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Molecular and Morphological Characterization of Green Mold, *Trichoderma* spp. isolated from Oyster Mushrooms

In-Young Choi*, Seung-Beom Hong[1] and Mahesh C. Yadav[2]

Jeollabuk-do Agricultural Research and Extension Services, Iksan 570-704, Korea
[1]Korean Agricultural Culture Collection, National Institute of Agricultural Biotechnology, Suwon 441-707, Korea
[2]National Research Centre for Mushroom, Solan-173213, H.P., India

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Isolates of *Trichoderma* spp. collected from *Pleurotus ostreatus* and *P. eryngii* beds, which included loosened substrate compactness and development of green colour, were grouped into three species. The occurrence of different species of *Trichoderma* was as *T.* cf. *virens* (70.8%), *T. longibrachiatum* (16.7%) and *T. harzianum* (12.5%). The conidia of *Trichoderma* spp. were ellipsoidal, obovoid and phialides were bowling pins, lageniform and the length of phialides was 3.5~10.0 × 1.3~3.3 μm. Phialides of *T.* cf. *virens* and *T. harzianum* were tending clustered, but it was solitary disposition in *T. longibrachiatum*. *T.* cf. *virens* was characterized by predominantly effuse conidiation, sparingly branched, and fertile to the apex and it was penicillate type. RAPD analysis could detect variability amongst three different species of *Trichoderma* using two newly designed URP-primers. However, intra-specific variation could not be detected in all the isolates except for rDNA sequence data classified *Trichoderma* isolates into three distinct groups representing three species. The profiles of rDNA sequences of isolates representing a species showed high similarity in *T.* cf. *virens* and *T. harzianum*. However, there was a variation in rDNA sequences of isolates representing *T. longibrachiatum*. The results of present study reveals that molecular techniques of RAPD and rDNA sequencing can greatly aid in classification based on morphology and precise identification of fast evolving species of *Trichoderma*.

KEYWORDS: ***Pleurotus* spp., RAPD, rDNA sequencing, *Trichoderma* spp.**

Oyster mushrooms are the most commercially grown mushroom, being cultivated on over 60% of mushroom farms, and had the highest income from mushroom in Korea (Oh *et al.*, 1999). Since 1986 when artificial cultivation techniques were developed using rice straw, cotton waste and sawdust, commercial production of oyster mushroom has been seriously affected by green mold epidemics. Amongst oyster mushrooms, *Pleurotus ostreatus* is the most popular cultivated mushroom in Korea (Bae *et al.*, 1996; Kang *et al.*, 2001). In the *Agaricus bisporus*, the seriousness of the disease is indicated by yield losses up to 30~100% experienced in 1995 in Clester county of Pennsylvania (Samuels *et al.*, 2002). The most serious outbreak of *Trichoderma* species on mushroom crops was caused by biotype Th-2 of *T. harzianum*, in Ireland in 1985-86 and resulted in loss of about 3~4 million pounds in mushroom industries in UK and Ireland (Fletcher, 1990).

So far, the main diseases reported in the oyster mushrooms are green mold caused by *Trichoderma*, black-gray velvet caused by *Trichrus spiralis*, fire mold caused by *Neurospora* sp., brown spot of *Pseudomonas tolaassi*, and virus in Korea (Kim *et al.*, 1995, 2000).

Trichoderma is a genus of filamentous Deuteromycetes and its habitats includes forest and agricultural soil and living plant. Some species of *Trichoderma* have been used to control other fungi under biological control of plant diseases, and to produce enzymes (Samuels, 1996). The genus *Trichoderma* causes disease in oyster mushroom by competition for nutriments and lysis of the mushroom cell by secretion of hydrolytic enzymes (Goltapeh and Dnaesh, 2000).

The first serious attempts to morphologically distinguish *Trichoderma* species was made by Rifai (1969), who divided them into nine taxa on the basis of conidiophore branching and conidia shape. The most detailed morphological studies of the anamorphs were carried out by Bissett (1984, 1991a, b), who distinguished about 21 texa in sect. *Pachybasium* and seven in sect. *Longibrachiatum*. Recently, other taxonomic methods supplementary to morphology showed a great diversity of *Trichoderma*. Samuels (1994, 2002) has used isoenzyme profiles as a taxonomic technique. ITS and 5.8S rDNA sequences and fingerprinting techniques have revealed the intra-generic relationships amongst species of *Trichoderma* (Fujimori and Okuda, 1994; Kim *et al.*, 2000).

The purpose of this study was to reduce damages caused by the green mold disease by quick diagnosis of species of *Trichoderma* involved and to establish phylogenetic relationship amongst *Trichoderma* spp. isolated from *Pleurotus* spp. by morphological and molecular characteristics. We report about occurrence of *Trichoderma* spe-

*Corresponding author <E-mail: choiiy@lycos.co.kr>

cies, morphological characteristics studied using scanning electron microscope and the phylogenetic analysis revealed by RAPD analysis and rDNA sequencing.

Materials and Methods

Fungal isolates. Twenty one isolates of *Trichoderma* spp. were isolated from *Pleurotus ostreatus* and *P. eryngii* cultivation beds having base materials *viz*; rice straw, cotton waste and sawdust from oyster farms. Three standard isolates used for identification were obtained from KACC (Korean Agricultural Collection, National Institute of Agricultural Science and Technology, Suwon, Korea) (Table 1).

Morphological observations. For the isolation of *Trichoderma* spp. from the infected samples collected from different oyster mushroom farms, fast growing mycelia with green colour and lower mycelial density than mushroom mycelia were picked up with help of pins from the symptom part of the beds. These isolates were cultured on MEA (malt extract agar, difco) and PDA (potato dextrose agar, difco) and were incubated in normal light for 2~3 days at 25°C. For morphological characterization of *Trichoderma* spp., observations on morphology of conidiophores, phialides and conidia were made using scanning electron microscope. The data include the degree and nature of aggregation of conidiophores, the pattern of branching of the conidiophores, the disposition of phialides, the shape and size of phialides, the shape and size of conidia and type of hyphae.

Table 1. List of *Trichoderma* spp. selected from oyster mushroom media and sawdust compost in this study

Isolate no.	Species name	Host	Compost base	Origin
1	*T.* cf. *virens*	*P. ostreatus*	Rice straw	Iksan
2	*T.* cf. *virens*	*P. ostreatus*	Cotton waste	Wanju
3	*T.* cf. *virens*	*P. ostreatus*	Cotton waste	Iksan
4	*T.* cf. *virens*	*P. ostreatus*	Rice straw	Iksan
5	*T.* cf. *virens*	*P. ostreatus*	Rice straw	Iksan
6	*T.* cf. *virens*	*P. ostreatus*	Cotton waste	Wanju
7	*T.* cf. *virens*	*P. ostreatus*	Rice straw	Wanju
8	*T.* cf. *virens*	*P. ostreatus*	Rice straw	Wanju
9	*T.* cf. *virens*	*P. ostreatus*	Rice straw	Wanju
10	*T.* cf. *virens*	*P. ostreatus*	Rice straw	Wanju
11	*T.* cf. *virens*	*P. ostreatus*	Cotton waste	Wanju
12	*T.* cf. *virens*	*P. ostreatus*	Rice straw	Wanju
13	*T.* cf. *virens*	*P. ostreatus*	Rice straw	Wanju
14	*T.* cf. *virens*	*P. ostreatus*	Rice straw	Wanju
15	*T.* cf. *virens*	*P. ostreatus*	Rice straw	Wanju
16	*T.* cf. *virens*	*P. eryngii*	Sawdust	Iksan
17	*T. harzianum*	*P. ostreatus*	Sawdust	Ikasn
18	*T. harzianum*	*P. eryngii*	Sawdust	Iksan
19	*T. longibrachiatum*	*P. ostreatus*	Sawdust	Iksan
20	*T. longibrachiatum*	*P. ostreatus*	Sawdust	Iksan
21	*T. longibrachiatum*	*P. eryngii*	Sawdust	Iksan
22	*T. longibrachiatum*	*P. eryngii*	Sawdust	KACC[a]
23	*T. harzianum*	*P. eryngii*	Sawdust	KACC
24	*T.* cf. *virens*	*P. eryngii*	Sawdust	KACC

[a]KACC (Korean Agricultural Culture Collection, National Institute of Agricultural Science and Technology, Suwon, Korea).

DNA extraction. Genomic DNAs from different isolates of *Trichoderma* spp. were extracted following the method of Zolan and Pukkila (1986). *Trichoderma* isolates were grown in shaking cultures of potato dextrose browth (PDB) for two weeks at 25°C. After harvesting mycelial from different isolates, the mycelial mats were freeze-dried. Mycelial mats were grinded with micro-pestle for DNA extraction microcentrifuge tubes (1.5 m*l*) and incubated in 400 μl lysis buffer[3% SDS, 50 mM EDTA, 50 mM Tris-HCl (pH 7.2), 1% 2-mercaptoethanol] at 68°C for 1 hour. After that, the mixture was gently extracted with chloroform : isoamylalcohol (24 : 1) containing 5% phenol and centrifuged at 12,000 rpm at room temperature for 1 min. The supernatant was transferred to a new tube and equal volume of absolute EtOH in the solution and centrifuged for pelleting the DNA. The pellet was washed with 70% EtOH and dissolved in TE buffer (10 mM Tris-HCl pH 8.0, 1 mM EDTA).

PCR Amplification and rDNA sequencing.

RAPD analysis: The primers for RAPD amplification were 20-mer URP-primers which were synthesized by KACC. PCR amplification was performed in a 50 μl reaction mixture containing 1 pmol of both primers, 2.0 units of *Taq* DNA polymerase (Promega), 0.1 mM dNTP, 5 μl 10×buffer and 100 ng template DNA. The parameters for PCR amplification were 94°C for 4 min; 35 cycles of 95°C for 1 min, 55°C for 1 min, 72°C for 2 min and final extension 72°C for 8 min. PCR product was analyzed on 1.2% agarose gel. UPGMA dendrogram was derived from RAPD profiles of genomic DNA in the 24 isolates of *Trichoderma* spp. with two URP-primers. The similarity coefficient (F) was calculated as the fraction of shared fragments between pairs of isolates. $F = 2Nxy/(Nx + Ny)$, where Nxy is the number of PCR products shared by isolates X and Y, while Nx and Ny are total number of PCR products in isolates X and Y, respectively. A dendrogram was constructed with NTSYS-pc (ver. 2.0) using the unweighted pair-group method with arithmetic mean (Rohlf, 1993).

rDNA sequencing: Amplified PCR products were ligated into pGEM T vector (promega co.) and was transformed with *E. coli* DH5α. The sequencing of rDNA was done using dideoxy chain termination method. The sequences of rDNA were compared with already published data of *Trichoderma* spp. and selected ITS regions sequences. ITS sequences data was aligned with clustal program and analysis was done for phylogenetic relationship

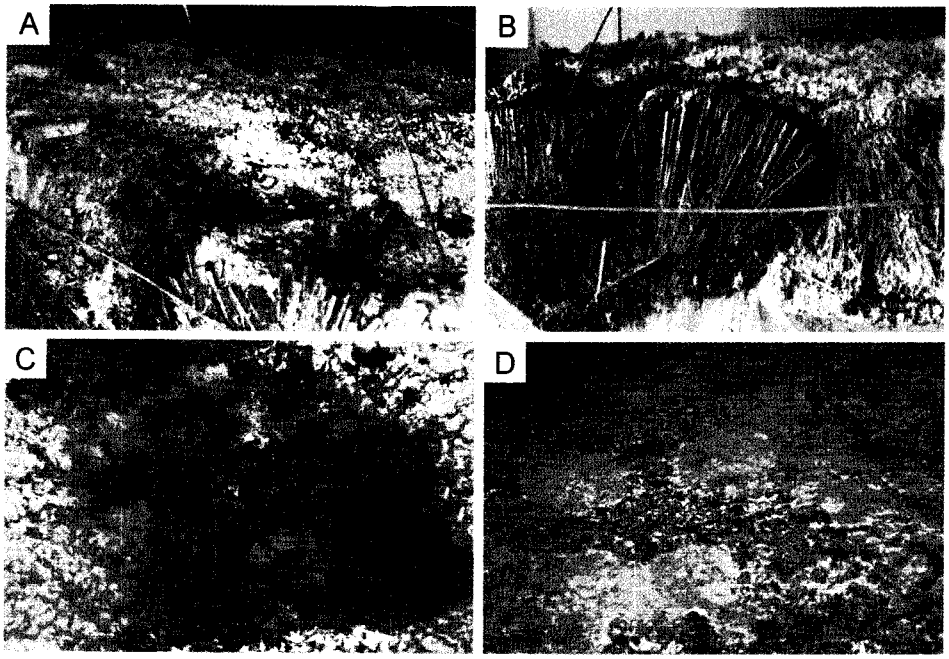

Fig. 1. Symptoms of *Trichoderma* infection on the beds of *Pleurotus ostreatus* (A and B) rice straw based substrates, (C and D) cotton waste based substrates.

by molecular evolutionary genetic analysis (MEGA, version 1.02, The Pennsylvania State University) and software (Kumar *et al.*, 1993). The distance was measured by Kimura's 2-parameter and phylogenetic tree was obtained from PCR products and ITS sequences of *Trichoderma* spp. Phylogenetic relationships were inferred by the neighbor-joining method (Saitou and Nei, 1987).

Results

Symptoms and occurrence. Symptoms of *Trichoderma* infection in *Pleurotus* beds were characteristic white mycelia which initially produces a denser compact (well-net) mycelia than that of *Pleurotus*. White mycelia of *Trichoderma* had many aerial mycelia and it gradually turns to green colour. Later on, the mycelia had deep green spread in substrates of mushroom beds (Fig. 1). The occurrence of different species of *Trichoderma* on *Pleurotus* beds was *T.* cf. *virens* (70.8%), *T. longibrachiatum* (16.7%) and *T. harzianum* (12.5%). *T. longibrachiatum* and *T. harzianum*

Table 2. Occurrence of *Trichoderma* spp. selected from mushroom beds

Species	*T.* cf. *virens*	*T. longibrachiatum*	*T. harzianum*
Ratio (%)	70.8	16.7	12.5

Table 3. Morphological characteristics of *Trichoderma* spp. observed by SEM (25°C, PDA, 3 days)

Morphological characteristics	*T.* cf. *virens*	*T. longibrachiatum*	*T. harzianum*
Conidia			
shape	ellipsodal, obovoid	ellipsodal, obovoid	ellipsodal, subglobose
colour	dark green	dilute green	pale green
size (μm)	1.5~4.0 × 1.0~2.5	1.0~4.8 × 1.0~3.1	1.3~3.3 × 1.3~2.5
L : W	3 : 2	3 : 2	3 : 2
Phialides			
shape	bowling pin, lageniform	bowling pin, lageniform	bowling pin, lageniform
disposition	tending clustered	solitary	tending clustered
Acropleurogenous	few	common	a few
size (μm)	3.5~10.0 × 1.7~3.0	4.3~9.6 × 1.3~2.7	4.4~7.2 × 2.6~3.3
L : W	3 : 1	5 : 3	5 : 3
Hyphae			
type	penicillate	tree branches	tree branches
width (μm)	2.0~6.0	1.5~4.1	1.5~6.0

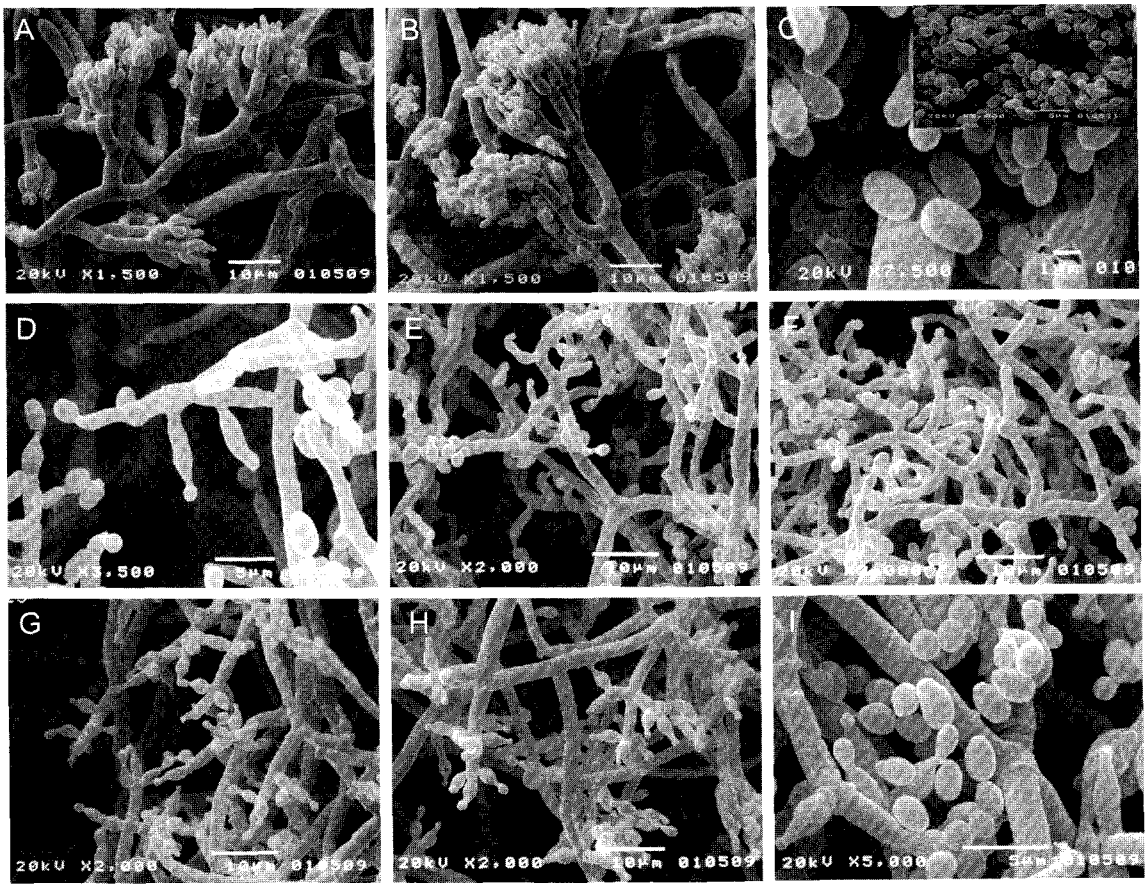

Fig. 2. Morphological characteristics of *Trichoderma* spp. observed by SEM. (A-C) Conidiophores, phialides and conidia respectively, of *T.* cf. *virens*. (D-F) Conidiophoers, phialides and conidia respectively, of *T. longibrachiatum*. (G-I) Conidiophores, phialides and conidia respectively, of *T. harzianum*.

were mainly isolated from sawdust based substrate and *T.* cf. *virens* was isolated from the rice straw and cotton waste substrates (Table 2).

Morphological characteristics. Differences in morphological characteristics of *Trichoderma* spp. are summarized in Table 3 and Fig. 2. The conidia were ellipsoidal and obovoid in *T.* cf. *virens* and *T. longibrachiatum*. However, it was ellipsoidal and subglobose in *T. harzianum*. The phialides were bowling pin, lageniform and the length was 3.5~10.0 × 1.3~3.3 μm. Phialides of *T.* cf. *virens* and *T. harzianum* were tending clustered, but *T. longibrachiatum* was solitary disposition. Especially, *T.* cf. *virens* was characterized by predominant conidiation, many divided branches, gathering all finger to top, and fertile to the apex. That was penicillate type. Conidiophores of *T.* cf. *virens* were smoothly bend, gather and not spread to top. Conidia broadly rounded to obovoid, both ends broadly rounded or with the base narrower. Phialides were hung like banana in the conidiophore, base and apex were more narrow than middle. *T. longibrachiatum* had conidiophores with long and flexuous, a few side branches and smoothly. Phialides was single, long, slightly wider in the middle than at the base. Conidia were ellipsoidal to obovoid, smooth and dilute green. Conidiophores of *T. harzianum* were spread to the top and smooth or rounded, wide near the base. Phialides of *T. harzianum* were arising mostly in crowded but had a angle with conidiophore, and had whorls of 2~6 on the terminal branches. Conidia subglobose to ellipsoidal, apex broadly rounded, base more narrowly rounded.

PCR fingerprinting of *Trichoderma*. Reproducible RAPD profiles of genomic DNAs of different *Trichoderma* spp. are presented in Fig. 3. Both the primers were useful in classifying *Trichoderma* spp. and the sizes of the PCR products ranged between 100 bp to 4.0 kb with a total of 6~13 bands in each RAPD profile. The isolates had a total of 435 bands with two primers, primer 1 had 168 bands and primer 2 had 267. The average number of bands with primer 1 and primer 2 was 7 and 11, respectively. *T. longibrachiatum* had 6~8 bands with primer 1 and had less bands than the other species, but the number of bands with the primer 2 was less in *T. longibrachiatum* and *T. harzianum* than those of *T.* cf. *virens*. The intra-species bands of *T.* cf. *virens*, *T. harzianum* and *T.*

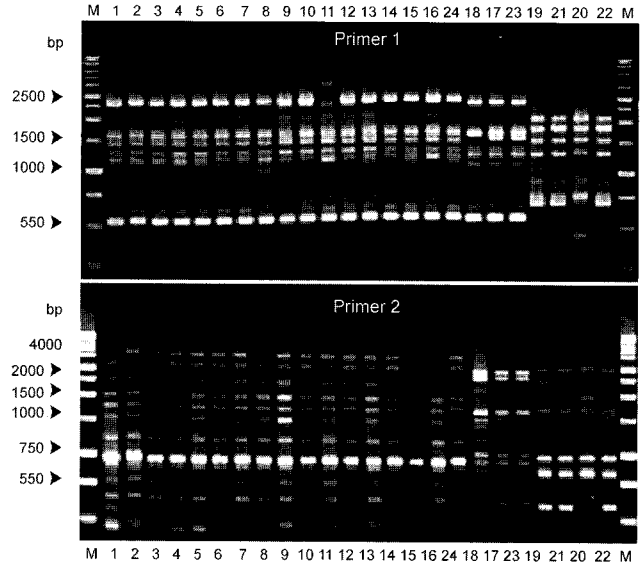

Fig. 3. Representative RAPD fingerprints using primer 1 and primer 2 of 24 isolates of *Trichoderma* spp. Lanes 1~16 and 24 : *T.* cf. *virens*; Lanes 17~18 and 23 : *T. longibrachiatum*; Lanes 19~22 : *T. harzianum*; M : 1 kb ladder marker (promega).

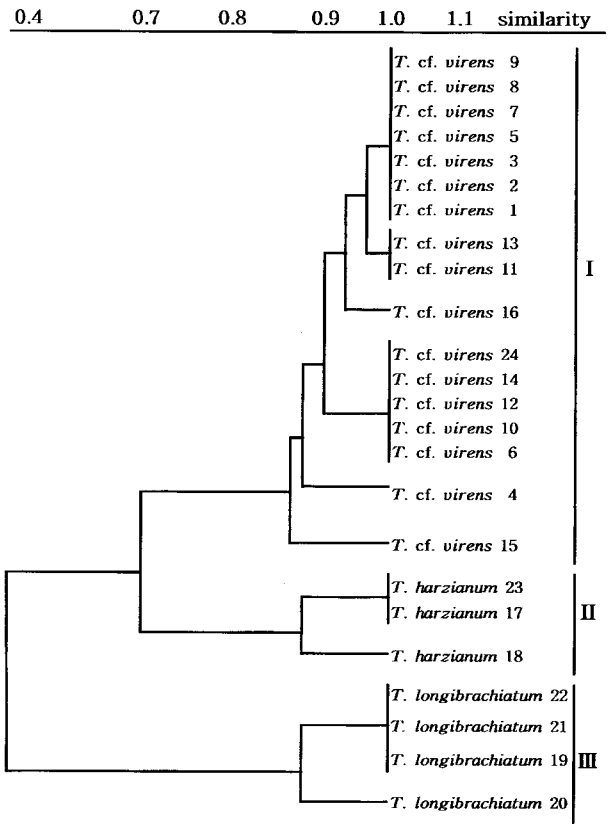

Fig. 4. UPGMA dendrogram derived from RAPD profiles of genomic DNA in the 24 isolates of *Trichoderma* spp. with two URP-primers

longibrachiatum were similar in both the primers. The UPGMA dendrogram based on RAPD profiles of genomic DNA divided the *Trichoderma* spp. into three groups, namely *T.* cf. *virens* in group I, *T. harzianum* in group III and *T. longibrachiatum* III (Fig. 4). The isolates of each *Trichoderma* spp. had about 90% similarity. Between *T.* cf. *virens* and *T. harzianum* there was about 70% similarity, but the similarity between these species and *T. longibrachiatum* was only about 40%.

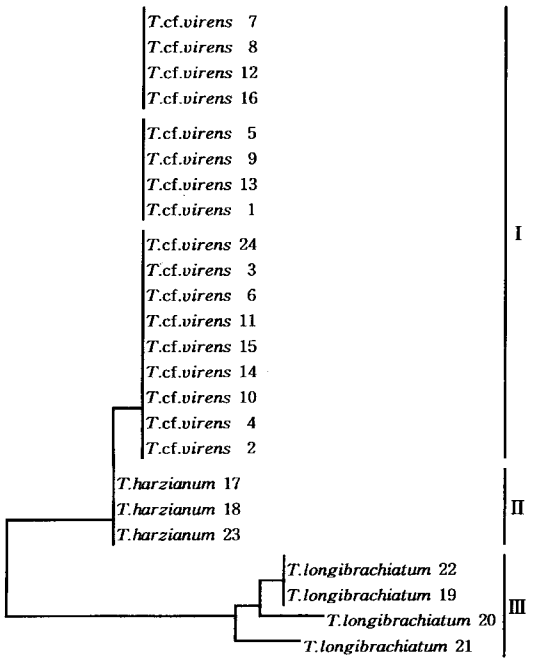

Fig. 5. UPGMA dendrogram derived from sequencing profiles of ribosomal DNA in the 24 isolates of *Trichoderma* spp.

Genetic relationship. Genetic relationship among different species of *Trichoderma* based on ribosomal DNA sequencing profiles is shown in Fig. 5. The 24 isolates tested were divided into three groups. *T.* cf. *virens* included in group I, *T. harzianum* and *T. longibrachiatum* included in group II and III, respectively. The first group included *T.* cf. *virens* isolates from rice straw, cotton waste and sawdust substrates. Seventeen isolates of *T.* cf. *virens* had 100% of DNA similarity. Also, 3 isolates of *T. harzianum* from sawdust had 100% of DNA similarity as well. *T.* cf. *virens* and *T. harzianum* had very close phylogenetic relationship. The third group which comprised of *T. longibrachiatum* isolates had more genetic distance than other groups. However, the isolates within subgroup had a high DNA sequences similarity.

Discussion

Green mold disease caused by *Trichoderma* spp. is a serious problem of oyster mushroom in Korea. It causes large economic losses to the mushroom growers. In the paper

attempts have been made to identified green mold, disease causing species of *Trichoderma* using a combination of morphological and molecular characteristics as the identification based on morphological traits alone is misleading (Kim *et al.*, 2000).

Trichoderma isolates from oyster mushroom beds were identified as three distinct species *viz.*, *T.* cf. *virens*, *T. longibrachiatum* and *T. harzianum*. Especially, occurrence of *T.* cf. *virens* was highest (70.8%) and hence it was an important species of *Trichoderma* causing green mold disease in oyster mushroom. Also, *T.* cf. *virens* is mostly associated with rice straw and cotton waste substrates which are the most popular substrates for the oyster mushroom cultivation in Korea. The occurrences of *T.* cf. *virens, T. longibrachiatum* and *T. harzianum* were similar in the sawdust based substrates. When the associations of *Trichoderma* spp. with oyster mushroom species were analysed, it was found that *T.* cf. *virens* is mostly associated with oyster mushroom species *Pleurotus ostreatus*. However, *P.eryngii*, the popular bottle mushroom, was damaged by the all three species of *Trichoderma*. The symptoms and the causal organisms of green mold disease in oyster mushroom is similar to that of button mushroom. However, Danesh *et al.* (2000) reported *T. harzianum*, *T. longibrachiatum*, *T. virens* and *Trichoderma* sp. as causing agent for green mold disease in *Agaricus bisporus* beds.

The symptoms of the green mold disease includes large coverage of mushroom substrates by the mold, lysis of the mushroom cell walls, and competence with mushroom mycelia for nutrients. Goltapeh and Danesh (2000) reported that *Trichoderma* is able to secrete hydrolytic enzymes such as chitinases, β-glucanases and cellulases, which lyse the mushroom cell walls and are supposed to play a basic role in the mycoparasitic activity of this fungus. *T. harzianum* colonizes mushroom compost, competes with mushroom mycelium for space and nutrients, and results in large areas of the growing beds that do not produce mushroom fruiting bodies. Yields losses from *Trichoderma* can be catastrophic therefore it is essential to study the epidemiology of *T. harzianum* (Bayer *et al.*, 2000). This is also one of the most serious disease of button mushroom as it initially produces a dense pure white mycelium, which resembles to mushroom mycelium and later on mold mycelial mat on casing layer gradually turns to green colour because of the heavy sporulation by the fungi (Danesh *et al.*, 2000).

The morphological characterization of *Trichoderma* spp. isolated from oyster mushroom growing substrates was done before based on morphology such as colonies, hyphae, conidiophores, phialides and conidia. However, in the present study, the emphasis was given on conidiophores spread type i.e gathering or non-gathering type and phialides-type fertile or non-fertile. *T.* cf. *virens* was characterized by more gathering of the fingers to top, many divided branches and more fertile phialides to the apex than other species. Conidiophores of *T.* cf. *virens* is smoothly bend, gather and not spread to top. Phialides were hung like banana in the conidiophore, and base and apex were more narrow than middle. However, *T. longibrachiatum* was solitary disposition into center point, solitary phialides from the top of mycelium, side branch borne at right angles but rarely put out further side branch and phialides were usually lageniform and bowling pin, subulate and slightly constricted at the base. *T. longibrachiatum* had long conidiophores, and a few conidiophores were branched. Conidiophores of *T. harzianum* were highly and regularly branched, and entire structure was pyramidal and were spread to the top and smooth or rounded, wide near the base. Phialides of *T. harzianum* were arisen mostly in crowded but had an angle with conidiophore, and had whorls of 2~6 on the terminal branches. Conidia were subglobose to ellipsoidal, apex broadly rounded and more narrowly rounded at base. *Trichoderma* section *Pachybasium* of Bissett (1991a, b) to which *T. virens* and *T. harzianum* belongs, is characterized by highly ramified conidiosphores that are often aggregated into compact fascicles or pustules and with short, broad branches bearing in flated phialides in crowded verticils. *Trichoderma* section *Longibrachiatum*, to which *T. longibrachiatum* belongs, is characterized by conidiophores with short, rarely rebranched side branches and smooth-walled ellipsoidal to oblong conidia. Phialides often arose singly, directly from the main axis, were cylindrical or narrowly flask-shaped and slightly wider in the middle than at the base lateral branches often comprised a single phialide subtended by an acropleurogenous phialide. Conidia were ellipsoidals to oblong, smooth and green (Samuels *et al.*, 1994). Bissett (1984) reported that *T. longibrachiatum* is characterized by conidiophore with long, main branches and relatively few, short, side branches. The final branches were very simply constructed. Conidiophores hyaline, smooth walled, arising from the substratum to form irregular tufts or arising primarily form the aerial mycelium in older colonies; main branches long and straight. Phialides solitary or in verticils of 2 or 3; usually broadly lageniform. Conidia one celled, dilute green, smooth walled, obovoid to ellipsoidal, apex broadly rounded, the base usually more narrowly and aggregating in minute heads at the tips of the phialides.

Molecular techniques such as RAPD and rDNA sequencing have been used widely for taxonomic conclusions in various organisms including *Trichoderma* spp. and oyster mushroom (Bae *et al.*, 1996; Kang *et al.*, 2001; Kim *et al.*, 2000; Samuels *et al.*, 1994, 2002). Therefore, it is necessary to correlate molecular phylogeny with morphological and other biochemical and physiological traits. RAPD fingerprinting and rDNA sequencing were also

used to classify *T. harzianum* strains that were antagonistic to the commercial production of mushroom (Muthumeenakshi *et al.*, 1994), *Trichoderma* species and the Ascomycetes *Hypocrea jecorina* (Kubicek and Harman, 1998). We observed genetic diversity within a collection of *Trichoderma* spp. isolates based on RAPD patterns. Both the primers were useful in classifying and grouping of *Trichoderma* isolates. The intra-species bands of *T.* cf. *virens, T. harzianum* and *T. longibrachiatum* were similar in both the primer. *T. longibrachiatum* had 6~8 bands with primer 1 and had less bands than the other species, but the number of bands with the primer 2 was less in *T. longibrachiatum* and *T. harzianum* than those of *T.* cf. *virens.* RAPD fingerprints using the URP-primers were helpful in classification and grouping of *Trichoderma* isolates and were in agreement with morphological observations. UPGMA dendrogram based on RAPD profiles of genomic DNA had high similarity within group but each group had long distance. The analysis of rDNA sequencing of *Trichoderma* spp. reveals the genetic variation in isolates representing *T.* cf. *virens* and *T. harzianum.* However, intra-species isolates has 100% DNA base sequence similarity. *T. longibrachiatum* isolates had more genetic distance than other groups. However, the isolates within subgroup had a high DNA sequences similarity. Also, *T.* cf. *virens* and *T. virens* are spread type of conidiophores. The shape and size of conidia and phialids are similar to each other. The analysis of rDNA sequencing reveals difference in rDNA base sequences of *T.* cf. *virens* and *T. virens.* However, *T.* cf. *virens* has more similarity with *T. harzianum* in rDNA sequences. Therefore, we have named the new species of *T. virens* as *T.* cf. *virens.*

Acknowledgments

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