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RESEARCH ARTICLE

Two New Records of Ascomycetes from Crop Field Soils in Korea

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Abstract

In the ongoing survey of fungal diversity in Korea, two Ascomycetes species, namely *Rhinocladiella similis* and *Toxicocladosporium irritans*, were isolated in 2016. These species were identified based on the internal transcribed spacer region and morphological characteristics. This is the first report of these species in Korea, and the morphological characteristics and images of the fungi are presented.

Keywords: Ascomycetes, Internal transcribed spacer, Phylogeny

Introduction

Sac fungi or Ascomycetes are the largest phylum of fungi, with over 64,000 species [1]. 'Ascus' is the distinct characteristic of this group of fungi. However, some species in this fungal group are asexual, and thus there is no formation of asci and ascospores.

Rhinocladiella is a widely distributed fungus that can be found in soil, herbaceous substrates, and decaying wood. *Rhinocladiella similis* belongs to the *Eurotiomycetes* class within the order *Chaetothyriales* of the *Herpotrichiellaceae* family. The typical morphology of *Rhinocladiella* consists of a profusely branched conidial apparatus of the same texture and pale-brown pigmentation as its mycelium [2]. *Rhinocladiella* is a genus of melanized fungi that can cause chromoblastomycosis. This order contains several clinically relevant species of the genera *Exophialia*, *Cladophialophora*, *Fonsecaea*, and *Phialophora*, which are possible etiologic agents of chromoblastomycosis and/or phaeohyphomycosis [3, 4]. The genus *Toxicocladosporium* (*Cladosporiaceae*, *Capnodiales*) was described by Crous et al. [5] as harboring cladosporium-like fungi consisting of distinct "dark, thick-walled conidial and conidiophore septa, and lacking the typical coronate *Cladosporium* scar type." *Toxicocladosporium* is widely distributed and has the capacity to colonize distinct substrates and plant families like *Cladosporium*. The type species of *T. irritans* was isolated

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from moldy paint in Suriname and named "*irritans*" because of the production of several volatile metabolites in culture that irritated skin exposed to the fungus [5]. The main objective of this study was to (i) describe the newly recorded isolates, *R. similis* and *T. irritans*, morphologically and molecularly, and (ii) compare the morphological features of these newly recorded isolates with those of previously reported isolates.

Materials and Methods

Sampling and isolation

Soil samples were collected from various locations in Gyeongnam, Gyeongsangnam-do, Korea. The GPS location was 35.071097 N, 127.580510 E for isolate KNU16-146 and 35.235877 N, 128.414059 E for isolate KNU16-332. Fungal isolation was performed using the conventional dilution technique [6]. After dilution, isolates were cultured on potato dextrose agar (PDA; Difco, Detroit, MI, USA) supplemented with 100 μg chloramphenicol (bacteriostat/L PDA) for 5~7 days at 25°C until growth of a fungal colony was observed. For further use, the isolate was preserved at 20°C on PDA slants.

Morphological characterization

The fungal macro-morphological characteristics were studied on PDA. The fungal specimens were single point inoculated and were incubated at 25°C for 7 days in darkness. Colony characteristics were recorded, and fungal materials were examined using an Olympus BX50F-3 light microscope (Olympus, Tokyo, Japan). For the micro-morphological examination, microscopic mounts of all isolates were made from colonies grown on PDA in lactic acid with a drop of alcohol added to remove air bubbles and excess conidia. Photomicrographs of the isolates were obtained with an HK 3.1 CMOS digital camera (KOPTIC, Seoul, Korea) attached to an Olympus BX50F-3 microscope. The microscopic structures of the isolates were also examined using a scanning electron microscope (LEO Model 1450VP Variable Pressure Scanning Electron Microscope; Carl Zeiss, Oberkochen, Germany).

Genomic DNA extraction, PCR amplification, sequencing, and phylogenetic analysis

For molecular genetic identification, isolates were grown on PDA for a week, and total genomic DNA was extracted using DNeasy Plant Mini Kit (Qiagen, Germantown, MD, USA) following the manufacturer's instructions. The internal transcribed spacer (ITS) region was amplified using primers ITS1 (5′-TCCGTAGGTGAACCTGCG-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) [7], and the amplified PCR products were sequenced using an ABI Prism 3730 DNA analyzer (Applied Biosystems, Foster City, CA, USA). All

sequence information was analyzed using the BLAST program National Center for Biotechnology Information (NCBI, http://www.ncbi.nlm.nih.gov/blast/). A phylogenetic tree was constructed using the neighbor-joining method and MEGA6.0 software [8]. Statistical confidence in the tree topology was evaluated based on a bootstrap analysis with 1,000 replicates. The newly recorded fungal isolates were deposited in the National Institute of Biological Resources (NIBR), Korea, under deposition numbers NIBRFG 0000499480 and NIBRFG0000499478 for isolates KNU16-146 and KNU16-332 respectively. The sequences of isolate KNU16-146 and KNU16-332 were also deposited in GenBank under accession numbers KY906219 and KY9066224, respectively (Table 1).

Table 1. Sequences of *Rhinocladiella similis* and *Toxicocladosporium irritans* along with their National Institute of Biological Resources and GenBank accession numbers

Isolates	NIBR no.	GenBank accession no.	Closest GenBank library strain	Similarity (%)
KNU16-146	NIBRFG0000499480	KY906219	*Rhinocladiella similis*	100
KNU16-332	NIBRFG0000499478	KY9066224	*Toxicocladosporium irritans*	99

Results

Morphology and micromorphology of isolate KNU16-146

***Rhinocladiella similis* de Hoog & Caligiorne, Journal of Clinical Microbiology 41 (10): 4777 (2003)**

The colony on PDA was slightly brown on the front side and black on the back side of the

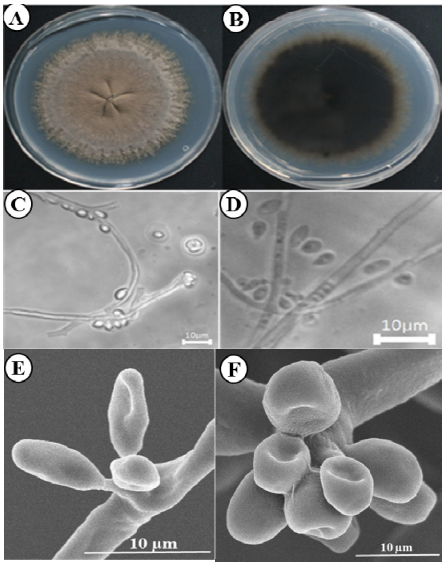

Fig. 1. Morphology of the isolate *Rhinocladiella similis* (KNU16-146) grown for 7 days on potato dextrose agar. A, obverse colony; B, reverse colony; C, D, simple microscopic images of conidiophores and conidia; E, F, scanning electron micrographs of conidiophores and conidia.

plate. It grew moderately, attaining a diameter of 60~65 mm in 7 days at 25°C (Fig. 1A, 1B). The colony was floccose. Sporulation was moderate to dense, the surface was rough, and the form was irregular. Diffusible pigments were not produced. Hyphae were pale olivaceous to brown and 1.3 mm wide. Conidiogenous cells were profusely branched, and the conidial color was brown. Conidiogeneous cells were cylindrical, 11~19 by 2 μm apically (Fig. 1C~1F). Conidia were subhyaline, noncatenate, cylindrical, and narrowed toward the base, and 3~6 by 1.3 μm of diameter. Budding cells were broadly ellipsoidal with a size of 1~5 μm. Morphology comparison of our study isolate (KNU16-146) with the previously reported *R. similis* isolate has been mentioned in Table 2.

Table 2. Comparison of the morphologies of isolate KNU16-146 and a previously described isolate of *Rhinocladiella similis*

Characteristics		**Study isolate** *R. similis*	*R. similis*[a]
Colony	Diameter	65～70 mm on PDA	NA
Hyphae	Color	pale olivaceous to brown	pale olivaceous to brown
	Size	1.4 μm wide	1.5 μm wide
	Shape	regular, thick walled and regularly septate	regular, thick walled and regularly septate
Conidiogenous cells	Structure	cylindrical with the apical part of variable length.	cylindrical with the apical part of variable length.
	Color	brown	brown
	Number and size	11～19 and 2 μm apically	11～19 and 2 μm apically
Chlamydospores	Presence	absent	absent
Conidia	Structure	subhyaline, noncatenate, cylindrical, narrowed toward the base	subhyaline, noncatenate, cylindrical, narrowed toward the base
	Size	3 to 6 by 1.4 μm	4 to 7 by 1.5 μm
Budding cells	Structure	broadly ellipsoidal	broadly ellipsoidal
	Size	1～5 μm	1～5 μm

PDA, potato dextrose agar; NA, Not available.
[a]Source of description [2].

Morphology and micromorphology of isolate KNU16-332

***Toxicocladosporium irritans* Crous & U. Braun, Studies in Mycology 58: 39 (2007)**

The colony color on PDA was black on both the front and back sides of the plate. It grew moderately, attaining a diameter of 65~70 mm in 7 days at 25°C. (Fig. 2A, 2B). Sporulation was moderate to dense, the form was irregular, the texture was floccose, and the surface was rough. It had branched mycelium that were finely verruculose. Conidiophores were macronematous and solitary. Conidiogenous cells were terminal or lateral with a slight taper towards the apex. Conidia were catenulate, ellipsoid to ovoid, and 0.4~1 μm wide (Fig. 2C, 2F). Morphology comparison of our study isolate (KNU16-332) with the

previously reported *T. irritans* isolate has been mentioned in Table 3.

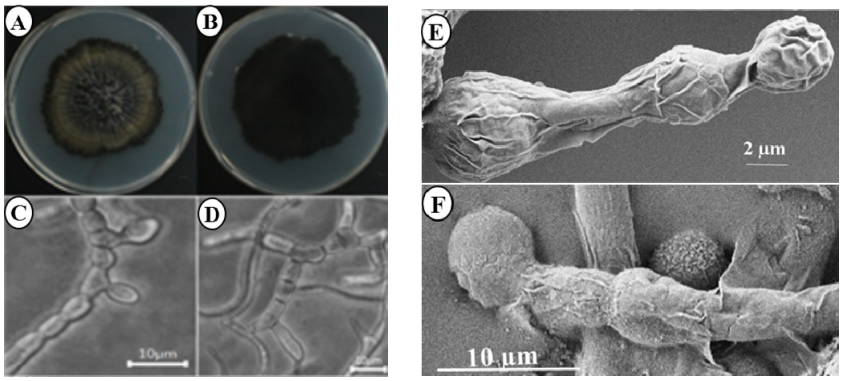

Fig. 2. Morphology of the isolate *Toxicocladosporium irritans* (KNU16-332) grown for 7 days on potato dextrose agar. A, obverse colony; B, reverse colony; C, D, simple microscopic images of conidiophores and conidia; E, F, scanning electron micrographs of conidiophores and conidia.

Table 3. Comparison of the morphologies of isolate KNU16-332 and a previously described isolate of *Toxicocladosporium irritans*

Characteristics		**Study isolate** *T. irritans*	*T. irritans*[a]
Colony	Diameter	65~70mm on PDA	NA
Mycelia	Color	dark brown	dark brown
	Shape	branched, septate, finely verruculose	branched, septate, finely verruculose
Hyphae	Structure	walls and septa becoming thickened and darkened with age	walls and septa becoming thickened and darkened with age
	Size	(2~)2.8~3.94 μm	(2~)3~4 μm
Conidiophores	Presence	present	present
	Structure	subcylindrical, straight to geniculate-sinuous, or irregularly curved, unbranched or branched above, 2~8-septate, dark brown, finely verruculose, walls thick, septa dark brown	subcylindrical, straight to geniculate-sinuous, or irregularly curved, unbranched or branched above, 2~7-septate, dark brown, finely verruculose, walls thick, septa dark brown
	Size	29.8~58.7 × 3.8~5.9 μm	30~60 × 4~6 μm
Conidia	Structure	catenulate in branched or unbranched chains, medium to dark brown, thick-walled, with dark, thick septa, smooth to finely verruculose; ramoconidia	catenulate in branched or unbranched chains, medium to dark brown, thick-walled, with dark, thick septa, smooth to finely verruculose; ramoconidia
	Size	(4~)6~8(~9) × (3~)4(~5) μm	(5~)6~8(~10) × (3~)4(~5) μm

PDA, potato dextrose agar; NA, Not available
[a]Source of description [5].

Molecular phylogeny of the fungal isolates

ITS regions were analyzed to determine the evolutionary relationships between isolates KNU16-146 (*R. similis*) and KNU16-332 (*T. irritans*) and previously described isolates of *R. similis* and *T. irritans*. Results obtained from the phylogenetic analysis suggested that isolate KNU16-146 was most closely related to *R. similis* (NR111244.1) (Fig. 3) with the bootstrap value of 100%. The type strain in GenBank was used to construct the phylogenetic tree. The sequence of isolate KNU16-332 matched that of *T. irritans* (EU040243.1), and these two strains formed a monophyletic group with a bootstrap value of 99% (Fig. 3). Type strains from GenBank were used as references for the construction of the phylogenetic tree. The molecular phylogeny strongly suggested that isolate KNU16-332 is *T. irritans*. Isolate KNU16-332 showed 99 % similarity with the type strain of *T. irritans* in GenBank (Fig. 3).

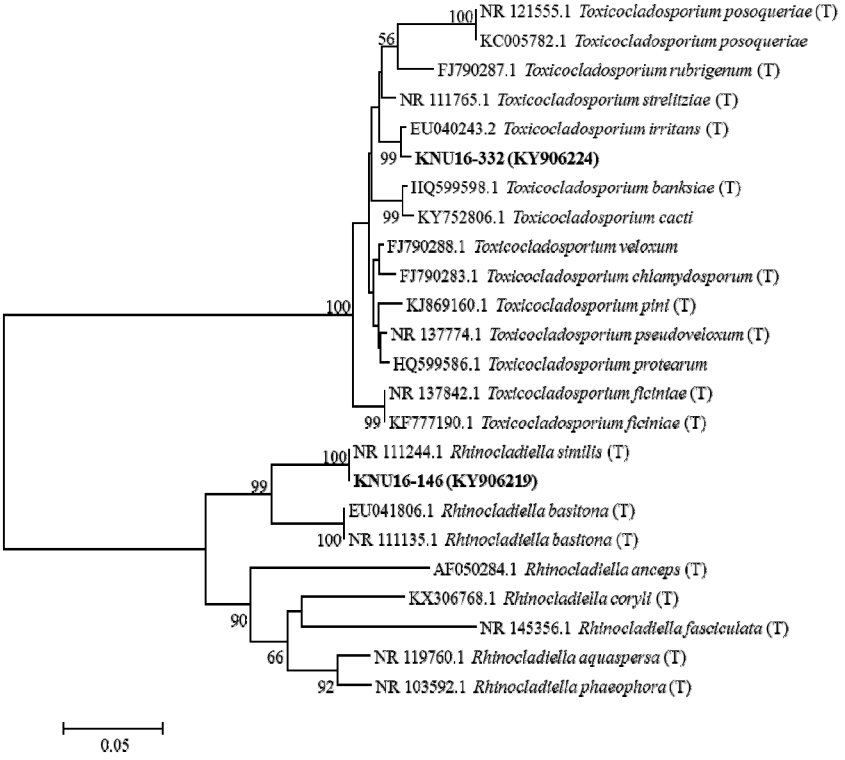

Fig. 3. Neighbor-joining phylogenetic analysis of the partial 18S-ITS1-5.8S-ITS2-28S rDNA sequences of KNU16-146 and KNU16-332 obtained from crop field soils in Korea. The phylogenetic tree was constructed using MEGA 6. The sequences obtained in this study are shown in bold. Numerical values (>50) on branches are the bootstrap values as percentages from a 1,000 bootstrap replicate analysis. The scale bar represents the number of substitutions per site. 'T' indicates the type strain. ITS, internal transcribed spacer.

Discussion

On the basis of the morphological features of isolate KNU16-146, this isolate was

assumed to belong to the genus *Rhinocladiella*. *R. similis* is in the Ascomycota division, under the family *Herpotrichiellaceae*. Previously identified specimens of *R. similis* and our newly recorded isolate *R. similis* had similar morphological and micromorphological structures [2], with a profusely branched conidial apparatus that was subhyaline, noncatenate, cylindrical, narrowed towards the base, and pale-brown pigmented, similar to the mycelium [2]. Similar conidia were observed in isolate KNU16-146 (Fig.1C~1H). In addition, the phylogenetic relationship between our isolate of *R. similis* and a previously reported *R. similis* indicated 100% similarity in the ITS region (Fig. 3). Type strains of *R. similis* were selected from GenBank for the similarity analysis, and results suggested that isolate KNU16-146 is 100% similar to an already reported *R. similis* (NR111244.1) (Fig. 3). This morphological and molecular analysis confirmed that our isolate is *R. similis*. *R. similis* has biotechnological importance. The different polarity of volatile organic compounds (VOCs) in gas-phase biofilters are degraded by *R. similis* [9]. Further study regarding its clinical and biotechnological value will be worthwhile. The other study isolate, KNU16-332, belongs to genus *Toxicocladosporium* of the family *Cladosporiaceae*. Isolate KNU16-332 was assumed to be a *Toxicocladosporium*, based on the shape and size of its hyphal conidia and conidiophores, respectively. Isolate KNU16-309 (*T. irritans*) was morphologically similar to the previously described *T. irritans* [5]. In addition, the phylogenetic analysis revealed that study isolate KNU16-332 was most closely related to *T. irritans*. Morphologically and molecularly, it is similar to *Cladosporium*. However, we used only *Toxicocladosporium* in the phylogenetic analysis. Conidia and conidiophores with septa and dark, thick walls and the absence of a typical coronate *Cladosporium* scar type [10] have been reported in the literature. Together, the morphological and molecular analysis confirmed that our study isolate KNU16-332 is *T. irritans*. Previously, *T. irritans* was reported to produce different volatile metabolites that causes irritation to skin. Our two newly recorded isolates, *R. similis* and *T. irritans*, are clinically relevant species. Morphologically and physiologically, they are different from each other. However, they both cause harm to humans if not handled safely. *R. similis* can cause chromoblastomycosis, which is a tropical and subtropical skin disease caused by accidental inoculation through the environment [11]. Likewise, the type strain of *T. irritans* was isolated from moldy paint and found to cause skin irritation upon exposure [5]. Further studies regarding these two newly recorded fungal isolates from Korea would be worthwhile.

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