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Title

The first assessment of cyanobacterial and diazotrophic diversities in the Japan Sea

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Abstract

The diversity of cyanobacteria and dizaotrophs in the Japan Sea was investigated by analyzing sequences of cyanobacterial 16S rRNA genes and nitrogen fixation genes (*nifH*) from seawater sampled at a depth ranging from surface to 100 m at two stations. Of the 107 cyanobacterial 16S rRNA gene sequences obtained, 97 sequences and 3 sequences were assigned to *Synechococcus* sub-cluster 5.1 and *Prochlorococcus* HL (II), respectively. Unlike other oceanic regions, compositional ratio of the sequences assignable to *Synechococcus* sub-cluster 5.3 was relatively high (8%). No sequences of diazotrophic cyanobacteria were found in the cyanobacterial 16S rRNA genes. In *nifH* clone library (36 sequences), 10 sequences were identified as a UCYN-A group of diazotrophic cyanobacteria. Residual 26 sequences (72%) were assigned to proteobacteria. These results suggest heterotrophic bacteria including UCYN-A dominated in diazotrophic community in the Japan Sea. Our study revealed the dominance of *Synechococcus* in cyanobacterial community and (photo)heterotrophic diazotrophs in diazotrophic community in the Japan Sea, suggesting its unique characteristics.

Key words: cyanobacteria, diazotroph, Japan Sea, *Synechococcus* sub-cluster 5.1, *Synechococcus* sub-cluster 5.3, UCYN-A, *nifH*

Introduction

Marine cyanobacteria, especially two genera, *Prochlorococcus* and *Synechococcus*, significantly contribute to 32-80% of the primary productivity in various oceanic regions [1–3]. Some other cyanobacteria retain ability for N_2 fixation which is the process of reducing N_2 gas to biologically available ammonium (referred to as diazotrophic cyanobacteria). The biological N_2 fixation is carried out by several species of bacteria and archaea, but mainly by diazotrophic cyanobacteria in the ocean [4]. Therefore, marine cyanobacteria play an important role in carbon cycle as well as in nitrogen cycle.

Among marine diazotrophic cyanobacteria, filamentous nonheterocystous *Trichodesmium* spp. are the most abundant diazorophs in the ocean. *Trichodesmium* spp. are observed throughout the tropical and subtropical Atlantic, Pacific, Indian oceans, and East and South China seas [5,6]. In North Atlantic Ocean, their contribution to nitrogen inputs can be as great or greater than nitrate advection from deep sea water [7]. In addition, heterocystous cyanobacterial diatom symbionts such as *Richelia* spp. and *Calothrix* spp. have also been considered to make a significant contribution to N_2 fixation in North Atlantic Ocean and North Pacific Ocean [8,9]. They are detected in tropical and subtropical regions in North Pacific Ocean and North Atlantic Ocean [10,11]. Recently, some unicellular cyanobacteria have been recognized as important diazotrophs in the ocean [12–14]. 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 bisphosphate carboxylase (Rubisco), and tricarboxylic acid cycle, therefore UCYN-A is considered to be photoheterotroph [15].

UCYN-A is shown to be more abundant than other two groups with broad latitudinal range in the North and South Pacific Oceans[10,14]. Unlike UCYN-A, UCYN-B including *Crocosphaera watsonii* WH8501 shows a patchy distribution in the ocean, and is detected in South Pacific, Eastern Atlantic, and North Pacific [10,14,16]. Compared to the other two unicellular diazotrophic cyanobacterial groups, UCYN-C including *Cyanothece* TW3 has less abundance in the open ocean [17,18].

The Japan Sea is a semi-enclosed marginal sea in the northwest Pacific 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 [19]. Its water exchange is limited in the upper layer because the Japan Sea is connected to adjacent seas with the shallow straits [19]. 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 Japan Sea.

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

Materials and Methods

Seawater collection

Sampling was conducted during Augaust-2011 on board the R/V Tajima at Station 2 (35°50.2N,

134°19.8E, 275 m depth) and Station 7 (37°20.2N, 134°19.8E, 2591 m depth) in the Japan Sea. Seawater was collected in 2l Niskin bottles mounted on a CTD (SBE-9 plus, Sea Bird Electronics) rosette from 0, 10, 30, 75, 100m, and deep chlorophyll maximum (DCM, near 50m). We measured profiles of temperature, salinity, and concentrations of chlorophyll a using the CTD.

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. [20] (Table 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 10x 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 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, we performed nested PCR using the degenerate oligonucleotide primer pair described by Zehr and Turner [21] (Table 1). The reaction mixtures were made with 5 µl of 10x 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). We sequenced a total of 107 and 36 clones containing cyanobacterial 16S rRNA gene and *nifH* inserts, respectively. Basic Local Alignment Search Tool (BLAST) comparisons were used to find the sequence closely related to those amplified from our samples with the National Center for Biotechnology Information (NCBI) database [22]. 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 [23].

Nucleotide sequence accession numbers

The 16S rRNA gene sequneces 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. 1). The water temperature in surface was relatively constant (25~26 °C) in Stn. 2 and Stn. 7, and water

113 temperature below 30 m depth declined gradually to 8.2 and 4.5 °C at 200 m depth, respectively (Fig. 1).
114 Salinity of surface water in Stn. 2 was around 32.7 psu, which was a bit lower than that in Stn. 7 (33.3
115 psu) (Fig. 1). This lower salinity of Stn. 2 was considered to be derived from freshwater input from the
116 terrestrial area. In each station, the concentrations of chlorophyll *a*, the maximum of which was observed
117 around 50 m depth, were extremely low (< 0.2 μM) compared with other oceanic regions [24].
118 We obtained a total of 107 clones of cyanobacterial 16S rRNA gene sequences from 12 samples in the
119 Japan Sea (Table 2). Of these, 96 sequences (90%) and 3 sequences (3%) were assignable (~99 %
120 nucleotide similarity) to *Synechococcus* sub-cluster 5.1, and *Prochlorococcus* HL-(high-light) (II),
121 respectively. In each station and depth, *Synechococcus* sub-cluster 5.1 dominated the cyanobactrial
122 community. Of the 96 sequences assignable to *Synechococcus* sub-cluster 5.1, 6 sequences showed a
123 100% identity with *Synechococcus* KORDI-19 which was isolated from the Japan Sea [25]. Residual 8
124 sequences (8%) were assignable to *Synechococcus* sub-cluster 5.3. This type of sequences was recovered
125 from samples ranging from 10-75 m depth in each station. In general, *Prochlorococcus* has two distinct
126 ecotypes, the high-light(HL)- and low-light(LL)-adapted ecotypes[26]. The sequences similar to a
127 subgroup of the HL ecotype, *Prochlorococcus* HL (II) were obtained only from surface water (0 m),
128 which is comparable with the previous reports that the HL-adapted ecotype generally distributes the upper
129 regions of the euphotic zone [26–28]. No sequences assignable to the LL-*Prochlorococcus* were obtained,
130 suggesting low abundance of LL-*Prochlorococcus* in the subsurface areas (Table 2).
131 We performed the *nifH*-targeting PCR amplification against the same 12 samples in the Japan Sea (Table
132 3). We 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* sequences were divided into five groups (Fig. 2). 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 30m, DCM, and 75m. This result is consistent with the observations that the peak abundances of UCYN-A was observed around 50 m depth in the Pacific Ocean [14]. 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). In β-Proteobacteria, the sequences (Group 3) were recovered from Stn. 2 at 10m and Stn. 7 at 10m with 100% amino acid identity to *Burkholderia vietnamensis* G4 and sequences recovered from North Pacific Ocean. *Burkholderia vietnamensis* were also observed in reservoirs and agricultural settings [29]. Of sequences assigned to γ- Proteobacteria, 12 sequences were highly similar to protein sequences recovered from Atlantic Ocean, South China Sea and Mediterranean Sea.

Discussion

Marine *Synechococcus* is phylogenetically subdivided into three major subclusters, 5.1, 5.2, and 5.3. *Synechococcus* sub-cluster 5.1 dominates *Synechococcus* community in the open ocean [30]. 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

153 conditions [2,31–33]. Although surface water of the Japan Sea is extremely nutrient-limited environment
154 (eg. under-detection limit for PO_4, NO_3, NO_2) [34], our data showed the dominance of *Synechococcus* in
155 the cyanobacterial community. This discrepancy suggests unknown factors controlling their distribution
156 in the Japan Sea, which will be the subject of future work.
157 *Synechococcus* sub-cluster 5.3 accounted for 8% of the clone library with vertical distribution in each
158 station [30, 35, 36] (Table 2). Huang et al. [30] report that their distribution may be restricted to certain
159 oceanic regions because few sequences assignable to *Synechococcus* sub-cluster 5.3 were detected in the
160 GOS database. However, their distribution and characteristics is not well understood.
161 No sequences belonging to diazotrophic cyanobacteria were detected in the cyanobacterial 16S rRNA
162 gene clone library. It is not surprising because the abundance of diazotrophic cyanobacteria is two to three
163 orders of magnitude lower than that of *Synechococcu*s or *Prochlorococcus* in most oceanic regions
164 [10,18,37,38]. In *nifH* clone library, sequences belonging to UCYN-A were obtained from Stn. 7 at 30m,
165 DCM, 75m depths. Due to the elevated phytoplankton biomass around DCM, the carbon source may be
166 more readily available for UCYN-A lacking the carbon fixation enzyme Rubisco. Sequences related to
167 other unicellular cyanobacteria (i.e. UCYN-B and C) were not detected in our *nifH* clone library.
168 Moisander et al. [37] suggest the possibility of limiting the growth of UCYN-A and B by differential
169 grazing pressure due to the smaller cell size of UCYN-A compared to UCYN-B. However, the factors of
170 limiting the distribution of the unicellular diazotrophic cyanobacterial groups have not unraveled yet well.
171 Sequences of filamentous cyanobacteria were also not detected in our clone libraries (Table 2).
172 Diazotrophic cyanobacteria require a lot of iron as cofactor of nitrogenase and phosphorus for energy

173 generation during diazotrophic growth [6]. As unicellular cyanobacteria have high surface/volume ratio
174 comparing to filamentous cyanobacteria, they may efficiently grow in oligotrophic environment because
175 phosphate uptake efficiency is dependent upon cell size [39]. Therefore, the presence of UCYN-A and no
176 detection of filamentous cyanobacteria may be due to the extremely nutrient poor condition in the Japan
177 Sea with iron repletion [34].
178 About two-thirds of *nifH* clones were derived from proteobacteria, and especisally sequences most
179 related to γ-Proteobacteria were abundant. Although the sequences belonging to γ-Proteobacteria are
180 detected in various oceans such as North Pacific Ocean and North Atlantic Ocean with accounting for a
181 substantial fraction of the DNA/RNA *nifH* clone libraries, it is still unknown what extent these
182 heterotrophic bacteria contribute to the net nitrtogen input in the ocean [40,41]. However, despite the low
183 N_2 fixation rates, heterotrophic bacteria dominated the diazotrophic community in the South Pacific with
184 significant nitrogen input into the ocean [42]. Therefore, given the absence of other diazotrophic
185 cyanobacteria, heterotrophic bacteria may account for substantial fraction of nitrogen input through N_2
186 fixation in the Japan Sea.
187 To our knowledge, this is the first study describing molecular cyanobacterial 16S rRNA genes and *nifH*
188 diversity in the Japan Sea. Our data showed wide distribution of *Synechococcus* sub-cluster 5.3 in the
189 Japan Sea vertically. Considering patchy distribution of *Synechococcus* sub-cluster 5.3 in other oceanic
190 regions [30], the Japan Sea may be one of the suitable environments for them to propagate. The
191 dominance of *Synechococcus* with oligotrophic well-stratified condition environment was contradict to
192 the general distribution of cyanobacteria in the ocean, therefore these results may reflect the unique

193 characteristics in the Japan Sea. We also showed that heterotrophic bacteria dominate the diazotrophic
194 community. Further studies are required for determining what factors affect the vertical and horizontal
195 distributions of different groups of these microorganisms in the Japan Sea.

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197

198 **Acknowledgments**

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330 **Figure Legends**

331

332 **Figure 1**

333 Vertical distributions (0-200 m) of temperature, salinity, chlorophyll *a* concentrations (mg m^{-3}) at Stn. 2

334 (solid line) and Stn. 7 (dotted line) in Japan Sea on August 2011. The asterisk shows that fluorescent

335 intensity was below zero.

336

337

338 **Figure 2**

339 Phylogenetic trees for *nifH*-deduced amino acid sequences from each sample constructed using the

340 neighbor-joining method. Bootstrap values greater than 50% for 1000 replicates are indicated at

341 respective nodes. Database entries for sequences are as follows: *Azotobacter chroococcum*

342 strain NRRL B-14637 (AAR11505); *Pseudomonas stutzeri* A1501 (YP_001171863); *Vibrio*

343 *diazotrophicus* clone CC1104A1 (AAP48983); *Sinorhizobium meliloti* bv (ABC67302);

344 *Azospirillum brasilense* (P17303); *Rhodopseudomonas* sp. 99D (BAC07290); *Rhodobacter*

345 *sphaeroides* 2.4.1 (YP_353614); *Burkholderia vietnamiensis* G4 (YP_001115195);

346 *Rhodoferax antarcticus* strain ANT.BR. clone 685K1_sp6 (ABI14697); *Sphingomonas*

347 *azotifigens* (BAE71134); *Bradyrhizobium japonicum* USDA 110 (BAC47034); *Xanthobacter*

348 *autotrophicus* Py2 (YP_001415004); *Crocosphaera watsonii* WH 8501 (AAP48976);

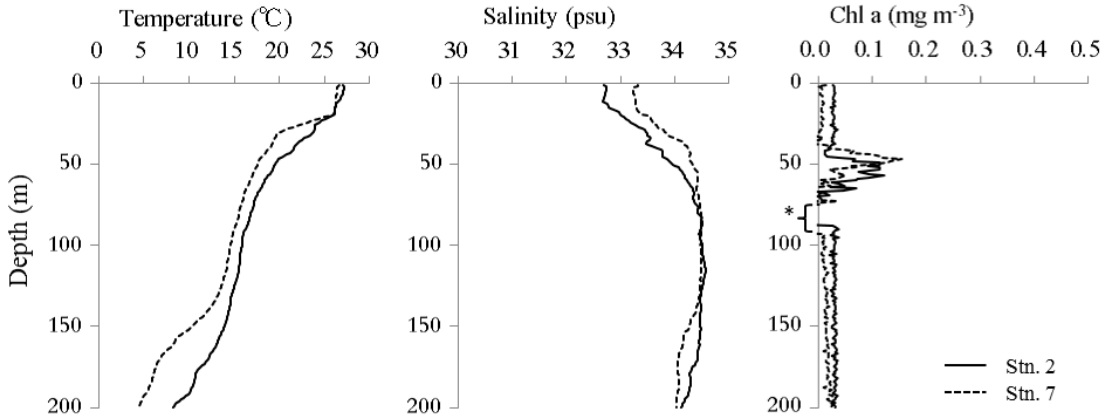
349 *Anabaena cylindrica* clone CC1085A1 (AAP48968); *Lyngbya lagerheimii* UTEX 1930

350 (AAA19029); *Cyanothece* sp. ATCC51142 (AAB61408); *Gloeothece* sp. SK40 (BAF51659);

351 *Gloeothece* 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)

Hashimoto et al., Figure 1



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387 Hashimoto et al., Figure 2

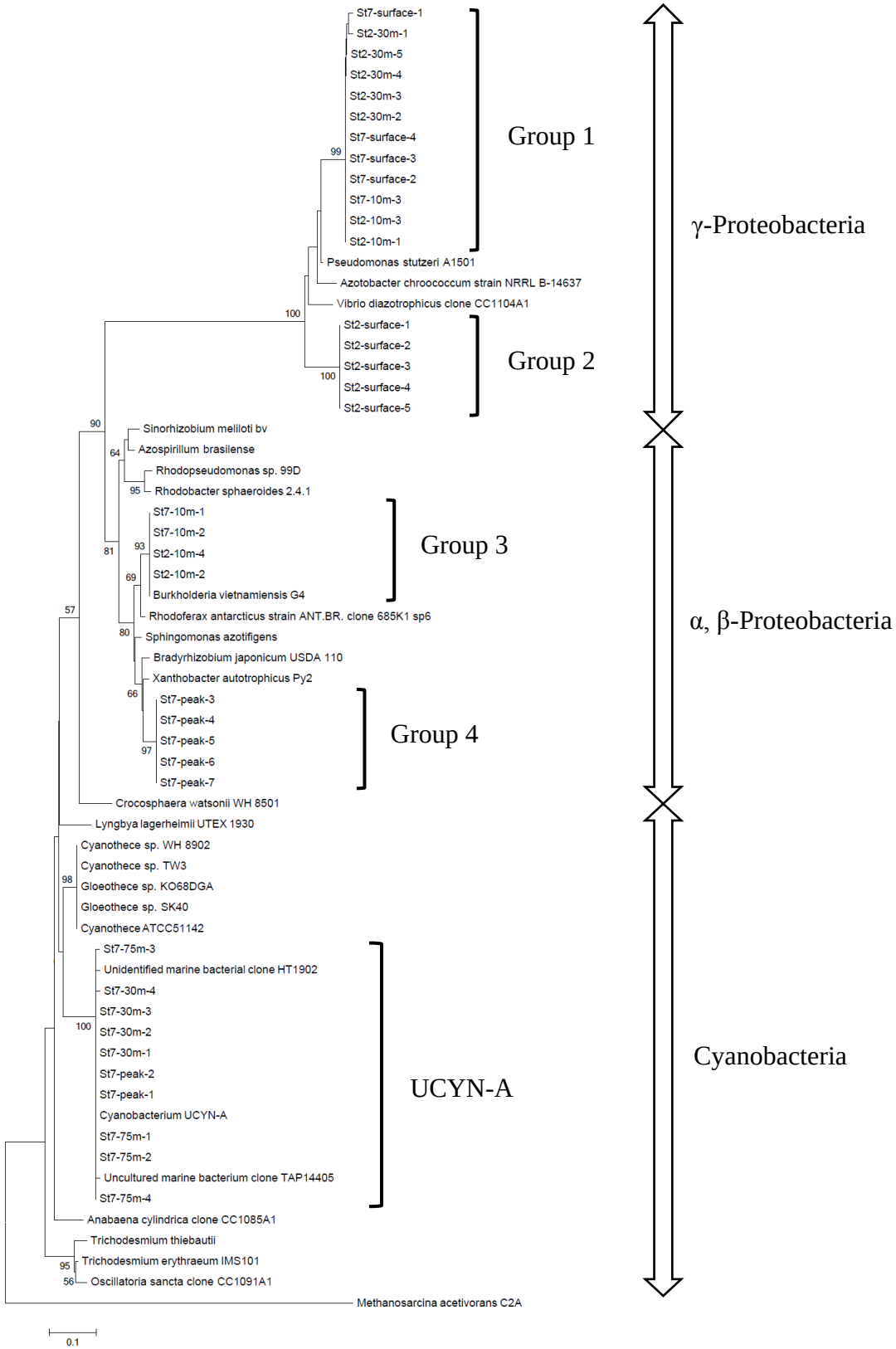

Table 1. Cyanobacterial 16S rRNA and *nifH* primers used in this study.

Primer	Target site	Sequence(5'-3')	Reference
CYA359F	16S rRNA of cyanobacteria	GGG GAA TYT TCC GCA ATG GG	Nübel et al. 1997
CYA781R(a)	16S rRNA of cyanobacteria	GAC TAC TGG GGT ATC TAA TCC CAT T	Nübel et al. 1997
CYA781(b)	16S rRNA of cyanobacteria	GAC TAC AGG GGT ATC TAA TCC CTT T	Nübel et al. 1997
nifH1	*nifH* of prokaryotes	TGY GAY CCN AAR GCN GA	Zehr and McReynolds 1989
nifH2	*nifH* of prokaryotes	AND GCC ATC ATY TCN CC	Zehr and McReynolds 1989
nifH3	*nifH* of prokaryotes	ATR TTR TTN GCN GCR TA	Zani et al. 2000
nifH4	*nifH* of prokaryotes	TTY TAY GGN AAR GGN GG	Zani et al. 2000

Table 2. Summary of cyanobacterial 16S rRNA gene clones obtained from each microbial group.

		Number of Clones			
Station	Depth	*Synechococcus* sub-cluster 5.1	*Synechococcus* sub-cluster 5.3	*Prochlorococcus* HL(II)	Total
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				-

Table 3. Summary of *nifH* clones obtained from each microbial group.

		Number of Clones			
Station	Depth	Cyanobacterium UCYN-A	α-,β-Proteobacteira	γ-Proteobacteria	Total
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				-

436

437