Characteristics and Diversity of Endophytic Bacteria in Endangered Chinese Herb *Glehnia littoralis* Based on Illumina Sequencing

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Abstract

Glehnia littoralis is an endangered medicinal plant growing in the coastal ecological environment and plays an important role in coastal ecosystems. The endophytes in the plant have a significant role in promoting plant growth and enhancing plant stress resistance. However, the endophytic bacterial structure associated with halophyte *G. littoralis* is still not revealed. In this project, the construction and diversity of endophytic bacterial consortium associated with different tissues of *G. littoralis* were illustrated with high throughput sequencing of the V3-V4 region of the bacterial 16S rRNA. The results resolved that the diversity and richness of endophytic bacteria were significantly higher in root than in leaf and stem. The operational taxonomic units (OTU) analysis demonstrated that the Actinobacteria and Proteobacteria were dominant in all the samples at the phylum level, and *Pseudomonas*, *Bacillus*, *Rhizobium* were the dominant genera. Our results unraveled that the bacterial communities differed among different tissues of *G. littoralis*. Endophytic bacterial communities in leaf and stem shared more similarity than that in the root. Furthermore, the difference of bacteria community and structure among different tissues were also detected by principal coordinate analysis. Taken altogether, we can conclude that the bacterial communities of different tissues are unique, which could facilitate understanding the diversity of endophytic bacteria in *G. littoralis*.

K e y   w o r d s: *Glehnia littoralis*, halophyte, endophytic bacteria, diversity, Illumina sequencing

Introduction

At a global level, over 800 million hectares of land are affected by salt. The increasing saline-alkali arable land poses a significant threat to global agricultural production (Rozema and Flowers 2008). Halophytes are adapted to saline soils and play significant ecological roles in preserving the intertidal ecosystem's balance. It is considered good practice for using halophytes to improving saline soils. To our best knowledge, all plants in natural ecosystems are symbiotic with endophytes,

and these habitat-adapted endophytes can improve the stress tolerance of host plants (Rodriguez et al. 2008; Redman et al. 2011). Many investigations have reported that endophytic bacteria isolated from halophytes have profound effects on their host plants' stress tolerance. For example, it was documented that the endophytic bacteria from *Arthrocnemum macrostachyum* could enhance the salt tolerance ability of the host plant (Navarro-Torre et al. 2017). Additionally, Hashem et al. (2016) elucidated that endophytic bacteria have beneficial effects on the growth and health of *Acacia gerrardii*

under salt stress. Moreover, these endophytic bacteria have been deemed to be useful in the improvement of saline soils (Syranidou et al. 2016).

Glehnia littoralis belongs to the Umbelliferae family, and it is an important medicinal plant in China. The dried roots of *G. littoralis*, generally called "Beishashen" are used as a necessary herbal medicine for approximately 650 years in China due to its definite effect on immune-mediated diseases (Yoon et al. 2010). The wild resources of *G. littoralis* are distributed in coastal areas of Japan, Russia, and China (Wang et al. 2016). *G. littoralis* is a precious germplasm resource with an important ecological function. It can be widely used in environmental protection, such as preventing sand erosion, improving the soil (Zhou et al. 2018). It can also be potentially used in agriculture as a bacterial fertilizer. It has been reported that endophytic fungi of *G. littoralis* showed a very strong antimicrobial activity (Hou et al. 2015). However, there was little knowledge about the endophytic bacterial diversity of the *G. littoralis* plant until now. Consequently, the diversity study of endophytic bacteria in *G. littoralis* will clarify the interactions between endophytic bacteria and salt tolerance of *G. littoralis*.

The present research's main idea was to gain a broad general view of the endophytic bacterial community in different tissues of *G. littoralis* using next-generation sequencing technology. It was the first study to illustrate the characteristic of endophytic bacteria related to the halophyte *G. littoralis* in a Chinese coastal area. This study will show a new perspective in endophytic diversity studies of salt-tolerance plants and provide a foundation for future research.

Experimental

Materials and Methods

Sample collection. Fresh samples of *G. littoralis* were collected from the Qingdao Laoshan coastal zone (N36°14'14.63" and E120°40'16.68"). Three sampling locations, approximately 1000 m apart, were selected, and four single healthy plant samples, were randomly gathered from each sampling location in September 2018. Samples collected included leaves, stems, and roots. All the plant samples were placed into the aseptic sample box immediately and stored at –80°C. The plant tissues were surface-sterilized following the previously described method (Correa-Galeote et al. 2014).

DNA isolation, PCR amplification, and Illumina sequencing. DN-14 Plant DNA Kit (Aidlab, Beijing, China) was used to extract total DNA from leaf, stem, and root samples following the operating manual. NanoDrop 2000 (Thermo Scientific, Wilmington, USA)

was used to measure the concentration and purity of DNA, and 1.5% agarose gel electrophoresis was used to examine the DNA quality. The specific primers 338F (5'-ACTCCTACGGGAGGCAGCAG-3') and 806R (5'-GGACTACHVGGGTWTCTAAT-3') were used to amplify V3-V4 hypervariable regions of the bacteria 16S rRNA gene (Mori et al. 2014). The acquired PCR products were purified from a 1.8% agarose gel using the DR01 TRIpure Reagent Kit (Aialab, Beijing, China). QuantiFluor™-ST (Promega, USA) was used to quantify the purified DNA according to the manufacturer's instructions. Illumina MiSeq platform (Illumina, San-Diego, USA) was used to sequence the purified amplicons using paired-end sequenced method following the standard procedures by Majorbio Bio-Pharm Technology Co. Ltd. (Shanghai, China).

Processing the raw data. Trimmomatic software was performed to handle the raw FASTQ files. Then the clean data were merged by FLASH (Magoc and Salzberg 2011) with the standard that has been described in the previous studies. Then we carried out the efficient tags after running QIIME v1.7 (Bais et al. 2006) processing and the UCHIME algorithm (Edgar et al. 2011). The online software UPARSE V7.1 (http://drive5. com/uparse/) was utilized to cluster the Operational taxonomic units (OTUs) with a 97% sequence identity. The RDP Classifier algorithm (http://rdp.cme.msu. edu/) containing the Silva (SSU123) 16S rRNA database was applied to annotate each OUT sequence with a 70% confidence threshold (Yan et al. 2020).

Statistical analysis. We run the Vegan package (Dixon 2003) in the R program to perform the statistical analyses. The alpha diversities of the observed OTUs together with Chao1, Shannon, ACE richness, and diversity indices were calculated using Tukey-Kramer tests and One-way ANOVAs. The "heatmap" program in R package was performed to create heatmap, and Venn-Diagram program was used to produce Venn diagrams. The principal coordinate analysis (PCoA) was carried out to estimate the relationships between bacterial community structures. In addition, the LEfSe software (v1.0) was used to distinguish abundant families among different plant specimens for biomarker discovery (Segata et al. 2011).

Results

Analysis of clean sequences. In total, 819,834 high-quality sequences were obtained after raw data quality trimming. The average length of the high-quality sequences ranged from 394 bp to 395 bp (Table I). The calculated rarefaction curves (Fig. 1) and coverage values (Table II) prompted that the libraries were abundant enough to the bacterial diversity analysis in

Table I
Characteristics of effective tags from samples of endophytic bacteria and rhizosphere bacteria associated with *G. littoralis*.

Sample	Sample site	Number of tags	Total length (bp)	Average length (bp)	Effective (%)
Leaf1	1	61,491	24,250,816	394	86.74
Leaf2	2	70,539	27,826,647	394	87.38
Leaf3	3	72,502	28,614,926	394	80.78
Leaf4	4	56,679	22,401,393	395	71.51
Stem1	1	74,176	29,259,670	394	85.41
Stem2	2	71,883	28,374,408	394	79.27
Stem3	3	73,232	28,877,138	394	66.46
Stem4	4	68,971	27,201,006	394	81.54
Root1	1	74,705	29,478,191	394	79.50
Root2	2	70,456	27,847,105	395	70.56
Root3	3	53,183	21,019,550	395	72.88
Root4	4	72,017	28,464,459	395	73.40

all the tissues that had been collected in our project. To our interest, the rarefaction curves have shown that the number of OTU in the root was higher than that of leaf and stem samples. In all libraries, 1,632 OTUs were observed, and 558 OTUs were shared by all the samples (Fig. 2). The numbers of OTUs that occurred only in leaf, stem, and root samples were 151, 211, and 270, respectively. The common bacterial OTUs in the samples were mainly distributed in *Proteobacteria* (73.12%), *Actinobacteria* (15.22%), *Firmicutes* (4.4%), and *Bacteroidetes* (4.32%) at the phylum level and in *Pseudomonas* (15.41%), *Pantoea* (13.32%), *Acidibacter* (3.29%), and *Bacillus* (2.57%) at the genus level.

Biological diversity and richness analysis. The diversity and richness of bacterial communities in all the samples are listed in Table II. Among the samples, the bacterial communities' richness and diversity in roots were highest, followed by stem and leaf.
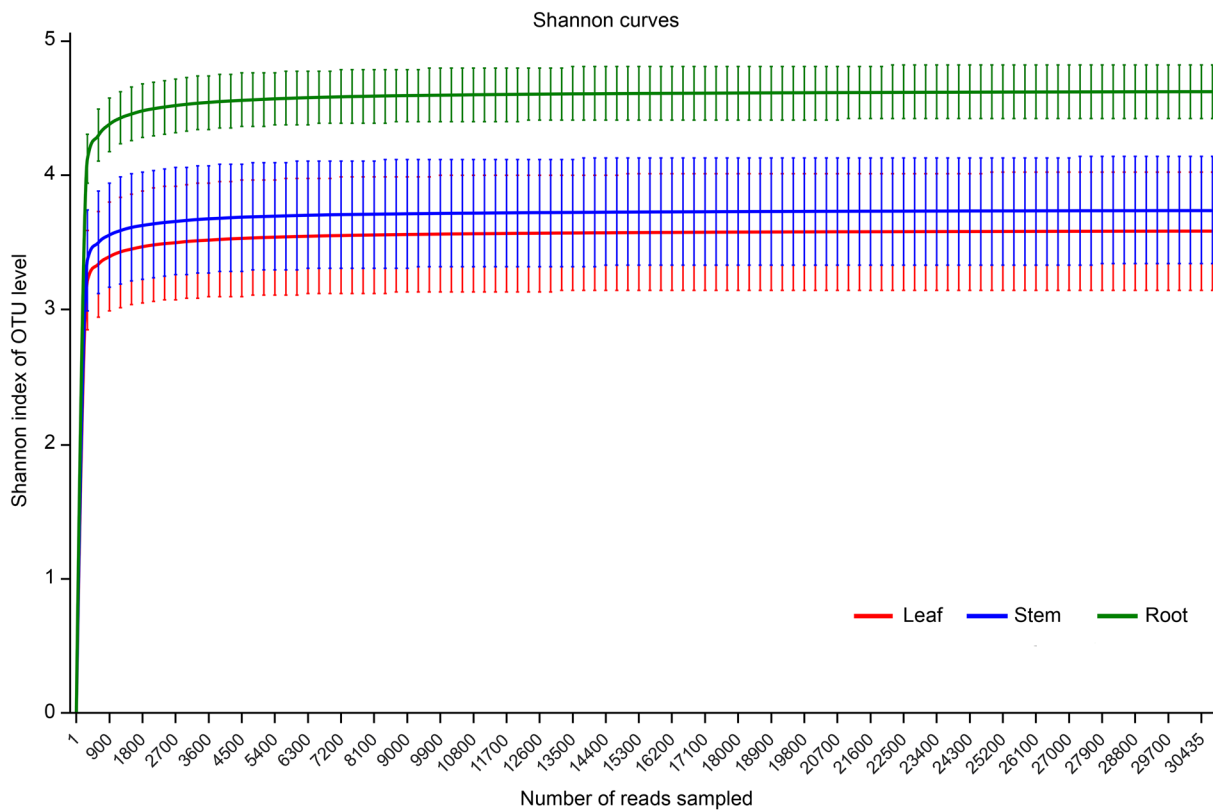

Fig. 1. Rarefaction curves based on the Shannon index OUT level. Error bars represent the standard error of four replicates.

Table II
Operational taxonomic unit (OTU) richness and diversity indices of different samples associated
with *G. littoralis* with a 97% similarity cut-off.

Sample name	OTUs observed	Shannon	Chao1	ACE	Coverage (%)
Leaf	526 ± 34 b	3.58 ± 0.43 b	600 ± 66 b	599 ± 69 b	99.6
Steam	555 ± 22 b	3.73 ± 0.39 b	613 ± 82 b	616 ± 77 b	99.6
Root	694 ± 19 a	4.60 ± 0.19 a	803 ± 40 a	818 ± 42 a	99.6

Values are the means of four replicates ± SD. Values within a column followed by different lowercase letters
are significantly different ($p < 0.05$).

In addition, the ACE and Chao1 richness values and Shannon index of the root were significantly higher than that of stem and leaf.

Bacterial taxonomic analysis at phylum level. High-throughput sequences annotated to the Bacteria domain were identified into 29 bacterial phyla. The relative community abundance on the phylum level of the top seven phyla is revealed in Fig. 3. Overall, the abundance of bacterial phyla varied among different tissues. Actinobacteria and Proteobacteria were the prominent in all samples, accounting for more than 61.19% and 6.84%, respectively. The abundance of Chloroflexi was 5.4% in the leaf samples and 5.9% in the stem tissues that was much higher than in the root (2.4%).

Bacterial taxonomic analysis at genus level. A heatmap of the top 50 genera was drawn based on the distributions and abundances of OTUs for all samples (Fig. 4). These identified bacterial genera were classified into the following four phyla: Proteobacteria, Bacteroidetes, Firmicutes, and Actinobacteria. Among the top 50 genera, 35 genera belonged to Proteobacteria, eight genera belonged to Actinobacteria, four to Bacteroidetes, and

three genera belonged to Firmicutes. The heatmap also showed that the endophytic bacteria were mainly concentrated in the leaf and stem samples. In addition, the distribution of endophytic bacteria was significantly different among the samples. *Acidibacter*, *Kibdelosporangium*, and *Steroidobacter* were mainly distributed in the root samples, while *Pantoea*, *Pseudomonas*, and *Enterobacteriaceae* were dominant in the leaf and stem samples. Four genera, *Aeromicrobium*, *Rhiobium*, *Roseateles*, and *Chryseobacterium* were predominantly distributed in the leaf samples, while *Methylophilus*, *Xanthomonas*, and *Cloacibacterium* were dominant in stem samples. The relative abundance of *Cloacibacterium* was higher in leaf samples (3%) than in the other samples.

Structures and varieties of the endophytic bacteria in different sample groups. The representatives of the endophytic bacteria communities of the three sample groups were remarkable distinct. As illustrated in Fig. 5, significant changes occurred in the endophytic bacteria communities between different sample groups. At the family level, Micromonosporaceae, Hyphomicrobiaceae, and Rhodospirillaceae were more abundant in the root samples. Only one family, Rhizobiaceae presented relatively higher abundance in the stem samples. Five families, such as Enterobacteriaceae, Pseudomonadaceae, Comamonadaceae, Flavobacteriaceae, and
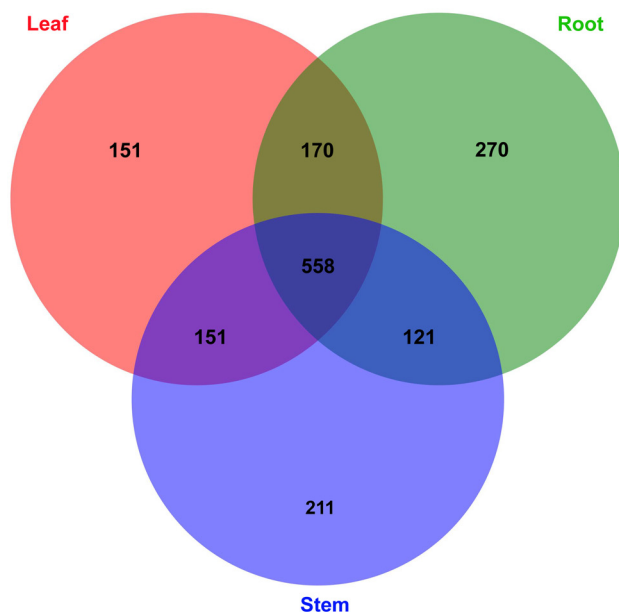

Fig. 2. Venn diagram showing the OTUs shared among different samples associated with *G. littoralis*.
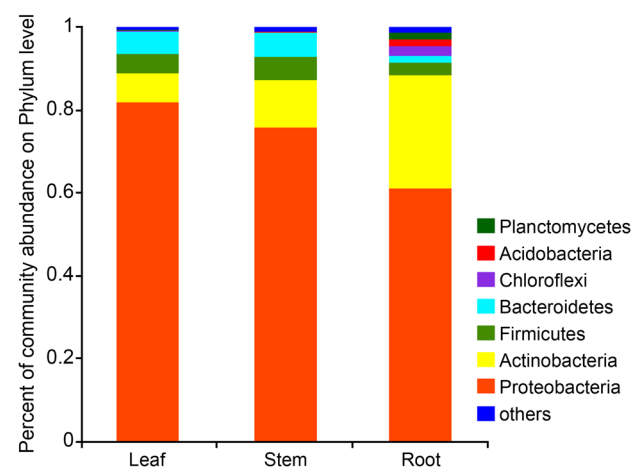

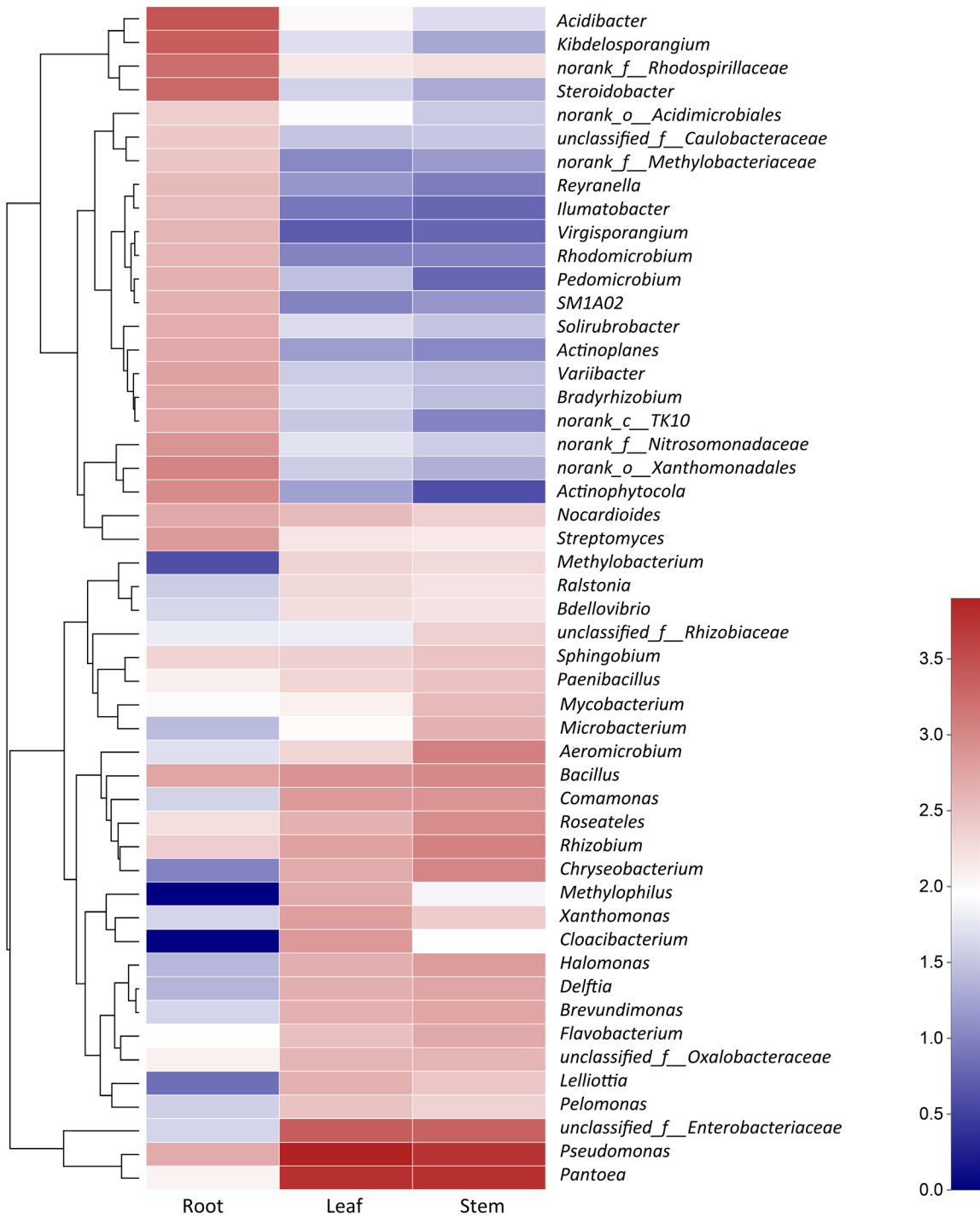Fig. 3. The bacterial abundance of different tissues at the phylum level.

Fig. 4. Heatmap displaying the relative abundances of the most dominant genera (top 50) in each sample. The dendrogram represents complete-linkage agglomerative clustering, based on Euclidean dissimilarities.

Rhizobiaceae, showed higher abundance in the leaf and stem samples that in root samples.

Correlation analysis of different samples. We carried out a similarity analysis in the species constructions of the three *G. littoralis* samples groups. Adonis analysis was utilized to define the mean differences and the correlation between two samples (Table III). We have found a significant difference between the root samples and stem samples ($R^2 = 0.46$, $p < 0.05$),

Table III
The Adonis analysis of the difference among samples associated with *G. littoralis*.

Tissues	Leaf	Stem
Root	$R^2 = 0.68$, $p = 0.027$	$R^2 = 0.46$, $p = 0.041$
Stem	$R^2 = 0.09$, $p = 0.748$	

The larger the value of R^2 (the ratio of group variance to total variance), the more significant the differences were among the tissues. $p < 0.05$ indicates a high reliability of the test.
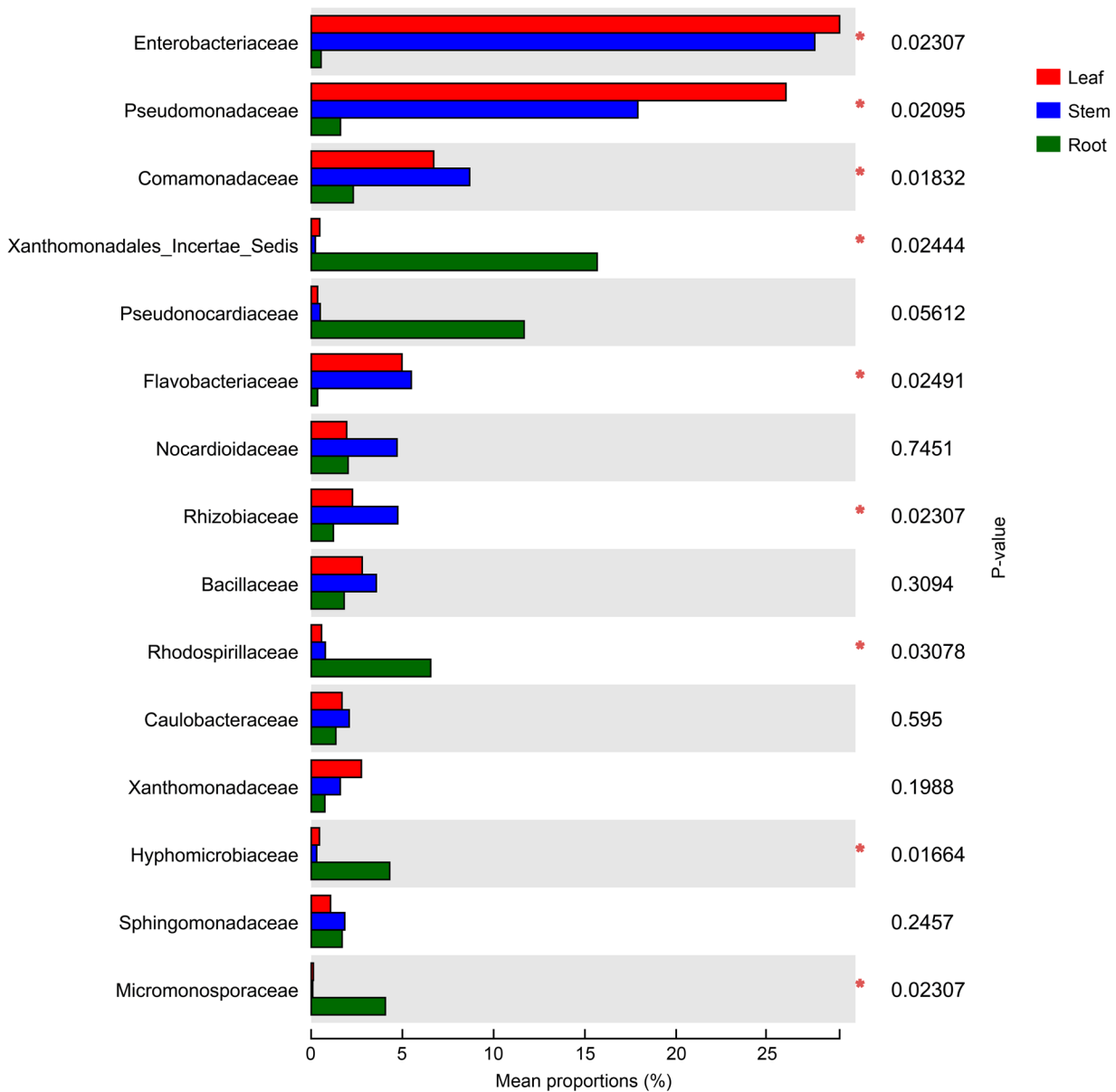
Fig. 5. Statistical comparison of the relative abundance at the family level by the Kruskal-Wallis H test.
$P < 0.05$ was considered statistically significant.

and leaf samples ($R^2 = 0.68$, $p < 0.05$). However, there was no significant difference between the leaf and stem samples. Based on the Adonis analysis, the number of endophytic bacteria in root samples was higher than in the leaf and stem samples.

The hierarchical clustering tree was constructed with the Unweighted Pair Group Method with Arithmetic Mean (UPGMA) to investigate the correlation between different tissues (Fig. 6). Consistent with the Adonis analysis results, two different clusters were found in the UPGMA tree based on the OTU level. All the root samples were clustered into group 1. The samples of leaf and stem were clustered into group 2. The UPGMA tree result clarified that the bacterial compositions of leaf and stem samples were more similar versus the root samples.

Furthermore, PCoA (principal coordinates analysis) disclosed the main changes in bacterial community components among all the samples (Fig. 7). The results showed that the root samples were relatively distinct from the leaf and stem samples. Moreover, the structure of the endophytic bacterial community in the leaf samples was similar to those in the stem tissues, but one sample from leaf and one from the stem was distinct from the group.

Discussion

There are no artificial culture methods for entirely isolating and identifying all endophytic bacteria from different tissues of the plant (Liu et al. 2017). The
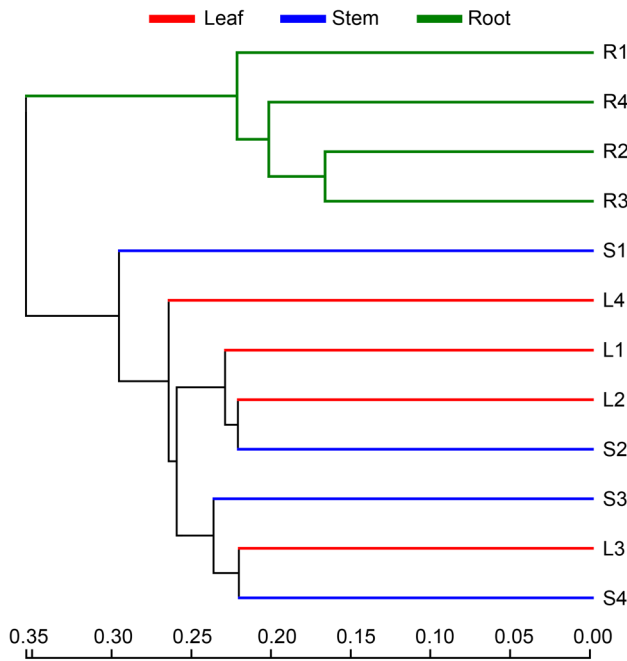
Fig. 6. Hierarchical cluster analysis of different microbiota in different samples using pairwise weighted UniFrac distances. L1, L2, L3, L4, four repetitions of the leaf; S1, S2, S3, S4, four repetitions of the stem; R1, R2, R3, R4, four repetitions of the root.
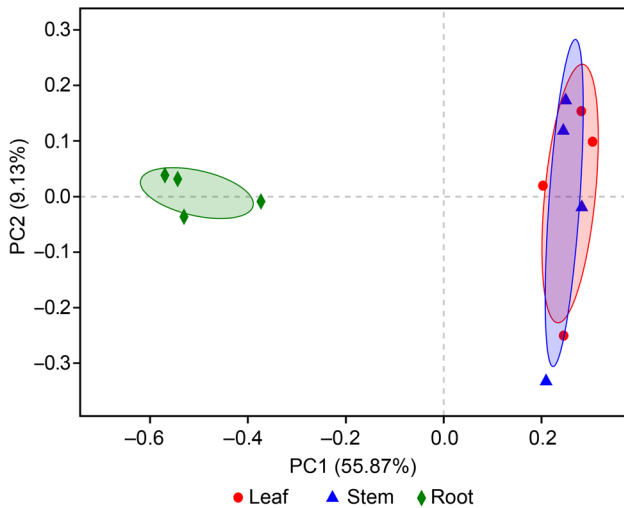

Fig. 7. Principal coordinate analysis (PCoA) based on Bray-Curtis dissimilarity between different samples.

high-throughput sequencing methods make it possible to identify the endophytic bacterial species without bacteria culturing (Ren et al. 2019). The 16S rDNA V3-V4 region sequencing method is more suitable for detecting and classifying the endophytic bacteria in the different tissues of *G. littoralis*. The results of our project show that the bacterial diversity and richness were higher in the root of *G. littoralis* than that of in the stem and leaf ($p < 0.05$, Table I) based on the results of the OTU analysis and the diversity indices such as ACE, Chao1 and Shannon's. Our results are consistent with previous endophytic bacterial studies, such as

halophyte *Phragmites australis* (Ma et al. 2013), *Oryza sativa* (Zhang et al. 2019), and *Messerschmidia sibirica* (Tian and Zhang 2017). We can find the changes of endophytic bacteria composition in different tissues of *G. littoralis*, and more bacteria communities inhabited in the root than in the leaf or stem. Previous studies have certified that most endophytic bacteria are derived from the soil. Due to the interaction between plants and soil, the diversity indices of root endophytic bacteria are higher than that of leaf or stem (Hardoim et al. 2011).

Previous studies have clarified that the plant bacterial communities based on high-throughput sequencing analysis constituted only a few dominant phyla, including Proteobacteria, Bacteroidetes, and Actinobacteria. Firmicutes were dominant in some studies (Miguel et al. 2016). In this project, the endophytic bacterial communities of *G. littoralis* were clustered into 29 phyla, and the dominant phyla were Proteobacteria, Bacteroidetes, and Actinobacteria, which is consistent with the studies quoted above (Miguel et al. 2016).

The cluster and heatmap analysis showed that structures of bacterial communities differed significantly across the different samples. At the phylum level, Proteobacteria, Actinobacteria, Firmicutes, and Bacteroidetes were prominent in all the tissues, but the proportions of the dominant communities are different (Fig. 3). This result is consistent with the bacterial communities' survey of halophyte *Salicornia europaea* (Szymanska et al. 2016). However, Actinobacteria is mainly distributed in the root samples, and the proportion of Bacteroidetes is higher in leaf and stem samples than in root samples. This result indicated that bacterial communities have some tissue specificity.

The detected genera in this study, including *Pseudomonas*, *Bacillus*, and *Rhizobium* are found commonly in other studies of plant endophytic bacterial communities (Ma et al. 2013; Eida et al. 2019). Many previous researches have clarified that endophytic Pseudomonas is widely distributed in plants' tissues (Feng et al. 2017). Many species of these genera have been reported to play significant roles in protecting hosts from diseases, promoting C or N cycling in the soil, and fixing nitrogen (Enya et al. 2007; Vepstaite-Monstavice et al. 2018). Furthermore, *Actinoplanes* is dominant in *G. littoralis* root samples, and *Sphingomonas* is common in all the samples, and some species of these two genera play important roles in the interaction of plants and microorganisms in halophytic ecosystems (Solans et al. 2011; Li et al. 2013). In total, we have observed many beneficial endophytic microorganisms in *G. littoralis*, and further investigation is required to investigate the specific interactions between the bacteria and *G. littoralis*.

Our results expound that compositions of bacterial communities are significantly different among all the tissues. These results are similar to previous researches

that different tissues of plants host different bacterial communities (Edwards et al. 2015). The bacterial structure of leaf and stem was more similar than that of the root as it was testified by Adonis analysis. This result is inconsistent with previous studies, which indicated that the endophytic bacteria of the leaf, stem, and root of coastal halophyte *M. sibirica* are similar (Tian and Zhang 2017). Many factors may contribute to these discrepancies, such as host genotypes, environmental factors, and other plant endophyte interactions.

Interestingly, the previous research reported by Jin et al. (2014) has mentioned that *Stellera chamaejasme* endophytic bacteria of the leaf and stem were clustered together but were different from those of root. Further proved by the PCoA analysis, the tissues that inhabited endophytic bacteria account for 55.87% of the variation in the community structure while sampling sites account for 9.13%. The results indicate that the tissues may exert an effect on endophytic communities.

Conclusions

Our study was first to show the endophytic bacteria diversity and composition of the coastal halophyte *G. littoralis* based on the 16S rDNA sequencing method. We have found that Proteobacteria, Actinobacteria, and Firmicutes were the dominant endophytic bacteria associated with *G. littoralis*. The results clarified that the composition of the endophytic bacterial communities was significantly distinct across the different habitats of leaf, stem, and root. Our study provides an in-depth understanding of the complex endophytic bacterial compositions that inhabited *G. littoralis*. We would further investigate the functional roles of those endophytic bacterial in plant-microbe interactions, such as the mechanism of promoting the plant growth in the inter-tidal zone.

Conflict of interest
The authors do not report any financial or personal connections with other persons or organizations, which might negatively affect the contents of this publication and/or claim authorship rights to this publication.

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