Chiang Mai J. Sci. 2006; 33(3) : 363 - 370
www.science.cmu.ac.th/journal-science/josci.html
Contributed Paper

Genetical Diversity of *Mastigocladus* in Ranong Hot Spring, Southern Part of Thailand

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Received: 1 February 2006
Accepted: 30 April 2006

ABSTRACT

Among photosynthetic prokaryotes, the cyanobacteria represent by far the largest and most diverse group. *Mastigocladus* is one of the thermophilic species found in hot springs throughout the world including Thailand. *Mastigocladus* mats occur dominant in Ranong Hot Spring, Ranong Province, Southern Thailand. Cyanobacterial mats have been studied in 40-60 ºC of temperature intervals. They were observed by using microscopy and molecular technique analysis. In this study, the denaturing gradient gel electrophoresis (DGGE) analysis of PCR-amplified 16S rDNA gene segments was used to profile cyanobacterial populations inhabiting different temperature regions in the mat community. From this technique, *Mastigocladus* - like cyanobacteria were found in every temperature range and every season, however there was no significant difference between cyanobacterial populations in the Ranong Hot Spring.

Keywords: *Mastigocladus*, thermophilic cyanobacteria, DGGE, hot spring, Thailand.

1. INTRODUCTION

Interesting cyanobacterial distribution includes the stigonematalean species *Mastigocladus laminosus*, found in many hot springs throughout the world, including Yellowstone National Park [1], British Columbia and Antarctica [2]. As described by Geitler [3], *Mastigocladus* form single filaments with long, narrow side branches, the branches generally arise from one side (reversed 'V'-shaped branching). Heterocysts are formed intercalary, rather than at the base of the branches, and their homogonia is not known. They form tough mats. The filaments are 4-8 μm wide with 3 μm wide side branches, and oriented at right angles to the main filaments. The cells of the main filaments are barrel shaped or short cylinders, and the cells of the side branches are long cylinders [4]. It is most likely dependent on the following environmental conditions: 45-60ºC, pH >7.5, low oxygen content, and low salinity. *M. laminosus* shows an extremely large degree of morphological variability suggesting several morphotypes and genotypes.

Mastigocladus is interesting because they are the most thermophilic nitrogen-fixing cyanobacteria [5]. The fatty acid composition of *Mastigocladus* differs from that of the other cyanobacteria. It lacks polyunsaturated fatty acids. This suite of fatty acids is quite similar to the eucaryotic alga *Cyanidium caldarium*, which also grows in the same temperature

range as *Mastigocladus*, but at an acidic pH [4]. Because *Mastigocladus* fixes nitrogen, it can be found in springs low in combined nitrogen.

The development of techniques for the analysis of 16S rRNA gene sequences in natural samples has greatly enhanced detection and identification of cyanobacteria in nature [7-9]. DGGE analysis of PCR-amplified 16S rRNA gene segments was used to profile microbial populations inhabiting different temperature regions in the cyanobacterial mat community and to infer the phylogenetic relationship of the community members and therefore allows the identification of unique strains in a culture collection. Many studies have generally utilized 16S rRNA gene data from environmental and cultures DNA demonstrate that genotypic diversity far exceeds phenotypic diversity as estimated by observation and culture techniques [10].

In Asia, studies of the cyanobacterial mats in hot springs have been minimally investigated [11-13]. Geographically, several hot springs were found throughout Thailand. One of the most famous hot springs in the southern part of Thailand is Ranong Hot Spring which is composed of 3 natural hot pools with the maximum surface discharge of 60-65ºC [14]. This hot spring is associated with Cretaceous granite. The natural surface manifestation are seepages and hot pools of neutral/alkaline pH (Elev: 40 m. above sea level, N 9°57′386″, E 98°39′270″). The hot water is clear, having a pH of 7-7.38, with some CO_2 bubbles, very little H_2S, minor algae and an alteration mineral of calcite. The general chemical constituents are as follows: Conductivity 490-2,190 $\mu s.cm^{-1}$, Hardness 61-65 $mg.l^{-1}CaCo_3$, Total alkalinity 132-142, Na 47.04-65.05, K 2.57-3.07, Ca 20.4-44.8, Mg 0.01-3, Fe 0-0.005, Mn 0-0.01, F 5.2-7.5, SO_4 24-28, Cl^{-1} 8.4-8.8, SRP 0.14-0.16, NO_3^-1.1-1.3, NH_4^+ 0.15-0.16 $mg.l^{-1}$ [15]. Thus, extensive research into the diversity of thermophilic cyanobacteria is needed as supporting knowledge in Thailand as well.

Here, we report the results of the genetical distribution of *Mastigocladus* within the Ranong Hot Spring of southern Thailand by using the environmental 16S rDNA (16S rRNA genes) analyses to characterize their cyanobacterial diversity.

2. MATERIALS AND METHODS

2.1 Sampling

Cyanobacterial samples were collected from Ranong Hot Spring, southern Thailand (Figure 1). The sampling sites were chosen with temperatures in the range of 40 to 60°C with 5°C intervals. Observation and collection of cyanobacteria samples were taken every 4 months for one year during a 10 am to 2 pm time period. Samples were collected using a cork borer pushed through the mat removing a small cylindrical core from which the top of each core was then selected and placed into a 1.5 ml microcentrifuge tube. Triplicate cores were collected from each sampling site [7].

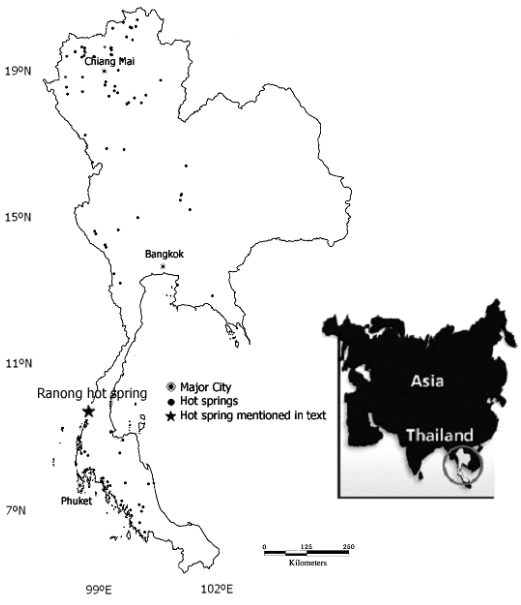


Figure 1. The hot spring districts in Thailand (after Raksaskulwong [16]), showing Ranong Hot Spring (RN) in southern Thailand.

2.2 Molecular Technique Analysis

2.2.1 DNA Extraction

DNA were extracted from cyanobacterial mat and from cultured isolates by the modified hot phenol method [17]. 500 ml of lysis buffer were added to each tube which contain a mat sample. The tubes were incubated for 1 hour at 60-65°C, after which 500 ml of hot phenol/ chloroform/ isoamylalcohol (25-24-1) was added. The supernatant was precipitated by absolute ethanol and the pellet washed with 70% ethanol and resuspended in 20-50 ml Tris-EDTA buffer. After a final gentle mixing and centrifuge for 5 min at 14,000 rpm, the DNA quality and quantity were determined using agarose gel electrophoresis.

2.2.2 PCR Amplification of Cyanobacterial 16S rDNA

Genes of 16S rDNA were amplified by PCR using cyanobacteria-specific primers CYA359F and CYA781R(a) [18] with a (GC) 40 clamp added to the forward primer. Each reaction contained 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.5 mM of each primer, approximately 10 ng template DNA, 0.1 $mg.ml^{-1}$ bovine serum albumin (BSA), 1.5U Taq polymerase and 1´buffer (Promega) in a total volume of 50 ml. The PCR amplification cycle was: 5 min at 94°C, then 30 cycles of 1 min denaturation at 94°C, 1 min annealing at 60°C, 1 min extension at 72°C, and a final extension of 7 min at 72°C. The PCR products were quantified by 1.5% (wt/vol) agarose gel electrophoresis with standard ehidium bromide staining to check for the recovery of products of the expected size (approximately 460 base pairs) and the product concentrations were estimated.

2.2.3 DGGE Analysis of Community DNA

DGGE was performed on a Bio-Rad Dcode system. An 8% polyacrylamide gel with a linear denaturant concentration from 40 to 60% was used (where 100% denaturant contains 7 M urea and 40% (v/v) formamide). Gels were electrophoresed for 17 h at a constant 60 V. Bands were excised. Then another PCR reaction was done from the band stab to ensure a clean product, using the reverse primer and a forward primer without GC-clamp. Reaction conditions were otherwise the same. PCR products were purified using Amicon microcon centrifugal devices (Millipore, Bedford, MA, USA) and served as sequencing templates. Sequencing was accomplished using the BigDye TM Terminator and an automatic sequencer 3730xl DNA analyzer (Macrogen Inc., Seoul, Korea). A BLAST search of the GenBank database was done to identify species or strains of the closest similarity.

2.2.4 Phylogenetic Analysis

An initial BLAST search of the NCBI GenBank database against the sequence data described above provided candidate sequences from which to compare the relatedness of the cyanobacteria to previously characterized species. Multiple sequence alignments were then created with reference to the selected GenBank sequences using BioEdit version 7.0.0. A phylogenetic tree was constructed from the aligned nucleotide positions corresponding to bases 359 to 781 of the *Escherichia coli* sequence. The Neighbor-joining (NJ) analysis using the Molecular Evolutionary Genetics Analysis (MEGA) package version 3.1 [19] was used to illustrate the relationship of partial 16S rRNA gene sequences to the representative cyanobacteria. The tree was then rooted using the *Escherichia coli* 16S rDNA sequence as an outgroup. To evaluate the robustness of branches in the inferred tree, one thousand replicates were used for bootstrap resampling.

2.2.5 Nucleotide Sequence Accession Number

The 16S rDNA sequences obtained in this study have been deposited in the GenBank database under accession no. DQ647799-DQ647803.

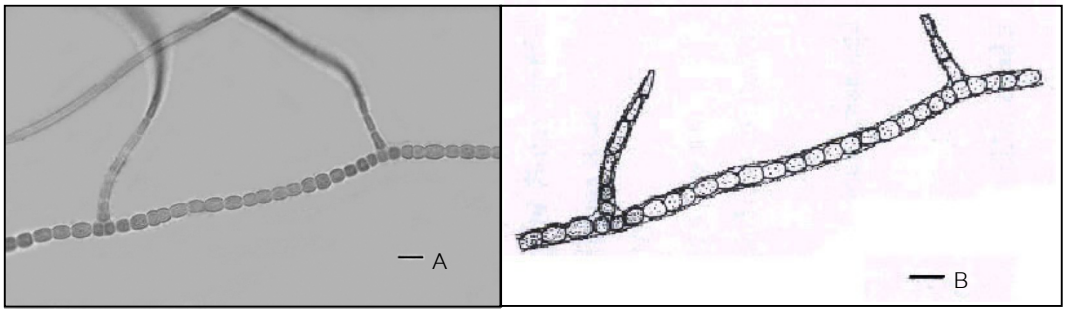
2.2.6 Community Evaluation

To compare the DGGE banding patterns

of cyanobacterial mat in Ranong Hot Spring in every range of temperatures and seasons, the Multivariate Statistical Package (MVSP) for window 3.1 was used. The MVSP was used to perform Principal Components Analysis (PCA) of the relationships between DGGE banding profile within each temperature range and season.

3. RESULTS AND DISCUSSIONS

The most common microbial mats in hot springs are those produced in neutral to alkaline springs by cyanobacteria [20]. A variety of different thermophilic cyanobacteria may produce mats, with distribution controlled by differences in temperature and water chemistry. In this study, we focused on the cyanobacterial mat population in Ranong Hot Spring in southern Thailand. *Mastigocladus* sp. was found to be the dominant species using microscopic observation (Figure 2). They form tough mats, green or dark green. The top layer of the cyanobacterial mat was a light green mat, but the bottom layer appeared as a yellow-green mat.



(scale bar: 10 μm)

Figure 2. Mastigocladus morphotypes from natural samples of Ranong Hot Spring.

The regional differences in morphotype diversity suggested the influences of geographical isolation, geological age and human activity on species disturbance in this hot spring environment may be examined more fruitfully using sensitive genetic tool. The sensitive genetic method is necessary to identify whether cyanobacteria are globally distributed or endemic populations [6, 20].

3.1 Molecular diversity of cyanobacterial 16S rDNA genes

We studied the 16S rDNA gene-defined community diversity in cyanobacterial mats from Ranong Hot Spring to characterize species composition of cyanobacteria in natural samples. A representative sample of DGGE separation of bulk cyanobacterial 16S rDNA genes is presented in Figure 3. Bands which migrated to the same position in the DGGE gel and displayed no ambiguous differences in nucleotide sequences were considered to represent unique 16S rDNA sequence types.

A total of 12 samples (bands) of DGGE amplified by PCR from environmental DNA samples were used and then 6 bands were reamplified and sequenced. All banding patterns were consistent between independent replicates from the varied seasons (Figure 3). DGGE results have revealed that 16S rDNA gene distributions change along the thermal gradient. All successfully sequenced 16S rDNA gene sequences were blasted against NCBI GenBank database, and those sequences found to share a high level of similarity were used to resolve alignment ambiguities and establish relationships for the sequences obtained in this study. For the major band, band A was conspicuous in every temperature

range (40-60ºC). They were found from the blast results, band DGGE RN55b position, it was *Mastigocladus*-like cyanobacteria with 95% similarity.

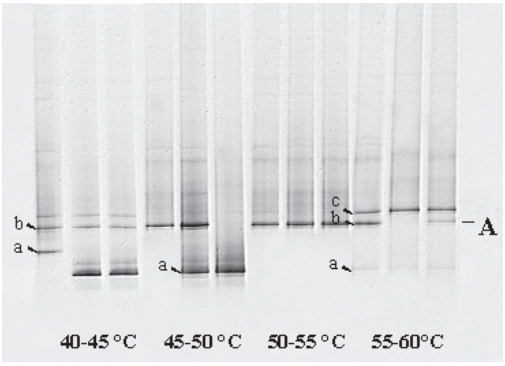


Figure 3. DGGE banding patterns of 16S rDNA gene-defined diversity among thermophilic cyanobacterial mats in Ranong Hot Spring. The first lane of each temperature range contains cyanobacterial mats from the rainy season, the second lane is from the cool dry season, and the third lane is from the summer season. Arrowheads to the left of the band indicate positions in the gradient at which defined bands were excised.

The DGGE banding patterns of cyanobacterial 16S rDNA gene analysis of Ranong Hot Spring in each season were put into MVSP to perform PCA analysis. The PCA analysis of DGGE banding patterns is presented in ordination diagrams or two-dimensional scatterplot. It showed the relationship between DGGE banding distribution in different ranges of temperature and seasons of this hot spring. The ordination diagram for the DGGE banding patterns of Ranong Hot Spring is shown in Figure 4. The eigenvalue for axis 1 was 8.413 and for axis 2 was 1.673. The cumulative percentage for both axes was 87.773, which indicates that two axes capture about 88% of total variance in the data set.

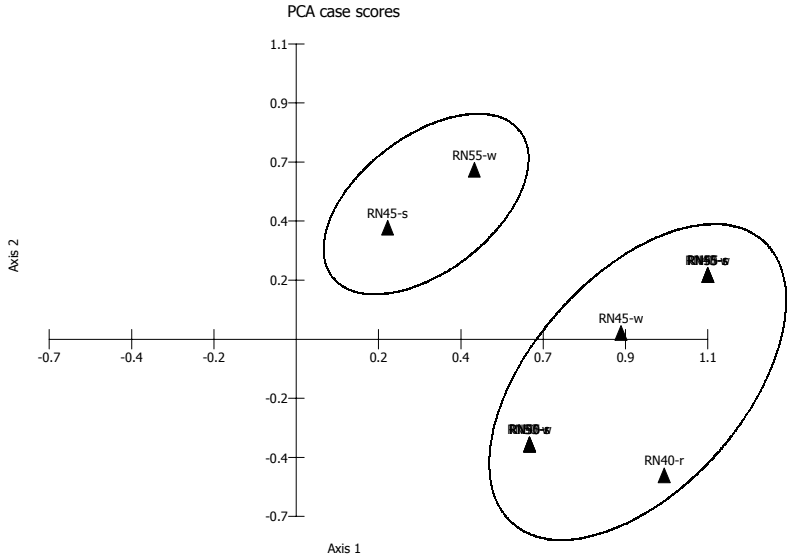


Figure 4. Principal Components Analysis (PCA) of cyanobacterial DGGE profile distribution in Ranong Hot Spring.

From the PCA scatter plot (Figure 4), it was found that the DGGE profile in this hot spring could be defined into 2 groups. The first group consisted of RN45-s (45-50°C temperature range in the summer season) and RN55-w (55-60°C temperature range in cool dry season), and another group which contains other sampling points, and have a positive correlation with axis 1. However, it was not clearly different between temperature ranges and each season. In another hand, the banding profiles of cyanobacterial population in Ranong Hot Spring are not differences.

The overall phylogenetic tree generated for the samples isolated in this study and the related sequences from the NCBI database is shown in Figure 5.

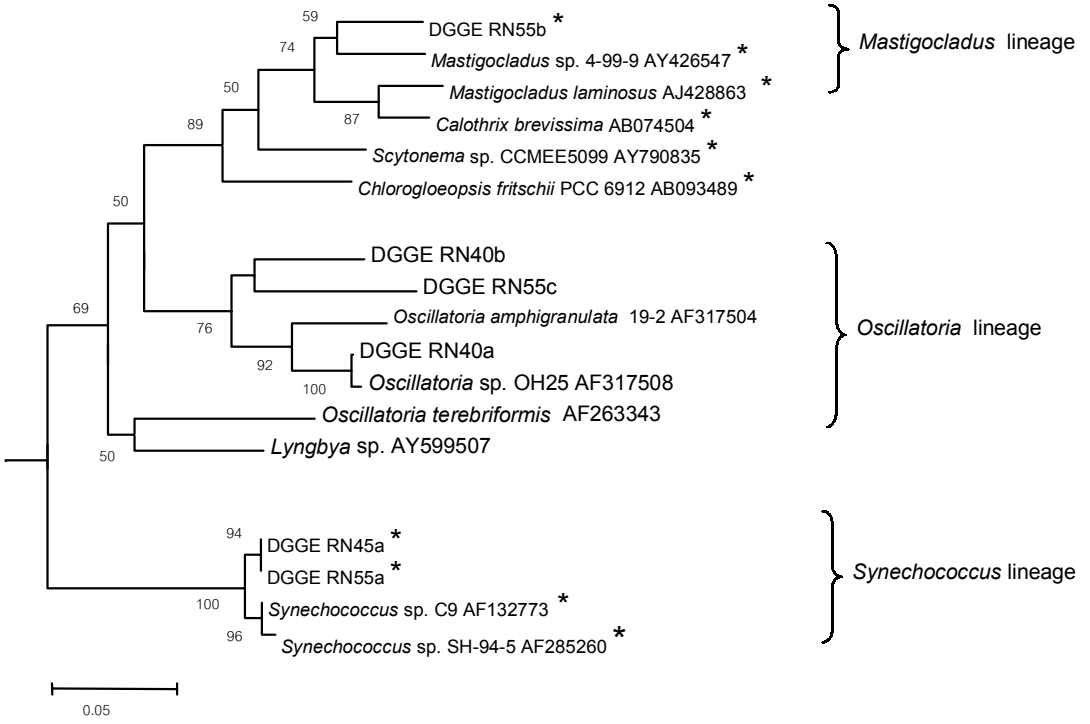


Figure 5. Phylogenetic relationships demonstrating *Oscillatoria*-like, *Mastigocladus*-like and *Synechoccoccus*-like cyanobacteria constructed on the basis of partial 16S rDNA gene sequences. Evolutionary distances were determined by the Neighbor-joining analysis. Sequence designations for DGGE samples are labeled by location, temperature (in 5°C intervals) and band position. The tree was rooted using *Escherichia coli* 16S rDNA sequence. Values at nodes indicate bootstrap percentages for 1,000 replicates. Values less than 50% were not reported. The branch also recovered using the UPGMA and Maximum Parsimony algorithms by adding an asterisk at the note.

The phylogenetic analysis of the partial 16S rDNA sequences of cyanobacteria in Ranong Hot Spring is classified into 3 lineages (Figure 5). There are clearly displayed differences between 3 clades (*Mastigocladus*, *Oscillatoria* and *Synechococcus*) with a high percent bootstrap value when analyzed by using Neighbor Joining tree.

The development of techniques for the analysis of 16S rDNA sequences in natural samples has already greatly enhanced detection and identification of cyanobacteria in nature ([7], [8], [9] and [18]). It should also be noted that in studies where near-complete 16S rRNA gene sequences have been used, conflicts between morphological and molecular identification of some cyanobacterial sequences have been found [13].

In conclusion, we have characterized community molecular diversity of cyanobacterial mats from the Ranong Hot Spring. It was found that the 16S rDNA gene-defined diversity of all mats. *Mastigocladus*-like cyanobacteria were found at every temperature range from 40-60°C. Moreover, others species were found in most temperature ranges, for instance *Synechococcus*-like and *Oscillatoria*-like cyanobacteria. And from the banding profile, there are no significant differences between temperature ranges and seasons.

ACKNOWLEDGEMENTS

The authors would like to thank the Royal Golden Jubilee Ph.D. Program (PHD/0087/2544), the Thailand Research Fund (BGJ4680015), the Graduate School of Chiang Mai University for financial support and the Center for Ecology and Evolution at the University of Oregon. We would also like to thank Julie A. Toplin and Dr. Tracy B. Norris (University of Oregon) for technical help with the molecular work done in Oregon.

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