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ORIGINAL ARTICLE

Investigation on diversity and population succession dynamics of endophytic bacteria from seeds of maize (*Zea mays* L., Nongda108) at different growth stages

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Abstract Plant seeds are carriers of both beneficial bacteria and pathogens. Using the 16S rRNA gene clone library technique, we conducted a preliminary study on the community diversity and population succession dynamics of endophytic bacteria in seeds of reciprocal cross hybrid maize at different seed developmental stages. In both hybrid lines (108A and 108B), more types of endophytic bacteria were found at the proembryo-forming stage than in the other two stages, including 29 and 23 bacterial operational taxonomic units (OTUs), respectively. *Undibacterium* (39.20 and 30.00 % in 108A and 108B, respectively) was the first dominant bacterium to appear. At the milky stage, fewer types of endophytic bacteria in 108A and 108B appeared, including 18 and 16 OTUs, respectively, and the abundance of the dominant genus *Burkholderia* in the two seed samples reached 73.38 and 80.43 %, respectively. *Limnobacter* appeared as the second and third endophytic dominant bacterium in 108A (4.55 %) and 108B (5.07 %), respectively, in both seed samples. At the dough stage, the abundance of the first dominant bacterium, *Burkholderia*, in 108A and 108B was 78.26 and 84.80 %, respectively. *Pantoea* appeared as the second endophytic dominant bacterium in the both seeds (9.42 and 4.80 % in 108A and 108B, respectively). This is the first study on endophytic bacteria present during several crucial stages of the dynamic grain growth process of plant seeds conducted using culture-independent methods.

Keywords Reciprocal cross maize seed · Endophytic bacteria · Bacterial diversity · Population succession · 16S rRNA gene library

Abbreviation

CTAB Cetyltrimethylammonium bromide
PGPB Plant growth-promoting bacteria

Introduction

Endophytic bacteria refer to those microbes that were able to colonize in the tissues of healthy plants and subsequently establish a harmonious relationship with the plant (Kloepper and Beauchamp 1992). They are able to influence plant growth directly or indirectly through biological control, plant growth-promoting effects, endophytic nitrogen-fixing activity, and other actions (Glick et al. 1999).

Plant seeds are not merely reproductive organs and/or important tool in agricultural production (Guan 2009), they are also carriers of various beneficial bacteria as well as pathogens. A number of studies have shown that there are

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many microbial communities on the seed surface and within the seed body (Nelson 2004). These seed and soil-born microbial communities are promoted during the seed germination process (Bacilio-Jiméne et al. 2001; Cottyn et al. 2001), resulting in significant impacts on plant health and soil fertility when the microbes interact positively with the plant (Barea et al. 2005).

It has been reported that endophytic bacterial communities of seeds could be influenced by seed status (Mundt and Hinkle 1976), nutrient components (Song et al. 2008), tissue parts (Cankar et al. 2005; Kutschera 2002), germination process (Coombs and Franco 2003; Ferreira et al. 2008), and plant genotype (Adams and Kloepper 2002; Mavingui et al. 1992; Michiels et al. 1989; Neal et al. 1973; Simon et al. 2001; Song et al. 1998). Moreover, a few studies have pointed out that during seed growth, seed microbial communities are dynamic, resulting in changes in the varieties, composition, and location of microbes. In addition to affecting seed development, the accumulation of and variation in nutrients (e.g., starch) also influence the composition of the indigenous microbial communities (Majewska-Sawka and Nakashima 2004; Mano et al. 2006; Nelson 2004). To date, except for plant pathogens, studies on bacteria associated with seeds have lagged far behind those on rhizosphere bacteria (Cankar et al. 2005). Johnston-Monje and Raizada (2011) found that seed endophyte community composition varied in relation to plant host phylogeny and that there was a core microbiota of endophytes that was conserved in maize seeds across boundaries of evolution, ethnography, and ecology. However, apart from some knowledge of the variety, genotype, and phylogeny of maize, there are even fewer studies focusing on the community diversity and succession of endophytic bacterial communities at different seed developmental stages. Studies on these aspects would contribute to future studies on the mechanisms of interaction between seeds and endophytic bacteria and establish the relationship between plant developmental stages and its endophytic bacterial community.

In the study reported here, we used the 16S rRNA gene clone library technique, in the absence of culture methods, to study community diversity and the succession of endophytic bacteria in self-cultivated seeds of the Chinese reciprocal cross hybrid maize (*Zea mays* L., Nongda108) at different seed developmental stages.

Materials and methods

Maize seed sampling and surface sterilization

In this paper the term test samples refers to the seeds of reciprocal cross maize (*Zea mays* L., Nongda108) at different seed growth stages, namely, 12 days (the proembryo-forming stage), 25 days (the milky stage), and 40 days (the dough stage) after pollination: Nongda108A (Huang C×178) and Nongda108B (178×Huang C) (supplied by Prof. Jianhua Wang, China Agricultural University). The plants were grown in open field of the Beijing Shangzhuang Experimental Station of China Agricultural University (40.13°N, 116.20°E), and seed samples (5.0 g for each sampling from 3 plants) were collected in August 2010, and 5.0 g seeds and stored at 4 °C.

Maize seeds of the same genotype were pooled as a single sample to average the deviations in the endophytic bacterial community among the seeds, The samples were then washed with sterile water, immersed in 70% alcohol for 3 min, washed with fresh sodium hypochlorite solution (2.5 % available Cl^-) for 5 min, rinsed with 70% alcohol for 30 s, and finally washed 5–7 times with sterile water (Sun et al. 2008). Aliquots of the final rinsing water were spread on Luria–Bertani (LB) solid medium plates and cultured for 3 days at 28 °C for detection of bacterial colonies in order to examine the effect of the surface sterilization. The seed samples that were not contaminated, based on the culture-dependent sterility test, were used for subsequent analysis.

DNA extraction and PCR amplification of the bacterial 16S rRNA gene

About 5.0 g of surface-sterilized maize seeds were frozen with liquid nitrogen, quickly ground into a fine powder in the precooled sterilized mortar, and then divided into five sterilized centrifugal tubes. The DNA of the maize seed samples was extracted using the CTAB procedure (Xie et al. 1999). The DNA was then resuspended in 30 μL sterile Milli-Q water.

The pair of primers 799f (5′-AACAGGATTAGATACCCTG-3′) and 1492r (5′-GGTTACCTTGTTACGACTT-3′) which can separate bacterial products and the maize mitochondrial product were used to amplify the seeds' indigenous bacterial 16S rDNA (Sun et al. 2008). A 50-μL volume of PCR reaction mixture contained 50 ng of DNA extract, 1×Taq reaction buffer, 20 pmol of each primer, 200 μmol each dNTP, and 1.5 units Taq enzyme (Fermentas, Thermo Fisher Scientific, Vilnius, Lithuania). The cycling procedure consisted of an initial denaturation cycle at 94 °C for 5 min, followed by 30 cycles of denaturation at 94 °C for 1 min, annealing at 52 °C for 1 min, and elongation at 72 °C for 1 min, with a final extension at 72 °C for 10 min. The PCR products were then electrophoretically separated and the target band excised and purified by Wizard SV Gel and the PCR Clean-up System (Promega, Madison, WI) as described by the manufacturer.

Construction of the 16S rRNA gene clone library

The purified PCR products were ligated into the T3 vector according to the protocol supplied by the manufacturer

(Transgen, China). *Escherichia coli* DH5α competent cells (Transgen) were transformed with the ligation products and spread onto LB agar plates containing ampicillin (100 μg/mL) and X-gal/IPTG on the surface for standard blue and white screening. White colonies were randomly picked and cultured in liquid LB overnight.

Sequencing and phylogenetic analysis

A total of 160 clones for each sample were randomly chosen for partial 16S rRNA gene sequencing in the ABI 3730 DNA sequencer (ABI, Foster City, CA). All of the nucleotide sequences, approximately 700 bases, were compared with the NCBI database using BLASTN or aligned by the identify analysis of EzTaxon server 2.1 (Chun et al. 2007). Sequences with a >97% similarity were assigned to the same species. The sequences were aligned using CLUSTALW (Thompson et al. 1994), and tree constructions were performed with the MEGA 4 program package using the neighbor-joining method (Tamura et al. 2007).

Evaluation of the size of the clone library

Two approaches were used to estimate the extent of the clone library. To estimate the representation of the library, we calculated the clone coverage with the following equation: $C=(1-n1/N)\times 100$ %, where n1 is the number of single clones, and N is the total number of clones in the clone library (Good 1953; Mullins et al. 1995). Diversity of the clone library was investigated by rarefaction analysis. Rarefaction curves were calculated using the freeware program aRarefactWin.

Results

The DNA extracted from surface-disinfected maize seeds was used to amplify the bacterial 16S rRNA gene fragments using primers 799f and 1492r with the aim of obtaining specific amplification of the bacterial 16S rRNA gene fragments and excluding contamination from plant mitochondrial (mt)DNA (Sun et al. 2008). Nevertheless. after electrophoresis, two bands of PCR products were displayed on the agarose gel. One band of between 1,000 and 1,500 bp was identified as the maize mtDNA through sequencing and alignment; a second band, between 700 and 800 bp, was considered to be the target band containing the bacterial 16S rRNA gene fragments. The purified PCR products were used to construct a 16S rRNA gene clone library of maize endophytic bacteria. In total, 160 clones were randomly chosen for sequencing, and the sequences of positive clones were submitted to GenBank (accession no. HQ402934—HQ403050).

The endophytic bacteria identified in 108A at different seed growth stages, namely, at 12, 25, and 40 days after pollination, included 29 , 18, and 16 bacterial operational taxonomic units (OTUs), respectively, and the calculated coverage of the three clone libraries was 87.20, 93.55, and 92.75 %, respectively. The endophytic bacteria in 108B at the three-grain growth stages included 23, 16, and 15 OTUs, respectively, and the calculated coverage of the three clone libraries was 87.96, 92.75, and 91.20 %, respectively. The rarefaction curves shown in Fig. 1, combined with the calculated coverage value, suggest that these libraries detected a large majority of the endophytic bacterial diversity in the maize seeds used in our study.

Among the endophytic bacteria in 108A at the three different seed growth stages, the sequences related to Pro-

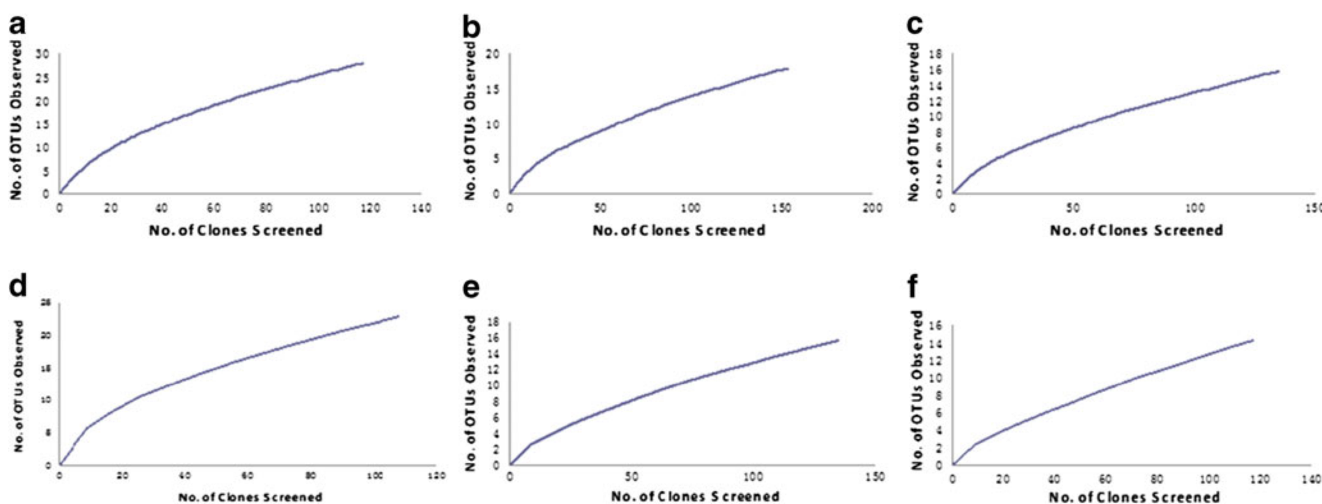

Fig.1 Rarefaction analysis of the seeds' endophytic bacterial 16S rDNA clone libraries at different growth stages of the reciprocal cross maize (*Zea mays* L., Nongda108). **a** 12 days, 108A, **b** 25 days, 108A, **c** 40 days, 108A, **d** 12 days, 108B, **e** 25 days, 108B, **f** 40 days, 108B. *108A* Nongda108A (Huang C×178), *108B* Nongda108B (178×Huang C), *OTU* operational taxonomic units

teobacteria made up the largest fraction of all three clone libraries (Tables 1, 2, 3). At 12 days of seed development (the proembryo-forming stage), among 125 clones analyzed, 116 clones (92.80 %) belonged to Proteobacteria, seven clones (5.60 %) to Bacteroidetes, one 1 clone (0.80 %) to Firmicutes, and one clone (0.80 %) to Actinobacteria. Among these, Proteobacteria, Bacteroidetes, Firmicutes, and Actinobacteria were made up of 24, three, one, and one bacterial OTU, respectively. *Undibacterium* (39.20 %), *Sphingomonas* (12.80 %), and *Acinetobacter* (9.60 %) were the dominant genera of the clone library. At 25 days of seed development (the milky stage), among 154 clones analyzed, 151 clones (98.05 %) belonged to Proteobacteria, two clones (1.30 %) to Firmicutes, and one clone, (0.65 %) to Bacteroidetes. Among these, Proteobacteria, Firmicutes, and Bacteroidetes were made up of 15, two, and one bacterial OTU, respectively. *Burkholderia* (73.38 %), *Pantoea* (12.34 %), and *Limnobacter* (4.55 %) were the dominant genera of the clone library. At 40 days seed development (the dough stage), among 138 clones, 131 clones (94.93 %) belonged to Proteobacteria, five clones (3.62 %) to Firmicutes, and two clones (1.45 %) to Actinobacteria. Among these, Proteobacteria, Firmicutes, and Actinobacteria were made up of 13, two, and one bacterial OTU, respectively. *Burkholderia* (78.26 %), *Pantoea* (9.42 %), and *Staphylococcus* (3.62 %) were the dominant genera of the clone library (Tables 1, 2, 3, 7).

Among the endophytic bacteria in 108B at the three different seed growth stages, the sequences related to Proteobacteria made up the largest fraction of all the three clone libraries (Tables 4, 5, 6). At 12 days of seed development (the proembryo-forming stage), among the 110 clones analyzed, 108 clones (98.18 %) belonged to Proteobacteria and two clones (1.82 %) to Firmicutes. Among these, Proteobacteria and Firmicutes were made up of 21 and two bacterial OTUs, respectively. *Undibacterium* (30%), *Serratia*

Table 1 Distribution of 16S rRNA clones detected from endophytes in the proembryo-forming stage of the reciprocal cross maize Nongda108A (Huang C×178)

Group	No. of OTUs	No. of clones	% Total clones	Closest NCBI match	% Identify
Proteobacteria	24	6	4.80	*Curvibacter gracilis* (AB109889)	100
		49	39.20	*Undibacterium* sp. EM 1 (GQ379228)	99
		8	6.40	*Pseudomonas lanceolata* (AB021390)	98
		1	0.80	*Pseudomonas plecoglossicida* (AB009457)	99
		16	12.800	*Sphingomonas echinoides* (AJ012461)	99
		7	5.60	*Acinetobacter beijerinckii* (AJ626712)	99
		2	1.60	*Acinetobacter radioresistens* (X81666)	99
		1	0.80	*Acinetobacter johnsonii* (X81663)	98
		1	0.80	*Acinetobacter* sp. (X81659)	99
		1	0.80	*Acinetobacter lwoffii* (X81665)	99
		2	1.60	*Rhizobium pisi* DSM 30132 (AY509899)	99
		6	4.80	*Burkholderia phytofirmans* (CP001053)	99
		1	0.80	*Burkholderia ginsengisoli* (AB201286)	97
		1	0.80	*Bradyrhizobium pachyrhizi* PAC48 (AY624135)	99
		2	1.60	*Pelomonas puraquae* (AM501439)	99
		2	1.60	*Achromobacter xylosoxidans* (Y14908)	100
		1	0.80	*Methylobacterium rhodesianum* (AB175642)	99
		1	0.80	*Xanthomonas sacchari* (Y10766)	99
		3	2.40	*Limnobacter thiooxidans* (AJ289885)	99
		1	0.80	*Agrobacterium larrymoorei* (Z30542)	99
		1	0.80	*Acidovorax facilis* (AF078765)	98
		1	0.80	*Pantoea agglomerans* (AJ233423)	99
		1	0.80	*Pantoea dispersa* LMG2603 (DQ504305)	99
		1	0.80	*Methylibium aquaticum* IMCC1728 (DQ664244)	97
Firmicutes	1	1	0.80	*Brevibacterium frigoritolerans* (AM747813)	99
Bacteroidetes	3	2	1.60	*Flavobacterium mizutaii* (AJ438175)	97
		1	0.80	*Flavisolibacter ginsengiterrae* (AB267476)	97
		4	3.20	*Chryseobacterium* sp. CPW406 (AJ457206)	98
Actinobacteria	1	1	0.80	*Arthrobacter nitroguaiacolicus* (AJ512504)	98

OTU, Operational taxonomic unit

Table 2 Distribution of 16S rRNA clones detected from endophytes in the milky stage of Nongda108A

Group	No. of OTUs	No. of clones	% Total clones	Closest NCBI match	% Identity
Proteobacteria	15	109	70.78	*Burkholderia phytofirmans* PsJN (CP001053)	100
		2	1.30	*Burkholderia gladioli* CIP 105410 (EU024168)	100
		2	1.30	*Burkholderia plantarii* (U96933)	99
		17	11.04	*Pantoea dispersa* LMG2603 (DQ504305)	99
		1	0.65	*Pandoraea sputorum* (AF139176)	98
		1	0.65	*Pantoea agglomerans* (AJ233423)	99
		7	4.55	*Limnobacter thiooxidans* (AJ289885)	100
		1	0.65	*Enterobacter cancerogenus* LMG 2693 (Z96078)	99
		1	0.65	*Pseudomonas hibiscicola* (AB021405)	99
		1	0.65	*Klebsiella pneumoniae* (Y17657)	99
		2	1.30	*Acinetobacter radioresistens* (X81666)	99
		3	1.95	*Methylophilus methylotrophus* (AB193724)	99
		1	0.65	*Massilia* sp. CCUG 58010 (FN814307)	98
		2	1.30	*Cronobacter turicensis* z3032 (EF059891)	100
		1	0.65	*Ochrobactrum pseudogrignonense* (AM422371)	100
Firmicutes	2	1	0.65	*Paenibacillus taejonensis* (AF391124)	99
		1	0.65	*Bacillus stratosphericus* (AJ831841)	99
Bacteroidetes	1	1	0.65	Uncultured *Saprospiraceae* bacterium (EU177734)	98

(17.27 %), and *Sphingomonas* (10.91 %) were the dominant genera of the clone library. At 25 days of seed development (the milky stage), all 138 clones analyzed belonged to Proteobacteria (100 %), including 16 OTUs. *Burkholderia* (80.43 %), *Limnobacter* (5.07 %), and *Cronobacter* (2.90 %) were the dominant genera of the clone library. At 40 days of seed development (the dough stage), among the 125 clones analyzed, 122 clones (97.60 %) belonged to Proteobacteria, two (1.60 %) to Firmicutes, and one (0.80 %) to Actinobacteria. Among these, Proteobacteria, Firmicutes, and Actinobacteria included 12, two, and one OTU, respectively. *Burkholderia* (84.80 %), *Pantoea* (4.80 %), and *Enterobacter*/*Escherichia*/*Acinetobacter* (1.60 %) were the dominant genera of the clone library (Tables 4, 5, 6, 7).

Table 3 Distribution of 16S rRNA clones detected from endophytes in the dough stage of Nongda108A

Group	No. of OTUs	No. of clones	% Total clones	Closest NCBI match	% Identity
Proteobacteria	13	108	78.26	*Burkholderia phytofirmans* PsJN (CP001053)	100
		1	0.72	*Klebsiella pneumoniae* subsp. *ozaenae* (Y17654)	99
		1	0.72	*Klebsiella pneumoniae* (Y17657)	99
		5	3.62	*Pantoea anthophila* LMG 2558 (EF688010)	99
		1	0.72	*Pantoea eucalypti* LMG 24197 (EF688009)	96
		7	5.07	*Pantoea agglomerans* (AJ233423)	99
		2	1.45	*Enterobacter cancerogenus* LMG 2693 (Z96078)	99
		1	0.72	*Pseudomonas* sp. C36 (AJ575816)	100
		1	0.72	*Vibrio* sp. LMG 20539 (AJ316193)	99
		1	0.72	*Salinivibrio costicola* (X95527)	99
		1	0.72	*Georgfuchsia toluolica* G5G6 (EF219370)	97
		1	0.72	*Steroidobacter denitrificans* FS (EF605262)	95
		1	0.72	*Thiobacter subterraneus* (AB180657)	93
Firmicutes	2	1	0.72	*Staphylococcus cohnii* subsp. *cohnii* (D83361)	100
		4	2.90	*Staphylococcus hominis* (X66101)	99
Actinobacteria	1	2	1.45	*Rothia amarae* (AY043359)	96

Table 4 Distribution of 16S rRNA clones detected from endophytes in the proembryo-forming stage of Nongda108B (178×Huang C)

Group	No. of OTUs	No. of clones	% Total clones	Closest NCBI match	% Identity
Proteobacteria	21	19	17.27	*Serratia marcescens* (AB061685)	99
		7	6.37	*Pseudomonas lanceolata* (AB021390)	98
		3	2.73	*Pseudomonas oryzihabitans* (AM262973)	100
		1	0.91	*Pseudomonas hibiscicola* (AB021405)	99
		1	0.91	*Enterobacter nimipressuralis* LMG 10245-T (Z96077)	99
		12	10.91	*Sphingomonas echinoides* (AJ012461)	99
		33	30.00	*Undibacterium* sp. EM 1 (GQ379228)	99
		1	0.91	*Acinetobacter beijerinckii* (AJ626712)	99
		1	0.91	*Acinetobacter schindleri* (AJ278311)	98
		1	0.91	*Acinetobacter lwoffii* (X81665)	98
		5	4.55	*Burkholderia phytofirmans* PsJN (CP001053)	100
		1	0.91	*Burkholderia ginsengisoli* (AB201286)	97
		11	10.00	*Curvibacter gracilis* (AB109889)	100
		1	0.91	*Achromobacter xylosoxidans* (Y14908)	99
		1	0.91	*Pantoea dispersa* LMG2603 (DQ504305)	99
		1	0.91	*Providencia rustigianii* (AM040489)	98
		1	0.91	*Perlucidibaca piscinae* IMCC1704 (DQ664237)	94
		1	0.91	*Brevundimonas* sp. BIO-TAS2-2 (FJ544245)	99
		3	2.73	*Limnobacter thiooxidans* (AJ289885)	100
		2	1.82	*Rhizobium pisi* DSM 30132 (AY509899)	99
		2	1.82	*Pelomonas puraquae* (AM501439)	99
Firmicutes	2	1	0.91	*Brevibacterium incertum* (Y14650)	99
		1	0.91	*Planomicrobium glaciei* 0423 (EU036220)	99

A consistent succession of community structures in endophytic bacteria could be seen in the transition from one growth stage to the next of 108A and 108B seeds, namely, 12 days (the proembryo-forming stage), 25 days (the milky stage), and 40 days (the dough stage) after pollination, (Tables 1, 2, 3, 4, 5, 6, 7). More types of endophytic bacteria in 108A and 108B (29 and 23 OTUs, respectively) appeared at 12 days of grain development than at the other two stages;

Table 5 Distribution of 16S rRNA clones detected from endophytes in the milky stage of Nongda108B

Group	No. of OTUs	No. of clones	% Total clones	Closest NCBI match	% Identity
Proteobacteria	16	111	80.43	*Burkholderia phytofirmans* PsJN (CP001053)	100
		4	2.90	*Cronobacter turicensis* z3032 (EF059891)	100
		1	0.72	*Acinetobacter radioresistens* (X81666)	99
		1	0.72	*Acinetobacter calcoaceticus* (X81661)	99
		1	0.72	*Pseudomonas hibiscicola* (AB021405)	99
		1	0.72	*Pseudomonas fragi* (AF094733)	99
		7	5.07	*Limnobacter thiooxidans* (AJ289885)	99
		1	0.72	*Enterobacter cancerogenus* LMG 2693 (Z96078)	99
		1	0.72	*Enterobacter oryzae* (EF488759)	98
		1	0.72	*Pantoea anthophila* LMG 2558 (EF688010)	99
		1	0.72	*Hydrocarboniphaga effusa* AP103 (AY363245)	93
		2	1.45	*Klebsiella pneumoniae* (Y17657)	100
		1	0.72	*Agrobacterium radiobacter* (AJ389904)	99
		2	1.45	*Achromobacter xylosoxidans* (Y14908)	99
		2	1.45	*Candidatus* Rhizobium massiliae (AF531767)	99
		1	0.72	*Psychrobacter urativorans* (AJ609555)	100

Table 6 Distribution of 16S rRNA clones detected from endophytes in the dough stage of Nongda108B

Group	No. of OTUs	No. of clones	% Total clones	Closest NCBI match	% Identity
Proteobacteria	12	105	84.00	*Burkholderia phytofirmans* PsJN (CP001053)	99
		1	0.80	*Burkholderia plantarii* (U96933)	98
		1	0.80	*Cedecea davisae* DSM 4568 (AF493976)	99
		5	4.00	*Pantoea anthophila* LMG 2558 (EF688010)	99
		1	0.80	*Pantoea agglomerans* (AJ233423)	99
		2	1.60	*Enterobacter amnigenus* (AB004749)	99
		1	0.80	*Serratia marcescens* (AB061685)	99
		2	1.60	*Escherichia hermannii* (AB273738)	99
		1	0.80	*Steroidobacter denitrificans* (EF605262)	96
		1	0.80	*Acinetobacter baumannii* (X81660)	100
		1	0.80	*Acinetobacter radioresistens* (X81666)	98
		1	0.80	*Enhydrobacter aerosaccus* (AJ550856)	99
Firmicutes	2	1	0.80	*Paenibacillus taejonensis* (AF391124)	99
		1	0.80	*Bacillus anthracis* str. *Ames* (AE016879)	99
Actinobacteria	1	1	0.80	*Corynebacterium* sp. CIP107291 (AJ438050)	99

dominance by any particular genus was not evident and the abundance of the three dominant genera in 108A and 108B was similar, with *Undibacterium* as dominant bacterium (Table 7). At 25 days of grain development, fewer types of endophytic bacteria in 108A and 108B were found compared with the previous stage; the abundance of the dominant genus *Burkholderia* in the two seed samples was remarkable, reaching 73.38 and 80.43 %, respectively; *Limnobacter* appeared as the second and the third endophytic dominant bacterium in 108A and 108B, respectively. At 40 days of seed development, the abundance of the dominant bacterium *Burkholderia* in 108A and 108B was similar to that in the previous stage, with 78.26 and 84.80 %, respectively; The dominance was still quite evident. Moreover, *Pantoea* appeared as the second endophytic dominant bacterium in 108A and 108B during this stage.

Table 7 Comparison of dominant genera from two seed samples at different growth stages

Growth stages	108A	108B
12	*Undibacterium* (39.20%)	*Undibacterium* (30.00%)
	Sphingomonas (12.80%)	*Serratia* (17.27%)
	Acinetobacter (9.60%)	*Sphingomonas* (10.91%)
25	*Burkholderia* (73.38%)	*Burkholderia* (80.43%)
	Pantoea (12.34%)	*Limnobacter* (5.07%)
	Limnobacter (4.55%)	*Cronobacter* (2.90%)
40	*Burkholderia* (78.26%)	*Burkholderia* (84.80%)
	Pantoea (9.42%)	*Pantoea* (4.80%)
	Staphylococcus (3.62%)	*Enterobacter* (1.60%); *Escherichia* (1.60%); *Acinetobacter* (1.60%)

Discussion

Seeds are the most important means of producing agricultural crops (Guan 2009; Hu 2006), but they are also carriers of various beneficial bacteria and pathogens. A large variety of microbial communities are found on the seed surface and within the seed body (Nelson 2004), and the physiological state of the seed influences the microbial communities which, in turn, have a significant impact on the health of the seeds and the plant (Gitaitis and Walcott 2007; Grum et al. 1998). However, compared to the large number of studies carried out on rhizosphere bacteria, few studies on bacteria associated with seeds have been reported (Cankar et al. 2005). Rijavec et al. (2007) reported the isolation of *Pantoea*sp., *Frigoribacterium*sp., *Microbacterium* sp., *Bacillus*sp., *Paenibacillus*sp., and *Sphingomonas*sp. from kernels of four different maize cultivars.

In order to ensure that the plant materials are as comparable as possible, the seeds of 108A and 108B, which are genetically related and produced in the same experimental plot, were selected as experimental material in this study. Both seed samples were pollinated and collected at the same time at each developmental stage. The reciprocal plant seed samples were first chosen to study the community diversity and population succession dynamics of their endophytes, in an attempt to understand the correlation between the differences in nutrient content, the agronomic traits of reciprocal plant seeds, and their endophytic bacteria. In this study bacteria present on the seed surface were washed off, sterilized, and consequently ignored in our analysis because the sources of such bacteria are very diverse and it is hard to prevent contamination from the environment. The different biological traits of reciprocal cross plants are mainly affected by cytoplasmic inheritance (Cui et al. 2010);

consequently, our results on whether or not our plants would be able to affect the endophytic community structure should provide new information on the influence of the cytoplasm on microbial endophytes of the seed.

Following fertilization of the pistillate flower of maize, the grain took shape and began to grow and develop. The developmental process usually consisted of four sequential stages, from fertilization to the mature grain:

1. The grain-forming stage, which occurred at 10–12 days after fertilization, is characterized by the appearance of the proembryo, a clear paste endosperm that is rich in water content and has little dry matter accumulation.
2. The milky stage occurred 15–35 days after fertilization and involves the general appearance of the embryo. The radicle, hypocotyl, and germ become evident, and the endosperm changes from gel to paste. The volume of the grain reaches a maximum, dry matter content increases linearly, and the water content decreases.
3. The dough stage occurred at 35–50 days after fertilization and is characterized by seeds with normal embryos. The endosperm changes from paste to wax, there is a continued accumulation of dry matter, and the grain begins to shrink. The volume became smaller, and dry matter accounts for over 70 % of the grain weight.
4. The late period occurs at 50–60 days after fertilization and is characterized by a slowing down of dry matter accumulation and a continued decrease in water content (Hu 2006; Wang and Yin 2005).

In order to be able to identify key developmental periods, we study selected three stages, namely, the proembryo-forming stage, the milky stage, and the dough stage, for study because grain changes were comparatively evident at these stages. In this way the relationship between grain changes and the community succession of endophytic bacteria could be established. Okunishi et al. (2005) isolated endophytic bacteria from rice seeds (*Oryza sativa*) sampled on days 10 (the early stage), 30 (the middle stage), and 60 (the late stage) after flowering (through culture-independent methods. These researchers found that at the early stage of maturation the endophytes were relatively diverse, including the genera *Bacillus*, *Sphingomonas*, and *Pantoea*. In contrast, at the middle and late stage the endophytes were all *Bacillus*, except for one isolate. This observation was confirmed by our results showing the lack of dominance of any one bacterial strain at the proembryo-forming stage when more kinds of endophytic bacteria were present than in the last two stages. In addition, the difference in abundance of the three endophytic dominant genera was less in the proembryo-forming stage than in other two stages, whereas the abundance of the dominant bacterium *Burkholderia* in both seed samples was very high at the milky and dough stages.

At the early stage of seed maturation, many more bacteria might be able to enter the seed because the seed is soft, thereby accounting for the relative diversity of endophytes at this stage. During the maturation process, the amount and concentration of water and dry substance in the seed change greatly, influencing the types of endophytic bacteria able to colonize the seeds. Endophytes that are more adapted to the new internal environment of the seed could survive and accumulate to become the dominant bacteria (Mano et al. 2006; Okunishi et al. 2005). Mano et al. (2006) reported that many endophytic bacteria resistant to high osmotic pressure are present at the late growth stage of rice seeds and that the abundance of endophytic bacteria with amylase activity increased significantly at this stage. Accordingly, the almost exclusive occurrence of *Burkholderia* inside the seeds of both hybrid maize lines at second and third stage might be result of adaptation to the internal environment and selection against less fit species. Because the seed inner environment at the milky stage was similar to that at the dough stage, the same predominant species was expected and indeed found at the second and third sampling.

To the best of our knowledge, this study is the first to focus on the community structure and succession of endophytic bacteria by tracing crucial stages in the dynamic process of grain growth using culture-independent methods. The results should be viewed as a contribution to our theoretical knowledge base of plant microbial ecology, particularly when the aim is to produce high-quality hybrid plant varieties.

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