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Analysis of the archaea communities in fields with long-term continuous cotton cropping

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This study was carried out in order to investigate the variations of the soil archaeal community structure in fields with continuous cotton cropping in Xinjiang Region, China. Soil samples were collected from four depths in fields with 7-year continuous cotton cropping. 16S rRNA-polymerase chain reaction-denaturing gradient gel electrophoresis (PCR-DGGE) was used to analyze the archaeal community structure in soil. The archaeal diversity indices of the soil samples at the same depth exhibited similar patterns of changes with the period of continuous cotton cropping. Cluster analysis indicated that although various timeframe of succession cropping had great effect on archaeal communities in the samples of soils at various depths, they all had the trend to restore spontaneously. Principal component analysis showed that the archaeal community structure varied similarly regardless of the period of continuous cotton cropping. Homology comparison of sequences recovered from the DGGE bands showed that the obtained sequences all originated from the archaea organisms not cultured. Continuous cotton cropping exerted significant influences on the structure of soil archaeal community in Xinjiang Region, which were largely determined by the soil depth and the period of continuous cotton cropping. The microbial diversity of soil archaeal communities gradually recovered after 5-year continuous cropping.

Key words: Cotton monoculture, Archaeal communities, 16S ribosomal RNA, denaturing gradient gel electrophoresis (DGGE).

INTRODUCTION

Xinjiang is one of the regions in which cotton was planted in the earliest time, and also now the only one planting base for long stapled cotton in China. Xinjiang region has all the natural environmental conditions suitable for planting cotton, which include rich heat energy, abundant sunshine, little rainfall, dry air, substantial differences between day and night temperature, and artificial irrigation of snow water (Zhu, 2013).

Consequently, a great amount of wasteland was reclaimed to plant cotton in the eighties of the last century; the seven indices such as planting area, total productivity, mean productivity per acre, and so on with Xinjiang cotton ranked first for 21 continuous years. Meanwhile, in Xinjiang, classes of crops are simple, and especially in the planting area within localities, the main cotton producing areas accounts for over 95%, and rotation cropping is almost impossible. Hence, long-term succession cotton cropping brings about productivity decrease, large-scale outbreak of plant diseases and insect pests (He et al., 2010; Wei et al., 2006), and continuous accumulation of pesticides, fertilizers and plastic film pollutant (Martin et al., 2007). However, along

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Abbreviations: DGGE, Denaturing gradient gel electrophoresis; **PCR,** polymerase chain reaction; **PCA,** principal component analysis; **BLAST,** Basic Local Alignment Search Tool.

with the increasing period of continuous cotton cropping, there are no clear trends in the continuous cropping obstacles while the crop yields remain high and stable. This has raised great interests of agriculturists.

Denaturing gradient gel electrophoresis (DGGE) technology has become more and more important to molecular microbiological ecologists (Huang et al., 2010), because it is not only powerful in monitoring the structure of microorganism communities in a specific environment, but also can be used for the qualitative and semi-quantitative analysis of microbial diversity (Hooper et al., 2001; Zoetendal et al., 2004).

DGGE was applied for the first time to microbial ecological research by Muyzer et al. (1993). Since then, it has been widely used in a variety of studies on microbial ecological environments, such as lakes, oceans, activated sludge, and particularly soil (Muyzer and Smalla, 1998; Jiang et al., 2010).

Due to long-term succession cropping, in Xinjiang there are various cotton fields of different succession cropping history in the range of 0-20 year. These cotton fields may provide very good research materials for researchers of microbiological ecology of soils. As among soil micro-organisms, quantity and classes of archaea are fewer than those of bacteria and fungus; few bands will appear on the DGGE gel so as to facilitate analysis. Therefore, in this paper, DGGE was used to study changes with timeframe of succession cropping in composition and evolution of archaeal communities in soils at various depths. Besides, this study was purposed to investigate the varying pattern of composition of archaeal communities during succession cotton cropping in this region, and correlation between cotton productivity decrease resulting from succession cropping and outbreak law of cotton insect pests.

MATERIALS AND METHODS

Sample collection

In August 2010, soil samples were collected from fields subjected to 0- (uncultivated), 1, 3, 5, 10, 15, and 20 year continuous cotton cropping in Shihezi, Xinjiang Autonomous Region, China (E 85º21′81″-25′55″, N 44º25′47″-26′96″). For each period of continuous cotton cropping, 40-cm-deep soil cores were collected from 5 plots by vertical drilling, and the samples were taken from 4 depths, that is, 1-10, 11-20, 21-30, and 31-40 cm. Soils from the same depth with the same continuous cotton cropping period were mixed in equal amounts to form a composite sample (n = 28). The samples were transported to the laboratory immediately after collection and stored at 4°C prior to experimental analyses conducted within seven days.

DNA extraction and polymerase chain reaction (PCR) amplification of 16S rRNA fragment

DNA was extracted using sodium dodecyl sulphate (SDS) based extraction method (Zhou et al., 1996). The only modification was an extra step of extraction using phenol, chloroform and isoamyl alcohol, prior to chloroform and isoamyl alcohol extraction. The DNA was purified using the Moreira (1998) method. During the first PCR reaction, 16S rRNA in almost full length was amplified using the archaeal universal primers PRA46f / RA1100r (5′(C/T)TAAGCCATGC(G/A)AGT/5′(C/T)TAAGCCATGC(G/A)AGT) (Ovreas et al., 1997). The 25 µL reaction mixture consisted of: 2.8 µL of 2.5 mmol/L dNTP, 2.5µL of 10xbuffer, 1 U Taq enzymes, 3 pmol of each primer, and 0.2 µL (10 ng) DNA template. The PCR conditions were as follows: 5 min at 94°C; 60 s at 94°C; 45 s at 65°C (0.5°C decrease per cycle for the first 20 cycles, finally down to 55°C); 120 s at 72°C, (1 s increase per cycle for 10 cycles) for a total of 30 cycles; 5 min for 72°C. During the second PCR cycle, the 16S rRNA V3 fragment from archaea was amplified using PARCH340f-GC / PARCH519r (5′(C/T)TAAGCCATGC(G/A)AGT-CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCACGGGGG G / 5′(C/T)TAAGCCATGC(G/A)AGT) (Ovreas et al.,1997; Muyzer et al.,1993) primers. The 25 µL reaction mixture consisted of 1 µL product of the first PCR cycle (1:100 diluted), 2.8 µL of 2.5 mmol/L dNTPs, 2.5 µL of 10 x buffer, 1 U of Taq enzymes, and 5 pmol each primer. The reaction protocol was as follows: 5 min at 94°C; 60 s at 94°C; 45 s at 65°C (0.5°C decrease per cycle for the first 20 cycles, finally down to 55°C); 60 s at 72°C; for a total of 30 cycles; and 5 min for 72°C.

DGGE analysis of PCR products

PCR products were separated using DGGE electrophoresis with the Dcode gel electrophoresis system (Bio-Rad, USA). The denaturing gradient gel in a final concentration of 6-8% was prepared in a denaturing gradient of 30-70% (7M urea and 40% formamide were fully denatured). The electrophoresis buffer was 0.5 x TAE. 25 µL PCR products were ran on the gel at 60°C, under 150 V for 4 h. And then the gel was stained by SYBR Gold (Bio Probe Products, Rockland, ME, USA). Images were taken using a FR-200 UV-visible analysis system (Furi Company, Japan).

The banding patterns of the DGGE profile were analyzed using Quantity One v4.62 software (BIO-RAD Company, USA), Gel-Pro analyzer 4.0 (Media Cybernetics Company, USA) and Excel software (Microsoft Company, USA). The position and intensity of each band was determined automatically by the program. Each band's intensity was divided by the average band's intensity of the sample to minimize the influence of the different DNA concentrations between samples.

Statistical analysis

The genetic diversity of the soil microorganism communities was evaluated on Shannon-Wiener index (*H*), abundance (*S*), and uniformity (E_H) based on the following equation (Hill et al., 2003):

$$H=-\sum_{i=1}^{s} p_i \ln p_i$$

$$E_{\mathrm{H}}=H/H\max=H/\ln S$$

Where, p_i is the ratio of the intensity of a single band to the total intensity of all bands within the same lane, and *S* is the total number of bands in each lane.

Cluster analysis was performed using the UPGMA method (Daniela et al., 2012). The bands data were subjected to principal component analysis (PCA) using 16.0 for Windows (SPSS Incorporated 2007). The principal component was then extracted from the correlation matrix. The standardized data were then

Table 1. Diversity of soil archea obtained using DGGE bands pattern data associated with different succession cropping history.

Soil depth (cm)	Cropping history (year)	Shannon-Wiener index (*H*)	Richness (*S*)	Uniformity (E_H)
1-10	0	1.30^{c}*	4^{a}	0.94^{cd}
	1	1.69^{d}	6^{b}	0.94^{cd}
	3	1.43^{c}	5^{a}	0.82^{bc}
	5	0.81^{a}	5^{a}	0.50^{a}
	10	1.09^{b}	5^{a}	0.67^{a}
	15	1.10^{b}	4^{a}	0.79^{b}
	>20	1.51^{d}	5^{a}	0.94^{cd}
11-20	0	1.49^{d}	5^{b}	0.92^{d}
	1	0.71^{b}	2^{a}	1.02^{d}
	3	0.40^{a}	3^{a}	0.37^{a}
	5	1.34^{d}	5^{b}	0.83^{cd}
	10	0.97^{c}	5^{b}	0.60^{b}
	15	0.73^{b}	3^{a}	0.67^{bc}
	>20	1.25^{d}	4^{ab}	0.90^{d}
21-30	0	0	0	NO
	1	1.50^{a}	5^{a}	0.93^{a}
	3	1.89^{b}	8^{b}	0.91^{a}
	5	1.69^{a}	6^{a}	0.94^{a}
	10	1.76^{ab}	7^{ab}	0.91^{a}
	15	2.11^{cd}	11^{c}	0.88^{a}
	>20	2.09^{c}	10^{c}	0.91^{a}
31-40	0	1.53^{d}	6^{a}	0.86^{c}
	1	1.78^{d}	8^{bc}	0.86^{c}
	3	0.97^{a}	4^{a}	0.70^{a}
	5	1.12^{b}	5^{a}	0.69^{ab}
	10	1.17^{b}	7^{b}	0.60^{a}
	15	0.78^{a}	4^{a}	0.57^{a}
	>20	1.56^{d}	6^{ab}	0.87^{cd}

*The following letters (a-d) indicate significant differences between different treatments at $P<0.05$.

projected onto the principal axes, plotted in two dimensions, and examined for clustering behavior (Figueiredo et al., 2012).

DNA cloning, sequencing, nucleotide sequence accession numbers, and phylogenetic analysis

High-density DGGE bands were recovered from the uncultivated sample, and specific bands were recovered from other continuous cropping samples. DNA band recovery and the subsequent cloning procedures were performed according to Daniela et al. (2012). Sequencing of the colonies containing the correct target gene was carried out with vector primers (PARCH340f-GC / PARCH519r). Sixteen sequences of environmental 16S rRNA genes acquired in this study have been submitted to the GenBank database. The closest relatives of 16S rRNA genes from soil DNA extract were obtained with Basic Local Alignment Search Tool (BLAST) software in the National Center for Biotechnology Information. Sequence alignment was conducted using the CLUSTAL X version 2.1 software. A phylogenetic tree of the 16S rRNA gene fragments was built using the neighbor-joining method. Bootstrap analyses were based on 1,000 replicates (Ge et al., 2008).

RESULTS

Microbial diversity indices

With extended continuous cotton cropping periods, microbial diversity indices (*H*, *S*, and E_H) of soil archaea at different depths showed various changes (Table 1). The three indices that increased sharply occurred within the first year of continuous cotton cropping. However, the increased rate gradually slowed down as the continuous cotton cropping periods increased and then decreased again after 5 years of continuous cropping. Among the different depths, the diversity indices of soil archaeal communities showed the largest reduction and rebound at the 21-30 cm depth. The three diversity indices of soil

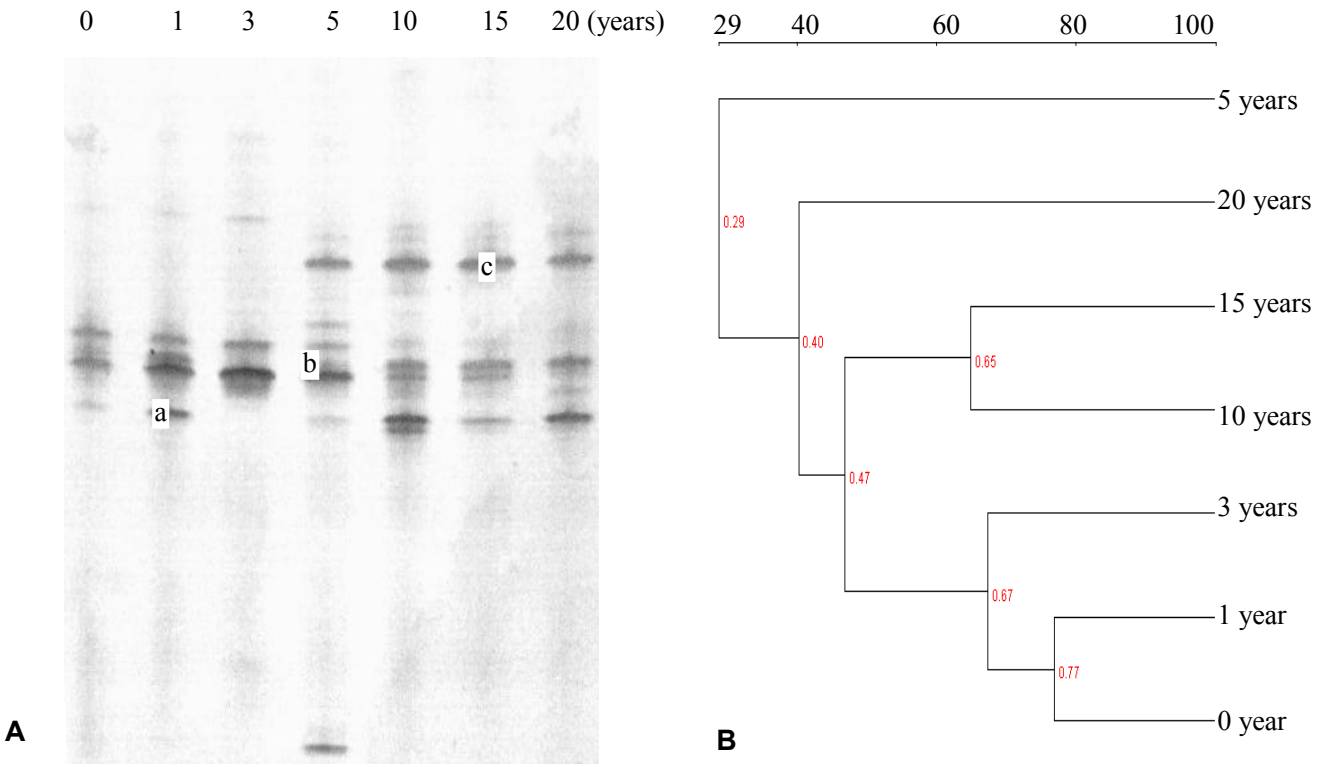

Figure 1. DGGE profiles (A) of PCR products from Archea in soils at the 1-10 cm depth and their cluster analysis (B) with 0, 1, 3, 5, 10, 15, and 20 year succession cropping of monoculture cotton (The a-c were excised bands). A and B: 1-10 cm deep soil samples years and Simility (%).

archaea gradually stabilized at levels slightly lower than those of uncultivated soils after 10 year continuous cropping, except that with the depth of 21-30 cm, no DNA band was amplified from the soil samples collected in the 0 planting year.

Similarity and cluster analysis

Cluster analysis showed that at the 1-20 cm depth, the uncultivated, 1 and 3 years continuously cropped samples were similarly clustered into a group, whereas the 10 and 15 years continuously cropped samples were clustered into a small group; and those from the samples of succession cropping for 5-year, were clustered into another third small cluster whose similarity was only more than 15% (Figures 1 and 2, right). This demonstrated that at this depth, cotton succession cropping for 5 years had the most effect on structure of archaeal communities in soils. At the 21-30 cm depth (Figure 3, right), the 3-year cropped samples had low similarity with other samples and was individually clustered into a small group, whereas those subjected to 1, 5, 10 15 and 20 years continuous cropping had high similarity and were clustered into a separate big group. At the 31-40 cm depth (Figure 4, right), the 15 and 20 year continuously cropped samples were similar and clustered into a small group, while the former were clustered into one big group.

PCA analysis

As shown in Figure 5, the first principal component was the most important at various soil depths, with a variance contribution rate > 41.14%. The first axis of the principal component analysis was positively correlated with the soil samples except for the 11-20 cm 1, 10, 15 year continuously cropped samples, and 31-40-cm 15 year continuously cropped soil samples. In particular, the soils had the highest correlations at the 1-10 and 21-30 cm depth and fluctuated between the positive and negative axes of the second principal component.

Sequences and phylogenetic analysis

A total of 28 sequences were recovered from 16 excised bands. The a-p bands were well isolated and they were high-density bands for soil samples with a different cropping history (Figures 1-4). For each isolated band, one sequence was selected and submitted to GenBank.

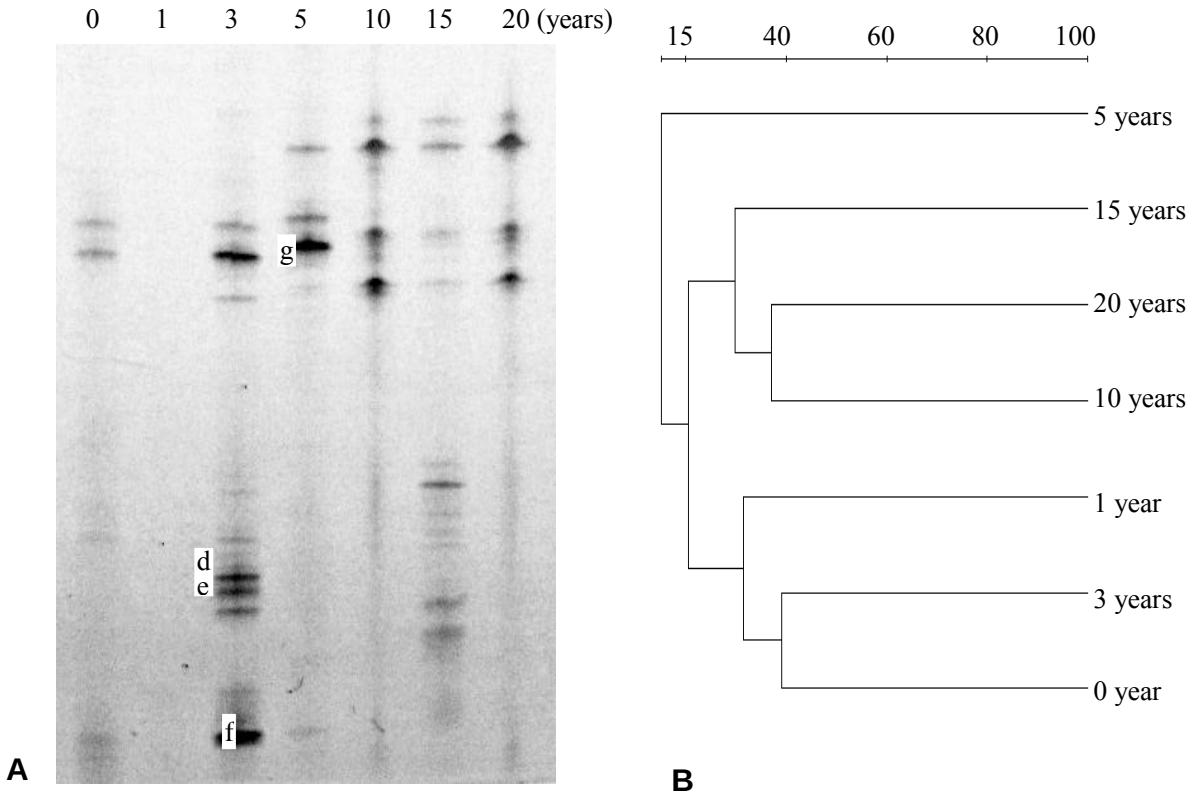

Figure 2. DGGE profiles (A) of PCR products from Archea in soils at depths of 11-20 cm and their cluster analysis (B) with 0, 1, 3, 5, 10, 15, and 20 year succession cropping of monoculture cotton (The d-g were excised bands). A and B: 11-20 cm deep soil samples Years and Simility (%).

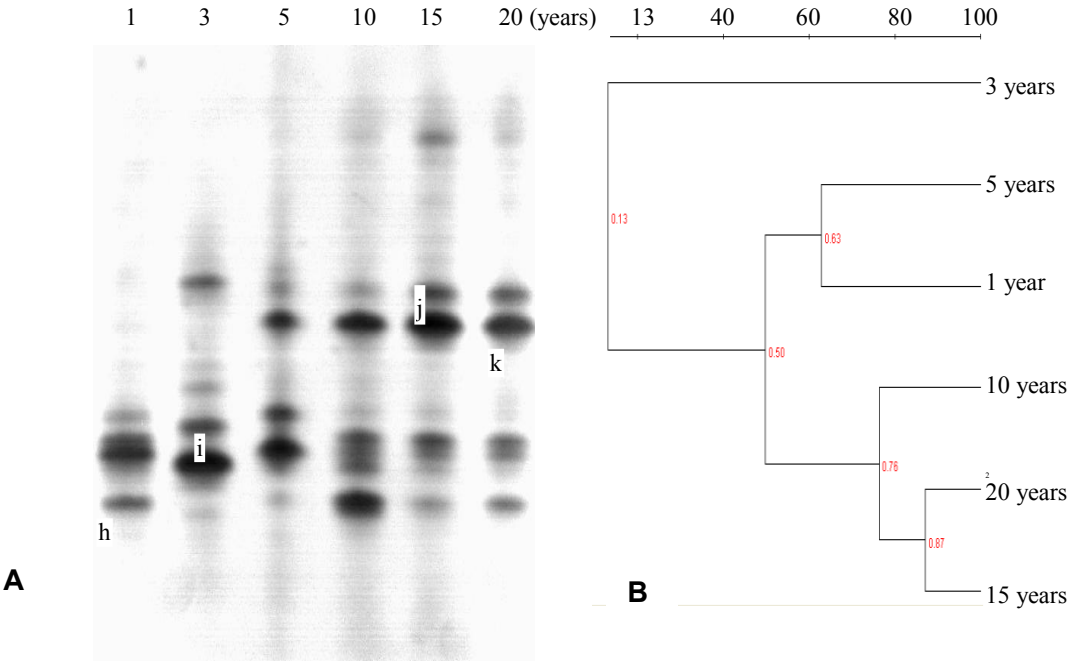

Figure 3. DGGE profiles (A) of PCR products from Archea in soils at depths of 21-30 cm and their cluster analysis (B) with 0, 1, 3, 5, 10, 15, and 20 year succession cropping of monoculture cotton (The h-k were excised bands). A and B: 21-30 cm deep soil samples Years and Simility (%).

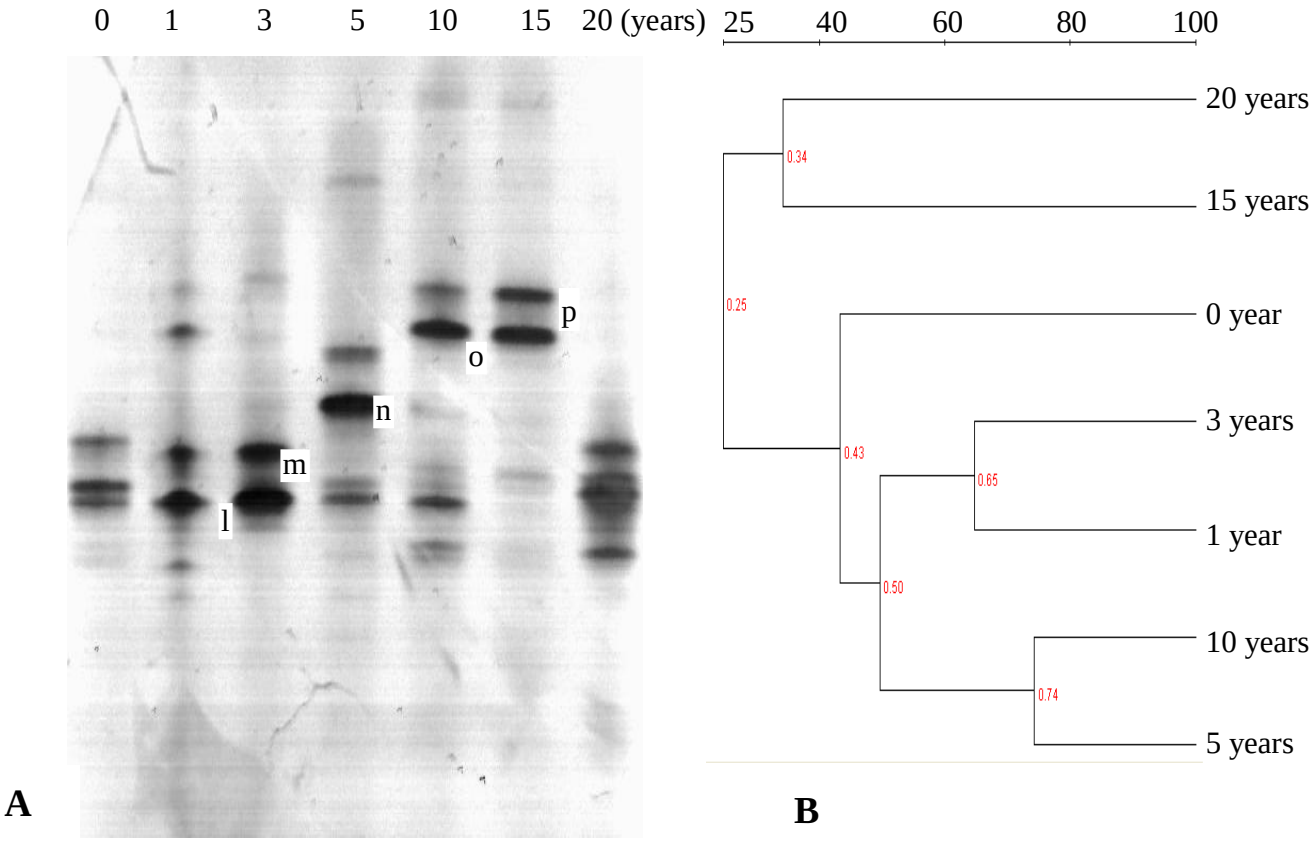

Figure 4. DGGE profiles (A) of PCR products from archea in soils at depths of 21-30 cm and their cluster analysis (B) with 0, 1, 3, 5, 10, 15, and 20 year succession cropping of monoculture cotton (The l-p were excised bands). A and B: 31-40 cm deep soil samples years and Similarty (%).

The acquired GenBank accessories were designated as JN572657-JN572672. Among the 16 sequenced fragments, 100% of them belonged to uncultured archaea microorganisms (Figure 6). Hence, those bands that were not analyzed probably contained a lot of 16S rRNA sequences that belonged to uncultured archaea microorganisms. Compared to the known sequences in GenBank, the 16 DNA sequences recovered from the DGGE gel shared 92-100% sequence homology, of which several sequences shared 97-100% homology with known microorganisms such as *Methanosarcina* sp. clone A686Methanosarcinaceae and *archaeon* clone 2-2A-04.

DISCUSSION

Increase in DGGE method accuracy in this study

DGGE is one powerful method for researching on microbiological ecology, but accuracy of the results obtained with this method was influenced by many factors (Székely et al., 2009). In this study, the 28 soil samples with different cotton succession cropping history and different soil depth were compared and analyzed. It was found that during extraction of total soil DNA and PCR amplification, many factors would result in increase in diversity of archaea in soil samples. For example, small fragments produced during DNA extraction and a large number of heterogeneous double-stranded DNA fragments would affect diversity of soil archaea. Furthermore, in this study there were several special design considerations to guarantee reliability of the data. Firstly, the collecting time of soil samples was mid-August, in which cotton growth was at the stage of harvest, and all the agricultural managements were the same in all of the crops studied. Since agricultural management activities (spraying pesticides, fertilizing, irrigating, among others) in the very year had ended, soil organism communities were stable (Wang et al., 2010). Secondly, the collecting site of soil samples was 15 cm away from cotton root (line distance, 60 cm). At the site, root of cotton had moderate effect on microorganism communities, and the soil samples were typical of the overall changes resulting from effect of cotton succession cropping on soil archaeal communities. Thirdly, Zhou and Moreira method were adopted for all the isolation and purification protocols of total soil DNA (Zhou et al., 1996;

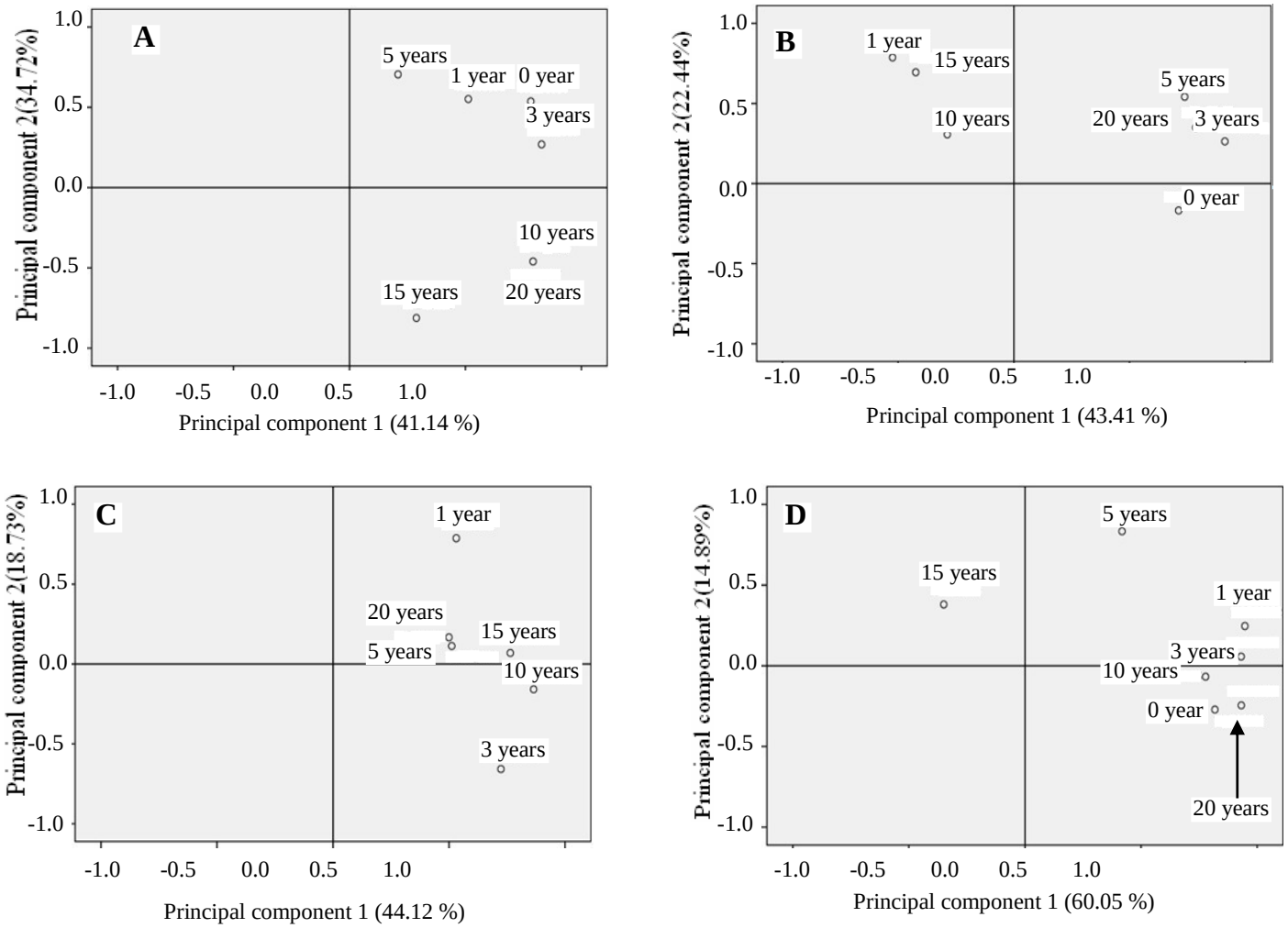

Figure 5. Principal component analysis (PCA) of the archaeal community structures obtained from the 1-10 cm (A), 11-20 cm (B), 21-30 cm (C) and 31-40 cm (D) depth soil layers, respectively.

Moreira, 1998), so as to avoid artificial difference between samples. Fourthly, touchdown PCR was adopted for both of the two DNA amplifications to ensure the same opportunity of amplification for 16S rRNA with various GC contents and the V3 region of 16S. This ensures isolation and analysis of characteristic fragments from most of archaeal species.

Effect of cotton succession cropping on composition of soil archaeal communities

The community structure of soil microorganisms is mainly determined by environmental factors such as soil properties (Girvan et al., 2003), whereas crop type and tillage management measures also cause shifts in the community structure of soil microorganisms (Acosta et al., 2008; Ge et al., 2008). In this study, according to the overall changes of the indices such as Shannon-Wierner index in Table 1, although long-term cotton succession cropping made structural diversity, abundance and uniformity of archaeal communities in soils at various depths decrease, the affecting extent and trend varied. With the depth of 11-20 cm in the cropping soil layer (0-30 cm deep), the indices decreased most and rebounded in the fifth planting year. With the depth of 1-10 cm, the indices decreased more and rebounded in the tenth planting year. With the depth of 21-30 cm, the indices decreased to the least extent and fluctuated once again. Differences in the dynamics of decreasing and rebound of diversity index through time in different time frame may be due to the interaction between cotton roots and soil. At the depth of 1-20 cm, substance exchange between soil and root is much frequent because most of the cotton roots are distributed in this area (especially at the depth of 11-20 cm). The frequent interaction has great influences on the structure and composition of soil archaeal communities at this depth and new communities can form more rapidly (expressed as shortened period of

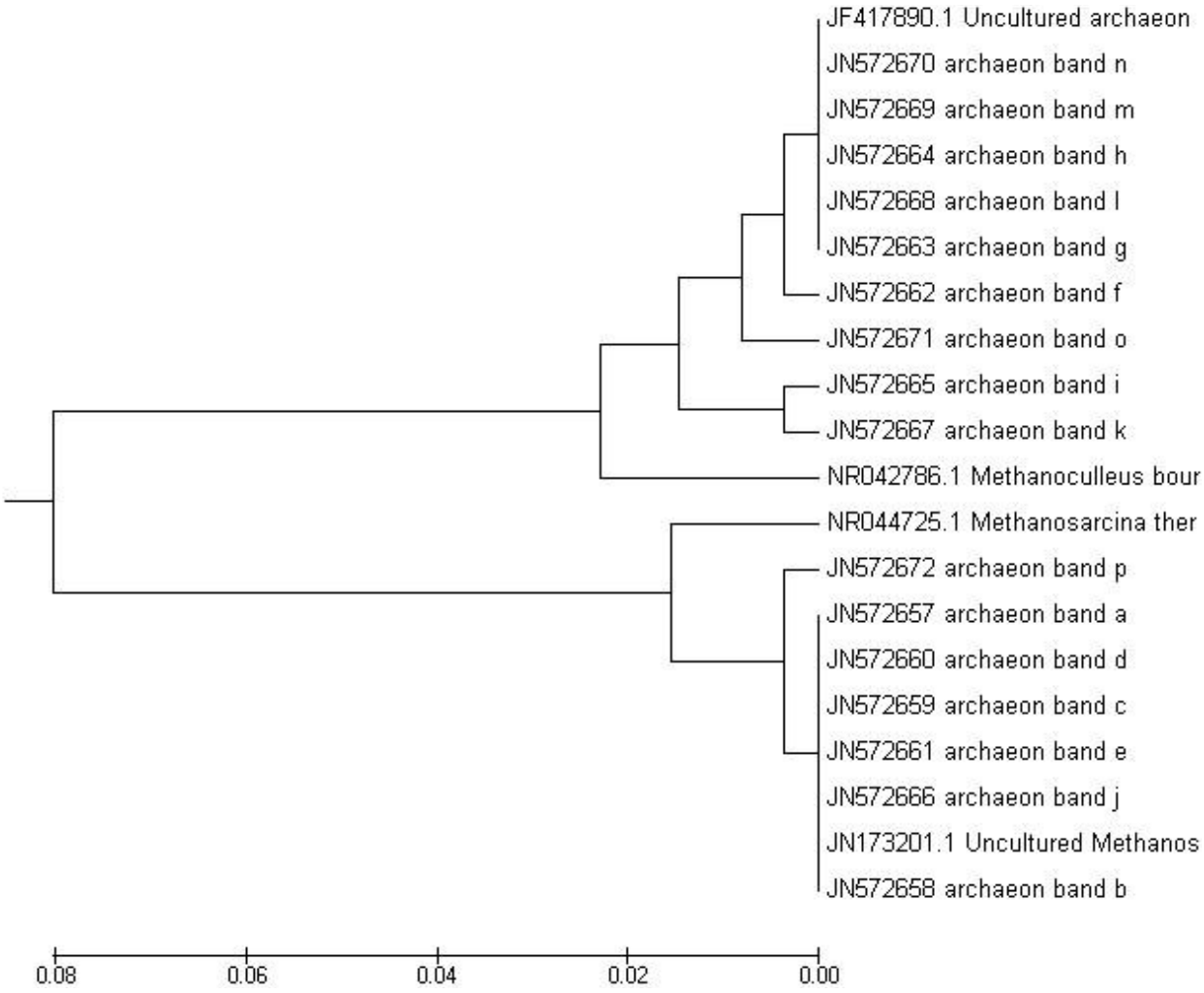

Figure 6. The closest sequence match of known phylogenetic affiliation with band sequences recovered from DGGE gel.

indices rebounding). However, at the depth of 31-40 cm which is tillage pan (never having been ploughed), the varying patterns of the indices show great similarity to that at the depth of 21-30 cm, although the extent of change was much less.

According to the results of cluster and PCA analysis (Figures 1-4 and 6), during 0-20-year period of cotton succession, cropping structure and composition of soil archaea at four different depths underwent first rapid change and afterwards partial restoration and then approached a new relatively stable state. And they at large exhibited some certain varying pattern with increase in soil depth.

This might have happened because when one soil microorganism community suffers long-term and stable selective pressure, its structure will be forced to change to some certain extent; the community has certain ability to be restored by itself, and can attains to one new relatively stable status as time goes on. Period of the restoration and similarity to structure and composition of the original microorganism community depend on strength of external selective pressure.

Information about soil archaeal species embodied on DGGE gel plates is much less than that about bacteria. Such information facilitates the analysis on effect of cotton succession cropping on structure of soil microorganism communities. After alignment with information in Genbank, it was found that all the sequences had association with uncultured archaea and the a-p sequences had no microbiological information associated with crop infection.

This was different from other related reports (Zhang et al., 2011; Zou et al., 2005). The reason may be that sequence information about soil microorganisms is too much, but the bands which we analyzed only represented a minority of all the archaeal species; maybe most of cotton soil-boring diseases may be caused by both bacteria and fungus. Therefore, it is so necessary to study on effect of cotton succession cropping on structure and composition of soil bacteria and fungus communities.

Similar variations in archaeal community structure and cotton yield during continuous cotton cropping

Similar to other cotton cultivation districts, newly reclaimed land in Xinjiang commonly shows serious continuous cropping obstacles such as reduced yields and pest-induced disease outbreaks. However, after extension of the continuous cropping period, not only are pest diseases spontaneously alleviated in cotton fields but crop yields are also recovered to their original high and stable state (Wei et al., 2006). By means of comparison and analysis, we found that cotton productivity and disease incidence had high correlation with history of cotton succession cropping. According to the data on cotton cropping for over 30 years in Xinjiang recorded by local institutions of agricultural technology service, during the history from unreclaimed land to cotton fields of 20 years succession cropping, cotton productivity and disease incidence exhibited strong regularity. In the first planting year, cotton productivity was very high, and nearly no plant diseases and insect pests occurred. Afterwards, with period of succession cropping, cotton productivity decreased continuously and plant diseases and insect pests occurred at high frequency, and the condition got worsen till the fourth-sixth planting year. Under the circumstance in which rotation cropping could not be conducted, dosage of pesticides had to increase. But with prolong succession cropping timeframe; this condition may allay sponta-neously. Till the ninth-eleventh planting year, no plant diseases and insect pests broke out at large scale and no cotton productivity decreased. This law is very similar to the varying pattern of how cotton succession cropping history affected structure of soil archaeal communities obtained in this study. This finding also implies that long-term continuous cropping may not cause continued destruction of soil archaeal community structures since the microbial communities can spontaneously adjust their composition to adapt to external environmental changes, thereby maintaining a new stable and healthy long-term community structure.

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