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Diversity surveys of soil bacterial community by cultivation – based methods and molecular fingerprinting techniques

LUO Hai-feng, QI Hong-yan*, ZHANG Hong-xun

(Department of Environ-Biotechnology, Research Center for Eco-Environmental Sciences, Chinese Academy of Sciences, Beijing 100085, China. E-mail: qihy@mail.rcees.ac.cn)

Abstract: By combining the cultivation methods with molecular fingerprinting techniques, the diversity surveys of soil bacterial community in 13 areas of China were carried out. The cultivable heterotrophic diversity was investigated by colony morphology on solid LB medium. Genetic diversity was measured as bands on denaturing gradient gel electrophoresis(DGGE) by the extraction and purification of the total soil DNA, and amplification of bacterial 16S rDNA fragments by polymerase chain reaction(PCR). The Shannon-Wiener indices of diversity(H), richness(S) and evenness(E_H) were employed to estimate the diversity of soil bacterial community. The results showed that there was an obvious diversification existed in soil from the different areas. However, the genetic diversity estimated by PCR-DGGE can provide more comprehensive information on bacterial community than the cultivation-based methods. Therefore, it is suggested to combine the traditional methods with genetic fingerprinting techniques to survey and estimate soil bacterial diversity.

Keywords: bacterial community; colony morphology; DGGE; diversity

Introduction

Diversity surveys of soil bacterial community by cultivation methods have severe limitations, since the majority of microbes observed microscopically in an environmental sample cannot be cultivated by standard methods. It was proved that only about 1% of total number of actively respiring bacterial cells can be cultured on laboratory media. In addition, less than 1% bacterial species have been isolated and characterized from soil samples (Ward, 1992; Amann, 1995).

The development of rapid and effective methods for recovery of DNA, directly from soil samples without prior cultivation (Torsvik, 1990; Tsai, 1992; Picard, 1992; Zhou, 1996) along with the use of genetic makers, of which the 16S rRNA genes (rDNA) are the most commonly used (Myuzer, 1993), have resulted in the development of a vast number of methods. These methods continuously try to circumvent the limitations of cultivation-based investigations by genetic fingerprinting of the microbial community (Frank, 1998; James, 1999; Muller, 2002). One such method, denaturing gradient gel electrophoresis (DGGE) that was primarily used to study a microbial community in 1993 (Myuzer, 1993) has become more and more popular, and has been widely used in recent years (Kowalchuk, 2002; Sigler, 2002).

Our work was designed to assess the genetic diversity and the diversity of the cultivable heterotrophic fraction (in the following referred to as heterotrophic diversity) of the soil bacterial communities in different areas of China. Genetic diversity was analyzed by total DNA extraction, PCR amplification of 16S rDNA fragments followed by separation of DGGE. Two approaches were taken to measure the diversity of heterotrophic population: (1) colony shape, size, color, etc.; and (2) colony appearance where isolates are grouped according the time of appearance on solid media. By comparing the diversity results obtained with different methods, the advantages of genetic fingerprinting techniques will be evaluated and the improvement of research methods of soil microbial ecology will be suggested.

1 Materials and methods

1.1 Soil samples and their physicochemical characteristics

Thirteen soil samples were obtained from different regions of China in February 2003. All the soils were collected between 2 and 20 cm in depth in the different farmlands. Their physicochemical properties are shown in Table 1.

1.2 Morphological examination of the cultivable bacteria

100 μl of appropriate soil dilutions were spread on agar plates and incubated at 25℃ for 14 d. Visible colonies were enumerated and marked daily throughout the incubation period. The bacterial colonies were grouped into classes according to the described method (De Leij, 1994). For typing of colony morphology, the colonies were grouped into morph types on the basis of visual differences such as colony color, diameter, edge, surface and other special characteristics. All morphological examinations were made on

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three replicate samples of each soil.

Table 1 Physicochemical characteristics of soils used in this study

Sample sites[a] (province/city/county)	Soil types[b]	Water, %	pH	Organic matter, %	Total N, %
Xinjiang/Wulumuqi	Vegetable	7.41	7.75	3.46	0.150
Hebei/Tang County	Wheat	16.78	8.21	0.69	0.050
Shaanxi/Fengxiang	Wheat	15.27	8.02	1.53	0.094
Zhejiang/Zhuji	Vegetable	19.92	6.16	1.28	0.069
Guizhou/Guiyang	Corn	27.17	6.95	4.16	0.157
Sichuan/Jiangan	Vegetable	24.49	6.34	1.99	0.120
Liaoning/Shenyang	Corn	18.56	7.52	2.71	0.110
Inner Mongolia/Xilinhaote	Turf grass	6.12	9.50	0.57	0.016
Qinghai/Xining	Wheat	2.54	8.39	0.24	0.030
Henan/Anyang	Wheat	12.71	6.80	0.76	0.048
Yunnan/Yun County	Rice	25.07	5.39	1.97	0.055
Fujian/Liancheng	Rice	20.58	6.42	2.98	0.132
Guangdong/Puning	Rice	21.66	5.65	2.46	0.082

Notes: a. the sample sites are abbreviated in the following tables, figures and statements, for example, XJ represents "Xinjiang" and so on; b. soil types are classified by the crops being planted or once planted in these soils in this study

1.3 Soil DNA extraction, purification and PCR-DGGE

A previous published DNA extraction method (Zhou, 1996) was used to extract the DNA of soil samples in this study. Silver Bead DNA Gel Recovery Kit (manufactured by Shanghai Sangon Co. Ltd.) was used to purify the crude DNA of soil samples. Primers (F_{357}GC and R_{518}) were used to amplify the bacterial 16S rRNA genes (16S rDNA fragments which are about 230 bp). The sequences of primers were as follows: F_{357} GC 5′-CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGG GCC TAC GGG AGG CAG CAG -3′ ; R_{518} 5′-ATT ACC GCG GCT GCT GG-3. The PCR temperature cycling conditions were as previously described (Myuzer, 1993). The DGGE was carried out using a DCode™ Universal Detection System according to the manufacture's instructions (Bio-Rad). The wells were loaded with equal amounts of DNA, and electrophoresis was performed with 10% (wt/vol) polyacrylamide (acrylamide-bisacrylamide, 37.5:1) and with a linear gradient of the denaturants urea and formamide from 30% to 50% at 60℃ for 5 h at 120 V. The gels were stained in an ethidium bromide solution and photographed.

1.4 DGGE band images analysis

After visualized and photographed, DGGE band patterns and average intensities of individual bands were analyzed using Quantity One image analysis software, Version 4.0 (BioRad laboratories). The software performed information concerning each ban's position and average intensity relative to the cumulative intensity value of all bands in the given lane. A band of DNA was detected if it accounted for greater than 0.2% of the total lane intensity.

1.5 Diversity of colony-forming population and genetic diversity

When analyzing of cultivable heterotrophic diversity and genetic diversity, richness (S), Shannon-Wiener (S-W) indices (H) and evenness (E_H) were used according to the following equations.

$$H = \sum_{i=1}^{s} p_i \ln p_i = -\sum_{i=1}^{s} (N_i/N)\ln(N_i/N);$$

$$E_H = H/H_{max} = H/\ln S,$$

where p_i is the ratio of the number in a specific group to the total number, S is the total number of morph types in cultivable heterotrophic diversity and p_i is the ratio of the specific band intensity to the total intensity of all bands, S is the total number of bands in each samples in genetic diversity. Additionally, the similarity coefficient (S_{xy}) was used to evaluate the similarity between any soil x and soil y in genetic diversity.

$$S_{xy} = N_{xy}/(N_x + N_y).$$

Here N_{xy} is the number of common bands in DGGE images between soil x and soil y, N_x is the number of bands in soil x and N_y is the number of bands in soil y.

2 Results and discussion

2.1 Enumeration of total colony-forming units (CFU)

The number of heterotrophic bacteria capable of forming colonies on solid medium was enumerated on LB agar plates. As it is shown in Fig.1, the total number of bacteria was much changed with sample sites, from 1.9 to 3.8×10^7 CFU g^{-1} soil. Fig.1 also indicated that the change tendency of fast-growing colonies appeared in first 2 d coincided with that of the total CFU.

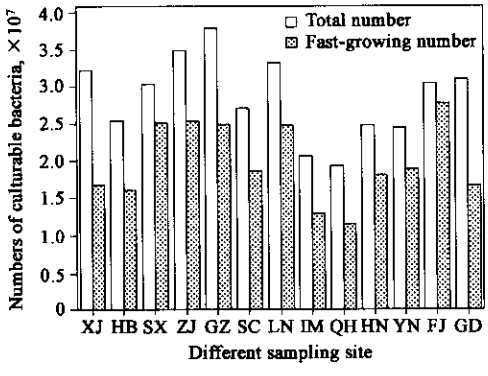

Fig.1 Total number of CFU during a 14-d period and the fast-growing colonies on agar plates of each soil

Fig.2 gave a colony appearance on agar plates each day during a 14-d period expressed in the percentage of the total CFU number, which revealed that most percentage of colonies appeared at the beginning of plating period in all soil samples, ten of them appeared at the first day, other three at the second day.

2.2 Diversity analysis of CFU

The diversity of cultivable heterotrophic fraction of the bacteria communities was examined by grouping the isolates according to colony morphology on the solid LB media.

Diversity analysis based on colony morphology (Table 2) showed that the types of cultivable heterotrophic bacteria in

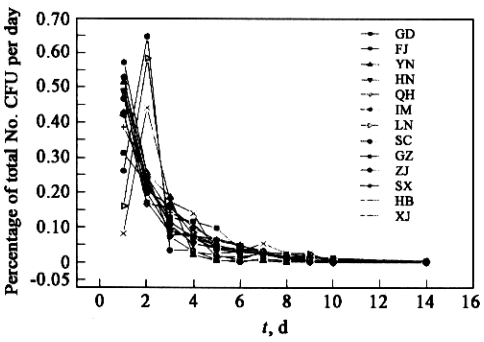

Fig.2 Colony appearance on agar plates each day during a 14-d period expressed in the percentage of the total CFU number

the soils of different regions in China had a great difference, the most abundant community among them was from Zhejiang soil, its value of richness reach from 8.1 to 9.3, while the lean soil was in Qinghai, which only changed from 1.7 to 2.9.

Table 2 Shannon-Wiener index (H), richness (S), and evenness (E_H) [mean ± *SEM*] of each soil samples estimated by the morph types of random 100 clones on agar plates

Soil	Shannon-Wiener index (H)	Richness[a] (S)	Evenness (E_H)
XJ	0.72 ± 0.11	6.7 ± 0.6	0.38 ± 0.02
HB	1.18 ± 0.14	4.7 ± 0.6	0.76 ± 0.07
SX	1.01 ± 0.09	6.0 ± 0.1	0.56 ± 0.03
ZJ	1.44 ± 0.11	8.7 ± 0.6	0.67 ± 0.02
GZ	0.39 ± 0.11	3.0 ± 0.5	0.42 ± 0.03
SC	1.22 ± 0.12	4.0 ± 0.5	0.90 ± 0.03
LN	1.06 ± 0.14	7.0 ± 0.5	0.55 ± 0.03
IM	0.80 ± 0.12	2.7 ± 0.6	0.85 ± 0.06
QH	0.71 ± 0.02	2.3 ± 0.6	0.89 ± 0.09
HN	0.67 ± 0.09	5.0 ± 0.5	0.42 ± 0.02
YN	0.93 ± 0.13	4.3 ± 0.1	0.64 ± 0.03
FJ	1.24 ± 0.13	7.7 ± 0.6	0.61 ± 0.03
GD	1.47 ± 0.12	6.3 ± 1.0	0.85 ± 0.02

Notes: a. Richness(S) was represented by the morphotypes of the 100 clones on agar plates

As far as the Shannon-Wiener index (H) and the evenness(E_H) of these soils are concerned, their variance ranged from 0.39 ± 0.11(H) in Guizhou and 0.38 ± 0.02 (E_H) in Xinjiang to 1.47 ± 0.12(H) in Guangdong and 0.90± 0.03(E_H) in Sichuan. In conclusion, the diversity index of CFU estimated by cultivation-based methods can reflect the major diversity tendency of cultivable bacterial community in these soils.

2.3 Genetic diversity analysis

The results in Table 3 showed that the genetic diversity was also similar to the diversity in term of CFU in these soils, while the richness (S) and Shannon-Wiener index (H) of each soil were bigger than those in diversity of CFU. The difference between the diversity of CFU and the genetic diversity for the same soil was just due to the different analysis methods used, since the traditional methods are based on the level of cell while molecular fingerprinting techniques are based on the level of nucleic acid. It was obvious that the molecular fingerprinting techniques, such as PCR-DGGE in this study, were superior to the traditional cultivation-based methods for discovering more comprehensive and precise information of soil microorganisms.

Table 3 Shannon-Wiener index (H), richness (S) and evenness (E_H) of each soil samples estimated by the DGGE bands patterns

Soil	Shannon-wiener index (H)	Richness[a] (S)	Evenness (E_H)
XJ	2.280	12	0.918
HB	2.121	10	0.921
SX	2.296	13	0.895
ZJ	2.155	11	0.899
GZ	2.220	13	0.866
SC	2.151	12	0.866
LN	2.466	16	0.899
IM	1.998	9	0.909
QH	1.853	8	0.891
HN	2.068	11	0.862
YN	2.133	11	0.890
FJ	2.138	11	0.892
GD	2.096	11	0.874

Notes: a. Richness(S) was represented by the band number in DGGE

2.4 DGGE band analysis

As evidenced by the number of DNA bands detected in the control lanes of the denaturing gradient gels, the 13 soil samples showed the abundant bacterial community. Between 8 and 16 dominant bacterial DNA bands were detected in the 13 soil samples. Many of the visually intense DGGE bands were unique to each of the individual soils suggesting that the 13 soil samples had different dominant bacterial population. Additionally, the common bands(band 1, 2 and 5 in Fig.3) in each lane revealed that some common types of bacteria existed in all the 13 soils.

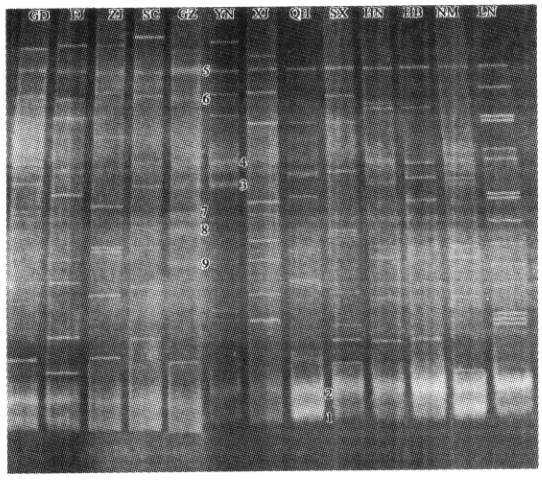

Fig.3 DGGE images of the 16S rDNA fragments of 13 soil samples

At the same time, the similarity in bacterial community of the different soil samples could be reflected by the similarity coefficient based on the common DGGE bands in these soils.

The comparison of similarity coefficient of these soils is given in Table 4, which showed that the bacteria community

in these soils had different similar extent of genetic information. For example, the similarity coefficient of genetic information between soil in Henan and soil in Inner Mongolia was 0.350, on the contrary, between in Liaoning and in Guizhou only 0.138.

Table 4 Similarity coefficient of soil bacterial community in different regions

Soil	XJ	HB	SX	ZJ	GZ	SC	LN	IM	QH	HN	YN	FJ	GD
XJ		0.182	0.280	0.261	0.240	0.167	0.214	0.238	0.200	0.217	0.260	0.174	0.217
HB	4		0.182	0.238	0.217	0.227	0.192	0.315	0.222	0.333	0.238	0.238	0.190
SX	7	4		0.167	0.192	0.240	0.172	0.182	0.238	0.167	0.167	0.208	0.167
ZJ	6	5	4		0.208	0.261	0.222	0.200	0.158	0.182	0.182	0.182	0.272
GZ	6	5	5	5		0.280	0.138	0.227	0.190	0.250	0.292	0.208	0.292
SC	4	5	6	6	7		0.214	0.190	0.200	0.304	0.261	0.217	0.304
LN	6	5	5	6	4	6		0.160	0.208	0.185	0.222	0.222	0.148
IM	5	6	4	4	5	4	4		0.176	0.350	0.150	0.250	0.300
QH	4	4	5	3	4	4	5	3		0.211	0.210	0.263	0.210
HN	5	7	4	4	6	7	5	7	4		0.227	0.227	0.272
YN	6	5	4	4	7	6	6	3	4	5		0.227	0.272
FJ	4	5	5	4	5	5	6	5	5	5	5		0.182
GD	5	4	4	6	7	7	4	6	4	6	6	4	

Notes: The data above diagonal are coefficient of community similarity and those down are number of common DGGE bands

3 Conclusions

By comparing of the results of soil bacterial community obtained by cultivable and genetic methods, it was clear that each method has its individual advantages. Although the cultivation-based method only covered a lower percentage of the whole microbes community, it can give more direct information on these microbes. While molecular fingerprinting techniques (PCR-DGGE) can give much phylogenic information on whole microbes community than the cultivation-based method did. Therefore, the combination of the traditional methods with the molecular biological techniques was the best way to study the diversity of bacterial community in environmental samples.

References:

Amann R I, Wolfgang L, Schleifer K H, 1995. Phylogenetic identification and in situ detection of individual microbial cells without cultivation [J]. Microbiological Rewiews, 59: 143—169.

De Leij F A A M, Whipps J M, Lynch J M, 1993. The use of colony development for the characterization of bacterial communities in soil and roots [J]. Microbial Ecology, 27: 81—97.

Frank S, Christoph C T, 1998. A new approach to utilize PCR single strand conformation polymorphism for 16S rRNA gene-based microbial community analysis[J]. Applied and Environmental Microbiology, 64: 4870—4876.

Kowalchuk G A, De Souza F A, Van Veen J A, 2002. Community analysis of arbuscular mycorrhizal fungi associated with *Ammophila arenaria* in Dutch coastal sand dunes[J]. Molecular Ecology, 11: 571—581.

James M T, Stella A B, Laus N *et al*., 1999. Open the black box of soil microbial diversity[J]. Applied Soil Ecology, 13: 109—122.

Muller A K, Westergaard K, Christensen S *et al*., 2002. The diversity and function of soil microbial communities exposed to different disturbances[J]. Microbial Ecology, 44: 29—58.

Myuzer G, Ellen C D W, Andre G U, 1993. Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction genes coding for 16S rRNA[J]. Applied and Environmental Microbiology, 59: 695—700.

Picard C, Ponsonnet C, Paget E *et al*., 1992. Detection and enumeration of bacterial in soil by direct DNA extraction and polymerase chain reaction[J]. Applied and Environmental Microbiology, 58: 2712—2722.

Sigler W V, Turco R F, 2002. The impact of chlorothalonil application on soil bacterial and fungal populations as assessed by denaturing gradient gel electrophoresis[J]. Applied Soil Ecology, 21: 107—118.

Torsvik V, Goksøyr J, Daae F L, 1990. High diversity in DNA of soil bacterial [J]. Applied and Environmental Microbiology, 56: 782—787.

Tsai Y, Olson B H, 1992. Rapid method for separation of bacterial DNA from humic substances in sediments for polymerase chain reaction[J]. Applied and Environmental Microbiology, 58: 2292—2295.

Ward D M, Bateson M M, Weller R *et al*., 1992. Ribosomal RNA analysis of microorganisms as they occur in nature[J]. Advance in Microbial Ecology, 12: 220—286.

Zhou J, Bruns M A, Tiedje J M, 1996. DNA recovery from soils of diverse composition[J]. Applied and Environmental Microbiology, 62: 316—322.

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