Phylogenetic analysis of major molds inhabiting woods and their discoloration characteristics. Part 1. Genus *Trichoderma*

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Abstract

The genus *Trichoderma* Persoon is a cosmopolitan genus consisting of more than 104 species and is commonly found on wood surfaces as a mold where it affects the appearance. Little attention has been given to wood-colonizing *Trichoderma* species and few studies are dedicated for identification of *Trichoderma* at the level of species. In the present study, up to 142 isolates were obtained from various wood samples. One *Gliocladium* and ten *Trichoderma* species were identified by morphological and molecular analysis. *T. atroviride* (30.3%) was the most abundant species followed by *T. citrinoviride* (26.8%) and *T. harzianum* (23.9%). The ability of *Trichoderma* species to discolor wood was also examined on sapwood blocks made of the commercially important radiata pine (*Pinus radiata*). *T. pleuroticola* caused the greatest discoloration of the wood surface.

Keywords: mold; *Pinus radiata*; translation elongation factor-1α; *Trichoderma*; wood discoloration.

Introduction

The genus *Trichoderma* (teleomorph Hypocrean, Ascomycota, Hypocreales) is characterized by rapidly growing colonies, the ability to colonize diverse substrates such as soil, decaying wood, and vegetable matter (Samuels 1996), and resistance to harmful chemicals (Kubicek and Harman 1998). The great economic importance of this species is based on the production of industrial enzymes and antibiotics; they are also pathogens of cultivated mushrooms and wood decay fungi (Rossman 1996; Harman and Kubicek 1998). They are also pathogens of cultivated mushrooms and predominant components of soil mycoflora, thus various basic and applied studies were dedicated to soil-associated *Trichoderma* (Widden and Abitbol 1980; Nelson 1982). Nevertheless, *Trichoderma* attracted relatively low interest. The combating of *T. harzianum* with chitosan and chitosan oligomers was investigated by Torr et al. (2005). Gradinger et al. (2008) tried to combat the colonization of pine wood with fungi with a white sporulating mutant of *T. harzianum* as antagonistic microorganism. Obanda et al. (2008) tested the resistance of *T. harzianum* to the biocide tebuconazol.

The genus *Trichoderma* also commonly colonizes wood surfaces. The mycelium produces cellulases, and this is the reason why the fungus grows easily on wood. Usually, the infestation appears in the form of black or greenish patches on the wood surface due to the colored spores. Probably, the little attention focused on wood-colonizing *Trichoderma* species can be explained by the fact that they cause little or no structural damage except for the disfiguration of the surface. Nevertheless, the economic damage is high as discolored wood losses in value.

Classification and identification of the genus *Trichoderma* at the species level based on morphological characteristics alone is difficult: many of them are very similar and the environmental conditions can change the morphological features. Therefore, the molecular approach for their identification is promising (Kindermann et al. 1998). The quoted authors analyzed the whole genus phylogenetically based on the internal transcribed spacer (ITS) region 1 of ribosomal DNA (rDNA). Recently, single gene sequence analysis was considered as insufficient for the determination of phylogenies (O’Donnell et al. 1998; Lieckfeldt and Seifert 2000).

The aim of the present study was to identify and characterize *Trichoderma* species colonizing wood substrates, based not alone on phylogenetic analyses using the multi-loci ITS region and translation elongation factor-1α gene (tef-1α) but also based on phenotypic features. In addition, the ability of this genus to discolor wood was investigated by observing the colonizing patterns of *Trichoderma* species on wood.

Materials and methods

Fungal isolation

Fungal isolation was carried out on the logs and timbers of eight different tree species mainly located in sawmills and log yards in Korea from 2000 to 2009 (Table 1). Wood species investigated included Douglas-fir (*Pseudotsuga menziesii*), Japanese red pine (*Pinus densiflora*), Korean pine (*Pinus koraiensis*), larch (*Larix kaempferi*), Norway spruce (*Picea abies*), radiata pine (*Pinus radiata*), Siberia spruce (*Picea jezoensis*), and tulip tree (*Liriodendron tulipifera*). Sampling sites included sawmills located in Bonghwa, Busan, Gyeongin, Gapyeong, Iksan, Incheon, and Yeouju, as well as a log yard located in Incheon. Further wood included chromated copper arsenate (CCA)-treated larch and Korean pine from Seoul, CCA-treated radiata pine from Incheon, creosote-treated wood from...
Table 1  Trichoderma strains identified by ITS and tef-1α sequences and their optimal growth temperatures.

<table>
<thead>
<tr>
<th>Isolate numbera</th>
<th>GenBank acc. no.</th>
<th>No. of isolates (%) frequencyb</th>
<th>Sourcec</th>
<th>Sample locationd</th>
<th>Closet fungal match (acc. no.)e</th>
<th>S (%)f</th>
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<th>CYA</th>
<th>MEA</th>
<th>PDA</th>
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<td>BH (4)</td>
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<td>LA (1), PD (3)</td>
<td>BH (3), TT (1)</td>
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<td>HM534652</td>
<td>43 (30.3)</td>
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<td>BH (9), BS (3), GM (1), GP (9), IC (9), IS (3), SU (1), YJ (8)</td>
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<td>BH (1), GP (1), IC (2), YJ (3)</td>
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<td>BH (12), BS (5), GI (1), GP (6), IC (11), YJ (3)</td>
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<td>T. citrinoviride</td>
<td>30</td>
<td>32.5</td>
<td>32.5</td>
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aKUC: Korea University Culture Collection, Seoul, Korea.
bGenBank accession numbers of the ITS region are in regular font and the tef-1α region in italic font.
cFrequency=(number of isolates/total isolates)×100.
dCCAKP, CCA-treated Korean pine; CCARP, CCA-treated radiata pine; CTW, creosote-treated wood; DF, Douglas-fir; JP, Japanese red pine; KP, Korean pine; LA, larch; NS, Norway spruce; RP, Radiata pine; SS, Siberia spruce; TT, tulip tree.
eBH, Bonghwa; BS, Busan; GP, Gapyeong; GM, Gwangmyeong; IS, Ikson; IC, Incheon; SU, Seoul; YI, Yongin; YJ, Yeouju.
fSimilarity scores from pairwise alignments of sample sequences with closest BLAST match or reference strains; ITS region in regular font and tef-1α region in italic font.
Gwangmyeong, and beams of CCA-treated Douglas-fir used in cooling towers in Yonjin. The wood surfaces were discolored by microorganisms in patches. To isolate fungi, small wood flecks were removed from each sample. The wood flecks were placed onto sterile malt extract agar (MEA; malt extract 20 g, agar 15 g, distilled water 1000 ml) containing 100 ppm streptomycin to inhibit bacterial growth. The plates were incubated at room temperature for 5–10 days. Subsequent subculturing onto fresh MEA plates ensured that pure cultures were obtained.

Cultures

The colony characteristics of all the isolates were examined from pure cultures grown in darkness at 25°C for 10–14 days on sterile MEA, potato dextrose agar (PDA, Difco potato dextrose agar 39 g, distilled water 1000 ml), and Czapek yeast autolysate agar (CYA, NaNO₃ 3.0 g, K₂HPO₄ 1.0 g, KCl 0.5 g, MgSO₄·7H₂O 0.5 g, FeSO₄·7H₂O 0.01 g, sucrose 30 g, yeast extract 5 g, agar 15 g, distilled water 1000 ml). To determine the optimal growth temperature, growth from the 5-day grown active margin of MEA cultures was inoculated onto fresh MEA and incubated in darkness at 20°C, 22.5°C, 25°C, 27.5°C, 30°C, 32.5°C, and 35°C for a representative strain of each species. Colony radii of three replicas of each isolate were measured after 3 days following incubation.

DNA extraction, PCR, and DNA sequencing

For DNA extraction, mycelia were harvested from the fungal cultures grown on 2% MEA with overlaying sterile cellophane paper. The nuclear rDNA gene cluster was amplified according to the manufacturer’s protocol. The nuclear rDNA gene cluster was amplified by polymerase chain reaction (PCR). The ITS region including the translation elongation factor-1α (tef-1α) region was undertaken according to Samuels et al. (2002). To determine the size of the PCR products, gel electrophoresis was performed on a 1% (w/v) agarose gel in 1× Tris-acetate-ethylenediamine tetraacetic acid buffer at 100 V for 20 min. The PCR products were stained with ethidium bromide and visualized under UV light with an Image Analyzer System. PCR products were purified with the Accuprep PCR purification kit (Bioneer) according to the manufacturer’s instructions, and the purified products were sequenced by MACROGEN (Seoul, Korea), a DNA sequencing facility. The sequences obtained in this study were deposited in GenBank under accession numbers HM534649–HM534663 (Table 1).

Phylogenetic analysis

Related sequences available to the public on the GenBank database were identified using the Trichoderma sequences and the Basic Local Alignment Search Tool (BLAST) algorithm. All the Trichoderma sequences were aligned with the ClustalX 2.0.12 (Larkin et al. 2007). The resulting alignments were corrected manually based on MacClade 4.08 (Maddison and Maddison 2005). The aligned rDNA sequences were phylogenetically analyzed with PAUP* 4.0b10 (Swofford 2002). The method used to find the most parsimonious trees was the close-neighbor-interchange (CNI) heuristic search (search level = 1). The initial trees for the CNI search were based on 10 replications of random addition trees. The trees generated were evaluated by maximum parsimony bootstrap proportions and neighbor-joining (NJ) bootstrap proportions with 1000 replications.

Wood discoloration characteristics

For the wood discoloration test, 100 mm glass Petri dishes containing two pieces of filter paper discs dampened with 3 ml distilled water were prepared. A single U-shaped glass rod was placed on the filter paper and they were sterilized together with sapwood blocks of radiate pine (50×60×50 mm³) by autoclaving at 121°C for 20 min. Moisture contents of sapwood blocks were generally adjusted to 60–80%. Following this, sterile sapwood blocks were placed onto the U-shaped glass rod. Radiata pine was chosen as it is one of the most commercially important tree species in Korea. The logs have been imported from Australia, Chile, and New Zealand. One representative isolate of each fungal species was inoculated onto the bottom of each wood sample from the margin of a 5–6 day grown MEA culture. The Petri dishes were then sealed with parafilm and incubated at 25°C. The degree of surface discoloration by mycelia and spores of Trichoderma species was estimated based on ratings from 0 (no discoloration) to 5 (completely discolored) following 3 weeks incubation. Discoloration characteristics, such as the color of spores and sporulation patterns, were also observed.

Results and discussion

Fungal identification

A total of 142 isolates from eight different tree species in Korea were sequenced for ITS and tef-1α regions and identified at the species level based on morphological, physiological, and genotypic characteristics (Table 1). As a result, 11 species were found in the following proportions: Trichoderma atroviride (30.3%), T. aureoviride (2.8%), T. citrinoviride (26.8%), T. dorotheae (0.7%), T. gamsii (0.7%), T. harzianum (23.9%), T. koningiopsis (2.8%), T. longibachiatum (3.5%), T. pleurotocola (2.8%), and T. viride (0.7%), as well as Gliocladium viride (4.9%), a genus closely related to Trichoderma. The most common species identified on woods, T. atroviride (43 isolates), is also a common species in soil.

Among these 11 species, four species have already been reported to cause plant diseases in Korea: T. harzianum causes black rot, T. longibachiatum causes black button disease, T. pleurotocola causes green mold, and T. viride causes blue mold (The Korean Society of Plant Pathology 2009). In addition, G. viride, T. atroviride, T. aureoviride, and T. citrinoviride have been isolated from soil or plant substrates in Korea, although their pathogenicity has not been proven (Kim et al. 1992; Park et al. 2005; Kim et al. 2010). In contrast, T. dorotheae, T. gamsii, and T. koningiopsis are reported here for the first time in Korea. It is interesting to note that T. dorotheae was obtained only from creosote-treat-
ed wood. This species might possess higher resistance to preservatives other than *Trichoderma* species.

**Phylogenetic analysis**

The maximum parsimony tree obtained from analysis of the ITS sequences is presented in Figure 1. Based on Bissett's research (1991), the genus was subdivided into five sections: *Longibrachiatum*, *Pachybasium*, *Trichoderma*, *Saturnisporum*, and *Hypocreanum*. As a result of our study, the 10 *Trichoderma* species identified were divided into three *Trichoderma* sections. *T. atroviride*, *T. dorotheae*, *T. gamsii*, *T. koningiopsis*, and *T. viride* were included in the section *Trichoderma*. Furthermore, of the two clades in this section, the above-mentioned five species are all in the 'Viride clade'. According to their ITS sequences, they are closely related to each other and show a very low degree of variability maximum: 6% of variable sites (Samuels et al. 2006); hence, it is difficult to separate the sequences within the 'Viride clade' at the species level by means of the single locus of ITS. As shown in Figure 1, it was not possible to differentiate between *T. gamsii* and *T. koningiopsis* on this basis. Therefore, the analysis of the partial sequence of the tef-1α region was used to establish a more detailed identification. In the tef-1α tree (Figure 2), the two species were clearly separated. The section *longibrachiatum* (Figure 1) including *T. longibrachiatum*, *T. citrinoviride*, and *T. citrinoviride* formed a single clade. The section *Pachybasium* contained *T. aureoviride*, *T. pleuroticola*, and *T. harzianum*. The latter has genetically variable characteristics and several phylogenetic species that are not distinguishable by morphology (Chaverri et al. 2003).

**Morphological and physiological characteristics**

Growth rates on MEA, PDA, and CYA were useful for the characterization of *Trichoderma* at the species level (Table 1). Although there were differences of approximately 12–20 mm in radial growth among the strains cultured under the same conditions, generally, growth rate patterns according to temperature were similar for the same strain grown on the three types of media. Mostly, hyphal growth was fastest on PDA media and slowest on CYA media. The most optimal growth temperatures were between 25°C and 27.5°C.
Wood-inhabiting *Trichoderma* species 261

**Figure 2** Phylogenetic tree based on tef-1α sequences of the ‘Viride clade’ in the *Trichoderma* section and parsimony criterion in PAUP*. The dataset is composed of 455 characters, of which 293 were constant, 78 were parsimony uninformative, and 84 were parsimony informative. The bootstrap values are placed on the branches (MP bootstrap proportions/NJ bootstrap proportions). The tree length is 104 steps with consistency index (CI) 0.9227 and retention index (RI) 0.8786. Genetic distances between strains are represented by branch length, and accession numbers are indicated in parentheses. Strains investigated in this study are indicated in bold font.

However, for *T. longibrachiatum* and *T. citrinoviride*, the optimal growth temperature was 32.5 ± 8°C on MEA and PDA media, whereas it was 30°C on CYA media. In general, *T. pleuroticola* grew the fastest at all temperatures.

Concerning colony morphology, in general, the morphologies were not sufficiently striking to distinguish between species. We observed considerable differences in conidial production or the color of the culture among strains of the same species (data not shown). However, among all species, only *T. citrinoviride* produced a yellow pigment that made all three media tested remarkably yellow and *T. gamsii* produced few or no spores on the three media utilized.

A distinctive coconut odor was detected emanating from some colonies. The odor of *T. gamsii* was the strongest, whereas that of *T. atroviride* was weaker. According to Claydon et al. (1987), the characteristic coconut odor of the species can be mostly attributed to an antifungal pyrone that might have potential as a biocontrol agent.

### Wood discoloration characteristics

The frequency of *Trichoderma* growth on wood and the degree of discoloration it causes has to be determined before the search for the most appropriate prevention. In our wood discoloration test on sapwood blocks of radiata pine, colonization developed the following patterns: spores scattered or flocked lumpy. The degree of discoloration was scored from 0 to 5 and the results are listed in Table 2. On the surface of radiata pines, *T. pleuroticola* showed the highest level of wood discoloration, in which small lumps of spores were scattered densely over the entire wood surface. Spores of *T. atroviride* and *T. harzianum* were scattered sparsely on the surface, but small lumps of spores were rarely seen. Although *T. citrinoviride*, *T. koningiopsis*, and *T. viride* produced lumpy spores that were scattered very sparsely on the wood surface, relatively bigger spore lumps were also observed. The color of the spores of *T. citrinoviride* was darker than that of other species. *T. aureoviride* produced light green colored spores, which were scattered over the entire surface. With the species *T. gamsii*, few or no spores on MEA, PDA, and CYA media were produced; however, spores were scattered very lightly over the entire surface, similar to *T. longibrachiatum* and *G. viride*. In contrast, *T. dorotheae*, produced few spores on all three media, and sporulation on the wood surface was very slow and weak, producing light green spores.

The most common *Trichoderma* species colonizing wood, such as *T. atroviride*, *T. citrinoviride*, and *T. harzianum*,...
showed partial and sparse sporulation on radiata pine. Sporulation patterns on the three media and wood surface seem to be not closely related. This might be due to the presence of wood extractives that are partly fungicidal. Moreover, differences in nutrients and environment influence the color. It is possible that Trichoderma species might produce different colonization patterns on other wood species; hence, further research is needed to study the fungal diversity and discoloration patterns of Trichoderma on other commercially important wood species.

Conclusions

In this study, a total of 142 fungal isolates from various wood samples were identified, one of them belonging to Gliocladium and ten Chaverri, P., Castlebury, L.A., Samuels, G.J., Geiser, D.M. (2003) Carbone, I., Kohn, L.M. (1999) A method for designing primer sets Bissett, J. (1991) A revision of the genus Trichoderma pleuroticola showed the strongest wood discoloration ability. This species is infrequently found on radiata pine, but it can cause a more serious problem on other wood species. In contrast, the most common species on wood – T. atroviride, T. citrinoviride, and T. harzianum, T. koningiiopsis, T. longibrachiatum, T. pleuroticola, and T. viride. Among the species, T. dorotheae, T. gamsii, and T. koningiiopsis were observed for the first time in Korea.

Trichoderma pleuroticola showed the strongest wood discoloration ability. This species is infrequently found on radiata pine, but it can cause a more serious problem on other wood species. In contrast, the most common species on wood – T. atroviride, T. citrinoviride, and T. harzianum – resulted in relatively less severe discoloration. However, it should be noted that these are commonly found fungi worldwide and are dominant fungi species on wood. Accordingly, discoloration of wood by Trichoderma is a global problem and, hence, the report of the present study is not limited to Korea.

Acknowledgements

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