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Comparisons of different hypervariable regions of *rrs* genes for fingerprinting of microbial communities in paddy soils

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ABSTRACT

The use of molecular approaches based on 16S rDNA-PCR in microbial ecology has revealed a tremendous prokaryotic diversity in environmental samples. However, there is little or no systematic evaluation of the impacts of hypervariable (V) regions of *rrs* genes choice on microbial community analysis in soil samples, especially the detailed information about the dominant groups preferentially amplified by different primer pairs. In the present study, eight primer pairs were detected to compare the different V regions for fingerprinting microbial communities in a paddy soil irrigated with petroleum-wastewater, using denaturing gradient gel electrophoresis (DGGE) and amplified ribosomal DNA restriction analysis (ARDRA) techniques. Results reveal the obvious PCR bias produced by different V regions. Both ARDRA analysis of 16S rDNA clone library and DGGE suggest that V_1–V_3 region amplified with primer pair 8f–519r produced the most informative fingerprinting profiles. Additionally, V_3–V_5 region amplified with 341f–907r was another preferable choice for microbial diversity in petroleum-contaminated soil. The V_4–V_5 region and single V region (V_1, V_3, and V_8) were not recommended for the future study of microbial diversity in soil samples. Phylogenetic analysis of 123 sequences from libraries constructed by amplicons generated from six different V regions suggests that different dominant groups were amplified with distinct primer sets. In detail, V_1–V_3 library (amplified with 8f–519r) and V_3–V_5 library were dominated by Actinobacteria (20.4%) (particularly in genus *Arthrobacter*), V_1–V_3 library (amplified with 63f–518r) was dominated by γ-Proteobacteria (25.0%) and α-Proteobacteria (22.0%) (particularly in genus *Brevundimonas*), V_3 library was dominated by β-Proteobacteria (22.3%) (particularly in genus *Gallionella*) and α-Proteobacteria (20.0%), V_6–V_8 library was dominated by Chlamydiae (20.4%) and β-Proteobacteria (20.4%), V_8 library was dominated by γ-Proteobacteria (27.2%) (particularly in genus *Acinetobacter*) and β-Proteobacteria (14.0%). The present work strongly recommends that primer pairs should be chosen cautiously in community diversity analysis based on PCR amplification of 16S rDNA, and involving at least two different 16S rDNA universal primer pairs would perform better.

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1. Introduction

Before the development of molecular techniques for estimating genetic diversity, studies on microbial community structure and diversity were restricted to cultivation-based methods, covering only the culturable fraction of the total bacterial population capable of forming colonies on a solid media in laboratory. Such an obvious limitation commonly causes underestimation of the population diversity present in natural environment (Torsvik et al., 1990; Amann et al., 1995). Recent advances in culture-independent molecular techniques have been applied to soil ecosystems, to enable researchers to study microbial diversity at the molecular level. The 16S rDNA genes are often used for such studies since these genes are present in all bacteria. The gene contains both conserved regions which can be used as primer for PCR amplification, and variable regions which can be used to distinguish sequences from each other (Ward et al., 1990). The PCR-amplified 16S rDNA segments could be further analyzed by some fingerprinting techniques, such as denaturing gradient gel electrophoresis (DGGE) (Muyzer et al., 1993; Li et al., 2005), temperature gradient gel electrophoresis (TGGE) (Brim et al., 1999; Cheung and Kinkle, 2001), terminal restriction fragment length polymorphism (T-RFLP) (Liu et al., 1997; Marsh, 1999), and amplified ribosomal DNA restriction analysis (ARDRA) (Martínez-Murcia et al., 1995; Smit et al., 1997). Among these methods, DGGE and ARDRA are now widely used and reported in microbial diversity studies. DGGE appears to be one of the best molecular community fingerprinting techniques in terms of predicting the actual Shannon–Wiener diversity index, richness, and evenness. The advantage of ARDRA is that the statistical information of community composition can be

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investigated by restriction analysis of the cloned 16S rDNA genes, and interesting clones can be sequenced.

However, in most of such analyses, 16S rDNA genes were amplified with only one primer set, either a single hypervariable (V) region or a combination of two or three V regions in *rrs* genes (Ferris et al., 1996; Röling et al., 2002; Andreoni et al., 2004; Lyautey et al., 2005; Gelsomino et al., 2006), and few authors have explained their justification of primer choice. In fact, a few researchers have noticed that PCR amplification of 16S rDNA is highly biased. Results described by Cocolin et al. (2001) showed that only the 41f–130r primer set gave PCR products that allowed differentiation by DGGE in their study of bacterial community in Italian sausages. Hansen et al. (1998) concluded that the PCR bias was dependent on the position of the primer sites, and suggested that community diversity analysis based on PCR amplification of 16S rDNA should involve in at least two different primer sets. The other researchers (Siciliano et al., 2000) also highlighted the importance of multiple primer pairs in 16S rDNA-DGGE analysis. Yu and Morrison (2004) compared the different hypervariable regions of *rrs* genes for fingerprinting of microbial communities in rumen and gastrointestinal by PCR–DGGE analysis, results showed that the PCR–DGGE profiles produced from the same community DNA samples were substantially different in terms of the number, resolution, and relative intensity of the bands.

In our previous work, the bacterial diversities in Shenfu irrigation area were evaluated by 16S rDNA–PCR–DGGE analysis. The Shenfu irrigation area is the largest petroleum wastewater irrigation area in northern China. The up- and mid-stream of the Shenfu irrigation area has been irrigated by the wastewater since 1940s (Li et al., 2005). Bacterial diversity analysis was performed with two different primer sets (341f–518r and 8f–519r), targeting the V_3 region and the V_1–V_3 region of *rrs* genes, respectively (Li et al., 2005, 2006). Results showed that the fingerprinting profiles produced from these two hypervariable regions were different in band number, band intensity, even the correlation between bacterial Shannon–Wiener diversity index (H') and total petroleum hydrocarbons (TPHs). The H' based on V_3 region showed a positive correlation with TPHs content, while the H' based on V_1–V_3 region showed a negative correlation with TPHs content.

Based on the facts described above, it is necessary to obtain a more fundamental understanding of the impacts of different hypervariable regions choice on community diversity analysis using 16S rDNA–PCR–based methods. Although this problem has received much attention, indeed, there is little or no systematic evaluation of how the choice of primer sets influence data quality and veracity on microbial community analysis in soil samples. In the present study, eight regions in *rrs* gene previously widely used for analysis of soil microbial diversity, have all been examined to compare the different hypervariable regions for fingerprinting of microbial communities in paddy soil irrigated by petroleum-wastewater in the Shenfu irrigation area. The results derived from this study will provide valuable references for soil microbial researchers in such kind of analysis.

2. Materials and methods

2.1. Site description and soil sampling

Soil samples were collected from the paddy fields in the Shenfu wastewater irrigation area that is located in the suburban area between Fushun and Shenyang city, Liaoning province, China. It is a dream site for microbial ecology study due to the fact that, different sites from up- to down-stream were under different irrigation history, for example, some paddy fields were changed to be irrigated by ground water at different irrigation regimes. Sampling sites differing in their relative position and irrigation history were selected. In the following text, the 11 sites are referred as soils **A**–**K** (Fig. 1). Top soil (0–20 cm) from each site was collected using a stainless steel auger. Five individual soil cores from each site were mixed completely for analysis. The soil samples were placed in airtight bags and stored on ice before being transported to the laboratory. Subsequently, all samples were kept at 4 °C until to be analyzed.

Physical and chemical properties of the studied soils were presented in Table 1. Soil moisture contents were determined by drying the soils at 110 °C for 48 h. Particle size analyses were performed by a modified hydrometer method, in which the clay content was determined after 8 h (Day, 1965). Soil pH was determined in a 1:5 ratio of soil to water. Organic carbon content was determined by the Walkley–Black method using $FeSO_4$ for titration (Nelson and Sommers, 1982).

2.2. Determination of total petroleum hydrocarbons and polycyclic aromatic hydrocarbons in petroleum-contaminated soils

Total petroleum hydrocarbons (TPHs) in 10 g of anhydrous sodium sulfate-dried soil were consecutively extracted with hexane, dichloromethane and chloroform (20 ml each) in an ultrasonic bath as described before (Li et al., 2005), and was determined gravimetrically.

Polycyclic aromatic hydrocarbons (PAHs) in 10 g of anhydrous sodium sulfate-dried soil were extracted with two batches of dichloromethane (20 ml each) by ultrasonication (20 °C, 20 min), followed by centrifugation for 10 min at 3500 rpm (Oleszczuk, 2006). The combined supernatants were concentrated to less than 1 ml under a gentle nitrogen stream in a fume hood. The residue was purified by 2 g of silica gel column with 10 ml 1:1 (v/v) elution of hexane and dichloromethane. The effluents were then evaporated to dryness, and dissolved in 1 ml methanol. All methanol extracts were filtered prior to analysis with 0.22-μm Teflon syringe

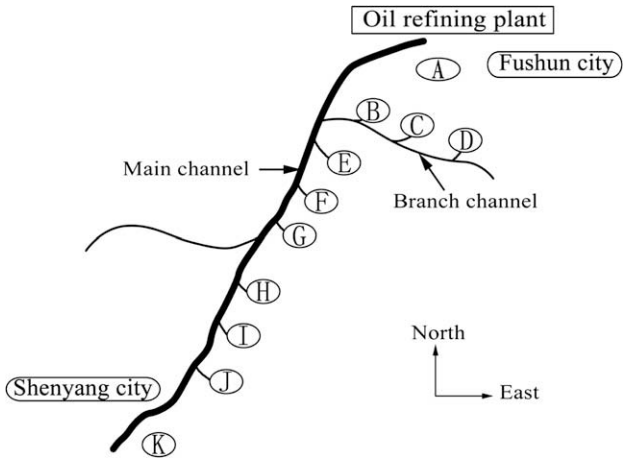

Fig. 1. Distribution and irrigated history of soil sampling sites in the Shenfu irrigation area. Site **A:** located at the up-stream and was changed to be irrigated by ground water for more than 30 years; site **B:** located at the third plot of branch channel in up-stream and has been irrigated by wastewater until present; site **C:** located at the sixth plot of branch channel in up-stream and has been irrigated by wastewater until present; site **D:** located at the ninth plot of branch channel in up-stream and has been irrigated by wastewater until present; site **E:** located at the upper reach of mid-stream and has been irrigated by wastewater until present; site **F:** located at the middle reach of mid-stream and has been irrigated by wastewater until present; site **G:** located at the lower reach of mid-stream and has been irrigated by wastewater until present; site **H:** located at the upper reach of down-stream and was changed to be irrigated by ground water 10 years ago; site **I:** located at the middle reach of down-stream and has been irrigated by wastewater until present; site **J:** located at the lower reach of down-stream and was changed to be irrigated by ground water 10 years ago; site **K:** located at the end of the channel and was changed to be irrigated by ground water 20 years ago.

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