Humus microhabitat affects distributions of soil fungi and bacteria in a temperate mountain forest

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Abstract
Humus layer has an important effect on the diversity and spatial distribution of microbes. However, the role of partitioning of humus layer microhabitats for soil microbial diversity has been poorly documented. In this study, 120 soil samples were collected from a 5-ha forest dynamic monitoring sample plot in a deciduous broad-leaved forest. Clustering analysis was used to delineate microhabitats by humus layer. We explored diversity and community composition of soil fungi and bacteria using Illumina sequencing of the ITS rRNA gene and 16S rRNA gene region on the MiSeq platform. We investigated the diversity, species composition, and the microhabitat preferences of fungi and bacteria and then analyzed the effects of environmental factors on soil fungi and bacteria. Our results showed that the diversity, species composition, and the indicator species of the fungal and bacterial community varied among different humus layer microhabitats. In network analysis, the specialization indexes showed that 16.20%/22.30% of the fungal/bacterial operational taxonomic units (OTUs) had the characteristics of distribution specialization for humus layer microhabitats. Mantel test showed that the main environmental drivers of fungi and bacteria were not consistent among the four microhabitats. Our findings indicated that the distribution of soil microbes among humus layer microhabitats is not random, but specialized. We furthermore found that there were differences in the relationship between soil microbes and environment among microhabitats defined by humus layer. Together, these findings suggest the importance of partitioning of humus layer microhabitats in maintaining local diversity in a soil microbial community.

KEYWORDS
16S rRNA gene, forest dynamics plot, humus layer microhabitats, ITS rRNA gene, microbial diversity, soil microbes
INTRODUCTION

Soil microorganisms are the primary drivers of ecosystem (Wu et al., 2019; Zeng et al., 2019). Microorganisms participate in the decomposition of soil organic matter and plant litter and mediation of carbon and nitrogen biogeochemical cycles in terrestrial ecosystems (Koozem et al., 2014; Zhao et al., 2018). Understanding soil microbial composition and diversity can reveal interrelations between soil microorganisms and local environments (Wang et al., 2019).

Humus are the colloidal substance formed by microbial decomposition and transformation of organic matter and the primary composition of organic matter (Sandrine, 2018). Numerous studies have shown that soil microbes are important participants to litter decomposition (Lin et al., 2019), and the chemical characteristics of litter also influence soil microbial diversity, resulting in a close relationship between soil microorganisms and humus. For instance, the thickness of humus layer and humus content are important factors to determine soil fertility (Li et al., 2018; Sandrine, 2018).

Niche theory emphasizes that species composition among different habitats is correlated with differences in environmental requirements of species and the distribution of environmental conditions in space (Chen et al., 2018; Chesson, 2000). The amount and composition of litters in different areas are distinct because of the differences of woody plants (Chen et al., 2018). The quality of litters has a direct influence on the formation of humus layer. Although ecologists have conducted a series of explorations about the factors that influence soil microbes, including abiotic factors and biological factors (Chen et al., 2019; Elloumi et al., 2008), the effects of humus microhabitats on microorganisms remain unclear.

Soil microbial communities are spatially dependent on and sensitive to environment (Ren et al., 2019). Moreover, the environmental conditions that fungi need for growth and reproduction are not similar to that of bacteria. Bacteria are considered to have high growth and turnover rates, whereas fungi have slow growth and turnover rates (Fierer et al., 2007). Many studies indicate that the coexisting mechanism of fungal communities is different from bacteria (Powell et al., 2015; Zhou et al., 2017). However, it is unknown whether the coexistence mechanisms of the two microorganisms are consistent among humus microhabitats.

Large-scale monitoring sample plots are important platforms to study the maintenance mechanism of forest biodiversity (Ma, 2017). Large forest sample plots have a lot of environmental spatial information, which is beneficial to the study of soil microorganisms (Ma, 2017). In recent years, significant progress has been made in the study of soil microorganisms based on the platform of forest dynamic sample plots. Gao et al. (2017) reported that the mechanisms responsible for maintaining the community composition of fungi differ between valley and ridge habitats. Gao et al. (2013) analyzed the relationship between ectomycorrhizal fungi and their host plants and then found that they had a good correlation at the genus level of host plants. Peng et al. (2019) studied the spatial distribution and drivers of soil microbial richness and diversity. The study of soil microorganisms based on forest sample plots is important to understand the mechanism of soil microbial diversity maintenance.

In the present study, we hypothesized that the coexistence mechanisms of soil microbes among different humus microhabitats were different. To test this hypothesis, we established a 5-ha forest dynamics plot in China, and clustering analysis was used to delineate microhabitats by humus layer for soil microorganisms. We investigated the diversity, species composition, and microhabitat preferences of microbes and then analyzed the relationship of soil microbes and environmental factors among different humus microhabitats by Mantel tests.

MATERIALS AND METHODS

2.1 Study area

The present study was conducted at a 5-ha permanent plot in Baiyun Mountain National Forest Park, China (111° 48′–112° 16′ E, 33° 33′–33° 56′ N), which is located in the transition zone from warm temperate zone to north subtropical zone. Baiyun Mountain National Forest Park had 1991 plant species, and the forest coverage rate was over 98.5%. The mean annual temperature of the study area is 13.5°C, and the mean annual precipitation is 1,200 mm.

The elevation of the 5-ha permanent plot varies from 1,538 m to 1,600 m above sea level, and the 20-m cell slopes vary from 4.3° to 55.5°. All trees with diameter breast height (DBH) ≥1 cm have been tagged, identified, measured, and georeferenced. In total, 17,963 individual trees belonging to 93 species and 34 families were identified in the 5-ha plot. Of the 93 woody plant species, Quercus aliena var. acuteserrata, Pinus armandi, Lauraceae obtusi-loba, and Toxicodendron vernicifluum are the dominant species in the plot (Chen et al., 2017).

2.2 Sampling design

In July 2018, 120 sampling points were selected on the basis of 20 m × 20 m subplots. The thickness of the soil humus layer was measured using rulers. Soil samples were collected three times as one sample at a 10 cm depth after litter removal using soil auger from 120 plots (20 m × 20 m), and the distance between each sampling point and the adjacent sampling point is about 10 meters. Moreover, all soil samples were immediately sieved through a 2-mm sieve, and the visible plant roots, stones and litter were removed. The sieved soil samples were divided into two portions to be processed and used for the following analyses: (a) stored at −80°C for DNA analysis and (b) immediately transported to the laboratory to determine soil pH, soil organic matter (SOM), available nitrogen (AN), available phosphorus (AP), and water content (WC).
2.3 | Soil DNA extraction, PCR amplification, and Illumina sequencing

Soil DNA was extracted from 0.5-g defrosted soil samples using the FastDNA Spin Kit (MP Biomedicals) according to the manufacturer’s instructions. The concentration of extracted DNA was detected by a fluorometer. All extracted soil DNA was stored at −80°C until PCR amplification and 16S/ITS sequencing.

Primers 515F (5′-GTGCGCACGCGCGCGTAA-3′) and 806R (5′GGACTACHVGGGTWTCTAAT-3′) targeting the V4 region of 16S rRNA gene. For fungi, the ITS1 region in the rRNA gene was amplified by PCR using the primers ITS1F (5′-CTTGGGTATCATTAGAGGAAGTAA-3′) and ITS2R (5′-GCTGGTTCCTTATCGATGC-3′) (Sarah et al., 2016). 30-ng DNA samples were taken, and fusion primers were used to configure the PCR system. To test the quality and concentration of PCR product, each mixed gene (16S rRNA gene and ITS rRNA gene) was detected by agarose gel electrophoresis. An equal amount of PCR product from each sample was placed in a single tube and sent to Illumina’s MiSeq platform at the Personal Biotechnology Co., Ltd., Shanghai, China.

2.4 | Bioinformatics analysis

To obtain more accurate and reliable results, the raw data were preprocessed to get clean data in following steps: (a) truncate sequences with average mass <20 bp; (b) remove joint contamination sequences; (c) remove ambiguous sequences; and (d) remove low complexity sequences. Then, the software FLASH (Fast Length Adjustment of Short reads, v1.2.11) was used to assemble the pair reads obtained from double-terminal sequencing into a sequence by using the overlapping relationship (Magoc & Salzberg, 2011), and the tags in the high-variation region were obtained. The splicing conditions are as follows: (a) minimal overlapping length: 5 bp and (b) mismatching ratio of overlapped region: ≤0.1. A total of 7,329,751 tags of bacteria and 8,502,219 tags of fungi were obtained from all samples. Finally, OTU representative sequences were taxonomically classified using Ribosomal Database Project (RDP) Classifier v.2.2 trained on the database UNITE_Version7, using 0.6 confidence values as cutoff. Filtered tags were clustered into OTU for species classification with 97% similarity using UPARSE (USEARCH version 7.0.1090) (Edgar, 2013).

2.5 | Topography and soil properties

The topography of the plot was quantified by measuring the elevation of the four corners of each 20 m × 20 m subplot. We calculated the mean elevation, convexity, and slope by following the methods described by Harms et al. (2001) and Valencia et al. (2004). Three topographic factors were measured for each 20 m × 20 m subplot in the field, namely elevation (with values from 1,538 m to 1,600 m), slope (with values from 4.31° to 55.50°), and convex–concave (with values from −45.72° to 59.26°). Soil samples were air-dried for 1 week to a constant weight and sieved using a 0.250-mm sieve. The potassium dichromate oxidation method was used to measure SOM (%) (Nelson & Sommers, 1982). The molybdenum-blue method (Olsen & Sommers, 1982) was used to measure AP (mg/kg). AN (mg/kg) was determined by alkaline hydrolysis diffusion method. Drying method was used for soil water content. Soil pH was measured in a 1:2.5 soil water extraction.

2.6 | Statistical analyses

In this study, cluster analysis was conducted to delineate humus layer microhabitat similarity in the thickness of the humus layer. According to the results of cluster analysis, humus microhabitats were divided and boxplot was made. The Kruskal–Wallis method was employed to explore the significant differences among the humus layer microhabitats (p < .05 level of significance). The Venn diagram was used to show the common and specific fungi and bacteria among different humus layer microhabitats. Microbial alpha diversity had two components (species richness and Shannon diversity), which were quantified using the vegan package in R 3.4.0. The Kruskal–Wallis method was used to test differences of the richness and diversity of fungi and bacteria among different humus layer microhabitats (p < .05 level of significance). The histograms of relative abundance of the dominant phyla of fungi and bacteria were plotted using R 3.6.1, and ANOVA was used to test whether the differences were significant.

Next, we assessed the impact of humus layer microhabitats on the beta diversity of the fungi and bacteria by running the beta-dispers function on the OTU matrix. The betadisper test was conducted using the betadisper command in the vegan package (Chen et al., 2018; Oksanen et al., 2012).

A correlation network approach was used to visualize strong potential relationships between fungi/bacteria and humus layer microhabitats. We evaluated the structure of the fungi/bacteria and habitat network using the H2’ metric of specialization and connectance index (Blüthgen et al., 2007). The architecture of the fungi/bacteria and habitat network was visualized using the “bipartite” package of R 3.4.0.

Indicator species analysis was conducted using the “indicspecies” package of R 3.4.0 to delineate the composition of fungi and bacteria among different humus layer microhabitats. The dependent variable in the indicator species analysis was the species abundance matrix of soil microbes.

Mantel test was performed to examine the linkage between environmental variables (AN, AP, SOM, WC, pH, slope, elevation, and convex–concave) and microbial community (fungi and bacteria). Spearman correlation analysis was performed to examine the autocorrelation among environmental factors. Correlation diagram is plotted in “ggcor” package in R 3.4.0.
3 | RESULTS

3.1 | Delineation of humus layer microhabitats

Cluster analysis classified the 120 sample plots into four habitat types based on the thickness of humus layer: A (0 ≤ thickness ≤ 3, \(n = 28\)), B (3 < thickness ≤ 5, \(n = 58\)), C (5 < thickness ≤ 7, \(n = 28\)), and D (7 < thickness ≤ 9, \(n = 6\)) (Figure 1). Soil physical and chemical properties of four microhabitats are shown in Table 1 and Figure S1. There were no significant differences in soil pH and AP among the four microhabitats. AN in habitats B and C was significantly different from that in habitats A and D (\(p < .05\)). SOM in habitat B was significantly different from those in habitats A and D (\(p < .05\)). There were significant differences in WC between A and B, as well as between A and C (\(p < .05\)) (Table 1; Figure S1).

3.2 | Fungal and bacterial diversity

A total of 6,266 fungal OTUs were detected in 120 soil samples, belonging to 17 phyla, 55 classes, 133 orders, 343 families, and 792 genera. A total of 3,471, 5,137, 4,007, and 6,180 OTUs of fungi were found in habitats A, B, C, and D, of which 5, 28, 9, and 52 OTUs were specific to these four microhabitats, respectively. Furthermore, 2,343 fungal OTUs were shared by four microhabitats, accounting for 37.39% of the total OTU number (Figure 2).

**FIGURE 1** Classification of humus layer microhabitats (a), information of humus layer (b), and maps of the humus layer microhabitats within the Baiyun Mountain permanent plot (c). The black solid line is the contour line of the plot. Blue is habitat A, yellow is habitat B, gray is habitat C, and red is habitat D.
TABLE 1  Soil physical and chemical properties of four microhabitats

| Habitat | AN (mg/kg)  | AP (mg/kg)  | SOM (%)  | WC    | pH    |
|---------|-------------|-------------|----------|-------|-------|
| A       | 133.75 ± 48.58b | 11.55 ± 8.47a | 5.73 ± 2.18b | 0.34 ± 0.09b | 6.37 ± 0.52a |
| B       | 174.76 ± 56.76a | 12.57 ± 7.99a | 6.85 ± 2.39a | 0.42 ± 0.12a | 6.37 ± 0.48a |
| C       | 186.00 ± 68.52a | 14.03 ± 7.21a | 6.79 ± 3.01ab | 0.43 ± 0.13a | 6.33 ± 0.51a |
| D       | 117.83 ± 31.75b | 12.44 ± 7.78a | 5.64 ± 2.40b | 0.40 ± 0.10ab | 6.48 ± 0.48a |

Note: Different letters indicate significant difference among fertilization treatments by one-way ANOVAs (LSD, p < .05).

Abbreviations: AN, mean alkali-hydrolyzable nitrogen; AP, available phosphorus; SOM, soil organic matter; WC, water content.

In addition, 7,441 OTUs of bacteria were shared among four microhabitats, which accounted for 57.90% of the 12,852 OTUs. All of the OTUs belonged to 51 phyla, 173 classes, 325 orders, 482 families, and 707 genera. The specific bacterial OTUs in habitats A, B, C, and D were 223, 641, 214, and 44, accounting for 1.74%, 4.99%, 1.67%, and 0.34% of total OTUs, respectively. The specific bacterial OTU number in habitat B were much higher than those in other microhabitats (Figure 2).

Notably, significant differences were observed not only in bacterial species richness (p = 0.0032) but also in bacterial Shannon diversity (p = 0.0017) among different humus layer microhabitats. Moreover, the two indices of fungal and bacterial alpha diversity increased along with the thickness of the humus layer (Figure 3).

3.3 | Differences in species composition of fungi and bacteria among different humus layer microhabitats

Of all the 17 fungal phyla detected in 120 soil samples, the dominant phyla were Basidiomycota (71.2%), Mortierellomycota (13.71%), and Ascomycota (11.98%). Other phyla including Mucoromycota, Rozellomycota, Cercozoa, Chytridiomycota, and Glomeromycota were present at lower levels. The mean relative abundance of Basidiomycota ranged from 61.85% to 76.61% among the four microhabitats. The mean proportion of Ascomycetes in habitats A and C showed significant differences (p < 0.05) (Figure 4).

The dominant phyla of all 51 bacterial phyla were Acidobacteria (28.82%), Proteobacteria (24.37%), Verrucomicrobia (18.76%), Planctomycetes (6.41%), and Nitrospirae (5.14%). The average abundance of these five phyla was different among the four microhabitats, and the difference of Verrucomicrobia (21.82% in A and 16.96% in C) was the most obvious. The average abundance of Acidobacteria in habitats A and D was significantly different. Significant differences were observed in the mean abundance of Proteobacteria between habitats A and C. The average abundance of Verrucomicrobia in habitat A was significantly different from that in habitats B, C, and D. The average abundance of Planctomycetes in habitat A was significantly different from that in habitats B and C (Figure 4).

Betadisper analysis followed by ANOVA test showed significant differences in the community composition of fungi (F = 3.854, p = 0.011) and bacteria (F = 2.287, p = 0.040) among different humus layer microhabitats (Figure 5).

Indicator species analysis showed that the indicator species of the fungal and bacterial community varied among different humus layer microhabitats. For example, the indicator species of fungi in habitat A were primarily composed of OTU 1061 (Ascomycota), OTU 2227 (Ascomycota), OTU 35 (Basidiomycota), etc. By contrast, the indicator species of fungi in habitat B were primarily composed of OTU 2097 (Cercozoa), OTU 3533 (unclassified), OTU 1957 (Ascomycota), etc. (Table S1). The bacterial community in habitat C was primarily composed of OTU 11822 (Proteobacteria), OTU 6744 (Planctomycetes), OTU 10394 (Planctomycetes), etc. However, the bacterial community in habitat D was primarily composed of OTU 12080 (Proteobacteria), OTU 9561 (Proteobacteria), OTU 12315 (Planctomycetes), etc. (Table S2).

In network analysis, 29.10% and 37.10% of possible interactions occurred on the observed interactions among bacteria/fungi and
humus layer habitat, respectively, based on the connectance index. The specialization index showed that 16.20%/22.30% of the fungal/bacterial OTUs had the characteristics of distribution specialization for humus microhabitats (Figure 6).

3.4 | Effect of environmental factors on fungal and bacterial community

The main drivers of fungi and bacteria were not consistent among the four microhabitats (Figure 7). In habitat A, AP, SOM, and WC have positive effects on fungal richness, and bacteria are greatly affected by CC and pH. In habitat B, SOM, AN, and WC have positive effects on fungal richness, and bacteria richness was negatively affected by AP and SL. In habitat C, both SOM and pH have significant positive effects on fungal and bacterial richness. In habitat D, all environmental factors greatly influenced the richness of fungi and bacteria. The data of topography and soil properties of 120 soil samples are shown in Table S3.

4 | DISCUSSION

In this study, the results showed that the characteristics of fungi and bacteria assemblages differed among different humus layer microhabitats, and soil microbe–environment relations depended on the different humus layer microhabitats in the temperate mountain forest. Together, these findings suggest the importance of partitioning of humus layer microhabitats in maintaining local diversity in a soil microbial community.
4.1 | Soil microbe–habitat associations

In the network analysis, the specialization indexes of fungi (16.20%) and bacteria (22.30%) in the four humus microhabitats are lower than the previously reported plant–fungus network (0.265; Toju et al., 2014) and the plant–seed diffusion network (0.354; Dicks et al., 2002). In this study, only the influence of humus layer microhabitats on soil microbes was considered. Terrain factors, light factors, and forest community also affect the distribution of soil microbes. Therefore, the specialization indexes of fungi and bacteria in our study are lower than that of the previous studies (Dicks et al., 2002; Toju et al., 2014). Hence, the distribution of soil microbes among humus layer microhabitats is not random, but specialized.

In the present study, we found that the characteristics of fungi and bacteria assemblages differed among different humus layer microhabitats (Tables S1 and S2). Such differences of microbial diversity among different humus layer microhabitats may be due to the changes in soil factors. As described in the results, there were significant differences in soil physical and chemical properties among the four microhabitats, except soil pH and AP. Niche theory states that species coexistence can be attributed to differences in the distribution of environmental requirements and spatial environmental conditions (Chen et al., 2018). Soil microhabitat varies in different humus layer microhabitats, and microorganisms will select the most suitable soil for their survival (Chen et al., 2018). After long-term habitat filtration, the differences of microbial species composition occurred among different humus layer microhabitats. Our study highlights the importance of partitioning of humus layer microhabitats in maintaining local diversity in a soil microbial community.

4.2 | Soil microbe–environment relations among different microhabitats

Our results indicated that the relationship between soil microbes and environment depended on the different humus layer microhabitats. Soil chemical properties and topography factors play important roles in the survival and reproduction of soil microorganisms (Wang et al., 2013; Guo et al., 2016; Wang et al., 2018; Huang et al., 2019). These factors have different effects on soil fungi and bacteria because of the differences in morphological structure and proliferation pattern. In different humus layer microhabitats, there are great differences in soil nutrients, so different physical and chemical properties have different effects on fungi or bacteria. Our result also well agrees with Shen et al. (2013) who showed that soil pH was a key factor in determining microbial diversity and community composition. Many studies have shown that pH has a negative effect on soil microorganisms (Li et al., 2019; Zhang et al., 2019). In our study, pH has a negative effect on fungi only in microhabitats with the thickest humus layer. Elevation gradients have different ecological conditions, leading to the environmental filtering and niche segregation of soil microbes (Sheng et al., 2019). With the increase of altitude, soil nutrient decreased significantly (Figure 7). Therefore, elevation has a negative effect on fungi and bacteria in the four microhabitats, except for fungi in the microhabitat with the thickest humus layer. AP,
AN, and SOM were also found to act as important factors influencing fungal and bacterial community composition (Van Geel et al., 2015; Li et al., 2019; Yoshimura et al., 2013). Liu et al. (2010) reported that soil water content indirectly influenced the soil microbes. In summary, the environmental drivers of soil microbes were not consistent in different humus layer microhabitats.

4.3 Differences in specialization characteristics between fungi and bacteria

The diversity and species composition of fungi and bacteria among different humus layer microhabitats are distinct. Some studies have shown that fungi possess stronger capacity to break down
recalcitrant broadleaf litter (De Graaff et al., 2010; Hunt et al., 1987), and most bacteria are only associated with the turnover of easily degradable substances (Paterson et al., 2008). In our study, microhabitat with thickest humus layer contained the largest number of fungal OTU species. However, the number of bacterial OTU species was not the largest in the microhabitats with the thinnest humus layer. This may be because AN, SOM, and WC in the microhabitat with the thinnest humus layer are lower.

The drivers of fungi and bacteria are different among different humus layer microhabitats in our study. For example, in habitat B, SOM was positively correlated with fungi, but negatively correlated with bacteria. In the habitat D, the effect of AP on fungi was greater than bacteria. AN and PH were positively correlated with bacteria, but negatively correlated with fungi in the habitat D. Although bacteria and fungi are involved in the formation and decomposition of humus layer, fungi are more related to plants and are strongly influenced by the diversity of trees (Peng et al., 2019).

5 | CONCLUSION

In conclusion, our study found that the distribution of soil microbes among different humus layer microhabitats is not random, but specialized. We further found that soil microbe-environment relations differ among microhabitats defined by humus layer. Overall, these findings highlighted the importance of partitioning of humus microhabitats in maintaining local diversity in a soil microbial community.

In this study, we only studied the effect of humus layer on soil fungi and bacteria and effects of topography and soil factors on them. The thickness of humus layer and soil physicochemical
properties were measured, and the relationship between them was analyzed. However, the chemical quality of humus may be an important factor for distribution and diversity of soil microbes. Therefore, studying the effect of chemical quality of humus on soil microbes would be necessary in future studies.

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CONFLICT OF INTEREST
None declared.

AUTHOR CONTRIBUTIONS
Nan Wang: Investigation (equal); Methodology (equal); Writing-original draft (lead). Qiang Fu: Investigation (equal); Methodology (equal). Ziyu Zhou: Investigation (equal). Yizhen Shao: Methodology (equal); Visualization (equal). Jing Wang: Writing-review & editing (equal). Wang Li: Funding acquisition (supporting); Writing-review & editing (equal). Yongzhong Ye: Supervision (equal); Validation (equal). Yun Chen: Data curation (equal); Funding acquisition (lead); Writing-review & editing (equal). Zhiliang Yuan: Conceptualization (supporting); Validation (supporting).

DATA AVAILABILITY STATEMENT
The microbial sequences obtained in this study were uploaded and made available on Dryad: https://doi.org/10.5061/dryad.rxwdbv8g.

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**SUPPORTING INFORMATION**

Additional supporting information may be found online in the Supporting Information section.

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