



Ectomycorrhizal fungal communities associated with *Quercus dentata* in a coastal broadleaf forest

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Abstract

Coastal forests are exposed to high salinity and drought stress, and plant growth is restricted under such harsh conditions. *Quercus dentata* is one of the most common species in coastal forests in northern Japan. We investigated the changing vegetation in a coastal forest, shoreline to inland, and examined the ectomycorrhizal (ECM) fungal communities associated with *Q. dentata*. We aimed to determine whether the ECM changes corresponded with the changes in vegetation. More than 300 m inland, broadleaf trees such as *Q. dentata* were dominant. An almost pure *Q. dentata* stand was formed in the area closest to the shoreline. By contrast, as the forest moved inland, the occurrence of other tree species increased and the density of *Q. dentata* gradually decreased, respectively. In the areas that were furthest inland, *Q. dentata*, *Q. crispula*, *Acer mono*, and *Tilia japonica* were equally dominant. Five sampling plots (20 m × 20 m, each) were set up in the forest 100 m apart, and soil cores (including the fine roots of *Q. dentata*) were sampled in each plot. The total ECM colonization rate was > 98% in each sampling plot. Morphological characterization and DNA sequencing of the root tips identified six taxa (*Tomentella* sp., *Russula* spp., *Tricholoma* sp., *Hebeloma* sp. and Boletales sp.). *Tomentella* sp. was relatively abundant near the shoreline and its abundance decreased as the density of *Q. dentata* decreased inland. Conversely, *Russula* sp. increased as the forest moved inland.

Keywords – coastal forest – ectomycorrhizal fungi – *Quercus dentata* – vegetation

Introduction

Coastal areas are exposed to high salinity and drought stress, and plant growth is restricted under such harsh conditions. Therefore, coastal vegetation is different from inland vegetation, with plant species tolerant to salinity and drought stress dominating near the shoreline. Plant species that are relatively less tolerant to these stresses dominate in inland forests. The factors underlying the formation of coastal forests remain unclear, and although past reports focus on the aboveground features, such as atmospheric salinity (Yura & Ogura 2006), underground symbioses may also determine the composition of the forest vegetation.

Ectomycorrhizal (ECM) fungi are important symbionts of plant roots of the dominant vegetation in boreal and temperate forests that includes Pinaceae, Betulaceae and Fagaceae (Smith & Read 1997; Van der Heijden et al. 2008). The ECM fungi typically inhabit forest soils and increase the plant uptake of soil water and nutrients to form an ECM network (Smith & Read

1997). These effects differ between fungal species (Baxter & Dighton 2001; Lehto & Zwiasek 2011). Moreover, different forest types support different ECM fungal communities (Smith & Read 1997).

The oak *Quercus dentata* (family Fagaceae), which is tolerant to salinity and drought, is one of the most common tree species of the coastal forests in Hokkaido, northern Japan. The fine roots of *Q. dentata* are covered with ECM fungi. From the eastern Asian coastal forests southward to temperate areas, the dominant tree species are typically *Pinus* spp., such as black pine, and some reports of the associated ECM fungal communities exist (e.g., Taniguchi et al. 2007; Obase et al. 2009; Matsuda et al. 2009; Aučina et al. 2011). Although coastal forests composed primarily of *Quercus* sp. are one of the most common forest types in northern Japan, reports on the ECM fungal communities of these forests are not available. In the present study, we investigated the changes in vegetation in a coastal forest, from the shoreline inland, and examined the changes in the ECM fungal communities associated with *Q. dentata* to determine if they corresponded with the changes in vegetation.

Materials and Methods

Study site

The study site was the Ishikari broadleaf coastal forest, Hokkaido, northern Japan (N43°14' and E141°19'). The mean annual temperature in the forest is 8.8°C, and the annual precipitation is 1,259.5 mm. The forest extends a few tens km along the shoreline and, at most, 600 m inland from the shoreline. The soil is sandy and covered with a layer of plant litter that is a few centimeters thick. The stand was primarily composed of *Q. dentata* and other broadleaf tree species that grew wild *Sasa senanensis* was dominant on the forest floor.

Vegetation and soil salinity survey

The vegetation and soil salinity were characterized to identify the differences between the shoreline and inland areas before the assessment of ECM colonization status. The survey was conducted from the shoreline inland, within a 5 m wide belt. The dominant tree species were evaluated for the percentage of basal area every 100 m. The soil salinity was measured every 100 m with an electrical conductivity (EC) meter (Twin Cond conductivity meter B-173, Horiba, Japan).

Sampling and observation of ECM colonization

Five sample plots (20 m × 20 m) were set up every 100 m in the forest. 16 cubic soil samples (25 cm × 25 cm × 25 cm) containing fine roots of *Q. dentata* were collected from each plot, and samples collected from each plot were combined. Although we could easily identify the roots in the soil samples as *Quercus* sp. by their external appearance, we could not distinguish the roots of *Q. dentata* from *Q. crispula*, because they were very similar to each other. Identification through DNA analysis was not possible because *Q. dentata* and *Q. crispula* often make hybrids. Because of this, the sampling in mixed forests was limited to areas immediately under and around *Q. dentata* specimens distant from *Q. crispula* trees to avoid the possibility of mixed-species sampling. The samples were stored in plastic bags at 4°C until further analysis. The fine roots were separated from the adhering soil by soaking and careful washing with tap water. All roots were observed under a microscope after being cut into 5–10 cm pieces. The viability of the ECM was assessed based on color, surface texture and the degree of mantle layer development. Non-viable ECM that appeared shrunk, discolored, and brittle were excluded from analyses. ECM fungi from each sample were categorized into morphological groups based on such criteria as the ramification system, the color, shape, texture, organization and the abundance of the emanating hyphae or cystidia, rhizomorphs, and the hyphal arrangement of the mantle surface, according to Agerer (1995) and Ingleby et al. (1990). The total ECM colonization rate and composition ratio of each ECM type were measured to compare the mycorrhizal status between plots. Total ECM colonization rate was calculated as follows: total ECM colonization rate (%) = (number of ECM

root tips)/(total number of root tips). The composition ratio of each ECM type was calculated as follows: ratio of specific ECM type = (number of the particular ECM type)/(total number of ECM root tips).

Identification of ECM fungal species

Two or three root tips were collected for each ECM morphotype, and the DNA was extracted with the DNeasy Plant Mini kit (QIAGEN, USA) according to the manufacturer's instructions. DNA amplification of the internal transcribed spacer (ITS) regions, including the 5.8S rDNA, was performed with a thermal cycler (2720 Thermal Cycler, Applied Biosystems). Because clamp connections were observed in all the ECM samples, the DNA was amplified via nested PCR using Basidiomycetes specific primer sets ITS1OF and ITS4OF (both developed by D. L. Taylor and available online at http://mercury2.iab.uaf.edu/lee_taylor/PCR_Primer_Orchid_Fungi.html) before the amplification with universal primers ITS1 (White et. al. 1990) and ITS4 (White et. al. 1990). The amplification was performed with the following program: initial denaturation at 95°C for 3 min, followed by 40 cycles of denaturation at 95°C for 15 s, annealing at 50°C for 40 s, extension at 72°C for 40 s, and final extension at 72°C for 7 min. The PCR products were analyzed by electrophoresis on 2% agarose gels and visualized with a UV light transilluminator after staining with EZ-Vision Three DNA Dye and Buffer (AMRESCO, USA). The sequences were compared with those deposited at GenBank database at the DNA Data Bank of Japan (DDBJ: <http://www.ddbj.nig.ac.jp/>) using the nucleotide–nucleotide basic local alignment search tool algorithm (BLAST). Assignment to taxonomic categories was performed as follows: sequence similarity of more than 97% signified identification to species level; sequence similarity of 95–97% equaled identification to genus level; sequence similarity of less than 95% meant identification to family or order level.

Statistic analyses

Analysis of variance followed by Tukey's test was used to compare mean soil EC of samples, significance level $p < 0.05$. To assess the sufficiency of sampling for each plot, species accumulation curves were generated. The curves were obtained by plotting the means of the total number of the expected species (Sobs, Mao Tau) in pooled samples, after 50 randomizations without replacement, using Estimate S version 8.0.0 (Colwell 2005).

Results

Vegetation and soil salinity differences between the near shoreline and inland areas

The area 50–300 m from the shoreline was dominated by herbs and shrubs, while broadleaf trees, primarily *Q. dentata*, dominated 300 m and more from the shoreline. *Quercus. dentata*, *Ulmus davidiana*, *Acer mono*, *Sorbus alnifolia*, *Maackia amurensis*, *Prunus sargentii*, *Morus bombycis*, *Kalopanax pictus*, *Q. crispula* and *Tilia japonica* were observed on the study site. The main tree species that formed a symbiotic association with ECM fungi were *Quercus. dentata* and *Q. crispula*. An almost pure *Q. dentata* stand formed in the area closest to the shoreline; however, the densities of other tree species increased, while the density of *Q. dentata* gradually decreased, as the forest moved inland, accordingly (Table 1). The EC values (mean \pm standard deviation) ranged from 19.33 ± 4.04 to 47.67 ± 13.32 $\mu\text{S}/\text{cm}$ and were higher near the shoreline, in the pure *Q. dentata* stand (Table 2).

ECM colonization status and identification from roots of Q. dentata

A total of 50,258 ECM root tips (13,752, 14,610, 9,556, 5,576 and 6,764 root tips for the 300, 400, 500, 600 and 700 m plots, respectively) were examined. The ECM colonization rates were more than 98% in all sample plots (Table 3). Six morphotypes of ECM root tips were characterized (Fig. 1). The species accumulation curves for ECM fungi in each sample plot tended to level off, which indicated that most ECM fungal taxa have been detected in the sample plots (Fig. 2).

Table 1 The observed tree species and the percentage of basal area (%) every 100 m from the shoreline on the study site

Distance from shoreline (m)	300–400	400–500	500–600	600–700	700–800
<i>Quercus dentata</i>	94.0	87.7	64.0	54.9	17.3
<i>Acer mono</i>	1.3	8.9	12.4	16.5	19.6
<i>Ulmus davidiana</i>	4.7				3.4
<i>Sorbus alnifolia</i>		1.8	0.9	2.4	3.7
<i>Maakia amurensis</i>		0.8	6.0	4.5	5.3
<i>Prunus sargentii</i>		0.4	0.8	1.9	
<i>Morus bombycis</i>		0.4		2.1	
<i>Kalapanax pictus</i>			12.1	10.3	11.3
<i>Quercus crispula</i>			3.8	7.4	17.1
<i>Tilia japonica</i>					22.3

Table 2 Soil EC every 100 m from the shoreline on the study site (values are means \pm SD, n=3). The letters indicate significant differences at $p < 0.05$ (Tukey's test).

Distance from shoreline (m)	100	200	300	400	500	600	700	800
EC (μ S/cm)	47.7 \pm 13.3 a	37.7 \pm 3.8 b	21.3 \pm 2.5 c	46.0 \pm 12.1 a	23.7 \pm 3.1 c	21.3 \pm 1.5 c	19.3 \pm 4.0 c	27.3 \pm 1.5 c

Table 3 ECM colonization rates (%) of *Q. dentata* in sample plots on the study site

Distance from shoreline (m)	300	400	500	600	700
ECM colonization rate (%)	98.8	98.1	99.8	99.2	99.7

Table 4 Identification of ECM fungi from the fine roots of *Q. dentata* from the study site

Identification	Accession no.	Closest match	Similarity
<i>Tomentella</i> sp.	AB979726	<i>Tomentella</i> sp. AF272912	534/556 (96%)
<i>Russula</i> sp.	AB979725	<i>Russula heterophylla</i> DQ422006	572/589 (97%)
Russulaceae sp.	AB979729	<i>Russula heterophylla</i> DQ422006	553/598 (92%)
<i>Tricholoma</i> sp.	AB979730	<i>Tricholoma album</i> AF241516	564/589 (95%)
<i>Hebeloma</i> sp.	AB979728	<i>Hebeloma</i> sp. FJ168594	371/383 (96%)
Boletales sp.	AB979727	<i>Xerocomus subtomentosus</i> JQ967281	489/564 (86%)

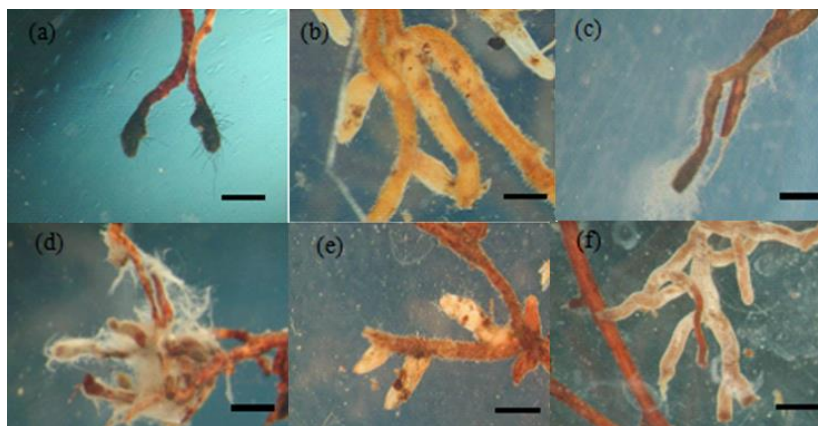


Fig. 1 – The appearance of ECM from fine roots of *Q. dentata* from the study site. (a) *Tomentella* sp., (b) *Russula* sp., (c) *Russulaceae* sp., (d) *Tricholoma* sp., (e) *Hebeloma* sp., and (f) *Boletales* sp. – Bars = 1 mm.

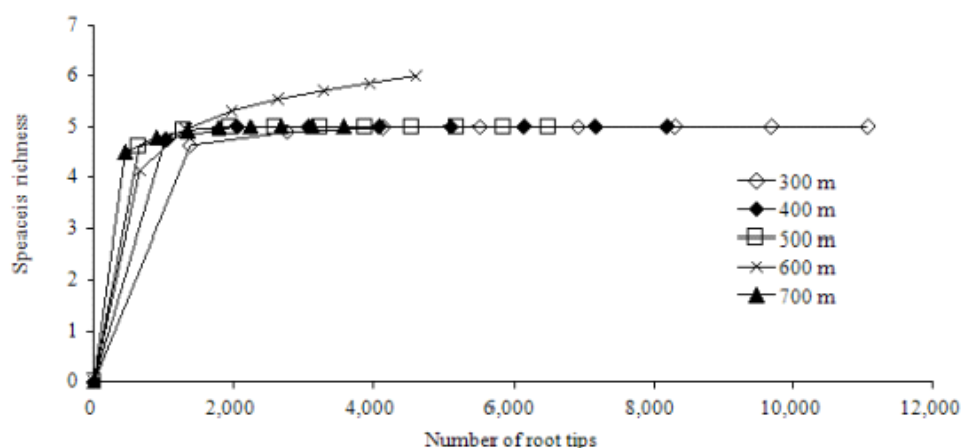


Fig. 2 – Species accumulation curves for ECM fungi of *Q. dentata* in the study site.

Composition of ECM fungi in each sample plot

The ITS regions were sequenced, with the similarities to sequences deposited in the DDBJ GenBank database ranging from 86% to 97% (Table 4). We identified six total taxa. *Tomentella* sp., *Russula* sp., Russulaceae sp., *Tricholoma* sp. and *Hebeloma* sp. were observed in all sample plots, while the species of Boletales were only observed in the plot 600 m inland, and their relative abundance was extremely low (0.1%, Table 5). *Tomentella* sp. was relatively abundant in the plot near the shoreline but the abundance gradually decreased inland (Table 5). In the 300 m plot, *Russula* sp. occurrence frequency was nearly the same as for *Tomentella* sp., but was higher than *Tomentella* sp. further inland. The remaining ECM fungal taxa did not show clear associations of abundance with the distance from the shoreline.

Table 5 The relative frequencies (%) of ECM fungal taxa in study plots on the study site

	Distance from shoreline (m)				
	300	400	500	600	700
<i>Tomentella</i> sp.	39.8	23.8	14.4	11.7	12.2
<i>Russula</i> sp.	39.7	73.7	68.8	66.5	65.7
Russulaceae sp.	10.7	0.8	7.0	11.2	10.5
<i>Tricholoma</i> sp.	9.0	0.6	3.2	1.0	3.2
<i>Hebeloma</i> sp.	0.8	1.1	6.6	9.5	8.4
Boletales sp.				0.1	

Discussion

In this study, we investigated the ECM fungal communities associated with *Q. dentata* in different stand structures in a coastal broadleaf forest of northern Japan. Aboveground, *Q. dentata*, which is tolerant to high salinity, was the dominant species nearest the shoreline, where the soil salinity was highest, while the frequency of other tree species increased inland. Underground, we classified the *Q. dentata* ECM fungi from different stand structures into six taxa, using morphological and genetic discrimination. The dominant ECM fungal species were *Tomentella* sp. and *Russula* sp. *Tomentella* sp. was relatively abundant near the shoreline and decreased as the density of *Q. dentata* decreased inland. Conversely, *Russula* sp. increased as the forest moved inland.

The ECM fungal communities of coastal forests have been examined in some studies. For example, the ECM fungal communities associated with *Pinus* sp. in eastern Asian coastal forests were investigated, with the dominant fungal species identified as *Cenococcum geophilum* (Taniguchi et al. 2007; Obase et al. 2009a; Matsuda et al. 2009). Indeed, *C. geophilum* is the most

common ECM fungal species in temperate and boreal forests and is often the dominant species in several forest types in addition to pine, such as poplar (Krpata et al. 2008), spruce (Baier et al. 2006) and oak (Valentine et al. 2004; Gebhardt et al. 2007). Moreover, previous reports showed that *C. geophilum* was salinity (Dixon et al. 1993; Matsuda 2006) and drought tolerant (Pigott 1982; Di Pietro et al. 2007). These characteristics are important for survival in coastal conditions and for forming stands; therefore, *C. geophilum* is thought to play an important role in the formation of coastal forests. However, we did not find *C. geophilum* in this study. Instead, the dominant ECM fungal species were *Tomentella* sp. and *Russula* sp., which differs from previous reports of the ECM fungal communities associated with pine in a coastal forest. A study by Richard et al. (2011) examined the ECM fungal communities associated with *Q. ilex* in a Mediterranean forest, and found the dominant fungal taxa to be Thelephoraceae and Russulaceae. These fungal taxa are also common in temperate and boreal forests (e.g., Lang et al. 2011; Kranabetter et al. 2011). Although these fungal taxa are common in various environments and thought to help growth of host plants, few studies of their actual effects on host plants have been conducted. Obase et al. (2009b) studied the effect of the mycorrhizal symbiosis of another *Tomentella* sp. and *Populus maximowiczii* seedlings in volcanic debris, and found that this symbiosis promoted the growth of host plants. Nevertheless, because only a few reports exist and conditions differ between studies, whether *Tomentella* sp. promotes the growth of host plants in a coastal forest remains to be investigated.

We found that the number of ECM fungal taxa in a coastal forest was low compared with inland forests, although our sampling effort was sufficiently robust to identify ECM fungal communities in a coastal forest. Matsuda et al. (2009) proposed that the coastal pine stands developed fewer ECM fungal associations and had simpler community structure than inland forests. The same phenomenon likely occurred in the *Quercus* sp. stand. The ECM fungal communities of other stands should be characterized to identify the factors that affect the formation of coastal vegetation.

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