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ORIGINAL PAPER

Composition of the soil fungal community is more sensitive to phosphorus than nitrogen addition in the alpine meadow on the Qinghai-Tibetan Plateau

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Abstract The alpine meadow on the Qinghai-Tibetan Plateau (QTP), which is sensitive to global climate change and human activities, is subjected to addition of nutrients such as nitrogen (N) and phosphorus (P) in the soil. The impacts of N or P on ecosystem structure and function depend at least partly on the response of soil fungal communities, although few studies have compared the effects of N and P addition, both separately and together. We examined the responses of composition of the soil fungal community to 3-year experimental nutrient additions (control, N, N plus P, and P) in a typical alpine meadow of the QTP. We found that P addition, regardless of N addition, significantly reduced fungal species richness and changed fungal community composition, while the effect of N was undetectable. Nitrogen plus phosphorus caused a more distinct community than either N or P addition alone. Multivariate regression tree, canonical correspondence analysis, and distance-based multivariate linear model analyses all suggested available P was a key parameter determining the diversity and composition of the fungal community. Other parameters such as dissolved organic N, aboveground net primary productivity of forbs, and dissolved organic C played important but secondary roles. The results indicated an important role of P in structuring soil fungal communities in the alpine meadow. Our results suggest that fungal diversity loss and long-term changes in ecosystem stability can result from fertilization management in the fragile alpine environment.

Keywords Nitrogen addition · Phosphorus addition · Soil fungi · Alpine meadow

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Introduction

Nitrogen (N) and phosphorus (P) are two major elements limiting plant and microbial growth in most terrestrial ecosystems (Elser et al. 2007; Vitousek and Howarth 1991). Since the twentieth century, the biogeochemical cycles of N and P have been greatly changed by anthropogenic activities, such as pollution from fossil combustion and deliberate N and P fertilization. It is estimated that the global N deposition rate will double or triple in the coming decades (Lamarque et al. 2005), and P deposition is becoming an important P source in global ecosystems (Ahn and James 2001; Vicars et al. 2010). This N and P enrichment in soil may have a profound impact on the structure and functions of terrestrial ecosystems. Soil microbial communities play key roles in the terrestrial ecosystem by facilitating key biogeochemical processes and functions in soil (Nannipieri et al. 2003). These processes are fundamental for soil fertility and plant growth (Artursson et al. 2006; Kennedy 1999). Knowledge of how microbial communities in soil respond to N and P enrichments is of great value for

understanding the effects of global change and anthropogenic activities on terrestrial ecosystems.

Compared to the many studies on soil bacteria, relatively little research has been done on soil fungi, yet fungi in soil also play major roles in element cycling. For instance, plant litter is often first attacked by rot fungi, due to their relatively high capacity to degrade lignocellulose (Hammel 1997), and mycorrhizal fungi are important for the growth and health of plants by nutrient exchange in a symbiotic relationship (Schachtman et al. 1998). In general, the composition of the soil fungal community is affected by soil factors, such as pH, moisture, and temperature (A'Bear et al. 2012; Kivlin et al. 2011; Sautour et al. 2001), and by aboveground plants (Hedlund et al. 2003; Johnson et al. 2004; Waldrop et al. 2006b). Nitrogen or phosphorus addition to soil could alter the composition of the soil fungal community directly by supplementing the nutrient supply or indirectly through changes in other soil factors, plants, or soil invertebrates such as fungivores (Antoninka et al. 2011; Bindraban et al. 2015; Jiang et al. 2015). The effects of N or P addition on composition of the soil fungal community depend on nutrient types and the dose, soil properties, and ecosystem type (Bao et al. 2013; Beauregard et al. 2010; Chen et al. 2016; Sun et al. 2014; Weber et al. 2013). There have been some studies investigating the responses of composition of the soil fungal community to fertilization management including both N and P addition. However, these studies had one or more of these shortfalls that are roughly addressing the overall "microbial biomass" (Li et al. 2015; Liu et al. 2013b), or using low-resolution techniques to investigate the composition of the fungal community (Liu et al. 2013b; Luo et al. 2015), or lacking a solid experimental design to statistically compare the different effects of N, P, and their interaction (Cassman et al. 2016). Owing to the stoichiometric relationship between N and P, a correct N:P ratio in soil is important for soil fertility and growth of plants and fungi. Enrichment only in N might aggravate a pre-existing P limitation in soil (Vitousek et al. 2010), and in many cases both N and P fertilizers are added to the field together in soil fertility management. Thus, an investigation of the different effects of N, P, and their interaction, with a solid experimental design and using the updated molecular method (such as rDNA ITS MiSeq sequencing), could help in gaining a comprehensive understanding of the effects of nutrient addition on composition of the soil fungal community.

The Qinghai-Tibetan Plateau (QTP) is the highest plateau in the world. It plays important roles in the atmospheric circulation of Eurasia and has great influences on the regional and global environments by controlling hydrologic cycling across China and south Asia (Shi et al. 2010). The alpine meadow is the most extensive land cover type on the QTP, covering about 35 % of the area of the whole plateau, with an average elevation of more than 4000 m (Zhang et al. 2009). The high altitude and low temperature have led to a relatively fragile alpine meadow ecosystem, which is sensitive to global climate change and anthropogenic activities (Zeng et al. 2013). Over the past 30 years, the QTP has experienced increased N deposition (up to 10 kg N ha^{-1} $year^{-1}$ (Liu et al. 2013a; Lü and Tian 2007). Nitrogen and P fertilizers have also frequently been applied to increase soil fertility and primary productivity of the QTP meadow land (Zhao and Zhou 1999). However, the ecological consequences of N and/or P amendments in alpine meadow soil are not well understood. It has been reported that N and P addition could affect diversity and/or composition of the aboveground plant community in the alpine meadow (Yang et al. 2011). Although the effects of N and P additions on the arbuscular mycorrhizal fungi community has been reported (Liu et al. 2012b), little is known about how the composition of the overall fungal community respond to N and P addition alone and the combined effects of adding these two nutrients in alpine ecosystems on the QTP.

Here, we carried out a field N and P fertilization experiment in a typical alpine meadow of the eastern QTP. We aimed to understand the following: (1) the effects of N and P additions, alone and in combination, on the diversity and composition of the overall soil fungal community in the alpine meadow and (2) the key factors affecting the composition of fungal communities in response to fertilization. Specifically, our experimental design allowed us to discern how N and P affect the composition of the soil fungal community, both alone and in combination. The biological responses to N and P enrichments in alpine meadow soil could provide a proxy for understanding the ecological consequences of global change and anthropogenic fertilization on other fragile and sensitive ecosystems, for example, the world's semi-arid and high-latitude/high-altitude regions.

Materials and methods

Experimental setup

The experimental plots were set up in 2011 on a typical alpine meadow on the QTP (37° 37′ N, 101° 12′ E) at the Haibei National Field Research Station of Alpine Grassland Ecosystem, Chinese Academy of Sciences. The terrain of these plots is flat with an elevation of 3220 m. The mean annual temperature is at −1.7 °C, ranging during the year from a mean monthly temperature of −14.8 °C in January to 9.8 °C in July. The mean annual precipitation is 560 mm at this station, of which more than 80 % is concentrated from May to September (Fang et al. 2012). The soil is a clay loam averaging 65 cm thickness. The top 5–10 cm of the soil profile is classified as Mat-Gryic Cambisol according to the classification system of the Chinese National Soil Survey (Zhao et al. 2015). The plant community is dominated by *Kobresia humilis*, *Festuca ovina*, *Elymus nutans*, *Poa pratensis*, *Carex scabrirostris*, *Scripus distigmaticus*, *Gentiana straminea*, *Gentiana farreri*,

Leontop odiumnanum, *Blvsmus sinocompressus*, *Potentilla nivea*, and *Dasiphora fruticosa*, and all plant species can be divided into four functional groups: grasses, sedges, legumes, and forbs (Fang et al. 2014). Prior to the experimental setup, the original soil properties were determined at this experimental site (Online Resource 1). The experimental plots were constructed in a randomized block design. In each of the six blocks, six plots were randomly assigned to six nutrient treatments. Each plot was 6 m × 6 m, with between neighboring blocks (Online Resource 2). Five of the blocks with four types of treatment plots were used in this study: N addition (urea, 100 kg N ha^{-1} $year^{-1}$), P addition ($Ca(H_2PO_4)_2$, 50 kg P ha^{-1} $year^{-1}$), NP addition (urea and $Ca(H_2PO_4)_2$, 100 kg N and 50 kg P respectively), and a control. Nutrient addition was carried out three times per year, in each of the summer months of June, July, and August, with one third of the dose for the whole year added at each time. At each application, the dry powder of each nutrient was spread evenly over the respective plot late in the afternoon. We surmised that the relatively humid soil in the rainy season would facilitate adsorption of nutrients and that in the evening the relatively low temperature (usually <10 °C) would prevent evaporation loss of the fertilizer.

Sample collection

On 12 August 2014, we sampled the top soil layer (0–10 cm) in the control (CK), nitrogen (N), phosphorus (P), and N plus P (NP) treatment plots. In each plot, five replicate soil cores (0–10 cm) were collected from the four corners (about 1 m from the border) and the center of each plot. The soil samples were collected using a 3-cm-diameter soil auger and pooled together in an aseptic plastic bag. A total of 20 soil samples (five blocks × four treatments) were collected, put on ice, and transferred to the laboratory as soon as possible. In the laboratory, the soil samples were preprocessed to remove stones and big plant roots and sieved through a 2-mm mesh. One aliquot for DNA extraction was stored in a freezer at −40 °C. One aliquot for soil chemical analysis determination was kept in a refrigerator at 4 °C. The main soil properties, except for total C, total P, and total N, were determined within 10 days.

The plant biomass on each plot was estimated using the harvest method. In each plot, due to the even distribution of plant biomass, one randomly assigned 0.5 × 0.5 m quadrat was used to harvest the plants within it (Xu et al. 2015). The functional group (grasses, sedges, legumes, or forbs) of each plant was identified. Plants collected from the quadrat were clipped to estimate aboveground net primary productivity (ANPP). Three replicate soil cores (0–40 cm) were collected at random with a caution not to represent only one portion of the plot. The cores were collected using a 7-cm-diameter soil auger and pooled together in an aseptic plastic bag to estimate belowground biomass (BGB). The biomass for the aboveground plant and roots was determined after drying in the oven at 65 °C for 48 h.

Soil properties

Soil pH was determined using a pH meter (E20-FiveEasy™ pH, Mettler Toledo, German) in a 1:5 (fresh soil:deionized water, wt/v) suspension after shaking for 30 min. Soil moisture (%, water/dry soil) was determined by gravimetric method after drying at 105 °C for 24 h. Dissolved total N (DTN) and dissolved organic C (DOC) were extracted by treating 10 g fresh soil with 100 mL 2 M KCl solution and deionized water, respectively. After shaking for 1 h, the soil suspension was filtered through glass fiber filters (Fisher G4, 1.2 μm pore space). The exchangeable NH_4^+-N and NO_3^--N were analyzed using a continuous-flow analytical system (San^{++} System, Skalar, Holland). DOC was determined using a TOC analyzer (Multi N/C 3000, Analytik Jena, Germany). Dissolved organic N (DON) was calculated as follows: $DON = DTN - NH_4^+\text{-}N - NO_3^-\text{-}N$ (NO_2^--N in the soil samples was close to the low detection limit). Available P (AP) in soil was extracted with sodium bicarbonate and determined using the molybdenum blue method (Ståhlberg 1980). Available N (AN) in soil was determined using the alkaline hydrolysis method (Sahrawat and Burford 1982). To estimate the total C (TC), total N (TN), and total P (TP) contents, soils were air-dried and ball milled to pass through a 0.05-mm pore mesh, to obtain dry soil powders. Both TC and TN were measured using an elemental analyzer (Vario MAX, Elementar, Germany). TP was determined using the molybdenum blue method with an ultraviolet-visible spectrophotometer (UV-2550, Shimadzu, Kyoto, Japan).

Soil DNA extraction and fungal ITS sequencing

Fresh weight soil (0.5 g) was used to extract the nucleic acids from each soil sample ($n = 20$), using a FastDNA® SPIN Kit for Soil (MP Biomedicals, Santa Ana, CA), according to the manufacturer's instructions. The final DNA yield was quantified using a Nanodrop 1000 spectrophotometer (Thermo Scientific, Wilmington, DE) and then stored at −40 °C until use.

The ITS2 region of the rRNA gene of fungi was amplified using the ITS3 and ITS4 primer set (ITS3, 5′-GCATCGATGAAGAACGCAGC; ITS4, 5′-TCCTCCGCTTATTGATATGC) (Balajee et al. 2007). ITS2 gene tag-encoded high-throughput sequencing was carried out using the Illumina MiSeq platform (PE 2000) at Novogene Company (Beijing, China).

Tag processing and bioinformatics analyses

The paired-end reads from sample DNA fragments were merged using FLASH (Magoč and Salzberg 2011) and then processed by QIIME 1.91 (Caporaso et al. 2010) (split_libraries_fastq.py)

to obtain high-quality tags for each sample. The tags were then analyzed using MOTHUR 1.35 (Schloss et al. 2009) as suggested by "MiSeq_SOP" (http://www.mothur.org/wiki/MiSeq_SOP). Briefly, sequences were aligned against the UNITE fungal database (Abarenkov et al. 2010). The UNITE fungal database is itself unaligned, and we made an aligned fungal database using software MAFFT (Kazutaka and Standley 2013) before we conducted sequence alignment. The aligned sequences were then processed by some denoising steps (MOTHUR commands: screen.seqs, pre.cluster). Chimeras were picked out by UCHIME implemented in MOTHUR. The non-chimera sequences were assigned to operational taxonomic units at 97 % similarity ($OTU_{0.03}$) using the furthest neighbor method. The taxonomy for each sequence was classified against the UNITE fungal database using Wang's method (Wang et al. 2007) at a threshold of 60 % similarity. An OTU table was then generated by the "make.shared" function, and the OTUs with only one sequence (singletons) were discarded. For comparison of alpha diversity between different treatments, the rarefaction analysis was firstly done to check species richness along sequence number. To compensate for the uneven sequencing efforts of different samples, a resampling step was carried out using MOTHUR (sub.sample) to obtain the same sequence number (16,816) for all samples. The resulting OTU table was used for the estimation of alpha diversity (OTU richness, Pielou's evenness) and beta diversity (reflected by Bray-Curtis dissimilarity among different samples) index and other community analyses. The rarefaction analysis and alpha and beta diversity estimation were done with the "vegan" package (Oksanen et al. 2015) in R 3.0.1 (R Core Team 2015).

Statistical analyses

Multivariate regression trees (MRTs) were constructed to display important relationships between changes in the composition of fungal communities and environmental parameters De'Ath (2002). MRTs create dichotomies, where samples that share similar species patterns in relation to environmental factors are clustered together. It is a hierarchical method that aims to minimize the least sum squares of the response data within a cluster by repeatedly splitting the data based on environmental variables. A 1000 cross-validation process using the "1se" method was used to decrease the structure complexity of MRTs, which would predict the main relationships between fungal species data and environmental variables. MRT analysis was carried out using R and the "mvpart" package (Therneau and Atkinson 2014).

The canonical correspondence analysis (CCA) and the distance-based multivariate linear model (DistLM) method were also used to link environmental parameters with the fungal community composition. The Bray-Curtis distance matrix based on an $OTUs_{0.03}$ table and the log-transformed environmental parameters were used in these two methods. The CCA was done with the "vegan" package in R, and the DistLM was done with the software Primer-E 7 (Clarke et al. 2014). In addition, to determine important OTUs indicating different treatments, an indicator analysis was done based on an $OTUs_{0.03}$ table using the "indicspecies" package (Caceres and Legendre 2009) in R.

Significant changes in fungal alpha diversity among different treatments were tested using the Kruskal-Wallis method and a post hoc Dunn's test for pairwise comparisons, if necessary. The Kruskal-Wallis and Dunn's tests were carried out using the "stats" (R Core Team 2015) and "dunn.test" (Dinno 2015) packages in R, respectively. The grouping effects of treatments towards fungal community composition were tested by ANOSIM with the software Primer-E 7 and displayed in a non-metric multidimensional scaling plot. Comparisons between beta diversity values in different treatments were carried out using a permutation-based pairwise *t* test with the package "RVAideMemoire" (Hervé 2015) in R.

To test and compare the effects of N and P factors on composition of the soil fungal community, we also conducted two-way analysis of variance and two-way Adonis analysis on the datasets of alpha and beta diversity, respectively. The four treatments (CK, N, P, and NP) can be assigned with both N and P attributes (for example, CK (N: 0, P: 0); N (N: 1, P: 0); P (N: 1, P: 0); NP (N: 1, P: 1)). These assignments allow analyses of both the specific and interactive effects of N and P on composition of the soil fungal community. The Adonis analysis was done with the "vegan" package in R.

Data accession numbers

All the MiSeq sequencing data (.fq files) were uploaded to the National Center for Biotechnology Information sequence reads archive and can be accessed with the BioSample numbers SAMN04445755–SAMN04445774 under BioProject PRJNA310048 (http://www.ncbi.nlm.nih.gov/bioproject/PRJNA310048).

Results

Environmental parameters

The short-term N and P additions did not significantly change the contents of exchangeable NH_4^+-N, DOC and TN, pH and soil moisture and belowground biomass (Kruskal-Wallis tests, $P > 0.05$) (Table 1), while NO_3^- and DON contents were significantly increased in the N and NP treatments (post hoc Dunn's tests, $P < 0.05$), which might be caused by the significant effect of the N factor (Online Resource 3). TP and AP contents increased significantly in the P and NP treatments (post hoc Dunn's tests, $P < 0.05$), due to a significant effect of the P factor (Online Resource 3). Among the plant

Table 1 Effects of N and P addition on mean values of soil physicochemical ($n = 5$) and plant productivity ($n = 4$, from blocks 1, 2, 4, 5) properties

Variables	CK	N	NP	P
pH	7.14 (0.21)^{a}	7.32 (0.47)^{a}	7.10 (0.51)^{a}	7.68 (0.53)^{a}
NO_3^- (mg kg^{-1})	19.5 (0.6)^{b}	26.0 (1.6)^{a}	27.5 (5.5)^{a}	19.1 (2.8)^{b}
NH_4^+ (mg kg^{-1})	9.33 (2.06)^{a}	9.76 (1.42)^{a}	10.45 (5.05)^{a}	12.51 (4.23)^{a}
DON (mg kg^{-1})	32.0 (3.8)^{b}	42.6 (4.7)^{ab}	46.7 (10.1)^{a}	33.5 (7.1)^{ab}
DOC (mg kg^{-1})	182.4 (16.2)^{a}	197.6 (37.1)^{a}	170.7 (16.4)^{a}	166.7 (10.6)^{a}
TN (%)	0.657 (0.026)^{a}	0.606 (0.064)^{a}	0.668 (0.063)^{a}	0.636 (0.063)^{a}
TC (%)	6.58 (0.26)^{a}	5.96 (0.66)^{a}	6.78 (0.64)^{a}	6.40 (0.65)^{a}
SM (%)	27.0 (4.1)^{a}	20.7 (4.0)^{a}	24.3 (1.2)^{a}	24.0 (2.2)^{a}
TP (%)	0.079 (0.001)^{b}	0.081 (0.006)^{ab}	0.093 (0.006)^{a}	0.094 (0.006)^{a}
AP (mg kg^{-1})	6.13 (1.71)^{b}	4.46 (1.95)^{b}	46.62 (4.47)^{a}	35.16 (13.81)^{a}
AN (mg kg^{-1})	58.9 (6.3)^{a}	58.8 (6.0)^{a}	55.6 (6.4)^{a}	54.7 (3.4)^{a}
ANPP_total (g m^{-2})	322.6 (18.5)^{b}	411.5 (16.6)^{b}	668.0 (32.6)^{a}	409.6 (87.6)^{b}
ANPP_grass (g m^{-2})	232.5 (15.4)^{b}	265.0 (9.1)^{b}	414.4 (5.4)^{a}	272.2 (64.3)^{b}
ANPP_sedge (g m^{-2})	10.93 (4.96)^{a}	27.60 (4.56)^{a}	1.67 (7.35)^{b}	7.21 (17.51)^{ab}
ANPP_legume (g m^{-2})	21.20 (6.88)^{ab}	12.03 (8.18)^{bc}	4.38 (7.11)^{c}	28.05 (4.37)^{a}
ANPP_forbs (g m^{-2})	58.0 (15.3)^{b}	106.9 (25.4)^{ab}	247.5 (30.1)^{a}	102.2 (25.7)^{b}
BGB (kg m^{-2})	1.62 (0.35)^{a}	1.45 (0.38)^{a}	1.72 (0.40)^{a}	1.75 (0.25)^{a}

The values in parentheses are standard deviations (SD). Different superscript lowercase letters after the SD values indicate significant differences ($P < 0.05$, post hoc Dunn's tests) among different treatments. Variables significantly different among treatments are shown in italics. Soil moisture was examined from only three blocks (randomly) for each treatment and was not included in the analyses linking environmental parameters with fungal communities

DON dissolved N, *DOC* dissolved C, *TN* total N, *TC* total C, *TP* total P, *SM* soil moisture, *AP* available P, *ANPP* aboveground net primary productivity, *BGB* belowground biomass

parameters, the total ANPP was significantly elevated in the NP treatment (post hoc Dunn's tests, $P < 0.05$); specifically, the ANPP of grass and forb plants was elevated the most in the NP treatment, while the ANPP of sedge and legume plants decreased by NP treatment (post hoc Dunn's tests, $P < 0.05$). Besides the sole effect of the N or P factor, NP interaction had significant effects towards the total ANPP and the ANPP of sedge (Online Resource 3).

Alpha diversity of fungal communities

The rarefaction curves showed an obvious decreasing trend of fungal OTU richness in P and NP groups, while the effect of N was minimal, compared to those of the control plots (Fig. 1a). After the resampling step, there were a total of 3712 non-singleton fungal OTUs in all samples. The OTU richness was the highest in the control treatment (1097 ± 45, mean ± SD). Phosphorus addition reduced alpha diversity the most, leading to a mean of 850 (SD, 64) for OTU richness. There were significant changes in OTU richness between the control and NP and the control and P (Dunn's test, $P < 0.05$) (Fig. 1b). We also determined the index of Pielou's evenness: the NP treatment seemed to show a higher evenness value than the others, but the effect was not statistically significant (Dunn's test, $P > 0.05$) (Fig. 1c). For OTU richness, the P factor had a significant effect ($P < 0.01$), the N factor had no significant effect, and the interactive effect of N and P was marginally significant ($P = 0.068$). For Pielou's evenness, both N and P had no significant effects ($P > 0.05$) (Table 2).

Composition and beta diversity of fungal communities

All sequences could be classified into 23 classes (Online Resource 4) affiliated with 6 phyla. Dothideomycetes (Ascomycota) (23.6 %), Sordariomycetes (Ascomycota) (19.6 %), Leotiomycetes (Ascomycota) (17.5 %), Eurotiomycetes (Ascomycota) (13.7 %), and Agaricomycetes (Basidiomycota) (8.2 %) were the five most abundant classes in all sequences. The percentage of Sordariomycetes (Ascomycota) was significantly higher in the NP (mean ± SD, 27.52 % ± 10.38 %) and P (23.80 % ± 5.35 %) treatments than in the controls (13.31 % ± 1.57 %) (Online Resource 4, $P < 0.05$). In contrast, the percentage of Zygomycota_class_Incertae_sedis (Zygomycota) was significantly lower in the NP (2.53 % ± 1.41 %) and P (1.36 % ± 1.49 %) treatments than in the controls (Online Resource 4, $P < 0.05$). Ascomycota_class_Incertae_sedis (Ascomycota) (2.54 % ± 0.62 %) were especially fewer in the N groups, and Agaricomycetes (Basidiomycota)

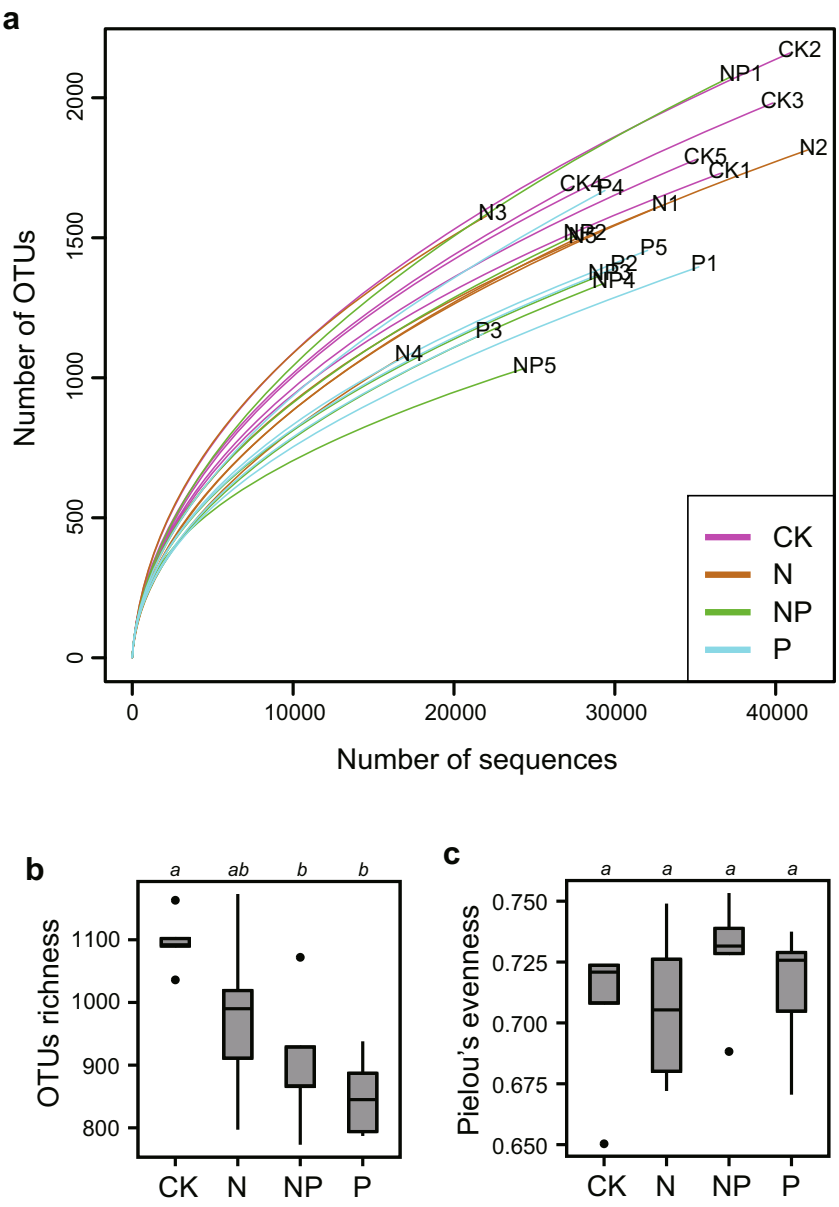

Fig. 1 Alpha diversity of soil fungi in different treatments. **a** Rarefaction curves. **b** OTU richness. **c** Pielou's evenness. Sharing at least one *lowercase letter* on top of the *box* indicates no significant difference between the two treatments (post hoc Dunn's tests, $P < 0.05$). Each *box* indicates 3rd and 1st quartiles of the value range for the top and bottom boundaries, respectively, and the *black line* inside each *box* represents the median value. *Ends of the whiskers* mark values within 1.5 times the interquartile range. Outliers shown as *solid circles* indicate values less or greater than 1.5 times the interquartile range

(1.81 % ± 0.23 %) were especially fewer in the NP group than in the other treatment groups (Fig. 2a, Online Resource 4).

At the $OTU_{0.03}$ level, nutrient additions also significantly changed the composition of the soil fungal community as a whole (Fig. 2b, Online Resource 5). The pairwise comparisons revealed that there were significant differences in OTU composition between the control and P, the control and NP, the NP and N groups, and the NP and P groups (Online Resource 5). Similar to the results for OTU richness, the P factor but not the N factor significantly affected fungal community composition, and the interactive effect of N and P was marginally significant (Table 2). The beta diversity (community turnover rate) in the NP treatment was significantly lower (Dunn's test, $P < 0.05$) than in the other treatments, implying that NP addition might lead to a more monotonous community composition of fungi with less variations between replicated plots in alpine meadow soil (Fig. 2b, Online Resource 6).

Indicator species for different treatments

We conducted indicator analyses to find the OTUs specific to a single treatment. There were more indicator species of statistical significance for CK (189) and NP (48) treatments than N (34) and P (7) treatments. The control and NP treatments also had higher indicator Stat values than the other two treatments (Online Resource 7). OTUs affiliated with *Conocybe echinata*, *Cistella* sp., *Rhytismatales* sp., uncultured *Laccaria*, and *Helotiales* sp. were the top five indicator taxa for the control treatment. *Chaetothyriales* sp., *Sordariales*, and *Nectriaceae* sp. were among the top five indicator taxa for the NP treatment

Table 2 The specific and interactive effects of N and P factors on the beta and alpha diversity of the fungal communities

	df	*F* values	*P* value	R^2
Adonis results for Bray-Curtis distance				
N factor	1	1.182	0.1947	0.0560
P factor	1	2.500	0.0006	0.1185
N × P	1	1.420	0.0604	0.0673
Residuals	16	0.758		
ANOVA results for OTU richness				
N	1	0.605	0.4482	
P	1	13.882	0.0018	
N × P	1	3.836	0.0678	
Residuals	16			
ANOVA results for Pielou's evenness				
N	1	0.375	0.5487	
P	1	1.331	0.2656	
N × P	1	0.272	0.6094	
Residuals	16			

N × P is the interactive effect of the N and P factors. The four treatments (CK, N, P, and NP) can be assigned with both N and P attributes. For example, CK (N: 0, P: 0); N (N: 1, P: 0); P (N: 1, P: 0); and NP (N: 1, P: 1)

(Online Resource 7), with *Sordariales* and *Nectriaceae* sp. corresponding to the high proportion of Sordariomycetes found in this treatment (Fig. 2a). For the sole N and P additions, the significance values for indicator species were all higher than 0.01, revealing less specific communities compared with the control and NP treatments (Online Resource 7).

Important environmental parameters for composition of the soil fungal community

MRT analysis was used to indicate the effects of environmental variables on composition of soil fungal communities. For alpha diversity, the MRT model could in total explain 66.5 % of the variance of the OTU richness dataset (Fig. 3). The tree was first split by AP, which accounted for 40.3 % of the variance. Another important environmental variable was DON, which accounted for 26.1 % of the variance. The rightmost eight samples on the tree with DON < 3.345 and AP < 16.75 had the highest means of diversity estimates (OTU richness). For beta diversity (community turnover), MRT analyses were carried out at both the class and $OTU_{0.03}$ levels. For the dominant lineage (class level) structure, the MRT model could in total explain 51.1 % of the variance of the original dataset (Fig. 4a). The tree was first split by ANPP of forbs, which accounted for 39.1 % of the variance. The second split was by ANPP of sedge, which accounted for 11.0 % of the variance. The three groups had a high proportion of Leotiomycetes (Ascomycota), Ascomycota_class_Incertae_sedis (Ascomycota), and Sordariomycetes (Ascomycota) for the left, middle, and right portions on the tree, respectively. For the $OTU_{0.03}$-level community composition, the MRT model gave a similar result to the $OTU_{0.03}$ richness dataset. The tree was first split by AP, which accounted for 12 % of the variance of the $OTUs_{0.03}$ table, and the second split by DON accounted for 9.2 % of the variance (Fig. 4b). However, most of the variation (79 %) in the $OTU_{0.03}$-level community composition could not be explained by the cross-validated MRT tree.

The DistLM method was used to test the relationship between environmental variables and the $OTU_{0.03}$-level community composition. Individual fitting showed that AP was the most important variable, which explained 21.08 % of the variation. Both TP and total ANPP contents were also significantly important (Online Resource 8). Fitting by sequential tests also showed that AP content was the essential variable, which explained far more of the variation in the composition of the fungal community than the other variables. DOC and pH were also important variables, but the effects were marginally significant (Online Resource 8). The CCA analysis confirmed that the AP was the most important factor influencing the $OTU_{0.03}$-level community composition. Besides, DOC, the ANPP of sedge, and DON played secondly important roles for the $OTU_{0.03}$-level community composition (Online Resource 9).

Discussion

In the alpine meadow environment of the QTP, it is already well established that N and P enrichment can affect some aspects of the general composition of soil biota directly, or indirectly by altering the growth and activity of plants (Stöcklin et al. 1998; Yang et al. 2011), with which many fungi have close interspecific relationships. However, it is not understood well how N and P enrichment specifically impacts the detailed composition of soil fungal communities in this region. This study provides fundamental data and perspectives on how the total fungal community composition responds to changes in N and P nutrition in soil. In particular, the experimental design allowed us to compare the effects of N and P separately and in combination. The results highlighted a role of P but not N in reducing fungal OTU richness and shaping the fungal community composition. This will be valuable for gaining an integrated understanding about the effects of N and P additions on the composition of the soil fungal community.

Phosphorus had a stronger influence on soil fungal OTU richness than nitrogen

Seldom have studies systematically compared the effects of N and P enrichments on soil fungal diversity. In this study, N addition alone had no significant effect on soil fungal

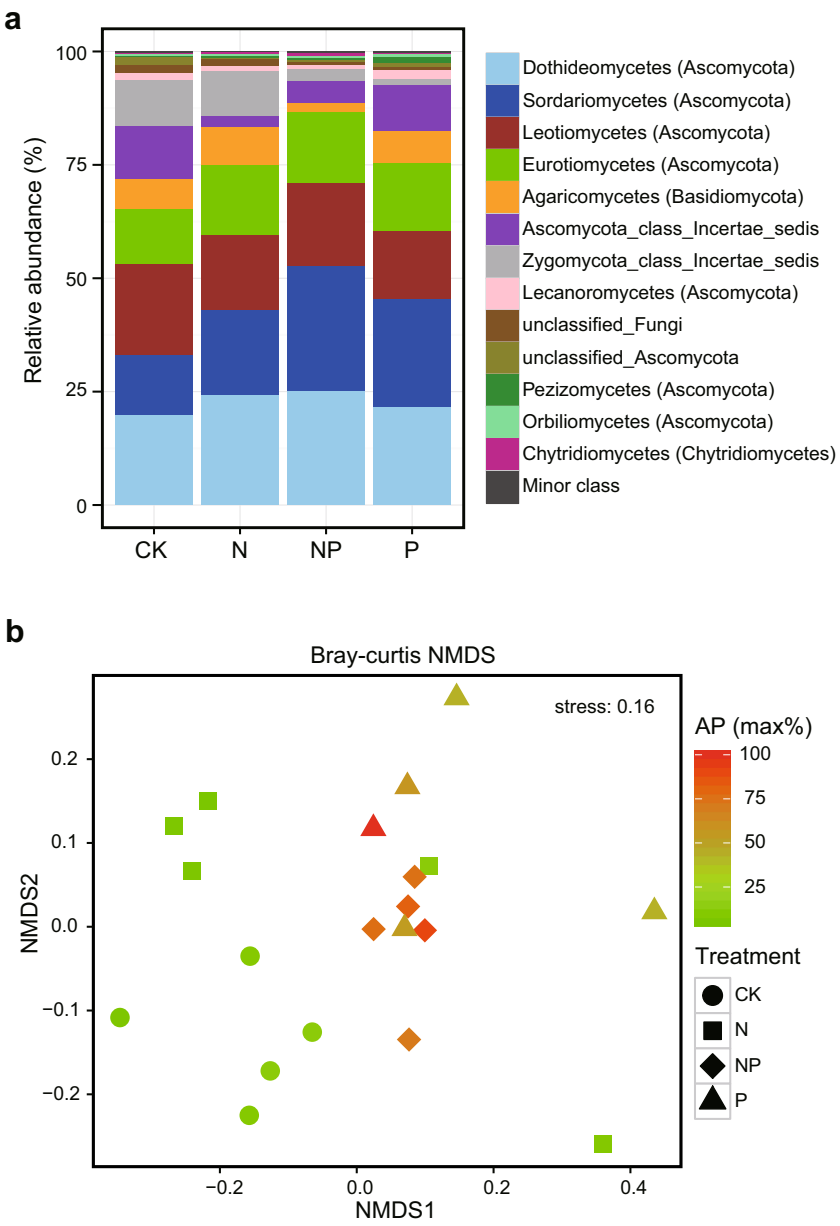

Fig. 2 Variation in soil fungi community compositions between different treatments. **a** Relative abundance of class taxonomy. **b** Non-metric multidimensional scaling plot of $OTU_{0.03}$-level changes in different samples based on Bray-Curtis dissimilarity

diversity, while P addition alone significantly reduced fungal OTU richness (Fig. 1). Two-way ANOVA confirmed the significant effect of the P factor on fungal diversity, and a marginal significance of N and P interaction (Table 2), which explained the also significantly lower richness in the NP treatment (Fig. 1). In previous studies, the effects of N or P on fungal diversity were not uniform. It has been reported that N addition increased fungal diversity in N-deficient soils (Mueller et al. 2014; Weber et al. 2013), but other studies showed that the effect may be opposite depending on ecosystem type and nutrient dose (Sun et al. 2014; Zhou et al. 2016). P addition was reported to reduce the species richness of AMF (Liu et al. 2012b) or the total fungal community (Bao et al. 2013). In old forest soils where N content was already relatively high and P was somewhat deficient, P addition could alleviate P deficiency of both plants and soil microbes and increase the soil fungal biomass (Liu et al. 2012a). In the alpine meadow soil on the QTP, AP was usually rather limited, regardless of the large quantity of total P (Online Resource 1; CK and N treatments in Table 1). However, no increase in fungal diversity was observed for both NP and P additions in our study, suggesting that the pre-existing P deficiency status could not be used directly to predict the positive or negative effects of P additions on fungal diversity.

The difference between N and P effects was also inferred from the MRT analysis, where AP content was the most important factor influencing fungal alpha diversity and DON content was of secondary importance (Fig. 3). In contrast to AP content,

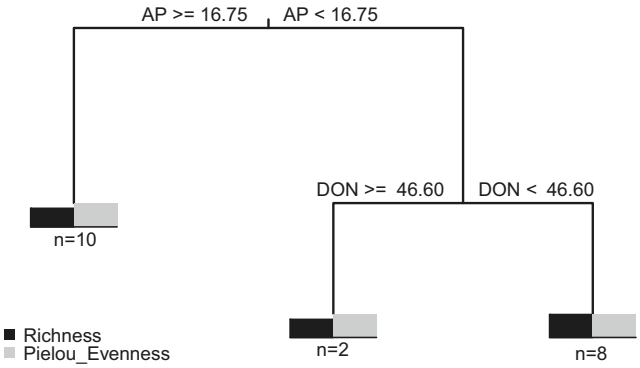

Fig. 3 Multivariate regression tree analysis of alpha diversity with environmental variables. The tree was constructed using the "1se" method with 1000 cross-validations to find the main variables significantly important for alpha diversity. See Table 1 for variable abbreviations. The values for both OTU richness and Pielou's evenness were normalized (divided by maximum values) before construction of the multivariate regression tree

there was no significant difference in measured available N contents between the different treatments (Table 1), possibly owing to the fact that N in soil can be utilized by plants more efficiently than P (Gerloff 1976). The smaller change in soil available N content might be one of the reasons why N addition has less effect on fungal diversity compared with P addition. The soil available N:P ratio was significantly reduced only by adding P (NP and P; Table 1). It has been reported that the available N:P ratio in soil may be a key factor influencing the competitive advantage of different fungal taxa. For instance, non-mycorrhizal fungi outperformed mycorrhizal fungi in a low N:P ratio situation (Chagnon and Bradley 2013). Our study found the abundance of some non-mycorrhizal fungi (e.g., Sordariomycetes) was substantially elevated following P addition (Fig. 2a, Online Resource 4). In addition, though our study was not designed to cover the whole AMF community, we also observed a significant decrease of the abundance of Glomeromycetes (the typical AMF group) in the P treatment (Online Resource 4). The modified competition patterns between different fungal groups might contribute partly to the overall diversity loss in P treatments.

The altered N or P nutrition situations in soil could have indirect effects on fungal diversity through influencing the interspecific relationships of fungi with other biota such as bacteria and soil fauna. Bacteria and fungi can form complex relationships in processes of nutrient acquisition and utilization (Pii et al. 2015). The antagonistic or synergic roles of bacteria to fungi could be changed accordingly to the nutrient type in soil (Meidute et al. 2008). In soils with a lower N:P ratio, bacteria could outperform fungi in decomposing

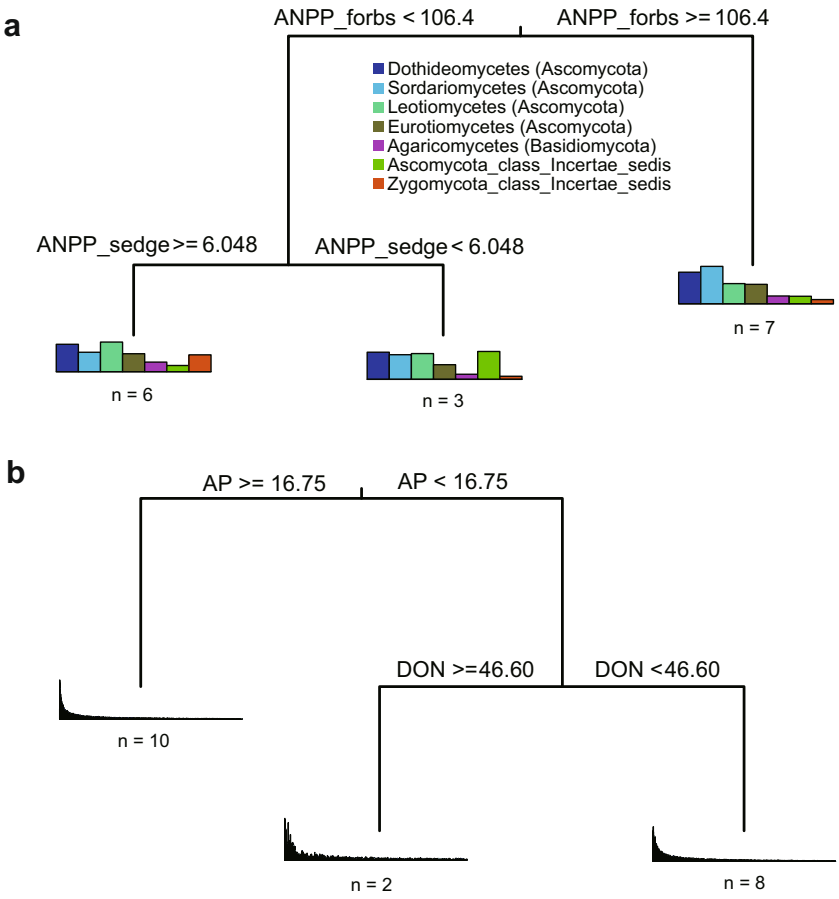

Fig. 4 Multivariate regression tree analysis of soil fungi community composition with environmental variables. **a** Using relative abundance (percent) of the main classes to represent community composition. **b** Using an $OTUs_{0.03}$ table to represent community composition. The tree was constructed using the "1se" method with 1000 cross-validations. See Table 1 for explanations of variable abbreviations

activities (Güsewell and Gessner 2008), which might contribute to a loss of soil fungal diversity. The abundance of soil fauna, including the fungivore, could also be influenced by the nutrient status (Jiang et al. 2015). For example, Wang et al. (2016) found that the typical fungivore collembola was highly positively correlated with DOC and AP contents of soil. Through increasing grazing effect or altering the competitive patterns between different fungal groups (Crowther et al. 2011), soil fauna might contribute to the loss of fungal diversity in the P addition treatments.

Soil fungal community composition as affected by N and P additions

The interactive effect between N and P was at least marginally significant ($P = 0.06$; Table 2, Online Resource 3). We observed the most marked changes of both aboveground plant biomass (Table 1) and the fungal community composition in the combined NP treatment (Fig. 2, Online Resource 5). Previous studies have stressed that N or P addition could change soil fungal community composition, even if the effect on alpha diversity is small (Beauregard et al. 2010; Frey et al. 2004). In studies of forest and tundra soils (Weber et al. 2013; Nemergut et al. 2008), the relative abundance of Basidiomycota was reduced by N addition. However, in the alpine meadow soil of our study, adding N alone reduced Ascomycota_class_Incertae_sedis; a significant reduction in Basidiomycota (class Agaricomycetes) by N addition required addition of P at the same time (Fig. 2a, Online Resource 4).

In the NP treatment, the total aboveground biomass was mostly increased (Table 1). Due to the stoichiometry relationship between N and P in biomass and biological processes, N and P addition alone might exaggerate the pre-existed N or P deficiency (Vitousek et al. 2010). Thus, only N and P combined addition could really alleviate the nutrient deficiency of plants and caused the most elevated aboveground biomass. However, the promotion by N and P was not evenly distributed among plant groups. Plants from sedge and legume groups might lose their competition advantages prominent in a situation of low nutrient status (Jiang et al. 2012), and by contrast, grasses and forbs increased substantially in the NP treatment (Table 1). This result is consistent with one previous study about the effects of fertilizer on plant communities in an alpine meadow (Sun et al. 2015). The greatest changes in plant biomass in the NP treatment could serve as a main reason for the most marked change of fungal community composition. For example, at the class level, changes in fungal community composition and plant parameters (i.e., ANPP of forbs and sedge) were strongly related in MRT analysis (Fig. 4a).

Forbs in the alpine meadow of the QTP contain high concentrations of secondary metabolites and are relatively unpalatable to animals (Meier et al. 2008). The secondary metabolites or root exudates of these forbs might have a strong selective effect on the community composition of soil fungi, resulting in a community with a lower turnover rate in the NP treatment (Online Resource 6). Some forbs contain high concentrations of tannins (Lukač et al. 2012), which can decrease the abundance of Agaricomycetes (Winder et al. 2013). Besides, the ratio of fungivore to bacterivores in the nematode community was highest under forbs than the other functional group in experimental grasslands (Viketoft et al. 2005). The increased fungi-feeding nematodes could also contribute to the diversity and composition changes in the soil fungal community. Both forbs and grasses, with their ANPP higher in the NP treatment than in the controls (Table 1), had a characteristic of low leaf N:P ratios than legumes and sedges in the alpine meadow (Xu et al. 2014). The N:P ratio could affect the enzyme activity in litter decomposing processes (Güsewell and Verhoeven 2006) and thus might change the composition of the soil fungal community, especially that of the saprophytic groups.

A set of indicator OTUs showed more response to the combined treatment than the sole N and P treatments (Online Resource 7). This result, combined with the lower beta diversity of the fungal communities in the NP treatment (Online Resource 6), suggests that adding N and P together could result in a specific fungal community with more spatial homogeneity than when N and P were added alone. OTUs from the orders *Chaetothyriales* and *Sordariales* and the family *Nectriaceae* were indicator species. Members of *Chaetothyriales* can live as endophytes on plant roots (Narisawa et al. 2007) or be ectomycorrhiza-associated fungi (Tedersoo et al. 2009). Members of *Sordariales* can degrade biopolymers, such as lignin, and humic compounds (Poggeler 2011). *Nectriaceae* includes numerous important plant pathogens, as well as several species extensively used as biodegraders or biocontrol agents (Lombard et al. 2015). There remains a lack of direct evidence about these indicator species in mediating plant community assembly. Studies from other grasslands have shown that endophytic fungi can help the host forb plants gain competitive advantage (Aschehoug et al. 2012), change plant community composition (Rudgers et al. 2010), or affect the litter degradation of the host grasses (Lemons et al. 2005). The pathogenic fungi might also contribute to alteration of plant community composition through their selectively pathogenic effects on different plant functional groups. In any case, these indicator fungal taxa in the combined NP treatment deserve further examination and may provide new clues explaining the marked changes of both plant and fungal community compositions caused by simultaneous N and P addition.

The edaphic factors important for fungi in alpine meadow soil

At the lower taxonomic ($OTU_{0.03}$) level of fungi, the measured plant parameters were found to have less influence than edaphic factors, such as AP and DON content, on fungal

community diversity and composition (Figs. 3 and 4b). The inconsistency between the class level and the $OTU_{0.03}$-level responses of diversity patterns to environmental changes has also been observed for bacterial communities (Ruiz-González et al. 2015). It is possible that there is a great deal of functional redundancy and equivalency among OTUs in a specific phylum or class of soil fungi (Allen et al. 1995; Rineau and Courty 2011), such that different OTUs within the group may react similarly to changes in the soil environment caused by plants. It is well known that the AP content is of key importance in the ecology and physiology of arbuscular mycorrhizal fungi (Covacevich et al. 2007; Alguacil et al. 2010). A recent study indicated that the denaturing gradient gel electrophoresis patterns of the total soil fungal community were shaped mostly by the AP content of an arable Andosol soil (Bao et al. 2013). Though the AP content could affect soil fungi indirectly by causing changes in plant biomass, there may also be extra ecological effects related to AP content, such as changing nutrient status for fungi, influencing bacteria and soil fauna, altering the competing scenarios between different fungal groups, or other unknown mechanisms, which would act profoundly to induce a corresponding community pattern at the $OTU_{0.03}$ level.

In addition to the AP content, the soil properties such as DON and DOC contents showed secondary important roles in shaping the composition of the soil fungal community (Online Resource 8, Online Resource 9). While little is known about the direct effects of DOC and DON contents on composition of the soil fungal community, there have been some lines that can be used to infer their relationships. The DOC containing readily decomposable substances such as organic acids and carbohydrates is probably mainly used by bacteria other than fungi (Marschner et al. 2003). However, Waldrop and Zak (2006a) found that the decrease of DOC content in forest soils was associated with the decrease of soil fungal abundance reflected by the ribosomal intergenic spacer analysis. In our study, the DOC contents were lower (though not significantly) in the N and NP treatments (Table 1), which might correspond to the less fungal richness compared with CK (Fig. 1). Pena et al. (2010) found that the diversity of ectomycorrhizal fungi was positively correlated to the DOC/DON ratio in forest soils. Since the ectomycorrhizal fungi are primarily formed by Agaricomycetes (Rinaldi et al. 2008; Tedersoo et al. 2010), it is possible that the decrease of Agaricomycetes observed here could also be ascribed to the specifically low DOC/DON ratio in the NP-treated soils (Table 1).

Conclusions

In our experiment, N and P fertilization had strong influences on the composition of the soil fungal community in alpine meadow soil on the Qinghai-Tibetan Plateau. Phosphorus had a stronger influence than N addition in affecting the soil fungal community composition. The interactive effect of N and P was also important, which contributed at least partly to the more specific soil fungal community composition in the NP treatment.

In future studies, the relative influence of N and P addition on fungal communities should be tested in a greater range of habitats, and the interactive effect of N and P needs to be stressed when examining the influence of fertilization on soil microorganisms and ecosystem functions. It is also necessary to study the effects of N and P addition on the natural baseline functioning of soil ecosystems and microbial communities, for example, the importance of these nutrients as limiting factors in microbial activity and biogeochemical fluxes.

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Compliance with ethical standards

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Conflict of interest The authors declare that they have no conflicts of interest.

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