Culturomics and Amplicon-Based Metagenomic Insights into the Bacteria of Soils with High Yield of Oryza sativa L. subsp. Japonica

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Abstract: The bacterial community in the paddy field agroecosystem has a pivotal role in the growth adaptability strategy of rice. Here, we studied the bacterial community structure composition of rhizosphere and non-rhizosphere soil samples from super rice in high-yield (920.99 kg/mu) and low-yield (785.30 kg/mu) fields of Japonica Chu 54 using both culturomics and amplicon-based metagenomics approaches. Using amplicon sequencing, a total of 54 phyla and 1167 genera of high-yield field bacteria were detected, while the low-yield field bacteria were distributed in 49 phyla and 865 genera. In addition, compared with low-yielding fields, there were significant differences in the composition and abundance of the same members in high-yielding fields. The node microorganisms in high-yield and low-yield fields were Anaeromyxobacterium and HSB_OF53-F07, respectively. Culturomics analysis unveiled a diverse array of bacterial taxa, encompassing four phyla, 113 genera, and 331 species, including 33 new undescribed lineages. The culturomics and high-throughput sequencing results indicate a widely adapted and highly abundant group of Exiguobacterium, which has broad prospects for application due to its extensive survival characteristics and plant growth-promoting functions. In summary, we analyze the bacterial community structure composition of rhizosphere and non-rhizosphere soil samples from super rice in high-yield and low-yield fields of Japonica Chu 54 using culturomics and amplicon sequencing techniques to better develop positive promotion strategies that adapt to its unique ecological environment.

Keywords: culturomics; 16S rRNA genes; super rice; rhizosphere; non-rhizosphere; bacteria; Oryza sativa L. subsp. Japonica

1. Introduction

According to the database of the Food and Agriculture Organization of the United Nations [1], for a period of 27 years from 1994 to 2021, rice ranked third globally compared with other produced commodities, with China having the largest rice production. Improving the self-sufficiency rate of rice on limited arable land will help China achieve its carbon neutrality target by 2060 [2].

Microorganisms have enormous potential to transform modern agriculture and help meet the growing global demand for food [3]. Soil is the seed bank of microorganisms, and plants establish healthy microbial communities from available microorganisms through
root exudates and immune responses. Plant microbial communities expand plant functions by participating in various processes, including nutrient absorption, growth promotion, and resistance to biotic and abiotic stresses [4].

Under the evolution of human intervention, *Oryza sativa* L. subsp. *Japonica* with high yield has a large number of undiscovered unique microbial communities, which are composed of bacteria, archaea, and small eukaryotes. Many studies have characterized the diversity and function of rice microbial communities using next-generation sequencing technology [5–10]. Previous research has established that *indica* rice can enrich more bacteria with nitrogen metabolism function through its own NRT1.1B gene [11]. In contrast to low-yield rice fields, the rhizosphere of ultra-high-yield fields has a richer bacterial community and higher nitrogen metabolism intensity of microorganisms [7]. Diazotrophic iron-reducing bacteria, mainly present in paddy soil, significantly contribute to maintaining soil nitrogen fertility and rice yield [12].

Meanwhile, the strains obtained exhibit a positive promoting effect on rice growth. For example, an increase in the special bacterial groups: *Bryobacter*, *Xanthobacteriaceae*, and *Gemmatimonas* can reduce the effective Cd content in rice fields [13]. Halotolerant microorganisms *Agrobacterium tumefaciens*, *Bacillus subtilis*, and *Lysinibacillus fusiformis* alleviate saline stress in rice, thus promoting rice growth in the salinized field [14,15]. Endophytic seed-associated bacteria (*Pantoee* sp. SS-1, *Pseudomonas* sp. SS-38, and *Pseudomonas* sp. S7-1) act as plant growth promoters of Cuban rice [16]. The combined application of silicate-solubilizing bacteria and plant growth-promoting bacteria can endow rice with heat stress tolerance [17]. *Paraburkholderia* and *Delftia* can promote rice growth and increase yield [18]. Endophytic bacteria in rice such as *Enterobacter cloacae* and *Klebsiella* sp. have both chlorpyrifos-mineralizing and growth-promoting abilities [19]. *Bacillus albus* has the potential to combat rice diseases caused by *Xanthomonas oryzae* [20]. Whether analyzing microbial community function in rice fields using next-generation sequencing technology or the specific role of strains in rice fields, studies ultimately focus on the development and application of strain resources.

Therefore, there is an urgent need to supplement the large-scale acquisition of bacterial resources and the composition of bacterial community structure for rice. By obtaining cultivable strains, sufficient research can be conducted on bacterial resources in characteristic rice fields to better develop positive promotion strategies that adapt crops to their unique ecological environment. In this study, we aim to obtain more abundant bacterial culturable resources in high-yield fields of the super rice (yield per mu > 900 kg) *Japonica Chu 54*, which is a unique variety developed in Yunnan, China, known for its “high altitude suitability and high yield”. Using various cultivation conditions, we aim to enhance the elucidation of the bacterial community structure of super rice in high-yield fields and provide a solid material foundation for subsequent research.

According to the search query: (soil) AND ((microbiome) OR (microflora) OR (microbiota) OR (bacteria) OR (fungi) OR (archaea) OR (virus) OR (protist)) on NCBI, the number of articles published has rapidly increased (Figure S4). With the development of next-generation sequencing (NGS) technology, this means that the research on soil microorganisms has reached unprecedented breadth and depth. The amplicon sequencing of 16S rRNA using the second-generation Illumina sequencing platform can explore “who is in it” at a genus level, while whole genome sequencing using third-generation PacBio sequencing can explore the “division of responsibilities” from both gene functional characteristics and species composition. Based on our research objectives, we chose 16S rRNA gene sequencing.

The existing classic microbial isolation and cultivation methods involve changing the culture medium according to research needs to obtain cultivable microbial resources, obtaining single colonies for 16S rRNA gene identification, and conducting research on physiological characteristics, ecological functions, and natural active products. The emergence of MALDI-TOF-MS has made large-scale screening work under many conditions possible. Currently, there are many technological innovations that allow nutrients and
metabolites in the native environment to freely diffuse through permeable membranes without allowing cell diffusion, aiming to mimic native conditions as much as possible, like iChip [21], hollow-fiber membrane chambers (HFMCs) [22], diffusion bioreactors [23], or soil substrate membrane systems (SSMSs) [24]. The combination of the manipulation of single-cell technology and droplet culture methods, like nanoporous multihole microbial incubators (NMMIs) [25], droplet cultivation [26], or the SlipChip [27], is able to move individual cells through laser tweeter Raman sorting or bind subsets of cells with specific functions or categories at the molecular level, such as Raman-activated cell sorting (RACS) [28], fluorescence in situ hybridization of live cells (Live-FISH) [29], or reverse genomics [30]. However, these innovative technologies are far from solving the problem of sustained growth of isolated single cells and subsequent research [31]. Therefore, we chose rapid and high-throughput culturomics as the edge tool to obtain high-yield rice field bacterial resources.

Culturomics is a high-throughput screening method designed based on the convenience and efficiency of MALDI-TOF-MS technology and the effective supplementation of 16S rRNA gene sequencing technology. It was first proposed by Lagier et al. [32] and first applied to the human intestinal tract. Based on diverse changes in traditional cultivation methods, this method has been widely applied [33–35]. One of the key technologies in culturomics, MALDI-TOF-MS, is a mass spectrometry soft ionization technology. Before this, there had been a large number of applications of chromatography–mass spectrometry in biological identification, laying the foundation for MALDI-TOF-MS technology to stand out.

Culturomics has emerged as an advantageous tool for isolating large-scale bacteria and identifying new species [36–39]. It has shifted the view on microbiota by supplying “new” bacterial diversity not previously captured using amplicon sequencing [40]. However, the culturomic methodology has not yet been comprehensively applied to describe the bacteria population in paddy soil. Thus, the objective of this study is to shed some light on bacteria diversity from soils sampled from super rice in both high- and low-yield fields using both culturomics and V3–V4 amplicon sequencing approaches. Previous studies have revealed the significant contribution of microbial communities in ultra-high yield fields at different growth stages to soil nitrogen metabolism, as well as the abundant nitrogen-metabolizing microorganisms [7], and Indica can enrich more bacteria with nitrogen metabolism through its own NRT1.1B gene [11]. Plant genes are important determinants of microbial community composition [41,42]. In comparison with previous studies, we aim to obtain more bacterial resources with regional characteristics using culturomics, including the rhizosphere soil and non-rhizosphere soil of super rice (Japonica) in high-yield fields from Chuxiong, Yunnan Province. Simultaneously, we reveal the rich bacterial taxa and key taxa in high-yield fields using amplicon sequencing technology. This information will guide us in discovering beneficial taxa that promote rice growth. The main question addressed by this research is the role of bacterial communities in the growth and adaptability of rice in paddy field agroecosystems, particularly in the context of high-yield and low-yield rice fields. It aims to understand the differences in bacterial community composition and abundance between these two types of fields using amplicon sequencing technology and explores the potential applications in promoting an efficient strategy for rice growth.

2. Materials and Methods

2.1. Material Source

The japonica rice new variety Chu 54 with an excellent taste and a yield of over 900 kg per mu is the fourth super-rice variety in Yunnan Province. Soil samples of rice fields were collected from Jinshan Town, Lufeng City, Chuxiong Yi Autonomous Prefecture, Yunnan Province (latitude: 25.1963889, longitude: 102.06083). The collection period was after harvest, and the yield was 949.5 kg/mu. In the central area of the paddy field, the five-spot-sampling method was used to select 5 sampling points (approximately 1 square meter). Each sampling point was mixed with 5 non-rhizosphere soils using a 20 cm sampler (soil
Similarly, in the central area of the paddy field, five sampling points (approximately 1 square meter) were used to collect five mixed rice plants with roots from each point. The samples were transported at low temperatures to the Beijing laboratory. Every sample was divided into two parts: 1. bacterial culture and 2. 16S rRNA gene sequencing. Rhizosphere soil was composed of 0~1 mm soil, according to previous studies [43,44], tightly attached to the root surface. Large pieces of soil were shaken off, and then the root system was transferred to 50 mL PBS (centrifuge tubes) with sterile forceps to collect the soil. Finally, 5 g of each sample (excluding plant residues) was immediately stored in a −80 °C refrigerator until testing.

2.2. Isolation and Purification of Strains

We set 5 pH values (5, 6, 7, 8, 9), 4 temperature values (20 °C, 25 °C, 30 °C, and 35 °C), and 3 dilution gradients (10⁻³, 10⁻⁴, 10⁻⁵) and selected two ecological niches (rhizosphere soil and non-rhizosphere soil), and 3 culture media (Table S1: R2A, LB, and Ashby), for a total of 180 cultivation conditions (Figure S1). Soil (10 g) and roots (10 g) were suspended in 20 mL sterile distilled water and incubated in a rotary stirrer for 8 h (160 rpm, 25 °C). The solution was continuously diluted, and then 100 mL of the diluted suspension was divided into three different freshly prepared growth solid media—R2A, LB, and Ashby. The media were incubated at five pH variables (pH5, 6, 7, 8, and 9) for one week under aerobic conditions at 20 °C, 25 °C, 30 °C, and 35 °C.

2.3. Analysis of Bacterial Diversity

The soil samples were transported to Shanghai Majorbio Company in dry ice for 16S amplification sequencing. The detection process was as follows: Total genome DNA from 0.5 g of fresh soil samples was extracted using an E.Z.N.A™ Mag-Bind Soil DNA Kit (OMEGA, Norcross, GA, USA) following the manufacturer’s instructions. After genomic DNA extraction, the extracted genomic DNA was detected using 1% agarose gel electrophoresis. The V3–V4 primers 341F and 805R (Table S5) were synthesized with barcode. The PCR used TransGen AP221-02: TransStart Fastpfu DNA Polymerase (TransGen Biotech, Beijing, China) with the PCR instrument: ABI GeneAmp® 9700 type (Applied Biosystems, Waltham, MA, USA). Mix The PCR products of the same sample were mixed, and 2% agarose gel electrophoresis was used for detection. The AxyPrepDNA gel recovery kit (AXYGEN Company, Union City, CA, USA) was used to cut and recover the PCR products. We used Tris_ HCl elution and 2% agarose electrophoresis detection. Referring to the preliminary quantitative results of electrophoresis, the PCR products were quantified using QuantiFluor™. The ST blue fluorescence quantitative system (Promega Company, Madison, WI, USA) was used for detection and quantification, followed by a corresponding proportion of mixing according to the sequencing requirements of each sample. We used the Building Illumina Library Using TruSeqTM DNA Sample Prep Kit (TransGen Biotech, Beijing, China), and after the PE reads obtained with Illumina sequencing were split into samples, the dual end reads were first quality controlled and filtered based on the sequencing quality. At the same time, the overlapping relationship between the dual-end reads was concatenated to obtain optimized data after quality control concatenation. Then, the sequence denoising method DADA2 [45] was used to process the optimized data and obtain ASV (Amplicon Sequence Variant) representative sequences and abundance information. Additionally, the alpha diversity and beta diversity of the samples were analyzed using OmicStudio [46,47]. Differences were tested using non-parametric Kruskal–Wallis testing. Pairwise comparisons were conducted using Dunne’s test (assuming unequal variance). Principal Coordinate Analysis (PCoA) was carried out using Bray Curtis [48] and Jaccard [49] distance matrices to calculate the distance between two samples and obtain the distance matrix. The co-occurrence network of soil in rice fields was analyzed using MENA [50,51] and Gephi (version 0.10.1). MENA constructs networks based on Random Matrix Theory (RMT). We combined the rhizosphere and non-rhizosphere soil samples from high-yield fields and the rhizosphere and non-rhizosphere soil from low-yield fields.
to meet the construction requirements of the co-occurrence network (with a minimum of 8 replicates), with correlation coefficient thresholds (Network Indexes) of 0.78. We kept the ASVs with 10 in total 10 samples, where each dot represents an ASV, the size of the dot reflects the degree of connectivity, and the color of the dot represents the ASV in a different module. A Venn and Venn network was created using Excel and Evenn [52,53]. A matrix heatmap was plotted using bioinformatics [54], an online platform for data analysis and visualization.

2.4. Species Identification

Using MALDI-TOF MS to identify bacteria, when the score is less than 6, 16S rRNA gene sequencing is performed to define new bacterial species with sequence similarity < 98.65% [55]. During 16S rRNA gene identification, the universal primers 27F and 1492R (Table S5) were used for bacterial amplification. The 25 µL PCR amplification reaction system contained 12.5 µL of 2× Pfu PCR Master Mix (TIANGEN Biotech Beijing Co., Ltd., Beijing, China), 8.5 µL ddH2O, 1 µL primer 27F, and 1 µL primer 1492R, as described in a previous study [56]. The bacterial 16S rRNA gene was amplified according to the following procedure: 5 min at 95 °C, 35 cycles: 20 s at 95 °C, 20 s at 55 °C, 60 s at 72 °C, 5 min at 72 °C). In addition, the similarity of 16S rRNA between species within the genus was also used as a reference for suspected new species.

The MALDI-TO-MS test was carried out using the microbiological detection system Autof ms 2000 (Beijing, China). To complete the test, a single colony (colony standard: size ≥ 1 mm, growth of logarithmic or stable wet colony) was scraped evenly coated in the center of the MALDI target sample point. The colony was not too thick and covered 80% of the MALDI target hole area. If the bacterial solution was too dry, 1 µL of lysate (pure formic acid and pure acetonitrile according to the solution obtained from the mixture of 4:3) was added and allowed to dry. Then, 1 µL of the substrate solution was taken as supernatant (the precipitate was not added to the sample wells of the MALDI targets). Then, the MALDI targets were put into the target chamber, and the peptide mass fingerprint was collected, saved, and compared. A database comparison score within [9.5, 10.0] represents the species level confidence, possible subspecies, within in [9.0, 9.5) represents species level confidence, within in [6.0, 9.0) represents horizontal confidence in the genus, and within [0.0, 6.0) stands for unbelievable.

2.5. Rice Growth Promotion Experiment in the Laboratory

The rice seeds were soaked on 3 September 2023 (Szebaok 4000 times solution; soaked for 24 h, room temperature; chest breaking in a humid environment and high temperature for 24 h, 38 °C; 30 °C germination for 24 h). The buds were about 1 cm long on 8 September, and about 3 cm on 10 September. Then, two seedlings were transplanted into a glass tube containing paddy soil (red soil) (4 replicates per treatment). The soil was previously passed through a 2 mm aperture sieve, mixed with water, and then divided into each glass tube, making the soil height in each test tube as high as possible. Bacterial liquid was added until the rice was in the growth stabilization period (on 21 September, the seedling length was about 15 cm). Three strains of a single colony were inoculated into LB liquid (150 mL) medium on 20 September. The bacteria were centrifuged and washed with sterile water on 21 September, and then 20 mL of 0.85% sterile NaCl was added to prepare the bacterial suspension. A total of 2 mL of liquid was added per treatment, and 0.85% sterile NaCl was used as the control group. After about 27 days of growth under sunlight (18 October), the following plant parameters were collected: height, leaf area index, stem diameter, and chlorophyll content. For statistical analysis, Student’s t-test was used for the laboratory experiment.
3. Results

3.1. Analysis of Bacterial Diversity Based on Amplicon Sequencing

To characterize the variations in root-associated microbiota assembly processes of Japonica Chu 54 rice cultivars, we analyzed the 16S sequencing data from two root-associated niches. After filtering, 682,823 high-quality reads were obtained, and those reads were clustered into 35,374 ASVs using the DADA2 clustering method. For alpha diversity of the two yields of rice, there was no significant difference between bacterial richness and low yield in high-yield rice soil (Chao 1 index, Kruskal–Wallis, Dunn’s test, \( p = 0.64 \), Figure 1a), there was no significant difference between high-yield rice soil bacterial diversity and low yield, and bacterial diversity in high-yield non-rhizosphere soil was higher than that in rhizosphere soil (Shannon index, Kruskal–Wallis, Dunn’s test, \( p = 0.1 \), Figure 1b). From the Bray Curtis and Jaccard PCoA (Principal Co-ordinates Analysis), it can be seen that there were significant differences in bacterial composition between high-yield and low-yield fields (\( p < 0.01 \), Figure 1d), and the same members had significant abundance differences (\( p < 0.01 \), Figure 1c).

Using amplicon sequencing, a total of 54 phyla, 173 classes, 383 orders, 621 families, and 1167 genera of high-yield field bacteria were detected, while the low-yield field bacteria were distributed in 49 phyla, 148 classes, 318 orders, 504 families, and 865 genera. In both rhizosphere and non-rhizosphere soils, the top 30 genera belonged to (Figure 2d) Pro-

![Figure 1. Diversity analysis of rhizosphere (R) and non-rhizosphere (N) soils in high-yield (H) and low-yield (L) fields. (a,b), Shannon and Chao1 index of the bacterial community in the rhizosphere and non-rhizosphere of two yield fields. The significance of difference was determined by Kruskal-Wallis test. (c,d), PCoA analysis grouped by the rhizosphere and non-rhizosphere based on Bray-Curtis and Jaccard distance matrices of bacterial communities of two yield fields.](image)

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*Figure 1. Diversity analysis of rhizosphere (R) and non-rhizosphere (N) soils in high-yield (H) and low-yield (L) fields. (a,b), Shannon and Chao1 index of the bacterial community in the rhizosphere and non-rhizosphere of two yield fields. The significance of difference was determined by Kruskal-Wallis test. (c,d), PCoA analysis grouped by the rhizosphere and non-rhizosphere based on Bray-Curtis and Jaccard distance matrices of bacterial communities of two yield fields.*
teobacteria, Acidobacteriota, Actinobacteria, Bacteroidota, Chloroflexi, Desulfobacterita, Firmicutes, and Myxococcota. The genus Exiguobacterium was abundant and especially enriched in the rhizosphere soil of high-yield fields, and it was followed by an unclassified genus in the family Micrococcaceae.

Figure 2. Based on within-module connectivity (Zi) and among-module connectivity (Pi) analysis, modules are distinguished by color, we evaluated the topological role of individual nodes in the networks and defined the keystone species in each network of two yield fields. (a) The network of high-yield paddy soil (nodes: 152, links: 212, cutoff (default): 0.780) represented by the bold part genus group, where cultivable strains were obtained using culturomics methods. (b) The phylum to which the ASV unit belongs in the network diagram. L—low-yield, H—high-yield. (c) The network of low-yield paddy soil; nodes: 120, links: 207, cutoff (default): 0.780. (d) The relative abundance of genus Top30 of rhizosphere (R) soil and non-rhizosphere (N) soil in high-yield (H) and low-yield (L) fields.

Diagrams of the bacterial network in high-yield paddy soil and low-yield paddy soil are shown in Figure 2. The connection degree of high-yield fields is higher than that of low-yield fields, indicating that they have relatively higher stability. Unfortunately, few
genus taxa can be annotated in this sequencing result. The nodes with max degree taxa in high-yield fields are *Anaeromyxobacter*, ASV143 (Phylum: *Chloroflexi*), ASV73 (Phylum: *Chloroflexi*), ASV81 (Phylum: *Acidobacteriota*), and the node with max betweenness is ASV664 (Phylum: *Chloroflexi*). The node with max degree and betweenness taxa of low-yield fields is HSB_OF53-F07. We chose betweenness centrality and degree centrality to confirm the most critical nodes [57,58]. The genus diagram that coincides with the genus obtained using cultureomics is boldly marked (Figure 2a), and genus taxa with a high degree of connectivity are not obtained. These taxa may be key factors affecting the entire paddy soil ecosystem. *Anaeromyxobacter* is a member of *Myxobacteria* that is able to grow anaerobically [59]. Previous studies indicate that *Bacillus*, *Anaeromyxobacter*, and HSB OF53-F07 are the predominant genera in rice fields (Arunrat et al. 2022 [9]). We counted the phylum of node taxa in the co-occurrence network (Figure 2b) and found that both high-yield and low-yield nodal microorganisms are distributed in *Firmicutes*, *Proteobacteria*, *Chloroflexi*, *Actinobacteriota*, *Desulfobacterota*, MBNT15, *Nitrospirota*, *Acidobacteriota*, *Myxococcota*, *Bacteroidota*, and *Gemmatimonadota* with differences in abundance. Among them, the beneficial effects of *Firmicutes* members in maintaining the sustainability of agricultural systems include [60]: plant growth promotion, biological control of plant pathogens, heavy metal phytoremediation, etc. *Proteobacteria* members are phenotypically diversified, showing extreme metabolic diversity [61]. *Chloroflexi* is known for the presence of a class of anaerobic phototrophic bacteria [62]. *Actinobacteriota* is known for its ability to produce secondary metabolites [63]. *Acidobacteriota* taxa often affect soil micronutrients and soil acidity [64].

### 3.2. Bacterial Diversity Based on Culturomics

This study designed a total of 180 cultivation conditions, and a total of 1706 strains were isolated from a single colony with different morphological characteristics on the culture medium. After identification, they belonged to four phyla, 10 classes, 26 orders, 46 families, 113 genera, and 331 species, and were distributed horizontally in *Proteobacteria*, *Firmicutes*, *Actinobacteria*, and *Bacteroidetes*. *Proteobacteria* was the main species, with 47 potential new species (33 after duplicate removal) (Table S2).

We counted the strains isolated from three different media (Figure S2). Among meR2A media, *Bacillus* accounted for 10% of all species, followed by *Pseudomonas*, *Microbacterium*, and *Flavobacterium*. In LB medium, *Bacillus* accounted for 20% of all species, followed by *Microbacterium*, *Pseudomonas*, *Stenotrophomonas*, and *Arthrobacter*. Among the strains with nitrogen fixation function isolated using Ashby medium (Figure S2b), *Pseudomonas* accounted for the highest proportion, followed by *Novosphingobium*, *Enterobacter*, *Rhizobium*, and *Sphingomonas*.

R2A medium is a low-nutrient medium, according to its component content, LB medium is a nutrient-rich medium, and Ashby medium is a nitrogen-fixing functional bacteria screening medium. The species isolated from these three media are presented in the Venn network (Figure 3). The strains that intersect oligotrophic (R2A) and eutrophic (LB) media are bacteria that can grow in trophic conditions, but unfortunately, we cannot define this as truly surviving under oligotrophic conditions and in eutrophic conditions. The taxa of the intersecting fractions of the three media can grow well without an additional nitrogen source and under eutrophic or oligotrophic conditions with a nitrogen source. It is clear that the number of bacteria with nitrogen-fixing function grown on oligotrophic medium (R2A) is higher than that of the eutrophication (LB) bacteria cultured. There is no bacterial group that can grow on these three media (eutrophic, oligotrophic, and nitrogen-free). LB medium can enrich more *Bacillus* taxa. Different media components may lead to different strains being isolated, and the reasons for the differences include the strain demand for nutrient type, the interaction relationship between strains, etc.
Figure 3. Culturable strains with different media as nodes. The Venn plot shows the number of culturable strains in each media.

Based on statistics on the number of validly published species in all bacterial phyla, as of 11 July 2023, there were 19,440 actively published bacterial species worldwide (https://lpsn.dsmz.de/text/numbers#species-names-validly-published-under-the-icnp-per-phylum-wo-synonyms, accessed on 19 October 2023), of which *Actinobacteria*, *Bacteroidetes*, *Firmicutes*, and *Proteobacteria* accounted for 90.58% of the total published bacterial species (Table 1). The use of culturomics methods increased the number of cultured bacterial species by 0.26%.

The number of strains isolated under different growth conditions in non-rhizosphere and rhizosphere soil was characterized with heat maps using culture temperature and pH as the horizontal and vertical coordinates (Figure 4c,d). It can be seen that more strains can be isolated at a temperature of about 30 °C, and more strains can be isolated at pH 6–8.
Table 1. Efficient publication of bacterial species worldwide.

<table>
<thead>
<tr>
<th>Phylum</th>
<th>Number of Species Effectively Published</th>
<th>Number of Potential New Species</th>
<th>Increase in Percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Proteobacteria</td>
<td>7484</td>
<td>17</td>
<td>0.23%</td>
</tr>
<tr>
<td>Actinobacteria</td>
<td>4298</td>
<td>9</td>
<td>0.21%</td>
</tr>
<tr>
<td>Firmicutes</td>
<td>3364</td>
<td>2</td>
<td>0.06%</td>
</tr>
<tr>
<td>Bacteroidetes</td>
<td>2463</td>
<td>18</td>
<td>0.73%</td>
</tr>
</tbody>
</table>

Figure 4. Statistics of isolated strains under different culture conditions. (a) Venn plot of isolated strains under 5 pH values. (b) Venn plot of isolated strains under 4 temperatures. (c) Heat map of isolated strains at 5 pH values and 4 temperatures in non-rhizosphere soil. (d) Heat map of isolated strains at 5 pH values and 4 temperatures in rhizosphere soil.
Bacterial species isolated at different temperatures and pH values were characterized using Venn diagrams (Figure 4a,b). It is interesting that *Exiguobacterium acetylicum* was isolated at all four pH values and five temperatures, indicating that this strain may be enriched in the rhizosphere of high-yielding rice while being able to adapt to a wide range of temperatures and pH changes. In addition, studies have shown that members of *Exiguobacterium* are distributed in a wide range of temperatures, salinities, and pH values, which are known as polyextremophiles [65].

Twenty-six bacteria were isolated at all four temperatures (Table S4), where *Bacillus* accounted for the majority. Thirteen bacteria were isolated at all five pH options. Eight bacteria were isolated at all four temperatures and five pH values, which can be called a group insensitive to environmental changes, which were also mostly *Bacillus*.

### 3.3. Culturoomics and Amplicon Sequencing Combined Analysis

Based on combined culturoomics and amplicon sequencing, among the top 30 ASV numbers in high-yield rhizosphere soils and non-rhizosphere soils, the number of *Exiguobacterium* was the most prominent, especially in the rhizosphere. Three *Exiguobacterium* members were isolated using the culturoomics method: *Exiguobacterium acetylicum*, *Exiguobacterium mexicanum*, and LGIW*s*. *Exiguobacterium* members have facultative anaerobic properties, which adjust to the flooded environment of rice fields, and some members have been reported to have plant growth-promoting effects [66,67]. *Exiguobacterium* is one of the beneficial genera that are essential for phosphate dissolution and Cd/Cu fixation in rice soil [68]. *Exiguobacterium acetylicum* can grow at 4–42 °C and has plant growth-promoting characteristics, which can promote the growth of wheat seedlings [69]. *Exiguobacterium mexicanum* is a slightly halophilic strain with HN-AD (heterotrophic nitrification and aerobic denitrification) function, according to Cui et al. [70]. Previous studies have pointed out that, the diverse utilization of polysaccharides and the family of transporters closely related to environmental stress resistance give members of this genus a wide range of adaptive capacities (salinity, temperature, and pH) [71]. Therefore, this genus has a good application prospect in paddy soil.

We obtained bacterial information using 16S rRNA sequencing at the genera level (removing no rank and unclassified) and compared it with the cultureomics results. Figure 5 shows the coverage of strains at the genus level between the high-throughput sequencing results and the cultureomics results in the rhizosphere and non-rhizosphere, respectively. Among them, 59 genus taxa obtained with culturoomics coincided with the sequencing results (5 genus taxa coincided with the Top30), and 55 genus taxa were independent of the sequencing results, supplementing the omission based on sequencing technology bacterial taxa [40,72]. In the unmatched taxa, many strains have been reported to have potential values, such as plant growth promotion, granting plants tolerance to diseases and extreme situations, pesticide degradation, and industrial substance synthesis (see Table S3).

### 3.4. Rice Growth Promotion Experiment in the Laboratory

Compared with the control group, the experimental group had a significantly increasing trend (Student’s test, $p < 0.05$, Figure 6). *Exiguobacterium acetylicum* showed extremely significant differences in plant height and the leaf area index, as well as significant differences in stem diameter and chlorophyll content. *Exiguobacterium LGIWs* showed significant differences in plant height, stem thickness, and the leaf area index, but no significant differences in chlorophyll content. *Exiguobacterium mexicanum* showed extremely significant differences in plant height, stem thickness, and the leaf area index, but no significant difference in chlorophyll content. In general, all three strains have a promoting effect, and *Exiguobacterium acetylicum* and *Exiguobacterium LGIWs* have a better effect on rice growth promotion than *Exiguobacterium mexicanum*. 
Figure 5. The isolated strains displayed in conjunction with the sequenced strains.

Figure 6. Plant parameter statistics. (a) Plant height, (b) stem diameter, (c) chlorophyll content (SPAD values), and (d) leaf area index. Colors represent different samples.
4. Discussion

Our study combines culturomics and high-throughput sequencing technology to explore the bacterial community (rhizosphere, non-rhizosphere) in high-yield and low-yield rice soil. According to amplicon sequencing, the top 30 bacterial members in the rhizosphere soil of high-yield rice fields are highly enriched in the genus *Exiguobacterium*. Using culturomics, three strains of *Exiguobacterium* were obtained: *Exiguobacterium acetylicum*, *Exiguobacterium mexicanum*, and LGIW_s. After adding pure cultures of these three strains to a tube system with rice seedlings, it was found that they have the function of promoting plant growth. Previous studies have shown that members of this genus possess facultative anaerobic and adaptive characteristics to extreme environments [68,69], making them suitable for use in flooded rice fields. At the same time, certain members of this genus have clear reports on phosphorus solubilization, heavy metal fixation, and nitrogen transformation in rice fields [66,67]. Based on these existing studies, this article conducted pot experiments on three obtained strains of *Exiguobacterium* and obtained positive results. It is expected that further research will be conducted on their mechanisms in stress resistance and growth promotion. The node microorganisms in high-yield and low-yield fields were *Anaeromyxobacterium* and HSB_OF53-F07, respectively, but we did not obtain these strains using culturomics methods. It is worth noting that previous studies on rice fields have also mentioned that *Bacillus*, *Anaeromyxobacter*, and HSB_OF53-F07 were the predominant genera in both rice-fish co-culture farming systems and rice monoculture farming systems [9]. So, they have great potential in the rice field ecosystem.

Among the large number of cultivable bacterial resources (331 species, 113 genera) isolated from high-yield fields of super rice using culturomics technology, 38 potentially new species were identified, which can increase the number of cultivated bacterial species worldwide by 0.26%. There are 55 taxa (genera) not part of the sequencing results. These missing groups may assist in isolating uncultured groups based on their metabolic exchange potential with key groups [73] or can be directly applied to agricultural production practices based on the metabolic function of the strains. Among all the isolated strains, there are about 96 functional strains reported so far. These strains exhibit diverse functions, including, but not limited, to plant growth promotion, environmental bioremediation, biological control, and extreme environmental tolerance. Bacteria with known and unknown functions provide a basis for better developing positive promotion strategies to adapt to their unique ecological environment.

It can be seen from the results that there are significant differences in the corresponding niches between the rhizosphere and non-rhizosphere and the low-yield and high-yield fields, which is consistent with our assumption. The node microorganisms and enrichment microorganisms are obviously different, while the bacteria screened with culturomics only account for a small part of the bacterial groups detected by amplicons, and there are still a large number of uncultivated and difficult-to-culture microorganisms. This explanation is supported by the results of recent research by William H et al. [31], including the key node microorganisms revealed by the network diagram in this study, which may play a decisive role in them [74]. Therefore, obtaining the target strain is the direction we should pay attention to in the future. The large number of unknown and untaxonized units obtained from the sequencing results leads to the incompleteness of the results, and higher throughput metagenomics may be selected in the future. In addition, this paper only isolated resources from bacteria to explore the structure of bacteria in paddy fields, and fungi and archaea are also important parts of paddy field ecosystems, which should also be paid attention to in the future.

Paddy ecosystems are a unique environment, which is an important part of global change, and also face threats such as salinization [75,76], acidification [77], poor nutrient utilization [78,79], organic pollutants, endocrine disruptors, antibiotics, and microplastic pollution [80–84]. In addition, the physiological and biochemical activities of microorganisms have far-reaching impacts on soil physicochemistry [85]. The plant microbiome can naturally and beneficially alleviate plant abiotic stresses [86]. In order to solve these
problems from microorganisms, we recommend exploring the interaction mechanism between single and combination strains that have been obtained and have positive effects on plants and soil. In the future, we should fully consider the relationship between rhizosphere metabolism and bacterial survival characteristics at different growth stages of rice, combined with multi-omics joint analysis, committed to isolating non-cultivable or difficult to cultivate microorganisms that were critical nodes in different stages, implementing phased and precise regulation strategies, and promoting green and sustainable agricultural development.

5. Conclusions

A deep understanding of microbial diversity and the functions of rhizosphere microbiota, as well as obtaining as many strain resources as possible, can further provide the comprehensive insights needed to manipulate these microbial communities. Based on this concept, our study for the first time starts from the structure of rhizosphere bacterial communities in high-yield and low-yield fields of super rice (Japonica), indicating that the enriched rhizosphere bacteria of *Exiguobacterium* in high-yield fields have a positive promoting effect on rice growth. This genus group has a wide range of physiological and biochemical characteristics that adapt to extreme environments. Importantly, we also analyzed *Anaeromyxobacterium* and HSB_OF53-F07 as key groups in rice fields, but the strains were not obtained using culturomics techniques. Based on the results of this study, we suggest strengthening research on uncultured microorganisms. In future agriculture, the use of microbial inoculants should select microorganisms that can participate in plant-microorganism interactions and that have adapted to similar environments, achieving precise application of single or synthetic microbiota to help maintain stability in rice field ecosystems. Our preliminary results will support further work to confirm the universality and implementation potential of these results in field trials and other agricultural systems.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/agronomy13122867/s1, Figure S1: The 180 cultivation conditions in this study; Figure S2: Statistics of strains isolated from three media (genus): (a) medium R2A (188 strains), (b) medium LB (164 strains), and (c) medium ASHBY (53 strains); Figure S3: Growth status of rice *Japonica Chu 54* on the 27th day after inoculation with a pure culture of the strains. (a) CK, (b) *Exiguobacterium mexicanum*, (c) *Exiguobacterium LGIW*, and (d) *Exiguobacterium acetylicum*; Figure S4: Number of articles with increasing age, (Left)Search query: (Root) AND ((microbiome) OR (microbiota) OR (bacteria) OR (fungi) OR (archaea) OR (virus) OR (protist))——NCBI PubMed.

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References


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