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Effects of Field-Grown Genetically Modified *Zoysia* Grass on Bacterial Community Structure

Lee, Yong-Eok[1]*, Sang-Hwan Yang[1], Tae-Woong Bae[2], Hong-Gyu Kang[2], Pyung-Ok Lim[3], and Hyo-Yeon Lee[4]

[1]*Division of Bio Science, Dongguk University, Kyungju, Kyongbuk 780-714, Korea*
[2]*Subtropical Horticulture Research Institute, Jeju National University, Jeju-Si, Jeju 690-756, Korea*
[3]*Faculty of Science Education, Jeju National University, Jeju-Si, Jeju 690-756, Korea*
[4]*Faculty of Biotechnology, Jeju National University, Jeju-Si, Jeju 690-756, Korea*

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Herbicide-tolerant *Zoysia* grass has been previously developed through *Agrobacterium*-mediated transformation. We investigated the effects of genetically modified (GM) *Zoysia* grass and the associated herbicide application on bacterial community structure by using culture-independent approaches. To assess the possible horizontal gene transfer (HGT) of transgenic DNA to soil microorganisms, total soil DNAs were amplified by PCR with two primer sets for the *bar* and *hpt* genes, which were introduced into the GM *Zoysia* grass by a callus-type transformation. The transgenic genes were not detected from the total genomic DNAs extracted from 1.5 g of each rhizosphere soils of GM and non-GM *Zoysia* grasses. The structures and diversities of the bacterial communities in rhizosphere soils of GM and non-GM *Zoysia* grasses were investigated by constructing 16S rDNA clone libraries. Classifier, provided in the RDP II, assigned 100 clones in the 16S rRNA gene sequences library into 11 bacterial phyla. The most abundant phyla in both clone libraries were Acidobacteria and *Proteobacteria*. The bacterial diversity of the GM clone library was lower than that of the non-GM library. The former contained four phyla, whereas the latter had seven phyla. Phylogenetic trees were constructed to confirm these results. Phylogenetic analyses of the two clone libraries revealed considerable difference from each other. The significance of difference between clone libraries was examined with LIBSHUFF statistics. LIBSHUFF analysis revealed that the two clone libraries differed significantly (P<0.025), suggesting alterations in the composition of the microbial community associated with GM *Zoysia* grass.

Keywords: Transgenic *Zoysia* grass, PCR, horizontal gene transfer, 16S rRNA gene, bacterial diversity

*Corresponding author
Phone: +82-54-770-2226; Fax: +;82-54-770-2287
E-mail: yelee@mail.dongguk.ac.kr

The global cultivating area of genetically engineered crops continues to increase because of the economic and agronomic benefits. Therefore, there are growing public concerns regarding the effects of genetically modified plants on the environment and human health [17]. The incorporation of antibiotic resistance genes in GM plants as selection markers has raised questions about the possible transfer of these genes to indigenous microbes in the soil. For this reason, environmental risk assessment of GM plants has been mainly focused on possible horizontal gene transfer (HGT) to relative plants or soil- and plant-associated microbial communities [39]. However, little is known about the consequence of the release of GM plants into the field on soil microbial biodiversity [3, 8]. The interactions between GM plants and soil microbial communities could change the microbial biodiversity and affect soil ecosystem processes. It is still a controversial issue whether GM plants exert any ecological effect on the microbial community in soils. Several studies have shown that GM plants can affect the rhizosphere microbial community [11, 12, 16, 31]. In other studies, however, the compositions of microbial communities in rhizospheres were not significantly affected by GM plants [24, 28, 30].

Since the majority of soil bacteria cannot be cultivated in the laboratory [2], cultivation-independent molecular approaches have been used to examine the microbial diversity in ecosystems. These include polymerase chain reaction (PCR)-based techniques such as restriction fragment length polymorphism (RFLP) analysis, denaturing gradient or temperature gradient gel electrophoresis (DGGE/TGGE), and analysis of 16S rRNA gene clone libraries. Among these, sequence analysis of 16S rDNA clones can provide detailed, reliable information about the microbial community structure and is therefore useful for bacterial community analysis in specific ecosystems [21].

Zoysia grass (*Zoysia japonica* Steud.) is one of the most popularly cultivated turf grasses for sports and recreational

environments in East Asia. To increase the commercial value of *Zoysia* grass, herbicide-tolerant *Zoysia* grass has been previously developed by introducing a *bar* gene, which confers tolerance to the herbicide Basta in transgenic plants [35]. The herbicide tolerance of the *bar* transgenic *Zoysia* grass was stably sustained for more than 5 years at the test field in Jeju Island, Korea [5]. In the previous studies, we assessed the environmental risk of GM *Zoysia* grass in the test field, focusing on horizontal gene transfer. However, no gene flow mediated by pollen flight was detected among the wild species and the non-GM *Zoysia* grasses within the test habitat [5]. Moreover, evidence for HGT mediated by insects' microflora or soil microorganisms was not found [6].

In a continuing effort to evaluate the effects of long-term cultivation of GM *Zoysia* grass on the environment, we focused our attention on soil microorganisms in the test field. In this study, we investigated the possibility of unintended HGT from GM grass to uncultured soil microorganisms with cultivation-independent method by PCR. We also analyzed the bacterial community structure of the rhizosphere of GM *Zoysia* grass and compared it with its non-GM counterpart using 16S rRNA gene sequence analysis.

Materials and Methods

Study Site, Sampling, and DNA Extraction

This study was conducted on a test field located in Sumangri, Jeju, Korea, which was approved for environmental risk assessment of GM plants by the Rural Development Administration/Korea Ministry of Agriculture and Forestry [5]. The test field consisted of two plots (24 m×12 m each) planted with GM *Zoysia* grass (GM plot) and non-GM *Zoysia* grass (non-GM plot). These plots were separated by 1 m of distance. The GM grass was sprayed with herbicide solution containing 2 g/l of bialaphos in order to provide selective advantage to the GM grass. The physicochemical properties of soils from GM and non-GM plots were studied previously and there was no statistically significant difference between these soils [5]. *Zoysia* grasses were planted in May 1999 and the sampling date for this study was 16 April 2010.

Three sampling sites, separated by >5 m radii, were randomly chosen from each plot, and samples from the top 15 cm of soil were taken. Each replicate sample was sieved through a 2-mm mesh sieve to remove any root debris and stones, and stored at 4°C prior to use.

Soil community DNAs were extracted directly from 0.5 g (dry weight) of each soil sample using the FastDNA SPIN Kit for Soil (MP Biomedicals, OH, USA) according to the manufacturer's protocol. Extracted DNAs from each replicate were pooled to provide representative samples from two plots, and quantified by agarose gel electrophoresis using a calibrated set of standard DNAs of known concentrations. Extracted community DNAs were stored frozen (−20°C) until further processing.

PCR Detection of *bar* and *hpt* Genes

The GM *Zoysia* grass contains the hygromycin resistance gene (*hpt*) and the bialaphos resistance gene (*bar*) as selection markers [35]. To test for the presence of *bar* and *hpt* genes in microbial community DNAs, the extracted soil DNA samples were subjected to PCR analysis using two primer sets specific for the *bar* and *hpt* genes (Table 1). Amplifications were performed in 50-μl reactions with *Taq* DNA polymerase (Enzynomics, Daejeon, Korea) as described previously [6]. pGPTV-HB DNA, which contains the *bar* and *hpt* genes [35], was used as a positive control.

PCR Amplification and 16S rDNA Clone Library Construction

The genomic DNAs directly extracted from soils of each plot were used as templates for PCR. Nearly full-length 16S rDNAs were amplified using the two bacteria-specific 16S rDNA primers 27F and 1522R (Table 1) [22]. PCR were carried out in 50-μl volumes, containing 25 pmol of each primer, 200 μM dNTP, 1× PCR buffer, 2.5 U of *Taq* polymerase (Enzynomics), and 100 ng of the extracted DNA. Reactions were carried out in a MyCycler thermal cycler (BIO-RAD, CA, USA) under the conditions as follows: initial denaturation at 95°C for 3 min, 25 cycles of denaturing at 94°C for 30 s, annealing at 54°C for 1 min, and extension at 72°C for 1 min, and a final extension step at 72°C for 7 min. Three PCR replicates of each sample were separately amplified and visualized on a 1% agarose gel. The resulting PCR products were gel-purified using a GEL SV Gel Extraction Kit (General Biosystem, Seoul, Korea) and combined separately for GM sample and non-GM sample. Two 16S rRNA gene libraries were constructed, one for each soil bacterial community from the GM plot and non-GM plot. The mixed populations of PCR products from each plot were ligated into the TOPcloner TA V2 vector (Enzynomics) and transformed into *E. coli* DH5α competent cells. Approximately 70 putative positive transformants from each clone library were randomly selected, and recombinant DNAs were purified using a Plasmid SV Mini Kit (General Biosystem) as recommended by the manufacturer. Restriction analysis using *Eco*RI was performed to ensure the presence of correctly sized inserts (about 1,500 bp). Clones that contained insert of incorrect size were excluded from sequencing. Sixty clones with the correct size were selected from each library for subsequent sequencing.

Table 1. Primers used in this study.

Primer	Sequences (5'→3')	Size (bp)	Target gene
bar-F	CATCGAAACAAGCACGGTCAACTTC	340	*bar* [6]
bar-R	TCCGAGCGCCTCGTGCATGCG		
hpt-F	CGCCGATGGTTTCTACAA	839	*hpt* [6]
hpt-R	ATAGTGGAAACCGACGCC		
27F	AGAGTTTGATCCTGGCTCAG	1,495	16S rDNA [22]
1522R	CATGCGGCCGCAAGGAGGTGATCCAACCGCA		

Detection of Chimeric Sequences and Sequence Analysis

A total of 120 purified DNAs were sequenced by a BigDye Terminator Cycle Sequencing Ready Kit with an automatic sequencer (ABI PRISM 3700 DNA Analyzer, Applied Biosystems, Foster City, CA, USA). Sequences with less than 500 bp or with multiple ambiguous bases were excluded from analysis. Sequences were manually edited to remove PCR primer and vector sequences. The remaining sequences were checked for chimeric artifacts by the program CHECK_CHIMERA at the Ribosomal Database Project II (RDP II) [9] and Bellerophon server [19], and all chimera sequences were eliminated. Cloned sequences were identified by performing BLAST analysis on the GenBank database at the National Center for Biotechnology Information (NCBI) [1], and the Classifier program [38] of the RDP II. In addition, 16S rDNA sequences of the two clone libraries were compared using the RDP II Library Compare Tool at the 80% confidence threshold.

Phylogenetic Tree Construction and Statistical Analysis

Alignments of 16S rRNA gene sequences and their closest relatives were performed with the ClustalW program (version 2.0) [34]. The aligned sequences were edited by using the BioEdit program [18]. Phylogenetic trees were constructed using MEGA (version 4) [33] by the neighbor-joining algorithm based on distance calculated by the Kimura-2 parameter model. Bootstrap analyses for 1,000 replicates were performed to provide confidence estimates for phylogenetic tree topologies.

To evaluate whether observed differences in bacterial community composition represented statistically significant differences, the two clone libraries were compared using the LIBSHUFF computer program [32]. The 16S rDNA sequences were aligned using ClustalW with a PHYLIP output. The aligned sequences were analyzed by LIBSHUFF after bootstrapping 1,000 times using the SEQBOOT program within PHYLIP (v. 3.69) [14] and generating distance matrices in the DNADIST program of PHYLIP using the Kimura-2 parameter model.

Nucleotide Sequence Accession Numbers

The partial 16S rRNA gene sequences determined in this study were deposited in the GenBank database under the accession numbers HQ121266 to HQ121365.

RESULTS AND DISCUSSION

DNA Extraction and Construction of 16S rDNA Clone Libraries

Environmental risk assessments of herbicide-tolerant plants have been focused on the possibility of HGT of transgenic DNA from GM plants to their wild relatives in the environmental habitat [39]. However, the effects of herbicide-tolerant plants and the associated herbicide application on the plant-associated microorganisms have rarely been addressed. The two most common environmental concerns associated with the effects of GM plants on soil microbial communities are the possibility of HGT of transgenic genes to soil microorganisms and the alteration of microbial diversity. Cultivation-based analysis of soil microbial diversity often leads to an underestimation of the true diversity, because less than 1% of the microorganisms present in soil can be cultivated [2]. A cultivation-independent technique based on PCR amplification of the 16S rRNA gene can overcome this limitation and might be a useful tool in environmental risk assessment studies to analyze the diversity of soil bacteria in the rhizosphere [20]. The first critical step in 16S rRNA gene library construction is the extraction of genomic DNA from a soil sample. DNA isolation from soil results in the co-extraction of humic acids, which interfere with further DNA works [41]. Although many soil extraction protocols have been described [23], DNA yields were varied depending on the soil types. The test field in Jeju consists of hydrothermally modified volcanic soils. An initial attempt to extract DNA using a mechanical-based bead-beating protocol [40] was ineffective. A chemical method using hexadecyltrimethylammonium bromide (CTAB) [4] also failed, as DNA yields were poor (data not shown). An efficient extraction of DNA was achieved from soils of the test field using the FastDNA SPIN Kit (MP Biomedicals). The yields of extracted DNAs were 2.0 to 5.0 μg/g dry weight soil by this commercial kit.

Detection of *hpt* and *bar* Genes in Total Community DNA from Soil

To assess the possibility of HGT from transgenic *Zoysia* grass to soil microorganisms, total community DNAs were extracted directly from the soils of GM and non-GM plots and analyzed by PCR. The transgenic *Zoysia* grass contained two marker genes, *hpt* and *bar*, conferring hygromycin and bialaphos resistance, respectively, to the plant [35]. Extracted DNAs from soil samples were subjected to PCR using the two different primer sets specific for the *bar* and *hpt* genes (Table 1). Fig. 1 shows the results of the PCR detection. DNA bands corresponding to the *bar* and *hpt* genes were observed in the positive control, but not in the genomic DNAs extracted from soil samples. Although there was no positive result, it is difficult to conclude that HGT of transgenic genes from GM grass to soil microorganisms did not occur under the environmental conditions, owing to the small sampling sizes and the limitations of the PCR method. Indeed, there is a need for more sensitive molecular biological methods to directly detect the transgenic genes in the soil ecosystem. Although there are few reports on HGT from plant to bacteria [10, 15], experimental evidence that HGT of transgenic DNA from plants to bacteria really occurs has been lacking [26, 29].

Taxonomic Identification of 16S rRNA Gene Sequences

In order to compare the bacterial community structures of the non-GM and GM plots, two separate 16S rRNA gene clone libraries were constructed by a culture-independent molecular method. Extracted genomic DNAs from soils of two plots were separately amplified using universal bacterial 16S rDNA primers 27F and 1522R (Table 1). The number

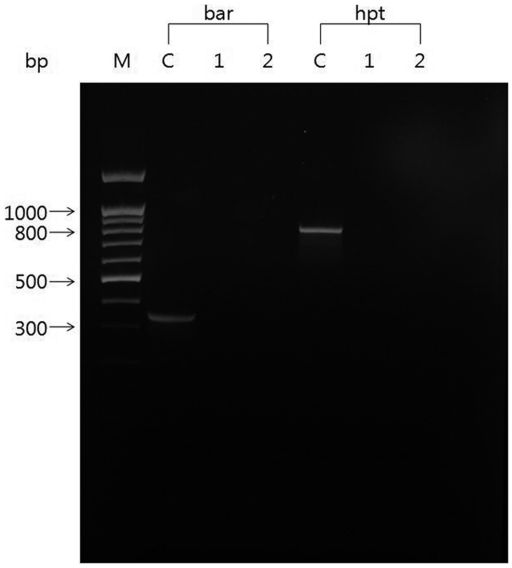

Fig. 1. PCR analysis for detecting *bar* and *hpt* genes in total community DNAs isolated from non-GM and GM plots.
Lane M, size marker (100 bp ladder); lane C, positive control (pGPTV-HB DNA); lane 1, DNA from non-GM plot; lane 2, DNA from GM plot.

of PCR cycles was reduced to 25 cycles to prevent bias towards one particular group [37]. The amplified fragments were cloned separately to produce two 16S rDNA libraries representing each plot. Sixty positive clones from each library were randomly selected and sequenced. Twelve poorly sequenced clones were excluded, and vector and primer sequences were removed manually. Chimeric sequences are frequently found in 16S rDNA clone libraries of environmental samples with mixed microbial populations [36]. The remaining 108 sequences (55 sequences from non-GM clones and 53 sequences from GM clones) were screened for chimeras using the program CHECK_CHIMERA at RDP II [9]. Eight chimeric sequences (5 from non-GM and 3 from GM) were identified and were omitted from further analysis. The absence of chimeras in the remaining 100 clones was confirmed with the Bellerophon program [19].

The partial 16S rDNA sequences were trimmed at fixed intervals, and only sequences approximately 620 bp in length, spanning between *E. coli* positions 775 and 1,400 and encompassing five hypervariable regions (V5-V9) [25], were used for further analysis. BLAST analysis showed that all sequences from both clone libraries displayed relationships with a wide range of environmental sequences from various uncultured bacteria. No sequence was found that was identical to any of the known 16S rDNA sequences in the database, and only 5 of 100 sequences (5%) showed more than 97% similarity to the nearest known strain, which was a criterion of same species (data not shown).

To compare the community structure and examine taxonomic representation within the community, we analyzed the 16S rRNA gene sequences data using the Classifier [38] and

Table 2. Phylotype distribution of 16S rRNA gene sequences detected in two clone libraries.

Taxonomic groups	% Phylotypes in the clone libraries (class)	
	non-GM plot	GM plot
Phylum Acidobacteria	44	64
Class Gp1	(14)	(18)
Class Gp2	(18)	(34)
Class Gp3	(8)	(4)
Class Gp4	(0)	(2)
Class Gp7	(4)	(4)
Unclassified Acidobacteria	(0)	(2)
Phylum Proteobacteria	24	24
Class Alphaproteobacteria	(6)	(4)
Class Betaproteobacteria	(10)	(16)
Class Gammaproteobacteria	(2)	(4)
Class Deltaproteobacteria	(4)	(0)
Unclassified Proteobacteria	(2)	(0)
Phylum Actinobacteria	4	0
Phylum Planctomycetes	4	0
Phylum Chloroflexi	4	0
Phylum Verrucomicrobia	2	0
Phylum Nitrospira	4	0
Phylum Gemmatimonadetes	0	4
Phylum OP10	0	2
Unclassified bacteria	14	6

Library Compare program of the RDP II. Results from the RDP II Library Compare showed that the two clone libraries had different bacterial community compositions. Table 2 summarizes the taxonomic distribution of all clones in both libraries. Based on the confidence threshold of 80%, the fifty 16S rRNA gene sequences obtained from the non-GM plot were assigned to 7 phyla and 8 classes, whereas only 4 phyla and 8 classes were detected in the sequences from the GM plot (Table 2). The most abundant taxonomic groups in both clone libraries were *Acidobacteria* and *Proteobacteria*. These two groups together made up 78% of all the obtained clones. *Acidobacteria* was the most diverse and abundant phylum in both libraries. This group made up 54% of the total clones. The phylum *Acidobacteria* is one of the most dominant bacterial groups in soil environments [7, 21]. Based on phylogenetic analyses, members of *Acidobacteria* were divided into eight subdivisions (Gp1–Gp8) [27]. However, relatively little is known about their function in soils and most of them are unculturable. Subgroups Gp1 and Gp2 dominated within the *Acidobacteria* in both clone libraries. These two subgroups together made up 78% of *Acidobacteria* from both clone libraries. The phylum *Proteobacteria* was the second most dominant group in both clone libraries. This group made up 24% of the total clones. The most abundant and diverse class within the *Proteobacteria* was *Betaproteobacteria*, followed by the *Alpha*-, *Gamma*-, and *Deltaproteobacteria*. Members of the *Deltaproteobacteria* were not detected in the GM

library. Five phyla were unique to the clones from the non-GM plot. These included *Actinobacteria*, *Planctomycetes*, *Chloroflexi*, *Nitrospira* (each with two clones), and *Verrucomicrobia* (one clone). In contrast, *Gemmatimonadetes* (two clones) and OP10 (one clone) were detected only in the clones from the GM plot (Table 2), suggesting that the non-GM plot might have more diverse bacterial communities than the GM plot. Some clone sequences were regarded as unclassified bacteria because they had less than 80% similarity to published sequences in GenBank databases, and thus their taxonomic positions were unclear. However, the non-GM library contains more unclassified bacteria than the GM library. Overall, the 16S rRNA gene sequences from the GM plot had a lower number of known phyla than those from the non-GM plot, possibly indicating a decrease in bacterial diversity.

Phylogenetic Analysis of 16S rRNA Gene Sequences

The 16S rDNA sequences obtained from the two clone libraries were also subjected to phylogenetic analysis. The phylogenetic tree of clone sequences from the non-GM plot and known strains belonging to each phylum is shown in Fig. 2, whereas the phylogenetic tree of clone sequences from the GM plot and known strains is shown in Fig. 3. The overall structures of the 16S rDNA phylogenetic trees were consistent with the expected taxonomy analyzed by Classifier of RDP II (Table 2), except that clone W1, which belonged to *Betaproteobacteria* in Classifier analysis, was related to the *Acidobacteria* group in the phylogenetic analysis.

Within the phylum *Acidobacteria* in the non-GM library, four clusters were found (Fig. 2). Nine clones (W9, W18, W25, W26, W30, W32, W41, W44, W45) belonged to the Gp2 cluster (supported by a 100% bootstrap value), and seven clones (W6, W14, W23, W28, W33, W35, W49) existed in the Gp1 cluster, supported by a 96% bootstrap value. Four clones (W7, W29, W36, W47) fell in a cluster representing Gp3, and two clones (W5, W42) belonged to class Gp7. Unclassified clone W11 occupied a separate position within the *Acidobacteria* clusters. Within the phylum *Proteobacteria* in the non-GM library, several clusters and subclusters were found (Fig. 2). Four clones (W10, W13, W37, W40) grouped with *Thiobacter subterraneus* in the *Betaproteobacteria* cluster. Within the *Alphaproteobacteria* cluster, we found two subclusters, one with W22 and *Phenylobacterium falsum* and another one containing W34 and W46. Unclassified clone W27 was related to the latter subcluster. Clone W43 had *Haliangium tepidum* as its closest relative, and clone W3 occupied a separate position in the *Deltaproteobacteria* cluster. Two clones (W16, W21) fell in a *Planctomycetes* cluster containing the type strain *Zavarzinella formosa*. Clones W8 and W20 clustered with the type strain of *Nitrospira moscoviensi*s. Two clones (W39, W48) clustered in the *Actinobacteria* group (supported by a 100% bootstrap value). Two clones (W15, W50) clustered with *Thermomicrobium roseum* in the *Chloroflexi* cluster. In the non-GM library, two unclassified clones (W17, W24) occupied separate positions close to the *Planctomycetes* cluster, and three unclassified clones (W19, W31, W38) were related to the *Chloroflexi* cluster (Fig. 2).

In the GM clone library, only four bacterial phyla were identified. Two subclusters were found in the *Acidobacteria*

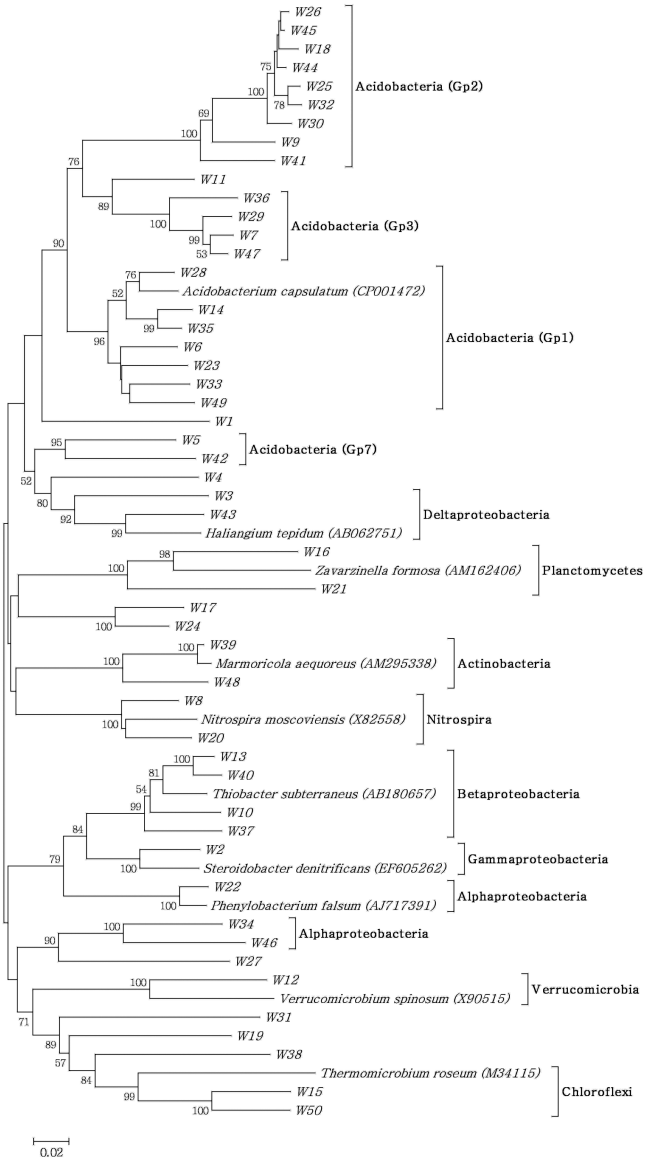

Fig. 2. Unrooted phylogenetic tree showing the relationship of 16S rRNA gene sequences from the clone library of the non-GM plot.

WT followed by a number means clones from the non-GM plot. Sequence analysis was performed using the neighbor-joining method with the Kimura-2 parameter model. Bootstrap values are based upon 1,000 replicates; only values greater than 50% are shown. The closest known sequences from GenBank were included as references for each phylum. GenBank accession numbers for reference sequences are given in parentheses. The scale bar indicates 0.02 substitutions per nucleotide position.

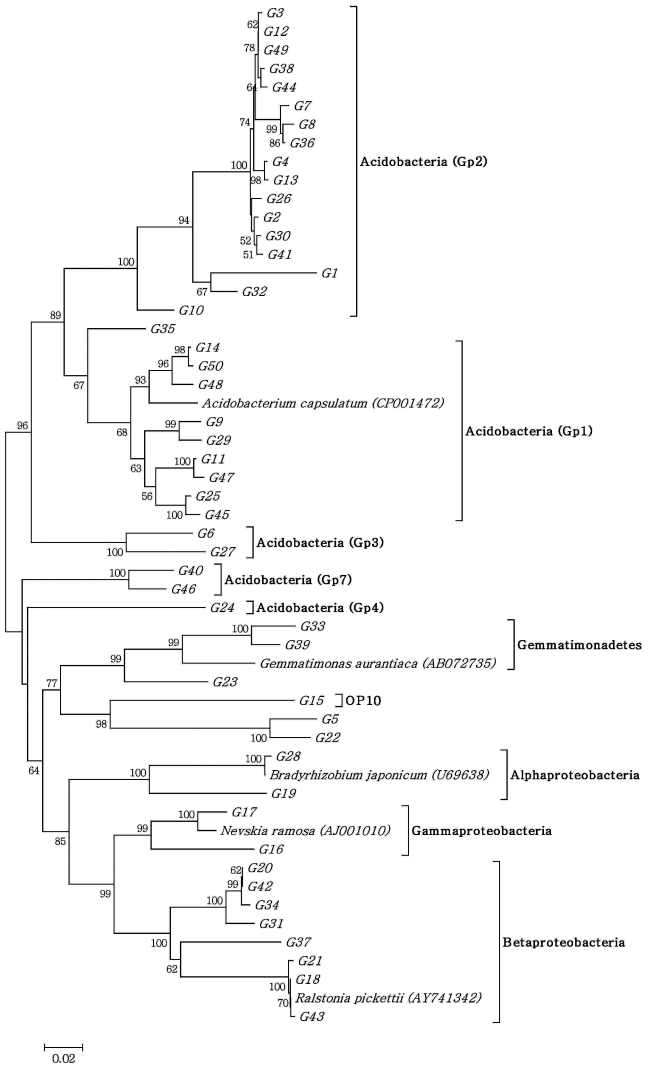

Fig. 3. Unrooted phylogenetic tree showing the relationship of 16S rRNA gene sequences from the clone library of the GM plot.
GM followed by a number means clones from the GM plot. Sequence analysis was performed using the neighbor-joining method with the Kimura-2 parameter model. Bootstrap values are based upon 1,000 replicates; only values greater than 50% are shown. The closest known sequences from GenBank were included as references for each phylum. GenBank accession numbers for reference sequences are given in parentheses. The scale bar indicates 0.02 substitutions per nucleotide position.

class Gp1. Two clones (G6, G27) fell in a cluster representing Gp3, and clones G40 and G46 constituted another Gp7 cluster (Fig. 3). In the *Betaproteobacteria* cluster, three subclusters were found. Amongst the clones of the *Betaproteobacteria* cluster, four clones (G20, G31, G34, G42) clustered (supported by a 100% bootstrap value) and another three clones (G18, G21, G43) were related to the type strain of *Ralstonia pickettii*. Clone G37 occupied a separate position. Two clones (G19, G28) grouped with *Bradyrhizobium japonicum* in the *Alphaproteobacteria* cluster. Clone G17 had *Nevskia ramosa* as its closest relative, and clone G16 occupied a separate position in the *Gammaproteobacteria* cluster (supported by a 99% bootstrap value). Two clones (G33, G39) clustered with the type strain of *Gemmatimonas aurantiaca*, and one unclassified clone (G23) occupied a separate position close to the *Gemmatimonadetes* cluster. Clone G15 belonging in the OP10 phylum clustered with two unclassified clones (G5, G22) occupying a separate position (Fig. 3).

Table 3. LIBSHUFF comparison of bacterial clone libraries from non-GM and GM plots.

Homologous (X) library	No. of clones	Heterologous (Y) library	*P* value[a]
Non-GM	50	GM	0.017[b]
GM	50	Non-GM	0.022

[a]Calculated using the LIBSHUFF program [32].
[b]Libraries are considered to be different from each other if either of the P values is less than 0.025.

The objective of this study was to determine the possible effects of GM grass and the associated herbicide application on soil bacterial community. An effect on the bacterial diversity might also be expected as a result of the herbicide Basta application. The application of Basta might cause the activation of *Acidobacteria* that was able to metabolize the herbicide. However, glufosinate ammonium (active ingredient of Basta) degrades rapidly in a soil environment [13] and may have little deleterious influence on soil ecosystems. In studies of Basta-tolerant maize, the herbicide did not affect the rhizosphere microbial community structure [30]. In contrast, other studies showed that glufosinate-tolerant transgenic oilseed rape may host altered rhizosphere microbial communities in comparison with the wild type [12, 16]. The result of this study was that the bacterial community structure in the GM plot was different from that in the non-GM plot in terms of phylogenetic distribution and phylotype composition of the 16S rDNA clones.

Comparison of the Bacterial Compositions Between the Clone Libraries

In an attempt to determine the significance of difference between the two clone libraries, LIBSHUFF statistics [32] was applied. Two clone libraries are assumed to be different if their homologous and heterologous coverage curves differ significantly. If the lower of the two *P* values returned by LIBSHUFF is less than or equal to 0.025, the two libraries are significantly different in community composition, with a confidence of 95% (p=0.05) [32]. Statistical comparison of both clone libraries demonstrated significant difference between the two bacterial community compositions (both *P* values were <0.025; Table 3). Consequently, the bacterial community composition of the GM plot was different from that of the non-GM plot, although the two plots were close to each other and probably had the same original substrate.

Sequence analysis of a PCR-derived clone library is useful for gaining an insight into the soil microbial communities. However, biases associated with the use of PCR, which may alter the abundance of identified bacteria, were also pointed out [36]. Nevertheless, it is unlikely that the difference found in this study was simply due to PCR and cloning bias, as the two clone libraries were created under identical conditions in our study to ensure that any biases occurred to the same degree across the samples. Therefore, statistical analysis could be used to make a relative comparison of the bacterial diversity between the two plots. However, as we only compared 50 clones from each library, it represents a rough estimate of the diversity of the dominant members of soil bacteria, and the conclusive result needs to be tested in studies of a larger number of clones.

In conclusion, analysis of 16S rDNA clone libraries indicated that bacterial community structures differed between the soils from a non-GM plot and a GM plot. The bacterial community of the GM plot seemed less diverse than that of the non-GM plot.

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