Investigating the endophytic bacterial diversity and community structures in seeds of genetically related maize (*Zea mays* L.) genotypes

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Abstract

This research aimed to investigate the composition and diversity of endophytic bacterial community in seeds of four hybrid maize and their parental lines, which was used to reveal the potential relationship and association of endophytic bacteria between maize genotypes and their genetic relevance. High-throughput sequencing (HTS) technology showed that a total of 1419 OTUs (46.6%) were parental lines unique and 1052 OTUs (34.5%) were hybrid varieties unique, with only 575 core OTUs revealed in all the samples. Most OTUs belonged to *Proteobacteria*. *Enterobacter* (23.2%), *Shigella* (21.2%), *Pseudomonas* (15.8%) and *Achromobacter* (10.1%) were the major genera; the bacterial community composition and diversity of endophytic bacteria were inconsistent among different seed genotypes. Based on principal component analysis (PCA), the results referred that the endophytic composition of hybrid sample showed obvious correlation with their female parental lines, and in ‘Jingke968’ and ‘MC738’ with the same female line the endophytic community was more similar than other hybrid samples. This was the first ever use of HTS technology for investigating the endophytic bacterial diversity and community structures in seeds of genetically related maize genotypes, it was shown that, there were core microbes shared among all genotypes of seed samples, and the female parental line was more significant to impact on the composition of their hybrid seeds than male parental line. This study would provide scientific clues for the future research on the vertical transmission of endophytes among maize generations.

Keywords Maize · Seeds · Endophytic bacteria · Diversity · HTS

Introduction

Maize is known as the queen of cereals which is the major source of carbohydrates, it is one of the significant crops around the globe (Zhao and Wang 2013; Bodhankar et al. 2017; Liu et al. 2017a). Seed is the basic source of reproduction in plants. It does not only preserve and transmit genetic

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information, but it also keeps ensured adaptive strategy for plants to reproduce under stress (Guan 2009). Further, it also carries many useful bacteria and other pathogenic organisms. According to many theorists, there exist many microbial communities over the surface and even inside the seeds (Nelson 2004; Liu et al. 2013). Studies on the physiology and pathology of seeds have confirmed that when the microbial community in a plant seed has a special relationship with the seed, it has a significant impact on plant health and soil fertility. (Bacilio-Jiméne et al. 2001; Cottyn et al. 2001; Barea et al. 2005).

Plant endophytes are microbes colonizing plant internal tissues and ubiquitously associated with plants; they play key role in plant growth and health, and subsequently establish a harmonious relationship with the host plant (Klopper and Beauchamp 1992; Yang et al. 2017). Endophytes can directly or indirectly affect the physiology and anatomy of plant, may do impact on other plant growth promoting activities (Liu et al. 2013). The endophytic bacteria in hybrid offsprings could be inherited from their parental lines through the vertical transmission, and also affected by other factors during the breeding and growth period. There have been reports that, there are two major sources of endophytes in plants, one is the external environment of the surface of plants, and the other is the seed (Hardoim et al. 2008; Mano and Morisaki 2008; Liu et al. 2018).

In recent years, with the rapid development in plant microecology and microbiology research, the biological functions and mechanism of endophytic bacteria on plant growth and development have become a research hotspot in agricultural biology. Deciphering the interaction between plants and their endophytes is also a hot topic in plant sciences, agronomy and ecology (Vandenkoornhuyse et al. 2015). At present, there are many reports on the integration of microorganisms with host plant seeds using culture-dependent method (Mastretta et al. 2009; Liu et al. 2009; Jiang et al. 2013), 16S rDNA clone library method (Liu et al. 2011, 2012a, 2013; Zou et al. 2012), denaturing gradient gel electrophoresis (DGGE) (Hardoim et al. 2012) and high-throughput sequencing (HTS) method (Liu et al. 2014, 2017a, 2018; Huang et al. 2016; Qin et al. 2016; Zhang et al. 2018). The subject and research progress on plant microecology is gradually developing (Liu et al. 2017a). Seed microbes may play an important role in plant productivity through influencing the primary composition of the plant microbiota. Nevertheless, the diversity and structure of seed-associated microorganisms have often been ignored, especially seed endophytes are still not well understood (Shade et al. 2017; Zhang et al. 2018).

Besides that, seeds serve as the initial inoculum for plant microbiota, transmitting microorganisms vertically from generation to another (Shade et al. 2017). The seed microbes transmitted across generations have profound

impacts on plant health, quality, productivity and microecology (Truyens et al. 2015; Nelson 2017). Although three main pathways of transmission between plant generations have been well described but the necessity and importance of vertical transmission remains unclear (Vandenkoornhuyse et al. 2015; Shade et al. 2017).

In this study, the seeds of four hybrid maize combinations and their parental lines independently developed and cultivated by China were used as research plant materials. To understand the composition and diversity of endophytic bacterial community of different seed genotypes and the vertical genetic correlation on endophytic bacteria among maize parental lines and their hybrid combination, high-throughput sequencing (HTS) technology based on Illumina Hiseq 2500 platform was used to study the community structure and diversity of their endophytic bacteria in hybrid combinations and their parental lines. This study provides a seed-source microbial ecological basis for the analysis of high quality and high yielding mechanism of hybrid maize, and supplies a theoretical basis for promoting the application of the idea of “plant and microbial symbiotic breeding (Wang et al. 2015; Liu et al. 2018)” in major and essential agricultural crops.

Materials and methods

Sample collection

Seeds were collected from four varieties of hybrid maize combinations (*Zea mays* L.) (Jingke665, Jingke968, MC738, Jingnongke728) and their parental lines (Jing 725, Jing 92, Jing 724, Jing 2416, MC01), which are self-developed and extensively planted in China, supplied by Professor Jiuran Zhao at Beijing Academy of Agriculture and Forestry Sciences (BAAFS). Among them, the variety ‘MC01’ is a unique low-heritability variety proven from previous breeding and genetics study. The detailed information about the maize seeds and the genetic relationships among the samples are shown in Fig. 1. All of the samples were collected from the scientific research base of BAAFS in Sanya, Hainan (18.36774333340131 N, 109.18169975280762 E, southern China) in March 2018; each seed sample was randomly collected from ten plants located in five sites and the base and the weight of each sample was approximately 1000 g; and the seed samples were stored at 4 °C in aseptic bags for 2 weeks prior to conduction of experiment.

Surface sterilization of samples

Firstly, the husks of each maize seed sample were removed by a small sheller. Then, under aseptic conditions the following operations were performed in the order listed: husked

Fig. 1 Genetic relationships among the four hybrid combinations. P1, Jing 725; P2, Jing 92; P3, Jing 724; P4, Jing 2416; P5, MC01; C1, Jingke665; C2, Jingke968; C3, MC738; C4, Jingnongke728

seeds were washed three times with prepared sterile water; 2.5 g of seeds were placed in a clean and sterile 50-mL tube containing 25 mL of phosphate buffer (Zhang et al. 2018), and the seeds were sonicated twice by an Ultrasonic Processor Scientz-IID sonicator (Ningbo Scientz Biotechnology Co., Ltd, China) at low power (237.5 W; 950 W×25%) in an ice bath for 5 min (alternating thirty 2-s bursts and thirty 2-s rests) (Lundberg et al. 2012; Zhang et al. 2018). To validate that the surface was sterilized, sterile tweezers were used to press surface-sterilized seeds into LB medium (LUQIAO), and the seeds were incubated at 30 °C for 72 h (Bodhankar et al. 2017; Zhang et al. (2018) and after incubation, there was no colony formed on LB medium, so the result of surface sterilization of samples met the requirements for subsequent experiments.

DNA extraction, 16S rRNA gene amplification and sequencing

DNA was extracted using the FastDNA® SPIN Kit for Soil (MP Biomedicals, Solon, OH, USA) according to the manufacturer's protocol after grounding into a fine powder in liquid nitrogen. Then 799F (AACAGGATTAGATACCCTG) and 1492R (GGTTACCTTGTTACGACTT) were used for the first-round amplification of 16S rRNA gene. Then the 750 bp fragment amplified from endophytic bacteria was cut and used as the template for the second-round amplification for V6-V8 regions (968F: AACGCGAAGAACCTTAC and 1378R: CGGTGTGTACAAGGCCCGGAACG). The amplified products were purified and then sequenced on MiSeq instrument using the MiSeq® Reagent Kit v3 (600 cycle) (Illumina).

Bioinformatics and statistical analysis

The assembled paired FASTQ files were performed by Mothur (version 1.39.0) (Schloss et al. 2011). Briefly, paired sequence reads were assembled after removing raw reads with low quality, such as read length less than 50 base pairs, or reads without the valid index. The high-quality DNA sequences were grouped into OTUs (Operational taxonomic units) under the threshold of 97% identity in comparison to SILVA reference database (V119) (Quast et al. 2013). Community richness, evenness and diversity analysis (Shannon, Simpson, ACE, Chao and Good's coverage) were

performed using Mothur. The t-test (with 95% confidence intervals) was used to determine whether the means of evaluation indices were statistically different and *p* values < 0.05 were considered as significant standard. Taxonomy was assigned using the online software RDP classifier (Wang et al. 2007) at default parameter (80% threshold) based on the Ribosomal Database Project (Cole et al. 2009). Genera and family abundance differences among samples were analyzed by Metastats (White et al. 2009). Spearman correlation coefficient between two variables was calculated using the R command 'cor.test'. The sequence data were submitted to NCBI Sequence Read Archive (<https://www.ncbi.nlm.nih.gov/sra/>) with BioProject and accession number.

Results

Sequencing data for diversity analysis

The raw high-throughput sequencing data was submitted to the NCBI database with BioProject number PRJNA510484 and accession number SRR8357070-SRR8357096. Nine seeds samples containing three repetitions for each were separately collected from five parental lines and four hybrid varieties (Fig. 1). A total of 2,513,380 (24,042—197,606) 16S rRNA genes were obtained by high-throughput DNA sequencing, and a minimum of 24,042 reads was applied as the criteria for data normalization in this study. Operational taxonomic unit (OTU), the group of 16S rRNA genes, was set at 97% similarity to estimate the diversity and richness of bacterial community, using a normalized read number 24,042. A total of 3046 OTUs (1994 of parents and 1627 of varieties) were obtained. The number of OTUs in the nine samples was roughly the same, indicating that the endophytic bacteria have some similarity at the host species level. The Good's coverage was both 99.4% for parental lines and hybrid varieties (Supplementary Table 1), that meant the sequencing depth was sufficient for microbiota investigation.

Endophytic bacterial populations in maize seeds

A total of 1419 OTUs (46.6%) were parental lines unique while 1052 OTUs (34.5%) were hybrid varieties unique (Supplementary Fig. 1a), with only 575 OTUs (core OTUs) existing in both groups. However, over 98% of sequenced

16S rRNA genes were contained in the core OTUs (Supplementary Fig. 1b), and the average reads number was 2.4 in each parental line unique OTUs and 2.9 in each variety unique OTUs. It can be seen from the experimental results that most of the core OTUs in hybrid offsprings are consistent with their parental lines, and it can be concluded that the core OTUs are inherited by the parental lines. However, the existence of a small number of unique OTUs may be affected by other factors such as reproduction and growth period.

Low quality and potentially contaminated DNA sequences were effectively removed through quality control and testing. At the phylum level, 544 out of the 575 core OTUs could be classified with 511 in Proteobacteria, 23 in Firmicutes and ten in Actinobacteria. While at the genus level, 35 genera were revealed, with *Enterobacter* (23.2%), *Shigella* (21.2%), *Pseudomonas* (15.8%) and *Achromobacter* (10.1%) as major microbiota. Besides these major microbiota, the proportion of other microbiota such as *Propionibacterium*, *Erwinia*, *Haemophilus* etc. was also similar to that of the female parent lines in the hybrid offspring (Fig. 2a). In addition, 34 OTUs were classified into species level (24 species), with *Enterobacter aerogenes*, *Pseudomonas veronii*, *Propionibacterium acnes* and *Erwinia dispersa* as most abundant (Fig. 2b). While on genus and species level, the bacterial community composition and group abundance of endophytic bacteria were obviously inconsistent from nine different seed genotypes, and the core seed microbiome was shared among different plant genotypes. There was a certain similarity among generations, and more emphasis was placed on the parental lines.

The correlation of endophytic composition between parents and hybrid varieties

Among the 575 core OTUs, the abundance of 11 OTUs showed significant difference (p value < 0.05) between male and female parents, while 17 OTUs showed significant richness difference between male parent line and hybrid variety, with only nine OTUs showing significant richness difference between female parent line and hybrid variety (Supplementary Fig. 2). The difference between hybrid offspring and female parent line was less than that of male parent line.

Principal component analysis (PCA) was performed to investigate whether the endophytic bacteria community was similar between parental lines and their hybrid varieties (Fig. 3 and Supplementary Fig. 3). Female parental line sample P3 showed similar bacterial composition with its two hybrid varieties sample C2 and C3, and female parent sample P1 located near its hybrid variety sample C1. The endophytic composition of hybrid sample C1, C2, C3 showed obvious correlation with their female parental lines. In particular, endophytic bacterial structure and diversity also showed differences among offspring with common

parents. The samples C2 and C3 with the same female parent were more similar than samples C1 and C2, and samples C3 and C4 with the common male parent. However, the female parent sample P5 contained different endophytic bacterial composition from its variety sample C4, which may be potentially due to the characteristic of low heritability of sample P5.

In addition, the hierarchical clustering heatmap (Fig. 4) also supported the above results that each variety had more similar bacteria community with its female parental line. Then the correlation coefficients were calculated among different samples using the Spearman test (Table 1) and it showed that except for sample C4, all the other three hybrid varieties showed the highest correlation with their female parental lines. From the results mentioned above, it is indicated that the composition of female parental lines might have a higher influence on the composition of hybrid progenies than the ones from male parental, and the heritability of the maize parental line would be a potential factor to influence on the vertical transmission of endophytes among maize generations.

Discussion

Compared with rice and soybean, in recent years, although investigations on bacteria associated with plants have been increased with each passing day (Liu et al. 2018). Research reports on endophytic bacteria in maize were relatively rare, and mainly concentrated in association with maize roots (Liu et al. 2013; Mehmood et al. 2018; Gao et al. 2018), while the existing research reports on endophytic bacteria in maize seeds are relatively limited (Liu et al. 2017a). Using culture-dependent method to investigate the endophytic bacterial communities in seeds of six hybrid maize varieties, *Pseudomonas*, *Microbacterium*, *Enterobacter*, *Bacillus* and *Pantoea* were isolated and identified from the seeds (Li et al. 2016). While *Enterobacter*, *Burkholderia*, *Pseudomonas*, *Sphingomonas*, and *Limnobacter* were detected by 16S rDNA clone library method from seeds of several hybrid maize and their parental lines (Liu et al. 2011, 2012a, b, 2013), and *Paenibacillus* sp. 5L8 isolated from the seeds of maize (*Zea mays* L., Jingke 968) had comprehensive and effective antagonistic activity against the common plant pathogens *Fusarium graminearum*, *Bipolaris maydis*, *Bipolaris sorokiniana* etc. (Liu et al. 2016a). High-throughput sequencing (HTS) contributes to gain more insight into rich endophytic bacteria in maize seeds, *Pantoea*, *Enterobacter*, *Aeribacillus*, *Sphingomonas* and *Halomonas*, were identified as the dominant species in different hybrid maize combinations (Chen et al. 2016; Liu et al. 2017a). It is worth mentioning that, there are also some new species of bacteria in maize seeds, *Paenibacillus zeae* sp. nov., *Paenibacillus*

Fig. 2 Endophytic bacterial distribution in different maize seeds. **a** The composition distribution and relative abundance on genus-level. **b** The composition distribution and relative abundance on species-level

chinensis sp. nov. and *Bacillus ciccensis* sp. nov. were isolated from maize seeds and proposed as new species through microbial polyphasic taxonomic approach (Liu et al. 2015, 2016b, 2017b).

In this study, 35 genera were revealed with *Enterobacter*, *Shigella*, *Pseudomonas* and *Achromobacter* as major microbiota in nine seeds samples. Among them *Enterobacter* and *Pseudomonas* as endophytic dominant genera were

frequently detected from seeds of several plants (Liu et al. 2009; Jiang et al. 2013; Sobolev et al. 2013; Zhang et al. 2018; Verma and White 2018). At present, there are some studies on the functions of endophytic bacteria (Ribeiro et al. 2018; Jai et al. 2019). Some species of *Enterobacter* and *Pseudomonas* genera have been previously found as the common plant growth-promoting bacteria (PGPB), which may directly or indirectly influence the growth, health

Fig. 3 Principal component analysis (PCA) analysis with Bray–Curtis dissimilarity based on OTUs. Points represent female parent lines, triangles represent male parent lines, and squares represent hybrid variety

Fig. 4 Heatmap of OTUs in nine seed samples constructed by hierarchical cluster analysis. The dendrogram at the top demonstrates the similarity of seed samples between parental lines (P) and hybrid varieties (C)

and development of host plants (Liu et al. 2012a, b). Yang et al. (2017) showed inoculation with *Enterobacter cloacae* induces rice self-resistance and thus resisting infection by subsequent pathogens. *Enterobacter* sp. SA187 had been isolated from the indigenous desert plant, and could be able to provide abiotic stress tolerance to *Arabidopsis thaliana* (Andrés-Barrao et al. 2017). A novel *Enterobacter cancerogenus* MSA2 was isolated from the rhizosphere of *Jatropha curcas* which is potentially important biofuel and feed stock plant (Jha et al. 2012). Recent research demonstrated that *Pseudomonas chlororaphis* subsp. *aureofaciens* strain M71 exerts beneficial effects on plant metabolism and primes tolerance mechanisms against biotic stresses and water stress in tomatoes (Brilli et al. 2018). *Pseudomonas fluorescens* DUS1-27 could increase the growth of *Brassica napus* L. (canola) over that of uninoculated control plants in a soil-based system. Endophytic bacterium *Pseudomonas fluorescens* Sasm05 may be attributed to its ability to produce indole-3-acetic acid and enhance phytoremediation (Chen et al. 2017) and *Pseudomonas* spp. OFT5 can improve nutrient uptake and plant growth under moderate salt-affected conditions by reducing stress-related ethylene levels and enhance NaCl stress tolerance by reducing stress-related ethylene production, resulting in improving growth, photosynthetic performance, and ionic balance in tomato plants (Win et al. 2018).

Shigella and *Achromobacter* are common soil bacterial groups (Santamaría and Toranzos 2003; Adam et al. 2014; Farhadkhani et al. 2018; Egea et al. 2017; Furlan and Stehling 2017; Gunasekera et al. 2018; Thulasi et al. 2018;

Table 1 Spearman correlation coefficients (R^2) between different samples

Sample	C1	C2	C3	C4	P1	P2	P3	P4	P5
C1	1	0.129	0.1655	0.1227	0.4912	0.1328	0.1714	0.1551	0.1414
C2	0.129	1	0.7434	0.6883	0.0663	0.5908	0.8582	0.7311	0.2644
C3	0.1655	0.7434	1	0.7201	0.0386	0.5816	0.9384	0.5421	0.3272
C4	0.1227	0.6883	0.7201	1	0.0603	0.6029	0.7354	0.5845	0.2772
P1	0.4912	0.0663	0.0386	0.0603	1	0.0996	0.0554	0.1561	0.0505
P2	0.1328	0.5908	0.5816	0.6029	0.0996	1	0.6235	0.6112	0.3713
P3	0.1714	0.8582	0.9384	0.7354	0.0554	0.6235	1	0.6263	0.3543
P4	0.1551	0.7311	0.5421	0.5845	0.1561	0.6112	0.6263	1	0.2638
P5	0.1414	0.2644	0.3272	0.2772	0.0505	0.3713	0.3543	0.2638	1

Garrido-Sanz et al. 2018; Fang et al. 2018), and they could colonize in plant tissues through plant roots (Liu et al. 2011). *Pantoea*, *Bacillus*, *Burkholderia*, *Sphingomonas*, *Limnibacter*, *Methylobacterium* and so on often separated or identified by high frequency from various plant seeds were also detected from this research (Mantica et al. 2010; Zhang et al. 2018), some species in these genera can also improve the plant growth and production (Liu et al. 2012a, 2018).

From the results of this study, the composition and diversity of endophytic bacterial community were obviously different among the seeds of four hybrid maize combinations and their parental lines. However, there was a certain similarity in endophytic diversity among genetically related varieties (Khalaf et al. 2016; Truyens et al. 2015), and the female parental line showed more affection on the endophytes of its hybrid variety. Although the endophytic diversity and group abundance were obviously different in each genotype, the great part of the seed microbiome was shared in the same plant. In recent years, during our research group excavating the endophytic microbial resources of maize seeds, progeny endophytic bacterial community diversity is affected not only by host genotype, but also by vertical transmission between generations (Liu et al. 2011, 2012a, b, 2013, 2017a, b; Frank et al. 2017). Besides, in this study it showed that the composition of female parental lines might have a higher influence on the composition of hybrid progenies than the ones from male parental, and several reports on the correlation of plant genotype and seed bacterial microbiome clearly achieved consistent research conclusions (Simon et al. 2001; Adams and Kloepper 2002; Sun et al. 2005; Picard and Bosco 2006; San et al. 2007; Chen et al. 2009; Zou et al. 2012; Liu et al. 2018; Zhang et al. 2018). This is the first time to select a unique low-heritability variety sample as a female parental line for hybridization, and it also reflected that the heritability of the maize parental line would be a potential factor to influence on the vertical transmission of endophytes among maize generations, and the related mechanisms need further research to be confirmed.

The 16S rDNA analysis using high-throughput sequencing first appeared in 2006, which applied to analysis

deep-sea microbial communities (Sogin et al. 2006), and this technology has since been widely used in the field of microbial ecology. HTS technology could overcome the shortcomings of traditional molecular biology methods, and analyze microbial community structure from the level of genome, breaking through the technical bottleneck that many endophytic microorganisms cannot or difficult to be isolated and cultured, and efficiently detect low-abundance but important endophytic bacteria in plant (Chen et al. 2016). This is first ever use of HTS technology for investigating the composition and diversity of endophytic bacterial community in seeds of hybrid maize varieties and their parental lines, and to reveal the endophytic bacterial composition in maize seeds and the correlation between endophytic bacterial diversity with seed genotypes. From this research, it also indicated that female parental line is more important to impact on the composition of their hybrid seeds than male parental line, and the heritability of the maize parental line would be a potential factor to influence on the vertical transmission of endophytes among maize generations. This study laid a necessary theoretical foundation for the further study on the interaction between maize plant generations and endophytic microbes during the vertical transmission process, and also provided a basis of microbial ecological research and practical applications for high quality maize breeding.

Conclusion

In this study, 575 core OTUs were revealed in all seed samples which assigned to the phylum Proteobacteria, Firmicutes and Actinobacteria; and *Proteobacteria*, and *Enterobacter* (23.2%), *Shigella* (21.2%), *Pseudomonas* (15.8%) and *Achromobacter* (10.1%) were the major endophytic genus. The results also referred that the endophytic composition of hybrid sample 'Jingke665', 'Jingke968', 'MC738' showed obvious correlation with their female parental lines, in particular the endophytic community structure and diversity of sample 'Jingke968' and 'MC738' with the same female

parent were more similar than sample 'Jingke665' and 'Jingke968', and sample 'MC738' and 'Jingnongke728' with the common male parent. Besides, a unique low-heritability variety sample 'MC01' was specially selected as a female parental line for hybridization, and unlike other varieties in this study, there was a significant difference of endophytic diversity shown between 'MC01' and its hybrid offspring 'Jingnongke728'.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Research involving human participants and/or animals This article does not contain any studies with human participants or animals performed by any of the authors.

Informed consent Not applicable.

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