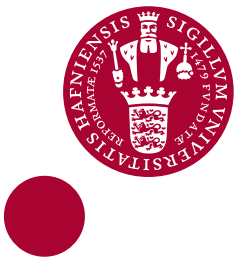

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Contrasting effects of long-term fertilization on the community of saprotrophic fungi and arbuscular mycorrhizal fungi in a sandy loam soil

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ABSTRACT

The changes of saprotrophic fungi (SF) and symbiotic arbuscular mycorrhizal fungi (AMF) in response to different fertilizers were investigated in a period of 21 years. Denaturing gradient gel electrophoresis profiles showed fungal community structure significantly changed after long-term fertilization. Long-term organic and mineral fertilization significantly increased the SF diversity, whereas mineral fertilization decreased the AMF diversity. SF quantity significantly increased in response to organic fertilizers, whereas the AMF propagules were down-regulated by nutrient-rich fertilization but induced by N, P or K-deficiency. Redundancy analysis showed that long-term fertilization differentially affected diversity and quantity of SF and AMF. Nutrient-rich organic fertilizers, resulting in higher contents of soil organic C (SOC), total N and mineral N, total and available P, regulated the quantity and diversity of SF positively and quantity of AMF negatively, respectively. The diversity of AMF was slightly down-regulated by SOC, total and mineral N and total P as well as the nutrient-rich mineral treatments, in contrast to the positive effects by available K and P. These results indicate that soil nutritional status and fertilizer types significantly affect SF and AMF. Our study of soil fungal community in response to the long-term fertilization can provide new strategies for agronomic practice.

Keywords: fungal rRNA gene; Shannon-Wiener diversity; fungal quantity; microbial communities; quantitative-PCR

Long-term organic fertilization has stronger impacts on microbial communities than mineral fertilization in agricultural soils with regards to improving soil fertility, microbial biomass and biodiversity (Mäder et al. 2002, Zhong et al. 2010, Zhang et al. 2014); similarly, long-term organic fertilization results in higher richness and diversity of bacteria (Ge et al. 2008). However, most investigations of the effects of fertilization on soil microbial communities were focused on bacterial community rather than fungi (Prévost-Bouré et al. 2011).

Fungi are one of the largest and most diverse kingdoms of eukaryotes and function as important biological components in terrestrial ecosystems (Cuadros-Orellana et al. 2013). Saprotrophic fungi (SF) are the largest group of fungi in soil, obtaining carbon by degrading organic compounds (Cuadros-

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Orellana et al. 2013). Arbuscular mycorrhizal fungi (AMF), as obligate symbionts with plants in soil, obtain carbon from plants assimilation products and supply mineral nutrients like phosphorus to plants as an exchange (Smith and Read 2008, Lin et al. 2012). SF community is strongly influenced by organic carbon contents in the soil (Zhong et al. 2010, Prévost-Bouré et al. 2011), whereas AMF are associated with different types of soil organic carbon (Hršelová et al. 1999, Verbruggen et al. 2010, Kiers et al. 2011), N and P fertilization (Johnson et al. 2003, Beauregard et al. 2013). Totally, previous studies mainly focused on the life styles of SF or AMF as well as the effect of mono-element such as soil N and P on SF and AMF communities. The impacts of multiple fertilization regimes on fungal communities have not been fully elucidated.

The aim was to investigate the contrasting effects of multiple fertilizers on SF and AMF under long-term fertilization regimes in a specific soil site. Accordingly, we sampled soils that had been supplied with organic and mineral fertilizers with different combinations over 21 years and analyzed community structures of SF and AMF, the relationships between fungal numbers and diversity, chemical properties, and fertilization treatments.

MATERIAL AND METHODS

Description of site and soil sampling. The site history and experiment design were described by Chen et al. (2011). Briefly, this study was established in 1989 in Fengqiu, China (35°00'N, 114°24'E). The soil is a typical sandy loam in North China. The planted crops were summer maize and winter wheat. Seven different treatments of fertilizers included organic manure (OM), one-half N from organic manure plus one-half N from mineral N fertilizer (1/2OMN), mineral fertilizers of NPK, NP, NK, PK, and a control (CK) without fertilizer. Four replicates for each treatment were prepared in fully randomized blocks.

Soil sampling and chemical analysis. In October 2010 after maize harvest, the soil was sampled at a depth of 0–15 cm. For each plot, 16 cores were sampled and mixed. The soil sample was air-dried for chemical analysis, most probable number (MPN) bioassay of infective propagules and molecular analysis. Soil chemical analysis was conducted as described by Chen et al. (2011).

DNA extraction and nested PCR. Genomic DNA from each soil sample was extracted using the Fast DNA ®SPIN Kit for Soil (Qbiogene, Carlsbad, USA). The extracted DNA diluted 10 times was used as the template for the first PCR. The first PCR product was 100-fold diluted and used as the template in the second PCR reaction. The PCR reaction mixtures (25 µL) contained 2.5 µL of 10 × Buffer, 2 µL of dNTP, 0.2 µL of $MgCl_2$, 0.4 µL of forward primer (10 µmol), 0.4 µL of reverse primer (10 µmol), 0.5 µL of Easy Taq DNA polymerase (5 U/µL), 1 µL of template and 18 µL of ddH_2O.

Fungal internal transcribed spacer (ITS) regions were amplified for detection of SF. SF-specific primers ITS1-F (Gardes and Bruns 1993) and ITS4 (White et al. 1990) were used in the first-round reaction, followed by the second-round amplification with primers ITS1-F and a GC clamp attached ITS2 (White et al. 1990, Muyzer et al. 1993).

AMF-specific primer AM1 (Helgason et al. 1998) and the universal eukaryotic primer NS31 (Simon et al. 1992) were used to amplify 18S rRNA gene of AMF in the first-round reaction. The second-round primers were Glo1 (Cornejo et al. 2004) and NS31-GC (Liang et al. 2008).

DGGE and Shannon-Wiener diversity. The PCR products were purified by the AxyPrep™ PCR Cleanup Kit and analyzed by DGGE with a Dcode™ Universal Mutation Detection System (Bio-Rad). The gels (SF 30–60% and AMF 30–50%; 8% polyacrylamide) were run in 1 × TAE buffer at 120 V for 5 min and 60 V for 16 h. Gels were stained by silver-staining containing 0.2% silver nitrate. The Shannon-Wiener diversity was performed according to DGGE patterns analyzed by the Quantity One Analysis Software version 4.62 (Bio-Rad, Hercules, USA). Three DGGEs were performed for three biological replicates of soil samples. Fungal sequences originally excised from the acrylamide gel (Liang et al. 2008) were submitted to the GenBank database and allocated accession numbers JQ 900263-JQ 900299 for SF and JQ433817–JQ433841 for AMF.

Quantification of saprotrophic fungi by quantitative-PCR. qPCR for quantification of SF was carried out with 10 µL of SYBR Green PCR master mix (Bio-Rad), 0.4 µL of ROX reference, 0.4 µL of forward primer ITS1-F and reverse primer ITS2 (10 µmol), 1 µL of DNA extract and Milli-Q water in a final volume of 20 µL using ABI 7300 Sequence Real-Time PCR System (Applied Biosystems, Foster City, USA).

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The qPCR was run in triplicates using the following program: 95°C for 30 s, 42 cycles (95°C for 5 s, 51°C for 45 s, 72°C for 31 s), and a final disassociation stage of 95°C for 15 s and 60°C for 15 s. The specificity of the PCR was determined by a melting curve analysis. Standard curves were created using a 10-fold dilution series of gDNA containing SF ITS region.

Determination of propagules of AMF. The number of infective AMF was analyzed by the most probable number of infective propagules (MPN) bioassay (Smith and Read 2008). Soil samples were diluted by the sterilized sandy soil (pH 8.6) in a 10-fold series ranging from 10^0 to 10^{-3}. Four technical replicates were prepared for each treatment. Trap plant was grown using semi-hydroponics in the diluted soil samples supplied with 10% of phosphorus Hoagland's nutrient solution and harvested after forty days. Roots were stained by trypan blue and examined by microscope to determine AMF propagules in soils.

Statistical analysis. Statistical procedures were performed using SPSS 16.0 (Chicago, USA). Significant differences were determined by the Duncan's multiple range test at $P < 0.05$. Redundancy analysis (RDA) was calculated by Canoco version 4.5 (Ithaca, USA). The results were derived from three or four replicates of soil samples.

RESULTS

Soil chemical properties. A summary of the soil pH and nutrient contents after long-term (21 years) fertilizer management was presented in Table 1 ($P < 0.05$). Long-term application of manure and inorganic NPK fertilizers significantly decreased soil pH. Soil organic C and total P contents significantly increased under all treatments except for the NK treatment. More than 10-fold increase in available P content was observed under treatments of OM, 1/2OMN and PK. Total N significantly increased under all treatments except for the NK and PK treatments, whereas mineral N significantly increased under organic treatments. Soil total K contents were not significantly affected by either long-term fertilization treatment, whereas available K significantly increased in all treatments except for in NP treatment.

Diversity of SF and AMF. The DGGE patterns of SF (Figure 1a) and AMF (Figure 1b) indicated that the long-term fertilization remarkably affected the structure of SF and AMF community. Phylogenetic analysis of SF sequences of PCR products extracted from 37 dominant gel bands (excepted for 2 chimeric sequences) were identified as four major SF groups, Ascomycota, Zygomycota, Basidiomycota and unknown clade (Figure 2), as well as 21 dominant AMF gel bands were identified as *Glomus* spp. and *Diversispora* spp. (Figure 3).

The Shannon-Wiener diversity (H') of SF and AMF from DGGE profiles were shown in Figure 4. The H' of SF significantly increased under six long-term fertilization treatments compared to the CK ($P < 0.05$), while the H' of AMF decreased in response to PK, NPK and 1/2OMN treatments.

Quantification of SF and AMF biomass. The standard fungal ITS DNA curve demonstrated a

Table 1. Soil chemical properties under long-term fertilization regimes

Treatment	pH	Organic C (g/kg)	Total N (g/kg)	Mineral N (mg/kg)	Total P (g/kg)	Available P (mg/kg)	Total K (g/kg)	Available K (mg/kg)
CK	8.81 ± 0.07^{bc}	4.73 ± 0.29^{a}	0.45 ± 0.03^{a}	4.00 ± 0.48^{a}	0.53 ± 0.03^{a}	0.77 ± 0.73^{a}	21.0 ± 0.7^{ab}	92.04 ± 2.70^{b}
NK	8.85 ± 0.07^{c}	4.58 ± 0.12^{a}	0.51 ± 0.03^{ab}	4.37 ± 1.78^{ab}	0.52 ± 0.03^{a}	0.74 ± 0.79^{a}	22.1 ± 0.5^{b}	344.76 ± 7.15^{e}
PK	8.81 ± 0.09^{bc}	5.45 ± 0.08^{b}	0.56 ± 0.04^{ab}	3.92 ± 1.37^{a}	0.90 ± 0.03^{c}	19.88 ± 0.60^{d}	21.6 ± 0.3^{ab}	293.28 ± 7.15^{d}
NP	8.68 ± 0.11^{ab}	5.81 ± 0.19^{b}	0.62 ± 0.00^{b}	4.38 ± 0.86^{ab}	0.72 ± 0.05^{b}	4.97 ± 1.79^{ab}	20.6 ± 0.3^{a}	71.76 ± 9.74^{a}
NPK	8.64 ± 0.13^{a}	5.86 ± 0.39^{b}	0.62 ± 0.03^{b}	4.48 ± 0.72^{ab}	0.78 ± 0.05^{b}	4.43 ± 1.42^{a}	22.0 ± 0.5^{b}	154.44 ± 12.38^{c}
1/2OMN	8.61 ± 0.03^{a}	7.85 ± 0.15^{c}	0.84 ± 0.11^{c}	6.17 ± 1.61^{bc}	0.75 ± 0.01^{b}	9.24 ± 1.76^{bc}	21.7 ± 0.4^{ab}	171.60 ± 2.70^{c}
OM	8.57 ± 0.08^{a}	9.45 ± 0.03^{d}	1.12 ± 0.02^{d}	6.48 ± 1.21^{c}	0.73 ± 0.02^{b}	13.50 ± 3.99^{c}	21.5 ± 0.8^{ab}	170.04 ± 7.15^{c}

Data reported as means ± SE followed by the different letters in each column are significantly different according to Duncan's multiple range test at the 0.05 level of probability. CK – no fertilizer; NK – mineral NK fertilizer; PK – mineral PK fertilizer; NP – mineral NP fertilizer; NPK – mineral NPK fertilizer; 1/2OMN – half organic manure plus half mineral fertilizer; OM – organic manure

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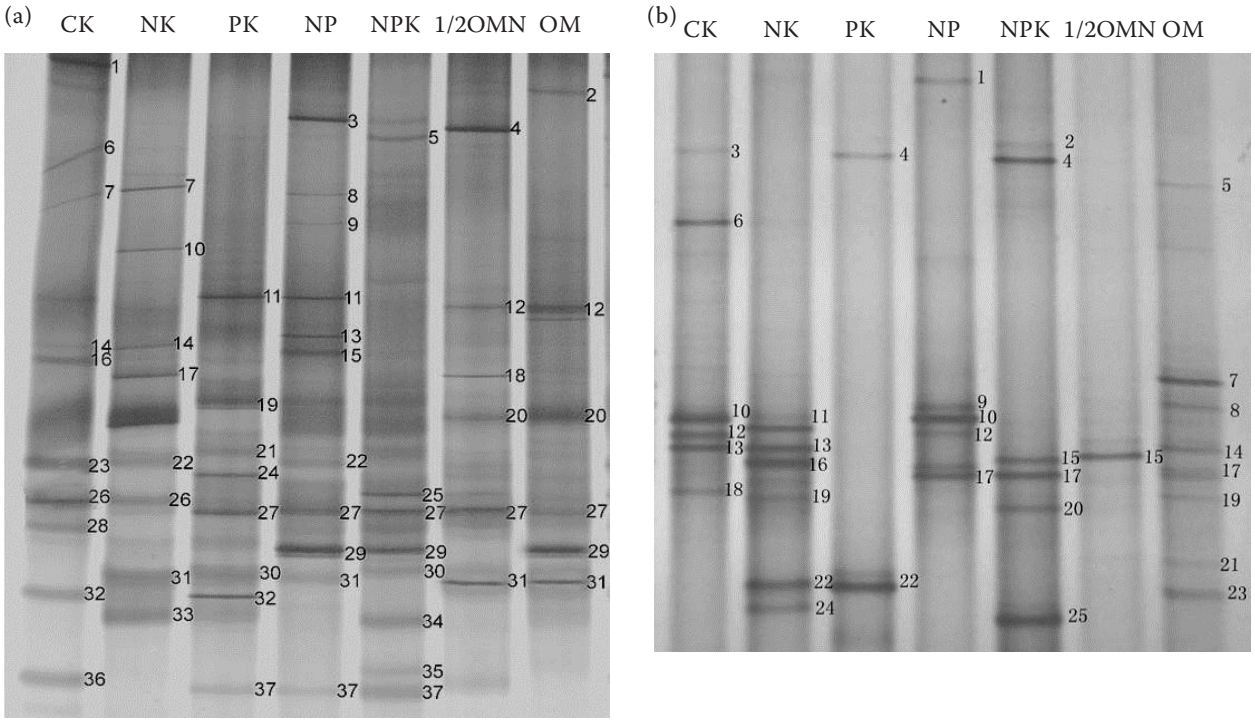

Figure 1. The representative PCR-DGGE profile of gene fragments of saprotrophic fungi (SF) and arbuscular mycorrhizal fungi (AMF) under long-term fertilization regimes. (a) SF internal transcribed spacer (ITS) gene fragments with the denaturing gradient from 30–60%. (b) AMF 18S rRNA gene fragments with the denaturing gradient 30–50%. Three DGGE profiles were performed for three replicates of soil samples. PCR products were extracted from marked bands on one gel and cloned for sequencing. CK – no fertilizer; NK – mineral NK fertilizer; PK – mineral PK fertilizer; NP – mineral NP fertilizer; NPK – mineral NPK fertilizer; 1/2OMN – half organic manure plus half mineral fertilizer; OM – organic manure

positive linear relation (R^2 = 0.998) between log values of fungal DNA and the Ct values (Figure 5a). The qPCR assays showed that SF ITS gene number varied from 1.28 × 10^9 to 9.58 × 10^9 copies/g soil (Figure 5b). The number of SF ITS marker sequence copies under the treatments of 1/2OMN and OM was significantly higher than that under mineral fertilizer treatments. The OM treatment resulted in the most abundant SF in soil (P < 0.05). The numbers of SF under mineral fertilizers had no significant differences among one another.

The MPN analysis revealed that noticeable differences occurred in AMF quantity. The infective propagules of AMF ranged from 2.5–7.75 (Figure 6). In addition, the propagule number was significantly lower under the nutrient-enriched fertilization (NPK, 1/2OMN and OM) than that under CK (P < 0.05). NK, PK and NP treatments resulted in increased AMF propagules, especially the highest propagule number observed in NK treatment of P-deficient stress (P < 0.05), compared to NPK treatment.

Statistic characterization of fungal diversity and quantity to chemical properties and fertilizer treatments. RDA clearly demonstrated that relationship of diversity and quantity of SF and AMF, soil chemical properties, six fertilization treatments and control (Figure 7). The long-term fertilization treatments affected the diversity and quantity of SF and AMF in different manners. Nutrient-rich organic fertilizers (OM and 1/2OMN), resulting in lower pH and higher contents of SOC, TN, MN, TP and AP than control (CK), regulated the quantity and diversity of SF positively and quantity of AMF negatively. In contrast, the diversity of AMF was down-regulated by long-term nutrient-rich mineral treatments NPK and 1/2OMN. The diversity of AMF was slightly negatively affected by the contents of SOC, TN, MN and TP but by AK and AP positively, respectively. Soil TK had smallest influence on both quantity and diversity of SF and AMF than the other nutrients.

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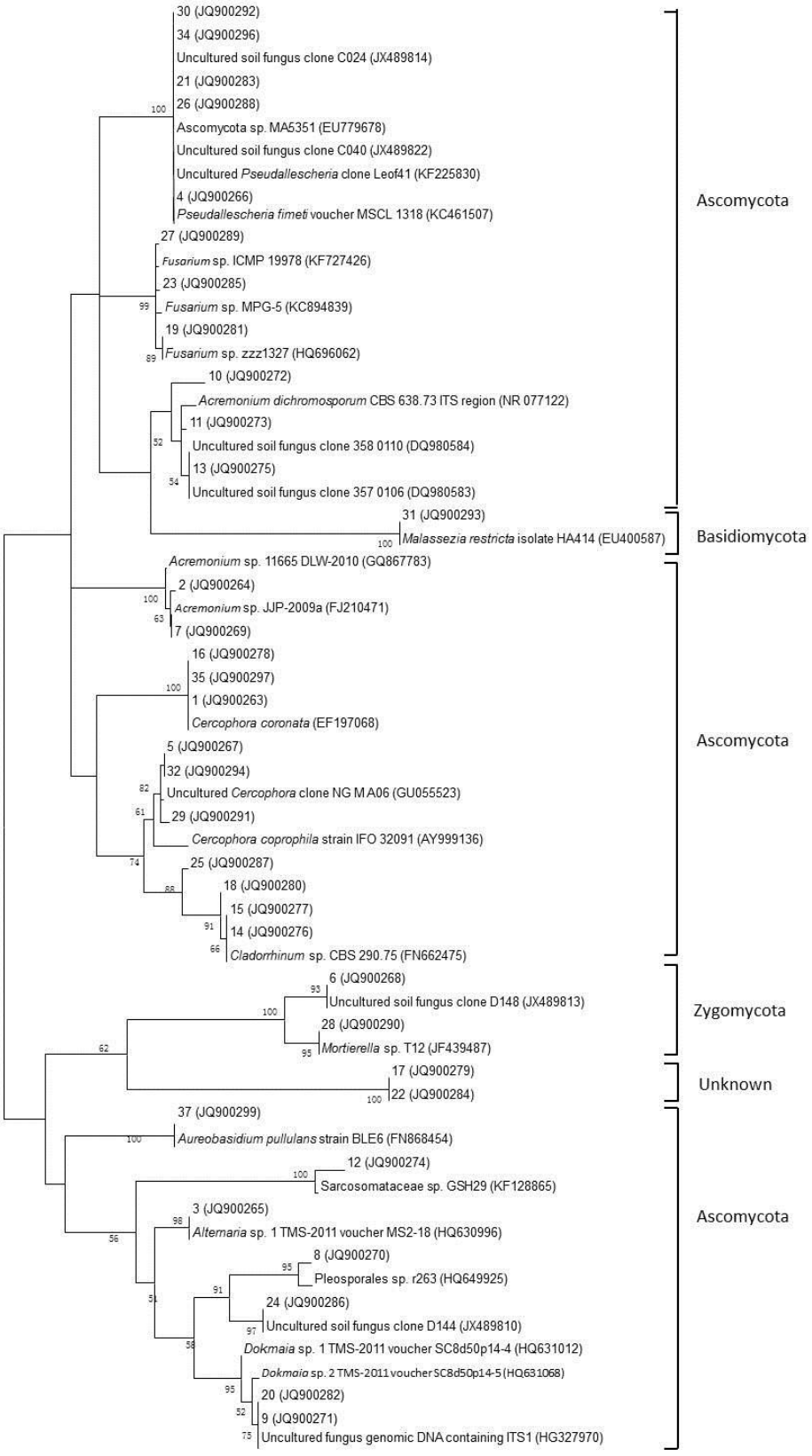

Figure 2. Neighbor-joining phylogenetic tree of saprotrophic fungi (SF) internal transcribed spacer (ITS) gene sequences. Bootstrap values over 50% from 1000 replications are shown on the branches. Numbers from 1–37 excepted for chimeric sequences 33 and 36 on the phylogenetic tree indicate gel bands marked in Figure 1a

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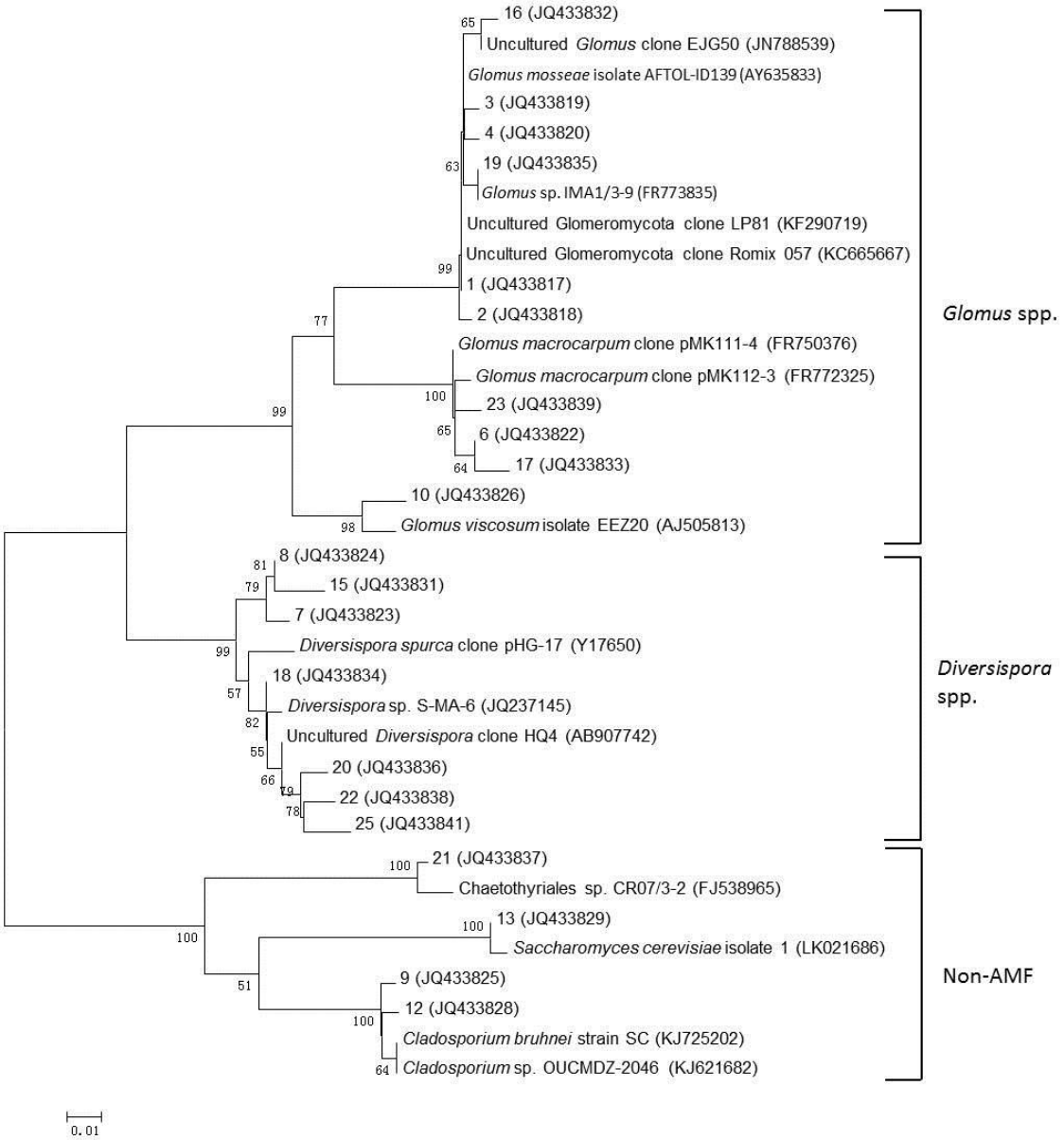

Figure 3. Neighbor-joining phylogenetic tree of arbuscular mycorrhizal fungi (AMF) 18S rRNA gene sequences. Bootstrap values over 50% from 1000 replications are shown on the branches. Numbers from 1–25 excepted for 4 chimeric sequences on the phylogenetic tree indicate gel bands marked in Figure 1b

DISCUSSION

SF growth stimulated by organic fertilization and AMF propagules increased by nutrition-deficient fertilization. Fertilization is one of the most important agricultural managements influencing the population densities and activity of soil microorganisms. Repeated application of organic fertilizers can increase microbial biomass and improve soil biological functions by a direct supply of exogenous organic C to the soil (Diacono and Montemurro 2010). Furthermore, organic fertilizers can indirectly affect soil microbial community by increasing plant growth and stimulating root exudation (Kumar et al. 2013). These findings are confirmed by our observation of a significant increase in SF diversity under both organic and mineral fertilizations (Figure 1) and in SF quantity under organic fertilization (Figure 5b). The reason for increased SF quantity under organic fertilizers (Figures 5b and 7) may be that SF can obtain carbon source from decomposing

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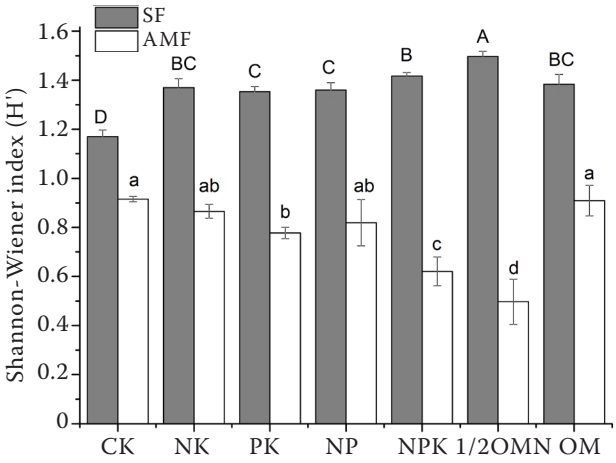

Figure 4. The diversity indices of saprotrophic fungi (SF) and arbuscular mycorrhizal fungi (AMF) under long-term fertilization regimes. Data are means of three replicates of DGGE based on replicates of soil samples. Different capital and lower letters above bars indicate significant differences among seven treatments ($P < 0.05$) for SF and AMF, respectively. CK – no fertilizer; NK – mineral NK fertilizer; PK – mineral PK fertilizer; NP – mineral NP fertilizer; NPK – mineral NPK fertilizer; 1/2OMN – half organic manure plus half mineral fertilizer; OM – organic manure

recalcitrant organic matters (Boddy et al. 2007), and that organic inputs directly benefit SF growth and facilitate the evolution of SF communities. This interpretation was further confirmed by SF quantity that increased significantly by the higher contents of SOC, TN and MN (Figure 7).

On the contrary, we observed a negative effect of long-term organic fertilization (1/2OMN and

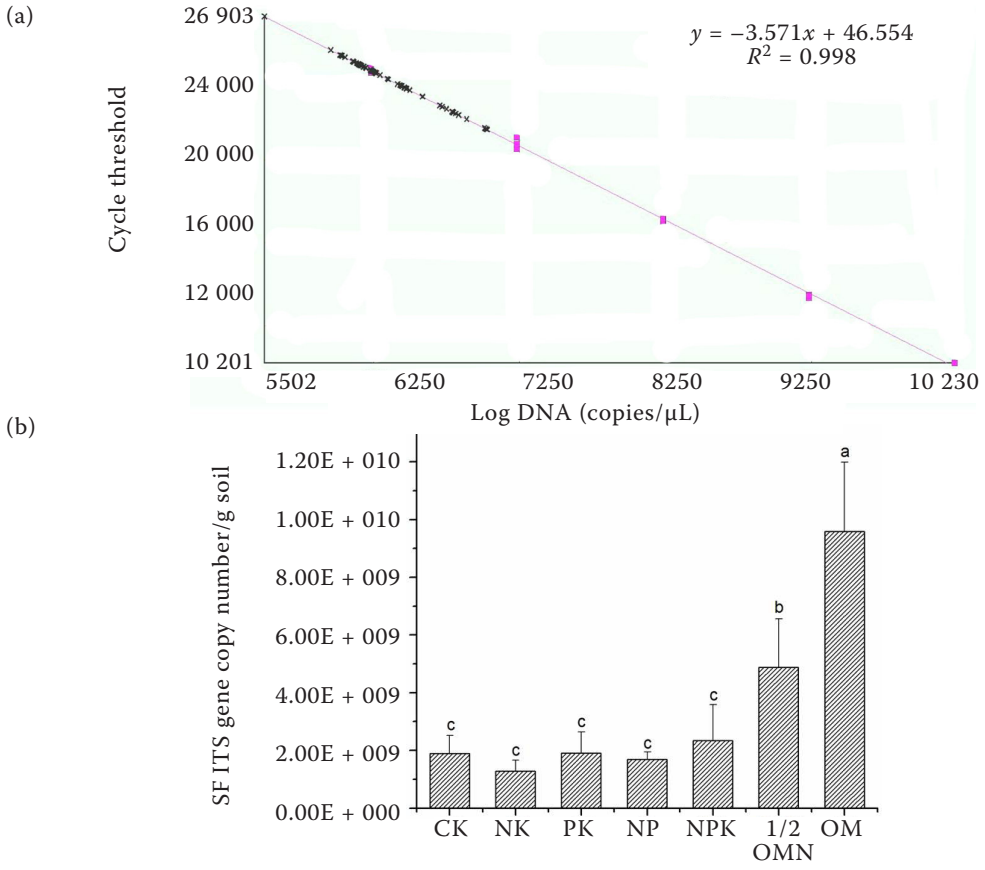

Figure 5. Saprotrophic fungi (SF) quantity under long-term fertilization regimes estimated by qPCR. (a) Standard curve was obtained by amplification of SF ITS gene using a 10-fold dilution series of template DNA. (b) ITS gene copy number in soil samples. Data are means of three replicates with standard deviation. Different letters above bars indicate significant differences among seven treatments ($P < 0.05$). CK – no fertilizer; NK – mineral NK fertilizer; PK – mineral PK fertilizer; NP – mineral NP fertilizer; NPK – mineral NPK fertilizer; 1/2OMN – half organic manure plus half mineral fertilizer; OM – organic manure

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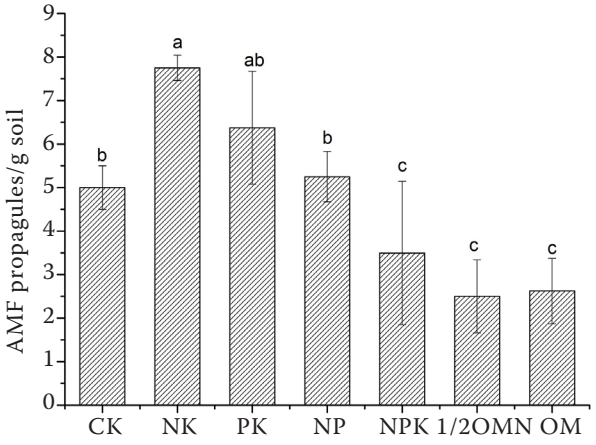

Figure 6. Arbuscular mycorrhizal fungi (AMF) propagules under long-term fertilization regimes estimated by the most probable number (MPN) method. Data are means of four replicates with standard deviation. Different letters above bars indicate significant differences among seven treatments ($P < 0.05$). CK – no fertilizer; NK – mineral NK fertilizer; PK – mineral PK fertilizer; NP – mineral NP fertilizer; NPK – mineral NPK fertilizer; 1/2OMN – half organic manure plus half mineral fertilizer; OM – organic manure

OM) as well as nutrient-enriched mineral fertilization (NPK) on the propagules of AMF (Figure 6). In general, plants with long-term stress of P- or N-deficiency stimulate the AMF population (Antunes et al. 2012). In accordance with it, we observed that the deficiency of mineral N, P or K fertilizer (NK, PK and NP) increased AMF propagules (Figure 6). Especially in NK treatment, mineral N most increased AMF propagules at P- and SOC-deficient status (Figure 6, Table 1). The possible reason is that higher ratio of soil N:P changes C allocation to AM structures, and low C availability can still support the explorative development of the AM mycelium when P availability is low (Johnson et al. 2003, Olsson et al. 2014). Under the nutrient-deficient environment, plants can detect, discriminate, and reward the appropriate fungal partners via releasing more soluble carbohydrates; in return, their fungal partners increase nutrients transfer to those roots providing carbohydrates (Kiers et al. 2011). Under a nutrient-enriched environment, mutualism is less important to plants and fungi due to the reduction of nutrients transfer (Smith and Read 2008), and thus the dependency of plant on AMF fades away (Lin et al. 2012). Hence, nutrient-stressed plants support mycorrhizae more than plants with ample nutrients (Johnson et al. 2003). RDA analysis (Figure 7) also clearly showed that AMF quantity

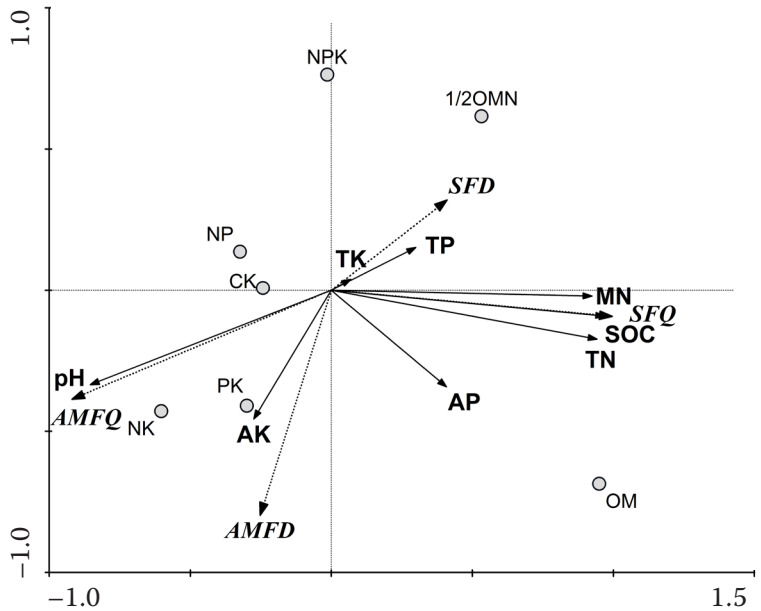

Figure 7. Redundancy analysis of the relationship between saprotrophic fungi (SF), arbuscular mycorrhizal fungi (AMF), fertilization treatments and soil chemical properties. The analysis was based on the data of qPCR of SF ITS gene (SFQ), propagules of AMF (AMFQ), diversity index of SF (SFD) and AMF (AMFD), and soil chemical properties under seven long-term fertilization regimes. SOC – organic C; TN – total N; MN – mineral N; TP – total P; AP – available P; TK – total K; AK – available K; CK – no fertilizer; NK – mineral NK fertilizer; PK – mineral PK fertilizer; NP – mineral NP fertilizer; NPK – mineral NPK fertilizer; 1/2OMN – half organic manure plus half mineral fertilizer; OM – organic manure

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was negatively affected by the contents of SOC, TN, MN and TP. Taken together, our results suggest that the quantity of AMF is not only related to SOC content, but also strongly influenced by the changes in soil nutritional status including N, P and K contents.

Increased SF diversity in contrast to decreased AMF diversity by most fertilization regimes. In addition to the alternation of fungal quantity, the diversity of SF and AMF were differentially regulated by the long-term fertilization. SF diversity increased in all fertilization treatments compared to control (Figure 4), probably due to the increased contents of soil nutrients such as SOC, TN, MN and TP (Figure 7). Previous study on AMF communities under different practices has demonstrated that organic management enhances the diversity of AMF assemblages, compared to conventional management practice (Verbruggen et al. 2010). However, we observed either a negative (1/2OMN) or no effect (OM) of long-term organic fertilization on the diversity of AMF, whereas mineral fertilization treatments (NP, PK, NK and NPK) caused a decrease in AMF diversity, compared to CK (Figure 4). Additionally, SOC, TN, MN and TP had a less negative impact on the diversity of AMF than the quantity of AMF (Figure 7), suggesting that the diversity and quantity of AMF respond to the nutritional status in the different manners.

In conclusion, a detailed analysis of SF and AMF community in response to the long-term organic and mineral fertilization was present. Higher contents of SOC, TN, MN and TP negatively affected AMF quantity and diversity. In contrast, organic fertilization stimulated SF growth. A possible reason of this difference is that SF depends on SOC from organic fertilizers in contrast with AMF dependent on plant C assimilation that is related to soil nutritional status and fertilizer types. A potential tradeoff between positive effects of AMF and SF communities on soil sustainability and negative effects on crop productivity has to be considered in order to provide new strategies for agronomic practice.

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