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RESEARCH ARTICLE

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A Novel Subspecies of *Didymella acutilobae* Causing Leaf Spot in East Asian Hogweed

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ABSTRACT

During disease surveys in 2021 and 2022, outbreaks of leaf spot were observed in East Asian hogweed (*Heracleum moellendorffii*) plants in fields located in Pyeongchang and Yeongwol, Gangwon Province, Korea. The disease incidence in the fields ranged from 2% to 50%. Based on the morphological and cultural characteristics, four single-conidium fungal isolates from the leaf spot symptoms were identified as *Phoma* sp. The phylogenetic analyses based on the combined sequences from the four genes (LSU, ITS, *TUB2*, and *RPB2*) indicated that the isolates clustered very closely with *Didymella acutilobae*. However, the morphological and cultural characteristics of the isolates exhibited somewhat distinct differences from those of *D. acutilobae*, suggesting that the isolates correspond to a novel subspecies. Pathogenicity tests revealed that the isolates caused leaf spot in East Asian hogweed plants. This is the first report of *D. acutilobae* subsp. *heraclei* subsp. nov. causing leaf spot in East Asian hogweed.

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1. Introduction

East Asian hogweed (*Heracleum moellendorffii* Hance) belongs to the family Apiacea and lives as a perennial plant. The plant grows primarily in the temperate regions and is native to many countries in East Asia including China, Korea, and Japan [1]. This plant has been used as an edible herb and known to have medicinal properties, providing anti-inflammatory effect [2] and immune enhancement [3].

During disease surveys in 2021 and 2022, outbreaks of leaf spot symptoms were observed in East Asian hogweed plants grown in fields in Pyeongchang and Yeongwol, Gangwon Province, Korea. Fungal isolates were obtained from the lesions and used to examine their morphological characteristics. Conidia in pycnidia of the isolates were generally hyaline, ellipsoidal, and aseptate, which corresponded to the typical characteristics of the genus *Phoma* [4].

Numerous *Phoma* species have been found in diverse substrates, functioning as saprobic, endophytic, and pathogenic fungi in relation to both plants and animals [5–7]. However, the limited morphological studies on the anamorphs and teleomorphs of *Phoma* spp. have resulted in a confusing group of taxa with ambiguous morphological boundaries among species [5]. Recently, many *Phoma* spp. have been reclassified as *Didymella* spp. using integrated approaches, including conventional methods and multigene phylogenetic analyses using 28S nrDNA (LSU), internal transcribed spacers and intervening 5.8S nrDNA (ITS), β-tubulin (*TUB2*), and RNA polymerase II second largest subunit (*RPB2*) genes [8–10]. Morphological features of the genus *Didymella* indicate eight hyaline to brown didymospores having one to multi-septa in cylindrical shaped bitunicate asci in a pseudothecium (teleomorph), and aseptate, hyaline conidia shaped in ellipsoidal to allantoid in a pycnidium (anamorph) [8].

The objective of this study is to discover and characterize unknown fungal isolates from the leaf spot symptoms of East Asian hogweed plants in Korea. The fungal isolates were investigated using multi-locus based phylogenetic analysis and examined for their mycological characteristics and pathogenicity.

2. Materials and methods

2.1. Investigation of disease and isolation of fungi

In June 2021 and July 2022, leaf spot outbreaks occurred in East Asian hogweed plants cultivated in four fields in Pyeongchang and Yeongwol, Gangwon Province, Korea. Diseased tissues were cut into pieces (5 × 5 mm) and surface-sterilized with 1% sodium hypochlorite solution for 1 min. The lesion pieces were placed on the 2% water agar and incubated at

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25 °C for 3–4 days. A single-conidium fungal isolates were obtained from pycnidia formed on the lesion pieces. Four single-conidium isolates (HEMO-2111, HEMO-2115, HEMO-2201, and HEMO-2203) were prepared to proceed for further identification and pathogenicity tests. A representative isolate (HEMO-2201) was deposited in the Korean Agricultural Culture Collection (KACC), Wanju, Korea.

2.2. Examination of mycological characteristics

Colonial morphology of the isolates was investigated using cultures grown on malt extracted agar (MEA), oatmeal agar (OA), and potato dextrose agar (PDA) according to the methods described in the previous studies [4,10]. Colony diameter was measured 7 days after incubation of the isolates on the media. Morphological characteristics were investigated 14 days after incubation of the isolates on OA. Thirty conidia and pycnidia from the OA cultures of each isolate and 15 sections of pycnidia prepared by the protocol in a previous study [11] were examined for their morphology using a light microscope (Nikon Eclipse Ci-L, Tokyo, Japan). NaOH spot test [4] was conducted using 1-week-old cultures on MEA.

2.3. Extraction of genomic DNA and PCR work

Extraction of genomic DNA of the isolates was performed using a method in the previous studies [11,12]. LSU, ITS, *TUB2*, and *RPB2* gene regions of the isolates were amplified using specific primer sets according to PCR protocols from a previous study [10]. The primers used were LR0R [13] and LR7 [14] for LSU, V9G [15] and ITS4 [16] for ITS, Btub2Fd and Btub4Rd [17] for *TUB2*, and RPB2-5f2 [18] and fRPB2-7cR [19] for *RPB2*. DNA Free-Taq Master Mix (CellSafe, Yongin, Korea) and Universal DNA Purification Kit (Tiangen, Beijing, China) were used in the PCR work under the manufacturer's protocol. Sequencing was processed at Bionics Co., Ltd. (Seoul, Korea) with the same primers. The sequence data were deposited in NCBI GenBank.

2.4. Phylogenetic analysis

The sequences of the isolates, along with relevant sequences of *Didymella* spp. from the previous studies [10,20,21] (Table 1), were aligned together using MUSCLE in MEGA 11 software [22]. *Coniothyrium palmarum* (CBS 400.71) was selected as an outgroup taxon. The multiple sequence alignments were processed and refined, if needed, using MEGA 11 software [22]. Maximum-likelihood estimation for the concatenated alignments was performed with a general time-reversible model, and 1,000 bootstrap replicates were conducted using MEGA 11 software [22]. Maximum-likelihood bootstrap values (BS) of 50% or higher were displayed at the nodes. The optimal

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. acutilobae subsp. *heraclei*	HEMO-2111	*Heracleum moellendorffii*	Korea	PP791476	PP791480	PP793989	PP793993
	HEMO-2115			PP791477	PP791481	PP793990	PP793994
	HEMO-2201			PP791478	PP791482	PP793991	PP793995
	HEMO-2203			PP791479	PP791483	PP793992	PP793996
D. aquatica	CGMCC 3.18349	Water	China	KY742209	KY742055	KY742297	KY742140
D. acutilobae	KACC 410302	*Angelica acutiloba*	Korea	OQ749983	OQ749981	OQ744071	OQ744073
D. brunneospora	CBS 115.58	*Chrysanthemum roseum*	Germany	KT389723	KT389505	KT389802	KT389625
D. chloroguttulata	CGMCC 3.18351	Air	China	KY742211	KY742057	KY742299	KY742142
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. gigantis	KACC 410301	*Angelica gigas*	Korea	OQ746316	OQ746336	OQ731405	OQ731407
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. macrophylla	CGMCC 3.18357	*Hydrangea macrophylla*	Italy	KY742224	KY742070	KY742312	KY742154
D. microchlamydospora	CBS 105.95	*Eucalyptus* sp.	UK	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. senecionicola	CBS 160.78	*Senecio jacobaea*	New Zealand	GU238143	GU237787	GU237657	MT018177
D. subrosea	CBS 733.79	*Abies alba*	France	MN943747	MN973540	MT005643	MT018174
D. suiyangensis	CGMCC 3.18352	Air	China	KY742243	KY742089	KY742330	KY742168
C. 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; KACC: Korean Agricultural Culture Collection, National Institute of Agricultural Sciences, Wanju, Korea.

[b]LSU: 28S large subunit of the nrDNA gene; ITS: internal transcribed spacer regions 1 and 2 including 5.8S nrDNA gene; *TUB2*: β-tubulin; *RPB2*: RNA polymerase II second largest subunit.

nucleotide substitution model for each gene was defined using MrModeltest version 2.4 software [23]. Then, Bayesian analysis was run by MrBayes version 3.2.4 software [24], based on the results of the model test.

The Bayesian estimation continued until the average standard deviation of split frequencies fell below 0.01. Generated trees underwent 25% burn-in procedure to calculate posterior probabilities (PP). Probabilities of 0.9 or higher were displayed at the nodes. The phylogenetic tree was visualized using FigTree version 1.4.4 software [25].

2.5. Pathogenicity test

Three isolates (HEMO-2115, HEMO-2201, and HEMO-2203) were used to corroborate their pathogenicity on the host plant leaves. A conidial suspension prepared from 1-month-old PDA cultures of each isolate was filtered through two layers of Miracloth (Sigma-Aldrich, St. Louis, USA) and diluted with sterile distilled water. East Asian hogweed plants were grown in circular plastic pots (14 cm in height, 15 cm in upper diameter, and 10 cm in lower diameter) filled with commercial bed soil in a vinyl greenhouse. A 25 mL of conidial suspension ($1–2 \times 10^6$ conidia/mL) of each isolate was sprayed onto leaves of each 6-month-old plant after shooting. The pots with inoculated plants were placed in plastic boxes in a room at 24–26 °C. Control plants were sprayed with the same amount of sterile distilled water and kept under the same conditions as the inoculated plants. After 4 days, the inoculated plants were taken out of the boxes and kept indoors. Pathogenicity of the isolates was checked based on leaf spot formation after 7 days

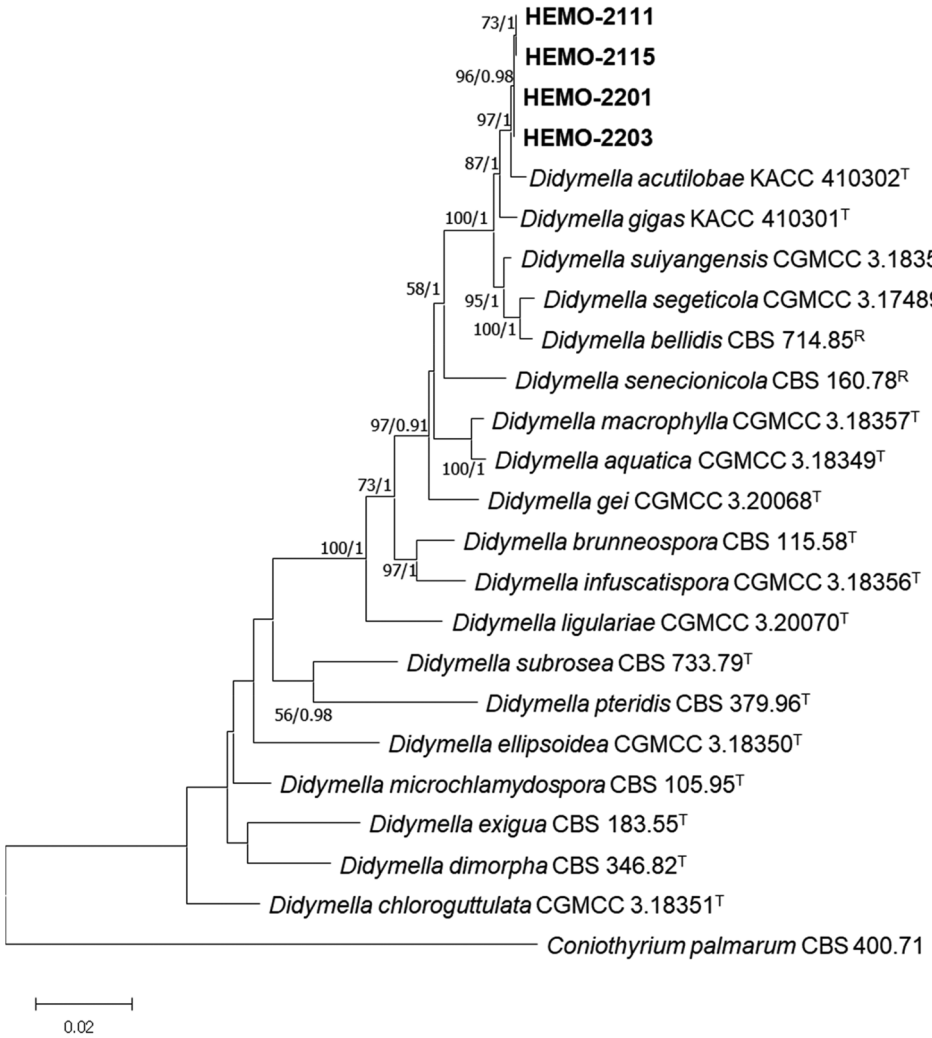

Figure 1. A phylogenetic tree constructed by the maximum-likelihood analysis with general time-reversible model based on concatenated alignments of partial large subunit nuclear ribosomal DNA, internal transcribed spacer regions 1 and 2 including 5.8S nrDNA gene, β-tubulin, and RNA polymerase II second largest subunit sequences of four isolates (HEMO-2111, HEMO-2115, HEMO-2201, and HEMO-2203) of *Didymella acutilbae* subsp. *heraclei* subsp. nov. and related *Didymella* spp. Bootstrap values (BS) and posterior probabilities (PP) are shown at nodes (BS/PP). The bar represents the number of nucleotide substitutions per site. The phylogenetic tree was rooted to *Coniothyrium palmarum* (CBS 400.71). T: ex-type strains; R: reference strains.

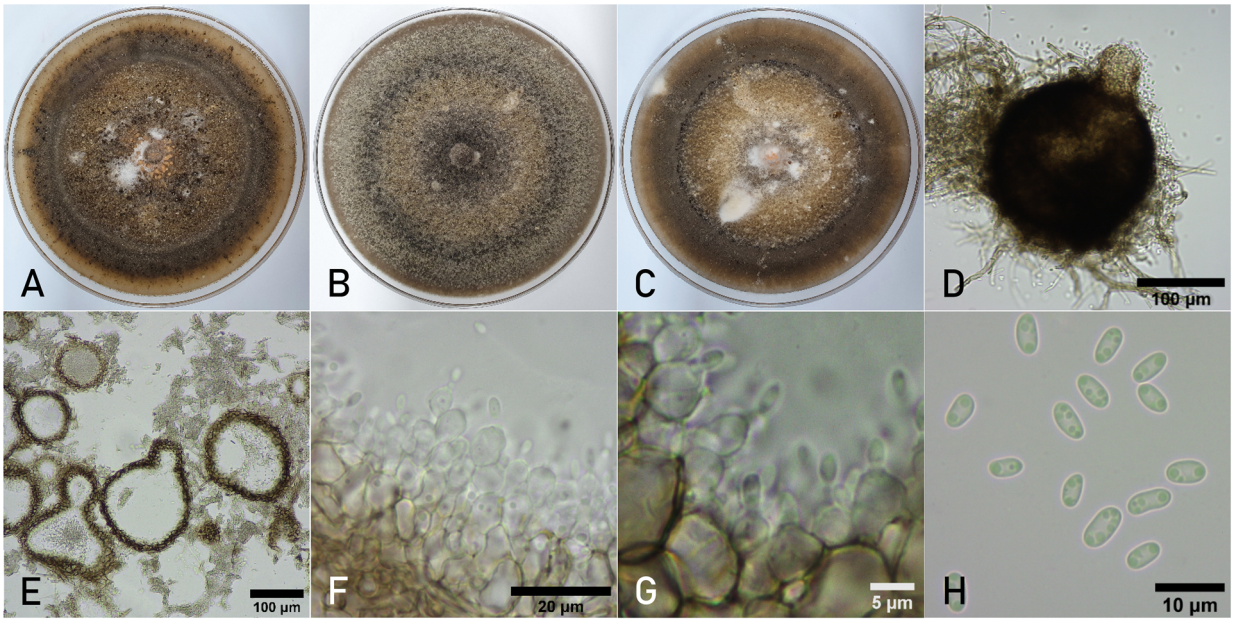

Figure 2. Cultural and morphological features of *Didymella acutilbae* subsp. *heraclei* subsp. nov. (A) Two-week-old colonies on malt extract agar; (B) oatmeal agar (OA); (C) potato dextrose agar. (D) A pycnidium produced in OA; (E) A section of pycnidia; (F,G) Conidiogenous cells and conidia; (H) Conidia.

from the inoculation. The pathogenicity test was conducted in triplicate.

3. Results

3.1. Phylogeny

According to the model test, the optimal models for alignments of LSU, ITS, *TUB2*, and *RPB2* were HKY+I, SYM+I+G, GTR+I+G, and GTR+I, respectively. The concatenated alignments of 20 ingroup taxa contained a total of 2,373 characters (939, 497, 337, and 600 characters for LSU, ITS, *TUB2*, and *RPB2*), respectively. No significant differences in topology were observed between two analyses (data not shown). Hence, the phylogenetic tree based on maximum-likelihood analysis with BS and PP at nodes was provided. The isolates belonged to the genus *Didymella* and were verified as the same species each other (Figure 1). In the phylogenetic analysis, the isolates clustered very closely to *Didymella acutilbae* G.B. Lee and W.G. Kim [26], showing a high bootstrap value and posterior probability. Additionally, the isolates were clearly distinguished from other closely related species such as *Didymella gigantis* G.B. Lee and W.G. Kim [11], *Didymella suiyangensis* Qian Chen, Crous & L. Cai [27], *Didymella segeticola* (Q. Chen) Q. Chen, Crous & L. Cai (synonym: *Phoma segeticola* Qian Chen) [27,28], and *Didymella bellidis* (Neerg.) Qian Chen & L. Cai (synonym: *Phoma bellidis* Neerg.) [10,29]. Genbank accession numbers of the isolates HEMO-2111, HEMO-2115, HEMO-2201, and HEMO-2203 are PP791476–PP791479, PP791480–PP791483, PP793989–PP793992, and PP793993–PP793996 for LSU, ITS, *TUB2*, and *RPB2*, respectively.

3.2. Taxonomy

Didymella acutilobae* subsp. *heraclei G.B. Lee and W.G. Kim, subsp. nov. (Figure 2).

MycoBank No.: MB 854063

Etymology: Subspecies name was derived from generic name of the host, *Heracleum moellendorffii*.

Holotype: Isolated from leaf of East Asian hogweed, Pyeongchang, Gangwon Province, Korea (37°37′50″N and 128°21′4″E), July 2022, W.G. Kim. The holotype culture (KACC 410752) deposited in the KACC was preserved in a metabolically inactive state.

Mycological characteristics: The diameter of 1-week-old cultures of the representative isolate (HEMO-2201) on MEA, OA, and PDA was 60–65 mm, 60–66 mm, and 63–65 mm, respectively. The culture on MEA showed brown to dark brown mycelium (Figure 2A). The culture on OA showed gray to dark olivaceous mycelium (Figure 2B). The culture on PDA showed brown to dark brown mycelium, which was similar to culture on MEA, but less brownish mycelium in center (Figure 2C). NaOH spot test on MEA was negative.

In the cultures, no teleomorph of the isolates was observed. Pycnidia 80–295 µm in diameter, globose, brown to black, solitary with 1–3 ostioles, papillate or non-papillate (Figure 2D and E). Pycnidial walls 3–5 layers, pseudoparenchymatous, consisting of round cells, 7–15 µm thick (Figure 2F). Conidiogenous cells globous to flask-shaped,

Table 2. Major morphological and cultural characteristics of *Didymella acutilbae* subsp. *heraclei* and closely related *Didymella* species.

Didymella spp.	Morphological characteristics		Colony on media[a] and result of NaOH spot tests	References
	Pycnidia	Conidiogenous cells and conidia		
D. acutilbae subsp. *heraclei*	80–295 µm in diameter. Solitary, globose, brown to black, with 1–3 ostioles, non-papillate or papillate.	Conidiogenous cells: 3.5–6.4×3.9–6.5 µm; phialidic, hyaline, ampulliform, globous to flask-shaped. Conidia 3.9–7.0×2.1–2.9 µm; ellipsoidal, smooth, aseptate, 1–8 polar guttules. Conidial matrix cream to salmon. Clamydospores absent.	MEA: brown to dark brown; 60–66 mm. OA: dark olivaceous gray to gray; 60–66 mm. PDA: brown to dark brown 63–65 mm. NaOH spot test: negative.	Present study
D. acutilbae (KACC 410302)	70–240 µm in diameter. Solitary or confluent, globose, brown to black, with 1–5 ostioles, non-papillate or papillate.	Conidia: 2.9–6.5×1.6–3.0 µm; ellipsoidal or slightly curved, aseptate with usually 2 bipolar guttules. Conidial matrix white. Chlamydospores absent.	MEA: brown to black with light concentric rings; 54–61 mm. OA: brown to dark olivaceous; 53–54 mm. PDA: white to light brown with concentric rings; 53–55 mm. NaOH spot test: negative.	[26]
D. gigantis (KACC 410301)	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.	[11]
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.	[10,29]
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 center; 64–66 mm. OA: white to grey; 56–65.5 mm. PDA: white to grey; 52–59 mm. NaOH spot test: negative.	[27,28]
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.	[27]

[a]Diameter of colonies on MEA, OA, and PDA was measured after incubation at 22 °C for 1 week. Other colony features were investigated after incubation at 22 °C for 2 weeks. MEA: malt extracted agar; PDA: potato dextrose agar; OA: oatmeal agar.

ampulliform, hyaline, phialidic, and 3.5–6.4×3.9–6.5 µm (Figure 2G). Conidia 3.9–7.0×2.1–2.9 µm (av. 5.5×2.4 µm), ellipsoidal, smooth, aseptate, multiple guttules mostly (Figure 2H). Conidial matrix cream to salmon. Chlamydospores absent. Major morphological and cultural characteristics of *D. acutilobae* subsp. *heraclei* and closely related *Didymella* spp. are summarized in Table 2. *D. acutilobae* subsp. *heraclei* showed slight differences in morphological and cultural characteristics from *D. acutilobae*.

3.3. Result of disease survey and pathogenicity test

During disease surveys conducted in June 2021 and July 2022, we found leaf spot symptoms on East Asian hogweed plants in the surveyed fields. The leaf spot symptoms initially appeared as small brown circular spots, and later developed into blight symptoms with enlargement (Figure 3A and B). The incidence of diseased leaves of the plants in the fields ranged from 2% to 50%.

The tested three isolates caused leaf spot symptoms in the inoculated plants (Figure 3C). However, no symptoms were observed in the control plants (Figure 3D). The induced lesions resembled those observed in the surveyed fields. Re-isolation of the isolates from the induced lesions was verified morphologically.

4. Discussion

Based on the phylogenetic analyses, four isolates of *D. acutilobae* subsp. *heraclei* were placed in the

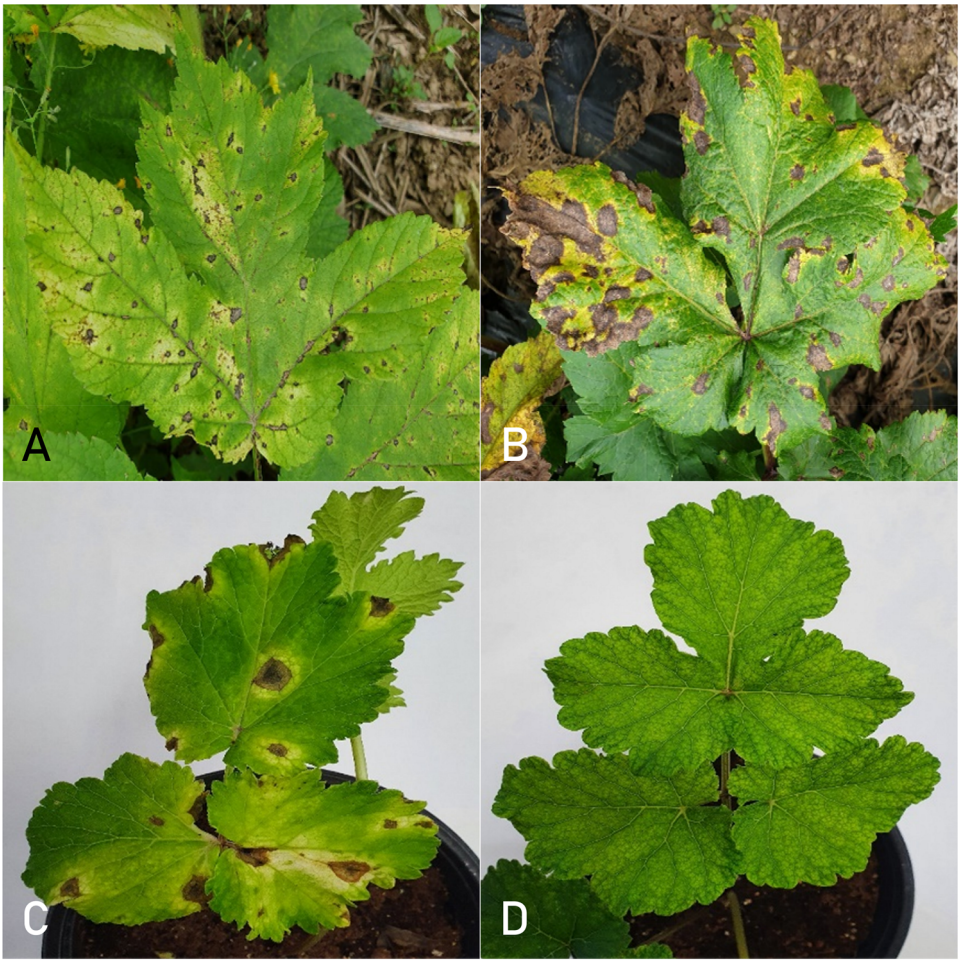

Figure 3. Leaf spot symptoms of East Asian hogweed plants. (A,B) Symptoms on the leaves observed in the investigated fields; (C) Induced symptoms on the leaves by artificial inoculation with the isolate (HEMO-2201) of *Didymella acutilbae* subsp. *heraclei* subsp. nov. in pathogenicity test; (D) A non-inoculated control plant.

genus *Didymella* clade and formed a cluster closely to *D. acutilobae*. Compared with the morphology of closely related *Didymella* spp., *D. acutilobae* subsp. *heraclei* produced longer conidia and more guttules than those of *D. acutilobae*. Pycnidial size of *D. acutilobae* subsp. *heraclei* was the biggest among those of related *Didymella* spp. Additionally, number of pycnidial ostioles of *D. acutilobae* subsp. *heraclei* was 1–3, which was identical to that of *D. gigantis*, whereas those of *D. suiyangensis*, *D. segeticola,* and *D. bellidis* were 1, 1–2, and 1–5, respectively. The growth rates of *D. acutilobae* subsp. *heraclei* on MEA, OA, and PDA were very similar to those of *D. gigantis*.

It has been reported that *Didymella* spp. cause leaf spot, stem rot, blossom rot and seed rot, etc. in various plants and are isolated from diverse substrates [30–34]. To date, reported fungal pathogens in *H. moellendorffii* include only *Erysiphe heraclei*, *Puccinia heraclei*, and *Ramularia heraclei* [35]. In this study, phylogenetic analyses confirmed the isolates from the diseased East Asian hogweed plants are clustered with *D. acutilobae*. Despite of the closeness to *D. acutilobae* in the phylogenetic analysis, *D. acutilobae* subsp. *heraclei* exhibited distinct morphological traits, including longer conidia, more number of guttules, colony colors, and faster growth rates on media compared with those of *D. acutilobae*. It is suggested that pathogenicity of *D. acutilobae* in East Asian hogweed is needed to be confirmed in the future. In conclusion, based on these unique morphological and cultural features, we advocate *D. acutilobae* subsp. *heraclei* as a new *Didymella acutiobae* subspecies and a fungal pathogen responsible for the occurrence of leaf spot in East Asian hogweed.

Disclosure statement

No potential conflict of interest was reported by the authors.

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