RESEARCH ARTICLE

Morphological and phylogenic diversity of cyanobacterial populations in six hot springs of Thailand

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ABSTRACT: To characterize the phylogenetic and morphological diversity of cyanobacteria in Thailand, cyanobacteria were collected from mats in six hot springs with water temperatures ranging from 40 to 75 °C. Samples were collected for culture isolation, microscopic morphological examination, and molecular analysis of the 16S rRNA sequence to establish the levels of morphological versus genomic diversity. Fourteen cyanobacterial morphotypes were investigated using microscopy with the dominant species of cyanobacteria being *Synechococcus* spp., *Phormidium* cf. *boryanum* and *Leptolyngbya* spp. A total of 20 distinct isolates of cyanobacteria were grown in culture medium, and an additional 79 samples were examined using denaturing gradient gel electrophoresis and DNA sequence analysis to establish phylogenetic relationships. The observed decrease in morphological diversity with increasing water temperature was found to be compensated for by an increase in molecular sequence diversity of the morphologically indistinguishable cyanobacterial species. Molecular clines were found to span both temperature and geophysical boundaries for samples from the northern and southern Thailand regions.

KEYWORDS: thermophilic cyanobacteria, DGGE, hot springs, Thailand, phylogenetics

INTRODUCTION

As the only oxygenic photosynthetic bacteria, the cyanobacteria are of interest due to their ability to grow in high temperatures and in other extreme environments. In addition, cyanobacteria are among the most versatile photosynthetic organisms on earth since they occur abundantly in virtually all known photic habitats, and can thrive in markedly extreme levels of humidity, light, salinity, temperature, availability of oxygen or carbon dioxide, and solar radiation[1]. Classifying these bacteria though has presented difficulties due to the inability to culture some taxa and the limited range of morphological features upon which these bacteria could be distinguished. In this study we examine the morphological and phylogenetic diversity of cyanobacteria in six hot springs of Thailand using molecular methods to obtain 16S rRNA gene sequences from both culturable and unculturable isolates. The high-temperature environments most useful for these kinds of studies are those associated with volcanic activity, such as in most hot springs, since these natural habitats have probably existed throughout most of the time in which organisms have been evolving on earth and present a stable niche in which such organisms could evolve[2–4].

Thermophilic cyanobacterial mat communities occur in geothermal springs of neutral/alkaline pH and at temperatures of up to ~ 74 °C. Mat community composition is largely temperature-defined, and mats have been clearly differentiated on the basis of the cyanobacterial taxa[5]. The main problems in applying morphological criteria in cyanobacterial classification arise from morphological features that vary with environmental conditions[6]. Sometimes microscopy and enrichment cultures have limited usefulness since distinct species of cyanobacteria can share similar simple morphological and cultivation limitations[7]. One way to better characterize the phylogenetic relationships between these morphologically similar species is to use molecular diversity information. The development of techniques for the analysis of 16S rRNA sequences in natural samples has already greatly enhanced detection and identification of cyanobacteria in nature[6,8–10]. 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[11]. Denaturing gradient gel electrophoresis (DGGE) analysis of PCR-amplified 16S rRNA gene segments has also been used to profile microbial populations inhabiting different temperature regions in the cyanobacterial mat community. The use of DGGE reveals the phylogenetic relationship of all community members, thus allowing the identification of unique strains[3]. Studies using 16S rRNA gene data from environmental samples and cultures have demonstrated that genotypic diversity can far exceed phenotypic diversity estimated by observation and culture techniques[12]. Although a few locations worldwide have been studied, distinct phylogeographic groups have been extensively studied only in the continental USA, Iceland, New Zealand, and some countries in Asia[11,13,14]. To date, studies of the cyanobacterial mats in hot springs in Thailand have been minimally investigated[11,15–17].

Here, we report the results of a polyphasic study of the cyanobacterial communities in six hot springs in Thailand. For this study, we combined the use of environmental 16S rRNA gene analysis and microscopic examination of culture isolates to characterize cyanobacterial diversity within some of the hot springs of Thailand.

MATERIALS AND METHODS

Sampling

Cyanobacterial samples were collected from 6 hot springs in San Kamphaeng, Pong Deud, Theppanom, Pra Rueang, Raksawarin Public Park, and Khaochaison (Fig. 1 and Table 1). The physico-chemical properties of the water from each sampling site were measured each visit[18], and the physico-chemical ranges for each spring are summarized in Table 2. At each hot spring, the site was surveyed to characterize the temperature profile range, and water
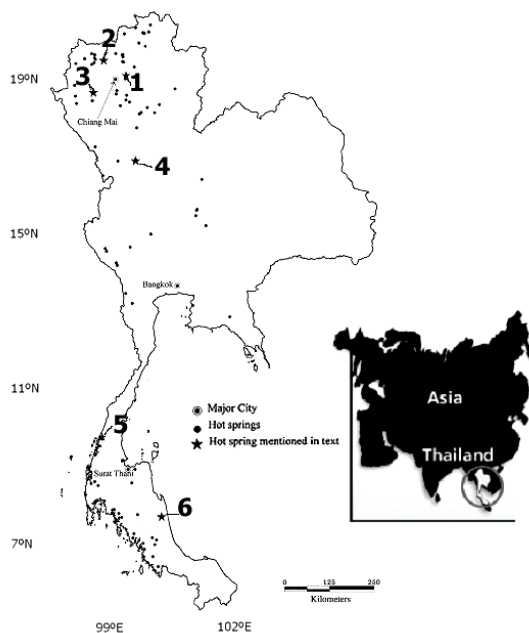

Fig. 1 The hot spring districts in Thailand after Raksaskulwong[38], showing the six locations sampled (the spring descriptions are listed in Table 1).

1. San Kamphaeng, Chiangmai (SK)
2. Pong Dued, Chiangmai (PD)
3. Theppanom, Chiangmai (TP)
4. Pra Rueang, Kamphaeng Phet (KP)
5. Suan Raksawarin, Ranong (RN)
6. Khaochaison , Phatthalung (PL)

temperature clines were established in the range of 40–75 °C using 5 °C intervals. Observation and collection of cyanobacteria samples were carried out every 4 months for one year between the 10 a.m. and 2 p.m. Samples for culture isolation and microscopic examination were picked with sterile forceps and spectula from the sampling sites and were then added to sterile polyethylene tubes. Samples for DGGE analysis 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. The samples used for molecular analysis were placed in ice or dry ice during transportation, and at the Chiangmai University laboratory all samples were stored at –20 °C for a maximum of two months before being analysed[8].

Morphological Classification

Morphological classification of natural and cultivated cyanobacteria was based on characters observable under a light microscope (400–1,000×), using an Olympus CH30RF200 compound microscope.

Table 1. Site descriptions and characteristics for each spring

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

Table 2. Physical and chemical properties of the hot spring waters

Spring	Air temp. °C	Max. temp. °C	pH	Conductivity Us.cm^{-1}	Total alkalinity mg.l^{-1}	Hardness mg.l^{-1} CaCo$_3$	Ca mg.l^{-1}	Mg mg.l^{-1}	Iron mg.l^{-1}
SK	32-34	64-67.5	8.0-8.5	752-1,120	253-260	160	24.5	31.5	<0.005
PD	28-30	67-68	8.0-9.3	528-530	143-154	160	64.1	34.2	<0.005
TP	30-32	67-72	8.4-9.0	536-674	188.5-222	160-196	22.5	25.6	<0.005
PL	26-36	51-51.5	7-7.8	474-1,740	189-222	170	48.9	11.7	<0.005
RN	30.5-32	56-58	7-7.4	490-2,190	132-142	63	20.4	3.0	<0.005
KP	27-34	46.5-50	7.5-8.3	539-788	246.5-264	28	6.0	3.2	<0.005

Spring	SRP mg.l^{-1}	NO3- mg.l^{-1}	NH4+ mg.l^{-1}	Sodium mg.l^{-1}	K mg.l^{-1}	Cl^{-1} mg.l^{-1}	Sulphides mg.l^{-1}	Sulphate mg.l^{-1}
SK	0.12-0.26	6.3-25.9	0.72-2.38	125.69-132.14	9.94-11.30	10	0.011-6.02	16-220
PD	0.28-0.95	0.6-1.6	nd-0.01	76.43-91.89	4.03-4.44	10-10.4	0.003-0.036	21-27
TP	0.41-1.68	0.8-13.6	nd-0.03	88.46-128.75	3.67-5.24	11.6-12.4	0.013-0.09	4-34
PL	0.18-0.34	1.3-1.4	0.13-0.21	25.93-28.13	1.71-2.29	6.8-10.1	nd-0.18	1-14
RN	0.16	1.1-1.3	0.15-0.16	47.04-65.05	2.57-3.07	8.4-8.8	0.001	24-28
KP	0.06-0.25	0.7-1.0	nd -0.08	114.07-147.25	3.85-5.11	20.1-22.8	nd	16-18

Morphologies of cyanobacteria were recorded using drawings and photomicrographs. References by Desikachary[19], Anagnostidis and Komarek[20–22], Hoffmann[23], Kovacik[24], Komárek and Anagnostidis[25,26] and Castenholz[27] were used for classification.

Sample cultivation and isolation

Small amounts of the cyanobacterial samples were dispersed by a tissue grinder into a set of tubes each of which was diluted serially into 8–10 tubes (dilution to extinction) with medium D or ND[28]. The most diluted tubes which showed positive growth were used as an indicator for the most abundant culturable cyanobacterial taxon. The successful cultures were inoculated into flasks containing medium D, ND or spread on agar-solidified plates using the same medium. Culture purification and cloning used streak plate dilution or whole filament isolation. All tubes, flasks and plates were incubated in photochambers at 40, 50 and 58 °C under fluorescent lamps (~3,000 lux)[28]. When cyanobacterial growth or movement was observed, one single filament or colony was transferred to 10 ml of fresh medium in a tube and these tubes were then incubated under the same conditions for 7 days or until growth occurred. This clonal culture was then transferred to 75 ml of fresh medium in 125 ml flasks.

DNA extraction

The bulk DNA of each cyanobacterial mat from the environmental samples and of each culture isolate was extracted by modification of the hot phenol method[29]. After centrifugation of the samples,

500 µl of lysis buffer containing CTAB[30] with lysozyme and Proteinase K were added to each tube. The tubes were then incubated for 1 h at 60 °C, after which 500 µl of hot phenol/chloroform/isoamylalcohol (25:24:1 prewarmed at 56–60 °C) was added. After mixing gently for a few minutes, the samples were centrifuged for 5 min at 14,000 rpm. The supernatant was then transferred to a new tube and 500 µl of chloroform/isoamylalcohol (24:1) was added. After a final gentle mixing and centrifugation for 5 min at 14,000 rpm, the supernatant was precipitated in absolute ethanol and the pellet washed with 70% ethanol and resuspended in 20–50 µl Tris-EDTA buffer. All DNA from the environmental samples were treated with RNAse to remove RNA from the samples.

PCR amplification of cyanobacterial 16S rRNA

For both DGGE and isolate DNA amplification, the 16S rRNA gene regions, approximately 400 bp in length, were amplified by PCR using the cyanobacteria-specific primers CYA359F and CYA781R(a)[9]. For the DGGE PCR amplification a (GC) 40 clamp was also added to the forward primer. Each reaction contained 1.5 mM MgCl$_2$, 0.2 mM dNTPs, 0.5 µM of each primer, approximately 10 ng template DNA, 0.1 mg/ml bovine serum albumin, 1.5 U *Taq* polymerase and 1×buffer (Promega) in a total volume of 50 µl.

For both the DGGE and cultured isolates, the PCR amplification cycle using the cyanobacterial primers 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.

DGGE analysis of community DNA

DGGE was performed on a Bio-Rad Dcode system. An 8 % polyacrylamide gel (Promega) with a linear denaturant concentration from 40 to 60 % was used (where 100 % denaturant contains 7 M urea and 40 % (v/v) formamide) and 100–120 ng of template was used in each lane. Gels were electrophoresed for 17 h at a constant 60 V. Bands were stabbed with a pipette tip, rinsed into a PCR reaction tube containing 10 µl sterile, nuclease-free water[10]. Then, another PCR reaction was done from the band stab to ensure a clean product, using the reverse primer and a forward primer without the GC-clamp. Reaction conditions were otherwise the same. PCR products were purified using Amicon microcon centrifugal devices (Millipore, Bedford, MA, USA) and sequenced. Sequencing was accomplished using the BigDye TM Terminator and an automatic sequencer 3730xl DNA analyzer (Macrogen Inc., Seoul, Korea). Some bands could not be sequenced directly and were cloned using the T/A cloning kit (Fermentas INC., Hanover, MD). A BLAST search of the NCBI GenBank database was then performed to identify species or strains of closest similarity.

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 alignments were then created with reference to the selected GenBank sequences using BioEdit version 7.0.0[31] which implements the ClustalW multiple alignment algorithm[32]. Alignment positions at which one or more sequences had gaps or ambiguities were omitted from the analysis. Using the remaining informative sites, a phylogenetic tree was constructed from the 422 multiple aligned nucleotide positions corresponding to bases 359 to 781 of the *Escherichia coli* sequence. A maximum parsimony (MP) max-mini branch-and-bound analysis using MEGA 3.1[33] was used to illustrate the relationship of partial 16S rRNA gene sequences for the representative cyanobacteria, where the MP tree branch lengths were estimated using the average pathway method for unrooted trees[34]. The tree was then 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 from which the overall MP consensus trees were generated. Subgroups with greater than 50 % consistency in the consensus tree are labelled at the respective nodes.

RESULTS AND DISCUSSION

Cyanobacterial morphotypes

In this study, 14 distinct morphotypic species of cyanobacteria were characterized using microscopy (Fig. 2). The mat samples from Pong Dued (PD) hot spring showed the most diverse morphotype assemblages, followed by those of Theppanom (TP), San Kamphaeng (SK), Raksawarin Public Park (RN), Khaochaison (PL), and Pra Rueang (KP), in that order (Table 3). The most abundant forms in all northern hot springs (SK, PD and TP) were *Cyanothece* sp. (Fig. 2D) and *Synechococcus* cf. *lividus* (Fig. 2C), which dominated the 60–75 °C range, and formed a thin brown green to yellow green mat on the surface of the flowing hot spring waters. *Phormidium* cf. *boryanum* (Fig. 2I), which occurred in all temperature intervals up to 60 °C and dominated the 45–50 °C,
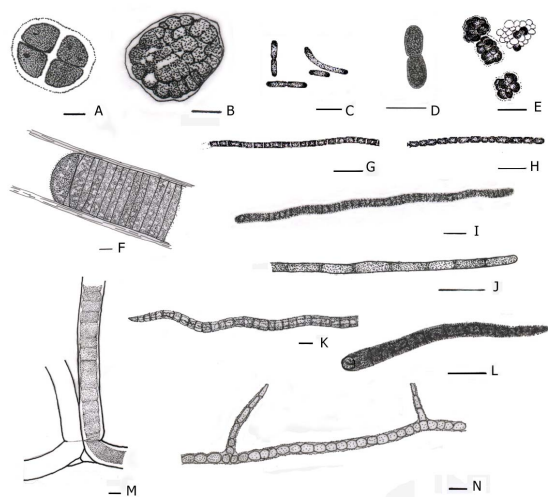

Fig. 2 Cyanobacterial morphotypes from natural samples in six hot springs of Thailand (A-D) Chroococcales; (A) *Chroococcus* sp., (B) *Cyanosarcina* sp., (C) *Synechococcus* cf. *lividus* Copeland, (D) *Cyanothece* sp., (E) *Pleurocapsales; Chroococcidiopsis* sp., (F-I) Oscillatoriales; *Lyngbya* sp., (G) *Leptolyngbya* sp., (H) *Pseudanabaena* sp., (I) *Oscillatoria* sp., (J) *Phormidium* sp., (K) *Phormidium* cf. *boryanum* (Bory ex Gomont) Anagnostidis and Komárek, (J-N) Nostocales; (L) *Calothrix* cf. *thermalis* (Schwabe) Hansgirg, (M) *Scytonema* cf. *coactile* Montagne, (N) Stigonematales; *Mastigocladus* cf. *laminosus* Cohn. The images were drawn by one of us (Udomluk Sompong) using fresh materials examined with a 400× and 1000× magnification compound microscope. (scale bars: 10 µm)
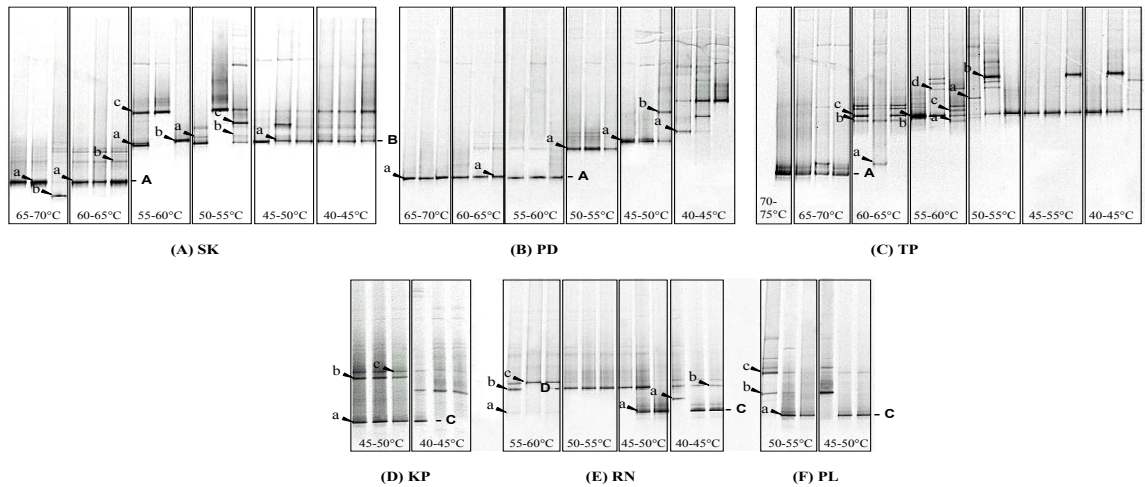
Fig. 3 DGGE banding patterns of 16SrRNA gene-defined diversity among thermophilic cyanobacterial mats in six hot springs of Thailand. The first lane of each temperature range contains cyanobacterial mats from the rainy season, the second lane is from the winter season, and the third lane is from the summer season. Arrowheads to the left of the band indicate positions in the gradient at which defined bands were excised. For each temperature range, at each site, excised bands are labelled as lower case letters (abcde). Conserved bands found across multiple temperature ranges or intervals are labelled with capital letters (ABCD).

50–55 °C, and 55–60 °C ranges in Pong Deud, San Kamphaeng and Theppanom Hot Springs, formed a dull green mat on the top of the surface[17]. *Phormidium* cf. *boryanum* was not found in any of the hot springs outside of the Chiangmai region.

For the enrichment cultures, not all of the cyanobacteria could be grown in the medium used. A total of 55 clones of cyanobacteria were grown. *Synechococcus* spp., *Chroococcidiopsis* spp., *Leptolyngbya* spp., and *Phormidium* spp. were conspicuous in all samples, but only 20 clones were found to contain distinct isolates. Some of the other dominant species such as other strains of *Synechococcus* and *Phormidium* cf. *boryanum* were not successfully cultured and therefore were identified using DGGE.

Molecular diversity of cyanobacterial 16S rRNA genes

To characterize those species of cyanobacteria that failed culture attempts in the lab, we studied the 16S rRNA gene-defined community diversity in cyanobacterial mats from the six hot springs, using DGGE. A representative sample of DGGE separation of bulk cyanobacterial 16S rRNA is presented in Fig. 3. Bands that migrated to the same position in the DGGE gel and displayed no ambiguous differences in nucleotide sequences were considered to represent unique 16S rRNA sequence types. Occasionally, some bands appeared to be heteroduplex molecules. Such bands form during mixed-template PCR when

annealing occurs between similar but non-identical products[35]. The original heteroduplex products may then have reformed, migrating higher in the gradient, since base pair mismatches weaken hydrogen bonding between the double strands[36].

A total of 79 samples of DGGE amplified by PCR from environmental DNA samples were used with 38 distinct bands then reamplified and sequenced. Most DNA samples were sequenced directly from excised bands, but some samples that generated ambiguous sequences were cloned before sequencing. Two major bands, intense band A (Fig. 3) from the high temperature intervals and band B from the moderate temperature intervals were conspicuous in three northern hot springs (SK, PD and TP). For two of the other major bands, intense band C was conspicuous in only the KP and PL hot springs and band D was conspicuous in only the RN hot spring.

Temperature and spatial distribution of sampled bacteria

Twelve taxa were restricted to the temperature range below 60 °C, while six of these were restricted to temperatures below 50 °C (Table 3). At this lowest temperature range *Scytonema* and *Calothrix* dominated most likely because these species are known to have the lowest thermal tolerance[17]. In addition, there was a geographic distribution of thermophilic cyanobacteria with only four species, *i.e. Cyanosarcina* sp. (Fig. 2B)*, Chroococcidiopsis* sp. (Fig. 2E), *Synechococcus* cf. *lividus* (Fig. 2C) and *Leptolyngbya* sp.

Table 3. Cyanobacterial morphotypes present in each range of temperatures at different hot springs

Cyanobacterial morphotypes	Bacterial Abundance																											
	SK						PD							TP							KP		RN				PL	
	40-45	45-50	50-55	55-60	60-65	65-70	40-45	45-50	50-55	55-60	60-65	65-70	70-75	40-45	45-50	50-55	55-60	60-65	65-70	70-75	40-45	45-50	40-45	45-50	50-55	55-60	45-50	50-55
Chroococcus sp.	+						++	++						+									+					
Cyanosarcina sp.	+							+						+							++	+		+				
Chroococcidiopsis sp.	++	+					++	++	++	+	+			+++	+	++					++	++	+	++	++		+	+
Synechococcus lividus Copeland	+	+	+++	++	++	+		+	++	+++	+++	++	+		+	++	++	+++	++	+		++	+	++	++	+++	+	++
Synechococcus sp.		+	++	+++	+++	+++			++	+++	+++	+++	++		+	++	+++	+++	+++									
Lyngbya sp.							+++	++						+														
Leptolyngbya sp.	+						+	+						++	+							+	++	+++	++	+++	+++	+++
Oscillatoria sp.	++																											
Pseudanabaena sp.	+	+					++	+							+	++											++	
Phormidium boryanum (Bory ex Gom.)Anagn. et Kom.	+++	+++	+++	+++				++	+++	+++				+++	+++	+++												
Phormidium sp.	+								+	+				++	+	+					+++	+++	+++	+	+	+		++
Calothrix thermalis (Schwabe) Hansg.							+++	+++						++														
Scytonema coactile Montague							++							++														
Mastigocladus laminosus Cohn	+							+															+++	+++	++	++		

(Fig. 2G), occurring in all six hot springs.

Essentially all banding patterns were similar from different seasons within the same springs with the possible exceptions being Pong Dued (PD) at 40–45 ºC, Theppanom (TP) at 55–60 ºC and Khao-chaison (PL) at 45–50 ºC. Due to this homogeneity, all seasonal data was pooled for each hot spring.

Phylogenetic Analysis of the cyanobacterial 16S rRNA

All successfully sequenced 16S rRNA gene sequences (Genbank accessions DQ640315-7, DQ647783-805, EF451976-2007) were blasted against the complete non-redundant NCBI GenBank database, and those sequences found to share a high level of similarity were used to resolve alignment ambiguities and to establish relationships for the sequences obtained in this study. The phylogenetic tree, generated for the samples isolated in this study and the related sequences from the NCBI database is shown in Fig. 4, The *Escherichia coli* 16S rRNA sequence was used as the out-group to root the tree. Members of the three form-genera (*Synechococcus*, *Phormidium/Pseudanabaena*, and *Oscillatoria*) were indistinguishable using morphological analysis alone, and each of these groups separated into at least two distinct molecular lineages. The 16S rRNA sequence data used to build this phylogenic tree were based on 370–425 bp per sample, where less than 97 % sequence identity is generally considered to represent different species, and <88% identity suggests separate genera[11,37]. These, of course, are arbitrary values based on well-known pathogenic bacteria.

The overall phylogenetic tree generated from these sequences suggested that several very similar morphotypes may be distinct species with similar morphology. The *Phormidium*-like cyanobacteria were found to contain two distinct molecular variants designated P1 and P2 (Fig. 4). The five sequences in P1, found in three Thailand springs (RN, PD, KP), share a 94.1 % average identity to each other whereas the nine sequences in P2 (excluding *Phormidium mucicola* which occurred exclusively in northern Thailand) were much more closely related to each other with a 98.1% average identity. The phylogeny clearly shows that the P1 *Phormidium* are more closely related to the morphologically distinct species *Leptolyngbya* sp. CNP1-B1-4 (89.1 %) and *Synechococcus elongatus* D83715 (87.5 %) than to the P2 *Phormidium* (77.7 %). Although both P1 and P2 were morphologically indistinguishable and clearly segregated into two molecularly distinct groupings, the sequences in P2 are highly related to the *Pseudanabaena* cyanobacteria which share nearly 100 % identity to some *Phormidium* sequences in Genbank. The classification of this morphologically indistinguishable group clearly must be done using molecular methods.

For the *Synechococcus* groups, three distinct lineages (S1, S2, and S3) were found with over 99 % average identity within each group. Lineage S1, exclusively found in hot springs outside of the Chiangmai region, was also present in all temperature intervals lower than 60 °C. Lineage S2, which was more closely related to lineage S1 (94.2 %) than to any other *Synechococcus* lineage, was found only in San Kamphaeng (SK) and Pong Dued (PD), Chiangmai province, and was found in all temperature ranges from 55 °C and higher. All *Synechococcus* lineages excluding S3 were related at the 93 % average identity level which places S1 and S2 at the same phylogenetic distance as *Synechococcus* sp. SK5, OH34, and OH20. lineage S3, also found exclusively in the Chiangmai region, shared a closer relationship to *Oscillatoria* cf.*terebriformis* (88.6 %)
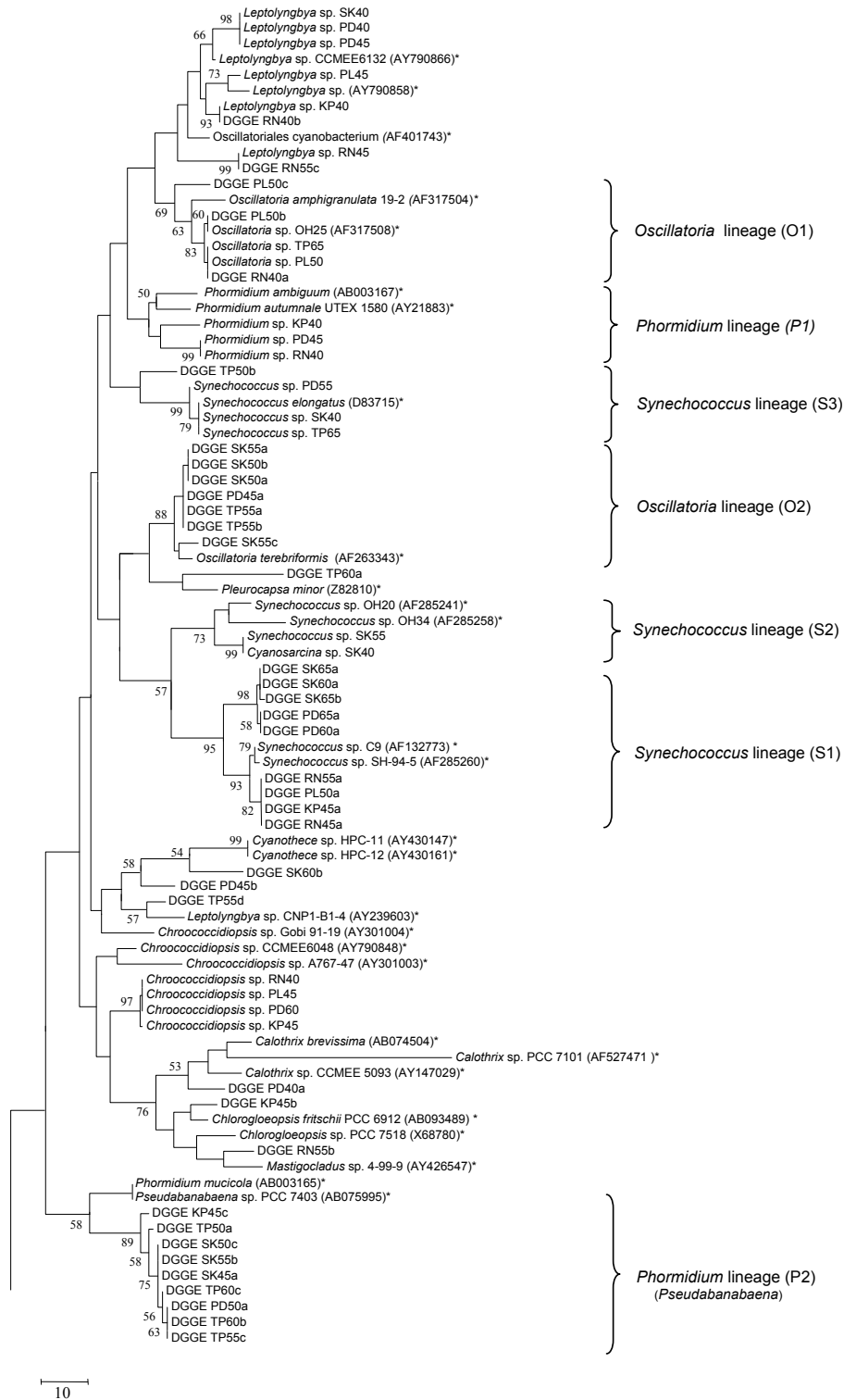
Fig. 4 Phylogenetic relationships for the thermophilic cyanobacteria were constructed using partial 16S rRNA gene sequences, where the tree was rooted using the *Escherichia coli* 16S rRNA sequence as an outgroup. Evolutionary distances were determined using the Maximum Parsimony analysis with 1,000 replicate bootstrap resampling to form the overall consensus tree. Sequence designations for DGGE samples are labelled by location, temperature (in 5 °C intervals) and band position. Values at nodes indicate bootstrap percentages for the 1,000 replicates, where values less than 50 % are not reported. Sequences marked with a (*) were obtained from NCBI with the accession number given in parentheses.

and *Calothrix brevissima* (87.6 %) than to any of the other *Synechococcus* lineages (81.9 %). Although *Synechococcus* lineage S3 is morphologically similar to the other *Synechococcus* species groups, it is clearly distinct and more closely related to other morphologically distinct species.

The *Oscillatoria* group was found to break into two distinct lineages each of which was more closely related to morphologically distinct cyanobacteria than to the other lineage. For these two lineages, subgroups O1 and O2 were found to be related at the 99 % identity level within each subgroup and between the two subgroups there was only a 79.5 % average identity level. Both subgroups were only found at temperatures lower than 60 °C, where *Oscillatoria* subgroup O1 was found to have no geographical barriers and *Oscillatoria* subgroup O2 was found to be geographically limited to the northern region of Thailand.

The phylogenetic analysis of the 16S rRNA 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 S3, which was only found in northern hot spring mats, is phylogenetically distinct from the *Synechococcus* spp. within lineages S1 and S2 (Fig. 4). In addition, many of the cyanobacterial sequences found in this study were generally quite different from the sequences found in the public databases. This suggests that further study of previously unexamined hot springs in addition to the hot springs in this study could reveal additional cyanobacterial species. This is important since many hot spring sites in Thailand are open to the public and have become locations for tourism as well as places the local people use for cooking[38]. Since many of the sequences from this study are unlike 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.

As our results show, there is still quite a bit of room to improve the NCBI taxonomy within the cyanobacteria, but at present these are the currently accepted taxonomic relationships. As additional studies are published, the taxonomy at NCBI is updated to reflect an improved understanding of the phylogenetic relationships of the species in question. Our results clearly show the problem with basing taxonomic relationships on morphological versus molecular based methodologies. Since we were mainly focused on establishing the overall level of diversity versus the absolute taxonomic relationships, our results do clearly establish that there is significant diversity in Thailand's hot springs and some of this cyanobacterial diversity has not yet been characterized outside of our work.

In conclusion, we have characterized community molecular diversity of thermophilic cyanobacterial mats from six geothermal springs in Thailand. It was found that the 16S rRNA gene-defined diversity of all mats exceeded that observed by microscopy alone, showing that the molecular data complements established morphological methods, and reveals an increased cyanobacterial diversity over that seen using microscopy alone. From this increased molecular diversity, several morphologically similar yet molecularly distinct cyanobacterial clusters were characterized which displayed both temperature and phylogeographic clines. In particular, the three morphologically defined species groups *Phormidium*, *Synechococcus*, and *Oscillatoria* were found to contain molecularly distinct species, each more closely related to cyanobacteria with dissimilar morphologies. As an untapped cyanobacterial resource, the six Thailand hot springs sampled in this study present a wide range of possibly unique species groups which could provide further insight into cyanobacterial evolution.

ACKNOWLEDGEMENTS

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