RESEARCH NOTE

Identification and Characterization of *Pseudocercospora cornicola* Causing Leaf Spots on *Cornus officinalis*

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ABSTRACT

Cornus officinalis plants that grow in several locations in Korea have been found to be infected with leaf spot disease. Symptoms include necrotic lesions, which are angular, irregularly shaped, vein-limited, and dark brown, on both sides of the leaves. The causal agent of the disease was identified to be *Pseudocercospora cornicola* based on the morphological characteristics of the fungus and molecular phylogenetic analysis of the obtained multi-locus DNA sequence data. This is the first report investigating *P. cornicola found* on *C. officinalis* in Korea.

Keywords: Fungal isolation, Multi-locus DNA dataset, Pathogenicity, Plant pathogen

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The genus *Pseudocercospora* Speg. (Mycosphaerellaceae) is a group of hyphomycetous fungi that are mainly phytopathogenic. This genus causes leaf spots, fruit spots, and blight diseases in a wide range of host plants, including angiosperms, gymnosperms, and ferns [1,2]. In Korea, studies on the diversity of the genus *Pseudocercospora* date back to the early 1990s [3]. Since then, new records on the cercosporoid group of fungi have been published [4-8]. However, the list of *Pseudocercospora* species found in Korea is incomplete. Thus, a study investigating *Pseudocercospora* and their effects on host plants is warranted.

Cornus officinalis Siebold & Zucc. (Cornaceae), commonly known as the Japanese cornelian cherry, is a deciduous plant native to China, Japan, and Korea and is widely used in traditional medicine [9]. Annual infections between September and November starting from the year 2000 were found to occur on the leaves of this plant with 100% disease incidence in the cities of Jeonju, Jinju, and Iksan in Korea. The symptoms were observed as necrotic leaf spots. Infection initially occurred on older leaves before spreading to younger leaves, and severe defoliation was observed after disease progression. The voucher specimens KUS-F17995 (Oct 29, 2000, Jinju, leg and det. H.D. Shin) and F28661 (May 20, 2015, Iksan, leg and det. H.D. Shin) were deposited at the Korea University Herbarium, Korea.

To observe sample morphology, small cuts were taken from fresh samples, mounted in a drop of sterile water, and examined under an optical microscope (Carl Zeiss AX10, Göttingen, Germany) equipped with a KCS-3.1C imaging system. At least 30 measurements were obtained for each diagnostic structure. To obtain a pure conidial culture, conidia were collected from the lesions using sterile forceps and placed in Eppendorf tubes containing sterile water. The conidial suspension was streaked onto 2% water agar (WA, Junsei, Japan) plates supplemented with 100 mg/L streptomycin sulfate and incubated at 25℃ for four days. The colonies were then transferred onto potato dextrose agar (PDA; Difco, Le Pont de Claix, France) plates. The isolates were obtained from KUS-F17995 and F28661 and were housed in the Korean Agricultural Culture Collection of Rural Development Administration (accession number: KACC48076) and Jeollabuk-do Agricultural Research and Extension Services (JBARES72), Korea, respectively. For molecular phylogenetic analysis, genomic DNA was extracted from cultures grown on PDA using a DNeasy Plant Mini Kit (Qiagen Inc., Valencia, CA, USA), as described by Nakashima et al. [10]. Multi-locus rDNA sequences of internal transcribed spacer (ITS) regions, partial actin (*actA*), partial translation elongation factor 1-alpha (*tef1*), and partial DNA-directed RNA polymerase II second largest subunit (*rpb2*) were amplified and sequenced using the primer pairs ITS4/ITS5, ACT-512F/ACT-2Rd, EF1-728F/EF-2, and RPB2-5f2/fRPB2-7cR, respectively [2,11-13]. The resulting fragments were sequenced in both directions using the PCR primers. The reactions were monitored using BigDye Terminator Cycle Sequencing Kits (Applied Biosystems Life Technologies, Madison, WI, USA), and the products were purified using a purification kit (Bioneer, Daejeon, Korea). Purified products were analyzed on an ABI3130 automated DNA sequencer (Applied Biosystems, Waltham, Massachusetts, USA).

Leaf spots were amphigenous, angular, irregularly shaped, 5-10 mm in diameter, vein-limited, dark brown, and confluent (Fig. 1A and B). The external hyphae were branched, septate, brownish, and 2-3 μm wide. Stromata were well-developed, erumpent, epiphyllous, pale to light brown, and up to 70 μm in diameter (Fig. 1C and D). Conidiophores were numerous, in dense fascicles, brown to pale brown, smooth-walled, cylindrical, straight or slightly curved, aseptate or with a single septum, and 3-5 μm wide and 30 μm long (Fig. 1E and F). Conidia were solitary, narrowly obclavate to cylindrical, hyaline to olivaceous, straight or slightly curved, 2-4 septate, obtuse at apex, truncated at base, 25-70 μm long, and 2-3 μm wide with unthickened and undarkened hila (Fig. 1G). One-week-old fungal colonies grown on PDA were creamy in color and developed moderate aerial mycelia with smooth undulating margins (Fig. 1H). These characteristics are consistent with those of *Pseudocercospora cornicola* (Tracy & Early) Y.L. Guo & X.J. Liu [14,15].

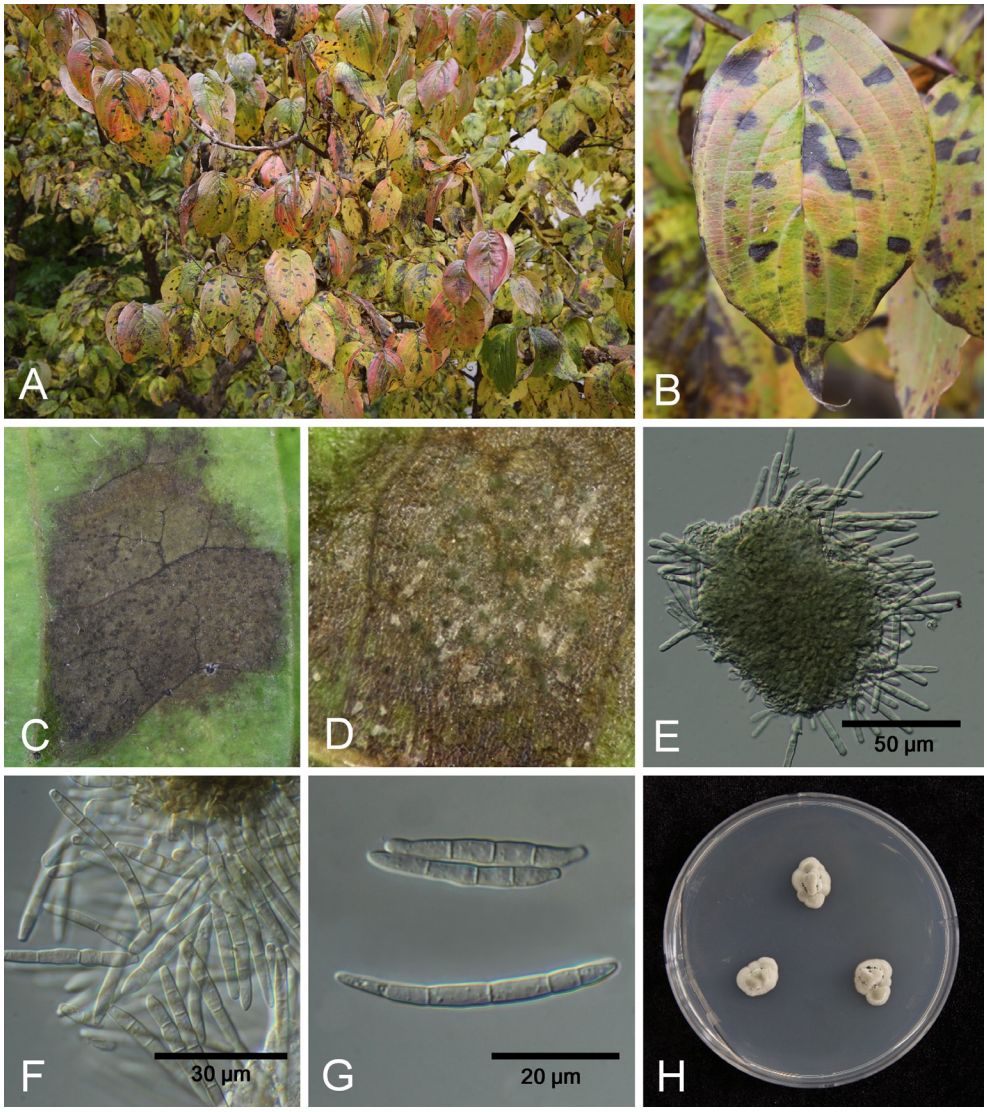

Fig. 1. Leaf spots caused by *Pseudocercospora cornicola* on *Cornus officinalis*. (A) General view of infected *C. officinalis* plant. (B) Close-up view of symptoms on leaves. (C, D) Fructification of the fungus on the lesion, showing grayish patches on the lesion due to heavy fructification. (E) Conidiophores. (F, G) Conidia. (H) One-week-old colony of *P. cornicola* growing on a potato dextrose agar at 25℃.

The sequences obtained for ITS (343 bp), *actA* (134 bp), *rpb2* (657 bp), and *tef1* (308 bp) were deposited in GenBank (accession numbers MT666074, MT711893, MT711894, and MT711895, respectively). A basic local alignment search tool (BLAST) search for sequences showed 100% identity with the sequences of *Pseudocercospora cercidis-chinensis* (MG733154), *P. fukuokaensis* (GU384429), and *P. pini-densiflorae* (LC587041 and LC587042) for *actA*, *tef1*, and ITS, respectively. The results for *rpb2* were 100% identical to the sequences of *P. fukuokaensis* (KX462632) and *P. imazekii* (KX462638). Although BLASTn search did not reveal percentage similarity between our sequences and other *P. cornicola*, we compared our sequences with existing *actA* (GU320389) and *tef1* (GU384400) sequences for *P. corniocola* in GenBank using MEGA and found 9 bp differences in *actA* and a 1 bp difference in the *tef1* gene. Significant variability was observed in the ITS region between our sequence and the GU269683 sequence from *P. cornicola*. For phylogenetic analysis, the newly obtained sequences were combined, assembled, and aligned using the SeqMan software (Lasergene; DNASTAR, Madison, WI, USA), with 18 sequences retrieved from GenBank. A phylogenetic tree was generated using the combined data of multi-locus DNA sequences of ITS regions and the *tef1*, *actA,* and *rpb2* genes in MEGA7 [16] using the neighbor-joining method. *Cercospora* cf. *nicotianae* (CPC 15918) and *Pallidocercospora heimioides* (CBS 111190) were selected as the outgroups. The robustness of the tree was evaluated using 1,000 bootstrap replications. The resulting tree shows that representative sequences of *P. cornicola* found in *C. officinalis* formed a well-supported, distinct clade from other sequences of *Pseudocercospora* species with the maximum bootstrap value (Fig. 2).

For the pathogenicity test, a mixed suspension of conidia and mycelial fragments obtained from a two-week-old isolate (KACC48076) was prepared. The colonies were flooded with sterile distilled water, and the surface of the colonies was carefully scraped with a sterile disposable loop without breaking the agar. The conidial concentration of the suspension was adjusted to approximately 1×10^4 propagules/mL. Ten healthy three-year-old plants were selected for the inoculation test. Five of the plants were sprayed with a mixed fungal suspension and the remaining five plants were selected as controls and sprayed with sterile water. All plants were individually covered with polyethylene bags to maintain 100% relative humidity for 24 h, and then kept outdoors. Typical leaf spots were observed on the inoculated plants 14 days post-incubation. No symptoms were observed in the control plants. The morphological characteristics of the fungus isolated from the lesions of the inoculated plants were identical to those observed in the field, which fulfilled Koch's postulates.

P. cornicola has previously been reported in *Cornus alba* var. *sibirica*, *C. brachypoda*, *C. controversa*, *C. kousa*, and *C. officinalis* in Japan; *C. florida* in the USA and Japan; and *Cornus* sp. in China [17]. To our knowledge, this is the first report on *P. cornicola* in Korea, and the first record of *C. officinalis* as a host for this fungus globally. The pathogen could be a potential threat to the safe production of *C. officinalis* fruits because of the rapid spread of the disease.

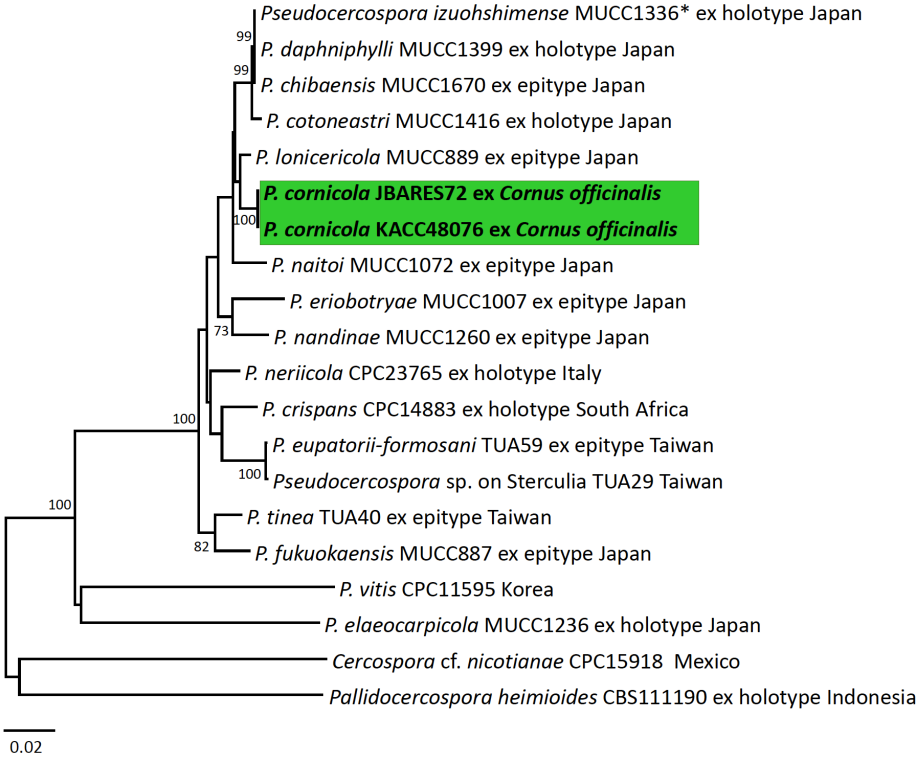

Fig. 2. Neighbor-joining tree of *Pseudocercospora cornicola* based on the multigene dataset of internal transcribed spacer (ITS) of rDNA region, *tef1*, *actA* and *rpb2* genes. The isolates obtained in this study are shown in bold. Bootstrap values (>70%) are provided on relevant branches.

CONFLICT OF INTERESTS

No conflict of interest was reported by the author(s).

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