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Morphological Characteristics of Ectomycorrhizas Found in Willow and Poplar Seedlings Established in the Area Devastated by the Volcanic Eruption of Mt. Usu, Hokkaido, Japan in 2000

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Abstract
The ectomycorrhizas (ECMs) were collected from the land devastated by the volcanic eruption of Mt. Usu, in Hokkaido, Japan, in 2000. ECM from the roots of dominant seedlings, Populus huleni var. angustifolia, Salix sachalinensis and Salix hultenii var. angustifolia, were examined and classified based on their morphotypes confirmed by PCR-RFLP banding patterns. And the ITS rDNA sequences of each morphotype were assigned with registered sequence in DDBJ. Nine ECM types were assigned (Laccaria amethystea, Inocybe lacera, Hebeloma mesophaeum, Scleroderma bovista, Thelephora terrestris, Thelephoraceae 1, Thelephoraceae 2, Tiber sp. and unidentified fungi). ECM formed by a given ECM fungal species displayed the same morphological characteristics in multiple host plant species. ECMs were described on the basis of both morphological and molecular characteristics.

Key words: Volcano, Ectomycorrhizal fungi, Morphology, molecular analysis, Salicaceae

Introduction
Exposed areas derived from volcanic eruption are vulnerable to further sedimentary disasters such as debris avalanches, due to instability of the land surface (e.g. Kadomura et al. 1983, Lecointre et al. 2004); therefore, it is essential to promote vegetation recovery quickly in denuded areas. Although various stressful growth conditions such as low soil nutrients, instability of the soil surface, and drought may persist after volcanic eruption, pioneer woody plants, in particular willows and poplar, often invade immediately and become established in devastated areas (e.g. Haruki and Tsuyuzaki 2001, Tsuyuzaki 1989).

Most woody plant species in natural forest ecosystems associate with mycorrhizal fungi in their root systems. Plants in the family Salicaceae associate with either ectomycorrhizal fungi and/or arbuscular mycorrhizal fungi (Wang and Oiu 2006). In general, mycorrhizal fungi assist host plants by enhancing nutrient acquisition (Colpaert et al. 1999, Tibbet and Sanders 2002, Moyersen et al. 1998) and drought tolerance (Lamhamedi et al. 1992). In this way they serve to ameliorate stressful growing conditions that encountered by their host plants during primary succession.

Classification of ECM morphological features is a standard method that be used to identify ECM fungal flora. Further characterization by molecular analysis of DNA from ECM facilitates more precise identification (Egger 1995). Taken together, morphological and molecular characterizations could provide fundamental knowledge for investigating ECM fungal species of interest. Several research groups have reported on the morphological characteristics of multiple ECM in the root systems of various woody plant species (e.g. Agerer 1987-2002, Ingleby et al. 1990). In this study, we reported on the morphological and molecular findings to identify and describe the ECM formed in the roots of willow and poplar seedlings during primary succession after the volcanic devastation in 2000, at Mt. Usu, in Hokkaido, Japan.

Materials and Methods
Study sites
Mt. Usu (42° 32’ N, 140° 50’ E, 773.1 m elevation) is an active volcano located in southwest Hokkaido, Japan. After twenty-two years from last eruption, Mt. Usu was erupted on March 31, 2000, and formed a number of small craters at the foot of the Nishiyama and Konpira areas. The study was conducted around the N-a crater at Nishiyama, where volcanic activity such as ejection of volcanic debris and thermal activity subsided in the autumn of 2000 (Obase et al. 2007). Before the eruption in 2000, the natural vegetation consisted mainly of secondary forests of broadleaf trees such as Betula spp., Acer spp. and Quercus spp., and partially of artificial forests of Larix kaempeferi (Lamb.) Carr. and Abies sachalinensis (Fr. Schm.) Carr. Masters. These forests were almost completely destroyed by the volcanic deposition of fine volcanic ash and pumice, one to three meters in depth. Approximately 71 hectares of the forest surrounding the craters were destroyed. In 2005, the climatic data for Date city (42° 30’ N, 140° 54’ E, 84.7 m elevation), which near Mt. Usu, was a mean annual precipitation of 982 mm, and an average annual temperature of 7.9°C, with a range from -4.6°C to 22.4°C, in February and August, respectively (Sapporo meteorological station).
Sampling and mycorrhizal assessment

The study site was established around the N-a crater where natural vegetation was almost completely destroyed by volcanic deposition in 2000 (Obase et al. 2007). In September 2005, lateral roots from three species of one year old willow and poplar seedlings, dominantly recruited and established in the area, were randomly sampled. Lateral roots were collected from 12 to 20 seedlings each of Populus maximowiczii A. Henry, Salix sachalinensis Fr. Schm. and Salix hultenii var. angustifolia Kimura and separated from the adhering soil by soaking and carefully washing in tap water. The isolated ECMs were examined by using dissecting and differential interference microscopy to characterize the gross morphology and the mantle and Hartig net, respectively. ECMs were classified by color, general shape, surface texture, hyphal structural arrangement of the mantle, and the frequency and shape of their emanating hyphae. Descriptive terminology for each characteristic was based on Goodman et al. (1996-2000).

One ECM root tip of each morphotype from each seedling (3 to 5 samples from each morphotype) was categorized individually by PCR-RFLP. Each sample was stored in 1.5 ml sterilized microtubes at -80°C until using. ECM fungal DNA was extracted from 5-10 mg ground, lyophilized tissue using the DNeasy Plant Mini kit (QIAGEN, USA) according to the manufacturer's instructions.

The ITS region, including the 5.8S rDNA, was amplified using a specific primer for higher fungi (ITS1-f; White et al. 1990) and a universal primer (ITS4; White et al. 1990). The following PCR amplification conditions were used: 94°C for 3 min, followed by 30 cycles of 94°C for 1 min, 50°C for 1 min and 72°C for 3 min, then a final extension at 72°C for 10 min (Landeweert et al. 2005). Single enzyme digests using Hinf I and Afl I were performed on PCR products from samples of each ECM morphotype. Using 2.5% agarose gel electrophoresis, we estimated the size of sample. The ECMs in Types 1-5 were distinguished by color, sometimes light-reddish grey at the tips, and the ECM shape, often monopodial-pinnate. It formed net synenchyma composed of irregularly arranged hyphae. Type 2 was distinguished by the presence of a hole-like structure in the center of the clamp connections in emanating hyphae. It formed net synenchyma composed of hyphae in parallel arrangement. Type 3 was characterized by ECM surrounded by dense, cottony emanating hyphae. It formed net synenchyma composed of hyphae in parallel arrangement. Type 4 was easily distinguished by its velvety textured appearance, possibly due to air adhering to emanating hyphae. The mantle showed distinct felt synenchyma with clamp connections. Type 5 was characterized by long, slender shaped ECM. It formed net synenchyma composed of hyphae in ring-like arrangements. The ECM in Type 6-9 were characterized by black color. Type 6 was easily distinguished by its graduated color, from white at the very tip (youngest cells) to brown in the less distal areas (older cells), and the presence of bristle-like awl-shaped cystidia occurring frequently at the mantle surface. It formed several different structures in the mantle, and one at the root tip. Some net synenchyma were composed of irregularly arranged hyphae, while other synenchyma were irregular and non-interlocking. Type 7 was characterized by distinctly verrucose emanating hyphae. It formed net synenchyma composed of irregularly arranged hyphae, while other synenchyma were irregular and non-interlocking. Type 8 and 9 were difficult to distinguish macroscopically. Examination showed a difference in the structure of the mantle surface; interlocking irregular synenchyma, and non-interlocking irregular synenchyma, respectively. In this work, PCR-RFLP band patterns were specific for each different ECM type and each ECM type was assigned different ECM fungal species or taxa by DNA sequences (Table 1).

Other research groups have described five ECM fungal species: Laccaria amethystea (Cuvelier 1991, Brand 1988), Inocybe lacer a (Agerer 1995, Ingleby 1990, Cripps 1997, Cripps and Miller 1995), Hebeloma mesophaeum (Agerer 1995, Ingleby 1990), Scleroderma bovista (Jakue 1999), and Thelephora terrestris (Agerer and Weiss 1989, Agerer and Weiss 1990, Ingleby 1990). The ECM types reported here showed morphological characteristics and fungal species assignments similar to those of previous reports. Examination of ECM Types 1-9 showed no significant difference in morphological characteristics among different host plant species. Each fungal species displayed the same characteristics independent of the host species. This finding is consistent with others where mycorrhizas formed in different woody plants by the same fungal species were found to be broadly similar (Ingleby 1990). However, other researchers have reported slight morphological differences in ECMS that were formed by same fungi among different host plant species (e.g. Beenken and Agerer 1996). It might due to the morphological variation was attributable to differences in environmental conditions and host plants.
<table>
<thead>
<tr>
<th>ECM type</th>
<th>ECM host</th>
<th>Color</th>
<th>General shape</th>
<th>Texture</th>
<th>Mantle (outer layer)</th>
<th>Emanating hypha</th>
<th>Possible identity (Accession No. of highest similarities)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Type 1</td>
<td>Pm, Sh, Ss</td>
<td>Light-reddish grey to light-reddish brown</td>
<td>Monopodial pinnate, straight to bent</td>
<td>Loosely wooly</td>
<td>Net synenchyma</td>
<td>Infrequent, smooth, 3-4µm in width, with clamp connection</td>
<td><em>Laccaria amethystea</em> AB211270 (557/558: 99%)</td>
</tr>
<tr>
<td>Type 2</td>
<td>Pm, Sh, Ss</td>
<td>Very pale brown to brown</td>
<td>Unbranched, straight</td>
<td>Smooth to shiny</td>
<td>Net synenchyma</td>
<td>Infrequent, smooth, 2-4µm in width, with clamp connection that possess a hole-like structure in the center</td>
<td><em>Inocybe lacera</em> AY750157 (595/601: 99%)</td>
</tr>
<tr>
<td>Type 3</td>
<td>Pm, Ss</td>
<td>White to brown</td>
<td>Unbranched, straight</td>
<td>Cottony</td>
<td>Net synenchyma</td>
<td>Abundant, 2-4µm in width, smooth or verrucose, with clamp connection</td>
<td><em>Hebeloma mesophaeum</em> AF126100 (453/453: 100%)</td>
</tr>
<tr>
<td>Type 4</td>
<td>Pm</td>
<td>Pale yellow to yellow</td>
<td>Unbranched to irregularly pinnate, dichotomous-like</td>
<td>Velvety</td>
<td>Felt prosenchyma</td>
<td>Abundant, 3-4µm in width, with clamp connection</td>
<td><em>Scleroderma bovista</em> AB099901 (531/538: 99%)</td>
</tr>
<tr>
<td>Type 5</td>
<td>Pm, Sh, Ss</td>
<td>White to very pale brown</td>
<td>Unbranched, straight to bent, slender</td>
<td>Smooth to loosely wooly</td>
<td>Net synenchyma</td>
<td>Infrequent, 3-4µm in width, with clamp connection</td>
<td>Thelephoraceae 1 DQ195392 (529/522: 94%)</td>
</tr>
<tr>
<td>Type 6</td>
<td>Pm, Sh, Ss</td>
<td>White (very tip) to Brown (older part)</td>
<td>Unbranched, straight to bent</td>
<td>Short-spiny</td>
<td>Net synenchyma to non-interlocking irregular synenchyma with bristle-like awl-shaped cystidia</td>
<td>Infrequent, 2-3µm in width, with clamp connection</td>
<td><em>Thelephora terrestris</em> AY230241 (544/521: 98%)</td>
</tr>
<tr>
<td>Type 7</td>
<td>Ss</td>
<td>Dark reddish brown</td>
<td>Unbranched, straight to bent</td>
<td>Felty</td>
<td>Net synenchyma to non-interlocking irregular synenchyma</td>
<td>Verrucose, 3-4µm in width, with clamp connection</td>
<td>Thelephoraceae 2 AF272913 (486/521: 93%)</td>
</tr>
<tr>
<td>Type 8</td>
<td>Ss</td>
<td>Dark reddish brown to black</td>
<td>Unbranched, straight to bent</td>
<td>Smooth</td>
<td>Interlocking irregular synenchyma</td>
<td>Rare, with no clamp connection</td>
<td><em>Tuber sp.</em> AJ534706 (522/526: 99%)</td>
</tr>
<tr>
<td>Type 9</td>
<td>Pm, Sh</td>
<td>Dark reddish brown to black</td>
<td>Unbranched, straight to bent</td>
<td>Smooth</td>
<td>Non-interlocking irregular synenchyma</td>
<td>Rare, with no clamp connection</td>
<td>Unidentified fungi AB096869 (508/513: 98%)</td>
</tr>
</tbody>
</table>

*Pm, Populus maximowiczii; Sh, Salix huilennii var. angustifolia; Ss, Salix sachalinensis.
**Accession No. that presented highest similarities with DNA sequences from each ECM morphotype and value of similarity (No. of matched bases / No. of total bases analyzed; percent similarities) were presented.
materials. In the present study, samples were obtained from *Salix* and *Populus* seedlings established in same environment that could account for the morphological uniformity observed in ECMs among different woody plant species.

In this work, PCR-RFLP band patterns were nearly identical for samples within a given ECM type, but were unique for each different ECM type. Furthermore, based on a comparison of DNA sample sequences with reference databank sequences or/and those from sporocarps that were collected from field survey, the DNA sequence from each ECM type (Types 1-8) was corresponded to a different ECM fungal species (Obase et al. 2007). So, this work demonstrated that molecular analysis combined with the standard method of morphological characterization may afford precise investigation and identification of ECM fungal flora. Also, by using together with examining relative abundance of each morphotype of root tips, composition of ECM fungal species that actually associated with plants in fields could be revealed more in detail.

![Fig. 1. Appearance (left photo) and structure of mantle surface (Right photo) of each ECM type observed in roots of willow and poplar seedlings at Mt Usu, Hokkaido, Japan. Blue and black bars indicate 0.5 mm and 10 μm, respectively.](image_url)
Acknowledgements
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Reference