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Patterns of Microbial Genetic Diversity and the Correlation Between Bacterial Demographic History and Geohistory

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1. Introduction

Microbial diversity is commonly represented by genotypic frequency of the whole gene pool (metagenomes) of a microbial community. The biological community structure is determined by the environment, species competition, and the evolutionary histories of the species living in the community (Aravalli et al., 1998). Because microorganisms are highly sensitive to environmental changes, they can be used as indicators of the properties of their environment (Aravalli et al., 1998). Therefore, the demographic history of a microbial population may indicate changes that have occurred in the local habitat.

Traditionally, the 16S ribosomal RNA genes (16S rRNA) are widely used as genetic barcodes for identifying and recording the microbial organisms of a specific “microbial community” (waters, soils, digestive tracts, etc.) (Liao et al., 2007; Kulakov et al., 2011). Characteristic of the 16S rRNA gene in species differentiation provides as good genetic tool for ecological survey. The comparison of the genome data of two *Prochlorococcus* ecotypes revealed a genetic differentiation in niches which is also reflected in the 16S rRNA differentiation (Rocap et al., 2003). Recently, the advanced (meta)-genomic survey provides more novel insights into the microbial ecology and niche differentiation (Rocap et al., 2003; Shanks et al., 2006; Staley, 2006; Avarre et al., 2007; Biddle et al., 2008; Kalia et al., 2008; Banfield et al., 2010; Benson et al., 2010; Wang et al., 2010; Morales & Holben, 2011). However, the price for the metagenomic survey even by the next-generation sequencing technologies (e.g. the 4 (Chistoserdova, 2010). Therefore, studies for the goals of microbial diversity of a community are still favoring the 16S rRNA genes as genetic barcodes.

In the past decades, the rapid development of the population genetic and phylogeographic analyses based on the coalescent theory leads advancement of the field of molecular evolution in eukaryotes (Avise, 2009; Hickerson et al., 2010). Until recently, the coalescent theory is found to be used for testing the microbial spatiotemporal hypothesis (Gray et al., 2011). The coalescence theory that was firstly proposed by Kingman (1982) provides a practical framework to model genetic variation in a population. This involves tracing backward through time in order to identify events that occurred since the most recent

common ancestor (MRCA) of the samples (Fu & Li, 1999; Kingman, 2000). This theory is sample-based and the speculation of evolutionary processes is more relevant than the classical population genetics theory that describes the properties of the entire population (Fu & Li, 1999). Three essential concepts comprise the coalescent process (Kingman, 2000): (1) the idea of identity-by-descent (Nagylaki, 1989), (2) selective neutrality and a constrained population size (Ewens, 1972, 1972), and (3) independent mutations of genealogy (Kingman, 1980). Along with advanced molecular techniques, approaches developed from the coalescence model can provide a sketch of the demographic history of microorganisms (Perez-Losada et al., 2007). Studies in this field have increasingly supported the reliability of molecular dating by microbial genetic analyses, such as estimating the TMRCA and the radiating time of bacteria and archaea by comparing 16S rRNA gene sequences (Sheridan et al., 2003) and exploring the early evolutionary history of phototrophy and methanogenesis in prokaryotes by the use of a relaxed molecular clock (Battistuzzi et al., 2004). There have also been reports of the successful use of DNA viruses to track recent and ancient local human histories (Pavesi, 2003, 2004, 2005; Kitchen et al., 2008).

2. The problematic definition of species in microbial diversity

Although the genetic diversity inferred by 16S rRNA gene or by genome data well display the entire microbial diversity of a community, the degree of biodiversity that just considers the appearance (birth) and the extinction (death) of “lineages” in phylogeny, i.e. the diversification rate, and ignores the concept of “species diversity” is still difficult to be accepted by traditional biologists. Therefore, species definition (species concept) of microorganisms, especially in prokaryotes, is still perplexed many microbial ecologists and environmental microbiologists, although some of them usually skirt this knotty problem.

However, the use of coalescent theory cannot prevent to discuss this knotty issue because the “monophyletic” species is necessary to be defined firstly (due to the assumption of identity-by-descent) to confirm the accurate coalescent inferences. Several papers discussed the species concept of prokaryotes in different points of view but most of them adopted a concept of “genetic similarity” as the principle (Ward, 1998; Vellai et al., 1999; Rossello-Mora & Amann, 2001; Cohan, 2006; Konstantinidis et al., 2006; Staley, 2006; Wilkins, 2006; Ward et al., 2008; Zimmer, 2008; Doolittle & Zhaxybayeva, 2009; Ereshefsky, 2010; Klenk et al., 2010) rather than the concept of monophyly. Achtman and Wagner (Achtman & Wagner, 2008) summarized five categories of species concepts in microbiology in which none of each has been generally accepted:

1. Monophyletic and genomically coherent cluster of organisms showing a high degree of overall similarity (Rossello-Mora & Amann, 2001)
2. An irreducible cluster of organisms of a common ancestor (Staley, 2006)
3. Having much greater degree of lateral gene transfer between each other than between other groups (Wilkins, 2006; Doolittle & Zhaxybayeva, 2009; Ereshefsky, 2010)
4. Forming a natural cluster (Nesbø et al., 2006)
5. Metapopulation lineages (Ereshefsky, 2010)

The first concept for delimiting species by monophyly should be used for the application of coalescent theory, such as inferring the demographic history (Fu & Li, 1999; Rosenberg & Nordborg, 2002; Rosenberg, 2003). In addition, the degree of genetic similarity is also a key

factor to determine the length of coalescent time. In other words, lineages of a clade with shorter genetic distance reflect relatively recent coalescent history (of this clade). In contrast, the clade composed of lineages with long genetic distance can reflect relatively long-historical demography but with wider variance and larger inaccuracy.

In general, the genetic similarity of microbes $\leq 98.7\%$ estimated by 16S rRNA genes are considered as different species but the opposite is not necessary true, i.e. the genetic similarity $\geq 98.7\%$ might not be the same species (Stackebrandt & Ebers, 2006; Achtman & Wagner, 2008). This value matches to the threshold of 70% reassociation value in DNA-DNA hybridization (Stackebrandt & Ebers, 2006). The value $< 98.7\%$ (or to round off $< 99\%$) identity in 16S rRNA gene overturns the old threshold of $< 97\%$ identity for delineating species of microorganisms. Therefore the definition of “a species” in microbiology, for the purpose for phylogeographic or demographic inferences by the coalescent theory, is concluded as an integration of monophyly and genetic distance lower than 1%.

3. A case study: Simple microbial composition of a volcanic pond and a demographic association of demographic history of microbes with geographic history

In this case study, the microbial composition of an acidic sulfidic lake located in the northern Taiwan is exemplified by genetic barcodes to represent the microbial diversity of a community and the microevolution of the dominant bacteria is further explored by the application of the coalescent theory. The sulfate lake is a special water type, since only certain chemical autotrophic microbes are able to utilize sulfides or sulfates as energy source (Moreira & Amils, 1997). Our study site, the “Niunai (Milky) Lake”, is a small crater pond composed of sulfate substrates, located in a volcanic mountain, Mt. Datun, in the Yangmingshan National Park (YMSNP) in the northern part of Taipei County in Taiwan (Fig. 1). The source of the water is a mineral spring and the abundant rainfall from the northeast monsoon in North Taiwan (4526.4 mm per year on average at Chutzuhu Station in YMSNP, Table 1). This lake has never dried up since records have been kept. Due to the neutralization by rainfall, the water is mildly acidic (approximate pH 6–7) and the water temperature is approximately 38–40°C. The crater took shape during a volcanic eruption in the Quaternary Period and two major eruptions have made the recent topography of the Datun Volcano Groups. The first eruption was 2.8 to 2.5 mega annual before present (Ma BP) and the last time a Datun volcano erupted was approximately 0.8–0.2 Ma BP. The volcanoes have been reposed since. Similar to Yellowstone National Park, sulfur is rich in the nearby soil, rocks, and waters in the Datun Volcanoes. It has been reported that the endolithic microbes preserved the geological history of Yellowstone National Park (Walker et al., 2005). Very few natural microbial communities, especially those in extreme environments like the Datun Volcanoes, have been reported in Taiwan, which is a young island that took shape less than 5 Ma BP (Shen, 1996). Therefore, the sulfate-rich pool, Niunai Lake, serves as an excellent template to explore the microbial community structure and the evolutionary history of the dominant species in the volcanic mountains of Taiwan. Through the analyses of the phylogenetic community structure, which can assess the community assemblages (Kraft et al., 2007), and the population genetic structure, we present

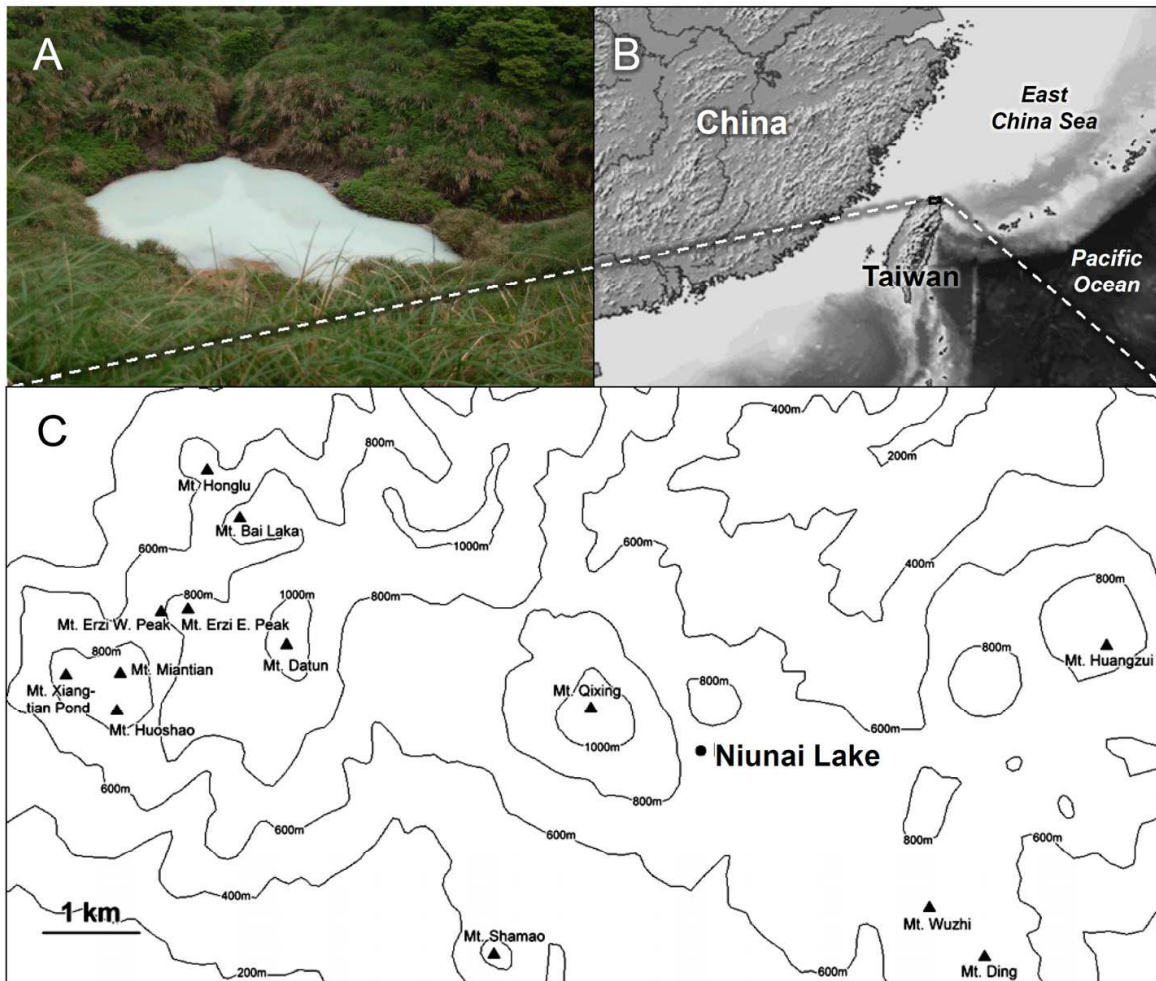


Fig. 1. Picture and map of the Niunai Lake. (A) The panorama of the Niunai Lake; (B) the location of the Niunai Lake in the northern Taiwan; (C) the contour map of the Yangmingshan National Park where the Niunai Lake located.

here the microbial community and population structures of a sulfur-rich environment. Several well-known studies that were based on culture-independent approaches (because less than 1% of microbial species are cultivable) have indicated that several unknown and unexpected taxa were discovered in the microbial communities (Giovannoni & Stingl, 2005). For example, 37% of the clones isolated from an extremely acidic (\sim pH 1) endolithic microhabitat in the Yellowstone geothermal environment were identified as *Mycobacterium* spp., which are pathogens of humans. (Walker et al., 2005). Additionally, while the long-considered dominant cyanobacteria comprised less than 5% of the clones isolated from the microbial community of the stromatolites of Hamelin Pool in Shark Bay, Western Australia, unknown proteobacteria comprised 28% of the clones. (Papineau et al., 2005). These studies suggest an unknown field of environmental microbiology that requires further investigation. According to the model proposed by Stackebrandt and Ebers (2006) and Acinas et al. (2004), 16S rRNA sequences of microorganisms that were more than 98.7% \sim 99% similar could be treated as an operational taxonomic unit (OTU). These OTUs, as defined by Stackebrandt and Ebers (2006) and Acinas et al. (2004), are 'microdiverse ribotype clusters' and are considered an important differentiation unit in natural bacterial

communities. In other words, 99% similarity delineates different microbial species in nature. Therefore we assumed that the observed ribotypes may represent the species or the categories of the microorganisms in the collected samples and that their colony frequencies are indicative of the composition of the microbial species. As such, instead of using microbial culture methods, we used culture-independent techniques (Perez-Losada et al., 2007) to examine the composition of the microbial community in Niunai Lake.

	Jan	Feb	Mar	Apr	May	Jun	Jul	Aug	Sep	Oct	Nov	Dec	Mean
Accumulated precipitation (mm)	269.3	277.3	240.3	207.8	275.3	294.7	248.3	446	588.1	837.3	521.9	320.1	377.2
Precipitation days	20	18	18	15	16	14	10	13	15	19	21	20	16.6
Mean temp. (°C)	11.7	12.2	14.6	18.1	20.9	23.5	24.8	24.5	22.7	19.8	16.4	13.3	18.5
Maximum temp. (°C)	15.3	15.8	18.8	22.4	24.9	27.5	29.4	29	26.9	23.4	19.7	16.8	22.5
Minimum temp. (°C)	9.2	9.6	11.7	15.1	18.2	20.9	21.9	21.8	20.3	17.7	14.2	10.9	16
Relative humidity (%)	88	89	88	87	87	87	84	84	85	87	88	88	86

Table 1. Statistical records (1971 ~ 2000) of weather at the Chutzuhu Station in YMSNP, sources from Central Weather Bureau, Taiwan (<http://www.cwb.gov.tw/>).

In this case study, we report the results of our study examining the composition of the microbial community in Niunai Lake using a 16S rRNA gene library. In addition, the long-term demographic history of the dominant taxon (*Thiomonas* sp.) of this community and the factors that influenced it are presented. We try to make a connection between the geological and demographic history of microbes in terms of molecular ecology and this should be helpful in understanding the relationship between environmental factors and the microbial composition.

3.1 Methods

3.1.1 Sampling and molecular techniques

The water samples were collected one meter below the water surface from Niunai Lake (25°10'00"N, 121°33'52"E) in the Datun Volcano Group in Yangmingshan National Park (YMSNP) in Taipei, Taiwan. The weather records in YMSNP are listed in Table 1. The Niunai Lake is a sulfate-rich (20–40% sulfide) pond at an altitude of 700 m. Water samples were collected in sterile bottles and immediately incubated on ice until filtration and metagenomic DNA extraction.

DNA extraction was immediately carried out in order to prevent a bias from the foraging of microfauna. The DNA extraction protocols have been previously described (Liao et al., 2001). The water samples were pre-filtered through Nuclepore PE filters with pore size of 11 µm. The filtered water was then passed through 0.22 µm filters and these filters were cut into pieces, soaked in the extraction buffer (200 mM Tris-Cl pH 7.5, 250 mM NaCl, 25 mM EDTA, and 0.5% SDS), and shaken for homogenization. The metagenomic DNA was extracted with phenol-chloroform-isoamyl alcohol buffer and the total extracted DNA was dissolved in ddH₂O for subsequent analysis.

16S rRNA gene fragments were selectively amplified from the genomic DNA using the following two PCR primers (Field et al., 1997): forward primer 27F (5'-AGAGTTTGATCMTGGCTCAG-3', nucleotides 8-27 relative to the *Escherichia coli* 16S rRNA gene) and reverse primer 1522R (5'-AAGGAGGTGATCCANCCRCA-3', nucleotides 1522-1541 relative to *E. coli*). This primer set is universal in amplifying most bacterial 16S rRNA genes. The PCR reactions (50 μ L) contained 0.4 μ L of extracted DNA sample, 1 μ M each primer, 0.1 mM each deoxynucleoside triphosphate, 20 μ g BSA, and 2.5 U of Super *Taq* polymerase (Violet) in 10X PCR buffer (Violet). The Super *Taq* polymerase was used for enlarging the yields of PCR product and decreasing the rate of PCR error. PCR parameters were as follows: an initial denaturation at 94°C for 5 min, followed by 35 cycles of denaturation (94°C for 60 sec), annealing (55°C for 60 sec), and extension (72°C for 90 sec), with a final extension step (72°C for 7 min). The PCR products were resolved in an ethidium bromide-stained 1% agarose gel in TBE. DNA fragments of the expected size were purified from the gel using the Gel Extraction System Kit (Viogene).

The 16S rRNA gene library was constructed by cloning the amplified PCR products into the *p*T&A vector (Yeastern Biotech). Competent DH5 α cells (*E. coli*) were transformed with the vector by heat shock transformation at 42° for 45 sec. The transformed cells were spread onto LB agar plates containing ampicillin, 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside, and isopropyl- β -D-thiogalactopyranoside (LB Ampicillin/X-gal/IPTG). Positive clones (which contained 16S rRNA gene PCR inserts) were confirmed using the M13F and M13R primers and were picked for further sequence analysis. Both directional sequencing was done at Genomics BioScience & Tech Co., Ltd. The sequences obtained in this study were deposited in GenBank under the accession numbers DQ145964-DQ146147.

3.1.2 Data analyses

The 16S rRNA gene sequences obtained in the study and those of known microorganisms in the NCBI database were aligned using the program Clustal X (Thompson et al., 1997), and then manually edited with the program BioEdit (Hall, 1999). All sequences were tested for possible chimeric artifacts using the Bellerophon software (Kelly et al., 2007). Putative chimeras were excluded from further analyses. Phylogenetic analysis (neighbor-joining (NJ) method) of the aligned data sets was then performed using TOPALi version 2.17 (Milne et al., 2004). The substitution model and rate model used for constructing the NJ tree were F84 (transition/transversion = 1.10) and gamma distribution (alpha = 0.69), respectively. The bootstrap analysis was conducted with 1,000 replications. From the analysis of the phylogenetic tree, the 16S rRNA gene clones of Niunai Lake were classified and the relative frequencies of taxa were counted. The species affinity of the 16S rRNA gene clones were identified through comparison with the Ribosomal Database Project (<http://rdp.cme.msu.edu/>).

After barcoding with the molecular characteristics by phylogenetic method (Liao et al., 2007), the number of microbial species in the Niunai Lake was estimated using the definition of microbial species proposed by Acinas et al. (2004) and Stackebrandt & Ebers (2006). Additionally, the "expected" richness was estimated using the S_{Chao1} index (Chao, 1984) and the rarefaction was estimated using the Rarefaction Calculator (<http://www2.biology.ualberta.ca/jbrzusto/rarefact.php>). The S_{Chao1} index is a nonparametric estimator of species

richness (Chao, 1984). In addition, the genetic polymorphisms of the dominant microbial species, *Thiomonas* sp. in the Niunai Lake were calculated. The haplotype diversity (H_d) and the π (i.e., the average number of pairwise nucleotide differences) and θ ($\theta = 4N\mu$, where N is the effective population size and μ is the mutation rate estimated by the total number of mutations) indices of nucleotide diversity (Nei, 1987) were calculated in order to understand the style of genetic variation in this microorganism.

In order to infer the demographic history, the mismatch distribution of the 16S rDNA of *Thiomonas* sp. was calculated using DnaSP 4.0 (Rozas et al., 2003). The Tajima's D (Tajima, 1989) and Fu's F_s (Fu, 1997) tests were used to assess the effect of demographic changes and were calculated using Arlequin 3.11 (Excoffier et al., 2005). Generally speaking, these tests are based on the frequency distributions of variations. With the exception of these tests, the demographic inferences were carried out using the Bayesian skyline plot (BSP) analysis (Drummond et al., 2005) and the software BEAST 1.4.8 (Drummond & Rambaut, 2007) in order to estimate fluctuations in the effective population size. This method estimates a posterior distribution of effective population sizes backward through time until the most recent common ancestor using MCMC procedures. The constant population size coalescent model was the basic assumption used for this approach. The model developed by Jukes and Cantor (Jukes & Cantor, 1969) was used for distance matrix correction. A uniform rate across all branches (strict molecular clock) and the general time reversible substitution model were used for this calculation. In order to obtain the correct parameters and a higher effective sample size for BSP analysis, six pre-runs were performed and the parameters were modified according to the suggestions of the runs. Markov chains were run for 1×10^7 generations for pre-runs and 3×10^7 generations for the final run and were sampled every 1,000 generations, with the first 10% of the samples discarded as burn-in. The other parameters were set as default. The TRACER v1.4 program (Rambaut & Drummond, 2007) was used to visualize the posterior probabilities of the Markov chain statistics and to calculate a statistical summary of the genetic parameters.

3.2 Results

3.2.1 Phylogenetic assemblage of the microbial community

A 1625 base pair, after sequence alignment, partial 16S rRNA gene sequence was used in the analysis. Among these 1625 sites (characters), 790 were constant, 171 were variable characters that were parsimony-uninformative, and the remaining 664 sites were parsimony-informative characters. A total of 181 haplotypes (considering gaps) or 148 haplotypes (not considering gaps) were obtained from the 184 clones in our 16S rRNA gene library. The 16S rRNA gene library derived from Niunai Lake samples was analyzed by NJ comparisons (Fig. 2) in which 13 species were identified using the species definition proposed by Acinas et al. (2004) and Stackebrandt & Ebers (2006). One microbial species belonging to the genus *Thiomonas* (Burkholderiales; 91.85% in abundance), four species belonging to the genus *Thiobacillus* (Burkholderiales; 2.72% in abundance), one species belonging to the genus *Acidiphilium* (Acetobacteraceae; one clone), and one species belonging to the genus *Escherichia* (Enterobacteraceae; one clone) were identified. Additionally, eight clones from unknown species were identified (Fig. 3). One of the eight unknown taxa belonged to the epsilonproteobacteria and the others were betaproteobacteria, based on the grouping of the neighbor-joining tree (Fig. 2).

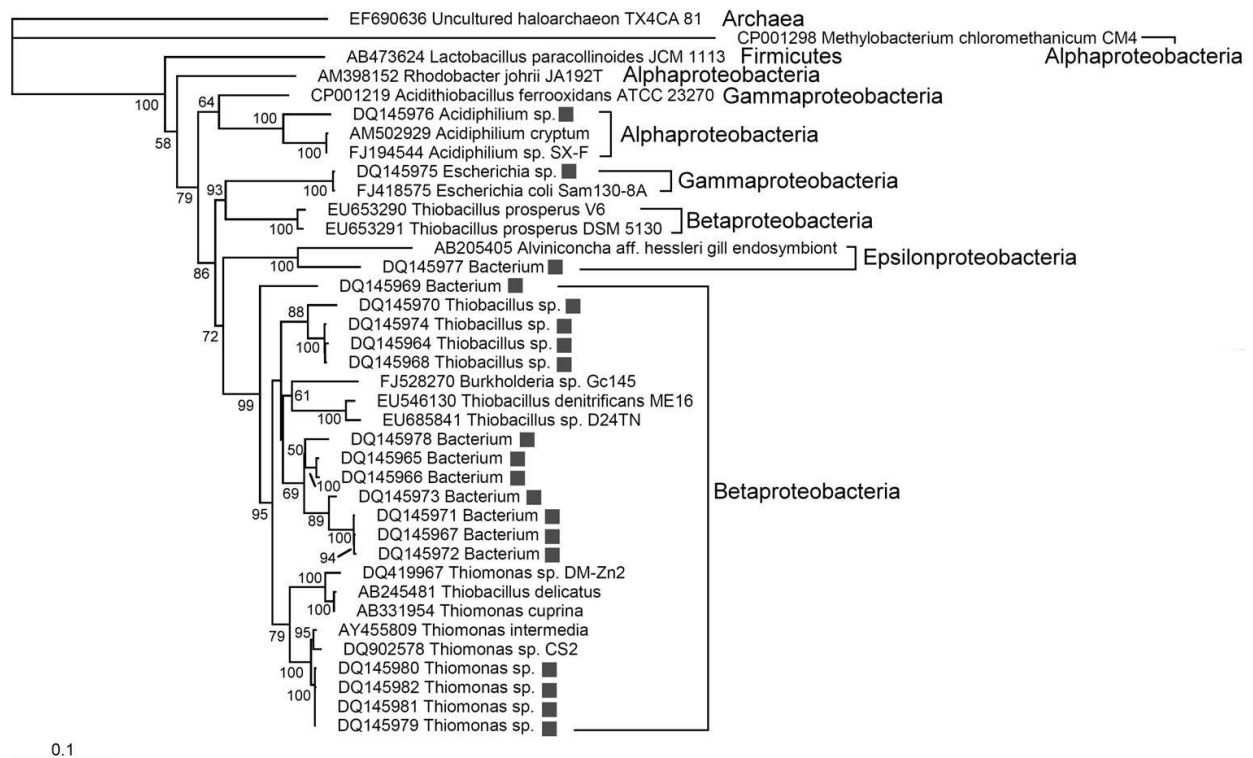


Fig. 2. Phylogenetic analysis of the 16S rRNA genes obtained from the Niunai Lake-derived clones and from the NCBI database. The reference sequences from NCBI were obtained by BLAST search. The tree was constructed by the neighbor joining method with the F84+G model and 1,000 bootstrapping replicates. The paraphyletic grouping is due to the genetic similarity of the sequences and cannot be explained as phylogenetic affinity. The lineages indicated in squares are sequences obtained in this study. The scale bar is the expected substitutions per site.

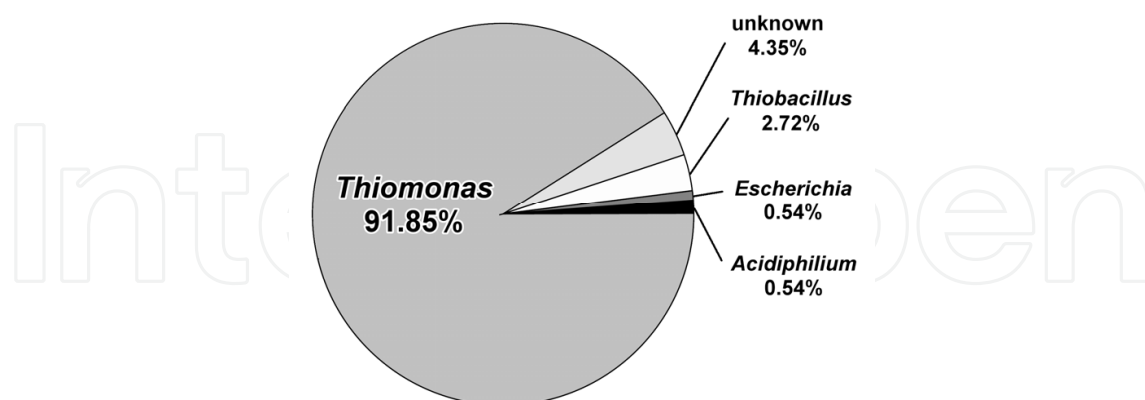


Fig. 3. Microbial species composition of the Niunai Lake community. The frequencies were estimated by the relative frequencies of the colony sizes of the 16S rRNA gene library.

Even though seven taxa of known genera were identified in the microbial community of Niunai Lake, they were all undescribed species. Additionally, although the relative sample size of the unknown taxa was small (8/184) compared to the known genera (176/184), almost half of the taxa identified in this research were previously unknown (6/13). Acinas et

al. (2004) have reported that for microorganisms, there was a 70% decrease in the number of OTUs (from 1633 to 520 OTUs), when only 99% sequence identity (as opposed to 100% sequence identity) was used as the cut-off. The dramatic decrease in the OTU number using the cut-off of 99% 16S rRNA gene sequence similarity may be due to variations within species and PCR errors. Based on the definition proposed by Acinas et al. (2004), the number of OTUs versus cluster similarity of our findings is shown in Fig. 4. The similarity in genetic composition was also demonstrated by a pairwise comparison where most variations fell into a 0.02% genetic distance (Fig. 4, inset). This similarity in microbial composition indicates that microbial species were selected by the acidic environment of Niunai Lake and that the composition of microbial community was simple.

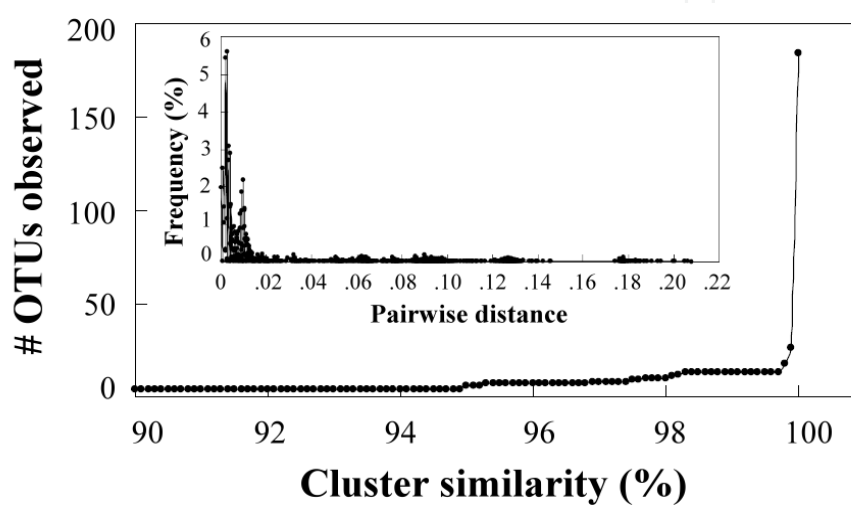


Fig. 4. Cluster-similarity curve of OTUs based on Nei's (1987) genetic distance. The uplift in 99% similarity indicates most microbial organisms in Niunai Lake belong to a single species. The inner plot indicates the frequency distribution of pairwise distance that demonstrates that most haplotypes are similar to each other within 0.02 genetic distances.

In our 16S rRNA gene library, there could be an inevitable amplification bias due to primer specificity. As a result, the unamplified 16S ribotypes would not be seen in this study (Moore et al., 1998; Morris et al., 2002). In order to estimate the probable species richness, both the S_{Chao1} and species-accumulation-curve methods were used. Both analyses indicated a higher number of species than what was detected in the 16S rRNA gene library. The S_{Chao1} index was 26.5 ± 9.418 species and the species-accumulation curve suggested a maximum number of 19.5 species (Fig. 5). Therefore, we could expect a greater microbial richness in Niunai Lake, even in such a harsh environment full of acidic, sulfurous, and *Thiomonas*-rich competitive stresses.

3.2.2 Genetic diversity and demographic history of *Thiomonas* sp.

The dominant bacterium *Thiomonas* sp. had a haplotype diversity (Hd) of 0.993, which indicates that most of the clones are different in genetic composition. The pairwise diversity (π) and genetic diversity index (θ) estimated from the segregating site (S) are 0.0146 ± 0.0077 and 0.0340 ± 0.0065 , respectively (Table 2). Both Tajima's D and Fu's F_s indices, which reflect the demography, have significant negatives ($D = -2.648$, $P < 0.00001$; $F_s = -23.746$, $P = 0.004$,

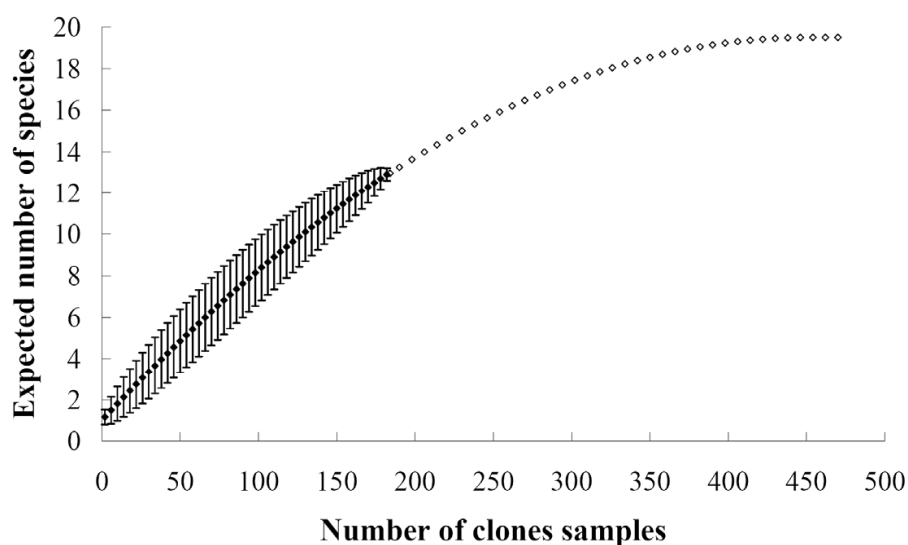


Fig. 5. Cumulative numbers of OTUs (the rarefaction analysis) as a function of the number of clones sequenced. The species-accumulation curve (black diamond dots) is not saturated, which suggests that the estimated number of microbial species of Niunai Lake is underestimated. The curve following the black diamond curve is a simulated curve that achieves a maximum number of species of 19.5 at 454 clones.

respectively) and indicate population expansion events (Table 2). We then used these nucleotide differences to calculate the distribution of pairwise differences (mismatch distribution). There was a left skew curve in the mismatch distribution plot, which had higher observed values than the expected values (Fig. 6). The differences in allelic frequency indicate that the observed values of the first four differences are lower than the expected values (Fig. 6, inset). In addition, clones of *Thiomonas* sp. are most pairwise different from each other in 5–10 nucleotide differences and illustrate an event of rapid population growth in the recent past, but not at the present.

Hd	π ($\times 10^3$)	S.D. ($\times 10^3$)	θ ($\times 10^3$)	S.D. ($\times 10^3$)	Tajima's D	P	Fu's Fs	P
0.993	14.617	7.678	33.974	6.463	-2.648	<0.00001	-23.746	0.004

Table 2. Genetic diversity of *Thiomonas* sp. in Niunai Lake. Hd: haplotype diversity (Nei, 1987); π : nucleotide diversity (Nei, 1987); θ : nucleotide diversity estimated by total number of mutations (Nei, 1987).

Except for the mismatch distribution, the BSP analysis was performed to depict the demographic history of *Thiomonas* sp. in Niunai Lake. Initially, the time to MRCA (TMRCA) of the *Thiomonas* sp. of the Niunai Lake was calculated. The strict-molecular-clock mode was selected because the known nodes for suitable molecular-dating were not acquired. Thus, the commonly used substitution rate of 4.5×10^{-9} per nucleotide site per year for prokaryotic SSU rDNA (estimated from *Escherichia coli*) suggested by Ochman and colleagues (Ochman & Wilson, 1987; Ochman et al., 1999) was used for calculating the TMRCA of *Thiomonas* sp. The coalescent time was estimated to be 7 Ma BP (6–9 Ma BP at the 95% confidence interval; Table 3), which is shorter than the coalescent time of 12 Ma BP (7.6–16.4 Ma BP at the 95% confidence interval) for whole *Thiomonas* species. In addition to the TMRCA, the demographic

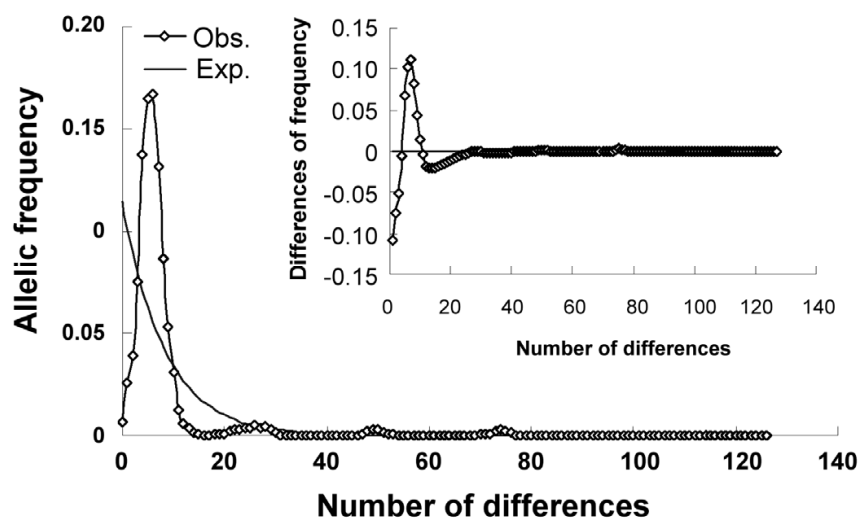


Fig. 6. Mismatch-distribution plot of pairwise differences of nucleotides. The right-skewed peak from the expected curve indicates a past-population-expansion of *Thiomonas* sp. in Niunai Lake. The diamond-line is the observed allelic frequency from the obtained sequences; the straight-line is the expectation by letting $\theta_{\text{initial}} = 8.971$ (per seq) and infinite θ_{final} . The inner plot indicates the differences of the observed- and expected-allelic-frequency curves.

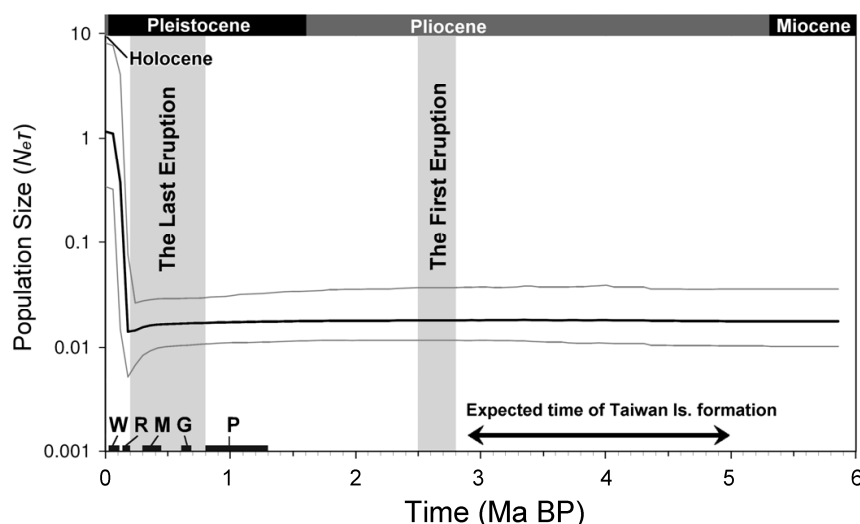


Fig. 7. Bayesian skyline plot for the population of *Thiomonas* in Niunai Lake. The plot was estimated by using the 16S rRNA sequences and was generated with a mutation rate of 4.5×10^{-9} per site per year (Ochman et al., 1999). The background effective population size of the *Thiomonas* population before the rapid expansion that occurred approximately 0.18 Ma BP was a result of the ancestral polymorphisms of *Thiomonas*. The x -axis is the time (Ma BP) and the y -axis is the scaled effective population size. The black line represents the medium value and the 95% confidence interval is shown by the gray lines. The geological time scale is presented in the top of graph. The shaded area indicates the eruptions of the Datun Volcanoes (Kim et al., 2005). The bars indicated as W, R, M, G, and P on the x -axis represent the Pleistocene glacial epochs Würm, Riss, Mindel, Günz, and Pre-Pastonian Stages, respectively, named according to the Alps glaciation events.

history was estimated by the BSP analysis. A constant population size through time was estimated until 0.35 Ma BP. The population size of *Thiomonas* sp. declined slightly from 0.35 Ma to 0.18 Ma BP and a rapid population growth followed until approximate 60 millennia BP when the growth rate gradually decreased (Fig. 7). The rapid population growth is approximate at two orders of magnitude.

3.3 Discussion

In this case study, two aspects were discussed: the microbial composition of the Niunai Lake community and the demographic history of the dominant bacteria *Thiomonas* sp. of Niunai Lake. We will try to illustrate the interactions between microorganisms and geologic history in terms of both of these aspects.

	Likelihood		TMRCA			
	<i>Thiomonas</i> sp. (Niunai Lake)	<i>Thiomonas</i> (NCBI)	<i>Thiomonas</i> sp. (Niunai Lake)		<i>Thiomonas</i> (NCBI)	
Mean	-5329.602	-1902.352	0.0332	(7.38)	0.0537	(11.93)
Stdev of mean	0.564	0.202	4.96×10 ⁻⁵	(11.03×10 ⁻³)	3.80×10 ⁻⁴	(8.45×10 ⁻³)
Median	-5329.21	-1901.978	0.0330	(7.33)	0.0522	(11.60)
95% HPD lower	-5347.411	-1911.269	0.0266	(5.91)	0.0343	(7.62)
95% HPD upper	-5312.535	-1894.13	0.0400	(8.89)	0.0737	(16.38)
Effective sample size (ESS)	253.488	504.472	4821.903		765.687	

Table 3. Summary statistics of maximum likelihood values for Bayesian skyline plot (BSP) analysis and the time to most recent common ancestor (TMRCA) estimated by BSP analysis. The unit of TMRCA is substitutions per nucleotide site and the parentheses are the dating by dividing the substitution rate (unit: Ma BP).

3.3.1 Microbial composition in the Niunai Lake community

The species composition in the microbial community was estimated using the phylogenetic approach. Even though the genera of most of the microbes were identified, the definite species are unknown, similar to other studies using this method (Blank et al., 2002; Walker et al., 2005; Omoregie et al., 2008). In spite of this drawback, the phylogenetic approach is still reliable for microbial identification and for inferring the microbial composition of the sample. In this study, a severe paraphyletic grouping was obtained (Fig. 2). The nucleotide compositions of these microbes, especially the rRNA nucleotide composition (Rudi, 2009), were not only shaped by heredity but could be affected by the properties of environment that they inhabited (Foerstner et al., 2005; Rudi, 2009). This may result in a similar genetic composition (e.g., GC content), a close genetic distance of distantly related microbes, and the disordered grouping of the phylogenetic tree.

The species composition of the microbial community of Niunai Lake is simpler than other environments (Table 4). Only 13 microbial species were detected in the 16S rDNA library,

Site	Area	Environmental properties	pH	Temp.	Dominant microbes	Species richness ^a	Reference
Chefren mud volcano, Nile Deep Sea Fan	Eastern Mediterranean	Iron- and Sulfide-rich	-	-	<i>Candidatus</i> Arcobacter sulfidicus (24%) in white mats; neutrophilic Fe(II)-oxidizing betaproteobacterium <i>Leptothrix ochracea</i> in orange mats	Very diverse	(Omoregie et al., 2008)
Soap Lake, lower Grand Coulee in E Washington State	USA	Saline and alkaline	9.8	7.3° ~ 16.3°C	Proteobacteria	508 (653)	(Dirnithri et al., 2008)
Stromatolites of Hamelin Pool in Shark Bay	Western Australia	Hypersaline	-	17° ~ 27°C	Novel proteobacteria (28%), planctomycetes (17%), and actinobacteria (14%)	71 (178) of surface, 124 (505) of interior, and 90 (566) of irregular sampling	(Papineau et al., 2005)
Danshui River Estuary, mangrove ecosystem	Taiwan	Salinity 7 ~ 25 PSU, dissolved nitrogen 0.15-6.59 (2.88 on average) mg L ⁻¹ ; dissolved phosphorus 0.02-1.53 (0.28 on average) mg L ⁻¹ ; suspended solid 42.75 mg L ⁻¹	~7.7	12.9° ~ 32°C (18°C on average)	Rhodobacteraceae (28.65%)	84 (447)	(Liao et al., 2007)
Rowley River in Plum Island Sound salt marsh, NE Massachusetts	USA	Marsh grass <i>Spartina alterniflora</i> , 20% ~ 34‰ salinity	-	-	Desulfobacteriaceae, Desulfobulbaceae and Desulfovibrionaceae (46.91%)	200 (332)	(Klepac-Ceraj et al., 2004)
Charca Verde pond, the forested university campus of Orsay	France	Freshwater suboxic pond, particles of organic material rich	7.07 ~ 7.8	7.8° ~ 9.9°C	Candidate division OD1 and beta-Proteobacteria	100 ~ 120 (170 ~ 198)	(Briée et al., 2007)
Volcanic lake in Deception Island in	Antarctica	140.1 S cm ⁻¹ of conductivity, low soluble reactive	6.7 ~ 7.7	1.1° ~ 6.5°C	Bacillariophyceae (58.70%)	46	(Llames & Vinocur, 2007)

Site	Area	Environmental properties	pH	Temp.	Dominant microbes	Species richness ^a	Reference
South Shetland Islands		phosphorus (69.2 g L ⁻¹) but high total phosphorus (418 g L ⁻¹), low dissolved inorganic nitrogen (20 µg L ⁻¹) and total nitrogen (75 µg L ⁻¹)					
Norris Geyser Basin, Yellowstone National Park	USA	Geothermal, high concentrations of sulfuric acid, metals, and silicates	~1	~35°C	<i>Mycobacterium</i> spp. (37%) and <i>Cyanidium</i> spp. (26%)	~40	(Walker et al., 2005)
Seafloor of Sagami Bay, Hatsushima Island	Japan	Cold-seep sediments in the deep sea (1168 ~1174 m in depth); hypersaline	-	-	<i>Calymptogena</i> spp. (64%)	>24	(Fang et al., 2006)
Niunai Lake, Datun Volcanoes	Taiwan	Sulfide-rich (20%–40% sulfide)	~6.5	38° ~ 40°C	<i>Thiomonas</i> sp. (91.85%)	13 (26.5)	This study
Saline mud volcano at San Biagio-Belpasso, Mt. Etna	Italy	High salinity brines (up to 100 g/L) with high concentrations of Na ⁺ and Cl ⁻ ions (93–95%); CO ₂ -rich gases (85–87% of total gas composition); lower amounts of methane (7 ~ 10%), nitrogen (1.8 ~ 2.3%), and oxygen (0.3 ~ 0.5%)	6.58	15.8°C	<i>Marinobacter</i> sp. (20%), <i>Propionibacterium acnes</i> (18.57%), and <i>Methylobacterium alcaliphilum</i> (15%)	19	(Yakimov et al., 2002)
Norris Basin, Yellowstone National Park	USA	Arsenite-oxidizing acidic thermal spring with arsenic concentration ~33 µM	3.1	58° ~ 62°C	<i>Hydrogenobacter acidophilus</i> (79% ~84%)	>8	(Jackson et al., 2001)

^a Numbers in parentheses are the expected species richness estimated by the Chao1 (Chao, 1984) equation.

Table 4. Comparisons of the dominant species and species richness of the Niunai Lake microbial community with other environments. This table is ordered by the species richness.

while 19.5 or 26.5 species were calculated to be expected. We compared the environmental properties and microbial species richness between the Niunai Lake and other locations (Table 4). In contrast to the highly complex microbial communities in nutrient-rich bodies of water (e.g., the Soap Lake in eastern Washington State (Dirnithiu et al., 2008), Charca Verde pond in the University of Orsay campus in France (Bri e et al., 2007), and the mangrove ecosystem of the Danshui River estuary in northern Taiwan (Liao et al., 2007)), most of the harsh environments have a lower species richness. When compared to the microbial communities in these volcanic or geothermal environments (e.g., the saline mud volcano at Mt. Etna in Italy (Yakimov et al., 2002) and the acidic thermal spring in Yellowstone National Park (Jackson et al., 2001)), the small species richness of Niunai Lake is not surprising. In addition, the dominant microbes metabolically correspond to the chemical properties of the environment. The simple microbial communities could be a consequence of long-term environmental selection and these dominant microbial species could be bio-indicators of the strict environments.

In the 16S rRNA gene library from Niunai Lake, 169 of 184 sequences (91.85%) were contributed to the genus *Thiomonas*, each as a different genotype. A similar dominance by a single microbial taxon in a strict environment has been reported. For example, 79–84% of the identified strains from the acidic thermal spring in the Norris Basin in Yellowstone National Park were *Hydrogenobacter acidophilus* (Jackson et al., 2001) and 64% of the strains from the deep-sea cold-seep sediments in the seafloor of Sagami Bay in Japan were *Calyptogenia* spp. (Fang et al., 2006). However, the abundance of *Thiomonas* sp. (91.85%) is higher than these cases. This suggests that a series of periodic selection events purged the diversity of the microbial community (Cohan, 2006) or just the conditions that favor *Thiomonas* sp. from the onset, and the remnant *Thiomonas* sp. may play an important ecofunctional role in Niunai Lake. The slightly diversified ecotypes could be the descendants of the surviving variant of the last selection event (Cohan, 2006). The existence of multiple ecotypes, or forms, is a general phenomenon in the microbial world for adaptation and survival in a broad range of extreme environments (Moore et al., 1998). For example, in a study by Walker et al. (Walker et al., 2005) that examined the composition of the microbial endolithic community in a geothermal environment (~35°C, pH 1) in Yellowstone National Park, the abundant and diversified microbes (37% *Mycobacterium* spp. and 26% *Cyanidium* spp. in abundance), and most of other microbes, were those that could adapt to the acidic and thermal environment. Although the geologic condition of YMSNP is not as well defined as that of Yellowstone National Park, the microbial communities in Niunai Lake may still be a representative of those dwelling in a volcanic environment similar to YMSNP. The most abundant microbes in Niunai Lake, *Thiomonas* sp., occur widely with the presence of thiosulfate, tetrathionate, H₂S, and elemental sulfur. This genus also contains facultative chemolithoautotrophs (Moreira & Amils, 1997). The genus *Thiomonas* was discovered and classified by Moreira and Amils in 1997 (Moreira & Amils, 1997) and was split from *Thiobacillus* because of characteristics identified in the phylogenetic analysis. However, *Thiomonas* also share some physiological features with *Thiobacillus*, such as the capability of oxidizing sulfides to sulfuric acid as metabolic products (Temple & Colmer, 1951). The detailed metabolic properties of *Thiomonas* spp. were described by Kelly et al. (2007). Niunai Lake is a sulfate pond in a stratum of volcano. Due to the high sulfate level in the substrate, the dissolved sulfate or sulfide in Niunai Lake results in a special and extreme environment that would

select for the microbes that have the ability to gain energy from the oxidation of a reduced sulfur compound. Another taxon present in Niunai Lake is *Acidiphilium* sp. (or related taxon), which plays an important role in sulfur-oxidization similar to *Thiobacillus* and *Thiomonas*. *Escherichia* is another genus found in Niunai Lake. While we cannot exclude the probability of contamination, the existence of *Escherichia* may also be due to the activities of wildlife around the Niunai Lake.

3.3.2 Climatic change, volcanism, and demographic history of *Thiomonas* sp.

In this part of the study, the evolutionary history of *Thiomonas* sp. was explored instead of the short-term population dynamics. The short-term population dynamics of microbial organisms, which are influenced by nutrients, toxins, temperature, and other biotic and abiotic factors, are commonly described through empirical studies (Tang et al., 1997; Lee et al., 2007; Lee et al., 2008; Ying et al., 2008). However, the long-term population demographics of bacteria and viruses are relatively few and most are relative to the evolutionary history of the host (Pavesi, 2003, 2004, 2005; Kitchen et al., 2008). However, some studies of molecular dating on microorganisms have been reported (Franzmann, 1996; Sheridan et al., 2003; Battistuzzi et al., 2004; Acquisti et al., 2007) and these studies provide excellent details on the molecular clock of microbial organisms.

Three independent analyses all indicate a population-growth event of *Thiomonas* sp. in Niunai Lake. The significant negatives of both Tajima's D and Fu's F_s indices, especially the high sensitivity in population expansion of negative Fu's F_s (Fu, 1997), suggest an increase in the population size. In addition, the mismatch distribution also demonstrates a left-skewed curve with a higher difference of frequency than expected, which is commonly explained by a population growth after a bottleneck event (Hwang et al., 2003; Cheng et al., 2005; Johnson et al., 2007). However, the number of differences is lower in the observed than the expected. This indicates that the event of population growth occurred in the recent past but not very recently. This speculation was supported by the Bayesian calculation according to the coalescent theory. The BSP analysis illustrates a similar demographic history of a bottleneck event by mismatch distribution resulting in a slight population decline (bottleneck effect) approximately 0.35 Ma to 0.18 Ma BP that was followed by a rapid expansion of population size until 60 millennia BP. From this point, the growth rate of the population decreased.

Similar to other studies of demographic histories (Flagstad & Roed, 2003; Carnaval & Bates, 2007; Lin et al., 2008), when compared with the paleoclimatic change, we noticed that the timing of the population decline seems to match the Mindel glacial epoch in the middle Pleistocene and the population expansion started at the beginning of Riss glacial epoch. Moreover, the timing of the gradual decrease in the population growth rate started at the beginning of the Würm glacial epoch. Therefore, the demographic history of *Thiomonas* sp. in Niunai Lake seems to perfectly match the glacial cycles. However, the effect of climatic changes on the demographic history of bacteria is full of paradoxes. First, it is difficult to explain the relationship between population growth and the Riss glacial period. Logically, the postglacial population expansion was reasonable, like other studies (Mikheyev et al., 2008), instead of expansion during the cold glacials. Second, the lowlands of SE Asia did not ice during the glacial periods, but became drier and colder. There is no evidence of the ice-covering of Niunai Lake, which is located at a low altitude (~700 m above sea level) in

northern Taiwan, during the glacials. Thus the influence of the glacial cycles on the demographies of microorganisms was less clear. The optimal temperature for the growth of *Thiomonas* spp. ranges from 20°C to 53°C (estimated from six *Thiomonas* species) but they can adapt to a wide range of temperatures (some strains can slowly grow at less than 4°C and up to 65°C) (Kelly et al., 2007). Although the temperature records of Mt. Datun during the glacials are not available, the temperature during the last glacial maximum was estimated to be approximately 8°C on average ($-4.1 \pm 0.68^\circ\text{C}$ in January and $20.3 \pm 0.31^\circ\text{C}$ in July) at Jih-Yueh Tan (approximate 750 m in altitude, 23°52'N lat., 120°55'E long., in central Taiwan) (Tsukada, 1966). Therefore, even in the relatively cold temperatures during the glacial maximum, *Thiomonas* spp. still have the ability to survive. For this reason, we do not think that the population size of *Thiomonas* sp. would be greatly influenced by climatic change. Despite some studies suggesting that the demographic histories of other microbes are consistent with climatic changes, they are host-dependent and influenced by the demographies of the hosts, which were affected by climatic changes (e.g. Mikheyev et al., 2008).

Alternatively, the activity of the Datun Volcanoes could have influenced the demographic history of *Thiomonas* sp. in Niunai Lake. According to the geological history, the Datun Volcanoes began to erupt approximately 2.8–2.5 Ma ago in a compressional tectonic environment (Song et al., 2000). These volcanic events are thought to have ceased during the late Pliocene and early Pleistocene and were followed by a second major eruption approximately 0.8–0.2 Ma BP. The Datun Volcanoes developed gradually during the episodic volcanic events that occurred between 2.8 and 0.2 Ma ago (Kim et al., 2005). While the volcanoes are believed to be currently inactive (Song et al., 2000), a recent study has suggested that these volcanoes may still be active because of continuing hydrothermal activities and gas fumaroles (Kim et al., 2005). The last eruption (approximately 0.8 Ma–0.2 Ma BP) formed large volcanoes in this area (Kim et al., 2005). The rapid expansion of the *Thiomonas* sp. population occurred approximately 0.18 Ma BP, just after the last eruption of the Datun Volcanoes. The geological events of the Datun Volcanoes match the demographic history of *Thiomonas* sp. Therefore, a more likely explanation for the rapid increase in the population size of *Thiomonas* sp. is that the volcanism created a sulfide-rich environment around Mt. Datun. Although the demographic changes affected by the earlier eruption events were difficult to examine because most ancestral variations were eliminated during the catastrophes, the population size change after the last eruptions was recorded in the genetic variations. The sulfide-rich lake water would have provided a hotbed for the growth of thiobacteria, like *Thiomonas* spp., which have ability to catabolize sulfur-containing compounds.

Of these two potential explanations for the demographic history of *Thiomonas* sp. (i.e., long-term climatic changes and volcanism), we prefer the model of volcanism affecting the bacterial evolution. This is because substrates in Niunai Lake could be directly used as materials for the catabolism of thiobacteria and the chemical properties of the environment are directly decided by the microbial composition (Munster et al., 1998; Mills et al., 2003). The sulfide-rich environment of the Datun Volcanoes has been maintained for very long time since the last volcanic eruption. This extremely harsh substrate could be a stress for other microbes but could be relatively suitable for the growth of thiobacteria (e.g., *Thiomonas*, *Thiobacillus*, and *Acidiphilium*). Although many cases of eukaryotic demographic histories have been reported to be influenced by glacial periods, especially studies detailing postglacial expansion (Bartish et al., 2006; Aoki et al., 2008; Lin et al., 2008), and although the

short-term population dynamics of microbes are easily influenced by temperature (Grisi et al., 1998; Skirnisdottir et al., 2000), the climatic change model seems inappropriate for explaining the long-term demographic history of *Thiomonas* sp. in this case.

In summary, based on a survey of the 16S rRNA gene, a very simple microbial composition was detected in the sulfide-rich Niunai Lake in a volcanic mountain of the northern Taiwan. Only 13 microbial taxa were detected and these were predominantly *Thiomonas* species (over 90%) with a small amount of *Thiobacillus*, *Escherichia*, and *Acidiphilium* species and approximately 4% unknown proteobacteria. While the expected microbial species richness was greater than what was observed (19.5 or 26.5 taxa), the species richness was still less than other bodies of water and revealed a simple microbial community structure. The dominant bacteria (belonging to genera *Thiomonas*, *Thiobacillus*, and *Acidiphilium*) function as sulfur oxidizers and may contribute to some of the lake's physical and chemical properties. In addition, we used population genetic approaches to explore the long-term demographic changes of the dominant species, *Thiomonas* sp. The observed significant negatives of both Tajima's *D* index and Fu's *F_s* index suggest a rapid population expansion. This suggestion was further supported by the left-skewed curve of the differences in allelic frequency between the observed and expected values in pairwise comparisons of sequences (mismatch distribution). The result of the mismatch distribution indicates a past event of rapid population growth. The Bayesian skyline plot that was analyzed according to the coalescent theory also suggests a bottleneck event followed a rapid increase of population size of *Thiomonas* sp. in Niunai Lake. The time of population expansion was estimated to be approximately 0.18 Ma BP until 60 millennia BP and this timing approximately matches the end of the last eruption of the Datun Volcanoes (~ 0.2 Ma BP), where Niunai Lake is located. We eliminated the hypothesis of glacial cycles influencing the bacterial demography and prefer volcanism as the underlying mechanism for the observed bacterial demography. While the sulfide-rich substrates created by the volcanism could directly accelerate the growth of thiobacteria, the climate change model could not account for the population growth during the cold Riss glacial epoch. In conclusion, the periodic selection by the sulfide-rich environment simplified the microbial community and resulted in the dominance of *Thiomonas* sp. Additionally, the geological history corresponds to the demographic history of *Thiomonas* sp. in Niunai Lake.

4. Conclusion

In conclusion, there is a correlation between microbial composition and environmental change (Aravalli et al., 1998). Thus, the environmental properties can directly affect the microbial composition (Ptacnik et al., 2008) and even shape their genetic composition (Foerstner et al., 2005; Rudi, 2009). A harsh environment simplifies the composition of the microbial communities by strong selection forces (i.e., ecotypes of microbes are recurrently purged of their diversity by periodic selection for a long time) (Cohan, 2006). This kind of periodic selection under thermal, acidic, and sulfide-rich conditions causes a high abundance of *Thiomonas* sp. with greater than 99% genetic identity that limits the microbial species richness and simplifies the microbial community in Niunai Lake. The long-term periodic selection of the microbes could last as long as 0.18 Ma. Since the end of the last volcanic eruption of the Datun Volcanoes (0.2 Ma BP), the level of the sulfide-rich substrates steadied and were a hotbed for the growth of thiobacteria. The periodic selection since 0.2

Ma BP resulted in the current microbial communities of the Datun Volcanoes. This study represents the tight connection between environmental-microbial demographic history and the *in situ* geologic characteristic and geohistory. In this case study, the 16S rRNA gene was used as the genetic marker for tracing the demographic history of microorganisms. Recently, the rapid development of the genomic technologies and theories and models for eliminating the effects of recombination or horizontal gene transfer helps for decreasing the variance of coalescence estimation. The use of multilocus (or genomic) makers for exploring the microevolution of microorganisms is expectedly going to be a future issue soon.

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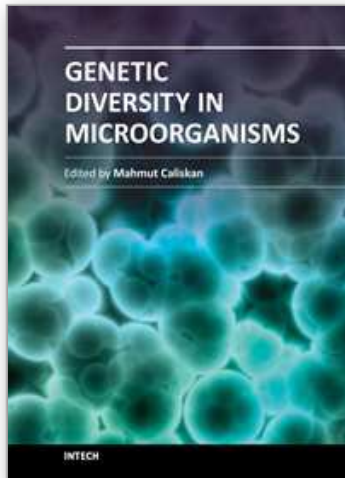
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Genetic Diversity in Microorganisms presents chapters revealing the magnitude of genetic diversity of microorganisms living in different environmental conditions. The complexity and diversity of microbial populations is by far the highest among all living organisms. The diversity of microbial communities and their ecologic roles are being explored in soil, water, on plants and in animals, and in extreme environments such as the arctic deep-sea vents or high saline lakes. The increasing availability of PCR-based molecular markers allows the detailed analyses and evaluation of genetic diversity in microorganisms. The purpose of the book is to provide a glimpse into the dynamic process of genetic diversity of microorganisms by presenting the thoughts of scientists who are engaged in the generation of new ideas and techniques employed for the assessment of genetic diversity, often from very different perspectives. The book should prove useful to students, researchers, and experts in the area of microbial phylogeny, genetic diversity, and molecular biology.

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