Diversity Analysis of Endophytic Bacterial Community in Two Citrus Cultivars With Different Resistance to Citrus Canker

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Abstract

The selective infection of *Xanthomonas citri* pv. *citri* to citrus cultivars is universally known, but it is not clarified whether there is a relationship between endophytic bacteria and the resistance of host variety to canker disease. In order to explore the relationship, Satsuma mandarin and Newhall navel orange were collected respectively as samples of resistant or susceptible cultivars to citrus canker disease, and endophytic bacterial community of two citrus cultivars were analyzed by using a next-generation, Illumina-based sequencing approach. Simultaneously, the seasonal dynamics of endophytic bacterial community and dominant genera were analyzed. The results showed that there were four dominant groups including *Proteobacteria*, *Firmicutes*, *Actinobacteria* and *Bacteroidetes* in all samples at phylum level. Endophytic bacteria were the most abundant in spring samples, then in summer and autumn samples. There were some differences between endophytic bacterial community of resistant citrus and that of susceptible citrus to canker disease, and the endophytic bacteria of Satsuma mandarin are more abundant than that of Newhall navel orange. According to the analysis of dominant bacteria in two citrus cultivars, it was found that some endophytic bacteria with antagonistic characteristics existed universally in all samples, although the dominant bacteria in different seasonal sample were different. However, in Newhall navel orange of susceptible citrus to canker disease, there were not only some bacteria against *Xanthomonas citri* pv. *citri,* but also some cooperative bacteria of canker occurrence like *Stenotrophomonas*.

Introduction

Plant endophytic bacteria are a class of microorganisms that live within the tissues and organs of plants which causing no symptoms. Both endophytic bacteria and host plants are adapted to each other and interact with each other to form a complex symbiotic relationship. The genetic background and genes of the plant have a significant effect on the species, quantity and activity of the bacteria (Hamilton et al., 2010; Gundel et al., 2012). Plant endophytic bacteria also have a degree of feedback on the growth of the host (Nair and Padmavathy, 2014). Recent years, the endophytic bacteria have being payed close attention in enhancing the tolerance of host plants to stressful environments, promoting plant growth and improving plant protection (Bulgarelli et al., 2014; Ying et al., 2006). However, the above considerable knowledge especially about inhibiting the growth of plant pathogens and resisting the disease, was mostly confined to culturable endophytes (Yuan et al., 2005; Liu et al., 2011; Zhao et al., 2016; Mnasri et al., 2017; Akbaba and Ozaktan, 2018; Yousefi et al., 2018). In fact, the most bacteria in plants which cannot be cultured artificially on media possess a vital influence on the growth of host including the resistance to diseases (Sun and Song, 2006). Therefore, it is necessary that the diversity of endophytic bacteria community is explored in order to deep understand the interaction between endophytes and hosts.

Citrus bacterial canker disease caused by *Xanthomonas citri* pv. *citri* (*Xcc.*) is an important quarantine disease in China. The disease caused an enormous economic losses and increased the trend of spread in the domestic and foreign navel orange cultivated areas (Furman et al., 2013), and has the capacity to

demolish citrus industries because of handicap of prevention and control (Gottwald 2007), so is called “citrus cancer” (Das 2003). There is a selective infection of *Xcc* to citrus cultivars, that is, citrus canker does not occur in some cultivars such as Nanfeng tangerine, Satsuma mandarin, Kumquat citrus and so on, but is serious in the other citrus cultivars of sweet orange. It had been proved that the resistance of citrus cultivars to canker disease were connected with many factors including citrus epidermal tissue especially stomatal frequency (Wang et al., 2011; Li et al., 2013), physiological components and content (Gogo et al., 1979), oxidase (Wang et al., 2011), phytoalexin (Boddu et al., 2004), resistance genes (Shiotani et al., 2008) as well as possible contribution of culturable endophytic bacteria from citrus (Liu et al., 2013). But for the relationship between the endophytic bacterial community from citrus and the resistance of their host cultivars to canker disease, few literature has studied at present.

In this study, we collected different citrus samples which belong to two cultivars i.e. Newhall navel orange and Satsuma mandarin with the characteristic of resistant or susceptible to canker disease respectively in different time. We made the following hypotheses: (i) the resistant activity of hosts to canker disease determine the diversity and function of endophytic bacteria communities associated with citrus cultivars; (ii) the predominant endophytic bacteria communities from different citrus cultivars with inconsistent resistance to canker disease have distinctive operational taxonomic units (OTUs); and (iii) endophytic bacteria have the potential to be beneficial to host plants to resist the canker disease. To test these hypotheses, we examined endophytic bacteria communities in the leaves and fruits of *Citrus sinensis* Osbeck ‘Newhall’(susceptibility to canker) and *Citrus unishiu* Marc. (resistance to canker) in spring, summer and autumn using high-throughput sequencing of the 16S rDNA V3-V4 region.

1. Materials And Methods

1.1 Samples collection

The endophytic bacterial communities in Satsuma mandarin (*Citrus unshiu*) which resistance to canker, and Newhall navel orange (*Citrus sinensis*) which susceptibility to canker were analyzed in this study. The young fruits and tender leaves without disease symptoms of the two citrus cultivars were randomly collected from the citrus orchard in Jiangxi Agricultural University, Jiangxi province of China. The tested samples comprise of young fruits of the Newhall navel orange and Satsuma mandarin (recorded as CSN.F and CU.F), and tender leaves of two citrus (recorded as CSN.SP, CSN.SU, CSN.AU and CU.SP, CU.SU, CU.AU) in spring, summer and autumn respectively. Each sample was pooled with six leaves or fruits from six independent plants, and stored at 4 ℃ in sterile plastic bags for further analysis.

1.2 Samples preparation and DNA extraction

To ensure that the bacterial communities were endophytes, before DNA extraction, the samples were surface-sterilized using 70% alcohol for 1 min and 1% sodium hypochlorite solution for 1 to 5 min. Samples were then washed 4 times with sterilized water, and dried on sterile paper. About 0.1 mL of the final eluate was collected to check for bacterial contamination.

Microbial DNA was extracted from plant tissues by using the previously reported methods (Jiao et al., 2010; Wu et al., 2018). Plant tissues were ground with liquid nitrogen and well-mixed. In order to exclude the interfere of plant chloroplast in the following extraction of the genomic DNA of bacteria, 3g ground tissue powder was suspended in 15 ml of respective enzyme solution (1.5% macerozyme R-10, 1.5% cellulase R-10, 0.12% N-morpholino ethane sulfonic acid, 0.36% $CaCl_2 \cdot 2H_2O$, 12.8% D-mannitol 0.011% NaH_2PO_4, pH=5.6), incubated at 37 ℃ with gentle agitation for 3 hours. Subsequently, the mixture was centrifuged at 200 g for 5 min and repeated three times. The supernatant was collected and centrifuged at 16500 g for 20 min. The pellet was collected. Finally, DNA was extracted using the EasyPure Bacteria Genomic DNA kit according to the manufacturer's instructions. Each sample was repeated in triplicate.

1.3 PCR amplification and next-generation sequencing

Purified DNA was used as the template for PCR amplification. The V3-V4 variable regions of 16S rRNA gene were amplified by the primer pair 341F (5'-CCTAYGGGRBGCASCAG-3') and 806R (5'-GGACTACNNGGGTATCTAAT-3') (Caporaso et al., 2011). PCR reaction were conducted with a 25 μl reaction system which consists of 2 μl diluted template DNA, 8.5 μl sterilized water, 1 μl of each primer and 12.5 μl PCR Mix (TaKaRa Corporation, Dalian, China). The PCR conditions were as follows: an initial denaturation of 5 min at 94 ℃; followed by 35 cycles of 94 ℃ for 30 s, annealing at 54 ℃ for 60 s, and extension at 72 ℃ for 2 min; then a final extension at 72 ℃ for 10 min.

The quality of the amplified PCR products was checked by electrophoresis in 2% agarose gel. Samples with a bright main strip between 400 bp-500 bp were chosen for further experiments. The PCR products were purified with a Qiagen Gel Extraction Kit (Qiagen, Germany), and then sequenced by Novogene Bioinformatics Technology Co., Ltd (Beijing, China) using the HiSeq 2500 platform (Illumina, SanDiego, CA, USA).

1.4 Sequence data treatment and Statistical analyses

Sequencing reads were assigned to each sample according to the unique barcode of each sample. Then, pairs of reads from the original DNA fragments were merged by using FLASH (Caporaso et al., 2010). The raw tags generated with FLASH were filtered with the QIIME (Quantitative Insights Into Microbial Ecology) software package (Caporaso et al., 2011) and subjected to a quality control procedure using Uparse software (Uparse v7.0.1001). The clean reads were clustered to generate operational taxonomic units (OTUs) at the 97% similarity level. We picked a representative sequence for each OTU and used the RDP (ribosomal database project) classifier to assign taxonomic data to each representative sequence (Wang et al., 2007). The MUSCLE software was used to analyze alpha- (within samples) and beta- (among samples) diversity (Edgar 2004).Heatmap analysis and rarefaction curve were conducted using R3.1.0. The histogram were created using Microsoft Excel 2010. All sequences have been deposited in the NCBI Sequence Read Archive database (accession number PRJNA515173).

2. Results

2.1 OTU number analysis

After quality filtering and chimera sequences removal, total 1197612 effective sequences of eight groups were obtained in this study. The OTUs of different samples were analyzed based on Venn diagrams. The OTUs were abundantly presented in the fruit and three seasonal leaves of Satsuma mandarin and Newhall navel orange (Fig.1, Fig.S1). The results showed that these eight samples shared 74 OTUs, indicating that they maybe the relatively stable components in endophytic bacterial communities associated with the two citrus cultivars. And the more unique OTUs were obtained in CSN.SP, CSN.SU, CU.SP and CU.SU samples, which indicated that the endophytic bacterial community was richer in leaf samples of spring and summer (Fig.S1).

Compared with the different seasonal samples, there were more unique OTUs in CSN.SP and CU.SP samples, which were 332 and 340, respectively. The OTUs of leave samples were the most in spring, followed by summer and autumn. And in the same seasonal leave samples, the total OTUs in Satsuma mandarin were more than in Newhall navel orange, except autumn leave sample (Fig.1 A and B). For fruit samples, the unique OTUs of CU.F sample was more than others samples (Fig.1C). Compared with different citrus species samples in the same season, the shared OTUs in spring leaves was 410, but only 160 in summer and autumn leaves. The unique OTUs of mandarin orange in spring and summer samples were more than that of the navel orange, whereas which was opposite in the autumn (Fig.1 D-F).

2.2 Endophytic bacterial community composition

All the obtained sequences in this study were classified from phylum to genus according to the program Mothur using the default setting. There were mainly ten phyla were identified from these samples, as showed in Fig.2 and Table S1. *Proteobacteria*, *Cyanobacteria* and *Firmicutes* were the most groups, and accounted for more than 90% of the reads at phylum level. *Actinobacteria* phylum accounted for a large proportion of the sequences in CSN.SP, CU.SP, CSN.SU, CU.SU, CSN.AU and CU.AU. In addition, *Bacteroidetes* were high in CU.SP, CSN.SU, CU.SU and CSN.AU sample, accounting for 1%-4%. We also found a similar trend of microbial growth and decline was presented in leave samples of every citrus cultivar with the seasonal variation. However, there were some differences in the contents of endophytic bacteria group. For example, the *Firmicutes* contents of the two citrus leaves were the highest in autumn and the lowest in summer, and the bacterial contents in the leaves of Satsuma mandarin were higher than that in Newhall navel orange.

The bacterial composition of the 8 samples at class, order, family level were showed in Fig.S2. At the genus level, “Others” (the sum of the relative abundance of the genus except for the top ten) occupied a large percentage. The most abundant genus was *Bacillus* in leaves and fruits samples except for CU.F and CSN.SU. *Stenotrophomonas*, *Halomonas*, *Shewanella* and *Brevundimonas* were also abundant relative to other genera in different samples (Fig.3, Table.S2). The heat map was used to analyze the variety of the abundance of most genera in these samples (Fig.4). In CSN.SP, the main genera were *Curtobacterium*, *Knoellia* and *Rhizobbradyium*. In CSN.SU sample, the predominant genera were *Propionibacterium*, *Gardnerella* and *Brevundimonas*. In CU.SP, the predominant genera included

Bifidobacterium, *Faecalibacterium*, *Blautia* and *Lactobacillus*. In CU.SU, *Brucella*, *Rhizobium* and *Pseudomonas* were abundant. In CU.AU sample, *Bacillus* was predominant genus. For fruit and CSN.AU samples, the dominated genus was not outstanding. These indicated that the bacterial community in three seasonal leaves of two citrus cultivars had obvious difference in dominant genera.

2.3 Diversity analysis of endophytic bacterial community

There were the upper and lower values in the same box, which represent the Weighted Unifrac and Unweighted Unifrac. The number in the chart was the phase coefficient between the samples, the smaller the different coefficients, the smaller the diversity of species. The coefficient of dissimilarity between the two citrus cultivars was 0.228 (0.283) in spring and 0.253 (0.636) in summer and 0.226 (0.500) in autumn, which indicated that the difference of endophytic bacterial community between the two citrus cultivars in summer was the maximal (Fig.5). The dissimilarity coefficient of two citrus indicated that the differences of endophytic bacteria in leaves of Satsuma mandarin and Newhall navel orange had changed greatly with the seasonal variation. Similarly, the 0.355 (0.598) of coefficient revealed the dissimilarity of endophytic bacterial community between two citrus fruit samples.

Discussion

At present, some researchers reported the relationship between endophytic bacteria and host resistance to disease, which were focus on culturable endophytic bacteria (Lacava et al., 2007; Flores et al., 2013). But the microorganisms identified by traditional methods of isolation and cultivation only account for 0.1%~10.0% of the total environmental organisms, which cannot truly reflect the structure of endophytic bacteria (Gangaiah et al., 2009; Magajna and Schraft, 2015). There were different bacterial communities in different citrus cultivars. Liu et al. (2013) found that the quantity and proportion of culturable endophytic bacteria in resistant citrus to canker disease were more than that in susceptible citrus. However, with regard to unculturable endophytic bacteria, few studies were reported. In this study, the diversity and relationship of bacterial community in the citrus including Satsuma mandarin (resistant to citrus canker) and Newhall navel orange (susceptible to citrus canker) were analyzed based on the high-throughput sequencing.

High-throughput sequencing technology, a milestone in the development of sequencing technology, makes it possible to get accurate analyze of the transcripts and genomes of species, and named deep sequencing or next-generation sequencing techniques (Endrullat et al., 2016; Montoya et al., 2016; Yong et al., 2017). Currently, the 16S rDNA amplicon sequencing has been widely used in microbial community analysis in different environments, involving multiple fields (Sun et al., 2014; Shen et al., 2015; Lu et al., 2016; Zhao et al., 2017). In this study, high-throughput sequencing of 16S rDNA method was used to study the differences of endophytic bacterial community in two citrus cultivars. However, in preliminary tests, because of high homology of chloroplast, mitochondria and bacteria in phylogenetic sequence, most sequences attributable to host chloroplasts and mitochondria after high-throughput sequencing of 16S rDNA V3-V4 variable region. In order to decrease the proportion of the host pollution phenomenon, we

conducted enrichment treatment of endophytic bacteria that plant cell wall were removed and more endophytic bacteria were released by using cellulase and macerozyme, followed that plant protoplasts and bacteria were separated by differential centrifugation in this experiment (Wu et al., 2018). However, the proportion of chloroplast and mitochondria in the peel of Satsuma mandarin fruit was still high, and the host pollution phenomenon was also still appeared in other samples. Therefore, it was required to explore a proper method of enriching endophytic bacteria or select more differential primers or formulate a better sequencing strategy in order to avoid the interfere of chloroplast and mitochondria in 16S rDNA amplicon based high-throughput sequencing technique.

The results showed that there were shared 74 OTUs in all samples, which indicated that same endophytic bacteria were presented in the samples. The order of endophytic bacterial abundance in leaves was spring > summer > autumn. We speculated the main reason is that spring can provide more nutrition. In summer, the growth of citrus was influenced by high temperature, which may lead to the decrease of endophytic bacterial abundance. The physiological activity of orange trees slow down in autumn and the quantity of endophytic bacteria decrease again (Liang et al., 2005; Rodríguez et al., 2017). Although the OTU number of Satsuma mandarin has a large decline as the season progresses, it is still higher than that of navel orange in spring and summer samples. However, in autumn samples the OTU number trend of two citrus cultivars was opposite. Obviously, in different cultivars, the abundant characteristic of endophytic bacteria was different. So, is there a relationship between the dynamic change of citrus endophytic bacteria and the host's resistance to canker? It remains to be further explored and may be verified by analyzing the endophytic bacteria from resistant or susceptible citrus to canker disease.

There were significant differences in endophytic bacterial community of samples, and the microorganisms in citrus leaves mainly distribute in *Proteobacteria*, *Firmicutes*, *Actinobacteria* at phylum level, which is correspond to the results of many literature reports (Gagne-Bourgue et al., 2013; Li et al., 2009). It has been reported that some species of *Firmicutes* have inhibitory effect on *Xcc* causing the citrus canker disease by producing antimicrobial peptides, bacteriolysis, niche competition, and so on (Chen et al., 2008; Huang et al., 2012; Chen et al., 2014; Liu et al., 2015). In this experiment, the bacterial content of *Firmicutes* was significantly higher in Satsuma mandarin leaves than that of Newhall navel orange during the growth of citrus, which is in accord with the viewpoint of Liu et al. (2013). The content of the *Firmicutes* bacteria in summer sample of all samples was the least, and the number of bacteria in Satsuma mandarin decreased more. We speculated that summer is the most serious occurrence period of citrus canker disease, and in order to resist the invasion of pathogenic bacteria, the sacrifice of the *Firmicutes* bacteria in competing position and interacting with pathogenic bacteria resulted in a sharp reduction in their population. Therefore, we thought that the content difference and the variation trend of endophytic bacteria attributed to *Firmicutes* may reflect the difference of resistance or sensitivity of citrus cultivars to canker disease. And the dynamic change of endophytic bacterial community may indicate the course of resisting to canker disease in citrus, which indicated that the difference of endophytic bacterial community in citrus was related to the ability of host resistance or susceptibility to canker disease.

At genus level, endophytic bacteria from oranges detected were mainly distributed in *Bacillus* and *pseudomonas* in this study. It is reported that *Bacillus* has strong resistance to other harmful factors, and is the main functional group for improving host disease resistance and is able to produced active substances such as subtilisin, polymyxin, nystatin, gramicidin that have a significant inhibitory effect on the pathogens (Rakotoniriana et al., 2013; Ji et al., 2015; Zhang et al., 2016). Our study showed that the content of *Bacillus* in two citrus leaves fluctuated with the seasonal change manifested as decreasing in summer and increasing in autumn, but the microorganism quantity in Satsuma mandarin was higher than in navel orange (Table S2, Fig.S2). Especially, *Bacillus* was only relatively abundant in the autumn leaves of Satsuma mandarin, and perhaps the presence of the bacterium was one of the factors that interfered the pathogenetic bacteria to infect citrus in autumn. Some studies have shown that *Pseudomonas* has antagonistic effect on *Xcc.* (Zhang et al., 2007; Murate et al., 2015; Michavila et al., 2017). In this study, the content of *Pseudomonas* in summer samples were the highest, and which in Satsuma mandarin samples were higher than in Newhall navel samples. It may be one of the reasons why Satsuma mandarin is not infected by *Xcc.* in summer. In addition, *Curtobacterium* sp. have been reported that abundantly existed in the roots of sweet orange uninfected by HLB pathogen (Trivedi et al., 2011), and *Curtobacterium flaccumfaciens* was able to control effectively citrus variegated chlorosis (CVC, caused by *Xylella fastidiosa*) (Araujo et al., 2002). *Curtobacterium* were found largely in spring leaves of Newhall navel orange but not in Satsuma mandarin. There may also be an inhibition of *Curtobacterium* to the occurrence of canker disease in Newhall navel orange. There was a synergistic effect reported between the number of *Stenotrophomonas* and occurrence of canker disease[55]. And it was found that *Stenotrophomonas* was the most abundant in the leaves of Newhall navel orange in summer during which the most serious canker disease occurred. Interestingly, *Acinetobacter* reported existing widely in the surface of plant leaves and possessing a potential control effect on canker disease (Gao 2016). In this study, *Acinetobacter* was found greater relative abundance in the leaves of Newhall navel orange in summer, which may be a microbial community adjustment in citrus after infecting of *Xcc.*. From the above, we concluded that the differences of endophytic bacteria in citrus cultivars may be related to the ability of host resistance or susceptibility to canker disease.

Declarations

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Ethical approval: This article does not contain any studies with human participants or animals performed by any of the authors.

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Authors' contributions

Conceived of or designed study: Bing Liu; Jiahao Lai; Junxi Jiang

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Contributed new methods or models: Junxi Jiang; Weigang Kuang

Wrote the paper: Bing Liu, Jiahao Lai

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Figures

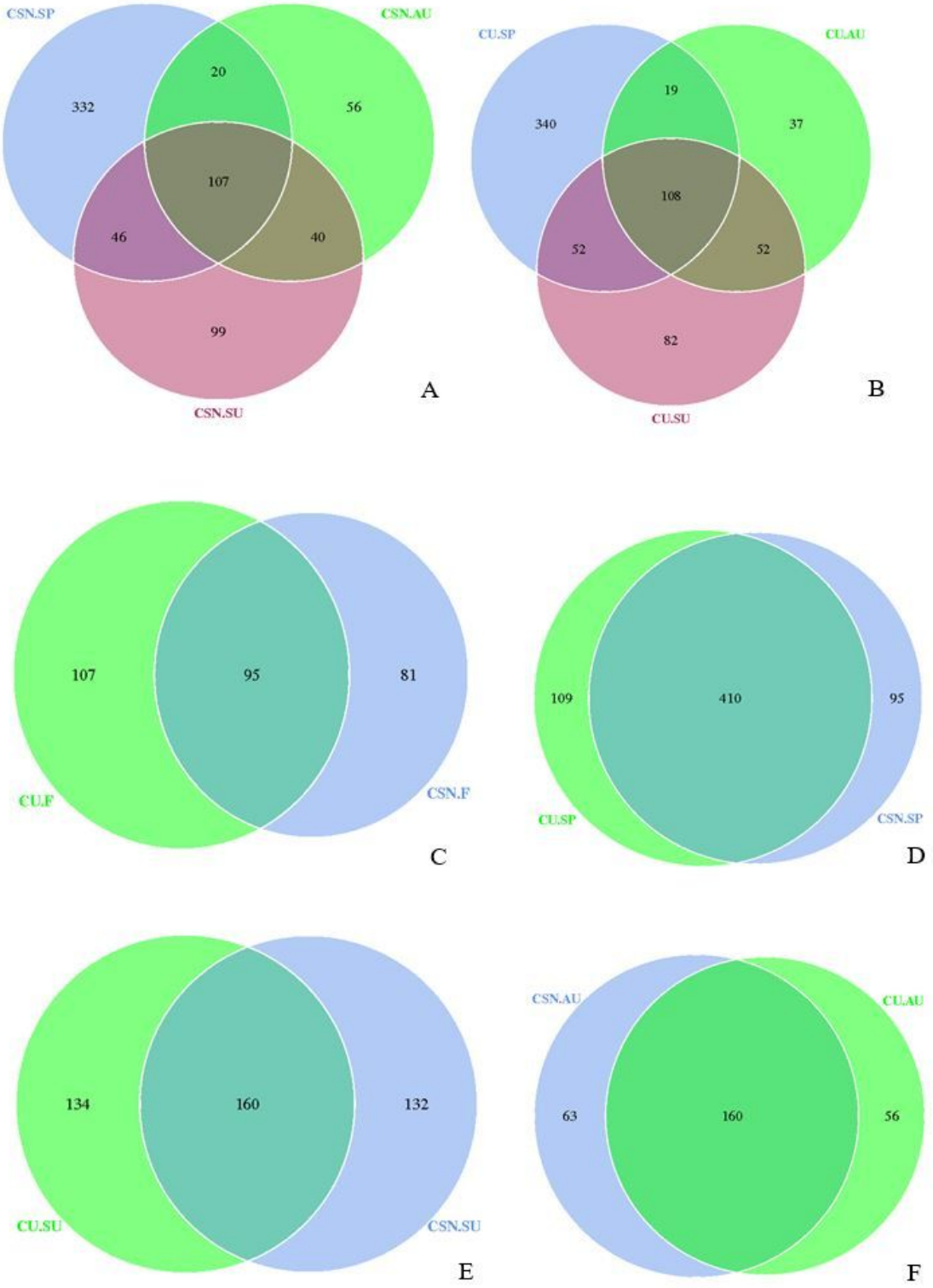

Figure 1

Venn diagram showing the unique and shared OTUs among the different samples.

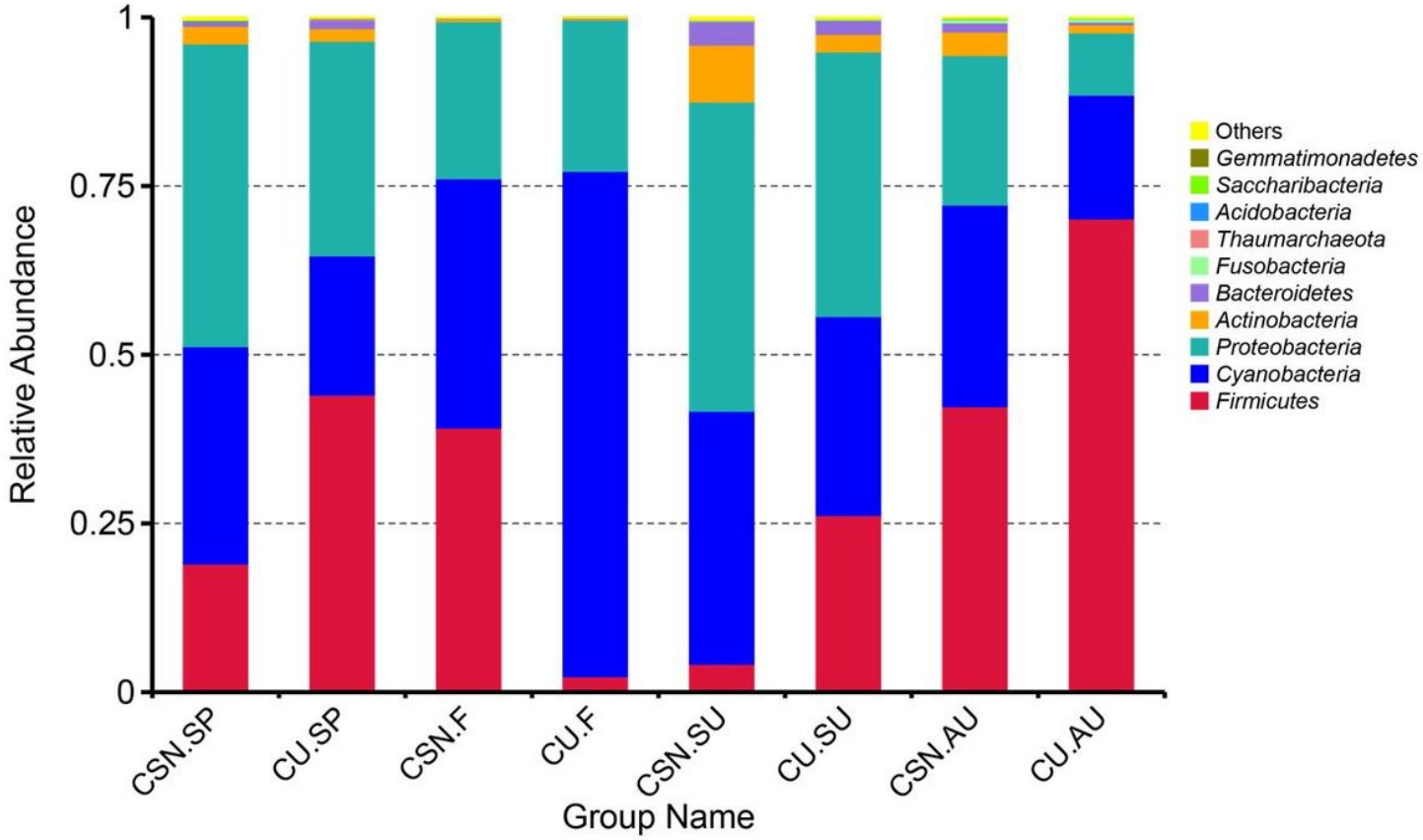

Figure 2

Relative abundances of different phylum in the eight samples.

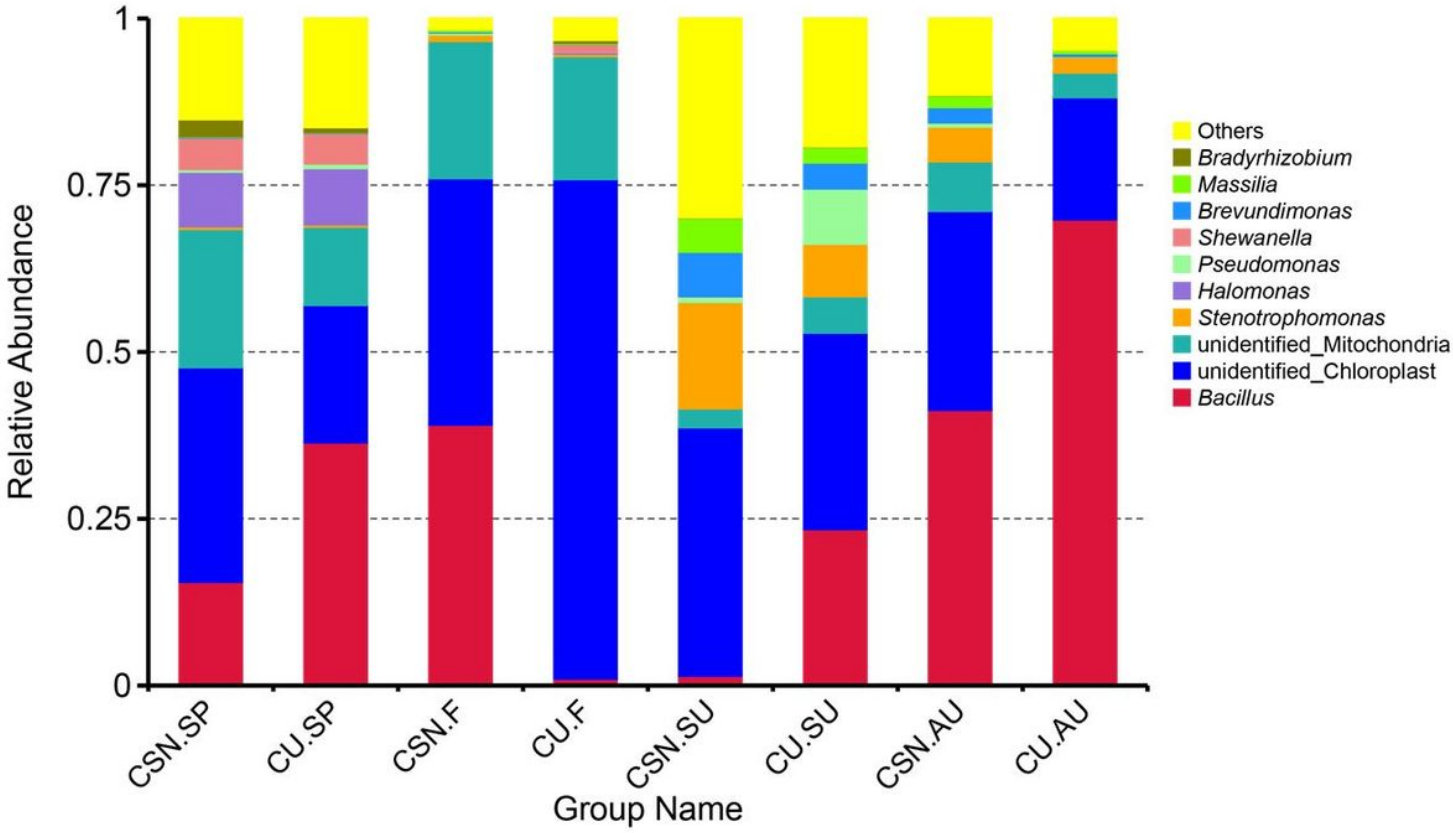

Figure 3

Relative abundances of different genus in the eight samples (Top ten genus).

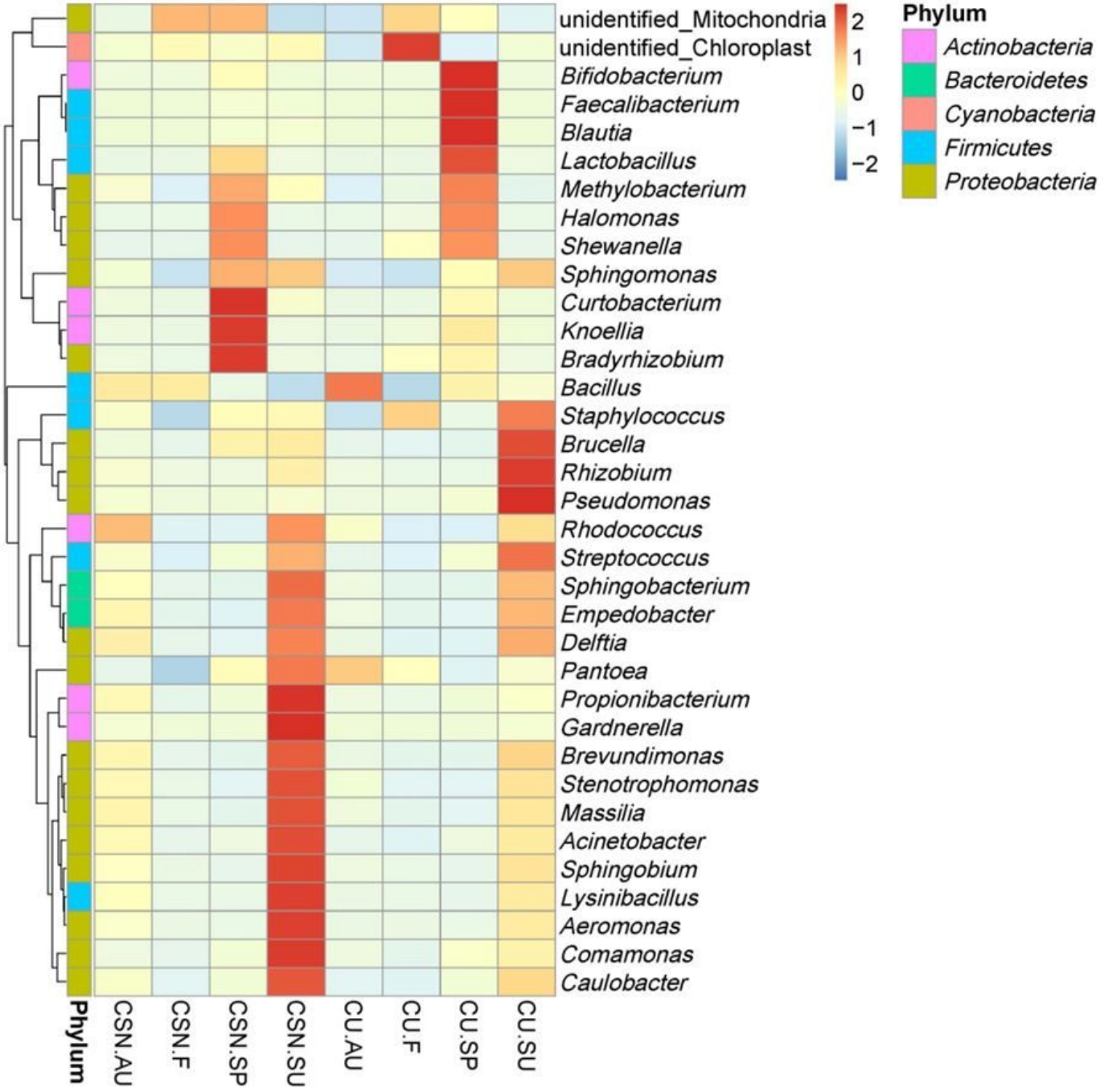

Figure 4

Heatmap showing the relative abundances of the top 35 genera in all samples. The 35 genera belonged to five phyla, as shown by different colors. The color intensity with the legend at the right of the figure indicates the relative values for each genus. The cluster tree at the left side of the graph indicates the clustering between same phylum.

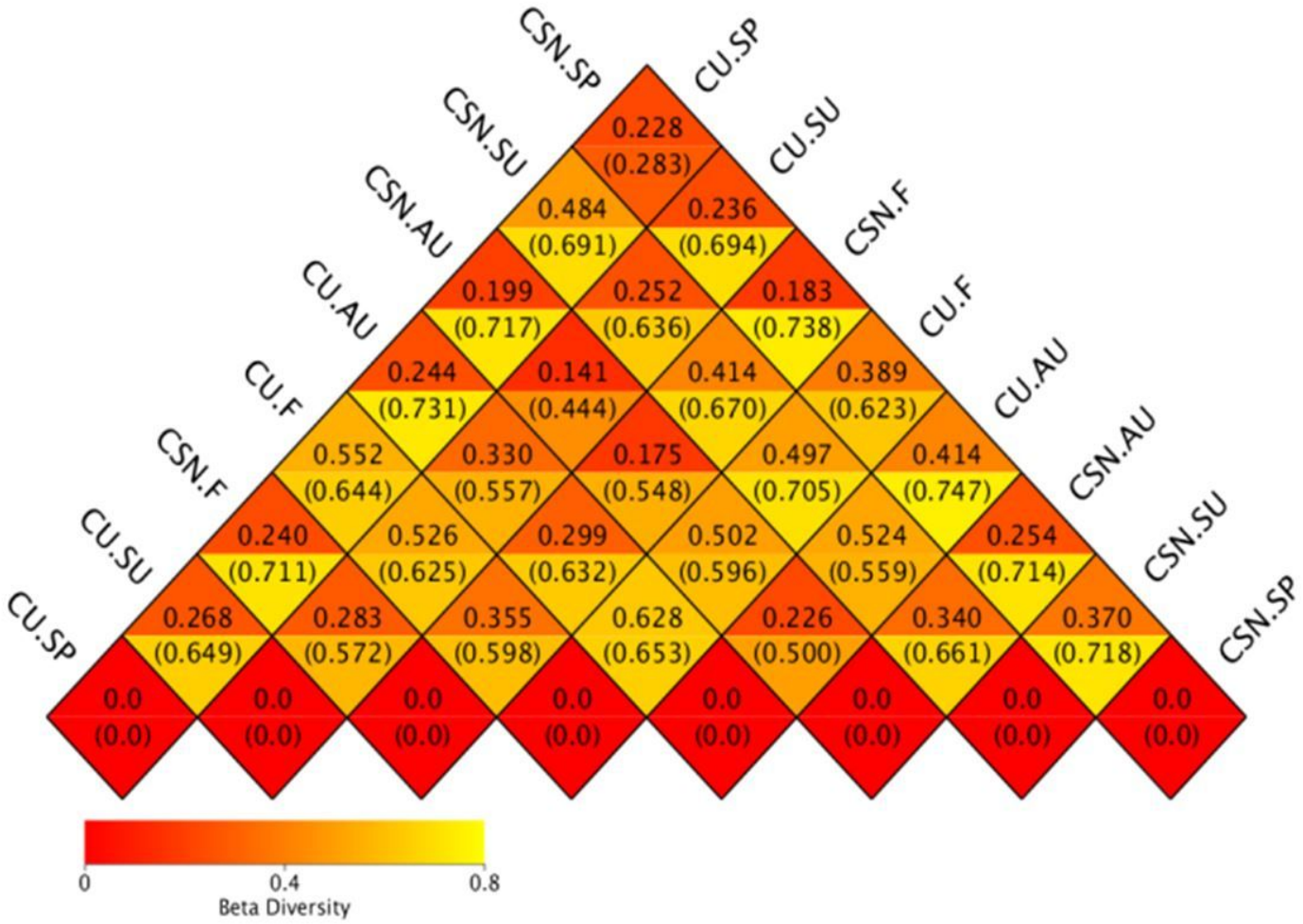

Figure 5

Beta diversity analysis of endophytic bacterial community.

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