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Molecular Phylogenetic Analyses of Three *Synechococcus* Strains Isolated from Seawater near the Ieodo Ocean Research Station

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Abstract – Three *Synechococcus* strains were isolated from seawater near the Ieodo Ocean Research Station (IORS), and their 16S rDNA genes and the internal transcribed spacer (ITS) between the 16S and 23S rRNA genes were sequenced to investigate their phylogenetic relationships. Phylogenetic trees based on the 16S rDNA and ITS sequences showed that they clustered in the main MC-A *Synechococcus* group (subcluster 5.1), but formed branches differentiating them from the described clades. As the IORS is located in an area affected by diverse water masses, high *Synechococcus* diversity is expected in the area. Therefore, the IORS might be a good site to study the diversity, physiology, and distribution of the *Synechococcus* group.

Key words – *Synechococcus*, Phylogeney, 16S rRNA, ITS gene, Ieodo

1. Introduction

Marine picophytoplankton (cells < 2–3 μm in diameter) belong mainly to two genera of cyanobacteria, *Synechococcus* and *Prochlorococcus*, and diverse eukaryotic picoplankton. The phycoerythrin-containing cyanobacteria of the genus *Synechococcus* are ubiquitously distributed in huge numbers and are dominant contributors to primary production and biomass in a vast area of the world's oceans (Waterbury *et al.* 1979; Partensky *et al.* 1999; Agawin *et al.* 2000). Despite their importance, few studies have examined the phylogenic relationships among *Synechococcus* species. Marine *Synechococcus* have been classified into three major clusters: MC-A, MC-B, and MC-C (Waterbury and Rippka 1989). Of these, MC-A contains diverse *Synechococcus* strains isolated from coastal and open oceans, and their classification is well supported by 16S rRNA and 16S-23S rRNA internal transcribed spacer (ITS) phylogenies (Rocap *et al.* 2002; Fuller *et al.* 2003).

The East China Sea (ECS), a marginal sea of the Northwest Pacific Ocean is influenced by diverse water masses, including the Kuroshio Current, Tsushima Warm Current, Yellow Sea cold water, and freshwater from the Yangtze River (Beardsley *et al.* 1985). In addition, the seasonal changes in solar radiation strongly affect the water temperatures of the ECS (Chen *et al.* 1993), and seasonal differences in the freshwater discharge from the Yangtze River strongly affect salinity in the study area (Zhang *et al.* 1994). These environmental differences greatly influence the biological conditions of phytoplankton (Guo 1994). According to recent studies, *Synechococcus* is the overwhelmingly dominant group of autotrophic picoplankton in the ECS. During warm periods, *Synechococcus* can reach 3.0×10^4 cells ml^{-1}, although cell abundance seems to be synchronized with changes in environmental conditions (Jiao *et al.* 2002, 2005; Noh *et al.* 2005). Although there are indications that *Synechococcus* distribution studies in the ECS using flow cytometry are being carried out, very few taxonomic studies have examined this genus in the ECS.

In this study, we investigated the molecular phylogenic relationships of three *Synechococcus* strains isolated from water near the Ieodo Ocean Research Station (IORS) with other previously described strains. We describe the strains and show that the strains isolated from the IORS form novel clades within the MC-A cluster. This study provides

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fundamental, important information on *Synechococcus* diversity in the ECS, and demonstrates the importance of the IORS in studying the ECS ecosystem.

2. Materials and Methods

The Ieodo Ocean Research Station (IORS) is a comprehensive ocean monitoring and research tower station located on the continental shelf of the East China Sea (ECS) at 32°07'22.63"N, 125°10'56.81"E (Fig. 1). For our phylogenic study, three strains of *Synechococcus* were isolated from seawater near the IORS in March (strains KORDI 36 and KORDI 49) and July (KORDI 38) 2004. Seawater samples were filtered through 3.0 μm polycarbonate filters by gravity. Nutrients based on f/2-Si medium supplemented with 100 μM (final conc.) NH_4Cl solution were added to the samples at about 1:10 dilution, and then the samples were incubated at 25°C under illumination at ca. 10 μE m^{-2} s^{-1}. These cultures were maintained by transferring small quantities of the culture to fresh medium roughly every 4 weeks for 2.5 years. Cycloheximide was added to the cultures to prohibit the growth of picoeukaryotes. Axenic cultures were obtained using dilution methods.

Target DNA was amplified by PCR using 2 μl of cell suspension or DNA extracted using a bead-beating protocol involving zirconium beads, chloroform, and isopropanol (McBain *et al.* 2003). The 16S rRNA gene segments were amplified using several primers (27F, Oxy359F, Syn1017R, Oxy1313R, and 1522R) according to the protocol described in Fuller *et al.* (2003). The ITS segments were amplified using a primer set (16S-1247f and 23S-1608r) according to the protocol described in Rocap *et al.* (2002). After purifying the PCR products with an AccuPrep PCR purification kit (Bioneer, Daejeon, Korea), the amplified DNA was sequenced using an Applied Biosystems (Foster City, CA, USA) automatic sequencer at Macrogen (Seoul, Korea).

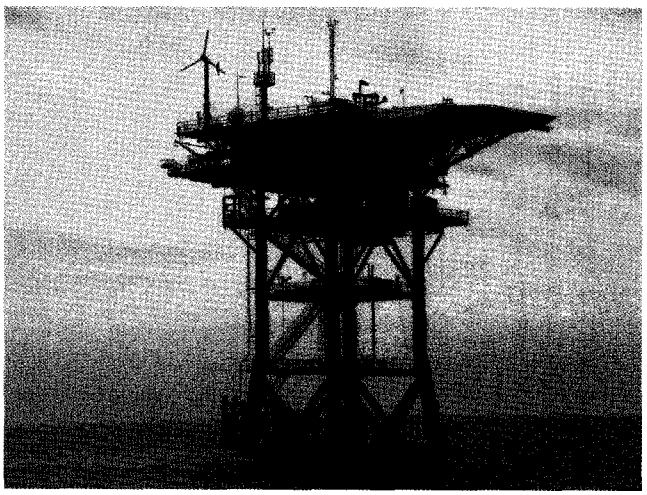

Fig. 1. A picture of the Ieodo Ocean Research Station taken from the R/V Eardo.

The 16S rRNA gene sequences were aligned with those of other *Synechococcus* strains obtained from the GenBank database based on the known 16S rRNA gene secondary structure information using the program *j*PHYDIT (Jeon *et al.* 2005). The ITS sequences were aligned using the program ClustalW.

Phylogenetic trees were obtained using the neighbor-joining, maximum-parsimony, and maximum-likelihood methods. An evolutionary distance matrix for the neighbor-joining method was generated according to the model of Jukes and Cantor. The robustness of tree topologies was assessed by bootstrap analyses based on 1,000 replications for the neighbor-joining and maximum-parsimony methods and 100 replications for the maximum-likelihood method. Phylogenetic analyses were carried out using MEGA 3 (Kumar *et al.* 2004) and PAUP* 4.0 (Swofford, 1998). Likelihood parameters were estimated using the hierarchical ratio tests in MODELTEST version 3.04 (Posada and Crandall 1998).

3. Results and Discussion

Almost complete 16S rDNA sequences were obtained for the three IORS strains. The 16S rDNA sequence similarity between strains KORDI 36 and KORDI 49 was 99.9%. The 16S rDNA sequences of strain KORDI 36 and KORDI 49 showed 98.8% and 99.0% similarity with that of strain KORDI 38, respectively. The phylogenetic relationships between these strains and other marine *Synechococcus* and *Prochlorococcus* sequences already in the database are shown in Fig. 2. Regardless of the tree-building method, the *Synechococcus* strains other than the three IORS strains fell into the ten distinct clades designated by Fuller *et al.* (2003), which are supported by high bootstrap values. The IORS strains clustered into subcluster 5.1 of the MC-A cluster. However, they were not clustered in any of the ten recognized clades. Although the 16S rDNA sequences of strains KORDI 39 and KORDI 49 showed high similarities (98.7–99.2%) to those of

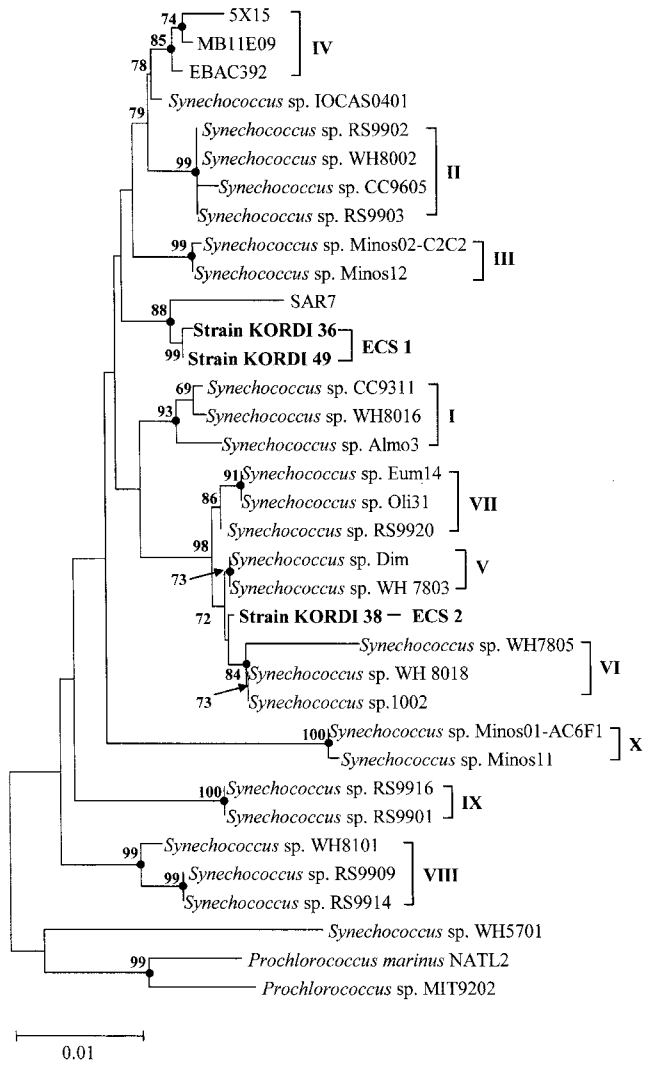

Fig. 2. Neighbor-joining tree showing the relationships between the IORS strains and other *Synechococcus* strains inferred using 16S rDNA sequences. Only bootstrap values (expressed as percentage of 1,000 replications) greater than 60% are shown at the nodes. Solid circles indicate that the corresponding nodes are also recovered in the maximum-likelihood and maximum-parsimony trees. The clade designation of Fuller *et al.* (2003) is shown to the right. *Synechococcus elongatus* PCC 7942 was used as the outgroup. Bar, 0.01 nucleotide substitutions per site.

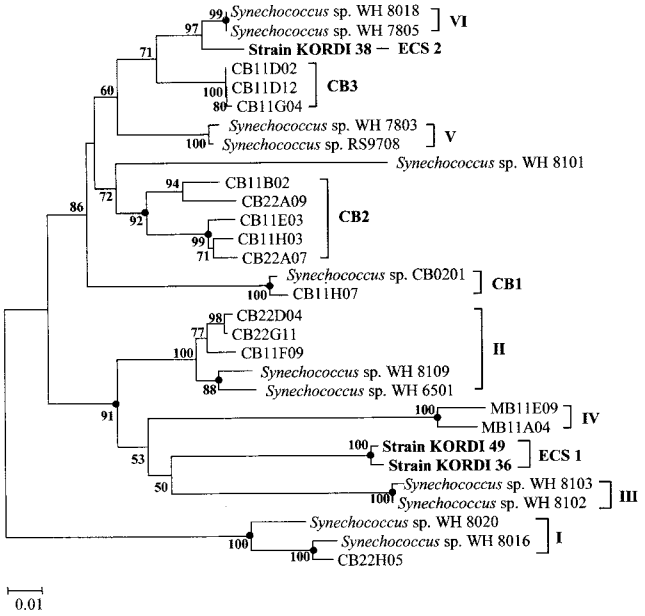

Fig. 3. Unrooted neighbor-joining tree based on ITS sequences showing the relationships between the IORS strains and other *Synechococcus* strains. Only bootstrap values (expressed as a percentage of 1,000 replications) greater than 60% are shown at the nodes. Solid circles indicate that the corresponding nodes were also recovered in the maximum-likelihood and maximum-parsimony trees. The clade designations of Rocap *et al.* (2002) and Chen *et al.* (2006) are shown on the right. Sequences with the prefix CB are clones obtained in Chesapeake Bay. Bar, 0.01 nucleotide substitution per site.

several *Synechococcus* strains belonging to clades II, III, and IV, they did not cluster into those clades. Similarly, strain KORDI 38 showed high sequence similarity (99.7–99.9%) to strains belonging to clades V, VI, and VII, except for the strain *Synechococcus* sp. WH7805, but strain KORDI 38 did not form a robust clade with them. Two robust novel clades based on the 16S rDNA sequences were found using all three tree-building methods.

As with the 16S rDNA sequence phylogeny, the IORS strains fell into two distinct novel clades in the phylogenetic analyses of the ITS sequences (Fig. 3). In the ITS phylogeny by neighbor-joining method, strain KORDI 38 formed a strong sister clade with clade IV, which consists of strains lacking phycourobilin (Rocap *et al.* 2002). Strain KORDI 38 has phycourobilin (data not shown), differentiating it from the members of clade IV. The IORS strains were distinguished from the other previously described clades, including cultured strains (Rocap *et al.* 2002) and sequences from culture and clone libraries from Chesapeake Bay (Chen *et al.* 2006). Furthermore, the classification based on ITS sequences was identical to that based on the 16S rDNA sequences. Therefore, it is evident that the IORS strains represent members of novel clades in the *Synechococcus* lineage. Interestingly, strains KORDI 36 and KORDI 49 formed a robust sister clade to a branch including a single noncultivated cyanobacterial sequence from the Sargasso Sea. Therefore, it would be intriguing to investigate the physiological and ecological characteristics and distribution of such small, abundant, locally and regionally distinct, but

important plankton.

For example, the ITS phylogeny recently showed that Chesapeake Bay is populated with diverse, unique *Synechococcus* strains (Chen *et al.* 2006). Chen *et al.* (2006) found three novel clades, two of which had environmental clones only that did not cluster into the six reported clades. The two novel clades in our study did not overlap the new clades found in Chesapeake Bay. Therefore, the marine *Synechococcus* isolates belonging to the novel clades reported here clearly support the high genetic diversity within the genus *Synechococcus*. In this respect, effort to find novel isolates and to elucidate their physiological and ecological characteristics may be indispensable to understanding their roles in the marine ecosystem. As the ECS is affected by diverse water masses, resulting in very variable physical, chemical, and biological conditions, high *Synechococcus* diversity is expected in the area. Therefore, the IORS might be a good site for isolating novel strains and studying the physiology, diversity, and ecology of the cyanobacterial genus *Synechococcus*.

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