Heterogeneity of Soil Bacterial and Bacteriophage Communities in Three Rice Agroecosystems and Potential Impacts On Nutrient Cycling

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Abstract

**Background:** As genetic entities infecting and replicating only in bacteria, bacteriophages can regulate the community structure and functions of their host bacteria, but they are often overlooked because of their relatively low abundance. The ecological roles of bacteriophages in aquatic and forest environments have been widely explored, but those in agroecosystems remains limited. Here, we used metagenomic sequencing to analyze the diversity and interactions of bacteriophages and their host bacteria in soils from three typical rice agroecosystems in China: double cropping in Guangzhou, southern China, rice–wheat rotation cropping in Jiamusi, northeastern China, and early-maturing single cropping in Nanjing, eastern China. Bacteriophages were isolated and their functions on soil nitrogen cycling and effect on soil bacterial community structure were verified in pot inoculation experiments and Illumina MiSeq sequencing.

**Results:** Soil bacteria and viral diversity and functions varied among the three agroecosystems. Genes detected in communities from the three agroecosystems were associated with typical functions; soil bacteria in Jiamusi were significantly enriched in genes related to carbohydrate metabolism, in Nanjing with xenobiotics biodegradation and metabolism, and in Guangzhou with virulence factors and scarce in secondary metabolite biosynthesis, which might lead to a significant occurrence of rice bacterial diseases. In the three ecosystems, 368 species of virus were detected. Notably, over-represented auxiliary carbohydrate-active enzyme (CAZyme) genes were identified in the viruses, which might assist host bacteria in metabolizing carbon, and 67.43% of these genes were present in Jiamusi. In bacteriophage isolation and inoculation experiments, Enterobacter bacteriophage-NJ reduced the nitrogen fixation capacity of soil by lysing N-fixing host bacteria and changed the soil bacterial diversity and community structure.

**Conclusions:** Our results showed that diversity and function of paddy soil bacteria and viruses varied in the three agroecosystems. Soil bacteriophages can affect nutrient cycling by expressing auxiliary metabolic genes (AMGs) and lysing the host bacteria that are involved in biogeochemical cycles. These findings form a basis for better understanding bacterial and bacteriophage diversity in different rice agroecosystems, laying a solid foundation for further studies of soil microbial communities that support ecofriendly production of healthy rice.

**Background**

Rice, the primary staple food for about 60% of China's population [1] and planted in about 29.5 million ha in China, accounts for 35% of the total area planted in grain crops [2]. Rice-growing areas in China are quite diverse and distributed throughout most of the country and differ in ecological environment, cultivation methods and production levels. The three most important rice agroecosystems are rice double cropping in southern China (22% of total rice area), rice rotation with other crops in central and eastern China (59% of total) and early-maturing single cropping in northeastern China (9% of total). Southern China has a subtropical and marginal tropical monsoon climate, which is warm and rainy all year with long summers and short winters [3], 5800–7500°C annual accumulated temperature, and about 300 days for rice growth, which is enough for double cropping. Central and eastern China belong to the climate in transition from subtropical to temperate zone, which is hot and rainy in summer but cold with less rain in winter with 4500-6500°C annual accumulated temperature and 210–260 days for plant growth, which is sufficient for rice rotation with other crops (wheat, oil rape, green manure or vegetables). However, northeastern China is at a high latitude with a temperate monsoon climate; it has a cold, long winter and warm, short summer. The annual accumulated temperature in this region is 2000-3700°C, and the rice production season is 150–180 days, long enough for only one rice crop. Flat terrain and fertile soil in this area, however, produce higher quality and yields of rice.

Different ecological environments and farming methods lead to different soil physicochemical properties and soil microbial diversity [4–6]. The hundreds of millions of soil microbes in soils include bacteria, fungi, archaea, viruses, that compete for nutrients and living space in the soil. Determining the physicochemical properties of soils and their effects on the soil microbial composition and diversity in different regions can help in utilizing local natural resources effectively and improving cultivation techniques to safely produce higher-yielding rice. Soil microbes not only breakdown organic matter and minerals, they also secrete secondary metabolites that can regulate plant growth [7–9]. Microbes in soil can cause disease or help protect against disease [10, 11]. Soil microbial communities and functions have strong spatial variability [12]. Bahram et al. (2018) found contrasting patterns in bacterial diversity in top soils across a latitudinal gradient around the world, with the highest diversity in temperate habitats, and environmental variables caused greater variation in microbial gene diversity than geographic distance did [13]. Soil microbial diversity and multifunctionality (decomposition and nutrient cycling, plant productivity) were demonstrated to be spatially specific and positively correlated in 78 global drylands and 179 locations across Scotland [14]. Elucidating spatial and temporal patterns of soil microbial diversity, community composition, and function will thus contribute to a better understanding of the generation and maintenance of microbial diversity, which will help to harness beneficial microbial resources for full use of their ecological functions in promoting healthy growth of plants in environmentally safe ecosystems.

Bacteriophages, which can infect and replicate within particular bacterial hosts, are also important components of soil microbial community, but are often overlooked due to their relatively low abundance [15]. However, they can regulate the community structure and function of bacteria and even the whole soil microbes [16]. Bacteriophages can modulate host bacterial populations and diversities by lysing bacterial cells [17], driving host evolution via bacteriophage-mediated horizontal gene transfer [18], and altering host bacterial function by reprogramming their metabolism via the expression of virus-encoded auxiliary metabolic genes (AMGs) [19]. AMGs have a variety of metabolic functions, including in carbon, nitrogen and sulfur metabolism [20, 21], and photosynthesis [22]. However, most studies on the function of bacteriophages have focused on marine ecosystems or the human intestinal tract rather than soils in agroecosystems.

Our knowledge about paddy soil microbial diversity and function in different agroecosystems is also limited, and even less is known about viral communities and their roles. Studies of viruses in glacier soil [23], mangrove soil [24], mud volcanic soil [25], and Antarctic soil [26] revealed differences in soil viral community structure and largely uncharacterized viral assemblages. In the present study, we used metagenomic sequencing to analyze bacterial and viral community composition, diversity and ecological functions in paddy bulk soils in the three most important rice agroecosystems, double cropping in southern
China, rice-wheat rotation cropping in central China, and early-maturing single cropping in northeastern China. We also tested the effects of bacteriophages on soil bacterial community structure and function in amended soils in pot experiments.

## Results

### Soil microbial communities and differences in three rice agroecosystems

In the metagenomic sequencing, 1.10 billion raw reads were obtained. After trimming, 1.07 billion clean reads remained. In Guangzhou, Nanjing and Jiamusi, 511,481, 593,018, 848,794 contigs were obtained, respectively, after clean reads were assembled. Based on similarities to entries in the NCBI-NR database, 97.81% of sequences were classified as bacteria, 1.73% as Archaea, 0.27% as Eukaryota, and 0.026% as viruses. In total, 82 phyla, 1904 genera and 12,455 microbial species were identified. At the phylum level, Proteobacteria, Actinobacteria, Chloroexi, Acidobacteria and Bacteroidetes were the top five most-abundant phyla, accounting for more than 78% of all the sequences (Fig. S1A). According to the LEfSe analysis (LDA > 3.5, \( p < 0.05 \)), soils in Guangzhou and Nanjing were enriched in microbes belonging to Proteobacteria and Actinobacteria, respectively, while soils in Jiamusi were enriched in bacteria belonging to Chloroflexi, Acidobacteria and Bacteroidetes (Fig. 1A). At the generic level, there were significant differences in the dominant genera (with abundance greater than 1% and without unclassified genera). There were 10 dominant genera in Guangzhou, 13 in Nanjing and nine in Jiamusi, only 20% of the total dominant genera were shared by the three rice agroecosystems, and 45% were unique to a single rice agroecosystem (Fig. 1B, Table S3). The top three most-dominant genera were Gemmatimonas, Anaeromyxobacter and Candidatus Solibacter in Guangzhou, Nocardioides, Gemmatimonas and Solirubrobacter in Nanjing, Bradyrhizobium, Candidatus Solibacter and Anaeromyxobacter in Jiamusi (Fig. S1B). It is worth noting that none were shared by the three rice agroecosystems. Our results that the microbial community composition differed among the three rice agroecosystems was confirmed by PCoA analysis. The principal coordinate axis 1 (PCo1) and the principal coordinate axis 2 (PCo2) explained, respectively, 26.66% and 19.22% of the variation in bacterial species (Fig. S2).

### Complicated relationship between bacteriophage and bacterial abundance and diversity

Viral and bacterial genes were selected out to analysis their relationship. Because of the low abundance of viruses, metagenomic sequencing only detected viruses that were relatively abundant. In total, 14 families, 29 genera and 368 species of viruses were identified. As in the most-often characterized viromes in various habitats, the annotated viruses in three rice agroecosystems belonged to families Siphoviridae, Podoviridae, Myoviridae, Phycodnaviridae and Herpesviridae. The relative abundance of Phycodnaviridae was highest in Jiamusi, and Siphoviridae, Podoviridae, Myoviridae and Herpesviridae were highest in Nanjing (Fig. 2A). Similar with the trend for the number of bacterial species, the number of viral species was highest in Nanjing (218 species), followed by Guangzhou (192 species) and Jiamusi (155 species). The three rice agroecosystems shared 13.39% of the total viral species, and 27.05% of the total viral species were shared by two agroecosystems; however, 59.56% of the viral species were specific to one agroecosystem (Fig. 2B).

Bacteriophages that infect the same bacterial genus were grouped together. We analyzed the correlation between the relative abundance of dominant bacteriophage groups (relative abundance greater than 1%) and their host bacterial genera (Table 1). There were 19 dominant bacteriophage groups, the relative abundance of the hosts for three bacteriophage groups was greater than 1%, less than 0.01% for four bacteriophage groups, and between 0.01% and 1% for 12 bacteriophage groups. Thus, the dominant bacteriophages were mainly distributed in bacteria with moderate abundance. The relationship between the relative abundance of the bacteriophages and that of their hosts is complex. The ratio of viral relative abundance to bacterial relative abundance (VBR) varies widely, between 0 and 27,382. But when VBR was greater than 100 in at least one of the three cropping systems, the abundance of bacteriophage and host bacteria was negatively correlated \( R^2 < -0.5 \).
genes, presumably to help decompose pesticides in the soil. As a result, pesticide residues in the soil were relatively high, and soils were significantly enriched in bacteria with xenobiotics biodegradation and degradation, especially for genes involved in biodegradation and metabolism of nitrotoluene, aminobenzoate, naphthalene, steroid and xylene. While soil bacteria in Nanjing were significantly enriched in genes associated with xenobiotics biodegradation and metabolism, nucleotide metabolism, and repair, biosynthesis of other secondary metabolites which were positively correlated with a high soil organic carbon (SOC) content (Fig. 3B; Table S2).

| Phages          | NJ phage | NJ bacteria | NJ VBR | GZ phage | GZ bacteria | GZ VBR | JMS phage | JMS bacteria |
|-----------------|----------|-------------|--------|----------|-------------|--------|-----------|-------------|
| Achromobacter phage | 1.247±0.512 | 0.845±0.035 | 1.476  | 0.252±0.057 | 1.370±0.039 | 0.184 | 0         | 1.854±0.073 |
| Vibrio phage    | 4.007±1.022 | 0.024±0.001 | 164.991| 3.067±0.668 | 0.031±0.0003 | 98.523 | 0.254±0.149 | 0.036±0.001 |
| Gordonia phage  | 3.759±1.168 | 0.080±0.003 | 47.181 | 0.507±0.577 | 0.058±0.002  | 8.705 | 20.029±2.19 | 0.043±0.001 |
| Streptomyces phage | 0.865±0.365 | 1.977±0.056 | 0.438  | 2.955±0.823 | 1.127±0.059  | 2.622 | 2.915±1.688 | 1.136±0.066 |
| Enterobacter phage | 15.889±0.484 | 0.005±0.0006 | 3050.137| 0.128±0.101 | 0.008±0.0007 | 15.398 | 0         | 0.007±0.001 |
| Stenotrophomonas phage | 7.319±1.331 | 0.040±0.001 | 184.654| 3.905±1.262 | 0.045±0.001  | 87.197 | 0         | 0.044±0.0005 |
| Salmonella phage | 5.390±0.478 | 0.002±0.0003 | 2515.275| 0.739±0.326 | 0.003±0.0005 | 252.934 | 0         | 0.003±0.0003 |
| Paramecium bursaria phage | 0.401±0.276 | 0.0009±0.0002 | 469.215 | 1.542±0.206 | 0.0003±0.000007 | 5020.489 | 10.980±1.297 | 0.0004±0.000005 |
| Rhodococcus phage | 1.827±1.079 | 0.393±0.012 | 4.652  | 3.983±2.79 | 0.196±0.009  | 20.372 | 3.930±1.682 | 0.181±0.012 |
| Burkholderia phage | 7.139±1.112 | 0.226±0.003 | 31.647 | 3.873±0.770 | 0.347±0.005  | 11.153 | 0.232±0.186 | 0.232±0.002 |
| Caulobacter phage | 0.374±0.278 | 0.087±0.004 | 4.293  | 0.972±0.531 | 0.099±0.002  | 9.751 | 0.701±0.201 | 0.058±0.002 |
| Mycobacterium phage | 8.404±7.98 | 2.405±0.133 | 3.495  | 5.269±0.545 | 1.278±0.087  | 4.122 | 5.679±1.066 | 0.438±0.027 |
| Bacillus phage   | 2.181±0.428 | 0.214±0.002 | 10.170 | 1.106±0.518 | 0.240±0.002  | 4.609 | 2.670±1.117 | 0.282±0.005 |
| Synechococcus phage | 1.318±0.377 | 0.059±0.0005 | 22.252 | 2.990±0.831 | 0.082±0.001  | 36.361 | 3.436±1.065 | 0.063±0.001 |
| Pseudomonas phage | 6.964±2.194 | 0.247±0.013 | 28.207 | 5.904±1.422 | 0.334±0.007  | 17.651 | 2.486±1.049 | 0.207±0.002 |
|Ralstonia phage  | 0.065±0.089 | 0.063±0.002 | 1.029  | 1.046±0.516 | 0.117±0.0008 | 8.933 | 0.501±0.102 | 0.076±0.001 |
|Rhodoferax phage | 0        | 0.027±0.001 | 0      | 1.392±0.386 | 0.054±0.004  | 25.709 | 0         | 0.044±0.0007 |
|Actinoplanes phage | 1.025±0.482 | 0.298±0.007 | 3.446  | 0.045±0.089 | 0.147±0.009  | 0.303 | 0.523±0.295 | 0.173±0.011 |
|Arthrobacter phage | 4.391±0.892 | 0.400±0.015 | 10.969 | 2.286±0.579 | 0.118±0.004  | 19.392 | 16.613±1.227 | 0.547±0.079 |

GZ: rice double cropping system in Guangzhou; JMS: rice single cropping system in Jiamusi; NJ: rice–wheat rotation cropping ecosystem in Nanjing. VBR: th abundance to bacterial relative abundance.

Metagenomic analysis of richness (represented by the Chao index) and diversity (represented by the Shannon index) revealed a significant difference in the bacterial and bacteriophage communities of paddy soils in different cropping systems. The Chao index showed that the bacterial richness in Nanjing was significantly higher than in Jiamusi and Guangzhou, while the difference between Jiamusi and Guangzhou was not significant. The viral richness was positively correlated with that of bacteria ($R^2 = 0.662, p = 0.019$) (Fig. 3A), and the viral richness of Nanjing was significantly higher than that of Jiamusi and Guangzhou. The Shannon index analysis found that the diversity of Nanjing and Guangzhou did not differ significantly, but both were significantly higher than in Jiamusi. Viral diversity was negatively correlated with that of bacteria ($R^2 = -0.876, p = 0.001$) (Fig. 3B).

**Soil bacterial function and its relationship with environment in three agroecosystems**

In the functional annotation of the soil metagenomic reads for the bacterial community using the Kyoto Encyclopedia of Genes and Genomes (KEGG) database, most sequences were associated with metabolism ($70.24-72.38\%$), and far fewer were involved in genetic information processing ($8.44-8.93\%$), environmental information processing ($6.79-7.26\%$), cellular processes ($5.49-6.01\%$), human diseases ($3.93-4.57\%$), and organismal systems ($2.71\%-2.85\%$). According to the LEfSe analysis (Fig. 4A), soil bacteria in Jiamusi were significantly enriched in genes associated with carbohydrate metabolism, replication and repair, biosynthesis of other secondary metabolites which were positively correlated with a high soil organic carbon (SOC) content (Fig. 4B; Table S2). While soil bacteria in Nanjing were significantly enriched in genes associated with xenobiotics biodegradation and metabolism, nuclotide metabolism, folding sorting and degradation, especially for genes involved in biodegradation and metabolism of nitrotoluene, aminobenzoate, naphthalene, steroid and xylene (Fig. S3). The annual frequency and dosage of chemical pesticide application especially herbicides in Nanjing were higher than those in other two areas (Table S2). As a result, pesticide residues in the soil were relatively high, and soils were significantly enriched in bacteria with xenobiotics biodegradation genes, presumably to help decompose pesticides in the soil.
Soil bacteria in Guangzhou were significantly enriched in genes associated with bacterial virulence, energy metabolism, translation, cell growth and death, which were positively correlated with annual accumulated temperature and annual precipitation. In the field disease evaluation, we found that the disease indices for rice brown spot, brown streak, bacterial blight, bacterial leaf streak and foot rot were the highest in Guangzhou (Fig. 5A). Similarly, the relative abundance of the pathogenic bacteria *Pseudomonas syringae*, *Acidovorax avenae*, *Xanthomonas oryzae* and *Dickeya zeae* was significantly higher in Guangzhou than in Jiamusi and Nanjing (Fig. 5B) and was positively correlated with temperature and humidity ($R^2 > 0.5$, $p < 0.05$). In addition, the analysis of the virulence factor database VFDB showed that the abundance of genes related to factors (adherence-related genes) in the Guangzhou region was significantly higher than that in Nanjing and Jiamusi, especially for genes for flagella, Twa type IV secretion system, fimbriae, Myf/pH6 antigen, and PI-2, accounting for more than 70% of the total adherence-related genes (Fig. 5C). Functional analysis of biosynthesis of other secondary metabolites showed that the genes related to antimicrobial antibiotic secretion (such as biosynthesis of streptomycin, penicillin and cephalosporin, and neomycin, kanamycin and gentamicin) in Guangzhou were significantly lower compared with that of Nanjing and Jiamusi (Fig. 5D). Bacterial diseases may be more serious in Guangzhou due to the higher temperature and humidity and lower secretion of antibiotics, which may be conducive to the reproduction of pathogenic bacteria and to the higher relative abundance of adherence-related genes might aid their infection of the plant host.

**Abundant auxiliary carbohydrate metabolic genes in paddy viruses**

In the functional annotation of viruses in paddy soils using the KEGG database (Fig. 6A), almost all functions in pathway level 2 were found, but the sequences were notably enriched in a limited number of functions; the top three assigned functions accounted for 70% of the annotated sequences. The top two most-abundant functions were nucleotide metabolism, replication and repair, which are critical for the reproduction and survival of viruses. In addition, viruses have genes related to carbohydrate metabolism and the biosynthesis of secondary metabolites; the relative abundances of genes related to carbohydrate metabolism in viruses were negatively correlated ($R^2 = -0.498$, $p = 0.334$) with those in soil bacteria (Fig. S4A). Similarly, the abundances of genes related to biosynthesis of secondary metabolites in viruses were negatively correlated ($R^2 = -0.500$, $p = 0.333$) with those in soil bacteria (Fig. S4B). These two types of genes carried by bacteriophages might assist host bacteria in carbon metabolism and secondary metabolite synthesis. In the annotation of carbohydrate-active enzymes (CAZymes) using the CAZy database (Fig. 6B), the species and abundance of viral CAZymes differed significantly among the soils of the three cropping systems. Notably, 67.43% of the CAZymes-related genes were present in Jiamusi, which was 9.83 and 2.62 times higher than in Guangzhou and Nanjing. In addition, there were two classes CAZymes (glycoside hydrolases, glycosyl transferases) in Guangzhou, two classes (glycoside hydrolases, carbohydrate-binding modules) in Nanjing, but four classes (glycoside hydrolases, carbohydrate-binding modules, glycosyl transferases, polysaccharide hydrolase) in Jiamusi. Remarkably, polysaccharide lyases (pectate lyase, exopolypgalacturonate lyase, thiopeptidoglycan lyase) were unique to Jiamusi, implying potential roles in the decomposition of soil organic carbon.

**Enterobacter bacteriophages can reduce the nitrogen fixation capacity of soil by lysing host bacteria**

In Nanjing, the most abundant bacteriophage was *Enterobacter* phage, with a relative abundance of 15.28%, compared with 0.13% in Guangzhou and 0% in Jiamusi (Fig. S5A). They were also negatively correlated with the abundance of host bacteria *Enterobacter* ($R^2 = -0.805$, $p = 0.404$) (Fig. S5B). To study its function, the bacteriophage was isolated from the soil in Nanjing and named *Enterobacter* phage-NJ (Fig. S6A). In the plate lytic experiment, the bacteriophage had lytic activity against nitrogen-fixing *Enterobacter* (Fig. S6B).

To assess the functional effects of *Enterobacter* phage-NJ on nitrogen fixation and uptake, we added *Enterobacter* phage-NJ to the sterilized soil to which nitrogen-fixing *Enterobacter* had been added. The results showed that the nitrogen-fixation capacity decreased by 55%, and plant height, fresh mass and nitrogen content of the rice plants were 14.27%, 32.25%, and 37.17%, respectively, lower than in the controls (Fig. 7B). Similar results were found when *Enterobacter* phage-NJ was added to untreated field soil; the nitrogen-fixation capacity decreased by 16.49%, and plant height, fresh mass, and nitrogen content were 9.29%, 17.97%, and 18.58%, respectively, lower than in the controls (Fig. 7C).

Rhizosphere microbial diversity in untreated field soil after the addition of *Enterobacter* phage-NJ was then studied using 16S amplicon sequencing. After the addition of *Enterobacter* phage-NJ, the richness and diversity of rice rhizosphere microorganisms was 29.97% and 19.17%, respectively, lower than in the control (Fig. S7A). Moreover, the community structure of rhizosphere microorganisms significantly changed; PCoA demonstrated separate clustering (Fig. S7B). In addition, the metabolic function of rhizosphere microorganisms also significantly decreased, especially carbohydrate metabolism, energy metabolism and biosynthesis of secondary metabolites (Fig. S7C).

**Discussion**

**Functional differences of soil bacteria in the three rice agroecosystems**

Soil bacteria in three rice agroecosystem performed multiple functions simultaneously and were significantly enriched in typical functions that were specific for an agroecosystem. Soil bacteria in Jiamusi and Nanjing were significantly enriched in genes associated with carbohydrate metabolism, xenobiotics biodegradation and metabolism, respectively, while soil bacteria in Guangzhou were enriched in genes associated with virulence factors, but scarce in genes associated with secondary metabolite biosynthesis, which might lead to the significant occurrence of rice bacterial diseases. These significant differences are likely related to the local environment and cropping system. The effects of environment on soil bacterial function can be divided into direct and indirect effects. Environmental factors especially temperature can directly influence the functions by accelerating the activity microbes [27, 28] or indirectly affect functions by altering the composition of microbial communities [29]. Soils in Jiamusi were significantly enriched in genes associated with carbohydrate
metabolism, which were positively correlated with the high content of soil organic matter. Soil organic matter can provide nutrients and promote multiplication of microbes, ultimately, indirectly promoting the capacity of carbon metabolism. With lower inputs of plant-derived organic carbon, desert communities have lower relative abundances of genes associated with nutrient cycling and the catabolism of plant-derived organic compounds compared with forests, grasslands, and tundra [30]. Our correlation analysis of environment and function showed that soil bacteria in Nanjing were significantly enriched in genes associated with xenobiotics biodegradation and metabolism, which were positively correlated with the large number of pesticide applications. Because of the rice–wheat rotation system in Nanjing, more types and applications of chemicals are used than in the double cropping system in Guangzhou and in the single cropping rice in Jiamusi. Chemical residues are beneficial to the survival of pesticide-degrading microbes and ultimately result in significant enrichment of the xenobiotics biodegradation and metabolism functions of soil bacteria in Nanjing. The genes associated with bacterial virulence factors in Guangzhou soils were positively correlated with higher annual accumulated temperature and annual precipitation. One of the most important conditions for rice bacterial diseases is high temperature and humidity [31]. In addition, double cropping rice in Guangzhou provides two growing seasons for hosts of bacterial pathogens, so pathogens have more time and opportunity to survive than Nanjing and Jiamusi. These results indicate that functions of soil bacteria vary in different agroecosystems and that environmental factors play important roles through affect microbial community composition.

Differences in soil viruses among the three agroecosystems and their relationship with host bacteria

In our study, there were significant differences in the species and composition of paddy soil viruses among the three agroecosystems. In Nanjing, 40.64% more virus species were detected than in Jiamusi and 13.54% more than in Guangzhou. A previous study showed that the bacterial host species and abundance may be a key factor controlling viral abundance and distribution [32]. Similarly, we found that soil viral richness was significantly positively correlated with bacterial richness ($R^2 = 0.662, p = 0.01$). Only 13.39% of the viral species were shared by all three rice agroecosystems, while 59.56% of the viral species were environmentally specific, which is likely associated with the significant differences in temperature, annual precipitation and soil physicochemical properties in the three rice agroecosystems because viral diversity and community structure have been reported to be controlled by factors such as temperature [33], soil moisture [34], and pH [35].

In soils, most viruses are bacteriophages, which can be divided into lysogenic or lytic bacteriophages. The abundance and distribution of bacteriophages and host bacteria influence each other in a complex relationship based on our results. Bacteriophages are obligate intracellular parasites, so their fitness and range limits are influenced by their hosts, and the bacteriophages in turn can affect host abundance and even the community structure and diversity of the whole bacterial community [36–38]. Lytic bacteriophages can modulate host bacterial populations and diversities by lysing their hosts [16], but lysogenic bacteriophages can increase the host abundance by promoting horizontal gene transfer by lysogenic conversion or transferring packaged bacterial genes to new hosts, which may expand the host metabolic profile and enhance microbial environmental adaptability [39]. The ratio of viral abundance to bacterial abundance (VBR) has been used as means to infer relationships between viruses and their potential hosts [40]. Lower VBRs means low virion production, and lysogenic is thus considered to be common. Conversely, higher VBR means higher virion production, and lysogenic is considered to be less prevalent; bacteriophages may thus be primarily lytic [41]. In cold deserts, the VBR ranged from 0.15 to 1.66, and the prevalence of bacterial lysogens was between 4.6 and 21.1% [42, 43], while in hot deserts, the VBR ranged from 170 to 8,200, and the prevalence of bacterial lysogens was 84%, much higher than in the cold desert soils [44]. In our analysis of the ratio of viral relative abundance to bacterial relative abundance, similar to the results of previous studies [41, 42, 44]; when the ratio was greater than 100, there was a significant negative correlation between the abundance of bacteriophage and bacteria, indicating that bacteriophages may be lytic, which negatively regulates the abundance of host bacteria. However, when the ratio was less than 100, the correlation between bacteriophage and bacteria abundance was not significant, perhaps because the bacteriophages may be lysogenic, which have little effect on host bacteria abundance, and/or because bacteriophages overlap in their distribution with multiple susceptible hosts in natural environment, while bacteria can often be infected with several different types of bacteriophages. Furthermore, many factors influence the abundance of bacteriophages and host bacteria, making the relationship between them more complex.

Bacteriophages inhibit soil nitrogen fixation by lysing host bacteria

Lytic bacteriophages have direct and indirect effects on nutrient cycling. They can directly lyse bacteria, which releases organic matter, including nutrients such as carbon, nitrogen and phosphorus. The carbon cycle driven by viruses accounts for 25% of the total carbon cycle in marine agroecosystems [16]. Addition of bacteriophages to soil also increases the NH$_4^+$ concentration via lysis, resulting in a release of inorganic nitrogen, followed by mineralization [45]. Indirectly, bacteriophage lysis can also slow nutrient transformations by reducing the population of key bacteria involved in nutrient cycling [45]. Higher abundance of T4-like phages increases bacterial death, thereby suppressing soil organic carbon mineralization [46]. In wetlands, bacteriophages can infect sulfate-reducing and methanogenic bacteria, which can potentially repress sulfate reduction (and associated carbon mineralization) and methane production, respectively [47]. However, indirect inhibition of biogeochemical cycling by bacteriophages has mostly been inferred by sequencing analysis without relevant experimental verification. In our study, Enterobacter phage-NJ, which was isolated from the soil in Nanjing, lysed three species of nitrogen-fixing Enterobacter bacteria. When Enterobacter phages-NJ were added to untreated field soil or sterilized soil with nitrogen-fixing bacteria, the soil nitrogen-fixation capacity decreased, and rice growth and nitrogen content decreased also. This result provides direct evidence that bacteriophages can inhibit biogeochemical cycling through lysis. Enterobacter phage phiEap-2 was the most abundant in the tested soils from Nanjing, with a relative abundance of 15%. When we extracted all viruses from the Nanjing soils and added them into natural field soil, the result was similar to adding bacteriophages alone; that is, soil nitrogen-fixation capacity was reduced. In addition, the soil microbial community changed, and the bacterial metabolic functions such carbon metabolism and energy metabolism decreased also. These results indicate that bacteriophages can regulate soil bacterial community structure and ecological function and that the differences in soil bacterial function were related to differences in the bacteriophages in the different agroecosystems.

Potential effects of auxiliary metabolic genes of phages on soil microbial function
Bacteriophages can affect nutrient cycling not only by lysing the host but also by metabolically reprogramming their hosts via the horizontal transfer of ecologically important genes and the expression of virus-carried auxiliary metabolic genes (AMGs) [48]. AMGs have been most extensively explored in marine ecosystem and are involved in photosynthesis, carbon metabolism, nitrogen metabolism and phosphate uptake [21]. Those genes are hypothesized to increase viral survival and replication by augmenting key steps in host metabolism. Cyanobacteria can photosynthesize in the ocean to obtain energy for growth, but they are susceptible to photoinhibition when sunlight is too intense. Cyanobacteria bacteriophage S-PM2 carries psbA and psbD genes, which encode proteins D1 and D2, key components of photosystem II, which are crucial sites of damage in photoinhibition. Bacterial infection with bacteriophage S-PM2 ensures that photoinhibition is prevented, thereby maintaining photosynthetic activity and energy production for the continued replication of the bacteriophage [18]. Marine viruses contain carbon metabolic genes that encode numerous enzymes involved carbon metabolism and have been shown to increase metabolic flux when glucose is limited [49, 50]. Bacteriophages in different ocean environments also harbor niche-specific AMGs. For example, in sunlight ocean water, photosynthesis-related genes are the main AMGs, but in dark ocean water, genes involved in the tricarboxylic acid cycle and electron transport chain prevail [49].

In mangrove soil [24], permafrost soil [51], and maize-barley rotation soil [20], AMGs involved in carbohydrate metabolism are notably over-represented. In the maize-barley rotation soil, three classes of genes—carbohydrate-binding modules, carbohydrate esterases, glycoside hydrolases—were identified. Similarly, in paddy soil, four classes of AMGs of bacteriophages involved in carbohydrate metabolism were identified in our study (glycoside hydrolases, glycosyl transferases, polysaccharide lyases, and carbohydrate-binding modules, with the abundance of glycoside hydrolases the highest), indicating that paddy soil viruses primarily participate in the decomposition of organic carbon, breakdown of complex carbohydrates to increase energy production and boost bacteriophage replication. The species and abundance of AMGs also differed among the three agroecosystems we studied. Compared with the bacteriophages in the other two agroecosystems, the bacteriophages in Jiamusi had the greatest diversity of AMGs genes related to carbon metabolism; there were four classes of CAZymes in Jiamusi (polysaccharide lyases, glycoside hydrolases, glycosyl transferases, carbohydrate-binding modules), but only two in Guangzhou (glycoside hydrolases, glycosyl transferases) and in Nanjing (glycoside hydrolases, carbohydrate-binding modules). However, soil bacteria in Jiamusi were significantly enriched for carbon metabolism function, likely due to the large diversity of bacteriophage AMGs genes related to carbon metabolism. Remarkably, polysaccharide lyases (pectate lyase, exopolysaccharide hydrolases, thiopeptidoglycan lyase) were unique to Jiamusi. Complex carbohydrates or polysaccharides such as cellulose, xylan, pectin, starch, alginate, mannan, and chitin are major components of plant cell walls, which are difficult to degrade. In Jiamusi, the soil organic matter content is the highest, perhaps because the bacteriophages carry genes for polysaccharide lyases, which might assist host bacteria in degrading organic matter in the soil.

**Conclusions**

In our analyses to identify and compare the bacterial and viral communities in soils from the three most important rice agroecosystems in China, the communities in Jiamusi were significantly enriched in genes related to carbohydrate metabolism, in Nanjing with xenobiotics biodegradation and metabolism, and in Guangzhou with genes associated with virulence factors, but those involved with secondary metabolite biosynthesis were scarce, which might lead to a significant incidence of rice bacterial diseases. The 368 virus species that were detected from the three agroecosystems likely affect the soil microbial community structure and function. Bacteriophage isolation and inoculation experiments showed that *Enterobacter* phage-NJ not only changed the soil bacterial diversity and community structure, but also reduced the nitrogen-fixation capacity by lysing nitrogen-fixing host bacteria. In addition, auxiliary carbohydrate-active enzyme (CAZyme) genes were notably over-represented in soil viruses, and 67.43% of all CAZyme genes were present in Jiamusi soils, which might assist host bacteria in metabolizing carbon. Our study will contribute to a better understanding of the microbial diversity in the typical rice agroecosystems in China, viral mechanisms that may be involved in this diversity and inform management programs for the safe production of healthy rice.

**Methods**

**Sampling sites and sample collection**

Paddy soil samples were collected during heading of rice in 2019 from long-term (more than 20 years) rice farms in Jiamusi (46°48’ N, 130°22’ E, 130 m a.s.l.) in Heilongjiang Province, Nanjing (30°02’ N, 118°46’ E, 30 m a.s.l.) in Jiangsu Province, and Guangzhou (23°13’ N, 113.27’ E, 871 m a.s.l.) in Guangdong Province in China. In Jiamusi, Nanjing and Guangzhou, the annual precipitation is 550 mm, 1090 mm and 1720 mm, respectively, with 67%, 73% and 77% mean humidity, respectively, and the annual accumulated temperature of 2700°C, 5400°C and 7500°C, respectively (http://www.weather.com.cn). The three regions differ in their rice planting systems (single rice cropping in Jiamusi, rice-wheat rotation in Nanjing, double rice cropping in Guangzhou). Rice was grown at each farm using local practices for cultivation, fertilizer application, and field management. Chemical pesticides were applied as shown in Table S1. Four plots (5 × 10 m) were randomly selected for collecting 30 cores of bulk soil samples (0–15 cm) at each farm. At the same time, plants were inspected for naturally occurring diseases in four randomly chosen, representative 5-m lengths of rows from each plot. The severity of rice leaf diseases (rice brown spot, brown streak, bacterial blight, bacterial leaf streak and foot rot) was visually scored on a 0 to 5 scale based on the estimated percentage leaf area with lesions: 0, no symptoms; 1, 0.1-6% of total leaf area; 2, 7-12%; 3, 13-25%; 4, 26-50%; 5, 51-100% [52]. A disease severity index was then calculated as \( \sum \) (Number of diseased plants with each score × Highest score) / (Total number of plants × Highest score) × 100. Disease incidence of rice bacterial foot rot was investigated as the (Number of plants with blackened, rotting roots / Total number of examined plants) × 100. Soil samples were transported to the laboratory on ice in a cooler. A portion of the soil sample was stored at -80 °C for DNA extraction, and the remainder was stored at 4 °C for analyses of soil chemical properties.

**Analyses of soil chemical properties**

Soil pH was determined using a Mettler-Toledo FE20-Five Easy PlusTM pH meter (Schwerzenbach, Switzerland) in a 1:5 soil–water (w/v) suspension. Soil organic carbon was measured using the chronic acid titration method [53]. Soil available nitrogen was determined by the alkali diffusion method, soil
available phosphorus was determined using the sodium hydrogen carbonate solution-Mo-Sb anti spectrophotometric method, soil available potassium was measured by the ammonium acetate method, followed by flame photometric detection [54].

DNA extraction

Microbial genomic DNA was extracted from 0.5 g soil with the E.Z.N.A. Soil DNA Kit (Omega Bio-TEK, Norcross, GA, USA) according to the manufacturer's instructions. Concentration and purity of extracted DNA were determined with a TBS-380 Mini-Fluorometer (Turner Biosystems, CA, USA) and NanoDrop2000 UV-Vis spectrophotometer (Thermo Scientific, Wilmington, DE, USA), respectively. DNA quality was confirmed using 1.2% agarose gel electrophoresis, with 1 x TAE buffer (40 mM Tris-Cl, 40 mM acetic acid, 1.0 mM EDTA) and ethidium bromide (0.5 µg mL⁻¹) under ultraviolet light [55].

Shotgun metagenomic analysis

The extracted DNA was fragmented to an average size of about 400 bp using Covaris M220 (Gene Company Limited, Hong Kong, China). A paired-end library was constructed using NEXTFLEX® Rapid DNA-Seq (Bioo Scientific, Austin, TX, USA). Paired-end sequencing was performed using the Illumina HiSeq 4000 platform (Illumina Inc., San Diego, CA, USA) at Majorbio Bio-Pharm Technology Co., Ltd. (Shanghai, China), HiSeq 3000/4000 PE Cluster Kit and HiSeq 3000/4000 SBS Kit according to the manufacturer's instructions (www.illumina.com). Sequence data associated with this project were deposited in the NCBI Short Read Archive database (accession PRJNA732820). The paired-end Illumina reads were trimmed of adaptors, and low-quality reads (length < 50 bp or with a quality value < 20 or having N bases) were removed by fastp version 0.20.0 (https://github.com/OpenGene/fastp). Metagenomics data were assembled using MEGAHIT (https://github.com/voutcn/megahit, version 1.1.2). Contigs of >300 bp were selected for further gene prediction and annotation. BLASTP against the NCBI NR database was used for taxonomic annotations of the representative sequences with an e-value cut-off 1e⁻⁵ using Diamond (http://www.diamondsearch.org/index.php, version 0.8.35). The KEGG annotation was conducted using Diamond against the Kyoto Encyclopedia of Genes and Genomes (KEGG) database (http://www.genome.jp/kegg/) with an e-value cutoff of 1e⁻⁵. Virulence factors were annotated using the VFDB database (http://www.mgc.ac.cn/VFs/) with an e-value cutoff of 1e⁻⁵. Carbohydrate-active enzymes were identified using HHMscan tools and the Carbohydrate-active enzyme Database (CAZY) with an e-value cutoff of 1e⁻⁵. We also selected the bacterial and viral genes and built a viral set for taxonomic, KEGG, and CAZY annotations.

Isolation of soil viruses

Soil sample (1 kg) was suspended in 1 L of SM solution (100 mM NaCl, 8 mM MgSO₄·7H₂O and 50 mM Tris/HCl, pH 7.5) and shaken for 2 h at 30°C. The soil particles were removed by centrifugation at 4000 × g for 15 min. The supernatant was filtered sequentially through 0.45 and 0.22 µm filters. Then, virus particles in the filtered supernatant were concentrated into 1 mL by centrifugation at 4000 × g in 100 kDa centrifugal ultrafiltration tubes.

Isolation of Enterobacter bacteriophage from paddy soil

Soil sample (10 g) was suspended in 50 mL Luria Bertani (LB) broth (1% tryptone, 0.5% yeast extract, and 1% NaCl) and dispersed by shaking for 12 h at 30°C. The soil particles were removed by centrifugation at 4000 × g for 15 min, and the supernatant was filtered-stereiled through a 0.22 µm filter. One milliliter of filtered supernatant was added to 10 mL of an overnight suspension culture of Enterobacter cloacaec (proven to be nitrogen-fixing and provided by Agricultural Culture Collection of China) and incubated for 12 h at 30°C [56]. Then bacterial cells were removed by centrifugation at 4000 × g for 15 min, and the supernatant was sterilized through a 0.22 µm filter. Then 0.1 mL of the filtered supernatant was mixed with 1 mL of the E. cloacae suspension (OD600 = 1). This mixture was then added to 2.5 mL of soft agar (0.35% agar prepared in 1% tryptone, 0.5% NaCl, 3 mM MgCl₂, 3 mM CaCl₂, and 0.04% [wt / vol] glucose) and poured in a uniform layer onto an LB agar plate. Bacteriophage plaques were identified after an overnight incubation at 30°C. Bacteriophages were purified via five consecutive transfers of them from individual plaques to new bacterial cell lines. The purified bacteriophage titer was determined by mixing 100 µL of host cells (A600 of 1) and 100 µL of bacteriophage dilution with soft agar and pouring the mixture over LB agar in a plate. Plaques were scored after an overnight incubation at 30°C. Bacteriophages were kept in buffer (10 mM Tris [pH 7.6], 5 mM MgSO₄·7H₂O, 0.01% gelatin) at 4°C.

Each isolated Enterobacter phage was analyzed for its ability to lyse E. ancerogenus and E. ludwigii (proven to be nitrogen-fixing and provided by Agricultural Culture Collection of China) using a plate lytic experiment with 0.1 mL bacteriophage mixed with 1 mL of overnight-cultured Enterobacter strain and shaken (200 rpm) at room temperature for 20 min, then 3 mL of soft nutrient agar was added, and the mixture was poured on LB agar plates and incubated overnight at 30°C. Lytic activity was indicated by transparent plaques [57].

Impact of Enterobacter bacteriophages on soil nitrogen-fixation capacity

In a greenhouse experiment, the influence of Enterobacter phages on soil nitrogen fixation capacity in untreated field soil and in sterilized field soil was investigated. Rice seeds were surface-sterilized and pregerminated, then seedlings with a primary root length of 1–2 cm were planted in pots (6 seeds per pot) with the untreated field soil. Three treatments with four pots each (each pot filled with 1 kg soil) were set up: (1) N+JV virus, virus isolated from 1 kg Nanjing soil was diluted 50 times with ddH₂O and added to each pot after planting rice; (2) N+phage, 1 mL Enterobacter bacteriophages (10⁷ PFUs) diluted 50 times with ddH₂O was added after planting rice; (3) NCK, 50 mL ddH₂O was added as control. After treatment, rice plants were grown in a greenhouse at 26°C with 14 h of fluorescent light and 10 h of darkness for 7 weeks and watered with 20 mL ddH₂O every 3 days.

Untreated field soil was autoclaved using the liquid cycle for 30 min at 121°C, then cooled at room temperature, then we repeated this procedure, sterilized soil was stored at 4°C until use. Mixed Enterobacter species (E. cloacae-211, E. ancerogenus-212, E. ludwigii-213, ratio 1:1:1) were cultured in LB broth overnight at 30°C, followed by centrifugation at 1,000 × g for 5 min. The pellet was resuspended in ddH₂O and diluted to an optical density OD600 of 0.2. Rice seeds were surface-sterilized and pregerminated, then seedlings with primary root length of 1–2 cm) were planted in pots (6 seeds per pot) with sterilized soil. Four
The study does not contain any studies with human participants or animals that require specific ethical approval.

Ethics approval and consent to participate

The study does not contain any studies with human participants or animals that require specific ethical approval.
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**Figures**

**Figure 1**

Differential analysis of soil microbial composition in three rice agroecosystems in China. (A): Circos analysis of distribution and proportion of the microbial dominant genera. In top left half of circle, numbers are relative abundance of genera in the three agroecosystems (GZ: rice double cropping in Guangzhou in blue; JMS: rice single cropping in Jiamusi in red; NJ: rice–wheat rotation cropping in Nanjing; others: genera with abundance less than 1% were merged and indicated as others). In lower right, the axis represents the distribution proportion of a genus in each agroecosystem. (B): Linear discriminant effect size analysis identified remarkable enrichment of taxa from phylum to genus level. Different color nodes represent the microbial groups that are significantly
higher in the corresponding groups and have a significant influence on the difference between the groups. Light yellow nodes represent the microbial groups that contribute no significant difference in different groups or influence on the difference between groups.

Figure 2
Differential analysis of soil viral composition in three rice agroecosystems in China. (A) Relative abundance of main soil viral families in the three rice agroecosystems in China. (B) Venn diagram showing the number of viral species shared and unique among different rice agroecosystems. GZ: rice double cropping in Guangzhou in blue; JMS: rice single cropping in Jiamusi in red; NJ: rice–wheat rotation cropping in Nanjing.

Figure 3
Alpha diversity of soil bacterial and viral communities. Variation in alpha diversity of soil bacterial community and viral community and (C) Chao index and (D) Shannon index. GZ: rice double cropping in Guangzhou in blue; JMS: rice single cropping in Jiamusi in red; NJ: rice–wheat rotation cropping in Nanjing.
Figure 4
Analysis of differences in soil bacterial functions and correlation analysis with environmental factors in the three rice agroecosystems. (A) Linear discriminant analysis of soil bacterial function at KEGG level 2. (B) Redundancy analysis showing the influence of environmental factors on soil bacterial function at KEGG level 2. GZ: rice double cropping in Guangzhou in blue; JMS: rice single cropping in Jiamusi in red; NJ: rice–wheat rotation cropping in Nanjing. SOC: soil organic carbon; AK: available potassium, AN: available nitrogen; AP: available phosphorus; TEM: annual accumulated temperature; AAR: annual precipitation; CA: chemical pesticides application.

Figure 5

Rice bacterial diseases and differences in soil bacterial function related to bacterial disease severity in the three agroecosystems in China. (A): Disease index for four diseases. Relative abundance of (B) potentially pathogenic bacteria and genes related to (C) virulence factors and (D) secondary metabolite biosynthesis. Different letters above the bars indicate a significant difference ($p < 0.05$) according to Kruskal-Wallis H test. GZ: rice double cropping in Guangzhou in blue; JMS: rice single cropping in Jiamusi in red; NJ: rice–wheat rotation cropping in Nanjing.

Figure 6

Abundant auxiliary carbohydrate metabolism genes in paddy soil viruses. Functional annotation of paddy soil virome. (A) Functional annotation was predicted with KEGG database ($E$-value < $1 \times 10^{-5}$). KEGG function class was ordered according to their hit frequency. (B) Annotation of viral carbohydrate metabolism-related ORFs in the CAZY database. GZ: rice double cropping in Guangzhou in blue; JMS: rice single cropping in Jiamusi in red; NJ: rice–wheat rotation cropping in Nanjing. CBM: carbohydrate-binding modules; GH: glycoside hydrolases; GT: glycosyl transferases; PL: polysaccharide lyases.
Figure 7

Addition of Enterobacter bacteriophage-NJ to soil reduced nitrogen-fixation capacity of Enterobacter species and inhibited rice growth. (A) (top row) Representative phenotypes of rice grown in sterile field soil amended with N-fixing Enterobacter (S+NF), virus isolated from Nanjing soil and N-fixing Enterobacter (S+NF+Virus), Enterobacter bacteriophage and N-fixing Enterobacter (S+NF+Phage), or in sterile field soil (SCK) and (B) effects of the treatments on N fixation, rice N content, plant height and dry mass. (A) (bottom row) Representative phenotypes of rice grown in sterile field soil from Nanjing (NCK) or in Nanjing soil amended with virus isolated from Nanjing soil (N+NJ Virus) or with Enterobacter bacteriophage (N+Phage) and (C) effects of treatments on N fixation, rice N content, plant height and dry mass. Different letters above the bars indicate a significant difference ($p < 0.05$) according to Kruskal-Wallis H test.

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