

**Ecological and genomic studies on diazotrophic
cyanobacteria in coastal seas**

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Chapter 1

General introduction

Cyanobacteria have developed the ability to harvest light energy and utilize it to fix carbon dioxide (CO_2) into organic matters, which is called to oxygenic photosynthesis. Emergence of cyanobacteria had brought environmental change from anaerobic to aerobic condition on primordial Earth's atmosphere (1). Their oxygenic photosynthesis and carbon fixation have significant influence on Earth's biogeochemical cycle (1). In addition to oxygenic photosynthesis, many cyanobacterial species also have the capacity of nitrogen fixation (referred to as diazotrophic cyanobacteria) which is the process of reducing nitrogen (N_2) gas to biologically available ammonium (2). Atmospheric N_2 that is the most abundant gas in the Earth, is hard to be utilized as biological nitrogen source due to its high unreactivity. Nitrogenase is a key enzyme complex for the biological reduction of N_2 into ammonia (3). In the open ocean, nitrogen availability often regulates plankton productivity because of the oligotrophic conditions (4). The revised $^{15}\text{N}_2$ tracer method has revealed that the global marine nitrogen fixation rate equals $177 \pm 8 \text{ T g N yr}^{-1}$ (5). This biological N_2 fixation is carried out mainly by diazotrophic cyanobacteria in the ocean (2). Therefore, marine cyanobacteria play an important role in nitrogen cycle as well as in carbon cycle.

1. General introduction

Although diverse taxa of diazotrophic cyanobacteria have been observed in the marine ecosystems, the early studies have focused on the filamentous cyanobacterium *Trichodesmium* because of its ability to form large surface aggregations which are enough to form a red tide even in oligotrophic open oceans (6). This blooming is observed even in satellite image (7). It is suggested that *Trichodesmium* is a major source of new nitrogen in the North Atlantic Ocean with annual nitrogen input of at least 1.6×10^{12} mol N (8). However, *Trichodesmium* accounted for only a quarter to one-half of N₂ fixation rates in various ocean basins (4). This disparity is responsible for the presence of other diazotrophic organisms. Heterocyst-forming cyanobacteria such as *Richelia* spp. and *Calothrix* spp. are also frequently observed in the open oceans and have symbiotic relationships with oceanic diatoms, including *Hemiaulus*, *Rhizosolenia* and *Chaetoceros* (2, 9). Heterocysts are specialized cells for nitrogen fixation, and thereby considered to provide fixed nitrogen to the diatom partners. These cyanobacterial symbionts are distributed in several oceans and the expression of their *nifH* genes are also observed (10, 11).

These filamentous diazotrophic cyanobacteria are easily identified from morphology. However, unicellular cyanobacteria are difficult to determine whether they have the ability of nitrogen fixation or not based on their morphology, and their diversity was a longstanding research question in the open ocean (2). Recent studies have revealed a substantial contribution of the small size fraction of plankton (< 10 μ m) including unicellular diazotrophic cyanobacteria of which abundances and nitrogen

fixation rates can be equal to or even greater than those of *Trichodesmium* (12–14). A genetic approach targeting the nitrogenase reductase gene (*nifH*) has revealed the diversity of diazotrophic cyanobacteria in the ocean (15). This study identified previously unrecognized unicellular diazotrophic cyanobacteria (UCYN). The UCYN are phylogenetically sub-divided into three groups based on amino acid sequences of *nifH* genes. UCYN-A is an uncultivated cyanobacterium and lacks key metabolic pathways for cyanobacteria, such as oxygenic photosystem II, ribulose biphosphate carboxylase (Rubisco), and tricarboxylic acid cycle (16, 17). These characteristics suggest their photoheterotrophic lifestyle and the possible partner organisms which supply carbon sources to UCYN-A (16, 17). A recent work has showed the symbiotic relationships between UCYN-A and an eukaryotic prymnesiophyte cell: UCYN-A provides fixed nitrogen to the host and the host provides fixed carbon to UCYN-A in return (18). The potential endosymbiotic relationship between UCYN-A and prymnesiophyte *Braarudosphaera bigelowii* was observed in coastal areas of Japan (19). UCYN-B (type strain *Crocospaera watsonii* WH8501) is a photoautotrophic diazotrophic cyanobacteria and possesses phycoerythrin as one of photosynthetic pigments. *C. watsonii* are divided into two different phenotype: larger cells that produce large amounts of exopolysaccharide and smaller cells that do not produce noticeable exopolysaccharide (20). UCYN-C was first identified in the North Atlantic Ocean (21). The *nifH* genes of UCYN-C includes *Cyanothece* sp., *Gloeotheca* sp., and *Gloeocapsa* sp., which were mainly established from coastal areas (e.g., intertidal zone, mangrove

1. General introduction

forest), with the exception of TW3 isolated from the open ocean (22, 23). A UCYN-C strain, *Cyanothece* sp. ATCC51142, is received much attention because of the capacity to generate high amounts of hydrogen under aerobic conditions (24).

These diazotrophic cyanobacteria has been intensively studied in the open ocean (25–27). Meanwhile, some studies have revealed the distribution of filamentous and unicellular cyanobacteria in coastal regions of Mediterranean Sea and Chesapeake Bay (28, 29). However, the diversity and distribution of diazotrophic cyanobacteria still remains obscure in coastal seas. Unexplored coastal areas lead me to expect that the communities of diazotrophic cyanobacteria are distinct to those of the open oceans. The insight into diazotrophic cyanobacteria would help the understanding to the ecological roles of them in coastal seas.

Thus, I performed an ecological study of diazotrophic cyanobacteria in two unexplored sea areas, the Japan Sea and the Seto Inland Sea. In particular, I revealed that the seasonal dynamics of diazotrophic cyanobacteria in the Seto Inland Sea. This study provided insight into the distribution and diversity of unicellular diazotrophic cyanobacteria in a coastal sea. Finally I succeeded in isolation of unicellular diazotrophic cyanobacteria from the Seto Inland Sea and characterized their genomic aspects.

Chapter 2

Ecological studies on diazotrophic cyanobacteria in coastal seas

Introduction

Diazotrophic cyanobacteria are important organisms in the ocean, affecting not only to primary production but also to nitrogen input (2). Although many studies have revealed the diversity and distribution of diazotrophic cyanobacteria in the open ocean (e.g. the Pacific Ocean and the Atlantic Ocean) (25, 27, 30, 31), little is known about them in a coastal sea, where is nitrogen-rich condition compared to the open ocean. Thus, to expand our understanding of diazotrophic cyanobacteria in coastal seas, I focused on two unexplored sea areas, the Japan Sea and the Seto Inland Sea.

In chapter 2-1, I investigated the diversity and vertical distribution of diazotrophic cyanobacteria in the Japan Sea. This first assessment have revealed the unique diazotroph community with the dominance of heterotrophic diazotrophs in the Japan Sea.

In chapter 2-2, I examined the seasonal dynamics of unicellular diazotrophic cyanobacteria in the Seto Inland Sea to provide insight into their characteristics in a coastal sea.

Chapter 2-1

Diversity and vertical distribution of diazotrophic cyanobacteria in the Japan Sea

Interoduction

Marine cyanobacteria, especially two genera, *Prochlorococcus* and *Synechococcus*, significantly contribute to 32-80 % of the primary productivity in various oceanic regions (32–34). Some other cyanobacteria retain ability for N₂ fixation which is the process of reducing N₂ gas to biologically available ammonium. This biological N₂ fixation is carried out by several species of bacteria and archaea, but mainly by diazotrophic cyanobacteria in the ocean (2). Therefore, marine cyanobacteria play an important role in nitrogen cycle as well as in carbon cycle.

Among marine diazotrophic cyanobacteria, filamentous nonheterocystous *Trichodesmium* spp. are the most abundant diazotrophs in the ocean. *Trichodesmium* spp. are observed throughout the tropical and subtropical Atlantic, Pacific, Indian oceans, and East and South China seas (6, 35). In North Atlantic Ocean, their contribution to nitrogen inputs can be as great or greater than nitrate advection from deep sea water (8). In addition, heterocystous cyanobacterial diatom symbionts such as *Richelia* spp. and *Calothrix* spp. have also been considered to make a significant contribution to N₂ fixation in North Atlantic Ocean and North Pacific Ocean (36, 37). They are detected in tropical and subtropical regions in North

Pacific Ocean and North Atlantic Ocean (38, 39). Recently, some unicellular cyanobacteria have been recognized as important diazotrophs in the ocean (13, 14, 40). The unicellular diazotrophic cyanobacteria (UCYN) are phylogenetically sub-divided into three groups, UCYN-A, B and C based on amino acid sequences of NifH (dinitrogenase reductase), a subunit of the nitrogenase complex enzyme. UCYN-A is an uncultivated cyanobacterium lacking oxygenic photosystem II, ribulose biphosphate carboxylase (Rubisco), and tricarboxylic acid cycle, therefore UCYN-A is considered to be photoheterotroph (17). UCYN-A is shown to be more abundant than other two groups with broad latitudinal range in the North and South Pacific Oceans (13, 38). Unlike UCYN-A, UCYN-B including *Crocospaera watsonii* WH8501 shows a patchy distribution in the ocean, and is detected in South Pacific, Eastern Atlantic, and North Pacific (13, 38, 41). Compared to the other two unicellular diazotrophic cyanobacterial groups, UCYN-C has less abundance in the open ocean (27, 42).

The Japan Sea is a semi-enclosed marginal sea in the northwest Pacific Ocean surrounded by the Japanese islands and Asian continent. The Japan Sea is often referred to as “the miniature ocean” because the situation, which the course of the western branch of the Tsushima Current and the cold water generated in north and the warm water in east, is similar to those of the global ocean (43). Its water exchange is limited in the upper layer because the Japan Sea is connected to adjacent seas with the shallow straits (43). The Japan Sea harbors a water mass, which called “Japan Sea Proper Water” underlies the thermocline shallower than 200 m (44). This is

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characterized with water temperature lower than 1 °C (45). Although many studies have examined the diversity of cyanobacteria in various oceanic regions, less is known about the diversity of cyanobacteria, especially diazotrophic cyanobacteria in the unique sea area, the Japan Sea.

In this study, I examined the diversity of cyanobacteria and diazotrophic cyanobacteria vertically in the Japan Sea using polymerase chain reaction (PCR) approach targeting cyanobacterial 16S rRNA and *nifH* genes.

Materials and Methods

Seawater collection

Sampling was conducted during August 2011 on board the R/V Tajima at Stn. 2 (35°50.2N, 134°19.8E, 275 m depth) and Stn. 7 (37°20.2N, 134°19.8E, 2591 m depth) in the Japan Sea (Fig. 2-1-1). Seawater was collected in 2 L Niskin bottles mounted on a CTD (SBE-9 plus, Sea Bird Electronics) rosette from 0, 10, 30, 75, 100 m, and deep chlorophyll maximum (DCM, near 50 m). I measured profiles of temperature, salinity, and concentrations of chlorophyll a using the CTD.

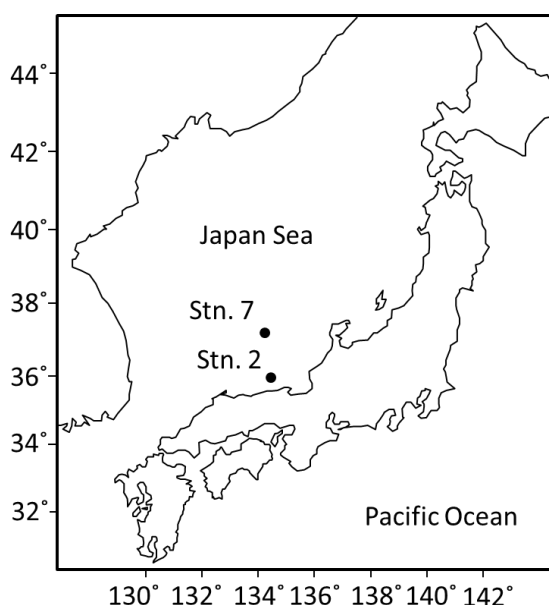


Fig. 2-1-1. Sampling stations in the Japan Sea.

DNA extraction

Each 300 ml sample was filtered through 0.2 μm pore-size polycarbonate filters (ADVANTEC, Tokyo, Japan), and then filters were wrapped in aluminum foil and were stored at 4 °C until stored at -80 °C in laboratory. Genomic DNA was extracted from the 0.2 μm filters using ISOIL kit (Nippon Gene, Tokyo, Japan).

PCR amplification

The cyanobacterial 16S rRNA gene sequences were amplified by PCR using primers described by Nübel et al. (46) (Table 2-1-1). The genomic DNA sample (1 μL) recovered from each station was added to each reaction mixture. The reaction mixtures were made with 5 μL of 10 $\times$ PCR Buffer for Blend Taq (TOYOBO, Osaka, Japan), 5 μL of dNTPs (2 mM each), 0.4 μL of forward primer, 0.2 μL of each reverse primer, 0.5 μL of Blend Taq (2.5 U μL^{-1} ; TOYOBO), and 37.7 μL of sterile Milli-Q water. After the initial denaturation

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Table 2-1-1. 16S rRNA and *nifH* primers used in this study.

Primer	Target site	Sequence(5'-3')	Reference
CYA359F	16S rRNA of cyanobacteria	GGG GAA TYT TCC GCAATG GG	(46)
CYA781R(a)	16S rRNA of cyanobacteria	GAC TAC TGG GGTATC TAA TCC CAT T	(46)
CYA781(b)	16S rRNA of cyanobacteria	GAC TAC AGG GGTATC TAA TCC CTT T	(46)
nifH1	<i>nifH</i> of prokaryotes	TGY GAY CCNAAR GCN GA	(47)
nifH2	<i>nifH</i> of prokaryotes	AND GCC ATC ATY TCN CC	(47)
nifH3	<i>nifH</i> of prokaryotes	ATR TTR TTN GCN GCR TA	(47)
nifH4	<i>nifH</i> of prokaryotes	TTY TAY GGNAAR GGN GG	(47)

step (2 min at 94 °C), the samples were amplified for 30 cycles (30 s at 94 °C, 30 s at 60 °C, and 1min at 72 °C). To amplify *nifH* genes, I performed nested PCR using the degenerate oligonucleotide primer pair described by Zehr and Turner (47) (Table 2-1-1). The reaction mixtures were made with 5 µL of 10× PCR Buffer for Blend Taq (TOYOBO), 5 µL of dNTPs (2 mM each), 2 µL of nifH3, 2 µL of nifH4, 0.5 µL of Blend Taq (2.5 U µL⁻¹; TOYOBO), and 34.5 µL of sterile Milli-Q water. The first round of PCR consisted of an initial 5 min denaturation at 95 °C, then 30 cycles of 1 min at 94 °C, 1 min at 50 °C and 1 min at 72 °C, and final elongation of 7 min at 72 °C. Of this reaction product, 1 µL was transferred into a second 50 µl reaction mixture containing the same reagent mixture, and primers were replaced with nifH1 and nifH2. The second round PCR consisted of an initial 5 min denaturation at 95 °C, then 30 cycles of 1 min at 94 °C, 1 min at 57 °C and 1min at 72 °C, and final elongation of 7 min at 72 °C.

Cloning, sequencing, and phylogenetic analysis

The amplified fragments were purified and cloned into pTac-1 vector (TOYOBO). I sequenced a total of 107 and 36 clones containing

cyanobacterial 16S rRNA gene sequences and *nifH* inserts, respectively. Basic Local Alignment Search Tool (BLAST) comparisons were used to find the sequence closely related to those amplified from samples with the National Center for Biotechnology Information (NCBI) database (48). The NifH phylogenetic tree based on the deduced amino acid sequences was constructed by the neighbour-joining method with Poisson correction using MEGA version 5 (49).

Nucleotide sequence accession numbers

The 16S rRNA gene sequences and *nifH* sequences reported in this paper have been deposited in the DDBJ under accession numbers AB727363-AB727505.

Results

The vertical thermal stratification was observed in surface water (0-30 m) of both Stn. 2 and Stn. 7 (Fig. 2-1-2). The water temperature in surface was relatively constant (25-26 °C) in Stn. 2 and Stn. 7, and water temperature below 30 m depth declined gradually to 8.2 and 4.5 °C at 200 m depth, respectively (Fig. 2-1-2). Salinity of surface water in Stn. 2 was around 32.7 psu, which was a bit lower than that in Stn. 7 (33.3 psu) (Fig. 2-1-2). This lower salinity of Stn. 2 was considered to be derived from freshwater input from the terrestrial area. In each station, the concentrations of chlorophyll a, the maximum of which was observed around

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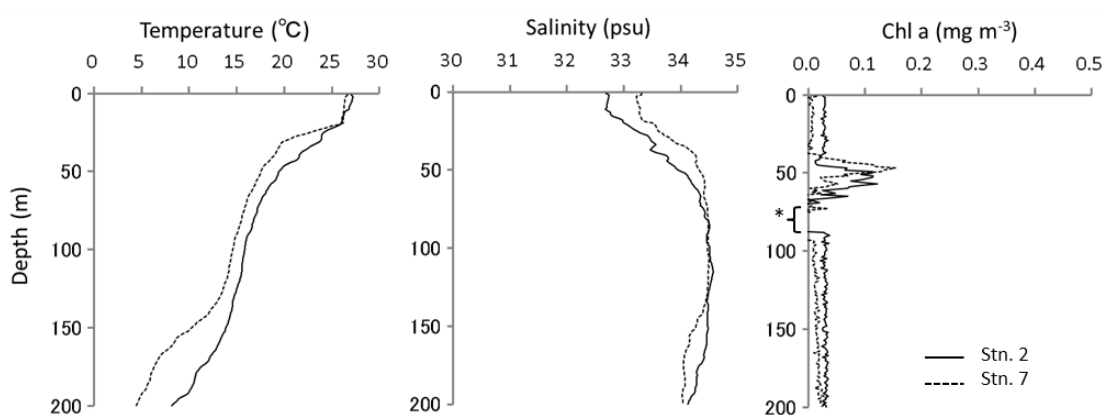


Figure 2-1-2. Vertical distributions (0-200 m) of temperature, salinity, chlorophyll *a* concentrations (mg m⁻³) at Stn. 2 (solid line) and 7 (dotted line) in August 2011. Asterisk indicates that fluorescent intensity was below zero.

50 m depth, were extremely low (< 0.2 μ M) compared with other oceanic regions (50).

I obtained a total of 107 clones of cyanobacterial 16S rRNA gene sequences from 12 samples in the Japan Sea (Table 2-1-2). No sequence of diazotrophic cyanobacteria was obtained in this clone library. Of these, 96 sequences (90 %) and 3 sequences (3 %) were assignable (~99 % nucleotide similarity) to *Synechococcus* sub-cluster 5.1, and *Prochlorococcus* HL-(high-light) (II), respectively. In each station and depth, *Synechococcus* sub-cluster 5.1 dominated the cyanobacterial community. Of the 96 sequences assignable to *Synechococcus* sub-cluster 5.1, 6 sequences showed a 100% identity with *Synechococcus* KORDI-19 which was isolated from the Japan Sea (51). Residual 8 sequences (8 %) were assignable to *Synechococcus* sub-cluster 5.3. This type of sequences was recovered from samples ranging from 10-75 m depth in each station. In general, *Prochlorococcus* has two

Table 2-1-2. Summary of cyanobacterial 16S rRNA genes obtained from each microbial group

Station	Depth	Number of Clones			Total
		<i>Synechococcus</i> sub-cluster 5.1	<i>Synechococcus</i> sub-cluster 5.3	<i>Prochlorococcus</i> HL(II)	
2	0m	7		2	9
	10m	9	1		10
	30m	9	1		10
	DCM	8	2		10
	75m	8			8
	100m	9	1		10
7	0m	9		1	10
	10m	10			10
	30m	9	1		10
	DCM	9	1		10
	75m	9	1		10
	100m				-

distinct ecotypes, the high-light (HL) and low-light (LL) adapted ecotypes (52). The sequences similar to a subgroup of the HL ecotype, *Prochlorococcus* HL (II) were obtained only from surface water (0 m), which is comparable with the previous reports that the HL-adapted ecotype generally distributes the upper regions of the euphotic zone (52–54). No sequences assignable to the LL-*Prochlorococcus* were obtained, suggesting low abundance of LL-*Prochlorococcus* in the subsurface areas (Table 2-1-2).

I performed the *nifH*-targeting PCR amplification against the same 12 samples in the Japan Sea (Table 2-1-3). I obtained a total of 36 clones of *nifH*. However, no PCR products were detected from DNA samples of Stn. 2 at DCM, 75 m, 100 m, and Stn. 7 at 100 m, suggesting low abundance of diazotrophs in these areas. On the basis of phylogenetic analysis, the *nifH*

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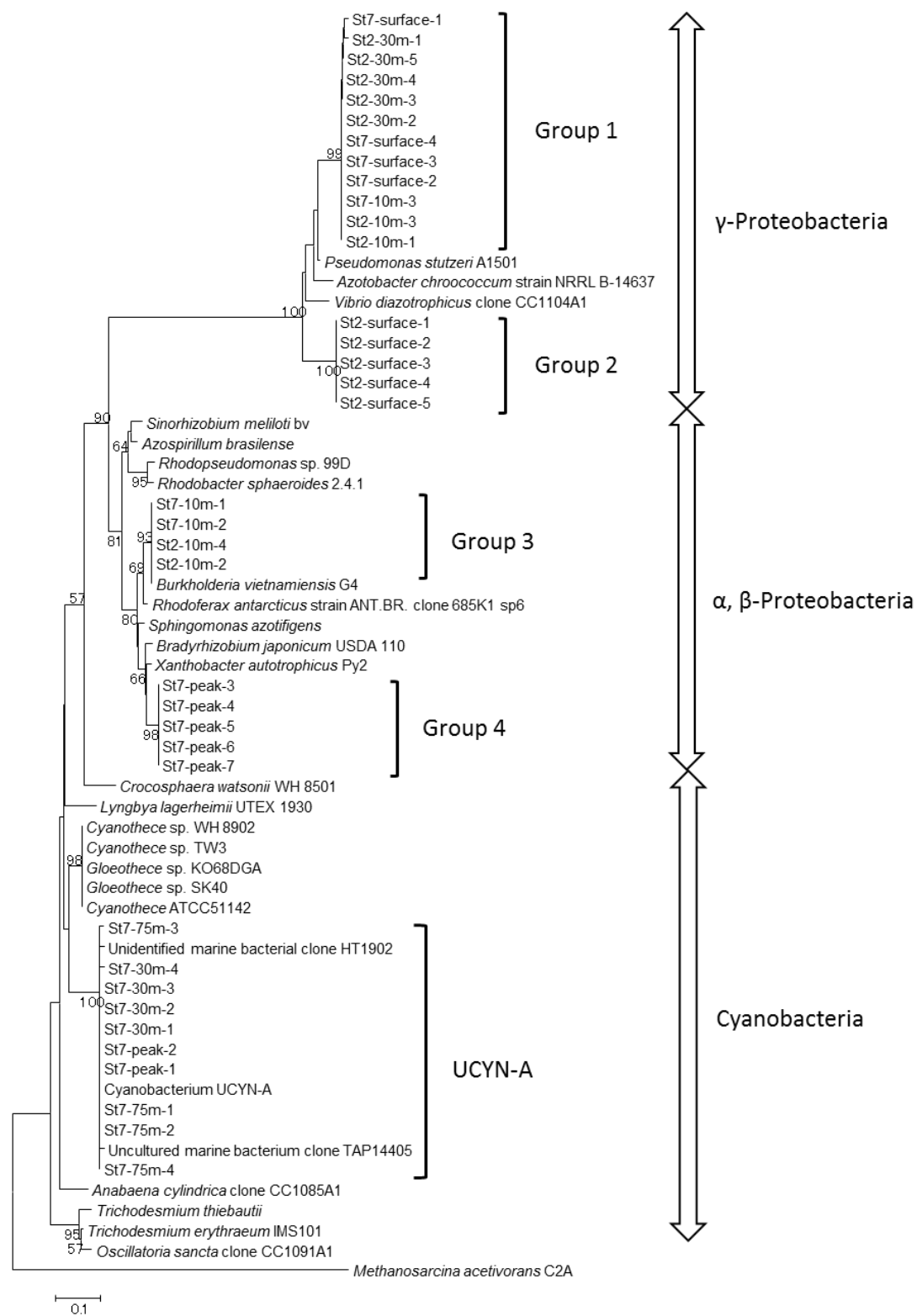
sequences were divided into five groups (Fig. 2-1-3). Sequences assigned to cyanobacteria showed a similarity to the sequences within UCYN-A, and accounted for 28 % of the *nifH* clone library. All sequences assignable to UCYN-A were recovered from Stn. 7 at 30 m, DCM, and 75 m. This result is consistent with the observations that the peak abundances of UCYN-A was observed around 50 m depth in the Pacific Ocean (13). Other sequences (72 %) were grouped within α -, β -, and γ -Proteobacteria. Sequences assigned as α -Proteobacteria (Group 4) showed 100% amino acid identity with sequences recovered from Baltic Sea and Chesapeake Bay (Fig. 2-1-3). In β -Proteobacteria, the sequences (Group 3) were recovered from Stn. 2 at 10 m and Stn. 7 at 10m with 100% amino acid identity to *Burkholderia vietnamensis* G4 and sequences recovered from North Pacific Ocean. *Burkholderia vietnamensis* was also observed in reservoirs and agricultural settings (55). Group 1 belonging to γ -Proteobacteria includes sequences which were completely identical to protein sequences recovered from Atlantic Ocean, South China Sea, and Mediterranean Sea.

Table 2-1-3. Summary of *nifH* genes obtained from each microbial group.

Station	Depth	Number of Clones			Total
		Cyanobacteria UCYN-A	α,β -Proteobacteria	γ -Proteobacteria	
2	0m			5	5
	10m		2	2	4
	30m			5	5
	DCM				-
	75m				-
	100m				-
7	0m			4	4
	10m		2	1	3
	30m	4			4
	DCM	2	5		7
	75m	4			4
	100m				-

Figure 2-1-3. Phylogenetic tree for *nifH*-deduced amino acid sequences from each sample constructed using the neighbor-joining method. Bootstrap values greater than 50% for 1000 replicates are indicated at respective nodes. Database entries for sequences are as follows: *Azotobacter chroococcum* strain NRRL B-14637 (AAR11505); *Pseudomonas stutzeri* A1501 (YP_001171863); *Vibrio diazotrophicus* clone CC1104A1 (AAP48983); *Sinorhizobium meliloti* bv (ABC67302); *Azospirillum brasilense* (P17303); *Rhodopseudomonas* sp. 99D (BAC07290); *Rhodobacter sphaeroides* 2.4.1 (YP_353614); *Burkholderia vietnamiensis* G4 (YP_001115195); *Rhodferax antarcticus* strain ANT.BR. clone 685K1_sp6 (ABI14697); *Sphingomonas azotifigens* (BAE71134); *Bradyrhizobium japonicum* USDA 110 (BAC47034); *Xanthobacter autotrophicus* Py2 (YP_001415004); *Crocospaera watsonii* WH 8501 (AAP48976); *Anabaena cylindrica* clone CC1085A1 (AAP48968); *Lyngbya lagerheimii* UTEX 1930 (AAA19029); *Cyanothece* sp. ATCC51142 (AAB61408); *Gloeotheca* sp. SK40 (BAF51659); *Gloeotheca* sp. KO68DGA (BAF51663); *Cyanothece* sp. WH 8902 (AAV28035); *Cyanothece* sp. TW3 (BAJ21365); Unidentified marine bacterial clone HT1902 (AAK83129); Uncultured marine bacterium clone TAP14405 (AAY60051); Cyanobacterium UCYN-A (ACJ53724); *Oscillatoria sancta* clone CC1091A1 (AAP48970); *Trichodesmium thiebautii* (AAA77023); *Trichodesmium erythraeum* IMS101 (AAA77022); *Methanosarcina acetivorans* C2A (NP_616144)

2-1. Diazotrophic cyanobacteria in the Japan Sea



Discussion

Marine *Synechococcus* is phylogenetically subdivided into three major subclusters, 5.1, 5.2, and 5.3. In the open ocean, most *Synechococcus* belong to sub-cluster 5.1 (56). In general, *Synechococcus* are more abundant in eutrophic coastal and mesotrophic open ocean waters with mixing conditions in water column, while *Prochlorococcus* is abundant in oligotrophic waters with stratified conditions (33, 57–59). Although surface water of the Japan Sea is extremely nutrient-limited environment (e.g. under-detection limit for PO_4 , NO_3 , NO_2) due to the water stratification and the absence of large rivers that deliver continental particles (60), the data showed the dominance of *Synechococcus* in the cyanobacterial community. This discrepancy suggests unknown factors controlling their distribution in the Japan Sea, which will be the subject of future work.

Synechococcus sub-cluster 5.3, which was recently established lineage, accounted for 8 % of the clone library with vertical distribution in each station (56, 61, 62) (Table 2). Huang et al. (56) report that their distribution may be restricted to certain oceanic regions because few sequences assignable to *Synechococcus* sub-cluster 5.3 were detected in the GOS database. However, their distribution and characteristics is not well understood.

No sequences belonging to diazotrophic cyanobacteria were detected in the cyanobacterial 16S rRNA gene clone library. It is not surprising because the abundance of diazotrophic cyanobacteria is two to three orders of magnitude lower than that of *Synechococcus* or *Prochlorococcus* in most

2-1. Diazotrophic cyanobacteria in the Japan Sea

oceanic regions (27, 38, 63, 64). In *nifH* clone library, sequences belonging to UCYN-A were obtained from Stn. 7 at 30m, DCM, 75m depths. Due to the elevated phytoplankton biomass around DCM, the carbon source may be more readily available for UCYN-A lacking the carbon fixation enzyme Rubisco. Sequences related to other unicellular cyanobacteria (i.e. UCYN-B and C) were not detected in *nifH* clone library. Moisander et al. (63) suggest the possibility of limiting the growth of UCYN-A and B by differential grazing pressure due to the smaller cell size of UCYN-A compared to UCYN-B. However, the factors of limiting the distribution of the unicellular diazotrophic cyanobacterial groups have not unraveled yet well.

Sequences of filamentous cyanobacteria were also not detected in clone libraries (Table 2-1-2). Among environmental parameters, iron and phosphorus are often referred to limit the development of diazotrophic cyanobacteria because they require a lot of iron as cofactor of nitrogenase and phosphorus for energy generation during diazotrophic growth (35). As unicellular cyanobacteria have high surface/volume ratio comparing to filamentous cyanobacteria, they may efficiently grow in oligotrophic environment because phosphate uptake efficiency is dependent upon cell size (65). Therefore, the presence of UCYN-A and no detection of filamentous cyanobacteria may be due to the extremely nutrient poor condition in the Japan Sea with iron depletion (60).

About two-thirds of *nifH* clones were derived from proteobacteria, and especially sequences most related to γ -Proteobacteria were abundant. Although the sequences belonging to γ -Proteobacteria are detected in various

oceans such as North Pacific Ocean and North Atlantic Ocean, it is still unknown what extent these heterotrophic bacteria contribute to the net nitrogen input in the ocean (66, 67). However, despite the low N_2 fixation rates, heterotrophic bacteria dominated the diazotrophic community in the South Pacific with significant nitrogen input into the ocean (68). Therefore, given the absence of other diazotrophic cyanobacteria, heterotrophic bacteria may account for substantial fraction of nitrogen input through N_2 fixation in the Japan Sea.

In this chapter, I revealed the diazotrophic community in the Japan Sea. The next chapter explores the distribution and diversity of unicellular diazotrophic cyanobacteria in a coastal sea, the Seto Inland Sea.

Chapter 2-2

Diversity and distribution of unicellular diazotrophic cyanobacteria in the Seto Inland Sea

Introduction

Biological nitrogen fixation is a key process in the nitrogen biogeochemistry in the ocean. The revised $^{15}\text{N}_2$ tracer method revealed that the global marine nitrogen fixation rate equals $177 \pm 8 \text{ T g N yr}^{-1}$ (5). In early studies on nitrogen fixation in the open ocean, the filamentous cyanobacteria *Trichodesmium* was thought to be a major nitrogen fixer (6); however, recent studies have revealed a wide distribution and a substantial contribution of unicellular diazotrophic cyanobacteria, of which the abundance and nitrogen fixation rates can be equal to or even greater than those of *Trichodesmium* (12, 13, 40). Unicellular diazotrophic cyanobacteria are systematically divided into UCYN-A, B and C based on the phylogeny of the *nifH* genes (nitrogen fixation enzyme dinitrogenase reductase) (2). UCYN-A is widely distributed throughout tropical and sub-tropical oligotrophic oceans with a greater abundance than the other unicellular cyanobacteria (27, 38). UCYN-A is an uncultured pico-size cyanobacterium that lacks the carbon fixation enzyme Rubisco and photosystem II and establishes a symbiotic relationship with eukaryotic algae (17). UCYN-B, type strain *Crocospaera watsonii*, is also distributed in various oceanic regions (69, 70). In contrast to UCYN-A and B, less information is available about the distribution of

UCYN-C in the open ocean; however, UCYN-C was detected in the subtropical Atlantic Ocean, and the highest abundance was observed in the sampling stations influenced by the Amazon River plume with 1.4×10^5 *nifH* gene copies L⁻¹ (31, 39, 66).

While importance of these diazotrophic cyanobacteria is noted in the open ocean due to the nitrogen-poor conditions, these cyanobacteria also distribute in sea areas with relatively nitrogen-rich conditions, such as coastal areas. The potential endosymbiotic relationship between UCYN-A and premnesiophyte *Braarudosphaera bigelowii* was demonstrated in coastal areas of Japan (19). Some studies have revealed the distribution of filamentous and unicellular cyanobacteria and active transcription of the *nifH* genes in the Mediterranean Sea and Chesapeake Bay (28, 29, 71–73), indicating the potential importance of unicellular diazotrophic cyanobacteria on nitrogen input.

The Seto Inland Sea (Fig. 2-2-1) is one of the largest semi-enclosed seas in Japan, with a size of approximately 23,300 km² and an average water depth of approximately 38 m, in which nutrient-rich freshwater inputs mainly through rivers. On the other hand, this coastal area is connected to the Pacific Ocean through Kii and Bungo Channels, and extremely oligotrophic surface water from the open ocean often affects the oceanographic conditions of the Seto Inland Sea (74, 75). Seasonal dynamics drastically change the environment parameters, with low nutrient conditions during the summer and high nutrient conditions during the winter season.

2-2. Diazotrophic cyanobacteria in the Seto Inland Sea

In this study, I characterized the diversity and distribution of unicellular diazotrophic cyanobacteria and their seasonal dynamics in the eastern Seto Inland Sea based on molecular biological methods over the course of two years. I used two methods (i) to reveal the diversity of unicellular diazotrophic cyanobacteria by constructing clone libraries targeting the *nifH* and their 16S rRNA genes; and (ii) to quantify their abundances using a quantitative PCR (qPCR) approach targeting the *nifH* genes.

Materials and Methods

Seawater collection

Figure 2-2-1 shows the five sampling points in this study. Sampling was conducted during monthly research cruises of the research vessel *Shin-Hyogo* (Hyogo Prefecture, Japan) during April 2012 – January 2014. Seawater was collected in 2 L HDPE (High Density Polyethylene) bottles from the surface and at a depth of 10 m using a Kitahara water sampler. The samples from Osaka Bay and the Kii Channel (Stn. A5, A12, K4, and K5) were collected during the even months (April, June, August, October, December, and February) and those from the Harima Nada (Stn. H3) were collected during the odd months (May, July, September, November, January, and March). The seawater samples from Stn. K5 and K4 were collected from April 2012 to February 2013 and from April 2013 to December 2013, respectively. Stn. A12 is located in the inner part of Osaka Bay. Stn. K4 and

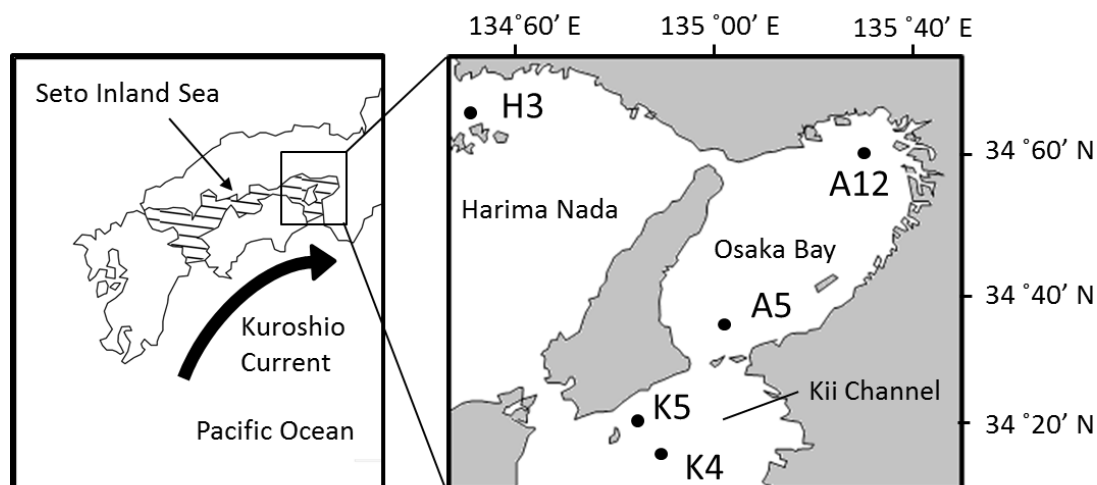


Figure 2-2-1. Sampling stations in Osaka Bay, Kii channel, and Harima Nada.

K5 are situated in the Kii Channel. Stn. A5 is located between Stn. A12 and K4. Stn. H3 is located in Harima Nada.

Biogeochemical analysis

Vertical profiles of temperature and salinity were measured using a CTD (Rinko-Profiler, JFE Advantech Co., Ltd, Hyogo, Japan). The water samples for nutrients (inorganic nitrogen (DIN; $\text{NO}_3 + \text{NO}_2 + \text{NH}_4$), phosphate (PO_4) and silicate (SiO_2)) were filtered through a GA 200 filter (Advantec Toyo, Tokyo, Japan). Concentrations of dissolved inorganic nitrogen (DIN; $\text{NO}_3 + \text{NO}_2 + \text{NH}_4$), phosphate (PO_4), and silicate (SiO_2) were analyzed using an automated continuous segmented flow analyzer (Quatro, BL TEC K.K., Osaka, Japan) within 2 days after sampling. The water samples for Chlorophyll a (Chl-a) were filtered through a GA 200 filter (Advantec Toyo, Tokyo, Japan), and then filters were stored at $-30\text{ }^\circ\text{C}$ until

analysis in laboratory. Chlorophyll a (Chl-a) concentration was determined with a fluorometer (10-AU, Turner Designs, CA, USA) after the seawater samples were filtrated and extracted with 90% acetone according to the protocols of Manabe and Tanda (76).

DNA extraction

Three hundred mL of each sample was filtered through 47-mm-diameter 0.2 μ m pore-size polycarbonate filters (ADVANTEC, Tokyo, Japan) under pressure less than 0.03 MPa, and then the filters were wrapped in aluminum foil and stored at -80 °C. Genomic DNA was extracted from the filters collected during April–September 2012 using ISOIL kit (Nippon Gene, Tokyo, Japan) according to the manufacturer's instructions. For the samples collected during October 2012 – January 2014, the extraction of genomic DNA was followed by the protocol of Church et al. (26) (referred to as XS method). This method are known to be effective for DNA extraction from cyanobacterial cells (77, 78). Therefore I used this methods for DNA extraction of samples collected during October 2012–January 2014. (26). Briefly, the filters were placed in 1.5 mL microcentrifuge tube and then added 500 μ L TE buffer and 500 μ L of potassium xanthogenate buffer (2% w/v potassium ethyl xanthogenate; 200mM Tris-HCl, pH 7.4; 2 mM EDTA, pH 8.0; 2% sodium dodecylsulfate; 1.6 M ammonium acetate). The tubes were incubated at 70 °C for 120 min, followed by incubation on ice for 30 min. After centrifugation, DNAs were obtained from the supernatant fractions with 2-propanol precipitation. The purity of extracted DNAs were evaluated

at the optical density of 230, 260, and 280 nm using spectrophotometry (Ultrospec 2100 pro, Amersham BioScience, Tokyo, Japan). XS method provided higher-purity DNA than ISOIL kit. There was a possibility that cyanobacterial abundance was underestimated during April – September 2012 due to template DNA extracted by a commercial kit. Therefore, the qPCR results obtained using the DNAs extracted by these methods were separately analyzed.

PCR amplification

To amplify the *nifH* genes, I performed nested PCR using the degenerate oligonucleotide primer pair described by Zehr and Turner (47) (Table 1). The primers nifH3/nifH4 and nifH1/nifH2 were used for the first and second rounds of PCR. The reaction mixtures contained 5 μ L of 10 $\times$ Ex Taq Buffer (Takara, Japan), 4 μ L of dNTPs (2 mM each), 2 μ L of forward primer (1 μ M), 2 μ L of each reverse primer (1 μ M), 0.25 μ L of ExTaq (2.5 U μ L⁻¹; TOYOBO), and 35.75 μ L of sterile Milli-Q water. The first round of PCR consisted of an initial 5 min denaturation at 95 °C, then 30 cycles of 1 min at 94 °C, 1 min at 50 °C and 1 min at 72 °C, and a final elongation of 7 min at 72 °C. Of this reaction product, 1 μ L was transferred into a second 50 μ L reaction mixture containing the same reagent mixture. The second round PCR consisted of an initial 5 min denaturation at 95 °C, then 30 cycles of 1 min at 94 °C, 1 min at 57 °C and 1 min at 72 °C, and a final elongation of 7 min at 72 °C.

The 16S rRNA gene sequences of unicellular diazotrophic

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cyanobacteria were amplified by PCR using the primers described by Mazard et al. (79) and Nübel et al. (46) (Table 2-2-1). PCR amplification was performed with reaction mixtures containing 5 μ L of 10 $\times$ Ex Taq Buffer (Takara, Otsu, Japan), 4 μ L of dNTPs (2 mM each), 1 μ L of forward primer (0.2 μ M), 1 μ L of reverse primer (0.2 μ M), 0.25 μ L of ExTaq (2.5 U μ L⁻¹; TOYOBO), and 37.75 μ L of sterile Milli-Q water. After the initial denaturation step (5 min at 95 °C), the samples were amplified for 30 cycles (1 min at 95 °C, 30 s at 57 °C, and 40 s at 72 °C), and a final elongation of 6 min at 72 °C.

Table 2-2-1. Primers used for clone analysis

Name	Target site	Oligonucleotide sequence(5'-3')	Reference	Direction
nifH1	<i>nifH</i> genes	TGYGAYCCNAARGCNGA	(47)	Forward
nifH2	<i>nifH</i> genes	ADNGCCATCATYTCNCC	(47)	Reverse
nifH3	<i>nifH</i> genes	ATRTTTRTTNGCNGCRTA	(47)	Forward
nifH4	<i>nifH</i> genes	TTYTAYGGNAARGGNGG	(47)	Reverse
CYA359F	16S rDNA of cyanobacteria	GGGGAATYTTCCGCAATGGG	(46)	Forward
Nitro821	16S rDNA of UCYN	CAAGCCACACCTAGTTTC	(79)	Reverse

Cloning, sequencing and phylogenetic analysis

The amplified fragments were purified and cloned into pTac-1 vector (TOYOBO). Sequence analysis was performed with a model 3130 genetic analyzer (Applied Biosystems, Foster City, CA). I sequenced a total of 127 and 44 clones containing *nifH* inserts and unicellular diazotrophic cyanobacterial 16S rRNA gene sequences, respectively. The sequences obtained were aligned using MEGA5 (49); the primer sequences were removed from all sequences. The phylogenetic tree of *nifH* genes and

unicellular diazotrophic cyanobacterial 16S rRNA gene were constructed by the neighbour-joining method with Poisson correction using MEGA version 5 (49).

qPCR amplification

I designed new primers targeting the *nifH* genes of UCYN-A and C using Primer3Plus software (<http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi/>) (Table 2-2-2). The new primers for UCYN-C were designed based on the *nifH* sequences from both uncultured UCYN-C and cultured UCYN-C (e.g., *Cyanothece* ATCC51142, TW3). Specificity of these primers were checked against the NCBI nr database with BLAST search. This search gave no sequence matches of the primers except for the targeted phylotypes. The amplification efficiencies and standard curves of these primers were analyzed by qPCR reactions with 5 µL of serial dilutions of each target PCR product added to 25 µL mixtures (Fig. 2-2-2). I used least-squares linear regression analyses of Ct versus $\log_{10}$ *nifH* gene copies for quantifying the target genes in the DNA samples. The least-squares linear regressions were $Ct = -3.498 (\log_{10} \text{ *nifH* gene copies}) + 39.33$ ($r^2=0.973$) and $Ct = -3.300 (\log_{10} \text{ *nifH* gene copies}) + 40.24$ ($r^2=0.981$) (Fig. 2). The detection limit was 10^2 *nifH* gene copies. The range of target gene concentrations was from 10^2 to 10^7 *nifH* gene copies. Melting curves of all of the environmental real-time PCR products had a peak at approximately 82 and 81 °C corresponding to the melting temperature of UCYN-A and C PCR products, showing no non-specific amplification (data not shown).

The qPCRs with the newly designed primers were run on a Dice thermal cycler (Takara, Otsu, Japan). The qPCR reactions were carried out with a minimum of duplicate reactions (mostly triplicates) per each sampling point, in 25 μL reaction volumes containing 5 μL of environmental DNA sample, 1 $\times$ SYBR Premix Ex Taq II, and primers at a final concentration of 10 $\mu\text{mol L}^{-1}$. The thermal cycling conditions for the primers targeting UCYN-A were 2 min at 95°C, and 35 cycles of 95°C for 15 s, 58°C for 15 s, and 78°C 30 s. For the primers targeting UCYN-C, the thermal cycling conditions were the same with the exception of the final elongation at 76°C for 30 s. I conducted statistical comparison of the environmental parameters and the abundances of UCYN-A and -C using linear regression in two periods: April – September 2012 and October 2012 – January 2014.

Nucleotide sequence accession numbers

The 16S rRNA gene sequences and *nifH* sequences reported in this paper have been deposited in the DDBJ under accession numbers LC63723-LC63766 and LC63901-LC64027, respectively.

Table 2-2-2. Primers used for RT-PCR

Primer	Target site	Sequence(5'-3')
UCYN-A_nifH_2F	<i>nifH</i> gene of UCYN-A	ACCACTGTTTTACATTTAGCTGC
UCYN-A_nifH_2R		CAGTAATAATACCACGACCAGCA
UCYN-C_nifH_1F	<i>nifH</i> gene of UCYN-C	AGCAGCAGAACGGGGAACCG
UCYN-C_nifH_1R		CCGGACTCAACACACTTRAYTCCTTGG

2-2. Diazotrophic cyanobacteria in the Seto Inland Sea

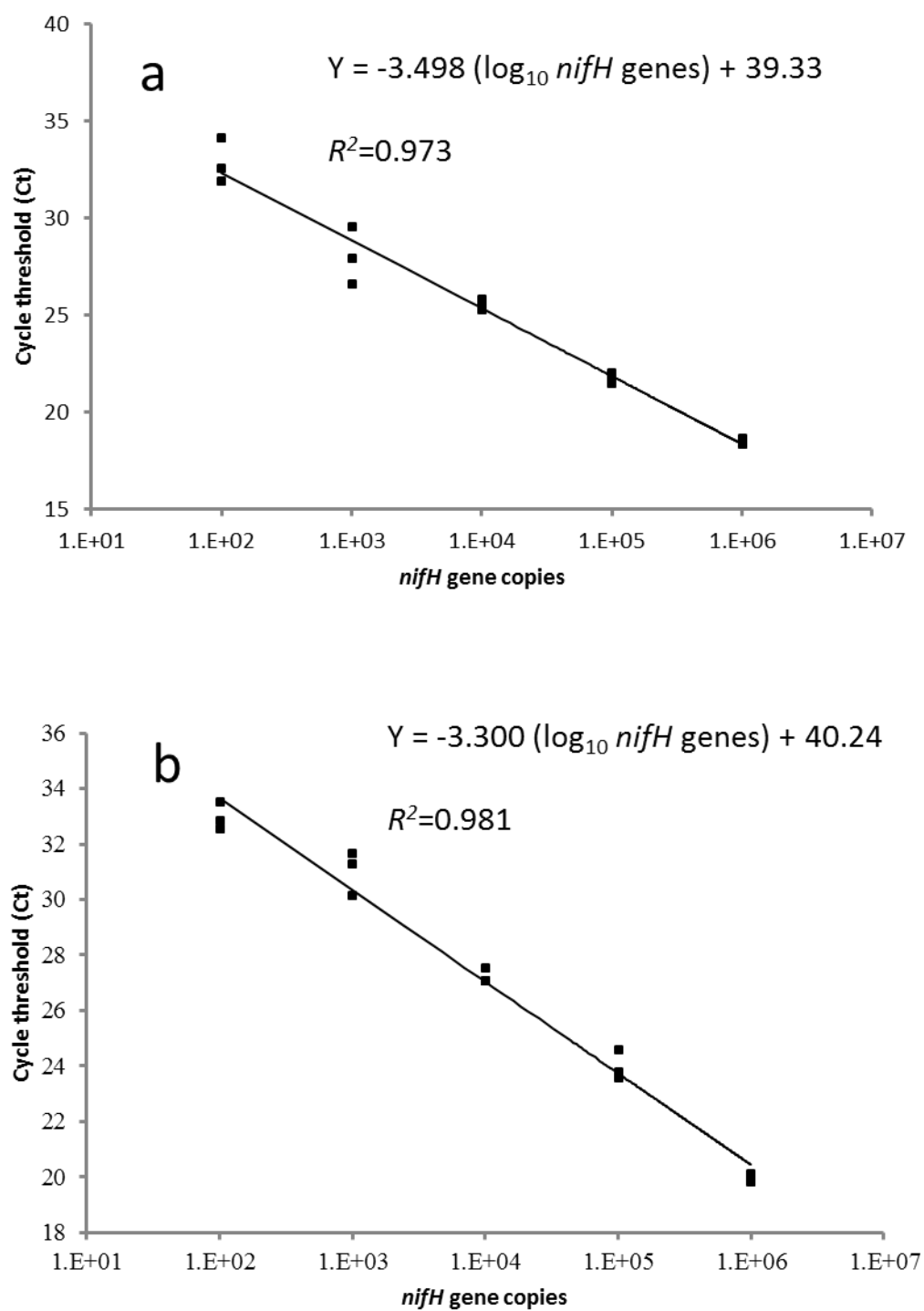


Figure 2-2-2. Standard curves of *nifH* QPCR primers used in this study.

(a) UCYN-A; (b) UCYN-C

Results

Environmental conditions

Hydrographic parameters of each sampling point differed by the influence of freshwater and open ocean water input (Table 2-2-3). The annual average surface salinity of Stn. A12 was the lowest at 28.8 among five sampling stations, while Stn. K4 had the highest average surface salinity at 33.1 ± 0.08 . The low salinity of Stn. A12 was due to the influence of freshwater from the Yodo River, the largest river flowing into the Seto Inland Sea. The high salinity of Stn. K4 was considered to be the influence of high-salinity open ocean water because Stn. K4 was the closest to the Pacific Ocean. Warmer water temperatures were observed during the summer and fall seasons (June-August and September-November), ranging from 18°C to 28°C at all of the sampling points. The water temperature decreased to 8-16°C during the winter and spring seasons (December-February and March-May). The DIN concentrations ranged 0.2-2.0 μM during the summer seasons (Table 2-2-3). PO_4 concentrations ranged 0.01-0.5 μM in the summer seasons. During the winter season, DIN and PO_4 concentrations were increased with the highest concentration measured at Stn. A12 (37.4 μM and 1.3 μM), respectively. In the Osaka Bay, Harima Nada, and Kii channel, the stratified and the well-mixed conditions were observed in summer and winter seasons, respectively (80–82). The elevated nutrient concentrations during the winter seasons were due to the well-mixed water conditions, whereas the high water temperatures during the summer seasons caused water stratification, which led to low nutrient concentrations in the surface water.

Table 2-2-3. Temperature, salinity, nutrient concentrations and diazotroph abundances in each station in the Osaka Bay, Kii channel, and Harima Nada.

Station	Depth	Month	Year	Salinity (p.s.u.)	Temperature (°C)	chlorophyll a (µg/L)	NH ₄ (µM)	NO ₂ (µM)	NO ₃ (µM)	DIN (µM)	PO ₄ (µM)	SiO ₂ (µM)	<i>nifH</i> gene copies l ⁻¹	
													UCYN-A	UCYN-C
A5	0 m	April	2012	30.1	12.6	6.8	0.5	0.0	0.2	0.7	0.0	0.6	UD	DNQ
		June		32.5	19.1	6.6	0.3	0.3	0.5	1.0	0.1	8.4	DNQ	DNQ
		August		31.5	26.4	1.2	0.3	0.8	0.4	1.5	0.3	12.5	UD	UD
		October		32.9	24.4	2.0	0.1	0.9	1.1	2.9	0.3	7.8	UD	5.2E+04
		December		30.2	11.6	0.9	0.1	1.9	3.7	5.7	0.5	7.5	UD	3.2E+04
		February	2013	32.8	11.4	0.5	1.3	0.5	3.0	4.8	0.4	6.3	UD	2.3E+05
		April		32.3	12.7	1.1	0.6	0.3	0.8	1.8	0.2	6.4	UD	UD
		June		33.2	20.0	1.3	0.1	0.6	0.7	1.3	0.2	10.0	UD	UD
		August		33.2	23.9	3.3	0.2	0.5	0.9	1.5	0.3	7.3	UD	DNQ
		October		32.8	23.7	3.3	0.2	0.8	1.6	2.6	0.3	5.0	UD	DNQ
		December		32.5	15.0	1.2	0.8	0.8	5.1	6.7	0.6	8.2	UD	UD
		April	2012	30.8	12.2	9.0	0.1	0.0	0.1	0.2	0.0	1.3	UD	7.7E+03
		June		32.6	19.1	4.6	0.1	0.4	0.4	0.9	0.1	8.6	5.6E+03	UD
		August		32.0	26.5	1.1	0.1	0.8	0.3	2.0	0.3	10.8	UD	UD
		October		32.9	24.4	1.5	0.0	1.1	0.4	3.4	0.3	8.5	*	*
		December		31.0	12.5	1.0	0.1	1.9	3.7	5.7	0.5	7.4	UD	3.2E+04
A12	0 m	February	2013	32.8	11.4	0.6	1.1	0.5	2.9	4.4	0.4	6.2	UD	6.9E+05
		April		32.3	12.7	1.1	0.7	0.3	0.8	1.8	0.2	6.4	UD	DNQ
		June		33.2	19.7	1.3	0.2	0.6	0.7	1.5	0.2	10.7	UD	UD
		August		33.2	23.3	2.2	0.1	0.5	1.0	1.6	0.2	7.5	UD	5.0E+03
		October		32.9	23.7	3.4	0.2	0.8	1.7	2.7	0.3	5.4	UD	UD
		December		32.8	15.1	1.4	0.6	0.8	5.3	6.7	0.6	8.4	UD	UD
		April	2012	26.6	14.2	20.5	0.1	0.5	1.2	1.9	0.0	0.8	UD	DNQ
		June		29.8	18.7	43.4	0.1	0.2	4.4	1.7	0.1	18.3	DNQ	DNQ
		August		25.8	27.9	95.3	1.1	0.8	0.1	2.0	0.4	9.7	DNQ	UD
		October		30.9	23.7	23.8	0.0	0.0	0.0	0.1	0.0	1.5	UD	4.7E+03
		December		31.8	14.7	2.6	3.2	3.4	14.0	20.6	0.9	18.6	UD	1.1E+04
		February		26.8	8.8	4.6	12.1	1.9	23.4	37.4	1.1	22.4	UD	8.6E+05
		April		28.1	14.8	7.5	0.4	0.6	10.5	11.4	0.1	8.3	UD	UD
		June		31.4	21.2	5.3	0.0	0.0	0.0	0.1	0.0	6.3	DNQ	6.6E+03
		August		27.8	30.3	30.8	0.2	0.0	0.1	0.3	0.1	15.1	UD	UD
		October		29.3	22.2	6.0	5.2	1.4	10.6	17.2	1.2	19.8	UD	DNQ
		December		28.4	11.9	0.5	7.7	1.9	24.5	34.0	1.3	19.9	UD	UD

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Table 2-2-3. Continued.

A12	10 m	April	2012	31.6	11.8	4.0	1.7	0.2	0.3	3.8	0.1	8.8	UD	DNQ
		June		32.2	18.3	9.5	0.1	0.8	3.2	0.4	0.2	12.8	5.0E+03	DNQ
		August		31.0	26.8	1.0	0.4	0.2	9.2	0.7	0.5	9.8	UD	DNQ
		October		31.5	23.6	10.1	0.1	0.1	0.3	0.5	0.1	2.4	UD	DNQ
K5	0 m	December		31.8	14.6	6.4	0.8	2.8	9.8	13.5	0.6	12.1	UD	1.5E+04
		February	2013	31.7	9.3	5.8	1.9	0.5	3.7	6.1	0.3	3.8	UD	6.9E+05
		April		31.9	12.7	5.6	0.2	0.1	0.4	0.6	0.1	1.8	UD	DNQ
		June		32.6	19.0	4.5	0.0	0.1	0.1	0.2	0.1	8.3	4.4E+03	DNQ
		August		32.5	24.4	1.4	0.1	0.7	1.1	1.9	0.3	10.5	UD	4.0E+03
		October		31.6	23.3	2.6	1.1	0.8	1.6	3.4	0.4	4.2	UD	DNQ
		December		31.9	14.6	1.3	2.0	1.0	6.6	9.6	0.6	9.0	UD	UD
		April	2012	32.0	13.0	5.4	0.7	0.2	5.2	1.7	0.1	6.6	UD	DNQ
		June		33.5	21.9	0.3	0.1	0.0	0.3	0.2	0.1	4.6	6.6E+03	UD
		August		32.2	27.1	0.9	0.0	0.1	0.8	0.3	0.1	7.8	UD	UD
		October		32.9	24.4	1.5	0.0	1.1	0.4	3.1	0.3	8.0	UD	DNQ
		December		32.8	16.1	1.0	0.1	1.7	3.6	5.3	0.5	7.2	UD	2.5E+04
	10 m	February	2013	32.8	11.4	0.2	1.0	0.5	3.0	4.6	0.4	6.2	UD	4.1E+04
		April	2012	32.2	12.9	1.6	1.0	0.2	1.4	2.9	0.2	8.4	UD	DNQ
		June		33.9	22.3	0.3	0.1	0.0	0.3	0.2	0.1	3.5	9.4E+03	8.9E+03
		August		32.2	26.6	0.7	0.0	0.6	0.1	1.4	0.3	9.5	DNQ	DNQ
K4	0m	October		32.9	24.3	2.2	0.1	1.0	1.1	3.1	0.3	7.9	UD	DNQ
		December		32.8	16.1	1.0	0.2	1.7	3.6	5.5	0.5	7.3	UD	7.2E+03
		February	2013	32.8	11.3	0.3	0.9	0.5	3.0	4.5	0.4	6.3	UD	6.1E+04
		April	2013	33.4	15.5	1.4	0.1	0.2	0.5	0.8	0.2	6.5	UD	UD
		June		33.0	20.8	1.6	0.0	0.1	0.0	0.1	0.1	8.3	7.7E+03	DNQ
		August		33.2	25.6	0.6	0.0	0.0	0.1	0.2	0.1	4.7	UD	5.5E+03
		October		32.9	23.4	2.9	0.2	0.8	1.7	2.6	0.3	5.1	UD	UD
		December		33.2	15.3	2.3	0.5	0.6	3.8	5.0	0.4	7.3	UD	UD
	10m	April	2013	33.8	15.9	1.1	0.7	0.2	0.8	1.7	0.2	6.3	UD	UD
		June		33.2	20.0	0.9	0.1	0.1	0.1	0.3	0.1	7.1	4.4E+03	4.2E+04
		August		33.1	23.4	0.7	0.1	0.0	0.1	0.3	0.1	5.6	UD	DNQ
		October		32.9	23.4	3.2	0.1	0.8	1.7	2.6	0.3	5.1	UD	UD
		December		33.2	15.3	2.6	0.5	0.7	4.1	5.3	0.4	6.2	UD	UD

Table 2-2-3. Continued.

H3	0 m	May	2012	31.0	15.4	4.5	0.1	0.0	0.0	0.2	0.1	4.0	1.2E+04	2.0E+04
	July		26.4	24.1	9.1	0.3	0.2	6.3	6.8	0.1	41.0	DNQ	DNQ	DNQ
	September		30.6	28.6	2.8	0.0	0.0	0.0	0.1	0.2	7.3	DNQ	1.6E+05	1.6E+05
	November		31.3	22.1	8.3	0.2	0.3	0.7	1.3	0.4	3.5	UD	UD	UD
	January	2013	30.9	8.6	6.1	0.0	0.0	0.1	0.1	0.1	1.7	UD	2.6E+04	2.6E+04
	March		31.9	8.0	1.3	1.0	0.0	0.1	1.1	0.2	0.4	UD	5.8E+04	5.8E+04
	May		31.6	15.1	1.2	0.0	0.0	0.1	0.1	0.1	3.2	UD	UD	UD
	July		30.4	23.6	2.2	0.0	0.0	0.0	0.1	0.0	10.4	6.1E+03	UD	UD
	September		31.3	27.5	1.1	0.1	0.1	0.1	0.3	0.3	6.4	UD	UD	UD
	November		30.6	21.0	3.2	0.2	1.1	0.6	2.0	0.5	6.9	UD	UD	UD
	January		31.7	10.8	1.6	1.0	0.3	1.3	2.6	0.4	9.1	UD	UD	UD
10m	May	2012	31.3	14.0	5.3	0.2	0.0	0.1	0.4	0.1	4.2	5.9E+03	1.3E+04	1.3E+04
	July		31.1	21.1	3.3	0.0	0.0	0.1	0.2	0.1	11.2	※	※	※
	September		31.1	27.8	1.3	0.3	0.1	0.3	0.7	0.4	9.9	DNQ	5.5E+04	5.5E+04
	November		31.3	22.1	8.8	0.2	0.3	0.7	1.1	0.4	3.4	UD	DNQ	DNQ
	January	2013	31.4	9.6	4.5	0.2	0.3	0.4	0.9	0.3	3.5	UD	3.3E+04	3.3E+04
	March		32.0	7.8	2.0	0.1	0.0	0.0	0.2	0.1	0.9	UD	1.7E+05	1.7E+05
	May		32.0	13.9	0.7	0.1	0.0	0.1	0.1	0.1	2.8	DNQ	UD	UD
	July		31.4	22.0	1.4	0.1	0.0	0.0	0.2	0.1	8.6	DNQ	UD	UD
	September		31.6	27.0	2.0	0.2	0.1	0.4	0.8	0.4	6.3	UD	UD	UD
	November		30.7	21.2	3.5	0.3	1.5	0.6	2.4	0.5	7.1	UD	UD	UD
	January	2014	31.7	10.8	1.7	1.1	0.2	1.3	2.6	0.4	9.0	UD	UD	UD

Date were not available (※), undetected (UD) or detected but below the limit of quantification (DNQ).

Genetic diversities of unicellular diazotrophic cyanobacteria

A total of 127 *nifH* sequences were obtained from 4 stations (Table 2-2-4, Figs. 2-2-3, 2-2-4). These *nifH* genes were clustered with sequences from Cluster I (most proteobacteria and cyanobacteria including UCYN-A, B and C), Cluster II (second alternative nitrogenases, *anfH* and archaea) and Cluster III (diverse anaerobic bacteria such as sulfate reducers, clostridia). No sequence of UCYN-A, B and C was detected from any of the seasons and sampling points in the *nifH* clone library (Fig. 2-2-3).

Table 2-2-4. Summary of *nifH* clones obtained from each microbial group

Station	Date	Year	Depth (m)	Number of Clones			Total
				Cluster I	Cluster II	Cluster III	
A5	April	2012	0	3			3
			10	1		4	5
	August	2012	0	4			4
			10	3		2	5
	February	2013	0	5		1	6
			10	8			8
A12	April	2012	0	3		1	4
			10	5			5
	August	2012	0	4		2	6
			10			5	5
	February	2013	0	3		3	6
			10	4	2	3	9
K5	April	2012	0	2		2	4
			10	4			4
	August	2012	0	6		2	8
			10	7		1	8
	February	2013	0	6			6
			10	7			7
H3	May	2012	0	2			2
			10	3			3
	September	2012	0	5			5
			10	5			5
	March	2013	0	4			4
			10	2		3	5
Total				96	2	29	127

2-2. Diazotrophic cyanobacteria in the Seto Inland Sea

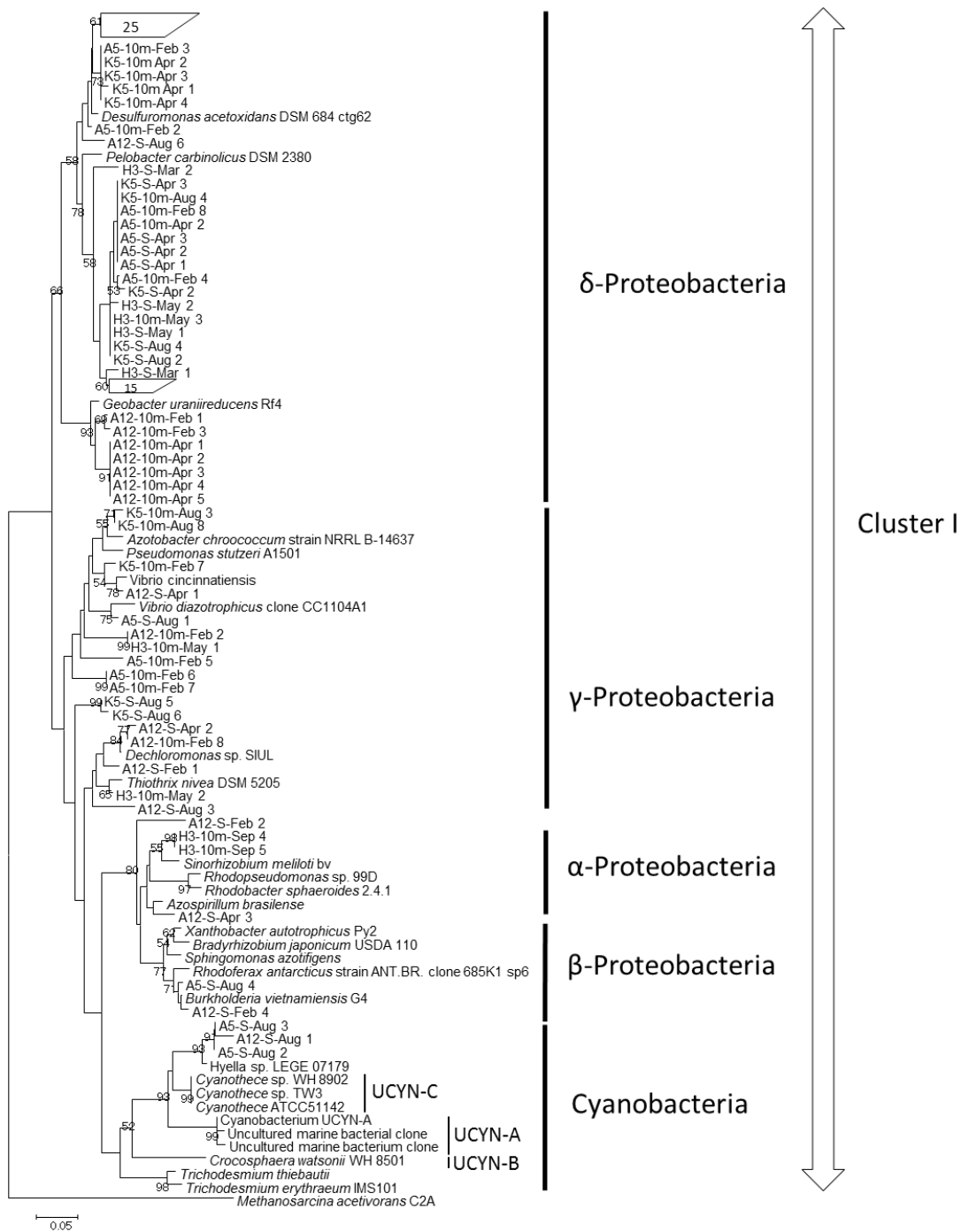


Figure 2-2-3. Phylogenetic tree for *nifH* genes using the neighbor-joining for Cluster I amino acid sequences. Bootstrap values greater than 50% for 1000 replicates are indicated at respective nodes. Numbers in polygons indicate the numbers of sequences in each group. Sequences obtained in this study are referred to (1) the station they were sampled (A5, A12, K5, K4 and H3), (2) the depth (S: surface and 10: 10m depth), (3) the month and (4) their clone number. Scale bar indicates 0.05 substitutions per site.

2-2. Diazotrophic cyanobacteria in the Seto Inland Sea

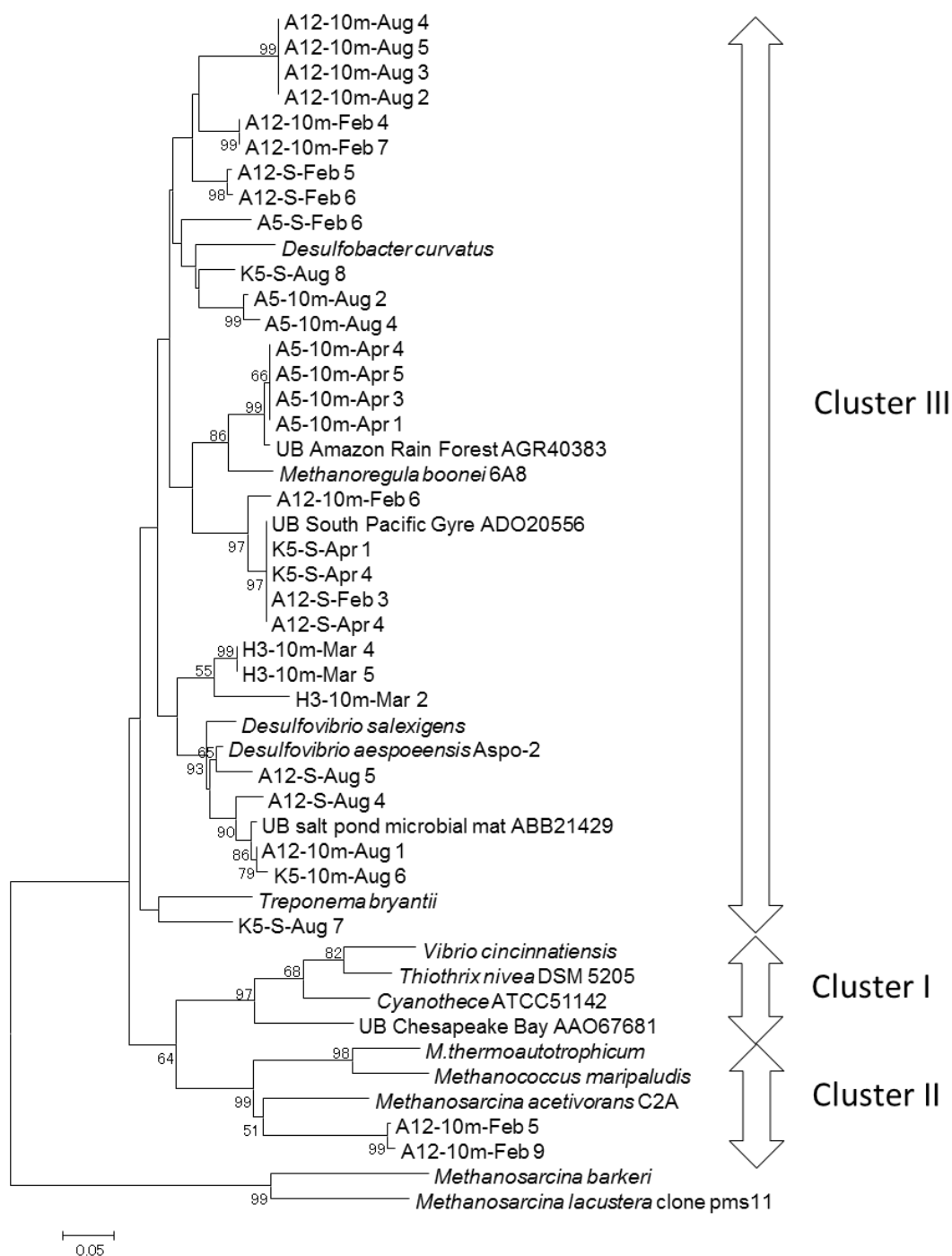


Figure 2-2-4. Phylogenetic tree for *nifH* genes using the neighbor-joining for Cluster II and III amino acid sequences. Bootstrap values greater than 50% for 1000 replicates are indicated at respective nodes. Sequences obtained in this study are referred to (1) the station they were sampled (A5, A12, K5, K4 and H3), (2) the depth (S: surface and 10: 10m depth), (3) the month and (4) their clone number. Scale bar indicates 0.05 substitutions per site.

I tried to obtain the sequences of unicellular diazotrophic cyanobacteria by amplifying their 16S rRNA genes. I were successful to retrieve 44 sequences by the PCR amplification (Table 2-2-5). These sequences were divided into four groups of unicellular diazotrophic cyanobacteria (Fig. 2-2-5). Of these, twelve sequences fell into the UCYN-A clade, which include the UCYN-A genome from Stn. ALOHA (CP001842) and the endosymbiont of *Braarudosphaera bigelowii* (AB847983) (17, 19). These UCYN-A sequences were detected at Stn. H3 in May and September (Table 2-2-5). Twenty-six sequences clustered with *Cyanothece* SKTU126, which was isolated from Sotobanari Island in Japan (22). The remaining five and three sequences were grouped with *Cyanothece* ATCC51142 and the endosymbiont of *Rhopalodia gibba*, respectively. *Cyanothece* ATCC51142, *Cyanothece* SKTU126 and the endosymbiont of *Rhopalodia gibba* have 95% similarity with each other and are diverged based on the 16S rRNA gene phylogeny (Fig. 2-2-5); however, they form a monophyletic group with UCYN-C in the *nifH* gene phylogeny (2, 23). Therefore, I referred these three groups as UCYN-C ribotype-1, 2, and 3, respectively.

2-2. Diazotrophic cyanobacteria in the Seto Inland Sea

Table 2-2-5. Summary of 16S rRNA clones obtained from each microbial group

Station	Month	Year	Depth (m)	Number of Clones		
				UCYN-A	UCYN-C	Total
A5	April	2012	0			
			10		5	5
	August	2012	0		5	5
			10			
			0			
A12	April	2012	0			
			10			
	August	2012	0		4	4
			10			
			0			
K5	April	2012	0			
			10			
	August	2012	0		5	5
			10			
			0			
H3	May	2012	0	5		5
			10	5		5
	September	2012	0	2	4	6
			10			
	March	2013	0		5	5
10				4	4	
Total				12	32	44

Distribution of unicellular diazotrophic cyanobacteria

The spatial distribution and temporal change of UCYN-A and C was examined at the surface and 10 m depth samples from five stations in the Seto Inland Sea through the year (Fig. 2-2-6, 2-2-7). UCYN-A was detected from May to September at each sampling point with abundance ranging from 4.4×10^3 to 1.2×10^4 *nifH* gene copies L⁻¹. UCYN-A was not detected from October to March.

2-2. Diazotrophic cyanobacteria in the Seto Inland Sea

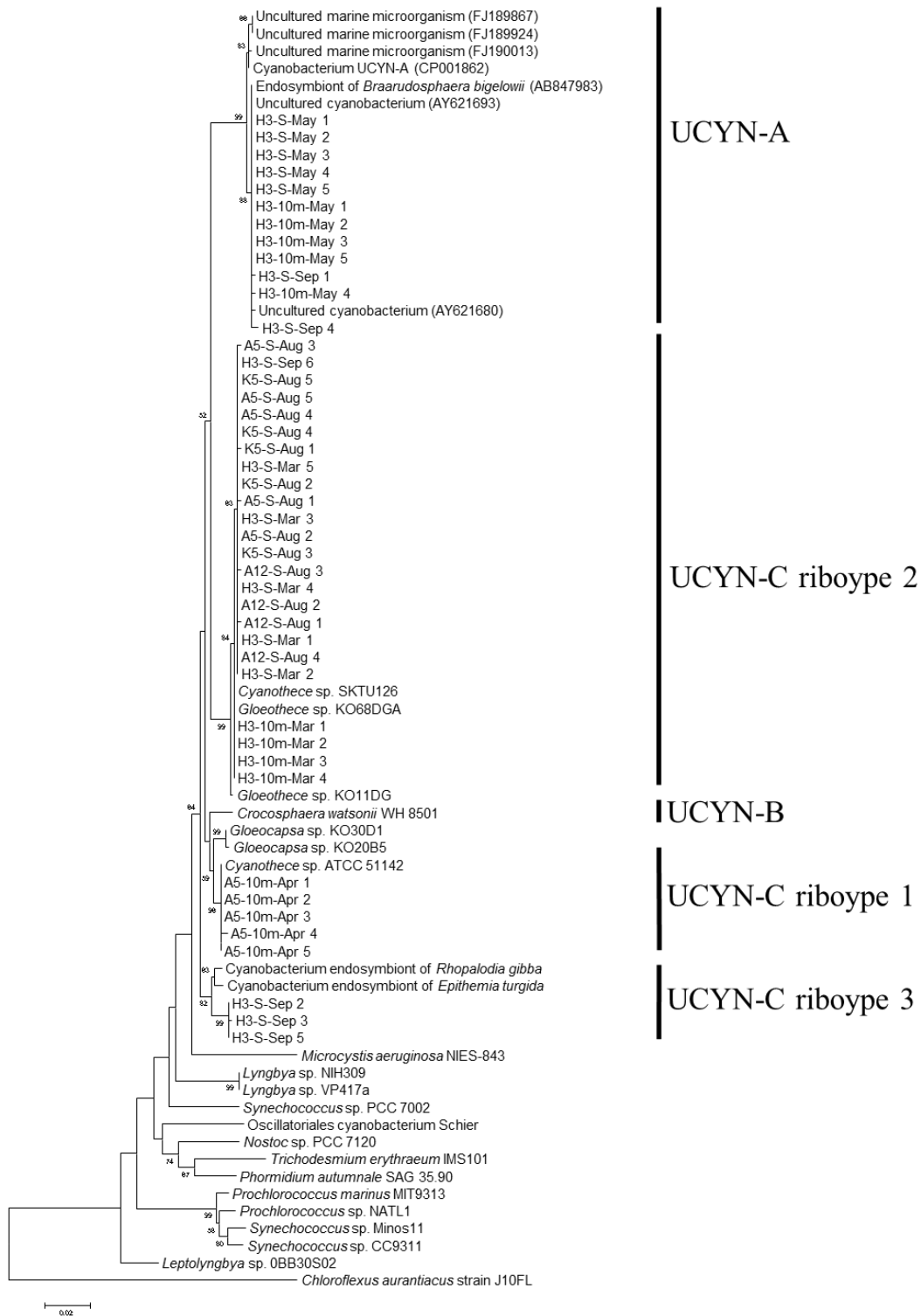


Figure 2-2-5. Phylogenetic tree of cyanobacterial 16S rDNA sequences constructed using the neighbor-joining method. Bootstrap values greater than 50% for 1000 replicates are indicated at respective nodes. Sequences obtained in this study are referred to (1) the station they were sampled (A5, A12, K5, K4 and H3), (2) the depth (S: surface and 10: 10m depth), (3) the month and (4) their clone number. Scale bar indicates 0.02 substitutions per site.

UCYN-C was consistently detected in the samples of Stn. A5, A12 and K5 from October 2012 to February 2013 ($> 10^4$ *nifH* gene copies L⁻¹), with the highest abundance of 8.6×10^5 *nifH* gene copies L⁻¹ at Stn. A12 in February 2013 (Fig. 2-2-7). However, the quantities were below the limit of detection in the same season in 2013. In Stn. H3, UCYN-C was detected from May 2012 to March 2013.

The *nifH* gene copies were plotted as a function of temperature and DIN to provide better insight into how the parameters may control their distribution (Fig. 2-2-8). UCYN-A was distributed in waters with temperatures ranging 14-28°C and nitrogen-depleted conditions. Meanwhile, UCYN-C was detected with temperatures ranging 8-29°C and even in nitrogen-rich conditions.

I conducted statistical comparison of environmental parameters and the qPCR results. No significant ($P > 0.05$) relationships was observed between UCYN-A and environmental parameters (Table 2-2-6). UCYN-C abundances were demonstrated a positive correlation with NO₂ ($R_2 = 0.948$, $P = 0.005$, $n = 5$) during April – September 2012. For the samples collected during October 2012 – January 2014, UCYN-C showed a negative correlation with salinity ($R_2 = 0.217$, $P = 0.025$, $n = 23$) and temperature ($R_2 = 0.182$, $P = 0.042$, $n = 23$), and a positive correlation with NO₃ ($R_2 = 0.263$, $P = 0.012$, $n = 23$), NH₄ ($R_2 = 0.484$, $P = 0.000$, $n = 22$), DIN ($R_2 = 0.012$, $P = 0.008$, $n = 23$), and PO₄ ($R_2 = 0.180$, $P = 0.044$, $n = 23$) (Table 2-2-6).

2-2. Diazotrophic cyanobacteria in the Seto Inland Sea

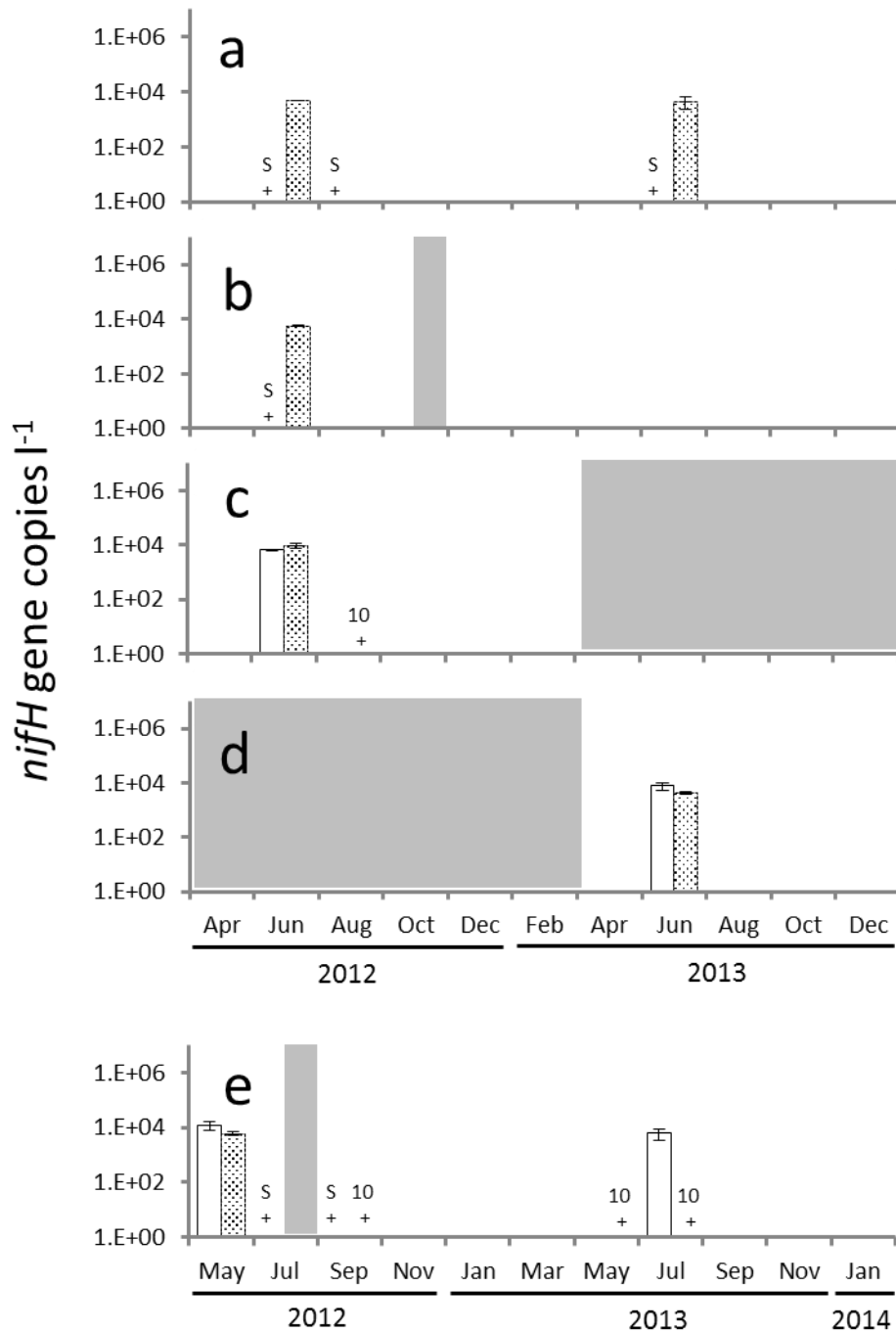


Figure 2-2-6. Abundances (*nifH* gene copies L⁻¹) of UCYN-A in five sampling stations. The white and dotted box indicate the abundance of surface and 10 m depth, respectively. The S+ and 10+ show that the abundances are detected but below the limit of quantification in surface and 10m depth, respectively. The grey box means that data were not available. (a) A12; (b) A5; (c) K5; (d) K4; (e) H3

2-2. Diazotrophic cyanobacteria in the Seto Inland Sea

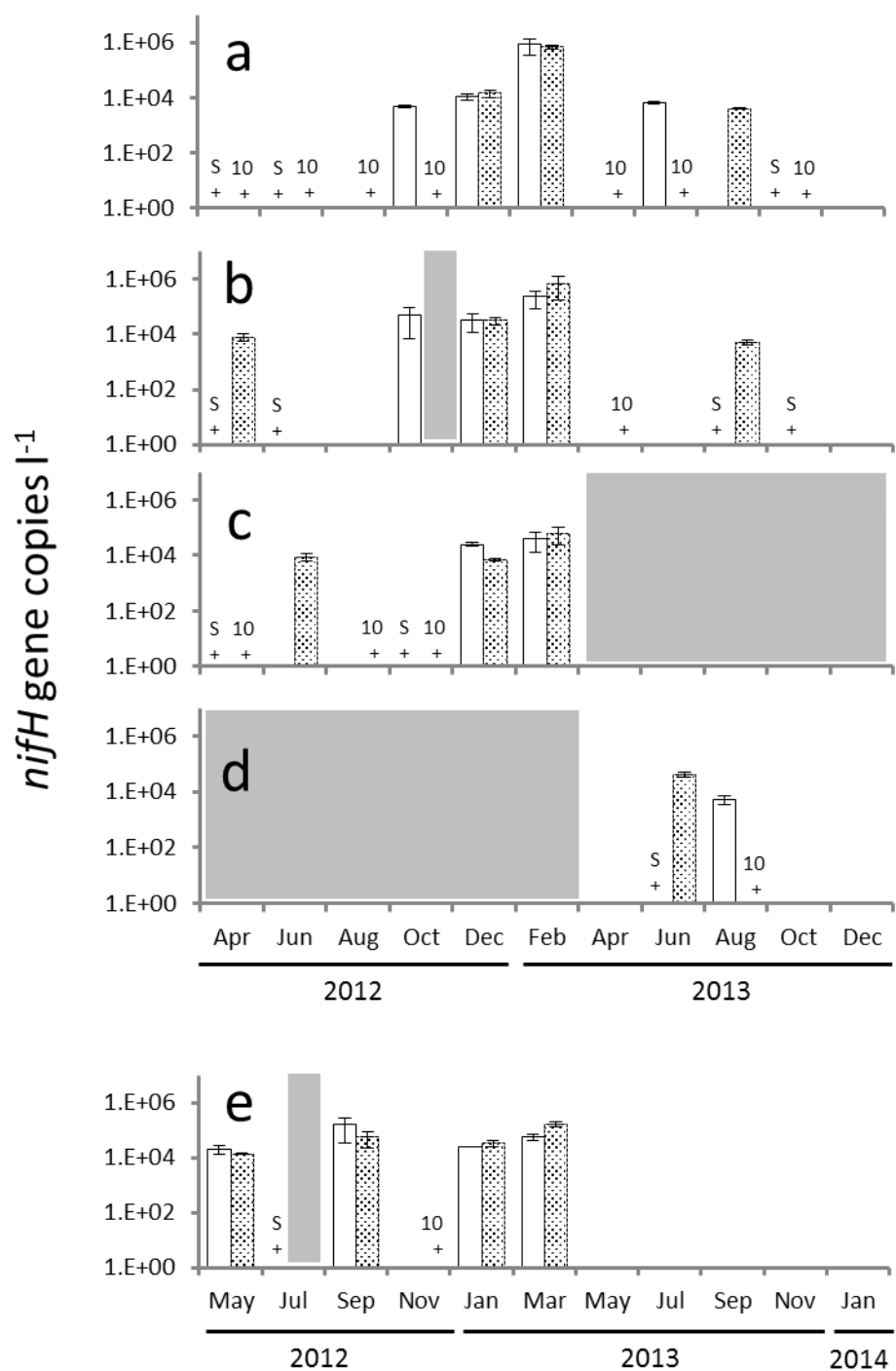


Figure 2-2-7. Abundances (*nifH* gene copies L^{-1}) of UCYN-C in five sampling stations. The white and dotted box indicate the abundance of surface and 10 m depth, respectively. The S+ and 10+ show that the abundances are detected but below the limit of quantification in surface and 10m depth, respectively. The grey box means that data were not available. (a) A12; (b) A5; (c) K5; (d) K4; (e) H3

2-2. Diazotrophic cyanobacteria in the Seto Inland Sea

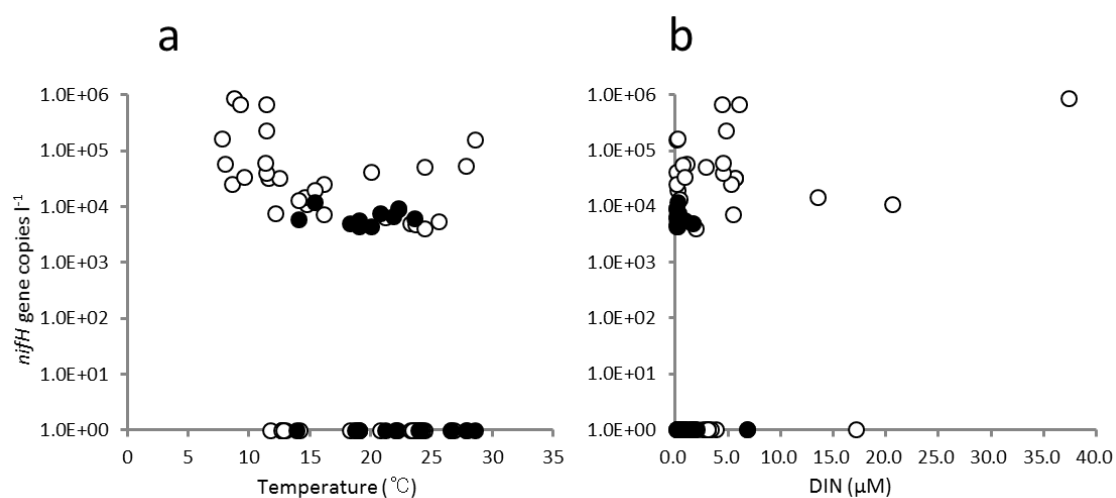


Figure 2-2-8. Plot of *nifH* gene copies L⁻¹ of UCYN-A and C as a function of (a) temperature and (b) dissolved inorganic nitrogen (DIN). Closed and open circles indicate abundances of UCYN-A and C, respectively. Samples with DNQ are designated as 1 *nifH* gene copies L⁻¹.

Table 2-2-6. Linear regression analysis between abundances of UCYN-A and C and each environmental parameter.

Variable	UCYN-A						UCYN-C					
	May - September 2012			October 2012 - January 2014			May-September 2012			October 2012 - January 2014		
	<i>R</i> ₂	<i>p</i>	<i>n</i>	<i>R</i> ₂	<i>p</i>	<i>n</i>	<i>R</i> ₂	<i>p</i>	<i>n</i>	<i>R</i> ₂	<i>p</i>	<i>n</i>
Salinity (psu)		> 0.05	6		> 0.05	4		> 0.05	6	0.217-	0.025	23
Temperature (°C)		> 0.05	6		> 0.05	4		> 0.05	6	0.182-	0.042	23
Chl a (μg/L)		> 0.05	6		> 0.05	4		> 0.05	6		> 0.05	23
NO ₃ (μM)		> 0.05	6		> 0.05	4		> 0.05	6	0.263+	0.012	23
NO ₂ (μM)		> 0.05	6		> 0.05	4	0.948+	0.005	5		> 0.05	23
NH ₄ (μM)		> 0.05	6		-	2		> 0.05	6	0.484+	0.000	22
DIN (μM)		> 0.05	6		> 0.05	4		> 0.05	6	0.012+	0.008	23
PO ₄ (μM)		> 0.05	6		> 0.05	4		> 0.05	5	0.180+	0.044	23
SiO ₂ (μM)		> 0.05	6		> 0.05	4		> 0.05	6		> 0.05	23
UCYN-A (gene copies L ⁻¹)		-			-			> 0.05	3		-	1
UCYN-C (gene copies L ⁻¹)		> 0.05	3		-	1		-			-	

Discussion

In open ocean environments, UCYN-C is usually not considered to be largely responsible for nitrogen fixation because of limited detection (27, 66). Compared with the wide and abundant distributions of UCYN-A and B, UCYN-C was detected sporadically or below the levels of detection in the sampling points examined in the Atlantic Ocean (27, 66).

In the Seto Inland Sea, the results from the clone analysis of the 16S rRNA gene sequences of unicellular diazotrophic cyanobacteria indicate the presence of three groups of UCYN-C, which I referred as ribotype-1, 2, and 3 (Fig. 2-2-5). Ribotype-1 includes *Cyanothece* sp. and *Gloeocapsa* sp., while isolates belonging to ribotype-2 include *Cyanothece* sp. and *Gloeotheca* sp. All isolates of ribotype-1 and 2 are photoautotrophs, while ribotype-3 contains cyanobacterium endosymbionts of *R. gibba* and *E. turgida* (Fig. 2-2-5). Endosymbionts of *R. gibba* and *E. turgida* were obtained from freshwater lakes (83, 84). These endosymbionts were called a “spheroid body,” with a close relationship with *Cyanothece* ATCC51142 (85). The spheroid bodies have a reduced genome and symbiotic relationships with diatoms and they lack the ability of photosynthesis but the ability of nitrogen fixation (85, 86).

Both isolates clustered to ribotype-1 and 2 were mainly isolated from coastal areas (e.g., intertidal zone, mangrove forest), with the exception of TW3 belonging to ribotype-1 isolated from the open ocean (22, 23). In this study, I showed that sequences of ribotype-2 were detected at all sampling points and in August, September and March, while sequences of ribotype-1

and 3 were limited at Stn. A5 in April and Stn. H3 in September, respectively. Although their physiological differences are not revealed, the different patterns of distribution provide the possibility of their physiological and ecological differences.

In addition to the detection of UCYN-C in the clone library, qPCR results suggest a wide and abundant distribution of UCYN-C in the Seto Inland Sea, reaching 8.6×10^5 *nifH* gene copies L⁻¹ at Stn. A12 in February 2013. This value is higher compared with other studies (27, 39, 66). UCYN-C was detected in the winter season, which was characterized by low water temperature and elevated nutrient concentrations of DIN and PO₄ (Table 2-2-3). The highest abundance of UCYN-C in the Atlantic Ocean was also detected at a sampling point with a low-temperature (14.9°C) and high nutrient conditions (PO₄ = 0.8 μM; NO₃+NO₂ = 20.6 μM) (27). It had been reported that UCYN-C was detected in seawaters with a wide temperature range from 16°C to 28°C and even in Arctic seawater (66, 87). However, low temperature enhances the respiratory cost and affects the growth rate and the timing of nitrogen fixation of UCYN-C, which suggests a diazotrophic growth disadvantage in low temperature conditions (88). These results highlight the incomplete understanding of the ecology of UCYN-C, and underlines the need for further investigations.

In contrast to the sporadic distribution of UCYN-C in the open ocean, I demonstrated that water columns of the Seto Inland Sea harbor high UCYN-C abundance throughout the year, suggesting the possibility that UCYN-C is a coastal-adapted phylotype and contribute to nitrogen input. To

estimate the potential contribution of UCYN-C to nitrogen input in the Seto Inland Sea, I used nitrogen fixation rate of the UCYN-C culture TW3 (7.31×10^{-15} mol N cell⁻¹ day⁻¹) and volumes of Osaka Bay (23). Assuming that UCYN-C has a single *nifH* gene per cell, their contribution to nitrogen input in Osaka Bay with the maximum abundance (8.62×10^5 cell L⁻¹), was calculated to be 0.0013 t N day⁻¹. This estimate deserves less than 0.01 % of nitrogen input to Osaka Bay from the rivers (89). This amount of nitrogen input through nitrogen fixation by UCYN-C seems to be less important in this coastal area. However, unlike the nitrogen input from rivers, UCYN-C may have the potential influence on the ecosystem through the biological interaction such as symbiosis and predation.

Clone analysis and qPCR results suggest that the detection of UCYN-A was restricted in early summer season, May, June, July, August and September, with temperatures ranging 14-28°C and lower nitrogen conditions (0.1-2.0 µM) (Fig. 2-2-5). UCYN-A was also observed in other coastal areas of the Mediterranean Sea and Chesapeake Bay (28, 29, 71, 90). The distribution of UCYN-A is mostly limited to the summer seasons, including June-August in the Seto Inland Sea (Fig. 2-2-6). Le Moal and Biegala (90) also reported that the blooming of UCYN-A-like picocyanobacteria reaching 10³ cells mL⁻¹ was confirmed during June-July in the Mediterranean Sea. The analysis in the Pacific Ocean suggested that UCYN-A *nifH* abundances had a weak but positive relationship with temperatures and a weak but negative correlation with nitrogen concentrations (NO₃⁻ + NO₂⁻) (38). High UCYN-A abundances were observed

at water temperatures around 22-23°C in the Atlantic Ocean (66), and the *nifH* transcripts were detected at water temperatures ranging from 12-19°C (28, 42). In the Seto Inland Sea, the DIN concentrations decreased in June-August compared with the winter season, and the N/P ratio (approximately 5) was less than the Redfield ratio (Table 2-2-3), suggesting that the availability of nitrogen may be the growth-limiting factor in these environments (91). Considering less abundance of UCYN-A (10^3 - 10^4 *nifH* gene copies L⁻¹) than the open ocean (13, 27, 66), UCYN-A may be transferred into the coastal area from the open ocean, rather than inhabiting in the Seto Inland Sea.

In conclusion, the results presented here provide the first evidence of the diversity and seasonal dynamics of unicellular diazotrophic cyanobacteria in the Seto Inland Sea. The results from qPCR showed the highest abundance of UCYN-C under conditions of low temperature and elevated nitrogen concentrations. Although this study did not provide a reasonable explanation for these observations, a significant contribution to the understanding of the ecological aspects of UCYN-C has been made. Our study suggested that UCYN-C were divided into three groups based on the 16S rRNA genes in the clone analysis; ribotype-1, 2, and 3. These phylotypes were detected during different months, suggesting that they have different ecological and physiological characteristics. High UCYN-A abundance was detected by qPCR only during the early summer. Detection of UCYN-A during the early summer may be explained by the relatively nitrogen-depleted conditions. Further studies are required to unravel the

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ecological importance of these unicellular diazotrophic cyanobacteria in the Seto Inland Sea.

In the next chapter, I succeeded to isolate unicellular diazotrophic cyanobacteria from the Seto Inland Sea and performed the genomic and phylogenetic analysis.

Chapter 3

Isolation and genomic analysis of unicellular diazotrophic cyanobacteria

Introduction

Unicellular diazotrophic cyanobacteria plays an important role in global biogeochemical cycles in the ocean through photosynthesis as well as nitrogen fixation (13, 27, 40). Global researches have revealed their abundance and wide distribution across the world's oceans, suggesting their significant contribution to the oceans as carbon and nitrogen sources (13, 27, 40).

Oceanic unicellular diazotrophic cyanobacteria have been recognized through environmental clone sequences of the nitrogenase reductase gene (*nifH*). The sequences are conveniently divided into major three groups; UCYN-A, B, and C (2). Until now, culturable strains of UCYN-A have yet not been established. The UCYN-A draft genome is constructed with UCYN-A cells sorted from seawater samples using flow cytometry (17). The genome of UCYN-A is significantly reduced and lacks key metabolic features for cyanobacteria: photosystem II complex and Rubisco, suggesting the dependent to an outer carbon source (16, 17). A recent study shows that UCYN-A are sub-divided into three groups; UCYN-A1, A2, and A3 based on nucleotide sequence of *nifH* gene (92). A comparative genomics of UCYN-A1 and A2 reveals that there are no remarkable metabolic differences between

3. Genomic analysis of UCYN-C

them but genome reduction have not occurred in the same manner, which may reflect their adaptations to different hosts (93).

UCYN-B (including a sole culturable species *Crocosphaera watsonii*) has various genetic mechanisms to adapt to oligotrophic oceanic environments; e.g. iron conservation by reduction of metalloenzyme and phosphorus scavenging by high-affinity transport of phosphate and hydrolysis of phosphomonoesters (94, 95). A comparative genomic analysis of six *C. watsonii* strains revealed that two sub-groups in this species are characterized by cell size and genome contents: small-cell strains with smaller genomic sizes and apparent gene loss, larger-cell strains with more metabolic capabilities (96).

Cyanothece sp. TW3 is reported as a sole authentic strain of the UCYN-C group isolated from the open ocean (23). However, many cyanobacteria show phylogenetic linkages to UCYN-C. Some unicellular cyanobacteria isolated from coastal waters, such as *Cyanothece* sp. from *Gloeotheca* sp, and *Gloeocapsa* sp. form monophyletic group with UCYN-C in the phylogenetic tree of the *nifH* gene (22, 23). The *nifH* genes of endosymbionts of *Rhopalodia gibba* and *Epithemia turgida* are also clustered with UCYN-C (2). Genomic and transcriptomic analysis of *Cyanothece* sp. ATCC51142 from intertidal zone of Gulf of Mexico, have characterized the metabolic cycling of photosynthesis in the day and nitrogen fixation in the night (97, 98). This strain also possesses a linear chromosome responsible for fermentative processes (97). The genome of the endosymbiont of *Epithemia turgida* is reduced compared with closely related unicellular diazotrophic

cyanobacteria and has lost key metabolic pathways including photosynthesis (86).

In chapter 2-2, I obtained four groups of 16S rRNA gene sequences of unicellular diazotrophic cyanobacteria from the Seto Inland Sea: UCYN-A and three groups of UCYN-C, which I referred to as ribotype-1, 2, and 3. UCYN-C ribotype-1 includes *Cyanothece* sp. ATCC51142, CCY0110 and *Gloeocapsa* sp. KO20B5, KO30D1, and KO38CU6. UCYN-C ribotype-2 contains *Cyanothece* sp. SKTU126, WH8904 and *Gloeotheca* sp. KO68DG, KO11DG, and SK40. UCYN-C ribotype-3 includes endosymbionts of *Epithemia turgida* and *Rhopalodia gibba*.

Some strains of UCYN-C ribotype-1 and 3 have been characterized their genomic features (86, 97). Although isolates of assigned to UCYN-C ribotype-2 such as *Cyanothece* sp. SKTU126 and *Gloeotheca* sp. KO68DG, KO11DG, and SK40 were characterized with their morphological features and phylogenetic analyses of 16S rRNA and *nifH* genes (22), their genomic analyses have never been conducted.

In this chapter, I succeeded to establish two strains belonging to UCYN-C ribotype-2. To obtain insight into the genomic feature of UCYN-C ribotype-2, I determined the draft genome sequence of the two strains and characterized their phylogeny and metabolic features. Their genomic analysis will provide the further understanding to their ecological and physiological aspects.

Materials and Methods

Isolation and culture

Samples were collected from intertidal zones of Goshiki beach located in Awaji island, Hyogo Pref., Japan (34.353 N, 134.741 E) and coastal area of Yura-cho, Wakayama Pref., Japan (33.984 N, 135.090 E) (Fig. 3-1). Samples from water columns were collected from Stn. A5 (34.190 N, 134.584 E), A6 (34.328 N, 135.072 E), A12 (34.360 N, 135.178 E), K2 (34.092 N, 135.032 E), K4 (34.073 N, 134.566 E), K5 (34.104 N, 134.517 E), H3 (34.424 N, 134.335 E), H6 (34.366 N, 134.432 E), H10 (34.310 N, 134.321 E), H13 (34.285 N, 134.467 E), H15 (34.205 N, 134.366 E), and H27 (34.389 N, 134.538 E) (Fig. 3-1). Cells in samples were collected on 47 mm of 0.2 μ m polycarbonate membrane filters (ADVANTEC, Tokyo, Japan). Each filter was then put into 300-ml Erlenmeyer flasks containing 150 mL of sterilized ASP2 or ASN III medium without nitrogen (99, 100) and incubated at 30°C under

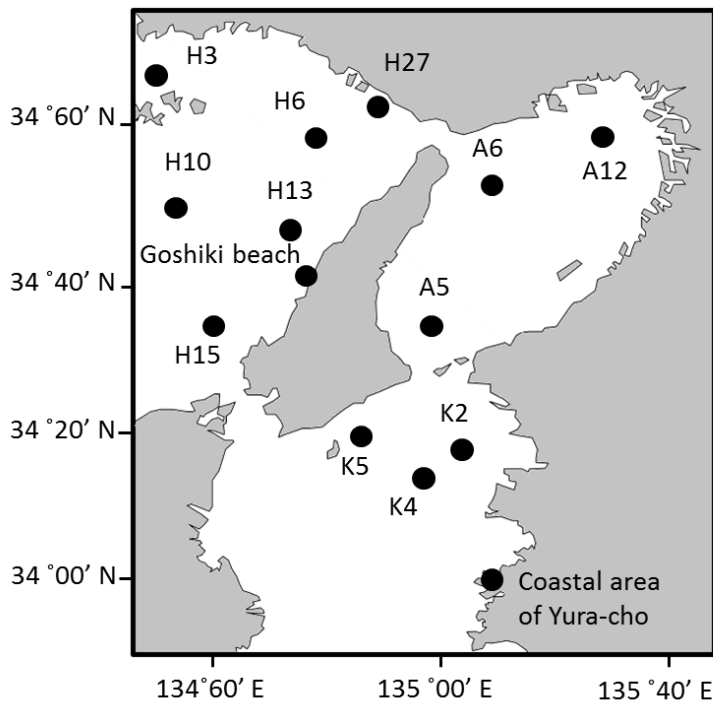


Figure 3-1. Sampling stations of this study.

illumination of 12:12 L:D cycles. After 4 to 8 weeks from the incubation, green cell clumps were observed in the samples from intertidal zones of Goshiki beach and coastal area of Yura-cho. Each cell clump was picked up by a Pasteur pipette and transferred to Erlenmeyer flasks containing ASP2 or ASN III medium. After the transfer was repeated twice, a single cell of the clumps were picked up by a Pasteur pipette using a microscope, and transferred to 150 × 15 mm test tubes containing 5 mL of ASP2 or ASN III medium. The test tubes were incubated at 30°C under illumination of 12:12 L:D cycles.

To establish axenic cultures, I conducted nutrient enrichment incubations according to a method described by Taniuchi et al. (23). Each single colony picked up by Pasteur pipette was incubated with ASP2 or ASN III medium added four nutrients (glucose, sucrose, tryptone, and yeast extract) individually to final concentration of 1 %. After incubation for 2 weeks, the cultures were checked by a fluorescence microscope after SYBR Gold staining. I established two clonal and axenic cultures of unicellular diazotrophic cyanobacteria, GS-1 and YR-1. GS-1 and YR-1 were maintained 10 mL of ASN III medium in 150 × 15 mm test tubes with incubation at 20°C under illumination of 12:12 L:D cycles.

Genomic DNA isolation, sequencing, assembly, and annotation

Genomic DNAs of GS-1 and YR-1 were obtained according to a method described previously (26). Briefly, each 30 mL of the cultures was filtered through 47-mm-diameter 0.2 µm polycarbonate membrane filter

3. Genomic analysis of UCYN-C

(ADVANTEC, Tokyo, Japan) under pressure less than 0.03 MPa. The filters were placed in 1.5 mL microcentrifuge tube and then added 500 μ L TE buffer and 500 μ L of potassium xanthogenate buffer (2% w/v potassium ethyl xanthogenate; 200mM Tris-HCl, pH 7.4; 2 mM EDTA, pH 8.0; 2% sodium dodecylsulfate; 1.6 M ammonium acetate). The mixtures were incubated at 70°C for 120 min, followed by incubation on ice for 30 min. After centrifugation, DNAs were obtained from the supernatant with 2-propanol precipitation. A genomic library was prepared using Nextera XT (Illumina) according to a manufacture's protocol and sequenced using MiSeq (Illumina) with MiSeq Reagent Kit v2 paired-end sequence. The sequencing run yielded a total of 8,546,324 and 2,593,228 paired-end 151-bp reads for GS-1 and YR-1, respectively. The sequence reads were assembled using the Velvet version 1.2.10 (101). After the assembly, the genomes were submitted to the Microbial Genome Annotation Pipeline ver1.060 (MiGAP) annotation server (<http://www.migap.org/index.php/en>) for subsystem classification and functional annotation (102). Genes were assigned to clusters of orthologous groups of proteins (COGs) based on RPS-BLAST (103). K number for protein sequences were assigned by using Blast KOALA (104). Metabolic pathways of each strain were predicted by using KEGG mapper (105).

Genome sequences of *Cyanothece* sp. ATCC51142 (AY728386), *Cyanothece* sp. CCY010 (AAXW000000000), *Crocospaera watsonii* WH8501 (AADV000000000), *Crocospaewa watsonii* WH0003 (AESD000000000), UCYN-A (CP001842), endosymbiont of *Epithemia turgida* (AP012549) were

downloaded from GenBank database. We used an Reciprocal Blast Hit (RBH) approach to identify all of the orthologous pairs among the genomes of unicellular diazotrophic cyanobacteria (106, 107).

Plasmid-like contigs in the draft genomes of GS-1 and YR-1 were predicted by following procedures. All contigs of GS-1 and YR-1 were searched against plasmid sequences of *Cyanothece* sp. ATCC51142 (plasmid A (CP000808), B (CP000809), C (CP000810), and D (CP000811)) using BLASTN with a $1e-5$ value cutoff (97). When a contig showed significant BLAST hit to the plasmid sequence of *Cyanothece* sp. ATCC51142, all genes of the contig were searched against NCBI nr database with BLASTP search. When at least one gene of the contig showed the highest similarity to plasmid sequences of *Cyanothece* sp. ATCC51142 and other genes in the contig did not show the highest similarity to chromosomal sequences of *Cyanothece* sp. ATCC51142, I defined this contig as a plasmid-like contig.

Phylogenetic analysis

For phylogenetic analysis, sequences of 16S rRNA, *nifH*, *adk*, *gyrB*, *recA*, *rpoB*, *secY*, and *treT* were downloaded from GenBank database. Alignments were done using ClustalW and the phylogenetic tree was created using the maximum likelihood method of MEGA5.0 (49). Bootstrap values were inferred from 100 replicates. Genomic Similarity Score (GSS) distance matrix was used to build by a neighbor-joining tree (108). GSS comprises two steps (108): (1) calculating the bit-scores of all the proteins in one organism with their orthologs in another organism based on Reciprocal Blast Hit

3. Genomic analysis of UCYN-C

(RBH); (2) normalizing them against the sum of bit-scores of the compared genes against themselves (self-bit-scores). The maximum GSS is 1, meaning the genomes are identical. For the phylogenetic analysis based on GSS, plasmid-like contigs of GS-1 and YR-1 predicted as above were excluded from this analysis.

Results and Discussion

Morphological characteristics

I isolated two strains of unicellular diazotrophic cyanobacteria (GS-1, YR-1) from intertidal zones of Goshiki beach and Yura-cho, respectively (Fig. 3-2). Cells of GS-1 and YR-1 were coccoid to rod shaped, with 2.8-5.9 and 3.1-6.7 μm in cell size, respectively. These two strains were sheathless, and divided by transverse binary fission. Based on the results and the classification of cyanobacteria (109), I assigned these strains to the genera of *Cyanothece*.

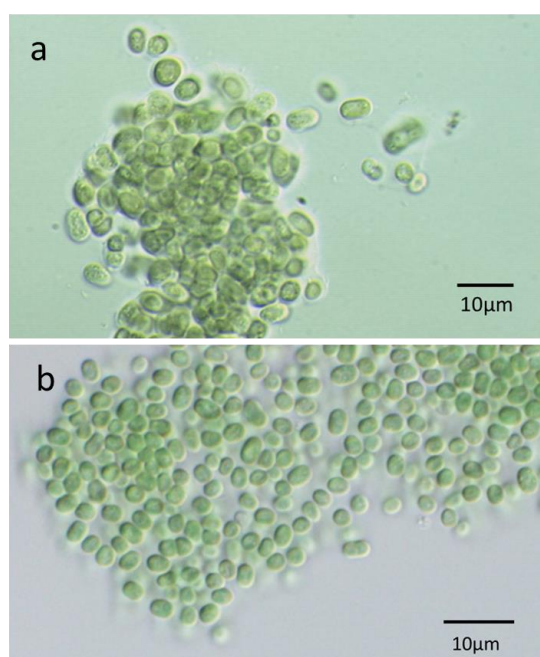


Figure 3-2. Light micrographs of unicellular diazotrophic cyanobacteria isolated from coastal waters around Japan. (a) GS-1; (b) YR-1.

Genome characteristics

Overall, draft assemblies for strains GS-1 and YR-1 yielded 490 and 207 contigs, respectively (Table 3-1). Genome sizes of GS-1 and YR-1 were 5.5 and 5.38 Mbp in size, with a G + C content of 36.9 % and 36.7 %, respectively (Table 3-1). The genome sizes of GS-1 and YR-1 were comparable to *Cyanothece* sp. ATCC51142 (Table 3-1). *Cyanothece* sp. CCY0110, *Crocospaera watsonii* WH8501, and WH0003 possessed larger genome sizes at 5.88, 6.24, and 5.90 Mbp, respectively (Table 3-1). The total numbers of ORFs are 5,376 for GS-1 and 5,120 for YR-1 (Table 3-1). Six and three contigs in the draft genome of GS-1 and YR-1, respectively, were predicted as plasmid-like contigs (Table 3-1, 3-2, 3-3). All of the plasmid-like contigs of both strains encoded *xerD* genes, which encode a site-specific recombinase.

Table 3-1. Genome characteristics of isolates, *Cyanothece* sp., and *Crocospaera watsonii*

	<i>Cyanothece</i> ATCC51142	<i>Cyanothece</i> CCY0110	GS-1	YR-1	<i>Crocospaera</i> <i>watsonii</i> WH8501	<i>Crocospaera</i> <i>watsonii</i> WH0003
Site of isolation	Coastal sea, America	Coastal sea, Tanzania	Coastal sea, Japan	Coastal sea, Japan	Atlantic Ocean	Pacific Ocean
Genome Size (bp)	5.46	5.88	5.50	5.38	6.24	5.90
G+C content (%)	37.1	36.7	36.9	36.7	37.1	37.7
Number of ORFs	5,303	5,542	5376	5,120	5,958	6,145
Number of Contigs	1	163	490	207	320	1,130
Number of Plasmids	4	N.D.	6 contigs	3 contigs	N.D.	N.D.
Reference	(97)	Stal et al. 2007 unpublished	This study	This study	(96)	(96)

N.D.: not determined

3. Genomic analysis of UCYN-C

Table 3-2. Characteristics of genes in plasmid-like contigs of GS-1.

ORF	Product length (amino acids)	COG code	COG function
NODE_29_orf_0001	99	L	Nuclease subunit of the excinuclease complex
NODE_29_orf_0002	119	-	-
NODE_29_orf_0003	128	-	-
NODE_29_orf_0004	172	-	-
NODE_29_orf_0005	75	-	-
NODE_29_orf_0006	127	-	-
NODE_29_orf_0007	107	-	-
NODE_29_orf_0008	74	-	-
NODE_29_orf_0009	138	R	Prophage maintenance system killer protein
NODE_29_orf_0010	326	-	-
NODE_29_orf_0011	164	-	-
NODE_29_orf_0012	808	-	-
NODE_29_orf_0013	129	-	-
NODE_50_orf_0001	103	-	-
NODE_50_orf_0002	197	-	-
NODE_50_orf_0003	133	R	Predicted nucleic acid-binding protein, contains PIN domain
NODE_50_orf_0004	78	-	-
NODE_50_orf_0005	194	L	Site-specific recombinase XerD
NODE_50_orf_0006	626	L	Predicted ATP-dependent endonuclease of the OLD family
NODE_50_orf_0007	207	-	-
NODE_50_orf_0008	46	-	-
NODE_124_orf_0001	286	T	Response regulator containing CheY-like receiver, AAA-type ATPase, and DNA-binding domains
NODE_124_orf_0002	251	-	-
NODE_124_orf_0003	206	-	-
NODE_124_orf_0004	139	-	-
NODE_124_orf_0005	582	-	-
NODE_124_orf_0006	93	-	-
NODE_345_orf_0001	54	-	-
NODE_345_orf_0002	114	-	-
NODE_345_orf_0003	88	-	-
NODE_345_orf_0004	176	R	Predicted acetyltransferase
NODE_345_orf_0005	101	S	Uncharacterized protein conserved in bacteria
NODE_345_orf_0006	287	-	-
NODE_703_orf_0001	170	-	-
NODE_703_orf_0002	68	-	-
NODE_703_orf_0003	52	-	-
NODE_703_orf_0004	50	-	-
NODE_804_orf_0001	105	-	-

Table 3-3. Characteristics of genes in plasmid-like contigs of YR-1.

ORF	Product length (amino acids)	COG code	COG function
NODE_3_orf_0001	629	-	-
NODE_3_orf_0002	129	-	-
NODE_3_orf_0003	74	-	-
NODE_3_orf_0004	165	R	Predicted nucleic acid-binding protein, contains PIN domain
NODE_3_orf_0005	80	-	-
NODE_3_orf_0006	192	L	Site-specific recombinase XerD
NODE_3_orf_0007	132	-	-
NODE_3_orf_0008	218	-	-
NODE_3_orf_0009	88	-	-
NODE_3_orf_0010	189	-	-
NODE_3_orf_0011	66	-	-
NODE_3_orf_0012	119	-	-
NODE_3_orf_0013	123	-	-
NODE_3_orf_0014	805	-	-
NODE_3_orf_0015	314	-	-
NODE_3_orf_0016	717	-	-
NODE_12_orf_0001	202	D	ATPases involved in chromosome partitioning
NODE_12_orf_0002	72	-	-
NODE_12_orf_0003	76	-	-
NODE_12_orf_0004	90	-	-
NODE_12_orf_0005	160	-	-
NODE_12_orf_0006	352	-	-
NODE_12_orf_0007	148	L	Nuclease subunit of the excinuclease complex
NODE_12_orf_0008	125	-	-
NODE_12_orf_0009	140	-	-
NODE_12_orf_0010	164	-	-
NODE_12_orf_0011	318	L	Site-specific recombinase XerD
NODE_12_orf_0012	82	-	-
NODE_12_orf_0013	233	-	-
NODE_13_orf_0001	120	-	-
NODE_13_orf_0002	561	-	-

Phylogenetic analysis

To identify the phylogenetic relationships between two strains isolated in this study and other unicellular diazotrophic cyanobacteria, I performed phylogenetic reconstructions based on *nifH*, 16S rRNA, five core genes, and GSS. First, the *nifH* genes of GS-1 and YR-1 were clustered with UCYN-C phylotype, clearly indicating that both strains were assigned to

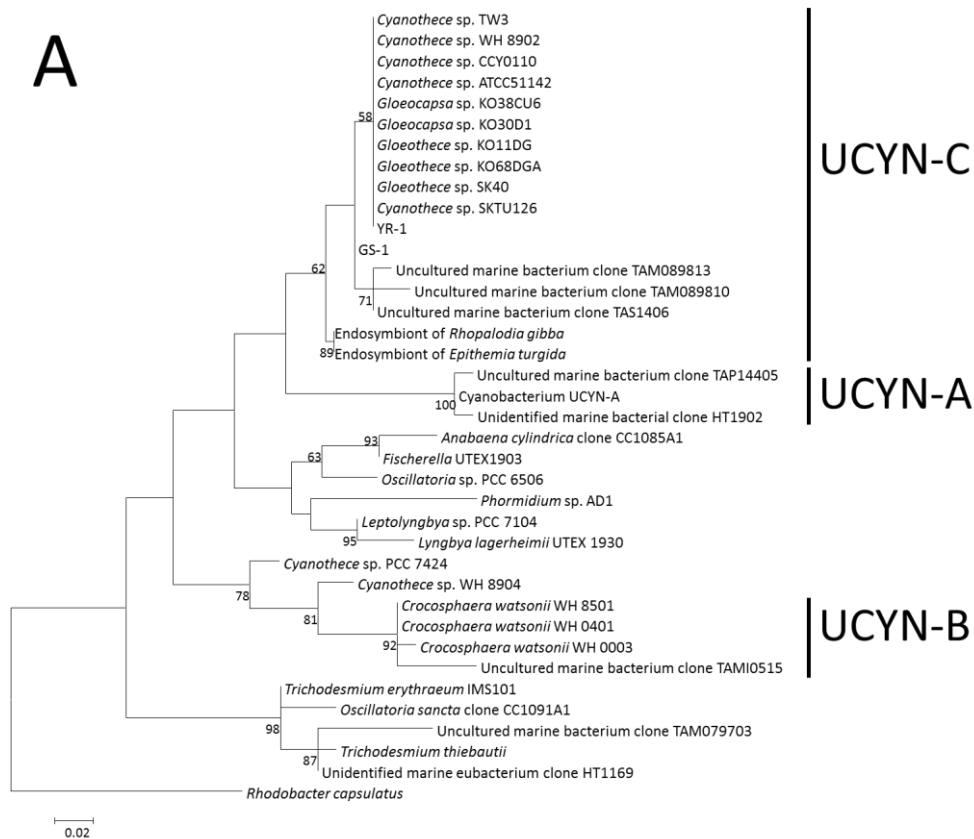
3. Genomic analysis of UCYN-C

UCYN-C (Fig. 3-3-A). In the phylogenetic tree of 16S rRNA gene, UCYN-A and B formed individual monophyletic groups (Fig. 3-3-B). However, UCYN-C was divided into three clades (Fig. 3-3-B). I referred these three groups as UCYN-C ribotype-1, 2, and 3, respectively (Fig. 3-3-B). UCYN-C ribotype-1 contains *Cyanothece* sp. ATCC51142 and CCY0110, and *Gloeocapsa* sp. KO38CU6, KO20B5, and KO30D1 (Fig. 3-3-B). UCYN-C ribotype-2 includes *Cyanothece* sp. SKTU126 and WH8904 and *Gloeotheca* sp. SK40, KO68DGA, and KO11DG (Fig. 3-3-B). UCYN-C ribotype-3 contains cyanobacterium endosymbionts of *Rhopalodia gibba* and *Epithemia turgida* (Fig. 3-3-B). The 16S rRNA genes of GS-1 and YR-1 were grouped into UCYN-C ribotype-2 (Fig. 3-3-B). In the phylogeny using five concatenated core proteins (*adk*, *gyrB*, *recA*, *rpoB*, and *secY*), GS-1 and YR-1 formed a distinct clade from the clade including *Cyanothece* sp. ATCC51142, CCY0110, *Crocospaera watsonii* WH8501, WH0401, and WH0003 (Fig. 3-3-C). Note that there are no available genomic sequence of UCYN-C ribotype-2, which includes *Cyanothece* sp. SKTU126 and WH8904 and *Gloeotheca* sp. SK40, KO68DGA, and KO11DG.

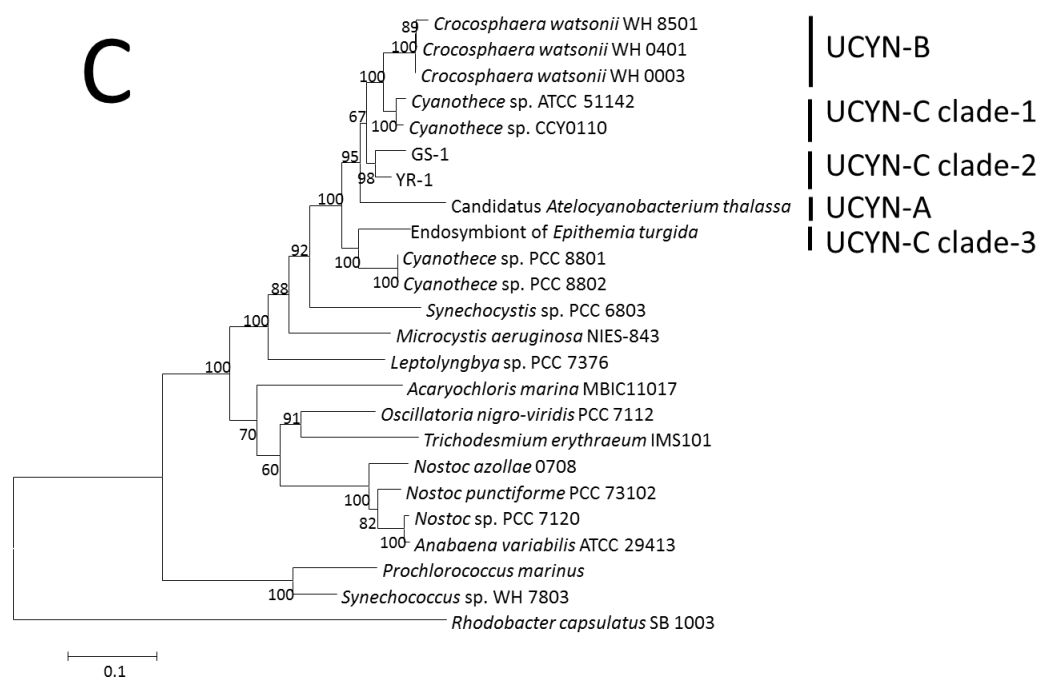
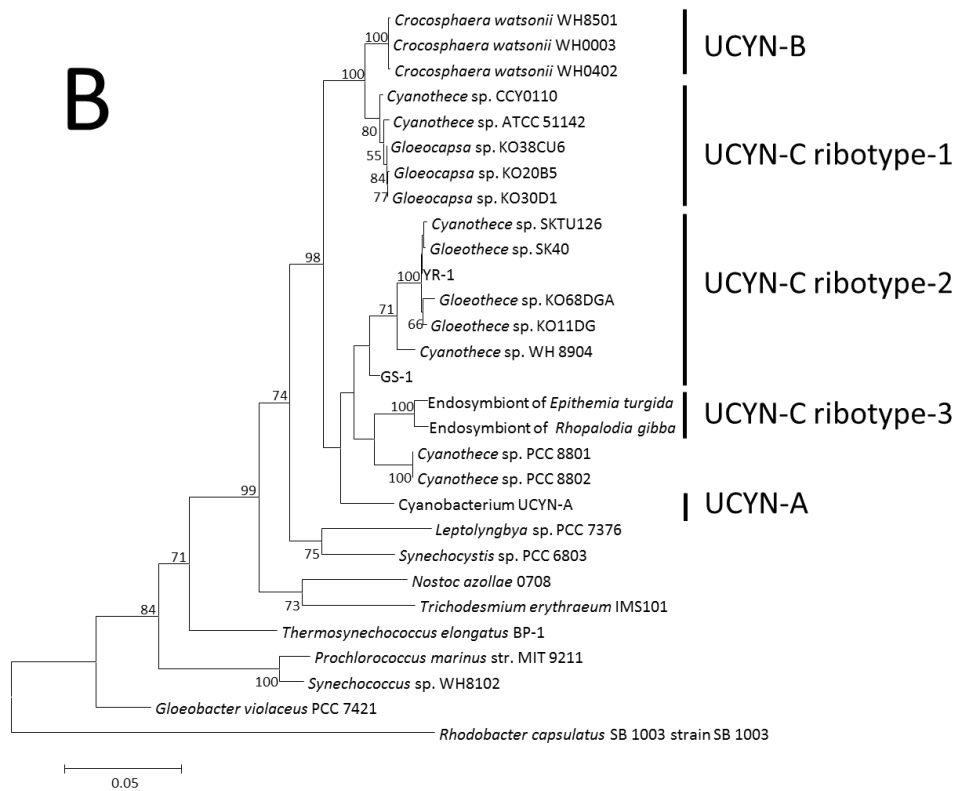
The neighbor-joining tree was reconstructed using a distance matrix of the GSS scores of GS-1, YR-1, *Cyanothece* sp. ATCC51142, CCY0110, UCYN-A, *Crocospaera watsonii* WH8501 and WH0003 of which genomes were available on public database (Fig. 3-3-D). In this phylogenic tree where UCYN-A was used as an outgroup, GS-1 and YR-1 formed a monophyletic clade branched from the endosymbiont of *Epithemia turgida* and diverged from *Cyanothece* sp. ATCC51142, CCY0110 and *C. watsonii* WH8501,

WH0401, and WH0003. *Cyanothece* sp. ATCC51142 and CCY0110 formed a cluster branched from *C. watsonii* WH8501, and WH0003. Thus, this phylogenetic analysis also supported the three clades in UCYN-C.

I referred to these groups as follows; UCYN-C clade-1 including *Cyanothece* sp. ATCC51142 and CCY0110; UCYN-C clade-2 including GS-1 and YR-1; UCYN-C clade-3 including endosymbiont of *Epithemia turgida* (Fig. 3-3-C, 3-3-D).



3. Genomic analysis of UCYN-C



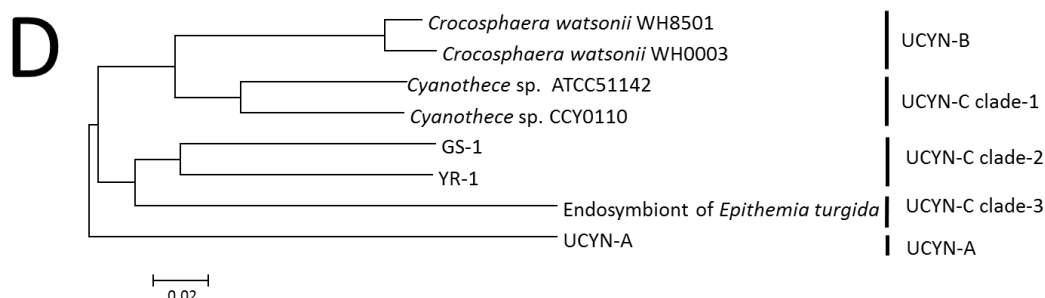


Figure 3-3. Phylogenetic reconstruction for unicellular diazotrophic cyanobacteria. **A** phylogenetic tree for *nifH* genes based on amino acid sequences. The tree was constructed by the maximum likelihood method. The bootstrap values are greater than 50% for 100 replicates are indicated at respective nodes. **B.** Phylogenetic trees for 16S rRNA genes. The tree was constructed by the maximum likelihood method. The bootstrap values are greater than 50% for 100 replicates are indicated at respective nodes. **C.** Phylogenetic trees for five core protein sequences. The tree was constructed by the maximum likelihood method. The bootstrap values are greater than 50% for 100 replicates are indicated at respective nodes. **D.** Genome Similarity Score (GSS) distance matrix plotted as a Neighbor-Joining tree.

Shared genes in unicellular diazotrophic cyanobacteria

GS-1 and YR-1 assigned as UCYN-C clade-2 formed an independent phylogenetic group from other marine unicellular nitrogen-fixing cyanobacteria. To gain insight into the characteristics of UCYN-C clade-2, I compared the genomes of UCYN-C clade-1 (*Cyanothece* sp. ATCC51142 and CCY0110), 2 (GS-1 and YR-1) and UCYN-B (*Crocospaera watsonii* WH8501 and WH0003). One thousand thirteen homologous genes were identified among them. They shared 31.1-37.4 % of ORFs in each genome. Of these ORFs, 1081 (56.5 %) were assigned to functional categories in Kyoto

3. Genomic analysis of UCYN-C

Encyclopedia of Genes and Genomes (KEGG) orthology (KO). All genomes possessed almost all of key metabolism for cyanobacteria including photosynthesis, nitrogen fixation, and central carbon metabolism (Fig. 3-4). They also contained genes encoding chlorophyll biosynthesis, Calvin cycle pathways, and carbon fixation pathways (Fig. 3-4). They possessed a large nitrogenase (*nif*) gene cluster including genes of *nifHDK*, key enzymes in nitrogen fixation (Fig. 3-5). Generally, cyanobacteria possess an incomplete tricarboxylic acid (TCA) cycle without 2-oxoglutarate dehydrogenase (110). Using 2-oxoglutarate dehydrogenase and succinyl-CoA synthetase, cyanobacteria complement the pathway by directly conversion of 2-oxoglutarate to succinate (110). The genome of the six strains used in this study possessed the genes involved in incomplete TCA cycle, 2-oxoglutarate dehydrogenase and succinyl-CoA synthetase.

Unique genes in the genome of GS-1 and YR-1

To unveil the characteristics of UCYN-C clade-2, I identified unique genes of UCYN-C clade-2 compared with genes encoded in the genomes of *Cyanothece* sp. ATCC51142, CCY0110, *Crocospaera watsonii* WH8501, and WH0003. The genomes of two UCYN-C clade-2 isolates harbored 239 unique genes, which made up 4.4 and 4.7 % of protein-coding genes in the genome of GS-1 and YR-1, respectively. Of these genes, 125 genes (52.3 %) were classified into COG categories (Fig. 3-6). Except for two categories, R (general function prediction only) and S (function unknown), the number of genes assigned to K category (Transcription) (11 genes) and T (Signal

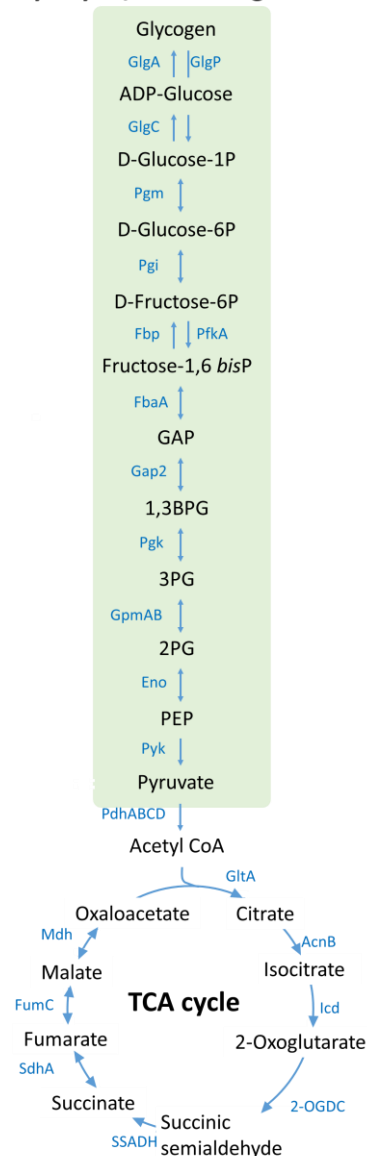
Glycolysis/Gluconeogenesis

Fig. 3-4. Schematic overview of key metabolic processes. Each arrow shows the direction of the reaction.

transduction mechanisms) (10 genes) were superordinated. Categories K and T involve in the mechanisms for sensing and responding to environmental stimuli. UCYN-C clade-2 strains may have an extensive range of cellular responses to environmental stimuli by these unique genes. Two hundred sixty-one unique genes were identified in the genome of UCYN-C clade-1.

3. Genomic analysis of UCYN-C

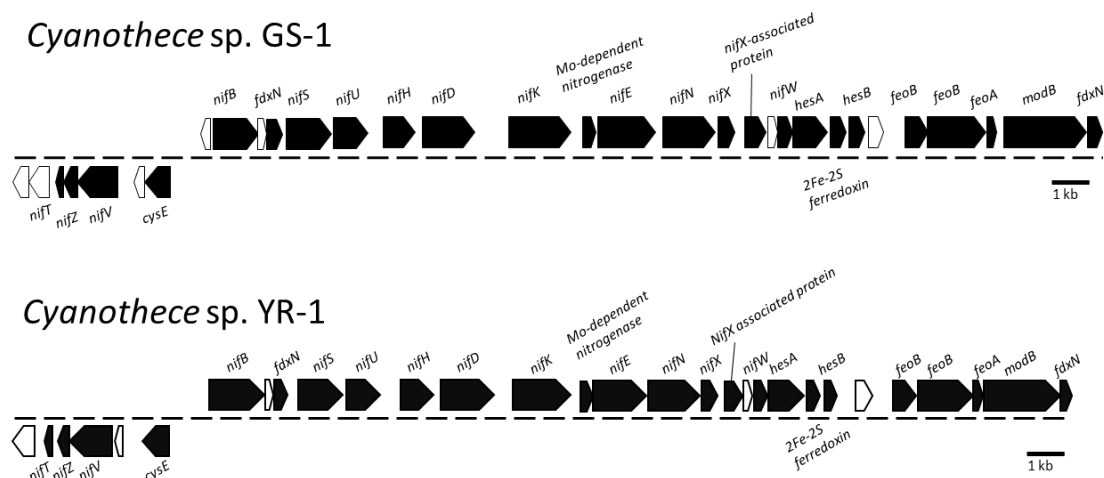


Figure 3-5. Alignment of clusters of nitrogen fixation-related genes in the two strains.

Black arrows indicate genes assigned to functional categories and white arrows correspond to hypothetical genes.

These correspond to 4.7-4.9 % of protein-coding genes in the genome of *Cyanothece* sp. ATCC51142 and CCY0110. This group contained larger number of genes assigned to category L (Replication, recombination and repair) than UCYN-C clade-2 (Fig. 3-6). Both strains of UCYN-C clade-1 are known to have phosphoenolpyruvate carboxykinase (PEPCK) which involved in the conversion step of oxaloacetate to phosphoenolpyruvate in gluconeogenesis (97). In contrast to these UCYN-C clade-1 strains, this gene was not encoded in the genomes of UCYN-C clade-2 and UCYN-B.

UCYN-B group possessed larger number of unique genes (846 genes) compared to UCYN-C clade-1 and 2. The most remarkable difference in the number of COG between UCYN-B and two UCYN-C groups were category L (Replication, recombination and repair) (Fig. 3-6). UCYN-B possessed numerous transposase genes in this category. The genes of phosphorus and iron transporter in category P were also abundant in the genomes of

UCYN-B (Secondary metabolites biosynthesis, transport and catabolism). These genes may be involved in the adaptation to the oligotrophic open ocean (94).

The genome of UCYN-C clade-2 was characterized with various metabolisms of compatible solutes. Compatible solutes are small molecules that accumulate in the cells to equilibrating the external osmotic pressure (111). Cyanobacteria are known to have five substances as compatible solutes: sucrose, trehalose, glucosylglycerol, glucosylglycerate, and glycine betaine (111).

The genomes of UCYN-C clade-1 and 2 encoded the sucrose-phosphate phosphatase (SPP) which supplies sucrose and inorganic phosphate by hydrolysis of sucrose-6-phosphate, suggesting their ability of sucrose biosynthesis (112) (Fig. 3-7). Ability of sucrose biosynthesis is widespread in both freshwater and marine cyanobacteria, e.g. *Anabaena*, *Nostoc*, *Cyanothece*, and *Synechococcus* (113). Both UCYN-B strains did not harbor the sucrose biosynthesis pathway. GS-1 and YR-1 possessed the gene encoding sucrose synthase (SuS) which catalyzes a reversible reaction but preferentially converts sucrose into fructose and UDP-glucose (114, 115). No gene encoding SuS was found in the UCYN-C clade-1 and UCYN-B strains. In cyanobacteria, the SuS gene is often observed in genomes of filamentous nitrogen-fixing cyanobacteria, but rarely in genomes of unicellular cyanobacteria (116). The SuS activity in unicellular cyanobacteria, such as *Anabaena* sp., *Microcystis aeruginosa* sp. PCC7806 and *Gloeobacter violaceus* PCC7421 are increased after addition of salt, suggesting the

3. Genomic analysis of UCYN-C

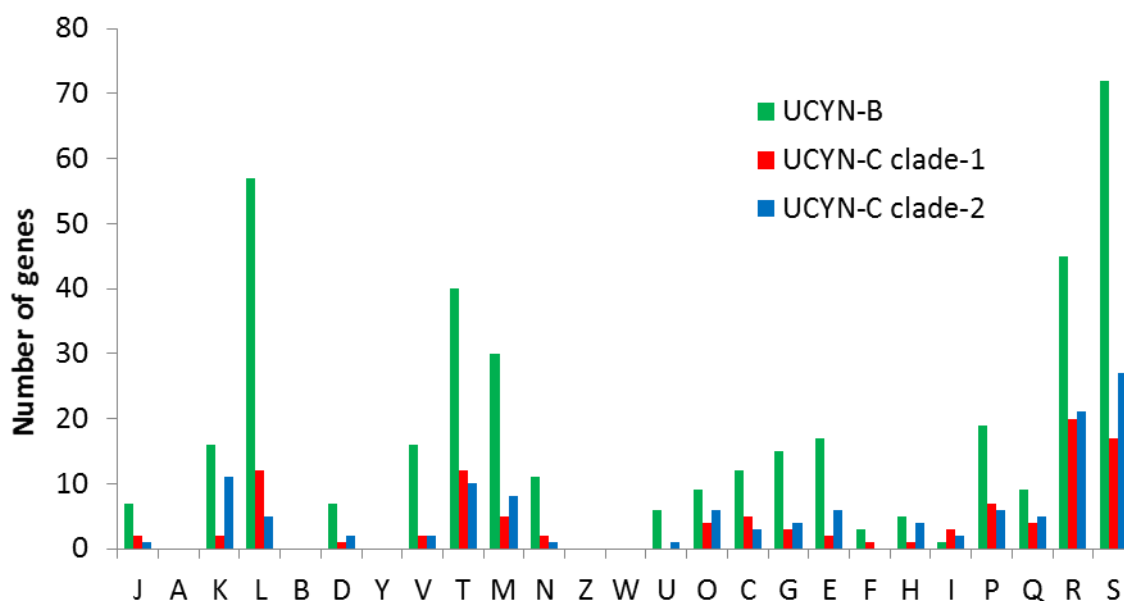


Figure 3-6. Numbers of genes assigned to COG functional categories in unique genes of UCYN-C clade-1, 2, and UCYN-B. The one letter code for COG categories is the following: ribosomal structure and biogenesis, (J); RNA processing and modification, (A); Transcription, (K); Replication, recombination and repair, (L); Chromatin structure and dynamics, (B); Cell cycle control, cell division, chromosome partitioning, (D); Defense mechanisms, (V); Signal transduction mechanisms, (T); Cell wall/membrane/envelope biogenesis, (M); Cell motility, (N); Intracellular trafficking, secretion, and vesicular transport, (U); Posttranslational modification, protein turnover, chaperones, (O); Energy production and conversion, (C); Carbohydrate transport and metabolism, (G); Amino acid transport and metabolism, (E); Nucleotide transport and metabolism, (F); Coenzyme transport and metabolism, (H); Lipid transport and metabolism, (I); Inorganic ion transport and metabolism, (P); Secondary metabolites biosynthesis, transport and catabolism, (Q); General function prediction only, (R); and Function unknown, (S).

potential importance of SuS enzyme in the salt-stressed conditions (116, 117). The simultaneous expression of sucrose biosynthetic and lytic genes may enable cells to perform high turnover of sucrose production and degradation in response to sudden changes of salt stress (117). Additionally, YR-1 possessed sucrose-phosphate synthase (SPS). The SPS enzyme is well characterized in *Anabaena* sp. and *Synechocystis* PCC 6803 (112, 118, 119).

This enzyme catalyzes sucrose-phosphate biosynthesis pathway from A/U/GDP-Glucose and fructose-6-phosphate (112, 118, 119).

Trehalose is also utilized as compatible solutes and the biosynthesis pathways are found in many cyanobacteria (113). For trehalose biosynthesis pathway, the genome of GS-1 and YR-1 contained TreY/TreZ pathway (Fig. 3-7), whereas UCYN-C clade-1 and UCYN-B strains lacked this pathway. This pathway was also found in cyanobacteria such as *Nostoc* 7120, *Anabaena variabilis* and *Nostoc punctiforme* (111). UCYN-B strains harbored *otsAB* gene predicted as an alternative pathway for trehalose biosynthesis (Fig. 3-7). In *Crocospaera watsonii* WH8501, expression of the *otsAB* gene is promoted under salt stress condition (120). YR-1 possessed an

	UCYN-C clade-1		UCYN-C clade-2		UCYN-B	
	ATCC51142	CCY0110	GS-1	YR-1	WH8501	WH0003
SPS (GTD)						
SPS (GTD-PHD)						
SPP						
SuS						
TreX						
TreY						
TreZ						
TreA						
TreT						
otsAB						
GgpS						
GgpP						
GpgS						
GpgP						
GsmT						
Dmt						

Figure 3-7. Genes related to metabolism of compatible solutes. Gray indicates presence and white indicates absence. ATCC51142, *Cyanothece* sp. ATCC51142; CCY0110, *Cyanothece* sp. CCY0110; WH8501, *Crocospaera watsonii* WH8501; WH0003, *Crocospaera watsonii* WH0003

3. Genomic analysis of UCYN-C

additional gene, *treT* gene for possible trehalose biosynthesis (Fig. 3-7). The TreT enzyme catalyzes the reversible formation of trehalose from NDP-glucose (ADP-, UDP-, and GDP-glucose) and glucose (121–123). The *treT* gene was contiguous to the other genes involved in TreY/TreZ pathway in the YR-1 genome (Fig. 3-8). In the corresponding region of GS-1 genome, *treT* was absent and the context was *treZtreY-treA* (Fig. 3-8). The contiguous cluster including *treT*, *treY*, and *treZ* genes is also observed in other bacteria such as *Synthrophobacter fumaroxidans*, *Pelotomaculum thermopropionicum*, and *Moorella thermoacetica* (123). The *treT* gene was first identified in a gene cluster of hyperthermophilic archaea *Thermococcus litoralis* (121). Until now, this gene have been found in hyperthermophilic archaea *Pyrococcus horikoshii*, the halotolerant thermophilic bacterium *Rubrobacter xylanophilus*, and delta Proteobacterium *Myxococcus xanthus* (Fig. 3-9) (123). However, no study has referred to *treT* gene in cyanobacteria. The residues in the active site (T49, D134, H155, D274/D275, and E326) were conserved among YR-1 and *P. horikoshii*, suggesting active catalysis in the cells of YR-1 (Fig. 3-10) (122).

Despite the absence of biosynthesis pathway of glucosylglucerate in other three UCYN-C strains, YR-1 harbored *gpgS* and *gpgP* genes, which synthesize glucosylglucerate from NDP-glucose and glycerate-3-phosphate (124). In contrast to other compatible solutes, glucosylglucerate is negatively charged, thereby considered to serve as counterion to the increase of K⁺ instead of glutamate under salt-stressed and nitrogen-deficient conditions (125). These versatile biosynthesis pathways for compatible solutes suggest

tolerance of YR-1 in highly salt-stressed environments. In conclusion, the present study revealed that UCYN-C was subdivided into three groups (UCYN-C clade-1, 2, and 3) based on phylogenetic analysis of five core genes and GSS. In particular, two strains (GS-1 and YR-1) of UCYN-C clade-2 had versatile metabolic pathways of compatible solutes, which enable them to cope with various environmental stresses. This characteristics may be involved in adaptation to coastal areas, where require them to be responsive to sudden changes and extreme conditions such as high light, osmotic changes, and oxidative stress.

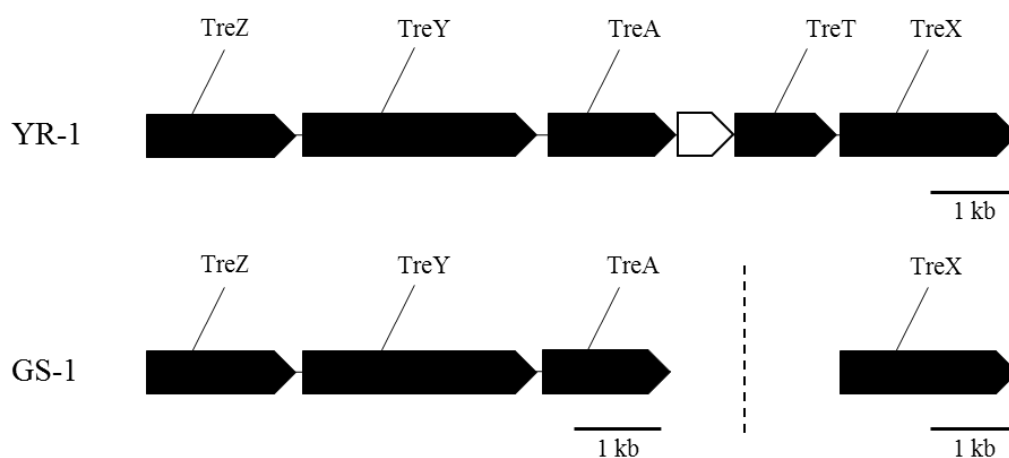


Figure 3-8. Trehalose biosynthesis gene cluster in YR-1 and GS-1. Black arrows indicate genes assigned to functional categories and white arrow correspond to hypothetical genes.

3. Genomic analysis of UCYN-C

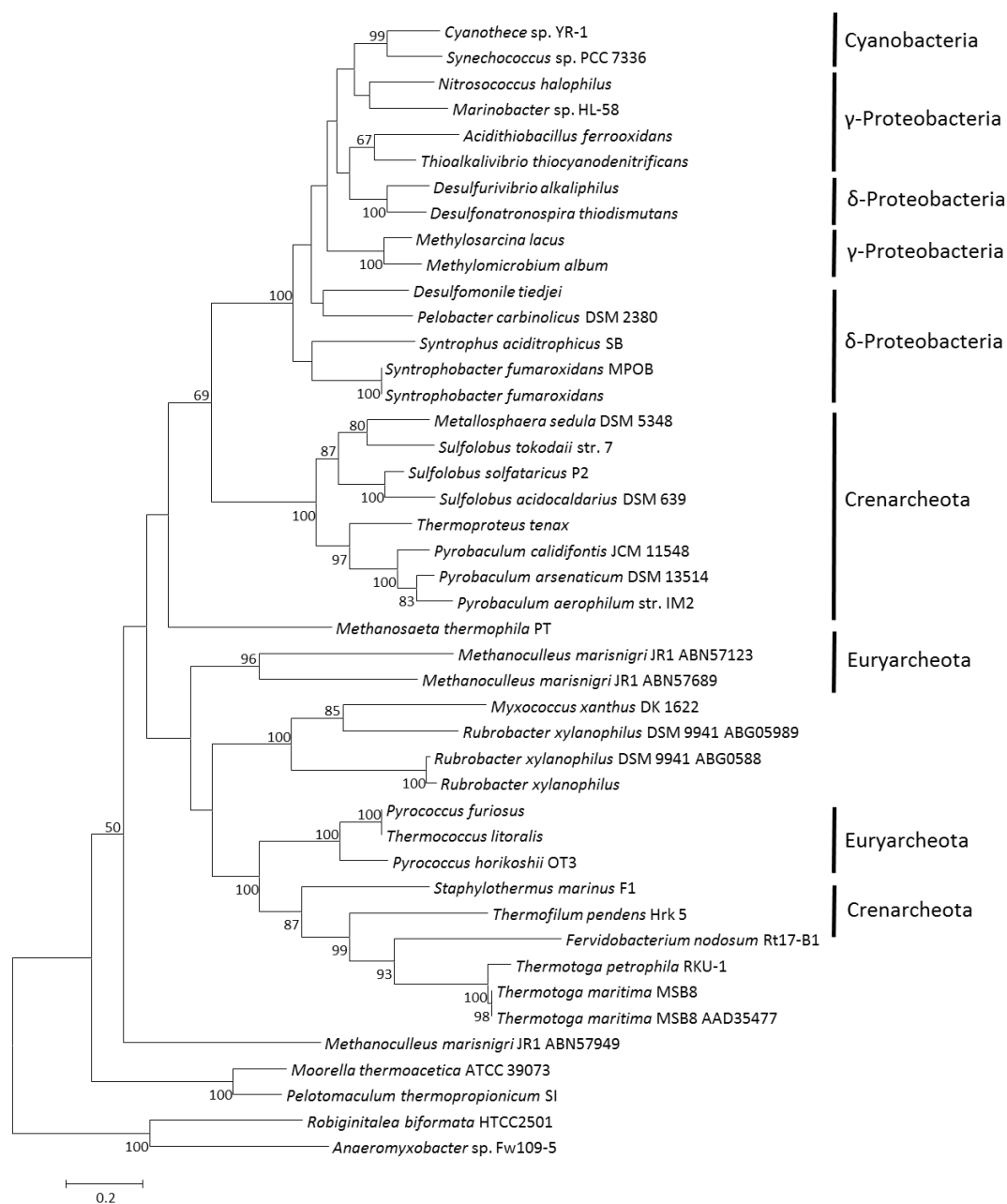


Figure 3-9. Phylogenetic tree of deduced amino acid sequences from TreT enzymes. The tree was constructed by the maximum likelihood method. The bootstrap values are greater than 50% for 100 replicates are indicated at respective nodes.

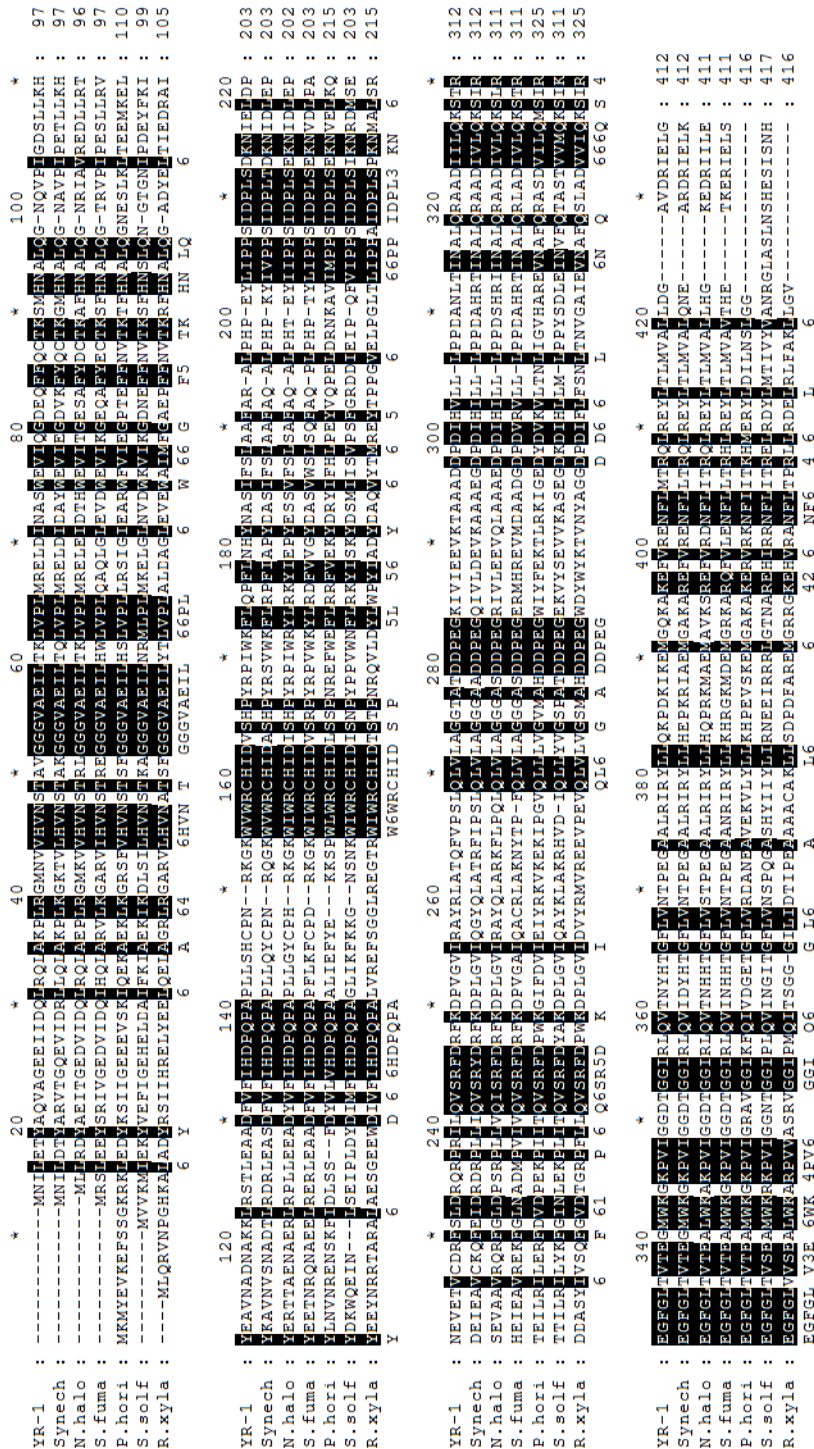


Figure 3-10. Alignment of amino acid sequences of *treT* gene. The conserved motifs are shown in black. The accession numbers for these sequences are as follows: WP_017327448 (*Synechococcus* PCC7336), WP_013034002 (*Nitrosococcus halophilus*), ABK18029 (*Syntrophobacter fumaroxidans*), BAA30133 (*Pyrococcus horikoshii*), AAK42730 (*Sulfolobus solfataricus* P2), ACJ76775 (*Rubrobacter xylanophilus*)

Chapter 4

Integration and outlook

Unicellular diazotrophic cyanobacteria are of great interest for their ability to provide carbon and nitrogen sources in the open ocean. Whereas their importance is well known in the open ocean, little is known about them in the coastal area.

In chapter 2, I examined the diversity and distribution of unicellular diazotrophic cyanobacteria in the Japan Sea and the Seto Inland Sea. Heterotrophic diazotrophs including UCYN-A were dominated in the Japan Sea. In the Seto Inland Sea, UCYN-C was detected with higher abundances compared to the open oceans through the year, suggesting that UCYN-C was inhabit in the coastal sea. Although UCYN-C is a monophyletic group in the *nifH* gene phylogenetic tree, the clone analysis of 16S rRNA gene suggested that UCYN-C consisted of three sub-groups (ribotype-1, 2, and 3).

In chapter 3, I succeeded to isolate two unicellular diazotrophic cyanobacteria (GS-1 and YR-1) from intertidal zones of the Seto Inland Sea. The *nifH* genes of these two strains were clustered with UCYN-C. Phylogenetic analysis based on five core genes and Genomic Similarity Score (GSS) strongly suggested that UCYN-C diverged into three groups (UCYN-C clade-1, 2, and 3) and two strains were assigned to UCYN-C clade-2. The genomes of GS-1 and YR-1 were characterized with versatile metabolic pathway of compatible solutes, suggesting their adaptation to coastal areas

where require microorganisms to be responsive to sudden changes of environmental conditions such as salinity.

This study revealed the ecological and genomic characteristics of UCYN-C in the coastal sea. It becomes important to provide insight into the evolutionally relationships of unicellular diazotrophic cyanobacteria.

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