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A N I N T E R N A T I O N A L J O U R N A L

Diversity of the Potential 2-Methylisoborneol-Producing Genotypes in Thai Strains of *Planktothricoides* (Cyanobacteria)

Wittaya Tawong[1]*
[1] *Department of Agricultural Science, Faculty of Agriculture Natural Resources and Environment, Naresuan University, Thailand.*

ABSTRACT

The genus Planktothricoides *Suda & Watanabe is considered as a 2-methylisoborneol (MIB) producer, affecting water quality and aquatic animal products worldwide. To date, there is limited information about the diversity of this genus from Thailand. In this study, Thai Planktothricoides strains were isolated from fish ponds and reservoirs in North, Northeast and Central regions for morphological examination, phylogenetic analyses based on 16S rRNA, rbcLX and MIB synthase genes as well as GC/MS/MS analyses. The morphological results and the 16S rRNA and rbcLX phylogenies of Thai* Planktothricodes *strains enabled them to be designated as* Planktothricoides raciborskii. *Cell dimensions of Thai strains tested were in 1.86 to 5.96 µm length (L), 2.83 to 13.70 µm width (W), and the L/W ratio ranged from 1:6 to 1:1. Among* Planktothricoides *strains, the 16S rRNA phylogenies demonstrated that three subclades (A, B and C groups) were apparently divided. The similarity of 16S rRNA genes between subclades were 96-98%. From the detection of MIB synthase genes and GC/MS/MS analyses, some strains grouped into A group were considered as MIB-producers. In this study, most Thai* Planktothricoides *strains belonging to the A group were found in all three regions, while the strains forming the B and C groups were not distributed in the North region. To our knowledge, the present study is the first report investigation and characterization of the potential MIB-producing* Planktothricoides *from Thailand. Therefore, providing a valuable tool as a model for the early prediction and detection of taste and odor event is necessary.*

Key word: 2-methylisoborneol; filamentous cyanobacteria; 16S rRNA; MIB synthase; Thailand

*Author for correspondence: wittayat@nu.ac.th

INTRODUCTION

Increasing eutrophication of water resources with changing climate are making cyanobacterial blooms more common, which can produce taste and odor episodes [1-4]. These undesirable effects are not only restricted to drinking water sources but are also significant causes of economic losses in the aquaculture industry due to their partitioning into fish flesh [5]. The two most common compounds known to cause earthy and musty off-flavors are the organic chemicals geosmin and 2-methylisoborneol (MIB) [6-8], which are secondary metabolic products of certain species of cyanobacteria and actinomycete bacteria. In Thailand, the occurrences of off-flavor episodes were frequently found in aquaculture [9-14]. These episodes erode consumer confidence in the quality and safety of production. Currently, accurate diagnostic tools for the detection of taste and odor producers are important. It would be invaluable to use this information for early detection of potentially odor-producing cyanobacterial blooms and thus leading to management solutions.

The cyanobacterial genus *Planktothricoides* Suda and Watanabe has also been reported as one of the most important MIB-producing genera and water bloom-forming cyanobacteria that could be widely distributed in aquatic and terrestrial environments, including fish ponds, rivers and reservoirs [15-17]. This genus includes two species: *P. raciborskii* Suda and Watanabe and *P. attenuate* Komárek and Komáarkova-Legnerová as currently accepted taxonomically. Nevertheless, the morphological characters of the genus *Planktothricoides* are frequently similar to those of the genus *Planktothrix*. One of the most important dissimilarities is the fact that *Planktothricoides* have solitary trichomes attenuated towards their ends and are sometimes slightly bent near the apex [18, 19]. Though both genera are morphologically similar, the genus *Planktothricoides* comprises an independent phylogenetic cluster from the genus *Planktothrix* based on 16S rDNA sequence analysis [15, 19].

Molecular analysis is recommended to clarify ambiguity in taxonomy based upon morphology, and cyanotoxin investigations are also widely used [20]. Several genetic markers such as the small-subunit rRNA (16S rRNA) gene, phycocyanin encoding locus and the conserved gene encoding D-ribulose 1,5-bisphosphate carboxylase-oxygenase large subunit (*rbcLX*) have been used for assessing cyanobacterial diversity and phylogenetic relationship [15, 21-24] . Particularly, the 16S rRNA gene is highly conserved between different species of cyanobacteria and has become an important molecular marker for phylogenetic analysis [25]. Recently, Giglio et al.[26] discovered the MIB biosynthetic genes in cyanobacteria from *Pseudanaaena limnetica* (Castaic Lake) and was further confirmed in *P. limnetica* NIVA- CYA 111 (Lake Biwa) and *Oscillatoria limosa* LBD 305b. More recently, Wang et al.[16] reported that *Pseudoanabaena* sp. dqh15 and *P. raciborskii* CHAB 3331 from China were confirmed to be 2-MIB producing organisms by using the 2-MIB synthesis-associated genes and GC-MS analyses. Afterwards, there were several cyanobacterial strains (e.g., *Oscillatoria*, *Planktothrix* and *Letolyngbya bijugata*) reported as the MIB producers [4, 17]. These studies provided fundamental information for the development of molecular technologies to monitor odor-producing microorganisms and investigate the relationship between odor production and the corresponding synthesis genes. Several protocols for the detection of geosmin have been reported along with the elucidated genetic background. At present, real-time PCR assays have been established for quantification of geosmin-producing *Streptomyces* [27] and *Anabaena* species [3, 28]. However, no molecular technique for MIB-producing cyanobacteria has been established.

Understanding the diversity of off-odor-producing groups is essential for the monitoring and management of off-flavor events [3, 4, 28, 29]. Although off-odor has been described as a common problem for cultured fish, there are very few molecular

genetic studies of cyanobacterial species producing off-odor from Thailand because of difficulties in the isolation and purification of strains. To evaluate diversity based on morphological and molecular analyses as well as ability of MIB production, Thai *Planktothricoides* strains were isolated in this study from several fishponds and reservoirs in Thailand. Then, microscopic morphological examination, pigment analysis and molecular diversity analysis based on 16S rRNA, *rcbLX* and MIB synthase genes between MIB-producing and non-MIB-producing strains were performed. Furthermore, GC/MS/MS analysis was also conducted for confirmation.

MATERIALS AND METHODS

Isolation, culturing condition and morphological characterization of Thai *Planktothricoides*

The *Planktothricoides* filaments were isolated from several freshwater ponds, most of which are used for fisheries or reservoirs from ten provinces of Thailand (Fig. 1). The strains were picked under a BX51 light microscope (Olympus, Tokyo, Japan) using the Pasteur Micropipette method [30]. The filaments were subsequently transferred to double concave sides and washed twice with distilled water. Then, these strains were maintained in capped tubes containing 10 ml of BG11 medium[31]. The cultural condition was kept under temperature of 28º C, a light intensity of 20 µmol photons $m^{-2}s^{-1}$ and a light-dark cycle of 16:8 h. During the exponential growth phase (around 28 days old), successfully grown *Planktothricoides* strains were observed for their morphological characters under a BX51 light microscope (Olympus, Tokyo, Japan) with digital camera for species identification according to the descriptions of Suda et al.[15] and Komárek and Komárková-Legnerová [32]. At least 50 filaments of strain tested, morphometric information as length (L) and width (W) were measured using the AxioVision software (Carl Zeiss, Jena, German). The L/W ratio was also calculated. Aberrant cell morphology was discarded for morphometric analyses.

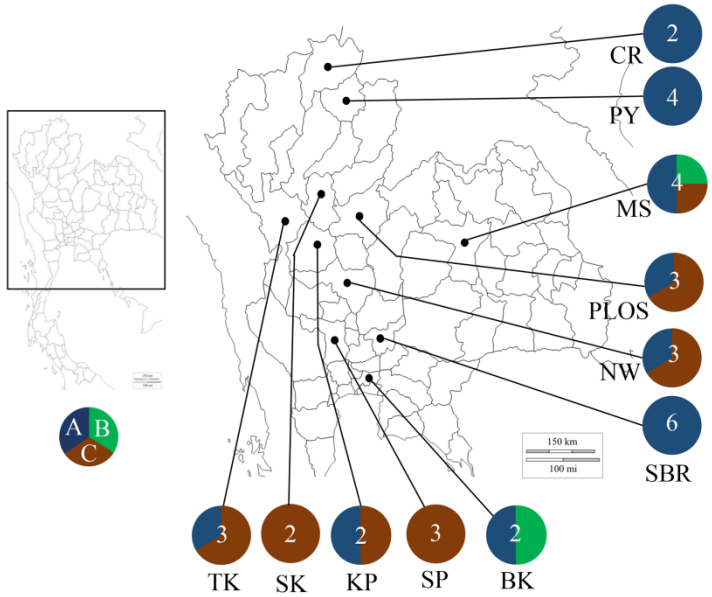

Figure 1. Geographic distributions based on the 16S rRNA gene sequences of *Planktothricoides* plotted on map showing upper part of Thailand. The numbers in each pie indicated the number of strains used for phylogenetic analyses. Each color in pies corresponds to a subclade in 16S rRNA phylogenetic trees, i.e. dark blue: Group A, green: Group B, brown: Group C. Each province is marked as CR: Chiang Rai, PY: Phayao, SK: Sukhothai, TK:

Tak, KP: Kamphaeng Phet, PLOS: Phisanulok, MS: Maha Sarakham, NW: Nakhon Sawan, SP: Suphanburi, SBR: Saraburi, BKK: Bangkok (reported by Suda et al.[15]).

In vivo and phycobilin pigment absorption spectra determination

Thai *Planktothricoides* strains were used for determination of *in vivo* absorption and phycobilin pigment absorption following the method described by Suda et al. [15]. An aliquot of culture was collected and the *in vivo* absorption spectra was measured from 350 to 850 nm by using UV-VIS HACH DR/4000 spectrophotometer (HACH, Loveland, USA). Then, the cells were harvested by centrifuging at 1,500 × g for 15 min at room temperatures. Then, the pellet cell was suspended in 0.1 M of phosphate buffer (pH 7), followed by five successive freezing and thawing cycles in order to disrupt the cell wall and consequently to isolate the phycocyanin (PC). The broken cell suspension was centrifuged at 1,500 × g for 15 min to remove unbroken cells. The absorbance of the supernatant was measured from 350 to 750 nm as explained above.

DNA extractions, PCR and sequencing

The cells were harvested by centrifugation for 10 min at 1,500 × g. DNA extraction was performed using a modified CTAB (cetyl-trimethyl-ammonium bromide)-based extraction method adapted for cyanobacteria[33]. The partial 16S rRNA, *rbcLX* and MIB synthase genes were amplified and sequenced by using the primers set showed in Table 1. The PCR reaction mixture (25 µl) for all genes consisted of 10 µl of nanopure water, 0.25 µl of each primer (0.10 µM), 2 µl of genomic DNA (20 ng), 12.5 µl of 2× Go Taq Green Master Mix (Promega, Madison, WI, USA) containing *Taq* DNA polymerase, magnesium chloride (3 mM), dNTPs (400 µM each) and reaction buffers (pH 8.5). The PCR conditions for 16S rRNA, *rbcLX* and MIB synthase genes were carried out using a Bio-Rad MyCycler (Bio-Rad, Hercules, CA, USA) with an initial denaturation at 94 °C for 2 min followed by 30 cycles of 94° C for 30s, 50° C for 30s and 72° C for 1 min, followed by the extension at 72° C for 5 min with a final hold at 4° C until needed. The PCR product were purified using the PCR purification kit (Omega, Norcross, GA, USA) and directly sequenced using an automated ABI Prism 3730XL DNA sequencer (Applied Biosystem, Foster, CA, USA). All sequences were submitted to GenBank and accession numbers (LC157916 – LC157948 for the partial16S rRNA genes, LC157949 – LC157985 for the *rbcLX* genes and LC157986 – LC157992 for the MIB synthase genes) are shown in Figs 3A–C.

Table 1. PCR primer sets used in this study

Primer names	Sequence (5'-3')	Synthesis direction	References
16S rRNA gene			
27F	AGA GTT TGA TCC TGG CTC AG	Forward for PCR and sequencing	Neilan et al. (1997)
809R	GCT TCG GCA CGG CTC GGG TC GAT A	Reward for sequencing	Jungblut et al. (2005)
1492Rc	TAC GGC TAC CTT GTT ACG AC	Reward for PCR	Neilan et al. (1997)
rbcLX gene			
CW	CGT AGC TTC CGG TGG TAT CCA CGT	Forward for PCR and sequencing	Rudi et al. (1998)
DF	GGG CAR YTT CCA CAK NGT CCA	Reward for PCR and sequencing	Rudi et al. (1998)
MIB synthase gene			
MIB3313F	CTC TAC TGC CCC ATT ACC GAG CGA	Forward for PCR and sequencing	Suurnäkki et al. (2015)

MIB4226R	GCC ATT CAA ACC CGC CGC CCA TCC A	Reward for PCR and sequencing	Suurnäkki et al. (2015)

Alignments and Phylogenetic analyses

The obtained sequences of 16S rRNA, *rbcLX* and MIB synthase genes in this study were separately aligned together with reference sequences of each gene retrieved from GenBank. The 16S rRNA alignment was made using MUSCEL implemented in MEGA V5.2[34], while the *rbcLX* and MIB synthase alignments were performed with ClustalW in BioEdit[35]. The hypervariable intergenic region between *rbcL* and *rbcX* genes [36] was removed from the analyses.

The phylogenetic trees were generated according to the Neighbor-Joining (NJ), Maximum Likelihood (ML) and Bayesian Inference (BI) methods. The phylogenetic trees according to the NJ analyses [37] were constructed with Tamura-Nei [38] as a genetic distance model implemented in program MEGA 5.2 [34]. The support values for the internal branches of NJ trees were estimated using the bootstrap method with 1000 replicates[39]. Substitution models as the best-fit model for ML and BI analyses were estimated with the Akaike Information Criterion (AIC) using MrModeltest v. 2.3[40]. The best-fit models of 16S rRNA, *rbcLX* and MIB synthase gene sequences were the GTR+ *I* + *G*. All parameters of selected modes are summarized in Table 2.

The ML analysis was conducted using PhyML 3.0 [41] with 100-fold bootstrap analysis. The BI was performed with MrBaye 3.1.2 [42] using Markov chain Monte Carlo algorithm. The program was run 12,000,000 generations for the partial16S rRNA data set, 4,000,000 generations for the *rbcLX* data set and 2,000,000 generations for the MIB synthase gene data sets, sampling trees every 100 generations until the standard deviation of split frequencies reached 0.01. To calculate Bayesian posterior probabilities (pp), the burn-in periods were determined at 105,000 trees for the partial 16S rRNA data set, 29,000 trees for the *rbcLX* data set and 5,000 trees for the MIB synthase gene sequence.

Table 2. Details of selected models of each datasets for ML and BI phylogenetic analyses.

Gene	Length (bp)	Alignment	Parameters for GTR model[a]		Substitution rates [b]				
			G	I	A-C	A-G	A-T	C-G	C-T
16s rRNA	746	MUSCLE	0.8434	0.3975	1.2553	1.5170	1.2827	0.6696	3.8381
rbcLX	756	ClustalW	0.8790	0.1961	1.6099	4.0284	1.3644	1.2734	5.3386
MIB synthase	988	ClustalW	1.4338	0.0354	2.1892	4.1593	2.5113	3.6829	3.3340

[a] AIC favored GTR model for all dataset.
[b] Calculated as G-T =1.0000.

Gas Chromatography-Mass Spectrometry

The pellet cells of Thai *Planktothricoides* strains tested were taken up in 1 ml of methanol and vortexed following the method of Kakimoto et al.[43] with a slight modification. The aliquot of each sample was sonicated at 30 °C for 20 min and then centrifuged at 2,000 × g for 5 min. The supernatant was transferred to a 1.5 ml of glass tube and directly used for gas chromatography (GC) analysis. A Varian (Varian Inc. CA, USA) Saturn 2200 tandem mass spectrometer (MS/MS) connected to a Varian 3800 GC was utilized to identify the geosmin and MIB

compounds from the Thai strains as described by Kakimoto et al[43]. Full scan electron impact mass spectra were recorded at a range of 40 to 200 m/z in a 0.55 total scan time. Peak identifications were aided by computerized mass spectra library and by interpretation of mass fragments. Furthermore, the mass spectrometers were also run in Selected-Ion Monitoring (SIM) in order to confirm the target compounds. The selected ions at m/z 95, 107, 121, 135, 150 were monitored for MIB, whilst m/z 112, 125, 126, 149, 182 were monitored for geosmin.

RESULTS

Morphological examination

The isolated *Planktothricoides* cells from cultures were morphologically examined using light microscopy. Most of the isolates in this study belong to the genus *Planktothricoides* based on the descriptions of Suda et al.[15] and presented typical characteristics of the *Planktothricoides raciborskii* Suda and Watanabe (Fig. 2). The trichomes of Thai strains were solitary, planktonic, or sometimes formed mats, settled to the bottom of the cultures tubes (Fig. 2). Most of the trichomes were unsheathed, straight, consisting of cylindrical cells, in terminal parts sometimes slightly curved, apical cells conical-rounded or rounded and without calyptra. They were constricted or indistinctly constricted at cross-walls. The color of the trichome was dark blue-green or yellow-green. The thick sections were also shown in a single trichome. These filaments were similar in cells that were much greater in width than in length.

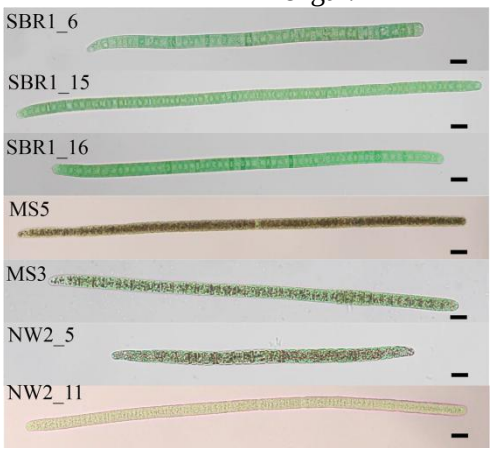

Figure 2. Photographs of Thai *Planktothricoides raciborskii* strains. Scale bar = 10 µm.

When measuring cell dimension, Thai *Planktothricoides* strains showed high morphometric variability, 1.86 to 5.96 µm in length and 2.83 to 13.70 µm in width. The length and width ratio (L/W ratio) ranged from 1:6 to 1:1. The average cell width (mean ± standard deviation) ranged from an average of 5.14±0.74 µm to 11.18±1.16 µm. The average cell length (mean ± standard deviation) ranged from 2.62±0.31 µm to 3.67±0.70 µm (data not shown).

In vivo and phycobilin pigment absorption spectra

The results indicated that the Thai strains used in this study followed somewhat the same patterns. *In vivo* absorption spectra had a large valley around 550 nm and two peaks at about 615 nm and 680 nm, respectively (data not shown). The first peaks were considered as phycocyanin (PC). These results were confirmed by the phycobiliprotein absorption spectrum presenting the maximum peak of PC at 615 nm (data not shown).

Phylogenetic analyses

Thirty-three 16S rRNA gene sequences from this study, 9 previous 16S rRNA sequences of *Planktothricoides* strains and other genera in GenBank were used to construct the phylogenetic trees using the NJ, ML, and BI methods. The final alignment of partial 16S rRNA sequences (746 bp) produced 173 variable sites and 127 parsimony informative sites. The phylogenetic trees derived from NJ, ML and BI analyses were nearly identical in topology; therefore, only the NJ tree is shown for clarity of illustration (Fig. 3A). In the partial 16S rRNA phylogeny, all Thai *Planktothricoides* strains formed a clade with moderate support values from the three analyses (NJ=93, ML= 88 and BI=0.51). Among *Planktothricoides* strains, phylogenetic trees based on the 16S rRNA sequences revealed that the *P. raciborskii* clade comprised two previously well-supported subclades, designated as A and B groups in this study. The B group was the earliest diverging species of the genus *Planktothricoides* clade containing two strains from Thailand (OR1-1 and MS4) with the well support value (NJ=86, ML= 81 and BI=1.00). The A group comprised with the published *P. raciboiskii* strains from Japan (type strain NIES-207, koyamaA and INBaOR), China (DP7.5.46 and CHAB3331), Australia (NSLA3 and NSLA4) and Thailand (T1-6-2) together with 18 new Thai strains (NJ=80, ML=61, BI=1.00) (Fig. 3A). Interestingly, there was a novel subclade containing 14 Thai strains (KP1_11, KP1_14, SK1, SP1_10, SK4, PLOS17, PLOS19, MS3, SP1_7, NW2_5, NW2_11, NW2_13, TK3_12, and TK1_14). The significantly supported values of this subclade obtained from NJ, ML and BI analyses were 98, 92 and 1.00, respectively. This subclade belonged to A group and was thus designed as a new subclade, termed as C group (Fig. 3A). Among the three different groups of *P. raciborskii* based on the partial 16S rRNA gene sequences, the similarity ranged from 96.8% to 98.4% and the genetic distances ranged from 0.016 to 0.031. In contrast, at the intraspecific level within each group, the similarity and genetic distances were around 98.4% to 99.9% and 0.001 to 0.016.

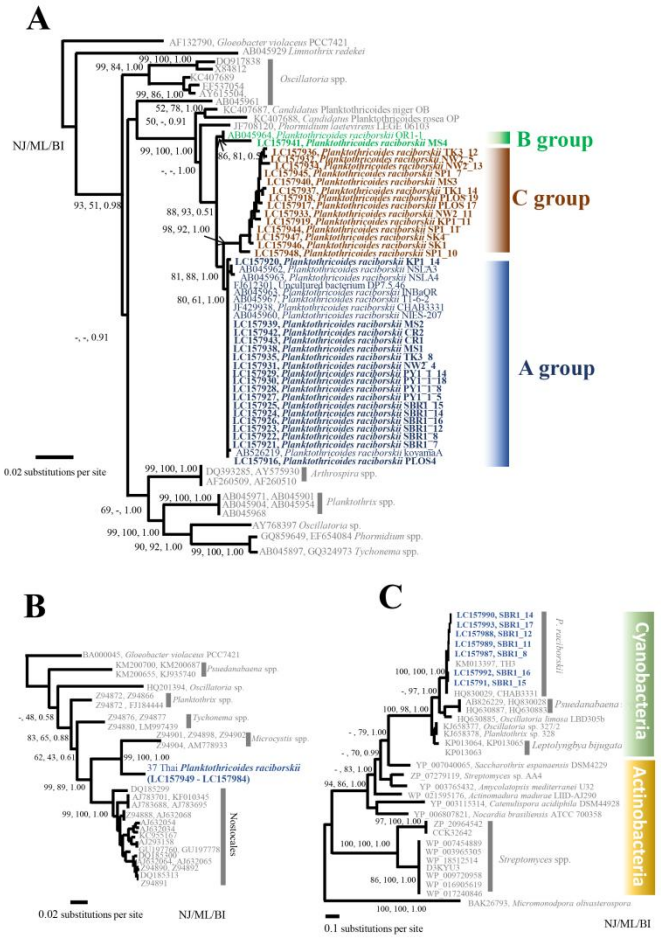

Figure 3. Phylogenetic analyses of Thai *Planktothricoides* strains. Node robustness was assessed by performing bootstrap values from NJ and ML analyses and posterior probability from BI analysis, respectively. Values below 50 aren't shown. A: NJ trees based on the alignment of partial 16S rRNA gene sequence (746 bp) of *Planktothricoides* strains with other cyanobacterial taxa. *Gloeobacter violaceus* PCC7421 was used as out group. Each color corresponds to a subclade of *Planktothricoides* in 16S rRNA phylogenetic trees, i.e. dark blue: Group A, green: Group B, brown: Group C.; B: ML trees based on the alignment of *rbcLX* gene sequence (757 bp) of *Planktothricoides* strains with other cyanobacterial taxa. *Gloeobacter violaceus* PCC7421 was used as out group. Thai strains used in this study are marked in dark blue and bold; C: ML based MIB synthase gene from cyanobacteria included Thai strains and closest actinobacteria. *Micromonospora olivasterospora* NRRL 8178 strain was used as an out group.

For phylogenetic analyses based on the *rbcLX* genes, 37 *rbcLX* gene sequences obtained from the Thai strains were obtained and aligned with reference sequences from GenBank. The final alignment containing the regions for about 256 bp *rbcL* and 500 bp *rbcX* produced 500 variable sites and 467 parsimony informative sites. The *rbcLX* trees made by NJ, ML and BI methods revealed that almost all topology positions were the same; therefore, only the ML tree is presented in Fig. 3B. The phylogenetic trees based on *rbcLX* sequences indicated that all Thai *Planktothricoides* strains formed a monophyletic clade with significantly supported values by NJ (99), ML (100) and BI (1.00) methods, respectively. This *Planktothricoides* clade was phylogenetically positioned between Nostocales and *Tychonema borurrellyi* clades with strong support values (NJ= 99, ML = 89 and BI = 1.00).

An approximately 988 bp of new MIB synthase gene sequence in the strains from Saraburi province was successfully amplified and subsequently aligned with MIB

synthase gene sequence of other MIB-producing cyanobacteria and actinomyces. The final alignment produced 848 variable sites and 740 parsimony informative sites. The MIB synthase phylogenetic trees determined from NJ, ML and BI methods revealed that almost all topology positions were the same; therefore, only the ML tree is presented in Fig. 3C. In the MIB synthase phylogeny, the monophyletic cyanobacteria branch formed a clade with other actinobacteria group. Among MIB-producing cyanobacteria strains, *Oscillatoria, Planktothrix* and *Leptolyngbya* branch was a basal sister branch, respectively. Subsequently, seven Thai *Planktothricoides* strains from Saraburi and two previous *Planktothricoides* sequences from Genbank (TH3 and CHAB3331) formed a clade with the robustly supported values from NJ=100, ML=100 and BI=1.00. This clade was the sister to the branch containing three strains of *Pseudanabaena* spp. and a strain of *Oscillatoria limosa* LBD305b. The MIB synthase gene sequences of Thai *Planktothricoides* strains had similarity of 99% compared with other MIB-producing *Planktothricoides* sequences.

Chemical analysis

To confirm the results of olfactory and MIB synthesis gene detections, three strains (SBR1_11, SBR1_15 and SBR1_16) representing the potential MIB-producing strains from Saraburi were selected for GC/MS/MS analysis. The odorous compounds were identified and confirmed according to the retention time and the ion chromatogram in the scan mode and SIM mode. Their total ion chromatography and mass spectrum revealed the compound peak with a retention time of 17.42 min according to MIB (Fig. 4). The specific mass spectrum ion for MIB from all three tested strains monitored by SIM mode revealed the molecular mass of 95, 107, 121, and 135, while the molecular mass for the geosmin did not show. As the results of GC/MS/MS, it can conclude that Thai *Planktothricoides* strains from Saraburi can produce the MIB compound.

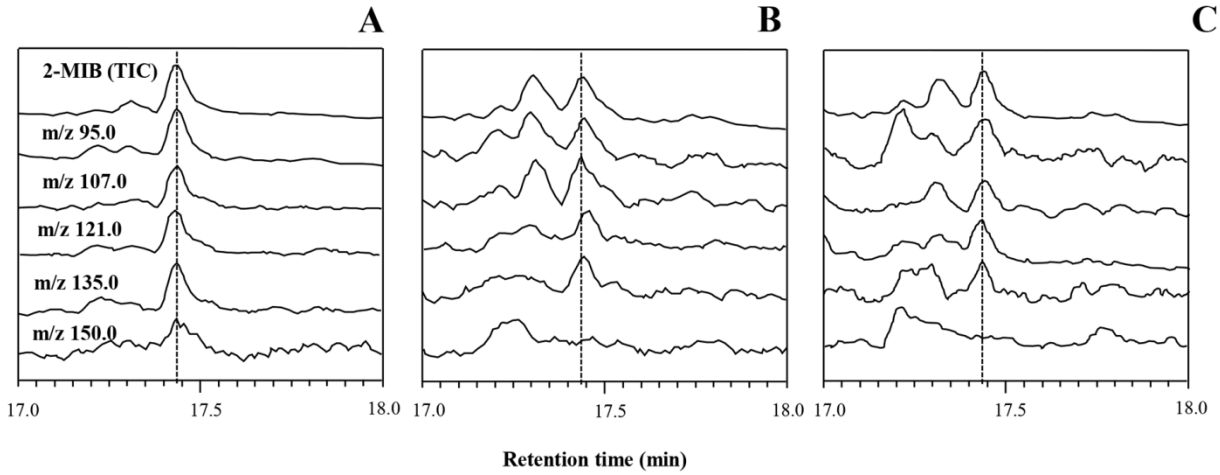

Figure 4. Total Ion Chromatograms (TIC) and Selected-Ion Monitoring (SIM) chromatogram according to MIB (m/z 95.0, 107.0, 121.0, 135.0 and 150) of Thai *Planktothricoides* strains SBR1_11 (A), SBR1_15 (B) and SBR1_15 (C) detected at around 17.42 min.

Distribution

All *Planktothricoides* strains used in this study were collected from fish ponds and fresh water resources in Northern (Chiang Rai, Phayao, Sukhothai, Tak, Kamphaeng Phet and Phisanulok), Northeastern (Maha Sarakham) and Central (Nakhon Sawan, Suphanburi and Saraburi) areas of Thailand (Fig. 1). The generic composition of *Planktothricoides* sp. based on 16S rRNA gene sequence was plotted onto a map of

Thailand. Furthermore, two previous strains isolated from a reservoir in Bangkok[15] were also included. During sampling, the temperature and pH in each sampling sites ranged from 26° C to 29° C and 7.5 to 9.0, respectively. Based on the 16S rRNA gene phylogenetic trees, most Thai *Planktothricoides* strains belonging to the A group were distributed in all three areas of Thailand. In this study, the strains forming the B and C groups were distributed in the Northeast and Central regions. The *Planktothricoides* strains isolated from Chiang Rai, Phayao and Saraburi only fell in the A group, while strains isolated from Sukhothai and Suphanburi belonged only to the C group.

DISCUSSION

In this study, during the period of cultivation, all strains (SBR1_6, SBR1_7, SBR1_8, SBR1_11, SBR1_12, SBR1_14, SBR1_15, SBR1_16 and SBR1_17) from Saraburi province were considered as potential odor-producing strains after olfaction. However, these strains do not differ morphologically from non-odor-producing strains. The *Planktothricoides* strains from Thailand were morphologically identical to the *Planktothricoides racborskii* corresponding to original description by Suda et al.[15]. Trichomes of Thai *Planktothricoides* strains appeared generally straight or slightly bent near the apex and attenuated towards the ends. These characteristics are different from *P. attenuate Komárek and Komárková-Legnerová* of which trichomes are always straight, wide in the middle and very gradually tapered towards ends[32]. Meanwhile, the genus *Planktothrix* showed trichomes that were not attenuated or attenuated towards the ends[32]. The type of phycobilin pigment composition is the morphological character corresponding to the original description of the genus *Planktothricoides*. Phycocyanin is a blue-colored pigment protein complex with the absorption maxima for phycocyanins found between 610 and 635 nm[44]. As the results of the pigment analyses, this study has demonstrated that Thai strains contained phycocyanin as dominant pigments belong to a group I-pc as with the designation of Suda et al.[15]. Meanwhile, cell dimensions are largely heritable across a range of organisms, including prokaryotes and eukaryotes [45- 48]. The results of morphometric information indicated that cell length, cell width and length to width ratio of Thai strains tested mostly overlapped with those of *P. raciborskii* strain NIES207, a type strain[15]. However, using cell size as a taxonomic criterion for cultured isolated of cyanobacteria can be problematic, since the character may vary according to the growth condition. This makes it difficult to define and to establish taxonomic limits for its identification [49-51]. Considering all results of morphological analyses, all Thai *Planktothricoides* strains tested were identified as *P. raciborskii*. Furthermore, this study suggests that both non-odor-producing and odor-producing *Planktothricoides* strain cannot be distinguished clearly using the basic information of morphological characters. Thus, in order to confirm these observations, it is necessary to increase the number of morphological analyses in the genus *Planktothricoides*.

The partial 16S rRNA genes cover approximately the first half of the gene, where most of the variable sites reside [52]. The results, based on the partial 16S rRNA gene sequences trees using the cyano-specific primers [53], are in accord with the earlier studies [15, 54], indicating a monophyletic clade of the genus *Planktothricoides*. Corresponding to the 16S rRNA phylogenetic result of Suda et al. [15], the previous two subclades, named in this study as A and B groups, were also obtained from Thai *Planktothricoides* strains. Interestingly, an outcome of the present study was the discovery of a novel subclade and designated as C group. However, the 16S rRNA gene phylogeny results have revealed inconsistencies with the morphological

classification of each group. This may be found in several cyanobacterial species in which differences in morphology are not reflected in analyses of 16S rRNA gene [55, 56, 57]. The cut-off points of 97.5% and 95% 16S rRNA gene sequence similarity have been suggested for bacterial species and genus definition, respectively (Ludwig et al., 1998). Bosshard et al. [59] and Janda and Abbott [60] mentioned that the given ≥99% similarity of 16S rRNA gene sequence is regarded to be the type strain or reference strain of species. According to those definitions, the existence of cryptic species was also suspected from Thai strains in this study when 16S rRNA gene similarity of < 97.5% was observed. This finding suggests that the *Planktothricoides* strains in three subclades within the genus *Planktothricoides* probably evolved separately. Moreover, cryptic diversity can be expected as still limited sequences from a few geographical areas are available for this species. Thus, the examination based on the morphological features, ecology and secondary metabolite profile of each subclade in detail are necessary.

In this study, the result of the *rbcLX* phylogeny was incongruity compared with that the partial 16S rRNA phylogeny with the absence of three subclades. This difference may be because the *rbcLX* genes are too conservative to determine at the subclass level [61, 62], while the 16S rRNA genes can provide a much higher degree of resolution among cyanobacterial taxa than either morphological or chemical traits [63]. Rudi et al.[36] also found that the topologies of the 16S rRNA gene and *rbcLX* trees were not congruent for genetically closely related *Nostoc* and *Anabaena* strains. Different regions of the genome may have experienced different evolutionary pressure, promoting an incomplete lineage sorting, leading to a divergence in the fixation rates among the locus and the taxon itself [64]. Considering the phylogenetic analysis results in this study, the 16S rRNA genes are more suitable for distinguishing genetic diversity within the genus *Planktothricoides* at the subclade level.

Genes (MIB synthase) amplified in this study showed sequence homology with other MIB-producing cyanobacterial species from previous studies [16, 26, 65-67]. Corresponding to the suggestion of Wang et al.[16], all 2-MIB-producing genes in cyanobacteria may have a common origin showing that homologous genes can be found between *Pseudanabaena* sp. dqh15 and *P. raciborskii* CHAB 3331. Moreover, the results of 16S rRNA gene phylogenies revealed that all MIB-producing *P. raciborskii* strains from Thailand and China (CHAB3331)[16] were grouped together with non-MIB-producing strains into the A group. This present study demonstrated that a single distinct group of MIB-producing strains cannot be supported as analyzed by 16S rRNA genes including *rbcLX* genes. The results can only indicate that the *Planktothriocoides* strain grouped in the A group should probably be considered as a potential MIB producer. On other hand, this study has hypothesized that MIB-producing strains within the genus *Planktothricoides* can coexist and distribute the ability of MIB production with non-MIB-producing strains due to horizontal gene transfer (HGT); for example, Nakasugi et al. [68]. Previous studies implied that the process of horizontal gene transfer may play a role in the distribution of hepatoxic strains within the genus *Microcystis* [69-71]. However, there is no clear experimental evidence which favors horizontal gene transfer as a mechanism to explain the distribution of MIB-producing strains in cyanobacteria. Alternatively, the lack of the MIB synthase genes of non-MIB-producing Thai *Planktothricoides* strains may consequently be due to losses of this gene during evolution. Similarly, preliminary data suggest that the loss of *mcy* genes seem to be a continuous process starting with a loss of function by mutation[72]. Thus, future research should also be focused on finding appropriate genetic markers to

conclusively investigate evolutionary relationships of MIB-producers within the genus *Planktothricoides*.

The qualitative confirmation of MIB compounds in GC/MS/MS analyses is needed with a minimum of four corresponding Selected-Ions Monitoring (SIM) analysis. The results of SIM mode in this study showed that peaks of m/z values according to MIB compound[73] were detected from Thai *Planktothricoides* strains isolated from Saraburi province. Thus, the results of the olfaction in the initial cultivation and GC/MS/MS analysis corresponded with those of the MIB synthase gene phylogenetic analyses, considering that Thai *Planktothricoides* strains isolated from Saraburi province were MIB producers. These results are useful to provide genetic information from Thai *Planktothricoides* species in order to prevent the risk of taste-odor events caused by the cyanobacterial origin of MIB. Future research could use molecular technologies to monitor odor-producing cyanobacterial species and investigate the relationship between odor production and the corresponding synthesis gene.

An interesting characteristic of cyanobacteria is their broad geographical distribution, which reflects the group's genotypic and phenotypic variation. The dispersal capability of the genus *Planktothricoides* in each region of Thailand is still poorly understood. Based on the 16S rRNA genes, the present study expected that the *Planktothricoides* strains belonging in the A group is the cosmopolitan species which can be found in all areas of Thailand. Depending on the aquatic environmental factors associating to chemical and physical parameters, the concentration of cyanobacteria can increase and produce unpleasant odor-causing compound in drinking water and aquaculture production. Addition research is needed to determine the distribution, abundance as well as environmental factors affecting growth of *Planktothricoides* species, in particular with MIB-producing strains, in several freshwater resources.

CONCLUSIONS

In conclusion, the results of morphological and phylogenetic (16S rRNA and *rbcLX* gene sequences) analyses revealed that all Thai *Planktothricoides* strains used in this study could be designated as *Planktothricoides raciborskii* (Woloszynska) Suda & Watanabe. MIB-producing and non-MIB-producing strains could not be distinguished using morphological features. The 16S rRNA gene sequences of Thai *Planktothricoides* strains fell into three different subclades (A, B and novel C groups), indicating the genetic variation into the genus *Planktothricoides*. From the detection of MIB synthase genes and GC/MS/MS analysis, strains from Saraburi were considered as MIB-producing strains. Moreover, the 16S rRNA phylogeny showed that the MIB-producing strains including Thai strains from Saraburi fell entirely into the A group. This information is provided for the development of quantitative molecular technologies to monitor odor-producing cyanobacterial species in environmental samples. In-depth studies on the environmental factors affecting the growth characterization and MIB bioaccumulation will also be required.

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Erratum

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