1 **Genetic analysis on *Dolichospermum* (Cyanobacteria; sensu *Anabaena*)**
2 **populations based on the culture-independent clone libraries revealed the**
3 **dominant genotypes existing in Lake Taihu, China**

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Abstract

Lake Taihu has been severely eutrophied during the last few decades and dense cyanobacterial blooms have led to a decrease in phytoplankton diversity. The cyanobacterial blooms in Lake Taihu were mainly composed of unicellular colony-forming *Microcystis* and filamentous heterocystous *Dolichospermum* (formerly known as planktonic species of *Anabaena*). In contrast to that of *Microcystis* spp., the fundamental knowledge about diversity, abundance and dynamics of *Dolichospermum* populations in Lake Taihu is lacking. The present study was conducted to understand genotypic distribution, dynamics and succession of *Dolichospermum* populations in Lake Taihu. By sequencing 688 internal transcribed spacer (ITS) regions between the 16S and 23S rRNA genes of *Dolichospermum*, we were able to confirm that all the sequences were *Dolichospermum* rather than *Aphanizomenon*. 118 different genotypes were identified from the obtained sequences, and two genotypes (W-type and L-type) were found to dominate in the lake, representing 36.6% and 26.2% of the total sequences respectively. These two dominant genotypes of *Dolichospermum* displayed the significant seasonal pattern. Stepwise regressions analysis revealed that water temperature was associated with the two dominant genotypes. The combined results implied the possible existence of ecotypes in bloom-forming cyanobacteria, probably triggered by water temperature in the lake.

Keywords: cyanobacteria, *Dolichospermum*, genotype, ITS sequence, Lake Taihu

1. Introduction

Harmful algae blooms (HABs) have become a serious issue in aquatic ecosystems worldwide (Ha et al., 2009; Okello et al., 2010; Paerl and Huisman, 2008). Cyanobacterial blooms not only produce different kinds of toxins (microcystins, anatoxin-a, cylindrospermopsin etc.), but also synthesize and release odor compounds (2-methylisoborneol (2-MIB), Geosmin) (Briand et al., 2008; Graham et al., 2010; Wang et al., 2011). Among the water bloom forming cyanobacterial genera, *Microcystis*, *Planktothrix*, *Dolichospermum* (formerly as planktonic species in *Anabaena* genus) (Wacklin et al., 2009), and *Aphanizomenon* are usually the rulers governing the freshwaters and producing numerous bioactive secondary metabolites over the world (Pan et al., 2009; Vaitomaa et al., 2003).

To elucidate the genetic diversity and the genotypic composition within populations of cyanobacterial blooms, an increasing number of studies have recently been conducted on the unicellular cyanobacterium *Microcystis* (Briand et al., 2009; Wang et al., 2012; Xu et al., 2011). Briand et al. (2009) revealed 306 different genotypes from 784 ITS sequences in a France Reservoir, and they further found the marked changes in the ITS genotype composition of *M. aeruginosa* populations during the development of the bloom. Bozarth et al. (2010) described 61 ITS genotypes in the 155 obtained sequences of *Microcystis* populations in the Copco Reservoir of Klamath River, US, and they indicated that different ITS and *cpcBA* genotypes dominated at the two population peaks in the reservoir. Zhu et al. (2012) found relatively higher genetic diversity from ITS genotypes of *Microcystis* dominated blooms in a pond system, compared with the studies from the reservoirs. Such results provided a new insight into the genetic diversity of blooms dominated by *Microcystis*, leading to a better understanding of the spatiotemporal dynamics of *Microcystis* populations during bloom seasons. However, those above studies on genetic diversity of bloom forming cyanobacteria have only targeted

on *Microcystis* organisms, and it is well known that filamentous cyanobacteria may coexist with *Microcystis* or even dominate in certain blooms in many lakes. Therefore, studying the composition and genetic diversity of filamentous heterocystous cyanobacteria would help us to explicitly understand the trend of cyanobacteria blooms, thereby allowing us to predict and control their occurrence (Briand et al., 2009).

Lake Taihu has been hypereutrophicated in the last few decades (Chen et al., 2003; Qin et al., 2007). Dense cyanobacterial blooms, mainly composed of *Microcystis* and some filamentous cyanobacteria such as *Dolichospermum*, *Aphanizomenon* and *Planktothrix*, annually occurred in a large area of the lake. *Aphanizomenon* and *Planktothrix* were found to be restricted at the north of the Lake Taihu, and *Dolichospermum* was observed as the second dominated cyanobacterial group, following *Microcystis* (Liu et al., 2011; McCarthy et al., 2007). Otten et al. (2012) predicted that reducing eutrophication in Lake Taihu could lead to the shift from *Microcystis* dominance to *Dolichospermum* dominance. Apparently, the occurrence of *Dolichospermum* species in Lake Taihu was increasingly concerned, however the fundamental knowledge about diversity, abundance and dynamics of *Dolichospermum* species in Lake Taihu is lacking. Our previous study indicated that *Dolichospermum flos-aquae* occurred throughout the entire Taihu lake with relatively higher abundance in western and southern parts (Wang et al. 2013). In attempts to better understand the cyanobacterial blooms in Lake Taihu, it is necessary to perform studies on genotypic composition and spatiotemporal pattern of genotypes in *Dolichospermum* populations. In this study we designed PCR primers that included one end of the 16S rRNA gene and the other at the end of the 23S rRNA gene so as to amplify the ITS region of filamentous cyanobacteria. The shorter fragment product with about 278 bp (ITS-S) was used for further genotypic analysis. From the obtained 688 ITS sequences, two dominant

genotypes of *Dolichospermum* populations in Lake Taihu were identified for the first time. We also studied the correlations between environment factors and ITS genotypes to find out the primary associated factor for the dominant genotypes.

2. Materials and methods

2.1. Study area and sample processing

Lake Taihu, which is located in the south portion of the Yangtze River delta, is the third largest freshwater lake (surface area: 2338 km², mean depth: 1.9 m) in China. It is strongly affected by the subtropical monsoon climate with plentiful optothermal and rainfall. Taihu is a regional important body of water due to its roles in tourism, fisheries and transportation. It has however been seriously polluted by human activities in the last half century, transitioning from a meso-oligotrophic lake into its current hypereutrophic state.

Our study sites included four stations, as shown in the Fig. 1. Sampling was performed during April, September, and November in 2009. Water quality parameters (water temperature, dissolved oxygen, pH) were measured by a YSI 6600 multiprobesonde (OH, USA). Nutrients (total phosphorus (TP), total nitrogen (TN), phosphate (PO₄³⁻), nitrate (NO₃⁻), ammonium (NH₄⁺)) were measured by an automated wet chemistry analyzer (SAN+ ++, Skalar, Holland). Samples (each sites 300 ml) for DNA extraction were filtered on 0.22 µm filter (Millipore, New Bedford, MA, USA), and maintained at -20 °C. Samples for chlorophyll a (chl a) analysis were filtered onto Whatman (NJ, USA) GF/F filters (25 mm) and determined according to the method described by Wang (2000).

2.2 DNA extraction and PCR amplification

The membrane filters with total genomic DNA were cut into small pieces by a sterilized scissor and suspended in 2mL of the lysis buffer (40 mM EDTA, 400 mM NaCl, 50 mM Tris-hydrochloride, pH 9.0). Procedures were performed as described by Rinta-Kanto et al. (2005). Purified DNA was resuspended in sterile 50uL deionized distilled water (ddH₂O). The concentration and purity of extracted DNA was determined using spectrophotometric measurements at 260 and 280 nm. The DNA was stored at -20°C until being applied to PCR amplification. Extracted DNA templates were amplified for ITS using following primers : ana-its (5' – TACACAGCGATGTGATGCAAATCTCAAAAACCGTAG –3')(this study), ULR (5'–CCTCTGTGTGCCTAGGTATC –3') (Iteman et al., 2000; Neilan et al., 1997). PCR reactions were performed in a volume of 50 µl containing 5-10 ng genomic DNA, 1×PCR buffer with 1.5 mmol L⁻¹ MgCl₂, 0.2 mmol L⁻¹ dNTPs, 2 mmol L⁻¹ for each primer and 1 U Taq DNA polymerase (Takara, Japan), and 150 ng ml⁻¹ BSA. The reactions was run in a BioRad Thermal Cycler (BioRad, USA) with one cycle at 94 °C for 5 min, 35 cycles at 94 °C for 30 s, 55 °C for 30 s, 72°C for 30 s, and finally 72°C for 5 min. The filamentous heterocystous cyanobacteria have usually multiple copies in ITS regions, the smallest product (ITS-S) was choosed as our target regions. After electrophoresis, the amplified ITS-S product was obtained and then purified by using QiaQuick DNA Remove Kit (Qiagen, Germany).

2.3. Cloning and sequencing of the ITS and Operational Taxonomic Units (OTUs) analysis

The purified ITS-S products were cloned in a pMDTM18-T vector system (TaKaRa) according to the manufacturer's instructions (2:1 to 5:1 molar ratio of PCR products to vectors). Between 50 and 60

white colonies from each clone library were sequenced using an ABI 3730XL automated sequencer (PerkinElmer Biosystems, USA). All nucleotide sequences were stored in the GenBank database under accession numbers KC151273 - KC151960.

The ITS sequences were firstly aligned using ClustalW in BioEdit software (vision 7.0.9.1) and manually corrected. The genotypes were obtained by using the DnaSP software (version 5.10.01) (Librado and Rozas, 2009). A minimum spanning tree (parsimony network analysis), which connects main genotypes together was constructed using TCS software (version 1.21)(Clement et al., 2000). In attempts to assess whether the picking colonies (50 to 60 clones) could cover the taxonomic diversity, the rarefaction curve was calculated using Mothur (Schloss et al., 2009).

2.4. Phylogenetic analysis

The alignment was manually corrected, and the aligned bases (269 bp) were used for constructing the phylogenetic trees. The neighbor-joining (NJ) algorithmic were constructed by MEGA4 program package with 1000 bootstrap analysis (Tamura et al., 2007). The maximum likelihood (ML) algorithmic were processed by PhyML on line (<http://www.atgc-montpellier.fr/phyml/>) with the following parameters (substitution model = HKY85; transversion ratio = 1.5147; proportion of invariable sites = 0; number of substitution rate categories = 2; gamma shape parameter = 1.1901; perform bootstrap = 1000). Bayesian analysis were performed in MrBayes version 3.1 (Ronquist and Huelsenbeck, 2003) with the following parameters (GTR/HKY model of DNA substitution; Nst = 2/6; rates = gamma; Ngen = 5,000,000; Nruns = 2; Nchains = 4; Temp = 0.2; Samplefreq = 1000; Printfreq = 1000; Burnin = 2500; Burninfrac = 0.4).

2.5. Statistical analysis

The associations between the main genotypes with environmental parameters were determined by Pearson's correlation coefficient with the software SPSS 13.0 for Windows (SPSS Inc., 221 Chicago, IL, USA).

3. Results

3.1. Phylogenetic analysis on ITS sequences of *Dolichospermum* and *Aphanizomenon*

Of 720 raw DNA reads we determined that 688 ITS amplicons passed our quality standards. 118 different genotypes were identified, and each genotype was defined as differing at least one bp from other genotypes. The obtained 118 ITS sequences belonging to *Dolichospermum* and 39 previous ITS sequences of *Dolichospermum*, *Aphanizomenon* strains cited from GenBank were used to construct the phylogenetic tree (Fig. 2). The phylogenetic grouping result showed that the 118 genotypes were grouped with all other *Dolichospermum* species, specially closed to two strains of *Dolichospermum* from the Lake Taihu (JX072918 and JX072921). The similarities of ITS sequences in these 118 genotypes were 97.8% ~ 99.6%. In contrast, the similarities of these 118 ITS sequences with those from *Aphanizomenon* sp. in Lake Taihu only were 81% ~ 83%.

3.2. Genotypes diversity and succession of *Dolichospermum* populations in Lake Taihu

Rarefaction curves were made to testify whether the sequencing number could estimate species

richness. Sixty clones were picked and sequenced in each sampling site every month in the present study, and the curves, as shown in Fig. S1, reflected the prediction showing that additional sampling will not lead to significantly increased estimates of total diversity. Therefore, picking and sequencing 50 to 60 clones can nearly cover the diversity of each site. As shown in the Table 1, the numbers of both ITS sequences and genotypes of each sampling site in each month, revealed the genetic diversity. Among these genotypes, 14 genotypes were detected at more than two different nucleotide sites, and a maximum parsimony network using the 14 genotypes was constructed to analyze the phylogenetic relationships among these genotypes. The minimum spanning tree as shown in Fig. 3 revealed the presence of two dominant genotypes as L-type and W-type, containing 252 and 180 sequences respectively, within total 688 sequences,. The genotypes containing 2 ITS sequences were hap11, hap26, hap42, hap44, hap59, hap76, hap78 and hap114, and the hap10, hap56, hap64 and hap116 had 4, 15, 19 and 3 ITS sequences respectively.

The spatiotemporal dynamics of genotypes of *Dolichospermum* populations in each sampling site during our survey period were shown in Fig. 4. It was shown that each site reflected different genotype composition and changes in the genotype. Similarly, the genotype of the same site is also different during the three months. However, it is evidently shown that the L-type and W-type were almost present in each site during the sampling time, and the dynamics for the both types revealed a trend. In April and September, the W-type appeared in all sites and has high proportions, but the L-type only has a high proportion in N1 site. Nonetheless, in November, the L-type increased rapidly, and the W-type only took small proportions in each site, even was not detected at the W4 and S2 sites. From the total numbers of the two dominating types on the basis of time (Fig. 4), We found that the two dominating types existed in the months of April and September. However, only 2 ITS sequences of

W-type were detected in all sites, whereas 168 ITS sequences of L-type were found in November.

3.3 Correlations between the Ratios of two dominant genotypes with Environmental parameters

Lake Taihu is a large shallow lake and dominated by a subtropical monsoon climate. Therefore, the environmental parameters showed a strong seasonal variation (Table 2). The correlations between the parameters and the ratios of the two dominant genotypes were analyzed. The W-type was shown to be positively associated with water temperature, dissolved oxygen and NH_4^+ , however the L-type as negatively related with water temperature even the significance value was not so high (Table S1). Major environmental parameters were taken to estimate the contributions to the quantities of the two dominant genotypes by stepwise regressions analysis (Xu et al., 2010), and the obtained equations were given as below:

$$Y1 = 1.83 - 0.055X1 - 0.158X2;$$

$$Y2 = -1.576 + 0.047X1 + 0.127X3;$$

where X1 is the factor of water temperature, X2 is the concentration of TN, X3 is the factor of pH, Y1 is the ITS sequence numbers of L-type, Y2 is the ITS sequence numbers of W-type.

4. Discussion

Rapid developments in elucidating the genetic diversity of cyanobacterial blooms by sequencing the environmental rRNA genes, such as internal transcribed spacer-ITS, have provided a new insight into the genetic diversity of *Microcystis* populations in freshwaters. In this study, ITS sequences from

244 *Dolichospermum* populations in Lake Taihu were successfully obtained, and the 688 obtained
245 sequences were confirmed to be *Dolichospermum* derived based on the alignment and phylogenetic
246 analysis (Fig. 2), implying the specificity of the designed primer for *Dolichospermum*. *Microcystis*
247 blooms in the Lake Taihu have been largely studied including researches on interactive effects among
248 the dynamics of *Microcystis*, microcystin production, and environmental factors (Otten and Paerl, 2011;
249 Wang et al., 2012). Recently, an increasing number of studies have been performed on the genotypic
250 composition of *Microcystis* blooms worldwide, including Lake Taihu (Wang et al., 2012). However,
251 genetic structure and diversity of blooms dominated by filamentous heterocystous cyanobacteria such
252 as *Dolichospermum* have been hardly performed (Wang et al., 2013). Through sequencing the
253 environmental libraries of the ITS region on *Dolichospermum* populations in Lake Taihu, we elucidate
254 the ITS based genetic diversity and succession of *Dolichospermum* in the Lake. Furthermore, we
255 identify the two dominant genotypes (W-type and L-type) of *Dolichospermum* existing in the lake, and
256 this is the first time to report the dominant genotypes existing in *Dolichospermum* blooms. This finding
257 added a better understanding the spatiotemporal dynamics of cyanobacterial blooms dominated by
258 filamentous cyanobacteria in freshwater ecosystems. Comparative results from ITS sequences between
259 *Microcystis* and *Dolichospermum* blooms in Lake Taihu, demonstrated that genetic diversity
260 (represented as values of genotype number/ITS sequence number) from *Microcystis* blooms was higher
261 than that from *Dolichospermum* blooms (Bozarth et al., 2010; Sabart et al., 2009). These studies on
262 genotypes of *Microcystis* dominated blooms, from river-based reservoirs with large surface areas and
263 certain degrees of water flow and pond systems, indicated that strong hydrodynamics influence may
264 trigger the selection of the main genotypes. It is well known that Chinese large shallow lakes, such as
265 Lake Taihu, are characterized with strong wind and hydrodynamics movement, and such features may

266 explain the existence of dominant genotypes in *Dolichospermum* populations in Lake Taihu from the
267 present study. From the spatiotemporal dynamics of *Dolichospermum* genotypes, the distribution
268 pattern and succession of the two dominant genotypes were significantly displayed (Fig. 4). The
269 W-type was found to dominate in both April and September at each sites. Although some site (N1)
270 showed the coexistence of both W- and L- types, the tendency for succession from W- to L-types was
271 very evident (Fig. 4). It is striking to note that much less or no W-type appeared in November. In
272 attempts to investigate environmental factors triggering the dominance and succession of the two
273 dominant genotypes, correlation and stepwise regulations analyses were undertaken. It was
274 demonstrated that the water temperature was the chief factor affecting the succession of the two
275 dominating types (Table S1), since W-type and L-type were respectively shown to positively and
276 negatively associated with water temperature. These two dominant genotypes of *Dolichospermum*
277 displayed the significant seasonal pattern and temperature associated feature, implying the existence of
278 possible ecotypes of bloom forming cyanobacteria in freshwater shallow lakes. It is well known that
279 picocyanobacteria *Prochlorococcus* in ocean is composed of several clades which are phylogenetically
280 and physiologically distinct with respect to their optimal light and temperature environments, and these
281 clades have been referred to as “ecotypes” (Malmstrom et al., 2010; Moore et al., 1998). Further
282 confirmation on the existence of bloom forming cyanobacterial ecotypes in shallow lakes should await
283 for the availability of the culture strains with these two genotypes and following physiological
284 examination. Therefore, the future study will concentrate on culture strains isolation and exploration of
285 physiological differences and ecological distribution of *Dolichospermum* populations in Lake Taihu at
286 the genotype scale, hopefully obtaining the ecotypes equivalent to the genotypes. The results based on
287 the culture-independent clone libraries provided a better understanding for the genetic diversity of

water bloom -forming *Dolichospermum* populations.

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Figure Legends

Fig. 1. Sampling stations in Lake Taihu.

Fig. 2. Phylogenetic tree based on the 118 different ITS sequences obtained in this study and those ITS sequences cited from Genbank Local bootstrap supports for branches present in more than 50% of 1,000 replications are indicated at each node as the order ML/Bayes/NJ algorithm. *Planktothrix agardhii* (EU226210) was used as the outgroup.

Fig. 3. Parsimony network (minimum spanning tree) of ITS genotypes in *Dolichospermum* populations in Lake Taihu. The oval stands for the genotype.

Fig. 4. Dynamics in the proportions of different ITS genotypes of *Dolichospermum* populations in Lake Taihu during the survey period.

Fig 1

Fig 2

Fig 3

Fig 4

Table 1 The numbers of ITS sequences and genotypes of *Dolichospermum* in all the samples.

Sampling Months	N1		W2		W4		S2	
	NO. of sequences	NO. of genotypes	NO. of sequences	NO. of genotypes	NO. of sequences	NO. of genotypes	NO. of sequences	NO. of genotypes
April 2009	60	13	51	17	57	18	60	25
September 2009	60	14	53	19	58	16	60	14
November 2009	60	6	51	10	58	8	60	10

Table 2 The measured environmental variables in the four sample sites of three months in Lake Taihu.

Data	sites	TN (mg/L)	TP (mg/L)	NO₃⁻ (mg/L)	NH₄⁺ (mg/L)	PO₄³⁻ (μg/L)	T (°C)	pH	DO (mg/L)	Chl a (μg/L)
April	N1	2.52	0.04	1.65	0.17	21.49	17.0	8.61	9.9	5.95
	W2	5.13	0.05	2.22	0.12	15.04	17.0	8.60	10.7	9.75
	W4	5.44	0.08	2.18	0.13	20.43	16.5	8.70	10.7	10.65
	S2	4.37	0.06	1.08	0.16	26.19	16.0	8.60	10.4	7.35
September	N1	1.63	0.08	0.60	0.60	36.15	22.0	8.24	9.7	73.65
	W2	3.42	0.09	0.70	0.18	45.43	21.8	7.92	8.8	18.95
	W4	3.22	0.08	0.78	0.06	43.36	21.8	7.71	8.7	11.12
	S2	3.10	0.08	0.90	0.12	36.15	21.7	8.12	9.6	14.87
November	N1	3.09	0.11	0.49	0.05	53.2	12.7	8.28	11.9	7.12
	W2	3.60	0.05	1.28	0.09	50.15	11.9	7.91	11.6	14.21
	W4	3.40	0.06	1.37	0.07	55.35	11.8	7.96	11.7	12.35
	S2	1.80	0.18	1.20	0.06	33.72	12.1	8.08	11.9	7.21