Mycobiology

Research Article

A Novel *Alternaria* Species Isolated from *Peucedanum japonicum* in Korea

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Abstract We isolated and examined a new *Alternaria* sp., which causes leaf spots on *Peucedanum japonicum* in Korea, by using molecular and morphological methods. Phylogenetic analysis based on a combined internal transcribed spacer region analysis and two protein-coding genes (*gpd* and *Alt a1*) demonstrated that the causal fungus was most closely related to *A. cinerariae* and *A. sonchi*, and relevant to *A. brassicae*. However, conidial morphology indicated that it is a novel species within the genus *Alternaria*, and therefore we have assigned the fungus a new name in this study.

Keywords *Alternaria*, Morphology, Phylogenetic analyses, Taxonomy

Alternaria is a ubiquitous fungal genus associated with a wide variety of substrates including seeds, plants, agricultural products, animals, soil, and the atmosphere. Most species are well-known and significant plant pathogens causing a range of diseases and post-harvest rots of numerous agronomic and ornamental plants [1]. Some species are commonly found as saprophytes in soil and decaying plant tissues, or as endophytes in various plants [2, 3]. In addition, several taxa have emerged as animal or human pathogens [4]. *Alternaria* species have been generally identified and classified based on cultural and conidial morphology [5-7]. Phylogenetic analyses of multiple gene loci have also been used to identify *Alternaria* species [8-10], which revealed eight nine lineages (sections) (*aternantherae*, *alternaria*, *brassicicola*, *gypsophilae*, *panax*, *porri*, *radicina*, and *sonchi*) and one species group (*infectoria*) [10]. Recently, *Alternaria* has been redefined with its allied genera based on sequence analysis of six gene regions [11], which demonstrated that the *Alternaria* clade is comprised of 24 lineages (sections) and six monotypic lineages. In the complex clade, 13 other genera have been reported as synonymies of *Alternaria*.

Peucedanum japonicum Thunb. is a medicinal plant belonging to the family Apiaceae and is widely distributed in southern and eastern Asia, including Korea [12]. The root is used in the treatment of cough and headache in Korea [13], and the leaf is a medicinal herb used for treating cough in Japan [14]. Young leaves and stems are also used as vegetables in Korea. During our studies on the genus *Alternaria* in Korea, a fungus believed to be an *Alternaria* sp. based on its morphological characteristics, was repeatedly isolated from *P. japonicum* leaf spot lesions [5]. We aimed to identify the *Alternaria* sp. that we isolated from *P. japonicum* by using morphological and molecular methods.

Mycobiology 2014 March, **42**(1): 12-16
http://dx.doi.org/10.5941/MYCO.2014.42.1.12
pISSN 1229-8093 • eISSN 2092-9323

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Received October 24, 2013
Revised October 31, 2013
Accepted January 20, 2014

MATERIALS AND METHODS

Isolates. The fungus-infected leaves of *P. japonicum* were collected from Yesan, Chungnam, Korea, in August 2011. Leaf spots were round or irregular, pale brown to dark brown with a pale gray spot in the center, and sometimes surrounded with yellow halos (Fig. 1A). Segmented leaf lesions were placed in Petri dishes with moist filter paper and incubated at 25°C to induce sporulation. Single spores were collected using a sterile glass needle and transferred onto potato dextrose agar (PDA; Difco, Montreal, Canada) plates to establish pure cultures (CNU 111485). In addition, the *Alternaria* isolate CNU 3010 (Jinan, Korea; September

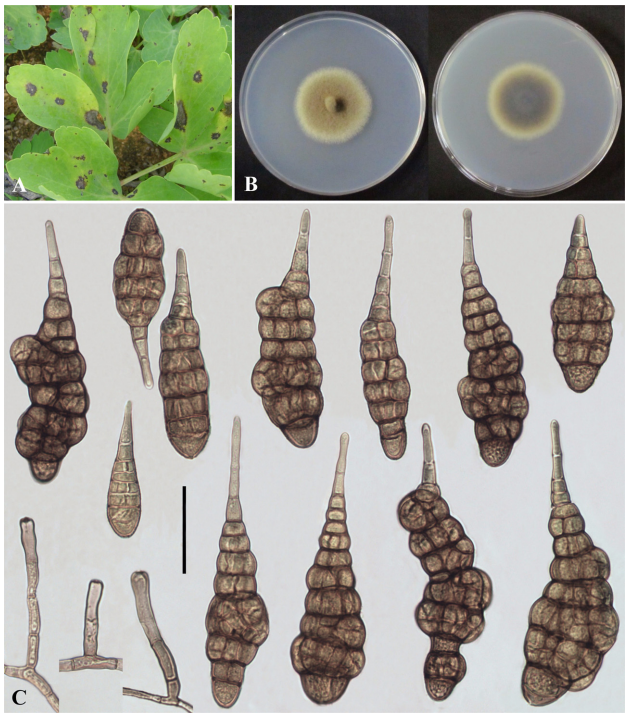

Fig. 1. Symptomatology and morphology of the present fungus. A, Symptoms on *Peucedanum japonicum* leaves; B, Obverse (left) and reverse (right) of colony on potato dextrose agar at 25°C for 7 days; C, Conidiophores and conidia produced on potato carrot agar at 22°C for 7 days (scale bar: C = 50 μm).

1999), derived from *P. japonicum* and isolated by Yu [5], was used for comparison. Isolates were deposited in the Culture Collection of Chungnam National University (CNU) in Daejeon, Korea, and the Korean Agricultural Culture Collection (KACC) in Suwon, Korea.

DNA extraction and PCR amplification. Isolates were grown on PDA for 5~7 days. Mycelia were collected from the growing colonies on PDA plates for DNA extraction. Genomic DNA was extracted following a previously described method [15] with some modifications. The internal transcribed spacer (ITS) region with the primers ITS5 and ITS4 [16], glyceraldehyde-3-phosphate dehydrogenase (*gpd*) gene with the primers *gpd*1 and *gpd*2 [17], and *Alternaria* allergen a 1 (*Alt a1*) gene with the primers Alt-a1-for and Alt-a1-rev [9] were amplified in a 50 μL reaction mixture using *Taq* DNA polymerase (Solgent, Daejeon, Korea) in a GeneAmp PCR System 2700 thermo cycler (Applied Biosystems, Carlsbad, CA, USA). The resultant products were purified using a Wizard PCR prep kit (Promega, Madison, WI, USA) and sequenced with FS DyeTerminator using an ABI automated DNA sequencer. The obtained sequences (ITS, *gpd*, and *Alt a1*) were deposited in GenBank with the accession numbers KF728230, KF728242, and KF889362 for CNU 3010 and KF728231, KF889361, and KF889363 for CNU 111485.

Phylogenetic analyses. The obtained and relevant sequences available in the GenBank database (Table 1) were aligned using CLUSTAL_X ver. 2.0.11, and were adjusted manually [18]. The combined dataset of ITS, *gpd*, and *Alt a1* gene sequences was analyzed using RAxML software [19]. Maximum likelihood analysis with 1,000 bootstrap replicates was performed using the GTRGAT model of nucleotide substitution. *Stemphylium botryosum* Wallroth ATCC 42170 was used as an outgroup for phylogeny analysis.

Morphological observations. The isolate CNU 111485 was used for subsequent morphological descriptions. Colony

Table 1. *Alternaria* isolates used for phylogenetic analyses in this study

Species	Isolate	GenBank accession No.		
		ITS	*gpd*	*Alt a 1*
A. alternantherae	EGS 52-039	JN383496	KC584096	JN383511
A. alternata	EGS 34-016	AF347031	AY278808	AY563301
A. longipes	EGS 30-033	AY751457	AY278811	AY563304
A. brassicicola	EEB 2232	AF229462	AY278813	AY563311
A. gypsophilae	CBS 107.41	KC584199	JQ646304	JQ646387
A. infectoria	EGS 27-193	AF347034	AY278793	FJ266502
A. eryngii	EGS 41-005	JQ693661	AY562416	AY563313
A. panax	EGS 29-180	JQ693662	JQ646299	JQ646382
A. porri	EGS 48-147	JF331450	JF331481	JF331538
A. dauci	ATCC 36613	AF229466	AY278803	AY563292
A. radicina	ATCC 96831	AF229471	AY278797	AY563286
A. brassicae	EGS 38-032	JQ693663	AY562414	AY563309
A. cinerariae	CBS 116495	KC584190	KC584109	AY563308
A. sonchi	EGS 46-051	JN383484	AY562412	AY563307
Present isolates	CNU 3010	KF728230	KF728242	KF889362
	CNU 111485	KF728231	KF889361	KF889363
Stemphylium botryosum	ATCC 42170	AF229481	AY278820	AY563274

characteristics (color, size, and texture) were assessed after 7 days of growth on the PDA plates at 25°C in the dark. Conidial morphology was examined under standard conditions as per Simmons [7]. Isolates were inoculated on potato carrot agar (PCA; 20 g white potato, 20 g carrot, and 20 g agar in 1 L). The plates were stored in a chamber without humidity control (a gradually drying atmosphere in unsealed plates) at 22°C under a fluorescent light/dark cycle of 8/16 hr for 7 days. Conidia were mounted in lactophenol picric acid solution (Fluka, Washington, USA) and measured using an OLYMPUS BX50 light microscope (Olympus, Tokyo, Japan) with an Artcam 300MI digital camera (ARTRAY, Tokyo, Japan). Randomly selected conidia (50) were counted for morphological descriptions.

RESULTS

Phylogenetic analyses. The combined ITS, *gpd*, and *Alt a1* datasets resulted in an alignment containing a total of 1,538 characters including alignment gaps, which comprised 524, 564, and 450 characters of the three genes, respectively. Maximum likelihood analysis was used to develop the phylogenetic tree shown in Fig. 2. The two isolates CNU 111485 and CNU 3010 had identical sequences in each of the three genes, and formed a strongly supported clade (100% bootstrap values) in the phylogram. The two isolates were most closely related to *A. cinerariae* Hori & Enjoji and *A. sonchi* Davis, although the relationship was supported by low bootstrap values (< 70%). The two isolates were also relevant to *A. brassicae* (Berkeley) Saccardo, as they fell in a well-supported clade (96%).

Morphological observations. The cultural and morphological characteristics of the two present isolates CNU 111485 and CNU 3010 were consistent. Conidial morphology differed from any known *Alternaria* species, including those of the phylogenetically relevant species *A. cinerariae*, *A. sonchi*, and *A. brassicae* (Table 2). Therefore, the present fungus described here is a novel species, *Alternaria peucedani* S. H. Yu, sp. nov.

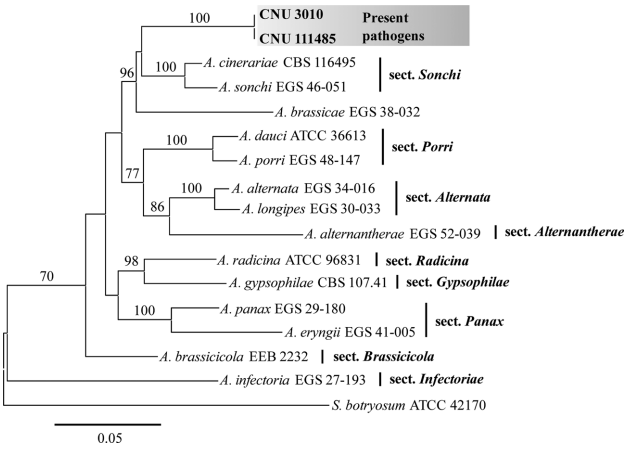

Fig. 2. Phylogenetic tree obtained from maximum likelihood analysis of combined internal transcribed spacer, *gpd*, and *Alt a1* gene sequences from present isolates and relevant species. Bootstrap values (≥ 70%) based on 1,000 replicates are shown above branches. The bar indicates nucleotide substitutions per site. Lineages were referenced from Woudenberg *et al.* [11].

Taxonomy.

Alternaria peucedani S. H. Yu, sp. nov. (Fig. 1).

MycoBank: MB805939

Description: Colonies on PDA (Fig. 1B) were well developed, 35~40 mm in diameter after 7 days incubation at 25°C in the dark, mycelium immersed and partly superficial, effuse, cottony, obverse olivaceous buff to olivaceous, reverse olivaceous buff to dark olivaceous. Conidiophores (Fig. 1C) arose singly, laterally, or terminally from hyphae, were pale brown, straight, or slightly curved, sometimes slightly swollen at the apex, commonly only one pigmented terminal conidiogenous site, smooth-walled with 1~7 transverse septa, 25~100 (~135) μm long, 4.5~9 (~10) μm wide. Conidia (Fig. 1C) on PCA were solitary, obclavate, smooth or roughed, pale brown to dark brown, 50~120 (~127) × 15~50 (~59) μm in size, 3~11 transverse septa, 2~9 longitudinal septa, mature conidia containing excessive swelling cells, constricted at eusepta. Conidial beaks were blunt-tapered, pale brown, 15~40 (~47) μm long with 0~2

Table 2. Morphological characteristics of present isolates and relevant species

Species	Conidial body		Catenation	References
	Size (mm)	Transverse septa		
Alternaria brassicae	150~200 (~250) × 20~35 (~40)	10~12	1~3	Simmons [7][a]
A. cinerariae	160~230 × 32~42	8~11	1~2	Simmons [7][b]
A. sonchi	50~90 (~100) × 18~30	5~7	1~2 (~3)	Simmons [7][a]
Present isolates				
CNU 111485	50~120 (~127) × 15~50 (~59)	3~11	Solitary	Current study[a]
CNU 3010	75~125 × 20~40 (~50)	6~14	Solitary	Yu [5][c]

[a]Conidia produced on potato carrot agar at 22°C.
[b]Conidia produced on V8 juice agar at 22°C.
[c]Conidia produced on V8 juice agar at 20°C.

septa, 3.5~6 (~8) μm wide.

Etymology: *Peucedani* refers to the genus of the host plant.

Holotype: Korea, Chungnam, Yesan, from *Peucedanum japonicum* Thunb. Leaf, August 2011, by S. H. Yu and J. X. Deng, CNUMH 11023 (dried cultures), ex-type cultures deposited in CNU (CNU 111485) and also in KACC.

DISCUSSION

The fungus we isolated from the leaf spots of *P. japonicum* was characterized based on phylogenetic assessment and morphological characteristics. The results indicated that the fungus is a novel species belonging to the genus *Alternaria*, consistent with a previous report by Yu [5], who considered it a new *Alternaria* taxon based on morphological characteristics without prior naming. Here, we propose a new name, *Alternaria peucedani* S. H. Yu, sp. nov., to accommodate the species. To determine the pathogenicity of the fungus to *P. japonicum*, a conidial suspension (1×10^5 conidia/mL) was dropped onto surface-sterilized leaves. Disease spots were seen after two days, which spread quickly and within days of inoculation. The results indicate that the species is the causal agent of leaf spots disease in *P. japonicum* (data not shown).

Fourteen selected species representing nine phylogenetic species groups of *Alternaria* [10] were analyzed with the *A. peucedani* isolates. The resultant phylogram generated from the three-gene combined dataset (ITS, *gpd*, and *Alt a1*) exhibited similar topology as reported previously (Fig. 2) [8-10]. Recently, the nine species groups have been shown as 10 lineages (nine sections and one monotypic lineage) among the 30 lineages in the Alternaria complex clade, and some section names were changed by Woudenberg *et al.* [11]. Phylogenetic analysis revealed that *A. peucedani* is closely related to *A. cinerariae* and *A. sonchi* in the sect *Sochi*, and comparative to *A. brassicae*, a monotypic lineage. However, *A. peucedani* is distinguishable from both the *Sochi* sect and the monotypic lineage (Fig. 2).

Based on conidial morphology, *A. peucedani* is most similar to *A. cinerariae*. Their mature conidia are characterized by cell hypertrophy with no predictable patterns and strongly inflated conidia. A similar situation is also seen in *A. panax* Whetzel [7], which is phylogenetically distant from *A. peucedani* and *A. cinerariae* (Fig. 2). *A. peucedani* conidia are solitary in culture, as shown by Yu [5] and in the present study, while those of relevant species (*A. cinerariae*, *A. sonchi*, and *A. brassicae*) are often catenulate with 2~3 conidia [7]. Additionally, the conidial body of *A. peucedani* is wider and shorter than that of *A. brassicae* and *A. cinerariae*, but larger than that of *A. sonchi*. *A. peucedani* also produces chlamydospores (12~15 μm wide) on V8 juice agar [5], but this structure is not observed on PCA.

ACKNOWLEDGEMENTS

This work was supported by National Institute of Biological Resources, Republic of Korea.

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