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Afforestation effects on soil microbiomes modulated by changing soil physicochemical properties in temperate grassland

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Grassland afforestation dramatically affects the abiotic, biotic, and ecofunction properties of the original ecosystems. Interference from afforestation might disrupt the stasis of soil physicochemical properties and the dynamic balance of microbiota, although some studies have suggested low sensitivity of soil properties and a small response of bacterial community to afforestation. However, this “small response” is probably due to the confounding effects of the generalist habitat and rare microbes. In this study, soil physicochemical and prokaryotic properties in a 30-year-old Mongolia pine (*Pinus sylvestris* var. *mongolica* Litv.) afforested region and adjacent grassland in Inner Mongolia were classified and quantified. Our results indicate that the high richness of rare microbes accounts for the alpha-diversity of the soil microbiome, whereas generalist (core microbiota) and habitat-specialist microbes present in few numbers but high abundance govern the beta-diversity of the grassland and afforested land microbiomes. Afforestation has changed the soil physicochemical properties, thus indirectly affecting the soil microbial composition rather than richness. The contents of soil P, Ca, and Fe account for the ecofunctional changes in soil microbiomes due to grassland afforestation. We conclude that grassland afforestation has changed the physicochemical properties and composition of the soil and ecofunctions of the soil bacterial community and that these effects of afforestation on the microbiome have been modulated by changes in soil physicochemical properties.

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Short title: Afforestation effect on soil microbes

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Conflict of Interest

The authors declare that they have no conflict of interest.

Abstract

Grassland afforestation dramatically affects the abiotic, biotic, and ecofunction properties of the original ecosystems. Interference from afforestation might disrupt the stasis of soil physicochemical properties and the dynamic balance of microbiota, although some studies have suggested low sensitivity of soil properties and a small response of bacterial community to afforestation. However, this "small response" is probably due to the confounding effects of the generalist habitat and rare microbes. In this study, soil physicochemical and prokaryotic properties in a 30-year-old Mongolia pine (*Pinus sylvestris* var. *mongolica* Litv.) afforested region and adjacent grassland in Inner Mongolia were classified and quantified. Our results indicate that the high richness of rare microbes accounts for the alpha-diversity of the soil microbiome, whereas generalist (core microbiota) and habitat-specialist microbes present in few numbers but high abundance govern the beta-diversity of the grassland and afforested land microbiomes. Afforestation has changed the soil physicochemical properties, thus indirectly affecting the soil microbial composition rather than richness. The contents of soil P, Ca, and Fe account for the ecofunctional changes in soil microbiomes due to grassland afforestation. We conclude that grassland afforestation has changed the physicochemical properties and composition of the soil and ecofunctions of the soil bacterial community and that these effects of afforestation on the microbiome have been modulated by changes in soil physicochemical properties.

Keywords: grassland afforestation; microbial composition; microbial ecofunction; soil physicochemical properties

Introduction

After a decline in forest coverage in China to only 8.6% due to war, urban construction, and reclamation, efforts by the Chinese government in the last 30 years to promote afforestation have increased forest coverage to nearly 20% (State Forestry Administration of China 2011). However, these plantations are not exclusively located at the original sites of deforestation. Consequently, these deforestation and afforestation events have greatly changed the landscape and ecosystems of China (Ahrends et al. 2017). Mongolia pine (*Pinus sylvestris* var. *mongolica* Litv.), an endemic tree in Inner Mongolia, is one of the main forestation species in temperate regions of China. Scots pine (*P. sylvestris* L.), a relative of Mongolia pine that is widespread from Western Europe to Eastern Siberia, is also an important tree in forestry (Krakau et al. 2013). Studies of Mongolia pine are scarce, but several studies have revealed that Scots pine is genetically and physiologically sensitive to environmental pollution (Chudzińska et al. 2014), geographic weather variation (Oleksyn et al. 2003) and climate change (Hurme et al. 1997; Savolainen et al. 2004). Phenological variation in response to climatic adaptation has also been suggested to be linked to quantitative trait loci (Hurme et al. 2000). These studies have demonstrated that Scots pine and, by reasonable extension, Mongolia pine have broad, plastic adaptability in response to environmental heterogeneity, supporting the wide use of these trees in afforestation.

Grassland afforestation is an artificial and direct change in vegetation that alters both the above- and underground ecosystems, including abiotic changes [e.g., land surface temperature (Li et al. 2016; Peng et al. 2014), hydrological connectivity, litterfall and litter decomposition (Khamzina et al. 2016; Lafleur et al. 2015), and soil physicochemical properties (Chen et al. 2008; Lafleur et al. 2015; Wang et al. 2016; Zheng et al. 2017)], biotic changes [e.g., flora(Ma et al. 2013) and fauna compositions(Márquez et al. 2015; Pedley et al. 2014) and soil microbiota (Gunina et al.

2017; Šnajdr et al. 2013; Xiao et al. 2017)], and ecofunctional changes [e.g., plant-soil and plant-microbe interactions (Lu et al. 2017; Ren et al. 2016) and functional diversity of communities (Cibils et al. 2015)]. Land use is a significant determinant of runoff and soil redistribution processes (Arnáez et al. 2015), and [illegible]tic responses could be sensitive to compositional changes in both species and ecofunctions (Xiao et al. 2017). Therefore, changes in microbial composition and function in soil can be quantified as indicators to monitor microenvironmental changes due to afforestation or reforestation.

[illegible]e afforestation process may alter the original environment. Although the environmental microbial composition may be sensitive to environmental variation, the effects of these microbial compositional changes on ecofunction remain unclear. A comprehensive review demonstrated that the soil microbial composition varies to alter acquisition, metabolism, and degradation processes in response to changes in soil [illegible]ue to grassland afforestation (Chen et al. 2008). The [illegible]N:P ratio of soils with different vegetation types also reflect different transformation processes and rates between plant and soil organic matter, and these biogeochemical processes are mediated by soil microbial community structure and functions (Zechmeister-Boltenstern et al. 2015). More importantly, environmental conditions and existing microbial diversity determine the ecological function and nutrient transformation efficiency of soil microbiota (Zechmeister-Boltenstern et al. 2015). Therefore, quantifying the effects of afforestation on changes in soil properties, microbial composition, and ecofunctions would accelerate the understanding of plant-soil-microbe interactions.

Afforestation has been suggested to have a greater impact on soil biological properties than soil chemical properties (Gunina et al. 2017; Jangid et al. 2011). These underground biological

changes are mainly ascribed to changes in litter amount and chemistry, which stimulate the development of the fungal community rather than the bacterial community (Klein et al. 1995). The response of the prokaryotic microbiome to changes in vegetation type is small, likely because microbial assemblies are based mainly on functional genes rather than species (Burke et al. 2011), and the changes in soil properties due to afforestation are relatively small and occur slowly (Gunina et al. 2017; Jangid et al. 2011). In addition, abundant microbial "core species" with highly conserved core functions (Falkowski et al. 2008) and rarely occurring species (Ai et al. 2013) may act as confounders in statistical analyses of the effects of afforestation or soil properties on microbiome change. Therefore, habitat generalists (core species) and specialists (divergent species) in microbial communities must be classified before analysis, particularly when using high-throughput sequencing (HTS) technology (e.g., 16S rRNA metagenomic sequencing), which can generate huge amounts of data, to outline the composition and structure of microbial communities (Székely & Langenheder 2014).

In this study, to quantify the effect of afforestation on soil properties and soil microbial composition and functions, the soil physicochemical and microbial compositions were measured in an afforested region and adjacent grassland. Based on previous studies that have demonstrated functional assembly of bacterial communities (Burke et al. 2011) and a small response of bacterial communities to afforestation, with low sensitivity to soil properties (Gunina et al. 2017; Jangid et al. 2011; Klein et al. 1995), we hypothesized that (1) the soil prokaryotic microbiome is indirectly affected by afforestation mediated by soil physicochemical properties and (2) soil microbial ecofunctions are more sensitive to changes in vegetation type than changes in soil microbial composition. To test these hypotheses, the 16S rRNA metagenomes of the afforested and adjacent grassland soil microbiomes were sequenced, and the relative abundances of

118 microorganisms and predicted ecophysiological functions were quantified by multivariate and
119 regression analyses. Based on these analyses, we provide a possible explanation of the link
120 between soil physicochemical properties and microbiome changes in grassland afforestation.

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122

123 ## Materials and Methods

124 **Study sites and sampling**

125 Mongolia pine is native to Honghua'erji in Inner Mongolia in China and is widely planted as a
126 forestation tree in temperate Asia. The study site was located 6 km west of the town of
127 Honghua'erji, in an artificial forest produced by seedling afforestation in a large area of thin
128 grassland (savanna). Field experiments were approved by the Honghuaerji Nature Reserve
129 (permit number 200/66150221). The tree ages of the Mongolian pines in this afforestation forest
130 ranged from 27 to 33 years, with a DBH of 21.21±4.19 cm (12.09~32.10 cm) and tree height of
131 12.86±0.86 (11.2~14.6 m). In this seedling afforestation area, almost no seedlings were found
132 (personal observation), implying that this region lacks naturally symbiotic fungi to assist the
133 germination and growth of Mongolian pines except in the planted soils. Although the lack of
134 fungal symbionts of Mongolian pines requires further investigation and confirmation, the
135 absence of seedlings suggests that this afforestation area was likely not a Mongolian pine forest
136 in the past.

137 At the study site, we collected soil samples from 20 quadrants, including 10 inside the forest (i.e.,
138 forest soils) and 10 in the adjacent grassland (i.e., grassland soils). At each location, soil samples
139 were collected at a depth of 50 cm from 10 quadrants located 10 to 100 m from each other. The

soil samples were separated into two parts: one part was dried for quantification of the soil physicochemical properties, and the other was used to quantify the microbiome. The latter samples were stored in RNAlater stabilization solution on ice immediately after collection until transfer to the laboratory, where the samples were stored at -20 °C before metagenomic DNA extraction.

Quantifying soil physicochemical properties

Organic carbon (C) was measured by the external-heat potassium dichromate oxidation method, and total nitrogen content (N) was measured by the Kjeldahl distillation method. The inductively coupled plasma (ICP) method was used with soils digested in a mixture of HF–$HClO_4$–HNO_3 to quantify the contents of the soil elements K, P, Ca, Fe, Mg, and Na. Soil pH was determined using a Sartorius pH meter PB-10 (Germany). All soil properties were determined and quantified by the State Key Laboratory of Vegetation and Environmental Change, Institute of Botany, Chinese Academy Sciences.

16S rRNA metagenome sequencing

Microbial metagenomic DNA was extracted with an EZNA® Soil DNA Kit (Qiagen, Valencia, CA, USA), and the concentration was adjusted to 50 ng/μL. The metagenomic DNA was quantified using Qubit® 2.0 (Invitrogen, Life Technologies, CA, USA). Primers 341F and 805R were used to amplify the V3-V4 hypervariable 16S rRNA region, and the PCR products were used to construct a DNA library with the Roche GS FLX Titanium emPCR kit (Roche Applied Science). The DNA libraries were then sequenced by Sangon Biotech Co. (Shanghai, China) on

an Illumina MiSeq 2X300. The sequencing procedures followed the manufacturer's instructions.

Before analysis, the raw HTS data were cleaned by removing sequence fragments shorter than 200 bp or with missing barcodes or polyN or polyA/T in Ribosomal Database Project (RDP) (Cole et al. 2007). We also discarded reads with PHRED quality scores <Q25 (Ewing & Green 1998; Ewing et al. 1998). The Mothur package was used to remove non-prokaryotic sequences and de-noise and trim the sequences (Mothur ver. 1.30.1, http://www.mothur.org/wiki/MiSeq_SOP (Schloss et al. 2009)). Chimeric sequences were removed using Uchime (Edgar et al. 2011). After quality filtering, we clustered sequences using a criterion of >97% sequence similarity as operational taxonomic units (OTUs) defined as representing the same species by the RDP classifier (Cole et al. 2007). The rarefied OTU table was generated using Qiime (Caporaso et al. 2010) and deposit in Mendeley (doi:10.17632/gjskh8wswz.1). Each OTU was annotated and classified according to the RDP classifier and SILVA database. The raw sequence data were deposited in NCBI GenBank under Bioproject PRJNA317430 (Accession number: SAMN04607375).

Predictive functional profiling of microbial communities

PICRUSt v. 1.0.0, a functional prediction tool for estimating shared gene content according to the corresponding microbiome phylogeny, was used to predict the molecular functions of each sample (Langille et al. 2013). PICRUSt generates the composition of gene families for each metagenome using an extended ancestral-state reconstruction algorithm. The online version of PICRUSt implemented in Galaxy (https://huttenhower.sph.harvard.edu/galaxy/) was used to assist the algorithms. The quality-filtered sequences were assigned to a closed reference OTU table against the Greengenes v. 13.5 OTU database (DeSantis et al. 2006) for PICRUSt

prediction implemented in QIIME v. 1.8.0 (Caporaso et al. 2010). Each OTU was normalized by its copy number. The functional contribution of each OTU member was reconstructed and predicted by mapping the 16S sequences to their nearest reference genome. 'Virtual' metagenomes with gene content abundance were then generated using the Kyoto Encyclopedia of Genes and Genomes (KEGG) Ortholog and Clusters of Orthologous Groups (COGs) databases.

Statistical analyses

This study included two types of measurements, the soil physicochemical properties and soil microbiomes (i.e., relative abundances of microbial OTUs), and one treatment, the grassland afforestation (i.e., vegetation type). We first assessed the differences in soil properties between vegetation types by the Kruskal-Wallis (KW) test. A logistic regression model was further used to determine if the soil properties predicted the grassland-afforestation treatment. The likelihood ratio test (LRT) was performed to identify the best-fitting model of the logistic regression. To test the hypotheses of a stochastic process of microbial assembly (random distribution model) or resource-governed assembly (niche-based mechanism), we used Rank-Abundance Dominance (RAD) analysis to display logarithmic species abundances against species rank order (McGill et al. 2007). Bray-Curtis distance-based redundancy analysis (dbRDA) was used to examine the explanatory proportions of the treatment for these two measurements. Because soil physicochemical properties can also affect the soil microbiome (Stutter & Richards 2012), we further performed a partial dbRDA to assess the effects of grassland afforestation on the soil microbiome conditioned by soil physicochemical properties and the effects of the soil physicochemical properties on the soil microbiome conditioned by vegetation type. Type II

ANOVA was used to evaluate the fit of the model of each constraint factor. We further identified divergent microbes (i.e., forest and grassland specialists) using the supermajority rule (>2/3 difference in abundance) with the assistance of the multinomial species classification method (CLAM) test (Chazdon et al. 2011). The soil elements that significantly explained the soil microbiome by partial dbRDA were then used as independent factors to predict the abundance of microbial specialists by the generalized linear model (GLM). Because the ecophysiological functions (COGs and KEGG modules) of the soil microbiomes were also predicted, all tests were repeated by replacing the soil microbial OTUs with the COGs and KEGG modules.

Results

Effects of afforestation on soil physicochemical properties

To characterize the soil physicochemical properties, the contents of eight elements (organic C, total N, P, K, Ca, Mg, Fe, and Na) and the pH value of each soil sample were determined (Table 1). Among these soil variables, the contents of C, P, Ca, Mg, and Fe were significantly higher in the forest soils than in the grassland soils (P = 0.0003, 0.008, 0.003, 0.0004, and 0.00006, respectively), and the pH of the grassland soils was significantly higher than the pH of the forest soils (P = 0.004) (Fig. 1). To estimate the explanatory proportions of the afforestation effect on the variance of soil properties, we performed dbRDA using vegetation type (i.e., forest and grassland) as the independent factor. Vegetation type significantly explained 50.18% of the variance of soil properties (P = 0.002, Table 2), indicating that afforestation has changed the soil properties.

To determine if the contents of soil elements can predict the vegetation type (i.e., afforestation

effect), we compared the simple logistic regression (SLR) model using a single soil element as the independent factor and the multivariate logistic regression (MLR) model using all soil elements as independent factors (i.e. the full model M1) to the empty (null) model M0. Most of the SLR models and M1 rejected the null model M0 ($P < 0.005$), except the SLR models with K (LRT: $P = 0.084$) or Na (LRT: $P = 0.108$) as the independent factor. We further compared the SLR models with each single soil element (C, N, P, Ca, Mg, Fe, and pH) as the independent factor to the MLR model using all C, N, P, Ca, Mg, Fe, and pH as independent factors (full model M2) by LRT. The SLR models with Mg or Fe as the independent factor could not be rejected by M2 ($P = 0.278$ and 0.130, respectively, Fig. 2). Although the other SLR models with C, N, P, Ca, or pH as the independent factor were rejected by M2, none of the independent factors in M2 could significantly predict the presence of forestation ($Z < 10^{-6}$, $P > 0.9999$ in each term). These results indicated that the contents of Mg (0.456±0.099 and 0.313±0.035 g/kg in forest and grassland soils, respectively) and Fe (8.355±1.047 and 5.607±1.308 g/kg in forest and grassland soils, respectively) could singly reflect the changes in soil properties due to afforestation (Table 1 and Fig. 2).

Afforestation effects on the relative abundances of soil microorganisms

We further examined how afforestation affected the biological properties of soils. We estimated the relative abundances (RAs) of the soil microbiome according to 16S rRNA metagenome sequencing. The RAs of microbial OTUs were estimated from the reads of the 16S rRNA gene with >97% similarity. Divergence of the soil microbiome was inferred by significant or marginal differences in the diversity indices (Shannon–Wiener index H, $KW\chi^2 = 3.291$, $P = 0.070$; Reciprocal Simpson's index $1/D$: $KW\chi^2 = 4.166$, $P = 0.041$; Pielou's evenness J: $KW\chi^2 = 9.864$,

$P = 0.002$, Table 3). However, no difference was estimated in the species richness of the soil microbiome between forest and grassland (KW$\chi^2 = 0$, $P = 1$, Table 3). The significant differences in H, $1/D$, and J but not richness between the grassland and forest soil microbiomes suggest that the change in the soil microbiome is species composition (RA) rather than species number. Hence, we used RA as an indicator to compare the microbiomes of the soil samples. Hierarchical cluster analysis (H-cluster) and DAPC (five first PCs of PCA used, which conserved 70% of the variance of the microbial RA) presented similar patterns of clear divergence of soil microbiomes between the forest and grassland (Fig. 3). These results confirm that the soil microbial composition has changed due to grassland afforestation despite no change in microbial richness.

Because most of the 16S rRNA sequences with <97% sequence identity and hence assigned as different OTUs were rarely found among different samples, we performed the CLAM test (Chazdon et al. 2011) to classify these OTUs as four types of microbes using the supermajority (2/3) rule: too-rare microbes, generalist microbes, and grassland- and forest-specialist microbes. In this classification, a large proportion of OTUs (95.46%) belonged to the too-rare microbes, which accounted for 38.42% and 36.73% of the abundance of grassland and forest soil microbes, respectively; only 2.98% of the OTUs were generalists, but they accounted for 35.55% and 35.88% of the abundance of grassland and forest soil microbes, respectively. Only 1.56% of the OTUs belonged to specialists, of which 1061 (0.7%) and 1224 (0.8%) microbial OTUs were identified as forest and grassland specialists, respectively. The RA of grassland-specialist microbes was 22.84% in grassland soils and 2.84% in forest soils, while the RA of forest-specialist microbes was 3.19% in grassland soils and 24.55% in forest soils.

We further used the RAs of the microbial OTUs as the dependent response to access the impact

of afforestation on the soil microbiome. When vegetation type was used as the categorical independent factor, 16.3% of the variance of microbial RA was explained significantly (P = 0.001). However, the significant explanatory effect of vegetation type was lost after removing (conditioning on) soil properties (P = 0.466, Table 2). When soil properties were used as a constraint factor, 64.4% of the variance of microbial RA was explained by soil properties, in which the organic C (11.4% explanation), P (10.0%), Ca (9.0%), Mg (7.1%), and Fe (10.5%) significantly fit the model by type II ANOVA (Table 2). Similar significant explanations by C (6.7%), P (9.5%), Ca (8.8%), Mg (6.7%), and Fe (5.4%) were obtained when the effect of vegetation type was removed (i.e., partial dbRDA, Table 2). These analyses suggested that afforestation has changed the soil physicochemical properties, which has indirectly affected the soil microbiome.

Effects of soil properties on the divergence of microbial phyla between forest and grassland soils

To identify the divergent soil microorganisms, the habitat-specialist microbial phyla were classified under the supermajority rule (i.e., 2/3 majority). At the phylum level, five phyla were habitat specialists, including one forest specialist (Thaumarchaeota, 2.3%) and four grassland specialists (Chloroflexi, Fibrobacteres, Nitrospirae, and Parcubacteria, 9%). The forest-specialist phylum Thaumarchaeota belongs to Archaea, while the four grassland specialists are Eubacteria.

We further tested the effects of soil properties on the abundance of these habitat specialists by GLM. Because organic C, P, Ca, Mg, and Fe significantly explained the RA of soil microbes in

dbRDA, those five soil variables were used as independent predictors in GLM. The variances of the microbial abundance of the sampled soils were significantly or marginally significantly greater than the means, suggesting overdispersion of these responses (P = 0.056, 0.001, 0.051, 0.002, and 0.002; alpha = 52.675, 25.871, 1.065, 23.101, and 76.612 in Thaumarchaeota, Chloroflexi, Fibrobacteres, Nitrospirae, and Parcubacteria, respectively). Hence, we used the quasi-Poisson model for GLM, which led to the same coefficient estimates as the standard Poisson model but with adjustment of the dispersion parameter for overdispersion. Under the quasi-Poisson model, Fe content was marginally correlated with the abundance of the forest specialist Thaumarchaeota; for the grassland specialists, the P content was correlated with the abundances of all four phyla (Table 4). In addition, C, Ca, and Mg were significantly correlated with Chloroflexi, Fe was significantly correlated with Nitrospirae, and Ca and Mg were marginally correlated with Parcubacteria (Table 4).

Afforestation effects on the soil ecophysiological functions predicted by soil microbiome

To understand the ecophysiological functions of the soil microbiomes, we predicted their functional composition using 16S rRNA gene and databases of reference genomes. A total of 4659 clusters of orthologous groups (COGs) and 306 KEGG modules (Level-3 KEGG orthology) were identified. Similar to the analyses for testing the effects of afforestation and soil properties on the soil microbiome, we used vegetation type and soil elements as predictors to test the explanatory proportion and significance of each predictor on the RAs of the COGs and KEGG modules. dbRDA indicated that 22.0% and 21.2% of the variation of COGs and KEGG modules was significantly explained by vegetation type, respectively, whereas the explanatory proportion decreased to 3.4% and 4.5% when conditioning the soil-property effect (Table 5). Soil properties

explained 83.0% and 82.4% of the variation of the COGs and KEGG modules in dbRDA, respectively, in which C, P, Ca, Mg, Fe, and N significantly or marginally fit the model according to type II ANOVA (Table 5). When conditioned on vegetation type, the explanatory proportion decreased slightly to 64.4% and 65.8% for the COGs and KEGG modules, respectively, and the remaining significant fitting factors were P, Ca, and Fe (Table 5).

Among all COGs, 44 and 21 were identified as forest and grassland specialists, respectively, whereas among all KEGG modules, only two forest specialists and no grassland specialists were identified. Further testing of the correlation of soil elements with these specialist COGs and KEGG modules revealed that 50 of the 65 COGs and both KEGG modules were significantly correlated with at least one soil element under Poisson or quasi-Poisson regression. These significant correlations indicate that soil properties, particularly P, Ca, and Fe, account for the changes in ecophysiological functional due to grassland afforestation.

Discussion

The closure of the tree canopy and increased litter accumulation that accompany the ecosystem change from grassland to forest may directly alter the soil environment (Bond & Midgley 2012; Cunningham et al. 2015). The significantly higher contents of soil physicochemical factors (C, P, Ca, Mg, Fe, and pH) in the Mongolian pine plantation areas than in the unplanted region suggest a great influence of grassland afforestation on secondary salinization. Soil mineral elements are usually increased in tree-plantation regions where groundwater is insufficient to meet water requirements (Nosetto et al. 2008). High contents of soil elements in a forest suggest not only a larger amount of litter biomass but also a rapid decomposition rate of pine litters compared to

343 other broadleaf flora (Berger et al. 2015). However, despite significant differences in the
344 contents of soil elements between forest and grassland, these soil elements, except Mg and Fe
345 (Fig. 2), could not singly predict the ecosystem change due to afforestation by logistic regression
346 analysis.

347 Significantly high predictable contents of Fe and Mg in forest soils reflect the characteristics of
348 litter and humus accumulation in forests (Song et al. 2008). Litter decomposition accelerates the
349 conversion and accumulation of soil non-organic elements (Fenchel et al. 2012b). In 2nd-to-3rd-
350 year needle litters of *P. sylvestris* L., a decrease in the rate of biomass loss but an increase in the
351 release of Fe and Mg were recorded (De Marco et al. 2007). Consequently, we suggest that the
352 high contents of Fe and Mg in the forest soils of our study sites are due to the long, steady
353 accumulation and decomposition of needle litter. The soil element cycling affects and is affected
354 by the composition of the soil microbiota (Fenchel et al. 2012b). For example, the iron bacteria
355 *Siderocapsa* (Siderocapsaceae) and *Leptothrix* (Comamonadaceae), which are able to deposit
356 iron metal oxides under natural conditions, can grow rapidly in iron-rich and acidic substrates
357 (Fenchel et al. 2012a; Hanert 2006). The distributions of the soil microbial abundances in our
358 samples best fit to Zipf and Zipf–Mandelbrot rank abundance models (Table S1 and Fig. 4). The
359 Zipf and Zipf–Mandelbrot rank abundance models belong to the family of random-branching
360 processes; these models suggest that individuals are always derived from ancestor individuals
361 (McGill et al. 2007) and that microbial community assembly is explained by the niche-based
362 mechanism (McGill et al. 2007; Mendes et al. 2014). These models indicate that decades of
363 grassland afforestation have generated soil properties that provide a divergent but stable resource
364 supply for the soil microbial community.

365 Classification by the supermajority rule revealed that generalist microbes represented more than

1/3 of the RA but only <3% of microbial OTUs. The low richness but relatively high abundance of generalists suggests that a great proportion of residents utilize broad resources or are highly tolerant of the environment (Verberk 2011). It has been suggested that microbes that are present in all or the majority of microbial communities with high abundance represent the core set of genes responsible for key elements of most metabolic pathways (Falkowski et al. 2008). Similarly, specialist microbes exhibited <1% richness but accounted for approximately 1/5 to 1/4 of the RA in the grassland and forest soils. These specialist OTUs with low richness and high abundance are probably more susceptible than generalists to environmental change. Since the original vegetation was scattered grasses, the grassland specialists rarely found in forest soils were those selected against by the afforestation effect; by contrast, forest specialists should be enriched after forestation. The environmental differences (e.g., the contents of soil C, P, Ca, Mg, and Fe, Table 2) could result in resource (niche) divergence to differentiate the microbial composition descended from the original microbiome, reflecting the microbial abundance distribution in the Zipf and Zipf–Mandelbrot models (Table S1).

In particular, the Fe content was significantly correlated with the abundance of the forest-specialist Archaea phylum Thaumarchaeota, and P was correlated with the four grassland-specialist Eubacteria phyla Chloroflexi, Fibrobacteres, Nitrospirae, and Parcubacteria (Table 4), suggesting that these two soil elements are key factors differentiating soil microbial composition. encodes the genes ammonia monooxygenase A (*amoA*, encoding subunit A of AMO) and *amoB*, which are distantly related to one another (Stieglmeier et al. 2014). The high abundance of *amoA* and its transcripts suggests that ammonia-oxidizing Archaea (AOA) outnumber ammonia-oxidizing bacteria (Shen et al. 2008) and that nitrogen cycling is enhanced in the forest (Konneke et al. 2005; Stieglmeier et al. 2014). A high abundance of AOA with a high content of Fe (e.g.,

ferrate, an ammonia oxidation reagent) could accelerate ammonia oxidation (Sharma et al. 1998).

Soil P is closely related to plant growth (Shen et al. 2011). However, much of soil P is stable and insoluble and hence recalcitrant to uptake by plants (Holford 1997). Microbes transform P to improve uptake by plants, which is also influenced by a combination of factors, including plant species and soil type (Chen et al. 2008). However, the specialists that were correlated with soil P content were primarily not responsible for P transformation but were light and aerobic thermophiles (e.g., Chloroflexi), symbionts with ruminant animals (e.g., Fibrobacteres), or involved in the nitrogen cycle (e.g., Nitrospirae). Although not directly linked to P content, these microbial ecofunctions reflect the differences between grassland and forest ecosystems. The major changes in the canopy, amounts of litterfall, plant composition, and root-microbe interactions due to grassland afforestation may explain the differences in soil P content as well as the abundances of these microbial phyla (Chen et al. 2008; Li et al. 2004).

By contrast, a high proportion of “too-rare” microbial OTUs (95% richness) occupied roughly >1/3 of the RA of soil microbes, reflecting transient changes in microorganisms in the environments. This rarity could result from stochasticity (Ai et al. 2013), fitness trade-off (Gobet et al. 2012; Gudelj et al. 2010), or biological interactions (García-Fernández et al. 2004; Narisawa et al. 2008; Schluter et al. 2015). These rare microbes are still relevant in ecological functions, including microbiome assembly and function and biogeochemical cycling (Jousset et al. 2017). The high richness of rare microbes contributes to the alpha-diversity of the soil microbiome. Although the ecological functions of these rare species in grassland or forest are not known, the high rarity provides adequate options for selection under environmental change, and these species may play an important role in maintaining fundamental ecosystem functions.

Based on the dbRDA of the relationships among vegetation type, soil properties, and microbial

412 composition, we concluded that the grassland afforestation affected the soil physicochemical
413 properties (Table 2) and soil microbiome composition and ecofunctions. In addition, these effects
414 of afforestation on the microbiome were modulated by changes in soil physicochemical
415 properties (Table 2 and Table 5). This conclusion was reached because the explanation of soil
416 microbiome by vegetation type decreased or was even lost when conditioned on soil properties
417 (Table 2). Albeit indirectly, afforestation indeed altered the ecofunction of the soil microbiome
418 (Table 5).

419 As discussed above, soil physicochemical properties interact with the soil microbiome. Because
420 the original vegetation before forestation was grassland, the changes in the soil properties are
421 probably attributable to the vegetation changes produced by afforestation. Several studies have
422 suggested that afforestation can influence biotic and abiotic changes in micro- and macro-
423 ecosystems (Jousset et al. 2017; Nosetto et al. 2008; Wang et al. 2016; Zheng et al. 2017). Here,
424 we suggest that the underground biotic change was indirectly affected by forestation mediated by
425 soil property changes (Table 2), especially the contents of soil P, Ca, and Fe, which are further
426 related to ecofunctional changes in soil microbiomes for different vegetation types (Table 5 and
427 Table S2). These results are similar to the bacterial abundance changes and compositional shifts
428 reported for a long-term poplar plantation, which were suggested to be highly correlated with the
429 changes in soil properties caused by afforestation (Zheng et al. 2017). Soil bacterial composition
430 has been suggested to be more closely related to plant diversity-controlled abiotic soil properties
431 because of the highly resilient characteristics of bacterial communities due to their fast life cycle
432 (de Vries et al. 2012; Lange et al. 2014).

433 ## Conclusions

434 The change in vegetation type was linked, at least in part, to ecofunctional changes in the soil

435 microbiome, despite an indirect impact on microbial composition. Aboveground changes in the
436 canopy, the composition and abundance of litterfall, and biotic activities (e.g., succession rate,
437 fauna activity, and root-microbe interactions) may be responsible for these changes in
438 underground ecofunctions. However, the relatively small proportion of microbial specialists and
439 high proportion of microbial generalists with respect to ecofunction compared to microbial
440 OTUs indicates that the vegetation change still preserved a high proportion of the core functions
441 of the soils (Li et al. 2004). This preservation occurred because the core functional genes were
442 distributed widely across a variety of microbial taxa. However, the high proportion of functions
443 that were correlated with changes in soil properties indicates that microbial ecofunctions are
444 highly sensitive to environmental change.

445

446 ## Acknowledgments

447 This research was financially supported by the Fundamental Research Funds for the Central
448 Universities (to SHW) and supported by the Ministry of Science and Technology, Taiwan
449 (MOST 105-2628-B-003-001-MY3 and MOST 105-2628-B-003-002-MY3) (to PCL). This
450 article was also subsidized by the National Taiwan Normal University (NTNU). We also thank
451 Dr. Dawn Schmidt for English editing of the manuscript.

452

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650 **Figure legend**

651 **Fig. 1.** Tukey's Honest Significant Differences (HSD) test revealing significant differences in soil properties, i.e.,
652 contents of organic C, P, Ca, Mg, and Fe and pH, between the forest (Fr) and grassland (Gr) soils.

653 **Fig. 2.** A simple logistic regression model revealing significant prediction of the absence (0) and presence (1) of
654 forestation based on the contents of the soil elements (a) Fe and (b) Mg.

655 **Fig. 3.** Divergence of the microbiome between the forest and grassland soils as revealed by (a) hierarchical cluster
656 analysis and (b) discriminant analysis of principal components (DAPC).

657 **Fig. 4.** Rank-abundance dominance (RAD) model tests showing that the sampled microbial communities were best
658 fit to the Zipf or Zipf–Mandelbrot model. The blue dots and the line in each panel are the observed values and the
659 expectations simulated from the observed data, respectively. Gr and Fr indicate samples from the grassland and
660 forest soils, respectively. The Zipf and Zipf–Mandelbrot models are both niche-based models. Rejection of the null
661 model indicates rejection of the hypothesis of a stochastic process of microbial assembly.

662

663

664

Figure 1

key's Honest Significant Differences (HSD) test

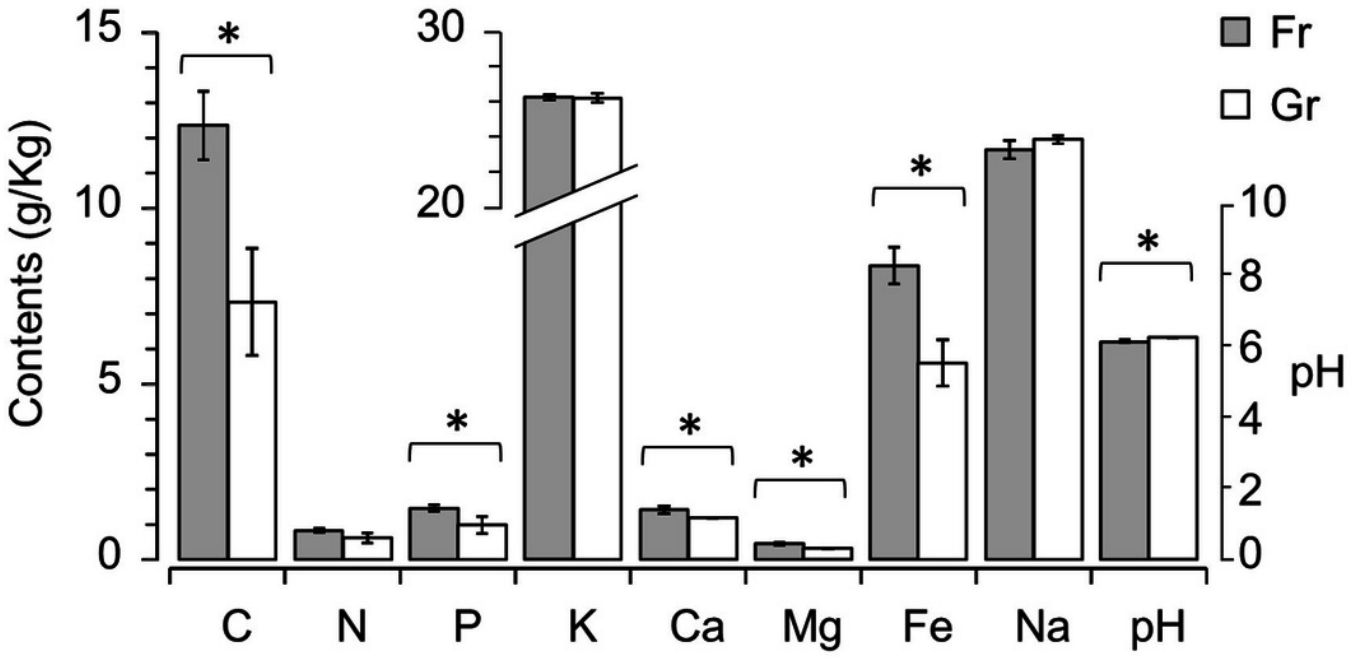

Figure 2

Simple logistic regression model revealing significant prediction of the absence (0) and presence (1) of forestation based on the contents of the soil elements (a) Fe and (b) Mg.

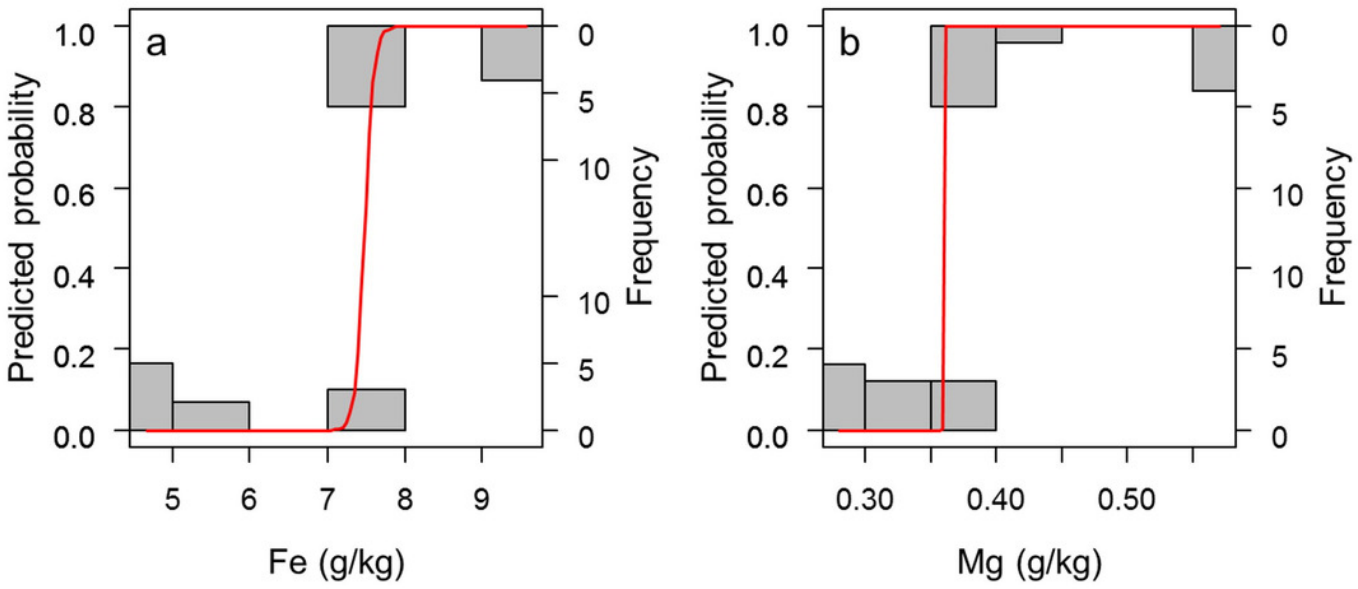

Figure 3

Convergence of the microbiome between the forest and grassland soils as revealed by (a) hierarchical cluster analysis and (b) discriminant analysis of principal components (DAPC).

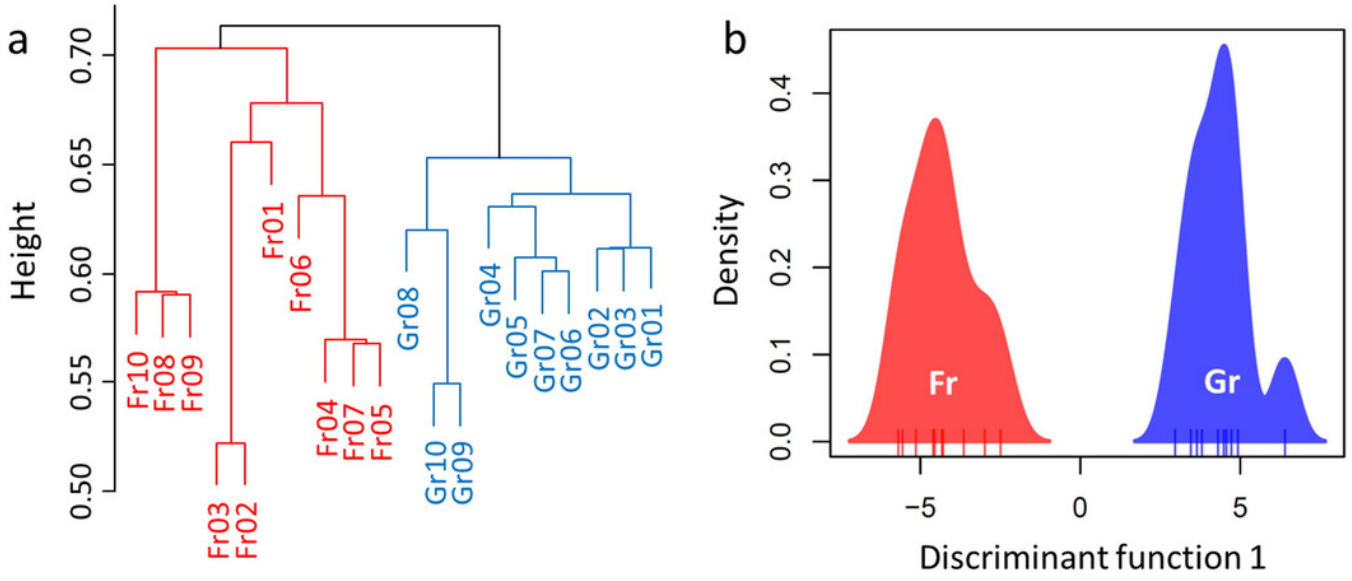

Figure 4

Rank-abundance dominance (RAD) model tests showing that the sampled microbial communities were best fit to the Zipf or Zipf–Mandelbrot model.

The blue dots and the line in each panel are the observed values and the expectations simulated from the observed data, respectively. Gr and Fr indicate samples from the grassland and forest soils, respectively. The Zipf and Zipf–Mandelbrot models are both niche-based models. Rejection of the null model indicates rejection of the hypothesis of a stochastic process of microbial assembly.

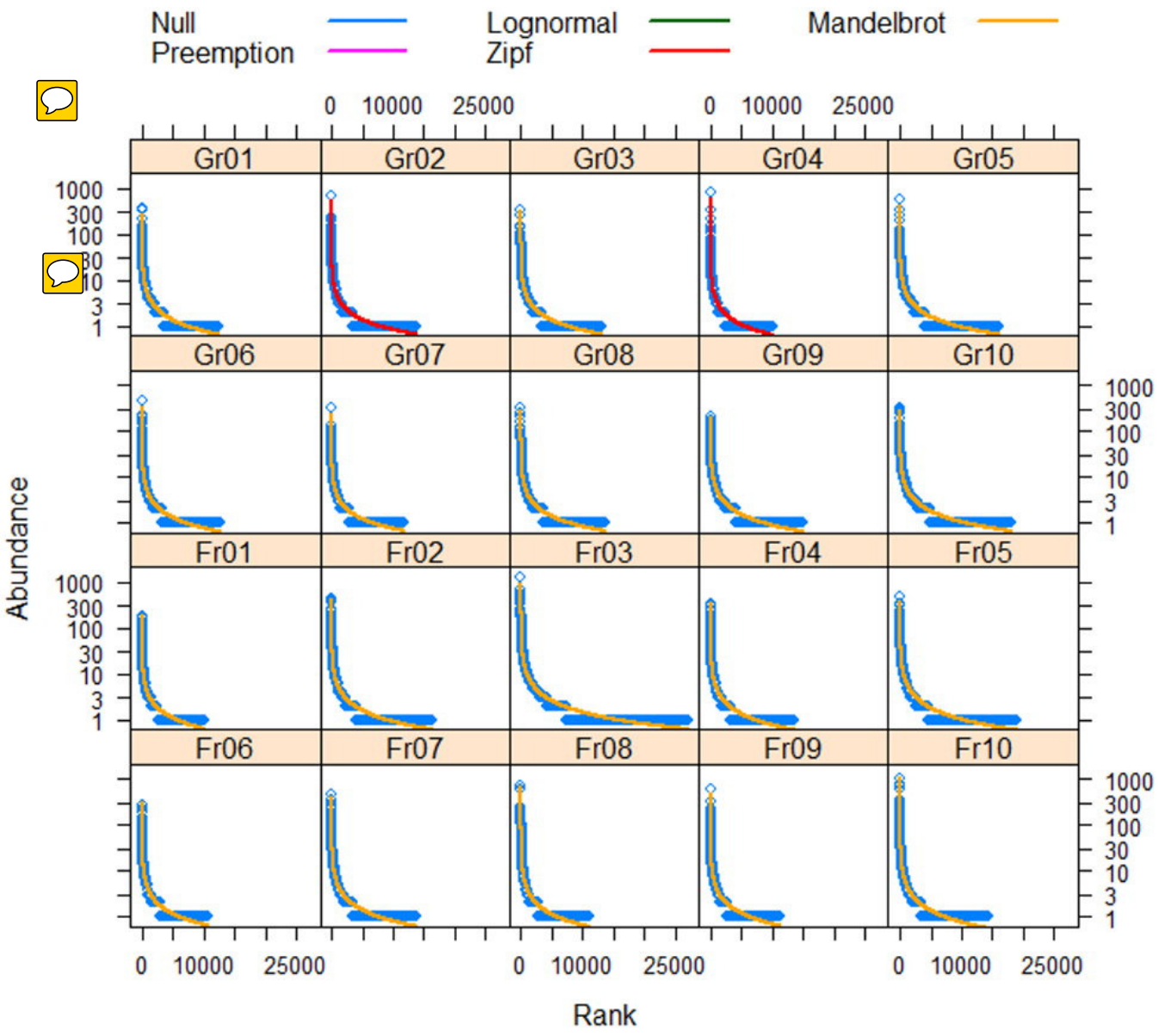
Null
Preemption
Lognormal
Zipf
Mandelbrot
Gr01
Gr02
Gr03
Gr04
Gr05
Gr06
Gr07
Gr08
Gr09
Gr10
Fr01
Fr02
Fr03
Fr04
Fr05
Fr06
Fr07
Fr08
Fr09
Fr10
Abundance
Rank
0
10000
25000
1000
300
100
30
10
3
1

Table 1(on next page)

Contents of soil elements (g/kg) and pH of the forest and grassland soils

Table 1. Contents of soil elements (g/kg) and pH of the forest and grassland soils

Group	C*	N	P*	K	Ca*	Mg*	Fe*	Na	pH*
Forest	12.358±1.950	0.835±0.116	1.469±0.199	26.303±0.341	1.424±0.222	0.456±0.099	8.355±1.047	11.665±0.531	6.216±0.095
Grassland	7.339±3.025	0.627±0.292	0.990±0.463	26.269±0.563	1.183±0.031	0.313±0.035	5.607±1.308	11.960±0.243	6.318±0.029

* Significant difference between the forest and grassland soils

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Table 2(on next page)

Summary of the (partial) dbRDA results for the afforestation effect (i.e., vegetation type) on soil properties and on the relative abundance (RA) of soil microbes.

The effect of soil properties on the microbial RA was also tested. Type II ANOVA was used to test the model fitting for each independent variable.

Table 2. Summary of the (partial) dbRDA results for the afforestation effect (i.e., vegetation type) on soil properties and on the relative abundance (RA) of soil microbes. The effect of soil properties on the microbial RA was also tested. Type II ANOVA was used to test the model fitting for each independent variable.

	dbRDA		ANOVA	
	S.S.	Proportion	*F*	*P*
Soil property ~ Vegetation type				
Constrained	0.027	0.502	18.13	**0.002**
Unconstrained	0.027	0.498		
Microbial RA ~ Vegetation type				
Constrained	0.849	0.163	3.504	**0.001**
Unconstrained	4.362	0.837		
Microbial RA ~ Vegetation type + Condition (Soil property)				
Conditional	3.356	0.644		
Constrained	0.194	0.037	1.053	0.466
Unconstrained	1.661	0.319		
Microbial RA ~ Soil property				
Constrained	3.356	0.644		
C	0.595	0.114	3.210	**0.001**
P	0.524	0.100	2.823	**0.001**
Ca	0.467	0.090	2.516	**0.001**
Mg	0.368	0.071	1.986	**0.003**
Fe	0.546	0.105	2.940	**0.001**
pH	0.228	0.044	1.232	0.145
N	0.249	0.048	1.343	0.070
K	0.188	0.036	1.012	0.438
Na	0.190	0.037	1.026	0.365
Unconstrained	1.855	0.356		
Microbial RA ~ Soil property + Condition (Vegetation type)				
Conditional	0.849	0.163		
Constrained	2.701	0.518		
C	0.350	0.067	1.899	**0.005**
P	0.493	0.095	2.673	**0.001**
Ca	0.457	0.088	2.477	**0.001**
Mg	0.350	0.067	1.896	**0.004**
Fe	0.283	0.054	1.534	**0.009**
pH	0.203	0.039	1.102	0.238
N	0.183	0.035	0.994	0.490
K	0.190	0.037	1.031	0.362
Na	0.190	0.036	1.029	0.378
Unconstrained	1.661	0.319		

Table 3(on next page)

Diversity indices of soil microbiomes

Table 3. Diversity indices of soil microbiomes

	Forest (mean ± SD)	Grassland (mean ± SD)	KW χ^2	*P*
Shannon-Wiener *H*	8.429±0.244	8.612±0.174	3.291	0.070
Reciprocal Simpson's index 1/*D*	877.783±281.326	1190.206±364.143	4.166	0.041
Species richness (*S*)	14685.0±4846.82	13664.6±2107.68	0	1
Pielou's evenness (*J*)	0.883±0.017	0.905±0.008	9.864	0.002

Table 4(on next page)

Effect of soil properties on the abundance of divergent soil microbial phyla inferred by the generalized linear model (GLM)

Table 4. Effect of soil properties on the abundance of divergent soil microbial phyla inferred by the generalized linear model (GLM)

	Thaumarchaeota		Chloroflexi		Fibrobacteres		Nitrospirae		Parcubacteria	
	t value	*P*	*t* value	*P*	*t* value	*P*	*t* value	*P*	*t* value	*P*
Intercept	0.068	0.947	-3.280	0.005	-1.087	0.295	-1.176	0.259	-1.590	0.134
C	-0.288	0.778	3.501	**0.004**	0.632	0.538	1.471	0.164	1.714	0.109
P	-0.420	0.681	4.848	**0.0003**	3.735	**0.002**	4.108	**0.001**	3.326	**0.005**
Ca	-0.083	0.935	3.857	**0.002**	1.267	0.226	1.667	0.118	2.007	**0.065**
Mg	0.031	0.976	-3.727	**0.002**	-1.174	0.260	-1.374	0.191	-1.934	**0.074**
Fe	2.088	**0.056**	-0.332	0.745	-1.263	0.227	-2.900	**0.012**	-0.575	0.574

Table 5(on next page)

Summary of the (partial) dbRDA results for the afforestation effect (i.e., vegetation type) and soil-property effect on the COGs and KEGG modules estimated from the soil microbiome.

Type II ANOVA was used to test the model fitting for each independent variable. The soil properties include the soil elements C, P, Ca, Mg, Fe, N, K, and Na and the pH value.

Table 5. Summary of the (partial) dbRDA results for the afforestation effect (i.e., vegetation type) and soil-property effect on the COGs and KEGG modules estimated from the soil microbiome. Type II ANOVA was used to test the model fitting for each independent variable. The soil properties include the soil elements C, P, Ca, Mg, Fe, N, K, and Na and the pH value.

	dbRDA		ANOVA		dbRDA		ANOVA	
	S.S.	Proportion	*F*	*P*	S.S.	Proportion	*F*	*P*
	COGs ~ Vegetation type				**KEGG ~ Vegetation type**			
Constrained	0.005	0.220	5.081	**0.001**	0.001	0.212	4.834	**0.001**
Unconstrained	0.016	0.780			0.002	0.788		
	COGs ~ Vegetation type + Condition (Soil property)				**KEGG ~ Vegetation type + Condition (Soil property)**			
Conditional	0.017	0.830			0.003	0.824		
Constrained	0.001	0.034	2.267	0.078	1E-04	0.045	3.140	**0.038**
Unconstrained	0.003	0.136			4E-04	0.130		
	COGs ~ Soil property				**KEGG ~ Soil property**			
Constrained	0.017	0.830			0.003	0.824		
C	0.002	0.094	5.541	**0.001**	3E-04	0.097	5.5051	**0.001**
P	0.003	0.140	8.234	**0.001**	0.001	0.165	9.3958	**0.001**
Ca	0.003	0.140	8.247	**0.001**	3E-04	0.107	6.0938	**0.001**
Mg	0.002	0.072	4.252	**0.005**	1E-04	0.042	2.4047	0.063
Fe	0.002	0.073	4.273	**0.010**	2E-04	0.066	3.738	**0.013**
pH	5E-04	0.023	1.347	0.283	9E-05	0.028	1.5886	0.177
N	0.005	0.258	15.157	**0.001**	0.001	0.292	16.615	**0.001**
K	2E-04	0.010	0.575	0.653	4E-05	0.012	0.6817	0.580
Na	4E-04	0.020	1.174	0.312	5E-05	0.016	0.914	0.453
Unconstrained	0.004	0.170			0.001	0.176		
	COGs ~ Soil property + Condition (Vegetation type)				**KEGG ~ Soil property + Condition (Vegetation type)**			
Conditional	0.005	0.220			0.001	0.212		
Constrained	0.013	0.644			0.002	0.658		
C	0.001	0.027	1.776	0.152	7E-05	0.021	1.478	0.257
P	0.004	0.172	11.371	**0.001**	5E-04	0.157	10.877	**0.001**
Ca	0.001	0.071	4.723	**0.006**	2E-04	0.061	4.226	**0.018**
Mg	0.001	0.030	1.963	0.133	8E-05	0.027	1.869	0.175
Fe	0.005	0.251	16.630	**0.001**	0.001	0.264	18.238	**0.001**
pH	0.001	0.032	2.094	0.110	1E-04	0.038	2.650	0.066
N	4E-04	0.018	1.162	0.302	9E-05	0.028	1.946	0.130
K	3E-04	0.014	0.928	0.406	7E-05	0.022	1.510	0.223
Na	0.001	0.030	2.017	0.141	1E-04	0.039	2.691	0.070
Unconstrained	0.003	0.136			4E-04	0.130		