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Prokaryotic community profiles of soils from Mayon volcano, Philippines based on 16S ribosomal RNA gene sequences

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Abstract

Mayon Volcano is the Philippines' most active volcano. Despite extensive pedological, ecological and ethnobotanical studies, no published information is known about its soil microflora. In this study, to determine the microbial community profiles, 16S rRNA gene was amplified and sequenced from genomic DNA isolated from volcanic soils collected from altitudinal gradients of Mayon Volcano. Phylogenetic analyses revealed 10 bacterial phyla, including an unclassified group, with *Acidobacteria* (40.6%) as the most dominant phylum. *Archaea* were distributed into three phyla and an unclassified archaeal group (53.9%), which comprised the majority. The composition of the prokaryotic community suggests roles in the cycling of organic and inorganic nutrients in Mayon Volcano ecosystem. At $p<0.1$, soil pH and organic matter content showed significant correlation with species richness of *Archaea* and diversity of *Bacteria*. In contrast, altitude, soil temperature and soil moisture content showed no significant influence on the composition and distribution of microorganisms in Mayon Volcano. This study provides the first known information on the prokaryotic composition of Mayon Volcano, including the soil properties that influence the structuring of these communities.

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Introduction

Mayon Volcano, also known as Mount Mayon, is a perfect stratovolcano rising to 2,462 m in the province of Albay on the island of Luzon in the Philippines. Its almost symmetric conical shape is famous world-wide. Mayon Volcano is the most active volcano in the country, registering over 50 eruptions over four centuries. Despite extensive pedological (Aberin, 2004), ecological (Dayao, 1994), and ethnobotanical studies (Buot *et al.*, 2009) conducted in and around Mayon Volcano, there is a dearth of information on the microbiological diversity of its soil.

The prokaryotes, which are distributed into domains *Bacteria* and *Archaea*, are ubiquitous, comprising the majority of organic matter on earth (Whitman *et al.,* 1998). They exhibit great metabolic and genetic diversity, and are major environmental determinants, responsible for the cycling of organic and inorganic compounds. They can also influence above-ground ecosystems by contributing to plant nutrition, plant health, soil structure, and soil fertility (Kirk *et al.*, 2004). Microbial biomass in soil and their activities are frequently used as an early indicator of changes in soil chemical and physical properties resulting from soil management and environmental stresses (Trasar-Cepeda *et al.*, 1998). Several environmental factors such as carbon and energy sources, available water, temperature, and pH can affect the ecology, activity and population dynamics of microorganisms in soil (Nannipieri *et al.*, 2003).

This study aims to determine the bacterial and archaeal community profiles in soils of Mayon Volcano. The information on the number of species of microorganisms, and their respective phylogenetic distribution, can lead to understanding the pattern and tempo of microbial diversification, as well as the complexity of this ecosystem. In addition, species-rich phylogenies may find practical application in ecosystem management, agriculture, drug discovery and medicine (McLaughlin *et al.*, 2009). The study also aims to correlate soil parameters such as temperature, pH, organic matter, moisture content, and altitude with the diversity of prokaryotes, to gain insight on the abiotic factors that may influence the assembly of these microbial communities in Mayon Volcano.

Materials and methods

Volcanic soil sample collection

Soil samples were collected from 500 meters above sea level (m asl), 1000 m asl, and 1500 m asl of Mayon Volcano. Elevation and coordinates of the study sites were determined using a Global Positioning System tracker (Garmin, USA). Each sampling site was 20 m away from the main trail. Within each site, three areas, 10 m away from each other, were measured and marked. In each of the three areas, soil samples were collected in 10 different points at 10 cm depth. Collection points were 1 m apart in a zigzag manner. Collected soil samples per elevation were mixed using trowel and pail from which three sterile bags of soil were obtained as replicates. During transport, the soil samples were kept at room temperature but kept at 4°C in the laboratory until testing. Representative samples for DNA extraction were stored at below 20°C.

Physico-chemical parameters of volcanic soils

Volcanic soil samples were submitted for analysis of organic matter (OM) content to the Analytical Services Laboratory, Soils and Agro-Ecosystem Division of Agricultural Systems Cluster, College of Agriculture, University of the Philippines Los Baños. Environmental soil temperature was measured using a thermometer during the time of collection.The pH of the soil mixture was determined using Milwaukee pH600 pocket-sized pH pen (Milwaukee Instruments, USA). To measure the moisture content of the soil, samples were placed on pre-weighed petri plate, dried in 50°C oven, and weighed every other day until the soil had dried to a constant weight.

Extraction of total genomic DNA

The total genomic DNA was extracted from each soil sample by using the FastDNA® SPIN Kit for Soil (MP

Biomedicals, LLC, Illkrich, France) by following the manufacturer's protocol. However, 20 mg of skim milk were added to the Lysing Matrix E tube with the soil sample.

Amplification of prokaryotic 16S rRNA Gene by polymerase chain reaction (PCR)

Bacteria

Amplification of bacterial 16S rRNA gene was performed in a 50 µL reaction mixture containing 1X KAPA HiFi buffer, 0.3 mM dNTPs, 0.3 µM of each of primers 27F (AGAGTTTGATCMTGG CTCAG) (Lane, 1991) and 1492R (GGGTTACCTTG TTACGACTT) (Stackebrandt and Liesack,1993), 1 U KAPA HiFi HotStart DNA polymerase, and 4.0 µL of template DNA. The thermal cycling program was run on GeneTest PCR machine (Bio-Gener Technology, China) as follows: 5 min of initial denaturation at 95°C; 35 cycles of 20 sec denaturation at 95°C; 20 sec annealing at 57°C; 1 min and 30 sec extension at 72°C; and 5 min final extension at 72°C with holding temperature of 4°C.

Archaea

Amplification of archaeal 16S rRNA gene was performed in 25 uL of PCR reaction mix containing 1X Q5 Reaction Buffer, 0.2 mM dNTPs, 0.5 µM each of primers 21F (TTCCGGTTGATCCYGCCGGA where Y= C or T) (DeLong, 1992) and 1391R (GACGGGCGGTGTGTRCA) (Barns *et al*., 1994; Reysenbach *et al*., 1994), 1X Q5 enhancer, 0.5 U Q5® High Fidelity DNA polymerase, and 4.0 µL template DNA. The thermal cycling program was run on GeneTest PCR machine (Bio-Gener Technology, China) as follows: 1 min initial denaturation at 98°C, 35 cycles of 10 sec denaturation at 98°C, 20 sec annealing at 62°C, 1 min extension at 72°C, and 5 min final extension at 72°C with holding temperature of 4°C.

Purification of PCR amplicons

Amplification products were purified by using the QIAquick PCR Purification Kit (QIAGEN Inc., California, USA), following the manufacturer's instructions.

Construction of prokaryotic 16S rRNA gene libraries

Purified 16S rRNA genes of *Bacteria* and *Archaea* were each cloned into pENTR™/D-TOPO® vector (Invitrogen, Life Technologies, California, USA). A ligation mixture containing 0.2 mM salt solution, 10 ng of purified 16S rRNA gene, and 20 ng of vector was gently mixed and incubated at 23˚C in the thermal cycler for 15 min. Two microliters of the ligation mixture were added into tube containing freshly thawed *E. coli* DH5α cells, which were then allowed to stand on ice for 20 min. Cells were heat-shocked by incubating the tubes at 42˚C water bath for 90 sec, and immediately incubated back on ice for 3 min. To permit growth of cells, 950 µL of SOC medium were added to tubes, which were incubated at 37˚C with shaking at 30 x g for 1 h. One hundred microliters of the SOC medium were plated on SOB plates with 50 µg/mL kanamycin. The remaining 900 µL SOC medium were pelleted by centrifugation at 665 x g for 3 min, which were then plated on SOB medium with 50 µg/mL kanamycin. All plates were incubated overnight at 37˚C.

Analysis of transformants by PCR

Transformants on SOB plates with kanamycin were individually picked and resuspended into 20 µL PCR mix containing 1X PCR buffer, 0.25 mM dNTPs, 0.2 uM of each of primers M13F and M13R (Thermo Fisher Scientific, California, USA), 3.0% DMSO, and 1 U Taq polymerase. The thermal cycling program was run on GeneTest PCR machine (Bio-Gener Technology, China) as follows: 5 min initial denaturation at 94˚C, 35 cycles of 30 sec denaturation at 94˚C, 30 sec annealing at 57˚C, 1 min and 30 sec extension at 72˚C, and 7 min final extension at 72˚C with holding temperature of 4˚C.

Transformants showing the expected size of the amplified insert on agarose gel electrophoresis were selected and inoculated to LB broth tubes with kanamycin for plasmid isolation. The tubes were incubated overnight with shaking at 37˚C.

Isolation of plasmid DNA putative clones

Plasmids bearing correct insets were isolated using the Perfectprep® Plasmid Midi Kit (Eppendorf, California) by following the manufacturer's protocol.

Analysis of DNA and measurement of DNA concentration

The presence and purity of DNA were verified on a 1.5% agarose gel run at 90 V for 1 h in 0.5x Tris-acetate-EDTA (TAE) buffer (20 mM Tris, 10 mM acetate, 0.5 mM EDTA, pH 8.0). The gel was stained with 0.5X Goodview™ nucleic acid stain (SBS Genetech Co., Ltd, China). DNA concentrations were estimated using NanoDrop™ 8000 Spectrophotometer (Thermo Scientific, USA).

Analysis of DNA sequence

Plasmids were sent to 1st Base DNA Sequencing Services, Malaysia for sequencing. Obtained sequences were manually evaluated using ChromasPro version 2 software to remove the low quality regions at both ends of the fragment. The sequences obtained were compared to references in the BLAST Database server of the National Centre for Biotechnology Information, allowing either specific phylogenetic classification or proposal of novel taxa when a clone is sufficiently divergent from known groups. Typically, cloned sequences were assigned to phylum, class, order, family, subfamily or species at sequence similarity cut-off values of 80, 85, 90, 92, 94 or 97%, respectively (Desantis *et al*., 2007). ClustalW software was used for multiple sequence alignment. Phylogenetic trees were inferred using neighbor-joining (NJ) tree algorithms (Saitou and Nei, 1987) performed in MEGA 6 software (Tamura *et al*., 2013). The robustness of the tree was evaluated by bootstrap analyses based on 1,000 reiterations.

Statistical analysis

The Kruskal-Wallis test was performed to determine significant differences in the physicochemical parameters of the soil samples. Phylogenetic data were analyzed using the *vegan* package in R softwareversion 3.1.1 to measure Shannon index to estimate diversity (Hill *et al*., 2003) and species richness. Analysis of variance was performed to determine the correlation between fungal diversity (Shannon diversity) or species richness and physicochemical properties of the soil.

Results and discussion

Physicochemical parameters of soils

Soils from all sampled altitudes of Mayon Volcano were acidic and statistically varied in moisture content. The soil at 1500 m asl, the closest to the crater, was significantly most acidic, and contained the least moisture and least organic matter (Table 1). The organic contents of soil at lower altitudes were significantly higher than at higher altitude.

Table 1. Physicochemical parameters of soil along altitudinal gradients of Mayon Volcano.

Altitude (m asl)	Temperature (˚C)	pH	Moisture content (%)	Organic matter content (%)
500	26.33 ± 0.58^{A}	5.93 ± 0.06^{A}	56.44 ±2.46^{A}	4.26 ± 0.32^{A}
1000	22.33 ± 0.58^{B}	5.53 ± 0.12^{A}	40.34 ±1.15^{B}	3.32 ± 0.83^{A}
1500	22.00 ± 0.00^{B}	4.10 ± 0.36^{B}	14.47 ±2.27^{C}	0.35 ± 0.13^{B}

p-value = 0.05. Values within a column with the same letter are not significantly different.

Analysis of bacterial 16S rRNA gene sequences

Twenty-four molecular operational taxonomic units (MOTUs) were generated from analysis of 16S rRNA gene sequences of a library of 64 bacterial clones. Majority of the bacterial population is represented by the phylum *Acidobacteria* (Table 2). This phylum has been consistently present in soils, comprising an average of 20% of the global soil bacteria (Janssen, 2006; Barns *et al*., 2007). The abundance of these bacteria in soils indicates their crucial roles in the

sustainability of this ecosystem. Recent physiological characterization of representative species within this phylum revealed that *Acidobacteria* can contribute to global hydrogen cycling (Greening *et al*., 2015) and to carbon cycling in forests (García-Fraile *et al*., 2015; Lladó*et al*., 2015). It is likely that the *Acidobacteria* enumerated in this study contribute to the cycling of nutrients in Mayon Volcano ecosystem. The profile of bacterial community in Mayon Volcano appears similar to that of Changbai Mountain in China (Shen *et al*., 2013) and Atlantic Rainforest in Brazil (Lima-Perim *et al*., 2016). Interestingly, Mount Fuji in Japan, also an active volcano, harbors similar bacterial profile, except for *Firmicutes*, *Planctomycetes*, and *Armatimonadetes* (Singh *et al*., 2012*)*.

Table 2. BLAST search results of the molecular operational taxonomic units (MOTU) of bacterial 16S rRNA gene sequences from soils of Mayon Volcano.

MOTU	Phylum	BLAST search result	Accession number	Identity (%)	Number of isolates at altitude (m asl)			Frequency (%)
					500	1000	1500	
1	*Acidobacteria*	*Holophaga foetida*	*NR 036891.1*	97	-	-	1	1.6
2		*Thermoanaerobaculum aquaticum*	*NR 109681.1*	100	-	2	-	3.1
3		*Candidatus Koribacter versatilis*	*NR 074350.1*	100	9	-	-	14.1
4		*Acidobacterium capsulatum*	*NR 043386.1*	99	-	1	-	1.6
5		*Granulicella paludicola*	*NR 115072.1*	98	2	-	-	3.1
6		*Candidatus Solibacter usitatus*	*NR 074351.1*	97	-	5	-	7.8
7		*Blastocatella fastidiosa*	*NR 118350.1*	97	4	2	-	9.4
								40.6
8	*Firmicutes*	*Bacillus acidiceler*	*KF150386.1*	98	1	-	-	1.6
9		*Paenibacillus contaminans*	*NR 044325.1*	98	3	-	-	4.7
10		*Streptococcus thermophilus*	*NR 074827.1*	97	2	2	2	9.4
								15.6

Table 2 continued.

11	*α-Proteobacteria*	*Rhodobacter azotoformans*	*NR 113300.1*	97	-	-	1	1.6
12		*Mesorhizobium chacoense*	*NR 025411.1*	98	-	-	1	1.6
								3.1
13	*β-Proteobacteria*	*Massilia kyonggiensis*	*NR 126273.1*	97	1	-	-	1.6
14	*γ-Proteobacteria*	*Arenimonas malthae*	*NR 043670.1*	97	-	1	-	1.6
15		*Thermomonas brevis*	*NR 025578.1*	97	-	3	-	4.7
16		*Lysobacter ginsengisoli*	*NR 112563.1*	99	-	2	-	3.1
								9.4
17	*δ-Proteobacteria*	*Haliangium ochraceum*	*NR 074917.1*	100	1	-	-	1.6
18	*Planctomycetes*	*Zavarzinella formosa*	*NR 042465.1*	98	1	-	1	3.1
19	*Armatimonadetes*	*Fimbriimonas ginsengisoli*	*NR 121726.1*	99	-	-	1	1.6
20	*Bacteriodetes*	*Empedobacter haloabium strain*	*NR 125708.1*	97	2	1	-	4.7
21	*Actinobacteria*	*Ferrimicrobium acidiphilum*	*NR 041798.1*	99	1	-	-	1.6
22		*Acidothermus cellulolyticus*	*NR 114693.1*	99	1	-	-	1.6
								3.1
23	Unclassified	Uncultured bacterium	*LN572827.1*	95	2	4	-	9.4
24		Uncultured bacterium clone	*GQ247079.1*	96	-	-	4	6.3
								15.6

Note: Numbers in boldface represent the total frequency per phylum.

Analysis of archaeal 16S rRNA gene sequences

Analysis of the 16S rRNA gene sequences of a library of 26 archaeal clones revealed nine MOTUs. Majority of the clones (53.8%) belonged to the unclassified group of *Archaea* (Table 3) as seen in soils of the Atlantic Rainforest in Brazil (Lima-Perim *et al*., 2016). The significance of this dominant group of *Archaea* in these environments remains unknown but their consistent presence in soil indicates important physiological role in this environment. Members of other archaeal phyla, namely, *Crenarchaeotea, Thaumarchaeota, and Euryarchaeota*, had also been

detected in soils of Mayon Volcano.These *Archaea*, especially the ammonia-oxidizing archaea (AOA) in the phylum *Thaumarchaeota*, have been demonstrated to play major roles in global biogeochemical cycles of nitrogen (Francis *et al*., 2007) and carbon elements (Zhang *et al*., 2015). AOA are also likely involved in nitrate leaching from soils, resulting to nitrogen loss from ecosystems and cause surface and groundwater contamination. Moreover, the activity of AOA may be a significant source of greenhouse gas (nitrous oxide) emissions from the soil (Kowalchuk and Stephen, 2001).

Table 3. BLAST search results of the molecular operational taxonomic units (MOTU) of archaeal 16S rRNA gene sequences from soils of Mayon Volcano.

MOTU	Phylum	BLAST search result	Identity (%)	Accession number	Number of isolates at altitude (m asl)			Frequency (%)
					500	1000	1500	
1	*Crenarchaeota*	*Uncult. crenararchaeote ArcB cF03*	97	*EU306968.1*	-	2	3	19.2
2	*Thaumarchaeota*	*Candidatus Nitrosopumilus koreensis*	98	*NR 102904.1*	-	2	1	11.5
3		*Candidatus Nitrosophaera gargensis*	99	*NR 102916.1*	-	-	1	3.8
								15.4
4	*Euryarchaeota*	*Methanomassiliicoccus luminyensis*	99	*NR 118098.1*	2	1	-	11.5
5	Unclassified	Uncult. Archaeon clone kaa66	96	*FJ936629.1*	3	-	-	11.5
6		Uncult. Arcceon lone Arc-CS39	96	*FJ584331.1*	-	1	-	3.8
7		Uncult. Archaeon clone D92	97	*FJ174726.1*	3	2	-	19.2
8		Uncult. Archaeon YL-S-A	97	*KC841495.1*	1	1	-	7.7
9		Uncult. Archaeon Clone A P3K3f	96	*GU127873.1*	1	2	-	11.5
								53.8

Note: Numbers in bold represent the total percentage per phylum.

Table 4. Relationship of environmental parameters to Shannon diversity index and species richness of *Bacteria* in Mayon Volcano.

Environmentalparameter	Shannon diversity index		Species richness	
	r^2	p-value	r^2	p-value
Altitude	0.08	0.21	0.05	0.29
Soil Temp	0.76	0.54	0.72	0.62
pH	0.02	0.01*(0.77)	0.11	0.09*(0.09)
Moisture Content	0.14	0.16	0.05	0.24
Organic Matter Content	0.02	0.02*(36.7)	0.02	0.10

p-value = 0.1; * Environmental parameter with significant effect on Shannon diversity index and/or species richness. Number after the asterisk (*) represents the magnitude of the effect.

Relationship between physicochemical properties of soil and microbial community

In Mayon Volcano, the pH of soil significantly ($p<0.1$) affected the prokaryotic species richness and bacterial diversity (Tables 4 and 5). The organic matter content of soil also influenced bacterial diversity as well as species richness of *Archaea*. In contrast, altitude, soil temperature, and soil moisture content had no effect on the composition and distribution of prokaryotic community. Studies on the influence of soil edaphic properties on the composition of microbial communities in the Atlantic Rainforest (Santos *et al*., 2014) and Sonoran Desert (Andrew *et al*., 2012) underline pH and organic matter concentrations as among the main soil properties influencing microbial community composition and diversity in these soils.

Soil pH has been commonly correlated with variations in bacterial and archaeal communities (Nicol *et al.*, 2008; Faoro *et al.*, 2010), and often the best predictor of diversity and composition of *Bacteria* (Fierer and Jackson, 2006; Tripathi *et al.*, 2012) and *Archaea* (Bengtson *et al.*, 2012; Tripathi *et al.*, 2013).

The null effect of altitude on prokaryotic diversity and species richness in Mayon Volcano were consistent with the findings of Fierer *et al.* (2011) on organic soil, mineral soil, and leaf surface but in contrast with those of Siles *et al.* (2016) where higher altitudes resulted to significant increase in microbial abundance, and Zhang *et al.* (2009) where altitude and abundance of ammonia oxidizing archaea in Mt Everest showed significant negative correlation. The effect of altitude is not independent, being influenced by other factors such as availability of soil organic matter (Siles *et al.*, 2016).

Table 5. Relationship of environmental parameters to Shannon diversity index and species richness of *Archaea* in Mayon Volcano.

Environmentalparameter	Shannon diversity index		Species richness	
	r^2	p-value	r^2	p-value
Altitude	0.49	0.34	0.04	0.28
Soil Temp	0.51	0.67	0.50	0.62
pH	0.98	0.14	0.05	0.08*(0.06)
Moisture Content	0.62	0.29	0.03	0.23
Organic MatterContent	0.06	0.15	0.01	0.09*(2.9)

p-value = 0.1; *Environmental parameter with significant effect in Shannon diversity index and /or Species Richness; value after the asterisk (*) represents the magnitude of the effect.

Despite significant differences in moisture content in soil from various site, surprisingly, soil moisture did not affect prokaryotic diversity and richness.

These results are in contrast to several reports where moisture was the most significant predictor of bacterial diversity at the genus level (Geyer *et al.*, 2014) and one of the key factors in shaping soil microbiome (Crits-Christoph *et al.*, 2013). Soil temperature also showed no significant effect on the microbial composition of Mayon Volcano.

Conclusion

The bacteria in soils of Mayon Volcanoare distributed into 10 known phyla and an unclassified group, and dominated by *Acidobacteria* while the Archaea were represented by three phyla and an unclassified archaeal group, which comprises the majority. Soil pH and organic matter content are important factors that influence the diversity and richness of the prokaryotes in Mayon Volcano. However, altitude, soil temperature and soil moisture content have no effect on the composition and distribution of microorganisms in Mayon Volcano.

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