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Cha, Joo Young

Field Science Center for Northern Biosphere, Hokkaido University

Im, Sangjun

Department of Forest Sciences, College of Agriculture and Life Sciences, Seoul National University

Lee, Sang Yong

Division of Forest Resource, College of Forest and Environmental Sciences, Kangwon National University

Ohga, Shoji

Laboratory of Forest Resources Management, Division of Forest Environment Sciences, Department of Agro-Environment Sciences, Faculty of Agriculture, Kyushu University

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Diversity of Fungal Species Isolated from Litter–mycelial Mats in the Litter Layer of a Korean Deciduous Forest

Joo Young CHA¹, Sangjun IM^{2*}, Sang Yong LEE³
and Shoji OHGA

Laboratory of Forest Resources Management, Division of Forest Environmental Sciences,
Department of Agro–environmental Sciences, Faculty of Agriculture,
Kyushu University, Fukuoka, 811–2415, Japan
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We isolated and identified the fungal species associated with litter–mycelial mat formation in the litter layers of a deciduous forest. A total of 43 isolates, belonging to 17 fungal taxa, were identified, and included 14 ascomycetes, 1 zygomycete, and 1 basidiomycete. The most abundant species were *Biscogniauxia* sp. followed by *Chaetomium globosum*, *Hypoxylon* sp., *Mortierella* sp., and *Xylaria* sp. *Biscogniauxia* sp., *Hypoxylon* sp., and *Xylaria* sp., but not *Mortierella* sp. or *C. globosum*, produced a colored zone on guaiacol medium, thus demonstrating lignin–degrading ability. Six of seven ligninolytic fungi were ascomycetes associated with the formation of a litter–mycelial mat in the litter layer of a deciduous forest. These data suggest that ascomycetes play a very important role in the formation of litter–mycelial mats in litter layers and that these mats are formed during the early to middle stages of decomposition in forest ecosystems.

INTRODUCTION

Litter decomposition plays an important role in nutrient cycles and controls primary production in forest ecosystems (Petersen and Luxton, 1982; Vitousek, 1982; Aerts, 1997; Clark *et al.*, 1998; Onyekwelu *et al.*, 2006; Pandey *et al.*, 2007) because it is a key ecological process for the transformation of organic matter and energy from the vegetation to the soil (Xu *et al.*, 2011). Animals living in soil, bacteria, and fungi are the three main decomposer groups (Swift *et al.*, 1979). Of these, fungi are arguably the most important (Cooke and Rayner, 1984) because they can decompose plant–derived lignin–rich polymers and humus (Ayato *et al.*, 2005). The profile and structure of decomposing fungal communities in forest litter and the frequency of occurrence of fungi have been reported as key factors in the process (Osono and Takeda, 2002). The temporal–spatial distribution pattern of fungi, however, is determined by the quality of litter (Swift, 1976).

However, fungi are well known to the general public as being responsible for the formation of water–repelling aerial molds and elaborate fruit–bodies such as mushrooms and polypores (Wosten *et al.*, 1999). Filamentous fungi colonize moist substrates such as wood and must breach the water–air interface to grow in the air (Wosten *et al.*, 1999). Moreover, organic substances leached from plant litter and produced by microbial mycelia can induce repellency (Hudson *et al.*, 1994; DeBano *et al.*, 1998). Most studies of water repellency in forest soils, however,

have focused on ectomycorrhizal fungi (Unestam, 1991; Smits *et al.*, 2003); in contrast, few have assessed the water repellency of saprophytic fungi (Wosten *et al.*, 1999). Smits *et al.* (2003) found that deuteromycetes were generally hydrophilic, while ascomycetes and basidiomycetes were mainly hydrophobic. Thus, greater knowledge of fungal composition and function is required for effective use of the water resources of forest ecosystems.

In Korea, most forest management operations, such as thinning and pruning, are no longer performed because of the increased use of fossil fuels instead of forest products (such as woody derivatives and litter) since the rapid economic expansion of the 1970s. As a result, the forest litter layer has gradually increased in thickness. Therefore, litter layers have formed litter–mycelial mats that increase soil water loss by blocking movement into soil. Moreover, the hydrophobicity of filamentous fungi associated with litter–mycelial mats is an important factor in the movement of rainfall, similar to the effect of surface water on litter layers in forest stands. So, this study aimed to clarify the fungal species associated with litter–mycelial mat formation. The term “litter–mycelial mat” means that the fresh to fermented litter layers were fixed by the decomposition of fungal mycelia, including white–rot humus, as described by Hintikka (1970).

MATERIALS AND METHODS

Study site

The study site is located in the Kwangrung Forest of the National Arboretum, Pochun–kun, Kyongki Province, Korea (37°44' N, 127°8' E). The site is a natural forest composed of deciduous trees with a density of 700/ha (more than 5 cm in diameter at a breast height of 1.3 m) with shrubs in the understory. The tree layer consisted of *Quercus serrata* Thunb. ex Murray, *Q. dentate* Thunb. ex Murray, *Carpinus laxiflora* (Siebold & Zucc.) Blume, and *Styrax japonicus* Siebold & Zucc. The shrubs were

¹ Field Science Center for Northern Biosphere, Hokkaido University, Nayoro, 096–0071, Japan

² Department of Forest Sciences, College of Agriculture and Life Sciences, Seoul National University, Seoul, 151–921, Korea

³ Division of Forest Resource, College of Forest and Environmental Sciences, Kangwon National University, Chuncheon, 200–701, Korea

* Corresponding author (E–mail: junie@snu.ac.kr)

Meliosma oldhamii Maxim. and *Cornus kousa* Buerger ex Miquel.

The organic layer of this forest developed well over the mineral soil with granite gneiss in places and consisted of litter, fermentation, and humus layers ranging from 4 to 6 cm in thickness.

Litter sampling and fungal isolation

Twenty samples of litter–mycelial mats ranging in thickness from 1 to 3 cm were collected using sterilized tweezers and placed in plastic bags. Sampling locations were randomly selected.

Fungi were isolated from the litter particles in size of about 0.2×0.5 cm using a modified washing method (Arnold *et al.*, 2007). Particle surfaces were sterilized by sequential immersion in 70% ethanol (2 min), 0.5% NaOCl (2 min), and sterilized distilled water (5 s). Particles were surface-dried under sterile conditions and on sterilized filter paper before placement on either 2% malt-extract agar (MEA) or 2% MEA + benomyl ($1 \mu\text{l ml}^{-1}$), which suppresses hyphal growth except for that of basidiomycetes. Six particles from each sample were placed on both kinds of medium for fungal isolation. Plates (9 cm in diameter) were sealed, checked daily for hyphal growth, and incubated at 25 °C in darkness for up to 2 weeks. Any fungal hyphae appearing on the plates were isolated onto PDA plates and incubated. Four weeks after isolation, pure cultures on PDA plates were used in DNA extraction.

Identification of fungal species

Fungal DNA from the mycelia of isolates was extracted using a DNeasy Plant Mini kit (QIAGEN, USA) according to the manufacturer's instructions. DNA amplification of the ITS regions, including the 5.8S rDNA, was performed on a MyCycler thermal cycler (BioRad, USA) using Takara Ex Taq (TAKARA, Japan) with a specific primer for higher fungi ITS 1f (Gardes and Bruns, 1993) and the universal primer ITS4 (White *et al.*, 1990). We used the following PCR amplification conditions: 94 °C (3 min), followed by 30 cycles of 94 °C (30 s), 50 °C (30 s), and 72 °C (2 min), and a final hold at 72 °C (10 min). We determined the quality and quantity of the PCR products by agarose gel electrophoresis using QA-Agarose™ (MP Biomedical, USA).

Each PCR product was purified using a QIA quick

PCR purification kit (QIAGEN, USA) according to the manufacturer's instructions, and sequenced with primers ITS 1f and ITS4. Sequencing reactions were conducted by Solgent Co., Ltd. (Daejeon, Korea). Fungal sequences were compared with the GenBank database at the National Center for Biotechnology Information (NCBI; <http://www.ncbi.nlm.nih.gov>) and UNITE (<http://unite.ut.ee>) using the nucleotide–nucleotide basic local alignment search tool algorithm (BLAST program). Sequences were aligned with closely matched reference sequences and submitted to neighbor-joining (NJ) analysis at the NCBI for taxonomic interpretation at the species, genus, and family levels. Sequences were considered to be identified at the species level when more than 97% identity with reference sequences derived from sporocarps of the same species was obtained and made a single clade with the specimen in NJ trees. When less than 97% identity with reference sequences derived from sporocarps was obtained, sequences were considered to be identified at the genus or family level based on phylogenetic tree interpretation.

Test of lignin-degrading ability

Ligninolytic activity was estimated by plate tests on wood powder medium supplemented with guaiacol (Nishida *et al.*, 1988; Cha, 1995; Miyamoto *et al.*, 2000). Fungal discs were cut with a 4-mm diameter corkborer from the actively growing colony margins of isolates maintained on PDA. These discs were incubated on agar plates containing 0.01% (v/v) guaiacol, 0.2% (w/v) birch (*Betula platyphylla* var. *japonica* Hara) wood powder (100-mesh-pass), and 1.8% (w/v) agar. Plates were incubated at 25 °C. Medium pH was adjusted to 5.0 before autoclaving at 121 °C for 15 min, and guaiacol was added after the medium had cooled to 50–60 °C. All tests were performed in 9-cm diameter disposable Petri dishes containing 15 ml of medium per dish, and four replicates were performed. After 2 weeks, the diameters of the colonies and surrounding colored zones were measured in two orthogonal directions.

RESULTS

Identification of fungal species

A total of 43 isolates representing 17 fungal taxa were identified from the litter–mycelial mat of deciduous

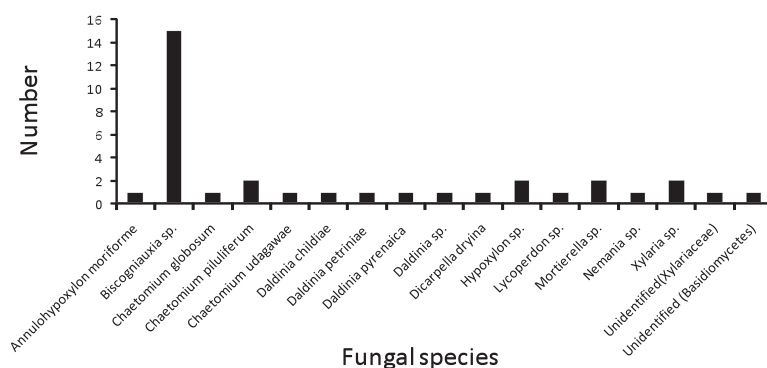


Fig. 1. Number of fungal species isolated from litter–mycelial mats in litter layer of deciduous forest.

Table 1. Possible identities of fungal species from litter-mycelial mats in litter layer of deciduous forest

Possible identity based on BLAST and phylogenetic analysis	BLAST match by the highest similarity		
	Definition	Accession no.	Overlapped sequence
Basidiomycetes			
<i>Lycoperdon</i> sp.	<i>Lycoperdon perlatum</i> isolate	EU622257	533/555 (96%)
Unidentified (Basidiomycetes)	Uncultured Basidiomycota clone	GU328584	539/670 (80%)
Ascomycetes			
<i>Annulohypoxyton moriforme</i>	<i>Annulohypoxyton moriforme</i> isolate	DQ322104	469/489 (95%)
<i>Biscogniauxia</i> sp.	<i>Biscogniauxia atropunctata</i> strain	EU715651	446/482 (92%)
<i>Chaetomium globosum</i>	<i>Chaetomium globosum</i> isolate	FJ772001	570/574 (99%)
<i>Chaetomium piluliferum</i>	<i>Chaetomium piluliferum</i> strain	GU183112	494/512 (96%)
<i>Chaetomium udagawae</i>	<i>Chaetomium udagawae</i> strain	GU183108	522/537 (97%)
<i>Daldinia childiae</i>	<i>Daldinia childiae</i>	AM292043	532/535 (99%)
<i>Daldinia petriniae</i>	<i>Daldinia petriniae</i> isolate	AY354238	474/489 (96%)
<i>Daldinia pyrenaica</i>	<i>Daldinia pyrenaica</i>	AM749927	508/520 (97%)
<i>Daldinia</i> sp.	<i>Daldinia concentrica</i> isolate	AY616681	456/486 (93%)
<i>Dicarpella dryina</i>	<i>Dicarpella dryina</i> isolate	FJ598616	552/555 (99%)
<i>Hypoxyton</i> sp.	<i>Hypoxyton serpens</i>	AY781226	501/534 (93%)
<i>Nemania</i> sp.	<i>Nemania</i> sp.	EF682111	412/453 (90%)
<i>Xylaria</i> sp.	<i>Xylaria</i> sp.	AB255244	552/560 (98%)
Unidentified (Xylariaceae)	<i>podosordaria tulasnei</i>	AY572970	513/581 (88%)
Zygomycetes			
<i>Mortierella</i> sp.	<i>Mortierella</i> sp.	FJ810149	607/610 (99%)

Table 2. The ligninolytic ability of fungal species from litter-mycelial mats in litter layer of deciduous forest

Fungal species	Colony diam ^a	Coloration zone diam ^b
<i>Annulohypoxyton moriforme</i>	≤20.3±0.5	20.3±0.5
<i>Biscogniauxia</i> sp.	≤33.7±1.5	33.8±1.5
<i>Chaetomium globosum</i>	8.8±0.5	0±0
<i>Chaetomium piluliferum</i>	0±0	0±0
<i>Chaetomium udagawae</i>	0±0	0±0
<i>Daldinia childiae</i>	0±0	0±0
<i>Daldinia petriniae</i>	0±0	0±0
<i>Daldinia pyrenaica</i>	0±0	0±0
<i>Daldinia</i> sp.	0±0	0±0
<i>Dicarpella dryina</i>	0±0	37.3±0.5
<i>Hypoxyton</i> sp.	≤20.8±5.9	20.8±5.9
<i>Lycoperdon perlatum</i>	0±0	0±0
<i>Mortierella</i> sp.	0±0	0±0
<i>Nemania</i> sp.	0±0	6±0
<i>Xylaria</i> sp.	≤17.3±0.5	17.3±0.5
Unidentified (Xylariaceae)	0±0	0±0
Unidentified (Basidiomycetes)	≤44.5±1	44.5±1
<i>Armillaria sinaula</i> *	≤30±0	30±0
<i>Bondarzewia montana</i> *	≤15.3±0.5	15.3±0.5

^a The mean of four replications in colony diameter (mm) on agar medium containing wood powder and guaiacol after 2 wk (±SD)

^b The mean of four replications in coloration zone diameter (mm) on agar medium containing wood powder and guaiacol after 2 wk (±SD)

* White rotting fungus of wood for comparison

forest (Table 1). Of these, we identified eight and seven samples to the species and genus levels, respectively, and two unidentified fungal species by matching sequences with those of known sporocarps. The 17 species comprised 14 ascomycetes (belonging to eight genera and 1 unidentified fungal species), 1 zygomycete,

and 2 basidiomycetes. The most abundant species was *Biscogniauxia* sp. (15 isolates) followed by *Chaetomium globosum* Kunze (3 isolates), *Hypoxyton* sp. (3 isolates), *Mortierella* sp. (3 isolates), and *Xylaria* sp. (3 isolates). All other fungal species were isolated only once each (Fig. 1). Both *Mortierella* sp. and unidentified

basidiomycetes also exhibited mycelial growth on benomyl-containing medium.

Lignin-degrading ability

Seven of 17 identified species produced dark red zones on guaiacol-containing media (Table 2). *Annulohyphoxylon moriforme* (Henn.) Y. M. Ju, J. D. Rogers & H. M. Hsieh; *Biscogniauxia* sp.; *Hypoxyylon* sp.; *Xylaria* sp.; and unidentified basidiomycetes showed mycelial growth with coloration on this medium. All others did not exhibit mycelial growth on guaiacol-containing medium, with the exception of three species, (*C. globosum*, mycelial growth; *Dicarpella dryina* Belisario & M. E. Barr and *Nemania* sp., coloration only). All species that produced coloration were either ascomycetes or unidentified basidiomycetes. Species that did not exhibit coloration represented basidiomycetes and *Lycoperdon* sp., zygomycetes (*Mortierella* sp.), and seven species of ascomycetes (*C. piluliferum* J. Daniels, *C. udagawae* Sergejeva ex Udagawa, *Daldinia childiae* J. D. Rogers & Y.-M. Ju, *D. petriniae* Y.-M. Ju, J. D. Rogers & San Martín, *D. pyrenaica* M. Stadler & Wollw, *Daldinia* sp., and unidentified *Xylariaceae*). The fungal species that possessed lignin-degrading ability, namely *Armillaria singular* Cha and Igarashi and *Bondarzewia montana* (Qué.) Sing., which are well known to cause white-rot of wood (Cha, 1995; Igarashi, 2009), exhibited both mycelial growth and coloration on guaiacol-containing media.

DISCUSSION

A wide variety of species of litter-decomposing fungi have been identified in different forest ecosystems (Christensen, 1989; Yao and Yang, 1997). Fifty-seven taxa, consisting of 18 ascomycetes and 39 deuteromycetes, were identified in rain forest using direct identification methods (Parungao *et al.*, 2002). From a subtropical zone forest, Song *et al.* (2004) reported 67 fungal taxa, comprising 56 deuteromycetes, 3 zygomycetes, 5 ascomycetes, and 3 unidentified fungi. Seventy fungi in a temperate forest and 77 in a subalpine coniferous forest in Japan were discovered by Osono *et al.* (2002) and Ando and Suzumura (1986), respectively. The species composition included non-basidiomycetes such as ascomycetes, zygomycetes, and deuteromycetes. Although basidiomycetes are well-known lignin decomposers (Miyamoto *et al.*, 2000) and associated with production of white-rot humus in forest litter layers (Hintikka, 1970), the non-basidiomycetes in this study are considered to play an important role in formation of litter-mycelial mats and will gradually be replaced by basidiomycetes during the late stage of decomposition.

In this study, a total of 17 species were isolated from the litter-mycelial mat of a deciduous forest in Korea. These data suggest that ascomycetes are involved in litter-mycelial mat formation in deciduous forests. *Biscogniauxia* sp. was the most frequently detected taxon (15 isolates), followed by *C. globosum*, *Hypoxyylon* sp., *Mortierella* sp., and *Xylaria* sp. (3 isolates each).

Notably, these species are considered to play important roles in fermentation as well as litter decomposition, because ascomycetes, deuteromycetes, and zygomycetes are involved in weight loss in litter (Osono and Takeda, 2002; Song *et al.*, 2010). *Biscogniauxia*, a genus of the family Xylariaceae, was the most dominant fungal taxon in this study. Therefore, clarification of the role of *Biscogniauxia* sp. in litter decomposition and litter-mycelial mat formation in litter layers is necessary.

The dominant species (*Biscogniauxia* sp., *Hypoxyylon* sp., and *Xylaria* sp., but not *Mortierella* sp. or *C. globosum*) produced colored zones after growth on guaiacol medium, demonstrating lignin-degrading ability as described by Nishida *et al.* (1988). Six of seven ligninolytic fungi were ascomycetes associated with formation of litter-mycelial mats in the litter layer of deciduous forests. In contrast, other species were non-ligninolytic; the hyphal growth of these cellulolytic and sugar fungi is generally faster than that of ligninolytic fungi (Osono, 2007). Of the ligninolytic fungi, Osono (2007) reported that ascomycetes are more abundant than basidiomycetes in freshly fallen leaves, but ascomycetes gradually decrease in abundance during decomposition to be replaced by basidiomycetes in the later stages. Therefore, ascomycetes play an important role in the formation of litter-mycelial mats in litter layers, particularly during the early to middle stages of decomposition. This will continue until they are replaced by basidiomycetes, and depends on soil moisture levels and the activity and distribution of soil-dwelling animals.

Although filamentous fungi have been largely excluded from hydrophobicity measurements (Hazen, 1990), some have been classified as hydrophilic or hydrophobic based on indirect observations such as their resistance to flooding (Stenstrom, 1991), growth behavior on agar plates, excretion of phenols, and production of aerial hyphae and spores. Smits *et al.* (2003) conducted a quantitative analysis of the hydrophobicity of the mycelial mats of filamentous fungi based on contact-angle measurements. Two groups classified as deuteromycetes were generally hydrophilic, whereas the ascomycetes and basidiomycetes were predominantly hydrophobic. In this study, ascomycetes represented 14 of the 17 species associated with litter-mycelial mat formation; future work will focus on characterizing these isolates, including their hydrophobicity to rainfall.

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