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Diversity of fungi in creosote-treated crosstie wastes and their resistance to polycyclic aromatic hydrocarbons

Min-Ji Kim · Hwanhwi Lee · Yong-Seok Choi · Gyu-Hyeok Kim · Na-Yoon Huh · Sangjoon Lee · Young Woon Lim · Sung-Suk Lee · Jae-Jin Kim

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Abstract This study was conducted to generate information regarding the diversity of fungi inhabiting creosote-treated wood in a storage yard for crosstie wastes in Gwangmyeong, Korea. Additionally, the resistance to polycyclic aromatic hydrocarbons (PAHs) of indigenous fungi that mainly occupy creosote-treated wood was evaluated. We isolated fungi from the surface and inner area of crosstie wastes and identified them using a combination of traditional methods and molecular techniques. Overall, 179 isolates including 47 different species were isolated from 240 sampling sites. The identified fungal species included 23 ascomycetes, 19 basidiomycetes, and 5 zygomycetes. Three species, *Alternaria alternata*, *Irpex lacteus*, and *Rhizomucor variabilis*, were the most frequently isolated ascomycetes, basidiomycetes, and zygomycetes, respectively. The results of this study showed that there was a large difference in the fungal diversity between the surface and the inner area. Additionally, zygomycetes and ascomycetes were found to have a greater tolerance to PAHs than basidiomycetes. However, two basidiomycetes, *Heterobasidion annosum* and *Schizophyllum commune*, showed very high resistance to PAHs, even in response to the highest concentration (1,000 ppm), which indicates that these species may play a role in the degradation of PAHs.

Keywords Creosote-treated wood · Crosstie waste · Fungal diversity · Polycyclic aromatic hydrocarbons · White-rot fungi

M.-J. Kim · H. Lee · Y.-S. Choi · G.-H. Kim · N.-Y. Huh · S. Lee · J.-J. Kim (✉)
Division of Environmental Science & Ecological Engineering, College of Life Sciences & Biotechnology, Korea University, 5-1 Anam-dong, Seongbuk-gu, Seoul 136-701, Korea
e-mail: jae-jinkim@korea.ac.kr

Y. W. Lim
National Institute of Biological Resources, Incheon, Korea

S.-S. Lee
Division of Forest Bioenergy, Korea Forest Research Institute (KFRI), 57 Hoegiro, Dongdaemun-gu, Seoul 130-712, Korea

Introduction

It is generally accepted that creosote is a major wood preservative. Creosote-treated wood (CTW) has commonly been used for railroad crossties and landscape facilities in Korea, as well as in the rest of the world. As a result, creosote contamination of surface soils, segments, and groundwater is commonly observed in areas in which CTW was used (Mueller et al. 1989). Due to the adverse affects of creosote, the recycling of CTW has been restricted in

Korea. In addition, an amendment of the Enforcement Decree of the Wastes Control Act strengthened the restriction of the use of CTW in February 2009.

Creosote is a chemical mixture composed of approximately 85% polycyclic aromatic hydrocarbons (PAHs), 10% phenolic compounds, and 5% *N*-, *S*- and *O*-heterocyclics (Mueller et al. 1989). PAHs not only occur naturally in fossil fuels, but also form during the incomplete combustion of organic materials (Freeman and Cattell 1990; Lim et al. 1999). PAHs are also ubiquitous environmental pollutants that are commonly found in soil at wood preservation plants and gasworks (Potin et al. 2004). Many PAHs cause harmful effects to both humans and animals due to their carcinogenic and mutagenic properties.

As a result, many remediation methods have been developed to detoxify environmental pollutants, including PAHs. They can be removed or converted into less harmful products by the indigenous microbiological community of the contaminated environment (Galli et al. 2008). Indeed, fungi are known for their remarkable ability to degrade complex and persistent natural materials such as lignin and microcrystalline cellulose. Additionally, many fungi produce extracellular enzymes involved in the transformation of lignin and the degradation of xenobiotic compounds with aromatic structures (Vyas et al. 1994; Collins et al. 1996; Rabinovich et al. 2004). White-rot fungi have generated a great deal of interest because they possess a ligninolytic enzyme system. For example, Atagana et al. (2006) evaluated the ability of various fungi from creosote-contaminated soil to remove PAHs. They found that a white-rot fungus belonging to the genus *Pleurotus* was better able to degrade creosote than other organisms studied, and that mixed fungal cultures more effectively degraded creosote than did pure cultures.

There is currently insufficient information regarding the diversity of microorganisms collected directly from contaminated environments to facilitate their use in degrading creosote. As a result of some studies, only a few fungal species have been used as major degraders in bioremediation. Consequently, it is necessary to identify indigenous fungi for bioremediation applications.

Here, we report on the diversity of CTW-inhabiting fungi. The information provided in this study may be integrated with existing research regarding indigenous fungal communities in CTW, and should serve as a foundation of useful information for future studies. The resistance of fungi to various concentrations of PAHs was also examined for identifying indigenous fungal species useful for biodegradation.

Materials and methods

Fungal isolation

Five creosote-treated tropical hardwood timbers (15 × 24 cm and 250 cm in length) were collected from the storage yard for crosstie wastes (CWs) containing creosote in Gwangmyeong, Korea. The samples had been used as railroad crossties for over 20 years prior to disposal. Weathering and/or deterioration of the CW samples were detected by the naked eye. Each CW piece was cut horizontally into approximately 10 cm blocks and then six rectangular pieces were randomly selected from each CW (Fig. 1). To isolate fungi, eight small wood flecks (about 0.5 × 0.5 cm and 1 cm in length) were removed from each piece. Four flecks were taken from the surface, and the remaining four samples were then aseptically detached from the treated area inside the cracked sections after dividing the pieces perpendicularly and horizontally. Inner area samples were obtained from areas treated with creosote. The wood flecks were placed on 2% Difco malt extract

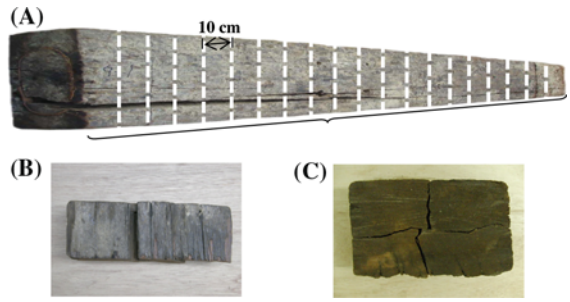

Fig. 1 Schematic diagram for fungal isolation from crosstie wastes. In the example shown, a crosstie material was cut horizontally into 10 cm slices and then six pieces were randomly selected from each CW (**a**). Eight small wood flecks were removed from each piece. Four flecks were taken in each of the four directions from the surface (**b**), and the remaining four samples were then aseptically detached from the treated area inside the cracked sections after dividing the pieces perpendicularly and horizontally (**c**)

agar (MEA, 20 g Difco malt extract and 15 g Difco agar per liter) with 100 ppm streptomycin (SMEA) and 2% SMEA with 4 ppm benomyl to isolate general fungi and basidiomycetes, respectively. The plates were incubated at room temperature for several weeks, and fungi were routinely subcultured from the mycelial margins to new plates to obtain pure cultures.

DNA extraction, PCR and sequencing

DNA was extracted from the mycelium following the method described by Lim et al. (2005). To achieve PCR amplification of the 28S rDNA and the internal transcribed spacer (ITS) region, two primer sets, LROR/LR3 (Vilgalys and Hester 1990) and ITS4/ITS5 (White et al. 1990), respectively, were used. In general, the ITS region was used to identify ascomycetes and zygomycetes, while the partial large subunit was used to precisely identify basidiomycetes due to sequence conservation in this taxon. Briefly, PCR amplification was conducted as described by Lee et al. (2000), after which the PCR products were purified using a PCR purification kit (Bioneer Inc., Daejeon, Korea). Amplified fragments were sequenced at the MACROGEN DNA Synthesis Sequencing Facility (Seoul, South Korea). Fungi were identified through a BLAST search of the GenBank database (Altschul et al. 1994). The closest matching species from the databases were over 97.5% homologous to those in ascomycetes and zygomycetes, or 98.5% homologous with those in basidiomycetes. All of the sequences determined in this study have been deposited in GenBank, and their accession numbers are shown in Table 1.

Analysis of PAHs in creosote-treated wood samples

For analysis of the PAHs in the CW samples, five different samples of the inner (treated) portion and surface of the tested wood were hammer-milled and then ground to ensure passage through a US standard 40-mesh screen (425 μm). The sawdust was then extracted in 100 ml methylene chloride in an ultrasonic bath for 45 min. Next, the extract was analyzed using a gas chromatography mass spectrometer (GC–MS) equipped with a VB-1 capillary column (VICI). The injector temperature was 330°C, and helium was supplied as the mobile phase at rates of 2 ml/min. The GC program consisted of an initial temperature of 100°C that was increased to 300°C at 10°C min^{-1}, then to 335°C at 15°C min^{-1}, where it was held for 10 min.

Determination of PAH resistance

Fungal resistance to a mixture of six PAH compounds, anthracene, fluoranthene, phenanthrene, pyrene, fluorene, and acenaphthene, was evaluated (Sigma–Aldrich, USA); these compounds were selected based on the composition of PAHs in the CW samples. All fungi were tested on 2% MEA supplemented with four total creosote concentrations (0, 250, 500, and 1,000 mg l^{-1}) containing these PAHs. The six PAH compounds were simultaneously dissolved in acetone prior to addition to the agar. Additionally, a known volume of acetone was used to spike the agar to provide a constant for the controls and all concentrations of PAHs (Lamar et al. 1999). A small fungal disc removed from the actively growing edge of each of the cultures was placed on the PAH media, and the samples were then incubated at room temperature until the fungi were fully grown on three replicate control plates (0 ppm PAH medium). During the incubation period, the growth rate was measured on a daily basis as the diameter of the colony.

Results and discussion

Fungal isolation and identification

A total of 179 fungal isolates was recovered from 240 sampling points in 30 block pieces randomly selected from five CWs (Table 1). One hundred and twenty and 59 isolates were obtained from the surface and the inner area, respectively. The macro- and microscopic characterization of the isolates allowed us to recognize 47 groups that were allotted to 23 ascomycete, 19 basidiomycete, and 5 zygomycete taxa. In order to further assign the isolates to the species level, the LSU rDNA region in basidiomycetes and the ITS region in ascomycetes and zygomycetes were sequenced from one selected isolate from each macro- and microscopically characterised group. Sequences of LSU rDNA regions have been used

Table 1 Fungi isolated from discarded creosote-treated wood

Isolate No.	GenBank acc. No.	No. of isolates[a]		Closest fungal match (acc. No.)	Similarity (%)[b]	Fungal identity
		S	I			
Ascomycetes						
KUC5005	GQ241273	24[c]		*Alternaria alternata* (AY433814)	548/550 (99.6)	*Alternaria alternata*
KUC5006	GQ241274	1		*Ampelomyces* sp. (EF672292)	514/515 (99.8)	*Ampelomyces* sp.
KUC5008	GQ241275		3[d]	*Byssochlamys nivea* (DQ322215)	577/583 (99.0)	*Byssochlamys nivea*
KUC5009	GQ241276	6[c]	1	*Cladosporium cladosporioides* (EU030342)	524/524 (100.0)	*Cladosporium cladosporioides*
KUC5010	GQ241277	3		*Curvularia trifolii* (AF455446)	584/586 (99.7)	*Curvularia trifolii*
KUC5011	GQ241278	1		*Epicoccum nigrum* (EU232716)	518/524 (98.9)	*Epicoccum nigrum*
KUC5012	GQ241279	1		*Exserohilum fusiforme* (AF163063)	544/546 (99.7)	*Exserohilum fusiforme*
KUC5013	GQ241280	3		*Fusarium culmorum* (AY147336)	522/523 (99.8)	*Fusarium culmorum*
KUC5014	GQ241281	1		*Hyalodendriella betulae* (EU040233)	533/545 (97.8)	*Hyalodendriella* sp.
KUC5007	GQ241282		1	*Paecilomyces lilacinus* (AB103380)	576/577 (99.8)	*Paecilomyces lilacinus*
KUC5016	GQ241283		2	*Paecilomyces carneus* (AB258369)	548/581 (93.8)	*Paecilomyces* sp.
KUC5015	GQ241284		5[d]	*Paecilomyces variotii* (FJ011547)	573/574 (99.8)	*Paecilomyces variotti*
KUC5017	GQ241285	3		*Penicillium griseofulvum* (EU497956)	534/534 (100.0)	*Penicillium griseofulvum*
KUC5018	GQ241286	3		*Penicillium janthinellum* (EU833221)	567/569 (99.6)	*Penicillium janthinellum*
KUC5019	GQ241287	1		*Pestalotiopsis photiniae* (AY682939)	528/532 (99.3)	*Pestalotiopsis photiniae*
KUC5020	GQ241288	1		*Pestalotiopsis vismiae* (FJ481027)	586/587 (99.8)	*Pestalotiopsis vismiae*
KUC5021	GQ241289		4[d]	*Phaeoacremonium rubrigenum* (AF197978)	559/562 (99.5)	*Phaeoacremonium rubrigenum*
KUC5023	GQ241290	6[c]	2	*Phialocephala dimorphospora* (AF486121)	544/548 (99.3)	*Phialocephala dimorphospora*
KUC5029	GQ241291	1		*Phialophora mustea* (AF083194)	543/545 (99.6)	*Phialophora mustea*
KUC5024	GQ241292	1		*Phoma glomerata* (FJ481024)	515/520 (99.0)	*Phoma glomerata*
KUC5025	GQ241293	1		*Pteris tremula* (AM920398)	478/483 (99.0)	*Pteris tremula*
KUC5026	GQ241294	7[c]	2	*Trichoderma atroviride* (EU272523)	587/588 (99.9)	*Trichoderma atroviride*
KUC5027	GQ241295	7[c]	3[d]	*Trichoderma koningiopsis* (EU280141)	583/585 (99.7)	*Trichoderma koningiopsis*
Zygomycetes						
KUC6001	GQ241268	2		*Mucor racemosus* (AJ271061)	605/613 (98.7)	*Mucor racemosus*
KUC6003	GQ241269	6[c]	9[d]	*Rhizomucor variabilis* (EF583637)	594/601 (98.8)	*Rhizomucor variabilis*
KUC6004	GQ241270	2		*Umbelopsis isabellina* (AJ876493)	587/603 (97.3)	*Umbelopsis* sp.
KUC6006	GQ241271	3	2	*Umbelopsis ramanniana* (AB193533)	568/590 (96.3)	*Umbelopsis* sp.
KUC6002	GQ241272		2	*Mucoromycote* sp. (EU076944)	614/631 (97.3)	*Mucoromycotina* sp.
Basidiomycetes						
KUC9001	GQ241249	2		*Coprinellus domesticus* (AY663837)	583/585 (99.7)	*Coprinellus domesticus***
KUC9004	GQ241250	6[c]		*Crustoderma* sp. (AY858355)	588/589 (99.8)	*Crustoderma* sp.**
KUC9009	GQ241251	2		*Heterobasidion annosum* (AF287866)	580/588 (98.6)	*Heterobasidion annosum**
KUC9010	GQ241252		2	*Hyphodontia flavipora* (AY858363)	583/583 (100.0)	*Hyphodontia flavipora**
KUC9011	GQ241253	1	2	*Hypochnicium cremicolor* (DQ677506)	584/591 (98.8)	*Hypochnicium cremicolor**
KUC9013	GQ241254	13[c]	2	*Irpex lacteus* (AY858353)	560/560 (100.0)	*Irpex lacteus**
KUC9015	GQ241255	3		*Phanerochaete sordida* (AY858368)	566/582 (97.3)	*Phanerochaete* sp.*
KUC9017	GQ241256	1		*Pheniophora* sp. (AY858367)	539/539 (100.0)	*Pheniophora* sp.*

Table 1 continued

Isolate No.	GenBank acc. No.	No. of isolates[a]		Closest fungal match (acc. No.)	Similarity (%)[b]	Fungal identity
		S	I			
KUC9020	GQ241257	2	7[d]	*Phlebia radiata* (AY858369)	582/582 (100.0)	*Phlebia radiata**
KUC9022	GQ241258		2	*Phlebiella* sp. (AY858371)	581/582 (99.8)	*Phlebiella* sp.*
KUC9023	GQ241259		2	*Platygloea disciformis* (AY629314)	545/571 (95.4)	*Platygloea* sp.**
KUC9026	GQ241260	3		*Schizophyllum commune* (AY858374)	581/582 (99.8)	*Schizophyllum commune**
KUC9029	GQ241261		1	*Sistotrema brinkmannii* (DQ898704)	576/578 (99.7)	*Sistotrema brinkmannii***
KUC9027	GQ241262	1		*Tilletiopsis albescens* (AJ235289)	582/583 (99.8)	*Tilletiopsis albescens***
KUC9028	GQ241263	2		*Trametes versicolor* (AY858378)	582/582 (100.0)	*Trametes versicolor**
KUC9006	GQ241264		2	*Grifola frondosa* (AY629318)	540/571 (94.6)	Unidentified basidiomycota sp.*
KUC9025	GQ241265		1	*Rigidoporus microporus* (AY333795)	567/585 (96.9)	Unidentified basidiomycota sp.*
KUC9007	GQ241266		1	*Auriculoscypha anacardiicola* (DQ419920)	551/568 (97.0)	Unidentified basidiomycota sp.**
KUC9008	GQ241267		1	*Helicogloea* sp. (AY512847)	551/574 (96.0)	Unidentified basidiomycota sp.**
Total isolates	120	59				
No. of points sampled	120	120				

[a] S, surface of creosote-treated wood; I, interior of creosote-treated wood

[b] Similarity (%) was derived from matched nucleotide/compared nucleotide in GenBank using rDNA sequences including the ITS sequence for ascomycetes and zygomycetes, and the LSU sequence for basidiomycetes. * White rot. ** Brown rot

[c] Dominant species on the surface. A species is considered dominant if $Pi > 1/S$, where Pi is the proportion of total samples represented by species i, and S (species richness) is the number of competing species present in the community (Camargo 1993)

[d] Dominant species in the interior

for fungal identification in many ecological studies (Kernaghan et al. 2003; Hunt et al. 2004; Kim et al. 2005, 2009). In addition, a wealth of LSU rDNA sequence information has been compiled in international databases as a result of several phylogenetic studies of basidiomycetes fungi and the advent of major taxonomic projects (Hibbett et al. 2007; Matheny et al. 2007). Therefore, it is now possible to identify fungi at a far higher resolution using the LSU rDNA region sequence. Moreover, although there are no rules for identifying fungi based on the similarity of LSU rDNA sequences, we previously demonstrated that basidiomycete isolates with greater than 98% similarity are conspecific (Kim et al. 2005, 2009).

ITS sequence comparison is regarded as an excellent tool for assigning unknown fungi to broad species groups or genera (Horton and Bruns 2001; Schmidt and Moreth 2002) and to identify even very closely related zygomycete (Meyer and Gams 2003; Lutzoni et al. 2004; Kwasna et al. 2006) and ascomycete species (Lutzoni et al. 2004; Lim et al. 2005; Arenz et al. 2006). Although Landeweert et al. (2003) suggested that over 95% ITS region sequence identity was enough to confidently group taxa into a genus, some fungi showed higher intraspecific variation in the ITS region sequence. For examples, ITS sequences from *Aspergillus* species ranged from 94 to 100% (Balajee et al. 2009). Because there are no rules for determining fungal identification at the species level based on the ITS sequences similarity, we assigned species names to our isolates when pairwise similarity scores from BLAST searches were greater than 98.0% in this study. The LSU rDNA and ITS sequence data enabled 47 taxa to be linked to established genera or species (Table 1). For determining the fungal diversity from CWs, direct sequence analysis from the wood samples might be

efficient and reveal even greater fungal diversity as compared to the plate culturing technique (Giraffa and Neviani 2001). However, further studies such as PAH resistance tests certainly require fungal isolation.

According to Camargo's index, the dominant ascomycetes on the surface were *Alternaria alternata*, *Cladosporium cladosporioides*, *Phialocephala dimorphospora*, *Trichoderma atroviride*, and *Trichoderma koningiopsis*, while *Rhizomucor variabilis* was the dominant zygomycete, and a *Crustoderma* sp. and *Irpex lacteus* were the dominant basidiomycetes. The dominant ascomycetes in the inner area were *Byssochlamys nivea*, *Paecilomyces variotii*, *Phaeoacremonium rubrigenum*, and *Trichoderma koningiopsis*, while *R. variabilis* was the dominant zygomycete, and *Phlebia radiata* was the dominant basidiomycete.

Among 94 ascomycete isolates (23 species), 71 (18 species) were detected on the surface and 23 (nine species) were recovered from the inner area. Four species were isolated from both surface and inner areas. Most of the species isolated in this study have been reported in other studies that have evaluated the diversity of fungi recovered from CTW and/or creosote-contaminated soil. Specifically, *A. alternata*, *C. cladosporioides*, *Curvularia trifolii*, *Epicoccum nigrum*, *Paecilomyces lilacinus*, *P. variotti*, *Penicillium janthinellum*, *P. dimorphospora*, *T. atroviride*, and *T. koningiopsis* have been identified in previous studies (Zabel et al. 1985; Launen et al. 1995; Lamar et al. 1999). *A. alternata* and *P. variotti* were recovered with the highest frequency, but the former was isolated only on the surface area and the latter was recovered from the inner area. Additionally, species found on the surface with a high frequency, such as *C. cladosporioides*, *P. dimorphospora*, and *T. atroviride*, were found at low frequencies in the inner area. Similarly, species that were recovered from the inner area at a high frequency, such as *B. nivea*, *P. variotii*, and *P. rubrigenum*, were not recovered from the surface. These results demonstrate that there were different optimized conditions for each individual fungus. As shown in Table 2, a much higher amount of PAHs was present in the inner area than on the surface. It is believed that the frequency at which fungi occur varies in response to different resistances to toxicity. Therefore, fungi recovered from the inner area with a high frequency might have a high resistance to toxicity or contaminants. *T. koningiopsis* was recovered from both inner and surface areas. This fungus is common in tropical America, but has also been isolated from natural substrates in East Africa, Europe and East Asia, and not only in creosote-treated wood (Samuels et al. 2006). Additionally, other fungi recovered with lower frequency may have accidently colonized the surface in creosote-treated sites.

Table 2 Chemical composition of creosote-treated wooden crosstie wastes (μg/g)

PAHs	Inner	Surface
Naphthalene	52.8 (13.8)[a]	12.0 (2.5)
Acenaphthylene	52.1 (33.8)	15.6 (10.1)
Acenaphthene	394.3 (59.1)	150.0 (78.2)
Fluorene	320.3 (10.8)	122.7 (81.8)
Phenanthrene	826.4 (72.3)	361.0 (43.2)
Anthracene	435.1 (87.3)	207.1 (51.0)
Fluoranthene	452.6 (33.7)	288.5 (62.5)
Pyrene	395.7 (47.8)	266.5 (59.1)
Benz[a]anthracene	143.4 (18.6)	109.7 (24.9)
Chrysene	100.9 (17.4)	75.6 (10.9)
Benzo[b]fluoranthene	67.8 (23.9)	27.1 (5.1)
Benzo[k]fluoranthene	24.7 (20.1)	14.4 (1.0)
Benzo[a]pyrene	54.3 (32.1)	40.8 (5.5)
Indeno[1,2,3-d]pyrene	5.8 (7.2)	–
Dibenz[a,h]anthracene	10.8 (7.7)	3.1 (4.3)
Benzo[ghi]perylene	–	6.2 (8.8)

[a] Values are the mean of five replicates; standard error is in parenthesis

A total of 26 zygomycete isolates (five species) were obtained; 13 isolates (four species) were detected on the surface, and 13 isolates (three species) were recovered from the inner area. Two zygomycetous taxa were identified as *Mucor racemosus* and *Rhizomucor variabilis*. Another two taxa were identified as belonging to the *Umbelopsis* genus. The taxon could not be identified at the species level because of insufficiency in the zygomycetes ITS sequence databases. Therefore, two isolates (one species) were treated tentatively as belonging to an unidentified *Mucoromycotina* species. *R. variabilis* was the dominant species at both sampling sites. In addition, members of the genus *Umbelopsis* were found at both sampling sites. Zygomycetes are commonly isolated from creosote-contaminated soil,

and the genus *Mucor* is frequently represented (Atagana 2003). But this genus was found only on the surface in this study.

A total of 59 basidiomycete isolates (19 species) was obtained from CTW, of which 36 (11 species) were found at the surface and 23 (11 species) were found in the inner area. Moreover, 20 isolates belonging to eight species were obtained only from the surface. Among these species, six white-rot fungi and two brown-rot fungi were identified. Similarly, 12 isolates found only in the inner area were classified into eight species including four unknown species. *H. cremicolor*, *I. lacteus*, and *P. radiata* were obtained from both sampling areas. Among the species found only on the surface, *Crustoderma* sp. was the only organism recovered with a high frequency.

The results of this study demonstrated that white-rot fungi represented 76.3% (45 isolates) of the isolates, while brown-rot fungi represented only 23.7% (14 isolates) of the isolates. These findings are similar to those of other studies, in which white-rot fungi were obtained as often, or more frequently than, brown-rot fungi from wood products that were in contact with the ground (Zabel and Moore 1958; Duncan and Lombard 1965; Butcher 1968; Kim et al. 2005). Additionally, Kim et al. (2005) reported that white-rot fungi commonly precede brown-rot fungi during the colonization of untreated softwoods in contact with the ground. When combined with the results of these previous studies, our results suggest that white-rot fungi are involved in the biodegradation of untreated wood and treated wood. Indeed, *I. lacteus* and *P. radiata* were dominant species in the surface and the inner area, respectively, and it was reported that these species have a high efficiency for the biodegradation of PAHs via the production of ligninolytic enzymes (Kamei et al. 2005; Cajthaml et al. 2008). Ligninolytic enzymes produced by white-rot fungi, which include lignin peroxidase and manganese peroxidase, are secreted extracellularly, after which they oxidize organic matter via a non-specific radical-based reaction (Bamforth and Singleton 2005). Additionally, *Crustoderma* spp., *I. lacteus*, and species such as *Sistotrema* spp., *Phanerochaete* spp., and *Hypodontia* spp. have been isolated from CTW by Zabel et al. (1985). They also reported that *T. versicolor* was the most frequently isolated fungus; however, this organism was not found to be prevalent in our study.

PAH resistance and fungal colonization

One isolate of each fungal species identified in this study was examined for its ability to resist four concentrations of PAHs (Table 3). The majority of fungi tested was sensitive to PAHs and was inhibited by treatment with 250 ppm when compared to 0 ppm. A total of 21 strains isolated in this study grew in the presence of 1,000 ppm PAHs. Among these strains, 13 ascomycetous isolates were more resistant to PAHs than three zygomycetous and five basidiomycetous isolates. The resistance of many of the fungal species to the four different concentrations differed significantly.

Six dominant strains in ascomycete species identified in this study, *A. alternata*, *B. nivea*, *C. cladosporioides*, *P. variotti*, *T. atroviride*, and *T. koningiopsis*, were found to have a greater tolerance of PAHs than did other fungal species. Lamar et al. (1999) found that *A. alternata* obtained from creosote showed a low growth rate after 2 weeks in the presence of 100 ppm creosote. They also found that *A. alternata* significantly degraded fluoranthene and pyrene as compared with bioremediation by other white-rot fungi. *C. cladosporioides*, which showed great tolerance to PAHs in this study, has been reported to be able to remove high levels (800 ppm) of phenanthrene. *Aspergillus niger* has also been reported as a degrader of phenanthrene (Cortés-Espinosa et al. 2006), but this species secretes mycotoxin as a secondary metabolite (Blumenthal 2004). Therefore, our result suggests that biodegradation using *C. cladosporioides* isolated in this study may offer an alternative to *A. niger*. However, further studies are required to determine the optimum conditions for its application in bioremediation.

Additionally, two strains of *Trichoderma* species obtained in this study, *T. atroviride* and *T. koningiopsis*, were strongly resistant to concentrations of PAHs ranging from 250 to 1,000 ppm. The growth of *T. atroviride* showed little difference in its sensitivity to PAHs at concentrations below 500 ppm, while the extension of mycelia was somewhat decreased at 1,000 ppm. The growth of *T. koningiopsis* did not differ significantly in the presence of 500 and 1,000 ppm PAHs. In a previous study, various *Trichoderma* spp. were found to contribute to the degradation of PAHs, with a maximum of 75%

Table 3 Growth of isolated fungi in the presence of increasing concentrations of polycyclic aromatic hydrocarbons

Isolate number	Fungal identity	Mycelia extension rate (mm/day) at the indicated creosote concentration (μg/ml)			
		0 ppm	250 ppm	500 ppm	1,000 ppm
Ascomycetes					
KUC5005	*Alternaria alternata*	9.1[1]a[2]	2.2b	2.0b	0.8c
KUC5006	*Ampelomyces* sp.	8.7a	0.0b	0.0b	0.0b
KUC5008	*Byssochlamys nivea*	11.2a	6.4b	4.7c	4.0d
KUC5009	*Cladosporium cladosporioides*	3.6a	1.3bc	1.2c	1.3b
KUC5010	*Curvularia trifolii*	7.8a	1.0b	0.7b	0.0c
KUC5011	*Epicoccum nigrum*	5.3a	0.8b	0.7b	0.6c
KUC5012	*Exserohilum fusiforme*	13.0a	2.5b	1.9c	0.0d
KUC5013	*Fusarium culmorum*	3.2a	0.8b	0.7b	0.6c
KUC5014	*Hyalodendriella* sp.	4.0a	0.5c	0.6b	0.4c
KUC5007	*Paecilomyces lilacinus*	3.8a	0.7b	0.7b	0.6b
KUC5016	*Paecilomyces* sp.	1.8a	1.4b	1.3c	0.0d
KUC5015	*Paecilomyces variotti*	5.9a	5.1b	4.5c	3.1d
KUC5017	*Penicillium griseofulvum*	2.2a	0.0c	0.7b	0.0c
KUC5018	*Penicillium janthinellum*	6.0a	2.2b	2.2b	2.1c
KUC5019	*Pestalotiopsis photiniae*	1.3a	1.1b	1.1b	0.0c
KUC5020	*Pestalotiopsis vismiae*	10.7a	0.6b	0.7b	0.0c
KUC5021	*Phaeoacremonium rubrigenum*	2.2a	1.2b	1.2b	0.6c
KUC5023	*Phialocephala dimorphospora*	2.9a	1.3b	0.9c	0.0d
KUC5029	*Phialophora mustea*	5.2a	2.3b	1.5c	0.8d
KUC5024	*Phoma glomerata*	5.0a	0.6c	0.9b	0.0d
KUC5025	*Pteris tremula*	4.2a	0.8b	0.0c	0.0c
KUC5026	*Trichoderma atroviride*	18.1a	16.1a	15.3b	3.4c
KUC5027	*Trichoderma koningiopsis*	19.1a	11.9b	8.9c	8.9c
Zygomycetes					
KUC6001	*Mucor racemosus*	13.7a	0.6b	0.5b	0.5b
KUC6003	*Rhizomucor variabilis*	14.4a	1.4b	0.0c	0.0c
KUC6004	*Umbelopsis* sp.	6.4a	1.1c	1.5b	1.2b
KUC6006	*Umbelopsis* sp.	5.6a	0.7b	0.6b	0.0c
KUC6002	Unidentified zygomycota sp.	5.5a	2.2b	2.3b	1.3c
Basidiomycetes					
KUC9001	*Coprinellus domesticus*	6.5a	1.3b	1.2c	0.0d
KUC9004	*Crustoderma* sp.	12.2a	0.6b	1.0c	0.0d
KUC9009	*Heterobasidion annosum*	5.5a	2.7b	2.4c	2.6bc
KUC9010	*Hyphodontia flavipora*	3.8a	0.0b	0.0b	0.0b
KUC9011	*Hypochnicium cremicolor*	7.7a	1.7c	2.2b	0.0d
KUC9013	*Irpex lacteus*	9.9a	5.6b	5.2b	1.5c
KUC9015	*Phanerochaete* sp.	15.7a	1.4c	2.4b	0.0d
KUC9017	*Pheniophora* sp.	8.0a	1.5b	1.6b	0.0c
KUC9020	*Phlebia radiata*	7.4a	4.2c	5.2b	0.0d
KUC9022	*Phlebiella* sp.	5.8a	2.1b	2.3b	0.0c
KUC9023	*Platygloea* sp.	0.9a	0.6b	0.6b	0.5c
KUC9026	*Schizophyllum commune*	4.6a	3.7b	3.4bc	3.3c

Table 3 continued

Isolate number	Fungal identity	Mycelia extension rate (mm/day) at the indicated creosote concentration (μg/ml)			
		0 ppm	250 ppm	500 ppm	1,000 ppm
KUC9029	*Sistotrema brinkmannii*	10.9a	4.8b	4.1c	0.0d
KUC9027	*Tilletiopsis albescens*	6.9a	0.6c	1.0b	0.0d
KUC9028	*Trametes versicolor*	12.3a	1.1c	1.5b	0.0d
KUC9006	Unidentified basidiomycota sp.	6.2a	0.7b	0.9b	0.0c
KUC9025	Unidentified basidiomycota sp.	7.7a	0.0b	0.0b	0.0b
KUC9007	Unidentified basidiomycota sp.	2.3a	0.8b	1.0b	0.0c
KUC9008	Unidentified basidiomycota sp.	8.0a	1.5b	1.6b	0.9c

[1] Values are the mean of three replicates

[2] Numbers followed by the same letter in each row are not significantly different ($\alpha = 0.05$) according to Duncan's method

pyrene (a 4-ring PAH) at 50 mg l^{-1} being removed by axenic cultures of *Trichoderma* spp. when pyrene was provided as the sole carbon source (Saraswathy and Hallberg 2002). Based on these results, *T. atroviride* and *T. koningiopsis* likely have the potential for use in later stages of bioremediation.

We also recovered two *Penicillium* species, *P. griseofulvum* and *P. janthinellum* from the surface of the samples, one of which showed strong resistance to PAHs in this study. *Penicillium* species are commonly dominant at PAH-contaminated sites (Launen et al. 1995). Specifically, *P. janthinellum* was not inhibited by PAHs, and it even grew on media spiked with 1,000 ppm PAHs. Ravelet et al. (2000) observed degradation of 50% of the initial concentration of PAHs (50 mg l^{-1}) by *P. janthinellum* during a 28-day incubation period. Combined with their results, our findings suggest that *P. janthinellum* from creosote-treated crossties plays an important role as a major slow degrader. On the other hand, all of the zygomycetes had a similar sensitivity to PAHs, regardless of the concentrations evaluated.

Although all basidiomycetes isolated were found to have different sensitivities to PAHs, four specific strains were resistant to up to 500 ppm. Among the basidiomycetes isolated in this study, most of them cause white rot in wood and are known for possessing a unique lignin-degrading enzymatic system. Two white-rot fungi, *Heterobasidion annosum* and *Schizophyllum commune*, which were isolated only from the surface, showed a high growth rate on media containing 1,000 ppm PAHs. *S. commune* was strongly resistant to PAHs, and it produced fruiting bodies on CW. Therefore, it is expected that these two species may be strong creosote-degraders during the initial stages of remediation. *I. lacteus*, which was found to be dominant on CW in this study, is known to produce ligninolytic enzymes, particularly when grown on malt extract-glucose medium that contains pyrene, and to be an efficient degrader of PAHs that possess 3–6 aromatic rings (Cajthaml et al. 2008). *P. radiata*, which was isolated most frequently from the inner area, showed a high mycelial extension rate in the presence of 500 ppm PAHs, but had no resistance to 1,000 ppm PAHs. Finally, *T. versicolor* was found to be very sensitive to PAHs in our study, while it showed the highest removal rate of phenanthrene in studies conducted by Han et al. (2004) and Morgan et al. (1991).

Although many studies have been conducted to develop useful methods of bioremediation using basidiomycetes, there is a limited number of species that can be used. Therefore, further studies must be conducted to identify various species and fungal communities capable of bioremediation. Fungi identified in this study that were found in the inner area and had a high resistance to PAHs may be useful for the degradation of creosote-treated crosstie wastes. In fact, Atagana et al. (2006) pointed out that the best growth on solid media spiked with creosote and selected PAHs was observed for *Cladosporium* sp. and not for the best PAH degrader, *Pleurotus* sp. Accordingly, we anticipate that our results will provide initial data for the selection of fungal species in the oxidation of PAHs.

Conclusions

This study is the beginning of an effort to collect fungal species from CW for application to the biodegradation of PAHs. A large difference in fungal diversity was observed between the surface and inner area. However, the real diversity of fungi in CW may be greater than that detected by the culture-based methodology chosen for this study. Nevertheless, among the fungi isolated, specific strains that were resistant to a mixture of 1,000 ppm PAHs were selected as applicable for bioremediation. Consequently, these isolates may play important roles as indigenous degraders of PAHs.

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