Seasonal changes in picocyanobacterial diversity as revealed by pyrosequencing in temperate waters of the East China Sea and the East Sea

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ABSTRACT: To elucidate the seasonal and spatial changes in picocyanobacterial diversity in temperate waters, the abundance and genetic diversity of picocyanobacteria and environmental variables were investigated at 3 stations located in the East China Sea and the East Sea. Barcoded amplicon pyrosequencing of 16S-23S internal transcribed spacer sequences was applied to study picocyanobacterial diversity using 36 samples. Through pyrosequencing, sequences belonging to 27 distinct *Synechococcus* and *Prochlorococcus* clades were retrieved, which showed high picocyanobacterial diversity and obvious seasonal variation in marginal waters. The coastal and cold water-adapted *Synechococcus* Clades I and IV were dominant during winter and spring, whereas the warm water-adapted *Synechococcus* Clade II and *Prochlorococcus* HLII ecotype were dominant during the summer and autumn. Further, *Synechococcus* Clades III, V, VII and 5.3-I as well as *Prochlorococcus* LLI opportunistically occupied distinct niches in the summer and early winter. In these temperate marginal seas, the seasonal distribution of picocyanobacterial diversity was mainly controlled by temperature and nutrient level. In addition, the seasonal circulation pattern of adjacent water masses was a key determinant of the physicochemical properties of water and consequently of picocyanobacterial diversity.

KEY WORDS: *Synechococcus* · *Prochlorococcus* · Diversity · East China Sea · East Sea · Pyroseqencing

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INTRODUCTION

In marine environments, *Synechococcus* and *Prochlorococcus* are the dominant cyanobacteria, and they are known to be important primary producers (Li 1994, Liu et al. 1997, Jardillier et al. 2010). *Prochlorococcus* is more abundant in oligotrophic and tropical open oceans, whereas *Synechococcus* dominates in mesotrophic coastal waters and in upwelling regions (Partensky et al. 1999).

Picocyanobacterial diversity has been studied using various genetic markers, such as 16S rRNA (Fuller et al. 2003), the 16S-23S internal transcribed spacer (ITS; Rocap et al. 2002, Ahlgren & Rocap 2006, Choi &

Noh 2009, Mella-Flores et al. 2011, Huang et al. 2012), nitrogen regulatory gene (*ntcA*; Penno et al. 2006, Post et al. 2011), nitrate reductase (*narB*; Jenkins et al. 2006, Paerl et al. 2008), RNA polymerase (*rpoC1*; Toledo & Palenik 1997, Mühling et al. 2005) and cytochrome b6 subunit (*petB*; Mazard et al. 2012). These studies have revealed the existence of diverse *Synechococcus* and *Prochlorococcus* clades or ecotypes. Most studies of picocyanobacterial distribution in marine environments have been conducted using dot-blot hybridization and real-time quantitative polymerase chain reaction (qPCR) methods using clade-specific probes (Fuller et al. 2003, Zwirglmaier et al. 2008, Tai & Palenik 2009, Post et al. 2011, Ahlgren &

Rocap 2012) or terminal restriction fragment length polymorphism (Lavin et al. 2008). Recently, barcoded pyrosequencing methods that identify short taxonomic molecular markers via 454 sequencing technologies have been used to analyze microbial diversity in various environments (Binladen et al. 2007, Qian et al. 2011). This method allows many samples to be analyzed simultaneously and produces a large number of sequence reads for community analyses. Thus, using this method, one can elucidate microbial diversity based on sequences, as in a clone library. Three recent studies examined picocyanobacterial diversity via the pyrosequencing of 16S rDNA (Post et al. 2011) and 16S-23S ITS sequences (Choi et al. 2011, Choi 2012), but the pyrosequencing of 16S rDNA sequences could not discriminate all *Synechococcus* clades due to high sequence similarities among clades. However, a latitudinal distribution of picocyanobacterial diversity was successfully revealed via the pyrosequencing of 16S-23S ITS sequences (Choi et al. 2011).

Although many studies have examined the distribution of *Synechococcus* in various oceanic regions, few studies have investigated seasonal variation in picocyanobacterial diversity in marine environments (Fuller et al. 2005, Tai & Palenik 2009, Post et al. 2011). In seasonal studies conducted in the subtropical Gulf of Aqaba and in a coastal monitoring site off the California coast, *Synechococcus* Clade II and Clades I and IV, respectively, were dominant throughout the year. The East China Sea (ECS) and the East Sea/Sea of Japan (ES) show seasonal changes in water temperature typical of a temperate region and, therefore, exhibit dynamic changes in the physicochemical environment. In the present study, we used pyrosequencing of 16S-23S ITS sequences to investigate seasonal and spatial changes in marine picocyanobacterial diversity in temperate coastal waters and to determine the ecological niches of these diverse lineages.

MATERIALS AND METHODS

Study sites

Two stations, Stn H6 and Stn I, were located in the ECS, and Stn D5 was located in the ES (Fig. 1). The ECS comprises a wide continental shelf shallower than 200 m that connects to the Yellow Sea (YS) and to the ES. The Kuroshio Current (KC), a western boundary current in the northwestern Pacific Ocean, is considered the major source of 2 branches, the Korea/Tsushima Warm Current (KTWC) and the Yel-

low Sea Warm Current (YSWC) (Fig. 1). The former transports subtropical waters into the ES through the Korea/Tsushima Strait, while the latter flows into the YS (Ichikawa & Beardsley 2002). In addition, the effect of the Changjiang River Diluted Water (CRDW) is intensified during summer due to heavy rainfall. Circulation in the ECS shows great seasonal variability, mainly due to the monsoon wind and discharge from Changjiang River. Stn I is located in the center of a shallow (water depth of ca. 50 m) and mesotrophic shelf region and is seasonally affected by both CRDW and oligotrophic KC water. Stn H6 is located in a branch of the KC (Ichikawa & Beardsley 2002) and is affected by this warm water mass almost year-round. The KTWC splits into 2 or 3 branches and affects the current system of the ES: the East Korean Warm Current, which flows northward along the east coast of Korea, and the Offshore Branch, which flows east-northeastward along the Japanese coast. Circulation in the southwestern ES is dominated by intraseasonal variability in warm and cold currents (Chang et al. 2004, Mitchell et al. 2005), and thus, Stn D5 is seasonally influenced by either warm or cold currents.
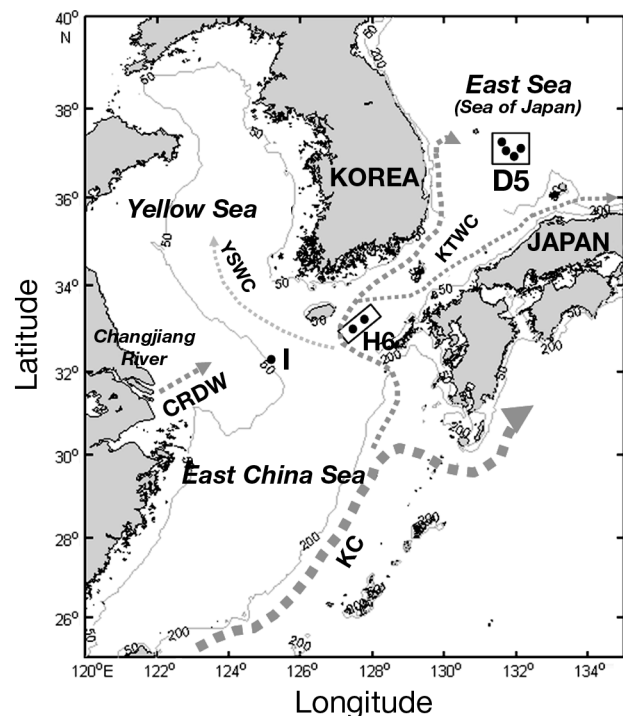

Fig. 1. The study area. Sampling sites at Stns H6 and D5 are located inside the boxes. Gray lines denote depth contour lines of 50 and 200 m. Gray dotted lines represent the Kuroshio Current (KC), Korea/Tsushima Warm Current (KTWC), Yellow Sea Warm Current (YSWC) and Changjiang River Diluted Water (CRDW) in the area

Sampling

Seawater samples were collected from the surface and the subsurface chlorophyll *a* (chl *a*) maximum (SCM) depth using 10 l Niskin bottles attached to a rosette sampler. If the development of a SCM was not observed, as may occur in the cold season, subsurface samples were collected at a depth of 20 m. In total, 36 samples were analyzed in the present study (Table 1).

Picocyanobacterial counts and environmental measurements

Synechococcus and *Prochlorococcus* abundances were determined via a Beckman-Coulter Altra flow cytometer. Chl *a*, extracted with 90% acetone, was quantified using a fluorometer (10 AU; Turner Designs; Parsons et al. 1984). Nutrient concentrations (NH_4, $NO_3 + NO_2$ and PO_4) were measured using a flow injection autoanalyzer (QuikChem AE; Lachat Instruments) and standard colorimetric procedures (Strickland & Parsons 1972). Temperature and salinity were measured using a conductivity, temperature and depth (CTD) sensor (SBE 9/11 plus; Sea Bird) mounted on a rosette sampler.

DNA extraction

Two-liter water samples were passed through a 0.2 µm Supor® filter (Gelman Sciences), and the fil-

Table 1. Summary of environmental variables and picocyanobacterial abundances at the surface and subsurface chlorophyll maximum. Samples are grouped by season but not in chronological order. nd: not detected; na: not available

Station	Season	Date	Depth (m)	Temp (°C)	Salinity	$NO_2 + NO_3$ (µM)	PO_4 (µM)	NH_4 (µM)	Chl *a* (µg l^{-1})	*Synechococcus* (×10^4 cells ml^{-1})	*Prochlorococcus* (×10^4 cells ml^{-1})
H6	Winter	Dec 2010	0	21.7	34.5	1.33	0.22	na	0.5	1.67	nd
			20	21.8	34.5	1.31	0.19	na	0.5	3.23	nd
		Feb 2009	0	16.5	34.5	4.27	0.61	0.91	0.6	0.12	nd
			30	16.5	34.5	4.56	0.51	0.95	0.6	0.08	nd
	Spring	Apr 2008	0	18.0	34.6	0.16	0.15	na	0.4	0.53	0.42
			25	16.5	34.6	0.11	0.09	na	1.6	0.44	nd
		Jun 2008	0	20.6	33.9	0.31	0.10	0.04	0.3	4.69	nd
			30	20.5	33.9	0.42	0.12	0.09	0.3	4.69	nd
	Summer	Aug 2008	0	30.0	31.5	nd	nd	nd	0.2	4.72	1.80
			37	20.9	33.8	10.68	0.73	nd	0.9	2.77	nd
	Autumn	Sep 2011	0	26.7	34.0	0.05	0.07	na	0.2	5.50	nd
			58	24.3	34.2	2.34	0.26	na	0.3	0.41	2.26
I	Winter	Dec 2010	0	16.9	32.0	11.23	0.37	na	0.8	1.48	nd
			20	16.9	32.0	10.59	0.37	na	0.8	3.40	nd
		Feb 2009	0	10.0	32.4	9.57	0.72	1.28	0.4	0.07	nd
			20	9.9	32.4	10.12	0.76	1.22	0.4	0.09	nd
	Spring	Apr 2008	0	11.6	32.5	3.70	0.23	na	1.7	0.62	nd
			20	10.9	32.6	6.13	0.32	na	0.4	0.31	nd
		Jun 2008	0	18.0	32.5	0.24	0.09	0.04	0.8	16.75	nd
			10	15.5	32.6	1.86	0.18	0.05	1.0	8.23	nd
	Summer	Aug 2008	0	27.8	29.5	0.66	0.02	0.02	1.1	3.61	nd
			15	24.7	29.9	1.70	0.02	0.02	1.4	11.69	nd
	Autumn	Sep 2011	0	23.4	32.1	0.56	0.05	na	0.7	2.46	nd
			20	23.6	32.2	0.49	0.06	na	0.7	2.77	nd
D5	Winter	Dec 2007	0	14.1	33.8	3.10	0.16	0.61	1.0	1.86	nd
			20	14.1	33.8	3.21	0.31	0.55	1.0	1.65	nd
		Feb 2008	0	10.9	34.3	4.76	0.42	0.59	1.8	0.20	nd
			30	11.0	34.3	4.88	0.36	0.64	1.5	0.19	nd
	Spring	Mar 2008	0	10.6	34.3	3.03	0.32	0.71	1.0	0.31	nd
			50	10.5	34.3	3.94	0.31	0.62	1.1	0.38	nd
		Apr 2006	0	10.7	34.4	4.25	0.38	na	0.2	0.02	nd
			50	9.1	34.3	5.20	0.49	na	0.4	0.04	nd
	Summer	Aug 2008	0	26.7	33.3	0.27	0.09	0.76	0.1	2.05	1.49
			50	15.3	34.4	0.23	0.16	0.29	0.8	2.24	0.06
	Autumn	Oct 2008	0	22.7	33.3	0.08	0.27	na	0.2	4.12	4.51
			34	17.8	34.2	2.04	0.29	na	0.4	1.12	1.27

ters were stored at –80°C after the addition of 1 ml
STE buffer (100 mM NaCl, 10 mM Tris-HCl and
1 mM EDTA, pH 8.0) for DNA analyses. To extract
DNA, the filters were cut into small pieces with ster-
ile scissors and, with the remaining storage buffer,
were placed in 50 ml sterilized conical tubes. After
adding 2 ml of fresh STE buffer, the cells were lysed
using lysozyme and proteinase K, and then DNA was
extracted using phenol/chloroform/isoamyl alcohol
according to Somerville et al. (1989).

Pyrosequencing of ITS sequences

To elucidate picocyanobacterial ITS sequence di-
versity, barcoded amplicon pyrosequencing using the
GS-FLX Titanium system was conducted. To amplify
partial picocyanobacterial ITS sequences, barcoded
primers were used. The barcoded primers consisted
of adapter A (forward) or B (reverse) + Key (TCAG) +
barcode (10 bp oligonucleotide, forward only) + pico-
cyanobacteria-specific primers. The ITS-af (5'-GGA
TCA CCT CCT AAC AGG GAG-3') and ITS-ar (5'-
GGA CCT CAC CCT TAT CAG GG-3') primers were
used as specific primers (Lavin et al. 2008). The ITS-af
and ITS-ar primers showed perfect matches to 99% of
the 2389 picocyanobacterial ITS sequences obtained
from cultures and clone libraries, indicating high
specificity to picocyanobacteria. The fused primer set
produced amplicons of ca. 250 to 330 bp.

One to 10 ng of template DNA was added to the
PCR reaction (total 50 µl), which contained Ex *Taq*
buffer (Takara Bio), 0.2 mM of each dNTP, 0.5 µM of
each primer and 2 U of Ex *Taq* (Takara Bio). PCR
amplification was conducted according to the follow-
ing cycle parameters: an initial denaturation step
(5 min, 94°C) was followed by 35 cycles consisting of
denaturation (45 s, 94°C), annealing (45 s, 52°C) and
extension (1.5 min, 72°C) steps and a final 10 min
extension step at 72°C. The size of the PCR products
was confirmed via agarose gel electrophoresis. When
PCR failed to amplify the DNA or produced a faint
band, nested PCR was applied (see Table 2). In this
case, the first PCR was conducted using the 16S-
1247F and 23S-241R primers under the suggested
PCR conditions (Rocap et al. 2002). Each PCR prod-
uct was quantified on agarose gels using DNA
QuantLadders (Lonza Rockland). Equal quantities of
each PCR product were pooled and then purified
using the AccuPrep PCR purification kit (Bioneer).
After resolution on a 2% agarose gel, the region
between 200 and 400 bp was excised, and DNA was
extracted using a Qiagen Gel Extraction kit (Qiagen).

Pyrosequencing (on a 1/8 PicoTiterPlate) of PCR
products was performed at Macrogen. Sequence
reads from the present study were submitted to the
NCBI sequence read archive (www.ncbi.nlm.nih.
gov/Traces/sra; accession number SRA089070).

Database for pyrosequencing data analysis

For pyrosequencing analysis, a database was con-
structed using ITS sequences from cultures and clone
libraries from 14 previous studies (Rocap et al. 2002,
Ahlgren & Rocap 2006, Chen et al. 2006, Haverkamp
et al. 2008, 2009, Choi & Noh 2009, Jing et al. 2009a,b,
Cai et al. 2010, Lavin et al. 2010, Mella-Flores et al.
2011, Huang et al. 2012, Jing & Liu 2012, Mazard et
al. 2012) as well as unpublished data (GenBank ac-
cession nos. HQ336805-HQ336939; D. H. Choi & J.
H. Noh unpubl. data). The sequences were aligned
using the MAFFT program (v. 7; Katoh & Standley
2013) with the FFT-NS-I strategy. After manual cor-
rection, the alignment was imported into the ARB
program (Ludwig et al. 2004). A neighbor-joining
(NJ) tree for full-length ITS sequences was built;
however, because the highly conserved tRNA region
was not informative in phylogenetic analysis, the
tRNA sequence was removed. The robustness of
nodes was examined using the bootstrap method.
Using the tree, the sequences were classified accord-
ing to previous clade designations (Rocap et al. 2002,
Ahlgren & Rocap 2006, Chen et al. 2006, Choi & Noh
2009, Mella-Flores et al. 2011, Huang et al. 2012).
Clades not clearly resolved in the bootstrap tree and
clades with relatively high similarities between clos-
est clades were grouped together (Fig. 2). In these
cases, the group was assigned the earliest published
clade name. Finally, the database contained 1651 ITS
sequences belonging to *Synechococcus* subcluster
(SC) 5.1 (814 sequences), *Synechococcus* SC5.2
(29 sequences belonging to marine clades CB4 and
CB5), *Synechococcus* SC5.3 (76 sequences) and *Pro-
chlorococcus* (732 sequences).

Next, we annotated the sequences with 4-rank tax-
onomic information (*Cyanobacteria*; *Synechococcus*
or *Prochlorococcus*; *Synechococcus* subclusters or
Prochlorococcus high-light adapted [HL]/low-light
adapted [LL]; clades). For the reference alignment,
the region between the forward primer and tRNA[Ile]
was cropped from the full-length alignment used in
the phylogenetic analysis above. In addition, a refer-
ence sequence file and its corresponding taxonomic
file (to be used for clade-assignment of pyrosequenc-
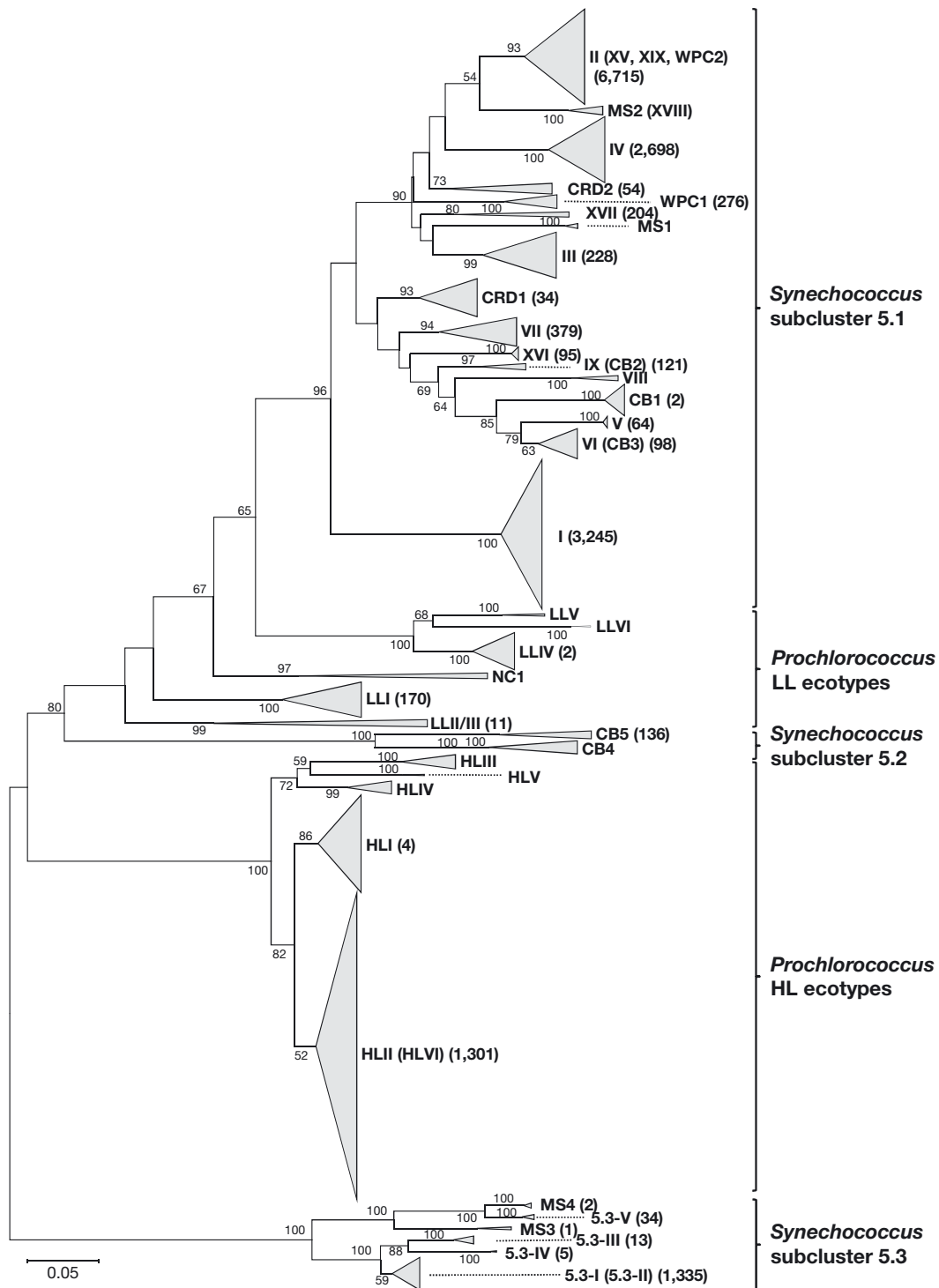ing reads) were exported from the ARB database.

Fig. 2. Compressed neighbor-joining tree based on 1220 positions of 16S-23S rDNA internal transcribed sequences showing the phylogenetic relationships among *Synechococcus* and *Prochlorococcus* genotypes. In total, 1619 sequences were used to construct the tree. Only bootstrap values >60% from 100 replicates are shown. Clade names are shown on the right of compressed branches. Clade names in parentheses represent clades grouped together with 1 representative clade on the basis of robustness of nodes or distances among closest clades. The numbers in parentheses following the clade name represent the total number of pyrosequencing reads belonging to each clade. The thickness and length of triangles are proportional to the number of sequences and distances among sequences, respectively. LL: low-light adapted; HL: high-light adapted. Scale bar = 0.05 nucleotide substitutions per site

Analysis of pyrosequencing data

Pyrosequencing data were mainly analyzed using the Mothur software (Schloss et al. 2009). To remove reads associated with errors, raw reads were filtered by allowing only those with a perfect match to barcode and forward primer sequences. The maximum allowed homopolymer length was 6 bp. Reads with an initial noisy signal (flow intensity: 0.5 to 0.7) before 150 flows were removed and flows beyond 350 were ignored. Then, flowgram data were grouped by samples based on their barcodes. The filtered reads were de-noised using 'shhh.flows' command, which is the Mothur-based re-implementation of PyroNoise (Quince et al. 2011). Then, the 'chimera.perseus' command was used to identify chimeric sequences. The remaining reads were aligned using the Needleman algorithm and the reference alignment. Subsequently, reads showing similarities < 90% to the reference sequences were screened to remove nonspecifically amplified reads. In addition, short reads not covering the full alignment were removed to avoid inconsistent classification by size difference. Using the 'pre.cluster' command (with the option of diffs = 4), the aligned reads were clustered to remove sequences that were likely due to pyrosequencing errors. Then, chimeric sequences were also removed using the 'chimera. uchime' command. The remaining reads were classified to each corresponding clade by means of the 'classify.seqs' command (k-nearest neighbor approach with an option of $k = 1$) using the reference sequence and its taxonomy files.

Statistical analyses

To view relationships among samples based on differences in clade diversity, a principal coordinates analysis (PCoA) was conducted using the FastUniFrac Web application based on NJ trees (Hamady et al. 2010). Samples were clustered based on the normalized weighted UniFrac distance. Canonical correspondence analysis (CCA) was performed using Canoco for Windows (v. 4.51) to clarify the factors controlling picocyanobacterial clades distribution. Explanatory variables (temperature, salinity, nitrate + nitrite, phosphate, chl a and *Synechococcus* abundances) were log(n + 1)-transformed, and response variables (clade %) were square root-transformed prior to analysis. The CCA model was produced with forward selection via Monte Carlo permutation significance tests ($p < 0.05$).

To determine the depth of coverage in each sample, Good′s coverage was calculated using Mothur software (Schloss et al. 2009).

RESULTS

Physicochemical characteristics of samples

At all stations, seawater temperature showed a seasonal pattern typical of temperate waters (Table 1). However, the ranges of values and vertical variation differed among stations. At Stn H6, the surface temperature was ~8°C higher during the winter and 3°C higher during the summer compared to other stations (Table 1). Surface temperatures were similar at Stns I and D5. Subsurface water temperatures showed large differences among stations (Table 1).

At Stn D5, salinity varied within a narrow range of 33.3 to 34.4 (Table 1). However, salinity in the ECS was lower and more variable than that observed in the ES, mainly due to runoff from the Changjiang River. This runoff appeared to influence Stn H6 in summer, resulting in a decrease in surface salinity from ~33.9 in June to 31.5 in August. In contrast, salinity at Stn I was consistently < 32.6, indicating the persistent influence of low salinity water from the Changjiang River.

The sum of nitrate and nitrite concentrations ranged from undetectable to 10.7 µM and tended to be higher at Stn I than at any other station (Table 1). During the winter, relatively high nutrient concentrations (3.1 to 10.1 µM) were observed at the surface due to vertical mixing. In the summer and autumn, nutrient concentrations were low at the surface but high at the subsurface due to thermal stratification. Phosphate concentrations showed similar seasonal trends as the sum of nitrate and nitrite, but the concentrations were more than an order of magnitude lower.

Chl a and picocyanobacterial abundances

Chl a concentrations varied from 0.1 to 1.8 µg l^{-1} during the present study, and overall ranges were similar at the 3 stations (Table 1). At Stn H6, which is influenced by oligotrophic KC water, chl a was lower than at Stn I during seasons in which vertical mixing was restricted due to thermal stratification. During summer, relatively high chl a values were observed at Stn I due to the inflow of the nutrient-rich CRDW (Table 1). The wide range in chl a concentrations

indicates that these regions shift seasonally from oligotrophic to mesotrophic states.

Synechococcus abundances varied by up to 3 orders of magnitude across seasons (Table 1). In February, *Synechococcus* abundance was extremely low, ranging from 0.07×10^4 to 0.20×10^4 cells ml^{-1}; the abundance then greatly increased and peaked between late spring and autumn (Table 1). The seasonal *Synechococcus* abundance patterns were similar at all 3 stations, but abundance was higher at Stn I during late spring and summer. At low temperatures, *Synechococcus* abundance increased with seawater temperature (Fig. 3). However, at ca. 20°C, *Synechococcus* abundances tended toward saturation, although their compositions changed dynamically (see section 'Seasonal and spatial compositions of picocyanobacterial assemblages').

When using flow cytometry, *Prochlorococcus* was detected in samples collected during summer and autumn but not in most other seasons (Table 1). However, failure to detect via flow cytometry does not indicate the total absence of *Prochlorococcus* given that *Prochlorococcus* sequences were successfully retrieved via pyrosequencing in several samples (Table 2). This discrepancy seems to have been due to a methodological failure, namely low abundance and dim fluorescence, as reported in other studies (Jacquet et al. 1998, Mella-Flores et al. 2011).

Characteristics of the pyrosequencing run

In total, 20 874 reads passed our trimming and screening procedures (barcode and primer matches and amplicon size). Additionally, 3556 reads were removed through the chimera screening and alignment procedures. Then, the remaining 17 227 reads were classified into 27 distinct *Synechococcus* and *Prochlorococcus* clades (Table 2). To determine whether the classification was reliable, a phylogenetic analysis using the aligned reads and 'add sequences into existing tree' method of the ARB program was also conducted. Between the 2 methods, at-odds or incorrect classifications were noted for only 37 sequences (0.2% of the total reads, data not shown), suggesting that clade classification using the Mothur pipeline is reliable. The read numbers per sample ranged from 105 to 1486, showing large variation. However, Good's coverage index estimated at a minimum read number of 105 was >97% for all samples (data not shown), indicating that sequence numbers were sufficient to assess picocyanobacterial diversity.
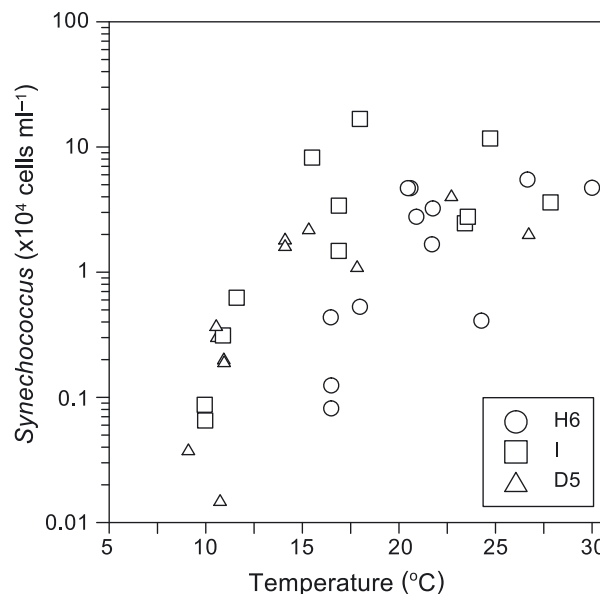

Fig. 3. Relationship between water temperature and *Synechococcus* abundances in the present study at 3 stations (H6 and I in the East China Sea and D5 in the East Seal/Sea of Japan)

Seasonal and spatial compositions of picocyanobacterial assemblages

In the ECS and ES, diverse *Synechococcus* and *Prochlorococcus* lineages appeared in time and space (Table 2). The number of clades found in each sample varied between 2 and 19, indicating large variation in picocyanobacterial diversity among samples. At Stns I and D5, markedly fewer lineages (2 to 6 clades) were observed during the cold season from late winter to late spring; however, the number increased in warmer water.

Thirteen of 17 clades belonging to *Synechococcus* SC5.1 were retrieved at fractions greater than 1% in at least 1 sample. At Stn D5, located in middle of the ES and thus far from the direct effects of freshwater and warm KC water, we observed apparently distinct picocyanobacterial compositions across seasons. At low seawater temperatures (~10°C), both *Synechococcus* Clades I and IV composed the greater part of all picocyanobacteria (82 to 100%). However, in surface water in the summer and autumn (23 to 27°C), Clade II comprised the dominant picocyanobacteria (50 to 62%), followed by the *Prochlorococcus* HLII ecotype (23 to 39%). However, Clades I and IV remained the dominant lineages at SCM depth in August and October, when seawater temperatures were consistently low due to thermal stratification (Table 1). At Stn I, seasonal picocyanobacterial compositions were similar compared to those at Stn D5. However, sev-

Table 2. Percentages of sequences belonging to each clade of *Synechococcus* Subcluster 5.1 (Syn-5.1SC), Subcluster 5.2 (Syn-5.2SC) and Subcluster 5.3 (Syn-5.3SC) and to *Prochlorococcus* (Pro). Minor clades of <1% abundance in all samples are not shown. Density of shading shows relative percentages of clades in each sample. The numbers in parentheses following clade name represent rRNA operon copy numbers obtained from genomes of culture strains. -: not detected; na: not available

Percentage of sequences belonging to each clade

Station		H6						I						D5					
Season		Winter		Spring		Summer	Autumn	Winter		Spring		Summer	Autumn	Winter		Spring		Summer	Autumn
Group / Month	Clade	Dec[a]	Feb[a]	Apr	Jun	Aug	Sep	Dec	Feb[a]	Apr	Jun	Aug	Sep	Dec	Feb	Mar[a]	Apr	Aug	Oct
Syn-5.1	I (2)	0.4	9.6	18.9	4.0	0.2	-	11.1	26.8	78.2	14.3	0.6	0.3	25.8	45.6	45.6	59.8	0.1	0.8
	II (2 or 3)	23.2	68.9	58.4	62.0	32.0	72.7	66.3	34.2	-	-	63.1	29.3	41.0	0.9	-	1.8	62.4	50.0
	III (2)	-	-	0.9	0.3	21.5	1.5	1.6	-	-	-	6.1	0.6	0.8	-	-	-	0.4	5.6
	IV (2)	-	0.2	3.6	28.3	-	0.1	-	25.0	20.4	85.7	-	-	13.6	41.7	54.3	38.0	3.2	-
	V (2)	-	-	-	-	-	-	2.5	0.2	-	-	-	12.7	1.0	-	-	-	-	-
	VI (2)	-	-	0.6	0.3	5.8	-	1.6	1.1	-	-	3.9	4.4	0.8	-	-	-	0.2	-
	VII (na)	33.8	1.4	1.5	0.3	-	1.4	0.4	-	-	-	-	3.0	-	-	-	-	-	0.2
	IX (2)	0.3	-	2.1	-	-	0.9	3.7	1.0	-	-	0.8	1.4	-	-	-	0.2	0.1	0.2
	XVI (na)	-	0.7	-	-	-	-	-	-	-	-	-	-	0.3	0.5	-	-	-	-
	XVII (na)	4.6	2.6	0.6	1.8	1.5	1.0	3.3	0.2	-	-	-	0.6	0.8	-	-	-	3.4	2.2
	CRD1 (na)	2.2	-	0.9	-	-	0.5	-	-	-	-	-	-	-	-	-	-	-	-
	CRD2 (na)	0.3	-	-	-	0.2	0.1	-	-	-	-	-	-	-	-	-	-	2.4	1.3
	WPC1 (2[b])	-	0.2	-	0.3	16.3	0.1	0.4	4.0	-	-	15.7	1.1	1.0	0.2	-	0.2	1.6	0.5
Syn-5.2	CB5 (2)	-	6.5	-	-	-	0.1	2.5	2.2	0.7	-	-	-	0.3	1.0	-	-	1.3	-
Syn-5.3	5.3-I (1)	29.7	5.0	4.5	1.8	4.1	2.2	5.8	4.8	-	-	9.8	46.7	11.7	4.3	-	0.2	2.1	-
	5.3-V (na)	3.8	0.2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Pro	HLII (1)	-	1.2	7.8	0.9	18.1	19.4	-	0.6	-	-	-	-	2.9	4.6	-	-	22.9	39.2
	LLI (1)	-	2.6	-	-	-	-	-	-	-	-	-	-	-	0.9	0.2	-	-	-
	LLII/III (1)	-	-	-	-	-	-	-	-	0.7	-	-	-	-	0.3	-	-	-	-
No. of reads		680	418	334	329	465	1486	243	628	147	105	510	362	383	585	564	666	1011	604
No. of clades		13	13	12	10	10	13	13	11	4	2	7	10	12	10	3	6	12	9

[a]Samples amplified by nested PCR
[b]Unpublished data (D. H. Choi & J. H. Noh)

Table 2 (continued)

Samples	Clade	H6 Winter Dec	Feb	Spring Apr[a]	Jun	Summer Autumn Aug	Sep	I Winter Dec	Feb	Spring Apr[a]	Jun	Summer Autumn Aug	Sep	D5 Winter Dec	Feb	Spring Mar	Apr	Summer Autumn Aug	Oct
											Subsurface chl a maximum								
Group							Percentage of sequences belonging to each clade												
Syn-5.1	I (2)	0.5	13.4	41.9	4.5	9.4	-	9.1	20.7	70.4	47.4	18.2	1.8	27.4	48.0	47.4	56.0	18.3	8.8
	II (2 or 3)	85.0	71.4	35.0	68.7	60.4	34.5	65.1	30.6	-	0.6	62.9	31.7	36.1	5.5	2.4	0.1	6.8	18.8
	III (2)	1.0	-	1.0	-	-	1.7	0.4	0.1	-	-	-	1.4	1.4	-	-	0.1	0.4	-
	IV (2)	-	0.5	14.3	20.0	6.8	-	4.8	29.2	11.2	52.0	1.9	-	10.6	33.5	41.6	43.7	66.9	22.0
	V (2)	-	-	-	-	0.1	-	-	-	-	-	-	1.1	-	-	-	-	-	-
	VI (2)	-	-	-	-	-	-	0.8	0.3	-	-	0.7	0.6	1.4	-	-	-	-	-
	VII (na)	7.3	2.7	0.3	0.3	0.7	0.7	-	-	-	-	0.2	7.7	-	-	-	-	-	-
	IX (2)	0.5	-	0.5	-	1.2	-	2.4	1.6	-	-	9.0	0.3	-	-	-	-	-	-
	XVI (na)	-	1.1	-	-	3.6	0.6	-	-	-	-	-	0.1	1.0	-	-	-	4.0	5.2
	XVII (na)	0.5	4.5	-	1.3	1.6	-	0.8	0.4	-	-	0.2	1.0	2.9	0.4	-	-	-	0.6
	CRD1 (na)	-	-	-	-	1.3	1.1	-	-	-	-	-	-	-	-	-	-	-	-
	CRD2 (na)	-	-	1.0	1.6	0.1	-	-	-	-	-	-	-	-	0.4	-	-	-	-
	WPC1 (2b)	0.5	0.7	-	-	0.7	0.6	-	2.4	-	-	1.0	3.1	0.5	-	-	-	-	0.4
Syn-5.2	CB5 (2)	0.5	1.8	-	-	0.5	-	10.3	2.3	-	-	0.7	-	-	-	1.4	-	0.7	0.2
Syn-5.3	5.3-I (1)	4.1	3.8	3.3	1.9	9.6	2.8	6.0	4.6	0.3	-	5.1	50.5	16.3	1.5	-	-	0.7	3.4
	5.3-V (na)	-	-	-	-	0.1	-	-	-	-	-	-	0.8	-	-	-	-	-	-
Pro	HLII (1)	-	-	1.8	1.0	2.4	42.9	-	1.3	14.7	-	-	-	2.4	10.9	4.8	-	1.4	30.2
	LLI (1)	-	-	0.5	-	1.0	14.1	-	6.6	3.2	-	-	-	-	-	2.4	-	0.7	9.3
	LLII/III (1)	-	-	0.3	0.3	0.1	1.7	-	-	0.3	-	-	-	-	-	-	-	-	0.2
	No. of reads	193	560	391	310	1069	177	252	702	348	323	412	796	208	275	209	668	278	536
	No. of clades	9	10	11	10	19	9	10	12	6	3	10	12	10	7	6	4	9	13

[a]Samples amplified by nested PCR
[b]Unpublished data (D. H. Choi & J. H. Noh)

eral differences are worth noting. At both the surface and SCM depths in autumn, Clade 5.3-I was dominant, comprising ~50% of the picocyanobacterial community. In addition, Clades V and WPC1 showed relatively high contributions (~15% each) at the surface in September and August, respectively. Furthermore, no *Prochlorococcus* sequence was retrieved from samples collected during the summer and autumn. In contrast to all other stations, *Synechococcus* Clade II was the dominant picocyanobacterium at Stn H6 in all seasons (Table 2). Although Clade I and IV sequences were also observed in late winter and spring samples, their contributions were relatively low compared to that of Clade II at Stn H6.

Clade CB5 belonging to *Synechococcus* SC5.2 ranged from undetectable to 10.3% of the sequences, and its overall sequence fraction tended to increase with decreasing temperature. Among the 6 clades in

Synechococcus SC5.3, clade 5.3-I was dominant, comprising up to 46.7% of the picocyanobacterial sequences. *Prochlorococcus* sequences were found in most samples from Stns H6 and D5 (Table 2). At Stn I, however, *Prochlorococcus* sequences were retrieved only in February and March. *Prochlorococcus* Clade HLII was dominant in both surface and SCM samples. The *Prochlorococcus* LLI ecotype was detected more frequently at the SCM depth than at the surface and appeared to make up a greater fraction of the total sequences in deeper water.

Relationships between picocyanobacterial community distribution and environmental factors

Based on PCoA, the greatest part of the observed sample variation (82.9%) could be accounted for by
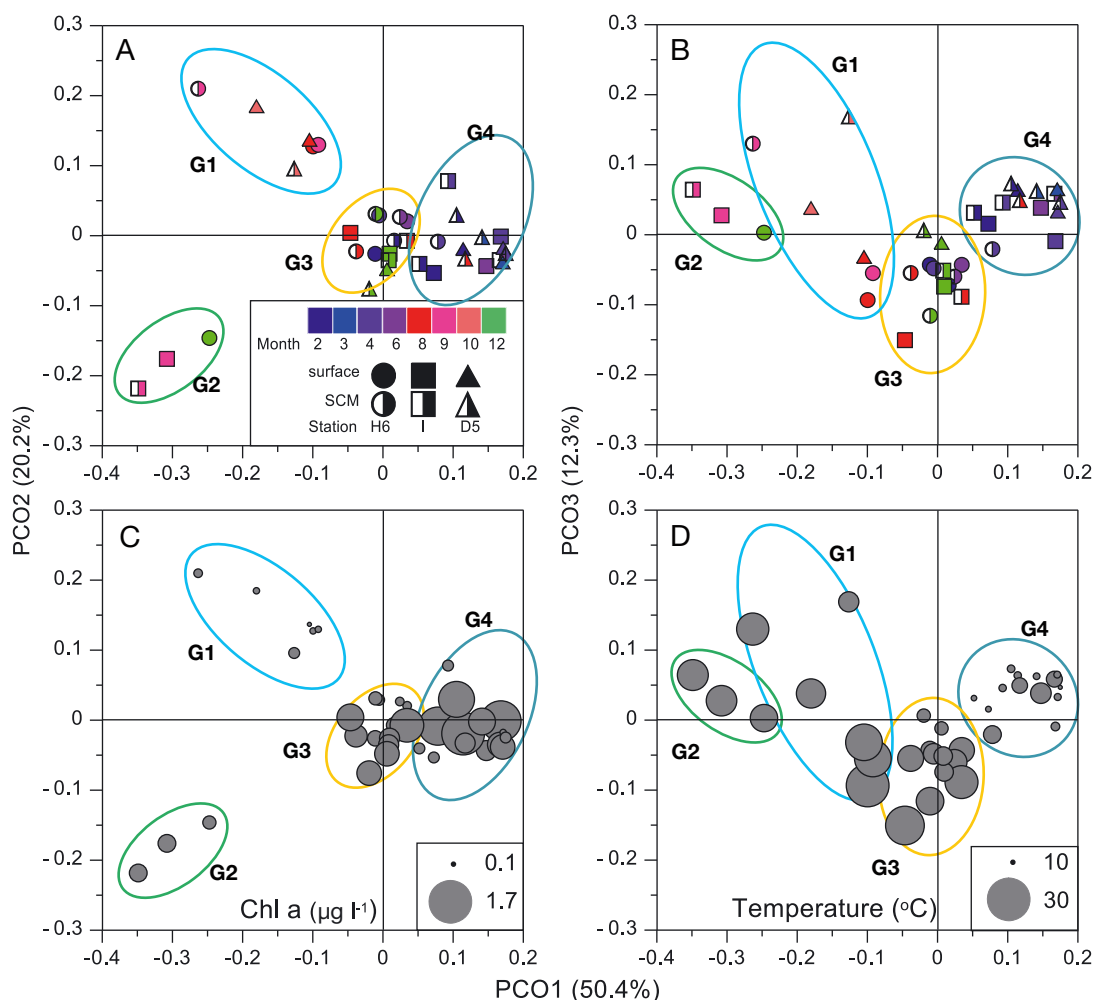

Fig. 4. Principal coordinates analysis of samples with the normalized weighted UniFrac. Plots of (A) the first 2 principal axes and (B) the first vs. third axes are shown. Plots (C) and (D) are the same plots as (A) and (B), respectively, but represent (C) chl *a* concentration and (D) temperature for each sample. The circles represent 4 groups classified on the basis of clustering patterns of samples

the first 3 principal components, and the samples could be divided into 4 groups (Fig. 4). Group G1 was made up of summer and autumn samples typified by high temperatures and low chl *a* levels (Fig. 4C,D). In these G1 samples, Clade II (19 to 73%) and *Prochlorococcus* HLII members (18 to 43%) were dominant, indicating the oligotrophic state of these samples. In a sample from the G1 group, Clades III and WPC1 showed their highest percentages, although these represented a low overall contribution (22 and 16%, respectively). In samples clustered within Group G2 (Fig. 4), *Synechococcus* 5.3-I was dominant (30 to 51%) over Clade II (23 to 32%). Compared to G1, these samples were typified by slightly lower temperatures (ca. 23°C) but relatively higher chl *a* levels (Figs. 4C,D). Another striking difference of G2 samples was the total lack of *Prochlorococcus* sequences. Clade VII was frequently found at Stn H6 influenced by oligotrophic KC water, and the highest contribution (34%) was observed in the sample belonging to Group G2 (Table 2, Fig. 4). Group G3 primarily included samples with temperatures between 14°C and 21°C. In these samples, Clade II was dominant (36 to 67%), although Clades I and IV also made up a considerable fraction of the sequences (7 to 38%). Thus, these ecologically contrasting clades were able to co-exist at intermediate water temperatures. Samples clustering in Group G4 exhibited the lowest temperatures (9 to 18°C). Samples obtained at Stns I and D5 during the winter to spring and samples from

SCM depth at Stn D5 in summer were mainly made up of Clades I and IV.

The CCA analysis also showed that the distribution of picocyanobacterial lineages was primarily related to temperature, $NO_3 + NO_2$ and salinity (Fig. 5). As noted above, Clades I and IV were situated on the right side of the biplot, indicating their preference for cold and relatively eutrophic waters. In contrast, the remaining clades were positioned opposite to Clades I and IV, suggesting that these *Synechococcus* and *Prochlorococcus* lineages favor warm and relatively lower nutrient conditions. However, the dynamic changes observed in the *Synechococcus* community, especially during the summer, suggest that individual *Synechococcus* lineages may respond to narrow ranges of temperature and nutrient concentrations and thus may have specific ecological niches (Tai et al. 2011).

DISCUSSION

Methodological considerations

To understand picocyanobacterial diversity in marine environments, clone libraries and specific probe/primer sequence-based quantitative approaches (southern hybridization or qPCR) have been widely used. However, analyzing clone libraries from tens of samples and performing qPCR for a multiple sets of primers or probes is laborious and expensive. In our experience, a single run of amplicon pyrosequencing using >100 barcodes on a 1/8 PicoTiter Plate could produce >600 high-quality reads per sample on the average (data not shown). Furthermore, only approximately a hundredth of this read number would be sufficient to study picocyanobacterial diversity at the clade level (see 'Results'). Thus, pyrosequencing is an appropriate tool for studying picocyanobacterial diversity in large numbers of samples due to its advantages in terms of time, cost and the quantity of data generated. However, note that pyrosequencing generates qualitative output and thus requires prior knowledge of the rRNA operon copy number and cell abundances to arrive at a quantitative estimation. To date, genomes have been sequenced for strains belonging to diverse *Synechococcus* and *Prochlorococcus* clades (see IMG-ER database; Markowitz et al. 2009). The rRNA operon copy numbers are either 1 or 2 in most strains (see Table 2). By applying these values to each clade or overall group means, the number of picocyanobacteria belonging to each clade can be approximated from *Synechococcus* and *Prochlorococcus* abundances without extreme bias.
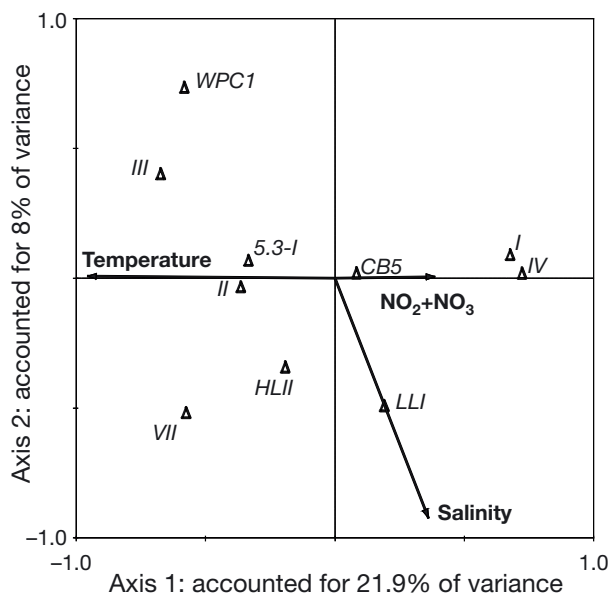

Fig. 5. CCA ordination plot for the first 2 CCA dimensions of the relationship between picocyanobacterial composition and environmental parameters. Only clades with >10% in at least 1 sample are shown. See Fig. 2 for clade identifications

Seasonal pattern of picocyanobacterial abundances

In the ECS and ES, *Synechococcus* abundances tended to increase markedly at temperatures below 20°C (Fig. 3), at which point saturation was reached. At Stn I, maximal *Synechococcus* abundances were similar to or slightly lower than those found at the Scripps pier over a 3 yr observation period (Tai & Palenik 2009). At the Scripps pier, a *Synechococcus* bloom was characterized by the codominance of Clades I and IV (Tai & Palenik 2009). Likewise, at Stn I in June with rapid increases in abundance, Clades I and IV exhibited dominance (Tables 1 & 2). Contrary to the observed codominance of Clades I and IV throughout the year at Scripps pier, however, the dominant *Synechococcus* was Clade II in summer in the present study. This dramatic change seems to be related to high temperatures (ca. 28°C) observed in August. In the subtropical Gulf of Aqaba in the Red Sea (Post et al. 2011), the surface of subtropical northwestern Pacific Ocean (Choi et al. 2011) and in coastal/continental shelf zones in subtropical/ tropical areas (Zwirglmaier et al. 2008), Clade II was dominant, suggesting that this lineage is highly competitive in warm waters. Similarly dramatic changes in *Synechococcus* diversity during periods of high abundance were also observed in surface waters of the ES (Table 2). Therefore, increases in *Synechococcus* abundance in temperate waters were attributable to Clades I and IV at intermediate temperature (~20°C), whereas at higher temperatures, changes in abundance were due to the warm-water-adapted clades.

At Stn H6 in February, *Synechococcus* abundances were 1 order of magnitude lower than those found at other stations at temperatures comparable to that of Stn H6 (Table 1). Stn H6 was likely to be affected by a branch of warm KC, and when the water temperatures decreased to 16°C in winter, the temperature would limit the growth of the dominant warm water-adapted Clade II *Synechococcus* (ca. 70%) (Table 2). Further, advection of cold-water-adapted Clades I and IV to Stn H6 through the shelf area would not be large in winter. Thus, the low abundances at Stn H6 seem to be related to both temperature and *Synechococcus* composition. In addition, the low abundances may have been due to limited light intensity as a result of active vertical mixing, considering that recent mesocosm experiments have shown that light plays an important role in the initiation of spring blooms (Sommer & Lengfellner 2008). Thus, in winter, *Synechococcus* abundances may be limited by temperature and light intensity.

Prochlorococcus is predominant in oligotrophic and tropical open ocean waters between 40° N and 40° S (Partensky et al. 1999). However, their distribution is suppressed in coastal areas and in upwelling waters (Partensky et al. 1999, Liu et al. 2004, Johnson et al. 2006). In the present study, we observed high summer and autumn abundances for *Prochlorococcus* (4.5×10^4 cells ml^{-1}; Table 1) in the central ES and the northeastern ECS influenced by the KC. In contrast, *Prochlorococcus* was not detected at Stn I. Thus, our work suggests that *Prochlorococcus* advected from the oligotrophic open ocean may continue to grow in marginal seas as long as conditions remain warm and oligotrophic; however, their growth appears to be inhibited in shallow shelf waters heavily influenced by the CRDW. Unexpectedly, *Prochlorococcus* sequences were found in several winter samples with low temperatures of ca. 10°C (Table 2). Because low temperatures have adverse effects on the growth of *Prochlorococcus* (Moore et al. 1995), its presence in winter samples was more likely due to advection from adjacent KC waters. Because *Synechococcus* abundances are low at that time, most likely due to light limitation and low temperatures, the advected *Prochlorococcus* would have a relatively high contribution to pico-cyanobacterial abundances. Thus, in the temperate marginal seas, the growth and distribution of *Prochlorococcus* appears to be controlled by temperature and the mixing of coastal and open waters.

Spatiotemporal variations of picocyanobacterial diversity

In the temperate waters, the spatiotemporal changes in picocyanobacterial composition were remarkable (Table 2). In studies of seasonal variation of *Synechococcus* diversity, either Clade I/IV or Clade II dominates throughout the year (Fuller et al. 2006, Tai & Palenik 2009, Post et al. 2011, Tai et al. 2011). However, our study showed that the dominant clade shifted from Clades I and IV (adapted to cold and coastal water) to the warm-water-adapted Clade II, demonstrating a distinct seasonal distribution in the ECS and the ES. In samples from cold waters (<18°C), Clades I and IV dominated, accounting for 50 to 100% of the picocyanobacterial community. Most of the samples were obtained at Stns I and D5 in winter and spring. Thus, Clades I and IV seems to be major *Synechococcus* lineages in winter and spring below ca. 18°C in temperate marginal waters. However, at Stn H6, Clade II was dominant

(58 to 85%), followed by Clades I/IV (10 to 25%). This difference may have been influenced by a branch of warm, oligotrophic water from the KC. On the contrary, in summer and autumn samples of Stns D5 and H6 affected by KC, *Synechococcus* Clades II and *Prochlorococcus* HLII dominated together, accounting for 49 to 92% of total pico-cyanobacteria (Table 2). Their dominance in summer and autumn was related to high temperature and oligotrophic conditions of the samples (Fig. 4C,D). However, in the summer sample of Stn I, Clade II was most dominant and *Prochlorococcus* was absent, likely due to the influence of Changjiang River water (Table 1).

Thus, in temperate waters, the seasonal change of picocyanobacterial diversity seems to be largely regulated by water temperature and trophic conditions. Changes in water properties and advection of pico-cyanobacteria derived by transport and mixing of adjacent water masses are also important in driving spatial variations of picocyanobacterial diversity in marginal temperate waters.

Ecological niches of *Synechococcus* clades

A higher total number of clades occurred in warm water. Clade III and, to a lesser extent, WPC1 were also abundant in surface water samples collected in the ECS during the summer (Table 2). The dominance of Clade III, followed by WPC1, was also reported in the oligotrophic Mediterranean Sea in the summer (Mella-Flores et al. 2011). The prevalence of Clade III in the Mediterranean Sea may be attributable to its competitive advantage in scavenging P under P stress (Scanlan et al. 2009, Mella-Flores et al. 2011). In the ECS, P limitation has been reported in association with salinity values <30.5 in summer (Wong et al. 1998). At Stn I in August, surface water showed a salinity of 29.5 and an N:P ratio of 33. Although the N:P ratio could not be estimated at Stn H6 due to undetectably low nutrient concentrations, the influence of CRDW was apparent considering the low salinity of 31.5. Thus, the relatively high proportion of Clades III and WPC1 in the samples may be explained by adaptation to P-limited conditions in the ECS. In the CCA analysis, Clades III and WPC1 were situated on the upper left (high temperature and low N preference), apart from all other clades (Fig. 5), suggesting that *Synechococcus* Clades III and WPC1 appear to be adapted to high-temperature and low-nutrient conditions, most likely P-limitation.

Clade 5.3-I was observed in all seasons, with the exception of several low-temperature samples collected in the spring, and this lineage often co-occurred with Clade II (Table 2). However, Clade 5.3-I members outnumbered Clade II members only in samples with temperatures of ca. 23°C and chl *a* concentrations of ca. 0.5 µg l^{-1}. A recent study showed that SC5.3 is prevalent at the SCM depth at 23°C in warm off-shore stations in the South China Sea as well as in surface waters in the North Atlantic with temperatures of ca. 20°C (Huang et al. 2012). Thus, Clade 5.3-I seems to occupy a niche similar to that of Clade II but may be more competitive under less oligotrophic (i.e. mesotrophic) and relatively low-temperature conditions.

The global distribution of Clade VII is unclear due to the widespread use of a probe targeting combined Clades V/VI/VII, but previous works on the distribution of the combined Clades V/VI/VII in tropical and subtropical waters showed that Clade VII is widespread in oligotrophic waters (Zwirglmaier et al. 2007, 2008). Similarly, Clade VII was frequently present in waters affected by the KC, suggesting that these picocyanobacteria are advected from warm, oligotrophic open waters. However, increased dominance (34%) in the December sample at Stn H6 suggests that Clade VII members may grow opportunistically under elevated nutrient conditions in boundary waters affected by coastal or upwelling waters.

Clades I and IV appeared to be dominant in samples collected in the winter and spring as well as in samples from SCM depths in the summer (Table 2). However, the relative contributions of Clades I and IV varied greatly in the present study, with I:IV ratios ranging between 0.03 and 40, suggesting that the 2 clades in fact occupy distinct niches in cold coastal waters. To date, the factors controlling the relative abundances of Clades I and IV have not been determined (Zwirglmaier et al. 2008). However, in the present study, the ratios of I to IV showed a weak but significant positive relationship with the sum of nitrate and nitrite (Fig. 6), suggesting that N availability may be one of the factors determining their relative abundances.

CONCLUSIONS

Barcoded amplicon pyrosequencing was successfully applied to elucidate picocyanobacterial diversity and its distribution in marginal seas. Pico-cyanobacterial composition changed dynamically
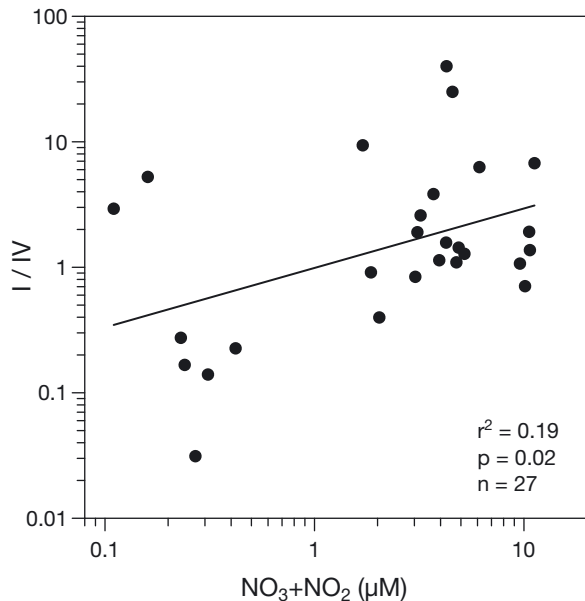
Fig. 6. Relationship between the sum of nitrate and nitrite concentrations and the abundance ratio of Clades I to IV in the present study

with season. In terms of an overall seasonal pattern, Clades I/IV and Clades II/HLII dominated during the cold and warm seasons, respectively. Furthermore, several picocyanobacterial clades were observed with less contribution. Temperature and nutrient levels likely play an important role in driving seasonal shifts in picocyanobacterial composition, and physical mixing between adjacent water masses is important in determining the physicochemical properties of water and thus the distribution of picocyanobacterial lineages. In addition, the distribution of *Prochlorococcus*, of which abundances could not be easily determined via flow cytometry, could be elucidated using the pyrosequencing method. Further application of this pyrosequencing method in high-throughput samples will broaden our understanding of the biogeography, ecological niches and adaptation to changing environments of various picocyanobacterial lineages.

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