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Biogeographic patterns of microbial association networks in paddy soil within Eastern China

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ABSTRACT

Understanding microbial associations and their responses to environmental changes are important topics in microbial ecology. Using an association network framework, here we compare the potential microbial association patterns within bacteria, fungi or diazotrophs in 198 paddy soil samples within Eastern China. All of three networks were non-random and possessed topological features of complex systems such as scale-free, small-world (i.e., high connectivity) and modularity, which could enable system stability and resilience. However, those networks exhibited distinct topological features. The fungal network was the most complex (based on average degree), the closest (based on average geodesic distance), the least modular (based on modularity) and contained the fewest positive links compared to bacterial and diazotrophic networks. In the fungal and diazotrophic networks, we detected 8 super-generalist OTUs (network hubs), which were the most important nodes in maintaining network structure. Further analyses showed that the bacterial network was mainly shaped by soil temperature, but the fungal network was mainly shaped by ammonia and the diazotrophic network was mainly shaped by volumetric water content, ammonia and soil temperature, signifying the importance of different environmental variables for each community. This network analysis approach provided new insights into microbial community responses to environmental perturbations by inferring bacterial, fungal and diazotrophic associations across a large spatial scale in paddy soils.

1. Introduction

Soil microorganisms play pivotal roles in agricultural ecosystems, including mediation of biogeochemical processes (e.g., carbon and nitrogen cycling) (Fierer et al., 2012; Xu et al., 2013), and promotion of above-ground plant health and productivity (Van Der Heijden et al., 2008; Wagg et al., 2011; Chaparro et al., 2012). Therefore, soil microorganisms have been considered as prospective approaches in agriculture management and ecosystem restoration (Chaparro et al., 2012; Hardoim et al., 2015). Increasing availability to generate datasets from high-throughput sequencing technologies has aided in our understanding on soil microbial diversity, composition and their potential functions. However, there have been a number of studies to analyze associations among microorganisms within a community only in recent years, which could be more important for ecological functioning than microbial diversity (Zhou et al., 2010; Ma et al., 2016).

Soil microorganisms coexist in complex ecological webs with diverse types of associations among species, including mutualism, competition, predation or neutral (Faust and Raes, 2012). However, microbial associations are poorly understood, owing to the lack of appropriate theoretical frameworks and experimental datasets. To address it, network inference approaches based on computational tools were developed to

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predict potential microbial associations from vast sequencing data (Zhou et al., 2010, 2011; Faust and Raes, 2012). Microbial networks are comprised of two components: (1) nodes representing individual microbial taxa derived from operational taxonomic units (OTUs); and (2) edges representing statistically significant, positive or negative associations between nodes, which might infer potential biological relationships. Despite lack of empirical evidence, microbial species with mutualistic relationships are assumed to positively associate with each other, while antagonistic microbes such as competition or predation may negatively associate (Weiss et al., 2016). Although it is challenging to experimentally validate associations among microbial taxa, network analysis may help unveil community and potential functional roles incapable of being approached through alpha- or beta-diversity analyses (Fuhrman and Steele, 2008). First, network analysis could reveal niche overlaps of microbes since similar responses of two taxa to environmental perturbation could result in co-occurrences, and vice versa. Second, association networks can be used to identify keystone species (that is, species highly associated with other species) (Berry and Widder, 2014), which may cause a large shift of microbial community composition as well as functions upon their removal (Berry and Widder, 2014). Third, network topological features (i.e., network complexity and modularity) can be affected by environmental perturbation. For example, microbial network complexity increased and then decreased during 269 days' emulsified vegetable oil amendment for uranium bioremediation (Deng et al., 2016), which can be used to characterize microbial responses to environmental perturbations.

Soil microorganisms can be classified into various taxonomic groups (e.g., archaea, bacteria, fungi and protists) or functional groups (e.g., nitrifiers, denitrifiers and methanogens). Recently, functional groups have gained increasing attention owing to inability to dissect biogeochemical cycling solely by taxonomic information (Louca et al., 2016). Diazotrophs, for example, occurring in a wide range of bacteria and archaea, are only small components of microorganisms (perhaps comprising less than 0.5% of the genomes), but important natural source of bioavailable nitrogen (N) input in many terrestrial habitats by fixing atmospheric N to biologically available NH_4^+-N (ammonia) (Zehr et al., 2003; Kumar et al., 2017). As N is usually the limiting factor for crop growth and productivity, especially in N-poor habitats (Elser et al., 2007; Shu et al., 2012), surveying diazotrophs provides more accurate resolution beyond taxonomic groups in assessing community function. Therefore, examining taxonomic groups of microbial communities may be insufficient to reveal microbial functions, which highlights the importance of incorporating functional groups into microbial ecology studies. However, association analyses of functional communities of microorganisms are much less understood compared to bacteria and fungi.

Microbial biogeography is the study of spatial distribution patterns of microbial communities and their responses to environmental changes (Martiny et al., 2006). Biogeographic patterns of microbial association networks can help unveil potential microbial associations in soils, and contribution of various environmental variables in shaping microbial communities, which could facilitate soil managements especially in agricultural soils. Here, we compared potential association patterns of bacterial, fungal and diazotrophic communities in environmentally heterogeneous paddy soils spanning 4 provinces along the Yangtze River within Eastern China, which is the major rice-producing region of China. As the largest anthropogenic wetland ecosystems and the longest cultivation lands on earth (Kögel-Knabner et al., 2010), paddy soils in China produce ~25% of grains for Chinese market. However, N loss is tremendous due to excessive use of fertilizers (Xing and Zhu, 2000; Zhu et al., 2011). Therefore, it is imperative to examine diazotrophic communities related to nitrogen fixation, which can be used to evaluate biological nitrogen fixation potentials in paddy soils. Specifically, we aim to test the following scientific questions - (i) whether microbial associations in paddy soils varied among bacteria, fungi and diazotrophs; and (ii) what environmental variables contributed to the association patterns of bacteria, fungi and diazotrophs. Given that soil pH has been reported to be a primary driver for soil bacterial spatial distribution (Fierer and Jackson, 2006; Shen et al., 2013), and microbial lifestyles differ between bacteria and fungi, we addressed two specific hypotheses in the current study - (i) association patterns and their environmental drivers in paddy soils vary among bacteria, fungi and diazotrophs; and (ii) soil pH links strongly to bacterial association patterns.

2. Materials and methods

2.1. Soil sampling

The detailed sampling information is shown in Fig. S1A. A total of 18 long-term paddy plots span 6 counties of Changxing (CX), Hefei (HF), Huangshi (HS), Jurong (JR), Xiantao (XT) and Tongcheng (TC), with 3 paddy plots evenly distributed within each county. Survey was performed from late October to early November in 2015. In each paddy plot, 11 topsoil samples, 20 cm in depth and 5 cm in diameter, were collected along the L-shape transects (Fig. S1B). Distances between two adjacent soil samples along both transects are 1 m, 5 m, 10 m, 20 m and 40 m, respectively. Our nested design of the experiment within and among paddy plots allows for data analyses across different spatial scales of plot-level, county-level and overall-level. Soil samples were packed into sterile polyethylene bags, immediately kept in a portable 4 °C refrigerator and then transported to the laboratory. Soils were used for DNA extraction (stored at −80 °C) or physiochemical variables measurement (stored at 4 °C).

2.2. Environmental variable collection and measurements

In this study, 9 soil physiochemical variables, including volumetric water content (VWC, %), soil temperature (Soil T, °C), soil pH (Soil pH), ammonia (NH_4^+-N, ppm), nitrate (NO_3^--N, ppm), dissolved total nitrogen (DTN, ppm), dissolved total carbon (DOC, ppm), microbial carbon (MBC, mg/kg) and microbial nitrogen (MBN, mg/kg), were measured (Table S1). In brief, VWC at the soil depth of 0–12 cm and soil T at the depth of 10 cm were measured *in situ* when soil samples were collected. VWC was measured for three times to generate mean values using a TDR 300 (Time-Domain Reflectometer, Spectrum Technologies, Inc., Aurora, IL, USA). Soil T was measured for three times to generate mean values using a mercurial thermometer. Soil pH was measured by a pH meter (E20-FiveEasyTM pH, Mettler Toledo, German) in a 1:5 ratio of soil to deionized water slurry after shaking for 30 min. To measure NH_4^+-N, NO_3^--N, DTN and DOC, 10 g of soil was added to a 250 ml plastic bottle with 100 ml of 2 mol/L KCl solution. NH_4^+-N and NO_3^--N concentrations were measured using a spectrophotometer. DTN and DOC were measured using a Multi N/C 2100 analyzer (Analytik, Jena, Germany). A total of 5 g vacuum-desiccated soil was added to 100 ml of 2 mol/L K_2SO_4 solution, vibrated, filtered, and the supernatant was used for MBC and MBN measurement (Wang and Wang, 2008). The concentration units for NH_4^+-N, NO_3^--N, DTN, DOC, MBC and MBN were uniformly transformed to mg/kg. In addition, geographical variables (longitude and latitude) and local climatic variables (monthly recorded ground surface temperature and monthly precipitations) were included in this study. The longitude and latitude of each sample were recorded by a portable GPS machine when soils samples were collected. Ground surface temperature at the soil depth of 5 cm and precipitation of each county were downloaded from public datasets generated by weather stations in China (http://data.cma.cn/site/index.html). These mean monthly values represent regional climate conditions.

2.3. DNA extraction, amplicon sequencing and sequencing data processing

Soil DNA was extracted using PowerMax Soil DNA Isolation Kit

(MOBIO Laboratories, Inc., Carlsbad, CA, USA) after freeze-grinding lysis (Ding et al., 2015). Extracted DNA for PCR amplification was diluted to 2 ng/μL. Three genes were amplified separately: (i) 16S ribosomal RNA (rRNA) genes for bacteria; (ii) internal transcribed spacer (ITS) for fungi; and (iii) bacterial introgenase subunit H (*nifH*) for diazotrophs. Universal primer pairs used were: 515F/806R (Caporaso et al., 2012) for 16S rRNA genes, ITS7F/ITS4R (Ihrmark et al., 2012) for ITS genes and PolF/PolR (Poly et al., 2001) for *nifH* genes. Two-step PCR was used to prepare amplicon libraries of these three genes as described previously (Wu et al., 2016). Specifically, 10 cycles were used in the first step and 20 cycles in the second step for both 16S rRNA and ITS genes, while 12 cycles were used in the first step and 23 cycles in the second step for *nifH* genes. PCR products were then quantified by PicoGreen, and equal amounts of DNA per sample were combined to generate pooled libraries. The pooled libraries were sequenced on a desktop MiSeq system (Illumina, San Diego, CA, USA) (2 × 250 bp paired ends) following the manufacture's protocols at the Institute for Environmental Genomics, University of Oklahoma after purification using QIAGEN Gel Extraction Kit (QIAGEN Inc., Valencia, CA, USA).

Raw amplicon sequencing data analysis was performed using the in-house Sequencing Analysis Pipeline (http://zhoulab5.rccc.ou.edu:8080/root) at University of Oklahoma, Norman, OK, USA. Briefly, chimeras were removed using Uchime (Edgar et al., 2011) after deleting reads of poor qualities by Btrim (Kong, 2011). The 16S rRNA gene and ITS sequences were then clustered into Operational Taxonomic Units (OTUs) with UPARSE (Edgar, 2013) at the 97% identity cutoff. The *nifH* sequences were clustered into OTUs with complete linkage clustering (Loewenstein et al., 2008) at the 95% amino acid identity (Penton et al., 2016; Afgan et al., 2018) with a hand-curated database of *nifH* protein sequences after corrected by Framebot software (Wang et al., 2013). A few samples were discarded due to poor quality in bacterial, fungal or diazotrophic sequence reads.

To normalize sequencing data prior to subsequent analysis, all sequences were randomly resampled to 23,000 sequences for bacteria, 2,000–10,000 sequences for fungi, and 10,000 sequences for diazotrophs, which were sufficient to capture the diversity of bacterial, fungal and diazotrophic communities, according to the rarefaction curves (Fig. S2).

2.4. Network analyses

After obtaining OTU tables for 16S rRNA gene, ITS and *nifH* gene, abundance data were transformed into relative abundance by dividing the sum reads of each sample as described previously (Wu et al., 2017). Only OTUs detected in more than half of samples were used for each network construction. In brief, pairwise similarities of relative abundance data across different OTUs were calculated based on Spearman correlation coefficients. The correlation matrix was subsequently transformed into a similarity matrix, and the random matrix theory (RMT) was used to automatically define the appropriate similarity threshold (*St*) prior to network construction (Yang et al., 2009). Once the *St* was determined, an adjacency matrix was obtained by keeping all the OTUs whose similarity values were larger than the determined *St*. Then the molecular ecological network (MEN) was constructed using the Molecular Ecological Network Analyses (MENA) Pipeline (http://ieg4.rccc.ou.edu/mena). Once the MEN was constructed, various network topological features were calculated based on the adjacency matrix using the MENA Pipeline. The topological features for individual nodes are useful in accessing nodes' roles in the network, and the topological features for the network are useful in comparing various MENs identified under different situations (Deng et al., 2012). To visualize association networks, the node represents a species or taxon and the edge linking two nodes represents positive or negative correlation between two nodes. Bacterial, fungal or diazotrophic communities in soil samples were categorized at the overall-level, county-level or plot-level. A few plot-level networks in bacteria were discarded because of excessive nodes for network constructions using the MENA pipeline. Topological features examined here included the total node number, total link number, average degree (avgK), centralization of degree (CD), average cluster coefficient (avgCC), average geodesic distance (GD), centralization of betweenness (CB), centralization of stress centrality (CS), density, total module number and modularity. Detailed biological implications of those network features were described previously (Table S2) (Wu et al., 2016). All networks were graphed using Cytoscape 3.7.0 (Shannon et al., 2003). The topological roles of nodes in the networks were classified into four categories according to within-module connectivity (*Zi*) and among-module connectivity (*Pi*): network hubs (highly connected nodes within entire network, $Zi > 2.5$ and $Pi > 0.62$), module hubs (highly connected nodes within modules, $Zi > 2.5$ and $Pi \leq 0.62$), connectors (nodes that connect modules, $Pi > 0.62$) and peripherals (nodes connected in modules with few outside connections, $Zi < 2.5$ and $Pi \leq 0.62$) (Deng et al., 2012).

2.5. Statistical analyses

One hundred random networks corresponding to each overall network were generated. In the random network, the numbers of nodes and links were constant, but all the links' positions were rewired randomly so that the rewired network was comparable to the empirical one (Maslov and Sneppen, 2002). For the 100 random networks, each network index was calculated with the average and the standard deviation. The *Z* test was employed to determine the significance of network indices between the empirical and random networks. For comparison of network indices from different networks, the student *t*-test was applied using the standard deviations derived from corresponding random networks. The importance of environmental variables for network topological features were estimated using multiple regression model (MRM) with the R 'ecodist' package. All environmental variables were standardized with function 'decostand' in R vegan package, and Euclidean distance matrices were used in the MRM model. Pearson's correlations were used to determine how environmental variables influence network topological features with the R 'cor.test' function. We further investigated the importance of environmental variables on microbial community composition using MRM model, in which Euclidean distance matrices for environmental variables datasets and Bray-Curtis distance matrices for community datasets were used. We also examined the relationships between microbial networks and environmental variables in an indirect way using OTU significance, which was defined as the square of Pearson correlation coefficient (r^2) of OTU abundance profile with environmental variables (Deng et al., 2012). After calculating OTU significance, Mantel tests were used to examine the relationships between OTU significance and network features for exploring relationships between potential interactions and environmental variables.

3. Results

3.1. Overall bacterial, fungal and diazotrophic communities in paddy soils

We obtained 32,014 bacterial OTUs (ranging from 1,199 to 5,838 OTUs across 188 samples), 11,341 fungal OTUs (ranging from 134 to 955 OTUs across 186 samples) and 17,677 diazotrophic OTUs (ranging from 252 to 2,235 OTUs across 183 samples). The most abundant phylum in bacterial community was *Deltaproteobacteria* (13.53%), followed by *Acidobacteria* (12.89%), *Alphaproteobacteria* (9.48%), *Betaproteobacteria* (8.76%), *Chloroflexi* (7.64%) and *Gammaproteobacteria* (5.00%) (Fig. S3A). Five classifiable phyla were identified for fungal communities: *Ascomycota* (45.75%), *Basidiomycota* (24.22%), *Zygomycota* (9.50%), *Chytridiomycota* (4.07%) and *Glomeromycota* (2.50%) (Fig. S3B). In contrast, the majority of diazotrophic communities in paddy soils were unclassified (80.91%) at the genus level, followed by *Bradyrhizobium* (5.18%), *Azotobacter* (3.71%), *Geobacter* (1.17%) and *Methylomonas* (1.06%) (Fig. S3C), suggesting that our knowledge about

diazotrophs was limited.

3.2. Overall network topological features from bacteria, fungi and diazotrophs

We constructed association networks at the overall-, county- and plot-levels, resulting in a total of 70 networks. All of them were non-random and displayed the scale-free feature, with R^2 of power law ranging from 0.711 to 0.967 (Table S3). The three overall networks also exhibited other general topological features, such as small-world and modularity (Table 1). Specifically, GD (geodesic distance) values were significantly higher than their corresponding randomized networks, indicating the small-world behavior. Modularity values were significantly higher than the modularity values from corresponding randomized networks, indicative of the modular feature. The association patterns among bacterial, fungal and diazotrophic communities were markedly different. The avgCC (average cluster coefficient), GD, and modularity were the highest ($p < 0.05$) for the bacteria, and the least ($p < 0.05$) for the fungi (Table 1). All county- (Fig. S4) and plot-level (Fig. S5) networks showed the modularity featureFig. S5, which was similar as the overall-level networks (Table S4). Most of county- and plot-level networks exhibited the small-world feature, except for 2 county-level (JR of bacteria and HF of diazotroph) and 3 plot-level (TC2 and JR1 of bacteria, and TC2 of diazotroph) networks (Table S4).

The overall-level network of bacterial communities tended to have positive correlations rather than negative correlations since positive links accounted for 57.91% of the potential interactions (Fig. 1). However, the overall-level networks of fungal (10.30% positive links) and diazotrophic (42.57% positive links) communities harbored fewer positive links than the bacterial network (Fig. 1). Similar pattern was also found in the networks at the county or plot level: bacterial networks contained more positive correlations (63.87% in the county-level and 69.86% in the plot-level), but fungal (32.9% in the county-level and 35.17% in the plot-level) and diazotrophic (31.14% in the county-level and 21.78% in the plot-level) networks contained fewer positive correlations (Table S3).

3.3. Putative keystone taxa

Networks hubs, modules hubs and connectors were identified in all networks (Fig. 1). For bacteria, no network hub was identified (Table 2).

Table 1
Topological features of empirical microbial networks and comparison with corresponding random networks.

	Network features	Bacteria	Fungi	Diazotrophs
Empirical networks	R^2 of power law	0.901	0.899	0.843
	Total nodes	271	86	169
	Total links	632	233	343
	Average degree (avgK)	4.664	5.419	4.059
	Average geodesic distance (GD)	**5.494**[b]	**3.035**	**3.909**
	Average clustering coefficient (avgCC)	**0.234**	**0.061**	**0.157**
	Modularity	**0.613**	**0.369**	**0.535**
Random networks[a]	Average geodesic distance (GD)	3.460 ± 0.039	2.801 ± 0.056	3.357 ± 0.063
	Average clustering coefficient (avgCC)	0.055 ± 0.009	0.134 ± 0.018	0.076 ± 0.013
	Modularity	0.426 ± 0.007	0.332 ± 0.010	0.445 ± 0.008

[a] Parameters of random networks were generated from 100 times of randomly rewired networks. Parameters presented here are mean values and standard derivations from random networks.

[b] Significant difference ($p < 0.05$) between any two of the 3 overall-level networks are shown in bold.

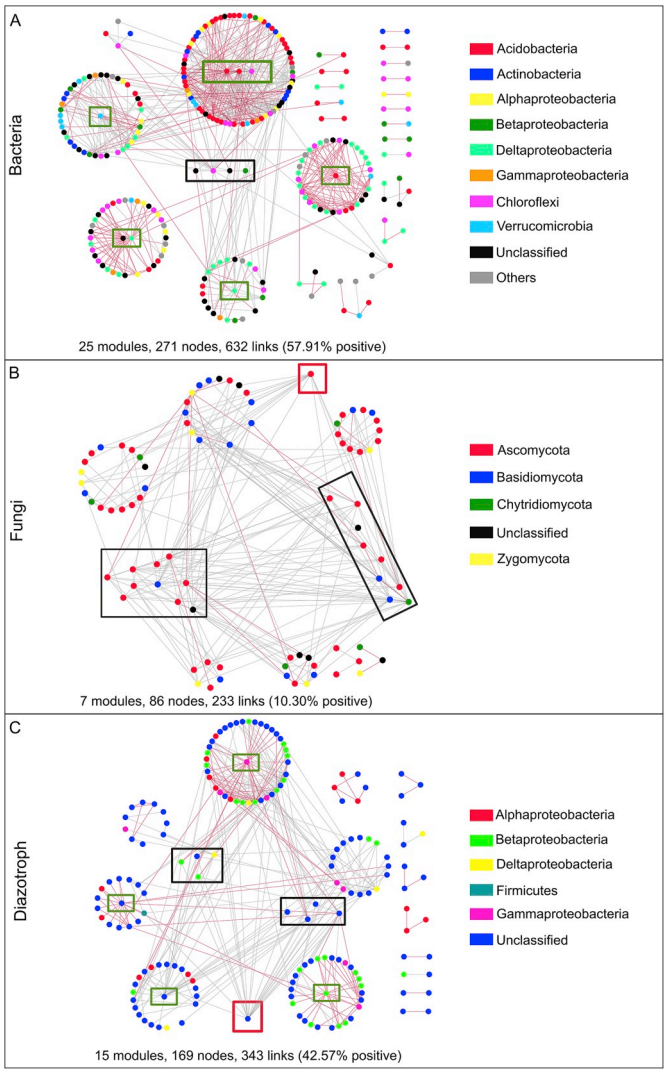

Fig. 1. An overview of microbial networks of bacteria (A), fungi (B) and diazotrophs (C). In the network graph, colors of nodes represent different phyla (classes for Proteobacteria); nodes in the red boxes are network hubs; nodes in the middle of modules in green boxes are the modules hubs; nodes in the black boxes are connectors; Red links represent positive correlations and grey links represent negative correlations. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Members from *Acidobacteria*, *Alphaproteobacteria*, *Deltaproteobacteria* and *Chloroflexi* together accounted for approximately half of all module hubs (49.80%) and connectors (48.31%) in the bacteria (Table S5). A total of 4 fungal network hubs belonged to *Podospora* (*Ascomycota*),

Table 2
Network hubs in the networks within bacterial, fungal and diazotrophic communities.

Network	Network hubs	Reads abundance (%)	Phylum	Genus
ITS	OTU_25	0.585	*Ascomycota*	*Podospora*
ITS-HF	OTU_583	0.049	*Ascomycota*	Unclassified
ITS-HS3	OTU_539	0.092	*Zygomycota*	*Mortierella*
	OTU_546	0.051	*Basidiomycota*	*Rhodotorula*
nifH	OTU_585	0.035	Unclassified	Unclassified
***nifH*-HF**	OTU_2691	0.021	Unclassified	Unclassified
***nifH*-TC**	OTU_46	2.089	Unclassified	Unclassified
***nifH*-CX1**	OTU_6565	0.019	Unclassified	Unclassified

Mortierella (*Zygomycota*), *Rhodotorula* (*Basidiomycota*) and an unclassified genus from *Ascomycota* (Table 2). Members of *Ascomycota* (35.29%–44.62%) and *Basidiomycota* (23.08%–30.88%) dominated the module hubs (66.18%) and connectors (67.70%) from fungal networks, and unclassified fungal phyla accounted for 10.29–11.54% of them (Table S5). A total of 4 network hubs in the diazotrophic network were identified, with all from unclassified phyla (Table 2). In addition, 69.23% of module hubs and 65.73% of connectors in diazotrophs were unclassified, with the rest belonging to *Betaproteobacteria* (10.26%–12.59%), *Alphaproteobacteria* (4.20%–7.69%), *Deltaproteobacteria* (6.41%–11.19%) and *Gammaproteobacteria* (4.90%–5.13%) (Table S5). Notably, most of module hubs (78.98%) and connectors (81.22%) were low in relative abundance (<0.099%), revealing potentially important roles of rare taxa in communities. In addition, few hubs and connectors were present in multiple networks.

To explore the interrelationships between two kingdoms, a bacteria-fungi network was constructed based on bacterial and fungal OTUs, which also exhibited typical topological features of scale-free, small-world and modularity (Table S3). There were 903 bacteria-bacteria links, overwhelmingly outnumbering the 3 bacteria-fungi links of *Bacteroidetes-Zygomycota*, *Chloroflexi-Ascomycota* and *Acidobacteria-Basidiomycota*. In the bacteria-fungi network, all hubs and connectors were bacteria, with one network hub belonging to *Deltaproteobacteria* (Table S6).

3.4. Association of network topological features with environmental variables

Associations of environmental variables with network topological features were assessed (The MRM analysis, Fig. 2 & Table S7). Collectively, these variables explained substantial variations in network topological features of fungi (46.54%, $p < 0.05$), but were insignificant for bacteria (62.74%, $p > 0.10$) or diazotroph (38.94%, $p > 0.10$). The most important variable linking to bacteria was soil temperature (8.88%, $p < 0.05$) (Fig. 2). In fungi, the most important variable was NH_4^+-N (18.51%, $p < 0.05$). In diazotrophs, the most important variables were VWC (volumetric water content) (15.19%, $p < 0.05$), NH_4^+-N (13.59%, $p < 0.05$) and soil temperature (11.01%, $p < 0.05$). Those environmental variables linking to network topological features also correlated with microbial community composition (Table S8).

In bacterial networks, total module number positively correlated with NH_4^+-N ($r = 0.68$, $p = 0.01$), but negatively correlated with soil pH ($r = -0.53$, $p = 0.06$) (Fig. 3). CB (centralization of betweenness) negatively correlated with DOC (dissolved total carbon) and NH_4^+-N ($r < -0.62$, $p < 0.05$). In fungi, network topological features correlated with NH_4^+-N, and soil temperature (Fig. 3). Total module number, modularity and GD positively correlated with NH_4^+-N ($r > 0.51$, $p < 0.05$). Also, modularity positively correlated with soil temperature ($r = 0.56$, $p < 0.05$). AvgK (average degree), CD (centralization of degree) and density negatively correlated with NH_4^+-N ($r < -0.56$, $p < 0.05$). CD and density negatively correlated with soil temperature ($r < -0.51$, $p < 0.05$). GD negatively correlated with NO_3^--N (nitrate) ($r = -0.52$, $p < 0.1$). In diazotrophs, network features correlated with NH_4^+-N and soil temperature (Fig. 3). Specifically, total module number, modularity, total node number, and GD positively correlated with NH_4^+-N ($r > 0.54$, $p < 0.05$). CD and density negatively correlated with NH_4^+-N ($r < -0.62$, $p < 0.05$). CS and density negatively correlated with soil temperature ($r < -0.53$, $p < 0.05$).

Trait-based OTU significance was also measured to evaluate associations between environmental variables and network features (Table S9). In the bacterial network, very strong correlations were observed between the node connectivity and the OTU significance of selected soil variables, based on all detected OTUs ($p < 0.05$) or several phylogenetic groups such as *Chloroflexi* ($p < 0.05$) and unclassified phyla ($p < 0.05$). In diazotrophs, the connectivity of all detected OTUs ($p < 0.05$) and unclassified OTUs ($p < 0.05$) significantly correlated with the OTU significance. However, no significant association was found in fungi.

4. Discussion

Given high complexity of microbial communities (Van Dijk et al., 2014), it is challenging to assess potential microbial associations. Therefore, association networks have been widely used to infer potential relationships among microorganisms (Zhou et al., 2010, 2011; Faust et al., 2012). Here, we compared association networks of bacteria, fungi and diazotrophs in paddy soils. We showed that potential association patterns of different microbial groups varied substantially in terms of topological features, keystone species, and correlations with environmental variables. As a result, there was no universal pattern of potential microbial associations across microbial taxonomic and functional groups.

All networks of paddy soil microbiome are scale-free and non-random. This is not surprising, given that non-random correlational networks are commonly documented for macro- and microorganisms (Horner-Devine et al., 2007; Prosser et al., 2007) as well as many social,

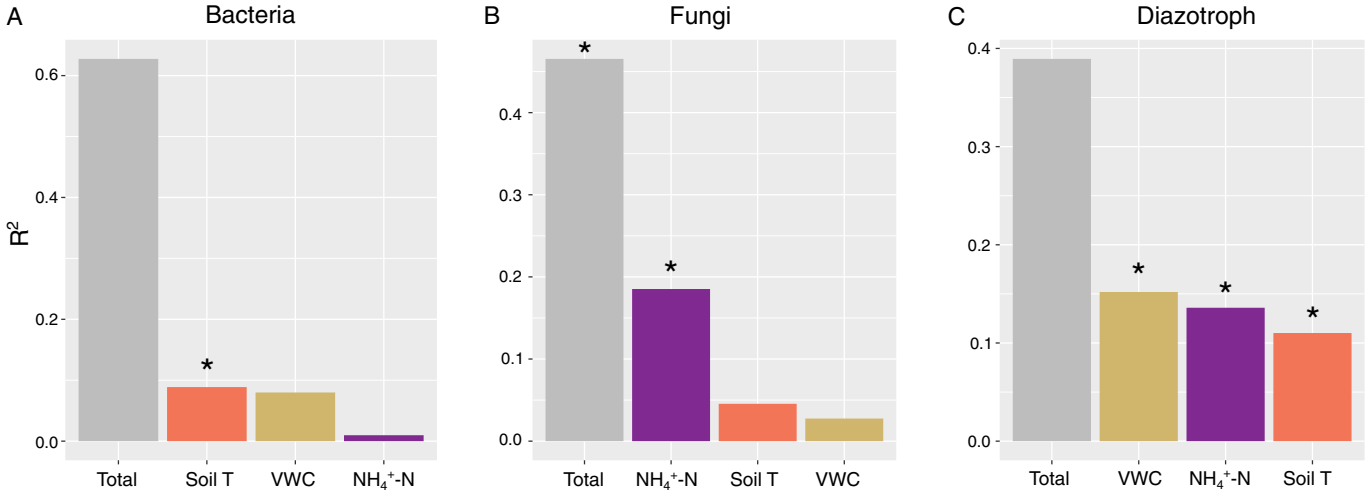

Fig. 2. The contribution (R^2 values) of the top four important environmental variables to topological features of overall-level networks in bacteria (A), fungi (B) and diazotrophs (C). Soil T, soil temperature; VWC, volumetric water content; NH_4^+-N, ammonia. * indicated the significant result at the $p < 0.05$ level. R^2 and p values were calculated using multiple regression of distance matrix analysis.

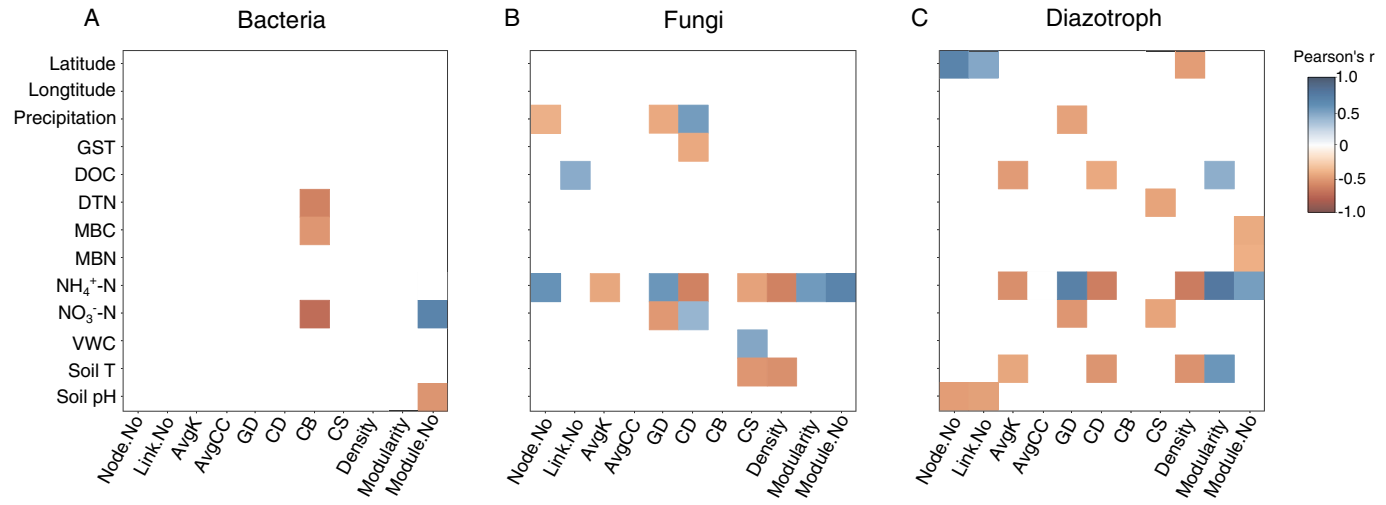

Fig. 3. Pearson correlation between network topological features and environmental variables. GST, ground surface temperature at the soil depth of 5 cm; DOC, dissolved total carbon; DTN, dissolved total nitrogen; MBC, microbial biomass carbon; MBN, microbial biomass nitrogen; NO_3^--N, nitrate; NH_4^+-N, ammonia; VWC, volumetric water content; Soil T; soil temperature. Node.No; the number of total nodes; Link.No; the number of total links; AvgK, average degree; AvgCC, average cluster coefficient; GD, average geodesic distance; CD, centralization of degree; CB, centralization of betweenness; CS, centralization of stress centrality; Modules.No; the number of total modules.

technological and molecular biological networks (Albert and Barabási, 2002; Barabási and Oltvai, 2004; Bullmore and Sporns, 2009). The findings of non-random network patterns in this study may suggest that paddy soil microorganisms tend to be correlational more than expected by chance, which could be attributed to roles of deterministic processes in shaping microbial community. All the networks showed topological features of scale-free, small-world and modularity. Those general features could have important implications for the stability and resilience of ecosystems. Biological networks with scale-free property show a pattern that few OTUs (hubs or connectors) have many associations whereas most OTUs (specialists) have few associations (Barabási, 2009). Consequently, the networks are robust in face of random removal to nodes, but are vulnerable to attacks against highly connected OTUs (hubs or connectors) (Barabási and Oltvai, 2004). For instance, it was shown that deleting a hub node in the yeast interactome network of protein-protein interactions could exert more phenotypic outcomes than other nodes (Yu et al., 2008). The small-world network means that any two OTUs can be connected with only a few associations, which could facilitate rapid responses of microbial community to environmental changes (Albert et al., 2000; Montoya et al., 2006). In microbial ecology, module means compartmentalization, i.e., a group of microbial species are intensively connected within themselves but loosely connected with nodes in other modules, which may result from habitat heterogeneity, niche differentiation and/or divergent selection (Olesen et al., 2007). Therefore, one module is expected to exert no or limited effect on other modules in the microbial community, which helps reduce microbial responses to environmental perturbations by constraining environmental perturbations within modules (Kitano, 2004). Here, we predict that stability of microbial associations in paddy soils remains robust in face of environmental perturbations, which may be important for soil ecosystem stability and functioning.

Species with a critical role in maintaining ecosystem stability, regardless of their abundance, are commonly defined as keystone species, which can often be detected as network hubs, module hubs and connectors by network analyses (Olesen et al., 2007; Faust and Raes, 2012). In this study, putative keystone microbial species were detected by the topology-based approach. Although the network hub is missing in many network studies (Barberán et al., 2012; Faust et al., 2012; Deng et al., 2016; Ma et al., 2016; Shi et al., 2016), a number of network hubs in the fungal and diazotrophic networks, as well as the bacteria-fungi network, were identified (Tables 2 and S6). However, all network hubs from the diazotrophic and bacteria-fungi networks are unclassified, which warrantee future work to characterize those keystone species. In contrast, it has been shown that *Podospora*, a fungal network hub, is one of the most abundant genera in healthy soils (Xu et al., 2012) and can control *Verticillium* wilt of tomato (Dutta, 1981). *Podospora* (0.585%) is present across 6 counties, suggestive of a crucial, prevalent role in paddy soil communities. Another network hub *Mortierella* is an important phosphate-solubilizing fungus in promoting soil enzyme activities and plant growth (Zhang et al., 2011), and the network hub *Rhodotorula* has been reported as a promising agent for *in situ* bioremediation of medium to high-level pesticide contaminated sites (Salam et al., 2013). Considering the critical role of keystone species in community complexity and stability, these species should receive high conservation priorities when environmental perturbation occurs. Keystone microbial species, such as mycorrhizal fungi, can mediate plant-plant communication by transferring signals of pathogenic fungal diseases from infected plants to their neighbor to invoke defenses (Babikova et al., 2013; van der Heijden and Hartmann, 2016), the presence of such keystone species could thus be essential for sustaining soil health and crops productivity. As a result, manipulating keystone species of microbial network structures, including isolating, characterizing, removing and adding putative keystone species, could provide a promising approach for agriculture management to improve crops productivity (Banerjee et al., 2018).

Only a few hubs or connectors show wide distribution across different plots, supporting the context dependency theory that keystone species does not dominate anywhere or any time, but play critical roles only under specific contexts (Salam et al., 2013). Our finding is in alignment with a previous observation that putative keystone species change with conditions (Lu et al., 2013; Lupatini et al., 2014). Interestingly, keystone species tend to be low in abundance (Shi et al., 2016; Wu et al., 2016), which is also observed in this study (Table 2, Table S5 & S6). Recent studies have showed that less abundant or rare species may play important roles in determining genetic diversity, functional diversity and ecosystem stability against environmental perturbation (Jousset et al., 2017). Therefore, a focus on abundant taxa, as proposed recently (Delgado-Baquerizo et al., 2018), would overlook some important species.

Positive correlations may indicate cooperative or mutualistic potential associations such as cross-feeding and/or syntrophic relationships, while negative correlations could infer antagonistic associations among species such as predation and/or competition for a limiting

resource (Faust and Raes, 2012). Here, we detected more positive correlations (57.91%) in bacterial networks than diazotrophic networks (42.57%) and fungal networks (10.30%) (Fig. 1). A possible explanation is that typical fungal lifestyles (e.g. heterotrophic, saprotrophic and spore-forming) are more universal and adapt to more niches than bacterial lifestyles. The weak niche differentiation of fungal communities possibly results in stronger competition (i.e., negative associations) for similar niches between soil microorganisms. However, negative feedbacks tend to stabilize processes while positive feedbacks conversely enhance ecosystem changes and destabilize the *status quo* (Simard et al., 2012), it is thus likely that fungal communities in the paddy soils may be more robust to environmental perturbations than bacteria.

As temperature can greatly increase metabolic rates and biochemical processes (Gillooly et al., 2001; Brown et al., 2004), temperature has strong effects on microbial metabolism and ecology. However, the temperature effect on potential microbial associations was rarely studied as compared to microbial diversity and composition (Lin et al., 2017). In this study, soil temperature rather than soil pH, was linked to microbial associations of bacteria and diazotrophs (Fig. 2 & Table S7). The finding was consistent with a previous study that temperature played crucial roles in affecting potential bacterial associations in anaerobic digestion processes (Lin et al., 2017), and reinforced critical roles of temperature in controlling microbial growth, activities and diversity (Davidson and Janssens, 2006; Zhou et al., 2016). In contrast, ammonia was correlated significantly to both diazotrophic and fungal associations but not bacterial associations (Fig. 2). In natural terrestrial ecosystems, bioavailable ammonia mainly originates from biological N fixation, which results from diazotrophic activities. Fungi, including ectomycorrhizal and arbuscular mycorrhizal fungi, are responsible for acquiring N for rice crop (Chalot et al., 2006; Guether et al., 2009), which form a strong link with paddy soil ammonia. Owing to significant correlations of temperature and ammonia in potential microbial associations (Fig. 2 & Table S7) and composition (Table S8), paddy soil processes may be sensitive to those environmental variables as well.

In summary, this study provides valuable insights into microbial associations in paddy soils at a large spatial scale, which go beyond the basic descriptions of microbial alpha- and beta-diversity by exploring potential, positive or negative relationships among community members. All association networks exhibited general topological features, such as scale-free, small-world and modularity. However, association patterns and their environmental drivers varied among different microbial groups. Compared to bacterial and diazotrophic associations, the fungal association was predicted to be the most complex, closest, antagonistic and had least niche differentiation (or selection). In addition, we identified 8 super-generalist OTUs in the fungal and diazotrophic associations, which may be important for maintaining microbial community structure.

Author's contribution

YY and JZhou designed this research. QG and YY collected the samples and performed the lab experiments. XW, QG, JZhao and JF completed bioinformatic analysis and further analysis. XW, YY and JZhou wrote the manuscript. QG, JZhao and JDVN helped write and improve the manuscript. All authors have contributed to the final manuscript.

Data accessiblity

MiSeq sequencing datasets of 16S rRNA gene, ITS and *nifH* gene are available in the NCBI Sequence Read Archive (SRA) with the accession number PRJNA438873.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

The authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.soilbio.2019.107696.

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