

Diversity of bacterial communities in the rhizosphere of the endangered plant, *Paeonia jishanensis*

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Abstract: The microbial community in the rhizosphere is thought to provide plants with a second set of genomes, which plays a pivotal role in plant growth. In the present study, soil samples were collected from the rhizosphere of an endangered plant, *Paeonia jishanensis*. The plants were divided into three groups: well-growing plants, poor-growing plants and dead plants. Metagenomic DNA was isolated from rhizosphere soil samples of these plants and 16S rRNA genes were sequenced by the PacBio-RS II system. The results of taxonomic analysis showed that the dominant phyla were Proteobacteria, Acidobacteria, Planctomycetes, Bacteroidetes and Actinobacteria in all three sample types. Linear discriminate analysis Effect Size (LEfSe) showed that 5 species, *Hirschia baltica*, *Arcobacter aquimarinus*, *Gimesia maris*, *Magnetococcus marinus* and *Pseudoxanthobacter soli*, were significantly enriched in the rhizosphere of well-growing plants. Additionally, the results of PCA, MDS and clustering analysis indicated that the bacterial community in the rhizosphere of living *P. jishanensis* plants was similar. With the death of plants, the bacterial community changed considerably. These findings suggest that the abundance of many beneficial rhizospheric microbes declined with the death of *P. jishanensis*. This is a potential way to preserve endangered plants by inoculating declining species with beneficial microbes.

Keywords: *Paeonia jishanensis*; endangered plant; rhizosphere; microbial communities; metagenomic; next generation sequencing

INTRODUCTION

Paeonia jishanensis is a wild species of the section Moutan that belongs to the genus *Paeonia*, which have decorative and medicinal values [1]. It has been proven that *P. jishanensis* is a significant ancestral species and many cultivated tree peony species were bred from it. *P. jishanensis* is also a traditional Chinese herbal medicinal plant, and many medically important chemical substances, including paeonol, paeoniflorin, benzoic acid, volatile oil and plant sterol, have been detected in the root bark of *P. jishanensis*. The plant is used to reduce inflammation, treat cardiovascular diseases, protect the liver, decrease blood sugar and lipids and for its anticancer activity [2].

P. jishanensis is distributed in a narrow area, where the Shaanxi, Shanxi and Henan provinces converge. It mainly grows in middle and low mountainous regions,

at altitudes above 850 m and below 1550 m [3]. Unfortunately, this species is facing extinction. Urgent measures are needed to conserve it. It has been reported that the potential extinction of *P. jishanensis* was mainly caused by four factors, including weak sexual propagation, habitat restriction, deficient competition and excessive exploitation. Accordingly, some researchers have suggested four strategies for conservation: building a nature reserve, off-site conservation, artificial breeding and the establishment of a gene bank of *P. jishanensis* [3].

The rhizosphere is a special zone defined by the interaction of plants and soil microorganisms. The microbes in the rhizosphere play a positive role in plant growth. It is thought that it provides plants with a second set of genomes. Rhizosphere microorganisms help plants to resist biotic and abiotic stress by improving soil nutrition status, reducing plant disease and degrading harmful substances. In addition, physi-

ological and metabolic activities such as exudates of plant roots could also improve or inhibit the growth of soil microorganisms [4]. Hence the rhizosphere microbial community differs in plant species. Previous studies have shown that the rhizosphere microbial community can affect plant health and seed germination [5-7]. Plant health could be improved by regulating the rhizosphere microbial community [8,9]. Moreover, rhizosphere microbes showed a more rapid response to environmental change when compared to plants. The rhizosphere microbial community structure can be used as a sensitive indicator to monitor soil quality and changes in plant health [10,11]. We speculated that a potential approach to preserving endangered plants is inoculation of beneficial microbes, with which we expect to develop a novel strategy for *P. jishanensis* conservation. As far as we know, there has been no research related to the rhizosphere microbial community of *P. jishanensis*.

In this study, we identified the bacteria present in the rhizospheres of *P. jishanensis* with different growth statuses by sequencing 16S rRNA genes from metagenomic DNA and comparing the corresponding communities. We hope to identify beneficial bacterial species in order to promote the growth of *P. jishanensis*. Moreover, these results should help in evaluating the health condition of *P. jishanensis* by monitoring the microbial community structure of the rhizosphere.

MATERIALS AND METHODS

Site description and sample collection

This research was carried out in the Majiagou forestry station (35°43'N, 110°58'E), Shanxi Province, China. A total of 27 soil samples from the rhizosphere of *P. jishanensis* were collected. According to the plant growth conditions, the samples were divided into three groups as follows: well-growing plants, poor-growing plants and dead plants. We defined the plants with strong vigor, thick green leaves and lush branches as “well-growing plants”, completely withered plants as “dead plants”, while plants with an intermediate growth status were defined as “poor-growing plants”. In each group, three samples were pooled randomly and then sequenced. Bulk soil was shaken off gently from the roots, and the residual soil closely bound to the root

was brushed and collected [12]. Samples were placed into aseptic bags and stored at 4°C in a refrigerator.

DNA extraction and sequencing

A TIANamp Soil DNA Isolation Kit (DP 336) (TIAN-GEN, China) was used to extract the total genomic DNA from 0.5-g soil samples, according to the manufacturer's instructions. The concentration and quality of the extracted DNA were assessed by a NanoDrop 2000 spectrophotometer (Thermo Scientific). The 16S rRNA genes were amplified by primers 27f (5'-AGAGTTTGA TCCTGGCTCAG-3') and 1541r (5'-AAGGAGGTGATCCAGCCGCA-3'). The polymerase chain reactions (PCR) were performed according to the described method [13]. PCR amplicons were extracted and purified using AMPureXp beads (Beckman Coulter) following the instructions of the manufacturer. Purified products were sent to Shanghai Personal Biotechnology Co., Ltd., China for PacBio sequencing.

Next-generation sequence data processing and taxonomic analysis

QIIME (version 1.8.0) was used to process the sequencing data [14]. Specifically, sequences were first sorted by barcode and trimmed to remove the primers and barcodes, then aligned by SILVA 16S reference alignments of sequences [15]. Chimeric sequences were identified and removed using UCHIME in USEARCH (v11, <http://www.drive5.com/usearch/>). The quality-checked bacterial sequences were clustered into operational taxonomic units (OTUs) at a 3% dissimilarity threshold. The most abundant sequence in each OTU was chosen as the representative sequence. The representative sequences were used for taxonomic identification. To identify the specific bacteria in plants with different growth statuses, LEfSe analysis was performed using the Galaxy platform (<http://huttenhower.sph.harvard.edu/galaxy/>) [16].

Alpha and beta diversity parameters

Alpha diversity values were estimated by calculating four indices (ACE, Chao1, Shannon's Index and Simpson's Index of Diversity). Additionally, rarefaction curves and rank abundance curves were constructed to

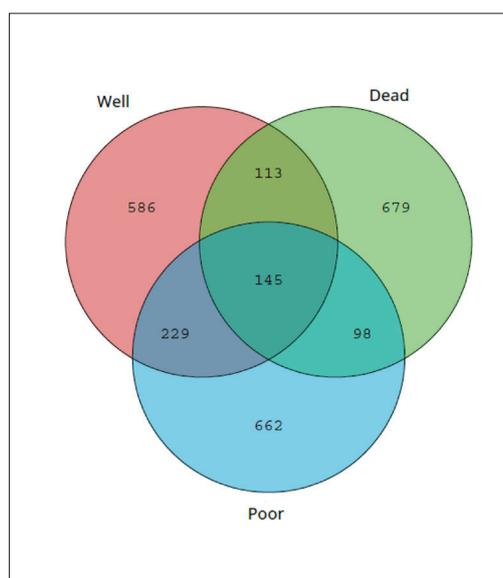


Fig. 1. Venn diagram illustrating the distributions of OTUs in three groups.

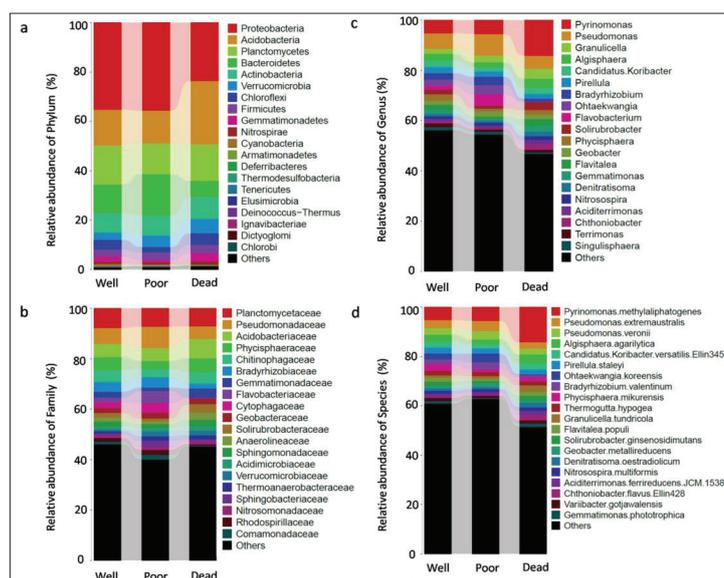


Fig. 2. The relative abundance at phylum, family, genus and species levels.

evaluate the microbial species richness and evenness using QIIME and R software [17]. To calculate beta diversity, principal component analysis (PCA), multidimensional scaling (MDS) and clustering analysis were calculated using QIIME software and R software. The PCA was performed on the community composition structure at genus level [18]. The MDS analysis was constructed by the principal coordinates analysis (PCoA) method [18]. A clustering assay was constructed by the hierarchical clustering analysis method [18]. The weighted UniFrac distance matrices were clustered and visualized using QIIME software [13].

Data availability

Raw data can be found in the NCBI database under accession numbers SRR8727413-SRR8727421.

RESULTS

16S rRNA metagenomics data and taxonomic analysis

After trimming and quality filtering, 54582 classifiable sequence reads were obtained from 9 samples. The mean number of classifiable sequences per sample was 6065 (dominant length: 1416–1602bp) (Supplementary

Fig. S1). These sequences were clustered into 2512 OTUs using a 3% dissimilarity threshold. A total of 145 OTUs were common for all groups, while 586, 662 and 679 OTUs were only detected in the well-growing plant group, the poor-growing plant group and the dead plant group, respectively. Total OTU numbers detected in the well-growing, poor-growing and dead plant groups were 1073, 1134 and 1035, respectively (Fig. 1). The representative sequence of each OTU was identified at different classification levels.

Microbial communities varied with plant growth status. Twenty-three phyla were detected in all of the three sample groups (Fig. 2a). Proteobacteria was the most abundant, followed by Acidobacteria, Planctomycetes, Bacteroidetes and Actinobacteria. The relative abundances of these 5 phyla in the rhizosphere of well-growing plants and poor-growing plants were 85.35% and 86.19%, respectively, and significantly higher than in the rhizosphere of dead plants (79.39%, $p < 0.05$). Fusobacteria was the least abundant (0.03%) phylum in the rhizosphere of well-growing plants. Deferribacteres and Chlorobi, both accounting for 0.03%, were the least present in the poor-growing plants' microbial community, while Cyanobacteria and Deinococcus-Thermus, both accounting for 0.04%, were the least abundant phyla in the rhizosphere of dead plants. At the family level, the relative abundance

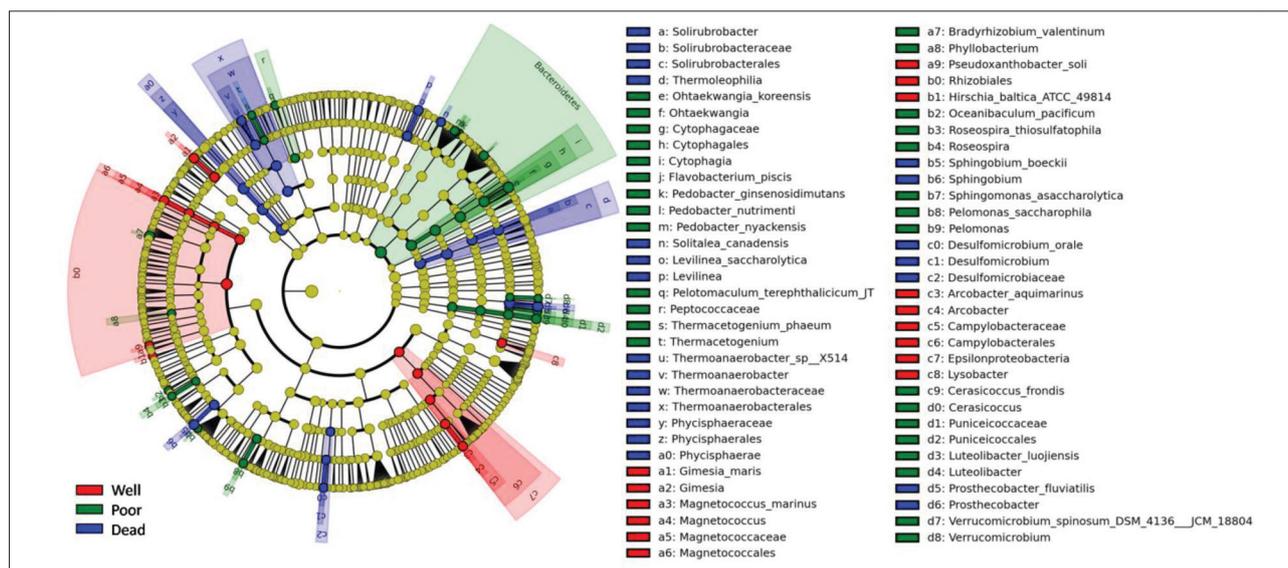


Fig. 3. LefSe comparison of bacterial taxa from the rhizosphere of three groups. The node size corresponds to the average relative abundance of the taxon. The yellow nodes represent taxa with no significant difference. Taxa with the highest relative abundance in the rhizospheres of well-growing, poor-growing and dead plant groups are shown in red, green, and blue, respectively ($P < 0.05$).

of Planctomycetaceae, Pseudomonadaceae, Acidobacteriaceae and unidentified family 1, was more than 5% (Fig. 2b, Supplementary Table S1). The relative abundance of unidentified family 1 was 16.60% in the rhizosphere of dead plants, which was significantly higher than observed in living plants ($p < 0.05$, Table S1). *Pseudomonas* and *Pyrinomonas* were the most abundant genera (Fig. 2c). *Pyrinomonas methylaliphatogenes* was the most abundant species in all samples, accounting for 5.1%, 5.5% and 14.2% in well-growing, poor-growing and dead plant groups, respectively (Fig. 2d). The relative abundance was more than 2% for *Pseudomonas extremaustralis*, *Pseudomonas veronii*, *Candidatus Koribacter versatilis* Ellin345, *Pirellula staleyi* and *Algisphaera agarilytica*. The number of species with an abundance above 1% was 25 in the rhizosphere of well-growing plants, while the numbers for poor-growing plants and dead plants were 18 and 16, respectively (Supplementary Table S2). A total of 617 species were identified and 3 species were unidentified in all of the 3 groups. The 3 unidentified species were only detected in well-growing and poor-growing plant groups (Supplementary Table S1).

LefSe was performed to analyze the statistical differences of the various taxa among samples (Fig. 3). According to LefSe analysis, the class Phycisphaerae was significantly enriched in the rhizosphere of dead plants

($P < 0.05$), while *Cytophagia* was significantly enriched in the poor-growing plant group ($P < 0.05$). At the genus level, in the dead plant group, *Sphingobium*, *Levilinea*, *Thermoanaerobacter*, *Solirubrobacter*, *Desulfomicrobium* and *Prosthecothecobacter* were significantly enriched ($P < 0.05$), while *Lysobacter*, *Gimesia*, *Magnetococcus* and *Arcobacter* were significantly enriched in the well-growing plant group ($P < 0.05$). *Phyllobacterium*, *Roseospira*, *Ohtaekwangia*, *Thermacetogenium*, *Pelomonas*, *Cerasicoccus*, *Luteolibacter* and *Verrucomicrobium* were significantly enriched in the poor-growing plant group ($P < 0.05$). At the species level, *Desulfomicrobium orale*, *Prosthecothecobacter fluviatilis*, *Levilinea saccharolytica*, *Sphingobium boeckii* and *Solitalea canadensis* were significantly more enriched in the dead plant group than in other groups ($P < 0.05$), whereas *Hirschia baltica*, *Arcobacter aquimarinus*, *Gimesia maris*, *Magnetococcus marinus* and *Pseudoxanthobacter soli* were most abundant in the rhizosphere of well-growing plants ($P < 0.05$). *Flavobacterium piscis*, *Oceanibaculum pacificum*, *Sphingomonas asaccharolytica*, *Pelomonas saccharophila*, *Cerasicoccus frondis*, *Luteolibacter luojiensis*, *Verrucomicrobium spinosum*, *Pedobacter nutrimenti*, *Pedobacter ginsenosidimitans*, *Pedobacter nyackensis*, *Ohtaekwangia koreensis*, *Pelotomaculum terephthalicum*, *Thermacetogenium phaeum*, *Bradyrhizobium valentinum* and *Roseospira thiosulfatophila* were significantly enriched in the poor-growing plant group ($P < 0.05$).

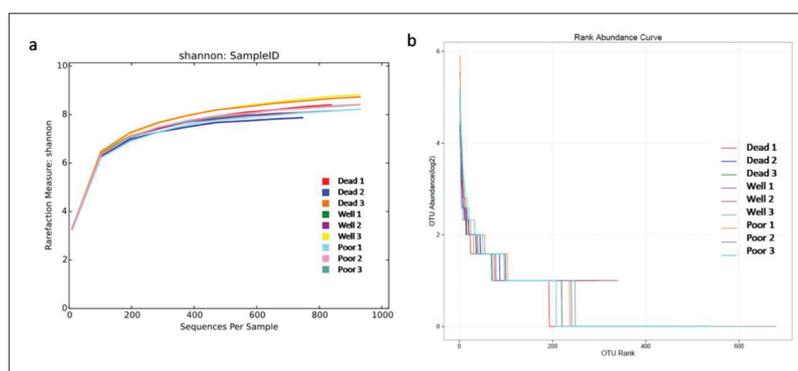


Fig. 4. Rarefaction curves (a) and rank abundance curves (b) of OTUs.

Table 1. Richness and evenness of the bacterial community in the rhizosphere of *P. jishanensis*.

Samples	Chao1	ACE	Simpson's Index of Diversity	Shannon's Index
Well-growing plants	611.5±505.2	660.3±589.8	0.997±0	8.40±0.5
Poor-growing plants	811.6±191.3	872.9±224.0	0.990±0	8.41±0.1
Dead plants	697.0±350.5	774.1±417.4	0.993±0	8.36±0.4

*The difference among all samples was not significant ($P>0.05$).

To evaluate the co-occurrence or co-exclusion interaction among the 50 most abundant species, Spearman's rank correlation coefficients were calculated. Dominant species with $P>0.6$ and $P<0.01$ were selected to build the association network. The results were visualized by Cytoscape (<http://www.cytoscape.org/>) software (Supplementary Fig. S2).

Alpha and beta diversity of the bacterial community

Alpha diversity analysis reflects the richness and evenness of species in a single sample. According to the rarefaction curves, species saturation was achieved and the sampling size was sufficient to estimate the bacterial diversity (Fig. 4a). Rank abundance curves revealed that the abundance of the OTUs differed greatly and had a low homogeneity in each sample (Fig. 4b).

To measure the alpha diversity, four indices were calculated: ACE, Chao1, Shannon's and Simpson's indices of diversity. The results showed that bacterial communities were rich and homogeneous in diversity for each sample. Also, the species richness and evenness do not significantly differ between the 3 plant groups (Table 1). This indicated that the growth status had no influence on the bacterial richness and evenness in the rhizosphere of *P. jishanensis*.

According to PCA, MDS and clustering analysis, the microbial community structure differed in each group. The difference was smaller between well-growing plants and poor-growing plants (Fig. 5a-c). To verify the significance of the difference, the weighted UniFrac distance between and within the groups was assayed, and showed that the difference between living plants and dead plants was significant, whereas the difference between well-growing plants and poor-growing plants was not significant (Fig. 5d). These results indicated that the bacterial community in the rhizosphere of living *P. jishan-*

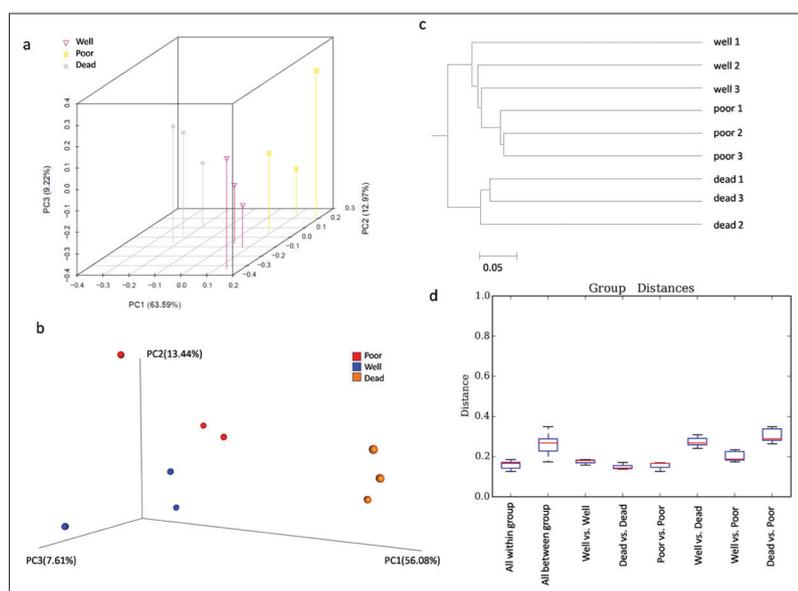


Fig. 5. Beta diversity assay. **a** – Three-dimensional PCA. **b** – PCoA based on the weighted UniFrac distance. **c** – Clustering assay based on the weighted UniFrac distance matrix. **d** – Box plots based on the weighted UniFrac distance.

ensis plants was similar; however, with the death of plants, the bacterial community changed considerably.

DISCUSSION

The edaphic condition is important for the distribution of local plants [19]. The interaction among plants, soils and microorganisms has been researched extensively. It has been proven that the rhizosphere microbial community differs with the plant species [20] and that environmental conditions can affect the microbial community more rapidly than the plants [21, 22]. A previous study showed that there was no significant difference in the concentrations of ten mineral elements in rhizosphere soil of *P. jishanensis* growing in different areas [23]. In the present study, we examined the bacterial communities of *P. jishanensis* with different growth status by PacBio sequencing. The results showed that the predominant phyla in the rhizosphere of *P. jishanensis* were Proteobacteria, Acidobacteria, Planctomycetes, Bacteroidetes and Actinobacteria, which was in accordance with peony cultivars (*P. suffruticosa*) [24].

The difference between the bacterial communities of well- and poor-growing plants was smaller than the difference between living and dead plants. LEfSe showed that four genera, *Lysobacter*, *Gimesia*, *Magnetococcus* and *Arcobacter*, and five species, *Hirschia baltica*, *Arcobacter aquimarinus*, *Gimesia maris*, *Magnetococcus marinus* and *Pseudoxanthobacter soli*, were significantly enriched in the rhizosphere of well-growing plants. Among these taxa, bacteria of the genus *Lysobacter* could be used to suppress disease of plants [25]. *Magnetococcus marinus* grow chemolithoautotrophically with thiosulfate or sulfide as the electron donors, and chemoorganoheterotrophically on acetate [26]. *Gimesia maris* accumulates α -glutamate, sucrose, ectoine and hydroxyectoine for osmoadaptation [27]. Accordingly, these bacterial species might enhance plant growth and tolerance to environmental threats.

Overall, this study suggests that the abundance of many beneficial rhizospheric microbes declined with the death of *P. jishanensis*. We speculate that it is helpful for the conservation of this endangered plant to inoculate with these beneficial microbes. The functions of these beneficial taxa have to be identified, and the presented hypothesis needs to be proven.

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Conflicts of interest disclosure: There is no conflict of interest.

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Supplementary Data

Available at: http://serbiosoc.org.rs/NewUploads/Uploads/Wang%20et%20al_3957_Supplementary%20Data.pdf