

## Analysis of Soil Fungal Community Related to *Rhododendron mucronulatum* in Biseul Mountain County Park, South Korea

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Researching the soil fungal community is important to understand the interaction between fungi and living plants. However, too few studies have examined the soil fungal community and their interactions with plants. *Rhododendron mucronulatum*, commonly known as Korean rosebay, is an important forest resource that has aesthetic, ecological, and potential pharmacological values. We used a pyrosequencing method to analyze the characteristics of fungal communities from *R. mucronulatum* soil samples from Biseul mountain county park, which is one of the famous places for large *R. mucronulatum* colonies in South Korea. We collected soil core samples in February and August at three sites in the Biseul Mountain County Park, taking into consideration the regional and seasonal conditions. We obtained 454,157 validated reads after pyrosequencing all six samples. The fungal communities from the first observation spot in August had the richest species diversity among the samples. Basidiomycota, Ascomycota, and Mortierellomycota were major phyla in the samples. Agaricales\_f, Mortierellaceae, and Clavariaceae were major families in the samples. The genus *Mortierella* was the most dominant in all six samples. Overall, 19 genera could be associated with *R. mucronulatum*. Sample 1 had 109 genera in sample 1, sample 2 had 111 genera, and sample 3 had 112 genera that were uniquely identified. The samples collected in August had 28 identified genera, that existed only in summer samples, indicating a weather effect. This study can be used as basic research to understand the relationship between soil fungi and plants.

**Key words** : Biseul mountain county park, fungal community, next-generation sequencing, pyrosequencing, *Rhododendron mucronulatum*

### Introduction

*Rhododendron mucronulatum* is a deciduous shrub which can be usually found in sunny places on highlands between 50 and 2,000 meters [24]. It is called 'azalea' or 'Korean rosebay' and blooms beautiful dark or light-pink flowers at the beginning of April, which are about 3-4.5 cm in diameter. It is not only cold- and disease-resistant, but also valuable for landscaping and gardening [25]. It is also edible, the flowers have been used to make wine, juice, and flower-patterned cakes by Koreans, but it can be weakly toxic

[22]. Some phenolic acids and flavonoids isolated from *R. mucronulatum* have antioxidative activities [18, 19]. *R. mucronulatum* stems and flowers' extracts also have antioxidant and anticancer activities [1, 8, 21]. Biseul mountain county park, Daegu, South Korea has a beautiful scenery with a large *R. mucronulatum* colony. Yearly, there is a local festival of *R. mucronulatum*, and many people visit to enjoy the scenery. Thus, *R. mucronulatum* is a vital forest resource with potential values that can be useful in various ways. However, there are few studies on the soil fungal community of *R. mucronulatum*. Kim *et al.*, analyzed fungal communities in the Korean red pine forest, the forest was mixed with few *R. mucronulatum* [17]. Also, Choi *et al.*, analyzed *R. mucronulatum*'s bacterial community [9].

Fungi play a major role in maintaining the soil ecosystem. They function in various ways, such as degradation of plant or animal residues and nutrient circulation in the soil [26, 31]. Also, they have a relationship with living plants by nutrient trade. Some soil fungi form a symbiotic relationship with plants and can be involved in the reduction of N<sub>2</sub>O emissions from soil [4]. They interact and affect each growth

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and form their microbial communities in soil. Some microbial communities can positively affect the relationship between plant diversity and the ecosystem [32]. Therefore, it is essential to research the soil fungal community to understand fungi and living plants' interaction. However, there are not enough studies on the soil fungal community and their plant interactions.

Next-generation sequencing (NGS) platforms make possible parallel sequencing of massive number of DNA sequences in one sample rapidly [3, 14, 16, 28, 29]. It provides low-cost and high-efficiency sequencing. Also, it can analyze hard-to-cultivate microbes.

In this study, we conducted the pyrosequencing analysis of fungal communities from soil samples of *R. mucronulatum* in Biseul mountain county park and analyzed their characteristics. Their diversity, species richness, relative abundance, taxonomic composition were analyzed, and the fungal genera concerned with *R. mucronulatum* were presented. We selected three sites in the Biseul mountain county park, and collected soil core samples in February and August considering regional and seasonal conditions.

## Materials and Methods

### Collection of samples

We collected soil samples in Biseul mountain from Daegu, South Korea. Three sites in the Biseul mountain county park related to the cluster of *R. mucronulatum*; the park entrance, the first observation spot, and the second observation spot (Fig. 1). The park entrance is a location where the colony of *R. mucronulatum* was formed thinly. The dense colony of *R. mucronulatum* was formed in the first observation spot and the second observation spot. The samples were collected at three sites in August, 2018 (summer) and February, 2019 (winter) considering the weather. The collected samples were numbered as follow; sample 1: collected in August at the entrance of the park, sample 2: collected in August at the first observation spot, sample 3: collected in August at the second observation spot, sample 4: collected in February at the entrance of the park, sample 5: collected in February at the first observation point, and sample 6: collected in February at the second observation point. For sampling, soil cores (depth: 15 cm, diameter: 2 cm) were collected using a soil sampler, and an equal amount of each sample was used for pyrosequencing.

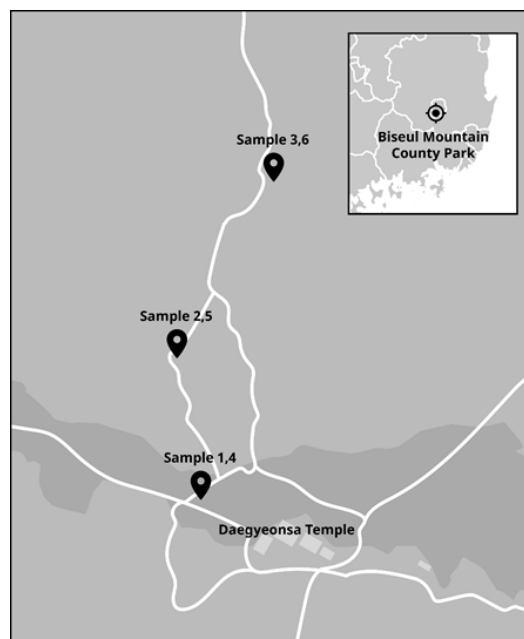


Fig. 1. The map of sampling sites in Biseul mountain county park from Daegu, South Korea. We selected three sites for investigation in various regions. Sample 1, 4 were collected at the entrance of the park. Sample 2, 5 were collected at the first observation spot. And sample 3, 6 were collected at the second observation spot.

### Extraction, purification and pyrosequencing of DNA

Pyrosequencing, one of the approaches for NGS, can analyze a large-scale sample of microbes from the soil [7, 10, 20, 23]. In NGS platforms, three common systems can be used: 454 GS-FLX (Roche, Branford, CT, USA), Illumina technology (Illumina, San Diego, CA, USA), and SOLiD systems (Applied Biosystems, Waltham, MA, USA) [14]. Here, the Illumina MiSeq Sequencing system was used, which can rapidly analyze the soil fungal communities' classification with DNA barcode sequences. For pyrosequencing, metagenomic DNA from the soil samples was extracted using the FastDNA SPIN Kit for soil (MP Biomedicals, Santa Ana, CA, USA) [20]. The quantified DNA was amplified by polymerase chain reaction (PCR) using primers targeting ITS2 region. The forward primer sequences were 5'-AATGATACGGCGACCACCGAGATCTACAC-XXXXXXXXX-TCGTCCG CAGCGTC-AGATGTGTATAAGAGACAG-GCATCGATGAAGAACGCAGC-3', and the reverse primer sequences were 5'-CAAGCAGAAGACGGCATAACGAGAT-XXXXXXXXX-GTCTCGTGGGCTCGG-AGATGTGTATAAGAGACAGTCC-TCCGCTTATTGATATGC-3'. "X" implies the barcode sequence varies from sample to sample. PCR was conducted using the PTC-200 Peltier thermal cycler (MJ Research,

Waltham, MA, USA). Agarose gel electrophoresis was performed for separating PCR products. The amplified PCR products were purified using the CleanPCR (CleanNA). The same concentrations of purified products were gathered and eliminated non-target fragments. And the quality and size of products were measured using an Agilent 2100 Bioanalyzer (Agilent Technology, CA, USA) with a DNA 7500 chip. Mixed amplicons were gathered and pyrosequencing was performed by Chunlab Inc. (Seoul, South Korea) using the Illumina MiSeq Sequencing system based on the manufacturer's instruction.

### Pyrosequencing data analysis and taxonomic identification

Here, all raw sequences for pyrosequencing were processed with the barcode sequences. Low-quality sequences were excluded by Trimmomatic 0.32 and not used for analysis [6]. Soil fungal communities were analyzed using the CLcommunity software (Chunlab, Inc.) for statistical and taxonomic analysis. The operational taxonomic units (OTUs) were analyzed using the CD-HIT clustering method at 97% sequence similarity [30]. We used the Mothur platform to generate the rarefaction curves and diversity indices. Also, taxon exclusive or (XOR) analysis was used to investigate unique fungal species [33, 34].

## Results and Discussion

### Pyrosequencing and statistical data analysis

The total number of reads was 463,429, of which 454,157 were valid reads. These were obtained after pyrosequencing for taxonomic analysis from all six samples (Table 1). The

lowest number of valid reads was 46,714 from sample 5, and the greatest was 106,548 from sample 3. The lowest number of OTUs was 461 from sample 5, and the greatest was 1,626 from sample 2. The valid reads and OTUs were significantly different between samples 1-3 and 4-6. The rarefaction curves showed at one view that the species richness of samples collected in August was greater than those in February (Fig. 2). It also implied that the fungal communities from the samples collected at the first observation point of the park in August were more diverse than the others. Chao1 estimation implied that sample 5 was the lowest (467) and sample 2 was the highest (1,640) in species richness. Shannon index indicated that sample 4 was the lowest (2.887) and sample 2 was the highest (5.114) in species richness. So, the fungal communities from the first observation point in August (sample 2) had the richest and the most diverse species.

### Taxonomic compositions of fungal communities related to *R. mucronulatum* at the phylum and the family levels

In sample 1, 25 phyla, 60 classes, 147 orders, 271 families, and 383 genera were identified. In sample 1, the dominant phyla were Basidiomycota (52.0%), Ascomycota (23.0%), and Mortierellomycota (9.0%). In sample 2, 29 phyla, 69 classes, 153 orders, 297 families, and 427 genera were identified. The dominant phyla were Ascomycota (39.4%), Basidiomycota (23.6%), and Mortierellomycota (11.1%). In sample 3, 25 phyla, 62 classes, 139 orders, 278 families, and 414 genera were identified. The dominant phyla were Ascomycota (42.6%), Basidiomycota (26.2%), and Mortierellomycota (14.5%). In sample 4, 15 phyla, 40 classes, 79 orders, 137 families, and

Table 1. Pyrosequencing and species richness analysis of the soil fungal communities associated with *R. mucronulatum* from samples collected in Biseul mountain county park

	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5	Sample 6
Number of total reads	92,046	89,661	107,771	71,729	47,927	54,295
Number of valid reads	91,024	87,221	106,548	70,192	46,714	52,458
Average read length (bp)	345.08	336.46	336.92	364.75	375.07	349.73
Maximum read length (bp)	475	460	478	479	460	478
Number of OTUs <sup>a</sup>	1,339	1,626	1,210	518	461	676
Chao1 estimation	1,344	1,640	1,217	529	467	681
Shannon index	3.731	5.114	4.812	2.887	3.010	3.768
Goods lib. coverage	99.95	99.89	99.96	99.93	99.93	99.92

<sup>a</sup>OTUs: Operational Taxonomic Units

Sample 1: the park's entrance in August, sample 2: first observation point in August, sample 3: second observation point in August, sample 4: the park's entrance in February, sample 5: first observation point in February, sample 6: second observation point in February.

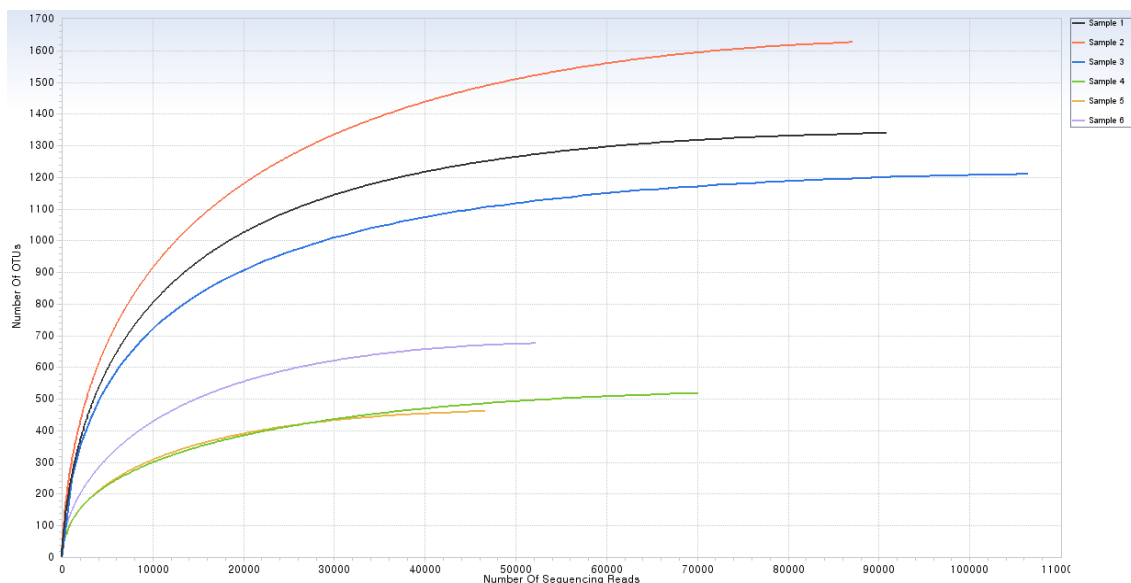


Fig. 2. The rarefaction curves for the relationship between sequencing reads and operational taxonomic units (OTUs) of fungi from all samples. The CD-HIT clustering method was used to cluster OTUs at 97% similarity. The fungal communities from sample 1-3 collected in August were more diverse than sample 4-6 collected in February.

196 genera were identified. The dominant phyla were Mortierellomycota (67.0%), Ascomycota (20.9%), and Basidiomycota (6.0%). In sample 5, 20 phyla, 49 classes, 91 orders, 145 families, and 185 genera were identified. The dominant phyla were Mortierellomycota (71.8%), Ascomycota (14.5%), and Fungi\_p (5.7%). In sample 6, 18 phyla, 48 classes, 95 orders, 154 families, and 206 genera were identified in total. The dominant phyla were Basidiomycota (51.2%), Ascomycota (27.2%), and Mortierellomycota (10.8%) in the sample 6 (Fig. 3).

At the family level, the dominant families in sample 1 were Agaricales\_f (46.6%) belonging to the class Agaricomycetes, and Mortierellaceae (8.7%) belonging to the class Mortierellomycetes and Fungi\_f (5.6%). The dominant families in sample 2 were Mortierellaceae (11.1%) and Helotiales\_f (10.6%) belonging to the class Leotiomycetes, and Clavariaceae (9.2%) belonging to the class Agaricomycetes. The dominant families in sample 3 were Mortierellaceae (14.5%), Helotiales\_f (12.2%), and Amanitaceae (8.1%) belonging to Agaricomycetes. The dominant families in sample 4 were Mortierellaceae (67.0%), Helotiales\_f (11.1%), and Clavariaceae (3.0%). The dominant families in sample 5 were Mortierellaceae (71.8%), Fungi\_f (5.6%), and Pseudeurotiaceae (4.4%) belonging to Leotiomycetes. The dominant families in sample 6 were Clavariaceae (43.6%), Helotiales\_f (12.7%), and Mortierellaceae (10.7%) (Fig. 4). Among all samples, Mortierellaceae was the most dominant family in four samples.

#### Characteristics of fungal community related to *R. mucronulatum* at the genus level

We identified common genera in all samples at the genus level to investigate important fungi related to *R. mucronulatum*. Overall, the genus *Mortierella* (total 119, 143 reads) was the most dominant genus in all samples. *Helotiales\_g* (38,295), *Agaricales\_f\_uc* (32,218), *Clavaria* (29,873), and *Fungi\_g* (16,121) followed.

Also, we selected 19 genera specifically identified in our samples only, compared to control samples unrelated to the county park and *R. mucronulatum* (Table 2). Overall, the genus *Moreaua* was the most common among the 19 genera, followed by *Clavariaceae\_g* and *Leohumicola*. *Moreaua* is a smut fungus found on sedges [27]. It has not been found in Korea, but *Moreaua terrima* was first discovered in 2014 [11, 12]. *Moreaua* was supposed to be related to *R. mucronulatum* in our samples, however, we only found unclassified *Moreaua* species. *Clavariaceae* is a family, including species that can produce resupinate sporocarps [5]. Some *Leohumicola* species can survive following heat treatment [15]. This implied for the first time that these 19 genera can be associated with *R. mucronulatum* by analysis of soil fungal community.

The top five genera were displayed in the most abundant order by analyzing the unique genera by location (Table 3). In sample 1, 109 genera were uniquely identified. *Agaricales\_f\_uc* was the most common, and *Clitopilus* and *Acremonium* were also found. In sample 2, 111 unique genera

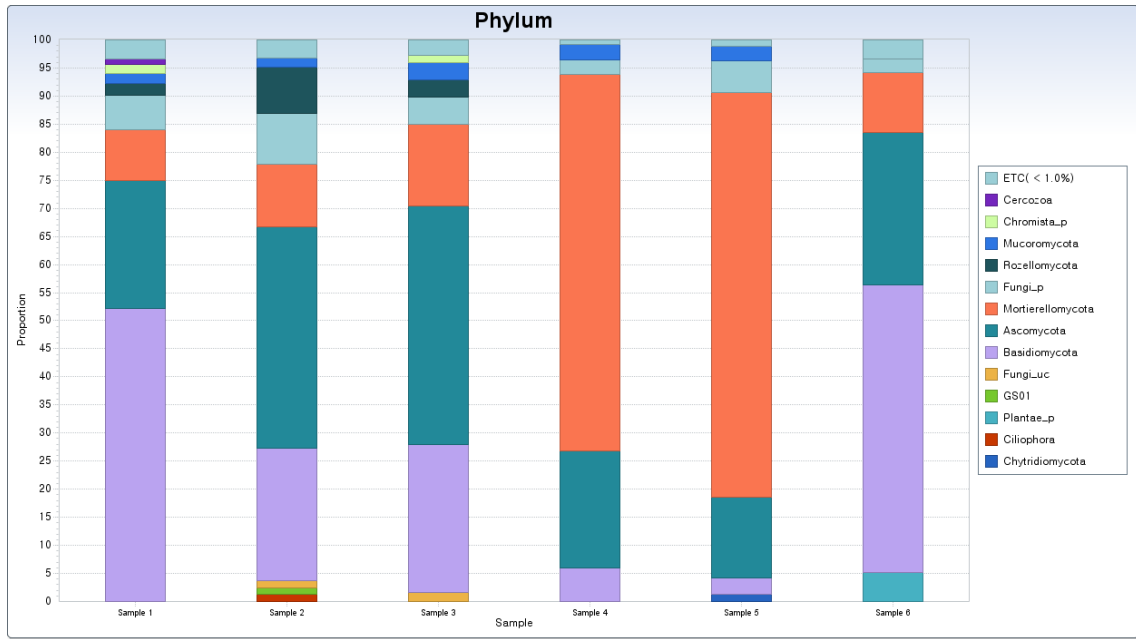


Fig. 3. Taxonomic composition at the soil fungal communities' the phylum level from all samples associated with *R. mucronulatum*. The fungal phyla are shown with more than 1% relative abundance in the samples, and the phyla less than 1% are marked as ETC. Basidiomycota was dominant phylum in sample 1 and 6, Ascomycota was dominant in sample 2 and 3, Mortierellomycota was dominant in sample 4 and 5.

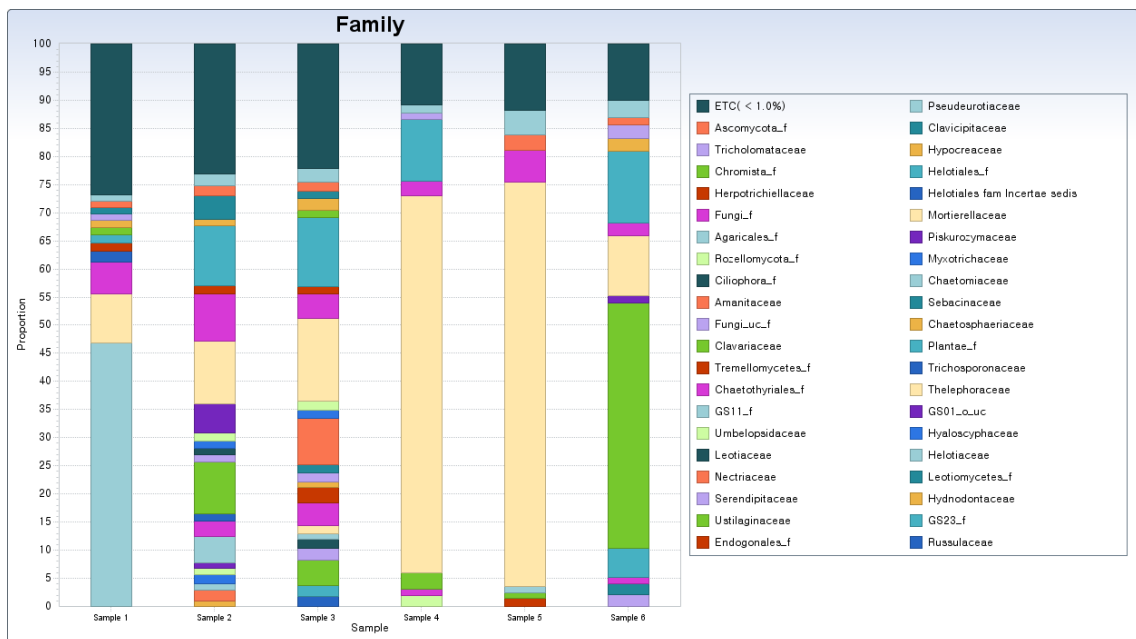


Fig. 4. Taxonomic composition at the family level of the soil fungal communities from all samples associated with *R. mucronulatum*. Forty-one major families are shown with different colors. The most abundant families in each sample were Agaricales\_f in sample 1(46.6%), Mortierellaceae in sample 2(11.1%), 3(14.5%), 4(67.0%), 5(71.8%), and Clavariaceae in sample 6(43.6%).

were identified. Hydnodontaceae\_g was the most common, and *Cutaneotrichosporon*, *Metazoa\_f\_uc*, *Auriculariales\_g*, *Lecanicillium*, *Clitopilus*, and *Acremonium* followed in order.

In sample 3, 112 unique genera were identified, and *Myxotrichaceae\_uc*, *Glomerales\_uc\_g*, *Ciliciodium*, *Cenococcum*, and *Rasamsonia* were identified. Unlike the previous analysis of

Table 2. The list in alphabetical order of 19 common genera in all samples from Biseul mountain county park compared with control samples

Name	
<i>Apiotrichum</i>	<i>Gymmostellatospora</i>
<i>Articulospora</i>	<i>Hyaloscypha</i>
<i>Cephalotheca</i>	<i>Ilyonectria</i>
Clavariaceae_g	<i>Leohumicola</i>
<i>Coniochaeta</i>	<i>Moreaua</i>
Entolomataceae_uc	<i>Paecilomyces</i>
<i>Geomyces</i>	<i>Pyrenochaetopsis</i>
GS05_g	<i>Rectipilus</i>
GS21_g	<i>Trichocladium</i>
GS22_g	

The genera can be associated with *R. mucronulatum* by analysis of soil fungal community.

common genus, it is obvious that the genus was specifically found depending on where it was sampled. However, they were the communities of *R. mucronulatum*-related soil fungus. There is a possibility of discovering a unique *R. mucronulatum*-related soil fungus that appears only at the spot.

Considering the weather's effect, we analyzed the genera that were uniquely identified only in samples collected in the summer. Twenty-eight unique genera were identified (Table 4). Among them, the most notable genus is the *Cladophialophora*, which has been most frequently found. This was followed by *Sordariales\_g*, *GS01\_o\_uc\_g*, and *Lecanoromyces\_g*. The genus *Cladophialophora* is a vital genus clinically and environmentally [2]. Some *Cladophialophora* species were found for their relationship with living plants in alkylbenzene-polluted soil [2]. We identified 17 *Cladophialophora* species and unclassified *Cladophialophora* species. *C. chaetospora* species are found in plant litter and were identified in all summer samples. Plant-associated species of *Cladophialophora* had appropriate growth temperature at 27°C - 30°C [2], which could be a reason for varying results dur-

Table 4. The genera identified in samples collected in August considering the effect of the weather

Name	Sample 1	Sample 2	Sample 3
Chaetosphaeriales_g	137	6	34
<i>Fusicolla</i>	11	3	3
Xylariales_g	17	31	38
Sordariales_g	32	53	1012
Agaricomycetes_f_uc	45	24	7
<i>Paraconiothyrium</i>	24	5	12
Dothideomycetes_uc_g	6	77	40
Chlorophyta_o_uc_g	6	162	96
<i>Didymella</i>	80	4	30
Russulales_uc_g	24	9	3
Lecanoromycetes_g	7	661	235
<i>Cladophialophora</i>	207	386	530
<i>Knufia</i>	59	21	18
<i>Alternaria</i>	54	12	72
Leucosporidiales_g	87	4	18
<i>Cyphellophora</i>	15	25	31
<i>Haptocillium</i>	21	4	6
Orbiliaceae_uc	55	7	4
Tremellales_uc_g	9	96	12
<i>Conlarium</i>	54	161	46
Stereopsidales_uc_g	2	5	3
<i>Exophiala</i>	113	34	201
Herpotrichiellaceae_uc	54	14	17
Spizellomycetales_f_uc	83	2	17
Dothideomycetes_g	75	30	329
GS01_o_uc_g	105	873	19
<i>Paraphoma</i>	1	2	10
Inocybaceae_uc	9	4	17

Twenty-eight unique genera were identified. They exist only in summer samples.

ing winter.

Here, six soil samples were collected from three spots and two weather of the Biseul mountain county park. Fungal communities of the samples were analyzed by pyrosequencing. Statistical analysis showed that 454,157 validated reads were pyrosequenced. The species richness of samples in

Table 3. The five unique genera in each sample collected at different spots in August

Sample 1		Sample 2		Sample 3	
Name	Number of reads	Name	Number of reads	Name	Number of reads
Agaricales_f_uc	32218	Hydnodontaceae_g	702	Myxotrichaceae_uc	1098
Hypocreales fam <i>Incertae sedis_g</i>	230	<i>Cutaneotrichosporon</i>	550	Glomerales_uc_g	661
<i>Clitopilus</i>	172	Metazoa_f_uc	223	<i>Ciliciopodium</i>	396
Chaetothyriaceae_g	161	Auriculariales_g	223	<i>Cenococcum</i>	368
<i>Acremonium</i>	153	<i>Lecanicillium</i>	162	<i>Rasamsonia</i>	214

They exist only in one sample.

summer was greater than samples in winter due to the proper temperature conditions. The richest and the most diverse fungal community was that of sample 2 from the first observation point in August. The genus *Mortierella* was the most dominant in all samples. The 19 genera can be associated with *R. mucronulatum*. One hundred nine genera in sample 1, 111 genera in sample 2, and 112 genera in sample 3 were uniquely identified.

To understand the relationship between fungi and living plants, it is vital to research the soil fungal community. They constantly interact, affecting each other's growth and forming microbial communities in the soil. By pyrosequencing, we showed that the fungal communities associated with *R. mucronulatum* could have unique or common fungi according to location and weather. Further studies are needed to determine the characteristics of specific or unclassified genera and species of these fungi.

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### The Conflict of Interest Statement

The authors declare that they have no conflicts of interest with the contents of this article.

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**초록 : 우리나라 비슬산군립공원 진달래나무(*Rhododendron mucronulatum*)와 관련된 토양 진균 군집의 pyrosequencing 분석**

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진달래(*Rhododendron mucronulatum*)는 우리나라에서 어렵지 않게 볼 수 있는 개화식물로 꽃이 아름다워 관상목으로 이용되고 있고 생태학적, 약리적으로 잠재력이 있는 중요한 산림자원이다. 우리나라의 대표적 진달래나무군락지인 비슬산군립공원에서 진달래나무 아래의 토양을 채집하여 그 진균 군집의 특성을 조사하였다. 위치와 계절에 따른 진균 군집의 차이를 확인하기 위하여 토양 샘플은 총 3개의 위치에서 2월과 8월에 각각 한번씩 채집하였다. Pyrosequencing을 통해 총 454,157개의 서열을 얻을 수 있었다. 첫번째 채집포인트에서 얻은 샘플의 진균 군집이 6개 샘플 중 가장 종 풍부도가 높았고 가장 다양한 진균들로 구성되어 있음을 확인하였다. 분류 단위 별 분석으로는, Basidiomycota, Ascomycota, Mortierellomycota가 대표적인 문(phylum)으로 나타났으며, Agaricales\_f, Mortierellaceae, Clavariaceae가 주요한 과(family)인 것으로 분석되었다. *Mortierella* 속(genus)은 모든 샘플중에서 가장 우점한 속이었다. 또한 총 진달래와 관련이 있는 것으로 추정되는 19개의 속이 확인되었다. 8월에 채집한 샘플에서 위치에 따라 각각 109개, 111개, 112개의 특이적인 속이 발견되었고, 2월의 샘플과 비교했을 때 2월의 샘플에는 존재하지 않는 28개의 공통된 속이 발견되었다. 이 연구는 추후 진달래나무에 특이적인 진균의 새로운 종을 규명하거나 토양 진균류와 식물의 상호작용을 규명하는 기초자료로 활용될 수 있다.