African Journal of Biotechnology Vol. 12(2), pp. 109-114, 9 January, 2013
Available online at http://www.academicjournals.org/AJB
DOI: 10.5897/AJB12.2355
ISSN 1684–5315 ©2013 Academic Journals

Full Length Research Paper

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

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Accepted 10 October, 2012

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

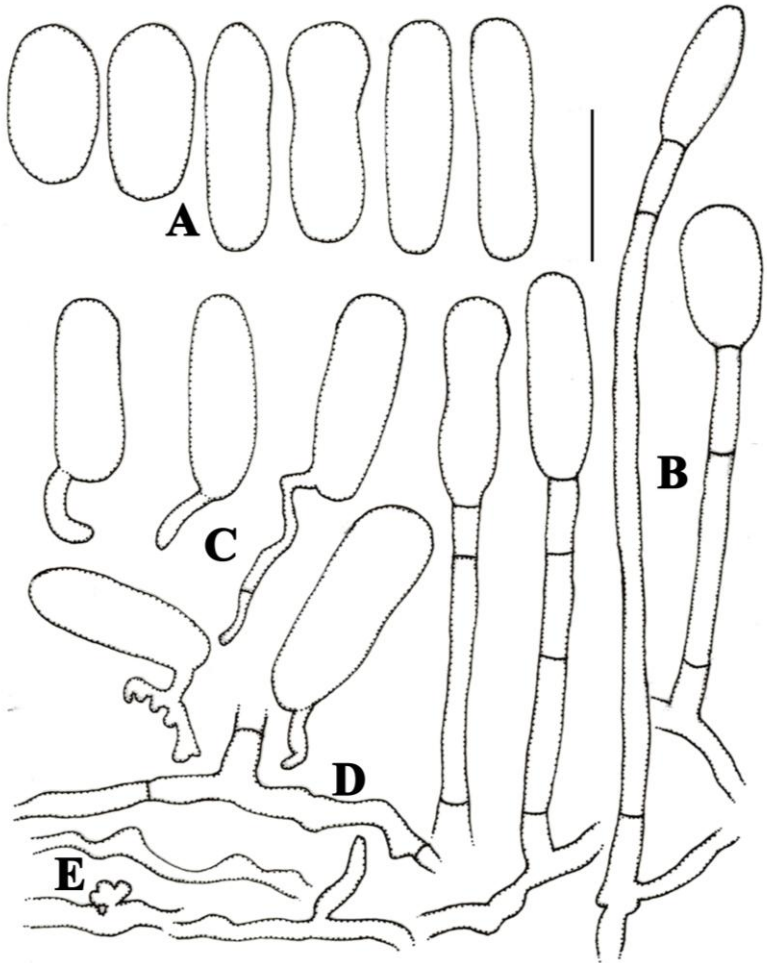

Figure 1. Morphological characteristics of *Ovulariopsis* anamorph of *P. cassiae-fistulae* on *C. fistula*, illustrated using a line drawing under a light microscope (400x). (A) Conidia (B) conidiophores (C) conidia with germ tubes of the *Pseudoidium* type (D) mother cell leading to conidiophores and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

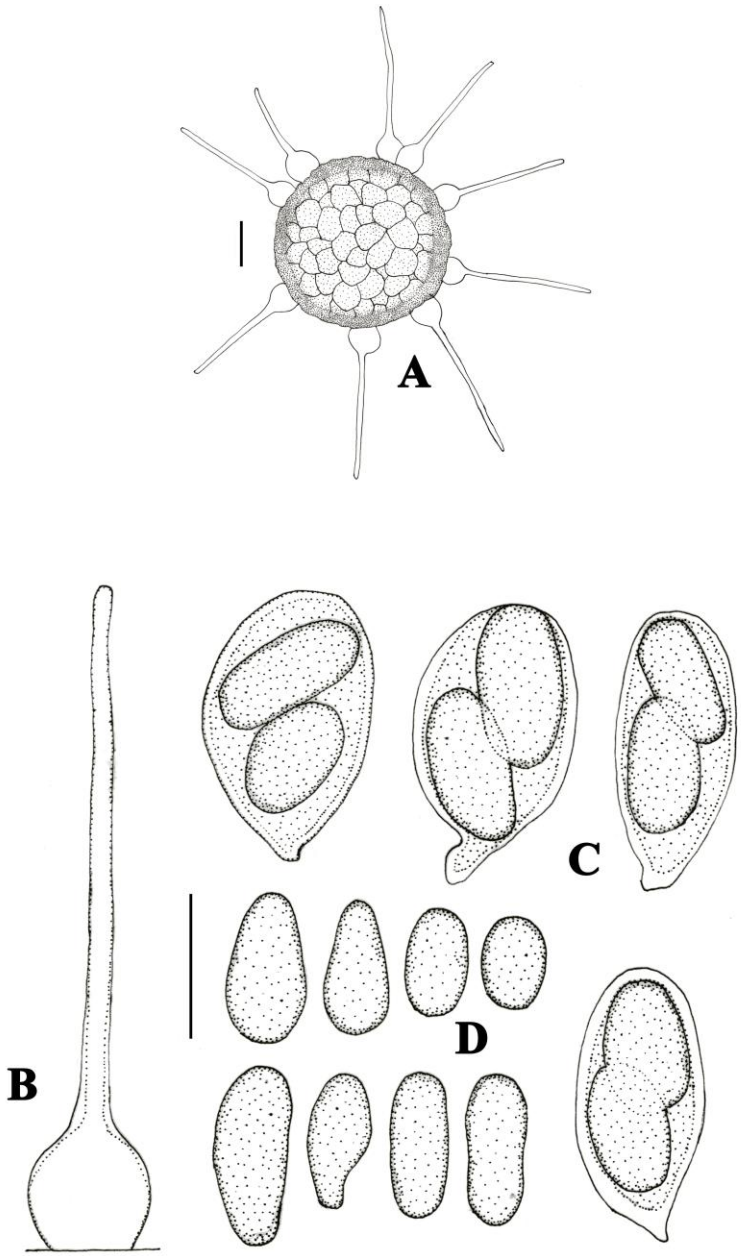

Figure 2. Drawing of teleomorph of *P. cassiae-fistulae* on *C. fistula*, illustrated using a line drawing under a light microscope (200 and 400X). (A) Chasmothecium (B) acicular appendage with bulbous base (C) asci (D) ascospores (bar 50 µm in A, bar 30 µm in B to D).

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

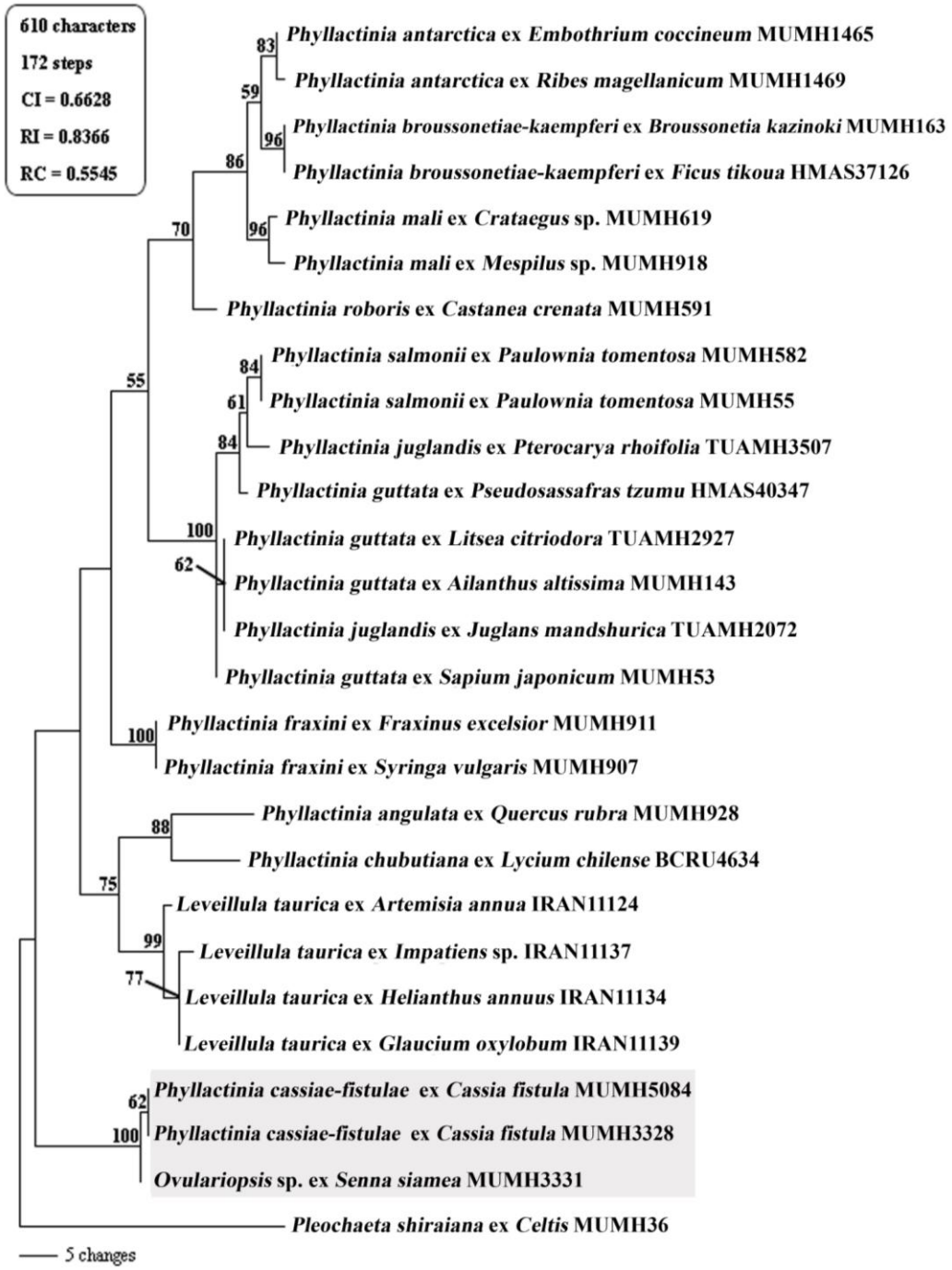

Figure 3. A parsimony ratchet tree based on the 28S rDNA sequences for 27 taxa, consisting of three sequences from *P. cassiae-fistulae* and 24 sequences of *Phyllactinia*, *Leveillula* and *Pleochaeta* retrieved from DNA database. Horizontal branch lengths are proportional to the number of nucleotide 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.

ACKNOWLEDGEMENTS

This research was supported by The Royal Golden Jubilee Ph. D. Program (Grant No. PHD/0125/2550), the Thailand Research Fund (DBG5380011) and a Grant-in-Aid for Scientific Research (C) from the Japan Society of the Promotion of Science.

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