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Distribution of ectomycorrhizal and pathogenic fungi in soil along a vegetational change from Japanese black pine (*Pinus thunbergii*) to black locust (*Robinia pseudoacacia*)

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Abstract The nitrogen-fixing tree black locust (*Robinia pseudoacacia* L.) seems to affect ectomycorrhizal (ECM) colonization and disease severity of Japanese black pine (*Pinus thunbergii* Parl.) seedlings. We examined the effect of black locust on the distribution of ECM and pathogenic fungi in soil. DNA was extracted from soil at depths of 0–5 and 5–10 cm, collected from the border between a Japanese black pine- and a black locust-dominated forest, and the distribution of these fungi was investigated by denaturing gradient gel electrophoresis. The effect of soil nutrition and pH on fungal distribution was also examined. *Tomentella* sp. 1 and *Tomentella* sp. 2 were not detected from some subplots in the Japanese black pine-dominated forest. Ectomycorrhizas formed by *Tomentella* spp. were dominant in black locust-dominated subplots and very little in the Japanese black pine-dominated forest. Therefore, the distribution may be influenced by the distribution of inoculum potential, although we could not detect significant relationships between the distribution of *Tomentella* spp. on pine seedlings and in soils. The other ECM fungi were detected in soils in subplots where the ECM fungi was not detected on pine seedlings, and there was no significant correlation between the distribution of the ECM fungi on pine seedlings and in soils. Therefore, inoculum potential seemed to not always influence the ECM community on roots. The distribution of *Lactarius quieticolor* and *Tomentella* sp. 2 in soil at a depth of 0–5 cm positively correlated with soil phosphate (soil P) and that of *Tomentella* sp. 2 also positively correlated with soil nitrogen (soil N). These results suggest the possibility that the distribution of inoculum potential of the ECM fungi was affected by soil N and soil P. Although the mortality of the pine seedlings was higher in the black locust-dominated area than in the Japanese black pine-dominated area, a pathogenic fungus of pine seedlings, *Cylindrocladium pacificum*, was detected in soil at depths of 0–5 and 5–10 cm from both these areas. This indicates that the disease severity of pine seedlings in this study was influenced by environmental conditions rather than the distribution of inoculum potential.

Keywords DGGE · Inoculum potential · Soil DNA extraction · Soil nitrogen · Soil phosphate

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Introduction

The distribution of ectomycorrhizal (ECM) fungal species is reportedly different and based on the host specificity (Molina et al. 1992), host age (Deacon et al. 1983), and distance of the roots from the tree stems (Ford et al. 1980). In addition, the ECM community is also influenced by nitrogen fertilization (Parrent and Vilgalys 2007), and soil chemistry seems to affect the distribution of ECM fungi.

In our previous study, dominant ECM fungal species on Japanese black pine seedlings (*Pinus thunbergii* Parl.) differed between a Japanese black pine-dominated forest and a black locust (*Robinia pseudoacacia* L.)-dominated forest (Taniguchi et al. 2007a). In the Japanese black pine-dominated forest, *Cenococcum geophilum* and *Russula* spp. were predominant, whereas *Tomentella* spp. were predom-

inant in the black locust-dominated forest. This distributional change seems to be influenced by soil N. Soil N may also influence the distribution of ECM fungi in soil, affecting the infection of pine seedlings; however, this phenomenon has not yet been elucidated.

Regeneration of pine seedlings was inhibited in a black locust-dominated forest (Taniguchi et al. 2007b), and *Cylindrocladium pacificum* was isolated from dead pine seedlings (Taniguchi et al. 2008b). By Koch's postulates, *C. pacificum* was shown to be a pathogenic fungus of pine seedlings. Distribution of this fungus may affect the disease severity of pine seedlings in black locust-dominated areas but has not yet been studied.

In this study, we examined the distribution of ECM fungi and a pathogenic fungus, *C. pacificum*, in soil by denaturing gradient gel electrophoresis (DGGE) to study the effect of these fungi on infection of pine seedlings. To examine the effect of environmental conditions on the distribution of ECM fungi and *C. pacificum* in soil, soil pH and nutrition were also assessed.

Materials and methods

Study site and sampling

The study site was located in the Arid Land Research Center near the Tottori Sand Dunes, Tottori, Japan (35°32′ N, 134°13′ E) and was the same as in our previous study (Taniguchi et al. 2007a). Japanese black pine and black locust are dominant in this site, and *Rhus succedanea*, *Mallotus japonicus*, and *Celtis sinensis* were also observed. This area was afforested with Japanese black pine and black locust about 50 years ago, although the ratio of planting density was not recorded. Pine wilt disease spread widely in the 1990s, which reduced the density of Japanese black pine and resulted in black locust dominance in some areas.

Two 10×20 m experimental plots were selected at the border between a Japanese black pine- and a black locust-dominated area. Each plot was divided into four 5×10 m subplots: I—Japanese black pine-dominated subplot, II—Japanese black pine-dominated subplot mixed with black locust, III—black locust-dominated subplot mixed with Japanese black pine, and IV—black locust-dominated subplot. Each subplot was then divided into 32 1.25×1.25 m sampling quadrats, and five quadrats were randomly selected from each subplot. In July 2005, after removing the litter layer at the center of each quadrat, about 5–10 g of soil samples were collected at depths of 0–5 and 5–10 cm. If pine seedlings were present near the sampling point, the point was displaced. After sieving (mesh size=5 mm) and removing long roots, approximately 0.5 g of soil from each soil sample was put into 2.0 ml tubes and stored at −80°C.

DNA extraction

The soil samples were ground in liquid nitrogen (1,800 rpm for 10 s) with a cell disruptor (MB400KUF; Yasuikikai Co., Osaka, Japan), added to 100 μl of 0.4% (*w*/*v*) skim milk powder solution, and ground again (1,800 rpm for 10 s). Skim milk prevents DNA degradation and adsorption by soil colloids (Volossiouk et al. 1995) and is also effective for DNA extraction from soil samples (Hoshino and Matsumoto 2004). After addition of 800 μl sodium dodecyl sulfate (SDS) buffer (0.3% SDS [*w*/*v*] in 0.42 M NaCl and 50 mM sodium acetate), the sample solution was incubated for 30 min at 65°C. Then, 500 μl of chloroform with isoamyl alcohol (49:1) was added to the sample solution, the mixture was centrifuged at 15,000 rpm for 15 min at 16°C, and the supernatant was transferred to a new tube. After adding 500 μl of phenol and chloroform (1:1), the mixture was centrifuged at 15,000 rpm for 20 min at 16°C, and the supernatant was transferred to a new tube. Then, chloroform and isoamyl alcohol extraction was conducted using 500 μl of chloroform with isoamyl alcohol (49:1). Following this, 40 μl of 3 M sodium acetate and 400 μl of isopropanol were added to the supernatant, which was incubated for 20 min at room temperature and then centrifuged at 15,000 rpm for 25 min at 16°C. The resultant DNA pellet was dissolved in 200 μl of TE buffer (10 mM Tris-HCl, pH 8.0; 1 mM ethylenediaminetetraacetic acid [EDTA], pH 8.0) and 200 μl of polyethylene glycol (PEG) 6000 solution (13% PEG [*w*/*v*] in 1.6 M NaCl) was added. After vortexing and incubating for 1 h at 4°C, the sample solution was centrifuged at 15,000 rpm for 25 min at 4°C. The resultant DNA pellet was rinsed with 70% ethanol and dried. Each dried DNA pellet was dissolved in 50 μl of 1/10 TE buffer (1 mM Tris-HCl, pH 8.0; 0.1 mM EDTA, pH 8.0). DNA concentration in the extract diluted by 5% with distilled water was determined using a spectrophotometer (UV Mini-1240; Shimadzu Co., Kyoto, Japan) at 260 nm.

Polymerase chain reaction amplification and DGGE

DNA was amplified in a 20-μl reaction mix containing approximately 0.3–0.7 ng of template DNA on a Mycycler thermocycler (BioRad Laboratories, Hercules, CA, USA). The internal transcriber spacer 1 (ITS1) region of rDNA was amplified with GC-clamped ITS1F (Gardes and Bruns 1993) and ITS2 primers (White et al. 1990). The thermal profile in touchdown polymerase chain reaction (PCR) was as follows: 3 min initial denaturation at 94°C, ten cycles of 1 min denaturation at 94°C, 30 s annealing at variable temperatures (55–65°C) decreased by 1°C in each cycle, and 1 min extension at 72°C, 30 cycles of 1 min denaturation at 94°C, 30 s annealing at 55°C, and 1 min extension at 72°C, and a 5 min

final extension at 72°C. All PCR products from each soil sample were inspected with agarose gel electrophoresis prior to DGGE.

To determine the distribution of inoculum potential of the dominant ECM fungi on the pine seedlings in 2004 (Taniguchi et al. 2007a), a mixture of the DNA of *Laccaria* sp. 1 (AB253515), *Lactarius quieticolor* (AB253518), *Russula* sp.1, *Russula* sp. 2 (AB253519), *Cortinarius* sp. (AB253520), *C. geophilum*, *Tomentella* sp.1 (AB253522), *Tomentella* sp. 2 (AB253523), and Unidentified ECM T01 (AB253524) was used as a reference marker of the ECM fungi. DNA of *C. pacificum* (AB287008) was used as a reference marker of the pathogenic fungi of the pine seedlings.

DGGE was performed in Dcode universal mutation detection system (Nippon Bio-Rad Laboratories Co., Tokyo, Japan) using 8% polyacrylamide gel with a 20–60% denaturing gradient (urea and formamide). Seventeen microliters of PCR products mixed with 17 μl of dye solution were loaded into the denaturing gel and electrophoresis was conducted at 75 V for 16 h at 60°C in 1× TAE buffer (40 mM Tris-acetate, 1 mM EDTA), following which band profiles were visualized by GelRed (Biotium, Inc., Hayward, CA, USA) staining. From the presence or absence of the band of a given fungus, the frequency of the fungus in each subplot was determined by dividing the number of quadrats with the fungus by the total number of quadrats examined for each subplot in the two plots.

Recovery of bands from DGGE gels and sequence analysis

To confirm whether the band at the same location as the DGGE band of the reference fungi was of the same species as the reference fungal species, sequencing analysis was conducted. DGGE bands of the soil sample at the same location as the reference marker were excised. Each excised band was placed in a 0.5-ml microtube to which 0.1 ml of TE buffer was added. The tubes were incubated at 4°C overnight. A 1-μl aliquot of the TE buffer containing DNA was used as the template for a PCR performed with one cycle of 95°C for 5 min, 30 cycles of 94°C for 30 s, 58°C for 30 s, and 72°C for 1 min, with a final extension of 72°C for 10 min. ITS1F and ITS2 were used as the primer sets of 18S rDNA. The PCR-amplified DNA fragment was purified, and direct sequencing was performed using a Big Dye Terminator v.1.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) with either ITS1F or ITS2 according to the manufacturer's instructions. Nucleotide sequences were determined by an Applied Biosystems 3300 sequencer (Applied Biosystems) and subjected to a Basic Local Alignment Search Tool (BLAST; National Center for Biotechnology) search to compare with sequences reported in the GenBank database.

Soil pH and nutrition

Soil pH was measured at a soil-to-water ratio of 1:10 (*v*/*v*). Available phosphate was extracted from 0.25 g of air-dried soil with 50 ml of Truog reagent (Truog 1930). Then, the soil P (P_2O_5 mg 100 g $soil^{-1}$) was determined by the molybdenum blue method (Kimura 1995). About 0.08 g of each soil sample was dried at 60°C for 3 days and ground using a cell disruptor (MB400KUF) for the analysis of total nitrogen concentration (soil N), total carbon concentration (soil C), and C/N ratio (soil C/N) using the Vario EL III CHNOS elemental analyzer (Elementar Analysensysteme GmbH, Hanau, Germany).

Statistical analysis

For soil pH and nutrition (soil C, soil N, soil C/N, soil P, and soil N/P), the analysis of variance ($P<0.05$) was used to determine differences between the four subplots. Then, the Tukey–Kramer test ($P<0.05$) was used to distinguish the differences, with computer program StatView 5.0 (SAS Institute Inc., Cary, NC, USA). Distribution of ECM fungi was analyzed by ALSCAL multidimensional scaling (MDS) with SPSS Version 10.0 J software (SPSS, Inc., Chicago, IL, USA).

To reveal what factors correlated with the distribution of fungi, Spearman's rank correlation coefficients between the presence or absence of each fungal species and soil pH or soil nutrition (soil N, soil C/N, soil P, and soil N/P) or total basal area of Japanese black pine (PBA) and black locust (RBA) within 2 m of the sampled locations were accounted. Correlation of the vertical distribution of each ECM fungal species in soil at depths of 0–5 and 5–10 cm was also analyzed by Spearman's rank correlation analysis. In order to compare the distribution between ectomycorrhizal fungi on pine seedlings in 2004 (Taniguchi et al. 2007a) and the ECM fungi in soil at the depth of 0–5 and 5–10 cm, Spearman's rank correlation analysis was conducted. The number of pine seedlings (Taniguchi et al. 2007a) and that of soils in each subplot, which each ECM fungal species was observed in, was used by this analysis. These analyses were performed using SPSS Version 10.0 J software.

Results

Soil pH and nutrition

Soil C and soil N (at depths of 0–5 and 5–10 cm) were significantly higher ($P<0.05$) in the black locust-dominated subplots (III and IV) than in Japanese black pine-dominated subplot I (Table 1). At both depths, soil C/N gradually

Table 1 Mean soil pH and nutrition (i.e., total soil nitrogen, total soil phosphate, total soil carbon, soil carbon–nitrogen ratio, and soil nitrogen–phosphate ratio) in each subplot (I–IV) at depths of 0–5 and 5–10 cm

	I	II	III	IV
Soil C (mg g^{-1} DW)				
0–5 cm	3.9±0.6a	5.7±0.9ab	15.1±4.1bc	23.2±4.0c
5–10 cm	2.0±0.1a	2.3±0.2ab	3.4±0.5bc	3.5±0.2c
Soil N (mg g^{-1} DW)				
0–5 cm	0.20±0.04a	0.34±0.06ab	0.95±0.25bc	1.52±0.28c
5–10 cm	0.09±0.01a	0.13 ±0.01a	0.21±0.04b	0.22±0.01b
Soil P (mg 100 g $soil^{-1}$)				
0–5 cm	0.9±0.1a	1.0±0.1a	1.6±0.4a	1.7±0.3a
5–10 cm	1.0±0.1a	1.0±0.0a	0.9±0.1a	1.0±0.1a
Soil C/N				
0–5 cm	20.1±1.4a	16.9±0.4b	16.1±0.4b	15.4±0.3b
5–10 cm	21.8±1.0a	17.9±0.5b	16.4±0.5b	16.1±0.4b
Soil N/P				
0–5 cm	0.22±0.04a	0.32±0.04ab	0.58±0.07b	0.93±0.13c
5–10 cm	0.10±0.01a	0.14±0.01a	0.23±0.03b	0.25±0.03b
Soil pH				
0–5 cm	5.3±0.1a	5.1±0.1ab	5.0±0.1bc	4.8±0.1c
5–10 cm	5.2±0.1ab	5.3±0.0a	5.2±0.1ab	5.1±0.1b

Values are means ± SE. Different letters in the same row indicate significant differences (Tukey–Kramer test; $P<0.05$)
soil N total soil nitrogen, *soil P* total soil phosphate, *soil C* total soil carbon, *soil C/N* soil carbon–nitrogen ratio, *soil N/P* soil nitrogen–phosphate ratio

decreased from subplot I to subplot IV. Soil P (0–5 cm depth) was higher in the black locust-dominated subplots (III and IV) than in the Japanese black pine-dominated subplots (I and II), but the differences were not significant. Soil P (5–10 cm depth) did not differ significantly between the four subplots. Soil N/P (at depths of 0–5 and 5–10 cm) was significantly higher ($P<0.05$) in subplots III and IV compared with subplot I. Soil pH (0–5 cm depth) was significantly lower in the black locust-dominated subplots (III and IV) than in Japanese black pine-dominated subplot I.

DGGE analysis

One of the DGGE profiles for fungal internal transcribed space (ITS) sequences from soil samples of each subplot at depths of 0–5 and 5–10 cm was shown in Fig. 1. In the DGGE analysis, nine to 20 (average=12.8), eight to 18 (average=13.2), 12–19 (average=13.2), and three to 20 (average=11.8) bands were observed in soil samples (depth=0–5 cm) collected from subplots I, II, III, and IV of plot 1, and nine to 15 (average=12.4), ten to 19 (average=13), 11–19 (average=14.8), and two to 18 (average=13.2) bands were observed in soil samples (depth=0–5 cm) collected from subplots I, II, III, and IV of plot 2. In the samples collected from a depth of 5–10 cm, four to 16 (average=11.2), eight to 18 (average=13.6), ten to 19 (average=14), and four to 16 (average=9.4) bands were observed in soil from subplots I, II, III, and IV of plot 1, and ten to 13 (average=11.4), five to 14 (average=10.6), eight to 17 (average=12.8), and one to 16 (average=9.6) bands were observed in soil from subplots I, II, III, and IV of plot 2.

Sequencing analysis revealed that the DGGE bands of *Laccaria* sp. 1 (a), *Russula* sp.1 (f), *Russula* sp. 2 (f), and *C. geophilum* (g) overlapped with those of the other fungi (Table 2). Therefore, these species were excluded from the following analysis.

Tomentella sp. 1 was not detected in samples from subplot I of plot 2 at the depth of 5–10 cm (Table 3); *Tomentella* sp. 2 was not detected in samples from subplot II of plot 1 and subplot I of plot 2 at the depth of 5–10 cm. However, the other ECM fungi were detected in samples from all subplots at both depths (i.e., 0–5 and 5–10 cm). The pathogenic fungus *C. pacificum* was detected in soil samples from all subplots at both depths (Table 3).

MDS analysis of the DGGE profile of ECM fungal DNA sampled at a depth of 0–5 cm did not separate the Japanese black pine-dominated subplots (I and II) from the black locust-dominated ones (III and IV; Fig. 2a). However, the DGGE profile of ECM fungal DNA (at a depth of 5–10 cm) from the Japanese black pine-dominated subplots (I and II) distributed in the third and fourth quadrat in Fig. 2b and that from the black locust-dominated subplots (III and IV) distributed in the first and second quadrat in the figure.

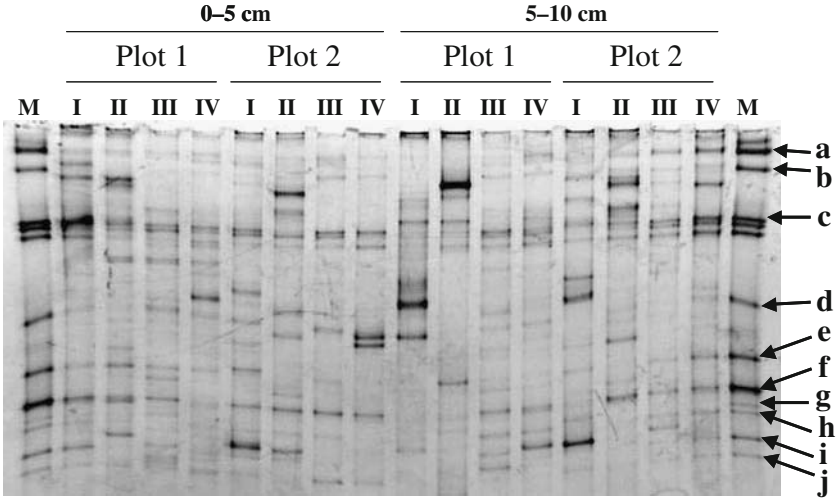

Fig. 1 One of the denaturing gradient gel electrophoresis profiles for fungal internal transcribed space sequences from soil samples of each subplot at depths of 0–5 and 5–10 cm. Reference fungi contained in the reference marker lane (*M*) are as follows: *a Laccaria* sp. 1, *b* Unidentified ECM T01, *c C. pacificum*, *d Cortinarius* sp., *e Tomentella* sp. 1, *f* Russula spp. (*Russula* sp. 1 and *Russula* sp. 2), *g C. geophilum*, *h Tomentella* sp. 2, *i Lactarius quieticolor*, and *j Tomentella* sp. 2

Correlation analysis

Distribution of *L. quieticolor* at the depth of 0–5 cm had a significantly positive correlation ($P<0.05$) with soil P (Table 4). The distribution of *Tomentella* sp. 2 positively correlated with soil P or soil N at the depth of 0–5 cm ($P<0.05$) and negatively correlated with PBA ($P<0.05$). The distribution of *Tomentella* sp. 1 at the depth of 0–5 cm positively correlated with RBA ($P<0.05$).

The distribution of *Cortinarius* sp., *Tomentella* sp. 1, Unidentified ECM T01, and *C. pacificum* at the depth of 0–5 cm positively correlated ($P<0.05$) with that at the depth of 5–10 cm, whereas the distribution of *L. quieticolor* and *Tomentella* sp. 2 at 0–5 and 5–10 cm had no significant correlation (Table 5). The distribution of ECM fungi (*L. quieticolor*, *Cortinarius* sp., *Tomentella* sp. 1, *Tomentella* sp. 2, and Unidentified ECM T01) on pine seedlings and in soils (at depths of 0–5 and 5–10 cm) had no significant correlation ($P>0.05$).

Discussion

The DGGE profile of DNA from soil samples at the depth of 0–5 cm analyzed by MDS analysis revealed that ECM communities did not differ between the two areas (Fig. 2a), whereas that at the depth of 5–10 cm tends to differ between the two areas (Fig. 2b). This is partially because the distribution of *Tomentella* spp. and *L. quieticolor* at the depth of 5–10 cm tends to differ between the Japanese black pine- and black locust-dominated forests (Table 3). The separation of the DGGE profile at the depth of 5–10 cm

Table 2 BLAST search from the DNA sequence of the DGGE band

Location of DGGE band	Accession no.	BLAST match (accession no.)	Overlap (bp)	Similarity (%)
a	AB467358	Uncultured *Rhizoctonia* isolate IT1B-10r (DQ061931)	75	100
b	AB467359	**Mycorrhizal basidiomycote isolate T01 (AB253524)**	149	97
c	AB467360	***Cylindrocladium pacificum (AB287008)***	121	100
d	AB467361	**Uncultured ectomycorrhiza (*Cortinarius*) (AB253520)**	147	97
e	AB467362	**Uncultured ectomycorrhiza (*Tomentella*) (AB253522)**	58	96
f	AB467363	Uncultured Russula clone 6S4.08.F04 (EF619752)	75	85
g	AB467364	Uncultured ectomycorrhiza (Thelephoraceae) clone STIL2RO50 (EU645603)	202	95
h	AB467365	**Uncultured ectomycorrhiza (*Tomentella*) (AB253523)**	161	98
i	AB467366	***Lactarius quieticolor* (AB253518)**	190	95
j	AB467367	Ectomycorrhizal root tip 81-sepB_Ny1.EB-17.1 (AF481369)	57	96

The bolding indicates that the sequence of the DGGE band was the same as that of the reference fungi

Table 3 Frequency of five ectomycorrhizal (ECM) fungi and a pathogenic fungus per five samples in each subplot (I–IV) at depths of 0–5 and 5–10 cm

	Plot 1				Plot 2			
	I	II	III	IV	I	II	III	IV
0–5 cm								
Lactarius quieticolor	0.2	0.6	0.4	0.4	0.2	0.4	0.6	0.6
Cortinarius sp.	0.6	0.4	0.6	0.4	0.6	0.4	0.6	0.4
Tomentella sp. 1	0.4	0.6	0.6	0.4	0.2	0.8	0.4	0.4
Tomentella sp. 2	0.2	0.4	0.6	0.6	0.2	0.4	0.4	0.2
Unidentified ECM T01	0.8	0.6	0.8	0.4	0.2	0.6	0.2	0.4
Cylindrocladium pacificum	0.6	0.6	1	0.6	1	0.8	0.6	0.8
5–10 cm								
Lactarius quieticolor	0.4	0.4	0.6	0.6	0.2	0.4	0.4	0.4
Cortinarius sp.	0.4	0.6	0.6	0.6	0.8	0.4	0.6	0.4
Tomentella sp. 1	0.8	0.6	0.4	0.4	0	0.6	0.4	0.6
Tomentella sp. 2	0.4	0	0.4	0.2	0	0.4	0.6	0.4
Unidentified ECM T01	0.6	0.4	1	0.4	0.2	0.2	0.6	0.4
Cylindrocladium pacificum	0.6	0.8	0.8	0.8	0.8	0.6	0.6	0.8

by MDS analysis may also be attributed to methodological restriction. The amount of DNA extracted from samples at the depth of 0–5 cm was 3.3 times more than that at the depth of 5–10 cm. Although we used approximately uniform amounts of DNA for PCR, the amount of fungal DNA from the samples at the depth of 5–10 cm, especially uncommon species, might be small and DGGE could not detect the presence of uncommon ECM fungi; however, the result seems to reflect the quantitative distribution of ECM fungi, and the distributional change of *Tomentella* spp. and *L. quieticolor* from Japanese black pine- to black locust-dominated areas may be detected in soil at the depth of 5–10 cm.

Distribution of three ECM fungi and one pathogenic fungus in soil at the depth of 0–5 cm has a positive correlation with that at the depth of 5–10 cm (Table 5). Dickie et al. (2002) reported that the vertical distribution of the ECM hyphal community differed among the litter layer, F-layer, H-layer, and B-horizon. In our study site, the soil in the Japanese black pine-dominated forest was sandy; the soil in the black locust-dominated forest was also sandy and covered with a 1 cm of humus. Therefore, the soil characteristics were not very different between both the depths (i.e., 0–5 and 5–10 cm), and the ECM fungal community may be similar between the depths.

Tomentella spp. were not detected from some subplots in the Japanese black pine-dominated forest (Table 3). Ectomycorrhizas formed by *Tomentella* spp. were dominant in black locust-dominated subplots (Taniguchi et al. 2007a) and very little in the Japanese black pine-dominated forest. Therefore, the distribution may be influenced by the distribution of inoculum potential. However, we could not detect significant relationships between the distribution of *Tomentella* spp. on pine seedlings and in soils. The other

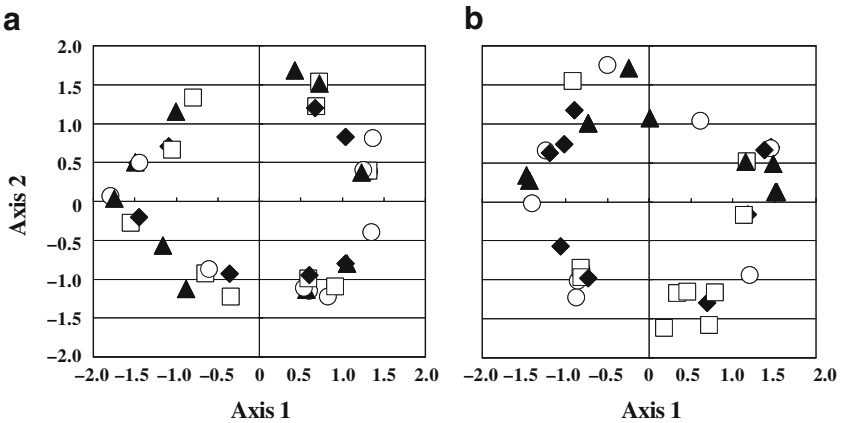

Fig. 2 Multidimensional scaling analysis of the denaturing gradient gel electrophoresis profile of ectomycorrhizal fungal DNA from soil samples at depths of **a** 0–5 cm and **b** 5–10 cm. *Open circles*, *open squares*, *closed triangles*, and *closed rhombi* indicate DGGE profiles from soil samples of subplots I, II, III, and IV, respectively

Table 4 Spearman's rank correlation coefficients between soil pH or nutrition (i.e., total soil nitrogen, total soil phosphate, total soil carbon, soil carbon–nitrogen ratio, and soil nitrogen–phosphate ratio), total basal area of Japanese black pine or total basal area of black locust, and occurrences of five ectomycorrhizal fungi or a pathogenic fungus

	Soil pH	Soil P	Soil N	Soil C/N	Soil N/P	PBA	RBA
0–5 cm							
Lactarius quieticolor	−0.02	0.37*	0.28	−0.25	0.25	−0.19	0.11
Cortinarius sp.	−0.13	0.07	0.02	0.12	0.04	−0.10	−0.01
Tomentella sp. 1	0.04	0.01	−0.03	−0.05	−0.02	−0.13	0.32*
Tomentella sp. 2	−0.18	0.35*	0.32*	−0.22	0.24	−0.33*	0.06
Unidentified ECM T01	0.22	−0.004	−0.18	0.16	−0.14	−0.03	0.05
Cylincrocladium pacificum	−0.09	−0.12	−0.20	0.23	−0.14	0.00	0.12
5–10 cm							
Lactarius quieticolor	0.06	−0.10	0.17	−0.10	0.13	−0.03	0.19
Cortinarius sp.	−0.18	0.02	0.13	0.04	0.13	−0.08	−0.12
Tomentella sp. 1	0.26	−0.02	0.06	−0.17	0.09	−0.18	0.03
Tomentella sp. 2	−0.17	0.12	0.18	−0.23	0.11	−0.21	−0.12
Unidentified ECM T01	−0.07	−0.02	0.12	−0.002	0.15	−0.18	0.12
Cylindrocladium pacificum	−0.25	−0.13	0.12	−0.13	0.16	−0.23	0.23

soil N total soil nitrogen, *soil P* total soil phosphate, *soil C* total soil carbon, *soil C/N* soil carbon–nitrogen ratio, *soil N/P* soil nitrogen–phosphate ratio, *PBA* total basal area of Japanese black pine, *RBA* total basal area of black locust, *ECM* ectomycorrhizal
*$P<0.05$ (correlation coefficient was significant)

ECM fungi were detected in soils in subplots where the ECM fungi was not detected on pine seedlings, and there was no significant correlation between the distribution of the ECM fungi on pine seedlings and in soils. Therefore, inoculum potential seemed to not always influence the ECM community on roots. Koide et al. (2005) also reported that the spatial distribution of ECM fungal species differ in mycelia and root-tip, which is partially supported by the result of this study.

Distribution of the inoculum potential of *Tomentella* sp. 2 in soil (depth=0–5 cm) positively correlated with soil N (Table 4). Although the effects of soil N on ECM fungi have been reported on the basis of ECM community structure of the sporocarps or ECM root tips (Peter et al. 2001; Parrent et al. 2006), this study indicates that soil N could influence not only the distribution of ECM fungi on roots but also the distribution in soil. Parrent and Vilgalys (2007) reported that extrametrical mycelia of ECM fungi were dynamic with a high degree of interspecific variation to N fertilization, and the distribution of *Tomentella* sp. 2 may be affected by soil N. Distribution of the inoculum potential of *L. quieticolor* in soil (depth=0–5 cm) and *Tomentella* sp. 2 positively correlated with soil P (Table 4). ECM root tips formed by the ECM fungi observed in a black locust-dominated forest have a higher ability to produce phosphatase than those in a Japanese black pine-dominated forest (Taniguchi et al. 2008a), and distribution of these ECM fungi may also be related to the distribution of soil P. However, the distribution of *Tomentella* sp. 1 in soil at the depth of 0–5 cm positively correlated with RBA and that of *Tomentella* sp. 2 negatively correlated with PBA. This indicates that other factors such as root density of Japanese black pine and black locust may affect the distribution; we could not explain the distribution only on the basis of soil N and soil P.

C. pacificum was detected in both the Japanese black pine- and black locust-dominated areas at both depths (Table 2). Although the mortality of pine seedlings was higher in a black locust-dominated area than in a Japanese black pine-dominated area (Taniguchi et al. 2007b), which seems to be caused by the damping-off disease (Taniguchi et al. 2008b), the distribution of this fungus had no relation to the high mortality of the pine seedlings. Because mortality of the pine seedlings increased under low-intensity light and high N conditions (Taniguchi et al. 2008b), the physiology of host plants and environmental conditions in the black locust-dominated area, where soil N increased and light intensity was low, may affect disease severity of the seedlings.

Table 5 Spearman's rank correlation coefficients between the presence and absence of each ECM fungus in soil at depths of 0–5 and 5–10 cm

Lactarius quieticolor	*Cortinarius* sp.	*Tomentella* sp. 1	*Tomentella* sp. 2	Unidentified ECM T01	*Cylindrocladium pacificum*
0.35*	0.53*	0.40*	−0.23	0.17	0.55*

*$P<0.05$ (correlation coefficient was significant)

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