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Characterization of *Trichoderma* spp. Associated with Green Mold of Oyster Mushroom by PCR-RFLP and Sequence Analysis of ITS Regions of rDNA

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Molecular profiles of PCR-RFLP and sequence analysis of internal transcribed spacer (ITS) regions of rDNA were compared between morphologically distinguishable species of *Trichoderma* isolated from substrates of oyster mushroom in Korea, *T. atroviride*, *T. citrinoviride*, *T. harzianum*, *T. longibrachiatum*, *T. virens*, and two unidentified species, *Trichoderma* sp. 1 and 2. PCR-RFLP analysis divided the *Trichoderma* spp. into six RFLP groups, A, B, C, D, E, and F. The RFLP groups were generally agreed with described morphological species, except that the RFLP group A containing the two unidentified species. A neighbor-joining tree based on ITS sequences well supported RFLP groups observed by RFLP analysis of ITS regions of rDNA. Additionally, the two unidentified species, *Trichoderma* sp. 1 and 2, which could not be distinguished by PCR-RFLP analysis, were separated in sequence analysis of ITS regions of rDNA.

Keywords : ITS regions of rDNA, oyster mushroom, PCR-RFLP, *Trichoderma*

Taxonomy of *Trichoderma* species has been defined on the basis of their morphological features; size and shape of conidia, phialide shape, patterns of conidiophore branching, growth rates in culture, and sporulation patterns when grown under specified condition. Rifail (1969) proposed the concept of 'aggregate' species and distinguished nine aggregates. Bissett (1984, 1991a, b, c) elevated Rifai's aggregate species to sectional level (sect. *Pachybasium*, *Trichoderma*, *Longibrachiatum*, *Hypocreanum*, and *Saturnisporum*). However, classification and identification of *Trichoderma* species by phenotype have been difficult, because morphological characteristics are easily changed by environmental influences (Ospina-Giraldo et al., 1998). In recent years, several studies of *Trichoderma* taxonomy have been carried out using molecular approaches, such as isozyme analysis (Samuels et al., 1994; Stasz et al., 1989), restriction fragment length polymorphism (RFLP) analysis (Gams and Meyer, 1998; Muthumeenakshi et al., 1994), random amplified polymorphic DNA (RAPD) analysis (Fujimori and Okuda, 1994; Kuhls et al., 1995; Schlick et al., 1994; Turner et al., 1997; Zimand et al., 1994) and sequence analysis (Kindermann et al., 1998; Kuhls et al., 1996, 1997). Sequence analysis of ITS regions of rDNA revealed section *Saturnisporum* could be merged with sect. *Longibrachiatum*. These sections formed a monophyletic group that was well supported by a bootstrap value of 100% (Kuhls et al., 1997). UP (universal primer)-PCR is a useful tool for the characterization and grouping of isolates in order to study their genetic relatedness (Bulat et al., 1998). More recently, *T. harzianum* biotypes Th 2 and Th 4 have been reidentified as new species and named as *T. aggressivum* f. *aggressivum* and *T. aggressivum* f. *europaeum*, respectively, on the basis of sequence analysis of ITS-1 region of rDNA and the translation elongation factor 1 alpha gene (EF-1α) and their morphological differences (Samuels et al., 2002). These molecular techniques have been used to distinguish species with specific group of strains that were defined by common morphology.

The objectives of this study was to establish classification of *Trichoderma* species associated with the green mold epidemic of commercially grown oyster mushroom (*Pleurotus ostreatus*) in Korea based on molecular characteristics including PCR-RFLP and sequence analysis of ITS regions of rDNA.

Materials and Methods

Fungal strains. A total of 118 isolates of *Trichoderma* species collected from various substrates of oyster mushroom were used in this study (Table 1). All cultures were maintained on potato dextrose agar (PDA, Difco, USA) slants at 4°C.

DNA extraction and PCR amplification. DNA was

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Table 1. Isolates of *Trichoderma* spp. collected from various substrates of the oyster mushroom

Species/ strain	Geographic origin	Substrate[a]	Culture type	RFLP pattern			RFLP group	Phylogenetic group
				Cfo I	*Hae* III	*Msp* I		
Trichoderma sp. 1								
CNU529	Jecheon, Chungbuk	WC	A	C1	H1	M1	A	A1
CNU571	Inje, Gangwon	RS	A	C1	H1	M1	A	A1
CNU601	Paju, Gyeonggi	S	A	C1	H1	M1	A	A1
CNU617	Yeoju, Gyeonggi	WC	A	C1	H1	M1	A	
CNU618	Paju, Gyeonggi	S	A	C1	H1	M1	A	
CNU646	Yeongi, Chungnam	S	A	C1	H1	M1	A	A1
CNU647	Yeongi, Chungnam	S	A	C1	H1	M1	A	A1
CNU648	Yeongi, Chungnam	S	A	C1	H1	M1	A	
CNU652	Yeongi, Chungnam	S	A	C1	H1	M1	A	
CNU657	Yeongi, Chungnam	S	A	C1	H1	M1	A	
CNU682	Jangseong, Chunnam	WC	A	C1	H1	M1	A	A1
C42	Buyeo, Chungnam	RS	A	C1	H1	M1	A	
C45	Buyeo, Chungnam	RS	A	C1	H1	M1	A	
C73	Dangjin, Chungnam	RS	A	C1	H1	M1	A	
C74	Dangjin, Chungnam	RS	A	C1	H1	M1	A	
CNU702	Yeosu, Chunnam	RS	A	C1	H1	M1	A	
CNU703	Yeosu, Chunnam	RS	A	C1	H1	M1	A	
CNU705	Yeosu, Chunnam	RS	A	C1	H1	M1	A	
CNU720	Hampyeong, Chunnam	RS	A	C1	H1	M1	A	
CNU724	Hampyeong, Chunnam	RS	A	C1	H1	M1	A	
CNU731	Hampyeong, Chunnam	RS	A	C1	H1	M1	A	
CNU782	Uiseong, Gyeongbuk	RS	A	C1	H1	M1	A	
CNU784	Uiseong, Gyeongbuk	RS	A	C1	H1	M1	A	
CNU788	Uiseong, Gyeongbuk	WC	A	C1	H1	M1	A	
CNU791	Uiseong, Gyeongbuk	WC	A	C1	H1	M1	A	
CNU794	Uiseong, Gyeongbuk	WC	A	C1	H1	M1	A	
CNU805	Yeongi, Chungnam	WC	A	C1	H1	M1	A	
Trichoderma sp. 2								
CNU501	Chuncheon, Gangwon	WC	B	C1	H1	M1	A	A2
CNU505	Inje, Gangwon	WC	B	C1	H1	M1	A	
CNU514	Inje, Gangwon	WC	B	C1	H1	M1	A	
CNU515	Inje, Gangwon	WC	B	C1	H1	M1	A	
CNU516	Taean, Chungnam	WC	B	C1	H1	M1	A	
CNU520	Okchun, Chungbuk	WC	B	C1	H1	M1	A	
CNU522	Boeun, Chungbuk	WC	B	C1	H1	M1	A	
CNU523	Boeun, Chungbuk	WC	B	C1	H1	M1	A	A2
CNU525	Koisan, Chungbuk	WC	B	C1	H1	M1	A	
CNU526	Koisan, Chungbuk	WC	B	C1	H1	M1	A	
CNU531	Danyang, Chungbuk	RS	B	C1	H1	M1	A	
CNU532	Danyang, Chungbuk	RS	B	C1	H1	M1	A	
CNU533	Chongwon, Chungbuk	WC	B	C1	H1	M1	A	A2
CNU537	Jincheon, Chungbuk	WC	B	C1	H1	M1	A	A2
CNU538	Jincheon, Chungbuk	WC	B	C1	H1	M1	A	A2
CNU539	Jincheon, Chungbuk	WC	B	C1	H1	M1	A	A2
CNU540	Jincheon, Chungbuk	WC	B	C1	H1	M1	A	
CNU542	Chongwon, Chungbuk	WC	B	C1	H1	M1	A	
CNU543	Chongwon, Chungbuk	WC	B	C1	H1	M1	A	A2
CNU544	Sanchong, Gyeongnam	WC	B	C1	H1	M1	A	A2

Table 1. Continued

Species/ strain	Geographic origin	Substrate[a]	Culture type	RFLP pattern			RFLP group	Phylogenetic group
				Cfo I	*Hae* III	*Msp* I		
CNU545	Namwon, Chonbuk	RS	B	C1	H1	M1	A	A2
CNU546	Namwon, Chonbuk	RS	B	C1	H1	M1	A	
CNU550	Namwon, Chonbuk	RS	B	C1	H1	M1	A	
CNU553	Namwon, Chonbuk	RS	B	C1	H1	M1	A	A2
CNU558	Juchon, Chonbuk	WC	B	C1	H1	M1	A	A2
CNU564	Yeongi, Chungnam	RS	B	C1	H1	M1	A	
CNU565	Yeongi, Chungnam	WC	B	C1	H1	M1	A	A2
CNU585	Seosan, Chungnam	S	B	C1	H1	M1	A	
CNU586	Gunja, Gangwon	WC	B	C1	H1	M1	A	
CNU587	Gunja, Gangwon	WC	B	C1	H1	M1	A	
CNU588	Kwangpan, Gangwon	WC	B	C1	H1	M1	A	
CNU595	Pyeongtaek, Gyeonggi	RS	B	C1	H1	M1	A	
CNU597	Yangju, Gyeonggi	S	B	C1	H1	M1	A	
CNU606	Paju, Gyeonggi	S	B	C1	H1	M1	A	
CNU608	Paju, Gyeonggi	WC	B	C1	H1	M1	A	
CNU610	Yeoju, Gyeonggi	RS	B	C1	H1	M1	A	A2
CNU624	Yeongi, Chungnam	WC	B	C1	H1	M1	A	
CNU629	Yeongi, Chungnam	WC	B	C1	H1	M1	A	
CNU640	Yeongi, Chungnam	S	B	C1	H1	M1	A	
CNU661	Yeongi, Chungnam	S	B	C1	H1	M1	A	
CNU672	Yuseong, Daejeon	WC	B	C1	H1	M1	A	A2
CNU676	Yuseong, Chungnam	WC	B	C1	H1	M1	A	
CNU683	Kyeongju, Gyeongbuk	WC	B	C1	H1	M1	A	
CNU701	Yeosu, Chunnam	RS	B	C1	H1	M1	A	
CNU707	Yeosu, Chunnam	RS	B	C1	H1	M1	A	
CNU713	Yeosu, Chunnam	RS	B	C1	H1	M1	A	
CNU716	Hampyeong, Chunnam	RS	B	C1	H1	M1	A	
CNU722	Hampyeong, Chunnam	RS	B	C1	H1	M1	A	
CNU728	Hampyeong, Chunnam	RS	B	C1	H1	M1	A	
CNU781	Uiseong, Gyeongbuk	RS	B	C1	H1	M1	A	
CNU785	Uiseong, Gyeongbuk	WC	B	C1	H1	M1	A	
CNU796	Annam, Chungbuk	WC	B	C1	H1	M1	A	
CNU798	Annam, Chungbuk	WC	B	C1	H1	M1	A	
CNU802	Yeongi, Chungnam	WC	B	C1	H1	M1	A	
CNU804	Yeongi, Chungnam	WC	B	C1	H1	M1	A	
T. harzianum								
CNU551	Namwon, Chonbuk	RS	C	C1	H1	M2	B	B
CNU554	Namwon, Chonbuk	RS	C	C1	H1	M2	B	
CNU556	Yeongi, Chungnam	WC	C	C1	H1	M2	B	B
CNU578	Inje, Gangwon	RS	C	C1	H1	M2	B	B
CNU625	Yeongi, Chungnam	WC	C	C1	H1	M2	B	
CNU662	Taean, Chungnam	WC	C	C1	H1	M2	B	
CNU663	Taean, Chungnam	WC	C	C1	H1	M2	B	
CNU681	Kyeongju, Gyeongbuk	WC	C	C1	H1	M2	B	
C40	Buyeo, Chungnam	RS	C	C1	H1	M2	B	
C93	Buyeo, Chungnam	S	C	C1	H1	M2	B	
C98	Buyeo, Chungnam	WC	C	C1	H1	M2	B	

Table 1. Continued

Species/ strain	Geographic origin	Substrate[a]	Culture type	RFLP pattern			RFLP group	Phylogenetic group
				Cfo I	*Hae* III	*Msp* I		
T. virens								
CNU639	Yeongi, Chungnam	WC	D	C1	H1	M3	C	C
C79	Dangjin, Chungnam	RS	D	C1	H1	M3	C	C
T. atroviride								
CNU503	Inje, Gangwon	WC	C	C2	H1	M4	D	D
CNU507	Yeosu, Chonnam	RS	C	C2	H1	M4	D	D
CNU511	Yeosu, Chonnam	RS	C	C2	H1	M4	D	
CNU534	Chongwon, Chungbuk	WC	C	C2	H1	M4	D	
CNU555	Namwon, Chonbuk	RS	C	C2	H1	M4	D	
CNU559	Yeongi, Chungnam	WC	C	C2	H1	M4	D	
CNU560	Yeongi, Chungnam	WC	C	C2	H1	M4	D	
CNU572	Inje, Gangwon	RS	C	C2	H1	M4	D	
CNU580	Seosan, Chungnam	S	C	C2	H1	M4	D	D
CNU582	Seosan, Chungnam	S	C	C2	H1	M4	D	
CNU583	Seosan, Chungnam	S	C	C2	H1	M4	D	
CNU674	Yuseong, Daejeon	WC	C	C2	H1	M4	D	
CNU779	Uiseong, Gyeongbuk	RS	C	C2	H1	M4	D	
T. citrinoviride								
CNU627	Yeongi, Chungnam	WC	E	C3	H2	M5	E	E
CNU638	Yeongi, Chungnam	WC	E	C3	H2	M5	E	
C114	Yuseong, Daejeon	RS	F	C3	H2	M5	E	
T. longibrachiatum								
CNU518	Taean, Chungnam	RS	E	C3	H3	M5	F	F
CNU577	Inje, Gangwon	RS	E	C3	H3	M5	F	
CNU592	Pyeongtaek, Gyeonggi	WC	E	C3	H3	M5	F	
CNU613	Yeoju, Gyeonggi	WC	E	C3	H3	M5	F	
CNU615	Yeoju, Gyeonggi	WC	E	C3	H3	M5	F	
CNU631	Yeongi, Chungnam	WC	E	C3	H3	M5	F	
CNU634	Yeongi, Chungnam	WC	E	C3	H3	M5	F	

[a] WC; waste cotton, RC; rice straw, S; sawdust

extracted from mycelia grown on PDA covered with a sterile cellophane disc. The scraped mycelia were lysed using a STES buffer (500 mM NaCl, 200 mM Tris-HCl (pH 7.6), 10 mM EDTA, 1% SDS). Following a phenol/ chloroform/isoamyl alcohol (25:24:1) extraction, genomic DNA was precipitated by ethanol in the presence of sodium acetate. DNA was stored at –20°C before use.

PCR amplification was carried out in a *i*-cycler (Bio-rad, USA) for 30 cycles of 94°C for 40 s denaturing, 55°C for 40 s annealing, and 72°C for 1min extension. Initial denaturing at 94°C was extended to 3 min and the final extension was at 72°C for 15 min. Amplification reactions contained 10 pmol of ITS5 and ITS4 (White et al., 1990), 250 μM dNTP, 10 mM Tris-HCl (pH 9.0), 40 mM KCl, 1.5 mM $MgCl_2$, 2 U of *Taq*-DNA polymerase (Bioneer, Korea), and 100 ng of template DNA in a final volume of 50 μl.

PCR-RFLP analysis. For RFLP analysis, 4 μl of a PCR product of ITS regions for each isolate was digested with 0.5 U restriction enzyme *Cfo* I (TakaRa, Japan), *Hae* III, and *Msp* I (Promega, USA) according to the manufacturer's instructions and separated on a 4% NuSieve 3:1 (BMA, USA) agarose, stained with ethidium bromide and visualized under UV light.

The patterns were normalized and further processed with GelCompar Version 2.5 pattern analysis software (Applied-Maths, Belgium) (Vauterin and Vauterin, 1992). Isolates were grouped using the Dice coefficient, as well as cluster analysis by UPGMA (unweighted pair group method with arithmetic average). The normalized densitometric traces obtained using each of the three restriction enzymes were then assembled to produce a single combined densitometric trace for each isolate, and analyzed as a single cumulative DNA.

Sequence determination and phylogenetic analysis. PCR product purification was conducted using a Wizard PCR prep kit (Promega, USA). Purified double-stranded PCR fragments were directly sequenced with BigDye terminator cycle sequencing kits (Applied Biosystems, USA.) following the manufacturer's instructions. The same primer sets for the PCR amplification were used to sequence both DNA strands. The gel electrophoresis and data collection were performed on an ABI Prism 310 Genetic Analyzer (Applied Biosystems, U.S.A.).

The sequences were compared with ITS sequence of rDNA available in the Genbank database by the BLAST search. Sequences generated from materials in this study and retrieved from GenBank were initially aligned using the program CLUSTAL X (Thompson et al., 1997), and then the alignment was refined manually using the PHYDIT program version 3.0 (Chun, 1995; available at http://plaza.snu.ac.kr/jchun/phydit). Ambiguously aligned regions were excluded from the subsequent analyses. A neighbor-joining tree was reconstructed with Kimura's 2-parameter distance model (Kimura, 1980) using the PHYLIP 3.57c package (Felsenstein, 1985). The bootstrap analysis using 1000 replications were performed to assess the relative stability of the branches.

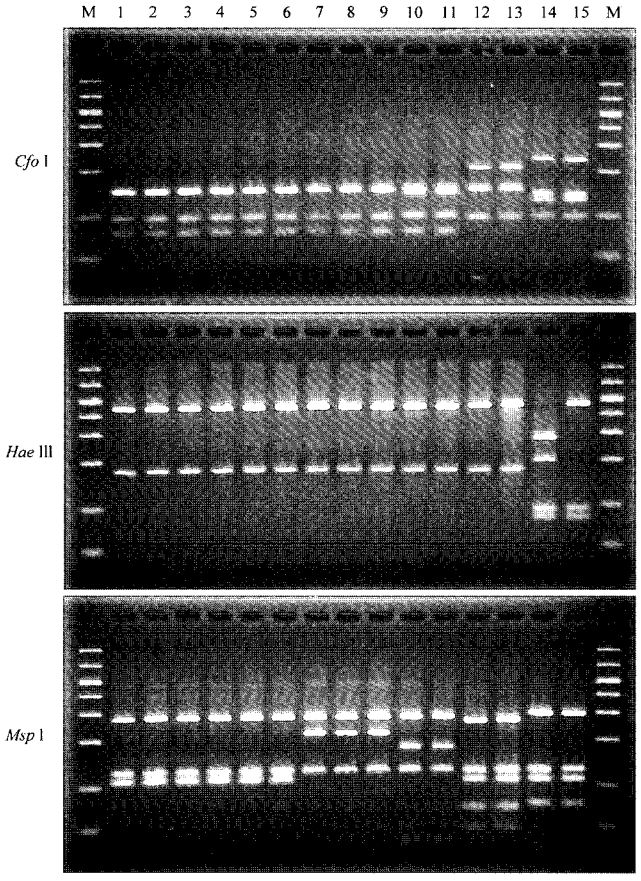

Fig. 1. PCR-RFLP patterns of ITS regions of rDNA of *Trichoderma* species isolated from oyster mushroom farms in Korea using endonucleases *Cfo* I, *Hae* III, and *Msp* I. Size Markers (M) are 50-1000 bp DNA markers. Lanes 1-3, *Trichoderma* sp. 1; lanes 4-6, *Trichoderma* sp. 2; lanes 7-9, *T. harzianum*; lanes 10-11, *T. atroviride*; lanes 12-13, *T. virens*; lane 14, *T. citriniviride*; lane 15, *T. longibrachiatum*.

Results

PCR-RFLP analysis. The primer ITS4 and ITS5 amplified ITS regions from all 118 isolates. The sizes of amplified ITS regions ranged from 580 to 610 bp. One hundred and eighteen isolates showed 3, 3, and 5 RFLP patterns on *Cfo* I(C), *Hae* III(H), and *Msp* I(M), respectively (Fig. 1). The RFLP groups A, B, C, D, E, and F were in general agreement with described morphological species, *Trichoderma* sp. 1 and 2, *T. atroviride*, *T. citrinoviride*, *T. harzianum*, *T. longibrachiatum*, and *T. virens*, however, the RFLP group A contained two unidentified species (*Trichoderma* sp. 1 and 2). There were no intraspecific differences among isolates on band patterns by *Cfo* I, *Hae* III, and *Msp* I. A UPGMA dendrogram from combined restriction pattern is shown in Fig. 2.

Phylogenetic analysis of ITS region. Representative strains of six RFLP groups were selected for sequences analysis of ITS regions to further prove the separation of *Trichoderma* isolates studied. *Hypocrea aureoviridis* CBS 103.69 was used as outgroup considering that it is a close relative of *Trichoderma* species. A distance analysis with Kimura's 2-parameter model based on ITS sequences

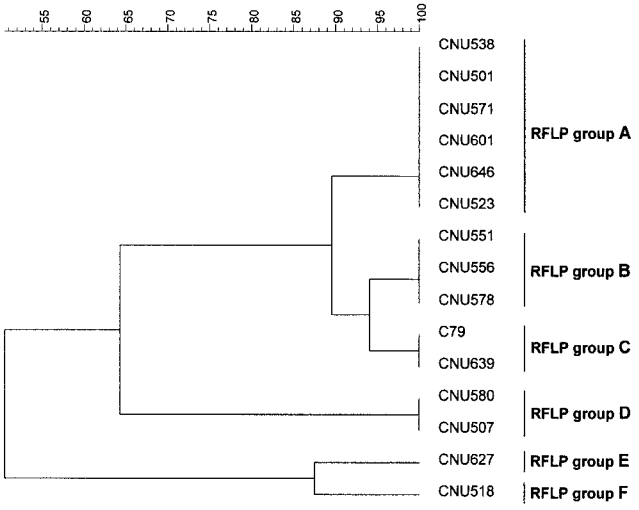

Fig. 2. Dendrogram showing the relationship among *Trichoderma* spp. isolated from various substrates of the oyster mushroom based on the PCR-RFLP profiles of ITS regions of rDNA. Dendrogram was constructed by the Dice coefficient and UPGMA in Gelcompar program.

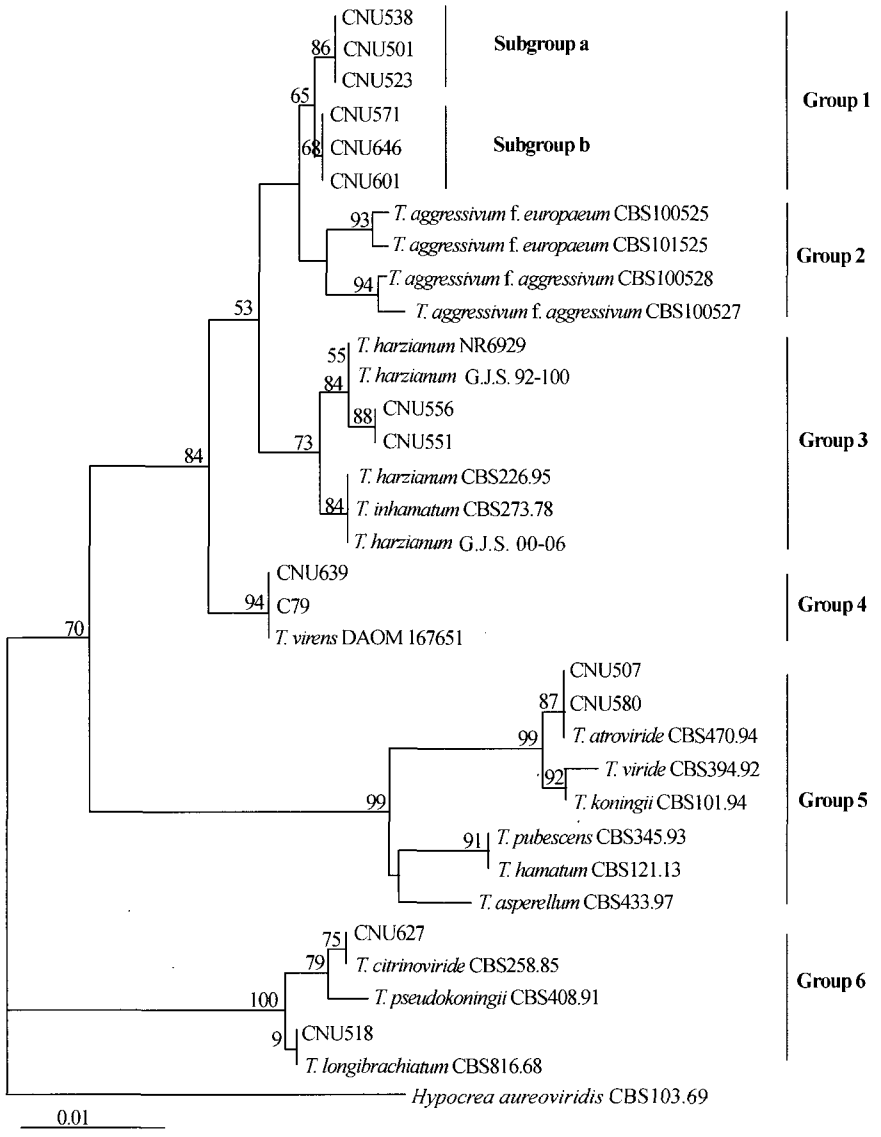

Fig. 3. Neighbor-joining tree based on ITS sequences showing relationships among *Trichoderma* spp. isolated from various substrates of the oyster mushroom and other species of the genus *Trichoderma*. The values above each branch indicate the percentage levels of bootstrap support (> 50%) for the branch point based on 1000 resamplings. The bar represents 0.01 substitutions per nucleotide position.

revealed that isolates of *Trichoderma* were divided into generated 6 distinct phylogenetic groups. This result well supported the RFLP groups of *Trichoderma* species observed by RFLP analysis of ITS regions (Fig. 3).

Group 1 including *Trichoderma* sp. 1 (isolates CNU501, CNU523 and CNU538 isolates) and *Trichoderma* sp. 2 (isolates CNU571, CNU601 and CNU646 isolates), were divided into two subgroups with subgroup 1 containing *Trichoderma* sp. 1 and subgroup 2 containing *Trichoderma* sp. 2. Group 2 contained *T. aggressivum* f. *europaeum* (Th 2) and *T. aggressivum* f. *aggressivum* (Th 4), which were known as the mushroom (*Agaricus bisporus*) aggressive groups in Europe and North America. No *Trichoderma* isolates from oyster mushroom farms in Korea were placed in this group. Group 3 included *Trichoderma* isolates CNU551 and CNU556, *T. harzianum* CBS 226.95 (ex-type strain) and *T. inhamatum* CBS 273.78 (ex-type strain). Group 4 was supported with a bootstrap value of 94% and included *Trichoderma* isolates CNU639 and C79 and *T. virens* DAOM167651 (ex-type strain). Group 5 comprised *Trichoderma* isolates CNU507 and CNU580, *T. atroviride* DAOM165779, *T. koningii* CBS 101.94, *T. viride* CBS 394.92, *T. pubescens* CBS 345.93, *T. hamatum* CBS 121.13, and *T. asperellum* CBS 433. 97, and was well supported with a bootstrap value of 99%. Group 6 contained *Trichoderma* isolates CNU518 and CNU627, *T.*

pseudokoningii DAOM 167678 (ex-type strain), *T. citrinoviride* CBS258.85 (ex-type strain) and *T. longibrachiatum* CBS816.68 (ex-type strain) and was well supported with a bootstrap value of 100%.

Discussion

Based on the PCR-RFLP analysis of the ITS regions with *Cfo* I, *Hae* III, and *Msp* I, *Trichoderma* species isolated from oyster mushroom farms in Korea were divided into 6 distinct groups. The dendrogram of RFLP patterns produce a phylogeny which was almost identical to those observed by ITS sequences. PCR-RFLP analysis, then, has proven quite useful for the first step of grouping the fungal isolates. Representative strain of each group can then be subjected to further analyses. PCR-RFLP analysis as a molecular marker can readily distinguish a given species or isolates from others (Gams and Meyer, 1998; Muthumeenakshi et al., 1994).

We previously investigated morphological and cultural characteristics of *Trichoderma* isolates associated with green mold of oyster mushroom farms in Korea and identified them as *T. atroviride*, *T. citrinoviride*, *T. harzianum*, *T. longibrachiatum*, *T. virens*, and two unidentified species, *Trichoderma* sp. 1 and 2. *Trichoderma* sp. 2 was the most commonly isolated species and exhibited strong colonization ability on mushroom compost (Park et al., 2005).

Trichoderma sp. 1 and 2 were characterized by RFLP group A (C1, H1, and M1) and group 1 from sequence analysis of the ITS regions. This monophyletic group is clearly distinguished from the previously reported *Trichoderma* species and comprises two subgroups, *Trichoderma* sp. 1 and 2. These subgroups differed from each other on the basis of sequence difference, growth rate at 35°C, colony morphology, conidial shape and branch pattern of phialides (Park et al., 2005). Phylogenetic analysis of ITS sequence revealed that *Trichoderma* sp. 1 and 2 were more closely related to *T. aggressivum* groups, which contained *T. aggressivum* f. *europaeum* (Th 2) and *T. aggressivum* f. *aggressivum* (Th 4) than to *T. harzianum* complex group and formed a monophyletic group supported by a bootstrap value of 78%. However, the result showed that *Trichoderma* sp. 1 and 2 were phylogenetically distinct not only from *T. aggressivum* but also from each other. In Korea, the predominant species associated with green mold epidemic of oyster mushroom was *Trichoderma* sp. 2 followed by *Trichoderma* sp. 1. On the basis of these results and morphological characteristics (Park et al., 2005), we propose that *Trichoderma* sp. 1 and 2 are new species.

Morphological species *T. harzianum* complex was characterized by RFLP group B (C1, H1, and M2) and group 2 from the sequence analysis of the ITS regions. This group included *T. harzianum* KCTC16976 (ex-type strain) and *T. inhamatum* KCTC6384 (ex-type strain) and was well supported with a high bootstrap value. Gams and Meyer (1998) concluded that on the basis of base difference in ITS1 region and RFLP and RAPD analysis, *T. harzianum* and *T. inhamatum* were different species. The present data based on the sequence analysis of the ITS regions showed that interspecific differences of *T. harzianum* complex are low (2-3 nt in ITS regions) and therefore, *T. harzianum* and *T. inhamatum* are indistinguishable since such small sequence differences have previously been shown to be characteristic for intraspecific variability within species of sect. *Longibrachiatum* (Kuhls et al., 1997). Kindermann et al. (1998) suggested that on the basis of sequence analysis of the ITS-1 region alone, *T. inhamatum* is fully contained within the genetic variability of *T. harzianum*. Recently, combined sequence analysis of ITS regions and endochitinase 42 (ech42) suggested that *T. harzianum* and *T. inhamatum* are phylogenetically indistinguishable (Kullning-Gardinger et al., 2002).

Morphological species *T. virens* was characterized by RFLP group C (C1, H1, and M3) and group 3 from the sequence analysis of the ITS regions. This group was clustered with *T. virens* DAOM167651 (ex-type strain) and was well supported with high bootstrap value.

Morphological species *T. atroviride* was characterized by RFLP group D (C2, H1, and M4) and group 4 from sequence analysis of the ITS regions. Group 4 including *T. atroviride*, *T. viride* and *T. koningii* showed very low sequence variation (0.3 to 2.0%). However, these species are distinguished from one another through conidial ornamentation and conidium morphology (Kindermann et al., 1998). CNU507 and CNU580 isolated from oyster mushroom farms in Korea exhibited a sequence identical to that of *T. atroviride* DAOM165779. Those isolates that have morphological characteristics of smooth and subglobose conidia were distinguished from *T. viride* and *T. koningii*.

Morphological species *T. citrinoviride* was characterized by RFLP group E (C3, H2, and M5) and morphological species *T. longibrachiatum* was characterized by RFLP group F (C3, H3, and M5) and exhibited a sequence identical to its ex-type strain, respectively. Kuhls et al. (1997) reported that all species sect. *Longibrachiatum* that were originally classified by morphological observation were clearly distinguished by the sequence analysis of ITS regions. Phylogenetic analysis showed that sect. *Longibrachiatum* forms a strictly monophyletic group within *Trichoderma*.

We propose that PCR-RFLP and sequence analysis of the ITS regions of rDNA in this study could be used as a

reference data for studies involving the identification and taxonomy of *Trichoderma* species isolated from oyster mushroom farms.

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