III. Biological Data

16. Cyanobacteria rDNA and rRNA density

(by Noriko Tomioka)

Sampling and sample filtration and DNA extraction

Lake water samples were collected from the surface to 2-m depth with a 2-m-long column sampler (diameter, 50 mm; RIGO, Tokyo, Japan). The 500-mL polyethylene bottle was placed in an ice cooler and immediately brought back to the NIES laboratory. The samples were filtered within 6 h after being collected. Twenty milliliters of each sample were filtered through an Omnipore membrane filter (diameter, 25 mm; pore size, 0.2 μm; type JG; Merck Millipore, Burlington, MA, USA), and the filters were stored at -80 °C until DNA extraction.

DNA extraction

From 1999 April to 2012 December

DNA was extracted from filter-fixed samples by using a FastDNA SPIN Kit (MP Biomedicals LLC, Illkirch, France). The membrane filters that had trapped the microorganisms in the water samples were cut into about 10 pieces, and all the pieces were placed in a tube with 1 mL of the isolation solution provided with kit. Homogenizing conditions were set at a speed of 4.0 for 20 s with 1/4" Sphere+Garnet Matrix with homogenizer (FastPrep; MP Biomedicals, LLC, Santa Ana, CA, USA) and other extraction operation was performed in accordance with the manufacturers' instruction.

Form 2013 January to 2018 December

DNA was extracted from filter-fixed samples by using an Extrap Soil DNA Kit Plus ver. 2 (Nippon Steel & Sumikin Eco-Tech Corp., Tokyo, Japan). Each filter was cut into small pieces and placed in a bead-beating tube, to which 950 μl of extraction buffer and 50 μl of lysis solution were added. Cells on filters were disrupted using a homogenizer (FastPrep; MP Biomedicals, LLC, Santa Ana, CA, USA) at 6.0 m s^{-1} for 40 s, after which DNA was purified by using magnetic beads to produce 100 μl of DNA extract.

RNA extraction

RNA was extracted from filter-fixed samples by using a FastRNA Pro Blue Kit (MP Biomedicals LLC, Illkirch, France). To reduce target RNA loss, 0.1 mg sodium ribonucleate (Wako, Tokyo, Japan) was added to each tube with the isolation solution. Homogenization was performed with a FastPrep (MP Biomedicals LLC) at a speed of 6.0 for 40 s. Other extraction operation was performed in accordance with the manufacturers' instruction.

Primer sets

16S DNA and 16S rRNA *of Microcystis aeruginosa*

Primer sequence (5'-3') Micro233F CTAATTGGCCTGRAGAAGAGC

Cyano342R GCTGCCTCCCGTAGGAGT

Amplicon size (bp) 145

Reference: Tomioka N, Imai A, Komatsu K. Effect of light availability on Microcystis aeruginosa blooms in shallow hypereutrophic Lake Kasumigaura. J Plankton Res. 2011; 33: 1263-1273.

16S DNA and 16S rRNA *of Planktothrix agardhii*

Primer sequence (5'-3') Plankto 650F TAAAGGCAGTGGAAACTGGAAGA

Plankto 734R GTGTCAGTTATAGCCCAGCAGAG

Amplicon size (bp) 129

Reference strain: Planktothrix agardhii NIES-204

Reverse transcription for rRNA measurement

First-strand complementary deoxyribonucleic acid (cDNA) was synthesized by using the PrimeScript RT reagent Kit (TaKaRa Bio, Siga, Japan), according to the manufacturer's Instructions. The primers used were Cyano342R and Plankto 734R

Real-time quantitative PCR

SYBR Green I PCR amplification was performed with a LightCycler 480 instrument (Roche, Mannheim, Germany). The 20-μL reaction mixture contained 1×LightCycler 480 SYBR Green I Master (Roche), 10 pmol of each primer and DNA extract or cDNA solution. Denaturation was performed for 5 min at 958C, followed by 40 cycles of repeated denaturation (10 s at 95°C), annealing (10 s at 60°C) with fluorescence acquisition (wavelength, 530 nm) and extension (10 s at 72°C). The temperature transition rate was 4.4°C s^{-1} for denaturation and extension and 2.2°C s^{-1} for annealing. A negative standard of Milli-Q water instead of the DNA solution was prepared in all experiments. After RT-PCR, a melting curve analysis was performed by continuous measurement of fluorescence while heating from 65 to 97°C at a transition rate of 0.11°C s^{-1} to calculate the Tm value. The crossing point was determined with the second derivative max method using LightCycler 480 software version 1.5 (Roche). A standard curve of dilutions of the PCR product was created with each analytical run to serve as a reference for copy numbers of cyanobacterial 16S rDNA.

Tm value calculation

In 2001, *Microcystis ichthyoblabe*, *Microcystis novacekii*, *Microcystis viridis* and *Microcystis*

wesenbergii, *Microcystis flos-aquae* and *Microcystis pseudofilamentosa* were integrated into M. aeruginosa based on 16S rDNA homology and DNA–DNA hybridizations(1). The primers used in this study were adjusted to amplify all of these. Although *M. aeruginosa* is genetically homogeneous, there are large variations in colony structure and cell size, which are thought to have a significant effect on growth. In addition, the GC content of PCR products differs slightly between these strains, and there are also differences in Tm values between strains. In this study, we have shown the transition between M. aeruginosa over time by listing the Tm values in addition to the density of M. aeruginosa. Since the Tm value differs depending on the PCR reagent, we cannot provide an absolute value, but for reference we have attached the approximate value calculated using the following formula published in Current Protocols in Molecular Biology, and the Tm value corrected using the average of the measured values of M. aeruginosa 843 (86.0) that was used as a standard curve.

Tm (℃) = 60.8 + 0.41 x (%GC) - (500/n)

GC content (%) is the GC content of the corresponding PCR amplified byproduct, and n is the length (bp) of the PCR product, which is all 143bp in this example.

	GC content (%)	Tm calculated value (℃)	Tm prediction (℃)
AB012336_Microcystis_novacekii_ TAC20	55.22	79.9	85.4
AF139319_Microcystis_aeruginosa UWOCC C5	55.97	80.3	85.7
AJ635434_Microcystis_ichthyoblabe_0BB35S	55.97	80.3	85.7
AB012338 Microcystis_ichthyoblabe_ TAC48	56.72	80.6	86.0
AB035549 Microcystis_aeruginosa NIES 843*	56.72	80.6	86.0
AB035550_Microcystis_ichthyoblabe	57.46	80.9	86.3
AF139316_Microcystis_aeruginosa UWOCC C4	57.46	80.9	86.3
D89034_Microcystis_wesenbergii	57.46	80.9	86.3
D89032_Microcystis_aeruginosa_NIES98	58.21	81.1	86.6

*Strains used for standard curve

1. Otsuka S, Suda S, Shibata S, Oyaizu H, Matsumoto S, Watanabe MM. 2001. A proposal for the unification of five species of the cyanobacterial genus *Microcystis* Kutzing ex Lemmermann 1907 under the Rules of the Bacteriological Code. 51:873-879.