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Enterobacter-infecting phages in nitrogen-deficient paddy soil impact nitrogen-fixation capacity and rice growth by shaping the soil microbiome

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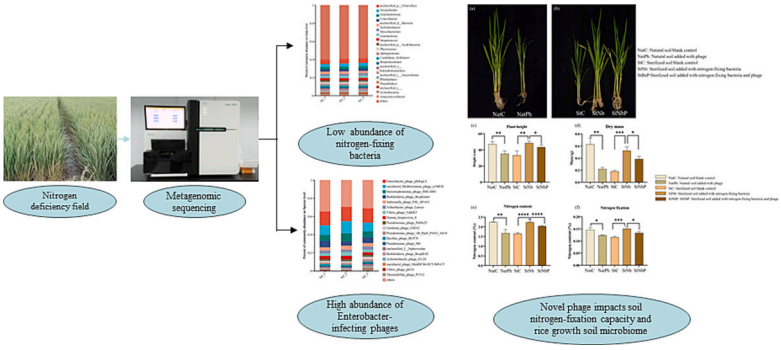
HIGHLIGHTS

- Low abundance of nitrogen-fixing bacteria and high abundance of *Enterobacter* phages were found in a field.
- A novel virulent phage (*Apdecimavirus* NJ2) in the same field that infects various *Enterobacter* species was identified.
- The phage impacts soil nitrogen-fixation capacity by changing bacterial community composition and biodiversity.
- Controlling phages in the soil could be a useful strategy for improving soil nitrogen fixation in the future.

GRAPHICAL ABSTRACT



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ABSTRACT

Bacteriophages ("phage") play important roles in nutrient cycling and ecology in environments by regulating soil microbial community structure. Here, metagenomic sequencing showed that a low relative abundance of nitrogen-fixing bacteria but high abundance of *Enterobacter*-infecting phages in paddy soil where rice plants showed nitrogen deficiency. From soil in the same field, we also isolated and identified a novel virulent phage (named here as *Apdecimavirus* NJ2) that infects several species of *Enterobacter* and characterized its impact on nitrogen fixation in the soil and in plants. It has the morphology of the *Autographiviridae* family, with a dsDNA genome of 39,605 bp, 47 predicted open reading frames and 52.64 % GC content. Based on genomic characteristics, comparative genomics and phylogenetic analysis, *Apdecimavirus* NJ2 should be a novel species in the genus *Apdecimavirus*, subfamily *Studiervirinae*. After natural or sterilized field soil was potted and inoculated with the phage, soil nitrogen-fixation capacity and rice growth were impaired, the abundance of *Enterobacter* decreased, along with the bacterial community composition and biodiversity changed compared with that of the unadded control paddy soil. Our work provides strong evidence that phages can affect the soil nitrogen cycle by

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changing the bacterial community. Controlling phages in the soil could be a useful strategy for improving soil nitrogen fixation.

1. Introduction

Most crops require a high nitrogen content in the soil to meet needs for photosynthesis, growth, high yields and quality, and to produce seeds with a high protein content (Cechin et al., 2022). Numerous soil microbes, including mycorrhizal fungi (Wang et al., 2020), rhizobia (Koskey et al., 2017), other nitrogen-fixing bacteria (e.g. *Azotobacter*, *Klebsiella*, *Rhodospirillum*) (Aasfar et al., 2021; Jack et al., 1999; Masters and Madigan, 1983), and nitrifying (e.g. *Nitrosomonas*, *Nitrobacter*) (Mellbye et al., 2016; Uemoto and Saiki, 1996) and denitrifying bacteria (e.g. *Pseudomonas*) (Best and Payne, 1965; Cameron et al., 1989) are involved in nitrogen fixation, nitrification and denitrification, critical processes in nitrogen cycling (Pashaei et al., 2022). Among the microbes involved in nitrogen cycling, bacteria in the genus *Enterobacter* (family *Enterobacteriaceae*) are involved in nitrogen fixation and have been isolated from the root nodules of certain crops, such as wheat and sorghum, and from the rhizospheres of rice (Chakraborty et al., 2019; Davin-Regli et al., 2019; James, 2000; Ji et al., 2020). Although some species of *Enterobacter* act as opportunistic pathogens of humans, other species of these common, widespread gram-negative bacilli are free-living or symbionts in terrestrial and aquatic environments including soil and sewers (Reitter et al., 2021; Singh et al., 2018). Some *Enterobacter* species promote plant growth by solubilizing phosphorus and potassium or producing indole-3-acetic acid (Kämpfer et al., 2005; Roslan et al., 2020).

Many soil bacteria are also host to viruses such as bacteriophages (hereafter, phages), the most abundant of soil viruses (Suttle, 2005; Wang et al., 2022b). Interactions between phages and their host bacteria play an important role in soil nutrient cycling by altering host abundance, the soil microbial community structure and function (Pratama and van Elsas, 2018). After phage infection, the host bacteria are lysed, thus significantly decreasing their abundance, but the abundance of other microbes increase as they compete for ecological niches and nutrients, thereby changing the microbial community structure (Escudero-Martinez et al., 2022; Yang et al., 2023; Zhang et al., 2022). Bacterial nitrification and denitrification are more sensitive to environmental disturbance; thus, alterations in the community of these bacterial species are more likely to impact the diversity of other microbes and thus the nitrogen content in the soil (Xu et al., 2021). After treatment with the polyvalent virulent phage ΦNF-1, a species of a *Nitrosomonas*, a genus of nitrifying bacteria, in vitro bacterial growth and NH_4^+ consumption was inhibited (Quirós et al., 2023). Nitrogen-fixing bacteria are most likely specifically lysed by soil viruses through a lysogenic-based strategy in urea-treated paddy soil (Li et al., 2019). On the other hand, when phage-infected bacteria are lysed, their cytoplasm is released into the soil, thus increasing elements such as nitrogen, phosphorus, potassium and calcium that are critical for plant growth (Daly et al., 2019). In addition, phages can be indirectly involved in soil element cycling by the expression of virus-encoded auxiliary metabolic genes (AMGs) in the host bacteria after infection, thus altering host processes that are involved in biogeochemical cycling of elements, thereby endowing the host with new functions and broadening its ecological niche (Rosenwasser et al., 2016). Therefore, the existence of abundant phages infecting nitrogen cycling associated bacteria holds a critical role in various environments.

During a field investigation, we found rice plants showing typical symptoms of nitrogen deficiency in a field in Nanjing, Jiangsu Province, China. We collected soil samples in the field for metagenomic sequencing, which showed low relative abundance of nitrogen-fixing bacteria and high relative abundance of phages infecting *Enterobacter* spp. We further isolated a novel phage from the soil and verified that the phage can affect nitrogen fixation in the soil and the nitrogen content and growth of rice plants in several experiments in which the soils were amended with a novel phage. These results provide strong evidence that this phage affects plant growth by altering the soil bacterial community structure and reduces soil nitrogen-fixation capacity in the rice rhizosphere, which is an important factor causing soil nitrogen deficiency. This provides a new perspective for understanding the potential biological mechanism of soil barren, and also provides a new idea for soil remediation by adding bacteriophage resistant bacteria.

2. Materials and methods

2.1. Paddy soil, bacterial strains and culture conditions

Paddy soil samples were collected in Nanjing, Jiangsu Province (30°02′ N, 118°46′ E, 30 m a.s.l.). Three regions (5 m × 10 m) were randomly selected as three repeats, thirty cores of bulk soil samples (0–15 cm) were randomly collected from each repeated region. Isolates of *Enterobacter*, *Escherichia*, *Dickeya*, *Erwinia*, *Pectobacterium*, *Pseudomonas* and *Yersinia* (purchased from the Agricultural Culture Collection of China) used in the present study are listed in Table S1. These bacteria were cultured in Luria Bertani (LB) broth (1 % tryptone, 0.5 % yeast extract, and 1 % NaCl) by shaking for 16 h at 37 °C.

2.2. Metagenomic sequencing

Genomic DNA was extracted from 0.5 g soil sample for each biological replicate using 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, Sunnyvale, CA, USA) and NanoDrop2000 UV–Vis spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA), respectively. The extracted DNA was fragmented to an average size of about 400 bp using a Covaris M220 Focused Ultrasonicator (Covaris, Woburn, MA, USA). 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, San Diego, CA, USA) at Majorbio BioPharm Technology Co., Ltd. (Shanghai, China), a HiSeq 3000/4000 PE Cluster Kit and a HiSeq 3000/4000 SBS Kit (Illumina) according to the instructions. All sequence data have been 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 using fastp version 0.20.0 (https://github.com/OpenGene/fastp). Metagenomics data were assembled using MEGAHIT version 1.1.2 (https://github.com/voutcn/megahit). Contigs ≥300 bp were selected for further gene prediction and annotation. The representative sequences were blastp against the NCBI NR database for taxonomic annotations of bacteria and viruses with an e-value cut-off $1e^{-5}$. The most abundant bacteriophage was isolated and studied for functions.

2.3. Isolation, purification and TEM observations of the phage

Phages were isolated from soil samples collected from the rice field in Nanjing. Each soil sample was sieved, then 10 g of the sieved sample was added to 50 mL of LB broth and cultured overnight at 37 °C. The culture was then centrifuged at 10,000 ×*g* for 20 min, and the supernatant was then filtered through a 0.22-μm filter to remove impurities and bacterial cells. This filtrate was used as the phage stock. One milliliter of the

filtrate was added to 10 mL of a culture of *Enterobacter cloacae* at the logarithmic growth stage and grown overnight at 37 °C. For the double agar overlay plaque assay to purify the phage, a 10-fold dilution series of the phage stock in PBS buffer was prepared and 10 μL of a dilution (10^{-1}–10^{-8}) was mixed with 100 mL of *E. cloacae* at the logarithmic growth stage at room temperature, then plated the mixture of phage and *E. cloacae* was mixed with 5 mL of soft agar (0.35 % agar prepared in 1 % tryptone, 0.5 % NaCl, 3 mM $MgCl_2$, 3 mM $CaCl_2$, and 0.04 % [wt/vol] glucose), then spread on a plate of solid LB agar plate and incubated overnight at 37 °C. Phages were purified via five consecutive transfers of them from individual plaques to new bacterial cell lawns. The purified phage was stored in buffer (10 mM Tris [pH 7.6], 5 mM $MgSO_4{\cdot}7H_2O$, 0.01 % gelatin) at 4 °C. The concentrated phage suspension (about 20 μL) was dropped on copper grids. After 15 min, a drop of 2 % (w/v) phosphotungstic acid was placed on each grid and incubated for 5 min, then removed the phosphotungstic acid and dried at room temperature. Phage particles on the grids were visualized with a H-7500 transmission electron microscope (TEM, Hitachi, Tokyo, Japan) at 80 kV as previously described (Summer et al., 2011).

2.4. Determination of phage host range

Seventeen strains representing species of *Enterobacter*, *Escherichia*, *Dickeya*, *Erwinia*, *Pectobacterium*, *Pseudomonas* and *Yersinia* (Table S1) were used to assess the host range of the phage in a phage lysis assay as described by Li and Tang (2011), indicating that the phage had lysed the bacterial cell.

2.5. Isolation of genomic DNA, genome sequencing and analysis

Phage DNA was extracted using a Phage Genomic DNA Extraction Kit (Norgen Biotek, Thorold, ON, Canada) according to the manufacturers' protocol. The quality and integrity of genomic DNA were assessed using 1 % agarose gel electrophoresis and densitometry and comparison to appropriate size standards. DNA yield and purity were measured as described earlier. Only high-quality DNA (OD260/280 = 1.8–2.0, >1 μg) was used. The phage DNA was sent to WinnerBio Technology Co., Ltd. (Shanghai, China) for third-generation sequencing of the genome using the Illumina NovaSeq 6000 platform (Illumina). Briefly, at least 1 μg genomic DNA was used to construct the sequencing library. DNA sample was sheared into 400–500-bp fragments using a Covaris M220 Focused-Ultrasonicator (Covaris) following the manufacturer's protocol. Illumina sequencing libraries were prepared from the sheared fragments (Ribarska et al., 2022). The prepared libraries were then used for paired-end Illumina sequencing (2 × 150 bp) on an NovaSeq 6000 (Illumina). The genome was assembled using SOAPdenovo2 (version 2.0.4, https://sourceforge.net/projects/soapdenovo2/) (Luo et al., 2012). Each assembled nucleotide sequence was used in a search against the National Center for Biotechnology Information (NCBI) non-redundant database (https://www.ncbi.nlm.nih.gov/), the UniProt database version 2023_04 (https://www.uniprot.org/), the protein families (Pfam) database version 36.0 (http://pfam.xfam.org/), the Clusters of Orthologous Group (COG) database version 2020 (https://www.ncbi.nlm.nih.gov/research/cog), and the Kyoto Encyclopedia of Genes and Genomes (KEGG) database version 92.0 (https://www.genome.jp/kegg/) for annotation. Tri-generation data were assembled using Canu software V21.1 (https://github.com/marbl/canu/releases/tag/) (Koren et al., 2017). Coding sequences for the assembled sequences were predicted using Glimmer 3 (http://ccbjhu.edu/software/glimmer/index.shtml) (Delcher et al., 2007). The sequence has been deposited in the NCBI BankIt database (GenBank accession: OR822025).

2.6. Impact of the novel phage on nitrogen-fixation capacity of the soil and on rice growth

To investigate the effect of the novel phage (*Apdecimavirus* NJ2) on the composition and diversity of the bacterial community, the nitrogen-fixing capacity of soil bacteria and rice growth, we set up experiments with a reciprocal transplant design using sterilized and natural field soils. Natural soil from same field was sterilized at 121 °C for 30 min, cooled to room temperature, sterilized again, then cooled and stored at 4 °C until use. *E. cloacae*, *E. cancerogenus* and *E. ludwigii* were cultured in LB Borth overnight at 37 °C. Then they were mixed together at a 1:1:1 ratio by volume, and collected by centrifugation at 6600 ×*g* for 10 min. The bacteria were then resuspended in ddH_2O to achieve an OD600 of 0.02. Rice seeds (cv. Nipponbare) were soaked in 75 % alcohol for 3 min, then washed with ddH_2O three times, then placed in a 25 °C incubator for 2–3 days to germinate. Seeds with sprouts that were 1–2 cm long were then selected and planted, five in each of three replicate pots containing either natural soil or sterilized soil that had been treated as follows:

The natural soils were either watered with 50 mL of ddH_2O (blank control 1) or inoculated with 50 mL of a phage suspension (2×10^5 plaque forming units [PFUs]) (natural soil with phage) per pot. The control pots with sterilized soil were either watered with 50 mL of ddH_2O (blank control 2) or inoculated with 50 mL of the suspension of the 1:1:1 mixture of nitrogen-fixing *Enterobacteria* (described above) (nitrogen-fixing bacteria control) per pot. Sterilized soil in another three pots was inoculated with 50 mL of the mixed *Enterobacteria* suspension per pot. After 1 week, the pots in blank control 2 and nitrogen-fixing bacteria were each watered with 50 mL ddH_2O, and each pot with sterilized soil with nitrogen-fixing bacteria was added with 50 mL of the phage suspension (2×10^5 PFUs).

All rice plants were grown in a greenhouse at 26 °C with 14 h of fluorescent light and 10 h of darkness, watering with 20 mL ddH_2O every 3 days per pot. After 7 weeks, rhizosphere soil samples were collected (Berlanas et al., 2019), plant height, dry mass were measured. Plant nitrogen concentrations were determined using the modified Kjeldahl digestion method (Nelson and Sommers, 1973). Nitrogen-fixation capacity of soil was assessed using the acetylene reduction assay measuring ethylene formation from acetylene (Lopez-Lozano et al., 2016).

2.7. DNA extraction from rhizosphere soil and 16S rRNA sequencing

Total DNA was extracted from the rice rhizosphere soils and its concentration and purity assessed as described above. The V3-V4 region of the 16S rRNA gene of soil bacteria was amplified by PCR in a thermocycler PCR system (GeneAmp 9700, ABI, Foster, CA, USA) using primer pair 338F (5′-ACTCCTACGGGAGGCAGCAGCAG-3′) and 806R (5′-GGACTACHVGGGTWTCTAAT-3′) designed by Biomarker Technologies. Co. LTD (Beijing, China). The PCR mixture contained 4 μL 5× TransStart FastPfu buffer, 2 μL 2.5 mM dNTPs, 0.8 μL 5 μM forward primer, 0.8 μL 5 μM reverse primer, 0.4 μL TransStart FastPfu DNA Polymerase, 10 ng template DNA, with ddH_2O added to reach 20 μL. PCR cycling conditions were initial denaturation at 95 °C for 3 min; 35 cycles of denaturing at 95 °C for 30 s, annealing at 55 °C for 30 s and extension at 72 °C for 45 s; and single extension at 72 °C for 10 min, and end at 4 °C. The amplified products were purified with the AxyPrep DNA Gel Extraction Kit (Axygen Biosciences, Union City, CA, USA), and a 16S rRNA library was constructed and used for high-throughput sequencing with the Illumina Novaseq 6000 platform by Biomarker Technologies. The raw sequencing reads were demultiplexed, quality-filtered (Benjamin et al., 2017). High-quality sequences from all samples were clustered into operational taxonomic units (OTUs) at 97 % sequence similarity using the default QIIME2 pipeline UCLAST (https://qiime2.org/) (Bolyen et al., 2019). The taxonomy of each gene sequence was analyzed using Silva reference gene data base (http://www.arb-silva.de/) with a confidence threshold of 70 % (Köljalg et al., 2013).

2.8. Statistical analyses

The alpha diversity of the bacterial community (including Shannon and Simpson index) was calculated using Mothur v1.30 software (https://mothur.org/wiki/download_mothur/) for the bacterial communities of the rhizosphere samples based on their OTUs levels (Schloss et al., 2009), Principal coordinate analysis (PCoA) analysis was performed in R using Bray-Curtis distances, which was used to compare the beta diversity of the bacterial community. Alpha diversity indices and relative abundance of major genus which were non-normally distributed were tested for significant differences date using nonparametric statistics Kruskal-Wallis test conducted in SPSS version 16.0 (Wickham, 2016).

The R package VennDiagram (Chen and Boutros, 2011) was used construct a Venn diagram to visualize the number and identity of shared and unique genera between the blank control samples and the samples with the phage. Species distribution analysis was performed by matplotlib version 1.5.1 (Hunter, 2007) in Python to show the distribution of bacterial relative abundance in the rhizosphere samples.

3. Results

3.1. Community composition of bacteria and viruses in nitrogen-deficient paddy soil

In the analysis of the community composition of soil bacteria and

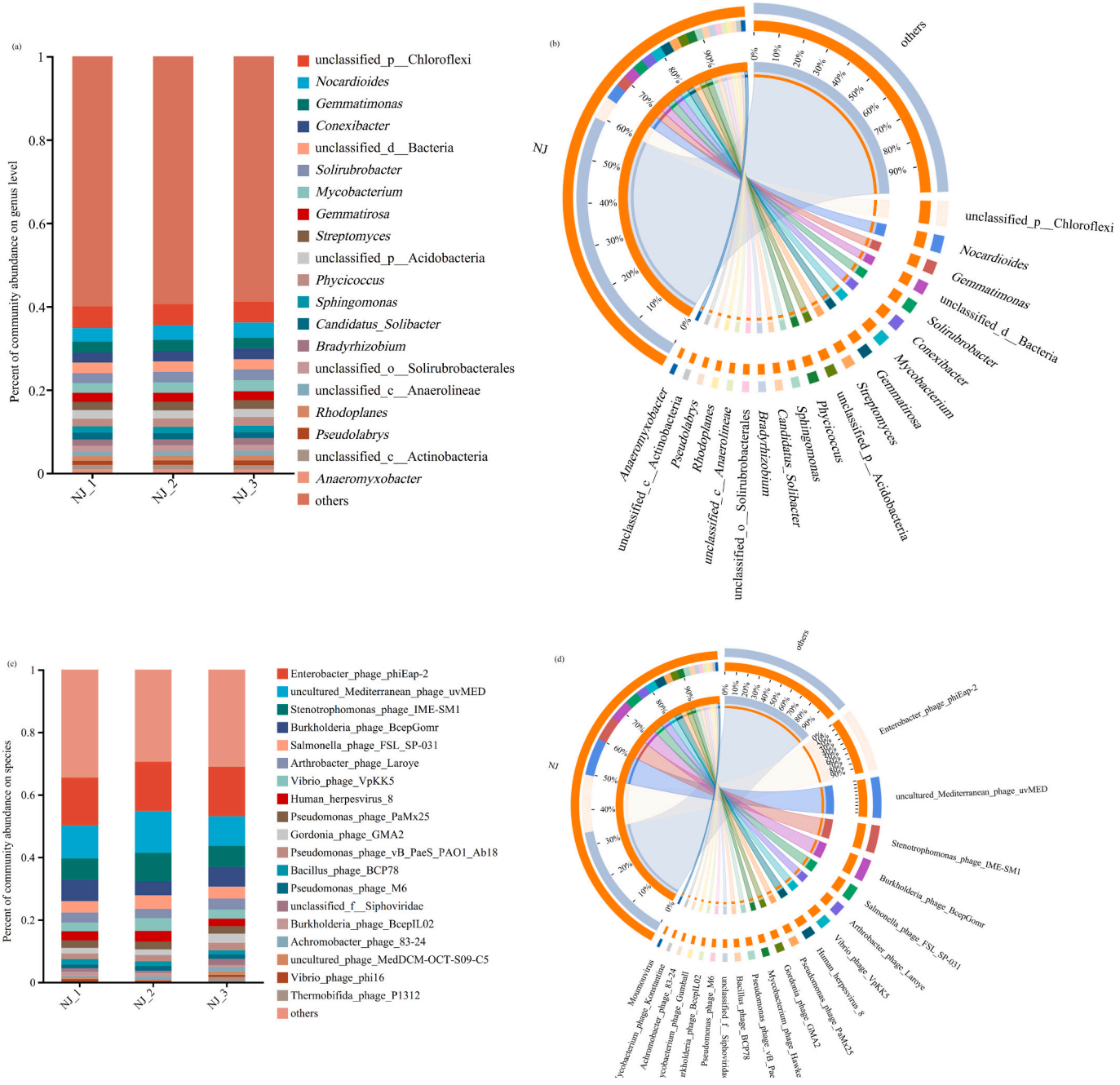

Fig. 1. Composition of soil bacterial and viral communities in nitrogen-deficient paddy soil. (a) Bacterial community at genus level. (b) Circos analysis of distribution and proportion of the dominant bacterial genera. (c) Composition of viral community at species level. (d) Circos analysis of distribution and proportion of the dominant viral species. NJ-1, NJ-2 and NJ-3 represent three soil samples.

viruses in the nitrogen-deficient soil in Nanjing, 1878 genera of bacteria and 218 virus species were detected. Of the bacterial genera, 20 were dominant (abundance >1 % of total). The four most abundant genera were unclassified-p-Chloroflexi, *Nocardioides*, *Gemmatimonas* and *Solirubrobacter*, with relative abundance of 5.1 %, 3.4 %, 2.5 % and 2.4 %, respectively (Fig. 1a and b). The abundance of *Enterobacter*, the potential host of *Enterobacter* phages, was extremely low (0.005 %). Of the 218 virus species, 19 were dominant with relative abundance >1 %. *Enterobacter* phages were the most abundant with a relative abundance of 15.89 % (Fig. 1c and d).

3.2. Phage isolation, visualization and host specificity

A novel phage was isolated from the collected soil samples. TEM micrographs revealed that the phage has an icosahedral head with a diameter of approximately 58 nm and a short tail approximately 15 nm long (Fig. 2a). Based on these characteristics and the 9th Report of the International Committee on Taxonomy of Viruses (ICTV), this phage was morphologically classified as a member of the *Autographiviridae* family. As shown in the phage lysis test, the phage infected *Enterobacter cloacae* (Fig. 2b). In the double agar overlay plaque assay of the 17 species of bacteria, the phage only infected *Enterobacter* species (*E. cloacae*, *E. cancerogenus*, *E. ludwigii*, *E. aerogenes* and *E. hormaechei*), meaning a narrow host range for it (Table S1).

3.3. Genome sequencing, annotation and analysis

Whole-genome sequencing of the novel phage followed by de novo assembly revealed a 39,605-bp linear genome with a GC content of 52.64 %. The NCBI ORF finder (www.ncbi.nlm.nih.gov/orffinder) predicted 47 putative ORFs in the genome, and a putative function was assigned to 27 predicted proteins (Table S2). The 27 proteins represented four main categories: (1) phage structure (tail tubular protein A, tail tubular protein B, internal virion protein A, internal virion protein B, internal virion protein C, internal virion protein D, major head protein, capsid assembly protein and head-to-tail connector protein), (2) package (terminase large subunit), (3) release (Rz-like lysis protein, amidase and holin) and (4) replication recombination (phage exonuclease, DNA-directed DNA polymerase, DNA helicase, endonuclease, single-stranded DNA-binding protein, DNA ligase, RNA polymerase and protein kinase).

In the Nucleotide BLAST (blastn) of the NCBI database (https://www.ncbi.nlm.nih.gov/), the genome sequence of the phage shared 73.76–78.91 % nucleotide sequence identity (query cover 34–72 %) with 100 phages, all of which are members of the subfamily *Studiervirinae*, family *Studiervirinae*. The genome sequence of the novel phage had the highest query coverage (72 %) with that of *Apdecimavirus* AP10, which then served as a closely related phage in subsequent analyses. The genome map of the novel phage and the blastn comparison of the phage nucleotide sequence with that of *Apdecimavirus* AP10 (KT852574) are shown in Fig. 2c.

For analyzing the genetic evolutionary relationship of the novel phage, nucleotide sequences of MHP (major head protein), RNAP (RNA polymerase) and terminase large subunit (terL) of related phages were downloaded from the NCBI database. The phylogenetic tree constructed using MHP sequences showed that the novel phage and *Apdecimavirus* AP10 shared the highest homology and were located on the same branch (Fig. 2d). The phylogenetic trees based on RNAP and terL had a similar topology to the tree based on MHP (Fig. 2e and f). All above results suggested that the isolated phage is a novel species in the genus *Apdecimavirus*, family *Autographiviridae*, and named it *Apdecimavirus* NJ2.

3.4. Apdecimavirus NJ2 reduced the nitrogen-fixation capacity of soil by lysing host bacteria

When *Apdecimavirus* NJ2 was added to untreated field soil, the nitrogen-fixation capacity of the soil was 15.36 % lower than in the blank control soil (Fig. 3f), and the height (Fig. 3a and c), dry mass (Fig. 3d), and nitrogen content (Fig. 3e) of rice plants was 25.21 %, 64.81 %, and 25.02 % lower than those of plants in the nature soil blank control respectively. When *Apdecimavirus* NJ2 was added to sterilized

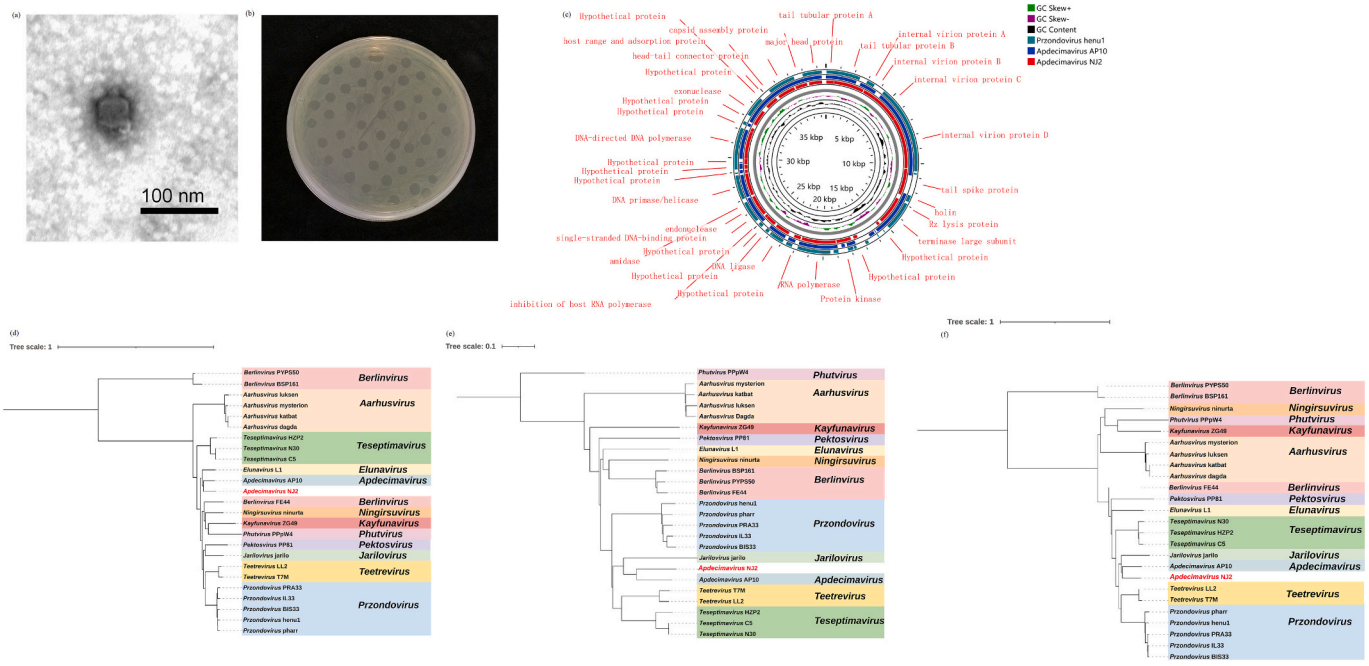

Fig. 2. Particle morphology, plaque lysis assay, and genomic structure of *Apdecimavirus* NJ2 and phylogenetic trees based on three genes in *Apdecimavirus* NJ2 and 24 other virus species in subfamily *Studiervirinae* using neighbor-joining method. (a) TEM of *Apdecimavirus* NJ2 particle. (b) Phage lysis assay for *Enterobacter cloacae*. (c) Complete gene map, numbering from outside inward: first circle represents open reading frames; the second and third circles are Blastn results for percentage similarity for *Apdecimavirus* NJ2 with *Apdecimavirus* AP10 and with *Przondovirus* henu1 DNA; the fourth circle indicates G + C skew of G-C/G + C; the innermost represents (G + C) mol%. (d–f) Phylogenetic analysis of (d) major head protein (MHP), (e) RNA polymerase (RNAP) and (f) terminase large subunit.

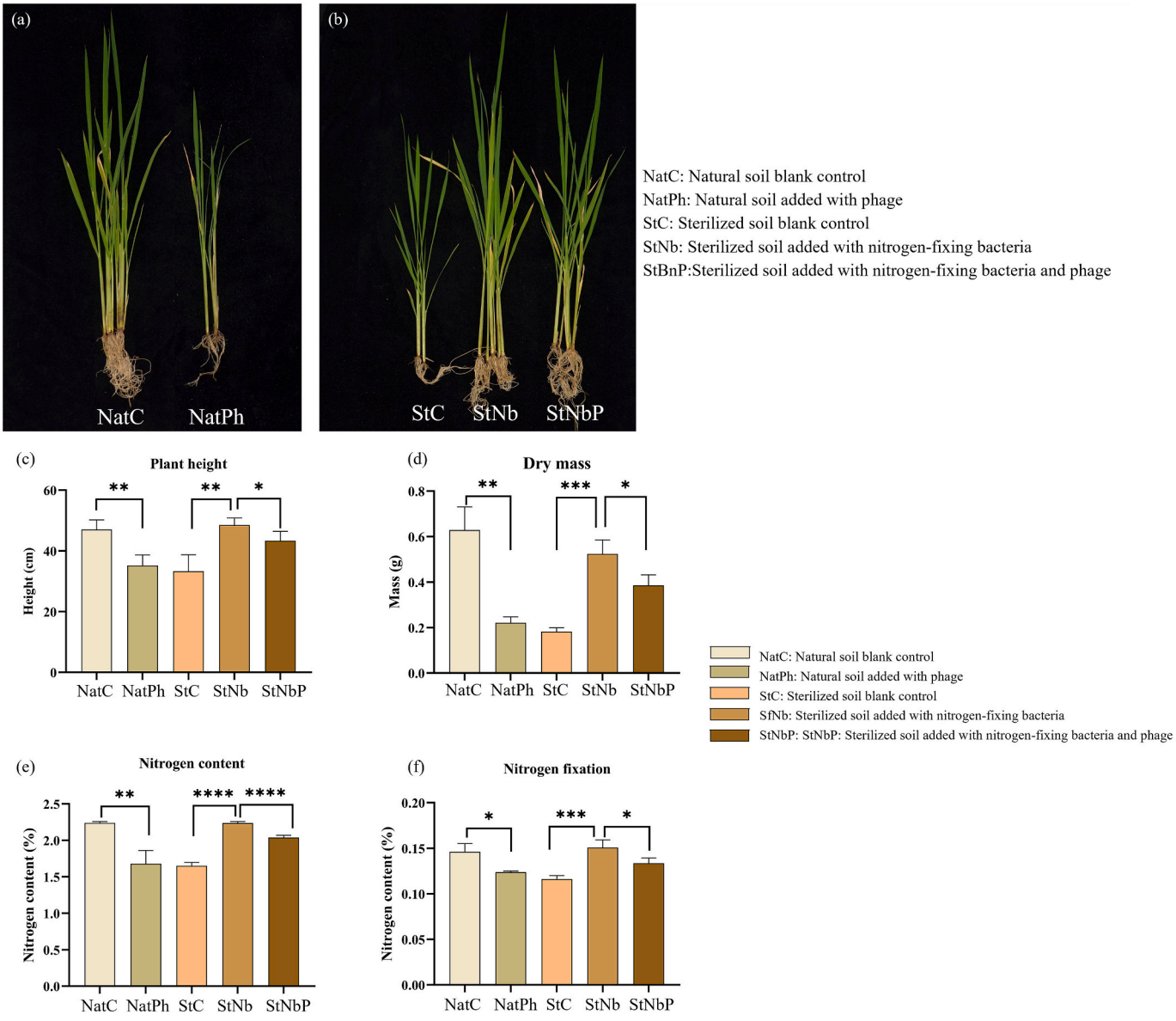

Fig. 3. Addition of *Apdecimavirus* NJ2 to soil reduced nitrogen fixation capacity of three *Enterobacter* species and inhibited rice growth. (a) Representative phenotypes of rice grown in natural field soil from Nanjing without (NatC) or with *Apdecimavirus* NJ2 (NatPh). (B) Representative phenotypes of rice grown in sterilized field soil (StC) or in sterilized soil with either three nitrogen-fixing *Enterobacter* species (StNb) or with nitrogen-fixing *Enterobacter* species and *Apdecimavirus* NJ2 (StNbP). Effects of the treatments on (c) plant height, (d) dry mass, (e) nitrogen content in rice, (f) nitrogen fixation capacity of *Enterobacter* species in soil. Asterisks above the bars indicate a significant difference between treatment means ($P < 0.05$) in a *t*-test.

soil that had been inoculated with the mixture of nitrogen-fixing *Enterobacter* strains (*E. cloacae*, *E. cancerogenus* and *E. ludwigii*), the nitrogen-fixation capacity was 11.35 % lower than in the sterilized soil added with nitrogen-fixing bacteria (Fig. 3f), and the height (Fig. 3b and c), dry mass (Fig. 3d) and nitrogen content (Fig. 3e) of rice plants were 10.68 %, 26.39 % and 8.79 % lower than those of plants in the sterilized soil amended with nitrogen-fixing bacteria respectively.

3.5. Treatment with Apdecimavirus NJ2 significantly changed the composition and diversity of rice rhizosphere bacteria

After quality control of the 16S rRNA sequence for all the samples, 1,439,290 high-quality sequences were obtained, 1,435,401 were valid sequences, accounting for 99.73 % of the total high-quality sequences. An average of 79,745 valid sequences were obtained for each sample, indicating that the sequencing data were sufficient to cover most of the rice rhizosphere bacteria. The rarefaction curve showed a rapid linear increase in the number of OTUs when the sequencing volume was small. However, when the sequencing volume was larger, the rate of increase in the OTUs gradually decreased, then leveled off, indicating that the amount of sequencing data obtained was sufficient to reflect the species diversity in the samples and thus ensured the reliability of the subsequent analyses (Fig. S1).

In the alpha-diversity analysis of the richness and diversity of the rice rhizosphere bacteria in the different treatments, the Chao1 index (735.03) for the natural soil amended with the novel phage was significantly lower (*t*-test, $P = 0.0461$) than for the natural soil control without the phage (740) (Fig. 4a). Similarly, the Shannon index for the natural soil amended with the phage (8.64) was significantly lower (*t*-test: $P = 0.0273$) than for the natural soil control (8.85) (Fig. 4b). The Chao1 index for the sterilized soil treated with nitrogen-fixing bacteria (640.12) or with nitrogen-fixing bacteria and phage (596.86) was higher (*t*-test: $P = 0.0160$, 0.5135, respectively) than for the sterilized soil control (588.19), and the index for the sterilized soil amended with

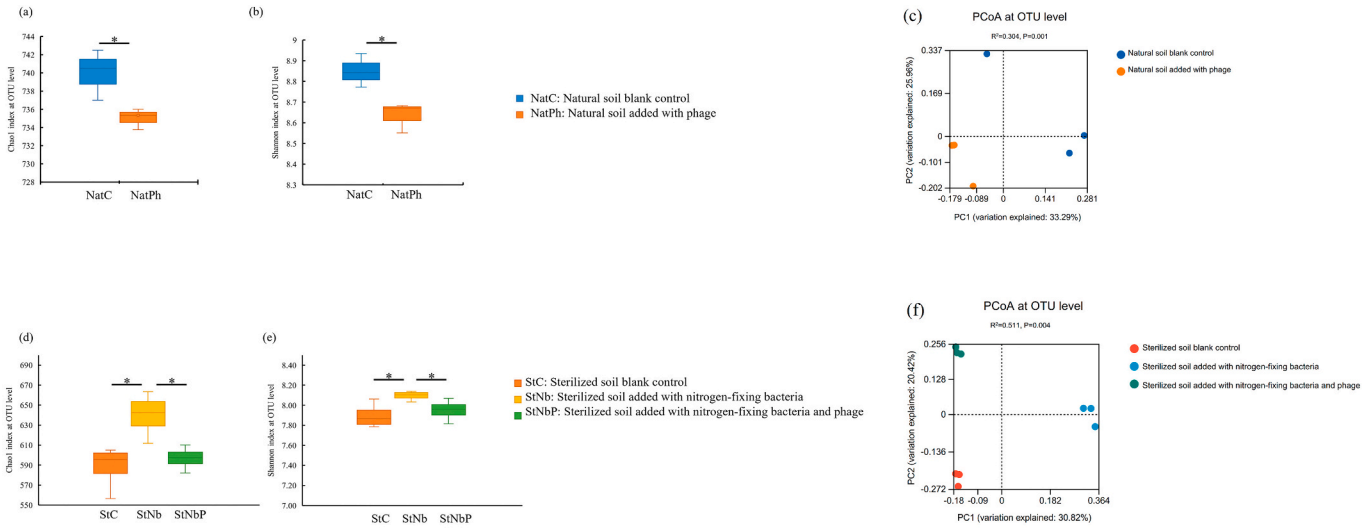

Fig. 4. Effects of *Apdecimavirus* NJ2 on bacterial diversity in natural and sterilized rhizosphere soil from rice. (a, b) Alpha diversity in natural field soil based on (a) Chao1 index and on (b) Shannon index. (c) PCoA of bacterial community in sterilized field soil. (d, e) Alpha diversity in (d) natural field soil based on Chao1 index and in (e) sterilized field soil based on Shannon index. (f) PCoA of bacterial community in sterilized field soil. Bars on a, b, d and e mean indicate standard error; an asterisk indicates a significant difference between treatments ($P < 0.05$) in a *t*-test.

nitrogen-fixing bacteria and phage was significantly lower (*t*-test: $P = 0.0137$) than for the sterilized soil amended with only nitrogen-fixing bacteria (Fig. 4d). Similarly, the Shannon index for the sterilized soil treated with nitrogen-fixing bacteria (8.09) or with nitrogen-fixing bacteria and phage (7.95) was higher (*t*-test: $P = 0.0229$, 0.5177, respectively) than for the sterilized soil control (7.89), and the index for the sterilized soil amended with nitrogen-fixing bacteria and phage was significantly lower (*t*-test: $P = 0.0460$) than for the sterilized soil amended with nitrogen-fixing bacteria (Fig. 4e). The diversity of soil bacteria in the sterilized soil should be very low, so the difference between the sterilized soil and the sterilized soil amended with nitrogen-fixing bacteria and phage was not significant.

The PCoA of the Bray-Curtis distances to assess the beta-diversity of rice rhizosphere bacteria composition revealed significant separation ($P < 0.05$) in the composition of rice rhizosphere bacteria between natural soil amended with the phage and natural soil control at the OTU level (PerMANOVA test: $R^2 = 0.304$, $P = 0.001$; Fig. 4c). Thus, the addition of the phage *Apdecimavirus* NJ2 significantly changed the bacterial community composition of rice rhizosphere in the natural and the sterilized soil.

3.6. The main changing bacterial taxa in rice rhizosphere after *Apdecimavirus* NJ2 addition

At the genus level, for natural soil, 399 genera were identified in the soil treated with the phage and 379 genera in the blank control soil; 354 genera were common to both treatment groups; 45 were unique to the soil with the phage (Table S3), and 25 were unique to the blank control (Fig. 5a). Among the 45 unique genera in the soil treated with *Apdecimavirus* NJ2, two genera have been hypothesized to be nitrogen-fixers and one to be a denitrifier (Aasfar et al., 2021; Gao et al., 2022; Gumaelius et al., 2001). In the sterilized soil, 360 genera were identified after nitrogen-fixing bacteria and the phage were added and 373 after only nitrogen-fixing bacteria were added; 311 genera were common to both treatment groups, 49 were unique to the soil with nitrogen-fixing bacteria and the phage (Table S4), and 62 were unique to soil with only nitrogen-fixing bacteria (Fig. 5b). Among 49 genera unique to sterilized soil with nitrogen-fixing bacteria and the phage, one genus is presumed to have a nitrogen fixation function and four genera to have a denitrification function.

For the top 30 most-abundant genera of rice rhizosphere bacteria, in natural soil after the addition of phage, the relative abundance of *Hydrogenophaga*, *Pseudomonas* and *Azospirillum* was 13.66 %, 48.19 % and 7.60 %, respectively, higher than in the blank control (Fig. 5c). For the sterilized soil after the addition of nitrogen-fixing bacteria and phage, the abundance of *Arenimonas*, *Hydrogenophaga* and *Azospirillum* was 23.55 %, 7.18 % and 76.53 %, respectively, lower than in the sterilized with nitrogen-fixing bacteria treated sterilized soil and the abundance of *Pseudomonas* was 176.19 % higher (Fig. 5d).

4. Discussion

Soil phage is an important component of soil ecosystems, and its abundance is closely related to the biogeochemical content and cycling (Kuzyakov and Mason-Jones, 2018; Wang et al., 2022a). Biogeochemical elements can influence the growth and diversity of bacteriophage host microbial communities, which in turn indirectly regulates the abundance and community structure of bacteriophages (Liang et al., 2024). Conversely, phages participate in host metabolism through their encoded auxiliary metabolic genes (AMGs), and regulate the abundance of bacteria associated with nutrient cycling which affect the biogeochemical cycling efficiency and content in soil (Tran and Anantharaman, 2021). In our study, low abundances of nitrogen-fixing bacteria and high abundances of *Enterobacter* phages were found where rice plants had symptoms of nitrogen deficiency in a field of Nanjing, China. While, in our previous study, a high abundance of nitrogen-fixing bacteria and low abundances of *Enterobacter* phages were found in was found in the soil of a field in Jiamusi, Heilongjiang Province which has fertile soil, high levels of nitrogen, and rice grows well (Wang et al., 2022a). Therefore, we hypothesized that the presence of *Enterobacter* phages leads to a decrease in the relative abundance of nitrogen-fixing bacteria and thus results in nitrogen deficiency and poor rice growth, and phage inoculation experiments confirmed this hypothesis. In nitrogen-poor environments, nitrogen-fixing bacteria are crucial for maintaining soil nitrogen levels (Yun et al., 2023). Our findings suggest that high bacteriophage abundance may exacerbate nitrogen deficiency by lysing nitrogen-fixing bacteria, potentially limiting plant productivity in nutrient-poor soils. This role of bacteriophages may explain why some barren soils struggle to naturally regain fertility. Understanding bacteriophages' impact is significant for agricultural and ecological restoration. Effective strategies could include using bacteriophage-resistant nitrogen-fixing strains or adjusting soil conditions (e.g., pH, moisture) to

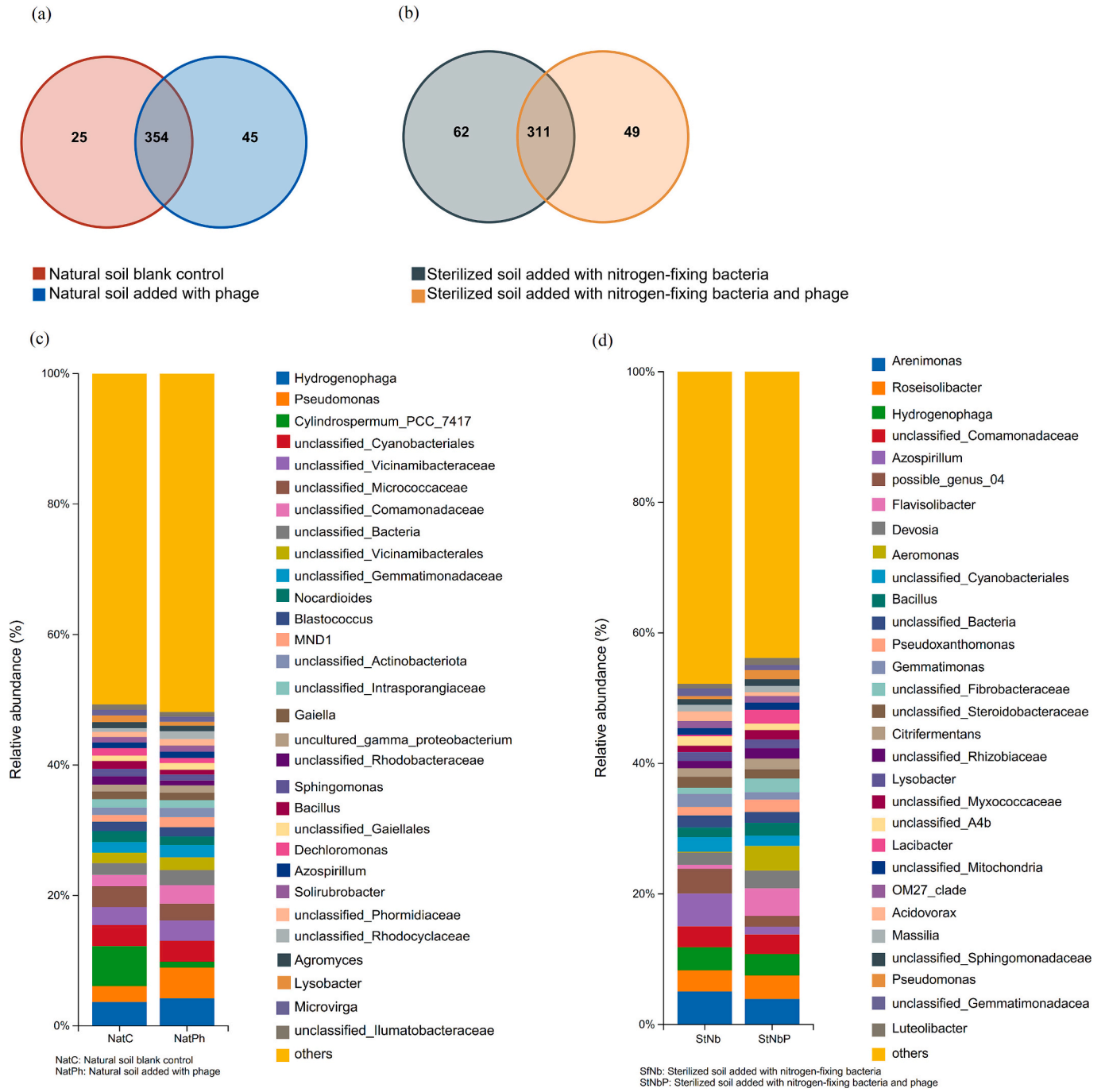

Fig. 5. Effects of *Apdecimavirus* NJ2 on bacterial community in rice rhizosphere. (a, b) Venn diagrams of the number of bacterial genera shared between treatments and unique to treatments. a, Natural soil blank control vs natural soil with phage *Apdecimavirus* NJ2; b, sterilized soil with nitrogen-fixing *Enterobacter* species vs sterilized soil with nitrogen-fixing *Enterobacter* species and phage *Apdecimavirus* NJ2. (c, d) Top 30 most-abundant genera of rice rhizosphere bacteria in (c) natural soil blank control and with phage *Apdecimavirus* NJ2, (d) sterilized soil with nitrogen-fixing *Enterobacter* species and with nitrogen-fixing *Enterobacter* species and phage *Apdecimavirus* NJ2.

reduce bacteriophage activity, thus improving nitrogen supply and supporting plant recovery.

Phages can influence the biogeochemical content in ways that directly or indirectly affect the structure and functions of bacterial communities (Rodriguez-Valera et al., 2009; Koskella and Brockhurst, 2014). In direct way, by causing the lysis of host bacteria, phages can directly control the abundance of host populations in soils, thus altering their biological functions (Braga et al., 2018; Koskella and Brockhurst, 2014; Rodriguez-Valera et al., 2009). For example, cyanophages can directly lyse host *Synechococcus* to reduce its abundance and play an important role in the cycling of nutrients and energy in the ocean (Suttle and Chan, 1994; Grasso et al., 2022). In wetlands bacteriophages infect and lyse sulfate-reducing bacteria and methanogenic bacteria, decreasing their abundance, potentially repressing sulfate reduction and methane production (Paula et al., 2018). In the present study, after adding the phage *Apdecimavirus* NJ2 to soils, and the abundance of the host *Enterobacter* bacteria was 35.62 % lower than in the control soil, the nitrogen fixation capacity was 15.36 % lower, and plant height, dry mass, and nitrogen content was 25.21 %, 64.81 %, and 25.02 % respectively, lower (Fig. 3). These results provide strong evidence that

virulent phages can lyse host bacteria, reducing their abundance and impairing their biological functions.

In indirect way, phages effectively lyse host bacteria, which makes space and resources available to other microorganisms, eventually altering the composition of the microbial community in the environments (Ankrah et al., 2014; Wang et al., 2019). Notably, in the present study, the composition and abundance of bacterial species that are not hosts of the phage *Apdecimavirus* NJ2 changed after the phage was added to nonsterilized paddy soil (Fig. 5). For example, the abundance of members of *Azospirillum* (Naqqash et al., 2022), *Kosakonia* (Gao et al., 2022), *Azotobacter* (Aasfar et al., 2021), which are involved in nitrogen fixation and of *Pseudomonas* (Daims et al., 2006), which is involved in nitrification and plant-growth promotion increased. Similarly, inoculation of *Pseudomonas syringae* phage in the soil significantly decreased the abundance of the host *Pseudomonas syringae* and increased that of the non-host *Pseudomonas* spp., because the decrease in the abundance of the host *Pseudomonas syringae* reduced the intraspecific competition between the host and the non-host bacteria of *Pseudomonas syringae* phage (Braga et al., 2020). We assume that, after the phage addition, the abundance of nitrogen-fixing host species of *Enterobacter* decreased, which opened ecological niches for other nitrogen-cycling bacteria (*Azospirillum* and *Pseudomonas*), leading to their increased abundance. Similar to our previous study (Wang et al., 2022a), the addition of the phage *Apdecimavirus* NJ2 decreased the nitrogen content in the soil and in rice plants and adversely affected plant growth, illustrating that phages can be responsible for substantially reduce nitrogen content in soil, but the self-recovery of soil through changing the soil microbial community structure cannot offset the nitrogen content reduced by phage, which ultimately leads to the decrease of soil nitrogen content and affects the growth of plants.

The selection of phage-resistant bacteria is closely linked to the characteristics of the phage, such as genome size, infection mechanisms, and host range (Labrie et al., 2010). Small-genome phages typically encode fewer functions and have simpler infection mechanisms (Stern and Sorek, 2011). Bacteria can acquire resistance through a single mutation or a few resistance mechanisms, making the selection of resistant strains relatively straightforward (Labrie et al., 2010). In contrast, large-genome phages may carry auxiliary metabolic genes (AMGs), multiple receptor-binding proteins, or immune evasion strategies (Dennehy and Abedon, 2021), requiring bacteria to develop complex resistance mechanisms, which complicates the selection process (Samson et al., 2013). Narrow-host-range phages infect only specific bacterial species, allowing for a more focused and straightforward selection of resistant strains. However, broad-host-range phages can infect various bacteria, complicating the selection of phage-resistant bacteria due to the need to account for different resistance mechanisms across multiple bacterial species (Hyman and Abedon, 2010). The novel phage NJ2 was isolated from the rice field in Nanjing and characterized here belongs to genus *Apdecimavirus* by morphology (Adriaenssens et al., 2020) and molecular analysis (Bujak et al., 2022; Lavigne et al., 2008). It has a narrow host range, capable of infecting only *Enterobacter* species among the 17 tested bacteria. This novel phage NJ2 has a dsDNA genome of 39,605 bp (Fig. 2c), which is small and simple, containing only the essential genes for phage structure, replication, assembly, and host lysis (Table S2). It lacks auxiliary genes related to complex infection mechanisms, such as cell wall-degrading enzymes, virulence factors, immune evasion genes, etc., suggesting a simple infection mechanism. These findings indicate that the phage NJ2 is a small, simple phage with a narrow host range, which suggests that it may be relatively easy to select phage-resistant nitrogen-fixing bacteria in future studies.

This study has identified a new bacteriophage NJ2, which has been shown to reduce soil nitrogen fixation and plant growth by lysing nitrogen-fixing bacteria, making it a major factor contributing to soil nitrogen deficiency and infertility. To control this phage and improve soil fertility, we will undertake the following work in the future: (1) Identify Key Genes for NJ2 Infection: We will identify the critical genes involved in NJ2 infection to elucidate its mechanism of recognizing and binding to specific receptors on nitrogen-fixing bacteria. This will provide theoretical support for developing phage-resistant nitrogen-fixing strains. (2) Determine Environmental Factors Affecting NJ2: We will identify environmental factors such as soil pH, temperature, humidity, and organic matter content that influence NJ2's distribution, activity, and infection. This will inform soil management strategies. (3) Screen for Phage-Resistant Nitrogen-Fixing Bacteria: We will select nitrogen-fixing strains resistant to NJ2 to provide microbial resources for improving nitrogen-deficient soils. (4) Implement Soil Fertility Improvement Strategies: Finally, we will use comprehensive measures such as adding phage-resistant nitrogen-fixing strains, applying organic fertilizers, and adjusting pH and moisture levels to suppress NJ2 activity, protect nitrogen-fixing bacterial communities, enhance soil fertility, and promote better plant growth conditions.

5. Conclusion

This work highlights the importance of phage communities in paddy soil for nitrogen cycle, because nitrogen-deficient rice plants was associated with low abundance of nitrogen-fixing bacteria in a field in Nanjing where the dominant viruses were *Enterobacter* bacteriophages. From the same field, we identified a novel bacteriophage belongs to genus *Apdecimavirus*, family *Autographiviridae*. By adding this phage to the soil with its host *Enterobacteria* species, the biodiversity and community structure of the soil bacteria changed, and the nitrogen-fixing capacity of the soil microbial community and thus the nitrogen availability for rice plants were reduced. Our results provide new insights on the contributions of phages in altering soil bacterial communities, ecological functions and plant growth. Controlling phages in the soil could be a useful strategy for improving soil nitrogen fixation in the future.

CRediT authorship contribution statement

Yu Liu: Writing – original draft, Methodology, Investigation, Conceptualization. **Yajiao Wang:** Writing – review & editing, Writing – original draft, Methodology, Investigation, Formal analysis, Conceptualization. **Wenchong Shi:** Software, Methodology. **Nan Wu:** Methodology, Investigation. **Wenwen Liu:** Methodology, Investigation. **Frederic Francis:** Writing – review & editing, Supervision. **Xifeng Wang:** Writing – review & editing, Writing – original draft, Methodology, Investigation, Funding acquisition, Conceptualization.

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.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2024.177382.

Data availability

Data will be made available on request. The GenBank accession

number of *Apdecimavirus* NJ2 is OR822025. The raw reads of metagenomic sequencing were deposited in the NCBI Sequence Read Archive database (accession number: PRJNA732820).

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