

## A New Approach to Analyzing Endophytic Actinobacterial Population in the Roots of Banana Plants (*Musa* sp., AAA)

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### INTRODUCTION

Bananas and plantains grown in the tropic and subtropic regions are among the important crops worldwide<sup>[1]</sup>. Nevertheless, fusarium wilt of banana (Panama disease) is one of the most devastating diseases of banana. It is a major problem throughout most of the banana production regions of the world. Since the *Fusarium* clamydospores persist in the soil for decades, no cultural or agronomic practices that are useful for the growth susceptible cultivars on infested soils have been identified<sup>[1]</sup>. No effective chemical method exists to control fusarium wilt of banana; however, biological control using endophytes provides promising perspective for sustainable control of fusarium wilt<sup>[2]</sup>.

Endophytes are thought to be a sub-population of the rhizosphere microbiome, but they also have characteristics distinct from rhizosphere bacteria<sup>[3]</sup>. The analysis of core *Arabidopsis thaliana* root microbiome suggested that although various different soil types altered the bacterial endophyte microbiome, actinobacteria were consistently enriched in the endosphere compared with the rhizosphere<sup>[4]</sup>. Endophytic non-filamentous actinobacteria had been isolated from growing shoot tips of banana<sup>[5,6]</sup>. Although the healthy-promoting roles of filamentous actinobacteria were widely accepted<sup>[7]</sup>, the diversity of actinobacteria among the banana root microbiota is still poorly understood.

The actinobacteria were bacteria with high G+C content, and

### ABSTRACT

**AIM:** To elucidate the endophytic actinobacterial populations of healthy banana plant roots.

**METHODS:** A surface sterilization protocol was adopted to remove rhizoplane bacterial DNAs and total DNAs of surface-sterilized roots were purified and analyzed by Illumina-based sequencing.

**RESULTS and CONCLUSION:** A total of 2152102 bacterial sequences remained with an average length of 448bp, 31952 different actinobacterial OTUs at the 97% similarity level were obtained, and the dominant families were *Nocardioidaceae*, *PseudoNocardiaceae* and *Nocardiaceae*, which accounted for 56.37%, 14.36% and 9.77%, respectively. At genus level, *Pseudonocardia* (11.83%), *Rhodococcus* (9.74%), *Nocardioides* (3.06%) were the most abundant taxa. The results indicated that surface sterilization approach with formaldehyde (36%) and Illumina-based sequencing is suitable for analysis of endophytic actinobacterial populations in banana roots.

they grow slowly than other bacteria, the cultivation methods and *E. coli* 16S rRNA genes might low estimate the actinobacterial taxa<sup>[8]</sup>. Deciphering the plant actinobacteriome is critical to identify actinobacteria that can be exploited for improving plant growth and health<sup>[9]</sup>. More comprehensive information on banana actinobacteriome would be obtained by high-throughput sequencing approaches.

The use of high-throughput sequencing technologies has been widely adopted as they allow identification of thousands to millions of sequences in a sample, revealing the abundances of even rare microbial species<sup>[3]</sup>. Illumina has fewer errors than 454 sequencing and it could provide a higher phylogenetic resolution than 454 based approaches<sup>[10]</sup>. The advantage of Illumina to provide 30 times more reads would enable us to perform in depth sequencing of samples in one run, making it an excellent tool for endophytic actinobacteria diversity.

To develop new approach to analyzing endophytic actinobacterial populations in banana roots, a new surface sterilization procedure and new actinobacteria specific primers were designed in the study. The Illumina-based analysis illustrated that the new approach was suitable for analyzing endophytic actinobacterial populations in healthy banana roots.

## MATERIAL AND METHODS

### Sample Collection

Four to five month-old field grown suckers of healthy banana plants (*Musa* sp., AAA, Giant Cavendish cv. Baxi,) were collected from a banana plantation in the suburbs of Guangzhou, China. The roots of healthy banana plants in fields without wilting symptoms were used for total DNA extraction. The plants ( $n = 8$ ) were dug out carefully to ensure that maximal amount of root materials was collected. The root samples were placed in plastic bags and processed after surface sterilization within 4 h of collection.

### Surface Sterilization

The root samples were washed with tap water to remove soil particles and sterilized by immersion in 36% formaldehyde solution for 7 min. Then, the sterilized roots were rinsed three times with demineralized sterile water (vortex for 2 min per rinse) to remove the surface sterilization agents.

### DNA Extraction

The total DNA was extracted using E.Z.N.A. HP Plant DNA Kit (Omega) and according to the manufacture's instruction. Total DNA concentration and purity were monitored on 1% agarose gels.

### Amplicon Generation and Illumina MiSeq sequencing

The actinobacteria specific primers 243F (5' - GGATGAGCCCG CGGCCTA - 3') and A3R (5' - CCAGCCCCACCTTCGAC - 3') were used to amplify actinobacteria 16S rRNA gene, and this could produce a 1.21 kb sequence fragment. The PCR reaction mixture (25  $\mu$ L) contained 1  $\times$  PCR buffer (Takara), 200  $\mu$ M dNTP, 0.2  $\mu$ M of each primer, 3 mM MgCl<sub>2</sub>, 2.5 U Taq DNA polymerase (Takara). Thermal cycling conditions were as follows: an initial denaturation at 94°C for 5 min, followed by 35 cycles at 94°C for 30 s, 56°C for 40 s, and 72°C for 90 s, with a final extension at 72°C for 10 min. The PCR products were further sequenced with the primers S-D-Bact-0341-b-S-17 (5' - CCTACGGGNGGCWGCAG - 3') and S-D-Bact-0785-a-A-21 (5' - GACTACHVGGGTATCTAATCC - 3') targeting the V3-V4 hyper variable regions of bacterial 16S rRNA genes<sup>[11]</sup>.

Both forward and reverse primers were tagged with adapter, pad and linker sequencing. Each barcode sequence was added to the reverse primer for pooling multiple samples into one run of sequencing. All PCR reactions were performed in a total volume of 30 $\mu$ L containing 15  $\mu$ L Phusion<sup>®</sup> High-Fidelity PCR Master Mix (New England Biolabs) and 0.5 units of AccuPrimer TM Taq DNA Polymerase (Life Technologies, USA); 0.2  $\mu$ M of forward and reverse primers, and 10 ng template DNA. Thermal cycling conditions were as follows: an initial denaturation at 98 °C for 1 min, followed by 30 cycles at 98 °C for 10 s, 50 °C for 30 s, and 72 °C C for 60 s, with a final extension at 72 °C for 5 min.

Following amplification, 5  $\mu$ L of PCR product was used to successful amplification using 1% agarose gel electrophoresis. The triplicate PCR reaction were combined and the pooled mixtures was purified with GeneJET Gel Extraction Kit (Thermo Scientific) and analyzed on an Agilent 2100 Bioanalyzer using High Sensitivity DNA Chips (Agilent Technologies, germany) for size distribution. The sequencing libraries were generated using NEB Next<sup>®</sup> Ultra<sup>™</sup> DNA Library Prep Kit for Illumina (NEB, USA) following manufacturer's recommendations and index codes were added. The library quality was assessed on the Qubit@ 2.0 Fluorometer (Thermo Scientific) and Agilent Bioanalyzer 2100 system (Agilent Technologies, Germany). Finally, the library was sequenced on an Illumina MiSeq platform at Magigen biotechnology Co. Ltd, Guangzhou, China.

### Combination and data preprocessing

Forward and reverse sequences were merged by overlapping paired-end reads using FLASH (V1.2.7, <http://ccb.jhu.edu/software/FLASH/>)<sup>[12]</sup>. All sequences reads with the same tag were assigned to the same sample according to the unique barcodes (raw tags). The raw tags were further strictly filtered by previous methods<sup>[13]</sup> and the quality of clean tags were detected by Qiime (V1.7.0, <http://qiime.org/index.html>)<sup>[11]</sup>, and the low quality tags were removed. The tags with chimera were detected and removed using UCHIME Algorithm, ([http://www.drive5.com/usearch/manual/uchime\\_algo.html](http://www.drive5.com/usearch/manual/uchime_algo.html))<sup>[14,15]</sup>. The effective sequences were then clustered into operational taxonomic units (OTU) at 97% sequence similarity using the UPARSE-OTU and UPARSE-OTUref algorithms of UPARSE software package (Uparse v7.0.1001, <http://drive5.com/uparse/>)<sup>[16]</sup>. Finally, the RDP classifier was used to assign representative sequence to the microbial taxa<sup>[17]</sup>. Sequence data have been deposited in the National Center for Biotechnology Information (NCBI) Sequence Read Archive (SRA) under the accession number SRP061867.

### Statistical analysis

Cluster analysis was preceded by principal component analysis (PCA) using the QIIME software package. QIIME calculates both weighted and unweighted unifrac distance, which are phylogenetic measures of beta diversity<sup>[18]</sup>, the phylogenetic relations among different microbial taxa were further displayed by KRONA<sup>[19]</sup>.

## RESULTS

### Validation of surface sterilization

To validate the surface sterilization, the sterilized roots were stirred in 10 mL sterile water. The copy numbers of whole bacterial 16S rRNA gene and bacterial 16S rRNA gene segments were further quantified by MPN (most probable number)-PCR and real-time PCR, respectively<sup>[20,21]</sup>. Results from MPN-PCR illustrated that sterilization by formaldehyde (36%) for 7 min removed 99.99% of rhizoplane bacterial whole 16S rRNA genes. Results from real-time

PCR indicated that the rhizoplane 16S rRNA gene copies of roots sterilized with formaldehyde (36%) for 7 min were similar to those in negative controls (without DNA template).

**Actinobacterial species richness and diversity**

After qualify filtering the raw reads, 2152102 bacterial sequences remained with an average length of 450 bp, 31952 different bacterial OTUs at the 97% similarity level were obtained (Table 1).

The alpha diversity indices calculated from bacterial OTUs of indicated that the banana roots contained more diverse actinobacteria (Table 2).

Bacterial representative sequences of each OTU were classified into the domain bacteria, more than 99.99% of the total data set was belonged to Actinobacteria (Figure 1).

The *Actinobacteria* was also the most dominant actinobacterial class observed, at 99.08%, and others belong to *Thermoleophilia* (0.82%) (Figure 2).

The dominant orders were *Actinomycetales* (99.07%) and *Solirubrobacterales* (0.82%) (Figure 3).

The *Nocardioideae* was the most domain family observed, at 56.37%, *PseudoNocardiaceae* was the second most abundant family, at 14.36%, others belonged to *Nocardiaceae*, (9.77%), *Microbacteriaceae* (3.77%), *Dietziaceae* (2.67%), *Dermabacteraceae* (1.35%), *Micrococcaceae* (1.18%), *Micromonosporaceae* (1.01%), *Mycobacteriaceae* (0.91%), *Actinosynnemataceae* (0.85%), *Corynebacteriaceae* (0.70%), *Kineosporiaceae* (0.30%), *Cellulomonadaceae* (0.10%) and *Promicromonosporaceae* (0.10%) (Figure 4).

The actinobacterial genera *Pseudonocardia*, were the most detected bacterial genera in the banana roots, at 11.83%. Other frequently detected generas belonged to *Rhodococcus* (9.74%), *Nocardioides* (3.06%), *Pimelobacter* (2.78%), *Dietzia* (2.04%), *Brachybacterium* (1.27%), *Microbacterium* (1.03%), *Mycobacterium* (0.91%), *Corynebacterium* (0.7%), *Citricoccus* (0.54%), *Micrococcus* (0.28%), *Quadrisphaera* (0.27%), *Agrococcus* (0.27%), *Arthrobacter* (0.22%) and *Leucobacter* (0.15%) (Figure 5).

About 64.48 % OTUs belonged to the unidentified actinobacterial taxa, they might be belonged to novel actinobacterial species.

**DISCUSSION**

Results from MPN-PCR and real-time PCR illustrated that surface sterilization protocol could remove all the rhizoplane bacterial 16S rRNA genes and the protocol with formaldehyde (36%) for 7 min could be used to remove rhizoplane bacterial whole 16S rRNA genes.

The actinobacteria are rich source of biologically active natural products and are widely distributed in different terrestrial and marine habitats<sup>[8]</sup>, their growth rates were lower than other endophytic bacteria and fungi<sup>[7]</sup>. The previous culture-dependent and culture-independent methods based on *E. coli* 16S rRNA gene might underestimate the diversity of endophytic actinobacteria. *Pseudonocardia* were the most detected bacterial genera in the banana roots, others belonged to *Rhodococcus* (9.74%), *Nocardioides* (3.06%). The actinobacterial taxa were not consistent with previous results. *Streptomyces* was the most frequently isolated genus from surface-sterilized banana roots<sup>[7]</sup>, nevertheless, it can not be isolated or detected in previous studies<sup>[1,6,22]</sup>. In the study, total 11 reads belonged to *Streptomyces* or Streptomycetaceae were detected by Illumina-based analysis. *Arabidopsis thaliana* hosts a genotype-specific core microbiome dominated by Actinobacteria, however, the removal efficiency of rhizoplane bacterial DNA was not demonstrated in these studies<sup>[4]</sup>. The relative abundance of sequences

**Table 1** The characteristics of effective tags in actinobacteria in banana roots identified by primers 243F and A3R.

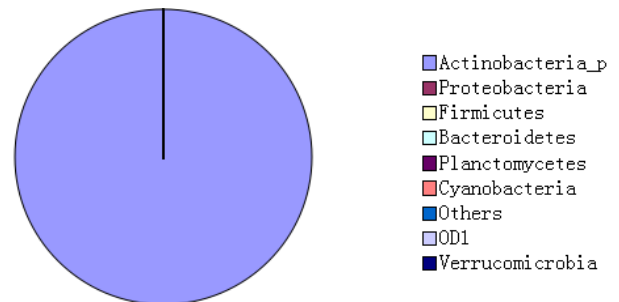
Sample	Banana roots			
	Numbers	Total length (bp)	Max length (bp)	Min length (bp)
Broot†	2345963	1051133372	490	337

† Broot, the extracted banana roots DNA was first amplified by primer set (243F and A3R primers ), and then the PCR products were sequenced by the primers targeting V3-V4 hyper variable regions of bacterial 16S rRNA genes.

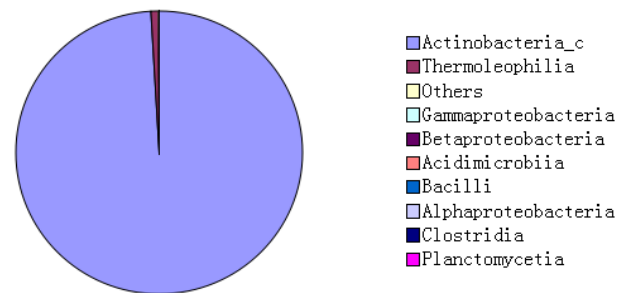
**Table 2** The alpha diversity indices of actinobacteria OTUs in actinobacteria in banana roots.

Strategies	Banana roots				
	Chao1‡	ACE	Shannon	Simpson	Coverage
Broot OTUs	31952	31952	6.66	0.86	1

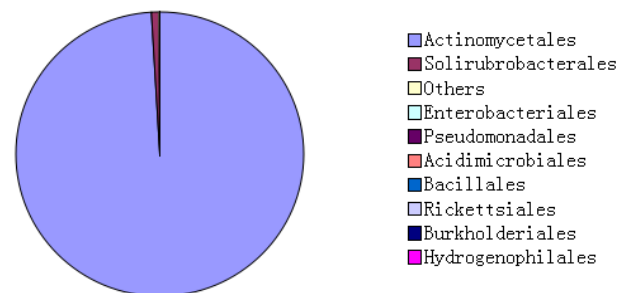
‡ Both Chao1 and ACE described an estimate of the total number of phylotypes in a source environment, and Chao1 is particularly appropriate for data sets in which most phylotypes are relatively rare in the community, ACE is appropriate for data sets in which some phylotypes occur more frequently. Both Shannon and Simpson index comprehensively reflect the richness and evenness of community, Shannon index is more sensitive to the richness of the community, and Simpson index is more sensitive to the evenness of the community. Coverage is a non-parametric estimator of the proportion of phylotypes in a library of infinite size that would be represented in a smaller library.



**Figure 1** Abundances of different bacterial phylums in banana roots identified by primers 243F and A3R.



**Figure 2** Abundances of different bacterial classes in banana roots.



**Figure 3** Abundances of different bacterial orders in banana roots.

identified as *Streptomyces* spp. was possibly biased by the extraction method as these actinobacteria have robust spores<sup>[23]</sup>. Our results indicated that the proportion of *Streptomyces* sequences was low

among the actinobacterial sequences in banana root. The dominance of actinobacteria in *Arabidopsis thaliana* roots did not derived from extraction methods, but from the rhizoplane *Streptomyces*, Other actinobacterial analysis based on 16S rRNA gene library showed that *Streptomyces* could be detected from wheat or rice roots<sup>[24,25]</sup>. However, hypochlorites were used to surface sterilize plant roots and the removal efficiency of rhizoplane bacterial DNA was not further demonstrated<sup>[26,27]</sup>. Probably, some rhizoplane *Streptomyces* spp. were misconsidered as endophytic streptomycetes in roots.

In the study, 99.99% of OTUs belonged to actinobacteria and high resolution actinobacteriome were obtained. The strategies of surface sterilization with formaldehyde (36%) and Illumina-based sequencing is suitable for analysis of endophytic actinobacterial populations in further studies.

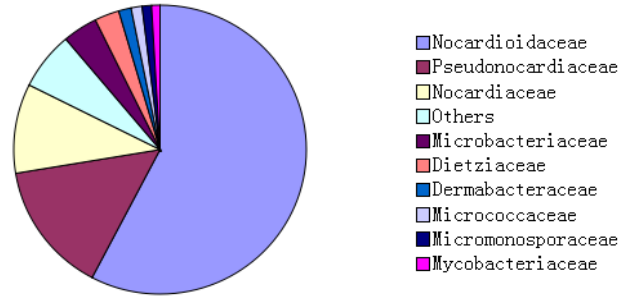


Figure 4 Abundances of different actinobacterial families in banana roots.

## CONFLICTS OF INTEREST

The authors have no conflicts of interest to declare.

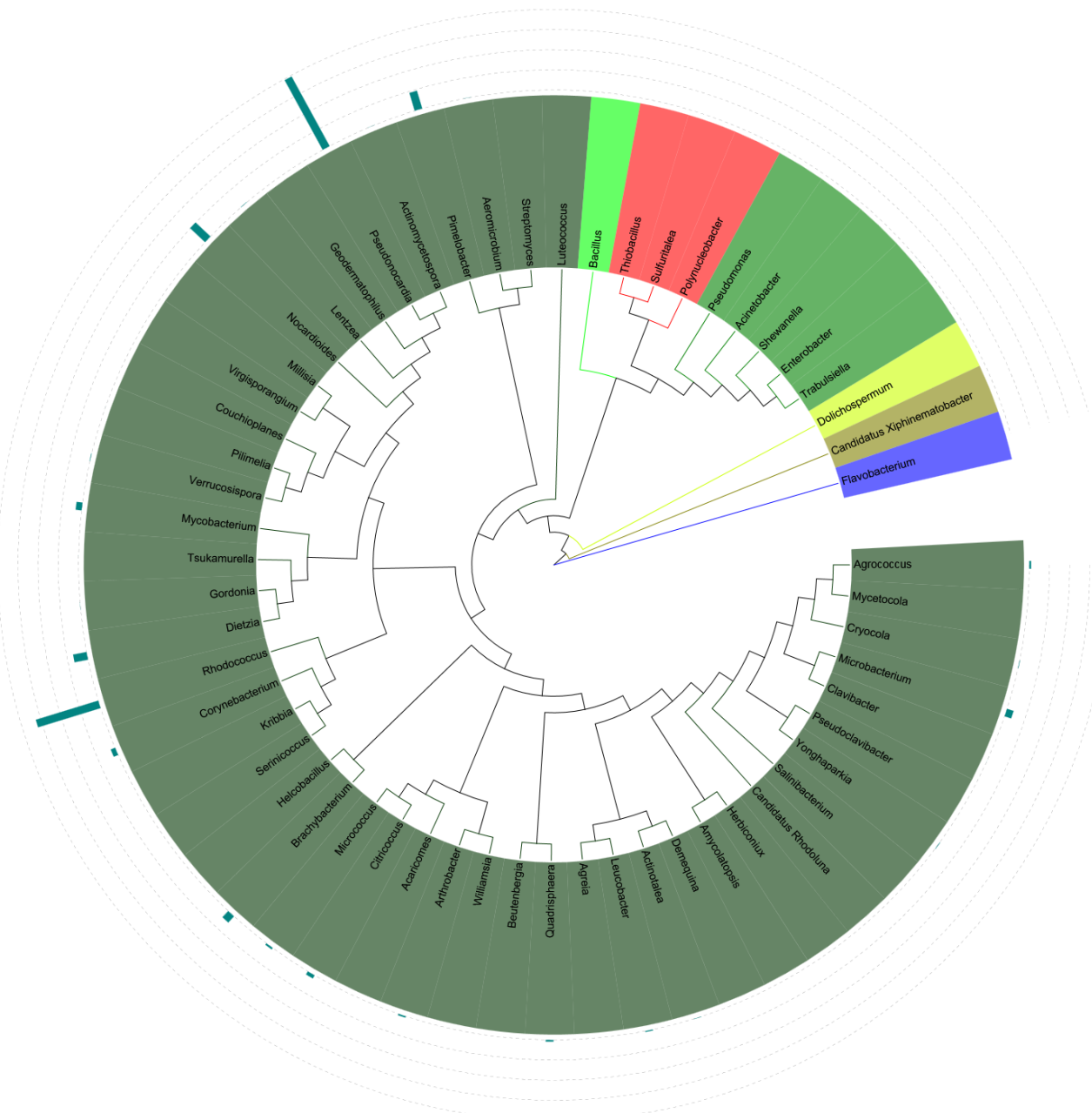


Figure 5 Phylogenetic distributions of actinobacterial taxa in in banana roots identified by illumina based analysis.



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