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Morphology, Molecular Phylogeny and Pathogenicity of *Colletotrichum panacicola* Causing Anthracnose of Korean Ginseng

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***Colletotrichum panacicola* isolates were obtained from anthracnose lesions of Korean ginseng and compared with four *Colletotrichum* species in morphology, molecular phylogeny and pathogenicity. Based on morphological characteristics, *C. panacicola* was easily distinguished from *Colletotrichum gloeosporioides* but not from *Colletotrichum higginsianum*, *Colletotrichum destructivum* and *Colletotrichum coccodes*. A phylogenetic tree generated from ribosomal DNA-internal transcribed spacer sequences revealed that *C. panacicola* is remarkably distinguished from *C. gloeosporioides* and *C. coccodes* but not from *C. higginsianum* and *C. destructivum*. However, molecular sequence analysis of three combined genes (actin + elongation factor-1α + glutamine synthatase) provided sufficient variability to distinguish *C. panacicola* from other *Colletotrichum* species. Pathogencity tests showed that *C. panacicola* is pathogenic to Korean ginseng but not to other plants. These results suggest that *C. panacicola* is an independent taxon distin-zguishable from *C. gloeosporioides* and other morphologically similar *Colletotrichum* species.**

Keywords : Anthracnose, *Colletotrichum gloeosporioides*, *Colletotrichum panacicola*, Korean ginseng

Korean ginseng (*Panax ginseng* Mey) is one of the most important medicinal crops being cultivated in Korea. The area of cultivation has been increased throughout the country due to high demand. Several diseases occur in ginseng during 5 to 6 years of cultivation. Most ginseng diseases are caused by fungi. Of these, anthracnose causing seedling blight in nurseries and leaf spot in permanent beds is one of the most destructive diseases (Chung and Bae, 1979).

In Korea, *Colletotrichum panacicola* recorded as a causal pathogen of Korean ginseng anthracnose (Takimoto, 1919) has been considered to be a synonym of *Colletotrichum gloeosporioides* without any basis that these two species are the same taxon. Accordingly, there is a need for a comprehensive approach for confirming the taxonomical position of *C. panacicola*. This study was conducted to compare morphology, molecular phylogeny and pathogenicity of *C. panacicola*, *C. gloeosporioides* and morphologically similar *Colletotrichum* species.

Materials and Methods

Isolation of pathogenic fungi. *Colletotrichum* isolates were obtained from leaves or fruits of Korean ginseng, Chinese cabbage, radish, soybean, potato, tomato and chili pepper with symptoms of anthracnose. The symptomatic tissues were surface-sterilized by immersion in 1% NaOCl for 1 minute followed by two rinses in sterile distilled water. The blotted tissues were transferred to water agar (WA) plates and incubated at 25°C for 3 days. Conidia produced on the tissues were suspended with sterile distilled water, streaked on WA plates and incubated at 25°C for 18 to 24 hours. Monoconidial isolates were obtained from germinating spores on WA and transferred to potato dextrose agar (PDA) slants.

Morphological and cultural characteristics. The *Colletotrichum* isolates were cultured on PDA plates in darkness at 23°C for 7 days. The culture plates were transferred into an incubator with alternating cycles of 12 h NUV light and 12 h darkness for another week to induce conidial production. The conidia harvested from each isolate were mounted in water and their morphological features were examined using a light microscope. To observe appressorial formation, a drop of conidial suspension (5×10^5 conidia·mL^{-1}) was dropped on sterilized slide glass in petri-plates with water-

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soaked filter paper and kept at 25°C for 16 to 24 hours. Mycelial growth and colony features of the isolates were examined on PDA plates kept in darkness at 26°C for a week. The colony colors were described using the Munsell renovation color system (Nippon Shikisai Co. Ltd.).

Extraction of genomic DNA, amplification and phylogenetic analysis. *Colletotrichum* isolates were cultured in potato dextrose broth at 25°C for 4 days. The mycelial mats were lyophilized for 16 hours and homogenized at 5,800 rpm for 40 seconds using the Precellys®24 (Bertin technologies). Genomic DNA was extracted with a modified CTAB method (Stewart and Via, 1993). The internal transcribed spacer (ITS) region of rDNA was amplified with primers ITS1 and ITS4 (White et al., 1990). Actin, elongation factor-1 alpha (EF-1α), and glutamine synthatase (GS) genes were amplified with primer sets ACT-512F/ACT-783R (Carbone and Kohn, 1999), EF1-526F/EF1-1567R (Rehner, 2001), and GSF1/GSR1 (Guerber et al., 2003), respectively. The PCR reactions were performed in a total volume of 50 µl including 100ng genomic DNA, 5 µl of 10× PCR buffer containing 25 mM $MgCl_2$, 0.2 mM dNTP, 0.2 µM of each primer and 1 unit of Taq DNA polymerase (Takara Bio Inc.) using a DNA Engine Tetrad®2 (Bio-Rad laboratories Inc.). Thermal conditions were programmed for 35 cycles of denaturation (94°C for 50 sec), annealing (60°C for 1 min) and extension (72°C for 1 min 20 sec). PCR products were purified with Wizard® SV gel and PCR clean-up system (Promega) following manufacturer's instructions. The eluted DNAs were cloned into pGEM-T easy vector (Promega) and transformed into *Escherichia coli* JM109. True transformants were selected and sequenced with 3730XL DNA Analyzer (Applied Biosystems). Two phylogenies were generated from the ITS region sequences and from the combined data of actin, EF1-α and GS genes. Sequence alignments were performed using Clustal X ver. 2.0.10 (Thompson et al., 1997). Nucleotide sequences of *Colletotrichum* isolates were deposited in GenBank (http://www.ncbi.nlm.nih.gov) and are listed in Table 1. Phylogenetic trees were constructed by neighbor-joining method (Saitou and Nei, 1987) with pairwise deletion parameters of Kimura's two-parameter model (Kimura, 1980) using MEGA version 4.0 (Tamura et al., 2007). Confidence of phylogenetic trees was supported with bootstrap method for 1,000 replicates (Felsenstein, 1985).

Pathogenicity assay. Tri-folia leaves of 4-year old ginseng, leaves of 3-week old tomato cultivar 'seokwang', leaves of 3-week old soybean cultivar 'daepoong' and leaves of 3-week old radish cultivar 'taegeun' were used for pathogenicity assays. Leaves were wounded with blunt pencil tips or left unwounded. Fifteen µl of conidia suspension (5×10^5 conidia·ml^{-1}) was used to inoculate wounded and unwounded sites of the leaves. The inoculated leaves were placed in plastic containers (230 × 295 × 55 mm) layered with moist paper towels, and kept at 26°C (± 1°C). Disease severity on ginseng, radish and tomato was based on lesion diameter measured 5 days after inoculation and disease severity on soybean was based on lesion diameter measured 10 days after inoculation on soybean.

Results

Morphological and cultural characteristics. Three *C. panacicola* isolates from leaves and fruits of Korean ginseng, two *Colletotrichum higginsianum* isolates from Chinese cabbage and radish, *Colletotrichum destructivum* isolate from soybean, two *Colletotrichum coccodes* isolates from potato and tomato, and two *C. gloeosporioides* isolates from chili pepper were used to compare their morphological and cultural characteristics (Table 1). Remarkable differences in conidial morphology between *C. panacicola*

Table 1. *Colletotrichum* species studied genetically and/or morphologically

Species	Isolate	Source of isolate	Accession no.			
			ITS	Actin	EF-1α	GS
C. panacicola	C08048	Korean ginseng	GU935867	GU944757	GU935827	GU935807
	C08061	Korean ginseng	GU935868	GU935791	GU935828	GU935808
	C08087	Korean ginseng	GU935869	GU944758	GU935829	GU935809
C. higginsianum	C97027	Chinese cabbage	GU935870	GU935792	GU935830	GU935810
	C08122	Radish	GU935873	GU935794	GU935833	GU935813
C. destructivum	C08077	Soy bean	GU935874	GU935795	GU935834	GU935814
C. coccodes	C07046	Potato	GU935877	GU935798	GU935837	GU935817
	C96002	Tomato	GU935875	GU935796	GU935835	GU935815
C. gloeosporioides	C07008	Chili pepper	GU935879	GU935800	GU935839	GU935819
	C07009	Chili pepper	GU935880	GU935801	GU935840	GU935820

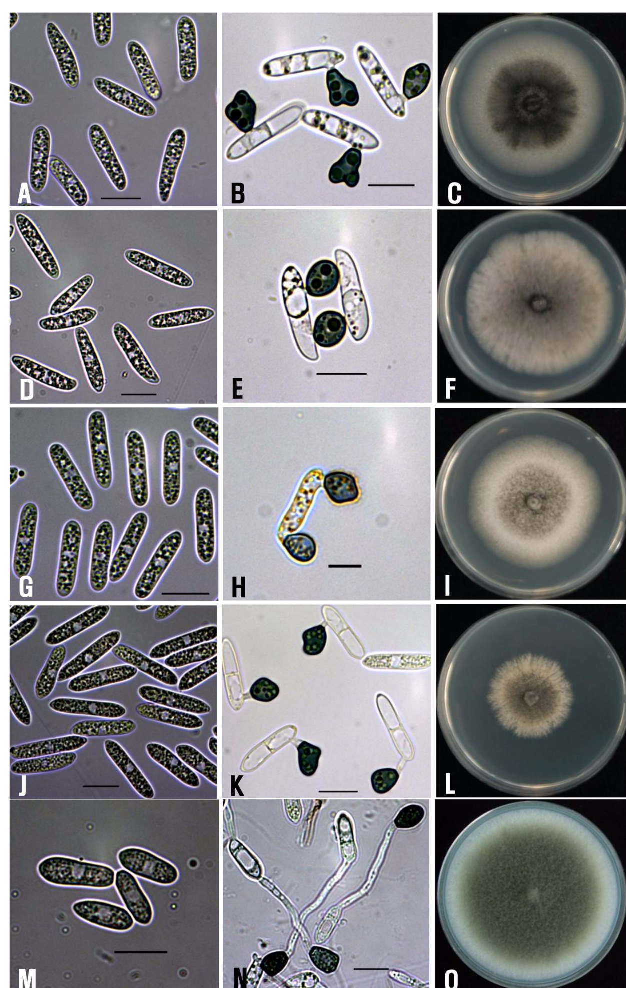

Fig. 1. Conidia, appressoria and colonies of *Colletotrichum* spp. A to C, *C. panacicola*; D to F, *C. higginsianum*; G to I, *C. destructivum*; J to L, *C. coccodes*; M to O, *C. gloeosporioides*. Three vertical rows from left to right denote conidia, appressoria and colonies, respectively. All scale bars represent 10 μm.

and *C. gloeosporioides* were observed (Fig. 1; Table 2). *C. panacicola* isolates were straight or slightly unilaterally curved, with a round apex and a subconical base, measuring 11.2–20.5 × 3.3–4.8 μm, with a mean range of 16.4–18.3 × 4.0–4.4 μm, matching well with previous descriptions of the fungi (Nakata and Takimoto, 1922). *C. gloeosporioides* isolates were usually straight, broad cylindrical with rounded both ends or pointed to one end, measuring 10.0–22.9 × 3.8–6.5 μm, with a mean range of 13.6–16.4 × 4.6–5.2 μm, mathching well with previous descriptions of the fungi (Gunnell and Gubler, 1992). There were no clear morphological differences among *C. panacicola*, *C. higginsianum*, *C. coccodes* and *C. destructivum.* The conidia of four species in common are elongated fusiform, usually pointed to one end and also in their size overlapped each other except *C. coccodes* in conidial width is somewhat wider than the other three species (Fig. 1; Table 2). The results of conidial morphology agreed well with previous descriptions for the respective species (Nakata and Takimoto, 1922; Sutton, 1980; 1992).

Morphology of conidial appressoria was compared among the *Colletotrichum* species. The appressorial shape of *C. panacicola* showed minor differences to that of *C. gloeosporioides* and their size overlapped to a significant degree. *C. panacicola* isolates were usually irregularly lobed, sometimes clavate and measured 6.0–11.3 × 4.4–7.9 μm, with a mean range of 7.8–8.6 × 5.5–6.3 μm. *C. gloeosporioides* isolates were clavate, ovate or irregularly lobed, and measured 6.5–10.1 × 5.3–7.8 μm, with a mean range of 8.1–8.5 × 6.0–6.9 μm. There were no clear differences in appressorial morphology among *C. panacicola*, *C. higginsianum*, *C. coccodes* and *C. destructivum* (Fig. 1; Table 2).

Colletotrichum colonies on PDA were compared. There were clear differences in colony morphology between *C. panacicola* and *C. gloeosporioides* (Fig. 1). *C. panacicola* isolates were 67 to 70 mm after 7 days, consisting of sparse aerial mycelium, grayish yellow brown to dark olive green in the central region and yellowish white towards the margin, whereas *C. gloeosporioides* were 83 to 85 mm after 7 days, consisting of floccose aerial mycelium and grayish yellow green on the surface. Colonies of *C. higginsianum*, *C. destructivum* and *C. coccodes* showed also distinct differences. *C. higginsianum* isolates C97027 and C08122 were 68 to 73 mm after 7 days, dark yellowish brown or yellowish gray on center of surface and dark yellowish brown or dull orange in reverse side. *C. destructivum* isolate C08077 was 62 to 63 mm, olive color in the central region and pale orange towards the margin on top side, yellowish brown on reverse side. *C. coccodes* isolates C07046 and C96002 were 42 to 49 mm and grew slowly, olive or dark olive gray on top and reverse side.

Analysis of rDNA-ITS and combined genes (actin + EF1-α + GS) sequences. Size polymorphism and sequence similarity derived from the sequences of rDNA-ITS region and combined multi-locus genes were investigated. The length of the rDNA-ITS from *Colletotrichum* species ranged from 487 bp to 500 bp and yield 507 aligned nucleotide positions for all species included in the alignment. The nucleotide size for alignment was 499 bp in both *C. panacicola* and *C. higginsianum*, 487 bp in *C. gloeosporioides* and 500 bp in *C. destructivum*. Comparative analysis of the rDNA region containing the two internal transcribed spacers (ITS1 and ITS2) and 5.8S rRNA gene revealed that nucleotide sequences of the 5.8S in all tested taxa were highly conserved but those of their ITS regions

Table 2. Morphology of *Colletotrichum panacicola* and other *Colletotrichum* species

Species	Isolate	Colony			Conidium			Appressorium		
		Diameter[a] (mm)	Color[b]		Shape[c]	Size (μm)		Shape[d]	Size (μm)	
			Top	Reverse		Range	Mean		Range	Mean
C. panacicola	C08048	69	DOG(138)	DOG(138)	F, TAB	14.3–19.9×4.0–4.7	17.0×4.4	IRL, CL	6.0–8.9×4.8–7.9	7.9×6.3
	C08061	68	DOG(138)	DOG(138)	F, TAB	11.2–19.1×3.3–4.7	16.4×4.0	IRL, CL	6.5–9.6×4.4–6.5	7.8×5.6
	C08087	68	DOG(138)	DOG(138)	F, TAB	15.1–20.5×3.8–4.8	18.3×4.3	IRL, CL	6.3–11.3×4.9–6.4	8.6×5.5
C. higginsianum	C97027	68	YG(68) DYB(76)	DYB(76)	F, TAB	15.7–22.7×3.9–4.9	19.0×4.4	IR, OV	6.2–9.2×4.9–6.9	7.8×6.0
	C08122	70	YG(68)	YG(68) DO(75)	F, TAB	15.3–22.2×3.7–5.0	19.1×4.4	OV, IR	5.8–10.1×4.8–7.0	7.4×5.5
C. destructivum	C08077	62	O(105) PO(60)	O(105) YW(92)	F, TAB	13.8–19.4×3.6–4.7	16.8×4.0	OV, CL	7.8–12.2×6.2–9.9	9.6×7.4
C. coccodes	C07046	45	O(105)	O(105)	F, TAB	13.2–23.0×4.0–5.7	18.4×4.7	IR, CL	7.0–12.2×4.5–7.4	8.7×5.9
	C96002	49	DOG(138)	DOG(138)	F, TAB	12.7–17.4×4.0–5.6	15.3×4.6	IR, CL	6.3–10.8×4.2–6.4	8.2×5.6
C. gloeosporio-ides	C07008	85	O(105)	DOG(138)	C, ROB	11.2–16.9×3.8–5.5	13.6×4.6	CL, OV	6.5–10.1×5.3–6.6	8.5×6.0
	C07009	83	DG (144)	DOG (138)	C, TAO	10.0–22.9×4.4–6.5	16.4×5.2	OV, IR	7.3–9.1×6.1–7.8	8.1×6.9

[a] Colony diameter was measured 7days after inoculation
[b] A serial number of Munsell renovation color system today's color/300, DG: dull green, DO: dull orange, DOG: dark olive green, DYB: dark yellowish brown, GYB: yellowish brown, PO: pale orange, O: olive, YB: yellowish brown, YG: yellowish gray, YW: yellowish white
[c] C: cylindrical; F: fusiform; TAB: tapered to both ends; TAO: tapered to one end; ROB: rounded both ends
[d] CL: clavate, IR: irregular, IRL: irregular lobed, OV: ovate.

were variable. In particular, nucleotide variations of the ITS1 (6.7%) were two times higher than those of ITS2 (3.2%). The difference of nucleotide variations between ITS1 and ITS2 was consistent with results of previous research (Cano et al., 2004; Sreenivasaprasad et al., 1996). The ITS sequences from *C. panacicola* isolates shared homology of 87.3–87.5% with *C. gloeosporioides* isolates, 92.9 to 93.1% with *C. coccodes* isolates However, the sequences showed high level of homology of 99.0–99.5% with *C. higginsianum* and *C. destructivum*.

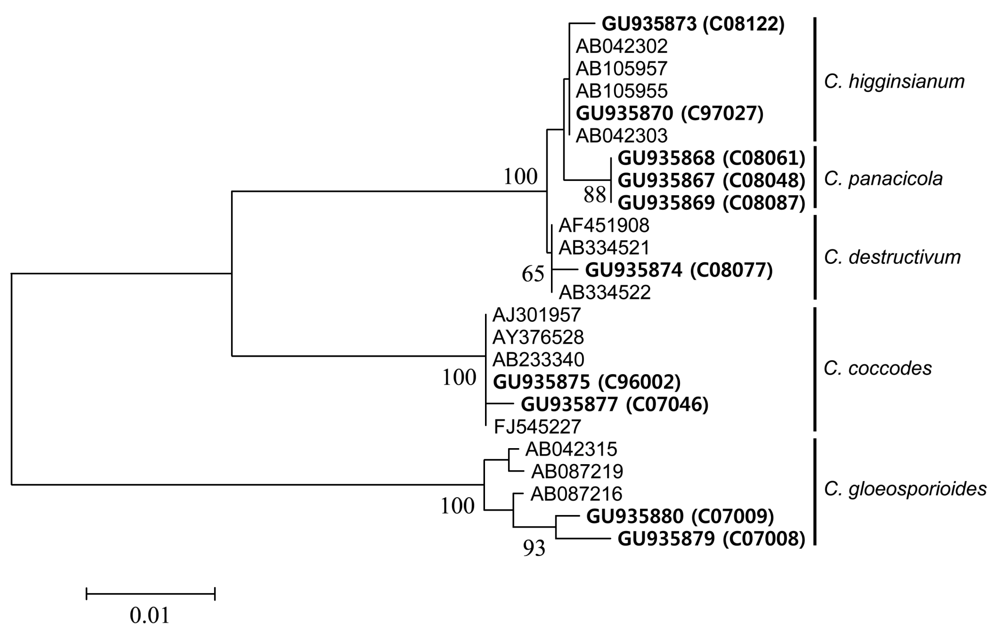

Fig. 2. A neighbor-joining tree derived from sequences of rDNA-ITS region of *Colletotrichum* spp. Numbers on nodes (> 60%) represent bootstrap values (%) from 1000 replicates. A phylogenetic tree was conducted using MEGA 4.0 with kimura-two parameter model. The letters in parentheses refer to isolate numbers. Bar represents 0.01 substitutions per site.

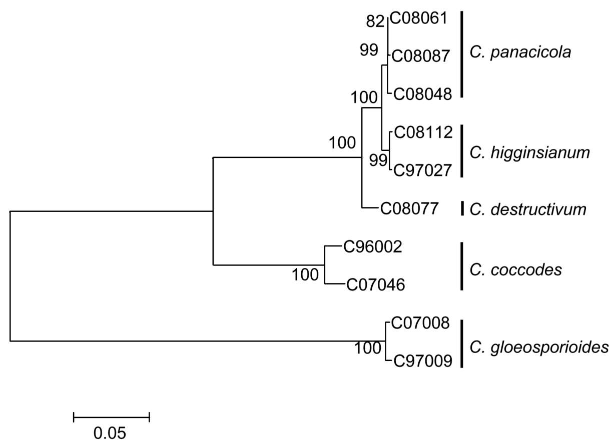

Fig. 3. A neighbor-joining tree derived from sequences of combined multi-locus gene (actin+EF1-α+GS) of *Colletotrichum* spp. Numbers on nodes (> 60%) represent bootstrap values (%) from 1000 replicates. A phylogenetic tree was conducted using MEGA 4.0 with kimura-two parameter model. Bar represents 0.05 substitutions per site.

Analysis of the combined data set of multi-locus sequences (actin + EF1-α + GS) revealed that the length of *Colletotrichum* species ranged from 2318 to 2377 bp, with alignment of 2553 bp. The size of the nucleotide sequence for alignment was 2349 bp in *C. panacicola* isolates, 2358 to 2365 bp in *C. higginsianum*, 2332 to 2341 bp in *C. gloeosporioides* and 2377 bp in *C. destructivum*. In the sequences of combined multi-locus gene, *C. panacicola* isolates showed similarity of 62.4–62.6% with *C. gloeosporioides*, 71.2–71.5% with *C. coccodes*, 97.8–98.1% with *C. higginsianum* and 95.2–95.3% with *C. destructivum*.

Phylogenetic relationships. Phylogenetic analysis based on the sequences of rDNA-ITS showed that *C. panacicola* is clearly differentiated from *C. gloeosporioides* and *C. coccodes* with a high bootstrap support of 100% but from *C. higginsianum* and *C. destructivum* with bootstrap support less than 60% (Fig. 2).

Sequences analysis of combined multi-locus genes provided higher resolution among the five *Colletotrichum* species than ITS (Fig. 3). *C. panacicola* was distinctly separated from the other *Colletotrichum* species with bootstrap support of 100% and was positioned far from *C. gloeosporioides* and *C. coccodes*. The *Colletotrichum* species which were not significantly differentiated in an ITS-based phylogenetic tree, *C. panacicola*, *C. higginsianum* and *C. destructivum* were also further separated each other with bootstrap support of 100%. Taken together, a phylogenetic tree derived from the sequences of combined multi-locus genes led to much higher resolution for the delimitation of *C. panacicola* from other species than ITS.

Pathogenicity. Pathogenicity of the five *Colletotrichum* species was tested on their host and on other plants (Table 3). *C. panacicola* isolates on the leaves of Korean ginseng induced circular dark brown lesions 4 days after inoculation. The lesions developed and no major differences in pathogenicity were observed among the isolates. Disease

Table 3. Pathogenicity of *Colletotrichum* spp. to leaves of several crops by artificial inoculation

Species	Isolate	Disease severity[a]							
		Ginseng		Radish		Tomato		Soy bean	
		W[b]	UW[c]	W	UW	W	UW	W	UW
C. panacicola	C08048	++	+	–	–	–	–	–	–
	C08061	++	++	–	–	–	–	–	–
	C08087	+++	++	–	–	–	–	–	–
C. higginsianum	C97027	+	–	+++	++	+	–	–	–
	C08122	–	–	++	+	–	–	+	–
C. destructivum	C08077	–	–	–	–	+	–	+++	–
C. coccodes	C96002	+	–	–	–	++	–	+	–
	C07046	–	–	++	+	+++	++	+	–
C. gloeosporioides	C07008	–	–	–	–	–	–	+	–
	C07009	–	–	–	–	–	–	–	–
Control		–	–	–	–	–	–	–	–

[a] Disease severity on ginseng, radish and tomato was determined on the basis of lesion diameter measured 5 days after inoculation and observed 10 days after inoculation on soybean, respectively. –, < 3.5 mm lesion; +, 3.5–7.0 mm lesion; ++, 7.0–10.5 mm lesion; +++, > 10.5 mm lesion
[b] wounded
[c] unwounded

severity on leaves with the wound treatment was slightly higher or similar to that of the unwounded leaves. No symptoms developed on the leaves of other hosts including radish, tomato and soybean.

The lesions on leaves of Korean ginseng inoculated with *C. gloeosporioides* isolates C07008 and C07009 did not appear. *C. higginsianum* C97027 and *C. coccodes* C96002 showed weak responses on wounded leaves of Korean ginseng, while *C. higginsianum, C. coccodes* and *C. destructivum* produced lesions on leaves of their host plants.

Discussion

C. panacicola was first described by Takimoto (1919). Later, Nakata and Takimoto (1922) provided a detailed description for the causal fungus of leaf blight of Korean ginseng. However, the species in Korea has been regarded as a synonym of *C. gloeosporioides*, a species complex encompassing diverse groups of strains and biotypes (Yu, 1992; Yu and Ohh, 1993). In addition, Mcpartland and Hosoya (1998) found that morphology of *C. panacicola* is similar to that of *C. coccodes* (Wallr.) Hughes.

C. panacicola could be distinguished from *C. gloeosporioides* using conidial morphology but not from *C. higginsianum*, *C. destructivum* and *C. coccodes*. *C. panacicola* could also be distinguished from *C. gloeosporioides* using colony features. The former grew slower than the latter, and both species showed different colony colors. Colony radial growth of *C. panacicola*, *C. higginsianum* and *C. destructivum* was not informative for their speciation, but colony colors revealed difference among them. In short, although conventional methods can be used for identification of the five *Colletotrichum* species, they provided limited information to delimitate *C. panacicola* from other morphologicaly similar *Colletotrichum* species (Talhinhas et al., 2005; Whitelaw-Weckert et al., 2007).

To overcome limitations of morphological approaches for determination of *Colletotrichum* species, molecular techniques have been employed (Guerber et al., 2003; Johnston and Jones, 1997; Lubbe et al., 2004; Talhinhas et al., 2002). Although ITS regions have known to be potentially informative regions for phylogenetic studies at species level, they were not informative enough to resolve relationships among the *C. panacicola* and its morphologically similar species. Moriwaki et al. (2002) revealed that *C. destructivum* was not separated from *C. higginsianum*, *C. linicola* and *C. fuscum* based on the sequences of ITS1 region. In addition, Sreenivasaprasad et al. (1996) reported that *C. destructivum* and *C. linicola* were not differentiated based on ITS1 sequences.

The sequence data from the three independent loci (actin + EF1-α + GS) were combined and used to complement the rDNA-ITS sequencing. A phylogenetic analysis derived from combined multi-locus sequences revealed that *C. panacicola* is not only differentiated from *C. gloeosporioides* and *C. coccodes* but also sufficiently differentiated from *C. higginsianum* and *C. destructivum* with 100% bootstrap support. Analysis using the combined dataset has been used for recognition of fungal species, including *Fusarium* spp. (O'Donnell et al., 1998). In this study, the phylogenetic tree based on sequences of the combined genes provided much better resolution for delimitation of *C. panacicola* from other *Colletotrichum* species than the rDNA-ITS regions.

Artificial inoculation of *Colletotrichum* species revealed that *C. panacicola* is pathogenic on leaves of Korean ginseng but *C. gloeosporioides* is not. Accordingly, *C. panacicola* was confirmed as a major pathogen causing anthracnose on Korean ginseng. *Colletotrichum* isolates from radish, tomato and soybean induced severe symptoms on their host plants by artificial inoculation but weak or no symptoms on other host plants. *C. panacicola* and other *Colletotrichum* species used in this study showed host specificity. Taxonomy of *Colletotrichum* species based on the host specificity has been accepted as a criterion to characterize species by previous workers (Farr et al., 2006; Lubbe et al., 2004; Nirenberg et al., 2002; Sutton, 1980). Although *C. coccodes* was isolated from soil planted with American ginseng (*Panax quinquefolius* L.) in Wisconsin, pathogenicity of the fungus on ginseng was not described (Mcpartland, 1985). *C. coccodes* isolate C96002 produced lesions on wounded leaves of Korean ginseng. However, whether the fungus in fields actually causes ginseng anthracnose or not is unclear and requires further investigation.

In conclusion, this work clearly demonstrates that *C. panacicola* is an independent taxon distinguishable from *C. gloeosporioides* and other morphologically similar *Colletotrichum* species based on their morphological, cultural and molecular characteristics and pathogenicity.

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