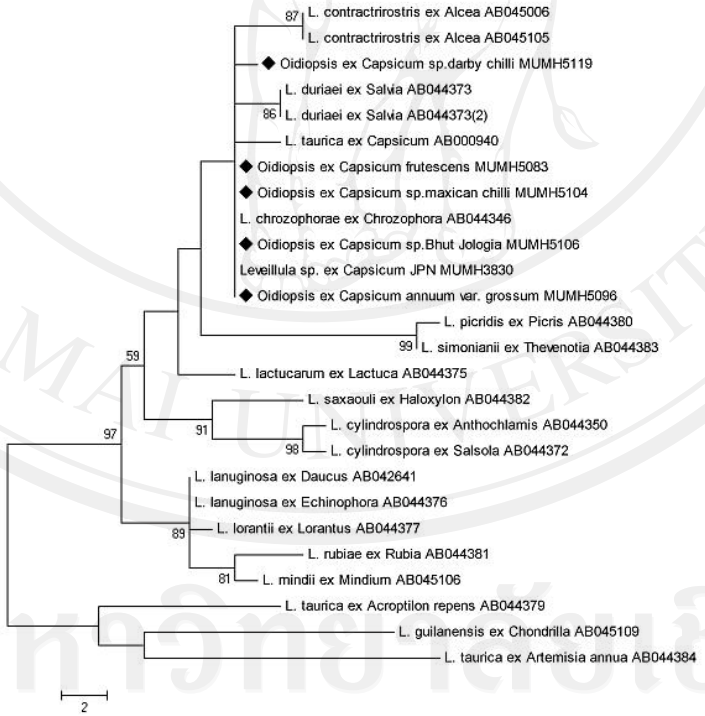

Fig 3. The Maximum parsimony phylogenetic tree based on fungal ITS gene sequences. Numbers above or below branches indicate bootstrap values (>50%) from 1,000 replicates. The tree length is 92, the consistency index (CI) is 0.708, the retention index (RI) is 0.832. Solid rhombus is represented as *Oidiopsis* that causing powdery mildew on chilli plants.

Discussion

The phylogenetic analysis represented by MP tree indicated that the five *Oidiopsis* specimens on chilli plants are located in a clade of *Leveillula* which confirms an anamorph-teleomorph connection of this fungus with *Leveillula*. *Leveillula* on *Acroptilon*, *Artemisia* and *Chondrilla* were used as the outer group based on Khodaparast *et al.* (2001).

The present phylogenetic result supported the morphological examination that showed no significance differences among five *Oidiopsis* found on *Capsicum* spp. (Braun, 1987 and Palti, 1988). Conidial germination type is *pseudoidium*-type (syn. *polygoni*-type) cited by Cook and Braun (2009). Thus, the morphological and phylogenetic analyses suggested strongly that the powdery mildew on *Capsicum* spp. is infected by *O. sicula* (teleomorph *L. taurica*) which agrees with the report of Goldberg (2004). Cunnington *et al*. (2003) revealed that *L. taurica* is a causal agent of powdery mildew disease on *C. annuum* in Australia. As a result, molecular analysis of rDNA ITS region is a strong tool to clarify taxonomy for species level in the genus. Species identification is an important information for accurate controlling this disease. In addition, this is the first report of taxonomy of powdery mildew on chilli plants by using morphological characteristics associated with molecular approach in Thailand.

This fungus attacks broadly range of plants (Glawe *et al*., 2005; Khodaparast *et al*., 2001). Future work such as pathogenicity test is necessary in order to determine pathogenicity of this fungus on differential varieties of chilli plants including other plants.

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Two new species of *Erysiphe* (*Erysiphales*, *Ascomycota*) from Thailand

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During a survey of powdery mildews in northern Thailand, two morphologically unique powdery mildews were collected on *Castanopsis* and *Lithocarpus*. Both powdery mildews have a thin, single layer of peridium cells of chasmothecia, which is a morphological character of the genus *Brasiliomyces*. However, recent molecular phylogenetic analyses indicates that *Brasiliomyces* is polyphyletic and shows that the two powdery mildews from Thailand belong to the *Erysiphe* lineage with *Oidium* subgenus *Pseudoidium* anamorphs. Therefore, they are described as *Erysiphe monoperidiata* sp. nov. and *E. asiatica* sp. nov.

Key words – *Brasiliomyces* – fungi – powdery mildew – taxonomy

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Introduction

The *Erysiphales* is a fungal group causing important plant diseases (powdery mildew) on about ten thousand angiosperm plants including many economically important cultivated plants (Amano 1986, Braun 2011). The biodiversity of the *Erysiphales* is less explored in tropical and subtropical regions compared with temperate regions of the Northern Hemisphere (Hirata 1976), probably because of fewer scientists working on this fungal group in these regions. In order to estimate the biodiversity of the powdery mildews in tropical regions, we have been working on the biodiversity of the *Erysiphales* in northern Thailand since 1999. This investigation revealed that there are still many undescribed and unique powdery mildew species in this region (To-anun et al. 2003, 2005). Therefore, exploring the *Erysiphales* in subtropical and tropical regions is important for further understanding of biodiversity, phylogeny and evolution of these organisms.

In this paper, we describe two new *Erysiphe* species recently found in northern Thailand. Both species have distinct morphological characteristics of the genus *Brasiliomyces*. However, recent molecular phylogenetic analyses revealed that *Brasiliomyces* is polyphyletic (Takamatsu *in litt*.) and the delimitation of this genus needs to be revised. Due to the phylogenetic position of the two new taxa within the *Erysiphe* clade, we prefer to assign them to *Erysiphe*.

Methods

Morphological examination

Specimens were collected in northern Thailand between November 2004 and March 2010. Details of host name, collection date, place, and collector were noted. Morphological

examinations were carried out as outlined in To-anun et al. (2003). Hyphae, chasmothecia, appendages, asci, and ascospores were stripped off from the leaf surfaces with a clean needle, mounted on a microscope slide, and examined in 3% NaOH using a light microscope with phase contrast 20×, 40×, and 100× objectives. The following data were recorded during the examination of the specimens: size and shape of chasmothecia, presence or absence of appendages, structure and size of peridial cells, number of asci per ascus, number of ascospores per asci, size and shape of asci and ascospores, and shape and position of hyphal appressoria. Thirty chasmothecia were measured per sample. Specimens were deposited at the National Museum of Nature and Science (TNS) and Mie University Mycological Herbarium (MUMH), Japan.

Phylogenetic analysis

Whole-cell DNA was extracted from chasmothecia by the chelex method (Walsh et al. 1991, Hirata & Takamatsu 1996). The rDNA internal transcribed spacer (ITS) region including 5.8S rDNA was amplified using primers ITS5 (White et al. 1990) and p3 (Kusaba & 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 & Kano (2001). The ITS5/PM6 and PM5/p3 fragments were sent to SolGent Co. (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) under the accession numbers of AB622211–AB622218.

Eight sequences of the rDNA ITS region determined in this study were aligned manually using MS Word ver.5.1 and colour-coded nucleotides with 23 sequences from the genus *Erysiphe*, including *Typhulochaeta japonica* and *Erysiphe trinae* (≡ *Brasiliomyces trini*) used in Heluta et al. (2009). This data set consisted of 31 sequences and 674 sites, of which 180 ambiguously aligned sites were removed from the following phylogenetic analysis. The alignments were deposited in TreeBASE (http://www.treebase.org/) under the accession number of S11366. Maximum parsimony analysis was done with the parsimony ratchet (Nixon 1999) in PAUP* 4.0 (Swofford 2002) and PAUPRat ver. 1 (Sikes & Lewis 2001) with the heuristic search option using 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). Tree scores, including tree length, CI, RI, and RC, were also calculated.

Results

Taxonomy

Erysiphe monoperidiata Meeboon, R. Divarangkoon & S. Takamatsu, **sp. nov.** Figs 1, 2
MycoBank 561124

Etymology – *monoperidiata*, refers to the chasmothecia of this species with a single peridium cell layer.

Erysiphes trinae similis, sed ascis 4–6-sporis distinguitur.

Typus – on *Castanopsis tribuloides* A.DC. (Fagaceae), THAILAND, Mae Hong Son Province, Huai Nam Dang National Park, 1 March 2010 (TNS-F-39216, holotype; MUMH 4988, isotype). rDNA sequence extype: AB622214 (ITS).

Colonies amphigenous, mainly epiphyllous, persistent, forming irregular white patches on the host surfaces. *Hyphae* hyaline, superficial, 4–6 µm wide, branching. *Appressoria* well developed, coral-like, single or occasionally opposite in pairs. *Conidiophores* and *conidia* unknown.

Chasmothecia scattered to gregarious, (55.5–)58–82.5(–85) µm diameter ($\bar{x}$ = 68.9 µm), containing 2–4 asci. *Peridium* thin, one conspicuous layer, yellowish to light brown, semitransparent, appendages present, poorly developed, often branched, rarely absent, mycelioid, (15.5–)18–66(–75) × (2.5–)3–6(–7.5) µm ($\bar{x}$ = 33.1 × 4.6 µm), colourless, aseptate, thin-walled, smooth. *Asci* sessile or short-stalked, (34–)36–58(–61) × (24–)28–49(–52) µm ($\bar{x}$ = 45.5 × 37.8 µm), 4–6-spored. *Ascospores* ellipsoid-ovoid, hyaline, (11–)12.5–25(–26) × (6–)7.5–13(–14.5) µm ($\bar{x}$ = 20.3 × 10.3 µm).

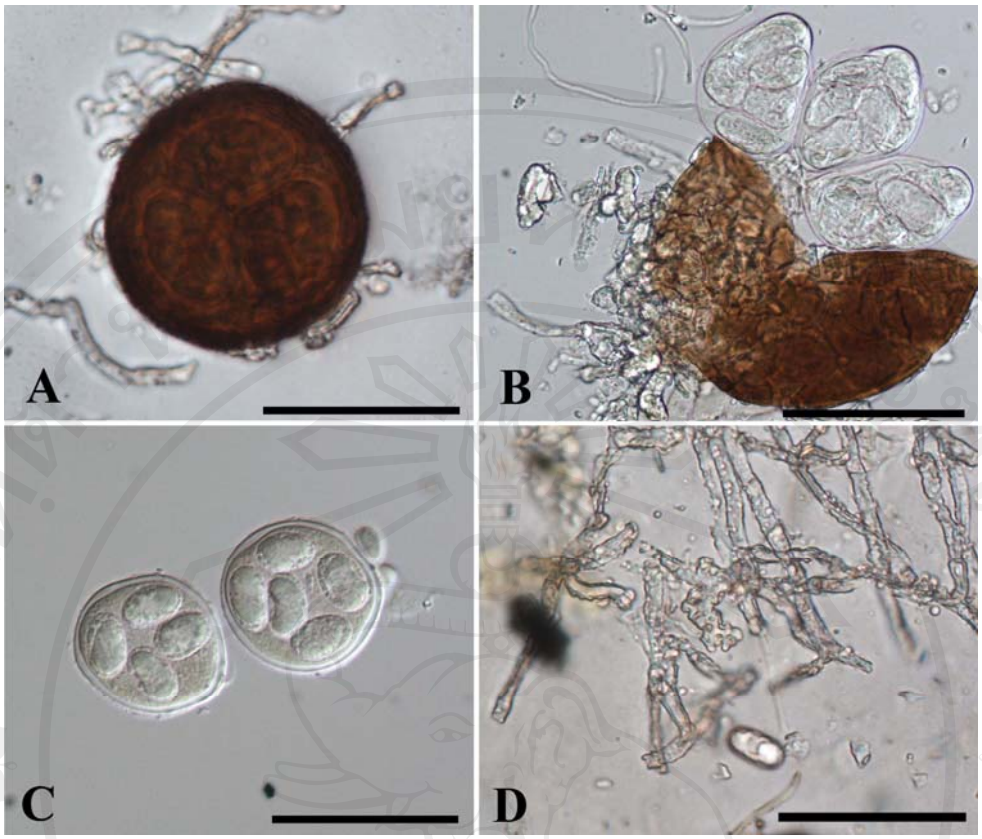

Fig. 1 – *Erysiphe monoperidiata*. **A** Chasmothecium. **B** Chasmothecia with asci and ascospores. **C** Asci and ascospores. **D** Appressoria. – Bars 50 µm.

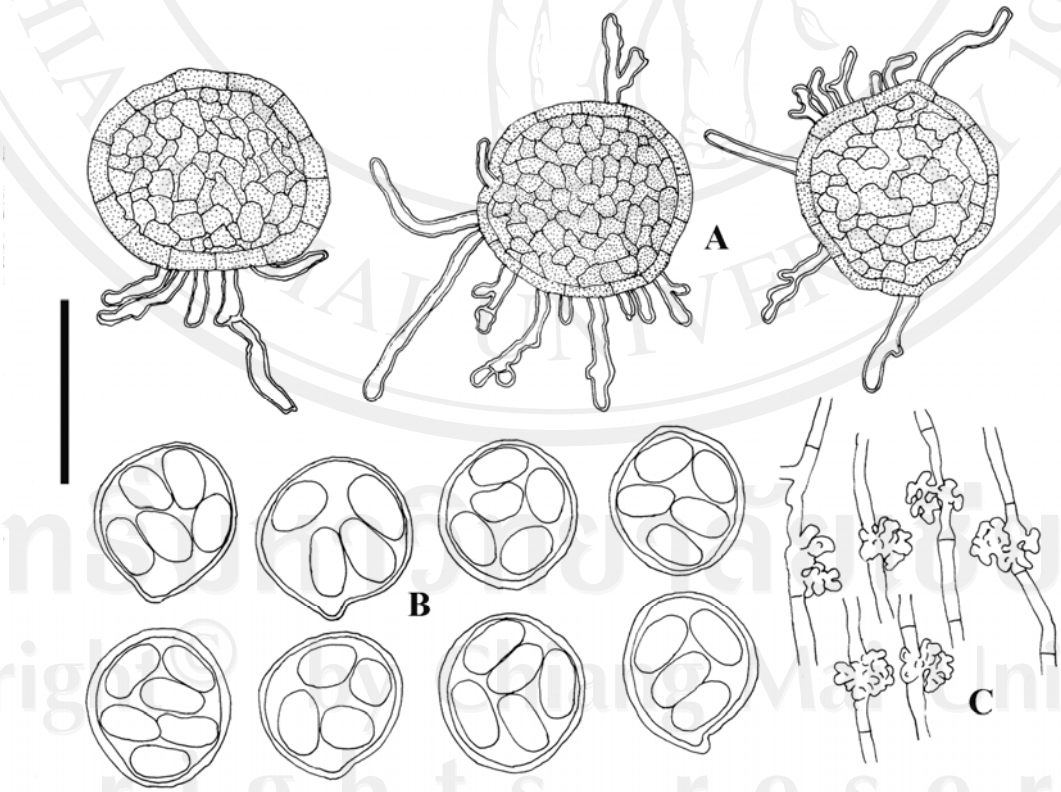

Fig. 2 – Drawing of *Erysiphe monoperidiata*. **A** Chasmothecia. **B** Asci and ascospores. **C** Appressoria. Bar – 50 µm.

Additional collections examined – on *Lithocarpus polystachyus* Rehder (Fagaceae), Thailand, Mae Hong Son Province, 1 March 2010 (MUMH 4986); on *Lithocarpus elegans* (Blume) Hatus. ex Soepadmo, Thailand, Mae Hong Son Province, 1 March 2010 (MUMH 4985); on *Castanopsis argyrophylla* King ex Hook.f. (Fagaceae), Thailand, Chiang Mai Province, Doi Khuntan, 21 March 2010 (MUMH 4987); on *Castanopsis indica* A.DC., Thailand, Chiang Mai Province, Botanical Garden, 10 March 2010 (MUMH 4990); on *Castanopsis calathiformis* Rehder & E.H.Wilson, Thailand, Chiang Rai Province, Khun Chae National Park, 5 March 2010 (MUMH 4991).

Host range and distribution – on *Castanopsis argyrophylla*, *C. calathiformis*, *C. indica*, *C. tribuloides*, *Lithocarpus elegans*, and *L. polystachyus* (Fagaceae), Asia, Thailand.

Erysiphe asiatica Meeboon, R. Divarangkoon & S. Takamatsu, **sp. nov.** Figs 3, 4
MycoBank 561125

Etymology – *asiatica*, a fungus found in Asia.

Erysiphes trinae similis, sed ascis 6–8-sporis distinguitur.

Typus – on *Castanopsis diversifolia* King ex Hook.f., THAILAND, Chiang Mai Province, Doi Pui National Park, 1 March 2010 (TNS-F-39215, holotype; MUMH 4992, isotype). rDNA sequence ex-type: AB622218 (ITS).

Colonies hypophyllous, persistent, forming irregular white patches on host surfaces. *Hyphae* hyaline, superficial, 4–6 µm wide. *Appressoria* well-developed, coral-like, single or occasionally opposite in pairs. *Conidiophores* and *conidia* unknown. *Chasmothecia* scattered, (51–)57–74(–78) µm diameter ($\bar{x}$ = 65.9 µm), containing only 2 asci. *Peridium* thin, one conspicuous layer, yellowish to light brown, semitransparent, chasmothecial appendages often absent or rudimentary, if present poorly developed, mycelioid, (31–)45–51(–66) × (4–)4.5–5(–5.5) µm ($\bar{x}$ = 48.6 × 4.8 µm), branched, hyaline, aseptate, thin-walled, smooth. *Asci* sessile or short-stalked, (45–)46–59(–62) × (38–)40–53(–57.5) µm ($\bar{x}$ = 51.5 × 45.6 µm), 6–8-spored. *Ascospores* ellipsoid-ovoid, olivaceous brown, (16–)18–25(–28) × (8.5–)9–15 (–16.5) µm ($\bar{x}$ = 21.5 × 12.2 µm).

Additional collections examined – *Castanopsis echinocarpa* Miq., Thailand, Chiang Mai Province, Phu Ping Palace, 19 March 2010 (MUMH 4989).

Host range and distribution – *Castanopsis diversifolia*, *C. echinocarpa* (Fagaceae), Asia, Thailand.

Phylogenetic analysis

Of the 494 total characters used in this analysis, 331 characters were constant, 61 characters were variable and parsimony-uninformative and 102 characters were parsimony-informative. A total of 174 equally parsimonious trees with 311 steps (CI = 0.675, RI = 0.799, RC = 0.539) were generated by the parsimony ratchet analysis. One of the best trees is shown in Fig. 5. *Erysiphe monoperidiata* and *E. asiatica* each formed separate clades with 97% and 100% bootstrap support, respectively. These two clades further formed a larger clade with 99% bootstrap support. This large clade grouped with *Typhulochaeta japonica*, *Erysiphe trinae* and *E. gracilis*, but with weak (54%) bootstrap support.

Discussion

Both *E. monoperidiata* and *E. asiatica* have a single layer of chasmothecial peridium cells, which is a morphological characteristic of the genus *Brasiliomyces* (Zheng 1984, Braun 1987). However, unpublished results of our recent phylogenetic study clearly indicate that the genus *Brasiliomyces* is polyphyletic, consisting of at least two independent lineages. This result urgently requires revision of the generic concept of *Brasiliomyces*. Because the current phylogenetic analysis indicates that both species belong to the *Erysiphe* clade with *Oidium* subgenus *Pseudoidium* anamorphs, together with *E. trinae* and *Typhulochaeta japonica*, we propose to assign these two new species to *Erysiphe*.

A total of eight *Brasiliomyces* species have been reported in the world, especially from subtropical and tropical regions (Harkness 1886, Viégas 1944, Marasas 1966, Boesewinkel 1980, Hanlin & Tortolero 1984, Hodges 1985, Kuo et al. 1992, Ahmad et al. 1998, To-anun et al. 2003). Three of the eight species occur on Fagaceae. Of these, *B. cyclobalanopsidis* is distinct from *E. monoperidiata*

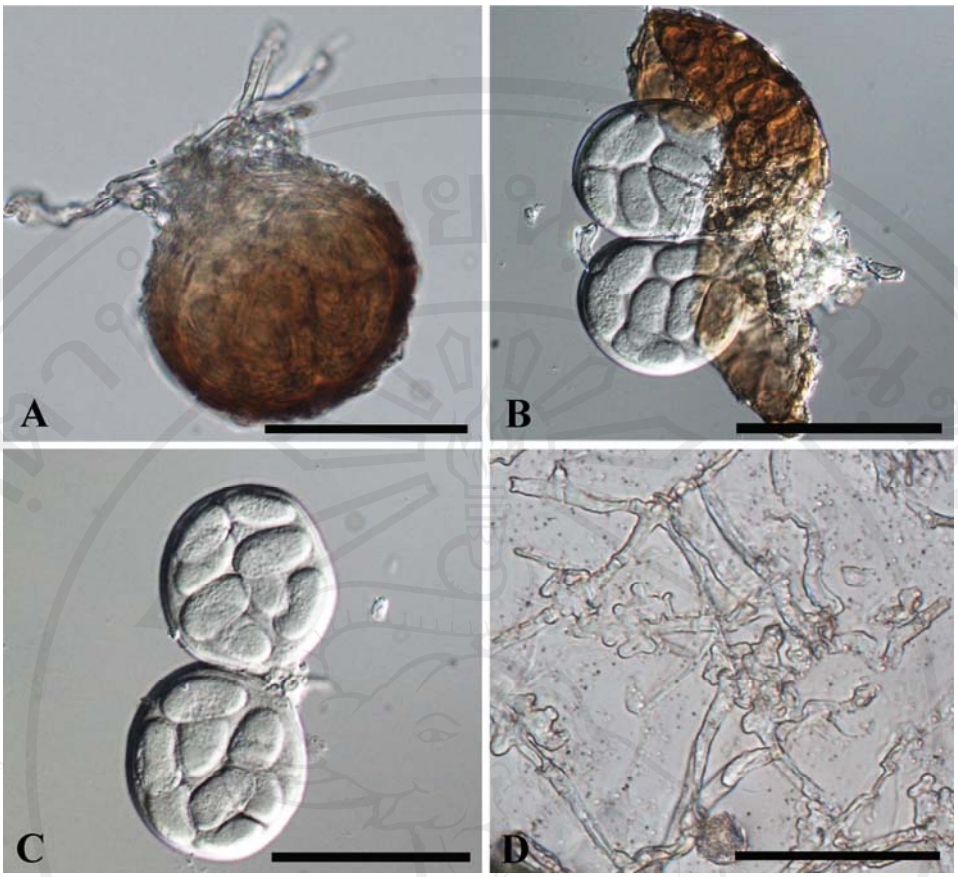

Fig. 3 – *Erysiphe asiatica*. **A** Chasmothecium. **B** Chasmothecia with asci and ascospores. **C** Asci and ascospores. **D** Appressoria. Bars = 50 µm.

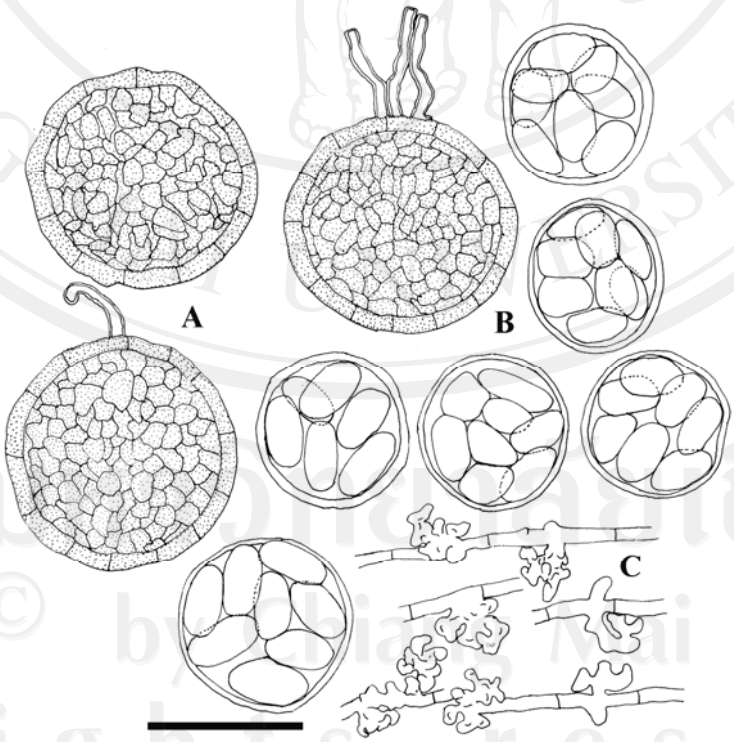

Fig. 4 – Drawing of *Erysiphe asiatica*. **A** Chasmothecia. **B** Asci and ascospores. **C** Appressoria. Bar = 50 µm.

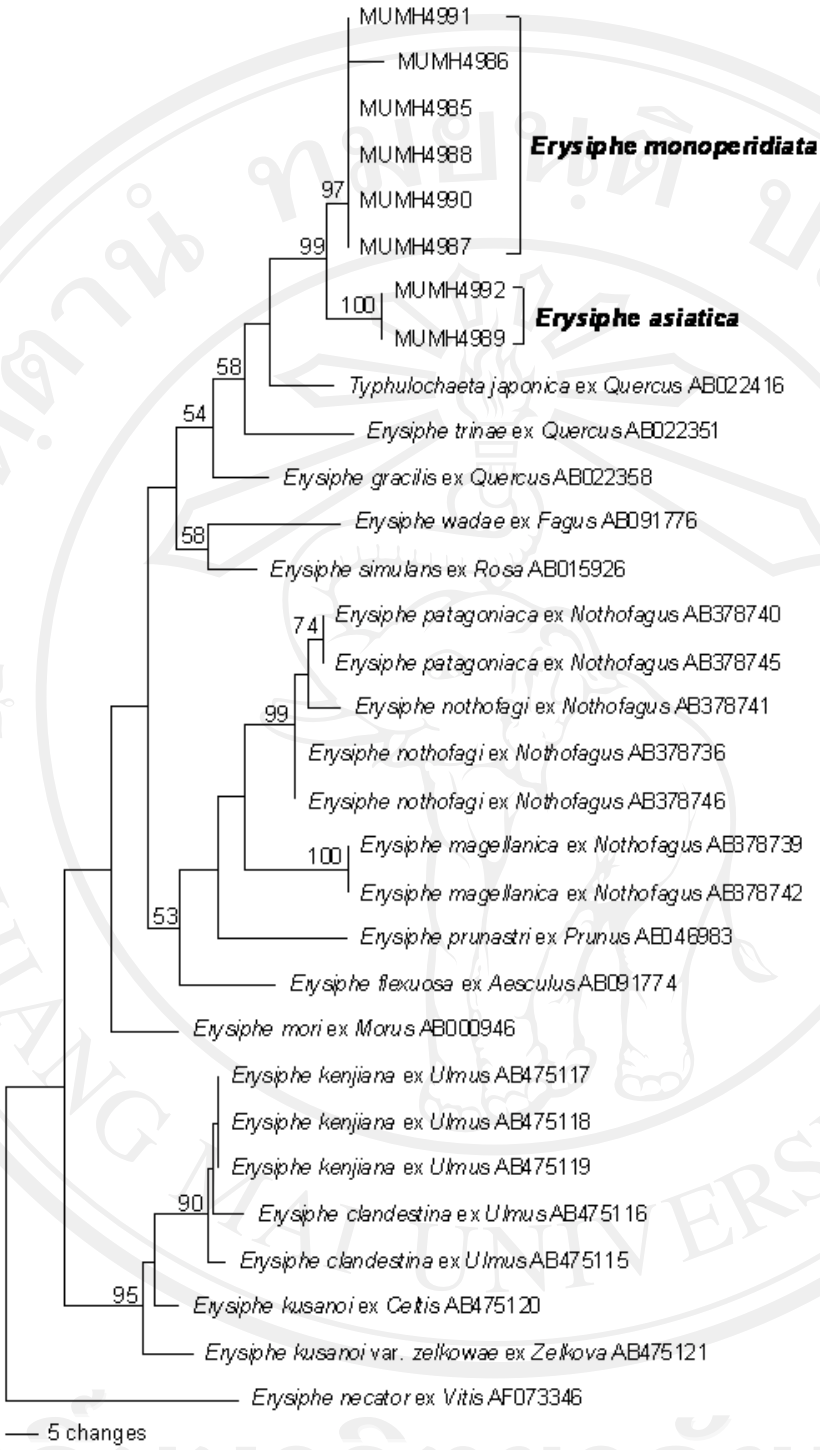

Fig. 5 – Phylogenetic analysis of the nucleotide sequences of the internal transcribed spacer (ITS) region including 5.8S rDNA for eight newly determined sequences and 23 sequences from *Erysiphe* species and *Typhulochaeta japonicae*. The tree is one of the 174 equally parsimonious trees with 311 steps, which was obtained by the parsimony ratchet method. Gaps were treated as missing data. Horizontal branch lengths are proportional to the number of nucleotide substitutions that were inferred to have occurred along a particular branch of the tree. Percentage bootstrap support (1000 replications; ≥50%) is shown on branches.

and *E. asiatica* by its much smaller ascospores. Epiphyllous mycelia of *B. kumanoensis* are shared by *E. monosperidiata*, but the former species differs from the latter one by its larger chasmothecia (80–90 μm). The present phylogenetic analysis indicates that *E. monoperidiata* and *E. asiatica* are closely related to *E. trinae* occurring on *Quercus agrifolia* in North America. However, they did not form a clade together in the phylogenetic tree (Fig. 5). In addition, *E. trinae* usually has 2-spored asci, which differs from *E. monoperidiata* and *E. asiatica* having 4–6-spored and 6–8-spored asci, respectively.

The present phylogenetic analysis indicates that *E. monoperidiata* and *E. asiatica* form a clade together with *T. japonica*, *E. trinae* and *E. gracilis* infecting *Fagaceae*. This clade belongs to a lineage consisting of fungi with uncinuloid appendages that formerly belonged to the genus *Uncinula*. Interestingly, *E. monoperidiata*, *E. asiatica*, *E. gracilis* and *E. trinae* have mycelioid appendages, and *T. japonica* has unique club-shaped appendages, which indicates that none of the species belonging to this clade has uncinuloid appendages. This result suggests that these different appendage shapes and a single layered peridium cells evolved on fagaceous hosts. Molecular phylogenetic analysis using more sequences from *B. cyclobalanopsidis* and *B. kumanoensis* is required for further and deeper discussions.

Acknowledgements

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Full Length Research Paper

Molecular and morphological characterization of *Phyllactinia cassiae-fistulae* (Erysiphaceae; Ascomycota) from Thailand

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Phyllactinia cassiae-fistulae **and its** ***Ovulariopsis*** **anamorph, a causal agent of powdery mildew on** ***Cassia fistula*****, have been found in Thailand for the first time. Phylogenetic analysis using the 28S ribosomal DNA sequences clearly demonstrated that** ***P. cassiae-fistulae*** **distinctly formed a unique clade at the basal part of** ***Phyllactinia*** **with 100% bootstrap support. This phylogenetic analysis supports the unique morphology of** ***P. cassiae-fistulae*** **anamorph having cylindrical-ellipsoil conidia and short conidiophores similar to** ***Oidium*** **species.**

Key words: Morphology, phylogeny, powdery mildew, *Cassia fistula*, *Senna siamea*.

INTRODUCTION

During the survey of powdery mildews from 2008 to 2011 in Northern Thailand, several interesting powdery mildews were discovered. One of them has been found on *Cassia fistula* and *Senna siamea* (Caesalpinioideae; Fabaceae) and was identified as *Phyllactinia cassiae-fistulae*. This species was first described by Paul and Thakur (2006) in India as a new variety, *P. bauhiniae* var. *cassia*, and later revised as *P. cassiae-fistulae* by Braun and Paul (2009). Kirschner and Chen (2008) demonstrated first record of this species on *C. fistula* in Taiwan (without teleomorphic stage) and reported detailed morphological characteristics of anamorphic stage. Anamorph of this fungus has a unique characteristic that is conspicuously distinct from all other species of *Phyllactinia*, but produced *Phyllactinia* teleomorph. Morphological observations showed conidiophore shorter than other *Ovulariopsis* species anamorph of *Phyllactinia* and showed production of cylindrical-ellipsoid conidia. This anamorphic feature is consistent with typical characteristic of *Oidium,* not *Ovulariopsis*.

In this study, molecular analysis combined with morphological analysis was performed to clarify taxonomy of *P. cassiae-fistulae*. This study is the first report of *P. cassiae-fistulae* from Thailand, and also the first report of this species on *S. siamea* in the world.

MATERIALS AND METHODS

Sample sources

Specimens were collected in the northern Thailand (Chiang Mai Province) from December to March during 2009. 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.

Morphological observation

Fresh specimens of powdery mildew on *C. fistula* leaves were examined by using a light microscope with 20 and 40x objective phase contras t lenses. Morphological observation on fungal

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colonies of anamorphic stage was stripped off from the leaf surfaces with clear adhesive tape or with a clean needle on teleomorphic stage, mounted on a microscope slide in distilled water. Morphological characteristics were measured in 30 replicates for each structure on anamorph: size and shape of conidia, conidiophore; position of the basal septum; shape and position of hyphal appressoria and presence or absence of fibrosin bodies (To-anun et al., 2005); on teleomorph: size and shape of chasmothecia, appendages, asci, ascospores (To-anun et al., 2003). Observation of conidial and ascospore germ tubes were carried out using the method of Hirata (1942).

Molecular phylogenetic analysis

Whole-cell DNA was extracted from mycelia or conidia using the chelex method (Walsh et al., 1991; Hirata and Takamatsu, 1996). The 28S ribosomal DNA (rDNA) including the domains D1 and D2, and ITS region including the 5.8S rDNA were amplified by the polymerase chain reaction (PCR) using nested primer sets. 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 30 s at 95°C followed by 30 s at 52°C for annealing, and 30 s at 72°C for extension; and a final extension cycle at 72°C for 7 min.

The following primer sets were used for amplified 28S rDNA (large subunit): PM3 (5'-GKGCTYTMCGCGTAGT-3') (Takamatsu and Kano, 2001), TW14 (5'GCTATCCTGAGGGAAACTTC-3'), NL1 (5'-AGTAACGGCGAGTGAAGCGG-3') and NLP2 (5'-GGTCCCAACAGCTATGCTCT-3') (Mori et al., 2000). 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 for amplification. A *Phyllactinia* and *Leveillula* specific primer Ph7 (TGTTGCTTTGGYAGGCCG) 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 nucleotide fragments of the second PCR products were sent to SolGent Co. (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.

The nucleotide sequences of rDNA were aligned with MUSCLE program (Edgar, 2004). 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). 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 50% support at that node. A tree with the highest likelihood value among the equal parsimonious trees was determined by PAUP 4.0 (Swofford, 2001).

RESULTS

Morphological observation

Symptoms

The symptoms appeared on the lower side of the leaves by produce effuse, thin to dense white colonies from the end of November. Chasmothecia production (perfect stage) was seen from mid-January. Chasmothecia have not been found every year in the same tree. The symptoms were only found on leaves. The severe infected leaves caused early leaf defoliation.

Anamorph

Mycelium hypophyllous, white, thin to dense, hyaline; appressoria nipple-shaped, rarely lobed to elongated; conidiophores arising from ectophytic hyphae, on upper surface of mother cells, position not central, rarely central, erect, straight or slightly bent (41–)86–173(–200) × (7–)10–15(–17) µm; mother cells forming conidia singly, (24–)31–80(–89) × 4–6 µm; foot-cells straight with a basal septum near branching point of mycelium up to away from it (28–)44–100(–151) × 3–6 µm; conidia cylindrical-ellipsoid, (32–)38–50(–54) × (10–)13–17(–20) µm, hyaline without conspicuous fibrosin-bodies, produce solitary on conidiophores and conidial germinates at the end, long branch, sometime rarely lobed, and formed *Pseudoidium* type (Figure 1).

Teleomorph

Chasmothecia scattered to gregarious, (126–)163–198(–210) µm, brown-blackish; appendages 5–13 in number, acicular with bulbous basal swelling, (67–)129–207(–305) × (20–)27–32(–34) µm, apex subacute or subobtuse, hyaline; penicillate cells in the upper part; asci numerous, sessile, (43–)49–63(–84) × (25–)27–32(–40) µm, 2-spored; ascospores ellipsoid-ovoid, rarely subglobose, (21–)24–40(–46) × (10–)13–16(–20) µm (Figure 2).

Phylogenetic analysis

The 28S rDNA sequences consisted of two sequences from *C. fistula* and one sequence from *S. siamea* were aligned with 24 sequences of *Leveillula*, *Phyllactinia* and *Pleochaeta* retrieved from DNA database (Takamatsu et al., 2008). *Pleochaeta shiraiana* was used as an outgroup taxon based on Takamatsu et al. (2008). The alignment data matrix consisted of 27 sequences and 610 total characters. Of these, 518 characters were constant, 26 characters were variable and parsimony-uninformative, and 66 characters were informative for parsimony analysis. A total of 201 equally parsimonious trees (CI = 0.6628, RI = 0.8366, RC = 0.5545) with 172 steps were constructed by the parsimony ratchet analysis. A tree with the highest likelihood value among the 201 trees is shown in Figure 3. *P. cassiae-fistulae* sequences deposited in DDBJ under the accession number AB691226 and AB691227 including *Ovulariopsis* anamorph on *S. siamea* AB691228 distinctly formed an

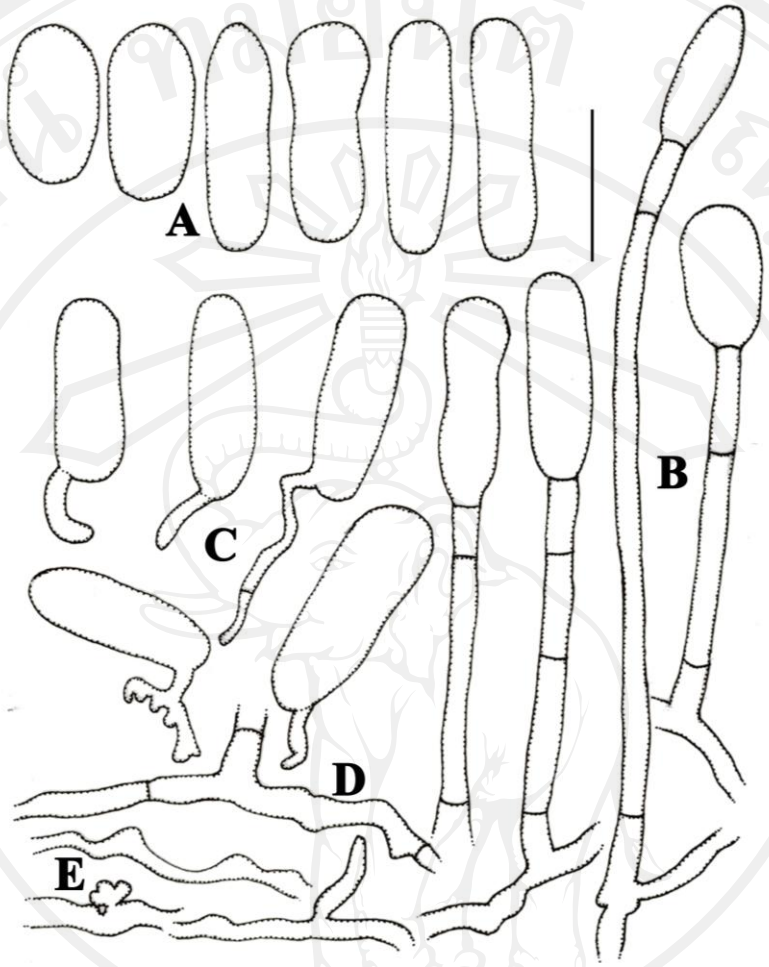

Figure 1. Morphological characteristics of *Ovulariopsis* anamorph of *P. cassiae-fistulae* on *C. fistula*, illustrated using a line drawing under a light microscope (400x). (A) Conidia (B) conidiophores (C) conidia with germ tubes of the *Pseudoidium* type (D) mother cell leading to conidiophores and (E) mycelia with appressoria (bar 30 µm).

independent clade at the basal part of *Phyllactinia/Leveillula* clade with bootstrap support (BS) of 100%. There was one base nucleotides substitution between isolates on *C. fistula* and *S. siamea* that suggest close relation to each other. However, specimens on *C. fistula* formed small clade from *S. siamea* which was supported by 62% BP value.

We also determined the rDNA ITS sequences for five samples of *P. cassiae-fistulae* on *C. fistula* and conducted FASTA search at the EMBL DNA database (http://www.ebi.ac.uk/embl/) using the sequences as queries. The highest similarities were obtained with *P. angulata* AB080566 (76.9%) and next with *P. chubutiana* AB243690 (75.8%). This result indicates that *P. cassiae-fistulae* is genetically isolated among *Phyllactinia* species. Because we could not obtain unambiguous alignment of *P. cassiae-fistulae* with other *Phyllactinia* species in ITS sequences, we did not conduct phylogenetic analysis of ITS sequences. Sequences analysis of ITS region further support the isolated phylogenetic situation of *P. cassiae-fistulae* among *Phyllactinia* species shown in the 28S rDNA analysis (Figure 3).

DISCUSSION

Several powdery mildew species have been reported on *Cassia* (Sattar and Hussain, 1976; Thaung, 2007; Zhao et al., 2010) in the world. However, there is no record of powdery mildew on *Cassia* in Thailand. This is the first report of powdery mildew on *Cassia* in Thailand. The morphological observations of anamorph of *P. cassiae-*

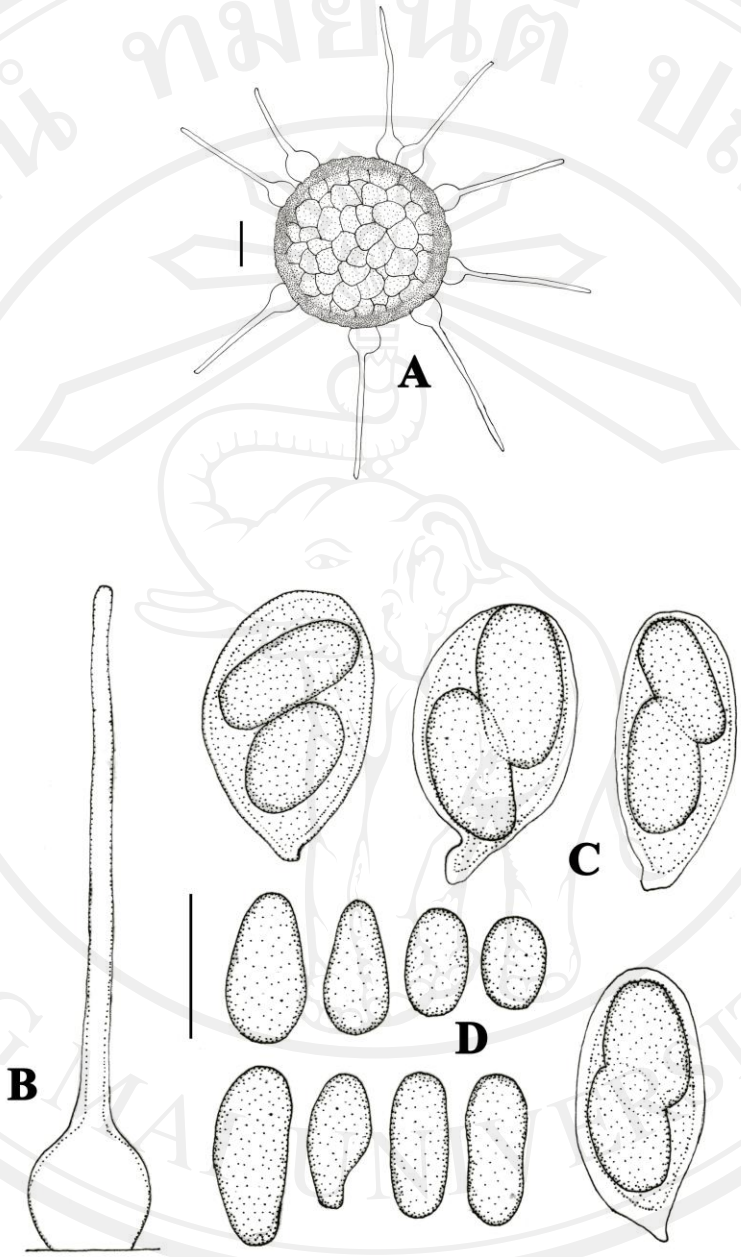

Figure 2. Drawing of teleomorph of *P. cassiae-fistulae* on *C. fistula*, illustrated using a line drawing under a light microscope (200 and 400X). (A) Chasmothecium (B) acicular appendage with bulbous base (C) asci (D) ascospores (bar 50 µm in A, bar 30 µm in B to D).

fistulae demonstrated that the cylindrical-ellipsoid conidia are quite distinct from other known *Phyllactinia* species having lanceolate conidia (Braun, 1987; Paul and Thakur, 2006; Braun and Paul, 2009). However, this fungus produced chasmothecia having acicular appendages with a bulbous swelling at the base that is a typical character of *Phyllactinia* (Braun, 1987).

Oidium cassiae-siameae has been recorded as a powdery mildew on *Cassia* (Amano, 1986; Braun, 1987). Kirschner and Chen (2008) described and illustrated a powdery mildew on *C. fistula* and compared it with *O. cassiae-siameae* specimen. The result revealed that the powdery mildew on *C. fistula* has morphology similar to *Oidium* species, but quite differs from *Oidium* species by

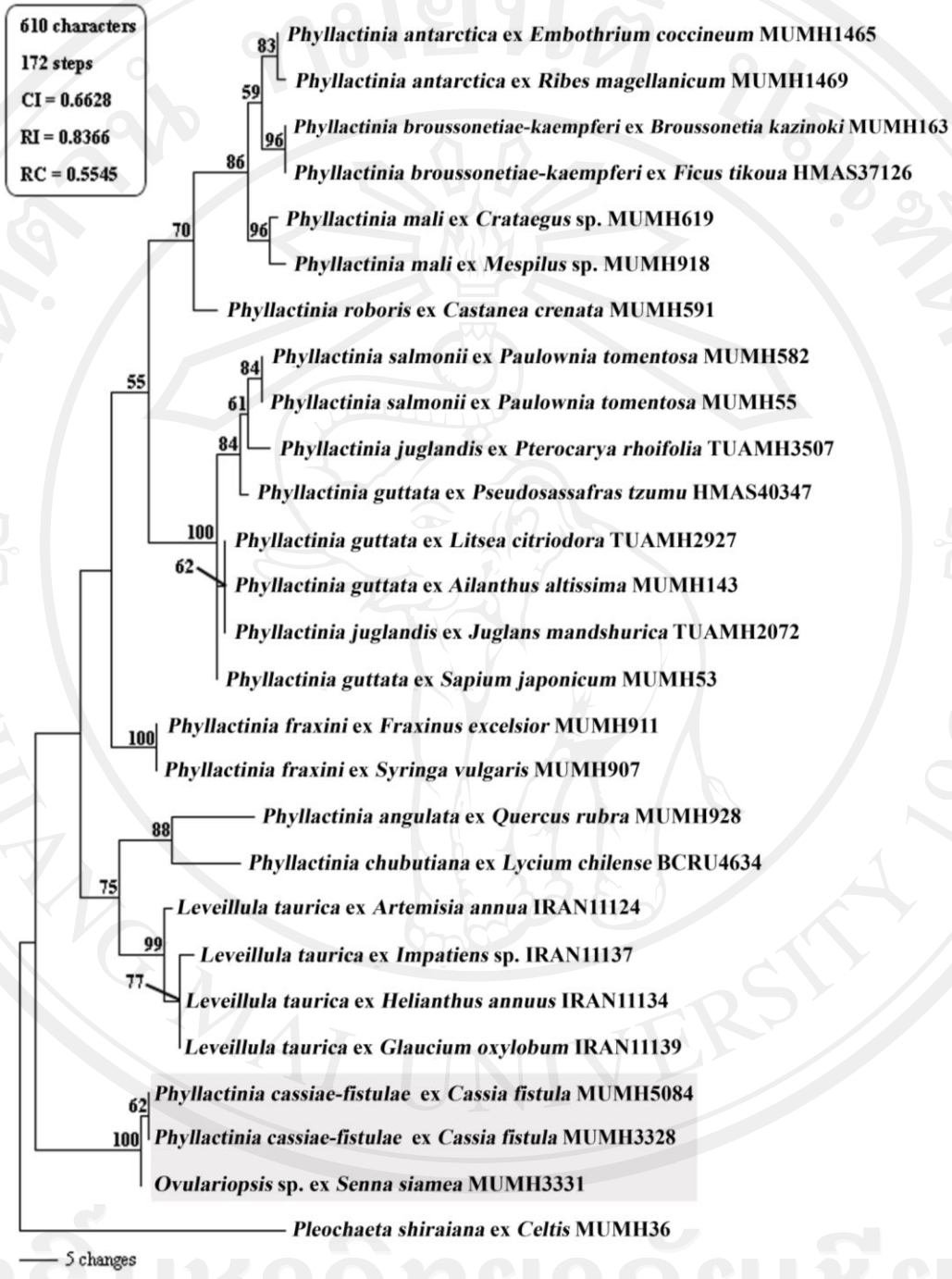

Figure 3. A parsimony ratchet tree based on the 28S rDNA sequences for 27 taxa, consisting of three sequences from *P. cassiae-fistulae* and 24 sequences of *Phyllactinia*, *Leveillula* and *Pleochaeta* retrieved from DNA database. Horizontal branch lengths are proportional to the number of nucleotide substitutions that were inferred to have occurred along a particular branch of the tree. Bootstrap values (≥50%) are shown above branches (CI = 0.6628, RI = 0.8366, RC = 0.5545).

produced endophytic hyphae. This endophytic behavior is typical appearance of the tribe *Phyllactinieae* and its host species is the same with that reported as a host of *P. cassiae-fistulae* (Braun and Paul, 2009).

The present study is the first report of phylogenetic analysis of *P. cassiae-fistulae*. The result indicated that the 28S rDNA sequences from three *P. cassiae-fistulae* isolates on *C. fistula* and *S. siamea* formed an independent

clade at the basal part of *Phyllactinia*/*Leveillula* clade with bootstrap support of 100%, and are sister to all other *Phyllactinia* and *Leveillula* sequences. This result may indicate that *Phyllactinia* is paraphyletic group as described by Takamatsu et al. (2008). Additionally, this phylogenetic clade showed the closest related between *P. cassiae-fistulae* on *C. fistula* and *S. siamea*. Therefore, molecular phylogenetic analysis based on the 28S rDNA sequences supported the unique anamorphic morphology of *P. cassiae-fistulae.* The isolated phylogenetic placement of *P. cassiae-fistulae* was also supported by the ITS sequence analysis.

A recent molecular phylogenetic study of the genera in the subtribe Cassiinae (Acharya et al., 2011) demonstrated that *C. fistula* and *S. siamea* are classified into *Cassia sensu lato.* The present study showing that both *C. fistula* and *S. siamea* are commonly infected by *P. cassiae-fistulae* supports the close relationship of the host plants.

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