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Diverse and Unique Picocyanobacteria in Chesapeake Bay, Revealed by 16S-23S rRNA Internal Transcribed Spacer Sequences†§

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rRNA internal transcribed spacer phylogeny showed that Chesapeake Bay is populated with diverse *Synechococcus* strains, including members of the poorly studied marine cluster B. Marine cluster B prevailed in the upper bay, while marine cluster A was common in the lower bay. Interestingly, marine cluster B *Synechococcus* included phycocyanin- and phycoerythrin-rich strains.

Phototrophic picoplankton (<3 μm) play an important role in the ocean's carbon cycle (18, 22, 33, 34). *Synechococcus* strains, which are small (1- to 3-μm) unicellular cyanobacteria, are a major component of marine picophytoplankton (32). New ecotypes and genotypes continue to emerge as the diversity of *Synechococcus* from different ecosystems is explored (3, 4, 6–10, 12–14, 20, 21, 24, 25, 30, 31), but in general, less is known about *Synechococcus* living in coastal and estuarine regions than about that in offshore regions (26). In Chesapeake Bay, picophytoplankton contribute 10 to 20% of total primary production during summer (1, 19, 23). Picophytoplankton can reach levels of 10^6 cells/ml and account for 56% of primary production in the lower bay (2). Recently, picocyanobacterial strains isolated from the bay were found to be dominated by marine *Synechococcus* (6), but knowledge about the diversity and distribution of picocyanobacterial populations in different Chesapeake habitats remains unclear.

Marine *Synechococcus* strains have been classified into three major clusters, i.e., marine clusters A, B, and C (MC-A, MC-B, and MC-C) (32). The MC-A cluster contains diverse *Synechococcus* strains isolated from coastal and open oceans, and its classification is supported by 16S rRNA and internal transcribed spacer (ITS) phylogeny (17, 19, 24). The MC-C cluster contains four closely related marine *Synechococcus* strains (11). In contrast to MC-A and MC-C, the phylogenetic position of MC-B is less understood.

Chesapeake Bay, the largest estuary in the United States, provides strong hydrological gradients and diverse habitats for picophytoplankton. In this study, we investigated the population structure of picocyanobacteria in Chesapeake Bay, based on the ITS sequences of isolates and environmental clones of picocyanobacteria.

Isolation and cultivation of Chesapeake Bay *Synechococcus* strains were as previously described (6). Water samples for DNA (2-m depth) were collected from three Chesapeake Bay stations (Table 1), using Niskin bottles, on board the R/V *Cape Henlopen* on 26 to 30 September 2002 and 4 to 8 March 2003. To concentrate microbial cells, 250 ml of water was filtered through 0.2-μm-pore-size filters (15). Nucleic acids from isolates and microbial communities were extracted using a method described elsewhere (27). ITS fragments of *Synechococcus* isolates were amplified as described by Rocap et al. (25). Clone libraries containing a large portion of the rRNA operon (16S rRNA-ITS-23S rRNA) from bacterioplankton within six surface water samples were constructed as previously described (28) with the following changes: (i) platinum HIFI polymerase mix (Invitrogen, Carlsbad, CA) was used to provide hot-start amplification, (ii) PCR products were A tailed using the QIAGEN A addition kit (QIAGEN, Chattsworth, CA), and (iii) products were cloned using the TOPO TA cloning kits for sequencing (Invitrogen). A minimum of 82 clones from each library were screened by a novel screening method adapted from the ITS-length heterogeneity-PCR method, which measures the length variation of two fragments amplified by PCR with fluorescence-labeled primers (29). Clones were screened based on the lengths of two regions of the ITS (SSU1406-tRNAala and SSU1406-LSU66). Representative clones were also sequenced to confirm the prescreening results. Plasmids were purified using the FastPlamid (Eppendorf, Westbury, NY) and Montage Miniprep96 (Millipore, Billerica, MA) kits. Sequencing was performed on an ABI Prism 3100 genetic analyzer using Big Dye V3.1 chemistry (Applied Biosystems, Foster City, CA). Phylogenetic analyses were conducted using the MacVector 7.2 program (Accelrys Software Inc., San Diego, CA) and the Molecular Evolutionary Genetics Analysis software, MEGA 3.1 (16).

Phylogenetic analysis of 82 picocyanobacterial ITS sequences (Fig. 1) included picocyanobacteria from freshwater lakes, brackish or estuarine waters, and coastal and oceanic waters. The majority of Chesapeake Bay cyanobacterial ITS

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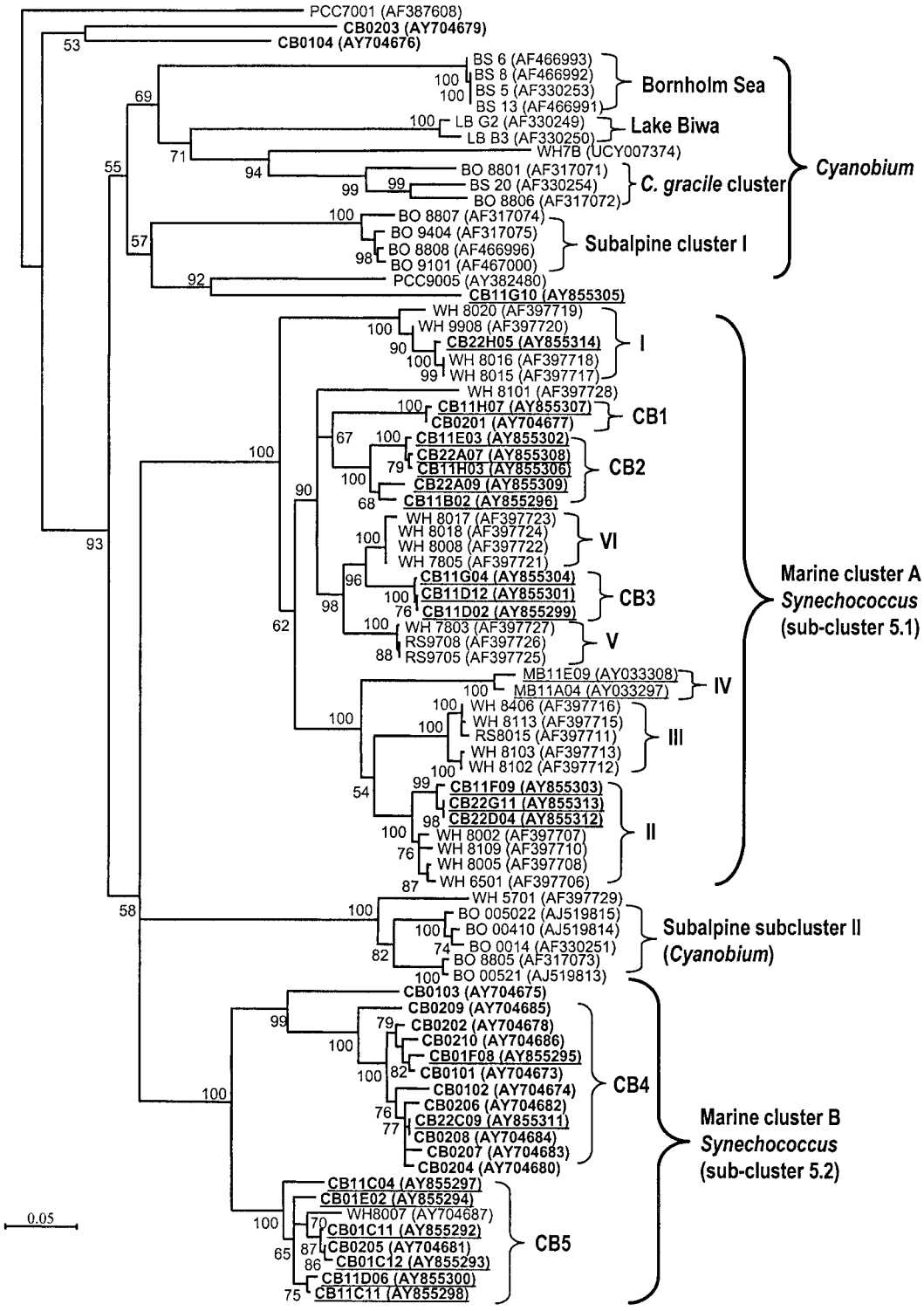

FIG. 1. Neighbor-joining tree based on ITS sequences (786 positions) of picocyanobacterial isolates (58 sequences, not underlined) and environmental clones (24 sequences, underlined) collected from lakes and from brackish, estuarine, coastal, and oceanic waters. The tree was rooted with PCC7001. Numbers at tree branches indicated the bootstrap values from 1,000 resamplings. Bootstrap values of less than 50 are not shown. The scale bar is equivalent to 0.05 substitution per site. Names in boldface represent the sequences from this study. Prefixes for the *Synechococcus* strains or environmental clones are as follows: CB, Chesapeake Bay; WH, Woods Hole; RS, Red Sea; MB, Monterey Bay; LB, Lake Biwa; BO, Lake Constance (Bodensee); and BS, Baltic Sea. The strains with the PCC prefix are the picocyanobacterial isolates collected by the Pasteur Culture Collection. The GenBank accession numbers are given in parentheses.

TABLE 1. Characteristics of three *rrn* operon clone libraries constructed from water samples collected in the upper, middle, and lower Chesapeake Bay in 2002[a]

Characteristic	Value for clone library		
	CB01 (upper bay)	CB11 (mid-bay)	CB22 (lower bay)
Station name	908	818	707
Location	39.0800N, 76.2000W	38.1800N, 76.1700W	37.0700N, 76.0700W
Water temp (°C)	23.3	23.9	24.2
Salinity (ppt)	15.5	19.4	27
Bacterial count (10^6 cells ml^{-1})	6.42	2.91	2.57
Synechococcus count (10^6 cells ml^{-1})	0.23	0.29	0.36
Synechococcus in total bacteria (%)	3.58	9.97	14.01
PC type in total *Synechococcus* (%)	86.7	47.8	18.4
Total clones	91	84	88
No. (%) of cyanobacterial clones	4 (4.4)	12 (14.3)	7 (8.0)
Prescreening size (bp) by FAM/HEX[b] of:			
943/458			CB22A09
954/464		CB11C11, CB11D02	
964/457		CB11B02, CB11E03, CB11H03	CB22A07
974/464	CB01C11, CB01E02, CB01C12	CB11C04, CB11D06	
996/460		CB11F09, CB11H07	CB22D04, CB22G11
1,007/460		CB11D12, CB11G04	CB22H05
1,056/486	CB01F08		CB22C09
1,123/487		CB11G10	

[a] Twenty-three of 263 clones were identified as cyanobacteria based on prescreening of ITS length. Relevant physical, chemical, and biological characteristics at these stations are shown.

[b] See reference 29 for an explanation of FAM/HEX.

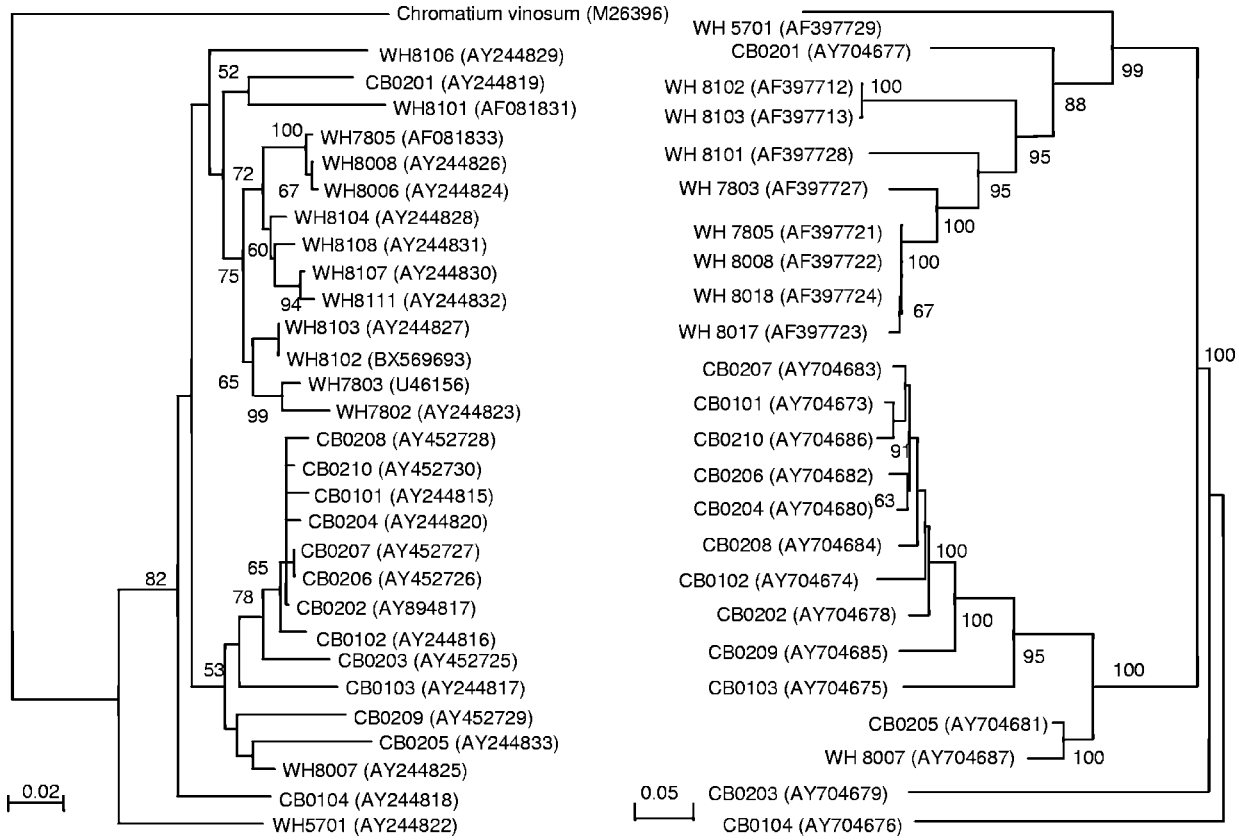

FIG. 2. Comparison of phylogenetic trees constructed from *rbcL* gene (left panel) and ITS (right panel) sequences from Chesapeake Bay and Woods Hole isolates. Bootstrap values were calculated based on 1,000 resamplings. Values lower than 50 are not shown. The scale bar is equivalent to 0.02 substitution per site for the *rbcL* phylogenetic tree and to 0.05 substitution per site for the ITS phylogeny.

sequences were affiliated with either MC-A or MC-B. Eleven of 14 Chesapeake isolates clustered with WH8007 (MC-B cluster). Among 22 environmental clones putatively identified as cyanobacteria, 13 clustered within MC-A, 9 clustered within MC-B, and 1 clone (CB11G10) formed a deep branch within the *Cyanobium* cluster. The discrepancy in MC-A distribution between culture and culture-independent methods is likely due to the salinity of media used for isolation (10 to 20 ppt) favoring the growth of estuarine MC-B rather than MC-A strains. MC-B strains are known to have an elevated salt requirement for growth. Regardless, both approaches confirmed that freshwater *Synechococcus* strains are rare in the bay, even in the upper bay where the salinity is in the range of 5 to 10 ppt. At least 16 subclusters (>95% sequence identity; bootstrap value, 100) could be identified across all the picocyanobacteria included in this study (Fig. 1). Eleven subclusters overlap with previously reported subclusters (8, 25), while at least four new subclusters (CB1 to CB4) were novel and unique to the Chesapeake Bay.

MC-B is a polyphyletic group containing both phycocyanin- and phycoerythrin-rich *Synechococcus* strains. At least two subclusters (CB4 and CB5) could be defined within the MC-B cluster. Within subcluster CB4, five phycoerythrin-rich *Synechococcus* strains (CB0206, CB0207, CB0208, CB0209, and CB0210) were closely related to four phycocyanin-rich *Synechococcus* strains (CB0101, CB0102, CB0202, and CB0204) (Fig. 1). A close relationship between phycocyanin- and phycoerythrin-rich *Synechococcus* strains in MC-B was also evident based on *rbcL* phylogeny (Fig. 2). The separation of MC-A and MC-B was also supported by *rbcL* phylogeny (Fig. 2).

Among six rRNA operon clone libraries, only three, constructed from the September samples, contained cyanobacterial sequences. The absence of cyanobacteria in the March clone libraries reflects a low abundance (typically, $<10^3$ cells/ml) of picocyanobacteria in the cold season. In the September clone libraries, all four clones from the upper bay were MC-B members, while only one of seven clones in the lower bay was an MC-B member. The mid-bay contained a mixture of both MC-A and MC-B members (Fig. 1 and Table 1). Despite the wide range of salinity along the bay, marine *Synechococcus* (MC-A and MC-B), not *Cyanobium*, dominated the Chesapeake picocyanobacterial community.

The ITS length among Chesapeake picocyanobacterial isolates and environmental clones varied widely, from 753 to 875 nucleotides and 606 to 913 nucleotides, respectively (see Table S1 in the supplemental material). The length heterogeneity of ITS is sufficient to differentiate various *Synchococcus* strains. Interoperon variation is not a concern for *Synechococcus*, which contains two identical rRNA operons (5). The average percent G+C of the ITS sequence for MC-B isolates and clones is 48.8% ± 2.5% (n = 19), which is lower than that for *Cyanobium gracile* PCC6307 (54%) and higher than those for *Prochlorococcus* (38.6% ± 2.0%) (25) and MC-A isolates and clones (44.1% ± 1.3%, n = 21) (see Table S1 in the supplemental material). Based on the ITS phylogeny and GC content, we suggest that WH8007, rather than WH5701 (10, 31), should be the reference strain for MC-B (or *Synechococcus* cluster 5.2 [11]).

Nucleotide sequence accession numbers. GenBank accession numbers are shown in Fig. 1.

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