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Mycorrhizal Symbioses Found in Roots of Fern and Its Relatives in Korea

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Mycorrhizal symbioses were found in the roots of 45 out of 59 species of pteridophytes collected in Korea. The mycorrhizal fungi were colonized in the root cortical cells, primarily in terrestrial species, but rarely in epiphytic or aquatic pteridophytes. Mycorrhizae that are typically found in orchid colonized the roots of the epiphytic pteridophytes, but not in other species. These were the first observations of orchid mycorrhizae in pteridophytes. Arbuscular mycorrhizal fungi were examined after staining, then confirmed with PCR, using a specific primer. This is the first report of arbuscular mycorrhizal colonization in the roots of pteridophyte species in Asia.

Keywords: Orchid mycorrhiza, PCR, pteridophytes, Symbiosis, VA mycorrhiza

The ecological and phylogenetic relationships of the fern and its relatives have not been widely studied because their commercial value is lower than that of other seed plants (Tryon and Lugardon, 1990; Pryer et al., 1995; Wolf, 1997; Rothwell, 1999). Mycorrhizae are symbiotic associations between plant roots and fungi, which stimulate plant growth and overcome environmental stresses (Harley and Smith, 1983). Ecological research has been conducted on the mycorrhizal relationships of more than 6,000 flowering plant species (Newman and Reddell, 1987; Gemma et al., 1992; Schmid and Oberwinkler, 1996). However, because only a few studies have included ferns (Gemma et al., 1992), little is known about the mycorrhizal symbiosis of these plants. Parihar (1996) has reported that some species of fern require fungi to promote spore germination. Symbiosis in ferns has been identified in the protonema stage of the prothallus, rather than in the roots of sporophytes. Mycorrhizae of fern sporophytes have not been demonstrated, although they are considered to be more important for spore germination (Kelly, 1950).

The relationship of mycorrhizal fungi and ferns might be related to the systematics of those species. This mycotrophism or mycorrhizal status could be used as a phylogenetic criterion for ferns, because it is related to their evolution (Nakai, 1933; Boullard, 1957, 1979; Gemma et al., 1992). In Korea, 272 species of fern and their allies have been identified (Nakai, 1952; Park, 1975), but their mycorrhizal association had not been reported. In this study, fern species and their allies were collected from various sites in Korea. Mycorrhizae were examined, then confirmed by PCR, using the specific primers. The various types of mycorrhizae we observed could be used as important features when grouping these fern species.

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MATERIALS AND METHODS

Plant Materials

We collected pteridophyte plants from various habitats, substrates, and geobotanical zones of the Korean Peninsula and on the islands of CheJu, Heuksan, and Ullung, from April 1998 to September 2000 (Table 1). Plant specimens were carefully preserved and deposited in the herbarium at Korea National University of Education, Republic of Korea. Identifications were made using several manuals (Nakai, 1952; Park, 1961, 1975; Lee, 1985), and were confirmed according to fern specimens previously collected for the herbaria at Seoul National University and Ajou University. The roots or rhizomes were carefully excised and stored in polyethylene bags at 4°C.

Root Staining

Roots or rhizomes were washed with tap water and placed in 100-mL bottles containing FAA solution. The roots were cut into 1- to 3-cm sections and

Table 1. Occurrence of arbuscular mycorrhizal colonizations in the roots of fern and its relatives collected in Korea.

Family	Species	Habitat		Mycorrhizal colonization		
		S[a]	Areas[b]	Type[c]	Freq[d]	Ratese
Lycopodiaceae	*Lycopodium obscurum*	T	F	N	0/5	0
Selaginellaceae	*Selaginella stauntoniana*	T	G	N	0/5	0
	Selaginella rossii	T	E	N	0/5	0
Equisetaceae	*Equisetum arvense*	T	E	V	4/5	1
	Equisetum hyemale	T	F	N	0/5	0
Ophioglossaceae	*Sceptridium ternatum*	T	E	VA	5/5	4
	Sc. multifidum var. *robustum*	T	B	A	5/5	4
Osmundaceae	*Osmunda japonica*	T	E	A	5/5	4
	Osmundastrum claytonianum	T	F	A	5/5	2
Gleicheniaceae	*Dicranopteris dichotoma*	T	B	VA	5/5	4
Hymenophyllaceae	*Hymenophyllum barbatum*	E	D	N	0/5	0
Pteridaceae	*Dennstaedtia wilfordii*	T	D	A	5/5	4
	Dennstaedtia hirsuta	T	D	A	4/5	4
	Sphenomeris chusana	T	D	A	5/5	4
	Pteridium aquilinum var. *latiusculum*	T	E	VA	5/5	4
	Pteris multifida	T	A	A	5/5	3
	Pteris cretica	T	A	A	5/5	3
	Coniogramme japonica	T	B	V	5/5	3
	Aleuritopteris argentea	T	A	VA	5/5	3
	Adiantum pedatum	T	B	A	5/5	3
Parkeriaceae	*Ceratopteris thalictroides*	A	G	N	0/5	0
Davalliaceae	*Davallia mariesii*	E	E	A	1/5	1
Aspidiaceae	*Matteuccia struthiopteris*	T	D	A	5/5	5
	Onoclea sensibilis var. *interrupta*	T	D	A	3/5	5
	Woodsia polystichoides	T	E	N	0/5	0
	Woodsia ilvensis	T	E	VA	4/5	3
	Polystichum lepidocaulon	T	A	VA	5/5	4
	Polystichum craspedosorum	T	D	A	5/5	4
	Polystichum tripteron	T	H	A	5/5	4
	Polystichum tsus-simense	T	H	A	5/5	4
	Cyrtomium falcatum	T	C	C	3/5	0
	Rumohra standishii	T	B	VA	4/5	4
	Dryopteris fragrans	T	G	A	5/5	4
	Dryopteris lacera	T	E	A	5/5	4
	Dryopteris amurensis	T	D	A	5/5	4
	Gymnocarpium dryopteris	T	E	VA	5/5	4
	Cyclosorus acuminatus	T	D	VA	5/5	3
	Athyrium conilii	T	E	A	5/5	4
	Athyrium japonicum	T	E	VA	4/5	4
	Athyrium niponicum	T	E	A	5/5	4
Aspleniaceae	*Asplenium prolongatum*	T	E	N	0/5	0
	Asplenium sarelii	T	E	N	0/5	0
	Asplenium incisum	T	E	V	2/5	2
	Asplenium unilaterale	T	E	N	0/5	0
	Camptosorus sibiricus	T	B	N	0/5	0
Polypodiaceae	*Lepisorus ussuriensis*	E	A	N	0/5	0
	Lepisorus thunbergianus	E	G	C	2/5	0
	Lemmaphyllum microphyllum	E	A	VA	1/5	1
	Pyrrosia linearifolia	E	E	C	3/5	0
	Pyrrosia lingua	E	C	C	3/5	0
	Pyrrosia petiolosa	E	C	N	0/5	0
	Pyrrosia hastate	E	C	N	0/5	0

Table 1. Continued

Family	Species	Habitat		Mycorrhizal colonization		
		S[a]	Areas[b]	Type[c]	Freq[d]	Ratese
Polypodiaceae	*Colysis elliptica*	T	A	N	0/5	0
	Crypsinus hastatus	E	E	VA	2/5	1
	Loxogramme saziran	E	A	N	0/5	0
Salviniaceae	*Salvinia natans*	A	F	N	0/5	0
Azollaceae	*Azolla japonica*	A	F	N	0/5	0

[a]Habitat substrates of the fern and its related species: A, aquatic type; E, epiphytic type, and T, terrestrial type.
[b]Collection sites on the islands of Cheju (A), Ullung (B), and Heuksan (C), as well as from Kyoungbuk (D), Chungbuk (E), Kyungki (F), Chunnam (G), and Kangwon (H).
[c]Cortical cells observed under light microscopy: A, Arbuscule type; V, vesicle type; VA, Vesicle and Arbuscule type; C, constricted hyphae (*Rhizoctonia*, might be related to orchid mycorrhizal hyphae); N, non-mycorrhizae.
[d]Number of root samples containing mycorrhizae/number of samples examined.
[e]0 = no mycorrhizal colonization in roots; 1 = 1-20%; 2 = 21-40%; 3 = 41-60%; 4 = 61-80%; 5 = 81-100%.

stained with trypan blue (Phillips and Hayman, 1970; Koske and Gemma, 1989). The stained mycorrhizal roots from the ferns were observed under a light microscope. Percent colonization rates for the roots were determined by the gridline intersect method (Giovanetti and Mosse, 1980).

DNA Extraction

Roots were ground with liquid nitrogen and mixed with a 650-μL lysis buffer [50 mM Tris-HCl (pH 7.5), 30 mM EDTA, 3% SDS, and 1% 2-mercaptoethanol; Brunel, 1992]. This mixture was then incubated at 65°C for 1 h, and centrifuged at 12,000*g* for 15 min at 4°C. The aqueous phase was transferred to a new microtube and mixed with an equal volume of PCI solution (25:24:1, TE-saturated phenol:chloroform: isoamylalcohol; Weising et al., 1995). After a brief vortexing for 1 or 2 min, the mixture was centrifuged at 12,000*g* for 5 min at 4°C. The aqueous phase was transferred to a new microtube, and the DNA was pelleted by isopropanol precipitation. This pellet was suspended in 500 μL of TEN buffer (10 mM Tris-HCl, 1 mM EDTA, and 100mM NaCl), then added to 50 μg/μL RNase A and incubated at 37°C for 30 min. The suspension was treated again with PCI, and the DNA was pelleted by ethanol (absolute alcohol) precipitation. This DNA pellet was re-suspended in 50 μL of TE buffer, and the concentration of DNA was estimated by absorbance at 260 nm.

DNA Amplification

Partial fungal small-subunit ribosomal DNA fragments were amplified by PCR using both a universal eukaryotic primer NS-31 (5'-TTG GAG GGC AAG TCT GGT GCC-3'; Simon et al., 1992) and a fungal primer AM-1 (5'-GTT TCC CGT AAG GCG CCG AA-3'; Helgason et al., 1998) that were designed to exclude plant DNA sequences. For amplification, 10 ng of the genomic DNA previously extracted was added to an *Accupower* PCR PreMix™ Kit (Bioneer Co, http://www.bioneer.com) with 10 pmole of each primer (Lee and You, 2000). PCR was carried out for 30 cycles (10 cycles at 95°C for 1 min, 58°C for 1 min, and 72°C for 2 min; 19 cycles at 95°C for 30 s, 58°C for 1 min, and 72°C for 3 min; and 1 cycle at 95°C for 30 s, 58°C for 1 min, and 72°C for 10 min) on a Personal Cycler™ (Biometra Co; Helgason et al., 1999). The PCR product was re-amplified following the same procedure. DNA bands amplified by PCR were displayed on 1.2% agarose gels, stained with ethidium bromide, and photographed under UV illumination. Band size was calculated via molecular-weight markers, using a 100-bp ladder.

RESULTS

Various types of fungal hyphae were associated with the root cortical cells in 39 out of 57 fern species (Fig. 1, Table 1). Both intracellular hyphae and haustoria were observed. The haustoria were formed along a long intra-hyphae located between the cortical cells in 29 species (Fig. 1, A-F and H). These structures were arbuscules. In addition, vesicles were observed in the cortical cells of 15 species (Fig. 1, G and K). Either arbuscules or vesicles, or both, were found in 35 fern plant species (Table 1).

However, a different type of intra-hyphae was found in the root cortical cells of only four species: *Lepisorus ussuriensis*, *Pteris cretica* (Fig. 1F), *Pyrrosia lingua* (Fig.

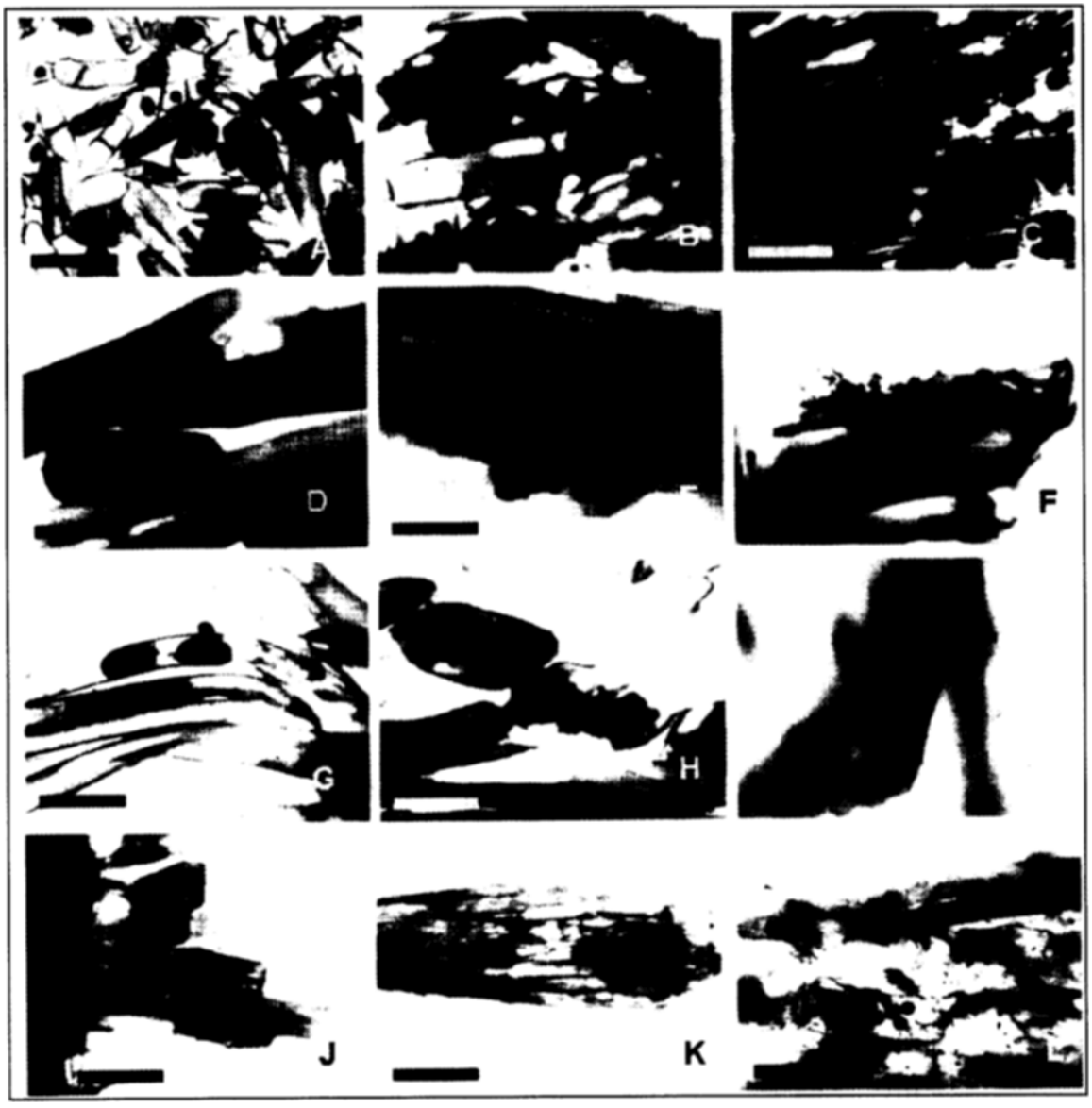

Figure 1. Mycorrhizal fungi colonized in the roots of fern and related species collected in Korea: A and B, *Sceptridium ternatum*; C, *Osmunda japonica*; D, *Dicranopteris dichotoma*; E, *Pteridium aquilinum*; F, *Pteris cretica*; G, *Coniogramme japonica*; H, *Athyrium conilii*; I, *Pyrrosia lingua*; J, *Polystichum craspedosorum*; K, *Aleuritopteris argentea*; and L, *Cyrtomium falcatum*. Scales: A, C, K = 200 μm; B, D, E, G, H, J, L = 100 μm; F, I = 50 μm.

1I), and *Pyrrosia linearifolia*. These constricted hyphae were similar to those seen in orchid roots. In addition, loosely coiled hyphae in the root cortical cells of *Polystichum craspedosorum* (Fig. 1J) were typical of the structure, "peloton", in orchid mycorrhizae. Therefore, we were able to identify two different types of mycorrhizae -- arbuscular and orchid -- in the roots of fern or its allies collected in Korea. Neither the ectomycorrhizal structures nor any mushroom related to ectomycorrhizal fungi were observed under the dissecting microscopes or at the collection sites.

PCR primers were used to confirm arbuscular mycorrhizae (AM) fungi in roots containing structures of arbuscules and/or vesicles. We did not use primers from genomic DNA that was extracted from the leaves (data not shown) if any bands had base pairs that matched those of the roots. Bands of approximately 550 bps were detected from roots of fern species having arbuscular mycorrhizal structures, but not from *Equisetum hyemale* and *Woodsia polystichoides*,

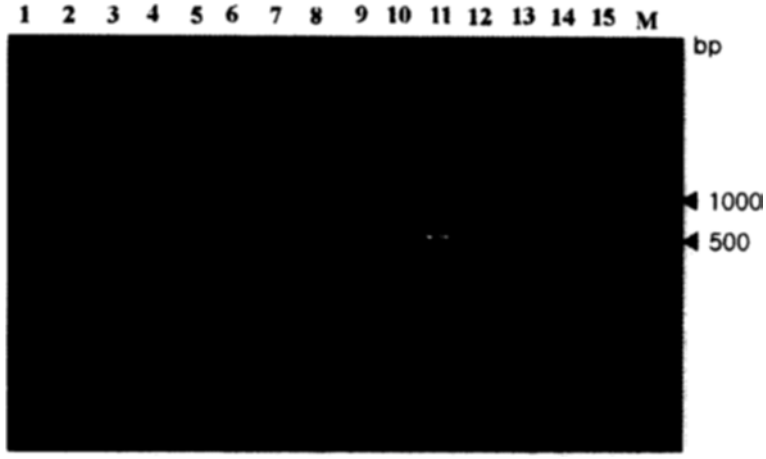

Figure 2. PCR bands for roots of fern and related species, with AM1 and NS31 primer pairs: Lane 1, *Asplenium incisum*; Lane 2, *Equisetum hyemale*; Lane 3, *Dryopteris fragrans*; Lane 4, *Gymnocarpium dryopteris*; Lane 5, *Polystichum craspedosorum*; Lane 6, *Osmunda japonica*; Lane 7, *Onoclea sensibilis*; Lane 8, *Polystichum tsus-simense*; Lane 9, *Woodsia polystichoides*; Lane 10, *Dennstaedtia wilfordii*; Lane 11, *Equisetum arvense*; Lane 12, *Pteridium aquilinum*; Lane 13, *Dicranopteris dichotoma*; Lane 14, *Sphenomeris chusana*; and Lane 15, *Sceptridium ternatum*; M, Molecular weight markers with the 100-bp ladder.

both of whose roots showed no such structures (Fig. 2).

Arbuscular mycorrhizae were abundant in the roots of terrestrial fern species in Botrychiaceae, Osmundaceae, Gleicheniaceae, Pteridaceae, and Aspidiaceae. In contrast, species in Lycopodiaceae or Hymenophyllaceae were not colonized by any type of mycorrhizal fungi, while only one species in Aspleniaceae showed colonization. None of the aquatic fern species were colonized by any type of mycorrhizal fungi (Table 1, Fig. 1).

Epiphytic ferns showed no or very low root colonization rates for AM fungi. Three species in Polypodiaceae were colonized by constricted hyphae, a specific feature of the orchid mycorrhizal fungi, *Rhizoctonia*. These structures were found in the roots of only one species in Aspidiaceae, *Cyrtomium falcatum*. The root morphology of two terrestrial species in Botrychiaceae (Ophioglossaceae) differed from other families, but was similar to that of the orchid root (Fig. 1). In addition, the swelling and coralloid shape of the arbuscules differed from those found in other Leptosporangiate roots. Finally, out of all the species of fern allies collected in families Lycopodiaceae, Selaginellaceae, and Equisetaceae, only one, *Equisetum arvense*, was colonized by mycorrhizal fungi.

DISCUSSION

This is the first report on the colonization of mycorrhizae in fern species and their allies collected in Korea. Previously in the fern allies, the Equisetaceae species had been considered nonmycotrophic (Kelly, 1950). In this study, however, AM fungal structures were observed in the roots of *E. arvense* under microscope, and their presence was confirmed by PCR, using AM primers (Figs. 1 and 2). Although another fern ally, the *Lycopodium* species, had been reported as being arbuscular mycorrhizal (Gemma et al., 1992), we did not observe any colonization in the roots we collected for the current research. Likewise, Gemma et al. (1992) had found AM colonization in the terrestrial species of *Selaginella*, but none of the epiphytic species studied here were colonized with any mycorrhizal fungi.

Schmid and Oberwinkler (1996) previously described atypical arbuscular mycorrhizal structures in fern roots of Ophioglossaceae. We also found these characteristic features in the roots of two species in Ophioglossaceae. Although our PCR with primers specific to AM fungi showed that this could be arbuscular mycorrhizae, further confirmation is required. The constricted hyphae we found in the roots of *P. lingua* and *C. falcatum* plants match that of a peloton found in the roots of orchid (Lee and You, 2000). The circled and coiled hyphae in the roots of *P. craspedosorum* are considered a form of digestive peloton also occasionally found in orchid roots. These orchid mycorrhizae are the first reported in pteridophytes.

The arbuscular mycorrhizal fungi observed in *Gigaspora* or *Scutellospora* do not produce the "vesicle" structure in plant roots, whereas the AM fungi in other

species produce both arbuscule and vesicle root structures. Therefore, we could identify two distinct types of AM structures in the pteridophytes, although some roots were colonized with one structure type only. It was unclear whether the colonization of AM fungi in the roots might have originated from different kinds of fungi. The composition of the fungi that colonized in the pteridophyte roots was revealed through cloning and sequencing for the overlapping, 550-bp bands (Fig. 2).

Our terrestrial pteridophytes were related to the AM fungi, but we found that the aquatic pteridophytes were nonmycotrophic. Epiphytic ferns were either nonmycorrhizal or orchid mycorrhizal. The mycorrhizal status of the collected fern species was related to their habitat substrate. Therefore, our results do not support mycotrophism as a phylogenetic criterion or Boullard's (1979) hypothesis that evolution in pteridophytes is accompanied by a decrease in mycotropy.

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