CHAPTER 4

CERCOSPOROID FUNGI ASSOCIATED WITH WEEDS

4.1. Introduction

Weeds continue to cause damage in agriculture, reducing yield and quality of crops by competing for water, nutrients, and sunlight with cultivated plants. With a growing interest in the use of pathogenic organisms for weeds control by both the classical and bioherbicide approaches, it is becoming increasingly important to compile a more complete record of these organisms. Because many of the weeds, exotic or non-exotic, become problematic in agricultural ecosystems and also in natural ecosystems, undoubtedly, natural parasites such as pathogenic fungi play an important role within the population dynamics of any weeds species. Of them, the cercosporoid fungi is one of many other groups of pathogenic fungi which are potential as a source of bioherbicide because this group is nearly universally pathogenic, occurring on a wide range of hosts in almost all major families of dicotyledonous most monocotyledonous families, some gymnosperms and ferns (Pollack, 1987). Therefore, preliminary surveys on exploitation and collection of such pathogenic fungi encompass database are always very important tasks to be carried out particularly for plant pathologists and quarantine researchers, even though, the process to the commercialization of the biological control agent from the pathogenic fungi is definitely a long way to go.

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Table 9 List of the cercosporoid fungi associated with weeds in this study.

Host	Pathogen
Christella parasitica	*Cercospora christellae* Meeboon, Hidayat, and To-anun
Conyza sumatrensis	*Cercospora nilghirensis* Govindu and Thirum.
Cosmos sulphureus	*Pseudocercospora cosmicola* (A. K. Kar and M. Mandal) Deighton
Datura alba	*Pseudocercospora daturina* (J. M. Yen) Deighton
Eupatorium adenophorum	*Cercospora eupatorii* Sacc.
Mikania cordata	*Cercospora mikaniicola* F. Stevens
Pueraria phaseoloides	*Pseudocercospora cruenta* (Sacc.) Deighton
Solanum verbascifolium	*Cercospora physalidis* Ellis
Solanum xanthocarpum	*Pseudocercospora egenula* (Syd.) U. Braun and Crous
Tridax procumbens	*Cercospora tridacis-procumbentis* Govindu and Thirum.
Vitex quinata	*Pseudocercospora viticicola* (J. M.Yen and Lim) J. M. Yen

In this study, 11 species of the cercosporoid fungi were recorded associated with weeds including one new species, *Cercospora christellae* associated with exotic weed *Christella parasitica* (Linn.) H. Lév. (table 9). *Christella parasitica* is a relatively small fern (stipe about 50 cm long) belongs to the plant family *Thelypteridaceae* (Tagawa and Iwatsuki, 1979). The fern is a common weed growth in several plantations and evergreen forests areas of Thailand, and also generally distributed in tropics and subtropics areas in Asia, from southern part of Japan to New Zealand (Tagawa and Iwatsuki, 1979). In this chapter, the new species is described

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and elucidated morphology and phylogenetically. The phylogenetic examination was carried out by analyzing internal transcribed spacer (ITS) region of nuclear ribosomal DNA (rDNA).

4.2. Materials and methods

Collection sites and morphological examination

Specimens of *Christella parasitica* was collected at several citrus plantations in Hang Dong district, Chiang Mai province, Thailand in February 2008. Specimens with disease symptoms of the cercosporoid fungi on leaves were collected during the course of field trips by using a ×10 and ×20 magnifying lens. Detailed observations of morphological characters were carried out by means of an OLYMPUS BX51 (OLYMPUS®, Japan) light microscope using oil immersion (×100). Specimens for microscopic observation were prepared by hand sectioning. Water and Shear's solution were used as mounting media. Thirty conidia, hila, conidiophores, conidiogenous loci, and 10 stromata were measured for each specimen. Line drawings were prepared at a magnification of ×400 and ×1000. Voucher specimen has been deposited at BIOTEC Bangkok Herbarium (BBH), Thailand. Cultures isolated from the specimens have also been deposited at BIOTEC Bangkok Culture Collection (BCC), Thailand and Molecular laboratory of Department of Plant Pathology, Chiang Mai University, Chiang Mai, Thailand.

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Molecular characterization

DNA extraction, Polymerase Chain Reaction (PCR) and sequencing

In this study, molecular characterization was carried out in order to elucidate the phylogenetic relationship with other related anamorphic taxa within *Mycosphaerella*. Total genomic DNA was extracted from fungal mycelia cultured on Malt Extract Agar (Difco, USA) following a 2 × cetyltrimethylammoniumbromide (CTAB) protocol (Cai *et al.*, 2005). DNA amplification of ITS region of nrDNA was performed by polymerase chain reaction (PCR) using ITS4 (5′-TCCTCCGCTTA TTGATATGC-3′) and ITS5 (5′-GGAAGTAAAAGTCGTAACAAGG-3′) primers (White *et al.*, 1990) to generate about 587 nucleotides from the complete ITS, including 5.8S rDNA region. The amplification condition was performed in a 50 ml reaction volume as follows: 1 × PCR buffer, 0.2 mM each dNTP, 0.3 mM of each primer, 1.5 mM $MgCl_2$, 0.8 units Amplitaq Taq Polymerase (Perkin-Elmer, Foster City, CA, U.S.A.), and 10 ng DNA. PCR parameters for all the regions were performed as follows: initial denaturation at 94ºC for 3 min, followed by 30 cycles of 94ºC for 1 min, 52ºC for 50 s, 72ºC for 1 min, and final extension of 72ºC for 10 min.

The characterization of PCR products was performed via agarose gel electrophoresis on a TAE 1% agarose gel containing Ethidium Bromide (EtBr) as the staining agent. The PCR product was purified using Qiaquick purification kit (Qiagen) and DNA concentration of the PCR products was subjected to automatic sequencing (ABI PRISM Dye Terminator Cycle Sequencing and ABI PRISM Sequencer model 377, Perkin Elmer). The new ribosomal DNA sequence has been deposited in GenBank under accession number FJ460222, and the GenBank accession numbers of the other sequences and taxa used to construct the phylogenetic trees were shown in

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figure 160. The nrDNA sequences of *Cladosporium cladosporioides* (Fresen.) G. A. de Vries and *C. oxysporum* Berk. and M. A. Curtis were assigned as outgroup.

Sequence alignment and phylogenetic analyses

Sequence obtained from the respective primers (ITS5 and ITS4) was aligned in Clustal X (Thomson *et al.*, 1997) and BioEdit (Hall, 1999). The sequences alignments were also refined by direct examination. Regions designated as ambiguously aligned were excluded from the analyses. Gaps were treated as missing data. Phylogenetic analyses were performed in PAUP version 4.0b10 (Swofford, 2002).

Unweighted Maximum Parsimony (UMP) analysis was performed in this study in order to confirm the phylogenetic relationship with related taxa. Trees were inferred using the heuristic search option with 1000 random sequence additions. Maxtrees were unlimited, branches of zero length were collapsed and all multiple parsimonious trees were saved. Descriptive tree statistics (tree length [TL]. consistency index [CI], retention index [RI], related consistency index [RC], homoplasy index [HI], and log likelihood [-ln L]) were calculated for trees generated under different optimality criteria. The Kishino-Hasegawa (KH) likelihood test (Kishino and Hasegawa, 1989) was carried out using PAUP to compare the best tree topology obtained by the nucleotide sequence data with a constrained tree. Clade stability was assessed in bootstrap analyses with 1000 replicates, each with 1000 replicates of random stepwise addition of taxa. Random sequence addition was used in the bootstrap analyses. Trees were figured in TreeView (Page, 1996). Other details are outlined in Cai *et al.* (2005).

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4.3. Results

Taxonomy

Cercospora christellae Meeboon, Hidayat and To-anun **sp. nov.** – **Figures 160**

Differt a C. apii sensu lato (C. cyclosori) et C. abacopteridis caespituli epiphylli, stromatibus bene evolutis, (10) 12.7 ± 2.1 (16) µm diam., conidiophoris abundis vel dense fasciculatis, conidiis basim truncata vel obconice truncata.

Etymology: *christellae*, derived from the genus name of the host plant.

Leaf spots amphigenous, distinct, irregular, rarely orbicular, brown throughout, often whitish to paler at the center, 1.5 - 5 mm diam., often limited by leaf veins *Caespituli* epiphyllous. *Stromata* intraepidermal, well-developed, (10) 12.7 ± 2.1 (16) µm diam. (n = 10), globose to subglobose, composed of 9-16-dark brown cells. – *Conidiophores* in densely fascicles of 5-14, cylindrical, arising through the plant epidermis, 3-13 septate, narrower toward the apex, unbranched, strongly geniculate throughout conidiophores, straight to slightly sinuose or curved, smooth, (68.9) 220 ± 106.2 (413.3) × (2.5) 4.5 ± 0.8 (4.9) µm (n = 30), brown at the base and paler towards the apex. *Conidiogenous cells* terminal or intercalary, holoblastic, polyblastic, integrated, proliferating sympodially, (14.8) 24.7 ± 9.6 (49.2) × (2.5) 4.1 ± 1.1 (4.9) µm (n = 30), pale brown. *Conidiogenous loci* protuberant, thickened, darkened, (2) 2.8 ± 0.3 (3) µm diam. (n = 30), 1-3 per cell. *Conidia* solitary, obclavate-filiform to acicular, straight to slightly curved, truncate to obconically truncate at base, acute to subobtuse at the apex, (35.8) 123.1 ± 53.4 (205.4) × (1.2) 2.7 ± 0.8 (3.7) µm (n = 30),

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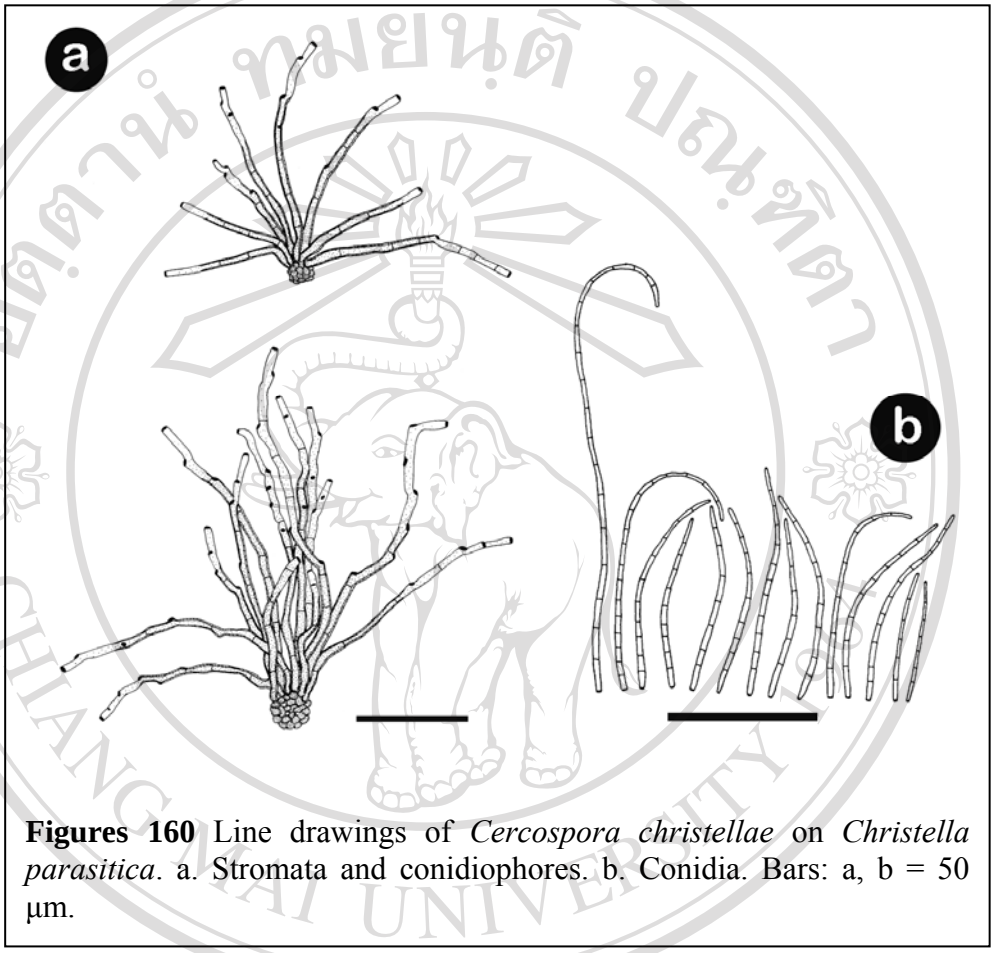
3-17-septate, hyaline, smooth, hila thickened, and darkened, (1.5) 1.8 ± 0.2 (2) μm diam. (n = 30).



Figures 160 Line drawings of *Cercospora christellae* on *Christella parasitica*. a. Stromata and conidiophores. b. Conidia. Bars: a, b = 50 μm.

On PDA slow growing, smooth to folded, dark brown, white to smoke gray at the surface, producing red pigment in the medium.

Specimen examined: THAILAND, Chiang Mai Province, Hang Dong, on living leaves of exotic weed *Christella parasitica* (Linn.) Lév. (*Thelypteridaceae*), 29 February 2008, Iman Hidayat (BBH 23574); type culture (BCC 32464 and 32465).

Habitat: Leaf spots of *Christella parasitica* (*Thelypteridaceae*).

Distribution: Chiang Mai, Thailand (type locality).

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4.4. Discussion

This fungus was identified as *Cercospora s. str.* due to having pigmented conidiophores, thickened and darkened conidiogenous loci, and hyaline filiform to scolecoid conidia (Crous and Braun, 2003). Recently, only four species of the cercosporoid fungi have been recorded associated with the plants of family *Thelypteridaceae*, viz, *Cercospora abacopteridis* J. M. Yen and Lim (Yen and Lim, 1973), *C. cyclosori* Goh and W. H. Hsieh (Hsieh and Goh, 1990), *Pseudocercospora abacopteridicola* (J. M. Yen and Lim) J. M. Yen (Yen and Lim, 1980), and *P. phyllitidis* (H. H. Hume) U. Braun and Crous (Crous and Braun, 2003).

Cercospora cyclosori is currently classified as *C. apii s. lat.* based on the present concept of the cercosporoid fungi (Crous and Braun, 2003), but *C. christellae* distinct from the plurivorous *C. apii s. lat.* (Crous and Braun, 2003) by having epiphyllous caespituli, well-developed stromata, numerous and densely fasciculate conidiophores with strongly geniculate throughout, and conidia obclavate-filiform under natural condition with obconically truncate base and smaller hila (1.5-2 μm wide) (Figures 160). Another species, *C. abacopteridis,* was described by Yen and Lim (1973) as having amphigenous symptom, hypophyllous caespituli, lacking of stromata, conidiophores solitary to 2-8 fasciculate (15-118 × 4-5 μm), and conidia acicular to filiform (62-400 × 2-4 μm). However, *C. christellae* is easily distinguishable from *C. abacopteridis* by its epiphyllous caespituli, well-developed stromata, and conidiophores in rich fascicle.

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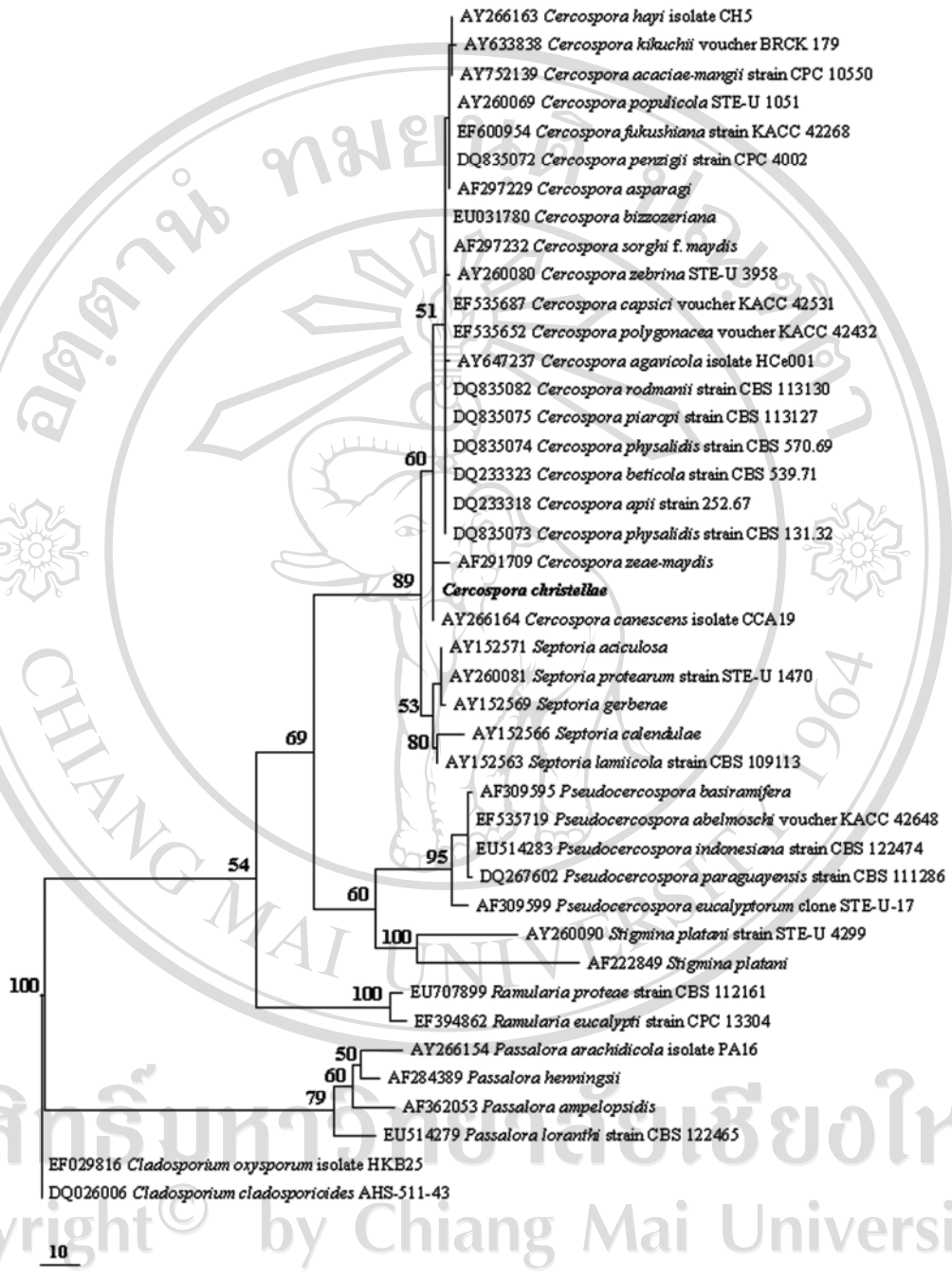

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Figure 161 Single parsimonious tree based on ITS nrDNA sequence data representing placement of *Cercospora christellae* within representatives of the family *Mycosphaerellaceae*. The tree is obtained from heuristic search with 1000 random taxon addition of the sequences alignment. Bootstrap values (>50%) from 1000 replicates of Unweighted Maximum Parsimony (UMP) analyses are shown above internodes

Since the combination of the morphological and phylogenetic analyses of new proposed taxa in *Cercospora* complex is quite important in order to avoid misidentification, therefore, molecular phylogenetic analysis of ITS region of nuclear ribosomal DNA of *C. christellae* was carried out in order to confirm the morphological elucidation of the fungus with related taxa, particularly the *Cercospora*-complex sensu Crous and Braun (2003) of the *Mycosphaerella* anamorphs.

The alignment of newly ITS sequences of *C. christellae* data matrix with 41 sequences retrieved from NCBI GenBank DNA database consists of 42 taxa. The data matrix yielded 486 total characters of which 327 characters were constant, 31 characters were variable and parsimony-uninformative, and 128 characters were parsimony-informative. Numbers of parsimonious trees retained from UMP analysis were 270, sum of minimum possible lengths is 215, and sum of maximum possible length was 762. The best parsimonious tree selected by using KH test was generated in 300 steps (CI = 0. 717, RI = 0. 845, RC = 0.605, HI = 0. 283).

Phylogenetic tree obtained from UMP analysis method was shown in figure 161. Based on this analysis, each of the three genera described as the true cercosporoid fungi by Crous and Braun (2003), such as, *Cercospora*, *Pseudocercospora*, and *Passalora*, formed well-supported clades with bootstrap support more than 59%. Of them, *Passalora* clade appeared as a basal group in the phylogenetic tree with 79% bootstrap support. This result is in a concordance with the phylogenetic tree generated from ITS region analysis reported by Stewart *et al.* (1999) that indicated the monophyletic of those tree genera, and *Passalora* clade remains as a

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basal group. The basal position of *Passalora* clade indicated that species in *Passalora* hold more plesiomorphic characters than other true cercosporoid clades.

The new species, *C. christellae*, together with other *Cercospora* species formed a monophyletic clade with 60% bootstrap support, and this clade appeared as a sister group to *Septoria* clade with 89% bootstrap support which is indicated a close relationship between the two genera. In addition, genus *Septoria*, a coleomycetous fungus, shares similar morphology characteristics to *Cercospora* in having holoblastic and sympodial of conidia proliferation, and hyaline, filiform to acicular and multiseptate conidia (Sutton, 1980). However, the two genera are well separated morphologically due to *Septoria* produced pycnidial conidiomata instead of stromata of genus *Cercospora*, although Verkley and Starink-Willemse (2004) insisted that conidiomatal structure seems to have little predictive value for phylogenetic relatedness. Therefore, more taxa are definitely required to analyze the relationship between the two genera. Another clade of the true cercosporoid fungi, *Pseudocercospora* clade, appeared as sister group to *Stigmina* clade with 60% bootstrap support. Based on morphology characteristics, the two taxa are similar in having holoblastic and terminal conidia proliferation, and obclavate to filiform-acicular with truncate base and multiseptate conidia, however, the two genera differ due to verrucose conidia of *Stigmina* with sometimes dark brown and produced longitudinal septation which is quite different to *Pseudocercospora s. str.* that having smooth and subhyaline conidia with only transverse septation, and unthickened conidial loci and hila.

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