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## ***Cercospora christellae*, a new cercosporoid fungus associated with weed *Christella parasitica* from northern Thailand**

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This report is recorded a further contribution to the survey of *Cercospora* and allied genera in Thailand which has been conducting since 2005. The main aim of the study was to provide a comprehensive database of this group of fungi in this region in order to assist the identification and detection. *Cercospora christellae*, found on weed *Christella parasitica*, is described here as a new species. The morphology and phylogenetic examination of internal transcribed spacer (ITS) region of nuclear ribosomal DNA (nrDNA) confirm the new proposed taxon.

**Key words:** diversity, hyphomycetes, leaf spot, phylogenetic, taxonomy, weeds

### **Introduction**

*Christella parasitica* (Linn.) H. Lév. is a relatively small fern (stipe about 50 cm long) that 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). Because many of the weeds, exotic or non-exotic, become problematic in agricultural ecosystems and also in natural ecosystems, therefore, natural parasites such as pathogenic fungi undoubtedly play an important role within the population dynamics of any weeds species. 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

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process to the commercialization of the biological control agent from the pathogenic fungi is definitely a long way to go.

The present work of the survey of biodiversity of *Cercospora* Fresen. and its allied genera in Thailand has been conducting since 2005, with the aim of providing a comprehensive database of this group of fungi in this region. To date, one hundred and six species of cercosporoid fungi associated with various hosts such as crops, weeds and ornamentals plants have been recorded from several locations in Thailand (Meeboon *et al.*, 2007a, b, c, d, 2008; Nakashima *et al.*, 2007). In this paper, one new species of *Cercospora sensu stricto* (s.str.) associated with *C. parasitica* is proposed. The new species is elucidated morphologically and also phylogenetically by examining ITS region of nuclear ribosomal DNA (nrDNA).

## **Materials and methods**

### ***Collection sites and morphological examination***

Specimens of *Christella parasitica* were collected at several citrus plantations in Hang Dong district, Chiang Mai province, Thailand in February 2008. The specimens with disease symptoms of cercosporoid fungi on leaves, detected by using a 10x and 20x magnifying lens, were collected during the course of field trips. Detailed observations of morphological characters were carried out by means of an OLYMPUS BX51 (OLYMPUS®, Japan) light microscope using oil immersion (1000x). 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 400x and 1000x. 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.

### ***Molecular characterization***

#### ***DNA extraction, Polymerase Chain Reaction (PCR) and sequencing***

In this report, molecular characterization was carried out in order to confirm the morphology-based identification, and to elucidate the phylogenetic relationship with other related anamorphic taxa within *Mycosphaerella* Johanson teleomorph. Total genomic DNA was extracted from fungal mycelia

cultured on Malt Extract Agar (Difco, USA) following a 2x cetyltrimethylammoniumbromide (CTAB) protocol (Rogers and Bendich, 1994). DNA amplification of ITS region of nrDNA was performed by polymerase chain reaction (PCR) using ITS4 (5'-TCCTCCGCTTATTGATA-TGC-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: 1x PCR buffer, 0.2 mM each dNTP, 0.3 mM of each primer, 1.5 mM MgCl<sub>2</sub>, 0.8 units AmpliTaq Taq Polymerase (Perkin-Elmer, Foster City, CA, USA), and 10 ng DNA. PCR parameters for all the regions were performed as follows: initial denaturation at 94 °C for 3 min, followed by 30 cycles of 94 °C for 1 min, 52 °C for 50 s, 72 °C for 1 min, and final extension of 72°C 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 nrDNA 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 figure 3. The nrDNA sequences of *Cladosporium cladosporioides* (Fresen.) G.A. de Vries and *Cladosporium oxysporum* Berk. and M.A. Curtis were assigned as outgroup.

### ***Sequence alignment and phylogenetic analysis***

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 analysis. 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 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).

## Results

### Taxonomy

*Cercospora christellae* To-anun, Hidayat and Meeboon, sp. nov. MycoBank No. MB 514196 (Fig. 1a-b)

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

Differ 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 truncatis vel obconice truncatis.

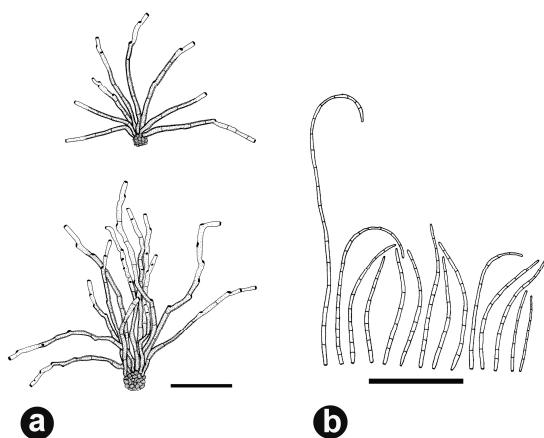
*Leaf spots* amphigenous, distinct, irregular, rarely orbicular, brown throughout, often whitish to paler at the center, 1.5 - 5 mm diam., often limited by leaf veins. *Caespituli* epiphyllous. *Stromata* intraepidermal, well-developed, (10) 12.7 ± 2.1 (16) µm diam. (n = 10), globose to subglobose, composed 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), 3-17-septate, hyaline, smooth, hila thickened, and darkened, (1.5) 1.8 ± 0.2 (2) µm diam. (n = 30).

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

*Habitat:* leaf spots of *Christella parasitica* (Linn.) H. Lév. (Thelypteridaceae).

*Distribution:* Chiang Mai, Thailand (type locality).

*Material examined:* THAILAND, Chiang Mai Province, Hang Dong, on living leaves of *Christella parasitica* (Linn.) Lév. (Thelypteridaceae), 28 February 2008, Iman Hidayat (BBH 23574: **holotype** designed here), (BCC 32464: **type cultures**, BCC32465)



**Fig. 1.** Line drawings of *Cercospora christellae* sp. nov. on *Christella parasitica* (from holotype). a. Stromata and conidiophores. b. Conidia. Bars: a, b = 50 µm.

## Discussion

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

*Cercospora cyclosori* is currently classified as *C. apii* *sensu lato* (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. 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) (Figs. 1, 2). Another *Cercospora* species from the family Thelypteridaceae, *C. abacopteridis*, was described by Yen and Lim

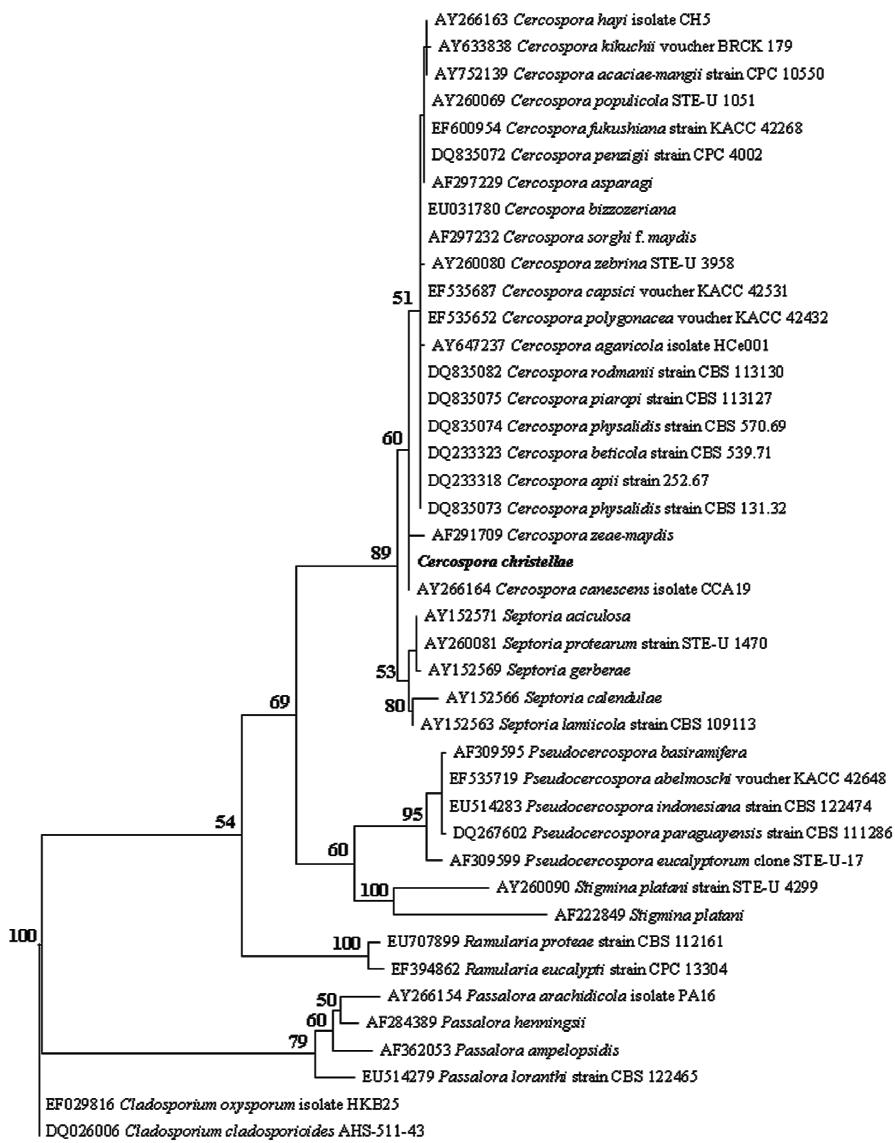
(1973) as having amphigenous symptom, hypophyllous caespituli, lacking of stromata, conidiophores solitary to 2-8 fasciculate ( $15\text{-}118 \times 4\text{-}5 \mu\text{m}$ ) and conidia acicular to filiform ( $62\text{-}400 \times 2\text{-}4 \mu\text{m}$ ). However, *C. christellae* is easily distinguishable from *C. abacopteridis* by its epiphyllous caespituli, well-developed stromata and conidiophores in rich fascicle.

Since the combination of the morphological and phylogenetic elucidation 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 morphology-based identification of the fungus with closely related taxa, particularly the *Cercospora*-complex anamorphs of the *Mycosphaerella* fide Crous and Braun (2003).

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 2. Based on this analysis, each of the three genera described as true cercosporoid fungi by Crous and Braun (2003), viz, *Cercospora*, *Pseudocercospora* Speg. and *Passalora* Fr., 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 with *Passalora* clade remain as a 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 (Fig. 2). This clade appeared as a sister group to *Septoria* Sacc. clade with 89% bootstrap support which is indicated a close relationship between the two genera. *Septoria*, a coelomycetous fungus, shares similar morphology characteristics to *Cercospora* in having holoblastic and sympodial of conidia proliferation, as well as hyaline, filiform to acicular and multiseptate conidia (Sutton, 1980). However, the two



**Fig. 2.** 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.

genera are well separated due to *Septoria* produced pycnidial conidiomata instead of stromata of the genus *Cercospora*. These genera are maintained as separate taxa, although Verkley and Starink-Willemse (2004) noted that conidiomatal structure seems to have little predictive value for phylogenetic relatedness. Therefore, more taxa are probably required to analyze the relationship between the two genera.

On the other hand, *Pseudocercospora* clade appeared as sister group to *Stigmina* Sacc. clade with 60% bootstrap support. Morphologically, these two genera are similar in having holoblastic and terminal conidia proliferation, as well as obclavate to filiform-acicular with truncate base and multiseptate conidia, however, they differs due to conidia of *Stigmina* are verrucose with sometimes dark brown and produced longitudinal septation, which is quite distinct to *Pseudocercospora* s. str. that having smooth and subhyaline conidia with having only transverse septation, and unthickened conidial loci and hila.

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