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Two Species of Endophytic *Cladosporium* in Pine Trees in Korea

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During our studies on the diverse endophytic fungi resident on conifer needles, many species of *Cladosporium* previously unreported in Korea were encountered. In this paper, we report on two species of *Cladosporium* from the needles of pine trees (*Pinus* spp.). Based on analyses of internal transcribed spacer gene sequence, and cultural and micromorphological characteristics, they were identified as *C. oxysporum* and *C. sphaerospermum*. Both species have not been hitherto reported in Korea.

KEYWORDS : *Cladosporium*, Endophytic fungi, ITS gene sequence, Morphological characteristics, Pine tree

Cladosporium constitutes one of the largest genera of hypomycetes, comprising more than 772 names (Dugan *et al.*, 2004). These species are among the most common fungi isolated from the environment almost anywhere in the world (Farr *et al.*, 1989; Flannigan, 2001; Mullins, 2001; Schubert, 2005). Many species are plant pathogens (Kwon *et al.*, 2001), while others are regularly encountered as contaminants and spoilage agents in food or industrial products, as well as being frequently associated with asthmatic complaints and endophytic fungi (Riesen and Sieber, 1985; Brown *et al.*, 1998; El-Morsy, 2000; Schubert, 2005). Few investigations to date have determined the incidence of endophytic *Cladosporium* from pine trees.

Endophytic fungi are taxonomically and biologically diverse but all share the character of colonizing internal plant tissues without causing apparent harm to their host (Wilson, 1995). They have proven to be promising sources of new and biologically active natural products, which are of interest for specific medicinal and agrochemical applications (Strobel, 2002).

During our studies on diversity of endophytic fungi from needles of pine trees in Korea, *Cladosporium* spp. was encountered frequently. They were identified based on the internal transcribed spacer (ITS) sequences, and cultural and morphological characteristics. In this paper, we report on two species of *Cladosporium* from the needles of pine trees that have hitherto not been reported in Korea.

Materials and Methods

Sampling. Healthy needles of pine trees (*Pinus densiflora*, *P. rigida*) were collected from mountain areas in Daejeon, Korea during July and August, 2006. Pine trees were selected randomly and needles were brought to the laboratory in separate sterile polyethylene bags.

Isolation and culture of endophytic fungi. Samples were cleaned under running tap water to remove debris and then air dried and processed within 5 h of collection. From each sample, 10 segments of 1 cm length were separated and treated as replicates. The segments were surface sterilized by immersion in 95% ethanol for 1 min, sodium hypochlorite (4% available chlorine) for 3 min and 95% ethanol for 30 s. The surface sterilized samples were washed in sterile water three times to remove the surface sterilization agents. Samples were allowed to dry on a paper towel in a laminar air flow chamber. Ten segments per sample were placed horizontally on separate Petri dishes containing potato dextrose agar (PDA) supplemented with the antibiotic streptomycin sulfate 0.4 mg/*ml* and dichloran rose bengal chloramphenicol agar (DRBC). After incubation at 25°C for 5, 10 and 25 days, individual hyphal tips of the developing fungal colonies were collected and placed onto PDA, incubated for 8~10 days and checked for culture purity. Eventually, cultures of *Cladosporium* were separated from other fungi based on their conidia. They were transferred to PDA slant tubes and 20% glycerol stock solution.

DNA extraction and polymerase chain reaction (PCR) amplification. For determination of the ITS region of the rDNA of the isolates, genomic DNA was extracted as previously described (Park *et al.*, 2005). PCR amplification was carried out for ITS1 and ITS4 (White *et al.*, 1990) in an i-cycler (Bio-Rad, Hercules, CA, USA) for 30 cycles of 94°C for 1 min (denaturing), 55°C for 1 min (annealing) and 72°C for 150 s (extension). Initial denaturing at 94°C was extended to 5 min and the final exten-

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sion was for 10 min at 72°C. PCR product was purified using a Wizard PCR prep kit (Promega, Madison, WI, USA). Purified double stranded PCR fragments were directly sequenced with BigDye terminator cycle sequencing kits (Applied Bipsystems, Foster City, CA, USA) by following the manufacturer's instructions. The same PCR primer sets as used for PCR amplification were used to sequence both DNA strands. Gel electrophoresis and data collection were performed on an ABI Prism 310 genetic analyzer (Applied Biosystems).

Phylogenetic analysis. The sequences were compared with the ITS sequence of rDNA available in the GenBank database by a BLAST search. Sequences generated from materials in this study and retrieved from GenBank were initially aligned using the CLUSTAL X program (Thompson *et al*., 1997) and the alignment was refined manually using the PHYDIT program version 3.2 (Chun 1995; available at http://plaza.snu.ac.kr/jchun/phydit). A neighbor-joining tree was reconstructed with Kimura's 2-parameter distance model (Kimura, 1980) using the PHYLIP 3.57c package (Felsenstein, 1985). Bootstrap analysis using 1000 replications was performed to assess the relative stability of the branches.

Morphological observations. *Cladosporium* isolates were inoculated on PDA and malt extract agar (MEA) media in three regions of 9 cm-diameter plastic Petri

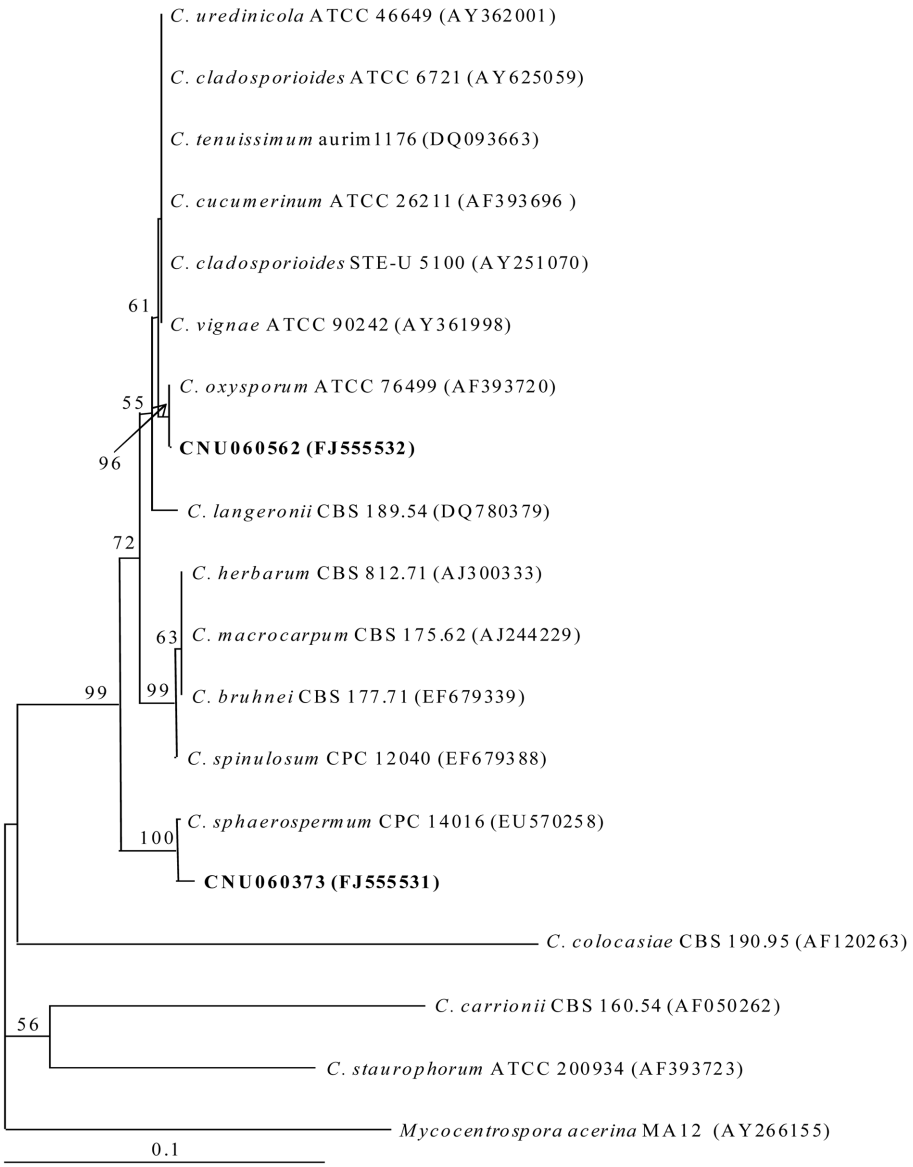

Fig. 1. Neighbor-joining tree based on the sequence of the ITS region showing the relationship between endophytic isolates and *Cladosporium* species. The number above each branch indicates bootstrap values obtained after a bootstrap test with 1000 replications. The bar represents 0.1 substitutions per site. Numbers in parenthesis are GenBank accession numbers.

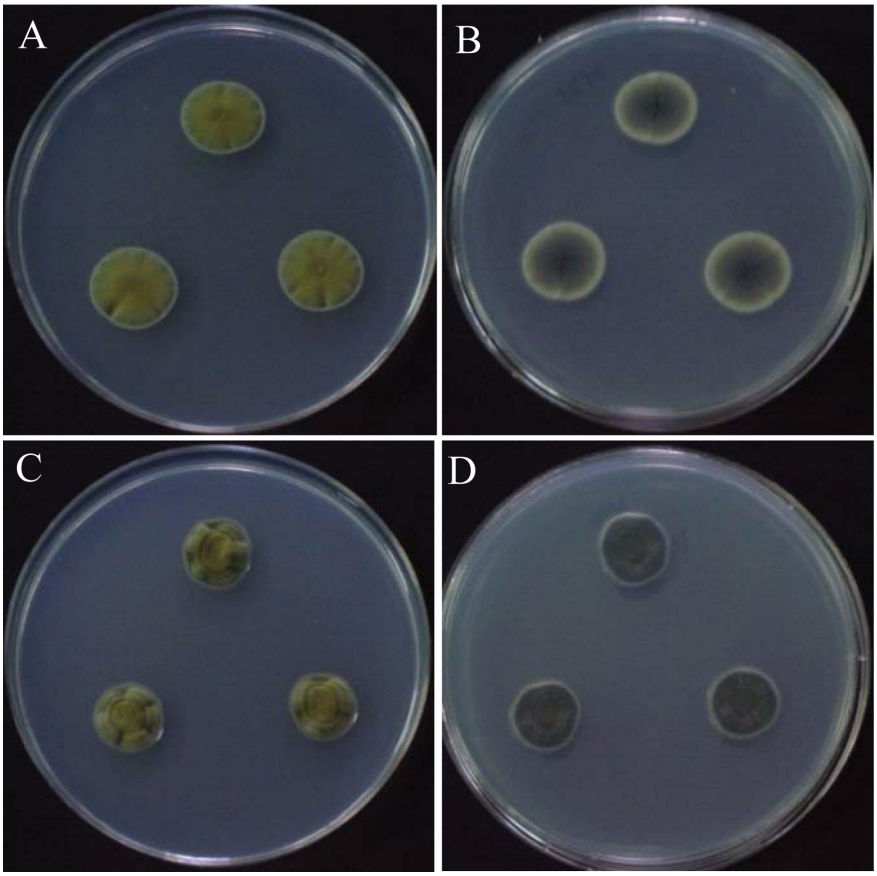

Fig. 2. *Cladosporium* colonies on MEA after 7 days of incubation at 25°C in the dark: *C. oxysporum* (A, B), *C. sphaerospermum* (C, D); obverse (A, C), reverse (B, D).

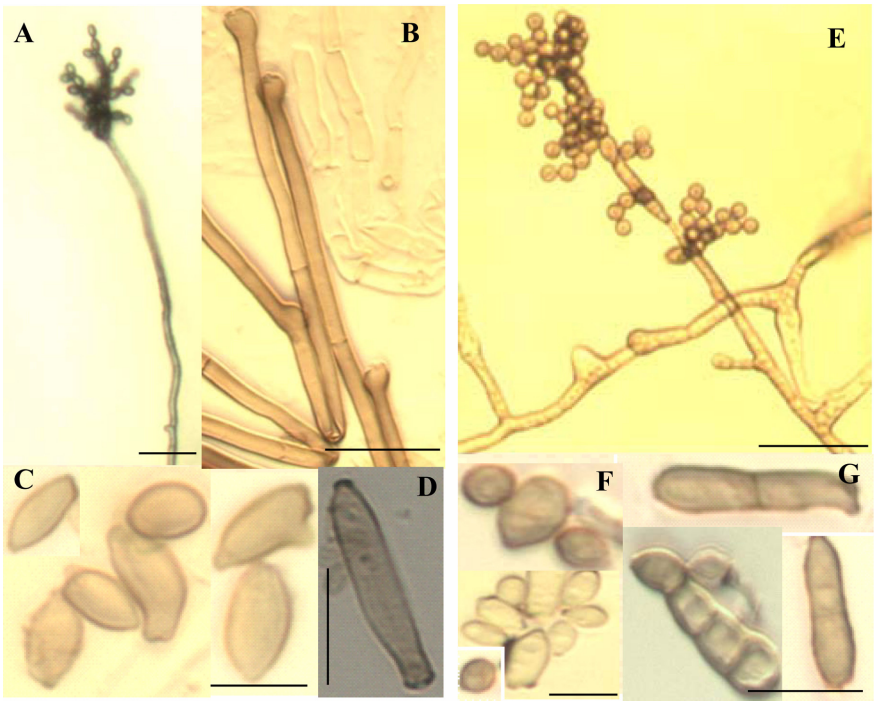

Fig. 3. Conidiophores (A, B, E), conidia (C, F) and ramoconidia (D, G) of *C. oxysporum* (A, B, C, D) and *C. sphaerospermum* (E, F, G). Scale bar = 20 μm (A, B, E, F), 5 μm (C, D, G).

dishes incubated for 7 days at 25°C in the dark. Colony appearance, exudate production, pigmentation and reverse coloration were assessed and colony diameters were recorded after one week at 25°C. These isolates were identified with the help of keys developed by Schubert (2005) and Zalar *et al*. (2007). The examination and measurements of conidiophores and conidia were made from slide preparations stained with lacto-phenol. Differential interference contrast microscopy was used for the observation and measurement of conidiophores and conidia.

Results and Discussion

Molecular analysis. The isolates assumed to be *Cladosporium* were confirmed on the basis of their ITS gene sequence analysis (Fig. 1). To determine the phylogenetic relationship between the *Cladosporium* isolates from the needles of pine trees and previously described species, ITS rDNA was sequenced and compared. The CNU060562 isolate and *C. oxysporum* ATCC 76499 clustered together in a group, which was well supported with a bootstrap value of 89%. The CNU060373 isolate was most closely related to *C. sphaerospermum* CBS 102045. CNU060373 and *C. sphaerospermum* CBS 102045 formed a monophyletic group, which was well supported with a bootstrap value of 100%; both were clearly distinguished from other *Cladosporium* species (Fig. 1).

Morphological observations. Two isolates selected as *Cladosporium* on the basis of their ITS gene sequence analysis were used for the morphological observations. According to cultural and morphological characteristics, the *Cladosporium* species were identified as *C. oxysporum* and *C. sphaerospermum* (Schubert 2005; Gugnani *et al*., 2006; Zalar *et al*., 2007; MycoBank (http://www.mycobank.org/). Taxonomic descriptions, photos of colonies and conidial and conidiophores structures of each species are given below.

***Cladosporium oxysporum* Berk. & Curt.**

Figs. 2(A~B), 3(A~D)

Colonies on MEA were moderately expanding, velvety and often floccose at the center, olivaceous to grey olivaceous; later the colonies turned dark olivaceous with reverse greenish-black. On PDA, the colonies were oliva-

Table 1. Comparison of morphological and cultural characteristics of *Cladosporium oxysporum* and the isolate of endophytic *Cladosporium*

Characteristics		Present isolate	*C. oxysporum* Berk. & Curt.[a,b]
Colony	Color	Olivaceous to grey-olivaceous	Olivaceous brown[a]
	Length	16~17.5 mm after 7 days on PDA	19~22 mm after 7 days on SDA[a]
Conidia	Size	2.5~10.0 × 2.0~4.5 μm	3~6 μm wide[b]
	Septa	Aseptate	0~1(-2) septate[b]
	Shape	Spherical to subspherical, limoniform, ovoid	Spherical to subspherical, subglobose, ovoid, limoniform, ellipsoid[a,b]
Ramoconidia	Size	14.5~21.5 × 3.5~4.5 μm	7~11 × 2.8~4.5 μm[a]
	Septa	0~1	0~1[a]
Conidiophores		Distinctly nodose with terminal and intercalary swellings, conidial chains were branched.	Straight, nodose with distinct, regular swellings, clearly separated and distinct from each other[a,b]

[a,b]Source of reference: [a]Gugnani *et al*. (2006), [b]Schubert (2005).

Table 2. Comparison of morphological and cultural characteristics of *Cladosporium sphaerospermum* and the isolate of endophytic *Cladosporium*

Characteristics		Present isolate	*C. sphaerospermum* Penz.[a]
Colony	Color	Olivaceous to grey-olivaceous	Olive-green to olivaceous-brown
	Length	9.5~11.0 mm after 7 days on PDA	21~44 mm diameter after 14 days on PDA
Conidia	Size	2.0~8.0 × 2.0~4.0 μm	2.5~7.0 × 2.0~4.5 μm wide
	Septa	Aseptate	Aseptate
	Shape	Elliopsoidal to cylindrical	Subspherical to spherical, less often short-ovoid
Ramoconidia	Size	8.5~20.0 × 3.0~6.0 μm	4.0~17.5 × 2.0~8.5
	Septa	0~4	0~4
Conidiophores		Conidiophores were variable in length, up to 300 μm long, 3~5 μm wide, smooth-walled or verrucose, not nodose.	Conidiophores erect or ascending, micronematous and macronematous, stipes of variable length, 10~300 × (2.5~6 μm).

[a]Source of reference: Zalar *et al*. (2007).

reverse dark green. The diameter of colonies on PDA and MEA were 16.5~17.5 mm and 15.0~17.0 mm, respectively. Conidiophores were straight to slightly flexuose, 3.5~5.5 μm wide, distinctly nodose with terminal and intercalary swellings, bearing branched conidial chains. Ramoconidia were present, single cells were common but double celled ramoconidia were rarely evident, with dimensions of 14.5~21.5 × 3.5~4.5 μm. Conidia were spherical to subpherical, smooth-walled, and the length and width of conidia were 2.5~10.0 × 2.0~4.5 μm.

Isolates examined: On needles of *P. densiflora*; CNU060562.

Notes: Colony characteristics and micromorphology of the fungus agreed well with the description of *C. oxysporum* (Schubert, 2005; Gugnani *et al.*, 2006). The species is closely related to *C. borassi* and *C. colocasiae*. *C. borassi* produces nodulose conidiophores but swellings are not regular, neither separated nor distinct from each other, and *C. calocasiae* bears 0~3 septate conidia, which makes them different from *C. oxysporum* (Schubert and Braun, 2004). The fungus has been reported from Cuba, Mexico, India and Texas (Schubert and Braun, 2004), but not hitherto in Korea.

***Cladosporium sphaerospermam* Penz.**

Figs. 1(C~D), 2(E~G)

Colonies on PDA and MEA were 9.5~11.0 mm and 10.0~13.0 mm in diameter, respectively, after 7 days at 25°C. Colonies were olivaceous to grey-olivaceous and powdery on PDA, and velvety to powdery, olive-green to grey-olivaceous on MEA; reverse side of the colonies were greenish-black, with a margin that was either regular or aracnoid, radially furrowed, having a wrinkled colony center and formed a crater-like structure. Conidiophores were variable in length, up to 300 μm long and 3~5 μm wide, smooth-walled or verrucose and not nodose. Secondary ramoconidia were present, possessed 0~4 septa, elongate, smooth-walled or verrucose, and the ramoconidial size were 8.5~20.0 × 3.0~6.0 μm. Conidia were spherical, ellipsoidal to cylindrical with sounded ends, single celled, verrucose with dimensions of 2.0~8.0 × 2.0~4.0 μm.

Isolates examined: On needles of *P. densiflora*; CNU060373.

Notes: Colony characteristics and micromorphology of the fungus agreed well with the description of *C. sphaerospermum* (Zalar *et al.*, 2007). The species is similar to *C. halotoleraus* and *C. velox* in colonial morphology (Zalar *et al.*, 2007). The fungus, however, has a white margin in colony, is usually irregular shaped and secondary ramoconidia possess 0~4 septa, which distinguishes it from other two fungi. The fungus has been reported from hypersaline water in the Mediterranean and the tropics, soil and plants in temperate climates, indoor wet cells and from humans (Zalar *et al.*, 2007). This is the first record of *C. sphaerospermum* in Korea.

Acknowledgements

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