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ORIGINAL PAPER

Ectomycorrhizal fungal communities associated with *Pinus thunbergii* in the eastern coastal pine forests of Korea

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Abstract We investigated the ectomycorrhizal (ECM) fungal colonization status of *Pinus thunbergii* mature trees and regenerating seedlings varying in age in coastal pine forests on the east coast of Korea. We established one 20 × 20-m plot at each of two study sites at *P. thunbergii* coastal forests in Samcheok. Fifty soil blocks (5 × 5 × 15 cm) were sampled at regular intervals, and ten *P. thunbergii* seedlings of age 0, 1–3, 3–5, and 5–10 years were sampled in each study plot. In total of 27 ECM fungal taxa, *Cenococcum geophilum* was dominant, followed by *Russula* sp., *Sebacina* sp., and unidentified *Cortinuris* sp. in mature trees. In 0-year-old seedlings, some fungal species such as *Sebacina* sp., *C. geophilum*, and unidentified *Cortinarius* sp. were dominant whereas only *C. geophilum* was dominant after 1 year, and there were no apparent succession patterns in ECM fungal compositions beyond a host age of 1 year. Most ECM fungal taxa that had colonized seedlings of each age class were also observed in roots of mature trees in each site. These taxa accounted for 86.7–100% and 96.4–98.4% of ECM abundance in seedlings and mature trees, respectively. The results indicate that the species composition of ECM fungal taxa colonizing seedlings of different age in forests is similar to that of surrounding mature trees. Our results also showed that *C. geophilum* is a common and dominant ECM fungus in *P. thunbergii* coastal forests and might play a significant role in their regeneration.

Keywords *Cenococcum geophilum* · Ectomycorrhizal fungi · *Pinus thunbergii* · Regeneration · Seedling

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Introduction

Pinus thunbergii Parl., which grows naturally in Japan and Korea and has been introduced in America, has excellent abilities to tolerate high salinity and drought stress and grows well in coastal areas. Therefore, this tree species has often been used for afforestation on coastal dunes (e.g., Lee et al. 2005) and became one of the major constituents of the coastal forests in Japan and Korea (e.g., Choi 1986; Park et al. 2002). However, *P. thunbergii* coastal forest has experienced a recent decline due to pine wilt disease (e.g., Konta 1999), with invasion of alien plant species or other hardwood plants in some cases (Konta 2001). In Korea, apart from these biological factors, many coastal pine forests have been cut down or maintained improperly due to the recent increase in developments for tourism, recreation, and military purposes. In recent years, several studies on understanding the process and mechanisms of establishment of coastal pine forests and projects such as afforestation have begun with the goal of the recovery and maintenance of coastal pine forest in Korea (e.g., Chun et al. 2008).

Previous investigation revealed that *P. thunbergii* are ectomycorrhizal (ECM) and recorded various ECM fungal partners such as basidiomycetes *Amanita*, *Laccaria*, *Lactarius*, *Inocybe*, *Pisolithus*, *Rhizopogon*, *Russula*, *Suillus*, *Tomentella*, and ascomycetes *Cenococcum geophilum* Fr. in *P. thunbergii* coastal forests (e.g., Matsuda et al. 2006,

2009; Taniguchi et al. 2007; Kataoka et al. 2008). It is known that the effect of ECM associations on host plant growth differs among different species of ECM fungi (e.g., Baxter and Dighton 2001). Also, recent studies have revealed that coinoculation with various ECM fungi can alter host growth and nutrient acquisition (Reddy and Natarajan 1997; Baxter and Dighton 2001). Thus, the ECM fungal community composition might influence establishment of host plant species, and it is necessary to know the species composition of ECM fungi for understanding how ECM associations relate to establishment of coastal pine forests.

In *P. thunbergii* coastal forests, *P. thunbergii* seedlings varying in age are often regenerating under mature trees and they appear to contribute to maintenance of coastal pine forest. It is likely that ECM colonization status of regenerating seedlings is affected by ECM fungal community of established trees because it is possible that expanding hyphae from ECM root tips of established trees and/or development of the other soil propagules such as spore and sclerotia can facilitate ECM formation of bare roots in seedlings. Several efforts have been conducted to understand the species composition of ECM fungi colonizing in mature trees and regenerating seedlings. They revealed that patterns of frequencies and/or abundance of each ECM fungi were similar between mature trees and seedlings (e.g., Jonsson et al. 1999; Matsuda and Hijii 2004; Teste et al. 2009). However, there is no study that dealt with the ECM fungal community of *P. thunbergii* established trees and congeneric seedlings in coastal pine forests.

Also, most of the previous studies investigated only seedlings of one age class, and developmental patterns of ECM fungal communities among regenerating seedlings of different age in forests had been less well studied (Jonsson et al. 1999). The changes in ECM colonization status throughout host plants' development give us rough but important information about what kind of fungi support host establishment in one period—the period from seedlings to saplings in which plants are vulnerable to environmental stresses and have a high risk of withering. Previous studies revealed ECM fungal successions along host plant age, but most of them were conducted in single cohort stands of different stand age class and detected clear succession in relatively long time interval such as several decades or longer (e.g., Visser 1995; Palfner et al. 2005; Gebhardt et al. 2007; Twieg et al. 2007). So, there is uncertainty whether there is clear succession of ECM fungal community composition in regenerating seedlings occurring over relatively short time periods in coastal pine forests.

In the present study, we revealed the ECM fungal community of mature *P. thunbergii* trees and regenerating seedlings of 0–10 years old and determined the degree of similarity of the ECM fungal community between mature trees and seedlings of each age class. We also studied the developmental patterns of ECM fungal colonization status in seedlings among different age classes in coastal pine forests.

Materials and methods

Study site

The study site was the *P. thunbergii* coastal forest adjacent to Maeng-Bang beach in Samcheok, on the east coast of Korea (37°23′ N, 129°13′ E). This pine forest is located ca. 50 m inland from the shoreline and is several hundred meters in width. The mature forests consist mainly of *P. thunbergii* that were planted in the 1950s. Although several woody and herbaceous plants, such as *Artemisia* sp., *Cassia mimosoides* L. var. *nomame* Makino, *Digitaria ciliaris* (Retz.) Koeler, *Lespedeza* sp., and *Rosa rugosa* Thunb., were sparsely observed on the ground, ECM hosts other than *P. thunbergii* were not observed at the study sites. The soils consisted entirely of maritime sand and shallow litter (L), fermentation (F), and humus horizon (H) of <5 cm thickness. In 2008, climatic data in Gangneung from the Korea Meteorological Administration indicated annual precipitation of 1,343 mm, ranging between 0 and 330.6 mm per month (February and August, respectively), and annual temperature of 14.0°C, ranging between 1.1°C and 26.7°C (January and July, respectively).

P. thunbergii has been known to exhibit poor shade tolerance, and its seedlings are often found in relatively open areas. To analyze the ECM fungal community, we selected two forest areas where seedlings varying in age occurred abundantly as compared to entirely closed areas. The two forest areas selected differed in their mature tree densities—11.5 and 6.0 (individuals/100 m^2) in relatively closed (site 1) and open sites (site 2), respectively. The soils were sandy and covered by a L layer of 1–2 cm thickness in both sites. In site 1, F and H layers of 4–5 cm thickness were present, whereas they were absent or existed only locally in site 2. We sampled three soil cores (5 cm in diameter and 5 cm in depth) from each study plot in September 2008 and determined soil water content (*w*/*w*), which showed 7.4 and 1.4% (*w*/*w*) in average in sites 1 and 2, respectively. The two study sites were located about 1.5 km apart.

Sampling methods and observation of ECM colonization

One plot (20×20 m) was established in each study site. In August to September 2008, we sampled 50 soil blocks (5×

5×15 cm length, width and depth, respectively) at regular intervals and ten whole *P. thunbergii* seedlings each of 0, 1–3, 3–5, and 5–10 years old in each plot. The seedlings in each age class were sampled in this way to avoid any bias of sampling points in the plots. We assumed that most of ECMs from soil blocks were associated with mature trees. Seedling age was estimated by counting knots and observed tree rings under microscopic observation. In site 1, seedling heights were 4.5±0.5, 8.0±1.4, 18.0±4.4, and 28.4±4.2 cm in 0, 1–3, 3–5, and 5–10 years old seedlings, respectively. In site 2, seedling heights were 4.4±0.5, 7.0±1.1, 14.1±3.9, and 31.6±6.9 cm in 0, 1–3, 3–5, and 5–10 years old seedlings, respectively. All samples were stored in plastic bags at 4°C until further analysis.

Roots were separated from adhering soil by soaking and washing carefully in tap water on a 0.5-mm sieve. All root segments were viewed under a dissecting microscope after being cut into 5–10 cm pieces. The viability of ECMs was interpreted based on color, texture of surface, and degree of development of the mantle layer. We excluded nonvital ECMs that looked shrunken, discolored, and brittle. ECMs from each sample were classified into morphological groups based on criteria such as ramification system, color, shape, texture, organization, and abundance of emanating hyphae or cystidia, rhizomorphs, and hyphal arrangement of mantle surface according to Agerer (1995) and Ingleby et al. (1990). ECMs of each morphotype were divided into two subsamples: One was placed in formaldehyde/acetic acid/ethyl alcohol solution with distilled water (1:1:9:9) for microscopic investigation, and the other was placed in 1.5-mL microtubes at −20°C for DNA extraction. We attempted to relate morphotypes between samples in each age class and sampling area only for morphotypes that were unique and easy to distinguish from others, such as *C. geophilum*. For morphotypes that did not have unique characteristics and were difficult to distinguish from others, we made no attempt to relate their presence between samples until molecular analysis was complete.

Identification of ECM fungal species

We selected one to six root tips in each ECM morphotype. Each selected root tips of one ECM morphotype was sampled from different seedlings and/or soil samples. Fungal DNA was extracted from one ECM root tip using the DNeasy Plant Mini kit (QIAGEN, USA) according to the manufacturer's instructions. To identify the fungal species of ECM root tips, DNA was also extracted from sporocarps of the following species that occurred in the study sites or surrounding areas: *Calvatia* sp., two *Inocybe* spp., *Lactarius akahatsu* Tanaka, *Lepista nuda* (Bull. ex Fr). Cooke, *Russula* sp., *Scleroderma bovista* Fr., and *Suillus granulates* (L. ex Fr.) Kuntze. DNA amplification of the internal transcribed spacer (ITS) regions, including the 5.8S rDNA, was performed on a My Cycler thermal cycler system (BioRad, USA) using Takara Ex Taq (TAKARA, Japan) with a specific primer for higher fungi ITS1-f (Gardes and Bruns 1993) and basidiomycete ITS4b (Gardes and Bruns 1993). In other cases, a primer set of ITS1f and universal primer ITS4 (White et al. 1990) was also used. We used the PCR amplification conditions described in Landeweert et al. (2005). Single enzyme digests using *Hin*fI (TAKARA, Japan) were performed on all polymerase chain reaction (PCR) products. We determined the quality and quantity of the PCR products by agarose gel electrophoresis using QA-Agarose TM (MP Biomedicals, USA), as well as the size of restriction fragments using MetaPhor agarose (BMA, USA).

Each PCR product arising from different ECM morphotypes and exhibiting differences in restriction fragment length polymorphism (RFLP) analysis was purified using the QIAquick PCR purification kit (QIAGEN, USA) according to the manufacturer's instructions and sequenced with primers ITS1f and ITS4. Sequencing reactions were entrusted to Solgent Co., Ltd. (Daejeon, South 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 NCBI for taxonomic interpretation at the species, genus, or family levels. Sequences were considered to be identified at the species level when more than 97% of identities with reference sequences derived from sporocarps of the same species were obtained and made a single clade with the specimen in NJ trees. When less than 97% of identities with reference sequences derived from sporocarps were obtained, sequences were considered to be identified at the genus or family level with interpretation of phylogenetic trees. As a result, total fungal DNA extraction and PCR-RFLP analysis were conducted on 85 ECM root tips, and 42 DNA sequences were obtained.

Estimation of mycorrhizal colonization

The ECM abundance, which was indicated by the number of ECM root tips of each morphotype, was recorded separately for each sample. The relative ECM abundance was calculated as the average value of a ratio of abundance of a given morphotype to total ECM abundance present on a given sample. The frequencies of ECM colonization, which were indicated by the ratios of the samples that contained a given ECM morphotype to total samples, were also recorded.

Species accumulation curves were drawn by plotting the mean of the accumulated number of expected species in pooled samples after 50 randomizations without replacement, using EstimateS version 7.51 (Colwell 2005) to estimate whether the sampling effort was enough to describe the ECM fungal flora in each age class of seedlings and mature trees. Estimated measurements of ECM fungal species richness in each age class of seedlings and mature trees were calculated by bootstrap, Jackknife 1, and Jackknife 2 estimators using EstimateS version 7.51 (Colwell 2005). Similarity of ECM fungal species composition using the Morishita–Horn index among mature trees and seedlings of each age class was also calculated using EstimateS version 7.51.

We used analysis of variance followed by Tukey's test to compare mean species richness per seedling between age classes. The variability of the fungal species composition among replicated seedlings from both sites was analyzed by principal coordinate analysis (PCoA) using CANOCO 4.5 (Ter Braak and Smilauer 2002). The Bray–Curtis index was chosen as the distance index. The ECM abundances were square root transformed to reduce the influence of abundant morphotypes.

Results

Identification of ECM fungi

A total of 61,491 mature tree and 27,917 seedling ECM root tips were observed and counted. We identified a total of 27 ECM fungal taxa from mature trees and seedlings (Table 1; Fig. 1). Of these, we identified seven, 12, and two

Table 1 Possible identities of ectomycorrhizal fungal species that formed each ECM type observed in the roots of *P. thunbergii* seedlings and soil samples in a *P. thunbergii* coastal forest in Samcheok, South Korea

Possible identity based on BLAST and phylogenetic analysis	Accession no.	BLAST match by the highest similarity		
		Definition	Accession no.	Overlapped sequence
Cenococcum geophilum	AB506084	Uncultured fungus clone	EF434154	949/979 (96%)
Clavulinaceae sp.	AB506106	Uncultured basidiomycete genes	AB285528	519/537 (96%)
Inocybe sp. 1	AB506085	*Inocybe umbratica*	AM882799	547/590 (92%)
Inocybe sp. 2	AB506086	*Inocybe* sp.	EU523565	599/619 (96%)
Laccaria amethystina	AB506087	*Laccaria amethystina* genes	AB211270	619/621 (99%)
Lactarius akahatsu	AB506088	*Lactarius akahatsu*	EF685045	670/671 (99%)
Otidea sp.	AB506108	*Otidea cochleata* voucher	EU784387	548/569 (96%)
Pseudotomentella sp. 1	AB506089	Uncultured *Pseudotomentella* isolate	EU668254	641/717 (89%)
Pseudotomentella sp. 2	AB506090	Uncultured Thelephoraceae clone	EU557327	702/709 (99%)
Pyronemataceae sp.	AB506091	Uncultured mycorrhizal fungus	AB428784	517/517 (100%)
Russula sp. 1	AB506092	Uncultured ectomycorrhizal fungus genes	AB354285	595/600 (99%)
Russula sp. 2	AB506093	Uncultured ectomycorrhiza (*Russula*) genes	AB253519	628/630 (99%)
Sebacina sp.	AB506094	*Sebacina incrustans*	EU819442	533/565 (94%)
Suillus granulatus	AB506095	*Suillus granulatus* gene	AB284447	589/589 (100%)
Suillus luteus	AB506096	*Suillus luteus* clone	DQ068969	627/628 (99%)
Tomentella ellisii	AB506097	Uncultured ectomycorrhiza (*Tomentella*) clone	FJ013069	596/598 (99%)
Tomentella sp. 1	AB506098	Uncultured Thelephoraceae genes	AB444654	625/629 (99%)
Tomentella sp. 2	AB506099	Uncultured ectomycorrhiza (*Tomentella*) isolate	EF218823	571/608 (93%)
Tomentella sp. 3	AB506110	Uncultured ectomycorrhiza (*Tomentella*) genes	AB253522	545/546 (99%)
Tuber huidongense	AB506100	*Tuber huidongense*	DQ486031	533/538 (99%)
Tuber sp.	AB506101	Uncultured mycorrhizal fungus	AY656958	543/582 (93%)
Unidentified (Atheliaceae) 1	AB506103	Uncultured Agaricomycetidae clone	FJ553461	517/538 (96%)
Unidentified (Atheliaceae) 2	AB506104	Uncultured *Tylospora* clone	EF619845	515/559 (92%)
Unidentified (Basidiomycete) 1	AB506109	–	–	–
Unidentified (Basidiomycete) 2	AB506105	–	–	–
Unidentified (*Cortinarius*)	AB506107	Uncultured ectomycorrhiza (*Cortinarius*) genes	AB253520	553/555 (99%)
Unidentified (Pezizaceae)	AB506102	Uncultured ectomycorrhizal fungus	AB218167	483/531 (90%)

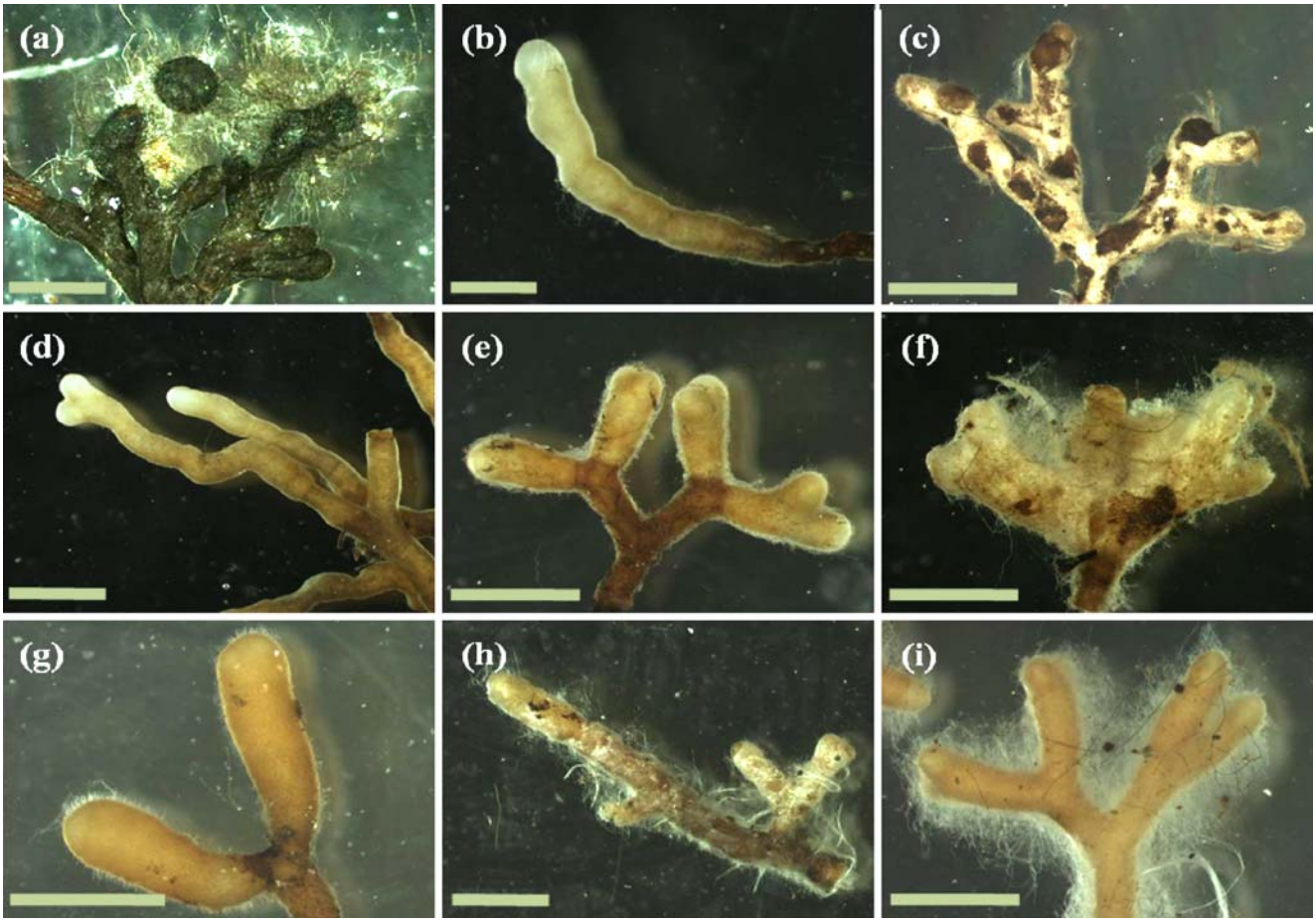

Fig. 1 Appearance of ectomycorrhizas in a *P. thunbergii* coastal forest in Samcheok, South Korea; **a** *C. geophilum* and its sclerotia, **b** *Inocybe* sp. 1, **c** *Pseudotomentella* sp. 2, **d** *Russula* sp. 1, **e** *Sebacina* sp., **f** *Suillus granulatus*, **g** *Tuber* sp., **h** unidentified Basidiomycetes 2, **i** unidentified *Cortinarius* sp. *Bars* indicate 1 mm

samples at the species, genus, and family levels, respectively, by matching sequences with those of known sporocarps. An ECM that was morphologically categorized as *C. geophilum* was matched with an uncultured fungus clone with the highest similarity of 96% and also with *C. geophilum*-like ECMs with 91–96% identities. It is known that this fungus has a high degree of genetic diversity (e.g., Farmer and Sylvia 1998; Jany et al. 2002); therefore, we considered this morphotype to be formed by *C. geophilum*. One morphotype was matched with *Trichophaea* and *Wilcoxina* by 88–90% identities, so we considered it to be formed by Pyronemataceae sp. Four fungal taxa could not be matched with sequences of known sporocarps but matched with those of estimated fungal taxa in other reports using such methods as phylogenetic trees, so one and three of these could be estimated to the genus and family levels, respectively (example, unidentified [Pezizaceae]). Two remaining fungal taxa could not be matched with sequences of known sporocarps or with estimated fungal taxa, so they were classified at the phylum level (example, unidentified [Basidiomycete]).

Status of ectomycorrhizal colonization on the roots of coastal pine

All soil samples contained roots of *P. thunbergii*, and a total of 23 fungal taxa were observed (Table 2). The numbers of fungal taxa observed in sites 1 and 2 were 20 and 16, respectively. Estimated species richness values by bootstrap, Jackknife 1, and Jackknife 2 estimators were 21.2, 22.9, and 25.8 in site 1 and 17.6, 19.9, and 22.8 in site 2, respectively. The species accumulation curves for ECM fungi in both sites tended to level off, indicating that most ECM fungal taxa were detected in our sampling effort (Fig. 2). Thirteen fungal taxa (*C. geophilum*, *Inocybe* sp. 1, *Pseudotomentella* sp. 2, *Pyronemataceae* sp., *Russula* sp. 1, *Russula* sp. 2, *Sebacina* sp., *Suillus luteus* (L.:Fr.) Roussel, *Tomentella* sp. 3, *Tuber* sp., unidentified Clavulinaceae sp., unidentified Basidiomycetes 2, and unidentified *Cortinarius* sp.) were shared between sites. Shared taxa accounted for 91.6% and 98.7% of the total abundance of ECMs in sites 1 and 2, respectively.

Table 2 Relative abundance and frequencies of each ectomycorrhizal fungi on the roots of *P. thunbergii* in each age class and soil samples collected in a *P. thunbergii* coastal forest, Samcheok, South Korea

ECM fungal taxa	Site 1										Site 2									
	0year		1–3years		3–5years		5–10years		Soil sample		0year		1–3years		3–5years		5–10years		Soil sample	
	RA	F	RA	F	RA	F	RA	F	RA	F	RA	F	RA	F	RA	F	RA	F	RA	F
Cenococcum geophilum	7.9	60	46.2	100	51.5	100	57.8	100	42.8	100	37.0	100	53.8	100	45.9	100	60.5	100	60.0	100
Clavulinaceae sp.			6.3	40			7.0	50	3.8	14									0.1	2
Inocybe sp. 1									0.2	6			1.9	20	10.2	50	1.9	60	1.7	40
Inocybe sp. 2													9.8	20						
Laccaria amethystina																			0.3	2
Lactarius akahatsu							2.7	20	0.2	2										
Otidea sp.	9.7	10			7.4	30			1.9	8										
Pseudotomentella sp. 1									0.2	2										
Pseudotomentella sp. 2			5.1	60	11.5	70	5.7	90	2.1	36					7.3	30	8.6	70	2.6	40
Pyronemataceae sp.									0.3	6									0.9	22
Russula sp. 1		4.0	10	10.3	60	6.6	30	16.6	76				5.3	10	3.1	30	3.3	30	2.1	24
Russula sp. 2									0.2	2			5.7	20					1.8	10
Sebacina sp.	52.4	70	2.0	10	9.7	50	9.2	70	12.8	44					12.2	30	14.7	80	8.5	32
Suillus granulatus	1.9	10	1.3	20	1.7	10	0.5	30	2.9	14			3.4	10			0.3	20		
Suillus luteus					<0.1	10	6.1	40	2.8	12									0.3	2
Tomentella ellisii											8.2	10							<0.1	4
Tomentella sp. 1											4.2	10							1.0	8
Tomentella sp. 2									1.1	20										
Tomentella sp. 3									1.1	24			0.7	10	3.1	30			0.3	14
Tuber huidongense									0.4	8										
Tuber sp.	11.3	40	5.8	30	2.4	20	4.3	40	5.3	42									0.1	2
Unidentified (Atheliaceae) 1			12.1	40					1.7	22							2.3	20		
Unidentified (Atheliaceae) 2																	6.1	30		
Unidentified (Basidiomycetes) 1											4.2	30								
Unidentified (Basidiomycetes) 2	16.8	50					0.1	10	1.3	50	6.5	10	8.7	50			0.3	20	5.8	72
Unidentified (*Cortinarius*)			17.4	30	5.4	30			2.1	32	40.0	70	10.6	40	18.2	80	1.8	30	14.4	86
Unidentified (Pezizaceae)																	<0.1	10		
Mean no. species per seedling[a]	2.4±0.8 a		3.4±0.7 ab		3.8±1.3 bc		4.8±1.1 c		–		2.3±0.7 a		2.8±0.9 ab		3.5±0.7 b		4.7±1.1 c		–	
Observed species richness	6		9		9		10		20		6		9		7		10		16	

Mean no. fungal species per seedling and total species richness were also represented

RA rela tive abundance, *F* frequencies, *ECM* ectomycorrhizal

[a] Averages and standard deviations are indicated.

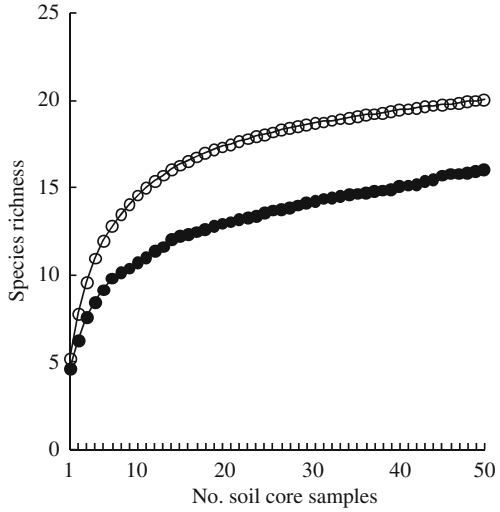

Fig. 2 Species accumulation curve for ectomycorrhizal fungi in site 1 (*open circle*) and 2 (*closed circle*) in a *P. thunbergii* coastal forest in Samcheok, South Korea

C. geophilum was observed in every soil sample and dominated in abundance in both sites; its relative abundance was 42.8% and 60.0% in sites 1 and 2, respectively (Table 2). In site 1, *Russula* sp. 1 and *Sebacina* sp. showed relatively high abundance (16.6% and 12.8%, respectively) and frequency (76% and 44%, respectively). *Tuber* sp. and unidentified basidiomycetes 2 showed relatively high frequency (42% and 50%, respectively). In site 2, unidentified *Cortinarius* sp. showed relatively high abundance (14.4%) and frequency (86%). *Sebacina* sp. showed relatively high abundance (8.5%) but low frequency (32%). *Inocybe* sp. 1, *Pseudotomentella* sp. 2, and unidentified basidiomycetes 2 showed relatively high frequency (40%, 40%, and 72%, respectively).

Ectomycorrhizal species composition on the roots of seedlings varying in age

A total of 22 fungal taxa were observed on the roots of seedlings in both sites (Table 2). The numbers of fungal taxa observed in sites 1 and 2 were 13 and 17, respectively, and eight fungal taxa were shared between sites. In total, ten, 14, 11, and 15 fungal taxa were observed in seedlings of 0, 1–3, 3–5, and 5–10 years old, respectively. Estimated species richness values were 11.5, 15.3, 11.9, and 15.8 by bootstrap; 13.8, 15.9, 12.9, and 15.4 by Jackknife 1; and 17.4, 14.3, 13.9, and 16.0 by Jackknife 2 estimator in seedlings of 0, 1–3, 3–5, and 5–10 years old, respectively. The species accumulation curves for ECM fungi in seedling age class of 1–3, 3–5, and 5–10 years old tended to level off, but that of 0 year old was ascending, indicating that most ECM fungal taxa were detected from age class of 1–3, 3–5, and 5–10 years old in this sampling effort but the probability of detection of rare fungal species is still high (Fig. 3). Observed species richness tended to increase in older age classes in both sites, from six taxa in 0-year-old seedlings to ten taxa in those 5–10 years old (Table 2); however, it seemed that the small sampling size affected on the determination of species richness in each age class, especially in those of 0-year-old seedlings. It was not clear whether species richness increase in seedlings of older age classes. Average species richness per seedling also gradually increased in older seedling age classes, from 2.4 and 2.3 taxa in 0-year-old seedlings to 4.8 and 4.7 taxa in 5–10-year-old seedlings in sites 1 and 2, respectively.

In site 1, *Sebacina* sp. dominated in relative abundance (52.4%) and frequency (70%) in 0-year-old seedlings, while *C. geophilum*, *Tuber* sp., and unidentified Basidiomycetes 2 showed relatively high frequency (60%, 40%, and 50%, respectively), but low relative abundance (7.9%, 11.3%, and 16.8%, respectively; Table 2). *C. geophilum* was observed in every seedling older than 0 years and dominated in relative abundance—46.2%, 51.5%, and 57.8% in the 1–3-, 3–5-, and 5–10-year-old age classes, respectively. Several fungal taxa, such as *Pseudotomentella* sp. 2, *Russula* sp. 1, *Sebacina* sp., unidentified Atheliaceae 1, and unidentified *Cortinarius* sp., showed relatively high abundance and/or frequency in one age class. In site 2, *C. geophilum* and unidentified *Cortinarius* sp. dominated in

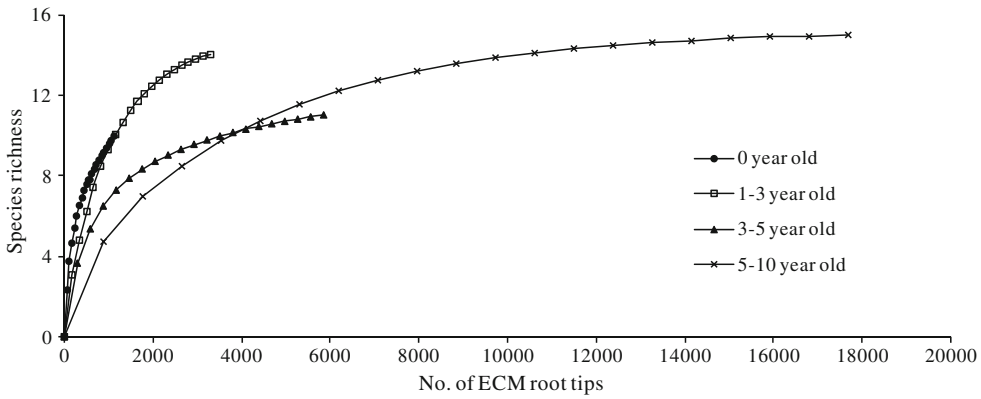

Fig. 3 Species-accumulation curve for ectomycorrhizal fungi in *P. thunbergii* seedlings of different age class in coastal pine forest in Samcheok, South Korea

relative abundance (37.0% and 40.0%, respectively) and frequency (100.0% and 70.0%, respectively) in 0-year-old seedlings. In seedlings older than 0 years, as the same pattern seen in site 1, *C. geophilum* was observed in every seedling and dominated abundance (53.8%, 45.9%, and 60.5% in age classes of 1–3, 3–5, and 5–10 years, respectively), followed by other fungal taxa, such as *Pseudotomentella* sp. 2, *Sebacina* sp., unidentified Basidiomycetes 2, and unidentified *Cortinarius* sp.

When looking at the variability of the ECM fungal community among seedlings using ordination analysis, the older age classes from both sites tended to be located on the positive and negative sides of the first and second PCOA axes, respectively (Fig. 4). The 0-year-old seedlings in both sites were located separate from each other, but the older age classes of both sites appeared to locate close together; in particular, 5–10-year-old seedlings from both sites were low in variability and colocated.

Comparison of ECM fungal community between mature trees and seedlings varying in age

In site 1, 20 different ECM fungal taxa were found in total, of which 13 taxa occurred on both mature trees and seedlings. All ECM fungal taxa in seedlings were observed in roots of mature trees and common ECM fungal taxa accounted for 96.4% of ECM abundance on mature trees. In site 2, 22 different ECM fungal taxa were found in total, of which 11 taxa occurred on both mature trees and seedlings. These common ECM fungal taxa accounted for 95.8%, 86.7%, 100%, and 91.2% of ECM abundance on seedlings of 0, 1–3, 3–5, and 5–10 years old and 98.4% on mature trees, respectively. Morishita–Horn similarity indices were 0.40, 0.75, 0.93, and 0.94 in site 1 and 0.80, 0.87, 0.97, and 0.96 in site 2 between mature trees and seedlings of 0, 1–3, 3–5, and 5–10 years old, respectively.

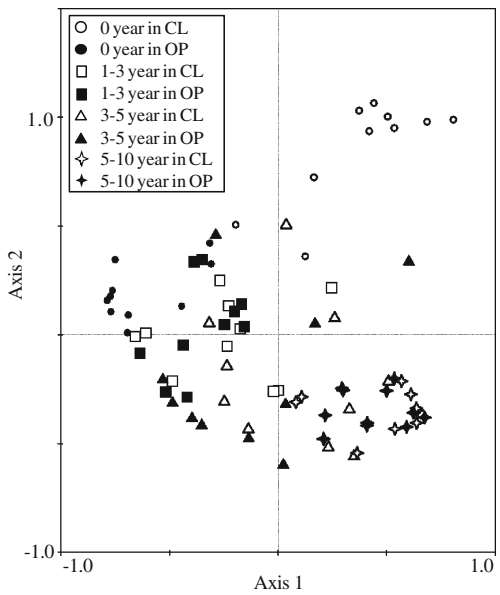

Fig. 4 The variability of ectomycorrhizal fungal composition among seedlings of different age classes and study sites as represented by PCoA. In total, the first two axes explain 38.3% of the overall variability

Discussion

We investigated the frequencies and abundance of ECM fungal taxa colonized seedlings varying in age and mature trees in *P. thunbergii* coastal forests and showed the ECM fungal communities and their similarities among seedlings of different age class and mature trees. However, to some degree, it appeared that small sample size made the results less robust; sampling effort was not enough to clarify the ECM fungal community in 0-year-old seedlings and the number of replicated plots was limited. Also, this study was carried out in a single *P. thunbergii* coastal forest; thus, the results are relevant only to this particular stand and cannot be extrapolated to coastal pine forests in general. Therefore, further investigations involving more intense sampling efforts are required for obtaining general information about the ECM fungal community in mature trees and regenerating seedlings and their similarities in coastal pine forests.

In mature trees, only a few ECM fungal taxa made up more than 10% of relative abundance, and the majority of ECM fungal taxa were detected from samples of a small number of tips in both sites. The existence of a few dominant fungi among a majority of rare fungi is a common pattern of species richness, as described in previous reports that were carried out in several kinds of forest ecosystems and taxonomic groups (e.g., Jonsson et al. 1999; Matsuda and Hijii 2004; Valentine et al. 2004). *C. geophilum* was the most dominant in abundance and frequency in both sites. *C. geophilum* is one of the most common ECM fungi in the temperate and boreal zones and is often observed as dominant in several kinds of forests, such as oak (e.g., Valentine et al. 2004; Gebhardt et al. 2007), pine (e.g., Jonsson et al. 1999; Douglas et al. 2005), *Populus* (Krpata et al. 2008), and spruce (e.g., Baier et al. 2006). Taniguchi et al. (2007) also detected this fungus as the dominant species in 0-year-old *P. thunbergii* seedlings in congeneric pine-dominated coastal forests. Matsuda et al. (2009) showed that *C. geophilum* is the dominant ectomycorrhizal fungus and is distributed ubiquitously in coastal pine forests of Japan by sampling soil cores. Several reports have shown the excellent adaptability of *C. geophilum* to variable environmental conditions: (1) Saleh-Rastin (1976) and Matsuda et al. (2006) showed that *C. geophilum* has the ability to resist salt stress, (2) several reports have shown that *C. geophilum* has higher drought tolerance than other

ECM fungi (e.g., Coleman et al. 1989; Di Pietro et al. 2007) and might protect host plants from drought stress by mycorrhization (e.g., Pigott 1982), (3) some reports have revealed that *C. geophilum* is commonly distributed in different soil layers, such as the O, F, H, and B layers, indicating high adaptability to different soil substrates (Dickie et al. 2002), and (4) it is known that *C. geophilum* makes sclerotia that might be tolerant to environmental stresses and could persist for long periods as active propagules in soils. The dominance of *C. geophilum* might be related to its excellent adaptations to the environmental conditions of coastal areas, where salt and/or drought stress is high and the status of soil substrates differs locally.

Russula sp. 1 and unidentified *Cortinarius* sp. were the second most dominant fungi, but their abundance and frequency differed between the study sites. *Russula* spp. is one of the most dominant and frequent taxa (e.g., Gardes and Bruns 1996; Taylor and Bruns 1999; Matsuda and Hijii 2004) and its sporocarps occur commonly in conifer communities (e.g., Matsuda and Hijii 1998; Peter et al. 2001). Sporocarps of *Russua* sp. 1 were observed in site 1, where the F and H layers are thick and water content is moderate and also in areas surrounding site 2 where tree densities are relatively higher than within site 2 itself and the F and H layers are almost absent. It seems that this fungus prefers relatively moderate soil water content, as found in site 1. Unidentified *Cortinarius* sp. was frequently and abundantly observed in site 2. This fungus was also detected in roots of current *P. thunbergii* seedlings at coastal pine forests in Japan (Taniguchi et al. 2007). This fungus, like *C. geophilum,* appears to be competitive in severe drought conditions.

Sebacina sp. was the third dominant fungus in both sites. The recent increasing use of molecular phylogenetic methods for the study of mycorrhizal fungal diversity has demonstrated that Sebacinaceae are present in orchid mycorrhizal (e.g., Dearnaley 2007), ericoid mycorrhizal (e.g., Selosse et al. 2007), and ectomycorrhizal associations (e.g., Urban et al. 2003). It has been reported that some species of *Sebacina* are commonly observed as ECMs in some forest ecosystems (e.g., Tedersoo et al. 2003; Urban et al. 2003; Mühlmann and Peintner 2008). This is the first report to detect *Sebacina* sp. in the roots of *P. thunbergii* in coastal pine forests. The ecological features of these fungi as ectomycorrhizal symbionts are still obscure, and further investigations are needed.

Because sample size of 0-year-old seedlings was inadequate, we excluded these data in discussion of transitional pattern of ECM fungal composition with host age. In older age classes from 1–3 to 5–10 years old, *C. geophilum* dominated, and no clear transitional pattern of ECM fungal composition with host age was observed in both study sites. The inconsistent occurrence and irregular pattern of abundance and frequency of fungi among different age classes might be partly explained by the small sample size; however, some other reasons should also be considered. Several studies have followed the ECM fungal community along a chronosequence of forest stand development during secondary succession and revealed that composition varies substantially along a large time scale (−50 or −100 years; e.g., Twieg et al. 2007). However, Palfner et al. (2005) reported a slight difference in ECM fungal composition among stands of low age (from <1 to 12 years old), and only *Tylospora fibrillosa* (Burt.) Donk dominated during the study period in Sitka spruce pure stands in northern England. In mixed age forest, Jonsson et al. (1999) investigated the status of ECM fungal colonization in self-regenerating seedlings of 1–10 years old and revealed no obvious difference in mycorrhizal frequency or abundance between the differently aged seedlings. These studies give us some indication that the succession in the ECM fungal community with seedling development on the limited time scale of 1–10 years from germination is not clear. However, because of scarcity of case studies, there is still much unclear about the presence or absence of ECM fungal succession in seedlings regenerating in forests, and this topic should be an important one for future work. As one of the reasons for no clear succession in the ECM fungal community with seedling development on the limited time scale of 1–10 years from germination in the coastal pine forest in our present study, the effects of existing ECM fungal community should be considered. Some reports showed the competition among ECM fungal taxa in occupying bare roots (Wu et al. 1999). In the present study, *C. geophilum* appears to be the most competitive in all stages of seedlings and also mature trees, which might inhibit mycorrhization by other fungi in seedlings regenerating in coastal pine forests.

Although this study employed a limited sampling effort, results suggested that ECM fungal taxa on regenerating seedlings in forests are largely the same present as on the surrounding mature trees. Also, our results showed that similarities of ECM fungal species compositions were high between mature trees and seedlings of each age class. Similarity of ECM fungal species composition between mature trees and regenerating seedlings has been often reported on temporal and boreal forest ecosystems (e.g., Fleming 1983; Jonsson et al. 1999; Matsuda and Hijii 2004; Teste et al. 2009). It seemed that the ECM fungal community of mature trees largely affects on the ECM colonization in regenerating seedlings of each age class in forests. In particular, dominant colonization by *C. geophilum* in both seedlings and mature trees and higher similarities of ECM fungal species compositions among seedlings and between sites in the older age classes are indirect indications that *C. geophilum* might play important

roles for the regeneration of *P. thunbergii* in the coastal pine forests.

The present study showed that *C. geophilum* is common and the most dominant ECM fungus associated with both mature trees and seedlings that have naturally regenerated under the pine forests and plays an important role in the dynamics and maintenance of the *P. thunbergii* coastal forests. The excellent ability of *C. geophilum* to colonize in severe environmental conditions and also in association with a wide range of host ages is advantageous for *P. thunbergii* seedlings that were planted in coastal areas where some environmental stresses, such as drought, make their establishment difficult. Further investigations are needed to examine the effects of ECM associations of *C. geophilum* on seedling establishment in the environmental conditions of coastal areas for the application of coastal pine reforestation.

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