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# Bacterial community structure of mangrove sediments in relation to environmental variables accessed by 16S rRNA gene-denaturing gradient gel electrophoresis fingerprinting

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SUMMARY: Bacterial community structure and the relationship between environmental variables and microbial communities in the surface sediments of tropical mangrove ecosystems were investigated in Sanya, Hainan Island, China. Profiles of bacterial communities were generated using culture-independent PCR-denaturing gradient gel electrophoresis (DGGE), and the results were interpreted with multivariate statistical analysis. Findings suggested that microbial communities varied with sample collection sites and seasons. The samples collected from the same sample sites at the same time had more similar microbial communities except samples SH3 and AB5, which also had unique sediment quality. Canonical correspondence analysis (CCA) revealed that the organic carbon concentration of the sediments accounted for a significant amount of the variability in the bacterial community composition. Phylogenetic analysis was used to identify the major groups of the predominant bacterial phylotypes. 16S rRNA gene-V3 fragments from 17 individual DGGE bands were sequenced and the corresponding bacteria were found in mangrove sediments for the first time based on BLAST results. Predominant bacterial phylotypes clustered with various taxonomic groups, including *Proteobacteria*, *Bacteroidetes*, *Gemmatimonadetes*, *Actinobacteria* and *Firmicutes*.

*Keywords*: bacterial community, mangrove sediments, denaturing gradient gel electrophoresis, multivariate statistics.

RESUMEN: Estructura de la comunidad bacteriana en sedimentos de manglares en relación a variables medioambientales mediante análisis de fragmentos del gen 16S rRNA y electroforesis en geles desnaturalizantes. – La estructura de la comunidad bacteriana y la relación entre variables ambientales y las comunidades microbianas de la superficie de sedimentos de los ecosistemas tropicales de manglares fueron investigados en Sanya, Hainan Island, China. Los perfiles de las comunidades bacterianas fueron obtenidos utilizando electroforesis de gradiente de gel desnaturalizante (DGGE), cultivo-independiente, y los resultados fueron interpretados con un análisis estadístico multivariante. Los resultados sugerían que las comunidades microbianas variaban con los lugares de muestreo y las estaciones. Las muestras recogidas en el mismo lugar de muestreo y al mismo tiempo tenían comunidades microbianas similares, excepto las muestras SH3 y AB5, que tenían diferente calidad de sedimentos. El análisis de correspondencia (CCA) mostró que la concentración de carbono orgánico en sedimentos explicaba una parte importante de la variabilidad en la composición de la comunidad bacteriana. Los análisis filogenéticos fueron usados para identificar los grupos más importantes de filotipos predominantes de bacterias. Se secuenciaron fragmentos 16 rRNA gene-V3 de 17 bandas individuales de DGGE y las bacterias correspondientes fueron encontradas por primera vez en sedimentos de manglares en base a resultados de BLAST. Los filotipos de bacterias predominantes se agrupaban con varios grupos taxonómicos que incluyen *Proteobacteria, Bacteroidetes, Gemmatimonadetes, Actinobacteria* y *Firmicutes*.

*Palabras clave*: comunidad bacteriana, sedimentos de manglares, electroforesis de gradiente de gel desnaturalizante, analisis estadistico multivariante.

## INTRODUCTION

Mangrove ecosystems are dominant ecosystems along tropical coastlines. They have important relationships with the regulation and optimisation of tropical marine environments. They are thought to be very important as primary producers of organic matter, providing the base for a large and complex food web. Although mangrove ecosystems are rich in organic matter, in general they are nutrient-deficient, especially with regard to nitrogen and phosphorus (Sengupta and Chaudhuri, 1991; Holguin *et al.,* 1992; Alongi *et al.,* 1993; Vazquez *et al.,* 2000). Microbial activity is responsible for major nutrient transformations within a mangrove ecosystem (Alongi *et al.,* 1993; Holguin *et al.,* 2001). It has been documented that microorganisms play an important role in the productivity, conservation and rehabilitation of mangrove ecosystems (Holguin *et al.,* 2001). Knowledge of microbial community diversity and the relationship between environmental factors and members of bacterial communities in mangrove sediments is important for understanding how the mangrove ecosystems function; this is necessary to formulate effective management and conservation strategies. To understand the community and diversity of bacteria in mangrove ecosystems, isolation and cultivation methods are often used. However, the culturable bacteria are not representative of total community. The major problem of cultivation-based analysis is that only a small part of the bacterial populations can be recovered from the sediments by traditional cultivation techniques (Staley and Konopka, 1985; Amann *et al.,* 1995; Bürgmann *et al.,* 2004). Most environmental bacteria cannot be cultured using current culture-based and traditional methods (Li *et al.,* 2006). Molecular techniques based on culture-independent techniques such as denaturing gradient gel electrophoresis (DGGE), automated ribosomal intergenic spacer analysis (ARISA) and terminal restriction fragment length polymorphism (TRFLP) have provided new tools for genetically identifying bacteria in all kinds of environmental systems, which has enhanced the knowledge of the in situ population structure of bacteria communities (Li *et al.,* 2006; Liang *et al.,* 2007; Muckian *et al.,* 2007). As DGGE can separate sequences of the same length with only a base difference, 16S rRNA gene-DGGE fingerprinting is particularly useful as an initial investigation to compare communities and identify the qualitative or most relatively abundant community members (Muyzer *et al.,* 1993).

The objectives of this study were (1) to determine the variability in microbial communities and environmental parameters from different tropical mangrove samples of Sanya Mangrove Nature Reserve, (2) to determine whether environmental variables are related to microbial community variability and (3) to identify the major bacterial phylogenetic groups present in the different samples investigated.

## MATERIAL AND METHODS

Sanya Mangrove Nature Reserve is a typical tropical mangrove ecosystem located at the southernmost part of Hainan Island in China, covering an area of about 250 $hm^2$, including Yalong Bay Qingmei, Yulin Bay Hongsha, Sanya River and Linwang Tielu Nature Reserve. The dominating tree species include *Rhizophora apiculata*, *Avicennia marina*, *Xylocarpus granatum*, *Rhizophora stylosa*, *Bruguiera gymnorhiza* and *Ceriops tagal*. The mangrove environment and the mangrove vegetation of Sanya Mangrove Nature Reserve have previously been described by Fu and Li (1999), Mo *et al.,* (1999) and Huang *et al.,* (2007). In this study the samples were collected from the swamp of Sanya Mangrove Nature Reserve. The swamp is inundated by tides twice a day. The mean relative humidity is about 80%, and the annual average air temperature and rainfall are 25.4°C and 1279.5 mm, respectively. The two sample collection sites were situated in the Sanya River (site B: 18°15′08.6″N; 109°30′51.1″E) and Yulin Bay Hongsha (site H: 18°15′52″N; 109°34′22″E) nature reserves, respectively.

Three kinds of soil samples were collected: bulk soil, roots with aggregates and decomposing leaves. Soil near the plant, but not in direct contact with any root, was designated as bulk soil (BS). Soil adhered loosely to roots and obtained by hand shaking for 1 min was designated as roots with aggregates (RA). Soil near the plant and covered by decomposing leaves and litter, but not in direct contact with any root was designated as decomposing leaves (DL). In summer, the three kinds of samples were collected from site H; in autumn, they were collected from both sites. For all samples, the top 10 cm of sediments with quintuplicate samples (over 1 $m^2$ area) was collected and mixed thoroughly. All the roots, leaves and litter in the samples were discarded, and then the soil samples were placed in sterile collection bags. The different samples at the same site

were collected at a distance from more than 10 m from each other. There were two duplicates: one for analysis of environmental parameters and one for DNA extraction. The samples for environmental parameter analysis were stored at 4°C prior to analysis, and the environmental variables including total organic carbon (OC), total nitrogen (TN), available soil phosphorus (AP), available soil potassium (AK) and pH were measured according to Bao (1999). The samples for DNA extraction were stored at -20 ºC prior to analysis.

Total community DNA was extracted from 1.0 g of wet samples using the E.Z.N.A.Soil DNA Kit (Omega Bio-tek) with some modification. To remove the inhibitors such as humic acids, a pre-extraction removal strategy was carried out according to Xi *et al.,* (2006). The inhibitor removal buffer (pH 8.0) contained (100 mM) Tris-HCl, (100 mM) EDTA, (100 mM) $Na_4P_2O_7$, (100 mM) NaCl, (1.0%) PVP, (0.05%) Triton X-100 and (4.0%) skim milk. The humic acids were washed out from the mangrove sediment samples successfully. Total DNA extractions were performed as recommended by the manufacturer (Omega Bio-tek). The precipitated DNA was resuspended in 20 μl of TE buffer (10 mM Tris-HCl, 1 mM $Na_2EDTA$, pH 8.0). For PCR purposes, the DNA concentration was measured spectrophotometrically (HITACHI) and adjusted to a concentration of 100 ng $\mu l^{-1}$.

Hot start PCR was used for 16S rRNA gene amplification using a PTC-200 thermal cycler (MJ Research). PCR was performed using primers 338F (5'-CCTACGGGAGGCAGCAG-3') and 518R (5'-ATTACCGCGGCTGCTGG-3') to amplify bacterial 16S rRNA gene-V3 segments. A 40 bp GC-clamp (cgc ccg ccg cgc gcg gcg ggc ggg gcg ggg gca cgg ggg g) was added to primer 338F to increase the separation of DNA bands in DGGE gel (Muyzer *et al.,* 1993). The PCR was carried out in 50 μl volumes, containing 1 μl DNA template, 1×Ex TaqTM Buffer, 4.95 mg $ml^{-1}$ BSA, 100 μM dNTP, 0.2 μM of each primer and 2 U Ex TaqTM. The Taq polymerase, dNTPs and PCR buffer were purchased from TaKaRa (TaKaRa Schuzo Co., Ltd., Biomedical Group, Japan). The thermal PCR profile was as follows: initial denaturation at 95°C for 5 min followed by 30 cycles of primer annealing at 55°C for 30 s, chain extension for 30 s at 72°C, denaturation for 30 s at 94°C and a final extension at 72°C for 10 min. To reduce possible inter-sample PCR variation, all PCR reactions were run in triplicate and pooled together before loading on DGGE gel. PCR products were analysed by electrophoresis in 2% agarose gels and ethidium bromide staining.

DGGE was performed using the Dcode universal mutation detection system as described in the manufacturer's manual (Bio-Rad, Hercules, CA, USA). Approximately 300 ng PCR products were loaded onto 8% (wt/vol) polyacrylamide gels in 1×TAE (40 mM Tris, 20 mM acetate, 1.0 mM $Na_2$-EDTA) with denaturant-gradient of 45-70% (100% denaturant was 7 M urea and 40% (wt/vol) deionised formamide). The gels were electrophoresed at a constant voltage of 100 V and 60 °C for 10 h. After electrophoresis, the DGGE gels were stained with ethidium bromide and visualised under UV light in an AlphaImager imaging system (Alpha innotech).

The major dense DGGE bands were excised from the gel, resuspended in 20 μL of TE buffer (10 mM Tris and 1 mM EDTA, pH 8.0) and left at 4°C overnight. The supernatant after centrifugation (12000 rpm, 5 min, 4 °C) was used as a template. Subsequently, 16S rRNA gene-V3 segments were amplified using the 338F and 518R primer pairs with GC-clamp, and was loaded again in a DGGE gel to confirm the position of the bands. Then 16S rRNA gene-V3 segments were amplified using the 338F and 518R primer pairs without GC-clamp, and the PCR product was purified with a PCR purification kit (Tiangen) and subsequently cloned into PMD18-T plasmid vector and *Escherichia coli* DHα cells using the PMD18-T cloning vector kit according to the manufacturer's instructions (Takara). Positive recombinants were then submitted for sequencing using an ABI3730 DNA Sequencer (USA) with M13 primer at the Shanghai Invitrogen Biotech Co. Ltd. Nucleotide sequences were compared with those in the GenBank database by BLAST algorithm for tentative identifications. Phylogenetic trees of 16S rRNA gene-V3 partial sequences were generated using the neighbor-joining algorithms in Mega II software. The level of support for the phylogenies derived from neighbor-joining analysis was gauged by 1000 bootstrap replicates.

DGGE digital images were analysed by the Gel Doc 2000 Quantity-One 4.5.2 gel documentation system (Bio-Rad, USA) to generate a densitometric profile. The peak areas of the fingerprint patterns were used to indicate the intensities. The bands were detected when the relative peak area to total peak area exceeded 1%. The Shannon index of bacterial diversity (Shannon and Weaver, 1963) and dissimi-

larity indices (Mouser *et al.,* 2005) were analysed according to the DGGE band profiles.

To assess changes in the genetic diversity of bacterial communities in different mangrove samples, DGGE banding patterns were analysed by multidimensional scaling (MDS) analysis (Muckian *et al.,* 2007). For this purpose, the dissimilarity indices were recorded in a binary matrix, which was then analysed with the program SPSS 15.0 for Windows. The resulting graphical representation, MDS map, showed every band pattern as one plot, and relative changes in community structure were visualised and interpreted as the distance among the plots. The closer the plots were to each other, the more similar were the DGGE banding patterns. Canonical correspondence analysis (CCA) (CANOCO 4.5; Biometris, Wageningen, the Netherlands) was performed on a statistical analysis of the DGGE profiles versus the environmental variables. The resulting ordination biplot approximated the weighted average of each species (in this case, band intensities) with respect to environmental variables, which were represented as arrows. The length of these arrows indicated the relative importance of that environmental factor in explaining variation in bacterial profiles, while the angle between the arrows indicated the degree to which they were correlated. A Monte-Carlo permutation test based on 199 random permutations was used to test the null hypothesis that bacterial profiles were unrelated to environmental variables.

## RESULTS

### Environmental parameters and DGGE patterns of different samples

The basic properties of the soil samples are shown in Table 1. The variabilities of these parameters in the different samples were obvious. For example, the OC concentration of samples SH3 (SH=Summer site H) and AB5 (AB=Autumn site B) was higher than that of the others, whereas the AK of sample SH3 was lower than that of the others. Figure 1 shows the bacterial community structure of different sediment samples. The banding patterns among all the samples were distinct. Analysis of DGGE gel (Fig 1.) resulted in a total of 338 detectable bands in 103 different positions (Table 2). The number of bands per sample varied between 24 and 29 (mean 26), indicating a diverse bacterial assemblage in mangrove sediments. A total of 17 bands was excised and successfully sequenced (see number and position in Fig 1.). The indices of *H*, reflecting the structural diversity of the bacterial community, were calculated on the basis of the number and relative intensities of bands on the gel track (Table 3). Based on Table 3, the Shannon index of samples ranked AH>SH>AB (AH=Autumn site H) among collected site and seasons, whereas the species abundance ranked SH>AH>AB. The results suggested that site H had a higher Shannon index and more microbial species than site B during our investigation. At site H, there was a higher Shannon index in autumn and more microbial species in summer. When we compared the difference among the sample types, the Shannon index of samples ranked RA>DL>BS, but the species abundance ranked DL>RA>BS. The results indicated that the samples collected from roots with aggregate had the highest Shannon index, and the samples collected from the soil near the plant and covered by decomposing leaves and litter had the most microbial species. The samples collected from soil near the plant, but not in direct contact with any root, had the lowest Shannon index and the fewest microbial species.

TABLE 1. – Main characteristics of the soil samples (value are given as mean, n=3).

| Samples | OC ($g\ kg^{-1}$) | TN ($g\ kg^{-1}$) | AP ($mg\ kg^{-1}$) | AK ($mg\ kg^{-1}$) | pH | Sample kind |
|---|---|---|---|---|---|---|
| SH1 | 28.9 | 0.90 | 26.7 | 284.3 | 6.50 | RA |
| SH2 | 55.8 | 1.25 | 18.5 | 304.4 | 6.85 | DL |
| SH3 | 102.8 | 0.87 | 22.5 | 225.6 | 5.02 | BS |
| SH4 | 51.2 | 1.26 | 22.49 | 479.6 | 2.68 | DL |
| SH5 | 23.8 | 0.95 | 10.27 | 288.6 | 4.73 | BS |
| AH1 | 35.0 | 0.78 | 28.9 | 258.6 | 4.65 | DL |
| AH2 | 37.5 | 0.86 | 27.4 | 276.3 | 4.40 | BS |
| AH3 | 43.2 | 1.33 | 39.8 | 370.5 | 5.22 | RA |
| AB1 | 36.2 | 1.60 | 4.89 | 436.5 | 3.65 | RA |
| AB2 | 49.7 | 1.14 | 11.99 | 259.7 | 3.8 | BS |
| AB3 | 56.9 | 1.29 | 27.8 | 432 | 4.39 | BS |
| AB4 | 26.3 | 1.84 | 12.63 | 295.6 | 2.96 | RA |
| AB5 | 122.3 | 1.02 | 10.98 | 268 | 3.86 | DL |

### MDS analysis of DGGE banding pattern

To compare broad-scale differences between bacterial community profiles, a multidimensional scaling (MDS) approach was employed to analyse DGGE banding patterns. Two-dimensional plots of MDS scores for mangrove samples are depicted in Figure 2. The results revealed that profiles from site B in autumn were differentiated from those from site H in autumn, with an MDS stress value

TABLE 2. – All the bands recovered on the DGGE gel of the samples collected from Site H in Summer (SH 1-5) and in Autumn (AH 1-3), and from Site B in Autumn (AB 1-5). The bands that were sequenced were in bold type. Black (+): presence, white (-): absence.

| | SH1 | SH2 | SH3 | SH4 | SH5 | AH1 | AH2 | AH3 | AB1 | AB2 | AB3 | AB4 | AB5 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| r1 | - | - | + | + | + | - | + | - | + | + | - | - | + |
| **S12** | + | + | - | + | - | - | - | - | + | - | + | + | - |
| r3 | - | - | - | - | - | + | - | - | - | - | - | - | - |
| r4 | - | - | - | - | - | - | - | + | - | - | - | - | - |
| r5 | + | + | - | + | - | + | + | - | + | + | - | - | - |
| r6 | - | - | - | - | - | - | - | - | - | - | - | - | + |
| r7 | - | - | + | + | + | - | - | - | - | - | - | - | - |
| r8 | - | + | - | - | - | - | - | - | - | - | - | + | + |
| r9 | + | - | - | - | - | + | + | + | - | - | + | - | - |
| r10 | - | - | - | - | - | - | - | - | + | - | - | - | - |
| r11 | - | - | + | - | - | + | - | + | - | - | - | - | - |
| r12 | + | + | - | + | - | - | + | - | + | - | - | + | + |
| r13 | - | - | - | - | + | - | - | - | - | - | - | - | - |
| **S14** | - | - | + | + | - | + | - | + | - | + | + | + | + |
| r15 | + | + | - | - | - | - | - | - | + | - | - | - | - |
| r16 | - | - | - | + | + | + | + | - | - | - | + | - | - |
| r17 | + | - | + | - | - | - | - | - | + | + | - | - | + |
| r18 | - | + | - | - | + | - | + | - | - | - | + | - | - |
| r19 | + | + | + | - | - | + | - | + | - | - | - | - | + |
| r20 | - | - | - | + | - | - | - | - | - | - | - | - | - |
| r21 | - | - | - | - | + | + | - | - | - | - | + | - | - |
| **S9** | + | + | + | + | - | - | + | - | + | + | - | - | - |
| r23 | + | - | - | - | + | + | - | - | - | - | + | + | + |
| r24 | - | - | - | + | - | - | - | - | - | - | - | - | - |
| r25 | - | + | + | - | - | - | - | + | - | - | - | + | - |
| r26 | + | - | - | - | + | - | + | - | + | + | + | - | - |
| r27 | - | - | - | - | - | + | - | - | - | - | - | - | + |
| r28 | - | - | - | - | - | - | - | + | - | - | - | - | - |
| r29 | - | - | + | + | - | - | - | - | + | - | - | + | - |
| **S1** | + | + | - | - | + | + | + | + | - | + | + | - | - |
| r31 | - | - | + | - | - | - | - | - | - | - | - | - | - |
| r32 | - | - | - | + | - | - | - | - | - | - | - | - | + |
| **S8** | - | + | + | - | - | - | - | - | - | - | - | + | - |
| **S11** | - | - | - | - | + | + | - | + | - | + | + | - | - |
| r35 | + | - | - | - | - | - | - | - | - | - | - | - | - |
| r36 | - | + | - | + | - | - | - | - | - | - | - | + | + |
| r37 | - | + | - | - | + | - | - | - | - | - | + | - | - |
| r38 | - | - | - | - | - | - | - | - | - | - | - | - | + |
| **S2** | + | + | + | + | - | - | + | - | - | - | + | + | - |
| **S19** | - | - | - | - | - | - | - | + | - | + | - | - | + |
| r41 | - | - | + | - | + | - | - | - | + | - | - | - | - |
| r42 | - | - | - | + | - | + | - | - | + | - | + | + | + |
| r43 | - | - | - | - | - | - | - | + | - | - | - | - | - |
| r44 | + | + | + | + | - | - | + | - | - | + | - | - | + |
| r45 | - | - | - | - | + | + | - | - | - | + | + | - | - |
| r46 | - | - | + | - | - | - | - | - | - | - | - | - | - |
| r47 | - | + | - | + | - | - | - | + | - | - | - | - | - |
| r48 | + | - | - | - | - | - | - | - | - | - | - | + | + |
| **S20** | - | - | - | - | + | + | + | + | - | + | + | - | + |
| r50 | + | + | + | + | - | - | - | + | + | - | - | - | - |
| r51 | - | - | - | - | - | - | - | - | - | + | - | + | - |
| r52 | - | - | - | - | + | + | + | + | + | - | + | - | - |
| **S3** | + | + | + | + | - | - | - | - | - | - | - | + | + |
| r54 | - | - | - | - | - | - | - | - | - | + | - | - | - |
| r55 | - | - | - | - | - | - | + | + | + | - | + | - | - |
| r56 | + | - | - | - | + | + | + | - | - | - | - | + | - |
| r57 | - | - | + | - | - | - | - | - | - | - | - | - | - |
| r58 | - | + | - | - | - | - | - | - | - | - | - | - | - |
| **S21** | - | - | - | - | + | - | - | + | + | - | - | - | + |
| r60 | + | - | - | - | - | + | - | - | - | - | - | - | - |
| **S15** | + | + | + | + | - | - | - | + | - | + | + | + | + |
| r62 | - | - | - | - | - | + | + | - | + | - | - | - | - |
| r63 | - | - | - | - | - | - | - | - | - | - | - | - | + |
| r64 | - | - | - | + | - | - | - | - | - | + | - | + | - |
| **S22** | - | - | - | - | + | - | - | - | + | - | + | - | - |
| **S18** | + | + | + | - | + | - | - | + | - | - | - | - | - |
| **S17** | - | - | - | + | - | + | + | + | - | - | + | + | - |
| r68 | - | - | - | - | - | - | - | - | - | - | - | - | + |
| r69 | - | - | - | + | - | - | - | - | - | + | - | - | - |
| **S4** | + | + | + | - | + | + | + | - | - | - | + | - | + |

TABLE 2 (cont.). – All the bands recovered on the DGGE gel of the samples collected from Site H in Summer (SH 1-5) and in Autumn (AH 1-3), and from Site B in Autumn (AB 1-5). The bands that were sequenced were in bold type. Black (+): presence, white (-): absence.

| | SH1 | SH2 | SH3 | SH4 | SH5 | AH1 | AH2 | AH3 | AB1 | AB2 | AB3 | AB4 | AB5 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| r71 | + | - | - | - | - | - | + | - | + | + | - | - | - |
| r72 | - | - | - | - | - | - | - | - | - | - | - | + | - |
| r73 | - | - | - | - | + | - | - | - | - | - | - | - | - |
| **S13** | - | - | + | + | - | - | - | + | + | + | - | - | - |
| r75 | + | - | + | - | - | + | - | + | - | - | - | - | - |
| r76 | - | - | - | - | - | - | - | - | - | - | - | + | - |
| r77 | - | - | - | - | + | - | + | - | + | - | - | - | - |
| r78 | - | - | + | + | - | + | - | + | - | - | + | - | + |
| r79 | - | + | - | - | - | - | - | - | - | - | - | - | - |
| r80 | + | - | - | - | - | - | - | - | - | - | - | - | - |
| r81 | - | - | - | - | - | + | - | - | - | - | + | + | + |
| r82 | - | - | - | - | - | - | - | - | - | + | - | - | - |
| r83 | - | - | - | - | + | - | - | - | - | - | - | - | - |
| r84 | - | - | - | - | - | + | - | - | - | - | - | + | - |
| r85 | + | + | + | - | - | - | + | - | - | + | + | - | - |
| r86 | - | - | - | + | - | + | - | - | - | - | - | - | - |
| r87 | - | - | - | - | - | + | - | - | - | - | - | - | - |
| r88 | - | - | - | - | - | - | + | - | + | + | - | - | - |
| r89 | - | - | - | - | - | - | - | - | - | - | + | - | - |
| r90 | - | - | + | + | - | - | - | + | - | - | - | - | + |
| r91 | + | + | - | - | - | - | - | - | - | - | - | + | - |
| r92 | - | - | - | - | - | - | - | - | + | + | - | - | + |
| r93 | - | - | - | - | - | - | + | - | - | - | - | - | - |
| r94 | - | - | - | - | - | - | - | + | - | - | - | - | - |
| r95 | - | - | + | - | - | - | - | - | - | - | - | + | - |
| r96 | - | + | - | - | - | + | - | - | - | - | - | - | + |
| r97 | - | - | - | - | + | - | - | - | - | - | - | - | - |
| r98 | - | - | - | - | - | - | - | - | + | - | - | - | - |
| r99 | + | - | - | - | - | - | + | - | - | - | - | - | - |
| r100 | - | - | + | - | - | - | - | - | - | - | - | - | - |
| r101 | - | + | - | + | - | - | - | - | - | - | - | - | - |
| r102 | + | - | - | - | - | - | - | - | - | - | - | - | - |
| r103 | - | - | - | - | - | - | - | - | - | + | - | - | - |

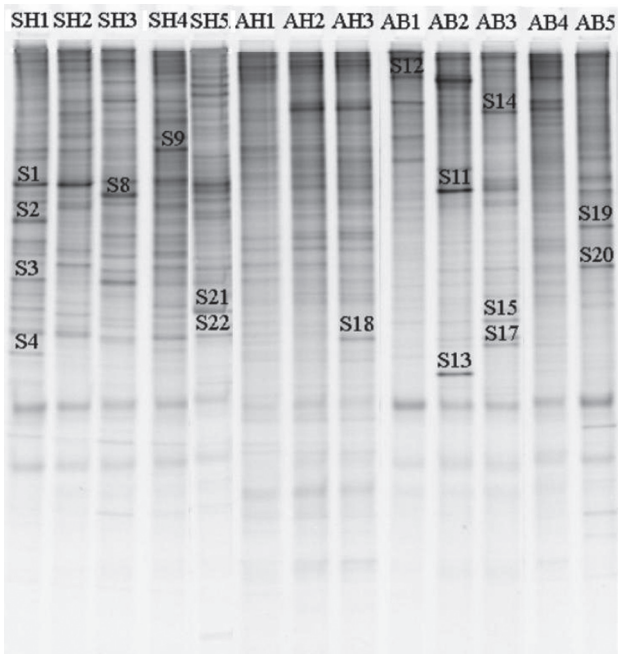


FIG. 1. – DGGE gels showing diversity of 16S rRNA gene fragments of the samples collected from Site H in summer (SH 1-5) and in autumn (AH 1-3), from Site B in autumn (AB 1-5). Lanes are labelled with sample numbers. Numbers on gels are the bands that were excised and sequenced and correspond to the list in Table 4.

TABLE 3. – Comparison of the microbial diversity of mangrove samples (the *H* was calculated on the basis of the number and relative intensities of bands on the gel track).

| samples | Shannon-weaver index (*H*) | DGGE bands number |
|---|---|---|
| SH1 | 1.293 | 29 |
| SH2 | 1.292 | 27 |
| SH3 | 1.078 | 28 |
| SH4 | 1.319 | 28 |
| SH5 | 1.251 | 24 |
| AH1 | 1.378 | 28 |
| AH2 | 1.148 | 24 |
| AH3 | 1.238 | 25 |
| AB1 | 1.229 | 24 |
| AB2 | 1.237 | 24 |
| AB3 | 1.304 | 25 |
| AB4 | 1.272 | 24 |
| AB5 | 1.039 | 28 |

of 0.17 (stress values of below 0.2 indicate that an MDS ordination plot is a good spatial representation of differences between data). The banding pattern of samples SH3 and AB5 were scattered from the other plots. Overall, MDS ordination plots indicated that the composition of bacterial communities varied with sampling sites and sampling time. Mangrove

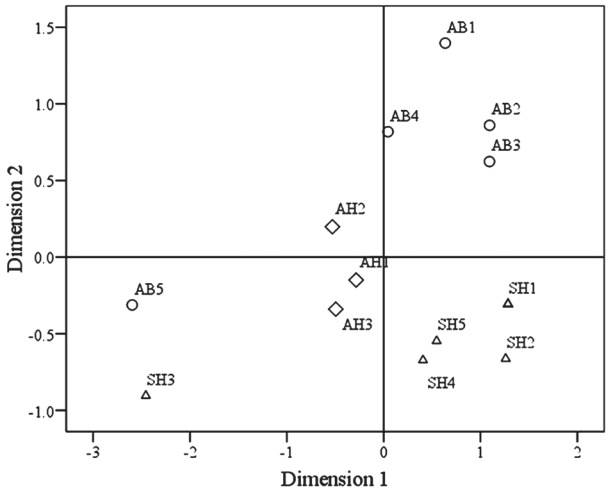


FIG. 2. – Two-dimensional plots of MDS analysis from DGGE patterns to compare broad-scale differences between bacterial community profiles of different mangrove samples (the MDS analysis was based on dissimilarity indices ($D$) and $D$ was given by:

$$D_{AB} = \sqrt{\sum_{i=1}^{P} (Ai - Bi)^2} \ ,$$

$A_i$: importance probability of each band on gel lane A. No band is treated as zero. $B_i$: importance probability of each band on gel lane B. No band is treated as zero. $P$: (total number of bands on lane A) + (total number of bands on lane B) – (total number of bands common to lane A and lane B).

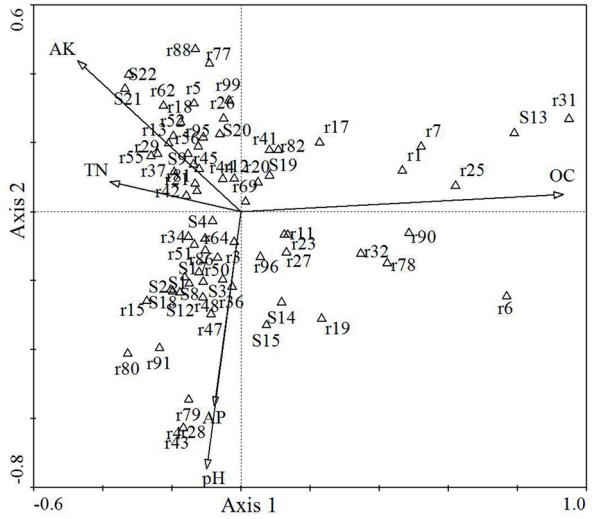


FIG. 3. – Canonical correspondence analysis (CCA) ordination diagram of DGGE data, with environmental factors as arrows (AK = available soil potassium, TN = total nitrogen, OC = total organic carbon content, AP = available soil phosphorus), and individual abundant bacterial phylotypes as triangles.

samples collected from the same site and in the same time had more similar microbial communities, except samples SH3 and AB5.

## Microbial community composition in relation to environmental variables

Multivariate statistical analysis was used to explore the relationships between environmental factors and the members of bacterial communities in more detail. Canonical correspondence analysis was carried out using abundant bacterial phylotypes (at least detected in three samples) within datasets together with environmental variables (Muckian *et al.,* 2007). Eigenvalues (indicating strength of the model) for the first two multivariate axes were 0.474 and 0.354. The sum of all canonical eigenvalues was 1.506. Axes 1 and 2 were found to explain 31.5% and 23.5% of the overall variance, respectively, indicating a strong gradient in the data set. Species environment correlations for both axes were more than 0.94, suggesting that bacterial phylotypes were strongly correlated with environmental factors. Monte-Carlo significance tests indicated that both axes explained a significant proportion of the variation in the data. The first axis was significantly correlated with OC ($P$<0.01), indicating that the first axis mainly showed the gradient of organic carbon concentration in the mangrove sediments. The second axis was correlated with pH and AP ($P$<0.05), which revealed the changes in bacterial community composition along the gradient of pH and available phosphate.

The CCA biplot is shown in Figure 3. Each environmental variable is represented by an arrow, which determines an axis. The projection of a taxon (indicated as open triangles) on this axis shows the level of the variable where the taxon is most abundant. More details about the interpretation of the biplot can be found in Ter Braak (1987). Based on Figure 3, it is clear that most bacterial phylotypes have a negative correlation with OC, which was distributed on the left side of the biplot, including the sequenced bacterial phylotypes S1, S2, S3, S4, S8, S9, S12, S17, S18, S22 and S21 (see number and position in Fig 1.). Bacterial phylotypes S14 and S15 were negatively correlated with AK. Bacterial phylotypes S19 and S20 showed a significant negative correlation with pH, AP. Furthermore, phylotypes r6, r78, r19, *et al.,* showed a significant negative correlation with AK and TN. Bacterial phylotypes r4, r28, r43 and r79 showed a significant correlation with AP and pH.

## Identification and phylogenetic analysis of the predominant bacterial phylotypes based on 16S rRNA gene sequences

Seventeen bands were excised from gel tracks, which were verified by DGGE three times to en-

TABLE 4. – Summary of the 16S rRNA gene sequences obtained from the respective bands in DGGE gel and the closest match to the National Centre for Biotechnology Information (NCBI) Nucleotide Sequence database (GenBank)

| Band | Accession no. | Database match | Origin | % Similarity |
|---|---|---|---|---|
| S1 | EF531307 | uncultured bacterium (AB099989) | inactive deep-sea hydrothermal vent chimneys | 98 |
| S2 | EF531308 | uncultured bacterium (EU499677) | freshwater sediment | 99 |
| S3 | EF531309 | *Lactococcus* sp. GA68 (EU260327) | tropical oligotrophic lakes | 99 |
| S4 | EF531310 | uncultured *Enhydrobacter* sp. (EU305591) | Sanwayao wastewater plant (WWTP) | 100 |
| S8 | EF531311 | uncultured *Sphingobacteria* bacterium (AY712439) | salt marsh | 97 |
| S9 | EF531312 | uncultured bacterium (AM234673) | environmental sample | 97 |
| S11 | EF531313 | uncultured bacterium (EU234087) | wastewater | 99 |
| S12 | EF531314 | uncultured deltaproteobacterium (DQ811831) | mangrove soil | 97 |
| S13 | EF531315 | uncultured bacterium (AY710524) | salt marsh | 94 |
| S14 | EF531316 | uncultured *Streptococcus* sp. (EU029521) | raw cow milk | 100 |
| S15 | EF531317 | uncultured bacterium (EF582536) | salt marsh sediment | 98 |
| S17 | EF531319 | uncultured bacterium (EU137496) | environmental samples | 98 |
| S18 | EF531320 | uncultured *Firmicutes* bacterium (EF462577) | high-temperature volcanic environment | 99 |
| S19 | EF531321 | Uncultured gammaproteobacterium (AM229469) | oil-polluted marine microbial mat | 96 |
| S20 | EF531322 | uncultured *Flavobacteria* bacterium (AY712396) | salt marsh | 94 |
| S21 | EF531323 | uncultured *Lactococcus* sp. (EU029397) | raw cow milk | 100 |
| S22 | EF531324 | uncultured bacterium (AM697164) | indoor dust | 100 |

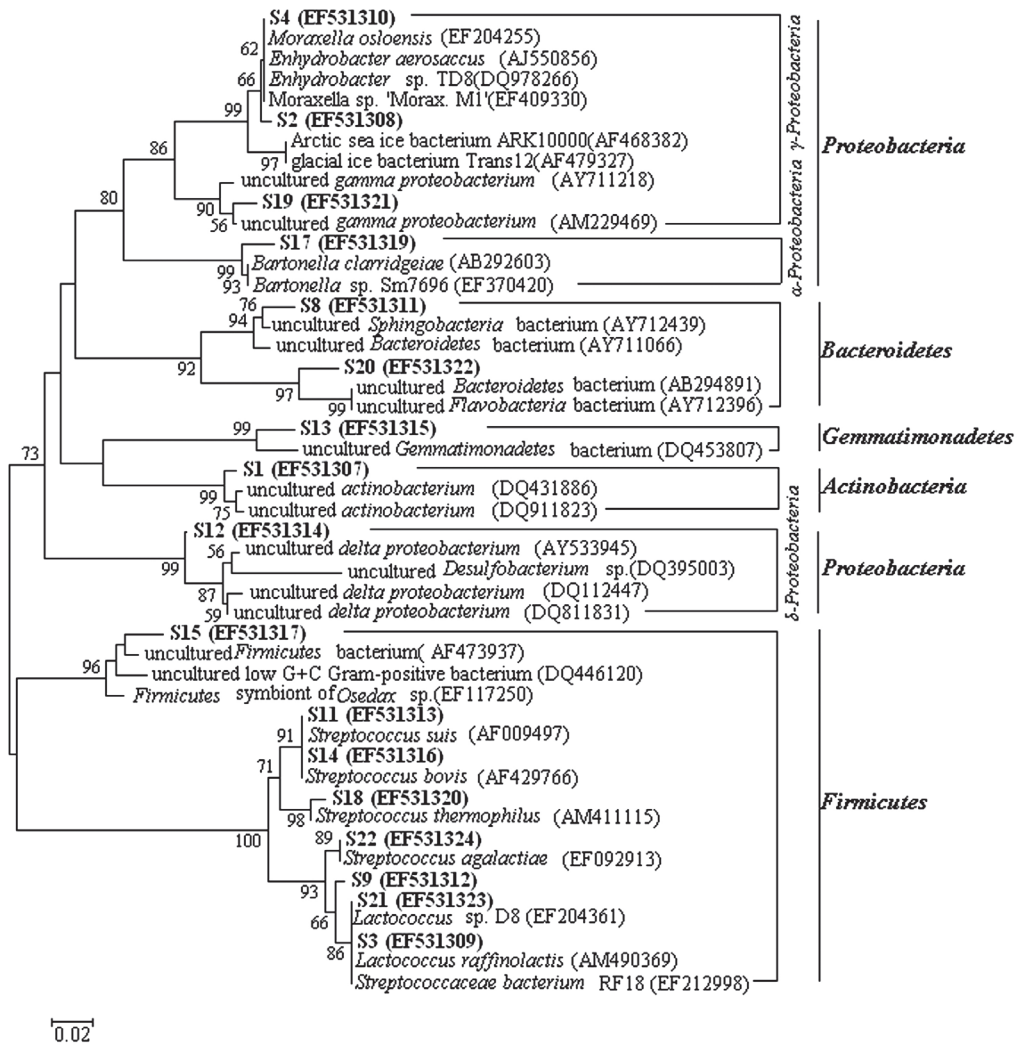


FIG. 4. – Unrooted phylogenetic tree based on 16S rRNA gene-V3 sequences representing the respective DGGE bands in Figure 1. Bootstrap analysis was based on 1000 replicates. Bootstrap values from distance analysis are depicted. Bootstrap values less than 50% are not shown. Scale indicates 2% sequence divergence.

sure a single band at the same location. Sequencing analysis of the predominant phylotypes that represents the excised DGGE bands is summarised in Table 4. All the sequences obtained in this study have been assigned to the GenBank nucleic acid sequence database with accession numbers EF531307-EF531317 and EF531319-EF531324. According to Table 4, most sequences were similar to 16S rRNA gene sequences reported from uncultured organisms present in environmental samples from sources such as mangrove soil, tropical oligotrophic lakes, salt marsh sediment, wastewater, high-temperature volcanic environment, oil-polluted marine microbial mat, inactive deep-sea hydrothermal vent chimneys and freshwater sediment. (Table 4).

For phylogenetic analysis, the 16S rRNA gene sequences of the 17 clones of this study were compared with those retrieved from GenBank database. The neighbour-joining analysis divided these sequences into five main groups, *Proteobacteria* (γ, α and δ subdivisions), *Bacteroidetes*, *Gemmatimonadetes*, *Actinobacteria* and *Firmicutes* (Fig. 4). According to Figure 4, ten sequences have high similarity or are even identical to cultivable bacteria. Two DNA bands clustered with γ-*Proteobacteria*, S2 and S4, and their 16S rRNA gene sequences were >99% identical to *Moraxella osloensis*. In the α-*Proteobacteria* cluster, band S17 had 98% similarity with *Bartonella clarridgeiae* at the DNA level. In the *Firmicutes* cluster most sequences were assigned to the *Streptococcus* and *Lactococcus* genera. 16S rRNA gene sequences of band S11 have 99% similarity with *Streptococcus suis*. Band S14 was 100% identical to 16S rRNA gene sequences of *Streptococcus bovis* and *Streptococcus suis*. The 16S rRNA gene sequence of S18 had 99% similarity with *Streptococcus thermophilus*. S22 was 100% identical to the 16S rRNA gene sequence of *Streptococcus agalactiae*. S21, S3 and S9 were clustered together with *Lactococcus raffinolactis*, and had 96-100% similarity.

However, the identity of seven DNA bands could not be verified with a strong credibility value to any defined genera in the phylogenetic analysis. All of them showed a high sequence similarity to uncultured bacterium from the GenBank database (Fig. 4). The 16S rRNA gene sequences of S19 had 96% similarity with uncultured γ-*Proteobacterium* retrieved from oil polluted marine microbial mat. In *Bacteroidetes* cluster, band S8 had 97% similarity with uncultured *Sphingobacteria* bacterium retrieved from Sapelo Island salt marsh samples, and band S20 had 94% similarity with uncultured *Flavobacteria* bacterium retrieved from salt marsh samples. In the *Gemmatimonadetes* cluster, the 16S rRNA gene sequence of S13 was 88% similar to uncultured bacterium retrieved from salt marsh. In the *Actinobacteria* cluster, S1 had 97% similarity with uncultured *Actinobacterium* retrieved from Mexico Island sediments. In the δ-*Proteobacteria* cluster, band S12 showed 97% similarity with uncultured deltaproteobacterium retrieved from mangrove soil samples. The 16S rRNA gene sequence of S15 had 97% similarity with uncultured *Firmicutes* bacterium associated with black band disease in coral.

## DISCUSSION

Although molecular approaches for assessing the microbial diversity in natural environments have been well developed and widely used in various types of environments (Urakawa *et al.,* 1999; Iwamoto *et al.,* 2000; Araya *et al.,* 2003; Bürgmann *et al.,* 2004; Li *et al.,* 2006; Sousa *et al.,* 2006; Muckian *et al.,* 2007), our knowledge of the microbiology of mangrove ecosystems is still largely based on cultivation-dependent studies (Holguin *et al.,* 2001). Recently, the 16S rRNA gene clone library was applied to investigate the bacterial composition in Futian mangrove swamp and the results showed that the surface sediments from subtropical mangrove swamp harbored a phylogenetically diverse population of organisms from the bacteria (Liang *et al.,* 2007). In this study, DGGE fingerprinting analysis provides an appropriate culture-independent approach for the rapid detection of the predominant microbial species and enables the simultaneous analysis of multiple samples. The banding patterns and numbers among all the samples indicated a diverse bacterial assemblage in tropical mangrove sediments.

Environmental parameters affecting the community of soil bacteria have been detailed over many years (Holguin *et al.,* 2001). The result from Alexander (1971) showed that the presence or absence of particular culturable bacterial genera may depend on soil parameters. Although a considerable amount is known about how culturable bacteria respond to environmental variables in mangrove ecosystems (Takizaqa *et al.,* 1993; Wu 1993; Wang and Hong 2005), little is known about how environmental variables influence the structure of

mangrove sediment bacterial communities in situ. In this study, CCA was employed to explore how the changes in microbial community composition varied with environmental conditions, which has been proven to be sensitive in detecting the relationship between bacterial community composition and environmental parameters (Iwamoto *et al.,* 2000; Mouser *et al.,* 2005; Jiang *et al.,* 2007; Zhang *et al.,* 2008). The previous studies reported that the cultivable bacteria composition has high similarity between mangrove soils collected from different mangrove areas (Shome *et al.,* 1995). While our results revealed that the in situ bacterial community compositions varied with different mangrove areas, and were strongly correlated with environmental factors. OC, pH and AP accounted for a significant amount of the variability in the bacterial community composition. This indicated that organic matter content, pH and available soil phosphors could influence the bacterial community structure in mangrove sediments. Bacteria were considered to be responsible for most of the carbon flux in tropical mangrove sediments, as they process most of the energy flow and nutrients, and act as a carbon sink (Alongi *et al.,* 1989; Boto *et al.,* 1979; Bano *et al.,* 1997; Alongi 1988, 1994). In tropical mangroves the resident bacterial community consumes much of the carbon dissolved in the interstitial water (Holguin *et al.,* 2001). Mangrove ecosystems depend on bacteria for nutrient recycling, as the nitrogen is consumed before it can be released into the atmosphere as $N_2$ (Rivera-Monroy and Twilley, 1996). The nitrate present in sediments and originating from the degradation of nitrogenous organic compounds is probably converted to ammonium ions by bacteria and is then assimilated by plants and bacteria. In turn, the bacteria seem to benefit from their association with the mangrove trees. In terrestrial environments, bacteria colonising the surface of plant roots induce root exudates, which can stimulate microbial activity in the rhizosphere (Lynch and Whipps 1990; Holguin *et al.,* 2001). In general, the relationship between bacteria community compositions and environmental factors were complicated in the mangrove ecosystem. Bacteria community compositions varied with environmental conditions and the variation of bacterial community composition may lead to the changes in nutrient condition in mangrove ecosystems.

In order to explore the phylogenetic and functional diversity of predominant bacteria in the mangrove habitat, the dense DGGE bands were excised and successfully sequenced. Phylogenetic analysis showed that the 17 16S rRNA gene sequences of this study clustered in various taxonomic groups, including *Proteobacteria*, *Bacteroidetes*, *Gemmatimonadetes*, *Actinobacteria*, and *Firmicutes* (Fig 4.). Based on our data, it appeared that the sequences clustering with the *Bacteroidetes*, *Gemmatimonadetes* and *Actinobacteria* group could not be verified with a strong credibility value to any defined species in the phylogetic analysis, which suggested that the bacteria in mangrove ecosystems are largely unexplored. Seven sequence phylotypes were identified with *Streptococcus* genera (gram-positive) of the phylum *Firmicutes*. Kathiresan (2003) reported that bacteria of *Streptococcus* genera were the predominant species of polythene and plastics-degrading microbes from the mangrove soil. Liao *et al.,* (2007) showed that *Proteobacteria* prevails in bacterial communities of Brazil mangrove soils. In our results γ-, α- and δ- *Proteobacteria* were detected in the *Proteobacteria* group. *Proteobacteria* have been suggested to play an important role in the N-, P-, and S- cycle in mangrove sediments, such as nitrogen fixation, phosphate solubility and sulfate reduction (Holguin *et al.,* 2001).

In conclusion, the surface sediments of tropical mangrove ecosystems harboured a phylogenetically diverse population of bacteria. The bacterial community compositions were related to environmental parameters, and the microbial communities varied with sample collection sites and seasons. Predominant bacteria in mangrove ecosystems clustered with various taxonomic groups. Some of them are related to genera or taxa that were classically identified in mangrove sediment and expected with known functions, but many are derived from uncultivable taxa. 16S rRNA gene-DGGE fingerprinting, in combination with comprehensive multivariate statistical approaches, is particularly useful as an investigation method for distinguishing between communities and obtaining the knowledge of relationship between microbial community and environmental parameters in mangrove ecosystems.

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