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Diversity of fungi associated with roots of *Calanthe* orchid species in Korea

Myung Soo Park[1], John A. Eimes[2], Sang Hoon Oh[3], Hwa Jung Suh[3], Seung-Yoon Oh[1], Seobihn Lee[1], Ki Hyeong Park[1], Hyuk Joon Kwon[4], Soo-Young Kim[4], and Young Woon Lim[1]*

[1]*School of Biological Sciences and Institute of Microbiology, Seoul National University, Seoul 08826, Republic of Korea*
[2]*University College, Sungkyunkwan University, Suwon 16419, Republic of Korea*
[3]*Department of Biology, Deajeon University, Daejeon 34520, Republic of Korea*
[4]*National Institute of Biological Resources, Incheon 22689, Republic of Korea*

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While symbiotic fungi play a key role in the growth of endangered *Calanthe* orchid species, the relationship between fungal diversity and *Calanthe* species remains unclear. Here, we surveyed root associated fungal diversity of six *Calanthe* orchid species by sequencing the internal transcribed spacer (ITS) region using 454 pyrosequencing. Our results revealed that *Paraboeremia* and *Coprinopsis* are dominant fungal genera among *Calanthe* species. In terms of overall relative abundance, *Paraboeremia* was the most common fungal genus associated with *Calanthe* roots, followed by *Coprinopsis*. Overall fungal diversity showed a significant degree of variation depending on both location and *Calanthe* species. In terms of number of different fungal genera detected within *Calanthe* species, *C. discolor* had the most diverse fungal community, with 10 fungal genera detected. This study will contribute toward a better understanding of those fungi that are required for successful cultivation and conservation of Korean *Calanthe* species.

Keywords: *Calanthe*, fungal diversity, next-generation sequencing, orchidaceae, orchid-related fungi

Introduction

The orchid genus *Calanthe* belongs to largest subfamily, the Epidendroideae, in the Family Orchidaceae, with about 21,000 species. *Calanthe* species are distinguished by their stunning morphological characteristics, including large white, orange, and yellow flowers often in tall, erect or arching inflorescence with large, plicate leaves (Teoh, 2005; Chen *et al*., 2009; Clayton and Cribb, 2013; Zhai *et al*., 2014). The *Calanthe* have a global distribution with approximately 150 species described worldwide; however, most are found in tropical and subtropical regions. Approximately 70 species of *Calanthe* are distributed in China, Taiwan, Japan, and Korea (Gale and Drinkell, 2007; Clayton and Cribb, 2013). In Korea, six species have been described in the southern regions of the peninsula as well as on Jeju Island, which is approximately 90 km from the mainland (Lee, 2013).

The *Calanthe* represent a significant economic resource for several reasons. First, many species of *Calanthe* are popular ornamental plants due to their impressive array of flower colors, attractive fragrance and a long history of cultivation (Kim *et al*., 2013). Indeed, *Calanthe* were the first orchids to be artificially hybridized in the 1850's (*Calanthe dominyi*) (Arditti *et al*., 1984). In addition, some *Calanthe* species have medicinal applications including anti-inflammatory, antibacterial, and anti-toxic activities (Yoshikawa *et al*., 1998). Moreover, methanolic extracts of *Calanthe* species have been used to promote blood circulation (Yoshikawa *et al*., 1998). Despite the important economic and health benefits of *Calanthe* species, cultivation of many species has proven to be difficult and many useful species are increasingly rare in the wild due to habitat disturbance and climate change, among other factors. Recently, several species of *Calanthe* have been categorized as endangered, and the decline in their populations has been attributed primarily to habitat loss and overharvesting (Lee and Choe, 2006). For example, two studies reported that over 90% of primary forest on the Malay Peninsula was destroyed in early twentieth century which led to the near extinction of many endemic species, including two *Calanthe* species (Corlett, 1991; Turner *et al*., 1994). Extinction risk has become a critical problem in South Korea as well. Among six reported *Calatnthe* species in Korea (*C. arisulifera*, *C. bicolor*, *C. discolor*, *C. kibanakirishima*, *C. reflexa*, and *C. sieboldii*) (Hyun *et al*., 1999a, 1999b; Kim *et al*., 2013), *C. discolor*, *C. sieboldii*, and *C. reflexa* were categorized as endangered species by the Ministry of Environment and the Korea Forest Service in 1997 (Lee, 2009).

The uncertain status of *Calanthe* species in the wild has spurred interest conservation. Protective measures that have been proposed include large scale cultivation in shade houses, restoration in natural forests (Liu *et al*., 2014), and genetic intervention to maintain higher diversity within species (Qian *et al*., 2013). These strategies, however, are problematic for several reasons. First, artificial cultivars produce products of lower value than those of wild orchids. Second, a basic lack of understanding of the orchid life cycle makes construction of optimal shade houses difficult (Arditti *et al*., 1990; Liu *et*

*For correspondence. E-mail: ywlim@snu.ac.kr; Tel.: +82-2-880-6708; Fax: +82-2-871-5191

al., 2014).

Several studies have focused on symbiotic fungi associated with *Calanthe* species in order to improve their cultivation and conservation. Several fungal genera are known to be symbiotic with orchid species, including *Armillaria*, *Erythromyces*, and *Rhizoctonia* (Terashita and Chuman, 1987; Otero *et al*., 2002; Yagame *et al*., 2007). Many of these fungal symbionts are important during the seed germination phase of orchids (Umata, 1995; Yagame *et al*., 2007). For example, several mycorrhizal fungi including *Ceratorhiza pernacatena* and *Epulorhiza repens*, were shown to be important symbionts of *C. rubens* and *C. rosea* (Athipunyakom *et al*., 2004). While studies of fungal diversity associated with *Calanthe* have been reported in other countries, little is known about fungal diversity associated with Korean species, and only an unknown *Tulasnella* species has been isolated from root tissue of *C. discolor* collected from Jeju Island (Youm *et al*., 2012). An improved understanding of native Korean fungus-orchid associations is crucial for cultivation of endangered or threatened *Calanthe* species as habitat loss and climate change continue to threaten the survival of native Korean orchid species.

The initial step for identification of fungi has traditionally been morphological analyses and culture methods. However, these methods have limitations since most of fungi associated with orchids cannot be cultured and morphological ambiguity makes identification of species unreliable. To overcome these limitations, molecular methods such as PCR-RFLP, t-RFLP, DGGE, and cloning have been used for more recent identification of fungi associated with host roots (Seifert, 2009; Swarts *et al*., 2010). Moreover, the more recent development of Next Generation Sequencing (NGS) has greatly improved the accuracy and efficiency of fungal diversity studies (Huang *et al*., 2014). In this study, we used NGS of the internal transcribed spacer region (ITS) to describe the fungal diversity associated with the roots systems of six different *Calanthe* species native to Korea: *C. aristulifera*, *C. bicolor*, *C. discolor*, *C. insularis*, *C. striata* and an unidentified species of *Calanthe*.

Materials and Methods

Collection and sample preparation

Twelve *Calanthe* individuals were collected during the flowering stage from six islands of the western and southern coastal areas in Korea. These specimens were identified as six *Calanthe* species: *C. aristulifera*, *C. bicolor*, *C. discolor*, *C. insularis*, *C. striata*, and an unknown *Calanthe* species (*Calanthe* sp.) (Fig. 1). One individual from four species was collected from Gageodo (GG), Hongdo (HD), and Jindo (JD). Three individuals of *C. striata* were collected from two islands: Gageodo (GG) and Hongdo (HD), and five individuals of *C. discolor* were collected from four sites: Jejudo (JJ), Geojedo (GJ), Jindo (JD), and Anmyeondo (AM) (Table 1). Since most wild *Calanthe* orchids are threatened or vulnerable species, limited samples were collected. Sampling was permitted by the National Park Authority of Korea. Orchid specimens were kept in the herbarium at Daejeon University and fresh root samples were transferred to the laboratory at Seoul National University on ice at 4°C for later DNA extraction. Roots were gently washed with running water to remove any debris and sterilized with 1% sodium hypochlorite for 2 min and then rinsed three times for 5 min with sterilized water. Next, surface-sterilized roots were cut into 5 cm fragments and air-dried.

DNA extraction, PCR, sequencing, and bioinformatics

Three fragments per 5-cm segment of root sample were ground with a mortar and pestle using liquid nitrogen. Genomic DNA was extracted using a modified CTAB extraction protocol described by Rogers and Bendich (1994). The ITS region was amplified using the primers ITS1F and ITS4 (White, 1990). Amplicon libraries were prepared using primers with a 454 pyrosequencing adaptor and multiple identifier (MID) tag. Each PCR was performed on a C1000TM thermal cycler (Bio-Rad) using the AccuPower PCR PreMix (Bioneer) in a final volume of 20 μl containing 10 pmol of each primer and 2 μl of DNA. PCR conditions included an initial denaturing step at 95°C for 5 min followed by 30 cycles of 95°C for 40 sec, 55°C for 40 sec, and 72°C for 1 min, and a final extension step of 72°C for 10 min. PCR products were electrophoresed through a 1% agarose gel with LoadingSTAR (Dyne Bio). To minimize PCR bias, three PCRs were pooled for each sample. PCR products were purified using the Expin PCR Purification Kit (GeneAll Biotechnology Co., Ltd.), and DNA concentrations were quantified using a NanoDrop ND-1000 spectrophotometer (Thermo).

Pyrosequencing was performed with flow pattern B on ¼ plate of a 454 GS FLX + System (Roche 454 Life Sciences) and sequenced in the reverse direction. All steps after library construction were performed at Macrogen. Raw sequence data have been deposited in the Sequence Read Archives

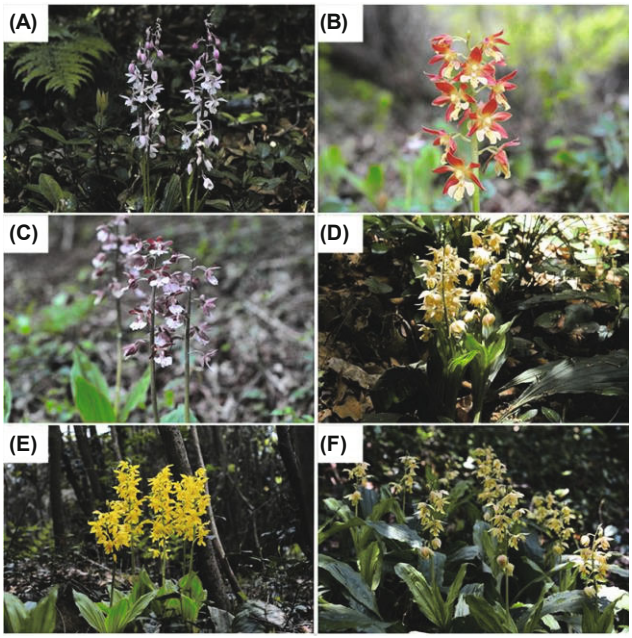

Fig. 1. Photographs of *Calanthe* in Korea. (A) *C. aristulifera*, (B) *C. bicolor*, (C) *C. discolor*, (D) *C. insularis*, (E) *C. striata*, (F) *Calanthe* sp.

Table 1. Sequence and Collection taxon information of *Calanthe* specimens used in this study

Calanthe Species	Seq Nos.	mOTU	Good's Coverage	Locality	Collection Date
Calanthe aristulifera	5,887	57	0.989	Hongdo-ri, Heuksan-myeon, Sinan-gun, Jeollanam-do (HD1)	May 2015
Calanthe bicolor	2,370	37	0.996	Susan-ri, Seongsan-eup, Seogwipo-si, Jeju-do (JJ1)	April 2015
Calanthe discolor	4,176	23	0.997	Susan-ri, Seongsan-eup, Seogwipo-si, Jeju-do (JJ2)	April 2015
	6,234	22	0.995	Susan-ri, Seongsan-eup, Seogwipo-si, Jeju-do (JJ3)	April 2015
	2,082	43	0.996	Dadae-ri, Nambu-myeon, Geoje-si, Gyeongsangnam-do (GJ)	May 2015
	1,892	47	0.993	Wau-ri, Jisan-myeon, Jindo-gun, Jeollanam-do (JD)	May 2015
	2,418	33	0.998	Jungjang-ri, Anmyeon-eup, Taean-gun, Chungcheongnam-do (AM)	May 2015
Calanthe insularis	3,773	39	0.995	Gageodo-ri, Heuksan-myeon, Sinan-gun, Jeollanam-do (GG1)	May 2015
Calanthe striata	2,913	44	0.993	Gageodo-ri, Heuksan-myeon, Sinan-gun, Jeollanam-do (GG2)	May 2015
	2,980	33	0.996	Gageodo-ri, Heuksan-myeon, Sinan-gun, Jeollanam-do (GG3)	May 2015
	5,304	49	0.991	Hongdo-ri, Heuksan-myeon, Sinan-gun, Jeollanam-do (HD2)	May 2015
Calanthe sp.	4,668	45	0.994	Hongdo-ri, Heuksan-myeon, Sinan-gun, Jeollanam-do (HD3)	May 2015

(Fritz *et al*., 2011) of NCBI under the accession number PRJNA381139.

Sequence reads from the library were trimmed and filtered with the following adjustments using QIIME v1.8.0 (Caporaso *et al*., 2010): minimum length of 300 bp, maximum length of 1,000 bp, minimum quality score of 25, no ambiguous bases, no mismatches in the primer sequence, and maximum homopolymer length of 6 bp. We applied these strict filtering requirements to minimize the effect of amplicon noise. Chimera detection and clustering were performed using USEARCH v5.2.236 (Edgar, 2010) implemented in QIIME. Molecular operational taxonomic units (mOTUs) were constructed with the QIIME clustering algorithm "average linkage" at 97% sequence similarity, and representative sequences were selected based on the sequence abundance. Singletons (mOTUs represented by a single sequence) were removed from analyses. BLAST 2.2.30 + (Altschul *et al*., 1997) was used to assign representative mOTU sequences to the most specific taxonomic designation possible (e.g. species, genus, family, order) against the UNITE v7.1 (22.08.2016) database (Koljalg *et al*., 2013) and sequences from the Seoul National University Fungal Collection.

Results

A total of 79,995 reads (range: 3,797–9,571 per amplicon) were recovered from 12 *Calanthe* individuals. After filtering, a total of 46,964 reads were used for analysis. For each sample, the number of sequences recovered after filtering ranged from 1,892–6,234 (mean = 3,612) (Table 1). In order to estimate relative abundance of fungal mOTUs associated with each sample, we normalized the read count to 1,800. Trend of diversity between samples did not change after normalization. The number of mOTUs per sample ranged from 22–57 (mean = 39, total = 151) (Table 1). For most cases, the taxonomic analysis of the mOTU from each sample allowed for classification into genus. A total of 70 fungal genera were identified among all the *Calanthe* samples. However, only 16 mOTUs had over 1% relative abundance: 13 mOTU were

Table 2. Relative abundance (%) of major genera

Major genera were selected based on the relative abundance in the total of samples (≥ 1%).

Phylum	Taxa	Total	*Ca*[a]	*Cb*	*Cd*					*Ci*	*Cs*			*C.*sp.
			HD1	JJ1	GJ	JD	JJ2	JJ3	AM	GG1	GG2	GG3	HD2	HD3
Ascomycota	*Cladosporium*	2.8	0.0	0.8	0.0	9.6	1.3	0.2	2.5	6.6	12.1	0.0	0.0	0.0
	Helotiales sp.	1.4	10.1	0.1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	1.3	4.9
	Hymenoscyphus	2.4	0.0	28.4	0.1	0.0	0.0	0.1	0.0	0.0	0.0	0.0	0.0	0.0
	*Ilyonectria**	3.1	30.4	0.8	0.2	0.0	0.1	0.0	0.0	0.1	0.0	0.0	0.9	4.4
	*Paraboeremia**	38.3	0.2	31.9	5.8	2.6	87.8	94.1	0.0	52.1	2.0	58.2	76.3	48.7
	Penicillium	1.5	0.0	0.0	16.9	0.1	0.0	0.0	0.1	0.1	0.0	0.0	0.4	0.0
	*Pestalotiopsis**	7.3	2.6	2.9	16.6	34.6	0.0	0.3	6.6	0.9	18.8	1.1	2.7	0.8
	Pseudogymnoascus	1.1	0.0	0.0	1.2	1.3	0.0	0.1	1.5	2.0	0.4	6.3	0.4	0.0
	*Pseudopestalotiopsis**	1.3	0.4	0.9	4.3	5.8	0.0	0.0	0.8	0.1	2.7	0.4	0.1	0.2
	Sordariales sp.	2	23.6	0.0	0.2	0.1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.7
	Tetracladium	2.6	0.0	7.4	0.0	0.0	4.7	0.3	0.0	15.6	0.0	3.1	0.0	0.0
Basidiomycota	*Coprinopsis**	22.4	13.3	6.8	42.9	33.4	0.1	0.1	70.8	16.8	52.4	26.4	4.8	1.1
	*Cryptococcus**	1.1	0.8	3.0	0.8	2.9	0.7	0.1	1.0	1.7	1.5	0.1	0.4	0.1
	Guehomyces	2.1	0.0	5.6	3.2	0.1	4.2	3.4	4.9	1.4	0.5	1.4	0.0	0.0
	Stereum	1.4	0.6	0.6	2.9	2.4	0.0	0.0	6.3	0.8	1.7	0.5	0.4	0.0
	Agaricomycetes sp.	2.5	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	29.9

[a] Species: *C. aristulifera* (*Ca*), *C. bicolor* (*Cb*), *C. discolor* (*Cd*), *C. insularis* (*Ci*), *C. striata* (*Cs*), *Calanthe* sp (*C.*sp).

* 6 fungal genera found all *Calanthe* species.

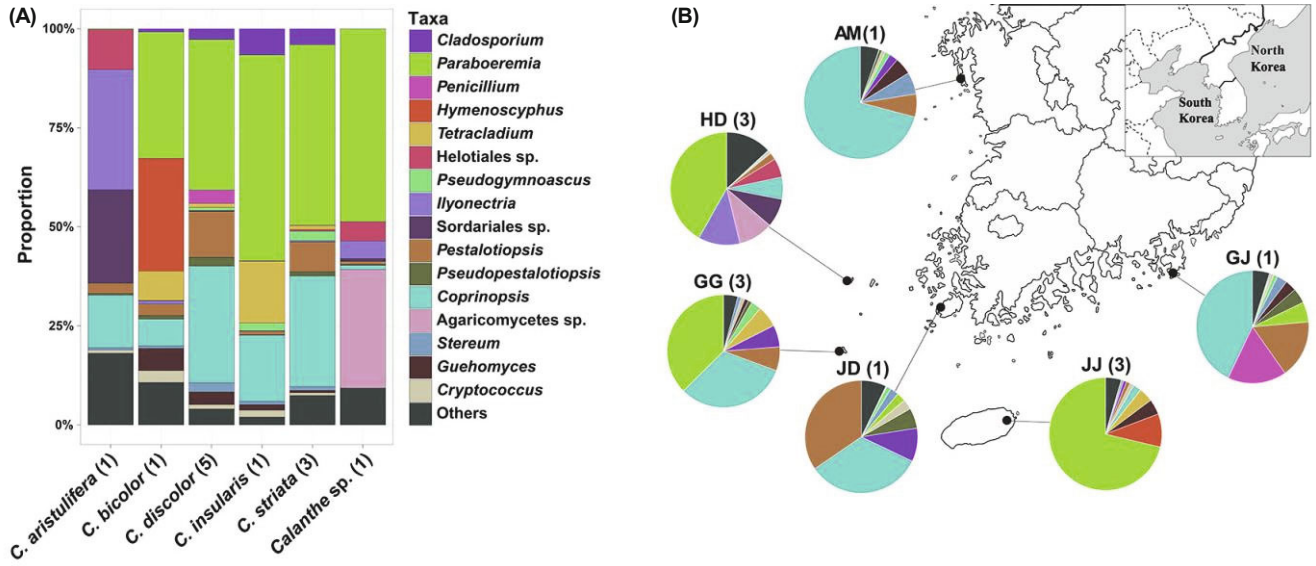

Fig. 2. Relative abundance of major fungal taxa according to *Calanthe* species (A) and locality (B). The numbers shown in parentheses indicate the number of samples.

identified at the genus and three mOTUs were identified at the family or order level. The major fungal genera (> 1% relative abundance) were *Paraboeremia*, *Coprinopsis*, *Pestalotiopsis*, *Ilyonectria*, *Cladosporium*, *Tetracladium*, *Hymenoscyphus*, *Guehomyces*, *Pseudopestalotiopsis*, *Stereum*, *Penicillium*, *Cryptococcus*, and *Pseudogymnoascus* (Table 2). In terms of overall relative abundance, *Paraboeremia* was by far the most common fungal genus associated with *Calanthe* roots, and was the dominant fungal genus in five of the six *Calanthe* species, representing over 30% of reads across these samples (range: 31.9–52.1) (Table 2). The next most common fungal genus was *Coprinopsis*, which was detected in all six *Calanthe* species at greater than 1% relative abundance (range: 1.1–21.5).

With regard to fungal dominance in individual *Calanthe* species, *Paraboeremia* was the dominant fungal genus in all *Calanthe* samples except for *C. aristulifera*, in which *Ilyonectria* was dominant (relative abundance: 30.4%). *Coprinopsis* was the second most dominant fungal genus in three *Calanthe* species (*C. discolor*, *C. insularis*, and *C. striata*). In the *C. bicolor* sample, the *Paraboeremia* and *Hymenoscyphus* were equally dominant, with relative abundances of 31.9% and 28.4%, respectively. In the *C. insularis* sample, *Tetracladium* had a relative abundance of 15.6%, which together with

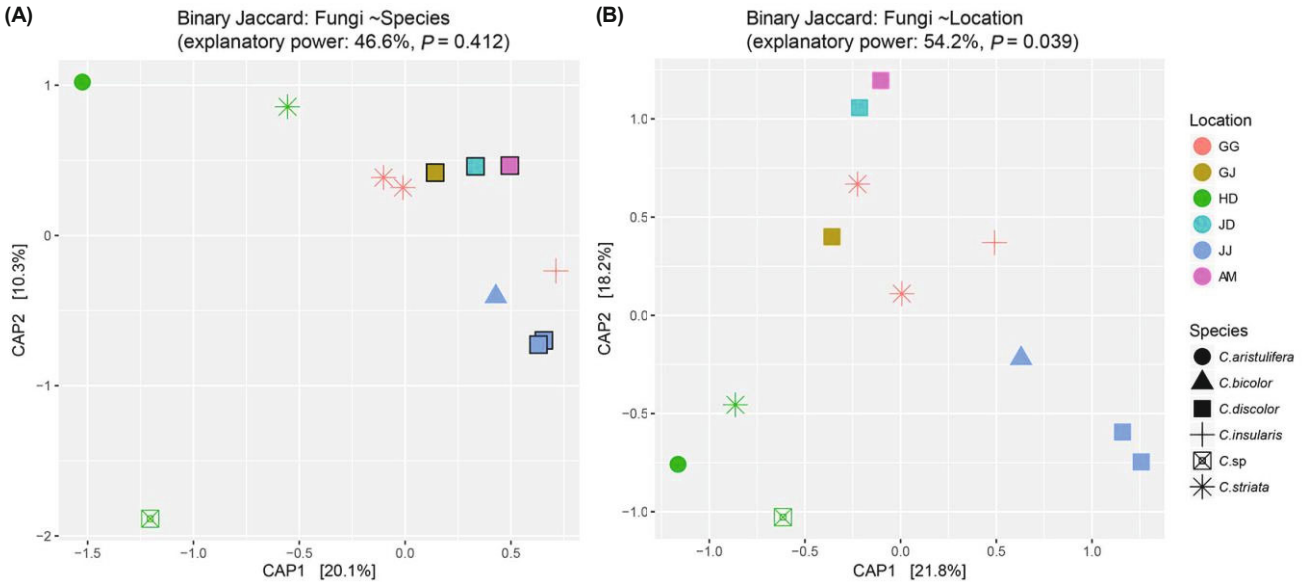

Fig. 3. CAP plots based on binary Jaccard distance with CAP models constrained by *Calanthe* species (A) and location (B). Fungal communities were significantly different between locations ($P = 0.039$), while no difference showed between *Calanthe* species ($P = 0.412$).

Paraboeremia and *Coprinopsis* represented ~85% of the reads detected in this species. *Paraboeremia* was the dominant genus in the unidentified *Calanthe* sample (*Calanthe* sp.) with a relative abundance of 48.7%, although an unidentified fungus in the class Agaricomycetes was also well represented (29.9%) (Table 2 and Fig. 2A).

The most diverse fungal community observed was in *C. discolor*, with 10 fungal genera detected (> 1% relative abundance); although we note that there were five samples of *C. discolor*, and so this may simply be the result of more thorough sampling. The overall fungal diversity among the different *Calanthe* species (> 1% relative abundance) ranged from 5–7 fungal taxa (usually genera, except as noted, Table 2). Within individual samples, one or two fungal genera appeared to be dominant in most cases (Fig. 2A and Table 2).

Some general patterns were also reflected with respect to location. In all sample locations, *Paraboeremia* and *Coprinopsis* were the most dominant fungal genera detected in terms of relative abundance, representing over 60% of total reads (Table 2). *Paraboeremia* was the most dominant in GG, HD, and JJ. *Coprinopsis* was dominant in GG, GJ JD, and TA. *Pestalotiopsis* was dominant in JD (Fig. 2B). While fungal communities were significantly different between locations based on binary Jaccard distance ($P = 0.039$), there was no significant difference in beta diversity between *Calanthe* species (Fig. 3).

Discussion

We surveyed the fungal diversity from the roots of six *Calanthe* orchid species native to Korea using high through-put DNA sequencing**.** Our results revealed two primary patterns regarding fungal diversity associated with these *Calanthe* species. First, there were two dominant fungal genera among *Calanthe* species: *Paraboeremia* and *Coprinopsis*. Second, there appears to be a significant degree of variation depending on both location and *Calanthe* species with respect to fungal presence and dominance. For example, *Paraboeremia* was overwhelmingly dominant in *C. discolor* at two sampling sites of Jeju Island (JJ2 and JJ3) while *Coprinopsis* was dominant in the other three sites (GJ, JD, TA). While four other fungal taxa were detected in all *Calanthe* species, some (e.g. *Pseudopestalotiopsis*) were often detected in only trace amounts (Table 2). Because the *Calanthe* are considered endangered species in Korea, sample size was limited. Despite these small sample sizes, there was notable fungal variation with respect to location and *Calanthe* species.

Paraboeremia species appeared to be the dominant fungal genus among most of the orchid species sampled. mOTUs of *Paraboeremia* aligned closely with *Paraboeremia selaginellae* (GenBank accession number: KT224856; 99–100%), which was previously been found in an endophytic fungal community from another orchid species (*Dendrobium officinale*) (unpublished data). Indeed, only six species of *Paraboeremia* have been described, and while the ecological roles of three of these species are unknown, the other three *Paraboeremia* species were known as plant pathogens (Jiang *et al*., 2017). The next most common fungal genus detected from the *Calanthe* species was *Coprinopsis*. Members in this genus are saprotrophic (Salvioli *et al*., 2016), and *Coprinopsis atramentaria* has previously been found in the roots of chlorophyllous orchids (Ouanphanivanh *et al*., 2013). Some species belonging to *Coprinellus* and *Psathyrella* have been isolated from the achlorophyllous orchid *Epipogium roseum* and are known to improve orchid seed germination rate and development of rhizomes and tubers (Yamato *et al*., 2005; Yagame *et al*., 2007, 2008). It is not surprising that *Coprinopsis* was the dominant fungal genus in *Calanthe* since *Coprinellus* and *Psathyrella* are closely related to *Coprinopsis*; however, further investigation is required to resolve the relationship between *Calanthe* and *Coprinopsis*.

Pestalotiopsis was detected in all *Calanthe* species, and is a well-known plant pathogen (Maharachchikumbura *et al*., 2014). Species in this genus produce various bioactive compounds which have been shown to possess antifungal and antimicrobial activities (Xu *et al*., 2014). A previous study found that *Pestalotiopsis* sp. isolated from the orchid species *Dendrobium officinale* showed cytotoxic and antifungal activities and produced monoterpenoids derivatives (Wu *et al*., 2015). Two fungal genera detected in this study that had moderate levels of abundance in three or four *Calanthe* species: *Cladosporium*, a common mold and plant pathogen, and *Tetracladium*, a genus that has been described as either marine (Roldan *et al*., 1989) or as a root endophyte (Sati *et al*., 2009). Thus, it is likely that the *Tetracladium* represent a poorly understood genus and further investigation (e.g. culturing and multi-locus sequencing for better taxonomic refinement) of Korean species associated with *Calanthe* roots is warranted. Of the two fungal genera that were dominant or at least well represented in a *Calanthe* species, *Ilyonectria* and *Hymenoscyphus* were detected in *C. aristulifera* and in *C. bicolor*, respectively. An unidentified fungal species in the class Agaricomycetes had a high relative abundance in the *Calanthe* sp. collected at HD. This species was not confidently identified because of low sequence similarity and unclear phylogenetic placement. Further specific identification is needed to reveal the relationship with *Calanthe* sp. Commonly found genera in this study were known pathogens, saprophytes, and symbionts (Vralstad *et al*., 2000; Huhndorf *et al*., 2004; Gross *et al*., 2014), and these different life histories are expected to play different ecological roles depending on environment. The same dominant fungal species were detected irrespective of *Calanthe* species or sampling site, thus, these dominant fungal genera likely have strong ecological relationships with *Calanthe* species.

While *Ceratobasidium* and *Tulasnella*, which are well known mycorrhizal fungi, have previously been identified in *Calanthe* species in Jeju Island (Dearnaley and Le Brocque, 2006; Youm *et al*., 2012), we did not detect any confirmed mycorrhizal fungi in this study. One possible reason is that the primers used for PCR did not amplify these fungal species. Taylor and McCormick developed new Tulasnellaceae-specific primers ITS1-OF/ITS4-OF that target all Basidiomycota including all *Tulasnella* clades of tulasnelloide fungi (Taylor and McCormick, 2008). However, *Tulasnella* species were also detected using universal primers although their PCR amplification efficiency was lower than that using Tulasnellaceae-specific primers ITS1-OF/ITS4-OF (Taylor and McCormick, 2008). ITS1F/ITS4 primers commonly amplify a wide range

of fungal taxa, thus we would expect this primer set to amplify fungal species in our samples. Further study is required to investigate whether tulasnelloid fungi are associated with Korean *Calanthe*.

Species specificity of root-associated fungi has been reported in some orchid species (Rasmussen, 2002; Bayman and Otero, 2006; Wang *et al*., 2017). In this study, however, fungal species composition was not different between orchid species, but was different between sampling sites (Fig. 3). Because we collected *Calanthe* species from islands that were distant from each other, factors such as meteorological conditions and vegetation composition likely contributed to the fungal distribution observed. A previous study showed that root-associated fungal communities can be different within orchid species depending on geographic distance (Taylor and Bruns, 1999).

This is the first study of fungal communities associated with some of Korea's most treasured ornamental plants, many of which are threatened with extinction. Indeed, given current trends of habitat loss, survival in the wild of some of these *Calanthe* species is difficult to predict, and cultivation of rare species may represent the only opportunity to preserve these species until environmental conditions improve. Ideally, future work will include more robust sampling of *Calanthe* root systems; however, the rarity and difficulty of these species means such sampling may not feasible. Thus, cultivation of Korean *Calanthe* species will likely be pursued in a "trial and error" method using candidate fungal species as potential symbionts.

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