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The diversity and organ distribution of endophytic bacteria of sweet cherry

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ABSTRACT: Endophytic bacteria are widely distributed in plants and play an important function and potential role in promoting plant growth and acting against stresses. To examine the diversity and distribution of the endophytic bacteria in the sweet cherry of Dalian, China, the community structures in sweet cherry varieties and organs were investigated using Illumina-HiSeq sequencing. A total of 18,797,077 effective tags corresponding to 16S rRNA gene V3-V4 regions were obtained from all the samples. Consequently, 512–1200 operational taxonomic units (OTUs) per sample and 24 prokaryotic phyla in total were revealed. Among these, Proteobacteria was the dominant phylum, followed by Actinobacteria, Firmicutes, and Bacteroidetes. Based on the alpha diversity and beta diversity analyses, a marked difference in endophytic bacterial diversity was evident among different organs; particularly, the diversity was higher in root than in stem or bark. Furthermore, there was significant correlation between the endophytic bacterial community and sweet cherry varieties. Identification of organ-specific OTUs, an indicator of organ-specific endophytic bacteria, and Linear Discriminant Analysis (LEfSe) revealed that there were 46 taxa differentially distributed among the three organs analyzed. In conclusion, by revealing the endophytic bacterial distribution among sweet cherry varieties and organs, our results suggested that organ type, but not genotype, can affect the composition of the endophytic microbiota of the sweet cherry.

KEYWORDS: sweet cherry varieties, endophytes, microbial diversity, organ distribution, high-throughput sequencing

INTRODUCTION

Endophytic bacteria are non-pathogenic and occur naturally in the internal tissues of plants [1]. In fact, some endophytic bacteria not only are non-pathogenic, but also can promote plant growth, be beneficial to the plant host (by producing a range of natural products, such as antibiotic substance, chitinase, and glucanase), contribute to enhanced biodegradation of environmental soil pollutants, and increase the resistance against pathogenic infection [2]. Almost 300,000 plant species identified have at least one species of endophyte [3]. In general, exploration and application of endophytic bacteria in agriculture will increase crop productivity through alleviating biotic and abiotic stresses of plant, such as pathogen invasion, biological nitrogen fixation, phosphate solubilization, and degradation of toxic substance in environment [4]. Endophytic bacteria can inhabit the whole body of most plant species and promote growth and productivity of plants through a variety of mechanisms [5, 6]. Treatment with a mixture of several rare endophytic bacteria instead of dominant strains altered plant phenotype including leaf and root mass fraction [7]. In addition, endophytic bacteria were isolated and applied on hosts to increase resistance to root rot disease in Chinese jujube [8], and to enhance tolerance to salinity stress in peanut [9]. Hence, beneficial endophytic bacteria and their interactions with plants have recently attracted much attention. Up to now, a lot of endophytic bacteria have been investigated in various plants, including sugar beet, cotton, banana, Chinese leek, maize, and the dicotyledonous flowering Acanthaceae plants [10–13]. However, relative studies have mostly focused on rhizospheric microorganisms [14–16]; and endophytic bacteria of sweet cherry (*Prunus avium* L.), a highly popular fruit worldwide, has not been reported.

Currently, the analysis of endophytic bacterial diversity is mainly done using traditional culture methods and culture-independent methods. To date, the available information on endophytic bacterial diversities has been obtained through culture-dependent surveys. However, only a small fraction, predicted no more than 1% of the bacterial species, has been identified with such conventional cultivation methods [17]. Culture-dependent studies inevitably miss numerous rare or culture-resistant species. Moreover, the current high-throughput sequencing method can discover many unknown minor members of a microbial community. In addition, the high sensitive detection method is robust and versatile. It has been successfully used for studying microbial diversities in several environments, including phyllosphere [18, 19], carposphere [20], rhizosphere [21], and soils [22].

The sweet cherry is quite a unique fruit with high nutritional value, appealing taste, and beneficial health benefits. Hence, the fruit is highly popular among consumers in the world [23]. Sweet cherries from Dalian, Liaoning Province, China are the most favorite because of their big size, bright color, thick flesh, and rich nutrients. The two main varieties, Jiahong and Summit, in Dalian region, are well known because of their excellent quality, good taste, and proper maturity period, and lead to a very high economic income for producers. Although both cultivars are delicious, they originate from totally different parents and possess totally different colors. Summit produces red fruits, while Jiahong produces yellow fruits.

To investigate the diversities of endophytic bacteria in different variety and different organs of sweet cherry, 30 samples from root, bark, and stem of the two sweet cherry cultivars were analyzed by high-throughput sequencing. The results showed significant differences between root, bark, and stem of the two varieties, hence, suggested that endophytic bacteria displayed diversity in tissue specificity but not in variety specificity.

MATERIALS AND METHODS

Plant sample collection

A sweet cherry field in Dalian, China was located for sampling of the plant's organs root: (SG and JG), bark (SP and JP), and stem (SZ and JZ) in April 2018.The samples were derived from two sweet cherry varieties Summit (S) and Jiahong (J). Sample codes are: SG, root of Summit; SP, bark of Summit; SZ, stem of Summit; JG, root of Jiahong; JP, bark of Jiahong; JZ, stem of Jiahong. Five samples were randomly selected per organ per variety from individual plant to a total of 30 samples. The surface of plant was washed with tap water to remove any attached clay and soil particles before taking samples. The surface sterilization of the samples was performed according to the method of Shi et al [17]. The samples were stored at 4 °C in the laboratory until further analyses.

Extraction of total DNA and amplification of 16S rRNA gene sequences

Total genomic DNA was extracted from the 30 sweet cherry organ samples using the PowerSoil DNA Isolation Kit (MO BIO Laboratories). The concentrations of the DNA samples were calculated by measuring their absorbance at 260 nm (A_{260}). Protein and organic solvent contaminations were assessed by the ratios of A260/A280 and A260/A230, respectively.

The 16S rRNA V3+V4 region was amplified using the following common primers specific: 338F (5′-ACTCCTACGGGAGGCAGCA-3′) and 806R (5′-GGACTACHVGGGTWTCTAAT-3′). The amplicons were ligated to adapters containing the barcode sequences for Illumina HiSeq high-throughput sequencing. PCR was carried out according to the method described by Dong et al [24]. Finally, all the PCR products were purified and quantified using Quant-iT™ dsDNA HS Reagent and then combined. The sequencing analysis was performed on the Illumina HiSeq 2500 platform (2×250 paired ends) by Biomarker Technologies, Beijing, China.

The obtained libraries of 16S rRNA gene fragments from endophytic bacteria of sweet cherry were deposited into the NCBI SRA database with the accession number PRJNA752228.

Data processing and statistical analysis

Paired-end reads were merged by FLASH [25], and the raw tags were strictly filtered using Trimmomatic v0.33. The sequences with chimera were detected and removed using UCHIME v4.2. Subsequently, sequence analysis was performed using the QIIME (Quantitative Insights Into Microbial Ecology) software package [26]. Effective sequences with similarities $\geqslant 97\%$ were clustered into the same operational taxonomic units (OTUs) [27]. Alpha diversity metrics (within a sample), including the rarefaction, Chao 1 richness, and Shannon and Simpson diversity indices; and ACE richness estimators were calculated. For beta (between samples) diversity analysis, based on un-weighted and weighted Unifrac distances, the level of dissimilarity among the bacterial communities was determined using the Principal coordinate analysis (PCoA) to assess the bacterial compositions of the samples by QIIME. Differences among the bacterial communities of the organs or varieties were evaluated using one-way analysis of variance (ANOVA). The significance of difference was tested by p-value; $p < 0.05$ was considered statistically significant. Functional and metabolic pathway prediction was performed on existing 16S rRNA sequencing data with PICRUSt software and compared to KEGG (Kyoto Encyclopedia of Genes and Genomes) database. The abundance differences of functional genes in biological metabolic pathways were compared to obtain the function prediction information of endophytic bacterial community in different organs of sweet cherry.

RESULTS AND DISCUSSION

Diversities of the endophytic bacterial communities

A single lane of a paired-end Illumina HiSeq 2500 was used to sequence the bacterial contents of the 30 sweet cherry samples, resulting in 22,351,077 reads. 18,797,077 effective tags were obtained after quality control through removing low quality or chimeric sequences. The number of different bacterial operational taxonomic units (OTUs) detected at 97% sequence similarity was up to 1395 in all the samples, and the

Table 1 Number of OTUs and alpha diversity indices of endophytic bacteria in sweet cherry.

Sample[#]	Threshold	No. of OTUs	Alpha diversity[*]			
			Chao1	ACE	Shannon	Simpson
SG	0.03	1302[a]	1003.0028[a]	1095.6851[a]	6.9617[a]	0.9758[a]
SP	0.03	1301[a]	919.7326[c]	1033.8553[b]	6.0717[b]	0.8718[b]
SZ	0.03	1229[a]	917.7703[c]	1044.6121[b]	6.3574[b]	0.9072[b]
JG	0.03	1240[a]	965.2095[b]	944.2696[c]	6.7668[a]	0.9683[a]
JP	0.03	1238[a]	805.8647[d]	857.7423[d]	5.6942[c]	0.9083[b]
JZ	0.03	1047[b]	789.8737[d]	836.7704[e]	5.5719[c]	0.8771[b]

[*] Different letters represent significant differences at $p < 0.05$ (ANOVA).
[#] SG, root of Summit; SP, bark of Summit; SZ, stem of Summit; JG, root of Jiahong; JP, bark of Jiahong; JZ, stem of Jiahong.

contents of the samples ranged from 512 to 1200 with an average of 776 (Table 1).

The diversity indices (Shannon and Simpson) indicated a higher diversity in root than in stem or bark. Additionally, Chao 1 and ACE based on 3% genetic distance suggested that more richness and more diverse bacterial OTUs were detected in Summit than in Jiahong ($p < 0.05$). Rarefaction curves based on the OTUs at 97% similarity appeared to level off (Fig. S1), indicating that all the recovered sequences could reasonably characterize the diversities of the endophytic bacterial communities associated with the three organs (barks, stems, and roots of the two sweet cherry varieties). Alpha diversity estimation demonstrated rich endophytic bacterial diversities in all these six organs (Table 1).

Bacterial composition and community structure

At the phylum level, 24 prokaryotic phyla were detected based on the 16S rRNA gene sequences (Fig. 1a). In all the samples, the most predominant phylum was Proteobacteria, comprising approximately 49.76%–77.78% of the reads, followed by Actinobacteria (3.71%–22.62%, average 12.03%), Firmicutes (0.74%–24.02%, average 11.18%), and Bacteroidetes (4.25%–13.50%, average 8.61%). The four phyla cumulatively corresponded to > 95% of the reads in the given sample, the other phyla accounted for ∼ 5%. The endophytic bacterial community compositions of SG and JG from roots were different from other samples. Sequences assigned to Actinobacteria were more abundant in root (22.6%) than in stem (5.0%) or bark (8.6%) ($p < 0.05$).

The dominant phyla in the three organs were Rhizobiales (23.40%), Sphingomonadales (13.21%), Clostridiales (6.15%), Xanthomonadales (7.82%), and Bacteroidales (5.57%), at the order level (Fig. S2). Rhizobiales and Sphingomonadales were more abundant in SP, SZ, JP, and JZ samples (40%, 36.94%, 53.48%, and 54.95% total proportion, respectively) than in SG or JG (21.79% and 29.84%, respectively). Xanthomonadales were more abundant in root (SG and JG) than in other four samples (SP, SZ, JP, and JZ). These results indicated a highly diverse endophytic bacterial community in sweet cherry.

The detected OTUs in six samples were distributed among 17 different bacterial genera and unclassified genera (Fig. 1b). Each of the genera was represented by more than one percent of the total OTUs. *Sphingomonas* and *Methylobacterium* were the most predominant bacterial genera in all the samples, altogether corresponding to 2.9%–30.2% of the microbial community of each sample. The other dominant genera were *Streptomyces* (average 2.9%), *Steroidobacter* (average 2.3%), *Bacteroides* (average 1.8%), and *Lactobacillus* (average 1.7%). However, *Sphingomonas* were more abundant in JZ (20.12%) than in other samples. *Methylobacteria* in SG (0.0056%) and JG (0.013%) were much rarer than in other samples. The abundance of *Steroidobacter* (6.2%) was significantly higher in the root than in the bark and the stem ($p < 0.05$). The hierarchical heatmap constructed at the genus level showed that the samples diverged into two clusters (Fig. S3). Samples JG and SG from root clustered together, which indicated a similar community structure between the two sweet cherry varieties' roots. Endophytic bacterial communities of other samples (from stem and bark) were clustered together. However, this group was then divided into two sub-branches seemingly according to the sweet cherry variety.

High-throughput sequencing of 16S rRNA genes has been successfully applied in the analysis of bacterial communities associated with the Chinese leek [28], soil [29, 30], animal gut [24, 31, 32], and water [33]. Here, we analyzed the endophytic bacterial communities in stems, barks, and roots of two sweet cherry varieties in Dalian, Liaoning province, China.

Based on Illumina sequencing of the V3-V4 regions of bacterial 16S rRNA genes and metagenomic library analysis, we found that different organs of sweet cherry were associated with different endophytic bacterial communities in terms of diversity and composition. Additionally, the endophytic bacterial structures of stem and bark samples were similar. To our knowledge, this is the first report about endophytic bacterial diversity of the sweet cherry using the PCR-based Illumina sequencing technology.

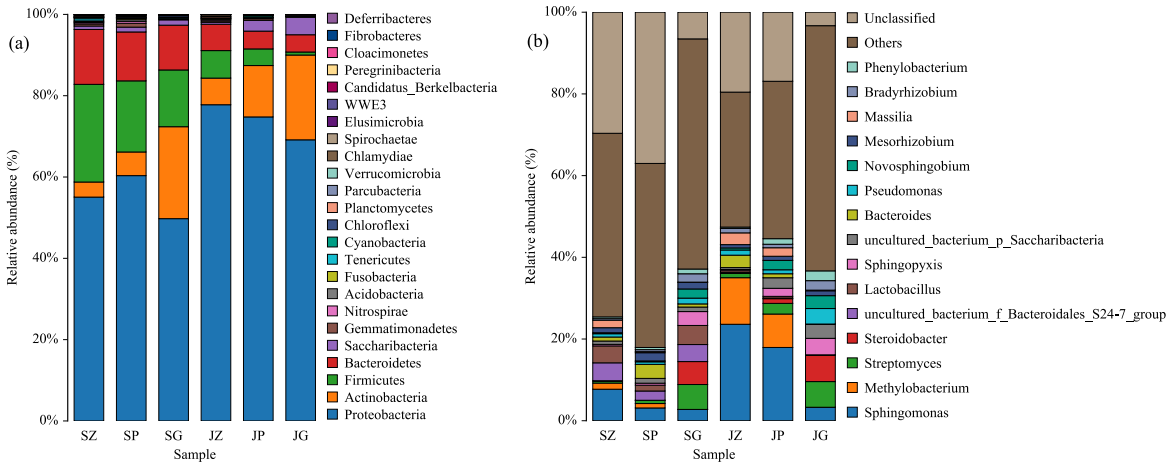

Fig. 1 Bacterial community composition at the phylum level, (a) and genus level, (b). Sequences that could not be classified into any known group were labeled 'Unclassified'. Less than 1% abundance of the phyla or genus was merged into 'Others'.

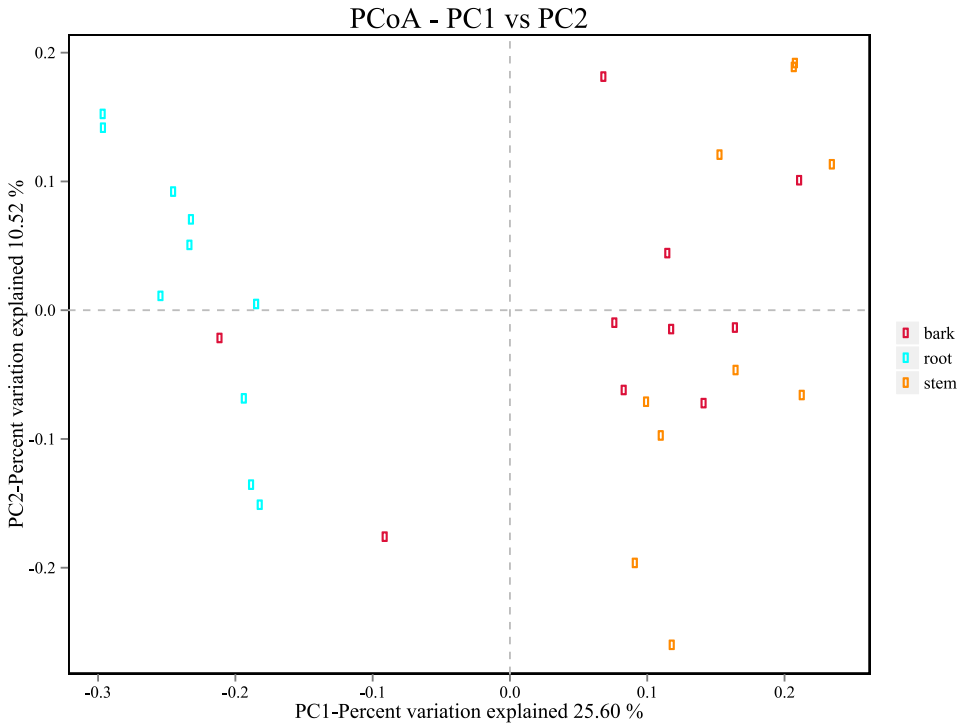

Fig. 2 Principal coordinates analysis (PCoA) of bacterial community structures using the un-weighted Unifrac distance matrix calculated according to the bacterial diversity.

Distribution of endophytic bacteria among sweet cherry organs

As shown in the Venn diagram (Fig. S4), OTUs were differentially distributed among the sweet cherry organs. The highest number of OTUs for a given organ was in the bark (1362 OTUs), followed by the root (1348 OTUs) and the stem (1300 OTUs). Among these, 1238 OTUs were found to be common to all the three organs; whereas 2, 4, and 12 OTUs were exclusive to the bark, root, and stem, respectively.

The relationships among the endophytic bacterial community structures of the sweet cherry samples were examined using the Principal Coordinate Analysis (PCoA) (Fig. 2). PCoA using the un-weighted Unifrac

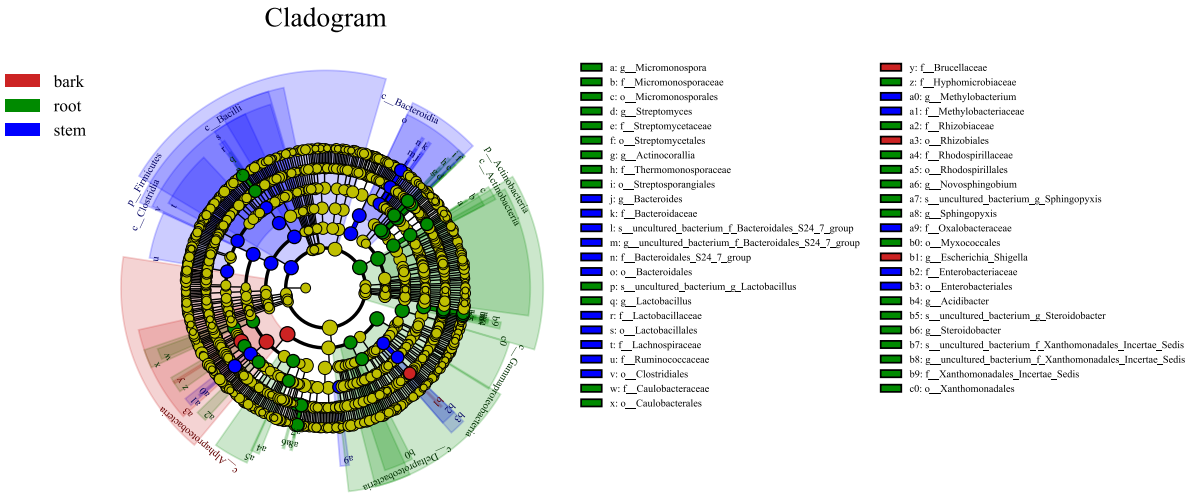

Fig. 3 Bacterial taxa significantly differentiated in different sweet cherry organs analyzed by linear discriminant analysis (LDA) effect size using the default parameters.

distances revealed clear differences among the bacterial communities of different organs. The first and the second axes showed that the cumulative percentage of the variance of species equaled to 25.60% and 10.52%, respectively, totaling 36.12%. The 10 samples from roots were clustered into one group, while the 20 samples from stems and barks were clustered together into another group. The PCoA analysis revealed that the bacterial communities of the barks and the stems were similar to each other but differed from the roots.

Furthermore, the species differentially present in each organ of the sweet cherry were analyzed using LEfSe (Linear discriminant analysis [LDA] effect size) (Fig. 3). The results suggested that 46 taxa (4 phyla, 7 classes, 12 orders, 16 families, 14 genera, and 4 species) differentially existed in the root, the bark and the stem (LDA scores = 4.0). Found significantly predominant in roots were the phyla Actinobacteria, Deltaproteobacteria, Gammaproteobacteria and several Alphaproteobacteria-associated taxa including the genera *Micromonspora* and *Streptomyces* and the orders Xanthomonadales and Myxococcales. In contrary, among the endophytic bacterial community of stems, Firmicutes such as Bacilli, Clostridia, Ruminococcaceae, and Lactobacillales were enriched at various levels. In addition, Bacteroidales (Bacterodida) and several taxa of Gammaproteobacteria were enriched in stems, while Alphaproteobacteria, including Brucellaceae and Rhizobiales, were the most predominant phyla in the barks.

To investigate the ecological implications of the endophytic bacteria associated with each sweet cherry organ, differences and changes in metabolic pathways of functional genes of microbial communities between different groups of all the samples were predicted using the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways. Among the three organs, the following 10 pathways were enriched in the root samples: cellular community, immune diseases, lipid metabolism, signaling molecules and interaction, sensory system, metabolism of terpenoids and polyketides, transport and catabolism, endocrine system, excretory system, and xenobiotics biodegradation and metabolism ($p < 0.05$) (Fig. S5). Cell growth and cell death, digestive system, bacterial infectious diseases, membrane transport, and metabolism of cofactors and vitamins were enriched in the stem samples ($p < 0.05$) (Fig. S5). Similarly, the different metabolic potentials between the root and the bark samples were explored. Seven pathways (endocrine system, cellular community, signaling molecules and interaction, sensory system, transport and catabolism, immune diseases, and excretory system, $p < 0.05$) were more enriched in the root samples, and only two pathways (metabolism of cofactors and vitamins, and membrane transport; $p < 0.05$) were more enriched in the bark samples (Fig. S5).

The colonization of endophytic bacteria is regulated by the host plant, and the accumulated nutrients are different with different organs structures. The root system is the entrance for endophytic bacteria into the plant because there are more secondary root, mechanical damage and wounds caused by pests and diseases, so more endophytic bacteria gathered.

Shi et al [16] have reported that the sugar beet has diverse endophytic bacteria at various stages of growth. The highest number of OTUs detected corresponds to the period of tuber growth and rosette formation. However, the endophytic bacterial diversity is reduced during seedling growth and sucrose accumulation. Li et al [34] reported that the diversity of peanut was highest in roots, followed by stem and leaf,

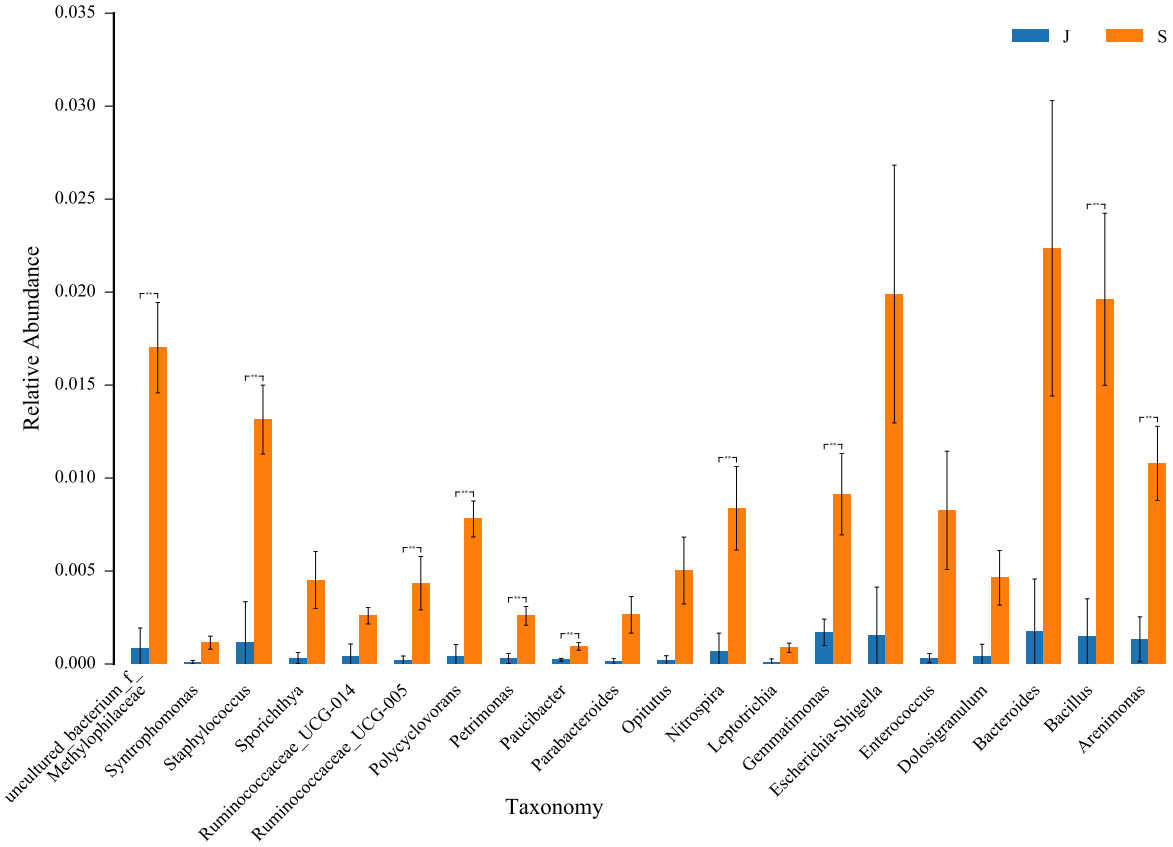

Fig. 4 Bacterial community composition in the two sweet cherry varieties at the genus level. Sequences that could not be classified into any known group were labeled 'Unclassified'. J represents the Jiahong variety, and S represents the Summit variety.

and the lowest was in flower. And the predominant phyla were Proteobacteria, Firmicutes, Actinobacteria, and Bacteroidetes. These results were similar to this paper's.

Endophytic bacterial community structures in two different sweet cherry varieties

To compare the bacterial communities associated with sweet cherry varieties, OTUs were examined. The number of OTUs detected in Summit and Jiahong sweet cherry samples based on the Veen diagram was 1395 in total (Fig. S6). The higher number of OTUs was detected in Summit. The number of unique OTUs in Summit and Jiahong was 58 and 9, respectively. Then, we compared the variation in bacterial species composition between the two varieties. Pyrosequencing results revealed that there were differences in endophytic bacterial community structure between Summit and Jiahong (Fig. 4). The *Polycyclovorans*, *Petrimonas*, *Arenimonas*, *Staphylococcus*, *Bacillus*, *Gemmatimonas*, *Nitrospira*, and *Paucibacter* were more abundant at the genus level in the Summit than the Jiahong. The difference between the two was statistically significant ($p < 0.05$).

The endophytic bacterial community structure can vary with host characteristics, including the elevation level of the habitat, associated vegetation, organ types [35–37], and organ age [38, 39]. Additionally, the growth stage may also lead to differences in endophytic bacterial communities, further affecting the distribution of endophytes. Interestingly, LEfSe analysis revealed that the bacterial communities were significantly different among roots, stems, and barks of the sweet cherry, and 46 taxa were differentially represented in the three organs. In addition, the parameters describing microbial richness in alpha diversity analysis indicated that both diversity and richness of endophytic bacteria in the roots were higher than in the stems and the barks. The observational presumably results from roots acting as a bridge that connects the host plant with the surrounding soil environment. Our results suggest that active endophytic bacteria have close relationships with sweet cherry organs during plant growth, and organ environment influences the endophytic bacterial community and distribution in sweet cherry. Interestingly, although

Summit and Jiahong grew up under almost uniform conditions including geographic locations, ecological environment, as well as irrigation conditions, the two varieties displayed obvious differences in dominant classes, richness, and bacterial OTU diversities of endophytic bacteria.

There was a high diversity of endophytic bacteria in the sweet cherry of DaLian, China. The distribution of endophytic bacteria showed clear differences among different organs and varieties, indicating that organs and host genotype affect the endophytic bacterial community structure. They probably occurred based on inherent differences of the cultivars. The type of host plant has a cumulative effect on the community structure and diversity of endophytes. Each cultivar develops their set of bacterial community related to other cultivars but uniquely distinct to one another. So, it is possible that the genotype, the phylogenetic relatedness, or other attributing factors are the effector shaping the endophytic bacterial communities of the sweet cherry. This finding suggests that a more in-depth study to understand the functional significance of endophytic bacterial diversity of the sweet cherry should be undertaken. The results of this study contribute to the isolation and characterization of sweet cherry-associated endophytic bacteria and provide a theoretical basis for the development and utilization of endophytic bacterial communities in sweet cherry farming.

Appendix A. Supplementary data

Supplementary data associated with this article can be found at http://dx.doi.org/10.2306/scienceasia1513-1874.2022.125.

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Appendix A. Supplementary data

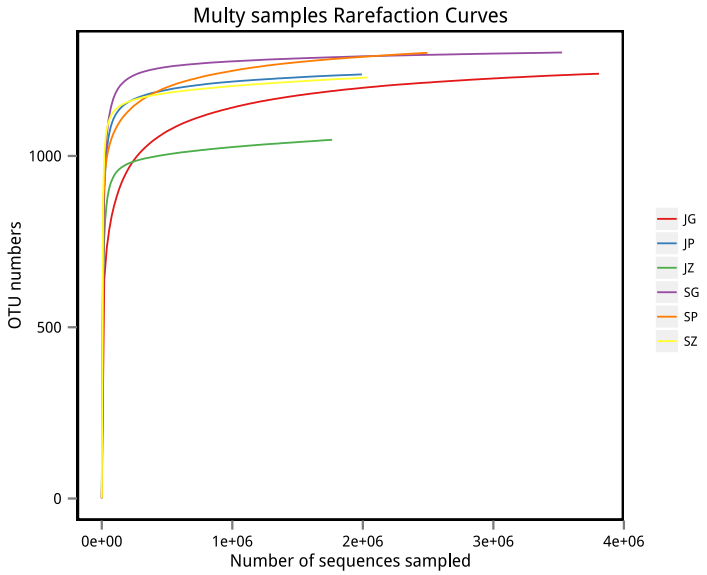

Fig. S1 Rarefaction curves depicting the effect of 3% dissimilarity on the number of OTUs identified.

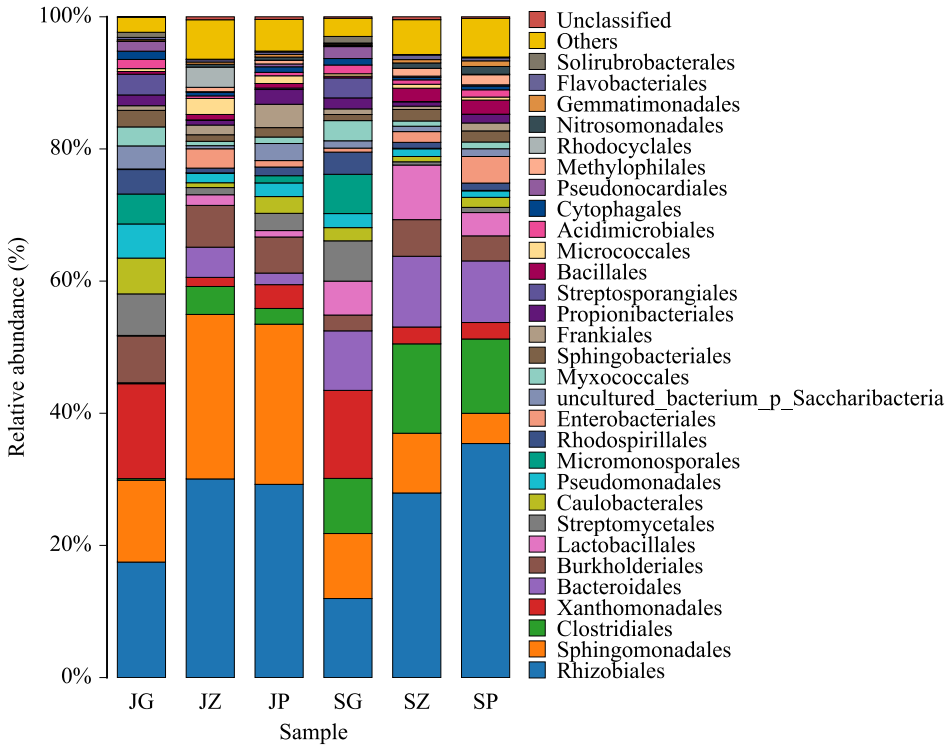

Fig. S2 Bacterial community composition at order level. Sequences that could not be classified into any known group were labeled 'Unclassified'. Less than 1% abundance of the phyla or genus was merged into 'Others'.

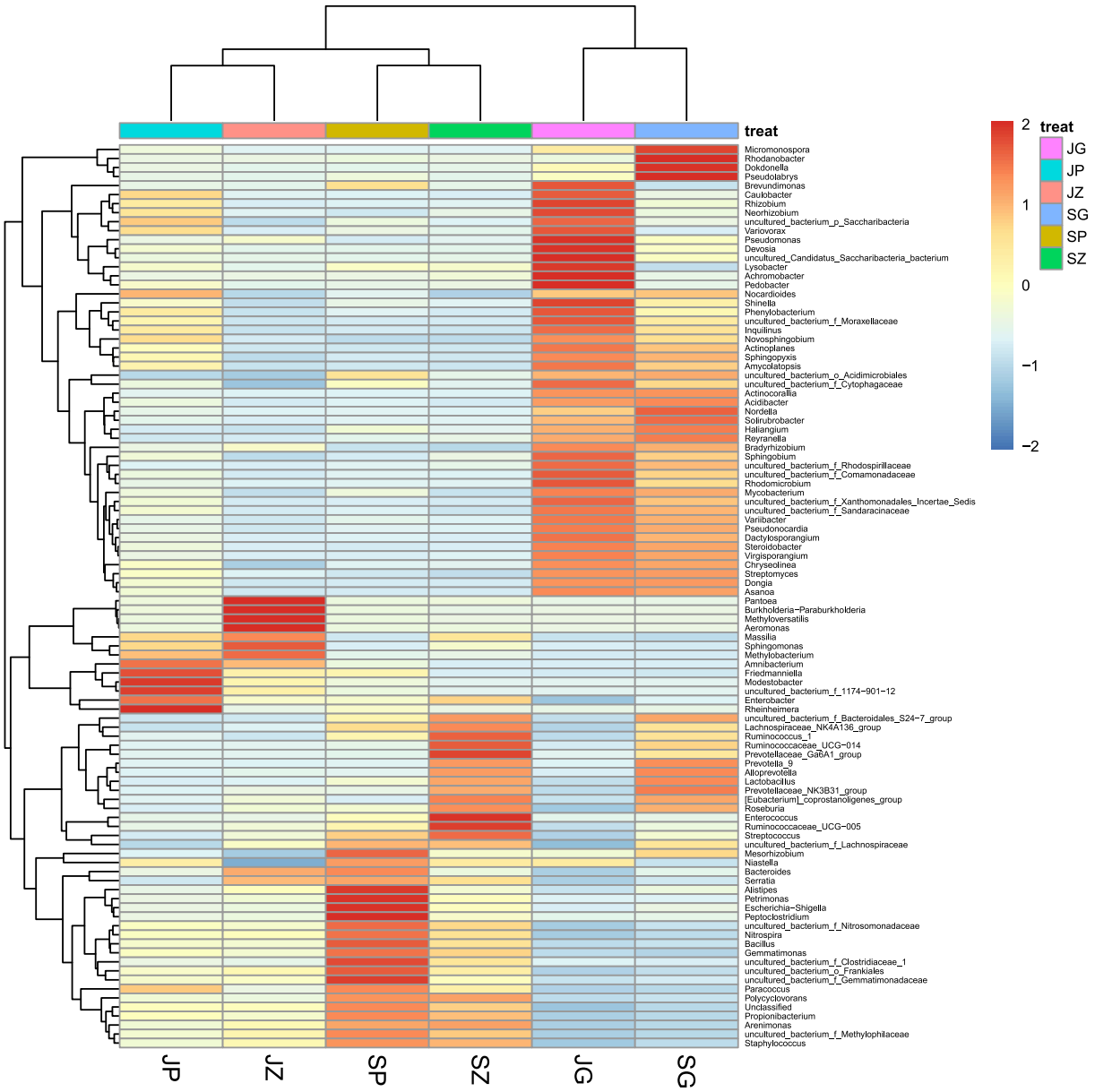

Fig. S3 Bacterial community heatmap analysis among the 6 samples at the genus level. Double hierarchical dendrogram shows the bacterial distribution. The bacterial phylogenetic tree was calculated using the neighbor-joining method and the relationship among samples was determined by Bray-Curtis distance and the complete clustering method. The heatmap plot depicts the relative percentage of each bacterial genus (variables clustering on the vertical-axis) within each sample (horizon-axis clustering).The relative values for bacterial genus are indicated by color intensity with the legend indicated at the top right corner.

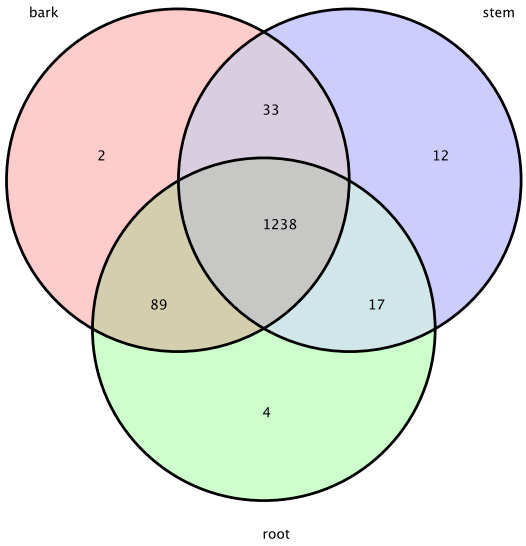

Fig. S4 Venn diagram describing the OTU distribution of the three organs samples.

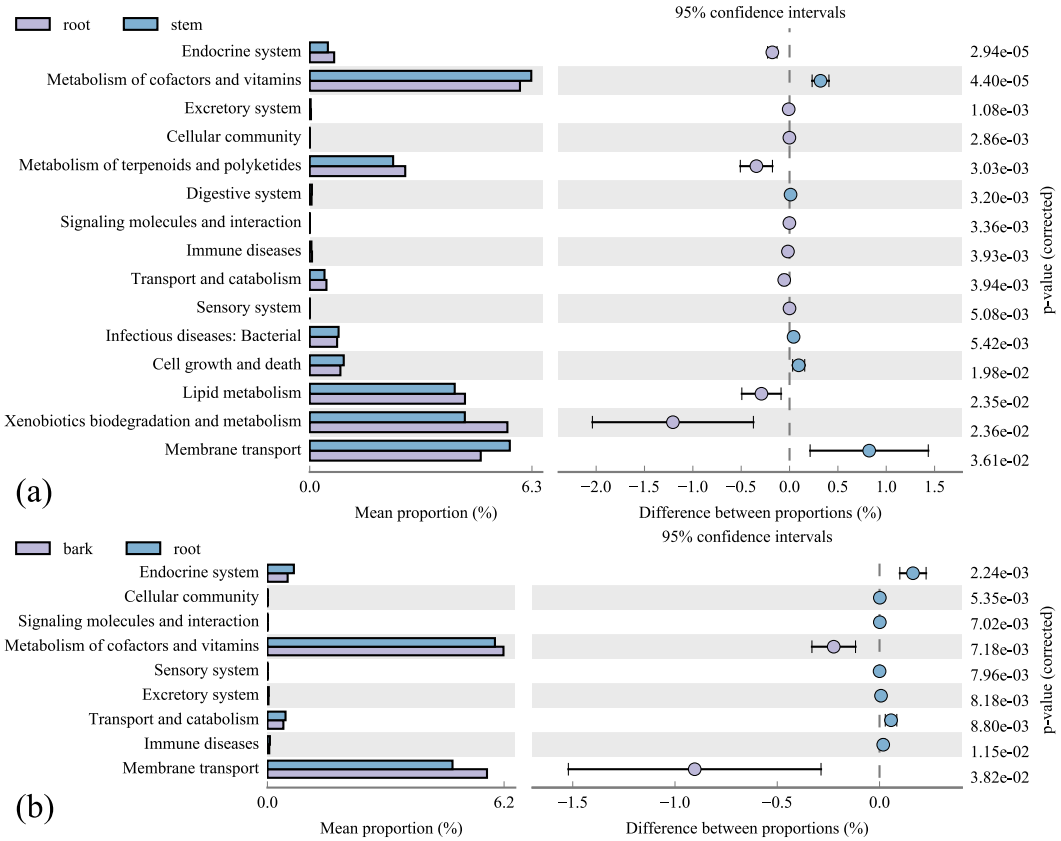

Fig. S5 Predicted function of endophytic microbiota between the root and stem (a), root and bark (b) from KEGG pathway.

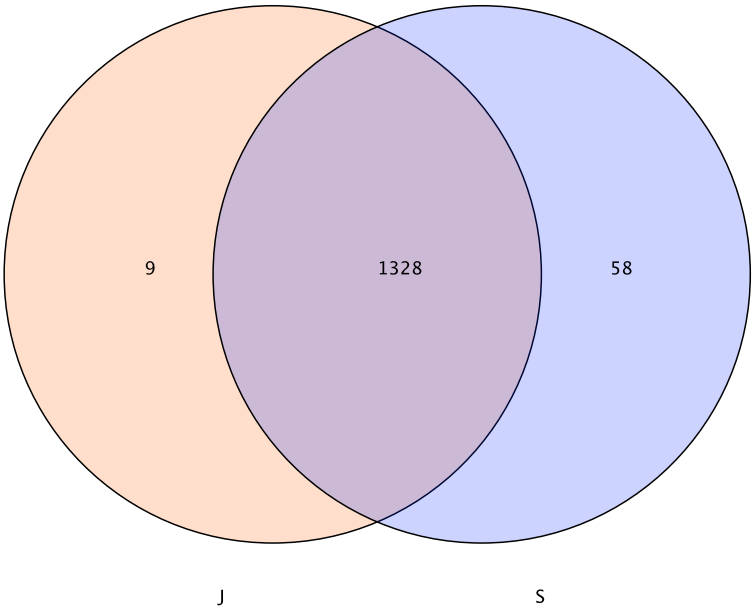

Fig. S6 Venn diagram describing the OTU distribution of the two cherry varieties.