Sporadic distribution and distinctive variations of cylindrospermopsin genes in cyanobacterial strains and environmental samples from Chinese freshwater bodies

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Running title: Distribution and variations of CYN genes
Abstract

Increasing reports of cylindrospermopsins (CYNs) in freshwater ecosystems have promoted the demand for identifying all the potential CYNs-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, CYNs 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 CYNs-producing *Cylindrospermopsis* in China and reveals the distinctive variations of *cyr* genes.

**Introduction**

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, 2, 3, 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, 6, 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, 9, 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). Mouse assay 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, 25, 26), *Anabaena* (27), *Umezakia* (28, 29),
*Oscillatoria* (30), and *Lyngbya* (31). CYNs-producing *Cylindrospermopsis*
from Australia and Asia have been reported, whereas *Cylindrospermopsis*
strains isolated from Europe and America are incapable of CYNs production
(32, 33, 34, 35, 36). However, no conclusion can be drawn about the
geographic distribution of the CYNs-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 CYNs
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 kinase CyrN providing the phosphoadenylylsulfate 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 *Aph. ovalisporum* (43). However, the protein–DNA interaction has not been verified in other CYNs-producing species. The effects of temperature, light, nitrogen, phosphate, and sulfate on
CYNs production are inconclusive because of uncertainties in strain dependence, release of CYNs, heterocyst formation, and combined effects of multi-factors in different experimental conditions (43, 44, 45, 46, 47, 48, 49, 50, 51). Davis et al. (51) highlighted the effects of the genetic diversity of CYNs producers on the concentration and composition of CYNs in aquatic ecosystems. Moreover, CYNs-producing and non-CYNs-producing genotypes often coexist in the same populations. Therefore, an overview of CYNs-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 CYNs producers is essential. The present work illustrates this issue 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 CYNs 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 (Table S1). Three strains including *C. raciborskii* AWT205, *C. raciborskii* cyDB-1, and *Aph. 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 photons m⁻² s⁻¹. Cyanobacterial cells were harvested at the exponential phase [optical density at 680nm (OD₆₈₀) = 0.8] by centrifugation (12000 g) and stored at -80 °C before further processing.

Collection of environmental samples

Water samples were collected in lakes and reservoirs of China from year 2006 to 2013 (Table 1). These water bodies were located between 22°N and 47°N in subtropical and temperate regions (Figure S1, Table S2). A volume of 300 ml to 500 ml of water was filtered using a membrane filter (MF-Millipore, 0.22 μm) in quadruplicate for each water body at 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 SDS lysis and
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 TE 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).

A primer pair PCβF/PCαR (54) with specificity targeting the phycocyanin operon (cpc) of cyanobacteria was used to confirm the validity of DNA templates for PCR reactions. Primers specific for cyrA, cyrI, and cyrJ genes of current known CYNs-producing species were designed (Figure S2A). Another primer set rpoC1F53/rpoC1R739 was designed to selectively amplify the rpoC1 genes of Cylindrospermopsis and Raphidiopsis (Figure S2B). PCR reaction 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, 1U of LA Taq (Takara, Japan), 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 Tm 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, Japan). Recombinant plasmids of 5 to 15 positive bacterial clones were extracted and the gene fragments were sequenced using the 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 using PCR primers in double direction.

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 Illumina 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 (38). The DNase-digested RNA extracts and cDNA were used as templates for transcription detection. The *cyrI* and *cyrK* genes were amplified using the primer sets cyrIF/cyrIR813 and RTcyrKF991/ RTcyrKR1379 (Table 2) respectively. Negative control without cyanobacterial cells was also subjected to the extraction and detection procedures. Genomic DNA of *C. raciborskii* AWT205 was used as 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 using the ClustalW (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 1000 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 2-parameter model with 1000 bootstrap replicates. The GenBank accession numbers of reference gene sequences were displayed in Table S3. Selection analysis of environmental cyrA, cyrI and cyrJ sequences were also performed as described (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 (61). Carbograph SPE cartridges (6.0 mL, 250 mg) were pretreated with 10 ml of elution solvent (dichloromethane:methanol, 1:4, v/v) acidified with 5% formic acid (v/v) and washed with 10 ml of water. The extracts were acidified with formic acid (1%, v/v) and ionic strength was
adjusted with 0.1% sodium chloride (w/v) before applying to the cartridges. Then, the cartridges were 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 water and the solution was filtered through an ultra centrifugal filter (Millipore, 100 kDa). Extracellular CYNs were also extracted from cell-free spent culture medium by the SPE method. A volume of 100 ml 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 LC-MS/MS using ESI-Q-TOF 6530 coupled with Infinity UHPLC 1290 (Agilent, USA). For LC conditions, a C18 column (4.6 mm by 250 mm, 5 μm) was applied with 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 mass spectrometer were set as follows: gas temperature 300 °C and flow rate 11 L min⁻¹, nebulizer pressure 45 psi, capillary voltage 3500 V, nozzle voltage 1000 V, and fragmentor voltage 175 V. Positive ions of m/z 100 to m/z 2500 were monitored and toxin analogues were determined 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, m/z 320.1, 274.1 and 194.1 for 7-deoxy-CYN). CYN and 7-epi-CYN could not be discriminated in this study and therefore CYN represented these two analogues.

An efficient 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 (v/v) in 50% aqueous methanol (v/v)] in solution A [0.05% aqueous trifluoroacetic acid (v/v)] during 0 min to 10 min, 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 manually collected from elution fractions and confirmed by LC-MS/MS. The standards were used for the identification of potential analogues in samples. Besides, 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

CYNs-producing cyanobacterial strains were first cultured to obtain original biomass (OD<sub>680</sub> = 0.2 to 0.4). The cyanobacterial cells were harvested onto a glass fiber filter (Whatman, GF/C) by gentle filtration (< 5 psi) at sterile conditions and washed three times using MA medium. Afterward, the cells were resuspended and diluted into six parallel cultures (100 ml MA for each) in 500 ml Erlenmeyer flasks with a cell density of OD<sub>680</sub> = 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 the third day and seventh day respectively. The cells and spent medium were separated by gentle filtration (< 5 psi) using membrane filters (MF-Millipore, 0.22 μm), and used for toxin extraction and detection as aforementioned.

Statistical analyses were performed by independent-samples t-test with SPSS 21.0 for Windows and the differences were taken as significant at \( P < 0.05 \).

Nucleotide sequence accession numbers

The nucleotide sequences obtained in this study are available under the following GenBank accession numbers: KJ139686-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. Those strains were collected from 38 freshwater bodies across China, except for several Lyngbya strains obtained from sward and hot spring. 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). Homologous 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. 10E6 (99%). By contrast, the cyrI sequences were found to have low similarities to the cyrI gene of *Aphanizomenon* sp. 10E6 (97%). The environmental cyr sequences and cyr genes from the C/R group and *Aphanizomenon* sp. 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%).

**Sequence analysis**

The cyr genes of *C. raciborskii* CHAB358 and *R. curvata* HB1 were sequenced and assembled into two complete gene clusters (Figure 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 (Figure 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 (Figure 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 529 bp (*C. raciborskii* AWT205 numbering, similarly hereinafter). Single-base mutations were also observed within *cyrI* sequences from TG and SY reservoirs (Figure S3). Six sequences had similar mutations to the *cyrI* gene of *C. raciborskii* CHAB358. Base transversions from guanine to thymine at 304 bp of two sequences were observed and also formed stop codons. In addition, four types of *cyrI* sequences were recognized according to intragenic sequence insertions compared with the *cyrI* gene of *C. raciborskii* AWT205, as depicted in Figure 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 622 bp. Besides, another insertion of a 30-nucleotide fragment, which is also a repeat copy of its upstream sequence, was observed in Itype3 after 494 bp. Moreover, Itype4a included two kinds of sequences (i.e., Itype4a’ and Itype4a”) that contained reverse complementary insertion sequences of a 92-nucleotide fragment after 85 bp, 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, and the cyrI gene of *R. curvata* HB1 was denominated as Itype4b with a long sequence insertion. Compared with 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 (Figure S4).

A 48-nucleotide fragment was found to be repeated within the cyrJ sequences. Three cyrJ sequence types, namely, Jtype1, Jtype2, and Jtype3, were identified based on copy number 1, 2, and 3 of this sequence repeat respectively (Figure 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 CYNs-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 (Figure 3). As displayed in Figure S5, 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 with 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 1,347 bp 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 (Figure 4). Thus, the CyrK mutant (451 aa) was shorter than the original CyrK (465 aa).

**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. Besides, the amplicons covered the gene regions with nucleotide mutation and deletions.

**Assessment of toxin release**

As depicted in Table S4, the cultures of four CYNs-producing cyanobacterial strains maintained exponential growth from low (OD$_{680}$ = 0.13) to high cell
density ($OD_{680} = 0.34$ to $0.61$). The concentrations and extracellular percentages of CYNs were analyzed (Table S4, Table 4). 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 the 7th day were significantly higher than the corresponding percentages on the 3rd day 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 the 3rd day. 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 the 3rd day. For the extracellular percentages of the total CYNs, *C. raciborskii* CHAB358 and *R. curvata* CHAB1150 remained to have lower values with the significantly lowest percentage for *R. curvata* CHAB1150 on the 3rd day (15 ± 1.0%) and the significantly highest percentage for *C. raciborskii* AWT205 on the 7th day (40 ± 3.0%).
Phylogenetics of potential CYNs 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 (Figure 5). High sequence similarities were displayed within four clades, namely, 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 than those between these two and other clades (93% to 97%). Besides, median values were found between Clade III and Clade IV (95% to 96%). Additionally, 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, CYNs-producing strains along with non-CYNs-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 contain both *cyr* genes and CYNs. However, CYNs-producing strains constituted only a small percentage of the total cyanobacterial strains in this study (1.7%). The *cyr* genes were also detected in eight freshwater bodies from which five CYNs-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 (Figure S6), 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 Itype4α$^f$ and Itype4α$^r$ 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. wolfei (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 (Figure S7). 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 \textit{cyrJ} genes among the C/R group and \textit{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, which is coincided with different arrangement patterns of the \textit{cyr} gene clusters. Therefore, HGT events were hypothesized to have occurred between local \textit{Cylindrospermopsis} and \textit{Raphidiopsis} species.

Neutral evolution has been demonstrated for most \textit{cyr} genes with low frequency of negatively selected codons (38), but purifying selection has also been found for the adenylation domain of \textit{Aph. ovalisporum}-like \textit{cyrB} sequences (66). Selection analysis of a large dataset of environmental \textit{cyr} sequences revealed evidence for neither recombination nor positive selection. Further, both \textit{cyrI} and \textit{cyrJ} sequences were not under neutral evolution (Tajima’s test, \(P < 0.01\)) with 1 to 11 negatively selected codons. Thus, these two \textit{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 \textit{C. raciborskii} strains were truncated at C-terminal due to single nucleotide deletions within the \textit{cyrK} genes. However, the transcription of mutant \textit{cyrK} and \textit{cyrI} genes could still be
detected. The transcription of *cyrI* genes may be ascribed to the co-transcription of polycistron (38), but *cyrK* gene was transcribed in the direction opposite to that of other *cyr* genes (Figure 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 *Cylindrospormopsis* strains is inserted into a hydrogenase gene cluster (*hyp*). The genome sequencing of *C. raciborskii* CHAB3438 also revealed a *hyp* gene cluster (Figure S8). 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 (Figure S8). As a result, the *cyrN* gene was only found in CYNs-producing strains, and the *cyrO* gene was observed in both CYNs-producing and non-CYNs-producing strains.
Therefore, cyrN gene, rather than cyrO gene, is likely to belong 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 CYNs-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 CYNs producers in this study was performed with cyrJ gene as a molecular probe for its higher specificity to CYNs-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, US (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 CYNs-producing Cylindrospermopsis in American continent.

The aquatic ecosystems that contained rpoC1 genes of C/R group included those with cyr genes, and are located in both subtropical and temperate regions. Thus, non-CYNs-producing species were more widely distributed than CYNs-producing species. The phylogenetics of potential CYNs producers (C/R group) were analyzed based on rpoC1 gene that
displays higher discriminatory power at the genus and species levels than 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 (Figure 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 multi-gene 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 existence of warm-adaptive species in the C/R group. The distribution of CYNs 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, Australia, Europe, and America, with African strains and the former two groups being closely related (71, 72, 73, 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. A 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, CYNs biosynthesis genes were found to be sporadically
distributed in cyanobacterial strains and freshwater ecosystems of China. All of the CYNs-producing strains and environmental cyr sequences in this study 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 CYNs-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 simultaneous spread of local and invasive species.

Acknowledgments

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References


39. **Stüken A, Jakobsen KS.** 2010. The cylindrospermopsin gene cluster of *Aphanizomenon* sp. strain 10E6: organization and recombination. Microbiology 156:


303–310.


70. Stucken K, Murillo AA, Soto-Liebe K, Fuentes-Valdés JJ, Méndez MA, Vásquez


Figure legends

Figure 1 Schematic structure of cyr gene clusters from CYNs-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.

Figure 2 Illustration of four sequence types of 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. Dash line, gaps introduced into the alignment; bold line, ITRs; arrow, beginning of the repeat sequences.

Figure 3 Illustration of three sequence types of 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. Dash line, gaps introduced into the alignment; arrow, beginning of the repeat sequences.

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

Figure 5 Phylogenetic tree of rpoC1 gene sequences from environmental samples and cyanobacterial strains (topology based on Bayesian tree). Bootstrap values above 50% were indicated at the nodes of the tree (Bayesian/ML/NJ). Aph. gracile ANA196-A and A. variabilis ATCC29413 were used as outgroups.
Table 1 Gene detection of environmental DNA samples from Chinese freshwater bodies. Abbr., abbreviation.

<table>
<thead>
<tr>
<th>Geographic origin</th>
<th>Abbr.</th>
<th>Date of sample</th>
<th>Gene regions&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>rpoC1</td>
</tr>
<tr>
<td>Longhu lake, Daqing, Heilongjiang</td>
<td>LH</td>
<td>Jul., 2012</td>
<td>ND</td>
</tr>
<tr>
<td>Jinyang lake, Taiyuan, Shanxi</td>
<td>JY</td>
<td>Aug., 2010</td>
<td>4</td>
</tr>
<tr>
<td>Fish pond, Qingdao, Shandong</td>
<td>FQ</td>
<td>Nov. 2013</td>
<td>7</td>
</tr>
<tr>
<td>Taihu lake, Wuxi, Jiangsu</td>
<td>TH</td>
<td>Aug., 2011</td>
<td>ND</td>
</tr>
<tr>
<td>Fish pond, Nanjing, Jiangsu</td>
<td>FN</td>
<td>Nov., 2007</td>
<td>3</td>
</tr>
<tr>
<td>Qiandao lake, Hangzhou, Zhejiang</td>
<td>QA</td>
<td>Oct., 2012</td>
<td>3</td>
</tr>
<tr>
<td>Xianghu lake, Hangzhou, Zhejiang</td>
<td>XH</td>
<td>Oct., 2012</td>
<td>5</td>
</tr>
<tr>
<td>Xihu lake, Hangzhou, Zhejiang</td>
<td>XL</td>
<td>Oct., 2012</td>
<td>5</td>
</tr>
<tr>
<td>Dongqian lake, Ningbo, Zhejiang</td>
<td>DQ</td>
<td>Jul., 2009</td>
<td>3</td>
</tr>
<tr>
<td>Donghu lake, Wuhan, Hubei</td>
<td>DH</td>
<td>Nov., 2006</td>
<td>3</td>
</tr>
<tr>
<td>Tangxun lake, Wuhan, Hubei</td>
<td>TX</td>
<td>Oct., 2012</td>
<td>ND</td>
</tr>
<tr>
<td>Liangzi lake, Ezhou, Hubei</td>
<td>LZ</td>
<td>Sept., 2011</td>
<td>4</td>
</tr>
<tr>
<td>Qiaodun lake, Daye, Hubei</td>
<td>QD</td>
<td>Sept., 2011</td>
<td>9</td>
</tr>
<tr>
<td>Chidong lake, Qichun, Hubei</td>
<td>CD</td>
<td>Aug., 2006</td>
<td>6</td>
</tr>
<tr>
<td>Lushui reservoir, Chibi, Hubei</td>
<td>LS</td>
<td>May, 2006</td>
<td>5</td>
</tr>
<tr>
<td>Poyang lake, Nanchang, Jiangxi</td>
<td>PO</td>
<td>Aug., 2012</td>
<td>ND</td>
</tr>
<tr>
<td>Erhai lake, Dali, Yunnan</td>
<td>EH</td>
<td>Aug., 2010</td>
<td>ND</td>
</tr>
<tr>
<td>Fish pond, Kunming, Yunnan</td>
<td>FK</td>
<td>Oct., 2006</td>
<td>3</td>
</tr>
<tr>
<td>Dongzhen reservoir, Putian, Fujian</td>
<td>DZ</td>
<td>Sept., 2011</td>
<td>2</td>
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<td>Fish pond, Panyu, Guangdong</td>
<td>FP</td>
<td>May, 2012</td>
<td>5</td>
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<tr>
<td>Shiyan reservoir, Shenzhen</td>
<td>SY</td>
<td>Jun., 2007</td>
<td>5</td>
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<tr>
<td>Qiankeng reservoir, Shenzhen</td>
<td>QK</td>
<td>Jun., 2007</td>
<td>5</td>
</tr>
<tr>
<td>Tiegang reservoir, Shenzhen</td>
<td>TG</td>
<td>Jun., 2007</td>
<td>9</td>
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<tr>
<td>Luotian reservoir, Shenzhen</td>
<td>LT</td>
<td>Jun., 2007</td>
<td>2</td>
</tr>
<tr>
<td>Changluopi reservoir, Shenzhen</td>
<td>CL</td>
<td>Jun., 2007</td>
<td>ND</td>
</tr>
</tbody>
</table>

<sup>a</sup> Number of unique sequences; D, detected; ND, not detected; minus symbol, not tested.
Table 2 Characteristics of primer pairs used for gene detection.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer</th>
<th>Sequence (5'-3')</th>
<th>Tm (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>cpcBA-IGS</td>
<td>PCβF</td>
<td>GGCTGCTTGTTTACGCGACA</td>
<td>50</td>
</tr>
<tr>
<td></td>
<td>PCαR</td>
<td>CCAGTACCACCAGCAACTAA</td>
<td></td>
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<tr>
<td>cyrA</td>
<td>cyrAF51&lt;sup&gt;a&lt;/sup&gt;</td>
<td>GATGGTTGTCGGGATTGCAGAT</td>
<td>57</td>
</tr>
<tr>
<td></td>
<td>cyrAR1167</td>
<td>GAAGCGAGAAACGCCATTGGT</td>
<td></td>
</tr>
<tr>
<td></td>
<td>cyrIF&lt;sup&gt;b&lt;/sup&gt;</td>
<td>CAGGCTTAATCTGCAACACATTTCT</td>
<td>56</td>
</tr>
<tr>
<td></td>
<td>cyrIR813</td>
<td>CGTTTATCATCTCCAGATGCATCCA</td>
<td></td>
</tr>
<tr>
<td>cyrI</td>
<td>cyrIF13</td>
<td>CGAATCAGAATGGTGCTGTGTC</td>
<td>59</td>
</tr>
<tr>
<td></td>
<td>cyrJR720</td>
<td>GACAAGATATAGCGCGCAACGACTCA</td>
<td></td>
</tr>
<tr>
<td>cyrJ</td>
<td>RTcyrKF991</td>
<td>GGAGCGTGGTGCTATTTTC</td>
<td>55</td>
</tr>
<tr>
<td></td>
<td>RTcyrKR1379</td>
<td>TGAGGAAGGCACGAGAAAG</td>
<td></td>
</tr>
<tr>
<td>cyrK</td>
<td>rpoC1F53</td>
<td>CACCAGAAGCTCCCGGCCT</td>
<td></td>
</tr>
<tr>
<td>rpoC1J</td>
<td>rpoC1R739</td>
<td>GGTGGAATGACTGGAATGGCTGA</td>
<td>60</td>
</tr>
</tbody>
</table>

<sup>a</sup>C. raciborskii CS-505 numbering.

<sup>b</sup>Targeting flanking sequence upstream *cyrI* gene.
Table 3 CYNs-producing cyanobacterial strains isolated from Chinese freshwater bodies.

<table>
<thead>
<tr>
<th>Strains</th>
<th>Geographic origin</th>
<th>cyrI</th>
<th>cyrK</th>
<th>CYN</th>
<th>7-deoxy-CYN</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>C. raciborskii CHAB357</td>
<td>Wenshan lake</td>
<td>M</td>
<td>M</td>
<td>ND</td>
<td>D</td>
<td>This study</td>
</tr>
<tr>
<td>C. raciborskii CHAB358</td>
<td>Wenshan lake</td>
<td>M</td>
<td>M</td>
<td>ND</td>
<td>D</td>
<td>This study</td>
</tr>
<tr>
<td>C. raciborskii CHAB3438</td>
<td>Xianghu lake</td>
<td>H</td>
<td>M</td>
<td>D</td>
<td>D</td>
<td>This study</td>
</tr>
<tr>
<td>C. raciborskii CHAB3440</td>
<td>Xianghu lake</td>
<td>H</td>
<td>M</td>
<td>D</td>
<td>D</td>
<td>This study</td>
</tr>
<tr>
<td>R. curvata CHAB114</td>
<td>Chidong lake</td>
<td>M</td>
<td>H</td>
<td>ND</td>
<td>D</td>
<td>This study</td>
</tr>
<tr>
<td>R. curvata CHAB1150</td>
<td>Chidong lake</td>
<td>M</td>
<td>H</td>
<td>ND</td>
<td>D</td>
<td>Jiang et al. (38)</td>
</tr>
<tr>
<td>R. curvata CHAB3416</td>
<td>Qiaodun lake</td>
<td>M</td>
<td>H</td>
<td>ND</td>
<td>D</td>
<td>This study</td>
</tr>
<tr>
<td>R. curvata HB1</td>
<td>Guanqiao Pond</td>
<td>M</td>
<td>H</td>
<td>D</td>
<td>D</td>
<td>Li et al. (22)</td>
</tr>
</tbody>
</table>

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.
Table 4 The percentages of extracellular CYNs in the cultures of four CYNs-producing cyanobacterial strains (%). Ex, extracellular.

<table>
<thead>
<tr>
<th>Strain</th>
<th>AWT205</th>
<th>CHAB358</th>
<th>CHAB3438</th>
<th>CHAB1150</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ex-percentage of CYN</td>
<td>27 ± 6.0&lt;sup&gt;b&lt;/sup&gt;</td>
<td>–</td>
<td>24 ± 3.0</td>
<td>–</td>
</tr>
<tr>
<td>Ex-percentage of 7-deoxy-CYN</td>
<td>39 ± 3.0</td>
<td>–</td>
<td>30 ± 6.0</td>
<td>–</td>
</tr>
<tr>
<td>Ex-percentage of total CYNs</td>
<td>24 ± 4.0</td>
<td>21 ± 1.0</td>
<td>47 ± 4.0</td>
<td>15 ± 1.0</td>
</tr>
<tr>
<td>Ex-percentage of total CYNs</td>
<td>45 ± 2.0</td>
<td>24 ± 1.0</td>
<td>51 ± 9.0</td>
<td>26 ± 2.0</td>
</tr>
</tbody>
</table>

<sup>a</sup> The strain numbers were described in the main text.

<sup>b</sup> Data (Mean ± SD) obtained at the 3rd day (regular) and the 7th day (boldface) after inoculation.