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Decay fungi from playground wood products in service using 28S rDNA sequence analysis

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Abstract

In order to establish integrated control strategies of wood degradation, a systematic survey of basidiomycete decay fungi colonizing various wood products in service is a prerequisite to increasing the service life of wood products. As a first step, we initiated a thorough survey of basidiomycete decay fungi colonizing playground wood products. For accurate fungal identification, traditional methods were complemented with molecular methods, including a BLAST search for large-subunit 28S rDNA sequences in Genbank and phylogenetic analysis. A total of 132 basidiomycete fungi, including 30 different fungal taxa, were isolated from 35 playgrounds in 15 different areas. Eight species, *Bjerkandera adusta, Ceriporia lacerata, Gloeophyllum trabeum*, *Peniophora* sp., *Phanerochaete sordida, Schizophyllum commune, Sistotrema brinkmannii*, and *Trametes versicolor*, were predominantly isolated. A combination of traditional and molecular tools allowed a more detailed identification and diversity.

Keywords: decay fungi; playground wood products; 28S rDNA sequence.

Introduction

Wood is employed extensively in industrial applications as structural or raw materials for buildings, utility poles, paper and other products (Zabel and Morrell 1992). However, unless naturally durable or sufficiently treated with preservatives, wood is susceptible to fungal decay in wet environments in which its moisture content rises above the fiber saturation level for extended periods of time. The damage of wood products caused by decay fungi gives rise to danger as well as to economic loss. Therefore, to prevent fungal attack, the extensive use of chemicals and replacement of materials has been applied (Eaton and Hale 1993; Kim et al. 2000). These are not always the best policies because of environmental problems and economic losses, and thus they are being replaced by environmental control or the use of fungal-resistant wood species such as western red cedar (*Thuja plicata* Don.) (Lim et al. 2005). These are strategies that are based on knowledge about the abiotic and biotic requirements of the decay fungi. To achieve this, researchers have examined the nature of decay, associations between fungi, and the damage they cause. Identification and physiology of the fungi have also been taken into account. Therefore, to establish integrated control strategies to prevent wood degradation, a systematic survey of basidiomycete decay fungi colonizing various wood products in service is a prerequisite.

Rapid and accurate identification of decay fungi is the most important step in systematic surveys. Traditionally, product fungi are identified by cultural characteristics (Duncan and Lombard 1965; Nobles 1965; Lombard and Chamuris 1990; Stalpers 1978; Wilcox and Dietz 1997). However, this approach has many limitations. Traditional methods cannot differentiate the mycelia of closely related species. Thus, several molecular methods have been applied to identify fungi on wood in service, i.e., randomly amplified polymorphic DNA (RAPD) analysis (Theodore et al. 1995; Schmidt and Moreth 1998), internal transcribed spacer-fragment restriction length polymorphism (ITS-FRLP) (Schmidt and Moreth 1999), polymerase chain reaction (PCR) with species-specific primers (Moreth and Schmidt 2000), and rDNA region sequence analyses (Schmidt and Moreth 2002, 2003; Schmidt et al. 2002; Högberg and Land 2004). Among these molecular techniques, procedures using DNA sequence analyses might be effective methods. The wealth of sequence information that has been compiled in the international databases GenBank, EMBL, and DDBJ means that it is now possible to identify fungi at a far higher resolution using molecular techniques. However, there is a potential risk when using BLAST searches. These international databases are open-source databases in which sequences have been deposited under incorrect species names or bad sequence profiles (cf. Högberg and Land 2004). Therefore, both phylogenetic and morphological studies should also be considered when trying to identify a particular species of fungus.

This study is the first report on the diversity of decay fungi from wood products in playgrounds in service in Korea. To identify fungi accurately, classical methods were complemented with molecular methods, including phylogenetic analysis and a 28S rDNA sequence BLAST search.

Materials and methods

Fungal isolation and identification using traditional methods

For fungal isolation, a number of wood cores were collected from various facilities (i.e., bench chairs, fence posts, picnic

tables, barriers, and palisades) on 35 playgrounds in 15 different areas of Korea (Table 1). Wood cores were obtained from points which, based on their visible characters, were expected to be possible sources of decay. These samples came from areas well above the ground level. All the wood products, which were mostly softwoods, were not previously treated with preservatives. A few hardwoods were also found from small towns or several areas near the seaside. Basidiomycete decay fungi were isolated following Wilcox and Dietz (1997) using 2% Difco malt extract agar (MEA, 20 g of Difco malt extract, 15 g of Difco agar, and 1000 ml of distilled water) and 2% Difco MEA with 4 ppm benomyl (BMEA) (Clubbe and Levy 1977). For light microscopy, fungal structures were mounted in water and observed using a Zeiss Axioplan light microscope. Isolates were organized into groups based on cultural characteristics described by Nobles (1965) and Stalpers (1978).

DNA extraction, PCR amplification and sequencing

DNA was extracted from mycelium as described by Lim et al. (2005). To achieve PCR amplification of the 28S rDNA region, primers LR0R and LR3 (http://www.biology.duke.edu/fungi/mycolab/primers.htm) were used. PCR amplification was performed as described by Kim et al. (2004). The PCR products were purified using a Qiaquick PCR Purification Kit (Qiagen, Mississauga, ON, Canada). Sequencing was performed on an ABI 3700 automated sequencer (Perkin-Elmer, Foster City, CA, USA) at the MACROGEN DNA Synthesis and Sequencing Facility (Seoul, South Korea). All of the nucleotide sequences determined in this work have been deposited in GenBank, and their accession numbers are shown in Table 1.

BLAST search and phylogenetic analysis

Identification of the decay fungi using the 28S rDNA region sequences was achieved via a BLAST search in GenBank (Altschul et al. 1994). The closest matching sequences for each species are shown in Table 1. For the phylogenetic analysis, closely matching sequences available in GenBank were downloaded and aligned with our 28S rDNA sequences using the Clustal X algorithm (Thompson et al. 1997). Then, manual adjustment of the alignments was carried out with the PHYDIT program version 3.2 (http://plasza.snu.ac.kr/~jchun/phydit/). The phylogenetic tree was constructed with PAUP*4.0b10 (Swofford 2002) using the parsimony method. Heuristic searches using tree-bisection reconnection branch swapping and 100 random-taxon sequence additions were also employed. Gaps were treated as missing data. Branch stability was assessed by 1000 replicate parsimony bootstrap replications implemented with PAUP*4.0b10. Based on the phylogenetic results of previous studies (Hibbett and Thorn 2001), *Auricularia auricular-judae* (AF291289) was chosen as an outgroup taxon.

Results

Isolation and morphological grouping of fungi

We investigated the fungal diversity of playground wood products. It was not our aim to consider any possible influence of the wood species colonized nor if the timber had been chemically preserved, but to obtain a general overview of the fungi present. A total of 132 basidiomycete fungi were isolated from 15 sampling sites (Table 1). The fungal diversity was highest in Seoul with 18 taxa, followed by Yangpyeung, Gwacheon, and Suncheon with nine, six, and five different taxa, respectively. Single taxons were collected from Bongwha, Chungju, Donghae, and Jinju (Table 1). Only three isolates, *Bjerkandera adusta, Hyphoderma praetermissum*, and *Schizophyllum commune*, were identified at the species level based on morphology (Noble 1965; Stalpers 1978). We grouped the remaining isolates by macro- and microscopic observations, i.e., mycelial shape and color, growth rate, hyphal type, presence of clamp-connections, etc. These allowed the recognition of 32 fungal taxa including the three species identified.

Molecular identification

The sequenced 28S rDNA sizes of representative isolates ranged from 617 to 668 bp. In most cases, two or more isolates from each group were sequenced. Two of the isolated sequences (KUC8138 *Ceriporia lacerata* and KUC80041 *S. commune*) were exactly the same as two of the other isolates (KUC8090 and KUC8016). Therefore, 30 representative sequences were analyzed for their identification. Sequence analysis could link most morphologically unidentifiable fungi to established genera or species (Table 1). The BLAST search in the databases showed that the sequences of our isolates have over 94.6% homology with their closest matching sequences. These closest matching species are listed in Table 1.

The phylogenetic analyses and morphological reassessment allowed a more detailed identification. In most cases, there was agreement concerning assignment to the species level when pairwise similarity scores from BLAST searches were over 97.9% (Table 1). However, some discrepancies were found between BLAST searches and final identification. KUC8075 matched with *Ceriporiopsis subvermispora* (99.8%), but it was identified finally as *Irpex lacteus*. The same results were shown for *C. lacerata* (KUC8090), *Schizopora flavipora* (KUC8006), and *Schizopora paradoxa* (KUC8140). We identified the species in question as different from the BLAST search result when we carried out phylogenetic analysis and morphology again. This is not surprising, because the phylogeny and morphology of the two species are very close (Table 1).

The identification of isolates was supported by establishing a phylogenetic tree (Figure 1). Phylogenetic positions of all isolates were consistent with previous studies (Hibbett and Thorn 2001; Lim 2001; Larsson et al. 2004). Two-thirds of the fungi isolated were grouped into the polyporoid clade. There were nine species of brown rot fungi and 21 of white rot fungi (Table 1). The fungal frequencies are summarized in Table 1, which also includes the dominant species. The dominant species, according to the index of Camargo (1993), were *B. adusta, C. lacerata, Gloeophyllum trabeum, Peniophora* sp., *Phanerochaete sordida*, *S. commune, Sistotrema brinkmannii*, and *Trametes versicolor*.

Discussion

For fungal identification, we used a combination of traditional and molecular methods. Species identification by morphology alone has been widely used in previous works (Duncan and Lombard 1965; Lombard and

Table 1 Basidiomycete decay fungi isolated from playground wood products in service.

Isolate number	GenBank accession	No. of isolates (% frequency)[a]	Sample location[b] (number of isolates)	Closest fungal match (accession number)	S (%)[c] (LSU)	Fungal identity	Rot type[d]	Family[e]
KUC8005	AY858349	1 (0.8)	SL (1)	*Amphinema byssoids* (AF518597)	98.1	*Amphinema byssoides*	W	C
KUC8002	AY858350	1 (0.8)	SL (1)	*Antrodia serialis* (AJ406519)	100.0	*Antrodia serialis*	B	P
KUC8011	AY858351	1 (0.8)	SL (1)	*Antrodia xantha* (AJ583430)	95.9	*Antrodia* sp.	B	P
KUC8072	AY858352	11 (8.3)*	SC (2), MP (1), YP (1), SL (4), GC (3)	*Bjerkandera adusta* (AF287848)	98.3	*Bjerkandera adusta*	W	P
KUC8075	AY858353	3 (2.3)	SL (3)	*Ceriporiopsis subvermispora* (AF287853)	99.8	*Irpex lacteus*	W	P
KUC8001	AY858354	2 (1.5)	SL (2)	*Crustoderma flavescens* (AY219387)	94.6	*Crustoderma* sp.	B	C
KUC8065	AY858355	1 (0.8)	SL (1)	*Crustoderma corneum* (AY219386)	95.3	*Crustoderma* sp.	B	C
KUC8020	AY858356	1 (0.8)	YJ (1)	*Cryptoporus volvatus* (AF393050)	98.6	*Cryptoporus volvatus*	W	P
KUC8090	AY858357	10 (7.6)*	SC (1), BS (1), GS (3), SL (2), GC (3)	*Cystidiodontia isabellina* (AJ406570)	97.9	*Ceriporia lacerata*	W	P
KUC8010	AY858358	1 (0.8)	SL (1)	*Fomitopsis pinicola* (AF287858)	97.9	*Fomitopsis pinicola*	B	P
KUC3030	AY858359	1 (0.8)	SL (1)	*Funalia trogii* (AJ457810)	99.8	*Funalia trogii*	W	P
KUC8053	AY858360	3 (2.3)	GL (3)	*Gloeophyllum sepiarium* (AF393059)	97.1	*Gloeophyllum* sp.	B	P
KUC8013	AY858361	12 (9.1)*	SL (11), SC (1)	*Gloeophyllum trabeum* (AF139948)	99.5	*Gloeophyllum trabeum*	B	P
KUC8031	AY858362	1 (0.8)	YP (1)	*Hyphoderma praetermissum* (AF518621)	99.7	*Hyphoderma praetermissum*	W	C
KUC8006	AY858363	4 (3.0)	SL (3), DH (1)	*Hyphodontia radula* (AJ406466)	99.8	*Schizopora flavipora*	W	P
KUC8140	AY858364	1 (0.8)	MP (1)	*Hyphodontia radula* (AJ406466)	99.5	*Schizopora paradoxa*	W	P
KUC8046	AY858365	1 (0.8)	YP (1)	*Hypochnicium eichleri* (AJ406508)	99.1	*Hypochnicium eichleri*	W	C
KUC8019	AY858366	3 (2.3)	BD (1), GL (1), SL (1)	*Hypochnicium eichleri* (AJ406508)	97.6	*Hypochnicium* sp.	W	C
KUC8015	AY858367	5 (3.8)*	SL (2), BH (1), GS (1), GC (1)	*Peniophora cinerea* (AF506424)	96.8	*Peniophora* sp.	W	C
KUC8037	AY858368	6 (4.5)*	SL (1), YP (4), BS (1)	*Phanerochaete sordida* (AJ406532)	98.5	*Phanerochaete sordida*	W	C

(Continued)

Isolate number	GenBank accession	No. of isolates (% frequency)[a]	Sample location[b] (number of isolates)	Closest fungal match (accession number)	S (%)[c] (LSU)	Fungal identity	Rot type[d]	Family[e]
KUC8034	AY858369	2 (1.5)	YP (2)	*Phlebia radiata* (AJ406541)	99.1	*Phlebia radiata*	W	C
KUC8041	AY858370	3 (2.3)	YP (2),GC (1)	*Phlebia subserialis* (AF141631)	98.6	*Phlebia subserialis*	W	C
KUC8093	AY858371	3 (2.3)	GC (3)	*Phlebiella griseofulva* (AJ406517)	99.5	*Phlebiella griseofulva*	W	C
KUC8048	AY858372	2 (1.5)	YP (1), GL (1)	*Rhizochaete filamentosa* (AY219393)	97.3	*Cystidiophorus castaneus*	W	C
KUC8073	AY858373	1 (0.8)	SL (1)	*Rhizochaete filamentosa* (AY219393)	95.9	*Phanerochaete* sp.	W	C
KUC8016	AY858374	20 (15.2)*	SL (5), CJ (1), YJ (1), GC (8), SC (1), AM (1), GS (1), MP (2)	*Schizophyllum commune* (AF261587)	99.7	*Schizophyllum Commune*	W	S
KUC8014	AY858375	10 (7.6)*	SL (2), GL (6), SC (1), BS (1)	*Sistotrema brinkmannii* (AJ406430)	98.2	*Sistotrema brinkmannii*	B	C
KUC8044	AY858376	1 (0.8)	YP (1)	*Stereum hirsutum* (AF393078)	99.8	*Stereum hirsutum*	W	C
KUC8018	AY858377	1 (0.8)	BD (1)	*Tapinella panuoides* (AF287879)	100.0	*Tapinella panuoides*	B	C
KUC8035	AY858378	20 (15.2)*	JJ (3), AM (1), BS (8), MP (6), YP(2)	*Trametes versicolor* (AF347107)	100.0	*Trametes versicolor*	W	P

[a]Frequency = (no. of isolates/total isolates) × 100.

[b]SL, Seoul; YP, Yangpyeung; SC, Suncheon; MP, Mokpo; GC, Gwacheon; BS, Busan; GS, Gunsan; YJ, Yeoju; GL, Gangleung; DH, Donghae; BD, Bundang; BH, Bongwha; CJ, Chungju; AM, Anmyeondo; JJ, Jinju.

[c]Similarity scores from pairwise alignments of sample sequences with closest BLAST match or reference strains.

[d]W, white rot; B, brown rot (Gilbertson and Ryvarden 1986, 1987; Ginns and Lefebvre 1993).

[e]C, Corticiaceae; P, Polyporaceae; and S, Schizophyllaceae (Gilbertson and Ryvarden 1986, 1987; Ginns and Lefebvre 1993).

*Dominant species. A species is considered dominant if Pi > 1/S, where Pi (frequency of species i/total frequency for all species) is the proportion of the total sample represented by species i, and S (species richness) is the number of competing species present in the community (Camargo 1993).

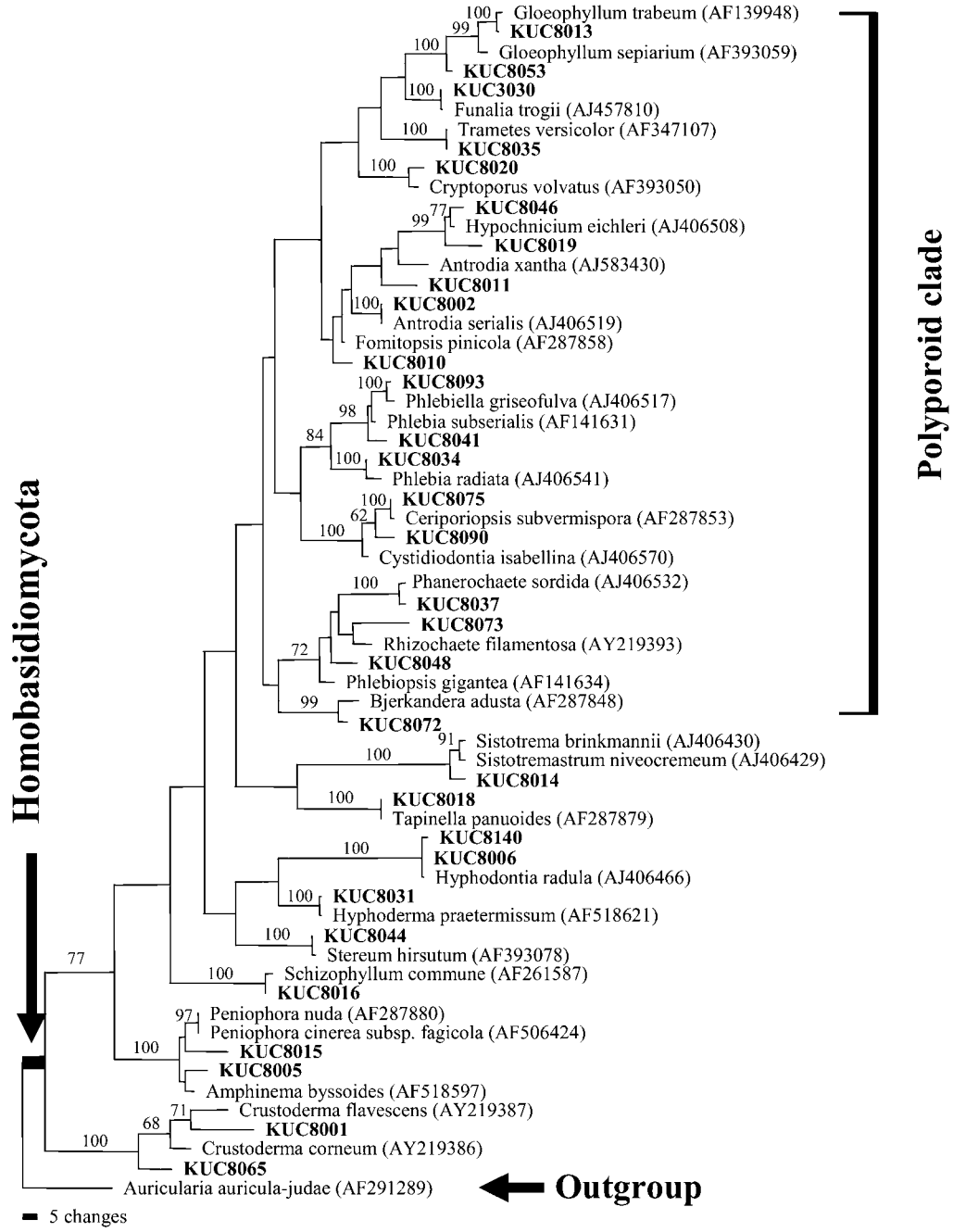

Figure 1 Phylogenetic tree based on partial 28S rDNA sequences of decay fungi isolated from the playground wood products service and reference strains from GenBank. This tree is one of 36 most parsimonious trees ($-\ln L = 6120.7409$) with 975 steps, a consistence index of 0.3928 and a retention index of 0.7427. Numbers above the branches are bootstrap values when numbers are greater than 60%. Newly produced sequences are in bold type. References, which were taken from GenBank, are in plain type. Accession numbers are indicated in parentheses.

Chamuris 1990; Wilcox and Dietz 1997). This method does not always give clear identification. In addition, it is time-consuming, labor-intensive and requires extensive knowledge. However, a combination of traditional and molecular methods is more effective for identifying fungal cultures. The identification using a combination of methods was carried out by as stepwise approach. First, all isolates were grouped based on their morphological characters. Second, one or two representative strains were selected from each group and sequenced. BLAST database searches were performed with partial 28S rDNA sequences as queries to reveal relationships to published sequences. Third, a phylogenetic tree was constructed to explore the sequence data within a phylogenetic context. These molecular approaches could link most morphologically unidentifiable isolate groups to established genera, clades or species. Finally, detail morphological features were used to confirm the final correct identification.

Most culturable bacteria and yeast can be identified from their nucleotide divergence only in the 16S rDNA and the D1/D2 domain of 26S rDNA, respectively (Olsen and Woese 1993; Kurtzman and Robnett 1995, 1998). In this study, the closest matching species from the data-

bases with over 97.9% sequence similarity were conspecific to our final identified fungi (Table 1). However, some exceptions were shown in *I. lacteus* (KUC8075), and two *Schizopora* species (KUC8006 and KUC8140). In case the sequences from related species that are correctly identified are not in the databases, the identification of the fungi cannot be determined using sequence data alone even though their sequence similarities are quite high (Table 1). Each corresponding species is very morphologically similar (Gilbertson and Ryvarden 1986, 1987) and phylogenetically related (Langer 1998; Paulus et al. 2000). In these cases, phylogenetic knowledge as well as morphological consideration is needed to confirm their identification.

The spectrum of isolates recovered from playground wood products might be biased, since not all decay fungi in playground wood products will grow equally well on both MEA and BMEA media. Furthermore, we did not attempt to sequence all of the individual isolates and other variable genes, such as the internal transcribed spacer. The ITS region has been preferentially used in the identification of fungi on wood in service, such as house-rot fungi, because of its high sequence and length variability (Schmidt and Moreth 2002; Högberg and Land 2004). This might have led to a further increase in the knowledge of different fungal taxa occurring on wood. Therefore, the number of decay fungi associated with playground wood products is certainly higher than is shown in the present study. The 28S rDNA sequences databases have grown greatly compared to ITS sequences of basidiomycete decay fungi. However, the 28S rDNA sequence data available for basidiomycete decay fungi are still incomplete. There have been several instances for which the closest GenBank match was only distantly related to the query sequence (Table 1).

Most of the decay fungi detected in this study have previously been reported with fruiting bodies in Korean forests (Jung 1994, 1995; Lee and Lee 2000; Lim 2001). However, *C. lacerata*, *Crustoderma* spp., *G. trabeum* and *H. praetermissum* are reported here for the first time. In other countries, *C. lacerata* and *Crustoderma* spp. have been reported from rotten wood (Ginns and Lefebvre 1993; Suhara et al. 2003) and *G. trabeum* and *H. praetermissum* from various wood products in use (Duncan and Lombard 1965; Viitanen and Ritschkoff 1991; Wilcox and Dietz 1997; Lim et al. 2005). These species might prefer decayed wood or dried wood products, and would thus rarely form fruiting bodies in the forest. Fruiting bodies are not commonly found on wood products, because decayed wood is usually discarded before the fungus has formed a fruiting body. This might be one of the reasons why these species have not been reported in previous surveys based on fruiting bodies. Consequently, these results might be useful in distinguishing between fungi that decompose mainly forest residues and those that significantly degrade wood after processing.

Many polypore Basidiomycete decay fungi have been reported in various wood products (Lemke 1964; Scheffer et al. 1984; Ginns 1986). A total of 13 polypore decay fungi were detected in this study. Among them, *B. adusta*, *C. lacerata*, *G. trabeum*, and *T. versicolor* were predominantly isolated from different sites. They have a reputation in wood products as well as standing and fallen trees due to their decay ability (Duncan and Lombard 1965; Eslyn and Highley 1976; Zabel and Morrell 1992; Wilcox and Dietz 1997; Suhara et al. 2003). In addition to polypore fungi, many corticioid fungi were also found in wood products in the present study. Corticioid fungi are characterized by resupinate fruit bodies. Many of these fungi are white-rot fungi capable of breaking down cellulose, hemicelluloses and lignin, whereas some, such as *Coniophora puteana* and *Serpula lacrymans*, are brown rotters that do not degrade lignin. They are distributed in all phylogenetic clades of Homobasidiomycetes (Hibbett and Thorn 2001; Lim 2001; Larsson et al. 2004). The most well-known corticioid fungi in wood in service are *Coniophora puteana*, *Phanerochaete chrysosporium*, *Serpula lacrymans*, and *Stereum sanguinolentum* (Duncan and Lombard 1965; Burdall 1985; Palfreyman et al. 1995; Schmidt et al. 2002; Högberg and Land 2004). Although many of the corticioid fungi cause serious heart or root rot of living and dead trees, they are often overlooked because of difficulty in identification. Nowadays, many different species have been reported from wood products because the sequence information for corticioid fungi has increased (Lim et al. 2005). Therefore, our survey supports the hypothesis that corticioid fungi might play an important role in deterioration of wood products.

Although most of the wood products used in this study were softwoods in contact with the ground, white rot fungi were approximately three times more prevalent than brown rot species (Table1). Similarly, several studies reported that, with respect to wood products in ground contact, white rot fungi were observed as often, and in some cases more frequently, on softwoods than brown rot fungi (Zabel and Moore 1958; Duncan and Lombard 1965; Butcher 1968). Given this, our results support the suggestion by Butcher (1968) that white rot fungi generally precede brown rot fungi in the colonization of untreated softwoods in contact with the ground. However, it is important to note that brown rot fungi cause advanced decay in softwood products in contact with the ground (Duncan and Lombard 1965; Butcher 1968; Stephan et al. 1996). For example, *Antrodia vaillantii* destroys timbers in contact with the ground, even if it was properly impregnated with chrome-copper salts (Stephan et al. 1996).

Nine brown-rot fungi (30% of total diversity) were detected, even though they were less frequently isolated in this study. Brown rot fungi rapidly and drastically reduce wood strength in the early stages of decay, while white rot fungi cause a slower, progressive decrease in wood strength (Wilcox 1978; Zabel and Morrell 1992; Kim et al. 1996). Identification of the presence of decay in its early stages (i.e., incipient decay) by microscopic examination can be uncertain (Wilcox 1968). Nevertheless, it is very important to note that incipient decay can cause a significant reduction in wood strength. For this reason many methods have been developed to detect incipient wood decay prior to the occurrence of significant strength loss (Clausen 1997; Jasalavich et al. 2000; Moreth and Schmidt 2000; Schmidt and Moreth 2000).

We also anticipate that our results will provide initial data for detecting both incipient decay and brown rot fungi.

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