CHAPTER 3

MATERIALS AND METHODS

3.1 Sampling

Cyanobacterial samples were collected from 6 hot springs which included San Kamphaeng, Pong Dued and Theppanom Hot Springs, Chiang Mai Province, northern Thailand, Pra Rueang Hot Spring, Kamphaeng Phet Province, central Thailand, Raksawarin Public Park, Ranong Province and Khaochaison Hot Spring, Phattalung Province, southern Thailand (Figure 5 and Table 1).

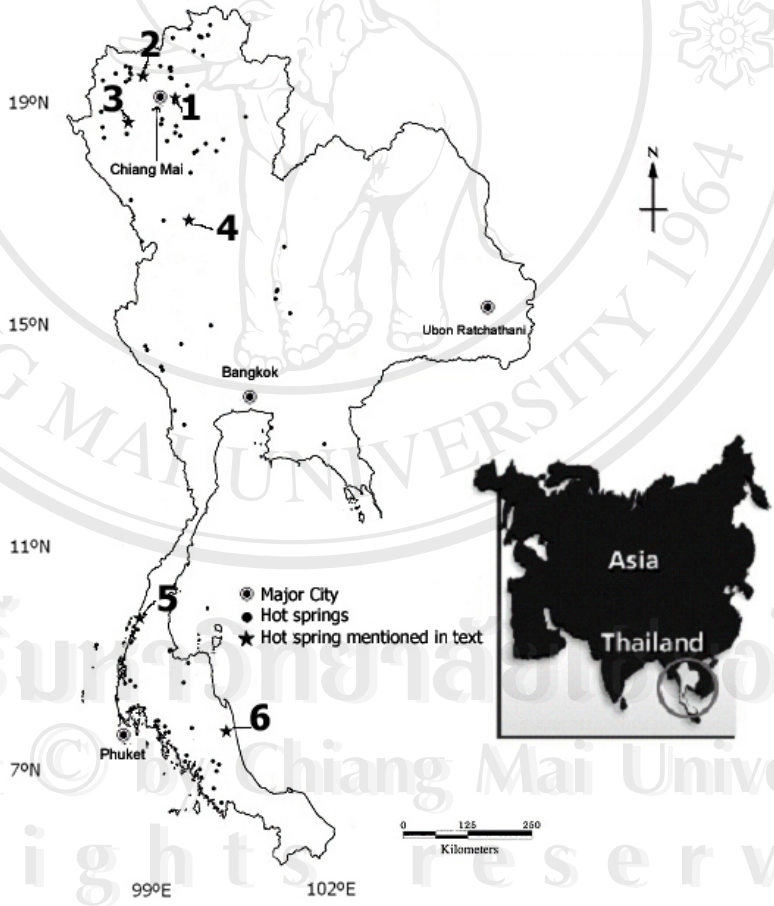

Figure 5 The hot spring districts in Thailand (after Raksaskulwong, 2000), showing the six locations sampled (the hot spring descriptions are listed in Table 1).

Figure 5 (continue)

1. San Kamphaeng, Chiang Mai (SK) 2. Pong Dued, Chiang Mai (PD)
3. Theppanom, Chiang Mai (TP) 4. Pra Rueang, Kamphaeng Phet (PR)
5. Raksawarin Public Park, Ranong (RS) 6. Khaochaison, Phatthalung (KC)

Table 1 Site descriptions and characteristics for each hot spring.

Site No.	Hot spring name	Location co-ordinates	Altitude (m)	Maximum temperature(°C)
1	San Kamphaeng (SK)	18°48′828″ N, 99°13′835″ E	363	98
2	Pong Dued (PD)	19°14′641″ N, 98°41′358″ E	760	98
3	Theppanom (TP)	18°16′175″ N, 98°23′974″ E	376	97
4	Pra Rueang (PR)	16°39′422″ N, 99°28′377″ E	86	50
5	Raksawarin Public Park (RS)	9°57′386″ N, 98°39′270″ E	40	60
6	Khaochaison (KC)	7°26′888″ N, 100°8′069″ E	55	51.5

3.1.1 Cyanobacterial mat sampling

At each hot spring, the area was surveyed to characterize the temperature profile. Sampling sites were chosen with temperatures in the range of 40 to 75°C with 5°C intervals. Cyanobacterial samples were collected every 4 months for one year from 10 am to 2 pm. Cyanobacterial mat were attached with soil, sand, cobble or concrete. Samples for culture isolation and microscopic examination were handled with sterile forceps and spectula from the sampling sites and were then added to sterile marked polyethylene tubes. Samples for DGGE analysis were collected using a cork borer (7.0 mm diameter) 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 the mats within areas of approximately 10 by 10 cm from each sampling site. The samples for molecular analysis were stored on ice or dry ice during transportation, and were stored at –20°C on arrival at the laboratory. (Ferris *et al*. 1997).

3.1.2 Water analysis

The physico-chemical properties of the water from each sampling site were measured in the field and at the laboratory. The velocity of hot spring was high in the PD, TP and RS Hot springs and lower in SK, PR and KC Hot Spring. The water level was lower in almost hot springs except PR Hot Spring because it was an artificial pool. The water samples were collected by polyethylene bottles for 3 replicates from the banks of the stream around the sampling sites. The bottles were kept in a cool box (5-7ºC). The water was collected along the stream in every season and every range of temperature during 40 to 75°C for a year.

3.1.2.1 Water temperature was measured in the field using a thermometer

3.1.2.2 pH was measured using a pH meter (Cole-Parmer Series 5986)

3.1.2.3 Conductivity was determined using a conductivity meter (Checkmate 90 Ciba Corning Ltd).

Water samples were collected in polyethylene bottles for chemical analysis:

3.1.2.4 Alkalinity was determined by the Titration method (APHA, 1998)

3.1.2.5 Nitrate-nitrogen (NO_3^-) was determined by the Cadmium reduction Method (APHA, 1998)

3.1.2.6 Ammonium-nitrogen (NH_4^+) was measured by the Nessler method (APHA, 1998)

3.1.2.7 Soluble reactive phosphorus (SRP) was measured by the Ascorbic acid Method (APHA, 1998)

3.1.2.8 The dissolved sulfide concentration (S^{2-}) was determined by the Methylene blue method (APHA, 1998)

3.1.2.9 Sulfate was measured by the Turbidimetric method (APHA, 1998)

3.1.2.10 Hardness, Calcium and Magnesium was measured by the EDTA titrimetric method (APHA, 1998)

3.1.2.11 Chloride was measured by the Potentiometric titration method (APHA, 1998)

3.1.2.12 Sodium, Potassium and Iron was measured by Atomic absorption spectroscopy

3.2 Morphological study

3.2.1 Morphological identification

Morphological classification of natural and cultivated cyanobacteria was based on characters observable under a light microscope (400-1,000X). Identification of cyanobacterial morphotypes was carried out microscopically using an Olympus CH30RF200 compound microscope. Morphologies of cyanobacteria were recorded using drawings and by taking pictures. Relevant keys such as Desikachary (1959), Anagnostidis and Komárek (1985; 1988; 1990), Hoffmann (1988), Kovácik (1988), Komárek and Anagnostidis (1989; 1999) and (Castenholz, 2001) were used as references for classification.

3.2.2 Isolation and cultivation

Small amounts of the cyanobacterial samples were dispersed by a tissue grinder and were diluted 10 fold serially into 8-10 tubes (dilution to extinction) with medium D or ND (Castenholz, 1981; Castenholz, 1988). The most diluted tubes which showed positive growth were used as an indicator for the most abundant cyanobacterial populations. The successful cultures were inoculated into 125 ml flasks containing medium D, ND or spread on agar-solidified plates of the same medium. Culture purification was conducted by streak plate technique. All tubes, flasks and plates were incubated in photochambers at 40, 50 and 58°C under fluorescent light (~3,000 lx) (Castenholz, 1988). When cyanobacteria growth was observed, one single filament or colony was transferred to 10 ml of fresh medium in a fresh tube and

incubated under the same conditions for 7 days or until growth occurred. This monoculture was then transferred to 75 ml of fresh medium flasks.

3.2.3 Cyanobacteria abundance evaluation

Cyanobacterial abundance was determined semi-quantitatively by counting the number of cells, filaments or colonies in replicate volumes of the homogenised pooled sample (0.02 ml). The mean abundance for each species for each temperature range was calculated as the arithmetic mean of the semi-quantitative occurrence data for that species from all samples from that temperature range. The percentage of cyanobacterial species was classified into 4 levels as follows;

Dominant = more than 30% of the total cell count
Common = 1–30% of the total cell count
Rare = less than 1% of the total cell count
Absent = below the detection

3.3 Molecular analysis

3.3.1 DNA extraction

The bulk DNA from the environmental samples and culture isolates were extracted using the modification of the hot phenol method (Giovannoni *et al*., 1990).

1) Pieces of cyanobacterial mat or colonies were collected and centrifuged (300 mg).

2) Mat was gently disrupted with a sterile grinding rod (this step may be omitted for “soft” samples).

3) 500 µl lysis buffer (1.4 M NaCl, 20 mM EDTA, 100 mM Tris-HCl (pH 7.5-8), 4 % CTAB, 1 % PVPP, 0.1 % β-mercaptoethanol, 50 mg/ml lysozyme and 20 mg/ml Proteinase K) was added.

4) The tubes were incubated at 60-65°C for 1 hour.

5) 500 µl hot phenol:chloroform:isoamylalcohol (25:24:1) (prewarmed at 56-60°C) was added.

6) After being mixed gently for a few minutes, the samples were centrifuged at 13,000 rpm for 5 minutes.

7) Aqueous layer (approx. 500 µl) was transferred to a new 1.5 ml eppendorf tube.

8) 500 µl chloroform:isoamylalcohol (24:1) was added, gently mixed and centrifuged at 13,000 rpm for 5 minutes.

9) The aqueous layer was transferred to a new 1.5 ml eppendorf tube. Step 8 were repeated until the aqueous phase was clear.

10) Approx. two volumes of cold absolute (100 %) ethanol was added and it was then incubated at -20°C overnight.

11) DNA pellets were collected by centrifugation at 13,000 rpm for 15 minutes in 4°C, washed with 70% ethanol or isopropanol and resuspended in 20-50 µl Tris-EDTA (TE) buffer.

12) All DNA were treated with RNAse A (10 mg/ml) at 65°C for 15 minutes to remove RNA from the samples.

13) 300 µl of TE buffer and 300 µl of phenol were added. The tubes were inverted for 5 minutes and centrifuged at 10,000-13,000 rpm for 5 minutes.

14) The aqueous phase were transferred to the new eppendorf tubes and extracted with the equal volume of chloroform:isoamylalcohol (24:1).

15) The tubes were inverted for 5 minutes and centrifuged at 10,000-13,000 rpm for 5 minutes.

16) The supernatant was removed to the new 1.5 ml eppendorf tube. Steps 10-11 were repeated.

19) The DNA quality and quantity were determined using agarose gel electrophoresis by compared with DNA mass ladder (Invitrogen, USA).

3.3.2 PCR amplification of cyanobacterial 16S rDNA

PCR amplification of cyanobacterial 16S rRNA genes from community DNA was performed using the cyanobacteria specific forward primer CYA359F (5′-GGGGAATYTTCCGCAATGGG-3′) (Y is a C/T nucleotide degeneracy) and the reverse primer CYA781R(a) (5′-GACTACTGGGGTATCTAATCCCATT-3′)

with a (GC) 40 clamp added to the forward primer (Nübel *et al*.,1997). The PCR products of the expected size were approximately 460 base pairs.

1) The PCR amplifications followed the protocol of Norris *et al*. (2002). Each reaction was performed in a total volume of 50 µl containing;

- 1.5 mM $MgCl_2$ (Promega)
- 0.2 mM dNTPs (dATP, dTTP, dGTP, dCTP) (Promega)
- 0.5 µM of each Cyanobacteria specific primer (obtained from QIAGEN Operon GmbH, Cologne, Germany)
- Approximately 10 ng template DNA
- 0.1 mg/ml bovine serum albumin (BSA) (Promega)
- 1.5 U Taq polymerase (Promega)
- 1× buffer (Promega)

2) The reaction tubes were placed in Perkin-Elemer thermal cycler (Gene Amp PCR system 2400) and the following PCR amplification cycle profile was used:

- 5 minutes at 94°C

- 30 cycles of 1 minute of denaturation at 94°C, 1 minute of annealing at 60°C, 1 minute of extension at 72°C

- A final extension of 7 minutes at 72°C

3) 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 and the product concentrations were estimated.

3.3.3 DGGE analysis of community DNA

DGGE analysis was performed on a Bio-Rad Dcode system according to the manufacturer. 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) (Table 2).

Table 2. Chemical ingredients of 40 and 60% denaturant concentration

Reagents	**40%**	**60%**
40% acrylamide	20 ml	20 ml
50× TAE	2 ml	2 ml
Formamide	16 ml	24 ml
Urea	16.8 g	25.2 g
H_2O	to 100 ml total volume	to 100 ml total volume

The special gradient maker (Hoefer SG 30 Gradient maker, Amersham Bioscience, Piscataway, New Jersey) was used according to the manufacturer to pour the gel.

Gels were electrophoresed for 17 h at a constant 60 V, 60°C. Gels were stained by using SYBR Green I (Molecular Probes, Eugene, OR, USA) and photographed using GEL DOC 2000 (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

3.3.4 Sequencing of DGGE bands

1. DGGE bands were isolated from DGGE gels by carefully tipping the band of interest with a sterile polypropylene pipette tip several times along the length of the band.
2. The tip was rinsed into a PCR reaction tube containing 10 µl sterile, nuclease-free H_2O.
3. The band was stored overnight at 4°C to allow the DNA to diffuse. The diffused DNA was used for subsequent reamplication or sequencing.
4. Another PCR reaction was done from 2 µl of the band stab to ensure a clean product.
5. The PCR reaction mixtures and the thermocycler program were the same as that used for the initial reactions, although the number of cycles varied from 25 to 30.
6. The quality of the band purification was checked by DGGE and re-amplification was repeated if necessary until a single band of sufficient purity was attained.

7. Another PCR reaction was done from the band stab which gave a clean product, but using the reverse primer CYA781R(a) and a forward primer without the GC-clamp, CYA359Fshort. Reaction conditions were otherwise the same.
8. PCR products were purified using Amicon microcon centrifugal devices (Millipore, Bedford, MA, USA) and used as sequencing templates.
9. Some bands could not be sequenced directly and were cloned using the T/A cloning kit according to the Manufacturer (Fermentas INC., Hanover, MD, USA).
10. Plasmid DNA preparation kit (QIAGEN, Valencia, California) was used to purify plasmid DNA and used as sequencing templates.
11. Sequencing was accomplished using the BigDye TM Terminator and an autometic sequencer 3730xl DNA analyzer (Macrogen Inc., Seoul, Korea).
12. A BLAST search of the GenBank database was done to identify species or strains of closest similarity.

3.3.5 Phylogenetic analysis

An initial BLAST search of the NCBI GenBank database provided candidate sequences from which to compare the relatedness of the cyanobacteria to previously characterized species.

Multiple alignments were created with reference to the selected GenBank sequences using BioEdit version 7.0.0 (Hall, 1999). Gaps or ambiguous positions were omitted from the analysis. Using the remaining informative sites, a phylogenetic tree was constructed from multiplying the aligned nucleotide positions corresponding to bases 359 to 781 of the *Escherichia coli* sequence.

The Neighbor-joining (NJ), Unweighted Pair-Group Method with Arithmetic Mean (UPGMA) and Maximum Parsimony (MP) analysis using the Molecular Evolutionary Genetics Analysis (MEGA) package version 3.1 (Kumar *et al*., 2004) were used to illustrate the relationship of partial 16S rRNA gene sequences to the representative cyanobacteria, where the MP tree branch lengths were estimated using the average pathway method for unrooted trees (Nei and Kumar, 2000).

Phylogenetic trees were inferred by using the neighbor joining (Saitou and Nei, 1987), UPGMA and maximum parsimony tree making algorithms. An evolution distance matrix was generated after Kimura (1980). The topologies of the resultant trees were evaluated by bootstrap analyses (Felsentein, 1985). The tree was rooted using the *Escherichia coli* 16S rRNA sequence as an outgroup. To evaluate the robustness of branches in the inferred tree, one thousand replicates were used for bootstrap resampling, where values at nodes indicate the bootstrap percentages and values less than 50% are not reported.

3.4 Data analysis

The computer statistical package, SPSS for Windows version 11.5 was used to perform statistical analysis: Categorical Principal Components Analysis (CATPCA) of the relationships between cyanobacterial morphotype abundance within each temperature range and season. CATPCA simultaneously quantifies categorical variables while reducing the dimensionality of the data. This technique is most useful when a large number of variables prohibit effective interpretation of the relationships between objects (subjects and units). By reducing the dimensionality, fewer components rather than a large number of variables can be interpreted.

Clustering technique is the study using the unweighted pair group method. Arithmetic averages (UPGMA) were applied to study to cluster of all hot springs within 3 seasons by using environmental parameters. Computation of the environmental parameters matrices was carried out using the Euclidean distance and Steinhaus coefficient, respectively, and UPGMA was selected as a clustering method for the presentation results (Forestier *et al*., 2002).

The Multivariate Statistical Package (MVSP) version 3.1 was used to perform Canonical Correspondence Analysis (CCA). CCA was used to test for relationships between the distribution of the cyanobacterial morphotypes and environmental variables. CCA is a multivariate method widely used in ecological studies to elucidate the relationship between community composition and recorded variation in the environment (Ter Braak, 1986). The resulting ordination diagram of CCA shows the main pattern of the biological assemblages as accounted for by the

environmental variables and the distribution of cyanobacterial morphotypes along each environmental gradient in three seasons of each hot spring. The MVSP was also used to perform Principal Components Analysis (PCA) of the relationships between DGGE banding profile within each temperature range and season. PCA is one of the ordination methods. Mathematically, PCA consists of an eigenanalysis of a covariance or correlation matrix calculated using the original measurement data. Graphically, it can be described as a rotation of a swarm of data points in multidimensional space so that the longest axis (the axis with the greatest variance) is the first PCA axis, the second longest axis perpendicular to the first is the second PCA axis, and so forth. Thus these first few PCA axes represent the greatest variation in the data set and hopefully contain some patterns of significance.