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Fungal diversity living in the root and sporophore of the endemic Korean fern *Mankyua chejuense*

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ABSTRACT

Ferns represent the basal group of vascular plants and are known to have fungal interactions with arbuscular mycorrhizal fungi, but diversity of endophytic fungi from ferns is rarely studied. Moreover, fungal diversity associated with ferns is likely underestimated as most studies have been performed based on a microscopic or culture-dependent approach. In this study, we investigated the endophytic fungal diversity within roots and sporophore of an endangered Korean fern (*Mankyua chejuense*), and compared it to fungi in surrounding soil using a metabarcoding approach. A high diversity of endophytic fungi (236 OTUs), mostly belonging to Ascomycota, was detected and fungal richness and composition were significantly different between habitats. Indicator species analysis showed that endophytic fungi have similar ecological characteristics to fungal species found from other land plants. Our results suggest that various fungal species are associated with ferns, thus understanding fern-associated fungal diversity can have a great implication for fern biology and conservation.

1. Introduction

Land plants have a long evolutionary history of interactions with fungi in terrestrial environments (Simon et al., 1993; Remy et al., 1994; Krings et al., 2012). Molecular and fossil evidence (Wang et al., 2010; Krings et al., 2012) suggest that fungi were one of the major drivers of the evolution of land plants, such as the transition to land (Simon et al., 1993; Strullu-Derrien et al., 2018). In fact, current plants show a high diversity of fungi living in plant tissues. These fungi can be classified to three types according to the pattern of interaction: Mycorrhizal fungi (positive), endophyte (neutral), and parasite (negative) (Johnson et al., 1997; Porras-Alfaro and Bayman, 2011; Selosse et al., 2018). Mycorrhizal fungi form specific structures in root systems, often with extramatrical hyphae in the soil, which serve to increase nutrient uptake (Smith and Read, 2008). Most land plants interact with mycorrhizal fungi and benefit from nutrient uptake and resistance to environmental stress (Lee et al., 2013; Willis et al., 2013; Corrales et al., 2018). In contrast to mycorrhizal fungi, endophytes do not form extramatrical hyphae and are typically found in or between plant cells in various organs such as leaves, stems, and roots (Saikkonen et al., 1998). The role of endophytes in the host plant is less clear. Endophytes can influence the physiology of host plants such as growth rate and resistance against environmental stress, pathogens, or herbivores (Jumpponen and Trappe, 1998; Rodriguez et al., 2009; Gundel et al., 2013; Khan et al., 2015; González-Teuber, 2016). However, the function of endophytes is not static but changeable depend on the context (e.g. environmental condition or host status) (Delaye et al., 2013; Selosse et al., 2018).

Ferns (Class Polypodiopsida) are early-diverging vascular plants that are the sister clade of seed plants (Smith et al., 2006). In several

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ecosystems, ferns play important ecological roles in nutrient cycling (Vitousek et al., 1995; Richardson et al., 2005), shaping of microhabitats (George and Bazzaz, 1999), influencing vegetation structure (Krueger and Peterson, 2009), and as food or medicine for animals (Solis et al., 2006; Lai et al., 2009). Like other land plants, ferns interact with arbuscular mycorrhizal fungi (AMF) (Read et al., 2000; Pressel et al., 2016; Lehnert et al., 2017; Hoysted et al., 2018) and endophytes (Petrini et al., 1992; Raviraja et al., 1996; Sati and Belwal, 2005; Sati and Arya, 2010; Del Olmo-Ruiz and Arnold, 2014). Various fungi have been detected from whole parts of ferns including gametophytes, leaf, and root habor (Petrini et al., 1992; Raviraja et al., 1996; Swatzell et al., 1996; Sati and Belwal, 2005 Muthukumar and Prabha, 2013), and they showed specific community structure depending on the host taxonomy and environments (Del Olmo-Ruiz and Arnold, 2014).

Most previous studies focusing on fern-associated fungi used microscopic observation, and little effort has been devoted to estimating fungal diversity (Pressel et al., 2016). Diversity of fern-associated fungi is likely underestimated because many of these fungi are neither distinguishable using microscopic characters nor culturable on artificial media. Recent culture-independent approaches, especially metabarcoding approaches using high throughout sequencing (HTS) technology (Kõljalg et al., 2013; Lindahl et al., 2013), allow the identification of fungal diversity, including unculturable fungi from various environments (Buée et al., 2009; Mar Rodríguez et al., 2015). These newer studies have led to the detection of high fungal diversity from a variety of plant species (Davey et al., 2013; Toju et al., 2013; Knack et al., 2015). A single study has used a metabarcoding approach to study fern-associated fungi while it only focused on the fungal diversity in fern pinae and did not try to find the fern specific fungi compared to surrounding soil (Younginger and Ballhorn, 2017). Nevertheless, the metabarcoding analysis found c.a. 260 OTUs of fungi associated with the fern which was much higher number of fungal taxa compared to it of previous study using culture-dependent method: two species (Swatzell et al., 1996), 10 species (Raviraja et al., 1996), 18 species (Sati and Belwal, 2005), 66 species (Petrini et al., 1992), 95 OTUs (Del Olmo-Ruiz and Arnold, 2014). Therefore, we can assume that the actual diversity of fern-associated fungi remains unexplored.

Mankyua chejuense is an endemic fern species belonging to the basal fern family Ophioglossaceae. Among the other taxa of Ophioglossaceae (e.g. *Botrychium*, *Helminthostachys*, and *Ophioglossum*) (Sun et al., 2001), *Mankyua* is inferred to have diverged early in the history of Ophioglossaceae (Shinohara et al., 2013; Gil and Kim, 2018). *Mankyua chejuense* is designated as an endangered species by the Korean government (Kang et al., 2010) and classified as Critically Endangered on the IUCN Red List of Threatened Species (Son et al., 2016). The range of habitats of *M. chejuense* is restricted to Jeju Island, and the population size is extremely small (Kim, 2004). Artificial cultivation of *M. chejuense* has been unsuccessful, possibly because of difficulties associated with obligate fungal symbionts, which were reported from other species in the Ophioglossaceae (Bidartondo, 2005).

An important step for the conservation and artificial cultivation of *M. chejuense* is to investigate the fungi that are associated with its living tissues. Because endophytes can enhance the growth of biomass or resistance ability against environmental stress or pathogens, fungi living in the fern can be the key drivers to solve the problem associated with the physiology and ecology of *M. chejuense*. These fungi can be recruited from the surrounding soil, and it may be possible to vertically transmit through spores, as it has been shown in seeds for other vascular plants (Shahzad et al., 2018). The objective of this study is to investigate the endophytic fungal diversity using a metabarcoding approach in roots and sporophores of *M. chejuense* compared with the surrounding soils. We expect a higher diversity of endophytic fungi from our metabarcoding approach compared to previous studies using microscopy or culture-dependent methods alone. The hypotheses of the community analysis were: (1) that diversity and community composition were different between habitats and sampling sites; and (2) that specific fungi were living in the roots and sporophore which were candidates of intimate symbiont of *M. chejuense*.

2. Materials and methods

2.1. Sample collections

Sampling was conducted in August 2017 on Jeju Island, South Korea (33°28′40.90″N, 126°43′41.82″E) (Fig. 1) with permission of the National Institute of Biological Resources (NIBRGR000083150). Habitats of *M. chejuense* include rock outcrops and swamps that are periodically submerged. The vegetation of sampling sites included shrubs and evergreen-deciduous broadleaf trees (*e.g.*, *Ulmus parvifolia* and *Camellia japonica*). The number of *M. chejuense* samples collected was limited to minimize the effect on the population, as *M. chejuense* is an endangered species and has a restricted range of habitats (Kim, 2004; Kang et al., 2010; Son et al., 2016). We chose five sites that were >100 m apart, and a total of three individuals of *M. chejuense* sporophytes were collected from each site. Each individual sampled was separated by more than 10 m to avoid autocorrelation (Peay et al., 2010; Bahram et al., 2016; Oja et al., 2017). If rhizomes were connected, we treated all connected samples as the same individual. From each individual, we collected roots and surrounding soil (depth: 5 cm) in triplicate based on above-ground structure and rhizome connections. In the case of sporophore samples, a single sample was collected from each site due to the small number of sporophores found. A total of 95 samples were prepared for DNA extraction (45 roots + 45 soil+ 5 sporophore samples). Samples were transferred to a lab in an ice box and stored at 4 °C until DNA extraction (within 2 d).

2.2. DNA extraction, PCR amplification, and sequencing

Root samples were rinsed using distilled water to remove organic debris, then surface sterilized to remove microorganisms and DNA fragments attached to the root surface (Menkis and Vasaitis, 2011). In an empty Petri dish, roots were divided into 2 cm pieces, immersed in 33% hydrogen peroxide for 30 s, and rinsed three times using sterilized distilled water. Five root pieces from each individual were homogenized using a mortar and pestle in 1 mL of cetyl trimethylammonium bromide (CTAB). Homogenized samples were used for DNA extraction based on a modified CTAB method (Rogers and Bendich, 1994). In the case of sporophores, whole tissue was used without cutting into smaller pieces and sterilized by the same method described above. Genomic DNA of soil samples was extracted from 500 mg of soil using the PowerSoil DNA extraction kit (MoBio, Carlsbad, CA, USA) following the manufacturer's protocol. Soil DNA was extracted in triplicate for each sample, and the three extracts were pooled to represent one sample.

PCR amplification of the fungal ITS2 region was conducted with ITS3 and ITS4 primers (White et al., 1990) ligated to Illumina sequencing adaptors. PCR was conducted three times for each sample using AccuPower PCR PreMix kit (Bioneer, Daejeon, South Korea) using the following conditions: 94 °C for 5 min, 30 cycles of 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 40 s, and 72 °C for 10 min as final extension. PCR products were checked on 1% agarose gel and purified using Expin™ PCR SV kit (GeneAll Biotechnology, Seoul, South Korea). The second round of PCR was conducted to

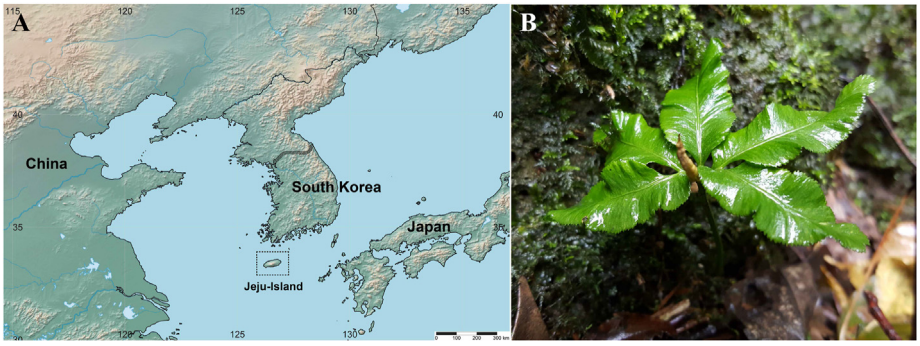

Fig. 1. (A) Map of sampling sites and (B) photograph of sporophyte of *M. chejuense*. The map was generated using SimpleMappr (Shorthouse, 2010). Photograph by Young Woon Lim.

attach multiple index delimiters (MID) following the Nextera XT Index kit protocol (Illumina, San Diego, CA, USA). After purification, concentrations of amplicon libraries were measured using a NanoDrop2000 (Thermo Fisher Scientific, Waltham, MA, USA) and pooled in equimolar quantities. Amplicon libraries were sequenced using an Illumina Miseq platform by Macrogen (Seoul, South Korea).

2.3. Bioinformatic analysis

Raw sequences were processed using QIIME v.1.8.0 (Caporaso et al., 2010). Paired-end sequences were merged using fastq-join implemented in QIIME and low quality sequences were removed (>25% of sequence length with Q < 20). The ITS2 region of the sequence was extracted using ITSx v.1.1b (Bengtsson-Palme et al., 2013). To create operational taxonomic units (OTUs), sequences were clustered at a 97% similarity threshold with an average linkage method using USEARCH v. 5.2.236 (Edgar, 2010). The most abundant sequence was selected as the representative sequence of an OTU and used for taxonomic assignment. NCBI BLAST was used to assign taxonomy against UNITE v. 7.2 (Kõljalg et al., 2013) and sequences from Seoul National University Fungal Collection (SFC) following the criteria of Tedersoo et al. (2014). Chimeric sequences were identified using UCHIME (Edgar et al., 2011) against the above databases; these sequences, as well as those that did not have match to fungi, were removed. Singleton OTUs were removed and all samples were rarefied to a minimum number of sequences (11,000 reads) before further analysis. To avoid the artifact from the sample cross-contamination, we retained only those OTUs with >0.5% relative sequence abundance in at least two samples for diversity and community analyses. Alpha diversity was calculated with Chao1 richness, Shannon's diversity and evenness in QIIME. Also Good's coverage was calculated and rarefaction curves were drawn for each individual sample in order to check whether the sequencing depth was enough for analysis in QIIME. Since all root and sporophyte samples were sterilized before DNA extraction, all OTUs, except for OTUs belonging to Glomeromycota (AMF), were treated as endophytes.

2.4. Statistical analysis

Statistical tests and graphics were done using R v. 3.1.0 (R Core Team, 2018). A Kruskal-Wallis test was used to compare the diversity indices between habitats (bulk soil, roots, and sporophore) and sampling sites with Dunn's test as post-hoc test adjusted by Benjamini and Hochberg method (Benjamini and Hochberg, 1995). Ordination analysis was conducted by non-metric multidimensional scaling (NMDS) analysis based on Bray-Curtis dissimilarities using phyloseq package (McMurdie and Holmes, 2013). Significance of difference of community compositions and homogeneity of group dispersion were tested using the function adonis and a betadisper respectively from the vegan package (Oksanen et al., 2018). The explanatory power of each category (habitats and sampling sites) was compared by variation partitioning analysis using varpart function in vegan. Indicator species analysis was performed to detect indicator species for each habitat (roots or sporophores) using indicspecies package (De Cáceres and Legendre, 2009). The aim of the analysis was to find the fungal candidates that have intimate relationship with the fern, thus we focused on the indicator species of roots and sporophores. The combination result of root and soil was also retained because many fungi in root were not unique but were shared with and originated from soil (Goldmann et al., 2016). Among the indicator species, we filtered OTU which was detected less than 20% of samples in the corresponding habitat (Root and Soil $\geq$ 9, Sporophore $\geq$ 2). Ecological information of indicator species was acquired from the reference search and metadata of the sequence in GenBank with high similarity ($\geq$97%) and coverage ($\geq$95%) by BLAST. Sequencing data were deposited in NCBI SRA under accession number PRJNA548016.

3. Results

3.1. Sequencing results and alpha diversity of fern-associated fungi

In total, 6,734,519 sequences were obtained as raw sequence reads, and 5,543,283 sequences were retained after filtering processing (range: 14,400–232,657 per samples). The average proportion of plant-derived sequences per samples was 0.011%, and it was removed before further analysis. All Good's coverage indices had high values (0.998–0.999), indicating that sequencing depth was enough to cover the diversity in the samples. Rarefaction curves verified adequate sequencing depth for analysis, especially for root and sporophore samples (Supplementary Fig. 1). A total of 1831 OTUs (range: 40–679 per sample) were detected: 12 phyla, 37 classes, 91 orders, 194 families, and 370 genera. After rarefaction of sequences, we compared the distribution of OTUs between habitats (bulk soil, roots, and sporophore) and sampling sites (Fig. 2). Among the abundant OTUs (>0.5% at least two samples), soil samples had the highest OTU richness (233), followed by root samples (206) and sporophore samples (52). Forty-eight OTUs were detected from all habitats (Fig. 2A). The number of unique OTUs was highest in soil samples (11.6%), but the OTUs unique to roots and sporophore samples were almost never detected (two and zero OTUs, respectively). A large number of OTUs detected from roots was shared with soil samples (98.5%). Among sampling sites, soil samples showed the highest number of OTUs shared among all

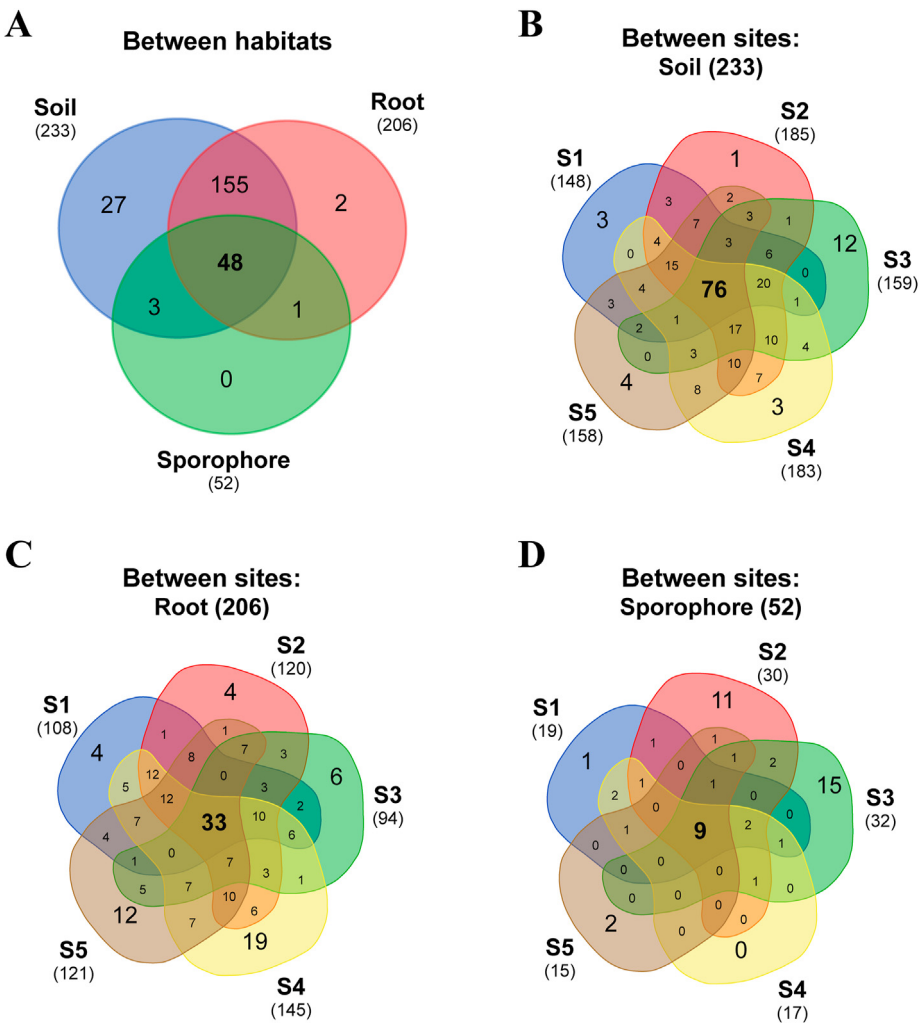

Fig. 2. Venn diagrams for the number of mOTUs in fungal communities of *M. chejuense* between (A) habitats and between sampling sites for (B) soil, (C) root, and (D) sporophore. The number of OTUs were calculated using the considerable abundant OTUs (>0.5% in at least 2 samples) after rarefication (14,400 reads).

sampling sites (32.6%), while root (16.0%) and sporophore (17.3%) samples had a lower proportion of shared OTUs (Fig. 2B–D). The number of unique OTUs for sample sites was low in soil and root relative to the shared ones (Fig. 2B–C), whilst the same occur for site S1, S4 and S5 for sporophores and the opposite for S2 and S3, that showed a higher proportion of unique species than shared ones (Fig. 2D).

All diversity indices were significantly lower in roots and sporophores than from soil ($P < 0.01$) (Fig. 3A). Among sampling sites, most samples showed similar values of alpha diversity indices, while S3 showed significantly lower diversity than both S2 and S4 samples ($P < 0.05$) and showed significantly lower evenness compared to S2, S4, and S5 samples ($P < 0.05$) (Fig. 3B). In a case of S4, the evenness was significantly higher than it of S1 site ($P < 0.05$).

3.2. Community structures of fern-associated fungi

Fungal community structures were compared between habitats and sampling sites using an ordination analysis (Fig. 4A). The PERMANOVA analysis showed that fungal community composition was significantly different between habitats ($R^2 = 12.0\%$, $P = 0.001$) and sampling sites ($R^2 = 20.5\%$, $P = 0.001$). Variation partition analysis showed that the explanatory power of sampling sites ($R^2_{adj} = 0.19$) was higher than for habitats ($R^2_{adj} = 0.12$), and there was no shared portion of variation (Fig. 4B). Betadisper analysis showed a significant difference in the dispersion of communities among habitats ($F = 8.136$, $P < 0.001$), and root communities had significantly higher variance than soil and sporophore communities (Supplementary Fig. 2). However, the dispersion of communities was not significantly different between sampling sites. Ascomycota, Basidiomycota, Glomeromycota, Mortierellomycota, and Rozellomycota were abundant (>1% in total abundance) (Fig. 4C). Among them, Ascomycota was most abundant (49.2–99.1%), followed by Basidiomycota (0.4–50.8%) and Mortierellomycota (0.0–20.9%). The sequence abundance of Glomeromycota was typically low (0.0–4.8%), but Glomeromycota was consistently more abundant in roots (0.9–4.8%) than in soil (0.3–0.9%) and sporophores (0.0%).

A total of 19 taxa, mostly at order level, were abundant (>1%) among all habitats and sites (Fig. 4D). The pattern of abundant major taxa was different between habitats. In soil samples, Chaetothyriales (18.1%) was the most abundant, followed by Sordariales (11.1%) and Geoglossales (10.2%). In root samples, Chaetothyriales (27.0%) was the most abundant, followed by Sordariomycetes OTU-11 (12.1%) and Helotiales (10.0%). In sporophore samples, Pleosporales (45.7%) was the most abundant, except for the sites S2 (Cystofilobasidiales, 35.2%) and S3 (Chaetothyriales, 90.0%).

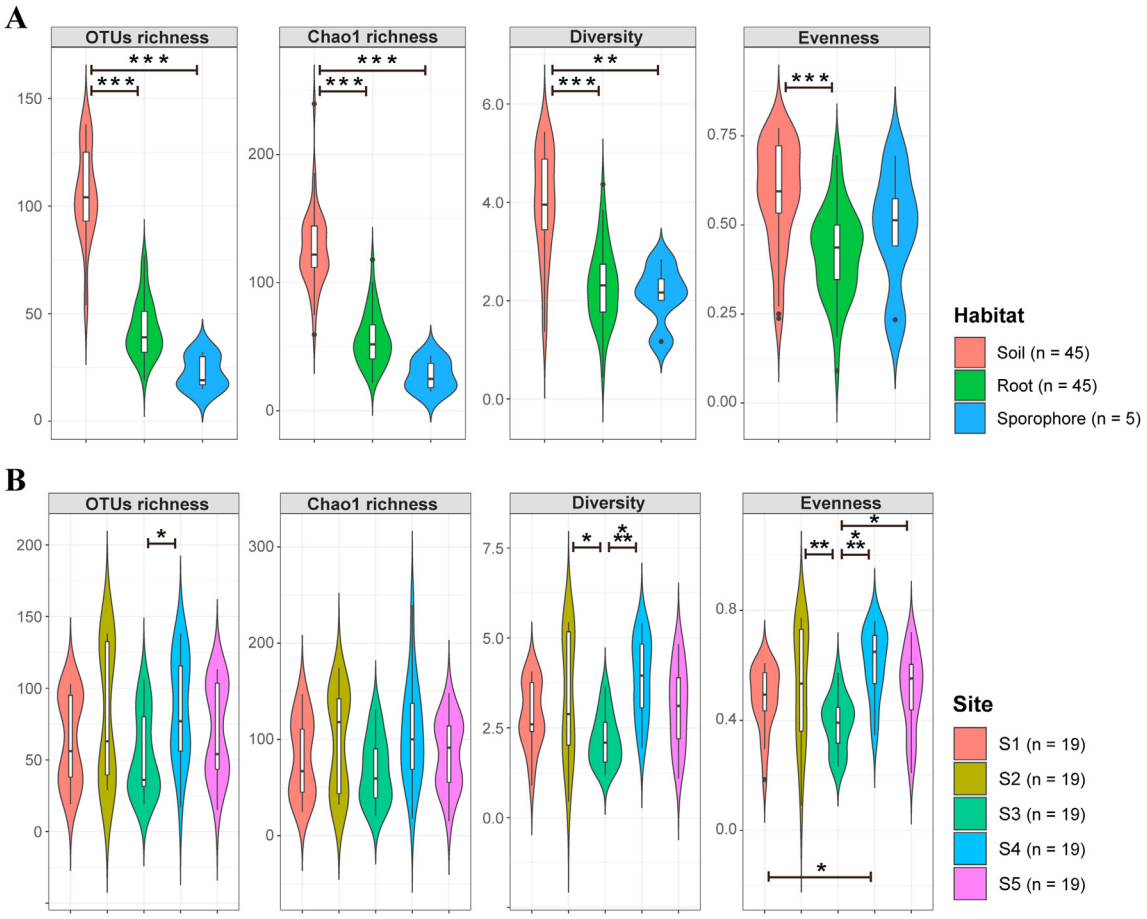

Fig. 3. Alpha diversity of fungal communities from *M. chejuense* between (A) the habitats and (B) the sampling sites. The density of values is shown as violin shape along the y-axis. Boxes represent the range of values from first-quartiles to third-quartiles with the median as the thick horizontal line. The number of OTUs were calculated using the considerable abundant OTUs (>0.5% in at least 2 samples) after rarefication (14,400 reads). OTUs richness: the number of OTUs as a richness estimation, Chao1 richness: Chao1 index, Diversity: Shannon's diversity, Evenness: Shannon's equitability.

Indicator species (OTU level) were detected for root and sporophore samples (Table 1). In root samples, Glomeraceae (4 OTUs) and Sordariomycetes (3 OTUs) were frequently detected. In root and soil samples, however, species of *Mortierella* (4 OTUs) were more frequently detected than sporophore samples. In sporophore samples, six OTUs belonging to Plesporales (*Didymella* - two OTUs, Massarinaceae, *Paraboeremia*, *Pyrenochaetopsis*, and *Stagonospora*) were frequently detected, and species of *Cyphellophora* and *Articulospora* were identified as indicator species. BLAST against NCBI GenBank showed that many indicator species matched highly with the sequences from the roots of various plants (liverwort, lycopod, and orchid species) and obligate or partial mycoheterotrophic plants (Supplementary Table 1).

4. Discussion

Unlike with other plants, the actual fungal diversity associated with the ferns is little studied, and diverse fungi are expected to be detected from the fern tissue, if a metabarcoding approach is used. As expected, our results showed high fungal diversity in roots and sporophores of *M. chejuense*. Also, fungal communities were significantly different between habitats and sampling sites. Many fungi, mostly ascomycetes, were specifically detected as an indicator species of the roots and sporophores, and they were suspected to have a specific relationship with *M. chejuense*.

4.1. Overall fungal diversity associated with M. chejuense

The diversity and community structure of fungi associated with *M. chejuense* are distinguished by both habitat and sampling site, which agrees with previous studies on the pattern of plant endophytes (Mishra et al., 2012; Del Olmo-Ruiz and Arnold, 2014; Li et al., 2020). Both alpha diversity and community composition differed significantly among habitats (soil, roots, and sporophores). Alpha diversity was lower from roots and sporophores than from the surrounding soil, which is consistent with previous studies comparing fungal diversity between plant roots and bulk soil (Goldmann et al., 2016; Oh et al., 2018). Generally, root-associated fungi are thought to be recruited from the surrounding soil (Jumpponen and Egerton-Warburton, 2005; Goldmann et al., 2016), and this seems to be the case for *M. chejuense* since 98.5% of fungi detected from roots were also found in soil samples. As most of the OTUs shared between roots and sporophores were also detected in soil, it is difficult to say whether these fungi were vertically transferred or not. Lower richness in the sporophore samples can result from short life times as a reproductive organ compared to roots, which agrees with the pattern of fungal endophytes in the fern pinnae showing lower richness in younger tissues (Younginger and Ballhorn, 2017).

Fungal communities were also significantly different between sampling sites, while alpha diversities were generally comparable. A previous study focusing on *Fagus sylvatica* trees showed that

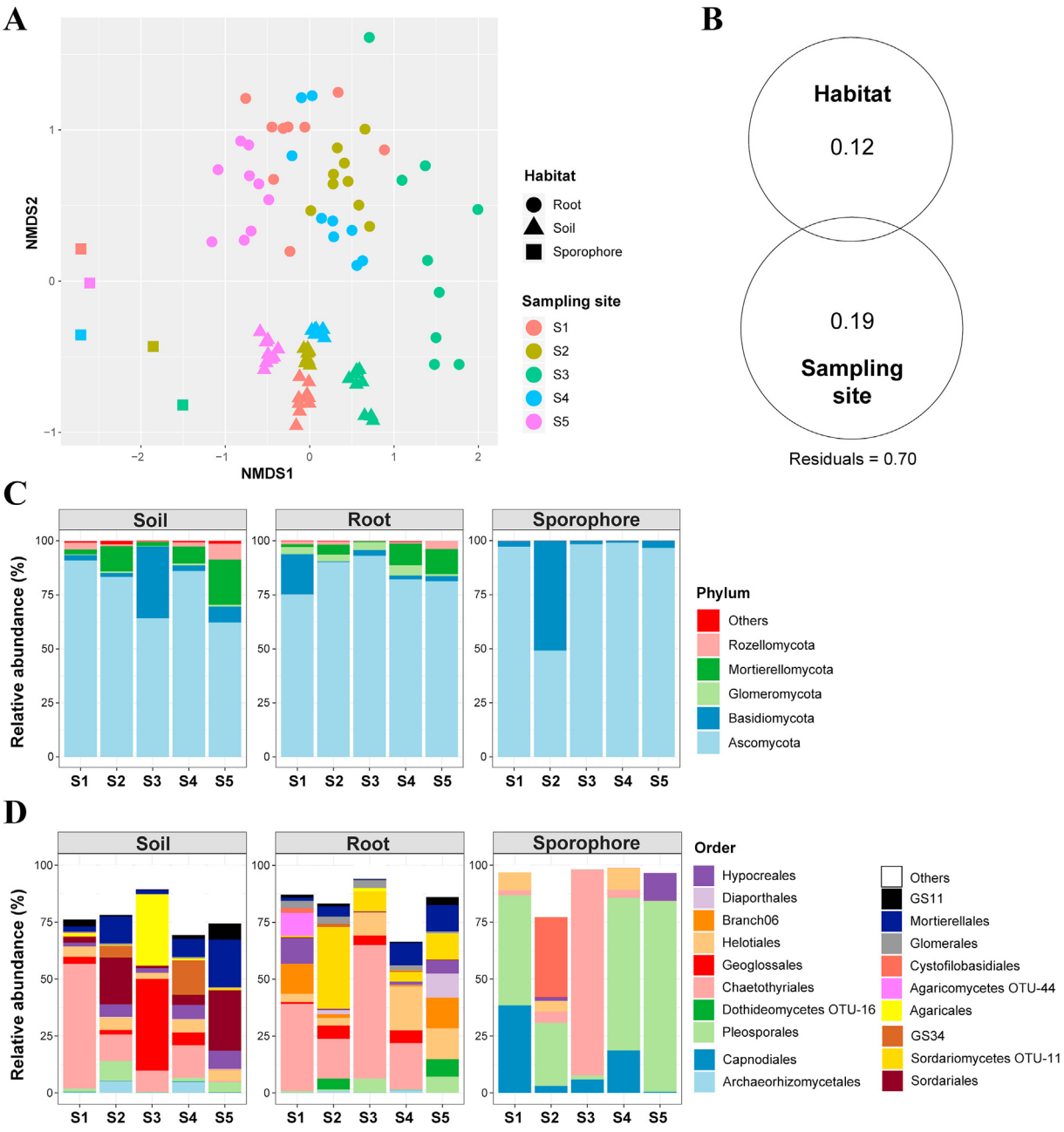

Fig. 4. Fungal community structures associated with *M. chejuense*. (A) NMDS plots based on Bray-Curtis dissimilarity. (B) Variation partitioning plot for effect size of habitat and sampling site. The relative abundance of (C) major phyla and (D) major orders. Taxa whose total abundance is more than 2% in each habitat were chosen as major taxa. OTUs with that high abundance (>2%) were included in the plot for a major order. Taxonomy hierarchy followed the UNITE database system. Branch06 belongs to Sordariomycetes, GS11 to Rozellomycota, and GS34 to Xylonomycetes.

spatial variation of root communities was lower because roots shape the specific environment and select specific communities (Goldmann et al., 2016). Here, in contrast, differences in OTU composition among sites were larger from roots and sporophores than from soil; Betadisper analysis also showed that community variance was higher in root communities. This may be explained by high genetic differentiation rates of the *M. chejuense* population (Chung et al., 2010). It has been reported that host genotype can influence the endophytic communities (Lamit et al., 2015; Albrectsen et al., 2018). Therefore, low dispersal rates of *M. chejuense* may have led to specialization of fungal communities associated with roots and sporophores. On the other hand, high spatial variation of fungal OTUs from roots and sporophores may indicate that *M. chejuense* does not form stable associations with fungi and/or that there is a wide range of taxa with which it can interact. Moreover, endophytic communities from roots and sporophores may be more heavily influenced by stochastic processes than fungal communities in soil because they need successfully to pass into the tissue systems, which can increase the spatial variation of fungal communities from root and sporophore compared to soil.

A total of 236 OTUs were detected, a higher diversity than found in previous studies investigating fern-associated fungal diversity (West et al., 2009; Rimington et al., 2015; Ogura-Tsujita et al., 2016; Lehnert et al., 2017). Similar to our results, previous studies using metabarcoding reported 264 OTUs from the pinae of *Polystichum*

Table 1
Indicator species of endophytic communities in *M. chejuense*. Frequency represents the number of occurrences in each habitat, and abundance represents the proportion of the sequence read number in each habitat (e.g. the OTU read number/the total read number of each habitat). Host information is acquired from the BLAST analysis, and detailed results are shown in Supplementary Table 1.

Group	OTU ID	Taxa name	Stat	Frequency			Abundance			Host information
				Soil (n = 45)	Root (n = 45)	Sporophore (n = 5)	Soil	Root	Sporophore	
Root	OTU-16	Dothideomycetes	0.666	8	20		p[a]	2.5		
	OTU-21	Herpotrichiellaceae	0.642	9	19		p	3.5		
	OTU-54	*Lachnum pygmaeum*	0.516	1	12		p	1.2		Vascular plant
	OTU-33	Branch06	0.88	27	35		p	5.7		
	OTU-14883	Sordariomycetes	0.644	10	19	1	p	1.0	p	Lycopod, Orchid
	OTU-11	Sordariomycetes	0.964	32	42		p	12.1		Lycopod, Orchid
	OTU-16478	Sordariomycetes	0.494		11			p		Lycopod, Orchid
	OTU-150	Glomeraceae	0.714	18	24		p	0.5		
	OTU-2238	Glomeraceae	0.665	9	21		p	0.1		
	OTU-5342	Glomeraceae	0.656	9	21		p	p		
	OTU-264	Glomeraceae	0.659	5	20		p	p		
Root + Soil	OTU-36	Dothideomycetes	0.888	42	29		p	1.5		
	OTU-17	Herpotrichiellaceae	0.809	37	22	1	2.5	1.5	p	Vascular plant
	OTU-38	*Rhexocercosporidium*	0.949	43	38		0.3	1.6		Orchid, Vascular plant
	OTU-115	*Tolypocladium album*	0.874	41	28	1	0.5	0.1	p	Orchid
	OTU-117	*Mortierella amoeboidea*	0.931	42	36		0.1	0.7		
	OTU-56	*Mortierella horticola*	0.937	45	34		0.8	1.5		
	OTU-14	*Mortierella rishikesha*	0.989	45	43	2	6.4	1.6	p	Vascular plant
	OTU-185	*Mortierella*	0.81	36	23		p	0.2		
	OTU-69	GS11	0.919	42	34		0.9	0.3		
Sporophore	OTU-34	*Cladosporium flabelliforme*	0.995	35	11	5	0.1	p	12.1	Fern, Vascular plant
	OTU-12946	*Cladosporium flabelliforme*	0.994	13	2	5	p	p	0.4	Orchid, Vascular plant
	OTU-121	*Pyrenochaetopsis leptospora*	0.768	21	7	3	p	p	1.3	Orchid, Vascular plant
	OTU-1981	*Didymella dimorpha*	0.994	10	6	5	p	p	1.4	Orchid, Vascular plant
	OTU-2474	*Didymella rumicicola*	0.996	10	10	5	p	p	2.5	Lycopod, Orchid, Vascular plant
	OTU-12902	*Paraboeremia selaginellae*	0.993	13	5	5	p	p	0.9	Lycopod
	OTU-43	*Stagonospora*	0.632	5	3	2	p	p	3.8	Moss
	OTU-23	Massarinaceae	0.999	22	6	5	p	p	14.0	Liverwort, Orchid, Vascular plant
	OTU-31	*Cyphellophora gamsii*	0.894	5	3	4	p		11.6	Orchid
	OTU-122	*Cyphellophora sessilis*	0.998	5	5	5	p	p	1.3	
	OTU-61	*Articulospora*	0.893	9	5	4	p	p	3.1	Orchid
	OTU-4597	*Articulospora*	0.631	1	1	2	p	p	0.5	Orchid
	OTU-505	*Fusarium*	0.621	16	1	2	p	p	0.3	Orchid, Vascular plant
	OTU-109	*Hyphodontia*	0.904		20	5		0.4	2	Orchid
	OTU-151	*Hymenochaete floridea*	0.858	2	14	4	p	p	0.7	
	OTU-175	*Hannaella*	0.97	14	12	5	p	p	0.6	Vascular plant

[a] p: rarely present (<0.01%).

munitum (Younginger and Ballhorn, 2017), a much higher number than previously reported using culture-dependent methods showing 2–95 species (Petrini et al., 1992; Raviraja et al., 1996; Swatzell et al., 1996; Sati and Belwal, 2005; Del Olmo-Ruiz and Arnold, 2014). Although more parts of the fern (roots and sporophre) were used, the number of OTUs detected from this study were lesser than it from fern pinnae (Younginger and Ballhorn, 2017), likely due to the difference of filtering procedure, only retained OTUs with >0.5% of abundance in at least two samples, or the difference of the fern species. Many previous studies reported that AMF associate with ferns (Read et al., 2000; Pressel et al., 2016; Lehnert et al., 2017; Hoysted et al., 2018). In the present study, however, sequences of AMF (Glomeromycota) comprised less than 5% of the fungi in roots of *M. chejuense*. We think this result may be partly due to the primers used for PCR amplification because ITS3 and ITS4 have mismatches for some members of Glomeromycota (Stockinger et al., 2010; Tedersoo et al., 2015).

On the other hand, endophytes were detected at high abundances in both roots and sporophores. Ascomycota was the most dominant phylum from all sampling sites, followed by Basidiomycota and Mortierellomycota. At the OTU level, the number of endophytes (225 OTUs) was 20-fold higher than that of AMF (11 OTUs). Although endophytes are often detected in fern roots with AMF (Petrini et al., 1992; Raviraja et al., 1996; Horton et al., 1998; Sati and Belwal, 2005; Muthukumar and Prabha, 2013), they were not found in Ophioglossaceae ferns (Pressel et al., 2016; Lehnert et al., 2017). The major endophytes of *M. chejuense* were members of Capnodiales, Chaetothyriales, Helotiales, and Pleosporales. Similar fungal composition was reported from other plants: Nothofagaceae leaf (Johnston et al., 2017), *Quercus* root (Toju et al., 2013), young pinae of fern *Polystichum* (Younginger and Ballhorn, 2017), strobili of lycopod *Lycopodium* (Pawłowska et al., 2014), and shoots of mosses (Kauserud et al., 2008). In previous studies, endophytes in ferns seem to be underestimated because they are difficult to observe under a microscope (Barrow, 2003; Barrow and Aaltonen, 2001; Yu et al., 2001).

4.2. Indicator species in roots and sporophores of M. chejuense

Many endophytic fungi were detected as indicator species with a small number of AMF. Four AMF OTUs belonging to Glomeraceae were identified as indicator species of roots. Although total abundance was low, the frequency of occurrence was high, suggesting an intimate interaction between AMF and *M. chejuense*, as reported by studies of other ferns (Pressel et al., 2016; Lehnert et al., 2017). Endophytic fungi were also frequently detected such as Herpotrichiellaceae sp. (OTU-21 and OTU-17) and *Cyphellophora* spp. (OTU-31 and OTU-122). Several species belonging to Herpotrichiellaceae (*e.g.*, *Cladophialophora*, *Exophiala*, and *Phialophora*) are known as dark septate endophytes (DSE) (Jumpponen and Trappe, 1998; Usui et al., 2016) and detected from the roots of several plants (Davey and Currah, 2007; Takashima et al., 2014). For example,

Cladophialophora species were isolated from roots of lycopod species in the genus *Huperzia* (Takashima et al., 2014). *Cladophialophora minutissima* was isolated from the gametophytes of moss species *Polytrichum juniperinum, Sphagnum fuscum*, and *Aulacomnium palustre* (Davey and Currah, 2007). *Cyphellophora* spp. (OTU-31 and OTU-122) were detected from sporophore samples, and *Cyphellophora* were isolated from roots of herbaceous plants such as *Juncus inflexus* and *Polygonum chinense* (Liu et al., 2017). Any mutualistic functions of non-AMF fungi associated with *M. chejuense* are unclear, although the increasing body of evidence suggests that DSE can form mutualisms with host plants (YuanlinZhanglong and cheng, 2010; Newsham, 2011), promoting the growth of roots and shoots (Berthelot et al., 2016), supplying nutrients or water (Mandyam and Jumpponen, 2005), protecting against plant pathogens (Wang et al., 2016), or increasing resistance against environmental stress such as drought or salinity (Santos et al., 2017). The role of edophyte in *M. chejuense* may be of latent decomposers; many endophytes exist in living tissue without symptoms, but they have been shown to start to decompose leaves or roots after they die (Voriskova and Baldrian, 2013; Guerreiro et al., 2018; Kohout et al., 2018).

Saprotrophic fungi were also frequently identified as indicator species in *M. chejuense*, especially in the sporophores. *Cladosporium, Didymella,* and *Paraboeremia* were most frequently detected in the endophytic communities of sporophores. These genera are also endophytes of other plant species (Marrs and Watt, 2006; Park et al., 2018; Tan et al., 2018). For example, *Paraboeremia* was found in the orchid roots, and were especially dominant in *Calanthe* species on Jeju Island, South Korea (Park et al., 2018). Although these genera are well-known plant pathogens (Grube et al., 2011; Lima et al., 2013; Jiang et al., 2017), it is unclear what their role is in *M. chejuense*. Previous studies showed that plant pathogenic fungi can also be present as non-pathogenic endophytes depending on both host species and environmental conditions (Backman and Sikora, 2008; Vieira et al., 2014), and even be beneficial to host plants (Hiruma et al., 2016; Wang et al., 2016). Further studies are needed to identify the exact role of these fungi in *M. chjuense*.

The metadata of sequences in GenBank matched by BLAST can expand the knowledge of ecological information of the endophytic fungal communities in *M. chejuense*. Many indicator OTUs matched to endophytic fungi of several vascular plant species such as *Carex*, Fagaceae, *Lonicera*, and *Rhododendron*. Endophytes from non-vascular plants were also detected; Sordariomycetes (OTU-11, OTU-14883, and OTU-16478), *Paraboeremia selaginellae* (OTU-12902), and Massarinaceae (OTU-23) were matched to leaf or root endophytic fungi from the lycopods (*Huperzia serrata* and *Selaginella kraussiana*) and liverwort (*Lophocolea* species). The orchid was the most frequently matched host species, and 15 OTUs were associated with the fungi from *Anoectochilus roxburghii*, *Gymnadenia nigra*, *Platanthera* species, and mycoheterotrophic *Gastrodia* species. These results suggest that endophytic communities of *M. chejuense* are shared with those of other plants, especially orchids. However, BLAST using ITS sequence cannot guarantee the identity between OTUs and match results, thus future confirmation is needed by long-read sequencing or a culture-dependent method.

4.3. Conservational implications of fungal diversity in M. chejuense

Several ferns exhibit mycoheterotrophy, the uptake of nutrients from associated fungi (Merckx, 2013; Pressel et al., 2016). In the Ophioglossaceae, to which *M. chejuense* belongs, mycoheterotrophic characteristics were found in gametophytes of *Botrychium* and *Ophioglossum*. Previous studies showed that young gametophytes of *Ophioglossum crotalophoroides* were free of fungal endophytes, thus endophytes may be recruited from the soil after germination (Mesler, 1976). However, mycorrhizal fungi are critical in the early growth of *Ophioglossum*, as the organisms cease to develop past the 3–4 cells status without mycorrhizal fungi (Whittier, 1984). In addition, sporophytes of *Ophioglossum vulgatum* can absorb nutrients from AMF *Glomus* species (Field et al., 2015), which implies that AMF is important to both gametophytes and sporophytes of Ophioglossaceae species. Gametophytes of *M. chejuense* have not been found, and there is no report of successful spore germination in axenic cultures (Kim, 2004). However, the diverse fungi observed in *M. chejuense* suggests that some of these can be a candidate for further research to find a fungal symbiont influencing on the physiology of *M. chejuense*. In addition, the high level of spatial heterogeneity observed in *M. chejuense* populations suggests that there has been long-term differentiation between populations (Chung et al., 2010) and that fungal-*M. chejuense* interactions are specialized based on geographical differences. Considering the endangered status of *M. chejuense,* the growth of gametophytes in axenic culture and transplantation of sporophytes are both important to conservation. Therefore, further studies on the role of the endophytic fungi found in this study will be useful for the successful conservation of *M. chejuense*.

5. Conclusions

In this study, we investigated the fungal diversity associated with *M. chejuense* using a metabarcoding approach and found a high abundance of ascomycetes from both roots and sporophores. Because this is the first study of fern-associated fungal diversity from the roots and sporophores using HTS technology, it is unclear if this extraordinary community is unique to *M. chejuense* or is a result of high-resolution HTS analysis. Nonetheless, our results suggest that non-mycorrhizal fungi as well as AMF might play an important role in fern ecophysiology. Furthermore, this investigation using metabarcoding improves our general understanding of the ecology and evolution of fungi-plant interactions. Finally, our findings make important contributions to the conservation of this endangered species since it shows a high diversity of fungi associated to the fern tissues, suggesting important functions for the fern species investigated.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.funeco.2020.101038.

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