RESEARCH ARTICLE

Didymella gigantis sp. nov. Causing Leaf Spot in Korean Angelica

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ABSTRACT

During a disease survey in October 2019, leaf spot symptoms with a yellow halo were observed on Korean angelica (*Anglica gigas*) plants grown in fields in Pyeongchang, Gangwon Province, Korea. Incidence of diseased leaves of the plants in the investigated fields ranged from 10% to 60%. Morphological and cultural characteristics of two single-spore isolates from the leaf lesions indicated that they belonged to the genus *Didymella*. Molecular phylogenetic analyses using combined sequences of LSU, ITS, *TUB2*, and *RPB2* regions showed distinct clustering of the isolates from other *Didymella* species. In addition, the morphological and cultural characteristics of the isolates were somewhat different from those of closely related *Didymella* spp. Therefore, the novelty of the isolates was proved based on the investigations. Pathogenicity of the novel *Didymella* species isolates was confirmed on leaves of Korean angelica plants *via* artificial inoculation. This study reveals that *Didymella gigantis* sp. nov. causes leaf spot in Korean angelica.

1. Introduction

Korean angelica (*Angelica gigas* Nakai) is a biennial or perennial belonging to the family Apiaceae. The plant is native to Korea and Manchuria and grows primarily in the temperate biome [1]. It has been used as a medicinal plant in Korea, which has been reported to have an anti-bacterial effect [2].

During a disease survey in October 2019, leaf spot symptoms with a yellow halo were observed on Korean angelica plants grown in fields in Pyeongchang, Gangwon Province, Korea. The symptoms occurred sporadically on the leaves, and the severely diseased leaves in the late stage blighted. We obtained fungal isolates from the leaf lesions and examined morphological characteristics of the isolates. The isolates produced ellipsoidal, hyaline, and aseptate conidia in pycnidia, which were fitted into the concept of the genus *Phoma* [3].

Many *Phoma* spp. were primarily named and revised based on their host relationships, morphological and cultural characteristics [3,4]. The genera *Didymella*, *Leptosphaeria*, *Mycosphaerella*, and *Pleospora* have been known as teleomorphs for the genus *Phoma* based on a morphological study [3]. Especially, *Didymella* was described as a type genus of the family *Didymellaceae* based on the phylogenetic

study and contained approximately 70% of described *Phoma* spp. [5]. It has been reported that the genus *Didymella* produces 8 hyaline or brown didymospores with 1 to multi-septa in cylindrical, clavate or saccate bitunicate asci within a pseudothecium in the teleomorphic state, and aseptate, ellipsoidal to allantoid hyaline conidia within a pycnidium in the anamorphic state [6,7]. Later, many *Didymella* species have been reported based on phylogenetic studies [7–9].

This study aims to identify the unknown *Phoma* sp. isolated from leaf lesions of Korean angelica in Korea using molecular phylogenetic analysis in conjunction with morphological and cultural characteristics. Also, the pathogenicity test of the isolates was conducted to determine that the isolates were responsible for the leaf spot symptoms observed in the fields.

2. Materials and methods

2.1. Disease survey and fungal isolation

In October 2019, we surveyed diseases occurring on Korean angelica plants grown in five fields in Pyeongchang, Gangwon Province, Korea. Fifty plants were observed in each field and investigated for disease incidence on the leaves of the plants. Diseased leaves of the plants were collected from the investigated

fields. Lesion pieces (5–6 mm²) were cut from the leaves and surface-sterilized with 1% sodium hypochlorite solution for one minute. The pieces were placed on 2% water agar (WA) and incubated at 25 °C for 3–4 days Then, actively growing mycelia from the pieces were transferred onto potato dextrose agar (PDA). The isolates were transferred onto oatmeal agar (OA), and single-spore isolates were obtained from 2-week-old OA cultures. Two isolates (ANGI-1914 and ANGI-1915) were selected among the single-spore isolates for identification and pathogenicity tests. A representative isolate was deposited in the Korean Agricultural Culture Collection (KACC), Wanju, Korea.

2.2. Investigation of cultural and morphological characteristics

Cultural and morphological characteristics of the isolates were investigated according to the methods described in the previous studies [3,7]. The cultural characteristics were investigated using three different media, malt extracted agar (MEA), OA, and PDA. The isolates were incubated on the media at 22 °C in the dark for 7 days to measure their colony diameter, followed by another week of incubation at 22 °C under alternating cycles of 13/11 hr of NUV light and dark to investigate their colony morphology. Thirty pycnidia and conidia of each isolate obtained from 2-week-old cultures on OA were examined for their morphological characteristics. The pycnidia and conidia mounted in sterile distilled water were observed and measured under the light microscope (Nikon Eclipse Ci-L, Tokyo, Japan). To observe the anatomic structure of pycnidial walls and the morphology of conidiogenous cells, pycnidia from the OA cultures were sectioned in 6 μm thick using the cryostat-microtome (KD-2950, Zhejiang Jinhua Kedee Instrumental Equipment Co., Jinhua, China). Fifteen pycnidial walls and conidiogenous cells mounted in lactic acid were observed and measured using the light microscope. NaOH spot test [3] was conducted on 1-week-old cultures on MEA.

2.3. DNA extraction, PCR, and sequencing

Genomic DNA of the isolates was extracted using the protocol in a previous study [10], with slight modifications. In polymerase chain reaction (PCR) experiments, partial large subunit nuclear ribosomal DNA (LSU), internal transcribed spacer regions 1 & 2 including 5.8S nrDNA (ITS), β-tubulin (TUB2), and RNA polymerase II second largest subunit (RPB2) gene regions were investigated with the primer sets of LR0R [11] and LR7 [12] for LSU, V9G [13] and ITS4 [14] for ITS, Btub2Fd and Btub4Rd [15] for TUB2, and RPB2-5f2 [16] and fRPB2-7cR [17] for RPB2. Conditions of PCR amplification for all the genes were followed as in the previous studies [6,7]. Takara Ex Taq (Takara Bio Inc., Shiga, Japan) was used to prepare PCR products of the two isolates following the manufacturer's instruction. The PCR products were purified using the Universal DNA Purification Kit (Tiangen, Beijing, China) following the manufacturer's protocol. The PCR products were sequenced at Bionics Co. (Seoul, Korea) with the same primers. The sequences were adjusted by SeqMan II (DNASTAR Inc., Madison, WI, USA) if necessary. The sequence data were deposited in Genbank.

2.4. Alignment and molecular phylogenetic analysis

The sequences of the isolates obtained from Korean angelica and the relevant sequences of Didymella spp. from the previous studies [6–8,18] (Table 1) were aligned together using MUSCLE [19]. Coniothyrium palmarum (CBS 400.71) was used as an outgroup taxon. The multiple sequence alignments were processed and improved, if necessary, with MEGA version 7 software [20]. The partition-homogeneity test was conducted by PAUP version 4.0 software [21]. A neighbor-joining (NJ) estimation and a maximum-likelihood (ML) estimation for concatenated alignments were conducted with a maximum composite likelihood model and a general time reversible model, respectively, performing 1,000 bootstrap replicates by MEGA version 7 software [20]. Neighbor-joining bootstrap values (NJBS) and maximum-likelihood bootstrap values (MLBS) equal to or greater than 50% were shown at nodes. Additionally, the model of the evolution of each gene was estimated by MrModeltest version 2.4 software [22]. Bayesian inference of the concatenated alignments was conducted by MrBayes version 3.2.4 software [23], using the model test results. The calculation ran until the average standard deviation of split frequencies reached a value of less than 0.01. Generated trees took a 25% burn-in process to calculate posterior probabilities (PP). The probabilities equal to or greater than 0.9 were shown at the nodes. The tree was visualized by FigTree version 1.4.4 software [24].

2.5. Pathogenicity test

Two isolates from leaf lesions of Korean angelica were used to confirm their pathogenicity on the

Table 1. Isolates of *Didymella* spp. and *Coniothyrium palmarum* used for molecular phylogenetic analyses in this study.

Species	Strain/Isolate[a]	Host/Substrate	Locality	Genbank accession number[b]			
				LSU	ITS	*TUB2*	*RPB2*
D. gigantis	ANGI-1914	*Angelica gigas*	Korea	OQ746315	OQ746335	OQ731404	OQ731406
	ANGI-1915	*Angelica gigas*	Korea	OQ746316	OQ746336	OQ731405	OQ731407
D. brunneospora	CBS 115.58	*Chrysanthemum roseum*	Germany	KT389723	KT389505	KT389802	KT389625
D. chloroguttulata	CGMCC 3.18351	Air	China	KY742211	KY742057	KY742299	KY742142
D. combreti	CBS 137982	*Combretum mossambiciensis*	Zambia	KJ869191	KJ869134	MT005626	MT018139
D. dimorpha	CBS 346.82	*Opuntiae* sp.	Spain	GU238068	GU237835	MT018158	GU237606
D. ellipsoidea	CGMCC 3.18350	Air	China	KY742214	KY742060	KY742145	KY742302
D. exigua	CBS 183.55	*Rumex arifolius*	France	EU754155	GU237794	GU237525	EU874850
D. gei	CGMCC 3.20068	*Geum* sp.	China	MT229675	MT229698	MT249266	MT239095
D. infuscatispora	CGMCC 3.18356	*Chrysanthemum indicum*	China	KY742221	KY742067	KY742309	KY742152
D. ligulariae	CGMCC 3.20070	*Ligularia sibirica*	China	MT229676	MT229699	MT249267	MT239096
D. microchlamydospora	CBS 105.95	*Eucalyptus* sp.	U.K.	GU238104	FJ427028	FJ427138	KP330424
D. pteridis	CBS 379.96	*Pteris* sp.	The Netherlands	KT389722	KT389504	KT389801	KT389624
D. segeticola	CGMCC 3.17489	*Cirsium segetum*	China	KP330455	KP330443	KP330399	KP330414
D. subrosea	CBS 733.79	*Abies alba*	France	MN943747	MN973540	MT005643	MT018174
D. suiyangensis	CGMCC 3.18352	Air	China	KY742243	KY742089	KY742330	KY742168
Coniothyrium palmarum	CBS 400.71	*Chamaerops humilis*	Italy	EU754153	AY720708	KT389792	KT389592

[a]CBS: Westerdijk Fungal Biodiversity Institute, Utrecht, The Netherlands; CGMCC: China General Microbiological Culture Collection, Beijing, China.
[b]LSU: 28S large subunit of the nrDNA gene; ITS: internal transcribed spacer regions 1 & 2 including 5.8S nrDNA gene; *TUB2*: ß-tubulin; *RPB2*: RNA polymerase II second largest subunit.

leaves of the host plants. Conidial suspension from 2-week-old OA cultures was filtered through two layers of Miracloth (Sigma-Aldrich, St. Louis, USA) and diluted with sterile distilled water. Korean angelica plants were grown in plastic pots (height: 14 cm; upper diameter: 15 cm; lower diameter: 10 cm) with commercial media in a vinyl greenhouse. A 30 ml of conidial suspension ($1–2 \times 10^7$ conidia/ml) of each isolate was sprayed onto the leaves of the 5-month-old plants after shooting. The inoculated plants were placed in plastic boxes ($63 \times 44 \times 47$ cm) under 100% relative humidity at room temperature (24–26 °C). Control plants were sprayed with the same quantity of sterile distilled water and placed under the same conditions as the inoculated plants. After 3 days, the inoculated plants were moved out of the boxes and kept indoors. Then, the pathogenicity of the isolates was determined based on the degree of leaf spot symptoms 12 days after inoculation. The pathogenicity test was performed in triplicate.

3. Results

3.1. Molecular phylogeny

Based on the model test, the best fitting models for alignments of LSU, ITS, *TUB2*, and *RPB2* were found to be HKY + I, HKY + I + G, GTR + I + G, and GTR + G, respectively. The partition-homogeneity test (*p* value

= 0.96) indicated that trees of the genes share a common underlying structure, which allowed us to combine the alignments of each investigated gene and construct phylogenetic trees. The concatenated alignments of 14 ingroup taxa contained a total of 2,218 characters (828, 453, 337, and 600 characters for LSU, ITS, *TUB2*, and *RPB2*), respectively. Three analyses indicated no significant difference in topology (data not shown). Thus, only the NJ tree with bootstrap values was provided with the posterior probabilities at nodes (NJBS/PP/MLBS). The isolates ANGI-1914 and ANGI-1915 were found to belong to the genus *Didymella* (Figure 1). In addition, the isolates were grouped together and determined as the same species because of no difference in their alignments. However, they were not grouped into the same species as any species in the genus. Moreover, a single clade containing the isolates showed a high bootstrap value and posterior probability that was distinct from other closely related species such as *Didymella bellidis* (Neerg.) Qian Chen & L. Cai (synonym: *Phoma bellidis* Neerg.) [7,25], *Didymella segeticola* (Q. Chen) Q. Chen, Crous & L. Cai (synonym: *Phoma segeticola* Qian Chen) [8,26], and *Didymella suiyangensis* Qian Chen, Crous & L. Cai [8]. Genbank accession numbers of the isolates ANGI-1914 and ANGI-1915 are OQ746315–OQ746316, OQ746335–OQ746336, OQ731404–OQ731405, and OQ731406–OQ731407 for LSU, ITS, *TUB2*, and *RPB2*, respectively.
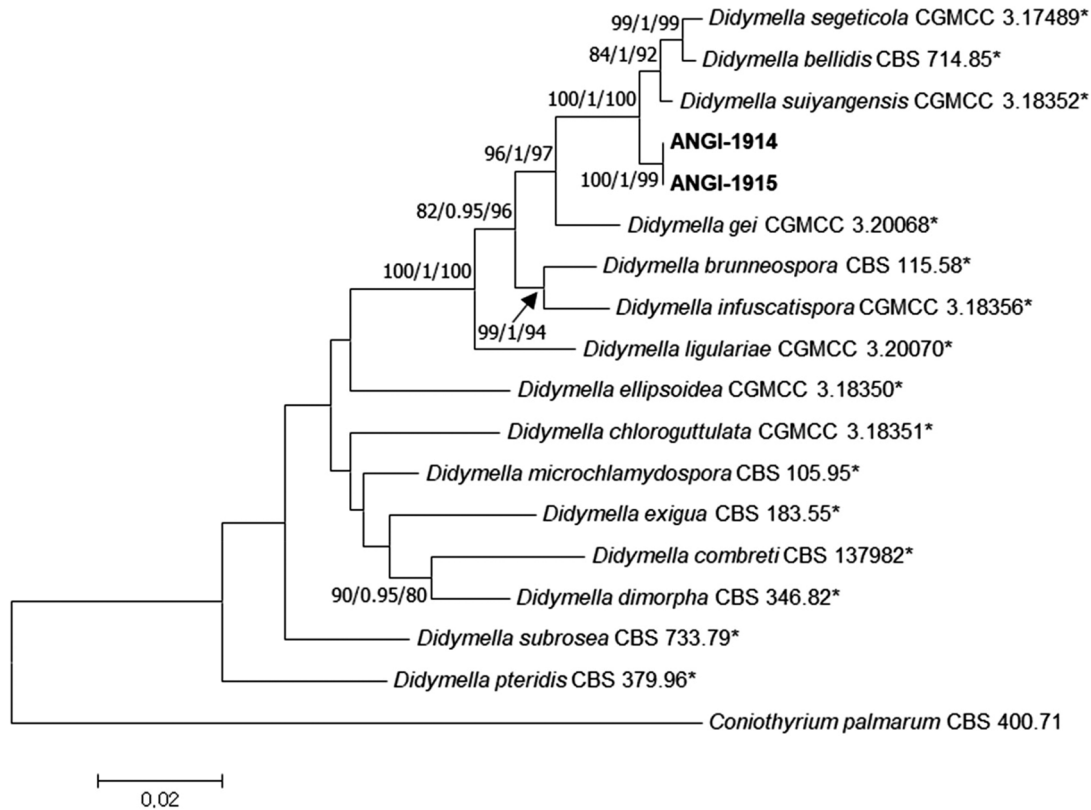
Figure 1. A phylogenetic tree constructed by the neighbor-joining analysis with maximum composite likelihood model based on a concatenated alignment of partial large subunit nuclear ribosomal DNA, internal transcribed spacer regions 1 & 2 including 5.8S nrDNA gene, β-tubulin, and RNA polymerase II second largest subunit sequences of two isolates (ANGI-1914 and ANGI-1915) of *Didymella gigantis* sp. nov. and related *Didymella* spp. Neighbor-joining bootstrap values (NJBS), posterior probabilities (PP), and maximum-likelihood bootstrap values (MLBS) are shown at nodes (NJBS/PP/MLBS). The bar represents the number of nucleotide substitutions per site. The phylogenetic tree was rooted to *Coniothyrium palmarum* (CBS 400.71). *The ex-type strains.

3.2. Taxonomy

Didymella gigantis G.B. Lee and W.G. Kim, sp. nov. (Figure 2)

MycoBank No.: MB 846811

Etymology: Named derived from the specific epithet of the host, *Angelica gigas*.

Holotype: Isolated from leaf of Korean angelica, Pyeongchang, Gangwon Province, Korea (37° 40′ 52” N and 128° 33′ 57”E), October 2019, W.G. Kim, ex-holotype culture (KACC 410301).

Cultural and morphological characteristics: The diameter of 1-week-old cultures of the isolates (ANGI-1914 and ANGI-1915) on MEA, OA, and PDA was 64.2–65.7 mm (av. 64.5 mm), 65.2–67.0 mm (av. 66.5 mm), and 65.0–66.2 mm (av. 65.4 mm), respectively. The culture on MEA showed a partly wrinkled colony with white to brown mycelium (Figure 2A). The culture on OA showed white mycelium of which the center was brown in color (Figure 2B). The culture on PDA showed a generally wrinkled colony with white to honey mycelium (Figure 2C). NaOH spot test on MEA was negative.

Teleomorph of the isolates was not found in the cultures. Pycnidia usually half submerged in the agar or on the surface (Figure 2D), 82–260 μm in diameter, solitary, glabrous, brown to black, with 1–3 ostioles, slightly papillate or non-papillate (Figure 2E and F). Pycnidial walls pseudoparenchymatous, composed of round cells, 3–5 layers, 12–32 μm thick, and outer cell layers pigmented (Figure 2G). Conidiogenous cells phialidic, hyaline, ampulliform, globous to flask-shaped, and 4.6–6.1 × 4.6–7.4 μm (Figure 2H and L). Conidia 4.0–8.5 × 1.7–3.6 μm (av. 6.1 × 2.5 μm), ellipsoidal or slightly curved long ellipsoidal, smooth, and aseptate with usually 2 bipolar guttules (Figure 2J). Chlamydospores absent. Major cultural and morphological characteristics of *D. gigantis* and closely related *Didymella* spp. are shown in Table 2. *D. gigantis* was somewhat dissimilar to the closely related *Didymella* spp. in the cultural and morphological characteristics.

3.3. Disease incidence and pathogenicity

During the disease survey in October 2019, leaf spot symptoms were observed on Korean angelica plants in the investigated fields. The symptoms occurred sporadically on the leaves, which were
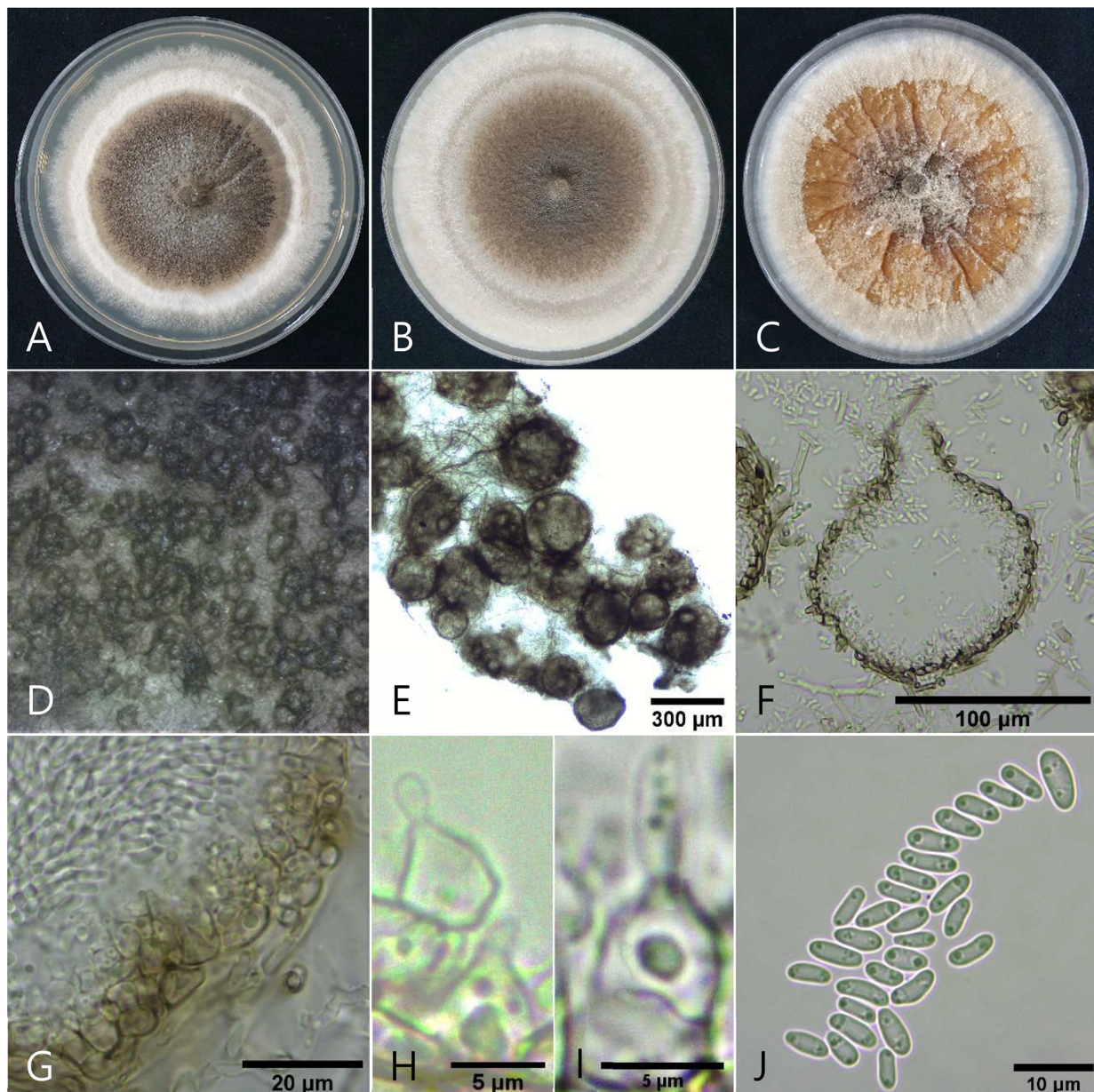
Figure 2. Cultural and morphological features of *Didymella gigantis* sp. nov. (A) Two-week-old colonies on malt extract agar; (B) oatmeal agar; (C) potato dextrose agar. (D, E) Pycnidia produced in oatmeal agar. (F) Section of a pycnidium. (G) Section of a pycnidial wall. (H, I) Conidiogenous cells and conidia. (J) Conidia.

circular, brown to dark brown with a yellow halo, and 1–3 mm in diameter in the early stage. They enlarged up to 20 mm as the disease progressed (Figure 3A and B). The severely diseased leaves in the late stage blighted. Incidence of diseased leaves of the plants in the investigated fields ranged from 10% to 60%.

The isolates (ANGI-1914 and ANGI-1915) from leaf spot lesions of Korean angelica induced leaf spot symptoms in the inoculated plants of Korean angelica (Figure 3C), but no symptoms were observed on the leaves of the control plants (Figure 3D). The induced lesions were similar to those observed in the fields during the disease survey. Re-isolation of the isolates from the induced lesions was confirmed.

4. Discussion

Based on the phylogenetic analyses, two isolates of *D. gigantis* were placed in the genus *Didymella* with the forming of an independent clade from other closely related taxa such as *D. bellidis*, *D. segeticola,* and *D. suiyangensis,* showing differences of 30 bp, 30 bp, and 19 bp in four loci from the related taxa, respectively. In addition, the morphological and cultural characteristics of *D. gigantis* were found to be somewhat different from those of other *Didymella* spp. Compared with the morphology of *D. bellidis*, *D. segeticola,* and *D. suiyangensis* [7,8,25,26], *D. gigantis* produced longer conidia than the three species. This fungus also produced much larger conidiogenous cells than *D. segeticola* and *D. suiyangensis*. The

Table 2. Major morphological and cultural characteristics of *Didymella gigantis* and closely related *Didymella* species.

| | Morphological characteristics | | Colony on media[a] and | |
Didymella spp.	Pycnidia	Conidiogenous cells and conidia	result of NaOH spot tests	Reference
D. gigantis	82–260 μm in diameter. Globose, glabrous, brown to black, with 1–3 ostioles, non-papillate or slightly papillate.	Conidiogenous cells: 4.6–6.1 × 4.6–7.4 μm; phialidic, hyaline, ampulliform, globous to flask-shaped. Conidia: 4.0–8.5 × 1.6–4.6 μm; ellipsoidal or slightly curved long ellipsoidal, smooth, aseptate with 2 bipolar guttules. Chlamydospores absent.	MEA: wrinkled, white to brown; 64.2–65.7 mm. OA: white to brown; 65.2–67.0 mm. PDA: wrinkled, white to honey; 65.0–66.2 mm. NaOH spot test: negative.	Present study
D. bellidis (CBS 714.85)	50–260 μm in diameter. Globose to irregular shape, glabrous, honey to black, with 1–5 ostioles, non-papillate or slightly papillate.	Conidiogenous cells: 3–6 × 4–8 μm; globose to bottle-shaped. Conidia: 3.8–6.4 × 1.8–2.6 μm; ellipsoidal, aseptate with 2 polar guttules. Conidial matrix salmon to saffron. Chlamydospores absent.	MEA: olivaceous to grey; 76–77 mm. OA: white to colorless, but salmon color in center; 68 mm. NaOH spot test: positive.	[7,25]
D. segeticola (CGMCC 3.17489)	90–105 × 75–95 μm. Subglobose, glabrous, pyriform to irregular shape in later, 1–2 ostioles, on an elongated neck.	Conidiogenous cells: 5–6.5 × 4–5.5 μm; phialidic, hyaline, simple, smooth, flask-shaped or sometimes isodiametric. Conidia: 4.5–7 × 2.5–4 μm; ellipsoidal to ovoid or cylindrical, aseptate with 1–6 polar guttules, Conidial matrix crème-white.	MEA: white and green in the center; 64–66 mm. OA: white to grey; 56–65.5 mm. PDA: white to grey; 52–59 mm. NaOH spot test: negative.	[8,26]
D. suiyangensis (CGMCC 3.18352)	90–240 × 55–180 μm. Globose to irregular shape, covered by some hyphal outgrowths, brown, 1 ostiole, slightly papillate or papillate.	Conidiogenous cells: 4–4.5 × 3–4 μm; phialidic, hyaline, smooth, ampulliform to doliiform. Conidia: 3.5–7 × 2–3 μm; ellipsoidal to oblong, smooth, aseptate with indistinct guttules. Conidial matrix cream.	MEA: grey to olivaceous; 59–64 mm. OA: white to buff; 52–55 mm. PDA: white to grayish brown; 57–61 mm. NaOH spot test: positive.	[8]

[a]Diameter of colonies on MEA, OA, and PDA was measured after incubation at 22 °C for one week. Other colony features were investigated after incubation at 22 °C for two weeks. MEA: malt extracted agar; PDA: potato dextrose agar; OA: oatmeal agar.
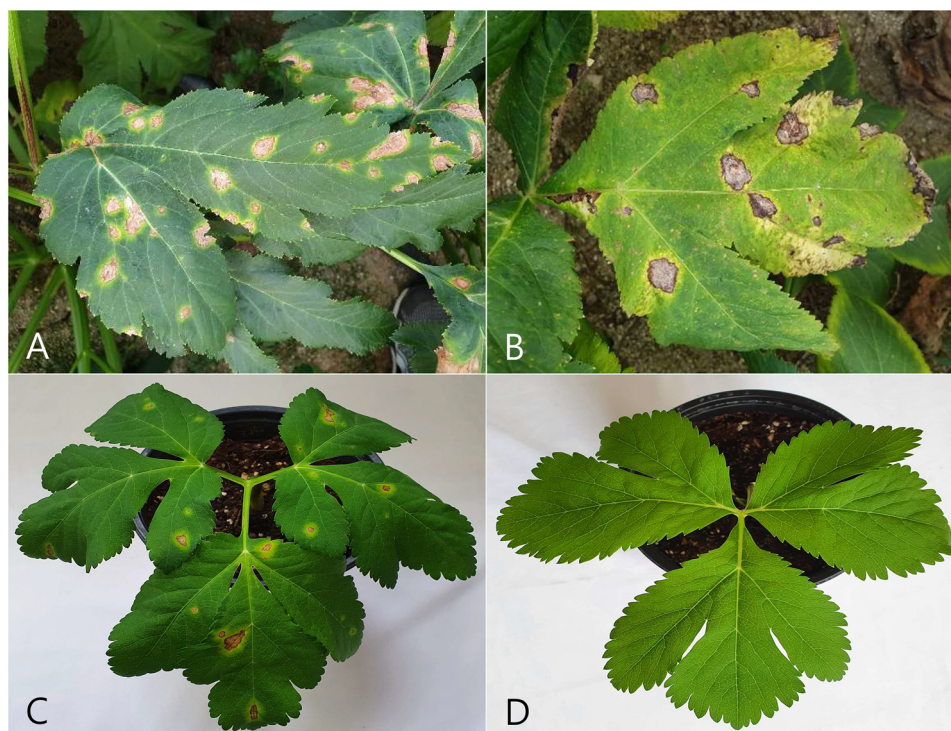
Figure 3. Leaf spot symptoms of Korean angelica plants. (A, B) Symptoms on the leaves observed in the investigated fields. (C) Induced symptoms on the leaves by artificial inoculation with the isolate (ANGI-1915) of *Didymella gigantis* sp. nov. in the pathogenicity test. (D) A non-inoculated control plant.

pycnidial size of *D. gigantis* was similar to that of *D. bellidis*, but much bigger than that of *D. segeticola* and *D. suiyangensis*. Additionally, number of pycnidial ostioles of *D. gigantis* was 1–3, but that of *D. bellidis*, *D. segeticola,* and *D. suiyangensis* 1–5, 1–2, and 1.

The colony of *D. gigantis* exhibited a cream-colored conidial matrix, which was similar to that of *D. segeticola* and *D. suiyangensis*. The growth rates of *D. gigantis* on MEA, OA, and PDA were similar to those of *D. segeticola*. *D. gigantis* formed a wrinkled colony on MEA and PDA, which was a unique characteristic of the fungus. The fungus showed a negative reaction in NaOH spot test, which was the same to only *D. segeticola* among the taxa. The differences between *D. gigantis* and other closely related *Didymella* spp. in morphological and cultural characteristics as well as the individual clustering in the phylogenetic tree analyses should be sufficient evidence to suggest the novelty of *D. gigantis*. Therefore, we propose *D. gigantis* sp. nov. as a fungal pathogen causing leaf spot in Korean angelica.

It has been reported that *Didymella* (anamorph: *Phoma*) spp. causes leaf spot, stem rot, seed rot, etc. in various plants [25,27,28]. In Korea, *D. bellidis* was reported to cause leaf spot of Korean angelica [29]. In the previous report, identification of the fungus was accomplished through BLASTn analysis with sequences of ITS and *TUB2* genes of the fungal isolate. However, multi-locus phylogenetic analyses using sequences of 4 genes (LSU, ITS, *TUB2*, and *RPB2*) were recommended to differentiate closely related species in the genus *Didymella* [7]. Accordingly, it is considered that the identification of *D. bellidis* in the previous report [29] should be reviewed again because multi-locus phylogenetic analyses were not performed. Further study is also needed to determine whether the reported species is the same as *D. gigantis*.

Disclosure statement

No potential conflict of interest was reported by the authors.

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