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Succession of genetic diversity of *Botryococcus braunii* (Trebouxiophyceae) in two Japanese reservoirs

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Abstract

Botryococcus braunii is a green colonial alga that produces large volumes of liquid hydrocarbon. Therefore, *B. braunii* is expected to be useful as an alternative fuel resource. Natural blooms of *B. braunii* have been recorded in several lakes and reservoirs. Elucidation of natural *B. braunii* blooming would provide important information for the development of an open-pond cultivation system. In this study, we periodically assessed the genetic diversity and colony density of *B. braunii* populations, along with several environmental parameters, in two Japanese reservoirs (provisionally called "N" and "S") from December 2008 to December 2009. Reservoir N had low numbers of *B. braunii* colonies whereas Reservoir S was characterized by periodic density increases that occurred in December 2008, and in March, September, and December 2009. Population genetics analysis using specific environmental sequences (PGA-SES method) was conducted for *B. braunii* populations for the first time. Among the *B. braunii*-dominated samples of Reservoir S, high levels of genetic diversity were observed in December 2008 and March 2009, whereas the diversity levels in September and December 2009 were low. The results suggest that *B. braunii* periodicity can be categorized into a high genetic diversity type and a low genetic diversity type. The high genetic diversity type may be caused by simultaneous growth of many genotypes, whereas the low genetic diversity type seems to be explained by increases in the cell density of only a few adapted genotypes.

Keywords: Biofuel; Bloom; *Botryococcus braunii*; Environmental sequence; Genetic diversity; Population genetics

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1. Introduction

The green colonial alga *Botryococcus braunii* Kützing (Trebouxiophyceae, Chlorophyta) is a cosmopolitan freshwater alga that is characterized by producing hydrocarbons that accumulate in the colony matrix. The hydrocarbon content of *B. braunii* colony reaches 75% of the dry weight, and this content level is significantly higher compared to other oil-producing microalgae [1]. Hydrocarbon derivatives from *B. braunii* have been identified in crude oils [2] and oil shales [3], indicating that *B. braunii* hydrocarbons are important sources of hydrocarbons found in a variety of oil-rich deposits [4]. Gudin et al. [5] reported that *B. braunii* could convert 3% of the solar energy to hydrocarbons. On account of these characteristics, *B. braunii* has received considerable attention as a potential source of renewable fuel. To date, many studies have been performed to evaluate *B. braunii* as an energy resource, e.g., understanding its growth characteristics [6, 7], developing methods for continuous culturing [8], and exploring means for effective harvesting [9]. In particular, the development of a mass cultivation system is considered a high priority [10].

Massive blooms of *B. braunii* have been observed in several aquatic environments [11]. For example, a maximum colony density of ca. 1.4×10^6 colonies/L was observed during a bloom in an English pond [12]. Although the size, density, and color of *B. braunii* colonies during blooms have been documented [12, 13], the mechanisms and processes of blooming remain unclear. Elucidation of the blooming process would provide important information for the development of a mass cultivation system.

It has been shown that succession of genetic diversity levels and genotype compositions is useful for analyzing the blooming process in freshwater cyanobacteria [14, 15]. Therefore, in this study, we periodically assessed the genetic diversity and colony density of *B. braunii* populations, along with several environmental parameters, in two reservoirs.

2. Materials and Methods

2.1. Study reservoirs and sampling

We investigated two reservoirs, provisionally called “N” and “S,” that are located in Okinawa Prefecture, Japan. Both reservoirs have a maximum depth >10 m, and the surface areas are 0.4 km^2 and 1.4 km^2 for reservoirs N and S, respectively. The total volume of Reservoir S (1.3×10^7 m^3) is approximately 3 times larger than that of Reservoir N (0.4×10^7 m^3). The two reservoirs are located approximately 5 km apart, and there is no aquatic connection between them. *B. braunii* was observed throughout the entire year in both reservoirs, and *B. braunii* blooms had been observed previously (unpublished data).

We collected 1 L of surface water from the middle of each reservoir at approximately 2-month intervals from December 2008 to December 2009. A total of 14 samples, 7 samples from each reservoir, were analyzed (Table 1). Water temperature, pH, specific conductance, and dissolved oxygen concentration were monitored using a 556 MPS multiparameter system (YSI Inc., Yellow Springs, OH, USA).

Five hundred milliliters of each surface water sample was fixed with Lugol’s solution (1% final concentration), and the density of *B. braunii* colonies was counted with the aid of an inverted light microscope (CK40; OLYMPUS, Japan). A portion of each sample (150 mL) was filtrated onto a 1.0-µm pore polycarbonate filter (Millipore), and the filter was immediately dried and maintained in a desiccator at room temperature until the DNA was extracted.

Concentrations of inorganic nutrients (NO_3^-+NO_2^-, NH_4^+, and PO_4^{3-}) were measured using an autoanalyzer (Traacs 800; Bran+Luebbe, Germany) according to the methods of Hansen and Koroleff [16].

Table 1 Study reservoirs and samples

Reservoir name and total volume (m^3)	Sampling date	Sample name	Number of sequences from clone libraries	Accession number
N 0.4×10^6	18 Dec, 2008	N-0812	24	AB602999–AB603022
	18 Mar, 2009	N-0903	27	AB603023–AB603049
	20 Apr, 2009	N-0904	33	AB603050–AB603082
	25 Jun, 2009	N-0906	28	AB603083–AB603110
	2 Sep, 2009	N-0909	34	AB603111–AB603144
	19 Oct, 2009	N-0910	27	AB603145–AB603171
	16 Dec, 2009	N-0912	32	AB603172–AB603203
S 1.3×10^7	18 Dec, 2008	S-0812	23	AB603204–AB603226
	18 Mar, 2009	S-0903	28	AB603227–AB603254
	20 Apr, 2009	S-0904	24	AB603255–AB603278
	25 Jun, 2009	S-0906	31	AB603279–AB603309
	2 Sep, 2009	S-0909	28	AB603310–AB603337
	19 Oct, 2009	S-0910	32	AB603338–AB603369
	16 Dec, 2009	S-0912	29	AB603370–AB603398

2.2. DNA amplification and sequencing

Total DNA from the filters was extracted using a DNeasy Plant Mini Kit (QIAGEN, Valencia, CA, USA) according to the manufacturer's protocol. PCR amplification was performed in an TaKaRa thermal cycler Dice (Takara Bio, Otsu, Japan) using a 25-μL reaction mixture containing 0.2 mM of each dNTP, 10× PCR buffer, 0.25 U Ex Taq DNA polymerase (Takara Shuzo, Osaka, Japan), 0.5 μM of each primer pair, and 0.5–5 ng of DNA extracted from the filter samples. A universal primer set, ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) [17], which was designed for the internal transcribed spacer (ITS) and the 5.8S rRNA gene, was used. The reaction profile for the PCR was as follows: 94°C for 10 min; 5 cycles at 94°C for 1 min, 65°C for 45 s, and 72°C for 1 min; 5 cycles at 94°C for 1 min, 60°C for 45 s, and 72°C for 1 min; 30 cycles at 94°C for 1 min, 55°C for 45 s, and 72°C for 1 min; and 72°C for 5 min as a final extension. We then performed a second PCR step using a *B. braunii*-targeted primer set, Bits1 (5′-AAGGATCATTGAACAYGTCWG-3′) and Bits4 (5′-TTAAGTTCAGCGGGTGCTC-3′), which was designed during this study. The second PCR amplification conditions were the same as the first. The secondary PCR products were cloned into the pGEM-T vector (Promega Corporation, Madison, WI, USA) and introduced into competent cells of *Escherichia coli*. An *E. coli* colony-PCR was carried out in a 25-μL reaction mixture containing 0.2 mM of each dNTP, 10× PCR buffer, 0.25 U Ex Taq DNA polymerase (TaKaRa), and 0.5 μM of the M13 forward and reverse primers with the following cycling conditions: 94°C for 10 min; 35 cycles at 94°C for 1 min, 55°C for 45 s, and 72°C for 1 min; and 72°C for 5 min as a final extension. We detected positive colonies using agar gel electrophoresis of the PCR product. Fragments from the positive colonies were sequenced by the Dragon Genomics Center, TaKaRa Bio Inc. (Otsu, Japan). We checked all sequences by nucleotide BLAST, BLASTN, provided on the National Center for Biotechnology Information web site (http://www.ncbi.nlm.nih.gov/).

2.3. Population genetics analysis

The genetic diversity (π) [18] for each sample and the genetic divergence (F_{ST}), based on the haplotype differences between the samples, were calculated in ARLEQUIN version 3.11 [19]. To examine genetic relationships among the 14 samples, a clustering tree was constructed using POPULATION version 1.2.30 (http://bioinformatics.org). The 14 samples were treated as the 14

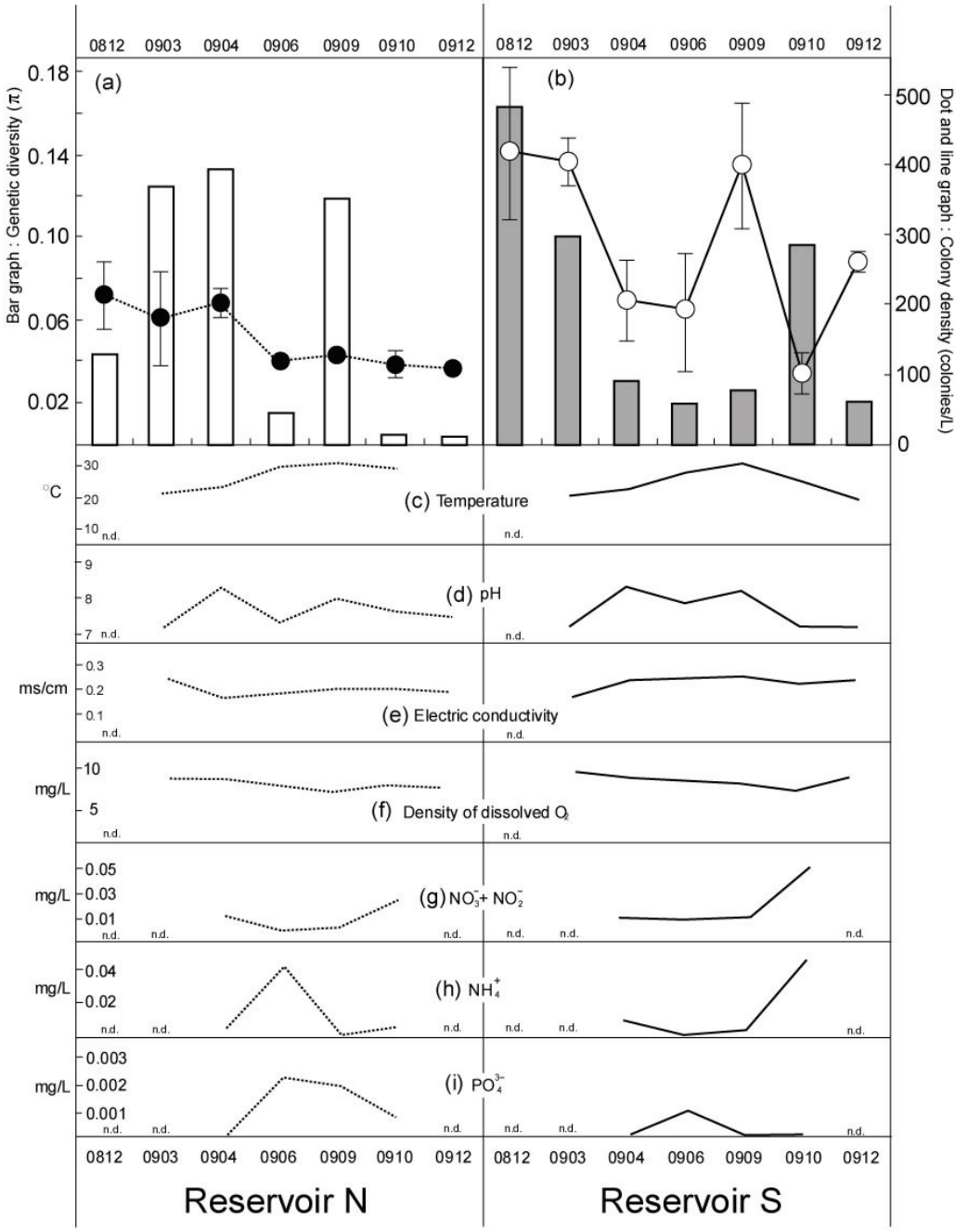

Fig. 1. Succession of colony density (dot and line graphs) and genetic diversity (bar graphs) of *Botryococcus braunii* in Reservoir N (a) and S (b). Error bars indicate the standard deviation from the mean. Fluctuations in the following environmental parameters were tracked: temperature (c), pH (d), specific conductance (e), dissolved oxygen concentration (f), nitrate and nitrite ion concentrations (g), ammonium ion concentrations (h), and phosphate ion concentrations (i). n.d. indicates no data. Dashed lines = Reservoir N, solid lines = Reservoir S.

"populations." POPULATION was also used to calculate the standard genetic distance [18] between the "populations" and to construct an unrooted tree using a neighbor-joining method. Bootstrap values were calculated with 1,000 replicates.

3. Results

3.1. Succession of B. braunii *colony density and environmental conditions*

The changes of *B. braunii* colony density in Reservoir N and S are shown in Fig. 1-a and 1-b. Maximum abundances were observed in Reservoir N sample N-0812 (213 ± 48.7 colonies/L) and Reservoir S sample S-0812 (420 ± 120 colonies/L). The colony density in Reservoir N gradually decreased from the first sample, while the density in Reservoir S increased and decreased several times. Although a *B. braunii* bloom was not observed during the research period, high densities of *B. braunii* occurred in December 2008, and again in March, September, and December 2009, in Reservoir S.

There was no statistically significant difference in the environmental conditions between Reservoirs N and S, except for the concentration of NH_4^+. The temperature range was 21.1–30.8°C in Reservoir N and 20.3–31.9°C in Reservoir S. The highest pH values (greater than 8) were observed in April 2009 and September 2009 (Fig. 1-d) in both reservoirs. The specific conductance (0.20 ± 0.017 ms/cm and 0.22 ± 0.022 ms/cm in Reservoir N and S, respectively) and the dissolved oxygen concentration (7.8 ± 0.56 mg/L in Reservoir N and 8.4 ± 0.50 mg/L in Reservoir S) were almost constant throughout the study. The highest values of the concentration of NO_3^- + NO_2^- (0.024 mg/L for Reservoir N and 0.049 mg/L for Reservoir S) and PO_4^{3-} (0.0020 mg/L for Reservoir N and 0.00086 mg/L for Reservoir S) were observed in October 2009 and June 2009, respectively. Only the concentration of NH_4^+ showed a discrepancy between the two reservoirs. The highest value (0.045 mg/L) of NH_4^+ in Reservoir N was observed in June 2009, whereas the highest value (0.045 mg/L) of NH_4^+ was observed in October 2009 for Reservoir S.

3.2. Succession of genetic diversity

Twenty-three to 34 sequences were obtained from each plasmid library (Table 1). In total, 205 and 195 sequences were determined in Reservoir N and S, respectively. The nucleotide length of the ITS fragments ranged from 675 to 708 bp and from 671 to 728 bp in Reservoir N and S, respectively.

The value of genetic diversity calculated by ARLEQUIN varied from 0.004 (N-0912) to 0.134 (N-0904) among the Reservoir N samples and varied from 0.020 (S-0906) to 0.164 (S-0812) among the Reservoir S samples (Fig. 1-a and 1-b). No significant relationship was determined between the colony density and the genetic diversity. Although the colony densities of Reservoir N samples N-0906 to N-0910 were almost identical, the genetic diversity increased by more than 6 times from June (N-0906) to September (N-0909) in 2009, but then decreased markedly in the October 2009 sample (N-0910). Reservoir S samples S-0812, S-0903, S-0909, and S-0912 showed relatively high densities of *B. braunii*, but genetic diversity varied among these four samples. The genetic diversity value for S-0812 was 0.164, which was the maximum value recorded during this study. The genetic diversity of S-0903 was also relatively high (0.101). On the other hand, the diversity values for samples S-0909 and S-0912 were 0.026 and 0.021, respectively.

3.3. Genetic relationship among samples

The neighbor-joining tree in Fig. 2 depicts the genetic relationship among 14 samples. Two major groups (Reservoir S and Reservoir N dominant groups) were recognized, but they were supported with low bootstrap values. Larger genetic distances were observed in Reservoir N samples than in Reservoir S samples. Values of genetic divergence (F_{ST}) between samples are summarized in Table 2. All pair-wise

F_{ST} values for Reservoir N samples were significantly higher than 0 ($p < 0.01$), and the average F_{ST} value was 0.520 ± 0.218. The F_{ST} values for Reservoir S samples were considerably lower than those for Reservoir N samples, and the average F_{ST} value was 0.092 ± 0.053.

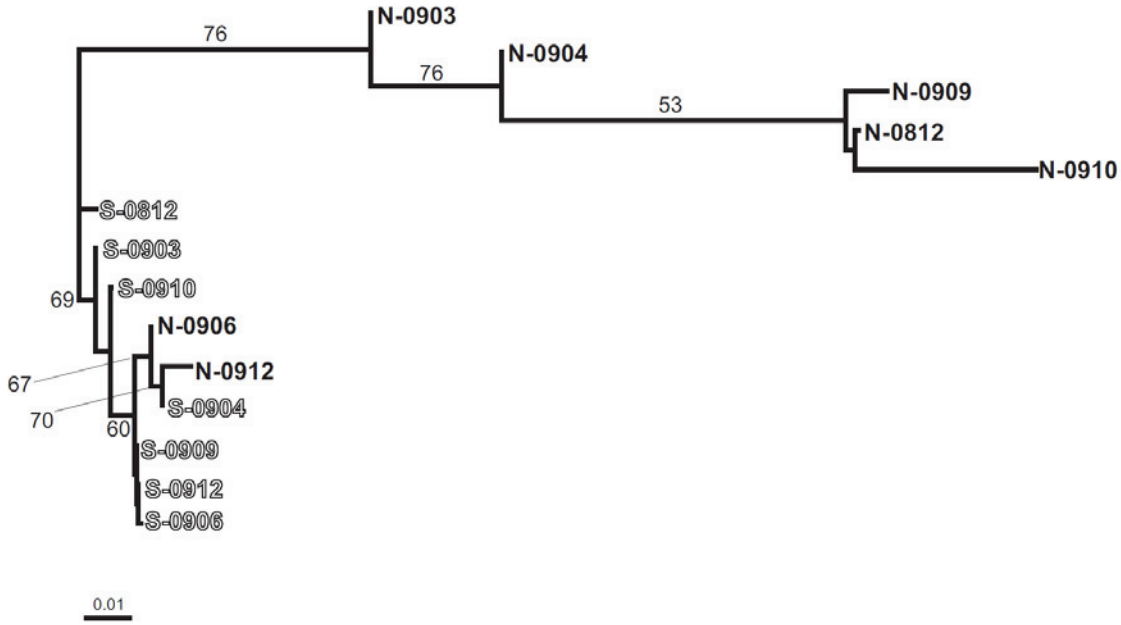

Fig. 2. Neighbor-joining tree for the 14 samples from Reservoir N and S. Bootstrap values (>50) are shown near the branches.

Table 2. Genetic divergence (F_{ST}) between samples in Reservoir N and S. Asterisks indicate values that are significantly ($p < 0.01$) different from 0

Reservoir N	N-0812	N-0903	N-0904	N-0906	N-0909	N-0910	N-0912
N-0812							
N-0903	0.339*						
N-0904	0.331*	0.021*					
N-0906	0.877*	0.434*	0.445*				
N-0909	0.119*	0.302*	0.299*	0.728*			
N-0910	0.665*	0.577*	0.428*	0.960*	0.425*		
N-0912	0.907*	0.465*	0.473*	0.386*	0.757*	0.982*	

Reservoir S	S-0812	S-0903	S-0904	S-0906	S-0909	S-0910	S-0912
S-0812							
S-0903	0.011						
S-0904	0.166*	0.155*					
S-0906	0.164*	0.115*	0.204*				
S-0909	0.148*	0.110*	0.103*	0.012			
S-0910	0.020	0.004	0.100*	0.067*	0.056*		
S-0912	0.157*	0.105*	0.144*	0.012	0.012	0.068*	

4. Discussion

4.1. PGA-SES method

As a part of this study, we propose novel methods for population genetics analysis. In previous population genetics studies of microalgae, it was necessary to establish a number of culture strains to obtain sufficient population components for DNA analysis [e.g., 20, 21]. Single-cell isolation and establishment of culture strains often requires considerable time, and, furthermore, there can be a selection of cells during the isolation procedure that introduces artificial bias [22]. However, with our new approach, the population genetics analysis carried out using specific environmental sequences (PGA-SES) may overcome these problems. DNA fragments that are amplified from environmental DNA using an organism-specific primer set should display a diversity of DNA sequences among targeted organisms in the environment. Therefore, we treated the sequences from the PCR amplification as sequences from "the population of the targeted organism" in the environment. DNA libraries of amplified environmental sequences have been used to assess genetic diversity of microbial communities [23-25]. In the case of the present study, only *B. braunii* sequences from environmental DNA were amplified using a *B. braunii*-specific primer set and were used for the population genetics analysis. This is the first time that the amplified sequences from environmental DNA have been applied to a population genetics study.

PCR error may cause overestimation of genetic diversity, whereas DNA polymerase with proofreading activity is known to reduce the PCR error. Since we used a DNA polymerase (Ex Taq) that did not have proofreading activity, our genetic diversity values may be overestimated because of PCR error. In addition, chimera formation during PCR has been shown to be a source of critical error in the analysis of amplified sequences [26]. However, it has also been suggested that small amplicons, less than 1 kbp in size, are resistant to PCR recombination [27]. Since the length of the targeted region in this study was ca. 700 bp, chimera formation should have been avoided.

4.2. Relationship among genetic diversity, colony density, and environmental parameters

The genetic diversity of *B. braunii* changed significantly throughout the monitoring period in both reservoirs. Despite colony densities that were relatively constant in Reservoir N, the genetic diversity fluctuated. Significant genetic divergence (F_{ST}) between samples was also observed in Reservoir N (Fig. 2 and Table 2). These results show that dynamic changes occurred in the *B. braunii* genotype composition in nature. Such dynamic succession of the genotype composition has been observed for other microalgae, e.g., *Microcystis* spp. [15, 28], *Planktothrix* spp. [14], and *Ditylum brightwellii* [20]. Therefore, marked fluctuations in genetic diversity seem to be a universal feature of microalgal populations.

An increase and domination of *B. braunii* colonies were observed in samples S-0812, S-0903, S-0909, and S-0912 from Reservoir S, and these samples can be categorized into 2 types. Samples S-0812 and S-0903 showed high genetic diversity levels, whereas the genetic diversity levels of S-0909 and S-0912 were low. This result suggests that there are two processes underlying *B. braunii* population increases. The high genetic diversity type may be caused by co-instantaneous growth of various genotypes, whereas the low genetic diversity type seems to be explained by population growth of a few adapted genotypes. In Reservoir S, prior to sample S-0912, sample S-0910 had a relatively high genetic diversity level. However, selection and growth of only adapted genotypes seemed to occur from October 2009 to December 2009, resulting in the low genetic diversity of sample S-0912. In Reservoir N, an increase of the low genetic diversity type probably occurred. Although samples N-0906, N-0909, and N-0910 had similar colony densities, the genetic diversity level increased markedly in sample N-0909, and it decreased rapidly in sample N-0910. Various genotypes appeared in September 2009; however, only selected genotypes appeared to survive and occur in the October 2009 sample. Therefore, two population growth types, the high genetic diversity type and the low genetic diversity type, seem to be involved in *B.*

braunii blooming. Further studies assessing *B. braunii* genetic diversity during blooms may confirm our conclusions.

Environmental parameters, such as nutrient concentrations, temperature, and pH, are important factors that affect aquatic microalgae density and the genetic diversity [15, 20]. For example, the abundance and genotype composition of the cyanobacterium *Microcystis* are influenced considerably by temperature [15]. In this study of *B. braunii*, no significant correlation was found between environmental parameters and cell density or genetic diversity. Furthermore, the factor that controlled switching between the two growth types was also unclear. Wake and Hillen [13] attempted to detect any critical environmental parameters associated with *B. braunii* blooming; however, they were unsuccessful. Because the parameters we monitored were only a small part of the environmental conditions and because our study spanned a relatively short time period, we may have missed some important factor or timing. Future analyses of additional biological/environmental factors, including predators, competition with other phytoplankters, and water circulation patterns, may provide new ideas for analyzing the key factors for *B. braunii* growth and blooming and for the switch between the two population growth types.

5. Conclusion

Natural blooms of *B. braunii* have been recorded in several lakes and reservoirs. Elucidation of natural *B. braunii* blooming would provide important information for the development of an open-pond cultivation system. The results of the present study suggest that there are two processes underlying *B. braunii* population increases. The high genetic diversity type may be caused by co-instantaneous growth of various genotypes, whereas the low genetic diversity type seems to be explained by population growth of a few adapted genotypes.

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