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Full Length Research Paper

Impact of different polymerase chain reaction (PCR) strategies on denaturing gradient gel electrophoresis-based analysis of bacterial communities in soils/sediments from the Northern Jiangsu Oil Field, China

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The selection of polymerase chain reaction (PCR) strategies is vital to a successful assessment of bacterial communities in soils/sediments by 16S rRNA gene-based denaturing gradient gel electrophoresis (DGGE) analysis. To obtain reliable information of the bacteria communities in soils/sediments from the Northern Jiangsu Oil Field (NJOF), the impact of six PCR strategies on DGGE analysis has been investigated. The results showed that one-step PCR approach with primer set 341f⊥GC/534r (Strategies 1 and 2) was not suitable for 16S rRNA gene amplification of the bacterial communities in the NJOF soils/sediments before DGGE analysis due to its non-specific DNA amplification and low efficiency of 16S rRNA gene amplification. Strategy 6 (one nested PCR approach with primer sets 27f/907r and 341f⊥GC/534r with a purification procedure) could be the most accurate assessment of community diversities, but only be suitable to perform DGGE analysis for a few samples and not for high-throughput DGGE analysis because it was time-consuming; and Strategies 5 (one nested PCR approach with a dilution procedure) and 3 (one two-step PCR approach with primer sets 341f /534r and 341f⊥GC/534r) could provide similar information on the bacterial diversity of the NJOF soils/sediments without a purification procedure comparing with Strategy 6. Therefore, we prefer to recommend Strategies 5 and 3 for high-throughput DGGE analysis, and have successfully obtained the useful information of bacterial communities in different NJOF soils/sediments by Strategies 5.

Key words: Polymerase chain reaction (PCR), denaturing gradient gel electrophoresis (DGGE) analysis, bacterial diversity, soils/sediments, Northern Jiangsu Oil Field (NJOF).

INTRODUCTION

Traditionally, the assessment of microbial communities in soils/sediments relied on culturing techniques using a variety of culture media designed to maximize the recovery of diverse microbial populations (Hill et al., 2000). However, it has been reported that more than 99% environmental microbes were uncultured in artificial media provided by conventional culturing methods (Janssen et al., 2002; Torsvik et al., 1990), which could lead to underestimation of actual cell numbers in soils/sediments. Recently, molecular techniques such as

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denaturing gradient gel electrophoresis (DGGE) (Muyzer et al., 1993), temperature gradient gel electrophoresis (Muyzer and Smalla, 1998), terminal restriction fragment length polymorphism (Terence, 1999), fluorescent *in situ* hybridization (Assmus et al., 1995) and single strand conformation polymorphism (Schwieger and Tebbe, 1998) have become popular in the assessment of microbial communities in soils/sediments, due to its specificity and accuracy.

Polymerase chain reaction (PCR)-DGGE was one of the most frequently-used molecular techniques for high throughput analysis of microbial communities in soils/sediments (Ferrari and Hollibaugh, 1999), and directly and rapidly provided a clear characterization of the community diversity. 16S rRNA gene-based PCR-DGGE community analysis mainly involves two steps: PCR amplification of 16S rRNA gene followed by DGGE analysis of PCR-amplified 16S rRNA gene fragments. The aim of PCR amplification is to prepare DNA samples for DGGE analysis, and is critical to the success of a community assessment. Several studies have been performed to assess the impact of DNA extraction methods (de Lipthay et al., 2004; Ferrera et al., 2010), PCR primers (Brons and van Ems, 2008; Muhling et al., 2008; Sanchez et al., 2007), and electrophoresis time (Sigler et al., 2004) on PCR-DGGE analysis of microbial community structures. However, rare report on the impact of different PCR strategies in DGGE analysis has been presented so far.

As with many molecular biological methods, the steps of PCR involved in DGGE-based community analysis are more or less consistent among different laboratories, but not standardized. To our knowledge, the reports focused on different PCR-amplified models in DGGE which influenced the analysis of bacterial community diversity by only two or three characterized PCR strategies (Shabir et al., 2005). A special PCR strategy should be used according to the sample source, humic acid content, and other sample characteristics before DGGE analysis. In this study, the impact of six PCR strategies on DGGE analysis has been evaluated to choose the most suitable PCR strategy for assessing bacterial communities in soils/sediments from the Northern Jiangsu Oil Field (NJOF), China. Here, we presented a standardized procedure to determine a suitable PCR strategy for DGGE analysis, and the advantages and disadvantages of these strategies were also discussed in detail.

MATERIALS AND METHODS

Reagents

Two soil samples were collected in the depth of 50 cm below the surface from two sites (sA: 119°51.13′E, 32°38.85′N; and sB: 119°51.40′E, 32°38.85′N) in NJOF, and then stored at -20°C for total DNA extraction. E.Z.N.A.™ soil DNA Kit was purchased from Omega Bio-Tek, Inc (Norcross, USA). Super *Taq* DNA polymerase was purchased from HaiGene Biotech Co., Ltd (Harbin, China). MiniBEST Agarose Gel DNA Extraction Kit Ver. 3.0 and deoxyribonucleotide triphosphates (dNTPs) mixture were purchased from TaKaRa Biotech Co., Ltd (Dalian, China). PCR primers were synthesized by Sangon Biotech Co., Ltd (Shanghai, China). SYBR gold was purchased from Invitrogen Biotech Co., Ltd (New York, USA). Other chemicals were commercial products with the analytical reagent grade. Deionized water was used to prepare all solutions.

Total DNA extraction

Total DNA was extracted with the E.Z.N.A™ soil DNA Kit from 0.5 g of dried soil samples. DNA isolation was performed according to the manufacturer's instruction, and modified as follows: completely grinding dried soils/sediments prior to the Kit procedure and adjusting the incubation time. During the extraction steps of the Kit, humic acid, proteins, polysaccharides, and other contaminants in soils/sediments were completely removed. Thus, the obtained DNA extract was suitable for PCR and other downstream applications without the need of further purification, and was checked by 0.8% agarose gel electrophoresis.

PCR strategies

Bacterial 16S rRNA genes were amplified by six PCR strategies with total DNA prepared from soil samples sA and sB as templates. Three sets of general bacterial primers were used in different PCR strategies, respectively, and their names, sequences and theoretical amplicon lengths are shown in Table 1. PCR for 16S rRNA gene was carried out in a 50 µl reaction mixture containing 1 µl of DNA template, 1 µl of 2.5 U μl^{-1} Super *Taq* DNA polymerase, 1 µl of dNTPs (10 mM), 2 µl of each primer (10 µM), 25 µl of 2 × HG PCR buffer, and 18 µl of DNA free grade water. PCR Program A was initially pre-heated at 95°C for 5 min for initial denaturation before 30 cycles composed of denaturation at 94°C for 1 min, annealing 1 min at 55°C, and elongation at 72°C for 1 min, and the final extension of PCR reactions was carried out at 72°C for 10 min. PCR Program B was initially pre-heated at 95°C for 5 min for initial denaturation before 30 cycles of amplification, which consisted of two steps. Step 1 was 10 touchdown cycles, which were composed of denaturation at 94°C for 1 min, annealing 1 min at temperature from 64 to 55°C with a 1°C decrement at each cycle, and elongation at 72°C for 1 min. Step 2 was carried out at the same conditions of PCR Program A amended with 20 cycles and without pre-denaturation.

With a combination of different primer sets and PCR programs, the impacts of six PCR strategies on DGGE analysis were assessed as follows:

Strategy 1: 16S rRNA gene in the DNA extraction was amplified by PCR Program A with primer set 1, and the obtained PCR products were used to DGGE analysis directly.

Strategy 2: The PCR products from Strategy 1 were concentrated to be three-fold, and then used to perform DGGE analysis. The concentration procedures include: addition of the 2-fold volume of absolute ethanol to 60 µl of PCR products obtained by Strategy 1, incubated at -20°C over night, precipitated DNA by centrifuging at 13,000 rpm for 10 min at 4°C, discarded the supernatant and add 20 µl of DNA free grade water to dissolve the DNA pellet.

Strategy 3: The 16S rRNA gene in the DNA extraction was firstly amplified by PCR Program B with primer set 3, and then 1 µl of the obtained PCR products was used as the template for PCR amplification with Program A and primer set 1. The PCR products

Table 1. Sequences and theoretical amplicon lengths of the general bacterial primer sets.

Primer set	Bacterial primer	Sequence (5'-3')	Amplicon length (bp)	Reference
1	341f⊥GC[a]	CCTACGGGAGGCAGCAG	234	Ellis et al. (2003)
	534r	ATTACCGCGGCTGCTGG		
2	27f	AGAGTTTGATCCTGGCTCAG	919	Marchesi et al. (1998)
	907r	CCGTCAATTCMTTTGAGTTT		Sanchez et al. (2007)
3	341f	CCTACGGGAGGCAGCAG	194	Ellis et al. (2003)
	534r	ATTACCGCGGCTGCTGG		

[a] Primer with a 40-bp clamp (CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCACGGGGG G) at the 5' end in order to increase the separation of DNA bands in DGGE analysis.

eventually obtained were used to perform DGGE analysis.

Strategy 4: The 16S rRNA gene in the DNA extraction was firstly amplified by PCR Program B with primer set 2, and then 1 µl of the obtained PCR products was used as the template for PCR amplification with Program A and primer set 1. The PCR products eventually obtained were used to perform DGGE analysis.

Strategy 5: The steps were the same as those of Strategy 4 except the template for PCR amplification with Program A, which was replaced by the PCR products obtained from PCR Program B in a 10-fold diluted concentration.

Strategy 6: The steps were the same as those of Strategy 4 except the template for PCR amplification with Program A, which was replaced by the purified PCR products obtained from PCR Program B. The purification was performed according to the manufacturer's instructions of TaKaRa MiniBEST Agarose Gel DNA Extraction Kit.

Denaturing gradient gel electrophoresis

18 µl of PCR products was loaded onto 8% (wt/v) polyacrylamide gels (40% acrylamide/bis solution, 37.5:1) with a 40 to 70% denaturant-gradient [100% denaturant contained 7 M urea, 40% (v/v) deionized formamide] in 1 × TAE buffer [40 mM Tris base, 20 mM acetate, 1.0 mM Na2-EDTA (pH 8.0)], and electrophoresis was run at 80 V for 17 h at 60°C. After electrophoresis, the gels were stained for 40 min in 1 × TAE buffer with SYBR gold (1:10,000 dilution) and rinsed with DNA free grade water, and photographed by UV transillumination.

DGGE patterns and statistical analyses

The diversity and similarity between the different PCR strategies for the soil samples were calculated according to the DGGE profiles. The DGGE patterns were also compared by clustering the different lanes with Quantity One software package 4.6.2 (Bio-Rad).

Species Richness (*R*) represents species numbers. In this study, *R* was calculated as band numbers shown in the DGGE profiles with a correction for crowded and obscure bands.

Dice similarity was assessed by band numbers and their positions, calculated as follows:

$$S_D = (2n_{AB}) / (n_A + n_B)$$

Where n_A and n_B represent the band numbers in lanes A and B, respectively; and n_{AB} represents the band numbers common to both lanes.

Shannon-Weaver diversity index (*H*) was used to determine the bacterial diversity, calculated as follows:

$$H = -\sum(P_i)(\log_2 P_i)$$

$$P_i = n_i/N$$

Where N represents the total species number in soil samples; n_i represents the number of species *i*; P_i is the proportion of species *i* in soil samples.

Index *H* may be used to evaluate the bacterial diversity by incorporating both the richness and distribution of types (Shannon, 1997). In this study, *H* was calculated on the basis of the intensities and band numbers shown in the DGGE profiles by Quantity One software package 4.6.2.

RESULTS

Total DNA extraction

As shown in Figure 1, a well-defined band of approximate 15 kb exhibited in the 0.8% agarose gel in lanes 1 to 4, which indicated the total DNA was successfully extracted from soil samples sA and sB, and suitable for the following PCR amplification. In addition, the target DNA band in lanes 3 and 4 was more luminous than that in lanes 1 and 2, indicating that the total DNA yield of sample sB was higher than that of sample sA.

16S rRNA gene-based PCR products

The results of 16S rRNA gene-based PCR with five strategies are shown in Figures 2a and b. The DNA extractions from samples sA and sB were used as the template for all PCR strategies. As shown in Figure 2a, the 16S rRNA gene fragment of approximate 200 bp was obtained by all PCR strategies. However, the 16S rRNA gene band obtained by Strategy 1 was weaker, indicating that Strategy 1 had a lower yield of PCR products. Moreover, a non-specific DNA amplification fragment of approximate 1,000 bp could also be obtained by Strategy

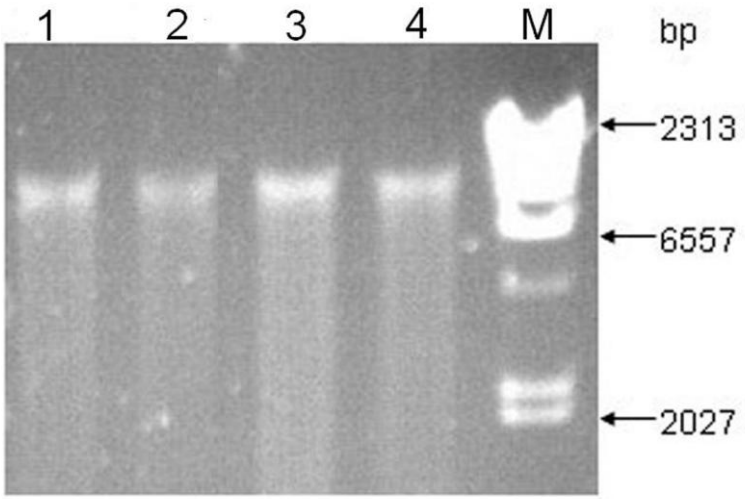

Figure 1. Total DNA extracted from two soil samples of sA and sB. M, λ-Hind III digest DNA marker; 1 and 2 are the duplicates of soil sample sA; 3 and 4 are the duplicates of soil sample sB.

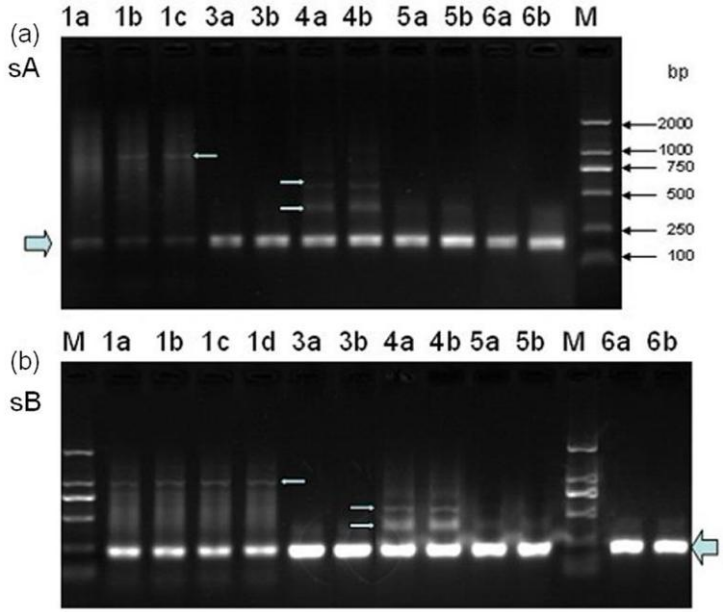

Figure 2. PCR products obtained from different PCR strategies. M, DL2000 DNA marker; 1, 3, 4, 5 and 6 represent the lanes with PCR-amplified strategies 1, 3, 4, 5 and 6, respectively; a, b, c, and d represent the replicates of each PCR-amplified strategy; and arrows indicate DNA bands.

1. The 16S rRNA gene band obtained by Strategy 4 was more luminous than that obtained by Strategy 1, but there were two non-specific DNA amplification fragments of approximate 400 and 500 bp. Strategies 3, 5 and 6 not only had higher yields of 16S rRNA gene PCR products, but also no non-specific DNA amplification fragments. In Figure 2b, the PCR products had shared the same features with those showing in Figure 2a.

DGGE profiles of 16S rRNA gene-based PCR products

DGGE profiles with the samples prepared by different PCR strategies showed that each strategy gave an autologous pattern (Figure 3). For soil sample sA, a dominant band 12 were common for all lanes with different PCR-amplified strategy. Band 13 with Strategy 1

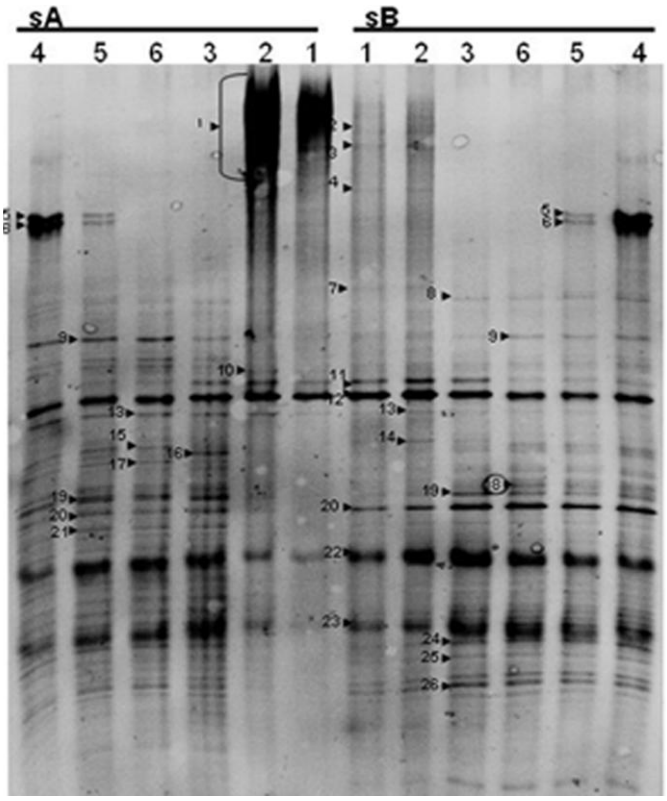

Figure 3. DGGE profiles of PCR 16S rRNA gene fragments from two soil samples. 1, 2, 3, 4, 5 and 6 represent the lanes with PCR-amplified strategies 1, 2, 3, 4, 5 and 6 respectively; and arrows indicate DNA bands.

Table 2. Diversity index (*H*) and Richness (*R*) calculated according to the digitized DGGE patterns from Figure 3.

PCR strategy		One-step PCR approach		Two-step PCR approach	Nested PCR approach		
		Strategy 1	Strategy 2	Strategy 3	Strategy 4	Strategy 5	Strategy 6
H	sA	2.25	2.79	5.05	5.09	5.06	4.92
	sB	4.19	4.28	4.62	4.93	4.78	4.67
R	sA	5	7	34	35	34	31
	sB	19	20	26	32	29	27

was faint, but its intensity was enhanced by other strategies. Compared with other strategies, Strategies 1 and 2 had fewer bands, and appeared a region with the strong intensity and fuzzy image (as arrow 1 indicated) in DGGE profiles. The results indicated that Strategies 1 and 2 were two inefficient methods for 16S rDNA amplification. For soil sample sB, dominant bands 12 and 20 were common for all lanes, but their intensities were enhanced by Strategies 3 to 6. In addition, bands 19, 24, 25 and 26 with Strategies 3 to 6 were brighter than those with Strategies 1 and 2. The results indicated that Strategies 1 and 2 owned their lower amplification efficiency. Bands 2, 3, 4 and 7 only appeared with Strategies 1 and 2, indicating that these bands were non-specific DNA amplification fragments. For samples sA and sB, bands 5 and 6 were strong bright with Strategy 4, while they were faint with Strategy 5, and absent with other strategies. The results indicated that Strategy 4 could produce non-specific DNA amplification fragments. Finally, bands 22 and 23 showed abnormal patterns in DGGE profiles, which may imply a crowd with many bands or something with abnormal products.

Analysis of bacterial communities

Diversity index (*H*) and richness index (*R*) of bacterial communities calculated according to the DGGE profiles in Figure 3 are shown in Table 2. Compared to Strategies

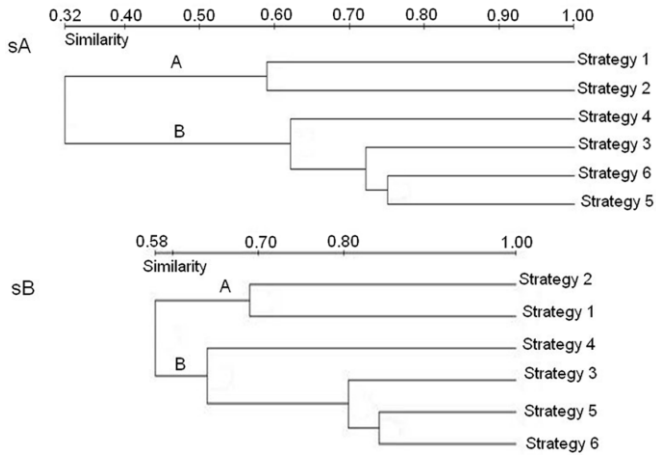

Figure 4. Dendrogram of the DGGE profiles from Figure. 3 clustering by the UPGAMA on the basis of the Dice similarity by the Quantity One software package 4.6.2.

1 and 2, Strategies 3 to 6 could obtain the higher values of *H* and *R* in soil samples sA and sB, indicating that Strategies 1 and 2 may underestimate the bacterial community diversity, and lead to DGGE analysis inaccurate. Although Strategy 4 has the highest values of *H* and *R*, it was still not suitable for 16S rDNA amplification of bacterial communities in the NJOF soils/sediments before DGGE analysis due to its false positive caused by non-specific DNA amplification.

The Unweighted pair-group method with arithmetic means (UPGMA) analysis of the DGGE patterns for six PCR-amplified strategies showed two distinct clusters in soil samples sA and sB (Figure 4). The patterns of Strategies 1 and 2 formed Cluster A, and the patterns of Strategies 3 to 6 formed Cluster B in two soil samples. Clusters A and B only shared 32% of the similarity in soil sample sA, while 58% in soil sample sB. For both sA and sB, their patterns with Strategy 4 formed a distinct sub-cluster separated from a joint sub-cluster with the patterns with Strategies 3, 5 and 6. The analysis also revealed that the patterns were more similar between Strategies 5 and 6, and the other patterns were more different in two soil samples.

We optimized the dilution steps of Strategy 5 for DGGE analysis, and obtained the very useful information of bacterial communities in NJOF soil samples. Figure 5 exhibited the DGGE pattern of PCR-amplified 16S rRNA gene fragments from NJOF soils/sediments by PCR-amplified Strategy 5 with 20-fold dilution, and the interested bands were excised from DGGE gel for sequencing to identify their phylotypes (Figure 5).

DISCUSSION

Strategies 1 and 2 belong to one-step PCR approach. In this study, we found that Strategy 1 yielded the lower diversity relative to that with Strategies 3 to 6. The reason may be attributed to that of the GC clamp influenced by the primer binding energy, and the yield of some specific products was too low to be detected on DGGE gels. In addition, Strategy 1 could produce non-specific DNA amplification bands, which could lead to the inaccuracy of bacterial community diversity. Although the concentration step (Strategy 2) enhanced the intensity of bands, it seemed to be not helpful for improving the bacterial diversity in DGGE profiles, which provided further evidence that the one-step PCR approach with primer set 341f⊥GC/534r was the low efficiency of 16S rRNA gene amplification, and could not eliminate non-specific DNA amplification bands. Therefore, the one-step PCR approach was failing to achieve an accurate assessment of bacterial diversity in the NJOF soils/sediments, and was not suitable for 16S rRNA gene amplification of bacterial communities before DGGE analysis.

It was reported that the nested PCR approach could enhance the yield of PCR products to reflect more accurate information of bacterial community diversity due to its trace DNA template and could be amplified successfully (Shabir et al., 2005). In this study, the nested PCR method (Strategies 4, 5 and 6) was tried to prepare the samples for DGGE analysis. DGGE analysis of Strategy 4 indicated that it owned the highest community diversity, but was not suitable for 16S rRNA gene amplification of bacterial communities in the NJOF soils/sediments due to its non-specific DNA amplification. Strategy 5 could eliminate the non-specific DNA amplification products by the dilution procedure of a PCR template, indicating that a suitable dilution was useful for DGGE analysis. Yet the dilution procedure should be performed carefully, because diluting DNA may make its reproducibility worse (Chandler et al., 1997). Strategy 6 was a reliable method that provided reasonable

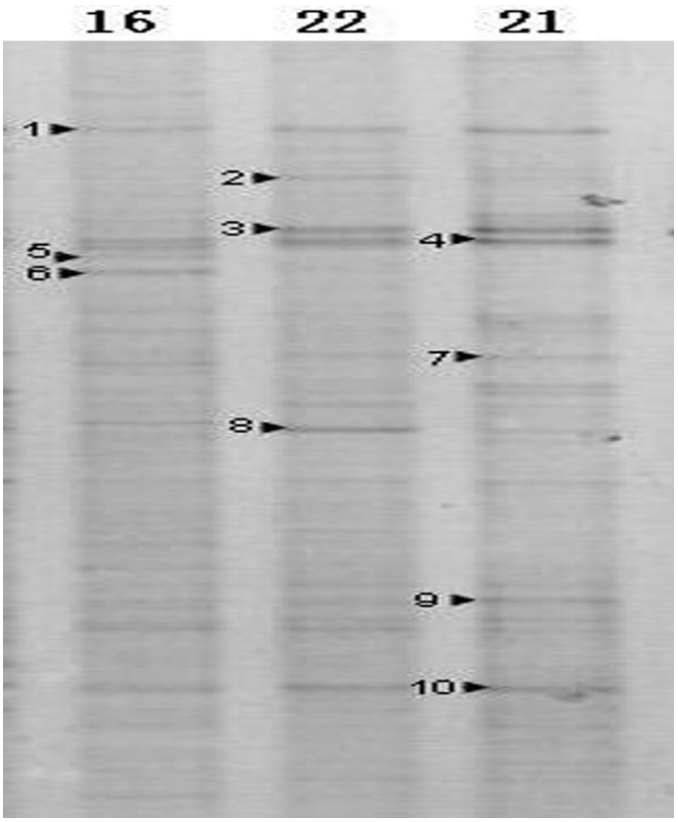

Figure 5. DGGE profiles of PCR-amplified 16S rRNA gene fragments from soils/sediments by PCR-amplified Strategy 5 with an improvement of 20-fold dilution. 16, 22 and 21 represent the sample numbers, and the three samples were randomly selected from the study area in NJOF. Isolates: 1, uncultured Myxococcales bacterium; 2, uncultured Caldilineaceae bacterium; 3, *Escherichia coli* W; 4, unknown bacterium; 5, uncultured *Desulfosporosinus* sp.; 6, uncultured Chloroflexi bacterium; 7, uncultured Acidobacteriales bacterium; 8, uncultured Anaerolineae bacterium; 9, uncultured Nitrospirae bacterium; and 10, uncultured delta-proteobacterium.

information on bacterial diversity, because its band patterns, to a great extent, are reproducible in Strategies 3 to 5. However, it should be taken into account that Strategy 6 including a purification step, seemed to be time-consuming and uneconomical for high throughput DGGE analysis. The cluster analysis revealed that the patterns were more similar between Strategies 5 and 6. Therefore, Strategies 5 and 6 were suitable to perform DGGE analysis for a great number of or a few samples, respectively.

To improve the yield of PCR products and reduce non-specific DNA amplification fragments, one two-step PCR approach (Strategy 3) was also tried to prepare samples for DGGE analysis. The two-step PCR approach (Strategy 3) was rarely performed in previous studies, and regarded as that it was not a strict method because the two-step PCR with the same primer set would enhance the potential of PCR bias (Sigler et al., 2004). However, in this study, the DGGE profiles of the two-step PCR approach showed to significantly reduce non-specific DNA amplification and shared more than 80% of the similarity with Strategy 6 in soil sample sB, implying that the two-step PCR approach sometimes is very useful for DGGE analysis due to its simple process.

It has been reported that the potential of PCR bias and artifact formation increased in the two-step and nested PCR approaches, and provided incorrect information on the abundance and diversity of microorganisms (Kanagawa, 2003). Due to the differences of primer binding energy (Polz and Cavanaugh, 1998), it led to an inexact population ratio in samples. In this study, bands 10 and 11 (Figure 3) had a bias of primer set 3, whereas band 9 had a bias of primer set 2. In addition, heteroduplex (Thompson et al., 2002), chimera (Judo et al., 1998) and the sequence error derived from amplification might produce the extra bands in DGGE

patterns to result in an overestimation of bacterial community diversity. Previous studies indicated that the PCR bias and artifact formation could be controlled and eliminated by optimization of PCR conditions (Kanagawa, 2003; Lenz and Becker, 2008; Smyth et al., 2010; Thompson et al., 2002). The optimization included many aspects such as primers, cycles, DNA polymerase, annealing temperature, elongation time and other factors in PCR conditions. Duplicate tests are also important, since the PCR bias and artifact formation occur by accident, neither of them is reproducible (Kanagawa, 2003). Therefore, the PCR conditions should be further optimized when the best strategy is chosen. In this study, we have successfully obtained the useful information on bacterial communities in different NJOF soils/sediments by PCR-amplified Strategies 5 with an improvement of 20-fold dilution.

Conclusions

In this study, the statistical analyses of DGGE profiles showed that different PCR-amplified strategies affected the diversity of bacterial communities in the NJOF soils/sediments. Strategies 1 and 2 achieved a low diversity for ignoring weak bacterial communities, and the non-specific DNA amplification products resulted in inaccurate information of bacterial communities in DGGE profiles. Strategy 4 was not suitable for 16S rRNA gene amplification of bacterial communities in the NJOF soils/sediments due to its non-specific DNA amplification. Strategies 3 and 5 may be recommended as two methods for high throughput DGGE analysis. Strategy 6 was considered to be a reliable method that provided reasonable information on bacterial diversity the NJOF soils/sediments, but it seemed to be time-consuming and uneconomical for high throughput analysis, and suitable for DGGE analysis of a small amount of samples.

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