How Do N Addition Affect Soil Fungal Community Assembly: Short- Versus Long-term Effects?

Jinhong He
South China Botanical Garden
Shuo Jiao
Northwest A&F University: Northwest Agriculture and Forestry University
Xiangping Tan
South China Botanical Garden
Hui Wei
South China Agricultural University
Xiaomin Ma
South China Botanical Garden
Yanxia Nie
South China Botanical Garden
Juxiu Liu
South China Botanical Garden
Xiankai Lu
South China Botanical Garden
Jiangming Mo
South China Botanical Garden
Weijun Shen (shenweij@scbg.ac.cn)
South China Institute of Botany: South China Botanical Garden https://orcid.org/0000-0001-7574-8839

Research

Keywords: Nitrogen addition, Fungal community, Stochastic processes, Deterministic processes, Tropical forest, Co-occurrence network

Posted Date: December 1st, 2020

DOI: https://doi.org/10.21203/rs.3.rs-113341/v1

ORIGINAL ARTICLE

How do N addition affect soil fungal community assembly: short- versus long-term effects?

Jinhong He[1], Shuo Jiao[2], Xiangping Tan[1], Hui Wei[3], Xiaomin Ma[1], Yanxia Nie[1], Juxiu Liu[1], Xiankai Lu[1], Jiangming Mo[1], Weijun Shen[1, *]

[1] Center for Ecological and Environmental Sciences, South China Botanical Garden, Chinese Academy of Sciences, 723 Xingke Rd., Tianhe District, Guangzhou 510650, China

[2] State Key Laboratory of Crop Stress Biology in Arid Areas, Shanxi Key Laboratory of Agricultural and Environmental Microbiology, College of Life Sciences, Northwest A&F University, Yangling 712100, China

[3] Department of Ecology, College of Natural Resources and Environment, South China Agricultural University, Guangzhou, 510642, China

*** Corresponding author:** Dr. Weijun Shen

Center for Ecological and Environmental Sciences,

South China Botanical Garden, Chinese Academy of Sciences

723 Xingke Road, Tianhe District

Guangzhou 510650, China

Tel.: + 86 20 3725 2950; Fax: + 86 20 3725 2950;

Email: shenweij@scbg.ac.cn

Abstract

Background: Soil fungi play critical roles in ecosystem processes and are sensitive to global changes. Elevated atmospheric nitrogen (N) deposition has been well documented to impact on fungal diversity and community composition, but how fungal community assembly respond to short- and long-term simulative N deposition remains poorly understood. Here, we carried out two field experiments to investigate the soil fungal community variations and assembly processes under short- (2 years) versus long-term (13 years) exogenous nitrogen addition (100 kg N ha^{-1} yr^{-1}) in a N-rich tropical forest of China.

Results: We observed that short-term N addition significantly increased fungal taxonomic and phylogenetic α-diversity, and shifted fungal community composition with significant increases in the relative abundance of *Ascomycota* and saprotrophic fungi, and decreases in the relative abundance of *Basidiomycota* and ectomycorrhizal (EcM) fungi. However, unremarkable effects were found under long-term N addition. The variations of fungal α-diversity, community composition, the relative abundance of major phyla, genera and functional guilds were mainly correlated with soil pH and the concentrations of NO_3^--N, and these correlations were much stronger under short- than long-term N addition. The results of null, neutral community models and the normalized stochasticity ratio (*NST*) index consistently revealed that stochastic processes played predominant roles in the assembly of soil fungal community under N addition in the tropical forest, and that the relative contributions of stochastic processes

were higher at short-term site. Furthermore, both short- and long-term N addition slightly loosened the co-occurrence networks of the fungal community.

Conclusions: These findings highlighted that the responses of fungal community structure to N addition were duration-dependent, i.e., the fungal community was sensitive to the short-term N addition but become acclimatized to long-term N enrichment.

Keywords: Nitrogen addition, Fungal community, Stochastic processes, Deterministic processes, Tropical forest, Co-occurrence network

Background

Fungi play vital roles in the structural and functional dynamics of terrestrial ecosystems. Saprotrophic fungi are the primary decomposers of plant litter and soil organic matter due to their relatively higher capability to degrade recalcitrant substances, such as cellulose and lignin [1, 2]. Mycorrhizal symbionts can promote plant nutrient acquisition and plant resistance to abiotic and biotic stresses, such as drought and pathogen attacks [3]. Mycorrhizal fungi can also shape plant community dynamics by affecting seed dispersal, seedling establishment and intra-/inter-specific interactions [4]. For these reasons, many studies have been devoted to understanding patterns in the diversity, composition and function of fungal communities in natural and managed ecosystems [5, 6], particularly in the past decade with the advance of the high-throughput sequencing technologies. However, we still have a very limited understanding on the assembly processes that structure fungal communities or microbial communities in general [7, 8], albeit such knowledge is critical for predicting ecosystem responses and for managing plant-fungi interactions under the context of global environmental changes and maintaining sustainable agriculture and forestry [9, 10].

It is now widely recognized that microbial community assembly is often determined by both deterministic and stochastic processes [11-13]. The deterministic processes mainly include selection via environmental filtering and biotic interactions. The stochastic processes consider that microbial species dynamics are controlled by

72 stochastic dispersal, ecological drift and diversification [12, 14]. While these are
73 conceptualized processes, new theoretical frameworks and experimental/analytical
74 methodologies are being developed to associate them with measurable ecological and
75 evolutionary processes, such as competition, predation, phylogenetic signals, network
76 interactions and trait variables [7, 15, 16]. In doing so, it is possible to quantify the
77 relative contribution of these assembly processes for different ecosystem types,
78 geographic scales and microbial categories [17-19], and to assess how the dominance
79 of the assembly process is affected by global or regional environmental changes [20-
80 22].

81 As one of the major global change drivers, anthropogenic reactive nitrogen (N) has
82 increased by 120% over the past few decades [23] and is expected to increase by 50-
83 100% from 2000 to 2030 [24]. The excessive loading of reactive N has been found to
84 have profound influences on soil microbial diversity, composition, activity and function
85 [25, 26]. Experimental N addition or fertilization generally reduces soil microbial
86 biomass and diversity directly by affecting microbial nutrition and indirectly by altering
87 edaphic conditions [26, 27]. In comparison with bacteria, fungi have been found to be
88 more responsive to N enrichment since they have lower N demands [28, 29]. Different
89 fungal guilds may exhibit different responses to N enrichment. For instance, the relative
90 abundance and diversity of mycorrhizal fungi decreased, whereas those of saprotrophs
91 increased with experimental N addition [30, 31], thus resulting in alterations in
92 community composition. More often, the N enrichment effects on fungal biomass and

93 diversity are positively related to the N application rate and duration, but we have very
94 limited knowledge on the duration effects, mainly because most N addition experiments
95 are short-termed and molecular approaches characterizing microbial diversity were not
96 available for early long-term experiments. Microbial communities are known for their
97 rapid evolution and adaptation to environmental disturbances [12, 32]. Understanding
98 the duration effects of N enrichment is therefore helpful for predicting the dynamic
99 responses of fungal communities to future environmental changes.

100 Nitrogen enrichment can also affect the processes underpinning fungal community
101 assembly but inconsistent results have been reported. For example, stochastic processes
102 dominate under the fertilization treatment in some studies [20, 21], whereas
103 deterministic processes were found to have a larger relative importance with
104 fertilization in other studies [31, 33]. Moreover, nitrogen fertilization resulted in
105 tightened network associations among fungal, bacterial and protists communities in
106 diverse agricultural soils [34], indicating increased importance of deterministic biotic
107 interactions in shaping microbiome. Contrastingly, nitrogen fertilization did not change
108 the fungal community networks in a temperate grassland soil [35]. The discrepancies
109 of fungal assembly process responses to N enrichment may be ascribed to the N
110 application rate, duration, ecosystem type and fungal categories of analysis; however,
111 existing studies were mainly carried out in cropland and grassland ecosystems.
112 Assessing the relative contribution of stochastic versus deterministic processes that

influence fungal community assembly under N enrichment in forest ecosystems remain scarce.

To assess the effects of N addition and application duration on the relative importance of deterministic versus stochastic processes in assembling forest soil fungal community, we took advantage of an existing long-term (~ 13 years) N addition experiment (one of the earliest N addition experiment in China) in a N-rich but phosphorus (P)-deficient subtropical forest [36], and initiated a new N addition experiment (hereafter the short-term (~ 2 years) experiment) in a nearby forest (~2 km away) with similar plant community composition and edaphic conditions [37]. Previous studies at these sites showed that N addition caused reductions in plant species diversity, soil acidification and alteration in N- transformation processes [37-39]. For this study, soil samples were taken at both sites in representative dry- and wet-season months and fungal diversity and community composition were assessed through Illumina Hiseq sequencing of internal transcribed spacer (ITS) region. We are aware that this is not an exact time-series experiment with which to assess the duration effects but forms a feasible comparison analogous to the space-for-time substitution principle. We hypothesized that i) both the short- and long-term N additions would impose negative impacts on soil fungal diversity and composition, and the negative effects would be stronger at the long-term site; ii) stochastic processes would dominate at the short-term site since it improves soil N availability for fungi, whereas deterministic processes would be prevalent at the long-term site since excessive N inputs create a deleterious nutritional

134 environment [25]; iii) both the short- and long-term N additions would change the
135 network structures of the soil fungal community, more clustered network structure
136 would be observed at the long-term site since it creates a more stressful environment
137 that selects more adaptive fungal guilds.
138
139 **Results**
140 **Response of soil physicochemical properties**
141 Soils from both sites were strongly acidic with an average pH of 3.93, and N addition
142 significantly decreased the soil pH by 0.1 unit at both sites (Additional file 1: Fig. S1a).
143 Nitrogen addition also significantly increased the soil NO_3^--N concentrations by 56.11%
144 at the short-term site, but only slightly (by 33.14%) increased at long-term site
145 (Additional file 1: Fig. S1b). Of all the soil physicochemical parameters measured in
146 this study, only the soil water content (SWC) was significantly different between the
147 short- and long-term sites ($P = 0.013$; Additional file 1: Table S2).
148
149 **Response of fungal diversity and community composition**
150 After a series of qualified and filtered steps, 2,942,016 sequences were retained and
151 clustered into 4,672 fungal OTUs. Of all the samples, a total of 96.8% of the sequences
152 could be assigned into 11 phyla and 261 genera (Fig. 1). At the phylum level,
153 *Basidiomycota* was predominant, followed by *Ascomycota*. The relative abundance of
154 both phyla was significantly impacted by the N addition and differed significantly

between the long- and short-term sites ($P < 0.05$; the insert text in Additional file 1: Fig. S2a, b). At the short-term site, the relative abundance of *Basidiomycota* was significantly decreased by 27.19% by N addition, whereas that of *Ascomycota* was significantly increased by 58.82% (Additional file 1: Fig. S2a, b). However, the relative abundances of these phyla were not significantly affected by long-term N addition ($P > 0.05$). At the genera level, the 4 dominant taxa were *Inocybe*, *Lactarius*, *Russula* and *Saitozyma* representing 13.5%, 10.8%, 9.1% and 6.0% of all sequences, respectively (Figs. 1b and Additional file 1: S2c-f). At both sites, the N addition decreased the relative abundance of *Inocybe* (by 90.93% and 79.32% at short- and long-term site, respectively) but increased that of *Russula* (by 368.04% and 283.98% at short- and long-term site, respectively), *Lactarius* (by 30.47% and 282.31% at short- and long-term site, respectively), and *Saitozyma* (by 87.13% and 60.44% at short- and long-term site, respectively). At the functional guild level, approximately 43.4% sequences were categorized into EcM fungi, and the relative abundance of this group significantly decreased by 26.13% at short-term site (Additional file 1: Fig. S3a). Nevertheless, only 3.5% of the sequences were classified as saprotrophic fungi, and the short-term N addition significantly increased their relative abundance of this group (Additional file 1: Fig. S3b).

The results from two-way ANOVA indicated the fungal taxonomical and phylogenetic α-diversity were significantly influenced by the duration of N addition ($P < 0.05$; the insert text in Fig. 2a-c). Specifically, the three fungal α-diversity

176 indices (richness, Shannon and phylogenetic diversity) increased significantly (by
177 more than 10%) at short-term N addition site ($P < 0.05$), but by only approximately
178 1% at long-term site (Fig. 2a-c). Moreover, the PERMANOVA indicated that the
179 duration, rate of N addition and their interactions significantly shaped the fungal
180 community composition ($P < 0.05$; the insert text in the top right corner of Additional
181 file 1: Fig. S4a). Specifically, the soil fungal community could be distinctly separated
182 by short- and long-term N addition based on the Bray-Curtis distance metric by
183 NMDS plots (Additional file 1: Fig. S4a). In addition, the dissimilarity of soil fungal
184 community was significantly decreased by short-term N addition (Fig. 2d). When
185 considering the variation in phylogenetic community composition (based on
186 βMNTD), the duration of N addition significantly changed the fungal phylogenetic
187 community composition, and the N addition significantly decreased βMNTD at the
188 short-term site, while significant changes were not found at the long-term site (Fig. 2e
189 and Additional file 1: Fig. S4b). Niche breadth analysis was conducted to explain the
190 distinct responses of fungal community composition, and revealed that fungal
191 community in N addition soils had a higher habitat niche breadth than that in control
192 soils at both sites (Additional file 1: Fig. S5).
193
194 **Factors affecting fungal diversity and community composition**
195 The linear regression indicated that the relative abundance of major functional guilds,
196 phyla and genera was mainly correlated with soil pH and NO_3^--N concentration, and

197 the correlations were more significant at short- than long-term site (Additional file 1:
198 Figs. S6 and S7). For instance, the relative abundance of EcM fungi and *Basidiomycota*
199 was significantly positively correlated with soil pH, and that of saprotrophic fungi and
200 *Ascomycota* was significantly negatively correlated with pH at the short-term site, while
201 these correlations were not significant at long-term site. The soil pH, not the NO_3^--N
202 concentration, was the dominant driver to predict the variations of fungal richness,
203 Shannon index and phylogenetic diversity (Fig. 3a-f). The aforementioned α-diversity
204 indices were negatively correlated with soil pH, and the correlations were more
205 significant at the short- than the long-term site. The CAP analysis also revealed that soil
206 pH was the most important variable for the shifts of fungal taxonomic and phylogenetic
207 community composition, and soil nutrients (such as DON, DOC and NO_3^--N) were also
208 important for fungal community composition (Fig. 3g-h, Additional file 1: Table S3).
209
210 **Response of assembly processes**
211 The mantel correlogram showed significant phylogenetic signals across relatively short
212 phylogenetic distances for the four sub-communities ($P < 0.05$, Fig. 4), indicating that
213 fungal N responses are phylogenetically conserved and that the closely related species
214 in fungal community exhibited more similar ecological preferences to the
215 environmental variables. Null model analysis revealed stochastic processes mainly
216 governed fungal community dynamics in the whole community and the four sub-
217 communities (Fig. 5a). In the sub-communities, the relative contribution of

stochasticity was increaed by short-term N addition from 71.2% (control) to 90.9% (N addition), while it was slightly decreased by long-term N addition from 78.8% (control) to 66.7% (N addition). The *NST* results also showed the fungal community were predominately governed by stochastic processes (*NST* = 81.8% and 82.7% for the control treatment at the short- and long-term sites and 89.0% and 79.2% the N addition treatment at the short- and long-term sites, respectively). Specifically, the *NST* increased significantly by short-term N addition ($P < 0.01$) but decreased slightly by long-term N addition (Fig. 5b). The neutral model explained a large fraction ($R^2 > 0.5$) of the variability in occurrence frequency of the fungal community, with more than 80% of species having frequencies within predicted ranges (Fig. S8).

Response of co-occurrence patterns

After data filtration, the remaining 405, 451, 369 and 461 OTUs were used to construct networks for the four sub-communities (Table 1). The networks showed topological properties of small-world, scale-free and modularity, and were significantly different from randomly generated networks (Table 1). The N addition slightly weakened the inter-connections among fungal species at both sites (Fig. 6, Table 1), with a lower average clustering coefficient (avgCC) and average connectivity degree (avgK), and a higher average path length (APL) and centralization of betweenness (CB). These results indicated that the networks were less clustered in the N addition soils. Additionally,

positive correlations were predominant in all networks and the number of positive correlations were decreased by N addition at both sites.

The topological roles of the OTUs identified in the networks were shown as a *Zi-Pi* plot in Figure 7. The majority of the OTUs were peripherals (also regarded as specialist) with most of their links being inside their modules. A total of 8, 15, 16 and19 OTUs were classified as module hubs, collectors and network hubs (putative keystone species) for the four sub-networks (Fig 7; Additional file 1: Table S4-S7). The control and N addition networks at short-term site shared 1 putative keystone species (OTU140; *Eurotiomycetes*, *Ascomycota*), while the two networks at long-term site did not share any keystone species. Most putative keystone species belonged to the classes *Eurotiomycetes*, *Agaricomycetes*, *Sordariomycetes*, *Dothideomycetes*, *Leotiomycetes*, *Mortierellomycetes* and *Tremellomycetes*.

Discussion

Soil fungal diversity and community composition were more sensitive to short-term N addition

In contravention to our first hypothesis, the soil fungal α-diversity was enhanced by N addition (100 kg N ha^{-1} yr^{-1}) at both sites (Fig. 2a-c). The increased fungal diversity at both sites may be ascribed to the following : i) the added N increased soil nutrient availability, modifying the range of ecological niches and favoring the survival, growth and reproduction of fungi [40]; ii) N addtion enhanced the plant net primary

productivity of the tropical forest [41, 42], leading to more plant-derived resources for soil fungal growth; iii) the rate of N addition (100 kg N ha^{-1} yr^{-1}) likely caused an intermediate disturbance, and led to many vacant ecological niches that facilitate fungal colonization, according to the intermediate disturbance hypothesis [43]; iv) the adaptation of soil fungal diversity to acidic environments, as their internal pH regulatory mechanism to maintain a relatively neutral pH in their cells [44], and thus the acidic soil induced by N addition would result in acidophilic or acid-tolerant fungi in the studied forest. However, some previous studies found that soil fungal diversity was decreased or not significantly affected by N addition [45-47]. The discrepancies in these findings are not entirely surprising given that variations in the ecosystem types, background N deposition, soil layers, duration of treatment, and N addition rate [26, 48, 49].

Additionally, we found that N addition significantly increased the fungal α-diversity at the short-term site (>10%), but little enhanced at the long-term site (<2%; Fig. 2a-c), suggesting that soil fungal species could be sensitive to short-term N addition, and long-term N enrichment may result in the acclimatization of the soil fungal community in the tropical forest soil. Our results are in line with some of the observations from other forest ecosystems [50, 51]. For instance, Zhao et al. [50] revealed that 5 years of N addition increased the fungal α-diversity in a temperate forest, while Entwistle et al. [51] showed there were no significant effects on fungal α-diversity under more than 15 years of N addition. We speculated that the different response patterns between short-

and long-term N addition in the present study could be attributed to the sensitivity of fungal species to N availability and the niche differentiation. For instance, short-term N addition significantly increased fungal richness (Fig. 2a), suggesting that some rare taxa were stimulated and appeared, while the prolonged N addition at the long-term site would form a relatively stable fungal community. Additionally, the forest age and tree size are lager at the long-term site (400 years and 8 cm of diameter at breast height) than at the short-term site (100 years and 6.6 cm) [39, 52], leading to more NO_3^--N leaching and plant uptake [53], and probably more niche differentiation at the long-term site. In contrast, the fungal species may be more equally distributed and have higher NO_3^--N concentrations to use under short-term N addition, resulting in a higher diversity.

Furthermore, nitrogen addition also altered the fungal community composition between short- and long-term site, and the dissimilarities were much greater under short-term than long-term N addition (Figs. 2d-e and Additional file 1: S4). This dissimilarity of fungal community composition may result from the changes of taxonomic structures. For example, N addition significantly decreased the relative abundance of *Basidiomycota* and EcM fungi, and increased the relative abundance of *Ascomycota* and saprotrophic fungi at the short-term site, while these taxa were not significantly impacted by long-term N addition (Additional file 1: Figs. S2 and S3), indicating the duration-dependent effects of nitrogen addition on fungal populations. Wang et al. [52] revealed that N addition (105 kg N ha^{-1} yr^{-1}) suppressed the fine root

301 production and turnover at the short-term site, and then would reduce the "mycorrhizal-
302 fine root system" and decrease the EcM colonization [54]. The positive effects of N
303 addition on relative abundance of saprotrophic fungi may be supported by the "Gadgil
304 effect", in which N competition between EcM and saprotrophic fungi could benefit to
305 saprotrophic growth [55, 56]. Such shifts in fungal community composition could affect
306 the soil enzymatic activities and litter decomposition, and thus modulate soil C, N, and
307 P cycling in forests exposed to atmospheric N deposition [57]. Recent studies
308 demonstrated that soil pH and NO_3^--N were the key drivers influencing fungal
309 community composition [58, 59]. The relative abundance of dominant functional guilds,
310 phyla and genera at the short-term site (Additional file 1: Figs. S4 and S5) was
311 significantly positively or negatively correlated with soil pH and NO_3^--N concentrations,
312 depending on their preference to N and tolerance to pH, which indicated the sensitive
313 responses of soil fungal community to short-tern N addition as well. Additionally, the
314 fungal community in N addition soils had a wider habitat niche breadth than that in
315 control soils at both sites (Additional file 1: Fig. S7), indicating the fungal community
316 has greater metabolic plasticity and governed less by environmental filtering under N
317 addition [60].
318
319 **Soil fungal community assembly was mainly shaped by stochastic processes**
320 The significant phylogenetic signals were found across relatively short phylogenetic
321 distances (Fig. 4), in line with previous studies on fungal communities [16, 61]. The

results suggested that closely related species exhibited more similar ecological preferences across environmental variations, and fungal responses to N addition exhibited phylogenetic conservation.

Revealing the assembly rules of microbial community is a central issue in microbial ecology [12]. Deterministic and stochastic processes are two dominant themes in the study of microbial community dynamics; therefore, it is critical to quantify the relative contributions of the two processes to community assembly [28, 62]. In the present study, the null model and *NST* analysis consistently showed that stochastic processes were more important than deterministic processes with respect to soil fungal community assembly under control and N treatments at both sites (Fig. 5), which were partially consistent with our second hypothesis. We speculated the short-term N addition enriched soil nutrients (e.g. NO_3^--N), widened the habitat niche breadth of fungal community, and weakened the effects of environmental filtering. However, the long-term N addition didn't change the soil properties much (Additional file 1: Fig. S1 and Table S2), and led to less habitat heterogeneity, resulting in a greater relative importance of stochastic events on community composition. Additionally, the hyphal networks, forming between fungi and the roots of their hosts, limit the dispersion of fungal species (i.e., dispersal limitation), since most of the dominant trees in the forest are ectomycorrhizal or arbuscular mycorrhizae [39, 63]. In general, our results are supported by prior studies reporting that stochastic processes predominate in fungal community assembly [13, 18, 21]. Furthermore, the neutral community model

343 explained a relatively large fraction (R^2 > 0.5) of fungal community variation,
344 indicating that the fungal community was more influenced by neutral (or stochastic)
345 processes.
346
347 **Co-occurrence patterns of microbial communities under short- and long-term N**
348 **addition**
349 Microorganisms can form complex interaction networks to coexist by occupying
350 specific ecological niches or responding similarly to environments [64]. The co-
351 occurrence networks can present the new insights into potential interactions and
352 assembly process of microbial communities under environmental disturbance [65]. The
353 N addition generated a looser and more random fungal networks at both sites (Fig. 6,
354 Table 1), indicating that the putative interactions between fungal species were
355 weakened by N addition and supporting our third hypothesis that N addition would alter
356 the fungal network structure. This is also supported by our observation that network
357 parameters such as *avgK* and *avgCC* were higher in control networks than N addition
358 networks. This result suggests that higher fungal diversity is not necessarily associated
359 with more complex co-occurrence networks. We speculated that higher fungal diversity
360 in N addition soils could lead to higher degree of functional redundancy, which
361 provides more opportunities for fungal species to correlate with their neighbours, as
362 microorganisms tend to interact with each other by function or metabolites preference
363 [66, 67]. As a result, N addition would lead to weak interspecies interactions and loose

co-occurrence networks of fungal community. However, Zhao et al. [34] suggested that the application of nitrogen fertilizers tightened bacteria–fungi–protist interactions in diverse agricultural soils. The discrepancies between these findings may result from our fungal network analysis and forest ecosystems. Additionally, positive correlations dominated all the networks (Fig. 6), implying that mutual cooperation rather than competitive exclusion played a dominant role in fungal community assembly.

Conclusions

In this study, we compared the variations and responses of fungal taxonomic and phylogenetic diversity and community composition between two nearby field experiments in a tropical forest which had received short- (2 years) and long-term (13 years) simulated N deposition (100 kg N ha^{-1} yr^{-1}). Our results indicated that fungal α-diversity was significantly increased by the short-term N addition rather than the long-term treatments. Additionally, the variations of fungal α-diversity and community composition were greater in short-term compared to long-term N addition. Soil pH and the concentrations of NO_3^--N were the major factors mediating the variations of fungal diversity and community composition. Stochastic processes played predominant roles in the assembly of soil fungal community under N addition at both sites, and the stochasticity was significantly increased by short-term N addition. Moreover, both short- and long-term N addition slightly loosened the co-occurrence networks of fungal community. Together, our results revealed that the soil fungal community structure

385 would be sensitive to short-term N addition, while become acclimatized to long-term
386 N enrichment. The study will contribute to our understanding of underlying
387 mechanisms that regulate the variations of soil fungal community, and facilitate the
388 prediction of fungal responses to the ongoing atmospheric nitrogen deposition in
389 tropical forests.
390
391 **Materials and methods**
392 **Site description**
393 The long- and short-term N addition experiments were established in Dinghushan
394 Biosphere Reserve (DHSBR) in the city of Zhaoqing, Guangdong province of south
395 China (112°10′ E, 23°10′N). The reserve lies in a subtropical/tropical moist forest
396 region, and covers about 1,155 ha with a monsoon climate. The mean annual
397 precipitation is 1,748 mm, mainly concentrated from April to September, and only a
398 small part falling from October to March. The mean annual temperature is 21.0 °C, with
399 a monthly average temperature ranging from 12.6 °C in January to 28.0 °C in July. The
400 wet N deposition in the reserve had reached 34.4 kg N ha^{-1} yr^{-1} in 2009-2010 [38, 39].
401 The forest type is evergreen broadleaf forest and it has been characterized as N-rich but
402 P-limited in previous studies [36, 39]. The dominant tree species being *Castanopsis*
403 *chinensis* Hance, *Machilus chinensis* (Champ. Ex Benth.) Hemsl., *Schima superba*
404 Chardn. et Champ., *Cryptocarya chinensis* (Hance) Hemsl., *Cryptocarya concinna*

405 Hance and *Randia canthioides* Champion ex Bentham. The forest ages are
406 approximately 400 and 100 years for the long- and short-term sites, respectively.
407
408 **Experimental design and soil sampling**
409 The long-term experiment was established in 2002, and nitrogen addition started in July
410 2003 with monthly spraying ammonium nitrate (NH_4NO_3) solution [36]. Three 10 × 20
411 m^2 plots were assigned for N addition (N; ambient + 100 kg N ha^{-1} yr^{-1}), and three
412 paired plots were designed as control (C; ambient nitrogen deposition). For each N
413 addition plot, the required NH_4NO_3 was weighed, dissolved in 20 L of water and
414 sprayed evenly below the canopy using a backpack sprayer. Control plots received 20
415 L of water, which is equivalent to an increase of 1.2 mm yr^{-1} precipitation. The short-
416 term experiment was established in October 2013; N addition began in September 2014,
417 with 0 and 105 kg N ha^{-1} yr^{-1} NH_4NO_3 for control (C) and N addition (N) treatments,
418 respectively. A total of 6 (3 replicates per treatment × 2 treatments) randomly scattered
419 plots (15 m × 15 m per plot) were set up. The required NH_4NO_3 was dissolved in 30 L
420 water and sprayed below the canopy monthly in each plot. The control plots received
421 an equivalent volume of water only, which corresponded to extra 1.6 mm of
422 precipitation occurred in each quadrat each year.
423 Surface soil samples (0-20 cm) were collected four times from each plot in wet (July
424 2015 and 2016) and dry (January 2016 and 2017) seasons. Within each plot, samples
425 were randomly taken using a soil auger (ϕ 5 cm) at 6 spots to form a composite sample

426 for analysis, which resulted in 24 soil samples for each site in the two years. Visible
427 roots and plant residues were removed from all samples, which were then sieved
428 through a 2-mm mesh. The sieved samples were divided into two groups. One group
429 was used to determine soil properties, and the other was kept at −80°C for subsequent
430 DNA extraction and microbial community analyses.
431
432 **Measurement of soil properties**
433 Soil water content (SWC) was measured gravimetrically using 10 g of fresh soil by
434 oven-drying at 105°C for 24 h. Soil pH was determined in a 1:2.5 air-dried soil:water
435 suspension (w:v) using a pH meter (F-71G, LAQUA, HORIBA, Japan). Soil
436 ammonium nitrogen (NH_4^+-N) and nitrate nitrogen (NO_3^--N) were extracted by 1 M
437 KCl from 20 g fresh soil samples, and detected using the Alpha-Naphthol Blue and dual
438 wavelength (220 nm and 275 nm) spectrophotometric methods (UV-6000, China),
439 respectively. Soil total organic carbon (TOC) was analyzed using an external heating
440 method with concentrated sulfuric acid and potassium dichromate (H_2SO_4-$K_2Cr_2O_7$).
441 To examine the contents of soil total N (TN) and phosphorus (TP), semi-micro Kjeldahl
442 digestion was carried out followed by Alpha-Naphthol Blue-spectrophotometer method
443 and the Mo-Sb anti-spectrophotometer method. Soil dissolved organic carbon (DOC)
444 and nitrogen (DON) were measured with a TOC analyzer (Shimadzu TOC-VCSH
445 Analyzer, Kyoto, Japan), after filtering the extracts of 10 g fresh soil in a 0.5 M K_2SO_4
446 solution.

447

448 **Soil DNA extraction and sequencing**

449 Total DNA from soils was extracted using the PowerSoil® DNA Isolation Kit (MoBio
450 Laboratories Inc., Carlsbad, CA, USA) according to the manufacturer's instructions.
451 The extracted DNA was examined for quantity and quality using a Nanodrop 2.0
452 spectrophotometer (Thermo Fisher Scientific, CA, USA). The paired primers ITS3-F
453 (5'- GCATCGATGAAGAACGCAGC-3') and ITS4-R (5'-
454 TCCTCCGCTTATTGATATGC-3') were used to amplify the ITS2 region of the
455 fungal rRNA gene [68]. The PCR and tag-encoded high-throughput sequencing of the
456 ITS2 were performed using the Illumina HiSeq platform (PE 250) (Guangdong
457 Magigene Biotechnology Co., Ltd. Guangzhou, China).

458

459 **Sequences processing and bioinformatics**

460 The raw sequences were cleaned to ensure high quality data; the reads with short
461 sequences (< 200 bp), barcodes, and poly base etc. were removed using the Fastp
462 (V0.14.1, https://github.com/OpenGene/fastp) and cutadapt
463 (https://github.com/marcelm/cutadapt/) [69, 70]. The high-quality paired-end reads
464 were then merged by USEARCH (V10.0.240, http://www.drive5.com/usearch/) with
465 minimal overlap of 16 bp. Then the sequences were split into operational taxonomic
466 units (OTUs) through the UPARSE pipeline, which performed chimera filtering and
467 OTU clustering simultaneously, based on a 3% dissimilarity level. OTUs with fewer

than two sequences were removed, and the representative sequence of each OTU was assigned to fungal taxonomic lineages by comparison with the UNITE database (V7.1; http://unite.ut.ee). All the non-fungal OTUs were removed before the downstream analyses. Eventually, to compensate for the uneven sequencing efforts of different samples, the OTU table was randomly subsampled to obtain the same sequence number (61,292) for all the fungal data samples using *vegan* package in R (V3.6.1; http://www.r-project.org/).

We classified the fungal OTUs into functional groups according to the FUNGuild database [71]. The taxa with confidence levels "highly probable" or "probable" and unique trophic guilds which belong to saprotrophic fungi, pathogenic fungi or EcM fungi were selected for further analyses.

Phylogenetic community assembly

The phylogenetic signals were assessed for the control and N addition treatments at each site to test whether fungal community responses to N addition were phylogenetically conserved. Phylogenetic correlogram was applied to test the phylogenetic signals, and measured by the "mantel.correlog" function in *vegan* package. The assembly processes of fungal community were evaluated using a null model analysis [15, 72, 73]. Based on the null model, ecological processes, which were classified into deterministic processes (e.g. variable and homogeneous selections) or stochastic processes (dispersal limitation, homogenizing dispersal, and undominated),

were evaluated by β-nearest taxon index (βNTI) in combination with Raup-Crick metric (Bray-Curtis-based Raup-Crick, RC_{bray}) [15, 28, 62, 72]. Briefly, pairwise phylogenetic turnover between communities was quantified by the MNTD metric (βMNTD), which calculated by the "comdistnt" function (abundance. weighted = TRUE). The deviations between observed βMNTD and the null βMNTD distribution were evaluated by βNTI. Based on Stegen et al. [72], βNTI >2 (βNTI <-2) between a pair of samples means the significantly higher (lower) phylogenetic turnover than expected (null distribution), indicating the predominance of variable selection (i.e., homogeneous selection). Subsequently, the RC_{bray} was calculated to estimate the pairwise comparisons which were $|\beta NTI| < 2$. The percentage of homogeneous dispersal was quantified as the fraction of pairwise comparisons with $|\beta NTI| < 2$ and $RC_{Bray} < -0.95$, while the dispersal limitation was quantified as the fraction of the pairwise comparisons with $|\beta NTI| < 2$ and $RC_{bray} > 0.95$. Finally, the remaining fractions ($|\beta NTI| < 2$ and $|RC_{bray}| < 0.95$) were treated as undominated processes [15, 28].

To further quantify the contribution of stochastic (neutral) processes to fungal community structure under different N treatments, a normalized stochasticity ratio (*NST*) and neutral community model were applied. The *NST*, an index developed with 50% as the boundary point between more deterministic (< 50%) and more stochastic (> 50%) assembly, was quantified using a pipeline based on the phylogenetic distance (http://ieg3.rccc.ou.edu:8080/; [74]). The neutral community model was fitted to

predict the relationship between the frequency taxonomic occurrence in a set of local communities and the abundance of those taxa across the wider metacommunity [75]. R^2 indicates the fit of the parameter based on nonlinear least-squares fitting.

Co-occurrence networks of microbial communities

Microbial interaction is one of the main drivers that contributes to deterministic process of community assembly. To estimate the interspecies interaction within the fungal community across the control and N treatments at each site, co-occurrence networks were constructed based on the Random Matrix Theory (RMT) using the Molecular Ecological Network Analysis Pipeline (http://ieg4.rccc.ou.edu/mena) [76]. Briefly, fungal OTU table was square-root transformed and then split into 4 sub-datasets according to duration and rate of N addition. For each sub-dataset, only those nodes detected more than half of the total samples (majority rule) were kept for subsequent network construction. More information on theories, algorithms, pipeline structure and procedures can be found in references [76, 77]. Network visualization was conducted using the interactive platform Gephi (WebAtlas, Paris, France). The "randomize the network structure and then calculate network" function was run to compare the properties of the empirical and randomly-generated networks. The connectivity of each node was determined based on its within-module connectivity (*Zi*) and among-module connectivity (*Pi*) [78]. Node topologies were organized into four categories: (1) module hubs (highly connected nodes within modules, generalists, $Zi > 2.5$); (2) network hubs

(highly connected nodes within the entire network, supergeneralists, $Zi > 2.5$ and $Pi > 0.62$); (3) connectors (nodes connecting modules, generalists, $Pi > 0.62$), and (4) peripherals (interconnected nodes in modules with few outside connections, specialists, $Zi < 2.5$ and $Pi < 0.62$) [76, 77].

Statistical analyses

The phylogenetic α-diversity was calculated with *picante* package in R. The taxonomic α-diversity (richness and Shannon indexes) was calculated by USEARCH. The richness was calculated by counting the observed OTUs in each sample. The Shannon diversity index was calculated according to the following equation:

$$Shannon\ index = -\sum_{i=i}^{S} p_i log_2 p_i$$

where S is the number of observed OTUs and p_i is the relative abundance of species i.

Three-way ANOVA was used to evaluate the effects of the duration, rate and season of N addition and their interactions on fungal taxonomic and phylogenetic α-diversity. As the results showed that season had no significant effect on fungal α-diversity, we considered the seasons as the replicates for downstairs analysis (Table S1). Therefore, two-way analysis of variance (ANOVA) was performed to analyze the effects of duration, rate of N addition and their interactions on soil properties, fungal α-diversity, and the relative abundance of major phyla, genera and functional guilds. Student's t-test was used to assess the differences of aforementioned parameters between the control

551 and N addition treatments in the short- and long-term sites, respectively. Before two-
552 way ANOVA and Student's t-test were performed, the data were checked for normality
553 (Shapiro test) and homoscedasticity (Bartlett test). If the datasets were not normal or
554 homoscedastic, they were subjected to the Box–Cox transformation, ensuring that they
555 met the assumptions of these tests. A linear regression model was used to express the
556 correlations between soil properties and community variables, such as α-diversity and
557 relative abundance of major phyla, genera and functional guilds.
558 The multivariate permutational analysis of variance (PERMANOVA) was
559 conducted to analyze the variances in taxonomic (based on Bray-Curtis distance) and
560 phylogenetic (based on βMNTD) community composition and visualized by non-
561 metric multidimensional scaling (NMDS). Analysis of similarities (ANOSIM) with
562 999 permutations based on Bray-Curtis dissimilarity and βMNTD was conducted to
563 identify differences in OTU composition within and between treatments. The
564 potential relationships between environmental variables and fungal taxonomic and
565 phylogenetic community composition were assessed using constrained analysis of
566 principal coordinates (CAP) analysis based on “capscale” function of the *vegan*
567 package in R. The significantly environmental factors were selected using “envfit”
568 function of the *vegan* package with 999 random permutations. The variables that
569 displayed significant effects ($P < 0.05$) were included in the ordination plot.
570 To help reveal the variation in species composition of the fungal community, the
571 Levins’ niche breadth (B) index [79] was determined with the following formula:

572 $$B_j = 1 \Big/ \sum_{i=1}^{N} P_{ij}^2$$

573 where B_j represents the habitat niche breadth of OTU_j in a metacommunity; N is the
574 total number of communities of each metacommunity; and P_{ij} is the proportion of OTU_j
575 in community i. A high B value represents wide habitat niche breadth. Community-
576 level B (*Bcom*) was calculated as the average B values from all the taxa in a single
577 community. The analysis was conducted using the "niche.width" function in *spaa*
578 package in R. All the statistical analyses were performed in R.

579

580 **Additional files**

581 **Additional file 1:**

582 **Table S1** The effects of duration (D), rate (R), season (S) of N addition and their
583 interactions on soil fungal taxonomic and phylogenetic α-diversity.

584 **Table S2** The effects of duration (D), rate (R) of N addition and their interactions on
585 soil properties.

586 **Table S3** ANOVA of environmental factors correlate with soil fungal taxonomic
587 (based on Bray-curits distance) and phylogenetic (based on βMNTD) community
588 composition at short- and long-term sites.

589 **Table S4** Summary of the basic taxonomies and the network connectivity of the
590 putative keystone species in control network at short-term site.

591 **Table S5** Summary of the basic taxonomies and the network connectivity of the
592 putative keystone species in N addition network at short-term site.

Table S6 Summary of the basic taxonomies and the network connectivity of the putative keystone species in control network at long-term site.

Table S7 Summary of the basic taxonomies and the network connectivity of the putative keystone species in N addition network at long-term site.

Supplementary Figures S1–S8

Fig. S1 The varaitions of soil pH and concentrations of NO_3^--N under N addition.

Fig. S2 The varaitions of relative abundance of major phyla and genera under N addition.

Fig. S3 The varaitions of relative abundance of major funtional guilds under N addition.

Fig. S4 Non-metric multidimensional scaling (NMDS) plots based on Bray-Curtis distance and βMNTD of fungal community.

Fig. S5 Comparison of mean habitat niche breadths (*Bcom*) in all taxa of fungal community between control and N addition at short- and long-term site, respectively.

Fig. S6 Relationships between soil pH and the relative abundance of major functional guilds, phyla and genera.

Fig. S7 Relationships between soil NO_3^--N concentrations and the relative abundance of major functional guilds, phyla and genera.

Fig. S8 Fit of the neutral community model of community assembly for control and N addition at the short- and long-term site, respectively.

Acknowledgements

We acknowledge the support of Dinghushan Forest Ecosystem Research Station, CAS; the assistance we received with field sampling and laboratory assays from Zhuang Ni and Drs. Xiaoge Han, Guangcun Hao and Dan He.

Funding

This work was supported by the National Natural Science Foundation of China (31425005, 31290222, and 31400420).

Availability of data and materials

All raw sequences from this paper have been submitted to the NCBI Sequence Read Archive (SRA) database under the BioProject number PRJNA664903.

Authors' contributions

WJS and JHH conceived, designed the study and wrote the manuscript. JHH, XPT, HW, XMM and YXN were responsible for performing the field and lab experiments. JHH and SJ analyzed the data. All authors discussed the results, critically reviewed the manuscript, and approved its publication. All authors read and approved the final manuscript.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References

1. Baldrian P, Valášková V. Degradation of cellulose by basidiomycetous fungi. FEMS Microbiol Rev. 2008;32:501-521.
2. Schneider T, Keiblinger KM, Schmid E, Sterflinger-Gleixner K, Ellersdorfer G, Roschitzki B, et al. Who is who in litter decomposition? Metaproteomics reveals major microbial players and their biogeochemical functions. ISME J. 2012;6:1749-1762.
3. Frey SD. Mycorrhizal fungi as mediators of soil organic matter dynamics. Annu Rev Ecol Evol Syst. 2019;50:237-259.
4. Tedersoo L, Bahram M, Zobel M. How mycorrhizal associations drive plant population and community biology. Science. 2020;367:eaba1223.

5. Mohamed DJ, Martiny JBH. Patterns of fungal diversity and composition along a salinity gradient. ISME J. 2011;5:379-388.

6. Tedersoo L, Bahram M, Põlme S, Kõljalg U, Yorou NS, Wijesundera R, et al. Global diversity and geography of soil fungi. Science. 2014;346:1256688.

7. Aguirre de Cárcer D. A conceptual framework for the phylogenetically constrained assembly of microbial communities. Microbiome. 2019;7:142.

8. Wang P, Li S, Yang X, Zhou J, Shu W, Jiang L. Mechanisms of soil bacterial and fungal community assembly differ among and within islands. Environ Microbiol. 2020;22:1559-1571.

9. Bissett A, Brown MV, Siciliano SD, Thrall PH. Microbial community responses to anthropogenically induced environmental change: towards a systems approach. Ecol Lett. 2013;16:128-139.

10. Dessaux Y, Grandclément C, Faure D. Engineering the Rhizosphere. Trends Plant Sci. 2016;21:266-278.

11. Caruso T, Chan Y, Lacap DC, Lau MCY, McKay CP, Pointing SB. Stochastic and deterministic processes interact in the assembly of desert microbial communities on a global scale. ISME J. 2011;5:1406-1413.

12. Nemergut DR, Schmidt SK, Fukami T, O'Neill SP, Bilinski TM, Stanish LF, et al. Patterns and processes of microbial community assembly. Microbiol Mol Biol Rev. 2013;77:342-356.

13. Zhao J, Gao Q, Zhou J, Wang M, Liang Y, Sun B, et al. The scale dependence of fungal community distribution in paddy soil driven by stochastic and deterministic processes. Fungal Ecol. 2019;42:100856.
14. Vellend BM. Conceptual Synthesis in Community Ecology. Q Rev Biol. 2010;85:183-206.
15. Stegen JC, Lin X, Fredrickson JK, Konopka AE. Estimating and mapping ecological processes influencing microbial community assembly. Front Microbiol. 2015;6:370.
16. Isobe K, Allison SD, Khalili B, Martiny AC, Martiny JBH. Phylogenetic conservation of bacterial responses to soil nitrogen addition across continents. Nat Commun. 2019;10:2499.
17. Chen W, Ren K, Isabwe A, Chen H, Liu M, Yang J. Stochastic processes shape microeukaryotic community assembly in a subtropical river across wet and dry seasons. Microbiome. 2019;7:138.
18. Jiao S, Lu Y. Abundant fungi adapt to broader environmental gradients than rare fungi in agricultural fields. Glob Chang Biol. 2020;26:4506-4520.
19. Kou Y, Wei K, Li C, Wang Y, Tu B, Wang J, et al. Deterministic processes dominate soil methanotrophic community assembly in grassland soils. Geoderma 2020;359:114004.

695 20. Zhang X, Johnston ER, Liu W, Li L, Han X. Environmental changes affect the
696 assembly of soil bacterial community primarily by mediating stochastic processes.
697 Glob Chang Biol. 2016;22:198-207.
698 21. Shi Y, Dang K, Dong Y, Feng M, Wang B, Li J, et al. Soil fungal community
699 assembly processes under long-term fertilization. Eur J Soil Biol. 2019;71:716-
700 726.
701 22. Ning D, Yuan M, Wu L, Zhang Y, Guo X, Zhou X, et al. A quantitative framework
702 reveals the ecological drivers of grassland soil microbial community assembly in
703 response to warming. Nat Commun. 2020;11:4717.
704 23. Galloway JN, Dentener FJ, Capone DG, Boyer EW, Howarth RW, Seitzinger SP,
705 et al. Nitrogen cycles: past, present, and future. Biogeochemistry. 2004;70:153-
706 226.
707 24. Reay DS, Dentener F, Smith P, Grace J, Feely RA. Global nitrogen deposition and
708 carbon sinks. Nat Geosci. 2008;1:430-437.
709 25. Janssens I, Dieleman W, Luyssaert S, Subke JA, Reichstein M, Ceulemans R, et
710 al. Reduction of forest soil respiration in response to nitrogen deposition. Nat
711 Geosci. 2010;3:315-322.
712 26. Zhang T, Chen HYH, Ruan H. Global negative effects of nitrogen deposition on
713 soil microbes. ISME J. 2018;12:1817-1825.
714 27. Treseder KK. Nitrogen additions and microbial biomass: a meta-analysis of
715 ecosystem studies. Ecol Lett. 2008;11:1111-1120.

716 28. Zhou J, Ning D. Stochastic community assembly: does it matter in microbial
717 ecology? Microbiol Mol Biol Rev. 2017;81:e00002-00017.
718 29. Wang Q, Ma M, Jiang X, Guan D, Wei D, Zhao B, et al. Impact of 36 years of
719 nitrogen fertilization on microbial community composition and soil carbon
720 cycling-related enzyme activities in rhizospheres and bulk soils in northeast China.
721 Appl Soil Ecol. 2019;136:148-157.
722 30. Morrison EW, Frey SD, Sadowsky JJ, van Diepen LTA, Thomas WK, Pringle A.
723 Chronic nitrogen additions fundamentally restructure the soil fungal community
724 in a temperate forest. Fungal Ecol. 2016;23:48-57.
725 31. Chen Y, Xu Z, Xu T, Veresoglou SD, Yang G, Chen B. Nitrogen deposition and
726 precipitation induced phylogenetic clustering of arbuscular mycorrhizal fungal
727 communities. Soil Biol Biochem. 2017;115:233-242.
728 32. Weese DJ, Heath KD, Dentinger BTM, Lau JA. Long-term nitrogen addition
729 causes the evolution of less-cooperative mutualists. Evolution. 2015;69:631-642.
730 33. Guo J, Ling N, Chen Z, Xue C, Li L, Liu L, et al. Soil fungal assemblage
731 complexity is dependent on soil fertility and dominated by deterministic processes.
732 New Phytol. 2020;226:232-243.
733 34. Zhao Z, He J, Geisen S, Han L, Wang J, Shen J, et al. Protist communities are
734 more sensitive to nitrogen fertilization than other microorganisms in diverse
735 agricultural soils. Microbiome. 2019;7:33.

35. Chen Y, Xu Z, Feng K, Yang G, Fu W, Chen B. Nitrogen and water addition regulate soil fungal diversity and co-occurrence networks. J. Soils Sediments. 2020;20:3192-3203.

36. Mo J, Brown S, Xue J, Fang Y, Li Z. Response of litter decomposition to simulated n deposition in disturbed, rehabilitated and mature forests in subtropical China. Plant Soil. 2006;282:135-151.

37. Han X, Shen W, Zhang J, Müller C. Microbial adaptation to long-term N supply prevents large responses in N dynamics and N losses of a subtropical forest. Sci Total Environ. 2018;626:1175-1187.

38. Lu X, Mo J, Gilliam FS, Zhou G, Fang Y. Effects of experimental nitrogen additions on plant diversity in an old-growth tropical forest. Glob Chang Biol. 2010;16:2688-2700.

39. Lu X, Vitousek PM, Mao Q, Gilliam FS, Luo Y, Zhou G, et al. Plant acclimation to long-term high nitrogen deposition in an N-rich tropical forest. Proc Natl Acad Sci U S A. 2018;115:5187-5192.

40. Di Lonardo DP, van der Wal A, Harkes P, de Boer W. Effect of nitrogen on fungal growth efficiency. Plant Biosyst. 2020;154:433-437.

41. Du E, de Vries W. Nitrogen-induced new net primary production and carbon sequestration in global forests. Environ Pollut. 2018;242:1476-1487.

42. Liang X, Zhang T, Lu X, Ellsworth DS, BassiriRad H, You C, et al. Global response patterns of plant photosynthesis to nitrogen addition: A meta-analysis. Glob Chang Biol. 2020;26:3585-3600.

43. Zhang X, Johnston ER, Barberán A, Ren Y, Wang Z, Han X. Effect of intermediate disturbance on soil microbial functional diversity depends on the amount of effective resources. Environ Microbiol. 2018;20:3862-3875.

44. Gross S, Robbins EI. Acidophilic and acid-tolerant fungi and yeasts. Hydrobiologia. 2000;433:91-109.

45. Yang X, Ma L, Ji L, Shi Y, Yi X, Yang Q, et al. Long-term nitrogen fertilization indirectly affects soil fungi community structure by changing soil and pruned litter in a subtropical tea (*Camellia sinensis* L.) plantation in China. Plant Soil. 2019;444:409-426.

46. Chen W, Xu R, Chen J, Yuan X, Zhou L, Tan T, et al. Consistent responses of surface- and subsurface soil fungal diversity to N enrichment are mediated differently by acidification and plant community in a semi-arid grassland. Soil Biol Biochem. 2018;127:110-119.

47. Cui H, Sun W, Delgado-Baquerizo M, Song W, Ma J, Wang K, et al. The effects of mowing and multi-level N fertilization on soil bacterial and fungal communities in a semiarid grassland are year-dependent. Soil Biol Biochem. 2020;151:108040.

774 48. Wang C, Liu D, Bai E. Decreasing soil microbial diversity is associated with
775 decreasing microbial biomass under nitrogen addition. Soil Biol Biochem.
776 2018;120:126-133.
777 49. Yang Y, Cheng H, Gao H, An S. Response and driving factors of soil microbial
778 diversity related to global nitrogen addition. Land Degrad Dev. 2020;31:190-204.
779 50. Zhao A, Liu L, Chen B, Fu W, Xie W, Xu T, et al. Soil fungal community is more
780 sensitive to nitrogen deposition than increased rainfall in a mixed deciduous forest
781 of China. Soil Ecol Lett. 2020;2:20-32.
782 51. Entwistle EM, Zak DR, Edwards IP. Long-term experimental nitrogen deposition
783 alters the composition of the active fungal community in the forest floor. Soil Sci
784 Soc Am J. 2013;77:1648-1658.
785 52. Wang W, Mo Q, Han X, Hui D, Shen W. Fine root dynamics responses to nitrogen
786 addition depend on root order, soil layer, and experimental duration in a
787 subtropical forest. Biol Fertil Soils. 2019;55:723-736.
788 53. Fang Y, Yoh M, Mo J, Gundersen P, Zhou G. Response of nitrogen leaching to
789 nitrogen deposition in disturbed and mature forests of southern China. Pedosphere.
790 2009;19:111-120.
791 54. Corrales A, Turner BL, Tedersoo L, Anslan S, Dalling JW. Nitrogen addition
792 alters ectomycorrhizal fungal communities and soil enzyme activities in a tropical
793 montane forest. Fungal Ecol. 2017;27:14-23.

794 55. Gadgil RL, Gadgil PD. Mycorrhiza and litter decomposition. Nature.
795 1971;233:133-133.
796 56. Fernandez CW, Kennedy PG. Revisiting the 'Gadgil effect': do interguild fungal
797 interactions control carbon cycling in forest soils? New Phytol. 2016;209:1382-
798 1394.
799 57. Ning C, Mueller GM, Egerton-Warburton LM, Wilson AW, Yan W, Xiang W.
800 Diversity and enzyme activity of ectomycorrhizal fungal communities following
801 nitrogen fertilization in an urban-adjacent pine plantation. Forests. 2018;9:99.
802 58. Francioli D, Schulz E, Lentendu G, Wubet T, Buscot F, Reitz T. Mineral vs.
803 organic amendments: microbial community structure, activity and abundance of
804 agriculturally relevant microbes are driven by long-term fertilization strategies.
805 Front Microbiol. 2016;7:1446.
806 59. Ullah S, Ai C, Ding W, Jiang R, Zhao S, Zhang J, et al. The response of soil fungal
807 diversity and community composition to long-term fertilization. Appl Soil Ecol.
808 2019;140:35-41.
809 60. Wu W, Lu H, Sastri A, Yeh Y, Gong G, Chou W, et al. Contrasting the relative
810 importance of species sorting and dispersal limitation in shaping marine bacterial
811 versus protist communities. ISME J. 2018;12:485-494.
812 61. Yang J, Jiang H, Sun X, Chen J, Xie Z, Dong H. Minerals play key roles in driving
813 prokaryotic and fungal communities in the surface sediments of the Qinghai-
814 Tibetan lakes. FEMS Microbiol Ecol. 2020;96:fiaa035.

62. Stegen JC, Lin X, Fredrickson JK, Chen X, Kennedy DW, Murray CJ, et al. Quantifying community assembly processes and identifying features that impose them. ISME J. 2013;7:2069-2079.

63. Gurmesa GA, Lu X, Gundersen P, Mao Q, Fang Y, Mo J. Species differences in nitrogen acquisition in humid subtropical forest inferred from ^{15}N natural abundance and its response to tracer addition. Forests. 2019;10:991.

64. Faust K, Raes J. Microbial interactions: from networks to models. Nat Rev Microbiol. 2012;10: 538-550.

65. Barberán A, Bates ST, Casamayor EO, Fierer N. Using network analysis to explore co-occurrence patterns in soil microbial communities. ISME J. 2012;6:343-351.

66. Levy R, Borenstein E. Metabolic modeling of species interaction in the human microbiome elucidates community-level assembly rules. Proc Natl Acad Sci U S A. 2013;110:12804-12809.

67. Tu Q, Yan Q, Deng Y, Michaletz ST, Buzzard V, Weiser MD, et al. Biogeographic patterns of microbial co-occurrence ecological networks in six American forests. Soil Biol Biochem. 2020;148:107897.

68. White TJ, Bruns T, Lee S, Taylor J. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: PCR protocols: a guide to methods and applications. 1990;18:315-322.

69. Martin M. Cutadapt removes adapter sequences from high-throughput sequencing reads. EMBnet J. 2011;17:10-12.

70. Chen S, Zhou Y, Chen Y, Gu J. Fastp: an ultra-fast all-in-one FASTQ preprocessor. Bioinformatics. 2018;34:884-890.

71. Nguyen NH, Song Z, Bates ST, Branco S, Tedersoo L, Menke J, et al. FUNGuild: An open annotation tool for parsing fungal community datasets by ecological guild. Fungal Ecol. 2016;20:241-248.

72. Stegen JC, Lin X, Konopka AE, Fredrickson JK. Stochastic and deterministic assembly processes in subsurface microbial communities. ISME J. 2012;6: 1653-1664.

73. Dini-Andreote F, Stegen JC, van Elsas JD, Salles JF. Disentangling mechanisms that mediate the balance between stochastic and deterministic processes in microbial succession. Proc Natl Acad Sci U S A. 2015;112:1326-1332.

74. Ning D, Deng Y, Tiedje JM, Zhou J. A general framework for quantitatively assessing ecological stochasticity. Proc Natl Acad Sci U S A. 2019;116:16892-16898.

75. Sloan WT, Lunn M, Woodcock S, Head IM, Nee S, Curtis TP. Quantifying the roles of immigration and chance in shaping prokaryote community structure. Environ Microbiol. 2006;8:732-740.

76. Deng Y, Jiang Y, Yang Y, He Z, Luo F, Zhou J. Molecular ecological network analyses. BMC Bioinform. 2012;13:113.

77. Zhou J, Deng Y, Luo F, He Z, Yang Y. Phylogenetic molecular ecological network of soil microbial communities in response to elevated CO_2. mBio. 2011;2: e00122-11.

78. Guimerà R, Amaral LAN. Functional cartography of complex metabolic networks. Nature. 2005;433:895-900.

79. Levins R. Evolution in changing environments : some theoretical explorations. Princeton University Press. 1968.

865 **Tables**

866 **Table 1** Detailed topological properties of the empirical and random networks of

867 fungal communities in soils under control and N addition at short- and long-term site,

868 respectively.

Network Index	Short-term site		Long-term site	
	Control	N addition	Control	N addition
Empirical networks				
Total nodes	405	451	369	461
Total links	596	638	630	770
Positive links (%)	65.94	55.96	72.06	65.58
Negative links (%)	34.06	44.04	27.94	34.42
Similarity threshold (St)	0.81	0.81	0.85	0.82
R^2 of power-law	0.848	0.894	0.91	0.805
Average connectivity(*avgK*)	2.943	2.829	3.415	3.341
Average clustering coefficient (*avgCC*)	0.159	0.149	0.152	0.122
Average path distance (GD)	7.55	8.589	5.803	7.662
Modularity (Module No.)	0.792 (50)	0.825 (48)	0.678 (49)	0.781 (40)
Dameter	21	22	14	24
Density (D)	0.007	0.006	0.009	0.007
Geodesic efficiency (E)	0.171	0.15	0.214	0.171
Harmonic geodesic distance (HD)	5.861	6.687	4.679	5.85
Maximal degree	20	13	21	43
Centralization of degree (CD)	0.042	0.023	0.048	0.087
Centralization of betweenness (CB)	0.081	0.159	0.083	0.169
Centralization of stress centrality (CS)	0.357	2.258	1.133	3.413
Random network				
Avg. clustering coefficient	0.015 (0.005)	0.007 (0.003)	0.020 (0.005)	0.021 (0.005)
Average path distance	4.619 (0.081)	5.309 (0.077)	4.202 (0.056)	4.332 (0.053)
Avg. modularity	0.625 (0.007)	0.658 (0.006)	0.555 (0.007)	0.573 (0.006)

869

870

871

872 **Figure legends**

873 **Fig. 1** Relative abundance of major soil fungal phyla and genera at the short- and
874 long-term N addition sites. Phyla and genera with the relative abundance > 0.2% are
875 included.

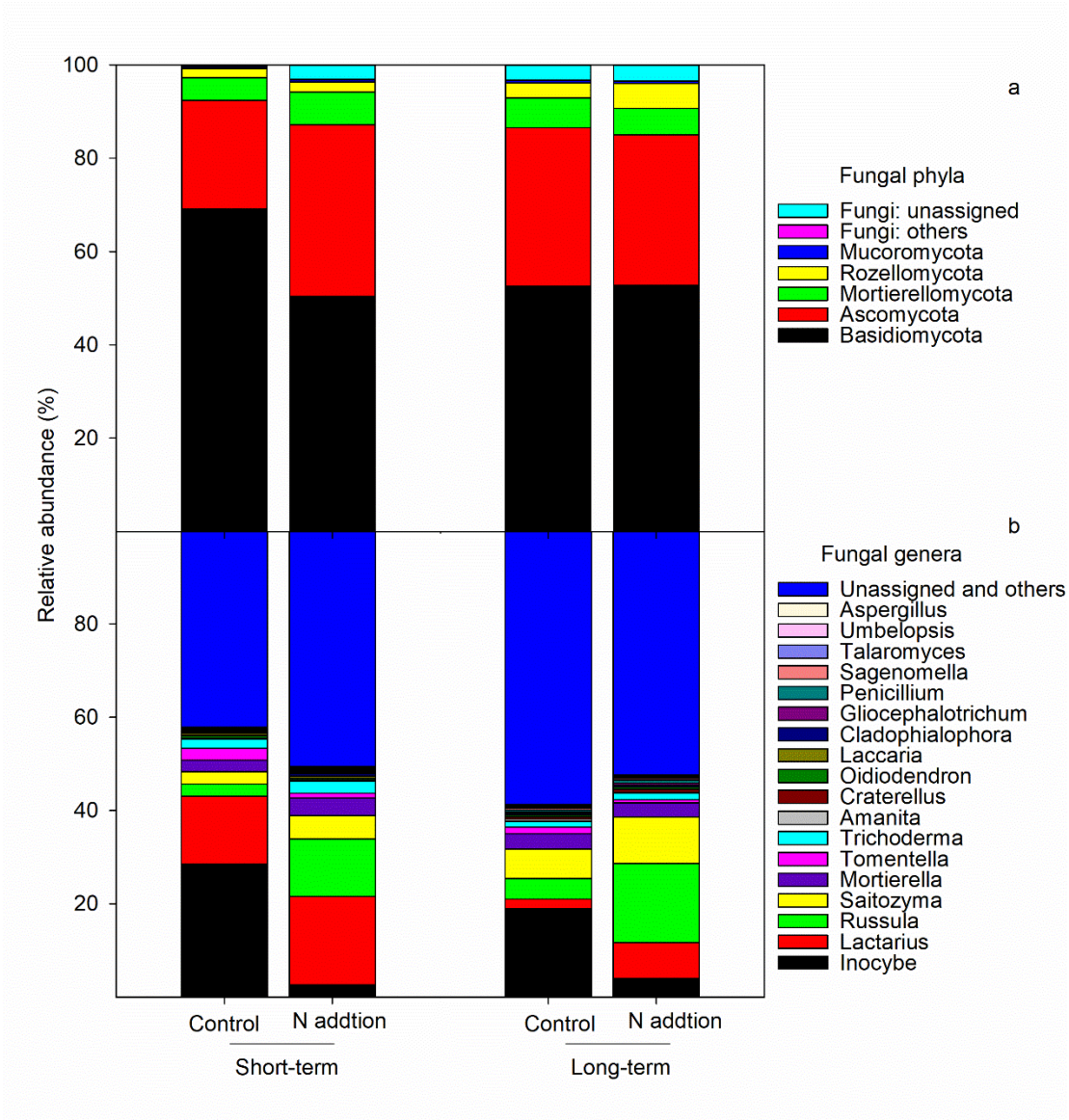
876 **Fig. 2** The varations of fungal α-diversity and community composition under short-
877 and long-term N addition. (**a-c**) The varaitions of fungal α-diversity under N addition
878 are indicated by student's t-test at short- and long-term sites, respectively. The bold
879 numbers denote the significantly differences ($P < 0.05$) of fungal α-diversity between
880 control and N addition at each site. The insert texts indicate the effects of duration
881 (D), rate (R) of N addition and their interactions on fungal α-diversity detected by
882 two-way ANOVA. (**d-e**) The boxplots show community dissimilarities between
883 control and N addition in short- or long-term site based on Bray–Curtis distance and
884 βMNTD, respectivly (*, $P < 0.05$; ***, $P < 0.001$; Wilcoxon rank-sum test).

885 **Fig. 3** Factors affecting fungal diversity and community composition. (**a-f**)
886 Relationships between fungal α-diversity and soil pH and NO_3^--N concentrations are
887 revealed by linear regression model. (**g, h**) CAP analysis show the community
888 dissimilarities based on Bray-Curtis distance and βMNTD against environmental
889 variables among samples. SWC, soil water content; NH_4^+-N, ammonium nitrogen;
890 NO_3^--N, nitrate nitrogen; TOC, total organic carbon; TN, total nitrogen; C/N, total
891 organic carbon / total nitrogen; TP, total phosphorus; DOC, dissolved organic carbon;
892 DON, dissolved organic nitrogen.

893 **Fig. 4** Phylogenetic Mantel correlogram between the Euclidean distance matrix of
894 OTU niche values and phylogenetic distance matrix shows significant phylogenetic
895 signal across short phylogenetic distances. Solid and open symbols denote significant
896 ($P < 0.05$) and non-significant ($P > 0.05$) correlations (phylogenetic signal),
897 respectively.
898 **Fig. 5** The assembly processes in shaping fungal communities. (**a**) The relative
899 contributions (%) of the five community assembly processes. (**b**) Boxplot shows the
900 variation in *NST* under control and N addition at short- and long-term sites (**, $P <$
901 0.01; Wilcoxon rank-sum test).
902 **Fig. 6** Co-occurrence patterns of OTUs of fungal community in control and N
903 addition treatment at short- and long-term site, respectively. The size of each
904 node is proportional to the number of connections (i.e. degree). The red and green
905 edges indicate positive and negative interactions between two individual nodes,
906 respectively.
907 **Fig. 7** Classification of nodes to identify putative keystone species within the
908 networks. The topological role of each OTU is determined according to the scatter
909 plot of within-module connectivity (*Zi*) and among-module connectivity (*Pi*).
910 Modules hubs have $Zi > 2.5$, whereas connectors have $Pi > 0.62$. The number of
911 module hubs and connectors is presented on the plot and the network hub is labeled
912 with the OTU number. The abbreviations of C and N are defined as control and N

addition, respectively. Detailed taxonomic information for module hubs and connectors is shown in Tables S4 to S8

915 **Fig. 1**



916

917

Fig. 2

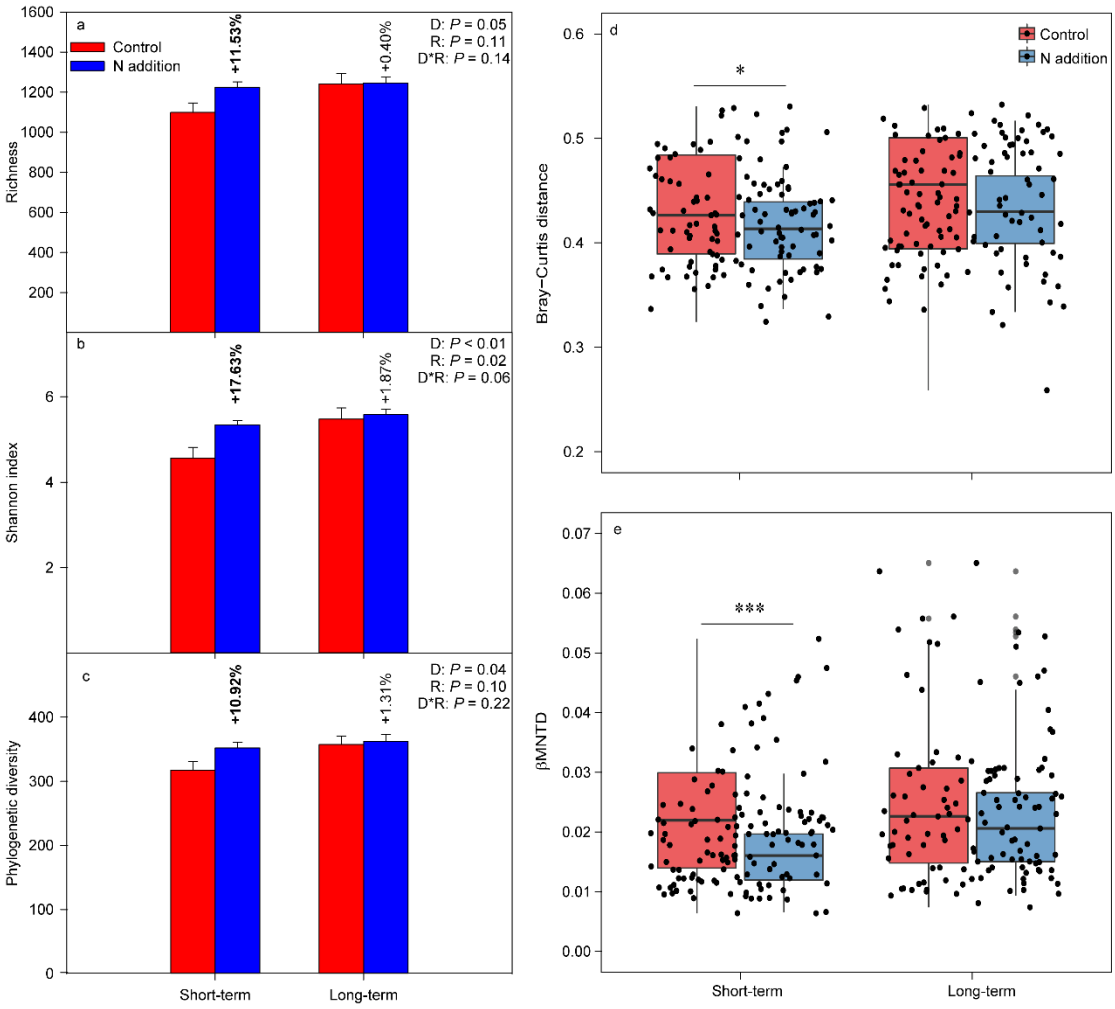
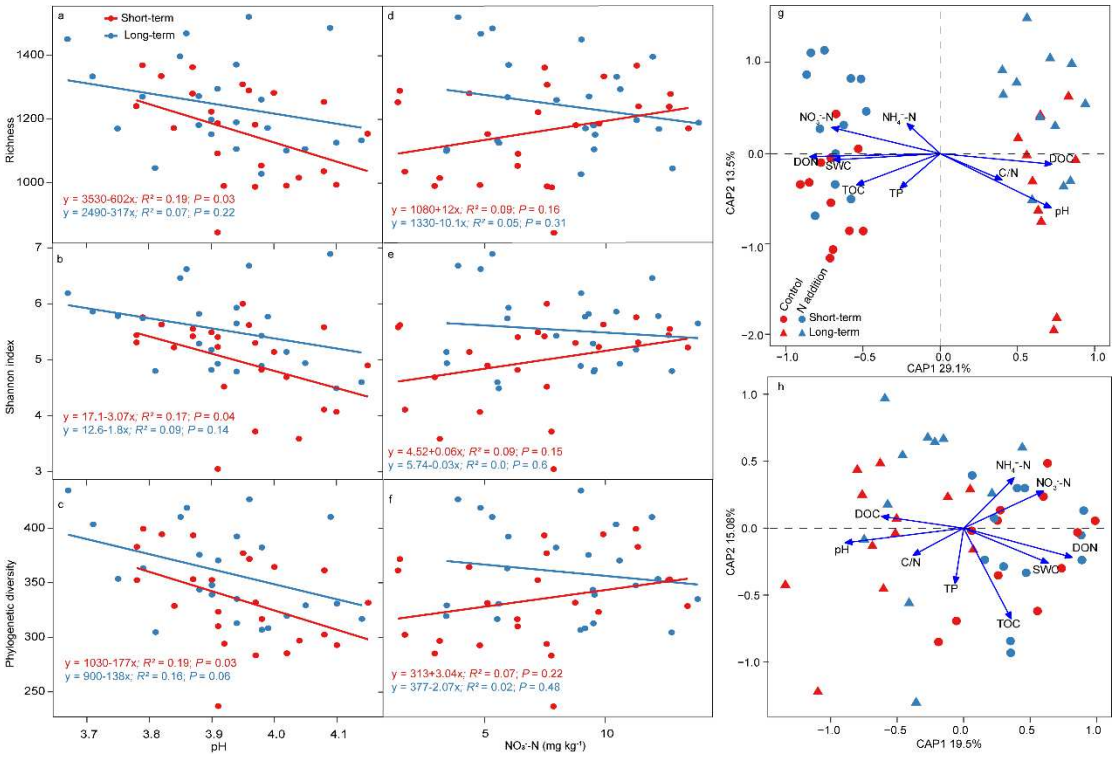
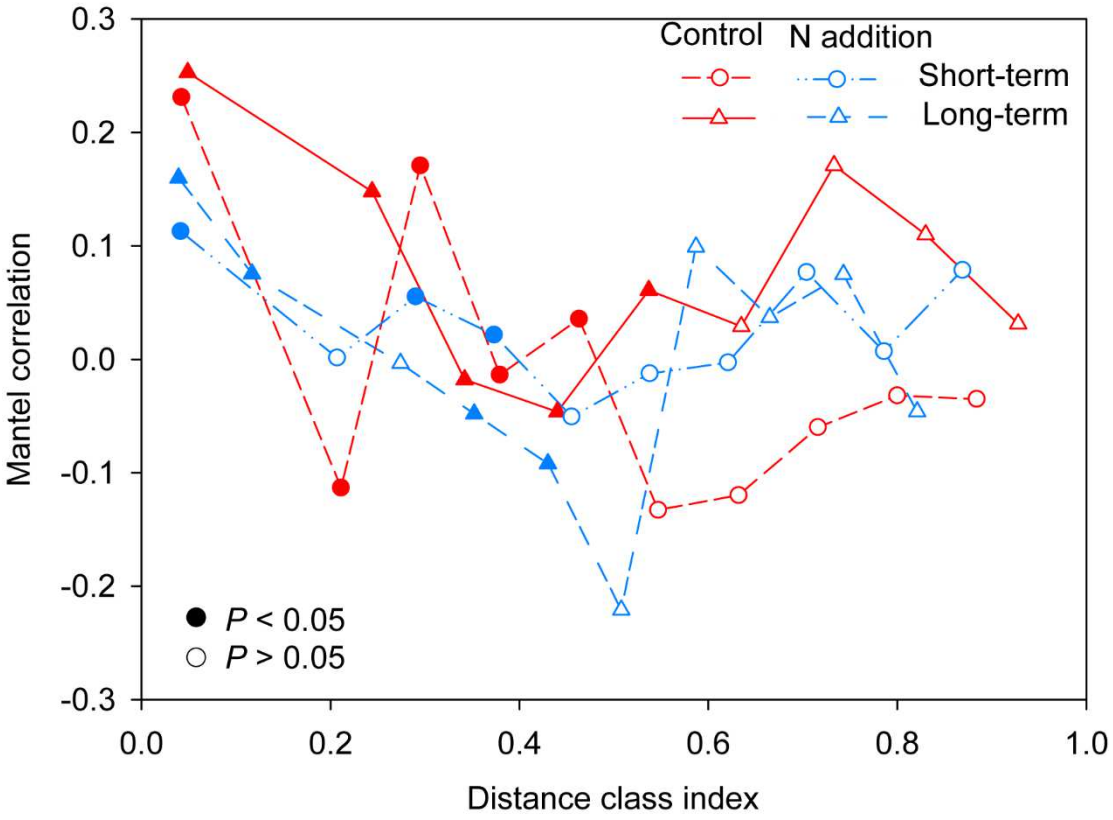

a
Control
N addition
+11.53%
+0.40%
D: P = 0.05
R: P = 0.11
D*R: P = 0.14
Richness
b
+17.63%
+1.87%
D: P < 0.01
R: P = 0.02
D*R: P = 0.06
Shannon index
c
+10.92%
+1.31%
D: P = 0.04
R: P = 0.10
D*R: P = 0.22
Phylogenetic diversity
Short-term
Long-term
d
Control
N addition
*
Bray–Curtis distance
e
***
βMNTD
Short-term
Long-term

Fig. 3


a
Short-term
Long-term
Richness
y = 3530-602x; R² = 0.19; P = 0.03
y = 2490-317x; R² = 0.07; P = 0.22
d
y = 1080+12x; R² = 0.09; P = 0.16
y = 1330-10.1x; R² = 0.05; P = 0.31
b
Shannon index
y = 17.1-3.07x; R² = 0.17; P = 0.04
y = 12.6-1.8x; R² = 0.09; P = 0.14
e
y = 4.52+0.06x; R² = 0.09; P = 0.15
y = 5.74-0.03x; R² = 0.0; P = 0.6
c
Phylogenetic diversity
y = 1030-177x; R² = 0.19; P = 0.03
y = 900-138x; R² = 0.16; P = 0.06
f
y = 313+3.04x; R² = 0.07; P = 0.22
y = 377-2.07x; R² = 0.02; P = 0.48
pH
NO3--N (mg kg-1)
g
NO3--N
NH4+-N
DON
SWC
TOC
TP
C/N
DOC
pH
Control
N addition
Short-term
Long-term
CAP2 13.5%
CAP1 29.1%
h
NH4+-N
NO3--N
DOC
pH
C/N
TP
SWC
DON
TOC
CAP2 15.08%
CAP1 19.5%

933 **Fig. 4**

934



935

936

937

938

939

940

941

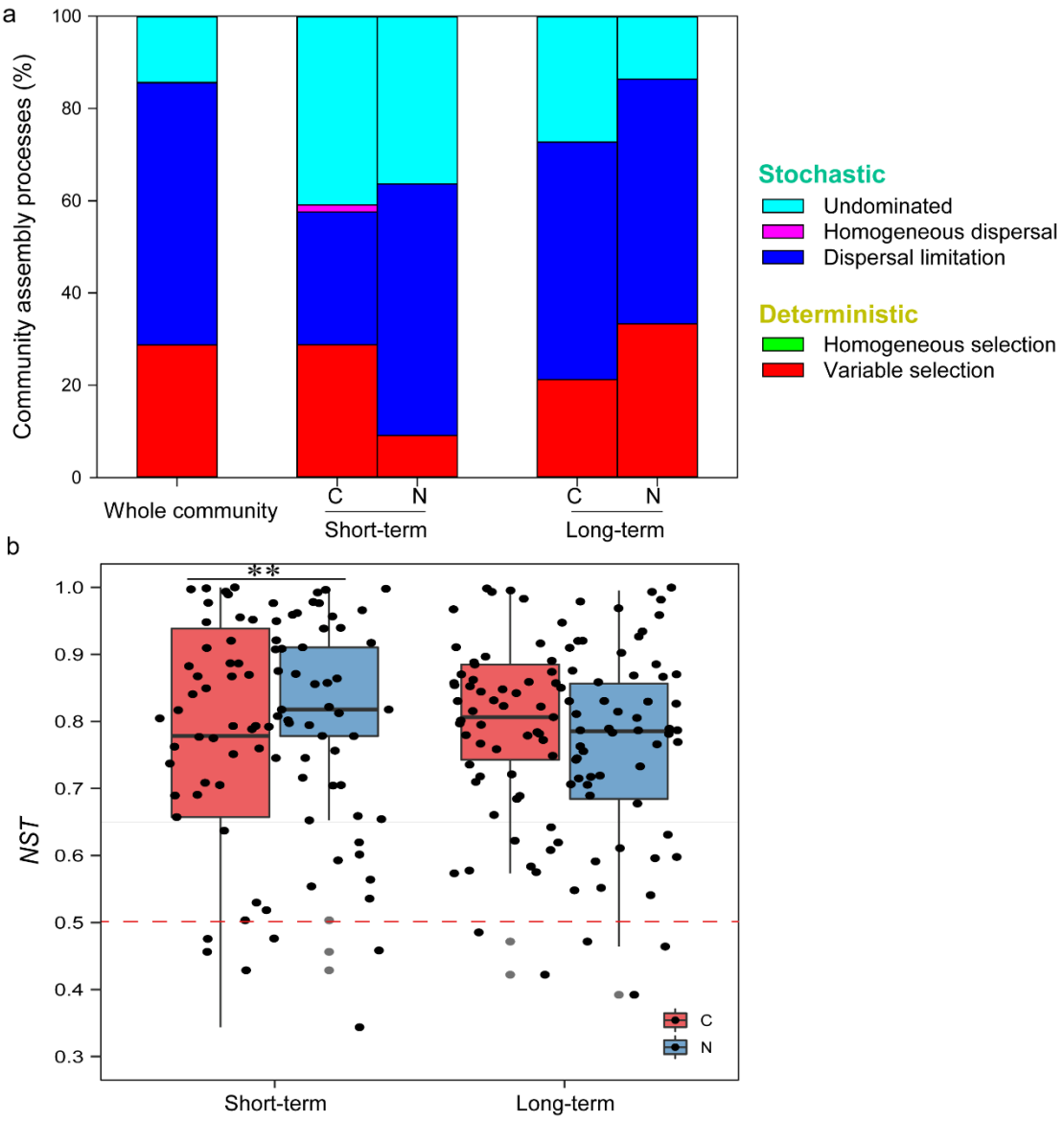
942

943

944

945

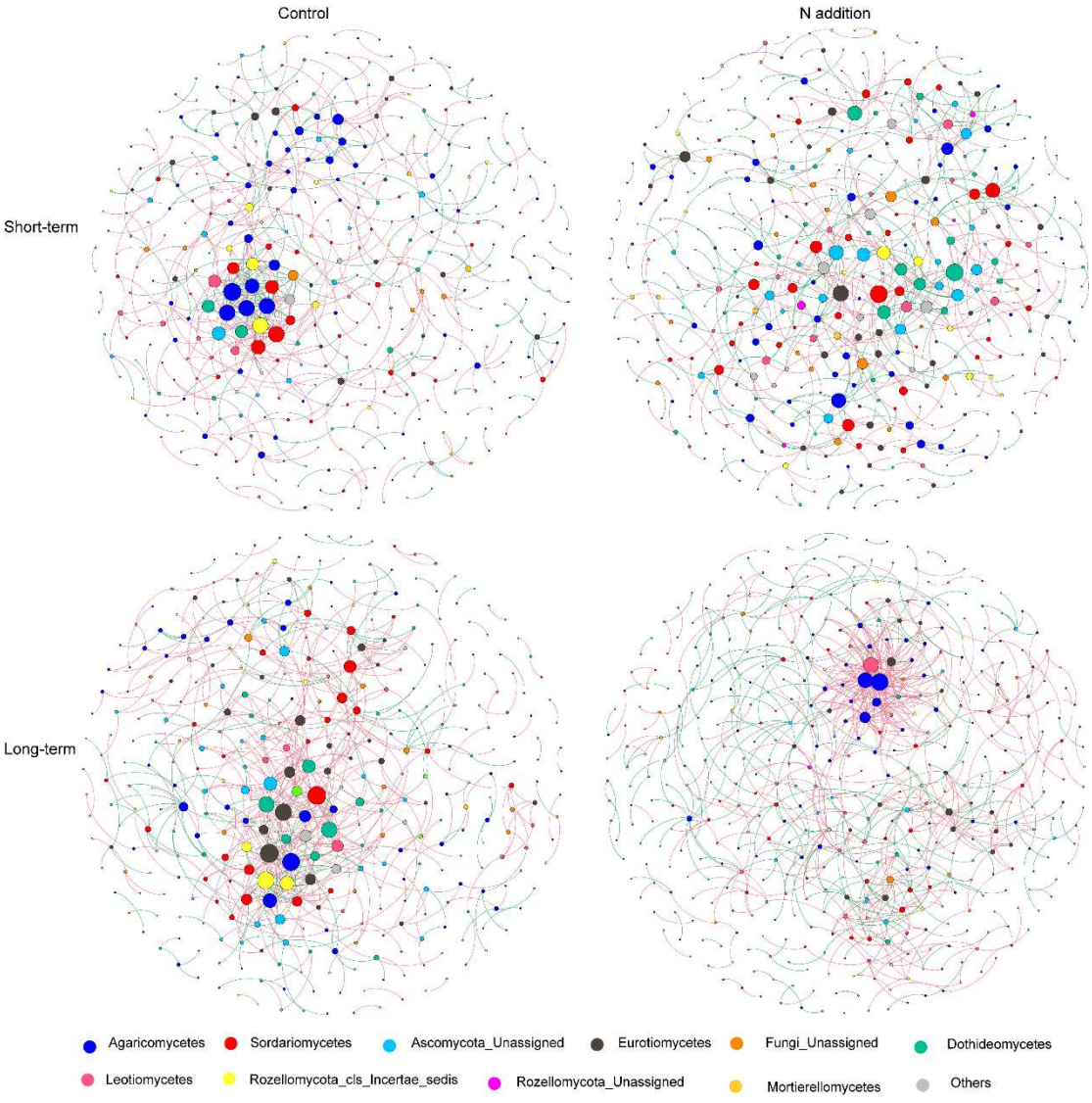
946 **Fig. 5**



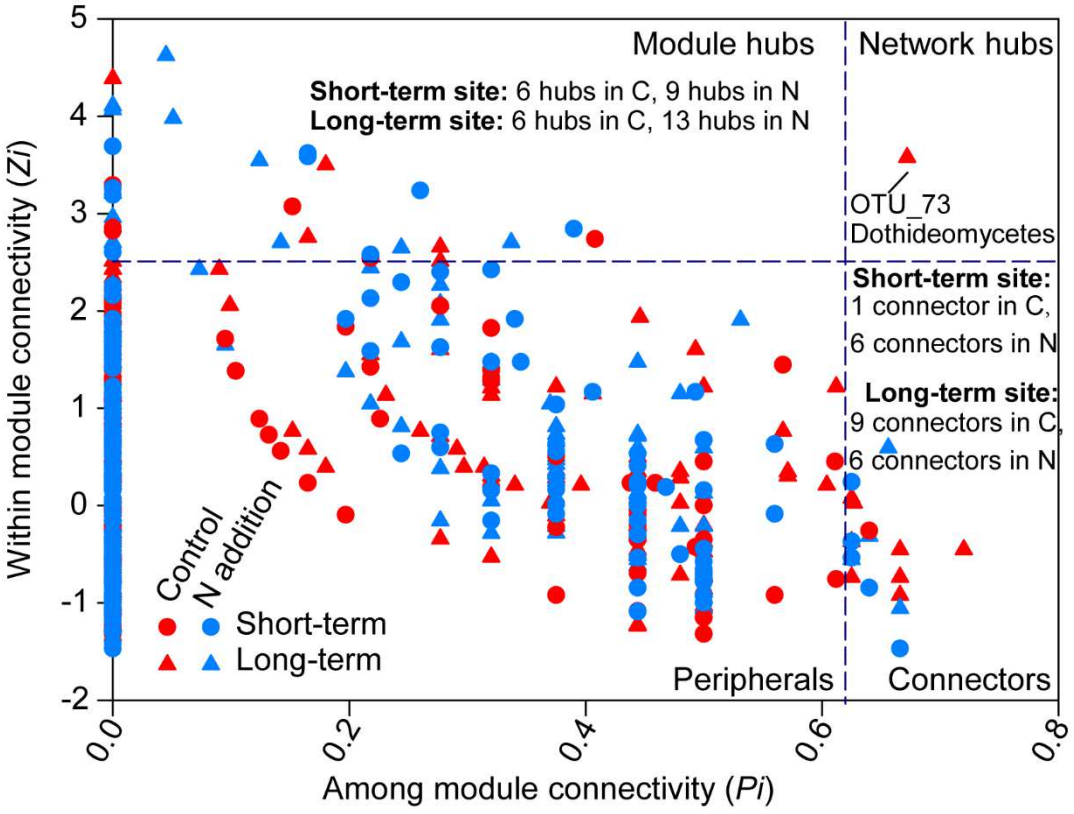
947

948

Fig. 6


Control
N addition
Short-term
Long-term
Agaricomycetes
Sordariomycetes
Ascomycota_Unassigned
Eurotiomycetes
Fungi_Unassigned
Dothideomycetes
Leotiomycetes
Rozellomycota_cls_Incertae_sedis
Rozellomycota_Unassigned
Mortierellomycetes
Others

957 **Fig. 7**


Module hubs
Network hubs
Short-term site: 6 hubs in C, 9 hubs in N
Long-term site: 6 hubs in C, 13 hubs in N
OTU_73
Dothideomycetes
Short-term site:
1 connector in C,
6 connectors in N
Long-term site:
9 connectors in C,
6 connectors in N
Control
N addition
Short-term
Long-term
Peripherals
Connectors
Within module connectivity (Zi)
Among module connectivity (Pi)
5
4
3
2
1
0
-1
-2
0.0
0.2
0.4
0.6
0.8

958

Supplementary materials for

How do N addition affect soil fungal community assembly: short- versus long-term effects?

Jinhong He[1], Shuo Jiao[2], Xiangping Tan[1], Hui Wei[3], Xiaomin Ma[1], Yanxia Nie[1], Juxiu Liu[1], Xiankai Lu[1], Jiangming Mo[1], Weijun Shen[1], *

[1] Center for Ecological and Environmental Sciences, South China Botanical Garden, Chinese Academy of Sciences, 723 Xingke Rd., Tianhe District, Guangzhou 510650, China

[2] State Key Laboratory of Crop Stress Biology in Arid Areas, Shaanxi Key Laboratory of Agricultural and Environmental Microbiology, College of Life Sciences, Northwest A&F University, Yangling 712100, China

[3] Department of Ecology, College of Natural Resources and Environment, South China Agricultural University, Guangzhou, 510642, China

* Corresponding author: Dr. Weijun Shen

Center for Ecological and Environmental Sciences,

South China Botanical Garden, Chinese Academy of Sciences

723 Xingke Road, Tianhe District

Guangzhou 510650, China

Tel.: + 86 20 3725 2950; Fax: + 86 20 3725 2950;

Email: shenweij@scbg.ac.cn

Supplementary Table S1–S7

Table S1 The effects of duration (D), rate (R), season (S) of N addition and their interactions on soil fungal taxonomic and phylogenetic α-diversity.

Table S2 The effects of duration (D), rate (R) of N addition and their interactions on soil properties.

Table S3 ANOVA of environmental factors correlate with soil fungal taxonomic (based on Bray-curits distance) and phylogenetic (based on βMNTD) community composition at short- and long-term sites.

Table S4 Summary of the basic taxonomies and the network connectivity of the putative keystone species in control network at short-term site.

Table S5 Summary of the basic taxonomies and the network connectivity of the putative keystone species in N addition network at short-term site.

Table S6 Summary of the basic taxonomies and the network connectivity of the putative keystone species in control network at long-term site.

Table S7 Summary of the basic taxonomies and the network connectivity of the putative keystone species in N addition network at long-term site.

Supplementary Figures S1–S8

Fig. S1 The varaitions of soil pH and concentrations of NO_3^--N under N addition.

Fig. S2 The varaitions of relative abundance of major phyla and genera under N addition.

Fig. S3 The varaitions of relative abundance of major funtional guilds under N addition.

Fig. S4 Non-metric multidimensional scaling (NMDS) plots based on Bray-Curtis distance and βMNTD of fungal community.

Fig. S5 Comparison of mean habitat niche breadths (*Bcom*) in all taxa of fungal community between control and N addition at short- and long-term site, respectively.

Fig. S6 Relationships between soil pH and the relative abundance of major functional guilds, phyla and genera.

Fig. S7 Relationships between soil NO_3^--N concentrations and the relative abundance of major functional guilds, phyla and genera.

Fig. S8 Fit of the neutral community model of community assembly for control and N addition at the short- and long-term site, respectively.

Supplementary Tables

Table S1 The effects of duration (D), rate (R), season (S) of N addition and their interactions on soil fungal taxonomic and phylogenetic α-diversity

	Taxonomic diversity				Phylogenetic diversity	
	Richness		Shannon index			
	F	*P*	*F*	*P*	*F*	*P*
Duration (D)	3.83	0.06	**7.90**	**<0.01**	**4.29**	**0.045**
Rate (R)	2.46	0.12	**4.60**	**0.04**	2.71	0.11
Season (S)	0.51	0.48	0.54	0.47	1.13	0.30
D*R	2.11	0.15	2.71	0.11	1.51	0.23
D*S	0.86	0.36	0.43	0.52	0.54	0.47
R*S	0.12	0.73	0.00	0.96	0.22	0.64
D*R*S	0.11	0.74	0.01	0.94	0.19	0.66

Notes: Three-way ANOVA was used to test the effects of duration (D), rate (R), season (S) of N addition and their interactions on soil fungal taxonomic and phylogenetic α-diversity. Significant effect ($P < 0.05$) is highlighted in bold.

Table S2 The effects of duration (D), rate (R) of N addition and their interactions on soil properties

	SWC (%)		pH		NH_4^+-N (mg kg^{-1})		NO_3^--N (mg kg^{-1})		TOC (%)		TN (%)		C/N		TP (%)		DOC (mg kg^{-1})		DON (mg kg^{-1})	
	F	*P*	*F*	*P*	*F*	*P*	*F*	*P*	*F*	*P*	*F*	*P*	*F*	*P*	*F*	*P*	*F*	*P*	*F*	*P*
Duration (D)	**6.67**	**0.01**	0.65	0.43	0.50	0.48	1.58	0.22	2.84	0.10	2.01	0.16	0.10	0.76	0.09	0.76	1.82	0.19	0.01	0.91
Rate (R)	0.24	0.63	**12.65**	**<0.01**	3.01	0.09	**9.47**	**<0.01**	0.15	0.70	1.51	0.23	2.27	0.14	0.18	0.67	0.12	0.74	0.50	0.48
D*R	1.1	0.3	0.003	0.95	0.00	0.99	0.19	0.66	1.02	0.32	1.03	0.32	0.42	0.52	0.58	0.45	0.17	0.68	0.24	0.63

Notes: The *F* and *P* values detected from a two-way ANOVA are listed. Numbers in bold indicate the significant effects ($P < 0.05$).

Abbreviation: SWC, soil water content; NH_4^+-N, ammonium nitrogen; NO_3^--N, nitrate nitrogen; TOC, total organic carbon; TN, total nitrogen; C/N, total organic carbon / total nitrogen; TP, total phosphorus; DOC, dissolved organic carbon; DON, dissolved organic nitrogen.

Table S3 ANOVA of environmental factors correlate with soil fungal taxonomic (based on Bray-curits distance) and phylogenetic (based on βMNTD) community composition at short- and long-term sites

	Factor	*df*	*Variance*	*Pseudo-F*	*p-value*
Bray-curits	pH	1	0.557	2.5326	0.001
	DON	1	0.3993	1.8157	0.001
	DOC	1	0.305	1.387	0.02
	NO_3^--N	1	0.2699	1.2272	0.048
	SWC	1	0.232	1.0549	0.28
	TOC	1	0.2381	1.0828	0.211
	C/N	1	0.2308	1.0494	0.293
	TP	1	0.2185	0.9934	0.442
	NH_4^+-N	1	0.197	0.8957	0.803
	Residual	38	8.3572		
βMNTD	pH	1	0.13107	1.7925	0.001
	NO_3^--N	1	0.08566	1.1715	0.046
	DON	1	0.08473	1.1588	0.058
	TOC	1	0.08363	1.1438	0.083
	SWC	1	0.0712	0.9737	0.585
	DOC	1	0.07331	1.0026	0.421
	NH_4^+-N	1	0.0719	0.9833	0.516
	C/N	1	0.07706	1.0538	0.211
	TP	1	0.07206	0.9855	0.542
	Residual	38	2.7786		

Abbreviation: SWC, soil water content; NH_4^+-N, ammonium nitrogen; NO_3^--N, nitrate nitrogen; TOC, total organic carbon; TN, total nitrogen; C/N, total organic carbon/ total nitrogen; TP, total phosphorus; DOC, dissolved organic carbon; DON, dissolved organic nitrogen.

Table S4 Summary of the basic taxonomies and the network connectivity of the putative keystone species in control network at short-term site

Network category	ID	Classification					No. module	Connectivity
		Phylum	Class	Order	Family	Genus		
Module hubs	OTU_967	*Basidiomycota*	*Agaricomycetes*	*Sebacinales*	Unassigned	Unassigned	0	6
	OTU_61	*Basidiomycota*	*Agaricomycetes*	*Trechisporales*	Unassigned	Unassigned	6	12
	OTU_477	*Ascomycota*	Unassigned	Unassigned	Unassigned	Unassigned	2	6
	OTU_344	*Basidiomycota*	*Agaricomycetes*	*Thelephorales*	*Thelephoraceae*	*Tomentella*	3	7
	OTU_66	*Ascomycota*	*Eurotiomycetes*	*Chaetothyriales*	*Herpotrichiellaceae*	*Cladophialophora*	5	7
	OTU_44	*Ascomycota*	*Eurotiomycetes*	*Eurotiales*	*Trichocomaceae*	*Sagenomella*	10	8
	OTU_11	*Mortierellomycota*	*Mortierellomycetes*	*Mortierellales*	*Mortierellaceae*	*Mortierella*	1	6
Connectors	OTU_140	*Ascomycota*	*Eurotiomycetes*	Unassigned	Unassigned	Unassigned	4	5

Table S5 Summary of the basic taxonomies and the network connectivity of the putative keystone species in N addition network at short-term site

Network category	ID	Classification					No. module	Connectivity
		Phylum	Class	Order	Family	Genus		
Module hubs	OTU_3025	*Ascomycota*	Unassigned	Unassigned	Unassigned	Unassigned	10	8
	OTU_706	*Ascomycota*	*Eurotiomycetes*	*Eurotiales*	Unassigned	Unassigned	3	12
	OTU_439	*Ascomycota*	*Dothideomycetes*	*Pleosporales*	*Didymosphaeriaceae*	Unassigned	9	11
	OTU_27	*Basidiomycota*	*Agaricomycetes*	*Boletales*	*Boletaceae*	Unassigned	2	11
	OTU_63	*Ascomycota*	*Sordariomycetes*	*Hypocreales*	*Hypocreaceae*	*Hypocrea*	2	13
	OTU_1014	*Ascomycota*	*Eurotiomycetes*	*Chaetothyriales*	*Herpotrichiellaceae*	*Cladophialophora*	17	8
	OTU_135	*Ascomycota*	*Sordariomycetes*	*Ophiostomatales*	*Ophiostomataceae*	*Ophiostoma*	6	11
	OTU_87	*Ascomycota*	*Sordariomycetes*	*Hypocreales*	*Hypocreaceae*	*Hypomyces*	5	7
	OTU_73	*Ascomycota*	*Dothideomycetes*	*Venturiales*	Unassigned	Unassigned	4	13
Connectors	OTU_470	*Ascomycota*	*Sordariomycetes*	Unassigned	Unassigned	Unassigned	1	4
	OTU_874	*Ascomycota*	*Sordariomycetes*	*Chaetosphaeriales*	*Chaetosphaeriaceae*	Unassigned	0	4
	OTU_140	*Ascomycota*	*Eurotiomycetes*	Unassigned	Unassigned	Unassigned	8	4
	OTU_240	*Ascomycota*	*Leotiomycetes*	*Helotiales*	Unassigned	Unassigned	11	4
	OTU_115	Unassigned	Unassigned	Unassigned	Unassigned	Unassigned	7	3
	OTU_7	*Mortierellomycota*	*Mortierellomycetes*	*Mortierellales*	*Mortierellaceae*	*Mortierella*	3	5

Table S6 Summary of the basic taxonomies and the network connectivity of the putative keystone species in control network at long-term site

Network category	ID	Classification					No. module	Connectivity
		Phylum	Class	Order	Family	Genus		
Module hubs	OTU_2359	Unassigned	Unassigned	Unassigned	Unassigned	Unassigned	9	6
	OTU_302	*Ascomycota*	*Dothideomycetes*	*Venturiales*	Unassigned	Unassigned	4	5
	OTU_295	*Ascomycota*	*Sordariomycetes*	*Xylariales*	Unassigned	Unassigned	1	14
	OTU_183	*Ascomycota*	Unassigned	Unassigned	Unassigned	Unassigned	11	11
	OTU_276	*Basidiomycota*	*Agaricomycetes*	*Russulales*	*Russulaceae*	*Russula*	4	6
	OTU_12	*Basidiomycota*	*Agaricomycetes*	*Russulales*	*Russulaceae*	*Russula*	3	10
Connectors	OTU_737	*Ascomycota*	*Eurotiomycetes*	Unassigned	Unassigned	Unassigned	4	4
	OTU_359	*Basidiomycota*	*Agaricomycetes*	*Sebacinales*	*Sebacinaceae*	*Sebacina*	1	3
	OTU_3153	*Ascomycota*	Unassigned	Unassigned	Unassigned	Unassigned	11	5
	OTU_539	*Rozellomycota*	*Rozellomycota_cls_Incertae_sedis*	*GS11*	Unassigned	Unassigned	2	4
	OTU_223	*Ascomycota*	*Leotiomycetes*	Unassigned	Unassigned	Unassigned	11	6
	OTU_84	*Ascomycota*	*Leotiomycetes*	*Helotiales*	*Myxotrichaceae*	*Oidiodendron*	4	3
	OTU_66	*Ascomycota*	*Eurotiomycetes*	*Chaetothyriales*	*Herpotrichiellaceae*	*Cladophialophora*	1	3
	OTU_85	*Ascomycota*	*Sordariomycetes*	Unassigned	Unassigned	Unassigned	4	4
	OTU_4	*Basidiomycota*	*Tremellomycetes*	*Tremellales*	*Trimorphomycetaceae*	*Saitozyma*	0	13
Network hub	OTU_73	*Ascomycota*	*Dothideomycetes*	*Venturiales*	Unassigned	Unassigned	2	18

Table S7 Summary of the basic taxonomies and the network connectivity of the putative keystone species in N addition network at long-term site

Network category	ID	Classification					No. module	Connectivity
		Phylum	Class	Order	Family	Genus		
Module hubs	OTU_1359	*Ascomycota*	*Sordariomycetes*	*Hypocreales*	*Cordycipitaceae*	*Lecanicillium*	1	12
	OTU_703	Unassigned	Unassigned	Unassigned	Unassigned	Unassigned	1	15
	OTU_2555	*Ascomycota*	*Sordariomycetes*	Unassigned	Unassigned	Unassigned	2	7
	OTU_353	*Ascomycota*	*Eurotiomycetes*	*Chaetothyriales*	Unassigned	Unassigned	1	13
	OTU_147	*Ascomycota*	*Leotiomycetes*	*Helotiales*	*Hyaloscyphaceae*	Unassigned	0	38
	OTU_308	*Rozellomycota*	Unassigned	Unassigned	Unassigned	Unassigned	9	6
	OTU_47	Unassigned	Unassigned	Unassigned	Unassigned	Unassigned	6	7
	OTU_39	*Basidiomycota*	*Agaricomycetes*	*Agaricales*	*Hydnangiaceae*	*Laccaria*	0	38
	OTU_158	*Ascomycota*	*Eurotiomycetes*	*Chaetothyriales*	*Herpotrichiellaceae*	*Cladophialophora*	8	15
	OTU_86	*Ascomycota*	Unassigned	Unassigned	Unassigned	Unassigned	5	6
	OTU_385	*Ascomycota*	*Eurotiomycetes*	Unassigned	Unassigned	Unassigned	2	7
	OTU_28	*Basidiomycota*	*Agaricomycetes*	*Russulales*	*Russulaceae*	Unassigned	0	43
	OTU_3	*Basidiomycota*	*Agaricomycetes*	*Russulales*	*Russulaceae*	*Lactarius*	7	11
Connectors	OTU_1020	*Ascomycota*	*Eurotiomycetes*	*Eurotiales*	*Aspergillaceae*	*Penicillium*	2	3
	OTU_315	*Basidiomycota*	*Agaricomycetes*	*Agaricales*	*Tricholomataceae*	Unassigned	2	5
	OTU_670	Unassigned	Unassigned	Unassigned	Unassigned	Unassigned	2	3
	OTU_175	*Ascomycota*	Unassigned	Unassigned	Unassigned	Unassigned	2	3
	OTU_464	*Ascomycota*	Unassigned	Unassigned	Unassigned	Unassigned	8	4
	OTU_5094	*Ascomycota*	*Leotiomycetes*	Unassigned	Unassigned	Unassigned	3	8

Supplementary Figures

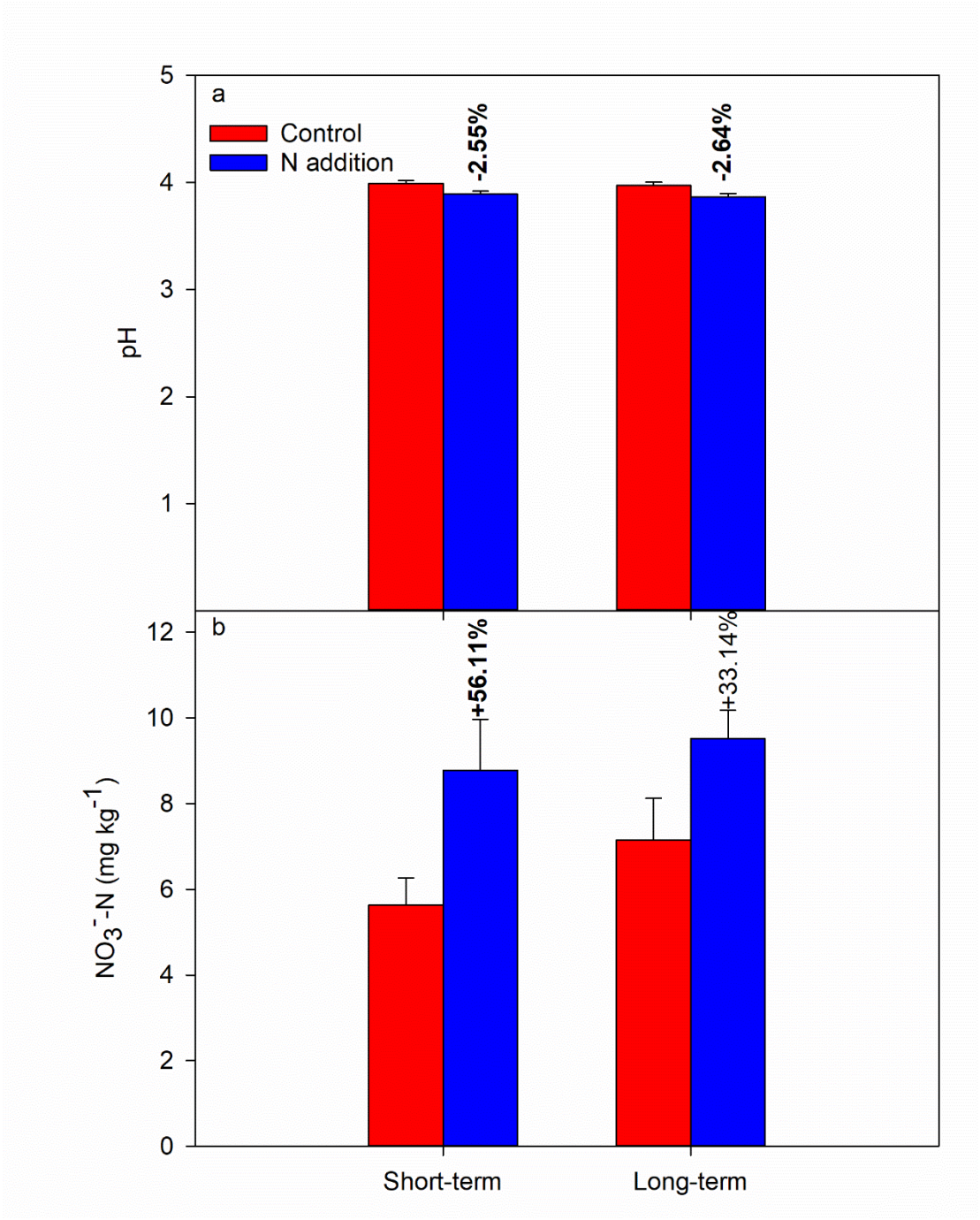


Fig. S1 The varaitions of soil pH (a) and concentrations of NO_3^--N (b) under N addition are indicated by student's t-test at short- and long-term sites, respectively. The bold numbers denote the significantly differences ($P < 0.05$) of aforementioned parameters between control and N addition at each site.

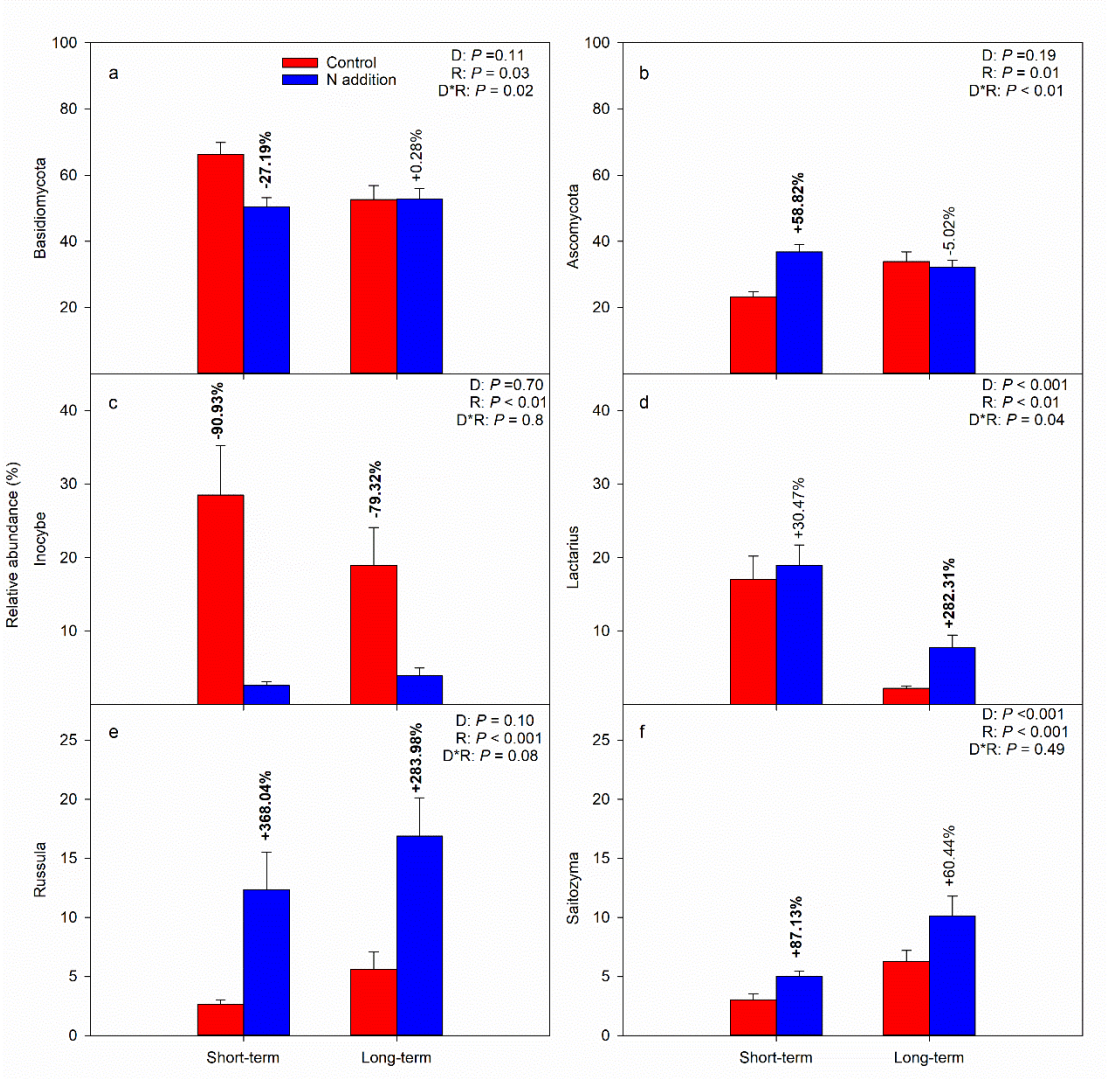


Fig. S2 The varaitions of relative abundance of major phyla (a, b), genera (c-f) under N addition are indicated by student's t-test at short- and long-term sites, respectively. The insert texts indicate the effects of duration (D), rate (R) of N addition and their interactions on the relative abundance of fungal major phyla and genera detected by two-way ANOVA. The bold numbers denote the significantly differences ($P < 0.05$) of aforementioned parameters between control and N addition at each site.

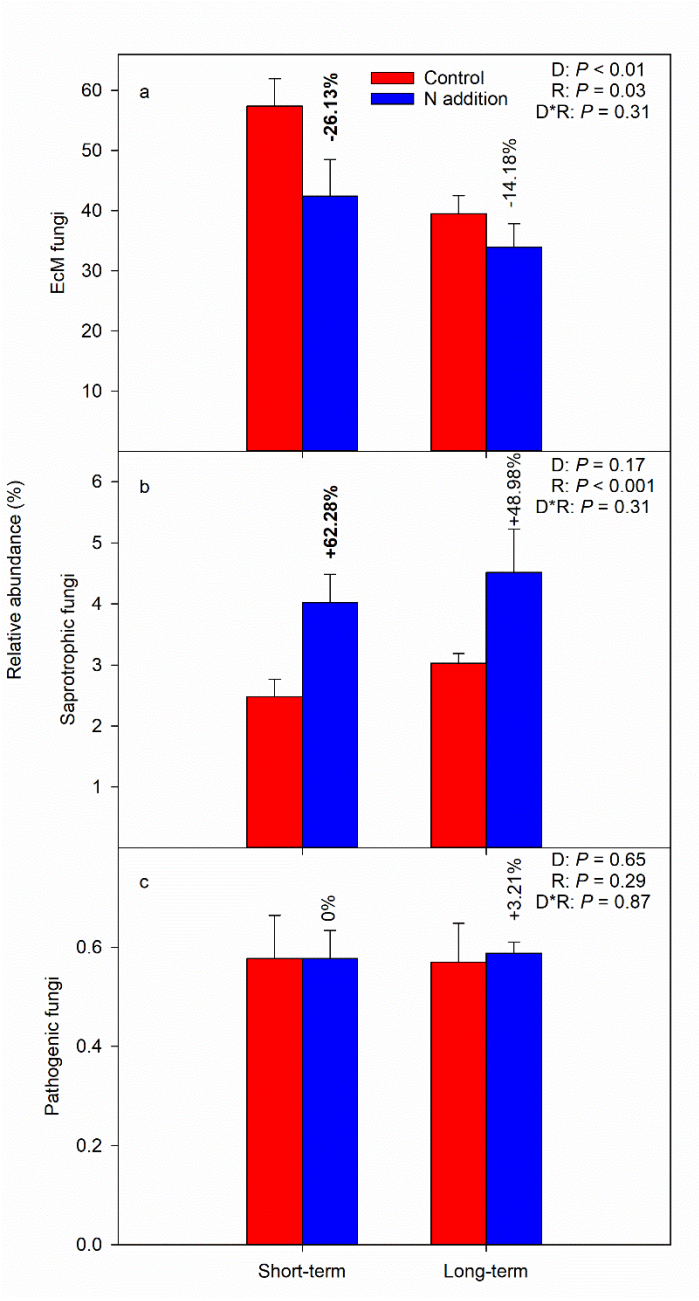
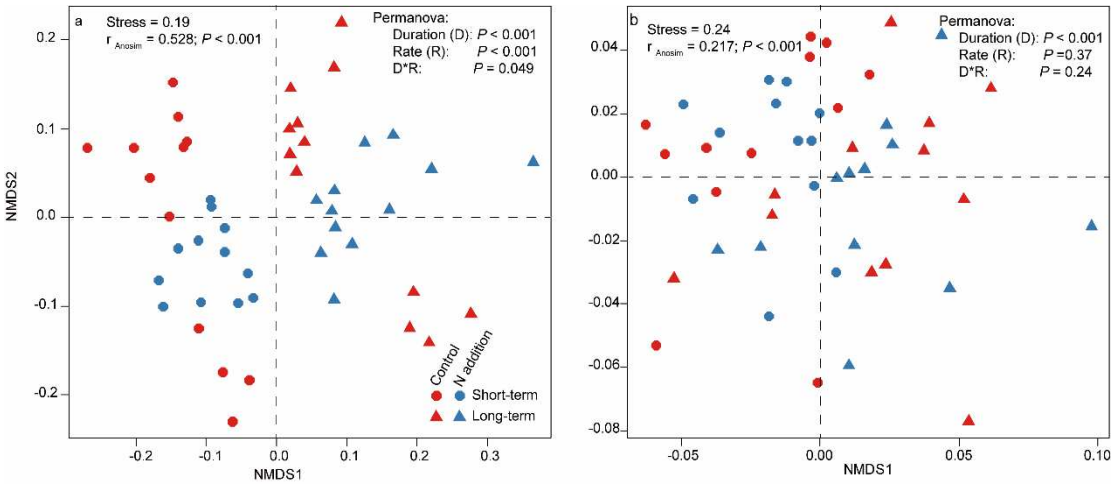


Fig. S3 The varaitions of relative abundance of major funtional guilds under N addition are indicate by student's t-test at short- and long-term sites, respectively. The insert texts indicate the effects of duration (D), rate (R) of N addition and their interactions on the relative abundance of fungal major funtional guilds detected by two-way ANOVA. The bold numbers denote the significantly differences ($P < 0.05$) of major funtional guilds between control and N addition at each site.



121

122 **Fig. S4** Non-metric multidimensional scaling (NMDS) plots based on Bray-Curtis

123 distance (a) and βMNTD (b) of fungal community. The results of Permanova show in

124 the graph to reveal the effects of duration (D), rate (R) of N addition and their

125 interactions on taxonomic and phylogenetic community dissimilarity.

126

127

128

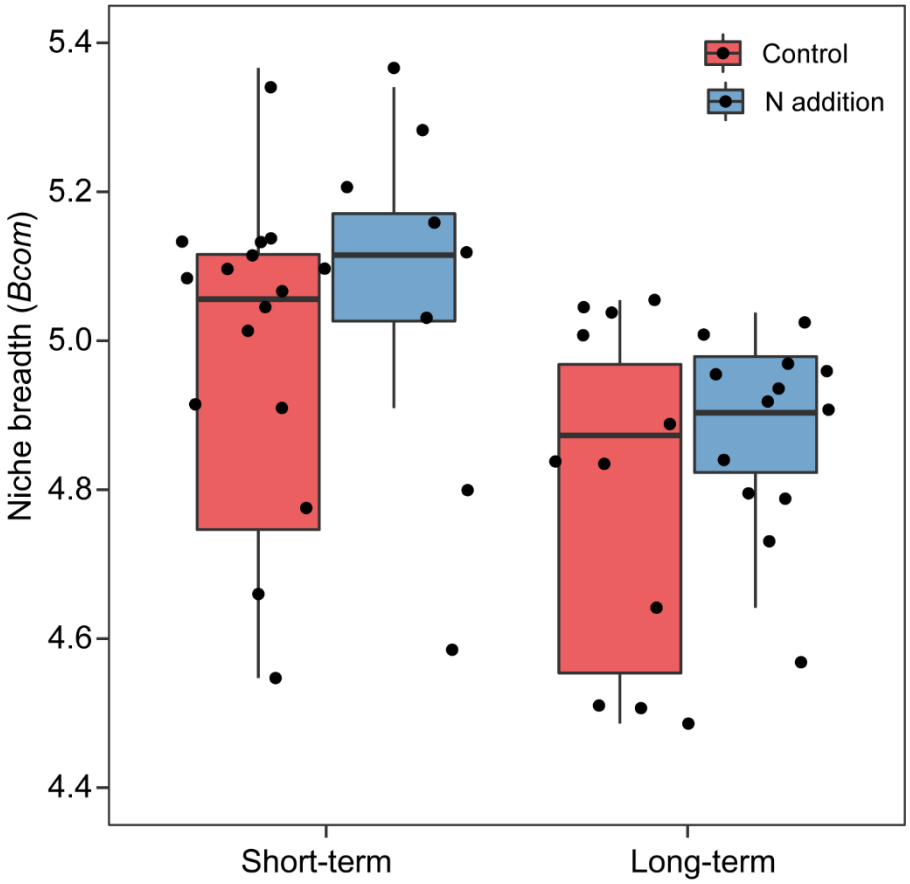
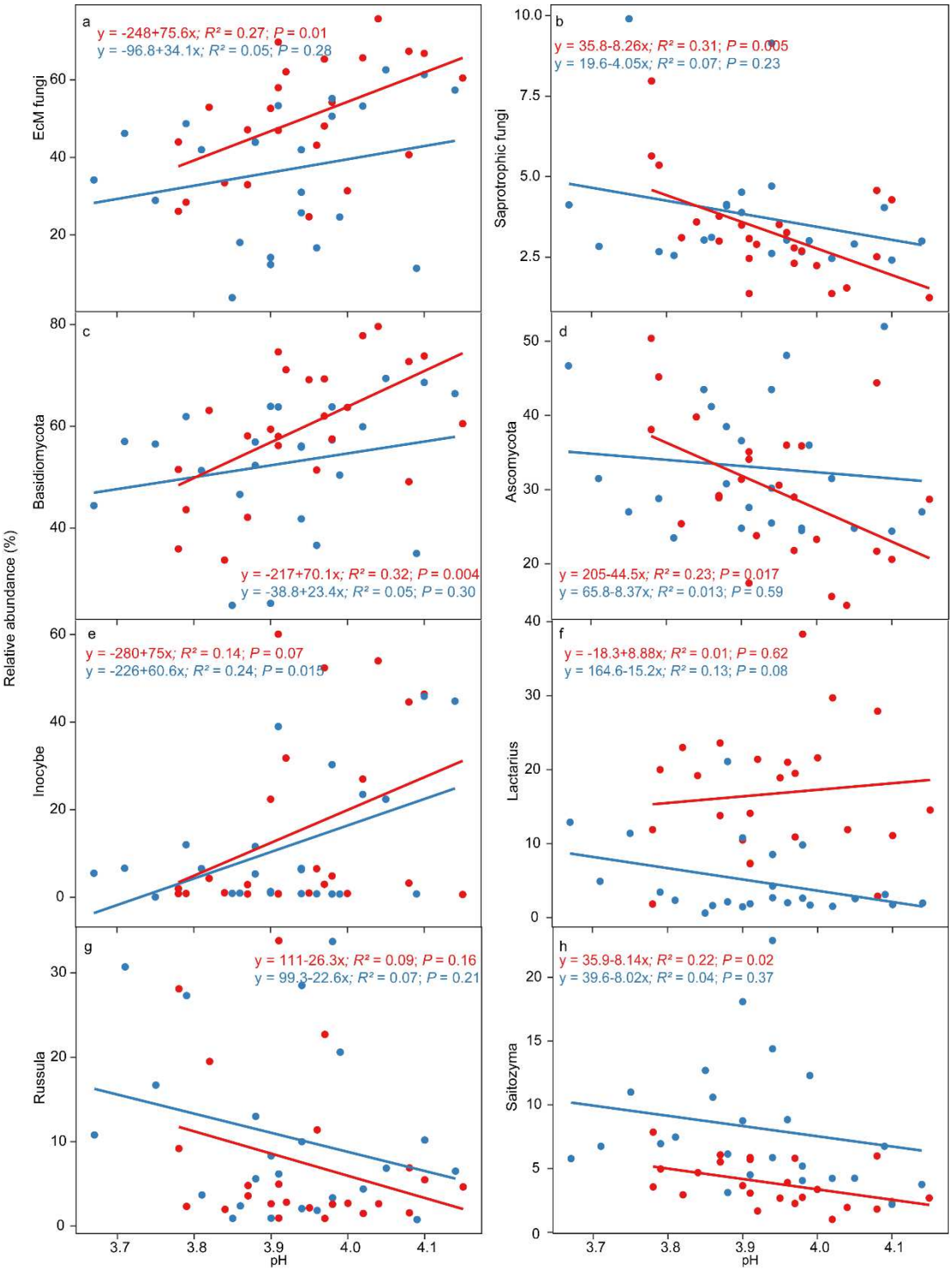


Fig. S5 Comparison of mean habitat niche breadths (*Bcom*) in all taxa of fungal community between control and N addition at short- and long-term site, respectively.



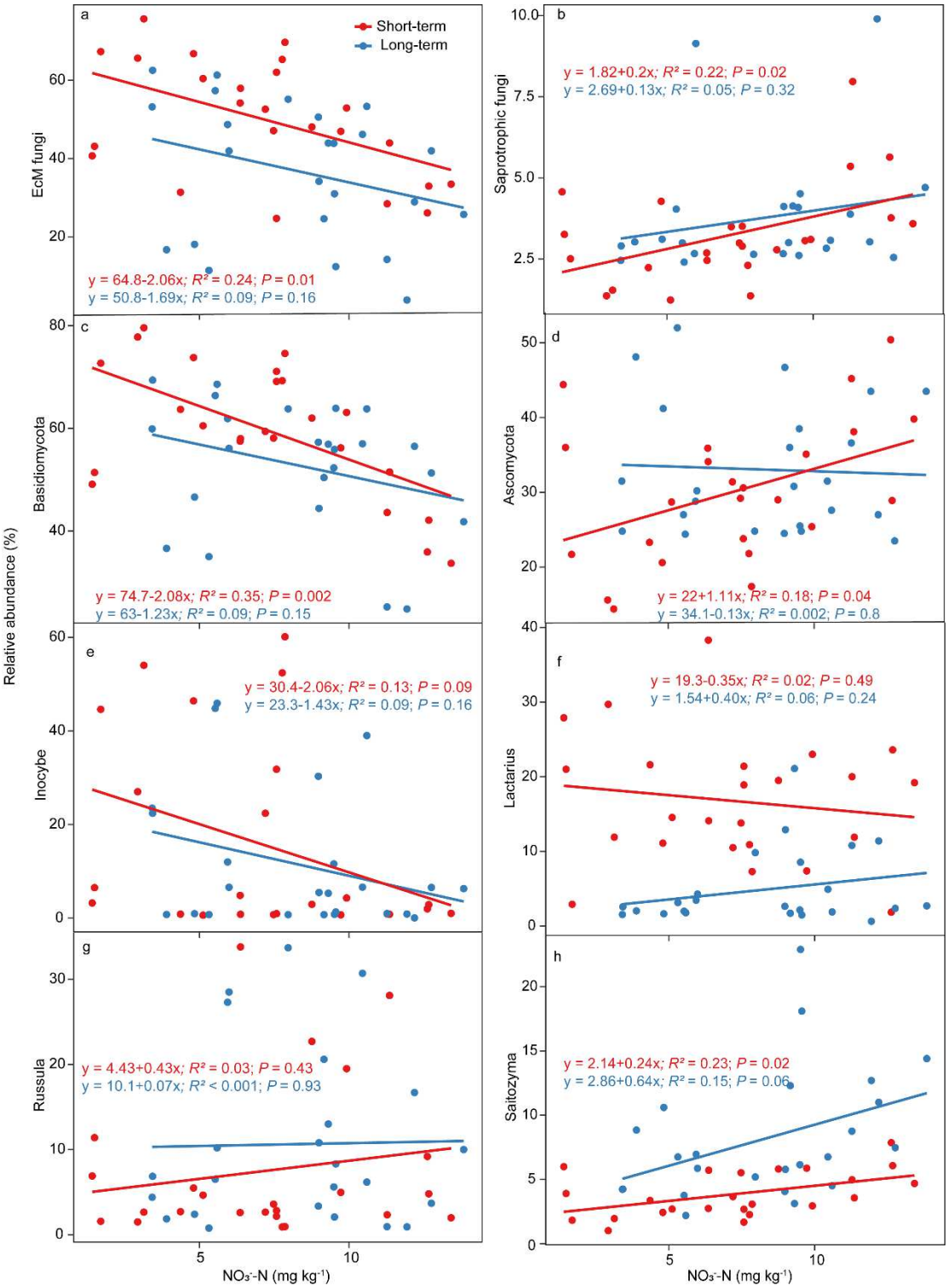
134

135 **Fig. S6** Relationships between soil pH and the relative abundance of major functional

136 guilds (a, b), phyla (c, d) and genera (e-h) are indicated by linear regressions model.

137

138



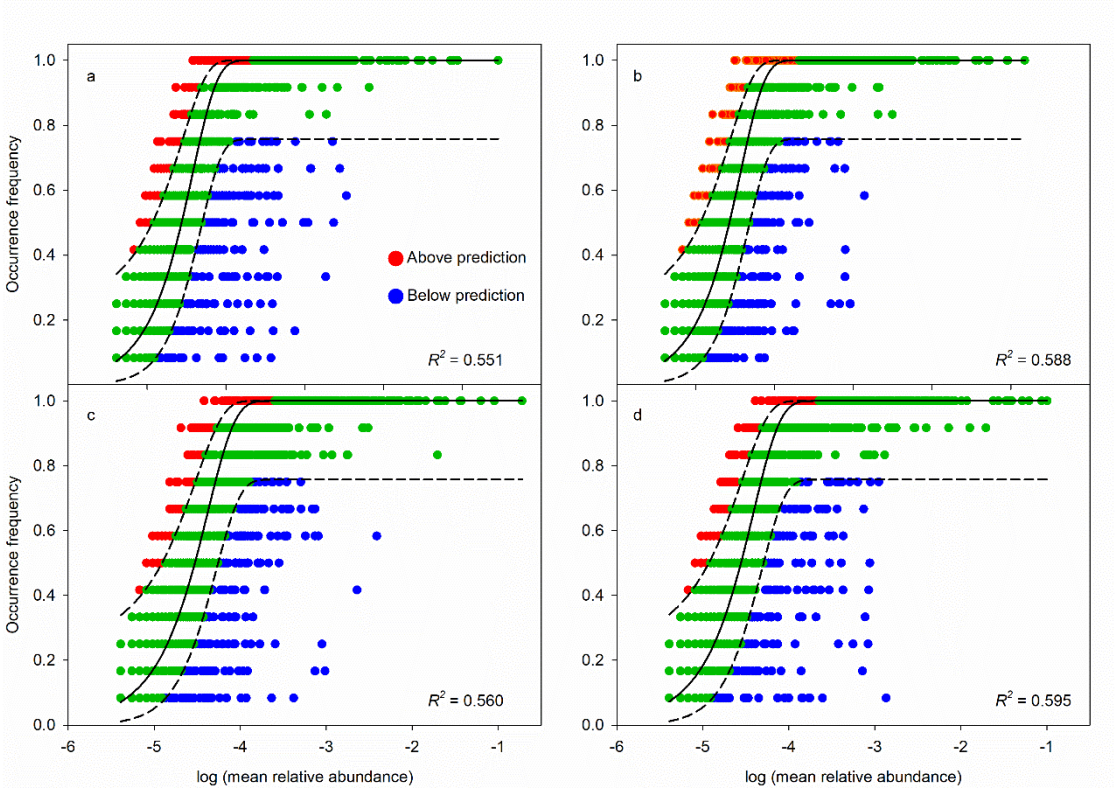
139

140 **Fig. S7** Relationships between soil NO_3^--N concentrations and the relative abundance

141 of major functional guilds (a, b), phyla (c, d) and genera (e-h) are indicated by linear

142 regressions model.

143



156

157 **Fig. S8** Fit of the neutral community model of community assembly for control (**a, c**)

158 and N addition (**b, d**) at the short- and long-term site, respectively. OTUs that

159 occurred more frequently than predicted by the model are shown in red, while those

160 that occurred less frequently than predicted are shown in blue. OTUs that occurred

161 within prediction are shown in green. Dashed lines represent 95% confidence

162 intervals around the model prediction (black line). R^2 indicate the fit to this model.

163

Figures

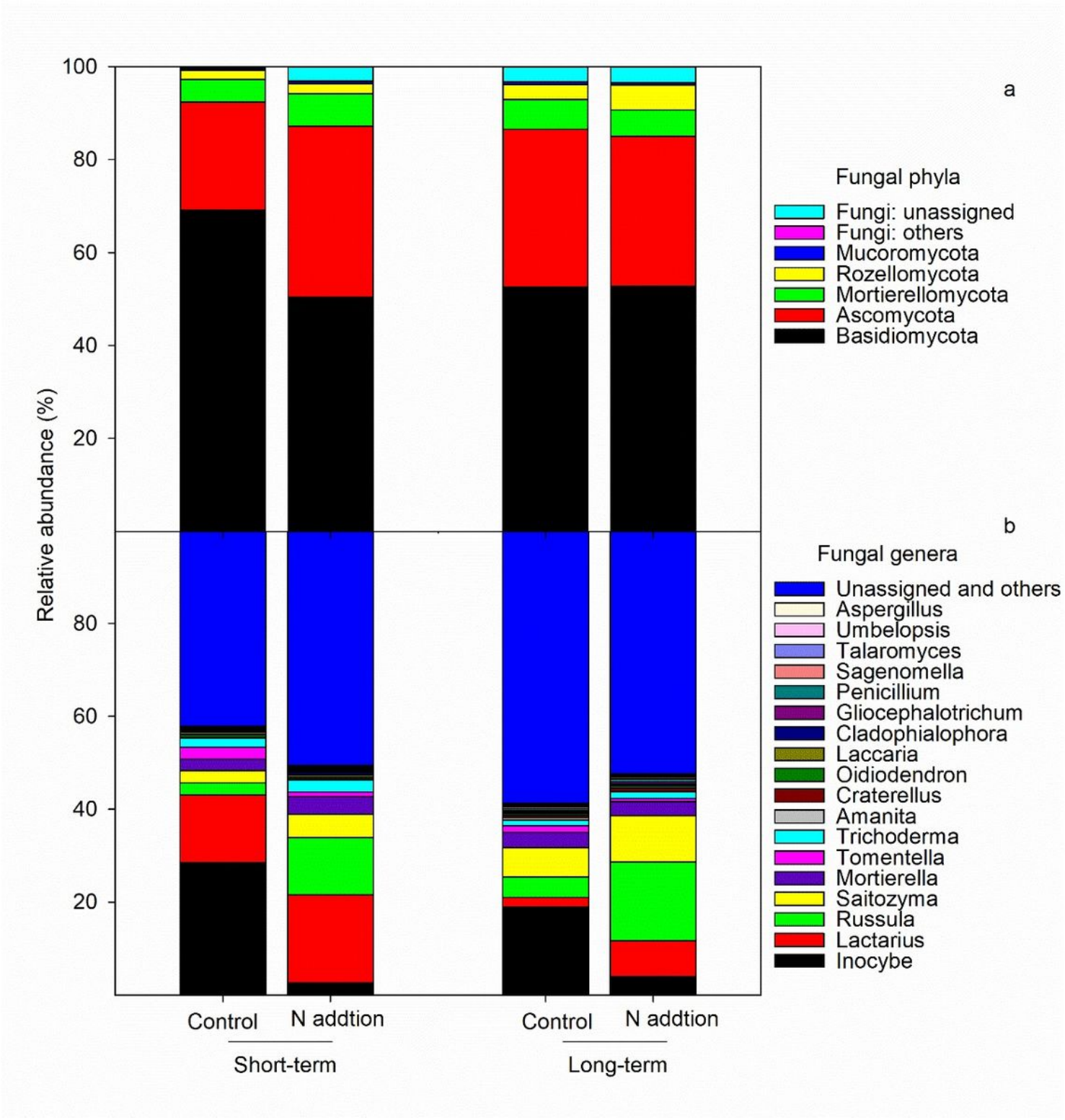


Figure 1

Relative abundance of major soil fungal phyla and genera at the short- and long-term N addition sites. Phyla and genera with the relative abundance > 0.2% are included.

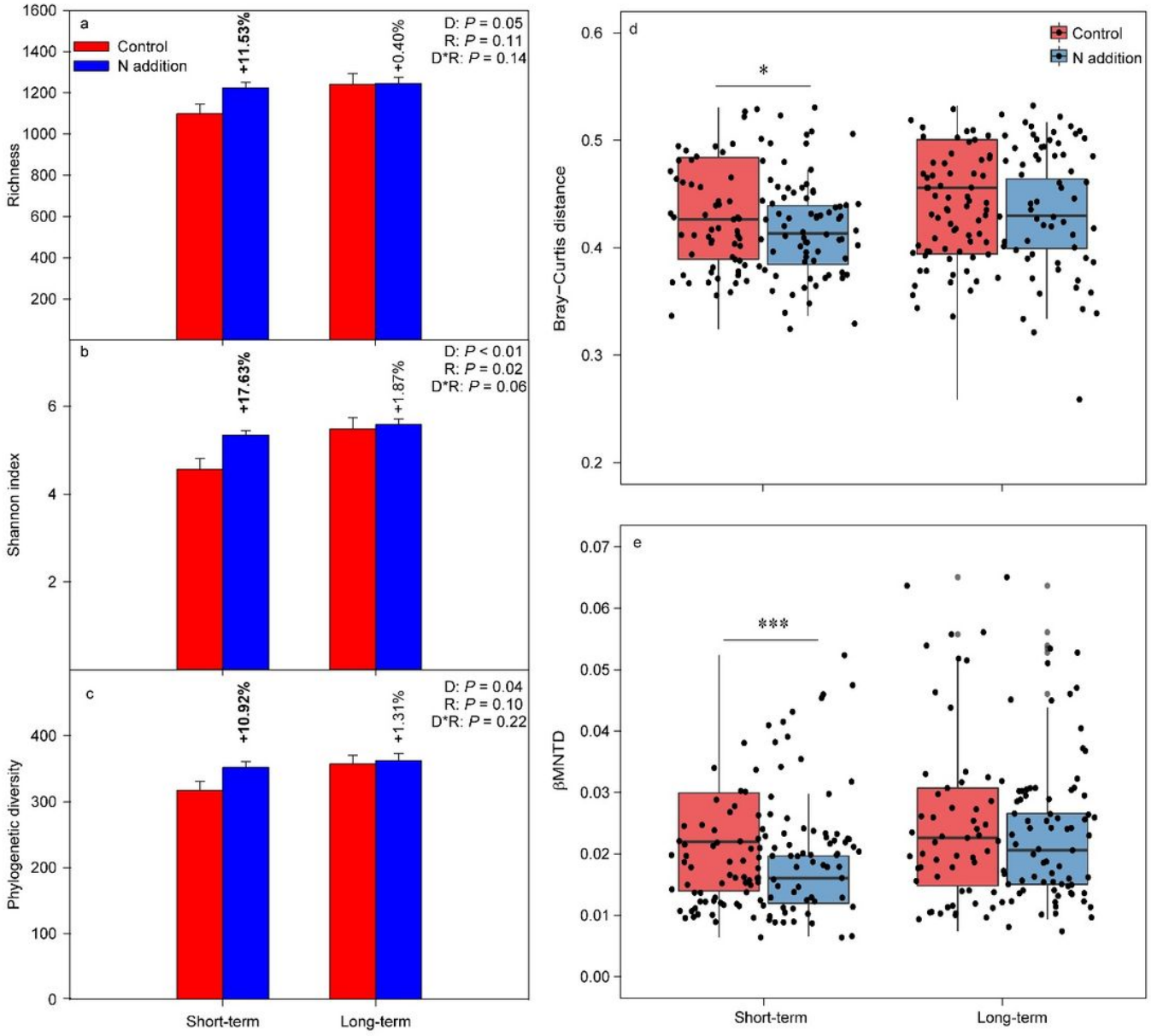


Figure 2

The varations of fungal α-diversity and community composition under short- and long-term N addition. (a-c) The varaitions of fungal α-diversity under N addition are indicated by student's t-test at short- and long-term sites, respectively. The bold numbers denote the significantly differences (P < 0.05) of fungal α-diversity between control and N addition at each site. The insert texts indicate the effects of duration (D), rate (R) of N addition and their interactions on fungal α-diversity detected by two-way ANOVA. (d-e) The boxplots show community dissimilarities between control and N addition in short- or long-term site based on Bray–Curtis distance and βMNTD, respectivly (*, P < 0.05; ***, P < 0.001; Wilcoxon rank-sum test).

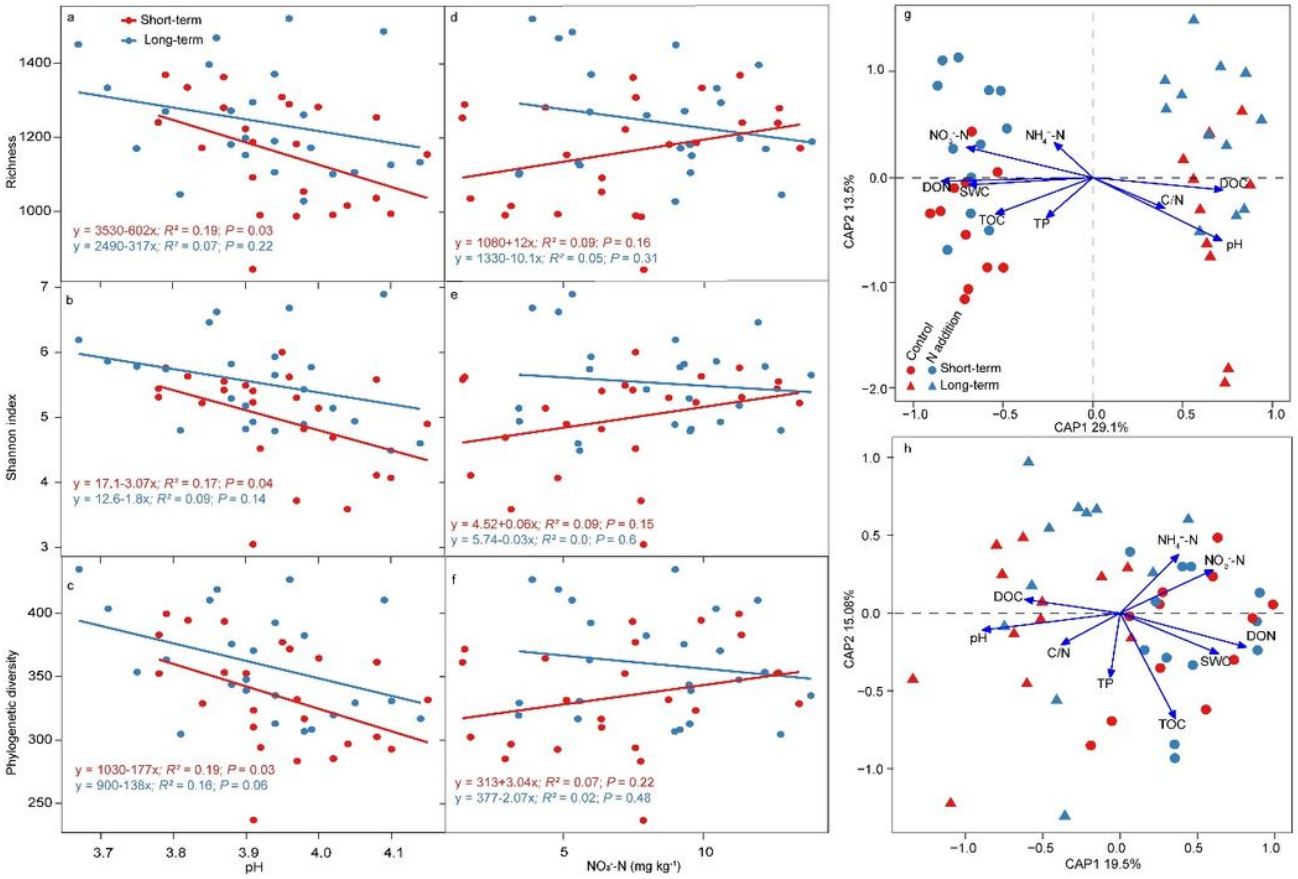


Figure 3

Factors affecting fungal diversity and community composition. (a-f) Relationships between fungal α-diversity and soil pH and NO3–N concentrations are revealed by linear regression model. (g, h) CAP analysis show the community dissimilarities based on Bray-Curtis distance and βMNTD against environmental variables among samples. SWC, soil water content; NH4+-N, ammonium nitrogen; NO3–-N, nitrate nitrogen; TOC, total organic carbon; TN, total nitrogen; C/N, total organic carbon / total nitrogen; TP, total phosphorus; DOC, dissolved organic carbon; DON, dissolved organic nitrogen.

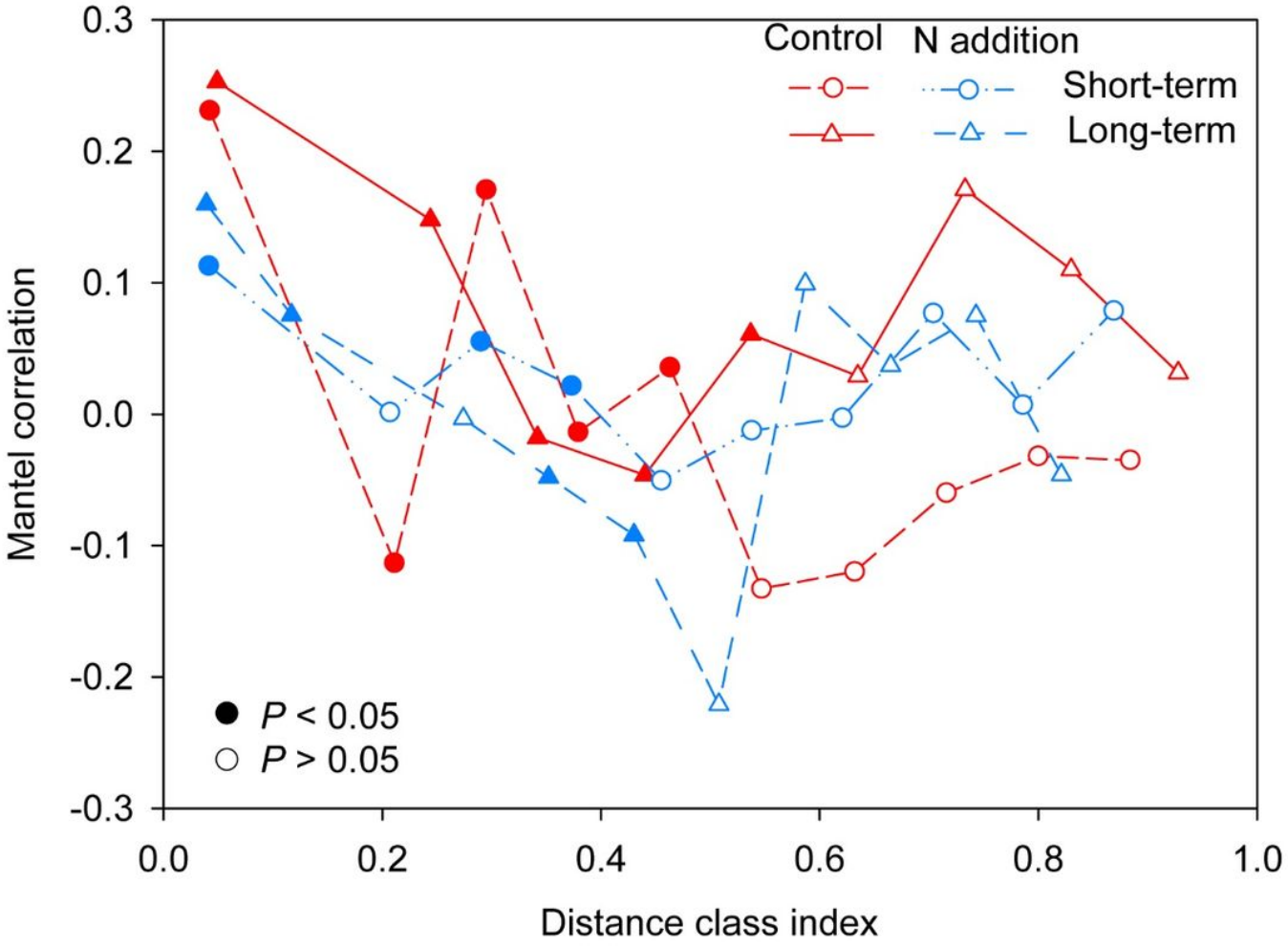


Figure 4

Phylogenetic Mantel correlogram between the Euclidean distance matrix of OTU niche values and phylogenetic distance matrix shows significant phylogenetic signal across short phylogenetic distances. Solid and open symbols denote significant (P < 0.05) and non-significant (P > 0.05) correlations (phylogenetic signal), respectively.

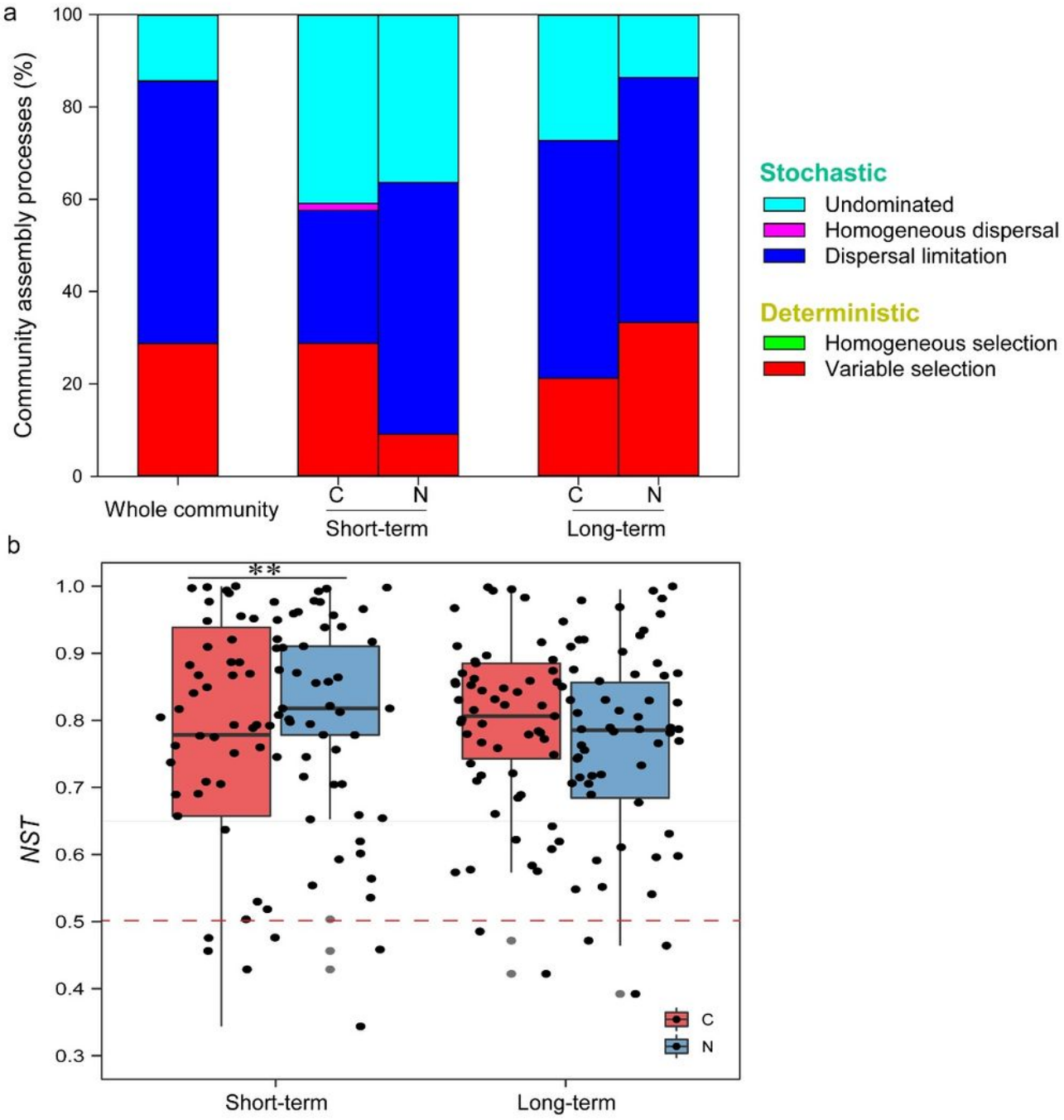


Figure 5

The assembly processes in shaping fungal communities. (a) The relative contributions (%) of the five community assembly processes. (b) Boxplot shows the variation in NST under control and N addition at short- and long-term sites (**, P < 0.01; Wilcoxon rank-sum test).

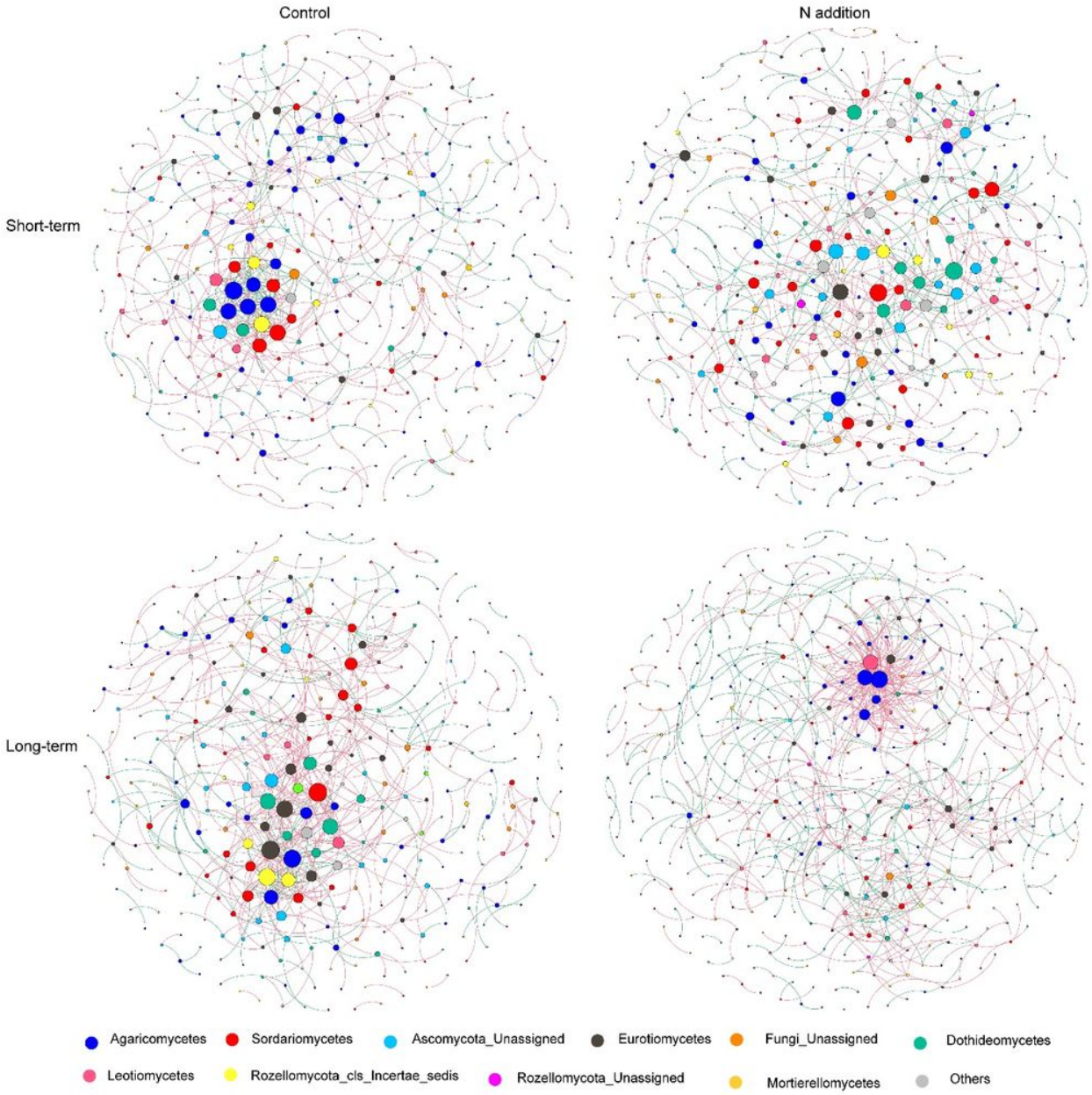


Figure 6

Co-occurrence patterns of OTUs of fungal community in control and N addition treatment at short- and long-term site, respectively. The size of each node is proportional to the number of connections (i.e. degree). The red and green edges indicate positive and negative interactions between two individual nodes, respectively.

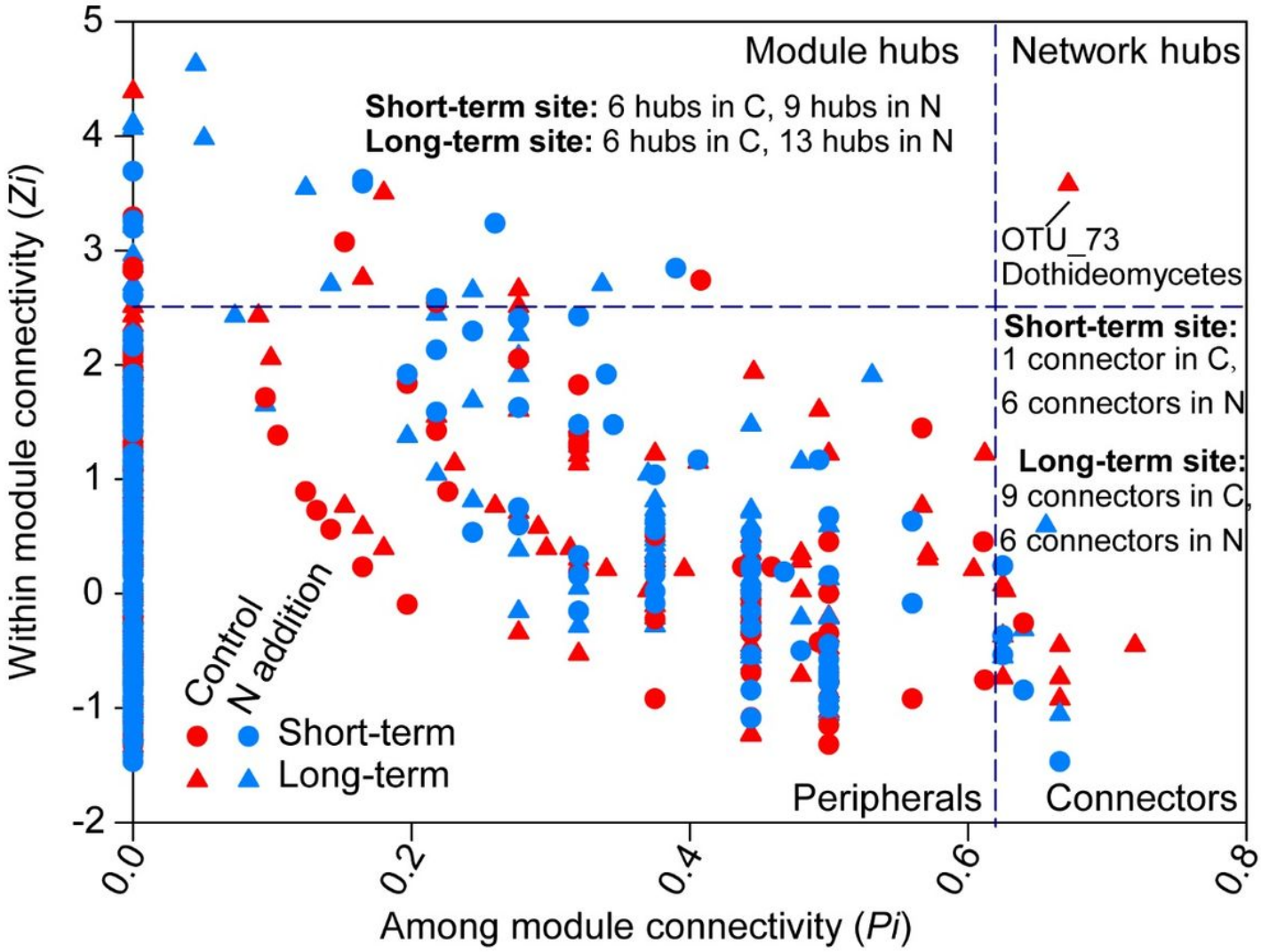


Figure 7

Classification of nodes to identify putative keystone species within the networks. The topological role of each OTU is determined according to the scatter plot of within module connectivity (Zi) and among module connectivity (Pi). Modules hubs have Zi > 5, whereas connectors have Pi > 0.62. The number of module hubs and connectors is presented on the plot and the network hub is labeled with the OTU number. The abbreviations of C and N are defined as control and N addition , respectively . Detailed taxonomic information for module hubs and module hubs and connectors connectors is shown in Tables S4 to S8