Taiwania 62(4): 399-406, 2017
DOI: 10.6165/tai.2017.62.399

Mycorrhizal fungi associated with Taiwanese *Pyrola morrisonensis* (Ericaceae) in a naturally regenerated forest

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(Manuscript received 16 June 2017; accepted 6 November 2017; online published 17 November 2017)

ABSTRACT: *Pyrola morrisonensis*, an evergreen herb in the family Ericaceae, is endemic to Taiwan. We examined mycorrhizal development and the associated fungi in this species. Nine plants were collected in a naturally regenerated forest in central Taiwan. The plants were genetically identical in their internal transcribed spacer (ITS) region, and their sequences matched the known sequence for *P. morrisonensis*. Fine roots of each plant were colonized by mycorrhizal fungi that formed mycorrhizas either with or without fungal mantles. DNA sequences of the ITS region of these fungi suggested that they belonged to mycorrhizal taxa that are common tree symbionts. Among them, members of Thelephoraceae were the dominant taxon in the host plants. These results indicate that *P. morrisonensis* is intimately associated with mycorrhizal fungi that might also connect with neighboring trees.

KEY WORDS: ITS, Molecular identification, Operational taxonomic unit, *Pyrola morrisonensis*, root morphology, Thelephoraceae.

INTRODUCTION

The genus *Pyrola* L. (Ericaceae) has two sections, *Pyrola* and *Ampliosepala*, each of which comprises three series; the former includes *Pyrola*, *Rugosae*, and *Ellipticae*, and the latter comprises *Japonicae*, *Scotophyllae*, and *Chloranthae* (Liu *et al.*, 2014). These plants are widely distributed on forest floors in various climatic regions, but mostly in the northern hemisphere's warm to cool temperate regions (Liu *et al.*, 2010, 2014). In addition, they are known to associate with mycorrhizal fungi to form arbutoid mycorrhizas (Peterson *et al.*, 2004; Smith and Read, 2008; van der Heijden *et al.*, 2015). The mycorrhizas form fungal mantles around fine roots as well as hyphal coils in their epidermal cells (Robertson and Robertson, 1985). However, recent studies indicated that the fungal mantles are rarely found in host species such as *P. chlorantha* (Massicotte *et al.*, 2008) and *P. japonica* (Matsuda *et al.*, 2012).

The advent of modern molecular genetics techniques has allowed researchers to identify the mycorrhizal fungi associated with various *Pyrola* species (Hashimoto *et al.*, 2012; Hynson and Bruns, 2009; Matsuda *et al.*, 2012; Tedersoo *et al.*, 2007; Toftegaard *et al.*, 2010; Vincenot *et al.*, 2008). These studies suggested that the arbutoid mycorrhizal fungi are mostly members of the Basidiomycota and Ascomycota and are ecologically identical to the groups of ectomycorrhizal fungi associated with woody plants in families such as the Fagaceae and Pinaceae.

These results suggest that *Pyrola* plants may connect with surrounding trees via common mycorrhizal networks (Selosse *et al.*, 2006, 2016; Simard *et al.*, 2012). In fact, some *Pyrola* species can be mixotrophic plants that obtain some of their carbon from photosynthates produced by neighboring woody plants via common mycorrhizal networks (Hynson *et al.*, 2009; Lallemand *et al.*, 2017; Matsuda *et al.*, 2012; Tedersoo *et al.*, 2007; Zimmer *et al.*, 2007). Since the Ericaceae includes species with various levels of fungal dependencies for nutrition, ranging from independent (autotrophy) through intermediate (mixotrophy) to completely dependent (mycoheterotrophy), the phylogenetic position of *Pyrola* species as well as their associated fungi are intriguing in terms of their implications for coevolutionary processes (Lallemand *et al.*, 2016).

In this framework, mycorrhizal associations have been investigated in *Pyrola* species, but one series *Rugosae* in section *Pyrola*, which is a sister taxa with series *Pyrola* (Liu *et al.*, 2014), has not yet been examined. Taiwanese *P. morrisonensis*, which belongs to the *Rugosae*, is an endemic species that was first found on Taiwan's Mount Morrison, now called Yu Mountain (Hayata, 1908). Because of its indigenous distribution, there have been few chemical and morphological studies of the species (Huang *et al.*, 1987; Takahashi, 1987), so little information is available on its autecology, including its mycorrhizal associations. Moreover, all mycorrhizal studies on *Pyrola* species have been conducted in cool temperate regions at higher latitudes than in Taiwan.

The purpose of this study was to characterize the morphological traits of *P. morrisonensis* mycorrhizas and to identify the mycorrhizal fungi associated with

the host plant. To accomplish this, we collected plants and examined their roots by microscopy; in addition, we sequenced fungal DNA derived from the fine roots of *P. morrisonensis*. To the best of our knowledge, this is the first study to clarify mycorrhizal associations in genus *Pyrola* growing in its southernmost habitat.

MATERIALS AND METHODS

Study site

We collected *P. morrisonensis* in a naturally regenerated forest in the Hehuan Mountain area of Taiwan (Figs. 1 A, B; 24°11'N, 121°22'E, 2535 m a.s.l.). The forest consisted of ectomycorrhizal *Quercus* spp. and *Tsuga sinensis* var. *formosana* trees, with ericaceous plants (*Rhododendron rubropilosum* and *Gaultheria itoana*), and dwarf bamboo (*Yushania niitakayamensis*) on the forest floor. Although some taxonomic and ecological studies on macrofungi were conducted (e.g. Tschen *et al.* 2004; Chou and Wang, 2005), no belowground studies on mycorrhizas has not done yet in this area. The study site lies in Taiwan's sub-tropical climatic zone, but at a sufficient elevation to have relatively cool temperatures (an annual average of 12.9°C in 2016 collected by the Meifeng weather station at elevation 2165 m).

Sampling of plants

We established three 10 m × 10 m plots within 100 m of each other. In each plot, we excised three plants and the surrounding soil (10 cm × 10 cm × 10 cm soil blocks). The samples were stored in a cooler until they could be brought back to the lab, at Taiwan's High Altitude Experimental Station of the Endemic Species Research Institute. On the same day, the plants were removed from the soil blocks by gentle rinsing with tap water and the rhizomes were placed in CTAB buffer solution (2% CTAB, 0.1 M Tris-HCl [pH 9.0], 1.4 M NaCl, 20 mM EDTA). They were then exported to Japan with permission from the Ministry of Agriculture, Forestry and Fisheries of Japan (26N-175). The root systems were examined for mycorrhization under a stereomicroscope at a maximum of 115× magnification (SZX16, Olympus, Tokyo, Japan). When hyphal coils were recognized in the epidermal cells or when mycelial mantles covered the root surface, the fungi were considered to be mycorrhizal. We removed four to eight samples of mycorrhizal root tips or 25-mm mycorrhizal segments and placed them in separate 1.5-mL centrifuge tubes for DNA extraction.

DNA analysis

Mycorrhizal samples were used for direct sequencing, with a focus on the fungal barcode site of the internal transcribed spacer (ITS) region, following the methods of Uesugi *et al.* (2016). In summary, 50 μL of Buffer A (0.1 M Tris [pH 9.5], 0.01 M EDTA [pH 8.0], 1 M KCl) was added to each centrifuge tube, and the root samples were then crushed with sterilized pestles and boiled at 95°C for 10 min. They were then centrifuged at 14 000 rpm for 1 min, and the supernatant was used as the PCR template.

For the PCR amplification, we used the KOD FX DNA polymerase kit (Toyobo, Osaka, Japan), following the manufacturer's recommendations. We used the primer pairs ITS1F (Gardes and Bruns, 1993) or ITSOF (Tedersoo *et al.* 2008) and TW13 (Taylor and Bruns, 1999). PCR conditions were an initial denaturation at 94°C for 3 min, followed by 35 cycles of 98°C for 10 sec, 52°C for 30 sec, and 68°C for 70 sec, with no final extension. Positive PCR products were sequenced using one of the abovementioned primers using the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) performed on the ABI 3130 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). When PCR products were difficult to sequence directly, they were cloned using TOPO TA Kits (Invitrogen, Carlsbad, CA, USA). Two to five clones were randomly chosen for each root segment, and DNA inserts were applied for sequencing as the above.

To confirm the identity of the plant samples, we extracted DNA from 5 mm × 5 mm dried leaf samples using the Qiagen DNeasy Plant Mini Kit (Qiagen, Tokyo, Japan). Since the ITS region has been used for phylogenetic analysis of *Pyrola* species (Freudenstein, 1999; Liu *et al.*, 2010), we conducted PCR amplification with a focus on the ITS region using the TaKaRa Ex Taq Kit (Takara, Otsu, Japan) with the primer pair ITS1/ITS4 (White *et al.*, 1990). Thermal cycles were an initial denaturation at 95°C for 3 min, followed by 30 cycles of 95°C for 30 sec, 52°C for 30 sec, and 72°C for 2 min, with final extension at 72°C for 10 min. The rest of the analysis followed the same processes described above for the mycorrhizal analyses.

Data analysis

Sequences obtained from the mycorrhizal and leaf samples were manually adjusted using MEGA ver. 6.06 (http://www.megasoftware.net/; Tamura *et al.*, 2013) and representative ones were submitted to the DNA Databank of Japan under accession numbers LC273388 to LC273426. After excluding chimera sequences inferred by UCHIME (Edgar 2010), the mycorrhizal samples more than 350 bp in length were divided into molecular operational taxonomic units (MOTUs) based on a threshold of 97% sequence similarity using version 1.36 of the Mothur software (https://www.mothur.org/; Schloss *et al.*, 2009). The sequence of representative MOTUs were subjected to a BLAST search to infer their taxonomic identities (Altschul *et al.*, 1997). The relative frequency of MOTUs in each plant was

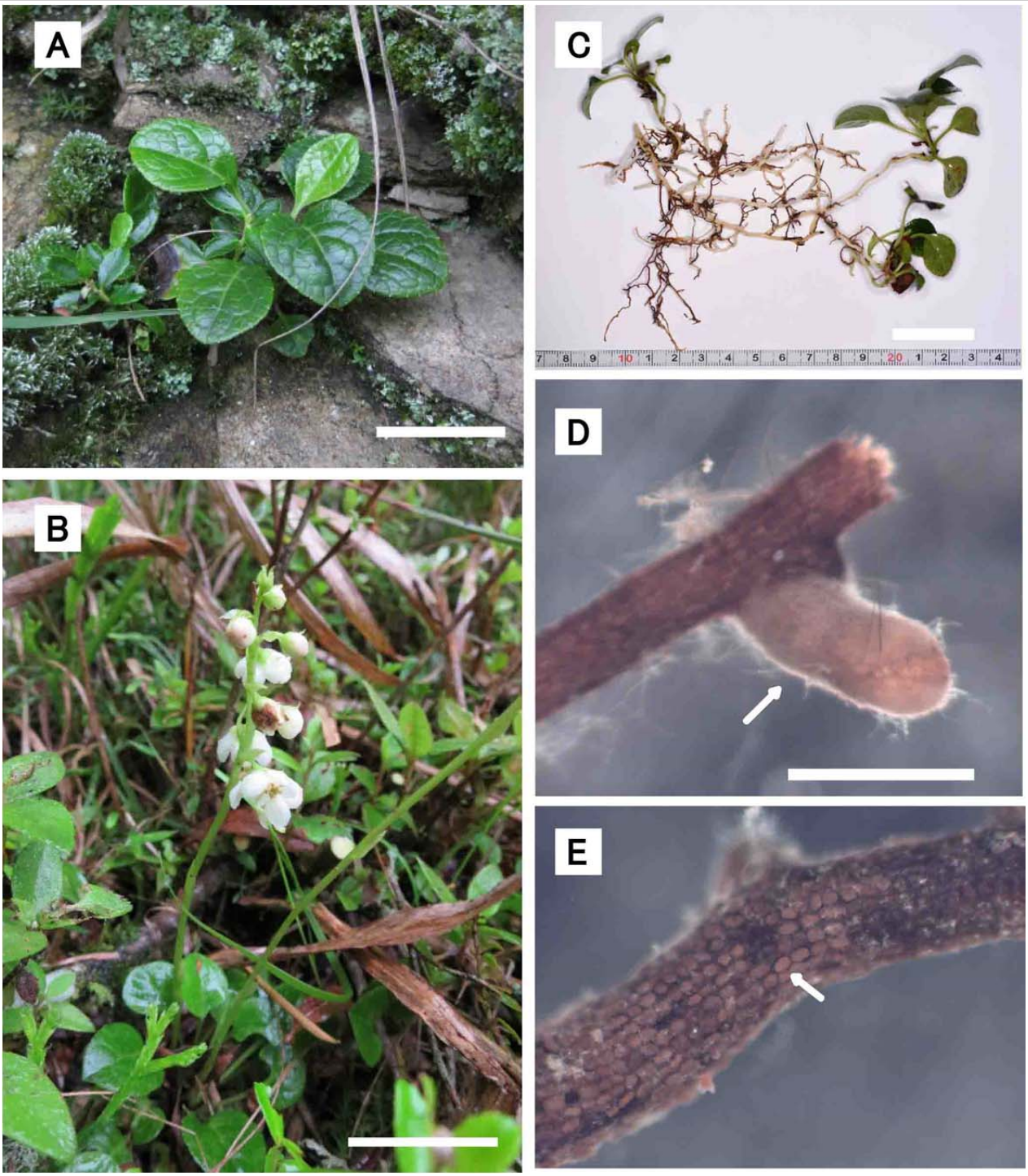

Fig. 1. ***Pyrola morrisonensis***. **A**: above ground part. **B**: scape with flowers. **C**: belowground rhizomes. **D**: fine root with fungal mantle colonized by Atheliaceae sp.1 **E**: fine root without fungal mantle colonized by ***Clavulina*** sp. Arrows in **D** and **E** indicate fungal mantle and hyphal coils, respectively. Bars indicate 3 cm (A, B and C) and 1 mm (D, E).

calculated as the number of mycorrhizal segments detected by each MOTU. Non-metric multidimensional scaling (NMDS) and PERMANOVA were also performed to test if MOTU community was structured in accordance with collected plots. Occurrence frequencies of each MOTU in each plant were used for ordination with the Chao dissimilarity index. These analyses were accomplished by R 3.12 (R Core Team, 2013) and the package vegan (Oksanen *et al.*, 2013).

The plant sequences were used to construct a

phylogenetic tree that included sequences derived from members of the genera *Pyrola*, *Chimaphila*, and *Orthilia* and that had been deposited in the DDBJ/EMBL/Genbank databases. The sequences were aligned using version 7 of the MAFFT software (http://mafft.cbrc.jp/alignment/software/; Katoh and Standley, 2013) using the L-ins-I strategy and 1PAM/κ = 2, but with all other settings at their default values. We performed maximum-likelihood analyses using the MEGA software with 1000 bootstrap replications and all other options set at their default values. We used the Kimura two-parameter and the Gamma distribution models with the lowest corrected value of Akaike's information criterion (cAIC) in MEGA. For this analysis, we used sequences from *Moneses uniflora* (AF133750 and FJ378568) as the outgroup.

RESULTS AND DISCUSSION

Pyrola morrisonensis had rhizomes that included fine branched roots (Fig. 1C). The roots showed two different types of mycorrhizas: forms with and without fungal mantles around the roots (Figs. 1D, E). Previous research reported no fungal mantle or poor mantle formation in *P. japonica* (Matsuda *et al.*, 2012) and *P. chlorantha* (Massicotte *et al.*, 2008). However, most *Pyrola* species previously examined formed clear fungal mantles (Massicotte *et al.*, 2008; Robertson and Robertson, 1985; Vincenot *et al.*, 2008).

The ITS region of all plants that had been morphologically identified as *P. morrisonensis* was identical and produced a fragment with a length of 657 to 689 bp. The phylogenetic positions of our samples clustered with a known *P. morrisonensis* sequence with a high bootstrap probability (97%, Fig. 2). Understanding the phylogenetic position of a species and its relationship with the associated mycorrhizal assemblages provides indispensable evidence to clarify evolutionary changes in the mycorrhizal association in certain plant taxa (Kinoshita *et al.*, 2016). Although the phylogenetic positions of *Pyrola* species within the Ericaceae have been controversial (Freudenstein *et al.* 2016; Kron *et al.*, 2002; Lallemand *et al.*, 2016), our samples were obviously clustered with *P. morrisonensis*. Since Liu *et al.* (2010) subdivided *Pyrola* into six series based on the combined sequences at four loci that focused on nuclear and chloroplast DNA, one locus is generally not sufficient to phylogenetically discriminate *P. morrisonensis* from other species. Based on the present results, however, the ITS region appears to provide sufficiently good discrimination for *P. morrisonensis*.

DNA analysis of the mycorrhizas produced successful PCR amplification for all root segments. After removing poor and chimeric sequences, 86% (43/50) of the segments produced 91 sequence data (Data not shown) that was divided into 30 MOTUs (Table 1). Among them, ectomycorrhizal Thelephoraceae species including the genus *Thelephora* and *Tomentella*, were dominant; they were detected in 7 MOTUs. In addition, 3 MOTUs belong to the member of Atheliaceae and Russulaceae (*Lactarius* and *Russula*). Four and three MOTUs matched best with *Oidiodendron* and Helotiales and were assumed to be endophytic fungi (e.g. Obase and Matsuda, 2014). The number of MOTUs detected were 10 to 13 per plot. Although MOTU compositions in each plot tended to be clustered in a NMDS scattering plot (Data not shown), the clustering was not significant (PERMANOVA, $p = 0.39$). Collectively, eighteen out of 30 MOTUs were assumed to be ectomycorrhizal and members of Thelephoraceae were dominant and found in all plots (Fig. 3 and Table 1).

To the best of our knowledge, this is the first study to define the mycorrhizal associations of *P. morrisonensis* at a low latitude compared with other studies of genus *Pyrola*. Although sampling efforts in this study were limited, our data nonetheless indicated that diverse mycorrhizal fungi, most of which were ectomycorrhizal, were associated with this host. This result supports previous suggestions that the species diversity of mycorrhizal fungi associated with *Pyrola* species might be higher due to a lack of species specificity in all species other than *P. japonica* (Matsuda *et al.*, 2012; Uesugi *et al.*, 2016). Apart from *P. morrisonensis* in this series, only *P. sumatrana* is distributed farther south; it grows in Indonesia under a tropical climate (Liu *et al.*, 2010). Learning whether diverse mycorrhizal fungi are associated with *P. sumatrana* would provide additional evidence of the degree of species specificity and its relationship with biogeography in this genus.

Among the mycorrhizal fungi detected from *P. morrisonensis*, members of Thelephoraceae, Atheliaceae and Russulaceae, as well as *Clavulina*, *Cortinarius* and *Tricholoma* species, were common ectomycorrhizal taxa reported from other *Pyrola* species (Hashimoto *et al.*, 2012; Hynson and Bruns, 2009; Matsuda *et al.*, 2012; Tedersoo *et al.*, 2007; Toftegaard *et al.*, 2010; Vincenot *et al.*, 2008). In addition, irrespective of the species, thelephoroid fungi were common symbiotic fungi in the genus *Pyrola*. Since the thelephoroid taxon is also a common group of ectomycorrhizal fungi associated with trees, including the species growing with *P. morrisonensis*, this fungal family may have an affinity for *Pyrola* species, although we do not have enough data to rule out the possibility that these associations only reflect local particularities of the mycorrhizal assemblages of adjacent tree symbionts.

In the roots of *P. morisonensis*, two fungal taxa (*Oidiodendron* in the Myxotrichaceae and Helotiales)

Table 1. Molecular operational taxonomic units and their representative sequences of mycorrhizal fungi associated with ***Pyrola morrisonensis***.

Taxa	Putative ecology [a]	Accession no.	Sequence length (bp)	Best match (Accession no.)	Score	e-value	No. of root segments colonized by each plant [b]								
							Plot 1			Plot 2			Plot 3		
							1 (5)	2 (4)	3 (6)	4 (8)	5 (6)	6 (4)	7 (6)	8 (6)	9 (5)
Atheliaceae sp.1	ECM	LC273404	447	*Tylospora asterophora* isolate VP-8 (KT692927)	335	7e-89	1								1
Atheliaceae sp.2	ECM	LC273408	424	Fungal sp. H401 (AB634276)	345	7e-92									1
Atheliaceae sp.3	ECM	LC273388	571	*Tylospora asterophora* isolate VP-8 (KT692927)	392	e-106									4
Cenococcum geophilum	ECM	LC273414	490	*Cenococcum geophilum* (LC095154)	860	0						1			
Chaetothyriales sp.	END	LC273398	900	Ascomycota sp. 4 RB-2011 (JQ272346)	478	e-131								1	
Clavulina sp.	ECM	LC273400	614	*Clavulina* sp. KA13-1242 (KR673708)	973	0			2						
Corticiaceae sp.	ECM	LC273389	555	Corticiaceae sp. BB-2010 (HM189734)	1084	0				2	1	1			
Cortinarius olivaceofuscus	ECM	LC273411	642	*Cortinarius olivaceofuscus* (AY669585)	1241	0								1	
Helotiales sp.1	END	LC273394	521	Helotiales sp. 5-NN-2017 (LC218318)	1017	0	2								
Helotiales sp.2	END	LC273402	682	Helotiales sp. DU60 (KM113762)	490	e-135					1	1			
Helotiales sp.3	END	LC273413	512	Helotiales sp. hig3uA2-NN-2017 (LC218734)	977	0	1								
Lactarius hirtipes	ECM	LC273416	670	*Lactarius hirtipes* (KF433007)	1189	0	1								
Leotiaceae sp.	END	LC273399	704	Leotiaceae sp. 19.L27 (KU057814)	507	e-140						1			
Mycelium radicis atrovirens	END	LC273407	518	*Mycelium radicis atrovirens* complex strain (AF486120)	971	0						1			
Oidiodendron sp.1	END	LC273412	496	*Oidiodendron* sp. 2 KO-2013 (AB846983)	761	0				1					
Oidiodendron sp.2	END	LC273410	532	*Oidiodendron* sp. isolate 15-17 (KX440134)	900	0				1					
Oidiodendron sp.3	END	LC273406	511	*Oidiodendron* sp. 2 KO-2013 (AB846983)	975	0									1
Oidiodendron sp.4	END	LC273409	520	*Oidiodendron* sp. isolate 15-17 (KX440134)	979	0		1							
Russula nitida	ECM	LC273395	665	*Russula nitida* (KU205349)	977	0	1				1				
Russula sp.	ECM	LC273415	633	*Russula* sp. 11 ZWG-2011(JN129409)	1114	0							1		
Spirosphaera sp.	END	LC273396	558	*Spirosphaera beverwijkiana* (EF029215)	706	0						1			
Thelephora terrestris	ECM	LC273391	619	*Thelephora terrestris* (AB634267)	1219	0			2				2		1
Thelephoraceae sp.1	ECM	LC273393	625	Thelephoraceous ectomycorrhiza S68 (AF430259)	1066	0				1					
Thelephoraceae sp.2	ECM	LC273403	623	Thelephoraceae sp. 24 CG-2012 (HE814080)	1096	0	2								
Tomentella fuscocinerea	ECM	LC273392	624	*Tomentella fuscocinerea* (KX497202)	1136	0		2	1			1			
Tomentella sp.1	ECM	LC273405	627	*Tomentella* sp. LM1634 (KM576636)	1041	0					1				
Tomentella sp.2	ECM	LC273401	644	*Tomentella* cf. *ramosissima* SE-2015 (KT275611)	1051	0							1		
Tomentella sp.3	ECM	LC273417	641	*Tomentella* sp. 2 BB-2010 (HM189969)	1124	0					1				
Tricholoma saponaceum	ECM	LC273390	635	*Tricholoma saponaceum* var. *saponaceum* (KJ705251)	1181	0								4	
Verrucariales sp.	END	LC273397	755	Verrucariales sp. RB-2011 (JQ272347)	589	e-165								1	1

a: ECM and END indicate ectomycorrhizal and endophytic fungi, respectively.
b: Parentheses in each plant indicate number of root segments. Total number of fungal taxa in some plants was more than the root segment owing to the detection of multiple fungi from single segments.

Fig. 2. Phylogenetic tree of ***Pyrola*** species inferred by the maximum likelihood method based on sequences of the internal transcribed spacer region. Sequences of ***P. morrisonensis*** in this study are shown in boldface, and ***Moneses uniflora*** was used as an outgroup.

were also detected. Because these taxa were reported from other *Pyrola* plants (e.g. Toftegaard *et al.*, 2010; Uesugi *et al.*, 2016) and deemed as endophytic nature with ericaceous plants (Obase and Matsuda, 2014), they may be a transitional root residents inferring "waiting room hypothesis" (van der Heijden *et al.*, 2015). If this is the case, two morphological types with or without fungal mantles may assume to be the differentiation process of root morphologies in evolutional mycorrhizal interactions.

In conclusion, we have provided the first details of mycorrhizal formation on *P. morrisonensis* in Taiwan. The detected fungi obtained from roots of the host were mostly taxa that are known to be ectomycorrhizal fungi associated with woody plants. The present study was conducted at only one site, so additional sites should be surveyed to provide a more complete picture of the mycorrhizal associations with Taiwanese *P. morrisonensis*. This knowledge will provide insights into mycorrhizal symbioses and the coevolutionary pathways in genus *Pyrola*.

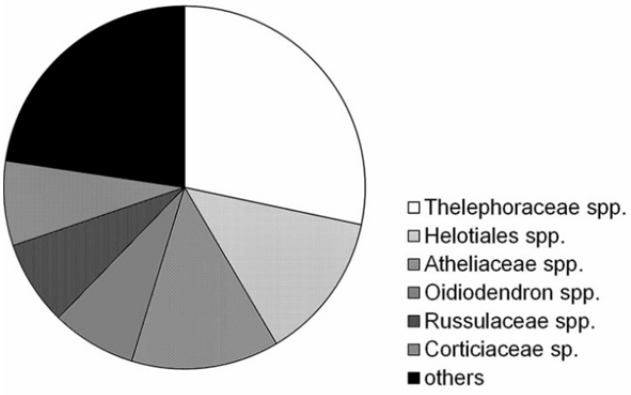

Fig. 3. Relative abundance of mycorrhizal fungi associated with ***Pyrola morrisonensis***. Successfully sequenced data from 9 plants over 3 plots were involved (n = 53).

ACKNOWLEDGEMENTS

We are grateful to two reviewers who provided valuable comments on earlier versions of this work, and thank for T. Chikada (Life Science Research Center, Center for Molecular Biology and Genetics, Mie University) for helping with the DNA analyses. This study was partly supported by KAKENHI grants (22688011, 23658124, 25304026) to YM.

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