

16S rRNA-Based PCR-DGGE Analysis of Actinomycete Communities in Fields with Continuous Cotton Cropping in Xinjiang, China

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Abstract The purpose of this study was to examine the variations in the microbial community structure of soil actinomycetes in fields with continuous cropping of cotton in Xinjiang Autonomous Region, China. Soil samples were collected from four depths in fields with 7-year continuous cotton cropping. The community structure of soil actinomycetes was examined using the 16S rRNA-based polymerase chain reaction–density gradient gel electrophoresis (PCR-DGGE) techniques. The microbial diversity indices of the soil samples from different depths generally decreased along with the period of continuous cotton cropping. When the period of continuous cropping of cotton reached 5 years, the diversity indices rose again and gradually stabilized at a level slightly lower than that of soils with original ecology (i.e., 0-year cotton cropping). Cluster analysis showed that at the 1–20-cm depth, the actinomycete community structure of the soil subjected to 1-year cotton cropping was similar to that of soil subjected to 0-year cotton cropping, whereas that of soils after 3-year continuous cotton cropping showed high similarity. At the 21–40-cm depth, the actinomycete community structure showed various changes but generally recovered to its original pattern after repeated fluctuations. Principal component analysis showed that at the 1–30-cm depth, the actinomycete community structure varied similarly regardless of the period of continuous cotton cropping. In contrast, there were no clear actinomycete community structure variation trends at the 31–40-cm soil depth. Homology comparison of sequences

recovered from the DGGE bands showed that the obtained sequences shared similarities >88 %. Alignment with the known homologous sequences indicated a lack of microorganisms related to soil-borne cotton diseases. Continuous cotton cropping exerted significant influences on the community structure of soil actinomycetes in Xinjiang Autonomous Region, which were largely determined by the soil depth and the period of continuous cotton cropping. The microbial diversity of soil actinomycete communities gradually recovered after 5-year continuous cropping. Thereafter, a new actinomycete community structure that was beneficial for continuous cropping of cotton was formed and stabilized each year.

Introduction

Cotton, a major economic crop in China, has an important position in the national economy. Xinjiang Autonomous Region is suitable for cotton cultivation for a variety of reasons, e.g., little rainfall, abundant sunshine, and substantial differences between day and night temperatures (annual rainfall of 110–200 mm, 1,000–1,500 mm evaporation, average temperature of 6–6.6 °C, 2,900 h of sunshine, altitude of 300–500 m, and temperature of 15–20 °C in August). Due to continuous cropping obstacles, cotton cultivation areas have substantially decreased in the middle and eastern parts of China. Consequently, a vast area of uncultivated land in Xinjiang has been reclaimed for cotton cultivation since the 1980s. For the 20 years up until 2011, the Xinjiang region raised China in cotton cultivation area, total yield, and yield per unit area. Due to the single crop species in Xinjiang (e.g., >90 % coverage of cotton in local major cultivation districts), crop rotation and/or intercropping is unlikely, leading to serious continuous cropping obstacles [1]. Similar to other cultivation areas, the continuous cropping of cotton in Xinjiang initially caused a large-scale

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outbreak of soil-borne diseases that resulted in substantial crop yield and quality losses. However, along 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 from agriculturists.

Continuous cropping is common in agricultural production and can cause obstacles such as soil quality degradation, crop yield reduction, and aggravated crop pest-induced plant diseases [2, 3]. The fundamental reason for continuous cropping obstacles is related to rhizosphere soil microstructure disorders or deterioration. Crop rotation, fallow, and manure application have commonly been used to solve continuous cropping obstacles in agriculture [4–6]. These methods attempt to improve the soil microstructure, but their effects are temporary [7, 8]. A healthy and stable soil microstructure is essential to maintaining long-term continuous cropping and stable high crop yields. This study aimed to examine the shifts in soil microstructure in fields subjected to continuous cotton cropping (from the occurrence to the disappearance of continuous cropping obstacles, further to the stable long-term high cotton yield) in Xinjiang, China. The results showed that continuous cropping of a single species both exerted strong influences on soil microstructure and affected the community structure of soil microorganisms at different depths [9]. Although soil microbial communities at different depths responded to environmental changes at different rates, the associated soil microstructure was capable of spontaneous adjustment according to the consistent long-term environmental changes, thereby forming a new stable and healthy structural pattern that is beneficial for continuous cropping.

Materials and Methods

Sample Collection

In August 2010, soil samples (gray desert soil: pH 8.59; total organic matter, 13.2 gkg⁻¹; clay, 243.5 gkg⁻¹; total N, 0.611 gkg⁻¹; total P, 0.987 gkg⁻¹; total K, 44.9 gkg⁻¹; exchangeable N, 39.4 mgkg⁻¹; exchangeable P, 20.9 mgkg⁻¹; exchangeable K, 212.0 mgkg⁻¹) 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 85°21'81"–25°55", N 44°25'47"–26°96"). For each period of continuous cotton cropping, 40-cm-deep soil cores were collected from five plots by vertical drilling, and the samples were taken from four depths, i.e., 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 7 days.

DNA Extraction and 16S rRNA Gene Polymerase Chain Reaction Amplification

DNA was extracted from 0.5 g of soil from each sample using a MoBio Ultra-Clean™ soil DNA kit (MoBio Laboratories, USA). Using the Moreira method [10], we analyzed the total DNA in the next step. During the first polymerase chain reaction (PCR) cycle, the 16S rRNA fragment was amplified using the actinomycete-specific primers 27F/16Sact1114r (5'-AGAPTTTGATCCTGGCTCAG-3'/5' -GAGTTGAC CCCGGCRGT-3') [11, 12]. PCR was performed in a total volume of 25 μL in 200-μL microtubes, which contained 0.15 μL each primer (60 pmol each), 2.8 μL 2.5 mmol/L dNTP mixture, 2.5 μL 10× buffer, 0.5 μL (2.0 U/μL) Taq enzymes, 0.2 μL (about 10 ng) DNA template, and 18.85 μL Milli-Q water. 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 for a total of 30 cycles; and 72 °C for 7 min. During the second PCR cycle, the actinomycete 16S rRNA V3 fragment was amplified using P338fGC/P518r primers [13]. PCR was performed in a total volume of 50 μL in 200-μL microtubes, which contained 1 μL of the product of the first PCR cycle (1:100 diluted), 0.25 μL each primer (100 pmol each), 5.6 μL 2.5 mmol/L dNTP mixture, 5 μL 10× buffer, 1 μL (2.0 U/μL) Taq enzymes, and 37.15 μL Milli-Q water. The reaction protocol was as follows: 5 min at 94 °C; 60 s at 94 °C, 45 s at 55 °C, 60 s at 72 °C for 30 cycles; and 72 °C for 5 min.

Polymerase Chain Reaction–Density Gradient Gel Electrophoresis Analysis and Band Identification

PCR samples were loaded onto 6–8 % (w/v) polyacrylamide gels in 0.5× TAE buffer (20 mM Tris–acetate, 1 mM ethylenediaminetetraacetic acid; pH 8.0). The polyacrylamide gels were made with denaturing gradients of 30–70 % (where 100 % denaturant contained 7 M urea and 40 % formamide). The gels (length, 18 cm) were run for 4 h at 150 V and 60 °C, after which time they were soaked for 1 h in SYBR Green I nucleic acid stain solution (1:10,000 dilution; Molecular Probes, Leiden, the Netherlands) and immediately photographed using an FR-200 UV–visible analysis system (Furi Company, Japan).

The banding patterns of the density gradient gel electrophoresis (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 were 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 [14]. All data were the average of three trials.

Statistical Analysis

The diversity index of the soil actinomycetes communities was evaluated using the Shannon–Wiener index (H), richness (S), and evenness (E_H) based on the following equation [15]:

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

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

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 unweighted pair group method with arithmetic mean. The band data were subjected to principal component analysis (PCA) using the Statistical Package for the Social Sciences version 16.0 for Windows (SPSS Incorporated 2007). The principal components were then extracted from the correlation matrix. The standardized data were then projected onto the principal axes, plotted in two dimensions, and examined for clustering behavior [16].

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 [16]. Sequencing (conducted by Sheng Gong Company, China) of the colonies containing the correct target gene insert was conducted using vector primers (P338fGC/P518r). The obtained sequences were submitted to the GenBank database under accession numbers JN572602–JN572631 and JN572673. A BLAST search was used to explore the sequence similarity with others in the GenBank database. Sequence alignment was conducted using the Clustal X version 2.1 software. A phylogenetic tree of the 16S rRNA gene V3 fragments was built using the neighbor-joining method.

Results

Microbial Diversity Indices

With extended continuous cotton cropping periods, microbial diversity indices (H , S , and E_H) of soil actinomycetes at

different depths showed various changes (Table 1). The three indices were highest for soil actinomycetes in uncultivated soils at various depths, and rapid decreases occurred within the first year of continuous cotton cropping. However, the decreasing rate gradually slowed down as the continuous cotton cropping periods increased and then rose again after 5 years of continuous cropping. Among the different depths, the diversity indices of soil actinomycete communities showed the largest reduction and rebounds at the 21–30-cm depth. The three diversity indices of soil actinomycetes gradually recovered at levels slightly lower than those of uncultivated soils after 10-year continuous cropping, except that the E_H value was slightly higher than that of uncultivated soils at the 31–40-cm depth.

Similarity and Cluster Analysis

Cluster analysis showed that at the 1–20-cm depth, the uncultivated and 1-year continuously cropped samples were similar and clustered into a single small group, whereas those 3-, 5-, 10-, 15-, and 20-year continuously cropped samples were similar and clustered into one big group (Figs. 1, 2, 3, and 4, right). At the 21–30-cm depth, the uncultivated sample had low similarity with other samples and was individually clustered into a small group, whereas those subjected to 3- and 15-year continuous cropping had high similarity and were clustered into a separate small group. The third cluster included four samples from the fields subjected to the shortest and longest continuous cropping periods, i.e., 1, 5, 10, and 20 years. At the 31–40-cm depth, the 20-year continuously cropped sample had the least similarity with the other samples, while the latter were clustered into one big group.

PCA Analysis

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

Homologous Sequence Similarity Analysis

DGGE bands 1–24 shown in Fig. 1, 2, 3, and 4 (left) represent the high-density bands from the uncultivated soil, whereas bands a–h represent the high-density bands from

Table 1 Diversity of soil actinomycetes obtained using density gradient gel electrophoresis band pattern data associated with different succession cropping histories

Soil depth	Cropping history (year)	Shannon–Wiener index (H')	Richness (S)	Evenness (E_H)
1–10 cm	0	2.38d	22a	0.77d
	1	1.98b	18a	0.69c
	3	1.93ab	21a	0.63c
	5	1.76a	23ab	0.56b
	10	2.07b	21a	0.68c
	15	1.22c	17a	0.43a
	20	2.10b	20a	0.70d
11–20 cm	0	2.46d	18ab	0.85d
	1	2.10d	15a	0.77c
	3	1.24a	18ab	0.43a
	5	1.67bc	17a	0.59b
	10	1.32a	18ab	0.46a
	15	1.35a	21b	0.44a
	20	1.33a	13a	0.52a
21–30 cm	0	2.63d	19b	0.89d
	1	1.03a	12a	0.41a
	3	1.79b	18b	0.62bc
	5	2.11c	15a	0.78d
	10	2.26cd	18b	0.78d
	15	2.11c	15a	0.78d
	20	2.00c	14a	0.46a
31–40 cm	0	2.39d	18c	0.81a
	1	2.19c	18c	0.76a
	3	1.84a	8a	0.89ab
	5	2.08bc	12b	0.84a
	10	2.30cd	14bc	0.87ab
	15	2.34cd	14bc	0.89ab
	20	1.67a	6a	0.93b

Means in a column are significantly different ($P < 0.05$) if followed by different letters (a–d)

Fig. 1 Density gradient gel electrophoresis profiles (*left*) of the polymerase chain reaction products from actinomycetes in soils at the 1–10-cm depth and their cluster analysis (*right*) with 0-, 1-, 3-, 5-, 10-, 15-, and 20-year successive monoculture cotton cropping (The 1–7 and *a–c* bands were excised)

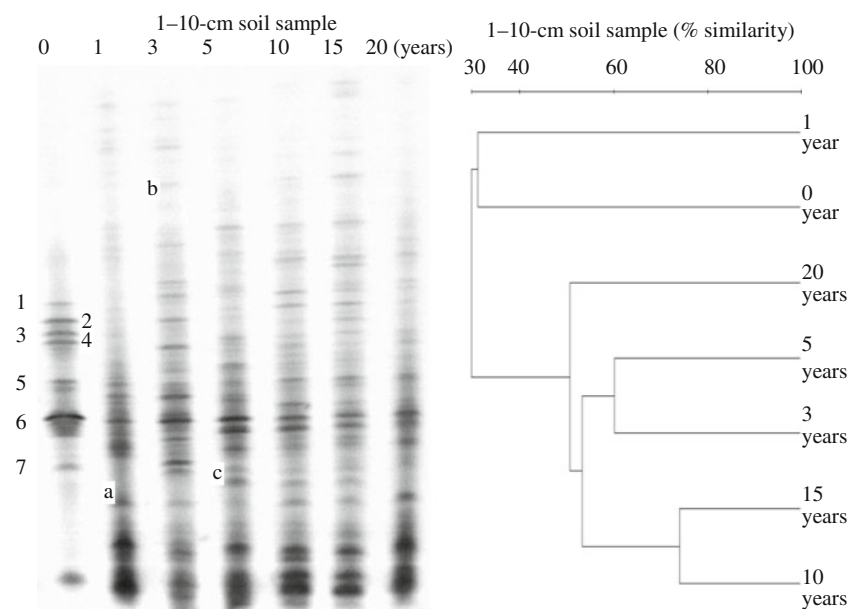
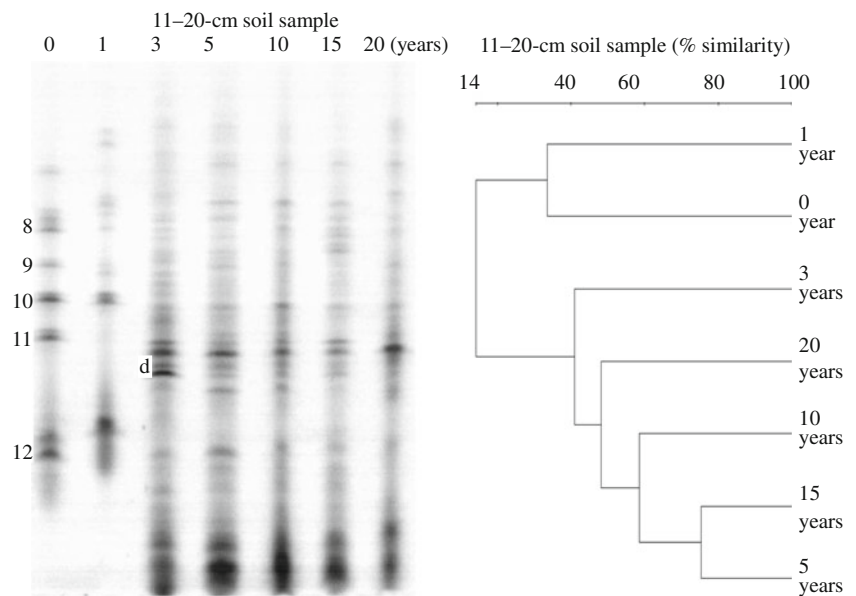


Fig. 2 Density gradient gel electrophoresis profiles (*left*) of polymerase chain reaction products from actinomycetes in soils at the 11–20-cm depth and their cluster analysis (*right*) with 0-, 1-, 3-, 5-, 10-, 15-, and 20-year successive monoculture cotton cropping (The 8–12 and *d* bands were excised)



soils subjected to different continuous cotton cropping periods. A phylogenetic tree was constructed based on the selected DNA sequences recovered from the DGGE gel as well as their known relatives retrieved from GenBank (Fig. 6). The 32 DNA sequences recovered from the DGGE gel shared 88–100 % sequence homology, of which several sequences shared 100 % homology with known microorganisms such as *Rhodococcus* sp. Pc2 *Fnocardiaceae*, *Bacterium Ellin* 5004, and unclassified *Actinobacteridae* of different genera. Further inspection of the known microorganisms that are mostly related to DNA sequences recovered from the DGGE gel indicated a lack of microorganisms related to soil-borne diseases such as cotton blight and verticillium wilt in the tested soils. This finding differed

from those results reported by other studies in terms of continuous cropping obstacles [17, 18].

Discussion

Community Structure of Soil Actinomycetes Shifted Due to Long-Term Continuous Cotton Cropping

The community structure of soil microorganisms is mainly determined by environmental factors such as soil properties [19], whereas crop type and tillage management measures also cause shifts in the community structure of soil microorganisms [20, 21]. In this study, 16S rRNA-based PCR-

Fig. 3 Density gradient gel electrophoresis profiles (*left*) of the polymerase chain reaction products from actinomycetes in soils at the 21–30-cm depth and their cluster analysis (*right*) with 0-, 1-, 3-, 5-, 10-, 15-, and 20-year successive monoculture cotton cropping (The 13–17 and *e–f* bands were excised)

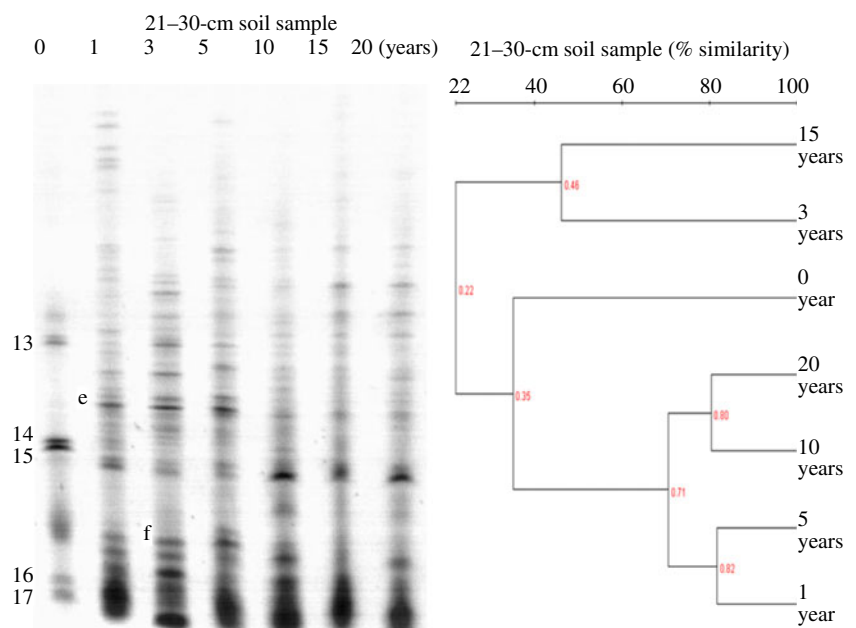
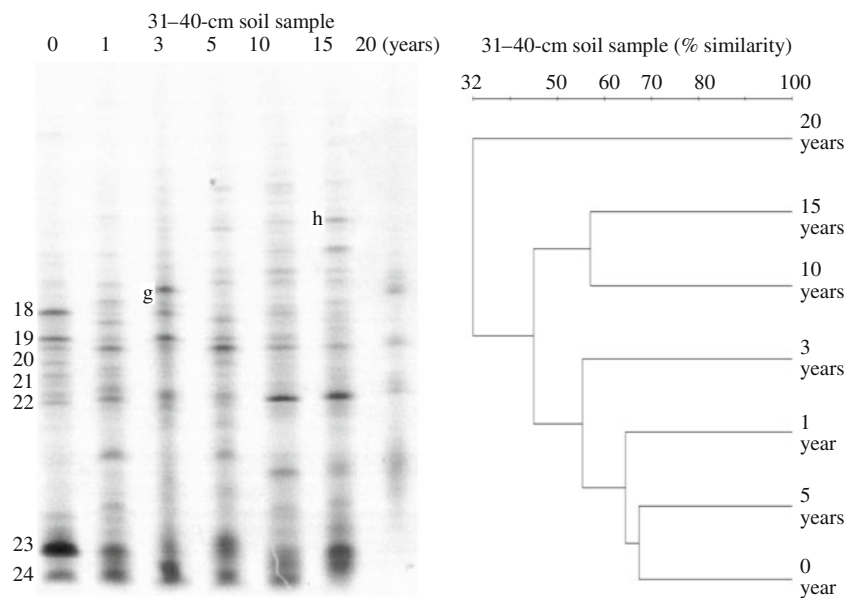


Fig. 4 Density gradient gel electrophoresis profiles (*left*) of the polymerase chain reaction products from actinomycetes in soils at the 31–40-cm depth and their cluster analysis (*right*) with 0-, 1-, 3-, 5-, 10-, 15-, and 20-year successive monoculture cotton cropping (The 18–24 and *g*–*h* bands were excised)

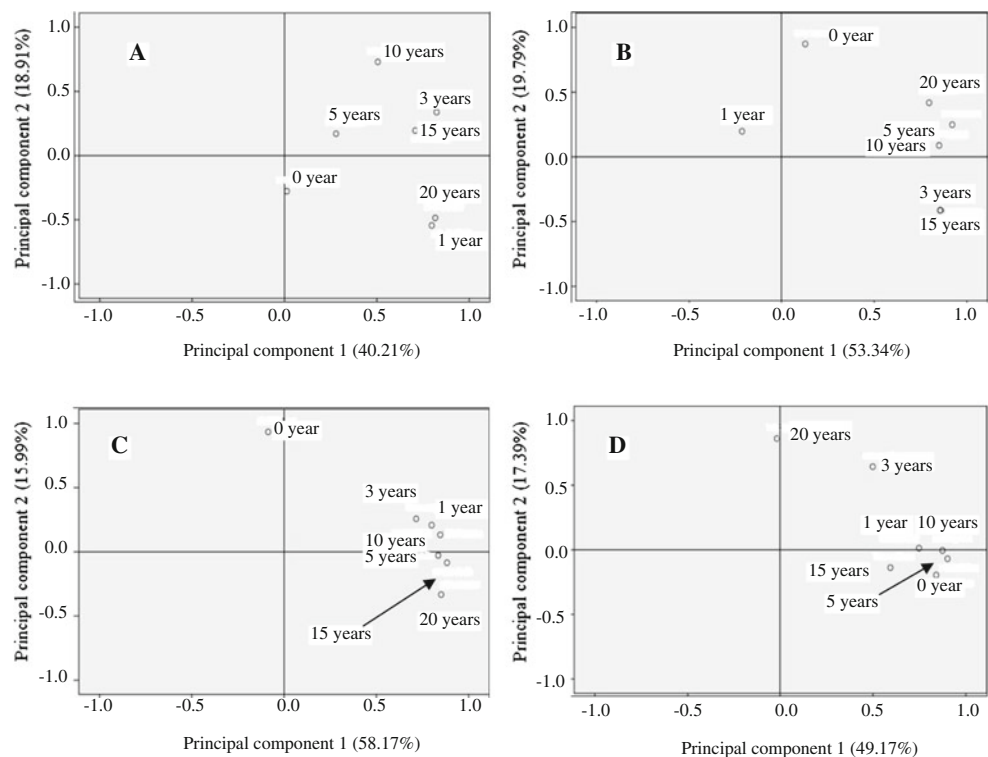


DGGE fingerprinting demonstrated that long-term continuous cotton cropping resulted in continuous changes in the community structure of soil actinomycetes, which shifted from the original ecological structure to a relatively stable and healthy pattern suitable for long-term continuous cotton cropping. In this process, the community structure of soil actinomycetes underwent the most rapid changes during the first year of cotton cultivation, mainly showing reduced microbial diversity that possibly benefited cotton growth and the appearance of some species that could induce the

obstacle of cotton continuous cropping [18]. This finding suggested that cotton cultivation had a strong influence on the community structure of soil actinomycetes.

With extension of the continuous cropping from 1 to 3 years, three microbial diversity indices started to rebound in the major distribution area of the cotton root at the 11–31-cm depth. When the field was subjected to 5-year continuous cotton cropping, microbial diversity indices rebounded at both 1–10-cm depth (surface layer) and 30–40-cm depth (plow pan). These findings indicated that continuous cotton

Fig. 5 PCA of the actinomycetes 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



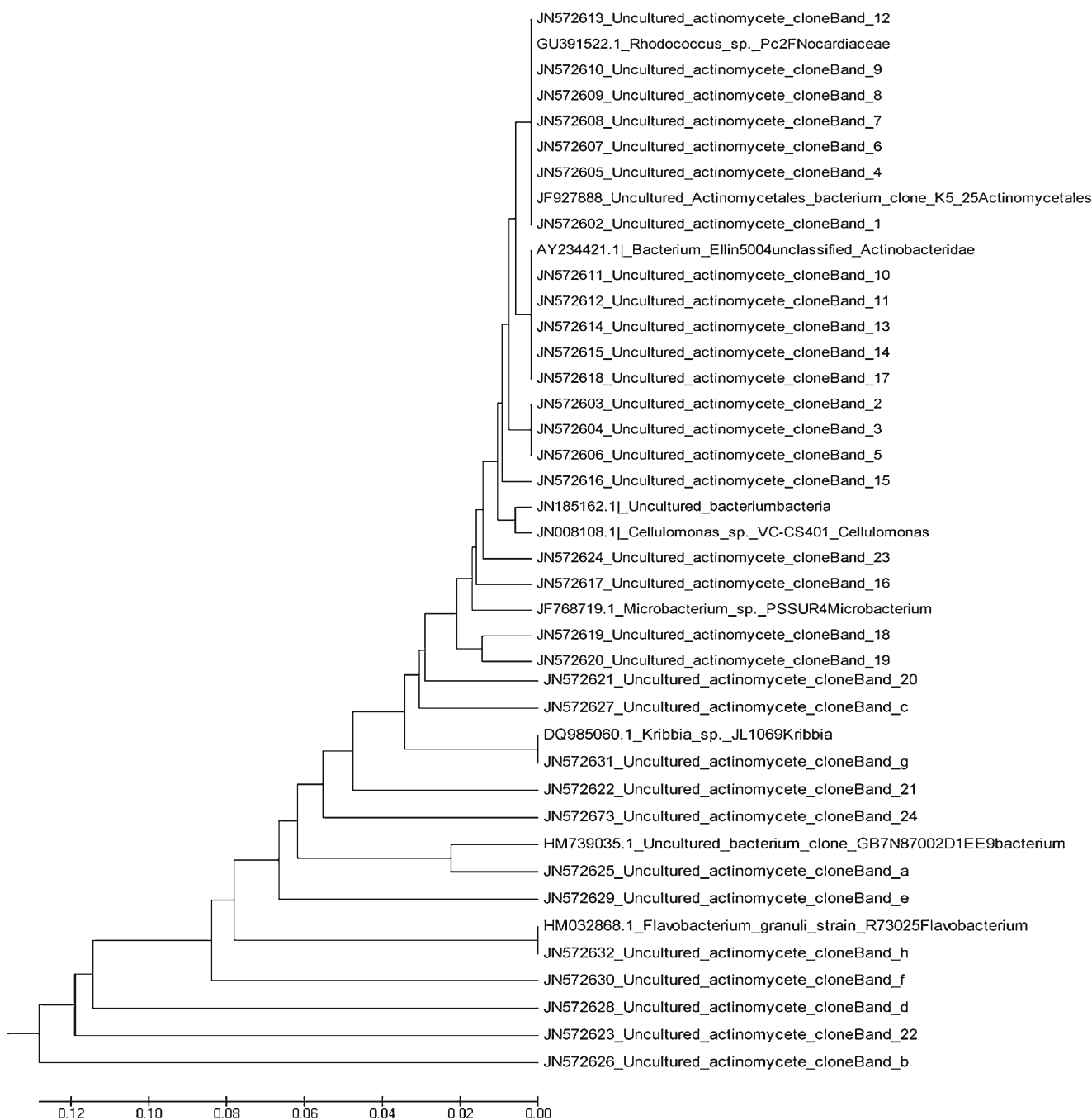


Fig. 6 The closest sequence match of known phylogenetic affiliation with band sequences recovered from the density gradient gel electrophoresis gel (bootstrap analyses were based on 1,000 replicates)

cropping had different influences on the community structure of soil actinomycetes regarding soil depth. It was likely that the interaction between cotton roots in the major distribution zone and the soil microorganisms was intense. The microbial diversity indices gradually stabilized in the field subjected to 10-year continuous cotton cropping, while cluster analysis showed that the soils subjected to multi-year continuous cropping were largely grouped together. These findings demonstrated that the soil microbial communities spontaneously

formed a new and relatively stable community structure under the stress of a long-term external environment.

Similar Variations in Actinomycete Community Structure and Cotton Yield During Continuous Cotton Cropping

Similar to other cotton cultivation districts (probably due to the large amount of endosulfan, monocrotophos, cypermethrin, lyphosate, foliar fertilizer, urea and diamine), 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. Results from the present study showed that during the process of continuous cotton cropping, the actinomycete community structural variations could be related to the occurrence and alleviation processes of continuous cropping obstacles. In particular, the reduction of soil actinomycete diversity and its rebound period, the occurrence of cotton pest diseases [8, 22, 23], the loss of crop yields, and the subsequent rebound period were nearly consistent at the 11–30-cm depth (arable layer), suggesting a close relationship between soil microbial community structure and function and crop yield [24]. This finding also implies that long-term continuous cropping may not cause continued destruction of soil microbial 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.

The Choice of Research Methods and Other Possible Factors May Cause Soil Actinomycete Community Structure Shift

Using the 16S rRNA-based PCR-DGGE technique [25], this study examined the entire shifting process of actinomycete community structures at different soil depths during continuous cotton cropping in Xinjiang for up to 20 years. Soil actinomycetes were targeted at different depths since previous research has shown that long-term cotton cropping can lead to the shifting of bacterial soil types to fungal soil types [26]. Despite the distribution of different actinomycete communities at different depths, variations in composition occurred during the entire continuous cropping period. In addition, the number of bands on the DGGE gel was between those of bacteria and fungus, thereby facilitating the data collection process. Soil sample collection was carried out at 15-cm line intervals (line spacing, 60 cm) since the soil microbes were affected by the crop root at this position, reflecting the composition of the microbial community structure of the entire soil environment.

Our results showed that the shift in the actinomycete community composition was most sensitive in the major distribution zone of the cotton roots, i.e., 11–31-cm depth, where they first showed the rebound and stabilization tendencies. Regardless of soil depth, the actinomycete community structure variations were quite similar, ultimately forming a relatively stable community composition. With regard to the spontaneous formation of the healthy microbial community structure in cotton fields subjected to long-term

continuous cotton cropping, we speculated that it was primarily related to the local saline–alkali soil type, dry climate, and cropping patterns of half-year plough and half-year fallow in Xinjiang.

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