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Polysaccharide biosynthesis-related genes explain phenotype-genotype correlation of *Microcystis* colonies in Meiliang Bay of Lake Taihu, China

Shutu Xu[1,2], Qianqian Sun[3], Xiaohua Zhou[3], Xiao Tan[3], Man Xiao[4], Wei Zhu[3] & Ming Li[2,5]

The 16S rDNA, 16S-23S rDNA-ITS, *cpc*BA-IGS, *mcy* gene and several polysaccharide biosynthesis-related genes (epsL and *TagH*) were analyzed along with the identification of the morphology of *Microcystis* colonies collected in Lake Taihu in 2014. *M. wesenbergii* colonies could be distinguished directly from other colonies using *espL*. *TagH* divided all of the samples into two clusters but failed to distinguish different phenotypes. Our results indicated that neither morphology nor molecular tools including 16S rDNA, 16S-23S ITS and *cpc*BA-IGS could distinguish toxic and non-toxic species among the identified *Microcystis* species. No obvious relationship was detected between the phenotypes of *Microcystis* and their genotypes using 16S, 16S-23S and *cpc*BA-IGS, but polysaccharide biosynthesis-related genes may distinguish the *Microcystis* phenotypes. Furthermore, the sequences of the polysaccharide biosynthesis-related genes (*espL* and *TagH*) extracted from *Microcystis* scums collected throughout 2015 was analyzed. Samples dominated by *M. ichthyoblabe* (60–100%) and *M. wesenbergii* (60–100%) were divided into different clade by both *espL* and *TagH*, respectively. Therefore, it was confirmed that *M. wesenbergii* and *M. ichthyoblabe* could be distinguished by the polysaccharide biosynthesis-related genes (*espL* and *TagH*). This study is of great significance in filling the gap between classification of molecular biology and the morphological taxonomy of *Microcystis*.

Microcystis spp. is a common genus of bloom-forming cyanobacteria, which generates *Microcystis* blooms worldwide[1]. *Microcystis* blooms is one of the serious harmful algae blooms because many *Microcystis* species produce microcystins having high toxicity[2]. These blooms also cause fish mortality due to depletion of oxygen[3] and loss of biodiversity and affect the cycles of biogenic elements in freshwater ecosystems[1,4]. Thus, an insight into the distribution, succession and diversity of *Microcystis* species is important to understand the life-cycle of *Microcystis* as well as ecology of *Microcystis* blooms.

During the past decades, many studies have been carried out to investigate the processes of *Microcystis* bloom formation[5,6]. Multiple *Microcystis* species have been recorded according to their morphological characteristics, especially their colonial morphology[7]. The life cycle[8], spatial distribution[9], seasonal succession[10] and physiology of *Microcystis*[11] has been well studied based on this morphological taxonomy. In addition, the competition between *Microcystis* spp. and other algae and also the competition among different *Microcystis* species have been investigated to reveal the ecology of *Microcystis* bloom formation[12,13].

Recently, *Microcystis* has been well documented having high phenotypic plasticity[14,15]. Otsuka *et al.*[16] demonstrated that the colonial morphology of *Microcystis* in culture could change from time to time. Sun *et al.*[17] indicated that colonies with colonial morphology of *M. aeruginosa* under culture conditions could change their

[1]College of Agronomy, Northwest A & F University, Yangling 712100, PR China. [2]College of Resources and Environment, Northwest A & F University, Yangling 712100, PR China. [3]College of Environment, Hohai University, Nanjing 210098, PR China. [4]Australian Rivers Institute, Griffith University, Nathan, Qld 4111, Australia. [5]Key Laboratory of Plant Nutrition and the Agri-environment in Northwest China, Ministry of Agriculture, PR China. Correspondence and requests for materials should be addressed to W.Z. (email: zhuweiteam.hhu@gmail.com) or M.L. (email: lileaf@163.com)

| Primer	For sequence (5′–3′)	Rev sequence (5′–3′)	Reference
16S	ATGTGCCGCGAGGTGAAACCTAAT	TTACAATCCAAAGACCTTCCTCCC	Gan et al.[26]
ITS(A)	TCAGGTTGCTTAACGACCTA	(G/T)TTCGCTCGCC(A/G)CTAC	Otsuka et al. (1999a)
ITS(S)	CCAGTGAAGTCGTAACAAGG	GGGTT(T/G/C)CCCCATTCGG	Otsuka et al.(1999a)
cpcBA-IGS	GGCTGCTTGTTTACGCGACA	CCAGTACCACCAGCAACTAA	Otsuka et al. (1999b)
mcyB	CTATGTTATTTATACATCAGG	CTCAGCTTAACTTGATTATC	Neilan et al. (1995)
epsL	CGATGGGTGCGTTATCTTCC	GCCGATTACTGGCTGTCCTG	Gan et al.[26]
TagH	CCGACAAAGGGACAGGTGAGA	CGCAAATCCTAAACGAGCCAC	Gan et al.[26]

Table 1. List of primer pairs for the amplification and sequencing of *Microcystis*.
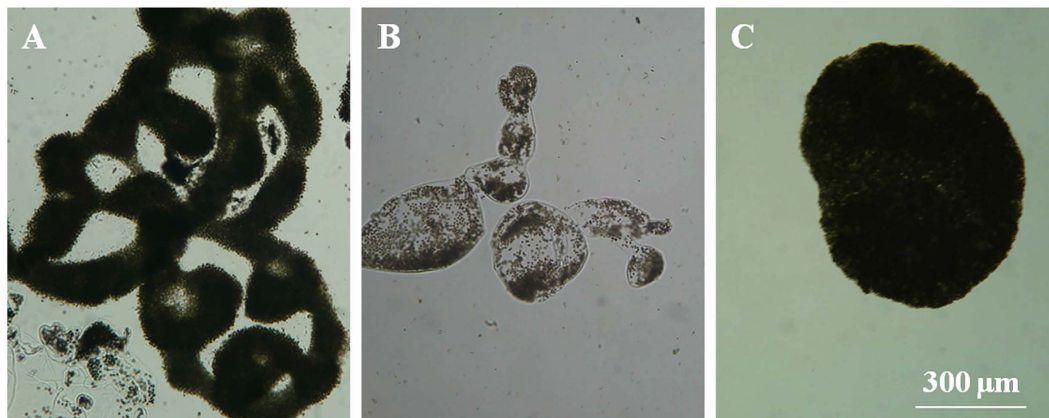
Figure 1. Micrographs of *Microcystis* species collected in Lake Taihu. (A) *M. aeruginosa*; **(B)** *M. wesenbergii*; **(C)** *M. ichthyoblabe*.
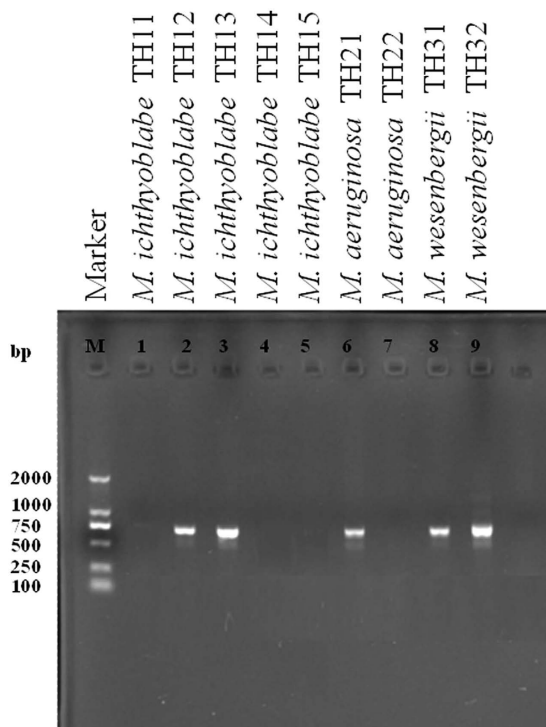
Figure 2. Electropherogram of the PCR products with the primer of *mcy*B.
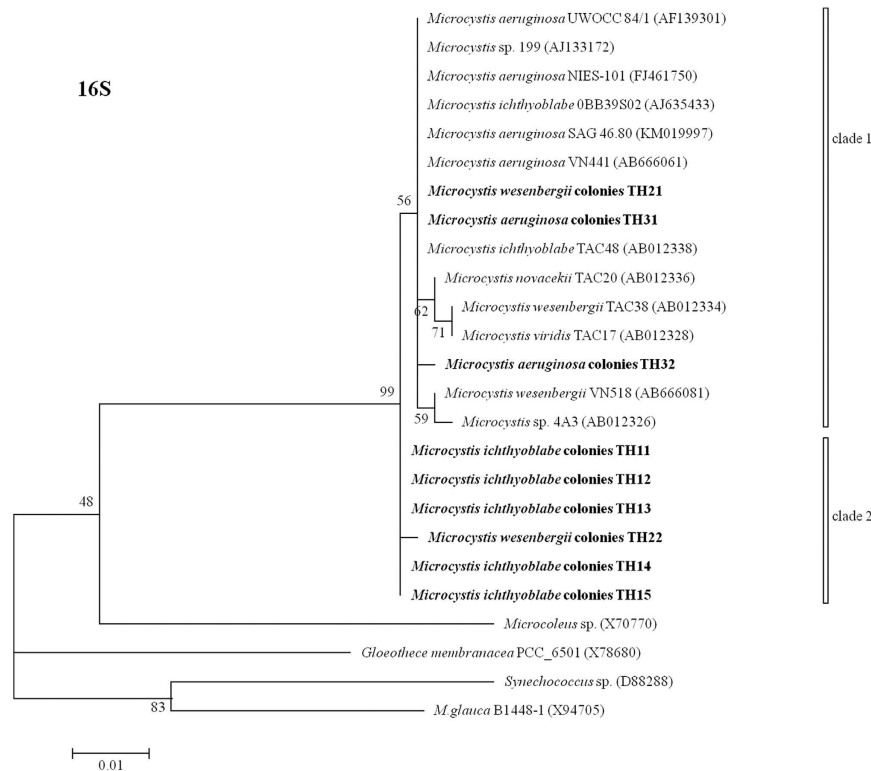
Figure 3. Phylogenetic tree based on the analysis of the 16S gene sequences.

morphology to that of a typical *M. novacekii*. Li *et al.*[18] illustrated that solubilization of mucilage could induce changes in colonial morphology and the authors suggested that seasonal succession of *Microcystis* species was due to morphological changes. Therefore, the taxonomy of this genus should be re-evaluated via molecular genetic analyses.

The phenotype-genotype correlation of *Microcystis* is helpful in filling the gap between classification of molecular biology and the morphological taxonomy of *Microcystis*. The phylogenetic analysis based on 16S rDNA was considered as one of the most reliable criteria for determining relationships among organisms with close relation[19]. However, the similarity of colonies in different morphology was high as measured by 16S rDNA sequencing[20,21], and thus the unification of five species of *Microcystis* has been proposed[22]. In addition, the events of horizontal gene transfer would cause flexibility of several informative genes including 16S rDNA of *Microcystis*[23]. A more reliable gene sequence should be explored to analyze the phenotype-genotype correlation of *Microcystis*. Otten and Paerl[24] indicated that *M. wesenbergii* could be identified from four different *Microcystis* morphospecies using 16S-23S rDNA-ITS sequences, but the other four morphospecies could not. Tan *et al.*[25] indicated *cpc*BA-IGS could be used as an effective tool to identify *M. wesenbergii*. Several polysaccharide biosynthesis-related genes were also found to identify morphospecies of *Microcystis*[26]. Thus, these genes were hypothesized to be significantly related to *Microcystis* colonial morphology, and this hypothesis has been preliminarily verified by Xu *et al.*[27].

In addition, microcystin-producing genes were also postulated to divide *Microcystis* into toxic species and non-toxic species[28]. The morphospecies was considered to relate to the toxicity of *Microcystis*. Generally, *M. ichthyoblabe* was considered as non-toxic species[29], while *M. aeruginosa* and *M. wesenbergii* as toxic species[30–32]. The microcystin synthetase (*mcy*) gene cluster in different *Microcystis* morphospecies was thus analyzed to reveal the phenotype-genotype correlation of *Microcystis* colonies[33]. However, it was still poorly understood whether there was a relationship between the phenotype and microcystin-producing genes.

The current study aimed to gain insight into the phenotype-genotype correlation of *Microcystis*. The 16S rDNA, 16S-23S rDNA-ITS, *cpc*BA-IGS, *mcy* gene (*mcy*B)[34] and several polysaccharide biosynthesis-related genes were analyzed along with the identification of the morphology of *Microcystis* colonies collected in the field. This study also attempted to resolve that polysaccharide biosynthesis-related genes might distinguish the *Microcystis* morphospecies as EPS played great roles in colony formation and morphological changes of *Microcystis*[18,35].

Materials and Methods

Experimental design. This study has two parts. (I) Seeking novel functional gene which may distinguish the *Microcystis* morphospecies. Individual *Microcystis* colonies were isolated from natural samples and then axenically cultured for PCR amplification and sequencing. Afterwards, phenotype-genotype correlation of *Microcystis* colonies was investigated and the function gene was identified. (II) Confirming the functional gene. *Microcystis* "scum" at different seasons were collected and divided into varying classes consisting of various *Microcystis*
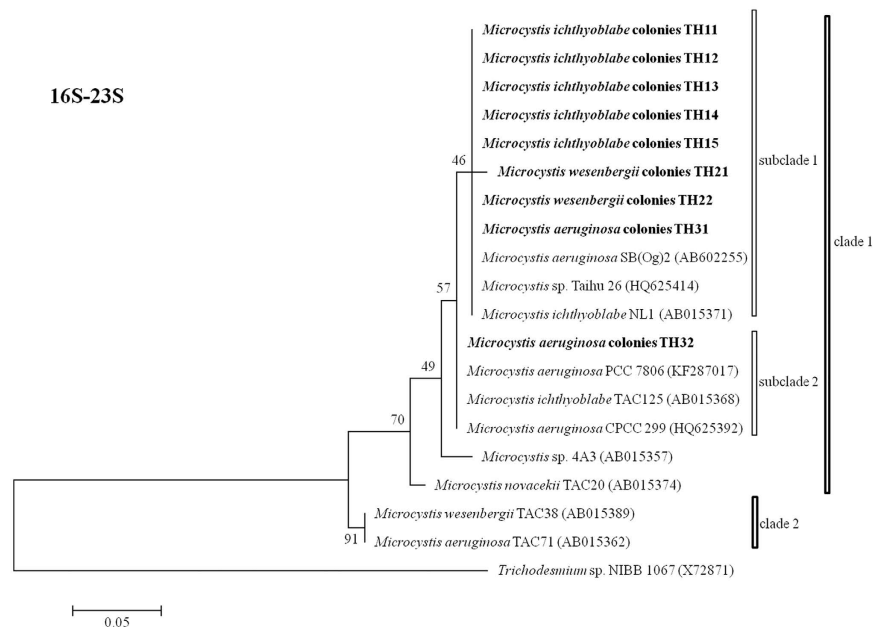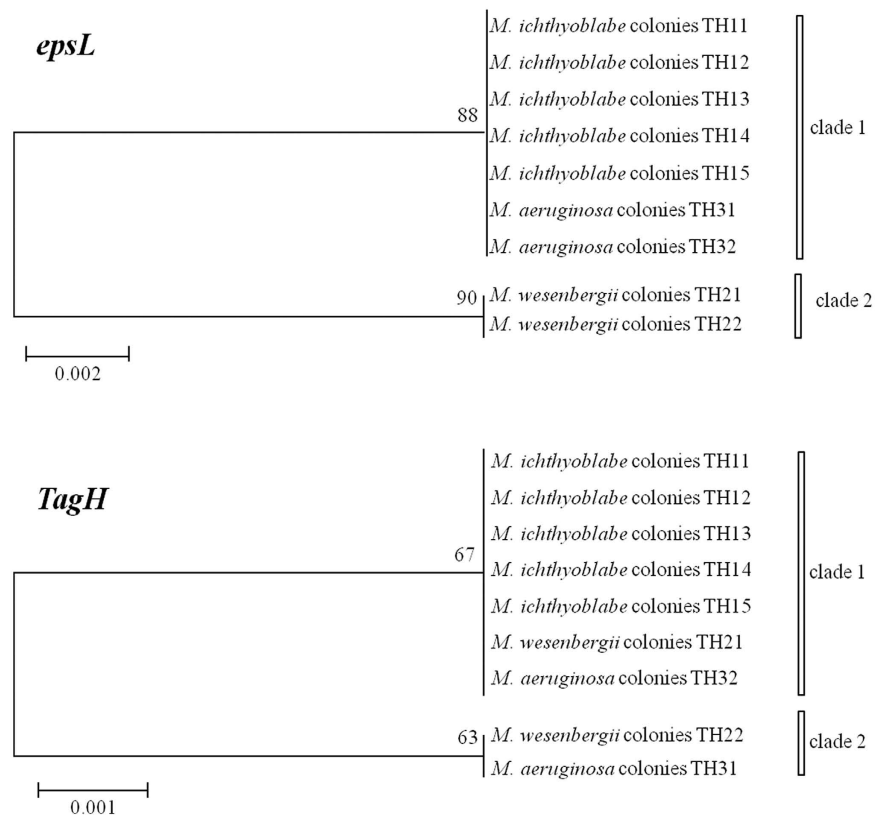
16S-23S

Figure 4. Phylogenetic tree based on the analysis of the 16S-23S gene sequences.
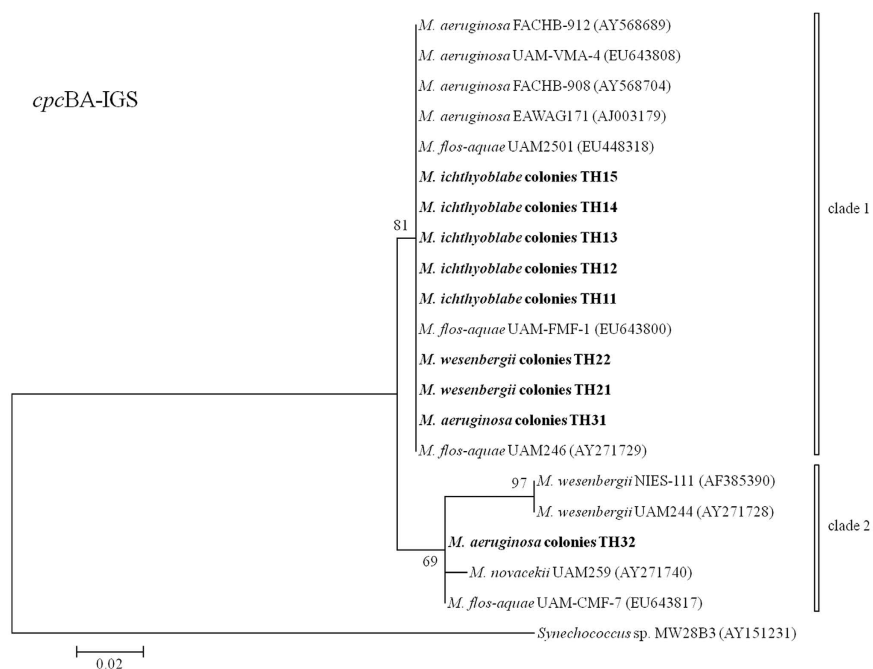
*cpc*BA-IGS

Figure 5. Phylogenetic tree based on the analysis of the *cpc*BA-IGS gene sequences.

morphospecies according to colony size. The functional genes of the subsamples were then analyzed to confirm that this gene succeed in distinguishing the *Microcystis* morphospecies.

Sample collections. Algal samples for colony isolation and culture in part I were collected during a *Microcystis* bloom in Meiliang Bay in northern Lake Taihu (China) on 15 August and 1 November 2014. Lake Taihu was selected in the current study because *Microcystis* spp. is the dominant species at most of the time and heavy *Microcystis* blooms occurs frequently[10]. In addition, the colony morphology and phylogenetic inference of *Microcystis* species has been well investigated in this lake[8,24,36], which could be referred to. The water samples containing abundant *Microcystis* colonies were collected directly from the lake surface (30 cm depth) and were transferred into plastic bottles with a capacity of 5 L. The samples were then stored in a cold closet and transported
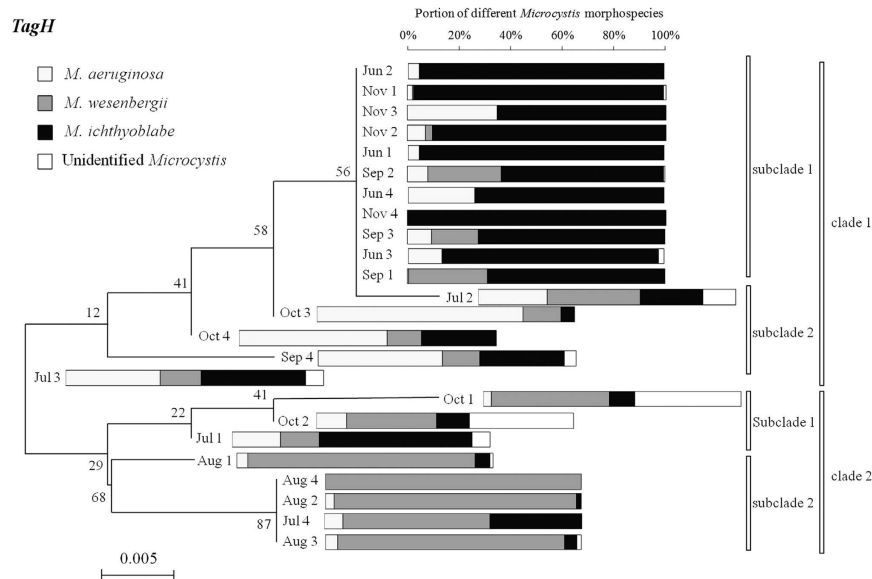
Figure 6. Phylogenetic tree based on the analysis of the polysaccharide biosynthesis-related gene sequences (*espL* and *TagH*).
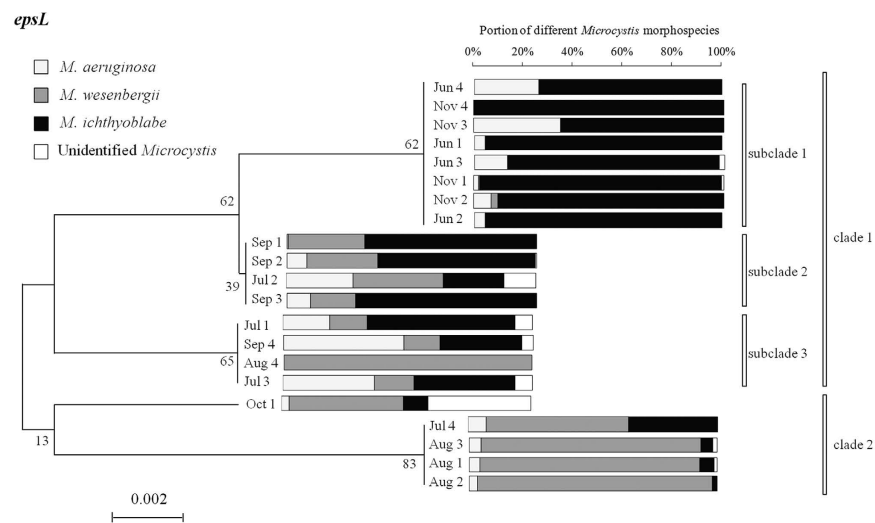
Figure 7. Phylogenetic tree based on the analysis of the sequences of *espL* genes extracted from *Microcystis* scums collected in different months with different morphospecies composition.

to the laboratory as soon as possible for culture. Algal samples for confirming the functional gene in part II were collected on 4 June, 16 July, 17 August, 29 September, 15 October and 15 November, 2015, respectively.

Microcystis colony separation. Water samples for part I were diluted with BG-11 culture medium until a single *Microcystis* colony could be separated by a pipette. The separated colony was examined under a microscope (×100), and the colonial morphology was recorded. *M. aeruginosa* and *M. wesenbergii* were found in the sample collected on 15 August. *M. ichthyoblabe* was found in the sample collected on 1 November. Five colonies of each morphology were separated for culture. *M. ichthyoblabe* colonies were named *M. ichthyoblabe* colonies TH11, TH12,

Figure 8. Phylogenetic tree based on the analysis of the sequences of *TagH* genes extracted from *Microcystis* scums collected in different months with different morphospecies composition.

TH13, TH14 and TH15. *M. aeruginosa* colonies were named *M. aeruginosa* colonies TH21, TH22, TH23, TH24 and TH25. *M. wesenbergii* colonies were named *M. wesenbergii* colonies TH31, TH32, TH33, TH34 and TH35.

Single colony culture. Each colony was washed with BG-11 medium three times. Then, the colonies were cultured in 10 mL of BG-11 medium in glass tubes at 25 °C under a 12 h:12 h light-dark cycle with a light density of approximately 45 μmol m^{-2} s^{-1}. After one month of culture, the *M. ichthyoblabe* colonies TH11, TH12, TH13, TH14, TH15, the *M. aeruginosa* colonies TH21 and TH22 and the *M. wesenbergii* colonies TH31 and TH32 grew well but the others died. The DNA of the growing *Microcystis* was extracted.

DNA extraction. The DNA extraction method was referred to Sun *et al.*[17]. *Microcystis* pellets were dispersed into 0.8 mL extraction buffer (1.5 M NaCl, 1% CTAB, 100 mM Tris-HCl, 100 mM Na$_2$EDTA, 100 mM Na$_3$PO$_3$, pH 0.8) and 20 μL of proteinase K (30 mg mL^{-1}). Afterwards, they were incubated at 37 °C for 30 min and then, 0.48 mL of 20% SDS was added to each sample, incubating at 65 °C for 1 h. The samples were extracted using phenol-chloroform-isoamyl (25:24:1) and chloroform-isoamyl (24:1) successively. Centrifuged at 8000 × g for 5 min, the supernatant was transferred to new tubes. Thereafter, 0.6 mL pure isopropyl alcohol was injected to purify the DNA sample. After 20-min centrifugation at 16000 × g, 70% ethanol was used to rinse the DNA sample. Each DNA sample was dried and dissolved in 100 μL of Tris-EDTA (10 mM Tris and l mM EDTA, pH 8.0). Finally, the DNA sample was analyzed using a Nanodrop-2000.

PCR amplification and sequencing. Seven pairs of primers targeting the 16S rRNA, 16S-23S ITS(A)/(S), *cpc*BA-IGS, *mcy*B, *Tag*H and *eps*L genes were used for the amplification and sequencing of all of the samples (see Table 1). A total volume of 50 μL containing 25 μL of 2 × PCR mixture buffer with tag enzyme (Bioteke, Beijing, China), 1.2 μL of each primer (10 μM), 2 μL DNA (10–20 ng μL^{-1}) and 21.8 μL ddH$_2$O was used for the PCR amplifications. The PCR amplification was run with an initial denaturation of the DNA at 94 °C for 5 min, followed by 34 cycles of 50 s at 94 °C, 50 s at 42 °C (*mcy*B) or 30 s at 50 °C (16S, 16S-23S) or 30 s at 52 °C (*cpc*BA-IGS) or 30 s at 55 °C (*Tag*H, *eps*L), and 1 min at 72 °C. The reaction was completed after 10 min at 72 °C. The detection and the size of the amplicons were determined by agarose (1.0%) gel electrophoresis compared with a DL2000 DNA Marker (Tiangen, Beijing, China). The amplicons with the correct length were used for sequencing by the Tianyihuiyuan biotechnology company (except *mcy*B gene).

Treatment of samples for part II. The sample for part II was poured gently through sieves (divided into four classes: >500 μm, 300–500 μm, 150–300 μm and 75–150 μm). Each class was re-suspended in BG-11 medium. For each subsample from sieving, the photomicrographs were taken using an Olympus C-5050 digital camera coupled with an optical microscope (Olympus CX31). The length and width of *Microcystis* colonies was analyzed using the UTHSCSA ImageTool (v3.00, University of Texas Health Science Center, San Antonio, TX, USA). The biovolume of *Microcystis* colony was calculated as volume = π/6 (length × width)$^{3/2}$ as it is hard to measure the thickness of colonies. A total of 300 colonies were analyzed in each sample. Afterwards, the percentage of different *Microcystis* morphospecies in the total *Microcystis* biovolume of each subsample was calculated. *Microcystis* morphospecies was identified according to Yu *et al.*[7]. In the current study, *M. ichthyoblabe*, *M. aeruginosa* and *M. wesenbergii* was identified as in Fig. 1 and other *Microcystis* colonies were defined as unidentified *Microcystis*.

For each subsample, DNA for PCR templates was extracted. Only *epsL* and *TagH* were used for amplification and sequencing according to the results of part I. All the procedure and method was as same as those described for part I.

Data analysis.   Alignment for all of the sequences was determined by Muscle and edited by software Bioedit[37]. Some related sequences in the NCBI database were also used for alignment. MEGA5 was used to construct neighbor-joining tree of phylogeny analysis[38], with bootstrap for 1000 replications, Maximum Composite Likelihood, and d: Transitions + Transversions.

Results and Discussion

Relationship between species and toxicity.   Figure 2 shows an electropherogram of the PCR products with the primer of *mcy*B. Our results showed that one *M. aeruginosa* colony contained *mcy*B but the other did not. Two out of five *M. ichthyoblabe* colonies contained *mcy*B in this study. Mazur-Marzec *et al.*[39] showed similar results in the Vistula Lagoon (southern Baltic Sea). However, *M. aeruginosa* colonies are generally considered as toxic species[30,40]. *M. ichthyoblabe* has never been reported to produce microcystins[29,41,42]. *M. wesenbergii* was classified as a non-toxic species[31], but our results showed that both two *M. wesenbergii* colonies contained *mcy*B. Nevertheless, some investigations[32,42] also illustrated that *M. wesenbergii* is toxic. All of the conflicting conclusions above indicated that there is not an exact relationship between the phenotype and microcystin-producing genes.

Yoshida *et al.*[32] divided 47 strains of *Microcystis* into three clusters based on the sequences of 16S-23S rDNA-ITS. Their results showed that the first cluster contained both non-toxic and toxic strains, the second only had toxic ones, and the last only had non-toxic strains. This result implied that the 16S-23S gene may distinguish the toxic and non-toxic *Microcystis* species, which was also reported by Janse *et al.*[43]. On the contrary, our results demonstrated that the 16S-23S gene sequences failed to distinguish nine strains with different phenotypes, four of which possessed the *mcy*B gene. This result suggested that 16S-23S rDNA-ITS gene failed to distinguish toxic and non-toxic strains. Yoshida *et al.*[44] suggested that 16S rDNA could used to identify toxic and non-toxic *Microcystis* species in some bloom stages. However, our results did not reach a similar conclusion. Therefore, the *Microcystis* species identified by morphology or molecular tools (16S rDNA, 16S-23S ITS and *cpc*BA-IGS) could not be used to distinguish toxic and non-toxic species.

Phylogenetic trees based on 16S, 16S-23S and cpcBA-IGS.   The phylogenetic trees referring to 16S, 16S-23S and *cpc*BA-IGS are illustrated in Figs 3, 4 and 5, respectively. The 16S sequences divided all of the samples into two clusters. All of the *M. ichthyoblabe* colonies were in the same clade, but this clade also included *M. wesenbergii* colony (TH22). Both of the *M. aeruginosa* colonies and *M. wesenbergii* colonies were found in clade 1. However, these colonies had high homozygosity in 16S with *M. ichthyoblabe* 0BB39S02 (AJ635433), *Microcystis novacekii* TAC20 (AB012336) and *Microcystis viridis* TAC17 (AB012328). 16S rDNA sequences could not be used to distinguish different phenotypes of *Microcystis*[20]. Lepère *et al.*[21] also reported that the 16S rDNA sequences of six *Microcystis* strains assigned to four different morphospecies based on colonial morphology were similar.

Sanchis *et al.*[45] used both the 16–23S rDNA ITS and the cpcBA-IGS sequences to identify *Microcystis*. Their results suggested that *M. novacekii* could be distinguished from *M. wesenbergii*, but there was a close relationship between *M. novacekii* and *M. aeruginosa*. Otten and Paerl[24] also indicated that *M. wesenbergii* could be identified within four different *Microcystis* morphospecies based on the 16S-23S rDNA-ITS sequences. Similarly, Yoshida *et al.*[32] found that *M. aeruginosa* could be distinguished from *M. wesenbergii* and *M. viridis* by the 16S-23S rDNA-ITS sequences. Do Carmo Bittencourt-Oliveira *et al.*[46] successfully distinguished the *M. aeruginosa* morphospecies from the morphospecies of *M. wesenbergii* and *M. viridis* based on the DNA sequences of *cpc*BA-ITS.

All the above studies considered that *M. wesenbergii* could be distinguished using the 16–23S rDNA ITS and the cpcBA-IGS sequences. Conversely, in the current study, the sequences displayed high homozygosity for each 16S-23S and *cpc*BA-IGS in all of the samples except for the *M. aeruginosa* colony, TH32 (Figs 4 and 5). Similarly, the phylogenic tree for the 63 *Microcystis* strains in China based on the *cpc*BA-IGS gene sequences showed that this gene did not always succeed in identifying different morphospecies[47]. These occasional failures may be resulted from genetic variations among the strains of *Microcystis*[48]. One *Microcystis* genotype was reported to have more than one phenotype[29,49]. In East Africa, 24 isolated strains of *M. aeruginosa* could be separated into 10 genotypes based on the DNA sequences of the PC-IGS and ITS1 rDNA regions[50]. Thus, there was no obvious relationship between these phenotypes and the phenotypes of *Microcystis* based on *16S, 16S-23S and* cpc*BA-IGS* because of the significant genetic variations among the strains of *Microcystis*.

Polysaccharide biosynthesis-related genes.   Figure 6 shows a phylogenetic tree based on the analysis of the sequences of the polysaccharide biosynthesis-related genes (*espL* and *TagH*). The results demonstrate that the *M. wesenbergii* colonies could be divided directly from other colonies using *espL*. Xu *et al.*[27] suggested that the polysaccharide biosynthesis-related gene *TagH* may explain the diversity of the *Microcystis* morphospecies. In the current study, *TagH* divided all of the samples into two clusters but failed to distinguish the different phenotypes.

Since very small amount of colonies were tested and cultured, there would be a risk that the final *Microcystis* morphotype would change compared with the initially identified *Microcystis* due to intraspecific competition. Therefore, part II was carried out to confirm as the polysaccharide biosynthesis-related genes could distinguish the *Microcystis* phenotypes. The phylogenetic tree based on the analysis of the sequences of the polysaccharide biosynthesis-related genes (*espL* and *TagH*) extracted from *Microcystis* "scum" collected from June and

November 2015, was shown in Figs 7 and 8, respectively. The gene *espL* divided all of the samples into two clusters and the first cluster was divided into three subclades (Fig. 7). The samples in clade 2 was dominated by *M. wesenbergii* (60–100%). The samples in subclade 1 of clade 1 was dominated by *M. ichthyoblabe* (60–100%). As shown in Fig. 8, the gene *TagH* divided all of the samples into two clusters. All the samples collected in June and November were brought into subclade 1 in clade 1 and samples in August were brought into subclade 2 in clade 1. The former samples was dominated by *M. ichthyoblabe* (60–100%) and the latter samples was dominated by *M. wesenbergii* (60–100%). In consequence, it was confirmed that *M. wesenbergii* and *M. ichthyoblabe* could be distinguished by the polysaccharide biosynthesis-related genes *espL* and *TagH*. However, the two polysaccharide biosynthesis-related genes (epsL and TagH) may not be qualified for identifying all the species of *Microcystis*. These two genes combined with some other functional genes may succeed in identifying all the *Microcystis* species based on further researches.

Extracellular polysaccharide (EPS) was considered to be the material basis of *Microcystis* colony formation. A positive relationship between colony size and EPS content has been reported during recent years[51,35]. Li *et al.*[18] illustrated that solubilization of mucilage, which consists of EPS, induced changes in *Microcystis* colonial morphology. Forni *et al.*[52] indicated that the composition of EPS in different *Microcystis* species varied. The EPS content of various *Microcystis* morphospecies was also different[53]. Therefore, the content and composition of EPS has been postulated to be related to *Microcystis* colony morphology. In conclusion, the polysaccharide biosynthesis-related genes could distinguish the *Microcystis* phenotypes.

Conclusions

(1) *Microcystis* species identified by morphology or molecular tools (16S rDNA, 16S-23S ITS and *cpc*BA-IGS) could not be distinguished as toxic and non-toxic species.

(2) There was no obvious relationship between the phenotypes of *Microcystis* species based on *16S, 16S-23S and* cpc*BA-IGS* because of the significant genetic variations among the strains of *Microcystis*.

(3) It was confirmed that polysaccharide biosynthesis-related genes could distinguish the *Microcystis* phenotypes.

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Author Contributions

M.L. and W.Z. designed the experiments, M.L., S.X., Q.S., X.Z. and X.T. carried out the experiments, M.L., S.X., W.Z. and M.X. analyzed the data, M.L., S.X., Q.S., X.Z. and M.X. draw all figures, M.L., S.X. and W.Z. wrote this paper.

Additional Information