1 **Molecular separation of two long taxonomically debated cyanobacterial genera**
2 ***Cylindrospermopsis* and *Raphidiopsis* (Nostocales) based on the ITS-L phylogeny**

3

4 Xiaochuang Li^{a, b}, Shouchun Li^c, Renqiu Kong^a, Renhui Li^{a, *}

5

6 ^aKey Laboratory of Algal Biology, Institute of Hydrobiology, Chinese Academy of
7 Sciences, Wuhan, Hubei 430072, China

8 ^bUniversity of Chinese Academy of Sciences, Beijing 100049, China

9 ^c College of Life Science, Jiangxi Normal University, Nanchang 330027, China

10 ^{*}Corresponding author. Tel.: +86 27-68780067.

11 E-mail address: reli@ihb.ac.cn (R. Li).

12

13

14

15

16

17

18

19

20

21

Abstract: The cyanobacterial genera *Raphidiopsis* and *Cylindrospermopsis* morphologically resembled each other and are difficult to distinguish, especially when heterocytes do not form in *Cylindrospermopsis* species. The phylogeny based on multiple gene loci sequences in previous studies cannot divide the strains of these two genera into separate clusters. In the present study, **four gene loci 16S rRNA**, ITS-L, *cpcBA*-IGS and *rpoC1*, sequenced from many Chinese strains (44 *Cylindrospermopsis* strains and 16 *Raphidiopsis* strains), were analyzed to infer the phylogenetic relationship between the two genera. According to the **16S rRNA**, *cpcBA*-IGS and *rpoC1* gene phylogeny, *Cylindrospermopsis* and *Raphidiopsis* strains are intermixed and divide into two or three groups. By contrast, the ITS-L analysis implied that they respectively form two separate and unique clusters. A fragment of 7 bp (RAGAAACT) in ITS-L was conserved for *Raphidiopsis* strains, which could be distinguished from *Cylindrospermopsis* strains. A pair of primers targeting the ITS-L fragment specific to *Raphidiopsis* strains were designed and further verified.

Keywords: *Cylindrospermopsis*, ITS-L, Phylogeny, *Raphidiopsis*, Taxonomy

1 Introduction

2 Blooms of the cyanobacterial genus *Cylindrospermopsis* Seenayya et Subba Raju
 3 have recently been exacerbated because of the eutrophication caused by anthropogenic
 4 activities and global climate change (Chapman and Schelske, 1997; Padisák, 1997;
 5 Briand et al., 2004; Gemelgo et al., 2008; Wu et al., 2011; Dobberfuhl, 2013;
 6 Karadžić et al., 2013; Lei et al., 2014; Recknagel et al., 2014). This genus
 7 morphologically resembles another cyanobacterial bloom-forming genus,
 8 *Raphidiopsis* Fritsch et Rich (Li et al., 2001; Moustaka-Gouni et al., 2009; Alster et
 9 al., 2010; Wu et al., 2011; Komárek, 2013). These two genera mainly differ in terms
 10 of heterocytes, which are absent in *Raphidiopsis*. Extensive public attention has been
 11 devoted to these genera because of their potential ability to produce cyanobacterial
 12 toxins, such as cylindrospermopsin, deoxy-cylindrospermopsin, **anatoxin-a**,
 13 **homoanatoxin-a** and saxitoxin (Hawkins et al., 1985; Lagos et al., 1999; Li et al.,
 14 2001; Griffiths and Saker, 2003; Namikoshi et al., 2003; Mohamed, 2007; Gemelgo et
 15 al., 2008; Yunes et al., 2009; McGregor et al., 2011; Soto-Liebe et al., 2012; Lei et al.,
 16 2014).

17 *Cylindrospermopsis* strains were originally assigned to the genus *Anabaenopsis*
 18 Elenkin (Woloszyńska, 1912) and *Cylindrospermum* Kützinger ex Bornet et Flahault
 19 (Horecká and Komárek, 1979), but have been subsequently classified as belonging to
 20 a new genus *Cylindrospermopsis*, given their **diacritical** character with obligatory
 21 formation of terminal heterocytes on one or both sides, and unique pattern of

1 heterocyte development, in which terminal heterocytes develop primarily from
2 terminal cells (Seenayya and Subba Raju, 1972). The genus *Raphidiopsis* is highly
3 morphologically similar to *Cylindrospermopsis*; as such, the taxonomical validity of
4 this genus has been questioned (Komàrkova et al., 1999). Systematically, the genus
5 *Raphidiopsis* has been classified into the family Nostocaceae (Desikachary, 1959; Li
6 et al., 2008), Oscillatoriaceae (Frémy, 1938; Jao, 1951) or Rivulaiaceae (Fritsch and
7 Rich 1929) depending on different aspects of morphological characteristics. The
8 genus *Raphidiopsis* has not yet been given a valid published name in the new edition
9 of Bergey's Manual of Bacteriology (Rippka et al., 2001) The phylogeny based on
10 16S rRNA gene sequences shows that *Raphidiopsis* and *Cylindrospermopsis* strains
11 cluster together and share high sequence similarities, suggesting that the former
12 belongs to the family of Nostocaceae (Li et al., 2008).

13 These two heterocytous cyanobacterial genera *Raphidiopsis* and *Cylindrospermopsis*,
14 are difficult to distinguish, especially in cases where heterocytes are not present in
15 *Cylindrospermopsis* strains (Li et al., 2001; Moustaka-Gouni et al., 2009; Alster et al.,
16 2010). They share similar ecological niches, in which *Raphidiopsis* species have been
17 found to co-occur with *C. raciborskii* (Woloszyńska) Seenayya et Subba Raju in a
18 range of waters (Moustaka-Gouni et al., 2009; Wu et al., 2011; Hill, 1970). McGregor
19 and Fabbro (2000) proposed that *Raphidiopsis*-like trichomes are only environmental
20 morphotypes and the heterocyst-omission phase of *C. raciborskii*. Moustaka-Gouni et
21 al. (2009) suggested that *R. mediterranea* Skuja represents non-heterocytous

1 life-cycle stages of *C. raciborskii* in Lake Kastoria (its type locality), Greece, as
2 evidenced by morphological and 16S rRNA phylogenetic analyses.

3 In previous studies, on the 16S rRNA phylogeny, *Cylindrospermopsis* could not be
4 distinguished from *Raphidiopsis* (Li et al., 2008). Moreover, inferring from the
5 phylogenetic analysis of seven concatenated gene loci sequences, namely, *psbA*, 16S
6 rDNA, *rbcL*, *rbcS*, *rpoC1*, *cpcB*, and ITS1 that contain no tRNAs (i.e., internal
7 transcribed spacer between 16S and 23S rDNA), Wu et al. (2011) found that
8 *Cylindrospermopsis* and *Raphidiopsis* strains are intermixed and grouped into a large
9 cluster. Through a genomic comparison between *C. raciborskii* strain CS-505 and *R.*
10 *brookii* strain D9, Stucken et al. (2010) found that these two share a set of 2539 genes,
11 with an average sequence identity greater than 90%. The authors suggested that these
12 two genera, *Cylindrospermopsis* and *Raphidiopsis*, are congeneric.

13 In the present study, four gene loci sequences, namely, 16S rRNA, *cpcBA*-IGS,
14 *rpoC1* and ITS-L, were amplified from 44 strains of *Cylindrospermopsis* and 16
15 strains of *Raphidiopsis* from Chinese waters to further identify genetic markers that
16 can distinguish the morphologically similar genera *Raphidiopsis* and
17 *Cylindrospermopsis*.

18

19

1 **Materials and methods**

2 *Strain isolation and maintenance*

3 In this study, 44 strains of *Cylindrospermopsis* and 16 strains of *Raphidiopsis* from
 4 Chinese freshwaters were isolated and maintained at the Biology of Harmful Algae
 5 (HAB), Institute of Hydrobiology, Wuhan, China. These strains were obtained from a
 6 survey of numerous lakes in China from 2006 to 2015. Information on these strains is
 7 shown in Table S1. These isolates were separated through the micropipette washing
 8 method (Rippka, 1988), and then kept in CT medium (Ichimura, 1979) at 25 °C under
 9 a 12:12 (L:D) cycle with a photon flux density of $40 \mu\text{mol} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$ provided by
 10 daylight fluorescent lamps. The *Raphidiopsis* strains were verified using primers
 11 nifHf (5'-CGTAGGTTGCGACCCTAAGGCTGA-3') and nifHr
 12 (5'-GCATACATCGCCATCATTTTCACC-3') according to the procedure of Gugger
 13 et al. (2005).

14

15 *DNA extraction, PCR amplification and sequencing*

16 The cultures in the exponential growth phase were harvested for total genomic DNA
 17 extraction according to the procedure of Neilan et al. (1995). Four gene loci, namely,
 18 16S rRNA, *cpcBA*-IGS (phycocyanin intergenic spacer between the bilin subunit
 19 genes *cpcB* and *cpcA*), *rpoCI* (RNA polymerase), and ITS-L (ITS containing both
 20 tRNAs), were amplified from the 56 strains to identify genetic markers that can
 21 distinguish the genera *Raphidiopsis* and *Cylindrospermopsis*. Information on the

1 primers for these three gene loci is shown in Table 1. Polymerase chain reaction
2 (PCR) was performed in an MJ mini personal thermal cycler (Bio-Rad, Hercules,
3 California, USA). Sequencing was conducted with an ABI 3730 automated sequencer
4 (Perkin-Elmer, USA). The resulting sequences of the three gene loci were deposited
5 in GenBank, and the accession numbers were shown in Table S1.

6

7 *Sequence alignment, phylogenetic analysis and primer design*

8 The correct amplification of the sequences was verified through BLAST analysis in
9 NCBI. Sequence alignment was performed with BioEdit Sequence Alignment Editor
10 version 7.1.3.0 (Hall, 1999). For phylogenetic analysis, trees were constructed with
11 neighbor-joining (NJ) and maximum likelihood (ML) algorithms implemented in
12 PAUP V4.0b10 (Swofford, 2003). Bayesian inference (BI) was executed in MrBayes
13 version 3.1.2 (Huelsenbeck and Ronquist, 2001). For NJ and ML analyses, tree
14 robustness was estimated using bootstrap percentages (BPs) with 1000 replications for
15 NJ, and 100 replications for ML because of the limited computing capability. The
16 most appropriate substitution models for ML and BI analysis were selected using
17 MrModeltest 2.3 (Nylander, 2004). The selected models are shown in Table S2. For
18 BI analysis, four chains were run twice for 5×10^6 generations, with sampling
19 conducted for every 1000 trees. In each run, the first 25% of the samples were
20 discarded as the burn-in phase. The obtained consensual phylogenetic trees were

1 visualized in TreeView 1.6.6 (Page, 1996) and edited in Adobe Photoshop CS6
 2 version 13.0.
 3 For ITS-L analysis, sequences containing tRNA^{ILE} and tRNA^{ALA} were aligned using
 4 MUSCLE on the website (<http://www.ebi.ac.uk/Tools/msa/muscle>), and then
 5 manually edited in Bioedit version 7.1.3.0. The primers were designed using Web
 6 Primer on the website (<http://www.yeastgenome.org/cgi-bin/web-primer>).
 7

8 **Results**

9 The 44 *Cylindrospermopsis* strains all featured with terminal heterocytes located at
 10 one or both ends (Fig. 1 A-K). Sixteen *Raphidiopsis* strains were characterized by
 11 their trichomes, which were continuously narrowed to both ends with usually
 12 elongated and pointed or needle-like ends. These characteristics are typical of the
 13 genus *Raphidiopsis* (Fig. 1 L-T). Heterocytes did not form in *Raphidiopsis* strains.
 14 *C. catamaco* strains with pointed filaments' ends, which were typical characteristics
 15 of the genus *Raphidiopsis*, were unable to distinguish from *Raphidiopsis* strains when
 16 heterocytes do not form in *Cylindrospermopsis* strains (Fig. 2 A-D). Non-typical
 17 morphology of the genus *Raphidiopsis* always occurred in *Raphidiopsis* strains that
 18 were featured with rounded filaments' ends at one or both sides (Fig. 2 E-N), were
 19 highly morphologically similar to those of *Cylindrospermopsis* strains. Furthermore,
 20 cell dimensions overlapped in *Cylindrospermopsis* and *Raphidiopsis* strains, making
 21 them difficult to distinguish from each other (Table 2).

1 We failed to amplify *nifH* genes that coded nitrogenase reductase from *Raphidiopsis*
 2 strains, whereas they could be retrieved from all *Cylindrospermopsis* strains (Fig. S1).
 3 As shown by 16S rRNA phylogeny, *Raphidiopsis* strains scattered into three
 4 subclusters, and grouped into a large cluster with 44 *Cylindrospermopsis* strains (Fig.
 5 3). Inferred from the phylogeny based on *cpcBA*-IGS, 44 *Cylindrospermopsis* strains
 6 were grouped into three clades, one of which contained a mixture of
 7 *Cylindrospermopsis* and *Raphidiopsis* strains (Fig. 4). Based on the *rpoC1*
 8 phylogenetic analysis, all *Cylindrospermopsis* strains were gathered into a large
 9 cluster (*Cylindrospermopsis* I), except for strain *C. catemaco* CHAB 1390, which
 10 represented a cluster (*Cylindrospermopsis* II) that was unique and separate from all
 11 *Cylindrospermopsis* strains (Fig. 5). All *Raphidiopsis* strains were divided into two
 12 clusters (cluster *Raphidiopsis* I and II), one of which (cluster *Raphidiopsis* I) showed
 13 a closer relationship to *Cylindrospermopsis* strains than *Raphidiopsis* II cluster (Fig.
 14 5). By contrast, the ITS-L phylogeny showed that *Cylindrospermopsis* and
 15 *Raphidiopsis* strains were divided into two separate clades with high bootstrap values
 16 (97/100/1.00 for NJ/ML/BI analysis) (Fig. 6).
 17 Based on the ITS-L sequence alignment, all *Cylindrospermopsis* and *Raphidiopsis*
 18 strains were classified into six types, two of which were shown in the
 19 *Cylindrospermopsis* strains (Fig. 7 I and II) and four (Fig. 7 III, IV, V, and VI) in the
 20 *Raphidiopsis* strains. Similar D1-D1' helices were shared among *Cylindrospermopsis*
 21 and *Raphidiopsis* strains. They were more variable in the *Cylindrospermopsis* strains

than that in the *Raphidiopsis* strains. Six types were found in the *Cylindrospermopsis* strains (Fig. S2, A-F), and four were identified in the *Raphidiopsis* strains (Fig. S2, G-J). The Box-B helices were conserved and divided into two types in all *Cylindrospermopsis* and *Raphidiopsis* strains (Fig. S3, A and B), the terminal loop of which contained five unpaired nucleotides that mainly differed in sequence composition. The V2 helix was absent in all *Cylindrospermopsis* strains and the majority of *Raphidiopsis* strains but was divided into two types in four *Raphidiopsis* strains (Figs. 7 and S4). The V3 helices were present in several *Raphidiopsis* strains (*R. curvata* CHAB 114, CHAB 341, CHAB 1150, CHAB 3416 and CHAB HB1) and absent in *C. catemaco* CHAB 1390 and *C. ellipsoidea* CHAB 3424. However, the secondary structure of the V3 helices was not found in the remaining *Raphidiopsis* strains, and most of the *Cylindrospermopsis* strains as a result of the natural deletion of several nucleotides (Figs. 7 and S5).

As shown in Fig. 7, a fragment of 7 bp (RAGAAACT) displayed in the box was conserved for the *Raphidiopsis* strains, but was more variable in the *Cylindrospermopsis* strains. A pair of primers (Raphi-F and Raphi-R) targeting the ITS-L fragment that was specific to *Raphidiopsis* strains was designed (Table 3). As shown in Fig. 8, all *Raphidiopsis* strains produced a single amplification product ranging from 275 to 343 bp because the V2 helix was absent in some of them. The V2 helix was not retrieved from all *Cylindrospermopsis* strains.

1 Discussion

2 The genus *Raphidiopsis* is highly morphologically similar to *Cylindrospermopsis*.
 3 The overlapping features of these two genera lead to difficulty in distinguishing them
 4 from each other (Moustaka-Gouni et al., 2009; Wu et al., 2011). They mainly differ in
 5 terms of heterocytes, which are absent in *Raphidiopsis* strains. However, distinction
 6 between these two genera is difficult when heterocytes are not shown in
 7 *Cylindrospermopsis* strains (Li et al., 2001; Moustaka-Gouni et al., 2009; Alster et al.,
 8 2010). The co-existence of *Cylindrospermopsis* and *Raphidiopsis* in the same waters
 9 has been widely reported (McGregor and Fabbro, 2000; Moustaka-Gouni et al., 2009;
 10 Wu et al., 2011). *Raphidiopsis*-like trichomes are regarded as only environmental
 11 types of *Cylindrospermopsis* (McGregor and Fabbro, 2000). In Lake Kastoria,
 12 Greece, Moustaka-Gouni et al. (2009) found that three dominant trichome
 13 morphotypes are present: the typical and non-heterocytous morphotype of *C.*
 14 *raciborskii* and the typical morphotype of *R. mediterranea*. The range of the cell
 15 dimension in these three morphotypes significantly overlaps. Trichome reproduction
 16 of *R. mediterranea* strains can also result in both *R. mediterranea* and *C. raciborskii*
 17 non-heterocytous morphotypes. Furthermore, these *Cylindrospermopsis/Raphidiopsis*
 18 morphotypes show high 16S rRNA sequence similarity to *R. mediterranea* HB2
 19 (99.54%) and *C. raciborskii* form 1 (99.19%). Based on these three aspects,
 20 Moustaka-Gouni et al. (2009) reported that *R. mediterranea* represents the
 21 non-heterocytous life-cycle stages of *C. raciborskii*. This claim was further

1 corroborated by the finding that *Raphidiopsis* trichomes are almost undetectable (less
 2 than 5% of *C. raciborskii* density) in Lake Kastoria when the N:P resource ratio drops
 3 to below the critical ratio of Redfield in summer (Moustaka-Gouni et al., 2007).
 4 The taxonomical validity of the genus *Raphidiopsis* had been questioned in recent
 5 studies (Komàrkova et al., 1999). According to Wu et al. (2011), Stucken et al. (2010),
 6 and Moustaka-Gouni et al. (2009), these two genera, *Cylindrospermopsis* and
 7 *Raphidiopsis*, are congeneric. They based this claim mainly on the following aspects.
 8 First, the molecular phylogeny based on multiple gene loci sequences failed to
 9 distinguish between *Cylindrospermopsis* and *Raphidiopsis* strains. They are
 10 phylogenetically intermixed, and both of them can be grouped into two or more
 11 clusters, indicating that the two genera are polyphyletic in evolutionary origin (Wu et
 12 al., 2011). Second, a genomic comparison showed that strain *C. raciborskii* CS-505
 13 and strain *R. brookii* D9 share a set of 2539 genes, with an average sequence identity
 14 greater than 90% (Stucken et al., 2010).
 15 In previous studies, genetic markers have been explored to distinguish between genera
 16 *Cylindrospermopsis* and *Raphidiopsis*, but the results are always unsatisfactory
 17 (Gugger et al., 2005; Stucken et al., 2009; Alster et al., 2010). Wu et al. (2011)
 18 concatenated a set of seven gene loci sequences (i.e., *psbA*, 16S rDNA, *rbcL*, *rbcS*,
 19 *rpoC1*, *cpcB*, and ITS1 between 16S and 23S rDNA) but failed to distinguish
 20 *Cylindrospermopsis* and *Raphidiopsis*. Similar results were obtained from the 16S
 21 rRNA, *cpcBA*-IGS and *rpoC1* phylogeny in the present study, that is, the 44

1 *Cylindrospermopsis* strains and 16 *Raphidiopsis* strains from China are
 2 phylogenetically intermixed and grouped into two or three clusters (Figs. 3-5),
 3 thereby supporting the result of previous studies that these two genera are
 4 polyphyletic in evolutionary origin (Gugger et al., 2005; Li et al., 2008; Stucken et al.,
 5 2009; Wu et al., 2011). On the contrary, based on the ITS-L phylogeny,
 6 *Cylindrospermopsis* and *Raphidiopsis* strains are divided into separate and unique
 7 clusters (Fig. 6), suggesting that they should be regarded as separate and distinct
 8 generic units. Their divergence is further reflected by the fact that ecologically
 9 important heterocytes that could fix nitrogen were absent in the *Raphidiopsis* strains.
 10 After nearly 10 years of laboratory observation, heterocytes have never formed in
 11 strains *R. curvata* HB1 and *R. mediterranea* HB2 (Li et al., 2008). Additionally,
 12 Gugger et al. (2005) and Li et al. (2008) found that *Raphidiopsis* strains turn from
 13 green to yellow within a week and are unable to survive in nitrogen-free media,
 14 indicating that they cannot form heterocysts to fix nitrogen. This phenomenon may be
 15 explained by strain *R. brookii* D9, which has only 39 of the 55 heterocyte-related
 16 genes found in strain *C. raciborskii* CS 505 after genomic comparison by Stucken et
 17 al. (2010). The amplification of the *nifH* gene that codes for nitrogenase reductase has
 18 not been successfully implemented in any of *Raphidiopsis* strains (Gugger et al., 2005;
 19 Stucken et al., 2010; Wu et al., 2011). The same results were also obtained in the
 20 present study, that the 16 Chinese *Raphidiopsis* strains failed to amplify the *nifH* gene
 21 (Fig. S1). Stucken et al. (2010) believed that the ability to produce heterocytes *R.*

1 *brookii* D9 was secondarily lost, along with the capacity to fix nitrogen; such a
 2 phenomenon has been observed in the species *Cuspidothrix issatschenkoi* (Usačev)
 3 Rajaniemi et al (Hodoki et al., 2013). Two distinct ecotypes that prefer different
 4 environments are present in *C. issatschenkoi* strains from Japanese lakes. All
 5 heterocytous *C. issatschenkoi* strains cannot produce **anatoxin-a** (ATX), whereas
 6 ATX can be produced in any of the non-heterocytous *C. issatschenkoi* strains.
 7 Moreover, the genera *Cylindrospermopsis* and *Raphidiopsis* also differ in terms of the
 8 morphology of their terminal cells (Fritsch and Rich, 1929; Seenayya and Subba Raju,
 9 1972; Komárek, 2013). In *Cylindrospermopsis* strains, terminal cells are conical and
 10 bluntly or sharply pointed, whereas in *Raphidiopsis* strains, they are always elongated
 11 with pointed or needle-like ends (Fig. 1).
 12 Previous efforts to identify genetic markers that can distinguish between genera
 13 *Cylindrospermopsis* and *Raphidiopsis* have been unsatisfactory (Gugger et al., 2005;
 14 Stucken et al., 2009; Alster et al., 2010). In the present study, based on the ITS-L
 15 alignment, a fragment of 7 bp (RAGAAACT) in ITS-L labeled in the box was
 16 conserved for the *Raphidiopsis* strains but was more variable in the
 17 *Cylindrospermopsis* strains (Fig. 7). Based on this fragment, a pair of primers
 18 targeting the ITS-L fragment that is specific to *Raphidiopsis* strains was designed
 19 (Table 3). The electrophoresis image shows that single amplification products ranging
 20 from 275 to 343 bp were obtained from all *Raphidiopsis* strains but were absent in all

1 *Cylindrospermopsis* strains (Fig. 8), indicating that the primers designed here are
 2 specific to the genus *Raphidiopsis*. Primers cyl2 (5'-GGCATTCCCTAGTTATATTG
 3 CCATACTA-3') and cyl4 (5'-GCCCCGTTTTTGTCCCTTTCGTGC-3'), which are
 4 specific to *C. raciborskii* isolates, were designed by Wilson et al. (2000) and further
 5 verified for *Raphidiopsis* and *Cylindrospermopsis* strains from China. Amplicons
 6 were shown in most of the *Cylindrospermopsis* strains but were absent in strain *C.*
 7 *taverae* CHAB 143 (Fig. S6). Notably, amplicons were retrieved from strains *R.*
 8 *curvata* CHAB 3360 and HB1 (Fig. S6). Amplicons based on the primers nifH and
 9 nifHr were obtained from *C. taverae* CHAB 143 but were not shown in *R. curvata*
 10 CHAB 3360 and HB1 (Fig. S1), indicating that the primers cyl2 and cyl4 designed by
 11 Wilson et al. (2000) are not specific to all *C. raciborskii* isolates.

12

13 **Conclusion**

14 Based on the ITS-L phylogeny, 44 *Cylindrospermopsis* strains and 16 *Raphidiopsis*
 15 strains from Chinese freshwaters were grouped into separate and unique clusters. A
 16 fragment of 7 bp (RAGAAACT) in ITS-L was conserved for *Raphidiopsis* strains that
 17 could be distinguished from *Cylindrospermopsis* strains. A pair of primers specific for
 18 *Raphidiopsis* strains were designed and further verified. Given that the ecologically
 19 important heterocytes were absent in the *Raphidiopsis* strains and part of the *nif*
 20 gene cluster was lost in the *Raphidiopsis* strains, the genera *Raphidiopsis* and
 21 *Cylindrospermopsis* should be regarded as separate and distinct generic units.

1

2 **Acknowledgement**

3 The research is funded by National Science Foundation China (NSFC 31170189) and
 4 State Key Laboratory of Freshwater Ecology and Biotechnology Grant (2015FB06).

5

6

7

8

- 9 1. Alster, A., Kaplan-Levy, R.N., Sukenik, A., Zohary, T., 2010. Morphology and
 10 phylogeny of a non-toxic invasive *Cylindrospermopsis raciborskii* from a
 11 Mediterranean Lake. *Hydrobiologia* 639, 115-128.
- 12 2. Briand, J.F., Le Boulanger, C., Humbert, J.F., Bernard, C., Dufour, P., 2004.
 13 *Cylindrospermopsis raciborskii* (Cyanobacteria) invasion at mid-latitudes:
 14 selection, wide physiological tolerance, or global warming? *J. Phycol.* 40,
 15 231-238.
- 16 3. Chapman, A.D., Schelske, C.L., 1997. Recent appearance of *Cylindrospermopsis*
 17 (Cyanobacteria) in five hypereutrophic Florida lakes. *J. Phycol.* 33, 191-195.
- 18 4. Desikachary, T.V., 1959. Cyanophyta, 1st ed. Indian Council of Agricultural
 19 Research, New Delhi, p. 686.

- 1 5. Dobberfuhl, D.R., 2003. *Cylindrospermopsis racihorskii* in three central florida
2 lakes: population dynamics, controls, and management implications. *Lake Reserv.*
3 *Manage.* 19, 341-348.
- 4 6. Frémy, P., 1938. Sur *Raphidiopsis mediaterranea* Skuja, etc. *Congr. Soc. Savants*
5 78, 157-161.
- 6 7. Fritsch, F.E., Rich, E., 1929. Contributions to our knowledge of freshwater algae
7 of Africa. 7. Freshwater algae (exclusive of diatoms) from Griqualand West. *R.*
8 *Soc. S. Afr.* 18, 1-92.
- 9 8. Gemelgo, M.C., Sant'Anna, C.L., Tucci, A., Barbosa, H.R., 2008. Population
10 dynamics of *Cylindrospermopsis raciborskii* (Woloszynska) Seenayya and Subba
11 Raju, a Cyanobacteria toxic species, in water-supply reservoirs in São Paulo,
12 Brazil. *Hoehnea* 35, 297-307.
- 13 9. Griffiths, D.J., Saker, M.L., 2003. The Palm Island mystery disease 20 years on: a
14 review of research on the cyanotoxin cylindrospermopsin. *Environ. Toxicol.* 18,
15 78-93.
- 16 10. Gugger, M., Molica, R., Le Berre, B., Dufour, P., Bernard, C., Humbert, J.F.,
17 2005.
- 18 11. Genetic diversity of *Cylindrospermopsis* strains (Cyanobacteria) isolated from
19 four continents. *Appl. Environ. Microbiol.* 71, 1097-1100.

- 1 12. Hall, T.A., 1999. BioEdit: a user-friendly biological sequence alignment editor
2 and analysis program for Windows 95/98/NT. Nucleic Acids Symposium Series.
3 41, 95-98.
- 4 13. Hawkins, P.R., Runnegar, M.T.C., Jackson, A.R.B., Falconer, I.R., 1985. Severe
5 hepatotoxicity caused by the tropical cyanobacterium (blue-green alga)
6 *Cylindrospermopsis raciborskii* (Woloszynska) Seenaya and Subba Raju isolated
7 from a domestic water supply reservoir. *Appl. Environ. Microbiol.* 50, 1292-1295.
- 8 14. Hill, H., 1970. A new form of *Raphidiopsis mediterranea* Skuja found in
9 Minnesota lakes. *Phycologia* 9, 73-77.
- 10 15. Hodoki, Y., Ohbayashi, K., Kobayashi, Y., Takasu, H., Okuda, N., Nakano, S.I.,
11 2013. **Anatoxin-a**-producing *Raphidiopsis mediterranea* Skuja var. *grandis* Hill is
12 one ecotype of non-heterocytous *Cuspidothrix issatschenkoi* (Usačev) Rajaniemi
13 et al. in Japanese lakes. *Harmful algae* 21, 44-53.
- 14 16. Horecká, M., Komárek, J., 1979. Taxonomic position of three planktonic blue
15 green algae from the genera *Aphanizomenon* and *Cylindrospermopsis*. *Preslia* 51,
16 289-312.
- 17 17. Huelsenbeck, J.P., Ronquist, F., 2001. MrBayes: Bayesian inference of
18 phylogeny. *Bioinformatics* 17, 754-755.
- 19 18. Ichimura, T., 1979. Media for freshwater cyanobacteria. In: Nishizawa, K.,
20 Chihara, M. (Eds.), *Methods in Phycology*. Kyouritsu Shuppan, Tokyo, America,
21 pp. 295-296.

- 1 19. Jao, C.C., 1951. A discussion of *Raphidiopsis curvata* Fritsch et Rich. *Sinensia* 2,
2 11-23.
- 3 20. Karadžić, V., Simić, G.S., Natić, D., Ržaničanin, A., Ćirić, M., Gačić, Z., 2013.
4 Changes in the phytoplankton community and dominance of *Cylindrospermopsis*
5 *raciborskii* (Wolosz.) Subba Raju in a temperate lowland river (Ponjavica,
6 Serbia). *Hydrobiologia* 711, 43-60.
- 7 21. Komárkova, J., Laudaes-Silva, R., Senna, P.A.C., 1999. Extreme morphology of
8 *Cylindrospermopsis raciborskii* (Nostocales, Cyanobacteria) in the Lagoa do Peri,
9 a freshwater coastal lagoon, Santa Catarina. Brazil. *Arch. Hydrobiol./Algol. Stud.*
10 94, 207-222.
- 11 22. Komárek, J., 2013. Cyanoprokaryota-3. Teil/3rd Part: Heterocytous Genera. In:
12 Büdel, B., Krienitz, L., Gärtner, G., Schagerl, M. (Eds.), Süßwasserflora von
13 Mitteleuropa Freshwater flora of central Europe 19/3. Elsevier/Spektrum,
14 Heidelberg, pp. 672-685.
- 15 23. Lagos, N., Onodeta, H., Zagatto, P.A., Andrinolo, D., Azevedo, S.M.F.Q.,
16 Oshima, Y., 1999. The first evidence of paralytic shellfish toxins in the freshwater
17 cyanobacterium *Cylindrospermopsis raciborskii*, isolated from Brazil. *Toxicon* 37,
18 1359-1373.
- 19 24. Lei, L., Peng, L., Huang, X. Han, B.P., 2014. Occurrence and dominance of
20 *Cylindrospermopsis raciborskii* and dissolved cylindrospermopsin in urban

- 1 reservoirs used for drinking water supply, South China. *Environ. Monit. Assess.*
2 186, 3079-3090.
- 3 25. Li, R., Carmichael, W.W., Brittain, S., Eaglesham, G.K., Shaw, G.R., Liu, Y.,
4 Watanabe, M.M., 2001. First report of the cyanotoxins cylindrospermopsin and
5 deoxy-cylindrospermopsin from *Raphidiopsis curvata* (Cyanobacteria). *J. Phycol.*
6 37, 1121-1126.
- 7 26. Li, R., Wilhelm, S.W., Carmichael, W.W., Watanabe, M.M., 2008. Polyphasic
8 characterization of water bloom forming *Raphidiopsis* species (cyanobacteria)
9 from central China. *Harmful Algae* 7, 146-153.
- 10 27. McGregor, G.B., Sendall, B.C., Hunt, L.T., Eaglesham, G.K., 2011. Report of the
11 cyanotoxins cylindrospermopsin and deoxy-cylindrospermopsin from
12 *Raphidiopsis mediterranea* Skuja (Cyanobacteria/Nostocales). *Harmful Algae* 10,
13 402-410.
- 14 28. Mohamed, Z.A., 2007. First report of toxic *Cylindrospermopsis raciborskii* and
15 *Raphidiopsis mediterranea* (Cyanoprokaryota) in Egyptian fresh waters. *FEMS*
16 *Microbiol. Ecol.* 59, 749-761.
- 17 29. Moustaka-Gouni, M., Vardaka, E., Tryfon, E., 2007. Phytoplankton species
18 succession in a shallow Mediterranean lake (L. Kastoria, Greece): steady-state
19 dominance of *Limnothrix redekei*, *Microcystis aeruginosa* and
20 *Cylindrospermopsis raciborskii*. *Hydrobiologia* 575, 129-140.
- 21 30. Moustaka-Gouni, M., Kormas, K.A., Vardaka, E., Katsiapi, M., Gkelis, S., 2009.

- 1 *Raphidiopsis mediterranea* SKUJA represents non-heterocytous life-cycle stages of
- 2 *Cylindrospermopsis raciborskii* (Woloszynska) Seenayya et Subba Raju in Lake
- 3 Kastoria (Greece), its type locality: evidence by morphological and phylogenetic
- 4 analysis. *Harmful Algae* 8, 864-872.
- 5 31. Namikoshi, M., Murakami, T., Watanabe, M.F., Oda, T., Yamada, J., Tsujimura,
- 6 S., Nagai, H., Oishi, S., 2003. Simultaneous production of **homoanatoxin-a**,
- 7 **anatoxin-a**, and a new non-toxic **4-hydroxyhomoanatoxin-a** by the
- 8 cyanobacterium *Raphidiopsis mediterranea* Skuja. *Toxicon* 42, 533-538.
- 9 32. Neilan, B.A., Jacobs, D., Goodman, A.E., 1995. Genetic diversity and phylogeny
- 10 of toxic cyanobacteria determined by DNA polymorphisms within the
- 11 phycocyanin locus. *Appl. Environ. Microbiol.* 61, 3327-3332.
- 12 33. Nylander, J.A.A., 2004. MrModeltest, version 2.3. Available at:
- 13 <http://www.abc.se/sci-hub.org/~nylander> [accessed 12 Apr. 2010].
- 14 34. Padisák, J., 1997. *Cylindrospermopsis raciborskii* (Woloszynska) Seenayya et
- 15 Subba Raju, an expanding, highly adaptive cyanobacterium: worldwide
- 16 distribution and review of its ecology. *Arch. Hydrobiol. Suppl.* 107, 563-593.
- 17 35. Page, R.D.M., 1996. TreeView: An application to display phylogenetic trees on
- 18 personal computers. *Comput. Appl. Biosci.* 12, 357-358.
- 19 36. Recknagel, F., Orr, P.T., Cao, H., 2014. Inductive reasoning and forecasting of
- 20 population dynamics of *Cylindrospermopsis raciborskii* in three sub-tropical
- 21 reservoirs by evolutionary computation. *Harmful Algae* 31, 26-34.

- 1 37. Rippka, R., 1988. Isolation and purification of cyanobacteria. *Method Enzymol.*
2 167, 3-27.
- 3 38. Rippka, R., Castenholz, R.W., Herdman, M., 2001. Subsection IV. In: Boone,
4 D.R., Castenholz, R.W. (Eds.), *Bergey's Manual of Systematic Bacteriology*, 2nd
5 ed., vol. 1. Springer, New York, pp. 562-589.
- 6 39. Seenayya, G., Subba Raju, N., 1972. On the ecology and systematic position of
7 the alga known as *Anabaenopsis raciborskii* (Wolosz.) Elenk. and a critical
8 evaluation of the forms described under the genus *Anabaenopsis*. In: Desikachary,
9 T.V. (Ed.), *First international symposium on taxonomy and biology of blue-green*
10 *algae*. Madras University, Madras, India, pp. 52-57.
- 11 40. Soto-Liebe, K., Méndez, M.A., Fuenzalida, L., Krock, B., Cembella, A., Vásquez,
12 M., 2012. PSP toxin release from the cyanobacterium *Raphidiopsis brookii* D9
13 (Nostocales) can be induced by sodium and potassium ions. *Toxicon* 60,
14 1324-1334.
- 15 41. Stucken, K., Murillo, A.A., Soto-Liebe, K., Fuentes-Valdés, J.J., Méndez, M.A.,
16 Vásquez, M., 2009. Toxicity phenotype does not correlate with phylogeny of
17 *Cylindrospermopsis raciborskii* strains. *Syst. Appl. Microbiol.* 32, 37-48.
- 18 42. Stucken, K., John, U., Cembella, A., Murillo, A.A., Soto-Liebe, K.,
19 Fuentes-valdés, J.J., Friedel, M., Plominsky, A.M., Vásquez, M., Glöckner, G.,
20 2010. The smallest known genomes of multicellular and toxic cyanobacteria:

- 1 comparison, minimal gene sets for linked traits and the evolutionary implications.
 2 *PLoS ONE* 5, e9235.
- 3 43. Swofford, D.L., 2003. PAUP*: Phylogenetic analysis using parsimony (*and other
 4 methods), version 4.0b10. Sinauer associates, Sunderland, Massachusetts,
 5 Thomazeau.
- 6 44. Wilson, K.M., Schembri, M.A., Baker, P.D., Saint, C.P., 2000. Molecular
 7 characterization of the toxic cyanobacterium *Cylindrospermopsis raciborskii* and
 8 design of a species-specific PCR. *Appl. Environ. Microb.* 66, 332-338.
- 9 45. Woloszynska, J., 1912. Das phytoplankton einiger Javanian Seen mit
 10 Berücksichtigung des Sawa-Planktons. *Bull. Int. Acad. Sci. Cracoviae Ser. B* 6,
 11 649-709.
- 12 46. Wu, Z., Shi, J., Xiao, P., Liu, Y., Li, R., 2011. Phylogenetic analysis of two
 13 cyanobacterial genera *Cylindrospermopsis* and *Raphidiopsis* based on multi-gene
 14 sequences. *Harmful Algae* 10, 419-425.
- 15 47. Yunes, J.S., De La Rocha, S., Giroldo, D., da Silveira, S.B., Comin, R., da Silva
 16 Bicho, M., Melcher, S.S., Sant'anna, C.L., Vieira, A.A.H., 2009. Release of
 17 carbohydrates and proteins by a subtropical strain of *Raphidiopsis brookii*
 18 (Cyanobacteria) able to produce saxitoxin at three nitrate concentrations. *J.*
 19 *Phycol.* 45, 585-591.

Figures and figures legends

Fig. 1 Typical morphology of genera *Cylindrospermopsis* and *Raphidiopsis*: (A–K) strains of *Cylindrospermopsis* with terminal heterocytes, (L–T) strains of *Raphidiopsis* with pointed filaments' ends. Scale bars: 10 μm .

Fig. 2 Non-typical morphology of genera *Cylindrospermopsis* and *Raphidiopsis*:

(A–D) strains of *Cylindrospermopsis catemaco* with pointed filaments' ends; (E–N) strains of *Raphidiopsis* with rounded filaments' ends at one or both sides. Scale bars: 10 μm .

Fig. 3 ML phylogenetic tree based on the 16S rRNA gene sequences (1132 bp) from 44 *Cylindrospermopsis* strains and 16 *Raphidiopsis* strains from China, and strains from other heterocytous cyanobacteria species (obtained from NCBI). Bootstrap values greater than 50% are given in front of the corresponding nodes for ML and NJ analyses followed by Bayesian posterior probabilities (≥ 0.5 are illustrated).

Cylindrospermopsis + *Raphidiopsis*

Fig. 4 ML phylogenetic tree based on the *cpcBA*-IGS gene sequences (508 bp) from 44 *Cylindrospermopsis* strains and 16 *Raphidiopsis* strains from China, and strains from other heterocytous cyanobacteria species (obtained from NCBI). Bootstrap values greater than 50% are given in front of the corresponding nodes for ML and NJ analyses followed by Bayesian posterior probabilities (≥ 0.5 are illustrated).

Fig. 5 ML phylogenetic tree based on the *rpoC1* gene sequences (516 bp) from 44 *Cylindrospermopsis* strains and 16 *Raphidiopsis* strains from China, and strains from other heterocytous cyanobacteria species (obtained from NCBI). Bootstrap values greater than 50% are given in front of the corresponding nodes for ML and NJ analyses followed by Bayesian posterior probabilities (≥ 0.5 are illustrated).

Fig. 6 ML phylogenetic tree based on the ITS-L (ITS containing both tRNAs) gene sequences (380 bp) from 44 *Cylindrospermopsis* strains and 16 *Raphidiopsis* strains from China, and strains from other heterocytous cyanobacteria species (obtained from NCBI). Bootstrap values greater than 50% are given in front of the corresponding nodes for ML and NJ analyses followed by Bayesian posterior probabilities (≥ 0.5 are illustrated).

Cylindrospermopsis

Raphidiopsis

Fig. 7 Sequence alignment of the ITS-L sequences from 44 *Cylindrospermopsis* strains and 16 *Raphidiopsis* strains from China. 6 types with their representatives were represented, 2 types in *Cylindrospermopsis* strains and 4 types in *Raphidiopsis* strains. Strains in type I: *C. catemaco* CHAB 1390, *C. ellipsoidea* CHAB 3424. Strains in type II: the remaining 42 *Cylindrospermopsis* strains. Strains in type III: *R. curvata* CHAB 114, CHAB 1150. Strains in type IV: *R. curvata* CHAB 3416, CHAB HB1. Strains in type V: *R. curvata* CHAB 341. Strains in type VI: *R. curvata* CHAB 3360, CHAB 3405, CHAB 3406, CHAB 3407, CHAB 3413, CHAB 3415; *R. mediterranea* CHAB 1353, CHAB 1355, CHAB 3326, CHAB 3408, CHAB HB2. Note: *Raphidiopsis* indicated by *R*; *Cylindrospermopsis* indicated by *C*.

Fig. 8 The image of electrophoresis for verifying the specificity of the primers designed for ITS-L gene sequences of *Raphidiopsis* strains: (lane 1) DNA marker; (lane 2-9) strains of *R. curvata* CHAB 114, *R. curvata* CHAB 141, *R. mediterranea* CHAB 1355, *R. curvata* CHAB 3360, *R. curvata* CHAB 3405, *R. curvata* CHAB 3415, *R. curvata* CHAB HB1, *R. mediterranea* CHAB HB2; (lane 10-18) strains of *C. taveriae* CHAB 143, *C. catemaco* CHAB 155, *C. helicoidea* CHAB 357, *C. raciborskii* CHAB 480, *C. africana* CHAB 1394, *C. raciborskii* CHAB 2138, *C. raciborskii* CHAB 2144, *C. raciborskii* CHAB 3318, *C. raciborskii* CHAB 3432.

Note: *Raphidiopsis* indicated by R; *Cylindrospermopsis* indicated by C.

Tables and tables legends

Gene	Primers 5'-3'	Annealing temperature	Product size (bp)	References
16S rRNA	TTGATCCTGGCTCAGGATGA	58 °C	1400	Gugger et al. (2002)
	AGTCCTACCTTAGGCATCCCCCTCC			
ITS-L	TGTACACACCGCCCGTC	55 °C	710	Iteman et al. (2000)
	CTCTGTGTGCCTAGGTATCC			
<i>cpcBA-IGS</i>	GGCTGCTTGTTTACGCGACA	57 °C	690	Neilan et al. (1995)
	CCAGTACCACCAGCAACTAA			
<i>rpoC1</i>	CACCAGAACGTATCCGCGCT	60 °C	700	This study
	ACGGAGATCGGGTGGAATGACT			

Table 1The primers and their products sizes from four gene loci 16S rRNA, ITS-L, *cpcBA-IGS* and *rpoC1* used in the study.

Genus and Species	Strain/NP	Vegetative cells (µm)			Heterocysts (µm)			Akinetes (µm)			Data Source
		Length	Width	L:W ratio	Length	Width	L:W ratio	Length	Width	L:W ratio	
<i>C. raciborskii</i>	CHAB 477	7.17-14.55	1.69-4.1	1.91-6.28	4.59-10.85	2.26-4.21	1.69-3.91	5.88-16.88	1.89-4.66	1.73-7.4	Present study
	CHAB 3443	5.47-12.2	1.48-2.93	2.14-5.76	3.04-7.52	2.04-3.71	1.29-2.38	9.43-18.96	2.52-4.14	2.37-6.28	Present study
	CHAB 3446	5.3-11.21	1.68-3	2.47-5.82	3.53-7.55	2.17-3.66	1.35-2.88	7.96-14.52	3.38-4.99	1.62-3.63	Present study
<i>C. catemaco</i>	CHAB 148	4.05-11.56	2.07-3.66	1.64-4.4	5.03-10.73	2.42-4.39	1.53-3.33	7.1-17.32	3.28-5.39	1.76-4.97	Present study
	CHAB 151	5.64-15.03	2.15-3.66	1.81-4.98	3.61-8.2	2.02-3.61	1.58-3.23	7.93-13.71	3.66-5.49	1.61-3.75	Present study
<i>C. philippinensis</i>	CHAB 5180	5.3-13.5	2.75-4.02	1.43-3.67	5.15-11.89	2.32-4.41	1.47-4.56	8.4-24.42	3.1-6.9	1.53-6.63	Present study
<i>R. curvata</i>	CHAB 114	5.95-9.83	2.08-3.2	2.13-4.16	—	—	—	6.24-16.57	3.12-7.04	1.14-3.4	Present study
	CHAB 1150	5.52-13.02	2.11-4	1.73-4.63	—	—	—	8.02-19.01	2.93-5.4	2.14-6.49	Present study
	CHAB 3406	4.07-10.99	1.91-3.87	1.84-4.58	—	—	—	10.7-16.56	2.62-4.79	2.66-5	Present study
	CHAB 3413	8.82-24.51	2.15-3.65	2.66-7.96	—	—	—	9.82-17.71	2.76-4.94	2.27-5.16	Present study
<i>C. raciborskii</i>	NP	4.25-16.40	0.9-2.7	2.2-12.3	3.9-8.9	1.2-2.8	—	5.2-15.5	1.7-2.9	—	Moustaka-Gouniet al., 2009
<i>R. mediterranea</i>	NP	4.1-12.5	0.8-2.3	2.3-10.2	—	—	—	5.8-18.1	1.5-2.7	—	Moustaka-Gouniet al., 2009
<i>R. mediterranea</i>	HB2	9.8-10.8	1.9-2.7	—	—	—	—	8.3-12.1	2.2-3.1	—	Li et al., 2008

Table 2 Morphometric features of *Cylindrospermopsis* and *Raphidiopsis* strains used in the study in comparison with those described from previous studies.

Note: *Raphidiopsis* indicated by *R*; *Cylindrospermopsis* indicated by *C*; Natural populations indicated by NP.

Primer name	Target gene	Sequence 5'-3'	Temperature	Product size (bp)
Raphi-F	ITS-L	CCATTGARGAATCGAAAGC	50.8 °C	275-343
Raphi-R		CCTAGTTGCTAGTTTCT	47.4 °C	

Table 3 Specific primers designed for *Raphidiopsis* strains based on ITS-L gene sequences.