CHAPTER 5

DISCUSSIONS

5.1 Cyanobacterial morphotypic diversity

Cyanobacterial mats collected from six hot springs in Thailand were observed. The 14 distinct morphotypic species of cyanobacteria were found. The most abundant forms in all northern hot springs were *Cyanothece* sp. and *Synechococcus* cf. *lividus* Copeland which dominated the 60–75°C range, and *Phormidium* cf. *boryanum* (Bory ex Gomont) Anagnostidis and Komárek, that dominated the 45–50°C, 50–55°C and 55-60°C ranges. *Phormidium* cf. *boryanum* and *Cyanothece* sp. were not found in any of the hot springs outside of the northern region. However, the most abundant forms in all southern hot springs were *Leptolyngbya* sp. and *Mastigocladus* cf. *laminosus* Cohn, which dominated the 40-60°C range. In central hot spring, the most abundant form was *Phormidium* sp., which dominated in lower temperatures (40-50°C). The modifier "cf.," meaning "carried forward," between the genus and species names indicates that the *Synechoccocus* cf. *lividus* Copeland in question resembles that described initially by Copeland on the basis of morphology (Castenholz, 1973; Castenholz, 1981; Ward *et al*., 1998). Cyanobacterial morphotype diversity in northern hot springs is more diverse than central and southern hot springs, because their hot spring waters have more range of temperature or higher temperatures than three other hot springs, the northern hot springs have relatively larger sizes than central and southern hot springs and some hot springs are natural environments, Human activities may not have disturbed or disrupted the habitats, especially in Pong Dued and Theppanom Hot Springs.

5.2 Plasticity of the hot spring

Physical disturbance of the mat can create gaps in the cyanobacterial mat, providing windows of opportunity for colonist species (Ward *et al*., 1998). Fluctuation in the temperature or discharge volume from the spring in the weeks or months preceding the sampling is another kind of disturbance that would vary the spatial

position of physical and chemical gradients within the stream and thus influence the diversity and abundance of the mat forming cyanobacteria (Sompong *et al*., 2005).

All the hot springs are accessible to the public, some are popular tourist destinations and some are used as cooking resources such as boiling eggs or bamboo shoots by the local village communities. Therefore, human activities may have caused disturbance or disruption to the mats. The sampling design did not identify the extent to which human activities altered the physical environment and contributed to the diversity of the mat community.

An important physical factor, temperature, was pre-eminent in determining the distribution of common mat forming thermophilic cyanobacteria in the hot springs of Thailand (Sompong *et al*., 2005). Although, the chemical environments in the hot springs were different, not many chemical variables were the significant determinant of the distribution, except for conductivity which was high and significantly correlated to *Mastigocladus* cf. *laminosus* distribution (Figure 33). Although dissolved sulfide, sulfate and inorganic nitrogen forms in SK Hot Spring were high, they were not high significant determinant of the cyanobacterial diversity. Nutrient availability is usually an important factor of species diversity and success in aquatic systems but this was not highly significant in the hot springs because the ground water sources contained adequate nitrogen and phosphorus for cyanobacterial requirements.

The species composition in the six hot springs of Thailand was compared to determine whether geographic isolation at this regional scale was influencing the mat communities. The multivariate analyses showed that the distribution of sites in a multidimensional space was dominated by the taxonomic diversity of each site and the taxonomic diversity was predominantly controlled by the thermal tolerance of the cyanobacteria.

Because the springs were geographically separated and their water chemistries differed, the distribution of these common taxa along the temperature gradient is a useful predictor of thermal tolerances of these common taxa. Therefore, the combination of morphotypes and the thermal tolerance data is useful to identify which

seem to occupy broad ecological niches and potential to disguise multiples genotypes with more restricted tolerances.

The morphological characterization of thermophilic cyanobacterial taxa can disguise several genotypes which have specific environmental adaptation abilities; the genotypic differentiation of *Synechococcus* cf. *lividus* reported by Miller and Castenholz (2000) for temperature and by Ward *et al*. (1998) for pH, are examples. The regional differences in morphotype diversity suggested the influences of geographical isolation, geological age and human activity on species disturbance in these hot spring environments may be examined more fruitfully using sensitive genetic tools. The sensitive genetic methods are necessary to unequivocally identify whether cyanobacteria are globally distributed or endemic populations (Ward *et al*., 1998; Papke *et al*., 2003).

5.3 Molecular diversity of thermophilic cyanobacterial 16S rRNA genes

A total of 55 clones of cyanobacterial enrichment cultures from six hot springs were extracted and sequenced. *Synechococcus* spp., *Chroococcidiopsis* spp., *Leptolyngbya* spp., *Oscillatoria* spp., *Mastigocladus* spp., *Scytonema* spp. and *Phormidium* spp. were conspicuous in all samples. Many clones are very morphologically similar or identical, in the case of the largely morphologically-defined genus *Phormidium, Oscillatoria* and *Scytonema*. It is often difficult to be confident of their diagnosis. However, it is unsound to assume that morphologically similar cyanobacteria are the same genotype at the species or genus level, such as unicellular *Synechococcus*. Not all of the cyanobacteria could be grown in the medium used, such as other strains of *Synechococcus, Cyanothece* and *Phormidium* cf. *boryanum*, because they are uncultivated species. The explanation is an inability to understand and reproduce the real microenvironmental niches defined by physiochemical and also biotic features, e.g. in symbiotic relationships which influence naturally occurring cyanobacteria (Ward *et al*., 1998). Cyanobacterial DNA from natural mats were also obtained from each hot spring in each temperature range and season to study community diversity. To obtain a better understanding of the role of microbial diversity in the maintenance of ecosystems, 16S rRNA genes were used to explore the

microbial diversity and to analyse the structure of microbial diversity (Olsen *et al*., 1986; Amman *et al*., 1995, Muyzer, 1999). Therefore, the genotypic differentiation such as *Synechococcus* cf. *lividus* in this study could be revealed by using cyanobacterial 16S rDNA gene sequences.

A 16S rRNA gene diversity was detected in bacteria and archaea in sulfide-rich hot spring microbial mats with neutral to alkaline pH (Skirnisdottir *et al*., 2000). Bacteria that thrive in such springs seem to vary depending on the sulfide concentration, pH, temperature and other chemical and physical factors. The molecular diversity analysis showed that bacterial diversity was lower in the sulfur mat than in the *Chloroflexus* (low-sulfide) mat. The majority of all sequences from mats showed more than 95% similarity to currently known sequences.

Recently, Kanokratana *et al.* (2004) detected cyanobacterial species in the Bor Khlueng Hot Spring (Ratchaburi, Thailand) from their 16S rDNA clone library. One of a single clone (PK13), distantly related (89% sequence identity) to *Nostoc entophylum* strain IAM M-267, was found. This low cyanobacterial diversity found in the Bor Khlueng Hot Spring is somewhat unusual because cyanobacteria are prominent components of the biota in geothermal habitats (Ward and Castenholz, 2000).

Hybridization techniques using specific 15-20 nucleotide probes were more appropriate for studying population dynamics, but the probes rely on sequence data and were either too specific, targeting only one particular population, or too general, overlooking closely related but ecologically different populations. In particular, cloning and sequencing of rRNA genes have been very useful for describing the compositions of microbial assemblages (DeLong, 1992; Fuhrman *et al*., 1992). However, analysis of clone libraries is time-consuming, simply because it is too laborious, expensive and not suitable when many different samples are analyzed. For example, in studies focusing on changes in microbial assemblages exposed to a perturbation or on how the microbial composition changed along environmental gradients, such as depth in water column, gradients across oceanographic features, or temporal changes with different time scales (Diez *et al*., 2001). Fingerprinting techniques such as DGGE, offer the best compromise between the number of samples processed and the information obtained. It provided both rapid comparison data for many communities and specific phylogenetic

information derived from excised bands (Muyzer *et al*., 1997). Three different approaches; DGGE, terminal restriction fragment length polymorphism (T-RFLP) and gene cloning were compared to quantify the relative levels of several marine picoeukaryote populations in one sample by Diez *et al*. (2001). They used three different primer sets and different PCR protocols. It was found that the relative levels of rDNA were different within three approaches. Moreover, differences in community structure could be easily discerned with both DGGE and T-RFLP analysis. More importantly, the DGGE method allowed us to discover populations of cyanobacteria that could not be confidently identified by cloning (Ferris *et al*., 1996b).

From these reasons, DGGE analysis was used to characterize those species of cyanobacteria mats from hot springs. DNA from enrichment cultures and from natural samples were identified using DGGE. The result was the simultaneous detection of many individual 16S rDNA molecules as a profile of bands, each of which could be reamplified and sequenced. DGGE results have revealed that 16S rDNA gene distributions change along the thermal gradient. Studies of DGGE application of cyanobacterial hot springs in other countries were done since 10 years ago, Ferris *et al*. (1996b) studied DGGE of PCR-amplified 16S rRNA gene fragments to profile the distribution of microbial populations inhabiting regions with different temperatures in a hot spring cyanobacterial community of Octopus Spring, Yellowstone National Park, USA. Different profiles were found for samples from sites with different temperatures indicating different populations. The distributions of several cyanobacterial populations compared with results obtained previously by oligonucleotide probe analyses and suggest that adaptation to temperature has occurred among cyanobacteria which are phylogenetically very similar.

Moreover, DGGE was used to evaluate seasonal distributions of bacterial populations along thermal gradients in a hot spring microbial mat of Octopus Spring, Yellowstone National Park (Ferris and Ward, 1997). Similar DGGE patterns were found for samples collected at the same site and for sites with the same temperature, regardless of the season. However, different profiles were seen for samples from sites with different temperatures. When compared cyanobacterial populations along the seasonal intervals, the temperature ranges at sampling sites were quite constant seasonally, even

though the ambient temperature was 0 and 25ºC during winter and summer sampling times, respectively. The stability in community composition at one site was consistent. Many conserved bands still found across multiple ranges or intervals with only one type that was not detected in all seasons. Overall, the order of sequence types along the thermal gradient was consistent throughout each seasonal series. Their DGGE results were shown similar with the study of DGGE of hot spring cyanobacterial communities of Thai Hot Springs, even though the ambient temperature was quite similar in all season.

The population of unicellular cyanobacteria (*Synechococcus*) ecotypes of a microbial mat in a hot spring effluent channel in Yellowstone National Park, Wyoming, was also examined (Ferris *et al*., 2003). Patterns of variation at the internal transcribed spacer locus separating 16S and 23S rRNA genes were used to suggest the existence of closely related but genetically distinct populations corresponding to different functional populations occurring at different depths. However, neither DGGE nor cloning analysis of PCR-amplified 16S ribosomal DNA (rDNA) fragments from the mat's surface and subsurface environments revealed a pattern consistent with vertical stratification of cyanobacterial genotypes.

The DGGE pattern could not be taken directly as a profile of community composition because of the occurrence of heteroduplex bands formed as artifacts from the reannealing of closely related single strands. More bands than actual populations were expected. The formation of heteroduplex bands also indicated that PCR amplification was non quantitative as a result of interference from template-primer binding by template-template interactions (Suzuki and Giovannoni, 1996). Therefore, it could not be presumed that DGGE band intensities reflect true gene abundances. However, it could be believed that the detected sequences were from dominant members of the mat community and that the appearance or disappearance of bands in DGGE profiles indicated large-scale (order-of-magnitude or greater) increases or decreases in the densities of these community members along the thermal gradient (Ferris and Ward, 1997).

DGGE, like cloning and sequencing (Ward *et al*., 1994), detects the occurrence of sets of phylogenetically related populations as a consistent pattern of community

structure. In the case of cyanobacterial populations, which are both phylogenetically and physiologically related, it was suggested that evolutionary specialization (e.g., temperature adaptation) may have led to the guild structure observed in the mat community (Ferris *et al*., 1996a). As with all methods used to study microorganisms in nature, DGGE is not without limitations. Obviously, there are many possible reasons why all populations might not be detected (Ward *et al*., 1992), and limited sequence data do not permit a robust evaluation of phylogenetic relationships of the sequence types detected. Still, DGGE offers a rapid means of detecting predominant populations which are PCR amplifiable. Because DGGE bands are sequenced directly, cloning is eliminated because it may cause bias (Rainey *et al*., 1994). Furthermore, as separation of different sequences is achieved by electrophoresis, laborious screening necessitated by redundancy of clones in a library is also eliminated. Thus, the increase in the number of samples that can be evaluated and the ability to simultaneously detect many populations enable a more effective pursuit of the ecology of native cyanobacteria.

5.4 The need for a polyphasic characterization

From the analysis, it has been demonstrated that significant components of cyanobacterial biodiversity can be underestimated when a single method for community description is used. A polyphasic study of the cyanobacterial communities growing on different soil desert crusts from Arches National Park, USA, were studied by Garcia-Pichel *et al*. (2001). They combined the use of environmental 16S rRNA gene analysis, microscopy and cultivation to characterize their cyanobacterial components and to prove the importance of the soil substrate in determination of community structure. The results showed that significant differences in community structure were found among soil types, indicating that soil characteristics may select for specific cyanobacteria and they could be provided an excellent classification. Study on cyanobacterial associated with hot spring_travertines ($CaCO_3$ deposition) was made by Pentecost (2003). He identified fossilized cyanobacteria using microscopy. However, he mentioned that the classical taxonomy (morphology) combined with molecular taxonomy will provide a superior classification.

Recently, similar observation was reported by Ballot *et al*. (2004) for cultured cyanobacterial strains of species of some Oscillatoriales from Kenyan and Indian

waterbodies. The investigation of a polyphasic approach including morphological characters and molecular sequence analysis of 16S rRNA gene, ITS region and PC-IGS locus was carried out. The results demonstrated that several distinct morphotypes may be genetically similar and vice versa.

When compared with this studied, microscopy clearly underestimated the diversity of morphological simple, unicellular, *Synechococcus*-like and filamentous, *Leptolyngbya*, *Phormidium* and *Oscillatoria*-like cyanobacterial forms. Molecular methods of DNA analysis, by contrast, failed to detect the presence of heterocystous cyanobacteria, *Scytonema* sp., which were conspicuous on microscopic observation. This was probably not due to a failure in the PCR amplification, or in the DGGE steps. Their extracellular sheath investments, which provide protection from high temperature and excessive UV radiation, probably prevented efficient extraction of their nucleic acids, even though the tougher disruption treatments may also result in shear damage to the DNA and consequently reduce the overall sensitivity of the procedures (Garcia-Pichel *et al*., 2001).

5.5 Phylogenetic analysis of the cyanobacterial 16S rDNA

A perfect match of the tree topologies cannot be expected (Rossello-Mora and Amann, 2001). No method is ideal for all performance criteria or the best for all circumstances. The method of choice depended both on the size and complexity of the data set (Hall, 2001). Some of the criteria that have been considered are efficiency, robustness, computational speed, and discriminating ability (Hillis *et al.*, 1996). Bootstrapping method was used to assess the reliability of the groupings (clades) in the tree. Bootstrapping involves repeatedly creating trees from a subsample of sites in the alignment and determining the fraction of the time the same clade appear. A minimum of 100 bootstrapping replicas was recommended, with 1000 preferred. However, it would take too long if this was created with the maximum likelihood method, although it would require even 100 replicates to bootstrap (Hall, 2001).

Sets of phylogenetically related cyanobacterial populations have also been observed in 16S rRNA studies of several other habitats and might reflect the generality of such an ecological basis for the evolution of microbial biodiversity. The phylogenetic

analysis of the 16S rDNA sequences and the morphological microscopy analysis of these cyanobacteria clearly displayed differences in biodiversity between northern and southern hot springs. However, hot springs with maximum temperatures above 60°C occur only in the Northern region which makes these differences less meaningful. There were clear geographic boundaries. However, some lineages occurred only at lower temperatures (e.g. *Calothrix* cf. *thermalis* and *Scytonema* cf. *coactile* in the northern region only). *Synechococcus* lineage C which was only found in northern hot spring mats is phylogenetically distinct from the *Synechococcus* spp. within lineages A and B (Fig. 48b). Phylogenetic trees of *Synechococcus* isolates from Oregon Hot Spring (Oregon, USA) were constructed by Miller and Castenholz (2000). They combined a comparative physiological approach with phylogenetic analyses to study the evolution of thermotolerance *Synechococcus*. The topology of *Synechococcus* clade is consistent for all algorithms and is strongly supported by bootstrap analysis data in all cases. Sequence groups II, III and IV are part the larger clade which includes *Synechococcus* sp. strains C9 and SH-94-5 which were also found to share a high level of similarity with *Synechococcus* spp. in this study. Another observation of cyanobacterial in hot spring was done by Ballot *et al*., 2004. They constructed phylogenetic trees of three genera of Oscillatoriales isolates from Kenyan Hot Spring by using sequence of partial 16S rRNA gene and other regions. A phylogenetic tree of 16S rDNA showed a tight cluster of the *Arthrospira* strains (AY575923-AY575932) and a closely related *Phormidium* cf. *terebriformis* strain (AY575933). Despite their differences in morphology and habitats, all sequences of Kenyan *Arthrospira* strains are grouped together.

It should be noted, that even where near-complete 16S rRNA gene sequences have been used, conflicts between morphological and molecular identification of some cyanobacterial sequences may be found (Hongmei *et al*., 2005). From the studied by Fox *et al*. (1992), they found that 16S rDNA sequence data are not sufficient to absolutely identify species. A 99.5% sequence similarity among three strains of *Bacillus* were clearly shown to fall into three different species based on DNA–DNA hybridization experiments. When strains have <97.5% similarity, the sequence data provide strong evidence that the strains are separate species. For strains with >97.5%

similarity, the molecular data are simply uninformative for making decisions at species level. However, many researchers have used values of >97.5% as criteria for collapsing strains into a single species (Palinska *et al*. 1996), even though this has clearly been shown to be arbitary (Casamatta *et al*., 2005).

In addition, many of the cyanobacterial sequences found in this study were generally quite different from the sequences found in the public databases. This is important since many of the hot spring sites are open to the public and have become locations for tourism as well as places the local people use for cooking. Many sequences may represent new species. Further taxonomic characterization is needed to clarify their exact taxonomic status. Since many of the sequences from this study are different from those recovered from other previously studied regions of the Earth, it would be important to study the human impact on these sites and possibly enact measures to preserve this natural resource.