Effect of karst rocky desertification on soil fungal communities in Southwest China

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ABSTRACT. Karst mountainous ecosystems are associated with karst rocky desertification (KRD), which can greatly impact soil structure and function. Despite the importance of soil microbes as a major factor maintaining ecosystem stability, we know little about the effect on soil fungal communities of KRD in karst regions. We investigated this relationship across a gradient of KRD soils from Guizhou, China by polymerase chain reaction and denaturing gradient gel electrophoresis (PCR-DGGE). Fungal diversity indices (Shannon-Wiener, richness, and evenness) significantly differed ($P < 0.05$) based on KRD severity, being lowest in moderately affected areas. Cluster analysis showed that the five sites examined clustered into two main groups according to KRD grade (high and low). Moreover, a homology search using sequences recovered from PCR-DGGE bands showed that the dominant fungi in each community varied remarkably, and included *Aspergillus*, *Aphanoascus*, *Blastomyces*, *Fusarium*, *Glomus*, *Geomyces*, *Gibberella*,

Mortierella, *Tetracladium*, and *Tumularia* species, and an unclassified group. In conclusion, these findings demonstrate that KRD has a significant impact on soil fungal communities.

Key words: Fungi; PCR-DGGE; Karst; Rocky desertification; 18S rRNA

INTRODUCTION

Karst mountainous regions are fragile environments highly sensitive to change. The karst mountainous region of Southwest China, an area spanning approximately 540,000 km^2, is among the world's three largest continuous karst regions (Huang et al., 2009; Qi et al., 2013; Yang et al., 2013). Over the past decades, inappropriate agricultural practices and over-exploitation of sloping lands have caused these areas to suffer greatly from karst rocky desertification (KRD), resulting in severe soil erosion and widespread exposure of basement rocks (Wu et al., 2011). KRD events can have a large impact on the structure and functioning of ecosystems through a variety of effects on soil, including structure deterioration, texture and porosity degeneration, decline in water capacity, and leanness (Huang et al., 2009; Peng et al., 2013; Tang et al., 2013). Therefore, these events may lead to serious economic constraints on the development of affected regions. Research on changes to soil structure in typical KRD regions has been limited to the study of basic physical and chemical properties (Zhou et al., 2012; Peng et al., 2013; Tang et al., 2013). Few studies, however, have investigated the effect on soil microbes.

Soil microbes play key roles in controlling land degradation processes such as soil loss, nutrient depletion, and reduced water holding capacity (Bossio et al., 2005; Souza et al., 2014). Several aspects of microbial activity have been proposed as soil quality measures due to their sensitivity, and have been used as bioindicators of degradation processes (Ros et al., 2003, 2009). Fungi are a fundamental component of soil microbial communities (Hagn et al., 2003; Orlandelli et al., 2012), and play important roles in many essential processes, including organic matter decomposition, elemental release by mineralization, and protection against leaching by elemental storage in biomass (Hagn et al., 2003; Malosso et al., 2006; Premalatha et al., 2015). Moreover, their mycelia contribute to soil aggregate stability, thereby preventing erosion (Rao et al., 2012). Therefore, changes in the composition of the fungal community can be used to assess soil quality, and research into such alterations may provide helpful information to assess the effectiveness of ecological restoration in karst regions. However, little is known about the relationship between soil fungal communities and KRD in karst mountainous regions exposed to considerable human activity.

Polymerase chain reaction coupled with denaturing gradient gel electrophoresis (PCR-DGGE) has been widely used to determine the species richness and structure of fungal communities in a variety of environments (Sigler and Turco, 2002; Costa et al., 2006; Yu et al., 2013; Zhang et al., 2013; Dieng et al., 2014). Primarily, this technique is carried out by amplifying specific sequences of interest, such as ribosomal RNA (rRNA) genes (Cahyani et al., 2004; Zhao et al., 2012; Yan et al., 2013; Yoshitake et al., 2013). It is a quicker and less labor-intensive approach than sequencing clone libraries for comparing

community composition across samples (Oros-Sichler et al., 2006; Liang et al., 2008). Besides, PCR-DGGE enables the detection of unculturable microorganisms and is better suited to the investigation of soil microbial diversity (Liang et al., 2008; Yan et al., 2013; Yoshitake et al., 2013; Najnin et al., 2015). Therefore, it also formed an important part of this study.

In order to address the relative lack of knowledge regarding the diversity and composition of soil fungal communities in karst ecosystems affected by KRD, our study aimed to characterize the relationship between soil fungi and KRD severity in the karst mountainous region of Southwest China.

MATERIAL AND METHODS

Field surveys and soil sampling

Five areas affected by typical degrees of KRD were selected in October 2014 at experimental sites established in Puding County, Guizhou Province, Southwest China. Puding has a northern subtropical wet monsoon climate with a mean annual temperature of 15.1°C (minimum and maximum monthly mean temperatures are 5.2°C in January and 23°C in July, respectively), and an annual rainfall of 1374.3 mm, of which 83.6-90.2% falls in May-October. The terrain surface undulates lightly, with an average slope of 6.9°. The average elevation is 1291 m, with a maximum height of 1551 m and a minimum of 1219 m. Yellow soil is the main soil type. Various types of natural vegetation are present, with evergreen and deciduous broad-leaved forest predominating (Zhang et al., 2010).

Xiong et al. (2002) proposed classification of KRD based on four factors: vegetation (%), slope (°), bare rock (%), and average topsoil depth (cm). According to these factors, we chose five sites representing typical KRD grades, namely, A) potential, B) slight, C) moderate, D) strong, and E) extremely strong. Basic characteristics of the selected sites and the dominant species present are described in Table 1.

Table 1. Description of the selected karst rocky desertification (KRD) sites.

KRD type	Code	Exposed rock (%)	Vegetation coverage (%)	Land use/type (dominant species)
Potential	A	20-30	80-70	Sloping cropland (maize + soybean intercropping)
Slight	B	31-50	69-50	Shrubland (*Coriaria sinica* + *Cotoneaster adpressus* + *Rosa cymosa* + *Pyracantha fortuneana*)
Moderate	C	51-70	49-30	Middle-coverage grassland (*Themeda japonica* + *Eremochloa ophiuroides* + *Arthraxon hispidus*)
Strong	D	71-90	29-10	Shikala land* (maize monoculture)
Extremely strong	E	>90	<10	Deserted shikala land (*Digitaria sanguinalis* + *Bidens bipinnata* + *Conyza canadensis*)

*In most KRD regions, the remaining cultivated fields are extremely small in size, dotted irregularly among exposed rocks and in fissures; this is referred to as shikala land (Li et al., 2009).

For each sampling site, five to eight cores were taken from surface soil (0-15 cm) using a 3-cm diameter auger along an S-shaped transect, before being bulked and mixed as a single soil sample. Each soil sample was kept in sealed plastic bag at 4°C and immediately transferred to -70°C storage after transportation to the laboratory, where they remained until used for determination of fungal phylogenetic diversity.

Soil DNA extraction

To extract total microbial DNA from soil, 5 g each sample was used for genomic DNA isolation. Total genomic DNA was extracted and purified using a PowerSoil DNA Isolation Kit (MO BIO, Carlsbad, CA, USA) following the manufacturer protocol. The quality and quantity of genomic DNA was examined by agarose gel electrophoresis and using a NanoDrop 1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA), respectively.

PCR

Amplification of 18S rRNA genes was conducted with the primers GC-Fung and NS1 (Table 2). The 25-μL reaction mixture contained 50 ng template DNA (a 50X dilution of extracted DNA), 10 mM each primer, 2.5 mM each deoxynucleotide, 1X *Taq* buffer (200 mM Tris-HCl, pH 8.4, and 500 mM KCl), and 2.0 U *Taq* DNA polymerase (Invitrogen, Waltham, MA, USA). Amplification conditions consisted of a long denaturation at 94°C for 5 min, before 30 cycles of 94°C for 30 s, 60°C for 30 s, and 72°C for 15 s. This was followed by a single extension step at 72°C for 10 min.

Table 2. Primers used for amplification of 18S ribosomal RNA genes.

Primer	Sequence (5'-3')
NS1	GTA GTC ATA TGC TTG TCT C
Fung	CAT TCC CCG TTA CCC GTT G
GC-Fung	CGC CCG CCG CGC CCC GCG CCC GGC CCG CCG CCC CCG CCC CAT TCC CCG TTA CCC GTT G

Amplified PCR products were separated by 1% agarose gel electrophoresis. DNA fragments of the correct size were excised from the gel and purified using a TIANgel Midi Purification Kit (TIANGEN, Beijing, China) following the manufacturer protocol. Gel-purified amplicons were verified by 1% agarose gel electrophoresis.

DGGE analysis

The DCode Universal Mutation Detection System (Bio-Rad, Hercules, CA, USA) was used to generate DGGE fingerprints. An 8-μL aliquot of each purified PCR product was loaded on a 16 x 16-cm DGGE gel, comprising 8% polyacrylamide (w/v) and a 35-60% denaturing gradient of urea plus formamide. The products were resolved by electrophoresis in 1X Tris-acetate-ethylenediaminetetraacetic acid (TAE) buffer at 60°C under a constant voltage of 60 V for 12 h. Gels were stained for 30 min in 1X TAE buffer containing SYBR Green I (Sigma, St. Louis, MO, USA), and visualized by ultraviolet transillumination (Gel Doc; Bio-Rad). The position and intensity of each band were determined using the Quantity One software v4.62 (Dice, 1945; Tang et al., 2013).

Cluster analysis of DGGE banding patterns was performed automatically by the Quantity One software. Similarities among soil samples were displayed graphically as

a dendrogram. The clustering algorithm used to calculate the dendrogram employed an unweighted pair group method with arithmetic mean, based on the Dice coefficient. Diversity of soil fungus communities was evaluated using the Shannon-Wiener index (H), richness (S), and evenness (E_H), based on Equations 1 and 2, as follows (Saikaly et al., 2005):

$$H = -\sum_{i=1}^{S} Pi \ln Pi \qquad \text{(Equation 1)}$$

$$E_H = H / H_{max} = H / \ln S \qquad \text{(Equation 2)}$$

where Pi 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.

Sequencing and phylogenetic affiliation

From the DGGE profiles, unique bands and one common band were selected for sequencing. The selected gene sequences were aligned against those of reference strains using the Basic Local Alignment Search Tool (http://www.ncbi.nlm.nih.gov/). All sequences recovered from DGGE bands and those of their closest reference strains were included in a maximum likelihood phylogenetic analysis carried out using PhyML v3.0. The general time-reversible + gamma distribution + invariant sites substitution model was chosen on the basis of the Akaike information criterion implemented in jModelTest 2 (Guindon et al., 2010; Darriba et al., 2012). Settings used in PhyML were as follows: BioNJ starting tree, four substitution rate categories, estimated proportion of invariable sites, and subtree pruning and regrafting and nearest neighbor interchange tree improvement algorithms. To assess support, 1000 nonparametric bootstrap replicates were used. Trees were drawn using the MEGA5 software (Saitou and Nei, 1987; Tamura et al., 2011; Tang et al., 2013).

RESULTS

Fungal diversity indices

We analyzed PCR-DGGE profiles of soil fungal communities at sites of potential, slight, moderate, strong, and extremely strong KRD in Guizhou, China. Fungi-specific amplification of 18S rRNA genes with GC-Fung and NS1 primers gave a clear PCR product of the expected size for all soil samples under investigation. The DGGE patterns exhibited differences between sites regarding the number, intensity, and migration rate of bands, suggesting shifts in fungal community structure in the bulk soils (Figure 1).

Each soil fungal diversity index (H, S, and E_H) showed the same varying trend as KRD grade increased (Figure 2). During the rocky desertification process, fungal diversity increased under slight KRD, but demonstrated the lowest values at the moderately affected site, showing the least complex banding pattern. Under strong KRD, all fungal diversity indices began to rise. Significant differences in the three indices were detected between degrees of KRD ($P < 0.05$). Moreover, measures of fungal diversity were lowest at the site of moderate KRD, indicating that this is an unstable stage in the karst ecosystem.
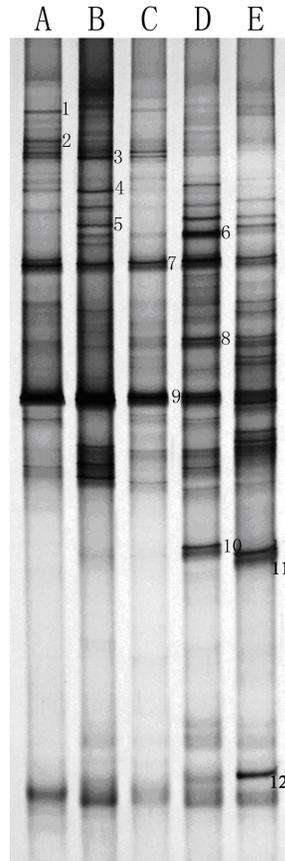
Figure 1. Denaturing gradient gel electrophoresis profiles of PCR products from fungi in potential (A), slight (B), moderate (C), strong (D), and extremely strong (E) karst rocky desertification soils. Labels indicate bands excised successfully for sequencing.
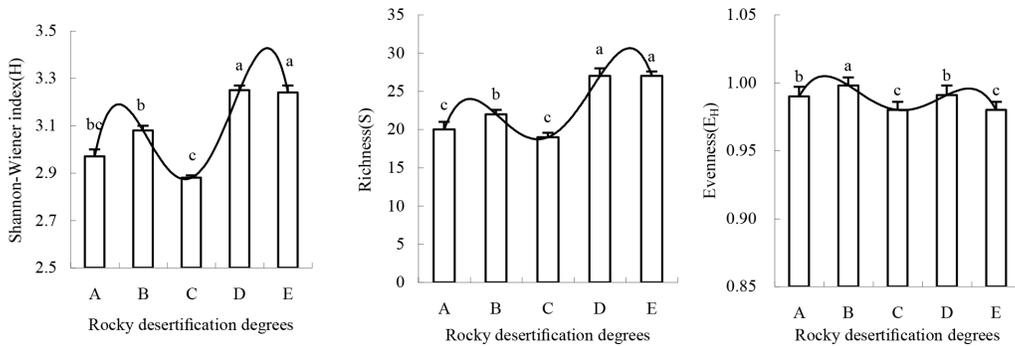

Figure 2. Genetic diversity indices of fungal communities in soils with potential (A), slight (B), moderate (C), strong (D), and extremely strong (E) karst rocky desertification. Means labelled with different letters (a–c) significantly differ (P < 0.05).

Similarity and cluster analysis

Cluster analysis showed that soil fungi DGGE patterns formed two main clusters, namely, high- and low-degree KRD groups (Figure 3). The former included sites D and E, and the latter sites A, B, and C, of which A shared 61% similarity with C, and (A, C) 57% similarity with B.

Fungal community analysis and phylogenetic affiliation

Twelve bands resulting from DGGE of amplified 18S rRNA genes from different KRD soils were excised for sequence analysis (Table 3). The phylogenetic tree constructed from partial 18S rRNA gene sequences is shown in Figure 4. The 12 DNA sequences recovered from the DGGE gel shared 96-100% sequence homology with reference sequences retrieved from the National Center for Biotechnology Information GenBank database, and two sequences shared 100% homology with characterized microorganisms such as *Geomyces destructans*. The fungal community in soil from site A was dominated by *Geomyces* (bands 1 and 2), that of B by *Geomyces*, *Aspergillus*, and *Tetracladium* (bands 3-5), that of C by *Mortierella* and *Tetracladium* (bands 7 and 9), that of D by *Gibberella*, *Fusarium*, and an unclassified group (bands 6, 8 and 10), and that of E by *Blastomyces*, *Aphanoascus*, *Mortierella*, and *Glomus* (bands 11 and 12). Compared to site A, bands unique to KRD-affected soils (bands 3-12) were associated with *Geomyces*, *Aspergillus*, *Tetracladium*, *Mortierella*, *Gibberella*, *Fusarium*, *Blastomyces*, *Aphanoascus*, and *Glomus* species, and an unclassified group.
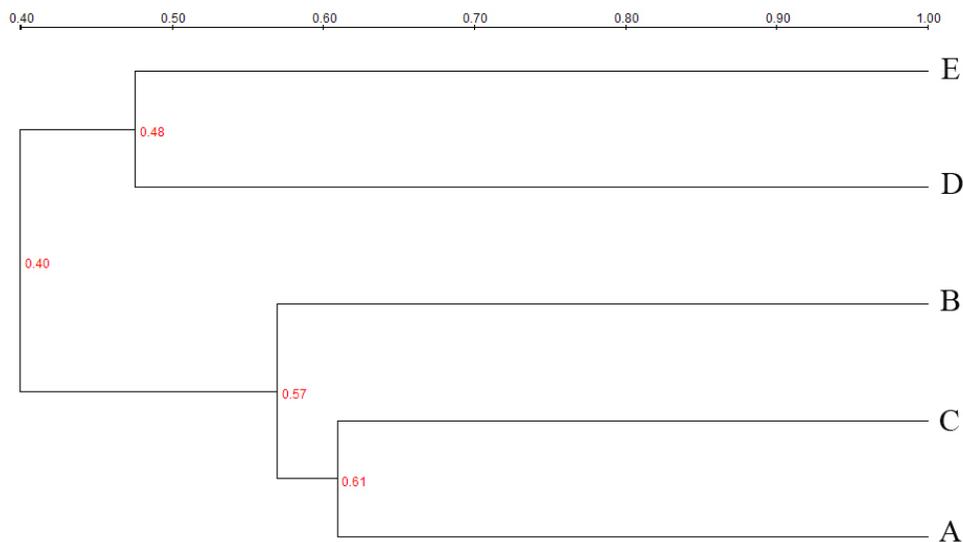

Figure 3. Cluster analysis of denaturing gradient gel electrophoresis profiles of fungi in soils affected by potential (A), slight (B), moderate (C), strong (D), and extremely strong (E) karst rocky desertification.

Table 3. Putative taxonomic affinities of 18S ribosomal RNA gene sequences generated from selected bands of denaturing gradient gel electrophoresis soil profiles.

Band	Accession No. of related sequence	BLAST match	Similarity (%)	Putative phylum
1	GU350434	*Geomyces destructans*	100	Dikarya
2	GU350434	*Geomyces destructans*	100	Dikarya
3	FJ231102	*Geomyces destructans*	98	Dikarya
4	ASNRR5SS	*Aspergillus fumigatus*	98	Dikarya
5	AY204617	*Tetracladium marchalianum*	99	Dikarya
6	KF357103	Uncultured eukaryote clone	99	Environmental sample
7	EU736290	*Mortierella alpina*	98	Fungi *incertae sedis*
8	JN236216	*Gibberella intermedia*	99	Dikarya
	KJ126877	*Fusarium oxysporum*	96	Dikarya
9	AY204617	*Tetracladium marchalianum*	99	Dikarya
10	AY357287	*Tumularia aquatica*	99	Dikarya
11	M63096	*Blastomyces dermatitidis*	99	Dikarya
	JN941595	*Aphanoascus verrucosus*	99	Dikarya
12	EU736292	*Mortierella minutissima*	99	Fungi *incertae sedis*
	FR750376	*Glomus macrocarpum*	98	Glomeromycota

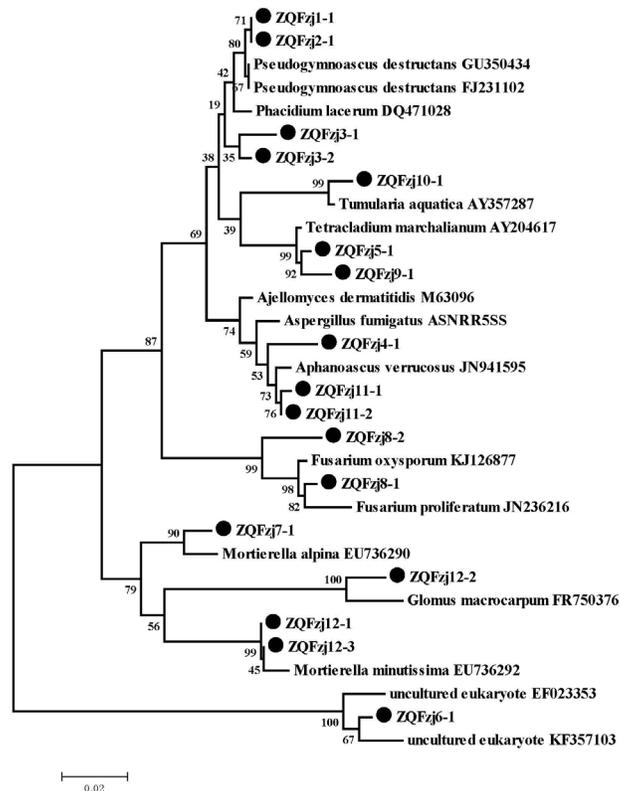BLAST = Basic Local Alignment Search Tool.

Figure 4. Maximum likelihood tree of partial 18S ribosomal RNA gene sequences from denaturing gradient gel electrophoresis bands and reference strains. Phylogenetic affiliations and accession numbers of reference strains are indicated. Bootstrap values >50% are shown at the nodes. The scale bar represents estimated number of substitutions per site.

DISCUSSION

Understanding shifts in soil fungal communities under complex environmental variations is of special importance in predicting responses of ecosystems to future degradative processes (Wang et al., 2014). Karst ecosystems are subject to rapid degradation events such as deforestation, soil loss, nutrient depletion, and reduced water holding capacity, resulting in KRD that can be difficult to reverse (Huang et al., 2009; Peng et al., 2013; Tang et al., 2013). In this study, we evaluated the ways in which KRD impacts soil fungal communities. Using molecular techniques, we assessed the structure and composition of such communities across a gradient of KRD stages.

Species richness and diversity in soil fungal populations are significantly altered in forest ecosystems according to the intensity of human disturbance (Wang et al., 2014). In this study, the three fungal diversity indices demonstrated the same varying trend across degrees of KRD severity (Figure 2), being lowest in the moderately affected site. This is probably because the population sizes of many species were reduced by disturbance, but gradually became stable by the strong KRD stage. Such variation is in agreement with the shifts in fungal communities associated with vegetation elimination (Ros et al., 2009) and succession (Zhu et al., 2012). In addition, cluster analysis showed that the five sites clustered into two main groups (Figure 3), consisting of high- and low-KRD categories, indicating a great difference between the 18S rRNA gene pools of the studied soils.

DGGE analyses of 18S rRNA gene fragments provide a measure of the composition and structure of the dominant species within a fungal community, and generate highly reproducible, site-specific profiles (Bossio et al., 2005). In the present study, these profiles primarily varied with regard to site, indicating that moderate KRD seems to have the strongest impact on fungal community structure, demonstrated by the least diverse DGGE pattern, with fewer bands and lower intensities. Soils exposed to strong and extremely strong KRD, on the other hand, showed DGGE bands of greater intensity and those not observed in soil from the moderate KRD site (Figure 1). These results are consistent with the findings of several other studies (Ros et al., 2009; Zhu et al., 2012). Smaller secondary influences were also found to result from differing KRD severity. We used a clone library approach to investigate the composition of soil fungi at each degree of KRD. Sequencing of DGGE bands revealed that KRD led to an increase in the proportion of some fungi (*Tumularia aquatica*, *Blastomyces dermatitidis*, *Aphanoascus verrucosus*, *Mortierella minutissima*, and *Glomus macrocarpum*), and a decrease in that of other common species such as *G. destructans*, compared with the site of potential KRD (Table 3). Taken together, these results suggest that KRD has a strong influence on soil fungal community structure. In addition, the unidentified group may play an important role in KRD-affected soil. However, even the functions of many well-studied genera in these ecosystems are not completely understood (Artz et al., 2007). Further investigations are required to characterize their association with karst degradation processes.

In conclusion, we have demonstrated that soil fungal communities are altered to different degrees according to KRD severity, and that this process has a negative long-term effect on such communities in karst ecosystems. Changes in fungal community composition are particularly apparent in increased KRD degrees. More importantly, shifts in the dominant fungal species are also associated with variations of KRD grade. Further investigations

should be carried out with a higher number of soil samples, and making use of other novel fingerprinting methods.

Conflicts of interest

The authors declare no conflict of interest.

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