Molecular Characterization and Morphology of Two Endophytic Peyronellaea Species from Pinus koraiensis in Korea

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Species of Phoma and its allies were isolated during a survey on the diversity of endophytic fungi associated with pine trees in Korea. Based on the phylogenetic analyses of internal transcribed spacer and β-tubulin gene sequences, two Phoma-like species from the needles of Pinus koraiensis were identified as Peyronellaea calorpreferens and P. glomerata. They were also morphologically identified based on the previous descriptions. Here, we report P. calorpreferens and P. glomerata being present in Korea as endophytic fungi in Pinus koraiensis.

KEYWORDS : Endophytic fungi, Morphology, Peyronellaea, Pinus koraiensis, Sequence analysis

Introduction

Peyronellaea is a proposed new genus [1] that includes species originally classified in genus Phoma defined by Boerema et al. [2] and its related species of pleosporalean genera. Based on morphology, Peyronellaea is considered as one of the nine sections of the Phoma genus and is characterized by possession of a multicellular chlamydosporal structure, which may occur in based classification with unicellular chlamydospores [2]. However, the morphological-and physiological-based classification is ambiguous, artificial and time-consuming [3-5] and requires highly skilled experience, which can make successful identification difficult for researchers including plant pathologists [6]. Therefore, the classification of Phoma and Phoma-like species are still controversial. Molecular phylogenetic analysis provides the potential for aiding the identification to understand the diversity of these species [1, 7, 8].

Fungal endophytes are microfungi that asymptotically colonize and live in healthy plant tissues [9] and the endophytic communities are ecologically important in forest ecosystems [10]. While endophytic fungi have been studied from needle, bark, xylem, and seed of conifers [11, 12], abundant endophytic fungi are found in needles of many conifer species [13]. The Korean pine (Pinus koraiensis) is one of the most important afforestation trees in Korea with its distribution in China, Russia, Japan and Europe [14, 15].

During a survey to understand the diversity of endophytic fungi associated with needles of pine trees in Korea, many hyaline-spored pycnidial fungi were obtained. In this study, two clamydospore-producing species from genus Peyronellaea were characterized in Korea. These two species (P. calorpreferens and P. glomerata) are reported for the first time in Korea as endophytic fungi from Pinus koraiensis.

Materials and Methods

Isolation of endophytic fungi. During July and August 2006, needle samples were collected from randomly selected Korean pines (Pinus koraiensis) in mountainous areas of Daejeon, Korea. Each sample was placed into a sterile polyethylene bag and processed for isolation within 5 hr of collection. Needles were washed with tap water to remove dust, dried in air and cut into 1 cm segments. For surface sterilization, the segments were soaked into 95% ethanol for 1 min, sodium hypochlorite (4% available chlorine) for 3 min, and 95% ethanol for 30 sec. They were rinsed in sterile distilled water for three times and dried in a laminar air flow chamber. Ten segments per sample were placed horizontally on dichloran rose bengal
chloramphenicol agar (Difco, Detroit, MI, USA) and potato dextrose agar (PDA; Difco) supplemented with the antibiotic streptomycin sulfate to inhibit bacterial growth. Five replicates were conducted for either medium. Developing hyphal tips of each emerged colony were collected after incubation at 25°C for 5, 10, and 25 days, and sub-cultured on PDA for 8–10 days. Pure cultures of isolates were maintained in PDA slant tubes and 20% glycerol stock solution, and deposited in the Culture Collection of Chungnam National University Fungi Herbarium. In this study, two isolates from this collection (CNU 060202 and CNU 060219) were used to characterize morphologically and molecularly.

DNA extraction and PCR amplification. For DNA extraction, mycelial tufts of the isolates were scraped from the surface of 7–10 day old colonies on PDA and transferred to 1.5 mL tubes. The mycelia were lyophilized, ground and the genomic DNA were extracted [16]. For PCR amplification, the internal transcribed spacer (ITS) region of the ribosomal DNA (rDNA) gene was amplified with primers ITS1 and ITS4 [17], and the β-tubulin gene was obtained with primers Bt2a and Bt2b [18]. PCR was conducted in a 50 µL reaction mixture and carried out in a GeneAmp PCR System 2700 thermo cycler (Applied Biosystems, Foster City, CA, USA). PCR products were purified with a PCR Clean-up System (Promega, Madison, WI, USA) and directly sequenced in both directions.

Sequence analysis. The obtained sequences of the ITS and β-tubulin regions were aligned with sequences of representative isolates of *Peyronellaea* species obtained from Genbank using the PHYDIT program ver. 3.2 [19]. Phylogenetic analysis was implemented with the program of PAUP v4.0b10 [20] by a heuristic search for the most parsimonious trees. It was conducted with 1,000 random stepwise additions and branch swapping by tree bisection reconnection. Parsimonious tree was constructed using the combined dataset (ITS and β-tubulin). The bootstrap values were assessed by 1,000 replications using a heuristic search with simple sequence addition.

Morphology. Morphological characteristics of the isolates were examined on oatmeal agar (OA) and malt extract.

Fig. 1. One of the parsimonious trees based on a combined dataset of internal transcribed spacer (ITS) and β-tubulin gene sequences of the two endophytic *Peyronellaea* species from Korean pine and their relevant species. Numbers at the nodes indicate bootstrap values from a test of 1,000 replications. The scale bar indicates the number of nucleotide substitutions and the mark 'T' indicates type strain or ex-type strain [1]. CI, consistency index; RI, retention index.
agar (MEA) media [2]. Small discs (0.5 cm diameter) were cut from the margin of developing cultures, inoculated on the center of agar plates and incubated at 22°C for 7 days in the dark to measure the diameter of the colonies. Then, the plates were placed in an incubator with light cycles (13 hr near visible ultraviolet light and 11 hr darkness) for 7 days to stimulate the formation of pycnidia. The features of 2-wk-old colonies were determined using color charts [21]. The pycnidia, chlamydospores and conidia formed on OA medium were observed using a BX50 microscope (Olympus, Tokyo, Japan). The size of pycnidia, conidia and chlamydospores were measured with the help of an Artcam 300MI digital camera (Artray, Tokyo, Japan). Morphological characteristics of the two isolates were compared with previous descriptions [1, 2].

Results and Discussion

Molecular characterization. Sequence analyses of the ITS region and β-tubulin gene revealed 100% sequence similarity between each of the two endophytic fungal isolates (CNU 060219 and CNU 060202) of Korean pine (P. koreensis) and its relevant sequences in GenBank by NCBI BLAST search. For isolate CNU 060219, the sequences of ITS (accession No. HM769278) and β-tubulin (accession No. HM769280) were identical to the representative isolate of Peyronellaea calorpreferens CBS 109.92. For isolate CNU 060202, the ITS sequence (accession No. HM769279) was identical to the representative isolate of P. glomerata CBS 528.66, but one nucleotide difference was found in the β-tubulin gene sequence (accession No. HM769281). However, the two gene sequences of CNU 060202 were identical to those of the isolate Peyronellaea glomerata D/034 from Glycine max with accession No. FJ427012 (ITS) and FJ427123 (β-tubulin) [8].

After the alignment of both gene sequences of two present isolates (CNU060219 and CNU060202) and other isolates of Peyronellaea species, parsimony analyses were performed with a total of 704 characters in the final dataset. The analyses revealed 47 most parsimonious trees (48 parsimony-informative characters, steps = 136, retention index = 0.8131, consistency index = 0.7279) by a heuristic search. The topology of the parsimonious tree (Fig. 1) distinguished between each of these two endophytic fungi isolates and its relevant Peyronellaea species by forming monophyletic clusters. Isolate CNU 060219 and P. calorpreferens isolates were placed in a clade highly supported by a bootstrap value (91%). The isolate CNU 060202 and P. glomerata isolates were grouped together with a high bootstrap value (82%), which was closely related to P. aurea. Meanwhile, the isolate CNU 060202 and D/034 formed a subclade in the P. glomerata clade with 55% bootstrap value. The two endophytic fungal isolates were identified as P. calorpreferens and P. glomerata respectively.

Morphology. Morphological observation of two isolates also verified them as different Peyronellaea species, P. calorpreferens and P. glomerata. Taxonomic descriptions, microphotographs of morphological structures of each species are shown in Figs. 2 and 3.

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Fig. 2. Morphology of the isolate CNU 060219 (Peyronellaea calorpreferens). Colonies on oatmeal agar (A) and malt extract agar (B) media for 14 days at 22°C, pycnidia (C-E), chlamydospores (F-L), conidia (M) (scale bars: C-E = 40 μm, F-L = 20 μm, M = 10 μm).
Two Endophytic *Peyronellaea* Species from *Pinus koraiensis*

Two endophytic *Peyronellaea* species were isolated from *Pinus koraiensis*

### *Peyronellaea calorpreferens* (Boerema, Gruyter & Noordel.) Aveskamp, Gruyter & Verkley 2010 (Table 1, Fig. 2)

Colonies were very fast growing at 22°C. The diameter of colonies on OA and MEA was 5.5–9 cm and 6–9 cm after 7 days, respectively. Colonies were honey, greenish olivaceous or whitish in tufts. The reverse of the colonies was often yellowish tinges, with dark olivaceous or blackish stains in the center. Colony grew well at 30°C. Pycnidia were subglobose with papillates and slightly papillates, 100–350 µm in diameter and usually solitary.

Conidia were hyaline, mostly oblong-ellipsoidal, 3.5–6.5 (-7) × 2–3 (-3.5) µm in size. Chlamydospores were often unicellular and multicellular, pale to dark brown.

**Isolate examined:** On needles of *Pinus koraiensis*; CNU 060219.

Cultural and morphological characteristics of the fungus agreed with the description of *P. calorpreferens* [1, 2]. The species was considered as a variety (*Phoma pomorum* var. *calorpreferens*) of *Ph. pomorum* because it is similar in

### Table 1. Comparison of morphological characteristics between the endophytic fungal isolate CNU 060219 from Korean pine and *Peyronellaea calorpreferens* described previously

| Character                  | CNU 060219                          | *P. calorpreferens*                      |
|----------------------------|-------------------------------------|-----------------------------------------|
| Colony Color               | Honey, greenish olivaceous whitish in tufts | Whitish in tufts’                      |
| Colony Size                | 5.5–9 cm diam. on OA 6–9 cm diam. on MEA | 4.5–6 cm diam on OA 5.5–7.5 cm diam. on MEA |
| Colony Others              | Grow well at 30°C                   | Grow very well at 30°C                  |
| Pycnidia Shape             | Subglobose with papillate or slightly papillate, smooth | Subglobose no-papillate, usually smooth and not furrowed’ |
| Pycnidia Size              | Usually solitary 100–350 µm diam.    | Slightly papillate ostioles’            |
| Conidia Shape              | Oblong-ellipsoidal, hyaline          | (70-) 100–200 (~250) µm diam.           |
| Conidia Size (µm)          | 3.5–6.5 (-7) × 2–3 (-3.5)            | (4–) 5–8.5 (~12) × 2–3 (~3.5)’          |
|                            |                                     | (3.5–) 4–8.5 (~12) × 2–3.5 (~4.5)’      |
| Chlamydospore              | Unicellular and multicellular-dictyosporous | Highly variable, unicellular and multicellular-dictyosporous |

OA, oatmeal agar; MEA, malt extract agar.

a Described by Boerema *et al.* [2] as *Phoma pomorum* var. *calorpreferens*.

b Described by Aveskamp *et al.* [1] as *Peyronellaea calorpreferens*.

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**Fig. 3.** Morphology of the isolate CNU 060202 (*Peyronellaea glomerata*). Colonies on oatmeal agar (A) and malt extract meal (B) media for 14 days at 22°C, pycnidia (C–F), chlamydospores (G–L), conidia (M) (scale bars: C–F = 40 µm, G–L = 20 µm, M = 10 µm).
their morphology with *Ph. pomorum* var. *pomorum* [2]. However, the fungus grew very well at 30°C when compared with *Ph. pomorum* var. *pomorum* and produced relatively larger conidia. Due to the phylogenetic placement of *Ph. pomorum*, which was distantly related with *Ph. pomorum* var. *calorpreferens* in phylogenetic analysis, it was elevated to species level as *Ph. calorpreferens* [5]. Recently, the species was renamed as *P. calorpreferens* after the systematic and polyphasic study of *Phoma* and related pleosporalean genera [1]. Simultaneously *Ph. heteroderae* was treated as a basionym of *P. calorpreferens* due to its similarity in morphological and molecular characteristics.

The fungus has been reported from food materials, soil (as soil and seedborn opportunist), indoor environment, and also from eggs of *Heterodera glycines* as *Ph. heteroderae* [1]. However, this study is the first report of *P. calorpreferens* as endophyte from Korean pine (*Pinus koraiensis*) which had been reported in Europe and America earlier [1-2].

**Peyronellaea glomerata** (Corda) Goid. ex Togliani 1952 (Table 2, Fig. 3).

Colonies were relatively fast growing at 22°C. The growth rate of colonies on OA and MEA was similar, 5–7.5 cm in diameter after 7 days. Colonies were olivaceous buff, olivaceous, greenish olivaceous, dense and woolly in places. The reverse of colonies was olivaceous to dark olivaceous under the area and paler elsewhere. Colonies did not grow at 34°C. Pycnidia were subglobose to obpyriform with papillate or long necks, 50–250 µm diam., mostly solitary, sometimes confluent. Conidia were highly variable in shapes, oblong, obovate, elliptical, hyaline or subhyaline, 5–10 (~12.5) × 2–3.5 (~4) µm in size. Chlamydospores were usually unbranched, solitary-terminal alternarioid, brown to dark brown.

**Isolates examined:** On needles of *Pinus koraiensis*; CNU 060202.

Pylogenetically, the species was placed in a clade comprising chlamydospore forming species and were recommended as a synonym of *P. glomerata* [1]. The species is closely related to *P. aurea*, but they can be easily differentiated by the characteristic of chlamydospore production.

The fungus is well-known for its worldwide from soil, various plants (pathogen or second invader), animal (human), inorganic material, and food. It was also reported as a mycoparasite of powdery mildew [22]. *P. glomerata* is also the first record as an endophyte from Korean pine (*Pinus koraiensis*) in Korea.

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