Soil Biology & Biochemistry 113 (2017) 1–13

Contents lists available at ScienceDirect

Soil Biology & Biochemistry

journal homepage: www.elsevier.com/locate/soilbio

Evident variations of fungal and actinobacterial cellulolytic communities associated with different humified particle-size fractions in a long-term fertilizer experiment

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ARTICLE INFO

Article history:
Received 8 September 2016
Received in revised form
24 May 2017
Accepted 26 May 2017

Keywords:
Particle-size fraction
Fertilization
Humus
Cellulolytic fungi
Cellulolytic actinobacteria

ABSTRACT

Cellulose is the dominant form of carbon (C) existing in arable soils, however the ecology of its degradation in soil is still relatively poorly understood. Here, community abundance and composition of fungal and actinobacterial cellulolytic genes (*cbhI* and *GH48*) from glycoside hydrolase family 7 and 48 together with characterization of fulvic acid (FA) and humic acid (HA) determined by cross polarization magic angle spinning (CPMAS) ^{13}C nuclear magnetic resonance (NMR) spectroscopy were explored in five soil particle-size fractions (large macroaggregate, coarse sand, fine sand, silt and clay), collected from a 33-yr mineral and organic fertilizer experiment. The results revealed the significant effects of particle-size fraction and fertilization on the distribution of soil humus and cellulolytic microbial community abundance. Strong correlations were detected between C content and structure of soil humus with cellulolytic microbial abundance. Generally, larger fractions (>63 μm) especially fine sand, which showed a lower degree of humification with higher aromaticity, lower HA/FA ratio, aliphaticity and alkyl/O-alkyl ratio of HA, were associated with greater abundance of cellulolytic microbes. However, smaller fractions (<63 μm), especially the clay fraction, showed lower *cbhI* and *GH48* gene abundances with a greater degree of humification indicated by ^{13}C NMR spectra. Phylogenetic analysis of the obtained nucleotide sequences revealed undiscovered sequences of both fungal and actinobacterial cellulolytic microbes. However, no clear clustering of sequences from particular particle-size fraction or fertilizer treatment was observed, even though combined application of chemical fertilizer and manure significantly increased cellulolytic gene abundances.

1. Introduction

Soils, with non-uniform distribution of nutrients (Balser et al., 2006) across different particle-size fractions, create heterogeneous environments and diverse habitats for microorganisms (Stemmer et al., 1998). Particle-size fractionation, which allows for the separation of soil organic matter pools with varying degrees of microbial alteration and mineral association, might help elucidate microbially-mediated soil carbon (C) cycling characteristics (Joliveta et al., 2006; Ling et al., 2014). Studies that focused on bulk soil might ignore these variations and lead to ambiguous conclusions around the mechanisms of soil C transformation. Soil contains the largest portion of organic C (~1500 Pg C in the first meter of soil) in global terrestrial ecosystem, which exceeds the cumulative pool of atmospheric C (760 Pg) and biotic C (560 Pg) (Post et al., 1982; Batjes, 1996; Jobbágy and Jackson, 2000; Janzen, 2004). Humus, mainly composed of fulvic acid (FA), humic acid (HA) and humin (HM), are the most ubiquitous non-living natural organic compounds in the environment (Stevenson, 1994). As an essential part of soil organic matter that is mostly derived from the decomposition of animal and plant litter, soil humus components might be altered by ambient shifts like fertilization regarding their chemical structure and properties (Jindo et al., 2011). Although the literature is replete with studies about variations in humus under different land uses (Reddy et al., 2012), soil types (Schöning and Kögel-Knabner, 2006) and organic amendments (Jindo et al., 2011), limited reports are available in terms of detailed chemical

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http://dx.doi.org/10.1016/j.soilbio.2017.05.022
0038-0717/

and spectroscopic characteristics of soil humus after long-term fertilizations, especially at soil particle-size fraction level.

Soil humification is primarily a microbially-mediated process. The influence of exogenous nutrients addition such as nitrogen deposition and fertilization on quantity and quality of humus formed in soil, might sensitively reflect on biogeochemical C transformation characteristics (Haase et al. 2007; Edwards et al., 2011; Weber et al., 2012). Cellulose decomposition is a critical process of soil C transformation as cellulose is the most abundant polysaccharide in plant litter that enters soil (Šturšová et al., 2012). It is well known that decomposition of organic matter is carried out by a group of enzymes having the capability to catalyze the hydrolysis of the substrate (Ryckeboer et al., 2003; Tuomela et al., 2000). Likewise, the decomposition of cellulose is mainly accomplished through the synergistic activities of three major groups of enzymes: (i) endoglucanases that cleave cellulose into smaller oligosaccharides, (ii) cellobiohydrolases that cleave cellobiose from the reducing and non-reducing ends of cellulose oligosaccharides, and (iii) β-glucosidases that cleave cellobiose into its glucose constituents (Kubicek et al., 2010). Among them, cellulolytic enzymes encoded by the fungal glycoside hydrolase family 7 cellobiohydrolase I gene (*cbhI*) and bacterial glycoside hydrolase family 48 (*GH48*) were generally considered to catalyze the rate-limiting step of cellulose decomposition (Baldrian and Valášková, 2008). Amplification with PCR primers that target the catalytic region of the fungal *cbhI* in *Ascomycota* and *Basidiomycota* allows a representative group of cellulolytic fungi to be detected and monitored in soil ecosystems (Edwards et al., 2008; Weber et al., 2011). Recently developed primers for the determination of the abundance and diversity of *GH48* gene from actinobacteria showed great promise in elucidating the ecological role of these organisms in terrestrial C cycling (de Menezes et al., 2015). As a widely accepted strategy to sustain or even improve crop yield and soil fertility, reasonable fertilizer management generally emphasizes significant effects on soil biological characteristics (Ahn et al., 2012). Altered C input such as manure application influences competitive interactions among soil microorganisms, thereby changing community composition and/or richness and potentially impacting soil C cycling. Therefore, systematic investigation of the key functional genes corresponding to specific microbes involved in cellulose decomposition after long-term fertilizations would not only provide essential information about targeted microbial communities capable of cellulolytic activity, but also facilitate the potential explanation of C transformation mechanisms after fertilization (Yeh et al., 2013).

In this study, we focused on the abundance and phylogenetic analysis of *cbhI* and *GH48* genes across bulk soil and five particle-size fractions under 33-yr fertilizer treatments through quantitative PCR, cloning and sequencing. Solid-state cross-polarization and magic angle spinning ^{13}C nuclear magnetic resonance (CPMAS ^{13}C NMR) spectra was also applied to illustrate the chemical and structural characteristics of soil humus (FA, HA and HM). Since soil particle-size fractions provide spatially heterogeneous microclimatic conditions for microorganisms, cellulolytic microbial communities were expected to respond differently to them. Specifically, we hypothesized that, after 33-yr of fertilization, (i) chemical and structural characteristics of soil humus would show characteristic variability among different fertilizer treatments and soil particle-size fractions, (ii) fungal and actinobacterial cellulolytic community abundance and phylogenetic affiliation would be influenced by fertilization, particle-size fraction and their interaction effects, and (iii) correlations would be observed between cellulolytic microbial abundance with soil humus chemical and structural characteristics.

2. Experimental procedures

2.1. Site description and experimental setup

The long-term fertilizer experiment was initiated in 1981 at South Lake station (30°37′N, 114°20′1″E), Hubei Province, China, where rice–wheat rotation is the common cropping system. The site is located in the northern subtropical to middle subtropical transitional geographic climate zone with an annual average temperature and precipitation of 16.4 °C and 1300 mm, respectively. The tested yellow-brown paddy soil with a clay loam texture belongs to Udalfs (USDA soil classification). At the beginning of the experiment, the soil had a pH (H_2O) of 6.3, 27.4 g kg^{-1} organic matter, 1.8 g kg^{-1} total N, 1.0 g kg^{-1} total P, 30.2 g kg^{-1} total K and 5.0 and 98.5 mg kg^{-1} of available P and K, respectively. Six treatments (three replicates each) were randomly implemented in 18 plots (8 m × 5 m) under a rotation of winter wheat and middle-season rice. Each plot was separated with cement block (40 cm) to avoid the interference between different fertilizer treatments. Treatments consisted of no fertilizer application (control, CK), fertilizer N (N), fertilizer N and P (NP), fertilizer N, P and K (NPK), manure plus fertilizer N, P and K (NPKM) and manure (M). Mineral fertilizers were applied as annual rate of 150 kg N ha^{-1}, 75 kg P_2O_5 ha^{-1}, 150 kg K_2O ha^{-1}. The N, P and K fertilizers were applied as urea, superphosphate and potassium chloride, respectively. For the NPKM treatment, the same rate of chemical fertilizers as NPK treatment were used plus 22,500 kg ha^{-1} organic fertilizer per year. Organic fertilizer was applied as pig manure (H_2O 69%) with properties of 15.1 g kg^{-1} N, 20.8 g kg^{-1} P_2O_5 and 13.6 g kg^{-1} K_2O.

Sixty percent of mineral fertilizers were applied to rice and the other 40% were applied during wheat season, while manure was applied equally (50:50) to the two crops. All fertilizer P and K and manure were applied once as basal dressing during wheat season and middle-rice season, while 40% of fertilizer N was applied as a basal fertilizer, 40% during tillering stage and 20% during booting stage in the middle–rice season. The amounts of N fertilizer applied to winter wheat were 50% as basal fertilizer, 25% for overwintering period and 25% during the jointing stage. Manure and mineral fertilizers were evenly applied onto the soil surface and immediately incorporated into soil (0–20 cm depth) before sowing.

2.2. Soil collection and particle-size fraction procedure

Undisturbed soil samples from the three replicates of each treatment were collected in May 17th and September 20th, 2014, one week before wheat and rice harvesting. Three soil cores (5 × 10 × 18 cm) at a depth of 0–20 cm from each plot were collected and equally merged as representative soil sample for one replicate of each fertilizer treatment. Moist soils were gently broken apart along the natural breakpoints and passed through a 5-mm sieve to remove visible organic debris. The 5-mm sieve was used rather than a 2-mm sieve because of the unique viscid characteristic of the paddy soil; were forced through a 2-mm sieve, the natural structure of the soil would be destroyed. After thorough mixing, different particle-size fractions were separated according to Stemmer et al. (1998), as the procedure described below. Briefly, the soil–water suspension was dispersed by low-energy sonication (output energy of 0.2 kJ/g) and subsequently fractionated by a combination of wet sieving and repeated centrifugation to avoid disruption of microaggregates. Finally, five fractions were obtained for each sample: large macroaggregate (>2000 μm), coarse sand-sized fraction (2000–200 μm), fine sand-sized fraction (200–63 μm), silt-sized fraction (63–2 μm), and clay-sized fraction (2–0.1 μm). Field-moist soils (35 g equivalent dry weight for each sample) were suspended in 100 mL of distilled water and then

placed into 150 mL glass beaker. The large macroaggregate, coarse and fine sand particle size fraction (>63 μm) were separated by manual wet sieving with a maximum of 700 mL of cooled distilled water. Silt-sized particles were separated from the clay fraction by four centrifugation steps at 150 × *g* for 5 min and at 15 °C. Between each centrifugation the pellets were suspended in cold water and centrifuged again to purify the silt fraction. The combined supernatants were centrifuged at 3900 × *g* for 30 min to obtain clay-sized particles and the resulting same size soil fraction from the glass beakers of the same sample were pooled together. The above procedure was repeated until we got enough soil samples for all the tests. The fractions were then stored at room temperature for chemical analysis, at 4 °C for extracellular enzyme analysis and −80 °C for the molecular analysis. The soil organic carbon, total nitrogen concentrations and enzyme activities of all the soil samples were reported in Zhang et al. (2016).

2.3. Extraction and purification of soil humus

The extraction of different humus fractions from different soil samples was performed with 5 g dry soil and 50 mL of 0.1 mol L^{-1} NaOH in 0.1 mol L^{-1} sodium pyrophosphate under a N_2 atmosphere and it was repeated several times until colorless supernatants were obtained. The suspensions were centrifuged at 5000 × *g* for 15 min and the pooled alkali extract was acidified to pH 2.0 with H_2SO_4, and kept for 24 h at room temperature. The soluble FA was separated from coagulation (HA fraction) by centrifugation (Stevenson, 1994). The residue, which was the precipitate in the centrifuge tube, was collected to provide HM. Total carbon concentrations of FA and HA were tested by MultiB/C3100TOC/TN and those of HM were measured using a vario MACRO cube element analyzer (Elementar Analysensysteme GmbH, Hanau, Germany).

The FA were purified by eluting the supernatant solution through a glass column filled with an XAD-8 resin; they were adsorbed on the resin, whereas nonhumic material passed through the column. The adsorbed FA were eluted using one bed volume of 0.1 mol L^{-1} NaOH. The Na fulvates were passed through a strongly acidic cation-exchange resin (Amberlite 120, Rohm and Haas Co., Philadelphia, PA) to obtain H^+-saturated FA. The dialysate was freeze-dried for chemical characterization. The HA were purified by treatment with 10 vol of a dilute 0.5% HF + HCl solution (5 mL L^{-1} HCl + 5 mL L^{-1} HF). This procedure was repeated three times. After centrifugation at 4000 × *g* for 15 min, the sample was washed repeatedly with water, followed by dialysis against deionized water using a 12- to 14-kDa cutoff membrane. Finally, the FA and HA samples were freeze-dried to keep the material stable until use (Jindo et al., 2011).

2.4. CPMAS solid-state ^{13}C NMR spectroscopy

Take the previous results about C, N and humus fractions contents for reference, chemical composition of purified FA and HA fractions of soil samples under CK, NPK, NPKM treatments were via CPMAS solid-state ^{13}C NMR spectroscopy and expressed as the relative abundance of the major C types. The solid-state ^{13}C NMR spectra were measured on a Bruker Avance III 400 NMR spectrometer (Germany) conducting at a spinning speed of 5 kHz and a contact time of 1 ms, with a ^1H 90° pulse length of 4 μs and a recycle delay of 0.8 s. The chemical shift regions 0–45, 45–95, 95–165 and 160–200 ppm were referred to alkyl C, O-alkyl C, aromatic C and carboxylic C, respectively (Zhang et al., 2015b). The areas of the spectral regions were measured though the integration routine of the spectrometer and expressed as percentages of the sum of all spectral areas respectively (Pane et al., 2013). The degree of aromaticity (ARO%) and aliphaticity (AL%) of the FA and HA were calculated according to the equations below:

$$ARO(\%) = \frac{\text{Aromatic C (95 – 165 ppm)}}{\text{C signal (0 – 165 ppm)}} \times 100 \quad (1)$$

$$AL(\%) = \frac{\text{Aliphatic C (0 – 95 ppm)}}{\text{C signal (0 – 165 ppm)}} \times 100 \quad (2)$$

The alkyl C/O-alkyl C ratio was calculated as an indicator of the degree of organic matter decomposition (Baldock et al., 1997).

2.5. DNA extraction and quantitative PCR (qPCR) assay

Soil DNA was extracted from 0.5 g fresh soil using the FastDNA® SPIN Kit (MP Biomedicals, Illkirch, France) and a Fast Prep-24 Homogenization System (MP Biomedicals, Irvine, CA) according to the manufacturer's instructions. Successful DNA extraction was characterized by electrophoresis on 1% (wt/vol) agarose gels. The quantity and quality of DNA were checked using Nanodrop spectrophotometer (Nanodrop, PeqLab, Germany).

Genes encoding fungal glycoside hydrolase family 7 cellobiohydrolase I gene (*cbhI*) and bacterial glycoside hydrolase family 48 (*GH48*) were selected as biomarkers of cellulolytic fungi and actinobacteria, respectively. All qPCR assays were carried out in an iCycler system (BioRad, USA) using SYBR Green I chemistry and the data were analyzed by Bio-Rad iQ5 v2.0, as described previously (Fan et al., 2011). The primers for qPCR and cloning of *cbhI* and *GH48* also the amplification protocols were listed in Table 1. Each 20 μl reaction cocktail of qPCR contained 10 μL of 2 × SuperMix (Bio-Rad, USA), 1.6 μL of 10-fold diluted DNA, 0.4 μL each primer (10 μM) and 7.6 μL sterilized water. The amplification specificity of *cbhI* and *GH48* was confirmed by generating a melting curve, respectively. Standard curves ranging from 10^2 to 10^8 copies were prepared by 10-fold serial dilution of known copy numbers of plasmid DNA possessing the genes of interest. qPCR was performed in triplicate and amplification efficiencies of 89.5%–92.5% were obtained with r^2 values > 0.99 for *cbhI* and 99.9%–107.5% with r^2 values > 0.99 for *GH48*.

2.6. Cloning, sequencing and phylogenetic analysis

Briefly, each PCR used 1 μL of 10-fold diluted soil DNA as template, 12.5 μL 2 × EasyTaq PCR SuperMix (TransGen Biotech, Beijing, China), 0.5 μL of each primer diluted to a final volume of 25 μL. After the agarose gel purification, PCR products were ligated into the pGM-T vector according to the manufacturer's instruction (Tiangen Biotech, Beijing, China) at 16 °C overnight and transformed into *Escherichia coli* TOP10. Transformants were selected via blue-white screening. Sequencing of positive colonies was conducted on an ABI 3730 sequencer using BigDye terminator cycle sequencing chemistry (Applied Biosystems, CA, USA). Six clone libraries for *cbhI* and *GH48* genes retrieved from bulk soil and the five particle-size fractions were created respectively. PCR products of the three treatment (CK, NPK and NPKM) of each soil fraction were mixed and served as the target clone fragments. After purification and clone, approximately 60 randomly selected clones from each clone library (333 clones in total for *cbhI* gene and 329 clones in total for *GH48* gene) were sequenced. The sequences of *cbhI* and *GH48* determined in this study were deposited in the GenBank database under accession numbers KX820305-KX820331 and KX768665-KX768695, respectively.

2.7. Statistical analysis

Differences of soil humus carbon contents and cellulolytic

Table 1
Primers used for the clone library and real-time PCR quantification of maker genes for *cbhI* and *GH48* microbial community.

Primer Name	Primer sequence (5′-3′)	Target gene	Thermal Profile	Molecular analysis	Reference
fungcbhIF	ACCAAYTGCTAYACIRGYAA	*cbhI*	95 °C, 5min; 40 × (94 °C, 30s; 48 °C, 45s; 72 °C, 90s; data collection at 84 °C for 10s);	qPCR in Fig. 2; clone library in Fig. 3	Edwards et al., 2008
fungcbhIR	GCYTCCCAIATRTCCATC				
GH48_F8	GCCADGHTBGGCGACTACCT	*GH48*	94 °C, 4min; 40 × (94 °C, 45s; 57 °C, 30s; 72 °C, 60s; data collection at 81 °C for 10s)	qPCR in Fig. 4	de Menezes et al., 2015
GH48_R5	CGCCCCABGMSWWGTACCA				
GH48_F1	RRCATBTACGGBATGCACTGGCT	*GH48*	95 °C, 2min; 2 × (each at 95 °C, 65 °C and 72 °C); 2 × (30s each at 95 °C, 62 °C and 72 °C); 3 × (30s each at 95 °C, 59 °C and 40s for 72 °C); 4 × (30s each at 95 °C, 56 °C and 45s for 72 °C); 5 × (30s each at 95 °C, 53 °C and 50s for 72 °C); 30 × (95 °C, 30s; 50 °C, 45s; 100s for 72 °C); 72 °C, 10 min	clone library in Fig. 5	
GH48_R1	VCCGCCCCABGMGTARTACC				
GH48_F1_cell	AYGTCGACAACRTSTACGGMTWCG			clone library in Fig. 5	
GH48_R1_cell	CCGCCCCASGCSWWRTACC				

community abundances were analyzed by SAS statistical software, where between and within-subject variation was determined for fertilization regimes and particle-size fractions respectively using Fisher's significant difference (LSD) at $P < 0.05$. The translated amino acid sequences of cellulolytic gene sequences obtained from our soils and related protein sequences obtained from the National Center for Biotechnology Information (NCBI) GenBank database were aligned with CLUSTALW. Phylogenetic tree was constructed using Neighbor-joining method in MEGA 6 (JCVI, Rockville, MD, USA) with 1000 replicates to produce bootstrap values (Tamura et al., 2013). Pearson's correlation analyses were performed to assess the linear correlation among soil properties, enzyme activities, C groups of FA and HA with *cbhI* and *GH48* gene abundances.

3. Results

3.1. Soil humus composition

Particle-size fractions and fertilization significantly influenced the distribution of humus components (FA, HA, HM and HA/FA), but their interaction effect on HA and HA/FA was not significant (Table 2). The highest C concentration of humus (FA, HA and HM) was found in fine sand, and the lower concentrations were in silt and clay fraction (63–0.1 μm) (Fig. 1a–c). This result was similar to that of soil organic carbon (SOC) concentrations in our previous report (Zhang et al., 2016). Compared with chemical fertilizer treatments, organic fertilizer application (NPKM and M) significantly improved FA and HM C concentrations in bulk soil, >200 μm and clay fractions (Fig. 1a and c), whereas the improvement of HA C concentration in 2000–2 μm fractions was not significant (Fig. 1b). NPKM and M treatments also showed significant impacts on FA C concentration of fine sand fraction (Fig. 1a) and HM of silt fraction (Fig. 1c). When compared with CK treatment, organic fertilizer input reduced HA/FA ratios of all the fractions except clay fraction which showed the highest HA/FA (Fig. 1d). Unlike the other four fractions, only fine sand soils contained higher FA than HA.

3.2. CPMAS solid-state ^{13}C NMR spectroscopy

The relative abundance of different C groups of FA varied with particle fractions (Table 3). The results showed that large macroaggregate and fine sand fractions contained more alkyl C and O-alkyl C groups (Table 3). However, carboxylic C was most abundant in coarse sand, silt and clay fractions, which resulted in an ultimately higher ARO% of the FA extracted from these fractions. The proportion of aromatic C group was not substantially different among the five fractions and fertilizer treatments. Alkyl/O-alkyl ratios of FA in fine sand and silt fractions were lower than in the other fractions.

Generally, HA showed higher abundance of alkyl C and lower carboxylic C than FA of each soil sample. Unlike FA, the dominant C group of HA extracted from all the soil samples was alkyl C, followed by O-alkyl C and aromatic C groups, with the lowest relative abundance of carboxylic C except in the fine sand fraction where the most abundant C group was aromatic C (Table 3). This resulted in the higher ARO% and lower AL% of HA in fine sand fraction. Fertilizer application, especially the NPKM treatment, reduced the alkyl/O-alkyl ratio of soil HA and this ratio was lowest in fine sand fraction and higher in silt and clay fractions.

Table 2
Two-way ANOVA analysis of soil humus, *cbhI*, *GH48* gene abundances in the five soil particle-size fractions, fertilizer treatments each with three replicates.

	Particle-size fraction		Fertilization		Particle-size fraction × Fertilization	
	F	*P*	*F*	*P*	*F*	*P*
FA	985.82	<0.0001	92.57	<0.0001	4.67	<0.0001
HA	229.74	<0.0001	32.53	<0.0001	**1.40**[1]	0.1584
HM	1237.91	<0.0001	54.07	<0.0001	4.58	<0.0001
HA/FA	85.56	<0.0001	4.61	0.013	**0.94**[1]	0.3677
Wheat season						
cbhI abundance	127.66	<0.0001	146.13	<0.0001	4.56	0.001
GH48 abundance	295.82	<0.0001	30.38	<0.0001	12.95	<0.0001
Rice season						
cbhI abundance	190.40	<0.0001	65.07	<0.0001	5.15	0.0004
GH48 abundance	3.67	0.0158	4.74	0.0169	**0.79**[1]	0.6048

Note: [1] The data in bold indicated the parameter was not significantly affected by soil particle-size fractions, fertilizer treatments or their interaction ($P < 0.05$).

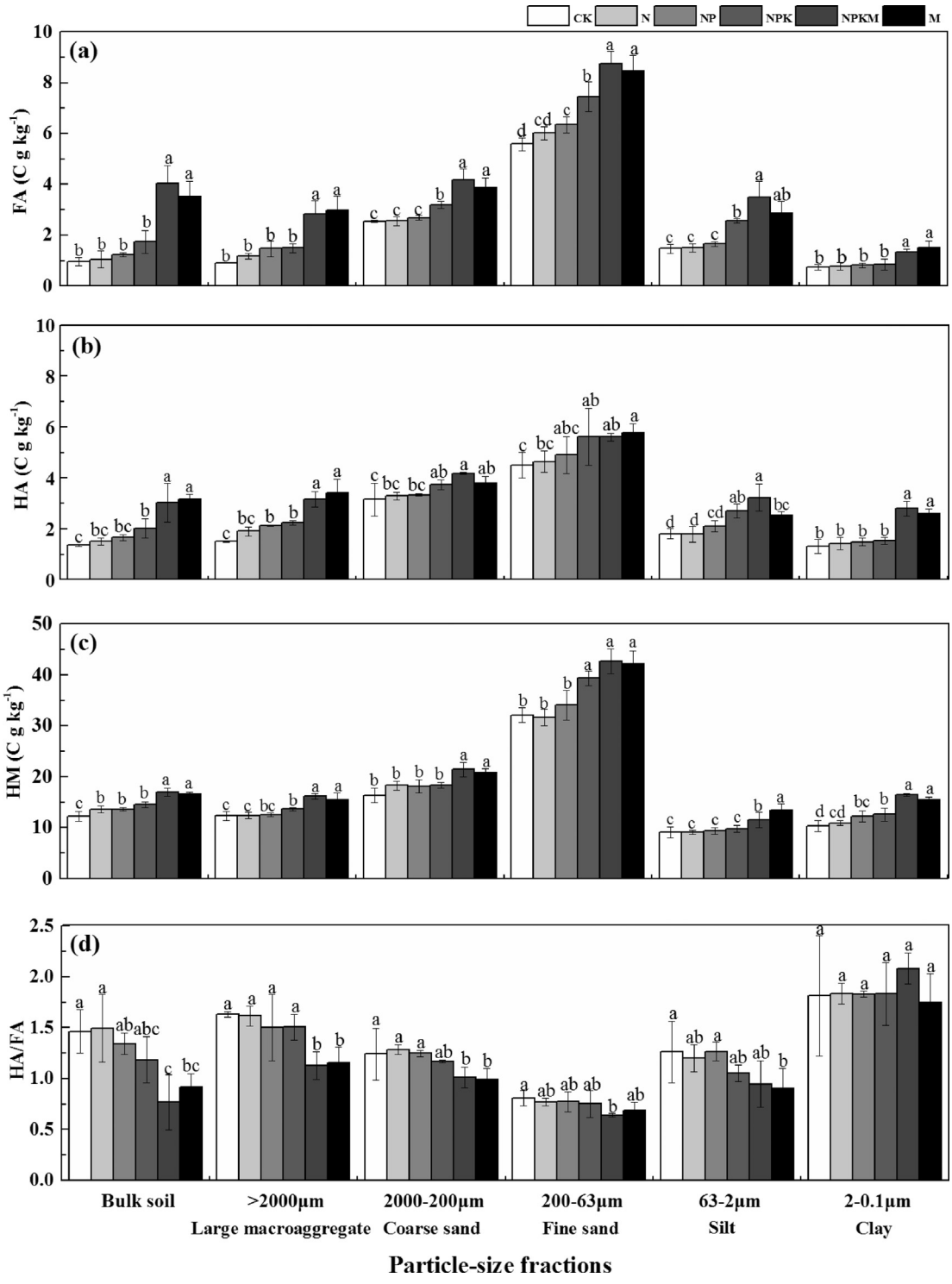

Fig. 1. Fulvic acid (FA), humic acid (HA), and humin (HM) contents and HA/FA ratios of soil samples from different fractions and the six treatments. Error bars represent standard error (n = 3) and are followed by a lowercase letter indicating a significant difference among fertilizer treatment within each fraction (Fisher's LSD test).

3.3. *cbhI* gene abundance and phylogenetic analysis

Soils under CK, NPK and NPKM treatments were used for molecular analyses. Abundance of the *cbhI* gene was in the range of 1.69–6.45 × 10^7 copies g^{-1} soil in wheat season and 0.54–8.60 × 10^7 copies g^{-1} soil in rice season across all samples (Fig. 2). Particle-size fraction and fertilizer treatments individually and interactively impacted *cbhI* abundance in both seasons ($P < 0.001$; Table 2). The *cbhI* gene abundance of bulk soil and larger fractions (>63 μm) was higher in rice season than in wheat season, but that of smaller fractions (<63 μm) showed opposite trend especially clay fraction. Although the lowest *cbhI* abundance of these two seasons were both observed in clay fraction, the *cbhI* abundance in sand and silt fraction (2000–2 μm) of wheat season

Table 3
Contribution of the different C types, aromaticity (ARO%), alphaticity (AL%) and alkyl/O-alkyl ratio determined by CPMAS ^{13}C NMR in the FA and HA extracted from the different particle-size fraction soils under studied three fertilizer managements.

Particle-size fraction	Treatments	0-45 ppm	45-95 ppm	95-165 ppm	160-200 ppm	ARO%	AL%	alkyl/ O-alkyl
		Alkyl C	O-alkyl C	Aromatic C	Carboxylic C			
FA								
>2000 μm (Large macroaggregate)	CK	40.4	20.2	22.3	17.1	36.9	73.1	2.01
	NPK	28.7	29.3	23.7	18.4	40.8	71.0	0.98
	NPKM	32.4	27.8	21.4	18.5	35.5	73.8	1.16
2000–200 μm (Coarse sand)	CK	22.0	15.1	21.6	41.3	58.3	63.2	1.45
	NPK	18.8	12.3	22.8	46.1	73.1	57.8	1.53
	NPKM	20.6	18.9	22.2	38.4	56.1	64.1	1.09
200–63 μm (Fine sand)	CK	28.1	31.5	23.3	17.1	39.2	71.9	0.89
	NPK	28.7	27.7	24.6	19.0	43.5	69.7	1.04
	NPKM	27.9	31.9	23.8	16.5	39.8	71.5	0.87
63–2 μm (Silt)	CK	14.83	16.1	15.8	53.2	51.1	66.2	0.92
	NPK	13.7	20.5	25.8	40.1	75.5	57.0	0.67
	NPKM	13.3	19.9	23.5	43.4	70.7	58.6	0.67
2–0.1 μm (Clay)	CK	22.9	14.3	22.9	40.0	61.6	61.9	1.60
	NPK	23.5	11.5	30.2	34.9	86.2	53.7	2.05
	NPKM	26.6	16.7	30.0	26.7	69.4	59.0	1.60
HA								
>2000 μm (Large macroaggregate)	CK	51.6	28.0	11.4	9.1	14.4	87.4	1.85
	NPK	40.5	32.1	15.3	12.2	21.0	82.6	1.26
	NPKM	31.9	27.9	27.7	12.5	46.4	68.3	1.14
2000–200 μm (Coarse sand)	CK	31.4	29.6	27.9	11.1	45.7	68.6	1.06
	NPK	35.1	28.7	24.5	11.7	38.5	72.2	1.22
	NPKM	29.4	29.4	28.1	13.2	47.8	67.7	1.00
200–63 μm (Fine sand)	CK	24.6	28.1	33.0	14.3	62.6	61.5	0.87
	NPK	24.4	28.4	32.2	15.1	61.0	62.1	0.86
	NPKM	24.6	28.5	32.0	14.9	60.4	62.4	0.86
63–2 μm (Silt)	CK	49.7	21.0	19.8	9.6	28.0	78.2	2.37
	NPK	36.2	28.9	21.6	13.3	33.1	75.1	1.25
	NPKM	36.0	28.1	24.5	11.4	38.3	72.3	1.28
2–0.1 μm (Clay)	CK	43.6	21.7	19.2	15.6	29.0	77.3	2.01
	NPK	40.0	27.1	25.0	7.9	37.2	72.9	1.48
	NPKM	44.0	30.6	20.8	4.6	27.9	78.2	1.44

and sand fraction (2000–63 μm) of rice season were relatively higher *cbhI* abundance than those in the other fractions. Compared with CK, we clearly observed that long-term fertilizer treatments (NPK and NPKM) significantly enhanced *cbhI* abundance of bulk soil and five particle-size fractions. Furthermore, *cbhI* gene abundance under NPKM was significantly higher than under NPK in most particle-size fractions except the large macroaggregate and fine sand of wheat season, and bulk soil and silt fraction of rice season.

Phylogenetic analysis showed that the *cbhI* gene sequences obtained could be tentatively clustered into 5 groups with strong phylogenetic association with: *Eurotimycetes*, *Dothideomycetes*, *Sordariomycetes* in *Ascomycetes*, *Agricomycetes* (I, II and III) in *Basidomycota* and an unclassified group (Fig. 3). A portion of the sequences was located in clusters without any cultured representative. No obvious cluster of sequences derived from same fertilizer treatment or particle-size fraction was observed.

3.4. GH48 gene abundance and phylogenic analysis

The abundance of the *GH48* gene across all the samples ranged 2.07–8.53 $\times$ 10^6 copies g^{-1} soil in wheat season and 0.38–16.58 $\times$ 10^6 copies g^{-1} soil in rice season, which was about ten times lower than the abundance of *cbhI* gene (Fig. 4). Both particle-size fraction and fertilizer treatment individually and interactively affected *GH48* abundance in wheat season but the interaction effect was not significant in rice season (Table 3). Unlike the varying pattern of *cbhI* gene, *GH48* gene abundance was generally higher in wheat season than in rice season except for the large macroaggregate and fertilized fine sand fractions. Fine sand fraction showed highest *GH48* abundance in both seasons, while for the rest fractions, *GH48* abundances of coarse sand and silt fraction were higher than the other fractions in wheat season. In rice season, the results showed slightly different with higher *GH48* abundance observed in >200 μm fraction. When compared with CK, NPKM significantly increased *GH48* gene abundance of >200 μm and clay-sized fractions in wheat season and also that of sand and clay fractions in rice season. However, compared with CK, the effect of NPK was not significant on most tested samples.

The phylogenetic tree (Fig. 5) revealed that some of the obtained sequences clustered with known actinobacterial *GH48* genes like *Streptomyces* and *Kitasatospora*, while the majority of clones formed new clusters without any cultured representative. Phylogenetic analysis did not show any pronounced clustering of sequences arising from either fertilizer treatment or particle-size fraction.

3.5. Correlation analysis

Significantly positive correlations were obtained between β-glucosidase and β-cellobiohydrolase activities with *cbhI* and *GH48* abundances ($P < 0.0001$; Table 4). However, both *cbhI* and *GH48* abundances showed negative correlations with the HA/FA ratio. Except for the above negative correlation and the unobvious correlation between *cbhI* gene abundance with total N, the abundances of cellulose-degrading microbes were significantly

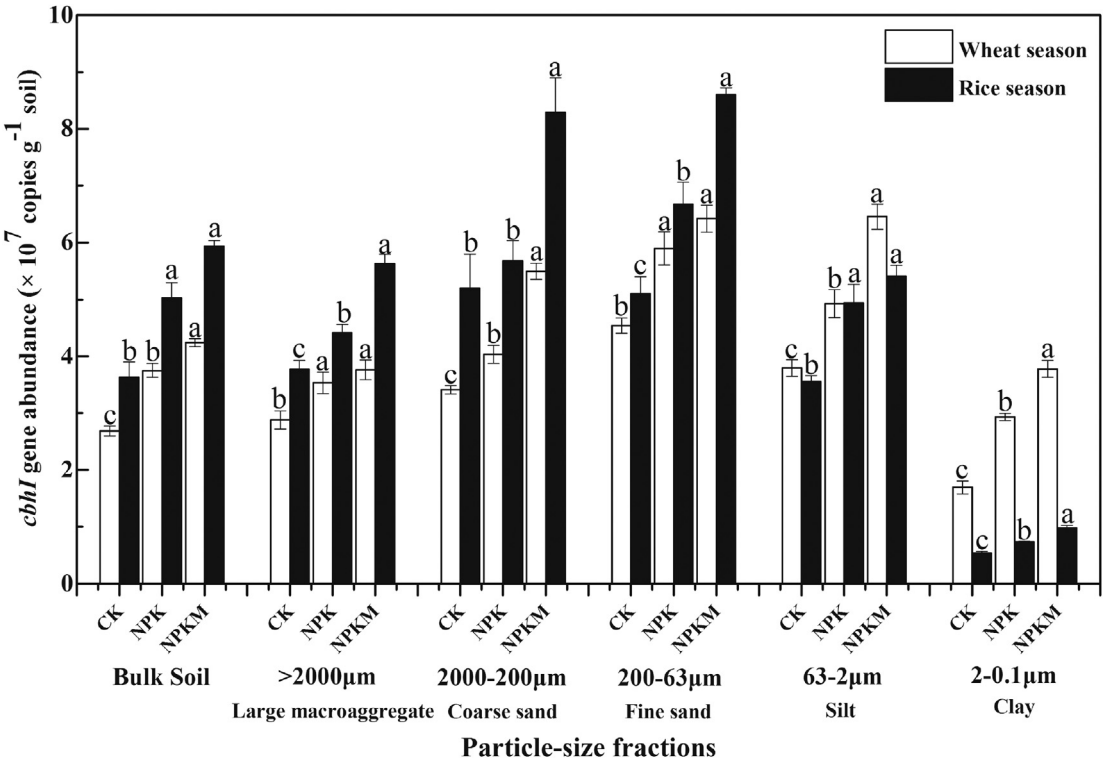

Fig. 2. *cbhI* gene copy numbers in bulk soil and different particle-size fractions under long-term fertilizations in wheat season and rice season. Error bars represent standard error (n = 3) and are followed by a lowercase letter indicating a significant difference among fertilizer treatment within each fraction ($P < 0.05$; Fisher's LSD test).

correlated with soil properties and soil humus components (Table 4). As we found, not all of the functional C groups of FA and HA were correlated with cellulose-degrading microbial community abundance. Interestingly, C groups like alkyl C and aromatic C of HA were negatively and positively correlated with cellulose-degrading microbial abundance respectively, however, no relationships were detected between the same C groups of FA with the abundance of cellulolytic microbes (Table 5). Also, *cbhI* and *GH48* abundance behaved differently to humus C groups. For example, *GH48* abundance was negatively correlated with carboxylic C of FA and *cbhI* abundance was positively correlated with that of HA. Even though both *cbhI* and *GH48* abundances showed negative correlations with alkyl/O-alkyl ratio of HA, no correlation was detected between *GH48* abundance with the alkyl/O-alkyl ratio of FA (Table 5).

4. Discussion

Fertilization, which is widely used to improve soil fertility and crop yield, strongly influences soil biological properties. The direction and extent of the shift in soil C cycling following fertilization can occur via more organic C input to soil by improved plant net primary productivity or altered microbial decomposition like heterotrophic mineralization of C (Edmeades, 2003; Carreiro et al., 2000). As a functional group, the cellulolytic community has been reported from litter or wood (Melillo et al., 1989; Dickie et al., 2012), but there is limited understanding of the development of cellulolytic community in soil particle-size fractions of different fertilized agricultural soils. To bridge the gap between fertilization practices and cellulose degradation, cellulolytic communities were explored with DNA-based molecular methods targeting the *cbhI* gene in fungi and the *GH48* gene in actinobacteria (Edwards et al., 2008; de Menezes et al., 2015). Soil organic matter humification might be hampered differently by varied decomposition within different particle-size fractions. The determination of cellulolytic microbial diversity and abundance patterns across certain samples, in concert with the determination of soil properties of different C components, is important for clarifying the action mechanism of cellulolytic microbial community in environmental process.

4.1. Soil humus variations across fertilizations and particle-size fractions

Soil organic matter is a complex, heterogeneous mixture of various materials with a continuum of decomposition and stabilization in the soil profile (Senesi and Loffredo, 1999). MacCarthy and Rice (1991) considered the heterogeneity of soil organic matter a major factor in explaining its persistence in the environment and suggested that its molecular irregularity presents a 'confusing molecular panorama' to microorganisms. Such disorder could preempt soil organic matter from serving as nutrient source for organisms capable of rapidly utilizing it as energy. Humus typically represents a large portion of natural organic matter distributed in soils, sediments and waters, which are often encountered as a result of their ubiquity (Morales et al., 2012; Matilainen et al., 2011). In this study, humus apparently accounted for >80% of SOC, which corresponds to previously reported values (Lehtonen et al., 2001). Generally, as the active components of humus, FA and HA differ in molecular weight, elemental and functional C group contents. HA is higher in molecular weight and contains less oxygen-containing functional groups when compared with FA (Güngör and Bekbölet, 2010), which was also true in this study with higher alkyl C proportion and lower carboxylic C proportion of HA observed than FA in all soil fractions except fine sand (Table 3). The aromatic C region is typical in spectra of lignin structural units and it is attributed to oxygen-substituted aromatic ring C, such as those of syringyl and guaiacyl units (Keeler et al., 2006). It was reported that the ARO% of

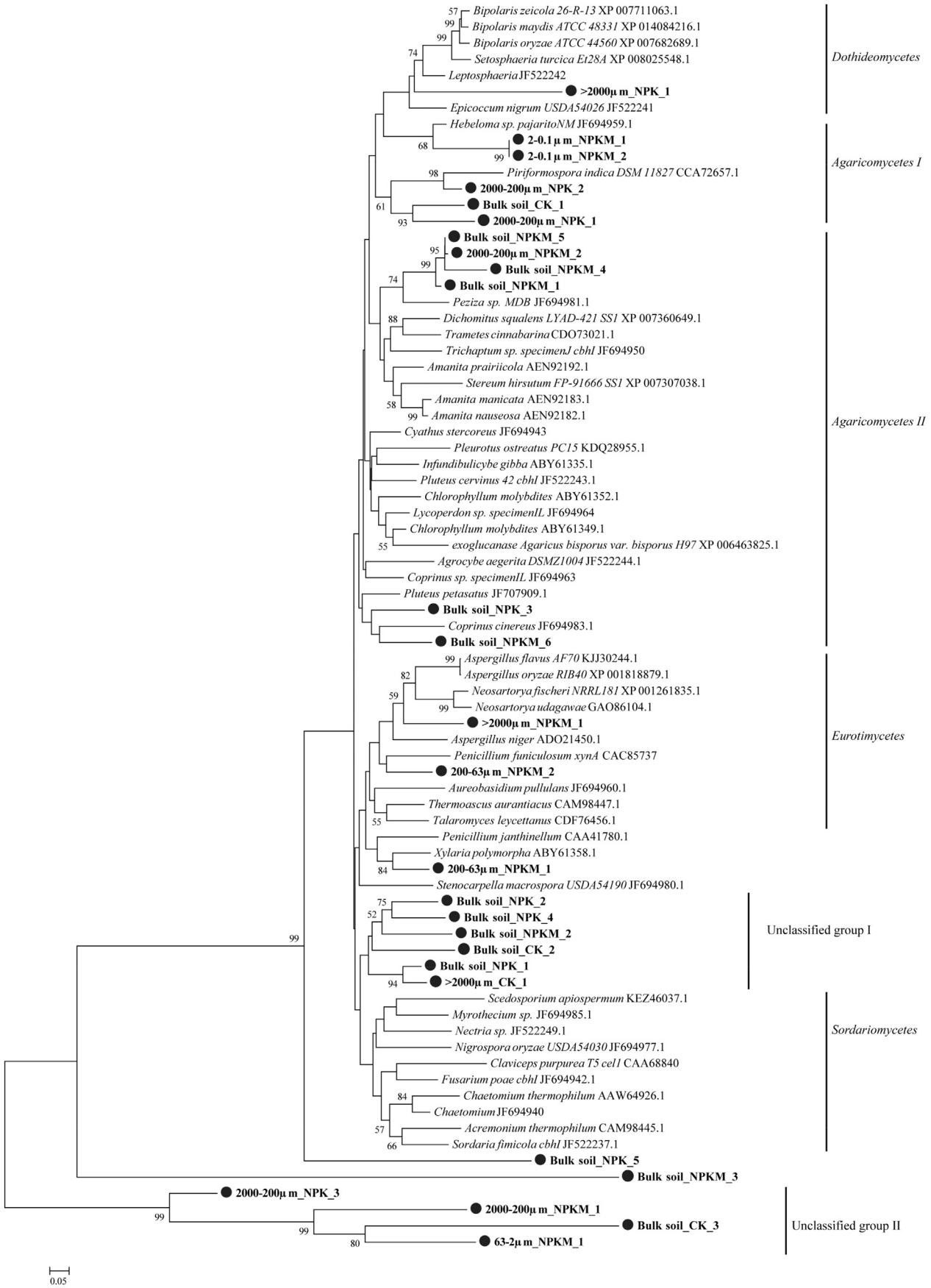

Fig. 3. Neighbor-joining phylogenetic analysis of the partial fungal cellobiohydrolase (*cbhI*) protein sequences retrieved from clone libraries together with sequences with sequences obtained from the NCBI database in soils across different particle-size fractions and fertilizations. Bootstrap analysis was performed with 1000 replicates and only values > 50% are shown. Sequences from this experiment were highlighted as a close circle.

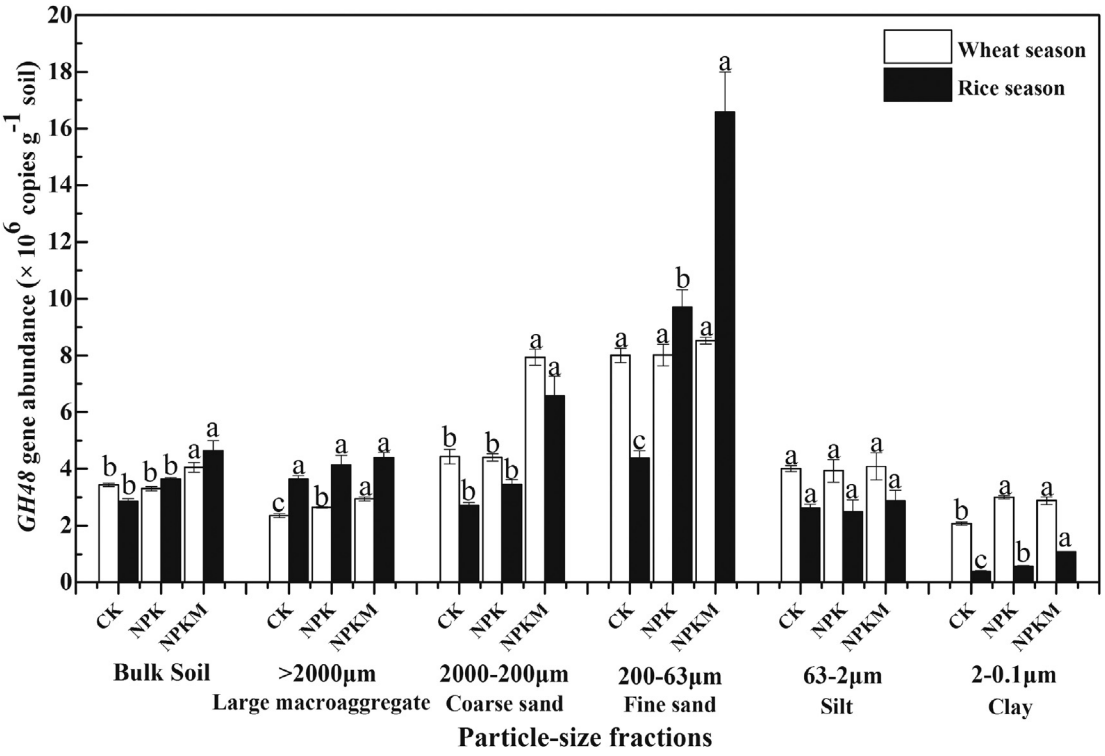

Fig. 4. *GH48* gene copies numbers in bulk soil and different particle-size fractions under long-term fertilizations in wheat season and rice season. Error bars represent standard error (n = 3) and are followed by a lowercase letter indicating a significant difference among fertilizer treatment within each fraction ($P < 0.05$; Fisher's LSD test).

HA was two or three times higher than that of FA (Abakumov et al., 2015), but we only observed the relatively higher ARO% of HA in fine sand fraction which probably meant that the chemical structure of different humus components might also depend on the heterogeneous environment that formed in different particle-size fractions.

The chemical structure of FA and HA varied across soil particle-size fractions as we hypothesized (Table 3). For FA, coarse sand, silt and clay fractions held larger contents of carboxylic C and higher aromaticity level than the other two fractions. However, the alkyl/O-alkyl ratio, which has been proposed as a sensitive index of soil organic matter decomposition (Baldock et al., 1997; Rosenberg et al., 2003), was lower in fine sand and silt fractions but higher in clay fraction. For HA, the dominant C group of HA in fine sand fraction was aromatic C and it was very different from the other soil fractions that were dominated by alkyl C, thus resulting in the highest ARO% and lowest AL% in fine sand fraction. Together with the highest soil C/N ratio, lowest HA/FA and alkyl/O-alkyl ratio of HA, these results further suggested the relatively lower humification degree of fine sand than the other fractions (Yang et al., 2004; Doran, 1980). On the contrary, higher alkyl/O-alkyl ratios of HA in silt and clay fractions (63–0.1 μm) suggested more stable organic matter and a possible accumulation of recalcitrant compounds within these fractions (Anderson and Paul, 1984).

Compared with the effect of particle-size fraction, fertilization showed relatively smaller effects on the structure of soil humus (Table 2). But still, compared with CK, HA/FA ratios of soils receiving fertilizers especially organic fertilizer (NPKM) except clay fraction were significantly reduced (Fig. 1d). This indicated a faster C turnover rate and lower humification in the organic fertilized soils which probably because labile compounds in manure served as an important source of energy and C for soil microorganisms (Sánchez-Monedero et al., 2004; Bastida et al., 2008). These results were further confirmed by the reduced alkyl/O-alkyl ratios of HA under NPKM than the other treatments (Table 3).

4.2. Cellulose-degrading fungi distribution among particle-size fractions under fertilizations

Cellulose is the major polysaccharide in plant litter, and cellulose-degrading enzymes are thus an appropriate target for the study of the decomposing microorganisms. Among these, cellobiohydrolase catalyzes the rate-limiting step of cellulose decomposition (Baldrian and Valášková, 2008) and the *cbhI* gene represents a suitable marker for the study of cellulolytic fungi (Edwards et al., 2008; Weber et al., 2011). These primers used in this study amplify *cbhI* genes belonging to the fungal glycoside hydrolase family 7 from the *Basidiomycota* and *Ascomycota* unless the template contains introns in the primer sequence (Šturšová et al., 2012). As hypothesized, abundances of *cbhI* gene showed significant variations in different particle-size fractions and fertilizer treatments with clear increases in *cbhI* abundance under mineral and organic fertilizer application (Fig. 2; Table 2). However, another long-term experiment indicated that cellulolytic gene abundance was decreased by 39% with mineral fertilizer application for 18 years (Fan et al., 2012). Furthermore, the distribution of *cbhI* gene abundance was strongly related to the pattern of SOC and C/N ratio which was higher in larger fractions (>63 μm) but lower in smaller fractions (63–0.1 μm). The lowest *cbhI* abundance and higher total N concentration were present in clay fraction, thus no significant relationship was observed between them (Table 4). Activities of β-cellobiohydrolase and β-glucosidase were highly correlated with changes of *cbhI* gene abundance (Table 4), suggesting that shifts in abundance of these cellulolytic fungi are of functional relevance in terms of C transformation in agricultural ecosystem. These strong relationships were also confirmed in another long-term fertilizer experiment (Fan et al., 2012) and a study about the responses of soil cellulolytic fungal communities to

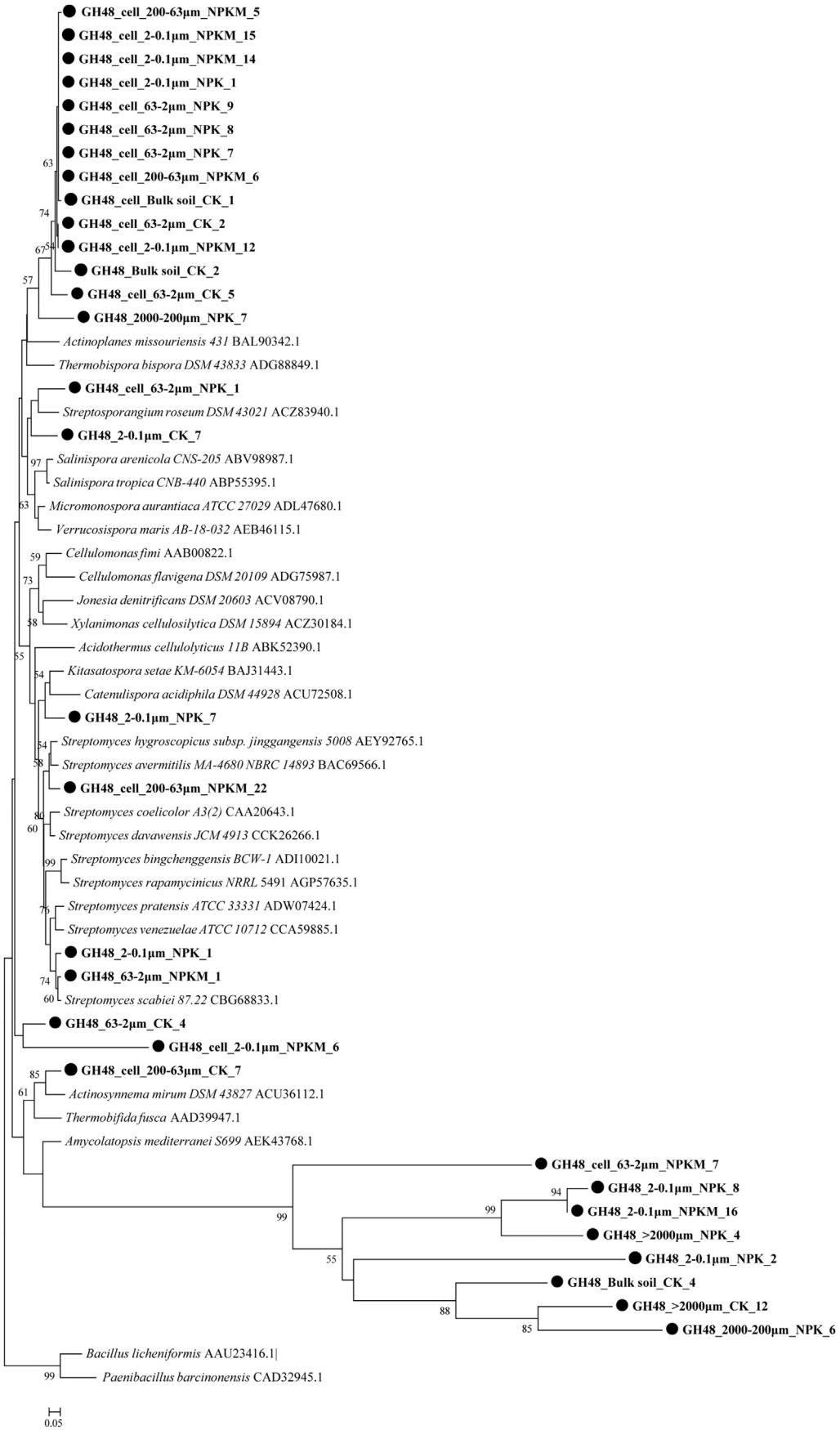

Fig. 5. Neighbor-joining phylogenetic trees of actinobacterial *GH48* protein sequences retrieved from clone libraries together with sequences with sequences obtained from the NCBI database in soils across different particle-size fractions and fertilizations. Bootstrap analysis was performed with 1000 replicates and only values > 50% are shown. Sequences from this experiment were highlighted as a close circle.

Table 4
Correlation coefficients (*r*) between soil nutrients, humic substances, enzyme activities and *cbhI* and *GH48* abundances of soils from different particle-size fractions under different fertilizer treatments in rice season. All the correlations are significant at the $P < 0.0001$ level.

	SOC	Total N	C/N	FA	HA	HM	HA/FA	βCB[2]	βG[3]	*GH48*
cbhI	0.609	n.s.[1]	0.745	0.746	0.721	0.585	−0.783	0.717	0.724	0.753
GH48	0.840	0.518	0.560	0.862	0.743	0.846	−0.604	0.921	0.868	

Note: [1] n.s. indicated no significant relationship was detected. [2] βG, β-glucosidase; [3] βCB, β-cellobiohydrolase.

Table 5
Correlation coefficients (*r*) between chemical composition of C groups of FA and HA with *cbhI* and *GH48* abundances of soils from different particle-size fractions under different fertilizer treatments in rice season.

	Alkyl C	O-alkyl C	Aromatic C	Carboxylic C	ARO%	AL%	alkyl/O-alkyl
FA							
cbhI	n.s.[1]	0.678**	n.s.[1]	n.s.[1]	−0.539*	0.539*	−0.600*
GH48	n.s.	0.698**	n.s.[1]	−0.530*	−0.547*	0.553*	n.s.[1]
HA							
cbhI	−0.707**	n.s.[1]	0.553*	0.546*	0.613*	−0.590*	−0.666**
GH48	−0.630*	n.s.[1]	0.533*	n.s.[1]	0.624*	−0.573*	−0.551*

Note: *$P < 0.05$; **$P < 0.01$.[1] n.s. indicated no significant relationship was detected.

elevated atmospheric CO2 at an aspen plantation site (Edwards and Zak, 2011).

This *cbhI* gene survey represents the most comprehensive cross-particle comparison of a fungal functional gene conducted to date. Based on prior cultivation studies, we expected to detect previously studied cellulolytic fungal taxa in these soil samples (Edwards et al., 2008; Weber et al., 2011). On the contrary, the vast majority of the dominant sequences obtained were not identifiable with high confidence using current databases (BLAST), indicating that many of the dominant cellulolytic fungi in soils are not the prototypical cellulose degraders that have been much studied. Rather, they represent novel taxa or unknown taxa, in which cellulose-degrading capabilities have not been recognized. According to previous reports, fungi from the phylum *Ascomycota* decrease along with the process of degradation as they are gradually replaced by fungi from *Basidiomycota* at the later stages of decomposition (Osono, 2007) because of their ability to synthesize the enzymes required for the degradation of complex polymers (Baldrian, 2008). This was also true in our study with most of the clustered sequences affiliated with *Agaricomycetes* in *Basidiomycota* after this 33-yr fertilization.

4.3. Cellulose-degrading actinobacteria community among particle-size fractions under fertilizations

Compared with bacteria, multicellular actinomycetes and fungi are well known as efficient cellulose decomposers. Members of the phylum *Actinobacteria* are abundant in soils and are thought to have an important role in organic matter turnover and the breakdown of recalcitrant molecules such as cellulose (Goodfellow and Williams, 1983; McCarthy, 1987) and polycyclic aromatic hydrocarbons (de Menezes et al., 2012). In this study, actinobacterial *GH48* gene abundance which showed significant variations in different particle-size fractions and fertilizer treatments, was ten times lower than *cbhI* gene abundance (Fig. 2; Table 2). However, the qPCR primers did not cover all actinobacterial *GH48* gene diversity from cultured actinobacterial strains and therefore probably provided an underestimation of the actual actinobacterial *GH48* gene abundance (de Menezes et al., 2015). Even so, we still observed the variability in *GH48* gene abundance among particle-size fractions following a pattern similar to that seen for SOC heterogeneity, which was highest in fine sand fraction (200–63 μm) and lower in silt and clay fractions (63–0.1 μm) (Fig. 2; Table 4). Correlation analysis showed that SOC, total N, C/N ratio and cellulase activities were significantly correlated with *GH48* abundance (Table 4). These results were confirmed by previous reports. For example, C/N ratio was thought to influence litter C availability, and high C/N ratio had a negative influence on extracellular hydrolytic enzymes during litter decomposition (Leitner et al., 2012; Geisseler and Horwath, 2009). It is not surprising that higher enzyme activities synthesized by cellulolytic microbes were observed under NPKM treatment in particle-size fractions with lower soil C/N ratios (Zhang et al., 2016). The clone library of soil *GH48* gene revealed new sequences that were not present in current gene databases. The majority of sequences were located in clusters without any cultured representative (Fig. 5), although a small number of soil clones clustered with *Streptomyces* and *Kitasatospora*. *GH48* gene sequence data are available for only 17 actinobacterial genera so far (de Menezes et al., 2015), and this low coverage of *GH48* gene diversity from cultured actinobacteria preclude a better understanding of the phylogenetic identity of the soil clones obtained here.

Even though differences in cellulolytic community abundance across particle-size fractions and fertilizer treatments were obvious, we did not observe any clearly clustered group of the *cbhI* or *GH48* sequences based on any particular fertilizer treatment or particle-size fraction which was contrary to our hypothesis. This likely suggests that although fertilization and particle-size fraction strongly affected cellulolytic community abundances, they did not have substantial influence on shaping their community structure. de Menezes et al. (2015) studied *GH48* gene community in soils from three paired pasture-woodland sites by using T-RFLP and clone libraries and similarly did not get an obvious cluster of sequences derived from woodlands or pastures, or from a specific site. However, particle-size fractions and fertilizations were comparatively more important in explaining community composition shifts of broader groups (i.e., bacteria, fungi) than those of cellulolytic community (Zhang et al., 2015a; 2016). As the primers developed here target a more specific microbial functional group (i.e., actinobacterial saprotrophs), the lower explanatory power of other ambient variation might simply reflect the narrow physiological range of the cellulolytic community (de Menezes et al., 2015).

4.4. Relations among soil humus with cellulose-degrading microbial community

Soil humification process is primarily a microbially-mediated

process and is generally accompanied by changes in the amount and chemical structure of humus (Ikeya et al., 2004). Although soil microbial diversity and metabolic activity in agricultural ecosystem have gained attention recently, it is not yet exactly clear how the abundance of microbes involved in C cycling are specifically related to humus components. The strong and variable correlations between cellulolytic microbial abundances with soil humus structural characteristics confirmed our hypothesis (Table 5). As previously reported (Shindo et al., 2005), HA was more active and sensitive to environmental changes than FA, which showed high correlations with cellulolytic community abundance in our study (Table 5). Meanwhile, the naturally different characteristics of humus components might result in the opposite relationships detected between the same chemical region of FA and HA with cellulolytic gene abundances (Table 5, Li et al., 2016). The signals in the O-alkyl C region, typically assigned to carbohydrates derived mostly from cellulose and bacterial biomass, were generally considered to be an easily bio-decomposable organic component (Preston and Trofymow, 2000). The greater value of the alkyl C/O-alkyl C ratio in clay fraction indicated that the organic matter associated with it was in advanced humification (Chavez-Vergara et al. 2014; Panettieri et al. 2014), which was consistent with its lower C/N ratio and higher HA/FA ratio. The positive correlation between O-alkyl C of FA with cellulolytic community abundance and the negative relationship between alkyl/O-alkyl C ratio with cellulolytic community abundance confirmed that the cellulose-degrading microbial community prefer less humified fractions such as fine sand, rather than smaller fractions especially clay fraction that contained more stable and recalcitrant substrates (Table 3; Figs. 2 and 4). The aromatic C of FA and O-alkyl C of HA, which did not vary that much in soil samples, had no pronounced correlation with both *cbhI* and *GH48* gene abundances (Table 5).

To the best of our knowledge this study is the first report describing the obviously different distribution of a functional microbial group across soil particle-size fractions mediating soil C transformation using culture-independent methods. We demonstrated that the abundance and structure of the cellulolytic community varied across five soil particle-size fractions under inorganic and organic fertilizer treatments. Organic fertilizer application could accelerate soil C transformation with improved humus composition, enhanced enzyme activities and cellulolytic community abundance. But we still could not tell whether the enhancement was caused by the extra C associated with manure application or the acceleration of natural soil C decomposition based on this study. More detailed studies employing stable isotope label at DNA or RNA level might help to solve this complicated picture of microbial mechanisms underpinning soil C transformation in agroecosystems. Also, future studies focusing on *in situ* expression of glucoside hydrolase genes should provide a better insight into the ecological role of these organisms in soil C cycling and their interactions with other soil saprotrophs.

5. Conclusion

In this long-term fertilizer experiment, distribution of soil humus extracted from different particle-size fractions and calculated HA/FA ratio suggested lower humification degree of fine sand and organic fertilized soils but higher humification degree of clay fraction. The abundance of fungal and actinobacterial cellulolytic microbes varied across different particle-size fractions. The abundances of *cbhI* and *GH48* genes, which were relatively higher in fine sand but lowest in clay fraction, were generally increased under organic fertilizer treatment. Several novel exocellulase *cbhI* and *GH48* lineages were also identified. Strong relationships were detected between cellulolytic community abundance with chemical composition of FA and HA, which indicated that cellulolytic microbes preferred less humified soils with higher aromaticity, lower aliphaticity and alkyl/O-alkyl ratio of HA, such as fine sand fraction, rather than highly humified soils such as clay fraction with higher aromaticity of FA and alkyl/O-alkyl ratios of FA and HA. Given the ubiquity and abundance of fungi and actinobacteria in soils, their role in soil C cycling clearly merits further attention.

Acknowledgements

We are grateful to three anonymous reviewers for their constructive suggestions on this manuscript. We also appreciate Sami Ullah for his help in language improvement. This research was financially supported by the National Natural Science Foundation of China (Grant no. 31471943), National Key Research and Development Program (2016YFD0200109), National Basic Research Program of China (2013CB127405, 2013CB127406), and the earmarked fund for China Agriculture Research System (CARS-01-31).

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