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Structure and functions of soil microbial communities and tree composition are more closely associated with keystone microbes than rare microbes in a subtropical forest

Xian Wu[1], David C. Deane[2], Hua Xing[1], Jiarong Yang[1], Junfang Chen[1], Xiaolin Liu[1], Shu Dong[1], Fangliang He[1,3] and Yu Liu[1,4,*]

[1]ECNU-Alberta Joint Lab for Biodiversity Study, Tiantong Forest Ecosystem National Observation and Research Station, School of Ecology and Environmental Sciences, East China Normal University, Shanghai 200241, China, [2]Research Centre for Future Landscapes and School of Environment and Genetics, La Trobe University, Bundoora, VIC 3083, Australia, [3]Department of Renewable Resources, University of Alberta, Edmonton, Alberta T6G 11 2H1, Canada, [4]Shanghai Institute of Pollution Control and Ecological Security, Shanghai 200241, China

*Corresponding author. E-mail: yuliu@des.ecnu.edu.cn

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Abstract

The significance of microbes for ecosystem functioning is well known; however, within a single system, the relative contributions of keystone and rare taxa to soil microbial functions are less well quantified, as are their shared or unique responses to abiotic conditions. Furthermore, their associations with tree community composition in natural forest ecosystems are not well understood. In this study, a total of 1287 soil samples were collected from a 20-ha subtropical forest plot and analyzed using high-throughput sequencing. Based on co-occurrence network analyses, we conducted a comparison of the associations between keystone and rare taxa with the structure, functions and stability of soil microbial communities. Additionally, we examined their associations with tree community composition. Results showed that keystone taxa made a significantly greater contribution than rare taxa in all comparisons of microbial functions and stability. Keystone taxa had direct effects on microbial community structure and also mediated indirect effects of abiotic conditions. Neither effect was evident for rare taxa. The importance of keystone taxa also extended to aboveground composition, where tree community composition was more closely associated with keystone taxa than with rare taxa. While it may still be premature to establish causality, our study represents one of the initial attempts to compare the relative importance of keystone and rare microbial taxa in forest soils. These findings offer the potential to improve natural forest ecosystem functioning and tree diversity through the manipulation of a small number of keystone soil microbial taxa, as has been demonstrated in agroecosystems.

Keywords: community stability, ecological network, ecosystem functioning, keystone taxa, rare taxa

亚热带森林土壤微生物群落以及树木组成与关键微生物的关系强于与稀有微生物的关系

摘要：微生物在参与碳氮循环和能量流动以及分解有机物质等方面对生态系统功能具有重要作用。然而，在单一生态系统中，关键类群和稀有类群对土壤微生物功能的相对贡献尚未得到充分量化。此外，它们对非生物因素的共同或独特响应缺乏深入研究，并且它们与森林生态系统中树木群落组成的关联尚不明确。因此，本研究依托浙江天童20公顷亚热带森林动态监测样地，采集了1287份土壤样本，并进行了高通量测序分析以明确微生物群落组成。基于共现网络分析，我们比较了微生物关键类群和稀有类群与土壤微生物群落结构、功能和稳定性间的关系。此外，我们还探究了它们与树木群落组成之间的关系。结果显示，在所有微生物功能和稳定性的比较中，微生物关键类群的贡献程度均高于稀有类群。关键类群对微生物群落结构产生了直接影响，并在一定程度上调节了非生物因素的间接作用。然而，稀有类群并未表现出类似的影响。此外，关键类群的重要性同样体现在地上树木群落的组成上，即树木群落组成与关键类群的关系明显强于与稀有类群的关系。上述研究结果表明，通过对少数关键土壤微生物类群的调控，有望提升森林生态系统的功能及树木多样性。

关键词：群落稳定性，生态网络，生态系统功能，关键类群，稀有类群

INTRODUCTION

Soil microbes are ubiquitous and indispensable ecosystem components (Xun *et al.* 2021) that are critical for ecosystem functioning (Bardgett and van der Putten 2014). In forest ecosystems, soil microbes also shape aboveground patterns of tree biodiversity through their influence on plant growth and community assembly (Hannula *et al.* 2021). Much research has sought to understand how highly diverse soil microbial communities regulate ecosystem structure and functions (Bardgett and van der Putten 2014). This has identified disproportionately high contributions to community structure and ecosystem functions made by both keystone and rare microbial taxa (Banerjee *et al.* 2018; Chen *et al.* 2020; Herren and McMahon 2018; Lynch and Neufeld 2015; Pester *et al.* 2010; Xiong *et al.* 2021; Xun *et al.* 2021). However, individual studies have tended to either focus solely on keystone taxa or compare abundant and rare taxa without differentiating keystone taxa. Even within a single ecological context, the relative importance of keystone and rare taxa for ecosystem functioning remains uncertain. Moreover, the role of abiotic conditions in structuring these critical components of soil microbial diversity is not well understood either. Such understanding could provide a more holistic view of the role of soil microbial diversity in ecosystem functioning, potentially informing new management interventions.

As 'ecosystem engineers' (Banerjee *et al.* 2018; Mills *et al.* 1993; Yue *et al.* 2019), keystone taxa are highly connected within the microbiome. By definition, keystone taxa have a huge impact on the structure and function of microbiome, regardless of their abundance across space and time (Banerjee *et al.* 2018; Lynch and Neufeld 2015; Paine 1995). Given their importance in soils, microbial keystone taxa have been extensively studied in agricultural ecosystems (Banerjee *et al.* 2019; Shi *et al.* 2020), for their roles in supporting ecosystem functions (Shi *et al.* 2020), maintaining soil microbiome stability and generalist metabolism (Fan *et al.* 2018; Xun *et al.* 2021), predicting nitrogen-cycling processes (Dai *et al.* 2021; Yue *et al.* 2019) and protecting crops against pathogens (Trivedi *et al.* 2017). The association between soil keystone taxa and agricultural production has been an important line of research (Banerjee *et al.* 2019), enabling their use to improve crop productivity (Wang *et al.* 2022; e.g. by adding the screened keystone taxa to the soil as biofertilizers; Fan *et al.* 2021). In contrast to agricultural systems, however, the role of keystone taxa in forest ecosystems remains largely unknown, despite considerable research on soil microbes (Barberán *et al.* 2015; Chen *et al.* 2019; Lladó *et al.* 2017). If microbial keystone taxa were found to have an influence on soil and tree communities, it would be reasonable to assume that the promotion of ecosystem functioning in natural forests could potentially be achieved by regulating keystone soil microbes, similar to successful approaches applied in agroecosystems (Wang *et al.* 2022; Zheng *et al.* 2021).

As with keystone taxa, numerous studies have revealed that the contributions of rare taxa to ecosystem services are disproportionate to their abundance (Jia *et al.* 2018; Pester *et al.* 2010;

Yang 2021). Indeed, the importance of rare taxa in performing keystone-like roles in microbial co-occurrence networks and community structure is increasingly recognized (Lynch and Neufeld 2015; Ramirez *et al.* 2018; Xiong *et al.* 2021). Additionally, the large number of species found with very low abundances in most microbial communities (Pedrós-Alió 2012; Xiong *et al.* 2021) collectively provide a reservoir of genetic traits associated with a wide range of ecosystem functions (Elshahed *et al.* 2008; Pascoal *et al.* 2021). This creates the potential for rare taxa to rapidly respond to environmental perturbation (e.g. conditionally rare taxa; Shade *et al.* 2014), thus providing resilience in ecosystem functioning (Pascoal *et al.* 2021) and explaining the disproportionately high contribution of rare taxa in driving ecosystem multifunctionality (Chen *et al.* 2020). Specific examples of the importance of rare microbial taxa include maintaining the stability of mycobiome networks (Xiong *et al.* 2021) and mediating the negative impacts of land degradation on ecosystem functioning (Wu *et al.* 2021).

Since both keystone and rare taxa have been associated with disproportionate contributions to soil microbial community structure, functions and stability, their relative abundances do not truly reflect their importance (Banerjee *et al.* 2018; Lynch and Neufeld 2015). Thus, the shared importance and, in some cases, overlapping roles of keystone and rare microbial taxa raise questions about their relative contributions and how they assemble into communities within a single ecological system. This is of particular interest in natural ecosystems, rather than agricultural ones, where relative abundances also reflect minimal anthropogenic impacts, such as soil disturbance or nutrient addition.

In this study, we collected 1287 soil samples from a 20-ha stem-mapped subtropical forest plot to analyze the network structure of soil bacterial and fungal communities. Our main interest was in comparing the relative influence of keystone and rare microbes on microbial community functions and stability, their respective sensitivities to abiotic conditions and their relationships with aboveground (tree community) composition. Given the strong influence of abiotic factors on keystone taxa (Resetarits *et al.* 2018; Yang *et al.* 2020) and the lesser influence on rare taxa (Ramirez *et al.* 2018), it was expected that there was a closer association between keystone taxa and abiotic (soil and topographic) factors. Specifically, we hypothesized that (i) the community structure of keystone taxa would be more strongly associated with abiotic (soil and topographic) factors than that of rare taxa, which would result in a greater association with aboveground composition; (ii) both keystone and rare taxa would contribute substantially to ecosystem functioning, but the former would dominate through their known linkages with biotic and abiotic factors; and that (iii) the high richness of rare taxa would enable them to make greater contributions to community stability. However, we found that keystone taxa dominated all comparisons, suggesting that rare taxa play a relatively limited role in this natural forest.

MATERIALS AND METHODS

Study site and soil sampling

The study site is located at Tiantong National Field Observation Station for Forest Ecosystems (29°48′ N, 121°47′ E), which is a representative subtropical evergreen broadleaved forest in Zhejiang Province, East China. The study region has a subtropical monsoon climate with humid, hot summers and dry, cold winters (Zhou *et al.* 2020). The mean annual precipitation is 1374 mm, and the mean annual temperature is 16.2 °C (Hu *et al.* 2020). The soil has a clay loam texture with 6.8% sand, 55.5% silt and 37.7% clay (Zhou *et al.* 2020). Common tree species include *Castanopsis fargesii* Franch., *Schima superba* Gardn and *Castanopsis carlesii* (Hemsl.) Hayata (Hu *et al.* 2020). The studied forest plot was established in 2008 as part of the Forest Global Earth Observatory network (https://forestgeo.si.edu/) and covered a total area of 20 ha (500 m × 400 m) (Qiao *et al.* 2020). The plot topographic data (elevation, slope and convexity) were collected and converted into topographic data for each sampling point using the Kriging interpolation method.

In 2018, we collected a total of 1287 soil samples over the 20-ha study area using a gridded sampling design (Supplementary Fig. S1; Wu *et al.* 2024). Following the criterion for soil sample collection established by the Center for Tropical Forest Sciences (John *et al.* 2007), we selected the intersections of each quadrat as the base sampling points and chose two additional sampling points randomly positioned 2, 5 or 8 m away from 70% of the base points in each selected direction. After removing surface litter and the organic layer, we took four soil cores from the mineral layer (0–10 cm depth) using a soil auger with a 10-cm inner diameter within 0.5 m around each selected sampling point to form a composite sample. In total, 1287 soil samples were collected around all selected sampling points. Soil samples were transported to the laboratory in iceboxes and immediately passed through a 2-mm sieve. Each composite sample was divided into three subsamples: one for molecular analysis (stored at −80 °C), another for the analysis of soil physicochemical properties (partially air- or oven-dried) and the third for measurements of ammonium (NH_4^+-N) and nitrate (NO_3^--N) contents (stored at 4 °C).

DNA extraction, PCR amplification and sequencing

High-throughput bacterial and fungal DNA analyses have been well described previously (Wu *et al.* 2023, 2024). Briefly, DNA was extracted from 0.5 g of soil per sample using a MagPure Soil DNA KF Kit (Magigene Biotechnology Co., Ltd, Guangzhou, China) according to the manufacturer's instructions. DNA quality was assessed using 1% agarose gels, and its concentration and purity were determined using a NanoDrop One (Thermo Fisher Scientific, Waltham, MA, USA). The bacterial 16S rRNA V4–V5 hypervariable regions were amplified using the universal primers 515F and 907R (Biddle *et al.* 2008), and the fungal second internal transcribed spacer region was targeted using the universal primers ITS3 and ITS4 (White *et al.* 1990). The PCR program was as follows: 94 °C for 5 min, followed by 30 cycles of denaturation at 94 °C for 30 s, annealing at 52 °C for 30 s and elongation at 72 °C for 30 s. Operational taxonomic units (OTUs) with a 97% similarity cutoff were clustered using the USEARCH software (Edgar 2010), and singleton OTUs and chimeric sequences were identified and removed. The representative sequences for each bacterial and fungal OTUs were taxonomically assigned using Silva (v.138.1; https://www.arb-silva.de/) and Unite (v.9.0; http://unite.ut.ee/index.php) databases, respectively. The sequences of all samples were rarefied according to the minimum sequence number (11 155 for bacteria and 24 400 for fungi) to correct for differences in sequencing depth among samples (Weiss *et al.* 2017). In total, there were 8373 bacterial OTUs and 11 961 fungal OTUs (after deleting OTUs with sequence numbers across all samples less than 20; Jiao and Lu 2020).

Identifying keystone taxa based on co-occurrence network analysis

Ecological interactions of within-group species in bacterial and fungal communities were analyzed using the Molecular Ecological Network Analysis Pipeline (MENAP; http://ieg4.rccc.ou.edu/mena/login.cgi; Deng *et al.* 2012; Zhou *et al.* 2010, 2011) with a log-transformed Pearson correlation matrix. We selected OTUs detected in more than 50% of the samples (644 of the total 1287) for network construction to ensure correlation reliability for the analysis. In ecological count data, a large portion of the sequencing data consists of zeros. Zero-value matching of two taxa can produce false strong correlations. A conventional way to address this problem is to remove taxa that occur in a few samples to avoid spurious correlations (Faust 2021). Although removing rare OTUs may disrupt the network structure, a high rate of false positive results would be more destructive (Weiss *et al.* 2016). Additionally, we compared the network structures that were constructed based on OTUs detected in over 50%, 40%, 30% and 20% of all samples. Despite having the smallest number of nodes, the networks, based on OTUs detected in more than 50%, demonstrated the highest values for average degree, average clustering coefficient, geodesic efficiency, centralization of degree, density and transitivity. On the contrary, they exhibited the lowest values for average path distance, harmonic geodesic distance and centralization of eigenvector centrality (Supplementary Fig. S2 and Table S1). These findings indicated that the presence of false strong correlations, resulting from retaining more zero values (i.e. including OTUs merely occurring in a few samples), could compromise the complexity and stability of the network.

In each molecular ecological network, nodes represented the OTUs, and edges denoted the correlations between one OTU and another. The network nodes were clustered into identical modules based on their relatively similar ecological niches and functions (Zhou *et al.* 2010), with each module representing species' environmental preferences and the heterogeneity of their habitats (Ma *et al.* 2020; Shi *et al.* 2016). In this study, we assessed the topological role of each node by considering two key properties: the relative within-module degree Zi, which measured the connectivity of a node within its module, and the participation coefficient Pi, which quantified the extent to which a node connects to different modules (Guimerà and Amaral 2005). Based on within-module (Zi) and among-module (Pi) connectivity, nodes in each network can be placed into four categories: peripherals ($Pi \leq 0.62$ and $Zi \leq 2.5$), module hubs ($Pi \leq 0.62$ and $Zi > 2.5$), connectors ($Pi > 0.62$ and $Zi \leq 2.5$) and network hubs ($Pi > 0.62$ and $Zi > 2.5$) (Olesen *et al.* 2007). The latter three 'hub' categories are proposed as keystone taxa owing to their dramatic effects on microbiome (Banerjee *et al.* 2018; Deng *et al.* 2012). We therefore adopted this approach to identify keystone taxa in our networks. All networks were visualized with Cytoscape (version 3.8.2) and Gephi (version 0.9.2).

Rare taxa analysis

Possibly due to the subjective understanding of 'rarity' (Galand *et al.* 2009; Pedrós-Alió 2012; Xue *et al.* 2018), there is no consistent threshold for defining rare microbial taxa in soil microbiology, with most studies using a value of 0.1% or 0.01% relative abundance. Consistent with previous studies (Galand *et al.* 2009; Logares *et al.* 2014; Xue *et al.* 2018; Zhao *et al.* 2022), we adopted the lower threshold of 0.01% of relative abundance across all samples. This decision was partly to make comparison with keystone taxa tractable, because using 0.1% as the threshold resulted in 74% of bacterial and 50% of fungal taxa (thousands of OTUs) being defined as rare. It is worth noting that the aforementioned literature did not include research on keystone species. Indeed, there has been an increasing number of studies reporting the relative abundance of keystone taxa so far, but the distribution of their relative abundances is uneven. The average relative abundance of keystone taxa ranges from 0.009% to 3.66% (Supplementary Table S2). Most previous studies classified rare OTUs into two categories: *always rare* taxa and *conditionally rare* taxa (Liang *et al.* 2020; Xue *et al.* 2018). In this study, we refer to always rare taxa, again for pragmatic reasons, because the high sample size of more than 1000 resulted in 97% of bacterial and 85% of fungal taxa being defined as conditionally rare taxa. Rare microbes are typically defined by their relative abundance, while keystone taxa are identified based on their roles within the network structure (Banerjee *et al.* 2018). Currently, there is no consistent threshold for the relative abundance of keystone taxa, possibly due to variations in keystone communities acquired based on different ecosystem functions (Yang *et al.* 2020). Note, however, that the average relative abundance of all identified keystone taxa far exceeded 0.01% across all samples (the average relative abundance of keystone taxa being 0.50% for bacteria and 0.27% for fungi; Supplementary Table S3). That is, by our definitions in this study system, rare and keystone taxa were mutually exclusive subsets in both bacterial and fungal microbial communities.

Statistical analyses

Influence of abiotic factors on individual taxa and community structure

We first calculated the Spearman correlation between abiotic and biotic factors in R using the *psych* package (Revelle 2022) to estimate the importance of specific abiotic factors on the relative abundance of individual keystone and rare taxa, as well as bacterial and fungal taxa. Additionally, we built multiple linear regression models of abundance as a function of edaphic and topographic (slope, elevation, convexity) predictors. Measurement of soil properties (pH, organic carbon (OC), total nitrogen (TN), total phosphorus (TP), available phosphorus (AP), available potassium (AK), NH_4^+-N, NO_3^--N, Al, Ca, Cu, Fe, Mg, Mn, Zn and soil moisture content (SM)) was based on three replicates for each sample and is described in detail in Supplementary Methods (see Supplementary Information I). All abiotic variables were standardized (mean = 0, SD = 1) prior to analysis.

To find the most parsimonious model, we started with a global model that included all predictors and compared all possible subset models using the *stepAIC* function from the R package *MASS* (Venables and Ripley 2002). The model with the lowest Akaike Information Criterion (AIC) was selected as the top-ranked model (Burnham and Anderson 2002). To quantify the explained variation for each of the predictors retained in the top-ranked model, we used variance decomposition implemented in the *relaimpo* package (Grömping 2006). Regression models were fitted by using the *lm* function from the R package *stats* (Field *et al.* 2012).

Having established the taxon-level responses to abiotic factors, we aimed to quantify the major determinants of microbial community structure. This was done by developing a causal path model that depicts the hypothesized direct and indirect effects of abiotic factors and keystone/rare taxa on microbial community structure. Prior to conducting the path analysis, we initiated the process with a global model that encompassed all predictors. Through the utilization of the *stepAIC* function from the R package *MASS* (Venables and Ripley 2002), we compared all possible subset models to identify the most parsimonious model for explaining variations in keystone and rare taxa. The model with the lowest AIC was selected as the top-ranked model (Burnham and Anderson 2002). Each taxonomic group was assumed to have a direct influence on community structure. However, due to the influence of abiotic factors on the composition of keystone and rare taxa, an indirect pathway was anticipated between abiotic factors and community structure mediated by the two taxonomic subsets (see Fig. 2 for causal path model structure). We implemented four separate causal path analysis models for keystone and rare taxa, as well as bacterial and fungal taxa, using the *lavaan* package (Rosseel 2012).

Soil and topographic predictors used in the causal path model are described above. To create biological predictors for keystone and rare taxa composition, as well as for overall community structure, we used the first principal coordinate from a principal coordinate analysis on the relevant Bray–Curtis dissimilarity matrix, which was performed using the *vegan* package (Oksanen *et al.* 2022). Community structure was calculated based on all taxa, including the taxonomic group involved in the direct causal pathway. To ensure this did not introduce circularity in the results, we repeated the analysis by calculating community structure after excluding the related taxonomic group. As results were essentially unchanged, we present the version excluding the related taxonomic group in Supplementary Information I. For the path analysis, all predictors were standardized (mean = 0, SD = 1) to improve normality. Support for the causal path models was evaluated using the following criteria: a non-significant Chi-square test ($P > 0.05$), goodness-of-fit index >0.90, and root mean square error of approximation <0.08 (Schermelleh-Engel *et al.* 2003).

Microbial community functions and network stability

Bacterial metabolic function profiles were performed with the *Phylogenetic Investigation of Communities by Reconstruction of Unobserved States* (PICRUSt2) approach (Douglas *et al.* 2020), and fungal OTUs were assigned to functional guilds with a confidence level of 'highly probable' or 'probable' based on the FUNGuild database (Nguyen *et al.* 2016). To identify the major predictors of microbial functions, we used random forest models, implemented with the *randomForest* package (Liaw and Wiener 2002). The contribution of each taxon to microbial functions was quantified as the percentage increase in mean squared error (MSE) of out-of-bag predictions when that taxon was removed, implemented with the *rfPermute* package (Archer 2023), where higher increases in MSE imply more important taxa (Breiman 2001; Jiao *et al.* 2018). Model cross-validated R^2 values (= 1 − MSE/observed variance) were assessed with 1000 permutations of the response variable.

Network stability was characterized by estimating measures of robustness and vulnerability, where higher robustness indicates a more stable network, and higher vulnerability has the opposite interpretation (Yuan *et al.* 2021). Robustness was defined as the proportion of remaining taxa in the MENs after a certain proportion of nodes was randomly removed (Dunne *et al.* 2002; Montesinos-Navarro *et al.* 2017). The abundance-weighted mean interaction strength of nodes was calculated to test the effects of taxa removal on the remaining taxa (see Yuan *et al.* 2021 for the specific calculation method). Vulnerability was measured as the maximum node vulnerability in each network (Deng *et al.* 2012; Yuan *et al.* 2021). Additionally, average variation degree (AVD) was used to evaluate microbial community stability, where a higher AVD represents lower microbial community stability (Xun *et al.* 2021). In addition to comparing the stability of bacteria and fungi, correlations between the average relative abundance of keystone and rare taxa and variation degree of microbial communities were explored using local regression smoothing models with *loess* function to fit the models in R.

Relationships between microbial community and aboveground tree community

We finally tested the relationships between the aboveground tree community composition and keystone *vs.* rare soil microbial taxa. This was done using partial Mantel tests to assess the correlations between Bray–Curtis dissimilarity matrices for tree assemblages and microbial communities. Partial Mantel tests were conducted using the *mantel.partial* function of the *vegan* R package (Oksanen *et al.* 2022), which utilized 1000 permutations after controlling for the potential effects of soil and topographic factors. Tree distributions were derived from the 2020 census for the Tiantong 20 ha stem-mapped plot, where all stems ≥1.0 cm in diameter at breast height were mapped, measured, tagged and identified to species. Tree species composition around each corresponding soil sampling site was calculated for four circular quadrats with radii of 5 m, 10 m, 15 m and 20 m.

Repeated keystone analyses on matching rare taxa samples

Although we had a total of 1287 samples available, rare bacteria taxa were only found in 535 samples and rare fungi were present in 1126 samples. As these samples with no defined rare taxa were omitted, the inference of keystone and rare taxa was based on different sample sizes. To test for any sensitivity to sample size on the resulting inference, we repeated the analyses for keystone taxa using only those samples that also contained rare taxa. As the results remained largely unchanged, we report the full sample sizes available for each analysis in the main text. For completeness, we present the results of the path analysis and the correlations between the tree community and keystone taxa limited to the same samples that were available for rare taxa in the Supporting Information (see Supplementary Information I and II for detail).

In order to enhance the universality and representativeness of the findings from the Tiantong plot, a comparative analysis of keystone and rare taxa from the Baishanzu plot was also conducted (Supplementary Information III). The Baishanzu plot is at a significantly higher elevation (1437.3–1659.8 m) than the Tiantong plot (312.4–592.2 m). Overall, it was found that the results from the Baishanzu plot were similar to those obtained from the Tiantong plot.

RESULTS

Prevalence of keystone and rare taxa

In total, 10 bacterial and 8 fungal OTUs were identified as keystone taxa (Supplementary Fig. S3), with keystone bacteria mainly belonging to the phyla Acidobacteria and Proteobacteria, and keystone fungi were predominantly from Ascomycota (Supplementary Table S3). One fungal keystone taxon, OTU106, was classified as a saprotroph. However, the majority of fungal keystone taxa exhibited compound guilds. For instance, OTU125 and OTU494 showed a combination of saprotrophic and symbiotrophic guilds, while even OTU37 and OTU38 belonged to compound guilds that encompassed pathotrophic, saprotrophic and symbiotrophic characteristics. Generally, keystone taxa had a very low average abundance across all samples (range: 0.03%–0.60%), but bacterium OTU25 (belonging to *Acidibacter*) was a notable exception, comprising an average of 2.31% of abundance in all samples (Supplementary Table S3). Nonetheless, keystone groups such as OTU10, OTU391, OTU34, OTU25 and OTU16685 in bacteria, as well as OTU73, OTU38, OTU125, OTU106, OTU20 and OTU37 in fungi, exhibited high node stress, indicating their roles as critical intermediaries in the network (Supplementary Table S3). A total of 29 bacterial (average relative abundance range: 1.5 e-04%–2.4 e-04%) and 102 fungal rare taxa (average relative abundance range: 6.9 e-05%–2.8 e-04%) were detected (Supplementary Table S4).

Taxon-specific effects of abiotic factors on keystone bacteria and fungi

Soil physicochemical properties and topographic factors were associated with the relative abundance of individual keystone bacterial OTUs, explaining ~23%–51% of variation (Fig. 1a). Across all groups analyzed, edaphic factors were consistently more influential than topographic factors. Bacterial keystone taxa showed consistently strong correlations with pH, Mn and OC, although the direction and magnitude of these correlations varied within and among taxa (Fig. 1a). Abiotic factors were also correlated with keystone fungal OTU abundance but accounted for less variation (~6%–35%, Fig. 1b). Notably, the direction of association with abundance for many influential edaphic factors was opposite for bacteria and fungi (especially Mn, Fig. 1a). In contrast, while several statistically significant associations with abiotic factors were evident in rare bacterial (Supplementary Fig. S4) and fungal (Supplementary Fig. S5) taxa, the magnitude of these associations was much weaker than for keystone taxa.

Keystone taxa mediate indirect abiotic influences on soil microbial community structure

There were differences in the supported causal pathways between keystone and rare taxa (Fig. 2; Supplementary Fig. S6). Most notably, keystone taxa (both bacteria and fungi) had direct significant effects on microbial community structure that were not evident for rare taxa (Fig. 2), and abiotic factors also had strong direct effects on taxonomic composition for keystone bacteria ($R^2 = 0.69$) and fungi ($R^2 = 0.65$), similarly absent in rare taxa (both $R^2 \leq 0.05$; Fig. 2). This resulted in an indirect causal pathway between abiotic factors and community structure mediated by keystone but not rare taxa (Fig. 2). As a result, keystone bacteria and fungi explained much higher variation in community structure (overall $R^2 = 0.92$ and 0.82, respectively) than rare bacteria or fungi ($R^2 = 0.44$ and 0.48, respectively; Fig. 2). Conclusions from path analysis were qualitatively identical when community structure was calculated after excluding the related keystone or rare taxa (Supplementary Fig. S7 and when the analysis was repeated for the subset of samples containing rare taxa (Supplementary Information II).

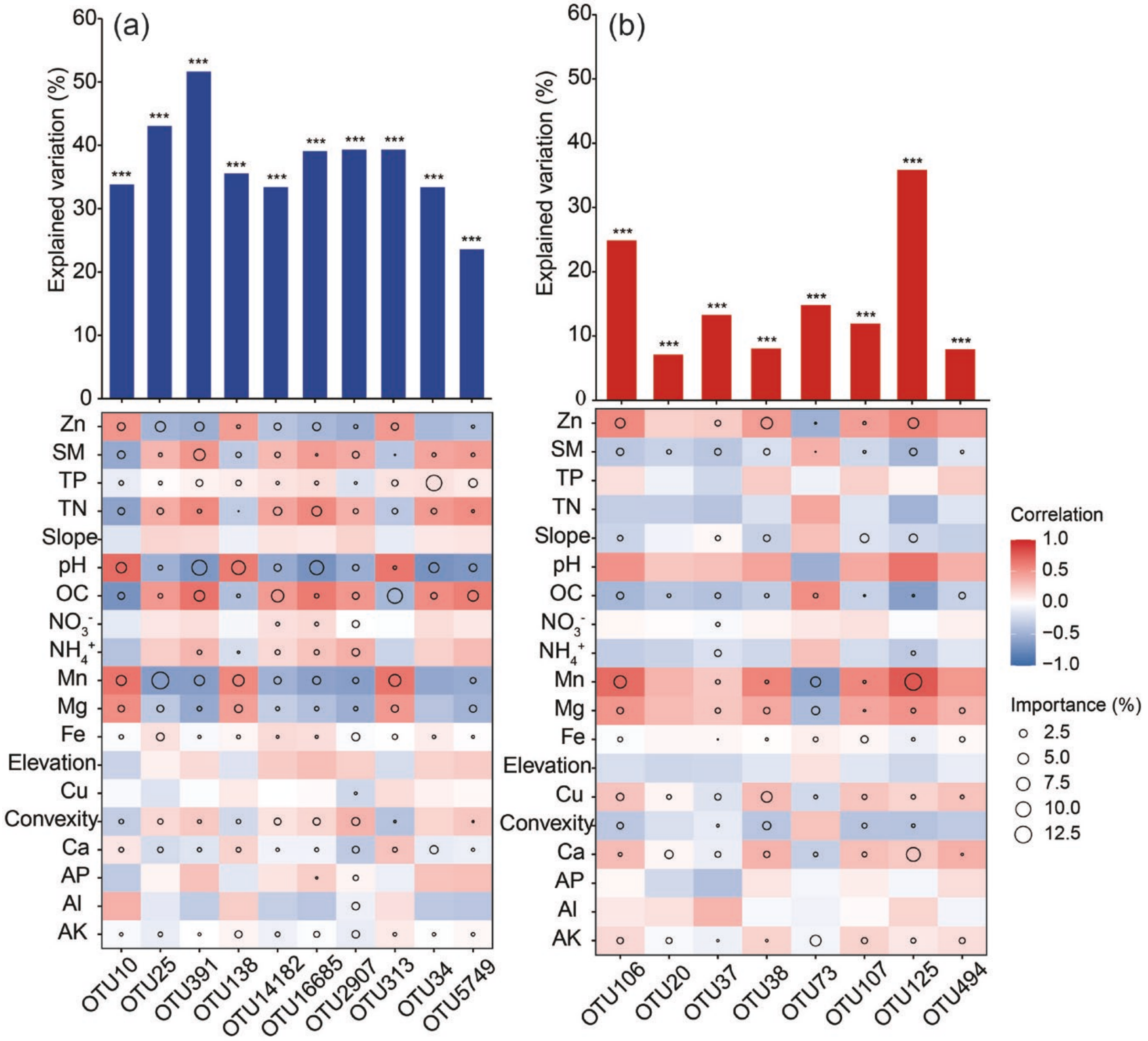

Figure 1: Contributions of soil and topographic factors to the differences in relative abundance of (a) keystone bacteria and (b) keystone fungi based on correlation and top-ranked regression models. We examined the correlations of these values with the differences in soil and topographic factors for each pairwise set of soil samples and identified the major predictors. The top bar chart represents the total contributions of soil and topographic factors to explain microbial variation (calculated by multiple regression modeling). All of the models had a type 1 error <0.001. Circle size in the bottom heatmap represents variable importance, and colors represent Spearman correlations.

Microbial community functions and network stability

A greater proportion of variation in microbial function was explained by all keystone taxa (48.7%–82.3%) than all rare taxa (2.7%–28.0%; Figs 3 and 4; Supplementary Figs S8 and S9). Bacterium OTU25 and fungus OTU73 were identified as the most important keystone taxa for all analyzed functions (Fig. 3). Bacterium OTU3723 was the most important rare taxon for predicting bacterial functions (Supplementary Fig. S8). The best predictors for symbiotrophic, pathogenic and saprotrophic fungi (excluding wood saprotrophs) were rare OTU36708, OTU14499 and OTU36708, respectively (Supplementary Fig. S9).

Co-occurrence networks showed that bacterial keystone taxa were distributed in six modules, and fungal keystone taxa were distributed in only three modules (Fig. 5a and b; Supplementary Fig. S10). Significantly higher stability was observed in

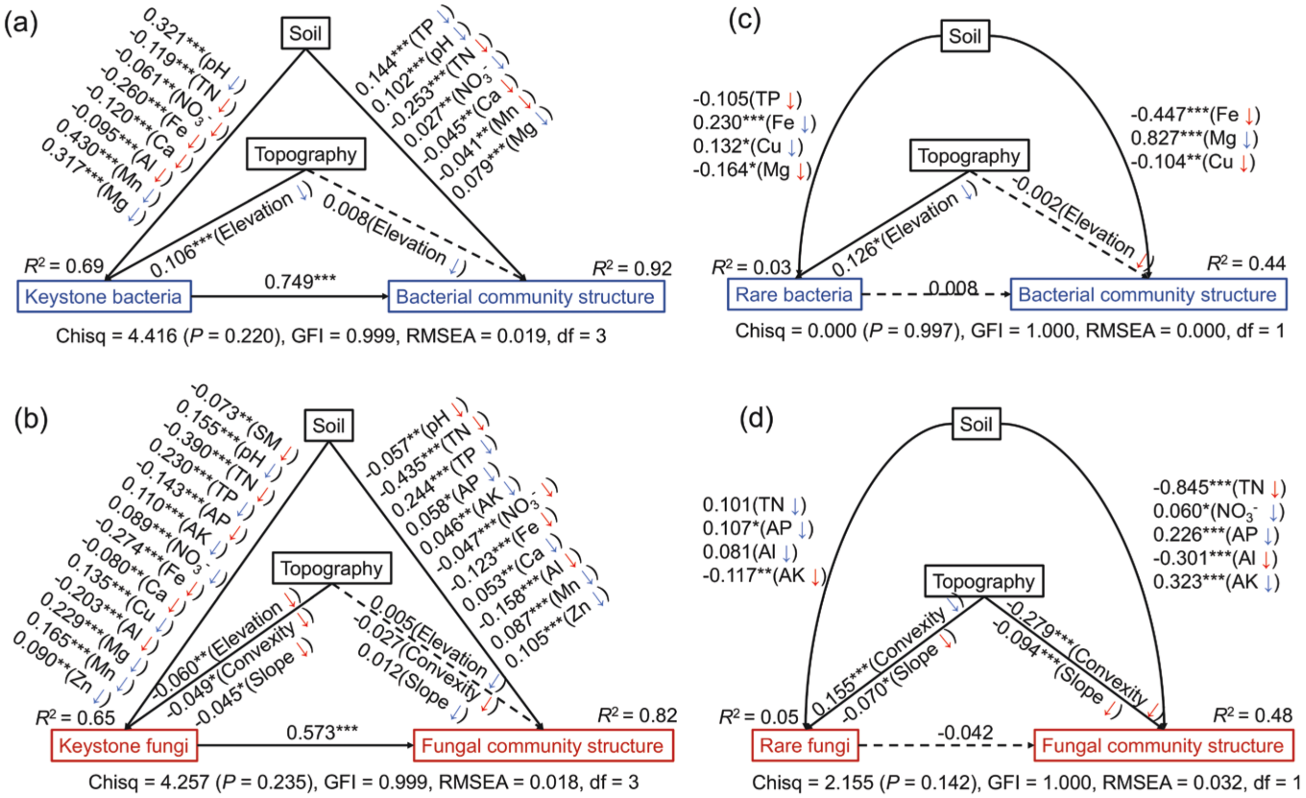

Figure 2: Causal path models for direct and indirect effects of soil properties, topographic factors and microbial groups on community structure. (a) for keystone bacteria, (b) for keystone fungi, (c) for rare bacteria and (d) for rare fungi. Solid and dashed arrows represent significant and non-significant relationships, respectively. R^2 values denote the proportion of variance explained for each variable. *** indicates $P < 0.001$; **, $P < 0.01$; *, $P < 0.05$. For keystone taxa, the response variables are calculated based on all soil samples (1287), but only 535 soil samples for rare bacteria and 1126 soil samples for rare fungi are selected because some samples have no defined rare taxa. That is why keystone and rare taxa are not analyzed together in the same model. After the sample size of keystone and rare taxa was unified, the results of SEMs had no major change (see Supplementary Information II for details).

the fungal network than in the bacterial network (Wilcox test, $P = 2.58e{-}32$, Fig. 5c and d; note higher robustness and lower vulnerability both indicate higher stability). Similarly, the stability (as inferred from AVD) of the fungal community was higher than that of the bacterial community (Fig. 5e; higher AVD means lower stability). Moreover, correlations between the relative abundance of rare taxa and the stability of the overall microbial community were slightly weaker than those between the relative abundance of keystone taxa and the stability of the overall microbial community (Supplementary Fig. S11).

Associations of keystone microbes and rare microbes with tree community composition

After controlling for the influence of soil and topographic factors, tree community composition within all radii of soil sampling sites was significantly correlated with microbial keystone taxa (all $P \le 0.001$, Table 1). In contrast, no significant associations were observed between tree community composition and microbial rare taxa (all $P \ge 0.175$, Table 1).

DISCUSSION

Keystone, not rare taxa, dominated ecosystem structure, functions and stability

Keystone and rare soil microbes can both make disproportionate contributions to ecosystem functions and community stability in diverse ecosystems (Banerjee *et al.* 2018; Herren and McMahon 2018; Lynch and Neufeld 2015; Pester *et al.* 2010). In this study, we found little evidence that rare soil microbial taxa contributed disproportionately to the structure, function and stability of soil microbial communities or to tree community composition. As expected, keystone taxa were more influenced by

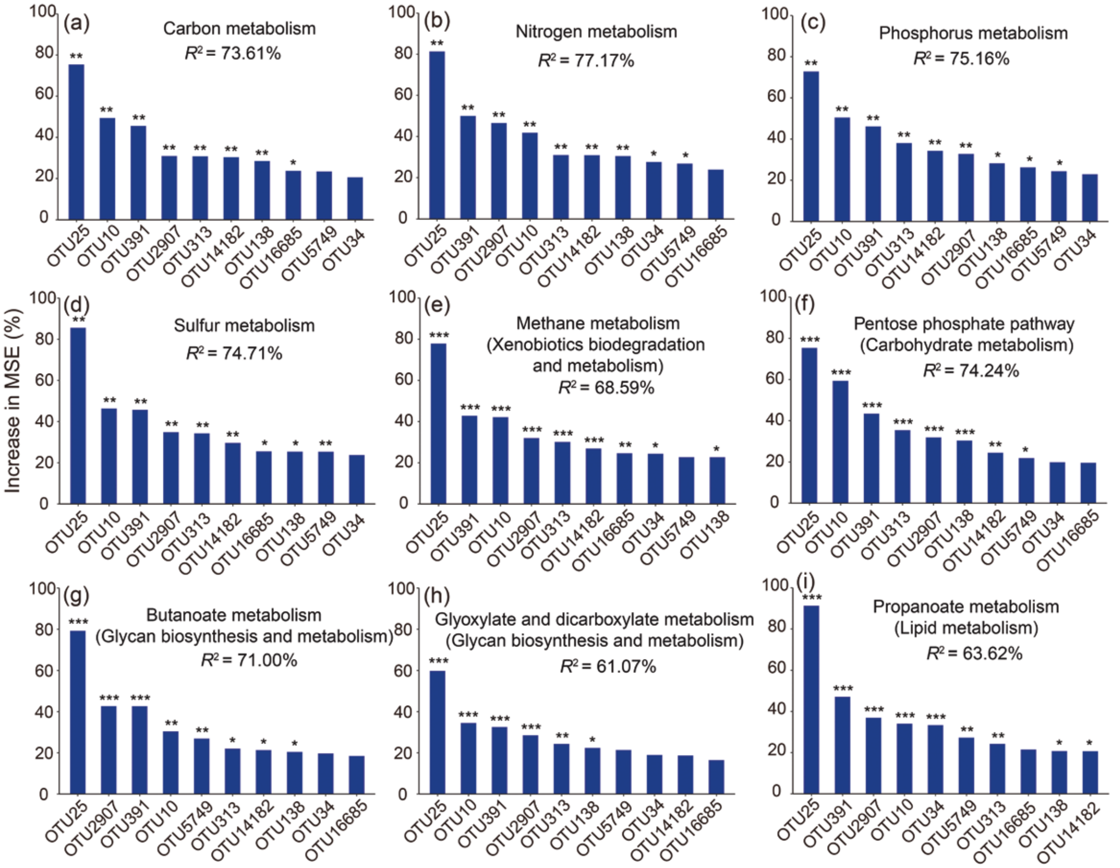

Figure 3: Keystone operational taxonomic unit (OTU) and their influence on bacterial functions. Bacterial contributions of variation in metabolic functions, including (a) carbon metabolism, (b) nitrogen metabolism, (c) phosphorus metabolism, (d) sulfur metabolism, (e) methane metabolism, (f) pentose phosphate pathway, (g) butanoate metabolism, (h) glyoxylate and dicarboxylate metabolism and (i) propanoate metabolism. The accuracy importance measure was computed for each tree and averaged over the forest (1000 trees). Percentage increases in the mean squared error (MSE) of variables were used to estimate the importance of these predictors, and higher MSE% values imply more important predictors. Significance levels are as follows: $^*P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$.

abiotic filtering than rare taxa and contributed more to soil community functions. Unexpectedly, in this forest ecosystem, keystone taxa also contributed more to community stability than rare taxa despite the higher diversity of the latter (Supplementary Fig. S11). This contrasts with the role played by rare taxa in sustaining crop mycobiome stability and ecosystem functions (Shade and Gilbert 2015; Xiong *et al.* 2021). As such, these findings raise questions over the relative importance of microbial keystone and rare taxa in unmanaged forest soils.

As anticipated, the effects of abiotic filtering on keystone taxa were greater than those on rare taxa. This was clearly illustrated by the indirect influence of abiotic factors on microbial community structure that was mediated by keystone taxa, but not by rare bacteria and fungi (Fig. 2). Ramirez *et al.* (2018) also found no evidence for abiotic filtering on rare soil bacteria across a variety of natural and anthropogenic habitats at the global scale, although they found that rare taxa dominated community structure, which contrasts with our results. Such inconsistency is likely due to our specific comparison of rare taxa with keystone taxa, without overlap between the two groups. It is possible that some influential rare taxa in the study of Ramirez *et al.* (2018) would have been classified as keystone taxa using our approach.

Keystone taxa constrain community composition through their influence on other taxa (Paine 1969). In our study site, abiotically filtered keystone taxa may interact with other taxa and alter the abundance of their partners, thereby regulating the entire soil microbial community structure (Banerjee *et al.* 2018). Bacterial keystone taxa were more influenced by abiotic factors than fungal keystone taxa (Figs 1 and 2), which might be attributed to the acidity of

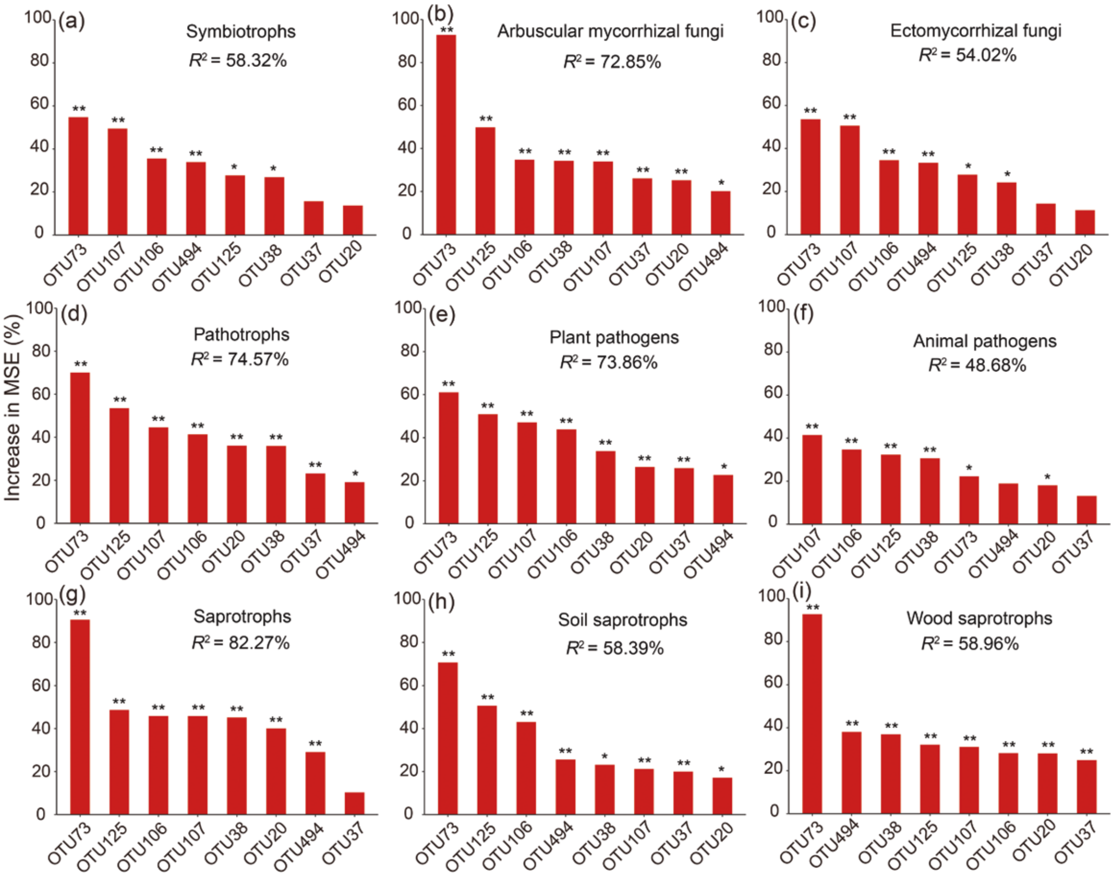

Figure 4: Keystone operational taxonomic unit (OTU) and their influence on fungal functions. Fungal contributions of variation in symbiotrophic, pathogenic, and saprotrophic functions, including (a) symbiotrophs, (b) arbuscular mycorrhizal fungi, (c) ectomycorrhizal fungi, (d) pathotrophs, (e) plant pathogens, (f) animal pathogens, (g) saprotrophs, (h) soil saprotrophs and (i) wood saprotrophs. The accuracy importance measure was computed for each tree and averaged over the forest (1000 trees). Percentage increases in the mean squared error (MSE) of variables were used to estimate the importance of these predictors, and higher MSE% values imply more important predictors. Significance levels are as follows: *$P < 0.05$ and **$P < 0.01$.

the soil (pH: 2.79–5.79), given that the community composition of bacteria is more sensitive to soil pH than that of fungi (Bahram *et al.* 2018; Fierer and Jackson 2006; Ni *et al.* 2021). Additionally, other studies have also shown that bacteria are more influenced by abiotic factors than fungi in a variety of ecosystems (de Dieu Habiyaremye *et al.* 2021; Millard and Singh 2010; Uroz *et al.* 2016).

Keystone taxa accounted for a larger proportion of functions of soil communities, although rare taxa did contribute to each function (Figs 3 and 4; Supplementary Figs S8 and S9). Keystone taxa have strong predictive power for variations in microbial community composition (Herren and McMahon 2018), and here we found keystone taxa explained more than five times the percentage of bacterial functions than that explained by rare taxa (Fig. 3; Supplementary Fig. S8). Microbial keystone taxa are positively associated with multiple functional genes related to nutrient cycling (Fan *et al.* 2021). The bacterial network with lower robustness and higher vulnerability suggested its stability was significantly lower than that of fungi (Fig. 5c and d). This might be attributed to the discrepancy in the body size of bacteria and fungi, which range from 0.2–20 μm (Young 2006) to 5–50 μm (Ingold 1971) in diameter, respectively.

Generally, diverse subsets of soil microbial taxa (e.g. bacterial keystone taxa *vs.* fungal keystone taxa; keystone taxa *vs.* rare taxa) have different life strategies and occupy various ecological niches (Pedrós-Alió 2012). Keystone taxa, on the one hand, have greater connectivity and play a stabilizing role in microbial community composition (Herren and McMahon 2018). On the other hand, keystone taxa, occupying wider niches than rare taxa, are likely

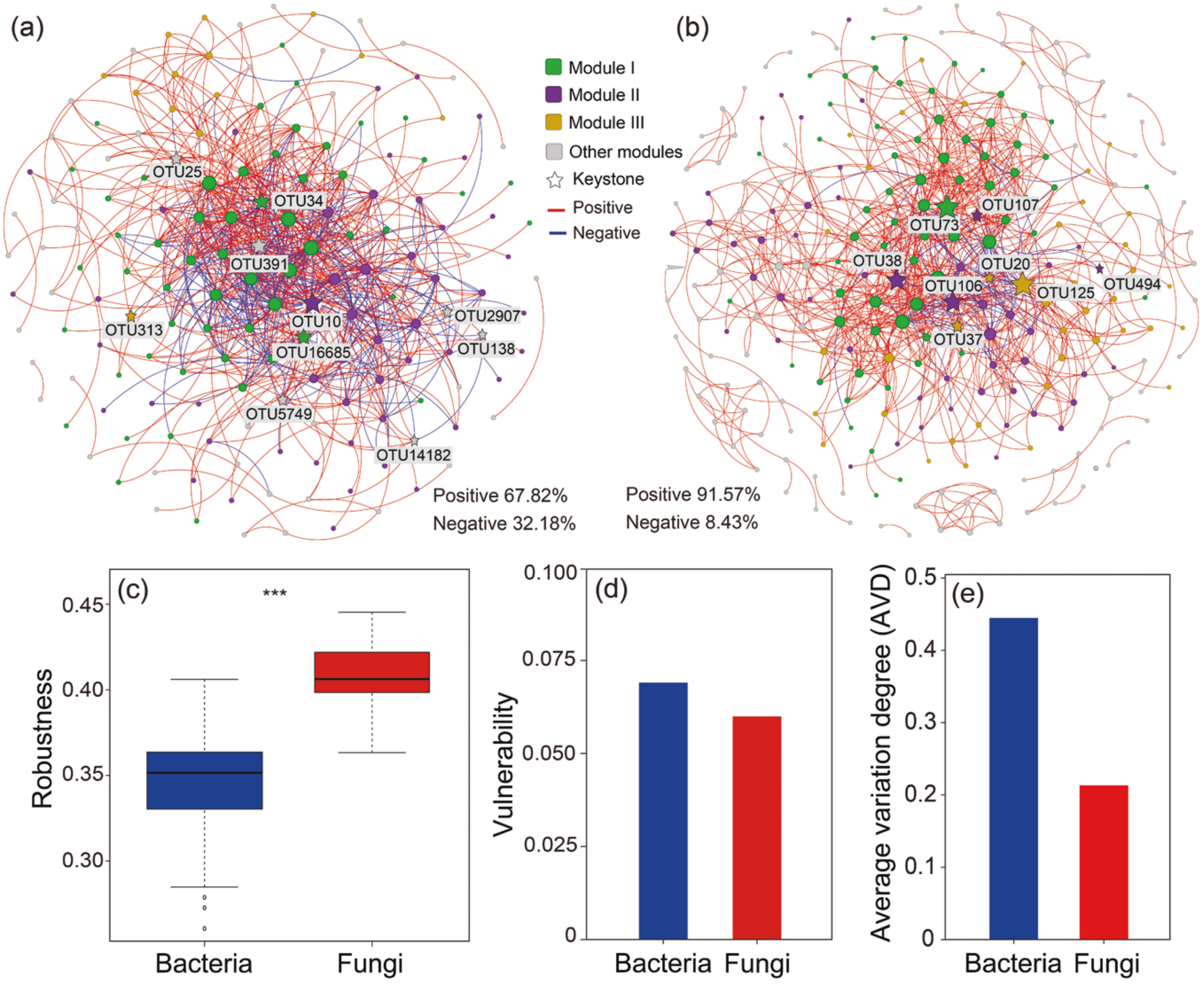

Figure 5: Co-occurrence network of operational taxonomic units (OTUs) for (a) bacterial and (b) fungal communities and network stability. The nodes were colored according to modularity, with node size proportional to the number of degrees. The edges in the networks depict correlations (red color = positive correlations; blue color = negative correlations). Keystone OTUs in the networks are indicated by star symbols. Since the correlations between OTUs that occur only in a small number of samples when constructing the networks can result in a high rate of false positives, the networks in this study did not contain rare taxa. (c) Robustness measured as the proportion of taxa remained when 50% of the taxa were randomly removed from each of the empirical molecular ecological networks, *** indicates $P < 0.001$. (d) Network vulnerability is measured by maximum node vulnerability in each network. (e) Average variation degree values of bacterial and fungal communities.

more able to utilize the resources that support their persistence. Microbial rare taxa, with their narrow niche breadth, exhibit greater habitat specificity (Jousset *et al.* 2017; Zhang *et al.* 2022).

Potential natural forest management applications of keystone soil microbes

We also found that tree composition was more strongly associated with keystone taxa than with rare taxa (Table 1). It is not surprising that soil microbes are known to strongly influence plant diversity (van der Heijden *et al.* 2008), resulting in significant correlations between aboveground tree communities and soil microbes (Barberán *et al.* 2015). Given that keystone taxa comprised relatively few species, if these correlations proved to be causal, it would simplify management applications analogous to those in agroecosystems. In such systems, it has been found that microbial keystone groups can be utilized to enhance crop productivity by modifying the aboveground-underground flow of mineral elements in the soil (Wang *et al.* 2022). Therefore, it is

Table 1: Relationships between keystone and rare microbial taxa with tree community composition

Radius (m)	Variable	*r*	*P*
20	Bacterial keystone taxa	0.134	<0.001
	Fungal keystone taxa	0.196	<0.001
	Bacterial rare taxa	0.004	0.209
	Fungal rare taxa	0.004	0.190
15	Bacterial keystone taxa	0.135	0.001
	Fungal keystone taxa	0.200	<0.001
	Bacterial rare taxa	0.005	0.175
	Fungal rare taxa	0.004	0.239
10	Bacterial keystone taxa	0.138	0.001
	Fungal keystone taxa	0.199	0.001
	Bacterial rare taxa	0.004	0.239
	Fungal rare taxa	0.003	0.265
5	Bacterial keystone taxa	0.116	0.001
	Fungal keystone taxa	0.177	0.001
	Bacterial rare taxa	0.001	0.411
	Fungal rare taxa	0.003	0.297

Statistics correspond to partial Mantel tests controlling for differences in soil and topographic factors. Differences among tree assemblages and microbial communities estimated using Bray–Curtis distances and differences among abiotic factors estimated using Euclidean distance. Radius refers to the distance around the corresponding soil sampling site used to define the neighborhood of trees, *r* is the Mantel test statistic showing the strength and direction of association, and *P* is the type 1 error estimate from permutation tests.

anticipated that a similar effect could be achieved in forest ecosystems. Collectively, these findings suggest that it could be potential, as with agroecosystems (Fan *et al.* 2021; Wang *et al.* 2022), to enhance the stability and resistance of soil microbial communities by regulating relatively few keystone taxa or a subset of microbial taxa to maintain or improve ecosystem functioning in natural forests. This may involve introducing them into forest soils as biofertilizers as follows (Zheng *et al.* 2021): (1) conducting a comprehensive analysis of the forest microbiomes using high-throughput sequencing techniques to elucidate their composition and interactions; (2) identifying keystone microbes by employing network analysis to reveal those with pivotal roles in forest soils; (3) screening for cultivable keystone groups that can be manipulated to exert beneficial effects; (4) selecting candidate isolates by comparison to the identified keystone taxa; (5) producing microbial fertilizer and applying it to forest soils (Supplementary Fig. S12).

Bacterial keystone taxa mostly belonged to Acidobacteria and Proteobacteria, which are among the most widely occurring phyla in soil globally (Ramirez *et al.* 2018). Consistent with the global pattern (Fierer and Jackson 2006), soil pH was strongly influential on keystone bacteria, and it is known to regulate the abundance of Acidobacteria (Rousk *et al.* 2010). Proteobacteria have also been found to be important in carbon cycling within forest ecosystems, as they preferentially consume the labile pool of organic carbon (Lladó *et al.* 2017). Additionally, certain taxa within the order Rhizobiales, involved in nitrogen-fixation within the N cycle (Chen *et al.* 2021), are well-known for their beneficial interactions with plants (Erlacher *et al.* 2015). In agroecosystems, Rhizobiales and *Candidatus Solibacter* have also been used as biomarkers (Zhang and Lv 2020). Fungal keystone taxa mostly belonged to Ascomycota, which are typical saprophytic fungi (Treseder *et al.* 2014) capable of degrading lignin-rich carbon sources in soils (Ye *et al.* 2020). Moreover, *Mortierella* is widely distributed in soils (Werner *et al.* 2016) and has been found to associate with both plant roots (Jiang *et al.* 2011) and leaves (Persoh 2013). While *Mortierella* is commonly isolated from forest litter as a saprophyte, it is now extensively used in agricultural soil for decomposition (Ozimek and Hanaka 2021). The keystone order Mortierellales also plays a critical role in decomposing complex organic matter (Wu *et al.* 2020).

Furthermore, the interaction between bacteria and fungi can have a profound impact on plant health, with bacterial commensals being able to regulate fungal growth (Getzke *et al.* 2019). Specifically, *Candidatus Solibacter* and *Acidibacter*, identified as two pivotal bacterial keystone genera, exert synergistic effects on ectomycorrhizal fungi and serve as positive predictors for the relative abundance of ectomycorrhizal fungi (Berrios *et al.* 2023). In the meanwhile, fungal keystone taxa with specific functions can engage in a range of interactions with bacteria, profoundly influencing the dynamics of bacterial communities. For example, fungal hyphae serve as a growth substrate for bacteria by releasing exudates as carbon resources (Shi *et al.* 2023; Zhang *et al.* 2021) and facilitate the spread of bacteria (Toljander *et al.* 2006). By mediating the dispersal of bacteria, fungal hyphae can also facilitate horizontal gene transfer among bacterial cells (Ruan *et al.* 2022), thereby promoting the rapid evolution of bacteria (Arnold *et al.* 2022; Shi *et al.* 2023). Therefore, the interactions between bacteria and fungi have the potential to shape the diversity and stability of microbiomes. This, in turn, ultimately influences ecosystem functioning, including soil carbon sequestration, plant productivity and pathogen suppression (Shi *et al.* 2023). Additionally, it has been noted that the variations in soil properties and topographic characteristics have the greatest impacts on the saprotrophic keystones (e.g. OTU125 and OTU106; Fig. 1), which play prominent roles in nutrient decomposition and redistribution (Hättenschwiler *et al.* 2005).

The multiple roles played by a relatively small number of keystone taxa in this forest raise the potential that keystone taxa could be manipulated to help maintain tree community diversity and regulate soil microbial processes. The identification of keystone species in forest soils could inform new approaches to sustainably manage forests to help ameliorate some impacts of global change (Bonan 2008). These findings could have the potential to guide future conservation priorities.

LIMITATIONS

While stronger associations were observed between keystone microbes, rather than rare microbes, and the structure and functions of soil microbial communities as well as tree composition, establishing definitive causality remains a challenge inherent in large-scale field investigation studies. Moreover, as with any study of this nature, patterns of co-occurrence in soil microbial networks must be interpreted with caution, as the correlations on which they are based might not translate to true interactions (Goberna and Verdú 2022). Ideally, the roles of keystone taxa inferred from this study should be tested using targeted follow-up experiments (Banerjee *et al.* 2018; Faust 2021). However, the test process could be complicated and time-consuming, such as the validation of the 'comammox' or complete nitrifiers (Costa *et al.* 2006; Daims *et al.* 2015). Moreover, it is currently unknown to what degree network stability, measured with a single parameter (Yuan *et al.* 2021), truly represents the stability of the study system. While this is a commonly adopted approach in network analysis, a variety of model calculations and experimental verifications of community stability are still necessary. Although the results from the Tiantong subtropical forest plot have been validated using another subtropical forest plot (Baishanzu), it is important to note that these findings may be limited to the specific forest type and environmental conditions. Therefore, future research should be conducted to test the universality and representativeness of these findings, including a wider range of forest types.

Supplementary Material

Supplementary material is available at *Journal of Plant Ecology* online.

Supplementary Information I

Table S1: The topological properties of networks constructed from operational taxonomic units (OTUs) that are present in more than different proportions of the samples.

Table S2: The relative abundance of keystone taxa reported in some studies.

Table S3: Relative abundances of keystone taxa in intra-trophic networks.

Table S4: The general information of rare microbial taxa.

Table S5: Topological properties of the MENs.

Figure S1: Distribution profile of quadrats and sampling sites.
Figure S2: Co-occurrence network of operational taxonomic units (OTUs) that are present in more than different proportions of the samples for (a) bacterial and (b) fungal communities.
Figure S3: Distribution of microbial operational taxonomic units (OTUs) based on their network roles.
Figure S4: Spearman correlation between abiotic factors and the relative abundance rare bacterial taxa.
Figure S5: Spearman correlation between abiotic factors and the relative abundance of rare fungal taxa.
Figure S6: Results of multiple regressions after the selection process for (a) keystone bacteria, (b) keystone fungi, (c) rare bacteria and (d) rare fungi on all measured variables.
Figure S7: Causal path models for direct and indirect effects of soil properties, topographic factors and microbial groups on community structure, where this structure was calculated after excluding the relevant keystone or rare taxa used in the path model.
Figure S8: Potential rare taxa drivers of variation in carbon metabolism, nitrogen metabolism, phosphorus metabolism, sulfur metabolism, methane metabolism, pentose phosphate pathway, butanoate metabolism, glyoxylate and dicarboxylate metabolism and propanoate metabolism of bacteria in the forest ecosystem.
Figure S9: Potential rare taxa drivers of variation in symbiotrophic (including arbuscular mycorrhizal fungi and ectomycorrhizal fungi), pathogenic (including plant pathogen and animal pathogen) and saprotrophic (including soil saprotroph and wood saprotroph) functions in the forest ecosystem.
Figure S10: Large modules (with ≥3 nodes) shown in circular layout for (a) bacterial and (b) fungal networks.
Figure S11: Relationships between average relative abundance of keystone and rare microbial taxa and variation degree.
Figure S12: Conceptual process for identifying and applying keystone soil microbial taxa in forests (adapted from Figure 7 in Zheng *et al.* 2021).

Supplementary Information II

Results of relevant analysis of keystone taxa from the same samples as rare taxa (535 samples for keystone bacteria and 1126 samples for keystone fungi).

Supplementary Information III

Results of relevant analysis of Baishanzu forest plot.

Authors' Contributions

Xian Wu and Yu Liu contributed to the study conception and design. Material preparation, data collection and analysis were performed by Xian Wu, Hua Xing, Jiarong Yang, Junfang Chen, Xiaolin Liu and Shu Dong. The first draft of the manuscript was written by Xian Wu. David C. Deane, Fangliang He and Yu Liu revised the manuscript. All authors read and approved the final manuscript.

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Conflict of interest statement. The authors declare that they have no conflict of interest.

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