

# GENETIC AND FUNCTIONAL DIVERSITY OF SOIL BACTERIA AND FUNGI FROM DIFFERENT MICROHABITATS IN A KARST REGION IN SOUTHERN CHINA

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## Abstract

Little is known about the difference between bacterial and fungal genetic and functional diversity in karst regions of south China. In this study, the genetic and functional diversity of bacteria and fungi in nine types of soil microenvironments in the karst region in Maolan National Nature Reserve in Guizhou were investigated by PCR-DGGE (Denaturing Gradient Gel Electrophoresis) and BIOLOG EcoPlates. Maolan National Nature Reserve is a UNESCO Biosphere Reserve and plays an important role in protecting the karst forest ecosystem and rare and endangered wild animals and plants in central Asia. The results showed that the diversity of both bacteria and fungi was high and the main factors influencing the diversity of bacteria and fungi were different. The bacterial community structure from different microhabitats under the same vegetation type had higher similarity than similar microhabitats in different vegetation types, which could indicate that the bacterial community structure was mainly controlled by vegetation. For fungi, similar microhabitat species under different vegetation types had higher similarities than different microhabitats species under the same vegetation type, which could indicate that the fungal community structure is mainly controlled by microhabitats. In addition, the metabolic patterns of similar microhabitats in different vegetation were different, while the metabolic patterns of different microhabitats in the same vegetation were not obviously different. In conclusion, the effect of vegetation types on soil microbial functional diversity was greater than that of microhabitats, and this difference was reflected by the different degrees of influence on soil microbial genetic diversity and community structure.

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## INTRODUCTION

The karst in southern China represents the most typical tropical-subtropical karst in the world (Yan et al., 2020; Zhou et al., 2020). It is a unique environmental unit in the subtropical climate zone, with a large elevation gradient and carbonate rock area, complex topography, and ecological fragility (Bai et al., 2013; Tian et al., 2016; Deng et al., 2020). Because of the natural conditions and human influence, the karst regions in China now have some special features including uncertain hydrological conditions, soil degradation, and low environmental recoverability (Zeng et al., 2018; Bai and Zhou, 2020; Wang et al., 2020). Rocky desertification may be the most typical environmental problem in the karst region in Guizhou Province in southeast China. Karst rocky desertification means that under the background of the fragile karst environment in the subtropical climate zone, unreasonable human social and economic activities have already caused serious soil erosion, large-scale exposure of bedrock, serious decline in land productivity, and soil degradation that is similar to desert landscapes (Zhan et al., 2013; Wang, 2020). Rocky desertification is a biogeochemical process that could be influenced by bacteria and fungi, and the effect is interactive (Wei et al., 2011; Hui et al., 2019).

Microorganisms are widely distributed in soil and can affect many kinds of soil biochemical reactions (Chen et al., 2019; Gregorutti and Caviglia, 2019). Soil microbial diversity is the basis and guarantee of the soil microorganism ecological function and plays an important role in maintaining soil quality and ecosystem stability and health. Soil microbial diversity can provide lots of information on soil and human influence, so it can also be used as a sensitive indicator of evaluating ecosystem stability and monitoring the change of soil quality. Therefore, the study of microorganisms in karst regions has attracted lots of attention. By studying the soil microbial diversity in mountains and grasslands in karst regions in Guizhou, it indicated that soil fertility, microbial biomass, and diversity were closely-related to each other (Jin et al., 2019). Another study chose arbuscular mycorrhizal fungi (AMF) as the research subject and found that microhabitats in karst regions in Guangxi Province could influence the richness of AMF by changing physical and chemical properties of soil and the vegetation types (Zhao et al., 2019). Some studies have shown that in karst regions, the microbial diversity can be affected by the vegetation and microhabitats (Dassen et al., 2017; Xue et al., 2017; Yang et al., 2018). However, previous studies ignored the discussion of genetic and functional diversity, especially on the comparison between the fungi and bacteria in microhabitats of karst regions. The role of soil microorganisms became more obvious when rocky desertification control and ecological restoration became more and more urgent. Thus, the characteristics of soil microorganisms from different kinds of microhabitats in karst region needs to be discussed due to the key role of soil microorganisms in soil succession, properties, and fertility. The microhabitat is a different small unit that can be

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clearly distinguished in external form. There are various types of microhabitats, including stone surfaces, stone pits, stone trenches, and soil surfaces, which make the distribution of exposed rocks and soil uneven (Du and Wang, 2010).

We studied the genetic diversity of soil bacteria and fungi in karst regions in Guizhou by PCR and denaturing gradient gel electrophoresis (DGGE). We analyzed the functional diversity of soil bacteria and fungi using average well color development (AWCD) using BIOLOG EcoPlates to show the main carbon sources of soil microorganisms by Principal Component Analysis (PCA). Few previous studies have investigated the genetic and functional diversity of bacteria and fungi from different microhabitats. The specific goals of this study were: (1) to understand the distribution and physiological features of soil microorganisms in karst soil more comprehensively; (2) to identify which factors were important in driving bacterial and fungal community distribution in karst; and (3) to identify the main factors influencing soil microbial communities.

## MATERIALS AND METHODS

### Site Description and Sampling

Maolan Biosphere Reserve (25° 09' to 25° 20'N 107° 52' to 108° 05'E) is situated in Libo County, Guizhou Province in southeast China. In April 1996, Maolan was included in the UNESCO International Man and Biosphere Reserve Network (MAB) and became a world biosphere reserve (<https://en.unesco.org/mab>). The main protection objects of the biosphere reserve are the mid-subtropical karst forest ecosystem and rare and endangered wildlife. The total area of the reserve is 21,330 ha, of which the core area covers 5,150 ha. The elevation here is 630–1075 m above sea level. According to the record of the weather station in Libo (altitude: 423.9 m), the average annual temperature and precipitation of the reserve is 18.6 °C and 1760 mm respectively. The annual precipitation from April to October is 1162 mm, accounting for 80 % of the annual precipitation, is the annual rainy season (Zhou, 1987). The rocks in Maolan karst forest are mainly limestone and dolomite, with quartz sandstone and a small amount of shale in some places (Han et al., 2008). The region is famous for its virgin forest that is well-preserved on the karst landscape. The ecosystem types in Maolan include subtropical and temperate rainforests and agroecosystems (with rice, rape, sweet potato and medicinal plants). Every year there are about 60,000 visitors coming here because of its karst landscapes, streams, ponds, and waterfalls. There are vegetation succession communities with different degrees of degradation in this area, and the vegetation communities are relatively complete under each degree of degradation, which are conducive to the comparative study of different vegetation types in the same area.

According to the main feature of karst vegetation succession, three representative vegetation types were selected (secondary forest, shrubbery, and primeval forest) (Table 1). In each forest succession vegetation type, four kinds of microhabitats were studied, namely, soil surface, stone ditch, stone seam, and stone surface. The stone ditch has a wider opening and U-shape cross section. There are well-developed tree and shrub layers in the stone ditch with some herbs, ferns, and litters. The stone seam has a narrow opening and V-shaped cross section. There are more ferns, litters, and semi-decomposed organic matter in the stone seam. All the microhabitats were natural rather than man-made. In fact, only the samples of stone seam, stone ditch, and soil surface were collected during the sampling process, because the vegetation and soil of the stone surface microhabitat rarely met our experimental needs (Fig 1). The sample of a microhabitat was composed of three parallel samples of the same microhabitats. In our sampling sites, the rock type is mainly limestone.

Our research was conducted in August 2018. When sampling, we determine appropriate sample sizes according to the area of each microhabitat. We mixed samples of the same microhabitats from each forest site. We used disinfected shovels to remove the topsoil when sampling. The sample was sieved through a 2 mm sieve to ensure thorough mixing, and the samples were stored at 4 °C and –20 °C for later use. The soil in the study area is dominated by rendzina, a humus-rich soil formed from parent limestone and dolomite that are almost pure carbonate rocks. Except for the rainy season, the soil is relatively dry. The soil here is rich in Ca<sup>2+</sup>, Mg<sup>2+</sup>, and HCO<sub>3</sub><sup>-</sup>, with a pH of 7.5 to 8.0, an organic matter content of 75.5 g kg<sup>-1</sup> to 380 g kg<sup>-1</sup>, and a total nitrogen content of 6.06 g kg<sup>-1</sup>.

### PCR-DGGE Analysis of Bacterial Diversity

DGGE can distinguish the sequence differences of target fragments with the same or similar molecular weight, and can be used to detect single base changes and genetic diversity, as well as polymorphism of PCR amplified DNA fragments. The principle of DGGE technology to detect nucleic acid sequences is that the DNA fragments of different sequences are denatured at their respective denaturant concentrations and eventually stagnate at their denaturant gradient position. Comparing PCR-DGGE dyed bands can determine the number and diversity of microorganisms in the soil samples (Muyzer and Smalla, 1998; Ma et al., 2003). Compared with other research methods of microbial diversity, this technology has some advantages. For example, PCR-DGGE can detect the whole microbial community (Zheng et al., 2013; Ling et al., 2020). Compared to modern molecular biology techniques, PCR-DGGE can analyze other microorganisms that coexist in the environment. By combining PCR-DGGE with other molecular biology techniques, we can get a more comprehensive analysis of microbial diversity.

**Table 1. The Background information of three types of vegetation in Maolan reserve.**

Vegetation Types	Slope	Bedrock Exposed Rate (%)	Vegetation Coverage (%)	Vegetation Characteristics
Primeval Forest	30-40	60-90	90-100	<p>The hierarchical structure is relatively complete, and the differentiation of plants between the tree layer, shrub layer, and herb layer is clear. The coverage of the tree layer is more than 80 % and the height is 10-20 m; the height of the shrub layer is 3-8 m and the coverage is 5-10 %.</p> <p>The dominant species are mainly round fruit incense, calyx haitong, small fruit Runan, green sandalwood, light leaf haitong, Rhizophora chinensis, ten major merits, celestial fruit and so on. The surface layer is covered with lichen and moss. In addition, understory is covered with 3-5 cm litter layer.</p>
Secondary Forest	30-40	50-80	90-100	<p>The hierarchical structure of the stand is clearly differentiated. The tree layer and shrub layer are relatively developed. The tree layer is 5-12 m high, while the coverage rate is over 80 %. The shrub layer is 2-3 m high, while the coverage is about 10 %.</p> <p>The dominant species mainly include Caragana yungui, Quercus glauca, Castanopsis fargesii, Pinus massoniana, Haitong, gerbera, etc. There are also a small number of vine thorns, ferns, lichens and other mosses. The thickness of the litter layer under the forest is 1-2 cm.</p>
Shrubbery Forest	20-30	70-80	80-100	<p>The vertical structure of the stand is simple, with few trees. It is mainly dominated by shrub layers, with a height of 2.5-3 m, a coverage rate of over 80 %, and a large degree of canopy closure.</p> <p>The dominant species are: Nandina domestica, Huaxiang, Gerbera, tiger thorn, Libo hornbeam, multi-veined Cyclobalanopsis glauca, rose hip etc. The litter cover under the forest is about 1-2 cm.</p>

The whole PCR-DGGE processes were divided into sample DNA extraction, gene amplification, separation, and strip recovery sequencing. Total Soil DNA was extracted using the Power Soil DNA Isolation Kit (MO BIO) Kit. All the



Figure 1. Different karst microhabitats in Maolan National Nature Reserve: A = Soil surface, B = Stone seam, and C = Stone ditch.

kit processes were strictly followed. For bacterial diversity analysis, the extracted DNA was used as templates for PCR. The amplification of bacterial DNA was performed using the universal 16S rDNA primers F338GC (5'-CGCCCGCCGCGCGC-GGCGGGCGGGGCGGG-GGCACGGGGGGCCTAC-GGAGGCAGCAG-3') and R518 (5'-ATTACCGCG-GCTGCTGG-3') (Shanghai Shenggong Biological Engineering Technology Service Company). The pre-denaturation condition was 94 °C for 5 min using the Model 475 Gradient Delivery System (Bio-Rad Laboratories Company). The first 20 cycles were 94 °C for 1 min, 65-55 °C for 1 min, and 72 °C for 3

min (the annealing temperature of each cycle decreased by 0.5 °C), and the last 10 cycles were 94 °C for 1 min, 55 °C for 1 min and 72 °C for 3 min, and finally, at 72 °C for 7 min. After electrophoresis in 1.5 % agarose gel, the products of the PCR reaction were stored at -20 °C degrees and analyzed by denaturing gradient gel electrophoresis. The denaturation gradient was from 30 % to 60 %. The concentration of polyacrylamide gel was 8 % in 1XTAE. Electrophoresis was conducted at 200 V and 75 V for 10 min and 10 h, respectively. After electrophoresis, the gel was dyed with silver dye (Bassam et al., 1991). The dyed gel was analyzed by BioRAD Gel doc-2000 Gel image analysis system, and all the electrophoresis bands were photographed.

### PCR-DGGE Analysis of Fungal Diversity

The total DNA of soil samples was extracted and purified using the Mo BIO kit according to the manufacture's instruction, and the quality of extracted DNA was evaluated by 1 % agarose gel electrophoresis. The product was stored at -80 °C. The purified genomic DNA was used as a template for polymerase chain reaction (PCR). The PCR amplification was performed on the Mastercycler PCR machine. The primers of the fungi were U1: 5'-GTGAA ATTGTTGAAA GGGAA-3', U2-GC:5'-CGCCCGCCGCGCGGGCGGGGCGGGGGCACGGGGGGGACTC CTTGGTCCGT-GTT-3'. All the primers used in this study were provided by Shanghai ShengGong Bioengineering Technology Service Company. PCR reaction conditions of the fungi were as follows: the pre-denaturation conditions were 94 °C for 3 min, and then 35 cycles were performed at 94 °C for 30 s, 53 °C for 30 s, 72 °C for 1 min and finally at 72 °C for 10 min. All subsequent treatments were the same as for bacteria.

### Biolog Ecoplates Functional Diversity

The fresh soil samples were weighed to 10 g of dry soil and placed in 100 mL sterile water, then oscillated for 20 min at 220 rpm. After dilution to 10<sup>-3</sup> with sterile water, 125 µL of diluted suspension was added to each well of Biolog ECO micropore plate (Biology, Hayward, CA, USA) using an 8-channel sample filler. After 168 hours of cultivation, Microlog Rel 4.2 software was used to read the absorbance value at 590nm wavelength on the BIOLOG Emax<sup>TM</sup> automatic plate reader (BIOLOG, Hayward, CA, USA) every 24 hours (Scuhutter and Dick, 2001). We have listed the carbon sources in Table 2.

### Statistical Analysis

DGGE profiles were analyzed by Bio - Rad QUANTITY ONE 4.4.0 software. Comparisons of banding profiles were established by the dice coefficient ( $C_s$ ) that was drawn by the unweighted pair group method with arithmetic mean (UPGMA) plot

$$C_s = \frac{2j}{(a + b)}, \quad (1)$$

where  $j$  is the band common to samples A and B and  $a$  and  $b$  are the respective number of bands in samples A and B.

Shannon's index (H), richness (S), Simpson index (D), McIntosh index (U) and evenness (E) were used to characterize microbial diversity using the equations of Agryzkov et al., (2018). Average well color development (AWCD) could be used to access the microbial community's overall ability to exploit carbon sources.

**Table 2. Correlation coefficients between main source of carbon and PC1 or PC2.**

Carbon Source Type (PC1)	Carbon Source	Load Value	Carbon Source Type (PC2)	Carbon Source	Load Value
Carbohydrate	D-galactonic acid-γ-lactone	0.750	Carbohydrate	β-methyl-D-glucoside	0.614
Amino acids	L-Arginine	0.719	Sugar	D-galactonic acid γ-lactone	0.553
Carboxylic acids	D-galacturonic acid	0.454	Amino acids	L-Arginine	0.383
Amino acids	L-asparagine	0.448	Polymer	Tween 40	0.404
Polymer	Tween 40	0.566	Sugar	N-acetyl-D glucosamine	0.648
Carbohydrate	i-erythritol	0.435	Sugar	D-cellobiose	0.443
Amino acids	L-phenylalanine	0.776	Sugar	α-D-lactose	0.476
Polymer	Tween 80	0.649	Sugar	D,L-α-glycerol phosphate	0.626
Carbohydrate	D-mannitol	0.523	Carboxylic acids	D-malic acid	0.502
Parent compound	4-hydroxybenzoic acid	0.596	...	...	...
Amino acids	L-serine	0.598	...	...	...
Polymer	Glycogen	0.441	...	...	...
Carboxylic acid	D-glucosamine	0.490	...	...	...
Amino acids	Glycyl-L-glutamic acid	0.698	...	...	...
Amine	phenethylamine	0.507	...	...	...
Sugar	α-D-lactose	0.441	...	...	...
Amine	Putrescine	0.688	...	...	...

$$AWCD = \frac{\sum(C - R)}{n}, \tag{2}$$

where *C* is the optical density value of each well with culture medium, *R* is the optical density value of the control well, *n* is the data of the culture medium, and the *n* value of the ECO plate is 31 (Guo et al., 2015; Miao et al., 2019).

Analysis of Variance (ANOVA) was performed on microorganisms and carbon sources uptake data using SPSS 16. The correlation matrix method is used to compress the data to obtain the different principal components and the distance between samples. The principal component values obtained after PCA analysis were used to analyze the differences between the inspection groups and correlate with environmental factors.

## RESULTS

### Genetic Diversity Characteristics of Bacteria in Karst Microhabitats

The bacterial communities of different soil samples were observed by DGGE (Fig. 2A). All nine lanes had a large number of bands, which show that each microhabitat was rich in bacterial diversity (Fig. 2B). At the same time, every lane had specific bands, which could illustrate that the diversity of bacteria among different microhabitats was quite different. From the DGGE profile, the shrubby forest (lane 4, 5, and 6: line 34, 38, and 37 bands out of 78, respectively) exhibited the most abundant bands, while the abundance of secondary and primeval forest were similar. Lane 1 from the shrubby forest had the lowest number of bands; 24 bands out of 72. The secondary forest stone ditch (lane 8) and the secondary forest stone seam (lane 9) had relatively fewer bands (28 and 30 bands out of 72, respectively). The bacterial abundance from the soil surface, stone ditch, and stone seam may be very similar.

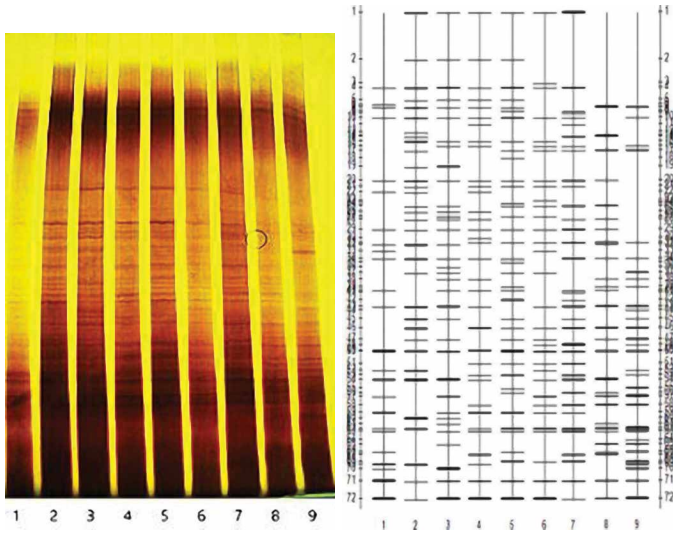


Figure 2. DGGE results of bacterial communities in different karst microhabitats samples: A = DGGE profile of amplified of bacterial communities in different karst microhabitats samples, and B = DGGE sketch map of bacterial communities in different karst microhabitats samples. Numbering is: 1. Primeval forest surface; 2. Primeval forest stone ditch; 3. Primeval forest stone seam; 4. Shrubby soil surface; 5. Shrubby stone ditch; 6. Shrubby stone seam; 7. Secondary soil surface; 8. Secondary forest stone ditch; and 9. Secondary forest stone seam.

stone ditch 5 (51.3 %) and the shrubby forest stone seam 6 (44.6 %), which could illustrate that the similarity among different microhabitats of the same vegetation may be higher.

### Diversity and Richness Analysis

We then calculated the diversity index of soil bacterial communities in different karst microhabitats samples, by Shannon index (*H*), richness (*S*) and evenness (*E*) (Table 4). The Shannon index and richness of shrubby soil stone ditch were highest among all microhabitats, which were 3.65 and 39, respectively. The Shannon index and richness of primeval forest surface were only 3.13 and 24, which were the lowest. The mean value of bacterial Shannon index among different microhabitats was 3.46. In terms of evenness index, except for the primeval forest surface and secondary forest stone ditch, which were slightly lower, the others remained at 1.00. The trends of bacterial Shannon index and the richness of primeval forest microhabitats were: stone seam > stone ditch >

### Similarity Analysis of Bacterial Communities in Different Karst Microhabitats

Based on the similarity of bacterial communities in different microhabitat samples, cluster analysis results were shown by a dendrogram, along with the similarity index between lanes (Fig. 3, Table 3). The dendrogram gave rise to two main branches of clusters and shared a similarity of 0.39. One branch cluster contains the secondary forest stone seam, soil surface and stone ditch, with the similarity of 0.47. The bacterial communities between primary forest soil surface and stone seam stayed closer with a similarity of 0.51. From the similarity index, the primary forest soil surface 1 had related with the primary forest stone ditch 2 (20.2 %) and the primary forest stone seam 3 (29.4 %). The shrubby forest soil surface 4 had high correlation with the shrubby forest

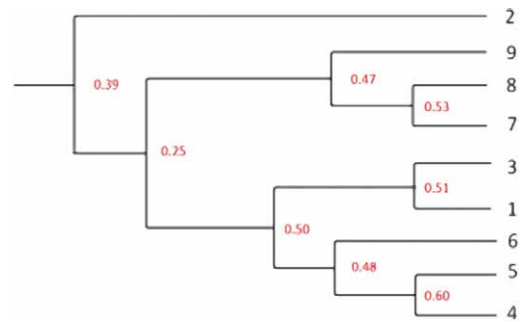


Figure 3. Similarity cluster of microbial communities among different karst microhabitats samples. Numbering is: 1. Primeval forest surface; 2. Primeval forest stone ditch; 3. Primeval forest stone seam; 4. Shrubby soil surface; 5. Shrubby stone ditch; 6. Shrubby stone seam; 7. Secondary soil surface; 8. Secondary forest stone ditch; and 9. Secondary forest stone seam.

**Table 3. The similarity index between different microhabitat samples lanes of microbial communities (%).**

Lane	1	2	3	4	5	6	7	8	9
1	100.0	20.2	29.4	33.5	22.1	35.9	24.2	25.6	22.2
2	20.2	100.0	48.3	40.5	48.5	43.1	47.0	25.1	30.7
3	29.4	48.3	100.0	58.4	45.1	44.1	42.5	29.5	34.7
4	33.5	40.5	58.4	100.0	51.3	44.6	39.6	35.7	33.5
5	22.1	48.5	45.1	51.3	100.0	52.1	39.6	26.7	39.9
6	35.9	43.1	44.1	44.6	52.1	100.0	40.1	34.1	29.4
7	24.2	47.0	42.5	39.6	39.6	40.1	100.0	25.0	37.9
8	25.6	25.1	29.5	35.7	26.7	34.1	25.0	100.0	25.0
9	22.2	30.7	34.7	33.5	39.9	29.4	37.9	25.0	100.0

Note: 1. Primeval forest surface; 2. Primeval forest stone ditch; 3. Primeval forest stone seam; 4. Shrubbery soil surface 5. Shrubbery stone ditch; 6. Shrubbery soil stone seam; 7. Secondary forest surface; 8. Secondary forest stone ditch; and 9. Secondary forest stone seam.

**Table 4. Shannon Index, richness and evenness of bacterial communities in different microhabitats samples.**

Microhabitat Samples	Shannon Index (H)	Richness (S)	Evenness (E)
Primeval forest soil surface	3.13	24	0.99
Primeval forest stone ditch	3.48	33	1.00
Primeval forest stone seam	3.54	35	1.00
Shrubbery forest soil surface	3.51	34	1.00
Shrubbery forest stone ditch	3.65	39	1.00
Shrubbery forest stone seam	3.48	33	1.00
Secondary forest soil surface	3.57	36	1.00
Secondary forest stone ditch	3.38	30	0.99
Secondary forest stone seam	3.42	31	1.00
Mean value	3.46	32	0.99

surface, and the order of fungal Shannon index and richness of secondary forest microhabitats and shrubbery were: soil surface > stone seam > stone ditch and stone ditch > soil surface > stone seam, respectively. However, the diversity index and abundance of different microhabitats did not show the same trend.

### Genetic Diversity Characteristics of Fungi in Karst Microhabitats

#### DGGE Analysis of Soil Fungal Communities

The fungal communities of different soil samples were observed by DGGE (Fig. 4A). Similar to bacteria, all the lanes had a large number of bands that showed each karst microhabitat was rich in fungal diversity (Fig. 4B). There were both the conserved and specific bands among all lanes, indicating that there were both similar and specific fungal populations in the soil in different types of microhabitats in karst. It was obvious that there were more fungal bands than bacterial bands, which suggested different abundance and diversity of fungal and bacterial species in karst microhabitats. From the DGGE profile, the shrubbery forest (lane 4, 5, and 6; 52, 64, and 46 bands out of 129, respectively) exhibited the most abundant bands, and the abundance of secondary and primeval forest was similar, which was also similar to bacteria (line 34, 38, and 37 bands out of 78, respectively). Lane 8 from the primeval forest had the lowest quantity of bands (42 bands out of 129). The average fungal bands in stone seam were higher than those in stone ditch and soil surfaces (52 bands,

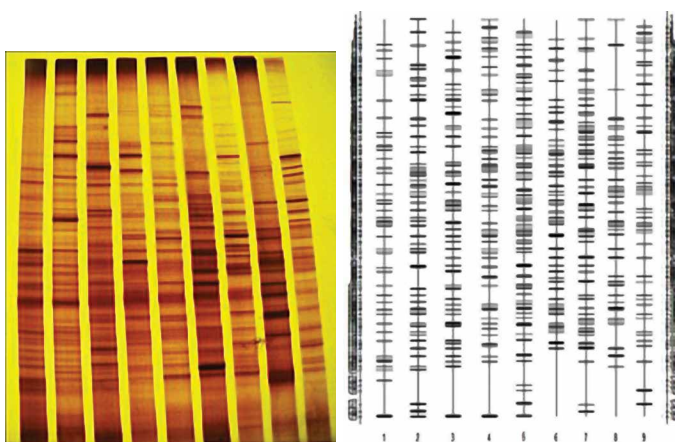


Figure 4. DGGE results of fungal communities in different karst microhabitats samples: A = DGGE profile of amplified of fungal communities in different karst microbial samples, and B = DGGE sketch map of fungal communities in different karst microhabitats samples. Numbering is: 1. Secondary forest stone seam; 2. Secondary forest stone ditch; 3. Secondary forest soil surface; 4. Shrubbery stone seam; 5. Shrubbery stone ditch; 6. Shrubbery soil surface; 7. Primeval forest stone seam; 8. Primeval forest stone ditch; and 9. Primeval forest soil surface.

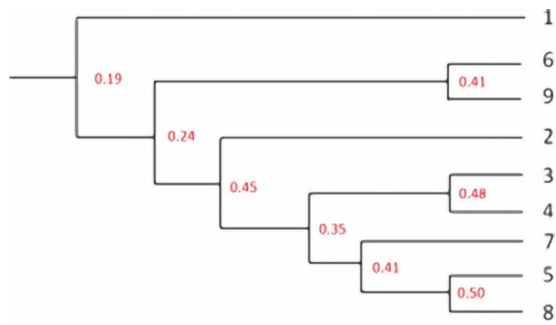


Figure 5. Similarity cluster of fungal communities among different microhabitats samples. Numbering is: 1. Secondary forest stone seam; 2. Secondary forest stone ditch; 3. Secondary forest soil surface; 4. Shrubbery stone seam; 5. Shrubbery stone ditch; 6. Shrubbery soil surface; 7. Primeval forest stone seam; 8. Primeval forest stone ditch; and 9. Primeval forest soil surface.

45 bands, and 49 bands, all out of 127), but the bacterial bands in the three microhabitats were similar.

### Similarity Analysis of Fungal Communities in Different Microhabitats

As shown in Figure 5, the dendrogram gave rise to two main branches of clusters and shared a similarity of 0.19 (Fig. 5, Table 5). The fungal communities between secondary forest stone ditch and shrubbery stone ditch stayed closer with a high similarity of 0.50. The similar indexes of the stone ditch of three microhabitats were similar. The primeval forest surface 9 had correlation with secondary forest surface 3 (16.9 %) and shrubbery soil surface 6 (19.0 %), which were a little low but very close. In general, the similarity index of community structure among different microhabitats was very low, indicating that there were differences in fungal community structure among different karst microhabitats.

Table 5. The similarity index of different microhabitats sample lanes of microbial communities (%).

Lane	1	2	3	4	5	6	7	8	9
1	100.0	29.0	26.3	41.2	35.5	18.0	17.5	21.8	17.9
2	29.0	100.0	39.0	21.6	49.9	28.8	30.4	33.6	19.1
3	26.3	39.0	100.0	26.8	43.9	42.4	31.5	25.8	16.9
4	41.2	21.6	26.8	100.0	32.7	19.3	22.6	27.2	18.5
5	35.5	49.9	43.9	32.7	100.0	34.7	34.4	38.2	33.7
6	18.0	28.8	42.4	19.3	34.7	100.0	48.2	33.2	19.0
7	17.5	30.4	31.5	22.6	34.4	48.2	100.0	34.7	30.1
8	21.8	33.6	25.8	27.2	38.2	33.2	34.7	100.0	16.5
9	17.9	19.1	16.9	18.5	33.7	19.0	30.1	16.5	100.0

Note: 1. Secondary forest stone seam; 2. Secondary forest stone ditch; 3. Secondary forest surface; 4. Shrubbery soil stone seam; 5. Shrubbery stone ditch; 6. Shrubbery soil surface; 7. Primeval forest stone seam; 8. Primeval forest stone ditch; 9. Primeval forest surface.

### Fungal Diversity and Richness Analysis

We then calculated the diversity index of soil fungal communities in different karst microhabitats samples by Shannon index (H), richness (S) and evenness (E) (Table 6). The highest diversity index of karst microhabitats fungi was primeval forest stone seam (4.11 and 62), shrubbery forest stone ditch (4.32 and 76) and stone seam (4.10 and 61). The lowest diversity index of all the microhabitats were primeval forest stone ditch (3.75 and 43) and secondary forest stone seam (3.78 and 44). The mean value in fungal Shannon index among different microhabitats was 3.99. There was no difference among the evenness from the nine types of microhabitats. The trend of fungal Shannon index and the richness of primeval forest microhabitats was: stone seam > stone ditch > surface, and the trends of fungal Shannon index

Table 6. Shannon index, richness and evenness of fungal communities in different karst microhabitats samples.

Microhabitat samples	Shannon index (H)	Richness (S)	Evenness (E)
Primeval forest surface	3.90	50	1.00
Primeval forest stone ditch	3.75	43	1.00
Primeval forest stone seam	4.11	62	1.00
Shrubbery forest surface	3.96	53	1.00
Shrubbery forest stone ditch	4.32	76	1.00
Shrubbery forest stone seam	4.10	61	1.00
Secondary forest surface	3.98	54	1.00
Secondary forest stone ditch	4.00	55	1.00
Secondary forest stone seam	3.78	44	1.00
Mean value	3.99	55	1.00

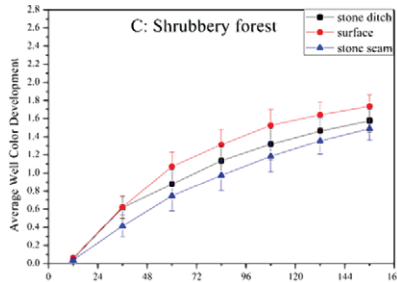
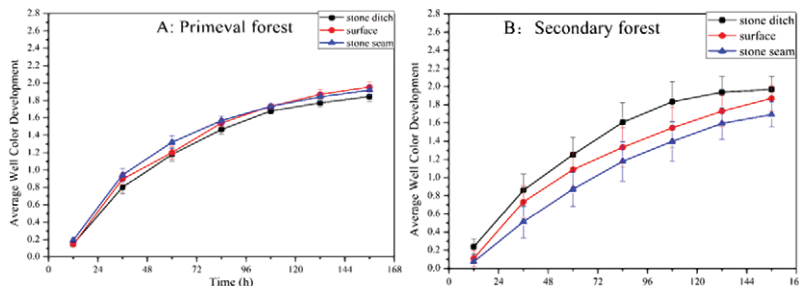


Figure 6. AWCD changes with incubation progress and comparisons among different vegetation.

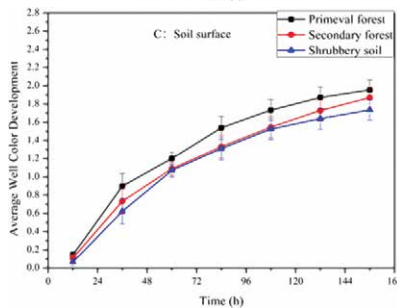
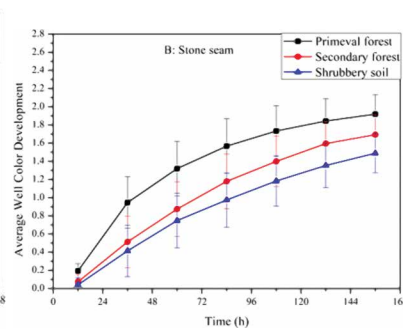
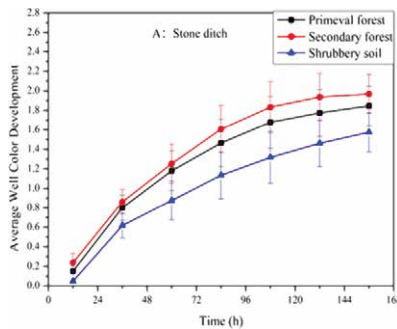


Figure 7 AWCD changes with incubation progress and comparisons among different microhabotats.

and richness of secondary forest microhabitats and shrubbery were: stone ditch > surface > stone seam and stone ditch > stone seam > surface, respectively.

### AWCD Analysis of Microbial Diversity in Karst Microhabitats

Average Well Color Development (AWCD) using Biolog EcoPlates is used to indicate the activity of soil microorganisms and the ability of soil microorganisms to use single carbon sources, and it could reflect soil microbial activity and microbial community physiological functional diversity (Miao et al., 2019). The density and activity of bacteria increased with the AWCD. Conversely, the lower the AWCD value would indicate lower bacterial density and lower activity. We accessed the different microhabitats of the same sample plot (Fig. 6). The AWCD values of the primeval forest including stone ditch, stone seam, and soil surface did not show differences, which indicated that the activity values were basically the same (Fig. 6A). The AWCD values and microbial activity of the secondary forest were obviously different during the growth period and the trend of three microhabitats was: soil surface > stone ditch > stone seam (Fig. 6B). The study accessed the same microhabitats of different sample vegetation (Fig. 7A–C). The AWCD values of three different types of vegetation in the stone ditch were different and the trend was: secondary forest > primeval forest > shrubbery forest (Fig. 7A). The three AWCD curves of the stone seam and soil surface were clearly different and the trend of microbial activity was the same: primeval forest > secondary forest > shrubbery forest (Fig. 7E–F).

### Diversity Index of Soil Microbial Community Metabolism Function

Shannon's diversity index indicates functional diversity, which means that the number of carbon sources used by soil microbial communities of all the ecosystems when the color change rate is consistent. For example, the bigger the Shannon index is the higher is the soil microbial community functional diversity. McIntosh index includes the number of species (richness) and the evenness of individual distribution in species. The diversity will become higher as the number of microbial types increases. Similarly, an increase in the evenness of individual distribution among species will also increase the functional diversity. The Simpson index is always used to access the dominant species (Ma, 2019). Table 7 shows the diversity index based on 96 hours of data. In the primeval forest, the Shannon's diversity indexes of three kinds of microhabitats were: stone ditch > soil surface > stone seam (Table 7). As for secondary and shrubbery forest, the trends of Shannon's diversity index were stone ditch > soil surface > stone seam and soil surface > stone ditch > stone seam, respectively. On the whole, the highest Shannon's diversity index was the secondary forest stone ditch (3.29), while the lowest was the secondary stone seam (3.15). The Simpson indexes of all investigated karst microhabitats were between 0.95–0.96, which were not different. The McIntosh indexes of karst microhabitats in primeval forest were different. The trend was: stone seam > soil surface > stone ditch. In the secondary forest microhabitats, the McIntosh index of stone ditch > soil surface > stone seam. The trend of McIntosh index of shrubbery forest was: soil surface > stone ditch > stone seam, which was also different among primeval and secondary forest. Overall, the highest and lowest evenness index was the primeval stone seam (10.28) and shrubbery stone seam (6.79).



**Table 7. Shannon's diversity index, Simpson index and McIntosh index of nine karst microhabitats.**

Sample	Shannon Index	Simpson Index	McIntosh Index
Primeval forest soil surface	3.25 ± 0.05	0.958 ± 0.002	10.19 ± 0.61
primeval forest stone ditch	3.26 ± 0.01	0.959 ± 0.000	9.62 ± 0.90
Primeval forest stone seam	3.19 ± 0.04	0.955 ± 0.002	10.65 ± 0.71
Secondary forest soil surface	3.20 ± 0.04	0.955 ± 0.002	9.15 ± 0.46
Secondary forest stone ditch	3.29 ± 0.04	0.960 ± 0.002	10.28 ± 1.14
Secondary forest stone seam	3.13 ± 0.04	0.951 ± 0.002	8.39 ± 0.60
Shrubbery soil surface	3.21 ± 0.02	0.956 ± 0.001	8.79 ± 0.56
Shrubbery stone ditch	3.18 ± 0.05	0.955 ± 0.003	7.78 ± 0.94
Shrubbery stone seam	3.15 ± 0.06	0.952 ± 0.004	6.79 ± 0.86

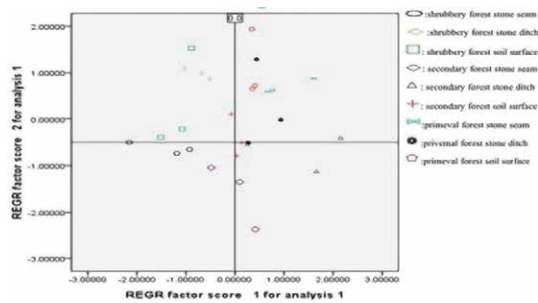


Figure 8. PCA of soil microbial carbon source utilization diversity of different karst microhabitats.

### Principal Component Analysis (PCA) of Soil Microbial Carbon Source Utilization

Different microhabitats showed differences on PC1 and PC2, indicating that their soil microorganisms do use different carbon sources (Fig. 8, Tables 2 and 8). The significance value less than 0.05 have been marked in red. The significance value is less than 0.05, indicating that the two objects have significant differences. Further variance analysis of the scoring coefficients of the principal components of carbon sources in different microhabitat habitats showed that on PC1, the soil microbial metabolism patterns in three types of microhabitats (stone seam, stone ditch, and soil surfaces of shrubbery forest) were not significantly different. In the secondary forest plots, there were significant

differences in the metabolic patterns between the stone seam and stone ditch, and between the stone ditch and the soil surface, but there was no significant difference in the metabolic patterns between the stone seam and the soil surface. On PC2, there were significant differences between the stone seam and the soil surface of the shrub forest, and there were no significant differences between the stone seam and the ditch, and between the stone ditch and the soil surface. There was no significant difference in the soil surface, stone seam, and stone ditch in the primeval forest. As for carbon resources analysis, there were five kinds of amino acids, four kinds of carbohydrates, three kinds of polymers, two kinds of carboxylic acids and amines, and one kind of amphiphilic compound showing significant relation to PC1, which indicated that the main carbon resources influenced PC1 were amino acids, carbohydrates, and polymers. Similarly, among the carbon sources that were significantly related to PC2, the carbon source affecting PC2 was mainly the carbohydrates because of six carbohydrates of PC2. In summary, the carbon sources that differentiated the microhabitat soil microbial metabolism patterns were mainly carbohydrates, amino acids, and polymers.

## DISCUSSION

Karst microhabitats are special and unique environmental units and have different effects on ecological environments in karst regions. Soil microorganisms are very important to the ecological restoration in karst regions. At different stages of desertification, the number and distribution of microorganisms are also different. Therefore, the soil microbiome can be a good indicator of soil quality and soil health in the karst rocky desertification regions (Tang et al., 2019). In fact, soil microorganisms in different microhabitats have different distributions and structural features that may indicate the role of soil microorganisms, such as bacteria and fungi, would also be different. The relationship between genetic and functional diversity in different microhabitats and soil microbial diversity is more direct. Some existing studies have ignored the various characteristics and roles of soil microorganisms in the microhabitats (Yu et al., 2004; Wei et al., 2008). Our experiments made up for this shortcoming to some degree and more comprehensively considered and studied the genetic and functional diversity of bacteria and fungi in different karst microhabitats, which may provide new insights into ecological restoration and rocky desertification management in karst regions.

There many ways to study microbial diversity in different microhabitats. Our study showed that the diversity of bacteria and fungi in microhabitats of the karst could be detected by PCR-DGGE. Wu et al. (2019) used phospholipid fatty acid (PLFA) technology to study the bacterial and fungal diversity in Maolan Natural Nature Reserve. The trends of diversity were: the stone trough > the stone ditch > the soil surface > the stone cave > the stone seam, which were similar to our result. Liao et al. (2013b) used substrate-induced respiration to access the amount of the soil microbial biomass in karst regions in Huajiang dry hot valley watershed in Guizhou Province. The results showed that the substrate-induced respiration of soil from their stone ditch was relatively high in all microhabitats investigated.

Table 8. The Principle Component scores for different microhabitats.

Dependent Variable	(I) VAvR00001	(J) VAR00001	Mean Difference (I-J)	Std. Error	Significance	95% Confidence Interval	
						Lower Bound	Upper Bound
REGR factor score 1 for analysis 1	1	2	0.46562679	0.40115217	0.261	-0.3771627	1.3084162
		3	0.64492381	0.40115217	0.125	-0.1978656	1.4877133
	2	1	-0.46562679	0.40115217	0.261	-1.3084162	0.3771627
		3	0.17929703	0.40115217	0.660	-0.6634924	1.0220865
	3	1	-0.64492381	0.40115217	0.125	-1.4877133	0.1978656
		2	-0.17929703	0.40115217	0.660	-1.0220865	0.6634924
	4	5	-1.34571883 <sup>a</sup>	0.40115217	0.004 <sup>a</sup>	-2.1885083	-0.5029294
		6	-0.02245966	0.40115217	0.956	-0.8652491	0.8203298
	5	4	1.34571883 <sup>a</sup>	0.40115217	0.004 <sup>a</sup>	0.5029294	2.9387222
		6	1.32325917 <sup>a</sup>	0.40115217	0.004 <sup>a</sup>	0.4804697	2.1885083
	6	4	0.02245966	0.40115217	0.956	-0.8203298	0.8652491
		5	-1.32325917 <sup>a</sup>	0.40115217	0.004 <sup>a</sup>	-2.1660486	-0.4804697
	7	8	-0.26144823	0.40115217	0.523	-1.1042377	0.5813412
		9	-0.68163703	0.40115217	0.106	-1.5244265	0.1611524
	8	7	0.26144823	0.40115217	0.523	-0.5813412	1.1042377
		9	-0.42018880	0.40115217	0.309	-1.2629782	0.4226006
	9	7	0.68163703	0.40115217	0.106	-0.1611524	1.5244265
		8	0.42018880	0.40115217	0.309	-0.4226006	1.2629782
REGR factor score for analysis 1	1	2	0.43734062	0.50234562	0.395	-0.6180484	1.4927296
		3	-0.41109415	0.50234562	0.424	-1.4664831	1.6442948
	2	1	-0.43734062	0.50234562	0.395	-1.4927296	0.6180484
		3	-0.84843477	0.50234562	0.108	-1.9038238	0.2069542
	3	1	0.41109415	0.50234562	0.424	-0.6442948	1.4664831
		2	0.84843477	0.50234562	0.108	-0.2069542	1.9038238
	4	5	-0.88944052	0.50234562	0.094	-1.9448295	0.1659485
		6	-1.19088983	0.50234562	0.029 <sup>a</sup>	-2.2462788	-0.1355009
	5	4	0.88944052	0.50234562	0.094	-0.1659485	1.9448295
		6	-0.30144931	0.50234562	0.556	-1.3568383	0.7539397
	6	4	1.19088983 <sup>a</sup>	0.50234562	0.029 <sup>a</sup>	0.1355009	2.2462788
		5	0.30144931	0.50234562	0.556	-0.7539397	1.3568383
	7	8	-0.93958746	0.50234562	0.078	-1.9949764	0.1158015
		9	-1.61499360 <sup>a</sup>	0.50234562	0.005 <sup>a</sup>	-2.6703826	-0.5596046
	8	7	0.93958746	0.50234562	0.078	-0.1158015	1.9949764
		9	-0.67540615	0.50234562	0.195	-1.7307951	0.3799828
	9	7	1.61499360 <sup>a</sup>	0.50234562	0.005 <sup>a</sup>	0.5596046	2.6703826
		8	0.67540615	0.50234562	0.195	-0.3799828	1.7307951

<sup>a</sup> The mean difference is significant at the 0.05 level.

Note: 1. Primeval forest stone seam; 2. Primeval stone ditch; 3. Primeval soil surface; 4. Secondary forest stone seam; 5. Secondary forest stone ditch; 6. Secondary soil surface; 7. Shrubbery stone seam; 8. Shrubbery stone ditch; 9. Shrubbery soil surface;

### Effects of Karst Microhabitats on Soil Microbial Genetic Diversity

In our study, the karst microhabitats investigated had abundant bacterial and fungal communities and functional diversity. The bacterial communities among nine research sites do have differences. Cluster analysis (Fig. 3) indicated that the bacterial community structure of different microhabitats under the same vegetation type was more similar than that of similar microhabitats under different vegetation types. The vegetation, rather than microhabitats, was more important to the distribution of bacteria in karst. In previous studies, we could demonstrate that the differences in the karst microhabitats soil heterogeneity were manifested by changes in clay particles, micro-aggregates, and effective nutrient quantities (Zhou and Lei, 2007; Wakerlin et al., 2008; Zhang et al., 2015). All the factors could influence the bacterial diversity on a high level, which was also the reason why the bacterial community structure was different among all kinds of microhabitats. Vegetation is an important source of soil nutrients. Microorganism distribute by the types and amount of plant litter and the rate of water loss from the soil surface. Under the same vegetation, the structure and kind of soil carbon and nitrogen resources are basically consistent, while other factors including soil water content, temperature, pH, aeration, physical structure, and the organic content could be different (Murugan et al., 2014; Ren et al., 2018). Therefore, our study shows that the bacterial community structure was mainly controlled by the composition of carbon source at the micro-geomorphic scale of microhabitats.

The distribution of fungi was sensitive to changes in environmental factors that may lead to changes in diversity. The heterogeneity of different microhabitats is high in water content, heat, light, and soil fungal diversity. The heterogeneity may be the reason why the fungal diversity may be lower than bacterial diversity in different microhabitats. According to the phylogenetic tree (Fig. 5) for fungi, similar microhabitats in different vegetation types were more similar than those in the same vegetation type. The impact of microhabitats on its diversity and community structure was greater than the impact of vegetation types on its diversity, which was opposite to bacterial diversity. Our research indicated that the spatial heterogeneity had different influences on the bacteria and fungi. Many studies had illustrated that the soil organic content could significantly influence the soil fungal community structure (Fan et al., 2012; Chen et al., 2016; 2017).

Hu et al. (2016) employed site comparisons of Chinese toona reforestation to study the relationship among soil microbial biomass carbon (MBC), microbial biomass nitrogen, and other organic matter and soil microbial community structure and diversity. Their results showed that the microbial biomass and activity of reforested soil were higher, which meant that the vegetation could increase the microbial diversity by increasing the content of soil organic matter. The complexity of vegetation carbon sources, the organic matter imported, and the spatial and temporal differences of carbon sources in the same system are three important factors influencing soil fungal functional diversity. The spatial and temporal differential may have a closer connection with the fungal community functional diversity in karst regions because the fungal community structure and abundance of different microhabitats of the same vegetation type were both different. In fact, the dominant species of fungi among different microhabitats were very different, which indicated that for a single karst vegetation type or ecosystem, the microhabitats within it could play all kinds of roles on the whole material cycle by changing microbial structure and dominant species. In the future, when carrying out karst ecosystem protection and rocky desertification restoration, it is necessary not only to pay attention to the role of the microhabitat, but also to take into account differences in the ecological effectiveness of the microhabitat in different restoration stages.

### Effects of Karst Microhabitats on Soil Microbial Functional Diversity

Among the three vegetation types, the metabolic activities of the three types of microhabitat in each plot were different. The differences were not obvious in the primary forest, but reached a significant level in the secondary and shrub forest. On the whole, the karst microhabitats had a significant impact on the soil microbial activity. The trend of microbial activity of three types of microhabitats was: stone ditch > soil surface > stone seam, possibly due to structural differences among the karst microhabitats. The stone seam is relatively closed with small openings and weak material accumulation, leading to lower microbial activity than other microhabitats. The stone ditch and soil surface are relatively open to the environment with a large area and a large amount of litter accumulation. Litter could provide rich carbon sources during the decomposition process (Zhu et al., 2012) that is beneficial to the microbial growth and material cycle (Liao et al., 2012; 2013a). The changing trends of AWCD of three samples were basically maintained in primary forest >> secondary forest > shrubbery forest, indicating that the vegetation degradation or reverse succession would decrease soil microbial activity of all microhabitats, even the whole ecosystem microbial activity consistent with the study by Zhu et al. (2012). The microbial metabolism patterns of the three types of microhabitats under the same vegetation type showed basically no significant differences, while the microbial metabolism patterns of the same vegetation type under the same microhabitats showed significant differences. According to the results of AWCD, for samples of the same vegetation type, the heterogeneity brought by different microhabitats was mainly reflected in the number of single populations of microorganisms rather than microbial community structure, so the metabolic pattern did not change. However, the change of vegetation types would affect the community structure and the number of single populations of microorganisms simultaneously, which made the metabolic patterns different from each other. In conclusion, the vegetation may be the main factor influencing soil microbial metabolic patterns in karst regions.

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