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Decreased occurrence of carbon cycle functions in microbial communities along with long-term secondary succession

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ABSTRACT

The succession of microbial community structure and function is a central ecological topic; however, the mechanisms that underlie community assembly and promote temporal succession remain unclear. We studied microbial community-associated functional dynamics in a well-established secondary successional chronosequence that spans approximately 160 years of ecosystem development on the Loess Plateau of China, by sequencing both 16S and ITS rRNA genes and soil metagenomes, resulting in a total of 132.5 Gb of data. Notably, both bacterial and fungal communities shifted with succession, but the microbial community changed little from the pioneer forest stage (approximately 110 years) to the latter successional forest stage. Fungi showed higher variability with succession than bacteria, and the shift of both the bacterial and fungal communities was related more to the soil characteristics than to the litter characteristics. Shifts in soil microbial functions were associated with microbial phylogenetic changes, but microbial gene function also showed changes in the absence of phylogenetic changes at the late successional stages. The reduction in microbial C cycle genes was related to a decrease in litter decomposition ability, thus resulting in a steady state of nutrient cycle in the ecosystem. In addition, high microbial respiration in nutrient-rich soil does not necessarily indicate high microbial decomposition functions; the latter also depend on the abundance of related genes, on enzyme activity and on the physicochemical properties of the litter. Our study provides a metagenome profile of a successional chronosequence and provides insight into the mechanisms underlying the soil microbe-driven functional changes in nutrient cycles during succession.

1. Introduction

Secondary succession occurs in disturbed areas and is characterized by changes in plant coverage, composition, biomass, soil nutrient level, and soil erodibility (Finegan, 1984), which have attracted increased attention due to the increased amount of abandoned cropland (Grime, 2006; Van der Maarel and Franklin, 2012; Zhang et al., 2013). Microorganisms play an important role in the Earth's global biogeochemical cycles (Palomo et al., 2016) and are essential to soil functions, especially organic matter decomposition and nutrient cycling; they are therefore important in the regulation of plant productivity and community dynamics and in soil formation (Harris, 2009). Integrating microbial community structure and function into the study of ecological succession could provide considerable knowledge regarding the mechanisms that drive successional dynamics and that underlie microbe-driven nutrient cycles (Fierer et al., 2010), especially considering that little is known of the belowground microbial community that occurs with long-term succession in an ecosystem.

Associations between plants and soil microorganisms are essential and unavoidable (Chabrerie et al., 2003). Some studies have addressed the changes in microbial communities that occur during succession; however, these works reported inconsistent results regarding community-level diversity. Kuramae et al. (2010) observed a decrease in microbial richness with field age, whereas Jangid et al. (2011) reported that bacterial diversity remained unchanged after > 50 years of succession, and Zhang et al. (2016) described an increase in diversity after 10 years of secondary succession. Furthermore, a few studies reported that fungal communities are more easily changed than bacterial communities by plants along a successional gradient (Cutler et al., 2014). In addition, due to the variation in soil substrates and the heterogeneity of ecosystems, the existence of common patterns of change in microbial communities undergoing succession remains unclear, as do the roles of the bacterial and fungal communities in this process. Moreover, whether microbial communities stabilize in the latter successional stage is

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unclear (Fierer et al., 2010). Thus, the complex patterns of microbial succession remain to be revealed.

Numerous studies have addressed how soil physicochemical properties change with succession and have reported that succession can enhance the nutrient availability of degraded soil and maintain soil fertility, which is strongly related to microbe functions (Cline and Zak, 2015; Cong et al., 2015; Kuramae et al., 2010). However, it remains unclear how microbial metabolic functions and soil properties develop concurrently develop during succession. Previous studies have mainly focused on microbial community structure (Banning et al., 2011; Jangid et al., 2011; Zhang et al., 2016), whereas functional shifts at the genetic level have attracted little attention. In terrestrial ecosystems, microorganisms act as major players in the C and nitrogen (N) cycles (Harris, 2009) and mediate the transfer of photosynthetic products from plants into soil (Heimann and Reichstein, 2008; Högberg et al., 2001). Voříšková and Baldrian (2013) reported that fungi are the key players in litter decomposition because of their ability to produce a wide range of extracellular enzymes, whereas bacteria are mainly involved in soil nutrient cycles, which easily shift with changes in soil physicochemical properties during succession (Dini-Andreote et al., 2016). However, it remains unclear whether changes in the abundance of specific microbial taxa in succession are related to changes in community functioning due to the close association of plant and soil microbial communities through bidirectional exchanges (Bever, 1994). Zhang et al. (2013) reported a decreasing decomposition rate during secondary succession from grassland to forest; however, the relationship between decomposition rate change and microbial functions remains unclear. In addition, the role of microbial function shifts in the C and N cycles along the successional gradient remains unknown.

Succession provides an opportunity to study changes in plant communities and in belowground microbial processes. However, studies of long-term succession usually involve primary succession (Schmidt et al., 2008; Williams et al., 2013), whereas studies of changes in the composition and function of belowground microbial communities during secondary succession have only been conducted on relatively small scales (Banning et al., 2011; Zhang et al., 2016). To the best of our knowledge, intact, long-term secondary succession over a 100-year period from abandoned land to grassland to shrub and forest and finally to predominantly stable plant communities has rarely been studied; as a result, the establishment of microbial communities and shifts in microbial function during such long-term succession are poorly understood. Here, we use a chronosequence in the Ziwuling forest region on the Loess Plateau of China with approximately 160 years of secondary succession to investigate the patterns of bacterial and fungal community succession that are associated with functional change. These patterns are investigated using microbial rRNA genes, shotgun metagenomic sequencing of total soil DNA and a suite of complementary approaches to reveal the responses of the structure and function of soil microbial communities to long-term secondary succession. In addition, we assessed the litter decomposition rate, the microbial respiration rate, the ratio of carbon (C)-use efficiency to N-use efficiency (CUE:NUE), and the related potential enzyme activity, all of which are related to litter decomposition, in the long-term secondary succession.

The major aims of this study are to assess how the microbial community and its potential functions change during secondary succession and how they subsequently influence litter decomposition and the process of succession and to identify the mechanisms driving nutrient cycling over the course of succession. We ask (1) What are the patterns of microbial community composition and functional shifts that occur along the long-term successional gradient? (here, we chose a site that was naturally regenerated on abandoned land and that has been transformed from grassland to shrubland and latter successional forest over approximately the past 160 years) (2) Which component (the bacterial community or the fungal community) is more sensitive to succession, and which factor (soil or litter properties) is more associated with soil microbial shifts? (3) How does microbial potential function change with succession, and do shifts in microbial genes affect nutrient cycling and drive succession as feedback?

2. Materials and methods

2.1. Study area and soil sampling

This study was conducted at the Lianjiabian Forest Farm in the Ziwuling forest region in Gansu Province, China (35°03′-36°37′N, 108°10′-109°18′E, 1211–1453 m a.s.l.). This region covers a total area of 23,000 km^2 (Supplementary Figure S1). In this area, the annual temperature is 10 °C, the annual rainfall is 587 mm, and the soil pH ranges from 7.92 to 8.31. The region is covered in species-rich uniform forests with a forest canopy density ranging from 80% to 95%. The soil in the region is calcareous cinnamon soil (Ustalfs) that evolved from primary or secondary loess 50–100 m in depth (Jia et al., 2005; Zou et al., 2002).

Based on previous research in the study area, the secondary succession has naturally regenerated on abandoned cropland (maize and foxtail millet were the main rotational crops grown in these stands for > 15 years before afforestation). The arable land was abandoned at different times after the local inhabitants emigrated from this area due to famine, war and other disasters that have occurred since 1842; thus, various successional stages from grassland to shrubland and latter successional forest (*Q. liaotungensis*) have been observed in this region over approximately the past 160 years (Wang et al., 2010). Chen (1954) investigated the vegetation recovery in this area in the 1950s and found that P. *davidiana* made up 70% of the vegetation cover after approximately 100 years. Zou et al. (2002) investigated the vegetation succession in this area three times (in 1962, 1982 and 2000) and found that the *P. davidiana* forests were replaced by *Q. liaotungensis* forests after approximately 50 years; thus, the recovery period for *Q. liaotungensis* forests was approximately 160 years. The ages of shrub and herbaceous communities that have undergone less than 60 years of recovery were estimated by consultation with local elders and by taking into account land contracts between farmers and the government; the age of forest communities older than 60 years was also determined by evaluating tree rings and consulting relevant written sources (Zhong et al., 2017). Our group investigated the vegetation succession in 2005 (Wang et al., 2010) and 2015. We chose four vegetation recovery stages as the subject of the study: (1) the grassland stage (approximately 30 years, S1), in which *Bothriochloa ischaemum* (Linn.) Keng is the main herbaceous species; (2) the shrubland stage (approximately 60 years, S2), in which *Hippophae rhamnoides* (Linn.) is the main shrub species; (3) the pioneer forest stage (approximately 110 years, S3), which is dominated by *Populus davidiana* Dode; and (4) the latter successional stage (approximately 160 years, S4), which is dominated by *Quercus liaotungensis* Koidz.

The degree of temporal variability also plays an important role in soil microbial communities; however, because soil sampling is inherently destructive, the exact same location cannot be sampled repeatedly (Fierer, 2017). Furthermore, in the few cases in which temporal variation has been explicitly quantified, temporal variation is typically lower than spatial variation (Docherty et al., 2015; Lauber et al., 2013). Thus, in this study, we performed one-time sampling in late September 2015. When the leaves began to senesce, four independent replicate plots not less than 300 m apart and not more than 5 km apart were established for each stage; the difference in elevation between any two samples was less than 120 m. The sizes of the sample plots in the forest, shrub and herbaceous communities were 20 m × 20 m, 5 m × 5 m and 2 m × 2 m, respectively. All surveyed soils had developed from the same parent materials and had been vegetative for varying lengths of time.

Nine soil samples were randomly sampled and combined from the 0–20 cm soil layer (the humus layer was removed at the forest sites) in each replicate plot at each stage using a soil-drilling sampler (5 cm

inner diameter), resulting in 16 soil samples in total. All of the samples were sieved through a 2-mm screen to remove roots and other debris. A portion of each soil sample was collected in a 50-mL centrifuge tube, which was frozen with liquid N, placed in a dry-ice box and transferred to the laboratory. The tubes were maintained at −80 °C until soil DNA extraction. The remaining fresh soil was stored in an icebox and transferred to the laboratory for incubation and the measurement of soil physicochemical properties. Newly senesced leaf litter was collected from each plot and combined. In the grassland sites, standing dead, senesced *B. ischaemum* Keng litter was collected. The litter was air-dried at room temperature for the subsequent incubation study and measurement of physicochemical properties.

2.2. Litter decomposition incubation and respiration measurements

The incubation experiments were performed using PVC collars to detect the litter decomposition rate and soil mineralization. The detailed incubation methods, respiration measurements and soil and litter physicochemical measurements are provided in the Supplementary Methods section of the Supporting Information. The basic litter chemical properties and soil physicochemical properties are shown in Supplementary Tables S1 and S2, respectively.

2.3. DNA extraction, Illumina HiSeq 2500 sequencing and data processing

Microbial DNA was extracted from 0.5-g soil samples using the E.Z.N.A. Soil DNA Kit (Omega Bio-tek, Norcross, GA, USA) according to the manufacturer's protocol. The V3-V4 region of the bacterial 16S rRNA gene and the ITS1 region of the fungal rRNA gene were amplified by PCR (95 °C for 2 min followed by 27 cycles of 95 °C for 30 s, 55 °C for 30 s, and 72 °C for 45 s and a final extension at 72 °C for 10 min) using the primers 341F (5′-barcode-CCTAYGGGRBGCASCAG-3′) and 806R (5′-GGACTACNNGGGTATCTAAT-3′) for 16S rRNA and the primers ITS1F (5′-barcode-CTTGGTCATTTAGAGGAAGTAA-3′) and ITS2R (5′-GCTGCGTTCTTCATCGATGC-3′) for ITS, where the barcode is an eight-base sequence that is unique to each sample. The PCRs were performed in triplicate 20-μL mixtures, each containing 4 μL of 5 × FastPfu Buffer, 2 μL of 2.5 mM deoxynucleotide triphosphates (dNTPs), 0.8 μL of each primer (5 μM), 0.4 μL of FastPfu polymerase, and 10 ng of template DNA. The relative abundances of the bacterial and fungal rRNA gene copies were quantified using the method described by Fierer et al. (2005). Amplicons were extracted from 2% agarose gels, purified using the AxyPrep DNA Gel Extraction Kit (Axygen Biosciences, Union City, CA, USA) according to the manufacturer's instructions and quantified with QuantiFluor-ST (Promega, USA). The purified amplicons were pooled at equimolar concentrations and paired-end sequenced (2 × 250) on an Illumina HiSeq 2500 platform (Illumina Int., San Diego, CA, USA) according to standard protocols.

The raw FASTQ files were demultiplexed and quality-filtered using QIIME (version 1.17) with the following criteria (Bokulich et al., 2013): (i) 250-bp reads were truncated at any site receiving an average quality score of < 20 over a 10-bp sliding window, and any truncated reads shorter than 50 bp were discarded; (ii) reads with exact barcode matching, 2 nucleotide mismatches in primer matching, or containing ambiguous characters were removed; and (iii) only sequences that overlapped by more than 10 bp were assembled according to their overlapping sequences; any reads that could not be assembled in this way were discarded. The sequences were submitted to the SRA (Sequence Read Archive) at the National Center for Biotechnology Information (NCBI) under accession number PRJNA340057 for 16S sequences and PRJNA340058 for ITS sequences. Operational taxonomic units (OTUs) were clustered with a 97% similarity cutoff using UPARSE (version 7.1 http://drive5.com/uparse/), and chimeric sequences were identified and removed using the UCHIME algorithm. The phylogenetic affiliation of each rRNA gene sequence was analyzed using the Ribosomal Database Project (RDP) Classifier (http://rdp.cme.msu.edu/) against the SILVA (SSU117/119) database for 16S rRNA and the UNITE database for ITS rRNA using a confidence threshold of 80% (Wang et al., 2007). On average, 69,799 high-quality 16S sequences and 59,245 high-quality ITS sequences were obtained per sample. To assess the microbial diversity among the samples in a comparable manner, a normalized dataset was used for the subsequent analyses. To normalize the data, a subset of the lowest number of sequences across all samples (58,066 for bacteria and 44,988 for fungi) from each sample was randomly selected using the mothur software package. The Shannon diversity index was calculated at a sequence dissimilarity (D) of 0.03. The rarefaction curves and Shannon-Wiener curves for the bacteria and fungi demonstrated that our sequencing data were representative of most of their compositions (Supplementary Figure S2).

2.4. Methods of library construction and metagenome sequencing

We chose three replicate samples from each stage for metagenomic sequencing. A total amount of 1 μg of DNA per sample was used as input material for the DNA sample preparations. Sequencing libraries were generated using the NEBNext® Ultra™ DNA Library Prep Kit for Illumina (NEB, Ipswich, MA, USA) following the manufacturer's recommendations, and index codes were added to attribute sequences to each sample. Briefly, each DNA sample was fragmented by sonication to a size of 300 bp. The DNA fragments were then end-polished, A-tailed, and ligated to the full-length adaptor for Illumina sequencing with further PCR amplification. Finally, the PCR products were purified (AMPure XP system), and the size distribution of the libraries was analyzed using an Agilent 2100 Bioanalyzer and quantified via real-time PCR. The clustering of the index-coded samples was performed on a cBot Cluster Generation System according to the manufacturer's instructions. After cluster generation, the library preparations were sequenced on an Illumina HiSeq 2000 platform (2 × 150) (Illumina Int., San Diego, CA, USA), and paired-end reads were generated.

2.5. Metagenome sequence assembly, construction of a nonredundant gene catalog and quantification of reference gene abundance

The length of each read was trimmed with Sickle. Reads that aligned to the human genome were removed. This set of high-quality reads was then used for further analysis. An average of 11 Gb of paired-end reads were obtained for each sample, totaling 132.5 Gb of high-quality data that were free of human DNA and adaptor contaminants (Supplementary Table S1). The high-quality reads from each sample were assembled into scaftigs using IDBA-UD (Peng et al., 2010) with the default parameters. Due to the complex soil microbial genomes, a mixed sequence assembly of all samples was used to increase the utilization of reads, resulting in a large dataset of contigs (25,278,358) and a high assembly rate that was significantly higher than that of the single-sample assembly (Supplementary Table S1). The sequences were submitted to the SRA at NCBI under the accession number PRJNA343355. Genes were predicted on the contigs using MetaGeneMark (Zhu et al., 2010). A nonredundant gene catalog was constructed with CD-HIT (Li and Godzik, 2006) using a sequence identity cutoff of 0.95 and a minimum coverage cutoff of 0.9 for the shorter sequences. This catalog contained 23,980,850 unique genes. The high-quality reads of the sequence data from each sample were compared to the nonredundancy catalog to obtain the gene set using SOAP align (identity = 0.95).

We aligned putative amino acid sequences translated from the gene catalog against the Kyoto Encyclopedia of Genes and Genomes Database (KEGG), focusing on C and N cycle genes, and against the Carbohydrate-Active Enzyme (CAZy) Database using BLAST (version 2.2.21) (e-value ≤ 1e-5 with a bit-score higher than 60). The KEGG includes the major basic functional genes of microbes, including genes related to cellular processes, environmental information processing,

genetic information processing, metabolism (C and N cycles), and organelle systems. The CAZy database includes the six conventional CAZy classes: glycoside hydrolase (GH), glycosyl transferase (GT), polysaccharide lyase (PL), carbohydrate esterase (CE), auxiliary activity (AA) redox enzymes and carbohydrate-binding modules (CBMs). The gene abundances were normalized to the number of recombinase A copies detected in each metagenome.

2.6. Calculations and statistical analyses

All of the statistical analyses were performed using the R software package (version 3.1.1) (R Core Team, 2014). ANOVA analyses were used to determine the significance of differences in the microbial diversity index (Shannon), abundances and soil and litter physicochemical properties among successional stages. The significant differences were determined at the 95% confidence level. When significance was detected at the $P < 0.05$ level, a post hoc Duncan's multiple range test was used for multiple comparisons. Principal component analysis (PCA) was used to test for differences in the litter chemical characteristics (litter C and N content, cellulose and hemicellulose, etc.) and soil physicochemical characteristics (total soil organic C, total N, dissolved organic C, pH, etc.) among the successional stages. Similarities in the samples based on the microbial taxon (bacteria and fungi) and the profiles of gene family relative abundance (KEGG and CAZy databases) were measured using the unweighted UniFrac distance for phylogenetic relationships and the Bray–Curtis dissimilarity index for gene families, respectively. Both the microbial taxa and the gene family profiles were compared using principal coordinate analysis (PCoA) based on the dissimilarity distance (R vegan package) (Oksanen et al., 2016). Spearman correlation coefficients were used to test for relationships between the environmental factors (soil properties and litter properties) and the microbial communities. ANOSIM analysis was used to test for differences within a group and among different groups in terms of both microbial taxon and gene family (R vegan package). Mantel test results were used to discern correlations among the metagenome gene family distances (CAZy and KEGG) and microbial community unweighted UniFrac distances (bacteria and fungi) and the soil physicochemical characteristics, litter characteristics and functional Euclidean distances based on the Spearman correlation coefficient (R vegan package). A redundancy analysis (RDA) was implemented to elucidate the relationships between the microbial gene changes and each of the litter and soil factors in the succession. Monte Carlo permutation tests were used to test for significant associations between litter and soil factors and gene changes. Because of the large number of CAZy gene families, we used a random forest (RF) classification analysis with 5000 trees (Breiman, 2001). In our RFs, the different gene abundances were included as predictors of the different successional stages. These analyses were conducted using the RandomForest package in R, and the significance of the importance measure of each predictor on the response variable was assessed using the rfPermute package in R.

3. Results

3.1. Soil physicochemical and litter chemical characteristics during long-term secondary succession

With succession, the dominant vegetation, litter properties and soil physicochemical characteristics showed progressive shifts. The vegetation community composition varied significantly at each stage (Table 1); the highest and lowest Shannon indexes were observed in S4 and S2, respectively. The litter characteristics changed significantly as well (Supplementary Table S2), including the nutrient contents (litter C, N, P, and K), lignin content, and cellulose and hemicellulose contents. In addition, the soil physicochemical properties showed significant differences across the successional gradient (Supplementary Table S3). The PCA analyses based on the litter chemical characteristics (Supplementary Figure S3a) and the soil physicochemical characteristics (Supplementary Figure S3b) showed that the litter composition differed among the four stages, whereas the soil properties of S3 and S4 were similar to each other but differed from those of S1 and S2.

3.2. Overall pattern of microbial composition during secondary succession

Quantitative PCR of the bacterial 16S and fungal ITS rRNA gene copies revealed that bacterial and fungal abundances increased along the successional gradient from S1 to S3 and then stabilized in S4 (Supplementary Figure S4a). Interestingly, the microbial diversity as measured by the Shannon index did not show a corresponding pattern across the successional stages; the lowest bacterial diversity was observed in S1, and the lowest fungal diversity was observed in S4 (Supplementary Figure S4b). At all stages, the bacterial communities were predominantly composed of the phylum Proteobacteria (ranging from 33.34% to 37.13%) (Fig. 1) followed by Actinobacteria (ranging from 17.23% to 27.46%). The main differences in the bacterial and fungal taxa among successional stages were analyzed by ANOVA, and the results are shown in Supplementary Table S4. The abundance of Verrucomicrobia increased during the process of succession, and Nitrospirae and Bacteroidetes showed an increase from S1 to S3 and a decrease in S4. At the class level, Alphaproteobacteria, Deltaproteobacteria and Gammaproteobacteria (Supplementary Figure S5a) showed similar trends with succession, and these trends were different from those observed for Thermoleophilia and Actinobacteria; however, no consistent patterns were observed for the other bacterial taxa. The fungal community showed a clearer pattern with succession; the abundance of Ascomycota, Zygomycota and class Agaricomycetes (Supplementary Figure S5b) first increased at S2 and thereafter decreased, and the phylum Basidiomycota and the class Agaricomycetes increased from S1 to S4.

The ANOSIM results (Supplementary Figure S5a) showed that both the bacterial and fungal communities differed significantly ($P = 0.001$) among the successional stages. The PCoA results (Fig. 2a) showed a difference between the bacterial and fungal communities. The composition of the microbial community differed between S1 and S2, and the composition at both of those stages differed from the compositions at S3 and S4, which were similar. Analysis of the UniFrac distances between samples showed that the phylogenetic distance for microbes increased with successional stage from S1 to S3, with similar distances in S3 and S4 (Fig. 2b). The results also showed that the fungal community displayed larger phylogenetic distances than did the bacterial community.

Based on the Mantel tests for the microbial UniFrac distance matrix and the Bray–Curtis distance matrix for the environmental variables, both the soil and the litter characteristics were significantly related to shifts in the microbial community (Table 2). The soil characteristics during succession explained 67.2% and 69.7% (r^2) of the variation in the bacterial and fungal communities, respectively, and the litter characteristics explained 17.4% and 15.6% of the variation in the bacterial and fungal communities, respectively. Significant correlations based on the Spearman correlation coefficient were also found between soil or litter characteristics and microbial taxa; these are consistent with the Mantel test results and are shown in Fig. 3.

3.3. Functional shifts in microbial communities during secondary succession

The litter decomposition rate, the microbial respiration rate with and without litter addition, the ratio of C-use efficiency to N-use efficiency (CUE:NUE) (the calculation of this ratio is shown in the Supplementary Methods), and the relevant potential enzyme activities (alkaline phosphatase (AP), β-glucosidase (BG), and phenol oxidase (PO)) were assessed along the succession (Supplementary Table S5). The results showed that soil respiration increased with succession during incubation without the addition of litter, indicating that soil microbial activity increased with succession. Interestingly, the litter

Table 1
Geographical and vegetation characteristics of different successional stages in the Ziwuling forest region on the Loess Plateau, China. S1 represents the grasslands stage, S2 represents the shrublands stage, S3 represents the pioneer forests stage, and S4 represents the latter successional forests stage. The successional stages represent the number of years since cropland abandonment.

Study site	Location	Successional stage (in years)	Biome	Altitude (m)	Slope (°)	Coverage (%)	Shannon- Wiener index	Main plant species
S1	36°05′ N, 108°31′E	30	Grass	1365	8	95	1.86	*Bothriochloa ischcemum*
S2	36°04′ N, 108°32′ E	50	Shrub	1354	18	83	0.93	*Hippohae rhamnoides*
S3	36°02′ N, 108°31′ E	110	Forest	1450	13	89	2.34	*Populus davidiana*
S4	36°02′ N, 108°32′ E	160	Forest	1449	18	87	2.59	*Quercus sliaotungensis*

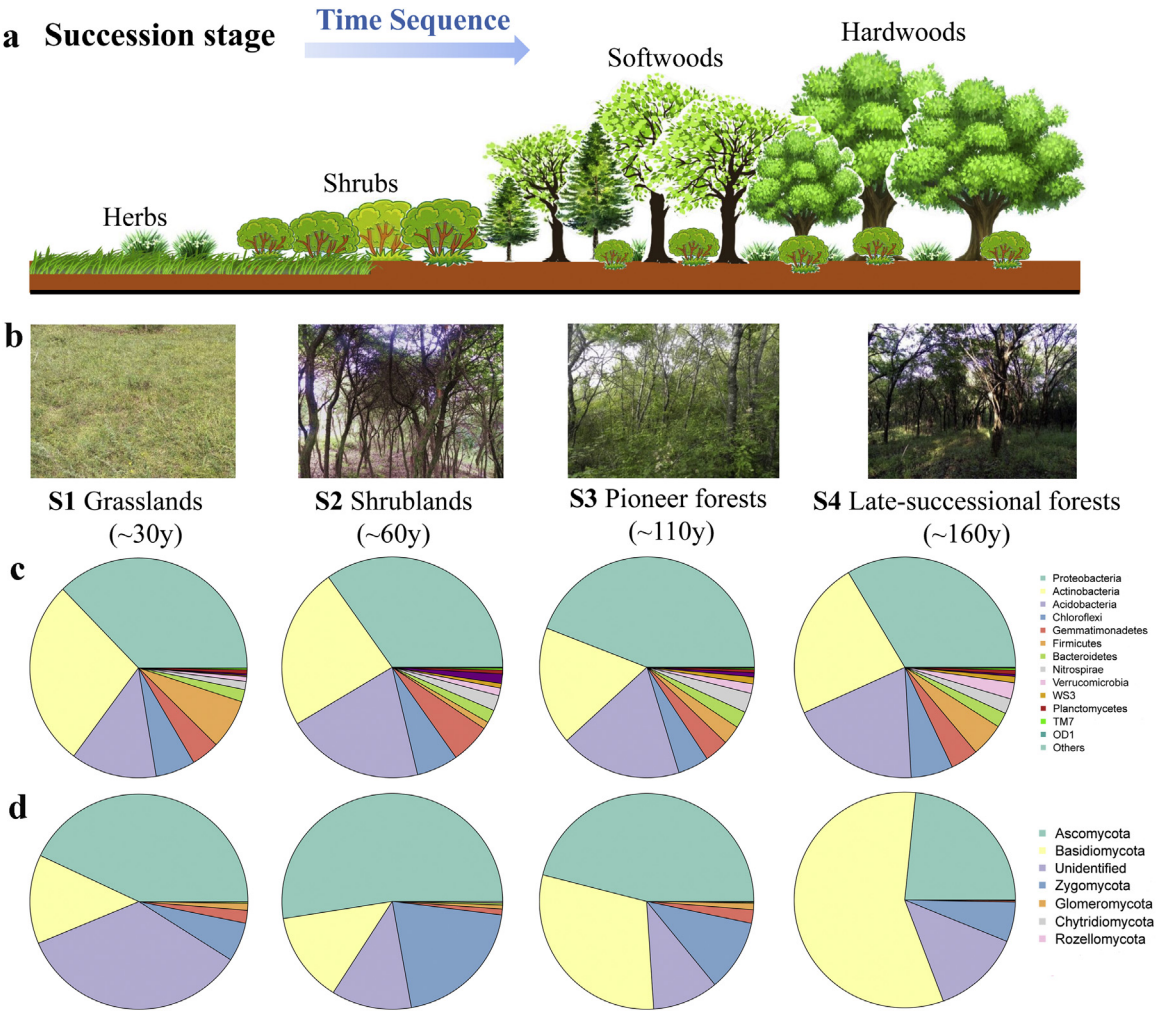

Fig. 1. A conceptual diagram of the major stages during the natural secondary succession process (a) and photographs of the study site at each successional stage (b). The microbial community composition of major taxa (abundance > 0.1%) of bacteria (c) and fungi (d) at the phylum level for each stage is shown.

decomposition rate was higher (lower litter mass remaining ratio) in S1 and S2 than in S3 and S4; furthermore, the cellulose-degrading enzymes (BG) and lignocellulose-degrading enzymes (PO) decreased with successional stage, whereas AP was higher in S3 and S4 than in the other stages.

The soil metagenomics reads were annotated using the KEGG database (Fig. 4, Supplementary Table S6) and the CAZy database (Fig. 5) to compare the relative abundances of functional genes of the C and N cycles among the successional stages. In total, 7776 KO genes and 304 CAZy genes were screened in all metagenomes. Functional changes among the successional stages were investigated via PCoA (Fig. 2c), which showed significant changes in functional genes (both KEGG and CAZy databases). Functional distance differed among the successional stages, which increased linearly up to S2 based on the KEGG gene distances and increased from S1 to S3 based on the CAZy gene distances; for both gene categories, functional distance decreased in S4 (Fig. 2d).

The genes identified as related to the C cycle based on the KEGG database (C cycle and C degradation) showed the highest relative abundances in S1 and decreased in abundance with succession (Figs. 4 and 6a), consistent with the total abundance of CAZy genes, which also decreased with years of succession (Figs. 5a and 6b). The results revealed 33 CAZy genes that were the strongest predictors of succession (Fig. 5b) and an obvious functional shift in each stage related to the C decomposition process; these genes were strongly associated with succession based on their importance values (mean decrease in accuracy) in the RF analysis as predictive genes and were best for classifying the samples according to successional stage. In addition, the relative abundances of the genes related to N metabolism and N fixation increased from S1 to S3 and then decreased in S4 (Fig. 6c).

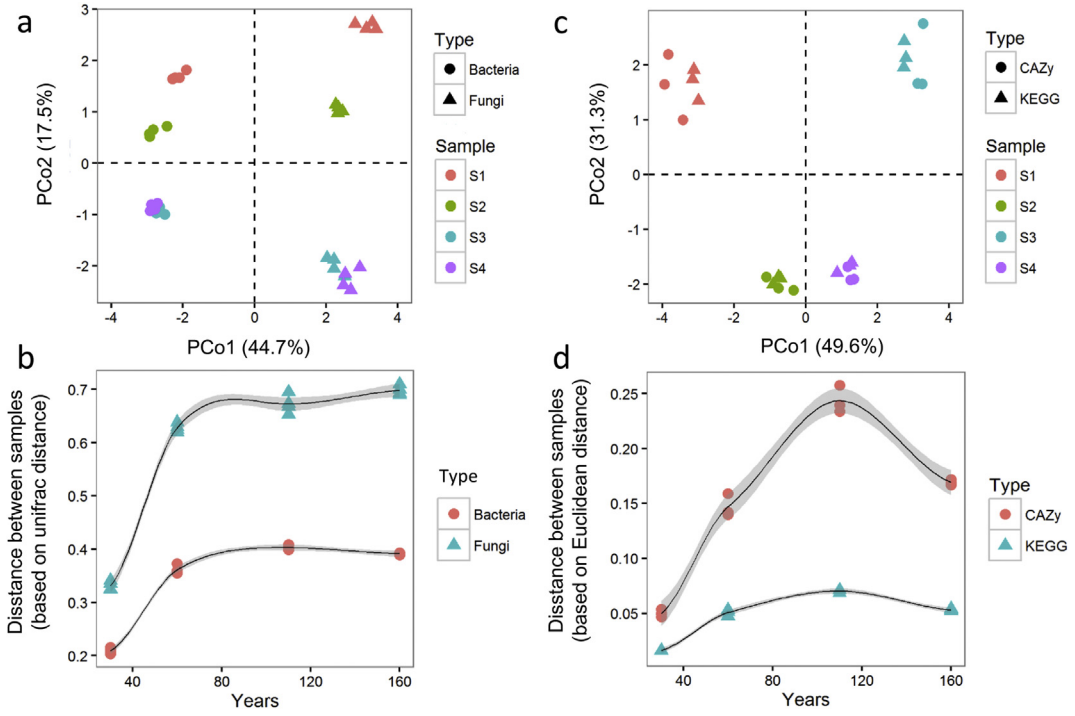

Fig. 2. Principal coordinate analysis (PCoA) plot based on the unweighted UniFrac distance of samples for the bacterial and fungal communities at all successional stages (a). Regression analysis of succession years and microbial distance at each stage (based on unweighted UniFrac distances) (b). PCoA plot of functional gene families (CAZy and KEGG database) (c). Regression analysis of succession years and functional gene distance in each stage (based on Euclidean distances) (d).

The phylogenetic and metagenomic parameters related to litter decomposition and environmental factors were significantly correlated with each other in nearly all cases (Table 2). It is important to note that the metagenomic shifts showed significant correlations with the parameters related to litter decomposition (r = 0.6928 and 0.6944 for CAZy and KEGG genes, respectively) and were more relevant to changes in the bacterial communities than to changes in the fungal communities, which were also significantly related to soil properties. The RDA analysis (Supplementary Figure S7) showed that most of the litter and soil characteristics had effects on microbial gene changes; however, soil TN and litter hemicellulose content showed highly significant correlations with microbial gene changes ($P < 0.001$) at the different successional stages for both KEGG and CAZy genes.

4. Discussion

4.1. Patterns of microbial community composition and functional change during long-term secondary succession

The intact secondary succession in the Ziwuling forest region provides a unique dynamic landscape for the investigation of patterns in microbial community succession and related factors. The microbial biomass of both bacteria and fungi increased with succession until the pioneer forest stage (S3) (Supplementary Figure S4a); this can be partially attributed to the increase in soil nutrient availability (Supplementary Table S3) (Cline and Zak, 2015). In addition, analysis of saturated 16S and ITS rRNA sequences revealed that the compositions of both the bacterial and fungal communities also changed significantly with successional stage (Fig. 2a). Bacterial diversity was higher in S2, whereas fungal diversity was the highest in S3 (Supplementary Figure S4b). At the phylum level, Verrucomicrobia, Nitrospirae and Bacteroidetes (bacteria) and Ascomycota and Zygomycota (fungi) showed different trends with succession, as did Alphaproteobacteria, Deltaproteobacteria, Gammaproteobacteria, Thermoleophilia and Actinobacteria at the class level; these trends were controlled by different factors (Dini-Andreote et al., 2014) and are shown in Fig. 3.

The results of our study, which included deep sequencing of the soil metagenome during long-term succession, provide a large dataset (132.5 Gb), a gene catalog of more than 2 million nonredundant genes,

Table 2
Mantel test results that were used to discern correlations among the metagenome gene family distances (CAZy and KEGG), microbial community UniFrac distances (bacterial and fungi), soil physicochemical characteristics (pH, BD, NO_3^-, NH_4^+, Olsen-P, SOC, TN, P, DOC, and DON), litter characteristics (C, N, P, K, cellulose, hemicellulose, lignin, and lignin:N ratio) and parameters related to litter decomposition Euclidean distances. The values shown are correlation coefficients based on the Spearman method. * indicates $P < 0.05$; ** indicates $P < 0.01$.

	Soil characteristics	Litter characteristics	Bacterial communities	Fungal communities	Litter decomposition parameters	CAZy genes	KEGG genes
Soil characteristics	1						
Litter characteristics	0.4091*	1					
Bacterial communities	0.8196**	0.4176**	1				
Fungal communities	0.8348**	0.3944**	0.7738**	1			
Litter decomposition parameters	0.7190**	0.1406	0.7723**	0.8634**	1		
CAZy genes	0.6203**	0.2697*	0.8250**	0.6383**	0.6928**	1	
KEGG genes	0.5643**	0.3292*	0.8223**	0.5657**	0.6944**	0.9417**	1

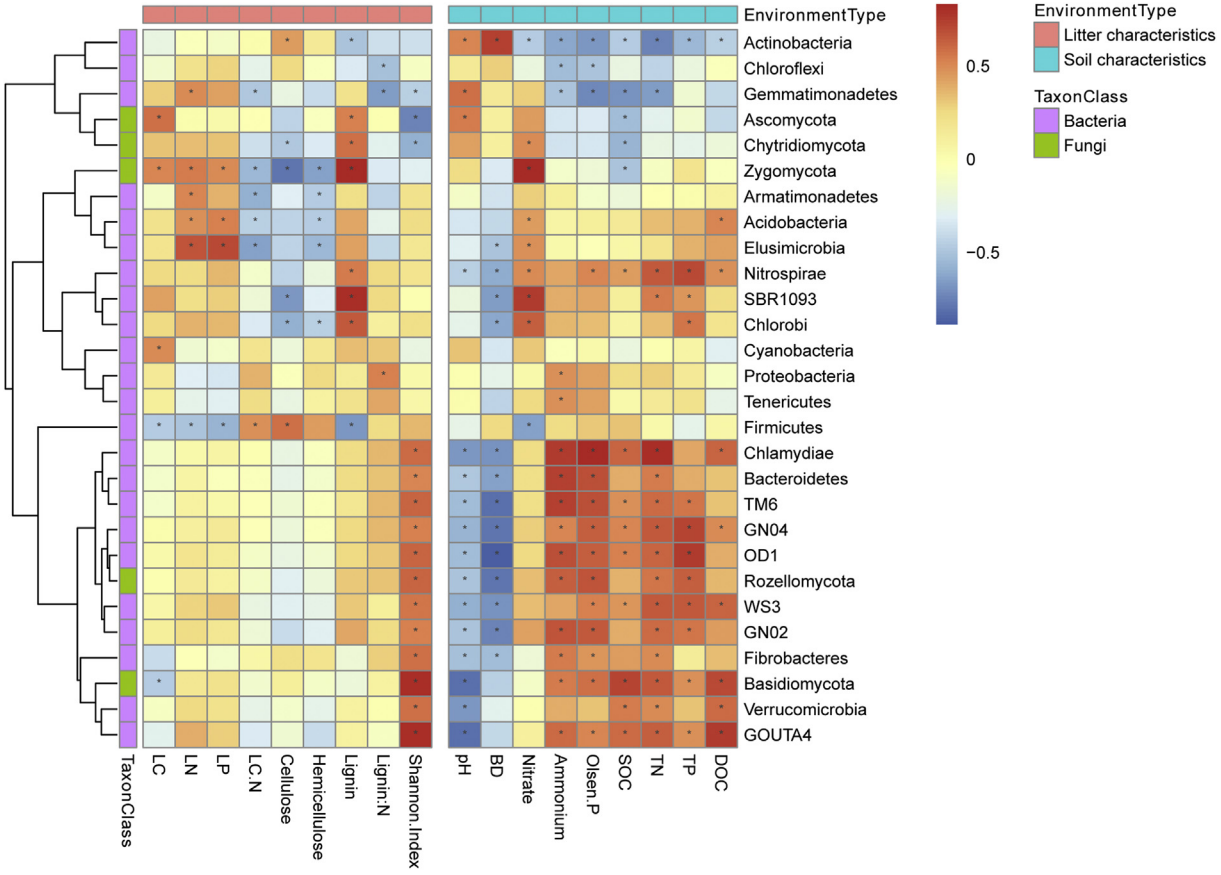

Fig. 3. Correlations between environmental factors and significantly changed microbial taxa based on the Spearman correlation coefficient. The color represents the value of the Spearman correlation coefficient; red indicates a positive correlation, and blue indicates a negative correlation. * indicates a significant correlation at $P < 0.05$. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

and the screening of two gene databases as a basis for determining the potential functions of microbes at different successional stages. The gene families changed significantly at different successional stages in both databases (Fig. 2c) because changes in microbial community function are often associated with variation in community composition (Fierer et al., 2007; Salles et al., 2009). Microbial functions were significantly correlated with the plant community, soil properties and the microbial community during the process of succession (Table 2), indicating the interconnectivity of microbial community assembly and the temporal dynamics therein.

One interesting finding was that the functional and compositional changes in microbial communities diverged during the latter succession stage. The microbial community changed little after the pioneer forest stage (S3), as evidenced by the lack of difference in composition between S3 and S4 in either the bacterial or fungal communities (Fig. 2a and b). However, the gene families present at these stages showed changes (Fig. 2c and d), indicating that a functional shift continued even when the community composition showed little change. The lack difference in microbial community might be because the similar soil properties of S3 and S4 resulted in a balance between the microbial community and the ambient environment. Dini-Andreote et al. (2014) investigated changes in a microbial community over a 105-year succession and found that the buffering effects of soil were more dominant at latter succession; these effects reduced the amplitude of variation and resulted in lower phylogenetic turnover, allowing the microbial community to achieve a balance with the soil environment. However, a similar microbial community does not always represent similar microbial function, as the same species can also perform different functions due to the environment. This phenomenon was also proven by Cong et al. (2015), who reported that the potential function significantly differed when there were no differences in microbial community between coniferous forest and mixed broadleaf forest during succession, but contrasts with primary succession, as shown in a recent meta-analysis (Ortiz-Álvarez et al., 2018), in which consistent changes in the taxonomic structure and functional attributes of bacterial communities during primary succession were reported.

4.2. Different variability of bacteria and fungi with succession and their relationships to soil and litter properties

In the present study, fungi showed a higher UniFrac distance than did bacteria, indicating that the fungal community was more sensitive to successional change than the bacterial community. This finding is consistent with other work on secondary succession (Dini-Andreote et al., 2016), in which it was reported that fungi may outcompete bacteria during succession because they may more effectively utilize the C available from plants and because they establish close associations with plants. In addition, Cutler et al. (2014) reported that plant community changes are significant for fungal communities but less relevant for bacterial communities in primary succession due to the close relationship between the plant and fungal communities because plants drive pedogenic change during early soil development and then drive the selection of soil bacterial communities (Williams et al., 2013).

Both litter characteristics and soil properties were closely associated with the soil microbial communities and their gene functions (Table 2, Fig. 3), which is consistent with the results obtained in studies of primary succession along a developmental sand-dune soil chronosequence (Williams et al., 2013). The associated relationship may be because the soil provides the substrates for microbial and plant growth, and plants affect the microbial community through the input of organic matter

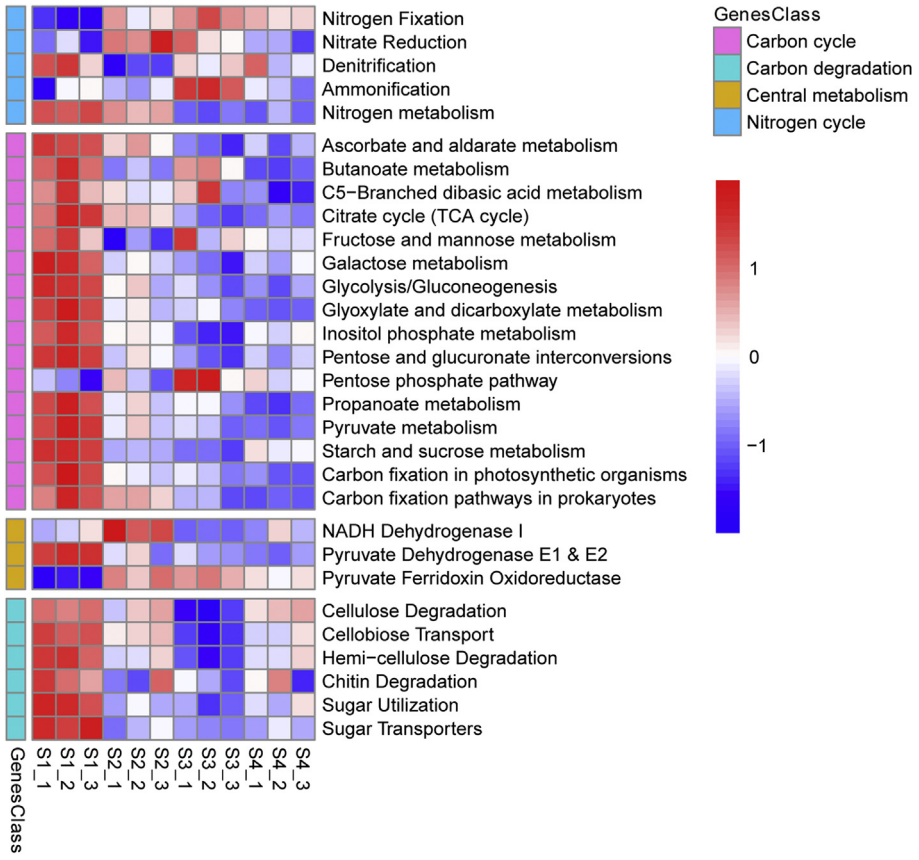

Fig. 4. Heatmaps indicating differences in the relative abundances of functional genes of the C and N cycles in the KEGG database for all successional stage metagenomes.

(Cleveland et al., 2014; Wedin and Tilman, 1990). Typically, pH affects the microbial communities substantially (Banning et al., 2011; Kuramae et al., 2010), but it is not a strong factor in this study because of the little change of pH among all the succession stages. Therefore we can discern the changes that occur related to other variables, such as SOC, TN and BD, which were the most important soil factors affecting microbial community and change in function (Supplementary Figure S7). Previous studies across other chronosequences have shown that changes in soil C and N often impact bacterial community composition and function (Edwards et al., 2006; Tscherko et al., 2004). However, BD may influence soil porosity and oxygen in the soil, after which it can affect microbial community and function or cause differences in soil temperature and moisture, both of which affect the microbial community structure and function (Cong et al., 2015). Soil P has effects on bacterial communities and functions (KEGG gene profiles), as soil P availability often limits bacterial growth (Correll, 1999; Schmidt et al., 2011) and these effects are more efficient in bacteria than in other microorganisms (Brasell et al., 2014; Thingstad et al., 1993). Due to higher N availability in the latter stage, P may be become the main limiting element for microbes (Ortiz-Álvarez et al., 2018); thus, microbe would synthesize AP enzymes to increase the Olsen-P content.

It is often difficult to establish specific connections between the microbial community and soil and litter properties. In the present study, the results of the Mantel test and correlation analysis (Table 2, Fig. 3) revealed a higher correlation coefficient between the microbial community and functions of the soil properties than litter quality in our secondary succession. These observations are consistent with the results of Cline and Zak (2015), who reported that soil properties appear to be a more important environmental factor for soil bacteria in secondary succession and indicated a high dependency of microbes on soil nutrients due to the easy utilization of soil nutrients by soil microbes. Thus, the microbial communities of S3 and S4 showed high similarity due to the similar soil properties (Supplementary Figure S3, Table S3). In our secondary succession, the fungal community showed higher variability with succession, but compared with the litter properties, the soil properties exhibited larger effects on microbial community and functional shift.

4.3. Decreased occurrence of C cycle genes and litter decomposition ability over long-term succession

Plant litter is a major source of soil organic C in ecosystems and as such plays a key role in nutrient recycling (García-Palacios et al., 2016). Given the fundamental role of microbial communities in ecosystems, their responses to environmental change may lead to shifts in litter decomposition and the soil nutrient cycle (Castro et al., 2010; Gutknecht et al., 2012; Singh et al., 2010). Without litter addition, the soil respiration was higher in the latter succession stage, which is in contrast to the CUE:NUE (Supplementary Table S5), potentially due to an increase in soil nutrient availability (Supplementary Table S3) (Zhong et al., 2015). However, with litter addition, the highest soil respiration was observed in S1 and S2, indicating higher microbial decomposition ability in the early successional stages. This finding is consistent with the measured decomposition rate (litter mass remaining) and the potential soil enzyme activities (BG and PO enzyme activities) and indicates that litter addition could play an important role in shaping microbial function due to the supply of a new C source. These findings are consistent with the work of Zhang et al. (2013), who reported a decreasing litter decomposition rate with succession from grassland to mature forest (approximately 100 years) and showed that it was mainly caused by changes in soil nutrient availability rather than by changes in litter quality. These results indicate that the high

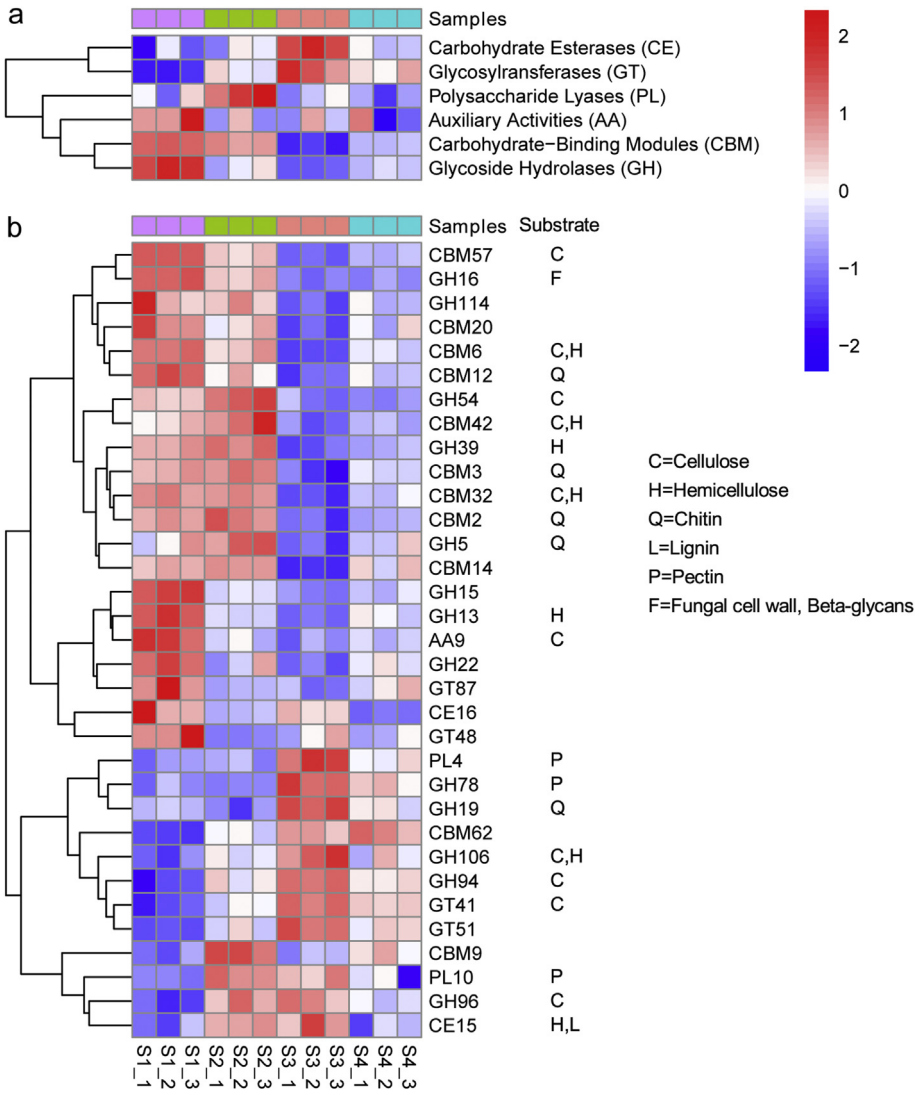

Fig. 5. Distribution of gene families in the CAZy database for each successional stage (a) and the relative abundances of the 33 CAZy gene families that were the strongest predictors of succession (b). Predictors were identified by random forest analysis (average Z-scores of 5000 runs). The heat maps show the scaled relative abundances (normalized and centered) of gene families. Substrate utilization is based on the CAZy database and on the literature.

microbial respiration in nutrient-rich soil does not necessarily indicate high microbial decomposition functions.

Regarding potential gene function, the CAZy genes in the whole metagenome changed more significantly than did the KEGG genes during the succession (Fig. 2d), indicating greater variability of C cycle genes during succession. The CAZy database mainly focuses on genes related to carbohydrate decomposition (Lombard et al., 2014), whereas the KEGG database also contains genes coding for ribonucleases, RNA polymerases, and enzymes involved in carbohydrate metabolism, C fixation, N metabolism and microbial metabolism (Kanehisa et al., 2008). Screening of the metagenomes for changes in the genes involved in the C cycle in the KEGG database, including genes associated with carbohydrate-, (hemi)-cellulose-, chitin- and sugar-processing systems, revealed a significantly higher relative abundance of such genes in S1 (Fig. 4), and the relative abundance of C cycle genes decreased with successional stage (Fig. 6a). In addition, we observed the highest potential for ammonia and nitrate assimilation in S3, and the relative abundance of genes associated with the N cycle increased from S1 to S3. The latter observation could explain the relative increase in soil N availability with succession (Waldrop et al., 2006; Zak et al., 1990) and is consistent with the work of Cline and Zak (2015), who also found genetic and enzymatic potential changes in the soil microbial community with succession that, in turn, have direct consequences for the decomposition of plant litter.

The relative abundance of CAZy genes decreased with years of succession (Fig. 6b), and the abundance of genes encoding GH, CBM and AA was higher in S1 than in the other stages. The AA and GH genes are related to litter decomposition, particularly the decomposition of lignin and humic matter; the higher abundance of these genes could promote decomposition (Cardenas et al., 2015; Paul, 2006). The distribution of gene families predictive of successional stages (Fig. 5b) indicates that succession specifically diminishes the genetic potential for hemicellulose, cellulose, lignin and pectin degradation, thereby potentially altering the enzyme activities related to decomposition (Table S7). These findings differ from those related to the primary succession in that enzyme activity increased up to an age of 50 years and then reached a temporary steady state up to 150 years (Tscherko et al., 2004) but are consistent with the results of our incubation experiment and the work by Zhang et al. (2013). All of these changes indicate that a decline in litter decomposition with succession might be primarily due to the observed decrease in associated gene abundance.

One intriguing interpretation of these results is based on the “nutrient limitation theory”, which states that resource availability is likely to be a fundamental driver of microbial succession (Cherif and Loreau,

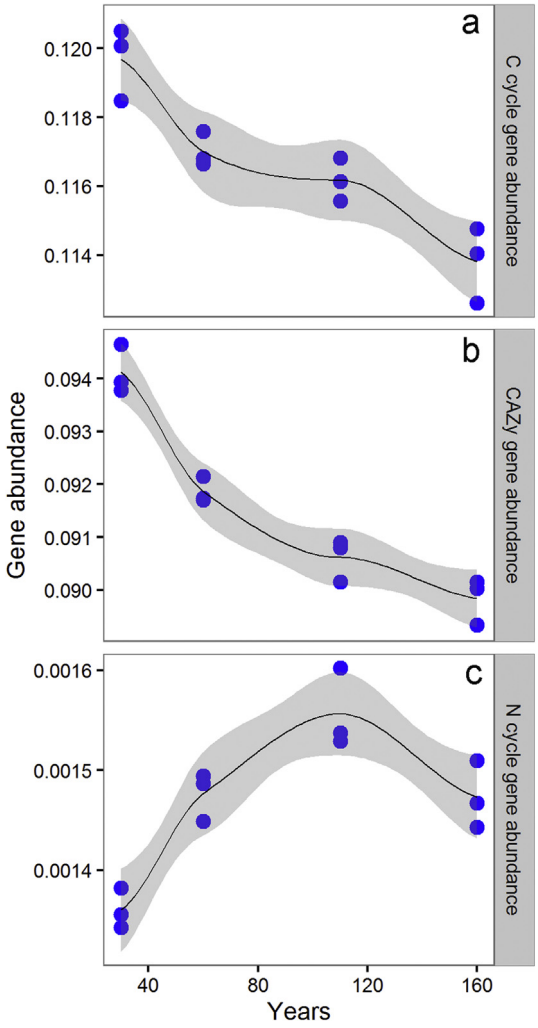

Fig. 6. Regression analysis of the association between years of succession and the relative abundance of selected functional genes: C cycle genes from the KEGG database (a), CAZy genes (b), and N cycle genes (c). All regressions were significant at the $P < 0.01$ level.

2007). Generally, microbial community growth in the early successional stage is primarily limited by soil nutrients (Gomez-Alvarez et al., 2007; Schmidt et al., 2008), but the high abundance of genes associated with C decomposition and the increasing abundance of N genes of microbes during early succession (Fig. 6) (Cline and Zak, 2015; Zak et al., 1990) could lead to the rapid and efficient decomposition of litter (Table S5) and increase the level of soil nutrients (such as C and N), thereby promoting plant growth and leading to plant community succession. Subsequently, the main resource limitation may have changed from a limitation in soil nutrients to another type of environmental (nonresource) limitation, such as water availability or UV radiation (Fierer et al., 2010). However, with sufficient soil nutrients in the latter successional stage, microbes readily obtained nutrients (this was shown by the fact that DOC was significantly higher in S4 than S3), and this led to a decline in the abundance of C decomposition genes. The balance among the soil nutrients created by these functional shifts gradually slows the process of succession, finally approaching a steady state in which the vegetation change is slowed. However, whether these decreasing C cycle genes are limited by other factors, such as P availability, a common limiting element in latter successional stages, remains to be determined in future studies. The results provide evidence that the genetic potential for biomass decomposition diminished over the succession, leading to a decrease in the decomposition rate.

Overall, the study of long-term secondary succession provides insight into how microbial communities evolve along a successional gradient, and the results highlight the interconnectivity of microbial community assembly and the temporal dynamics. Both the bacterial and fungal communities changed during succession; the fungi exhibited greater variability with succession, whereas the microbial community changed little from the pioneer forest stage (approximately 110 years of succession) to the later successional forest stage. The results also indicate that changes in soil properties during the process of secondary succession play major roles in microbial community establishment and succession. In addition, shifts in the soil metagenome were associated with microbial phylogenetic changes, but functional shifts continued to occur even when the community composition became stable. The reduction in microbial C cycle genes was related to a decrease in litter decomposition ability, thus resulting in a steady state of nutrient status in the ecosystem. In addition, the high microbial respiration in nutrient-rich soil does not necessarily indicate high microbial decomposition functions; these also depended on the abundance of related genes, on enzyme activity and on the physicochemical properties of the litter. Our study provides a metagenomic profile of a successional chronosequence and provides insight into the mechanisms underlying soil microbe-driven functional changes in nutrient cycles during succession.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.soilbio.2018.05.017.

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