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Full Length Research Paper

Dynamics of fungal diversity in different phases of *Pinus* litter degradation revealed through denaturing gradient gel electrophoresis (DGGE) coupled with morphological examination

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Fungal diversity in *Pinus sylvestris* var*. mongolica* litter was investigated by PCR-DGGE coupled with a traditional cultivation method. Twenty-one fungal strains were isolated by traditional cultivation methodology, most being filamentous fungi. Total DNA was extracted directly from the L, F1, F2 and H litter layers respectively using the bead-beating method. About 460 bp rDNA fragments were obtained by Nested-PCR and analyzed by denaturing gradient gel electrophoresis (DGGE). PCR-DGGE analysis recovered seven operational taxonomic units (OTUs) from the different decomposing litter layers. Six OTUs belonged to Ascomycetes and one to Basidiomycota. *Sporobolomyces inositophilus* was revealed by both methods, but there was no overlap in other species detected. A major shift of fungal communities on litter occurred during decomposition. The Shannon-Weaver index, which measures diversity in categorical data, was maximal in the F1 layer, then decreased with substratum degradation and reached it lowest value in the H layer.

Key words: Fungal diversity, polymerase chain reaction and denaturing gradient gel electrophoresis (PCR-DGGE), Traditional culture, Litter.

INTRODUCTION

Litter decomposition is an essential process involving organic matter and nutrient cycling in forests (Shirouzu et al., 2009; Osono et al., 2009). Microorganisms, especially fungi are the main degraders and effect litter decomposition rates (Curlevski et al., 2010; Swift et al., 1979). The fungi are more important because they produce specific enzymes that degrade lignocellulose matrices in litter, which other microorganisms are unable to assimilate (Cooke and Rayner, 1984; Kjoller and Struwe, 1982). Some fungi are also important as biocontrol agents and producers of bioactive compounds (Aly et al., 2010; Bucher et al., 2004; Duarte et al., 2006; Xu et al., 2010). Therefore attention should be given to studying fungal diversity in the natural environment.

Mycologically, succession is more precisely the sequential occupation of the same site by thalli (normally mycelia) either of different fungi, or of different associations of fungi (Rayner and Todd, 1979). Fungi replace one another as their dynamic communities of mycelia alter in space and time, each species adapted for occupation of particular niches (Frankland, 1998). Fungal succession has been observed by several authors (Frankland, 1976; Ho et al., 2002; Shirouzu et al., 2009; Osono et al., 2009; Zhou and Hyde, 2002). Zhou and Hyde (2002) found that the fungal communities on bamboo baits can be categorized into early colonisers, middle-stage colonisers, later colonisers, regular inhabitants and sporadic inhabitants. Osono et al. (2009) reported that *Aspergillus* sp. and *Trichoderma asperellum* occurred most frequently throughout *Shorea obtusa* leaf decomposition in a tropical seasonal forest. The

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prevalence of *Nigrospora* sp., *Cladosporium oxysporum*, and *Talaromyces* sp. decreased, whereas the frequency of occurrence of an unidentified species in the *Amphisphaeriaceae* increased during decomposition. However, above most fungal succession research were based on mycelial isolations from surface sterilized samples (Kinkel and Andrews, 1988; Tokumasu, 1996).

Molecular techniques circumvent the cultivation problem and have become a powerful tool for the identification of nonsporulating morphospecies (Giordano et al., 2009; Promputtha et al., 2005; Wang et al., 2005). These methods use ITS rRNA gene sequences to characterize species variation (Fatehi and Bridge, 1998). The small subunit 18S rRNA gene region is more conserved and has limited variation within species and is a promising alternative. RFLP analysis requires a laborious cloning step in situations where environment samples are inhabited by more than one species (Jonsson et al., 1999a; b). Molecular fingerprinting techniques such as temperature and denaturing gradient gel electrophoresis (TGGE and DGGE) analysis of rDNA fragments amplified from total community DNA could analyze several different sequences from a single environmental sample simultaneously and have been mainly used to analyze the composition of bacterial population communities (Muyzer et al., 1993; Muyzer and Smalla, 1998; Smit et al., 1997; Weidner et al., 1996). Several studies also have successfully used this approach to document fungal communities (Duong et al., 2006; Nikolcheva et al., 2003, 2005; Vainio et al., 2005). However, the fungal diversity of *Pinus sylvestris* var*. mongolica* litter in China using PCR-DGGE coupled with morphological examination has not been reported.

The purpose of this study is 1) to characterize the fungal communities in *Pinus sylvestris* var. *mongolica* litter, as this is one of the fast-growing timber and tree coniferous species in Northeast China 2) to compare if the results of PCR-DGGE and traditional culturing are in correspondence, and 3) to reveal if the dynamics of fungal community composition changed with litter further degradation.

MATERIALS AND METHODS

Sampling

The study site was *Pinus sylvestris* var. *mongolica* natural forest in Maoer Mountain, which is located in Xiaoxing'an Mountain, northwest of the Zhangguangcai Mountains in Heilongjiang Province, China (127° 32′ E, 45° 21′ N) (Xu et al., 2009). The average elevation of this area is 300 m and has a marked continental monsoon climate characterized by four distinct seasons.

Litter was collected from the freshly fallen litter layer (L), fermentative layer 1 (F1) and fermentative layer 2 (F2) and humus horizon (H) at the experimental site. L layer consists of the upper, dry layer and is composed of freshly fallen needles, twigs, stems and bark. F layer occurs below the L layer, a further sub-division of the fermentation layer into F1 and F2 layers is clearly recognizable. F1 needles being dark in colour and frequently still intact and the F2 needles greyish, fragmentary and compressed together. H layer occurring in humus consists of well-decomposed organic matter of unrecognizable origin (Brandsberg, 1969). Samples were collected on 19 June 2009. Five sampling points were selected according to various direction of exposure, slope gradient, elevation, humidity in a 'zigzag' transect, and each litter layer from the five sampling points were mixed with their equivalent layers and taken to laboratory for further study.

Incubation and observation

The every mixed litter layer from five sampling points was treated with surface sterilization and cultivated (25 ± 2°C) on rose Bengal medium separately. Pure culture of fungus was cultivated on PDA plates at (25 ± 2°C) and preserve on PDA slant at 4°C. Morphological identification was based on fungal culture colony or hyphae characters, spore characteristics, and reproductive structures where discernible (Huang et al., 2008). All cultivations and observations were conducted in duplicate and repeated at least twice.

DNA extraction

Extraction of DNA directly of every mixed litter layer from five sampling points was carried out using an improved glass bead beating method (Vainio and Hantula, 2000; Yeates et al., 1998). As L and F1 layer litter samples were longer, they were cut into 1 to 2 cm fragments before DNA extraction. Extraction buffer (100 mM Tris-HCl [pH 8.0], 100 mM sodium EDTA [pH 8.0], and 200 mM NaCl, 1%PVP, 2%CTAB) was mixed with litter layer samples. Glass beads (diameter 3 mm) were added and mixtures were shaken at 37°C. After 40 min sodium dodecyl sulphate (SDS) was added (20%) and shaking continued for a further 10 min. The samples were incubated at 65°C for 1 h, transferred and centrifuged at 6000 g for 15 min. The supernatants were collected, then mixed with polyethylene glycol (30%) /sodium chloride (1.6 M) and incubated at room temperature for 2 h. Samples were centrifuged (10,000 g for 20 min) and the partially purified nucleic acid pellet resuspended in TE buffer (10 mM Tris-HCl, 1 mM sodium EDTA, pH 8.0). Sodium acetate (4 M [pH 5.4]) was added to a final concentration of 0.3 M. The mixtures were extracted for equal volume with phenol/ chloroform/ isoamyl alcohol (25:24:1, by vol.) and centrifuged (10,000 g for 5 min). Finally, the DNA was selectively precipitated by adding 0.6 volume isopropanol for 2 h, pelleted by centrifugation in a microcentrifuge (15000 rpm for 20 min), washed with 70% ethanol, dried under vacuum and resuspended in TE buffer.

Isolates were grown on PDA and mycelium was scraped from the surface (Prihastuti et al., 2009). Genomic DNA of 5 unknown isolates, which could not be identified by morphological identification, was extracted using fungal DNAout Kit (Tiandz, China) according to the manufacturer's instructions.

PCR amplification and denaturing gradient gel electrophoresis (DGGE)

Partial fungal SSU rDNA fragments were amplified with the universal primer pairs (nu-SSU-0817-5'-TTA GCA TGG AAT AAT RRA ATA GGA and nu-SSU-1536-3'-ATT GCA ATG CYC TAT CCC CA) and nested pairs (nu-SSU-0817-5' and nu-SSU-1196-3'-TCT GGA CCT GGT GAG TTT CC). The amplified regions of two primer pairs were carried out as described in Borneman and Hartin (2000). A GC clamp (CCC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GCC G) was added to nu-SSU-1196-3' in order to stabilize the melting behavior of the DNA fragment and efficient separation in DGGE. In first round of amplification all reagents were

Table 1. Identification of 21 fungal isolates.

Taxon	Species	Family	Phylum	GenBank No.
UF-1	*Irpex lacteus*	Steccherinaceae	Basidiomycota	HQ641118
UF-2	*chaetomium* sp.	Chaetomiaceae	Ascomycota	HQ651814
UF-3	*Hebeloma radicosum*	Hymenogastraceae	Basidiomycota	HQ641119
UF-4	*Acremonium* sp.	Hypocreaceae	Ascomycota	HQ641121
UF-5	*Colletotrichum* sp.	Phyllachoraceae	Ascomycota	HQ651813
IS-1	*Alternaria sp. 1*	Pleosporaceae	Ascomycota	
IS-2	*Alternaria sp. 2*			
IS-3	*Alternaria sp. 3*			
IS-4	*Aspergillus niveoglaucus*	Trichocomaceae	Ascomycota	
IS-5	*Glomerularia* sp.	Platygloeaceae	Basidiomycota	
IS-6	*Mucor corticolus*	Mucoraceae	Zygomycota	
IS-7	*Paecilomyces varioti*	Trichocomaceae	Ascomycota	
IS-8	*Penicillium simplicissimum*	Trichocomaceae	Ascomycota	
IS-9	*P. janthinellum*	Trichocomaceae	Ascomycota	
IS-10	*Pestalotiopsis* sp.	Amphisphaeriaceae	Ascomycota	
IS-11	*Pilaira saccardiana*	Mucoraceae	Zygomycota	
IS-12	*Rhizopus hangzhou*	Mucoraceae	Zygomycota	
IS-13	*Sporobolomyces inositophilus*	Ustilaginaceae	Basidiomycota	
IS-14	*Trichoderma koningii*	Hypocreaceae	Ascomycota	
IS-15	*Trichocladium alopallonellum*	Bionectriaceae	Ascomycota	
IS-16	*Verticillium* sp.	Plectosphaerellaceae	Ascomycota	

combined and heated at 94°C for 4 min. Thirty-five cycles of PCR were performed by using 94°C for 1 min, 54°C for 1 min, and 72°C for 2 min, followed by 72°C for 7 min. In the second amplification round the purification of the first PCR product were used as a template and the annealing temperature was 51°C.

The partial SSU rDNA fragments amplified were analysed by the denaturing gradient gel electrophoresis system, DGGE-2401 (CBS scientific company, Del Mar, CA) using 8% (w/v) acrylamide/ bisacrylamide (37.5:1) gels. The gels were run at 150V in 1x TAE-buffer (40 mm Tris/Acetate, pH 8; 1 mM EDTA) at a constant temperature of 60°C for 7 h. The rDNA fragments were visualised by silver staining. The DGGE mobilities of different samples were compared in parallel on the same gels in order to reveal differences or to confirm similarity.

Universal primers ITS1 (TCC GTA GGT GAA CCT GCG G) and ITS4 (TCC TCC GCT TAT TGA TAT GC) (White et al., 1990) were used to amplify the rDNA ITS of 5 unknown fungal isolates. PCR amplifications were performed as described in Chen et al. (2007).

Sequencing of DGGE fragments

DGGE fragments were excised with a sterile scalpel. The DNA of each fragment was rapidly rinsed with 100 mL of sterile water and eluted in 20 mL of sterile water overnight at 4°C. One microliter of the eluted DNA of each DGGE band was re-amplified by using the conditions with nested pairs described above. The success of this procedure was checked by electrophoresing 3 mL portions of the PCR products in DGGE gels as described earlier.

Analysis of the DGGE patterns

Scanned gels were analyzed with the bioRad quantityone 4.6.2 software package by using the strategy proposed by Omar and Ampe (2000). The patterns were analyzed in two ways, as follows: Firstly after bands were assigned to the gel tracks and the corresponding bands in independent tracks were matched, Dice's coefficients of similarity and the unweighted pair group method with arithmetic averages were used to calculate dendrograms. Secondly two parameters were used to assess the structural diversity and evenness in the microbial community studied. After bands were assigned to the gel tracks, the Shannon-Weaver index of general diversity (*H'*) was calculated with the following equation: $H' = -\sum P_i \cdot \ln P_i$. *H'* was calculated on the basis of the bands in the gel tracks by using the intensities of the bands as judged by peak heights in the densitometric curves. Using the same data, evenness was calculated by using the following function: $E_H = H'/\ln S$, where S is the number of bands in gel tracks.

RESULTS

Isolates obtained by pure culture

We obtained 21 isolates by pure culture, 16 isolates were identified morphologically and 5 isolates which could not be distinguished morphologically were identified by ITS region analysis. They are all listed in Table 1.

Fungal diversity by DGGE

After experiments were repeated and refined, stable and clear denaturant gradient gel electrophoresis profiles

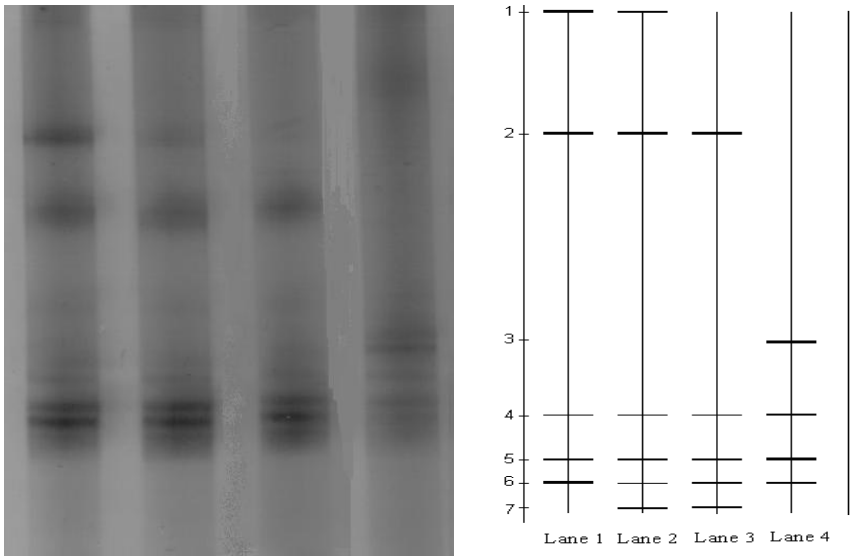

Figure 1. DGGE profile (a) of 18S rDNA sequences amplified from DNA extracted directly from L, F1, F2 and H layer in *Pinus sylvestris* var. *mongolica* litter and its schematic drawing (b). Symbols: Lane1, L layer; 2, F1 layer; 3, F2 layer; 4, H layer.

were obtained and contained high similarity among replicates (performed on DNA separately extracted from different specimens). Only one of three DGGE gel is shown in Figure 1 and is reported as schematic drawing (Agnelli et al., 2004). In Figure 1 the number of bands in the F1 layer is the largest. Some bands (for example, Lane 1-1 and Lane 2-1; Lane 2-7 and Lane 3-7) shared similar electrophoretic mobility. Bands 4-6 were detectable throughout the whole litter decomposition profile, indicating the dominance of a few populations. Thus it can be observed that the fungal community may be correlated with nutrient substrate during the litter decomposition.

All of the sequences retrieved corresponded to portions of the 18S rDNA gene (Table 2). With the exception of *Sporobolomyces inositophilus*, all other taxa were identified as Ascomycota. These species, *Desmazierella acicola*, *Geopyxis majalis* and *Pulchromyces fimicola*, appeared in all litter layers, and thus considered to be main decomposers of freshly fallen litter through to heavily decayed litter. In addition, *Sporobolomyces inositophilus* was detected by DGGE. This taxon only appears only in the H layer during leaf decomposition.

Analysis of similarity

The similarity between the DGGE patterns of the fungal community was evaluated using an unweighted pair group method with arithmetic averages (UPGMA). UPGMA analysis revealed that the DGGE patterns for each layer clustered from three DGGE analyses as showed in Figure 2. The patterns from the F1 and F2 layers were most similar (81% similarity). A major shift occurred in the bands from the L and H layers: The DGGE patterns from the L layer belonged to a second cluster that was clearly separated from profiles obtained from the F1 and F2 levels. There was also only 34% similarity between the patterns of H layer with other layers.

Shannon-Weaver index and evenness

Analysis of DGGE patterns was determined by estimation of the biodiversity and evenness indices (Shannon and Weaver, 1963). The Shannon-Weaver index of biodiversity on fresh fallen litter was 1.61, and increased from initial decomposition and reached its maximum value in the F1 layer. This index decreased with substratum consumption and further decomposition, and was minimal in the H layer when litter was completely broken down into debris and was similar to soil. The fungal diversity decreased significantly with substratum consumption (Table 3). The evenness values remained at about 1.0 throughout the process and not changed significantly with litter decomposition stage (Table 3).

DISCUSSION

This is the first report on the fungal diversity of *Pinus* litter

Table 2. Fungal 18S rDNA blast results of bands in DGGE profile.

Band number[a]	Species	Identity (%)	GenBank No.	Family	Phylum	Frequency of occurrence (%)
Band 1	*Aureobasidium pullulans*	99	GU451241	Dothioraceae	Ascomycota	50
Band 2	*Ascomycota* sp.	95	GU451244		Ascomycota	75
Band 3	*Sporobolomyces inositophilus*	99	GU451243	Ustilaginaceae	Basidiomycota	25
Band 4	*Desmazierella acicola*	99	GU451240	Sarcosomataceae	Ascomycota	100
Band 5	*Pulchromyces fimicola*	99	GU451242	Not assigned	Ascomycota	100
Band 6	*Geopyxis majalis*	99	HM068870	Pyronemataceae	Ascomycota	100
Band 7	Uncultured Hypocreales	99	GU451241	Hypocreales	Ascomycota	50

[a] Bands were extracted from the DGGE gel showed in Fig. 1(b).

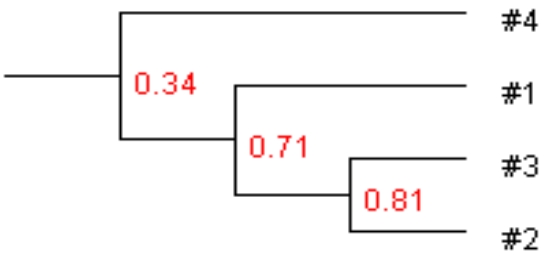

Figure 2. Dendrogram derived from DGGE analysis of the fungal community on the basis of the Dice's coefficient of similarity with the unweighted pair group method with arithmetic averages. Symbols: #1, L layer; #2, F1 layer; #3, F2 layer; #4, H layer.

Table 3. Shannon-Weaver index of diversity and evenness.

Litter layer	Shannon-Weaver index	
	Diversity	Evenness
L layer	1.609 ± 0.02b	1.000 ± 0.11a
F1 layer	1.792 ± 0.06a	0.999 ± 0.01a
F2 layer	1.609 ± 0.01b	0.994 ± 0.06a
H layer	1.386 ± 0.08c	1.003 ± 0.02a

Mean ± SE (n=3); small letters in the same column indicate significant differences among layers ($p<0.05$).

in China using combined traditional culture and molecular methods especially DGGE.

Primer performance

DGGE has been used extensively for examination of fungal communities in different ecological systems such as grass, wheat, wood and soil (Laforgue et al., 2009; Nikolcheva et al., 2005; Tao et al., 2008; Vainio and Hantula, 2000). DGGE is over-reliant on the PCR technique, so primer pairs with strong specificity for fungal rDNA sequences are considered especially important. Many primer pairs used for amplification of fungal 18S rRNA gene fragments were reported in previous studies such as EF4-EF3, EF4-fung5 and EF4-NS3. Smit et al. (1999) found that the primer pair EF4-EF3 was slightly biased in amplifying *Basidiomycota* and *Zygomycota*, whereas the primer pair EF4-fung5 amplified mainly *Ascomycota* as predicted by computer analysis. Borneman and Hartin (2000) reported that above primer pairs amplified some non fungal templates, and primers EF4-NS3 produced no amplification products with any of the templates except *Amoebidium parasiticum* and *Mucor rouxii*. It is important to develop specific primer sets with a satisfactory fungal amplification range and a sufficiently low 18S homology to other eukaryotes. However, as results described in Borneman and Hartin (2000) report, the newly designed PCR primers, nu-SSU-0817-5', nu-SSU-1196-3' and nu-SSU-1536-3', could overcome the shortcomings discussed and are regarded as useful tools for identifying fungi in environmental samples. They can also be used in the construction of fungal rDNA libraries from other environmental samples, for denaturation gradient gel electrophoresis analysis. In this paper nu-SSU-0817-5', nu-SSU-1196-3' and nu-SSU-1536-3' are first reported for analysis of fungal communities directly from environmental samples. In addition, nested PCR with above these two pairs was used in this study increases further specific amplification.

Species of fungi detected by PCR-DGGE in this study have been reported previously. *Aureobasidium pullulans,* black yeast in Dothioraceae, has frequently been found in soil and water, especially relying upon decaying leaf litter, wood and other plant life (Leathers, 2002). *Sporobolomyces inositophilus* was first isolated from a dead leaf of *Sasa* sp. in Japan by Nakase and Suzuki (1987). Van and Gourbiere (1997) concludes that abundance of *Verticicladium trifidum* (the anamorph of *Desmazierella acicola*) is maximal (near 100% needles colonized) around a latitude of 45° N, which is in accord with latitude of sampling field in this study. *Desmazierella acicola* was also isolated from litter sections of Norway

spruce in mixed forest with 18% frequency of occurrence (Przyby et al., 2008).

Analysis of fungal community

Seven OTUs were successfully recovered by DGGE in this study revealing some typical fungal colonization in litter (for example, *Aureobasidium pullulans* and *Desmazierella acicola*) as well as taxa that appear unable to grow on artificial media. The phylogenetic placement of Band 2 could not be resolved, which was also found in a previous study. O'Brien et al. (2005) reported on litter microorganisms from a mixed deciduous forest in the Southeastern United States. Taxa were sequenced and identified using available data from GenBank, and about 12% of the sequences could not be identified even to phylum level. Most isolation of fungi from fallen leaves using traditional techniques is filamentous fungi (Song et al., 2004; Wang et al., 2008). The numbers of fungi identified by DGGE, however, is small as compared to traditional culture (Duong et al., 2006; Promputtha et al., 2005).

Fungal communities revealed in the *Pinus* litter layers (F1, F2, L and H) by PCR-DGGE differed significantly from those yielded by traditional culture metholodogy study, with *Sporobolomyces inositophilus* appearing in both methodologies. The common taxa commonly found using traditional cultural studies were not detected through DGGE methodology. There may be several reasons for this. It has been suggested that some species are not abundant in leaves, while others occupy spaces as small as single cells (Ghimire and Hyde, 2004; Varma et al., 2004). In such cases it might be plausible that these fungi present in small amounts could not provide enough fungal DNA to be detected by DGGE. Fast-growing fungi are usually abundantly on artificial media (Duong et al., 2006). At the same time fast-growing fungi may occupy ecological niches ahead of others and inhibit the growth of slow and moderately slow-growing genera which may lead to species loss in diversity study. Jayasinghe and Parkinson (2008) found that fast growing genera of the litter decomposer fungi were more tolerant of actinomycete antagonism than slow and moderately slow-growing genera (for example, *Cladosporium*, *Mortierella*). Consequently, *Mucor*, *Penicillium* and *Trichoderma* are easy to isolate on media. Besides, DGGE is considered to be a PCR-based technique. The usage of nested PCR increased the specificity of amplified production, while also may increase biases due to differences in amplification efficiency. It is indicated that nested PCR maybe support the low number of DGGE-bands observed during this study.

Litter fungal colonization is regulated both by exogenous or environmental factors and endogenous factors. Environmental factors include climate and soil nutrient availability (Cortez et al., 1996). The sample site used in this study is located in cold temperature zone and this may lead to lower fungal diversity than those in subtropical and tropical zones. Endogenous factors are leaf litter nutrient content (for example, C, N, P) and secondary metabolites (for example, phenolic compounds) content (Koukoura et al., 2003).The phenolic compounds were involved imbue tissues with antifungal and antioxidant properties. This is confirmed by Ormeno et al. (2006) who showed that a negative significant correlation between total phenolic compounds and ergosterol concentrations, an indicator of fungal biomass, of leaf litter. The phenolic compounds concentration in pinus needle is higher in broad leaves, so the fungal diversity on pinus needle litter is lower relatively. Meanwhile the concentration of phenolics depends on abiotic and biotic factors, such as pollution stress, nitrogen concentration, and insect grazing (Lieutier et al., 1991; Sokolski et al., 2006). Thus it is necessary to carry out two methods, DGGE and traditional culture to investigate fungal diversity during litter decomposition.

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