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Mariana Forearc Serpentinite Mud Volcanoes Harbor Novel Communities of Extremophilic *Archaea*

ANDREA C. CURTIS[1], C. GEOFFERY WHEAT[2], PATRICIA FRYER[3], and CRAIG L. MOYER[1*]

[1]*Biology Department, Western Washington University, Bellingham, Washington, USA*
[2]*Global Undersea Research Unit, University of Alaska Fairbanks, Fairbanks, Alaska, USA*
[3]*Hawaii Institute of Geophysics and Planetology, School of Ocean and Earth Science and Technology, University of Hawaii, Honolulu, Hawaii, USA*

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Extremophilic archaeal communities living in serpentinized muds influenced by pH 12.5 deep-slab derived fluids were detected and their richness and relatedness assessed from across seven serpentinite mud volcanoes located along the Mariana forearc. In addition, samples from two near surface core sections (Holes D and E) at ODP Site 1200 from South Chamorro were subjected to SSU rDNA clone library and phylogenetic analysis resulting in the discovery of several novel operational taxonomic units (OTUs). Five dominant OTUs of *Archaea* from Hole 1200D and six dominant OTUs of *Archaea* from Hole 1200E were determined by groups having three or more clones. Terminal-restriction fragment length polymorphism (T-RFLP) analysis revealed all of the dominant OTUs were detected within both clone libraries. Cluster analysis of the T-RFLP data revealed archaeal community structures from sites on Big Blue and Blue Moon to be analogous to the South Chamorro Hole 1200E site. These unique archaeal community fingerprints resulted from an abundance of potential methane-oxidizing and sulfate-reducing phylotypes. This study used deep-sea sediment coring techniques across seven different mud volcanoes along the entire Mariana forearc system. The discovery and detection of both novel *Euryarchaeota* and Marine Benthic Group B *Crenarcheaota* phylotypes could be efficacious archaeal indicator populations involved with anaerobic methane oxidation (AMO) and sulfate reduction fueled by deep subsurface serpentinization reactions.

Keywords: *Archaea*, community structure, molecular ecology, subsurface microbiology

Introduction

The Mariana convergent plate margin, a nonaccretionary subduction zone (Uyeda 1982), lies along the eastern boundary of the Philippine plate in the western Pacific (Figure 1). Since the Eocene (∼45 Ma) the Pacific plate has been subducting beneath the Philippine plate, ultimately giving rise to the Mariana forearc, a region of serpentinite mud volcanoes formed between the trench and volcanic arc (Fryer et al. 1999; Maekawa et al. 1993). Dehydration reactions, induced by increasing pressure and temperature within the down-going slab, transform the overriding plate into a hydrated composite of serpentinite, muds and fluids that extrude from fault-derived conduits resulting in giant (reaching sizes up to 50 km in diameter and 2 km in height) mud volcanoes (Fryer et al. 1999; Mottl et al. 2003, 2004). These mud volcanoes form a zone of seamounts from 30 to 120 km behind the trench axis and often have active springs that deliver water to the seafloor that is typically fresher than seawater and highly alkaline with pH ∼12.5 (Fryer et al. 2006; Hulme et al. 2010; Mottl et al. 2003, 2004; Wheat et al. 2008).

In 1996, springs were discovered on the summit of South Chamorro Seamount (the southernmost mud volcano on the forearc) 85 km arcward of the Mariana Trench (Fryer and Mottl 1997). In 2001, as part of Ocean Drilling Program (ODP) Leg 195, advanced piston core (APC) samples were recovered and whole-round core sections were acquired from Site 1200 near one of the active springs to investigate the structure and diversity of microbial populations that exist in the shallow subsurface (Shipboard Scientific Party 2002). In 2003, we obtained piston, trigger and gravity cores (in addition to using the ROV Jason II to collect push-core samples) from seven mud volcanoes (Fryer et al. 2006). The sampled mud volcanoes include (from north to south) Pacman, Blip, Nip, Quaker, Big Blue, Blue Moon and South Chamorro (Figure 1).

We are grateful to the captain and crew of both the R/V *JOIDES Resolution* and R/V *Thomas G. Thompson* for their assistance in collecting samples as well as the Ocean Drilling Program. This project was funded in part by Western Washington University's Office of Research and Sponsored Programs, U.S. Science Support Program (USSSP) and the Consortium for Ocean Leadership, and by National Science Foundation award OCE-0727086 (to CM).

*Address correspondence to Craig L. Moyer, Biology Department MS#9160, Western Washington University, 516 High Street, Bellingham, WA 98225, USA; Email: cmoyer@hydro.biol.wwu.edu

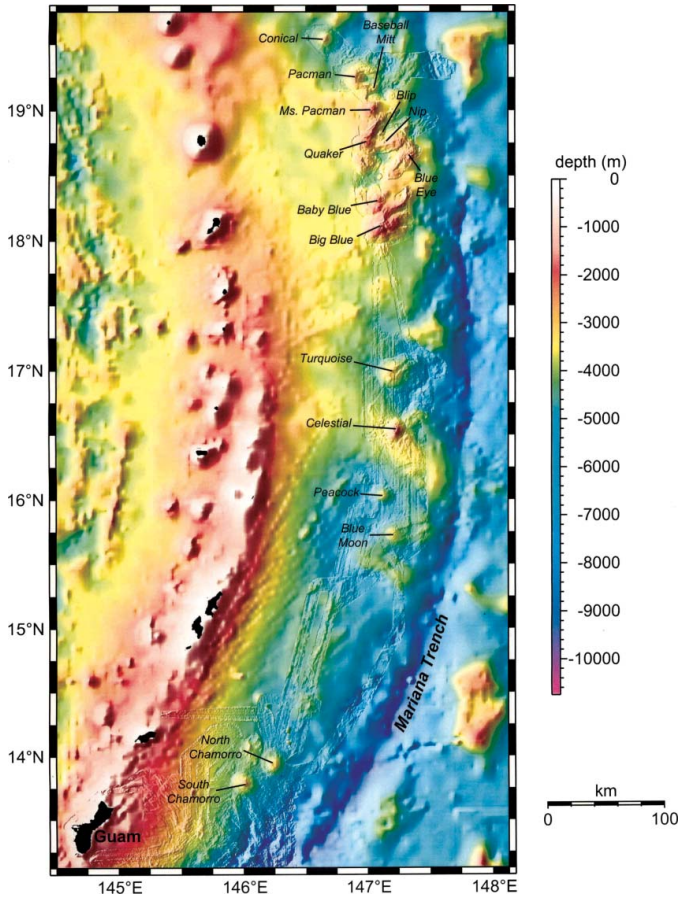

Fig. 1. Bathymetric map of the southern Mariana arc and forearc, ranging from northernmost mud volcano (Conical Seamount) to southernmost (South Chamorro Seamount) nearest to Guam (after Fryer et al. 1999) (color figure available online).

Estimates for the chemical composition of fluids at depth at these sites reveals a consistent variation in the fluid composition with distance from the trench axis, which is a proxy for the depth to the subducting slab (Hulme et al. 2010). In general, these upwelling fluids contain elevated levels of dissolved sulfate that are enriched in sulfide, which is attributed to both anaerobic methane oxidation (AMO) and sulfate reduction in a microbial habitat overwhelmingly dominated by Archaea (Hulme et al. 2010; Mottl et al. 2003, 2004; Wheat et al. 2008). The Mariana forearc's mud volcanoes thus provide a unique view into the process of devolatization of a subducting slab and the serpentinization processes within the overriding plate as a mechanism for fueling subsurface communities of extremophilic Archaea.

Fluids from the subducting slab rise at rates of at least tens of cm/yr (maximum measured is 36 cm/yr at Big Blue Seamount), faster than the rock matrix, based on systematic variations in pore water chemical profiles and an advection-diffusion model, providing methane, hydrogen, and sulfate for microbial consumption (Fryer et al. 1999; Hulme et al. 2010).

The deep subsurface biosphere retains the largest biomass of microorganisms compared to any other habitat on Earth and the extent of microbial diversity in the subsurface continues to remain the least understood (Orcutt et al. 2011; Whitman et al. 1998). Biotic and abiotic chemical reactions as well as geological processes associated with microbial populations in the deep subsurface biosphere are interlinked, but little is known of the complexity of those linkages (Gold 1992). Seamounts are also now being recognized as environments hosting enhanced microbial diversity and biomass (Emerson and Moyer 2010). Previous studies have elucidated the importance of hydrogen-driven microbial communities; hydrogen is a by-product of the serpentinization process (Takai et al. 2004a).

Fluids emanating from mud volcanoes along the Mariana forearc are ultimately derived from the subducting slab (yielding an ultrahigh pH) and to date, the Izu-Bonin-Mariana forearc represents the only known example of slab-derived rocks being returned to the surface in an active subduction zone; however in geologic time such processes were common (Fryer et al. 2006; Maekawa et al. 1993). As such, this subsurface system not only offers the possibility of uncovering an unique microbial ecosystem based on chemolithotrophic primary producers, but additionally yields the potential for finding possible analogs for extraterrestrial life (Schulte et al. 2006) and better understanding origins of life on Earth (Martin and Russell 2007; Teske et al. 2003).

The goal of this study was to detect and discover extremophilic archaeal populations, and determine the richness and relatedness of these populations within their respective communities. Archaeal communities from seven seamounts were assessed with T-RFLP, while two sites atop the southernmost seamount, South Chamorro, were additionally investigated using clone library analysis (Figure 1). T-RFLP is a rapid and reproducible SSU rDNA fingerprinting technique that has the potential for reliably reflecting changes in community structure and diversity allowing for comparisons of richness and evenness among samples (Hartmann and Widmer 2008; Marsh 2005).

Although a bacterium (*Marinobacter alkaliphilus*) was previously isolated from subseafloor muds (Takai et al. 2005), no bacteria were detected using PCR-based molecular methods from any of the samples in this study. In a previous study (designed to gain insights into microbial biomass) samples from South Chamorro (ODP Hole 1200E) were examined via phospholipid fatty acid (PLFA) analysis which showed two subsurface peaks that ranged from ~500 to 1000 times more archaeal biomass than bacterial in a setting with essentially no sedimentary organic carbon input (Mottl et al., 2003). Geochemical and lithological properties of muds and sediments (e.g., mineral composition, pore water geochemistry and methane concentration) have been shown to affect microbial habitats in deep marine settings (Inagaki et al. 2006). Thermodynamic modeling of ultramafic-hosted microbial habitats indicates that nearly twice as much chemical energy is available to chemolithotrophs in an ultramafic setting than in basalt-hosted habitats (McCollom 2007). The investigation of samples from Mariana serpentinite seamounts using molecular biological techniques provides an excellent opportunity for microbial exploration to study phylogenetic diversity and ecological significance in an extreme subsurface environment representing the highest pH limit for life (Takai et al. 2005).

Materials and Methods

Sample Collection and Site Descriptions

Mud samples were collected from the southern Mariana forearc on the summit of South Chamorro Seamount (Figure 1) aboard the drill-ship R/V *Joides Resolution*, during ODP Leg 195 (March through May, 2001). Collections occurred at ODP Site 1200, Holes D & E, at a depth of ~3 km below sea level. An advanced hydraulic piston corer (APC) was used to collect whole-round samples (n = 18 [Hole 1200D] and n = 21 [Hole 1200E]), which were processed immediately after splitting of the core liner. Core samples were extruded and then broken from the core rather than cut to minimize the possibility of contamination. Aseptic handling was conducted for all samples and processed within 30 min under laminar flow.

Subsamples were collected and stored at 4°C in sterile PBS for fluorescent and light microscopy. The remaining samples were quick frozen in liquid nitrogen and maintained at −80°C for land-based molecular analyses. Core samples were additionally subjected to tracer tests during collection to assess potential seawater intrusion during drilling. A perfluorocarbon tracer was used during drilling of ODP Hole 1200D, whereas fluorescent microspheres were used entirely during ODP Hole 1200E sampling and in core sections five through nine of Hole 1200D (e.g., Smith et al. 2000). More detailed site descriptions and sample processing procedures are addressed in Mottl et al. (2003).

Core samples were also collected from seven seamount summits (from south to north – South Chamorro, Blue Moon, Big Blue, Quaker, Nip, Blip, and Pacman; Figure 1) along the Mariana forearc during R/V *Thomas G. Thompson* cruise TN 154 (March through May, 2003). Piston, trigger and gravity core samples were obtained and processed immediately following the splitting of core liners on deck (max core depth ~6 m). Push core samples were obtained by the ROV Jason II and processed immediately upon surfacing (max core depth ~30 cm). Samples of whole core sections (0 to 10 cmbsf) were collected and aseptically processed within 30 min of retrieval. More detailed site descriptions and sample processing procedures of these same core samples are addressed in Hulme et al. (2010). Deeper core samples were tested in all cases (during both expeditions), but none were found to contain sufficient biomass to warrant further nucleic acid-based study.

Genomic DNA Extraction and Purification

DNA extractions from the uppermost core samples from ODP Holes 1200D and 1200E (0 to 10 cmbsf) were accomplished using the method described by Zhou et al. (1996) with approximately 10 g of sample ground until nearly powder-like with a mortar and pestle that was partially submerged in liquid nitrogen (without the use of bead-beating, otherwise the extraction process was the same as described below for T-RFLP). DNA extracts were visualized in a 1% agarose gel and concentrated with Microcon 50 ultrafiltration units (Millipore, Bedford, MA, USA) to ~100 μl in volume. Extracts were then quantified by UV spectrophotometry at 260 nm. Unfortunately, this method (as well as multiple others) proved unsuccessful at isolating sufficient gDNA at deeper depths from ODP core samples.

PCR Amplification of SSU rDNA for Clone Library

PCR primers for the initial amplification of archaeal SSU rDNAs templates were analogous to those used by DeLong (1992); priming sites were identical. The 21F primer [5′-TTC YGG TTG ATC CYG CCR GA] was synthesized where Y = pyrimidine analog "P" and R = purine analog "K" (Glen Research, Sterling, VA, USA). The 958R primer [5′-YCC GGC GTT GAN TCC AAT T] was synthesized where Y = pyrimidine analog "P" and N = an equal mixture of analogs "P" and "K" at a single position. The PCR parameters were: 2 min denaturation at 94°C followed by 25 to 30 cycles of 94°C for 1 min, 56°C for 1.5 min and 72°C for 3 min. Cycles were followed by a final hold at 72°C for 7 min. PCR conditions included: 50 ng template gDNA, 1 U JumpStart *Taq* DNA polymerase (Sigma-Aldrich, St. Louis, MO, USA), 5 μl 10X PCR reaction buffer, 2.5 mM $MgCl_2$, 1 μM each forward and reverse primer, 200 μM each dNTP, 10 μg BSA and molecular grade water to a total volume of 50 μl. Negative control reactions were performed with each round of amplification. Amplified products were examined against a 1 kb ladder DNA size standard using 1% agarose gel electrophoresis to determine size and efficiency.

Screening of Clone Libraries

For each of the two clone libraries, five replicate PCRs were generated and reactions were concentrated and desalted with Microcon 50 ultrafiltration units (Millipore) and eluted with 30 μl molecular grade water. The PCR amplicons were cloned with a TA cloning kit following manufacturer's instructions (Invitrogen, Carlsbad, CA, USA). All putative clones were streaked for isolation and the inserts assayed for correct size using PCR with M13F and M13R primers (Moyer 2001). All reactions were sized with a 1 kb ladder DNA size standard by 1% agarose gel electrophoresis.

OTU Determination and Rarefaction

Partial sequence data from all clones were quality controlled and aligned using the program BioEdit (Hall 1999). Sequences were trimmed to exclude the archaeal 21F primer and thereafter include ~300 bp of the 5′-end of the SSU rRNA gene. The program DOTUR was used to determine the number of unique operational taxonomic units (OTUs) for each clone library using a 97% similarity cutoff (Schloss and Handelsman 2005).

SSU rDNA Sequencing

Representative SSU rDNA clones from OTUs containing three or more clones were fully sequenced (Moyer 2001) using an ABI 3100 automated sequencer (Applied Biosystems, Foster City, CA, USA). Internal sequencing primers were the same as those of Moyer et al. (1998). SSU rDNA gene

Table 1. Summary of dominant archaeal OTUs from ODP Hole 1200D

Lineage, Group	Similarity Values[1]	Number of Clones[2]	Representative out
Crenarchaeota, Marine Group I	0.92 – 0.98	19	DSA OTU 1
Crenarchaeota, Marine Group I	0.92 – 0.98	12	DSA OTU 2
Crenarchaeota, Marine Group I	0.91 – 0.98	7	DSA OTU 3
Crenarchaeota, Marine Group I	0.93 – 0.97	7	DSA OTU 4
Crenarchaeota, Marine Group I	0.93 – 0.97	6	DSA OTU 5

[1]Range of pair-wise comparisons across entire group.
[2]Total number of SSU rDNA clones representing the library was 62.

sequences were contiguously assembled using BioNumerics (Applied Maths, Saint-Martens-Latem, Belgium) and checked for chimeras using both the Bellerophon server (Huber et al. 2004) and Mallard, which allows comparisons to any potential parent sequence (Ashelford et al. 2006).

Phylogenetic Analysis

Sequences were compared to those in GenBank using BLAST (Altschul et al. 1990; Benson et al. 2000). SSU rDNA sequences with the closest similarity to each OTU as well as other representatives were used in phylogenetic comparisons. All sequences were imported into ARB and aligned to the SSU_Jan04 database using the ARB fast aligner (Ludwig et al. 2004). Phylogenetic analyses were restricted to regions of moderately to highly conserved nucleotide positions that were unambiguously aligned for all sequences. Phylogenetic placements were calculated using fastDNAml version 1.2.2 (Olsen et al. 1994) using the general two-parameter model of evolution (Kishino and Hasegawa 1989) and allowing for the global swapping of branches. The search for an optimal tree was repeated within these parameters until the best log likelihood tree was calculated in at least three independent tree calculations.

Each phylogenetic tree was bootstrapped 500 times allowing for the global swapping of branches. The search for each bootstrap was repeated until the best log likelihood score was calculated for at least two independent bootstrap calculations. Using the program BioEdit, similarity values were determined from an identity matrix for all group members using the 5′-end ~300 bp data. A pair-wise comparison across each group was possible by determining the proportion of identical residues between aligned sequences (Tables 1 and 2).

Nucleotide Sequence Accession Numbers

The SSU rDNA sequences representing the OTUs used in this analysis have been submitted to GenBank and assigned accession numbers EF414497 through EF414507.

Genomic DNA Extraction and Purification for T-RFLP

DNA extractions of core samples (0 to 10 cmbsf) from all seven forearc seamounts were performed with modifications using the combined protocols of Miller et al. (1999) and Zhou et al. (1996). Approximately 5 g of sample and 5 g of acid-washed zirconium-silica beads were placed into a 50 ml centrifuge tube. To this tube, 13.5 ml DNA-extraction buffer (100 mM Tris-HCl, 100 mM Na_2EDTA, 100 mM sodium phosphate, 1.5 M NaCl and 1% CTAB [pH 8.0]), 1 mg proteinase K and 3.75 mg achromopeptidase were added. Samples were placed at 37°C and shaken horizontally at 225 rpm for 30 min. Next, 300 μl of 20% SDS was added and samples were incubated in a 65°C water bath for 2 h with gentle inversion every 15 to 20 min.

Each mixture was then transferred to a 30 ml sterile container and placed on a bead-beater at maximum speed for 3 min (BioSpec Products, Bartlesville, OK, USA). The mixture was next transferred proportionally into two 50 ml tubes and centrifuged at 6,000 × *g* for 10 min at room temperature. The supernatant was removed and placed into two fresh 50-ml tubes and gDNA was extracted an additional time with 5 ml of DNA-extraction buffer followed by incubation at 65°C for 15 min and centrifugation at 12,000 × *g* for 10 min.

All supernatants were combined, mixed with an equal volume (~30 ml) of phenol-chloroform-isoamylalcohol (24:24:1 v/v) and centrifuged at 6,000 × *g* for 10 min at room

Table 2. Summary of dominant archaeal OTUs from ODP Hole 1200E

Lineage, Group	Similarity Values[1]	Number of Clones[2]	Representative out
Crenarchaeota, Marine Group I	0.92 – 0.98	19	ESA OTU 1
Euryarchaeota, *Methanobacteria*	0.79 – 0.99	13	ESA OTU 2
Crenarchaeota, Marine Benthic Group B	0.82 – 0.97	7	ESA OTU 3
Euryarchaeota, *Methanosarcinales*	0.85 – 0.99	4	ESA OTU 4
Euryarchaeota, *Methanobacteria*	0.80 – 0.99	3	ESA OTU 5
Crenarchaeota, Marine Benthic Group B	0.81 – 0.97	3	ESA OTU 6

[1]Range of pairwise comparisons across entire group.
[2]Total number of SSU rDNA clones representing the library was 60.

temperature. The aqueous phase was transferred to two 50 ml tubes and linear acrylamide (20 μg/ml final) was added to each. The gDNA was precipitated with 0.6 volume isopropanol for 1 h at room temperature and was pelleted via centrifugation at 12,000 $\times$ *g* for 20 min at room temperature. The supernatant was decanted and the remaining pellet was rinsed with 70% EtOH and dissolved in 200 μl of TE (10 mM Tris, 0.1 mM EDTA, pH 8) buffer. All extracts were further cleaned using Qiaex II (Qiagen, Valencia, CA, USA) with elution in 50 μl of TE and then quantified by UV spectrophotometry at 260 nm.

T-RFLP Preparation

Three replicate archaeal PCRs were performed, each using 50 ng of gDNA, with the same priming sites as described above for clone library generation. The forward primer was 5′-end-labeled with 6-FAM with the amplification conditions and subsequent treatment with eight tetrameric restriction endonucleases as described by Davis and Moyer (2008). All reactions were desalted using Sephadex superfine G-75 (Amersham Biosciences, Uppsala, Sweden) and dehydrated. Reactions were resuspended in 15 μl formamide with 0.33 μl ROX-500 internal size standard, denatured at 95°C for 5 min and separated by capillary electrophoresis using an ABI 3100 genetic analyzer with a 50-cm capillary array and POP-6 (Applied Biosystems). Each reaction was separated and visualized at least twice to ensure reproducibility.

T-RFLP Normalization and Analysis

Terminal-restriction fragments (T-RFs) were sized against the ROX-500 internal size standard using GeneMapper v.3.7 (Applied Biosystems). Electropherograms were imported into the program BioNumerics (Applied Maths, Sint-Martens-Latem, Belgium). Community fingerprints were compared in BioNumerics using average Pearson product moment correlation (Häne et al. 1993) and unweighted pair group method with arithmetic mean (UPGMA) cluster analysis of all eight restriction digests using the relative fluorescent proportions of each electropherogram. The cophenetic correlation coefficient was calculated to assess the robustness of the cluster analysis groupings. Peak detection was limited to peaks between 50 and 500 base pairs in size and with height at least 3% of the maximum value of the fingerprint (Davis and Moyer 2008).

Direct Cell Counts

All samples underwent an examination of total cell counts in an attempt to visualize the distribution and abundance of subsurface microorganisms. Shipboard-prepared subsamples fixed in 2.5% glutaraldehyde were vigorously vortexed then 10 to 25 μl was removed with a wide-bore pipet tip and applied to pre-blackened, 25 mm diameter, 0.2 μm pore size, polycarbonate filters (Shipboard Scientific Party 2002). Duplicate filters were stained with 5 μM of Syto 13 (Molecular Bioprobes, Eugene, OR, USA) and 10 fields from each filter were examined using epifluorescence microscopy. Cell enumeration included normalization to the filtered volume.

Results

Clone Library Analysis

Archaeal SSU rRNA gene sequences were determined from 62 clones obtained from South Chamorro Seamount, ODP Hole 1200D. Using the DOTUR program, a total of 13 Hole 1200D subsurface *Archaea* or DSA OTUs were identified based on $\geq$97% sequence similarity and 5 dominant DSA OTUs were chosen as representatives of the community based on their group containing three or more clones (Table 1). Archaeal SSU rDNAs were determined from 60 clones obtained from South Chamorro Seamount, ODP Hole 1200E. Again using the DOTUR program, a total of 20 Hole 1200E surface *Archaea* or ESA OTUs were identified and 6 dominant ESA OTUs were chosen as representatives of the community, based on the same three or more clone criteria (Table 2).

To compare observed taxa richness between the libraries with respect to the absolute richness within the communities, a sample-size independent method was required. Rarefaction estimates the number of OTUs in a community of finite size by random sampling without replacement. The estimated number of OTUs were plotted against the number of clones analyzed for each clone library. Estimates of OTU richness (number of OTUs predicted) yielded ODP Hole 1200E having approximately 33% greater richness than ODP Hole 1200D (Figure 2).

Phylogenetic Analysis

Each OTU from ODP Holes 1200D and 1200E had a representative clone that was fully sequenced and phylogenetic

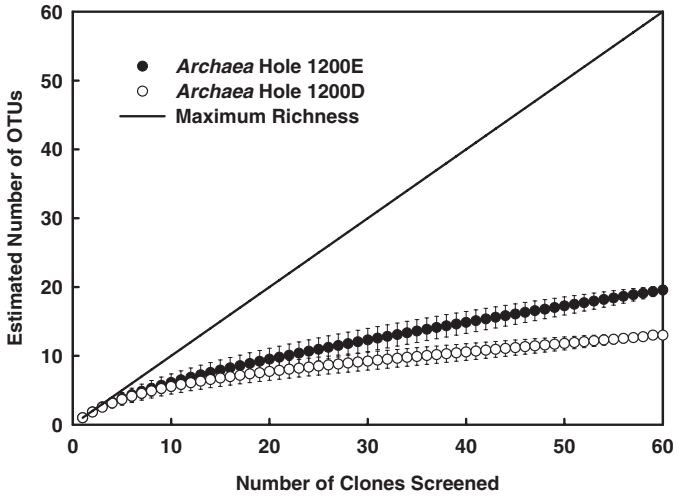

Fig. 2. Rarefaction curves comparing the estimated population richness of two clone libraries from South Chamorro Seamount, ODP Site 1200 Holes D and E. Estimates indicate an increase of ~33% in OTU richness in the 1200E archaeal community sampled over that of 1200D. Standard deviation confirms significant difference between the two sample sites. Curve of slope = 1 represents theoretical maximum richness. Error bars represent standard error estimates.

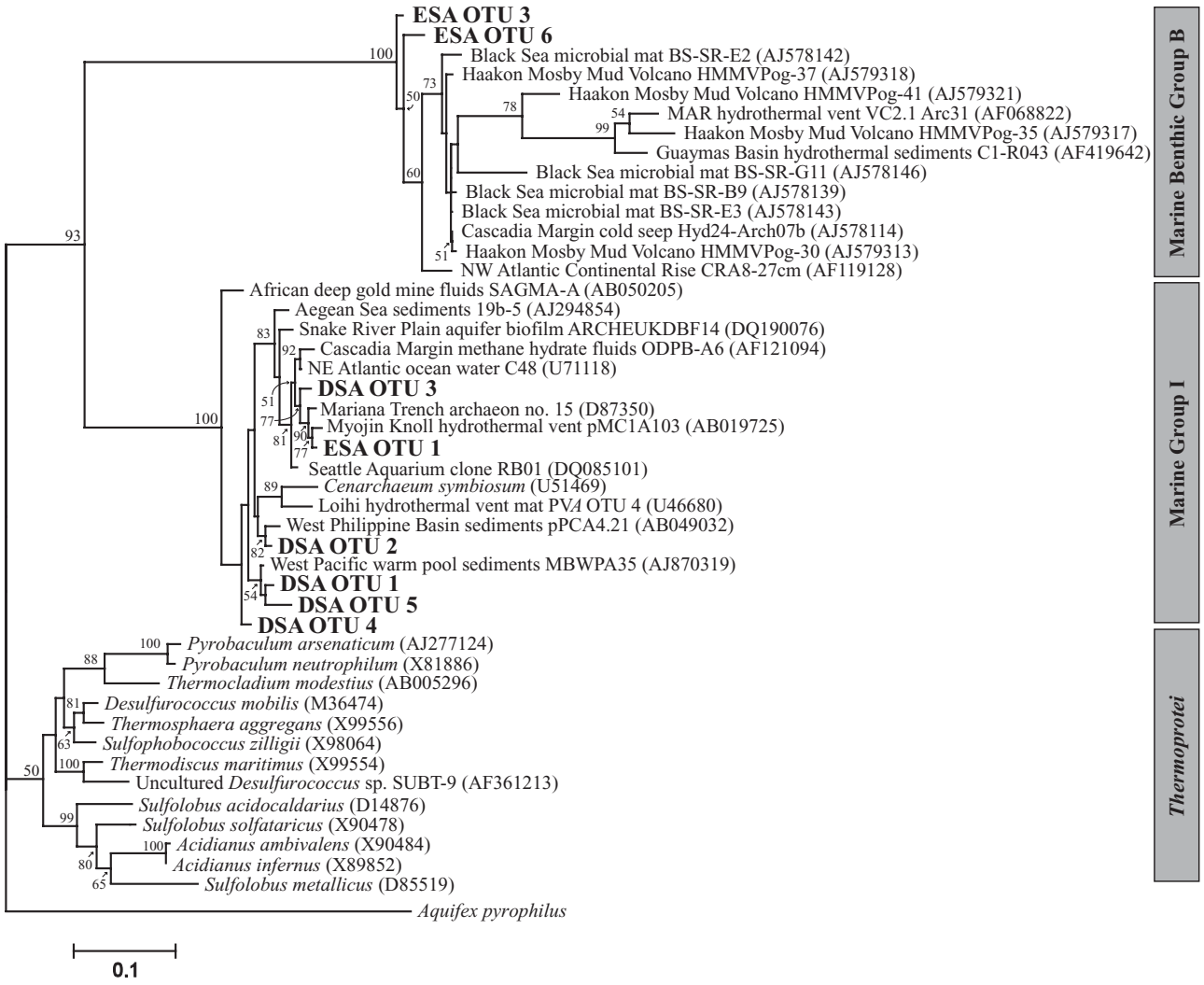

Fig. 3. Phylogenetic tree showing relationships of DSA OTU and ESA OTU phylotypes within the Marine Benthic Group B and Marine Group I (*Crenarchaeota*) as determined by maximum likelihood analysis of aligned SSU rDNA sequences. Numbers at nodes represent bootstrap values based on 500 bootstrap resamplings. The outgroup is represented by *Aquifex pyrophilus*. The scale bar represents 0.10 fixed mutations per nucleotide position. Bootstrap values are shown for frequencies at or above a threshold of 50%.

affiliations were determined (i.e., a phylotype). For ODP Hole 1200D, all dominant DSA OTUs resided within the Marine Group I *Crenarchaeota* (Figure 3). DSA OTU 1 comprised 31% of the clone library. The closest match to known database sequences for this OTU belongs to an uncultured crenarchaeote, clone MBWPA35 (AJ870319), originally detected from sediment in the western Pacific near China (Wang et al. 2005). DSA OTU 5 comprised 10% of the clone library and clustered closest with the nearest relative to DSA OTU 1. DSA OTU 4 comprised 12% of the clone library and grouped most closely with the same phylotype closest to DSA OTU 1, clone MBWPA35. DSA OTU 2 comprised 19% of the clone library and had a closest match to an uncultured archaeon labeled pPCA4.21 (AB049032) obtained from a subseafloor environment off the coast of Japan (Inagaki et al. 2001). DSA OTU 3 comprised 12% of the clone library with a closest match to an unidentified archaeon (D87350) detected from the Mariana Trench at a depth of 10,898 m (Kato et al. 1997).

The most dominant ESA OTU (1) at ODP Hole 1200E resides within the Marine Group I *Crenarchaeota*, comprising 32% of the clone library (Figure 3). Three of the remaining five dominant ESA OTUs (i.e., 34% of the clone library) were affiliated with the *Euryarchaeota* and were found within either the *Methanobacteria* or *Methanosarcinales* (Figure 4). ESA OTUs 3 and 6 were found most closely related to Marine Benthic Group B *Crenarchaeota* (OTU 3, representing 12% of the library, and OTU 6, representing 5% of the library, respectively). ESA OTU 1 had a closest match belonging to an uncultured marine archaeon (pMC1A103) detected from sediment obtained from a clear smoker chimney at Myojin Knoll (Takai and Horikoshi 1999).

ESA OTU 2 comprised 22% of the clone library, and had a closest match to an uncultured archaeon CRA13-11cm (AF119124) collected from deep-sea sediment within the Atlantic Ocean (Vetriani et al. 1999). ESA OTU 5 represented 5% of the clone library and was most closely related to the nearest

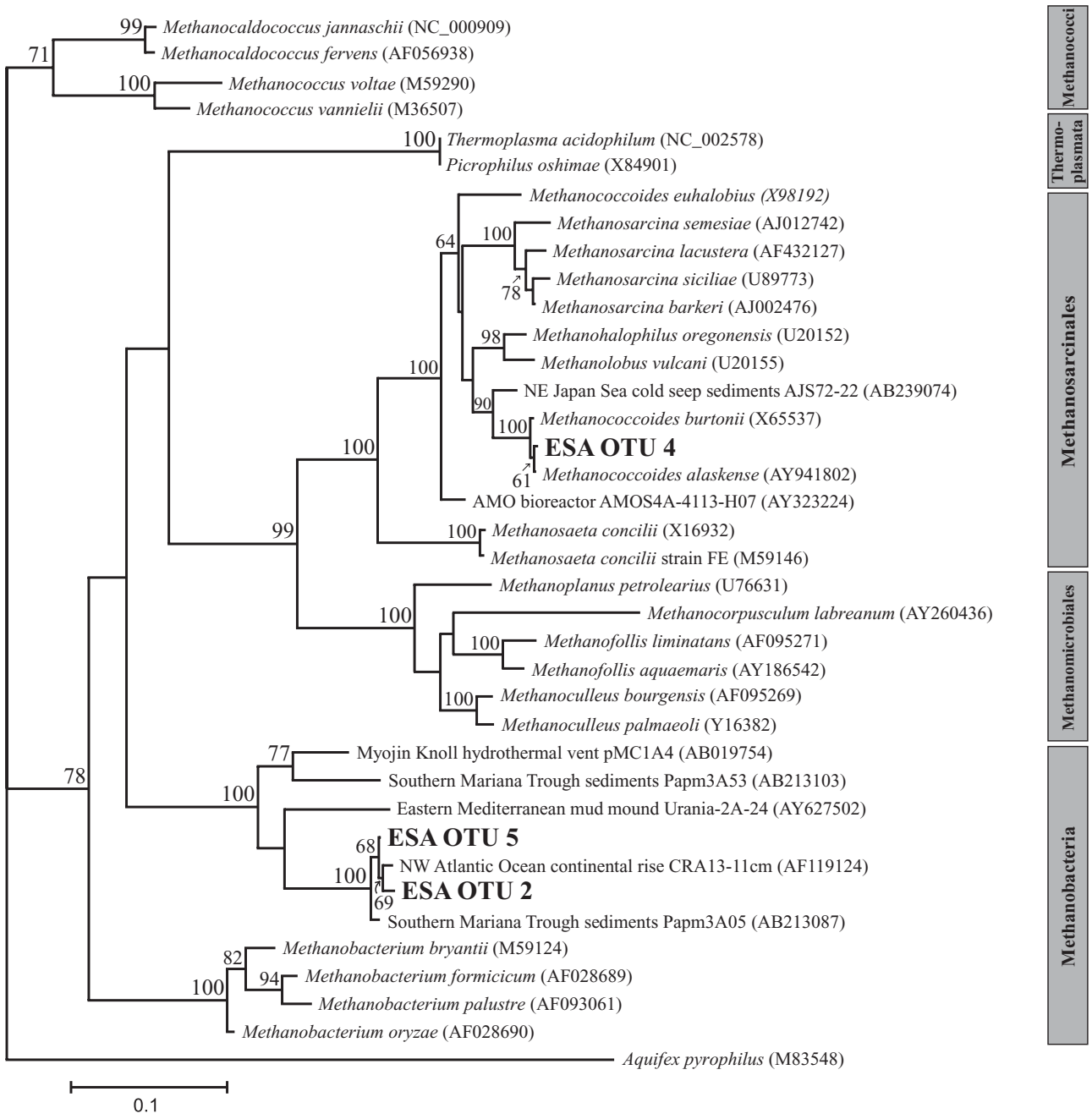

Fig. 4. Phylogenetic tree showing relationships of ESA OTU phylotypes within the *Methanobacteria* and *Methanosarcinales* (*Euryarchaeota*) as determined by maximum likelihood analysis of aligned SSU rDNA sequences. Numbers at nodes represent bootstrap values based on 500 bootstrap resamplings. The outgroup is represented by *Aquifex pyrophilus*. The scale bar represents 0.10 fixed mutations per nucleotide position. Bootstrap values are shown for frequencies at or above a threshold of 50%.

relative to ESA OTU 2, both among the *Euryarchaeota* order *Methanobacteria* (Figure 4). ESA OTU 4 comprised 7% of the clone library and was most closely related to a methylotrophic methanogen, *Methanococcoides alaskense* (AY941802) detected from anoxic marine sediment in Skan Bay, Alaska (Singh et al. 2005) among the *Euryarchaeota* order *Methanosarcinales* (Figure 4). ESA OTUs 3 and 6 were both most closely related to a deep-sea sediment archaeon, clone CRA8–27 cm (AF119128), detected from the NW Atlantic Ocean (Knittel et al. 2005).

T-RFLP Analysis

Selected electropherograms (treatments with *Hae*III and *Hha*I) from ODP Hole 1200D show T-RFs that match either all or a majority of the DSA OTUs detected within the

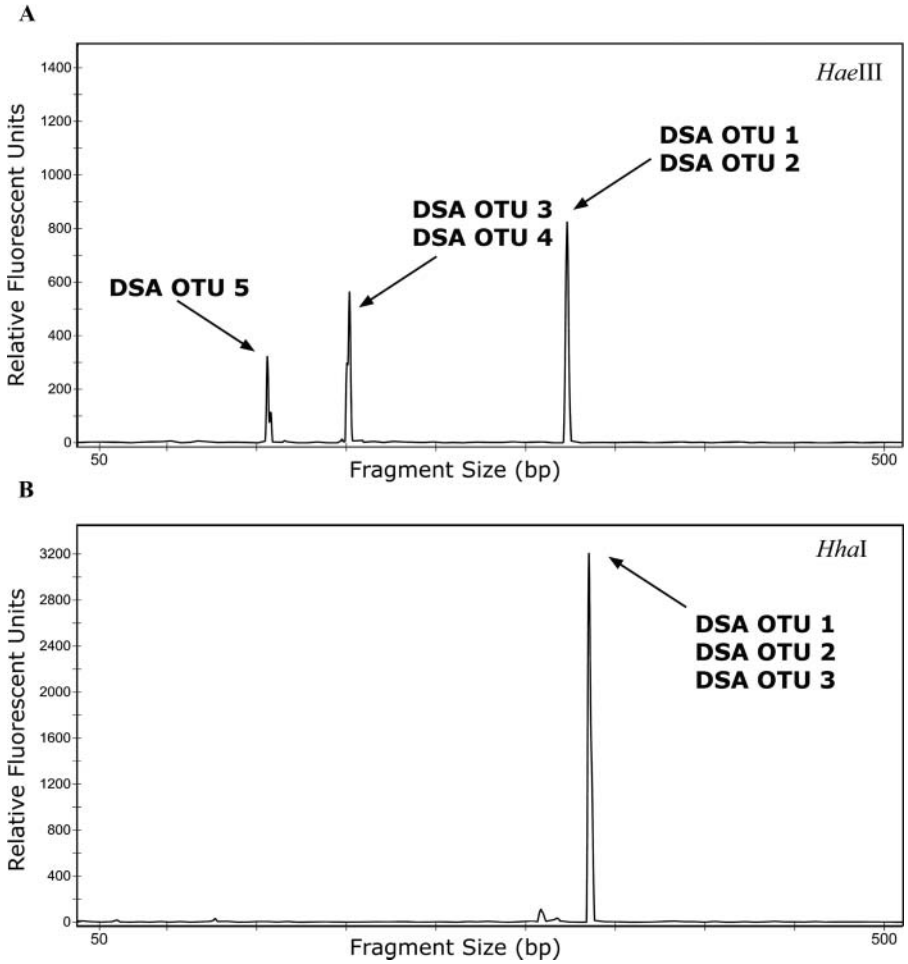

Fig. 5. T-RFLP electropherogram of (A) *Hae*III and (B) *Hha*I digests from ODP Hole 1200D at South Chamorro Seamount. Arrows indicate T-RFs matching DSA OTUs, all from within the Marine Group I (*Crenarchaeota*).

Hole 1200D clone library as determined by *in silico* digestion of Marine Group I *Crenarchaeota* phylotypes (Figure 5). Selected electropherograms (treatments with *Hae*III and *Hha*I) from ODP Hole 1200E also show T-RFs that when combined match all of the ESA OTUs within the Hole 1200E clone library, which contained both *Crenarchaeota* and *Euryarchaeota* phylotypes (Figure 6). To ensure maximal detection of OTUs, a total of eight restriction endonucleases were used when analyzing all samples. Similar T-RF patterns that matched the dominant phylotypes (found in the two ODP Site 1200 clone libraries) were also detected using the remaining six restriction enzymes: *Alu*I, *Bst U*I, *Msp*I, *Rsa*I, *Hinf* I, and *Sau*96I (data not shown).

Cluster analysis of samples (collected with multiple coring techniques) from seven different forearc seamount locations revealed archaeal communities similar to the ODP Hole 1200E library (i.e., ESA OTUs) which contained novel phylotypes (*Euryarchaeota* from within either the *Methanobacteria*, *Methanosarcinales* or the *Crenarchaeota* Marine Benthic Group B). These unique archaeal communities were detected in samples from the summits of South Chamorro (Hole 1200E), Big Blue and Blue Moon Seamounts (Figure 7). Total cell counts from these samples, as determined by epifluorescent microscopy, ranged from 9.5×10^6 cells/ml (at South Chamorro and Big Blue) to 1.1×10^8 cells/ml (at Pacman), falling within the range of approximately one order of magnitude from the lowest to the highest cell counts found across the seven seamounts examined (data not shown).

Discussion

The goal of this study was to discover and detect the most abundant microbial populations, as well as study their richness and relatedness, within the serpentinized mud volcanoes on the Mariana forearc using SSU rDNA clone library and T-RFLP analyses. In a previous study, phospholipid fatty acid or PLFA results from ODP Hole 1200E indicated an exceptionally high subsurface archaeal biomass (500 to 1000 times higher than bacterial biomass) collocated in zones of dissolved sulfide maxima and sulfate minima. This was taken to indicate the presence of an archaeal dominated community potentially capable of both AMO and sulfate reduction (Mottl et al., 2003).

Under the highly productive waters off Peru, subsurface archaeal consortia using AMO have been shown to occur in marine sediment habitats where methane is consumed at the

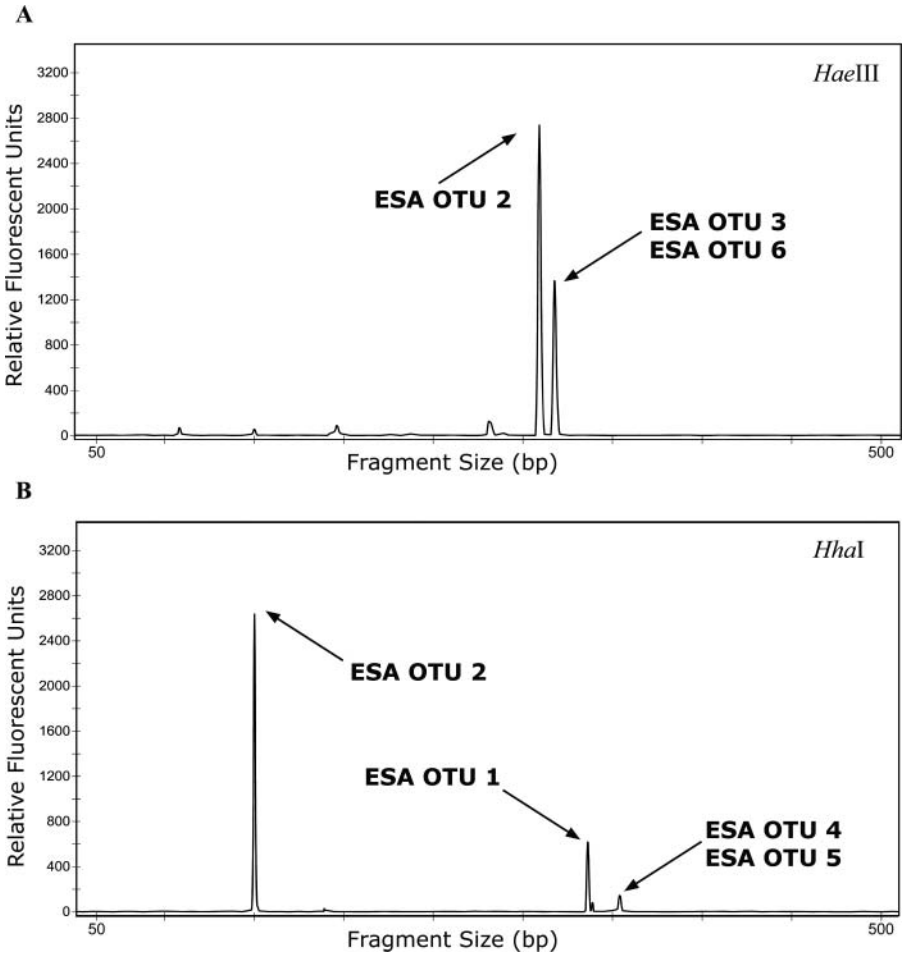

Fig. 6. T-RFLP electropherogram of (A) *HaeIII* and (B) *HhaI* digests from ODP Hole 1200E at South Chamorro Seamount. Arrows in (A) indicate T-RFs matching ESA OTU 3 and 6 from within the Marine Benthic Group B (*Crenarchaeota*) and ESA OTU 2 from within the *Methanobacteria* (*Euryarchaeota*). Arrows in (B) indicate T-RFs matching ESA OTU 2, 4 and 5 from within the *Methanobacteria* and *Methanosarcinales* (*Euryarchaeota*) and ESA OTU 1 from within the Marine Group I (*Crenarchaeota*).

expense of sulfate (Biddle et al. 2006). The highly alkaline (up to pH ~12.5) deep-sourced fluids from serpentinized mud volcanoes of the Mariana forearc provide the opportunity to investigate archaeal dominated communities and their ecological significance within the subsurface biosphere. The incorporation of seven seamounts in this study offers the benefit of analyzing archaeal communities from novel ecosystems with unique geochemical parameters spread across a wide geographic range (Figure 1).

Rarefaction curves comparing the estimated population richness of two clone libraries from South Chamorro Seamount (ODP Holes 1200D and 1200E) indicated that while

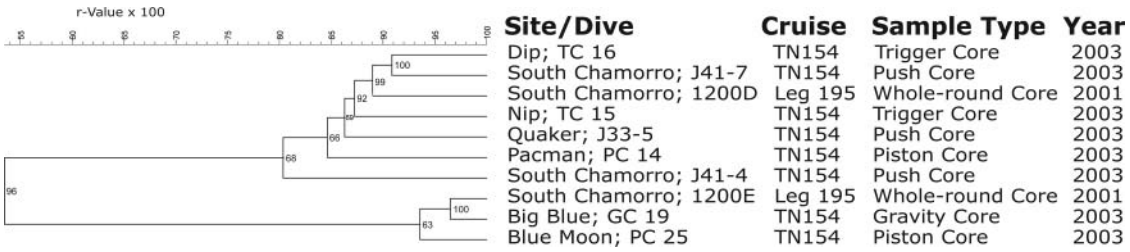

Fig. 7. UPGMA/Pearson product-moment cluster analysis of T-RFLP archaeal community fingerprints spanning seven serpentinized mud volcanoes along the Mariana forearc using eight restriction digest treatments on each of 10 samples. Unique archaeal community fingerprints resulted from an abundance of putative methane-oxidizing and sulfate-reducing phylotypes clustered from three sites: South Chamorro (ODP Hole 1200E), Big Blue and Blue Moon Seamounts. The numbers at nodes are the cophenetic correlation coefficients.

organismal diversity was low, saturation of either community had not yet been reached (Figure 2). The significant increase in richness of the Hole 1200E clone library (~33% greater than that of Hole 1200D) is thought to be due to the additional presence of the indicator *Euryarchaeota* phylotypes (Figure 6), which is likely dependent in part on its geographic proximity to an active spring located at the summit of South Chamorro Seamount. Upwelling of sediment pore fluids slows with distance from the spring; Hole 1200D, located approximately 80 m from the spring, had a pore fluid upwelling speed of 0.2 cm per year relative to the mud matrix.

In contrast Hole 1200E, located only a few meters from the spring, had a pore fluid upwelling speed estimated at 3 cm/yr (Fryer and Salisbury 2006). More recently, maximum upwelling speeds of 10 and 36 cm/yr have been modeled for Blue Moon and Big Blue, respectively (Hulme et al. 2010), which have shown similar archaeal community structures (Figure 7). The upward flow of pore fluids from depth coupled with active serpentinization processes transports slab-derived nutrients that appears to fuel archaeal chemolithotrophic metabolisms and faster flow appears to correlate with the added complexity of the archaeal community.

Between the two sampling locations at South Chamorro Seamount (ODP Holes 1200D and 1200E) 33 OTUs were detected among the 122 archaeal clones examined with a total of 11 OTUs detected as dominant community members (Tables 1 and 2). Sequence comparisons of representative clones indicated that all DSA OTUs (1 through 5) and a single ESA OTU (1) grouped within the Marine Group I (MGI) *Crenarchaeota* (Figure 3). T-RFLP data from Hole 1200D allowed for discrimination among all these MGI populations (Figure 5). The efficacy of this approach was confirmed by the comparison of T-RFLP data from Hole 1200E, where only a single MGI phylotype was detected (Figure 6), supporting our phylogenetic analysis (Figure 3).

These MGI phylotypes appear to represent the most abundant archaeal populations within the microbial community at South Chamorro Seamount. This is consistent with previous reports that the MGI *Crenarchaeota* dominate the global ocean biosphere (Karner et al. 2001; Takai et al. 2004b). The serpentinite muds investigated at South Chamorro revealed geochemical gradients caused by the rising fluids that originate at depth and mix with seawater near the surface (Fryer and Salisbury 2006). The persistence of the MGI phylotypes among all the Mariana forearc samples is likely an indication of this near surface mixing phenomenon within all of the mud volcanoes.

Phylogenetic analysis of representative clones from ODP Hole 1200E grouped ESA OTUs 3 and 6 within the Marine Benthic Group B (MBGB) *Crenarchaeota* (Figure 3). Additional comparisons revealed euryarchaeotal classifications with ESA OTUs 2 and 5 grouping within the *Methanobacteria* and ESA OTU 4 grouping within the *Methanosarcinales* (Figure 4). This discovery is intriguing because of all the *Methanosarcinales* isolates known, none have been able to grow above pH 10 (Boone et al. 2001). The MBGBs, *Methanobacteria* and *Methanosarcinales* phylotypes were only detected in the Hole 1200E clone library (Tables 1 and 2).

T-RFLP data from Hole 1200E allowed for discrimination among each of the MBGB *Crenarchaeota* and *Euryarchaeota* populations detected, again supporting our phylogenetic analysis (Figure 6). Distinctly separate from the other *Crenarchaeota* phylotypes discovered, recent findings suggest the MBGBs may have a syntrophic relationship with these *Euryarchaeota* with respect to AMO. Their relatively high abundance in association with benthic cold seeps indicates that they may be working in conjunction with AMO *Euryarchaeota*, acting as a newly discovered type of sulfate reducer (Biddle et al. 2006; Knittel et al. 2005; Sørensen and Teske 2006; Tourova et al. 2002).

Cluster analysis of the T-RFLP data from across the Mariana forearc system revealed the detection of additional archaeal communities from sites on Blue Moon and Big Blue Seamounts similar that of South Chamorro Seamount ODP Hole 1200E (Figure 7). These unique archaeal fingerprints resulted from an abundance of indicator *Euryarchaeota* phylotypes, which may be associated with methane-oxidizing and sulfate-reducing chemolithotrophic metabolisms. The combination of our phylogenetic analysis of the dominant populations within the archaeal communities at ODP Site 1200 in conjunction with the T-RFLP cluster analysis from multiple locations indicates that the distribution of these archaeal populations within near surface habitats is most likely due to environmental parameters (e.g., flow rates, pH, availability of methane, sulfate, etc.). Samples from Blue Moon and Big Blue Seamounts that had corresponding archaeal communities to South Chamorro Seamount, ODP Hole 1200E are hypothesized to have occurred at similar regions of high flow and ensuing pore water geochemistry (e.g., Hulme et al., 2010; Mottl et al. 2003). Serpentinite seamount environments are dynamic systems, and it is likely that the summit locations of high flow conduits are not only patchy but change over time (Fryer et al. 2006).

This study allowed for the discovery and detection of novel *Euryarchaeota* as well as Marine Benthic Group B *Crenarcheaota* phylotypes, which were determined as possible efficacious archaeal indicator populations involved with AMO and sulfate reduction fueled by deep subsurface serpentinization reactions. These indicator populations are apparently living (in relatively high numbers) on the edge of the habitable zone for life in this near-surface serpentinite mud habitat. In the future, we plan to use additional molecular microbial techniques such as fluorescent *in situ* hybridization (FISH) to aid in the localization of metabolically active methane-oxidizing and sulfate-reducing archaeal populations (e.g., Biddle et al. 2006; Knittel et al. 2005; Schippers et al. 2005; Schrenk et al. 2004) and quantitative-PCR (q-PCR) in an effort to determine the location and identity of both phylogenetic groups and their functional genes within these archaeal communities (e.g., Dhillon et al. 2005; Inagaki et al. 2004; Lloyd et al. 2006; Takai et al. 2004b). Further localization of these extremophilic archaeal populations along with a better understanding of their corresponding metabolic potential will aid in determining if these populations are actually endemic to high flow systems along the Mariana forearc or if they have the ability to exist in other habitats.

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