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) (atermantherae, 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 synonyms 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.

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
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 gpd1 and gpd2 [17], and Alternaria allergen a1 (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 Dye Terminator 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 a1   |
|-----------------------|------------|-----------------------|---------|---------|----------|
| A. alternantherae     | EGS 52-039 | JN383496              | JN383496| KC584096| JN383511 |
| A. alternata          | EGS 34-016 | AF347031              | AF347031| AY278808| AY563301 |
| A. longipes           | EGS 30-033 | AY751457              | AY751457| AY278811| AY563304 |
| A. brassicicola       | EEB 2232   | AF229462              | AF229462| AY278813| AY563311 |
| A. gysophila          | CBS 107.41 | KC584199              | KC584199| JQ646304| JQ646387 |
| A. infectoria         | EGS 27-193 | AF347034              | AF347034| AY278793| FJ266502 |
| A. cryngii            | EGS 41-005 | JQ693661              | JQ693661| AY562416| AY563313 |
| A. panax              | EGS 29-180 | JQ693662              | JQ693662| JQ646299| JQ646382 |
| A. porri              | EGS 48-147 | JF331450              | JF331450| JF331481| JF331538 |
| A. dauci              | ATCC 36613 | AF229466              | AF229466| AY278803| AY563292 |
| A. radicina           | ATCC 96831 | AF229471              | AF229471| AY278797| AY563286 |
| A. brassicae          | EGS 38-032 | JQ693663              | JQ693663| AY562414| AY563309 |
| A. cinerae            | CBS 116495 | KC584190              | KC584190| KC584109| AY563308 |
| A. sonchi             | EGS 46-051 | JN383484              | JN383484| AY562412| AY563307 |
| Present isolates      | CNU 3010   | KF728230              | KF728230| KF728242| KF889362 |
|                       | CNU 111485 | KF728231              | KF728231| KF889361| KF889363 |
| Stemphylium botryosum | ATCC 42170 | AF229481              | 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). 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.

**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

![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].](image)

**Table 2.** Morphological characteristics of present isolates and relevant species

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

*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 x 10^6 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 al*) 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 from *sonchicola* to *brassicarum*, 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. brassicarum*) are often catenulate with 2–3 conidia [7]. Additionally, the conidial body of *A. peucedani* is wider and shorter than that of *A. brassicarum* 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.

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