Analysis of Microbial Communities on Recent Volcanic Deposits along a Vegetation Gradient in the Island of Miyake, Japan

2014.3

Symbiotic Science of Environment and Natural Resources

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Abstract

New substrates derived from volcanic activities, such as lava, tephra, and volcanic ash, are habitats opening to invasion by microorganisms and the microbial immigration might drive the initial soil genesis and ecosystem formation. Prior to colonization by plants, the pioneer microbes were found to play a significant role in fixation of carbon and nitrogen from the atmosphere, resulting in the input of organic matter to the deposits. Plant colonizers might influence the early microbial community on the new deposits through litter input, root exudates, and dead root tissues. Reciprocally, specific microbes can associate with plants through the root-microbe symbiosis or give a negative effect on plants by microbial interfering actions. Such plant-microbe interaction will accelerate a primary ecosystem succession on the volcanic deposits.

The Island of Miyake (Miyake-jima) is a volcanic island situated on the western rim of Pacific Ocean. The last eruption of Miyake-jima Island from the Mount Oyama in 2000 ejected large amounts of volcanic ash and formed a collapsed crater. About 60% of vegetation on the island was initially influenced by heavy deposition of volcanic ash. After the crater formation, large amount of volcanic gas containing SO_2 and H_2O have been emitted and caused widespread defoliation, particularly on the leeward side of Mount Oyama. Vegetation has been recovering from the damage at the foothill of mountain and windward sides. The purpose of this study was to characterize the microbial communities on the Miyake-jima volcanic deposits at a windward side where vegetation recovery gradually occurred, and to analyze how the early microbial community responds to the first colonizer plants. To this end, we established three sites along an elevational transect, representing sparsely grass-covered (site IG7), fully grass- and partially

shrub-covered (site IG8), and fully grass- and shrub-covered lands (site IG9). The volcanic deposits (C1) and the buried soils (2A) beneath them at each site were sampled in July, 2009 and September, 2011. The investigation was designed to compare bacterial and fungal communities between these deposits by molecular approaches using the T-RFLP, clone library, and PCR-based pyrosequencing methods, as well as conventional measurements of population density, respiratory activity and substrate utilization profile.

The results showed that all samples of the deposits and the buried soils were acidic [pH (H_2O), 4.2-4.7], and the deposit samples contained trace of total organic carbon (TOC, 0.2-0.7 g kg^{-1}) and total nitrogen (TN, 0.1-0.9 g kg^{-1}), which were significantly lower than those in buried soil samples (TOC, 48.9-100.1 g kg^{-1}; TN, 4.2-8.3 g kg^{-1}). Although the deposit samples contained approx. 100 times low TOC than the buried soil samples, differences in bacterial population density were not as large as the differences in TOC values. Total direct counts (TDC) and plate counts of culturable bacteria of the volcanic deposit sample were range in 1.0-4.0×10^8 cells g^{-1} (dry soil) and 0.1-1.2×10^7 CFUs g^{-1} (dry soil), respectively. However, no detectable amount of ergosterol and low counts of fungal propagules for the volcanic deposits and buried soil samples indicated that fungi constituted low fractions of their microbial community. Despite the differences in the vegetation cover, the volcanic deposit samples displayed low among-site variation for chemical properties (pH, TOC, and TN) and microbial population densities (TDC and culturable counts).

Statistical analyses of T-RFLP, clone library, and pyrosequencing data reveal that the microbial communities of volcanic deposit samples were phylogenetically diverse, in spite of very low-carbon environmental conditions, and their diversity was comparable to

that in the buried soil samples. Comparing with the microbial communities in buried soils, the volcanic deposit communities were characterized by the presence of *Betaproteobacteria* and *Gammaproteobacteria* as the main bacterial classes, *Deinococcus-Thermus* as the minor bacterial phylum, and Ascomycota as the major fungal phylum. Because the volcanic deposit samples display low among-site variation for chemical properties, there is no apparent factor other than the aboveground vegetation cover to explain difference in microbial community among the different site volcanic deposits. Multivariate analysis revealed that a positive correlation of *Oxalobacteraceae*, *Gallionellaceae*, and *Micrococcaceae* with a grass *Carex oshimensis* but a negative correlation of *Xanthobacteraceae* and *Gemmatimonadaceae* with the grass. The presence of *Sphingobacteriaceae*, *Burkholderiaceae*, and *Acetobacteraceae* correlated positively with a tree, *Camellia japonica*. Fungi thriving in the site IG7 volcanic deposits, such as Sordariomycetes, Saccharomycetes, Pezizomycetes, and Lecanoromycetes in the phylum Ascomycota, and Dacrymycetes in the phylum Basidiomycota showed a highly negative relationship with the major plants, *Miscanthus condensatus* and *Alnus sieboldiana*. Agaricomycetes in the phylum Basidiomycota correlated positively but Eurotiomycetes in the phylum Ascomycota negatively with a shrub, *Rubus trifidus*. These results, therefore, suggested that the aboveground vegetation feature significantly influenced the early microbial communities on Miyake-jima volcanic deposits, but their influence on the microbial population was not distinct. In conclusion, these findings give a better understanding of how belowground microbial communities develop and interact with the establishment of the first aboveground plants in newly exposed volcanic deposits.

Acknowledgements

I would like to express my deep gratitude to Professor Hiroyuki Ohta, my direct supervisor, for his patient guidance, enthusiastic encouragement and useful critiques of this research work, and also for his sincere concern and warm help in my life in Japan. I would also like to thank Professor Kazuhiko Narisawa and Professor Yoko Katayama, my academic supervisors together with Professor Ohta, for their valuable discussion and helpful advice to this research. My grateful thanks are also extended to Professor Yasurou Kurusu and Professor Hitoshi Sekimoto for their detailed, valuable comment and advice during the final examination. I would like to express my warm gratitude to Professor Takashi Kamijo, University of Tsukuba, and the members at his laboratory, for their earnest collaborative research in this work, and their kind assistance with the sample collection. Along these same lines, my special thanks are also addressed to Professor Masahira Hattori, University of Tokyo, and the staffs Dr. Wataru Suda, Dr. Seok-won Kim, and Dr. Kenshiro Oshima at his laboratory for their valuable discussion and technical help with 454-pyrosequencing and data processing. In addition, this research was conducted with a scholarship from Ministry of Education, Culture, Sports, Science, and Technology, Japan. I would also like to take this opportunity to give my sincere gratitude to the institution for providing the financial support to conduct this research.

Next, my special thanks are extended to Associate Professor Tomoyasu Nishizawa, Dr. Yoshinori Sato, Dr. Reiko Fujimura and Dr. Zhihua Bao who worked at Laboratory of Environmental and Toxicological Chemistry (Lab. of ETC), for their technical support, constructive discussion and suggestion contributed hugely to the process of present research. I would also like to thank the precursors and friends in Lab. of ETC, including

Masafumi Umezu, Kazuto Tsuruda, Purba Islam, Tomowaki Yasuda, Shinya Sato, Tomonori Sugawara, Masahi Ishi, Akihiro Kawamura, Miho Kanemoto, Megumi Akaike, Tatusya Imai, Masahiro Naraoka and Kei Yamada, Momoko Sato, Ayumu Nishimura, Shun Ijima, Manami Akiyama, Miyuki Ishikawa, Shoko Oshima, Makoto Shimabukuro, Saki Mizukami, Kento Uehara, Akinori Hirano and Miyu Kindaichi, and other Chinese and international friends studying in Ibaraki University and Tokyo University of Agricultural and Technology who supported in my life and experiments.

Lastly and most specially, the deepest appreciation goes to my dear parents, and also to my family, for their endless patience, pray and support in all way possible, and for their unconditional love and unwavering faith in me. For my aunty, Yubo Han who is also a graduate of Lab. of ETC, thanks for her grate help, advice and encouragement to me for having a study abroad. Finally, I would like to express my heartfelt thanks to my dear girl friend for her selfless and endlessly love, company and support to me.

Chapter 1

General Introduction

Soil formation is a long and slow process combined to the effects of climatic, mineral, biological, topological, and anthropogenic factors on parent materials. Pristine land, such as volcanic desert and glacier forefront, always consist of a mosaic of succession states, and presents an ideal opportunity to study the early development of soil genesis and terrestrial ecosystem (Crocker & Major, 1955; Rejmanek*, et al.*, 1982; Matson, 1990; Crews*, et al.*, 1995; Chadwick*, et al.*, 1999). Previous studies have documented that microorganisms can rapidly colonize the newly exposed mineral materials, and the microbiota in the new substrates contributes to catalyze the earliest stages of biogeochemical cycling (King, 2003; Sato*, et al.*, 2004; King*, et al.*, 2008; Sato*, et al.*, 2009).

Prior to colonization by plants, microbial community is the most important factor in the early development of ecosystem, as their function in accumulation of soil nutrient pool, particularly soil carbon and nitrogen (Nemergut*, et al.*, 2007; Schmidt*, et al.*, 2008; Fujimura*, et al.*, 2012). Subsequent colonization of plant directly influences the early microbial community through providing soil microbiota with organic substrates such as leaf litter, root exudates, sloughed-off cells, auto-lysed cortex cells, and dead root tissues (Bardgett*, et al.*, 2005; Dennis*, et al.*, 2010). Reciprocally, the specific soil microbes can also affect plant, as both the positive effects of root symbionts and negative effects of soil pathogens (Bardgett*, et al.*, 2005; Ehrenfeld*, et al.*, 2005; Bever*, et al.*, 2010; Bever*, et al.*, 2012). In addition, the quantity and type of organic substrates from plant differs according

to the aboveground vegetation feature. In grassland soils, ratios of above- and below-ground productivity varied from 1:1 to 1:9 (Warembourg & Paul, 1977), and the larger root density in such soil has been equated with their comparatively large microbial biomass (Lynch & Panting, 1980). But in a deciduous woodland soil, the measured annual input of litter was 5,446 kg ha^{-1}, while root litter production was estimated at 928 kg ha^{-1} and root exudates production at 687 kg ha^{-1}, giving a ratio of about 3:1 for above- and below- ground production (Gary*, et al.*, 1973). Hence, the soil microbial community may responds to the changes in plant productivity and diversity during the vegetation succession. Recently, the glacier forefront ecosystem has well characterized as the primary microbial succession model on newly exposed substrates (Jumpponen, 2003; Brankatschk*, et al.*, 2011; Knelman*, et al.*, 2012; Zumsteg*, et al.*, 2012). For instance, Knelman *et al.* (2012) reported plant specific differences in the bacterial community composition of vegetated soils, and how such differences promote shifts in biogeochemical processes, such as asymbiotic N_2 fixation, in the early stage of primary succession where low N availability may limit bacterial and plant growth. Concerning about volcanic environments, the information on such interactions is limited, but several studies on a volcanic desert and young volcanic deposits showed that the soil microbial biomass and community structure were influenced by colonizer plant (Nüsslein & Tiedje, 1999; Nara*, et al.*, 2003; Yoshitake*, et al.*, 2013).

To obtain a comprehensive understanding about plant-microbe interaction in initial development of terrestrial ecosystem, this study aimed to characterize the early soil microbial communities developed on recent volcanic deposits derived from the eruption of the Island of Miyake (Miyake-jima) in July 2000.

1.1 Introduction of Miyake-jima and the eruptive event in 2000

The Island of Miyake (Miyake-jima; 55.5 km^2 in area; the highest point, 775 m), an active basalt volcano, is situated in the western rim of Pacific Ocean (34°05′N, 139°11′E), about 180 km south of Tokyo, and belongs to the Fuji volcanic southern zone in the East Japan volcanic belt (Fig. 1). Miyeka-jima is characterized by a humid warm-temperate climate. The meteorological data in 1953-2012 reported by the MetBroker Weather Station 476770, available at http://www.tutiempo.net/en/Climate/MIYAKEJIMA/476770.htm, was as follows: mean total annual precipitation, 3,001 mm (ranged from 2,340 mm [2007] to 3,951 mm [2010]), and mean annual average temperature, 17.9 °C. The isolated island is exposed to strong winds (annual wind speed, 5.1 m s^{-1}) with frequent wind directions in southwest, west-southwest, and northeast.

The recent geological eruptions of Miyake-jima occurred in 1940, 1962, 1983, and 2000, with about 20-year intervals (Kato*, et al.*, 2005). The last eruption occurred at Mount Oyama, the summit of the island, from July to September in 2000, ejecting large amounts of volcanic ash and gas (Nakada*, et al.*, 2001). The total volume of tephra deposits in the 2000 eruptive evens is about 9.3×10^6 m^3 dense-rock equivalent or about 2.3×10^{10} kg (Nakada*, et al.*, 2005). The monthly average emission rate of SO_2 peaked at 54 kt d^{-1} in December 2000, and gradually decreased to 7 kt d^{-1} in the end of 2002 (Kazahaya*, et al.*, 2004), and gas emission has continued to this day. The data for on-going SO_2 flux measurements by Kazahaya are available at http://staff.aist.go.jp/kazahaya-k/miyakegas/COSPEC.html.

About 60% of vegetation on the island was initially damaged by the heavy deposition of volcanic ash (Yamanishi*, et al.*, 2003). After the crater formation, large amounts of

volcanic gas containing SO_2 and H_2S caused widespread defoliation, particularly on the leeward side of Mt. Oyama (Kamijo & Hashiba, 2003; Kato*, et al.*, 2005).

1.2 Ecosystem development after the 2000 eruption of Miyake-jima

Multifarious recovery of vegetation has occurred with the decreasing SO_2 emission after 2001, which included stem sprout of defoliated trees, sprout from buried vegetative organ, germination of buried seeds, and colonization of seeds and spores (Kamijo & Hashiba, 2003). According to the satellite remote sensing study and the field survey, vegetation recovery on the fresh deposits markedly proceeded in the windward side (west, northwest, north, and northeast), where SO_2 concentrations were lower than that in the leeward side (Kamijo*, et al.*, 2008; Takahashi*, et al.*, 2011). Two grass species, *Miscanthus condensatus* and *Carex oshimensis*, increased notably after the 2000 eruption, which were not dominant species in early stage of primary succession before the eruption. Increasing tendencies were also found in two shrub species, *Eurya japonica* and *Alnus sieboldiana* (Kamijo, *et al.*, 2008).

Several studies reported that the abundance of beetles and birds temporarily increased after the eruption but prior to 2006 (Katoh & Higuchi, 2011; Makihara*, et al.*, 2011). Katoh & Higuchi (2011) revealed that a lot of dead standing trees caused the plague of xylophage such as *Chlorophorus muscosus* and *Anomala japonica izuensis*. Consequently, the population density of entomophagous birds increased with the beetle bloom. The amount of dead standing tree in Miyake-jima significantly decreased after 2006 (Kamijo*, et al.*, 2011), causing the decreasing in the population densities of beetles and birds. Although there is little direct report about the recovery of animal community, Makihara *et*

al., (2011) found that the avian abundance positively correlated with the increasing in vegetation coverage. This may imply that the recovery of animal community is in progress with the vegetation recovery in Miyake-jima.

1.3 Previous studies of microbial ecology in early development of ecosystem on Miyake-jima

New terrestrial substrates such as lava, tephra, and volcanic ash, that are laid down by volcanic eruptions generally lack carbon and nitrogen. Microorganisms play an important role in the entrance of these elements into terrestrial ecosystem. Carbon monoxide-oxidizing bacteria and hydrogen-oxidizing bacteria were well demonstrated as the carbon-fixer on recent Hawaiian volcanic deposits (King, 2003; Dunfield & King, 2004; Dunfield & King, 2005; King & Weber, 2008). These bacteria were also found to contribute to the carbon-fixation into Mt. Pinatubo volcanic mudflow deposits and Miyake-jima scoria deposited in 1983 (Sato*, et al.*, 2004; King*, et al.*, 2008). Additionally, the predominant bacteria of the microbial community on the 22-year-old scoria at site KP (Fig. 1.1), a cluster related to *Herbaspirilium*, potentially fixed CO_2 from atmosphere (Lu*, et al.*, 2008). The CO_2-absorbing activities were confirmed on the unvegetated 22-year-old scoria at site KP and on the unvegetated 5-year-old volcanic ash at site OY near the crater, respectively, by the measurement of *in vitro* respiration activity (Fujimura, unpublished data), proofing the findings in Lu, *et al*., (2008). On the other hand, the nitrogenase activity was twice higher on the unvegetated volcanic ash deposited in 2000 (site OY) than that on the forest soil (site CL, > 800-year-old), suggesting relatively higher nitrogen-fixing activity of microbiota in the fresh volcanic deposits. The further

investigations of early bacterial communities on volcanic deposits at site OY found that the microbial community was dominated by autotrophic, N_2-fixing Fe(II) oxidizers, *Acidithiobacillus ferrooxidans* and the *Leptospirillum* groups, by clone library analysis of 16S rRNA genes (Sato*, et al.*, 2009; Fujimura*, et al.*, 2012; Fujimura*, et al.*, 2012). These findings suggested that the chemolithotrophic bacteria catalyze the initial biogeochemical cycling on the recent Miyake-jiama volcanic deposits.

Concerning about how the early microbial community in the volcanic deposits changes with the deposit age, metagenomic analyses of total microbial DNAs from unvegetated 3.5-, 6.6-, and 9.5-year-old volcanic deposits at site OY (sample ID; OY04, OY05, and OY10, respectively), and from another 9-year-old deposits with low influence of volcanic gas at site IG7, and from >800-year-old forest soil at site CL were carried out (Fujimura, 2012). That study showed that chemolithotrophic Fe-oxidizing bacteria dominated the early microbial community and contributed to the accumulation of carbon and nitrogen. But the early microbial community rapidly changed with deposited age. Initially, *Acidithiobacillus ferrooxidans* and *Leptospirillum ferrooxidans* dominated the 3.5-year-old deposit, but the abundances of these bacteria decreased with the deposit age. Moreover, the analyses of *rbcL* and *porA* genes showed that *Acidithiobacillus* and *Leptospirillum* genera dominated carbon-fixing communities in all volcanic samples. However, the analyses of *nifH* gene showed that genera *Acidithiobacillus* and *Leptospirillum* initially dominated the 3.5-year-old deposits, whereas the abundance of heterotrophic N_2-fixing microbes increased with the deposit age. Additionally, more diverse denitrifying genes (*narG*, *nirS*, *nirK*, *norB*, and *nosZ*) were detected in the low volcanic gas influenced deposits, which were comparative to those in the forest soil in

Miyake-jima. These findings provided a comprehensive understanding of the succession of early microbial community before the plant colonization in the volcanic deposits on Miyake-jima.

1.4 Purpose of this study

Plant establishment is the important marker of the formation and development of terrestrial ecosystem, because that almost all of the energy flowing in the terrestrial ecosystem comes from the photosynthetic products of plant (Begon*, et al.*, 1990). A number of studies had showed that the activities of chemoautotrophs before vegetation establishment, particularly asymbiotic N_2-fixing bacteria, might create a relatively suitable habitat for the initial plant colonizer. As the consequence of plant colonization, a large amount of organic input providing by the plant colonizer may give rise to a dramatic influence on the early soil microbiota. However, the detailed information about how the plant colonizer affects the early microbial community on fresh volcanic deposit of Miyake-jima is still little known.

Additionally, in spite of several ecological researches on early soil microbiota on Miyake-jima Island, no study of the development of the fungal community in volcanic deposits responded to deposited age or vegetation succession has been conducted, except for the distribution of ectomycorrhizal fungi in soil at different depths was investigated to examine the effect of the 2000 eruption (Yamanaka & Okabe, 2006). As a large fraction of soil microbiota, fungi play an important role in the terrestrial ecosystem. For instance, mycorrhizal symbioses with plant root are universal in terrestrial ecosystem and contribute to uptake of mineral nutrients, nutrient mobilization from organic substrate,

and mediation of plant responses to stress (Roger, 2004). Such these functions of fungi may have been fundamental to land colonization by plants (Read & Perez-Moren, 2003; Martin*, et al.*, 2008). Hence, the study of the early fungal communities in unvegetated and vegetated volcanic deposits would conduct a better understanding of establishment and early development of terrestrial ecosystem.

For a comprehensive understanding of the early development of terrestrial ecosystem, the objects of this research were:

1. To analyze the soil physicochemical properties, microbial population and activity on the 9- and 11-year-old volcanic deposits at poorly to fully vegetated sites in Miyake-jima. This study aimed to examine the effect of vegetation type on the early development of soil physicochemical properties (pH, water content, water holding capacity, total organic carbon, and total nitrogen) and microbial properties (microbial population density, respiratory activity, and organic substrate utilization).
2. To investigate the bacterial communities on the 9-year-old volcanic deposits at poorly to fully vegetated sites in Miyake-jima by T-RFLP and clone library analyses targeting the 16S rRNA gene. This study aimed to examine the effect of vegetation type on the early development of soil bacterial community.
3. To investigate the bacterial and fungal diversities and communities on the 9- and 11-year-old volcanic deposits at poorly to fully vegetated sites in Miyake-jima by pyrosequecing of 16S rRNA gene and 18S rRNA genes, respectively. This study aimed to explore the relationships of first plant colonizers and specific microbial groups in the new volcanic deposits.

In this research, the buried soils beneath the volcanic deposits at each sampling site were also analyzed to determine the difference in change trajectory of early soil and mature soil in response to a same vegetation succession.

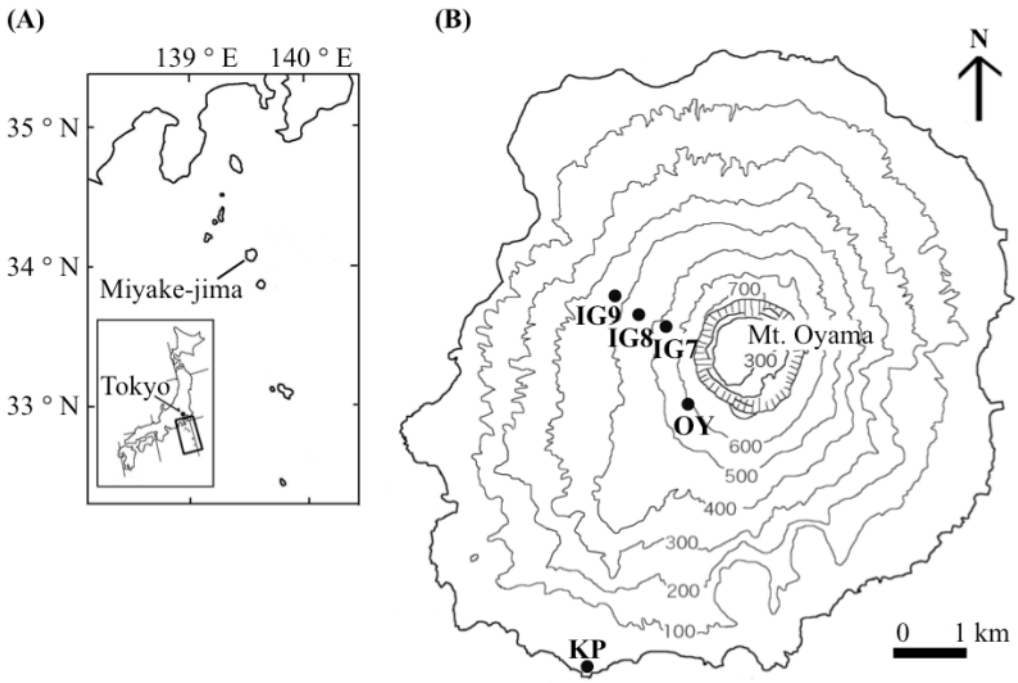


Fig. 1.1. Maps showing the location of the Island of Miyake (Miyake-jima) in the western rim of the Pacific Ocean (A) and the sampling sites in previous researches and in this research (B).

Chapter 2

Physicochemical and Microbiological Properties of Poorly to Fully Vegetated Volcanic Deposits in Miyake-jima

2.1 Introduction

New substrates (lava, tephra, and volcanic ash) originated from volcanic activity are infertile for soil microorganism and terrestrial plant, generally lacking organic matter and nitrogen. The accumulation of these substances takes an important part in soil genesis and terrestrial ecosystem development. Previous studies on chronosequence have showed that carbon and nitrogen content of new substrates increased with the exposure age, and microorganisms contribute to the early process of C and N accumulation (Crews*, et al.*, 1995; Hodkinson*, et al.*, 2003; King, 2003; Lilienfein*, et al.*, 2003; Fujimura, 2008; Zumsteg*, et al.*, 2012). Plant colonization can accelerate the accumulation of organic matter through litter input, root exudate, and dead root tissues, also give a big influence on soil microbial properties (Lynch & Panting, 1980; Zak*, et al.*, 2003; Wardle*, et al.*, 2004). Although a number of studies have characterized the effect of primary succession on soil chemical and microbial properties (Nierop*, et al.*, 2001; Schipper*, et al.*, 2001; Schmidt*, et al.*, 2008), only a few of such study concerning primary succession on volcanic environment were conducted.

In this chapter, the physicochemical and biochemical properties of the recent volcanic deposit along a vegetation gradient representing sparsely grass-covered site, fully grass-covered site, and fully grass-covered and shrub-covered site in Miyake-jima Island, Japan were examined: analyses included the determinations of soil pH, water content,

water holding capacity, carbon content, nitrogen content, microbial population densities, respiration activity, and utilization profile of organic compounds.

2.2 Materials and methods

Site description and sample collection

Miyake-jima Island is an active volcano island situated in the western rim of the Pacific Ocean. Mt. O-yama in the island last erupted in 2000 with the ejection of large amounts of volcanic gas (mainly SO_2) and ash. About 60% of forest area in the island had been destroyed by the eruption in 2000. The fresh volcanic ash had been characterized by high contents of fine sand (36-76%), acidity [pH (H_2O), 3.1-4.0], and high amounts of exchangeable Ca^{2+} (33.5-115 cmolc kg^{-1}) and Al^{3+} (0.8-10.2 cmolc kg^{-1}) (Kato*, et al.*, 2002). An on-site vegetation survey in 2003 showed that vegetation recovery was found in the windward area and characterized by stem sprout of defoliated trees, sprout from buried vegetation organ, germination of buried seeds, and colonization of seeds and spores (Kamijo & Hashiba, 2003).

Site IG7 to IG9 (Fig. 1.1) were established along an elevational transect in the northwest side of Mt. Oyama (altitude: site IG7 540 m; site IG8, 437 m; and site IG9, 380 m). The thickness of volcanic deposit derived from the eruption in 2000 was 450, 330, and 280 mm at sites IG7, IG8, and IG9, respectively (Fig. 2.2.1). The percentages of vegetation cover on these sites are summarized in Table 2.2.1 and the detailed vegetation profiles are shown in Fig. 2.2.2. From analysis using satellite data, the study sites were completely (site IG7) or partially (sites IG8 and IG9) unvegetated in November 2000 (Yamanishi*, et al.*, 2003). In 2009, the deposit at site IG7 supported very limited growth

of low grass species (<1 m in height), *Miscanthus condensatus* and *Calamagrostis autumnalis*. The percent coverage of low grass was 20% in 2009 and increased to 45% in 2011, accompanied with the growth of other low grass species, *Polygonum cuspidatum* var. *terminale*, *Carex oshimensis*, and *Carex okuboi*. High grass species (>1 m in height) and shrub species did not occur on the deposit at the site. Sites IG8 and IG9 were characterized by the vigorous growth of *M. condensatus* up to 2 m in height and its high coverage (90 to 100%). A deciduous broad-leaved tree, *Alnus sieboldiana*, established at the sites after the eruption and its coverage at site IG2 was 15% and 25% in 2009 and 2011, respectively, and that at site IG3 was 65% and 60% in 2009 and 2011, respectively.

The upper volcanic deposits (C1) derived from the eruption in 2000 and the buried soils (2A) beneath the deposit were sampled in July 27, 2009 (deposit age, 9 y) and September 5, 2011 (deposit age, 11 y). After removing the surface litter layer, the upper volcanic ash deposits were taken from 10-200 mm in depth at site IG7, 30-140 mm in depth at site IG8, and 50-160 mm in depth at site IG9. The buried soil was also taken from 0-50 mm in depth under the volcanic layer at each site. At sampling, several core samples were taken from each layer up to a total of about 1 kg, mixed in sterile plastic bags, and immediately stored on ice. Finally, samples were divided into two portions and kept at 4°C and -20°C until bacteriological analyses and DNA extraction were done, respectively. Major roots and debris were removed from all samples prior to analysis and extraction.

Physicochemical analyses

Total organic carbon (TOC) and inorganic carbon (IC) were determined using a

Shimadzu TOC-L (Shimazu, Kyoto, Japan). Total nitrogen (TN) was determined using a Yanaco CHN Corder type MT-6 (Yanaco Analytical Instruments, Kyoto, Japan). Slurry consisted of 1:2.5 mass ratio of sample and deionized water was used to determine pH value. The volumetric water content was analyzed by drying the materials at 105 °C overnight. The saturated water capacity was measured by the weight of water that air-dried materials can absorb in an hour.

Respiratory activity and substrate utilization profile

To measure *in vitro* respiratory activity (as CO_2 evolution), 200 g of volcanic deposit sample or 100 g of buried soil sample were placed together with a portable wireless infrared CO_2 monitor (C2D-W01TR or C2D-W02TR; UDOM, Mito, Japan) in a sealed 1,100-mL volume plastic box. Carbon dioxide concentration in the box was recorded continuously at room temperate (27-30°C) for 90 minutes and initial CO_2 production rate was calculated. The assay was performed within several hours after sampling. ECO MicroPlate (BiOLOG, CA, USA) was used for organic substrate utilization profiling as described previously (Fujimura*, et al.*, 2012). In brief, 1 g of sample was suspended in 99 mL sterile water and then the suspensions were shaken on a reciprocal shaker at 220 strokes min^{-1} for 20 minutes. After centrifugation at 500×*g* for 10 minutes, 150 μL subsamples were inoculated to each well on the plate (triplicate). The inoculated plates were incubated at 30°C for 8 days.

Enumeration methods and soil ergosterol quantification

Total direct microscopic counts (TDC) of bacteria were determined using ethidium bromide with fluorogenic dye as described previously (Ohta*, et al.*, 2003). In brief,

triplicate membrane filters were prepared and bacteria were counted in at least 50 randomly selected microscopic fields of each filter preparation. Culturable bacteria were enumerated on full-strength nutrient broth (NB, medium composition showed in Table 2.2.2) and 1:100 diluted nutrient broth (DNB, medium composition showed in Table 2.2.3) as the plating agar medium (Ohta & Hattori, 1980; Lu*, et al.*, 2008). Four replicates of sample dilutions were plated and incubated at 30ºC for 28 days. Fungal propagules were counted on rose bengal agar medium (Table 2.2.4) in four replicates (Martin, 1950), and the plate media incubated at 23ºC for 7 days**.** Ergosterol was determined as an indicator of fungal biomass by the method of vibration-assisted extraction followed by HPLC quantification (Gong*, et al.*, 2001; Zhaorigetu*, et al.*, 2008). The HPLC system (Tosoh Corporation, Tokyo, Japan) was essentially the same as described previously (Zhaorigetu*, et al.*, 2008). A soil sample taken at a forest site not suffering from the 2000 eruption in Miyake-jima and two agricultural soils from Field Science Center, Ibaraki University College of Agriculture were used as the control.

Statistical analysis

Statistical analyses were conducted using R 3.0.2 for Mac OS X Cocoa GUI (http://r-project.org). Multivariate analysis of variance (MANOVA) was used to test for significant differences in the chemical and biological properties of samples and Tukey's honestly significant difference (HSD) test was performed to determine the rank order. Significance was defined at $P<0.05$.

Table 2.2.1. Vegetation cover profiles at site IG7 to site IG9 in Miyake-jima Island.

Site	Date	Low grass layer		High grass layer		Shrub layer		Total plant species	Dominant plant species
		Height (m)	Coverage (%)	Height (m)	Coverage (%)	Height (m)	Coverage (%)		
IG7	2009	1	20	-	-	-	-	5	*Miscanthus condensatus* (2)[a], *Calamagrostis autumnalis* (1)
	2011	1	45	-	-	-	-	6	*Miscanthus condensatus* (2), *Polygonum cuspidatum* var. *terminale* (2), *Calamagrostis autumnalis* (1), *Carex oshimensis* (1)
IG8	2009	0.5	10	3	100	6	15	15	*Miscanthus condensatus* (5), *Rubus trifidus* (1), *Alnus sieboldiana* (2), *Eurya japonica* var. *japonica* (1), *Camellia japonica* (1), *Dryopteris caudipinna* var. *caudipinna* (1)
	2011	0.5	20	3	100	6	25	14	*Miscanthus condensatus* (5), *Rubus trifidus* (2), *Alnus sieboldiana* (2), *Smilax china* (1), *Eurya japonica* var. *japonica* (1), *Machilus thunbergii* (1), *Carex oshimensis* (1)
IG9	2009	0.7	20	3	100	6.5	65	21	*Miscanthus condensatus* (5), *Alnus sieboldiana* (4), *Rubus trifidus* (1), *Carex oshimensis* (2), *Dryopteris caudipinna* var. *caudipinna* (1), *Ardisia japonica* (1)
	2011	0.5	30	3	90	6.5	60	21	*Miscanthus condensatus* (5), *Alnus sieboldiana (4), Smilax china* (1), *Ligustrum ovalifolium* var. *pacificum* (1), *Rubus trifidus* (1), *Camellia japonica* (1), *Microtropis japonica* (1), *Hydrangea macrophylla* (1), *Carex oshimensis* (1), *Ardisia japonica* (1), *Kadsura japonica* (1)

[a] Numbers in parentheses show the species abundance represented in Braun-Blanquet cover-abundance scale: 5, $c \geq 75\%$; 4, $50\% \leq c \leq 75\%$; 3, $25\% \leq c \leq 50\%$; 2, $5\% \leq c \leq 25\%$; 1, $0.1\% \leq c \leq 5\%$; +, $c \leq 0.1\%$.

Table 2.2.2. Nutrient broth agar medium (NB)

Meat extract (Wako)	10 g
Peptone (Bacto™)	10 g
NaCl (Wako)	5 g
Distilled water	1000 ml
Agar (Wako)	15 g

pH, 7.0 – 7.2

Table 2.2.3. 100-fold diluted nutrient broth agar medium (DNB)

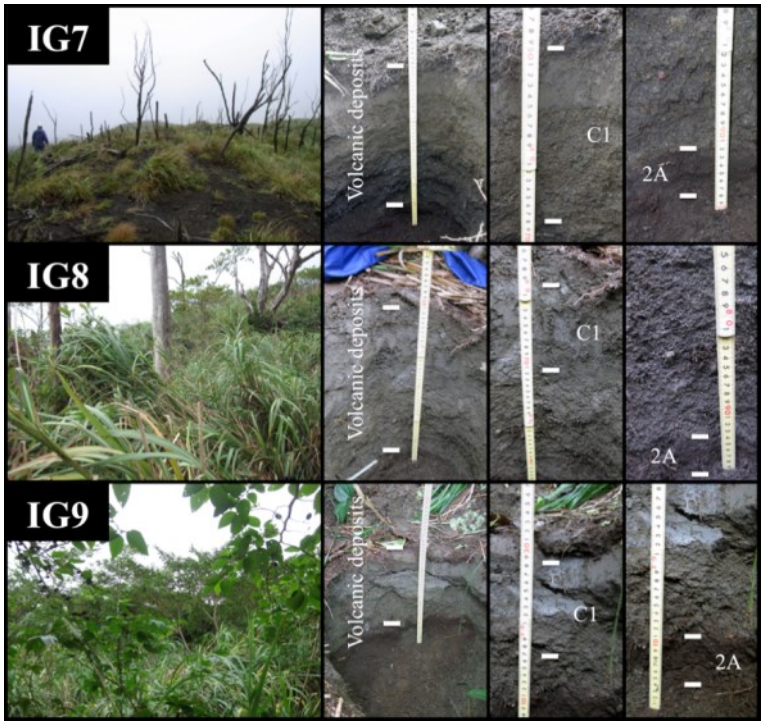
Sterile agar-free nutrient broth (pH: 7.0 – 7.2)	10 ml
Distilled water	Approx. 990 ml
Agar (Wako)	15 g

Table 2.2.4. Martin medium

KH_2PO_4 (Wako)	1.0 g
$MgSO_4 \cdot 7H_2O$ (Wako)	0.5 g
Peptone (Bacto™)	5.0 g
Glucose (Wako)	10.0 g
Rose bengal (Wako)	0.033 g
Streptomycin (Wako)	30 mg
Distilled water	1000 ml
Agar (Wako)	15.0 g

pH, 6.8

(A)



(B)

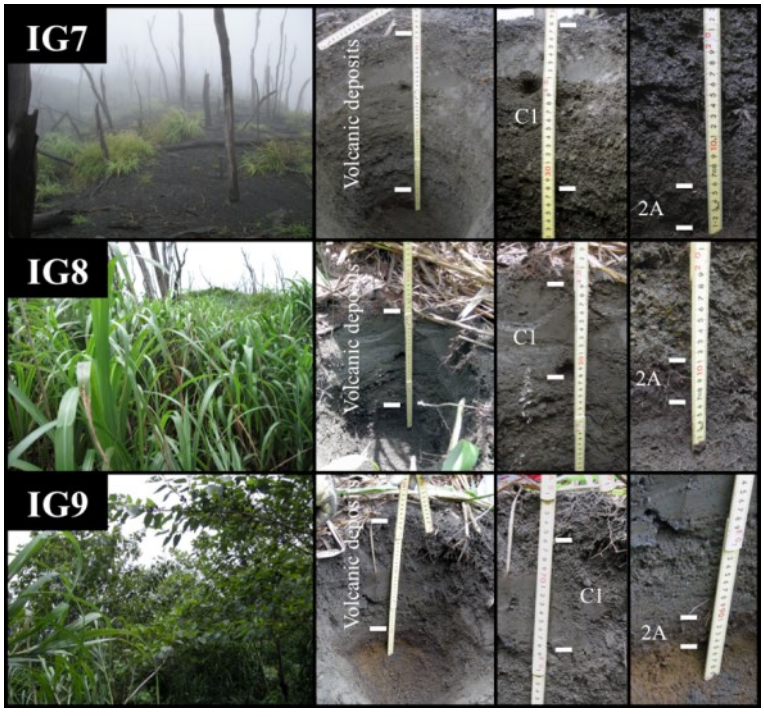


Fig. 2.2.1. Photographs showing the vegetation cover and soil layer profiles of volcanic deposit (C1) and buried soil (2A) at sites IG7, IG8, and IG9 in 2009 (A) and 2011(B).

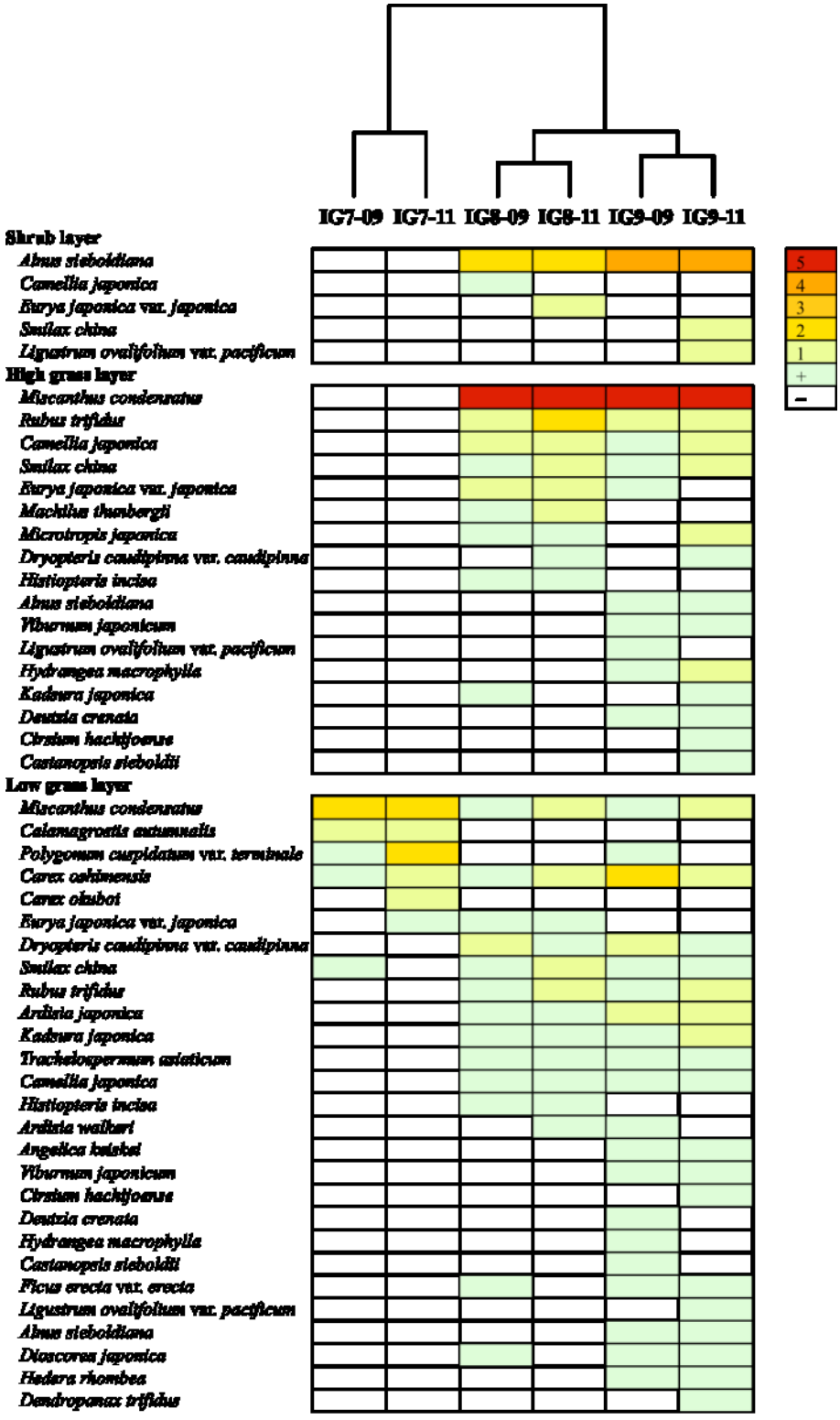


Fig. 2.2.2. Heat map showing the abundances and distributions of plant species at site IG7, IG8, and IG9 in 2009 and 20011. The color key relates to species abundance represented in Braun-Blanquet cover-abundance scale: 5, c ≥ 75%; 4, 50% ≤ c ≤ 75%; 3, 25% ≤ c ≤ 50%; 2, 5% ≤ c ≤ 25%; 1, 0.1% ≤ c ≤ 5%; +, c ≤ 0.1%; -, not detected.

2.3 Results

Physicochemical and biochemical characteristics

Physicochemical properties of the volcanic deposit and buried soil samples are shown in Table 2.3.1. The pH value of volcanic deposits was similar to that of buried soils and varied between 4.2 and 4.7, with no significant difference between the samples in 2009 and 2011. TOC (0.2-0.7 g kg^{-1}) and TN (0.2-0.9 g kg^{-1}) contents were significantly lower in the volcanic deposit samples and not significantly different between the sites, comparing with the buried soils. TOC values of buried soils varied from 48.9 g kg^{-1} for the sample from site IG9 in 2011 (sample ID, IG9-2A-11) to 100.1 g kg^{-1} for the sample from site IG7 in 2011 (sample ID, IG7-2A-11). In a parallel tendency with TOC, TN contents of the buried soils varied from 4.2 to 8.3 g kg^{-1}. Trace amounts of total inorganic carbon (0.10 g kg^{-1}) was detected for IG7-2A-09, IG7-2A-11, and IG8-2A-09, but undetectable in all deposit samples and the other soil samples. C/N ratios of volcanic deposit samples were significantly lower than those of buried soil samples. Water contents and saturated water capacities of volcanic deposit samples were also significantly lower than those of soil samples, and not significantly different between the sites and the years.

In vitro respiration activities of the volcanic deposit samples (0.15-0.57 μg CO_2-C g^{-1} h^{-1}) were significantly lower than those of the buried soil samples (0.98-1.60 μg CO_2-C g^{-1} h^{-1}) (Tukey's HSD test, $P<0.05$). The stable results of organic substrate utilization profiling with ECO MicroPlate were obtained by incubating the plates for 8 days (Table 2.3.2). In the case of volcanic deposits, the IG7-C1-09 sample used the fewest substrates (13 substrates) and the IG8-C1-09 sample showed the highest utilization (29 substrates).

In contrast, the buried samples used more substrates (22-28 substrates) than the volcanic deposit samples, except for the IG7-2A-09 sample (15 substrates).

Microbial populations

Microbial population densities of the volcanic deposit samples ranged from about 1.0×10^8 cells g^{-1} (dry soil) for IG7-C1-09 to 4.0×10^8 cells g^{-1} (dry soil) for IG8-C1-09, which were about one order of magnitude lower than levels in the buried soil samples (Fig. 2.3.1A). Similarly, plate counts on DNB were 7 to 10 times lower in the volcanic deposit samples (1.0×10^6 to 3.0×10^6 CFU g^{-1} [dry soil]) than those in the soil samples (7.0×10^6 to $1.2\times10^7 g^{-1}$ [dry soil]) (Fig. 2.3.1B). This was also the case for plate counts on NB (Fig. 2.3.1C). The plate counts on DNB were 1.2 to 1.9 times higher than those on NB for all the tested samples, except for the IG7-C1-09 sample giving 5.5 times higher counts on DNB than on NB. The difference between plate counts on DNB and NB can be explained partly by the presence of oligotrophic bacteria (Ohta & Hattori, 1980; Ohta & Hattori, 1983). The ergosterol content was undetectable levels (<0.01 μg g^{-1} [dry soil]) for all the tested volcanic deposit and buried soil samples (Table 2.3.1). Because the reference soils from the forest in Miyake-jima and the arable land contained 0.09 and 0.35 μg g^{-1} (dry soil), the results indicated that the fungal populations in both the volcanic deposits and buried soils were far lower than those in normal environmental soils. Actually, for the samples taken in 2011, the fungal propagule counts were as low as 10^2 to 10^3 g^{-1} (dry soil) for the volcanic deposit samples and 10^3 to 10^4 g^{-1} (dry soil) for the buried soil samples (Fig. 2.3.1D).

Table 2.3.1. Physicochemical and biochemical properties of Miyake-jima volcanic deposit and buried soil[*].

Sample ID[†]	pH	TOC ($g\ kg^{-1}$)	IC ($g\ kg^{-1}$)	TN ($g\ kg^{-1}$)	C:N ratio	WC (%)	WHC ($g\ H_2O\ g^{-1}$)	Respiratory activity ($\mu g\ CO2\text{-}C\ g^{-1}\ h^{-1}$)	Ergosterol content ($\mu g\ g^{-1}$)
Volcanic deposit									
IG1-C1-09	4.3	0.2 ± 0.0^{a}	-	0.2 ± 0.0^{a}	0.9	22	0.5	0.15 ± 0.02^{a}	< 0.01
IG1-C1-11	4.7	0.2 ± 0.0^{a}	-	0.1 ± 0.0^{a}	1.6	25	ND	0.53 ± 0.07^{abc}	< 0.01
IG2-C1-09	4.3	0.5 ± 0.1^{a}	-	0.9 ± 0.0^{a}	0.6	21	0.5	0.37 ± 0.11^{abc}	< 0.01
IG2-C1-11	4.5	0.3 ± 0.1^{a}	-	0.3 ± 0.1^{a}	0.8	26	ND	0.57 ± 0.10^{abcd}	< 0.01
IG3-C1-09	4.2	0.3 ± 0.0^{a}	-	0.8 ± 0.1^{a}	0.4	23	0.5	0.25 ± 0.05^{ab}	< 0.01
IG3-C1-11	4.4	0.7 ± 0.0^{a}	-	0.5 ± 0.1^{a}	1.3	27	ND	0.32 ± 0.06^{abc}	< 0.01
Buried soil									
IG1-2A-09	4.5	94.5 ± 4.3^{d}	0.1 ± 0.0	7.3 ± 0.1^{d}	12.9	50	1.1	0.98 ± 0.16^{bcde}	< 0.01
IG1-2A-11	4.3	100.1 ± 1.5^{d}	0.1 ± 0.0	8.3 ± 0.6^{d}	12.1	55	ND	1.56 ± 0.19^{e}	< 0.01
IG2-2A-09	4.4	69.0 ± 7.5^{c}	0.1 ± 0.0	5.3 ± 0.2^{c}	12.9	45	0.9	1.06 ± 0.03^{cde}	< 0.01
IG2-2A-11	4.5	60.3 ± 7.1^{bc}	-	5.1 ± 0.3^{bc}	11.9	46	ND	1.60 ± 0.28^{e}	< 0.01
IG3-2A-09	4.4	62.6 ± 2.2^{bc}	-	4.6 ± 0.2^{bc}	13.6	52	0.9	1.37 ± 0.17^{de}	< 0.01
IG3-2A-11	4.6	48.9 ± 0.8^{b}	-	4.2 ± 0.1^{b}	11.8	44	ND	1.41 ± 0.30^{e}	< 0.01

[*] TOC, total organic carbon; IC, inorganic carbon; TN, total nitrogen; WC, water content; WHC, water holding capacity; -, not detected; ND, not determined. Data for TOC, IC, TN, and respiratory activity represents the mean and STD of triplicate determinations. Different letters indicate significant differences between mean values within a given comparison (MANOVA with Tukey's HSD test, $P<0.05$).

[†] C1, volcanic deposit; 2A, buried soil; -09, sampled in 2009; -11, sampled in 2011.

Table 2.3.2. ECO MicroPlate reactions for water extracts from the Miyake-jima volcanic deposits and soils

		Volcanic deposit at site[a]:			Soil at site[a]:		
Substrate		IG1	IG2	IG3	IG1	IG2	IG3
Amines	Phenyl ethylamine	-/+	+/+	w/w	-/+	+/+	+/+
	Putrescine	-/-	+/w	w/-	-/+	+/+	w/+
Amino acids	L-Arginine	w/+	+/+	w/+	w/+	+/+	+/+
	L-Asparagine	-/+	+/+	w/+	-/+	+/+	+/+
	Glycyl-L-Glutamic acid	-/-	+/+	w/-	-/+	w	+/+
	L-Phenylalanine	+/+	+/+	+/+	-/+	+/+	+/+
	L-Serine	-/+	+/+	w/w	w/+	+/w	+/+
	L-Threonine	w/w	+/+	-/+	-/+	+/+	+/+
Carbohydrates	D-Cellobiose	-/w	+/+	-/-	w/+	+/+	+/+
	Erythritol	-/-	w/+	-/-	-/+	-/+	w/-
	D-Galactonic acid, γ-lactone	+/+	+/+	w/+	w/+	+/+	w/+
	N-Acetyl-D-Glucosamine	w/w	+/+	w/+	w/+	+/w	+/+
	Glucose-1-Phosphate	-/-	w/w	-/-	-/-	-/-	-/+
	β-Methyl-D-Glucoside	-/w	+/+	-/w	-/-	-/w	w/w
	D,L-α-Glycerol phosphate	w/-	+/-	-/w	-/+	w/-	-/+
	α-D-Lactose	-/w	w/+	w/-	w/+	+/w	+/w
	D-Mannitol	-/+	+/+	+/w	w/+	+/w	+/+
	D-Xylose	-/-	-/-	-/-	w/-	w/-	-/-
Carboxylic acids	D-Galacturonic acid	w/+	+/+	w/+	-/+	+/-	+/+
	D-Glucosaminic acid	-/w	+/+	+/-	-/+	+/w	+/+
	γ-Hydroxybutyric acid	w/+	+/+	w/w	-/+	w/-	-/w
	α-Ketobutyric acid	-/+	+/+	w/w	-/-	+/-	+/+
	Itaconic acid	-/+	w/+	-/-	-/+	-/-	-/w
	D-malic acid	+/-	+/+	w/+	-/+	+/w	+/+
	Pyruvic acid methyl ester	+/+	+/+	+/+	+/+	+/+	+/+
Phenolic compounds	2-Hydroxy benzoic acid	w/-	+/+	w/-	w/+	-/-	-/w
	4-Hydroxy benzoic acid	w/+	+/w	w/-	+/+	+/-	+/+
Polymers	α-Cyclodextrin	-/-	w/-	-/-	w/-	-/+	w/-
	Glycogen	-/-	-/-	-/-	w/-	-/+	-/+
	Tween 40	-/+	+/+	-/+	+/+	+/+	+/+
	Tween 80	w/+	+/+	+/+	+/+	+/+	+/+
Positive reaction		4/15	14/24	5/11	4/25	20/14	19/23
Weakly positive reaction		9/6	5/3	15/7	11/0	4/8	5/5
Total No. of utilized substrates		13/21	29/27	20/18	15/25	24/22	24/28

[a] Reactions were scored positive (+), weakly positive (w), or negative (−) relative to control wells. Assays were examined separately in 2009 and 2011 (2009 sample/2011 sample).

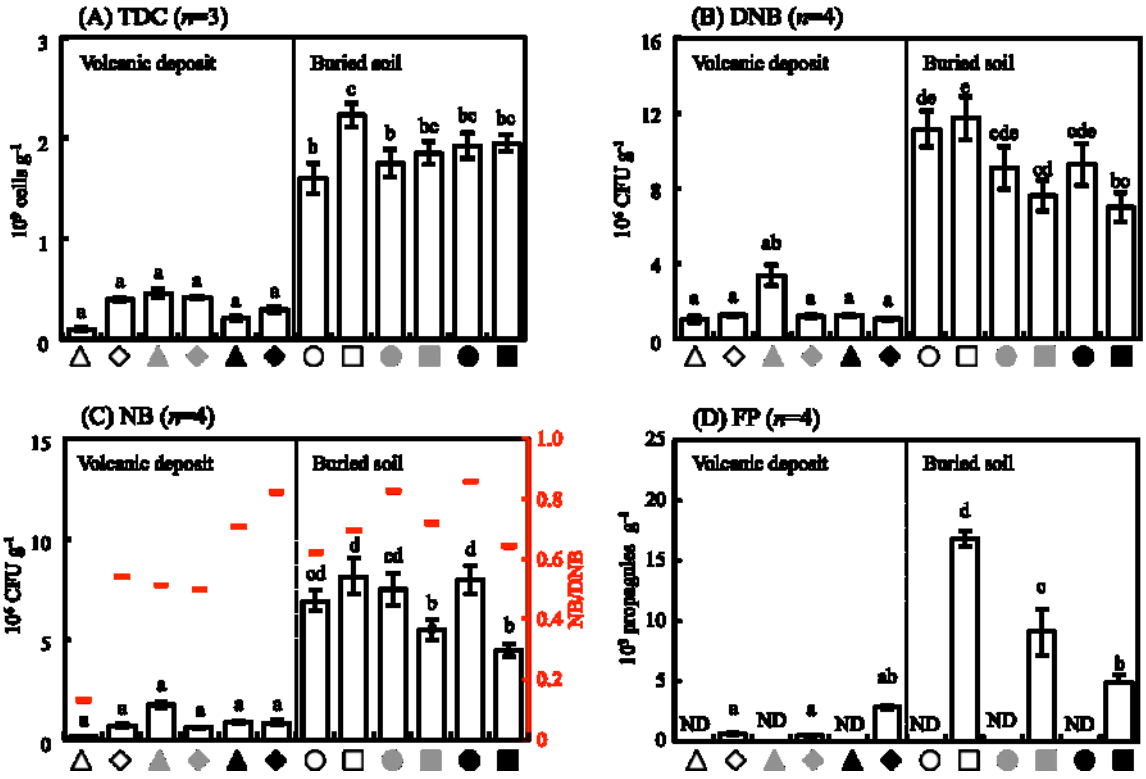


Fig. 2.3.1. Microbial populations of Miyake-jima volcanic deposit and buried soils. TDC, total direct count (A); DNB, bacterial plate count on 100-fold diluted nutrient broth (B); NB, bacterial plate count on full-strength nutrient broth (C); FP, fungal propagule count on Martin medium (D). The red lines in (C) indicate the ratio of NB to DNB. ND, not determined. △, IG7-C1-09; ▲,IG8-C1-09; ▲,IG9-C1-09; ◇,IG7-C1-11; ◆, IG8-C1-11; ◆, IG9-C1-11; ○, IG7-2A-09; ●, IG8-2A-09; ●, IG9-2A-09; □, IG7-2A-11; ■, IG8-2A-11; ■, IG9-2A-11.

2.4 Discussions

Accumulation of nutrients in newly exposed materials has much studied in aspect of soil formation (Crews*, et al.*, 2001; Nierop*, et al.*, 2001; Tscherko*, et al.*, 2003). A number of studies have indicated that early development of terrestrial ecosystem on recent volcanic deposits (Nara*, et al.*, 2003; Gomez-Alvarez*, et al.*, 2007; Mark Ibekwe*, et al.*, 2007; Yoshitake*, et al.*, 2013) and deglaciated soils (Nemergut*, et al.*, 2007; Schmidt*, et al.*, 2008; Schutte*, et al.*, 2009) was associated with pioneer colonizer plants. In a study with volcanic desert on Mount Fuji, total carbon (TC), total nitrogen (TN), and soil organic matter (SOM) contents increased with the vegetation development, and soil microbial biomass was strongly correlated with TC, TN, and SOM contents (Yoshitake*, et al.*, 2013). These findings suggested that the belowground accumulation of organic nutrients along a vegetation development was a determinant for soil microbial biomass. In our study sites, the sites IG8 and IG9 were covered fully by grass plants and partly or mostly by shrub plants. In spite of vegetation development, the TOC values of all volcanic deposit samples (0.2-0.7 g kg^{-1} in Table 2.3.1) were much lower than those of the samples in the above-mentioned studies [8.1-28.9 g kg^{-1} for a subalpine volcanic desert on Mount Fuji (Yoshitake*, et al.*, 2013); 2.5-3.0 g kg^{-1} for a glacier forefield (Knelman*, et al.*, 2012)]. From the data of the 16-, 38-, 60-, and 125-years-old Volcanogenous Regosols in Miyake-jima (Kato*, et al.*, 2005), a positive linear relationship is noted between volcanic deposit age and TC, which is approximated by an equation, TC (g kg^{-1}) = 0.023 t (r^2 = 0.98), where t is volcanic deposit age (y). The TOC values (0.2-0.7 g kg^{-1}) of our volcanic deposit samples (age, 9-11 y; total inorganic carbon, undetectable levels) are substantially fit to the equation. From the equation, it will take

>43 years to accumulate TC >1.0 g kg^{-1} in the volcanic deposit. Therefore, our study reveals the earliest change in belowground microbial community at the onset of vegetation cover development.

Although the TOC content of volcanic deposit samples was approx. 100 times lower than those of the buried soil samples, differences in microbial population density were not as large as the differences in TOC values (Table 2.3.1 and Fig. 2.3.1). This can be explained partly by differences in the content of available organic matters. When the *in vitro* respiratory activity of all samples are plotted against the corresponding TOC values, the respiration per unit of organic carbon decreased sharply at higher TOC values (Fig. 2.4.1), suggesting a relative reduction in available substrate in the samples. This organic matter dynamics was noted previously by a study with 18- to 300-years-old Hawaiian volcanic deposits (King, 2003). Interestingly, a recent study with deglaciated soils showed that soil carbon along the chronosequence was of microbial origin and inputs of organic matter were dominated by microbial carbon and nitrogen fixation (Schmidt*, et al.*, 2008). Generally, microbial biomass is characterized by low C:N ratio and biomass debris is readily consumable for soil microbes, which can result in high activity of respiration per unit of organic carbon. Indeed, the C:N ratio was much lower in the volcanic deposit samples (0.4 to 1.6) than the buried soil samples (11.8 to 13.6) (Table 2.3.1).

Although the composition of organic matter in the volcanic deposits was not directly analyzed, ECO MicroPlate profiles (Table. 2.3.2) implied that the organic composition in the volcanic deposits was different between poorly and fully vegetated site. The potential changing in organic matter might result in a changing in the microbial community in the volcanic deposit. In addition, the ratio of NB to DNB jumped from 0.13 for IG7-C1-09 to

0.82 for IG9-C1-11 (Fig. 2.3.1C), supporting that the potential changing in early microbial community during the vegetation development in Miyake-jima Island.

No detectable amount of ergosterol and low counts of fungal propagules for the Miyake-jima volcanic deposit and buried soil samples (Table 2.3.1 and Fig. 2.3.1D) indicated that fungi constituted low fractions of their microbial communities. From the contents of ergosterol in upland soils (about 1.0-2.2 μg ergosterol g^{-1} [dry soil]) (Zhaorigetu*, et al.*, 2008), the fungal population of Miyake-jima samples is estimated to be less than one-tenth of those in the upland soils. It has been reported that fungi are more influenced by vegetation type than prokaryotes, because fungi directly associated with plant (Nielsen, *et al.*, 2010). Hence, further study would focus on the changing in fungal community in Miyake-jima volcanic deposits with vegetation type.

Chapter 3

Analysis of Early Bacterial Community along a Vegetation Gradient in Miyake-jima Island by 16S rRNA Gene-targeted PCR-T-RFLP and Clone Library Analyses

3.1 Introduction

Although the recent Miyake-jima volcanic deposits were characterized by low content of nutrient (TOC, 0.2-0.7 g kg^{-1} and TN, 0.2-0.9 g kg^{-1}), the deposits harbored relatively high bacterial population densities (TDC, 1.0-4.0×10^{9} cells g^{-1} [dry soil]) corresponding to one-tenth of those in buried soil, which contained 49-100 g kg^{-1} of TOC and 4.2-8.3 g kg^{-1} of TN. Further, organic carbon substrate utilization profile showed the vegetated volcanic deposits were able to use wider variety of substrates than those by unvegetated deposits used. This implied that the bacterial community might be affected by aboveground vegetation.

Determination by using culture-based methods gives limited information on the microbial diversity and community structure, because of the fact that large fractions of the microorganism existing in nature appear to be refractory to cultivation (Torsvik*, et al.*, 1990; Ward*, et al.*, 1990; Amann*, et al.*, 1995). Culture-independent DNA fingerprinting, such as amplified rDNA restriction analysis (ARDRA), denaturing gradient gel electrophoresis (DGGE), terminal restriction fragment length polymorphisms (T-RFLP), and clone library analysis are commonly used to asses both of diversity and structure of microbial community (Muyzer*, et al.*, 1993; Moyer*, et al.*, 1994; Liu*, et al.*, 1997). Particularly, since the use of an automated sequencer outputs the results quickly in a digital numerical format to compare different samples in an easy way, T-RFLP method in

general provides a useful tool for microbial ecology research (Dunbar*, et al.*, 2000). In addition, clone library analysis can provide more detailed information on what microbial members inhabit in the environments.

To clarify whether is the early microbial community in volcanic deposits affected by the aboveground vegetation feature or not, we analyzed the bacterial 16S rRNA genes in the volcanic deposit and buried soil samples by using T-RFLP and clone library analyses.

3.2 Materials and methods

Samples

The study sites were described in the Materials and Methods in Chapter 2 (Page 15-16). The 9-year-old Miyake-jima volcanic deposits and the buried soils from sites IG7, IG8, and IG9 were used for the extraction of environmental DNA. In this experiment, the lower volcanic deposits (layer C2, Fig. 3.2.1) were also analyzed to examine the effect of aboveground vegetation on the bacterial community in the lower volcanic deposits.

DNA extraction and PCR amplification

Five gram of the volcanic deposit samples (layers C1 and C2) was used for DNA extraction, according to a method based on lysis with a high-salt extraction buffer (1.5 M NaCl) and extended heating of the sample suspension in the presence of sodium dodecyl sulfate, hexadecyltrimethyl ammonium bromide (CTAB), and Proteinase K (Zhou*, et al.*, 1996; Fujimura*, et al.*, 2012). The detailed protocol was described previously (Guo, 2011). DNA extraction from the buried soil samples (layer 2A, 0.5-1.0 g) was performed by ISOIL for Bead Beating (Nippon Gene, Tokyo, Japan) with skim milk powder (Wako,

Osaka, Japan) according to a minor modified manufacturer's instructions (Nishizawa*, et al.*, 2008). The extracts were confirmed by electrophoresis in the 1.0% agarose gel. The 16S rRNA gene was amplified using universal bacterial primers 10F and 907R (Table 3.2.1) with an amplification reaction mixture (Table 3.2.2). Amplification was carried out under the thermal cycling conditions (Table 3.2.3) on a TaKaRa PCR Thernal Cycler Dice TP600 (TaKaRa Bio, Otsu, Japan). PCR products were confirmed by electrophoresis in the 1.5% agarose gel. After the confirmation of amplicons, the PCR was performed again using the fluorescently labeled 10F (Q-10F, Table 3.2.3) and 907R primers.

Terminal-restriction fragment length polymorphism (T-RFLP) analysis

The fluorescently labeled-PCR amplicons were purified using a QIAquick PCR Purification Kit (QIAGEN, GmbH, Hilden, Germany) according to the manufacture's protocol and eluted in 40 µl of Buffer EB. The purified amplicons were separately digested with *Hae*III, *Hha*I, and *Msp*I (TaKaRa Bio). The restriction endonuclease reaction mixture were made according to Table 3.1.4 and incubated at 37°C for 2 h. After incubation, the labeled fragments were purified using the QIAquick PCR Purification Kit according to the manufacture's instruction and eluted in 30 µL of Buffer EB. The accurate lengths of terminal-restriction fragments (T-RFs) from the amplified rRNA gene products were determined on a 3130xl PE Applied Biosystems Automated DNA Sequencer (Applied Biosystems). For the measurement, 2 µL of purified T-RFs was mixed with 7.9 µL of Hi-Di formamide and 0.1 µl of DNA fragment length standard LIZ® (-250)500 (Applied Biosystems) for standardization. The mixture was then denatured at 96°C for 2 min and immediately chilled on ice before the electrophoresis with the automated DNA

sequencer in the GeneMapper mode. After electrophoresis, the lengths of fluorescently labeled T-RFs were determined by comparison with internal standards by using the GeneMapper software (version 3.7, Applied Biosystems). Relative similarities of T-RFLP profiles data sets (relative abundance of the T-RF height and length) were examined using non-metric multidimensional scaling (NMDS) based on R software. NMDS uses an iterative algorithm to reduce multidimensional similarity data to a low dimensional ordination that indicates relative similarity of samples through relative ordination distance. Hence, the very similar samples are close together.

Clone library

The DNA extracted from the upper volcanic deposits (layer C1) and the buried soils (layer 2A) collected at sites IG7, IG8, and IG9 in July, 2009 were performed to build clone libraries. The 16S rRNA gene was amplified using bacterial universal primers 10F and 907R according to the above-mentioned method. PCR products were cleaned and purified using a QIAquick PCR Purification kit (QIAGEN, GmbH, Hilden, Germany) according to the manufacture's protocol. The purified amplicons were cloned using a pGEM®-T Easy Vector Systems (Promega, A1360). The mixtures of ligation reaction were set up as described in Table 3.1.5 and incubated at 16°C for 40 min. After incubation, 1 μL of Solution III (TaKaRa) and 50 μL of competent cells (*Escherichia coli* DH5α, TaKaRa) were added into the mixture, and then the mixture was incubated on ice for 20 min and heat-shocked at 42 °C for 45 s. After chilling on ice for 2 min, the ligation reaction transformations were transferred to 800 μL of SOC liquid medium (TaKaRa) and shaking-incubated at 37°C for 30 min. One hundred microliters of transformation culture

was plated onto LB/Ampicillin/IPTG/X-Gal plates (Table 3.2.6). The plates were incubated at 37°C overnight. The white single colonies were selected to shaking-incubate in 1 mL of liquid LB medium with 0.075 mg mL^{-1} ampicillin at 37°C for 18-20 h. The plasmid was extracted using TENS [TE:10% SDS:2N NaOH = 18:1:1 (by volume)] and 3M sodium acetate (pH, 5.5) in the following way. After the cell suspension was centrifuged, 900 μL of the supernatant was removed and the pellet was re-suspended and mixed in 300 μL of TENS until the solution become viscous. Thereafter, 150 μL of 3M sodium acetate was added to the suspension and mixed by vortex then centrifuged. The resulting supernatant was transferred to a sterile 1.5 mL-tube and mixed with 1000 μL of ethanol. After centrifugation, the pellet was washed with 1000 μL of 70% ethanol, suspended in 20 μL of TE, and treated with RNase. Finally, an agarose electrophoresis was performed to check whether the target gene was inserted successfully or not. The sequence reaction was carried out under the condition described in Table 3.2.7 and 3.2.8. After ethanol precipitation, the purified pellet was suspended in 17 μL of Hi-Di formamide and sequenced on an automated DNA sequencer (model 3130xl, Applied Biosystems).

Phylogenetic analysis of clone sequences

The clone sequences were assembled and edited using GENTYX for Windows (version 5.0 and version 10 network) software (Genetyx, Tokyo, Japan) and Sequence Scanner software (version 1.0, Applied Biosystems), and compared with similar DNA sequences retrieved from the DNA Data Bank of Japan (DDBJ) and National Center for Biotechnology Information (NCBI) using the DDBJ-BLAST and NCBI-BLAST

programs. The clone sequences were grouped into unique operational taxonomic units (OTU) using the web-based bioinformatics platform FastGroupII with a 99 % sequence similarity cutoff value. Rarefaction curves and diversity index were also generated in the FastGroupII with a 97% sequence similarity cutoff value. Relative similarities of libraries were analyzed by weighted UniFrac-based hierarchical clustering on Mothur platform. Phylogenetic analysis of the OTUs at >97% sequence similarity level of the deposit samples was performed on MEGA5. Multiple alignment and calculation of genetic distances were performed with ClustalW. Representative clones of each OTU group and close relatives were used to construct the phylogenetic tree. Neighbor-joining trees were constructed on nucleotide positions 218-913 (*E. coli* numbering) of the 16S rRNA gene by using the Jukes-Cantor model with complete deletion of gapped positions. The robustness of inferred tree topologies was evaluated 1000 bootstrap replications of the data, and bootstrap values are showed where they exceed 50%.

Table 3.2.1. Universal primers for amplifying bacterial 16S rRNA genes

Name	Direction	Base sequences
10F	Forward	5′-AGTTTGATCCTGGCTCAG-3′
907R	Reverse	5′-CCGTCAATTCCTTTR*AGTTT-3′
Q-10F	Forward	5′-C**AGTTTGATCCTGGCTCAG-3′

*R, A or G; **C, the fluorescent molecular.

Table 3.2.2. Contents of PCR mixture (Total 30 μl)

10 × Ex Taq buffer (TaKaRa Bio)	3.0 μL
dNTP mixture (Mg^{2+} plus, TaKaRa Bio)	3.0 μL
10F primer (Operon)	0.5 μL
907R primer (Operon)	0.5 μL
DNA template	1 μL (0.1 mg μL^{-1}) or 2 μl (0.05 mg μL^{-1})
Ex Taq polymerase (TaKaRa Bio)	0.3μL (1.45U)
Sterilized MilliQ water	21.7μL or 20.7μL

Table 3.2.3. PCR thermal cycling conditions

95 °C	120 seconds	1 cycle
95 °C	30 seconds	
54 °C	45 seconds	23 cycles
72 °C	90 seconds	

Table 3.2.4. Compositions of the restriction endonuclease reaction mixture

	*Hae*III and *Hha*I	*Msp*I
Sterile MilliQ water	7 μL	5 μL
10 × T Buffer	2 μL	0 μL
10 × M Buffer (BSA-free)	0 μL	2 μL
0.1 % BSA	0 μL	2 μL
Endonuclease	1 μL	1 μL
Purified PCR amplicons	10 μL	10 μL
Total	20 μL	20 μL

Table 3.2.5. Contents of ligation reaction mixture (Total 10 μL)

2× Rapid Ligation Buffer, T4 DNA Ligase	5.0 μL
pGEM®-T Easy Vector (50 ng)	1.0 μL
Purified PCR amplicons	3.0 μL
T4 DNA Ligase (3 Weiss units/μL)	1.0 μL

Table 3.2.6. LB/Ampicillin/IPTG/X-Gal medium (Total 1000 mL)*

Tryptone (Bacto™)	10 g
Yeast Extract (Bacto™)	5 g
NaCl (Wako)	10 g
Distilled water	1000 mL
Agar (Wako)	15 g

*Ampicillin (final concentration, 75 μg/L), isopropyl β-D-1-thiogalactopyranoside (IPTG; final concentration, 200 μM), and 5-bromo-4-chrolo-3-indlyl--β-D-galactopyranoside (X-Gal; final concentration, 40 μg/L) were added into the autoclaved medium.

Table 3.2.7. Contents of sequence reaction mixture (Total 10 μL)

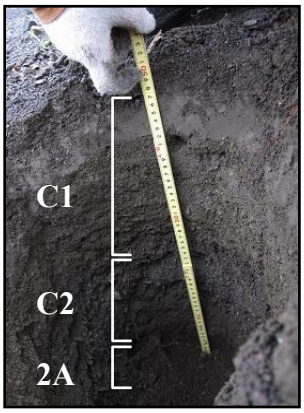
5 × Sequence Buffer (ABI)	1.5 μL
BigDye Terminator v.3 (ABI)	0.5 μL
*M4 or RV1 primer (3.2 pmol/μL)	1.0 μL
Plasmid DNA template	1.0 μL
Sterilized MilliQ water	6.0 μL

*Forward primer M4 5'-GTTTTCCCAGTCACGAC-3'

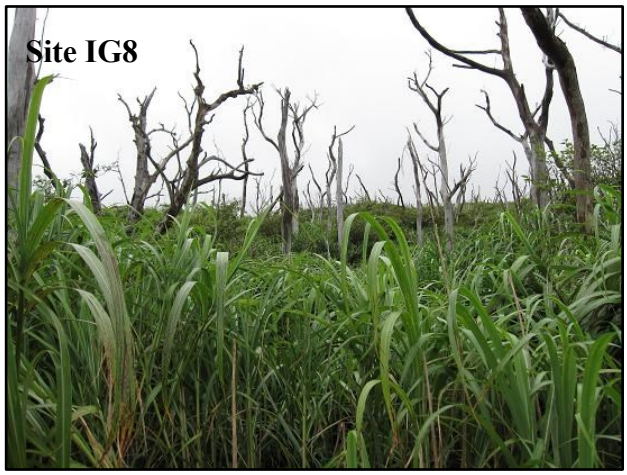
Reverse primer RV1 5'-CAGGAAACAGCTATGAC-3'

Table 3.2.8. Thermal cycling condition of sequence reaction

96 °C	120 seconds	1 cycle
96 °C	30 seconds	
50 °C	15 seconds	25 cycles
60 °C	240 seconds	



C1, 0-24 cm; C2, 14-44 cm; 2A, 44-50 cm



C1, 3-20 cm; C2, 20-33 cm; 2A, 33-38 cm

C1, 5-14 cm; C2, 14-27 cm; 2A, 27-32 cm

Fig. 3.2.1. Photographs showing study sites and soil profiles at sites IG7, IG8, and IG9 (July, 2009).

3.3 Results

16S rRNA gene-based T-RFLP analysis

Bacterial 16S rRNA genes recovered from the volcanic deposits and buried soils under the deposits were analyzed by PCR-based T-RFLP profiles. When the cut-off signal peak height of T-RF was set at 30 on the automated sequencer, the numbers of detected T-RFs were 49-112 in the *Hae*III digests, 53-129 in the *Hha*I digests, and 46-115 in the *Msp*I digests among the soil bacterial communities in volcanic ash deposits and the buried soils (Table 3.3.1). The number of detected T-RFs was the most abundant in the site IG8-C1 sample (*Hae*III-digested T-RFs 105, *Hha*I-digested T-RFs 129, *Msp*I-digested T-RFs 155) and the least in the site IG9-C1 sample (*Hae*III-digested T-RFs 49, *Hha*I-digested T-RFs 53, *Msp*I-digested T-RFs 46) among the volcanic deposits and buried soils.

The electropherograms obtained with *Hae*III, *Hha*I, and *Msp*I were illustrated in Figure 3.3.1-3.3.3. The major T-RFs of C1 samples from sites IG7, IG8 and IG9 were different from each other and different from those of C2 and 2A samples, while the major T-RFs of C2 and 2A samples in the three sites were similar. In the *Hae*III-digested T-RFLP profiles, the major T-RFs were at 193- and 219-base in the site IG7-C1 sample, at 193-, 198-, 205-, 253-, 259- and 380-base in IG8-C1, and at 198- and 243-base in the site IG9-C1 sample, respectively. The *Hae*III-digested T-RFLP profiles also revealed that the common major T-RFs with 41-, 191-, 193-, 252- and 324-base among the site C2 and 2A samples in the three sites (Fig. 3.3.1). Similarly, the major T-RFs detected in *Hha*I-digested electropherograms were at 186- and 573-base in the site IG7-C1 sample, at 91-, 186-, 200-, 213- and 447-base in the site IG8-C1 sample, and at 365- and 570-base in

the site IG9-C1 sample. The common major T-RFs digested by *Hha*I of C2 and 2A samples in the three sites were at 40-, 62-, 91-, 186-, 200-, 206- and 341-base (Fig. 3.3.2). The major T-RFs detected in *Msp*I-digested electropherograms were at 135- and 486-base in the site IG7-C1 sample, at 129-, 135-, 147- and 485-base in the site IG8-C1 sample, and at 485-base in the site IG9-C1 sample. The common major T-RFs digested by *Msp*I of C2 and 2A samples in the three sites were at 225-, and 262-base (Fig. 3.3.3).

To estimate the similarity in bacterial communities among the volcanic deposits and buried soils with different aboveground vegetation feature, NMDS analysis was performed based on the data sets of T-RFLP profiles. The scatter plot of NMDS analysis revealed that the bacterial communities in the volcanic deposits and buried soils were different according to the site and layer difference (Fig. 3.3.4). The positions of C1 samples spread more widely than those of C2 samples, suggesting that the influence of the aboveground vegetation on the structure of bacterial community was clearer in the upper volcanic deposit (layer C1) than the lower deposit (layer C2). Comparing with the layer C1 samples, the profiles of the layer C2 samples were close to those of the layer 2A soils.

16S rRNA gene clone library analysis

A total of 570 full-length sequences (102, 106, 104, 84, 88, 86 clone sequences from the site IG7-C1, IG8-C1, IG9-C1, IG7-2A, IG8-2A, and IG9-2A samples, respectively) and 307 unique OTUs identified at the 99% sequence similarity were obtained from the 6 clone libraries. When the sequence similarity was set at 97% with gaps, Chao1 richness were 196, 221, 95, 355, 169, and 261, Shannon-Wiener index were 3.96, 4.10, 3.71, 4.01,

4.04, and 3.92 on IG7-C1, IG8-C1, IG9-C1, IG7-2A, IG8-2A, and IG9-2A, respectively (Table 3.3.2). The rarefaction curve with a 97% sequence similarity showed the bacterial diversity was highest in the site IG7-2A sample and lowest in the site IG9-C1 sample (Fig. 3.3.5). Hierarchical clustering with weighted Unifrac distance showed that the libraries grouped into three clusters (Fig. 3.3.6). First cluster was grouped with the site IG7-C1 and IG8-C1 libraries; the second cluster with only IG9-C1 library; the third cluster by the buried soil libraries.

The distribution of phyla in the clone libraries was showed in Fig. 3.3.7. Clones from the site IG7-C1 sample grouped with closest relatives from *Acidobacteria* (3%), *Actinobacteria* (11%), *Firmicutes* (14%), *Gemmatimonadetes* (4%) and *Planctomycetes* (6%). *Bacteroidetes*, *Chloroflexi*, *Nitrospirae* and *Verrucomicrobia* were represented by less than 2% of total recovered sequences and were grouped together as 8%. In clones from the site IG7-C1 sample, *Proteobacteria* accounted for 47% of the sequenced clones and were composed of *Alphaproteobacteria* (19%), *Betaproteobacteria* (4%), *Gammaproteobacteria* (22%) and *Deltaproteobacteria* (2%). In addition, unclassified bacteria and unknown clone sequences were grouped together as other 4%. Clones from the site IG8-C1 sample were affiliated with the *Acidobacteria* (9%), *Actinobacteria* (8%), *Bacteroidetes* (6%), *Firmicutes* (4%), and *Proteobacteria* (56%), which included *Alphaproteobacteria* (18%), *Betaproteobacteria* (8%), *Gammaproteobacteria* (23%), and *Deltaproteobacteria* (7%). The other clones (11%) included *Chloroflexi*, *Nitrospirae*, unclassified bacteria, and unknown sequences. The site IG9-C1 clones belonged to *Acidobacteria* (6%), *Actinobacteria* (9%), *Firmicutes* (11%), and *Proteobacteria* (61%), which were composed of *Alphaproteobacteria* (7%), *Betaproteobacteria* (28%),

Gammaproteobacteria (25%) and *Deltaproteobacteria* (1%). Phyla less than 3% of recovered sequences included *Bacteroidetes*, *Nitrospirae*, and *Planctomycetes*. Unclassified bacteria and unknown clone sequences accounted for 8% of the site IG9-C1 clones. On the other hand, the taxonomic profile at phylum level in 2A samples was similar to each other. The major phyla in 2A libraries were *Acidobacteria* (20-29%), *Actinobacteria* (7-8%), *Chloroflexi* (17-20%), and *Proteobacteria* (26-32%), in which *Alphaproteobacteria, Betaproteobacteria*, and *Gammaproteobacteria* were 16-23%, 1-3%, and 7-8%, respectively. In addition, the 2A libraries contained 7-12% of unknown bacteria.

Phylogenetic analysis of the clone sequences recovered from the deposit libraries were showed in Fig. 3.3.8 and 3.3.9. Many clones from the site IG7 and IG9 libraries were close to *Fulvimonas soli* belonging to *Gammaproteobacteria*, which uses plasticized acetylated starch granules as a source of carbon. While many *Gammaproteobacteria*-related clones were close to a sulfur-oxidizing bacterium. The biggest cluster of woodland library related to *Sideroxydans paludicola* and *Herbaspirillum seropedicae* belonging to *Betaproteobacteria,* the former is an acid-tolerant Fe(II)-oxidizing bacterium isolated from rhizosphere of wetland plant. The latter was a root-associated N_2-fixing bacterium. Surprisingly, the predominant bacterial groups (*Leptospirillum* and *Acidithiobacillus*) on bacterial community at the completely unvegetated site OY were rare in the three clone libraries of this study.

Table 3.3.1. Number of T-RFs in the volcanic deposit and buried soil samples.

Restriction enzymes / Site / layer	*Hae*III			*Hha*I			*Msp*I		
	IG7	IG8	IG9	IG7	IG8	IG9	IG7	IG8	IG9
C1	89	105	49	74	129	53	79	155	46
C2	83	90	59	87	88	72	88	89	73
2A	72	112	86	96	99	76	98	131	106

All data were collected at a peak height over 30.

Table 3.3.2. Chao1 richness and Shannon-Wiener index of the volcanic deposit and buried soil samples.

	IG7-C1	IG8-C1	IG9-C1	IG7-2A	IG8-2A	IG9-2A
Chao1 richness	196	221	95	356	169	261
Shannon-Wiener	3.96	4.10	3.70	4.01	4.04	3.92

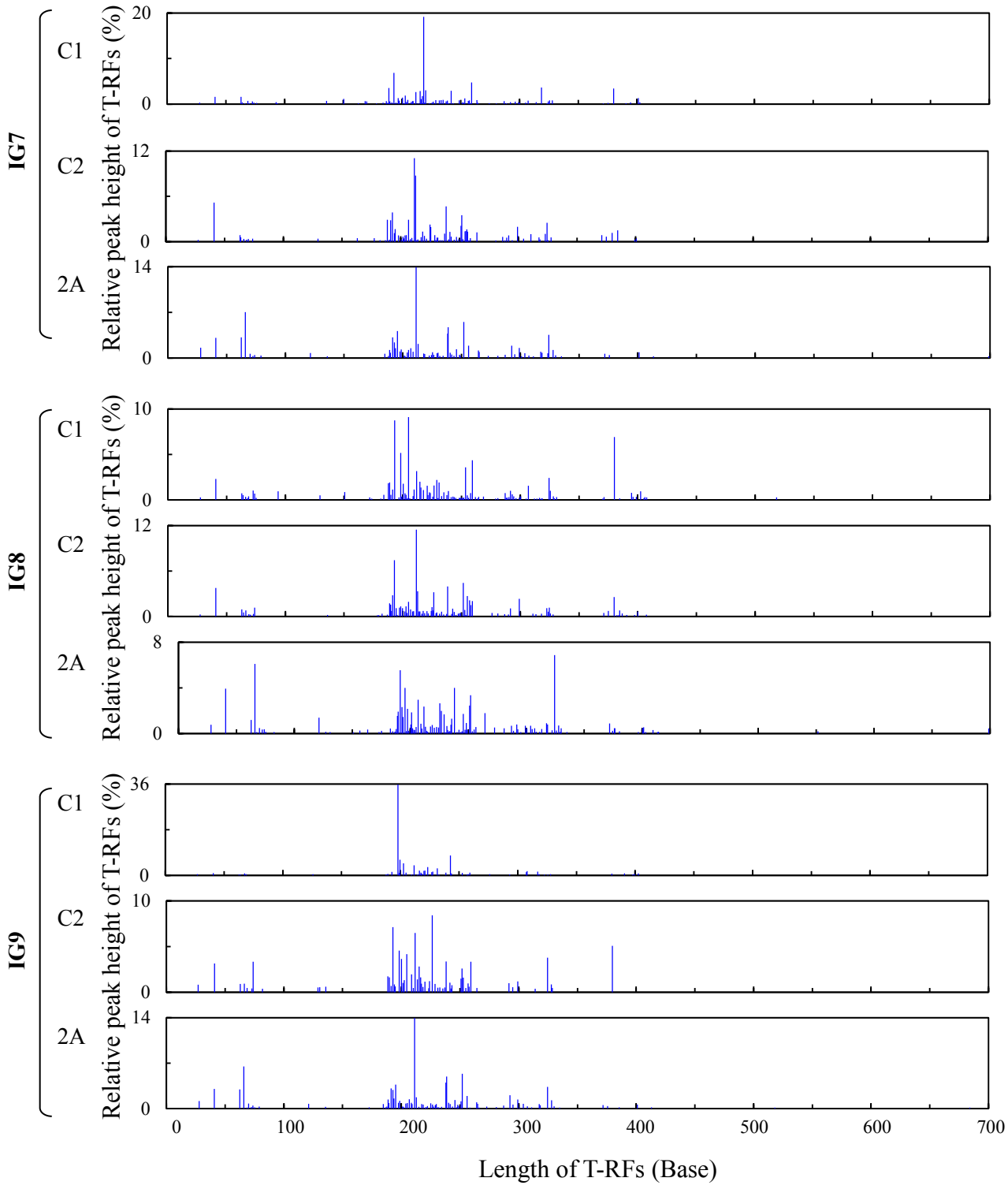


Figure 3.3.1. 16S rRNA gene-targeted PCR-T-RFLP profiles of the volcanic deposits (C1 and C2) and buried soils (2A) collected from sites IG7, 8, and 9. *Hae*III was used for digestion.

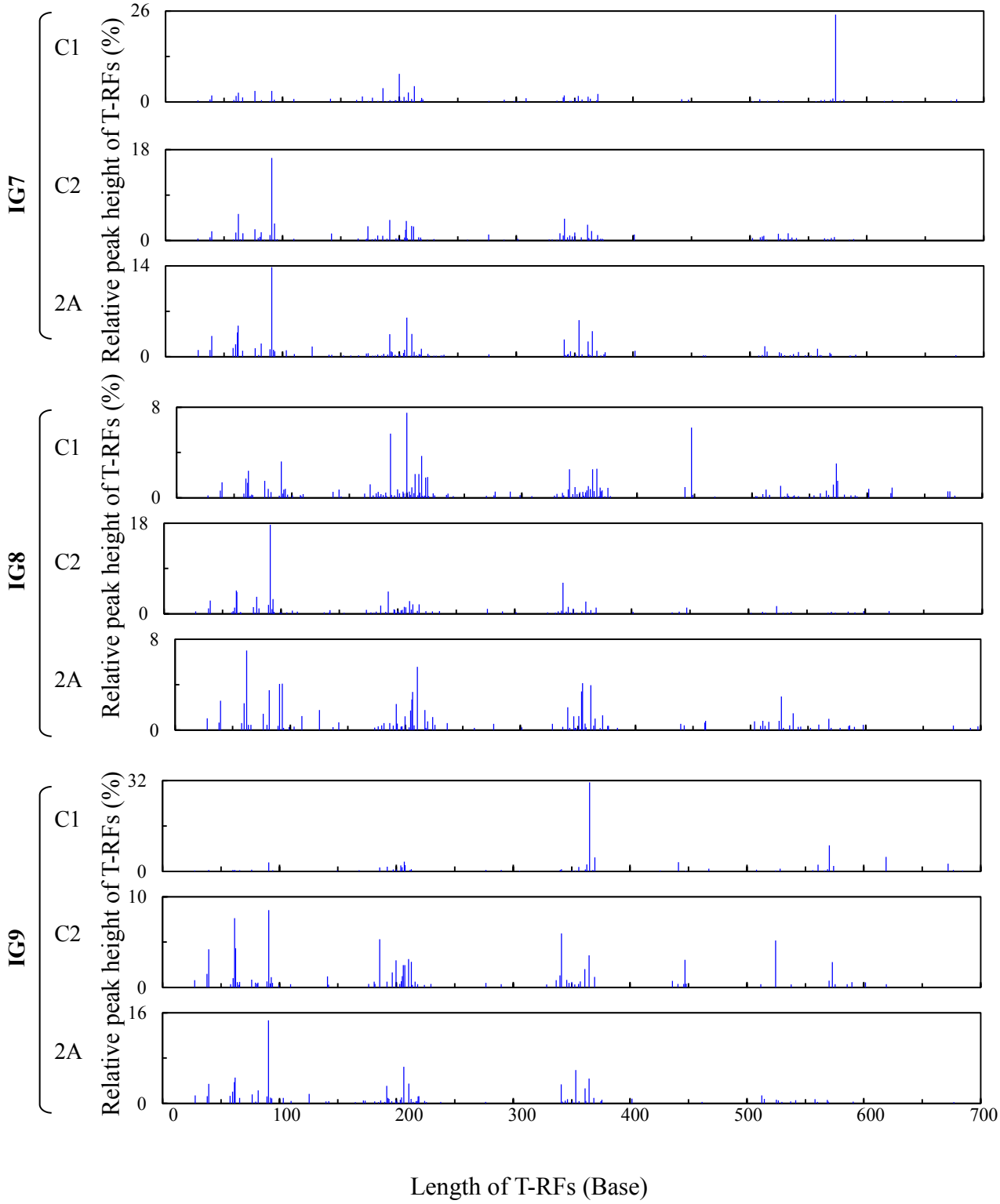


Figure 3.3.2. 16S rRNA gene-targeted PCR-T-RFLP profiles of the volcanic deposits (C1 and C2) and buried soils (2A) collected from sites IG7, 8, and 9. *Hha*I was used for digestion.

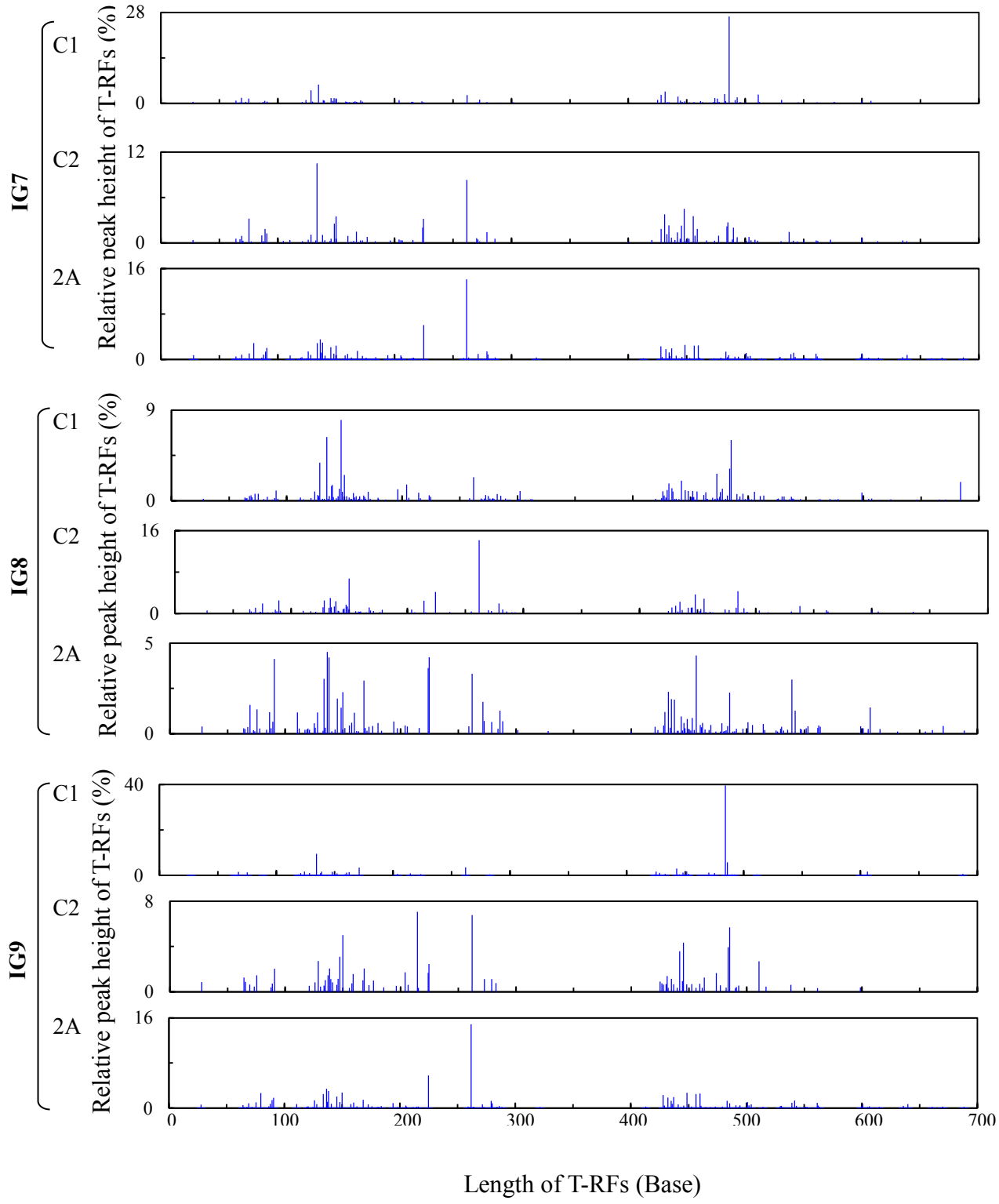


Figure 3.3.3. 16S rRNA gene-targeted PCR-T-RFLP profiles of the volcanic deposits (C1 and C2) and buried soils (2A) collected from sites IG7, 8, and 9..*Msp*I was used for digestion.

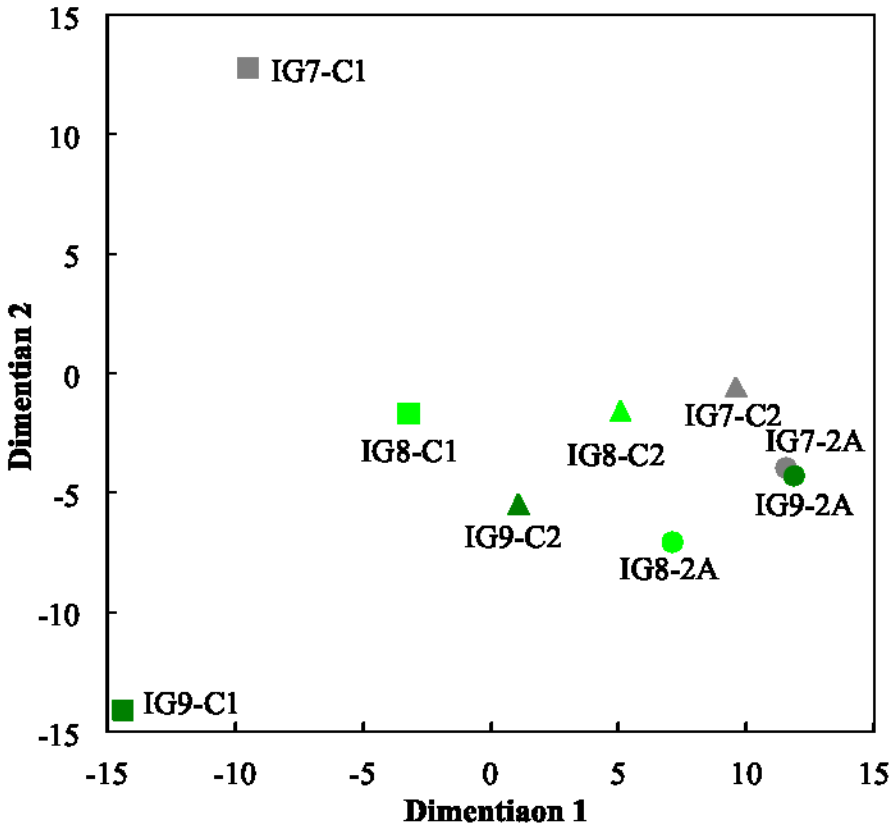


Fig. 3.3.4. Non-metric multidimensional scaling (NMDS) analysis based on the data sets of 16S rRNA gene-targeted T-RFLP profiles from the upper volcanic deposits C1, the lower volcanic deposits C2 and the buried soils 2A in sites IG7, IG8, and site IG9, collected in September 2009. NMDS was performed using the T-RFs profiles (relative abundance of the peak height and fragment length) combined *Hae*III, *Hha*I, and *Msp*I enzymes, respectively. Stress value was 0.0035.

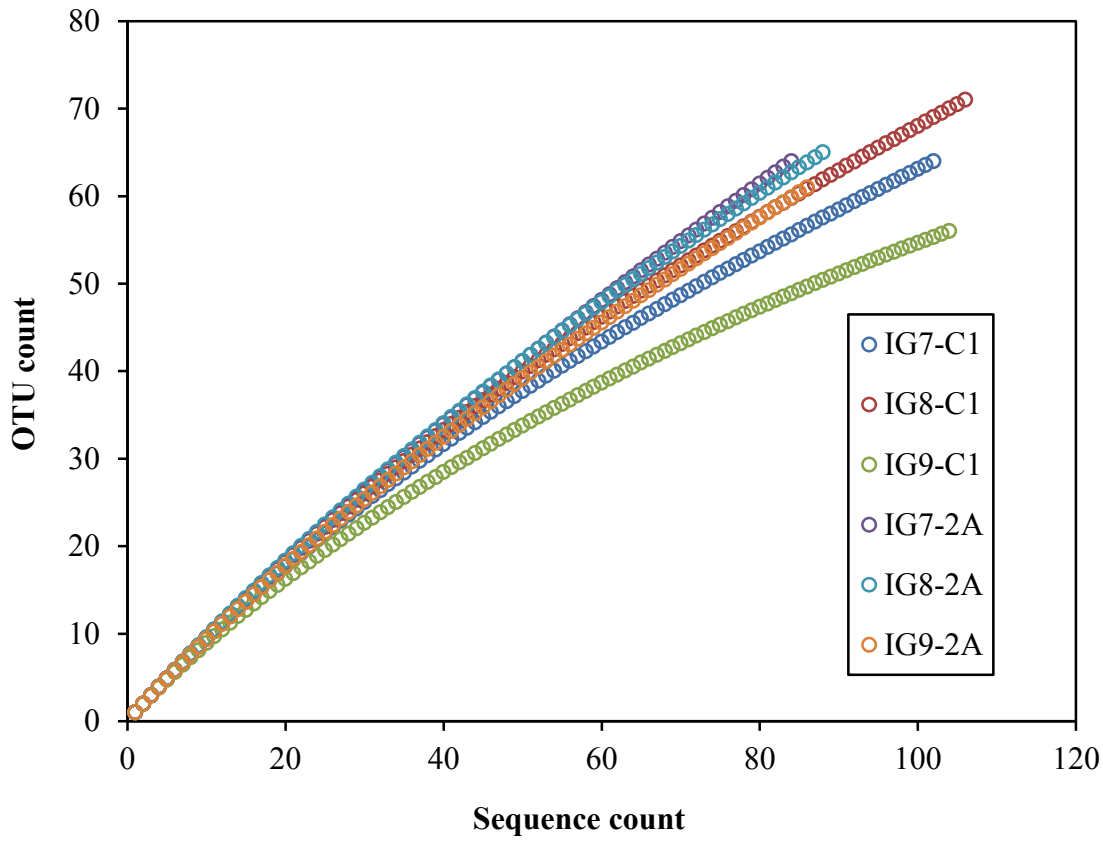


Fig. 3.3.5. Rarefaction curves for the volcanic deposit samples and the buried soil samples. Curves are shown at 97% sequence similarity.

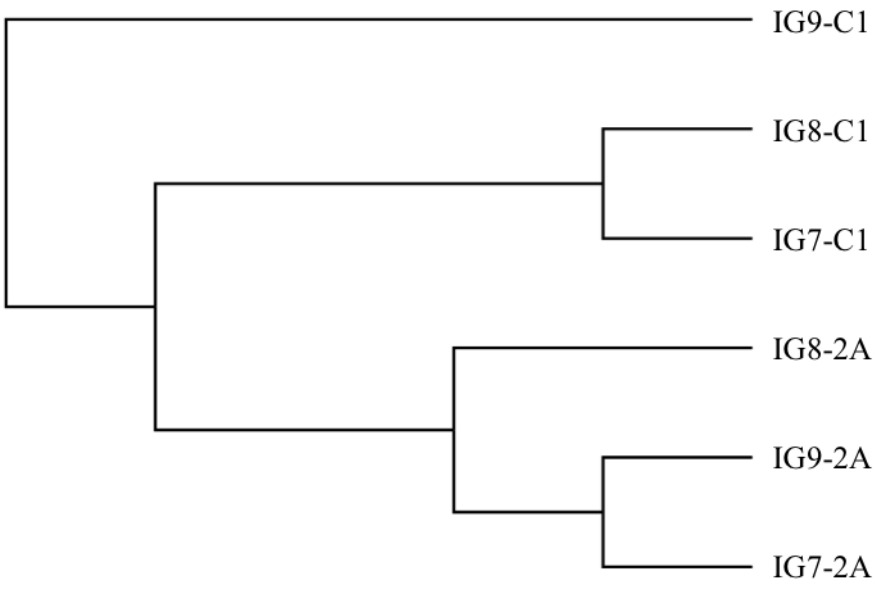


Fig. 3.3.6. Hierarchical clustering of bacterial clone libraries of the volcanic deposit (C1) and buried soil (2A) samples from sites IG7, IG8, and IG9, collected in September 2009, by weighted UniFrac. Silva bacterial tree was selected as the reference tree.

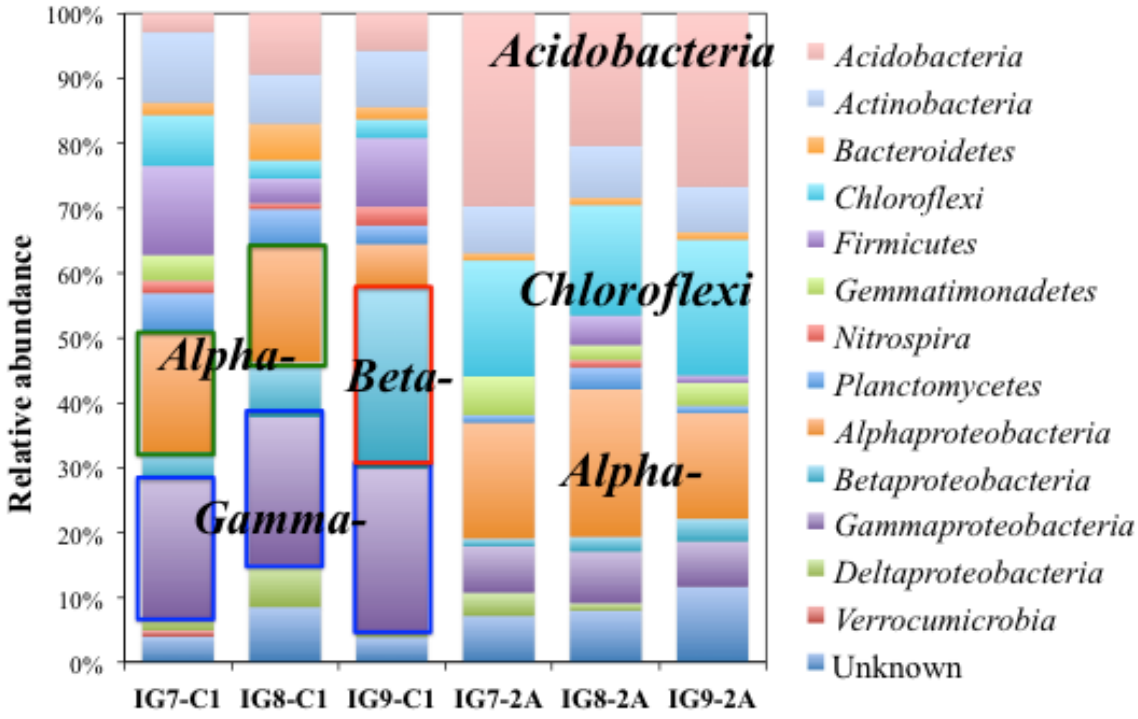


Fig. 3.3.7. Distributions of bacterial phyla and proteobacterial classes in the upper volcanic deposits C1 and the buried soils (layer 2A) from sites IG7, IG8, and IG9, collected in September 2009.

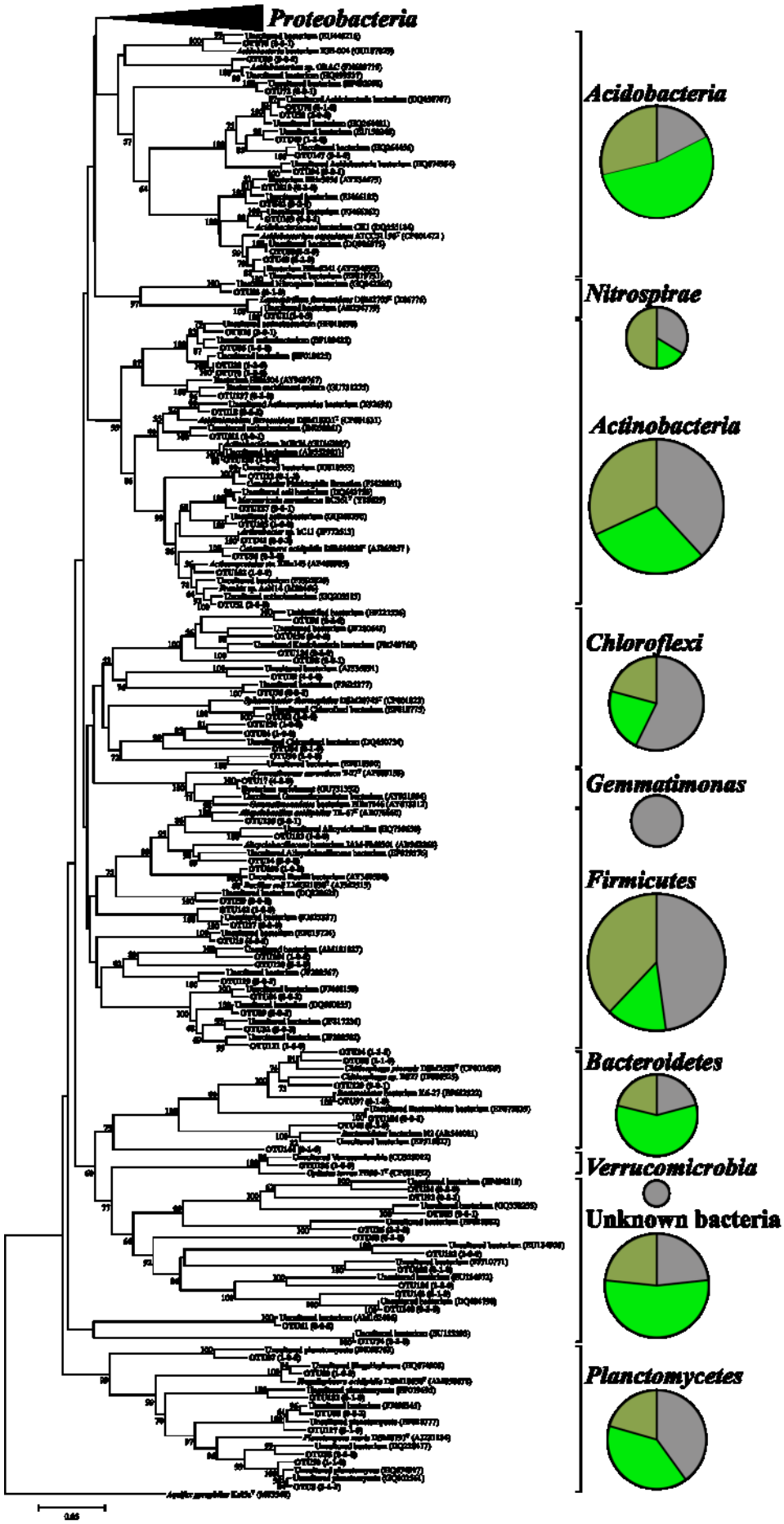

Proteobacteria
Acidobacteria
Nitrospirae
Actinobacteria
Chloroflexi
Gemmatimonas
Firmicutes
Bacteroidetes
Verrucomicrobia
Unknown bacteria
Planctomycetes

Fig. 3.3.8. Neighbour-joining phylogenetic tree of 16 rRNA gene clone sequences from volcanic ash deposits at sites IG7, IG8, and IG9, respectively. The tree was generated using nucleotide positions 218-913 (*E. coli* numbering). Clones were represented at OTU-level (defined at 97% sequence similarities by FastGroupII). OTU designation was followed in parenthesis by the number of clones represented by that OTU in the site IG7, IG8, and IG9 libraries, respectively. OTUs are highlighted in *bold.* The boxes indicated the clone sequences recovered from the volcanic deposits at site OY. Bootstrap values (1,000 replicates) are shown where they exceed 50%. The scale bar represents 5% estimated sequence divergence. *Aquifex pyrophilus* Kol5a^T was used as the out-group. Pie diagrams show clones count from three sites at phylum level. Gray, light green, and dark green indicates proportion of IG7-C1, IG8-C1, and IG9-C1 in each phylum, respectively.

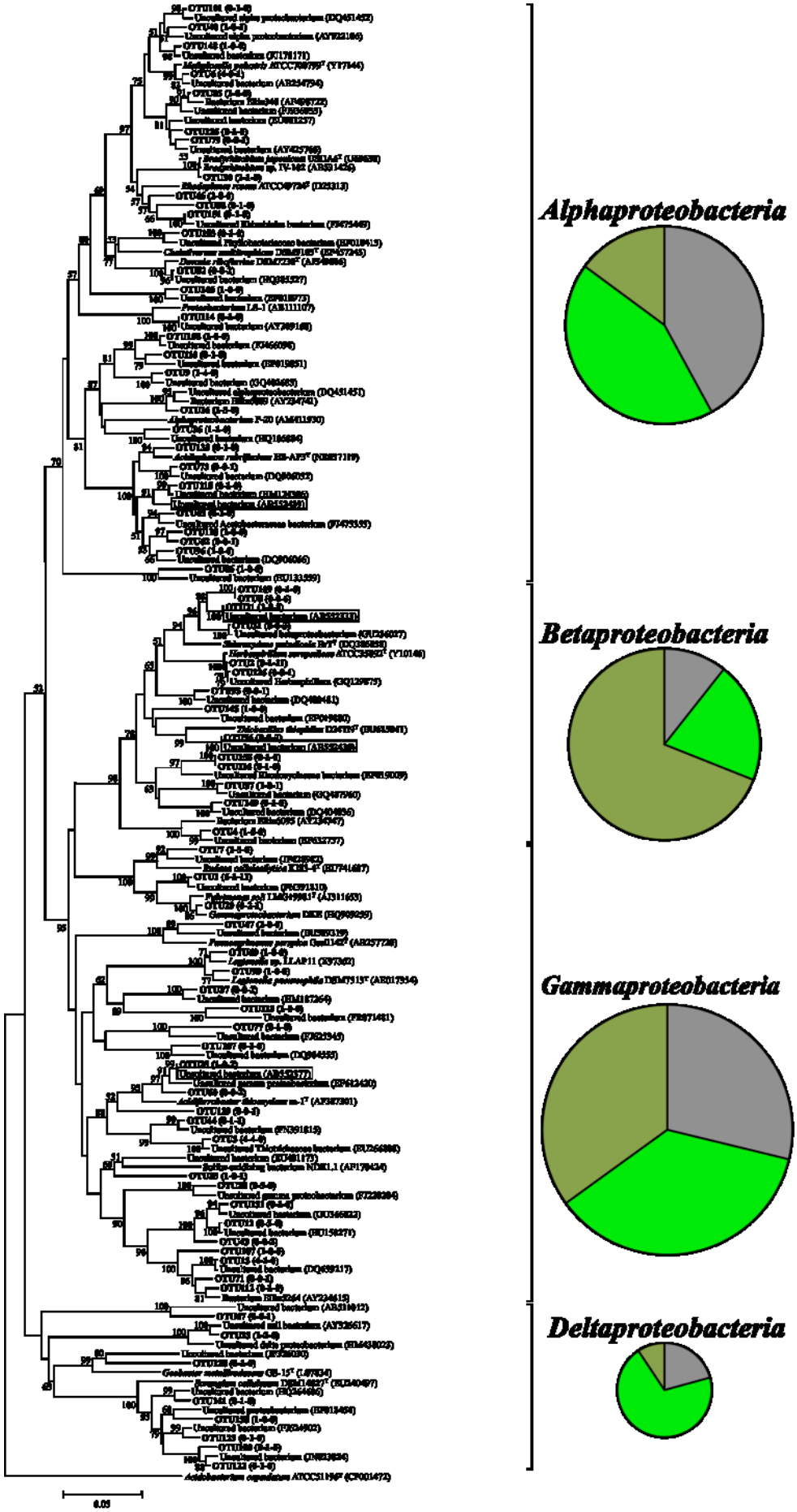

Alphaproteobacteria
Betaproteobacteria
Gammaproteobacteria
Deltaproteobacteria
0.05

Fig. 3.3.9. Neighbour-joining phylogenetic tree of proteobacterial 16 rRNA gene clone sequences from volcanic ash deposits at sites IG7, IG8, and IG9, respectively. The tree was generated using nucleotide positions 218-913 (*E. coli* numbering). Clones were represented at OTU-level (defined at 97% sequence similarities by FastGroupII). OTU designation was followed in parenthesis by the number of clones represented by that OTU in the site IG7, IG8, and IG9 libraries, respectively. OTUs are highlighted in bold. The boxes indicated the clone sequences recovered from the volcanic deposits at site OY. Bootstrap values (1,000 replicates) are shown where they exceed 50%. The scale bar represents 5% estimated sequence divergence. *Acidobacterium capsulatum* ATCC51196^{T} was used as the out-group. Pie diagrams show clones count from three sites at phylum level. Gray, light green, and dark green indicates proportion of IG7-C1, IG8-C1, and IG9-C1 in each proteobacterial class, respectively.

3.4 Discussion

A number of studies have reported the influence of the aboveground vegetation on soil microbial community (Ohtonen*, et al.*, 1999; Kowalchuk*, et al.*, 2002; Bardgett*, et al.*, 2005). In this chapter, we identified the microbial diversity and compared the similarity of microbial community on the 9-year-old Miyake-jima volcanic deposits and the buried soils from bare land and vegetation recovery land by T-RFLP profiles. The number of detected T-RFs in the volcanic deposits with grassland was the most one among the volcanic deposits, indicating that microbial diversity was highest in the grassland volcanic deposits (site IG8, Table 3.3.1). But the T-RFs of the volcanic deposits from woodland (site IG9) was the least one, suggested a probability of decreasing microbial diversity from grassland to woodland. Chao1 richness and Shannon-Wiener index calculated from the clone sequences at 97% similarity level also showed the highest diversity of bacterial community in the site IG8-C1 sample (Table 3.3.2). Interestingly, a similar result was obtained in a study on bacterial community dynamics across a floristic gradient in a temperate upland grassland ecosystem, which showed that bacterial numbers, diversity, microbial activity, and potential functional diversity followed a trend, increasing with decreasing plant diversity (Brodie*, et al.*, 2002). In addition, the number of T-RFs detected in the buried soils was higher than that in the adjacent volcanic deposits, indicating that diverse microorganisms inhabited in the buried soils. These microorganisms were the potential source to colonize the newly exposed volcanic deposits.

NMDS analysis of T-RFLP profiles showed a dramatic change in bacterial community of upper deposits from barren land (site IG7) to woodland (site IG9), but the differences

in lower volcanic deposits and buried soils were not significant (Fig. 3.3.4). Similarly, clone library analysis also showed that the difference among volcanic deposit community was higher than those among buried soil community. Many studies reported that early and late successional plant species effect on the microbial community in various environments such as glacier foreland in alpine ecosystem and from short grassland to early stage of forest in chalk grassland (Ohtonen*, et al.*, 1999; Chabrerie*, et al.*, 2003; Tscherko*, et al.*, 2005). Our findings suggest not only that the soil community was shaped by vegetation development in the new substrates, but also that it seemed to be influenced by the vertical movement of bacteria from the lower soils.

The clone sequences recovered from the volcanic deposits at barren land (site IG7), grassland (site IG8), and woodland (site IG9), were classified into 10, 9, and 9 phyla, respectively, which is comparable to those in buried soil and significantly more than those in the volcanic deposit near the crater (site OY) collected in 2004, 2005, and 2007, only 5 phyla were retrieved including *Firmicutes*, *Actinobacteria*, *Proteobacteria*, *Nitrospirae* and *Acidobacteria* (Fujimura, *et al.*, 2012). This result suggested that the early microbial communities in sites IG7 to IG9 were more complex than those in site OY.

Despite *Proteobacteria* dominated all the three clone libraries in this study, the profile of the proteobacterial class was different among the libraries. More clones of *Alphaproteobacteria* were found in the site IG7-C1 and IG8-C1 samples than those in the site IG9-C1 sample. *Alphaproteobacteria*, which include numerous plant symbionts such as nitrogen-fixing bacteria, and chemolithotrophic bacteria such as methane-oxidizing acidophilic bacteria, are common in soils and can cope with hostile environments that have extremes in pH, temperatures, salinity and nutrient levels (Madigan*, et al.*, 2009;

Rastogi, *et al.*, 2010) (Fig. 3.3.9). In contrast, more clones of *Betaproteobacteria* were found in the site IG9-C1 sample than those in the site IG7-C1 and IG8-C1 samples, including many clones related to genera *Sideroxydans* and *Herbaspirillum* (Fig. 3.3.9). The former includes many obligate lithotrophic Fe(II)-oxidizing bacteria. The closest relative strain of the *Sideroxydans*-clones recovered from the IG9-C1 sample was a neutrophilic Fe(II)-oxidizing bacteria isolated from the rhizosphere of wetland plant *Typha latifolia* and potentially abundant in rhizosphere (Weiss, *et al.*, 2007). Some strain related to *Sideroxydans* was able to tolerant the acidic condition (Ludecke, *et al.*, 2010). The bacteria related to *Herbaspirillum* genus were able to fixing nitrogen (Baldani, *et al.*, 1986). The *Gammaproteobacteria*-clones were found at similar proportion in the three clone libraries. The most clones related to *Fulvimonas soli* and uncultured gammaproteobacteria (Fig. 3.3.9). Surprisingly, only a few sequences related to *Acidithiobacillus ferrooxidans*, which was dominant in clone libraries recovered from site OY (Sato, 2006). *Frateuria*-like gammaproteobacteria can oxidize iron (Hallberg & Johnson, 2005) and was also capable of reducing ferric iron (Coupland & Johnson, 2008), while *Acidithiobacillus ferrooxidans* were able to using Fe(II), hydrogen, and some sulfur compounds (Drobner, *et al.*, 1990; Kelly & Wood, 2000). In addition, a high number of *Deltaproteobacteria* sequences were found in the site IG8-C1 library, and most of them belonged to *Sorangium cellulosum*.

Relatively abundant *Acidobacteria*-, *Actinobacteria*-, and *Firmicutes*-related clones were also retrieved from the three clone libraries (Fig. 3.3.8). *Acidobacteria* are a relatively new phylum, with members that are primarily acidophilic and found regularly in culture-independent studies of soils. The phylum *Actinobacteria* is common in soil

environments, includes members that usually prefer stable, non-extreme environments and are famous for their production of antibiotics, while most of *Firmicutes* have gram-positive cell wall structure, and produce endospores to resistant to extreme conditions. Notably, only 6 clones related to *Nitrospirae* phylum were detected in the three libraries (2 clones in the site IG7-C1, 1 clone in the site IG8-C1, and 3 clones in site IG9-C1) (Fig. 3.3.8), inversely *Leptospirillum ferrooxidans* belonging to *Nitrospirae* dominated the site OY clone library (39 %) constructed in 2004 (Sato, 2006). Hippe reported that *L. ferrooxiands* can grow in acidic environment (pH optimum at 2.5–3.0) on mineral Fe(II) medium and get energy from Fe(II)-oxidation (Hippe, 2000). Comparing that of the site OY volcanic deposits taken in 2004, the pH of the volcanic deposits in the present study was relatively higher (pH 3.5 for the site OY deposit in 2004; pH 4.2–4.5 for the site IG7, IG8, and IG9 deposits in 2009), suggesting that the increase in deposit pH might reduce the population density of *L. ferrooxidans*.

The clones related to *Gemmatimonadetes* and *Verrucomicrobia* were only recovered from the site IG7-C1 sample (Fig. 3.3.8). *Gemmatimonadetes* and *Verrucomicrobia* are new phyla described recently, and their metabolism is still unclear (Zhang*, et al.*, 2003; Wagner & Horn, 2006). Other phyla found commonly in the three clone libraries were *Bacteroidetes*, *Chloroflexi*, and *Planctomycetes* (Fig. 3.3.8). The *Bacteroidetes* are widely distributed in the environment, including in soil, sediments, seawater, and animal guts. The *Chloroflexi* strains are facultatively aerobic, yield energy from light but do not produce oxygen in photosynthetic process. *Planctomycetes* is a phylum of aquatic bacteria and found in samples of brackish, and marine and fresh water.

In conclusion, the composition of early bacterial community in 9-year-old volcanic deposits were different significantly among sites IG7, IG8, and IG9, suggesting that the bacterial community was affected by the aboveground vegetation profile.

Chapter 4

Characterization of Early Microbial Community along a Vegetation Gradient in Miyake-jima Island by PCR-based Pyrosequencing of 16S and 18S rRNA genes

4.1 Introduction

The eruption of Mt. Oyama in Miyake-jima Island in 2000 was characterized by the ejection of large amounts of volcanic gas and ash. The plant-soil ecosystem around the crater was damaged completely by the deposition of volcanic ash and acidic precipitation. Previous studies have characterized the early bacterial community and their succession on the completely unvegetated volcanic deposits derived from the eruption in 2000. Ohta*, et al.* (2002) reported that the population density of cultivable bacteria was about 10^4 cells g^{-1} (dry soil) in the 1-year-old Miyake-jima deposits, and 83 % of 36 bacterial isolates from the deposits were related to *Thiobacillus*. The bacteria related to the *Thiobacillus* cluster (OY cluster) were also isolated from the 4-year-old deposits on the completely unvegetated site (site OY). Sato (2006) performed bacterial 16S rRNA gene clone library analyses and reported that *Proteobacteria* (30%), *Nitrospirae* (41%), *Acidobacteria* (16%) and *Firmicutes* (7%) were abundant in the bacterial communities of volcanic deposits. Particularly, chemolithotrophic bacteria, *Acidithiobacillus ferrooxidans* in class *Gammaprotebacteria* and *Leptospirillum ferrooxidans* in phylum *Nitrospirae* dominated the volcanic deposit communities. Analysis of carbon dioxide-fixing gene (*rbcL*) and nitrogen-fixing gene (*nifH*)-targeted PCR-clone libraries showed that 25% of *rbcL* gene sequences were closely related to that of *A. ferrooxidans* and 58% and 31% of *nifH* gene sequences were closely related to those of *A. ferrooxidans* and *L. ferrooxidans*,

respectively (Fujimura, 2008). Sato*, et al.* (2009) successfully isolated the iron-oxidizing *L. ferrooxidans* strains from the site OY deposits collected in 2008 and demonstrated the nitrogenase activity (acetylene reduction) for one of the isolates. Stable isotope probing with $^{15}N_2$ gas ($^{15}N_2$-SIP) was performed to examine whether *A. ferrooxidans* and *L. ferrooxidans* were actually responsible for nitrogen fixation or not in the volcanic deposit. The results suggested that ^{15}N-DNA fractions recovered from the volcanic deposit sample incubated with $^{15}N_2$ gas contained DNA derived from *L. ferrooxidans* and *A. ferrooxidans* (Sugawara, *et al.*, 2008). The carbon and nitrogen metabolisms of *L. ferrooxidans* strain C2-3 isolated from the site OY deposit was predicted by its complete genome information (Fujimura*, et al.*, 2012). Subsequent studies showed that the early bacterial community on the unvegetated deposits changed rapidly with deposit aging (Fujimura, 2012; Fujimura*, et al.*, 2012).

In Chapter 3, to examine the influence of aboveground vegetation type on the early microbial community, we have analyzed the microbial populations, microbial activity, and microbial community on the Miyake-jima volcanic deposits along a vegetation gradient by both culture-based and culture-independent methods. The results have showed that the upper volcanic deposits displayed high among-site variation for the bacterial community, in spite of no significant difference in chemical properties (pH, TOC, and TN) and microbial populations (total direct count and culturable count). Clone library analysis based on 16S rRNA genes showed that many recovered sequences from the upper deposits were affiliated with *Gammaproteobacteria* in all three sites and *Alphaproteobacteria* in the barren land (site IG7) and grassland sites (site IG8), while sequences from the woodland site (site IG9) were closely related to those of

Betaproteobacteria.

When the 16S rRNA gene clone libraries constructed in this research were compared to the clone in previous studies on Miyake-jima volcanic deposits (Sato, 2006; Fujimura, 2012), we found that a relatively complex bacterial community inhabited in the volcanic deposits along the vegetation gradient. About 60% of 159 OTUs (>97% similarity level) defined in the 3 libraries from different sites were singletons, implying that a large fraction of undetected bacteria in the three clone libraries. Further, most of the OTUs were related to uncultured bacteria, limiting the characterization of the relationship between specific bacterial species and plant species. In addition, the information on the early fungal community in the deposits is still little known, despite low counts of fungal propagules for the Miyake-jima volcanic deposits. Therefore, the aim of study in this chapter was to characterize the microbial community on the 9- and 11-year-old Miyake-jima volcanic deposit at a windward side where vegetation recovery gradually occurred, and to analyze how the early microbial community responds to the first colonizer plant. The investigation was designed to compare bacterial and fungal communities between these deposits by molecular approaches using the PCR-based pyrosequencing of the 16S rRNA gene and 18S rRNA gene, respectively.

4.2 Materials and methods

Samples

The study sites and sample collection were the same as described in the Materials and Methods in Chapter 2 (Page 15-16). The 9- and 11-year-old Miyake-jima volcanic

deposits and the buried soils from site IG7, IG8, and IG9 were used for the extraction of environmental DNA.

DNA extraction, PCR amplification, and tag pyrosequencing

The DNA extraction was performed according to above-mentioned method in Chapter 3 (Page 28-29). DNA extraction was made in duplicate and the extracts were pooled together. All pooled DNA samples were purified using AMPure XP magnetic purification beads (Beckman Coulter, Brea, CA). The V1-V2 region in the 16S ribosomal RNA gene was amplified using universal primers 27Fmod (5'-CCATCTCATCCCTCGTGTCTCCG ACTCAGNNNNNNNNNNagrgtttgatymtggctcag-3') and 338R (5'-CCTATCCCCTGTG TGCCTTGGCAGTCTCAGtgctgcctcccgtaggagt-3'), where the capital letters represent the adaptors A and B, respectively, for 454 pyrosequencing (Kim*, et al.*, 2013). The NNNNNNNNNN indicates a unique 10-bp barcode sequence for each sample. PCR was performed in 1×Ex Taq PCR buffer (50 μL), deoxynucleoside triphosphate (2.5 mM), Ex Taq polymerase (Takara Bio, Otsu, Japan), each primer (10 μM), and 40 ng of extracted DNA under thermal conditions of 2 min at 96 °C, 20 cycles of 96 °C for 30 sec, 55 °C for 45 sec, and 72 °C for 1 min, and a final extension of 72 °C for 10 min on a 9700 PCR system (Life Technologies, Tokyo, Japan). Another universal primers set 817F (5'-CCATCTCATCCCTCGTGTCTCCGACTCAGNNNNNNNNNNttagcatggaataatrraat agga-3') and 1196R (5'-CCTATCCCCTGTGTGCCTTGGCAGTCTCAGtctggacctggtga gtttcc-3') were used to analyze the fungal community (Borneman & Hartin, 2000), where same pyrosequencing adaptors and barcode sequences to amplification of 16S rRNA gene were used. The 817F-1196R primers set has been shown to target a region of fungal 18S

ribosomal RNA gene, which is variable between major taxa and can permit phylogenetic analyses such as UniFrac (Rousk*, et al.*, 2010). PCR was performed in the same condition with PCR amplification of 16S rRNA gene described above, excepting that the annealing temperature was set to 56 °C.PCR products of 16S rRNA and 18S rRNA genes were confirmed by electrophoresis on 2% agarose gels, purified by the Beckman AMPure XP magnetic purification beads, and quantified using Quant-iT PicoGreen dsDNA Assay Kit (Life Technologies). A composite sample was prepared by pooling approximately equal amounts of PCR amplicons from each sample and subjected to pyrosequencing using the 454 GS FLX Titanium or 454 GS JUNIOR (Roche Applied Science, Penzberg, Germany) according to the manufacturer's instructions.

Sequence data processing and analysis

All the raw sequence data obtained from 454 pyrosequencing were assigned to each sample on the basis of their barcode sequence. Reads with an average quality value <25 and not having both universal primer sequences were filtered off. The trimmed reads were processed and analyzed in the Mothur platform. The selected reads were denoised using the 'pre.cluster' command (Huse*, et al.*, 2010). PCR chimeras were filtered off using Chimera Slayer (Haas*, et al.*, 2011). To remove the small portions of unexpected archaeal, the sequences of 16S rRNA genes were identified by the RDP Classifier (Wang*, et al.*, 2007), and filtered out the archaeal sequences. To remove the portions of unexpected un-fungal sequences, the effective sequences of 18S rRNA genes were aligned to the SILVA small subunit ribosomal RNA (SSU rRNA) database by basic local alignment search tool (BLAST) (Pruesse*, et al.*, 2007). Top hit of each sequence were

extracted by another self-written Python script. The unexpected sequences were filtered out. After above operation, the sequences of each sample were defined as qualified reads and each data set was rarefied to the smallest libraries using Daisy-Chopper (available at http://www.genomics.ceh.ac.uk/GeneSwytch). To define operational taxonomic units (OTUs), pairwise distances between sequences of the trimmed data sets were calculated at the average neighbor algorithm (Schloss & Westcott, 2011). Here OTUs were defined at an average intra-OTU sequence identity of 97%, which is the narrowest clustering distance recommended for 454 pyrosequences (Kunin*, et al.*, 2010). Good's coverage, abundance-based coverage estimator (ACE), Shannon-Wiener index (*H'*) and the inverse Simpson index (1/*D*) were calculated at 0.03-cutoff level. Pairwise dissimilarities between samples were calculated by the weighted UniFrac metric based on a relaxed neighbor-joining tree that was built with the representative sequence for each OTU using 'clearcut' command (Evans*, et al.*, 2006). The SILVA bacterial and eukaryotic trees provided by Mothur (available at http://www.mothur.org/wiki/Silva_reference_files) were used for the reference trees of UniFrac analyses. For classification, the sequences were compared to the SILVA SSU rRNA database using the Bayesian classifier and a confidence threshold of 80% (bootstrap) (Kuffner*, et al.*, 2012).

Statistical analysis

The influence of sampling site, sample type, and time factor on the microbial diversity indices (OTUs, abundance-based coverage estimator, Shannon index, and inverse Simpson index) was also evaluated using student *t*-test and MANOVA at 0.03-cutoff with 95% confidence intervals on R platform (available at http://www.r-project.org).

Similarities and differences in bacterial and fungal community structure among the samples were examined using the weighted UniFrac distance with the principal coordinate analysis (PCoA) ordination technique. Heat maps of the most abundant 50 bacterial OTUs and 30 fungal OTUs in each sample were used to compare the major compositions of the libraries. The heat maps were constructed using the function heatmap.2 from the R package gplots (available at http://cran.r-project.org/web/packages/gplots/index.html). For better visualization of heat maps, OTUs tables were $\log_2$-transformed (Lundberg*, et al.*, 2012). Hierarchical clustering of rows and columns in the heat maps was based on Bray-Curtis similarities and used group-average linkage. Canonical correspondence analysis (CCA) was employed to explore the relationship between microbial communities and environmental variables. The percentage abundances of bacterial families and fungal classes in each library were used as the species input, and the vegetation properties (Braun-Blanquet cover-abundance and count of plant species, and vegetation coverage) and chemical properties (pH, TOC, TN, and water content) served as the environmental input. Ordination plots of the results from CCA were performed using the function cca in the R package vegan (available at http://cran.r-project.org/web/packages/vegan/index.html).

Sequence data accession number

The pyrosequencing reads were deposited in the DDBJ Sequence Read Archive database under accession number DRA001160.

4.3 Results

Diversity of microbial communities

A total of more than 200,000 qualified reads (139,807 bacterial reads and 70,157 fungal reads), with average read length of 305 and 385 bp, for bacterial and fungal reads, respectively, were obtained from the Miyake-jima volcanic deposit and buried soil samples (Table 4.3.1). Community diversity, similarity, and structure were analyzed using the data rarefied at the smallest bacterial library from the volcanic deposit layer C1 sample from site IG9 in 2009 (sample ID, IG9-C1-09) (6,501 reads) and fungal library (IG7-C1-11, 4,084 reads). Total numbers of bacterial and fungal OTUs were 7,983 and 1,624, respectively. The number of bacterial OTUs per sample was in the range of 729 to 1,794, with average Good's coverage of 90.04%, while that of fungal OTUs per sample ranged from 109 to 332, with average Good's coverage of 97.12% (Table 4.3.1). Overall, the difference in the bacterial diversity was not related to the differences in vegetation cover and deposit age (*t*-test, $P>0.05$), but the bacterial diversity of the IG9-C1-11 sample was significantly higher than those in the other samples. As for the fungal diversity, the volcanic deposit samples at site IG9 (IG9-C1-09 and IG9-C1-11) showed high diversity, comparing with the samples form sites IG7 and IG8. The fungal diversity of the buried soil samples was clearly higher in the 2011 samples than in the 2009 samples.

Similarities between microbial communities

PCoA plots of bacterial and fungal OTU data sets are shown in Figs. 4.3.1A and 4.3.1B, respectively. Results of this analysis showed that all bacterial communities of the volcanic deposit samples clustered away from those of the buried soil samples (Fig. 4.3.1A). The bacterial communities of the buried soil samples showed higher among-site

variation than the volcanic deposit samples. As for the fungal communities, the two samples from the site IG7 (IG7-C1-09 and IG7-C1-11) clustered together and this cluster was separated from the other volcanic deposit and soil samples resulting three clusters (Fig. 4.3.1B). The fungal communities of buried soil samples were clustered together, implying a weak influence of vegetation cover on the soil fungal community beneath the volcanic deposit.

The 50 most abundant bacterial OTUs in each sample were selected (a total of 275 OTUs for all 12 samples), and their abundances were compared to those in other samples as shown in a heat map (Fig. 4.3.2A). The heat map cluster analysis showed two distinct clusters, which confirmed the difference between the bacterial communities of volcanic deposits and buried soils (Fig. 4.3.1A). This cluster analysis showed among-site variation of the bacterial community in the volcanic deposits by forming two separate clusters of the site IG7 (IG7-C1-09 and IG7-C1-11) and IG8 (IG8-C1-09 and IG8-C1-11) samples and separating them from the site IG9 samples (IG9-C1-09 and IG9-C1-11). On the other hand, the buried soil bacterial communities of the same sampling date but not the same site clustered together, in consistent with the results of the PCoA analysis (Fig. 4.3.1A).

Likewise, the 30 most abundant fungal OTUs in each sample were selected (a total of 161 OTUs for all 12 samples) and their heat map comparison is illustrated in Fig. 4.3.2B. Overall, the results of the heat map cluster analysis confirmed the three major clusters given by the PCoA analysis: (1) the site IG7 volcanic deposit samples, (2) the site IG8 and IG9 volcanic deposit samples, and (3) the buried soil samples from all sites.

Phylogenetic analysis of bacterial communities

High percentages of bacterial OTUs (79.6 to 90.9%) from the volcanic deposit samples could be assigned to known bacterial phyla, while the percentages of assignable OTUs from the buried soil samples were lower (42.7 to 67.4%). Major bacterial phyla that represented >1% of each community composition were *Acidobacteria*, *Actinobacteria*, *Bacteroidetes*, *Chloroflexi*, *Gemmatimonadetes*, and *Proteobacteria* (Fig. 4.3.3A). *Proteobacteria* was the most abundant phylum in the volcanic deposit bacterial communities (50.3 to 68.4%) and constituted the major group in the buried soil communities (14.7 to 34.9%). Relative abundance estimations of the underlying classes revealed differences between the volcanic deposit and the buried soil communities. Although *Alphaproteobacteria* was predominant in the communities of both the volcanic deposit and buried soil samples (Fig. 4.3.3B), *Betaproteobacteria*, dominated by the family *Oxalobacteraceae*, and *Gammaproteobacteria*, dominated by the family *Xanthomonadaceae*, represented the main classes in the volcanic deposit communities but quite minor classes (≤1.0%) in the buried soil communities (Figs. 4.3.3C and 4.3.3D). Except for the IG9-C1-11 community, the families *Oxalobacteraceae* and *Xanthomonadaceae* increased their relative abundance in response to changes in vegetation cover from grass (site IG8) to shrub (site IG9) plants. In contrast, the IG9-C1-11 sample harbored a higher proportion of *Alphaproteobacteria* and lower proportions of *Betaproteobacteria* and *Gammaproteobacteria* comparing with the other samples.

Further classification at the family level of the phylum *Actinobacteria* indicated that the family *Acidothermaceae* was exclusively present in all buried soil samples but not in all volcanic deposit samples (Fig. 4.3.3E). An inspection of minor bacterial populations

also indicated the difference between the volcanic deposit and buried soil bacterial communities (Fig. 4.3.3F). *Deinococcus-Thermus* accounted for 0.4-1.7% of the total OTU number of each volcanic deposit community but <0.05% in the buried soil communities, except for IG9-C1-11. In addition, the relative abundance of *Cyanobacteria* was higher in the volcanic deposit samples (0.8-1.9%) than in the buried soil samples (0.1-0.4%).

Phylogenetic analysis of fungal communities

Ascomycota was the most abundant phylum in the fungal communities of both volcanic deposit (24.5-72.1% of total OTUs in each sample) and buried soil (12.8-37.2%) samples, followed by Basidiomycota and Glomeromycota (Fig. 4.3.3G). It was noteworthy that the class Sordariomycetes in Ascomycota was the most abundant in the site IG7 volcanic deposits (IG7-C1-09 and IG7-C1-11) but few in the sites IG8 and IG9 volcanic deposits (Fig. 4.3.3H). As for Basidiomycota, Agaricomycetes was the main class in the volcanic deposit, especially in the IG8-C1-11 community, and buried soil communities (Fig. 4.3.3I).

Relationship between microbial community and environment

CCA was performed to discern possible linkages between statistically significant environmental factors including vegetation data and known bacterial and fungal taxonomic groups. To this end, data sets of assignable OTUs to known phyla were used for the analysis. For the bacterial data of volcanic deposits, the first axis separated the communities in the IG9-C1-09 and IG8-C1-11 samples from those in the others, while the

second axis separated those in the IG7-C1-11, IG7-C1-09, and IG8-C1-11 from those in the others (Fig. 4.3.4A), which was in accordance with the PCoA plot data (Fig. 4.3.1A). CCA showed a positive correlation of *Oxalobacteraceae*, *Gallionellaceae*, and *Micrococcaceae* with a grass *Carex oshimensis* but a negative correlation of *Xanthobacteraceae* and *Gemmatimonadaceae* with the grass. The presence of *Sphingobacteriaceae*, *Burkholderiaceae*, and *Acetobacteraceae* correlated positively with a tree, *Camellia japonica*. No strong positive correlation was found between any bacterial families and the most abundant grass, *Miscanthus condensatus* and the most abundant shrub, *Alnus sieboldiana* but *Thermaceae* and *Coxiellaceae* showed a negative correlation with these plants. No strong influence of the chemical properties (pH, TOC, and TN) on bacterial community was found, as expected from the low among-sites variation of the chemical properties showed in Chapter 2.

For the fungal data of volcanic deposits (Fig. 4.3.4B), the first axis separated the communities in the site IG7 samples (IG7-C1-09 and IG7-C1-11) from those in the site IG8 and IG9 samples and the second axis separated the site IG7 samples and IG8-C1-11 sample from those in the others, all consistent with the PCoA results (Fig. 4.3.1B). Fungi thriving in the site IG7 volcanic deposit, such as Sordariomycetes, Saccharomycetes, Pezizomycetes, and Lecanoromycetes in the phylum Ascomycota, and Dacrymycetes in the phylum Basidiomycota showed a highly negative relationship with the major plants, *Miscanthus condensatus* and *Alnus sieboldiana*. Agaricomycetes in the phylum Basidiomycota correlated positively but Eurotiomycetes in the phylum Ascomycota negatively with a shrub, *Rubus trifidus*.

In the case of buried soils, *Nocardioidaceae* showed a positive correlation with the

major grass, *Miscanthus condensatus* but *Oxalobacteraceae* and *Coxiellaceae* showed a negative correlation with the major grass (Fig. 4.3.5A). *Beijerinckiaceae*, *Acetobacteraceae*, and *Micrococcaceae* correlated negatively with *Carex oshimensis* and *Alnus sieboldiana*. In the fungal data, only negative correlation of Tremellomycetes with *Alnus sieboldiana* and *Carex oshimensis* was found (Fig. 4.3.5B). TOC and TN seemed to show no important impact on known bacterial and fungal groups (Figs. 4.3.5A and 4.3.5B).

Table 4.3.1. Qualified reads, OTU counts, alpha diversity indexes of microbial communities, and Good's coverage*

Sample ID	Bacteria						Fungi					
	No. of qualified reads	OTU count	ACE	*H′*	1/*D*	C (%)	No. of qualified reads	OTU count	ACE	*H′*	1/*D*	C (%)
IG7-C1-09	35,835	846	2,218	5.04	39.49	93.26	4,616	166	365	2.44	4.79	98.14
IG7-C1-11	8,310	757	1,731	4.88	39.25	94.15	4,084	187	640	2.44	3.80	97.58
IG8-C1-09	9,073	1,207	4,346	5.68	99.52	89.16	5,283	210	698	2.61	4.21	97.14
IG8-C1-11	9,732	729	1,778	4.93	53.89	94.34	5,912	134	471	1.96	3.12	98.11
IG9-C1-09	6,501	824	2,493	4.59	22.05	92.97	7,511	291	913	3.71	15.17	96.23
IG9-C1-11	12,256	1,794	5,579	6.50	208.51	83.99	8,741	332	1,016	3.73	16.26	95.64
IG7-2A-09	7,396	1,086	3,075	5.54	67.93	90.88	4,779	212	617	2.67	5.12	97.21
IG7-2A-11	9,336	1,276	4,257	5.62	68.88	88.36	7,992	242	1,172	3.38	14.02	96.55
IG8-2A-09	9,348	1,116	3,631	5.45	57.83	90.14	5,250	109	218	1.79	2.66	98.53
IG8-2A-11	9,844	1,254	4,621	5.60	63.69	88.48	6,080	244	909	3.14	8.45	96.45
IG9-2A-09	9,265	1,166	3,665	5.59	72.53	89.88	4,971	171	426	2.29	3.93	97.85
IG9-2A-11	12,911	1,549	6,243	5.86	93.91	84.88	4,938	280	1,059	3.18	8.04	95.98

*Samples were rarefied at the smallest bacterial library (IG9-C1-09, 6501 reads) and fungal library (IG7-C1-11, 4084 reads) for OTU count and statistical analyses. All indexes were calculated at cutoff level of 0.03 in the average neighbor method. ACE, abundance-based coverage estimator; *H′*, Shannon index; *1/D*, inverse Simpson index; C, Good's coverage.

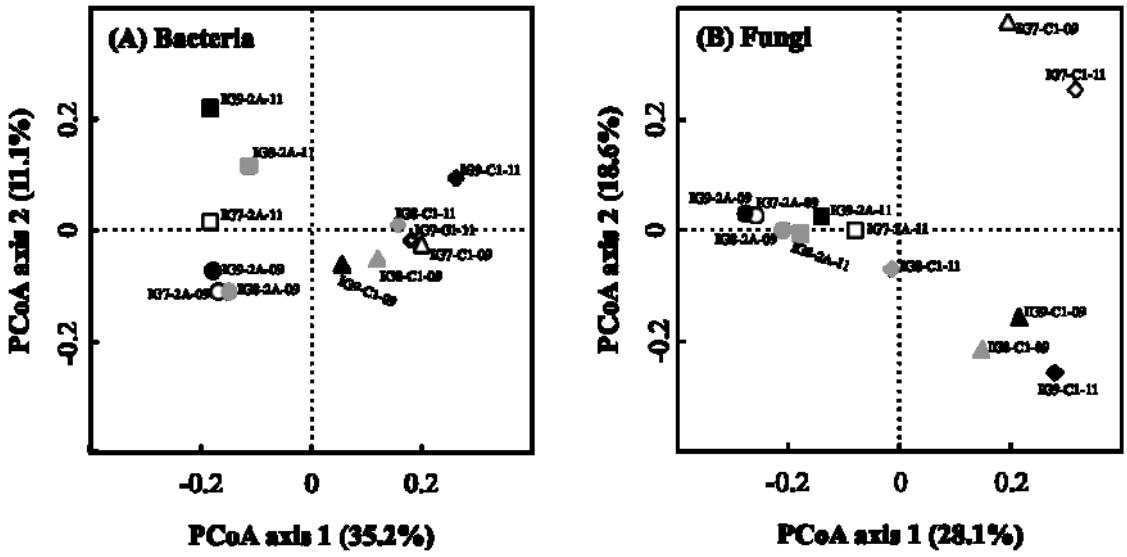


Fig. 4.3.1. Principal coordinate analysis (PCoA) plots of bacterial (A) and fungal (B) communities of the volcanic deposit (triangles and diamonds) and soil (circles and squares) samples by weighted UniFrac. Silva bacterial and eukaryotic trees were selected as the reference trees.

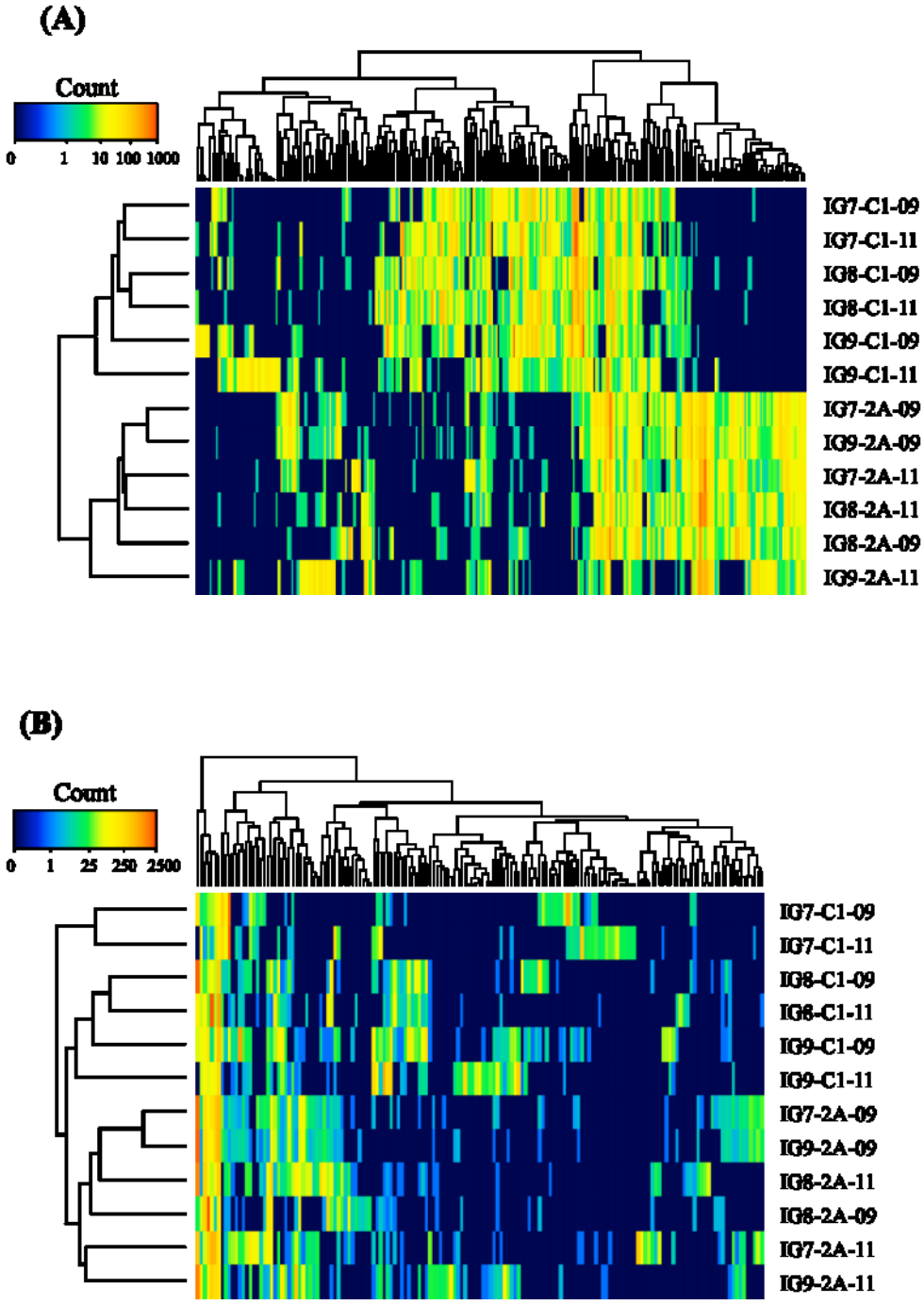


Fig. 4.3.2. Heat map presentations of the 50 most abundant bacterial OTUs in each sample (A) and the 30 most abundant fungal OTUs in each sample (B). The samples and OTUs were clustered on their Bray-Curtis similarities (group-average linkage). The key relates to the untransformed read counts.

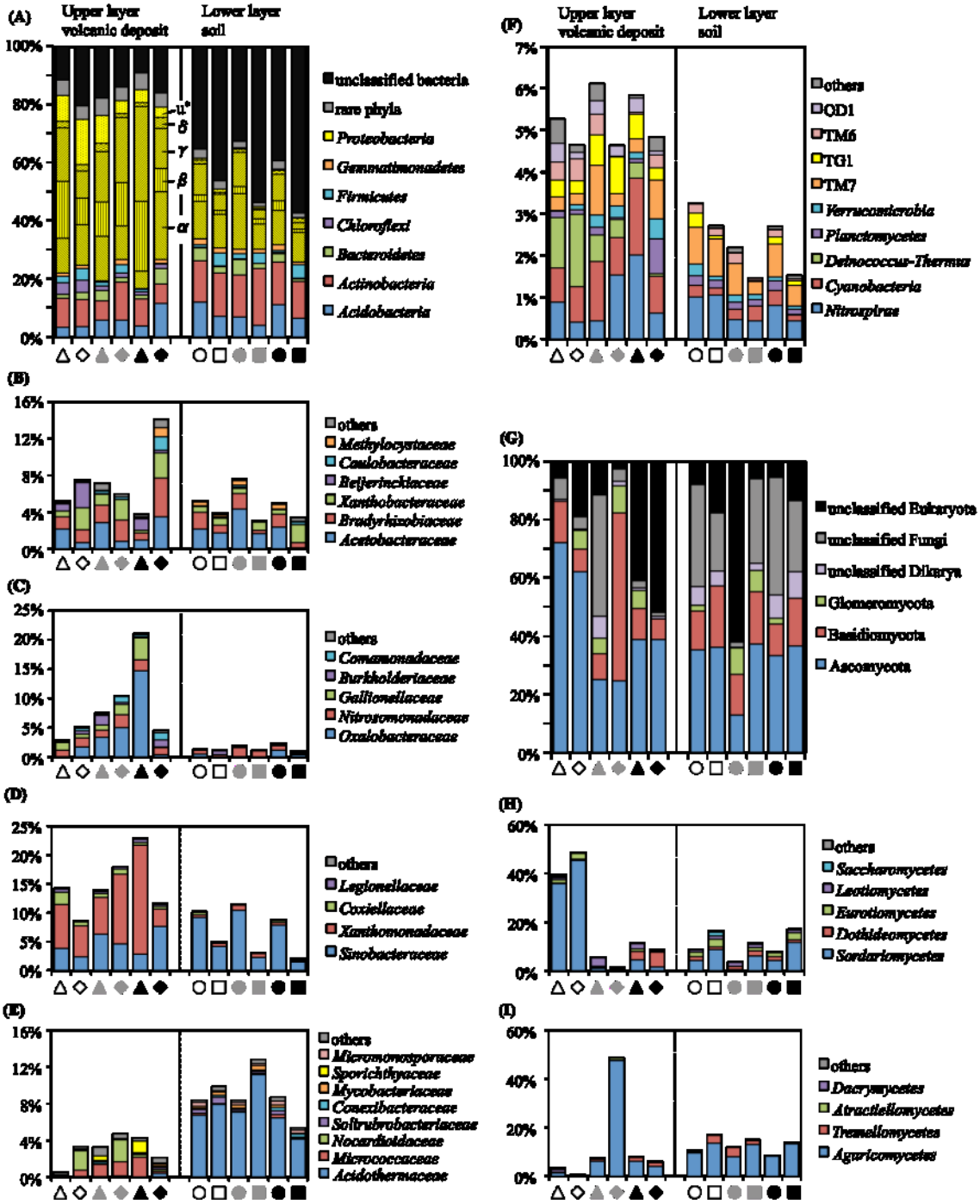


Fig. 4.3.3. Taxonomic classification of the pyrosequencing reads. Classification at the phylum and proteobacterial class level (*α*, *Alphaproteobacteria*; *β*, *Betaproteobacteria*; *γ*, *Gammaproteobacteria*; *δ*, *Deltaproteobacteria*; u, unclassified proteobacteria) for total bacterial OTUs (A), family-level classifications of the OTUs belonging to

Alphaproteobacteria (B), *Betaproteobacteria* (C), and *Gammaproteobacteria* (D), and *Actinobacteria* (E), and classification of low-abundance OTUs (<1% of total bacterial OTUs in each sample) into bacterial phyla (F). Classification at the phylum level for total fungal reads (G), class-level classifications of the reads of Ascomycota (H), and Basidiomycota (I). △, IG7-C1-09; ▲,IG8-C1-09; ▲,IG9-C1-09; ◇,IG7-C1-11; ◆, IG8-C1-11; ◆, IG9-C1-11; ○, IG7-2A-09; ●, IG8-2A-09; ●, IG9-2A-09; □, IG7-2A-11; ■, IG8-2A-11; ■, IG9-2A-11.

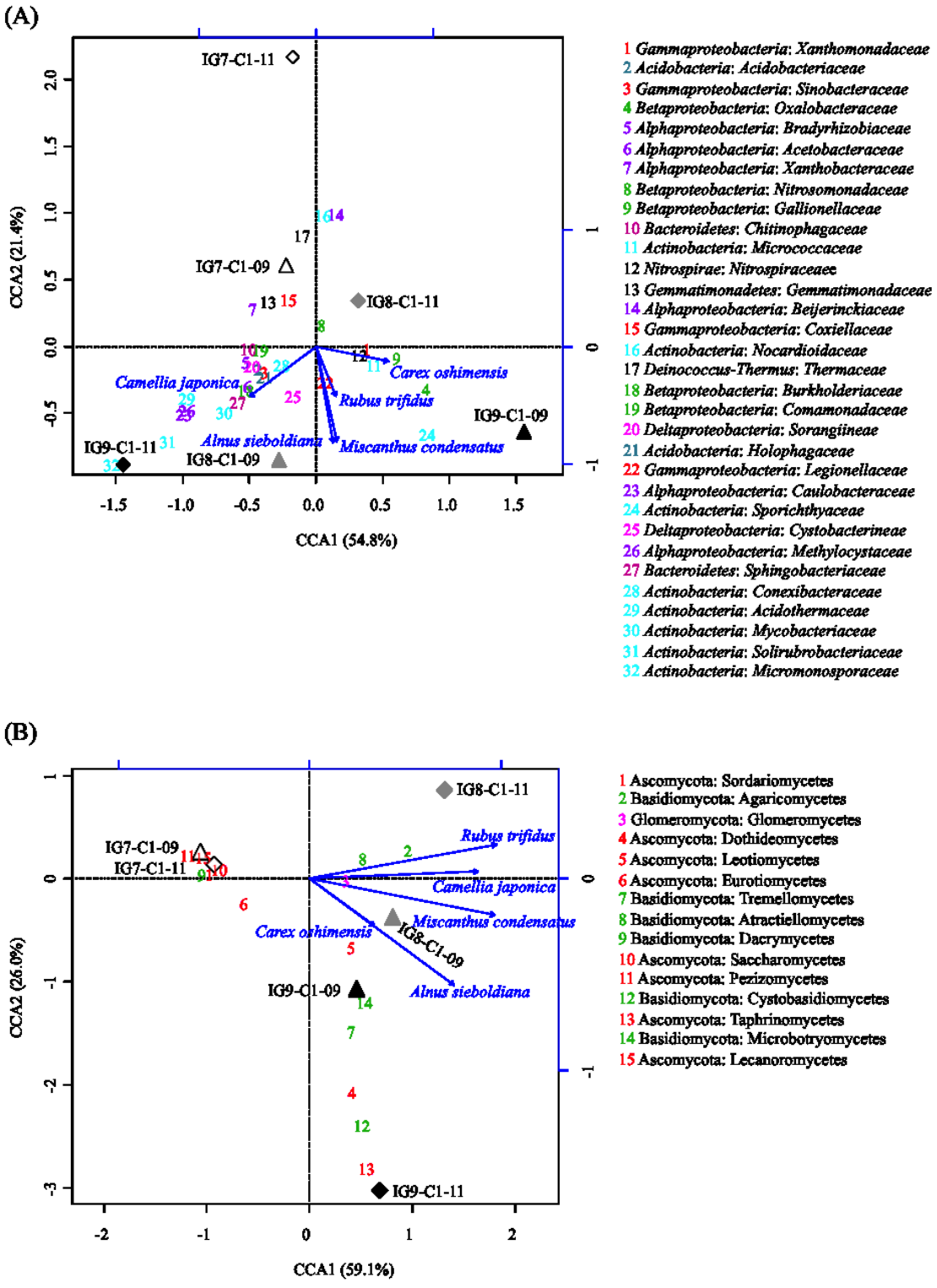


Fig. 4.3.4. Canonical correspondence analysis (CCA) ordination plots of bacterial (A) and fungal (B) communities of six volcanic deposits (triangles and diamonds) and results of the analysis of environmental factors affecting bacterial and fungal distribution, showing significant effects of the colonizer plants. The direction of the arrows for individual plant

species indicates an increasing coverage of that plant and the length of the arrows indicate the degree of correlation with the represented axes. The numbers correspond to the bacterial families (A) and fungal classes (B) in the keys at right and are ranked according to abundance.

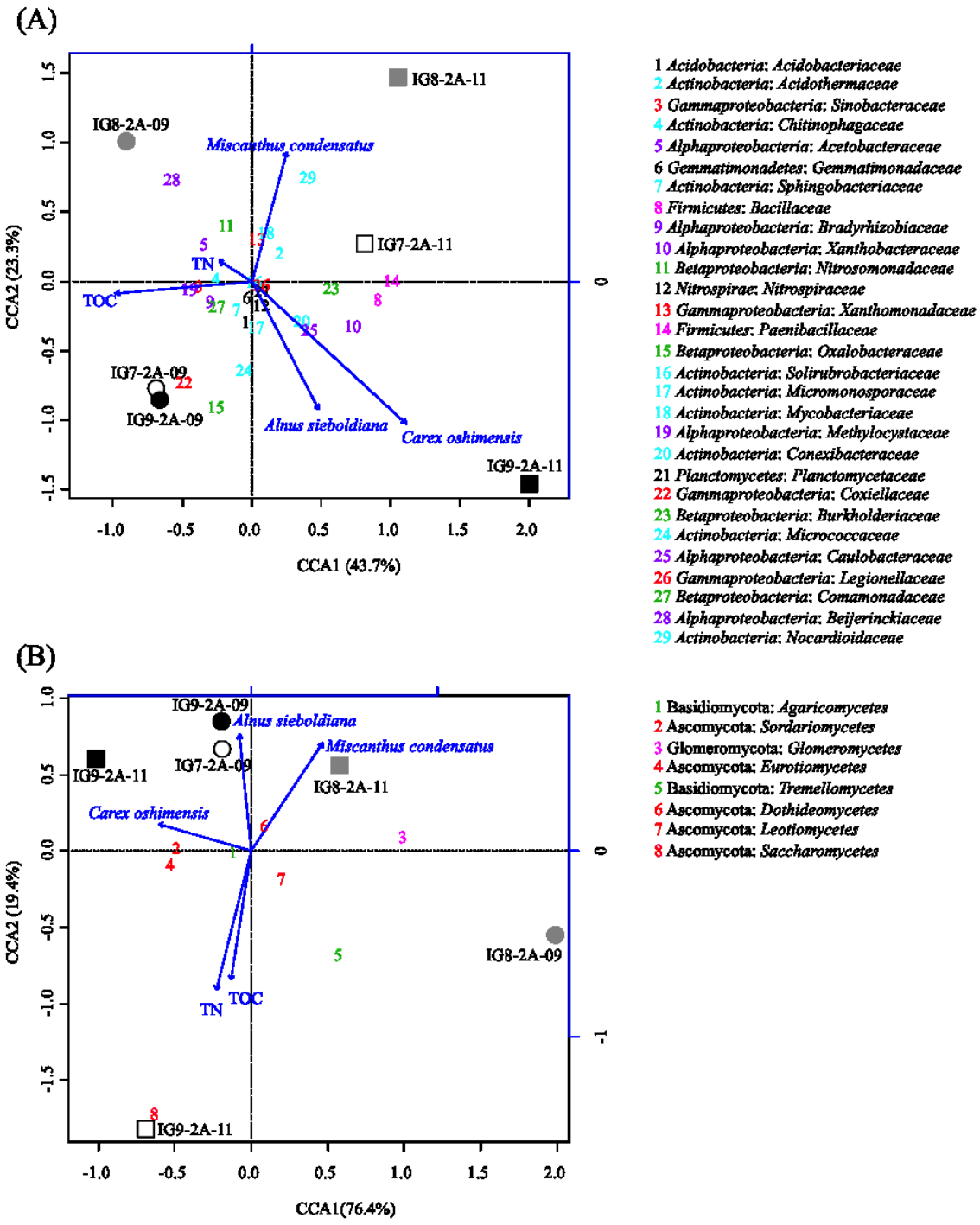


Fig. 4.3.5. Canonical correspondence analysis (CCA) ordination plots of bacterial (A) and fungal (B) communities of six buried soil samples (circles and squares) and results of the analysis of environmental factors affecting bacterial and fungal distribution, showing effects of the colonizer plants, TOC, and TN. The direction of the arrows for individual

factor indicates an increasing concentration of that factor and the length of the arrows indicate the degree of correlation with the represented axes. The numbers correspond to the bacterial families (A) and fungal classes (B) in the keys at right and are ranked according to abundance.

4.4 Discussions

A variety of preceding studies on volcanic deposits, deglaciated soils, and other newly exposed minerals have shown that the phylum *Proteobacteria* usually dominate the early bacterial community (Mark Ibekwe*, et al.*, 2007; Knelman*, et al.*, 2012; Zumsteg*, et al.*, 2012), because the bacteria in this phylum own advantageous traits such as phototrophy, photoheterotrophy, and chemolithotrophy, in early ecosystems with limited nutrient resources. Our results showed that although the vegetation has developed at different levels, *Proteobacteria* was still the most abundant phylum in the bacterial community of volcanic deposits. The inspection of the underlying families revealed the predominance of the family *Oxalobacteraceae* in *Betaproteobacteria* and *Xanthomonadaceae* in *Gammaproteobacteria* in the volcanic deposit communities (Figs. 4.3.3C and 4.3.3D). The family *Oxalobacteraceae* was reported as root-colonizing heterotrophic bacteria in succession of bacterial communities during early plant development (Green*, et al.*, 2006; Green*, et al.*, 2007). This can be expected to be true for the bacterial community of the Miyake-jima volcanic deposit because CCA showed a positive correlation of *Oxalobacteraceae* with a grass *Carex oshimensis* (Fig. 4.3.4A). The family *Xanthomonadaceae* was reported as a major component of pasture rather than woodland or broad-leaved forest (Chim Chan*, et al.*, 2008). In our data, the correlation of *Xanthomonadaceae* with *Carex oshimensis* also seemed to be positive in the CCA plot (Fig. 4.3.4A). In the IG9-C1-11 samples, *Oxalobacteraceae* and *Xanthomonadaceae* were replaced by *Alphaproteobacteria*, specifically the families *Acetobacteraceae*, *Bradyrhizobiaceae*, and *Xanthobacteraceae* (Figs. 4.3.3B to 4.3.3D). This succession seems to be supported by the notion that plant raised the proportion of

Alphaproteobacteria, particularly *Rhizobiales*, in various soil environments (Haichar*, et al.*, 2008; Knelman*, et al.*, 2012).

Actinobacteria are the second abundant phylum dominating the bacterial community in the volcanic deposits (Figs. 4.3.3A and 4.3.3E), which are generally known to decompose recalcitrant polymers in soils (Heuer*, et al.*, 1997). A recent study with Zimmerman sand created by glacial outwash indicated that host plant species and increasing plant richness altered the composition of *Streptomyces* communities, which were the most abundant among the *Actinobacteria* (Bakker*, et al.*, 2013). Similar to this finding, in our study, the family-level composition of *Actinobacteria* in the volcanic deposits was also found to differ distinctly at the different sites (Fig. 4.3.3E). On the other hand, the *Actinobacteria* composition in the buried soil samples was essentially invariant among the sites. As discussed in chapter 2, the respiration per unit of soil organic carbon seems to reflect the nature of soil organic matter and thus the very low respiration rates of the Miyake-jima buried soil samples suggest the presence of higher amounts of recalcitrant substrates than those of readily consumable substrates in the soil samples. This consideration may explain the steady population density of *Actinobacteria* in the buried soil.

Our results showed that the phylum *Cyanobacteria* was present at low levels (0.8-1.9% of bacterial communities) in the Miyake-jima volcanic deposits (Fig. 4.3.3F), which was not detected by clone library analysis of 16S rRNA gene in chapter 3. This organism is known to be primary colonizers on the newly exposed minerals and dominate the early microbial communities (Hodkinson*, et al.*, 2003; Gomez-Alvarez*, et al.*, 2007). In addition to this phototroph, filamentous anoxygenic phototrophic *Chloroflexi* was found

to be present at higher levels (about 4%) in the site IG7 volcanic deposit, comparing with *Cyanobacteria* (Fig. 4.3.3A). Although the population was low in the site IG8 and IG9 deposits, the *Chloroflexi* group may occupy the wider niche than *Cyanobacteria* in unvegetated sites. It is also noted that *Deinococcus-Thermus* was found at the IG7 volcanic deposits but not in the soil samples (Fig. 4.3.3F). The genus *Deinococcus* is known to show remarkable resistance to a range of damage caused by ionizing radiation, desiccation, UV radiation, and oxidizing agents (Makarova*, et al.*, 2001). The resistance to solar radiation or desiccation may be a secondly determinative factor for the survival of microbes in the volcanic deposits, particularly at unvegetated sites.

In spite of the low fungal populations, a substantial amount of fungal reads were obtained from the volcanic deposit samples. The results of PCoA showed high among-site variation for the fungal communities in the volcanic deposits (Fig. 4.3.1B). Further CCA plot showed that Ascomycota thriving in the site IG7 volcanic deposit, such as the class Sordariomycetes, showed a highly negative relationship with the major plants, *Miscanthus condensatus* and *Alnus sieboldiana* (Fig. 4.3.4B). Interestingly, Zumsteg *et al.* reported a similar observation, the succession from an Ascomycota-dominated community in unvegetated soils to a more Basidiomycota-dominated community in vegetated soils in the forefield of Damma glacier (Zumsteg*, et al.*, 2012). Members of Sordariomycetes are ubiquitous and represent pathogens and endophytes of plants, animal pathogens, and mycoparasites (Zhang*, et al.*, 2006). However, the mechanism of why Sordariomycetes negatively correlated with plant colonization in the new substrates is still little known. Within Basidiomycota, the class Agaricomycetes was exclusively present in the IG8-C1-11 sample (Fig. 4.3.3I). Members of Agaricomycetes are the most

important ectomycorrhizal fungi, which construct symbiotic associations with many territorial plants (Hibbett & Matheny, 2009). Further, investigations at glacier forefield showed that plant colonization could increase the proportion of mycorrhizal fungi in the early soil communities of alpine habitats (Mühlmann & Peintner, 2008; Zumsteg*, et al.*, 2012). Analogously, in our results, Agaricomycetes showed a positive correlation with a shrub, *Rubus trifidus* (Fig. 4.3.4B).

Comparing to the clone library analysis of 16S rRNA gene expounded in chapter 3, the PCR-based pyrosequencing of 16S rRNA gene shown a similar but more detailed results. In addition, our analysis of fungal community in the volcanic deposits by pyrosequencing of 18S rRNA gene is the first investigation of the early fungal community developing on the Miyake-jima volcanic deposits derived from the eruption in 2000. However, our analysis did not detect what group of bacterium and fungus in the volcanic deposits positively correlation with dominant plant species *Miscanthus condensatus* and *Alnus sieboldiana*. It may be resulted from the positively correlating microorganisms inhabits in the zone directly associated with the plant such as rhizosphere. To examine further these microbe-plant correlations, community analyses should focus on the rhizosphere of colonizer plants.

In conclusion, this chapter showed that microbial communities in recent Miyake-jima volcanic deposits were phylogenetically diverse despite low-carbon conditions. Because the volcanic deposit samples displayed low among-site variation for chemical properties (pH, TOC, and TN), there is no apparent factor other than the aboveground vegetation cover to explain the difference in microbial community among the different site volcanic deposits. Indeed, CCA could show several positive and negative relationships between

microbial groups and plant species. Our findings give a better understanding of how belowground microbial communities develop and interact with the establishment of the first aboveground plants in newly exposed volcanic deposits.

Chapter 5

General Discussion

Microorganisms play an important role in soil formation on newly exposed minerals from volcanic activities, such as lava, tephra, and volcanic ash. Prior to colonization by plants, free-living diazotrophs and some other chemolithotrophic bacteria are the most important agents in the early soil ecosystem, as their function in accumulation of organic carbon and nitrogen (Nemergut*, et al.*, 2007; Schmidt*, et al.*, 2008; Fujimura*, et al.*, 2012). Subsequent colonization of plants directly influences the early microbial community through litter inputs, root exudates, and dead root tissues (Bardgett*, et al.*, 2005; Dennis*, et al.*, 2010). Reciprocally, the microbial community influences the plant as both the positive effects of root symbionts and the negative effects of pathogens (Ehrenfeld*, et al.*, 2005; Bever*, et al.*, 2010; Bever*, et al.*, 2012). Such plant-microbe interactions will drive a primary ecosystem succession on the volcanic deposit.

Recently, the primary microbial succession on glacier forefront ecosystems was well characterized as the model showing early development of terrestrial ecosystems (Jumpponen, 2003; Brankatschk*, et al.*, 2011; Knelman*, et al.*, 2012; Zumsteg*, et al.*, 2012). Concerning about volcanic environments, in this study, the microbial population density, microbial activity, and microbial community on the 9- and 11-year-old Miyake-jima volcanic deposits along a vegetation gradient were investigated for a better understanding of plant-microbe interaction in early terrestrial ecosystems. In this chapter, the primary succession of plant-soil ecosystem on recent volcanic deposits derived from the eruption in 2000 on Miyake-jima is discussed.

5.1 Microbial ecosystem and primary succession prior to plant colonization on the Miyake-jima volcanic deposits

Miyake-jima Isalnd in the western rim of Pacific Ocean erupted in 1940, 1962, 1983, and 2000, with about 20-year intervals. The eruption in 2000 occurred at Mt. Oyama was characterized by ejecting large amounts of volcanic ash and acidic gas. About 60% of vegetation on the island was initially influenced by the heave deposition of volcanic ash. After the crater formation, large amounts of volcanic gas containing SO_2 and H_2S have been emitted and caused widespread defoliation, particularly on the leeward side of Mt. Oyama (Kamijo & Hashiba, 2003). The volcanic deposits derived from this eruption had been characterized by low contents of carbon (TC, 0.1-0.7 g kg^{-1}) and nitrogen (0.1-0.9 g kg^{-1}), high contents of fine sand (36-76%), strong acidity [pH (H_2O), 3.1-4.0], and high amounts of exchangeable Ca^{2+} (34-115 cmolc kg^{-1}) and Al^{3+} (0.8-10.2 cmolc kg^{-1}) (Kato*, et al.*, 2002). Such extreme environment was not suitable for plant colonization.

Chemolithotrophic bacteria such as the members related to *Thiobacillus*, *Leptospirillum*, and *Acidithiobacillus* firstly colonized and dominated the newly deposited volcanic ash (Ohta*, et al.*, 2002; Sato, 2006). These autotrophic N_2-fixing Fe(II) oxidizers and H_2-oxidizers drive the initially biogeochemical cycling and may result in the accumulation of organic carbon and then contribute to the development of complex microbial communities, including mixotrophic and heterotrophic microbes (Sato*, et al.*, 2009; Fujimura*, et al.*, 2012; Fujimura*, et al.*, 2012). Indeed, the primary microbial succession prior to plant colonization shown that the N_2-fixing genes in the deposits rapidly changed from autotrophic Fe(II) oxidizers (*Leptospirillum* and *Acidithiobacillus*)

dominated community to diverse N_2-fixers (*Leptospirillum*, *Acidithiobacillus*, *Methylococcus*, *Beijerinckia*, *Leptothix*, *Herbaspirillum*, *Bradyrhizobium*, *Burkholderia*, and *Azorhizobium*) inhabited community with deposit aging (Fujimura, 2012). In this context, the N_2-fixing activity of both autotrophs and heterotrophs may be important to supply fixed nitrogen essential for early vegetation development.

5.2 Secondary microbial succession correlated with the first plant colonizers on the Miyake-jima volcanic deposits

The initial vegetation recovery on the Miyake-jima volcanic deposits was characterized in the windward area in 2003, as stem sprout of defoliated trees, sprout from buried vegetative organ, germination of buried seeds, and colonization of seeds and spore (Kamijo & Hashiba, 2003). The vegetation development may cause the secondary microbial succession on the deposits. In this study, the three sites established along a vegetation gradient on the elevational transect in the northwest side of Mt. Oyama were selected to explore how the microbial community develops with the vegetation succession.

Several studies have indicated that the microbial biomass and community on recent volcanic deposits was strongly influenced by pioneer colonizer plants (Nara*, et al.*, 2003; Gomez-Alvarez*, et al.*, 2007; Mark Ibekwe*, et al.*, 2007; Yoshitake*, et al.*, 2013). In a study with volcanic desert on Mount Fuji, total carbon, total nitrogen, and soil organic matter (SOM) contents increased with the vegetation development, and soil microbial biomass was strongly correlated with TC, TN, and SOM contents (Yoshitake*, et al.*, 2013). These findings suggested that the belowground accumulation of organic nutrients along

the vegetation development was a determinant for soil microbial biomass. However, in despite of high vegetation development on the Miyake-jiama volcanic deposits, the total organic carbon content were much lower than those in the above-mentioned studies. Therefore, our research reveals the earliest stage of secondary succession in belowground microbial community at the onset of vegetation cover development. The above analyses in Chapter 2 have shown that the availability of organic substrate for early microbial community was relatively high in the vegetated deposits. This may be resulted from the organic carbon input from plants is easy to be decomposed by the microbes, particularly those from the herbaceous plants. Indeed, almost all of the plants growing at the study sites were grasses and small shrubs. No determinable accumulation of organic matter may limit the increasing of microbial biomass in the volcanic deposits.

Although the volcanic deposits contained a trace of TOC and TN, bacterial population density and *in vitro* respiratory activity was not as low as the TOC and TN value. In a pure culture experiment with a soil oligotrophic bacterium, the growth yield was calculated as 1.48×10^{9} CFU mg^{-1} (soil organic carbon) (Ohta & S., 1988). When the TDC values per unit soil organic carbon of the volcanic deposits are calculated, the values are 0.40×10^{9} to 1.95×10^{9} cells mg^{-1} (soil organic carbon), comparable to the yield value of the pure culture experiment. This implies that oligotrophic bacteria dominated the volcanic deposit, in despite of plant colonization on the volcanic deposit. The relatively high respiratory activities of volcanic deposits may be explained by their low C:N ratio. Generally, microbial biomass is characterized by low C:N ratio and biomass debris is readily consumable for soil microbes, which can result in high activity of respiration per unit of organic carbon.

Despite no significant influence of plant colonization on the microbial population density, we found that the microbial community was strongly affected by aboveground vegetation feature, which were revealed by all analyses of T-RFLP, clone library, and PCR-based pyrosequencing. Comparing to the completely unvegetated volcanic deposit, the poorly to fully vegetated volcanic deposits harbored more diverse bacteria, such as the members from phyla *Chloroflexi* and *Verrocumicrobia* recovered in the clone analysis of 16S rRNA genes. Statistical analyses of pyrosequencing data showed that the vegetated deposits harbored phylogenetically diverse microbial communities, which was comparable to that in the buried soils. Further multivariate analysis revealed that several bacterial classes correlated positively or negatively with specific plant species. Particularly, *Oxalobacteraceae*, *Gallionellaceae*, and *Micrococcaceae* positively correlated with a grass *Carex oshimensis,* but a negative correlation of *Xanthobacteraceae* and *Gemmatimonadaceae* with the grass. In addition, the presence of *Sphingobacteriaceae*, *Burkholderiaceae*, and *Acetobacteraceae* correlated positively with a tree, *Camellia japonica*. In the further vegetation development, it is notable that *Alphaproteobacteria*, specifically the families *Acetobacteraceae*, *Bradyrhizobiaceae*, and *Xanthobacteraceae*, replaced *Oxalobacteriaceae* in *Betaproteobacteria* and *Xanthomonadaceae* in *Gammaproteobacteria* as the major members. This succession seems to be supported by the notion that plant raised the proportion of *Alphaproteobacteria*, particularly *Rhizobiales*, in various soil environments (Haichar*, et al.*, 2008; Knelman*, et al.*, 2012).

On the other hand, the early fungal community of Miyake-jima volcanic deposits seemed to be more influenced by the vegetation feature than prokaryotes. This may

resulted from that fungi directly associated with plant (Nielsen*, et al.*, 2010). Despite the low fungal populations, the substantially diverse fungal communities were also detected in the poorly to fully vegetated deposits by pyrosequencing of 18S rRNA genes. The fungal communities showed high among-site variation in the deposits but not in the buried soils, suggesting the early fungal community were strongly shaped by the aboveground vegetation feature. Sordariomycetes belonged to Ascomycota was negatively correlated to the presence of *Miscanthus condensatus* and *Alnus sieboldiana*. Similar fungal succession, which changed from an Ascomycota-dominated community in unvegetated soils to a more Basidiomycota-dominated community in vegetated soils, was observed in the glacier forefield (Zumsteg*, et al.*, 2012). However, information on the Sordariomycetes in unvegetated volcanic deposits is not yet available. In addition, Agaricomycetes belonging to Basidiomycota positively correlated with a shrub, *Rubus trifidus*. Members of Agaricomycetes are the most important ectomycorrhizal fungi, which construct symbiotic associations with many territorial plants (Hibbett & Matheny, 2009). Possibly, this fungal group promoted some specific plants to colonize the infertile volcanic deposits.

5.3 Concluding remarks

This research focused on the microbial ecology in recent Miyake-jiama volcanic deposits along a vegetation gradient, and showed phylogenetically diverse microbial community developing on the 9- and 11-year-old deposits, despite low-carbon and low-nitrogen conditions. Because the volcanic deposits displayed low among-site variation for chemical properties (pH, TOC, and TN), there is no apparent factor other

than the aboveground vegetation cover to explain the difference in microbial community among different site volcanic deposits. Indeed, multivariate analysis showed several positive and negative relationships between microbial groups and specific plant species. These findings give a better understanding of how belowground microbial communities develop and interact with the establishment of the first aboveground plants in newly exposed volcanic deposits.

When we had been conducting this research, several new questions were unfolding incrementally. These questions were as follows:

1. What microbial groups is the decomposer of plant debris in recent Miyake-jima volcanic deposits? *Miscanthus condensatus*, the most abundant plant growing on the volcanic deposit, are perennial C4 grasses with rapid growth and high biomass features. We indeed found that this plant grown to more than 1.5 m in height on the volcanic deposits, implying a large amount of organic input to the deposit microbial community. It is, therefore, necessary to clear what microbial groups decompose the plant biomass。
2. What kind of microorganisms inhabit in the rhizosphere of the first plant colonizers on Miyake-jima volcanic deposit? Although several relationships between microbial groups and specific plant species were characterized, this research cannot detect such plant-microbe relationship with the dominant plants *Miscanthus condensatus* and *Alnus sieboldiana.* It may be resulted from the positively correlating microorganisms inhabits in the zone directly associated with the plant such as rhizosphere. To examine this further, community analyses should focus on the rhizosphere of colonizer plants.

3. What is the ecological role of predominant fungi related to Sordariomycetes in the unvegetated deposits, and why does this fungal group dramatically decrease in response to the establishment of first plant colonizers?

To answer the above-mentioned questions, much deeper and more extensive researches will be needed, such as characterization of the decomposers by *in situ* $^{13}CO_2$ stable isotope probing, metagenomics analysis of the rhizosphere of plant colonizers, and complete genome analysis of the dominant fungi Sordarimycete in the unvegetated deposits.

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