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New phylogenetically distinct cyanophages found in the coastal Yellow Sea by Qingdao

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Summary. – Genetic composition of natural cyanophage population in the coastal Yellow Sea area near Qingdao was investigated using the *g20* gene as a genetic marker. Amplification of this gene by PCR yielded sequences, which were cloned and subjected to the restriction fragment length polymorphism (RFLP) analysis. Sixteen clones exhibiting different RFLP patterns were sequenced and analyzed. The sequence analysis indicated a diversity of cyanophages covering sixteen *g20* genotypes. Their phylogenetic analysis revealed two distinct phylogenetic groups. One group (7clones) localized with the cyanophage P77 in the cluster III described before. Second group (9 clones) did not localize with any of the cyanophage described so far and was designated as a novel W cluster. The study revealed a genetic diversity of cyanophages in the coastal Yellow Sea and confirmed the genetic differences between cyanophage populations in Western and Eastern Pacific.

Keywords: cyanophages; genetic diversity; Yellow Sea

Introduction

Marine unicellular cyanobacteria are abundant in the photic zone of the world's oceans and their concentrations could reach up to 10^6/ml (Michael and Emile, 2004; Sandaa *et al*., 2006). Cyanobacteria (the genera *Synechococcus* and *Prochlorococcus*) are important contributors to the primary productivity in marine ecosystems (Sullivan *et al*., 2008). Together, they form the dominant phototrophs in many regions of the tropical and subtropical oceans, and thus contribute significantly to global carbon cycling (Zwirglmaier *et al*., 2007).

Cyanophages are viruses that infect cyanobacteria belonging to the genera *Synechococcus*, *Prochlorococcus* and other. They are now recognized as ubiquitous, abundant, and diverse biological entities just as their hosts in the marine ecosystems. In coastal waters their concentrations usually range from 10^3/ml to 10^5/ml and sometimes even rise up to 10^6/ml (Mann, 2003; Wang and Chen, 2004). Cyanophages, an important part of the aquatic microbial community, play a key role in the regulation of biological production and cycling of the carbon and other nutrients. They influence the dynamics of cyanobacteria populations and mediate a gene transfer among microorganisms in the sea (Fuhrman, 1999; Wommack and Colwell, 2000; Sullivan *et al*., 2008). Studies showed that cyanophages help to prevent and control algal bloom, what could help to reduce environmental pollution and be beneficial for the maintenance of the natural ecological balance (Suttle *et al*., 1990).

Cyanophages as a diverse biological entity in the marine ecosystems were seen as diverse also in terms of their morphology (Safferman *et al*., 1983; Mann 2003). Morphological studies showed that all cyanophages reported to date were tailed phages with dsDNA genome belonging to three viral families *Myoviridae*, *Siphoviridae*, and *Podoviridae* (Safferman *et al*., 1983). The majority of the isolated marine cyanophages belonged to the family *Myoviridae* (Sandaa and Larsen, 2006). However, very little was known about the genetic diversity and phylogenetic linkages of cyanophages in the natural marine environments. The main reason was that no gene could be used as a universal proxy to infer

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Abbreviations: NJ = neighbor-joining; RFLP = restriction fragment length polymorphism

cyanophage diversity (Paul *et al*., 2002). Now P60, P-SSP2, P-SSP4, P-SSP7, S-PM2, P-SSM2, P-SSM4, Syn9, Pf-WMP3, Pf-WMP4, and S-RSM4 have been isolated and their genome sequencing have been completed (Chen and Lu, 2002; Hardies *et al*., 2003; Sullivan *et al*., 2005; Mann *et al*., 2005; Weigele *et al*., 2007; Liu *et al*., 2007, 2008; Millard *et al*., 2009). Whole-genome comparisons showed that conserved genes that are shared among certain cyanophage taxonomic groups exist and could be used to examine the cyanophage diversity (Sandaa and Larsen, 2006). A conserved region found in most cyanomyoviruses (cyanophages that belong to the family *Myoviridae*) is *g20* gene that encodes the viral capsid assembly protein (Hambly *et al*., 2001). Several pairs of primers specifically targeting the *g20* gene have been developed and used in a variety of cyanophage diversity studies (Fuller *et al.,* 1998). Researches using RFLP or denaturing gradient gel electrophoresis with these primers have revealed that cyanophages are genotypically diverse in the sea and that their diversity vary among different marine environments (Lu *et al*., 2001; Zhong *et al*., 2002; Wang and Chen, 2004; File´ e *et al*., 2005; Short and Suttle, 2005**;** Sandaa and Larsen, 2006). Up to date, the cyanophage diversity based on the *g20* gene has been examined along the Norwegian coastal waters, Rhode Island's coastal waters, Georgia coastal rivers, Gulf Stream, Sargasso Sea, Arctic Chuckchi Sea, coast of British Columbia, Chesapeake Bay, Southern Ocean, western Arctic Ocean, and Eastern Pacific (Lu *et al*., 2001; Zhong *et al*., 2002; Wang and Chen, 2004; Short and Suttle, 2005; File´ e *et al*., 2005; Sandaa and Larsen, 2006). However, no data describing the cyanophage diversity has yet been reported for the coastal waters of Western Pacific.

The coastal Yellow Sea by Qingdao has characteristic of a shelf sea and is located at the edge of Western Pacific.

In this study, genetic composition of the population of cyanophages in the coastal Yellow Sea based on the *g20* gene was investigated by PCR amplification, cloning, sequencing, and phylogenetic analysis.

Materials and Methods

Sample collection. On 1 April 2009, a sample of 1,000 ml surface seawater was collected from Zhanqiao Pier (36°18´N, 120°19´E) of the coastal Yellow Sea by Qingdao, China. The salinity was 31.48 ppt and water temperature was 9.8°C. First the sample was filtered through a 0.45 μm pore-size hybrid cellulose membrane (Whatman) to remove zooplankton, phytoplankton, and some bacteria and the sample was concentrated to a final volume of 10 ml by tangential flow filtration (Millipore) through a cartridge with a 50 K cutoff membrane.

Concentration and purification of cyanophages. The method of virioplankton concentration proposed by Colombet *et al.* (2007) was adopted and further improved. Polyethylene glycol 8000 (Promega Corporation) together with NaCl were added to the ultrafiltered retentate to the final concentrations of 10% and 0.6%, respectively, and then incubated at 4°C in the dark for 22 hrs. The concentrate was then centrifuged at 10,500 × *g* for 40 mins at 4°C and resuspended in 300 μl of 0.1 mol/l NaCl buffer containing 8 mmol/l $MgSO_4$, 50 mmol/l Tris–HCl and 0.005% (w/v) glycerol adjusted to the neutral pH. Further, 100 μl of 1 mol/l KCl solution was added and the concentrate was incubated on ice for 30 mins and centrifuged at 11,500 × *g* for 10 mins at 4°C to obtain the concentrated and purified free viruses.

PCR. Total DNA was extracted by the phenol-chloroform extraction followed by ethanol precipitation. The oligonucleotide primers CPS1 and CPS4 were chosen based on the previous work and amplified to an expected product about 430 bp of the *g20* gene (Fuller *et al*., 1998; Marston and Sallee, 2003). The reaction mixture (25 μl) contained 35 ng of template DNA in 10 mmol/l PCR reaction buffer (Mg^{2+} free) supplemented with 250 μmol/l concentrations of each dNTP, 1.5 mmol/l $MgCl_2$, 1.25 U of DNA polymerase (Takara), and 5 μmol/l of each primer.

The reaction conditions were as follows: 94°C/3 mins, the reaction mixture was run for 35 cycles at 94°C/45 secs, 50°C/45 secs, and 72°C/45 secs, followed by 72°C/5 mins. 2 μl of the diluted PCR products was added to 50 μl of PCR mixture and the reaction was conducted as described above, except the number of amplification cycles was reduced to 25 and final elongation was extended to 30 mins. PCR amplification was carried out with a PTC-100 DNA Engine thermocycler (Biorad). Each reaction was run with a negative control (no template was added). Furthermore, the final PCR products were electrophoresed and photographed with a gel documentation system (JS-380, Peiqing Technology).

Cloning. Amplicons of the appropriate size were excised from agarose gels and purified with TIANgel midi purification kits (Tiangen Biotech). Then, they were ligated into pMD18-T cloning vectors (Takara) and the vector-amplicon products were transformed into DH5α competent cells (Takara) in accordance with the manufacturer's instructions. At last, 100 white clones were picked randomly and marked as ZQS-1 to ZQS-100.

Restriction analysis and sequencing. The clones were PCR amplified using similar protocol as above. Products of all PCRs were digested with restriction endonuclease *Rsa I* (MBI) according to the manufacturer's instructions (Marston and Sallee, 2006). The digested products were separated by electrophoresis on 15% polyacrylamide gels. Clones with the different PCR-RFLP profiles were picked and sequenced using an ABI Prism 377 automated sequence analyzer.

Phylogenetic analysis. BLAST analysis of the inferred DNA sequences were conducted after the removal of vector sequence through the NCBI website (Altschul, 1990). The regions of sequences with strongest identity were extracted for using in phylogenetic analysis. Sequences were aligned with Clustal W (Thompson, 1994). Neighbor-joining (NJ) analysis of the inferred DNA sequences was conducted using MEGA 4.0 software package. Coliphage T4 was used as outgroup. Support for the clades was estimated by means of bootstrap analysis using 1,000 replicates and percentages ≥50 were reported. Phylogenetic reconstruction using maximum parsimony approach in MEGA 4.0 yielded similar results (not shown).

Nucleotide sequence in Genebank. Nucleotide sequences determined in this study have been deposited in the GeneBank database and are listed under Acc. Nos. GU108178-GU108193.

Results

Restriction analysis

After two rounds of PCR, the fragments of approximately 430 bp in length were successfully amplified from the sample. We obtained 92 positive clones from the 100 randomly picked white clones. Among these clones, identical band patterns were encountered more than twice and this fact suggested that the diversity of cyanophages had been adequately sampled. The digestion of *g20* sequences by *Rsa I* resulted in 3 to 6 sub-bands per band type. Finally, 16 distinguishable RFLP patterns were identified visually (Fig. 1).

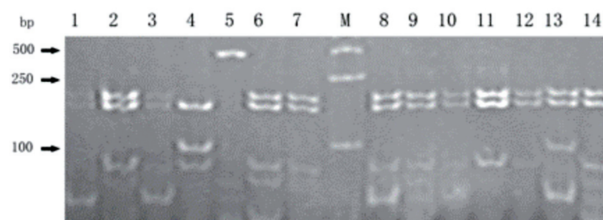

Fig. 1

Restriction analysis of g20 gene sequences of cyanophages from Yellow Sea

PAGE of part of *Rsa*I-digested gene clones (lanes 1–14). DNA size markers (lane M).

Phylogenetic analysis

Phylogenetic analysis of cyanophage *g20* sequences using both NJ and maximum parsimony analyses gave similar results and the phylogenetic tree emerging from NJ analysis is presented (Fig. 2). Some cyanophage sequences from Zhong *et al.* (2002) were included in the analysis to identify the phylogenetic clusters defined in that study. Phylogenetic analysis revealed that there were two clearly distinguished clusters in the examined coastal area. One group consisting of 7 sequences (ZQS-11, ZQS-14, ZQS-50, ZQS-51, ZQS-59, ZQS-70, and ZQS-93) massed together within Cluster III of the cultured *Synechococcus* phages, which displayed high nucleotide similarity with cyanophage P77 and S-WHM1. The paired similarity level among these sequences varied from 94.1% to 99.6% and GC content ranged from 42.2% to 43.1% ($x = 42.8$). The other group was signed as Cluster W and comprised 9 novel sequences (ZQS-7, ZQS-13, ZQS-45, ZQS-54, ZQS-58, ZQS-66, ZQS-77, ZQS-94, and ZQS-98). This group was not represented in the previous studies and did not group with any other known *Synechococcus* phages due to the lack of any closely related neighbors. However, it showed a high sequence similarity with those cyanophages used in the systematic tree. There was 94.5% to 99.2% sequence similarity in this cluster and the GC content ranged from 42.9% to 43.6 % ($x = 43.1$).

Discussion

Cyanophages distributed widely in the aquatic systems have a significant impact on the marine ecosystem dynamics (Sandaa and Larsen, 2006; Sullivan *et al.*, 2008). So far, most studies have focused on the cyanophage diversity in the Southern Ocean, Eastern Pacific, Arctic Ocean, and the North Atlantic (Lu *et al.*, 2001; Zhong *et al.*, 2002; Wang and Chen, 2004; Short and Suttle, 2005; File´ e *et al.,* 2005; Sandaa and Larsen, 2006). As far as we are aware, no information about cyanophage diversity in the coastal waters of Western Pacific has been reported. This study firstly revealed the phylogenetic affiliations of cyanophage community in Qingdao coastal waters.

Cyanophage community in the coastal Yellow Sea by Qingdao was genetically diverse and 16 distinct genotypes were identified in this area. In accordance with this, the relatively high diversity of cyanophages in other marine areas has already been reported. Zhong *et al.* (2002) and Sullivan *et al.* (2003, 2006) proposed that this phenomenon could be due to either phage-host genetic exchange through transduction or genetic exchange between co-infecting phages. Another complementary hypothesis supported the idea that this phenomenon might be linked to the rich abundance of host community (Dorigo *et al.*, 2004). In the studies of cyanophage diversity performed in Norwegian coastal waters, Sandaa *et al.* (2006) found that the cyanophage diversity varied according to the host abundance. Wang *et al.* (2004) showed similar results in the Chesapeake Bay. Their results demonstrated that the cyanophage diversity was highest, when *Synechococcus* abundance reached the annual maximum (Wang and Chen, 2004; Sandaa and Larsen, 2006). Research in our lab showed that the abundance of *Synechococcus* in the coastal Yellow Sea by Qingdao was lowest in the spring and was about 5.43×10^3/ml (Liang, 2008). However, low springtime cyanophages diversity might underestimate the overall cyanophage diversity in this area. Besides, the primers used in this study were originally designed to amplify the cyanomyoviruses (Fuller *et al.,* 1998). Similarly, recent studies showed that the other cyanophage families such as *Siphoviridae* and *Podoviridae* were also abundant and diverse in the marine ecosystems (Marston and Sallee, 2003; Sandaa and Larsen, 2006). Therefore, it was possible that using *g20* gene as a genetic marker could underestimate the cyanophage diversity of this area.

Phylogenetic analysis placed the 16 distinct *g20* genotypes of the identified cyanomyoviruses into two main clades. One of them was previously designated as Cluster III by Zhong *et al.* (2002), who studied 3 different marine ecosystems in the Eastern Pacific and grouped those distinct sequences

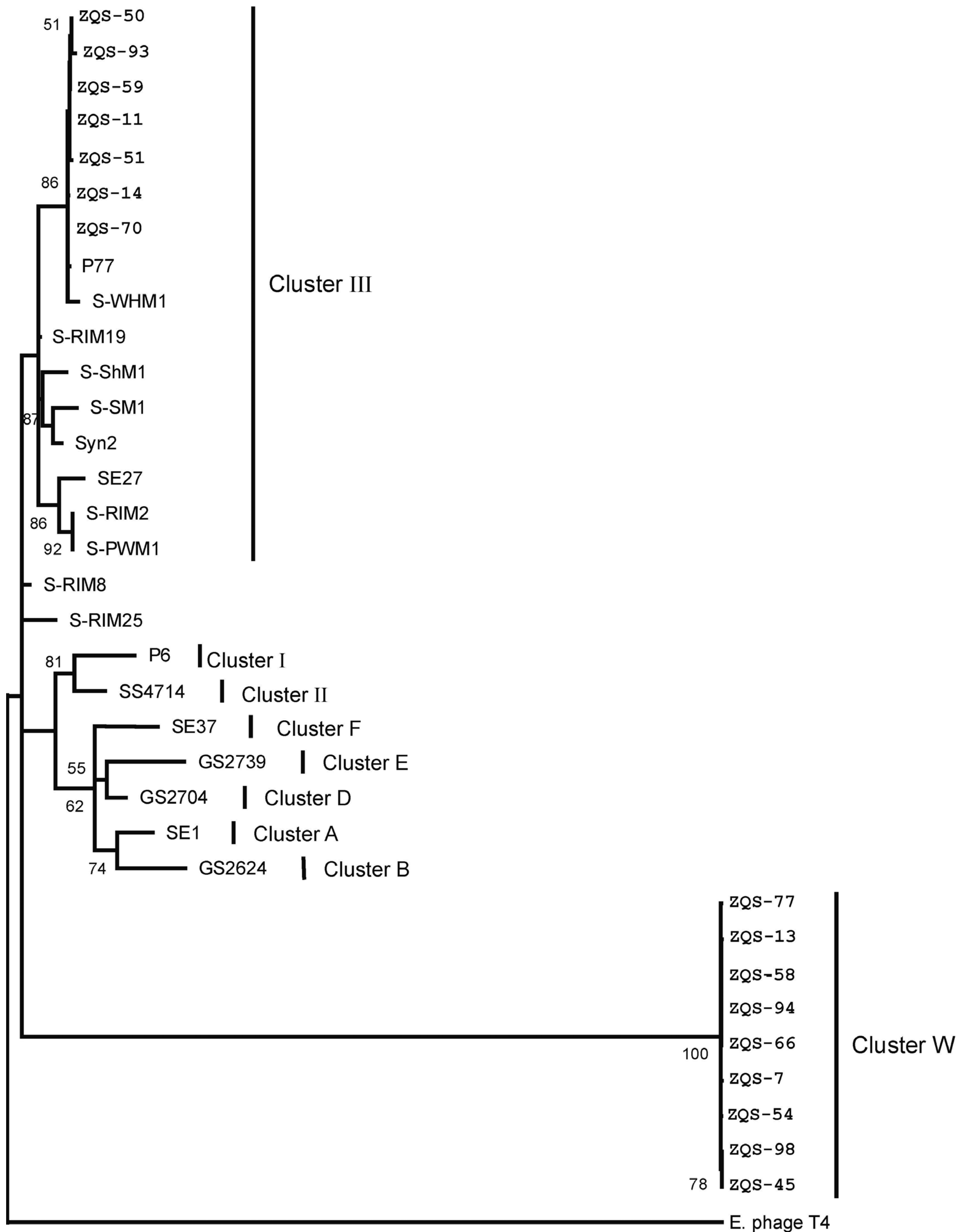

Fig. 2

The g20 gene-based phylogenetic tree of cyanophages from Yellow Sea

The tree refers to the scheme of Zhong *et al.* (2002). The cyanophages from the Yellow Sea are in bold. Scale bar indicates 0.1.

into 9 clusters signed as Cluster I, II, III, and Cluster A, B, C, D, E, F. Cluster III was determined to represent the lytic phages that infect WH7803-like *Synechococcus*. Cyanophages in this cluster were able to adapt to the different marine environments (Zhong *et al*., 2002). Our results demonstrated this conclusion too. In previous studies, the closely related cyanophages were detected over great distances. For example, genetically related cyanophages were found to be widely distributed in the Sargasso Sea and Gulf Stream and the cyanophages originating from different oceans were different only by 8 out of 165 bases of the *g20* gene fragment (Wilson *et al*., 1999; Zhong *et al*., 2002). In this study, 7 different cyanophage genotypes were classified as Cluster III and were grouped with P77 and S-WHM1. Nucleotide similarity analysis revealed that ZQS-70 shared 99% of the nucleotide similarity with cyanophage isolate P77, which was isolated from the Altamaha River estuary that flows into the Atlantic (Lu *et al*., 2001).

Identities of the members of Cluster W were enigmatic due to their lack of grouping with other *g20* sequences obtained from the previous studies. The sampled location was adjacent to the tourist-attractive Zhanqiao Pier, where the seawater was slightly polluted. Furthermore, we could not rule out the appearance of novel *Synechococcus* strains that could be resistant to the eutrophicated water (Zhong *et al*., 2002). This hypothesis, however, remains to be tested as the studies of *Synechococcus* diversity is done in this area. Isolation of more representative strains would provide an additional support for the overall topology of phylogenentic tree and such findings will shed light on the quest for phylogenetic affiliation of an unknown cluster.

Zhong *et al*. (2002) studied 3 different marine ecosystems in the Eastern Pacific and found that the cyanophages of Cluster E were present in all marine areas examined. These results implied that cyanophages of Cluster E were widely distributed in the Eastern Pacific. Nonetheless, in our study no cyanophage sequences were found to locate into Cluster E. On the other hand, we discovered several novel sequences that did not group with any cluster designated by Zhong *et al*. (2002). The distinct cyanophage population structures in the Eastern and Western Pacific indicated that the Pacific contains different cyanobacterial populations that respond accordingly to the differing levels of light, nutrients, and other physical conditions.

In addition, the PEG concentration method is less labor intensive and does not require access to the expensive centrifuge facilities (Colombet *et al*., 2006). Using PEG concentration and phenol-chloroform extraction showed that this method could successfully extract virioplankton genomic DNA (about 35 ng/μl) from seawater. This DNA was of high quality and suitable enough for the amplification of target fragments and for analysis of the cyanophage community composition.

Primers targeting the viral capsid assembly protein gene were successfully used in this study to investigate the genetic diversity and phylogenetic affiliation among natural cyanophage assemblages in the Yellow Sea coastal area around Qingdao. Phylogenetic analysis revealed that natural cyanophage populations are relatively diverse and a novel cyanophage cluster was discovered in this area. Further investigation of the cyanophage communities from distinct coastal waters will allow the exploration of the new virus-host systems.

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