

Metagenomic Analysis of Bacterial Communities in *Rhododendron mucronulatum* in Biseul Mountain County Park, Daegu, Korea

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Rhododendron mucronulatum, native to Korea, Mongolia, Russia and parts of northern China, is known not only for its medicinal properties but also as a tourist attraction. One of the most famous tourist destinations with *R. mucronulatum* is in Biseul Mountain County Park, Daegu, Korea. To investigate the relationship between *R. mucronulatum* and microbiome communities in the surrounding soil, three sites within the park were chosen for sampling in February and August. The soil samples were then passed through a pyrosequencing process for analysis of the bacterial communities, and a total of 404,899 sequencing reads were obtained. Between 2,349 and 4,736 operational taxonomic units (OTUs) were observed across the three sampling zones and two seasons; samples from the park entrance showed a higher number of OTUs than the other two sites, and samples from August had more OTUs than those from February. The sample from the second observation site displayed the fewest OTUs, particularly in February. According to Chao1 and Shannon indices, samples from the park entrance in August demonstrated the highest degree of species richness and diversity. Studying the bacterial communities across the six samples identified the common population as comprising 287 genera, 45 of which are only present in Biseul Mountain County Park and are expected to participate in the colonization of *R. mucronulatum*.

Key words : Bacterial community, Biseul mountain, metagenomics, *Rhododendron mucronulatum*

Introduction

Bacteria are a type of biological cell and constitute a large domain of prokaryotic microorganisms. As member of kingdom Monera, bacteria have various kind of shapes including the spheres, rods and spirals. Also bacteria are one of the first created life form on earth and are present in most of various habitats. Specially, bacteria inhabit in the soil environment are able to penetrate plants and affect to their host as parasitic or symbiotic. As such a relationship has been continued for millions of years, plants have been colonized by various kind of species including bacteria and the structural composition of bacterial communities also have been affected on the host plants. As symbiosis, bacteria contribute to plant growth promotion or biocontrol of pathogens. As many studies have been revealed about specific relationship

between bacterial communities and surrounded host, bacterial communities are expected to play important role for physiology of host [9, 11, 16, 23]. Because of symbiotic role from the bacterial communities, the composition of bacterial communities also being important. But even bacterial contribution to their host is well known, only 27 percent of the bacterial species have been grown in the laboratory environments. This limitation of culture result the lack of bacterial species diversity from communities and show the necessity of other experiment method not depend on the cultural experiment.

The metagenomics was first appeared in publication in 1998 by Jo Handelsman, Jon Clardy, Robert M. Goodman, Sean F. Brady, and others [21]. The term included the idea that collecting genes sequenced from the environment could be analyzed for the study of a single genome. And by Kevin Chen and Lior Pachter, the researchers at the University of California, Berkeley, metagenomics were defined as the application of modern genomics technique without isolation and lab cultivation of individual species. Not like gene sequencing was started with a culture of same cells as a source of DNA, early metagenomics expected the large groups of microorganisms would be existing in many environment

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that hardly be cultured and thus hardly be sequenced. In early studies, 16S ribosomal RNA sequences were used for analysis because of relative shortness, conservation within a species and general difference between species. The studies of ribosomal RNA (rRNA) genes collected from the environments showed the less than 1% of the bacterial and archaeal species in the sample under method of cultivation. Norman R. Pace and colleagues used PCR to explore the diversity of ribosomal RNA sequences and supported the presence of a complex community of unexplored species. Although the method of Norman R. Pace and colleagues showed the limitation of exploring highly conserved, non-protein coding genes, it supported early microbial morphology-based analysis that diversity was far more complex than was known by culturing methods. Based on this method, Healy F. G. and colleagues reported the metagenomics isolation of functional genes from complex culture of environmental organisms lived in laboratory [10]. Starting using environmental shotgun sequencing in 2002, the method of sequencing had been published newly and more of microorganisms were detected than previous. Recently in 2005, C. Schuster at Penn State University and colleagues published the first sequences with high-throughput sequencing and massively parallel pyrosequencing developed by 454 Life Sciences [17].

Rhododendron mucronulatum known as Korean rhododendron is colonized in slightly shady northern slope site. It belongs to *Rhododendron* species usually inhabit forested regions at 1,600 - 2,300 m in Korea, Mongolia, Russia and parts of northern China [5]. As edible plant, it has been used as medicine regulating circulatory system, respiratory system and gynecological system. Also *R. mucronulatum* is one of the most attractive tourist resource in Korea. Biseul mountain (35°43'00.8"N 128°31'29.5"E) located in border of Dalseong-gun, Daegu and Gachang, Gyeongsangbuk-do, is the one of the most famous recreation forest place. In May, in full blossom of *R. mucronulatum* is the best sights in Biseul Mountain County Park. As this colonization is only positioned in specific site of Biseul Mountain, association of microbiome is expected [20].

As a subject of the publication, *R. mucronulatum* has been attractive source for many studies. But most of studies published in the past were concerned about the floral color or the flavor of blossom. Even publications about diversity of *Rhododendron* species or phytochemical were also existing, investigating about metagenomic analysis has not been founded. In this study, metagenomic analysis to investigate

the characteristic of bacterial population in *R. mucronulatum* colony were in progress. The investigation was done by GS-FLX pyrosequencing platform. Bacterial population from the soil samples of Biseul Mountain County Park, Daegu, Korea were collected and analyzed based on the 16S ribosomal RNA sequences. These data would be useful for investigating the colonization of *R. mucronulatum*.

Materials and Methods

Sample collection

The soil samples were collected in Biseul mountain county park from the Biseul mountain in Daegu, Korea. During the study, three sampling sites in county park were selected based on colonization of relation with *R. mucronulatum* as follow; (i) entrance of park (ii) 1st observation point (iii) 2nd observation point. Also to recognize the influence of the weather, sampling was done in February and August, separately. In this study, sample number was decided as follow; Sample 1 - entrance of park in August; Sample 2 - 1st observation point in August; Sample 3 - 2nd observation point in August; Sample 4 - entrance of park in February; Sample 5 - 1st observation point in February; Sample 6 - 2nd observation point in February. For the sampling, ten soil cores (depth: 15, diameter: 2 cm) with soil sampler were collected and were homogenized separately for experiments [6, 8].

DNA Extraction, purification and pyrosequencing

Metagenomic DNA from the soil samples was extracted and quantified by the Quat-iT PicoGreen dsDNA Assay kit (Invitrogen, Carlsbad, CA, USA) for the pyrosequencing analysis [2]. Extracted and quantified DNA was then amplified using the barcoded fusion primers targeting the 468 base pair of the V3/V4 regions from 16S rRNA genes that contained V3-341F 5'-CCTACGGGNGGCWGCAG-3' and V4-805R 5'-GACTACHVGGGTATCT AATCC-3' [1, 7, 12, 14]. PCR was proceed by the PTC-200 Peltier thermal cycler (MJ Research, Waltham, MA, USA) under following conditions; Initial denaturation (3 min at 95°C); 25 cycles of denaturation (30 sec at 95°C), annealing (30 sec at 55°C) and extension (30 sec at 72°C); final extension (5 min at 72°C). While on PCR amplification, clean-up and size selection were done with AMPure bead XP (Beckman Coulter, CA, USA) in gel electrophoresis for amplicon pooling. And measurement of final concentration was done with Quat-iT Pico

Green dsDNA Assay kit and A260/A280 ratio. Using Agilent DNA 7500 kit, pooled sized short fragments were measured by Agilent 2100 Bioanalyzer (Agilent Technology, CA, USA). The pyrosequencing was done by Chunlab Inc. (Seoul, Korea) using the ver. 10 of Illumina iSeq 100 Sequencing System (Illumina, CA, USA) based on the instruction from website. With new sequencing chemistry, called One-Channel SBS Chemistry, iSeq 100 Sequencing System produced higher quality reads than the Illumina MiSeq that used before. All pyrosequencing data were matched with closely related species using the BLASTN search tool and calculating the 16S rRNA gene sequence similarities had been done by global alignment algorithm at the EzTaxon-e web server (<http://www.ezbiocloud.net/eztaxon>).

Taxonomic assignment of sequencing reads and analysis

With database version PKSSU4.0, Microbiome Taxonomic Profile (MTP) of pyrosequencing reads were browsed and informed [3, 6, 13]. The sequencing data was analyzed using the EzBioCloud 16S-based Microbiome Taxonomic Profiling (MTP) app. Also bacterial communities could be analyzed by CLcommunity software ver. 3.46 (Chunlab, Inc. Seoul, Korea), but in this study, MTP app was used for analysis. The operational taxonomic units (OTUs) were analyzed with CD-HIT program under 99% of sequence similarity [19]. The rarefaction curve and diversity indices, included Chao1 and Shannon, were created as chart by the Mothur platform. Also by taxon XOR analysis comparing system, unique species of bacteria were checked [22, 23].

Results

Pyrosequencing and statistical data analysis

From 6 samples, total 404,899 reads were pyrosequenced

and 320,575 reads were verified for taxonomical assignment. The number of validated reads ranged from 43,709 to 72,766 per sample and the average length of the read was about 410 bp. From 2,349 to 4,736 OTUs per sample were achieved separately at a 99% similarity level because the richness of bacterial OTUs was different by the sampling site and weather (Table 1). To analyze the species richness, the rarefaction curves were created and indicated that September had greater bacterial community than May (Fig. 1). Also in same month, samples from the entrance of the county park showed the greater bacterial community than the others. Chao1 estimation indicated that species richness decreased in following order: Sample 1 (4,858) > Sample 2 (4,201) > Sample 3 (3,542) > Sample 5 (2,625) > Sample 4 (2,575) > Sample 6 (2,402). Sannon index showed that species diversity decreased in following order: Sample 1 (7,051) > Sample 2 (6,827) > Sample 3 (6,649) > Sample 4 (6,077) > Sample 5 (5,791) > Sample 6 (5,789). Therefore, entrance site of county park in September showed the greatest species richness and diversity.

Composition of *R. mucronulatum*-associated bacteria according to weather

Diversity and compositions of the bacteria were different between site and weather. In Microbiome Taxonomic Profile, Sequences of bacterial V3V4 rRNA gene were classified from phylum down to the genus level. Taxonomic result about sample 1 showed association with 42 phyla, 105 classes, 211 orders, 447 families and 1145 genera of bacteria. Bacterial community of sample 1 was dominated by members of the 3 phyla Acidobacteria (31.0%), Proteobacteria (26.1%) and Verrucomicrobia (10.0%). The sample 2 population was concern with 33 phyla, 85 classes, 157 orders, 344 families and 872 genera of bacteria. Bacterial community of sample 2 was dominated by members of the 3 phyla Acidobacteria (36.6%),

Table 1. Summary of results from pyrosequencing and statistical analysis

	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5	Sample 6
Number of total reads	64,109	68,526	58,576	64,896	82,761	66,031
Number of validated reads	44,343	45,788	43,709	58,052	72,766	55,917
Mean read length (bp)	411.45	410.89	411.36	410.5	410.86	410.49
Maximum read length (bp)	451	440	448	446	452	436
Number of Operational Taxonomic Units (OTUs)	4,736	4,113	3,465	2,517	2,578	2,349
Chao1 estimation	4,858	4,201	3,542	2,575	2,625	2,402
Sannon index	7.051	6.827	6.679	6.077	5.791	5.789
Good's coverage	98.551	98.896	99.058	99.532	99.641	99.531

From 6 samples of Biseul Mountain County Park, total 404,899 reads were analyzed using ver. 10 of Illumina iSeq 100 Sequencing System.

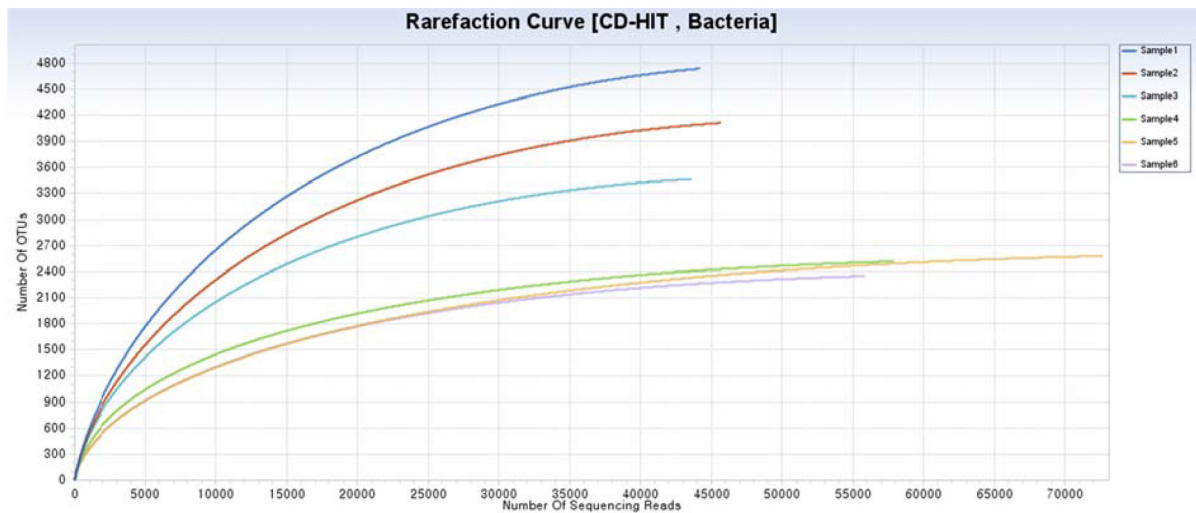


Fig. 1. Rarefaction curves for Operational Taxonomic Units (OTUs) at each 6 samples of *R. mucronulatum* were formed with 99% similarity of cutoff value. OTUs were clustered at 3% dissimilarity with CD-HIT. The bacterial community from entrance in August showed the highest degree of OTUs among the samples and prove the highest degree of species richness and diversity.

Proteobacteria (25.4%) and Verrucomicrobia (10.1%). The sample 3 population was concern with 29 phyla, 79 classes, 138 orders, 294 families and 767 genera of bacteria. Bacterial community of sample 3 was dominated by members of the 4 phyla Acidobacteria (33.1%), Proteobacteria (22.6%), Verrucomicrobia (12.8%) and Chloroflexi (10.5). The sample 4 population was concern with 29 phyla, 71 classes, 129 orders, 252 families and 592 genera of bacteria. Bacterial community of sample 4 was dominated by members of the 3 phyla Proteobacteria (33.8%), Acidobacteria (25.9%) and Actinobac-

teria (13.5%). The sample 5 population was concern with 30 phyla, 74 classes, 131 orders, 264 families and 602 genera of bacteria. Bacterial community of sample 5 was dominated by members of the 2 phyla Acidobacteria (43.0%) and Proteobacteria (27.5%). The sample 6 population was concern with 28 phyla, 66 classes, 114 orders, 233 families and 547 genera of bacteria. Bacterial community of sample 6 was dominated by members of the 3 phyla Proteobacteria (36.2%), Acidobacteria (28.0%) and Verrucomicrobia (11.9%) (Fig. 2).

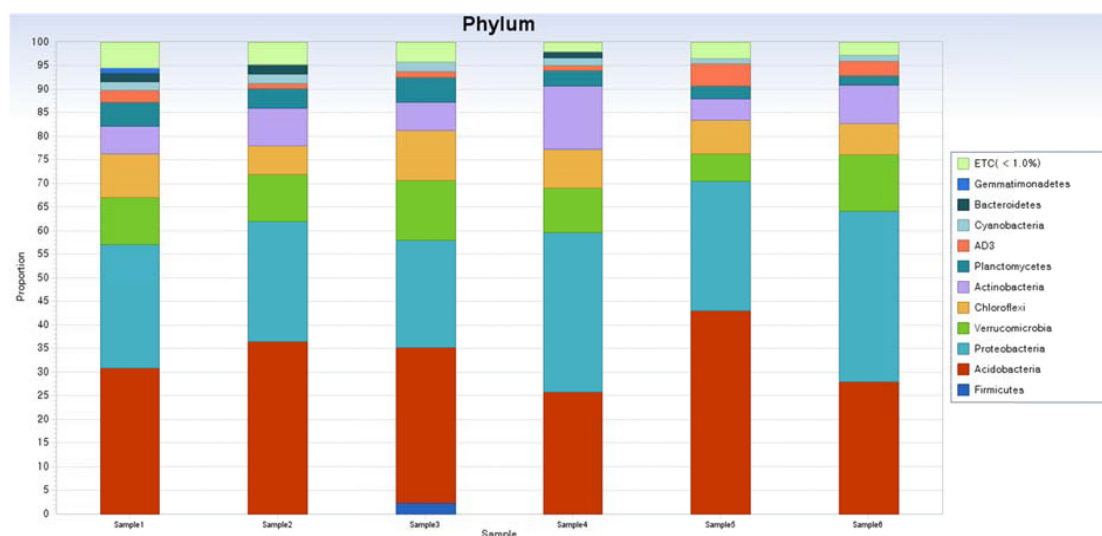


Fig. 2. Taxonomic classification of bacteria associated with 6 samples of *R. mucronulatum* was formed at phylum level (with the RDP classifier). The bacterial phyla are shown with a relative abundance of more than 1% in all samples. The 11 phyla dominated by Acidobacteria, Proteobacteria and Verrucomicrobia are shown.

In family level of taxonomic results, bacterial community of sample 1 was dominated by PAC000121_f (13.8%) belong to class Solibacteres, Acidobacteriaceae (8.4%) and Chthoniobacteraceae (8.0%). Bacterial community of sample 2 was dominated by PAC000121_f (15.0%), Acidobacteriaceae (12.7%), Chthoniobacteraceae (7.2%) and Bradyrhizobiaceae (7.1%). Bacterial community of sample 3 was dominated by PAC000121_f (15.2%), Chthoniobacteraceae (11.6%) and Acidobacteriaceae (9.5%). Bacterial community of sample 4 was dominated by PAC000121_f (11.2%), Bradyrhizobiaceae (10.8%), Acidobacteriaceae (8.7%) and Chthoniobacteraceae (8.0%). Bacterial community of sample 5 was dominated by PAC000121_f (25.9%) Acidobacteriaceae (11.8%) and Rhodospirillaceae (8.9%). Bacterial community of sample 6 was dominated by PAC000121_f (16.1%), Bradyrhizobiaceae (12.0%), Chthoniobacteraceae (11.5%), Rhodospirillaceae (10.7%) and Acidobacteriaceae (8.9%) (Fig. 3).

In genus level of taxonomic results, bacterial community of sample 1 was dominated by PAC000121_g (9.3%) and PAC002252_g (4.2%) belong to family Chthoniobacteraceae. Bacterial community of sample 2 was dominated by PAC000121_g (7.0%), PAC000030_g (5.0%) belong to class Solibacteres and PAC002252_g (4.5%). Bacterial community of sample 3 was dominated by PAC000121_g (9.6%), PAC002252_g (6.6%) and PAC001932_g (4.8%) belong to family Chthoniobacteraceae. Bacterial community of sample 4 was dominated by JADL_g (5.7%) belong to family Rhodospirillaceae, PAC002252_g (5.5%), PAC000166_g (5.2%) belong to

order Streptosporangiales, PAC000121_g (4.9%) and *Rhodoplanes* (4.7%). Bacterial community of sample 5 was dominated by PAC000121_g (15.1%), PAC000030_g (7.4%), JADL_g (5.7%) and HM748676_g (4.1%) belong to family Acidobacteriaceae. Bacterial community of sample 6 was dominated by PAC000121_g (9.0%), JADL_g (8.5%), PAC002252_g (6.4%), Rhodoplanes (5.9%), PAC001932_g (5.0%) and PAC000030_g (4.8%) (Fig. 4).

Population-specific bacterial OTUs at the genus level

In the study, 6 samples showed 287 common genus and to recognize unique bacterial population in the samples from Biseul Mountain county park, control soil sample that not belong to county park (specially not concern with *R. mucronulatum*) were collected and pyrosequenced [18]. By comparing in genus level, total 45 specific bacteria concern with *R. mucronulatum* were analyzed (Table 2). Among the specific genus *Methyloceanibacter* was the major common bacteria from all weather and site [4].

Genus *Methyloceanibacter* was first reported in 2014 by Takeuchi *et al.* This methanol-oxidizing bacterium a member of the family Hyphomicrobiaceae in the order Rhizobiales was known for regulating the biogeochemical cycling of one-carbon substrates [15]. In this study, total 4 species with unknown name in genus *Methyloceanibacter* were discovered. Also other present common specific genera were founded; *Glaciimonas*, *Nitrospira* and *Paucimonas*. But unlike *Methyloce-*

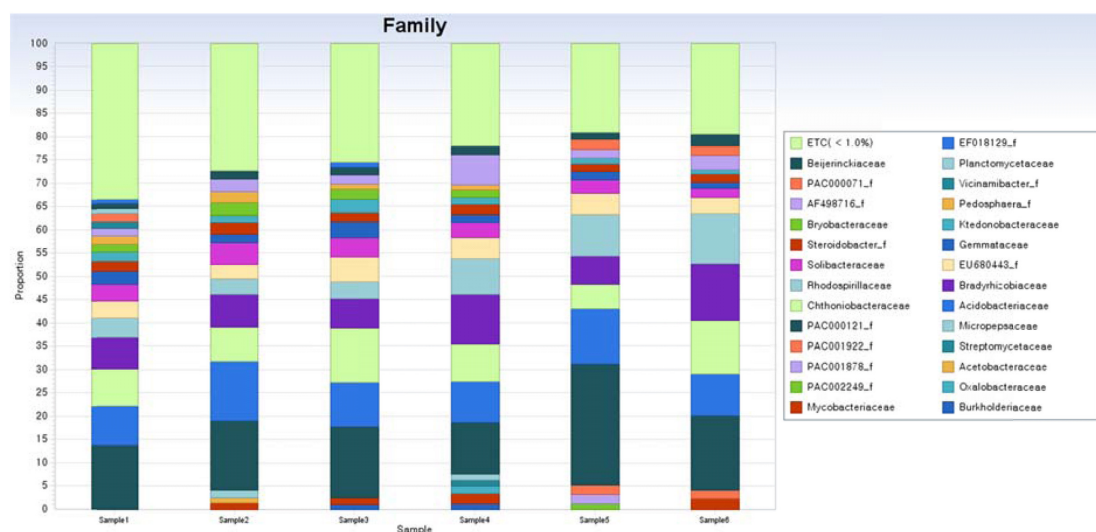


Fig. 3. Taxonomic classification of bacteria associated with 6 samples of *R. mucronulatum* was formed at family level (with the RDP classifier). The bacterial families are shown with a relative abundance of more than 1% in all samples. The 27 families dominated by PAC000121_f belong to class Solibacteres, Acidobacteriaceae and Chthoniobacteraceae are shown.

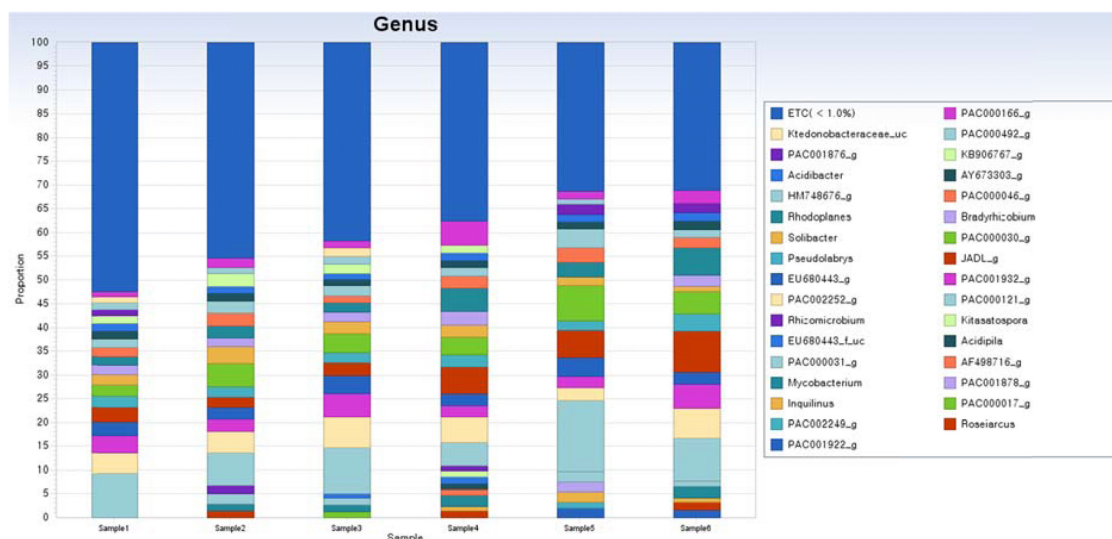


Fig. 4. Taxonomic classification of bacteria associated with 6 samples of *R. mucronulatum* was formed at genus level (with the RDP classifier). The bacterial genera are shown with a relative abundance of more than 1% in all samples. The 34 genera from 6 samples are shown.

Table 2. Population-specific *R. mucronulatum*-associated bacterial Operational Taxonomic Units (OTUs) in genus level were shown

Biseul Mountain county park				
PAC001336_g	EF018438_g	PAC001840_g	EF494362_g	PAC002281_g
AM997775_f_uc	PAC002318_f_uc	EU861837_g	EF018221_g	FJ479101_g
JN023844_g	EU335161_g	EF494344_g	GU599038_g	Paucimonas
PAC000287_f_uc	DQ413131_g	AY281358_g	AF234690_g	Nitrospira
PAC000324_g	PAC000323_g	EU135153_g	PAC002546_g	PAC001813_g
PAC001298_g	JX133647_g	DQ404819_f_uc	AY661982_f_uc	Methyloceanibacter
AY425783_g	PAC000149_f_uc	LBTE_f_uc	Glaciimonas	AY963418_g
EF516835_g	EF018129_f_uc	PAC002126_g	GU127807_g	PAC001956_g
EU881182_g	AJ318204_g	PAC000396_f_uc	FM253572_g	PAC002249_g

Including classified into scientific name and unclassified or unknown name for taxon, total 45 genera were recognized.

anibacter, other 3 genera were not dominating in all 6 samples.

Discussion

Because of various inhabit on earth, bacteria were participated in various life cycle of creatures including the plants. And the root of plants were closely related to the surrounding bacterial communities. Such bacterial communities showed different population depend on the unique environment factors. However comparing other metagenomic analysis, soil bacterial community structure may have more influence with geographical location than in environmental factors.

In this study, three different sites of Biseul Mountain County Park and two different weather were selected as comparison. Because of the appropriate temperature, sample

in August showed higher degree of species richness and diversity than in February. And the sample from the entrance showed more advantages in degree of species richness and diversity than other sites because of accessibility. But even the percentage of population is different, these six samples showed the common composition of population with 45 genera including *Methyloceanibacter*, *Nitrospira*, *Glaciimonas*, *Paucimonas* and *et al.* As these common genera were unique in samples from Biseul Mountain County Park, they were expected to participate in the colonization of *R. mucronulatum*. In conclusion, this study offer possibility to find out not only the composition of bacterial community regarding the host, but also the main influential bacteria for *R. mucronulatum*. Further studies concern with the fungi and archaea would be great helpful for creating the whole metagenomics of Biseul Mountain County Park.

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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 : 비슬산 군립공원의 진달래에 대한 박테리아 군집 metagenomics 분석 규명

최두호 · 정민지 · 권해준 · 김미경 · 김동현 · 김영국 · 김종국*

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한국, 몽골, 러시아, 중국 북반부에 서식하는 진달래는 예로부터 약재뿐 만이 아니라 관광 자원으로써도 잘 알려져 왔다. 이중 한국의 대구에 위치한 비슬산 군립공원 또한 진달래 관광지로 유명하다. 이러한 환경에서 진달래와 흙에 존재하는 미생물 군집 간의 관계를 조사하기 위해서, 2월과 8월에 걸쳐 군립공원 내 3 곳을 선택하여 실험을 진행하였다. 박테리아 군집 조사를 위해 확보된 흙들은 pyrosequencing 과정을 거쳤으며 총 404,899개의 sequencing reads를 확보하였다. 두 날씨에 따라 각기 다른 3 곳의 채집 장소로부터 operational taxonomic units (OTUs) 값이 측정되었으며 이는 2,349에서 4,736 수치를 보였다. OTUs 값 측정결과 채집 장소 중에서는 군립공원 입구 지역에서 가장 높은 값을 보였으며 계절로는 8월이 2월보다 높은 수치를 보였다. 반면 가장 낮은 수치의 OTUs 값을 보인 샘플은 제 2 관측지점, 특히 2월에 채집한 흙이었다. 또한 Chao1과 Sannon index 값 측정 결과, 8월에 채집한 군립공원 입구에서 유래한 샘플에서 가장 높은 생물 풍부도와 다양성 수치를 보였다. 연구를 통해, 6 샘플로부터 유래한 박테리아 군집은 비슬산 군립공원에서만 발견되는 45개의 속과 287개의 종으로 구성된 공통의 조성을 보였다. 이 발견된 공통 박테리아들은 진달래 군집 생성에 영향을 끼칠 것으로 기대되고 있다.