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Phylogenetic diversity of marine *Synechococcus* spp. in the Sea of Okhotsk

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ABSTRACT: The phylogenetic composition of marine *Synechococcus* spp. populations from 5 stations, 4 in the Sea of Okhotsk and 1 in the western subarctic Pacific, representing a wide range of hydrographic and ecological conditions, was examined using internal transcribed spacer (ITS) clone libraries and phylogenetic analysis. *Synechococcus* spp. at Stn G9, an estuarine station near the Amur River, belonged to Subcluster 5.2 phylotypes, while the other 4 stations were composed exclusively of Subcluster 5.1 *Synechococcus* spp. and dominated by phylotypes of Clades I and IV; this is in agreement with the general global distribution pattern of marine *Synechococcus* spp. lineages. Additionally, novel phylotypes that are not clustered to any existing clades were found, and subclades that exclusively contain phylotypes from the Sea of Okhotsk were also formed within respective clades, demonstrating the occurrence of unique *Synechococcus* spp. assemblages in the high-latitude marginal sea. Furthermore, distinct *Synechococcus* spp. populations were found at different stations with different hydrographic conditions, suggesting that the spatial variations in the phylogenetic composition of *Synechococcus* spp. were strongly affected by the physical-chemical parameters of the seawater. Overall, our study adds significant new insights into the diversity of *Synechococcus* spp. populations in the subpolar marginal sea and supports the conclusion drawn by Zwirglmaier et al. (2008; Environ Microbiol 10:147–161) that *Synechococcus* spp. phylogeography is driven by environmental conditions rather than by geographical proximity.

KEY WORDS: *Synechococcus* spp. · Phylogeny · Diversity · Sea of Okhotsk

INTRODUCTION

Chroococcoid picocyanobacteria *Synechococcus* spp. (<2 µm) are ubiquitously present in marine environments, from the equator to the Arctic, and from coastal to oceanic waters (Partensky et al. 1999). They span a range of different colors, depending on their pigment composition (Wood 1985, Olson et al. 1990, Stomp et al. 2007). *Synechococcus* spp. containing high concentrations of phycoerythrin (PE) absorb blue-green light effectively, and have a red appearance, whereas strains with high concentrations of phycocyanin (PC) absorb red light effectively, and have a blue-green color. PE-containing *Synechococcus* spp. are the dominant group of *Synechococcus* spp. in oceanic waters (Li et al. 1983, Platt et al. 1983, Olson et al. 1990), where green and particularly blue light penetrate deep into the water column. On the other hand, PC-containing cyanobacteria dominate in turbid waters, where red light prevails (Stomp et al. 2007). Coexistence of PE- and PC-containing picocyanobacteria can be found in waters of intermediate coloration, including estuarine and coastal waters (Murrell & Lores 2004, Stomp et al. 2007, Haverkamp et al. 2008).

Based on pigment content and physiological characterization, marine *Synechococcus* spp. have been divided into 3 distinct groups: marine clusters A (MC-A), B (MC-B), and C (MC-C) (Waterbury & Rippka 1989). MC-B contains mostly halotolerant strains isolated from coastal waters that possess PC but lack PE (Fuller et al. 2003). The MC-A group, which contains PE as their major light-harvesting pigment, is the dominant *Synechococcus* spp. group within the euphotic zone of both open oceans and coastal waters (Ferris &

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Palenik 1998, Wood et al. 1998, Wilmotte et al. 2002). Recently, the marine *Synechococcus* spp. in both MC-A and MC-B were reformed to a polyphyletic cluster, called Cluster 5, after being reclustered with *Prochlorococcus* and *Cyanobium* genera (Herdman et al. 2001), with Subclusters 5.1 and 5.2 replacing the former MC-A and MC-B, respectively. At the same time, MC-C was further divided into Clusters 3 and 4; the former contains 4 euryhaline, PE-lacking strains that are facultative photoheterotrophs, while the latter contains a single strain that is true marine, PE-containing, and capable of N_2 fixation (Herdman et al. 2001). A refinement of the more modern classification has recently been proposed by Dufresne et al. (2008) based on a comparative genomic analysis of the sequenced *Synechococcus* spp. Furthermore, after analysis of 11 marine *Synechococcus* spp. genomes, Six et al. (2007) demonstrated that there is no general relatedness between pigment composition and phylogenetic classification.

Molecular studies of microbial diversity typically use the most conserved 16S rRNA gene (Fuller et al. 2003); however, this does not provide enough resolution for the sub-species level. The structure and length of the internal transcribed spacer (ITS) region between the highly conserved 16S and 23S rRNA genes is inherently variable among microorganisms at species or inter-species levels and is an ideal target region for discrimination of fine-scale relationships between closely related gene clusters. A clone library encompassing the ITS rRNA operon allows the investigation of diversity of various marine *Synechococcus* spp. strains with high resolution (Rocap et al. 2002, Chen et al. 2006). Many studies have been done on the molecular diversity and ecology of cyanobacteria in various ecosystems (Scanlan & West 2002, Brown et al. 2005, Jing et al. 2006), but few have been conducted in polar or subpolar oceans (Vincent et al. 2000, Wilmotte et al. 2002, Waleron et al. 2007).

The Sea of Okhotsk is one of the marginal seas of the North Pacific Ocean, composed of a broad continental shelf along the Kamchatka and Siberian coasts and deep basins such as the Kuril and Derugina basins. Although the Sea of Okhotsk is geographically located at a temperate latitude, it has many characteristics of a polar ocean: namely, large seasonal variations in water temperature (~14°C) and large amounts of sea ice along the Siberian coast on the northwestern continental shelf to the east coast of Sakhalin every winter (Alfultis & Martin 1987). The Sea of Okhotsk is separated from the North Pacific Ocean by the Kuril Islands chain with important passages at Bussol (2300 m sill depth) and Kruzensterna (1900 m sill depth) straits. The Amur River, one of the longest rivers in the world, supplies fresh water and dissolved and suspended materials into the Sea of Okhotsk (Nakatsuka et al. 2004).

The Sea of Okhotsk is recognized as being the most productive marine basin among the world's oceans (Sorokin & Sorokin 1999). A shallow pycnocline and long periods of daylight create favorable conditions for intense phytoplankton growth throughout the growth season. However, no studies have been done on the population dynamics of *Synechococcus* spp. in the Sea of Okhotsk until recently (Liu et al. 2009), and their phylogenetic composition and diversity remain unknown. In the present study, by using the ITS clone library with restriction fragment length polymorphisum (RFLP) analysis as a DNA fingerprinting technique, we investigated the phylogenetic composition of marine *Synechococcus* spp. at 5 sampling sites in the Sea of Okhotsk.

MATERIALS AND METHODS

Sample collection. Surface seawater samples (1 to 4 m depth) were collected at 5 stations representing different oceanographic settings in the Sea of Okhotsk and the western subarctic Pacific during August and September 2006, using lever action-type Niskin bottles (General Oceanics) mounted to a CTD device (Fig. 1). Between 0.75 and 1.2 l of seawater were filtered on board first through a 3 µm and then a 0.22 µm pore-size polycarbonate membrane (47 mm diameter, Millipore). The membranes were stored at –80°C until DNA extraction was done on land.

Water samples were also taken for determining the concentrations of NO_3, PO_4, SiO_2, and chlorophyll *a* (chl *a*) and the abundance of *Synechococcus* spp. Chl *a* in 3 size-fractions (<2, 2 to 10, and >10 µm) was measured in surface water by filtering 300 to 500 ml seawater through 10 and 2 µm polycarbonate Nuclepore membrane filters (Whatman) and GF/F filters using a multilayer filtering tower consisting of polycarbonate filter holders (Sartorius Stedim Biotech). Filters were processed in 5 ml of N,N-dimethylformamide (DMF) for 24 h at –20°C, and chl *a* concentrations were measured with a fluorometer (Turner Design AU-10) using the non-acidification protocol of Welschmeyer (1994). For flow cytometric analysis of *Synechococcus* spp. abundance, samples of 1 ml seawater were fixed with 0.2% (final concentration) paraformaldehyde and stored at –80°C until analysis. Water temperature and salinity were measured using a Sea-Bird Electronics CTD system (SBE 911 plus). Macro-nutrients were analyzed using a BRAN+ LUEBBE autoanalyzer (QuAAtro).

Flow cytometry. A FACSCalibur flow cytometer equipped with a 15 mW laser exciting at 488 nm and

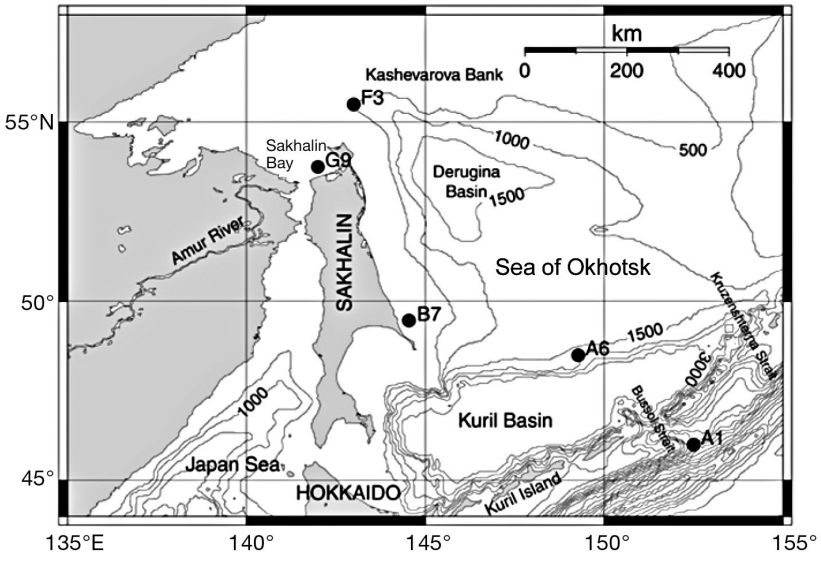

Fig. 1. Locations of the sampling stations in the Sea of Okhotsk. Detailed characteristics of each sampling station are described in 'Results'. Bathymetry is given in metres

with the standard filter setup was used to enumerate the picoplankton. Forward and right-angle light scatters and green (515 to 545 nm), orange (564 to 606 nm), and red (>650 nm) fluorescence were collected, saved, and analyzed with CYTOWIN software (Vaulot 1989). All signals were normalized to that of the 1 µm beads (Fluoresbrite YG beads; Polysciences) that were added to each sample. *Synechococcus* spp. were distinguished from eukaryotic picoplankton primarily by differences in light scatter and autofluorescence, particularly the strong orange fluorescence from PE.

DNA isolation and amplification. Total genomic DNAs were recovered from biomass collected with the 0.22 µm filters by phenol:chloroform:isoamyl alcohol (25:24:1) extraction at 60°C after lysis with CTAB buffer containing RNase A (10 mg ml^{-1}) and lysozyme (50 mg ml^{-1}). Extracted DNAs were stored at −80°C after precipitating with isopropanol and then used as template for the amplification of 1.5 kb ITS segments. The ITS segments cover the distal and proximal conserved flanking regions of the 16S rRNA gene, the ITS region, and the 23S rRNA gene. *Synechococcus* spp.-specific primers used were 16S-1247F (5′-CGT ACT ACA ATG CTA CGG-3′) (Rocap et al. 2002) and 23S-495R (5′-ACG GTT TCA GGT TCT ATT TCA CTC-3′) (Jing et al. 2009). PCR reaction was carried out with 50 µl master mix including 5 µl of 10× buffer, 2 µl of $MgCl_2$ (25 mM), 4 µl of deoxyribonucleotide triphosphates (dNTPs; 2.5 mM), 0.2 µl of *Taq* polymerase (5 U), and 1 µl of each primer (10 µM) with the following program: 95°C for 3 min; 30 cycles of 95°C for 1 min, 55°C for 50 s, 72°C for 1 min; and final extension at 72°C for 10 min. PCR products were stained with ethidium bromide and visualized on 1% agarose gel.

Clone library construction and RFLP screening. Independent PCR products from triplicate samples were pooled to reduce the chances of PCR artifacts and then purified by PureLink™ Quick Gel Extraction Kit (Invitrogen). Purified amplicons were cloned into the PCR4.0 vector by using the TOPO TA cloning kit (Invitrogen). In total 5 clone libraries were constructed, and from each, 46 to 75 white colonies were randomly picked. The correct DNA insertion was identified by direct amplification of the inserted DNA fragment with the original PCR primer set. Positive colonies were digested by 3 restriction enzymes: *Eco*R I (G↓AATTC), *Hae* III (GG↓CC), and *Hin*d III (A↓AGCTT) at 37°C for 3 h in 10× buffer M (Amersham Biosciences). The digestion products were separated by electrophoresis in 3% agarose gel. The restriction fragment banding patterns were visualized under UV radiation with digital imaging system of universal hood (Bio-Rad) and were normalized against the 1 kb Plus DNA Ladder (Invitrogen).

Sequencing and phylogenetic analysis. For groups of colonies showing the same RFLP pattern, 1 representative clone was randomly chosen. Plasmid DNAs of all colonies showing distinct RFLP fingerprints were extracted, purified, and then sequenced with an Applied Biosystems 3730 genetic analyzer using the BigDye Terminator Cycle Sequencing kit v3.1 (Applied Biosystems) with the QM13 forward primer.

BLAST searches of the GenBank database (www.ncbi.nlm.nih.gov) were performed to identify closely related sequences. For the purpose of the present study, 98% sequence similarity was used as a cut-off value. Sequences were also checked for chimeric properties by using Chimera_CHECK from the Ribosomal Database Project II (RDP II). Neighbor-joining (NJ) trees were generated using the program Phylip 3.63 (http://evolution.genetics.washington.edu/phylip.html) for all sequences and selected representative sequences from GenBank after aligning with Clustal X 1.80. Bootstrap values were obtained with 1000 resamplings and clades with >50% bootstrap value were shown on the nodes of branches.

Diversity analysis. Genetic diversity was assessed using the following indices: (1) individuals, or the total clone numbers examined; (2) species richness, or the number of different operational taxonomic units (OTUs); (3) coverage, derived from the equation:

Coverage = 1 – (*N*/individuals), *N* being the number of clones occurring only once (Kemp & Aller 2004); and (4) the Shannon-Wiener index (*H'*) and Simpson's index of diversity (1 – D) (Krebs 1989). In addition, rarefaction curves were plotted for each clone library with the software Analytic Rarefaction version 1.3 (www.uga.edu/~strata/software).

Nucleotide sequence accession numbers. ITS sequences of all the colonies obtained in the present study were deposited in GenBank under accession numbers EU497882 to EU497912.

RESULTS

Physical, chemical and biological characteristics of studied sites

The physical, chemical and biological characteristics of each sampling station are given in Table 1. Stn A1 was located on the western edge of the Western Subarctic Gyre (water depth >4000 m) in the North Pacific. Stn A6 was located on the north edge of the deep Kuril Basin (Fig. 1). Stn B7 was at the continental shelf (water depth 130 m) east of the Sakhalin Island. Stn F3 (water depth 350 m) was located in the Kashevarova Bank north of the Sakhalin Island, while Stn G9 was in the shallow water of the Sakhalin Bay (water depth <30 m), which was strongly influenced by the freshwater discharge from the Amur River, as indicated by low salinity (16.48). With the exception of Stn A1, nitrate concentrations in surface waters were below the detection limit (<0.2 µM). Chl *a* concentrations at Stns A1 and A6 were low and dominated by cells of <10 µm in size. The chl *a* concentration at Stn F3 was higher, but still composed mostly of <10 µm sized cells. The highest chl *a* concentration (17.25 mg m^{-3}) occurred at Stn G9 in the Sakhalin Bay, where both nitrate and phosphate were depleted, with 84% of the chl *a* derived from >10 µm sized phytoplankton.

Synechococcus spp. abundance and cytometric characteristics

Synechococcus spp. were clearly detected by flow cytometry at each station (Fig. 2). With the exception of Stn G9, *Synechococcus* spp. at all the stations were PE-rich strains that showed strong orange fluorescence (Fig. 2a). At Stn G9, both PE- and PC-rich cells were detected (Fig. 2b). The PC-containing *Synechococcus* spp. lack the signature orange fluorescence of PE. Moreover, the PE-containing *Synechococcus* spp. at Stn G9 had remarkably lower orange fluorescence relative to red (chlorophyll) fluorescence compared to the *Synechococcus* spp. at other locations (Fig. 2c).

Synechococcus spp. abundances ranging from 6.58×10^4 to 1.93×10^5 cells ml^{-1} were observed at study sites, except at Stn B7 on the continental shelf, where it was only 460 cells ml^{-1} (Table 1).

Analyses of diversity and comparison of ITS gene clone library

Five independent ITS clone libraries based on samples collected from each of the 5 spatially separated stations were constructed, and each library exhibited distinct *Synechococcus* spp. community structures after RFLP screening. A total of 271 positive clones were obtained, of which 31 clones as representatives corresponding to their distinct RFLP patterns were subjected to further phylogenetic analyses.

The composition of each ITS clone library is shown in Table 2. In terms of unique taxa (OTU), the highest number was found at Stn G9 (26.9% of total taxa), whereas the lowest number was derived from Stn B7 (7.7% of total taxa). Stn A6 was the most diverse one, reflected by both the Shannon-Wiener index and the Simpson index. In contrast, Stn B7 had the lowest diversity, since only 2 taxonomic groups were present. Five RFLP patterns occurred at both Stns A1 and A6, and they accounted for 55.6 and 62.5% of total RFLP pat-

Table 1. Water parameters and *Synechococcus* spp. abundance (only those containing phycoerythrin) at the sampling stations. nd = not determined; Sal = salinity; *Syn* = *Synechococcus* spp. abundance; Temp = temperature

Date	Stn	Temp (°C)	Sal	NO_3 (µM)	SiO_2 (µM)	PO_4 (µM)	Chl *a*				*Syn* ($\times 10^4$ cells ml^{-1})
							Total (mg m^{-3})	>10 µm (%)	2–10 µm (%)	<2 µm (%)	
22 Aug	A1	14.2	32.73	7.2	7.8	1.02	0.48	9	47	44	6.58
24 Aug	A6	14.3	32.24	0.0	11.9	0.29	0.44	6	17	77	19.35
26 Aug	B7	10.6	32.45	0.1	0.2	0.40	0.67	nd	nd	nd	0.46
2 Sep	F3	13.7	28.16	0.0	5.1	0.11	2.86	4	44	52	12.14
6 Sep	G9	15.8	16.48	0.0	24.6	0.00	17.25	84	11	5	8.13

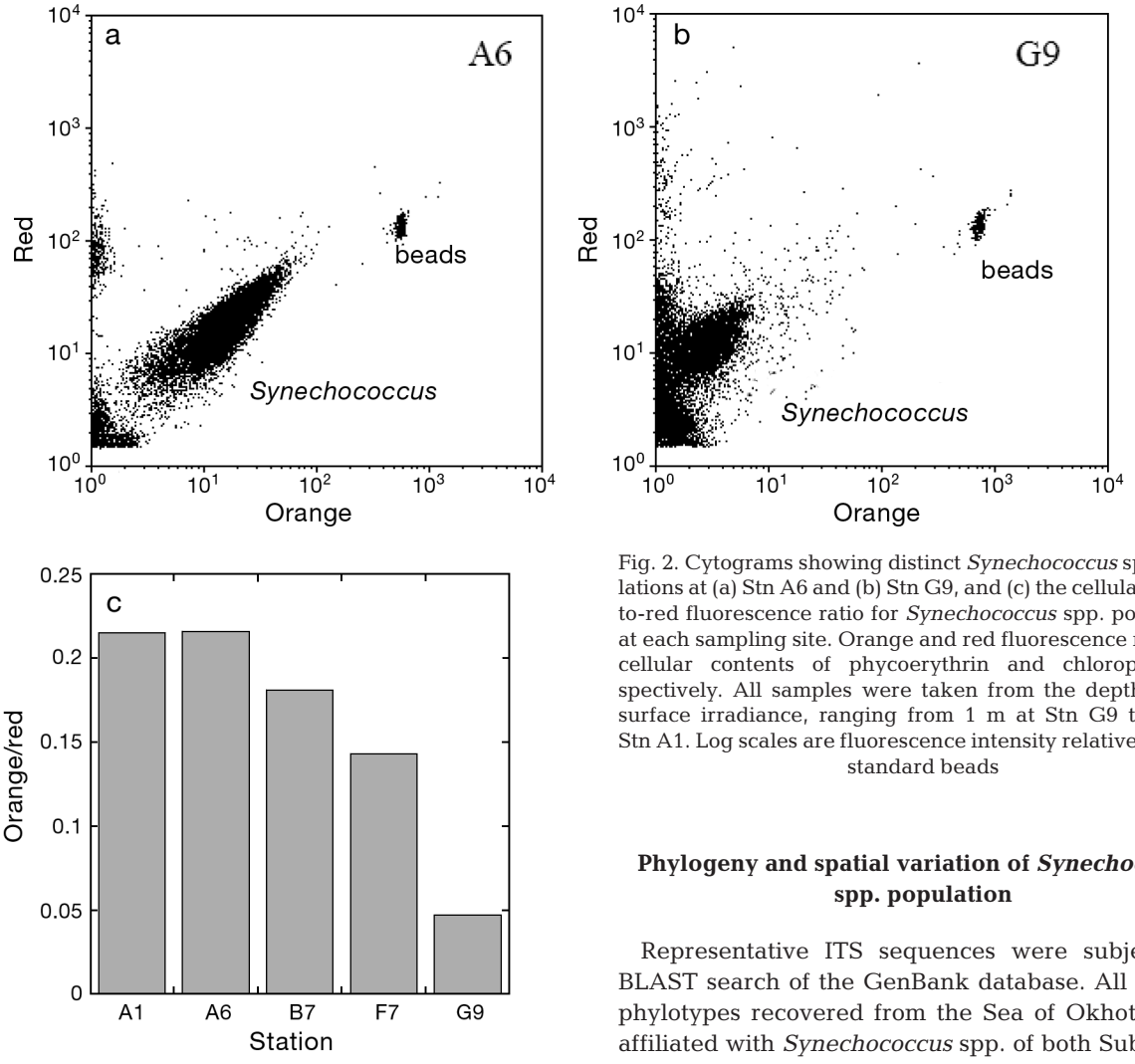

Fig. 2. Cytograms showing distinct *Synechococcus* spp. populations at (a) Stn A6 and (b) Stn G9, and (c) the cellular orange-to-red fluorescence ratio for *Synechococcus* spp. populations at each sampling site. Orange and red fluorescence represent cellular contents of phycoerythrin and chlorophyll, respectively. All samples were taken from the depth of 60% surface irradiance, ranging from 1 m at Stn G9 to 4 m at Stn A1. Log scales are fluorescence intensity relative to standard beads

terns and 66.0 and 80.0% of total clones obtained from each station, respectively. The remaining RFLP patterns obtained from the present study were all unique, suggesting that *Synechococcus* spp. populations at Stns B7, F3, and G9 were distinct from each other and were not present at Stns A1 and A6. Furthermore, as a relative estimation of how well the subsamples represented the environment, the coverage of all 5 clone libraries was >95% (Table 2), meaning that the clone numbers screened in each library were sufficient for diversity analysis and at least 96% of the natural *Synechococcus* spp. community diversity was recovered in the present study. This is in agreement with the tendency of the rarefaction curves (Fig. 3), which reflected the colony numbers picked yielding a sufficient fraction of the actual species.

Phylogeny and spatial variation of *Synechococcus* spp. population

Representative ITS sequences were subjected to BLAST search of the GenBank database. All the new phylotypes recovered from the Sea of Okhotsk were affiliated with *Synechococcus* spp. of both Subclusters 5.1 and 5.2, and most of them fell into several known marine *Synechococcus* spp. clades (Fig. 4).

The majority of our *Synechococcus* spp. phylotypes, 24 out of 31, belonged to Subcluster 5.1 and were distributed into marine Clades I, II, and IV. In addition, 3 phylogenetic groups composed exclusively of phylotypes from Stn A1 were also formed, but could not be

Table 2. Composition and diversity analysis of the 5 marine *Synechococcus* spp. populations' internal transcribed spacer (ITS) gene clone libraries. 1 – D = Simpson index of diversity; *H'* = Shannon-Wiener index of diversity; ind. = individuals; OTU = operational taxonomic unit

Stn	Species richness	No. of unique OTUs	No. of ind.	Coverage (%)	*H'*	1 – D
A1	9	4	50	96.0	1.79	0.775
A6	8	3	50	98.0	1.95	0.848
B7	2	2	46	100.0	0.68	0.485
F3	5	5	75	98.7	1.32	0.689
G9	7	7	50	96.0	1.48	0.718

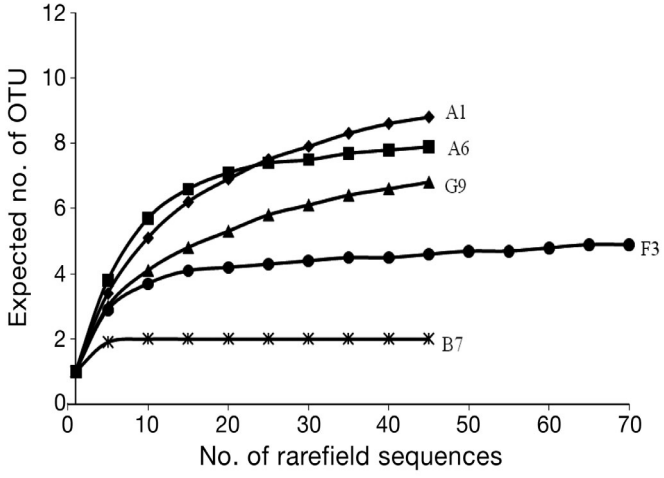

Fig. 3. Rarefaction analysis of the 5 marine *Synechococcus* spp. internal transcribed spacer (ITS) clone libraries. OTU = operational taxonomic unit

assigned into any existing marine *Synechococcus* spp. clades; therefore, they may represent the novel and unique *Synechococcus* spp. OTUs specific to the Sea of Okhotsk. The remaining phylotypes retrieved from Stn A1 were clustered together with those from Stn A6 and distributed into both Clades I and IV. Within Clade I, a distinct subclade composed entirely of phylotypes from Stn F3 was more closely related to *Synechococcus* sp. WH9908. Moreover, 2 distinct phylotypes obtained from Stn B7, OB7-17 (58.7% of the total phylotypes) and OB7-59 (41.3% of the total phylotypes), were not related to each other; the former belonged to Clade II, while the latter was clustered with Clades V and VI. Phylotype OB7-17 was the most abundant phylotype recovered in our study.

All the phylotypes retrieved from Stn G9 belonged to Subcluster 5.2. In contrast, no Subcluster 5.2 phylotypes were found at the other 4 stations. Five of our phylotypes, OG9-04, OG9-05, OG9-08, OG9-09, and OG9-

Fig. 4. Neighbor-joining tree showing inferred phylogenetic relationships of the *Synechococcus* spp. (internal transcribed spacer [ITS]) gene sequences cloned from the 5 stations. Sequences obtained as part of the present study are in **bold**. Scale bars indicate Jukes-Cantor distances. Bootstrap values >50 (for 1000 iterations) are shown. GenBank accession numbers are given in parentheses. Suffixes: BB = Blanes Bay; CB = Chesapeake Bay; CC = California Current; GM = Gulf of Mexico; LIS = Long Island Sound; MB = Monterey Bay; MS = Mediterranean Sea; SS = Sargasso Sea; WH = Woods Hole

40, were grouped together and clustered with the CB-5 clade, while 2 other phylotypes, OG9-17 and OG9-26, were aligned with the CB-4 clade; both clades were defined for *Synechococcus* spp. phylotypes found in Chesapeake Bay (Chen et al. 2006). Phylotypes OG9-26 and OG9-40 were equally abundant, and each accounted for 36% of the total Subcluster 5.2 phylotypes.

DISCUSSION

The marine picocyanobacteria *Synechococcus* spp. and *Prochlorococcus* spp. are important primary producers in the oceans (Partensky et al. 1999). There are only a few studies on the population dynamics of *Synechococcus* spp. in the subarctic Pacific Ocean (Neuer 1992, Liu et al. 2002a,b,c) Our report represents the first molecular study on marine picocyanobacteria in the Sea of Okhotsk, and reveals that genetically and potentially ecologically distinct *Synechococcus* spp. populations occur in different regions of the subarctic marginal sea.

Consistent with previous studies (Wood 1985, Ahlgren & Rocap 2006), most of our *Synechococcus* spp. phylotypes belong to Subcluster 5.1, and were mainly distributed in 3 (i.e. Clades I, II, and IV) of the 16 distinct phylogenetic clades previously described (Ahlgren & Rocap 2006, Penno et al. 2006). Based on the global scale analysis of existing data on the spatial partitioning for picocyanobacterial lineages, Clades I, II, and IV were normally the dominant clades in different global oceanic regions (Zwirglmaier et al. 2008). Clades I and IV were found co-occurring in high-latitude coastal and continental shelf zones, while Clade II was considered their 'counterpart' in the subtropical and tropical latitudes. All 3 clades are more abundant in surface layers of the euphotic zone (Toledo & Palenik 2003, Ahlgren & Rocap 2006, Zwirglmaier et al. 2008). Since the Sea of Okhotsk is a temperate sea with characteristics of a polar ocean, our results, which show the dominance of Clades I and IV in *Synechococcus* spp. populations in surface water, match well with the global distribution of *Synechococcus* spp. clades.

The only Clade II phylotype, OB7-17, was detected at Stn B7 on the continental shelf, which possesses very low *Synechococcus* spp. abundance and diversity. The occurrence of Clade II *Synechococcus* spp. in a high-latitude marginal sea does not fall into the general distribution pattern that shows that Clade II *Synechococcus* spp. usually dominates in subtropical and tropical waters such as the Red Sea (Fuller et al. 2003, 2005) and the Sargasso Sea (Ahlgren & Rocap 2006). The other phylotype found at Stn B7, OB7-59, is related to Clades V and VI, both of which are widely distributed in oceanic waters (Zwirglmaier et al. 2008) and contained strains with low phycourobilin:phycoerythrobilin (PUB:PEB) ratios and PUB-lacking strains respectively (Rocap et al. 2002). Interestingly, 6 novel phylotypes from Stn A1 cannot be grouped into any existing clades and are located in between Clades I and VI, with relatively closer phylogenetic relationships with Clade I, as supported by the high bootstrap values (Fig. 4).

Within respective clades, our *Synechococcus* spp. phylotypes formed several subclades that are only moderately or distantly related to other sequences reported from similar studies; this is also supported by the low sequence similarity values of 90 to 98% obtained from the BLAST search. For example, a distinct subclade composed entirely of phylotypes from Stn F3 was present in Clade I, and another subclade formed with phylotypes from Stn G9 that belong to Subcluster 5.2 existed within Clade CB-5, which contained *Synechococcus* spp. phylotypes found in Chesapeake Bay (Chen et al. 2006). These findings suggest that the *Synechococcus* spp. phylotypes obtained from the Sea of Okhotsk are unique and lack a close relatedness with previously reported phylotypes from other geographic locations, and therefore may represent species indigenous to the Sea of Okhotsk. Alternatively, it could be that similar environments have not been subjected to this analysis, and thus closely related sequences have yet to be deposited in GenBank. In addition to this, it is clear that there is not sufficient evolutionary resolution obtained for phylotypes recovered from Stns B7 and F3, indicated by the long branches, although GenBank sequences from freshwater lakes and coastal marine waters were used as sister groups in the phylogenetic tree.

Our results show high *Synechococcus* spp. abundance and diversity among different sampling sites in the Sea of Okhotsk that covers a broad range of hydrographic and trophic conditions. Stns A1 and A6, representing the high nitrate, low chlorophyll (HNLC) subarctic Pacific Ocean (Liu et al. 2004b) and nitrate-depleted Kuril Basin, respectively, showed marked similarity in *Synechococcus* spp. composition and diversity, suggesting efficient water exchange between the Sea of Okhotsk and the North Pacific through the passages along the Kuril Islands (Ohshima et al. 2002). *Synechococcus* spp. represented a significant portion of phytoplankton biomass at both stations (15 and 45% for Stns A1 and A6, respectively, in terms of chl *a* concentration; K. Suzuki et al. unpubl. data). Sequences from these 2 stations possessed high phylogenetic diversity (Table 2) and were mixed together to form 2 subclades within Clades I and IV, respectively (Fig. 4). This is consistent with the global distribution pattern of Clades I and IV dominating in the temperate mesotrophic waters (Zwirglmaier et al. 2007, 2008).

Stn G9, located at the bottom of Sakhalin Bay, was heavily influenced by the Amur River freshwater discharge (salinity in the upper 5 m of water was 16.5). The water was discolored due to a high concentration of humic organic matter from Siberia forest land. Both NO_2 + NO_3 and PO_4 were depleted, but the SiO_2 concentration was high (Table 1). The chl *a* concentration was high and phytoplankton was dominated by large diatoms (K. Suzuki et al. unpubl. data). A high number of unique OTUs was found at Stn G9; all the phylotypes recovered from Stn G9 belonged to Subcluster 5.2 and were related to those *Synechococcus* spp. strains and phylotypes recovered from Chesapeake Bay (Chen et al. 2006). Flow cytometric analysis revealed the existence of both PE- and PC-containing *Synechococcus* spp. populations, with the PE-containing population possessing much lower PE (likely strains lacking PUB or with a low PUB:PEB ratio that typically thrive in coastal and estuarine waters; Olson et al. 1988, 1990, Liu et al. 2004a) compared to populations in the oligotrophic open ocean (represented by Stn A6 in Fig. 2). These results confirm that Stn G9 was under strong influence of the Amur River plume and that a highly diversified *Synechococcus* spp. assemblage, including both PC- and PE-containing strains, occurred in high-latitude estuaries.

CONCLUSIONS

Our study adds significant new insights into the diversity of *Synechococcus* spp. population in subpolar waters. The majority of *Synechococcus* spp. phylotypes recovered from the Sea of Okhotsk fall into Clades I and IV, which is consistent with the general global distribution of *Synechococcus* spp. lineages. Furthermore, distinct *Synechococcus* spp. populations were found at different stations with drastically different hydrographic conditions, which were likely a result of niche differentiation, i.e. specific phylotypes with distinct physiological adaptations in specific hydrogeographical niches. Our results are in agreement with the conclusion drawn by Zwirglmaier et al. (2007, 2008) that *Synechococcus* spp. phylogeography is driven by environmental conditions rather than by geographical proximity.

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