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ORIGINAL PAPER

Phylogenetic diversity of nitrogen-fixing bacteria in mangrove sediments assessed by PCR–denaturing gradient gel electrophoresis

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Abstract Culture-independent PCR–denaturing gradient gel electrophoresis (DGGE) was employed to assess the composition of diazotroph species from the sediments of three mangrove ecosystem sites in Sanya, Hainan Island, China. A strategy of removing humic acids prior to DNA extraction was conducted, then total community DNA was extracted using the soil DNA kit successfully for *nifH* PCR amplification, which simplified the current procedure and resulted in good DGGE profiles. The results revealed a novel nitrogen-fixing bacterial profile and fundamental diazotrophic biodiversity in mangrove sediments, as reflected by the numerous bands present DGGE patterns. Canonical correspondence analysis (CCA) revealed that the sediments organic carbon concentration and available soil potassium accounted for a significant amount of the variability in the nitrogen-fixing bacterial community composition. The predominant DGGE bands were sequenced, yielding 31 different *nifH* sequences, which were used in phylogenetic reconstructions. Most sequences were from *Proteobacteria*, e.g. α, γ, β, δ-subdivisions, and characterized by sequences of members of genera *Azotobacter*, *Desulfuromonas*, *Sphingomonas*, *Geobacter*, *Pseudomonas*, *Bradyrhizobium* and *Derxia*. These results significantly expand our knowledge of the nitrogen-fixing bacterial diversity of the mangrove environment.

Keywords Mangrove sediments · Nitrogen-fixing bacteria · Denaturing gradient gel electrophoresis · Multivariate analysis

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Introduction

Mangrove communities are recognized as highly productive ecosystems that provide large quantities of organic matter to adjacent coastal waters (Holguin et al. 2001; Lugomela and Bergman 2002). Although mangrove ecosystems are rich in organic matter, in general they are nutrient-deficient, especially of nitrogen (Holguin et al. 1992; Vazquez et al. 2000). Nitrogen fixation was considered being the major source of combined nitrogen input in mangrove forest habitats (Hicks and Silvester 1985; Kyaruzi et al. 2003). High rates of nitrogen fixation have been found associated with dead and decomposing leaves, pneumatophores, the rhizosphere soil, tree bark, cyanobacterial mats covering the surface of the sediment, and the sediments themselves (Zuberer and Silver 1978, 1979; Hicks and Silvester 1985; Holguin et al. 1992, 2001; Lugomela and Bergman 2002).

Culture-dependent methods are known to be inadequate for analysis of nitrogen-fixing microbial community because only a small fraction of nitrogen-fixing bacteria in

environmental samples are culturable (Bürgmann et al. 2004). Molecular methods based on PCR amplification of *nifH* sequences are now widely used for providing new insights into nitrogen-fixing bacterial diversity and into structure and dynamics of diazotrophic communities. Particularly, fingerprinting methods such as DGGE of PCR-amplified *nifH* sequence have been used to examine the complexity and stability of the diazotrophic assemblage in various types of nitrogen limited environments, such as seagrass bed communities (Bagwell et al. 2002), salt marshes (Piceno et al. 1999), plant rhizosphere (Piceno and Lovell 2000a, b; Lovell et al. 2000), and acid forest soil (Rosado et al. 1998). However, there has been no comparable investigation of the nitrogen-fixing bacterial community composition in mangrove habitats, which have large ecological value.

Although a considerable amount is known about how environmental variables affect on nitrogen fixation rates in mangrove ecosystems (Zuberer and Silver 1978, 1979; Toledo et al. 1995; Holguin et al. 2001). Little is known on the relationship between environmental parameters and the structure of nitrogen-fixing bacterial communities in mangrove ecosystems. Multivariate techniques, such as canonical correspondence analysis (CCA) have been proven to be sensitive in detecting the relationship between microbial community composition and environmental parameters (Iwamoto et al. 2000; Mouser et al. 2005; Jiang et al. 2007).

In this study, we document the variability in nitrogen-fixing bacterial communities and environmental parameters from different tropical Mangrove samples of Sanya Mangrove Nature Reserve. Our primary objectives were (1) to assess the diazotrophic community structure and diversity, (2) to explore whether environmental variables are related to diazotraphic community composition in tropical mangrove sediments, and (3) to identify the major phylogenetic groups of organisms that are capable of contributing to nitrogen fixation in mangrove sediments. To our knowledge, this is the first time a direct molecular approach has been used to investigate the nitrogen-fixing bacterial composition in mangrove ecosystem.

Materials and methods

Study site and sampling

The sediments samples were collected from Sanya Mangrove Nature Reserve, which is a typical tropical mangrove ecosystem, located at the southernmost part of Hainan Island in China, including Yalong Bay Qingmei, Yulin Bay Hongsha, Sanya River and Linwang Tielu Mangrove Nature Reserve. In this study ten samples were collected from swamp of Yulin Bay Hongsha (site A), Yalong Bay Qingmei (site B), and Sanya River (site C) 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 the rainfall are 25.4°C and 1279.5 mm, respectively. The location of each site was recorded with GPS (Global Positioning System), which are listed in Table 1. For all samples, the top 10 cm of sediments with quintuplicate samples (over 1 m^2 area) were collected and mixed, then placed into sterile collection bags. There are two duplicates: one for analysis of environmental parameters and another for DNA extraction. The samples for environmental parameters 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°C prior to DNA extraction.

Total community DNA extraction and PCR amplification

Total community DNA was extracted from 1.0 g of wet sediment using the E.Z.N.A®. Soil DNA Kit (Omega Biotek) with some modification. To remove the inhibitors such as humic acids, 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 sediments samples successfully. Total DNA extractions were performed as recommended by the manufacturer (Omega Biotek). The precipitated DNA was resuspended in 20 µl of TE buffer (10 mM Tris–HCl, 1 mM Na_2EDTA, pH 8.0) and was stored at −20°C. For PCR purposes, the DNA concentration was measured spectrophotometrically (HITACHI) and adjusted to a concentration of 100 ng μl^{-1}.

Table 1 The location of collection sites and the summary of samples

Collection sites	Samples	Predominant Mangrove species
Site A	A1	*Xylocarpus granatum*
N: 18°15′52″;	A2	*Rhizophora stylosa*
E: 109°34′22″	A3	*Bruguiera gymnorhiza*
	A4	*Bruguiera gymnorhiza*
Site B	B1	*Kandelia obovata*
N: 18°13′50.2″;	B2	*Lumnitzera racemosa*
E: 109°37′15.8″	B3	*Ceriops tagal*
Site C	C1	*Avicennia marina*
N 18°15′08.6″;	C2	*Rhizophora apiculata*
E 109° 30′51.1″	C3	*Avicennia marina*

Several specific primers and different PCR protocols for the amplification of the *nifH* gene fragment were tested for optimization. A PolF/PolR primer set (Poly et al. 2001a) and nested PCR set-up was found to be the most efficient method for amplification of *nifH* gene (data were not shown). PCR amplification for *nifH* gene was performed on a PTC-200 thermal cycler (MJ Research). The first PCR was performed using primers PolF and PolR. The PCR was carried out in 25 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 1 U Ex TaqTM. The Taq polymerase, dNTPs and PCR buffer were purchased from TaKaRa (TaKaRa Schuzo Co., Ltd., Biomedical Group, Japan). All the primers were synthesized by Invitrogen (Shanghai Invitrogen Biotech Co. Ltd). 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. A total of 0.2 μl of the PCR product was used as template in a subsequent PCR, performed in 50 μl volume under the same conditions as above, except using different primers: PolF (with a 40 bp GC-clamp cgc ccg ccg cgc gcg gcg ggc ggg gcg ggg gca cgg ggg g attached to the 5′ end) and PolR. The annealing temperature in the PCR profile was increased to 60°C. To reduce possible inter-sample PCR variation, all the PCR reactions were run in triplicates and pooled together before loading on DGGE gel. In DNA extraction and PCR amplification, genomic DNA of *E. coli* was used as positive control and PCR mixture without DNA template was used as negative control. PCR products were analyzed by electrophoresis in 1.5% agarose gels and ethidium bromide staining.

Denaturing gradient gel electrophoresis

DGGE was performed using 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_2EDTA) with denaturant-gradient of 45–65% [100% denaturant was 7 M urea and 40% (wt/vol) deionized formamide]. The gels were electrophoresed at a constant voltage of 100 V and 60°C for 10 h. After electrophoresis, DGGE gels were stained with ethidium bromide and visualized under UV light in an AlphaImager imaging system (Alpha innotech).

Sequencing and phylogenetic analysis

DGGE bands were excised from gels, 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 (12,000 rpm, 5 min, 4°C) was used as a template for *nifH* sequence amplification using the same primer pairs without GC-clamp. Ten microliters of the PCR product was loaded again in a DGGE gel to confirm the position of the bands. The remaining PCR product was purified with the PCR Purification kit (Tiangen) and subsequently cloned into PMD18-T plasmid Vector and *E. coli* DHα cells using PMD18-T cloning vector kit according to manufacturer's instructions (Takara). Positive clones were identified by PCR amplification with PMD-18T Vector primer pairs T7 (5V-TAA TAC GAC TCA CTA TAG GG-3V) and M13 (5V-CAG GAA ACA GCT ATG ACC-3V), using the same program as *nifH* amplification. PCR products were affirmed in DGGE gel to confirm its position. Positive recombinants were then submitted for sequencing using an ABI3730 DNA Sequencer (USA) with M13 primer at the Shanghai Invitrogen Biotech Co.Ltd. Nucleotide and protein sequences were compared to those in the GenBank database by BLAST algorithm to identify sequences with a high degree of similarity. Phylogenetic trees of *nifH*-derived protein 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 1,000 bootstrap replicates.

Statistical analysis

DGGE digital images were analyzed by gel documentation system, Gel Doc 2000, Quantity-One 4.5.2 (Bio-Rad, USA) to generate a densitometric profile. The peak areas of the fingerprint patterns were used to indicate the intensities. Bands with a relative intensity of less than 0.5% of the sum of all band intensities were discarded. The diversity and dissimilarity indices were analyzed according to the DGGE banding profiles. The Shannon index of bacterial diversity, *H*, was calculated by using the following equation (Shannon and Weaver, 1963):

$$H = -\sum Pi \log Pi.$$

Where P_i is the importance probability of the bands in a track. It was calculated from $Pi = ni/N$. Where n_i is the area of a peak and *N* is the sum of all peak areas in the densitometric curve.

Canonical correspondence analysis (CCA) (CANOCO 4.5; Biometris, Wageningen, The Netherlands) was performed to explore the relationship between environmental variables and the members of nitrogen-fixing bacterial communities. The resulting ordination biplot approximated the weighted average of each species (in this case, bands 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 analysis

The basic properties of the soil samples were shown in Table 2. The variabilities of these parameters from different samples were obvious. For example, the concentration OC of samples from site A were higher than that of others, however, the AP concentration of sample A1 was lower than that of other samples. The sample A3 had the lowest AK concentration. And A4 had the highest AK concentration. Analysis of DGGE Gel (Fig. 1) resulted in a total of 215 detectable bands in 64 different positions. The number of bands per sample varied between 15 and 27, which indicated a diverse nitrogen-fixing bacterial assemblage in ecosystem sediments. A total of 31 bands were excised and successfully sequenced (see number and position in Fig. 1). The indices of *H*, reflecting the structural diversity of the nitrogen-fixing bacterial community, were calculated on the basis of the number and relative intensities of bands on the gel track. Based on Table 2, the lowest Shannox index was detected in the sample A4, while the highest Shannox index occurred in sample B2. The mean Shannox index of three sample sites were ranked site B > site A > site C.

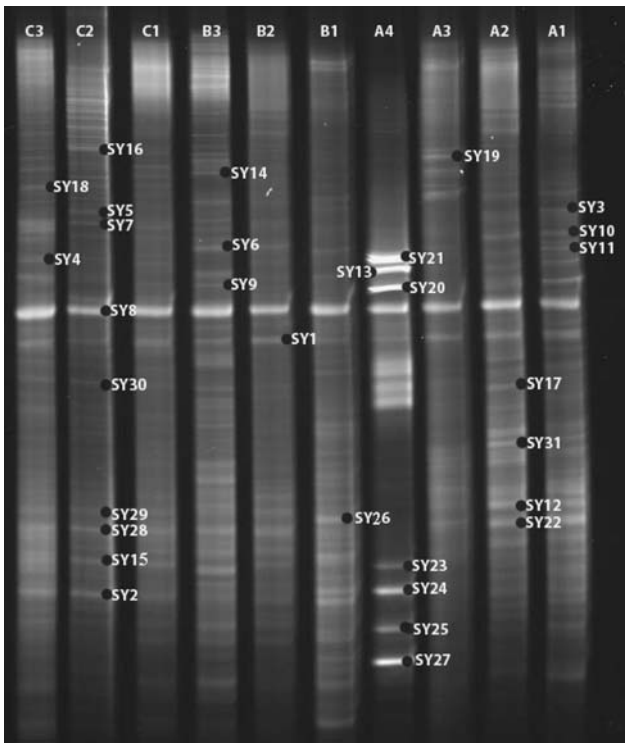

Fig 1 *NifH*-based DGGE fingerprinting of sediments samples from mangrove ecosystem, Lanes: A1-A4 obtained from site A; B1–B3 collected from site B; C1–C3 from site C

Relationship between environmental variables and nitrogen-fixing bacterial community structure

Canonical correspondence analysis was carried out using abundant DGGE bands (the relative intensity exceeded 0.5%) together with environmental variables. Eigenvalues (indicating strength of the model) for the first two multivariate axes were 0.488 and 0.373. The sum of all

Table 2 Main characteristics of the soil samples (value are giver as mean, $n = 3$)

Samples	OC (g kg^{-1})	TN (g kg^{-1})	AP (mg kg^{-1})	AK (mg kg^{-1})	pH	Shannon–Weaver index (*H*)	DGGE bands
A1	100.6	1.95	10.31	261.5	4.80	1.237	23
A2	113.1	3.99	18.32	301.2	6.86	1.196	22
A3	95.7	1.43	23.18	223.8	5.10	1.051	18
A4	83.6	1.17	22.23	512.6	5.23	1.031	15
B1	55.8	1.27	18.56	305.1	6.67	1.141	23
B2	85.9	1.88	12.65	294.8	2.91	1.249	27
B3	38.4	0.91	27.85	278.1	4.43	1.053	22
C1	53.1	0.96	22.37	268.5	2.66	1.153	23
C2	32.0	0.92	25.98	288.2	6.49	1.040	21
C3	29.3	1.85	12.61	286.4	2.89	1.123	21

canonical eigenvalues was 1.517. Axes 1 and 2 were found to explain 32.2 and 24.6% of the overall variance, respectively, indicating a strong gradient in the data set. Species environment correlations for both axes were more than 0.99, suggested that nitrogen-fixing bacterium were strongly correlated with environmental factors. Monte-Carlo significance tests indicated that both axes explained a significant proportion of the variation in the data. CCA biplot was shown in Fig. 2. Each environmental variable is represented by an arrow; the length of the individual arrow indicates how much variance is explained by that variable. The projection of a species (indicated as open triangles) on this axis indicated the level of the variable where the species is most abundant. The position of species relative to arrows indicates the extent to which a species is correlated with an environmental parameter. More details about the interpretation of the biplot can be found in Ter Braak (1987). Based on Fig. 2, it is clear that Axis 1 was significantly positively correlated with AK ($P = 0.984$), which suggested that Axis 1 showed a gradient in AK. Axis 2 had an important negative correlation with OC ($P = -0.938$), which revealed a negative OC gradient distribution. Most of the bacteria were negatively correlated with AK, and distributed along the OC gradient (Fig. 2). Bacteria SY19 were significant positive relative to TN. Another cluster of bacteria such as SY18, SY7, SY2 and SY15 were strong negatively correlated with OC and distributed upper of Axis 2. Bacteria SY21, SY27, SY20, and SY25, which

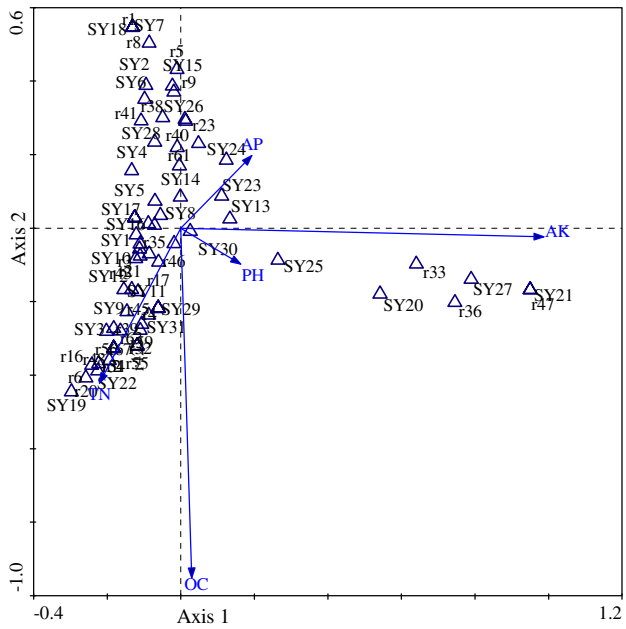

Fig 2 Canonical correspondence analysis (CCA) ordination diagram of DGGE data, with chemical factors as *arrows* and individual abundant nitrogen-fixing bacteria

were unique in sample A4, were positively correlated with AK. In general, nitrogen-fixing bacterial community composition had significant correlation with environmental parameters in tropical mangrove sediments.

Sequencing and identification of DGGE fragments

Most excised bands were detected in more than three samples, with the relative intensity exceeded 1%. Some bands, e.g. bands SY1 and SY8 were detected in all the samples. Occasionally, unique bands detected in only one sample and the relative intensity exceeded 3% were excised, e.g. bands SY21, and SY27. Thirty-one bands were excised from gel tracks, which were verified by DGGE three times to ensure a single band at the same location, were signed with SY1-31. Sequencing analysis of nitrogen-fixing bacterium that represents the DGGE bands of mangrove sediments was summarized in Table 3. All the sequences obtained in this study have been assigned to the GenBank nucleic acid sequence database with accession numbers EF178501–EF178502, EF185784–EF185791, EF191077, EF196648–EF196656, EF199926 and EF494084–EF494093. According to Table 3, most of the sequences were similar to *nifH* sequences reported from uncultured organisms present in environmental samples from sources such as mangrove roots, rhizosphere of smooth cordgrass, sea water, lakes, rivers and oil-contaminated marine sediments (Table 3). Most of the *nifH* clones had homologies lower than 90% sequence identity with *nifH* genes deposited in the GenBank database; however, at the protein level these clones shared 93–100% similarity with published *nifH* sequences (Fig. 3.). These results indicate a high diversity of potentially nitrogen-fixing bacteria in mangrove ecosystem, as previously demonstrated in other microenvironments (Ueda et al. 1995; Lovell et al. 2001; Reiter et al. 2003).

For phylogenetic analysis, the *nifH*-derived protein sequences of the 31 clones of this study were compared to those from GenBank database. The neighbor-joining analysis divided these nitrogen-fixing bacterial sequences into five main groups (Fig. 3). According to Fig. 3, two DNA bands were fallen into cluster A, SY9 and SY20, of which the deduced nitrogenase iron protein sequences were both related to those of *δ-Proteobacteria* members. However, the *nifH* sequences of bands SY9 and SY20 were 87–89% similar to uncultured nitrogen-fixing bacteria retrieved from oil-contaminated marine sediment and white spruce rhizosphere, respectively (Table 3). In cluster B, the protein sequence of band SY3 was 95% related to *Bradyrhizobium elkanii* USDA 76, which derived from *α-Proteobacteria*. The protein sequence of band SY10 exhibited 99% sequence identical with that of *Sphingomonas azotifigens*, which is also a member of *α-Proteobacteria* family. The protein sequence of band SY5

Table 3 Summary of the *nifH* sequences obtained from the respective bands in DGGE gels and the closest match to the sequence from GenBank database

Band	Accession no.	Database match with accession no. in parentheses	Origin	% Identity
SY1	EF178501	Nitrogen-fixing bacterium HS002(EF191079)	Mangrove roots	94
SY2	EF178502	Uncultured bacterium(AY224038)	Choptank River, USA	82
SY3	EF185784	Sphingomonas azotifigens (AB217474)	Roots of *Oryza sativa*	88
SY4	EF185785	Uncultured bacterium clone 2H16(DQ177020)	Mangrove roots	89
SY5	EF185786	Uncultured bacterium (AJ716352)	Mine soil under *Serianthes calycina*	87
SY6	EF185787	Uncultured bacterium clone 1H11 (DQ176985)	Mangrove roots	88
SY7	EF185789	Uncultured *Cyanobacterium* (DQ398399)	Falls Lake, USA	84
SY8	EF185788	Nitrogen-fixing bacterium HS002(EF191079)	Mangrove roots	93
SY9	EF185790	Uncultured bacterium clone OilUp-10321 (DQ078040)	Oil-contaminated marine sediment	87
SY10	EF185791	Uncultured alpha proteobacterium (EF470546)	Western Channel Observatory, English Channel	90
SY11	EF191077	Uncultured nitrogen-fixing bacterium (DQ402773)	Rhizosphere of smooth cordgrass	80
SY12	EF494084	Nitrogen-fixing bacterium HS002(EF191079)	Mangrove roots	92
SY13	EF196648	Uncultured nitrogen-fixing bacterium (DQ402888)	Rhizosphere of smooth cordgrass	84
SY14	EF196650	Uncultured nitrogen-fixing bacterium (AY196415)	Soil	90
SY15	EF196649	Uncultured bacterium (AY224038)	Choptank River, USA	82
SY16	EF196651	Uncultured nitrogen-fixing bacterium (AY196415)	Soil	90
SY17	EF196652	Uncultured bacterium (AY224038)	Choptank River, USA	82
SY18	EF196653	Uncultured nitrogen-fixing bacterium (AY196415)	Soil	90
SY19	EF196654	Uncultured nitrogen-fixing bacterium (AY196415)	Soil	90
SY20	EF196655	Uncultured soil bacterium (DQ776597)	White spruce Rhizosphere	89
SY21	EF196656	Uncultured bacterium (AM286448)	Microbial delta mat, France	77
SY22	EF494085	Nitrogen-fixing bacterium HS002 (EF191079)	Mangrove roots	94
SY23	EF494088	nitrogen-fixing bacterium HS002(EF191079)	Mangrove roots	94
SY24	EF494086	Nitrogen-fixing bacterium HS002(EF191079)	Mangrove roots	94
SY25	EF199926	Unidentified nitrogen-fixing bacteria (AF216920)	Rhizosphere of the smooth cordgrass	93
SY26	EF494087	Nitrogen-fixing bacterium HS002(EF191079)	Mangrove roots	93
SY27	EF494089	Nitrogen-fixing bacterium HS002(EF191079)	Mangrove roots	94
SY28	EF494090	Nitrogen-fixing bacterium HS002(EF191079)	Mangrove roots	94
SY29	EF494091	Uncultured bacterium(AY224038)	Choptank River, USA	82
SY30	EF494092	Uncultured bacterium(AY224038)	Choptank River, USA	82
SY31	EF494093	Uncultured bacterium(AY224038)	Choptank River, USA	82

was related for 94% to the sequence of *Derxia gummosa*, which belongs to *β-Proteobacteria* (Fig. 3). However, the *nifH* sequences of bands SY3, SY10 and SY5 were 87–90% similar to organisms present in environmental samples (Table 3). It is clear that numerous nitrogen-fixing bacteria fell into cluster C (Fig. 3). The most intensity band occurred in all samples was band SY8 (Fig. 1). The *nifH* sequence of band SY8 was 93% similar to nitrogen-fixing bacteria HS002 isolated from mangrove roots (Table 3). The protein sequence of band SY8 exhibited 99% sequence similarity with the protein sequence of *Azotobacter chroococcum*, which is belong to *γ-Proteobacteria*. Other bands, e.g. bands SY1, SY12, SY22-24 and SY26-28, share 92–94% identical nucleotide with nitrogen-fixing bacteria HS002, and share 96–99% identical protein sequences with *A. chroococcum* (strain mcd 1). Bands SY14, SY16, SY18 and SY19 showed 93% identity with the protein sequence of *A. chroococcum*. Moreover, the protein sequences of bands SY4, SY6 and SY25 shared 95–96% similarity with *γ-Proteobacteria Pseudomonas stutzeri*. However, the *nifH* sequences of bands SY4 and SY6 shared 88–89% identical to uncultured nitrogen fixing bacteria retrieved from mangrove roots, and the *nifH* sequence of band SY25 was 93% identical to unidentified nitrogen-fixing bacteria isolated from rhizosphere of the smooth cordgrass (Table 3; Fig. 3). In cluster D, the identity of DNA bands SY7, SY11, SY13, and SY21 could not be verified with a strong credibility value to any defined genera in the phylogenetic analysis. All of them showed a high protein sequence similarity (90–99%) with uncultured nitrogen-fixing bacterium from GenBank database (Fig. 3). Accoring to Table 3, the *nifH* sequence of band SY7 was 84% similar to uncultured

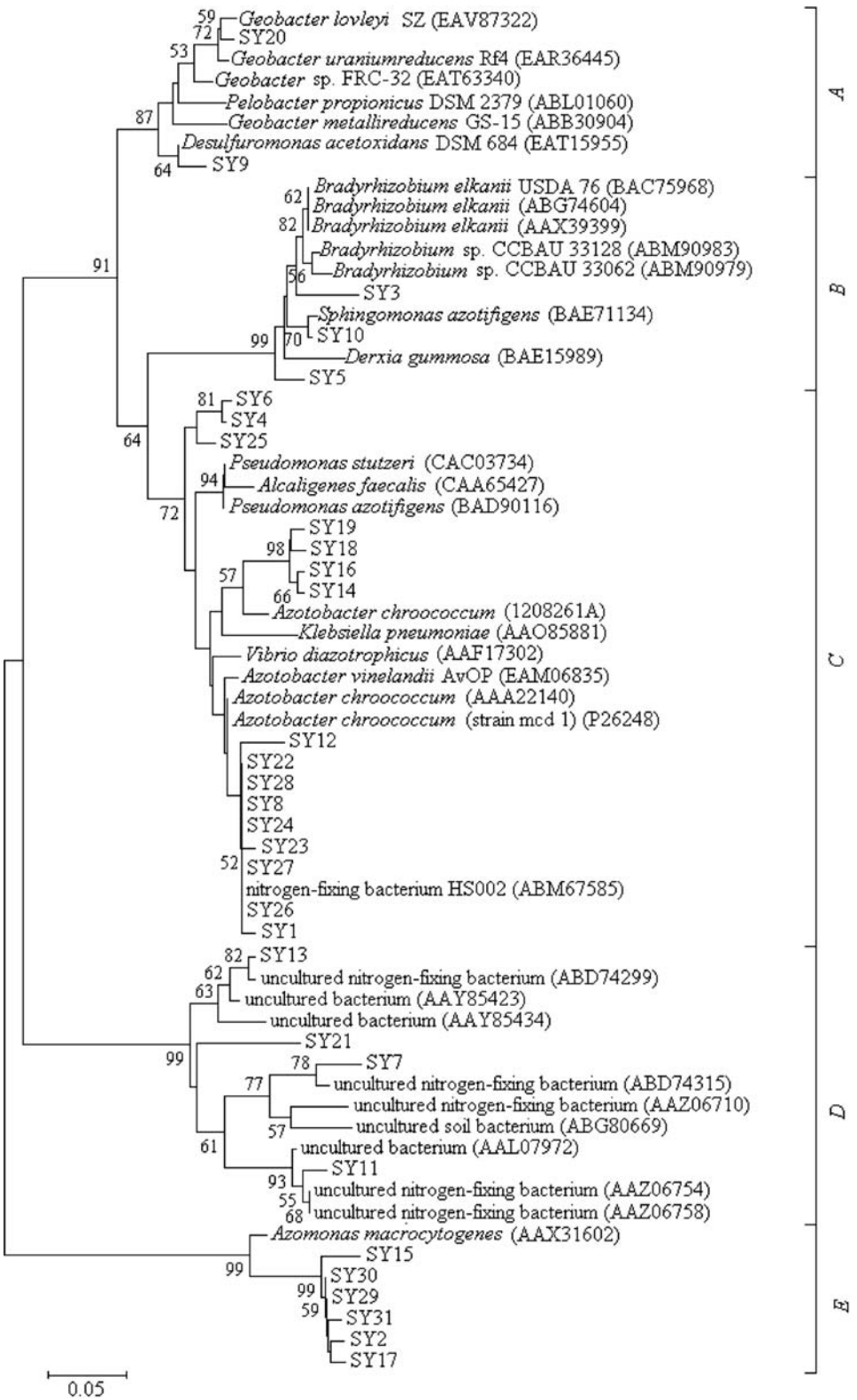

Fig 3 Unrooted phylogenetic tree based on *nifH*-derived protein sequences representing the respective DGGE bands in Fig 2. Bootstrap analysis was based on 1,000 replicates. Bootstrap values from distance analysis are depicted. Bootstrap values less than 50% are not shown. *Scale* indicates 5% sequence divergence

cyanobacteria retrieved from Falls Lake, USA. The *nifH* sequences of bands SY11 and SY13 were 80–84% similar to uncultured nitrogen-fixing bacteria retrieved from rhizosphere of smooth cordgrass. The sequence of band SY21 was only 77% similar to uncultured bacteria retrieved from microbial delta mat, France (Table 3). In cluster E, DNA bands SY2, SY15, SY17, and SY29-SY31 exhibiting 93–95% protein sequences similarity with *Azomonas*

macrocytogenes, fell into *γ-Proteobacteria* subdivision, whereas, their *nifH* sequences were 82% similar to uncultured bacteria retrieved from Choptank River, USA.

Discussion

In this study, culture-independent DGGE fingerprints of PCR-amplified *nifH* genes were used to study predominant nitrogen-fixing bacterial populations in ten mangrove sediments. To identify the complex, non-culturable nitrogen-fixing communities of mangrove microorganisms, the quantity of the extracted microbial community DNA was obviously very important in terms of obtaining satisfactory community profiles. It is well known that the sediments of mangrove ecosystems are rich in humic acids (Sengupta and Chaudhuri 1991; Holguin et al. 1992; Vazquez et al. 2000). To avoid interference from humic acids during PCR amplification, the very important step is removing humic acids from mangrove sediments in DNA extraction. A strategy of removing humic acids prior to DNA extraction was conducted in this study to resolve the problem. After that the E.Z.N.A®.Soil DNA Kit was applied and the large fragment DNA was recovered from mangrove sediments. The obtained total community DNA can be used as a template directly for *nifH*-PCR amplification; there is good reproducibility between triplicate experiments with this method. This is a useful method for DNA-based PCR analysis of mangrove sediments and other rich humic acid microorganisms.

Environmental parameters affecting the community of soil bacteria, especially diazotroph, have been detailed over many years (Riffkin et al. 1999; Poly et al. 2001b). The result from Alexander (1971) showed that the presence or absence of particular culturable bacterial genera may depend on soil parameters. In this study, CCA revealed that AK and OC accounted for a significant amount of the variability in the nitrogen-fixing bacterial community composition. This suggests that available soil potassium and organic matter content could influence the diazotrophic community structure in mangrove sediments. It is not surprising that organic matter content concentration influence the diazotrophic community structure because mangrove ecosystem accumulates biodegradable organic matter, which can supply the high energy for nitrogen fixation (Holguin et al. 2001). A positive correlation was found between nitrogen fixation rates and the availability of organic matter in mangrove sediments. Energy for nitrogen fixation can also be derived from leaves and roots decomposed by non-diazotrophic microfora that colonize dead mangrove leaves (Zuberer and Silver 1978, 1979). However, how the concentration of available soil potassium influence the diazotrophic community structure remained unexplained. In this study most bacteria were negatively correlated with AK, however, the bands which were unique in sample A4 were positively correlated with AK.

Nitrogen-fixing bacteria from *Proteobacteria* are common in marine environments. Bagwell et al. (2002) reported sequences affiliated with α- and (*γ*-+*β*-) *Proteobacteria* in oligotrophic tropical seagrass bed communities. Church et al. (2005) detected the *nifH* sequences were identical to uncultivated *γ-Proteobacteria* sequences in the oligotrophic North Pacific Ocean. The finding of Bird et al. (2005) suggests that *γ-Proteobacteria* are widespread and likely to be an important component of the heterotrophic diazotrophic microbial community of the tropical and subtropical oceans. In this study, most derived protein sequences of DGGE bands were similar or even identical to well-described organisms. Some of them were similar to *nifH* sequences retrieved from mangrove roots samples. However, many sequences of DGGE bands were similar to *nifH* sequences retrieved from uncultured nitrogen-fixing bacteria. Mangrove ecosystems often contain nitrogen-fixing cyanobacteria (Kyaruzi et al. 2003), however, only the sequence of band SY7 in this study was similar to uncultured cyanobacteria. The first possibility of detecting cyanobacteria by DGGE from mangrove sediments is small because the intensity of excised bands exceeded 1%. The second reason may be the use of primer sets. Several specific primers were tested in this study; the PolF/PolR primer set retrieved more stable and clear bands in DGGE profiles from our samples (data not shown).

DGGE has recently become a common technique for the analysis of microbial organism population ecology and dynamics, being particularly useful in studies that involve multiple samples (Henriques et al. 2006; Li et al. 2006). Despite the shortcomings that data obtained using DGGE should be analyzed taking into consideration several associated biases related to DNA extraction efficiency, rRNA gene copy number, PCR primer annealing efficiency, chimera formation, heteroduplex formation and cloning (Henriques et al. 2006), DGGE has been used to provide community profiles in over 700 publications since the year 2000 (Sousa et al. 2006) and has been proved to present a valid picture of the community and that the resulting profiles represent the majority of the nitrogen-fixing bacteria present in environment samples (Rosado et al. 1998; Piceno et al. 1999; Lovell et al. 2000; Piceno and Lovell 2000a, b; Bagwell et al. 2002). Our results suggested that *nifH* PCR based DGGE fingerprinting combined with gene sequencing and phylogenetic analysis is a useful tool for investigating the community diversity and identifying the predominant nitrogen-fixing bacteria. PCR based DGGE fingerprinting, in combination with multivariate statistical approaches are particularly useful as

investigation method for distinguishing between communities and obtaining the knowledge of relationship between functional bacterial community and environmental parameters in mangrove ecosystem.

Results reported here have obtained a fundamental diversity insight into the major nitrogen-fixing bacterial populations in the mangrove sediments and can be used as a starting point for further phylogenetic studies and functional analyses. This is, to our knowledge, the first time that a direct molecular approach has been used to investigate the nitrogen-fixing bacterial populations in mangrove sediments. Additional knowledge from these studies will help understand the relationship between the nitrogen-fixing bacterial community composition and environmental parameters.

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