Plant Pathol. J. 28(2) : 137-148 (2012)
http://dx.doi.org/10.5423/PPJ.OA.02.2011.0030
pISSN 1598-2254 eISSN 2093-9280

The Plant Pathology Journal

Identification of *Trichoderma*, a Competitor of Shiitake Mushroom (*Lentinula edodes*), and Competition between *Lentinula edodes* and *Trichoderma* species in Korea

Chang Sun Kim[1,2], Myung Soo Park[3], Seon Cheol Kim[4], Nitaro Maekawa[5] and Seung Hun Yu[1]*

[1]*Laboratory of Plant Pathology, College of Agriculture and Life Sciences, Chungnam National University, Daejeon 305-764, Korea*
[2]*The Course of Bioenvironment Science, The United Graduate School of Agricultural Sciences, Tottori University, Tottori 680-8553, Japan*
[3]*Bio-Control Research Team, Chemical Biotechnology Research Center, Korea Research Institute of Chemical Technology, Daejeon 305-600, Korea*
[4]*National Forestry Cooperatives Federation, Forest Mushroom Research Institute, Yeoju 469-803, Korea*
[5]*Fungus/Mushroom Resource and Research Center, Tottori University, Tottori 680-8553, Japan*

(Received on February 7, 2011; Revised on February 1, 2012; Accepted on February 15, 2012)

During investigating of shiitake mushroom competitors, 289 isolates of *Trichoderma* spp. were collected from shiitake mushroom farms in different districts and the Forest Mushroom Research Center of Korea, among which 29 representative strains were selected. Based on the DNA sequences of the *rpb2* and *tef1* genes and the ITS rDNA, and their morphological characteristics, they were identified as *T. atroviride*, *T. citrinoviride*, *T. harzianum*, *T. longibrachiatum*, and two undescribed species, *Trichoderma* spp. 1 and 2, which are considered to be the candidate of new species. Competition tests between *Lentinula edodes* (Sanjo302) and the *Trichoderma* species indicated that the six species of *Trichoderma* were significantly different from each other in terms of their ability to invade the mycelial blocks of shiitake. In both of dual cultures on potato dextrose agar and sawdust media, *Trichoderma* spp. 1 and 2 strongly invaded the mycelial blocks of shiitake. Our results suggest that the two *Trichoderma* species may cause potentially serious economic losses in shiitake cultivation of Korea.

Keywords **:** competition test, ITS, phylogenetic analysis, *rpb2*, *tef1*

Shiitake mushrooms (*Lentinula edodes*) are widely cultivated as a food source in East Asia and are dried and exported to many countries because of its special flavor and aroma (Chen, 2005; Luo, 2004). Recent research has indicated that the shiitake mushroom also has useful clinical effects, including an immunostimulant (Yamamoto et al., 1997). In Korea, the consumption of shiitake mushrooms is increasing annually since 1999, and there are now about 20 cultivars of shiitake, which were promoted and disseminated by the Forest Mushroom Research Center.

The genus *Trichoderma* is one of the most important pathogens in the cultivation of the shiitake mushroom, and often causes severe damage during its production (Miyazaki et al., 2009). *Trichoderma* species mainly attack the mycelia of *L. edodes* in bed logs and sawdust cultures. The identification of *Trichoderma* at the species level has proved difficult because of their interspecific morphological similarities (Chaverri and Samuels, 2003). It led to the establishment of "aggregate" species concept by Rifai (1969) that all *Trichoderma* species could be distinguished to nine aggregates. Later, Bissett (1984, 1991a, b, c) established a new system of *Trichoderma* classification based on the branching pattern of conidiophores and the characteristics of phialides and conidia. After the introduction of molecular methods in *Trichoderma* taxonomy, the species concept of *Trichoderma* has changed dramatically (Chaverri and Samuels, 2003; Jaklitsch, 2009). In addition, the phylogenetic data helped to establish the relationship between anamorph *Trichoderma* and their related teleomorph *Hypocrea* (Chaverri and Samuels, 2003; Samuels et al., 1998, 2002).

In recent years, Park et al. (2005, 2006) identified seven distinct species of *Trichoderma* from that *Trichoderma* isolates from green mold of oyster mushroom were identified as seven distinct species (*T. pleuroticola*, *T. pleurotum*, *T. atroviride*, *T. citrinoviride*, *T. harzianum*, *T. longibrachiatum* and *T. virens*). However, little is known about the species of *Trichoderma* associated with the green mold observed on the shiitake mushrooms in Korea, and only five species of *Trichoderma* (*T. citrinoviride*, *T. harzianum*, *T. polysporum*, *T. longibrachiatum* and *T. viride*) were

*Corresponding author.
Phone) +82-42-821-5762, FAX) +82-42-823-8679
E-mail) shunyu@cnu.ac.kr

reported (Anonymous, 2009; Kim et al., 2012). In case of Japan, most of *Trichoderma* species identified by morphological methods or based on internal transcribed spacer (ITS) sequences (Hashioka, 1973; Miyazaki and Tsunoda, 2003; Miyazaki et al., 2009; Tokimoto and Komatsu, 1975; Tokimoto, 1985; Watanabe et al., 2005). The use of phylogenies based on single gene sequences is now generally discredited, especially the ITS regions of the ribosomal RNA genes, because some fungi have been shown to contain paralogous copies of these sequences (O'Donnell et al., 1998; Lieckfeldt and Seifert, 2000). Therefore, Taylor et al. (1999) proposed basing phylogenetic species concepts on a concordance multigene approach. In this study we used the sequences of three genes for the phylogenetic analysis: ITS, *rpb2* (RNA polymerase II), and *tef1* (translation elongation factor 1-α).

This study was conducted to identify these *Trichoderma* species as shiitake mushroom competitors, based on molecular and morphological characteristics. A competition test between *L. edodes* and *Trichoderma* species was also performed in the laboratory.

Materials and Methods

Strains and grouping. In total, 289 of *Trichoderma* isolates were collected from shiitake mushroom farms from different districts and the Forest Mushroom Research Center in Korea. *Trichoderma* isolates were separated into about 20 groups based on their culture characteristics on potato dextrose agar (PDA) for 10 days at 25°C, and then 29 representative strains were selected from the groups based on locality of Korea for analysis. The reference strains and isolates examined are listed in Table 1, with their NCBI (National Center for Biotechnology Information) GenBank accession numbers.

DNA isolation. The isolates were grown in shaken liquid culture in potato dextrose broth for 3–4 days at 25°C. The mycelia were collected from the cultures by filtration and then transferred to 1.5 ml tubes. These samples were frozen at –70°C. The DNA was extracted with the method of Cubero et al. (1999).

PCR amplification and sequencing. For the amplification of ITS, *rpb2*, and *tef1* gene, three different primer sets were used; ITS5 and ITS4 (White et al., 1990), fRPB2-5F and fRPB2-7cR (Liu et al., 1999), and EF1-728F (Carbone and Kohn, 1999) and tef1-rev (Samuels et al., 2002), respectively. The PCR mixtures contained 0.5 pmol of each primer, 0.25 mM dNTPs, 10 mM Tris-HCl, 50 mM KCl, 1.5 mM $MgCl_2$, 2.5 U of *Taq* DNA polymerase, and 15 ng of template DNA. The PCR cycling conditions for ITS and *tef1* gene were as follows: an initial denaturation step at 94°C for 10 min, followed by 30 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 60 s; with a final elongation step at 72°C for 10 min. For *rpb2* gene, the number of cycles and annealing temperature were respectively modified to 40 cycles and to 50°C.

The PCR products were purified using a Wizard PCR Preps DNA Purification System (Promega, Madison, WI, USA). The purified double-stranded PCR fragments were directly sequenced with the BigDye Terminator Cycle Sequencing Kit (Applied Biosystems, Forster City, CA, USA), according to the manufacturer's instructions. The same primer sets used for PCR amplification were used to sequence both DNA strands. In the case of *rpb2* gene, two additional internal primers, RPB-432F and RPB-450R (Degenkolb et al., 2008), were used for the sequencing reactions. Gel electrophoresis and data collection were performed on an ABI Prism 310 Genetic Analyzer (Applied Biosystems).

Phylogenetic analysis. To determine the phylogenetic positions of the *Trichoderma* isolates, sequence alignments of the three gene fragments from 69 isolates of *Trichoderma* species, including 29 Korean isolates and 40 reference strains of sequences retrieved from GenBank, were analyzed with Neighbor-Joining and Bayesian inference (Table 1). The sequences were proofread, edited, and merged into comparable sequences using the PHYDIT program version 3.2 (Chun, 1995; available at http://plaza,sun,ac.kr/~jchun/phydit). The DNA sequences were aligned with Clustal X 1.81 (Thompson et al., 1997), and then visually corrected with a text editor. Ambiguously aligned regions were excluded from subsequent analyses. NJ tree were constructed using PAUP version 4.0b10 (Swofford, 2003) using the Kimura 2-parameter model. Bootstrap support (BS) values for nodes were computed from 1000 replicates for NJ analyze (Jeon et al., 2010). The general time-reversible model, under the assumption of a discrete gamma-shaped rate variation with a proportion of invariable sites (GTR + I + G), was estimated as the best-fit likelihood model for the combined-sequences dataset. Bayesian analyses were conducted with MrBayes 3.1.2 (Ronquist and Huelsenbeck, 2003). Posterior probabilities (PP) were approximated with the metropolis-coupled Markov chain Monte Carlo method. Two parallel runs were conducted with one cold and three heated chains for 5 million generations, starting with a random tree. The three chains were heated at 0.2 for the combined-sequences dataset (the heats for cold chain 1 and heated chains 2, 3, and 4 were 1.00, 0.83, 0.71, and 0.63, respectively). The trees were saved to a file every 100th generation. We deemed that the two runs had reached convergence when the average standard

Table 1. Information of *Trichoderma* isolates analyzed in this study

Species	Locality	Substrate	Strain No.	GenBank accession numbers		
				ITS	*rpb2*	*tef1*
T. aggressivum	–	–	DAOM 100525	AF057600	AF545541	AF348095
T. arundinaceum	–	–	CBS 119575 T	AY154921	EU338303	EU338275
T. atroviride	–	–	CBS 142.95	AF456917	EU341801	AF456891
	Yeoju, Gyeonggi	Bed log	CNU N006	HM769740	HM920159	HM920188
	Yeoju, Gyeonggi	Bed log	CNU N016	HM769741	HM920160	HM920189
	Gumi, Gyeongbuk	Bed log	CNU N020	HM769742	HM920161	HM920190
	Youngdong, Chungbuk	Bed log	CNU N022	HM769743	HM920162	HM920191
	Gumi, Gyeongbu k	Bed log	CNU N088	HM769744	HM920163	HM920192
	Cheongju, Chungbuk	Bed log	CNU N112	HM769745	HM920164	HM920193
	Yeoju, Gyeonggi	Bed log	CNU N121	HM769746	HM920165	HM920194
	Gongju, Chungnam	Bed log	CNU N192	HM769747	HM920166	HM920195
T. brevicompactum	–	–	CBS 109720 T	–	EU338317	EU338299
	–	–	CBS 121154	EU338330	–	–
T. citrinoviride	–	–	DAOM 139758	EU330960	EU338338	EU338334
	Jangsu, Jeonbuk	Bed log	CNU N066	HM769748	HM920167	HM920196
	Gangneung, Gangwon	Bed log	CNU N114	HM769749	HM920168	HM920197
	Goheung, Jeonnam	Bed log	CNU N152	HM769750	HM920169	HM920198
	Goheung, Jeonnam	Bed log	CNU N153	HM769751	HM920170	HM920199
	Yeoju, Gyeonggi	Sawdust	CNU N262	HM769752	HM920171	HM920200
T. erinaceus	–	–	DAOM 166121	DQ109534	EU248604	DQ109547
T. hamatum	–	–	DAOM 167057	Z48816	AF545548	AY750893
T. harzianum	–	–	IMI 393966	AF443915	AY391925	–
	–	–	CBS 226.95 T	–	–	AF348101
	Yecheon, Gyeongbuk	Bed log	CNU N034	HM769730	HM920149	HM920178
	Yeoju, Gyeonggi	Bed log	CNU N072	HM769731	HM920150	HM920179
	Gongju, Chungnam	Bed log	CNU N143	HM769732	HM920151	HM920180
	Yeoju, Gyeonggi	Bed log	CNU N169	HM769733	HM920152	HM920181
	Hwacheon, Gangwon	Bed log	CNU N171	HM769734	HM920153	HM920182
	Yecheon, Gyeongbuk	Bed log	CNU N180	HM769735	HM920154	HM920183
	Gumi, Gyeongbuk	Bed log	CNU N185	HM769736	HM920155	HM920184
	Gongju, Chungnam	Bed log	CNU N202	HM769737	HM920156	HM920185
	Yeoju, Gyeonggi	Bed log	CNU N300	HM769738	HM920157	HM920186
	Yeoju, Gyeonggi	Sawdust	CNU N305	HM769739	HM920158	HM920187
T. koningii	–	–	CBS 988.97	DQ323409	EU248600	DQ289007
T. longibrachiatum	–	–	DAOM 166989	EU330961	EU338339	EU338335
	Yecheon, Gyeongbuk	Bed log	CNU N086	HM769753	HM920172	HM920201
T. saturnisporum	–	–	ATCC 28023	X93977	–	–
	–	–	CBS 330.70	–	DQ087243	–
	–	–	IMI 146852	–	–	AY865642
T. scalesiae	–	–	G.J.S. 03–74	DQ841742	EU252007	DQ841726
T. strigosum	–	–	CBS 348.93 T	AF487657	–	–
	–	–	DAOM 166121	–	AF545556	AF487668
T. tomentosum	–	–	DAOM178713A T	DQ085432	AF545557	AY750882
T. virens	–	–	G.J.S. 00–108	DQ083023	–	–
	–	–	G.J.S. 01–287	–	EU341804	AY750894
T. viride	–	–	CBS 101526	X93979	EU248599	AY376053
T. viridescens	–	–	CBS 333.72	DQ315441	EU341802	DQ307523

Table 1. Continued

Species	Locality	Substrate	Strain No.	GenBank accession numbers		
				ITS	*rpb2*	*tef1*
Trichoderma sp. 1	Yeoju, Gyeonggi	Sawdust	CNU N309	HM769754	HM920173	HM920202
	Wanju, Jeonbuk	Sawdust	CNU N349	HM769755	HM920174	HM920203
	Yeoju, Gyeonggi	Sawdust	CNU N417B	HM769756	HM920175	HM920204
Trichoderma sp. 2	Jangsu, Jeonbuk	Bed log	CNU N109	HM769757	HM920176	HM920205
	Yeoju, Gyeonggi	Bed log	CNU N334	HM769758	HM920177	HM920206
Hypocrea candida	–	–	CBS 114249 T	AY737757	AY391899	AY737742
H. catoptoron	–	–	CBS 114232	AY737766	AY391900	AY737726
H. ceracea	–	–	CBS 114245 T	EU330953	AF545508	AY937437
H. ceramic	–	–	CBS 114576 T	AY737764	AF545510	AY737738
H. chlorospora	–	–	G.J.S. 98–1	AY737762	AY391906	AY737737
H. cinnamomea	–	–	G.J.S. 97–237	AY737759	AY391920	AY737732
H. cremea	–	–	CBS 111146	AY737760	AF545511	AY737736
H. cuneispora	–	–	CBS 111148	AY737763	AF545512	AY737727
H. decipiens.	–	–	G.J.S. 97–207	EF558548	–	EF550995
	–	–	G.J.S. 91–101	–	DQ835520	–
H. dingleyae	–	–	CBS 119053	DQ313151	EU341803	AF348117
H. intricate	–	–	G.J.S. 02–78	EU264002	EU241505	EU248630
H. jecorina	–	–	TUB F-833	AY857227	–	–
	–	–	TUB F-430	–	DQ087241	–
	–	–	ATCC 24449	–	–	DQ025754
H. melanomagna	–	–	CBS 114236	AY737770	AY391026	AY737751
H. nigrovirens	–	–	CBS 114330	AY737777	AF545518	AY737744
H. phyllostachydis	–	–	CBS 114071	EU330959	AF545513	AY737745
H. rodmanii	–	–	G.J.S. 89–120	EU330947	EU338323	EU338285
H. cf. *rufa VE*	–	–	IMI 352471	EQ315449	EU341808	DQ307530
H. semiorbis	–	–	DAOM 167636	AY737758	AF545522	AY737750
H. stilbohypoxyli	–	–	CBS 112888	AY380915	EU341805	AY376062
H. straminea	–	–	CBS 114248 T	AY737765	AY391945	AY737746
H. thailandia	–	–	CBS 114234	AY737772	AY391957	AY737748
H. victoriensis	–	–	G.J.S. 99–130	EU330952	EU338336	EU338331
*Nectria cinnabarina**	–	–	CBS 279.48	AF163025	–	–
	–	–	G.J.S. 91–111	–	AF545567	–
	–	–	G.J.S. 89–107	–	–	AF543785

*, out group; T, ex-type strain

deviation of the split frequencies dropped below 0.01. The trees obtained before convergence was reached were discarded with the burn-in command, and the remaining trees were used to calculate a 50% majority consensus topology and PP. PP values below 0.95 were not considered significant. Model parameter summaries after MCMC run and burning first samples were collected. For combined sequences set mean substitution rates were estimated as A↔C = 0.09, A↔G = 0.34, A↔T = 0.10, C↔G = 0.04, C↔T = 0.38, G↔T = 0.06; nucleotide frequencies were estimated as 0.22(A), 0.29(C), 0.24(G), 0.25(T); alpha parameter of gamma distribution shape was 0.65.

Analysis of phenotypes. To check the cultural characteristics of the *Trichoderma* isolates, we selected eight representative of *Trichoderma* species (*T. harzianum* CNU N169; *T. atroviride* CNU N088 and CNU N112; *T. citrinoviride* CNU N152 and CNU N153; *T. longibrachiatum* CNU N086; *Trichoderma* sp. 1 CNU N417B; and *Trichoderma* sp. 2 CNU N109), based on phylogenetic groupings (Fig. 1). The cultures used for the study of anamorph micromorphology were grown on cornmeal agar + 2% dextrose (CMD; Difco), synthetic low-nutrient agar (SNA; Nirenberg, 1976), or PDA (Difco) at 20°C or 25°C for 7–20 days. All measurements for the morphological analyses were made

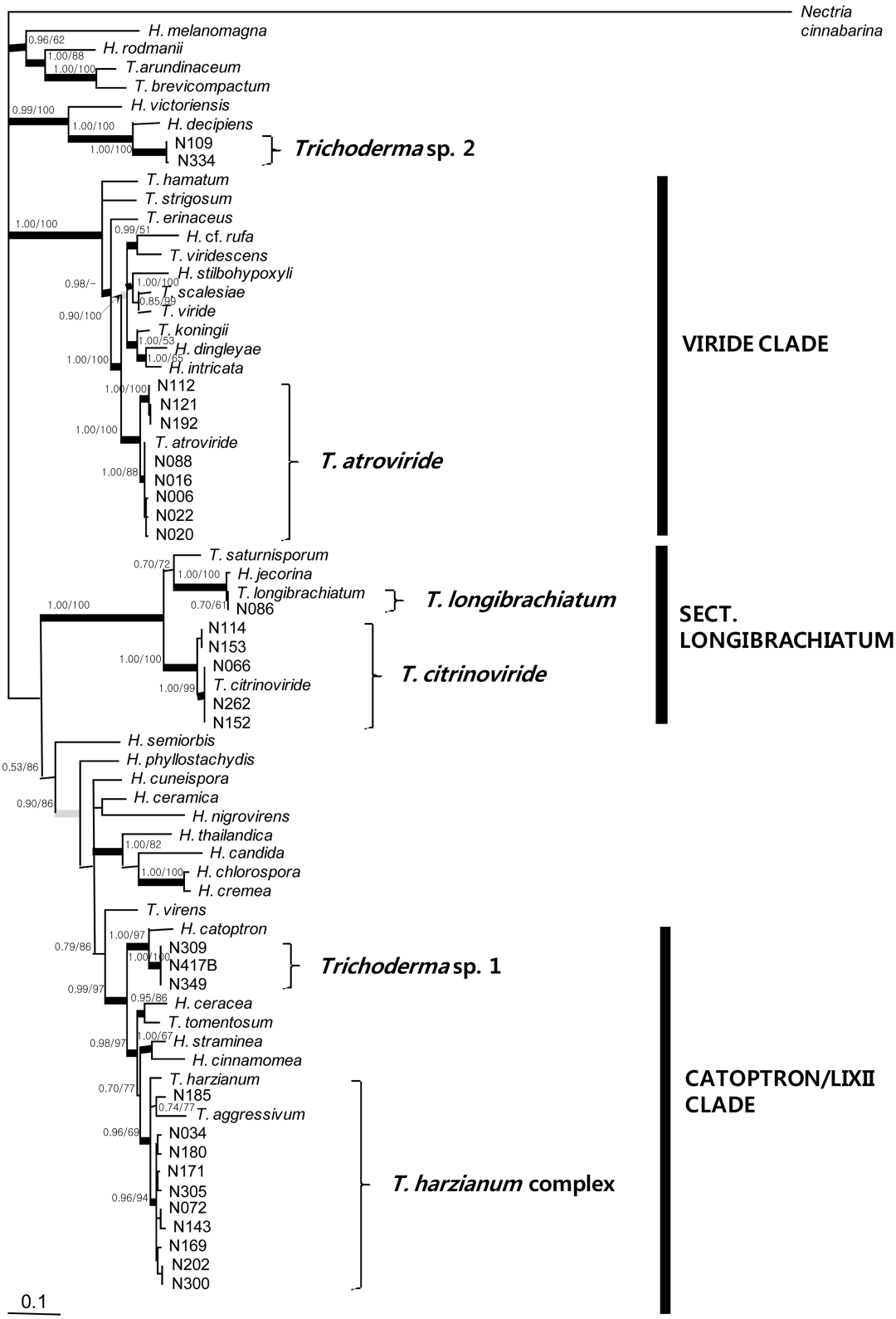

Fig. 1. Bayesian 50% majority rule consensus topology based on the combined sequence set (ITS, *rpb2*, and *tef1* genes). Bayesian posterior probabilities (PP), and bootstrap of 1000 replicates in NJ analysis are indicated as PP/NJBS above or below branches or at nodes. Only NJBS values > 50% are shown. Branching points supported by posterior probabilities > 0.94 are indicated by thick lines, and those supported by > 0.89 but < 0.95 by gray lines. *Nectria cinnabarina* was used as the outgroup.

in 3% KOH or water. Where possible, 50 units of each morphological parameter were measured for each collection. Growth rates were determined on PDA and SNA. After a few days, when the colony growth was visible on PDA and SNA, but before conidia production, a plug (5 mm diameter) was taken from the actively growing edge of the colony and inoculated onto freshly prepared medium. The inoculum plug was placed mycelia-side-down, approximately 1 cm from the edge of a vented Petri dishes (9 cm diameter) containing 20 ml of freshly made medium. The Petri dishes were incubated in the dark at 15–45°C (in increments of 5°C). They were examined at 24 h intervals when the colony radius, measured from the edge of the inoculum plug, and the colony appearance were recorded. Each growth trial consisted of a single Petri dish for each strain at each temperature. The growth trials were repeated three times at roughly weekly intervals, and the average radius was calculated from the three independent measurements.

Competition test. To assess the competition between the *Lentinula edodes* and *Trichoderma* species, we selected a shiitake mushroom race (Sanjo302) and six representative *Trichoderma* species (*T. harzianum* CNU N169, *T. atroviride* CNU N088, *T. citrinoviride* CNU N152, *T. longibrachiatum* CNU N086, *Trichoderma* sp. 1 CNU N417B, and *Trichoderma* sp. 2 CNU N109). On PDA, the shiitake inoculums were prepared 10 days before *Trichoderma* inoculation, and their appearance after invasion was observed 3, 10, and 15 days after *Trichoderma* species inoculation. To measure the growth rates and competition between the shiitake and *Trichoderma* species on sawdust medium, we modified the dual-culture method of Tokimoto et al. (1994). Glass tubes (30 mm in diameter and 200 mm in length) were filled with the sawdust medium (oak sawdust and rice bran cakes in a ratio of 4:1; moisture content = 60%), leaving 30 mm empty spaces at both ends. The ends of the glass tubes were capped with air-permeable silicon plugs. A 5 mm diameter disk of shiitake mycelia on PDA medium was inoculated at one end of the tube and allowed to grow at 25°C in the dark. Mycelial elongation (mm) was measured from day 14 to day 21. The other end of the tube was inoculated with a piece of *Trichoderma* species on day 21. After incubation at 25°C for seven days, the extent of *Trichoderma* mycelial invasion into the mycelial block of shiitake was measured.

Results

Phylogenetic analysis. In the Bayesian inference a set of four chains reached convergence after about 3,000,000 generations, and therefore the first 30,000 trees in each parallel run were discarded by setting the burnin command to 30,000; the remaining ca. 40,002 trees (representing ca. 2,000,000 generations) were used to calculate a 50% majority consensus tree and to determine PP. The likelihoods (ln L) of the best states for cold chains of the two runs were −11710.30 and −11731.14 respectively.

The combined sequences (1366 total characters: 599 of ITS; 311 of *rpb2*; 456 of *tef1*) (Fig. 1) show the clear separation of these isolates with strong statistical support. The *Trichoderma* isolates were identified as *T. harzianum*, *T. atroviride*, *T. citrinoviride*, *T. longibrachiatum*, and two unidentified species, each of which were supported by high PP and BS values above 0.95 and 60%. The isolated strains belong to section Longibrachiatum (*T. longibrachiatum* and *T. citrinoviride*), the Viride clade (*T. atroviride*), and the Catoptron/Lixii clade (*T. harzianum* and *Trichoderma* sp. 1), respectively. *Trichoderma* spp. 1 and 2 are closely related to *Hypocrea catoptron* and *H. decipiens,* respectively, but clearly differentiated from previously reported species of *Trichoderma* (Fig. 1).

Phenotypic analysis. The optimal temperature for the growth of all species on PDA or SNA was between 25°C and 30°C. However, the mycelial growth rate can be useful in distinguishing *Trichoderma* species. Samuels et al. (2006) applied the system for growth rate determination to as many isolates of each species as possible using subtle differences in growth rate on PDA and SNA to aid in species delimitation. Fig. 2 shows growth rates of six species of *Trichoderma* on PDA and SNA media, given as mm colony radius after 72 h at seven temperatures. We found that the mycelial growth rate of *Trichoderma* sp. 1 was slow and its optimal temperature range was narrow compared with those of the other *Trichoderma* species. The optimal temperature range for *Trichoderma* sp. 2 was also narrow, but this species grew quickly at the optimal temperatures. The growth rate patterns of *T. citrinoviride* and *T. longibrachiatum*, belonging to section Longibrachiatum, were almost identical to each other, with a wide range at optimal temperatures (Fig. 2).

Differences in micromorphological characteristics of six species were described in Table 2 - *Trichoderma* spp. 1 and 2 compared with related species in phylogenetic tree, respectively. The morphological characteristics of four identified *Trichoderma* species (*T. atroviride*, *T. citrinoviride*, *T. harzianum* and *T. longibrachiatum*) were almost the same as those previously reported species by Park et al. (2005) (data not shown).

Optimum temperature of *Trichoderma* sp. 1 for growth on SNA and PDA ca. 20–25°C. Colony radius on SNA after 3d at 15°C 5.5–6.0 mm, 20°C 20.1–21.8 mm, 25°C 21.1–22.6 mm, 30°C 3.0–3.4 mm and 35–45°C 0.0 mm

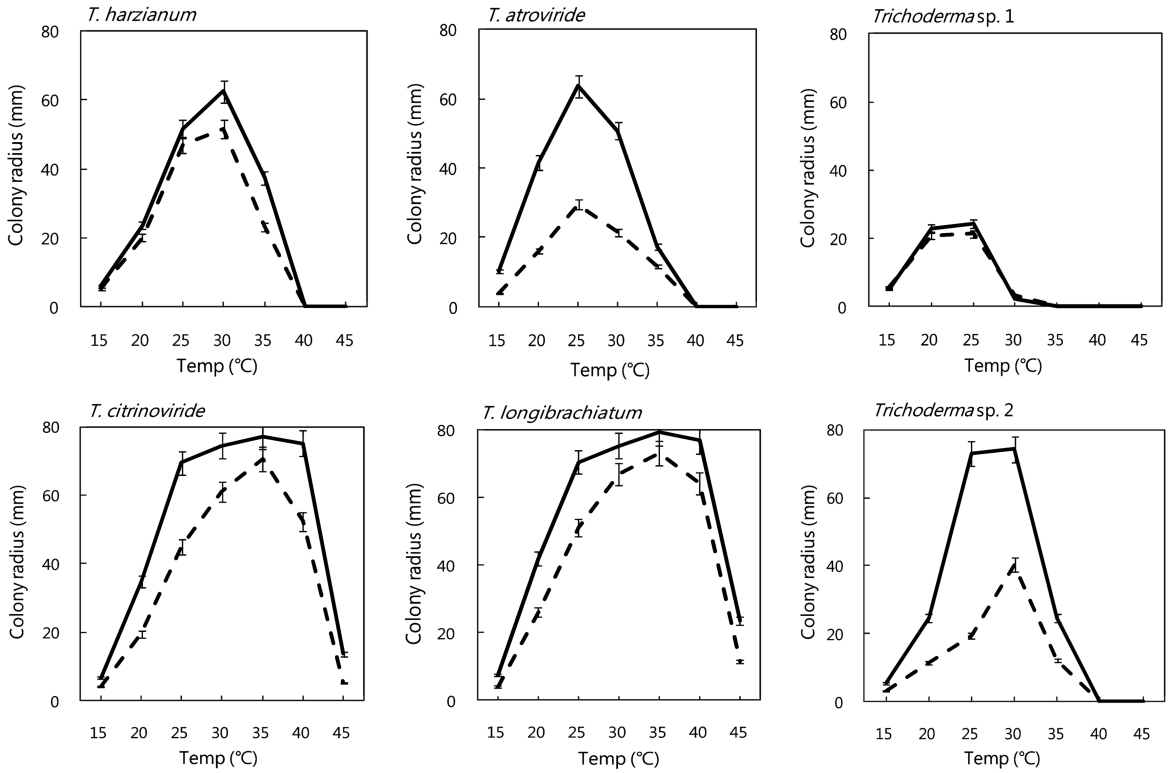

Fig. 2. Colony radii of *Trichoderma* species after 3 days on PDA or SNA at 15, 20, 25, 30, 35, 40, and 45 ℃. Solid line = PDA; dashed line = SNA. Bars indicate the standard errors of the means of three replicate cultures.

Table 2. Morphological characteristics of six *Trichoderma* species and related species

Species	Character				
	Conidiophore type	Phialides shape (size: μm)	Conidia shape (size: μm)	Coconut-like odor	Formation of chlamydospores
T. atroviride	trichoderma-like	straight or sinuous, sometimes hooked (5.8–14.3 × 2.4–4.5)	subglobose to ovoidal (2.5–3.8 × 2.1–3.3)	+	+
T. citrinoviride	trichoderma- to pachybasium-like	cylindrical or slightly enlarged (3.2–6.8 × 2.1–3.3)	oblong to ellipsoidal (2.1–3.5 × 1.4–2.1)	–	+
T. harzianum	pachybasium-like	flask-shaped (3.3–7.2 × 2.3–3.7)	subglobose to ovoidal (2.4–3.6 × 1.8–2.8)	–	+
T. longibrachiatum	trichoderma-like	cylindrical (4.9–12.2 × 1.9–3.4)	oblong to ellipsoidal (3.4–5.9 × 2.3–3.4)	–	+
Trichoderma sp. 1	verticillium- to gliocladium-like	ampulliform or lageniform (4.0–12.9 × 1.3–3.5)	ellipsoidal to rare reniform (2.3–5.2 × 1.8–3.0)	–	+
Hypocrea catoptron/ *T. catoptron**	pachybasium- to verticillium-like	ampulliform (5.5–7.2 × 3.2–4.2)	ellipsoidal to oblong (3.5–4.0 × 2.3–2.7)	–	–
*H. straminea**	pachybasium- to verticillium-like	ampulliform (2.7–5.0 × 3.0–5.0)	broad ellipsoidal, sometimes oblong (3.0–3.2 × 2.0–2.2)	–	–
Trichoderma sp. 2	NA	NA	NA	–	+
*H. decipiens***	irregularly verticillate	subulate (13–25 × 2.4–3.0)	subglobose or obovate to subellipsoidal (4.4–6.7 × 2.5–3.5)	ND	ND

+, detected; –, not detected; NA, not available due to lack of conidia; ND, not described
*, Chaverri and Samuels, 2003
**, Overton et al., 2006 (*H. decipiens* for the species interpreted as *H. farinosa*)

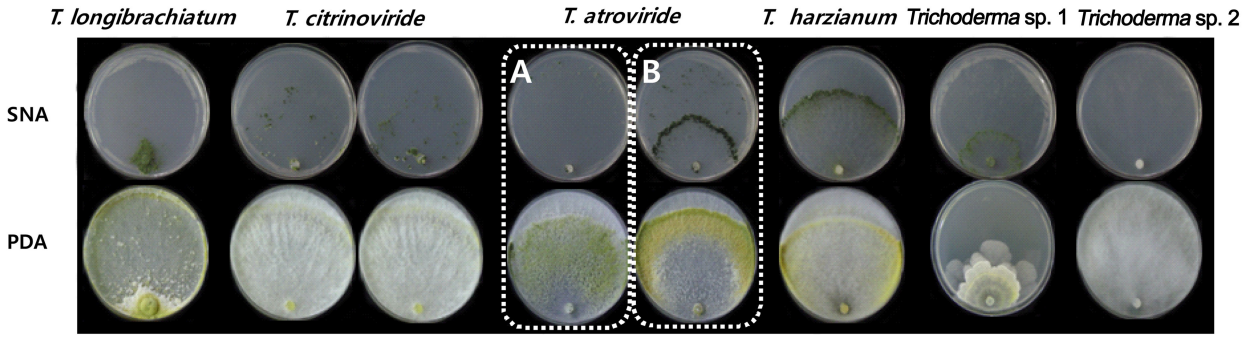

Fig. 3. Culture of *Trichoderma* species on SNA and PDA media for 10 days at 25 °C. A, *T. atroviride* strain CNU N112; B, *T. atroviride* strain CNU N088.

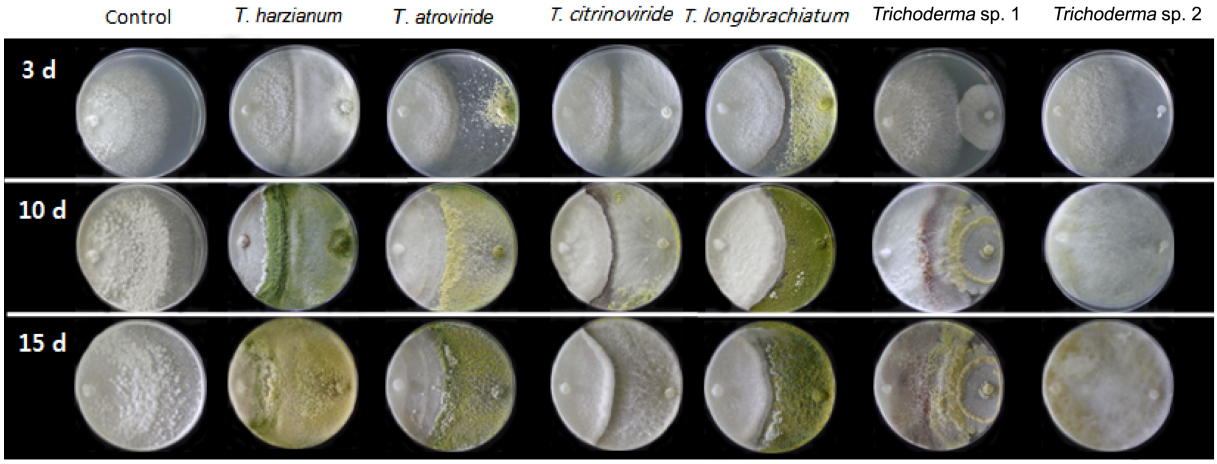

Fig. 4. Dual cultures of *Lentinula edodes* (Sanjo 302) and *Trichoderma* species on PDA at 25 °C. The shiitake mycelium inoculums were prepared 10 days before the *Trichoderma* inoculation and their invaded appearance was observed after 3, 10, and 15 days. Cultures of *L. edodes* were controls on left side (on PDA at 25 °C).

(n=3). Colony radius on PDA after 3d at 15 °C 4.9–5.5 mm, 20 °C 16.7–20.1 mm, 25 °C 17.3–25.2 mm, 30 °C 2.1–3.0 mm and 35–45 °C 0.0 mm (n=3) (Fig. 2). On SNA at 25 °C for 10d, colony formed a curcular band of light green and pustules. On PDA at 25 °C for 10 d, mycelia growth pattern were irregular appearance (Fig. 3). No odor detected. Conidia ellipsoidal to rare reniform, smooth, 2.3–5.2 × 1.8–3.0 μm, L/W ratio of 1.0–2.4. Chlamydospores formed. Conidiophores type veticillium- to gliocladium-like, and phialides shape ampulliform or lageniform, 4.0–12.9 × 1.2–3.5 μm, L/W ratio of 1.8–5.2.

Optimum temperature of *Trichoderma* sp. 2 for growth on SNA ca. 30 °C and on PDA 25–30 °C. Colony radius on SNA after 3d at 15 °C 2.1–5.7 mm, 20 °C 8.4–14.5 mm, 25 °C 15.2–23.7 mm, 30 °C 36.4–53.1 mm, 35 °C 11.3–13.0 and 40–45 °C 0.0 mm (n=3). Colony radius on PDA after 3d at 15 °C 5.2–5.7 mm, 20 °C 22.6–26.4 mm, 25 °C 72.7–73.6 mm, 30 °C 73.7–75.8 mm, 35 °C 18.8–30.2 and 40–45 °C 0.0 mm (n=3) (Fig. 2). On SNA and PDA at 25 °C for 10d, did not produce spore, but only white mycelial growth (Fig. 3). No odor detected.

Competition between *Lentinula edodes* and *Trichoderma* species. The competition test between the *Lentinula edodes* (Sanjo 302) and *Trichoderma* species indicated that the six species of *Trichoderma* were significantly different from each other in terms of their ability to invade the mycelial blocks of shiitake on PDA (Fig. 4). Of the *Trichoderma* species, *Trichoderma* spp. 1 and 2 and *T. harzianum* had strongly invaded the mycelial blocks of shiitake 15 days after *Trichoderma* inoculation. In contrast, *T. atroviride* had only moderately invaded the mycelial blocks of shiitake, and *T. citrinoviride* and *T. longibrachiatum* had only slightly invaded them or not at all.

The growth rate of *Trichoderma* species and *L. edodes* were investigated on sawdust medium. The growth of the *Trichoderma* species was approximately 3–5 times faster than that of the shiitake mycelia (Fig. 5). Among the *Trichoderma* species, the growth rate of *Trichoderma* sp. 2 was faster than those of the other species, whereas that of

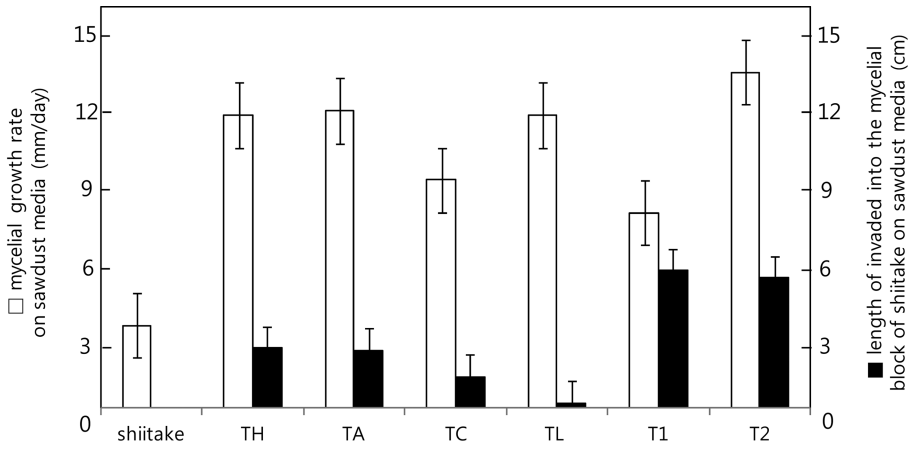

Fig. 5. Growth rate of *Lentinula edodes* (Sanjo 302) and *Trichoderma* species at 25°C on sawdust medium (left). Distance of *Trichoderma* species invasion into the mycelial block of shiitake, seven days after *Trichoderma* species inoculation in dual culture in sawdust medium at 25°C (right). TH, *T. harzianum*; TA, *T. atroviride*; TC, *T. citrinoviride*; TL, *T. longibrachiatum*; T1, *Trichoderma* sp. 1; T2, *Trichoderma* sp. 2. Bars indicate the standard errors of the means of three replicates.

Trichoderma sp. 1 was slowest. In the competition test on sawdust medium, *Trichoderma* spp. 1 and 2 strongly invaded the mycelial blocks of shiitake, and *T. harzianum* and *T. atroviride* moderately invaded them. However, *T. citrinoviride* and *T. longibrachiatum* invaded the mycelial blocks of shiitake only slightly or not at all (Fig. 5).

Discussion

Six species of *Trichoderma* were clearly distinguished in the present phylogenetic and phenotypic analyses. Nine *Trichoderma* species that cause economic losses during shiitake cultivation have previously been reported in Japan [*T. atroviride*, *T. citrinoviride*, *T. harzianum*, *T. longibrachiatum*, *T. pseudokoningii*, *T. polysporum*, *T.* cf. *stramineum*, *T. virens* and *Trichoderma* sp. (Miyazaki et al., 2009)]. However, we found only six species: *T. harzianum*, *T. atroviride*, *T. citrinoviride*, *T. longibrachiatum*, and two unidentified species. We speculate that still many additional species will be recognized in Korea.

Recently, Kim et al. (2010) reported *Gliocladium viride* (anamorph of *Hypocrea lutea*), isolated from bedlogs of shiitake mushroom. This species damaged to bedlogs of shiitake mushroom and were infested by mushroom flies. Although this species was morphologically similar to true *Gliocladium*, its phylogenetic position located in *Hypocrea*/*Trichoderma*, and teleomoph characteristics of this species is an undoubted species of *Hypocrea* (Samueals, 2006). For that reasons, the scientific name of this species was changed to *Trichoderma deliquescens* by Jaklitsch (2011).

Trichoderma sp. 1 is phylogenetically related to *Hypocrea catoptron*, but the two species could be easily distinguished by the branching pattern of the conidiophores; in the former species it is veticillium- to gliocladium-like, but in latter is pachybasium-like. In comparison of ITS sequences, this species was identical to a sequence of *T.* cf. *stramineum* (GenBank Accession No. AB298692), but we could find no morphological description of the latter isolate. However, the ex-type of *H. straminea* CBS 114248 (anamorph: *T. stramineum*) and *Trichoderma* sp. 1 are distantly related in phylogenetic tree (see Fig. 1). Morphologically, *H. straminea* CBS114248 (ex-type) is also clearly different from *Trichoderma* sp. 1 by forming pachybasium- and verticillium-like conidiophores, and by producing no chlamydospores on the medium, along with *H. catoptron* (Table 2). Therefore, *Trichoderma* sp. 1 remains an unidentified species.

The phylogenetic position of *Trichoderma* sp. 2 is close to *H. decipiens*, but they were clearly divided into two groups in combined tree (Fig. 1) and their individual gene trees also supported that (data not shown). Interestingly, the isolates of *Trichoderma* sp. 2 did not produce conidia on SNA, PDA, or CMD medium, and chlamydospores were only produced by an old colony on PDA medium. *Trichoderma* section Hypocreanum is characterized by verticillium- and acremonium-like anamorphs (Chaverri and Samuels, 2003). However, these anamorphs are difficult to find and sometimes they do not produce conidia on artificial medium. Previously, Kullnig-Gradinger et al. (2002) suggested that during its evolution, *Trichoderma* sect. Hypocreanum may have lost the ability to form *Trichoderma*-like anamorphs. For that reason, *Trichoderma* sp. 2 may be belongs to sect. Hypocreanum.

Phylogenetic analysis (Fig. 1) revealed the genetic variations within *T. harzianum*. It was well known from a report of Chaverri et al. (2003) that the *T. harzianum–H. lixii* species complex consists of several phylogenetic line-

ages. *Trichoderma citrinoviride* and *T. atroviride* are separated into two subgroups on the tree, but they share almost identical morphological characteristics (data not shown). Interestingly, we noted also genetic variation within *T. atroviride* (Fig. 1). In Fig. 1, *T. atroviride* was slightly divided into two groups: group A (N112, N121 and N192) and group B (N006, N016, N020, N022 and N088). These two groups could be distinguished on SNA medium. Group B formed a circular band of dark green with pustules, but group A did not form it (Fig. 3). This character allows the easy distinction of the two groups. Apart from this exception, their morphological characteristics are similar and they are phylogenetically closely related. Therefore, we consider that these groups belong to a species, with accumulating genetic variations.

Most *Trichoderma* species can secrete lytic enzymes (mainly 13-1,3-glucanases and chitinases) or antibiotics that inhibit the cell wall synthesis of other fungi (Chet, 1987; Claydon et al., 1987; Dennis and Webster, 1971a, b; Di Pietro et al., 1993; Howell, 1998; Schirmbock et al., 1994). For example, *T. atroviride* has a coconut-like odor attributed to 6-pentyl-a-pyrone (6PAP) that inhibits spore germination of plant pathogenic fungi and suppresses the mycelial growth of other fungi (Smith and Grula, 1982; Stefanova et al., 1999). In the present competition test, *Trichoderma* spp. 1 and 2 caused severe damage to the mycelia of the shiitake. These results suggest a possibility that they secrete powerful lytic enzymes or antibiotics. We hope to find new lytic enzymes or antibiotics in these species.

Conclusion

Of six species of *Trichoderma* associated with the green mold observed during shiitake mushroom cultivation, three species are newly reported as shiitake mushroom competitors in Korea: *T. atroviride* and two previously unknown species (*Trichoderma* spp. 1 and 2). Based on their molecular and morphological characteristics, we propose the two unidentified species as the candidate of new species. Given the result of competitive tests, *Trichoderma* spp. 1 and 2 could potentially cause large economic crop losses in shiitake mushroom cultivation. Therefore, more extensive research is required in the near future.

Acknowlegements

We are so grateful to reviewers and editors for their comments and proofreadings on the manuscript. This work was supported by a grant from Regional Subgenebank Support Program of Rural Development Administration, Republic of Korea.

References

Anonymous. 2009. List of Plant Diseases in Korea. 5th Edition. The Korean Society of Plant Pathologly, Seoul. 853 pp..

Bissett, J. 1984. A revision of the genus *Trichoderma*. I. Sect. *Longibrachiatum* sect. nov. *Can. J. Bot.* 62:924–931.

Bissett, J. 1991a. A revision of the genus *Trichoderma*. II. Infrageneric classificaition. *Can. J. Bot*. 69:2357–2372.

Bissett, J. 1991b. A revision of the genus *Trichoderma*. III. Sect. *Pachybasium*. *Can. J. Bot*. 69:2373–2417.

Bissett, J. 1991c. A revision of the genus *Trichoderma*. IV. Additional notes on section *Longibrachiatum*. *Can. J. Bot*. 69: 2418–2420.

Carbone, I. and Kohn, L. M. 1999. A method for designing primer sets for speciation studies in filamentous ascomycetes. *Mycologia* 91:553–556.

Chaverri, P. and Samuels, G. J. 2003. *Hypocrea*/*Trichoderma* (Ascomycota, Hypocreales, Hypocreaceae): species with green ascospores. *Stud. Mycol.* 48:1–113.

Chen, A. W. 2005. Chapter 1: What is shiitake. In: Mushroom Growers' Handbook 2. ed. by MushroomWorld. pp. 1–11. MushroomWorld, Seoul, Korea.

Chet, I. 1987. *Trichoderma* - application, mode of action and potential as a biocontrol agent of soil borne plant pathogenic fungi. In: Innovative Approaches to Plant Disease Control, ed. by I. Chet, pp.137–160. John Wiley and Sons, New York, USA.

Chun, J. 1995. Computer-assisted classification and identification of actinomycetes. Ph. D. thesis, University of Newcastle, Newcastle upon Tyne, UK.

Claydon, N., Allan, M., Hanso, J. R. and Avent, A. G. 1987. Antifungal alkyl pyrones of *Trichoderma harzianum*. *Trans. Br. Mycol. Soc.* 88:503–513.

Cubero, O. F., Crespo, A. N. A., Fatehi, F. and Bridge, P. D. 1999. DNA extraction and PCR amplification method suitable for fresh, herbarium-stored, lichenized, and other fungi. *Pl. Syst. Evol*. 216:243–249.

Degenkolb, T., Dieckmann, R., Nielsen, K. F., Gräfenhan, T., Theis, C., Zafari, D., Chaverri, P., Ismaiel, A., Brückner, H., Döhren, von H., Thrane, U., Petrini, O. and Samuels, G. J. 2008. The *Trichoderma brevicompactum* clade: a separate lineage with new species, new peptibiotics, and mycotoxins. *Mycol. Progr*. 7:177–219.

Dennis, C. and Webster, J. 1971a. Antagonistic properties of species groups of *Trichoderma*. I. Production of nonvolatile antibiotics. *Trans. Br. Mycol. Soc.* 57:25–39.

Dennis, C. and Webster, J. 1971b. Antagonistic properties of species groups of *Trichoderma*. II. Production of volatile antibiotics. *Trans. Br. Mycol. Soc*. 57:41–48.

Di Pietro, A., Lorito, M., Hayes, C., Broadway, K. and Harman, G. E. 1993. Endochitinase from *Gliocladium virens*. Isolation, characterization, synergistic antifungal activity in combination with gliotoxin. *Phytopathology* 83:308–313.

Hashioka, Y. 1973. Scanning electronmicroscopy on the mycoparasites, *Trichoderma*, *Gliocladium* and *Acremonium*. *Rept. Tottori Mycol. Inst.* 10:473–484.

Howell, C. R. 1998. The role of antibiosis in biocontrol. In: *Trichoderma & Gliocladium*, vol. 2. ed. by G.E. Harman and C. P. Kubicek, pp 173-184. Taylor & Francis, Padstow.

Jaklitsch, W. M. 2009. European species of *Hypocrea* Part I. The green-spored species. *Stud. Mycol.* 63:1−91.

Jaklitsch, W. M. 2011. European species of *Hypocrea* part II: species with hyaline ascospores. *Fungal Divers*. 48:1−250.

Jeon, Y.-A., Kim, W.-G., Kim, D.-H., Kwon, S.-W. and Hong, S. B. 2010. Taxonomic position of Korean isolates of *Rhizoctonia solani* based on RAPD and ITS sequencing of ribosomal DNA. *Plant Pathol. J.* 26:83−89.

Kim, J. Y., Yun, Y. H., Hyun, M. W., Kim, M. H. and Kim, S. H. 2010. Identification and characterization of *Gliocladium viride* isolated from mushroom fly infested oak log used for shiitake cultivation. *Mycobiology* 38:7−12.

Kim, J. Y., Kwon, H. W., Tang, L. and Kim, S. H. 2012. Identification and characterization of *Trichoderma citrinoviride* isolated from mushroom fly-infested oak log beds used for shiitake cultivation. *Plant Pathol. J.* 28:219.

Kullnig-Gradinger, C. M., Szakacx, G. and Kubicek, C. P. 2002. Phylogeny and evolution of the fungal genus *Trichoderma* – a multigene approach. *Mycol. Res.* 106:757−767.

Lieckfeldt, E. and Seifert, K. A. 2000. An evaluation of the use of ITS sequences in the taxonomy of the *Hypocreales. Stud. Mycol.* 45:35−44.

Liu, Y. J., Whelen, S. and Hall, B. D. 1999. Phylogenetic Relationships Among Ascomycetes: Evidence from an RNA polymerase II Subunit. *Mol. Biol. Evol*. 16:1799−1808.

Luo, X. C. 2004. Progress of xiang-gu (shiitake) cultivation in China. In: Mushroom Science XVI: Science and Cultivation of Edible and Medicinal Fungi. ed. by Romaine, Keil, Rinker and Royes, pp. 317−322. The Pennsylvania State University Press, USA.

Miyazaki, K. and Tsunoda, M. 2003. Application of DNA markers to research on *Trichoderma* in mushroom facilities of Japan (1): RAPD, SSCP marker. *Mushroom Sci. Biotechnol.* 11:65−70.

Miyazaki, K., Tsuchiya, Y. and Okuda, T. 2009. Specific PCR assays for the detection of *Trichoderma harzianum* causing green mold disease during mushroom cultivation. *Mycoscience* 50:94−99.

Nirenberg, H. I. 1976. Untersuchungen über die morphologische und biologische Differnzierung in der *Fusarium*-Sektion *Liseola*. *Mitteilungen aus der Biologischen Bundesanstalt für Land- und Forstwirtschaft Berlin- Dahlem* 169:1−117.

O'donnell, K., Cigelnik, E. and Nirenberg, H. I. 1998. Molecular systematic and phylogeography of the *Gibberella fujikuroi* species complex. *Mycologia* 90:465−493.

Overten, B. E., Stewart, E. L. and Geiser, D. M. 2006. Taxonomy and phylogenetic relationships of nine species of *Hypocrea* with anamorphs assignable to *Trichoderma* section *Hypocreanum*. *Stud. Mycol.* 56:39−65.

Park, M. S., Seo, G. S., Lee, K. H., Bae, K. S. and Yu, S. H. 2005. Morphological and cultural characteristics of *Trichoderma* spp. associated with green mold of oyster mushroom in Korea. *Plant Pathol. J.* 21:221−228.

Park, M. S., Bae, K. S. and Yu, S. H. 2006. Two new species of *Trichoderma* associated with green mold of oyster mushroom cultivation in Korea. *Mycobiology* 34:111−113.

Rifai, M. A. 1969. A revision of the genus *Trichoderma*. *Mycol. Pap*. 116:1−116.

Ronquist, F. and Huelsenbeck, J. P. 2003. MrBayes 3: Bayesian phylogenetic inference under mixed models. *Bioinformatics* 19:1572−1574.

Samuels, G. J., Petrini, O., Kuhls, K., Lieckfeldt, E. and Kubicek, C. P. 1998. The *Hypocrea schweinitzii* complex and *Trichoderma* sect. *Longibrachiatum*. *Stud. Mycol*. 41:1−54.

Samuels, G. J., Gams, W., Castlebury, L. A. and Petrini, O. 2002. *Trichoderma* species associated with the green mold epidemic of commercially grown *Agaricus bisporus*. *Mycologia* 94: 146−170.

Samuels, G. J. 2006. *Trichoderma*: Systematics, the sexual state, and ecolgy. *Phytopathology* 96:195−206.

Samuels, G. J., Dodd, S., Lu, B., Petrini, O., Schroers, H. and Druzhinina I. S. 2006. The *Trichoderma koningii* aggregate species. *Stud. Mycol.* 56:67−133.

Schirmbock, M., Lorito, M., Wang, Y. L., Hayes, C. K., Arisan-Atac, I., Scala, F., Harman, G. E. and Kubicek, C. P. 1994. Parallel formation and synergism of hydrolytic enzymes and peptaibol antibiotics, molecular mechanisms involved in the antagonistic action of *Trichoderma harzianum* against phytopathogenic fungi. *Appl. Environ. Microbiol.* 60:4364−4370.

Smith, R. J. and Grula, E. A. 1982. Toxic components on the larval surface of the corn earworm (*Heliothis zea*) and their effects on germination and growth of *Beauveria bassiana*. *J. Invertebr. Pathol.* 39:15−22.

Stefanova, M., Leiva, A., Larrinaga, L. and Coronado, M. F. 1999. Metabolic activity of *Trichoderma* spp. isolates for a control of soil-borne phytopathogenic fungi. *Revista de la Facultad de Agronomia*, *Universidad del Zulia* 16:509−516.

Swofford, D. L. 2003. PAUP*: phylogenetic analysis using parsimony (*and other methods). Version 4.0b10. Sunderland, Massachusetts: Sinauer Associates.

Taylor, J. W., Jacobson, D. J. and Fisher, M. 1999. The evolution of asexual fungi: speciation and classification. *Annu. Rev. Phytopathol*. 37:197−246.

Thompson, J. D., Gibson, T. J., Plewniak, F., Jeanmougin, F. and Higgins, D. G. 1997. The ClustalX windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Res*. 25:4876−4882.

Tokimoto, K. and Komatsu, M. 1975. Effect of carbon and nitrogen sources in media on the hyphal interference between *Lentinus edodes* and some species of *Trichoderma*. *Ann. Phytopathol. Soc. Jpn.* 45:261−264.

Tokimoto, K. 1985. Physiological studies on antagonism between *Lentinus edodes* and *Trichoderma* spp. in bedlogs of the former (in Japanese). *Rep. Tottori Mycol. Inst.* 23:1−54.

Tokimoto, K., Komatsu, M. and Fukumasa-nakai, Y. 1994. Establishing the selection method for the *Lentinula edodes* strains resistant to *Trichoderma* spp. *Proc. Japan Acad.* 70:112−116.

Watanabe, S., Kumakura, K., Kato, H., Iyozumi, H., Togawa, M. and Nagayama, K. 2005. Identification of *Trichoderma* SKT-

1, a biological control agent against seedborne pathogens of rice. *J. Gen. Plant Pathol.* 71:351−356.

White, T. J., Bruns, T. D., Lee, S. B. and Taylor, J. W. 1990. Amplification and direct sequencing of fungal ribosomal DNA for phylogenetics. In: PCR protocols: A guide to the methods and applications. ed. by M. A. Innes, D. H. Gelfand, J. J. Sninsky and T. J. White, pp. 315. Academic Press, New York, USA.

Yamamoto, Y., Shirono, H., Kono, K. and Ohashi, Y. 1997. Immunopotentiating activity of the water-soluble lignin rich fraction prepared from LEM--the extract of the solid culture medium of *Lentinus edodes* mycelia. *Biosci. Biotechnol. Biochem*. 61:1909–1912.