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Ectomycorrhizal Fungal Communities Associated with Dominant Tree Species in a Subarctic Limestone Area

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Authors' contributions

This research work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

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ABSTRACT

Limestone soils are stressful for plant growth. Plant-associated ectomycorrhizal (ECM) fungi may promote plant growth under stressful conditions, yet available information on ECM fungi in limestone areas is scarce. We investigated the ECM fungal communities associated with dominant tree species in a subarctic limestone area. We aimed to determine whether the ECM species differed between calcareous and non-calcareous areas, and the distribution property common to ECM fungi in limestone areas. Morphological characterization and DNA sequencing of root tips identified 57 ECM taxa. The ECM fungal compositions in the calcareous area differed from those in the non-calcareous area, even when comparisons were made between fungi on the same tree species. Rather, when ECM species were grouped at the genus level, they tended to be dissimilar

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between calcareous areas and between non-calcareous areas. Especially, Tomentella spp. and Sebacina spp. tended to be present more frequently in calcareous areas, while *Cenococcum geophilum* and Russula spp. tended to be present more frequently in non-calcareous areas.

Keywords: Alkaline soil; calcareous soil; subalpine forest; ectomycorrhiza; basidiomycete; ascomycete; Tomentella; Sebacina.

1. INTRODUCTION

Limestone, which consists primarily of calcium carbonate, is found throughout the world [1]. Although there are various reports, the calcareous soils show a high pH range of 7.0-8.5 [2,3]. That causes a shortage of exchangeable P, Fe, and Mn for plant growth [2,4,5]. Especially, P and Fe deficiencies can be fatal for plants. On calcareous soil, P is bound by Ca, and the uptake is greatly restricted [2,6]. And also, a lack of Fe causes lime-induced chlorosis [2,7]. Moreover, limestone is easily eroded by rain, and the weathered soil is unstable and arid [5]. Therefore, limestone flora, also called calcicolous flora, is different from other forest vegetation [8,9] and is often composed of specific tree species that are tolerant to these stresses [10]. The factors underlying the formation of limestone flora remain unclear, and although past reports focused on the characteristics of individual plant species, symbioses with other species may be critical for determining the flora type.

Ectomycorrhizal (ECM) fungi are important symbionts of plant roots in the subarctic and subalpine forests [11]. The ECM fungi usually inhabit forest soils, and promote the uptake of soil water and nutrients by the plant, by utilizing their hyphae network [12]. Consequently, ECM symbiosis somewhat alleviates host plant stress induced by environmental conditions and enables the host to survive. Fungal ability to support the plant host differs depending on the ECM species, and the composition of ECM species may be influenced by rhizosphere characteristics, such as the soil pH [11]. Therefore, the composition of ECM fungal species reflects the conditions of each habitat, and contributes to the host's establishment and expansion of its distribution.

Characteristic ECM fungal species are often found in environments that are stressful to the host. ECM fungi in serpentine soils [13], alpine areas [11,14], and coastal forests [15] have been investigated in the past. Similarly, it has been suggested that calcicolous plant species can only grow on calcareous soils in the presence of a symbiotic relationship with a specific ECM fungus [10]. On calcareous soil, exudation of organic acid from ECM fungal hypha results in the dissolution of insoluble P and the chelation of Fe, leading to more available for plant uptake [16-18]. However, few researchers have documented ECM fungal communities in natural limestone areas. Moreover, these limited data are regionally biased, in Europe [19-22], North America [23], Oceania [24]. They all concluded that a specific ECM fungal community was found in calcareous soil, however, their results did not provide a unified agreement about what ECM fungal species dominated and what factor determined the community composition. Related reports for Asia are rare. Fan et al. examined the ECM fungi associated with *Picea crassifolia* in Mt. Helan [25], however the soil type was uncertain. Hence, the distribution of ECM fungal species in Asian subarctic limestone areas remains poorly known.

In the present study, we investigated the community composition of ECM fungal species that grow symbiotically with dominant tree species in a subarctic limestone area, and determined the characteristics of the ECM fungal composition by comparing the composition in a calcareous area with that in a non-calcareous area. We hypothesized the following: ECM fungal community in a calcareous area differs from that in a non-calcareous area, in other words, an area covered with typical brown forest soil. In addition, on a world scale, there is a common distribution pattern of ECM fungal species in calcareous areas.

2. MATERIALS AND METHODS

2.1 Study Area

The study area was Mt. Kirigishi alpine forest preserve in Hokkaido, northern Japan (1,057 m above sea level at the highest point; N43°14' and E142°14'). The mean temperature at the nearest city (Furano, 174 m above sea level; N43°20' and E142°24') is 6.3 °C, with an annual precipitation of 969.6 mm. However, the study area is mountainous and, therefore, the actual

mean temperature might be lower. The flora represents the transitional area between the cool-temperate forest and the subarctic forest. Concerning the soil, limestone fragments and decomposition are widely distributed, centering on the peak.

The study areas (the gross area is 25,000 m²) were devised in the south of Mt. Kirigishi, at an altitude of 570–910 m. They mainly encompassed the typical mixed needleleaf and broadleaf forest, featuring *Quercus crispula* and *Abies sachalinensis*. With an increasing altitude, the ratio of subalpine tree species, for example, *Picea jezoensis*, *Betula ermanii* and *Acer ukurunduense*, to other tree species also increased. Around the peak, tall trees (> 20 m) are very rare, and characteristic limestone vegetation is observed [26,27]. Even in the highland meadow, there are a few tree species that tolerate the stressful environment. These environmental differences are easily identified by landscapes.

Based on a preliminary survey of the soil pH and flora, for convenience, the study areas were classified into calcareous areas (CL), non-calcareous (NO) areas, and buffer areas, separating the former two areas. With increasing altitude, the influence of limestone on the soil appeared to increase. Accordingly, the soil pH was the highest at the peak and became lower with a decreasing altitude.

2.2 Sampling and Evaluation of ECM Colonization

In October 2018, the fine roots of the dominant tree species were collected, tracing from the trunks, together with the surrounding soil. Seven tree species, growing in CL or NO areas, were selected for sampling, namely, *P. jezoensis*, *A. sachalinensis*, *Pinus pumila*, *Picea glehnii*, *B. ermanii*, *Q. crispula*, and *Carpinus cordata*. The root samples of some of these species (*P. jezoensis, A. sachalinensis, B. ermanii*, and *Q. crispula*), were collected in both CL and NO areas. At the same time, the others got strong preferences for particular soil conditions: *P. pumila* and *C. cordata* samples were only collected in the CL areas, and *P. glehnii* samples were only collected in the NO areas. In total, root samples were collected in 11 areas, in the central part of each tree species community. And in each sampling point, root tips were collected from multiple points, in a center circle, with the radius of 50 cm, of each tree community per area. The tree individuals were 10 years and over. The sampling areas are at least 30 m away from each other, because CL and NO areas were patchily distributed. All root and soil samples were stored in plastic bags at 4 °C until further analysis.

The fine roots were washed gently in tap water, selected at random, and observed under a microscope. Mature ECM root tips in each sample were classified into morphotypes, according to their morphological characteristics [28,29]. The frequency of each ECM type was calculated as follows: specific ECM type ratio = (number of ECM root tips of a particular type) / (total number of ECM root tips). Three root tips were collected for each ECM morphotype, and stored separately at –20°C for the ensuing molecular analysis.

2.3 Molecular Identification of the Mycorrhizal Fungal Root Tips

The internal transcribed spacer (ITS) region of rDNA was amplified by polymerase chain reaction (PCR) using the AmpDirect plus PCR kit (SHIMADZU Co., Sapporo, Hokkaido, Japan) and the 2720 Thermal Cycler (Applied Biosystems, Tokyo, Minato-ku, Japan). The fungus specific primer pair ITS1F/ITS4 [30,31] was used for PCR before an amplification step with the universal primer pair ITS1/ITS4 [30,31] to obtain a sufficient concentration of the target sequence. The amplification was performed using the following program: initial denaturation at 95°C for 10 min; followed by 30 cycles of denaturation at 94°C for 30 s, annealing at 48 °C for 30 s, and extension at 72°C for 1 min; and a final extension at 72°C for 7 min. The PCR products were mixed with 6-Agarose gel loading buffer (BiONEER Co., Daedeok-gu, Daejeon, Korea) analyzed by electrophoresis on 2% agarose gel, and visualized using a blue LED transilluminator after staining with Midori Green Direct (BiONEER Co.). The PCR products were purified using FastGene™gel/PCR Extraction kit (Nippon Genetics Co., Ltd., Bunkyo-ku, Tokyo, Japan). The sequences were assigned to taxonomic categories. The sequencing was performed at Macrogen Japan Inc. (Kyoto, Kyoto, Japan). The sequencing reaction involved BigDye Terminator v3.1 Cycle Sequencing kit, BigDye XTerminator Purification kit for sample purification, and Applied Biosystems 3730xl DNA Analyzer for sequencing. Then, the sequences were compared with those deposited in the GenBank database at the DNA Data Bank of Japan (DDBJ: http://www.ddbj.nig.ac.jp/), using the nucleotide basic local alignment search tool

(BLAST). The above procedure was based on a report of Arai et al 2017 [15] and was repeated to obtain reasonable results for each ECM morphotype.

2.4 Soil Analysis

The soil pH was determined for each sampling point to confirm the differences between the sampling areas (Table 1). After air-drying, each soil sample was sieved through a 2 mm mesh and suspended in tap water at 1:5 ratio. The soil pH was determined after stirring and leaving it to stand for 1 d, using a piercing type pH meter (PH-220Spear; Satoshoji Co., Ltd., Chiyoda-ku, Tokyo, Japan). The values of soil pH are the mean +/- Standard Deviation (SD) for five replicate soils.

2.5 Statistical Analysis

Unless stated otherwise, statistical analyses were performed in R v3.6.1 (R Core Development Team 2019). Tukey's HSD test was performed to test for differences in the soil pH value among the different sampling points, and the Wilcoxon rank sum test was conducted to test for differences in the soil pH value between the CL and NO areas, using the coin package of R [32]. The significance level was set at $p < 0.05$.

Very few fungal species were commonly found in all sampling points. Therefore, the ECM fungal composition data were considered at the genus level. Bray–Curtis index [33] was used to calculate the dissimilarities of ECM fungal compositions and soil pH values among the sampling points, using the vegan package v2.5-6 of R [34]. The same package was used for non-metric multidimensional scaling (NMDS) analysis to illustrate the patterns of ECM fungal composition at each sampling point. The association between the ECM fungal composition and soil pH was examined by Mantel test with Bray–Curtis index for each dataset [35,36]. After collating the ECM frequency data by the sampling area and normalizing the number of observed root tips at each sampling point, the similarity percentages (SIMPER) function [37] was used to identify species that contributed the most to the Bray–Curtis dissimilarity of ECM fungal composition in the CL and NO areas.

To assess the sufficiency of sampling for each sampling point, species accumulation curves were generated by sample-based rarefaction analysis using Estimate S v9.1.0 [38] with 1000 randomizations without replacement.

3. RESULTS

3.1 ECM Identification and Colonization Status at Each Sampling Point

Overall, 20178 ECM root tips were examined, and 57 ECM species were identified (Tables 1, 2). No common ECM species in the sampling areas (CL: calcareous area, and NO: non-calcareous area) or the host tree species, except for *Cenococcum geophilum*, a ubiquitous species, were apparent.

The species accumulation curves of ECM fungi at almost all sampling points tended to level off, indicating that most of the ECM fungal taxa at the sampling points have been detected (Fig. 1). However, few accumulation curves at specific sampling points, namely, *Betula ermanii* in CL, *Carpinus cordata* in CL, *Pinus pumila* in CL, and *Picea glehnii* in NO did not level off, which indicated the scarcity of root samples. If the number of root samples had been higher, then it is possible that more newcomer species would have been found at the sampling points.

In this study, 2038 and 2659 root tips of *Picea jezoensis* were examined in the CL and NO areas, respectively. Seven and six morphotypes of ECM root tips were identified in these areas, accordingly (Tables 1, 2). In the CL area, the frequencies of *Tomentella* sp.1, *Sebacina incrustans*, *Tomentella* sp.2, and *Sebacina* sp.1 were relatively high, and these four species accounted for 98.1% of total ECM colonization. By comparison, the frequencies of *Tyrospora asterophora*, *C. geophilum*, and *Pseudotomentella* sp.1 were high in the NO area, and these three species accounted for 97.1% of total ECM colonization therein.

Further, 1308 and 2034 root tips of *Abies sachalinensis* were examined in the CL and NO areas, respectively. Seven and five morphotypes of ECM root tips were identified in these areas, accordingly (Tables 1, 2). In the CL area, the frequencies of *Lycoperdon perlatum* and *Sebacina epigaea* were relatively high, and these two species accounted for 80.3% of total ECM colonization. By comparison, the frequency of *Russula* sp.3 was remarkably high in the NO area, and this species accounted for 66.7% of total ECM colonization in this area.

In addition, 2099 and 2216 root tips of *B. ermanii* were examined in the CL and NO areas, respectively. Six morphotypes of ECM root tips were identified in these areas (Tables 1, 2). In

the CL area, the frequency of Clavulinaceae sp. was relatively high, and the species accounted for 77.6% of total ECM colonization. By comparison, the frequencies of *Russula* sp.3 and *Pseudotomentella* sp.2 were remarkably high in the NO area, and these species accounted for 93.7% of total ECM colonization therein.

Furthermore, 1054 and 2058 root tips of *Quercus crispula* were examined in the CL and NO areas, respectively. Three and two morphotypes of ECM root tips were identified in these areas, accordingly (Tables 1, 2). In the CL area, the frequency of *Tomentella* sp.6 was remarkably high, and this species accounted for 76.4% of total ECM colonization. By comparison, the frequency of *C. geophilum* was high in the NO area, and this species accounted for 73.4% of total ECM colonization therein.

In the current study, 424 root tips of P. pumila were examined in the CL area. Six morphotypes of ECM root tips were identified (Tables 1, 2). The frequencies of *Tomentella* sp.7 and *Sebacina* sp. LM2566 were relatively high, and these two species accounted for 83.0% of total ECM colonization.

Further, 2195 root tips of *C. cordata* were examined in the CL area. Four morphotypes of ECM root tips were identified (Tables 1, 2). The frequency of *Tomentella* sp.8 was relatively high, and this species accounted for 83.0% of total ECM colonization.

Table 1. Soil pH and ECM root tip status

Host tree	Classification of soils	Soil pH	Root tips	ECM fungal species
P. jezoensis	CL	7.2 ± 0.01	2038	7
	NO	5.2 ± 0.01**	2659	6
A. sachalinensis	CL	6.8 ± 0.01	1308	7
	NO	5.3 ± 0.01**	2034	5
B. ermanii	CL	7.2 ± 0.01	2099	6
	NO	5.4 ± 0.01**	2216	6
Q. crispula	CL	7.1 ± 0.01	1054	3
	NO	5.5 ± 0.01**	2058	2
P. pumila	CL	7.1 ± 0.01	424	6
C. cordata	CL	7.4 ± 0.01	2195	4
P. glehnii	NO	5.6 ± 0.01	2093	10

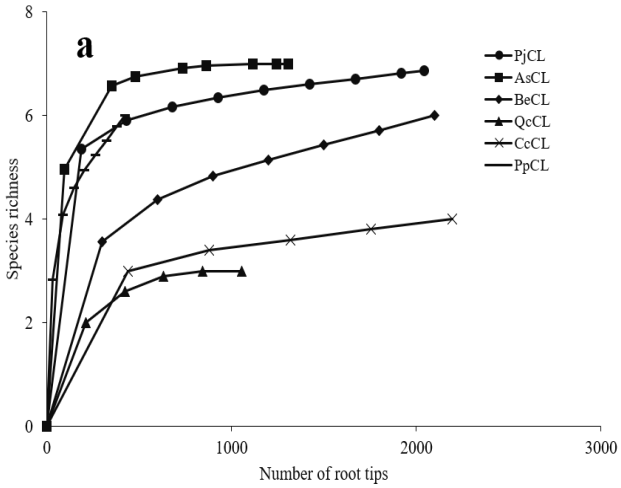
*Values of soil pH are the mean ± SD for five replicate soils. Significant differences between areas were determined using Tukey's HSD test. ** $p < 0.01$*

Table 2. Species identities of ECM fungi observed at each sampling point

Species	Accession no.	Observed areas (frequency [%])
Amphinema byssoides	LC523838	PgNO (12.2)
Cadophora sp.	LC523839	BeCL (2.9)
Cenococcum geophilum sp.1	LC523840	PgNO (30.3), PjNO (30.1)
Cenococcum geophilum sp.2	LC523841	BeCL (5.8)
Cenococcum geophilum sp.3	LC523842	QcNO (73.4)
Cladphialophora sp.	LC523843	PgNO (7.5)
Clavulinaceae sp.	LC523844	BeCL (77.5)
Cortinarius sp.	LC523845	PgNO (1.9)
Didymellaceae sp.	LC523846	BeCL (1.4)
Genea hispidula	LC523847	BeNO (24.3)
Helotiales sp.	LC523848	PpCL (4.5)
Humaria sp.1	LC523849	AsNO (2.6)
Humaria sp.2	LC523850	PpCL (0.7)
Inocybe sp.1	LC523851	AsCL (0.2)
Inocybe sp.2	LC523852	BeNO (45.0)
Inocybe sp.3	LC523853	QcNO (26.6)
Laccaria sp.	LC523854	BeNO (9.9)
Lactarius sp.	LC523855	PgNO (3.9)
Luteoamylascus aculeatus	LC523856	BeCL (0.4)
Lycoperdon perlatum	LC523857	PjCL (0.9), AsCL (40.7)
Peziza sp.	LC523858	PjCL (0.6), PpCL (8.7), AsCL (4.9)

Species	Accession no.	Observed areas (frequency [%])
Piloderma sp.	LC523859	AsNO (0.3)
Pseudotomentella sp.1	LC523860	PjNO (24.7)
Pseudotomentella sp.2	LC523861	AsNO (26.8)
Russula sp.1	LC523862	PjNO (0.6)
Russula sp.2	LC523863	AsCL (3.0)
Russula sp.3	LC523864	AsNO (66.9), PgNO (27.4)
Russula sp.4	LC523865	AsNO (3.5)
Sebacina epigaea	LC523866	AsCL (39.6)
Sebacina incrustans	LC523867	PjCL (25.6)
Sebacina sp. LM2566	LC523868	CcCL (9.3)
Sebacina sp.1	LC523869	PjCL (15.4)
Sebacina sp.2	LC523870	AsCL (5.8)
Sebacina sp.3	LC523871	BeNO (12.2)
Sebacina sp.4	LC523872	BeNO (2.7)
Sebacina sp.5	LC523873	PpCL (6.8)
Suillus sp.	LC523874	PpCL (0.7)
Thelephora sp.1	LC523875	CcCL (7.5)
Thelephora sp.2	LC523876	CcCL (0.2)
Tomentella liacinogrisea	LC523877	QcCL (22.8)
Tomentella sp.1	LC523878	PjCL (37.1)
Tomentella sp.2	LC523879	PjCL (20.0)
Tomentella sp.3	LC523880	PjCL (0.4)
Tomentella sp.4	LC523881	PjNO (1.1)
Tomentella sp.5	LC523882	BeCL (12.0)
Tomentella sp.6	LC523883	QcCL (76.4)
Tomentella sp.7	LC523884	CcCL (83.0)
Tomentella sp.8	LC523885	PpCL (78.5)
Tomentella sp.9	LC523886	PgNO (9.5)
Tomentella sp.10	LC523887	PgNO (6.3)
Tomentella sp.11	LC523888	PgNO (1.0)
Tomentella sp.12	LC523889	PgNO (0.1)
Tricholoma squarrulosum	LC523890	AsCL (5.8)
Tuber sp.1	LC523891	PjNO (0.5)
Tuber sp.2	LC523892	BeNO (6.0)
Tuber sp.3	LC523893	QcCL (0.9)
Tylospora asterophora	LC523894	PjNO (42.9)

PgCL and PgNO: P. jezoensis in the CL and NO areas, respectively; AsCL and AsNO: A. sachalinensis in the CL and NO areas, respectively; BeCL and BeNO: B. ermanii in the CL and NO areas, respectively; QcCL and QcNO: Q. crispula in the CL and NO areas, respectively; PpCL: P. pumila in the CL area, CcNO: C. cordata in the CL area, PgNO: P. glehnii in the NO area



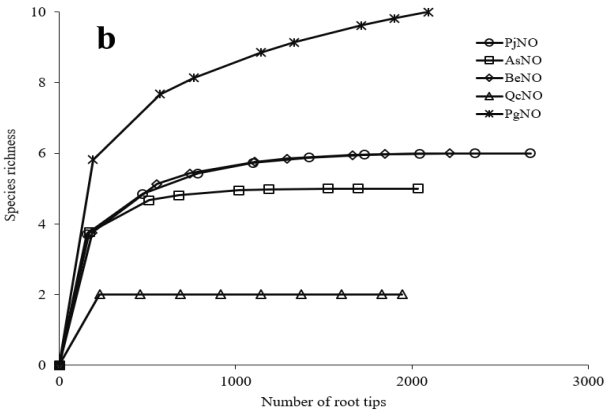


Fig. 1. Species accumulation curve for each sampling point in the CL areas (a) and NO areas (b). (CL: calcareous area, NO: non-calcareous area, Pj: *Picea jezoensis*, As: *Abies sachalinensis*, Be: *Betula ermanii*, Qc: *Quercus crispula*, Cc: *Carpinus cordata*, Pp: *Pinus pumila*, Pg: *Picea glehnii*)

Finally, 2093 root tips of *P. glehnii* were examined in the NO area. Ten morphotypes of ECM root tips were identified in each area (Tables 1, 2). The frequencies of *C. geophilum* and *Russula* sp.5 were relatively high; however, the differences in the frequencies of ECM fungal species were lower than those at other sampling points.

3.2 Factors Determining the ECM Fungal Composition

The NMDS analysis divided the sampling points into two groups, according to the ECM composition ($p < 0.01$ each pair of sampling points; Fig. 2). These two groups generally corresponded to the classifications by the sampling area (CL and NO area), except for *B. ermanii* in the NO area. This suggested that the presence of limestone was a more important factor determining the ECM fungal composition than the type of host trees present. Among the soil parameters, we focused on the soil pH. We conducted the Mantel test to evaluate the similarity of patterns between the soil pH and ECM composition. The analysis confirmed the above-mentioned conclusion ($p = 0.001$), even when excluding tree species collected only in one area ($p = 0.011$).

SIMPER analysis revealed the ECM fungal genera that contributed the most to the difference in ECM composition between the CL and NO areas. The top ten fungal genera, explaining 85.4% of the overall difference, were evaluated in detail (Table 3). Among these genera, *Tomentella* (28.9%), *Cenococcum* (11.8%), *Russula* (8.8%), *Sebacina* (8.0%), and *Inocybe* (6.5%) were present at three or more sampling points. This suggested that *Tomentella* and *Sebacina* were characteristic for the CL area, and *Cenococcum*, *Russula*, and *Inocybe* were characteristic for the NO area.

4. DISCUSSION

Comparison of the ECM composition of the same tree species in the CL (calcareous) and NO (non-calcareous) areas revealed no ECM fungal species in common in these two areas. Furthermore, we did not find any regularities in ECM frequency, even when the data were grouped at the genus or higher level. Accordingly, the NMDS analysis (Fig. 2) and Mantel test suggested that the ECM fungal composition is determined by the soil pH rather than by differences in the host tree species. High soil pH is a suitable indicator of the limestone effect [26]. Some researchers examined the ECM community in NO areas, associated with the same tree species as our study, on *Picea jezoensis*, *A. sachalinensis*, *Betula ermanii* [38], on *Quercus crispula* [39,40] on *Pinus pumila* [41] and on *Picea glehnii* [42,43]. However, their ECM fungal composition is greatly different from the results of the current study (Table 2). Our research suggested a specific ECM fungal composition in the CL area of Mt. Kirigishi, in accordance with the initial hypothesis.

Besides the current study, some researchers compared the ECM fungal community in the CL area with that of the surrounding area. They found high frequencies of specific ECM fungal

genera, *Tomentella* spp. [19,20,23,24] and *Sebacina* spp. [23,24] in CL areas. These reports are in agreement with the results of the current study in CL areas of Mt. Kirigishi (Table 3). In addition, some researchers documented the decline of ECM fungal diversity in CL areas [24], however, such a tendency was not found in our study. Instead, it may be possible that the ECM fungal diversity in CL areas was higher than those of NO areas, when we consider not leveling off accumulation curves of many CL samples.

Tomentella spp. tend to exhibit low host-specificity and to have adapted to growth in various environments [44,45]. Certainly, *Tomentella* spp. were associated with all tree species investigated in the current study, however, they were unevenly distributed in accordance with soil conditions. In addition, other instances of *Tomentella* spp. dominance in CL areas has been reported previously. Baier et al. [19] compared the ECM fungal communities in CL areas with those of NO areas, and documented the dominance of *Tomentella* spp. in a subalpine region. In the same way, Harrington, Mitchell [20] and Timling et al. [23] found the dominance of *Tomentella* spp. by comparative research in arctic regions, and Anne et al. [24] in a lowland area. At least some species from the genus *Tomentella* can adapt to CL soil. According to previous studies, *Tomentella* spp. preferentially dwell in the soil with high pH [19], mineral soil [20,46], and on a steep slope [22]. These environmental conditions are notable in the CL areas, and the characteristics of limestone may have generated conditions for the dominance of *Tomentella* spp.

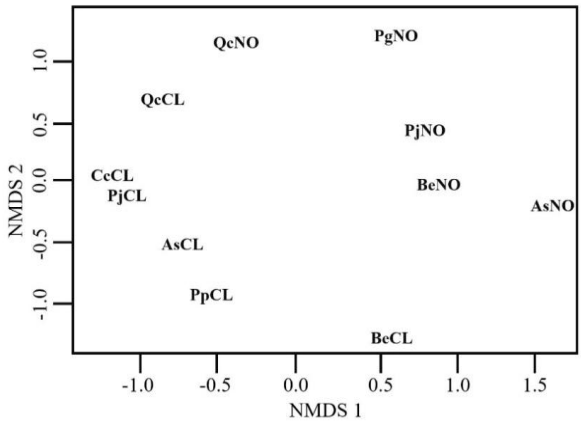


Fig. 2. NMDS analysis of ECM fungal composition at each sampling point

Stress value = 0.14. PgCL and PgNO: P. jezoensis in the CL and NO areas, respectively; AsCL and AsNO: A. sachalinensis in the CL and NO areas, respectively; BeCL and BeNO: B. ermanii in the CL and NO areas, respectively; QcCL and QcNO: Q. crispula in the CL and NO areas, respectively; PpCL: P. pumila in the CL area, CcNO: C. cordata in the CL area, PgNO: P. glehnii in the NO area. The differences between sampling points were based on the Bray-Curtis index

Table 3. Results of the SIMPER test showing the top ten fungal genera that contributed the most to the difference in ECM fungal composition between the CL and NO areas

Genera	Average contribution to overall dissimilarity (%)	Total frequencies in overall CL (%)	Total frequencies in overall NO (%)
Tomentella	28.9	55.0	2.4
Cenococcum	11.8	1.1	27.1
Russula	8.8	0.5	20.0
Sebacina	8.0	17.1	2.4
Clavulina	7.0	12.9	0.0
Inocybe	6.5	0.0	14.3
Pseudotomentella	4.7	0.0	10.3
Tylospora	3.9	0.0	8.6
lycoperdon	3.8	6.9	0.0
Genea	2.2	0.0	4.9
Total	85.6	93.5	90.1

With regards to *Sebacina* spp. in the study area investigated, their closest matches have been mainly found in alpine or subarctic regions, for example, *S. incrustans* found in Albert Bay, Canada [23], and *Sebacina* sp.1 found in the Helan Mountains, China [25]. *Sebacina* spp. tolerate cold climates [25]. The CL area evaluated in the current study contains many steep and bare slopes, and the ground tends to be frozen in winter because of a limited snow cover [47]. Under such conditions, *Sebacina* spp. may replace the common ECM fungal species of the warm-temperate forest because of their low-temperature tolerance. This point of view is supported by some reports documented that the frequencies of *Sebacina* spp. in CL areas were higher than those of NO areas.

By contrast, in the NO area of Mt. Kirigishi, the frequencies of *C. geophilum* and *Russula* spp. were characteristically higher than those in the CL area (Table 3).

C. geophilum shows great adaptability and is often the dominant species in various types of forests [24]. However, in the current study, the frequency of *C. geophilum* was low in the CL area, a highly stressful environment (Table 3). According to some previous reports, this species tends to prefer eutrophic conditions, such as soil rich in organic matter [17,48]. Hence, it may not prefer CL areas, where litter is scarce. Furthermore, *Russula* spp. are widely found in forests in the northern hemisphere; however, these species were rarely found in the CL area investigated in the current study. Mundra et al. suggested that *Russula* spp. preferentially dwell in the acidic and eutrophic soil, and this notion coincides with the findings of the current study [8].

As mentioned above, ECM fungi adapted to soil conditions and separate colonies/clusters developed on differing soils, at least, at the genus level. And there may be a common distribution pattern of ECM fungal species in calcareous areas around the world, as initially hypothesized. The general ECM fungi, living in NO areas, may not retain ecological superiority in unique CL areas.

5. CONCLUSION

This study was one of few attempts to investigate ECM fungal communities in limestone areas. In the current study, it was suggested that characteristic ECM fungi, *Tomentella* spp., and *Sebacina* spp., might be able to effectively support the growth of host trees in limestone areas, and these distribution patterns might be found throughout the world. These ideas indicate a potential to solve some environmental problems. Limestone is a valuable mineral resource and has a long history of mining throughout the world. Nevertheless, revegetation is perturbed in closed mines because of the characteristics of limestone, and practical greening is urgently needed [47,48]. Specifically, the slopes of a strip mine are difficult to green with general tree species [48]. ECM fungi, found in our research, could be used for field greening. Further, many rare plant and animal species were distributed in Mt. Kirigishi. The ECM fungi identified in the current study may be intricately connected with these species, and may be used for their conservation. The contribution of individual ECM fungi to the ecosystem remains unclear, and further research is needed to elucidate this [49].

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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