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Mycorrhizal Morphology and Symbiotic Fungi on the Root of *Pyrola renifolia*

Kei Kitamura [a], Yutaka Tamai [a*], Takashi Yajima [a] and Toshizumi Miyamoto [a]

[a] Graduate School of Agriculture, Hokkaido University, Sapporo Hokkaido, Japan.

Authors' contributions

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

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ABSTRACT

Introduction: *Pyrola renifolia* (*Ericaceae*) is a sciophyte that thrives in shaded forests. Although it is considered to be a mixotrophic plant, little is known about its root system and fungal associates.
Aims: This work aimed clarifying the root morphological feature and the symbiotic fungi.
Methodology: Subterranean parts of the pyroloid were collected from a Sakhalin fir (*Abies sachalinensis*) stand at Hokkaido, Japan, and analyzed the root system and mycorrhizal morphology and fungal symbionts with common methods.
Results: The sciophyte has a well-developed subterranean system with highly extended rhizomes and mycorrhizal short roots, which are categorized into three morphotypes. Typical arbutoid mycorrhizae with thick mantle structures were formed on the root tip. The main fungal ectomycorrhizal basidiomycete associates were identified as *Filobasidiella* spp., *Russula* spp., and *Tomentella* spp. A mantleless morphotype with Hartig net and the intracellular hyphal coil was mainly associated with root endophyte ascomycetes, viz. *Cladophiaphora* spp., *Didymella* spp., Helotiales spp., *Leptodontidium orchidicola*, and *Phialocephala fortinii*. Further, we observed ericoid-type mycorrhizae and dark septate endophyte colonization in the middle of the root.
Conclusion: Pyrolaceae have mycorrhizae that are similar to the ERM widely observed in Ericaceae, and also those similar to monotropoid mycorrhizae. Among Ericaceae, pyroloids

**Corresponding author: E-mail: ytamai@agr.hokudai.ac.jp;*

supplement any carbon source insufficiency from photosynthesis with the mycorrhizal fungal resources to adapt to the shaded growth environment, while monotropoids do not photosynthesize and completely rely on mycotrophy. P. renifolia is a mixotrophic plant that relies on both autotrophic photosynthesis and heterotrophic nutrition via mycorrhizal fungi.

Keywords: Sciophyte; mycorrhiza; arbutoid; ericoid; endophyte; rhizome; mixotroph; heterotrophy.

1. INTRODUCTION

Plants that grow in the shrouded forest floor environment include mixotrophs that rely on both autotrophy, through photosynthesis, and heterotrophy, mediated by mycorrhizal fungi. Mixotrophic plants have been discovered among *Orchidaceae* plants with green leaves [1] and *Pyrolaceae* (*Pyroloideae*), a perennial herb family [2,3]. There are reports that the light environment affects the plant dependence on mycorrhizal fungi [4].

The *Ericaceae* family includes autotrophic, heterotrophic, and mixotrophic plants with ericoid mycorrhiza (ERM), monotropoid mycorrhiza, and arbutoid mycorrhiza (ARM), which form mycorrhizal roots unique to *Ericaceae* plants. Ericoid mycorrhizal plants generally grow in areas of poor nutrition, such as the heathland, and alpine and sub-alpine zones. Here, mycorrhizal fungi invade extremely thin root epidermal cells (the hair roots) and form coiled hyphae in the inner root cells. Mycorrhizal fungi transport phosphoric acid, nitrogen, iron, and other nutrients from the soil to the host plant, and also protect the host plant from heavy metal stress [5]. Monotropoid mycorrhizal plants are achlorophyllous plants, such as *Monotropastrum humile* and *Monotropa hypopitys*, which do not photosynthesize and therefore depend on the mycorrhizal fungi for carbon sources. In this case, mycorrhizal fungi invade the epidermal cells of the root with peg-shaped hyphae, and form the mantle and Hartig net, which are the features of the ectomycorrhizal root. It is thought that mycorrhizal fungi form ectomycorrhiza with surrounding trees and transport carbon compounds obtained from the trees to monotropoid mycorrhizal plants [2,5]. Finally, ARM are found on *Arbutus*, *Arctostaphylos*, and *Pyrolaceae*, which are formed by mycorrhizal fungi that form ectomycorrhiza with trees. The *Pyrolaceae* family includes plants that receive carbon and nitrogen from mycorrhizal fungi, as well as mixotrophic species that also acquire carbon via photosynthesis [5,6]. In addition, the mycorrhizal form of *Pyrolaceae* is different depending on the host plant and mycorrhizal species, with some forming a thick mantle, although there are many reports of a mantle that is thin or missing [7,8].

One plant that grows in the shrouded environment of a forest floor is *Pyrola renifolia*. It is an evergreen perennial that is distributed in the subarctic region of Amur, north-eastern Honshu, Hokkaido, Minami Chishima, Sakhalin, and the Korean Peninsula [9]. It is common in Hokkaido, growing on the floor of *Abies sachalinensis* forests. Symbiosis with mycorrhizal fungi is thought to be important for this plant because it grows in a shaded environment; however, mycorrhiza formation has not been clarified. In the current study, we investigated the mycorrhizal morphology and symbiotic fungal flora of *P. renifolia* growing in an *A. sachalinensis* stand.

2. MATERIALS AND METHODS

2.1 Sample Collection

P. renifolia plants forming a colony were collected on October 18–19, 2013 in an *A. sachalinensis* stand at the Hiyama Research Forest of Hokkaido University. The material was collected with a soil block measuring 100 x 50 x 20 cm (length x width x depth). The sample was placed in a plastic bag and refrigerated at 4 °C until analysis. The ground portion of the sample was carefully flushed with tap water to avoid cutting a stem or a root. Then, the fungal symbionts were evaluated and identified as described below.

2.2 Microscopy

After the initial general examination of the whole underground portion, the root was studied using a stereomicroscope (SZX9; Olympus Corporation, Tokyo, Japan). Based on the morphological traits of mycorrhiza, type division was determined. All root tips were examined to obtain a count of each type. A compound microscope (BH2; Olympus Corporation) was used for a detailed evaluation, and the material

was processed, stained, and sectioned as described below, as needed. The preliminary analysis was based on the methods of Piercey et al. [10] and Fukuchi et al. [11].

First, the roots were cut into approximately 5 cm pieces. They were then soaked in a 10% KOH aqueous solution for decolorization, incubated at 80°C for 5 min, washed with water, dipped in 2.5% H_2O_2 for 5 min, and treated for a period of time before washing again with water. Next, the pieces were placed in 1% HCl, incubated at room temperature for 5 min, and immersed in 0.1% chlorazol black E at 80°C for 2 h. Then, the root material was dipped in 50% glycerol to remove excess dyeing solution.

Thin sections were prepared after tissue fixation, polyethylene glycol (PEG) embedding, and sectioning. Briefly, roots with different appearances were first examined under a stereomicroscope to select samples for analysis. The material was then immersed in a fixative (2.5% glutaraldehyde, 0.10 mol/l HEPES buffer, pH 6.8), overnight, at 4 °C. The samples were embedded, and thin sections were prepared following the method of Ogata and Slepecky [12]. First, 1.5% agarose was heated in a microwave oven to completely dissolve it and prevent it from solidifying. The root material was then placed in the dissolved agarose, cooled, and allowed to solidify, with fine adjustments under the stereomicroscope. After the agarose has completely solidified, excess agarose was removed using a razor, and the remainder was shaped into a block (a cube with a side length of approximately 3–5 mm). Next, an appropriate amount of PEG 4000 powder was placed in glass sample bottles and dissolved at 60 °C. Pure water (three times the volume of PEG 4000, the same volume as the PEG 4000, or one-third the volume of PEG 4000) was added to the solutions, which were dissolved again by warming, resulting in solutions of 25%, 50%, and 75% PEG, accordingly. After dissolution, the 25% and 50% PEG solutions were maintained at room temperature; 75% PEG was maintained at 40°C; and 100% PEG solution was maintained at 60°C. The agarose block was placed in a 1.5 ml microtube together with 25% PEG and shaken at room temperature for 2 h. After substitution with 25% PEG, the block was transferred to a 1.5 ml tube containing 50% PEG and shaken at room temperature for 2 h, which was followed by 75% PEG and 100% PEG substitutions. Because the two most concentrated solutions had to be kept warm, the appropriate samples were placed in a heating block and stirred manually several times. Replacement with 100% PEG was done twice. After the final replacement, the block was removed with a metal spatula preheated with a lighter. Excess PEG was wiped off before rapid cooling of the sample on an ice pack. Next, excess PEG was scraped off from the cooled and solidified block using a razor blade, so that the root material was on top. A wood block measuring 2 x 2 x 1 cm (length x width x thickness) was used as a base, and the sample block was fixed onto it with an instantaneous adhesive. Using a stainless-steel microtome blade, 10–20 μm-thick sections were prepared.

2.3 Molecular Analysis

The relationship between symbiotic fungal species and mycorrhizal morphology was elucidated by typing and by species determination by DNA analysis. Three samples of each mycorrhizal type were collected. When less than three types of samples were available, DNA was analyzed from one or two samples. Each sample type was cryopreserved at −20 °C until DNA extraction.

DNA was extracted using ISOPLANT II (Nippon Gene Co., Ltd., Tokyo, Japan), according to the manufacturer's instructions. The DNA solution was cryopreserved at −20 °C until polymerase chain reaction (PCR).

PCR was carried out using a LaboPass SP-Taq polymerase kit (Cosmo Genetech Co., Ltd., Seoul, Korea). Each reaction tube contained 16.5 μl master mix, 8 μl template DNA, 2 μl ITS 1F primer [13], 2 μl ITS 4 primer [14], and 21.5 μl sterile distilled water. Each tube was placed in a thermal cycler (2720 Thermal Cycler; Applied Biosystems, Foster City, CA, USA). PCR amplification involved initial denaturation at 95 °C for 2 min; followed by 40 cycles of denaturation at 95 °C for 20 s, primer annealing at 50 °C for 40 s, extension at 72°C for 1 min; and a final extension cycle at 72°C for 8 min.

The PCR products were checked by agarose electrophoresis and purified using a FastGene gel/PCR extraction kit (Nippon Genetics Co., Ltd.). When multiple products were obtained after an amplification reaction, the bands were cut from the gel, and DNA was purified from each band as described above. The purified PCR products were sequenced using forward and reverse primers, and a 3730xl DNA sequencer (Applied Biosystems) at Hokkaido System

Science Co., Ltd. The obtained sequences were then analyzed using the BLAST search algorithm against closely similar sequences retrieved from the DNA Data Bank of Japan (DDBJ).

3. RESULTS

The rhizomes developed length-wise in a horizontal direction, making it impossible to dig up all the underground parts of an individual plant. The roots branched from the developed rhizomes every several cm, and branched several times, with a length of approximately 5–10 cm. In the subsurface portion of the rhizome, the root occupancy was low. Our examination revealed that some of the aboveground community-forming portions belonged to the same individuals connected by rhizomes (Fig. 1A, B).

Close inspection revealed the presence of various differently shaped hyphae on the root, which precluded detailed type classification by observation only. Therefore, we roughly classified the mycorrhiza into the following three types: mantle-forming (type 1); mantleless, which looked like framed epidermal cells (type 2); and mantleless and non-framed epidermal cell type (type 3). Type 1 mycorrhizae were further roughly divided by mantle color, into ones forming an ochre mantle (type 1-Y) and ones forming a black mantle (type 1-B) (Fig. 1C, D). All types were apparent on every part of the root except for the root tip, at which only types 1 and 2 were present.

In type 1 mycorrhiza, which formed a mantle, the Hartig net and intracellular mycelium were present, with a clamp connection on the hyphae. The mantle color was ochre or black, or reddish-brown (Fig. 2). In type 2 mycorrhiza, the external mantle was missing and the epidermal cells appeared to be framed, with only the Hartig net and intracellular hyphae apparent. Stereomicroscopic analysis of the root confirmed that the developed Hartig net framed the epidermal cells (Fig. 3). Type 3 mycorrhiza, which did not form a mantle and where the epidermal cells were not framed, formed along the entire root except for the root tip.

Fig. 1. Morphological overview. A: Root and rhizome structure. B: Detailed structure of the root (a) and rhizome (b). C: Type 1-Y mycorrhiza with a yellow mantle. D: Type 1-B mycorrhiza with a black mantle

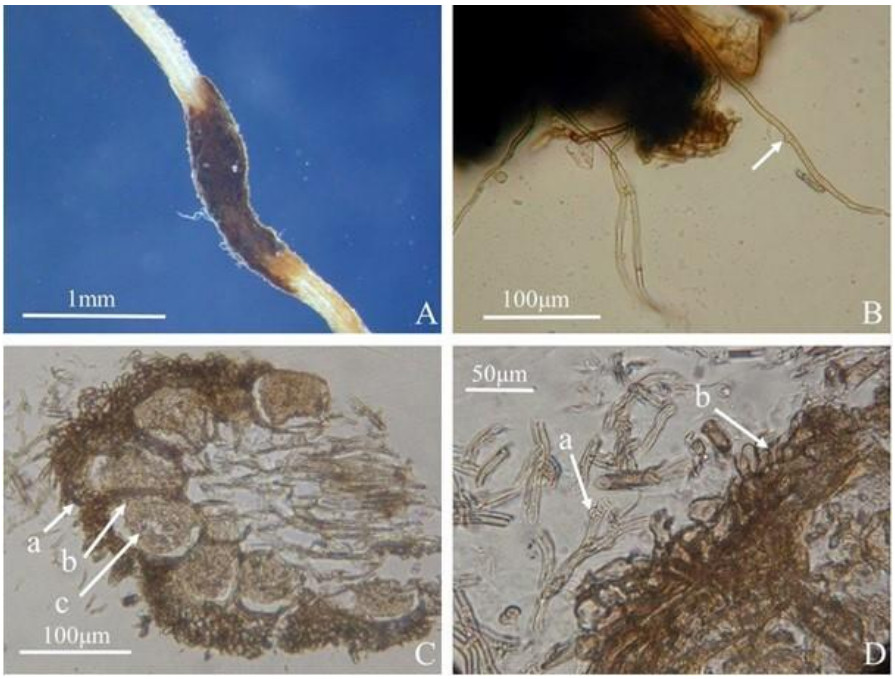

Fig. 2. Structure of mycorrhiza with a mantle (type 1). A: Mycorrhiza with a black mantle. B: External hypha with a clamp connection (arrow). C: Transverse section, with a thick mantle (a), a Hartig net (b), and internal hyphae (c) visible. D: Transverse section, with a clamp connection (a)and thick mantle (b)visible

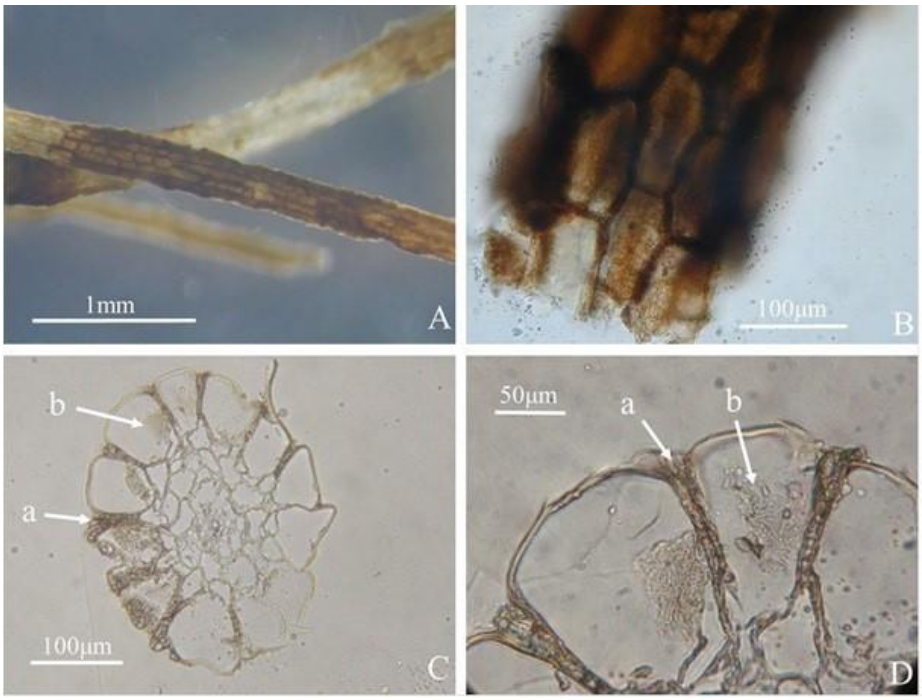

Fig. 3. Structure of mycorrhiza without a mantle (type 2). A: Mycorrhiza without a mantle. B: Epidermal cells framed with a brown Hartig net. C :Transverse section, with the Hartig net (a) and internal hyphae in the epidermal cells (b)visible. D :Transverse section detail showing (a)the Hartig net and (b)internal hyphae in the epidermal cells

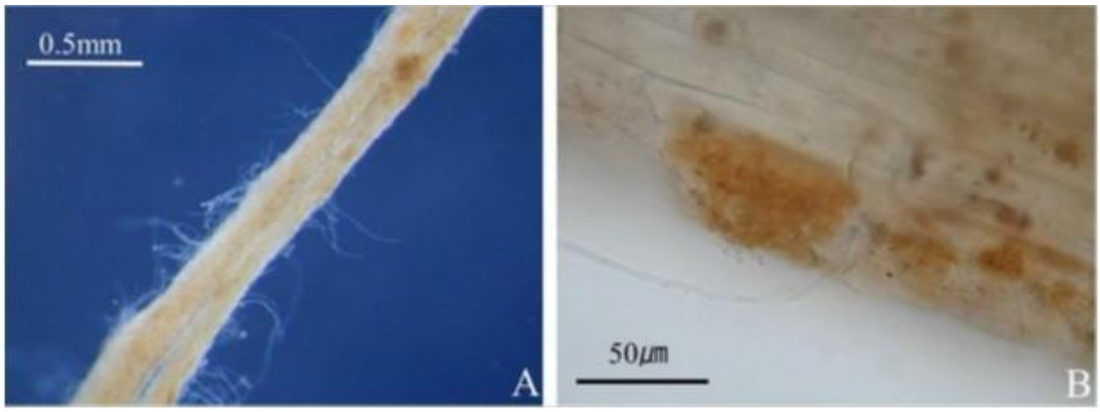

Fig. 4. Structure of mycorrhiza without a mantle and Hartig net (type 3). A: Mycorrhiza with external hyphae. B: External hyphae and internal hyphae in the epidermal cells

Table 1. Mycorrhizal type of root tips of *P. renifolia*

Type	Number (%)
Type 1: Arbutoid mycorrhiza with mantle	27 (10.4)
Yellow	17
Black	9
Red	1
Type 2: Arbutoid mycorrhiza without mantle	233 (89.6)
Total number of samples	**260**

Further, the root was white, with white external hyphae (Fig. 4A). Using a compound microscope, we confirmed the presence of dark septate endophyte (DSE)-like hyphae and intracellular hyphae, as well as the lack of the Hartig net. We observed DSE-like hyphae mainly in type 3 mycorrhizae, irrespective of the root fragment (Fig. 4B). They were also present in the fragments forming the mycorrhizal root types 1 and 2.

We counted the root tips for each mycorrhizal root type. Among all the counted roots (260), 233 were type 2, and 27 were type 1. Within type 1, 17 roots were ochre-coloured, nine were black, and one was reddish-brown (Table 1).

The DNA extraction and PCR amplification were successful for three samples each of type 1-Y, type 1-B, and type 2, collected at the root tip. For locations other than the root tip, we successfully analyzed one type 1-Y sample, three type 1-B samples, three type 2 samples, and two type 3 samples. For the root tip samples, we obtained a single PCR product from three type 1-Y samples, and two each of type 1-B and type 2 samples. For material sampled from the root at locations other than the root tip, we obtained a single PCR product from one type 1-B and two type 3 samples. All type 1-Y root tip and type 3 non-root tip samples yielded a single PCR product. All non-root tip type 1-Y and type 2 samples yielded multiple PCR products. Overall, we obtained multiple PCR products from almost half of the samples.

The molecular analysis revealed that *Russula* spp. formed a type 1-Y ochre mantle. For the other types, it was assumed that fungal species of each genus of each type were formed. In type 1-B mycorrhizae, forming a black mantle, we detected *Filobasidiella* spp. and *Tomentella* spp. In non-root tip type 1-B samples, we identified *Didymella* spp. and *Phialocephala fortinii*. In type 2 mycorrhizae, which do not form a mantle, we commonly observed *Didymella* spp., except for the root tip, where we identified *Agrocybe erebia*, *Cladophialophora* spp., *Filobasidiella* spp., *Helotiales* spp., *Leptodontidium orchidicola*, and *P. fortinii*. In type 3 mycorrhizae, we detected *Didymella* spp. and *P. fortinii* (Table 2). Among the 25 fungal symbionts, 12 were basidiomycetes (*A. erebia*, *Filobasidiella* spp., *Russula* spp., and *Tomentella* spp.) and 13 were ascomycetes (*Cladophialophora* spp., *Didymella* spp., *Helotiales* spp., *L. orchidicola*, and *P. fortinii*) (Table 2).

Table 2. Mycorrhizal fungi associated with *Pyrola renifolia*

Position in the root	Type	Putative species	Taxon	Putative ecology	Accession number	Score	e-value	Similarity	Closest match
Apical	1-Y	*Russula* sp. 1	B ● ◎	ECM	AB976609	953	0.0	97%	Russulaceae sp. 2 HE814089.1
		Russula sp. 2	B ● ◎	ECM	AB976610	335	9e–88	93%	Uncultured *Russula* sp. AB546563.1
		Russula sp. 3	B ● ◎	ECM	AB976612	998	0.0	97%	Russulaceae sp. 2 HE814089.1
	1-B	Ascomycetes sp. 1	A	UNK	AB976616	553	4e–153	90%	Uncultured fungus KC966062.1
		Filobasidiella sp. 1	B	UNK	AB976614	686	0.0	100%	*Cryptococcus* sp. FJ873462.1
		Filobasidiella sp. 2	B	UNK	AB976615	645	0.0	89%	*Cryptococcus* sp. MK186933.1
		Tomentella sp. 1	B ● ◎	ECM	AB976613	804	0.0	100%	Uncultured *Tomentella* LC175705.1
	2	*Cadophora orchidicola*	A ○	DSE	AB976619	1031	0.0	98%	*Cadophora orchidicola* MT294412.1
		Didymella sp. 1	A ○	END	AB976618	798	0.0	100%	*Didymella segeticola* strain YBZ221 MT530451.1
		Helotiales sp.	A ●○◎	ECM/END	AB976617	911	0.0	100%	Helotiales sp. 7-NN-2017 LC218322.1
		Phialocephala fortinii	A ●○	DSE	AB976620	907	0.0	99%	*Phialocephala fortinii* MT028045.1
Basal and intermediate	1-Y	*Filobasidiella* sp. 3	B	UNK	AB976622	708	0.0	94%	*Cryptococcus* sp. MK186933.1
		Russula sp. 4	B ● ◎	ECM	AB976621	883	0.0	94%	*Rusulla* sp. LM3944 KM576559.1
	1-B	*Didymella* sp. 2	A ○	END	AB976626	544	2e–150	92%	*Didymella segeticola* strain YBZ221 MT530451.1
		Filobasidiella sp. 4	B	UNK	AB976625	785	0.0	95%	Uncultured Basidiomycota GU328623.1
		Phialocephala fortinii	A ●○	DSE	AB976623	863	0.0	100%	*Phialocephala fortinii* KU516545.1
		Tomentella sp. 2	B ● ◎	ECM	AB976624	1011	0.0	98%	Uncultured *Tomentella* KC702624.1
	2	*Agrocybe erebia*	B ◎	ECM	AB976630	1122	0.0	99%	*Agrocybe erebia* KR673706.1
		Ascomycetes sp. 2	A	UNK	AB976632	525	9e–145	88%	Ascomycota sp. 2KM-2017 MG018069.1
		Cladophialophora sp.	A ○	DSE	AB976628	612	6e–171	96%	*Cladophialophora* sp. 2-NN-2017 LC229675.1
		Didymella sp. 1	A ○	END	AB976627	830	0.0	100%	*Didymella segeticola* strain YBZ221 MT530451.1
		Didymella sp. 3	A ○	END	AB976629	436	2e–118	100%	*Didymella segeticola* strain YBZ221 MT530451.1
		Filobasidiella sp. 5	B	UNK	AB976631	983	0.0	100%	*Cryptococcus* sp.MK045385.1
	3	*Didymella* sp. 1	A ○	END	AB976634	894	0.0	100%	*Didymella segeticola* strain YBZ221 MT530451.1
		Phialocephala fortinii	A ●○	DSE	AB976633	981	0.0	99%	*Phialocephala fortinii* MT028045.1

B Basidiomycetes, A Ascomycetes, ● taxon already found on other pyroloids, ○ taxon already found on ERM plants, ◎taxon already found on ECM plants, ECM ectomycorrhiza, END endophyte, DSE dark septate endophyte, UNK unknown

4. DISCUSSION

In the current study, we analyzed the root system and fungal associates of *P. renifolia*. We described three types of mycorrhiza, which underscore the mixotrophic lifestyle of this plant.

In the current study, most aboveground parts of the plant community were connected by rhizomes and, therefore, they may belong to the same individual. The *Pyrolaceae* seeds require symbiosis with fungi in order to germinate, and the germination rate is very low [15,16]. It is assumed that *P. renifolia* forms a community mainly by vegetative propagation. In addition, because the roots are not well developed and no root hairs are apparent, *P. renifolia* might rely heavily on mycorrhizal fungi for nutrient absorption.

Based on our analysis, type 1-Y mycorrhiza, which forms a yellow mantle, is formed by *Russula* spp. (*Russulaceae*), as has been already reported for other *Pyrolaceae* [8,15,17]. The fungus also forms ectomycorrhiza (ECM) with fir trees [18]. *Tomentella* spp. form the black mantle in type 1-B mycorrhiza. *Helotiales* spp., involved in type 2 mycorrhiza, have also been reported for other *Pyrolaceae* and form an ECM with trees [2,8,15]. *A. erebia*, detected in type 2 mycorrhiza, reportedly forms ECM with *Pinaceae* trees [19]. The mycorrhizae morphology greatly differs depending on the symbiotic mycorrhizal fungal species, some of which form ECM with trees but do not form a mantle in *P. renifolia*. *Pyrolaceae* are thought to obtain carbon sources from trees via mycorrhizal fungi [2]. Similarly, *P. renifolia* obtains carbon sources from *A. sachalinensis* via these mycorrhizal fungi and relies on photosynthesis to supplement any carbon deficits, thus adapting to the shaded environment of the forest floor. The mycorrhiza type forming a mantle was similar to the monotropoid mycorrhizal root formed in *Monotropastrum humile*, which forms the mantle, Hartig net, and intracellular hyphae. Symbiotic mycorrhizal species are known to form an ECM with trees.

Further, *P. fortinii*, detected in type 1-B, is a DSE but also forms an ECM. It might form a mantle on *P. renifolia*. *Didymella* spp. cause browning lesions, mainly on the plant leaf and stem, although endophytes have also been reported among these fungi [20]. We detected more symbionts in type 2 mycorrhiza than in the other types, many of which are DSE or endophytes (Table 2).

In type 1 mycorrhiza, which formed a mantle, we often detected only one fungal species, especially at the root tip. This type possesses ECM-like morphological traits and may be exclusive to type 1. Further, in a high proportion of type 2 mycorrhiza samples (which lack the mantle), we observed hyphae of different morphologies at the same site and identified numerous fungi. This indicates that multiple fungi coexist at the same location in this mycorrhiza type.

In type 3 mycorrhiza, we detected *Didymella* spp. and *P. fortinii*. The known characteristics of these two fungi are consistent with those noted by us during microscopic evaluation. In addition, only a single PCR band was amplified from the relevant root material, and it appears that *Didymella* spp. and *P. fortinii* form the mycorrhizal root in type 3 samples. DSE may form white external hyphae [20,21]. White hyphae observed in the current study may therefore be those of DSE. These two fungi have been previously found in ericoid mycorrhizal plants [5,22]. Further, the coiled hyphae and similarity to ERM (no mantle or Hartig net) suggest that type 3 mycorrhiza is a mycorrhizal root-like ERM.

Among the species reported in the current study, *Cladophialophora* spp., *Didymella* spp., *L. orchidicola*, and *P. fortinii* have been found in ERM plants [5,22]. It is generally thought that *Pyrolaceae* species form ARM roots and are associated with ectomycorrhizal fungi of trees [5]. Other than the ECM fungal species, the species identified in the current study were consistent with those reported for ERM-forming plants (Table 2). Based on these presumed mycorrhizal species and our observations, type 1 mycorrhiza is an ARM with a mantle, type 2 mycorrhiza is an ARM without a mantle, and type 3 mycorrhiza is an ERM. Further, the morphological features of type 2 mycorrhiza seem to be intermediate to those of type 1 and type 3 mycorrhizae. Finally, the fungal species denoted herein as *Ascomycetes* spp. were identified based on a comparable homology with several genera, as determined by the BLAST search, which suggests database or classification confusion.

5. CONCLUSION

Pyrolaceae have mycorrhizae that are similar to the ERM widely observed in *Ericaceae*, and also those similar to monotropoid mycorrhizae found

in the monotropoid mycoheterotrophic plants. Among *Ericaceae*, pyroloids supplement any carbon source insufficiency from photosynthesis with the mycorrhizal fungal resources to adapt to the shaded growth environment, while monotropoids do not photosynthesize and completely rely on mycotrophy. *P. renifolia* is a mixotrophic plant that relies on both autotrophic photosynthesis and heterotrophic nutrition via mycorrhizal fungi.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

REFERENCES

1. Bidartondo MI. The evolutionary ecology of myco-heterotrophy. New Phytologist. 2005; 167:335-352.
DOI:https://doi.org/10.1111/j.1469-8137.2005.01429.x
2. Tedersoo L, Pellet P, Kõljalg U, Selosse MA. Parallel evolutionary paths to mycoheterotrophy in understorey Ericaceae and Orchidaceae: Ecological evidence for mixotrophy in Pyroleae. Oecologia. 2007;151: 206-217.
DOI: 10.1007/s00442-006-0581-2
3. Zimmer K, Hynson NA, Gebauer G, Allen EB, Allen MF, Read DJ. Wide geographical and ecological distribution of nitrogen and carbon gains from fungi in pyroloids and monotropoids (Ericaceae) and in orchids. New Phytologist. 2007;175:166-175.
DOI:https://doi.org/10.1111/j.1469-8137.2007.02065.x
4. Matsuda Y, Shimizu S, Mori M, Ito SI, Selosse MA. Seasonal and environmental changes of mycorrhizal associations and heterotrophy levels in mixotrophic *Pyrola japonica* (Ericaceae) growing under different light environments. American Journal of Botany. 2012;99:1177-1188.
Available:https://www.jstor.org/stable/41548341
5. Smith SE, Read DJ. Mycorrhizal symbiosis. 3rd Edition. Amsterdam: Elsevier; 2008.
6. Hynson NA, Bruns TD. Evidence of a myco-heterotroph in the plant family Ericaceae that lacks mycorrhizal specificity. 2009; Proceedings of the Royal Society B, Biological Sciences. 276: 4053-4059.
DOI:https://doi.org/10.1098/rspb.2009.1190
7. Massicotte HB, Melville LH, Tackaberry LE, Peterson RL. A comparative study of mycorrhizas in several genera of Pyroleae (Ericaceae) from western Canada. Botany. 2008;86:610-622.
DOI: https://doi.org/10.1139/B08-027
8. Vincenot L, Tedersoo L, Richard F, Horcine H, Kõljalg U, Selosse MA. Fungal associates of *Pyrola rotundifolia*, a mixotrophic Ericaceae, from two Estonian boreal forests. Mycorrhiza. 2008;19:15-25.
DOI 10.1007/s00572-008-0199-9
9. Yonekura K, Murata J. Syllabus of the vascular plants of Japan arranged in the order of phylogeny-based system. Tokyo: Hokuryukan. Japanese; 2013.
10. Piercey SJ, Murphy DC, Mortensen JK, Paradis S, Creaser RA. Geochemistry and tectonic significance of alkalic mafic magmatism in the Yukon-Tanana Terrane, Finlayson Lake Region, Yukon. Canadian Journal of Earth Science. 2002;39:1729-1744.
DOI: https://doi.org/10.1139/e02-090
11. Fukuchi S, Obase K, Tamai Y, Yajima T, Miyamoto T. Vegetation and colonization status of mycorrhizal and endophytic fungi in plant species on acidic barren at crater basin of Volcano Esan in Hokkaido, Japan. Eurasian Journal of Forest Research. 2011;14:1-11.
Available: http://hdl.handle.net/2115/47114
12. Ogata Y, Slepecky NB. Immunohistochemical labeling of inner ear tissues embedded in polyethylene glycol 4000 –Comparative study with araldite and unicryl embedded sections. Nippon Jibiinkoka Gakkai Kaiho 1996;99:361-369. Japanese
DOI:https://doi.org/10.3950/jibiinkoka.99.361
13. Gardes M, Bruns T. ITS primers with enhanced specificity for basidiomycetes: application to the identification of mycorrhizae and rusts. Molecular Ecology. 1993;2:113-118.
DOI:https://doi.org/10.1111/j.1365-294X.1993.tb00005.x
14. White TJ, Bruns T, Lee S, Taylor J. Amplification and direct sequencing of fungal ribosomal RNA genes for

phylogenetics. In: Innis MA, Gelfand DH, Sninsky JJ, White TJ, Eds., PCR Protocols: a guide to methods and applications. San Diego: Academic Press. 1990;315-322.

15. Hashimoto Y, Fukukawa S, Kunishi A, Suga H, Richard F, Sauve M, Selosse MA. Mycoheterotrophic germination of *Pyrola asarifolia* dust seeds reveals convergences with germination in orchids. New Phytologist. 2012;195:620-630. DOI:https://doi.org/10.1111/j.1469-8137.2012.04174.x
16. Johansson VA, Eriksson O. Recruitment limitation, germination of dust seeds, and early development of underground seedlings in six Pyroleae species. Botany. 2013;91:17-24. DOI: https://doi.org/10.1139/cjb-2012-0153
17. Toftegaard T, Iason GR, Alexander IJ, Rosendahl S, Taylor AFS. The threatened plant intermediate wintergreen (*Pyrola media*) associates with a wide range of biotrophic fungi in native Scottish pine woods. Biodiversity Conservation. 2010; 19:3963-3971. DOI 10.1007/s10531-010-9940-8
18. Matsuda Y, Hijii N. Ectomycorrhizal fungal communities in an *Abies firma* forest, with special reference to ectomycorrhizal associations between seedlings and mature trees. Canadian Journal of Botany. 2004;82:822-829. DOI: https://doi.org/10.1139/b04-065
19. Yamada A, Katsuya K. Mycorrhizal association of isolates from sporocarps and ectomycorrhizas with *Pinus densiflora* seedlings. Mycoscience. 1995;36:315–323. DOI: https://doi.org/10.1007/BF02268607
20. Sun X, Ding Q, Hyde KD, Guo LD. Community structure and preference of endophytic fungi of three woody plants in a mixed forest. Fungal Ecology. 2012;5:624-632. DOI: https://doi.org/10.1016/j.funeco.2012.04.001
21. Barrow JR. Atypical morphology of dark septate fungal root endophytes of *Bouteloua* in arid southwestern USA rangelands. Mycorrhiza. 2003;13:239-47. DOI 10.1007/s00572-003-0222-0
22. Vanó I, Sakamoto K, Inubushi K. Phylogenetic relationships among non-pathogenic isolates of dark septate endophytes from Ericaceae plants. Hort Research. 2011;65:41-47. Available:https://opac.ll.chiba-u.jp/da/curator/900116132/6515hortresearch.pdf

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