Identification and Characterization of *Gliocladium viride* Isolated from Mushroom Fly Infested Oak Log Beds Used for Shiitake Cultivation

Jun Young Kim¹, Yeo Hong Yun¹, Min Woo Hyun¹, Myeong Ho Kim¹ and Seong Hwan Kim^{1,2*}

¹Department of Microbiology, ²Institute of Basic Sciences, Dankook University, Cheonan 330-714, Korea

(Received February 14, 2010. Accepted February 25, 2010)

A green mold species that has not previously been reported in Korea was isolated from oak log beds used for shiitake (*Lentinula edodes*) cultivation that were infested by mushroom flies. In this study, we identify the mold species as *Gliocladium viride* (an anamorph of *Hypocrea lutea*) and describe its mycological properties. The fungus was cottony on both potato dextrose agar (PDA) and Czapek yeast extract agar (CYA), but was colored white on PDA and became yellowish green and brown on CYA. Mycelial growth on PDA attained a diameter of 73 mm at 30°C after 5 days. The fungus grew faster on malt extract agar (> 80 mm, 5 days at 25°C) compared to CYA and PDA (< 68 mm, 5 days at 25°C). Penicillate conidiophores of the fungus are hyaline, smooth walled, branching above typically in four stages, and 120–240 µm in length. Club-shaped or slender phialides are formed on the metulae. Conidia of the fungus were ovate and elliptic, yellowish brown and green, and 2.5–3.0 µm × 1.8–2.3 µm in size. Typically, slimy conidia are formed in a mass and colored brown to dark green to almost black. The internal transcribed spacer rDNA and translation elongation factor 1 alpha gene sequences of the fungus isolated here show 99% identity with previously identified *G. viride* strains.

KEYWORDS: *Gliocladium viride*, ITS rDNA, Mushroom fly, Shiitake mushroom, *tef1-α* gene

Shiitake is a popular edible and medicinal mushroom in Korea. To produce high quality mushrooms, shiitake has been cultivated on oak log beds. Recently, many farmers in the shiitake mycoculture industry in Korea have been stressed by severe yield losses due to damage of mushroom log beds caused by infestations of mushroom flies. So far, the identity of the infesting mushroom flies has not yet been elucidated, despite them being frequently found in damaged oak log beds of shiitake on many mushroom farms across Korea. It has been postulated that the damage to the mushroom log beds are caused not only by mushroom flies, but also by green fungi. Some species belonging to the *Hypocrea*, *Trichoderma*, and *Gliocladium* genera have been known as agents of green mold disease, which affects cultivated mushrooms such as *Agaricus bisporus*, *Lentinula edodes* and *Pleurotus ostreatus* [1, 2]. However, no investigation has yet determined what kinds of green molds are present and which species are problematic in damaging shiitake log beds infested by mushroom flies. Therefore, we have examined green molds in the mushroom fly-infested shiitake log beds. In this study, we report the isolation and identification of *Gliocladium viride*, which has not been described before in Korea.

Materials and Methods

Fungal isolation. Samples of damaged oak log beds were obtained in the summer of 2009 from mushroom

farms in Cheonan city, Korea (Fig. 1). The sampled log beds were split into small pieces (0.5 cm × 0.5 cm × 0.2 cm), surface sterilized in 1% sodium hypochlorite solution for 2 min, washed three times with sterile water and put on potato dextrose agar (PDA) containing streptomycin (100 µg/mL) and incubated for several days at 25°C. Mycelia grown out from the small pieces were transferred to fresh PDA and single spore isolates were obtained from the PDA-grown fungi. The obtained single spore isolates were maintained on PDA for the duration of the experiment and stored either at –80°C in 10% glycerol for long-term storage or at 4°C for short-term storage.

Morphological studies. Colony appearance and sporulation were tested from cultures grown in darkness at 25°C for 5–7 days on PDA, Czapek yeast extract agar (CYA) and malt extract agar (MEA). The growth rates of the isolates were determined at 15°C, 20°C, 25°C, 30°C and 37°C on PDA for 5 days. Agar disks (5 mm diameter) taken from the edge of an actively grown colony were placed at the end of a PDA plate. Three replicate plates were tested for each temperature. Light microscope observations and mycelia length measurements were made in 0.05% KOH solution by a phase contrast Axioskop 40 (Karl Zeiss, Oberkochen, Germany). Mycelial measurements are reported as the maximum and minimum values of 30 measurements, given between brackets, as well as the mean ± standard deviation. For scanning electron microscopic observations, the fungal isolates were grown for 3–5 days at 25°C on PDA. Agar blocks were fixed with 2% glut-

*Corresponding author <E-mail: piceae@dankook.ac.kr>

Fig. 1. Examples of damaged shiitake log beds infested by mushroom flies. Arrow (A) and (B) indicates the spot where green molds were isolated in this study.

araldehyde in 0.1 M cacodylate buffer for 16 hr and 1% osmium tetroxide in 0.1 M phosphate for 1 hr. The fixed samples were subsequently washed with 0.05 M cacodylate buffer, dehydrated in a series of ethanol washes (50% for 20 min, 75% for 20 min, 90% for 20 min, 95% for 20 min and 100% for 20 min), passed through ethanol-isoamyl acetate, dried with a Hitachi critical point dryer and coated with platinum-palladium at 25 nm using an Hitachi E-1030 ion sputter (Hitachi Science Systems, Ltd., Tokyo, Japan). The prepared specimens were examined by a Hitachi S-4200 scanning electron microscope (SEM) operating at 10 kV.

DNA extraction, PCR amplification and DNA sequencing. Fungi were grown for 3–5 days on PDA at 25°C and fungal genomic DNA for PCR was obtained from the mycelia of the PDA-grown cultures using the drilling method described by Kim *et al.* [3]. The internal transcribed spacer (ITS) ribosomal DNA regions were amplified by PCR using the universal primer pair, ITS1 and ITS4 [4]. The *tef1- α* gene, encoding translation elongation factor 1- α , was amplified using the primers TEF728 and TEF1 [5]. Sequencing was performed on an ABI 3700 automated sequencer (Perkin-Elmer Inc., Waltham, MA, USA) at the DNA synthesis and sequencing facility, MACROGEN (Seoul, Korea). The determined nucleotide sequences were then compared to publicly available sequences by BLASTN against the GenBank database (<http://www.ncbi.nlm.nih.gov/BLAST>) and by *Tricho*-BLAST at the website of the International Subcommittee on *Trichoderma* and *Hypocrea* Taxonomy (ISTH, <http://www.isth.info/>). ISTH is a publicly available database with sequence diagnosis and similarity search tools, which covers all genetically characterized species of the *Trichoderma* and *Hypocrea* genera.

Molecular phylogenetic analysis. The determined nucleotide sequences were manually edited using the Chromas

v2.31 program (Technelysium Pty. Ltd., Helensvale, Qld, Australia) and aligned using the ClustalW2 program (European Bioinformatics Institute, Cambridge, UK). Reference sequences of related taxa were obtained from the GenBank database. The aligned sequences were analyzed with the PAUP 4.0 b10 program [6]. Phylograms based on ITS and *tef1- α* gene sequences were constructed by the neighbor-joining method [7]. Bootstrap values were generated with 1,000 replicates through heuristic searches. *Fusarium solani* was used as an outgroup.

Results and Discussion

Colony morphology. Initially several fungi were isolated from the damaged oak log beds (Fig. 1). When they were grown on PDA and MEA, they showed similar morphological properties. Thus, three of them were selected, coded as DKU002, DKU003 and DKU004, and used for this study (Table 1). Among these three isolates, DKU002 was used as a representative isolate for morphological description. The colony morphology and color of DKU002 grown on three different media are given in Fig. 2. The fungus was cottony on both PDA and CYA, but was colored white on PDA and became yellowish green and brown on CYA. The bottoms of the fungal cultures, seen on the reverse side of the plates, were colorless or light brown. Colony color was clearer on CYA than on MEA and PDA. On all three media, the fungus produced thin, transparent hyphae and its mycelia broadly extended over the entire medium. Mycelia were more densely formed on PDA and CYA than on MEA. These observations show that colony colors and mycelia growth patterns of DKU002 vary depending on type of media. On MEA, mycelia were flat with few aerial mycelia. Weak, unevenly spaced concentric rings resulted from different growth rate of mycelia also formed on MEA, but were not observed on PDA or CYA plates. On MEA a greenish color appeared along with concentric rings. On PDA and CYA, the green color

Table 1. Morphological characters of *Gliocladium viride* and the isolate DKU002

Characters	<i>Gliocladium viride</i> ^a (anamorph of <i>Hypocrea lutea</i>)	DKU002 (present study)
Colony color	Green	Yellowish brown and green
Conidiophore		
Color	Hyaline	Hyaline
Shape	Upper branching typically in four stage	Upper branching typically in four stage
Size	100~225 $\mu\text{m} \times 8\sim 10 \mu\text{m}$	120~240 $\mu\text{m} \times 8\sim 10 \mu\text{m}$
Conidia		
Color	Green to dark green	Yellowish brown, green
Shape	Green elliptic	Ovate, elliptic
Size	3.0~3.8 $\mu\text{m} \times 2\sim 2.5 \mu\text{m}$	2.5~3.0 $\mu\text{m} \times 1.8\sim 2.3 \mu\text{m}$
Fructification	Greenish, dark green to blackish slimy in mass	Greenish, dark green to blackish green slimy in mass

^aDescription referred from *G. deliquescens*, the synonym of *G. viride* [8].

Fig. 2. Colony patterns of DKU002 grown on potato dextrose agar (A), Czapek yeast extract agar (B) and malt extract agar (C) after 7 days of culturing at 25°C.

Fig. 3. Variations in mycelial growth of DKU002 on PDA at different temperature (A) and on different media at 25°C (B). CYA, Czapek yeast extract agar; MEA, malt extract agar; PDA, potato dextrose agar.

mostly appeared at the edge of the plates. DKU002 was a rapidly growing fungus, and grew faster on MEA and PDA than on CYA (Fig. 3). After 5 days of incubation on PDA, mycelia of the fungus grew to a diameter of 16 mm at 15°C, 39 mm at 20°C, 68 mm at 25°C, 73 mm at 30°C and 25 mm at 37°C (Fig. 3). These results show that the fungus grows well at 25~30°C at which shiitake cultivation normally occurs.

Morphological characters. Light and scanning electron microscopic images of DKU002 are given in Fig. 4. The fungus has an erect penicillate conidiophore structure (Fig. 4O and P). Conidia formed without chains at the apices of the conidiophores (Fig. 4E and K). These microstructures show characteristics of the genus *Gliocladium* (Fig. 4G~I and K). Conidiophores of the fungus are hyaline, smooth walled, typically upper branching, and 120~

Fig. 4. Morphological features of DKU002 observed by a light microscope (A~N) and scanning electron microscope (SEM) (O~Q). Conidiophores and wet slimy conidia masses formed on potato dextrose agar (A~C) and on malt extract agar (D~E). Conidia (F, Q), conidiophores (G~K), an extending root-like structure (L~M) and a chlamyospore-like structure (N) were all observed. Penicillate with four stage branches (O~P) were observed under SEM.

240 μm in length (Table 2). Conidiophores arise from both submerged and surface hyphae, with several growing from each point; both aerial and submerged stolons

are present at these points (Fig. 4A~D). Conidia fructification is shown typically in four stages (Fig. 4N~P). Three to five primary branches (ramus) arise from the conidio-

Table 2. *Gliocladium viride* isolates used in this study

Species	Collection no.	GenBank accession no.		Substrate/ host	Origin	Collector
		ITS	<i>tef1-α</i>			
<i>G. viride</i>	DKU 002	GU903310	GU903312	<i>Quercus</i> sp.	Korea	J Y Kim, S H Kim
	DKU 003	GU903311	GU903313	<i>Quercus</i> sp.	Korea	J Y Kim, S H Kim
	DKU 004	GU903315	GU903314	<i>Quercus</i> sp.	Korea	J Y Kim, S H Kim

ITS, internal transcribed spacer.

Fig. 5. Phylogenetic relationships of the DKU isolates to other related species. Cladograms based on analysis of a partial nucleotide sequence of the internal transcribed spacer region (A) and the *tef1-α* gene (B) was generated by neighbor-joining analysis. Numbers at nodes represent the percentage of 1,000 bootstrap resampling runs that result in the same branching. *Fusarium solani* was used as an outgroup.

phore stipe. Each primary branch bears a verticil of secondary branches, namely, verticils of metulae. Club-shaped or slender phialides are formed on the metulae (Table 2). The shape of the primary and secondary branches and metulae are elongate and oblong. Overall, each series of branches in the conidiophores are progressively smaller. Conidia were ovate and elliptic, yellowish brown and green, and $2.5\sim 3.0\ \mu\text{m} \times 1.8\sim 2.3\ \mu\text{m}$ in size (Table 2, Fig. 4F and Q). Conidia production is very abundant on CYA, usually enveloping the entire colony. Typically, slimy conidia are formed in a mass ranging in color from brown to dark green to almost black (Fig. 4B~E and K). Chlamydospore-like structures are seen (Fig. 4N). The conidiophore base has extending root-like (rhizoid-like) hyphae (Fig. 4 and M). Perithecia and sclerotia were not found.

According to the taxonomy key of the *Gliocladium* genus, species having conidial areas that are pale yellow-green to dark green belong to the *G. catenulatum* series or the *G. deliquescens* series [8]. If conidia are typically pale yellow-green and commonly remain in chains to form wet

columns, the species belongs to *G. catenulatum* series. However, if the conidia are typically dark green to almost black and collect into slime balls, the species belongs to *G. deliquescens* series. In this study, the observed morphological characteristics of DKU002 were similar to those of the *G. deliquescens* series, which includes *G. deliquescens*, *G. atrum* and *G. nigro-virescens*. Among the species in the *G. deliquescens* series, the description of *G. deliquescens* most matched the morphological characteristics of DKU002. The comparison of *G. deliquescens* and DKU002 is given in Table 2. *G. deliquescens* is a synonym of *Gliocladium viride* [9], which is the anamorph of *Hypocrea lutea* [10].

Molecular phylogenetic analysis. For further evidence to confirm whether DKU002 is the anamorph of *H. lutea*, its ITS rDNA and *tef1-α* gene sequences were analyzed. We amplified these target DNA sequences by PCR and determined the nucleotide sequences of the PCR amplicons. 573 (ITS rDNA) and 637 (*tef1-α*) bp nucleotide

sequences were determined from DKU002, DKU003, and DKU004, respectively. These three isolates shared 99% sequence identity in both the ITS rDNA and *tef1- α* genes. These results indicate that the three DKU isolates are the same species. When homologous sequences for these ITS rDNA sequences were mined from the GenBank DNA database, the experimentally determined sequences most matched (99% similarity) the ITS rDNA sequence of *Gliocladium deliquescens* (accession number GQ229478), *Gliocladium viride* (accession number EU076919), and *Hypocrea lutea* (accession number AB027384). In the case of the *tef1- α* gene, the sample sequences most matched (99% similarity) the *tef1- α* gene sequences of *H. lutea* (accession number FJ860772 and FJ860644). Phylogenetic analyses based on the ITS region (Fig. 5A) and *tef1- α* gene (Fig. 5B) sequences also placed DKU002~004 with *H. lutea* (teleomorph of *G. viride*). When we searched the ITS rDNA and *tef1- α* gene sequences of DKU002 through TrichoBLAST, the sequences matched with those of *H. lutea* and *H. melanomagnum*. Chaverri and Samuels [10] noted that the anamorph of *H. melanomagnum* (*Trichoderma melanomagnum*) is almost indistinguishable from the anamorph of *H. lutea* (*Gliocladium viride*). In their description of *H. melanomagnum/Trichoderma melanomagnum*, no chlamydospore was observed and colonies grown on PDA showed concentric rings with regular intervals. But in this study, DKU0002 produced chlamydospore-like structures and did not produce concentric rings on PDA. Thus, we could differentiate DKU002 from *H. melanomagnum/Trichoderma melanomagnum*. Our morphological and molecular data conclusively suggest that the green mold species from shiitake log beds is *G. viride*.

Overall, we identified the anamorph of *H. lutea* and described its morphological characteristics and its molecular and physiological properties. This is the first description of *Gliocladium viride* in Korea. This fungus is usually abundant in soils. Recently, it has been isolated from mushroom cultivation facilities in Japan (GenBank accession number AB298706). However, the significance of this fungus in mushroom cultivation has not yet been identified. Thus, further properties of this species need to

be explored, especially in Korea, to determine whether it plays a role in the mushroom fly associated deterioration of shiitake oak log beds.

Acknowledgements

This study was carried out with the support of Forest Science & Technology Projects (Project No. S120909L050000) provided by the Korea Forest Service.

References

1. Park MS, Seo GS, Lee KH, Bae KS, Yu SH. Morphological and cultural characteristics of *Trichoderma* spp. associated with green mold of oyster mushroom in Korea. *Plant Pathol J* 2005;21:221-8.
2. Savoie JM, Mata G. *Trichoderma harziaum* metabolites pre-adapt mushrooms to *Trichoderma aggressivum* antagonism. *Mycologia* 2003;95:191-9.
3. Kim SH, Uzunovic A, Breuil C. Rapid detection of *Ophiostoma piceae* and *O. quercus* in stained wood by PCR. *Appl Environ Microbiol* 1999;65:287-90.
4. White TJ, Bruns T, Lee S, Taylor J. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis MA, Gelfand DH, Sninsky JJ, White TJ, editors. *PCR protocols: a guide to methods and applications*. San Diego: Academic Press; 1990. p. 315-22.
5. Evidente A, Ricciardiello G, Andolfi A, Sabatini MA, Ganassi S, Altomare C, et al. Citranifidiene and citranifidiol: bioactive metabolites produced by *Trichoderma citrinoviride* with potential antifeedant activity toward aphids. *J Agric Food Chem* 2008;56:3569-73.
6. Swofford DL. PAUP*: phylogenetic analysis using parsimony (*and other methods). Version 4.0 b10. Sunderland: Sinauer Associates; 2002.
7. Kimura M. A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. *J Mol Evol* 1980;16:111-20.
8. Raper KB, Thom C. A manual of the Penicillia. Baltimore: Williams & Wilkins Co.; 1949. p. 674-87.
9. Matruchot L. Sur un *Gliocladium* nouveau. *Bulletin de la Société Mycologique de France* 1893;9:249-52.
10. Chaverri P, Samuels GJ. *Hypocrea/Trichoderma* (Ascomycota, Hypocreales, Hypocreaceae): species with green ascospores. *Stud Mycol* 2003;48:1-116.