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Morphological and genetic characterization of marine filamentous cyanobacterium *Geitlerinema* isolated from Thailand

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ABSTRACT: *Geitlerinema* is a dominant benthic filamentous cyanobacterium found in the Gulf of Thailand and the Andaman Sea. We investigate the diversity of *Geitlerinema* strains isolated from Thailand using morphological and molecular characterization. No morphological differences were observed in the *Geitlerinema* isolates. Nucleotide sequencing and phylogenetic analyses of the 16S rDNA, the 16S–23S rRNA internal transcribed spacer (16S–23S ITS) and the *cpcB-cpcA* intergenic spacer (*cpcB-cpcA* IGS) showed that the marine *Geitlerinema* isolates belong to a single cluster that includes marine *Geitlerinema* sp. Flo1 and *Geitlerinema* sp. PCC7105 but is distinct from a type species, *Geitlerinema splendidum* and *Geitlerinema* strains which were recently transferred to the genus *Anagnostidinema*. From our results, it is confirmed that marine *Geitlerinema* differs from true freshwater *Geitlerinema* and is proposed to be a new genus in Oscillatoriales. In addition, using random amplification of polymorphic DNA (RAPD), the marine *Geitlerinema* isolates in this study could be classified into eight clades; however, this classification revealed no correlation with the geographic locations.

KEYWORDS: marine *Geitlerinema*, morphology, genetic diversity, 16S rDNA, 16S–23S ITS, *cpcB-cpcA* IGS, RAPD

INTRODUCTION

Cyanobacteria are prokaryotes capable of oxygenic photosynthesis. They can be generally found in terrestrial, freshwater, brackish and marine ecosystems. Some cyanobacteria can also inhabit extreme environments such as hot springs, frozen lakes in Antarctica, hypersaline environments or hot deserts. Cyanobacteria can be taxonomically classified by genus based on colony or trichome morphology, ultrastructure character, cell physiology and biochemistry, culture conditions, habitat or ecology, and genetic characteristics[1].

Geitlerinema is a filamentous cyanobacterium belonging to the order Oscillatoriales[1]. *Geitlerinema* was originally classified in the LPP-B 'Oscillatorian' group[2] but was later simultaneously assigned to a subgenus of the genus *Phormidium*[3] and designated as "*Oscillothrix*"[4]. *Geitlerinema* was later re-classified as a new genus of oscillatorialean cyanophytes in the family Pseudanabaenaceae[5]. *Geitlerinema* can be found in different aquatic habitats such as freshwater or marine environments[2,6,7]. The morphology of *Geitlerinema* is a thin, highly motile, flexuous gliding trichome with rounded shape at both ends and the obligate absence of a sheath. Additional morphological characteristics such as cellular dimension and width, the number and localization of granules, and other ultrastructural characteristics have been well-studied in some strains of *Geitlerinema*[8–10]; however, morphological data are not sufficient for species-level identification.

Many molecular techniques have been used to investigate the genetic diversity and phylogenetic relationships among cyanobacteria. Variable nucleotide sequence regions such as the 16S–23S rRNA internal transcribed spacer region (16S–23S

ITS) and the intergenic spacer region of the phycocyanin locus (*cpcB-cpcA* IGS) have been targeted for phylogenetic analysis in cyanobacteria[11–15]. In addition, random amplification of polymorphic DNA (RAPD) has been used to examine the correlation of morphology, genetics, and geography among strains of the marine cyanobacterium *Leptolyngbya valderiana* (Pseudanabaenaceae)[16]. Molecular techniques, including 16S rDNA restriction analysis and nucleotide sequencing of 16S rDNA and the phycocyanin intergenic spacer, were employed to investigate genetic variation among *Geitlerinema* at the species or population levels[6,10,17]. Using both morphological and 16S rDNA molecular analysis, *Geitlerinema* has been verified as a polyphyletic microorganism[18–20].

Only few studies on cyanobacterial diversity in Thailand have been reported[21–23]. There have been studies on a diversity of cyanobacteria from soil ecosystems and agricultural areas in North, Central and Northeast part of Thailand[21], from hot springs in Thailand[22] and from a man-made solar saltern in Petchaburi province, Thailand[23]. Until now, the diversity of marine cyanobacteria isolated from Thailand has not yet been reported. Thailand is a Southeast Asian country harbouring two coasts, the Gulf of Thailand, connected to the Pacific Ocean, and the Andaman Sea, connected to the Indian Ocean. In this study, most of marine cyanobacteria isolated along the coastline of Thailand were found to belong in genus *Geitlerinema*. This study aimed to investigate morphological and phylogenetic diversity among *Geitlerinema* populations in two different coastlines of Thailand. It is possible that marine *Geitlerinema* isolates in this study might be morphologically or genetically diverse from each other and in addition, they might be distinct from the previously reported freshwater *Geitlerinema* strains. Hence morphology and molecular characterizations using 16S rDNA, 16S–23S ITS and *cpcB-cpcA* IGS nucleotide sequencing and RAPD pattern analysis of *Geitlerinema* isolates were investigated.

MATERIALS AND METHODS

Collection and isolation of cyanobacteria

Cyanobacterial strains in this study were isolated from samples of seawater, stones, sand, and shells collected from the Gulf of Thailand and the Andaman Sea of Thailand (Fig. 1). The samples were collected from 40 coastal locations of 6 provinces in Thailand during June to December in 2013. The approximately 20–40 samples from each location

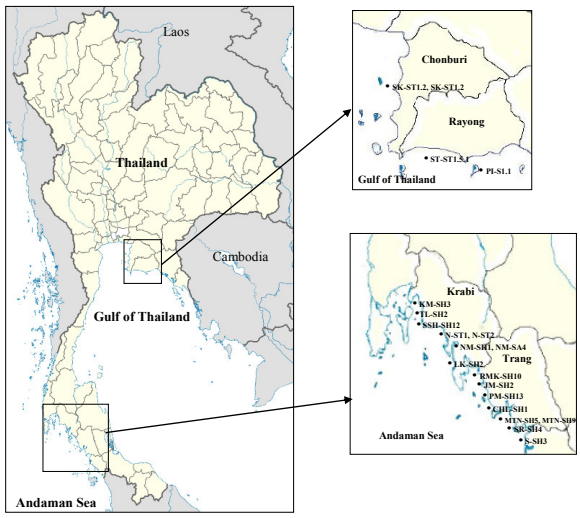

Fig. 1 Map of sampling collection locations.

were randomly collected. Planktonic cyanobacterial strains were isolated from seawater whereas benthic cyanobacterial strains were isolated from stones, sand, and shells. All samples from each environment were inoculated in flasks containing liquid ASN III medium[2]. The flasks were then incubated at 30 °C under light illumination of 30 μmol photons m^{-2} s^{-1} for 7–30 days or until green colour in medium appeared. In case of planktonic cyanobacterial isolation, seawater samples at 10–30 cm depth were filtered onto 20 μm mesh plankton net and the biomass on the filter was subsequently inoculated in medium and cultivated under corresponding conditions. Each filament or colony of cyanobacteria from stones, sand, shells, and water were isolated by a single cell isolation technique under a stereomicroscope (Nikon SMZ745T, Japan)[24]. The cyanobacterial cells were washed several times with autoclaved ASN III medium and transferred onto new solid ASN III agar plates. The monoclonal cyanobacterial culture was used in all experiments.

Cyanobacterial cultivation

Cyanobacterial isolates were cultivated in 250-ml Erlenmeyer flasks containing 100 ml of liquid ASN III medium. Cells were grown at 30 °C with shaking at 120 rpm under white-light illumination of 30 μmol photons m^{-2} s^{-1} for 14 days.

Morphological analysis

The trichome shape, cellular width and length, cell wall constrictions, shape of apical cells, the presence or absence of calyptrae, and the number of

cyanophycin granules were determined in *Geitlerinema* isolates under a light microscope (Olympus CX31, Japan). In each *Geitlerinema* isolate, dimension of ten vegetative cells per trichome was measured in twenty trichome cells by calculation magnification of images using the reference scale bar. Trichome cells were photographed under magnification with video camera system (Nikon Bx51, Japan) using NIS-Elements Ver. 3.2 software (Nikon, Japan).

Genomic DNA isolation

One loop (approximately 40 mg wet weight) of purified *Geitlerinema* cells grown on ASN III agar was suspended in 400 µl of TE buffer (10 mM Tris-HCl and 1 mM EDTA, pH 8.0) in a microcentrifuge tube. Genomic DNA was isolated according to the protocol of Golden et al[25].

PCR amplification and DNA sequencing

DNA fragments of 16S rDNA, 16S–23S ITS, and *cpcB-cpcA* IGS of twenty marine *Geitlerinema* isolates were amplified by PCR using primer pairs F16SrDNACyano (5′-GCTCAGGATGAACGCTGGC G-3′) and R16SrDNACyano (5′-CGGCTACCTTGTTA CGACTCCA-3′), F16S–23SITS (5′-TGTACACACCGC CCGTCAC-3′) and R16S–23SITS (5′-CTCTGTGTGC CTAGGTATCC-3′)[26], and FcpcB-cpcIGS (5′-TTGCC T(G/T)CGCGACATGGAAAT-3′) and RcpcB-cpcAIGS (5′-AGAGCTTCAAC(G/A)TACCAGCT-3′)[11], respectively. PCR reaction was performed as previously described by Phunpruch et al[27]. Each 50 µl PCR reaction contained 20 mM Tris-HCl (pH 8.5), 50 mM KCl, 1.5 mM $MgCl_2$, 200 µM dNTPs, 0.25 µM of each primer, 0.05 U of *Taq* DNA polymerase (Promega, USA) and 0.1 µg of *Geitlerinema* genomic DNA. The PCR program consisted of an initial denaturation step at 94 °C for 5 min, followed by 35 cycles of denaturation at 94 °C for 30 s, annealing at 55–60 °C for 1 min, and extension at 72 °C for 5 min with a final extension at 72 °C for 10 min. PCR products were purified using the Gel/PCR DNA fragments extraction kit (Geneaid, Taiwan) and sequenced in both directions with the Big-Dye terminator cycle sequencing ready reaction kit (Perkin Elmer, USA) using an ABI PRISM 3700 DNA analyser at First BASE Laboratories (Malaysia).

RAPD analysis

Twenty universal random 10-mer primers were screened for Random Amplification of Polymorphic DNA (RAPD) analysis of *Geitlerinema* strains. Only five primers, OPA-03 (5′-AGTCAGCCAC-3′), OPA-05 (5′-AGGGGTCTTG-3′), OPA-07 (5′-GAAACGG GTG-3′), OPA-10 (5′-GTGATCGCAG-3′), and OPA-19 (5′-CAAACGTCGG-3′), displayed polymorphism and were thus chosen for RAPD analysis. Amplification was conducted in 25 µl PCR reactions containing 20 mM Tris-HCl (pH 8.5), 50 mM KCl, 1.5 mM $MgCl_2$, 200 µM dNTPs, 0.25 µM of each primer, 0.05 U of *Taq* DNA polymerase (Promega, USA) and 0.05 µg of template DNA. The PCR program consisted of an initial denaturation step at 94 °C for 5 min, followed by 40 cycles of denaturation at 94 °C for 1 min, annealing at 40 °C for 1 min, and extension at 72 °C for 2 min with a final extension at 72 °C for 7 min. PCR products were analysed by 2% (w/v) agarose gel electrophoresis staining with 0.5 µg/ml ethidium bromide (Bio Basic, USA). A voltage gradient of 8 V/cm was applied into the gel. After electrophoresis, the gel was illuminated under a UV light.

Phylogenetic analysis

The 16S rDNA, 16S–23S ITS, and *cpcB-cpcA* IGS nucleotide sequences were compared with those of *Geitlerinema* or other cyanobacteria available in the GenBank nucleotide database by multiple sequence alignment analysis using the CLUSTALW[28]. Maximum likelihood (ML) trees were constructed with molecular evolutionary genetics analysis (MEGA) software version 6.06[29]. The nucleotide substitution with complete-deletion gap treatment using the evolutionary model of Tamura and Nei[30] was used for analysis. Bootstrap values were obtained from 1000 replicates for each database. For ML tree construction of 16S rDNA, 16S rDNA sequence of *Gloeobacter violaceus* was used as cyanobacterial outgroup and that of *E. coli* as distant outgroup. The ML phylogenetic tree of 16S–23S ITS sequences of 20 *Geitlerinema* isolates and other *Geitlerinema* strains was constructed using MEGA with 16S–23S ITS of another cyanobacterium, *Phormidium autumnale* CCALA 850, as an outgroup sequence. Each RAPD pattern was analysed from RAPD fingerprints generated on an agarose gel. To construct binomial matrix, the presence of a band at each position on a gel was scored as “1” while the absence was scored as “0”. The similarity index was used to calculate the genetic distance values and to construct the dendrogram. The data matrix based on Jaccard’s similarity coefficient and phylogenetic dendrogram was conducted with the unweighted pair-group method with arithmetic averages (UPGMA) analysis using NTSYSpc 2.01e program.

Table 1 Morphological characteristics comprising cellular width, cellular length, and number of cyanophycin granules per cell of marine *Geitlerinema* strains isolated from Thailand[†].

No.	Isolates	Width (μm)			Length (μm)			L:W	Granules				
		Min–Max	Mean	SD	Min–Max	Mean	SD		1	2	3	4	*n*G
1	CHL-SH1	2.22–2.50	2.28	0.11	4.09–6.80	5.09	1.01	2.23	15	7	2	0	24
2	JM-SH2	2.22–2.78	2.33	0.17	3.18–5.45	4.27	0.68	1.83	12	14	2	0	28
3	KM-SH3	1.67–2.22	1.82	0.19	1.82–3.18	2.59	0.46	1.42	18	9	1	0	28
4	LK-SH2	2.22–2.78	2.38	0.21	3.18–6.80	4.73	1.00	1.99	15	12	1	0	28
5	MTN-SH5	1.67–2.22	2.07	0.25	3.18–9.55	6.36	1.86	3.07	15	7	0	0	22
6	MTN-SH9	1.67–2.22	1.82	0.17	3.18–5.90	4.18	0.78	2.30	20	7	3	0	30
7	N-ST1	1.67–2.22	1.83	0.16	2.73–4.55	3.64	0.64	1.99	14	8	0	0	22
8	N-ST2	1.67–1.94	1.82	0.14	3.18–7.73	5.86	1.41	3.22	10	15	7	1	33
9	NM-SA4	1.67–2.22	1.89	0.28	3.64–5.45	4.45	0.60	2.35	16	8	1	0	25
10	NM-SH1	2.22–2.78	2.57	0.25	3.24–5.16	4.20	0.58	1.63	17	5	0	0	22
11	PI-S1.1	1.67–2.22	1.67	0.20	3.18–5.00	4.23	0.54	2.53	15	13	2	0	30
12	PM-SH13	1.94–2.22	2.10	0.14	3.64–5.45	4.86	0.58	2.31	18	6	1	0	25
13	RMK-SH10	1.67–1.94	1.82	0.14	2.73–4.55	3.64	0.54	2.00	12	14	2	0	30
14	S-SH3	1.67–2.22	2.13	0.16	2.73–5.45	4.18	0.86	1.96	15	11	2	0	28
15	SK-ST1.1	1.94–2.22	2.11	0.14	2.73–6.80	5.00	1.27	2.37	21	6	1	0	28
16	SK-ST1.2	1.67–2.22	1.94	0.13	3.64–6.80	5.45	0.86	2.81	11	7	4	1	23
17	SR-SH4	1.67–2.22	1.92	0.15	3.18–7.27	5.50	1.05	2.86	19	5	3	0	27
18	SSH-SH12	2.22–2.78	2.64	0.23	3.18–5.45	4.50	0.66	1.70	21	4	0	0	25
19	ST-ST1.5.1	1.67–2.22	1.88	0.24	3.18–5.00	4.05	0.66	2.15	12	8	0	2	22
20	TL-SH2	1.67–2.22	1.94	0.22	3.64–5.45	4.59	0.59	2.37	18	5	3	0	26

[†] Mean and SD of cell width and length were calculated from 20 measurements. L:W is the ratio of average length and width; *n*G is the total number of granules.

Secondary structure of transfer RNA based on the 16S–23S ITS region

The secondary structure of each tRNA was predicted by using MFOLD 3.2 (www.bioinfo.rpi.edu/applications/mfold/)[31] with the folding temperature at 37 °C.

RESULTS

Morphological characteristics

By isolation of marine cyanobacteria from samples of seawater, stones, sand, and shells, randomly collected from the Gulf of Thailand and the Andaman Sea, a total of 175 cyanobacterial strains belonging to seven different genera were isolated from 40 coastal locations. Among them, 147 isolates were identified by 16S rDNA sequencing analysis as *Geitlerinema*. Morphological and genetic characterization was performed on the representative 20 marine *Geitlerinema* isolates, which included 16 isolates from the Andaman Sea and four isolates from the Gulf of Thailand (Fig. 1). Using a light microscope, the morphology of these isolates was observed to be a flexuous or straight filament composed of a single trichome. The trichome was not constricted at the cross walls and was attenuated towards their ends. Each individual trichome contained one or more granules of cyanophycin (Table 1), one of which was close to the cross wall. In addition, the apical cells were rounded-cones without calyptrae at the outer cell wall (Fig. 2). Although all 20 *Geitlerinema* isolates showed the similar cellular shape, they appeared in a wide variety of trichome sizes. In this study, the average cellular length and width of the *Geitlerinema* strains were 2.59–6.36 μm and 1.67–2.64 μm, respectively, and the cellular length/width ratio ranged from 1.42–3.22 (Table 1). A single granule per cell was mostly observed in *Geitlerinema* (30–84%) whereas two or three granules per cell were less observed (16–50% and 0–21%, respectively). Four granules per cell were observed in only three strains (isolates N-ST2, SK-ST1.2 and ST-ST1.51) (Table 1).

Phylogenetic tree analysis of 16S rDNA

Fragments of 16S rDNA were amplified and sequenced for all twenty marine *Geitlerinema* isolates. The resulting 960 bp nucleotide sequences were deposited in GenBank under accession numbers KX955234–KX955253 and compared with those of other *Geitlerinema* and cyanobacterial strains. Maximum likelihood (ML) phylogenetic analysis of the

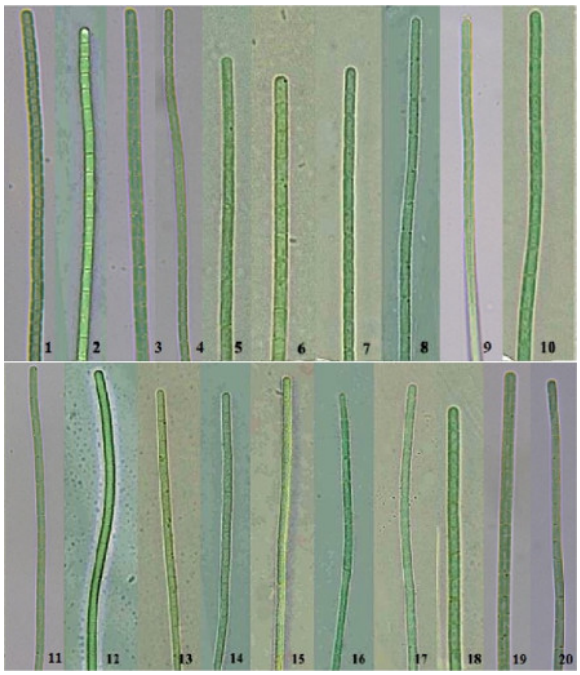

Fig. 2 Morphologies of marine *Geitlerinema* strains isolate from Thailand; *Geitlerinema* sp. CHL-SH1 (1), JM-SH2 (2), KM-SH3 (3), LK-SH2 (4), MTN-SH5 (5), MTN-SH9 (6), N-ST1 (7), N-ST2 (8), NM-SA4 (9), NM-SH1 (10), PI-S1.1 (11), PM-SH13 (12), RMK-SH10 (13), S-SH3 (14), SK-ST1.1 (15), SK-ST1.2 (16), SR-SH4 (17), SSH-SH12 (18), ST-ST1.5.1 (19), and TL-SH2 (20).

16S rDNA sequences was performed. The resulting phylogenetic tree shows three clades of this genus (Fig. 3). All *Geitlerinema* isolates from Thailand (both from the Gulf of Thailand and the Andaman Sea) were clustered in the "Marine *Geitlerinema*" (Fig. 3). Interestingly, *Geitlerinema* sp. A28DM, *Geitlerinema* sp. Flo1 and *Geitlerinema* sp. PCC7105 were also clustered into this clade. The second *Geitlerinema* clade comprised many strains of freshwater *Geitlerinema splendidum* (strain P014, P017, SERB48 and PSE0519C) (Fig. 3). The third clade was composed of many strains of freshwater *Geitlerinema* species and *Leptolyngbya* species which were recently designated as *Anagnostidinema pseudacutissimum* and *A. amphibium*[32] (Fig. 3).

Phylogenetic analysis of the 16S–23S ITS

The 570–580 bp DNA fragments of 16S–23S ITS of twenty marine *Geitlerinema* isolates were sequenced and the nucleotide sequences were deposited in the GenBank database under accession numbers KT186109–KT186128. The sequences showed 86.5–100% nucleotide similarity to each other. Compared with other *Geitlerinema* strains deposited in GenBank, the sequences showed 41.9–99% nucleotide similarity, with the highest similarity (86.6–99%) to *Geitlerinema* sp. Flo1 (accession No. FJ042948.1) and *Geitlerinema* sp. PCC7105 (accession No. FJ042946.1). The ML phylogenetic tree of the 16S–23S ITS sequences shows that *Geitlerinema* could be divided into four clades with a bootstrap value of $> 90\%$ (Fig. 4). All marine *Geitlerinema* strains isolated from Thailand, *Geitlerinema* sp. Flo1, and *Geitlerinema* sp. PCC7105 were clustered into clade I; *G. pseudacutissimum* and *G. carotinosum* were clustered into clade II; *G. unigranulatum*, *G. lemmermannii*, and *G. amphibium* were clustered into clade III; and *G. acuminatum* and *G. splendidum* were clustered into clade IV (Fig. 4).

The 16S–23S ITS nucleotide sequences among *Geitlerinema* sp. collected from Thailand (clade I) were subdivided into 4 groups. Sequences of each representative subgroup (*Geitlerinema* sp. RMK-SH10, LK-SH2, ST-ST1.5.1, and TL-SH2) were compared with that of *Geitlerinema* sp. PCC7105. The results showed that the 16S–23S ITS of *Geitlerinema* sp. RMK-SH10 is 9–10 nucleotides longer than that of other strains. All the sequences contain the conserved domains (D1, D1', D2, D3, D4, D5, and the antiterminator boxA), which are the regions involved in the formation of stem-loop structure (V2, V3 and the antiterminator boxB), and two tRNA sequences ($tRNA^{Ile}$ and $tRNA^{Ala}$) (Fig. 5). The $tRNA^{Ile}$ gene is located downstream of the 16S rDNA whereas the $tRNA^{Ala}$ is located upstream of the 23S rDNA (Fig. 5). Almost all $tRNA^{Ala}$ secondary structures of the *Geitlerinema* sp. assessed in this study have three loops (the small bubble above, the side loop, and the terminal loop), except for the structure of *Geitlerinema* sp. RMK-SH10, which contains only two loops due to the presence of 9–10 additional nucleotides.

Phylogenetic analysis of the *cpcB-cpcA* IGS

DNA fragments of 90–91 bp region of the *cpcB-cpcA* IGS of twenty marine *Geitlerinema* isolated in Thailand were sequenced and deposited in the GenBank database under accession numbers KT228281–KT228300. A multiple sequence alignment revealed that the obtained nucleotide sequences are highly similar (95.6–100%) to each other and show 42.7–99% similarity to other *Geitlerinema* strains reported in GenBank. The results showed that *Geitlerinema* could be divided into three clades with a bootstrap

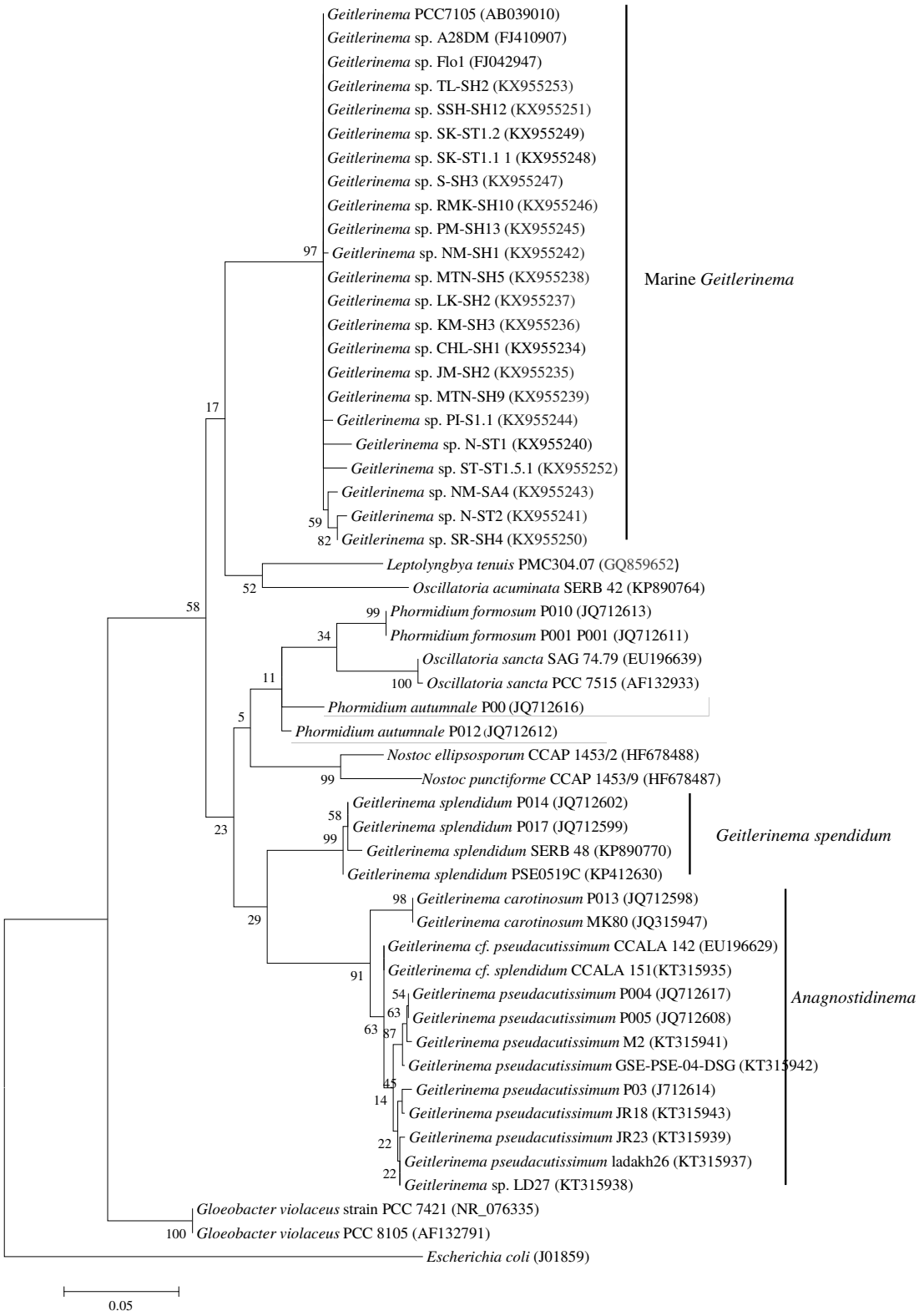

Fig. 3 The maximum likelihood (ML) phylogenetic tree based on the 16S rDNA sequences of twenty marine *Geitlerinema* isolates and other cyanobacterial strains with 1000 bootstrap replicates.

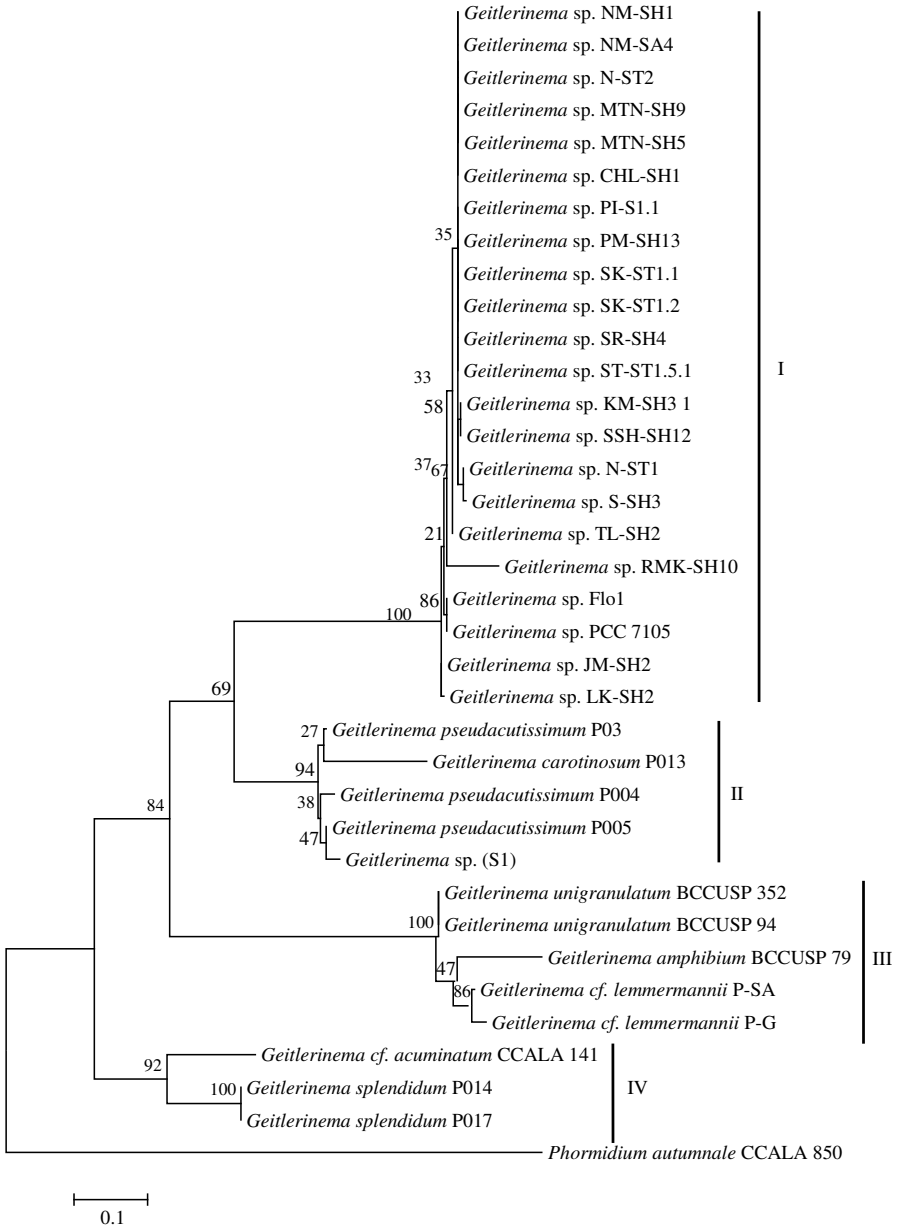

Fig. 4 The maximum likelihood (ML) phylogenetic tree based on the 16S–23S rRNA ITS sequences of twenty marine *Geitlerinema* isolates and other *Geitlerinema* strains with 1000 bootstrap replicates.

value of > 90% (Fig. 6). All *Geitlerinema* isolates from Thailand, *Geitlerinema* sp. Flo1 (accession No. FJ042941.1), and *Geitlerinema* sp. PCC7105 (accession No. FJ042942.1) were clustered into Clade I. In addition, *Geitlerinema* sp. LK-SH2 and *Geitlerinema* sp. JM-SH2 were genetically similar (Fig. 6). Clades II and III contained many strains of *G. unigranulatum* and *G. amphibium* (Fig. 6). The *cpcB-cpcA* IGS region of *Geitlerinema* species in clade I comprised 86–91 nucleotides in length, and was distinct from those of clade II and clade III that contained 83 and 291–294 nucleotides, respectively.

RAPD analysis

RAPD pattern analysis was performed for twenty marine *Geitlerinema* isolates using the selected five primers, constructing 85 different PCR product bands that yielded 100% polymorphism. Each individual primer generated 14–17 identical PCR product bands. The size of the PCR products ranged from

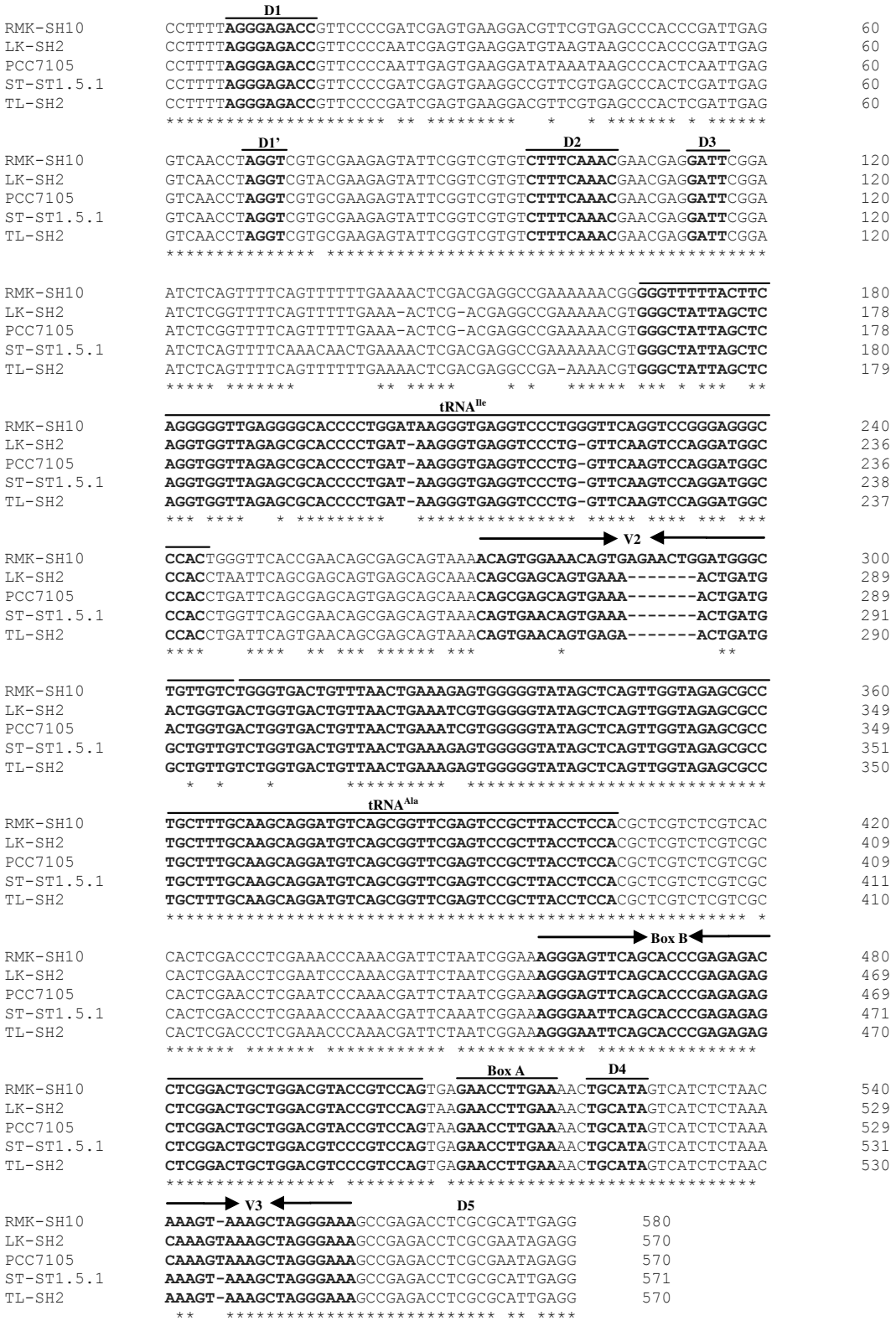

Fig. 5 Alignment of 16S–23S rRNA ITS sequences of *Geitlerinema* sp. TL-SH2, LK-SH2, ST-ST1.5.1 and RMK-SH10 compared to that of *Geitlerinema* sp. PCC7105. All sequences contain $tRNA^{Ile}$, $tRNA^{Ala}$, the consensus domain sequences (D1–D5), the antiterminator (boxB and boxA) and the major variable stems V2 and V3.

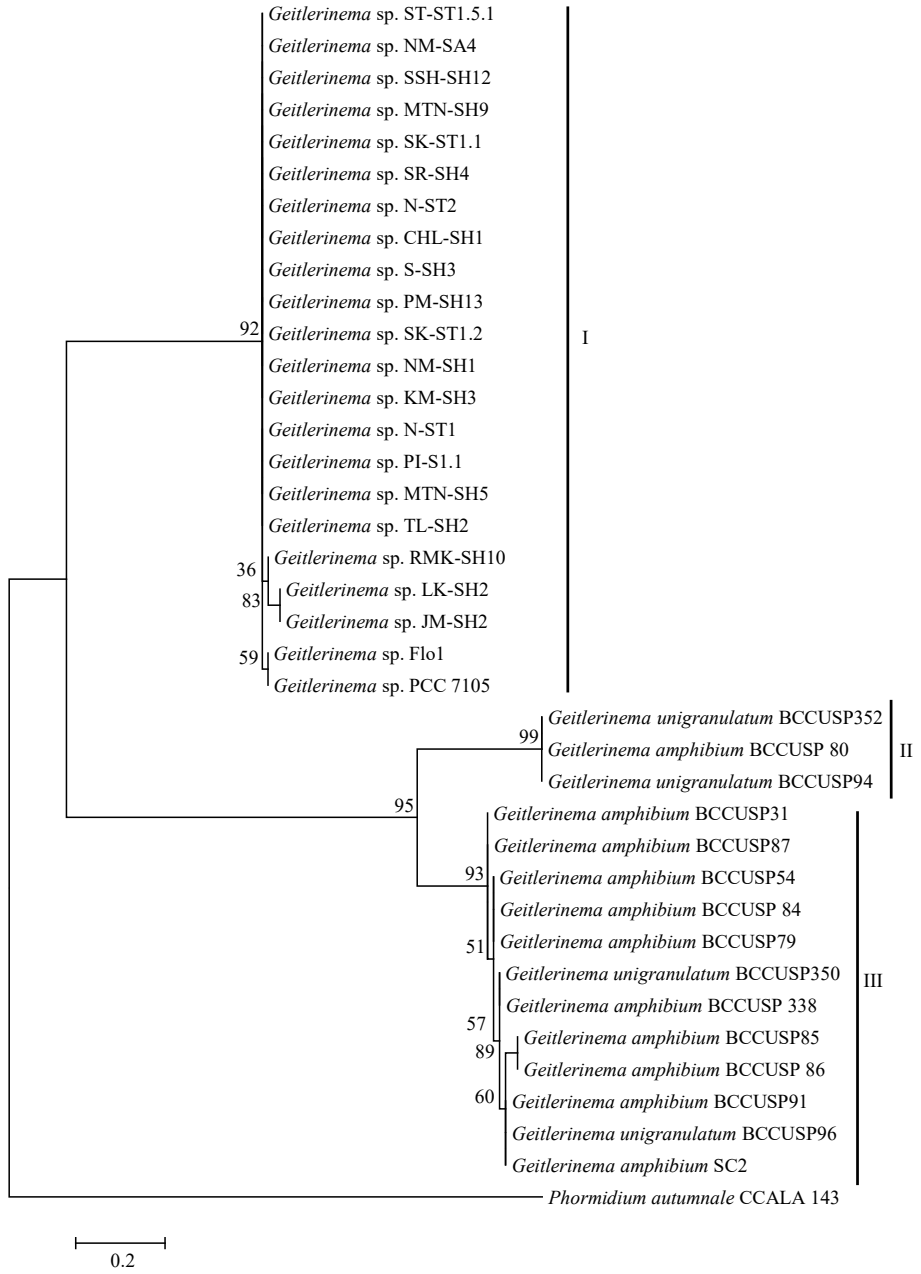

Fig. 6 The maximum likelihood (ML) phylogenetic tree based on the *cpcB-cpcA* IGS sequences in twenty marine *Geitlerinema* isolates and other *Geitlerinema* strains with 1000 bootstrap replicates.

600–4000 bp. The RAPD patterns and similarity matrix of *Geitlerinema* isolates using five selected primers. The RAPD tree exhibited eight clades with an average similarity coefficient of 0.71 (Fig. 7). Clade I contained *Geitlerinema* sp. KM-SH3 and clade II contained *Geitlerinema* sp. PI-S1.1. Clade III contained 12 *Geitlerinema* strains, including three *Geitlerinema* isolates (SK-ST1.2, ST-ST1.5.1 and SK-ST1.1) from the Gulf of Thailand and nine isolates from the Andaman Sea. The similarity within clade III ranged from 71.1–90%. The remaining six isolates from the Andaman Sea were classified into five clades: *Geitlerinema* sp. TL-SH2 in clade IV, *Geitlerinema* sp. N-ST1 in clade V, *Geitlerinema* sp. PM-SH13 in clade VI, *Geitlerinema* sp. CHL-SH1 in clade VII, and *Geitlerinema* sp. LK-SH2 and *Geitlerinema* sp. JM-SH2 in clade VIII (Fig. 7). The similarity within clade VIII was 74%.

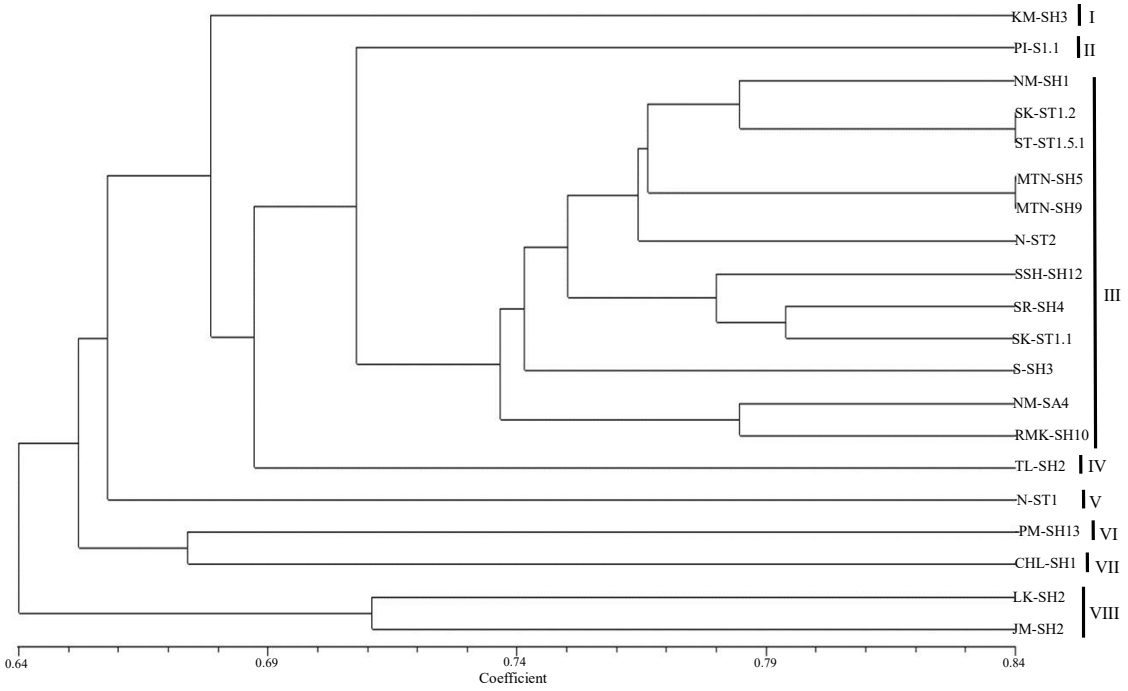

Fig. 7 Dendrogram showing the relationship of marine *Geitlerinema* isolates based on UPGMA cluster analysis of the RAPD profiles derived using five primers and Jaccard's similarity coefficient.

DISCUSSION

The marine cyanobacterial isolates from the Gulf of Thailand and the Andaman Sea mostly belonged to genus *Geitlerinema*. Although samples were randomly collected, it was quite easy for isolation benthic cyanobacteria attached to shells and stones due to the trichome structure and the obvious blue-green colour appearance. In this study, 20 *Geitlerinema* isolates, representatives from the different locations, were subjected to morphological and phylogenetic analyses. All isolates studied correspond to one single morphological type and all of them are similar to *G. pseudacutissimum* and *G. carotinosum* whose morphology has been previously reported by Hašler et al[10]. However, our isolates are different from the type species of *Geitlerinema*, *G. splendidum* P014 and *G. splendidum* P017 which are bent or screw-like at the ends with capitate or rounded apical cells[10]. Furthermore, *G. amphibium* and *G. unigranulatum* formed a flexuous to straight trichome with no constriction at the cross walls but with rounded-conical apical cells. Their hooked apical cells were observed in cultures[17]. In this study, the observed cellular length was similar to that of *G. amphibium* and *G. unigranulatum*, which are 2.2–7 μm long, but the observed cellular width was quite wider than those organisms, which are 1.02–1.91 μm wide, as well as the number of granules[17]. Since all *Geitlerinema* strains investigated show the similar morphological data, using genetic information might help to assess the variation among *Geitlerinema* strains in this study.

The 16S rDNA phylogenetic tree shows that all *Geitlerinema* isolates from Thailand were clustered in the same clade designated as "marine *Geitlerinema*" (Fig. 3). These results indicate that marine *Geitlerinema* isolates from Thailand are genetically similar to each other and closely related to many marine *Geitlerinema* strains, *Geitlerinema* sp. A28DM isolated from sandy shores parallel to the coast indented by the estuarine mouth of river Tapi, Gujarat, India[33], *Geitlerinema* sp. Flo1 (*Oscillatoria limnetica* Flo1), present in the culture collection of the Department of Marine Microbiology at the University of Bremen[34] and *Geitlerinema* sp. PCC7105, recognized as the reference strain for the marine species of this genus according to Bergey's Manual[1]. This clade was phylogenetically distant from other freshwater *Geitlerinema* clades which are consistent with previous reports[19,32]. Recently, Strunecký and coworkers revised the genus of *Geitlerinema* that *Geitlerinema splendidum* was only one species according to the morphological similarity

with its origin description but other *Geitlerinema* or *Leptolyngbya* species was proposed to transfer into *Anagnostidinema* gen. nov.[32]. Our result and the previous study clearly demonstrated that marine *Geitlerinema* is phylogenetically distant from other freshwater *Geitlerinema* and might be separated into other genera of cyanobacteria. The 16S rDNA sequences have been used as a target region for identification and phylogenetic analysis of several cyanobacteria; however, the data are insufficient to guarantee species identity and genetic diversity because of a lower evolutionary rate of variation[35]. With this reason, we investigated other variable nucleotide sequence regions such as 16S–23S ITS and *cpcB-cpcA* IGS.

From the Maximum Likelihood phylogenetic tree of the 16S–23S ITS sequences, marine *Geitlerinema* isolates from Thailand are genetically similar to each other and are closely related to *Geitlerinema* sp. Flo1 and *Geitlerinema* sp. PCC7105 but are different from other freshwater *Geitlerinema* strains found in central Europe such as *G. carotinosum*, *G. pseudacutissimum*, and *G. see acuminatum*. It was suggested that 16S–23S ITS sequence analysis could be used to primarily classify *Geitlerinema* strains at the species level based on the highly variable nucleotide sequences; however, this was not possible due to the limited genetic variation among the strains assessed in this study.

The 16S–23S ITS nucleotide sequences among marine *Geitlerinema* sp. collected from Thailand (clade I) were subdivided into four groups. Sequences of each representative subgroup (*Geitlerinema* sp. RMK-SH10, LK-SH2, ST-ST1.5.1, and TL-SH2) were compared with that of *Geitlerinema* sp. PCC7105. All the sequences contain the conserved domains (D1, D1′, D2, D3, D4, D5, and the antiterminator boxA), which are the regions involved in the formation of stem-loop structure (V2, V3 and the antiterminator boxB), and two tRNA sequences ($tRNA^{Ile}$ and $tRNA^{Ala}$) (Fig. 5).

Almost all $tRNA^{Ala}$ secondary structures of the *Geitlerinema* sp. assessed in this study have three loops (the small bubble above, the side loop, and the terminal loop), except for the structure of *Geitlerinema* sp. RMK-SH10, which contains only two loops due to the presence of 9–10 additional nucleotides. *Geitlerinema* sp. RMK-SH10 may be genetically diverse from other strains in the subgenus. A comparative analysis of tRNA secondary structure might be a useful tool for studying genetic diversity among individual species of cyanobacteria.

From the Maximum Likelihood phylogenetic tree of the *cpcB-cpcA* IGS sequences *Geitlerinema* could be divided into three clades. All twenty marine *Geitlerinema* isolates from Thailand, *Geitlerinema* sp. Flo1, and *Geitlerinema* sp. PCC7105 were classified into Clade I (Fig. 6). This result is similar to those based on 16S rDNA and 16S–23S ITS sequences. It also confirmed the close genetic relationship among these marine *Geitlerinema* species. It was previously reported that the nucleotide sequences of cyanobacterial *cpcB-cpcA* IGS ranged from 69–298 nucleotides[36]. Since the length of *cpcB-cpcA* IGS sequence is variable compared with that of the 16S–23S ITS sequence, the former might yield information enabling the differentiation at the strain level. However, this was not the case for the highly similar *Geitlerinema* isolates from Thailand assessed in this study.

In general, the DNA fingerprint obtained by RAPD is a molecular technique used for cluster analysis in order to study geographic patterns and to examine genetic diversity among cyanobacteria[16,37,38]. In this work, eight clades of marine *Geitlerinema* could be separated using by this method, revealing the genetic relationship among *Geitlerinema* populations. Within clade III, 12 *Geitlerinema* strains could be divided into many subgroups depending on the similarity coefficient. *Geitlerinema* sp. ST-ST1.5.1 was most closely related to *Geitlerinema* sp. SK-ST1.2 whereas *Geitlerinema* sp. MTN-SH5 was most closely related to *Geitlerinema* sp. MTN-SH9 (Fig. 7). Interestingly, *Geitlerinema* sp. LK-SH2 and JM-SH2 were not separated from other strains based on 16S–23S ITS sequence (Fig. 4) and *cpcB-cpcA* IGS sequences (Fig. 6) but could be separated by RAPD. This was because the RAPD technique uses one short primer to randomly amplify DNA fragments to generate many PCR bands in a pattern. The polymorphic profiles generated by RAPD analysis suggested a considerable degree of intra-species heterogeneity[39]. However, previous reports demonstrated that although RAPD patterns of the cyanobacteria *L. valderiana* and *P. retzii* showed genetic diversity, they did not correspond to the distinct geographic area of isolation[16,38]. Thus a combination of both morphological and molecular data may facilitate the establishment of taxonomic diversity.

In summary, twenty marine *Geitlerinema* strains isolated from the Gulf of Thailand and the Andaman Sea in Thailand could not be phylogenetically distinguished from each other using morphology and molecular analyses of the 16S rDNA, 16S–23S ITS and the *cpcB-cpcA* IGS sequences; however, DNA

fingerprinting using RAPD analysis could separate all twenty marine *Geitlerinema* strains isolated from Thailand into eight clades without the correlation with geographic locations. In addition, by 16S rDNA, 16S–23S ITS and the *cpcB-cpcA* IGS sequence analysis, *Geitlerinema* isolated in this study was clustered in "marine *Geitlerinema*" group which is suggested to classify into other or new genera of cyanobacteria.

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