

Full Length Research Paper

# Diversity of *nifH* gene sequences in the sediments of South China Sea

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In order to contribute to knowledge about structure of marine diazotrophic communities in the sediments of South China Sea, the molecular diversity of the *nifH* gene, which encodes the Fe protein of the nitrogenase complex, was assessed by polymerase chain reaction (PCR) amplification using PolF/R primers, followed by cloning and sequencing. Sequences of *nifH* genes were amplified from environmental deoxyribonucleic acid (DNA) samples collected during three stations including shallow sea (75 m, station L10), shelf (450 m, station L2) and deep sea (1000 m, station L21), and covering an area between 17 to 19°N and 111 to 119°E. Samples from shallow sea contained  $\beta$ -, and  $\delta$ -proteobacteria; the shelf contained  $\alpha$ -,  $\beta$ -,  $\delta$ -proteobacteria; the deep sea contained  $\alpha$ -,  $\delta$ -,  $\gamma$ -proteobacteria, firmicutes, and green nonsulfur (GNS) bacterium. These results suggested that diazotroph was significant component potentially contributing to nitrogen fixation in South China Sea.

**Key words:** *nifH*, diversity, sediment, the South China Sea.

## INTRODUCTION

The diazotroph, which is a fundamental component of ecosystems, catalyses the reduction of atmospheric N<sub>2</sub> gas to biologically available ammonium, providing an important source of fixed nitrogen for the biosphere (Moir, 2011). Most microorganisms that perform biological N<sub>2</sub> fixation with the nitrogen fixation (*nif*) gene cluster (Rees et al., 2005). The *nifH* gene, which encodes the iron protein of nitrogenase, is a highly conserved functional gene useful in phylogenetic studies (Zehr et al., 2003). Culture-independent and molecular methods were developed and applied in assessment of diazotroph diversity by amplifying, cloning and sequencing of the *nifH* gene from environmental DNA samples (Falcon et

al., 2002; Jenkins et al., 2004; Moisander et al., 2007, 2008; Langlois et al., 2008). The diversity of diazotrophs was accessed in many different habitats by this approach, including soils, freshwater and saltwater lakes, salt marshes, deep-sea vents and so on (Falcon et al., 2002; Jenkins et al., 2004; Moisander et al., 2007, 2008; Langlois et al., 2008).

The South China Sea (SCS) is one of the largest marginal seas in the tropical Pacific that potentially shares microbial community components from coastal and open ocean ecosystems (Moisander et al., 2008). The SCS has a deep basin with a maximum depth of 5000 m and a shelf less than 100 m deep, the conditions such as warm, permanently stratified, oligotrophic, and dust rich. The environment is favorable for nitrogen fixation (Karl et al., 2002; Moisander et al., 2008; Zhang et al., 2011).

In the present study, diazotrophic bacteria associated with sediments were investigated by the diversity analysis of sequences amplified by polymerase chain reaction (PCR) from deoxyribonucleic acid (DNA) extracted from

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different depths of the SCS. The amplified *nifH* products were characterized by DNA sequencing and were compared with the sequences of nitrogenase genes available in database from different environment. Investigations of *nifH* diversity and phylogenetic analysis in the SCS sediments may help to understand the distribution of diazotrophic bacteria.

## MATERIALS AND METHODS

### Sampling and DNA extraction

Samples were collected from the subsurface sediment of the SCS of different depths of water column in the range of 70-1,000 m. Samples from shallow sea (75 m, station L10), shelf (450 m, station L2) and deep sea (1000 m, station L21). Three sediment samples were collected during the month of July 2007; collection was three times in one sample. Undisturbed surface sediments down to 1-5 m depth were sampled using sterile techniques and stored in liquid nitrogen during the cruise and at -80°C after returning to the laboratory. Sediment DNA was extracted by a previously established procedure (Zhou et al., 1997).

### Polymerase chain reaction (PCR), cloning and restriction fragment length polymorphism (RFLP) analysis

Bacterial nitrogenase reductase genes were amplified with primers PoIF (5'-TGCGAYCCSAARGCBGAC TC-3') and PoIR (5'-ATS GCCATCATYTCRCCGGA-3') (Poly et al., 2001). Thermal cycling conditions were 95°C for 5 min, followed by 30 cycles of 94°C for 30 s, 53°C for 1 min, 72°C for 40 s, and a final extension step of 72°C for 5 min. The PCR products were then cloned using a TAKARA TA cloning kit (TOYOBO Shanghai, Shanghai, China). PCR amplification products containing the right-size (402 bp) insert were digested with 1 U of restriction enzymes *Mbo* I, *Rsa* I, *Msp* I (MBI) for 4 to 8 h at 37°C. The restriction profiles were evaluated by electrophoresis in 3% agarose gel. Clones that produced the same RFLP pattern were grouped together and considered representatives of the same operational taxonomic unit (OTU). The PCR products showing different RFLP patterns were randomly selected for sequencing. Plasmid DNA was prepared and sequenced, at least twice in both directions, by using an ABI PRISM 377 DNA sequencer (Perkin-Elmer Cetus Instruments, Norwalk, CT).

### Sequence alignment and phylogenetic analysis

The product of about 402 bp fragment was obtained by PCR reaction. The sequences of *nifH* gene from the NCBI GenBank database were selected on the basis of sequence similarity to one or more of the marine sequences. These diazotrophs sequences were utilized for phylogenetic reconstructions. Protein sequences for each major sequence cluster were aligned in Clustal W (Thompson et al., 1994). Maximum likelihood phylogenies were constructed in MEGA (ver. 5.0) by the Bootstrap method using pair wise deletion of gaps and missing data with 1000 bootstrap resamplings (Tamura et al., 2011).

### Nucleotide sequence accession numbers

The *nifH* sequences determined in this study are available in the GenBank database, accession numbers HM063747-HM063831.

## RESULTS AND DISCUSSION

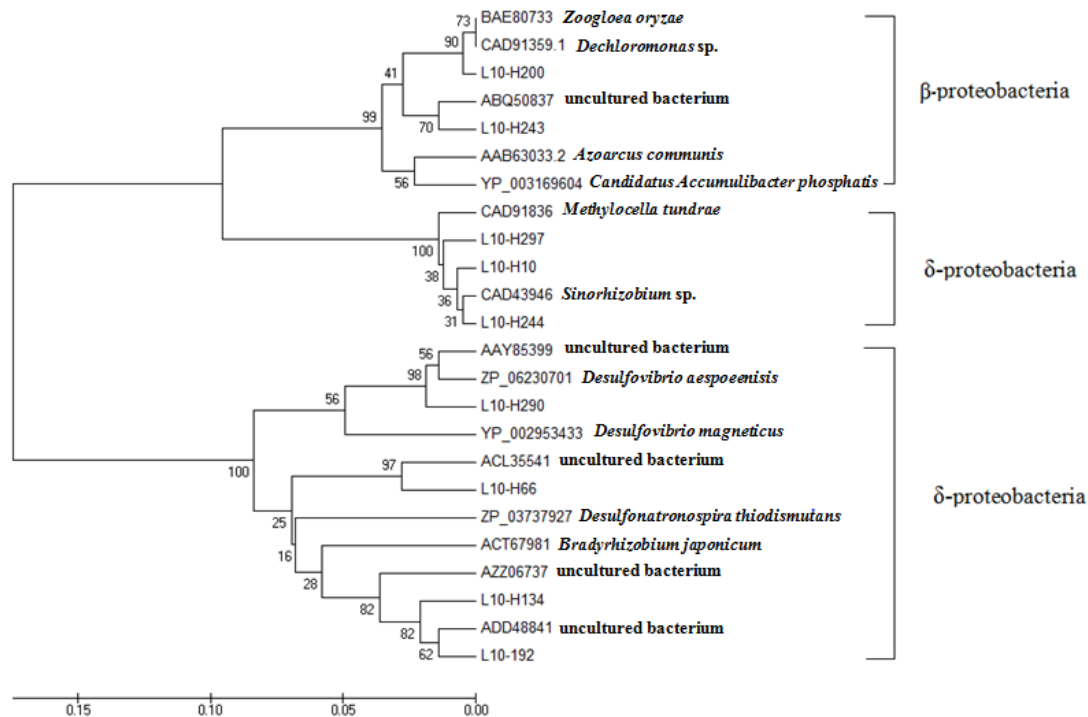
### Analysis of all *nifH* clones in three stations

The *nifH* gene has been one of the most important functional genes used when studying diversity in numerous habitats in last few years. In this study, the *nifH* gene was used as a molecular marker for studying the diazotrophic diversity and abundance in the SCS sediments in three stations. A total of 203 *nifH* clones from three sediment samples were obtained (station L10, 43 clones; station L2, 91 clones; station L21, 69 clones). The diversity of *nifH* sequences was analyzed by restriction fragment length polymorphism (RFLP), and 48 restriction profiles were obtained in this study. The deduced amino acid sequences shared 39% to 99% similarity to the closest match GenBank *nifH* and *nifH*-like sequences. Phylogenetic analysis indicated that most *nifH* protein sequences might be obtained from currently uncultured or uncharacterized bacteria, and covered diverse environments. The similarity of sequenced *nifH* genes ranged from 73 to 100% between each other.

### *nifH* diversity in station L10

In shallow sea (75 m, station L10), 9 protein sequences (43 clones) clustered into three major groups, including  $\beta$ - $\delta$ - subdivisions of proteobacteria (Figure 1). The most sequences were belonging to  $\delta$ -proteobacteria, including L10-H10, L10-H66, L10-H134, L10-H192, L10-H244, L10-H290 and L10-H297. These sequences were very similar to sequences previously recovered from various environments, including rhizosphere of plant, soil, corals, oligotrophic tropical sea grass bed communities, salt marsh, beach, marine sediment, and bay (Zhang et al., 2006; Musat et al., 2006; Coelho et al., 2008; Lovell et al., 2008; Teng et al., 2009). These sequences showed high homology with *nifH* genes of the *Desulfovibrio* sp. (*Desulfovibrio magneticus*, *Desulfovibrio aespoenensis*), *Desulfonatronospira thiodism*, *Sinorhizobium* sp., and *Bradyrhizobium japonicum*. The sequences L10-H134 and L10-H290 had high identity with sequences previously recovered from northern South China Sea (ADT90055.1), Chesapeake Bay (AAZ06761.1), Jiaozhou Bay (ACN77086.1), eastern Mediterranean Sea (ABQ50774.1, Man-Aharonovich et al., 2007). The L10-H10, L10-H244, and L10-H297 showed a high homology with the *nifH* gene of *Sinorhizobium* sp. TJ170 and *Methylococcus capsulatus* str.

The  $\beta$ -proteobacteria cluster contains L10-H200 and L10-H243. The protein sequence L10-H200 had a high identity with the *nifH* genes of *Dechloromonas* sp. SIUL and *Zoogloea oryzae*. The latter bacterium was isolated from rice paddy soil (Xie and Yokota, 2006). The protein sequence L10-H243 showed a high homology with the *nifH* gene of uncultured bacterium which was



**Figure 1.** Phylogenetic trees for *nifH* of the station L10 constructed in MEGA (ver. 5.0) based on amino acid sequences using the Bootstrap method. Phylogenetic relationships were bootstrapped 1000 times, and bootstrap values are shown.

found in the eastern Mediterranean Sea (Man-Aharonovich et al., 2007).

### *nifH* diversity in station L2

In shelf site (450 m, station L2), 20 protein sequences (91 clones) clustered into four major groups, including  $\alpha$ -,  $\beta$ -,  $\delta$ - subdivisions of proteobacteria (Figure 2). The  $\alpha$ -proteobacteria cluster contained a distinct sequence L2-H81. It had a 98% protein sequence similarity with the *nifH* sequences affiliated with *Azospirillum brasilense*, an aerobic, plant growth-promoting rhizobacteria (PGPR) isolated from cereal root (Umali-Garcia et al., 1980; Steenhoudt and Vanderleyden, 2000; Bashan et al., 2004; Cui et al., 2006).

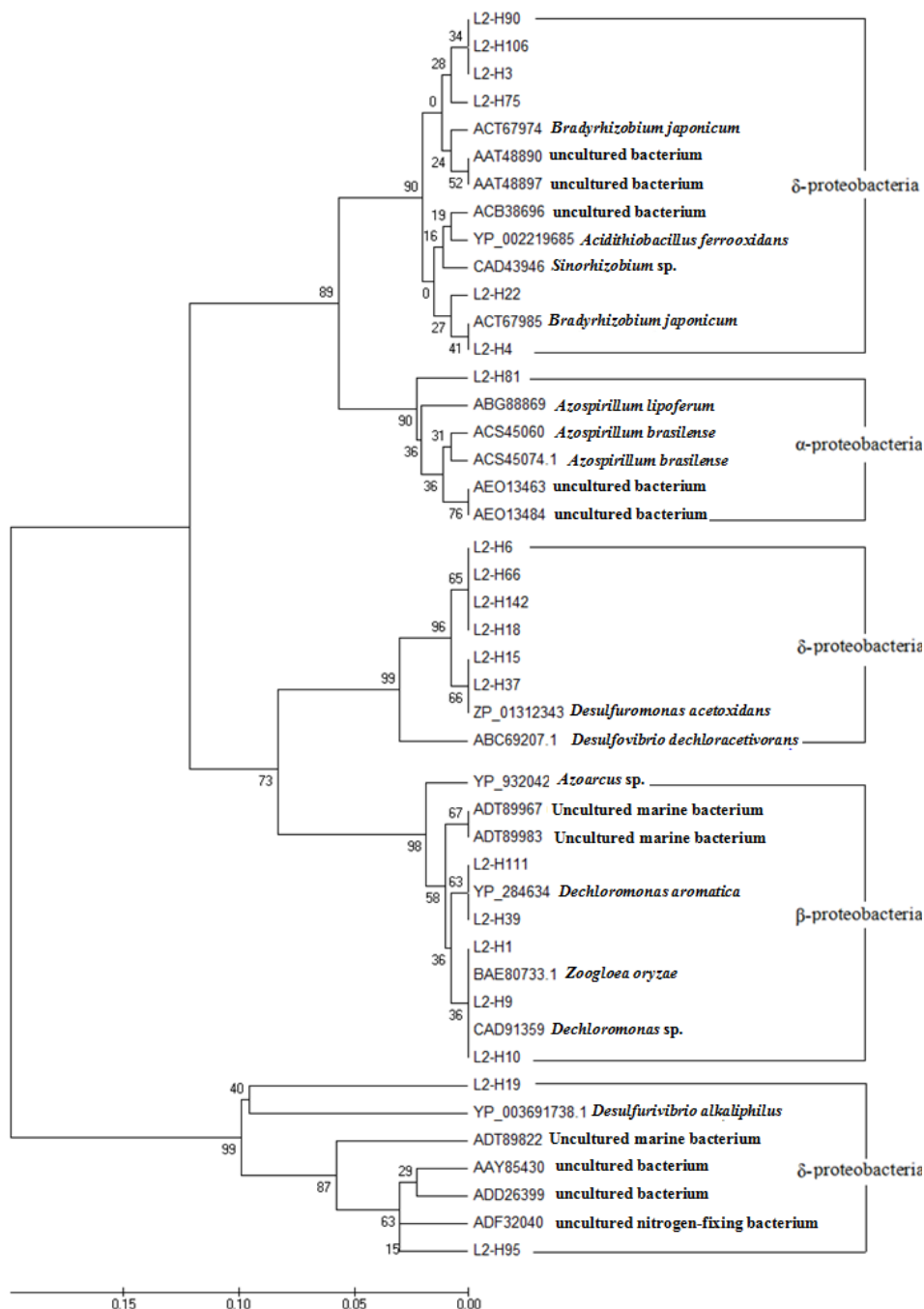
Among the  $\beta$ -proteobacteria, *Dechloromonas* sp. SIUL was the dominant diazotroph covering 50.5% (46/91) of the clone library. These protein sequences showed high similarity to sequences previously obtained from various environments, including rhizospheres, soil, corals, coast, salt marsh, eastern Mediterranean Sea, and wastewater (Man-Aharonovich et al., 2007; Bowers et al., 2008). The protein sequences L2-H1, L2-H9 and L2-H10 showed high identity with *Dechloromonas* sp. SIUL. The protein sequences L2-H39 and L2-H111 had 92.9% similarity with uncultured marine bacteria (ADT89967 and ADT89983) which were found in the northern SCS (Kong et al., 2011).

The most sequences belonging to  $\delta$ -proteobacteria, including L2-H3, L2-H4, L2-H6, L2-H15, L2-H18, L2-H19, L2-H22, L2-H37, L2-H66, L2-H75, L2-H90, L2-H95, L2-H106 and L2-H142. The protein sequences of L2-H3, L2-H75, L2-H90 and L2-H106 showed 89%-91% similarity with *nifH* sequences of uncultured bacterium (AAT48890, AAT48897) which were found in the Tibetan plateau (Zhang et al., 2006). L2-H4 and L2-H22 had high protein sequence identity with the *nifH* sequences of *B. japonicum* which is a species of legume-root nodulating, microsymbiotic nitrogen-fixing bacterium species (Dashti et al., 1997). L2-H6, L2-H15, L2-H18, L2-H37, L2-H66 and L2-H142 had high identity of protein sequences with *nifH* sequences of *Desulfuromonas acetoxidans* DSM 684 and *Desulfovibrio dechloracetivorans* (Ju et al., 2007).

### *nifH* diversity in station L21

In deep sea (1000 m, station L21), 19 protein sequences (69 clones) clustered into seven major groups, including  $\alpha$ -,  $\delta$ -,  $\gamma$ -proteobacteria, firmicutes, and green nonsulfur (GNS) bacterium (Figure 3).

The  $\alpha$ -proteobacteria cluster contained a single protein sequence L21-H146. It had a 91% protein sequence similarity with the *nifH* sequences of *A. brasilense* which is an important diazotroph isolated from cereal root (Umali-Garcia et al., 1980; Steenhoudt and

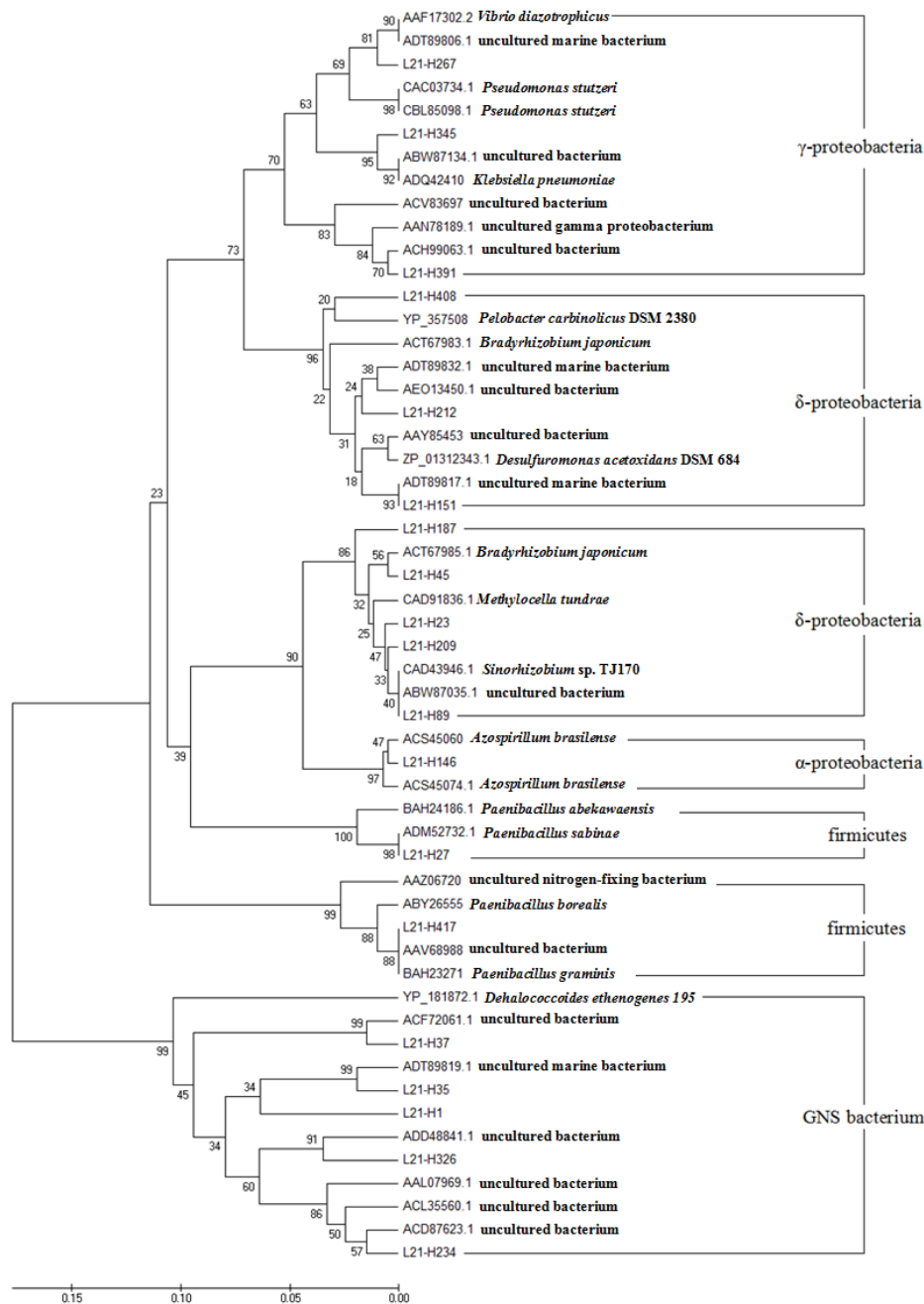


**Figure 2.** Phylogenetic trees for *nifH* of the station L2 constructed in MEGA (ver. 5.0) based on amino acid sequences using the Bootstrap method. Phylogenetic relationships were bootstrapped 1000 times, and bootstrap values are shown.

Vanderleyden, 2000; Bashan et al., 2004; Cui et al., 2006).

The most sequences belonging to  $\delta$ -proteobacteria, formed the dominant diazotrophic group, including L21-H23, L21-H45, L21-H89, L21-H151, L21-H187, L21-H209, L21-H212 and L21-H408. The L21-H212 had 90% similarity with *nifH* sequence of uncultured bacterium

(ADT89832.1) which was isolated from the northern SCS (Kong et al., 2011). The L21-H151 had high identity with *nifH* sequence of uncultured bacterium (ADT89817.1) which was also isolated from the northern SCS (Kong et al., 2011). The sequence L21-H408 showed 90% similarity with *nifH* sequence of *Pelobacter carbinolicus* DSM 2380. The sequences L21-23, L21-H45, L21-89,



**Figure 3.** Phylogenetic trees for *nifH* of the station L21 constructed in MEGA (ver. 5.0) based on amino acid sequences using the Bootstrap method. Phylogenetic relationships were bootstrapped 1000 times, and bootstrap values are shown.

L21-H187, and L21-H209 showed high identity with *nifH* sequence of *B. japonicum*, *Methylocella tundra* and *Sinorhizobium* sp. TJ170.

The protein sequences L21-H267, L21-H345 and L21-H391 belong to the  $\gamma$ -proteobacteria cluster. L21-H267 had 90% similarity of uncultured marine bacterium (ADT89806) which was found in the northern SCS (Kong et al., 2011). L21-H345 showed 90% protein similarity

with *Klebsiella pneumoniae* which was isolated from the root surface of rice (Liu et al., 2011). The L21-H391 had a 90% protein sequence similarity with different environmental sequences, including African sweet potato (AAN78189.1; Reiter et al., 2003), Italian white truffle *Tuber magnatum* (Barbieri et al., 2010), rhizosphere of mangrove, coastal microbial mats.

The L21-H27 and L21-H417 belong to firmicutes

cluster, which had high identity with *nifH* sequences of firmicutes *Paenibacillus* sp. The GNS bacterium cluster contained L21-H1, L21-H35, L21-H37, H21-H234 and L21-H326. These protein sequences showed high similarity with *Dehalococcoides ethenogenes* 195 (Seshadri et al., 2005).

In this study, we examined the phylogenetic diversity and abundance of diazotrophs in the SCS by analysis of *nifH* gene from three different stations. The results indicated that deep sea sediment had higher diversity of diazotrophic bacteria than those of shelf site and shallow sea.

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