Molecular characterizations of Thai *Raphidiopsis raciborskii* (Nostocales, Cyanobacteria) based on 16S rDNA, *rbcLX*, and cylindrospermopsin synthetase genes

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**Abstract:** *Raphidiopsis* (previously *Cylindrospermopsis*) *raciborskii* is considered as a cylindrospermopsin (CYN) producer in Thailand. There are no reports including molecular information on the genes associated with CYN production of cyanobacteria from Thailand. In the present study, 27 new *R. raciborskii* strains from Thailand were isolated. Their morphological features and phylogenetic positions based on the partial sequences of 16S rDNA and *rbcLX* were analyzed for identification. Morphological characteristics of all the strains tested, and the natural samples, were found to be similar to the original description of *R. raciborskii*. The *rbcLX* phylogenetic trees revealed that the clade of *R. raciborskii* is independent from those of *R. mediterranea* and *R. curvata*, whereas the 16S rDNA phylogenetic trees failed to discriminate these species. Thus, all the strains tested in the present study were identified as *R. raciborskii*. Furthermore, the LC/MS/MS analysis confirmed that some of the strains tested produced CYN and 7-deoxy-CYN. The presence of genes associated with CYN biosynthesis (*cyrA*, *cyrB*, *cyrC*, and *cyrJ*) in Thai *Raphidiopsis* strains was investigated by PCR and phylogenetic analyses. Results of PCR showed that the PCR amplicons of *cyrB* and *cyrJ* were obtained from all toxic strains, while the amplicons of *cyrA* and *cyrC* were not obtained in some toxic Thai strains. Our findings supported the observation that *cyrB* and *cyrJ*, especially the latter, may be useful for the detection of CYN-producing cyanobacteria in water resources for water supplies and fisheries.

**Key words:** 16S rDNA, Cylindrospermopsin biosynthesis gene, *Raphidiopsis raciborskii*, *rbcLX*, Thailand

**Introduction**

Toxins produced by cyanobacteria are well known to be potentially harmful compounds to human and animal health (Soares et al. 2006). Furthermore, these toxins have chronic effects on humans subjected to long-term contact with hepatic toxins and neurotoxins (Bittencourt-Oliveira et al. 2014). Currently, occurrences of toxic cyanobacterial blooms are frequently reported in Thai freshwaters. The main toxic cyanobacterial genera found in the freshwaters of Thailand are microcystin producers; *Microcystis aeruginosa* Kütz (Sengpracha et al. 2006, Somdee et al. 2013), and a cylindrospermopsin (CYN) producer, *Cylindrospermopsis raciborskii* Seenayya & Subba Raju (Chonudomkul et al. 2004). Recently, Aguilera et al. (2018) proposed the unification of the genera *Cylindrospermopsis* Seenayya & Subba Raju and *Raphidiopsis* Fritsch & Rich under the single genus *Raphidiopsis* due to their close evolutionary relationships. The name *Raphidiopsis* was retained as the...
genus name since it has nomenclatural priority over the name *Cylindrospermopsis* (Aguilera et al. 2018). Hereafter, *C. raciborskii* is referred to as *R. raciborskii*. Although monitoring the potential toxicity of these cyanobacteria in freshwater resources for drinking water is important, it has not been routinely conducted in Thailand up to now.

Cylindrospermopsis (CYN) is a tricyclic alkaloid produced by some cyanobacterial genera including the genus *Raphidiopsis* (previously reported as *Cylindrospermopsis*) (Ohtani et al. 1992, Li et al. 2001, Chonudomkul et al. 2004, Wimmer et al. 2014). Because CYN can accumulate in the water bodies and the aquatic food chain, it has the potential to cause serious human and aquatic animal health risks. CYN can interfere with multiple cellular metabolic processes (Frosicio et al. 2003) and cause injury in several organs including the liver, thymus, kidney, and heart in humans and other animals (Terao et al. 1994, Yang et al. 2017). Two naturally occurring epimers of CYN, 7-epicylindrospermopsis (Banker et al. 2000) and 7-deoxy-cylindrospermopsis (7-deoxy-CYN) (Norris et al. 1999) have also been identified. The first report of CYN and its epimers producing *R. raciborskii* in Thailand and South East Asia was reported by Li et al. (2001) who discovered that *R. raciborskii* (strain CY-Thai) could produce CYN and 7-deoxy-CYN. Then, Chonudomkul et al. (2004) reported that CYN was detected in some *R. raciborskii* strains isolated from various reservoirs in Thailand. Recently, it was demonstrated that the *R. raciborskii* strain can produce two new CYN analogs; 7-deoxy-desulfo-CYN, and 7-deoxy-desulfo-12-acetylcylinindrospermopsis (Wimmer et al. 2014). However, it is not clear whether these are CYN congeners, precursors or degradation products.

The cyanobacterial species *Raphidiopsis raciborskii* has been found present in the phytoplankton community of several freshwater environments (Figueroedo & Giani 2009, Sinha et al. 2012). The morphology of *R. raciborskii* is very similar to that of *Cylindrospermopsis* Skuja and *Raphidiopsis curvata* Fritsch & Rich and they can co-occur in nature (Moustaka-Gouni et al. 2009, McGregor & Fabbro 2000, Aguilera et al. 2018). This is a major problem for discrimination between these all species because they present coiled or straight trichomes, and develop akinetes in a single unit or adjacent pairs. The morphological difference between these species is the existence or not of heterocyte cells (Moustaka-Gouni et al. 2009): *R. raciborskii* with straight and coiled trichomes develop heterocyte cells, while *R. mediterranea*, characterized by the straight trichomes, and *R. curvata*, distinguished by the coiled trichomes, do not develop heterocyte cells. Several attempts have been made to elucidate the taxonomic relationships between these species in the genus *Raphidiopsis* using molecular techniques; however, their phylogenetic relationships suggest a monophyletic group (Wu et al. 2011, Komárek 2013, Li et al. 2016, Aguilera et al. 2018). In terms of toxicity, toxins produced by *R. raciborskii* are known to vary among strains: some Australian strains produce CYN, while some Brazilian strains produce saxitoxin (STX) (Hoff-Rissetti et al. 2013). Blooms of *Raphidiopsis* have been reported worldwide in tropical (Figueroedo & Giani 2009) and subtropical (Everson et al. 2011) to temperate climate zones (Sinha et al. 2012). The occurrence of *R. raciborskii* is very common in Thai freshwaters, which are extensively used for drinking water, recreational, and agricultural purposes (Somdee et al. 2013). One previous study reported that a Thai *R. raciborskii* strain CY-Thai had mouse toxicity (minimum lethal dose of 250 mg dry weight cells/kg body weight within 24 h and 125 mg/kg at 72 h) (Li et al. 2001). However, data on the toxic content and toxigenicity is still scarce.

The morphological characteristics of cyanobacteria do not always correspond to their taxonomic position and, therefore, the use of molecular markers for phylogenetic studies has become essential for purposes of identification/differentiation. At present, phylogenetic analyses utilize various genes; the 16S rDNA, 16S-23S rDNA internal transcribed spacer (ITS), rpoC1 locus, and the ribulose-bisphosphate carboxylase (rbc) genes (Wu et al. 2011, Sciuto & Moro 2016, Aguilera et al. 2018). Based on several phylogenetic markers (epcBA-IGS, niFH, and 16S rDNA), Panou et al. (2018) reported that *R. raciborskii* strains originating from the Mediterranean region shared a common ancestor with strains from the American continent, whereas the strains from Central Europe and Portugal, Asia, and Australia may have their origin in the African continent. Furthermore, DNA-based approaches for cyanotoxin detection have been widely employed and can be achieved by amplifying several genetic markers, such as genes encoding for microcystin, cylindrospermopsin, anatoxin-a, saxitoxin and nodularin (Hoff-Rissetti et al. 2013, Lorenzi et al. 2015). A biosynthetic pathway for CYN production has been proposed based on the deduced function of each CYN gene (*cyr*) (Mihali et al. 2008). In particularly, CYN-producing *R. raciborskii* is associated with the presence of the *cyr* gene clusters (*cyrA–O*) (Sinha et al. 2014), which present some variation among toxic strains (Jiang et al. 2014). A putative CYN biosynthesis gene cluster encoding amidinotransferase (*cyrA*), one mixed PKS/NRPS module (*cyrB*), four PKS modules (*cyrC–cyrF*) and additional tailoring enzymes has been described and a pathway for CYN synthesis has been proposed (Kellmann et al. 2006, Mihali et al. 2008). Moreover, microcystin synthetase genes were also present in *R. raciborskii* TAU-MAC 1414 from Greece, but the presence of microcystin-LR was not confirmed using LC-MS/MS (Panou et al. 2018). Although chemical analyses have been conducted to confirm the production of CYN and its analogues (Li et al. 2001, Wimmer et al. 2014), the genes responsible for the biosynthesis of CYN in Thai *R. raciborskii* strains have not been reported.

In the present study, to provide new insight into their CYN biosynthesis, *Raphidiopsis* strains were isolated from several freshwater sources in Thailand. Morphological and molecular analyses were carried out to identify the con-
Molecular characterizations of Thai Raphidiopsis species. Genes in the CYN gene clusters, \textit{cyr}A, \textit{cyr}B, \textit{cyr}C, and \textit{cyr}J were also investigated in the Thai \textit{R. raciborskii} strains. Furthermore, we analyzed their CYN and 7-deoxy-CYN production by LC/MS/MS analysis and tried to find molecular markers specific to toxic strains of \textit{R. raciborskii} that would facilitate their monitoring in Thailand.

### Materials and Methods

#### Strain isolation, cultivation, and microscopic observation

\textit{Raphidiopsis} strains were isolated, using the micropipette-washing method, from four freshwater sources in Thailand between 2016 and 2017: Mae La River in Sing Buri (SBR) province (14°56′41.3 N, 100°19′58.7 E); ponds in Phitsanulok (PLK) (7°12′45.2 N, 100°35′17.0 E), and

Table 1. List of Thai \textit{Raphidiopsis raciborskii} strains used in this study showing the results of cylindrospermopsin gene (\textit{cyr}) amplification based on \textit{cyr}A, \textit{cyr}B, \textit{cyr}C, and \textit{cyr}J and LC/MS/MS analysis based on cylindrospermopsin (CYN) and 7-deoxy-CYN.

| Strain code | Sampling location | 16S rDNA | \textit{rbcLX} | \textit{cyr} gene amplification | LC/MS/MS |
|-------------|-------------------|----------|---------------|-------------------------------|----------|
|             |                   |          |               | \textit{cyr}A \*1 \textit{cyr}B \*2 \textit{cyr}C \*3 \textit{cyr}J \*4 | CYN 7-deoxy-CYN |
| NUPLC1      | Fish Pond, Maung, Phitsanulok | +       | +  | -  | -  | -  | -  | -  | -  |
| NUPLC2      | Fish Pond, Maung, Phitsanulok | +       | -  | -  | +  | +  | -  | -  | -  |
| NUPLC3      | Fish Pond, Maung, Phitsanulok | +       | -  | -  | +  | +  | -  | -  | -  |
| NUPLC6      | Fish Pond, Maung, Phitsanulok | +       | -  | -  | -  | -  | +  | +  | +  |
| NUPLC7      | Fish Pond, Maung, Phitsanulok | +       | -  | -  | +  | +  | -  | -  | -  |
| NUPLC11     | Fish Pond, Maung, Phitsanulok | +       | -  | -  | +  | +  | -  | -  | +  |
| NUPLC12     | Fish Pond, Maung, Phitsanulok | +       | -  | -  | +  | +  | -  | -  | -  |
| NUPLC13     | Fish Pond, Maung, Phitsanulok | +       | -  | -  | +  | +  | -  | -  | -  |
| NUPLC20     | Fish Pond, Maung, Phitsanulok | +       | -  | -  | -  | -  | +  | +  | +  |
| NUPLC23     | Fish Pond, Maung, Phitsanulok | +       | -  | -  | -  | -  | +  | +  | +  |
| NUPLC26     | Fish Pond, Maung, Phitsanulok | +       | -  | -  | +  | +  | -  | -  | -  |
| NUPLC27     | Fish Pond, Maung, Phitsanulok | +       | -  | -  | +  | +  | -  | -  | -  |
| NUPLC32     | Fish Pond, Maung, Phitsanulok | +       | -  | -  | +  | +  | -  | -  | -  |
| NUSBR1      | Mae La River, Bang Rachan, Sing Buri | +     | + | + | + | + | + | + | + |
| NUSBR1_1    | Mae La River, Bang Rachan, Sing Buri | +     | + | + | + | + | + | + | + |
| NUSBR2      | Mae La River, Bang Rachan, Sing Buri | +     | + | + | + | + | + | + | + |
| NUSBR3      | Mae La River, Bang Rachan, Sing Buri | +     | + | + | + | + | + | + | + |
| NUSBR5      | Mae La River, Bang Rachan, Sing Buri | +     | + | + | + | + | + | + | + |
| NUSPBC2     | Bueng Chawak Lake, Doem Bang Nang Buat, Suphan Buri | +     | + | + | + | + | + | + | + |
| NUSPBC3     | Bueng Chawak Lake, Doem Bang Nang Buat, Suphan Buri | +     | + | + | + | + | + | + | + |
| NUSPBC4     | Bueng Chawak Lake, Doem Bang Nang Buat, Suphan Buri | +     | + | + | + | + | + | + | + |
| NUSPBC5     | Bueng Chawak Lake, Doem Bang Nang Buat, Suphan Buri | +     | + | + | + | + | + | + | + |
| NUSPBC7     | Bueng Chawak Lake, Doem Bang Nang Buat, Suphan Buri | +     | + | + | + | + | + | + | + |
| NUSLAC1     | Pond, Muang, Songkhla | +     | + | + | + | + | + | + | + |
| NUSLAC2     | Pond, Muang, Songkhla | +     | + | + | + | + | + | + | + |
| NUSLAC3     | Pond, Muang, Songkhla | +     | + | + | + | + | + | + | + |

\*1: primer set of CYLAT-F/CYLAT-R (Kellmann et al. 2006).
\*2: primer set of CPS-F/CPS-R (Kellmann et al. 2006).
\*3: primer set of A205PK-F/A205PK-R (Kellmann et al. 2006).
\*4: primer set of cyr-F/cyr-R (Mazmouz et al. 2010).

+ : gene or toxin presence; − : gene or toxin absence; nt: non-tested strain.
DNA extraction, PCR amplification, and sequencing

All PCRs were performed using genomic DNA extracted by the chelex-based DNA isolation method (Richlen & Barber 2005). Briefly, the cell pellets were dissolved in 100 μL of 10% (w/v) solution of Chelex100 resin (Bio-Rad, Hercules, CA) and incubated at 95°C for 20 min with thorough mixing every 10 min. Then, the tubes were centrifuged at 1,500 g for 1 min. The supernatant containing genomic DNA was used as a template for PCR amplification. The partial fragment of 16S rDNA was amplified using the primer set 27F1/1492Rc (Neilan et al. 1997). The region containing the ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit (rbcL) gene and the chaperonin-like protein (rbcX) gene was amplified with the primer set CW/CX (Rudi et al. 1998). In addition, the cyrA, cyrB, cyrC, and cyrJ genes, involved in the CYN biosynthesis, were also amplified using the primer sets CYLAT-F/CYLAT-R, CPSF-F/CPSF-R, A205PK-F/A205PK-R (Kellmann et al. 2006), and cyr-F/cyr-R (Mazmouz et al. 2010), respectively.

All PCR processes were conducted using the Tks-Gflex™ DNA Polymerase (TaKaRa, Otsu, Japan) according to the manufacturer’s protocol. All amplifications were performed under the thermal cycling conditions: 35 cycles at 98°C for 10 sec; 57°C for 15 sec; 68°C for 45 sec and subsequently 4°C. PCR products were purified using 30% (w/v) polyethylene glycol (PEG) 8000 precipitation (Hartley & Bowen 2003). PCR products were sequenced with the primers 27F1 and 809R (Jungblut et al. 2005) for the 16S rDNA and the same PCR primers for the rbcLX and the CYN biosynthesis genes. Direct sequencing was conducted using a Big Dye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems Japan, Tokyo, Japan) following the protocol provided by the manufacturer.

Phylogenetic and sequence analyses

The sequence data sets for each gene were separately aligned with the reference sequences derived from GenBank using ClustalW (Thompson et al. 1994) in the UniPRO UGENE program (Okonechnikov et al. 2012). Phylogenetic trees were analyzed by the Neighbor-Joining (NJ), Maximum likelihood (ML), and Bayesian inference (BI) methods. The NJ trees were constructed using the MEGA version 6.0 program package (Tamura et al. 2013) with the p-distance model and bootstrapping (1,000 replicates). As the best-fit model obtained from MrModeltest 2.3 (Nylander 2004), the GTR+G+I for 16S rDNA, GTR+I for rbcLX and cyrJ, K80+G for cyrA, GTR for cyrB, and GTR+G for cyrC were used. Synechococcus elongatus PCC6301 was used as an outgroup for both 16S rDNA and rbcLX phylogenies. Sequences used as the outgroups for the cyrA, cyrB, cyrC, and cyrJ trees were determined following Hoff-Rissetti et al. (2013) or McGregor & Sendall (2015).

The ML analyses were conducted using the PhyML 3.0 program (Guindon et al. 2010) with the model obtained from MrModeltest 2.3 and node confidence was assessed by 100 bootstrap (bt) replicates. The BI analyses were performed with MrBayes software 3.2 (Ronquist et al. 2012) with the same model used in the ML analyses. One partition was used for each analysis and with four runs of four chains, sampling from the chain every 100 generations. After the standard deviation values of the two runs dipped below 0.01, at least 10,000 trees were sampled to calculate the posterior probabilities (pp). In the present study, the NJ, ML, and BI phylogenetic trees based on each gene were identical. For reasons of clarity and simplicity, only the NJ tree has been shown in the present study. The uncorrected genetic distances (p) between the sequences were calculated using the p distance option in the program MEGA 6.0 (Tamura et al. 2013). The nucleotide sequences reported in the present study were deposited in the DDBJ database under the GenBank accession numbers: LC422957–LC422982 (16S rDNA), LC422983–LC422996 (rbcLX), LC423038–LC423043 (cyrA), LC423024–LC423037 (cyrB), LC423011–LC423023 (cyrC), and LC422997–LC423010 (cyrJ).

Cylindrospermopsis (CYN) analysis

For the preparation of crude extract, 1 mL of a culture containing Thai R. raciborskii cells at the exponential growth phase was frozen and thawed at least twice, as described by B-Bères et al. (2015). To confirm the presence of CYN and its analogues in the Thai strains, CYN and 7-deoxy-CYN concentrations were determined in the aqueous extract (intracellular and extracellular) by liquid chromatography-mass spectrometry analysis on a Triple Quadrupole Varian 320 MS (LC/MS/MS) (Varian, Palo Alto, CA, USA); 20 μL of each aqueous extract was in-
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subjected onto a Pursuit XRs 3 C18 column (150×4.6 mm; Agilent Technologies, Waldbronn, Germany) coupled to a double mass spectrometer in tandem (Varian 320MS-MS) at 50°C and a flow rate of 0.3 mL/min (solvent A: 0.1% formic acid/deionized water, solvent B 0.1% formic acid/acetonitrile). Based on the analysis of molecular ion masses and the target MS/MS spectrum, the mass spectrometer, equipped with an electrospray ionization (ESI) source, was operated in the total ion chromatograms (TIC) mode for scans m/z from 100 to 800 and the single ion monitoring (SIM) mode. The toxin analogues were determined by parent ions (m/z 416 for CYN and m/z 400 for 7-deoxy-CYN) and corresponding mass transition (m/z 416/336, 416/194 and 416/176 for CYN and m/z 416/176 and 416/194 for 7-deoxy-CYN) as per the description of Pekar et al. (2016).

Results

Morphological characterization

In the present study, 27 new Raphidiopsis strains were established from Thai freshwaters (Table 1). Of these, 15 selected Thai Raphidiopsis strains and three natural populations used in the present study displayed the following trichomes: solitary, straight, slightly tapered at one or both ends, no sheath external to trichome (Fig. 1). Vegetative cells were cylindrical (Fig. 1). The heterocyte cells were observed terminally at one or both ends of the trichome (Fig. 1). Furthermore, trichomes without heterocyte cells were also observed in almost all of the cultured strains (Fig. 1). The morphological characteristics of the sampled Thai strains and natural populations of Raphidiopsis were similar to the typical characteristics of the genus Raphidiopsis reported in the current classification system of Komárek & Komárková-Legnerová (2003). Meanwhile, cell dimensions in the tested strains and in natural populations were found to be highly variable. The vegetative

![Fig. 1](image1.png)

Micrographs of the Thai Raphidiopsis raciborskii strain NUPLKC32 in the present study, showing various morphological characters such as different length of trichome or non-terminal heterocyte cells. Arrow indicates the sharply pointed terminal cell as a non-common morphological feature, H: heterocyte, Scale bars: 10 μm.

![Fig. 2](image2.png)

Morphometric information of Thai Raphidiopsis raciborskii strains and natural populations observed in the present study, compared with those from previous studies. R.: Raphidiopsis, NP: natural population. Lines depict minimum to maximum values. Open circles are average values. Bars are standard deviation values.
cell dimensions of cultured strains ranged from 5.11 to 17.77 (average 10.03 ± 4.46) µm in length and from 1.75 to 5.12 (average 3.10 ± 0.29) µm in width. Their heterocyte cells were found to be ellipsoidal. The heterocyte cell dimensions of cultured strains ranged from 3.57 to 12.41 (average 6.55 ± 1.11) µm in length and from 1.78 to 4.26 (average 2.90 ± 0.32) µm in width (Fig. 2). The vegetative cell dimensions of natural populations were 5.41 to 12.78 (average 8.26 ± 0.09) µm in length and 2.10 to 3.82 (average 3.02 ± 0.70) µm in width (Fig. 2). All the cultured strains tested and the natural populations used in the present study did not form akinete cells. The straight trichomes of the cultured strains never became the coiled form and sharply pointed terminal cells, as a non-common morphological feature, were observed under laboratory culture conditions. Furthermore, cells without heterocyte cells isolated from the natural populations formed heterocyte cells under culture conditions (data not shown).

Phylogenetic analyses based on the partial 16S rDNA and rbcLX

To identify the Thai strains used in the present study, the partial 16S rDNA and rbcLX sequences were used for phylogenetic analyses. Based on the partial sequences of 16S rDNA, the results showed that all Thai strains and other strains of Raphidiopsis species were intermixed and formed a single Raphidiopsis clade (BI=0.98, ML=60, and NJ=99) (Fig. 3A). The average genetic (p) distance based on the partial sequences of 16S rDNA within the Raphidiopsis clade was 0.001 (data not shown).

Phylogenetic trees were also constructed based on the sequences of rbcLX using 14 new Thai Raphidiopsis strains and 26 reference cyanobacterial strains (Fig. 3B). The rbcLX trees showed that the Raphidiopsis clade was formed with robust support values (BI=1.00, ML=99, and NJ=100) (Fig. 3B). In this clade, three strains of R. curvata (CHAB114, CHAB341, and CHAB1150) from China formed an early diverged branch (BI=0.98, ML=95, and NJ=100). The branch was followed by a clade comprised of five strains of R. mediterranea (CHAB352, CHAB3326, CHAB2314, CHAB1355, and CHAB1350) from China (BI=1.00, ML=99, and NJ=99). It was followed by a R. raciborskii clade which could be well-differentiated from a R. mediterranea clade with moderate support values (BI=0.86, ML=0.85, and NJ=99) (Fig. 3B). Within the R. raciborskii clade (BI=0.89, ML=93, and NJ=98), two subclades were identified. The first clade (I) comprised two Thai strains (NUSLAC2 and NUSBRC3) and two strains (CHAB1331 and CHAB1355) from China with weak support values (BI=0.59, ML=0.50, and NJ=100) (Fig. 3B). The second clade (II) comprised two new Thai strains (NUSBRC1 and NUSBRC2) and two strains (CHAB114 and CHAB341) from China with strong support values (BI=1.00, ML=99, and NJ=100) (Fig. 3B).

Fig. 3. Neighbor-joining (NJ) phylogenetic trees based on the partial sequences of 16S rDNA (A) and rbcLX (B) of Thai Raphidiopsis raciborskii strains and other cyanobacterial strains from GenBank. The strains established in the present study are shown in bold and black color. Posterior probabilities (pp)>0.5 and bootstrap support values (bt)>50% for individual nodes are shown on the tree based on BI/ML/NJ analyses, respectively. The hyphen-minus (-) shown on some branches is the support value of pp<0.5 or bt<50%. Branch length is proportional to the number of substitutions per site. In the 16S rDNA phylogeny, the box indicates the strains of R. raciborskii having identical sequences. Black circles denote CYN-producing Thai strains and white circles denote non-CYN-producing Thai strains.
support values (Fig. 3B). The second subclade (II) was composed of Thai strains (NUPLKC1, NUSBRC3, NUSPBC3, NUSPBC6, NUSPBC7, NUPLKC11, NUPLKC12, NUPLKC27, NUPLKC2, NUPLKC3, NUPLKC32, NUPLKC27, NUSBRC1, NUSBRC1, NUSBRC1_1, NUSBRC6, and NUSBRC5) and strain CHAB353 from China with weak support values. Based on the *rbcL* sequences, the *p* value between the *R. raciborskii* subclades I and II was 0.004 (Table 2). Among the three *Raphidiopsis* clades, the *p* values ranged from 0.014 to 0.038 (Table 2). Considering the results of these phylogenetic analyses together with morphological observations described above, Thai strains used in the present study were identified as *R. raciborskii*.

**PCR amplification and phylogenetic analyses based on sequences of the CYN biosynthesis gene clusters**

Subsequently, the presence of genes responsible for CYN biosynthesis, including the regions of *cyrA*, *cyrB*, *cyrC*, and *cyrJ*, in *Raphidiopsis raciborskii* strains was analyzed by PCR amplification. The PCR results showed that the *cyrA* gene was amplified from six Thai strains (NUSBRC1_1, NUSBRC1, NUSBRC6, NUSBRC7, NUPLKC27, and NUPLKC32) (Table 1). The phylogenetic tree based on the *cyrA* sequences depicted a topology revealing that six of the Thai strains used in the present study were intermixed in a clade containing the cyanobacterial strains including *R. raciborskii*, *Aphanizomenon* sp., and *R. curvata*, respectively (BI=0.89, ML=99, and NJ=99) (Fig. 4A).

Fourteen Thai strains (NUPLKC7, NUPLKC13, NUSBRC6, NUPLKC12, NUPLKC11, NUPLKC27, NUPLKC32, NUPLKC2, NUPLKC3, NUSBRC1, NUPLKC26, NUSBPC7, NUSBRC1_1, and NUSBRC5) possessed a region with *cyrB* (Table 1). The *cyrB* phylogenetic tree revealed that Thai strains grouped together with previous strains of *Raphidiopsis raciborskii*, *Aphanizomenon* and *R. curvata*, respectively (BI=1.00, ML=88, and NJ=92) (Fig. 4B).

PCR amplicons of *cyrC* were obtained from 13 Thai strains (NUPLKC26, NUSBPC7, NUPLKC32, NUPLKC11, NUPLKC12, NUPLKC27, NUPLKC3, NUPLKC7, NUPLKC13, NUSBRC1, NUSBRC1_1, NUSBRC5, and NUSBPC6) (Table 1). Among the *Raphidiopsis raciborskii* strains, the phylogenetic relationship of the *cyrC* sequences indicated the presence of two subclades with moderate support values (BI= 0.61, ML=71, and NJ=99) (Fig. 4C). The first subclade (I), comprising 12 CYN producing strains and one non-CYN producing strain derived from GenBank, was formed with weak support values. All Thai *cyrC* sequences determined in the present study were clustered in the second subclade (II) together with two *Raphidiopsis curvata* strains, three *R. raciborskii* strains, and three *Aphanizomenon* sp. strains with weak support values (Fig. 4C).

In the present study, PCR amplicons containing the *cyrJ* sequence were amplified from the same strains that contained the amplicons of *cyrB* (Table 1). The *cyrJ* phylogenetic trees showed that the *Raphidiopsis raciborskii* strains analyzed belonged to three subclades, as evidenced by robust support values (BI=1.00, ML=100, and NJ=100) (Fig. 4D). The first subclade (I) was positioned as a base subclade of the CYN-producing *R. raciborskii* clade, consisting of six CYN-producing *R. raciborskii* strains from China (eyDB-1) and Australia (CYP011K, AQS, CS-506, FSS-127, and AWT205) (BI=0.99, ML=91, and NJ=95) (Fig. 4D). Then, the second subclade (II) was found to be a sister clade to a third subclade (III) with moderate support values (BI=1.00, ML=63, and NJ=66). The second subclade (II) was comprised of seven new sequences from seven Thai strains (NUPLKC26, NUSBPC7, NUSBRC5, NUPLKC32, NUPLKC27, NUPLKC7, and NUPLKC3) and the *cyrJ* sequences of the Chinese strains of *Aphanizomenon* sp. (10E6), *Raphidiopsis curvata* (CHAB150, HBI, and CHAB3416), and *R. raciborskii* (CHAB357 and CHAB358) (BI=0.99, ML=63, and NJ=61) (Fig. 4D). The last subclade (III) was composed of seven *cyrJ* sequences from the Thai strains (NUSBPC6, NUPLKC2, NUPLKC11, NUSBRC1_1, NUSBRC1, NUPLKC12, and NUPLKC13) obtained in the present study and *cyrJ* sequences from two Chinese *R. raciborskii* strains (CHAB3440 and CHAB3438). The third subclade (III) was moderately sup-

### Table 2. The genetic distance (*p*) values based on the sequences of *rbcL* used in this study.

| No. | Clade (subclade) | Within group | 1   | 2   | 3   | 4   | 5   | 6   | 7   | 8   | 9   |
|-----|------------------|-------------|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| 1   | *Raphidiopsis raciborskii* (II) |             | 0.002 |     |     |     |     |     |     |     |     |
| 2   | *Raphidiopsis raciborskii* (I)  |             | 0.005 | 0.004 |     |     |     |     |     |     |     |
| 3   | *Raphidiopsis mediterranea*     |             | 0.001 | 0.014 | 0.017 |     |     |     |     |     |     |
| 4   | *Raphidiopsis curvata*          |             | 0.005 | 0.036 | 0.038 | 0.036 |     |     |     |     |     |
| 5   | *Chrysosporum*                 |             | 0.000 | 0.232 | 0.233 | 0.231 | 0.228 |     |     |     |     |
| 6   | *Anabaenopsis*                 |             | 0.002 | 0.220 | 0.220 | 0.225 | 0.215 | 0.126 |     |     |     |
| 7   | *Sphaerorespermopsis*           |             | 0.042 | 0.218 | 0.218 | 0.217 | 0.222 | 0.168 | 0.152 |     |     |
| 8   | *Dolichospermum plantonicum*    |             | 0.000 | 0.208 | 0.208 | 0.209 | 0.212 | 0.169 | 0.155 | 0.118 |     |
| 9   | *Dolichospermum ucrainicum*     |             | 0.000 | 0.210 | 0.210 | 0.209 | 0.210 | 0.158 | 0.149 | 0.112 | 0.024 |
| 10  | *Synechococcus elongatus*       |             | nc   | 0.371 | 0.372 | 0.363 | 0.369 | 0.367 | 0.345 | 0.358 | 0.360 | 0.353 |

nc : not calculated
ported by ML (61) and NJ (66) analyses, but weakly supported by BI analysis (Fig. 4D).

Detection of CYN analogues by LC/MS/MS analysis

Analysis by LC/MS/MS confirmed the presence of CYN and 7-deoxy-CYN from 24 of the Thai Raphidiopsis raciborskii strains tested (Table 1 and Fig. 5). In the selected ion monitoring mode (SIM), the ion peaks for CYN (m/z 416) and 7-deoxy-CYN (m/z 400) of cell extracts from the 13 toxic Thai strains tested were detected at retention times of 12.5 and 11.8 min, respectively (Table 1, Figs. 5A and B). At identical retention times to CYN and 7-deoxy-CYN obtained in SIM mode, the thirteen toxic strains were also positively identified by transition m/z 416 > 176, 416 > 194, and 416 > 336 for CYN and by transition m/z 400 > 176 and 400 > 194 for 7-deoxy-CYN (Fig. 5A and B).

In terms of revealing relationships between CYN productivities and the existence of CYN genes in Thai Raphidiopsis raciborskii, the toxin productivities and CYN genes presence were compared; toxic strains NUPLKC2, NUPLKC3, NUPLKC7, NUPLKC11, NUPLKC12, NUPLKC26, NUPLKC27, NUPLKC32, NUSBRC1, NUSBRC3, NUSBRC2, NUSBRC6, and NUSBRC8 were used. The results showed that the toxin productivities and CYN genes presence were positively correlated, indicating that the presence of CYN genes in the strains was responsible for their CYN production.

Fig. 4. Neighbor-joining phylogenetic trees based on the sequences of cyrA (A), cyrB (B), cyrC (C), and cyrD (D) of Thai Raphidiopsis raciborskii strains and other cyanobacterial strains from GenBank. The strains established in the present study are shown in bold and black color. Posterior probabilities (pp) > 0.5 and bootstrap support values (bt) > 50% for individual branches are shown on the tree based on BI/ML/NJ analyses, respectively. The hyphen-minus (-) shown on some branches is the support value of pp < 0.5 or bt < 50%. Branch length is proportional to the number of substitutions per site. In the cyrA phylogeny, each box indicates the strains having identical sequences. Black circles denote the known CYN-producing strains and white circles are the known non-CYN-producing strains.
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BRC1_1, NUSBRC5, NUSPBC6, and NUSPBC7 at least possessed cyrB and cyrJ among the four genes tested as shown in Table 1.

Discussion

In the present study, we investigated the morphological and phylogenetic characteristics of new Thai Raphidiopsis strains; as well as whether or not they possessed CYN genes as determined/indicated by PCR/sequencing and CYN production using LC/MS/MS. Our results showed that the morphological features of the Raphidiopsis cells of Thai strains and natural population samples have high morphological variability in their cell dimensions comparing to those previously reported (Fig. 2). However, the average cell dimensions in length and width of the vegetative cells of Thai strains and the natural populations investigated corresponded to those found in the original description of Raphidiopsis raciborskii (2.5 to 16.0 and 2.5 to 4.0 µm for vegetative cells, respectively) (Woloszyńska 1912) and the type strain of R. raciborskii AWT205 (7 to 15 and 3 to 4 µm for vegetative cells, respectively) (Willis et al. 2017) (Fig. 2). The length and width ranges of vegetative cells of Thai strains and natural population samples overlapped among other Raphidiopsis strains (Fig. 2). R. raciborskii had been differentiated from Raphidiopsis mediterranea and Raphidiopsis curvata on the basis of the presence of heterocyte cells, which are absent in R. mediterranea and R. curvata (Komárek & Komáreková-Legnerová 2003, Wu et al. 2011). Meanwhile, the Thai R. raciborskii strains tested in the present study had straight trichomes both with and without heterocyte cells coexisting in the same culture. Thus, it may be easy to misidentify heterocyte-less R. raciborskii as R. mediterranea (Wu et al. 2011). The strains in the present study possessed morphological traits corresponding to the description of R. raciborskii (Komárek & Komáreková-Legnerová 2003).

The results of the phylogenetic trees based on the partial sequences of the 16S rDNA in the present study revealed that the Raphidiopsis raciborskii strains tested were not differentiable from other Raphidiopsis species and this result corresponds with that of previous studies (Gugger et al. 2005, Li et al. 2008). Molecular phylogeny based on multiple gene loci sequences (i.e., psbA, 16S rDNA, rbcL, rbcS, rpoC1, cpcBA-IGS, and 16S-23S rDNA) and secondary structures of the 16S-23S ITS region failed to distinguish among species in the genus Raphidiopsis (Wu et al. 2011, Aguilera et al. 2018). Recently, only the results of the ITS-L phylogeny have indicated a separation of Raphidiopsis species (Li et al. 2016). However, PCR amplification of ITS regions between the 16S rDNA and 23S rDNA of the cyanobacteria proved to be somewhat complicated by revealing two true ITS regions of different sizes. Our results, based on the rbcLX phylogeny, also showed that the Thai R. raciborskii could be differentiated from other Raphidiopsis species. For example, Raphidiopsis curvata CHAB1150 was clustered together with Thai R. raciborskii strains in the 16S rDNA phylogeny, but R. curvata CHAB1150 was clearly separated from Thai R. raciborskii strains in the rbcLX phylogeny. According to the results of PCR amplification producing a single size PCR amplicon and the facility of sequence alignment in the present study, it is possible that rbcLX may be utilized as a specific marker to differentiate between species in the genus Raphidiopsis. However, further analyses, using more strains from various areas, around the globe, are necessary to confirm this supposition. In the present study, the results of our 16S rDNA and rbcLX phylogenetic analyses showed that all Thai CYN-producing strains were intermixed with other non-CYN producing cyanobacterial strains, suggesting that the CYN biosynthesis gene may be horizontally transferred among cyanobacterial strains. Alternatively, some
genes in the CYN biosynthesis gene cluster were absent in some cyanobacterial strains (e.g., Moustafa et al. 2009). Thus, more studies about horizontal gene transfer of CYN biosynthesis genes are needed to confirm this hypothesis.

In order to characterize the genes responsible for CYN production in Thai \emph{R. raciborskii} strains, the presence or absence of the four \textit{cyr} genes (\textit{cyrA}, \textit{cyrB}, \textit{cyrC}, and \textit{cyrJ}) was examined, because this cluster of \textit{cyr} genes is directly implicated in the synthesis of CYN and has already been found in different cyanobacterial CYN-producing genera (Jiang et al. 2012). Hoff-Risseti et al. (2013) mentioned that \textit{cyrA}, \textit{cyrB}, \textit{cyrC}, and \textit{cyrJ} were sequenced from the Australian \textit{R. raciborskii} strain (CYP011K) that produced 7-deoxy-CYN and CYN. The \textit{cyrA} gene, encoding an L-arginine:glycine amidinotransferase enzyme responsible for the formation of guanidinoacetate, an ornithine form of L-arginine and glycine, in the first step of the CYN biosynthesis is found in the type strain \textit{R. raciborskii} AWT205. Thus, \textit{cyrA} amidinotransferase has been considered to be necessary for the metabolic pathway enabling biosynthesis of CYN (Jiang et al. 2012, Hoff-Risseti et al. 2013). On the other hand, our results showed that this gene was not amplified from most of the toxic strains we studied. This may partly be because the primer annealing site of the \textit{cyrA} used in the present study may have high site variation, resembling primer annealing sites in the microcystin gene (\textit{mcy}) regions (Tanabe et al. 2004, Kurmayer et al. 2005). This may result in non-amplification of target genes due to non-annealing of the oligonucleotide primers (Kurmayer et al. 2005, Mbedi et al. 2005, Lorenzi et al. 2015). Similarly, Lorenzi et al. (2015) reported that the positive results for the presence of microcystin in Brazilian reservoirs were not related to the results of PCR amplification of \textit{mcyA}. Moreover, Hoff-Risseti et al. (2013) found that the \textit{cyrA} sequence in the non-CYN-producing \textit{R. raciborskii} T3 had two nucleotide deletions in positions 525 and 1054, causing a frameshift mutation. Therefore, a new primer specific to \textit{cyrA} should be developed in the future.

The \textit{cyrB} sequence encodes the non-ribosomal peptide synthetase (NRPS) adenylation domain that is responsible for amino acid recognition and activation, and the \textit{cyrC} sequence adenylation domain uses guanidinoacetate as a substrate for subsequent polyketide extensions (Hoff-Risseti et al. 2013). Meanwhile, the \textit{cyrC} sequence contains the code for polyketide synthase, which is a potential enzyme involved in CYN biosynthesis. This \textit{cyrC}-encoded enzyme plays a key role in the incorporation of a further acetate unit into guanidinoacetate, while a subsequent ketoreduction provides the next intermediate (Jiang et al. 2012, Hoff-Risseti et al. 2013). It has been reported that CYN-producing cyanobacteria must possess the gene homologues of \textit{cyrA}, \textit{cyrB}, and \textit{cyrC} for the production of the toxin (Rasmussen et al. 2008, Lorenzi et al. 2015). However, recent studies have reported that non-CYN producers such as \textit{Raphidiopsis raciborskii} (from Brazil), \textit{Chryso-
the \textit{cyrB} and \textit{cyrJ} sequences might be considered to be signature molecular markers for detecting toxicogenic/potentially toxic cyanobacteria genotypes and can be used to assess the occurrence of CYN-producing cyanobacteria in water resources for water supplies and fisheries in Thailand. Nevertheless, the \textit{cyrB} gene can also be found in non-CYN-producing strains (e.g., Ballot et al. 2011, Hoff-Rissetti et al. 2013). Considering the results of previous reports worldwide and the present study, it has been shown that \textit{cyrJ} is the most important gene and should therefore be selected as the first molecular marker for determination of CYN production in cyanobacteria in aquatic environments worldwide.

In conclusion, the present study revealed that morphological and molecular analyses on the new \textit{Raphidiopsis} strains isolated from Thai freshwater allowed them to be identified as \textit{R. raciborskii}. Moreover, \textit{rbclX} may be utilized as a genetic marker to differentiate between species in the genus \textit{Raphidiopsis}. However, further phylogenetic analysis of \textit{rbclX} using strains isolated from various global areas are needed to confirm this possibility. The LC/MS/MS analysis confirmed that the 13 Thai \textit{R. raciborskii} strains showing PCR amplification of the \textit{cyrB} and the \textit{cyrJ} could produce CYN and 7-deoxy-CYN. Thus, our findings support the supposition that \textit{cyrB} and \textit{cyrJ}, especially the latter, may be useful for the detection of CYN-producing cyanobacteria in water resources for water supplies and fisheries for wider geographical areas, including Thailand. However, additional molecular and growth characterizations should be carried out to identify all potential CYN-producing species of cyanobacteria and to understand the environmental factors that can potentially influence the abundance of toxin producing genotypes and CYN production.

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