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A comparison of conventional and miniprimer PCR to elucidate bacteria diversity in Malaysia Ulu Slim hot spring using 16S rDNA clone library

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Abstract

The diversity of the bacterial community in Ulu Slim hot spring was examined using a 16S ribosomal DNA culture-independent approach. A total of 144 different 16S rDNA sequences were cloned and analyzed. The majority of sequences were found to be within the Betaproteobacteria and Gammaproteobacteria while the remaining sequences belonged to the Bacteroidetes, Acidobacteria, Cloroflexi, Nitrospira and candidate divisions. Some of the bacteria 16S sequences could be novel and indigenous to this hot spring as their sequences are low in similarity when compared with known sequences. The 16S ribosomal DNA clone library was assembled using conventional PCR amplification with degenerate 27F and 1492R primers specific for bacterial rDNA. A new miniprimer PCR assay was also used to construct a library. When compared, we conclude that the latter approach which using mutagenized Dynamo-II polymerase utilizing 10-mer primers was a better approach than the conventional PCR as the environmental humid acid inhibition effect was less. Ideally, to best understand the overall prokaryote diversity in the hot spring, it is better to use a combined conventional and miniprimer PCR approach.

Keywords: prokaryote biodiversity, thermophiles, hot springs, Restriction Fragment Length Polymorphism.

Introduction

The living communities that exist in extreme natural conditions like geothermal hot springs have always attracted much interest from taxonomists, microbiologists, and ecologists alike. But the most interest has come from the commercial industrial entities looking for biocatalysis that are stable under extreme conditions. One of the most successful research milestones in elucidating thermophile biodiversity was the study done at the Yellowstone hot spring (Hugenholtz et al. [1]). Since then, similar studies have been done all over the world in similar environments, for example in the Tibet, Indonesia and Thailand hot springs (LIU et al. [2]); AMININ et al. [3]; KANOKRATANA et al. [4]). The elucidation of thermophile biodiversity can either be done based on pure culture or direct identification using a 16S ribosomal DNA culture-independent approach. Culture-dependent approach can only isolate barely a few "leaves from the trees in a forest" and the actual numbers of thermophiles and their diversity may be better explored through culture-independent method. Most of the current sequences in the RDP database are uncultured environmental sequences while purely

isolated sequences constitute only a small fraction of the total (CHAI et al. [5]). This statistic concurs with the long-standing statement that most microorganisms are not cultivable.

The Titiwangsa Mountains are the main mountain range that forms the backbone of the Malay Peninsula. About 40 natural geothermal springs are found along this mountain range. A culture-independent approach to study the diversity of thermophiles in these hot springs has never been done before. The objective of this study is thus to elucidate the diversity of bacteria located in Ulu Slim hot spring, however, it is not intended to target all prokaryotic diversity. The 16S rDNA based biodiversity studies may sometime be prejudiced due to the limitation of so-called "universal primers" (20−25 nt) that are not perfectly conserved in all prokaryotes. Therefore, another main objective of this work was to demonstrate the use of a novel miniprimer PCR approach (ISENBARGER et al. [6]) in detecting the presence of taxa that may be missed by conventional PCR for several reasons that will be described in this work.

Materials and methods

Water analysis

Approximately 100ml of water from the springhead was sampled and the water analysis was done at the Universiti Teknologi Malaysia Chemistry Department servicing laboratory. HACH and APHA (American Public Health Association) standard water analysis methods were used to quantify the concentration of salts inside the sample. Two or more runs were conducted for the water analysis. The data presented here are the average values.

Bulk genomic DNA extraction and purification

The green-yellow layer on the top of clay pond bed in the hot spring was sampled and stored inside sterile bottles. Approximately 100 mg of the collected biofilm was then weighed and used directly for the extraction of bulk genomic DNA. Extraction of genomic DNA was done using the SoilMaster DNA Extraction kit (EPPICENTRE) following the supplied instructions. Initially, two other DNA extraction methods were utilized i.e. using established SDS/CTAB (ZHOU et al. [7]) and using a biofilm pretreated with aluminum ammonium sulfate ($AlNH_4(SO)_2$/SDS/CTAB) (DONG et al. [8]) approaches but neither yielded any PCR products.

Conventional PCR amplification

High purity grade universal primers 27F and 1492R were used for the conventional PCR approach. The 27F and 1492R primer sequences are 5'- AGR GTT YGA TYM TGG CTC AG -3' and 5'- GGT TAC CTT GTT ATG ACT T -3' respectively. Each 50-µl PCR cocktail contained 36.5 µl of sterile water, 1µl of a 100µM of the forward primer, 1 µl of a 100 µM of the reverse primer, 5 µl of 10× PCR reaction buffer, 1 µl of 10 mM dNTPs in an equimolar ratio, and 0.5 µl of *Taq* DNA polymerase (0.75 units/µl) and 5 µl of template genomic DNA. All PCR reagents and polymerase used were products of YEASTERN BIOTECH. The PCR program was run on a Bio-Rad thermal cycler and consisted of an initial denaturation at 94°C for 10 minutes. The first reaction cycle proceeded as follows: 1.5 minutes at 94°C, 1 minute at 55°C, and 2 minutes at 72°C. After a total of 35 cycles, an additional 5 minute extension at 72°C was programmed.

Miniprimer PCR amplification

The method suggested by ISENBARGER et al. [6] was followed for the miniprimer PCR amplification. Shorter (10 nt) primers 27F-10 and 1505R-10 with sequences 5'- AGA GTT TGA T -3' and 5'- CCT TGT TAC G -3' respectively were utilized. Miniprimer PCR amplification was carried out using a mutated *Thermus brockianus* polymerase (DynamoII from FINNZYMES - an unreleased product). Each 50-µl PCR cocktail contained 36.5 µl of sterile water, 1µl of a 100µM of the forward primer, 1 µl of a 100 µM of the reverse primer, 5 µl of 10× PCR reaction buffer (FINNZYMES), 1 µl of 10-mM dNTPs (YEASTERN

BIOTECH) in an equimolar ratio, and 0.5 µl of DyNAmo II DNA polymerase (0.75 units/µl) and 5 µl of template genomic DNA. The same amount of genomic template DNA was utilized in both the conventional and miniprimer PCRs.

Restriction Fragment Length Polymorphism

All PCR products were gel purified using the Wizard SV Gel and PCR Clean-Up kit (PROMEGA) following the instructions included. Cloning was done using the pGEM vector (PROMEGA) and subsequently transformed into *E. coli* DH5-α cells. Plasmids were isolated using the HiYield Plasmid Mini kit (YEASTERN BIOTECH). Approximately 400–500 ng of each purified plasmid clone was digested with *Hha*I and *Msp*I (NEW ENGLAND BIOLABS) for 1 hour at 37°C. As a control, the native pGEM vector was also digested concurrently with all samples.

Phylogenetic analysis

Chimera sequences and closest phylogenetic affiliation were identified using the CHECK_CHIMERA program and Classifier program provided in the Ribosomal Database Project (RDP release 10) (MAIDAK et al. [9]). Distance based on the Jukes-Cantor function was chosen to generate neighbor-joining (NJ) trees. A bootstrap setting of 1,000 replicates was used. MEGA 4 software (TAMURA et al. [10]) was used for all the phylogenetic tree analyses.

Results

Description of Ulu Slim hot spring

Ulu Slim hot spring is located at 3°53'58.5" N, 101°29'52" E in an undeveloped agricultural village. It is about 100 km north west of the Malaysian capital city of Kuala Lumpur. The hot spring can be described to be a small circular pond with diameter of approximately 100m. The main springheads are located in an inner pool enclosed by a circular fence and short concrete wall. Within the inner pool, there is a hard rock island at the center of the inner pool. From the rock, two major springheads channeled the underground water to the inner pool. When measured during three visits made from August 2008 to December 2009, the mean water temperature at the springheads was found to be 98ºC with standard deviation of 10. The average pH was found to be 7.8 with standard deviation of 0.5. Laboratory analysis of the springhead water showed that it has less than 2.0 g ml^{-1} total nitrogen and 0.9 g ml^{-1} sulfate. The total calcium, iron, magnesium, sodium and chloride concentration was determined to be 0.18, <0.05, <0.002, 15.1 and 4.1 g ml^{-1}, respectively. The chemical oxygen demand (COD) value was measured to be 81 mg ml^{-1}. The figures presented here are the average values while the standard deviation was in the range of 0.5−1.0.

Extraction of genomic DNA

Approximately 3 m away from inner pool springhead, a thin layer of less than half a centimeter of the green-yellow layer was sampled and analyzed for its bacterial community. The collected wet biofilm from the hot spring clay pond bed was used for the extraction of bulk environmental genomic DNA. Initially, a cell lysis method based on SDS and hexadecyltrimethyl ammonium bromide (CTAB) was used. This produced an intact and concentrated genomic DNA band when visualized with ethidium bromide after electrophoresis. Initial PCR amplifications of the genomic DNA using the 27F and 1492R primer pair were unsuccessful with commercial polymerases GoTaq (PROMEGA), NovaTaq (NOVAGEN), and YEAtaq (YEASTERN BIOTECH). None of the amplifications using these *Taq* polymerases generated any PCR products.

It is suspected from past studies that humid acid (from the breakdown of organic matter) frequently found in soil samples (DONG et al. [8]; BRAID et al. [11]) could have inhibited the PCR amplifications. Humic acid has similar chemical properties to DNA that make it a

hinderance to any microbial diversity research. Based on a recent real time PCR analysis, humid acid inhibited the amplification process through sequence specific binding to the template DNA, thus reducing the availability of template (OPEL et al. [12]).

Although using SDS/CTAB was a routine approach to isolating bulk genomic DNA from uncultured samples, it does not reliably remove humic acid (BRAID et al. [11]). Pretreatment of the collected samples using 100mM aluminum ammonium sulfate for 10 minutes (DONG et al. [8]) did not result in any viable PCR products. Switching to the use of a commercial kit i.e. SoilMaster DNA Extraction kit (EPPICENTRE) to isolate the bulk genomic DNA finally resulted in viable PCR products.

The same amount of genomic DNA was also used as template for comparing the amplification efficiency between the YEAtaq (conventional PCR) and DynamoII polymerases (miniprimer PCR). Interesting, the DynamoII polymerase generated approximately a tenfold higher yield of PCR product than normal *Taq* polymerase (YEASTERN BIOTECH) all other factors being equal. This suggested that the miniprimer PCR method which uses protein engineered DynamoII polymerase and shorter 10-nt primers may have the advantage of overcoming leftover traces of humid acid besides requiring less optimizations. It is also suspected that the purified genomic DNA contained traces of humid acid since other commercial DNA polymerases still failed to amplify any PCR products.

Conventional PCR usually requires 20–25-nt primers. The sequences of the universal 16S rDNA primers used have regions that are not entirely conserved for all bacteria (ISENBARGER et al. [6]; MAUKONEN and SAARELA [13]) and would thus not anneal to all bacterial rDNA sites. The inventors of miniprimer PCR method removed the degenerate portions in the 27F and 1492R primers (ISENBARGER et al. [6]) and denoted the 10-nt primers as 27F-10 and 1505R-10 which comprise only the most highly conserved region of the 16S rDNA. With these shorter primers, the DynamoII polymerase amplifies best with an annealing temperature of 40ºC (ISENBARGER et al. [6]). Compared to these miniprimers, the conventional PCR 27F and 1492R primers are thus more degenerate, longer, less specific and hence may only weakly anneal to dissimilar templates since they are less "specific" than the miniprimers in this case (MAUKONEN and SAARELA [13]).

Humid acid inhibition might be more significant in conventional PCR since the miniprimer PCR appears to generate a much higher yield of PCR products than conventional PCR. This observation is consistent with the results obtained using soil samples from other hot springs (data not shown). In order to reduce any bias in the PCR amplification and to test if the increased yield accounted for an increase in the number of different bacteria, two separate 16S rDNA clone libraries were constructed and compared, one each from conventional PCR and miniprimer PCR.

RFLP analysis

A total of 144 (72 from each) positive recombinant plasmids were isolated from both libraries. These were differentiated by restriction fragment length polymorphism (RFLP). Sequences that differed only slightly (<2%) were considered as a single phylotype and removed. A total of 39 unique clones were thus obtained. All full-length recombinants were inspected for the occurrence of chimeric sequences using CHIMERA_CHECK and 14 sequences were suspected to be chimeric. A total of 25 complete 16S rDNA sequences with the length of 1.4–1.5 kb have been deposited in the NCBI database (see Table 1) with accession numbers HM640987–HM641011.

Table 1. List of 16S rRNA bacterial clones obtained from genomic DNA extracts of Ulu Slim hot spring. Denotation "M" at the end, for examples US25M is a clone obtained using miniprimer PCR while, US21 refers to a clone obtained from conventional PCR.

US 16S clones	Bacterial division	Accession number	Identity to nearest hit by BLASTn (%)
US21	γ-*Proteobacteria*	HM641006	*Aeromonas veronii* (99)
US24	γ-*Proteobacteria*	HM641000	*Acinetobacter* sp. (99)
US26	*Chloroflexi*	HM640992	Uncultured green nonsulfur bacterium (91)
US27	*Chloroflexi*	HM641007	Uncultured green nonsulfur bacterium (91)
US210	β-*Proteobacteria*	HM641005	*Curvibacter delicatus* (98)
US213	γ-*Proteobacteria*	HM641004	*Acinetobacter* sp. WN4 (99)
US215	γ-*Proteobacteria*	HM641003	*Aeromonas veronii* (99)
US218	β-*Proteobacteria*	HM640990	*Vogesella* sp. u31 (99)
US227	γ-*Proteobacteria*	HM640991	*Aeromonas jandaei* (99)
US229	γ-*Proteobacteria*	HM640998	*Aeromonas punctata* (99)
US246	γ-*Proteobacteria*	HM640999	*Aeromonas veronii* (99)
US256	*Bacteroidetes*	HM640994	*Flavobacterium* sp. (98)
US24M	Unaffiliation	HM640987	Uncultured bacterium isolate S2R-45 (90)
US25M	γ-*Proteobacteria*	HM640988	*Vogesella* sp. u31 (99)
US26M	Unaffiliation	HM640989	Uncultured bacterium clone O1aA93 (86)
US211M	β-*Proteobacteria*	HM641008	*Curvibacter delicatus* (99)
US226M	β-*Proteobacteria*	HM640995	*Acidovorax* sp. (99)
US227M	Candidate division OP 10	HM640993	Uncultured bacterium clone G04b_L1_B06 (95)
US234M	Candidate division OP 10	HM640997	Uncultured candidate division OP10 (95)
US235M	β-*Proteobacteria*	HM640996	*Pseudorhodoferax soli* strain TBEA3 (95)
US236M	*Nitrospira*	HM641010	Unidentified *Nitrospira* group OPB67A (95)
US237M	*Acidobacteria*	HM641001	Uncultured *Acidobacteriaceae* bacterium A3DB-E10 (98)
US250M	Candidate division AC1	HM641002	Uncultured candidate division AC1 (94)
US263M	γ-*Proteobacteria*	HM641011	*Acinetobacter* sp. (99)
US271M	β-*Proteobacteria*	HM641009	*Acidovorax delafieldii* (99)

Phylogenetic analysis

Figure 1 shows the rooted phylogenetic tree of Ulu Slim (US) hot spring biofilm community. Throughout the whole report, denotation "M" at the end, for examples US25M is a clone obtained using miniprimer PCR while, US21 refers to a clone obtained from conventional PCR. A majority of the clones were identified as *Proteobacteria*. US clones that are grouped as γ-Proteobacteria were US271M, US226M, US235M, US211M, US210, US218 and US25M. A total of eight clones of Ulu Slim hot spring sequences were closely related to Acinetobacter and Aeromonas (β-subdivision). The Proteobacteria forms the biggest phylum and most phylogenetically diverse in the bacteria domain, however most of the representative species are mesophiles. In recent years, various culture-independent studies done at hot springs located in Tibet (LIU et al. [2]), Thailand (KANOKRATANA et al. [4]), and India (GHOSH et al. [14]) suggested that the Proteobacteria are prevalent in geothermal hot springs. Pure isolates of thermophilic β- and γ-subdivision Proteobacteria strains are limited, examples of such strains are *Tepidimonas ignave* and *Methylothermus* strain HB with maximum growth limit of 60–70ºC, respectively (MOREIRA et al. [15]; BODROSSY et al. [16]). Besides that, cloned 16S rDNA sequence (US256) closest BLASTn match showed it to be *Flavobacterium*. The *Flavobacterium* clones (AY555793 and AY555788) which previously identified from Thailand hot spring (KANOKRATANA et al. [4]) have similarity with the US256 clone sequence of only 75%. This could suggest that the US256 16S sequence may represent a new species.

Using the miniprimer PCR approach, the 16S rDNA from the US237M clone has the closest 98% similarity to that of an uncultured Acidobacteriaceae bacterium (DQ645247) previously reported by CONNON et al. [17]. This uncultured strain was identified as one from the Alvord hot spring with relatively high concentration of arsenic.

Subsequently, the cloned 16S rDNA sequence of sample US236M has a similarity of 95% with the unidentified *Nitrospira* group OPB67A (AF027001) and uncultured bacteria clone kab242 (FJ936959), identified at the Yellowstone hot spring and Kamtchatka volcano mud respectively. The similarity with other uncultured bacterium clones is lower at 88%, while the nearest pure isolates were *Nitrospira sp.* SRI-237 and *Thermodesulfovibrio yelloestonii* DSM 11347 (87 % and 87% respectively). Both strains are obligate anaerobic, thermophilic, sulfate-reducing bacteria placed into separate genera in the *Nitrospira* phylum.

The cloned 16S rDNA sequences obtained from the US26 and US27 samples clustered and formed a closely related lineage with that of uncultured *Chloroflexi* bacterium. This Green non-sulfur bacteria also known as *Chloroflexi* are obligate or facultative phototrophs and may contain some bacteriochlorophyll. Interestingly, the Ulu Slim 16S rDNA sequences were 93% similar with any uncultured *Chloroflexi*. Besides that, Ulu Slim clones sequences show only 72–76% similarity with known pure strains of thermophiles *Chloroflexi* members like the *Roseiflexus castenholzii* (AB041226) and *Chloroflexus aeaurantiacus* (AJ308500). Thus the Ulu Slim 16S rDNA sequences could be novel and indigenous to Ulu Slim hot spring. Further studies will be conducted in the near future to ascertain their identity.

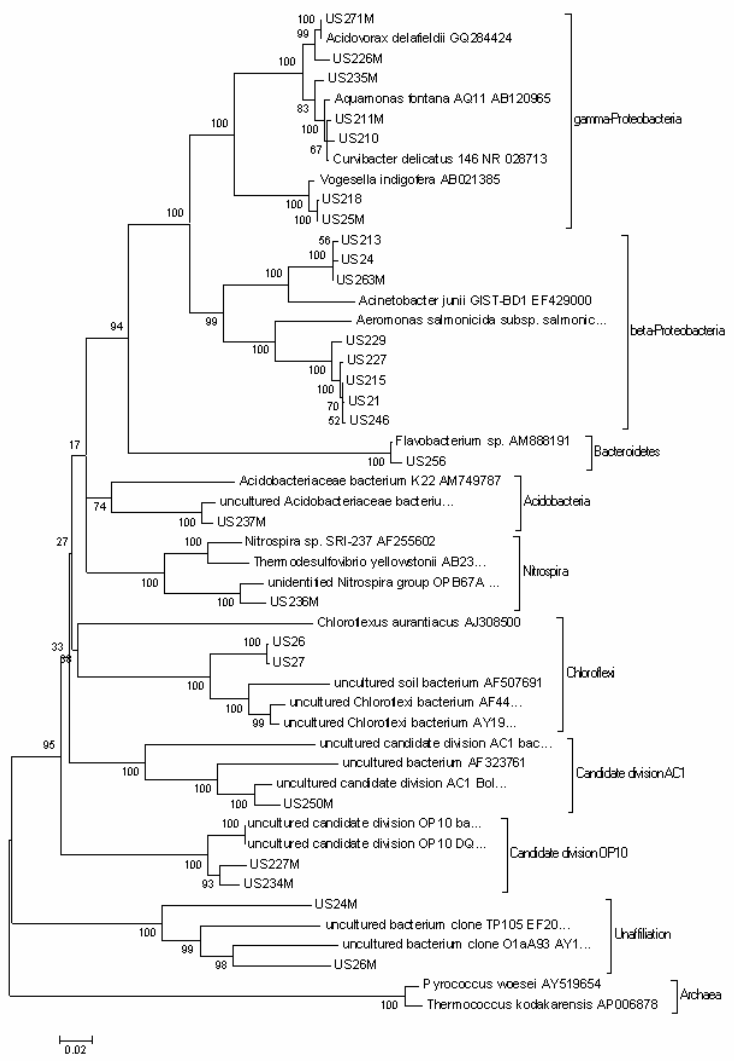

Figure 2. Rooted neighbor-joining 16S rRNA gene-based phylogenetic tree of uncultured bacteria. The scale bar corresponds to a 2% difference in nucleotide sequences. Archaea species were used as outgroups.

Some unaffiliated environmental sequences could be classified as a candidate division since limited information was available to describe them. A single clone sequence denoted as US250M is an unclassified bacterium. The closest match was uncultured candidate division AC1 bacterium clone Bol26 (AY193177) (94%). Unfortunately, this division is limited to only 12 known sequences found in NCBI database. Only two sequences were identified from hot springs. Two clones sequence denoted US227M and US234M were members of candidate division OP 10. This division has been given less attention due to lack of known sequences. Recently, PORTILLO and GONZALEZ [18] reviewed the deposited sequences in NCBI and concluded that OP 10 candidates were found in diverse environments –volcanic, geothermal sites, hypersaline systems and other sources. Most of the OP 10 candidates were detected via culture-independent approach. Interestingly, the first novel representative of OP 10 isolate was obtained from geothermal soil in New Zealand (STOTT et al. [19]), 10 years after the 16S rDNA from the first uncultured sequence was reported. The sampling site temperature was about 95°C with the pH value as high as 9.3. Under such state, the heated water should be anoxic. The described condition for culturing the novel strain was surprisingly. The medium was adjusted to acidic pH (3.8−5.5) and cultured at 60ºC under normal aerobic conditions. The authors claimed that the ingredient that finally enables the growth of the reported

unculturable strain was the addition of gellan to the growth media.

The last novel lineage found in this study was from clones US24M and US26M. The closest match clones were uncultured bacterium clones with no affiliation suggested by the NCBI or RDP databases. One of the closest uncultured bacterium clone pCOF_65.7_Toll with similarity of about 85% was identified from Black Pool Yellowstone National Park. HALL et al. [20] suggested the novel taxa as "Toll clade" and the organism that belong to this clade seemed to be a minor population in hot springs. As the members of this suggested candidate division is few, the NCBI taxonomy browser and RDP database have not recognized and assigned a proper candidate division name for this novel clade.

Discussion

This study did not intend to target all prokaryotic diversity in the Ulu Slim hot spring. However, a diverse bacterial community was observed and novel environmental clones were identified. Since the introduction of protein-engineered *Thermus brockianus* DNA-polymerase (ISENBARGER et al. [6]), at least 16 reports have cited this discovery. Several publications, for an example MAUKONEN and SAARELA [13] have theoretically agreed that miniprimer PCR may be a better approach in the elucidation of bacterial diversity. However, this has yet to be evaluated, as this mutated DNA-polymerase is not yet available as a commercial product. This current work was intended to assess the performance of miniprimer PCR and the outcomes have never been described. From the standpoint of the 16S rDNA PCR products and data collected from the two libraries, the miniprimer PCR approach exhibited several advantages:

(1) The miniprimer PCR was less inhibited by humid acid probably because the shorter 10-nt primers are more specific and thus anneal better than the conventional primers.

(2) As mentioned earlier, the PCR products obtained from the conventional PCR is much less than that in miniprimer PCR. To increase the PCR product yield, conventional PCR using normal commercial DNA-polymerase may require some PCR optimization i.e less template used (so to dilute the humid acid) or increase the number of amplification cycles. This may create a bias in amplifying certain strains in the environmental samples or creating artificial sequences caused by low replication fidelity of *Taq* polymerase. The miniprimer PCR has the advantage of being faster and being less tedious to set up and run.

(3) Besides producing a lower yield of PCR products, PCR artifacts (chimera) were found to be higher in conventional PCR (data not shown).

(4) In this particular sample used in this work, both conventional and miniprimer PCR were able to detect the abundance of *Proteobacteria* taxa but the 16S rDNA clones from the base of the phylogenetic tree were all obtained using the miniprimer PCR approach. This suggests that miniprimer PCR could detect a broader spectrum of environmental bacteria in the soil sample. *In silico* primer testing was done using RDP Probe Match program (MAIDAK et al. [9]). The program can accept primer sequence and use as a probe to search the deposited 16S sequences in the RDP database. The forward 27F-10 primer used in miniprimer PCR could theoretically complement approximately 40% more matches than the conventional 27F primer. This again suggests that miniprimer PCR is a better approach in detecting the presence of prokaryotes probably missed by conventional PCR.

Conclusion

The Ulu Slim hot spring was found to be a microbial rich geothermal environment. We concluded that using miniprimer-PCR is a powerful tool to elucidate a broader dimension of bacterial community in the environment where conventional PCR may be less sensitive. For

best results, we suggest using a combined conventional and miniprimer PCR approach to reduce any bias in targeting the total bacterial population.

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