World Journal of Microbiology & Biotechnology (2005) 21: 933–939
DOI 10.1007/s11274-004-6819-4

Archaeal community revealed by 16s rRNA and fluorescence *in situ* hybridization in a sulphuric hydrothermal hot spring, northern Taiwan

Chang-Chai Ng[1], Chen-Chin Chang[2] and Yuan-Tay Shyu[1,*]
[1]*Institute of Horticulture, National Taiwan University, No.140, Keelung Road Section 4, Taipei 10600, Taiwan ROC*
[2]*Department of Home Economics, Tainan Woman College of Arts and Technology, Tainan, Taiwan*
**Author for correspondence: Tel.: +886-2-23660727, Fax: +886-2-23661441, E-mail: tedshyu@ntu.edu.tw*

Received 29 July 2004; accepted 25 November 2004

Keywords: 16S rRNA, archaea, FISH, microbial community, thermoacidic hot spring

Summary

The archaeal community composition of Yangmingshan National Park in northern Taiwan was investigated by 16S rRNA and fluorescence *in situ* hybridization (FISH). Optimization of tetrameric restriction enzyme (TRE) was performed to achieve efficient digestion and differentiation in the restriction fragment length polymorphism (RFLP) fragments, and *Aci*I, *Bst*UI and *Rsa*I were shown to be the optimal TREs for TRE-RFLP. Nine clones were obtained in the studies, with clones M70 and M6 being found to be phylogenetically affiliated to *Sulfolobus* and *Caldisphaera* in domain Crenarchaeota, respectively, whereas seven other clones were found to be affiliated to an uncultured and unidentified archaeon isolated from thermoacidic environments. In FISH, soil and water region cells were hybridized with DAPI (4′, 6-diamidino-2-phenylindole) and specific fluorescently labelled probes. 15.69 and 7.16% of the DAPI-stained cells hybridized with universal archaeal probe ARC915 and sulphate-reducing bacterial probe SRB385, respectively.

Introduction

Investigations of the extremophile community and its exploitation in the industrial field have always been of high interest to microbiologists for at least a decade (Arahal *et al.* 1996; Canganella *et al.* 1998; Lévêque *et al.* 2000; Oren 2002; Sunna & Peter 2003). Culture-based methods are time consuming and most importantly, they do not exactly reflect the complexity of microbial diversity (Snaidr *et al.* 1997; Moter & Ulf 2000), thus various molecular techniques including fluorescence *in situ* hybridization (FISH) (Christensen *et al.*1999; Pernthaler *et al.* 2002), 16S rRNA gene cloning (Arahal *et al.* 1996; Dunbar *et al.* 1999); denaturing gradient gel electrophoresis (DGGE) (Ferris & Ward 1997), flow cytometry (Porter *et al.* 1997) and the immunological method (Schloter *et al.* 1995), have been applied to give more comprehensive and precise characterizations of Bacteria and Archaea in numerous extreme environments.

16S rRNA (operational taxonomic unit OTU) generated using tetrameric restriction enzyme (TRE) digestion has been applied for microbial community analysis and used to generate (restriction fragment length polymorphism RFLP) patterns for undefined bacterial clone library analysis (Moyer *et al.* 1994, 1996; Haddad *et al.* 1995). FISH, a popular tool for basic ecological research, improves the determination of active populations in the archaeal community, has been widely applied in long-term microorganism monitoring and investigation (Llobet-Brossa *et al.* 1998; Delong *et al.* 1999). Fluorescently labelled oligonucleotides are used to hybridize targeted regions on archaeal rRNA specifically, allowing direct enumeration of specific and living cells in the environment. However, deviations in counting non-growing and starving environmental microbes may be occurred (Oda *et al.* 2000).

Taiwan has many active geothermal vents and hot springs. Among these, Macao, located in Yangmingshan National Park, northern Taiwan, is a sulphuric geothermal hot spring. Successful isolation of thermoacidophilic *Sulfolobus yangmingensis* (Jan *et al.* 1999) and other thermophiles and halophiles from these areas (Tsen 2000) have indicated the opulence and richness of archaeal diversity in northern Taiwan, which possess high potential to be further studied and explored. In this study, the archaeal community was first described by TRE-RFLP of the 16S rRNA gene, and several fluorescently labelled oligonucleotide probes were used for epifluorescence microscopic investigation.

Materials and methods

Sampling

Soil from a 15-cm depth and water at the sampling site Macao (E121°31′, N25°10′) in Yangmingshan National Park were collected by core sampler on 14 June 2003.

Field measurement of soil temperature and pH was carried out by Cardy Twin pH meter (Cole Palmer, USA). Electrical conductivity of water samples was measured by Microprocessor conductivity meter (WTW LF96, Germany). The specimens were transported below 0 °C and processed immediately upon return to the laboratory.

Amplification and cloning of the 16S rRNA gene

The total DNA of the soil was extracted by FastDNA® Spin Kit (BIO101, USA) and further on purified by Wizard® DNA purification system (Promega, USA), primer pair F1 (5′-TCCGGTTGATCCTGCCGGA-3′) and R1 (5′-GAGGTGATCCAGCCGCAGG-3′) (Takayanagi *et al.* 2001) was used for the amplification of the 16S rRNA gene. The 25 μl PCR reaction mixture contained DNA template 5 μl, 5 μl of 1 mM dNTP, 0.5 μM of each primer, 0.5 μl *Taq* DNA polymerase (5U/μl, AmpliTaq; Perkin–Elmer, USA), 10 × PCR buffer 2.5 μl. The amplification was performed with Gene Amp® PCR system 9700, Applied Biosystems, USA) with the condition initial heating 95 °C for 2 min, 30 cycles consisting of 95 °C for 5 s, 60 °C for 30 s, 72 °C for 1.5 min, and final extension 72 °C for 10 min. The amplificates were purified with Gel Extraction kit (Viogene, Taiwan) and subsequently ligated into pGEM®-T Vector Systems (Promega, USA). The ligation product was transformed into ECOS® competent cell (Yeastern Biotech, Taiwan) and detection occurred through blue–white screening with IPTG and X-Gal.

TRE-RFLP and sequencing

White colonies were selected and plated on LB agar plate with the antibiotic Ampicillin at a concentration of 50 μg/ml. Colony-PCR was done by using primer pair TAF (5′-CAAGGCGATTAAGTTGGGTA-3′) and TAR (5′-GGAATTGTGAGCGATAACA-3′) provide by the pGEM®-T Vector Systems followed the PCR conditions described as manufacturer's manual. Inserts of the correct size were excized and purified by GM-T® Gel Extraction kit (Yeastern Biotech, Taiwan) and later digested by the TRE *Aci*I, *Bst*UI and *Rsa*I (New England Biolabs, USA). Clones exhibiting different restriction patterns were chosen and sequenced (Mission Biotech, Taiwan). The sequencing results were compared on National Center for Biotechnology Information (NCBI) database.

Phylogenetic analysis

Sequence alignment and phylogenetic analysis was performed with the multiple sequence alignment software CLUSTAL W ver. 1.82 (Higgins *et al.* 1994). The phylogenetic tree was constructed by the neighbour-joining and UPGMA with robustness of 1000 bootstrapping using PHYLIP package ver. 3.6b.

Sample fixation

Samples were fixed in 3.7% (wt/vol) formamide overnight at 4 °C. Cells from the soil region were extracted based on the method of Christensen *et al.* (1999), cells from the water region were filtered onto a 25 mm diameter, 0.2-μm-pore-size polycarbonate white GTTP filter (Millipore, USA) under vacuum 5 mm Hg. The vacuum was released and 1 ml of 2% (wt/vol) NaCl-50% (vol/vol) ethanol was incubated on the filter for 1 min and subsequently vacuumed and dried. The filters were stored under −20 °C for further processing.

FISH and microscopic observation

The filters were placed in a 5.5 cm diameter Petri dish and 1 ml of preheated hybridization solution was added. The hybridization solution contained 50–70% (vol/vol) formamide (Llobet-Brossa *et al.* 1998; Pernthaler *et al.* 2002) (Table 1), 10% dextran sulphate (Sigma, USA), 0.01% (wt/vol) poly A (Sigma, USA) and 5 × SET (1 × SET is 150 mM NaCl, 1 mM Na_2EDTA, 20 mM Tris–HCl, pH 7.8). Five hundred nanogram of fluor-labelled oligonucleotide probe was subsequently added. The Petri dishes were incubated overnight in a hybridization oven (Yih Der Co., Ltd, Taiwan) at the specific temperature stated in Table 1. After the incubation, filters were washed by 5 ml wash solution containing 0.2 × SET and 50% (vol/vol) formamide for 2 h at the appropriate temperature (see Table 1). For total cell densities hybridization, the filters were incubated in 1 × PBS (145 mM NaCl, 8.7 mM Na_2HPO_4, 1.5 mM

Table 1. Total DAPI cell count and relative percentages of hybridized cells with specific probes.

% of cells hybridized with probe[a]	Cell sample from soil region	Cell sample from water region
DAPI count (10^8 cell/cm^3)	2.16 ± 0.73	0.084 ± 0.0015
ARC915[b]	15.69	2.2
EUB338	3.12	0.05
EUK516	1.5	3.2
SRB385	7.16	1.42
CYAN785	0.7	0.03
ALF968	1.32	0.79
BET42	0.32	n.d

[a] Probe-specific count/DAPI count ×100%.
[b] ARC915, domain *Archaea*; EUB338 (Amann *et al.* 1990), domain *Bacteria*; EUK516 (Poppert *et al.* 2002), domain *Eukarya*; SRB385 (Amann *et al.* 1990), sulphate-reducing bacteria; CYAN785 (Knapp & David 2004), cyanobacteria; ALF968 (Detmers *et al.* 2004), alpha subclass of proteobacteria; BET42 (Detmers *et al.* 2004), beta subclass of proteobacteria. FISH conditions of different oligonucleotide probes with the hybridization temperature, formamide concentration for hybridization, and formamide concentration for wash were ARC915 (65 °C, 70%, 50%); EUB338 (65 °C, 60%, 50%); EUK516 (65 °C, 20%, 45%); SRB385 (65 °C, 20%, 45%); CYAN785 (60 °C, 35%, 50%); ALF968 (50 °C, 35%, 45%); BET42 (50 °C, 35%, 45%). n.d: not detected.

NaH_2PO_4, pH 7.4) containing DAPI at 1 μg/ml for 2 min at room temperature, and quickly rinsed in 1 × PBS. Filters were placed on a slice and 100 μl paraffin oil (Reidel-de Haeen, Germany) was added as antifading agent. The filters were viewed immediately but could be stored in the dark at 4 °C for several days.

Microscopic viewing was carried out with an Eclipse E600 microscope with Y-FL Epi-fluorescence attachment microscope (Nikon, Japan) equipped with Super High Pressure Mercury Lamp (HB-10103AF, Nikon, Japan) under 100 × Plain Fluor Objective (Nikon, Japan). The images were photographed by digital camera (Coolpix 4500, Nikon). The quantification of probe-positive cells was performed as described by Delong *et al*. (1999), briefly, 10 fields of approximately 100 cells per field were counted, a complementary probe as negative control was included for every sample and used to calculate the final probe-positive cell number. The fraction of probe-positive was calculated as the ratio of the number of probe-positive cells to the total number of DAPI-stained cells.

Results

Chemical properties of soil and vent water samples

Field measurement of soil temperature found a range of 84–94 °C with pH value of 2.0–3.5. The electrical conductivity of water samples was 2.1 mS/m.

Cloning and TRE-RFLP results

Two hundred and eighteen white colonies were selected and reconfirmed by colony-PCR, 192 were shown to be colony with correct size (1.5 kb) of insert (designated as NTU01-192). The clones were then digested by *Rsa*I, *Bst*UI and *Aci*I and RFLP fragments were thus generated (Figure 1). Before the digestion, we carried out the computer-simulated digestion of 16S rRNA gene by 24 TREs in order to optimize its efficacy for RFLP screening. A total of 180 archaeal SSU rRNA gene sequences was selected from (Ribosomal Database Project II RDP II), after the computer-simulated RFLPs digestion by 24 TREs, the average number of restriction sites per taxon for each of the 24 TREs is shown in Table 2. The distribution of the restriction fragment size-frequency pattern indicated that *Bst*UI, *Rsa*I and *Aci*I gave a 40% occurrence of band frequency ranging from 200 to 450 bp (data not shown), while the other group of TREs gave occurrence of band frequency < 200 bp or evenly dispersed in the > 600 bp (data not shown), which impede the RFLP analysis. TRE-RFLP digestion of 192 clones resulted in nine archaeal taxonomic groups and each of the representative taxonomic group was sequenced and the phylogenetic relationships were constructed as inferred by neighbour-joining and UPGMA methods.

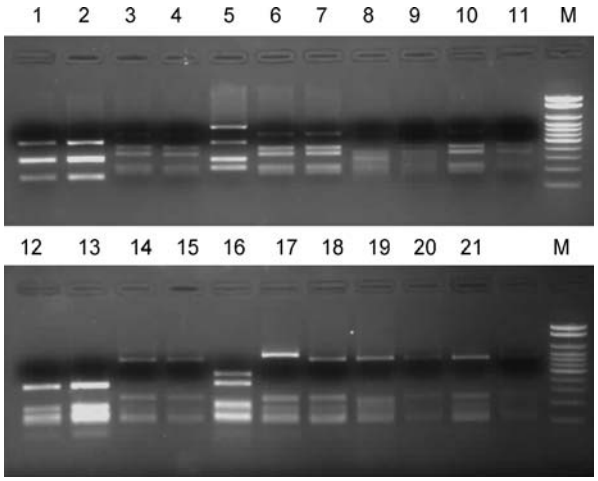

Figure 1. Restriction patterns of clones by tetrameric restriction enzymes *Rsa*I (lane 1–7), showing three archaeal RFLP patterns (lane 1,2; lane 3,4,6,7 and lane 5); *Bst*UI (lane 8–14), showing two archaeal groups (lane 8–11; lane 12, 13 and lane 14) and *Aci*I (lane 15–21), which showing two archaeal groups (lane 15–21; lane 16). M: molecular marker 2 kb–100 bp.

Phylogenetic analysis of archaeal 16S rRNA

The phylogenetic tree showed that clone M70 (accession no. AY627864) was grouped within the Crenarchaeota (98% similarity BLAST search), closely related to *Sulfolobus shibatae* (accession no. M32504), order Sulfolobales, whereas clone TA6 (accession no. AY627858) was related to *Caldisphaera lagunensis* (accession no AB087499) (97% similarity) (Figure 2). Clones M7, M8,

Table 2. Restriction sites per taxon of TRE in 180 archaeal SSU (font in bold indicate TRE used in this study).

RE	Site	Mean of restriction sites per taxon
***Aci*I**	C/CGC	7.65
*Alu*I	AG/CT	7.07
*Bfa*I	C/TAG	5.35
*Bsp*KT6I	GAT/C	5.41
***Bst*UI**	CG/CG	7.61
***Rsa*I**	GT/AC	5.51
*Cvi*AII	C/ATG	9.20
*Cvi*RI	TG/CA	8.02
*Hae*III	GG/CC	8.79
*Hha*I	GCG/C	5.48
*Hpa*II	C/CGG	9.14
*Hpy*CH4IV	A/CGT	7.23
*Mnl*I	$CCTC(N)_7$	4.12
*Mse*I	T/TAA	7.19
*Sth*132I	CCCG	7.72
*Taq*I	T/CGA	5.25
*Tsp*59I	/AATT	5.45
*Bss*KI	/CCNGG	10.26
*Dde*I	C/TNAG	7.41
*Fnu*4HI	GC/NGC	11.93
*Hinf*I	G/ANTC	5.47
*Hpy*188I	TCN/GA	6.71
*Hpy*CH4III	ACN/GT	4.76
*Mae*III	/GTNAC	7.45

M17 and M50 (accession nos. AY627859, AY627860, AY627861 and AY627862 with similarity of 95% or above, respectively) were closely related to an uncultured archaeon clone isolated from an arsenite-oxidizing acidic thermal spring (accession no. AF325184). Clones M4, M5 and M74 (accession nos. AY627856, AY627857 and AY627863 with similarity 96% or above, respectively) were clustered within the unidentified archaeon group (Figure 2).

Fluorescence in situ hybridization

The archaeal community was visualized *in situ* using the specific fluorescently labelled probes ARCH915, EUB338, EUK516, SRB385, CYAN785, ALF968 and BET42. Archaeal cells were visualized in the soil region and the water region of the sampling site (Figure 3). The DAPI-stained-cell count in the soil region had a higher number of archaeal cell than the water region ones, with the archaeal cells in soil cells 2.16×10^8 cell/cm^3, two orders of magnitude higher than cells in water region (8.4×10^6 cell/cm^3) (Table 1). 15.7% of DAPI-stained-cells hybridized with ARC915 and this result suggested a low cellular rRNA content in this site.

Discussion

TRE-RFLP

Although TRE-RFLP facilitates phylogenetic reconstruction (Moyer *et al.* 1996), there is often found an inability of RFLP to display the full extent of the microbial diversity in the sequences of the target gene or molecular marker. Accordingly, the careful selection of restriction enzymes in RFLP is found to be essential for more efficient and effective cutting and the differentiation of digested fragments.

Phylogenetic analysis of archaeal 16S rRNA

The phylogenetic placement of clone M70 inferred by neighbour-joining (Figure 2) was consistent with those inferred by UPGMA and discrete method Parsimony, but not by Maximum likelihood (data not shown). However, phylogenetic analyses using both the distance and discrete methods consistently placed clone M6 related to *Caldisphaera lagunensis,* a novel thermoacidophilic crenarchaeote isolated from a hot spring in the Philippines (Itoh *et al.* 2003), with high bootstrap support (Figure 2). Several novel species of *Sulfolobus*

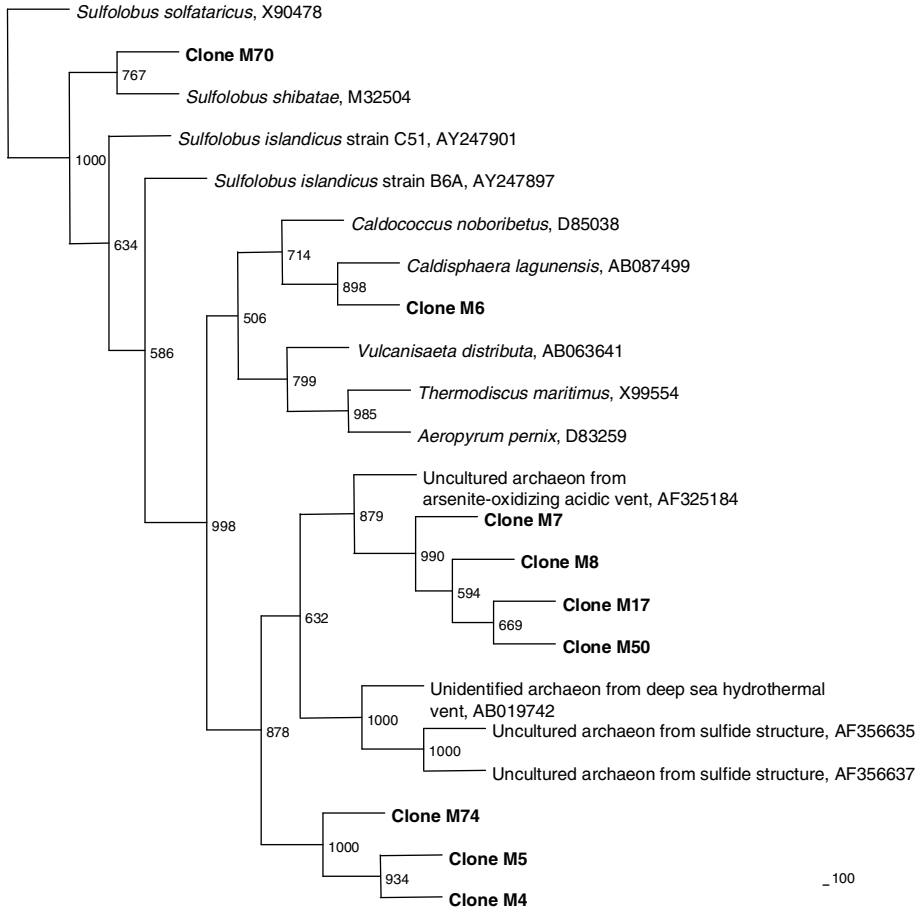

Figure 2. Neighbour-joining phylogenetic tree from analysis 1.5 kb of 16S rRNA gene sequence of clones obtained from Macao area, Yangming san National Park. Numbers at the node indicating the levels of bootstrap supports out of 1000 resampling. Scale bar represents 0.1 substitutions per nucleotide position.

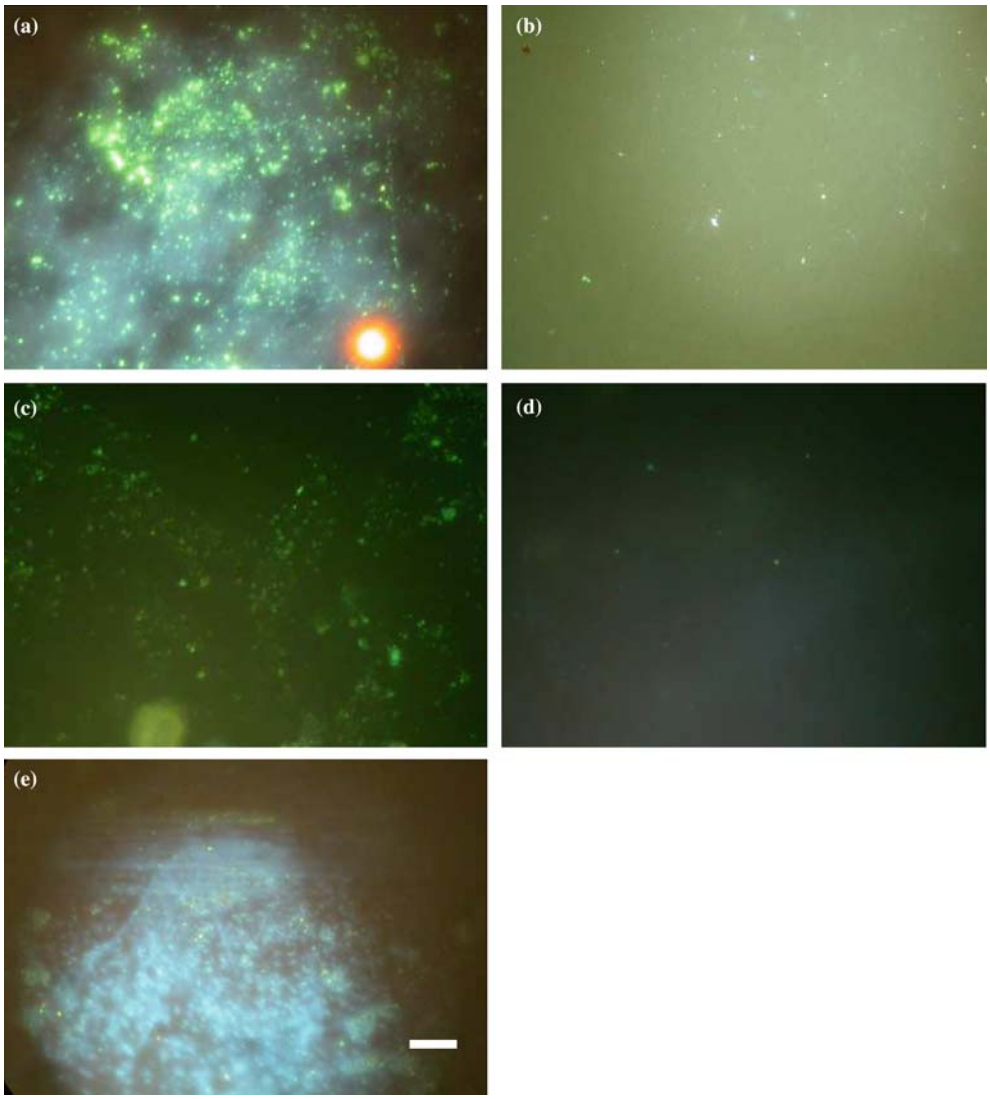

Figure 3. Epifluorescence micrographs cells samples from Macao area of Northern Taiwan. Samples soil sediment and water were hybridized with probe ARCH915 (a, b) and EUB338 (c, d), respectively. DAPI-stained cells (e). Bar, 10 μm.

discovered recently in North East Asian countries (Jan *et al*. 1999; Suzuki *et al*. 2002; Xiang *et al*. 2003) have suggested that the *Sulfolobus* genus is widely distributed in this region, owing to the similar thermoacidophilic environments investigated. Clones M7, M8, M17 and M50 had phylogenetic affiliations with an uncultured archaeon amplified from an arsenite-oxidizing acidic thermal spring (accession no. AF325184) (Jackson *et al*. 2001), and the phylogenetic placement is consistent with the result inferred by UPGMA, Parsimony and ML method. Another clone cluster M4, M5, M74 was grouped to an uncultured archaeon isolated from sulphide structure (accession no. AF356635) and an unidentified archaeon isolated from a deep-sea hydrothermal vent (accession no. AB019742) (Takai & Horikoshi 1999). The findings presented here were primarily crenarchaeal sequences, as indicated by Barns *et al*. (1994) that Crenarchaea have been reported from environmental samples including marine systems and hot springs. The archaeal community in Macao is relatively low, as this sampling site has encountered serious ecological invasions and disturbances due to the exploitation of the thermal vent water by local people for commercial use.

Archaea have been found mostly in anaerobic high-temperature volcanic vents on the ocean floor, continental hot springs, other volcanic environments and highly salty or acidic ponds. As Taiwan is located at the intersection of the Philippine and Pacific plate tectonics, certain geothermal events and the archaeal community are similar to those of Japan, China and the Philippines. In this study, those unidentified and uncultured archaeal clones have potential to be investigated further, not merely to note their numerical abundance, but also to examine exploitation of their uses in the biotechnological and industrial fields.

FISH detection

Total DAPI-stained cells in this study (Table 1) were one order of magnitude lower than that of marine sediment (Llobet-Brossa *et al*. 1998). The detection

yields of FISH by the labelled probes used here were considerably lower than those obtained from activated sludge (Snaidr *et al.* 1997), soil (Zarda *et al.* 1997) and marine water (Delong *et al.* 1999). The DAPI count obtained from 15-cm below the soil region was higher than that of the water region ones. Up to 15.7% of the DAPI-stained cells hybridized with ARC915 (Table 1). Additionally, the bacterial community (probe EUB338) was found to be low, with only 3.12% of cells being hybridized, whereas 7.16% of cells were hybridized with sulphate-reducing bacteria (probe SRB385) and proteobacteria comprised merely 1.64% in the community. The low cellular rRNA content in Macao is strongly influenced by human activities. The depth of sampling significantly influences the result of FISH due to the microbial distribution differing over the vertical profile of the soil. According to Llobet-Brossa *et al.* (1998), mud samples obtained from the uppermost layer (0.5 cm underground) have the highest fraction of detectable microbial community. Compared to those sampling depths of between 1 cm (Bintrim *et al.* 1997) and 2–10 cm (Barns *et al.* 1994), this study conducted sampling at a depth of 15 cm depth to avoid the human disturbance factor to our microbial community caused by water pipeline construction by local people. Overall detection of the archaeal community in Macao should be in direct relationship with environmental disturbance, so a long-term monitoring process of environmental and microbial community shifting needs to be carried out for this study site.

Future investigations will combine the use of a larger set of specific probes, and also other molecular techniques like DGGE to provide a clearer understanding of the structure and diversity of the archaeal communities in this area.

Acknowledgement

This work was supported by a Grant from Council of Agriculture, ROC. The authors are grateful for financial support of this research under contract no. F1 (1) 92A1092.

References

Amann, R.I., Binder, B.J, Olson, R.J, Chisholm, S.W., Devereux, R. & Stahl, D.A. 1990 Combination of 16S rRNA-targeted oligonucleotide probes with flow cytometry for analyzing mixed microbial populations. *Applied and Environmental Microbiology* **56**, 1919–1925.

Arahal, D.R., Floyd, E.D., Bruce, J.P., Benjamin, E.V. & Antonio, V. 1996 Phylogenetic analyses of some extremely halophilic archaea isolated from Dead Sea water, determined on the basis of their 16S rRNA sequences. *Applied and Environmental Microbiology* **62**, 3779–3786.

Barns, S.M., Ruth, E.F., Mathew, W.J. & Norman, R.P. 1994 Remarkable archaeal diversity detected in a Yellowstone National Park hot spring environment. *Proceedings of the National Academy of Sciences of the United States of America* **91**, 1609–1613.

Bintrim, S.B., Timothy, J.D., Jo, H., Gary, P.R. & Robert, M.G. 1997 Molecular phylogeny of archaea from soil. *Proceedings of the National Academy of Sciences of the United States of America* **94**, 277–282.

Canganella, F., William, J.J., Agata, G. & Garabed, A. 1998 *Thermococcus guaymasensis* sp. nov. and *Thermococcus aggregans* sp. nov., two novel thermophilic archaea isolated from the Guaymas Basin hydrothermal vent site. *International Journal of Systematic Bacteriology* **48**, 1181–1185.

Christensen, H., Michael, H. & Jan, S. 1999 Counting and size classification of active soil bacteria by fluorescence *in situ* hybridization with an rRNA oligonucleotide probe. *Applied and Environmental Microbiology* **65**, 1753–1761

Delong, E.F., Lance, T.T., Terence, L.M. & Christina, M.P. 1999 Visualization and enumeration of marine planktonic archaea and bacteria by using polyribonucleotide probes and fluorescent *in situ* hybridization. *Applied and Environmental Microbiology* **65**, 5554–5563.

Detmers, J., Strauss, H., Schulte, U., Bergmann, A., Knittel, K. & Kuever, J. 2004 FISH shows that *Desulfotomaculum spp*. are the dominating sulfate-reducing Bacteria in a Pristine Aquifer. *Microbial Ecology* **47**, 236–242.

Dunbar, J., Shannon, T., Susan, M.B., Jody, A.D. & Cheryl, R.K. 1999 Levels of bacterial community diversity in four arid soils compared by cultivation and 16S rRNA gene cloning. *Applied and Environmental Microbiology* **65**, 1662–1669.

Ferris, M.J. & Ward, D.M. 1997 Seasonal distribution of dominant 16S rRNA-defined populations in a hot spring microbial mat examined by denaturing gradient gel electrophoresis. *Applied and Environmental Microbiology* **63**, 1375–1381.

Haddad, A., Camacho, F., Durand, P. & Cary, S.C. 1995 Phylogenetic characterization of the epibiotic bacteria associated with the hydrothermal vent polychaete *Alvinella pompejana*. *Applied and Environmental Microbiology* **61**, 1679 -1687.

Higgins, D., Thompson, J. & Gibson, T.J. 1994 CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Research* **22**, 4673–4680.

Itoh, T., Suzuki, K., Sanchez, P.C. & Nakase, T. 2003 *Caldisphaera lagunensis* gen. nov., sp. nov., a novel thermoacidophilic crenarchaeote isolated from a hot spring at Mt Maquiling, Philippines. *International Journal of Systematic and Evolutionary Microbiology* **53**, 1149–1154.

Jackson, C.R., Langner, H.W., Donahoe-Christiansen, J., Inskeep, W.P. & McDermott, T.R. 2001 Molecular analysis of microbial community structure in an arsenite-oxidizing acidic thermal spring. *Environmental Microbiology* **3**, 532–542.

Jan, R.L., Jeffrey, W., Shu-Miaw, C., Chien-Wei, T. & Suh-Der, T. 1999 A novel species of thermoacidophilic archaeon, *Sulfolobus yangmingensis* sp. nov. *International Journal of Systematic Bacteriology* **49**, 1809–1816.

Knapp, C.W. & David, W.G. 2004 Development of alternate ssu-rRNA probing strategies characterizing aquatic microbial communities. *Journal of Microbiological Methods* **56**, 323–330.

Lévêque, E., Stefan, J., Bernard, H. & Abdel, B. 2000 Thermophilic archaeal amylolytic enzymes. *Enzyme and Microbial Technology* **26**, 3–14.

Llobet-Brossa, E., Ramon, R.M. & Rudolf, A. 1998 Microbial community composition of wadden sea sediments as revealed by fluorescence *in situ* hybridization *Applied and Environmental Microbiology* **64**, 2691–2696.

Moter, A. & Ulf, B.G. 2000 Fluorescence in situ hybridization (FISH) for direct visualization of microorganisms. *Journal of Microbiological Methods* **41**, 85–112.

Moyer, C.L., Dobbs, F.C. & Karl, D.M. 1994 Estimation of diversity and community structure through restriction fragment length polymorphism distribution analysis of bacterial 16S rRNA genes from a microbial mat at an active, hydrothermal vent system, Loihi Seamount, Hawaii. *Applied and Environmental Microbiology* **60**, 871–879.

Moyer, C.L., James, M.T., Fred, C.D. & David, M.K. 1996 A computer-simulated restriction fragment length polymorphism analysis of bacterial small-subunit rRNA genes: efficacy of selected tetrameric restriction enzymes for studies of microbial diversity in nature. *Applied and Environmental Microbiology* **62**, 2501–2507.

Oda, Y., Slagman, S.J., Meijer, W.G., Forney, L.J. & Gottschal, J.C. 2000 Influence of growth rate and starvation on fluorescent *in situ* hybridization of *Rhodopseudomonas palustris*. *FEMS Microbiology Ecology* **32**, 205–213.

Oren, A. 2002 Diversity of halophilic microorganisms: environments, phylogeny, physiology, and application. *Journal of Industrial Microbiology and Biotechnology* **28**, 56–63.

Pernthaler, A., Christina, M.P., Jacob, P., Edward, F.D. & Rudolf, A. 2002 Comparison of fluorescently labelled oligonucleotide and polynucleotide probes for the detection of pelagic marine bacteria and archaea. *Applied and Environmental Microbiology* **68**, 661–667.

Poppert, S., Andreas, E., Reinhard, M., Michael, W. & Matthias, H. 2002 Detection and differentiation of Chlamydiae by fluorescence in situ hybridization. *Applied and Environmental Microbiology* **68**, 4081–4089.

Porter, J., Daniel, D., Melanie, H., Clive, E. & Roger, P. 1997 Go with the flow – use of flow cytometry in environmental microbiology. *FEMS Microbiology Ecology* **24**, 93–101.

Schloter, M., Assmus, B. & Hartmann, A. 1995 The use of immunological methods to detect and identify bacteria in the environment. *Biotechnology Advances* **13**, 75–90.

Snaidr, J., Rudolf, A., Ingrid, H., Wolfgang, L. & Karl-Heinz, S. 1997 Phylogenetic analysis and in situ identification of bacteria in activated sludge. *Applied and Environmental Microbiology* **63**, 2884–2896.

Sunna, A. & Peter, L.B. 2003 A gene encoding a novel extremely thermostable 1,4-*b*-xylanase isolated directly from an environmental DNA sample. *Extremophiles* **7**, 63–70.

Suzuki, T., Toshio, I., Taketoshi, U., Kurt, H., Naoki, N., Takahide, K., Toshiaki, U., Akihiko, Y. & Tairo, O. 2002 *Sulfolobus tokodaii* sp. nov. (*Sulfolobus sp*. strain 7), a new member of the genus Sulfolobus isolated from Beppu Hot Springs, Japan. *Extremophiles* **6**, 39–44.

Takai, K. & Horikoshi, K. 1999 Genetic diversity of archaea in deep-sea hydrothermal vent. *Environmental Genetics* **152**, 1285–1297.

Takayanagi, S., Kawasaki, H., Sugimori, K., Yamada, T., Sugai, A., Ito, T., Yamasato, K. & Shioda, M. 1996 *Sulfolobus hakonensis* sp. nov., a novel species of acidothermophilic archaeon. *International Journal of Systematic Bacteriology* **46**, 377–382.

Tsen, S.D. 2000 *Isolation and identification of Archaea in Taiwan*. NSC89-2320-B010-028 National Science Council, Taiwan ROC. Watanabe, K., Kodama, Y. & Harayama, S. 2001 Design and evaluation of PCR primers to amplify bacterial 16S ribosomal DNA fragments used for community fingerprinting. *Journal of Microbiological Methods* **44**, 253–262.

Xiang, X.Y., Dong, X.Z. & Li, H. 2003 *Sulfolobus tengchongensis* sp. nov., a novel thermoacidophilic archaeon isolated from a hot spring in Tengchong, China. *Extremophiles* **7**, 493–498.

Zarda, B., Dittmar, H., Antonis, C., Wilhelm, S., Alexander, N., Rudolf, A. & Josef, Z. 1997 Analysis of bacterial community structure in bulk soil by in situ hybridization. *Archives of Microbiology* **168**, 185–192.