TÜBİTAK

Turkish Journal of Biology

http://journals.tubitak.gov.tr/biology/

Research Article

Turk J Biol
(2018) 42: 231-239

doi:10.3906/biy-1708-32

Biological influence of *cry1Ab* gene insertion on the endophytic bacteria community in transgenic rice

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Received: 13.08.2017 • **Accepted/Published Online:** 19.04.2018 • **Final Version:** 13.06.2018

Abstract: The commercial release of genetically modified (GMO) rice for insect control in China is a subject of debate. Although a series of studies have focused on the safety evaluation of the agroecosystem, the endophytes of transgenic rice are rarely considered. Here, the influence of endophyte populations and communities was investigated and compared for transgenic and nontransgenic rice. Population-level investigation suggested that *cry1Ab* gene insertion influenced to a varying degree the rice endophytes at the seedling stage, but a significant difference was only observed in leaves of Bt22 (Zhejiang22 transgenic rice) between the GMO and wild-type rice. Community-level analysis using the *16S rRNA* gene showed that strains of the phyla Proteobacteria and Firmicutes were the predominant groups occurring in the three transgenic rice plants and their corresponding parents. By contrast, the endophytic communities of Minghui63 and Xiushui11 showed a weaker response to *cry1Ab* gene insertion than did Zhejiang22, and the community results were consistent with the population-level investigation. The populations and communities of rice endophytes were affected by the *cry1Ab* gene to a different extent in different rice varieties and plant tissues. The results of this study broaden our understanding of unexpected transgenic influences on nontarget organisms.

Key words: 16S rRNA, community structure, endophytes, transgenic rice

1. Introduction

Rice (*Oryza sativa* L.) is one of the most important staple foods, as more than half of the world's population depends on it for daily sustenance (FAO, 2008). It has been estimated that rice production must increase by 40% to meet the greater needs of the projected human population in 2030 (Khush, 2005). Rice genetically modified through biotechnology to tolerate various biotic and abiotic stresses offers a potential strategy to meet the escalating food demands of growing populations worldwide, especially those of developing countries (Khush, 2005; Ansari et al., 2015). In China, the insecticidal proteins from *Bacillus thuringiensis* (*Bt*) have been developed to control several crop pests, namely lepidopteran stem-borers and leaf-folders (Chen et al., 2011). Although transgenic rice has not yet been approved for commercial release in China, the *cry1Ab* gene-modified rice Huahui1 and its hybrid line, Shanyou 63, were granted biosafety certificates and thus approved for limited release in select field trials in Hubei Province from 2009 to 2014 (MAPRC, 2009).

The major debates surrounding transgenic rice are food safety concerns and potential ecological threats to nontarget microorganisms, flora, and fauna. Therefore, the unexpected effects of *Bt* transgenic crops on nontarget microorganisms, flora, and fauna should be evaluated, both cautiously and systematically, before any commercialization (Dale et al., 2002; Craig et al., 2008). At the time of writing, various studies have evaluated the unexpected effects of *Bt* transgenic crops, including genetically modified (GMO) rice, on environmental microorganisms (Saxena and Stotzky, 2001; Zwahlen et al., 2003; Singh et al., 2013; Chen et al., 2017; Shu et al., 2017). Several investigations have shown that *Bt* transgenic plants can slightly alter the microbial communities of soil in agroecosystems; these negative impacts might arise from toxic Cry proteins released from root exudates or decomposed crop residues (Saxena and Stotzky, 2001; Zwahlen et al., 2003; Liu et al., 2016). The microbial community studies revealed that *Bt* transgenic eggplant, maize, or rice showed negative effects on soil organic carbon content and soil available

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N, P, or K that affected the microbial population sizes and communities in the rhizospheric soils (Singh et al., 2013; Zhu et al., 2014; Shu et al., 2017). Collectively, these studies suggest that *Bt* transgenic plants may, directly or indirectly, affect the environmental microorganism of agrosystems.

Endophytes are a group of microorganisms that inhabit plant tissues but do not harm their hosts (Hallmann et al., 1998). In recent years, endophytes have become a "hotspot" of research activity in microbiology because of their abundant secondary metabolites, effects on plant growth, and biological control applications—the way by which endophytes interact with their host plants probably entails a mechanism similar to that of plant growth-promoting rhizobacteria (Lodewyck et al., 2002; Feng et al., 2006). Likewise, rice endophytes have important roles to play in plant health and sustainable rice production (Rangjaroen et al., 2017). However, to the best of our knowledge, the influence of *Bt* transgenic rice on endophytic bacteria remains largely unknown. In this study, the population sizes and communities of endophytic bacteria in three *Bt* transgenic rice plants and their parents were investigated and compared, and the ecological safety of GMO rice for endophytes was given a preliminary evaluation.

2. Materials and methods

2.1. Plant material and field trials

Seeds of the wild-type Zhejiang22 (ZJ22) and its transgenic variety, Bt22, were obtained from the Institute of Crops and Nuclear Technology Utilization of the Zhejiang Academy of Agricultural Sciences. Other wild-type and transgenic varieties (Minhui63 versus TT51Bt; Xiushui11 versus KMDBt) were provided by the Center of Science and Technology Development of the Ministry of Agriculture of the People's Republic of China (Beijing, China). Under a natural field setting (located in Hangzhou, China), 150 plants of each wild-type or transgenic rice variety were grown in one pot by using potting technology (pot field trial size: 1.0 m long × 0.5 wide × 0.5 m height). The field trial of each rice variety consisted of three replicate plots, and the rice plants were grown from June to August in 2015. The wild-type plots were separated from the transgenic plots by a buffer area (5 m wide, Figure 1). Normal water and manure management was administrated until the end of the experiment and paddy soil from the local farm was used in the field trial. At the seedling and stooling stages of growth, samples of leaf, stem, and root were randomly collected and stored in sterile plastic bags at 4 °C. The endophytic bacteria were isolated from the collected samples within 24 h.

2.2. Isolation and purification of endophytes

Ten plants of each rice variety were randomly collected. The leaf, stem, and root samples were rinsed with sterile water for 10 min and then cut into 10-mm-long pieces before being sterilized. The leaf and stem fragments were surface-sterilized in 70% ethanol for 2 min, accompanied by gentle shaking, while the root was sterilized for 7 min, followed by rinsing twice with sterile water. After the surface-sterilization, 2.5 g of leaf or root tissue or 5 g of stem tissue was ground into homogenate by using a sterilized and precooled mortar, respectively. Each homogenate was diluted in 10 mL of sterile 0.1% peptone water, and a diluted solution of 100 µL was seeded onto the agar plates. Endophytic strains were cultured on plate count agar (PCA; Amresco, Shanghai, China). The seeded plates were incubated for 48 h at 30 °C for bacterial growth. After incubation, colonies were counted for the selected valid agar plates (i.e. those with 20–300 colonies on each plate) and the population sizes were calculated and compared. The colonies of bacterial strains were picked and repeatedly restreaked onto PCA agar until their purity was confirmed for the *16S rRNA* gene analysis.

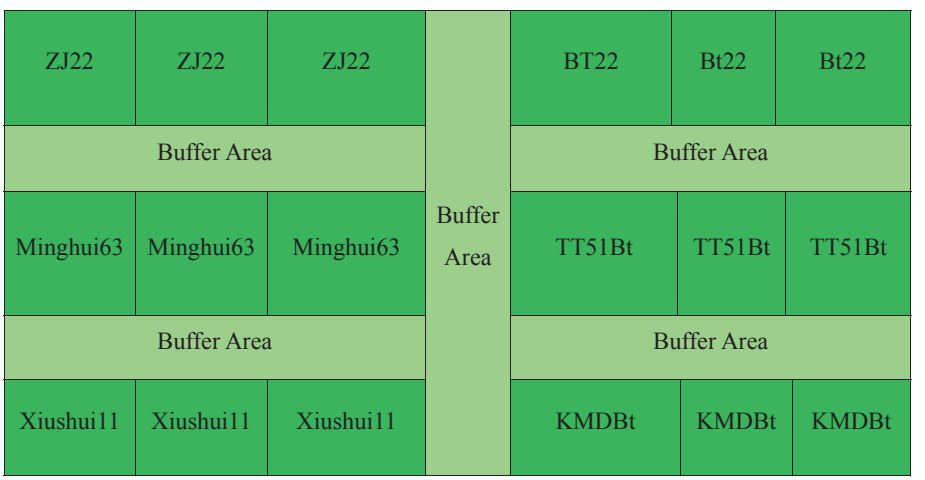

Figure 1. Schematic image showing the design of field trials.

2.3. DNA extraction and quantification, and PCR amplification

Genomic DNA of purified strains was extracted and purified by the ChargeSwitch gDNA Mini Bacteria Kit (Invitrogen, Shanghai, China) following the manufacturer's instructions, and the DNA extract was quantified at an absorbance of 260 nm. These quantified DNA extracts were stored at –20 °C before use. The universal bacterial specific primer sets 16SF (forward 8–27, AGAGTTTGATCCTGGCTCAG) and 16SR (reverse 1521–1540, GGTTACCTTGTTACGACTT) were selected for the 16S rRNA gene amplification (Brosius et al., 1978). The PCR amplification of the *16S rRNA* gene was performed as described by Li et al. (2007), and the PCR amplicons were purified by QIAquick PCR Purification Kit (QIAGEN, Hilden, Germany) and sequenced by Life Technologies Inc. (Shanghai, China) using the universal primers as PCR.

2.4. Phylogenetic analysis

Sequence-based identification and phylogenetic analysis were performed by submitting the obtained *16S rRNA* gene sequences to the RDP database (http://rdp.cme.msu.edu/) for BLAST searches. Sequences were aligned using the program BioEdit 7.01, and the overhanging ends were removed from both ends to ensure that all the sequences were of the same length (Hall, 1999). RDP 16S rRNA Training Set 9 was selected for taxonomical hierarchy categories analysis and the determination confidence interval was set to 80% (Pruesse et al., 2007; Cole et al., 2009; Schloss, 2009; McDonald et al., 2012). Partial least squares discrimination analysis (PLS-DA) at bacterial species level was performed as by Bevilacqua and Marini (2014).

2.5. Statistical analysis

Unless otherwise indicated, all tests were performed with three replicates, and the values are presented as means ± standard deviation (SD). Data were evaluated by one-way ANOVA between the wild-type and its transgenic variety with SPSS 11.0 (SPSS Inc., Chicago, IL, USA). Once a significant difference ($P < 0.05$) was detected among the means, the least significant difference test was applied to validate the pairwise differences of the means.

3. Results and discussion

3.1. Validating the sterilizing method for rice tissues

The surface-sterilizing method was first investigated to exclude the possible interference of phyllospheric microorganisms from the rice materials. The optimal surface-sterilizing methods were respectively investigated for the rice leaves, stems, and roots. Ma et al. (2013) excluded phyllospheric microorganisms by sterilizing the fragments of common reed (*Phragmites australis*) with 95% ethanol for 30 s with shaking, followed by a 10% bleach treatment (0.5% NaOCl) for 2 min and 70% ethanol for 2 min. Here, different concentrations of ethanol were evaluated for the surface sterilization of rice fragments, and 70% ethanol gave the best results. According to the surface disinfection results in this study, the phyllospheric microorganisms on the roots, stems, and leaves were thoroughly removed via soaking in 70% ethanol for a minimum sterilizing time of 7.0, 2.0, and 2.0 min, respectively (data not shown). Comparatively, the surface sterilizing methods as previous published could also obtain equal results for rice tissues, but 70% ethanol treatment in this study was simpler than those of previous studies (Oyebanji et al., 2009; Ma et al., 2013).

3.2. Populations of endophytes between *Bt*-transgenic and parental rice

The different rice tissues harbored quite different populations of endophytic bacteria. At the seedling stage, the population sizes in the *Bt* and non-*Bt* rice roots were greater than those of the stems. At the stooling stage, the opposite result was found, as the endophyte populations in the stems of both *Bt* and non-*Bt* rice exceeded those of both roots or leaves. For both stages, however, the endophyte populations of the *Bt* and non-*Bt* rice were lowest in the leaves (Figure 2). Comparing the two growth stages, the endophyte populations in leaf and stems at the stooling stage were slightly larger than those of the seedling stage. These results indicate that the population of endophytes increased with growth development of rice plants.

The bacterial populations in each tissue of the three *Bt* rice varieties were lower than those of corresponding non-*Bt* rice (i.e. the wild-type parent) at the seedling stage. Compared to the non-*Bt* rice varieties (i.e. ZJ22, Minghui63, and Xiushui11), the endophyte populations in the roots, stems, and leaves of ZJ22Bt were reduced by 11%, 18%, and 56%, respectively; likewise, those of TT51Bt were reduced by 7%, 4%, and 21% and those of KMDBt were reduced by 3%, 4%, and 23% (Figure 2A). While these population reductions were observed in all tissues of the tested *Bt* rice varieties, a significant difference was only obtained for the leaves of Bt22 ($P < 0.01$). However, the same result was not obtained at the stooling stage between the *Bt* and non-*Bt* rice varieties. For example, when compared with their parents, the endophyte populations in the leaves of Bt22 and TT51Bt increased by 23% and 10%, respectively (Figure 2B). These results suggest that the *cry1Ab* gene insertion exercised different influences on the endophyte populations of the rice, but significantly so only for leaves of Bt22 in the present study.

3.3. Endophytic bacterial communities of the *Bt*-transgenic and wild-type rice varieties

To investigate the influence of the *cry1Ab* insertion on the community (or diversity) of the endophytic bacteria in rice, a total of 1200 endophytic bacteria—600 isolates

from *Bt* and 600 from non-*Bt* rice varieties—were selected, and the *16S rRNA* genes were analyzed. We randomly picked 600 bacterial strains and confirmed their colony purity for each rice type (*Bt* or non-*Bt*) at the two plant growth stages. In this way, a total of 1059 high-quality *16S rRNA* gene sequences were obtained and 141 poor-quality sequences were discarded; the number of high-quality sequences per tissue for the different rice types is shown in Table 1. The high-quality sequences were submitted to the RDP database for BLAST searches and the identified species list is shown as Supplementary Material 1.

This yielded *1053 rRNA* gene sequences (99.4% of the total) belonging to recognized bacterial species and six sequences (0.6%) that were unclassified (Supplementary Material 1 and 2). Among the 1053 identified endophytes, 16 strains belonged to *Actinobacteridae*; 1 strain belonged

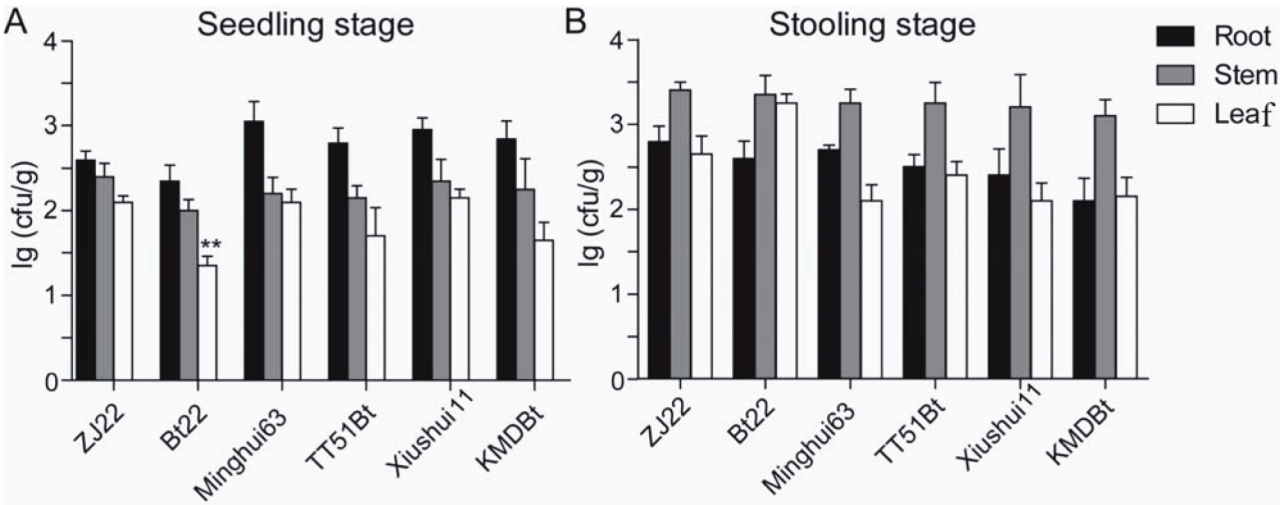

Figure 2. The populations of endophytic bacteria in the *Bt* transgenic and non-*Bt* parental rice varieties at two plant growth stages.

Table 1. The numbers of high-quality sequences obtained for different tissues of *Bt*-transgenic and their parental rice varieties (Sd, seedling stage; St, stooling stage).

Rice variety	Tissues			Total number
	Leaf	Stem	Root	
ZJ22 (Sd)	27	32	34	93
ZJ22 (St)	28	31	31	90
Bt22 (Sd)	32	32	31	95
Bt22 (St)	33	30	32	95
Minghui63 (Sd)	28	22	28	78
Minghui63 (St)	30	25	31	86
TT51Bt (Sd)	29	31	25	85
TT51Bt (St)	30	26	28	84
Xiushui11 (Sd)	31	29	24	84
Xiushui11 (St)	30	27	28	85
KMDBt (Sd)	31	32	27	90
KMDBt (St)	31	33	30	94

to *Bacteroidetes* (*Flavobacteriales*); 476 strains belonged to *Firmicutes*, including 474 strains of bacilli (472 *Bacillales* and two unclassified bacilli) and two unclassified *Firmicutes*; 547 strains belonged to *Proteobacteria*, which included 29 strains of Beta-*Proteobacteria* (28 Burkholderiales and 1 *Rhodocyclales*), 9 strains of Alpha-*Proteobacteria* (1 *Sphingomonadales* and 8 *Rhizobiales*), and 505 strains of Gamma-*Proteobacteria* (1 *Alteromonadales*, 9 *Aeromonadales*, 3 *Chromatiales*, 10 *Xanthomonadales*, 171 *Pseudomonadales*, 305 *Enterobacteriales*, and six species of unclassified Gamma-*Proteobacteria*), and four strains of unclassified *Proteobacteria*; and 13 unclassified bacterial strains (Supplementary Material 1).

On the basis of the community analysis results, *Proteobacteria* and *Firmicutes* were the two predominant bacterial groups in the three rice tissues for both non-*Bt* and *Bt* rice. The populations of the other taxonomic groups (e.g., *Actinobacteria* and *Bacteroidetes*) were obviously smaller than the groups of *Proteobacteria* and *Firmicutes*. Various studies have indicated that the majority of functional plant endophytes (e.g., nitrogen-fixing bacterial species of *Bacillales*, *Rhizobiales*, and *Pseudomonadales*) or plant pathogenic bacteria (e.g., pathogenic species of *Xanthomonadales*) belong to these two predominant groups in many rice varieties (Sun et al., 2008; Knief et al., 2012; Bruto et al., 2014). Therefore, the phylogenetic results of the *Bt* and non-*Bt* rice varieties in this study were similar to the findings of previous endophytic studies of rice.

When comparing the bacterial communities of *Bt* and non-*Bt* rice, the *cry1Ab* gene showed quite a different influence on the endophytic communities among the rice varieties and tissues. For example, the *Proteobacteria* strains in the leaves of Bt22 were significantly promoted by the insertion of the *cry1Ab* gene, whereas these strains were reduced significantly in the leaves of TT51Bt and KMDBt. By contrast, the *Proteobacteria* strains were promoted in the stems of the Bt22 and TT51Bt rice varieties, but slightly reduced in KMDBt rice. The *cry1Ab* gene insertion did little to reduce the *Proteobacteria* strains in the roots of Bt22, but it clearly promoted *Proteobacteria* strains in the roots of TT51Bt and KMDBt (Figure 3). Considering the community structure of the *Firmicutes* strains, the *cry1Ab* gene insertion slightly reduced *Firmicutes* strains in the leaves of Bt22, but it obviously promoted the *Firmicutes* strains in the leaves of TT51Bt and KMDBt; beyond increasing the *Firmicutes* strains in the leaves of KMDBt, the *cry1Ab* gene insertion also promote the *Actinobacteria* strains in the rice tissue. Nonetheless, the *cry1Ab* gene insertion reduced the *Firmicutes* strains in the stems of ZJ22Bt and TT51Bt, but it promoted them in the stems of KMDBt. In the roots, the *cry1Ab* gene insertion reduced the Firmicutes strains of TT51Bt and KMDBt, but not of Bt22 (Figure 3). However, the community-level influences of the *cry1Ab* gene insertion on the *Proteobacteria* and *Firmicutes* groups were similar between the TT51Bt and KMDBt rice, but these latter influences were unlike those for Bt22.

Based on the bacterial community analysis by the *16S rRNA* gene sequences from the rice varieties of the *Bt* and non-*Bt* materials, the phylogenetic similarity of the endophytic community for each rice tissue was analyzed. The communities of Minghui63 and Xiushui11 were more similar to each other, with both separated from the ZJ22 rice (Figure 4); this result is consistent with the population investigation of this study. The PLS-DA result at the species level indicated that the influence order of *cry1Ab* gene insertion into three rice varieties was ZJ22 > Minghui63 > Xiushui11 (Figure 5), and the PLS-DA also supported the above population investigation. Phylogenetic analysis showed that the endophytic communities of Bt22 roots, Bt22 leaves, TT51Bt roots, and KMDBt stems had a high similarity to their corresponding non-Bt rice types, indicating that the *cry1Ab* gene insertion had a limited influence on the endophytic communities in the roots and leaves of ZJ22, the roots of Minghui63, and the stems of Xiushui11. By contrast, the *cry1Ab* gene insertion clearly influenced the endophytic communities of the Bt22 stems, the TT51Bt stems, the TT51Bt leaves, the KMDBt leaves, and KMDBt roots—the phylogenetic clades of these endophytic communities were clearly separated from their parent rice clades (i.e. non-*Bt*) at the bottom of dendrogram. This result indicates that the *cry1Ab* gene insertion was able to somehow change the endophytic communities in the stems of ZJ22, the stems and leaves of Minghui63, and the leaves and roots of Xiushui11. In sum, the *cry1Ab* gene insertion showed less of an endophytic community influence on the plant material of ZJ22 than upon the other two rice varieties of Minghui63 and Xiushui11, and the unexpected influences of the *cry1Ab* gene on the rice endophytic communities differed variously from one rice variety to another.

Studies of transgenic plants for other environmental effects (e.g., soil and water microbial community, rhizospheric microorganisms, nematodes, earthworms, and other nontarget organisms) indicated that exogenous genes (including the *cry1Ab/c* gene) might have various unexpected effects on nontarget organisms. For example, *Bt* transgenic eggplant, maize, and rice showed negative effects on soil organic carbon content or soil available N, P, or K and thereby changed the microbial populations and communities in the planting soils (Singh et al., 2013; Zhu et al., 2014; Chen et al., 2017; Shu et al., 2017). The study of Shu et al. (2017) showed that soil available N, P, and K and the soil bacterial community were significantly influenced by Cry1Ab *Bt* maize, but the bacterial community of

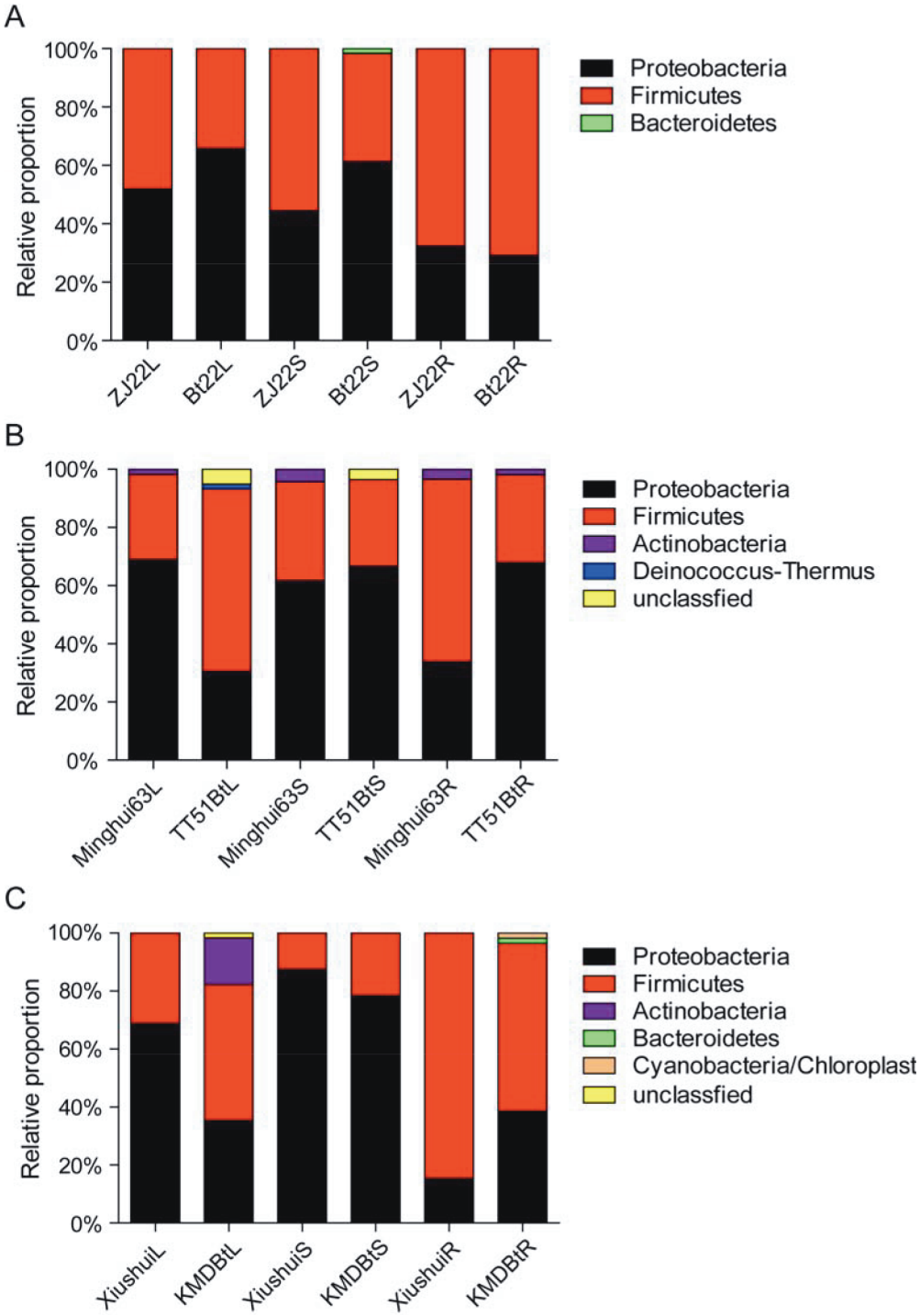

Figure 3. Endophytic bacterial communities in the leaves, stems, and roots of the *Bt* transgenic and non-*Bt* parental rice varieties. A) The communities of ZJ22 and Bt22. ZJ22L indicates the ZJ22 leaves, ZJ22S indicates the ZJ22 stems, ZJ22R indicates the ZJ22 roots, Bt22L indicates the Bt22 leaves, Bt22S indicates the Bt22 stems, and Bt22R indicates the Bt22 roots. B) The communities of Minghui63 and TT51Bt. Minghui63L indicates the Minghui63 leaves, Minghui63S indicates the Minghui63 stems, Minghui63R indicates the Minghui63 roots, TT51BtL indicates the TT51Bt leaves, TT51BtS indicates the TT51Bt stems, and TT51BtR indicates the TT51Bt roots. C) The communities of Xiushui11 and KMDBt. XiushuiL indicates the Xiushui11 leaves, XiushuiS indicates the Xiushui11 stems, XiushuiR indicates the Xiushui11 roots, KMDBtL indicates the KMDBt leaves, KMDBtS indicates the KMDBt stems, and KMDBtR indicates the KMDBt roots.

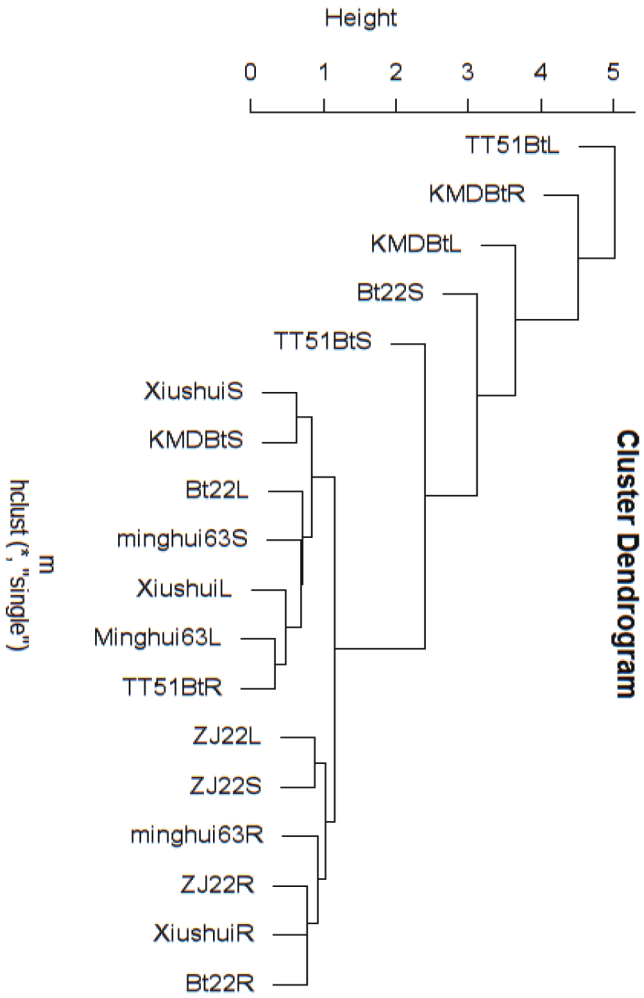

Figure 4. Cluster dendrogram based on the community analysis of ZJ22, Minghui63, Xiushui11, and their corresponding *Bt*-transgenic rice varieties. The abbreviations for the rice tissues are the same as those used in Figure 2.

earthworm guts in *Bt* and non-*Bt* maize fields showed no statistically significant difference. Chen et al. (2017) found that long-term *cry1Ab/1Ac Bt* rice planting reduced phytoparasitic nematode abundance but did not affect other nematode parameters in paddy fields. Comparatively, Cry1Ab *Bt* altered the endophytic communities in rice tissues in this study, but these influences likely differ substantially in different cultivars.

3.4. Conclusions

Many studies indicate that transgenic plants have the potential to cause unexpected effects on soil and rhizospheric microorganisms, but only a few such studies have focused on plant endophytes. This is rather surprising, given that plant endophytes are considered as a special group of organisms that directly encounter the Cry1Ab protein and some unexpected toxins. Hence, one might expect that plant endophytes should be the organisms most vulnerable to being affected by *cry1Ab* gene insertion. The population investigation of this study suggests that the *cry1Ab* gene insertion influenced rice endophytes at the seedling stage, but a significant difference was only observed in one tissue type of Bt22. Our community analysis shows that the *cry1Ab* gene may alter endophytic communities in several tissues of the tested rice varieties, but these influences likely differ substantially from variety to variety. Considering the limitations of this study (e.g., uncultured microorganisms were not considered, the numbers of isolates for the community analysis were small), the conclusions of this study require further verification.

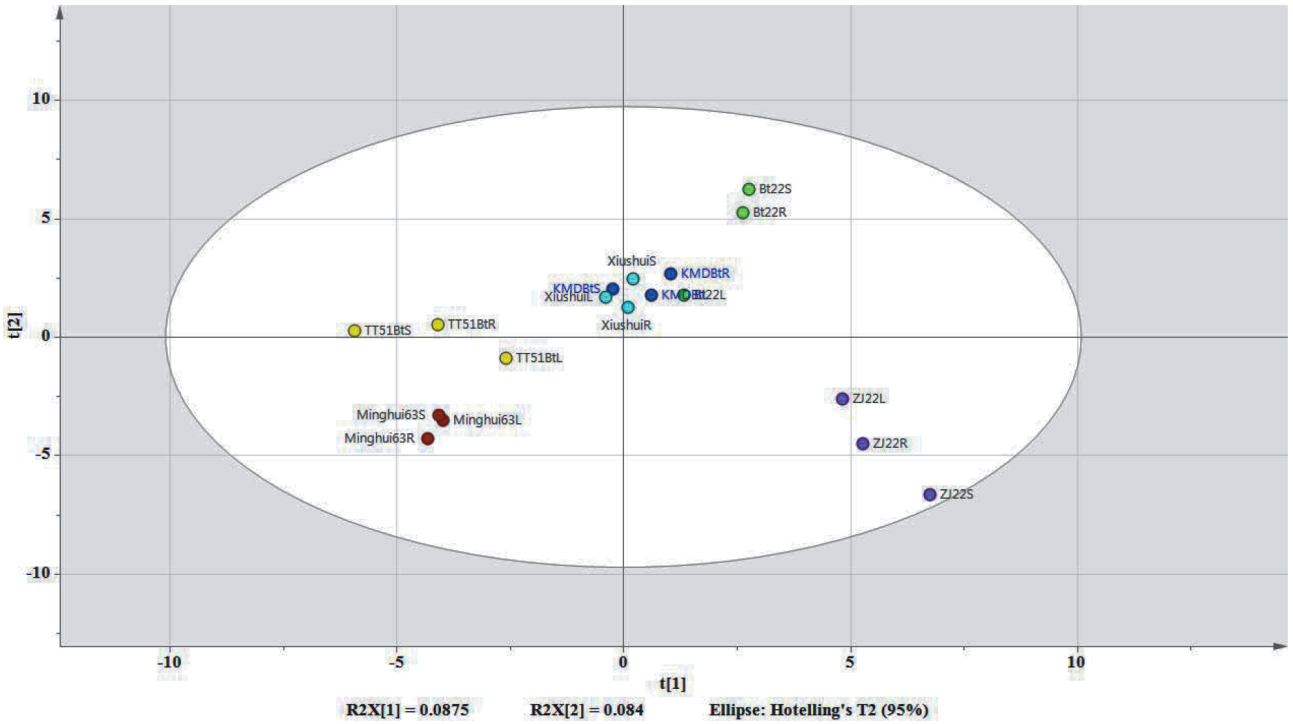

Figure 5. PLS-DA result of the endophytic isolates from ZJ22, Minghui63, Xiushui11, and their corresponding *Bt*-transgenic rice varieties under thespecies level.

Acknowledgments

We would like to thank the native English-speaking scientists of Elixigen Company (Huntington Beach, CA, USA) for editing our manuscript. We thank Dr Xiaoming Zhang at Zhejiang Academy of Agricultural Sciences for kindly providing the rice materials of Zhejiang22 and Bt22, and Dr Qingshan Xu at Anhui Agricultural University for PLS-DA assistance. This work was supported by grants from the National Natural Science Foundation of China (31671949) and the Anhui Natural Science Foundation (Nos. 1608085J08, 1608085QC57).

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Supplementary Material 1. The bacterial species identified from each rice material.

ZJ22L	**No.**	**ZJ22S**	**No.**	**ZJ22R**	**No.**
Bacillus altitudinis	2	*Acidovorax oryzae*	1	*Azospira oryzae*	1
Bacillus aryabhattai	3	*Acidovorax temperans*	1	*Bacillus altitudinis*	4
Bacillus beringensis	1	*Bacillus altitudinis*	1	*Bacillus aryabhattai*	5
Bacillus cereus	1	*Bacillus aryabhattai*	5	*Bacillus asahii*	1
Bacillus dabaoshanensis	1	*Bacillus butanolivorans*	1	*Bacillus cereus*	1
Bacillus firmus	2	*Bacillus glycinifermentans*	2	*Bacillus firmus*	1
Bacillus licheniformis	4	*Bacillus haikouensis*	2	*Bacillus gibsonii*	1
Bacillus marisflavi	2	*Bacillus marisflavi*	2	*Bacillus haikouensis*	2
Bacillus sp.	1	*Bacillus oryzaecorticis*	2	*Bacillus oryzaecorticis*	2
Bacillus thioparans	2	*Bacillus paralicheniformis*	1	*Bacillus* sp.	8
Bacillus vietnamensis	2	*Bacillus siamensis*	2	*Bacillus subtilis*	2
Bacillus vireti	1	*Bacillus* sp.	4	*Bacillus vietnamensis*	1
Curtobacterium plantarum	9	*Bacillus subterraneus*	3	*Ensifer adhaerens*	1
Fictibacillus barbaricus	1	*Bacillus vietnamensis*	4	*Ensifer sesbaniae*	5
Paenibacillus lautus	2	*Bacillus xiamenensis*	1	*Fictibacillus barbaricus*	9
Pantoea agglomerans	4	*Brevibacterium halotolerans*	1	*Fictibacillus phosphorivorans*	2
Pantoea allii	5	*Curtobacterium plantarum*	4	*Lysinibacillus xylanilyticus*	2
Pantoea ananatis	11	*Enterobacter* sp.	2	*Paenibacillus assamensis*	2
Sporosarcina luteola	1	*Fictibacillus barbaricus*	1	*Paenibacillus lautus*	1
Total number	**55**	*Herbaspirillum seropedicae*	1	*Pantoea ananatis*	13
		Lysinibacillus fusiformis	2	*Sporosarcina koreensis*	1
		Lysinibacillus mangiferihumi	1	**Total number**	**65**
		Moraxella osloensis	2		
		Pantoea agglomerans	3		
		Pantoea allii	1		
		Pantoea ananatis	6		
		Pseudomonas mendocina	1		
		Pseudomonas psychrotolerans	4		
		Rhizobium straminoryzae	1		
		Sphingomonas trueperi	1		
		Total number	**63**		

Bt22L	**No.**	**Bt22S**	**No.**	**Bt22R**	**No.**
Bacillus altitudinis	2	*Achromobacter mucicolens*	3	*Bacillus altitudinis*	5
Bacillus anthracis	1	*Aeromonas jandaei*	1	*Bacillus anthracis*	1
Bacillus aryabhattai	5	*Bacillus altitudinis*	8	*Bacillus aryabhattai*	2
Bacillus cereus	3	*Bacillus cereus*	4	*Bacillus cucumis*	1
Bacillus marisflavi	6	*Bacillus firmus*	3	*Bacillus megaterium*	17
Bacillus subtilis	1	*Bacillus marisflavi*	5	*Bacillus paramycoides*	5
Bacillus wiedmannii	1	*Bacillus vietnamensis*	1	*Bacillus* sp.	1
Bacillus xiamenensis	2	*Burkholderia latens*	1	*Bacillus velezensis*	1

Supplementary Material 1. (Continued).

Brevibacterium halotolerans	1	*Burkholderia vietnamiensis*	3	*Bacillus vietnamensis*	1
Burkholderia vietnamiensis	1	*Chryseobacterium gambrini*	1	*Curtobacterium plantarum*	3
Curtobacterium plantarum	18	*Curtobacterium plantarum*	1	*Kosakonia pseudosacchari*	3
Pantoea agglomerans	11	*Enterobacter* sp.	9	*Kosakonia* sp.	2
Pantoea allii	1	*Flavobacterium acidificum*	1	*Paenibacillus jamilae*	1
Pantoea ananatis	12	*Jeotgalibacillus marinus*	1	*Paenibacillus* sp.	6
Total number	**65**	*Klebsiella pneumoniae*	2	*Paenibacillus tyraminigenes*	1
		Klebsiella variicola	2	*Pantoea ananatis*	2
		Pantoea agglomerans	4	*Pantoea eucalypti*	1
		Pantoea ananatis	2	*Pseudomonas mendocina*	10
		Pseudomonas aeruginosa	1	**Total number**	**63**
		Pseudomonas alcaligenes	2		
		Pseudomonas mendocina	6		
		Staphylococcus saprophyticus	1		
		Total number	**62**		

Minghui63L	**No.**	**Minghui63S**	**No.**	**Minghui63S**	**No.**
Bacillus aquimaris	2	*Aeromonas cavernicola*	2	*Aeromonas cavernicola*	1
Bacillus aryabhattai	1	*Bacillus anthracis*	1	*Aeromonas media*	1
Bacillus fortis	1	*Bacillus aryabhattai*	1	*Arthrobacter* sp.	2
Bacillus isronensis	1	*Bacillus marisflavi*	1	*Bacillus anthracis*	15
Bacillus megaterium	1	*Bacillus pumilus*	1	*Bacillus aryabhattai*	1
Bacillus pumilus	2	*Bacillus* sp.	3	*Bacillus cereus*	1
Bacillus sp.	2	*Bacillus xiamenensis*	2	*Bacillus marisflavi*	1
Bacillus xiamenensis	2	*Curtobacterium plantarum*	5	*Bacillus paralicheniformis*	1
Curtobacterium citreum	1	*Exiguobacterium indicum*	3	*Bacillus* sp.	10
Curtobacterium plantarum	17	*Fictibacillus phosphorivorans*	1	*Bacillus xiamenensis*	5
Flavobacterium acidificum	5	*Leucobacter chromiiresistens*	1	*Delftia tsuruhatensis*	2
Unclassified Lachnospiraceae	1	*Microbacterium testaceum*	1	*Lysinibacillus cresolivorans*	2
Lysinibacillus fusiformis	1	*Pantoea ananatis*	1	*Lysinibacillus macroides*	1
Lysinibacillus macroides	1	*Pantoea* sp.	2	*Pantoea ananatis*	3
Paenibacillus barcinonensis	1	*Pseudomonas brassicacearum*	1	*Pseudomonas chlororaphis*	2
Pantoea agglomerans	12	*Pseudomonas chlororaphis*	1	*Pseudomonas kilonensis*	2
Pantoea ananatis	2	*Pseudomonas indoloxydans*	1	*Pseudomonas lini*	1
Pantoea sp.	2	*Pseudomonas rhodesiae*	1	*Pseudomonas mohnii*	1
Pantoea stewartii	1	*Pseudomonas umsongensis*	14	*Pseudomonas umsongensis*	7
Rhizobium larrymoorei	1	*Sporosarcina koreensis*	3	**Total number**	**59**
Sporosarcina koreensis	1	*Stenotrophomonas maltophilia*	1		
Total number	**58**	**Total number**	**47**		

Supplementary Material 1. (Continued).

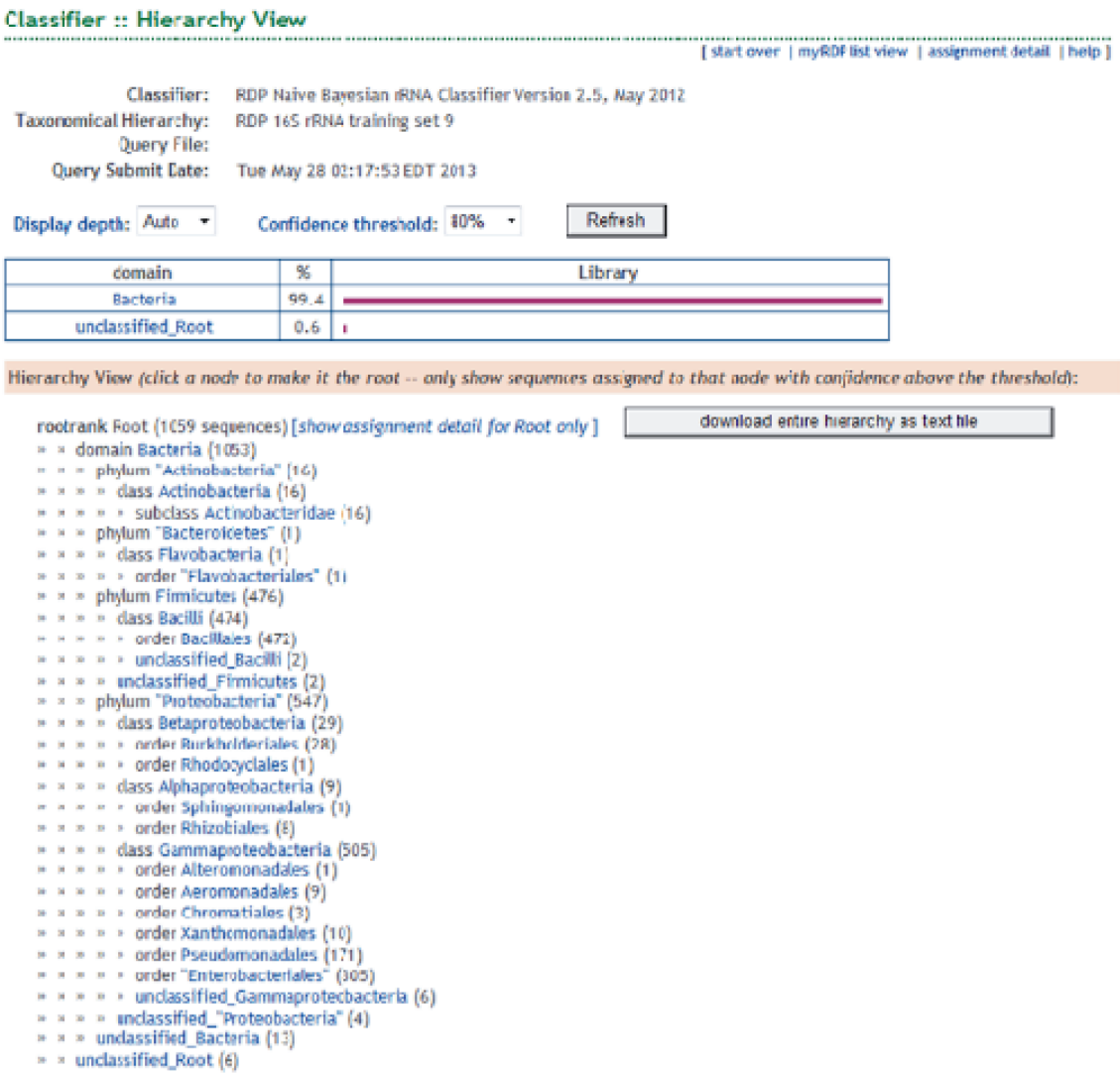
TT51BtL	**No.**	**TT51BtS**	**No.**	**TT51BtR**	**No.**
Bacillus aryabhattai	10	*Aeromonas caviae*	1	*Achromobacter insolitus*	2
Bacillus enclensis	1	*Aeromonas dhakensis*	2	*Achromobacter mucicolens*	1
Bacillus horikoshii	1	*Aeromonas rivipollensis*	1	*Bacillus altitudinis*	2
Bacillus haikouensis	1	*Bacillus firmus*	1	*Bacillus aryabhattai*	2
Bacillus indicus	1	*Bacillus indicus*	1	*Bacillus cereus*	1
Bacillus jeotgali	2	*Bacillus paranthracis*	1	*Bacillus firmus*	1
Bacillus marisflavi	1	*Bacillus* sp.	1	*Bacillus indicus*	1
Bacillus oryzaecorticis	3	*Bacillus subtilis*	1	*Bacillus* sp.	2
Bacillus safensis	1	*Bacillus vietnamensis*	1	*Bacillus thuringiensis*	1
Bacillus siamensis	1	*Bacillus xiamenensis*	3	*Bacillus vietnamensis*	1
Bacillus subtilis	2	*Burkholderia* sp.	1	*Bacillus zhangzhouensis*	2
Bacillus vietnamensis	2	*Cedecea neteri*	1	*Burkholderia* sp.	8
Bacillus xiamenensis	4	*Curtobacterium plantarum*	6	*Curtobacterium plantarum*	2
Curtobacterium plantarum	12	*Enterobacter sp.*	8	*Delftia tsuruhatensis*	2
Exiguobacterium indicum	1	*Exiguobacterium acetylicum*	5	*Fictibacillus barbaricus*	1
Fictibacillus phosphorivorans	1	*Exiguobacterium indicum*	1	*Flavobacterium acidificum*	1
Flavobacterium acidificum	3	*Exiguobacterium mexicanum*	1	*Halobacillus yeomjeoni*	1
Lysinibacillus cresolivorans	1	*Jeotgalibacillus malaysiensis*	1	*Microbacterium laevaniformans*	1
Lysinibacillus fusiformis	1	*Klebsiella variicola*	1	*Paenibacillus* sp.	1
Unclassified	3	*Pantoea agglomerans*	2	*Pantoea agglomerans*	1
Oceanobacillus profundus	2	*Pantoea* sp.	3	*Pseudomonas aeruginosa*	5
Paenibacillus konsidensis	1	*Pseudomonas brassicacearum*	1	*Pseudomonas frederiksbergensis*	6
Pantoea agglomerans	2	*Pseudomonas chengduensis*	1	*Pseudomonas mohnii*	1
Pantoea allii	1	*Pseudomonas frederiksbergensis*	3	*Pseudomonas prosekii*	1
Pantoea sp.	1	*Pseudomonas hunanensis*	2	*Pseudomonas umsongensis*	6
Total number	**59**	*Pseudomonas jessenii*	2	**Total number**	**53**
		Pseudomonas mohnii	1		
		Pseudomonas umsongensis	1		
		Pseudomonas vancouverensis	1		
		Stenotrophomonas maltophilia	1		
		Unclassified	1		
		Total number	**57**		

XiushuiL	**No.**	**XiushuiS**	**No.**	**XiushuiR**	**No.**
Bacillus altitudinis	4	*Aeromonas lacus*	1	*Bacillus altitudinis*	13
Bacillus anthracis	1	*Aeromonas veronii*	1	*Bacillus aryabhattai*	12
Bacillus aryabhattai	3	*Bacillus altitudinis*	2	*Bacillus cereus*	2
Bacillus cereus	4	*Bacillus cereus*	3	*Bacillus firmus*	1
Bacillus cytotoxicus	1	*Bacillus* sp.	1	*Bacillus fortis*	1
Bacillus indicus	1	*Curtobacterium plantarum*	7	*Bacillus indicus*	1
Bacillus megaterium	1	*Enterobacter ludwigii*	1	*Bacillus marisflavi*	1

Supplementary Material 1. (Continued).

Bacillus subtilis	1	*Pantoea agglomerans*	1	*Bacillus* sp.	1
Bacillus xiamenensis	2	*Pantoea allii*	1	*Bacillus vietnamensis*	4
Bacillus zhangzhouensis	1	*Pantoea* sp.	2	*Bacillus xiamenensis*	1
Curtobacterium plantarum	21	*Pseudomonas chengduensis*	20	*Bhargavaea cecembensis*	1
Enterobacter ludwigii	2	*Pseudomonas indoloxydans*	4	*Curtobacterium plantarum*	1
Enterobacter sp.	7	*Pseudomonas inxydans*	1	*Escherichia* sp.	1
Flavobacterium acidificum	3	*Pseudomonas oleovorans*	1	*Fictibacillus barbaricus*	3
Pantoea agglomerans	4	*Pseudomonas otitidis*	1	*Fictibacillus phosphorivorans*	3
Pantoea allii	3	*Shewanella xiamenensis*	1	*Pseudomonas brassicacearum*	1
Pantoea sp.	2	*Staphylococcus sciuri*	1	*Pseudomonas frederiksbergensis*	1
Total number	**61**	*Stenotrophomonas pavanii*	7	*Pseudomonas umsongensis*	4
		Total number	**56**	**Total number**	**52**

KMDBtL	**No.**	**KMDBtS**	**No.**	**KMDBtS**	**No.**
Bacillus altitudinis	4	*Achromobacter xylosoxidans*	1	*Bacillus altitudinis*	8
Bacillus aryabhattai	8	*Bacillus aryabhattai*	1	*Bacillus aryabhattai*	3
Bacillus coreaensis	1	*Bacillus indicus*	1	*Bacillus cereus*	2
Bacillus licheniformis	1	*Bacillus marisflavi*	1	*Bacillus firmus*	1
Bacillus marisflavi	1	*Bacillus* sp.	2	*Bacillus indicus*	1
Bacillus megaterium	1	*Bacillus tequilensis*	1	*Bacillus megaterium*	3
Bacillus rhizosphaerae	1	*Bacillus vietnamensis*	1	*Bacillus* sp.	2
Bacillus sp.	1	*Bacillus xiamenensis*	2	*Bacillus thioparans*	1
Bacillus vietnamensis	1	*Curtobacterium plantarum*	5	*Bacillus vietnamensis*	8
Bacillus xiamenensis	1	*Exiguobacterium profundum*	2	*Cronobacter dublinensis*	6
Bacillus zhangzhouensis	1	*Fictibacillus nanhaiensis*	1	*Cronobacter turicensis*	1
Curtobacterium plantarum	3	*Fictibacillus phosphorivorans*	1	*Fictibacillus enclensis*	1
Enterobacter ludwigii	1	*Lysinibacillus macroides*	1	*Fictibacillus phosphorivorans*	1
Enterobacter sp.	7	*Pantoea agglomerans*	3	Unclassified	2
Fictibacillus barbaricus	4	*Pantoea ananatis*	1	*Paenibacillus illinoisensis*	1
Fictibacillus nanhaiensis	1	*Pantoea* sp.	1	*Pseudomonas chengduensis*	7
Fictibacillus phosphorivorans	1	*Pseudomonas chengduensis*	15	*Pseudomonas flavescens*	1
Lysinibacillus xylanilyticus	2	*Pseudomonas indoloxydans*	1	*Pseudomonas indoloxydans*	3
Microbacterium paraoxydans	9	*Pseudomonas mendocina*	1	*Rheinheimera tangshanensis*	3
Pantoea agglomerans	4	*Pseudomonas oleovorans*	13	*Sporosarcina saromensis*	1
Pantoea sp.	7	*Pseudomonas otitidis*	5	*Stenotrophomonas pavanii*	1
Streptomyces vinaceusdrappus	1	*Pseudomonas toyotomiensis*	5	**Total number**	**57**
Unclassified	1	**Total number**	**65**		
Total number	**62**				



Classifier :: Hierarchy View

[start over | myRDP list view | assignment detail | help]

Classifier: RDP Naive Bayesian rRNA Classifier Version 2.5, May 2012
Taxonomical Hierarchy: RDP 16S rRNA training set 9
Query File:
Query Submit Date: Tue May 28 02:17:53 EDT 2013

Display depth: Auto Confidence threshold: 80% Refresh

domain	%	Library
Bacteria	99.4	
unclassified_Root	0.6	

Hierarchy View *(click a node to make it the root -- only show sequences assigned to that node with confidence above the threshold)*:

rootrank Root (1059 sequences) [*show assignment detail for Root only*] download entire hierarchy as text file

- domain Bacteria (1053)
  - phylum "Actinobacteria" (16)
    - class Actinobacteria (16)
      - subclass Actinobacteridae (16)
  - phylum "Bacteroidetes" (1)
    - class Flavobacteria (1)
      - order "Flavobacteriales" (1)
  - phylum Firmicutes (476)
    - class Bacilli (474)
      - order Bacillales (472)
      - unclassified_Bacilli (2)
    - unclassified_Firmicutes (2)
  - phylum "Proteobacteria" (547)
    - class Betaproteobacteria (29)
      - order Burkholderiales (28)
      - order Rhodocyclales (1)
    - class Alphaproteobacteria (9)
      - order Sphingomonadales (1)
      - order Rhizobiales (8)
    - class Gammaproteobacteria (505)
      - order Alteromonadales (1)
      - order Aeromonadales (9)
      - order Chromatiales (3)
      - order Xanthomonadales (10)
      - order Pseudomonadales (171)
      - order "Enterobacteriales" (305)
      - unclassified_Gammaproteobacteria (6)
    - unclassified_"Proteobacteria" (4)
  - unclassified_Bacteria (13)
- unclassified_Root (6)

Supplementary Material 2. The results of the bacteria subpopulations queried in the RDP database based on their 16S rRNA gene sequences.