Bacterial communities in soil samples from the Mingyong Glacier of southwestern China

Haoyu Li[1,2], Muhammad Kamran Taj[2], Xiuling Ji[2], Qi Zhang[2], Liangbing Lin[2], Zhimei Zhou[2] and Yunlin Wei[2*]

[1]Faculty of Environmental Science and Engineering, Kunming University of Science and Technology, Kunming, RP China
[2]Faculty of Life Science and Technology, Kunming University of Science and Technology, Kunming, RP China

Abstract: The present study was an effort to determine the bacterial diversity of soils in Mingyong Glacier located at the Meili Snow Mountains of southwestern China. Mingyong Glacier has different climatic zones within a very narrow area, and bacterial community diversity in this low temperature area remains largely unknown. In this study, soil samples were collected from four different climatic zones: M11A (dry warm valley), M14 (forest), M15 (grass land), and M16 (glacier zones). Phylogenetic analysis based on 16S rRNA gene V6 hypervariable region showed high bacterial abundance in the glacier. The number of Operational Taxonomic Units ranged from 2.24×10^3 to 5.56×10^3 in soil samples. Statistical analysis of 16S rRNA gene clone libraries results showed that bacterial diversity in zones M11A, M14 and M16 are higher than in zone M15. The bacterial community structures are clearly distinguishable, and phylogenetic analysis showed that the predominant phyla were Proteobacteria, Deinococcus-Thermus, Firmicutes, Actinobacteria, and Nitrospirae in Mingyong Glacier. Seventy-nine different orders from four zones have been isolated. Bacterial diversity and distribution of bacterial communities related to the anthropogenic perturbations in zone (M15) were confirmed by diversity index analysis, and the diversity index of other three zones was satisfactory through this analysis software. The results suggest that bacterial diversity and distribution analyses using bacterial 16S rRNA gene V6 hypervariable region were successful, and bacterial communities in this area not only had the same bacterial phyla compared to other glaciers but also had their own rare species.

Keywords: Bacterial Community; Bacterial Diversity; Mingyong Glacier

INTRODUCTION

The harshest environment for living organisms is glacier ice because of its high hydrostatic pressure, subfreezing temperatures and very low nutrient content (Margesin and Miteva, 2011, Miteva *et al*., 2009). Glaciers are a good example of dynamic ecosystems in which there are diverse thermal regimes with varying hydrological, physical and geochemical features in their deep ice or sub glacial portions and supra glacial areas (Hodson *et al*., 2008). Microbial cells that originate from geographically close or distant places are deposited with snow and progressively become embedded in the deeper layers of ice. Primary and very important sources of bacterial cells are marine surface aerosol, terrestrial dust and volcanic ashes. Depth and altitude of glaciers affect bacterial abundance, and thereby their abundance differs between glaciers. These variations are directly proportional to the load of dust in yearly snow drizzle with very high cell numbers deposited during colder environments. (Miteva *et al*., 2009). The current microbiological studies on non-polar and polar glaciers revealed extensively variable physiological, morphological and phylogenetic microbial ranges (Miteva, 2008). The glaciers were dominated by microorganisms, which include major bacterial phylogenetic groups such as Firmicutes, Actinobacteria, CFB (Cytophaga-Flavobacterium-Bacteroides) and Proteobacteria.(Simon *et al*., 2009).

In the present study, the bacterial diversity was studies Mingyong Glacier, located at Meili Snow Mountain of Yunnan Province, China. Meili Snow Mountains is part of the Hengduan Mountains, and it has high biodiversity (Yang, 1990). Different bacterial communities are distributed in different vertical climatic zones: glacier, forest, grassland and dry warm valley. The environment in each zone is quite different, varying in altitude, temperature and ultraviolet intensity. The bacterial communities play very important roles in maintaining matter circulation, transformation and ecological balance in this area. In the present study, bacterial diversity in the Mingyong Glacier was analyzed by the metagenome technique, which was originally proposed by American scientist Handelsman and his research group in 1998. Metagenomics can principally access 100% of genetic resources in a given environment, whereas the other hand traditional culturing methods and traditional genomics can access only 1% of these resources. In the majority of this study, the V3 region (approximately 60 bp) and the V6 region (approximately 70 bp) were used to identify the bacteria community (Huse *et al*., 2008, Huse *et al*., 2010, Liu *et al*., 2007).

In this study, we analyzed the bacterial communities by

**Corresponding author:* e-mail: homework18@126.com

using the V6 hypervariable region sequence of the 16S rRNA gene of the 16S rRNA gene, which is better than the pure culturing methods. This is the first ever report on the analysis of bacterial communities by using 16S rRNA gene V6 hypervariable region sequence.

MATERIAL AND METHODS

Sampling

In April 2012, soil samples were collected from four different altitude locations: M11A (dry warm valley), M14 (forest), M15 (shrubby) and M16 (glacier) (table 1). To avoid contamination, the soil samples were collected from 20 cm below the surface and placed in aseptic tubes with Teflon caps. Three different soil samples were collected and mixed together in each location. Samples were stored at 4°C for further analysis.

DNA extraction

Environmental DNA from soil samples were extracted by using the SoilMaster™ DNA Extraction Kit (Epicentre USA). The 100mg soil sample was weighed into the 1.5ml tube, then 250µl Soil DNA Extraction Buffer and 2µl Proteinase K were added into the tube. Then 50 µl Soil Lysis Buffer were added into the solution. The solution was incubated at 65°C for 10 minutes before centrifuged for 2 minutes at 1000×g. The 60µl Protein Precipitation Reagent was added into the supernatant and incubated on ice for 8 minutes, and centrifuged for 8 minutes, discarded the supernatant. DNA Precipitation Solution (6µl) was added. Vortexes the solution briefly, centrifuged for 5 minutes. The supernatant was carefully decanted. The pellet was washed with 500µl Pellet Wash Solution, decanted the supernatant carefully. The pellet was resuspended in 300µl TE Buffer. The integrity and yield of the DNA obtained were confirmed through electrophoresis by using 1% agarose gel.

PCR amplification

PCR was performed to amplify the 16S rRNA gene V6 hyper variable region from the isolated DNA. 967F and 1046R primers were used (Sogin *et al*., 2006) (table 2). Fifty micro liter reaction mixtures contained 1.5µl of template DNA, 1µl of Taq DNA polymerase (Thermo Fisher USA), 5µl dNTP Mix (0.2mM of each), 0.5µM of each primer, 5µl 10×Taq Buffer and 2.5mM $MgCl_2$, with nuclease-free water added to 50µl. The PCR amplifications used an initial denaturation step at 94°C for 4 min, followed by 30 cycles of 45 sec at 94°C, 45 sec at 56°C and 1 min at 72°C. After these cycles, the temperature was held at 72°C for 10 min.

To pellet the PCR product, 50µl sodium acetate (3 M) and 1.25ml dehydrated alcohol were added into the tube. The tube was kept at -80°Cfor 20 min and then centrifuged at 16,000g for 15min at 4°C. Then, 500µl dehydrated alcohol was added into the pelleted tubes and the tube was centrifuged at 16,000g for 15min at 4°C. The supernatant was poured out, and finally 100µl 1×TE buffer was added to dissolve the pellet. PCR amplification products were stored at -20°C (Silva, 2012).

PCR product sequencing

The sequencing for PCR amplification products had accomplished in BGI (Shenzhen China) company, which used the Qubit Fluorometer to disclosed the product, by the Paired-end sequencing of the Illumina platform (Gao *et al*., 2013).

Phylogenetic analyses of sequences

After Operational Taxonomic Units (OTU) analysis to derive the sequence information, phylogenetic trees were constructed using the Mothur (version 1.27.0) (Schloss *et al*., 2009). The relative abundance of genera in different samples was analyzed using BLASTn (Altschul *et al*., 1997)[14] and MEGAN(Huson *et al*., 2011). All the database information comes from RDP (http://rdp.cme.msu.edu/) (Sundquist *et al*., 2007), SILVA (http://arb-silva.de/), and NCBI (http://www.ncbi.nlm.nih.gov/index.html).

STATISTICAL ANALYSIS

Alpha diversity (α-diversity) refers to species diversity in a single sample analysis, including the Chao1 value, ACE value, Shannon and Simpson index. The Alpha diversity indexes of the samples were calculated using Mothur (version 1.27.0) (Schloss *et al.*, 2009).

RESULTS

The information of sequencing results

Detailed information for Paired-end Illumina sequencing of the four samples is shown in table 2. After stitching and filtering the clean reads, clustering 16S rRNA gene Tags were obtained for OTU species classification. The diversity of bacterial communities from the samples can be preliminarily indicated by the Tags and OTU numbers, and the details are shown in table 2.

Table 2 shows the details of the sequencing result. The data utilization ratios of the four samples were all acceptable. Even though the number of Tags in M16 was less than the other three samples, the number of OTUs was almost the same as M11A and M14. The number of OTUs identified in sample M15 was less than the others, and these results suggest, to some degree, that the diversity of bacterial communities from M15 may be lower than that of the other three samples.

Community diversity

The dimensions of the pie charts represent the number of OTUs and different colors represent the four samples (Claesson *et al*., 2009). At the phylum level, OTUs were

Table 1: List of sampling locations in this study

	M11A	M14	M15	M16
Altitude (m)	2016	2460	3636	3877
Longitude	E98°49'01"	E98°46'42"	E98°45'43"	E98°44'49"
Latitude	N28°28'08"	N28°27'48"	N28°24'10"	N28°24'28"
N (%)	0.091	0.536	0.560	0.560
S (mg/kg)	7.0	25.8	1.5	5.3
P (mg/kg)	2.4	4.4	1.0	1.0
K (mg/kg)	64	120	22	32
Ca (mg/kg)	260	258	259	250
Mg (mg/kg)	19.6	192	1.0	606
Fe (mg/kg)	14	0.6	8.4	13.9
Zn (mg/kg)	8.7	0.4	0.4	2.2
Cu (mg/kg)	5.4	2.6	2.0	5.1
Mn (mg/kg)	4.6	2.9	1.0	3.4

Table 2: The information of sequencing

Sample ID	Raw data (Mb)	Adapter (%)	N base (%)	Clean data (Mb)	Tags Number[a]	Unique Tags Number	OTU Number
M11A	260.45	10.400	0.500	227.29	64,464	12,414	5,563
M14	258.11	5.236	0.477	239.88	65,370	11,323	5,502
M15	231.74	1.448	0.470	224.91	68,917	4,729	2,248
M16	304.10	20.926	0.478	232.51	31,813	8,448	5,118

Table 3: Alpha diversity of the samples

Sample Name	Chao1	ACE	Shannon	Np Shannon	Simpson
M11A	10158.16	13688.37	6.511	6.618	0.007
M14	12002.72	17735.03	6.366	6.474	0.008
M15	3614.374	4560.342	2.589	2.693	0.246
M16	10714.99	15900.21	6.755	6.924	0.005

Table 4: Beta diversity of the samples

	M11A	M14	M15	M16
M11A	0	0.075	0.046	0.029
M14	0.075	0	0.094	0.075
M15	0.046	0.094	0	0.077
M16	0.029	0.075	0.077	0

principally distributed in Proteobacteria, Actinobacteria, Firmicutes and Bacteroidetes. The percentages of Proteobacteria OTUs were 13.97% in M11A, 20.99% in M14, 53.78% in M15 and 11.26% in M16. For the percentages of Actinobacteria OTUs, M11A was 53.79%, M14 was 18.31%, M15 was 6.83% and M16 was 21.06%. For the percentages of Firmicutes OTUs, M11A was 53.15%, M14 was 23.27%, M15 was 7.96%, and M16 was 15.46%. For the percentages of Bacteroidetes OTUs, M11A was 48.15%, M14 was 23.01%, M15 was 10.73%, and M16 was 18.12%. The OTUs distributed in Verrucomicrobia, Chlorofexi, Acidobacteria and Planctomycetes for each sample were all less than those of the other phyla.

At the order level, seventy-nine different orders were represented in the four samples (fig. 1). M14 had the highest number of orders in the four samples, followed by M11A and M16 with almost the same number, while M15 was the lowest. The OTUs of M14, M11A, and M16 were distributed across most orders, but M15 OTUs were mostly classified as Thermales and Enterobacteriales. The four samples had almost the same proportion of Pseudomonadales; this phenomenon is unique for all Pie charts.

With the exception of M15, each sample contained unique orders. M11A contained Bacteroidetes Order II. Incertae sedis, unclassified Bacteroidetes, Cardiobacteriales and Mycoplasmatales. M14 contained Herpetosiphonales and Sytrophobacterales. M16 contained Puniceicocales, unclassified Acidobacteria and Desufarculales. However, the proportion of these unique orders was a small proportion of all OTUs.

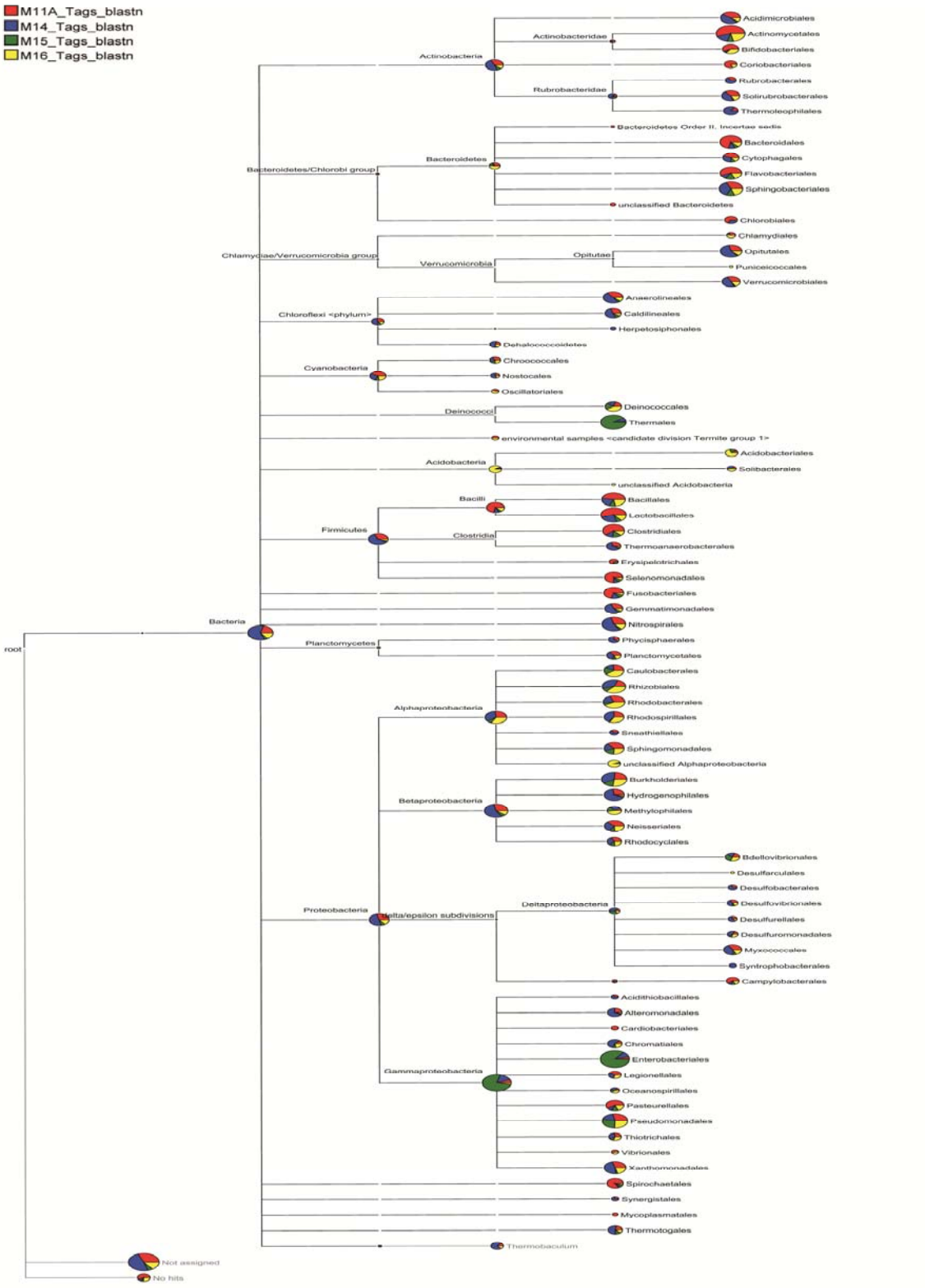

Fig. 1: V6 amplicon sequences from the four samples assigned with BLAST and MEGAN (at order level). Pie charts display the relative abundance for each sequence. 'Not assigned' indicates reads with BLAST hits below the cutoff value.

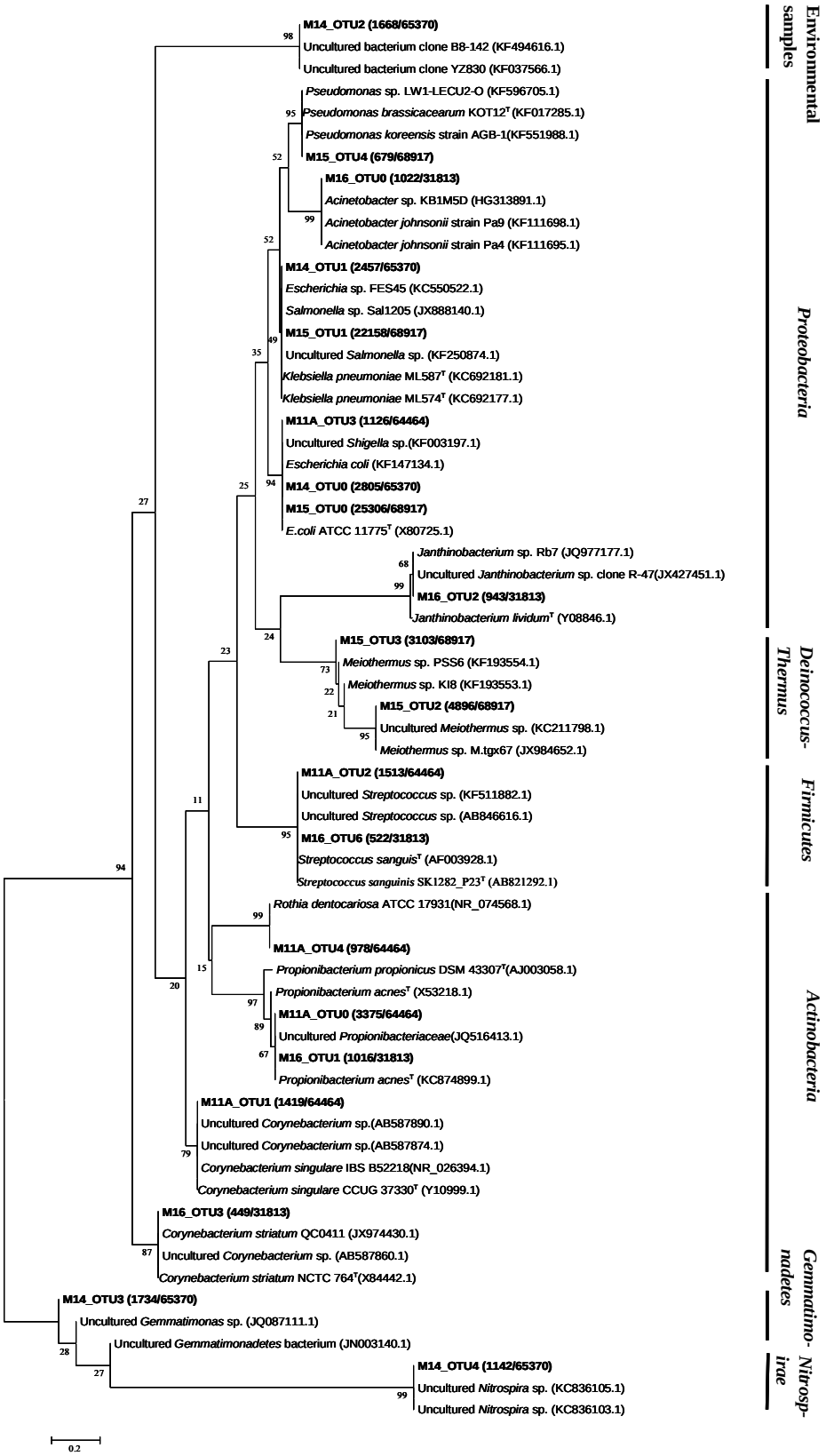

Fig. 2: Phylogenetic analysis based on partial OTUs from the sequencing results. Gen Bank accession numbers are listed after species names.

Phylogenetic analysis

Based on the sequencing results, the top five OTUs from each sample were chosen to build a phylogenetic tree. For example, M11A_OTU0 (3375/64484) means that OTU0 has 3375 of the total 64484 Tags in sample M11A; other samples are displayed in the same manner. The predominant bacterial community of each sample is revealed in this method.

Twenty OTUs from four samples were affiliated with the phyla Proteobacteria, Deinococcus-Thermus, Firmicutes, Actinobacteria and Nitrospirae. The OTUs were distributed irregularly, which means that the predominant bacterial community of each sample was quite different. This result also shows that none of the phyla can contain every OTU from all the samples (fig. 2).

Diversity index

Chaol (Kemp and Aller, 2004) and ACE(Chao, 1992) used the OTU number to forecast microbe communities, and it is also the standard for measuring species abundance. The Shannon index (Zhang, 2008) is the diversity index, which integrates OTU abundance and uniformity. The Np Shannon index is an algorithm based on the Shannon of another diversity index, and it is highly sensitive to rare species. When the values of these four indexes increase and the Simpson index decreases, it means that the diversity of the sample is high. From the Chaol and ACE index, the diversity of M14 is higher than that of the other three samples. However, from the Shannon, np Shannon and Simpson index, the M16 has the highest of the four samples. In other words, there are many more rare species in M16 than in the other samples (table 3).

Beta diversity analysis was used to compare the differences between samples. In this study, the presence of higher species diversity correlated with Beta diversity values closer to zero, which means that the difference between the two samples in the diversity of species present is smaller than expected.

DISCUSSION

Many researchers have explored the bacterial communities in Gulkana Glacier Alaska and East Rongbuk Glacier Everest. All of the phylum of bacteria from Alaska or Everest such as Proteobacteria, Deinococcus-Thermus, Firmicutes, Actinobacteria, and Nitrospirae have been found in Mingyong Glacier. Verrucomicrobia (Bergmann *et al*., 2011) was the characteristic phylum of bacteria in this study, which was not found in Gulkana Glacier and East Rongbuk Glacier. In this situation, we conjectured that the temperature and the altitude were perhaps different in these three glaciers. The data found in table 2 were reported by BGI Company and indicate that the PCR products were satisfactory and that the numbers of OTUs were higher than the numbers of culturable bacterial 16S rRNA gene clone libraries. Moreover, the fraction of coverage obtained in bacterial communities by this method is more than that of other traditional methods. The 16S rRNA gene clone libraries and culture-independent methods were used by Takahiro Segawa *et al* (2011).

The phyla Proteobacteria, Deinococcus-Thermus, Firmicutes and Actinobacteria were the principal bacterial communities in the Mingyong glacier compared to other glaciers (fig. 2). This finding may indicate that the low-temperature environment selected these bacterial communities. In Mingyong Glacier, several different vertical climatic zones such as glacier, forest and dry warm valley are distributed within a very narrow area. Therefore, the bacterial communities were more plentiful in the area with single climatic zone, and the biodiversity is also rich.

In the Pie charts (fig. 1), the unique orders found in M11A, M14 and M16 are shown. M11A had Bacteroidetes Order II. Incertae sedis, unclassified Bacteroidetes, Cardiobacteriales and Mycoplasmatales. M14 had Herpetosiphonales and Syntrophobacterales, while M16 had Puniceicoccales, unclassified Acidobacteria and Desulfarculales. Although the unique orders of each sample were only a small part of the whole, this finding can nonetheless demonstrate that the different climatic zones had their own particular bacterial communities. This phenomenon in Mingyong Glacier can be affected by light intensity, nutrients, and temperature, among other factors. These bacteria may be found in other locations not only in the glacier demonstrating that these bacteria tolerate a broad temperature range.

Using the diversity index (table 3), the diversity of dominant bacterial communities in M11A and M14 is richer than that of the other two samples. However, using the Shannon, np Shannon and Simpson indexes, the diversity of rare species in M16 is higher than that of the other samples. These statistical data indicated that the biodiversity of high altitude areas in Mingyong Glacier was rich. However, the diversity index of sample M15 is the lowest of the four samples, perhaps because sample M15 was collected from a basin inside the mountain that has been natural seasonal grassland for the local residents for many years. Therefore, the biodiversity might have changed with anthropogenic perturbations in this area. This phenomenon may explain why the OTUs of M15 contained a large proportion of Enterobacteriales, which may have come from the dung of poephogus grunniens (Krzic, 2000). In the geographical distribution, the results showed that the difference in the two samples regarding species diversity was no necessary connection exists from beta diversity analysis.

Comparing the diversity index and the type of species

with the four samples, the diversity index of M15 was less than that of the other samples in the index. After removing the different altitude of each sample, the relationship between elements and the biodiversity has been analyzed. Many other researchers were also interested in this phenomenon. After analyzing elements from all the samples (table 1), it was found that the elements S, Mg, Cu and Mn were markedly different than the others. The relationship between elements and the biodiversity in Mingyong Glacier is still not very clear.

The present work was intended to reveal the distribution of bacterial communities in Mingyong Glacier. Results indicated that the biodiversity was rich in this area and that the diversity of rare species is greater in higher altitude areas than in the lower ones. Comparison with the results of previous studies suggests that Proteobacteria, Firmicutes and Actinobacteria were abundant in Mingyong Glacier. The predominant bacterial Phylum was the same as in other glaciers. In this study, a more accurate result has been obtained by using metagenomic 16S rRNA gene V6 hyper variable region sequencing than by using other traditional methods.

This is the first report on the bacterial community abundance, ecological distribution and bacterial diversity isolation in Mingyong Glacier, which is a unique low-latitude, low-temperature, high-altitude and seasonal glacier. By comparison bacterial community in vertical climatic zone along glacier-forest-dry warm valley, it will help us to illustrate adaptation mechanisms between bacterial community structures and environment, and provide clue to further study on relationship of biological diversity and environment in Mingyong Glacier. All experiments are well designed and performed systematically and much valuable fundamental data and experience for further microbial ecology and biogeochemistry research are accumulated. The microbial resource collected in this area can be further studied and exploited in future.

Although we try our best to consider every climatic zone of the glacier before the start of sampling, the Kawakarpo peak is still a virgin peak; thus, there are still many unreachable locations. The sampling locations might not be sufficiently exhaustive to completely represent the Mingyong Glacier. Additionally, the environment for sampling location M15 may have changed with anthropogenic perturbations; this interference should be prevented as much as possible.

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