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Thirty four years of nitrogen fertilization decreases fungal diversity and alters fungal community composition in black soil in northeast China

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ABSTRACT

Black soil is one of the main soil types in northeast China, and plays an important role in Chinese crop production. However, nitrogen inputs over 50 years have led to reduced black soil fertility. It is unclear how N affects the fungal community in this soil type, so a long-term fertilizer experiment was begun in 1980 and we applied 454 pyrosequencing and quantitative PCR to targeted fungal ITS genes. There were five treatments: control (no fertilizer), N_1 (low nitrogen fertilizer), N_2 (high nitrogen fertilizer), N_1P_1 (low nitrogen plus low phosphorus fertilizers) and N_2P_2 (high nitrogen plus high phosphorus fertilizers). Soil nutrient concentrations (Total N, Avail N, NO_3^-, NH_4^+, etc.) and ITS gene copy numbers increased, whereas soil pH and fungal diversity decreased in all the fertilized treatments. Relationships between soil parameters and fungal communities were evaluated. Dothideomycetes, Eurotiomycetes, Leotiomycetes, Sordariomycetes, and Agaricomycetes were the most abundant classes in all soils. Principal coordinates analysis showed that the fungal communities in the control and lower-fertilizer treatments clustered closely and were separated from communities where more concentrated fertilizers were used. Fungal diversity and ITS gene copy number were dependent on soil pH. Our findings suggested that long-term nitrogen and phosphorous fertilizer regimes reduced fungal biodiversity and changed community composition. The influence of the more concentrated fertilizer treatments was greater than the lower concentrations.

1. Introduction

Nitrogen (N) inputs from anthropogenic sources are currently estimated to be 30%–50% greater than those from natural terrestrial sources and ten times greater than the anthropogenic inputs 100 years ago (Canfield et al., 2010). However, regular use of chemical fertilizers may result in a decrease in the soil nitrogen utilization efficiency (Singh et al., 2014) and may even impair soil quality and functionality (Fierer et al., 2012). N additions have resulted in a significant decrease in soil pH (Guo et al., 2010), which is the main predictor of bacterial diversity in soils (Zhalnina et al., 2015). Furthermore, these changes in soil quality are probably linked to the soil fungal community changes that mediate many ecological processes and influence plant growth and soil health (Klaubauf et al., 2010). The structure of soil fungal communities is the result of complex interactions among selection factors that may favor beneficial or detrimental relationships (Paungfoo-Lonhienne et al., 2015). Studies have shown that N fertilization decreases fungal biomass (Wallenstein et al., 2006), reduces diversity, and alters the community structure of fungi in soil (Edwards et al., 2011; Paungfoo-Lonhienne et al., 2015). An increase in N fertilizer addition was shown to increase the relative abundance of the phylum Ascomycota (Klaubauf et al., 2010), while simulated N deposition increased the proportion of basidiomycete sequences recovered from a forest floor, whereas the proportion of ascomycetes in the

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community was significantly lower under elevated N deposition (Edwards et al., 2011). Moreover, a seven year application of N and N plus phosphorus (P) fertilizer reduced ectomycorrhizal diversity (Wright et al., 2009). Soil pH has been shown to affect the alpha diversity (richness and phylodiversity within the community), but not beta diversity (the variation of community composition among different samples) of soil fungal community as altitude changes in a typical Tibetan forest ecosystem (Wang et al., 2015). However, whether the main drivers of fungal diversity are pH, nutrients, or other factors remains largely undetermined.

Chinese black soil belongs to the pachic Haploborolls subtype of Haploborolls in Borolls suborders. The so called 'Black soil' in China would better fit the Phaeozems than the Chernozems in the classification scheme of the World Reference Base (Yuri et al., 2011). The relatively high organic matter and clay content, the macroaggregate stability of the epipedon (about 70%) and the high cation exchange capacity, along with its favorable macronutrient status make the black soils fertile and productive (Xing et al., 2005). The black soil region occupies approximately 20% of the national arable land and contributes to up to 30% of the national staple food in China (Han et al., 2013) and it is thus considered the most important agricultural area for grain production and cultivation (Zhao et al., 2015). Extensive high inputs of inorganic fertilizers have occurred in this region which has seriously degraded the soil physicochemical properties and environmental health since large-scale reclamation in the 1950s (Yin et al., 2015). The nutrient-based alterations to associated microbiota were reflected in significant shifts in denitrifying bacteria communities (Fan et al., 2011; Yin et al., 2015) in black soil. Our previous study showed that long term N and N plus P fertilization reduced the bacterial diversity and 16S rRNA gene copy numbers, changed the community composition of bacteria in black soil, and that pH is the most important factor shaping the bacterial community structure (Zhou et al., 2015). However, the effect of long-term mineral fertilizer application on the fungal communities in the black soils of northeast China is not well understood.

To better understand how N and N plus P additions impact the fungal diversity and composition in black soil, since 1980 we have performed a manipulative field experiment with three N and N plus P addition levels in Harbin city in northeast China. Our objectives for this study were (i) to compare the changes in fungal community composition and diversity under different fertilization regimes, (ii) to determine the relationships between microbial diversity, dominant groups and soil parameters associated with these changes. To achieve our goals, we applied 454 pyrosequencing and quantitative PCR to characterize the fungal community, both quantitatively and qualitatively, and targeted the fungal internal transcribed spacer (ITS) gene. Given that inorganic nutrients from anthropogenic sources are predicted to increase in the agricultural system, understanding how these inputs will alter the soil fungal communities is critical if we are to accurately predict terrestrial ecosystem responses and identify approaches for ameliorating the negative effects.

2. Materials and methods

2.1. Site and soil characteristics

The experimental site and soils were described in greater detail in a previous study (Wei et al., 2008). The long-term fertilization experiment started in 1980 in Harbin city, Heilongjiang Province, P.R. China (45°40′N, 126°35′E) was used to evaluate the impact of fertilizers on plant productivity and soil properties including the diversity and composition of microbial communities in soil. Various combinations of inorganic fertilizers (N fertilizer was applied as urea, while the P fertilizer was calcium super phosphate and ammonium hydrogen phosphate) have been applied since the beginning of the experiment. There were five treatments in this study: CK (without fertilizer), N_1 (150 kg N ha^{-1} y^{-1}), N_2 (300 kg N ha^{-1} y^{-1}), N_1P_1 (150 kg N plus 75 kg P_2O_5 ha^{-1} y^{-1}), and N_2P_2 (300 kg N plus 150 kg P_2O_5 ha^{-1} y^{-1}). Each fertilization treatment had three replicated plots, with an area of 9 m × 4 m for each plot. Wheat, maize, and soybeans were continuously grown on the field before the long-term study began as described in our previous study (Wei et al., 2008). The wheat growing period was from early April to late September, and the samples of this study were collected in late September, 2013 after the wheat had been harvested. From each replicate plot, ten soil cores (5–25 cm depth, 10 cm diameter) were collected and pooled to minimize within-plot variation. After the soil was pooled in sterile plastic bags, they were taken back to the laboratory on ice. All the samples were sieved (2 mm), thoroughly homogenized, and divided into two subsamples: one was air-dried for soil analysis; the other was stored at −80 °C for DNA extraction and subsequent molecular analysis. The soil pH was determined using a glass combination electrode with a soil:water ratio of 1:1 (Li et al., 2013); total nitrogen (TN) was determined according to Strickland and Sollins (1987); soil KCl-extractable NO_3^- and NH_4^+ were determined by extraction with 2 M KCl, steam distillation and titration (Mulvaney, 1996); available N was determined according to the Kjeldahl method (Bremner and Mulvaney, 1982); total organic carbon (TOC) was determined using a Shimadzu TOC-5000 analyzer (Shimadzu, Kyoto, Japan); available phosphorus was assayed according to the method described by Olsen et al. (1954); and available K was determined by the ammonium acetate method (Helmke and Sparks, 1996).

2.2. Quantitative PCR

The total DNA was extracted from 0.25 g of soil using the PowerSoil DNA Isolation Kit (MOBIO Laboratories Inc., Carlsbad, CA, USA) according to the manufacturer's protocol. To minimize the DNA extraction bias, three successive extractions of microbial DNA from the same soil samples were combined and purified using a DNeasy Tissue kit (Qiagen, Valencia, CA, USA) (Guo et al., 2012). The qPCR method was used to measure the abundance of the soil fungi with the primer set (target for fungal ITS gene): ITS4 (TCCTCCGCTTATTGATATGC) and ITS5 (GGAAGTAAAAGTCGTAACAAGG) (Schoch et al., 2012). The reactions were carried out using an ABI Real-Time 7500 system (Applied Biosystems, Waltham, MA, USA) and SYBR Green detection (FastFire qPCR PreMix, TIANGEN BIOTECH, China). The standard for measuring the ITS gene quantity was developed from a clone with the correct insert. A plasmid DNA preparation was obtained from the clone using a Miniprep kit (Qiagen, Germantown, MD, USA). The R^2 of the standard curve was >0.99. The qPCR reactions were run in triple replicates with the DNA extracted from each soil sample. The abundances of the bacterial 16S rRNA gene copies were quantified using the same method as for the ITS gene. The bacteria to fungi ratio was calculated using the 16S rRNA and ITS gene copies (Wurzbacher et al., 2014).

2.3. Barcoded pyrosequencing

Amplicon libraries were produced by using the tagged fungal specific primers ITS1F (5′- CTTGGTCATTTAGAGGAAGTAA -3′) and ITS2R (5′-TCCTCCGCTTATTGATATGC-3′) (Lai et al., 2007), designed for the ITS region and for tag-encoded 454 GS-FLX amplicon pyrosequencing, as recommended by Abarenkov et al. (2010b). The PCR amplification procedure was as follows: 5 min at 94 °C, followed by 32 cycles of 1 min at 94 °C (denaturation), 45 s at 59 °C

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(annealing), and 1 min at 72 °C (extension), followed by the final extension step of 7 min at 72 °C. Pyrosequencing was performed using the 454 Genome Sequencer FLX Titanium platform (Roche, IN, USA) by the Research and Testing Laboratory (Personalbio, China). Fungal ITS sequence analysis was carried out in Mothur 1.31.1(Schloss et al., 2012). In brief, sequences outside of the range of 200–800 bases long were trimmed, and then assigned to 15 soil samples based on unique 7 bp barcodes. Reads with quality scores lower than 20, ambiguous bases, and improper primers were discarded before clustering. The resultant high-quality sequences were then clustered into operational taxonomic units (OTUs) at 97% similarity using UPARSE (Edgar, 2013). The UNITE + INSDC fungal ITS database (Abarenkov et al., 2010a) was used as a reference for classification. Sequence data were uploaded to the NCBI Sequence Read Archive under accession number SRX766215.

2.4. Statistical analysis

Alpha-diversity was assessed by calculating the ACE, Chao1, Shannon, and Simpson index values. The differences in soil properties, ITS gene copies and alpha-diversity among samples were tested using a one-way analysis of variance (ANOVA), and paired comparison of treatment means was achieved by Tukey's procedure using SPSS 19.1 statistical software (SPSS, Chicago, IL, USA) (Ahn et al., 2012). Spearman correlation coefficients between soil properties and the abundant phyla (classes and genera) were calculated using SPSS 19.1. In all tests, a P-value <0.05 was considered to be statistically significantly. Weighted Fast UniFrac distances between the samples were calculated and PCoA was performed on the basis of the distance measured. The coordinates were used to draw 2D graphical outputs.

3. Results

3.1. Long-term fertilization changed soil properties

The 34-year application of N and P significantly increased the soil total N, available N, available P, TOC contents ($P < 0.05$, Table 1), and wheat yield (2140–3352 kg ha^{-1} year^{-1}). However, 34 years of fertilization significantly reduced the soil available N:P ratio and soil pH steadily decreased with increasing fertilizer inputs from 6.36 to 4.64 (Table 1). The higher-mineral fertilizer treatments (i.e., N_2 and N_2P_2) had a lower pH and higher soil nutrient levels than the lower-mineral fertilizer treatments (i.e., N_1 and N_1P_1). Furthermore, wheat yield was significantly and positively correlated with TN and available P concentrations, but negatively correlated with the available N:P ratio ($P < 0.05$) (Table 2).

Effects of long-term fertilization on fungal ITS gene copy numbers and the bacteria-to-fungi ratio.

The 34-year fertilization regimes significantly ($P < 0.05$) increased the abundance of the soil fungi community according to the qPCR analysis of the fungal ITS gene (Fig. 1A). The ITS gene copy numbers ranged from 2.0×10^5–4.8×10^5 (per gram soil). The ITS gene copy numbers were negatively correlated (Spearman's rank correlation) with soil pH ($F = -0.661$, $P < 0.05$), and positively correlated with the NH_4^+ ($F = 0.727$, $P < 0.01$), NO_3^- concentrations ($F = 0.587$, $P < 0.05$), the C: N ratio ($F = 0.629$, $P < 0.05$), and TOC ($F = 0.675$, $P < 0.01$) in all experimental soils (Table S1). However, the bacteria-to-fungi ratio decreased significantly in all fertilized treatments (Fig. 1B). A significant rise in the bacteria-to-fungi ratio was observed with increasing soil pH ($F = 0.815$, $P < 0.01$), while an opposite trend was detected for soil TN, NH_4^+ and NO_3^-, and TOC concentrations, and the C: N ratio (Table S1).

Table 2
Spearman correlations of wheat yield and alpha diversity to soil properties.

Soil properties	Chao1	ACE	Simpson	Shannon	Wheat yield
pH	−0.171	−0.261	**−0.705****	**0.819****	−0.327
Total N	−0.114	0.063	**0.595***	**−0.825****	**0.587***
NH_4^+	0.183	0.252	**0.562***	**−0.611***	−0.071
NO_3^-	0.129	0.173	**0.594***	**−0.648****	0.124
Available N	−0.158	0.087	0.283	**−0.633***	0.414
Available P	−0.016	0.226	0.017	−0.395	**0.753****
TOC	−0.284	−0.103	0.310	**−0.591****	−0.030
Available K	0.082	−0.147	0.340	−0.021	−0.498
N: P	0.096	−0.141	−0.069	0.459	**−0.827****
C: N	0.130	0.068	**0.542***	−0.495	0.123

Soil factors indicated include Avail N (Available N), Avail P (Available P), Avail K (Available K), TOC (Total Organic Carbon), N: P (Available N: Available P), C: N (Total Organic Carbon: Available N).
**$P < 0.01$, *$P < 0.05$.

3.2. Long-term fertilizer application decreased fungal diversity

A total of 174,379 high quality sequences (71% of the total 246,462) were obtained in the fungal community analysis of the 15 soil samples and the mean read length was 575 bp. The Good's coverage values ranged between 0.98 and 0.99 with a 97% similarity cutoff, which indicated that the current numbers of sequence reads were sufficient to capture the fungal diversity in these soils. The numbers of OTUs in the 15 samples ranged between 356 and 420 (Table S2).

There were significant differences in microbial diversity ($P < 0.05$) for the Shannon and Simpson indexes. The Shannon index was lower and the Simpson index was higher for all fertilized treatments than for the unfertilized plots, which confirmed that the fungal biodiversity decreased after 34 yr N and N plus P fertilizer regimes. However, there was no significant difference in the Shannon and Simpson index between the four fertilized treatments (Table S2). Furthermore, there was no significant difference in fungal richness (ACE and Chao1), coverage and OTUs between unfertilized and fertilized treatments (Table S2). The relationships between α-diversity and soil properties are shown in Table 2. The

Table 1
Properties of soil samples under different fertilizer treatments.

Fertilizer regimes	pH (1:1H_2O)	Total N (g kg^{-1})	Avail N (mg kg^{-1})	NO_3^- (mg kg^{-1})	NH_4^+ (mg kg^{-1})	Avail P (mg kg^{-1})	TOC (mg kg^{-1})	N: P	C: N	Avail K (mg kg^{-1})	Wheat yield (kg ha^{-1})
CK	6.36 (0.03) d	1.20 (0.01) a	118.5 (4.0)a	7.68 (0.05)a	33.82 (1.09)a	9.29 (0.31)a	56.7 (2.1)a	12.8 (0.1)d	47.3 (2.2)a	176.6 (7.9)a	1548.1 (280.1)a
N_1	5.64 (0.03) c	1.28 (0.01) b	127.1 (0.1)b	8.47 (0.06)b	36.91 (2.51)ab	10.89 (0.64)b	65.3 (6.0)a	11.7 (0.7)c	50.96 (4.7)a	183.2 (20.4)ab	2140.0 (230.5)b
N_2	4.64 (0.03) a	1.42 (0.03) c	160.2 (3.1)d	24.54 (0.08)e	40.75 (1.15)c	15.30 (0.31)c	108.6 (4.1)c	10.5 (0.4)b	76.5 (1.1)c	216.6 (39.9)b	2155.2 (248.1)b
N_1P_1	5.59 (0.08) c	1.38 (0.04) c	139.9 (2.8)c	10.50 (0.06)c	34.77 (0.54)ab	70.88 (0.83)d	62.0 (0.5)a	2.0 (0.1)a	44.98 (0.99)a	152.2 (9.2)a	3352.0 (244.7)d
N_2P_2	4.79 (0.03) b	1.38 (0.03)c	157.3 (4.3)d	22.29 (0.23)d	39.07 (1.74)bc	84.97 (0.72)e	88.6 (8.6)b	1.9 (0.1)a	64.19 (7.3)b	143.8 (6.7)a	2617.0 (329.5)c

Values are mean ± standard deviation ($n = 3$). Values within the same column followed by the different letters indicate significant differences ($P < 0.05$).
Fertilizer regimes: CK (without fertilizer), N_1 (150 kg N ha^{-1}), N_2 (300 kg N ha^{-1}), N_1P_1 (150 kg N ha^{-1} plus 75 kg P_2O_5 ha^{-1}), N_2P_2 (300 kg N ha^{-1}plus 150 kg P_2O_5 ha^{-1}).
Soil factors indicated include Avail N (Available N), Avail P (Available P), Avail K (Available K), TOC (Total Organic Carbon), N: P (Available N: Available P), C: N (Total Organic Carbon: Available N).

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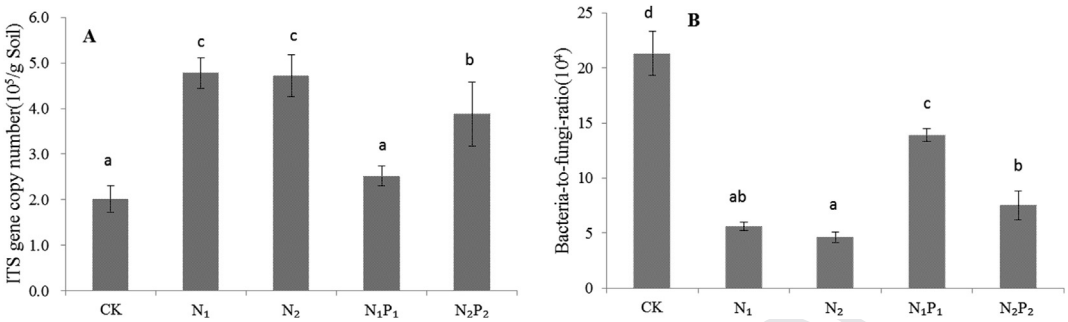

Fig. 1. Results of the quantitative PCR (qPCR). (A) The abundance of fungi as indicated by the number of ITS copies measured using qPCR. Same letters above columns denote no significant difference ($P < 0.05$, Tukey's test); (B) Bacteria-to-fungi ratio in different treatments.

Simpson index was negatively associated with soil pH and positively correlated with soil TN, NH_4^+, NO_3^- and the C:N ratio. The Shannon index significantly decreased as the pH value fell ($F = 0.819$, $P < 0.01$), while an opposite trend was detected for soil TN, NH_4^+, NO_3^-, and TOC concentrations (Table 2).

3.3. Fungal community composition

The relative abundances of the four most represented phyla (ITS > 0.1% of total reads) in the 15 samples are shown in Fig. 2A. Twelve abundant classes and 34 genera (with a relative abundance of ITS sequences higher than 0.1%) were identified (Fig. 2B and C). All 15 soils are dominated by Ascomycota, which are represented by 61.7–78.2% of the sequences, followed by Basidiomycota (7.1–20.6%), Zygomycota (2.4–4.6%) and Chytridiomycota (0.6–2.8%). Phylum Glomeromycota was only detected occasionally at low frequency.

There were significant ($P < 0.05$) differences between treatments for phyla Ascomycota, Basidiomycota, and Chytridiomycota, but there was no significant ($P > 0.05$) difference between treatments for phylum Zygomycota (Table S3). The relative abundance of Ascomycota was lower while that of Basidiomycota was higher in the N_2 treated plot than the other four plots (Table S3). The most abundant classes for all soils were Sordariomycetes and Dothideomycetes (Fig 2B).

The relative abundances of the genera *Cyphellophora*, *Penicillium*, *Chloridium*, *Trichoderma*, and *Acremonium* increased in all fertilizer treatments, compared to the unfertilized controls (Fig. 3A–E); and the relative abundance of those five genera increased to a greater extent in the N_2 and N_2P_2 treatments than in the N_1 and N_1P_1 treatments (Fig. 3A–E). Furthermore, the relative abundance of genera *Exophiala*, *Clonostachys*, and *Sarocladium* decreased in the N_1 and N_1P_1 treatments, but increased in the N_2 and N_2P_2 treatments (Fig. 3F–H). For N_2 and N_2P_2, the relative abundance of genera *Schizothecium*, *Magnaporthe*, and *Phaeosphaeriopsis* decreased to a greater extent than in the N_1 and N_1P_1 treatments (Fig. 3J–L). Notably, the relative abundance of genera *Coprinellus* and *Stropharia* in the unfertilized control plots were 2.5% and 2.0%, respectively, but they could not be detected in any of the fertilized treatments (Fig. 3I).

3.4. OTU-level fungal β-diversity analysis

We used PCoA of the weighted Unifrac distances to examine the association between fungal community and fertilizer (Fig. 4). The first two principal coordinates represented 43.86% (PC1) and 19.99% (PC2) of the variation in the fungal communities (Fig. 4). There were obvious separate groups in each of the samples. PC1 generally distributed the fungal communities along with soil pH. The fungal communities from plots with a relatively acidic pH (4.64 and 4.79) and the higher N inputs (i.e., N_2 and N_2P_2) were generally located in the right part; whereas those from plots with a slightly higher pH (5.59 and 5.64) and a lower N addition (i.e., $N_1$and N_1P_1) were in the left. The unfertilized control, with the highest pH (6.36), was in the far left (Table 1, Fig. 4). These results show that the fungal communities were affected by long-term N addition and soil pH was associated with their variation, other chemical properties showed no apparent pattern in this analysis. Furthermore, an obvious difference was also observed between the communities in the N_2 and N_2P_2 plots, indicating that high P fertilizer inputs also had important effects on the fungal community composition.

4. Discussion

This study explored the fungal communities in wheat field soils under long-term fertilizer treatment. Combined ITS gene abundance and fungal community composition analyses showed that fertilizer regimes had great impacts on the abundance, diversity, and composition of fungi.

4.1. ITS gene abundance

Quantitative PCR assays revealed that fungal ITS gene abundancies in the fertilized plots ranged from 2.5×10^5 to 4.8×10^5 g^{-1} soil and were significantly higher ($P < 0.05$) than those in non-fertilized soils (2.0×10^5 g^{-1} soil), which showed that N and P fertilization had a mildly stimulatory effect on the fungal populations, although the fertilization regimes significantly decreased soil pH. The reason is that fungi have a competitive advantage over many bacteria because of their ability to import N and P (Wu, 2011; Chen et al., 2014). The results showed that fungal growth was high at almost all pHs and this was in agreement with previous physiological studies that fungal species typically have a wide pH optimum, often covering 5–9 pH units without significant inhibition of their growth (Rousk et al., 2010). Furthermore, the observed increase in fungal ITS gene copies as the C:N ratio rose in the soil (Table S1) was not surprising because many fungal species are better adapted to N limitation than bacteria (Nilsson et al., 2012), a fact that is reflected in generally higher C:N ratios in fungi than in bacteria.

4.2. Bacteria-to-fungi ratio

In this study, we found that long term N and P inputs reduced the bacteria-to-fungi ratio, which was not in agreement with previous conclusions that high N deposition increased bacteria-to-fungi ratios (Zechmeister-Boltenstern et al., 2011). Here, the decrease in the ratio of bacteria-to-fungi was most likely due to the

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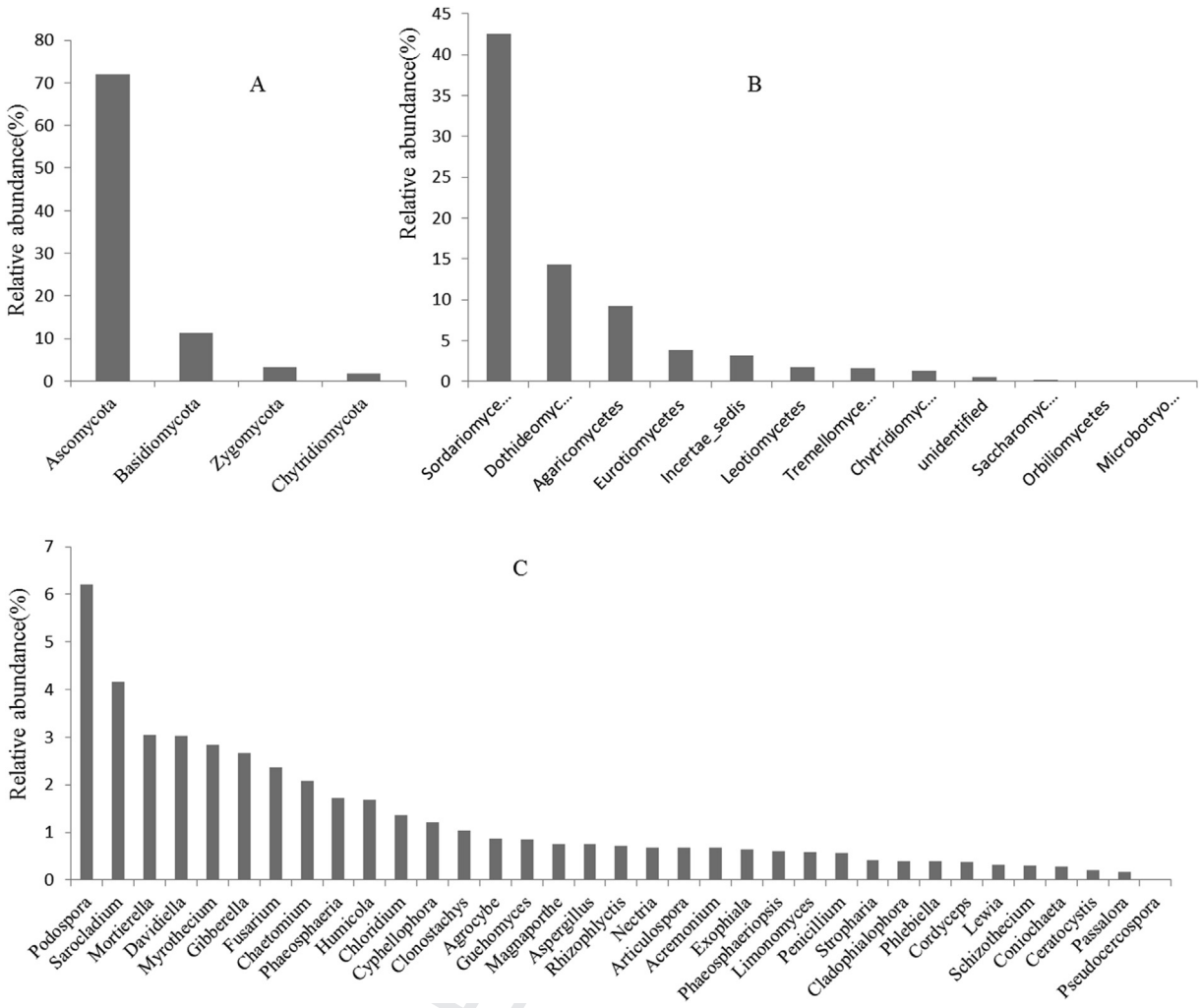

Fig. 2. Fungal community composition of long term fertilized soils. The relative abundance is represented as a proportion of ITS gene reads of the total number of reads. (A) Fungal phyla distribution. (B) The most abundant fungal classes with relative abundance >0.01% of total analyzed fungal community (C) The most abundant fungal genera with relative abundance >0.01% of the total analyzed fungal community.

strong inhibiting effect of decreased pH on bacterial abundance (from 4.3×10^9 to 2.2×10^9 copy g^{-1} soil). The results confirmed that the bacteria-to-fungi ratio was strongly and positively related with soil pH ($F = 0.815$, $P < 0.01$; Table S1). Furthermore, the bacteria-to-fungi ratio was negatively correlated with TOC and the C: N ratio ($F = -0.680$ and -0.639, respectively; both $P < 0.05$; Table S1), which was in good agreement with Fierer et al. (2009).

We found that high N fertilizer (N_2) rates led to a lower bacteria-to-fungi ratio, which suggests that long term high inputs of N fertilizer may directly or indirectly influence the composition of soil microbial communities (Strickland and Rousk, 2010; Zechmeister-Boltenstern et al., 2011) and the different wheat yields supports the concept that there are functional differences between microbial communities. In the N_2 treatment, with the lowest bacteria:fungi ratio, this fungal-dominated ecosystem led to a faster mineralization of N, and a slightly lower microbial retention of mineral N (NH_4^+) (Rousk and Frey, 2015); thus, the concentration of NH_4^+ in the N_2 treatment was far lower than two times of that in N_1, although the urea addition in the N_2 treatment was twice that in N_1. There was no obvious difference in the wheat yield between the N_2 and N_1 treatments, although the concentration of TOC and NH_4^+ in the N_2 treatment was higher than in N_1. Additionally, the wheat yield in the CK, N_1, and N_2 treatments were lower than in the N_1P_1 and N_2P_2 treatments, which may be related to the lower N:P ratio in the former three treatments, and available P was the limiting nutrient for wheat yields. Bacteria, which have been reported to retain more mineral N than fungi (Rousk and Frey, 2015), were more dominant than fungi in the N_1P_1 and N_2P_2 treatments than in the N_1 and N_2 treatments. The results indicated that a higher bacteria:fungi ratio in the N_1P_1 and N_2P_2 treatments may provide more inorganic N for wheat, while there was also adequate TOC and available P in these plots, which combined to produce wheat yields of 3352 and 2617 (kg ha^{-1}) in the N_1P_1 and N_2P_2 treatments, respectively (Table 1).

Furthermore, previous study proved that high N availability may reduce the need for plants to invest C in nutrient-absorbing systems, and by that induce a shift in C allocation in favor of aboveground tissue production at the expense of root production (Wei et al., 2013). For this reason, we found that N_2 and N_2P_2 soil samples had the higher concentration of TOC, with higher N availability (NO_3^- and NH_4^+) and slightly higher wheat production than no fertilizer treatment. But for N_1 and N_1P_1, there were relative lower N availability and then lower concentration of TOC, so they got the higher wheat yield than N_2 and N_2P_2 soil samples. In contrast, the three soil nutrients and wheat yield was the lowest in the control plots (Table 1).

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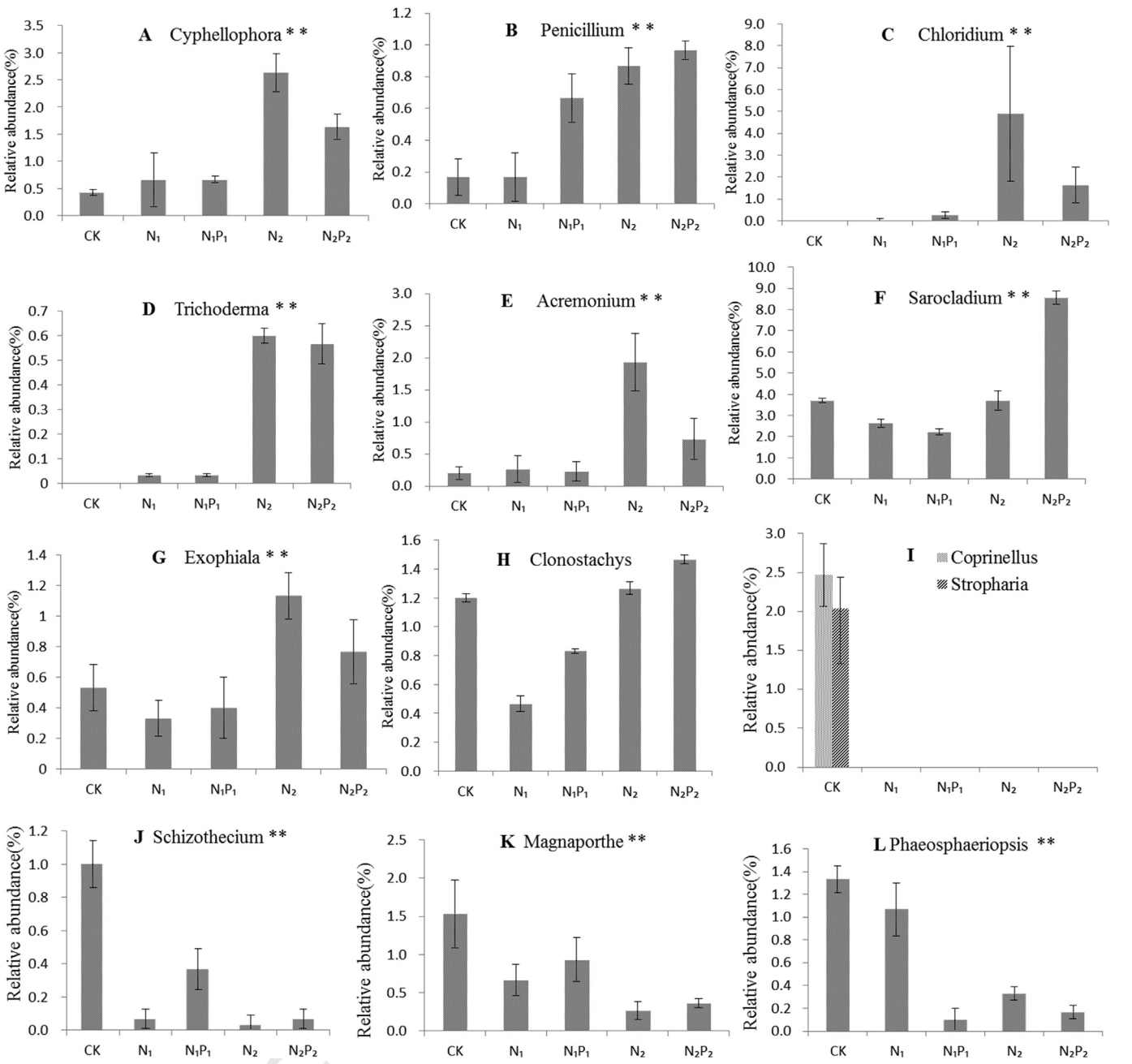

Fig. 3. Relative abundances of common genera under different long-term fertilizer treatments. Error bars indicate the standard deviation of relative abundance between three replicate samples. **P < 0.01, *P < 0.05.

4.3. Fungal alpha diversity

Greater biodiversity in soil can lead to a more stable system, and enhance the combination of vital microbial functions and processes (Chaer et al., 2009). However, all fertilized soil samples in this study had lower fungal diversity and showed a trend towards a negative response to N-amendment (Van Diepen et al., 2011), which was in agreement with the conclusion that N fertilizer reduced the diversity of active (Steven et al., 2007; Paungfoo-Lonhienne et al., 2015) and ectomycorrhizal (Wright et al., 2009) fungi. The loss of arbuscular mycorrhizal fungi biodiversity has been reported to decrease both plant biodiversity and ecosystem productivity, while increasing ecosystem instability (Maček et al., 2011). This study indicated that the decrease of fungal diversity may result in a less stable agroecosystem and may lead to unsustainable crop production.

A significant decreasing trend for fungal diversity with decreasing pH was observed ($R^2 = 0.819$, $P < 0.01$), which was quite similar to a recent investigation on fungal communities (Wang et al., 2015), soil ammonia oxidizers, and eukaryotes (Hu et al., 2013; Shen et al., 2014; Zhalnina et al., 2015). With the decreasing pH, some endurable taxa became rare or even vanished, and the balance of taxa quantity in previous habitats was broken, resulting in a decrease in the fungal community diversity. However, fungal diversity was negatively correlated with N inputs including total N, NH_4^+, NO_3^- and available N (Table 2). For this reason, we suspect the decrease in fungal diversity coincided with the inordinately high concentrations of N in

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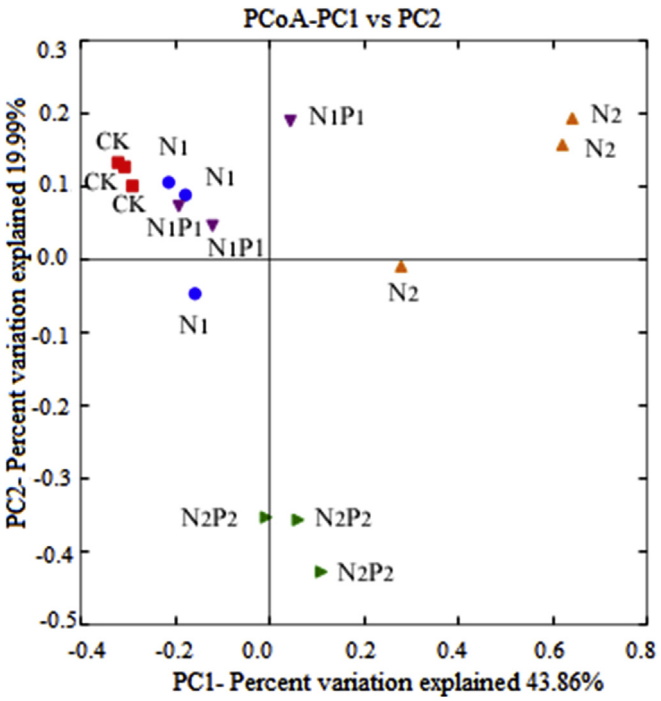

Fig. 4. Principal coordinates analysis (PCoA). PCoA of the pyrosequencing reads obtained from soils subjected to different fertilization regimes based on the weighted Fast UniFrac metric. The first two axes are drawn and the percentage of variance explained by each axis is given.

various forms (total N, NH_4^+, available N, and NO_3^-) associated with the ultra-rich soils created under experimental fertilizer treatments in this study.

There was no significant effect of the N and N plus P fertilizer regimes on fungal community richness in this study, which was consistent with previous reports that N fertilization has a neutral effect on AM fungal richness (Eom et al. 1999; Mueller and Bohannan, 2015). In contrast, previous studies have reported lower AMF species richness because of N (Wang et al., 2011) and P fertilization (Alguacil et al., 2010; Gosling et al., 2013).This stability of fungi community richness suggests that the 34 year N and N plus P fertilizer regimes do not negatively impact the number of fungal species.

4.4. Fungal community composition

The fungal community in this study was dominated by Ascomycota and their relative abundance in treatments N_1, N_1P_1, and N_2P_2 increased slightly, which agreed with findings that Ascomycota dominance is enhanced by relatively high soil N contents (Nemergut et al., 2008; Klaubauf et al., 2010; Paungfoo-Lonhienne et al., 2015). Previous research has revealed that the most diverse groups of saprotrophic fungi are Ascomycota (Xiong et al., 2014) in the alpine meadows of the Tibetan plateau and that their growth rate is correlated with N availability (Fontaine et al., 2011). However, Ascomycota, who are the key decomposers in agricultural soils clearly decreased in the N_2 treatment, while they flourished in response to straw additions to soil (Ma et al., 2013). The result indicated that members of the Ascomycota are particularly vulnerable to high nutrient levels, such as the high urea input in this study, and Wang et al. (2015) also found that excess N was harmful to the members of this phylum in a biological soil crust. The shift in their abundance may in turn dramatically affect the soil C decomposition (Xiong et al., 2014).

In our study most members of the phylum Ascomycota were found to be affiliated to the class Sordariomycetes (Fig. 2). Most species in the order Hypocreales in this class and Eurotiales in class Eurotiomycetes demonstrate N_2O producing activity (Mothapo et al., 2015). However, we found the abundance of Sordariomycetes and Eurotiomycetes was higher in high N (i.e., N_2) than in low N and N plus P treatments (i.e., N_1 and N_1P_1), as well as the no added fertilizer treatment (CK). Because N_2O is a potent greenhouse gas and ozone depleting substance (Mothapo et al., 2015), the increase in fungal species capable of denitrification in more concentrated N fertilizer treatments would stimulate N_2O emissions and may be not good for sustaining the biosphere.

The second most abundant Ascomycotal fungi in our study was class Dothideomycetes (Fig. 2B), which has been implicated in cell wall decay (Freedman et al., 2015) and is important to ecosystem health and global carbon cycling as a saprophyte and degrader of plant biomass (Goodwin and Kema, 2009). However, they decreased in all fertilized soils, similar with the result of Freedman et al. (2015), which indicated that long term N and N plus P addition may reduce the microbial contribution to the litter decomposition in black soil.

Leotiomycetes were relatively more abundant in the N_1P_1 and N_1 treatments, which was a similar result to that of a recent study (Freedman et al., 2015), whereas they were less abundant in the N_2P_2 and N_2 treatments. Among the sequences in this class, 84.4% were classified as order Helotiales, who are common root fungi, and shown to harvest and provide their hosts (plants) with nutrients immobilized in organic matter (Upson et al., 2009a). Indeed, the order Helotiales a declined dramatically with elevated N and N plus P in black soil (data not shown), as also reported by Dean et al. (2013). Because Helotiales from roots in cold climates are important to N uptake (Upson et al., 2009b; Newsham, 2011), it is perhaps not surprising that this group is highly sensitive to N enrichment in northeast China where there is a temperate continental monsoon climate with an average annual temperature of 3.5 °C (Zhou et al., 2015). Their declines perhaps indicated a loss of plant–fungal symbioses under N enrichment (Dean et al., 2013).

Furthermore, fungal variation between the same N inputs in N_2 and N_2P_2 showed that high P fertilizer also affected the fungal community structure. Similarly, the ectomycorrhizal fungal composition in N plus P plots was significantly different from the control or N (Wright et al., 2009). P fertility can directly influence soil microorganisms, but the effect of fertilization may also be attributable to changes in plant metabolism (Beauregard et al., 2010). Root exudation represents an important source of soil carbon for microorganisms and is influenced by plant P status.

4.5. Relationship between abundant groups and soil properties

The abundances of the Sordariomycetes have been reported to be significantly correlated with both the soil C:N ratio and extractable P concentrations (Lauber et al., 2008). In this study, we found that Sordariomycetes abundance was positively related to the available P, TOC, and N:P (Table 3), this maybe because the higher concentration of available P, TOC and the high N:P ratio can provide sufficient nutrients for them. Dothideomycetes abundance was positively and significantly correlated with soil pH (Table 3), which was in good agreement with Zanardo et al. (2015). With the increase in the amount of fertilizer, soil pH decreased from 6.36 to 4.64, and Dothideomycetes abundance decreased gradually. The result indicated that this fungal group was sensitive to soil pH. But they were negatively related with concentration of Total N and NO_3^- (Table 3).

Many studies have shown that soil pH was the strongest factor shaping bacterial community structures (Xiong et al., 2012; Zhao

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Table 3
Spearman correlations for the long term experiment between ITS relative abundance of the main classes and soil properties.

Class	pH	Total N	NO_3^-	NH_4^+	Avail P	Avail K	TOC	N: P	C: N
Dothideomycetes	**0.825****	**−0.755****	**−0.792****	−0.758	−0.014	−0.435	−0.847	−0.176	**−0.791****
Eurotiomycetes	**−0.901****	**0.715****	**0.961****	**0.812****	0.432	0.028	**0.866****	0.18	**0.834****
Leotiomycetes	**0.739****	**−0.795****	**−0.753****	**−0.568***	−0.**645****	0.083	−0.664	0.231	**−0.581***
Sordariomycetes	−0.199	0.425	0.140	−0.092	**0.638***	**−0.630***	**0.726****	**0.692****	−0.063
Agaricomycetes	−0.281	0.385	0.333	0.362	−0.265	**0.724****	0.077	0.012	0.431
Microbotryomycetes	0.451	−0.463	**−0.549***	−0.249	−0.372	0.072	−0.323	−0.303	−0.357
Tremellomycetes	−0.327	0.213	0.197	0.212	**0.519***	−0.397	0.288	0.253	0.037
Chytridiomycetes	0.027	−0.055	−0.027	0.266	**−0804****	**0.910****	−0.360	−0.477	0.268

Soil factors indicated include Avail P (Available P), Avail K (Available K), TOC (Total Organic Carbon), N: P (Available N: Available P), C: N (Total Organic Carbon: Available N).
$^{**}P < 0.01$, $^{*}P < 0.05$.

et al., 2014; Zhalnina et al., 2015). In this study, the PCoA results showed that the effects of N and N plus P addition on fungal community composition were probably strongly mediated by soil pH, which was in agreement with Kim et al. (2014).

5. Conclusion

In conclusion, after performing qPCR and barcode pyrosequencing analyses, we found that long-term N and N plus P fertilization increased fungal abundance, reduced fungal diversity and the bacteria-to-fungi ratio, and altered community structure. The compositions of the fungal communities in the plots receiving the high level fertilizer addition were significantly different from those receiving low level fertilizer treatments. The results showed that the 34 y inorganic fertilization regimes changed fungal community and increased N and P fertilizer dosage has a potential negative impact on carbon cycling and nitrogen in soil and promotes fungal genera with known pathogenic traits, uncovering a negative effect of intensive fertilization.

Conflict of interest

The authors have no conflict of interest to declare.

Uncited reference

Ten Hoopen et al., 2010.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.soilbio.2015.12.012.

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