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Sporadic Distribution and Distinctive Variations of Cylindrospermopsin Genes in Cyanobacterial Strains and Environmental Samples from Chinese Freshwater Bodies

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Increasing reports of cylindrospermopsins (CYNs) in freshwater ecosystems have promoted the demand for identifying all of the potential CYN-producing cyanobacterial species. The present study explored the phylogenetic distribution and evolution of *cyr* genes in cyanobacterial strains and water samples from China. Four *Cylindrospermopsis* strains and two *Raphidiopsis* strains were confirmed to produce CYNs. Mutant *cyrI* and *cyrK* genes were observed in these strains. Cloned *cyr* gene sequences from eight water bodies were clustered with *cyr* genes from *Cylindrospermopsis* and *Raphidiopsis* (C/R group) in the phylogenetic trees with high similarities (99%). Four *cyrI* sequence types and three *cyrJ* sequence types were observed to have different sequence insertions and repeats. Phylogenetic analysis of the *rpoC1* sequences of the C/R group revealed four conserved clades, namely, clade I, clade II, clade III, and clade V. High sequence similarities (>97%) in each clade and a divergent clade IV were observed. Therefore, CYN producers were sporadically distributed in congeneric and paraphyletic C/R group species in Chinese freshwater ecosystems. In the evolution of *cyr* genes, intragenomic translocations and intergenomic transfer between local *Cylindrospermopsis* and *Raphidiopsis* were emphasized and probably mediated by transposases. This research confirms the existence of CYN-producing *Cylindrospermopsis* in China and reveals the distinctive variations of *cyr* genes.

Harmful cyanobacterial blooms, along with eutrophication in freshwater ecosystems, global warming, and worldwide spread of invasive cyanobacterial species, have drawn great attention in recent years (1–4). Cyanotoxins, such as saxitoxins, anatoxins, microcystins, and cylindrospermopsins (CYNs), are toxic metabolites produced by cyanobacteria, and their syntheses are regulated by a series of genetic and environmental factors (5–7). The outbreak of hepatoenteritis in Palm Island (Queensland, Australia) in 1979 led to the discovery of CYN, which was first isolated from bloom-forming *Cylindrospermopsis raciborskii* and proved to be mainly hepatotoxic (8–10). CYN is a sulfate ester with high solubility in water and comprises a tricyclic guanidine group and a hydroxymethyluracil moiety (10). Two analogues of CYN have been described: 7-epi-CYN, an enantiomer of CYN (11), and 7-deoxy-CYN with no hydroxylation on C-7 (12).

CYN can damage the liver, thymus, kidney, and heart (13). The cytotoxicity of CYN may be mediated by inhibiting the syntheses of protein (14) and glutathione (15). CYN is also a potential carcinogen because of its genotoxic effects by inhibiting pyrimidine nucleotide synthesis (16) and inducing DNA strand breakage (17, 18). An assay performed in mice revealed that 7-epi-CYN has severe toxicity similar to CYN and that uracil moiety is required for their toxicity (19). However, 7-deoxy-CYN shows no toxicity to mouse, and thus hydroxylation at C-7 is also crucial for the toxicity of CYNs (12). The bioaccumulation of CYNs in the tissues of vertebrates and invertebrates has been reported (20, 21) as a great health risk for humans and animals.

To date, CYNs have been detected in *Nostocales* and *Oscillatoriales* species, including *Cylindrospermopsis*, *Raphidiopsis* (22, 23), *Aphanizomenon* (24–26), *Anabaena* (27), *Umezakia* (28, 29), *Oscillatoria* (30), and *Lyngbya* (31) spp. CYN-producing *Cylindrospermopsis* from Australia and Asia have been reported, whereas *Cylindrospermopsis* strains isolated from Europe and America are incapable of CYN production (32–36). However, no conclusion can be drawn about the geographic distribution of the CYN-producing genotype of *Cylindrospermopsis* before additional samples from each continent are investigated by molecular and chemical methods.

The *cyr* gene cluster that encodes amidinotransferase, peptide synthetase (PS), polyketide synthase (PKS), and tailoring enzymes involved in CYN production has been described in *C. raciborskii* (37), *R. curvata* (38), *Aphanizomenon* sp. (39), and *Oscillatoria* sp. (30). The amidinotransferase CyrA catalyzes a transfer of an amidino group from arginine to glycine, which results in the first-product guanidinoacetate (40). Five *cyr* genes (*cyrB* through *cyrF*) that encode multi-enzymatic PSs and PKSs are probably involved in the polyketide chain synthesis that incorporates five units of acetate (41). The uracil moiety results from *de novo* synthesis possibly catalyzed by CyrG and CyrH. The sulfate group is incorporated by a sulfotransferase CyrJ with a suggested adenylylsulfate

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TABLE 1 Gene detection of environmental DNA samples from Chinese freshwater bodies

Geographic origin	Abbreviation	Date of sample	Gene regions[a]			
			rpoC1	*cyrA*	*cyrI*	*cyrJ*
Longhu Lake, Daqing, Heilongjiang	LH	July, 2012	ND	–	–	ND
Jinyang Lake, Taiyuan, Shanxi	JY	Aug., 2010	4	–	–	ND
Fish pond, Qingdao, Shandong	FQ	Nov., 2013	7	–	–	ND
Taihu Lake, Wuxi, Jiangsu	TH	Aug., 2011	ND	–	–	ND
		Nov., 2011	ND	–	–	ND
Fish pond, Nanjing, Jiangsu	FN	Nov., 2007	3	–	–	ND
Qiandao Lake, Hangzhou, Zhejiang	QA	Oct., 2012	3	3	10	10
Xianghu Lake, Hangzhou, Zhejiang	XH	Oct., 2012	5	1	7	8
Xihu Lake, Hangzhou, Zhejiang	XL	Oct., 2012	5	–	–	ND
Dongqian Lake, Ningbo, Zhejiang	DQ	July, 2009	3	–	–	ND
Donghu Lake, Wuhan, Hubei	DH	Nov., 2006	3	–	–	ND
Tangxun Lake, Wuhan, Hubei	TX	Oct., 2012	ND	–	–	ND
Liangzi Lake, Ezhou, Hubei	LZ	Sept., 2011	4	2	2	D
Qiaodun Lake, Daye, Hubei	QD	Sept., 2011	9	1	5	8
Chidong Lake, Qichun, Hubei	CD	Aug., 2006	6	1	11	5
Lushui Reservoir, Chibi, Hubei	LS	May, 2006	5	–	–	ND
Poyang Lake, Nanchang, Jiangxi	PO	Aug., 2012	ND	–	–	ND
		Oct., 2012	ND	–	–	ND
Erhai Lake, Dali, Yunnan	EH	Aug., 2010	ND	–	–	ND
		Sept., 2010	ND	–	–	ND
		Oct., 2010	ND	–	–	ND
Fish pond, Kunming, Yunnan	FK	Oct., 2006	3	–	–	ND
Dongzhen Reservoir, Putian, Fujian	DZ	Sept., 2011	2	2	8	6
Fish pond, Panyu, Guangdong	FP	May, 2012	5	–	–	ND
Shiyan Reservoir, Shenzhen	SY	June, 2007	5	D	6	3
Qiankeng Reservoir, Shenzhen	QK	June, 2007	5	–	–	ND
Tiegang Reservoir, Shenzhen	TG	June, 2007	9	3	10	9
Luotian Reservoir, Shenzhen	LT	June, 2007	2	–	–	ND
Changliupi Reservoir, Shenzhen	CL	June, 2007	ND	–	–	ND

[a] The number of unique sequences is indicated where applicable. D, detected; ND, not detected; –, not tested.

kinase CyrN providing the phosphoadenylyl sulfate pool (37). CyrI has been proven to catalyze hydroxylation at the C-7 of 7-deoxy-CYN (42), and CyrK has been proposed to be a potential transporter. Although *cyr* genes are highly conserved, the rearrangements of the *cyr* gene cluster and the insertion mutation of the *cyrI* gene have been reported (38). The *cyrN* and *cyrO* genes are found only in the end of the *cyr* gene cluster of *C. raciborskii* and are suggested to be excluded from the core set of *cyr* genes (30, 38).

An AbrB-like protein has been reported to be involved in the transcription regulation of *cyr* genes in *Aphanizomenon ovalisporum* (43). However, the protein-DNA interaction has not been verified in other CYN-producing species. The effects of temperature, light, nitrogen, phosphate, and sulfate on CYN production are inconclusive because of uncertainties in strain dependence, the release of CYNs, heterocyst formation, and combined effects of multiple factors in different experimental conditions (43–51). Davis et al. (51) highlighted the effects of the genetic diversity of CYN producers on the concentration and composition of CYNs in aquatic ecosystems. Moreover, CYN-producing and non-CYN-producing genotypes often coexist in the same populations. Therefore, an overview of CYN-producing species in total phytoplankton is essential for the risk assessment of CYNs. Furthermore, a systematic investigation of the diversity of *cyr* genes has not been performed and is thus necessary.

Cyanobacterial blooms occur perennially in numerous freshwater ecosystems, and CYNs have been detected in some urban reservoirs of China (52). Therefore, a comprehensive understanding of the diversity and distribution of CYN producers is essential. We illustrate this issue here by investigating the presence of *cyr* genes in cyanobacterial strains and environmental samples from different parts of China. Specifically, phylogenetic analysis was performed to explore the diversity and evolution of CYN producers. The conservation and variation of *cyr* gene sequences were also characterized.

MATERIALS AND METHODS

Cyanobacterial strains and culture conditions. Cyanobacterial strains isolated from Chinese freshwater bodies were used for molecular and chemical analysis of CYNs (see Table S1 in the supplemental material). Three strains—*C. raciborskii* AWT205, *C. raciborskii* cyDB-1, and *Aphanizomenon ovalisporum* ILC-164—were isolated from Australia, Brazil, and Israel, respectively. Pure cultures of the cyanobacterial strains were grown in liquid MA medium (53) at 25°C under a 12-h/12-h light/dark cycle with constant white light intensity of 30 μmol of photons m^{-2} s^{-1}. Cyanobacterial cells were harvested at the exponential phase (optical density at 680 nm [OD_{680}] = 0.8) by centrifugation (12,000 × *g*) and stored at −80°C before further processing.

Collection of environmental samples. Water samples were collected in lakes and reservoirs of China from 2006 to 2013 (Table 1). These water bodies were located between 22°N and 47°N in subtropical and temperate regions (see Fig. S1 and Table S2 in the supplemental material). A volume of 300 to 500 ml of water was filtered using a membrane filter (MF-Millipore, 0.22-μm pore size) in quadruplicate for each water body at

TABLE 2 Characteristics of primer pairs used for gene detection

Gene	Primer	Sequence (5′-3′)	T_m (°C)
cpcBA-IGS	PCβF	GGCTGCTTGTTTACGCGACA	50
	PCαR	CCAGTACCACCAGCAACTAA	50
cyrA	cyrAF51[a]	GATGGTTGTCGGGATTGCAGAT	57
	cyrAR1167	GAAGCGAGAAACGCCATTGGT	57
cyrI	cyrIF[b]	CAGGCTTATCTGCAACAACATTTCT	56
	cyrIR813	CGGTTTATCAGTTCCAGAGTATCCA	56
cyrJ	cyrJF13	CGAATCGCAATGTGGTCTGTGC	59
	cyrJR720	GACAAGATATAGCGGCAACGACTCA	59
cyrK	RTcyrKF991	GGAGCGTGTTGGCTATTTC	55
	RTcyrKR1379	TGAGTCAAGGCACGAGAAG	55
rpoC1	rpoC1F53	CACCAGAACGTATCCGCGCT	60
	rpoC1R739	GGTGGAATGACTGGAATGGCTGA	60

[a] *C. raciborskii* CS-505 numbering.
[b] Targeting flanking sequence upstream *cyrI* gene.

each collection period. The filters were also stored at −80°C before DNA extraction.

DNA extraction, PCR, and sequencing. Genomic DNA of cyanobacterial cells were extracted by using sodium dodecyl sulfate lysis and a phenol-chloroform-isoamyl alcohol extraction method described previously (54). Environmental DNA was extracted from membrane filters using a water DNA extraction kit according to the manufacturer's protocol (Omega Bio-Tek, USA). The filters were cut into pieces first and then subjected to the extraction process of the kit. The purified DNA were dissolved in Tris-EDTA buffer (pH 8.0) and stored at −20°C. The purity and concentrations of DNA samples were determined by a Nanodrop 2000 spectrophotometer (Thermo Fisher Scientific, USA).

The primer pair PCβF/PCαR (54) with specificity targeting the phycocyanin operon (*cpc*) of cyanobacteria was used to confirm the validity of DNA templates for PCRs. Primers specific for *cyrA*, *cyrI*, and *cyrJ* genes of current known CYN-producing species were designed (see Fig. S2A in the supplemental material). Another primer set, rpoC1F53/rpoC1R739, was designed to selectively amplify the *rpoC1* genes of *Cylindrospermopsis* and *Raphidiopsis* (see Fig. S2B in the supplemental material). PCR mix were prepared in 50-μl volumes containing 5 μl of 10× PCR buffer (TaKaRa, Japan), 10 nmol of each deoxynucleotide triphosphate, 10 pmol of each primer, 1 U of LA *Taq* (TaKaRa), and 100 ng of DNA templates. The cycling conditions were as follows: 94°C for 3 min; 35 cycles of 94°C for 45 s, 50°C to 60°C for 1 min, and 72°C for 2 min; 72°C for 10 min; and a 4°C hold. The annealing temperatures depended on the T_m values of primers (Table 2).

The positive PCR products were amplified in triplicate and purified using a gel extraction kit (Omega Bio-Tek, USA). Purified gene fragments from environmental DNA were cloned into pMD18-T vector (TaKaRa). Recombinant plasmids of 5 to 15 positive bacterial clones were extracted, and the gene fragments were sequenced using an ABI 3730 automated sequencer (Applied Biosystems) in both directions. The primer regions of obtained sequences were deserted, and duplicated sequences in each water body were removed. The gene fragments from cyanobacterial strains were sequenced directly by using PCR primers in double directions.

Two methods were utilized to obtain the whole *cyr* gene clusters of cyanobacterial strains. First, the *cyr* genes and flanking sequences were amplified and sequenced according to the PCR methods described earlier (38). Second, genome sequencing was performed using a Hiseq 2000 (Illumina, USA) according to the manufacturer's instructions. A sequence library of 300 bp was constructed, and paired-end sequencing was carried out. After removing the low-quality reads, genome sequences were assembled by two software programs, including SOAPdenovo (v1.05) and Velvet (v1.0.09). The conservation of gene and protein sequences was verified by homologous search using BLAST on the website of the National Center for Biotechnology Information (NCBI). Open reading frames (ORFs) were determined by the ORF Finder tool implemented on the NCBI website.

Transcription detection. Cyanobacterial cells from 2 ml of culture at the exponential phase were harvested by centrifugation. RNA extraction, DNase digestion and cDNA synthesis were performed as described previously (38). The DNase-digested RNA extracts and cDNA were used as the templates for transcription detection. The *cyrI* and *cyrK* genes were amplified by using the primer sets cyrIF/cyrIR813 and RTcyrKF991/RTcyrKR1379 (Table 2), respectively. A negative control without cyanobacterial cells was also subjected to the extraction and detection procedures. The genomic DNA of *C. raciborskii* AWT205 was used for positive PCR templates.

Phylogenetic assignment. Four data sets, namely, *cyrA*, *cyrI*, *cyrJ*, and *rpoC1*, were constructed, including environmental sequences and reference gene sequences from cyanobacterial strains. Multiple sequence alignments were created by using the CLUSTAL W (v1.4) option in Bioedit v7.0.9.0 software and manually corrected. The best substitution models for gene evolution were selected by Modeltest v3.7 (55) and used for the inference of phylogenetic trees. Maximum-likelihood (ML) algorithm was used to carry out phylogenetic analysis by PHYML v3.0 (56) and PAUP v4.0b10 with 1,000 bootstrap replicates. Bayesian phylogenetic inference was performed using MrBayes v3.1.2 (57), and the parameters were set as described earlier (38). Neighbor-joining (NJ) trees were constructed by MEGA v4 (58) using Kimura two-parameter model with 1,000 bootstrap replicates. The GenBank accession numbers of reference gene sequences were displayed in Table S3 in the supplemental material. Selection analysis of environmental *cyrA*, *cyrI*, and *cyrJ* sequences were also performed as described previously (38). The secondary structures of protein sequences were predicted by PSIPRED v3.3 available online (59).

Toxin extraction and analysis. Intracellular CYNs were extracted from lyophilized cyanobacterial cells by a modification of a method reported previously (60). Briefly, 30 mg of dry cells were mixed with 1 ml of Millipore water, sonicated for 20 min in an ice bath and shaken for 1 h at room temperature, followed by centrifugation. A total of 2-ml supernatants were collected after the extraction step was repeated. The supernatants were further subjected to solid-phase extraction (SPE) as described previously (61). Carbograph SPE cartridges (6.0 ml, 250 mg) were pretreated with 10 ml of elution solvent (dichloromethane-methanol, 1:4 [vol/vol]) acidified with 5% formic acid (vol/vol) and washed with 10 ml of water. The extracts were acidified with formic acid (1% [vol/vol]), and the ionic strength was adjusted with 0.1% sodium chloride (wt/vol) before application to the cartridges. The cartridges were then washed with 10 ml of water, followed by air to remove excess liquid. The absorbed toxins were eluted by 10 ml of elution solvent, and the solvent was removed by rotary evaporation thereafter. The precipitate was redissolved in 2 ml of water, and the solution was filtered through a Millipore ultracentrifugal filter (100 kDa). Extracellular CYNs were also extracted from cell-free spent culture medium by the SPE method. A volume of 100 ml of acidified medium was applied with a flow rate of 5 ml min^{-1}, and the toxins were eluted by 20 ml of elution solvent.

CYNs were analyzed using two methods. First, CYNs were detected by liquid chromatography-tandem mass spectrometry (LC-MS/MS) using an ESI-Q-TOF 6530 coupled with Infinity UHPLC 1290 (Agilent, USA). For LC conditions, a C_{18} column (4.6 mm by 250 mm, 5 μm) was applied with a temperature of 35°C. Compounds were separated by two linear gradient stages, 5 to 15% methanol in water during 0 min to 10 min, and 15 to 50% methanol in water during 10 min to 20 min with a flow rate of 0.25 ml min^{-1}. The injection volume was 20 μl. The parameters of the mass spectrometer were set as follows: gas temperature, 300°C; flow rate, 11 liters min^{-1}; nebulizer pressure, 45 lb/in^2; capillary voltage, 3,500 V; nozzle voltage, 1,000 V; and fragmentor voltage, 175 V. Positive ions of *m/z* 100 to *m/z* 2,500 were monitored, and toxin analogues were deter-

mined by parent ions (*m/z* 416.1 for CYN and *m/z* 400.1 for 7-deoxy-CYN) and corresponding fragments (*m/z* 336.1, 274.1, and 194.1 for CYN and *m/z* 320.1, 274.1, and 194.1 for 7-deoxy-CYN). CYN and 7-epi-CYN could not be discriminated in the present study and therefore CYN represented these two analogues.

An efficient high-pressure liquid chromatography (HPLC) method was established by optimization of the HPLC-PDA method reported by Welker et al. (60). In brief, the SSI 1500 series system (SSI, USA) and a Synergi Polar-RP column (4.6 mm by 250 mm, 4 μm) maintained at 30°C was used. The elution conditions were as follows: a linear gradient of 10 to 30% solution B (0.05% trifluoroacetic acid [vol/vol] in 50% aqueous methanol [vol/vol]) in solution A (0.05% aqueous trifluoroacetic acid [vol/vol]) for 0 to 10 min, an isocratic stage of 30% solution B for 5 min, ramping to 100% solution B in 5 min, and final equilibration for 15 min. An injection volume of 20 μl and a flow rate of 0.8 ml min^{-1} were applied. UV absorption was detected at 262 nm. Standard CYNs were prepared by manual collection from elution fractions and then confirmed by LC-MS/MS. The standards were used for the identification of potential analogues in samples. In addition, commercial standard CYN (Enzo Life Sciences, USA) was used for quantification analysis, and the concentration of 7-deoxy-CYN was calculated as CYN equivalents.

Detection of toxin production in growth cultures. CYN-producing cyanobacterial strains were first cultured to obtain original biomass (OD_{680} = 0.2 to 0.4). The cyanobacterial cells were harvested onto a glass fiber filter (Whatman, GF/C) by gentle filtration (<5 lb/in^2) at sterile conditions and washed three times using MA medium. Afterward, the cells were resuspended and diluted into six parallel cultures (100 ml of MA for each) in 500-ml Erlenmeyer flasks with a cell density of OD_{680} = 0.13. The cultures were shaken manually three times every day. After inoculation, random cultures of each strain were used for toxin detection in triplicate at day 3 and 7, respectively. The cells and spent medium were separated by gentle filtration (<5 lb/in^2) using membrane filters (MF-Millipore, 0.22-μm pore size) and used for toxin extraction and detection as described above. Statistical analyses were performed by independent-sample *t* test with SPSS 21.0 for Windows, and the differences were taken as significant at a *P* of <0.05.

Nucleotide sequence accession numbers. The nucleotide sequences obtained in the present study are available under GenBank accession numbers KJ139686 to KJ139955.

RESULTS

Phylogenetic and geographic distribution of CYN genes. All DNA templates from cyanobacterial strains and the environmental samples were confirmed to be efficient for *cpc* gene amplification. A total of 362 cyanobacterial strains, belonging to 10 genera of three orders, namely, *Chroococcales*, *Nostocales*, and *Oscillatoriales*, were examined for the presence of *cyrJ* gene. Positive strains were then detected for *cyrA* and *cyrI* genes. These strains were collected from 38 freshwater bodies across China, except for several *Lyngbya* strains obtained from swards and hot springs. Four *Cylindrospermopsis* strains and two *Raphidiopsis* strains were confirmed to contain *cyr* genes. CYNs were detected in the cell extracts of these strains by LC-MS/MS (Table 3). *C. raciborskii* CHAB3438 and *C. raciborskii* CHAB3440 contained both CYN and 7-deoxy-CYN, but the other four strains produced only 7-deoxy-CYN. *C. raciborskii* CHAB357, *C. raciborskii* CHAB3440, and *R. curvata* CHAB114 were isolated from the same cyanobacterial populations as and shared highly similar *cyr* sequences and toxin production to *C. raciborskii* CHAB358, *C. raciborskii* CHAB3438, and *R. curvata* CHAB1150, respectively. In addition, *cyr* genes, CYN, and 7-deoxy-CYN were also detected in *C. raciborskii* cyDB-1.

The presence of *cyr* genes was also examined in environmental DNA samples from 25 freshwater bodies. Finally, 13 *cyrA*, 59 *cyrI*, and 49 *cyrJ* sequences were obtained from samples collected from eight lakes and reservoirs (Table 1). A BLAST search revealed high similarities between environmental *cyr* sequences and corresponding *cyr* genes from *Cylindrospermopsis* and *Raphidiopsis* (C/R group, 99%). The environmental *cyrA* and *cyrJ* sequences were also found to be highly similar to the *cyr* genes of *Aphanizomenon* sp. strain 10E6 (99%). In contrast, the *cyrI* sequences were found to have low similarities to the *cyrI* gene of *Aphanizomenon* sp. strain 10E6 (97%). The environmental *cyr* sequences and *cyr* genes from the C/R group and *Aphanizomenon* sp. strain 10E6 were clustered into an independent clade in phylogenetic trees (data not shown). This clade was separated from the *cyr* genes of other species by high bootstrap values in the trees of the *cyrI* and *cyrJ* genes (97 to 100%).

TABLE 3 CYN-producing cyanobacterial strains isolated from Chinese freshwater bodies

Strain	Geographic origin	Result[a]				Source or reference
		cyrI	*cyrK*	CYN	7-Deoxy-CYN	
C. raciborskii						
CHAB357	Wenshan Lake	M	M	ND	D	This study
CHAB358	Wenshan Lake	M	M	ND	D	This study
CHAB3438	Xianghu Lake	H	M	D	D	This study
CHAB3440	Xianghu Lake	H	M	D	D	This study
R. curvata						
CHAB114	Chidong Lake	M	H	ND	D	This study
CHAB1150	Chidong Lake	M	H	ND	D	Jiang et al. (38)
CHAB3416	Qiaodun Lake	M	H	ND	D	This study
HB1	Guanqiao Pond	M	H	D	D	Li et al. (22)

[a] M, mutant sequences compared to *cyr* genes of *C. raciborskii* AWT205; H, homologous to *cyr* genes of *C. raciborskii* AWT205; D, detected; ND, not detected.

Sequence analysis. The *cyr* genes of *C. raciborskii* CHAB358 and *R. curvata* HB1 were sequenced and assembled into two complete gene clusters (Fig. 1). The genome of *C. raciborskii* CHAB3438 was assembled using high-quality data with an average coverage of 220, and the *cyr* gene cluster was found to be located in two contigs. The gap was closed by PCR amplification and Sanger sequencing. The final contig had a length of 50,355 bp and contained the whole *cyr* gene cluster (Fig. 1). The *cyr* genes in these three gene clusters showed high similarities to those of *R. curvata* CHAB1150 (>99%). The gene arrangement patterns of the *cyr* gene clusters of the C/R group strains from China were conserved and divergent from that of *C. raciborskii* AWT205 from Australia. The *cyrN* and *cyrO* genes were absent in the *cyr* gene clusters of Chinese strains (Fig. 1).

The CyrI of *C. raciborskii* CHAB358 was found to be truncated because of an intragenic stop codon caused by a base transition from cytosine to thymine at bp 529 (according to *C. raciborskii* AWT205 numbering, used here and below). Single-base mutations were also observed within *cyrI* sequences from TG and SY reservoirs (see Fig. S3 in the supplemental material). Six sequences had similar mutations to the *cyrI* gene of *C. raciborskii* CHAB358. Base transversions from guanine to thymine at bp 304 of two sequences were observed and also formed stop codons. In addition, four types of *cyrI* sequences were recognized according to

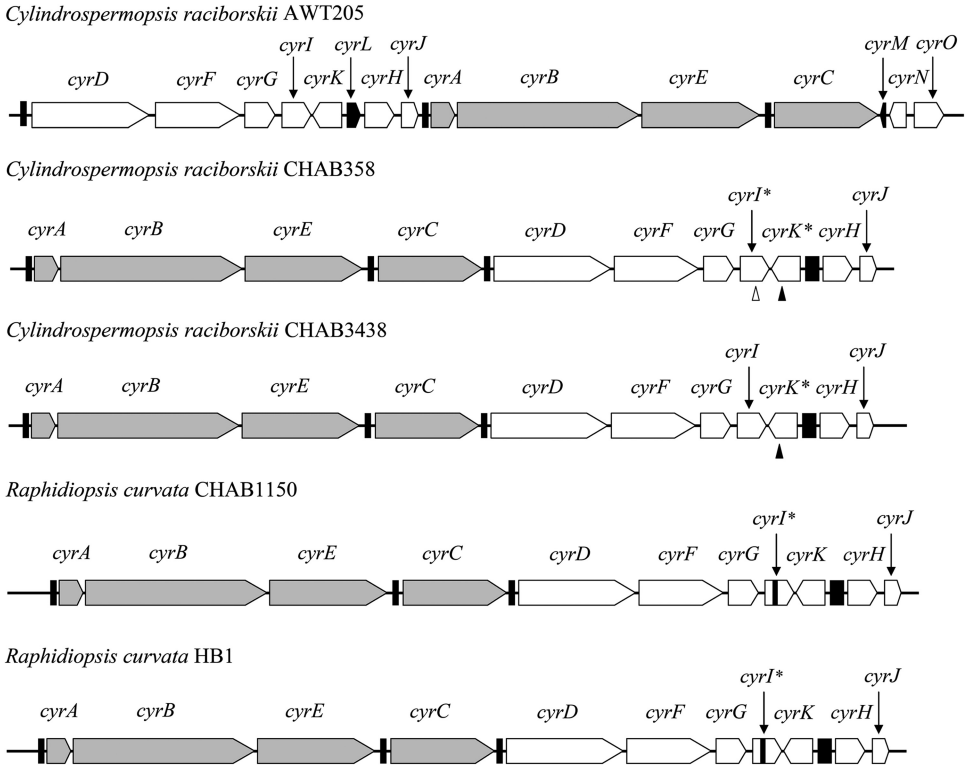

FIG 1 Schematic structure of *cyr* gene clusters from CYN-producing cyanobacterial strains. Gray and white bars, *cyr* genes; black bar, transposase sequences or vestiges thereof; open triangles, base mutation in this position; solid triangles, nucleotide deletion in this position.

intragenic sequence insertions compared to the *cyrI* gene of *C. raciborskii* AWT205, as depicted in Fig. 2. Itype1 contained no insertion sequence, but an insertion of a 6-nucleotide fragment, which is a repeat copy of its upstream sequence, was observed in Itype2 and Itype3 after bp 622. In addition, another insertion of a 30-nucleotide fragment, which is also a repeat copy of its upstream sequence, was observed in Itype3 after bp 494. Moreover, Itype4a included two kinds of sequences (i.e., Itype4a^{f} and Itype4a^{r}) that contained reverse complementary insertion sequences of a 92-nucleotide fragment after bp 85, and the insertion sequences contained identical inverted terminal repeats (ITRs). The *cyrI* genes of *R. curvata* strains and those of other strains were classified into Itype4 and Itype1, respectively. In particular, both *R. curvata* CHAB1150 and *R. curvata* CHAB3416 contained the *cyrI* genes of Itype4a^{f}, and the *cyrI* gene of *R. curvata* HB1 was denominated as Itype4b with a long sequence insertion. Compared to Itype1, the deduced protein sequences of Itype2 and Itype3 were extended with repeated amino acids. The sequence insertions in Itype4 caused stop codons within the gene sequences and resulted in truncated protein sequences (see Fig. S4 in the supplemental material).

A 48-nucleotide fragment was found to be repeated within the *cyrJ* sequences. Three *cyrJ* sequence types—Jtype1, Jtype2, and Jtype3—were identified based on copy numbers 1, 2, and 3 of this sequence repeat, respectively (Fig. 3). Jtype2 contained two subtypes, namely, Jtype2a with two intact repeats and Jtype2b with a 6-nucleotide deletion in the first repeat. Most *cyrJ* genes from CYN-producing strains belong to Jtype2a, and those of three *C. raciborskii* strains (i.e., AWT205, CS-505, and cyDB-1) and *R. mediterranea* FSS-150 belong to a third subtype Jtype2c with a different 6-nucleotide deletion in the second repeat (Fig. 3). As displayed in Fig. S5 in the supplemental material, the sequence repeats within the *cyrJ* genes of the C/R group and *Aphanizomenon* sp. 10E6 were conserved. The second repeats in these species were divided into two groups based on nucleotide variations. One group contained the C/R group from Australia and Brazil, and the other contained the C/R group from China and *Aphanizomenon* sp. 10E6. Compared to Jtype1, the deduced protein sequences of Jtype2 and Jtype3 were extended and contained peptide repeats.

The *cyrK* genes of *C. raciborskii* CHAB358 and *C. raciborskii* CHAB3438 lacked a thymine nucleotide at bp 1347 unlike those of *C. raciborskii* AWT205 (1,398-bp length). This lack of thymine nucleotide led to the truncation of the C-terminal sequence of CyrK (Fig. 4). Thus, the CyrK mutant (451 amino acids) was shorter than the original CyrK (465 amino acids).

Transcription analysis. The transcriptions of *cyrI* and *cyrK* genes were examined for *C. raciborskii* CHAB358 and *C. raciborskii* CHAB3438. *C. raciborskii* AWT205 was used as a positive strain. Pure RNA extracts were not contaminated by genomic DNA, and *cyr* gene fragments were obtained from all cDNA samples. In addition, the amplicons covered the gene regions with nucleotide mutation and deletions.

Assessment of toxin release. As depicted in Table S4 in the supplemental material, the cultures of four CYN-producing cyanobacterial strains maintained exponential growth from a low (OD_{680} = 0.13) to a high (OD_{680} = 0.34 to 0.61) cell density. The concentrations and extracellular percentages of CYNs were analyzed (Table 4; see also Table S4 in the supplemental material).

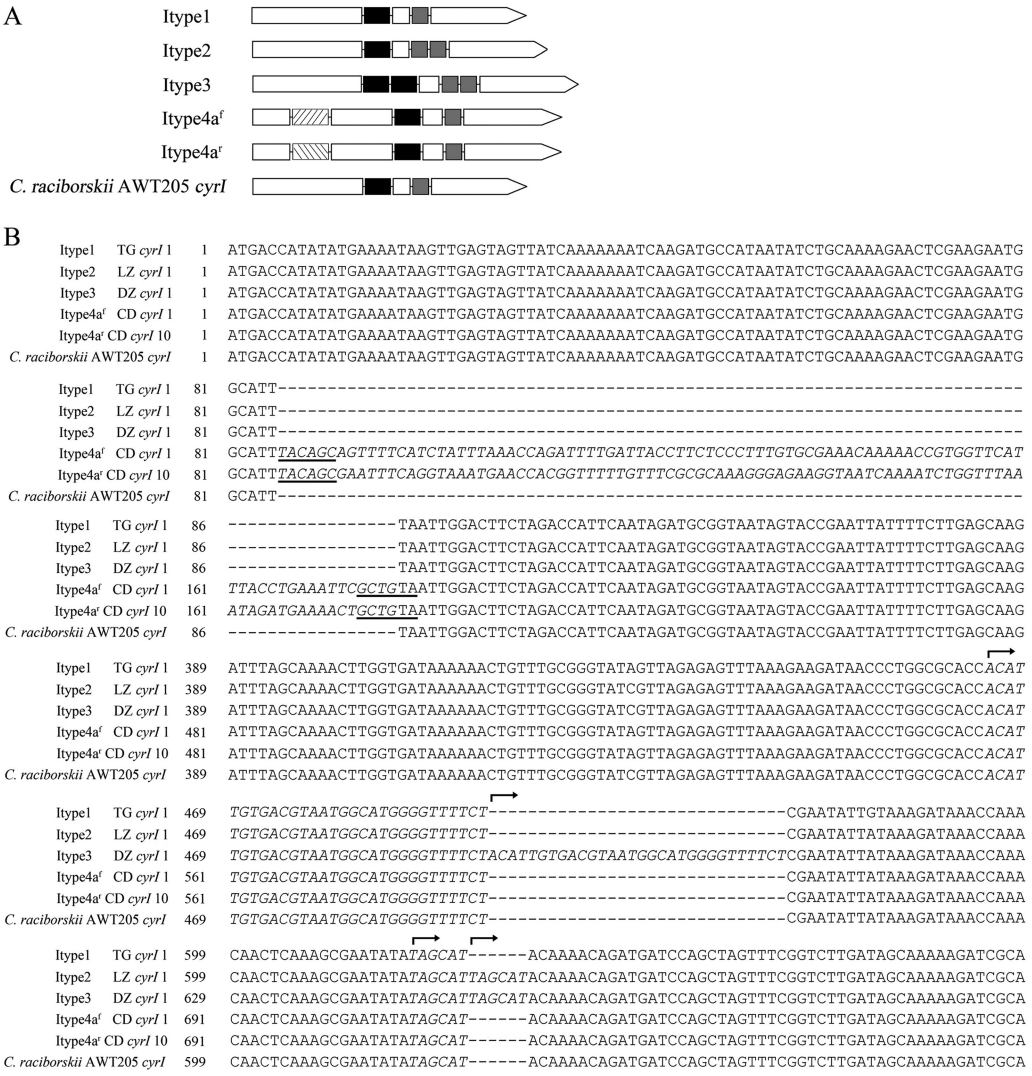

FIG 2 Illustration of four sequence types of the *cyrI* gene. (A) Schematic structures of *cyrI* sequence types. White bar, *cyrI* sequences; black and gray bars, repeat sequences; slash and backslash bar, insertion sequences; *C. raciborskii* AWT205, reference strain. (B) Partial alignment of representative *cyrI* gene sequences. Repeat sequences and insertion sequences were italicized. Dashed line, gaps introduced into the alignment; bold line, ITRs; arrow, beginning of the repeat sequences.

Only 7-deoxy-CYN was detected in *C. raciborskii* CHAB358 and *R. curvata* CHAB1150, and a high percentage of CYN was observed in both extracellular (92 to 96%) and intracellular (95 to 98%) CYNs of *C. raciborskii* AWT205 and *C. raciborskii* CHAB3438. The extracellular percentages of CYN (30 to 39%), 7-deoxy-CYN (24 to 51%), and total CYNs (24 to 40%) on day 7 were significantly higher than the corresponding percentages on day 3 for all strains except *C. raciborskii* CHAB3438. The extracellular percentages of CYN and 7-deoxy-CYN between *C. raciborskii* AWT205 and *C. raciborskii* CHAB3438 were not significantly different except those of 7-deoxy-CYN on day 3. The extracellular percentages of 7-deoxy-CYN in *C. raciborskii* CHAB358 and *R. curvata* CHAB1150 were similar and significantly lower than those of other two strains, except between *C. raciborskii* AWT205 and *C. raciborskii* CHAB358 on day 3. For the extracellular percentages of the total CYNs, *C. raciborskii* CHAB358 and *R. curvata* CHAB1150 had lower values, with the significantly lowest percentage for *R. curvata* CHAB1150 on day 3 (15% ± 1.0%) and the significantly highest percentage for *C. raciborskii* AWT205 on day 7 (40% ± 3.0%).

Phylogenetics of potential CYN producers based on *rpoC1* sequences. As displayed in Table 1, *rpoC1* genes were detected in 19 lakes and reservoirs by C/R group specific primers, and 88 *rpoC1* sequences were obtained. All of these sequences were confirmed to be derived from the C/R group by best BLASTn hits. Five independent clades were observed in the phylogenetic tree of *rpoC1* sequences, and high support values were obtained for the divergence of clade I and clade II (Fig. 5). High sequence similarities were displayed within four clades: clade I (>97%), clade II (>99%), clade III (>98%), and clade V (>98%). However, clade IV comprised sequences with low to high similarities (95% to 100%), which is consistent with the long branches of this clade in the tree. Sequence similarities among clades were also calculated. The values between clade I and clade II (96 to 98%) were higher

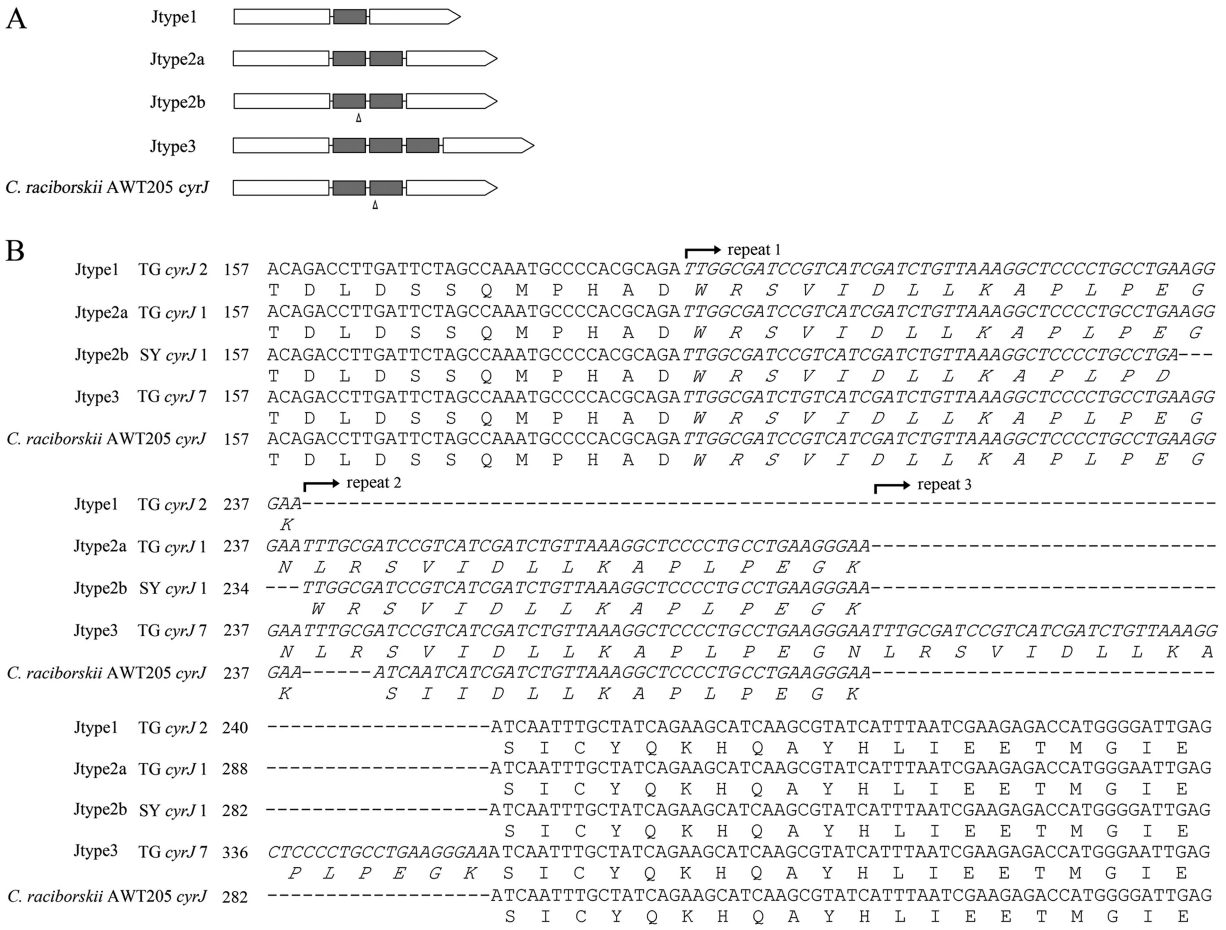

FIG 3 Illustration of three sequence types of the *cyrJ* gene. (A) Schematic structures of *cyrJ* sequence types. White bar, *cyrJ* sequence; gray bar, repeat sequences; triangle, nucleotide deletions; *C. raciborskii* AWT205, reference strain. (B) Partial alignment of representative *cyrJ* gene sequences and deduced protein sequences. Repeat sequences were italicized. Dashed line, gaps introduced into the alignment; arrow, beginning of the repeat sequences.

than those between these two and other clades (93 to 97%). In addition, median values were found between clade III and clade IV (95 to 96%). Also, clade V was the most divergent of all clades with lowest similarities (93 to 96%). Both clade I and clade V contained reference sequences from *Raphidiopsis*. However, the former was a *Raphidiopsis*-mix clade related to both *R. mediterranea* and *R. curvata*, whereas the latter was related to *R. curvata* only and thus was a *R. curvata*-like clade. Clade II contained reference sequences from *Cylindrospermopsis* and was denominated as a *Cylindrospermopsis*-like clade. For the closely related clade III and clade IV, no reference sequence was obtained for the former, but the latter included two reference sequences from *C. raciborskii* CHAB3409 and *R. brookii* D9. In addition, CYN-producing strains, along with non-CYN-producing strains, clustered together in clade II and clade V.

DISCUSSION

As displayed in Table 3, four *C. raciborskii* strains and four *R. curvata* strains from Chinese freshwater bodies were confirmed to

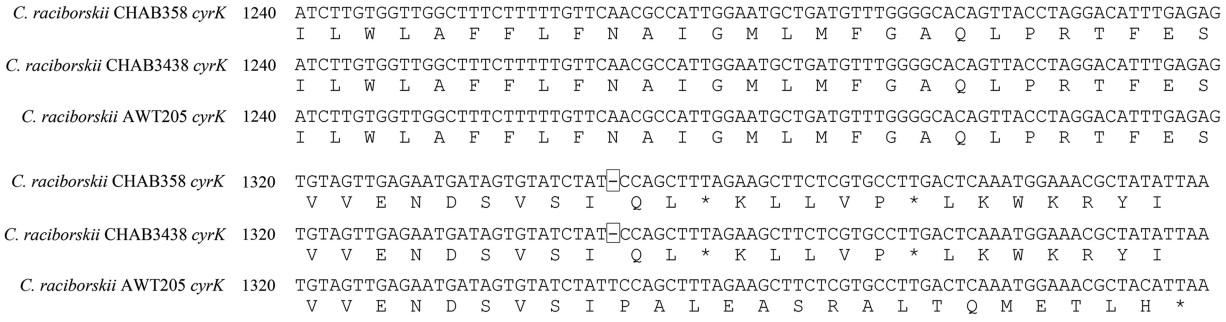

FIG 4 Partial alignment of mutant *cyrK* gene sequences and deduced protein sequences. Rectangle, nucleotide deletion; asterisk, stop codon.

TABLE 4 Percent extracellular CYNs in cultures of four CYN-producing cyanobacterial strains

CYN	Mean extracellular CYN content (%) ± SD[a]			
	AWT205	CHAB358	CHAB3438	CHAB1150
CYN				
Day 3	27 ± 6.0	–	24 ± 3.0	–
Day 7	39 ± 3.0	–	30 ± 6.0	–
7-Deoxy-CYN				
Day 3	24 ± 4.0	21 ± 1.0	47 ± 4.0	15 ± 1.0
Day 7	45 ± 2.0	24 ± 1.0	51 ± 9.0	26 ± 2.0
Total CYNs				
Day 3	27 ± 6.0	21 ± 1.0	25 ± 3.0	15 ± 1.0
Day 7	40 ± 3.0	24 ± 1.0	31 ± 6.0	26 ± 2.0

[a] The four strains are described in the text. Data obtained on days 3 and 7 after inoculation are shown. −, not detected.

contain both *cyr* genes and CYNs. However, CYN-producing strains constituted only a small percentage of the total cyanobacterial strains in the present study (1.7%). The *cyr* genes were also detected in eight freshwater bodies from which five CYN-producing strains were isolated. All of these aquatic ecosystems were located in the subtropical region.

Homologous and phylogenetic analyses revealed that the cloned *cyr* sequences from environmental samples were most likely to be derived from the C/R group. The mixed clade of *cyr* genes from the C/R group and *Aphanizomenon* sp. 10E6 was due to highly conserved sequences and few information sites (38, 39, 62).

The deduced protein sequences of Itype1 to Itype3 were conserved. The 6-nucleotide insertion in Itype2 and Itype3 formed two additional amino acids that belong to α-helix in the predicted secondary structures of CyrI proteins (see Fig. S6 in the supplemental material), and the 30-nucleotide insertion in Itype3 formed a duplicate peptide, including two residues involved in Fe^{2+} binding (42). The reverse complementary insertion sequences in Itype4a^{f} and Itype4a^{r} provided more evidence for the transposon origin of these insertions. Similarly, the insertions of transposable elements within microcystin genes have also been reported (63, 64). The *cyrI* genes of two *C. raciborskii* strains contained base mutations, and those of four *R. curvata* strains denominated as Itype4 contained insertion mutations (Table 3). All of these mutations caused truncated protein sequences of CyrI, and therefore five strains synthesized only 7-deoxy-CYN due to the lack of CyrI function, as discussed earlier (38). Likewise, the *cyrI* gene variations may explain the high concentrations of 7-deoxy-CYN rather than CYN in *L. wollei* (31), *C. raciborskii* ISG9 (65), and the field populations of *C. raciborskii* (49).

The 48-nucleotide repeats in Jtype2 and Jtype3 caused duplicate peptides that belong to α-helix in the predicted secondary structures of CyrJ proteins (see Fig. S7 in the supplemental material). CYNs have been detected in cyanobacterial strains with the *cyrJ* genes of Jtype2a and Jtype2c. Therefore, nucleotide deletion in one repeat of Jtype2 does not lead to the deficiency of CyrJ function. The conservation of sequence repeats within *cyrJ* genes among the C/R group and *Aphanizomenon* sp. emphasized the horizontal gene transfer (HGT) among these species as described by Jiang et al. (38). According to the second repeat, the C/R group strains differed between China and Australia/Brazil, a finding which coincided with different arrangement patterns of the *cyr* gene clusters. Therefore, HGT events were hypothesized to have occurred between local *Cylindrospermopsis* and *Raphidiopsis* species.

Neutral evolution has been demonstrated for most *cyr* genes with low frequency of negatively selected codons (38), but purifying selection has also been found for the adenylation domain of *Aphanizomenon ovalisporum*-like *cyrB* sequences (66). Selection analysis of a large data set of environmental *cyr* sequences revealed evidence for neither recombination nor positive selection. Further, both *cyrI* and *cyrJ* sequences were not under neutral evolution (Tajima's test, $P < 0.01$) with 1 to 11 negatively selected codons. Thus, these two *cyr* genes from the C/R group may be under weak purifying selection. The sequence variations may be anciently created during the formation of these genes.

The CyrK sequences of four *C. raciborskii* strains were truncated at the C-terminal ends due to single-nucleotide deletions within the *cyrK* genes. However, the transcription of mutant *cyrK* and *cyrI* genes could still be detected. The transcription of *cyrI* genes may be ascribed to the cotranscription of polycistron (38), but *cyrK* gene was transcribed in the direction opposite to that of other *cyr* genes (Fig. 2). The release of CYNs in four strains with the CyrK of different lengths was investigated during a short culture period. A minor proportion of the total CYNs were extracellular for each strain (15 to 40%), but the accumulation of extracellular CYNs during the exponential growth phase must result from active release as proposed by Preussel et al. (47). The release was probably mediated by the transporter protein CyrK (37). The extracellular percentages of CYNs were strain dependent and did not correlate with CyrK lengths. Therefore, the mutant CyrK may function as the original CyrK.

Stucken et al. (67) found that the *cyr* gene cluster of Australian *Cylindrospermopsis* strains is inserted into a hydrogenase gene cluster (*hyp*). The genome sequencing of *C. raciborskii* CHAB3438 also revealed a *hyp* gene cluster (see Fig. S8 in the supplemental material). Four ORFs were observed between *hypF* and *hupC* genes, including two transposases (T1 and T2), as well as *cyrN* and *cyrO* genes. Intergenic sequences between *hypF* and *hupC* genes in other strains of the C/R group were also characterized (see Fig. S8 in the supplemental material). As a result, the *cyrN* gene was only found in CYN-producing strains, and the *cyrO* gene was observed in both CYN-producing and non-CYN-producing strains. Therefore, the *cyrN* gene, rather than the *cyrO* gene, likely belongs to the *cyr* gene cluster. The whole *cyr* gene cluster was probably originally inserted into the *hyp* gene cluster and then translocated to other genomic loci, with *cyrN* being a remnant. On the other hand, the *cyr* genes may have experienced acquisition, loss, and reacquisition in Chinese CYN-producing strains. The transfer of the *cyr* gene cluster was probably mediated by transposases observed to surround the gene cluster and between *hyp* genes.

The screening detection of potential CYN producers in the present study was performed with *cyrJ* gene as a molecular probe for its higher specificity to CYN-producing species than PS/PKS genes (37). However, *Cylindrospermopsis*-like *cyr* fragments except *cyrJ* were detected in *Cylindrospermopsis* strains from Brazil and water samples from Florida (34, 36). The Brazilian *C. raciborskii* cyDB-1 showed the presence of both *cyr* genes and CYNs and thus provides strong evidence for the distribution of CYN-producing *Cylindrospermopsis* in the American continent.

The aquatic ecosystems that contained *rpoC1* genes of C/R group included those with *cyr* genes and are located in both sub-

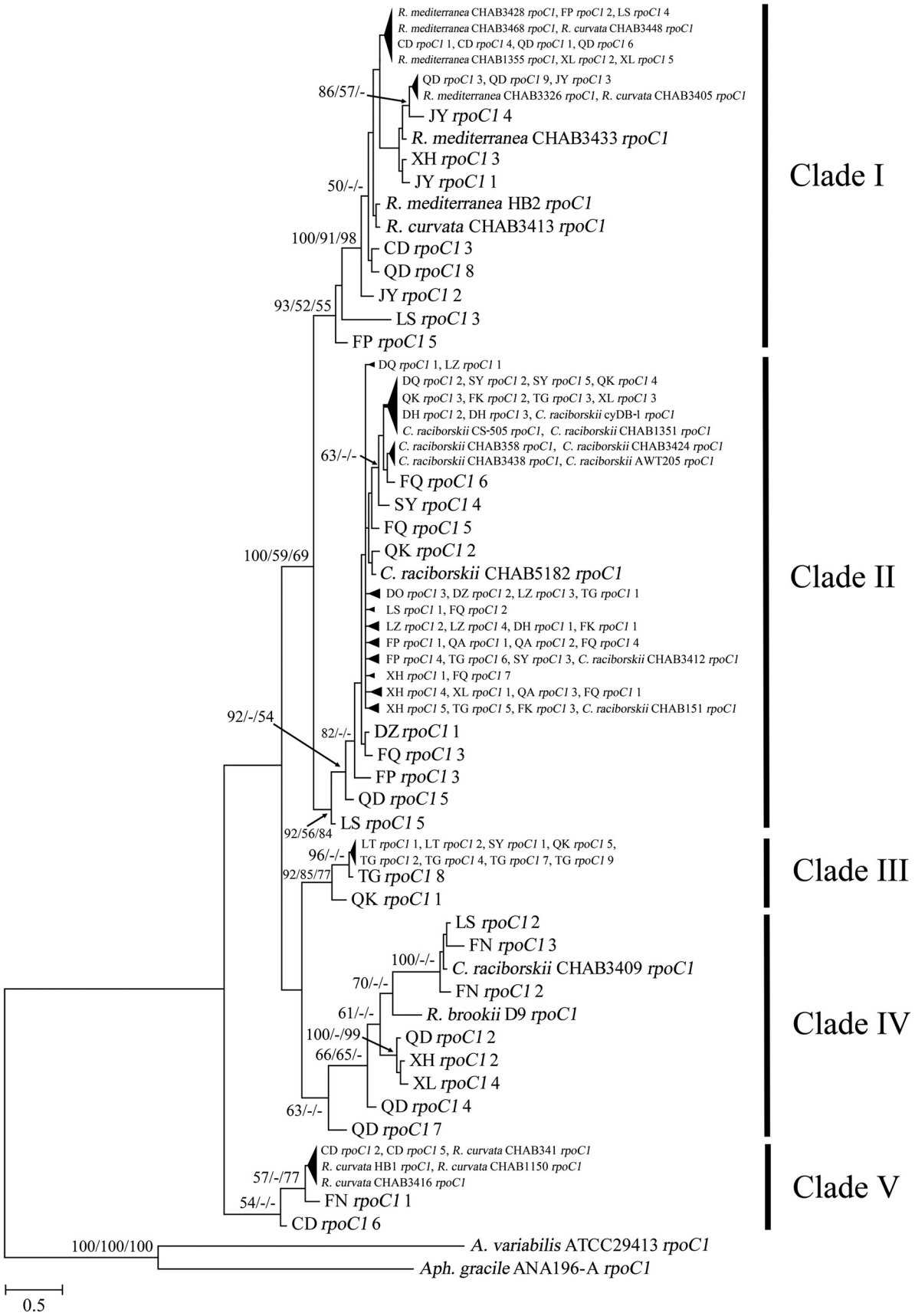

FIG 5 Phylogenetic tree of *rpoC1* gene sequences from environmental samples and cyanobacterial strains (topology based on a Bayesian tree). Bootstrap values above 50% are indicated at the nodes of the tree (Bayesian/ML/NJ). *Aphanizomenon gracile* ANA196-A and *Anabaena variabilis* ATCC 29413 were used as outgroups.

tropical and temperate regions. Thus, non-CYN-producing species were more widely distributed than were the CYN-producing species. The phylogenetics of potential CYN producers (C/R group) were analyzed based on the *rpoC1* gene that displays higher discriminatory power at the genus and species levels than does the 16S *rrn* gene (68). The sequences in each clade were homogeneous but a little divergent in clade IV. Low support values were obtained for most of the five clades (Fig. 5), but sequence similarities among clades were lower than those within each clade. *Raphidiopsis*-mix clade I, *Cylindrospermopsis*-like clade II, and *R. curvata*-like clade V were also observed in a phylogenetic tree based on multigene sequences (69). Clade III and clade IV indicated cryptic and intricate evolutionary clades in C/R group. Clade III, clade IV, and clade V contained sequences from only subtropical regions, indicating the existence of warm-adaptive species in the C/R group. The distribution of CYN producers in these clades was sporadic, as reported previously (70). *Cylindrospermopsis* and *Raphidiopsis* might be congeneric as previously described (67). Meanwhile, both genera are suggested to be paraphyletic and taxonomic reconsideration of the C/R group is necessary.

Previous phylogeographic studies have suggested that *Cylindrospermopsis* strains were separated into three distinct groups, namely, strains from Australia, Europe, and America, with African strains and the former two groups being closely related (71–74). However, inconsistent phylogenetics have been observed for Tunisian and Spanish strains clustered into the America group (75, 76), and for clade II with strains from China, Australia, and Brazil without geographical separation. The present hypotheses suggested that the worldwide dispersion of *Cylindrospermopsis* originated from the tropical zones of Africa and Australia (77) or the warm refuge areas of each continent (73). The invasion success of *Cylindrospermopsis* has been attributed to phenotypic plasticity and different ecotypes (2, 74). On the contrary, the adaptability of *Cylindrospermopsis* and closely related *Raphidiopsis* in different environmental conditions may imply that the two species have similar cosmopolitan distribution to *Microcystis* (78), instead of invasive colonization. Furthermore, the coexistence of local and invasive species is a probable reason for the inconsistent results of phylogeographic analyses. For instance, *R. curvata* CHAB3413 and *R. curvata* CHAB3416 were isolated from the same water body with highly similar morphology and clustered into clade I and clade V, respectively. Worldwide cooperation is suggested for further phylogeographic study of *Cylindrospermopsis* and *Raphidiopsis* with strains from all climate conditions of each continent and through more effective methods, such as comparative genomics. Particularly, evidence for the distribution and growth conditions of *Raphidiopsis* should be provided in the future.

In conclusion, CYN biosynthesis genes were found to be sporadically distributed in cyanobacterial strains and freshwater ecosystems of China. All of the CYN-producing strains and environmental *cyr* sequences described here belong to congeneric and paraphyletic *Cylindrospermopsis* and *Raphidiopsis* species. Distinctive sequence variations, including base mutations, repeat sequences, and transposon insertions in the conserved *cyr* genes, are likely to be created during the formation of these genes. The C-terminal sequence of CyrK is probably not crucial for its function as a transporter. The *cyrN* gene is likely to be a member of the *cyr* gene cluster and distant from other *cyr* genes in Chinese CYN-producing strains. The intragenomic translocations and HGT of the *cyr* gene cluster are related to flanking transposases. The worldwide dispersion of *Cylindrospermopsis* may result from the simultaneous spread of local and invasive species.

ACKNOWLEDGMENTS

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