DISSERTATION

PROKARYOTIC COMMUNITIES AND BIODIVERSITY IN THE HIGHLY ACIDIC HOT SPRINGS

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ABSTRACT

The prokaryotic microbial communities in hot springs have been extensively studied based on the 16S rRNA gene phylogenetic analysis. Although these pioneering studies have improved our understanding of prokaryotic communities living in high temperature environments, little is known about both bacterial and archaeal communities in the highly acidic aquatic environments. In addition, there were a few examples of reports that surveying and comparing the prokaryotic communies of distinct highly acidic hot springs displaying a wide range of environmental factors in a restricted field of geothermal area.

In this study, the 16S rRNA gene phylogenetic analysis was performed to reveal bacterial and archaeal community structures and biodiversity of four distinct highly acidic hot ponds displaying a wide range of the environmental factors in a restricted field of the Kirishima geothermal area, Kagoshima Prefecture, Japan. The four ponds were selected based on differences in temperature and the total concentration of examined chemical components: 1) Pond-A: 93°C and 58 mmol L^{-1}; 2) Pond-B: 66°C and 73 mmol L^{-1}; 3) Pond-C: 88°C and 6.7 mmol L^{-1}; and 4) Pond-D: 67°C and 11 mmol L^{-1}. Principal component analysis showed that these four ponds were clearly distinguished by different chemical compositions and temperatures.

In total, 372 clones of the bacterial 16S rRNA gene were analyzed and classified into 35 phylotypes. The dominant bacterial group was the class γ-Proteobacteria. The bacterial species diversity was greatest in Pond-D, and the dominant phylotype detected (37% of all clones) was closely related to *Acinetobacter junii*. Pond-B had the highest relative total concentration of examined chemical components, where the bacterial species diversity was the lowest despite the greatest gene diversity among the four ponds. The bacterial community was dominated by a

phylotype closely related to *Acidithiobacillus caldus* as well as an uncultured species of δ-Proteobacteria in this pond. Pond-A and Pond-C had the highest relative temperatures and were dominated by a phylotype closely related to *Acinetobacter johnsonii* (accounted for more than 57% of the identified clones). Pond-A had the highest relative temperature, where the bacterial gene diversity was the lowest among the four ponds.

On the other hand, in total, 431 clones of the archaeal 16S rRNA gene were classified into 26 phylotypes. In Pond-B, the archaeal gene diversity and the species diversity was the highest among the four, and the member of order Sulfolobales were dominant. The Pond-D also shown relatively high species diversity and the most freaquent group was uncultured thermoacidic spring clone group. In contrast to Pond-B and Pond-D, much less archaeal phylotypes were detected in Pond-A and Pond-C showing relatively high temperatures. Especially in Pond-A had the relatively high total concentration of examined chemical components, the archaeal gene diversity and the species diversity was the lowest among the four ponds.The dominant archaeal groups in these ponds were also different. The members of the order Sulfolobales shared of 89% of total clones in Pond-A, and the uncultured crenarchaeal groups shared 99% of total Pond-C clones.

This study highlights the different bacterial and archaeal species composition and biodiversity in highly acidic hot ponds of a restricted field in a geothermal area characterized by different temperatures and chemical compositions. Although other environmental factors could also have influenced the bacterial and archaeal community structure and biodiversity, the present data will be helpful for improving our understanding of the prokaryotic ecology in the highly acidic ponds.

Chapter I

General introduction

1.1. Prokaryotes consist of two domains -Bacteria and Archaea-

All organisms living on the earth are divided into major two groups, Eukaryotes and Prokaryotes, based on the presence or absence of nuclei and membrane-encased organelles in their cells. Most prokaryotes consist of a single cell, which typical sizes and structures are smaller and simpler than eukaryotic cells (Madigan *et al*., 2008). Although invisible to the naked eyes, prokaryotes are an essential component of the earth's biota. They catalyze unique and indispensable transformations in the biochemical cycles of the biosphere, produce important components of the earth's atmosphere, and represent a large portion of life's genetic diversity (Whitman *et al*., 1998). To estimate the number and total carbon of prokaryotes on the earth, several representative habitats were first examined by Whitman *et al*. This analysis indicated that most of prokaryotes reside in three large habitats: seawater, soil, and oceanic and terrestrial subsurface sediments (Table 1-1). Although many other habitats contain dense populations, their numerical contribution to the total number of prokaryotes is small. Thus, evaluating the total number and total carbon of prokaryotes on the earth became a solvable problem. The number of prokaryotes and the total amount of their cellular carbon on earth were estimated to be $4\text{-}6\times10^{30}$ cells and 350-550 Pg of C (1 Pg = 10^{15} g), respectively (Table1-2). The total carbon of prokaryotes on earth is enormous, approximately 60-100% of the total carbon found in plants and phytoplankton (Table 1-2). These results indicated that total carbon of prokaryotes on earth represent approximately half of estimates of total carbon stored in living organisms. And also signifying that prokaryotes play an important role in global CO_2 fixation. In addition, the earth's

prokaryotes contain large amounts of N and P, 85-130 Pg of N and 9-14 Pg of P, respectively. In all plants, amount of N and P were estimated 10 Pg of N and 1.05 Pg of P, respectively. Thus, prokaryotes contain about 10-fold more of these nutrients than do plants. Other essential nutrients are probably distributed similarity. Therefore prokaryotes may represent the largest living pool for these nutrients on the earth (Whitman *et al*., 1998). These studies indicated that prokaryotes are an essential component of the earth's biota and we cannot disregard the presence of prokaryotes in considering the relationships between organisms and environmental factors in the every environmental habitat.

Prokaryotes had been considered to be consisted of one type of organism because their cells were typically single, smaller and simpler and also has little genetic diversity. However Woese and Fox revealed that Prokaryotes consisted of two phylogenetically distinct lineages, Bacteria and Archaea, based on comparing sequences of 16S ribosomal RNA gene as the genetic marker (Woese and Fox, 1977; Woese *et al*., 1990). Ribosomes are structures to synthesize proteins in the cell and consisted of two subunits, small subunit (30S ribosomal subumit in prokaryotes) and large subunit (50S ribosomal subunit in prokaryotes). These two subunits are composed of RNAs and proteins. The RNA composing of small subunit of ribosome (small subunit ribosomal RNA, SSU rRNA) is 16S rRNA in Prokaryotes and its counterpart in Eukaryotes, 18S rRNA gene. The gene encoding 16S rRNA has a length of approximately 1500 bases. These SSU rRNA genes have been most widely used in molecular phylogenetic analysis of microorganisms. There are mainly five advantages to use SSU rRNA genes: these genes are 1) universally distributed in Bacteria, Archaea, and Eukaryotes; 2) functionally constant; 3) sufficiently conserved (that is, slowly changing); 4) had adequate length and 5) had existed large and growing databases.

The phylogenetic and evolutionary relationships of all exant living organisms on the earth were revealed by comparing the sequences of SSU rRNA genes. The universal phylogenetic tree created by Woese *et al*. showed that the primary groups of all exant living organisms on the earth were classified mainly three lineages, Bacteria, Archaea, and Eukaryotes (Woese *et al*., 1990) (Fig. 1-1). The phylogenetic tree was also showed that the lineages of Prokaryotes consisted of Bacteria and Archaea were phylogenetically more widely distributed than that of Eukaryotes. Thus, it is suggested that the genetic diversity of Prokaryotes is higher than that of Eukaryotes.

1.2. Chemotrophic thermoacidophilic prokaryotes in the highly acidic hot springs

The biosphere in aquatic environments showing highly acidic hot springs is only composed of prokaryotic communities. It is reported that eukaryotic microorganisms such as Protozoa, Algae, and Funji have not been found over 62^{o}C (Madigan *et al*., 2008). Kato *et al*. also showed that eukaryotic 18S rRNA genes were not detected from acidic hot springs (28^{o}C, pH2.5 and 78^{o}C, pH3.5) in Ohwakudani, Japan (Kato *et al*., 2011). Additionally, photosynthetic prokaryotes are not known to occur at temperature higher than ≈75^{o}C (Brock, 1967; Brock, 1978; Jannasch, 1985; Jannasch *et al*., 1985; Ward *et al*., 1998; Spear *et al*., 2005; Madigan *et al*., 2008; Ward *et al*., 2012). Moreover, Cox *et al*. revealed the transition to microbial photosynthesis in terrestrial hot springs (Cox *et al*., 2011). They observed visible pigments, coloring green, orange, brown, yellow (in association with green) and/or purple involved in photosynthetic cells forming biofilms in 996 hot springs at Yellowstone National Park. As the results, nowhere pigmented biofilms observed to occur in an environment at a temperature higher than 74^{o}C, consistent with previous observations of above mentioned other reserchers. Besides, there appears to be a pH

dependence on the upper temperature of the transition to photosynthesis. The transition to photosynthesis occurred at low temperature with decreasing pH below ~6.5 (Fig. 1-2). As an example, no strong evidence for photosynthesis was found above 60^{o}C at pH~4.

Microorganisms whose growth temperature optimum exceeds 45^{o}C are called "thermophiles" and those whose optimum exceeds 80^{o}C are called hyperthoermophiles. And also, microorganisms that grow optimally at low pH, typically below pH6, are called "acidophiles" and whose optimum falls below pH 3.0 are called "extreme aidophiles". This definition excludes many fungi and yeasts which, although often tolerant of extreme acidity, have pH optima nearer to neutrality (Norris and Johnson, 1998; Johnson, 1998; Madigan *et al*., 2008). These informations suggest that the biosphere in terrestrial hot springs with highly acid and high temperature are only composed of chemotrophic thermoacidophilic prokaryotic communities.

1.3. The previous studies of prokaryotic communities, gene diversity and species diversity in the highly acidic hot springs

Hot spring prokaryotic microbial communities have been extensively studied based on the 16S rRNA gene phylogenetic analysis in areas such as Yellowstone National Park in the United States (Barns *et al*., 1994; Barns *et al*., 1996; Hugenholtz *et al*., 1998; Reysenbach *et al*., 2000; Jackson *et al*., 2001; Blank *et al*., 2002; Meyer-Dombard *et al*., 2005; Mathur *et al*., 2007; Tin *et al*., 2011), Lassen Volcanic National Park in the United States (Siering *et al*., 2006; Wilson *et al*., 2008; Tin *et al*., 2011), Kamchatka hot springs in Russia (Perevalova *et al*., 2008; Reigstad *et al*., 2010; Tin *et al*., 2011; Mardanov *et al*., 2011; Burgess *et al*., 2012), Montserrat and Saint Lucia in the islands of the Lesser Antilles (Burton and Norris, 2000; Stout *et al*., 2009), Icelandic hot springs (Slirnisdottir *et al*., 2000; Perevalova *et al*, 2008; Kvist *et al*., 2007; Reigstad *et al*.,

2010), Mt. Unzen hot springs in Japan (Takai and Sako, 1999), Ohwakudani hot springs in Japan (Kato *et al*., 2011), Pisciarelli hot springs in Italy (Kvist *et al*., 2005), Bor Khlueng hot springs in Thailand (Kanokratana *et al*., 2004), the hot springs in northern Thailand (Purcell *et al*., 2006), the hot springs of White Island in New Zealand (Donachie *et al*., 2002) the Wai-o-tapu geothermal area in New Zealand (Childs *et al*., 2008), the Tengchong hot springs in China (Song *et al*., 2010), and the hot springs on the Tibetan Plateau, China. Although these pioneering studies have improved our understanding of prokaryotic communities living in high temperature environments, little is known about both bacterial and archaeal communities in especially highly acidic hot springs.

Here, I intoduce some examples indicating community strucrutes and the diversity index of bacteria and archaea derived from highly acidic hot springs showing above 60^{o}C at pH ~ 4.

Bacterial community structures and species diversity were investigated in 12 highly acidic hot springs distributed in Yellowstone National Park (Mathur *et al*., 2007). The temperature and pH of these hot springs were 60-70^{o}C and 1.0-1.2, respectively. The bacterial species diversity assessed by Shannon-Weaver index ranged from 0.83 to 3.67. The four samples with iron-rich deposites were dominated by rRNA gene sequences of uncultured bacteria related to gram-positive iron-oxidizing acidophiles despite a range of temperature. The mixed iron-sulfur sample was dominated by uncultured bacteria related to *Sulfobacillus disulfooxidans* affiliating to the class Clostridia. The seven samples with sulfur-rich deposites were dominated by *Hydrogenobaculum* spp. affiliating to the class Aquificae despite a range of temperature. The 16S rRNA clone analyses indicated that the class Clostridia was the most dominant bacteria in the highly acidic hot springs (78^{o}C, pH 1.5) of Montserrat in the islands of the Lesser Antilles (Burton and Norris,

2000). And also, the order Thermoplasmatales and Sulfolobales were the most dominat archaea in the highly acidic hot springs (78^{o}C, pH 1.5 and 98^{o}C, pH 3.0) of the same area.

1.4. The effects of temperature and chemical components on prokaryotic communities, gene diversity and species diversity

The activities of prokaryotes are greatly affected by the chemical and physical state of their environment. Many environmental factors can be considered. However, key environmental factors control the growth of all prokaryotes: temperature, pH, water availability, oxygen, and chemical components. Some other environmental factors also can potentially affect the growth of prokaryotes, such as pressure, oxidation redox potential, organic matter composition, and trophic relationships and so on (Madigan *et al*., 2008). Therefore, it could be considered that prokaryotic gene diversity, species diversity and community structures are also affected by the key environmental factors. However, there is little known about how temperature and chemical components especially dissolved element affect to bacterial and archaeal biodiversities and community structures.

Huang *et al*. studied that how bacterial and archaeal species diversity assessed by Shannon-Weaver index response to thermal stress in ten hot springs with a wide temperature range on the Tibetan Plateau, China (Huang *et al*., 2011). The temperature and pH of these hot springs were 26-81^{o}C and close to neutral, respectively. Statistical analysis showed that temperature was not significantly correlated with both bacterial and archaeal species diversity. However, the value of coverage was low because of the insufficient numbers of analyzed clones in this study. Mathur et al. also showed that bacterial genetic diversity derived from 12 highly acidic hot springs (60-75^{o}C, pH 1.02-1.23) in Yellowstone National Park, USA was not

significantly correlated with temperature (Mathur *et al*., 2007). They also focused about how mineral chemistry affected to bacterial gene diversity. Principal component analysis found that bacterial genetic diversity was most attributable to the variance of mineral chemistry. For example, sulfur-rich sediment samples contained a high diversity of uncultured bacteria related to *Hydrogenobaculum* spp., while iron-rich sediments were dominated by uncultured bacteria related to a diverse array of gram-positive iron oxidizers. Regrettably, nothing had been studied about archaea in this study. On the other hand, although only targeting on the phylum Crenarchaeota, Song *et al*. studied crenarchaeotal diversity in eight hot springs located in Tengchong, China (Song *et al*., 2010). The ranges of temperature and pH of these hot springs were 44-96^{o}C and 2.8-7.7, respectively. It was clearly showed that the *F*st values which assess the digree of differentiation between communities were linearly correlated with the temperature distances (R^2 = 0.55). However, pH calues do not have an obvious liner correlation with *F*st. Therefore, temperature may predominate over pH in affecting crenarchaeotal genetic diversity among Tengchong hot springs. Therefore, the complehensive study that surveying and comparing the both bacterial and archaeal communies of distinct highly acidic hot springs displaying a wide range of environmental factors in a restricted field of geothermal area have not been described. Futhermore there was no report that quantitatively assessed the species diversity and the gene diversity of bacterial and archaeal communities in the highly acidic hot springs.

1.5. The Kirishima geothermal area

The Kirishima geothermal area was selected as a study area in this study. The general spatical distribution of acidic groundwater around the Quaternary volcanoes in Japan was examined using a database of 9300 groundwater geochemistry (Asamori *et al*., 2002). The results

showed that acidic groundwater with pH < 4.0 occurred in the Kirishima geothermal area and are distributed to about 10 km from the Kirishima volcano. Kirishima volcano, which is one of the largest Quaterary volcanoes in Japan, is located in the border between Kagoshima and Miyazaki prefectures of southern Kyushu. And also, this volcano is part of the northern section of the Kagoshima graben, a volcano-tectonic depression caused by the subduction of the Philippine Sea plate (Tsuyuki, 1969). The volcano occupies a 20 km × 30 km area that is elongated in the nowthwest-to-southeast direction and contains more than 20 small volcanoes (Imura *et al*., 2001). The Kirishima geothermal region surrounding the Kirishima volcano has been characterized by extensive volcanic activity since the Pleistocene epoch and this is continuing; this activity has resulted in the deposition of thick pile of volcanic rocks (Goko, 2000).

1.6. Aims and scopes of this study

In this dissertation, the aims were to reveal and compare community structures, gene diversity and species diversity of bacteria and archaea based on 16S rRNA gene phylogenetic analysis among multiple distinct highly acidic hot springs displaying a wide range of temperature and chemical copositions in the Kirishima geothermal area. Moreover, I intended to reveal the correlations among bacterial and archaeal community structures, gene diversity, species diversity, temperature, and chemical components.

All organisms present in an ecosystem like highly acidic hot spring could be analyzed and assessed since the biosphere in the ecosystem is only composed of prokaryotic communities in this study. In addition, the effects of temperature and chemical components on bacterial and archaeal gene diversity, species diversity and communities could be revealed in this study.

1.7. Organization of the thesis

For the sake of simplicity, this thesis including this general introduction has been divided into five chapters. In this chapter, the abbreviations throughout the thesis have been explained on the table. In the second chapter, as the first step of this study, the geothermal area possessing highly acidic hot springs was selected and these ponds were statistically classified based on the environmental factors valiability. In the third chapter, bacterial community structures, gene diversity and species diversity in the highly acidic hot springs displaying a wide range of temperature and chemical components chosen in the second chapter were assessed and compared. In the forth chapter, archaeal community structures, gene diversity and species diversity in above mentioned highly acidic hot springs were assessed and compared. In the fifth chapter, prokaryotic gene diversity and species diversity in each highly acidic hot spring were summarized and overall prokaryotic gene diversity and species diversity in a geothermal area were assessed.

Pg	Peta gram
DNA	Deoxyribonucleic acid
RNA	Ribonucleic Acid
rRNA	ribosomal RNA
S	Svedberg coefficient
PCR	Polymerase chain reaction
Bp	base pair
Kb	kilobase
IPTG	Isopropylthio-β-D-galactoside
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactoside
Amp	Ampicillin
EDTA	Ethylene diamine tetra acetic acid
TE	Tris-EDTA
TAE	Tris-acetate-EDTA
RFLP	Restriction fragment length polymorphism
BLAST	Basic local alignment search tool
LB medium	Luria-Bertani medium
C	Carbon
N	Nitrogen
Fe	Iron
S	Sulfur
Al	Aluminum
Mg	Magnesium
Si	Silicon
Ca	Calcium
P	Phosphorus
Na	Sodium
K	Potassium
As	Arsenic
Li	Lithium
Cr	Chromium
Mn	Manganese
Ni	Nickel
Cu	Copper
Zn	Zinc
Rb	Rubidium
Sr	Strontium
Cd	Cadmium
Cs	Cesium
Pb	Lead
CO_2	Carbon dioxide
Phylotype	Phylogenetic type

Table 1-1. The number and amount of cellular carbon of prokaryotes in major three habitats

Ecosystem	Aquatic habitats		Soil		Unconsolidated subsurface sediments	
	Number of cells, $\times 10^{28}$	Amount of carbon, Pg	Number of cells, $\times 10^{28}$	Amount of carbon, Pg	Number of cells, $\times 10^{28}$	Amount of carbon, Pg
Terrestrial	0.023	0.0042	26	26	25-250	22-215
Marine	12	2.2	—	—	355	303
Total	12	2.2	26	26	380-605	325-518

Cited from Falkowski *et al.*, 1998; Whitman *et al.*, 1998

1 Pg = 10^{15} g

Aquatic habitats were divided into terrestrial fresh water ecosystem and marine water ecosystem. Terrestrial fresh water ecosystem was composed of lakes, rivers and saline lakes. Marine water ecosystem was composed of continental shelf and open ocean water which was represented by upper 200 m and below 200m water and 0-10 cm sediment from the surface. The upper 10 cm of sediment in the open ocean is included in the oceanic habitat because, as a result of animal mixing and precipitation, it is essentially contiguous with the overlying water column.

Soil habitats divided into forest soils and other soils. Forest soils were composed of tropical rain forest, tropical seasonal forest, temperature evergreen forest, temperate deciduous forest and boreal forest. Other soils were composed of woodland & shrubland, savanna, temperate grassland, desert scrub, cultivated land, tundra & alpine and swamps & marsh.

The subsurface is defined here as marine sediments below 10 cm and terrestrial habitats below 8 m. Unconsolidated sediments represent most of the marine subsurface and about 20% of the terrestrial subsurface. Marine subsurface sediments were composed of deep oceans (0.1-400 m below from the surface) and continental shelf & slope (0.1-3000 m below from the surface). Terrestrial subsurface sediments were composed of coastal plains (8-4000 m below from the surface). At 4 km, the average temperature reaches 125 ^{o}C, which is close to the upper temperature limit for prokaryotic life.

Table 1-2. Relationship between prokaryotes and plant biomass to primary productively

Ecosystem	Net primary productivity, Pg of C/yr	Total carbon content, Pg of C	
		Prokaryotes	Plant and phytoplankton
Terrestrial	48	241	560
Marine	51	305.2	1-1.8
Total	99	353-546	560-561.8

Cited from Falkowski *et al.*, 1998; Whitman *et al.*, 1998

1 Pg = 10^{15} g

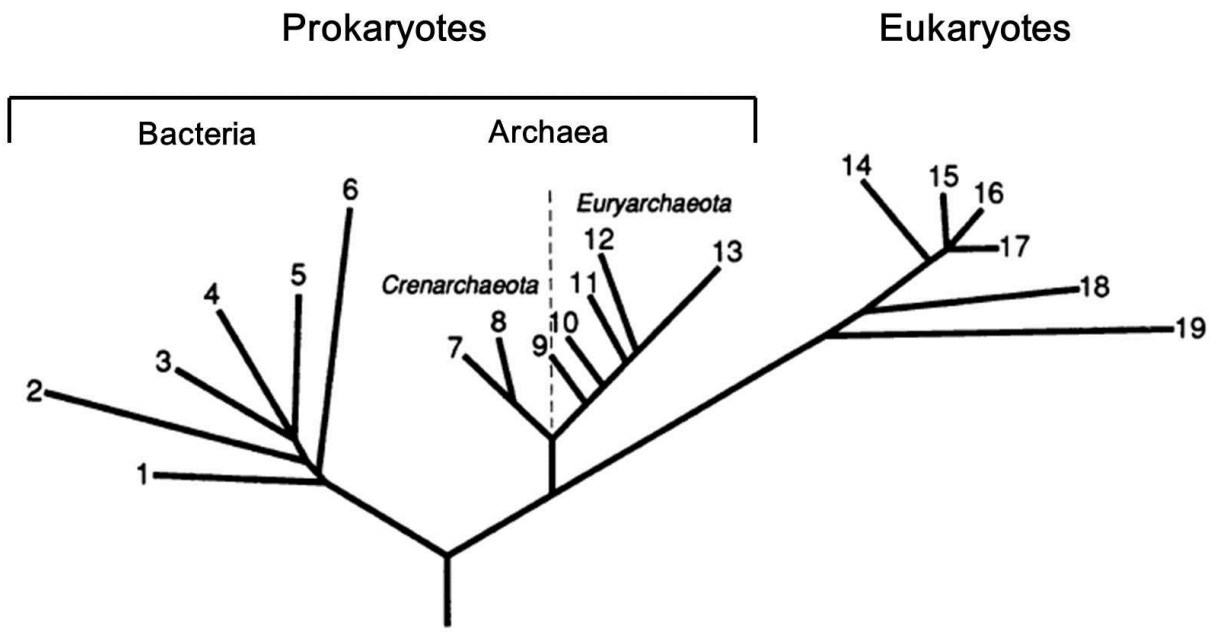

Fig. 1-1. Universal phylogenetic tree showing the domains of the Bacteria, the Archaea, and the Eukaryotes, cited from Woese *et al.* (1990). Branching order and branch lengths are based on SSU rRNA gene sequence comparisons. The position of the root was determined by comparing (the few known) sequences of pairs of paralogous genes that diverged from each other before the three primary lineages emerged from their common ancestral condition (Iwabe et al., 1989). The numbers on the branch tips correspond to the following groups of organisms. Bacteria: 1. Thermotogales; 2. flavobacteria and relatives; 3. cyanobacteria; 4. purple bacteria; 5. Gram-positive bacteria, and 6. green nonsulfur bacteria. Archaea: the phylum Crenarchaeota: 7. the genus Pyrodictium; and 8. the genus Thermoproteus; and the phylum Euryarchaeota: 9. Thermococcales; 10. Methanococcales; 11. Methanobacteriales; 12. Methanomicrobiales; and 13. the extreme halophiles. Eukaryotes: 14. the animals; 15. the ciliates; 16. the green plants; 17. the fungi; 18. the flagellates; and 19. the microsporidia. The names of these organisms were described as the time of publication.

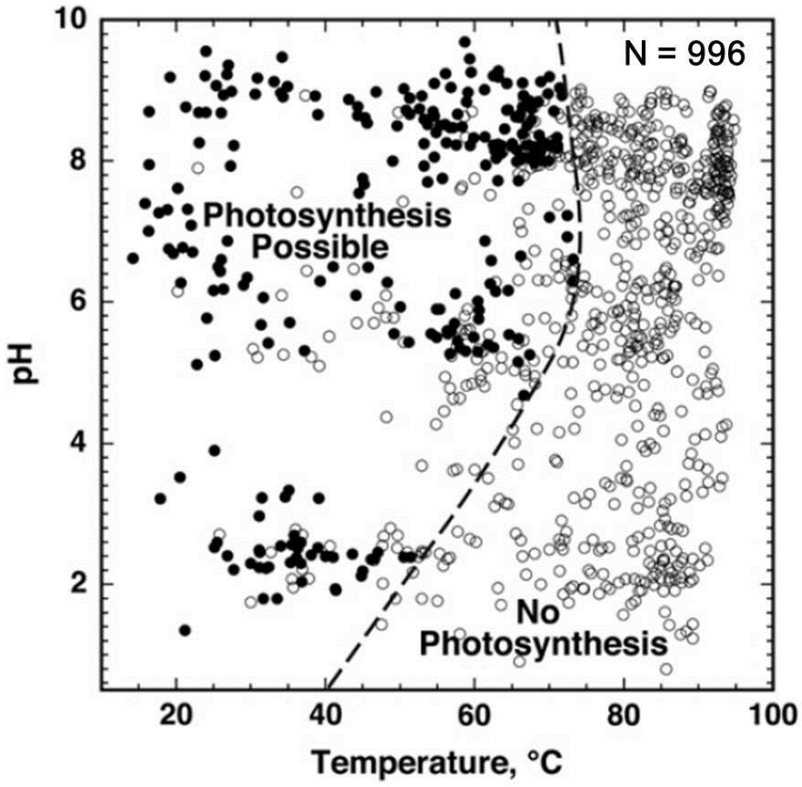

Figure 1-2. The transition to microbial photosynthesis in hot springs at Yellowstone National Park relative to pH and temperature, cited from Cox *et al*. (2011). Filled symbols show sample locations with visible photosynthetic pigments. Open symbols indicate locations with no visible pigments. The curve is drawn to incrude all the sites with photosynthetic pigments, and indicates the location of the photosynthetic fringe in terms of pH and temperature.

Chapter II

Chemical characteristics of ponds in the Kirishima geothermal area

2.1. Introduction

Terrestrial geothermal areas are located in various regions of our planet, most particulary around the borders of tectonic plates and in areas where the Earth's crust is relatively thin. In these cases, thermal springs, fumaroles and geysers may be commonplace. Solfatara fields may also be present; these are acidic hot springs, mud ponds and soils whose low pH derives from the oxidation of sulfur, formed by the condensation of volcanic gases (H_2S, SO_2 and HCl). The elevated temperatures and extreme acidity in solfatara fields accelerate the dissolution of minerals, so that these environments may also contain elevated concentrations of soluble metals and silica (Johnson *et al*., 2003).

The Japanese Islands lie at the junction of four major tectonic plates – the Pacific and the Philippine Sea oceanic plates and the North American and the Eurasian continental plates. The Pasific plate is subducted beneath the Kuril Arc and the Izu-Ogasawara Arc, where is called Kuril Trench, Japan Trench, and Izu-Ogasawara Trench. On the other hand, the Philippine Sea plate is subducting beneath Southwest Japan Arc and the Ryukyu Arc, where is called Nankai trough and Ryukyu Trench. The Quaternary volcanoes lie parallel to these trenches and form a "volcanic front" (NUMO, 2004). Volcanic geothermal areas exist around the Quaternary volcanoes and hot springs are distributed in these areas. The general spatical distribution of acidic groundwater around the Quaternary volcanoes in Japan was examined using a database of 9300 groundwater geochemistry (Asamori *et al*., 2002). The results showed that acidic groundwater with pH < 4.8 mainly occur in volcanic regions and are distributed from several kilomaters to about 10 km from

the Quaternary volcanoes. The pH value of groundwater tends to increase with increasing distance from a volcano. One geothermal area where highly acidic hot springs are distributed in Japan was selected based on these informations.

Kirishima volcano, which is one of the largest Quaterary volcanoes in Japan, is located in the border between Kagoshima and Miyazaki prefectures of southern Kyushu. And also, this volcano is part of the northern section of the Kagoshima graben, a volcano-tectonic depression caused by the subduction of the Philippine Sea plate (Tsuyuki, 1969). The volcano occupies a 20 km × 30 km area that is elongated in the nowthwest-to-southeast direction and contains more than 20 small volcanoes (Imura *et al*., 2001). The Kirishima geothermal region surrounding the Kirishima volcano has been characterized by extensive volcanic activity since the Pleistocene epoch and this is continuing; this activity has resulted in the deposition of thick pile of volcanic rocks (Goko, 2000).

This dissertation primary intended to reveal the prokaryotic community structures and biodiversity of distinct highly-acidic hot ponds displaying a wide range of the environmental factors in a restricted field of geothermal area. Therefore, as the first step of this study, a number of ponds located in a 1 km^2 field within the Kirishima geothermal area were ramdomly selected and the temperature, pH and chemical components of pond waters were measured as the representative of environmental factors. These results were statistically analyzed in order to classify the ponds based on the environmental factors variability in this chapter 2.

2.2. Materials and methods

2.2.1. Study area and sample collection

The ponds investigated in this study were all located in a 1 km^2 field in/near the region of the Tearai hot spring (Tsuyuki, 1980). This district is situated 3 km southwest of the Ohnami-Ike volcanic crater lake, in the Kirishima geothermal area, Kagoshima Prefecture (Fig. 2-1). The altitude in this area generally ranged from 800-1000 m (Goko, 2000). The Sampling location within the Kirishima geothermal area is located on private land, and thus, the area is not usually exposed to human activity. We obtained permission from an owner of the land to sample the hot springs and pond water as well as soil and various other samples of organisms native to the area. There are many hot springs and muddy ponds with several colors of sediments and waters present in the area. The sampling was conducted in July 2005, February and June 2006. Elemental sulfur is deposited around many fumaroles in this field. Surface muddy water samples including sediments from each pond were collected in sterile 100 mL glass bottles. The temperature and pH of the samples were measured at each sampling site. Samples were brought back to the laboratory and part of each sample was filtered using a 0.22 μm membrane filter (Asahi Glass). Water samples were stored at 4^oC after acid treatment for analysis of chemical component. Muddy water samples were stored at -25^oC for the prokaryotic community and biodiversity analysis.

2.2.2. Analysis of chemical components

The chemical components of pond waters in the Kirishima geothermal area were analysed by inductively coupled plasma optical emission spectroscopy (ICPS-7000 Ver.2, Shimadzu). The 12 elements (Fe, S, Al, Mg, Si, Ca, P, Na, K, As, Rb, and Cs) were chosen as the

major elements within the 72 elements that were preliminary surveyed by ICP analysis.

2.2.3. Statistical analysis

To clarify the relationships among temperature, pH, each chemical component, and total concentrations of examined chemical components of the 21 ponds in the Kirishima geothermal area, Pearson's correlation coefficients (*r*) were calculated. Principal component analysis was performed to reveal the environmental variables (Temperature and each chemical component) that most affecting to the difference of ponds using XLSTAT software (Addinsoft, New York, NY).

2.3. Results and Disccussion

2.3.1. Water chemistry

The sampling was conducted at 21 potisions of the Kirishima geothermal area (Table 2-1 and Fig. 2-2). The altitude of 21 ponds located in a 1 km^2 field ranged from 759-896 m. The colors of ponds were gray or brown and different by ponds. Temperature, pH and chemical components of 21 pond waters were shown in Table 2-2. Temperature ranged from 63-94^oC and average value was 83^oC. The pH value of the ponds ranged from 1.8-4.0 and average value was 2.5. Therefore ponds located in this field showed highly acidic and hot. Major elements showing over 1% of total concentrations of examined chemical components in all pond waters were S and Si (Table 2-3). And also, the element showing the lowest values of the coefficients of variation was Si (Table 2-2). Total concentrations of examined chemical components ranged from 3.1-82 mmol L^{-1} and average value was 26 mmol L^{-1}. The total concentrations of examined chemical components were plotted on the temperatures of 21 pond waters in Figure 2-3. A number of pond waters were plotted on reratively lower total concentrations of examined chemical components less than 20 mmol L^{-1} despite a range of temperature. The pond waters of seven sites were plotted on reratively higher total concentrations of examined chemical components over 30 mmol L^{-1}. Among them, the only one pond (St. 2) was characterized by a lower temperature and a higher total concentration of examined chemical components. The 21 ponds located in a 1 km^2 field within the Kirishima geothermal area were widely distributed based on the temperature and total concentrations of examined chemical components.

2.3.2. Correlation of the environmental variables

To clarify the relationships among temperature, pH, each chemical component, and

total concentrations of examined chemical components of the 21 pond waters in the Kirishima geothermal area, Pearson's correlation coefficients (*r*) were calculated (Table 2-4). Several chemical components were found to be statistically correlated with each other. For example, Fe was strongly and positively correlated with S, Al, P, As, Rb, and Cs with a significance level α= 0.10. The total concentration of the examined chemical components was also strongly and positively correlated with Fe, S, Al, Mg, P, As, Rb, and Cs. The pH was strongly and positively correlated with Ca and Na, and negatively correated with P. Temperature was not statistically correlated with any of the chemical components.

The relationships among temperature, pH, each chemical component/ Si ratio, and total concentrations of examined chemical components/ Si of the 21 pond waters in the Kirishima geothermal area, Pearson's correlation coefficients (*r*) were showed (Table 2-5). Temperature was strongly and positively correlated with Al/Si, Mg/Si, and Cs/Si ratio. The correlations among chemical components correspond approximately to the results that were not standardized in silicon. The trends of relationships among the environmental variables of the ponds in the Kirishima geothermal area were revealed in this study.

2.3.3. Principal component analysis

The principal component analysis was performed to distinguish the 21 ponds based on environmental variables (Fig. 2-4). The some variables characterizing the sites were explained by three principal factors: Factor 1 (F1) 47%, Factor 2 (F2) 15% and Factor 3 (F3) 13% (Table 2-6). F1 was strongly loaded by Fe, S, Al, Mg, P, As, Rb, and Cs (positively); F2 was strongly loaded by Fe, Mg, Si, Ca, Na, and K (positively); F3 was strongly loaded by Temperature and Ca (positively), and Si and K (negatively) (Table 2-7). In this study, we focused on the principal

components 1 and 3 (PC1 and PC3), which affected F1 and F3, respectively. Strong contributions to PC1 were made by Fe, S, Al, Mg, P, As, Rb, and Cs and weakly contributions were Si, Ca, Na and K. On the other hand, Temperature, Si, Ca and K strongly contributed to PC3 (Fig. 2-4a). The 21 ponds located in a 1 km^2 field within the Kirishima geothermal area were classified into four types based on the two principal components, PC1 and PC3 (Fig. 2-4b).

Table 2-1. Locations and sediment colors of the ponds in the Kirishima geothermal area

Station	Position		Altitude (m)	Color of sediments
	Latitude	Longitude		
1	31°54'52.60"N	130°48'50.20"E	844	light gray
2	31°54'52.40"N	130°48'50.30"E	842	brown gray
3	31°54'52.10"N	130°48'49.90"E	839	gray
4	31°54'52.90"N	130°48'50.30"E	847	light gray
5	31°54’37.90"N	130°49'15.70"E	859	brown gray
6	31°54'33.70″N	130°48'57.00"E	761	brown
7	31°54'37.70"N	130°49'00.60"E	759	brown
8	31°54'37.70"N	130°49'00.60"E	759	brown gray
9	31°54'37.70"N	130°49'00.60"E	759	gray
10	31°54'37.80"N	130°49'00.20"E	759	light gray
13	31°54'36.70"N	130°48'59.90"E	757	light gray
14	31°55'05.10"N	130°48'42.30"E	892	light gray
15	31°55'04.50"N	130°48'41.00"E	885	gray
16	31°55'05.00"N	130°48'41.10"E	884	light gray
17	31°55'04.80"N	130°48'40.90"E	884	light gray
18	31°55'05.10"N	130°48'41.20"E	885	light gray
19	31°55'05.20"N	130°48'40.80"E	884	gray
20	31°55'05.30"N	130°48'41.80"E	889	light gray
21	31°55'06.10"N	130°48'42.70"E	896	gray
22	31°55'05.80"N	130°48'42.50"E	894	gray
23	31°55'05.80"N	130°48'42.20"E	892	light gray

Table 2-2. Temperature, pH and chemical components of 21 pond waters in the Kirishima geothermal area

Station	Temp.	pH	Fe	S	Al	Mg	Si	Ca	P	Na	K	As	Rb	Cs	Total conc.
1	80	2.8	1173	3019	4331	0.041	2022	63	59	0.043	0.026	14	647	934	12261
2	66	2.0	20566	21913	10672	1924	1620	272	152	0.043	0.026	15	14052	2223	73410
3	91	2.5	10138	20501	2387	0.041	1241	153	132	0.043	0.026	12	6169	219	40952
4	90	2.2	4895	13350	8562	917	1585	218	107	43	0.026	13	2796	1773	34260
5	93	4.0	0.018	3944	866	1459	646	5950	41	569	0.026	11	47	401	13933
6	69	2.8	658	2617	699	0.041	2716	62	58	1213	0.026	13	371	156	8562
7	94	2.9	1012	4866	3338	1975	1316	2698	44	227	0.026	12	571	775	16833
8	93	2.6	6964	20681	16071	3568	1705	1367	92	0.043	0.026	14	4131	3341	57934
9	91	2.2	3569	34395	22388	3662	2129	1336	144	39	0.026	16	9950	4776	82403
10	94	1.9	4173	19367	15270	2497	1633	516	229	0.043	0.026	14	2407	3224	49329
13	88	2.4	4262	27228	18038	4808	3847	3704	75	326	0.026	15	2585	3908	68794
14	90	2.5	1608	2782	3921	0.041	1961	177	53	0.043	0.026	15	846	840	12201
15	67	2.3	487	1930	75	1783	5285	980	41	367	189	11	288	47	11482
16	88	2.4	172	1863	540	0.041	3700	187	41	0.043	0.026	12	92	118	6725
17	63	2.3	271	2166	945	757	4358	665	43	203	5.1	12	152	208	9784
18	94	2.4	490	2215	2358	0.041	4047	183	46	0.043	0.026	12	265	504	10119
19	93	1.8	279	5991	1994	5.3	4133	261	43	0.043	0.026	12	157	422	13296
20	82	2.3	73	1804	313	0.041	1161	131	40	0.043	0.026	12	16	70	3619
21	78	2.4	26	1541	264	0.041	1049	57	39	0.043	0.026	11	21	59	3067
22	75	2.3	172	1623	747	0.041	2143	235	41	0.043	0.026	12	109	167	5248
23	65	2.5	49	1523	307	0.041	1086	77	40	0.043	0.026	12	32	68	3191
mean	83	2.5	2906	9301	5433	1112	2351	919	74	142	9.3	13	2176	1154	25591
SD	11	0.45	4875	10285	6889	1481	1327	1490	51	292	41	1.5	3703	1461	25751
VC	0.14	0.18	1.7	1.1	1.3	1.3	0.56	1.6	0.69	2.1	4.4	0.12	1.7	1.3	1.0

Temperature is expressed in ^{o}C. The concentrations of each chemical component are expressed in μmol L^{-1}. The concentrations of each chemical component were the mean of values measured three times. The detection limit is 0.001 mg L^{-1}. Temp., Total conc., SD, and CV indicate Temperature, total concentration of examined chemical components, standard deviation, and coefficient of variation.

Table 2-3. Temperature, pH and the percentages of chemical components of 21 pond waters in the Kirishima geothermal area

Station	Temp.	pH	Fe	S	Al	Mg	Si	Ca	P	Na	K	As	Rb	Cs
1	80	2.8	9.6	25	35	0.000	16	0.51	0.48	0.000	0.000	0.11	5.3	7.6
2	66	2.0	28	30	15	2.6	2.2	0.37	0.21	0.000	0.000	0.021	19	3.0
3	91	2.5	25	50	5.8	0.000	3.0	0.37	0.32	0.000	0.000	0.029	15	0.54
4	90	2.2	14	39	25	2.7	4.6	0.64	0.31	0.13	0.000	0.038	8.2	5.2
5	93	4.0	0.000	28	6.2	10	4.6	43	0.29	4.1	0.000	0.081	0.34	2.9
6	69	2.8	7.7	31	8.2	0.000	32	0.72	0.67	14	0.000	0.16	4.3	1.8
7	94	2.9	6.0	29	20	12	7.8	16	0.26	1.3	0.000	0.069	3.4	4.6
8	93	2.6	12	36	28	6.2	2.9	2.4	0.16	0.000	0.000	0.025	7.1	5.8
9	91	2.2	4.3	42	27	4.4	2.6	1.6	0.18	0.048	0.000	0.019	12	5.8
10	94	1.9	8.5	39	31	5.1	3.3	1.0	0.46	0.000	0.000	0.029	4.9	6.5
13	88	2.4	6.2	40	26	7.0	5.6	5.4	0.11	0.47	0.000	0.021	3.8	5.7
14	90	2.5	13	23	32	0.000	16	1.4	0.43	0.000	0.000	0.12	6.9	6.9
15	67	2.3	4.2	17	0.65	16	46	8.5	0.36	3.2	1.6	0.10	2.5	0.41
16	88	2.4	2.6	28	8.0	0.001	55	2.8	0.61	0.001	0.000	0.18	1.4	1.8
17	63	2.3	2.8	22	9.7	7.7	45	6.8	0.44	2.1	0.052	0.12	1.6	2.1
18	94	2.4	4.8	22	23	0.000	40	1.8	0.45	0.000	0.000	0.12	2.6	5.0
19	93	1.8	2.1	45	15	0.040	31	2.0	0.32	0.000	0.000	0.089	1.2	3.2
20	82	2.3	2.0	50	8.7	0.001	32	3.6	1.1	0.001	0.001	0.32	0.44	1.9
21	78	2.4	0.83	50	8.6	0.001	34	1.9	1.3	0.001	0.001	0.36	0.68	1.9
22	75	2.3	3.3	31	14	0.001	41	4.5	0.78	0.001	0.000	0.22	2.1	3.2
23	65	2.5	1.5	48	9.6	0.001	34	2.4	1.2	0.001	0.001	0.36	0.99	2.1
mean	83	2.5	7.6	34	17	3.5	22	5.1	0.50	1.2	0.081	0.12	4.9	3.7
SD	11	0.45	7.5	10	10	4.7	18	9.4	0.34	3.2	0.36	0.11	5.1	2.
VC	0.14	0.18	0.99	0.30	0.60	1.3	0.82	1.8	0.69	2.6	4.4	0.89	1.0	0.58

Temperature is expressed in ^{o}C. The percentages of each chemical component are expressed in %. The concentrations of each chemical component were the mean of values measured three times. The detection limit is 0.001 mg L^{-1}. Temp., SD, and CV indicate Temperature, standard deviation, and coefficient of variation.

Table 2-4. Correlation matrix showing *r* values for Pearson's correlation among 15 environmental variables in the pond waters of the Kirishima geothermal area; N=21

Variables	Temp.	pH	Fe	S	Al	Mg	Si	Ca	P	Na	K	As	Rb	Cs	Total conc.
Temp.	—	0.13	-0.06	0.32	0.36	0.25	-0.19	0.32	0.24	-0.25	-0.34	0.15	-0.01	0.36	0.25
pH	—	—	-0.27	-0.27	-0.27	-0.03	-0.35	**0.69**	**-0.38**	**0.49**	-0.08	-0.25	-0.29	-0.24	-0.26
Fe	—	—	—	**0.64**	**0.45**	0.33	-0.24	-0.08	**0.64**	-0.20	-0.12	**0.55**	**0.89**	**0.42**	**0.72**
S	—	—	—	—	**0.91**	**0.78**	-0.13	0.20	**0.76**	-0.16	-0.17	**0.74**	**0.79**	**0.90**	**0.98**
Al	—	—	—	—	—	**0.85**	-0.08	0.21	**0.70**	-0.17	-0.18	**0.83**	**0.63**	**1.00**	**0.92**
Mg	—	—	—	—	—	—	0.06	**0.55**	**0.46**	0.06	0.10	**0.59**	**0.46**	**0.87**	**0.81**
Si	—	—	—	—	—	—	—	-0.12	-0.27	0.15	**0.52**	-0.07	-0.22	-0.08	-0.10
Ca	—	—	—	—	—	—	—	—	-0.09	0.35	0.01	-0.01	-0.04	0.25	0.22
P	—	—	—	—	—	—	—	—	—	-0.20	-0.15	**0.63**	**0.69**	**0.68**	**0.76**
Na	—	—	—	—	—	—	—	—	—	—	0.18	-0.03	-0.20	-0.15	-0.14
K	—	—	—	—	—	—	—	—	—	—	—	-0.21	-0.12	-0.18	-0.13
As	—	—	—	—	—	—	—	—	—	—	—	—	**0.68**	**0.83**	**0.80**
Rb	—	—	—	—	—	—	—	—	—	—	—	—	—	**0.61**	**0.84**
Cs	—	—	—	—	—	—	—	—	—	—	—	—	—	—	**0.91**
Total conc.	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—

Values in bold are different from 0 with a significance level $\alpha = 0.10$ ($r > 0.37$). Temp. and Total conc. indicate Temperature and total concentration of examined chemical components, respectively.

Table 2-5. Correlation matrix showing *r* values for Pearson's correlation among 14 environmental variables in the pond waters of the Kirishima geothermal area; N=21

Variables	Temp.	pH	Fe/Si	S/Si	Al/Si	Mg/Si	Ca/Si	P/Si	Na/Si	K/Si	As/Si	Rb/Si	Cs/Si	Total conc./Si
Temp.	—	0.13	-0.05	0.34	**0.37**	**0.40**	0.28	0.27	0.07	-0.34	0.18	-0.02	**0.38**	0.29
pH	—	—	-0.24	-0.12	-0.23	0.33	**0.82**	-0.09	**0.83**	-0.08	**0.56**	-0.27	-0.15	-0.06
Fe/Si	—	—	—	**0.74**	**0.47**	0.25	-0.12	**0.66**	-0.19	-0.12	0.23	**0.93**	**0.42**	**0.79**
S/Si	—	—	—	—	**0.82**	**0.62**	0.10	**0.83**	-0.06	-0.20	0.36	**0.83**	**0.79**	**0.97**
Al/Si	—	—	—	—	—	**0.73**	-0.01	**0.66**	-0.17	-0.19	0.19	**0.61**	**0.99**	**0.85**
Mg/Si	—	—	—	—	—	—	**0.61**	**0.48**	**0.41**	-0.08	**0.46**	0.35	**0.79**	**0.71**
Ca/Si	—	—	—	—	—	—	—	0.14	**0.87**	-0.06	**0.64**	-0.10	0.10	0.18
P/Si	—	—	—	—	—	—	—	—	0.01	-0.23	**0.56**	**0.65**	**0.64**	**0.81**
Na/Si	—	—	—	—	—	—	—	—	—	-0.02	**0.48**	-0.19	-0.07	0.02
K/Si	—	—	—	—	—	—	—	—	—	—	-0.32	-0.13	-0.19	-0.19
As/Si	—	—	—	—	—	—	—	—	—	—	—	0.22	0.24	**0.41**
Rb/Si	—	—	—	—	—	—	—	—	—	—	—	—	**0.56**	**0.86**
Cs/Si	—	—	—	—	—	—	—	—	—	—	—	—	—	**0.84**
Total conc./Si	—	—	—	—	—	—	—	—	—	—	—	—	—	—

Values in bold are different from 0 with a significance level $\alpha = 0.10$ ($r > 0.37$). Temp. and Total conc. indicate Temperature and total concentration of examined chemical components, respectively.

Table 2-6. The factor analysis of 13 environmental variables in the 21 ponds of the Kirishima geothermal area

Factor	Eigenvalue	Total variance (%)	Cumulative eigenvalue	Cumulative variance (%)
Factor 1	6.09	46.82	6.09	46.82
Factor 2	1.94	14.89	8.03	61.71
Factor 3	1.70	13.05	9.73	74.76
Factor 4	1.11	8.52	10.84	83.27
Factor 5	0.74	5.70	11.58	88.97
Factor 6	0.48	3.66	12.06	92.63
Factor 7	0.44	3.36	12.50	95.99
Factor 8	0.24	1.86	12.74	97.85
Factor 9	0.14	1.10	12.88	98.94
Factor 10	0.09	0.70	12.97	99.65
Factor 11	0.03	0.26	13.00	99.91
Factor 12	0.01	0.09	13.01	99.99
Factor 13	0.00	0.00	13.01	100

Table 2-7. Factor loadings for 13 environmental variables in the 21 ponds of the Kirishima geothermal area

Variables	Factor 1	Factor 2	Factor 3	Factor 4	Factor 5	Factor 6	Factor 7	Factor 8	Factor 9	Factor 10	Factor 11	Factor 12	Factor 13
Temperature	0.33	0.14	**0.72**	-0.36	0.12	0.35	0.23	0.16	0.04	0.04	-0.02	0.01	0.00
Fe	**0.68**	**-0.41**	-0.31	0.29	0.26	0.01	0.27	0.03	-0.11	0.16	0.02	-0.03	0.00
S	**0.96**	0.06	-0.03	-0.02	0.05	0.03	0.05	-0.12	0.20	-0.01	0.13	0.01	0.00
Al	**0.95**	0.18	0.04	-0.16	-0.13	-0.07	-0.11	-0.01	0.03	0.02	-0.02	-0.05	0.01
Mg	**0.78**	**0.55**	-0.04	-0.02	0.11	-0.16	-0.10	-0.06	-0.02	0.13	-0.05	0.06	0.00
Si	-0.21	**0.44**	**-0.57**	-0.50	-0.16	0.00	0.38	-0.13	-0.06	-0.04	-0.01	0.00	0.00
Ca	0.19	**0.72**	**0.39**	0.36	0.32	-0.12	0.11	-0.02	-0.13	-0.12	0.03	-0.01	0.00
P	**0.81**	-0.26	-0.08	0.00	0.02	0.41	-0.17	-0.22	-0.15	-0.07	-0.01	0.01	0.00
Na	-0.19	**0.55**	-0.17	0.62	-0.37	0.30	0.07	0.00	0.08	0.05	-0.02	-0.01	0.00
K	-0.23	**0.41**	**-0.67**	-0.17	0.41	0.21	-0.22	0.19	0.03	-0.01	0.01	-0.01	0.00
As	**0.84**	-0.02	-0.15	0.00	-0.39	-0.04	0.00	0.30	-0.13	-0.06	0.05	0.03	0.00
Rb	**0.81**	-0.31	-0.29	0.21	0.16	-0.05	0.18	0.07	0.14	-0.14	-0.09	0.02	0.00
Cs	**0.94**	0.22	0.05	-0.15	-0.13	-0.09	-0.12	-0.01	0.01	0.00	-0.04	-0.06	-0.01

Values in bold are different from 0 with a significance level $\alpha = 0.10$ ($r > 0.37$).

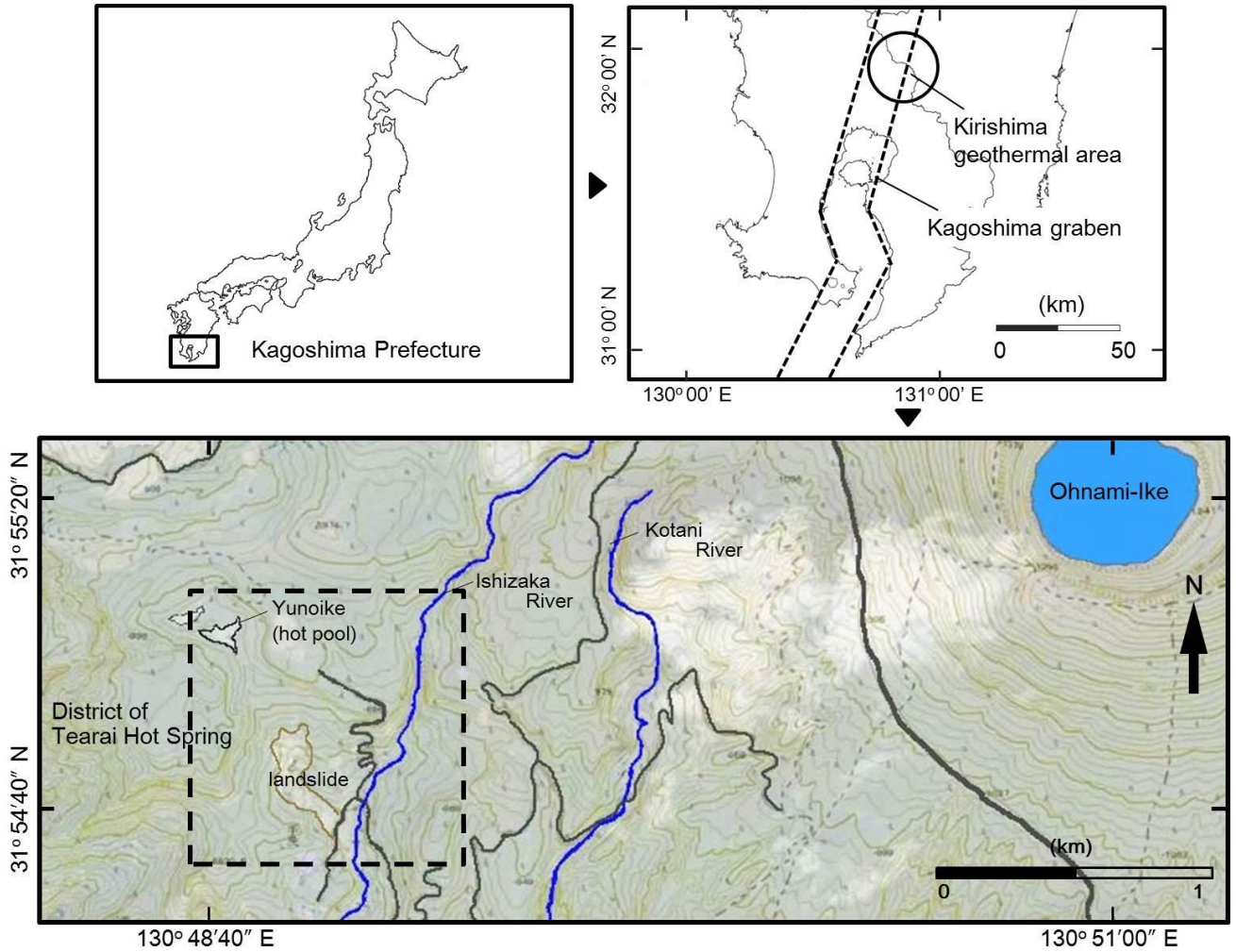

Figure 2-1. Map of the sampling sites in/near the region of the Tearai hot spring, in the Kirishima geothermal area, Kagoshima Prefecture, Japan. The dotted line showed the study area in this study

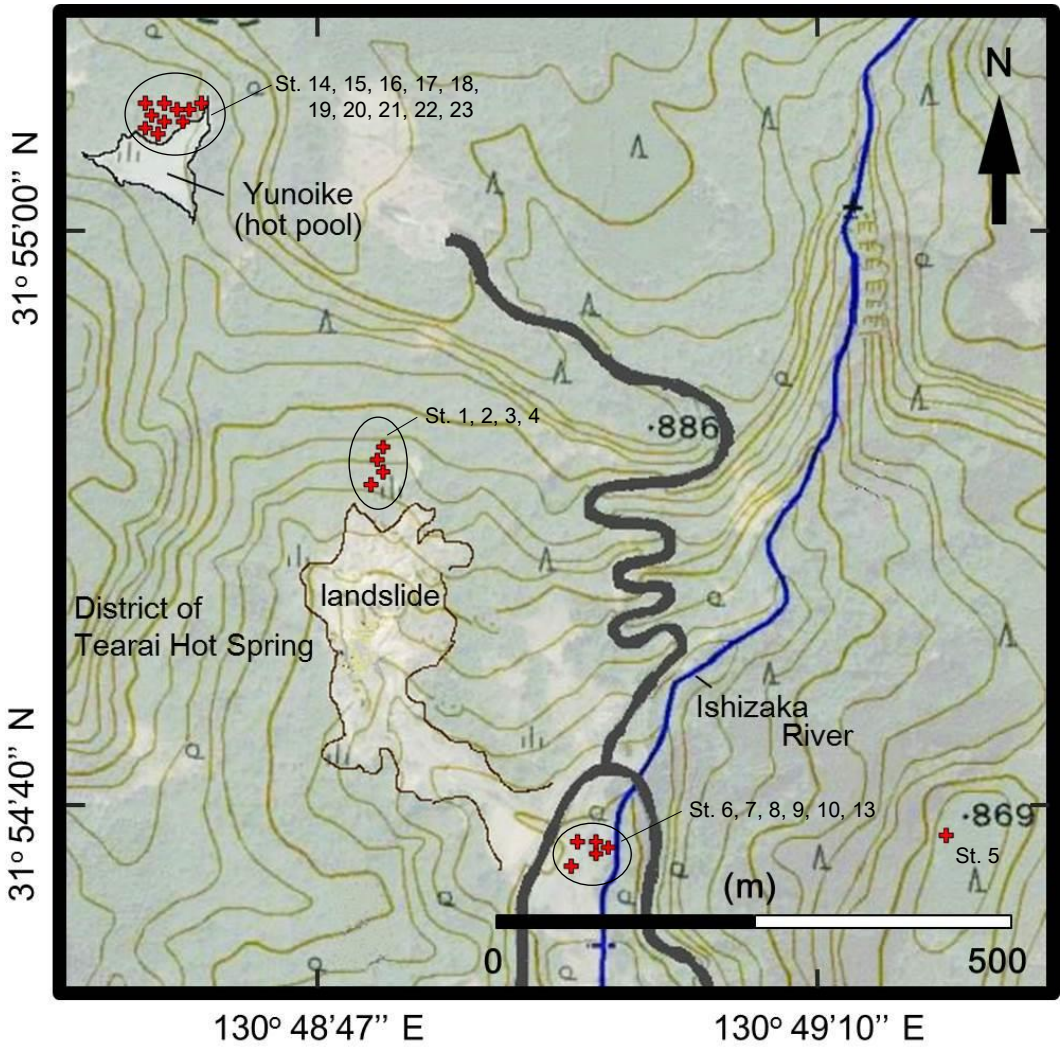

Figure 2-2. Map of 21 sampling sites in/near the region of the Tearai hot spring in the Kirishima geothermal area.

Crosses show the sampling positions in this study. The numbers showed each pond name.

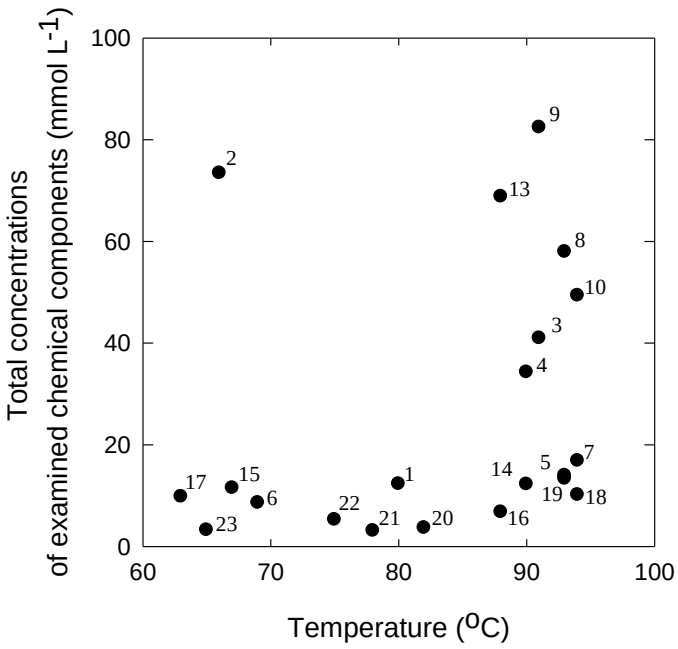

Figure 2-3. The relarionship between temperature and total concentrations of examined chemical components of 21 pond waters in the Kirishima geothermal area; N=21. The numbers showed each pond name.

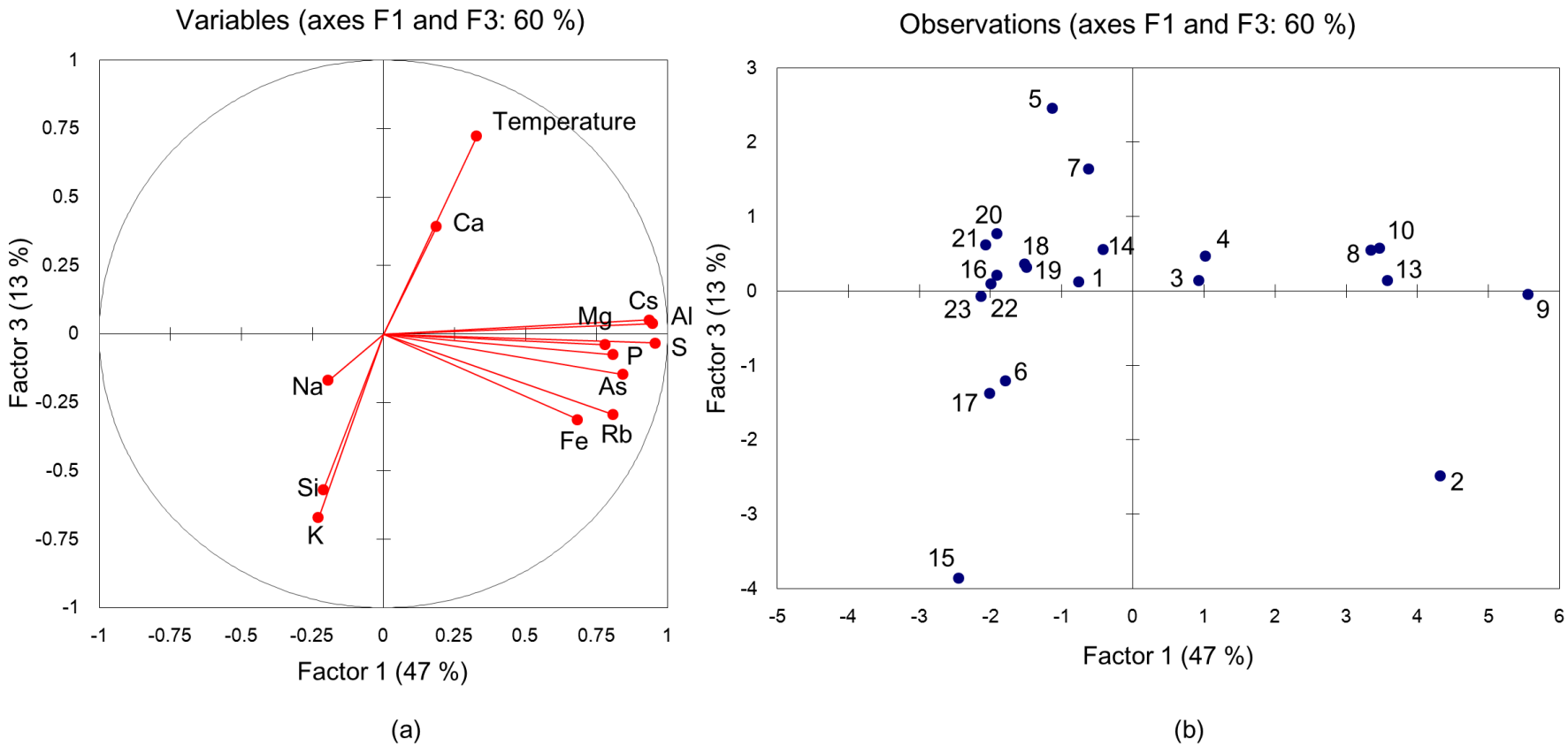

Figure 2-4. Principal components analysis showing the 13 environmental variables of the 21 ponds. (a) Factor loadings on principal components 1 and 3, (b) relationships between the 21 ponds and the principal components.

Chapter III

Bacterial community structures and biodiversity

in the highly acidic hot ponds with different temperatures and chemical components

3.1. Introduction

Prokaryotes are devided into bacteria and archaea, which show two phylogenetically distinct domains as mentioned in Chapter I “General introduction”. Altough bacteria and archaea are similar in their cell sizes, there are major several differences for phenotypic characteristics such as cell wall components, structure of cytoplasmic membrane lipids, subunit structure of RNA polymerase, and so on. In this chapter, I focused on the bacterial community structures and diversity of four distinct solfataric-acidic ponds in the Kirishima geothermal area.

The following places were subjected to study about bacterial community structures and/or its diversities in hot springs: Yellowstone National Park in the United States (Hugenholtz *et al*., 1998; Jackson *et al*., 2001; Reysenbach *et al*., 2000; Blank *et al*., 2002; Meyer-Dombard *et al*., 2005; Mathur *et al*., 2007; Tin *et al*., 2011), Lassen Volcanic National Park (Siering *et al*., 2006; Wilson *et al*., 2008; Tin *et al*., 2011), Kamchatka hot springs in Russia (Tin *et al*., 2011; Mardanov *et al*., 2011; Burgess *et al*., 2012), the island of the Lesser Antilles (Burton and Norris, 2000; Stout *et al*., 2009), Icelandic hot springs (Slirnisdottir *et al*., 2000), Bor Khlueng hot springs in Thailand (Kanokratana *et al*., 2004), the hot springs in northern Thailand (Purcell *et al*., 2006), the hot springs of White Island in New Zealand (Donachie *et al*., 2002), the Wai-o-tapu geothermal area in New Zealand (Childs *et al*., 2008) and the hot springs on the Tibetan Plateau, China (Huang *et al*., 2011). However, no study described about relationships between the bacterial communities/diversity and environmental factors although these pioneering studies have

improved our understanding of prokaryotic communities living in high temperature environments. On the other hand, we have surveyed a relatively wide geothermal field, the Kirishima geothermal area, and have found that this field contains many acidic ponds of various temperatures and chemical components as mentioned in Chapter II. The objective of this chapter is to reveal the bacterial communities and diversity in hot springs displaying a wide range of temperature and chemical copositions of Kirishima geothermal area, and to clearlify the relationships between the bacterial communities and environmental factors (temperature and chemical components).

3.2. Materials and methods

3.2.1 Sample collection and analysis of chemical components

The ponds investigated in this study were all located in a 1 km^2 field in/near the region of the Tearai hot spring (Tsuyuki, 1980). This district is situated 3 km southwest of the Ohnami-Ike volcanic crater lake, in the Kirishima geothermal area, Kagoshima Prefecture (Fig. 3-1, Table 3-1). The Kirishima geothermal region has been characterized by extensive volcanic activity since the Pleistocene epoch and this is continuing: this activity has resulted in the deposition of a thick pile of volcanic rocks (Goko, 2000). The Kirishima volcano, which is one of the largest Quaternary volcanoes in Japan, is part of the northern section of the Kagoshima graben, a volcano-tectonic depression (Tsuyuki, 1969) caused by the subduction of the Philippine Sea plate. The volcano occupies a 20 km × 30 km area that is elongated in the northwest-to-southeast direction and contains more than 20 small volcanoes (Imura *et al*., 2001).

The sampling location within the Kirishima geothermal area is located on private land, and thus, the area is not usually exposed to human activity. We obtained permission from an owner of the land to sample the hot springs and pond water as well as soil and various other samples of organisms native to the area. There are many hot springs and muddy ponds present in the area, and these have a variety of temperatures and elemental compositions.

Muddy water samples including sediments from each pond were collected in sterile 100 mL glass bottles. The temperature and pH of the samples were measured at each sampling site. Part of each sample was filtered using a 0.22 μm membrane filter (Asahi Glass) and used for analysis of the chemical component, which was performed by inductively coupled plasma optical emission spectroscopy (ICPS-7000 Ver.2, Shimadzu). We selected four ponds characterized by a wide range of temperatures and chemical components for the bacterial community analysis.

3.2.2 16S rRNA gene clone libraries and sequencing

Environmental DNA was extracted from 10 g of each muddy water sample including sediment using the UltraClean Soil DNA Kit Mega Prep (Mo Bio Laboratories) according to the manufacturer's instructions.

The purified DNA was then used as the template for amplification of the bacterial 16S rRNA gene using the bacteria-specific primer B27F (5'-AGAGTTTGATCCTGGCTCAG-3') and the universal primer U1492R (5'-GGYTACCTTGTTACGACTT-3'). The PCR conditions included an initial denaturation step at 94°C for 3 min, followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 58°C for 30 s, and extension at 72°C for 2 min using *Ex Taq* DNA polymerase (Takara Bio). This was followed by a final extension step at 72°C for 10 min.

The PCR products were purified using a GFX PCR DNA and Gel Band Purification Kit (GE Healthcare) and ligated into the pT7 Blue T-Vector (Novagen). *Escherichia coli* DH5α cells were transformed with the plasmid library and plated onto LB plates including 100 μg mL^{-1} ampicillin, 40 μg mL^{-1} X-gal, and 0.5 mM IPTG. Blue/white selection was performed by randomly picking and subculturing individual white colonies in 100 μL of 2× YT medium containing 100 μg mL^{-1} ampicillin in a 96-well plate at 37°C overnight. The inserted 16S rRNA gene was amplified using 1 μL of the culture as the template with the forward primer T7P-F (5'-TAATACGACTCACTATAGGG-3') and reverse primer T7U-R (5'-GTTTTCCCAGTCACGACGT-3'). About 800 bp of the 5'-region of each 16S rRNA gene clone was sequenced using the aforementioned bacteria-specific primer B27F and used for the taxonomic and phylogenetic analyses.

3.2.3 Identification of 16S rRNA gene clones and phylogenetic analysis

16S rRNA gene sequences were edited using MEGA5 (Molecular Evolutionary Genetics Analysis, http://www.megasoftware.net/) (Tamura *et al*., 2011). We also searched for chimera sequences by manually checking the sequence alignments using GENETYX Ver.10.0.3 software (Genetyx). Clones with 97% or greater sequence similarity were treated as a phylotype. The representative sequences of each phylotype were compared with 16S rRNA gene sequences published in the National Center for Biotechnology Information DNA database using BLAST (BLASTN; http: //www.ncbi.nlm.nih.gov/BLAST/) (Altschul *et al*., 1990) to identify individual clones. The representative sequences of each phylotype and related sequences in the GenBank database were aligned using CLUSTALW Ver.1.83 (Thompson *et al*., 1994). A maximum likelihood tree including bootstrap probabilities (1000 samplings) was constructed using MEGA5.

3.2.4 Statistical analyses

Measurements of diversity ideally include richness, which is the number of different species or groups present, and evenness, which is the distribution of those groups (Hurlbert, 1971; Stirling and Wilsey, 2001). The Shannon–Weaver index (Shannon *et al*., 1949), $H' = -\Sigma(pi)(\ln pi)$, and Simpson's reciprocal index (Simpson, 1949), $1/D$, where $D = \Sigma(pi)^2$ and pi is the proportion of phylotypes *i* relative to the total number of phylotypes, both consider richness and evenness (Stout *et al*., 2009; Stirling and Wilsey, 2001). In this study, the Shannon‑Weaver index and Simpson's reciprocal index were calculated using ESTIMATES 8.0 (Colwell, 2006). Evenness ($J' = H' / \ln S$) was also calculated (Pielou, 1969). ESTIMATES 8.0 was also used to calculate the Chao1 nonparametric richness estimator (Chao, 1987) and the abundance-based coverage estimator of species richness (ACE) (Chao *et al*., 2000). These coverage estimators determine the number of probable phylotypes in the environment compared with the number observed in the

sample. Homologous coverage (biodiversity coverage) *C* was determined using the following equation: $C = 1 - (N/n)$, where *N* is the number of phylotype sequences detected and n is the total number of clones analyzed (Good, 1953; Singleton *et al.*, 2001). Further analysis of bacterial libraries at the phylotype level included measurements of gene diversity, θ_π, using the software package ARLEQUIN (Excoffier *et al.*, 2005). θ_π is the average sequence divergence, or an estimate of the total genetic variation in a sample (Martin, 2002). Additionally, statistical analysis also included principal components analysis, which was used to determine correlations among bacterial diversity and environmental factors including temperature and chemical component. Canonical correlation analysis was also used to detect correlations between bacterial groups and temperature or chemical components using XLSTAT software (Addinsoft, New York, NY).

3.2.5 Nucleotide sequence accession numbers

Representative nucleotide sequences of the phylotypes are available in the DDBJ/EMBL/GenBank databases under accession numbers AB762419–AB762465.

3.3. Results and Discussion

3.3.1 Water chemistry

The four ponds in the Kirishima geothermal area were selected based on their different temperatures and total concentration of examined chemical components: 1) Pond-A: 93°C and 58 mmol L^{-1}; 2) Pond-B: 66°C and 73 mmol L^{-1}; 3) Pond-C: 88°C and 6.7 mmol L^{-1}; and 4) Pond-D: 67°C and 11 mmol L^{-1}. The characteristics of the sampling sites and these ponds are shown in Table 1. The pH value of the ponds ranged from 2.0–2.6. In the ponds with higher concentrations of the examined chemical components, the concentration and percentage of Fe, S, and Al, in particular, were higher than in the other ponds.

To clarify the relationships between temperature and chemical component or among the chemical components of the four ponds, Pearson's correlation coefficients (r) were calculated (Table 3-2). Several chemical components were found to be correlated with each other. For example, Fe was strongly correlated with P, As, and Rb and S was strongly correlated with Al, Si, P, As, and Cs. The total concentration of the examined chemical components was also strongly correlated with Fe, S, P, and As. Temperature was not statistically correlated with any of the chemical components.

We also attempted to perform the principal components analysis to distinguish the four ponds based on environmental variables (Fig. 3-2). All the variables characterizing the sites were explained by three principal factors: Factor 1 (F1) 63%, Factor 2 (F2) 20%, and Factor 3 (F3) 17% (Table 3-3). F1 was strongly loaded by S, Al, P, As, and Cs (positively) and Si (negatively); F2 was strongly loaded by Ca; and F3 was relatively strongly loaded by temperature (Table 3-4). In this analysis, we focused on the principal components 1 and 3 (PC1 and PC3), which affected F1 and F3, respectively. Strong contributions to PC1 were made by S, Al, P, As, Cs,

and Si and moderate contributions were made by Fe, Mg, Na, K and Rb. Temperature contributed relarively strongly to PC3 (Fig. 3-2a). These environmental variables were defining characteristics of the four ponds (Fig. 3-2b), and we therefore discuss the bacterial community structures by concentrating on these elements and the different temperatures of the ponds in the following sections.

3.3.2 16S rRNA gene clone libraries

The 16S rRNA gene clone libraries were successfully constructed using environmental DNA extracted from four muddy water samples including sediments. A total of 372 clones (Pond-A: 95, Pond-B: 94, Pond-C: 92, Pond-D: 91 clones) of the bacterial 16S rRNA gene were analyzed. No chimerical sequences were detected. The clones were classified into 35 phylotypes on the basis of the sequence similarity values, and these consisted of 10 classes: Flavobacteria, γ-Proteobacteria, β-Proteobacteria, α-Proteobacteria, Nitrospirae, δ-Proteobacteria, Bacilli, Actinobacteria, Thermotogae, and Aquificae (Table 3-5, Fig. 3-3).

3.3.3 Bacterial community in Pond-A

Pond-A was characterized by relatively high temperature and high total concentration of the examined chemical components. Analysis of 16S rRNA gene sequence similarities of the 95 clones derived from the pond revealed 14 phylotypes, which was the largest number of phylotypes detected of all four ponds (Table 3-6). Of the sequences derived from this pond, 87% were very similar to those of cultured species (>98.9%), including *Chryseobacterium aquaticum* (Kim *et al*., 2008) from the class Flavobacteria; *Acinetobacter johnsonii* (Bouvet and Grimont, 1986) and *Pseudomonas poae* (Behrendt *et al*., 2003) from the class γ-Proteobacteria; *Acidovorax*

temperans (Willems *et al*., 1990), *Curvibacter lanceolatus* (Ding and Yokota, 2004) and *Methylophilus leisingeri* (Doronina and Trotsenko, 1994) from the class β-Proteobacteria; and *Propionibacterium acnes* from the class Actinobacteria (Douglas and Gunter, 1946; Moore and Cato, 1963; Bojar and Holland, 2004). The largest number of clones was assigned to a single phylotype, ST8B3-2, which accounted for 67% of all clones derived from this pond. This phylotype was also dominant in Pond-C and was very similar to the sequence of *A. johnsonii* (98.9%), which is an aerobic, gram-negative, heterotrophic bacteria with an optimal growing temperature of 15–30°C; no growth occurs at 37°C. The genus *Acinetonbacter* is widely distributed in soil, water (Baumann, 1968), and sewage (Warskow and Juni, 1972). The second dominant phylotype was ST8B3-10, which was very similar to the *P. poae* sequence (99.4%). This species is an aerobic, gram-negative, heterotrophic, fluorescent bacteria that has an optimal growth temperature of 21°C; no growth occurs at 41°C. The bacteria was isolated from the phyllosphere of grasses (Behrendt *et al*., 2003). These were unexpected results because these mesophilic microbes should not have been able to grow in Pond-A given its high temperature. At the moment, we have no reasonable explanation for these findings, but it is known that some species with closely related 16S rRNA gene sequences have different optimal growth temperatures. For example, we recently described a novel *Paenibacillus* species that is the only thermophilic strain of the genus Paenibacillus, which has to date only consisted of mesophilic species (Ueda *et al*., 2013). On the other hand, the remaining 13% of all clones derived from Pond-A was classified into seven phylotypes, and they did not show any significant similarity with any cultured species. The Shannon–Weaver index score for Pond-A was the third highest among these four ponds; it was lower than those of Pond-C and Pond-D but higher than that of Pond-B (Table 3-6). The gene diversity index (θ_π) was the lowest among the four ponds (Table

3-6).

3.3.4 Bacterial community in Pond-B

Pond-B was characterized by a relatively low temperature and a high total concentration of the examined chemical components; it had the lowest Shannon–Weaver index diversity score of all four ponds (Table 3-6). However the gene diversity index (θ_π) was the highest among the four ponds (Table 3-6). A total of 94 clones were derived from Pond-B, and these were determined to constitute 11 phylotypes (Table 3-5). Nearly half of the sequences in this pond showed a significantly close relationship with one of the following four cultured species (>99.6%): *Acidithiobacillus caldus* (Hallberg and Lindstörm, 1994; Kelly and Wood, 2000) from the class γ-Proteobacteria; *Ralstonia pickettii* (Ralston *et al*., 1973; Yabuuchi *et al*., 1995) from the class β-Proteobacteria; *Acidicaldus organivorans* (Johnson *et al*., 2006) from the class α-Proteobacteria; and *Staphylococcus epidermidis* from the class Bacilli (Schleifer and Kloos, 1975). Almost all the clones were assigned to a single phylotype, ST2B3-1, which was very similar to the sequence of *A. caldus* (99.6%); these clones accounted for 44% of all clones derived from this pond. *A. caldus* is an aerobic, gram-negative, moderately thermophilic, sulfur-oxidizing acidophile with an optimal growth pH of 2–2.5 and temperature of 45°C. This species is also capable of chemolithotrophic growth on reduced sulfur and molecular hydrogen. On the other hand, 53% of the Pond-B clones constituted seven phylotypes that showed no significant similarity with any cultured species; this was the highest percentage of cultured species among the four ponds. Most of the uncultured clones were assigned to the phylotype ST2B3-15, which affiliated with the class δ-Proteobacteria (Fig. 3-3). This phylotype showed the closest match to a published environmental clone BA71, which was detected from a lithotrophic biofilm at an

extreme acid mine drainage site (DNA database Accession No. AF225447) (Bond *et al*., 2000). The clones of this phylotype were the most dominant in this pond, similar to the aforementioned ST2B3-1 phylotype.

3.3.5 Bacterial community in Pond-C

Pond-C was another pond with a relatively high temperature, and 92 clones were derived from this pond. These were classified into nine phylotypes, which is the lowest value of species richness among the four ponds (Table 3-6). The species diversity index score in Pond-C was the second highest among the four ponds, i.e., it was lower than that of Pond-D but higher than those of Pond-A and Pond-B. Ninety percent of the sequences from this pond were very similar to the following cultured species (>98.0%): *Elizabethkingia miricola* (Kim *et al*., 2005) from the class Flavobacteria; *A. johnsonii* from the class γ-Proteobacteria; and *A. temperans*, *Delftia tsuruhatensis* (Shigematsu *et al*., 2003), *Massilia alkalitolerans* (Kämpfer *et al*., 2011) and *Paracoccus marinus* (Khan *et al*., 2008) from the class β-Proteobacteria (Table 3-5). Most clones were assigned to a single phylotype, ST8B3-2, which was also dominant in Pond-A, and accounted for 57% of the clones derived from Pond-C. The second most dominant phylotype was ST8B3-23, which was very similar to the *A. temperans* sequence (99.2%). *A. temperans* is an aerobic, gram-negative bacteria that, has been reported as an abundant member of activated sludge microbial communities (Willems *et al*., 1990; Heijstra *et al*., 2009). On the other hand, the remaining 10% of the clones in Pond-C were allocated to three phylotypes that did not show any significant similarity with any cultured species.

3.3.6 Bacterial community in Pond-D

Pond-D, which was characterized by a relatively low temperature and a low total concentration of the examined chemical components, was the most diverse of the four ponds, as assessed by the Shannon-Weaver index and Simpson's reciprocal index (Table 3-6). A total of 91 clones were derived from this pond and these consisted of 13 phylotypes. Eighty-two percent of the sequences from this pond were very similar to those of the following cultured species (>98.0%): *E. miricola* from the class Flavobacteria; *A. junii* (Bouvet and Grimont, 1986) and *A. caldus* from the class γ-Proteobacteria; *D. tsuruhatensis* and *M. alkalitoleran*s from the class β-Proteobacteria; and *S. epidermidis* from the class Bacilli and *P. acnes* of the class Actinobacteria (Table 3-5). A single phylotype, ST15B2-3, contributed 37% of all clones derived from this pond. This phylotype was only detected in this pond and was very similar to the *A. junii* sequence (99.7%). *A. junii* is an aerobic, gram-negative, heterotrophic bacteria with an optimal growth temperature of 15–30°C; no growth occurs at 44°C. The second most dominant phylotype was ST2B3-24, which accounted for 14% of the clones in this pond. This phylotype was very similar to the *S. epidermidis* sequence (100.0%). *S. epidermidis* is an aerobic, gram-positive, heterotrophic bacteria that is ubiquitous in the environment. It has been isolated from human skin; animal products such as meat, milk, and cheese; and other sources including soil, sand, seawater, freshwater, dust, and air (Kloos *et al*., 1991; Wieser and Busse, 2000). On the other hand, 18% of the clones from Pond-D consisted of six phylotypes that did not show any significant similarity to any cultured species. Most of these uncultured clones were assigned to the ST15B2-55 phylotype, which is affiliated with the class Aquificae (Fig. 3-3). This phylotype was only detected in this pond and showed the closest match to a published environmental clone KOZ166 (97.6%) detected from Yellowstone National Park (DNA database Acc. No. EF156606).

3.3.7 Bacterial biodiversity and community structure in relation to different temperatures and different total concentrations of the examined chemical components

At least 85% of the 16S rRNA gene sequences from each pond could be analyzed since the homologous coverage values were 0.85 or above for all ponds (Table 3-6). When gene and species diversity was compared for ponds of different temperatures (Temp. approx. 90°C, Pond-A + Pond-C vs. Temp. approx. 70°C, Pond-B + Pond-D), the lower temperature ponds showed higher gene and species diversity according to θ_π and the Shannon-Weaver index values, respectively (Table 3-6). On the other hand, when ponds with different concentrations of the examined chemical components were compared, species diversity indices for the ponds with lower concentrations of the chemical components (Pond-C + Pond-D, Total conc. <15 mmol L^{-1}) were higher than those for the ponds with higher concentrations (Pond-A + Pond-B, Total conc. >55 mmol L^{-1}). Gene diversity showed almost the same value within ponds with different concentrations of the examined chemical components. As a result, the bacterial species diversity was highest in the pond characterized by a lower temperature and a lower concentration of chemical components (Pond-D). In contrast, the combination of higher temperature and higher total concentration of the examined chemical components (Pond-A) resulted in the lowest diversity in this study.

With regard to species composition and distribution, these were different in ponds characterized by different temperatures and different total concentrations of the examined chemical components. As shown in Table 3-5, the dominant bacterial group across all ponds was the class γ-Proteobacteria. At a lower taxonomic level, within the class γ-Proteobacteria, phylotype ST8B3-2, which is very similar to the *A. johnsonii* sequence (98.9%) and is affiliated with the order Pseudomonadales, was only detected in the higher temperature ponds (Pond-A +

Pond-C). On the other hand, phylotype ST2B3-1, which is very similar to the *A. caldus* sequence (99.6%) and is affiliated with the order Acidithiobacillales, was only detected in the lower temperature ponds (Pond-B + Pond-D). These results suggest that the species composition and distribution within the class γ-Proteobacteria differs for higher and lower temperature ponds. On the other hand, the species composition and distribution also varied between ponds with different total concentrations of the examined chemical components. Phylotype ST8B3-33, which is affiliated with the genus *Acinetobacter* spp., was only detected in the ponds with higher concentrations of the total examined chemical components (Pond-A + Pond-B). In contrast, phylotypes such as ST16B3-9, ST16B3-16, and ST16B5-42, which are affiliated with the order Burkholderiales, were only detected in ponds with lower total concentrations of the examined chemical components (Pond-C + Pond-D).

3.3.8 Geochemistry and bacterial biodiversity or group correlations

As shown in Table 3-2, bacterial species diversity was statistically correlated with Si, Na, and K. And also, bacterial gene diversity (θ_π) was statistically correlated with temperature, pH, and species diversity.

To clarify the relationships between bacterial groups and the temperature or chemical component of the four ponds, canonical correlation analysis was performed (Fig. 3-4, Table 3-7, 3-8, 3-9, and 3-10). Specific bacterial groups were found to be correlated with particular factors: the classes δ-Proteobacteria, α-Proteobacteria, and Nitrospirae were strongly correlated with Fe and Rb, the classes Bacilli and Flavobacteria with Si, Na, and K, and Actinobacteria with Na and K. On the other hand, the classes γ-Proteobacteria and Thermotogae were positively and negatively correlated with temperature, respectively. These statistical analyses

indicated that there are correlations between bacterial diversity and environmental factors, and these will allow us to begin tracing trends in environmental effects on bacterial diversity. However, linking these types of studies with culture-based studies will provide more insight into how specific elements affect bacterial communities.

Table 3-1. Characteristics of sampling sites and pond waters and sediments in the Kirishima geothermal area

	Pond-A		Pond-B		Pond-C		Pond-D	
Temp. (℃)	93		66		88		67	
pH	2.6		2.0		2.4		2.3	
Concentration (μmol L^{-1}) / Composition (%)								
Fe	6963.8	12	20566	28	172.43	3	486.64	4
S	20681	36	21913	30	1863.3	28	1930.1	17
Al	16071	28	10672.2	15	539.92	8	74.924	1
Mg	3567.9	6	1923.8	3	0.0411	0	1783.0	16
Si	1704.5	3	1620.4	2	3699.6	55	5284.6	46
Ca	1367.4	2	271.45	0	187.07	3	979.49	9
P	92.021	0	152.11	0	40.846	1	40.889	0
Na	0.0435	0	0.0435	0	0.0435	0	367.20	3
K	0.0256	0	0.0256	0	0.0256	0	188.84	2
As	14.408	0	15.173	0	11.736	0	11.430	0
Rb	4130.5	7	14052	19	92.265	1	288.38	3
Cs	3341.4	6	2223.3	3	117.937	2	46.968	0
Total	57934	100	73409	100	6725.2	100	11482	100
Latitude (N)	31°54'37.7"		31°54'52.4"		31°55'05.0"		31°55'04.5"	
Longitude (E)	130°49'00.6"		130°48'50.3"		130°48'41.1"		130°48'41.0"	
Altitude (m)	759		842		884		885	
Color of sediments	light brown		light brown		gray		gray	

Detection limit is 0.001 mg L^{-1}.

Table 3-2. Correlation matrix showing *r* values for Pearson's correlation among environmental variables in the pond waters of the Kirishima geothermal area; N=4

Variables	θπ	Shannon	Temp.	pH	Fe	S	Al	Mg	Si	Ca	P	Na	K	As	Rb	Cs	Total conc.
θπ	—	0.23	**-0.99**	**-0.91**	0.51	0.05	-0.23	-0.10	0.17	-0.34	0.40	0.44	0.44	0.12	0.54	-0.23	0.21
Shannon	—	—	-0.34	0.13	-0.68	-0.74	-0.68	-0.14	**0.93**	0.32	-0.73	0.96	0.96	-0.80	-0.67	-0.68	-0.71
Temp.	—	—	—	0.86	-0.40	0.05	0.31	0.12	-0.29	0.29	-0.29	-0.55	-0.55	-0.01	-0.43	0.31	-0.10
pH	—	—	—	—	-0.67	-0.16	0.17	0.29	0.07	0.65	-0.56	-0.07	-0.07	-0.27	-0.70	0.17	-0.31
Fe	—	—	—	—	—	0.84	0.62	0.34	-0.76	-0.26	**0.99**	-0.46	-0.46	**0.90**	**1.00**	0.62	**0.91**
S	—	—	—	—	—	—	**0.95**	0.71	**-0.93**	0.21	**0.91**	-0.58	-0.58	**0.99**	0.81	**0.95**	**0.99**
Al	—	—	—	—	—	—	—	0.82	**-0.90**	0.44	0.72	-0.57	-0.57	**0.90**	0.58	**1.00**	0.89
Mg	—	—	—	—	—	—	—	—	-0.49	0.82	0.44	-0.02	-0.02	0.61	0.30	0.83	0.66
Si	—	—	—	—	—	—	—	—	—	0.00	-0.83	0.84	0.84	**-0.94**	-0.74	-0.89	-0.89
Ca	—	—	—	—	—	—	—	—	—	—	-0.15	0.33	0.33	0.07	-0.29	0.44	0.12
P	—	—	—	—	—	—	—	—	—	—	—	-0.51	-0.51	**0.95**	**0.98**	0.72	**0.96**
Na	—	—	—	—	—	—	—	—	—	—	—	—	—	-0.62	-0.44	-0.57	-0.52
K	—	—	—	—	—	—	—	—	—	—	—	—	—	-0.62	-0.44	-0.57	-0.52
As	—	—	—	—	—	—	—	—	—	—	—	—	—	—	0.88	**0.90**	**0.99**
Rb	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	0.58	0.89
Cs	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	0.89
Total conc.	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—

Values in bold are different from 0 with a significance level $\alpha = 0.10$ ($r > 0.90$). The only Shannon-Weaver index for bacterial clone libraries is shown as species diversity.

θπ, Shannon, Temp., and Total conc. indicate the gene diversity index, Shannon–Weaver index, Temperature, and total concentration of examined chemical components, respectively.

Table 3-3. The factor analysis of 13 environmental variables in the four pond of the Kirishima geothermal area

Factor	Eigenvalue	Total variance (%)	Cumulative eigenvalue	Cumulative variance (%)
Factor 1	8.19	63.0	8.19	63.0
Factor 2	2.57	19.8	10.8	82.8
Factor 3	2.23	17.2	13.0	100.0

Table 3-4. Factor loadings for 13 environmental variables in the four pond of the Kirishima geothermal area

Variables	Factor 1	Factor 2	Factor 3
Temperature	0.11	0.54	0.83
Fe	0.85	-0.43	-0.31
S	**0.99**	0.10	-0.14
Al	**0.92**	0.38	0.01
Mg	0.60	0.70	-0.39
Si	**-0.97**	0.01	-0.23
Ca	0.08	**0.96**	-0.28
P	**0.91**	-0.31	-0.27
Na	-0.70	0.19	-0.69
K	-0.70	0.19	-0.69
As	**0.99**	-0.04	-0.11
Rb	0.82	-0.47	-0.32
Cs	**0.92**	0.39	0.00

Values in bold are different from 0 with a significance level α=0.10 (r > 0.90).

Table 3-5. Affiliation and closest published species or clones of 35 phylotypes of bacteria detected in the ponds of the Kirishima geothermal area

Phylotypes	Affiliation	Closest species or clones (accession number)	16S rRNA gene similarity (%)	Number of clones detected from each site			
				Pond -A	Pond -B	Pond -C	Pond -D
	class Flavobacteria						
ST16B10-59 (=ST15B2-2)	*Elizabethkingia miricola*	*Elizabethkingia miricola* (EU375848)	99.0			3	6
ST8B3-52	*Chryseobacterium aquaticum*	*Chryseobacterium aquaticum* (AM748690)	100.0	2			
	class γ-Proteobacteria						
ST8B3-2 (=ST16B3-5)	*Acinetobacter johnsonii*	*Acinetobacter johnsonii* (NR044975)	98.9	64		52	
ST15B2-3	*Acinetobacter junii*	*Acinetobacter junii* (NR026208)	99.7				34
ST8B3-33 (=ST2B3-83)	*Acinetobacter* sp.	Subsurface groundwater clone BANW433 (DQ264432)	96.8	1	1		
ST2B3-1 (=ST15B3-22)	*Acidithiobacillus caldus*	*Acidithiobacillus caldus* (NR026517)	99.6		41		4
ST8B3-10	*Pseudomonas poae*	*Pseudomonas poae* (AJ492829)	99.4	7			
ST8B3-13	Uncultured *Pseudomonadaceae*	Maple sap clone 100p3_613 (FJ934668)	95.4	1			
ST16B4-80 (=ST15B8-1)	Uncultured Pseudomonadales	Subsurface groundwater clone BANW563 (DQ264531)	97.1			1	2
ST15B8-95	Uncultured Pseudomonadales	Coastal urnaban watershed clone C01JMA (JF692239)	95.3				2
ST8B3-37	Uncultured Pseudomonadales	Subsurface groundwater clone BANW416 (DQ264418)	98.0	2			
ST8B3-15	Uncultured Pseudomonadales	Lake stream water clone D-79 (HQ860678)	94.4	1			
ST2B3-60	Uncultured γ-Proteobacteria	Bioleaching pulp with pH <2.0 clone zy-5 (EF672753)	92.5		1		
ST15B2-44	Uncultured γ-Proteobacteria	Acinetobacter junii (NR026208)	90.8				1
	class β-Proteobacteria						
ST8B3-23 (=ST16B3-18)	*Acidovorax temperans*	*Acidovorax temperans* (NR028715)	99.2	3		11	
ST16B3-9 (=ST15B2-50)	*Delftia tsuruhatensis*	*Delftia tsuruhatensis* (NR024786)	99.5			8	5
ST16B3-16 (=ST15B3-82)	*Naxibacter alkalitolerans*	*Massilia alkalitolerans* (AY679161)	98.0			3	3
ST16B4-10	*Paracoccus marinus*	*Paracoccus marinus* (AB185957)	98.4			6	
ST8B3-40	*Curvibacter lanceolatus*	*Curvibacter lanceolatus* (NR024702)	99.5	1			
ST2B4-26	*Ralstonia pickettii*	*Ralstonia pickettii* (NR043152)	100.0		1		
ST8B3-18	*Methylophilus leisingeri*	*Methylophilus leisingeri* (NR041258)	99.5	1			
ST16B5-42 (=ST15B2-13)	Uncultured *Comamonadaceae*	Spacecraft assembly clean room *Delftia* sp. clone GI5-13-D06 (FJ192433)	98.5			1	2
ST8B3-46	Uncultured *Methylophilaceae*	Uranium-contaminated aquifer clone 1013-28-CG9 (AY532564)	93.4	1			
ST8B3-58	Uncultured *Methylophilaceae*	River site β-proteobacterium clone RBE2CI-98 (EF111184)	91.1	1			
	class α-Proteobacteria						
ST2B3-49	*Acidicaldus organivorans*	*Acidicaldus organivorans* (AY140238)	99.6		1		
	class Nitrospirae						
ST2B3-57	Uncultured Nitrospirales	Acid mine drainage sediment clone H50 (DQ328622)	99.6		1		
	class δ-Proteobacteria						
ST2B3-15	Uncultured δ-Proteobacteria	Extreme acid mine drainage clone BA71 (AF225447)	97.3		41		
	class Bacilli						
ST2B3-24 (=ST15B2-14)	*Staphylococcus epidermidis*	*Staphylococcus epidermidis* (NR036904)	100.0		1		13
ST16B3-94	Uncultured *Paenibacillaceae*	Bacillus sp. YNPRH6P-1 (AF465647)	99.2			7	
ST2B3-86	Uncultured Bacillales	Drinking bulk water clone SW-3S_A04 (JX286150)	99.5		3		
ST8B3-7	Uncultured Bacillales	Banana plantation soil clone WB128 (JX133663)	88.0	5			
	class Actinobacteria						
ST8B3-32 (=ST15B9-35)	*Propionibacterium acnes*	*Propionibacterium acnes* (NR040847)	99.3	5			10
ST2B3-20	Uncultured Acidimicrobiales	Hot spring clone SK299 (AY882848)	99.2		1		
	class Thermotogae						
ST2B4-6 (=ST15B8-31)	Uncultured Thermotogae	Thermal spring sediment clone kma134 (HM149925)	98.6		2		1
	class Aquificae						
ST15B2-55	*Hydrogenobaculum* sp.	Hot spring Hydrogenobaculum sp. clone KOZ166 (EF156606)	97.6				8
Total				95	94	92	91

Table 3-6. Diversity index scores for clone libraries of bacteria detected in the ponds of the Kirishima geothermal area

Sample	Shannon	Simpson	Rich	Even	S_{ACE}	S_{Chao1}	θ_π	Coverage	Total clone number
Pond-A	1.38	2.14	14	0.521	23.8	26.3	115 ± 59	0.85	95
Pond-B	1.25	2.61	11	0.523	28.8	21.5	150 ± 79	0.88	94
Pond-C	1.48	2.83	9	0.676	10.2	10.0	121 ± 65	0.90	92
Pond-D	2.04	5.12	13	0.796	14.2	13.3	144 ± 74	0.86	91
Temp. approx. 90 °C (Pond-A + Pond-C)	1.66	2.51	21	0.544	31.0	41.3	119 ± 60	0.89	187
Temp. approx. 70 °C (Pond-B+ Pond-D)	2.23	6.42	21	0.733	29.4	29.2	144 ± 72	0.89	185
Total conc. > 55 mmol L^{-1} (PondA + Pond-B)	2.00	4.70	24	0.630	42.9	42.0	139 ± 69	0.87	189
Total conc. < 15 mmol L^{-1} (Pond-C + Pond-D)	2.33	7.09	17	0.823	17.9	17.5	139 ± 70	0.91	183

Diversity index scores measured were Shannon-Weaver index (Shannon), Simpson's reciprocal index (Simpson), Richness (Rich), Evenness (Even), the coverage estomators S_{ACE} and S_{Chao1}, the gene diversity index θ_π, and the homologous coverage. Temp. and Total conc. indicate temperature and total concentration of examined chemical components, respectively.

Table 3-7. Correlation matrix showing *r* values for Pearson's correlation between environmental factors and proportions of indivisual bacterial groups detected from the four ponds in the Kirishima geothermal area; N=4

Variables	Flavobacteria	γ-Proteobacteria	β-Proteobacteria	α-Proteobacteria	Nitrospirae	δ-Proteobacteria	Bacilli	Actinobacteria	Thermotogae	Aquificae
Flavobacteria	—	-0.22	0.29	-0.68	-0.68	-0.68	**0.98**	0.82	-0.24	0.91
γ-Proteobacteria	—	—	0.09	-0.50	-0.50	-0.50	-0.40	-0.08	-0.76	-0.45
β-Proteobacteria	—	—	—	-0.61	-0.61	-0.61	0.29	-0.28	-0.68	-0.09
α-Proteobacteria	—	—	—	—	**1.00**	**1.00**	-0.56	-0.43	0.87	-0.33
Nitrospirae	—	—	—	—	—	**1.00**	-0.56	-0.43	0.87	-0.33
δ-Proteobacteria	—	—	—	—	—	—	-0.56	-0.43	0.87	-0.33
Bacilli	—	—	—	—	—	—	—	0.76	-0.10	**0.93**
Actinobacteria	—	—	—	—	—	—	—	—	0.02	**0.90**
Thermotogae	—	—	—	—	—	—	—	—	—	0.17
Aquificae	—	—	—	—	—	—	—	—	—	—
Temperature	-0.19	**0.90**	0.50	-0.60	-0.60	-0.60	-0.33	-0.32	**-0.91**	-0.55
Fe	-0.79	-0.20	-0.74	**0.95**	**0.95**	**0.95**	-0.72	-0.41	0.75	-0.46
S	-0.80	0.34	-0.75	0.61	0.61	0.61	-0.84	-0.30	0.34	-0.58
Al	-0.69	0.62	-0.63	0.33	0.33	0.33	-0.79	-0.21	0.04	-0.57
Mg	-0.17	0.54	-0.76	0.05	0.05	0.05	-0.30	0.39	0.04	-0.02
Si	**0.94**	-0.44	0.46	-0.55	-0.55	-0.55	**0.98**	0.61	-0.14	0.84
Ca	0.35	0.62	-0.36	-0.50	-0.50	-0.50	0.18	0.70	-0.36	0.33
P	-0.82	-0.07	-0.76	0.89	0.89	0.89	-0.78	-0.41	0.66	-0.51
Na	**0.91**	-0.45	-0.09	-0.33	-0.33	-0.33	**0.93**	**0.90**	0.17	**1.00**
K	**0.91**	-0.45	-0.09	-0.33	-0.33	-0.33	**0.93**	**0.90**	0.17	**1.00**
As	-0.86	0.25	-0.72	0.70	0.70	0.70	-0.87	-0.40	0.41	-0.62
Rb	-0.78	-0.25	-0.73	**0.96**	**0.96**	**0.96**	-0.70	-0.42	0.77	-0.44
Cs	-0.69	0.62	-0.64	0.33	0.33	0.33	-0.78	-0.20	0.04	-0.57

Values in bold are different from 0 with a significance level α=0.10 ($r > 0.90$).

Table 3-8. The eigenvalues, the corresponding inertia, and the corresponding percentages of canonical correlation analysis between environmental variables and proportions of individual bacterial groups detected from the four ponds in the Kirishima geothermal area; N=4

Factor	Eigenvalue	Total variance (%)	Cumulative eigenvalue	Cumulative variance (%)
Factor 1	1.00	33.3	1.00	33.3
Factor 2	1.00	33.3	2.00	66.7
Factor 3	1.00	33.3	3.00	100.0

Table 3-9. Canonical factor loadings for proportions of individual 10 bacterial groups in the four pond of the Kirishima geothermal area

	Factor 1	Factor 2	Factor 3
Flavobacteria	-0.13	-0.39	**-0.91**
γ-Proteobacteria	0.05	-0.82	0.57
β-Proteobacteria	**0.90**	-0.29	-0.33
α-Proteobacteria	-0.25	0.88	0.41
Nitrospirae	-0.25	0.88	0.41
δ-Proteobacteria	-0.25	0.88	0.41
Bacilli	-0.10	-0.21	**-0.97**
Actinobacteria	-0.67	-0.39	-0.63
Thermotogae	-0.49	0.87	-0.04
Aquificae	-0.45	-0.09	-0.89

Values in bold are different from 0 with a significance level α=0.10 (r > 0.90).

Table 3-10. Canonical factor loadings for 13 environmental variables in the four pond of the Kirishima geothermal area

	Factor 1	Factor 2	Factor 3
Temperature	0.48	-0.75	0.45
Fe	-0.37	0.68	0.64
S	-0.47	0.16	0.87
Al	-0.43	-0.16	0.89
Mg	-0.80	-0.38	0.47
Si	0.11	-0.14	**-0.98**
Ca	-0.64	-0.77	0.04
P	-0.40	0.57	0.72
Na	-0.45	-0.09	-0.89
K	-0.45	-0.09	-0.89
As	-0.39	0.29	0.87
Rb	-0.36	0.71	0.61
Cs	-0.44	-0.16	0.88

Values in bold are different from 0 with a significance level α=0.10 (r > 0.90).

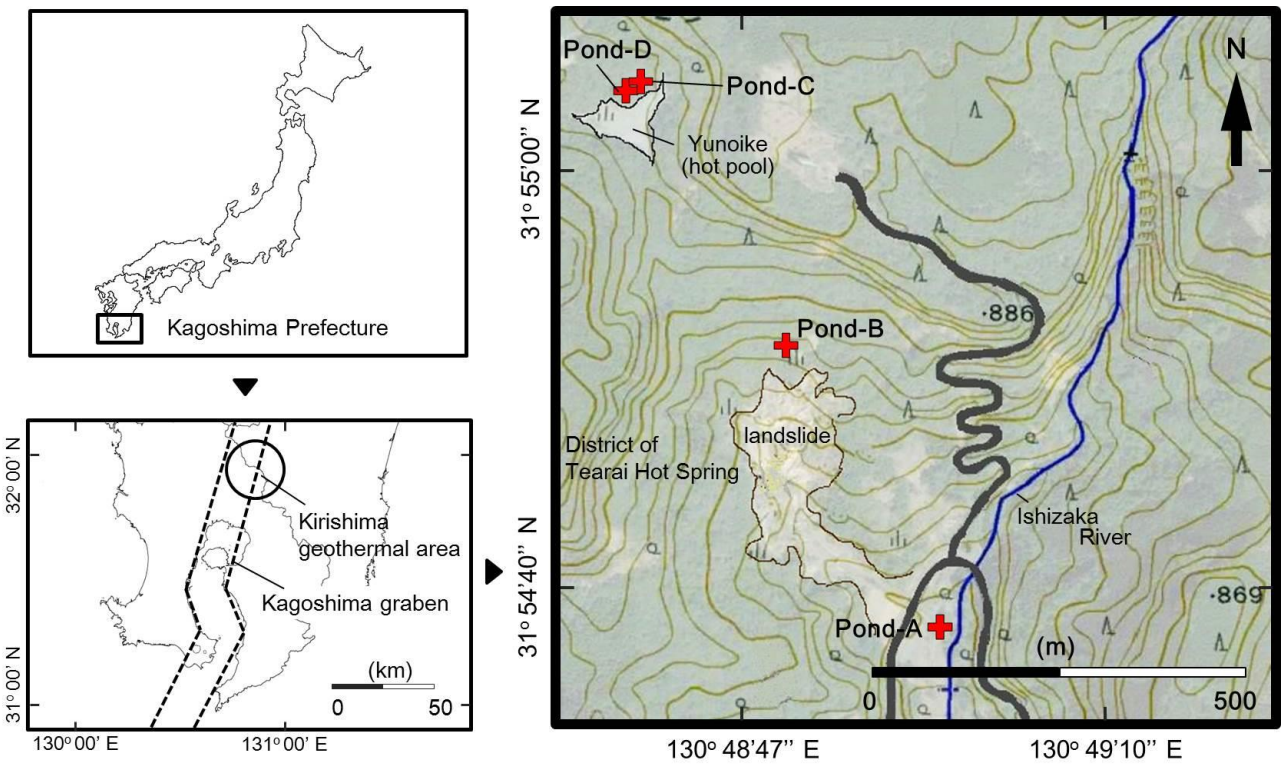

Figure 3-1. Map of the sampling sites in/near the region of the Tearai hot spring, the Kirishima geothermal area, Kagoshima Prefecture.

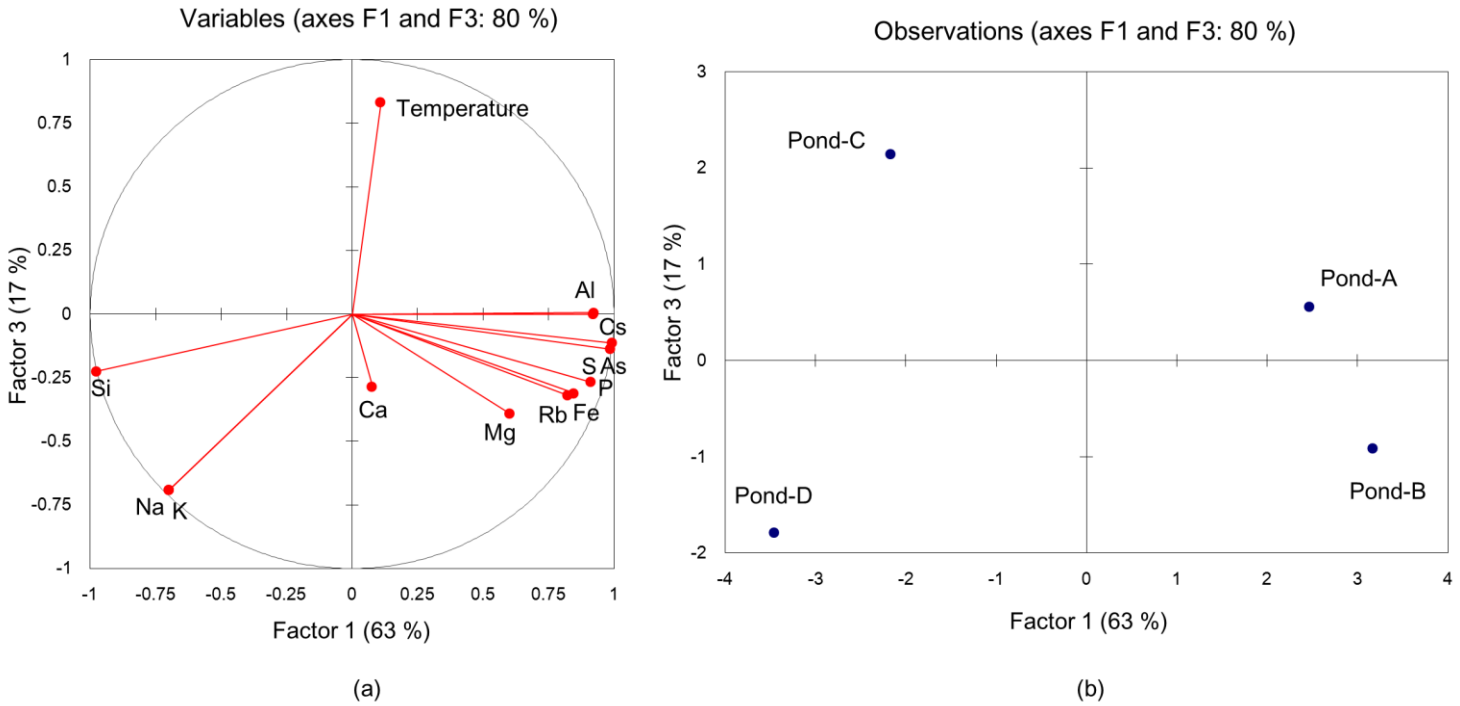

Figure 3-2. Principal components analysis showing the 13 environmental variables of the four ponds. (a) Factor loadings on principal components 1 and 3, (b) relationships between the four ponds and the principal components.

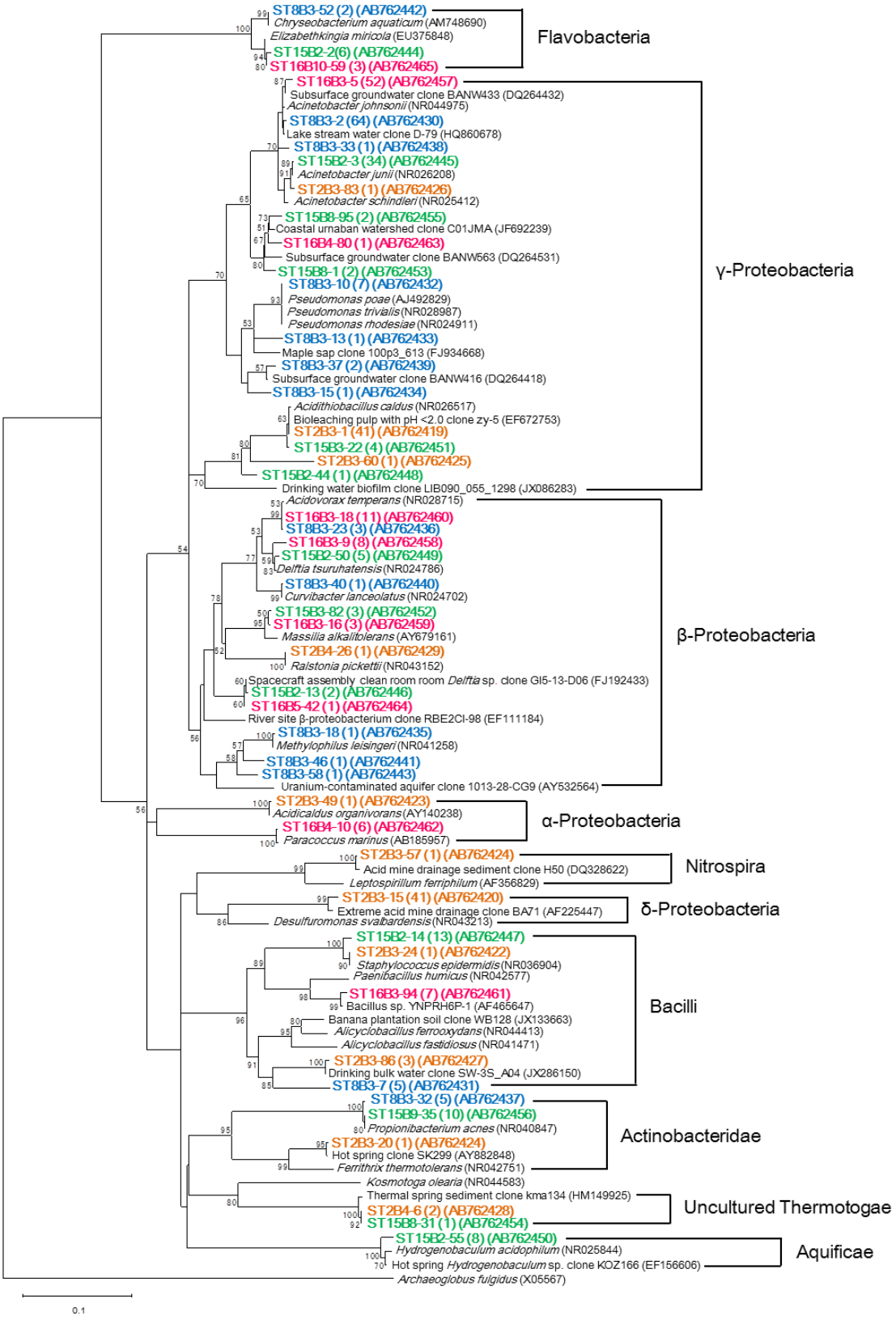
ST8B3-52 (2) (AB762442)
Chryseobacterium aquaticum (AM748690)
Elizabethkingia miricola (EU375848)
ST15B2-2(6) (AB762444)
ST16B10-59 (3) (AB762465)
Flavobacteria
ST16B3-5 (52) (AB762457)
Subsurface groundwater clone BANW433 (DQ264432)
Acinetobacter johnsonii (NR044975)
ST8B3-2 (64) (AB762430)
Lake stream water clone D-79 (HQ860678)
ST8B3-33 (1) (AB762438)
ST15B2-3 (34) (AB762445)
Acinetobacter junii (NR026208)
ST2B3-83 (1) (AB762426)
Acinetobacter schindleri (NR025412)
ST15B8-95 (2) (AB762455)
Coastal urnaban watershed clone C01JMA (JF692239)
ST16B4-80 (1) (AB762463)
Subsurface groundwater clone BANW563 (DQ264531)
ST15B8-1 (2) (AB762453)
ST8B3-10 (7) (AB762432)
Pseudomonas poae (AJ492829)
Pseudomonas trivialis (NR028987)
Pseudomonas rhodesiae (NR024911)
ST8B3-13 (1) (AB762433)
Maple sap clone 100p3_613 (FJ934668)
ST8B3-37 (2) (AB762439)
Subsurface groundwater clone BANW416 (DQ264418)
ST8B3-15 (1) (AB762434)
γ-Proteobacteria
Acidithiobacillus caldus (NR026517)
Bioleaching pulp with pH <2.0 clone zy-5 (EF672753)
ST2B3-1 (41) (AB762419)
ST15B3-22 (4) (AB762451)
ST2B3-60 (1) (AB762425)
ST15B2-44 (1) (AB762448)
Drinking water biofilm clone LIB090_055_1298 (JX086283)
Acidovorax temperans (NR028715)
ST16B3-18 (11) (AB762460)
ST8B3-23 (3) (AB762436)
ST16B3-9 (8) (AB762458)
ST15B2-50 (5) (AB762449)
Delftia tsuruhatensis (NR024786)
ST8B3-40 (1) (AB762440)
Curvibacter lanceolatus (NR024702)
ST15B3-82 (3) (AB762452)
ST16B3-16 (3) (AB762459)
Massilia alkalitolerans (AY679161)
ST2B4-26 (1) (AB762429)
Ralstonia picketti (NR043152)
Spacecraft assembly clean room room Delftia sp. clone GI5-13-D06 (FJ192433)
ST15B2-13 (2) (AB762446)
ST16B5-42 (1) (AB762464)
River site β-proteobacterium clone RBE2CI-98 (EF111184)
ST8B3-18 (1) (AB762435)
Methylophilus leisingeri (NR041258)
ST8B3-46 (1) (AB762441)
ST8B3-58 (1) (AB762443)
Uranium-contaminated aquifer clone 1013-28-CG9 (AY532564)
β-Proteobacteria
ST2B3-49 (1) (AB762423)
Acidicaldus organivorans (AY140238)
ST16B4-10 (6) (AB762462)
Paracoccus marinus (AB185957)
α-Proteobacteria
ST2B3-57 (1) (AB762424)
Acid mine drainage sediment clone H50 (DQ328622)
Leptospirillum ferriphilum (AF356829)
Nitrospira
ST2B3-15 (41) (AB762420)
Extreme acid mine drainage clone BA71 (AF225447)
Desulfuromonas svalbardensis (NR043213)
δ-Proteobacteria
ST15B2-14 (13) (AB762447)
ST2B3-24 (1) (AB762422)
Staphylococcus epidermidis (NR036904)
Paenibacillus humicus (NR042577)
ST16B3-94 (7) (AB762461)
Bacillus sp. YNPRH6P-1 (AF465647)
Banana plantation soil clone WB128 (JX133663)
Alicyclobacillus ferrooxydans (NR044413)
Alicyclobacillus fastidiosus (NR041471)
ST2B3-86 (3) (AB762427)
Drinking bulk water clone SW-3S_A04 (JX286150)
ST8B3-7 (5) (AB762431)
Bacilli
ST8B3-32 (5) (AB762437)
ST15B9-35 (10) (AB762456)
Propionibacterium acnes (NR040847)
ST2B3-20 (1) (AB762424)
Hot spring clone SK299 (AY882848)
Ferrithrix thermotolerans (NR042751)
Actinobacteridae
Kosmotoga olearia (NR044583)
Thermal spring sediment clone kma134 (HM149925)
ST2B4-6 (2) (AB762428)
ST15B8-31 (1) (AB762454)
Uncultured Thermotogae
ST15B2-55 (8) (AB762450)
Hydrogenobaculum acidophilum (NR025844)
Hot spring Hydrogenobaculum sp. clone KOZ166 (EF156606)
Aquificae
Archaeoglobus fulgidus (X05567)
0.1

Figure 3-3. Phylogenetic tree of bacterial 16S rRNA gene clones detected in the four ponds from the Kirishima geothermal area. Bootstrap values (>50%) based on 1000 replicates are indicated at nodes. The scale bar indicates the number of nucleotide substitutions per position. The number in parenthesis with next to the phylotype name represents the number of clones from each phylotype. The DNA database accession numbers are also indicated in parentheses. Archaeoglobus fulgidus was used as an outgroup species. The phylotype names derived from Ponds-A, B, C, and D are shown in blue, yellow, red, and green, respectively.

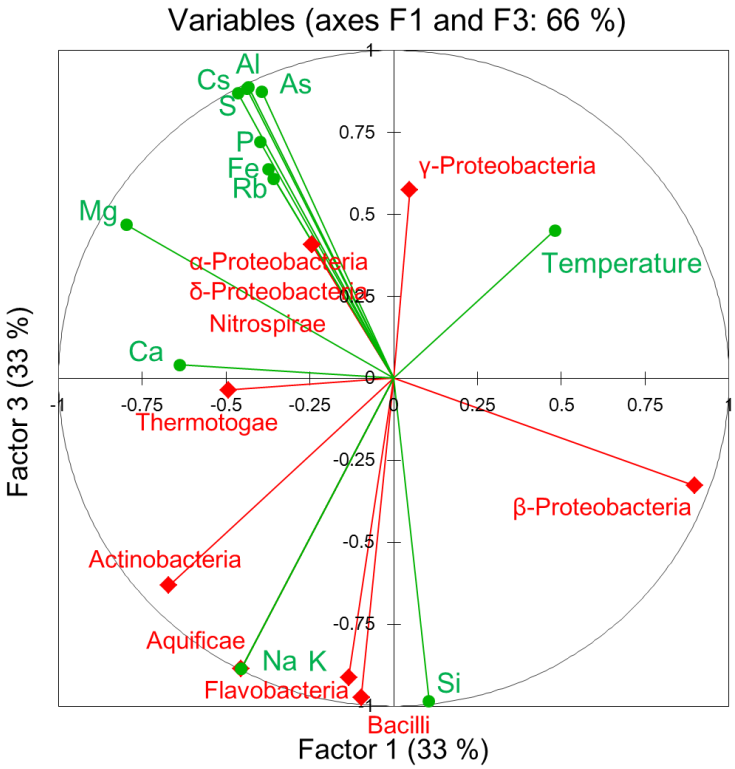

Figure 3-4. Canonical correlation analysis showing correlations between environmental factors and proportions of individual bacterial groups. Bacterial groups are shown in abbreviations in a red rhombus shape. Environmental factors are shown in green circles.

Chapter IV

Archaeal community structures and biodiversity

in the highly acidic hot ponds with different temperatures and chemical components

4.1. Introduction

The extreme environments are unique places to study how organisms interact with and adapt to the surroundings. A variety of microorganisms actively habit in the extreme environments and are called extremophiles. Archaea, the other domains of Prokaryote, includes many kinds of extremophiles such as thermophiles, halophiles and methanogens. Particularly, archaeal thermophiles are thought to have some similar characteristics with common ancestor of life. As mentioned in the introduction of Chapter III, archaeal species have distinguishable phenotypic characteriastics with bacterial ones. This Chapter focuses on the archaeal community structures and diversity of four distinct solfataric-acidic ponds in the Kirishima geothermal area.

The following places were subjected to study about archaeal community structures and/or its diversities in hot springs: Yellowstone National Park in the United States (Barns *et al*., 1994; Barns *et al*., 1996; Hugenholtz *et al*., 1998; Reysenbach *et al*., 2000; Blank *et al*., 2002; Meyer-Dombard *et al*., 2005), Kamchatka hot springs in Russia (Perevalova *et al*., 2008), the island of the Lesser Antilles (Burton and Norris, 2000; Stout *et al*., 2009), Icelandic hot springs (Kvist *et al*., 2007; Perevalova *et al*., 2008), Mt.Unzen hot springs in Japan (Takai and Sako, 1999), Ohwakudani hot springs in Japan (Kato *et al*., 2011), Pisciarelli hot springs in Italy (Kvist *et al*., 2005), Bor Khlueng hot springs in Thailand (Kanokratana *et al*., 2004), Wai-o-tapu geothermal area in New Zealand (Childs *et al*., 2008), and Tengchong hot springs in China (Song *et al*., 2010). As same as the bacterial studies, these pioneering works lack the analyses about

relationships between the archaeal communities/diversity and environmental factors. It might be important to reveal that how environmental factors affect archaeal community structures and diversity in individual hot spring habitats. The objective of this chapter is to reveal the archaeal communities and diversity in hot springs displaying a wide range of temperature and chemical copositions of Kirishima geothermal area, and to clearlify the relationships between the archaeal communities and environmental factors (temperature and chemical components).

4.2. Materials and methods

4.2.1 Sample collection and analysis of chemical components

The contents of this section are described in Section 3.2.1 (page 35).

4.2.2. 16S rRNA gene clone libraries and sequencing

Environmental DNA was extracted from 5 to 10 g of each muddy water sample including sediment using the UltraClean Soil DNA Kit Mega Prep (Mo Bio Laboratories) according to the manufacturer's instructions. The precipitated DNA was purified using the GFX PCR DNA and Gel Band Purification Kit (GE Healthcare).

Purified DNA was used as the template for the amplification of archaeal 16S rRNA gene by archaea-specific primer A21F: 5′-TTCCGGTTGATCCYGCCGGA and universal primer U1492R: 5′- GGYTACCTTGTTACGACTT. The PCR conditions included an initial denaturation step at 94°C for 3 min, followed by 35 cycles of denaturation at 94°C for 30 sec, annealing at 55°C for 30 sec, and extension at 72°C for 2 min using Ex Taq DNA polymerase (Takara Bio). This was followed by a final extension at 72°C for 10 min.

The PCR products were purified using the aforementioned GFX Kit and were ligated into the pT7 Blue T-Vector (Novagen). *E. coli* DH5α cells were transformed with the plasmid library and were plated onto LB plates including 100 μg mL-1 ampicillin, 40 μg mL^{-1} X-gal and 0.5 mM IPTG. Blue/white selection was conducted by randomly picking and subculturing individual white colonies in 100 μL of 2× YT medium containing 100 μg mL^{-1} ampicillin in a 96-well plate at 37°C overnight. The inserted 16S rRNA gene was amplified using 1 μL of the culture as the template with the same PCR procedure mentioned above. About 800 bp of the 5′-region of each 16S rRNA gene clone was sequenced by aforementioned archaea-specific

primer A21F and used for taxonomic and phylogenetic analysis.

4.2.3. Identification of 16S rRNA gene clones and Phylogenetic analysis

The contents of this section are described in Section 3.2.3 (page 36).

4.2.4. Statistical analyses

The contents of this section are described in Section 3.2.4 (page 37).

4.2.5. Nucleotide sequence accession numbers

The representatives of nucleotide sequences of the phylotypes are available in the DDBJ/EMBL/GenBank databases under the accession numbers AB753272-AB753298 and AB755799-AB755806.

4.3. Results and Discussion

4.3.1. Water chemistry

The contents of this section are described in Section 3.3.1 (page 39).

4.3.2. 16S rRNA gene clone libraries

16S rRNA gene clone libraries were successfully constructed using the environmental DNAs extracted from four muddy water samples including sediments. A total of 432 clones of archaeal 16S rRNA gene were analyzed. A chimerical sequence was detected during the analysis and was not used for further study. On the basis of the sequence similarity values, a total of 431 clones (Pond-A: 106, Pond-B: 112, Pond-C: 109, Pond-D: 104 clones) were classified into 26 phylotypes, consisting of 25 crenarchaeal phylotypes and a single euryarchaeal one (Table 4-1). The homologous coverage values were 0.88 or above for all ponds indicating that approximately 90% of the 16S rRNA gene clones in these ponds could be considered in this study (Table 4-2).

The guanine-plus-cytosine (G+C) content in the 16S rRNA gene sequences detected from 26 phylotypes in this study ranged from 56.6% to 69.0%, with an overall average of 62.4 ± 3.6%. According to Kimura *et al*, the growth temperature of archaea are strongly correlated with the G+C content, while the phylotypes containing this amount of G+C were grouped as the moderately thermophilic and hyperthermophilic archaea (Kimura *et al*., 2010). Therefore, all phylotypes detected in this study were could possibly related to moderately thermophilic and hyperthermophilic archaea.

4.3.3. Archaeal community in Pond-A

Pond-A was characterized by relatively high temperature and high total concentrations of the examined chemical components. Analysis of 16S rRNA gene sequence similarities of 106 clones derived from the pond revealed five phylotypes of Crenarchaeota, which was smallest number of phylotypes detected of all four ponds (Table 4-1). 5 and 6% sequences of this pond were highly similar to those of cultured species (>98.0%) of the order Thermoproteales, *Caldivirga maquilingensis* and *Vulcanisaeta distributa*, respectively. The type strains of both species were hyperthermophilic archaea optimally growing at above 85°C, and they were originally isolated from acidic hot springs in Philippines and Japan, respectively (Itoh *et al*., 1999; Itoh *et al*., 2002).

On the other hand, other 89% of Pond-A clones did not show significant similarities with any cultured species. Almost all the clones of them were assigned as a phylotype ST8A1-12 affiliated with the order Sulfolobales. This phylotype showed 95-96% sequence similarity with published environmental clones, NAKO74-07 and HS3wa_52 detected from Nakabusa hot spring, Japan (DNA database Accession No. AB366602) (Kimura *et al*., 2010) and Tatung Volcano hot spring, Taiwan (DNA database Acc. No. FJ797311). The species diversity assessed by the Shannon-Weaver index and Simpson's reciprocal index in the Pond-A was lowest among the four ponds (Table 4-2). And also, the gene diversity (θ_π) in this pond was the lowest among all four ponds (Table 4-2).

4.3.4. Archaeal community in Pond-B

Pond-B was characterized by a relatively low temperature and a high total concentration of the examined chemical components; it had the largest number of phylotypes, resulting that the species diversity indices and evenness value in this pond were highest among

the four ponds (Table 4-2). Additionaly, the gene diversity (θ_π) was the highest value among all four ponds (Table 4-2). A total of 112 clones consisted of 14 phylotypes that were classified into the following six groups; the order Sulfolobales, Acidilobales, Thermoproteales, and three uncultured crenarchaeal groups (Table 4-1, Fig. 4-1). 21% of the total clones were closely related to any of five cultured species (>98.0 %); *Sulfolobus solfataricus* (Zilling *et al*., 1980), *Metallosphaera sedula* (Huber *et al*., 1989), *Acidianus brierleyi* (Segerer *et al*., 1986), *Caldisphaera lagunensis* (Itoh *et al*., 2003) and *Caldivirga maquilingensis* (Itoh *et al*., 1999). *S. solfataricus*, *M. sedula* and *A. brierleyi* are facultatively chemolithoautotrophic aerobes and require elemental sulfur or sulfidic ores. These species and their close relatives have been isolated from acidic solfatara fields around the world (Huber and Stetter, 2001). *C. lagunensis* and *C. maquilingensis* are heterotrophic anaerobes. Their growths are stimulated or constrained by the presence of sulfur as an electron acceptor.

On the other hand, nine phylotypes sharing 79% in total of all Pond-B clones showed no significant similarity with any cultured species. Nearly half of these uncultured clones were assigned as a phylotype ST2A1-5. This phylotype was most dominant (35%) in Pond-B and was phylogenetically distant not only from any cultured species, but also from any published environmental clones. This novel phylotype belonged to a cluster in the order Sulfolobales (Fig. 4-1). This cluster also harbored another Pond-B phylotype ST2A1-32, which showed 98% 16S rRNA gene sequence similarity with published environmental clone, LH2wa_90 detected from Taiwanese hot spring (DNA database Acc. No. FJ797343).

The phylotype ST2A1-8 belonging to the uncultured thermoacidic spring clone group (UTSCG) (Kato *et al*., 2011) was secondary dominant in Pond-B and it shared 18% of the total Pond-B clones. Interestingly, phylotypes similar to ST2A1-8 were also frequently detected in

Pond-C and Pond-D, suggesting that this crenarchaeal species survive relatively wide range of temperature and chemical components in acidic hot springs. There might be unfavorable factors in Pond-A for the presence of UTSCG. The phylotypes ST2A1-2 and ST2A1-15 were placed in the sister cluster of UTSCG with published clones detected from Yellowstone National Park (DNA database Acc. No. DQ834245). We call this cluster as UTSCG II in this study.

The phylotype ST2A1-25 was thirdly dominant in Pond-B and was placed into the sister cluster of the hot water crenarchaeotic group II (HWCG II) (Takai and Sako, 1999; Schrenk *et al*., 2003; Nunoura *et al*., 2005) with phylotype ST2A1-52 and published environmental clone SK859 detected from acidic hot spring in Yellowstone National Park (DNA database Acc. No. DQ834111). We call this cluster as HWCG VI in this study. The phylotype ST2A1-25 was also dominant in Pond-C.

4.3.5. Archaeal community in Pond-C

Pond-C was another pond with a relatively high temperature and showed relatively low value of species richness as same as Pond-A (Table 4-2). A hundred and nine clones were classified into six phylotypes as follows; *Thermocladium modestius* of the order Thermoproteales, four uncultured crenarchaeal phylotypes and uncultured euryarchaeal phylotypes. The type strain of *T. modestius* was originally isolated from solfataric mud at Noji-onsen, Japan, and is an anaerobic heterotroph growing optimally around 75°C, pH 4.0 (Itoh *et al*., 1998).

As mentioned in the previous section, the uncultured phylotypes ST2A1-8 of UTSCG and ST2A1-25 of HWCG VI were dominant in the clone library constructed for this pond sample. These two phylotypes shared 81% in total of the Pond-C clones. Three phylotypes sharing 56% of Pond-C clones were affiliated with uncultured thermoacidic spring clone group (UTSCG) (Kato *et*

al., 2011).

4.3.6. Archaeal community in Pond-D

Pond-D was characterized by a relatively low temperature and a low total concentration of the examined chemical components. A hundred and four clones derived from this pond consisted of nine phylotypes. The species diversity indices in Pond-D were lower than those in Pond-B, but were higher than the values in Pond-A and Pond-C (Table 4-2). The phylotype sharing 20% of the total clones was related to *Caldisphaera draconis* with 95% sequence similarity of 16S rRNA gene. This species is chemoorganotrophic anaerobe isolated from acidic hot spring in Yellowstone National Park (Boyed *et al*., 2007).

Other phylotypes showed no significant similarity with any cultured species. Most frequent phylotype was ST2A1-8 affiliated with UTSCG and it shared 50% of the total clone in this pond. In contrast to the archaeal communities in other three ponds, the secondary dominant uncultured phylotype (ST16A1-50) was affiliated with Euryarchaeota and showed 99% sequence similarity of 16S rRNA gene with thermal spring clone kmc048 detected from Kamchatka hot springs in Russia (DNA database Acc. No. HM150106). This phylotype shared 16% of the total clones in this pond.

The phylotype ST15A1-26 together with ST15A2-137 and ST15A1-32 were barely detected in Pond-D, and were placed into the sister cluster of HWCG II (Takai and Sako, 1999; Schrenk *et al*., 2003; Nunoura *et al*., 2005). We call these clusters as HWCG V and HWCG VII, respectively in this study.

4.3.7. Archaeal biodiversity and community structure in relation to different temperatures and different total concentrations of the examined chemical components

When the species diversity was compared within ponds with different temperatures (Temp. approx. 90°C, Pond-A + Pond-C vs. Temp. approx. 70°C, Pond-B + Pond-D), assessed by the Shannon-Weaver index and Simpson's reciprocal index, the lower temperature ponds showed higher diversity (Table 4-2). The gene diversity (θ_π) showed higher value in the lower temperature pond as same as the species diversity. On the other hand, when comparing within ponds with different concentrations of the examined chemical components, the species diversity indices of the pond with higher concentration of the chemical components (Total conc. > 55 mmol L^{-1}, Pond-A + Pond-B), was higher than that of Pond-C + Pond-D (Total conc. < 15 mmol L^{-1}). The gene diversity (θ_π) showed almost the same value within ponds with different concentrations of the examined chemical components. As a result, the archaeal diversity was highest in the pond characterized as lower temperature and higher total dissolved elemental concentration (Pond-B). In contrast, the combination of higher temperature and lower total dissolved elemental concentration (Pond-A) caused lowest diversity in this study.

When focusing on the species composition and distribution, they were dissimilarity within ponds with different temperature and chemical components. As shown in Table 4-1, the phylotypes affiliated with the order Sulfolobales were only detected in the ponds showing higher concentrations of the examined chemical components (Pond-A + Pond-B). The members of the order Sulfolobales is generally characterized as facultatively or obligately chemolithoautotrophic S^0 metabolizers, some members oxidize ferrous iron and sulfidic ores, producing soluble metal sulfates (Huber and Stetter, 2001). Therefore, the presence of Sulfolobales makes sense in these ponds including higher concentrations of the examined chemical components, especially sulfur, as

we would expect to detect microbes that metabolically depend on sulfur as an electron donor. It is also interesting that the species composition within the order Sulfolobales between Pond-A and Pond-B was clearly different each other. We detected sequences closely related to Sulfolobales species (98.9% similarity) in Pond-B (66°C), but most of the clones detected from Pond-A (93^{o}C) were affiliated with uncultured Sulfolobales, forming a phylotype ST8A1-12. In addition, the members of the genus *Caldisphaera* of the order Acidilobales were frequently detected from lower temperature ponds (Pond-B + Pond-D), but not detected from higher temperature ponds (Pond-A + Pond-C). This may be due to the growth temperature limit of the members of the genus *Caldisphaera*, which is 85^{o}C (Prokofeva *et al*., 2009).

4.3.8. Geochemistry and archaeal biodiversity or groups correlations

As shown in Table 4-3, archaeal species diversity was statistically correlated with pH and relatively correlated with temperature. The gene diversity (θ_{π}) was relatively correlated with the species diversity (Table 4-3).

To clarify the relationships between archaeal groups and themperature or chemical component of the four ponds, canonical correlation analysis was performed (Fig. 4-2, Table 4-4, 4-5, 4-6, and 4-7). Specific archaeal groups were found to be correlated with particular factors: the order Sulfolobales with S, Al, and Cs and uncultured Euryarchaeota with Na and K, whereas UTSCG group was negatively correlated with S, Al, and Ca. In addition, the order Acidilobales and HWCG group were negatively correlated with Temperature and Mg, respectively. To date, the element requirements in archaea were conducted on certain cultured species and little were known on the uncultured archaea. However, the correlations between the uncultured archaeal groups and the dissolved elemental concentrations shown in this study could give more insights into how

specific elements affect uncultured archaeal communities.

Table 4-1. Affiliation and closest published species or clones of 26 phylotypes

Phylotypes	Affiliation	Closest species or clones (accession number)	16S rRNA gene similarity (%)	Number of clones detected from each site			
				Pond -A	Pond -B	Pond -C	Pond -D
	order Sulfolobales						
ST2A1-3	*Acidianus brierleyi*	*Acidianus brierleyi* (D26489)	98.9		3		
ST2A1-43	*Acidianus* sp.	*Acidianus ambivalens* (D85506)	95.5		1		
ST2A1-14	*Metallosphaera sedula*	*Metallosphaera sedula* (D26491)	100.0		7		
ST2A1-16	*Sulfolobus solfataricus*	*Sulfolobus solfataricus* (D26490)	99.1		2		
ST8A1-57	*Sulfurisphaera* sp.	*Sulfurisphaera ohwakuensis* (D85507)	95.1	1			
ST2A1-5	Uncultured Sulfolobales	Acidic hot spring clone HO78W21A35 (AB600386)	80.7		39		
ST2A1-32	Uncultured Sulfolobales	Acidic sulfuric hot spring clone LH2wa_90 (FJ797343)	98.3		6		
ST8A1-12	Uncultured Sulfolobales	Acidic sulfuric hot spring clone HS3wa_52 (FJ797311)	96.6	92			
ST8A1-52	Uncultured Sulfolobales	Acidic sulfuric hot spring clone HS3wa_52 (FJ797311)	94.1	1			
	order Acidilobales						
ST2A1-9	*Caldisphaera lagunensis*	*Caldisphaera lagunensis* (AB087499)	98.5		10		
ST2A1-27 (=ST15A1-7)	*Caldisphaera* sp.	*Caldisphaera draconis* (EF057392)	95.4		2		21
	order Thermoproteales						
ST8A1-8 (=ST2A1-31)	*Caldivirga maquilingensis*	*Caldivirga maquilingensis* (AB013926)	98.0	5	2		
ST8A1-40	*Vulcanisaeta distributa*	*Vulcanisaeta distributa* (AB063630)	98.9	7			
ST16A1-87	*Thermocladium modestius*	*Thermocladium modestius* (AB005296)	99.4			1	
	other crenarchaeal groups						
ST2A1-8 (=ST16A1-1, ST15A1-1)	UTSCG	Acidic sulfuric hot spring clone LH2wa_02 (FJ797332)	99.7		20	42	52
ST15A1-3	UTSCG	Acidic hot spring clone Uzon4-5d (HQ395709)	96.1				2
ST16A1-6	UTSCG	Acidic spring clone HO28S9A21 (AB600335)	96.9			6	
ST16A1-20 (=ST15A1-6)	UTSCG	Hot spring clone SK865 (DQ834117)	96.9			13	6
ST2A1-2	UTSCG II	Hot spring clone BW303 (DQ924843)	93.3		4		
ST2A1-15	UTSCG II	Hot spring clone SK993 (DQ834245)	99.7		1		
ST15A1-26	HWCG V	Acidic spring clone HO28S9A51 (AB600343)	96.3				1
ST15A2-137	HWCG V	Acidic spring clone HO28S9A51 (AB600343)	98.4				2
ST2A1-25 (=ST16A1-2, ST15A1-34)	HWCG VI	Hot spring clone SK859 (DQ834111)	95.8		13	46	2
ST2A1-52	HWCG VI	Hot spring clone SK859 (DQ834111)	96.0		2		
ST15A1-32	HWCG VII	Acidic sulfuric hot spring clone HS4sa_15 (FJ797318)	91.2				1
	euryarchaeal groups						
ST16A1-50 (=ST15A1-8)	Uncultured Euryarchaeota	Thermal spring clone kmc048 (HM150106)	99.2			1	17
Total				106	112	109	104

Table 4-2. Diversity index scores for clone libraries of archaea detected in the ponds of the Kirishima geothermal area

Sample	Shannon	Simpson	Rich	Even	S_{ACE}	S_{Chao1}	θ_π	Coverage	Total clone number
Pond-A	0.53	1.32	5	0.332	7.04	6.00	60.2 ± 36.8	0.95	106
Pond-B	2.06	5.41	14	0.780	15.4	14.2	121 ± 61.9	0.88	112
Pond-C	1.23	2.91	6	0.687	10.1	7.00	115 ± 67.1	0.94	109
Pond-D	1.45	3.10	9	0.659	10.9	9.25	112 ± 60.2	0.91	104
Temp. approx. 90 °C (Pond-A + Pond-C)	1.58	3.66	11	0.659	16.0	17.0	107 ± 56.2	0.95	215
Temp. approx. 70 °C (Pond-B+ Pond-D)	2.20	5.82	20	0.735	23.3	21.0	123 ± 61.5	0.91	216
Total conc. > 55 mmol L^{-1} (Pond-A + Pond-B)	1.99	4.37	18	0.689	21.0	19.5	111 ± 55.9	0.92	218
Total conc. < 15 mmol L^{-1} (Pond-C + Pond-D)	1.61	3.68	11	0.673	15.5	13.3	111 ± 58.2	0.95	213

Diversity index scores measured were Shannon-Weaver index (Shannon), Simpson's reciprocal index (Simpson), Richness (Rich), Evenness (Even), the coverage estomators S_{ACE} and S_{Chao1}, the gene diversity index θ_π, and the homologous coverage. Temp. and Total conc. indicate temperature and total concentration of examined chemical components, respectively.

Table 4-3. Correlation matrix showing *r* values for Pearson's correlation among environmental variables in the pond waters of the Kirishima geothermal area; N=4

Variables	θ_π	Shannon	Temp.	pH	Fe	S	Al	Mg	Si	Ca	P	Na	K	As	Rb	Cs	Total conc.
θ_π	—	0.88	-0.70	-0.80	0.13	-0.44	-0.70	-0.78	0.40	-0.83	-0.01	0.23	0.23	-0.32	0.17	-0.70	-0.30
Shannon	—	—	-0.87	**-0.99**	0.54	0.00	-0.32	-0.41	0.07	-0.71	0.42	0.14	0.14	0.11	0.58	-0.32	0.16
Temp.	—	—	—	0.86	-0.40	0.05	0.31	0.12	-0.29	0.29	-0.29	-0.55	-0.55	-0.01	-0.43	0.31	-0.10
pH	—	—	—	—	-0.67	-0.16	0.17	0.29	0.07	0.65	-0.56	-0.07	-0.07	-0.27	-0.70	0.17	-0.31
Fe	—	—	—	—	—	0.84	0.62	0.34	-0.76	-0.26	**0.99**	-0.46	-0.46	**0.90**	**1.00**	0.62	**0.91**
S	—	—	—	—	—	—	**0.95**	0.71	**-0.93**	0.21	**0.91**	-0.58	-0.58	**0.99**	0.81	**0.95**	**0.99**
Al	—	—	—	—	—	—	—	0.82	**-0.90**	0.44	0.72	-0.57	-0.57	**0.90**	0.58	**1.00**	0.89
Mg	—	—	—	—	—	—	—	—	-0.49	0.82	0.44	-0.02	-0.02	0.61	0.30	0.83	0.66
Si	—	—	—	—	—	—	—	—	—	0.00	-0.83	0.84	0.84	**-0.94**	-0.74	-0.89	-0.89
Ca	—	—	—	—	—	—	—	—	—	—	-0.15	0.33	0.33	0.07	-0.29	0.44	0.12
P	—	—	—	—	—	—	—	—	—	—	—	-0.51	-0.51	**0.95**	**0.98**	0.72	**0.96**
Na	—	—	—	—	—	—	—	—	—	—	—	—	—	-0.62	-0.44	-0.57	-0.52
K	—	—	—	—	—	—	—	—	—	—	—	—	—	-0.62	-0.44	-0.57	-0.52
As	—	—	—	—	—	—	—	—	—	—	—	—	—	—	0.88	**0.90**	**0.99**
Rb	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	0.58	0.89
Cs	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	0.89
Total conc.	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—

Values in bold are different from 0 with a significance level $\alpha = 0.10$ ($r > 0.90$). The only Shannon-Weaver index for archaeal clone libraries is shown as species diversity.

θ_π, Shannon, Temp., and Total conc. indicate the gene diversity index, Shannon–Weaver index, Temperature, and total concentration of examined chemical components, respectively.

Table 4-4. Correlation matrix showing *r* values for Pearson's correlation between environmental factors and proportions of indivisual archaeal groups detected from the four ponds in the Kirishima geothermal area; N=4

Variables	Sulfolobales	Acidilobales	Thermoproteales	UTSCG	HWCG	Uncultured Euryarchaeota
Sulfolobales	—	-0.41	0.89	**-1.00**	-0.61	-0.59
Acidilobales	—	—	-0.59	0.43	-0.39	0.83
Thermoproteales	—	—	—	-0.88	-0.51	-0.49
UTSCG	—	—	—	—	0.58	0.62
HWCG	—	—	—	—	—	-0.28
Uncultured Euryarchaeota	—	—	—	—	—	—
Temperature	0.35	**-0.90**	0.68	-0.35	0.21	-0.53
Fe	0.56	0.06	0.13	-0.58	-0.29	-0.50
S	**0.92**	-0.25	0.65	**-0.93**	-0.53	-0.62
Al	**1.00**	-0.41	0.86	**-1.00**	-0.58	-0.62
Mg	0.85	0.01	0.80	-0.83	**-0.92**	-0.07
Si	-0.87	0.55	-0.65	0.89	0.21	0.87
Ca	0.50	0.05	0.69	-0.46	-0.80	0.29
P	0.67	-0.02	0.27	-0.69	-0.35	-0.55
Na	-0.54	0.84	-0.46	0.57	-0.33	**1.00**
K	-0.54	0.84	-0.46	0.57	-0.33	**1.00**
As	0.87	-0.24	0.56	-0.88	-0.43	-0.67
Rb	0.52	0.09	0.09	-0.55	-0.26	-0.48
Cs	**1.00**	-0.40	0.86	**-1.00**	-0.59	-0.61

Values in bold are different from 0 with a significance level α=0.10 ($r > 0.90$). UTSCG: uncultured thermoacidic spring clone group (Kato *et al*., 2011), HWCG: hot water crenarchaeotic group (Barns *et al*., 1996; Takai and Horikoshi, 1999; Takai and Sako, 1999; Inagaki *et al*., 2003; Schrenk *et al*., 2003; Nunoura *et al*., 2005; Satoh *et al*., 2013)

Table 4-5. The eigenvalues, the corresponding inertia, and the corresponding percentages of canonical correlation analysis between environmental variables and proportions of individual archaeal groups detected from the four ponds in the Kirishima geothermal area; N=4

Factor	Eigenvalue	Total variance (%)	Cumulative eigenvalue	Cumulative variance (%)
Factor 1	1.00	33.3	1.00	33.3
Factor 2	1.00	33.3	2.00	66.7
Factor 3	1.00	33.3	3.00	100.0

Table 4-6. Canonical factor loadings for proportions of individual six archaeal groups in the four pond of the Kirishima geothermal area

	Factor 1	Factor 2	Factor 3
Sulfolobales	-0.66	0.04	0.75
Acidilobales	0.42	**-0.90**	-0.14
Thermoproteales	**-0.91**	0.18	0.83
UTSCG	0.64	-0.07	-0.77
HWCG	0.56	0.75	-0.36
Uncultured Euryarchaeota	0.13	-0.77	-0.63

Values in bold are different from 0 with a significance level α=0.10 (r > 0.90). UTSCG: uncultured thermoacidic spring clone group (Kato *et al*., 2011), HWCG: hot water crenarchaeotic group (Barns *et al*., 1996; Takai and Horikoshi, 1999; Takai and Sako, 1999; Inagaki *et al*., 2003; Schrenk *et al*., 2003; Nunoura *et al*., 2005; Satoh *et al*., 2013)

Table 4-7. Canonical factor loadings for 13 environmental variables in the four pond of the Kirishima geothermal area

	Factor 1	Factor 2	Factor 3
Temperature	-0.68	0.72	-0.17
Fe	0.24	-0.10	**0.97**
S	-0.32	-0.02	**0.95**
Al	-0.60	0.06	0.80
Mg	-0.76	-0.44	0.49
Si	0.27	-0.35	**-0.90**
Ca	-0.89	-0.45	-0.09
P	0.11	-0.08	**0.99**
Na	0.10	-0.80	-0.59
K	0.10	-0.80	-0.59
As	-0.20	0.03	**0.98**
Rb	0.29	-0.11	**0.95**
Cs	-0.61	0.05	0.80

Values in bold are different from 0 with a significance level α=0.10 (r > 0.90).

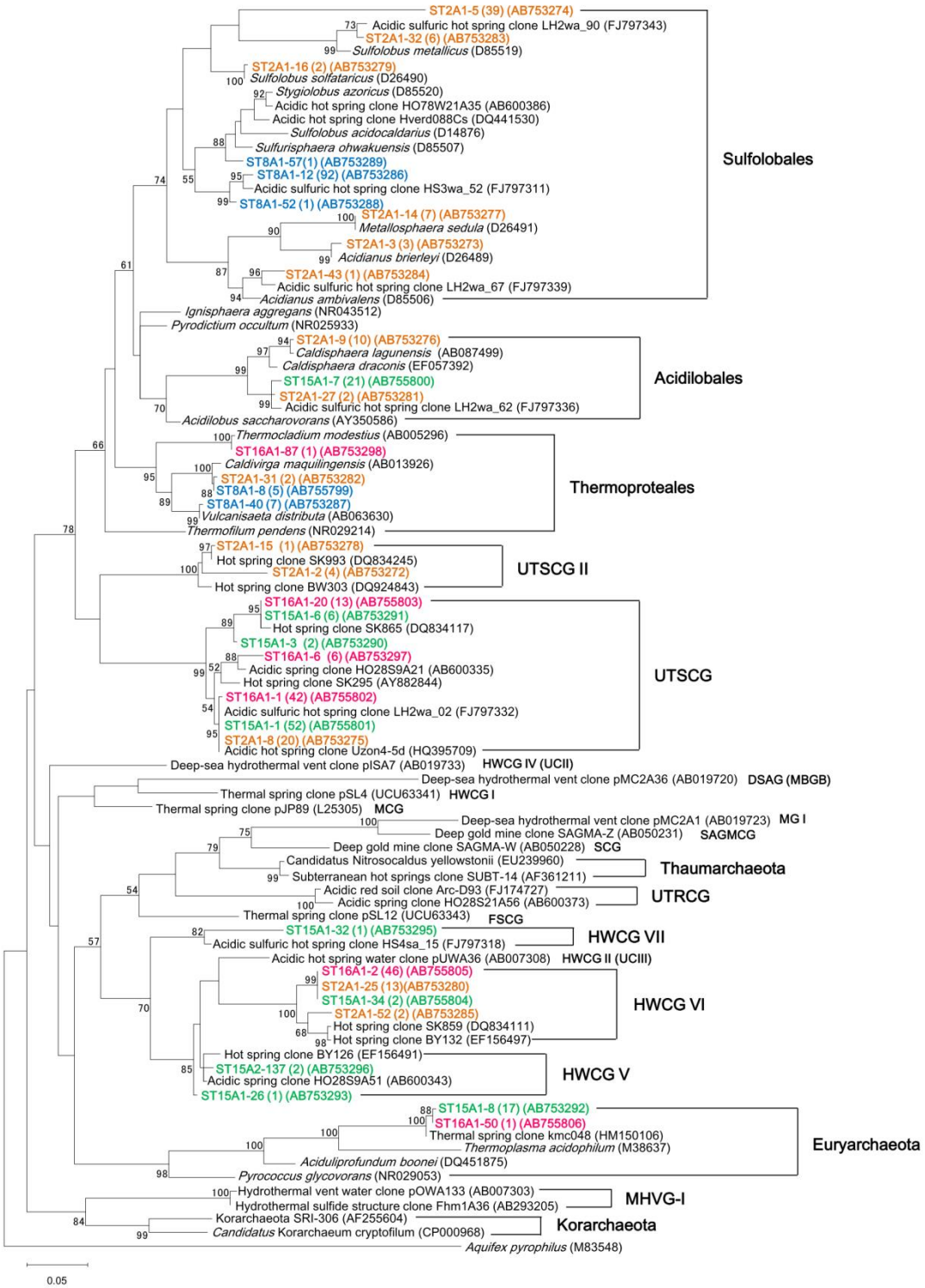
ST2A1-5 (39) (AB753274)
Acidic sulfuric hot spring clone LH2wa_90 (FJ797343)
ST2A1-32 (6) (AB753283)
Sulfolobus metallicus (D85519)
ST2A1-16 (2) (AB753279)
Sulfolobus solfataricus (D26490)
Stygiolobus azoricus (D85520)
Acidic hot spring clone HO78W21A35 (AB600386)
Acidic hot spring clone Hverd088Cs (DQ441530)
Sulfolobus acidocaldarius (D14876)
Sulfurisphaera ohwakuensis (D85507)
ST8A1-57(1) (AB753289)
ST8A1-12 (92) (AB753286)
Acidic sulfuric hot spring clone HS3wa_52 (FJ797311)
ST8A1-52 (1) (AB753288)
ST2A1-14 (7) (AB753277)
Metallosphaera sedula (D26491)
ST2A1-3 (3) (AB753273)
Acidianus brierleyi (D26489)
ST2A1-43 (1) (AB753284)
Acidic sulfuric hot spring clone LH2wa_67 (FJ797339)
Acidianus ambivalens (D85506)
Sulfolobales
Ignisphaera aggregans (NR043512)
Pyrodictium occultum (NR025933)
ST2A1-9 (10) (AB753276)
Caldisphaera lagunensis (AB087499)
Caldisphaera draconis (EF057392)
ST15A1-7 (21) (AB755800)
ST2A1-27 (2) (AB753281)
Acidic sulfuric hot spring clone LH2wa_62 (FJ797336)
Acidilobus saccharovorans (AY350586)
Acidilobales
Thermocladium modestius (AB005296)
ST16A1-87 (1) (AB753298)
Caldivirga maquilingensis (AB013926)
ST2A1-31 (2) (AB753282)
ST8A1-8 (5) (AB755799)
ST8A1-40 (7) (AB753287)
Vulcanisaeta distributa (AB063630)
Thermofilum pendens (NR029214)
Thermoproteales
ST2A1-15 (1) (AB753278)
Hot spring clone SK993 (DQ834245)
ST2A1-2 (4) (AB753272)
Hot spring clone BW303 (DQ924843)
UTSCG II
ST16A1-20 (13) (AB755803)
ST15A1-6 (6) (AB753291)
Hot spring clone SK865 (DQ834117)
ST15A1-3 (2) (AB753290)
ST16A1-6 (6) (AB753297)
Acidic spring clone HO28S9A21 (AB600335)
Hot spring clone SK295 (AY882844)
ST16A1-1 (42) (AB755802)
Acidic sulfuric hot spring clone LH2wa_02 (FJ797332)
ST15A1-1 (52) (AB755801)
ST2A1-8 (20) (AB753275)
Acidic hot spring clone Uzon4-5d (HQ395709)
UTSCG
Deep-sea hydrothermal vent clone pISA7 (AB019733) HWCG IV (UCII)
Deep-sea hydrothermal vent clone pMC2A36 (AB019720) DSAG (MBGB)
Thermal spring clone pSL4 (UCU63341) HWCG I
Thermal spring clone pJP89 (L25305) MCG
Deep-sea hydrothermal vent clone pMC2A1 (AB019723) MG I
Deep gold mine clone SAGMA-Z (AB050231) SAGMCG
Deep gold mine clone SAGMA-W (AB050228) SCG
Candidatus Nitrosocaldus yellowstonii (EU239960)
Subterranean hot springs clone SUBT-14 (AF361211)
Thaumarchaeota
Acidic red soil clone Arc-D93 (FJ174727)
Acidic spring clone HO28S21A56 (AB600373)
UTRCG
Thermal spring clone pSL12 (UCU63343) FSCG
ST15A1-32 (1) (AB753295)
Acidic sulfuric hot spring clone HS4sa_15 (FJ797318)
HWCG VII
Acidic hot spring water clone pUWA36 (AB007308) HWCG II (UCIII)
ST16A1-2 (46) (AB755805)
ST2A1-25 (13)(AB753280)
ST15A1-34 (2) (AB755804)
ST2A1-52 (2) (AB753285)
Hot spring clone SK859 (DQ834111)
Hot spring clone BY132 (EF156497)
HWCG VI
Hot spring clone BY126 (EF156491)
ST15A2-137 (2) (AB753296)
Acidic spring clone HO28S9A51 (AB600343)
HWCG V
ST15A1-26 (1) (AB753293)
ST15A1-8 (17) (AB753292)
ST16A1-50 (1) (AB755806)
Thermal spring clone kmc048 (HM150106)
Thermoplasma acidophilum (M38637)
Aciduliprofundum boonei (DQ451875)
Pyrococcus glycovorans (NR029053)
Euryarchaeota
Hydrothermal vent water clone pOWA133 (AB007303)
Hydrothermal sulfide structure clone Fhm1A36 (AB293205)
MHVG-I
Korarchaeota SRI-306 (AF255604)
Candidatus Korarchaeum cryptofilum (CP000968)
Korarchaeota
Aquifex pyrophilus (M83548)
0.05

Figure 4-1. Phylogenetic tree of archaeal 16S rRNA gene sequences detected in Kirishima hot springs. Bootstrap values (>50%) based on 1000 replicates are indicated at nodes. The scale bar indicates the number of nucleotide substitutions per position. Number in the parenthesis with phylotype name represents the number of clones of each phylotype. The DNA database accession numbers are also indicated in the parenthesis. *Aquifex pyrophilus* is used as an outgroup species. The phylotype names derived from Pond-A, B, C and D shown in blue, yellow, red and green, respectively. UTSCG: uncultured thermoacidic spring clone group (Kato *et al*., 2011), HWCG I: hot water crenarchaeotic group I (Barns *et al*., 1996; Nunoura *et al*., 2005), HWCG II (as known as UCIII: uncultured crenarchaeal group III) (Takai and Sako, 1999; Schrenk *et al*., 2003; Nunoura *et al*., 2005), HWCG IV (also known as UCII) (Takai and Horikoshi, 1999; Inagaki *et al*., 2003; Schrenk *et al*., 2003), DSAG (as known as MBGB): deep-sea archaeal group (marine benthic group B) (Takai and Horikoshi, 1999; Vetriani *et al*., 1999), MCG: miscellaneous crenarchaeal group (Barns *et al*., 1994; Takai *et al*., 2001; Teske *et al*., 2002; Inagaki *et al*., 2003), MG I: marine crenarchaeotic group I (DeLong *et al*., 1992; Takai and Horikoshi, 1999), SAGMCG: South Africa gold mine group (Takai *et al*., 2001), SCG: soil crenarchaeotic group (Takai *et al*., 2001), UTRCG: uncultured Thaumarchaeota-related clone group (Kato *et al*., 2011), FSCG: forest soil crenarchaeotic group (Barns *et al*., 1996; Jurgens & Saano, 1999) and MHVG-I: marine hydrothermal vent group I (Takai & Sako, 1999; Takai *et al*., 2001; Kato *et al*., 2011).

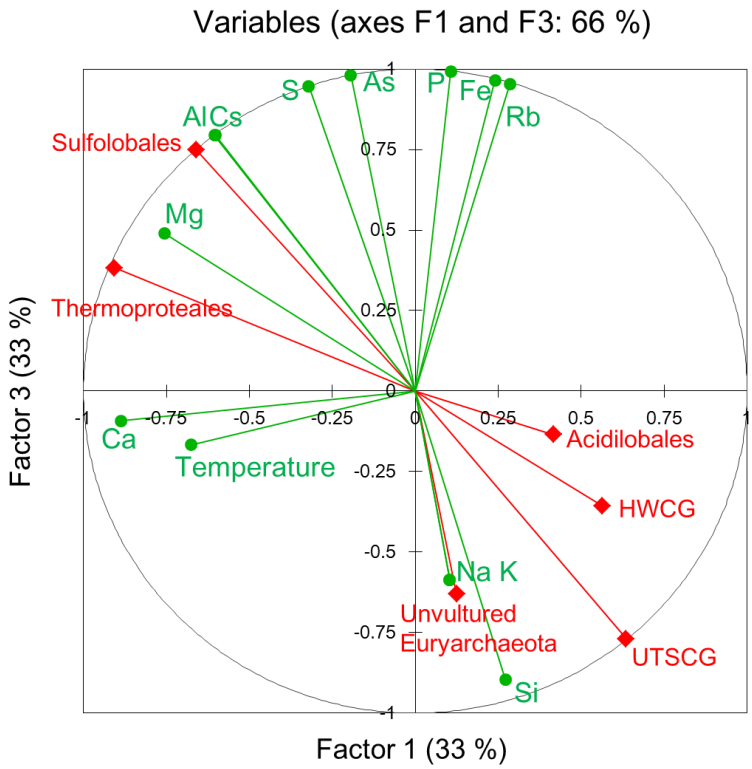

Figure 4-2. Canonical correlation analysis showing correlations between environmental factors and proportions of individual archaeal groups. Bacterial groups are shown in abbreviations in a red rhombus shape. Environmental factors are shown in green circles.

Chapter V

General conclusions

In this study, 16S rRNA gene phylogenetic analysis was performed to reveal the prokaryotic community structures, gene diversity and species diversity among four distinct highly acidic hot ponds displaying a wide range of temperature and chemical components in the Kirishima geothermal area, Kagoshima Prefecture, Japan. The correlations between the bacterial and archaeal community structures, gene diversity, species diversity, temperature, and chemical components were also examined. This study could be assessed all organisms in an environment since the highly acidic hot springs are only composed of chemotrophic prokaryotes.

The four ponds displayed a wide range of temperatures and chemical components. Principal component analysis showed that the selected four ponds were clearly distinguished by different chemical components and temperatures as follows: Pond-A: higher temperature and chemical components, Pond-B: lower temperature and higher chemical components, Pond-C: higher temperature and lower chemical components, Pond-D: lower temperature and chemical components. These ponds might be good exmples to discuss about the correlations between the microbial community structures, biodiversities, temperature, and chemical components.

In total, 803 prokaryotic clones of 16S rRNA gene were analyzed and classified into 61 prokaryotic species (bacterial 35 phylotypes and archaeal 26 phylotypes). The coverage value showed that this study successfully covered nearly 90% of prokaryotes existing in this habitat. In the bacterial community, the hot spring temperature clearly affected to the biodiversity, and the species compositons were affected by both chemical composition and temperature. On the other hand, the correlations between the archaeal communites and environmental factoers were slightly different to the bacterial ones. The archeal diversity was most affected by temperature as well as

bacterial one, and species composition was more affected by chemical components of the ponds than the temperature. Altoghether, these analyses indicated that the species diversity was affected by the temperature in Kirishima hot springs, and the relationships between community structures and environmental factors were depended on the groups of microorganisms.

Although other environmental factors than the temperature and chemical composition could also have influenced the bacterial and archaeal community structures and biodiversity, the present data will be helpful for improving our understanding of the prokaryotic ecology in the highly acidic ponds. Moreover, by taking into account of this study results, the effects of the environmental factors on the prokaryotic community structures, gene diversity and species diversity in the highly acidic hot springs will hopefully be generalized in studies relating to prokaryotic ecology in hot springs in the future. In addition, many 16S rRNA gene clones that showed no significant similarity with any cultured species in this study expect that we still have many chances to find novel bacterial and archaeal species via culturing experiments.

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APPENDICES

Page

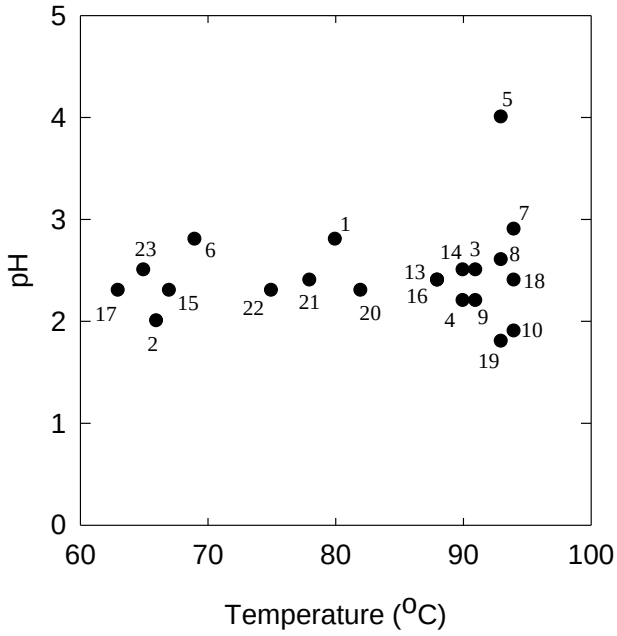


Figure 2-A. The relarionship between temperature and pH of 21 pond waters in the Kirishima geothermal area; N=21.

Table 2-A. Temperature, pH and the standardization of chemical components of 21 pond waters in the Kirishima geothermal area

Station	Temp.	pH	Fe/Si	S/Si	Al/Si	Mg/Si	Ca/Si	P/Si	Na/Si	K/Si	As/Si	Rb/Si	Cs/Si	Total conc. /Si
1	80	2.8	0.58	1.5	2.1	0.000	0.031	0.029	0.000	0.000	0.007	0.32	0.46	6.1
2	66	2.0	13	14	6.6	1.2	0.17	0.094	0.000	0.000	0.009	8.7	1.4	45
3	91	2.5	8.2	17	1.9	0.000	0.12	0.11	0.000	0.000	0.010	5.0	0.18	33
4	90	2.2	3.1	8.4	5.4	0.58	0.14	0.068	0.027	0.000	0.008	1.8	1.1	22
5	93	4.0	0.000	6.1	1.3	2.3	9.2	0.063	0.88	0.000	0.017	0.073	0.62	22
6	69	2.8	0.24	0.96	0.26	0.000	0.023	0.021	0.45	0.000	0.005	0.14	0.058	3.2
7	94	2.9	0.77	3.7	2.5	1.5	2.1	0.034	0.17	0.000	0.009	0.43	0.59	13
8	93	2.6	4.1	12	9.4	2.1	0.80	0.054	0.000	0.000	0.008	2.4	2.0	34
9	91	2.2	1.7	16	11	1.7	0.63	0.068	0.018	0.000	0.007	4.7	2.2	39
10	94	1.9	2.6	12	9.3	1.5	0.32	0.14	0.000	0.000	0.009	1.5	2.0	30
13	88	2.4	1.1	7.1	4.7	1.3	0.96	0.019	0.085	0.000	0.004	0.67	1.0	18
14	90	2.5	0.82	1.4	2.0	0.000	0.090	0.027	0.000	0.000	0.007	0.43	0.42	6.2
15	67	2.3	0.092	0.37	0.014	0.34	0.19	0.008	0.069	0.036	0.002	0.055	0.009	2.2
16	88	2.4	0.047	0.50	0.15	0.000	0.051	0.011	0.000	0.000	0.003	0.025	0.032	1.8
17	63	2.3	0.062	0.50	0.22	0.17	0.15	0.010	0.047	0.001	0.003	0.035	0.048	2.2
18	94	2.4	0.12	0.55	0.58	0.000	0.045	0.011	0.000	0.000	0.003	0.065	0.13	2.5
19	93	1.8	0.067	1.4	0.48	0.001	0.063	0.010	0.000	0.000	0.003	0.038	0.10	3.2
20	82	2.3	0.063	1.6	0.27	0.000	0.11	0.035	0.000	0.000	0.010	0.014	0.060	3.1
21	78	2.4	0.024	1.5	0.25	0.000	0.054	0.037	0.000	0.000	0.011	0.020	0.057	2.9
22	75	2.3	0.080	0.76	0.35	0.000	0.11	0.019	0.000	0.000	0.005	0.051	0.078	2.4
23	65	2.5	0.045	1.4	0.28	0.000	0.071	0.037	0.000	0.000	0.011	0.029	0.062	2.9
mean	83	2.5	1.7	5.1	2.8	0.60	0.73	0.043	0.083	0.002	0.007	1.3	0.60	14
SD	11	0.45	3.2	5.6	3.5	0.80	2.0	0.036	0.21	0.008	0.004	2.2	0.73	14
VC	0.14	0.18	1.8	1.1	1.2	1.3	2.7	0.83	2.5	4.4	0.51	1.8	1.2	1.0

Temperature is expressed in ^{o}C. Each chemical component was standardized at Si. The concentrations of each chemical component were the mean of values measured three times. The detection limit is 0.001 mg L^{-1}. Temp., Total conc., SD, and CV indicate Temperature, total concentration of examined chemical components, standard deviation, and coefficient of variation.

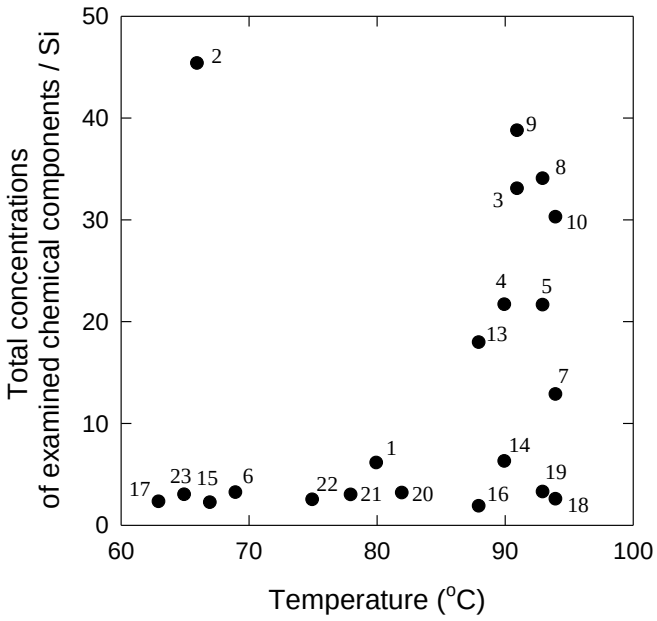


Figure 2-B. The relarionship between temperature and total concentrations of examined chemical components/Si of 21 pond waters in the Kirishima geothermal area; N=21.

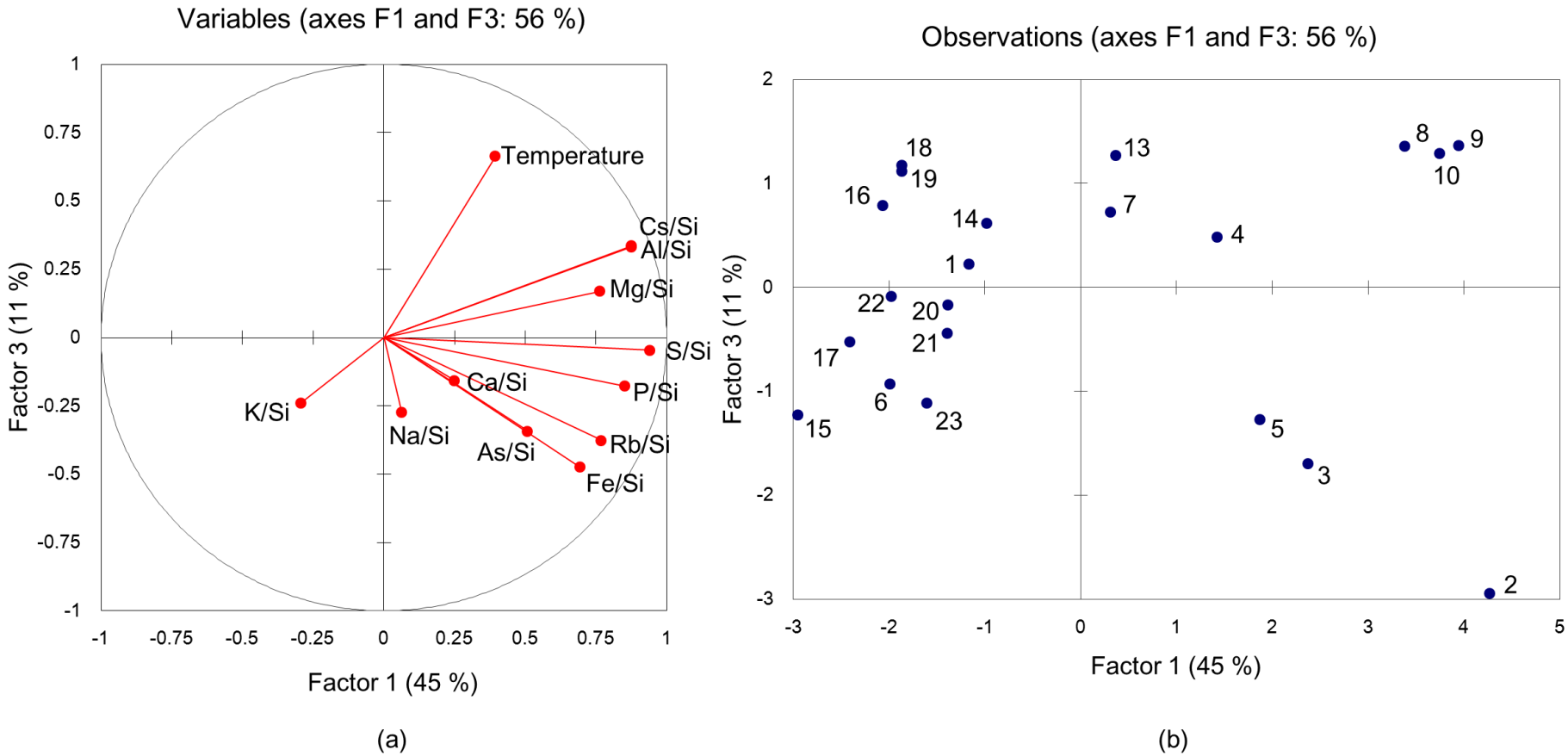


Figure 2-C. Principal components analysis showing the 12 environmental variables of the 21 ponds. (a) Factor loadings on principal components 1 and 3, (b) relationships between the 21 ponds and the principal components.

Pond-A

Pond-B

Pond-C

Pond-D

Figure 3-A. Sampling sites for prokaryotic diversity analyses of the highly acidic hot ponds in the Kirishima geothermal area.

Table 3-A. Correlation matrix showing *r* values for Pearson's correlation among environmental variables in the pond waters of the Kirishima geothermal area; N=4

Variables	θπ-B	Shannon-B	θπ-A	Shannon-A	θπ-P	Shannon-P	Temp.	pH	Fe	S	Al	Mg	Si	Ca	P	Na	K	As	Rb	Cs	Total conc.
θπ-B	—	0.23	0.70	**0.90**	0.87	**0.92**	**-0.99**	**-0.91**	0.51	0.05	-0.23	-0.10	0.17	-0.34	0.40	0.44	0.44	0.12	0.54	-0.23	0.21
Shannon-B	—	—	0.18	-0.03	0.11	0.41	-0.34	0.13	-0.68	-0.74	-0.68	-0.14	**0.93**	0.32	-0.73	0.96	0.96	-0.80	-0.67	-0.68	-0.71
θπ-A	—	—	—	0.88	**0.95**	0.88	-0.70	-0.80	0.13	-0.44	-0.70	-0.78	0.40	-0.83	-0.01	0.23	0.23	-0.32	0.17	-0.70	-0.30
Shannon-A	—	—	—	—	**0.98**	**0.90**	-0.87	**-0.99**	0.54	0.00	-0.32	-0.41	0.07	-0.71	0.42	0.14	0.14	0.11	0.58	-0.32	0.16
θπ-P	—	—	—	—	—	**0.94**	-0.85	**-0.94**	0.37	-0.19	-0.50	-0.56	0.24	-0.75	0.24	0.23	0.23	-0.08	0.41	-0.50	-0.04
Shannon-P	—	—	—	—	—	—	**-0.94**	-0.85	0.20	-0.32	-0.59	-0.44	0.47	-0.51	0.07	0.55	0.55	-0.24	0.24	-0.59	-0.17
Temp.	—	—	—	—	—	—	—	0.86	-0.40	0.05	0.31	0.12	-0.29	0.29	-0.29	-0.55	-0.55	-0.01	-0.43	0.31	-0.10
pH	—	—	—	—	—	—	—	—	-0.67	-0.16	0.17	0.29	0.07	0.65	-0.56	-0.07	-0.07	-0.27	-0.70	0.17	-0.31
Fe	—	—	—	—	—	—	—	—	—	0.84	0.62	0.34	-0.76	-0.26	**0.99**	-0.46	-0.46	**0.90**	**1.00**	0.62	**0.91**
S	—	—	—	—	—	—	—	—	—	—	**0.95**	0.71	**-0.93**	0.21	**0.91**	-0.58	-0.58	**0.99**	0.81	**0.95**	**0.99**
Al	—	—	—	—	—	—	—	—	—	—	—	0.82	**-0.90**	0.44	0.72	-0.57	-0.57	**0.90**	0.58	**1.00**	0.89
Mg	—	—	—	—	—	—	—	—	—	—	—	—	-0.49	0.82	0.44	-0.02	-0.02	0.61	0.30	0.83	0.66
Si	—	—	—	—	—	—	—	—	—	—	—	—	—	0.00	-0.83	0.84	0.84	**-0.94**	-0.74	-0.89	-0.89
Ca	—	—	—	—	—	—	—	—	—	—	—	—	—	—	-0.15	0.33	0.33	0.07	-0.29	0.44	0.12
P	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	-0.51	-0.51	**0.95**	**0.98**	0.72	**0.96**
Na	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	-0.62	-0.44	-0.57	-0.52
K	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	-0.62	-0.44	-0.57	-0.52
As	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	0.88	**0.90**	**0.99**
Rb	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	0.58	0.89
Cs	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	0.89
Total conc.	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—

Values in bold are different from 0 with a significance level α = 0.10 (*r* > 0.90). The only Shannon-Weaver index is shown as species diversity. The bacterial, archaeal and prokaryotic gene diversity were indicated as θπ-B, θπ-A, and θπ-P, respectively. The bacterial, archaeal and prokaryotic species diversity were indicated as Shannon-B, Shannon-A, and Shannon-P, respectively. Temp, and Total conc. indicate Temperature, and total concentration of examined chemical components, respectively.

Table 3-B. Correlation matrix showing *r* values for Pearson's correlation among factors in the pond waters of the Kirishima geothermal area; N=4

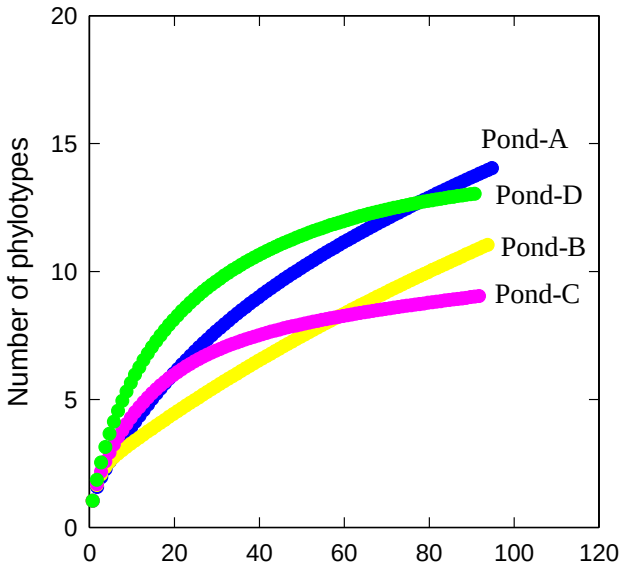
Variables	θπ-B	Shannon-B	θπ-A	Shannon-A	θπ-P	Shannon-P	Temp.	pH	Fe/Si	S/Si	Al/Si	Mg/Si	Ca/Si	P/Si	Na/Si	K/Si	As/Si	Rb/Si	Cs/Si	Total conc./Si
θπ-B	—	0.23	0.70	**0.90**	0.87	**0.92**	**-0.99**	**-0.91**	0.51	0.08	-0.20	-0.24	-0.54	0.33	0.44	0.44	0.04	0.54	-0.20	0.19
Shannon-B	—	—	0.18	-0.03	0.11	0.41	-0.34	0.13	-0.68	-0.75	-0.69	-0.51	-0.24	-0.76	0.96	0.96	-0.82	-0.67	-0.69	-0.75
θπ-A	—	—	—	0.88	**0.95**	0.88	-0.70	-0.80	0.14	-0.40	-0.68	-0.79	**-0.98**	-0.08	0.23	0.23	-0.37	0.18	-0.68	-0.28
Shannon-A	—	—	—	—	**0.98**	**0.90**	-0.87	**-0.99**	0.55	0.04	-0.29	-0.41	-0.76	0.35	0.14	0.14	0.04	0.59	-0.28	0.17
θπ-P	—	—	—	—	—	**0.94**	-0.85	**-0.94**	0.38	-0.16	-0.47	-0.58	-0.87	0.17	0.23	0.23	-0.15	0.42	-0.47	-0.03
Shannon-P	—	—	—	—	—	—	**-0.94**	-0.85	0.21	-0.29	-0.56	-0.59	-0.79	-0.01	0.55	0.55	-0.32	0.25	-0.56	-0.17
Temp.	—	—	—	—	—	—	—	0.86	-0.40	0.03	0.28	0.30	0.55	-0.22	-0.55	-0.55	0.07	-0.43	0.28	-0.09
pH	—	—	—	—	—	—	—	—	-0.67	-0.19	0.13	0.26	0.65	-0.49	-0.07	-0.07	-0.20	-0.70	0.13	-0.32
Fe/Si	—	—	—	—	—	—	—	—	—	0.85	0.64	0.50	0.05	**0.98**	-0.46	-0.46	0.86	**1.00**	0.64	**0.91**
S/Si	—	—	—	—	—	—	—	—	—	—	**0.95**	0.87	0.56	**0.95**	-0.58	-0.58	**0.99**	0.83	**0.95**	**0.99**
Al/Si	—	—	—	—	—	—	—	—	—	—	—	**0.97**	0.79	0.79	-0.57	-0.57	**0.93**	0.60	**1.00**	**0.90**
Mg/Si	—	—	—	—	—	—	—	—	—	—	—	—	0.89	0.67	-0.40	-0.40	0.84	0.46	**0.98**	0.81
Ca/Si	—	—	—	—	—	—	—	—	—	—	—	—	—	0.26	-0.23	-0.23	0.52	0.01	0.79	0.45
P/Si	—	—	—	—	—	—	—	—	—	—	—	—	—	—	-0.56	-0.56	0.95	**0.97**	0.79	**0.98**
Na/Si	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	**-0.66**	-0.45	-0.57	-0.56
K/Si	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	-0.66	-0.45	-0.57	-0.56
As/Si	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	0.83	**0.93**	**0.99**
Rb/Si	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	0.60	0.89
Cs/Si	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	**0.90**
Total conc./Si	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—

Values in bold are different from 0 with a significance level α = 0.10 (*r* > 0.90). The only Shannon-Weaver index is shown as species diversity. The bacterial, archaeal and prokaryotic gene diversity were indicated as θπ-B, θπ-A, and θπ-P, respectively. The bacterial, archaeal and prokaryotic species diversity were indicated as Shannon-B, Shannon-A, and Shannon-P, respectively. Temp. and Total conc. indicate Temperature, and total concentration of examined chemical components, respectively.

Table 3-C. Correlation matrix showing *r* values for Pearson's correlation among factors in the pond waters of the Kirishima geothermal area; N=4

Variables	θπ-B	Shannon-B	θπ-A	Shannon-A	θπ-P	Shannon-P	Primcipal component 1	Primcipal component 2	
							8 elements	Temp.	2 elements
θπ-B	—	0.23	0.70	**0.90**	0.87	**0.92**	0.19	**-0.99**	0.19
Shannon-B	—	—	0.18	-0.03	0.11	0.41	-0.74	-034	**0.94**
θπ-A	—	—	—	0.88	**0.95**	0.88	-0.30	-0.70	0.40
Shannon-A	—	—	—	—	**0.98**	**0.90**	0.16	-0.87	0.08
θπ-P	—	—	—	—	—	**0.94**	-0.04	-0.85	0.25
Shannon-P	—	—	—	—	—	—	-0.18	**-0.94**	0.48
8 elements	—	—	—	—	—	—	—	-0.09	**-0.90**
Temp.	—	—	—	—	—	—	—	—	-0.32
2 elements	—	—	—	—	—	—	—	—	—

Values in bold are different from 0 with a significance level α = 0.10 (*r* > 0.90). The only Shannon-Weaver index is shown as species diversity. The bacterial, archaeal and prokaryotic gene diversity were indicated as θπ-B, θπ-A, and θπ-P, respectively. The bacterial, archaeal and prokaryotic species diversity were indicated as Shannon-B, Shannon-A, and Shannon-P, respectively. 8 elements showed the concentration of the sum of Fe, S, Al, Mg, P, As, Rb, and Cs as the principal component 1. 2 elements showed the concentration of the sum of Si and K as the principal component 2. Temp.showed Temperature.



Number of bacterial 16S rRNA gene sequences detected from
the four highly acidic-solfataric ponds in the Kirishima geothermal area

Figure 3-B. Rarefaction curves for each bacterial clone lirary. Phylotypes were defined as 97.0%.

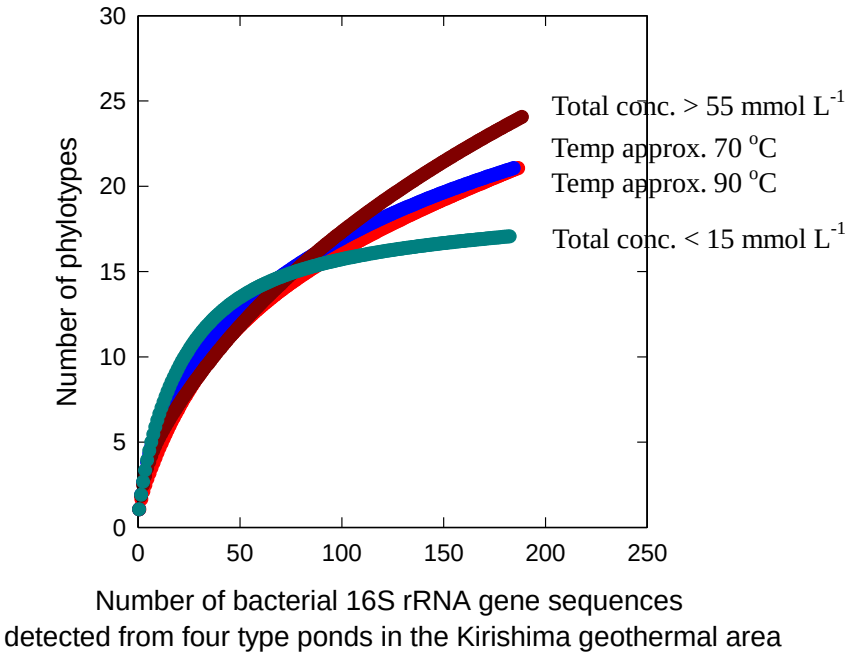
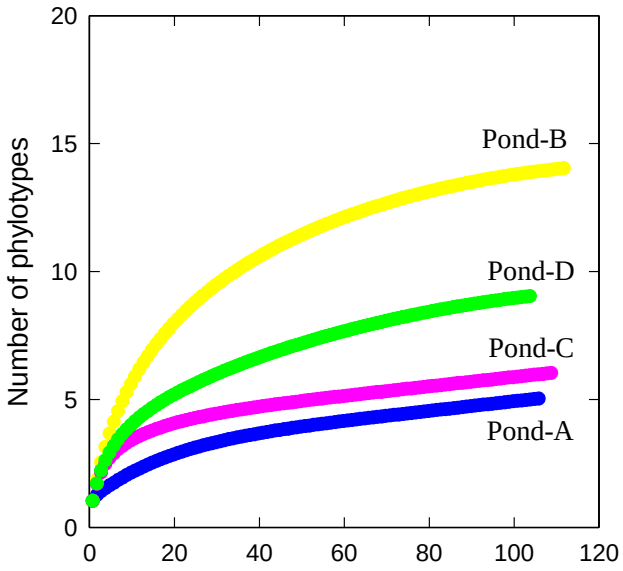


Figure 3-C. Rarefaction curves for each type of bacterial clone lirary. Phylotypes were defined as 97.0%.



Number of archaeal 16S rRNA gene sequences detected from
the four highly acidic-solfataric ponds in the Kirishima geothermal area

Figure 3-D. Rarefaction curves for each archaeal clone lirary. Phylotypes were defined as 97.0%.

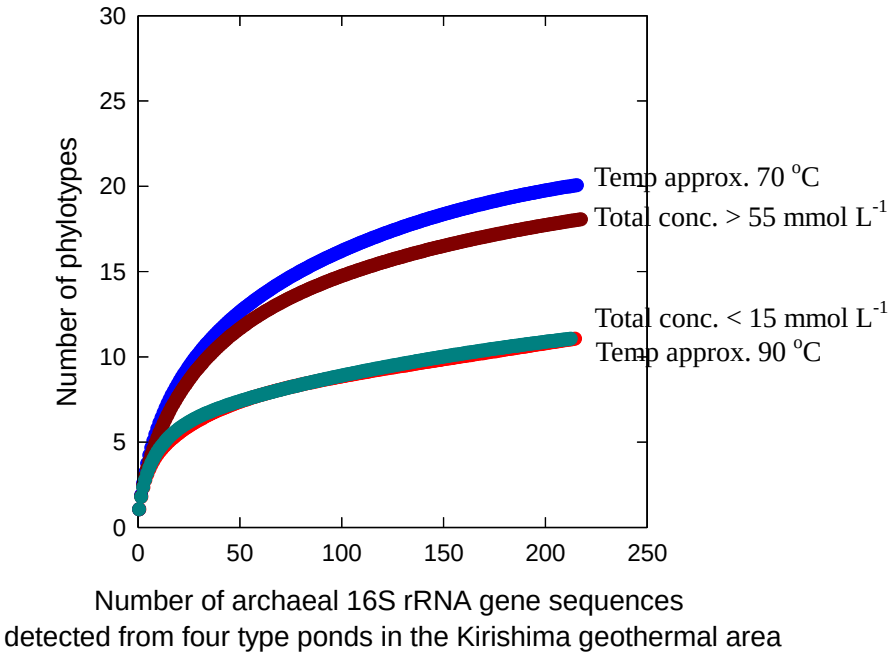


Figure 3-E. Rarefaction curves for each type of archaeal clone lirary. Phylotypes were defined as 97.0%.

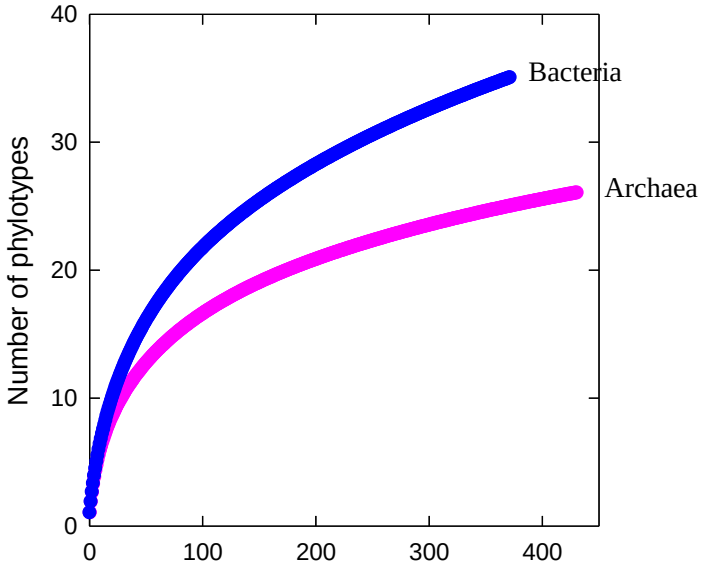


Figure 3-F. Rarefaction curves for each bacterial and archaeal clone lirary. Phylotypes were defined as 97.0%.

Table 3-D. Affiliation to the class level of bacterial 16S rRNA gene detected from each four ponds in the Kirishima geothermal area

Affiliation in the class level	Pond-A			Pond-B			Pond-C			Pond-D		
	Number of clones	%	Number of species	Number of clones	%	Number of species	Number of clones	%	Number of species	Number of clones	%	Number of species
γ-Proteobacteria	76	80	6	43	46	3	53	58	2	43	47	5
δ-Proteobacteria	0	0	0	41	44	1	0	0	0	0	0	0
β-Proteobacteria	7	8	5	1	1	1	29	31	5	10	11	3
α-Proteobacteria	0	0	0	1	1	1	0	0	0	0	0	0
Flavobacteria	2	2	1	0	0	0	3	3	1	6	7	1
Nitrospirae	0	0	0	1	1	1	0	0	0	0	0	0
Bacilli	5	5	1	4	4	2	7	8	1	13	14	1
Actinobacteria	5	5	1	1	1	1	0	0	0	10	11	1
Aquificae	0	0	0	0	0	0	0	0	0	8	9	1
Uncultured Thermotogae	0	0	0	2	2	1	0	0	0	1	1	1
Total	95	100	14	94	100	11	92	100	9	91	100	13

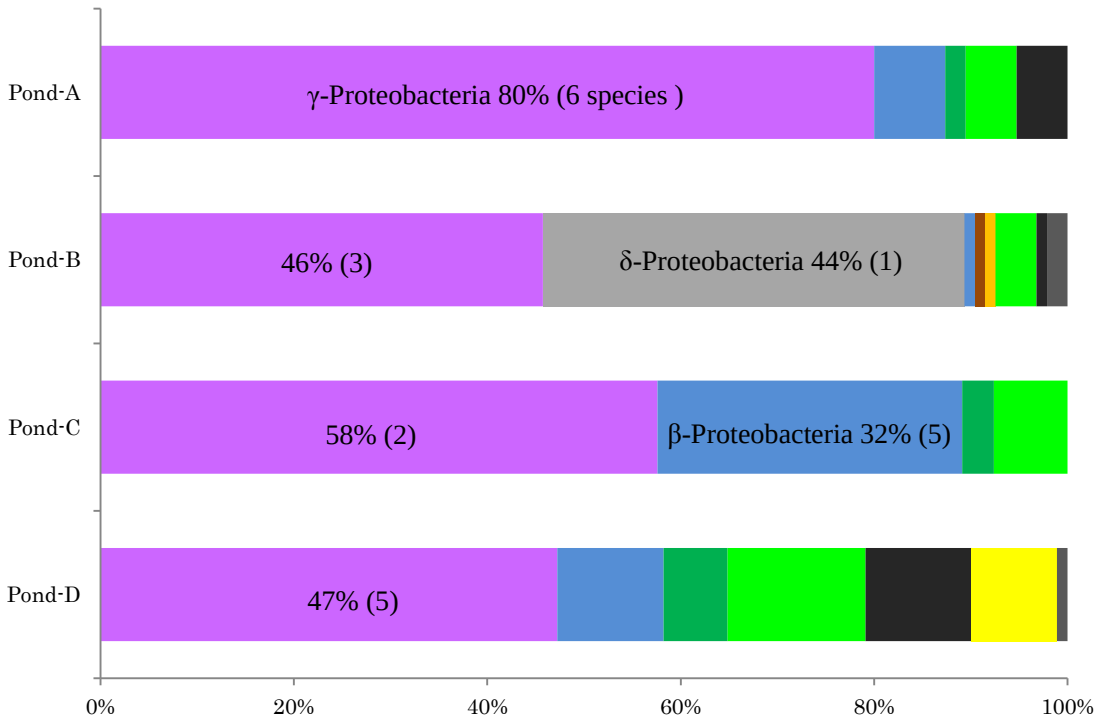


Figure 3-G. Phylogenetic distribution in the class level of bacterial 16S rRNA gene detected from each four ponds in the Kirishima geothermal area.

Table 3-E. Affiliation to the species level of bacterial 16S rRNA gene detected from each four ponds in the Kirishima geothermal area

Affiliation in the species level	Pond-A			Pond-B			Pond-C			Pond-D		
	Number of clones	%	Number of species	Number of clones	%	Number of species	Number of clones	%	Number of species	Number of clones	%	Number of species
Acinetobacter johnsonii	64	68	1	0	0	0	52	57	1	0	0	0
Acinetobacter junii	0	0	0	0	0	0	0	0	0	34	37	1
Acinetobacter sp.	1	1	1	1	1	1	0	0	0	0	0	0
Acidithiobacillus caldus	0	0	0	41	44	1	0	0	0	4	5	1
Pseudomonas poae	7	8	1	0	0	0	0	0	0	0	0	0
Uncultured *Pseudomonadaceae*	1	1	0	0	0	0	0	0	0	0	0	0
Uncultured Pseudomonadales	0	0	0	0	0	0	1	1	1	2	2	1
Uncultured Pseudomonadales	0	0	0	0	0	0	0	0	0	2	2	1
Uncultured Pseudomonadales	2	2	1	0	0	0	0	0	0	0	0	0
Uncultured Pseudomonadales	1	1	1	0	0	0	0	0	0	0	0	0
Uncultured γ-Proteobacteria	0	0	0	1	1	1	0	0	0	0	0	0
Uncultured γ-Proteobacteria	0	0	0	0	0	0	0	0	0	1	1	1
Uncultured δ-Proteobacteria	0	0	0	41	44	1	0	0	0	0	0	0
Acidovorax temperans	3	3	1	0	0	0	11	12	1	0	0	0
Delftia tsuruhatensis	0	0	0	0	0	0	8	9	1	5	6	1
Naxibacter alkalitolerans	0	0	0	0	0	0	3	3	1	3	3	1
Paracoccus marinus	0	0	0	0	0	0	6	6	1	0	0	0
Curvibacter lanceolatus	1	1	1	0	0	0	0	0	0	0	0	0
Ralstonia pickettii	0	0	0	1	1	1	0	0	0	0	0	0
Methylophilus leisingeri	1	1	1	0	0	0	0	0	0	0	0	0
Uncultured *Comamonadaceae*	0	0	0	0	0	0	1	1	1	2	2	1
Uncultured *Methylophilaceae*	1	1	1	0	0	0	0	0	0	0	0	0
Uncultured *Methylophilaceae*	1	1	1	0	0	0	0	0	0	0	0	0
Acidicaldus organivorans	0	0	0	1	1	1	0	0	0	0	0	0
Elizabethkingia miricola	0	0	0	0	0	0	3	3	1	6	7	1
Chryseobacterium aquaticum	2	2	1	0	0	0	0	0	0	0	0	0
Uncultured Nitrospirales	0	0	0	1	1	1	0	0	0	0	0	0
Staphylococcus epidermidis	0	0	0	1	1	1	0	0	0	13	14	1
Uncultured *Paenibacillaceae*	0	0	0	0	0	0	7	8	1	0	0	0
Uncultured Bacillales	0	0	0	3	3	1	0	0	0	0	0	0
Uncultured Bacillales	5	5	1	0	0	0	0	0	0	0	0	0
Propionibacterium acnes	5	5	1	0	0	0	0	0	0	10	11	1
Uncultured Acidimicrobiales	0	0	0	1	1	1	0	0	0	0	0	0
Uncultured Thermotogae	0	0	0	2	2	1	0	0	0	1	1	1
Hydrogenobaculum sp.	0	0	0	0	0	0	0	0	0	8	9	1
Total	95	100	14	94	100	11	52	100	9	91	100	13

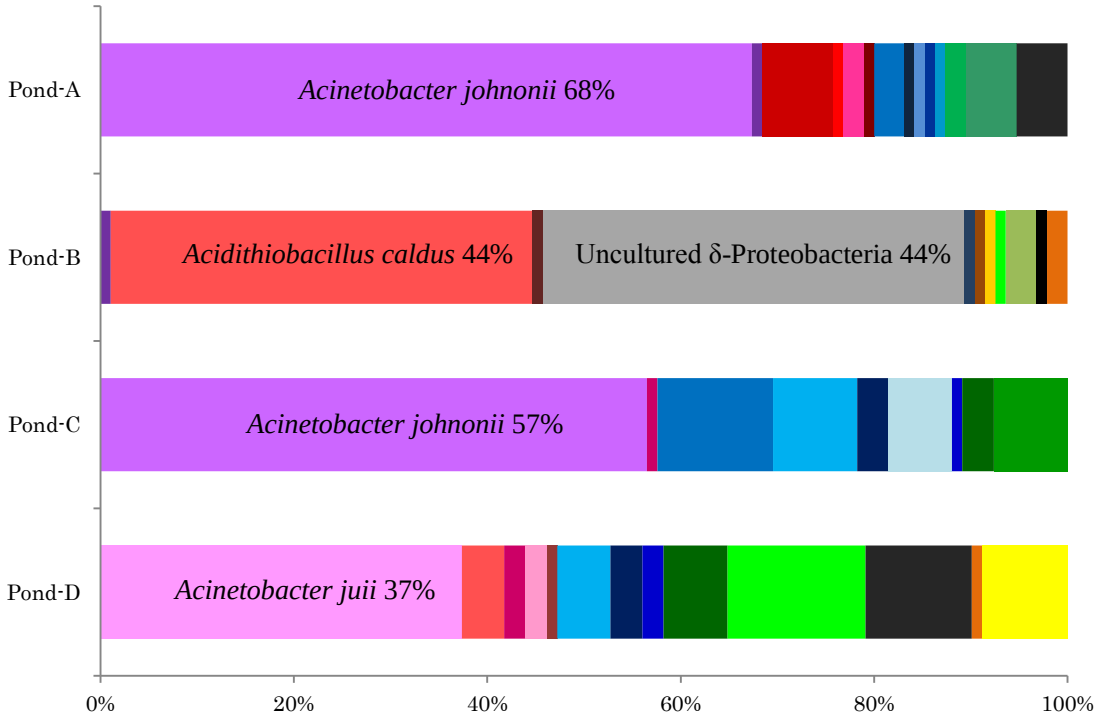


Figure 3-H. Phylogenetic distribution in the species level of bacterial 16S rRNA gene detected from each four ponds in the Kirishima geothermal area.

Table 3-F. Affiliation to the class level of bacterial 16S rRNA gene detected from ponds with diffetent temperarures and total concentrations of the examined chemical components in the Kirishima geothermal area

Affiliation in the class level	Temp. approx. 90 °C			Temp. approx. 70 °C			Total conc. > 55 mmol L^{-1}			Total conc. <15 mmol L^{-1}		
	Number of clones	%	Number of species	Number of clones	%	Number of species	Number of clones	%	Number of species	Number of clones	%	Number of species
γ-Proteobacteria	129	69	7	86	47	7	119	63	8	96	53	6
δ-Proteobacteria	0	0	0	41	22	1	41	22	1	0	0	0
β-Proteobacteria	36	19	9	11	6	4	8	4	6	39	21	5
α-Proteobacteria	0	0	0	1	1	1	1	1	1	0	0	0
Flavobacteria	5	3	2	6	3	1	2	1	1	9	5	1
Nitrospirae	0	0	0	1	1	1	1	1	1	0	0	0
Bacilli	12	6	2	17	9	2	9	5	3	20	11	2
Actinobacteria	5	3	1	11	6	2	6	3	2	10	6	1
Aquificae	0	0	0	8	4	1	0	0	0	8	4	1
Uncultured Thermotogae	0	0	0	3	2	1	2	1	1	1	1	1
Total	187	100	21	185	100	21	189	100	24	183	100	17

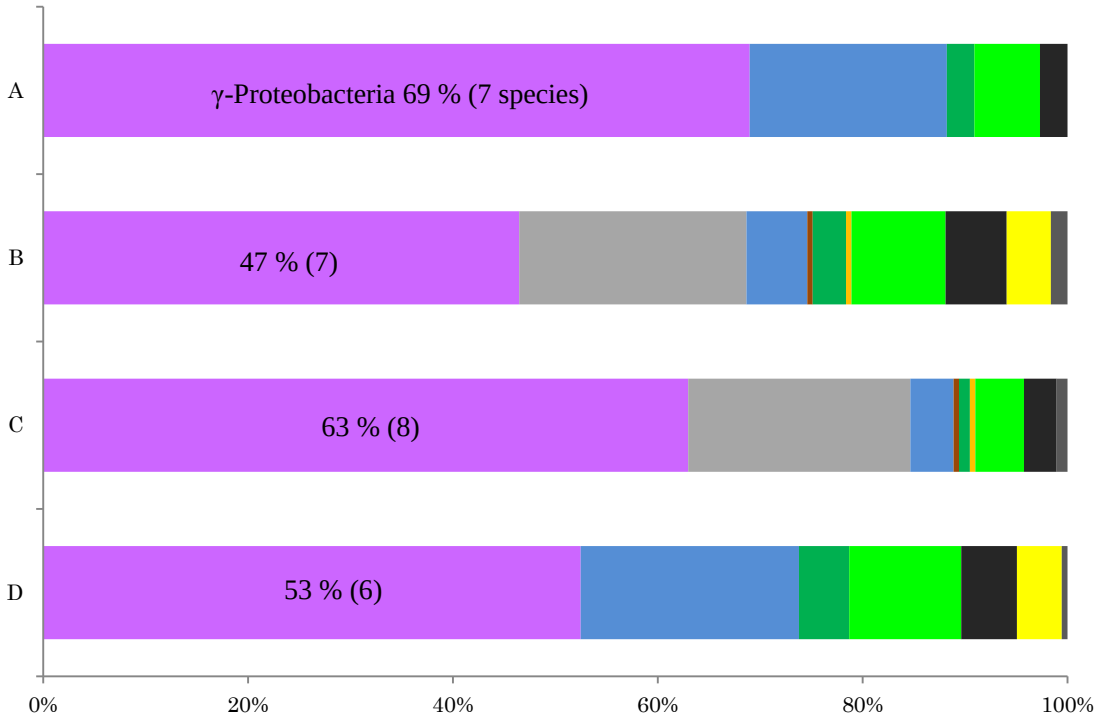


Figure 3-I. Phylogenetic distribution in the class level of bacterial 16S rRNA gene detected from ponds with diffetent temperarures and total concentrations of the examined chemical components in the Kirishima geothermal area. A: Temp. approx. 90 °C, B: Temp. approx. 70 °C, C: Total conc. > 55 mmol L^{-1}, and D: Total conc. < 15 mmol L^{-1}.

Table 3-G. Affiliation to the class level of bacterial 16S rRNA gene detected from all the four ponds in the Kirishima geothermal area

Affiliation in the class level	Number of clones	%	Number of species
γ-Proteobacteria	215	58	12
β-Proteobacteria	47	13	10
δ-Proteobacteria	41	11	1
Bacilli	29	8	4
Actinobacteria	16	4	2
Flavobacteria	11	3	2
Aquificae	8	2	1
α-Proteobacteria	1	0	1
Nitrospirae	1	0	1
Uncultured Thermotogae	3	1	1
Total	372	100	35

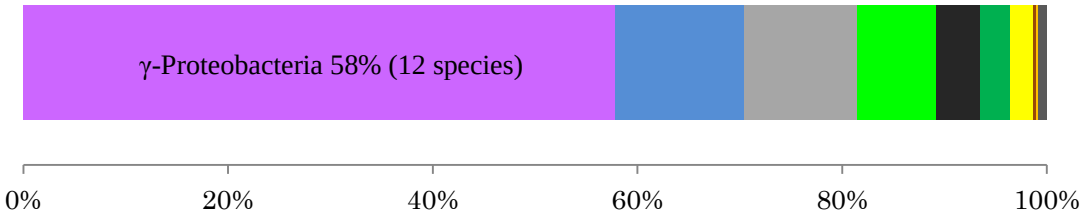


Figure 3-J. Phylogenetic distribution in the class level of bacterial 16S rRNA gene detected from all the four ponds in the Kirishima geothermal area.

Table 4-A. Affiliation to the order level of archaeal 16S rRNA gene detected from each four ponds in the Kirishima geothermal area

Affiliation in the order level	Pond-A			Pond-B			Pond-C			Pond-D		
	Number of clones	%	Number of species	Number of clones	%	Number of species	Number of clones	%	Number of species	Number of clones	%	Number of species
Sulfolobales	94	89	3	58	52	6	0	0	0	0	0	0
Acidilobales	0	0	0	12	11	2	0	0	0	21	20	1
Thermoproteales	12	11	2	2	2	1	1	1	1	0	0	0
UTSCG	0	0	0	20	18	1	42	39	1	52	50	1
UTSCG	0	0	0	0	0	0	0	0	0	2	2	1
UTSCG	0	0	0	0	0	0	6	6	1	0	0	0
UTSCG	0	0	0	0	0	0	13	12	1	6	6	1
UTSCG II	0	0	0	4	4	1	0	0	0	0	0	0
UTSCG II	0	0	0	1	1	1	0	0	0	0	0	0
HWCG V	0	0	0	0	0	0	0	0	0	1	1	1
HWCG V	0	0	0	0	0	0	0	0	0	2	2	1
HWCG VI	0	0	0	13	12	1	46	42	1	2	2	1
HWCG VI	0	0	0	2	2	1	0	0	0	0	0	0
HWCG VII	0	0	0	0	0	0	0	0	0	1	1	1
Uncultured Euryarchaeota	0	0	0	0	0	0	1	1	1	17	16	1
Total	106	100	5	112	100	14	109	100	6	104	100	9

UTSCG: uncultured thermoacidic spring clone group (Kato *et al*., 2011), HWCG: hot water crenarchaeotic group (Barns *et al*., 1996; Takai & Horikoshi, 1999; Takai & Sako, 1999; Inagaki *et al*., 2003; Schrenk *et al*., 2003; Nunoura *et al*., 2005; Satoh *et al*., 2013)

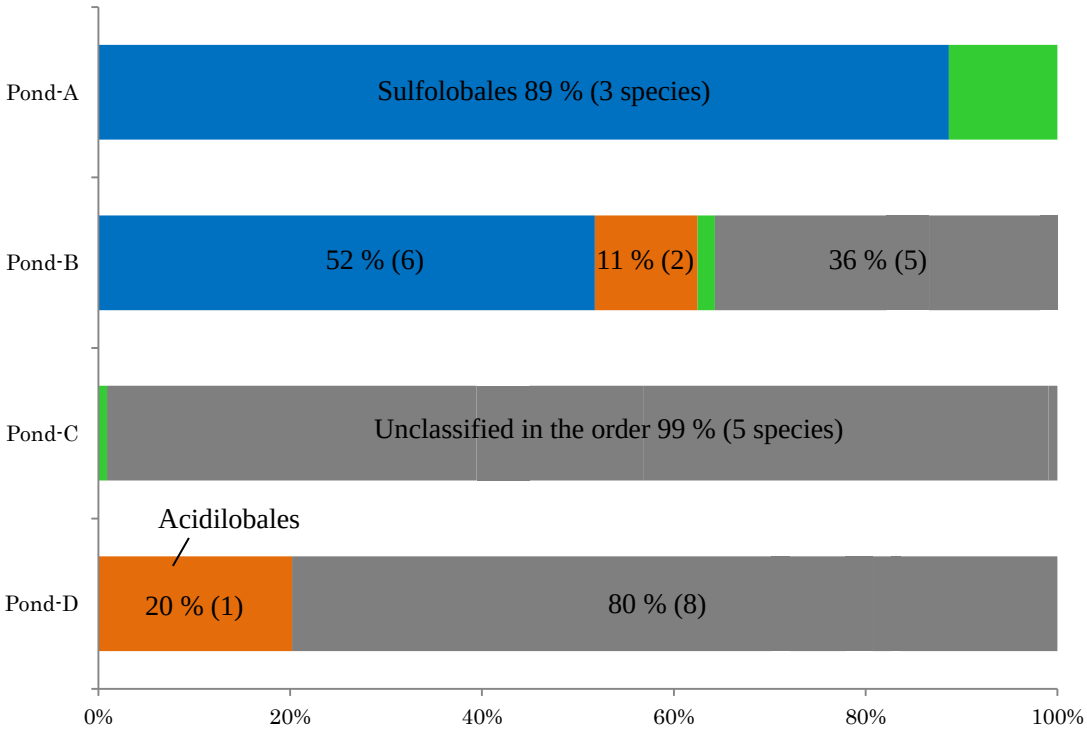


Figure 4-A. Phylogenetic distribution in the order level of archaeal 16S rRNA gene detected from each four ponds in the Kirishima geothermal area.

Table 4-B. Affiliation to the species level of archaeal 16S rRNA gene detected from each four ponds in the Kirishima geothermal area

Affiliation in the species level	Pond-A			Pond-B			Pond-C			Pond-D		
	Number of clones	%	Number of species	Number of clones	%	Number of species	Number of clones	%	Number of species	Number of clones	%	Number of species
Acidianus brierleyi	0	0	0	3	3	1	0	0	0	0	0	0
Acidianus sp.	0	0	0	1	1	1	0	0	0	0	0	0
Metallosphaera sedula	0	0	0	7	6	1	0	0	0	0	0	0
Sulfolobus solfataricus	0	0	0	2	2	1	0	0	0	0	0	0
Sulfurisphaera sp.	1	1	1	0	0	0	0	0	0	0	0	0
Uncultured Sulfolobales	0	0	0	39	35	1	0	0	0	0	0	0
Uncultured Sulfolobales	0	0	0	6	5	1	0	0	0	0	0	0
Uncultured Sulfolobales	92	87	1	0	0	0	0	0	0	0	0	0
Uncultured Sulfolobales	1	1	1	0	0	0	0	0	0	0	0	0
Caldisphaera lagunensis	0	0	0	10	9	1	0	0	0	0	0	0
Caldisphaera sp.	0	0	0	2	2	1	0	0	0	21	20	1
Caldivirga maquilingensis	5	5	1	2	2	1	0	0	0	0	0	0
Vulcanisaeta distributa	7	7	1	0	0	0	0	0	0	0	0	0
Thermocladium modestius	0	0	0	0	0	0	1	1	1	0	0	0
UTSCG	0	0	0	20	18	1	42	39	1	52	50	1
UTSCG	0	0	0	0	0	0	0	0	0	2	2	1
UTSCG	0	0	0	0	0	0	6	6	1	0	0	0
UTSCG	0	0	0	0	0	0	13	12	1	6	6	1
UTSCG II	0	0	0	4	4	1	0	0	0	0	0	0
UTSCG II	0	0	0	1	1	1	0	0	0	0	0	0
HWCG V	0	0	0	0	0	0	0	0	0	1	1	1
HWCG V	0	0	0	0	0	0	0	0	0	2	2	1
HWCG VI	0	0	0	13	12	1	46	42	1	2	2	1
HWCG VI	0	0	0	2	2	1	0	0	0	0	0	0
HWCG VII	0	0	0	0	0	0	0	0	0	1	1	1
Uncultured Euryarchaeota	0	0	0	0	0	0	1	1	1	17	16	1
Total	106	100	5	112	100	14	109	100	6	104	100	9

UTSCG: uncultured thermoacidic spring clone group (Kato *et al*., 2011), HWCG: hot water crenarchaeotic group (Barns *et al*., 1996; Takai & Horikoshi, 1999; Takai & Sako, 1999; Inagaki *et al*., 2003; Schrenk *et al*., 2003; Nunoura *et al*., 2005; Satoh *et al*., 2013)

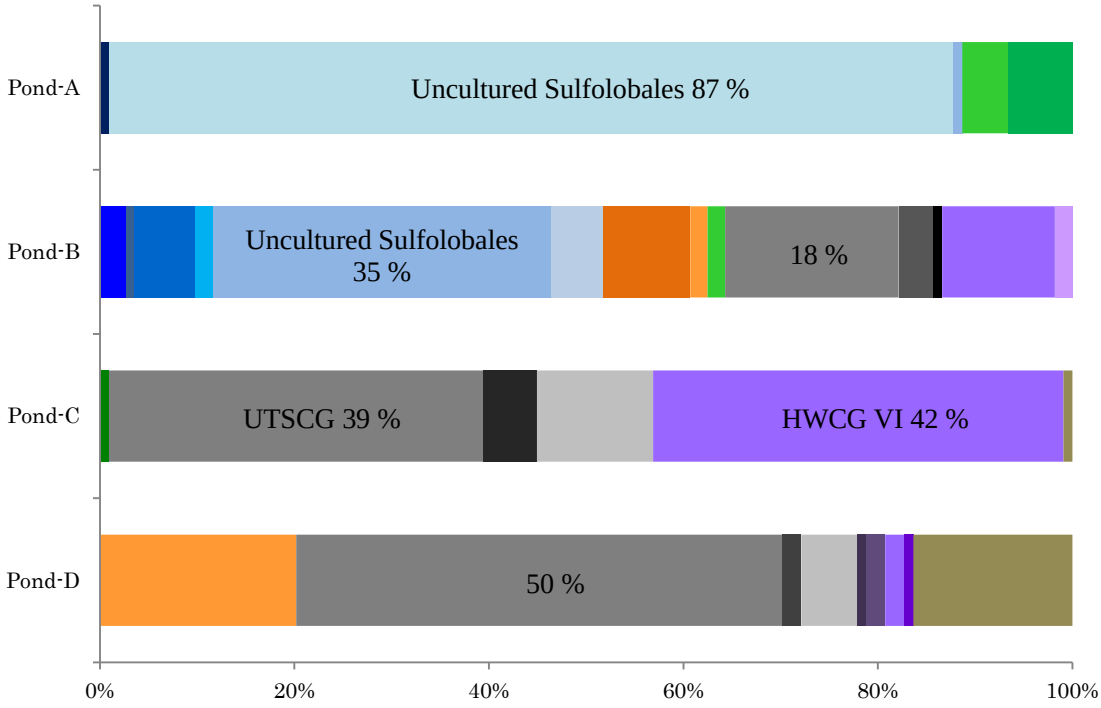


Figure 4-B. Phylogenetic distribution in the species level of archaeal 16S rRNA gene detected from each four ponds in the Kirishima geothermal area.

Table 4-C. Affiliation to the order level of archaeal 16S rRNA gene detected from ponds with diffetent temperarures and total concentrations of the examined chemical components in the Kirishima geothermal area

Affiliation in the order level	Temp. approx. 90 °C			Temp. approx. 70 °C			Total conc. > 55 mmol L^{-1}			Total conc. <15 mmol L^{-1}		
	Number of clones	%	Number of species	Number of clones	%	Number of species	Number of clones	%	Number of species	Number of clones	%	Number of species
Sulfolobales	94	44	3	58	27	6	152	70	9	0	0	0
Acidilobales	0	0	0	33	15	2	12	6	2	21	10	1
Thermoproteales	13	6	3	2	1	1	14	6	2	1	1	1
UTSCG	42	20	1	72	33	1	20	9	1	94	44	1
UTSCG	0	0	0	2	1	1	0	0	0	2	1	1
UTSCG	6	3	1	0	0	0	0	0	0	6	3	1
UTSCG	13	6	1	6	3	1	0	0	0	19	9	1
UTSCG II	0	0	0	4	2	1	4	2	1	0	0	0
UTSCG II	0	0	0	1	1	1	1	1	1	0	0	0
HWCG V	0	0	0	1	1	1	0	0	0	1	1	1
HWCG V	0	0	0	2	1	1	0	0	0	2	1	1
HWCG VI	46	21	1	15	7	1	13	6	1	48	23	1
HWCG VI	0	0	0	2	1	1	2	1	1	0	0	0
HWCG VII	0	0	0	1	1	1	0	0	0	1	1	1
Uncultured Euryarchaeota	1	1	1	17	8	1	0	0	0	18	9	1
Total	215	100	11	216	100	20	218	100	18	213	100	11

UTSCG: uncultured thermoacidic spring clone group (Kato *et al*., 2011), HWCG: hot water crenarchaeotic group (Barns *et al*., 1996; Takai & Horikoshi, 1999; Takai & Sako, 1999; Inagaki *et al*., 2003; Schrenk *et al*., 2003; Nunoura *et al*., 2005; Satoh *et al*., 2013)

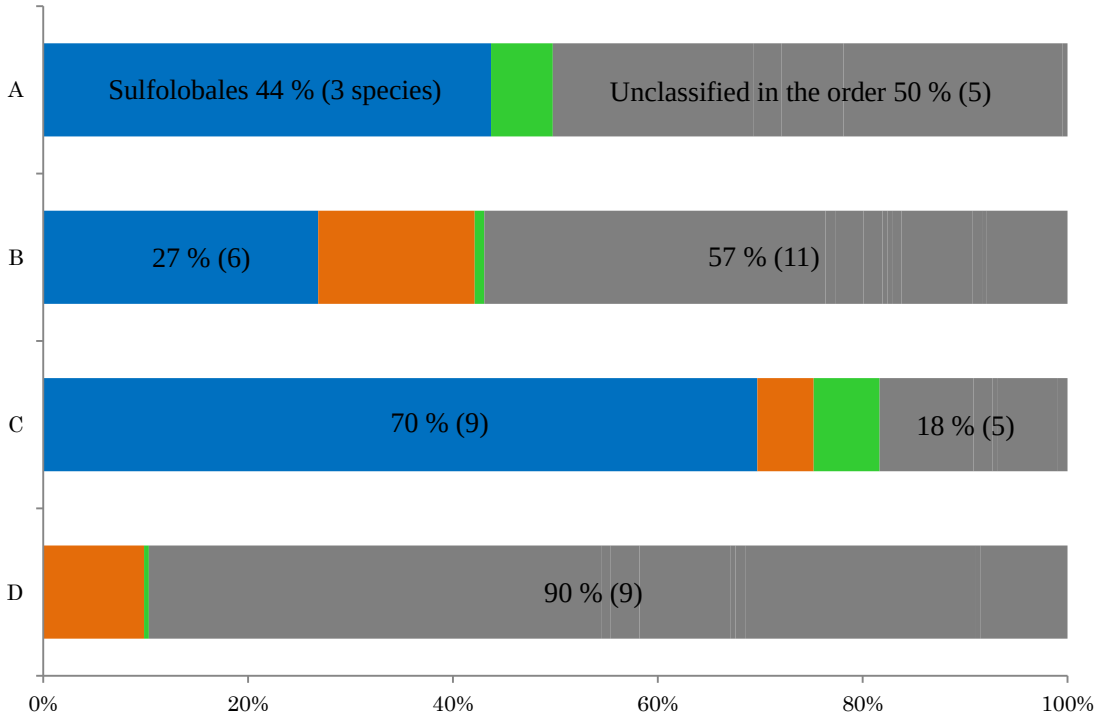


Figure 4-C. Phylogenetic distribution in the order level of archaeal 16S rRNA gene detected from ponds with diffetent temperarures and total concentrations of the examined chemical components in the Kirishima geothermal area. A: Temp. approx. 90 ^{o}C, B: Temp. approx. 70 ^{o}C, C: Total conc. > 55 mmol L^{-1}, and D: Total conc. < 15 mmol L^{-1}.

Table 4-D. Affiliation to the order level of archaeal 16S rRNA gene detected from all the four ponds in the Kirishima geothermal area

Affiliation in the order level	Number of clones	%	Number of species
Sulfolobales	152	35	9
Acidilobales	33	8	2
Thermoproteales	15	4	3
UTSCG	114	27	1
HWCG VI	61	14	1
UTSCG	19	4	1
Uncultured Euryarchaeota	18	4	1
UTSCG	6	1	1
UTSCG II	4	1	1
UTSCG	2	1	1
HWCG V	2	1	1
HWCG VI	2	1	1
UTSCG II	1	0	1
HWCG V	1	0	1
HWCG VII	1	0	1
Total	431	100	26

UTSCG: uncultured thermoacidic spring clone group (Kato *et al*., 2011), HWCG: hot water crenarchaeotic group (Barns *et al*., 1996; Takai & Horikoshi, 1999; Takai & Sako, 1999; Inagaki *et al*., 2003; Schrenk *et al*., 2003; Nunoura *et al*., 2005; Satoh *et al*., 2013)

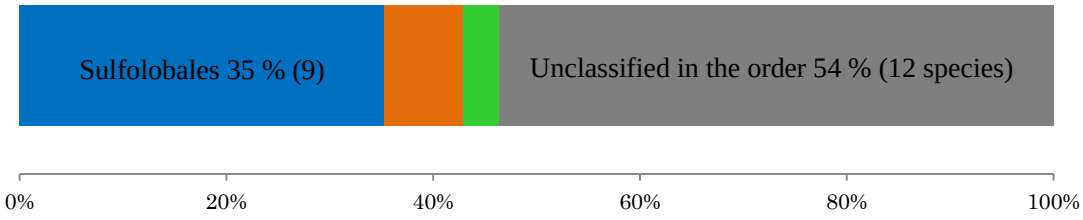


Figure 4-D. Phylogenetic distribution in the order level of archaeal 16S rRNA gene detected from all the four ponds in the Kirishima geothermal area.

Table 4-E. Affiliation to described species or uncultured species of prokaryotic 16S rRNA gene detected from each four ponds in the Kirishima geothermal area

Affiliation	Pond-A			Pond-B			Pond-C			Pond-D		
	Number of clones	%	Number of species	Number of clones	%	Number of species	Number of clones	%	Number of species	Number of clones	%	Number of species
Described species of bacteria	83	41	7	44	21	4	83	41	6	75	39	7
Described species of archaea	12	6	2	24	12	5	1	1	1	0	0	0
Uncultured species of bacteria	12	6	7	50	24	7	9	5	3	16	8	6
Uncultured species of archaea	94	47	3	88	43	9	108	54	5	104	53	9
Total	201	100	19	206	100	25	201	100	15	195	100	22

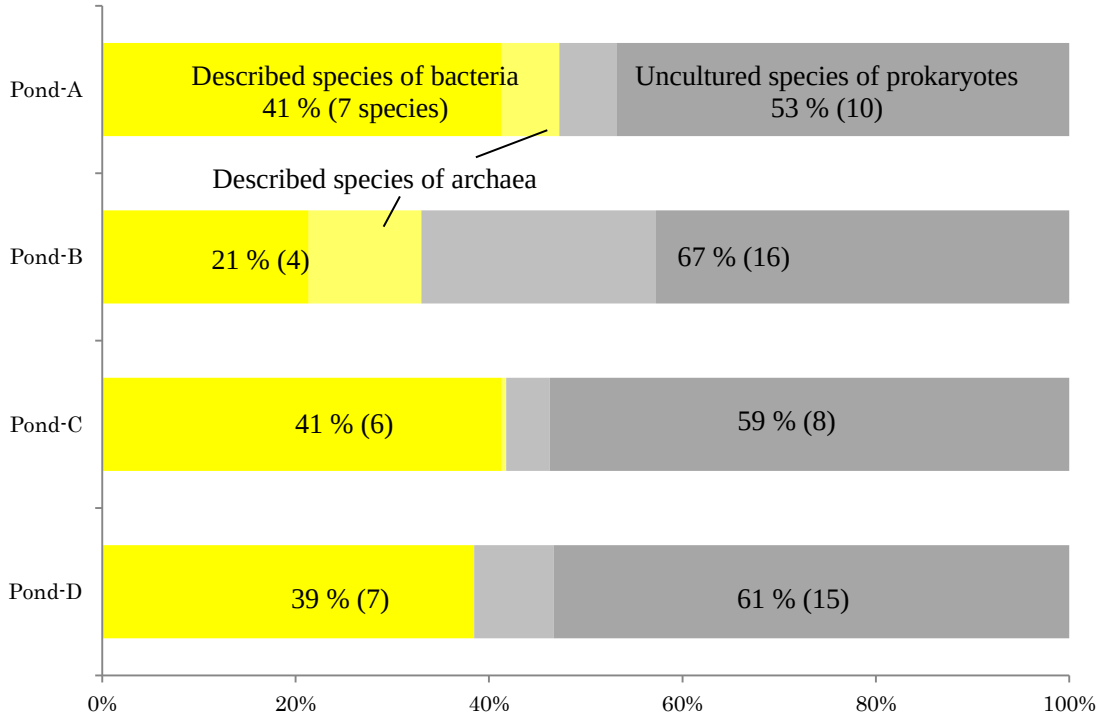


Figure 4-E. Affiliation to described species or uncultured species of prokaryotic 16S rRNA gene detected from each four ponds in the Kirishima geothermal area.

Table 4-F. Affiliation to chemoautotrophic or chemoheterotrophic species of prokaryotic 16S rRNA gene detected from each four ponds in the Kirishima geothermal area

Affiliation	Pond-A			Pond-B			Pond-C			Pond-D		
	Number of clones	%	Number of species	Number of clones	%	Number of species	Number of clones	%	Number of species	Number of clones	%	Number of species
Autptroph species of bacteria	0	0	0	41	20	1	0	0	0	4	2	1
Autotroph species of archaea	0	0	0	12	6	3	0	0	0	0	0	0
Heterotroph species of bacteria	83	41	7	3	2	3	83	41	6	71	36	6
Heterotroph species of archaea	12	6	2	12	6	2	1	1	1	0	0	0
Unknown auxotroph species of bacteria	12	6	7	50	24	7	9	5	3	16	8	6
Unknown auxotroph species of archaea	94	47	3	88	43	9	108	54	5	104	53	9
Total	201	100	19	206	100	25	201	100	15	195	100	22

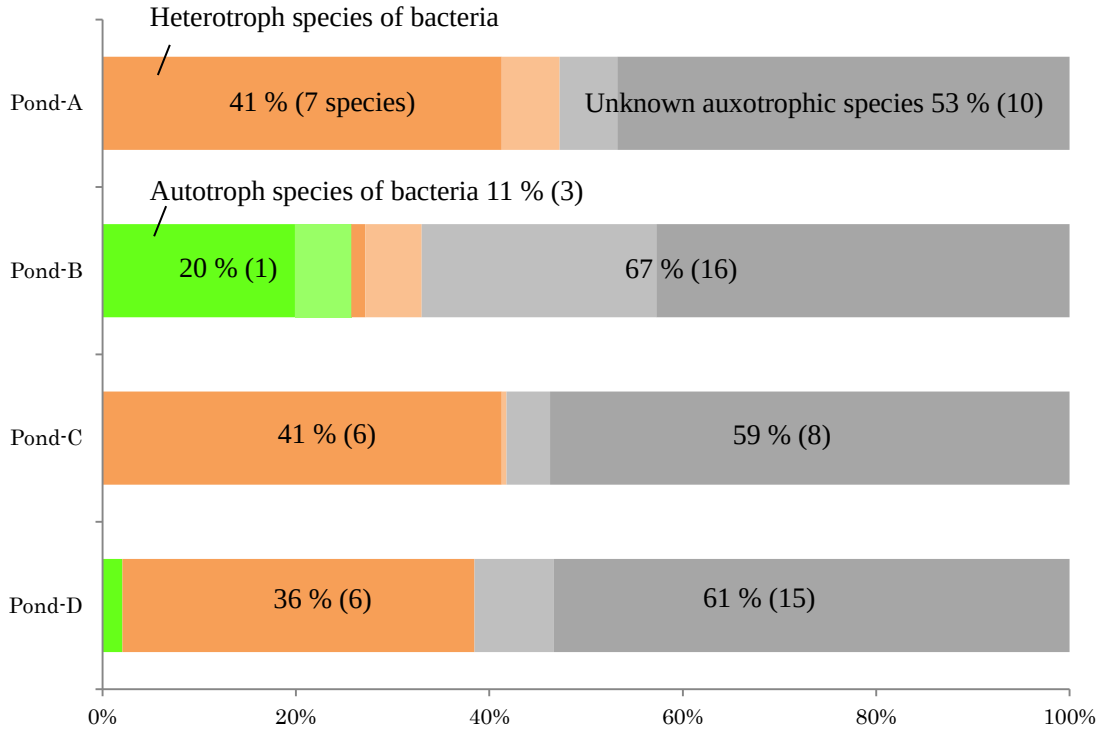


Figure 4-F. Affiliation to chemoautotrophic or chemoheterotrophic species of prokaryotic 16S rRNA gene detected from each four ponds in the Kirishima geothermal area.

Table 4-G. Affiliation to described species or uncultured species of prokaryotic 16S rRNA gene detected from all the four ponds in the Kirishima geothermal area

Affiliation in the phylum level	Number of clones	%	Number of species
Described species of bacteria	285	35	16
Described species of archaea	37	5	7
Uncultured species of bacteria	87	11	19
Uncultured species of archaea	394	49	19
Total	803	100	61

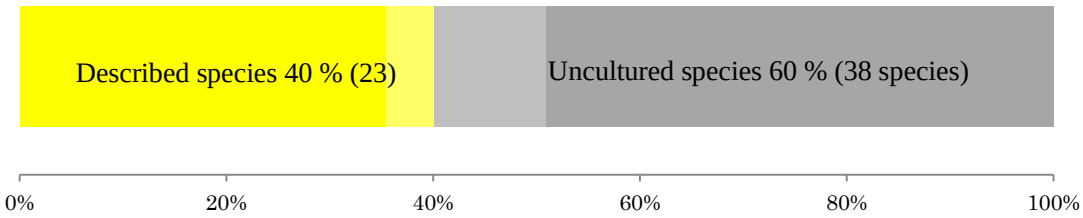


Figure 4-G. Affiliation to described species or uncultured species of prokaryotic 16S rRNA gene detected from all the four ponds in the Kirishima geothermal area.