第23卷 第5期
1999年10月

水生生物学报
ACTA HYDROBIOLOGICA SINICA

Vol.23, No.5
Oct., 1999

PHYLOGENETIC RELATIONSHIP AMONG SEVEN STRAINS OF *MICROCYSTIS* BASED ON THE RANDOMLY AMPLIFIED POLYMORPHIC DNA PCR*

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Abstract As a part of the molecular taxonomic revision of the genus *Microcystis* (Cyanophyceae), genome DNA divergence within and between three Microcystis species including seven strains from different areas were analyzed electrophoretically after using randomly amplified polymorphic DNA PCR with 24 primers. The main result was summarized as follows: (1)the genus *Microcystis* includes at least four taxonomic entities which differ in morphology and genetics: *M. aeruginosa* 101, *M. aeruginosa* 7820, *M. aeruginosa* 90 and *M sp.* (Dianshan); *M. viridis*; *M. wesenbergii*; *M. aeruginosa* 41. (2) M. aeruginosa comprises two heterogeneous taxonomic entities which could be viewed as separete species. (3) for their unique genotype and minor morphological variation, *M. viridis* and *M. wesenbergii* are regarded as well-established species. (4)as anticipated, *Anabaena* sp 7120 as the control exhibits entirely different genotype and farther genetic distance than those of other *Microcystis* strains.

The RAPD study on the genus *Microcystis* illustrates that it is possible to analyze intra- and inter-specific variation of Cyanophytes on the basis of genotype rather than phenotype and , therefore , offers significant and indispensable clues in solving taxonomic problems of cyanophytes, especially at the species and genus levels. Combined with the on-going research on special primers which sensitively and reliably distinguish the toxic and non-toxic strains of *Microcystis*, RAPD would be a helpful method to understand more explicitly the phylogenetic relationships between toxic and non-toxic strains of the genus *Microcystis*.

Key words *Microcystis*, RAPD, PCR, Phylogenetic Relationship.

Microcystis, blue-green alga (Cyanophyceae), are one of the common bloom-forming cyanophytes in eutrophic lakes, reservoirs and ponds of the world. Most of the *Microcystis* strains we chose in the research have been featured in toxin components and are being often selected in physiology and molecular basis for toxin production. The traditional taxonomic methods of *Microcystis* have largely relied on the morphology of colonies that includes cell arrangement and features of the mucilage. These morphological characteristics, however, cannot be easily applied in distinguishing strains of *Microcystis* in the long-term laboratory cul-

* Supported by National Natural Science fondation project(No. 39730380)
Received August 2, 1999; revised Sept. 13, 1999

tures or in some special conditions of natural environments. For example, *Microcystis viridis* was initially isolated and cultured as aggregated colony, but the colonial shape retained only for several months before displaying unicellular habit in culture[4]. A poor understanding of the taxonomy of this problematic genus has resulted from a low responsibility on the traditional criteria. In addition, precise circumscriptions of the *Microcystis* "species" still remain uncertain.

Because the massive growth of *Microcystis* has recently caused grave problems in entertainment and drinking water management as well as in aquatic environments, a well-defined taxonomic revision of the genus is now urgently needed not only by taxonomists, but also by researchers in limnology and its related fields. However, for the extremely simple morphological features of *Microcystis*, further taxonomic researches cannot merely depended on morphological investigation. It is indispensable to develop a new analytical approach that would lead to the solution of this problem.

Under these circumstances, we initiated the RAPD study (i.e. molecular taxonomic approach based on genome divergence) of the genus *Microcystis*. We adopted the RAPD study because this means involves some methodological advantages , which can provide significant clues to solving taxonomic problems in cyanophytes on the genetic basis ; that is, the RAPD study makes it possible to analyze intra-and inter-specific variation of cyanophytes on the basis of genotype rather than phenotype.

1 Materials and Methods

1.1 Organisms and culture methods Cyanobacteria *Microcystis* and *Anabaena* 7120 were cultured in medium MA[2] and in medium BG－11[3] respectively. Illumination was provided by cool－white fluorescent tubes. Light intensity was adjusted to 60μE $m^{-2}s^{-1}$. The strains used in this study are listed in table 1.

Tab.1 Cyanobacterial strains used in this study

Strain	Culture Collection	Source
Microcystis aeruginosa 41	FACHB－Collection	Donghu Lake, Wuhan, China
Microcystis aeruginosa 7820	UTEX－Collection	South Africa
Microcystis aeruginosa 90	NIES－Collection	Lake Kawaguchi, Japan
Microcystis aeruginosa 101	NIES－Collection	Lake Suwa, Japan
Microcystis sp.(Dianshan)	FACHB－Collection	Dianshan Lake, Shanghai, China
Microcystis viridis	FACHB－Collection	Dianchi Lake, Kunming, China
Microcystis wesenbergii	ACHB－Collection	Taihu Lake, Wuxi, China
Anabaena 7120	UTEX－Collection	Unknown

1.2 DNA extractions and RAPD－PCR analysis Total genomic DNA of the culture cells were extracted according to the method by Chisholm. The 24 random 10－mer oligonu-

cleotide(20 primers of I－01 to I－20 and 4 primers of M－11 to M－14), primers were obtained Operon Technologies(Alameda, Calif.). Optimized PCR were performed in a total volume of 50μL containing 3mM $MgCl_2$, 200μmol/L each deoxynucleotide triphosphate, 10 pmol of each PCR primer, 1U of Taq DNA polymerase. Thermal cycling were conducted in Geneamp 2400 (Perkin Elmer)as following program: initial denaturation at 94℃ for 5 min; 35 cycles of 94℃ for 30 s, 36℃ for 30 s , 72℃ for 60 s; and a final extention at 72℃ for 7 min. After PCR, 20μl aliquots of products were electrophoresed in 1.5% agarose gels containing 0.5μg/ml ethidium－bromide in the gel and the bands were observed in Gel Doc 1000(Biorad).

1.3 Calculation of genetic distance Bands visualized on a gel were considered as RAPD markers. Bands with same migration positions between the strains were regarded as homologous characteristics. The migration of bands between strains were used as indication of genetic relationship. The phenogram was constructed according to fussy cluster analysis.

2 Results and Discussions

2.1 Efficiency of amplification by PCR Initially, a total 24 primers were chosen to generate RAPD patterns for 8 strains of cyanobacteria, the number of 10－mer oligonucleotides was reduced to 10, these being the primers which produced informative and reproducible genetic markers for the strains(Tab 2). Total bands of each strain amplified by the above mentioned 10 primers were accounted and listed in Tab 3.

Tab.2 sequence of 24 primers and amplified efficiency

Primer	Sequences	Amplified efficiency	Primer	Sequences	Amplified efficiency
I－01	ACCTGGACAC	+	I－13	CTGGGGCTGA	+
I－02	GGAGGAGAGG	+ +	I－14	TGACGGCGGT	+ +
I－03	CAGAAGCCCA	+ +	I－15	TCATCCGAGG	+
I－04	CCGCCTAGTC	+	I－16	TCTCCGCCCT	+ +
I－05	TGTTCCACGG	－	I－17	GGTGGTGATG	+
I－06	AAGGCGGCAG	+ +	I－18	TGCCCAGCCT	+
I－07	CAGCGACAAG	+ +	I－19	AATGCGGGAG	+
I－08	TTTGCCCGGT	+	I－20	AAAGTGCGGG	+ +
I－09	TGGAGAGCAG	+	M－11	GTCCACTGTG	+
I－10	ACAACGCGAG	+ +	M－12	GGGACGTTGG	+
I－11	ACATGCCGTG	+	M－13	GGTGGTCAAG	+ +
I－12	AGAGGGCACA	+ +	M－14	AGGGTCGTTC	+

+ + : Clear and high copies of bands ; + : Low copies of bands; － : No bands

Tab.3 Total bands of each strain amplified by the 10 primers

Species	Total bands	I-02	I-03	I-06	I-07	I-10	I-12	I-14	I-16	I-20	M-13
M. aeruginosa 41	36	7	4	3	4	4	3	3	4	3	1
M. aeruginosa 7820	35	3	5	3	6	2	3	4	3	3	3
M. aeruginosa 90	33	3	6	3	6	2	3	1	3	3	3
M. aeruginosa 101	34	3	5	3	7	2	3	1	4	3	3
M. sp. (Dianshan)	37	6	6	4	3	3	3	4	2	3	3
M. viridis	33	4	5	6	5	1	3	2	2	3	2
M. wesenbergii	35	5	5	5	5	1	4	3	2	3	2
Anabaena 7120	31	2	3	5	4	3	3	3	5	2	1

2.2 The genetic distance of the strains The genetic distance between *Anabaena* and *Microcystis* was over 0.68, suggesting a very distinctive genetic divergence between these two genera; whereas the values ranged from 0.073 to 0.507 among 7 strains of *Microcystis*. It was very obvious that the strains *Microcystis aeruginosa* 7820 and *M. aeruginosa* 101 is closely related as the genetic distance between them was only 0.073(Tab4, 5, 6). The result indicated that genetic relatedness among the genera of toxic *Microcystis* was supported by the described RAPD markers and thus the sensitivity of RAPD provided a taxonomy and phylogeny for *Microcystis* which are comparable to those achieved by more traditional methods (Fig.1).

Tab.4 Number of same bands between strains

Strains	*M. aeruginosa* 7820	*M. aeruginosa* 90	*M. aeruginosa* 101	*M.* sp. (Dianshan)	*M. viridis*	*M. wesenbergii*	*Anabaena* 7120
M. aeruginosa 41	22	17	20	21	18	18	4
M. aeruginosa 7820		27	32	24	18	23	4
M. aeruginosa 90			27	22	20	21	8
M. aeruginosa 101				24	18	22	4
M. sp. (Dianshan)					23	22	8
M. viridis						21	10
M. wesenbergii							9

Tab.5 Genetic similarity(S) between strains

Strains	*M. aeruginosa* 7820	*M. aeruginosa* 90	*M. aeruginosa* 101	*M.* sp. (Dianshan)	*M. viridis*	*M. wesenbergii*	*Anabaena* 7120
M. aeruginosa 41	0.620	0.493	0.571	0.575	0.522	0.507	0.239
M. aeruginosa 7820		0.794	0.927	0.667	0.592	0.657	0.242
M. aeruginosa 90			0.806	0.679	0.606	0.618	0.250
M. aeruginosa 101				0.676	0.537	0.638	0.246
M. sp. (Dianshan)					0.657	0.611	0.235
M. viridis						0.618	0.313
M. wesenbergii							0.242

S=2Nxy/(Nx=Ny), Nxy: number of same bands between strain x and y;

Nx and Ny represent the total bands of strain x and strain y, respectively.

Tab.6 Genetic distance among strains

Strains	*M. aeruginosa* 7820	*M. aeruginosa* 90	*M. aeruginosa* 101	*M.* sp. (Dianshan)	*M. viridis*	*M. wesenbergii*	*Anabaena* 7120
M. aeruginosa 41	0.380	0.507	0.429	0.425	0.478	0.493	0.761
M. aeruginosa 7820		0.206	0.073	0.333	0.408	0.343	0.758
M. aeruginosa 90			0.194	0.321	0.394	0.382	0.750
M. aeruginosa 101				0.324	0.463	0.362	0.754
M. sp. (Dianshan)					0.343	0.389	0.765
M. viridis						0.382	0.687
M. wesenbergii							0.758

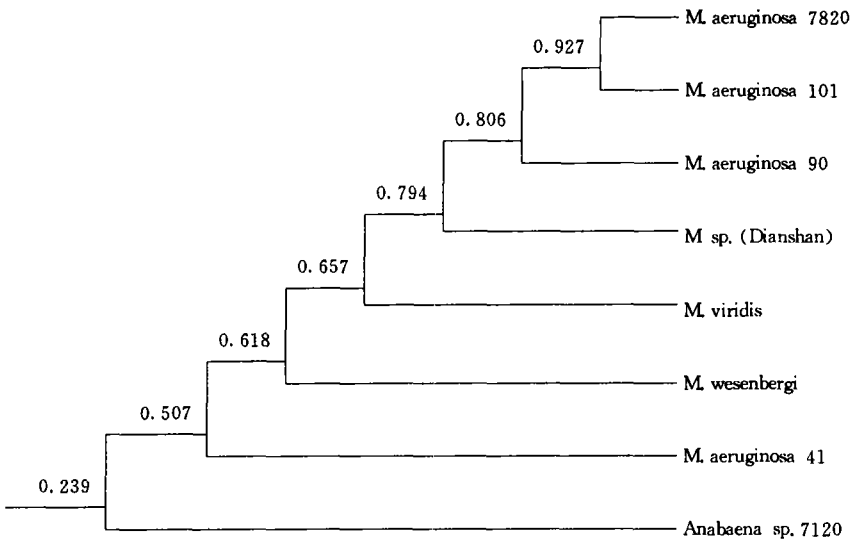

Fig.1 Phenogram based on RAPD markers amplified from 8 strains of *cyanobacteria*. A neighbor-joining tree was constructed with fussy cluster analysis.

2.3 Discussion In present study, as anticipated, *Anabaena* sp 7120 as the controll exhibits entirely different genotype and farther genetic distance with other *Microcystis* strains. The genetic distance between *Anabaena* and *Microcystis* is over 0.68, whereas the values ranged from 0.073 to 0.507 among 7 strains of *Microcystis*. According to fig 1, the genus *Microcystis* includes at least four taxonomic entities which differ in morphology and genetics: *M. aeruginosa* 101, *M.* a7820, *M.* a90and *M* sp. (Dianshan); *M. viridis*; *M. wesenbergii*; *M. aeruginosa* 41. The result also clearly demonstrates that two of morphotypes of *M. aeruginosa* are highly diverged at the genome level. It is concluded that *M. aeruginosa* comprises at lease two taxonomic entities which are greatly differentiated in their morpholohgy and genetics and thus could be viewed as separate species. For their unique genotypes, *M. viridis* and *M. wesenbergii* are regarded as well-established species that can be discriminated explicitly from strains of *M. aeruginosa*. Our results have shown that RAPD－PCR based classification of *Cyanobacteria* is an alternative and complementary approach to the traditional methods for studying *Cyanobacteria* systematics.

Recently, Japanese scientists have demomstrated that , for five morphological types of *Microcystis of Japanese origination, the result of* RAPD *analysis used to discriminate genotypes is in good agreement with those of the allozyme genotype study. This good accordance* indicated a high reliability of RAPD for discrimianting the affiliated groups of *Microcystis*[5]. Their consequence not only completes but also affirms our result.

Our study on *Microcystin synthetase genes* can definitely distinguish the toxic and non-toxic strains of the genus *Microcystis* (data not shown), it is thus of great necessity to develop a reliable and quick method in discriminating these strains. Sequences of *Microcystin synthetase genes* of all strains studied will be compared to show the phylogenetic relationships between toxic and non-toxic strains of the genus *Microcystis*. How the genus of *Microcystis* and the *Microcystin*-producing *Cyanobacteria strains* has differently evolved will consequently become an attractive issue. The two distinct evolving routes will help to explain the function of *Microcystin* in *Cyanobacteria*.

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七株微囊藻系统进化关系的 RAPD－PCR 分析

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摘要 应用 RAPD－PCR 的方法,选用 24 个随机引物,分析来自不同地区的 7 株微囊藻的基因组多态性. 结果显示, *Microcystis. viridis* 及 *M. wesenbergii* 明显与 *M. aeruginosa* 区分开. *M. aeruginosa* 分为两个可视为不同种的异源分类单位. 作为对照的 *Anabaena* sp. 7120 与其他微囊藻株表现出完全不同的基因型及更远的遗传距离.

此项研究表明,以基因型而不是表现型为基础,分析蓝藻种内及种间区别是可能的. 因此,为解决蓝藻分类问题,特别是在种和属的水平上,提供了重要的线索. 结合正在进行的用特异性及准确性强的引物区分微囊藻产毒及非产毒株的方法, RAPD－PCR 可望将微囊藻产毒及非产毒株进化关系澄清.

关键词 微囊藻, RAPD, PCR, 系统进化关系