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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 maximowiczii*, *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, *Tuber* 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, Tibbett and Sanders 2002, Moyersoen *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 kaempferi* (Lamb.) Carr. and *Abies sachalinensis* (Fr. Schm.) 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).

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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; Gardes and Bruns 1993) 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 *Hin*fI and *Alu*I were performed on PCR products from samples of each ECM morphotype. Using 2.5% agarose gel electrophoresis, we estimated the quality and quantity of the PCR products, as well as the size of restriction fragments.

We used the primer ITS1-f to sequence samples of each PCR product arising from different ECM morphotypes and exhibiting differences in RFLP analysis. Sequencing reactions were performed using the BigDye Terminator v3.1/1.1 Cycle Sequencing Kit (Applied Biosystems, USA), followed by ethanol precipitation and analysis with an ABI Auto Sequencer 310 (Applied Biosystems, USA). The DNA sequences were compared with the GenBank database at the DNA Data Bank of Japan (DDBJ) and also with the DNA sequences of ECM sporocarps reported in a preliminarily study (Obase *et al*. 2005). Species names were assigned to BLAST matches exhibiting more than 95% homology.

Results and Discussion

Analysis of ECM root tip samples revealed nine distinct ECM types (Table 1, Figure 1). Some ECM types could not be described due to insufficient amounts of sample. The ECMs in Types 1-5 were characterized by white color (Figure 1). Type 1 was 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 (Figure 1). 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 lacera* (Agerer 1995, Ingleby 1990, Cripps 1997, Cripps and Miller 1995), *Hebeloma mesophaeum* (Agerer 1995, Ingleby 1990), *Scleroderma bovista* (Jakucs 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 1. Morphological characteristics and the putative identity of associated fungal species of ECM collected from roots of willow and poplar seedlings established in an area devastated by volcano activity at Mt. Usu, in Hokkaido, Japan.

ECM type	ECM host	Color	General shape	Texture	Mantle (outer layer)	Emanating hypha	Possible identitiy (Accession No. of highest similarities)
Type1	Pm, Sh, Ss	Light-reddish grey to light-reddish brown	Monopodial pinnate, straight to bent	Loosely wooly	Net synenchyma	Infrequent, smooth, 3-4μ m in width, with clamp connection	*Laccaria amethystea* AB211270 (557/558; 99%)
Type2	Pm, Sh, Ss	Very pale brown to brown	Unbranched, straight	Smooth to shiny	Net synenchyma	Infrequent, smooth, 2-4μ m in width, with clamp connection that possess a hole-like structure in the center	*Inocybe lacera* AY750157 (595/601; 99%)
Type3	Pm, Ss	White to brown	Unbranched, straight	Cottony	Net synenchyma	Abundant, 2-4μ m in width, smooth or verrucose, with clamp connection	*Hebeloma mesophaeum* AF126100 (453/453; 100%)
Type4	Pm	Pale yellow to yellow	Unbranched to irregularly pinnate, dichotomous-like	Velvety	Felt prosenchyma	Abundant, 3-4μ m in width, with clamp connection	*Scleroderma bovista* AB099901 (531/538; 99%)
Type5	Pm, Sh, Ss	White to very pale brown	Unbranched, straight to bent, slender	Smooth to loosely wooly	Net synenchyma	Infrequent, 3-4μ m in width, with clamp connection	Thelephoraceae 1 DQ195592 (522/552; 94%)
Type6	Pm, Sh, Ss	White (very tip) to Brown (older part)	Unbranched, straight to bent	Short-spiny	Net synenchyma to non-interlocking irregular synenchyma with bristle-like awl-shaped cystidia	Infrequent, 2-3μ m in width, with clamp connection	*Thelephora terrestris* AY230241 (544/551; 98%)
Type7	Ss	Dark reddish brown	Unbranched, straight to bent	Felty	Net synenchyma to non-interlocking irregular synenchyma	Verrucose, 3-4μ m in width, with clamp connection	Thelephoraceae 2 AF272913 (486/521; 93%)
Type8	Ss	Dark reddish brown to black	Unbranched, straight to bent	Smooth	Interlocking irregular synenchyma	Rare, with no clamp connection	*Tuber* sp. AJ534706 (522/526; 99%)
Type9	Pm, Sh	Dark reddish brown to black	Unbranched, straight to bent	Smooth	Non-interlocking irregular synenchyma	Rare, with no clamp connection	Unidentified fungi AB096869 (506/513; 98%)

* Pm, Populus maximowiczii; Sh, Salix hultenii 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 combinated 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.

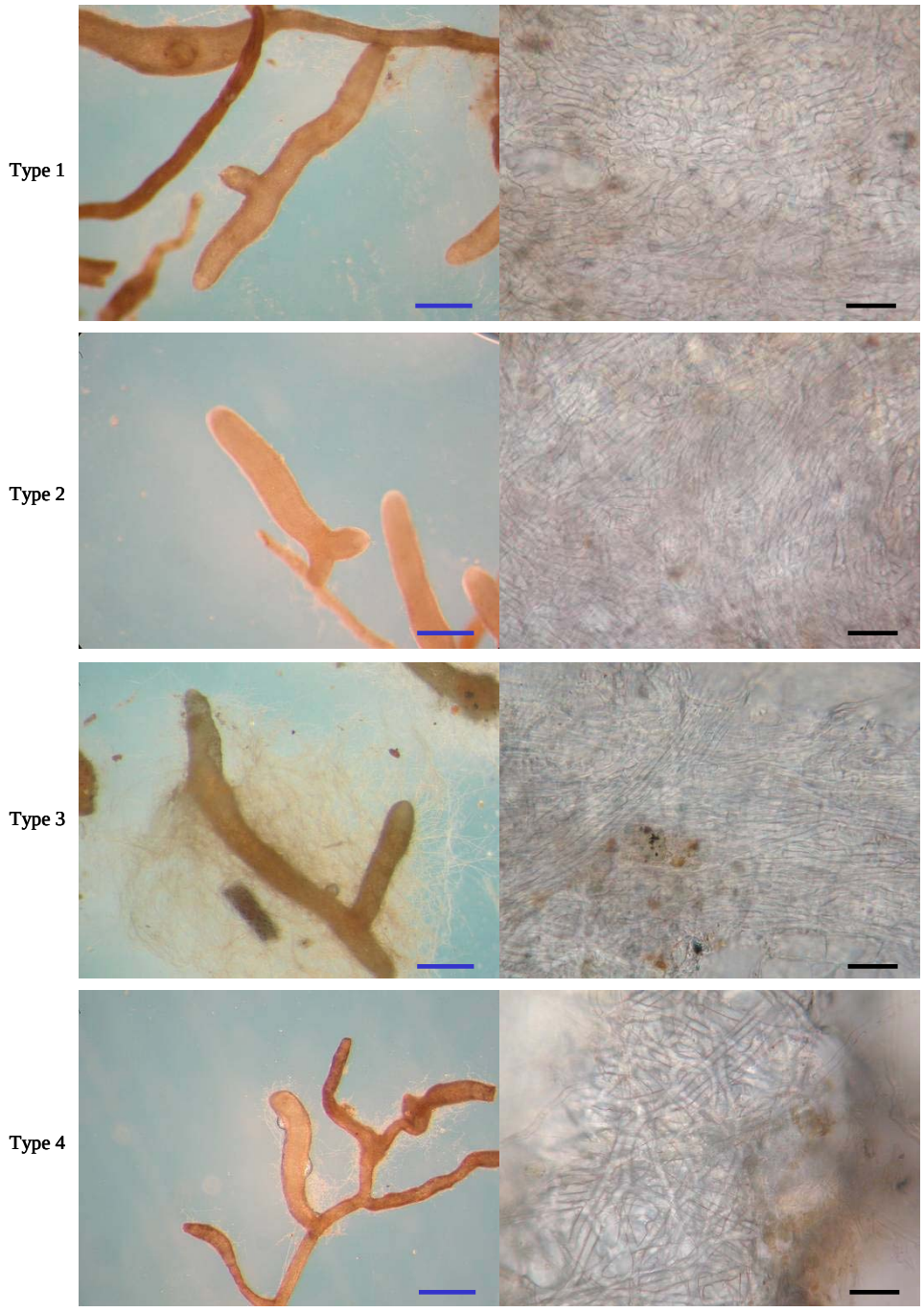
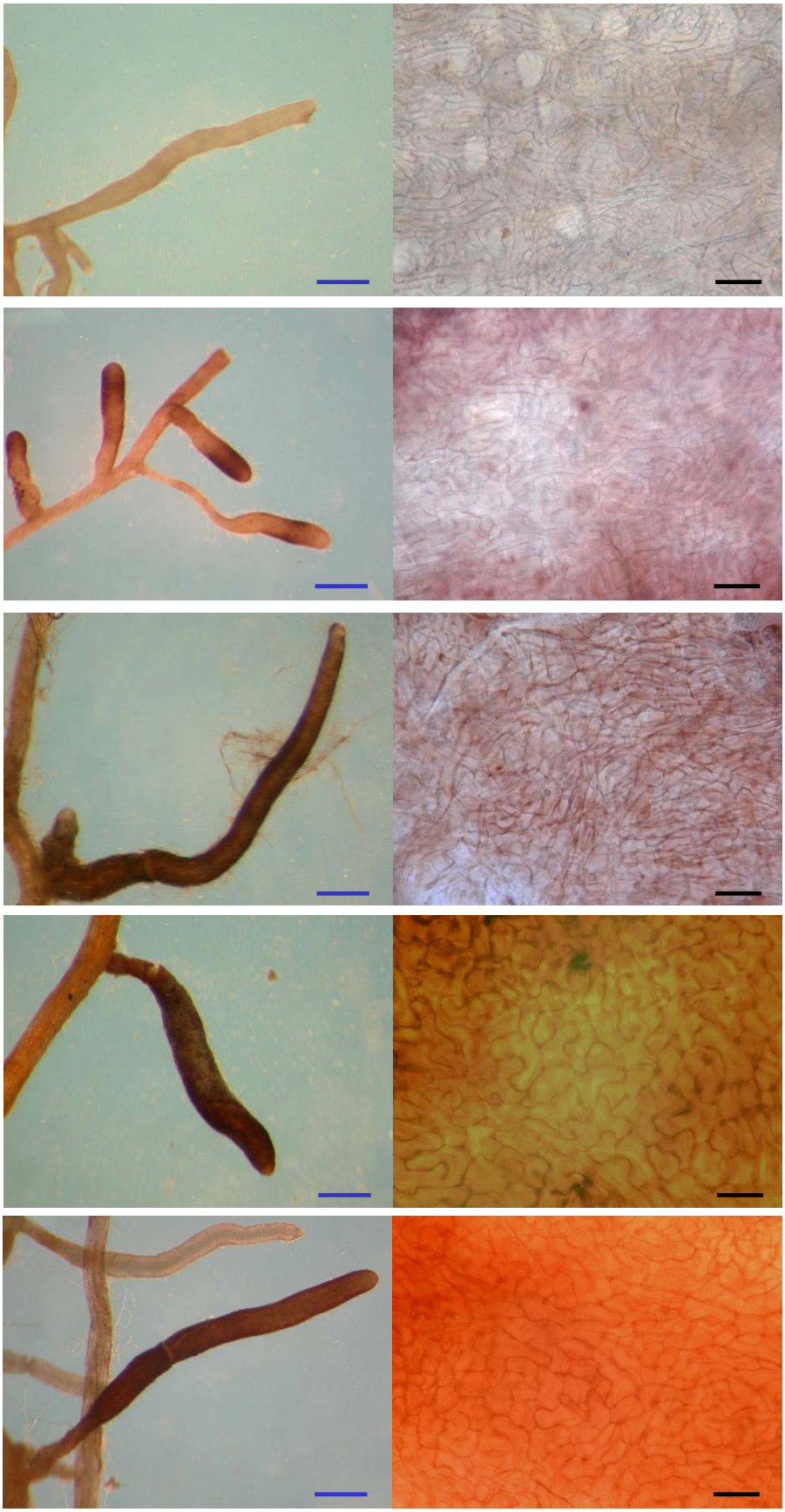


Fig. 1. Appearance (left photo) and structure of mantle surface (Right photo) of each ECM type observed in roots of willow and poplar seedlings at Mt Usu, Hokkaido, Japan. Blue and black bars indicate 0.5 mm and 10 μm, respectively.


Type 5
Type 6
Type 7
Type 8
Type 9

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