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Report on my visit at the Center for Ecological Researches (CER) of Kyoto University

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Abstract. This report summarizes my visit to the Center for Ecological Researches (CER) of Kyoto University, supported by the International Research Unit of Advanced Future Studies. The report presents the description of my one month activity at CER, and give a brief review paper on diversity, taxonomy and genome comparison of bloom forming *Microcystis* species, a cyanobacterial group causing serious environmental problems.

Key words: Cyanobacterial Bloom, *Microcystis*, Taxonomy, Diversity, Genome

1. General description of my activity at CER

I was invited by Prof. Nakano Shin-ichi, the director of the Center for Ecological Researches (CER) to work at CER as a visiting professor, from February 1, 2016 to February 29, 2016. This visiting professorship was supported by the International Research Unit of Advanced Future Studies. I appreciate the financial support from the International Research Unit of Advanced Future Studies, and invitation from Prof. Nakano, and the help from many colleagues at CER.

During my stay at CER this time, I was on the boat to have a ecological survey on Lake Biwa with staffs and students together at CER. Lab examination after the survey included the preparation of pico-cyanobacterial samples and observation under the fluorescence microscope, and identification of dominant phytoplankton species under the light microscope.

On February12, 2016, I presented a talk with the title "Cyanobacterial blooms -- not the expected blossom but the ecological disaster in water" in the International Symposium on Advanced Future Studies held at Yukawa Institute for Theoretical Physics, Kyoto University. It was my first time to talk my major in the front of experts from very different fields, and many discussions and comments after my presentation indicated the mutual interest we are sharing.

On February 17, I was travelling to Shinshu University at Matsumoto city, Nagano, to visit Prof. Park Ho-Dong at Department of Environmental Sciences. We have discussed and exchanged our shared points on cyanotoxins produced by cyanobacterial species, and reached our wish on future cooperation on the integrated approach of chemical and molecular detection on novel types of cyanotoxins. On February 22, I visited Kanazawa University to meet Prof. Sakamoto at lab of plant physiology, faculty of Sciences. I introduced our researches on UV-absorbing chemicals (MAAs) from bloom forming *Microcystis* isolated from Chinese lakes. Prof. Sakamoto gave some valuable suggestion for the analytic methods on MAAs in cyanobacterial organisms. And on

February 23, I moved to Tsukuba, Ibaraki, to visit Microbial culture collection at National Institute for Environmental Studies (NIES). Upon the discussion with NIES staff, we agreed to perform the joint studies on Genomic projects on cyanobacterial strains from both China and Japan waters.

2. Review paper on diversity of bloom forming *Microcystis* species (cyanobacteria)

My researches have been focused on the diversity of water bloom forming cyanobacteria. The cyanobacteria are photosynthetic prokaryotes found in most of types of featured environment. They are also quantitatively among the most important organisms on Earth since it is estimated that cyanobacterial global biomass is 3×10^{14} g C or a thousand million tons (10^{15} g) wet biomass (Garcia-Pichel *et al*., 2003). The cyanobacterial record may extend back to ~3,500 million years ago, and they had a key role in the origin of the atmosphere oxygen in the Earth. Among about 2000 currently existing species of cyanobacteria, planktonic cyanobacteria forming water blooms by massive development cause problems in many nutrient-rich water bodies around the world. Among the bloom forming cyanobacteria, *Microcystis* is the most common and notorious. *Microcystis* dominance in blooms attribute to its advantageous features including buoyancy control, storage strategy at the bottom of the water column, inorganic nitrogen strategy, higher requirements for some trace elements than eukaryotic phytoplankton and resistance to zooplankton grazing by forming large-sized colonies and producing hepatotoxic metabolites called microcystins (Carmichael, 1996). Toxin production in *Microcystis* blooms always leads to the problem in drinking water safety, posing the serious threat to human health. A large volume of studies on *Microcystis* species and their blooms have been performed during last several decades. Researches on diversity, taxonomy and molecular characterization of *Microcystis* were also largely conducted since these issues are very close related to recognization and monitoring of the *Microcystis* species. Using this chance of my stay in CER, Kyoto University, I took time to summarize the partial knowledge of *Microcystis* species, focusing on taxonomy, molecular phylogentics, genotypes within field *Microcystis* populations and the status of genomic information that is beginning to facilitate studies on the detailed controls of bloom dynamics, and the potential hazards caused by these blooms and their toxins.

2.1. Molecular conservation of the *Microcystis* genus by single genes.

In the *Microcystis* genus, About 30 species have been described in the current cyanobacteria (cyanophyta) taxonomic system (Komárek and Anagnostidis, 1999). Of these species, six species including *M. aeruginosa* (Kützing) Kützing, *M. flos-aquae* (Wittrock) Kirchner, *M.ichthyoblabe* Kü tzing, *M. novacekii* (Komárek) Compè re, *M. viridis* (A. Braun) Lemmermann, and *M. wesenbergii* (Komárek) Komárek in were the main water bloom forming species in Japan (Komárek, 1991), and ten species, including the above six plus *M. botrys* Teiling, *M. firma* (Kützing) Schmidle, *M. smithii* Komárek et Anagnostidis and *M. pseudofilamentosa* Crow were found to be dominant in China (Yu et al., 2007). *Microcystis* species compositions in blooms in other countries /regions were found to be similar, within the range of these two Asian countries. Limitations in morphology-based taxonomy in all kinds of microorganisms are always regarded to be compensated by molecular genetic approaches which have been introduced into phylogenetic and taxonomic issues. Considerable efforts have been made to examine genetic divergence in *Microcystis* species and strains with the purpose to find the linkage between genetic and morphological variations in *Microcystis* species, and (Kato *et al*., 1991; Bittencourt-Oliveira *et al.*, 2001; Kurmayer *et al*., 2003). The 16S rRNA gene, an effective tool mostly used in

prokaryotic systematics, has been used extensively in the molecular taxonomy of cyanobacteria (Wilmotte and Golubic, 1991; Nelissen et al., 1996). However, divergence, within 16S rRNA gene sequences at the species level in *Microcystis*, was found to be low, often much less than 1% (Rudi et al., 1997; Otsuka et al., 1998). In addition, other gene regions such as ITS of 16S–23S rDNA and the DNA-independent RNA polymerase (*rpoC1*) gene, were not able to differentiate species (strains) within the genus *Microcystis* (Otsuka et al., 2000; Yoshida et al., 2008). However, one exception was *cpcBA*-IGS sequences (phycocyanin intergenic spacer and flanking regions) with the capacity to distinguish *M. wesenbergii* from other species of *Microcystis,* and these other species were still not divided by the *cpcBA*-IGS sequence (Tan et al. 2010). Such high genetic homology among the species of *Microcystis* on the basis of the single genes mentioned above, plus additional evidence from DNA–DNA hybridization, it was proposed that *Microcystis* species should be integrated into one species as *Microcystis aeruginosa* (Otsuka et al., 2001).

2.2 Genetic diversity of *Microcystis aeruginosa* strains by MLST and genotype analysis within the *Microcystis* populations from blooms.

Genetic analysis based on single genes is considered to be not free from problems invoked by recombination, and a multilocus sequence typing (MLST) approach for *M. aeruginosa* was developed to index the genetic variation of seven housekeeping loci (*ftsZ,glnA, gltX, gyrB, pgi, recA* and *tpi*), each of which is free from vigorous selection pressure, and the selected loci are scattered around the chromosome to reflect overall genome evolution (Tanabe et al. 2007). The results from the MLST analysis demonstrated high genetic diversity, clonal population structure and substantial recombination in *M. aeruginosa* , and the MLST phylogeny further showed that microcystin-producing genotypes are not monophyletic, providing further evidence for the gain and loss of toxicity during the intraspecific diversification of *M. aeruginosa*, different from the result by Rantala et al. (2004) who suggested the microcystin production has been vertically inherited through the diversification of toxic cyanobacterial genera, and non-microcystin production occurred at the cases of repeated loss along evolutionary course. Thus, this established MLST scheme seemed to provide the potential for characterizing the population genetic diversity in *M. aeruginosa*. In the following studies, the MLST revealed a high level of genetic differentiation among locations, even with fine-scale spatial and temporal genetic differentiation pattern (Tanabe et al. 2009), and it is further suggested that geographic factors have far less impact on the fine-scale spatial genetic diversity of *M. aeruginosa* than local genetic drift or, possibly selection. Genotypes of numerous *M. aeruginosa* isolates from Japan based on the MLST were divided into at least seven distinct phylogenetic clusters partially corresponding to either colony morphology or microcystin production, and further genotyping analysis on a special cluster (Group G genotypes all found in Lake Kasumigaura, Japan) revealed an expansion of the possible adaptive lineage in a localized aquatic environment (Tanabe et al. 2009). Similarly, genotypes of *Microcystis* populations in blooms determined by ITS region in France, US and China water bodies also revealed that pattern. Significant spatiotemporal changes and selections of dominant genotypes exist in the genotype composition of *Microcystis* populations from eutrophic and hypereutrophic waters (Bozarth *et al.* 2010, Sabart *et al.* 2009). Xu *et al.* (2011) reported that different *Microcystis* genotypes occupied diverse sections and branches of Qinhuai River, a major eutrophic river network in China. However, another study from Zhu *et al*. (2012) who indicated no significant dominant genotypes existing in Xinghu Pond, a eutrophic pond in Wuhan, China. These latter two results imply that special circumstances and uncertain factors influence the genetic composition of *Microcystis* populations in aquatic ecosystems. Recent study by comparing Chinese five lakes, namely Taihu, Chaohu, Gucheng, Shijiu and Erhai Lakes, showed that the *Microcystis* ITS genotypes and genetic diversity were negatively correlated with eutrophication level, and the high genetic diversity of the *Microcystis*

populations in Erhai Lake, a plateau lake in Yunnan China, may have resulted from the effect of the early stage of eutrophication (Song *et al.* 2015).

2.3 Genomic comparision of *Microcystis* strains

Genomic information about *Microcystis* species was not available until 2007 when the complete genome sequence of *M.aeruginosa* NIES-843 was published (Kaneko et al. 2007), and the genome of *M. aeruginosa* PCC7806, the most studies *Microcystis* strain, was later published in 2008. Currently there are 17 genomes of *M. aeruginosa* strains available in NCBI. To my knowledge, this is the largest number of genomes published from one species in freshwater cyanobacteria, plus *M. panniformis* FACHB1757 recently released (Zhang et al. 2016). Among these 18 strains, only *M. aeruginosa* NIES 843, *M. aeruginos* NIES2549 and *M. panniformis* FACHB1757 have complete genomes, and the latter two were sequenced using PacBio RS II sequencer, so called the third generation sequencer. The genome size of the 18 *Microcystis* strains varied from 4.26-5.84 Mb, ranging from 4368 to 6360 genes, but with very close GC contents (from 42.3-43.2%). Zhang et al. (2016) compared *M. panniformis* FACHB1757 with three strains of *M. aeruginosa* PCC7806, NIES 843, and NIES 2549, and these four strains had total 8704 genes, but with 2699 orthologous genes representing the core-genome for them. Similarly, Yang et al. (2015) studied the genetic diversity within the pan-genome of *M. aeruginosa* by combining 14 strains, and the pan-genome contained more than 15000 genes, with only 2192 orthologous genes representing the core-genome for *Microcystis aeruginosa*, and total 4742 strain-specific genes for these *M. aeruginos*a were detected, implicating that *M. aeruginos*a is well able to be adapted and well develop in the different specific localities. Sequencing and comparing the genomes of *Microcystis* strains can easily obtain average nucleotide identity (ANI) value between any two strains of them. Before genome age, classical DNA-DNA hybridization technique is the recommended standard for genetically determining bacterial species. The ANI analysis is newly recommended to substitute DNA-DNA hybridization for bacterial species circumscription, and the ANI value with above the threshold (94%) in each pair indicates that both strains are belonging to same species (Richter and Rossello-Mora, 2009). It is well known that DNA-DNA hybridization is hardly applied in cyanobacterial species determination since this method needs axenic strains, which is one of the most difficult problem in cyanobacterial culture procedure. Therefore, using the ANI value may bring a great help to cyanobacterial taxonomy along the continuous genomes of cyanobacteria published in future. In the case of *Microcystis* strains, the ANI values in each pairs of the the 17 strains of *M. aeruginosa* were more than 94%, even *M. panniformis* FACHB1757 have over 94% ANI with these 17 *M. aeruginosa* strains by comparing their genomes. Such a result will also need more studies on *Microcystis* intraspecies differentiation by introducing and judging the ANI index in cyanobacterial species definition, based on the genomic information of cyanobacterial strains.

3. Conclusion

This visit to CER allowed me to have such a opportunity to meet and discuss with many excellent scientists and students at CER and KU, and even in Japan. The whole activities including my lake survey, presentation in the symposium and visit tours to different universities/institutes during my stay were very successful. The review paper on *Microcystis* I am writing here was greatly beneficial from this visit, allowing me to take time in reading papers and preparing and writing this manuscript. Once again I would like to express my thanks to the International Research Unit of Advanced Future Studies for the support, and to Prof. Nakano and his colleagues at CER for the helps of my visit.

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