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Contributed Paper

Molecular Phylogenetic and Morphological Analysis of a Powdery Mildew Found on *Dalbergia lanceolaria* in Thailand

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ABSTRACT

Phyllactinia dalbergiae and its *Ovulariopsis* anamorph, causing a powdery mildew fungus on *Dalbergia lanceolaria*, has been found for the first time in Thailand. Identification of this fungus using molecular analysis has not been previously reported. In this study, molecular phylogenetic analysis of the nuclear ribosomal DNA (28S, ITS regions including 5.8S) combined with morphology were used to clarify and confirm identification of this fungus. The phylogenetic analysis demonstrated that *P. dalbergiae* formed a distinct clade at the basal part of the *Phyllactinia* tree with 97% bootstrap support. The phylogenetic results supported the unique morphology of the *P. dalbergiae* anamorph that has twisted foot-cells. Additionally, the nucleotide sequences obtained from both of anamorphic and teleomorphic states were identical.

Keywords: phylogeny, *Phyllactinia dalbergiae*, *Ovulariopsis*, 28S rDNA, ITS

1. INTRODUCTION

Phyllactinia spp. are partly endo-parasitic fungi, within Tribe Phyllactinieae (Erysiphaceae) causing powdery mildew diseases [4]. The genus *Phyllactinia* is distributed worldwide, especially in the temperate regions of the Northern Hemisphere such as East Asia, Europe and North America. *Phyllactinia* is known to be parasitic on woody plants [1]. *Phyllactinia dalbergiae* has been reported to cause powdery mildew on the woody plant *Dalbergia* spp. in India and Pakistan [19, 22, 21]. *Phyllactinia dalbergiae* was reported by Indian mycologists for the first time in 1965 [20]. *Dalbergia sissoo* is also infected by this fungus [4, 18, 20]. Furthermore, Braun [4] has reported that *P. dalbergiae* also caused powdery mildew disease on *Dalbergia lanceolaria*. The anamorphic stage of *P. dalbergiae* has unique conidiophore foot-cells that have a sinuous to twisted form as is typical of conidiophore foot-cells in genus *Streptopodium*. This characteristic is distinct from other *Phyllactinia* species which have straight conidiophore foot-cells. There has been confusion in identification of this powdery mildew

because its anamorphic characteristics are identical to *Streptopodium* in terms of typically forming twisted foot-cells. Moreover, previous reports have described taxonomic problems regarding the powdery mildews resulting from morphological data [3, 4, 18, 20]. Therefore, this study re-examined the morphology of *P. dalbergiae* both of the anamorphic and teleomorphic states.

Molecular analysis is a useful tool in clarifying confused identification including the study of phylogeny and evolution [11]. Phylogenetic relationships based on the nucleotide sequences of the nuclear ribosomal DNA have been used to clarify the taxonomic systems of powdery mildews [15, 16, 28, 31]. In addition, this technique is useful in demonstrating the link between anamorphs and their teleomorphs [10, 14]. This is the first study of the molecular phylogeny of *P. dalbergiae* based on analyses of the nuclear large subunit of ribosomal DNA (28S) and internal transcribed spacers (ITS) including 5.8S rDNA region.

2. MATERIALS AND METHODS

2.1 Sample Sources

Powdery mildew specimens were collected in Chiang Mai, Thailand and 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. The DNA sequences of *P. dalbergiae* were deposited in DDBJ under the accession numbers as AB724092, AB724093 and AB724094.

2.2 Morphological Analysis

Fresh and herbarium specimens were examined for morphological characterization by using a light microscope. Fungal colonies containing the teleomorphic state were lifted from infected leaf surfaces of fresh specimens with clear adhesive tape or with a clean needle and mounted on a microscope slide in distilled water for observation. Herbarium specimens were examined using lactic acid following the method of Shin and La [24]. Morphological characteristics were recorded (n = 30) as follows: for the anamorph: size and shape of conidia, conidiophores; position of the basal septum; shape and position of hyphal appressoria and presence or absence of fibrosin bodies; for the teleomorph: size and shape of chasmothecia, appendages, asci, and ascospores including observation of conidial germination [9].

2.3 Molecular Phylogenetic Analysis

Whole-cell DNA was extracted from mycelia or conidia using the chelex method [10, 32]. The 28S ribosomal DNA (rDNA) including the domains D1 and D2, and Internal transcribed spacer (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) in a PCR thermal cycler SP (Takara, Kyoto, Japan) following the thermal cycling procedures of Takamatsu [29].

The 28S (large subunit) rDNA gene was amplified by PCR using primer PM3 (5′-GKGCTYTMCGCGTAGT-3′) [30], TW14(5′GCTATCCTGAGGGAAACTTC-3′) [16] for the first PCR. Nested primer sets NL1 (5′-AGTAAC- GGCGAGTGAA GCGG-3′) and NLP2 (5′-GGTCCCAACA GCTATGCTCT-3′) [16] were used for the second amplification using the first PCR product as a template.

Primer sets of ITS1, ITS4, ITS5, p3, PM6 and Ph7 were used for amplification of the ITS regions. A *Phyllactinia* and *Leveillula* specific primer Ph7 (5′-TGTTGCTTTG-

GYAGGCCG-3′) was designed in this study. Primers ITS5 [33] and p3 [12] 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 [33] as sequence primers of ITS regions.

The nucleotide sequences of rDNA were aligned with the MUSCLE program [5]. Maximum parsimony (MP) trees were constructed from the alignment data matrix using the parsimony ratchet method [17] in PAUP 4.0b8 [27] and PAUPRat ver. 1 [26]. The reliability of the internal branches of the inferred tree was estimated by bootstrap analysis [6] using 1,000 replications. Lack of bootstrap value indicates less than 50% support at that node. PAUP 4.0b8 [27] was used for finding a tree which obtained the highest likelihood value among the equally parsimonious trees.

3. RESULTS AND DISCUSSION

3.1 Morphological Analysis

The powdery mildew fungus forms thin to dense white colonies that are seen as white patches on the lower side of the leaves (hypophyllous) in *Dalbergia lanceolaria* (Figure 2). In this study, powdery mildews on *Dalbergia lanceolaria* are determined to be of both the anamorphic (imperfect) and teleomorphic (perfect) states. In the anamorph, hyphae are hyaline, white, sub-straight to flexuous, with appressoria nipple-shaped, rarely lobed to elongate. Conidiophores are erect or slightly bent, long and slender, (173-)208-313(-330)×(5-)6-12 (-22) µm ($\overline{X}$ = 248 × 9 µm), arising from ectophytic hyphae on the upper surface of mother cells, not positioned centrally, with a basal septum near the branching point of mycelium up to and away from it, (3-)4-9(-12) µm. Mother cells form conidia singly, (52-)65-76(-89) × (3-)4-5(-5.4) µm (= 70 × 4 µm). Foot cells are sinuous to twisted, (83-)97-213(-260) × (3-)4-5(-6) µm (= 150 × 4 µm). Conidia are lanceolate or clavate, (35-)67-81(-89) × (12-)13-19(-24) µm (= 73 × 16 µm), without conspicuous fibrosin-bodies, with a moderately long, rarely lobed (*Pseudoidium* type) appressorium formed on the germ tube of conidia (Figure 3 and 4).

At the end of year, this powdery mildew formed sexual structures, chasmothecia, on the lower side of leaves. Chasmothecia are yellowish when immature and become brown-blackish in color, scattered to subgregarious, (173-)198-215(-254) µm in diameter (= 208 µm). Appendages are 5-9 in number, acicular with a bulbous basal swelling, (133-)173-282(-307) × (29-)32-40 (-44) µm (= 230 × 34 µm), apex subobtuse and hyaline. Asci are sessile, numerous, (48-)54-72(-82) × (26-)29-38(-42) µm (= 69 × 34 µm), containing 2 ascospores ellipsoid-ovoid in shape, rarely subglobose and (28-)33-36(-46) × (14-)17-22(-26) µm (= 32 × 19 µm) (Figure 5 and 6).

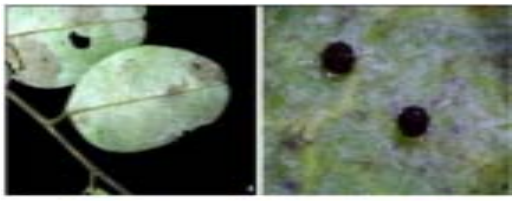

Figure 2. Powdery mildew caused by *Phyllactinia dalbergiae* on *Dalbergia lanceolaria* L. f. var. *lakhonensis* (Gagnep.) Niyo. & Ho. leaves. (A) Symptoms appeared on the lower surface of leaves. (B) A close-up of chasmothecia scattered on the lower surface of leaves.

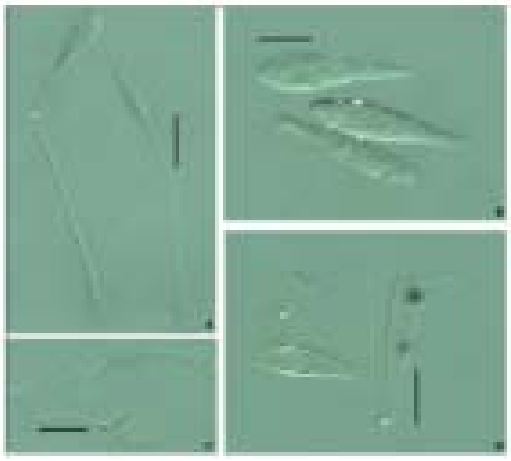

Figure 3. Morphology of the *Ovulariopsis* anamorph of *Phyllactinia dalbergiae*; (A) conidiophores with twisted foot-cells. (B) conidia (C) mycelia with appressoria and (D) conidial germination. (*Bar* = 50 µm in A, *Bar* = 27 µm in B-D).

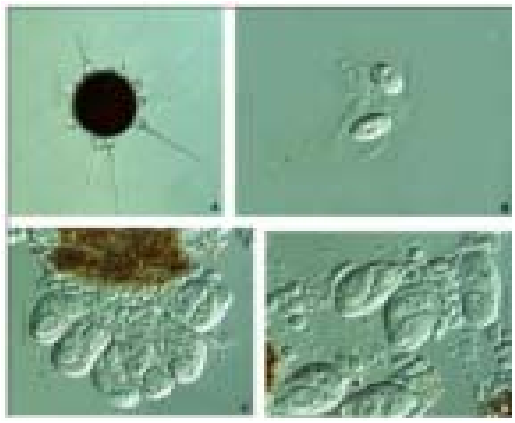

Figure 5. Teleomorph of *Phyllactinia dalbergiae*; (A) chasmothecium (B) ascospores (C) numerous asci per chasmothecium and (D) ascus consisting of 2 ascospores. (*Bar* = 111 µm in A, *Bar* = 27 µm in B-D).

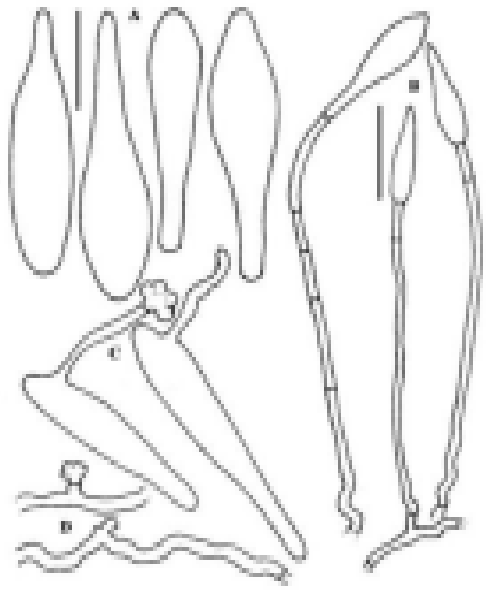

Figure 4. Illustration of the *Ovulariopsis* anamorph of *Phyllactinia dalbergiae*; (A) conidia (B) conidiophores with twisted foot-cells (C) conidial germination and (D) mycelia with appressoria. (*Bar* 30 µm).

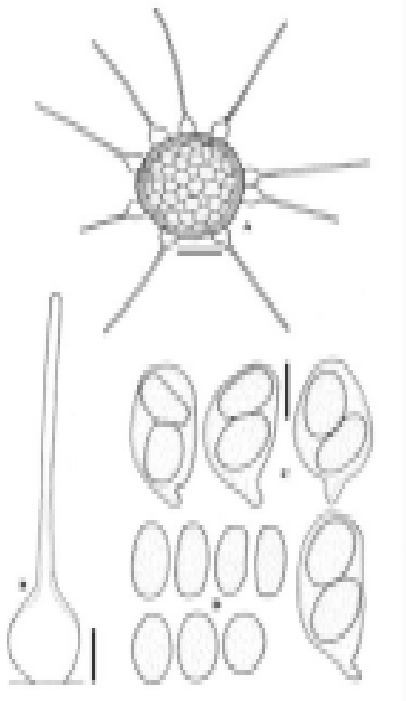

Figure 6. Teleomorph of *Phyllactinia dalbergiae* illustrated using line drawings; (A) chasmothecium (B) appendage (C) ascus consisting of 2 ascospores and (D) ascospores. (*Bar* = 111 □ m in A, *Bar* = 27 □ m in B-D).

Based on the characteristics of the anamorph and teleomorph, powdery mildew on *Dalbergia lanceolaria* in this study can be identified as *P. dalbergiae* because of consistent agreement with *P. dalbergiae* characteristics recorded by Pyrozynski [20]. Morphologically this fungus is distinct from other *Phyllactinia* anamorphs by its sinuous to twisted conidiophore foot-cells. Normally, the presence of twisted foot-cells is a unique anamorphic characteristic of the genus *Streptopodium* (*Pleochaeta* teleomorph) [4]. Nevertheless, four other 4 *Phyllactinia* anamorph species including *P. aceris*, *P. ampulliformis*, *Ovulariopsis durantae* and *O. lawsoniae* have been reported to have sinuous to twisted conidiophore foot-cells [2, 8, 23, 13].

However, this fungus produces chasmothecium that have acicular appendages with a bulbous swelling at the base, a typical characteristic of *Phyllactinia* teleomorphs. Morphology of appendages is an important characteristic for genera delimitation [4, 29]. Certain *Phyllactinia* species have unique appendages known as penicillate cells which gelatinize under moist conditions have apically digitated branches. Penicillate cells have been proposed as a useful characteristic for species delimitation within the genus *Phyllactinia* [4, 23, 25]. In this study penicillate cells were not clearly observed.

3.2 Molecular Phylogenetic Analysis

The 28S rDNA sequences data were combined with ITS data including 5.8S rDNA sequences to construct a phylogenetic tree that consisted of a 29 alignment data matrix. A total of 29 nucleotide sequences, of which 24 sequences from *Phyllactinia*, 4 sequences from *Leveillula taurica* and one sequence from *Pleochaeta shiraiana* that were used as out-group taxons based on Takamatsu [29]. Of the 880 total characteristics used in this analysis, 720 were constant and 50 were variable (uninformative). 110 characteristics were informative for parsimony analysis. A total of 201 equally parsimonious trees (CI = 0.6438, RI = 0.8078, RC = 0.5200) with 320 steps were constructed by parsimony ratchet analysis. A tree with the highest likelihood value among 201 trees is shown in Figure 6. *Phyllactinia dalbergiae* under the accession numbers, AB724092–AB724094 distinctly formed an independent clade at the basal part of the *Phyllactinia* and *Leveillula* clade with bootstrap support of 97%. However, *Phyllactinia cassiae-fistula* clade also clustered at the basal position. The sequences of *Phyllactinia dalbergiae* obtained from both developmental states (conidia and chasmothecia) were identical and grouped together in the clade. We believe that the current study has helped to resolve the taxonomic confusion surrounding indentification of *Phyllactinia dalbergiae* through morphology alone by the additional use of DNA techniques that involve PCR and DNA sequencing [7]. The phylogenetic analysis of powdery mildew on *Dalbergia lanceolaria* using detailed morphological descriptions is reported here for the first time. The phylogenetic analysis using combined sequence data of 28S and ITS regions including 5.8S rDNA demonstrated that *Phyllactinia dalbergiae* is distinctly set in the basal clade apart from other *Phyllactinia* species with bootstrap support of 97%. This in turn correlates well with the unique characteristic of the conidiophore foot-cells.

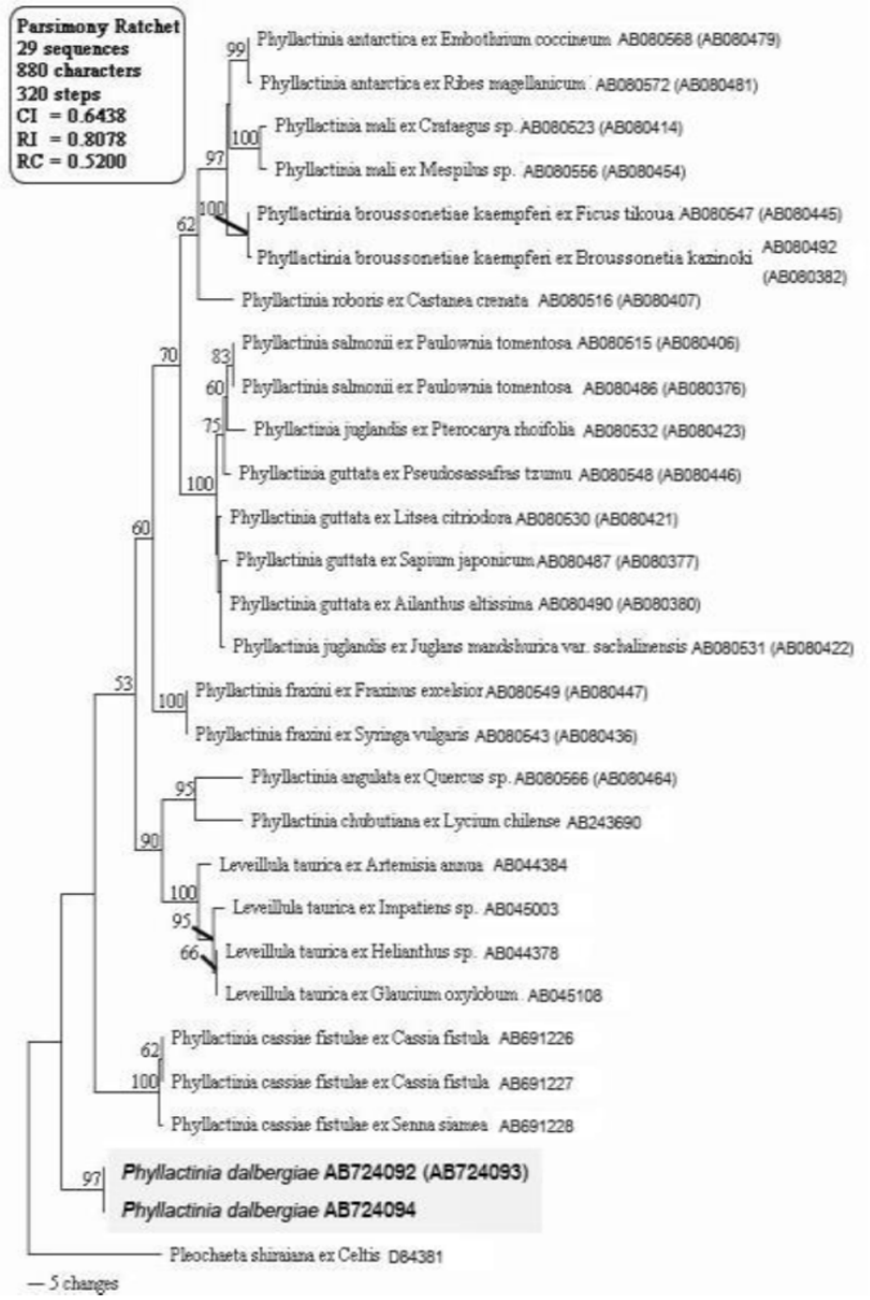

Figure 6. The phylogenetic relationship between *Phyllactinia dalbergiae* on *Dalbergia lanceolaria* L. f. var. *lakhonensis* (Gagnep.) Niyo. & Ho. and *Phyllactinia* species, *Leveillula taurica*, was inferred by the parsimony ratchet method using the combined dataset of the rDNA ITS regions and the divergent domains D1 and D2 sequences of the 28S rDNA. Bootstrap values (≥ 50%) are shown above branches. (Sequences of the 28S rDNA and internal transcribed spacer (ITS) were separately deposited in a DNA database under the accession numbers. Parenthesis means the accession number of 28S rDNA sequence.).

This phylogenetic analysis revealed that *Phyllactinia dalbergiae* on *Dalbergia lanceolaria* apparently split from other *Phyllactinia* at an early stage of evolution. Additionally, the phylogenetic tree indicated that *Phyllactinia* is paraphyletic group as also demonstrated by Takamatsu [29]. The aligned sequence data obtained from ITS regions including 5.8S rDNA could not be used because the nucleotide bases data differed significantly from other *Phyllactinia* species. These results indicate that the nucleotide sequences of ITS regions were variable in this phylogenetic study of *Phyllactinia dalbergiae*

In addition, the DNA sequence data obtained from both the anamorph (conidia, mycelia) and teleomorph (chasmothecia) were identical with each other. The current study supports the contention that the nucleotide sequence data obtained from either conidia or chasmothecia can be used for phylogenetic studies of powdery mildew fungi [10].

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