J. Microbiol. Biotechnol. (2005), **15**(5), 1087–1093

JOURNAL OF MICROBIOLOGY AND BIOTECHNOLOGY

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Molecular and Cultivation-Based Characterization of Bacterial Community Structure in Rice Field Soil

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Received: February 15, 2005
Accepted: April 13, 2005

Abstract The population diversity and seasonal changes of bacterial communities in rice soils were monitored using both culture-dependent approaches and molecular methods. The rice field plot consisted of twelve subplots planted with two genetically-modified (GM) rice and two non-GM rice plants in three replicates. The DGGE analysis revealed that the bacterial community structures of the twelve subplot soils were quite similar to each other in a given month, indicating that there were no significant differences in the structure of the soil microbial populations between GM rice and non-GM rice during the experiment. However, the DGGE profiles of June soil after a sudden flooding were quite different from those of the other months. The June profiles exhibited a few intense DNA bands, compared with the others, indicating that flooding of rice field stimulated selective growth of some indigenous microorganisms. Phylogenetic analysis of 16S rDNA sequences from cultivated isolates showed that, while the isolates obtained from April soil before flooding were relatively evenly distributed among diverse genera such as *Arthrobacter*, *Streptomyces*, *Terrabacter*, and *Bacillus/Paenibacillus*, those from June soil after flooding mostly belonged to the *Arthrobacter* species. Phylogenetic analysis of 16S rDNA sequences obtained from the soil by cloning showed that April, August, and October had more diverse microorganisms than June. The results of this study indicated that flooding of rice fields gave a significant impact on the indigenous microbial community structure; however, the initial structure was gradually recovered over time after a sudden flooding.

Key words: 16S rRNA, soil DNA, DGGE, bacterial community, rice field

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The soil environment includes numerous microhabitats within which diverse microbial populations interact. Soil microbial communities are quite complex and stable, but their structure and composition can be dramatically changed when exposed to sudden environmental changes, such as flooding and fertilization [30]. Soil microbiological experiments traditionally have focused on cultivation of individual microbial species, which can be used for further phylogenetic and physiological characterizations [13, 36]. However, cultivation has limited usefulness, since most of the soil microorganisms are known to be unculturable on laboratory media [2, 8].

Molecular methods based on total soil microbial community DNA analysis can overcome the limitation of culture-based techniques. Analysis of 16S rDNA sequences cloned from environmental DNA facilitated detection and identification of unculturable microorganisms and has confirmed the immense underestimation of naturally occurring bacteria and archaea in the environment [11, 12, 15, 19, 20]. Denaturing gradient gel electrophoresis (DGGE) of PCR-amplified 16S rRNA genes has been used to produce DNA banding patterns that represent the whole microbial community, allowing to detect significant changes of the microbial community structure in nature [10, 21, 26].

Unlike upland fields, the wet rice fields are characterized by submergence during part or all of the cropping period. Flooding changes the physicochemical properties and nutrient status of rice soil, leading to the differentiation of diverse microhabitats that can support the growth of a variety of microorganisms. In addition, the introduction of genetically modified crop plants might affect a microbial community and biogeochemical processes in agricultural soils [17, 25, 31]. There have been several investigations on the changes of microbial communities of rice soils using phospholipid

fatty acids and Biolog microplates [4, 29]. Recently, population dynamics of archeal bacteria and methane-oxidizing bacteria have been characterized by analysis of 16S rRNA genes and metabolic genes [11, 20]; nevertheless, limited information is available about the diversity and changes of total microbial community structure and their spatial distribution following sudden environmental changes in rice field soils.

In this study, we used both molecular and cultivation techniques to examine numerically dominant members of the bacterial community in GM rice experimental field soils during four different months throughout the year. Microbial community diversity was analyzed by DGGE of specific DNA fragments of 16S rRNA genes. 16S rDNA sequences obtained by direct cloning from soil DNA and isolated bacterial populations were analyzed and compared with each other. The objectives of this work were to investigate species diversity of bacterial populations, to study seasonal changes in the bacterial community, and to compare the results obtained by culture-based methods with those from DNA-based techniques between GM rice and non-GM rice soils.

Materials and Methods

Rice Soil Samples and Culture Media

The experiments were carried out in the risk assessment experimental area at the Agricultural Research site at Suwon, Gyunggido, Korea. The rice field, characterized as a clay loam soil, has been under normal agricultural practices for more than 20 years. The plot consisted of twelve subplots, each 2.0 by 5.0 m. Four different rice varieties (two glufosinate-resistant GM rice plants, Iksan 483 and Milyang 204; two control non-GM rice plants, Anjung and Dongjin) (T. S. Kim *et al.*, 2004. *Abstr. International Symposium on Rice Production Technology*, Suwon, Korea, PS1-60) were planted early in June and grown on the subplots in three replicates. Soil samples from the rice field were taken from each subplots in April (30 days before flooding), June (30 days after flooding), August, and October and kept at 4°C prior to use. A 10-g soil sample from each site was homogenized with 95 ml of sterilized 0.85% saline solution by shaking at 200 rpm on a rotary shaker (Vision Co., Bucheon, Korea). Total counts of bacteria, actinomycetes, and fungi were measured with tryptic soy agar, sodium caseinate agar, and malt extract agar medium, respectively [37]. Dominant bacterial strains were isolated by plating appropriate dilutions of soil suspensions onto peptone-tryptone-yeast extract-glucose (PTYG) medium [3]. All cultures were incubated at 28°C for 4–7 days.

Identification of Dominant Bacterial Isolates

Dominant bacterial isolates were subjected to repetitive extragenic palindromic (REP)-PCR procedure to distinguish different strains by comparison of their genomic DNA fingerprints [6, 35]. Computer-assisted analysis of genomic fingerprints was performed using the NTSYS program (Version 1.8; Exeter Software, New York, U.S.A.). Cluster analysis of similarity matrices was performed by the unweighted pair group method using arithmetic averages [33]. For taxonomic identification of the isolates, total genomic DNA was extracted from each strain with a Wizard Genomic DNA Purification Kit (Promega, Madison, U.S.A.). PCR amplification of nearly full-length 16S rRNA genes was performed with 27f and 1492r (*E. coli* 16S rRNA gene sequence numbering) as previously described [5, 18]. PCR products were purified from agarose gel slices using a QIAquick Gel Extraction Kit (QIAGEN, Hilden, Germany). Sequencing was performed with an ABI Prism BigDye Terminator Cycle Sequencing Ready Kit (Applied Biosystems, Foster City, U.S.A.) according to the manufacturer's instructions with the sequencing primers 27f and 519r. Approximately 400 unambiguous nucleotide positions were used for comparison with the data in GenBank using the Basic Local Alignment Search Tool (BLAST) [1]. Sequences from nearest relatives were identified from the Ribosome Database Project (RDP) using the Similarity-Rank program of the RDP [22].

PCR Amplification of Soil Bacterial 16S rDNA

Soil microbial community DNA was extracted using a FastDNA Spin Kit (Qbiogene, Carlsbad, CA, U.S.A.). PCR was carried out using the primers 27f and 1492r, which target the 16S rRNA genes of a wide range of members of the domain bacteria at positions 28 through 1,491 (*E. coli* 16S rRNA gene sequence numbering) [18]. PCR amplification was performed in 50-μl reaction mixtures containing 10× PCR buffer (200 mM Tris/HCl, 500 mM KCl, pH 8.4), 100 ng of template DNA, 25 pmol each of primer, 200 μM each of dNTP (Boehringer Mannheim, Indianapolis, IN, U.S.A.), and 2.5 U of *Taq* polymerase. PCR cycles consisted of an initial denaturation at 95°C for 5 min, followed by 30 cycles of 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min and a final extension at 72°C for 7 min. After PCR amplification, 10 μl samples of the PCR products were checked by electrophoresis on horizontal 1.5% agarose gels.

Cloning and Sequencing

The 16S rDNA PCR products obtained from the soil bacterial community DNA were cloned into the pGEM-T Easy Vector as recommended by the manufacturer (Promega, Madison, WI, U.S.A.). The preparation of randomly selected clones, followed by PCR amplification of cloned inserts and purification of PCR products, were carried out as described previously [14]. Sequencing was performed with an ABI Prism BigDye Terminator Cycle Sequencing Ready Kit (Applied Biosystems, Foster City, CA, U.S.A.)

according to the manufacturer's instructions with the sequencing primers 27f and 519r. Approximately 400 unambiguous nucleotide positions were used for comparison with the data in GenBank using the Basic Local Alignment Search Tool (BLAST) [1]. Sequences from nearest relatives were identified from the Ribosome Database Project (RDP) using the Similarity-Rank program of the RDP [22]. An unrooted phylogenetic tree was constructed by the neighbor-joining method, using PAUP 4.0 beta10 [34].

Denaturing Gradient Gel Electrophoresis (DGGE) Analysis

Soil bacterial community DNA was extracted using a FastDNA Spin Kit (Qbiogene). For pure culture, total genomic DNA was extracted from each strain with a Wizard Genomic DNA Purification Kit (Promega). PCR amplification of the 16S rRNA genes was performed with primers 1070f and 1392r (*E. coli* 16S rRNA gene sequence numbering) as previously described [9]. The PCR product contained a GC clamp of 40 bases, added to the reverse primer, and had a total length of 323 bp, including the highly variable V9 region. PCR reaction mixtures were prepared as previously described [26]. PCR cycles consisted of an initial denaturation at 95°C for 5 min, followed by 30 cycles of 95°C for 1 min, 55°C for 1 min, and 72°C for 1 min, and a final extension at 72°C for 7 min. PCR products were subjected to DGGE with the Dcode™ Universal Mutation Detection System (BIO-RAD, Hercules, CA, U.S.A.) according to the procedure previously described [9]. PCR samples were applied directly onto 8% (wt/vol) polyacrylamide gels with a denaturing gradient, ranging from 40% to 70% (where 100% denaturant contained 7 M urea and 40% formamide). The gels were electrophoresed for 17 h at 60°C and a constant voltage of 40 V. After electrophoresis, the gels were stained with SYBR Green I, rinsed for 10 min, and photographed with UV transillumination (302 nm).

Nucleotide Sequence Accession Numbers

The partial 16S rRNA gene sequences from the clone library have been deposited in the GenBank nucleotide sequence databases under accession numbers AY921477 through AY921570 and AY930431 through AY930463.

RESULTS AND DISCUSSION

Changes of Total Microbial Populations

The total counts of bacteria, fungi, and actinomycetes in rice soils during this study were in the range of 2.3×10^6–1.3×10^7 cells/g soil, 6.3×10^3–2.8×10^4 cells/g soil, 2.5×10^5–6.9×10^5 cells/g soil, respectively. The bacterial cell densities in April before flooding, which ranged from 2.3×10^6 to 4.1×10^6 cells/g soil in three replicate subplots, were substantially increased to 7.9×10^6–1.0×10^7 cells/g soil in June after flooding and rice planting. Flooding of rice soil appears to enhance the availability of nutrients for the indigenous bacterial community, thus resulting in increase of their populations over the twelve subplots in this study. The increased bacterial populations were stably maintained during the rest of the experiment. On the other hand, the fungal cell densities were slightly decreased in June after flooding and then recovered to the initial densities in October, when the flooded water was almost gone. The actinomycetes populations were relatively stably maintained throughout the experiment. The overall population densities of the soil microorganisms were similar to those observed from agricultural soils [16, 27]. The result indicated that the bacterial community among the indigenous soil microbial populations responded and adapted most efficiently to flooding, but that there were no significant differences in soil microbial population levels between GM rice and non-GM rice during this study.

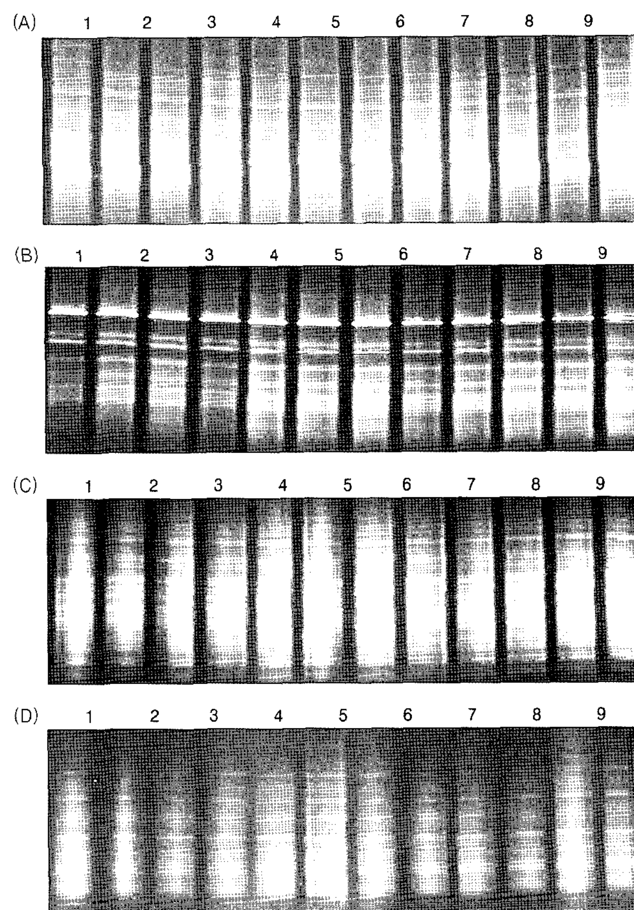

Fig. 1. DGGE analysis of 16S rDNA fragments obtained after PCR amplification with eubacterial primers 1070F and 1392R.
DGGE profiles for April (A), June (B), August (C), and October (D) paddy soils of Anjung rice (lanes 1, 7, and 10), Iksan 483 rice (lanes 2, 5, and 12), Dongjin rice (lanes 3, 8, and 11), and Milyang 204 rice (lanes 4, 6, and 9).

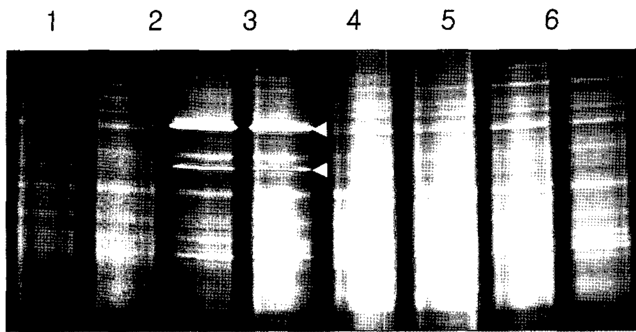

Fig. 2. DGGE analysis of 16S rDNA fragments obtained after PCR amplification with eubacterial primers 1070F and 1392R.
DGGE profiles of rice field soil samples taken in April (1, 2), June (3, 4), August (5, 6), and October (7, 8). Arrows point to the products corresponding with *Arthrobacter*, *Cellulomonas*, *Streptomyces*, and *Pseudomonas* species isolated from June rice soil.

DGGE Analysis

To investigate the impact of flooding and GM rice cultivation on the structure of the microbial community, total soil microbial DNAs extracted from rice field soils were analyzed by DGGE after PCR amplification of the variable V9 region of the 16S rRNA gene. For April soils before flooding and rice planting, very similar DGGE banding patterns were observed over the twelve subplots, indicating that initially there were no apparent differences in indigenous microbial community structure among these subplots (Fig. 1). Similarly, no differences were detected among June, August, and October rice soil samples (Fig. 1). However, the DGGE profiles of June soils after flooding and rice planting were quite different from those of the other months, as shown by the comparative DGGE banding profiles of four months throughout the year (Fig. 2). In particular, a few intense DGGE DNA bands were detected in June soil samples, compared with the other soil samples. When the bacterial isolates obtained from June soil were analyzed by DGGE, the intense 16S rDNA bands in the DGGE profiles were found to correspond with those of *Arthrobacter*, *Cellulomonas*, *Streptomyces*, and *Pseudomonas* species (Fig. 2, indicated by arrows). The dominance of the intense DNA bands was not observed in the DGGE profiles of subsequent August and October soil samples. Rather, the DGGE DNA band patterns of August and October rice soils became similar to those of April soil (Fig. 2). The dramatic changes of DGGE patterns from April to June indicated that the indigenous microbial community of April was significantly affected by flooding, and several microbial populations subsequently well adapted to flooding became dominant in June. Previously, soil microbial communities were found to be quite complex and stable, as shown by strong similarities between DGGE banding patterns from the different months throughout the year [7, 10, 32]. In our study, a sudden flooding apparently gave a substantial impact on the indigenous microbial community of the rice soil, leading to distinct DGGE profiles in June. However, the apparent similarity of DGGE profiles of August and October to those of April reflects that the soil microbial community is so stable as to gradually recover its initial structure over time after flooding. The results also indicated that there were no significant differences in the structure of the soil microbial populations between GM rice and non-GM rice during the experiment.

Analysis of Dominant Culturable Bacteria

To investigate species diversity of dominant culturable bacteria in rice soils before and after flooding, dominant colonies were isolated from each of the April and June soils that displayed a large difference in DGGE profiles. When the isolates were subjected to the REP-PCR procedure, which had been used to molecularly compare and type each strain based on its DNA fingerprint pattern [6, 35], the thirty two strains of April soil were grouped into twenty nine distinct groups (Fig. 3A). On the other hand,

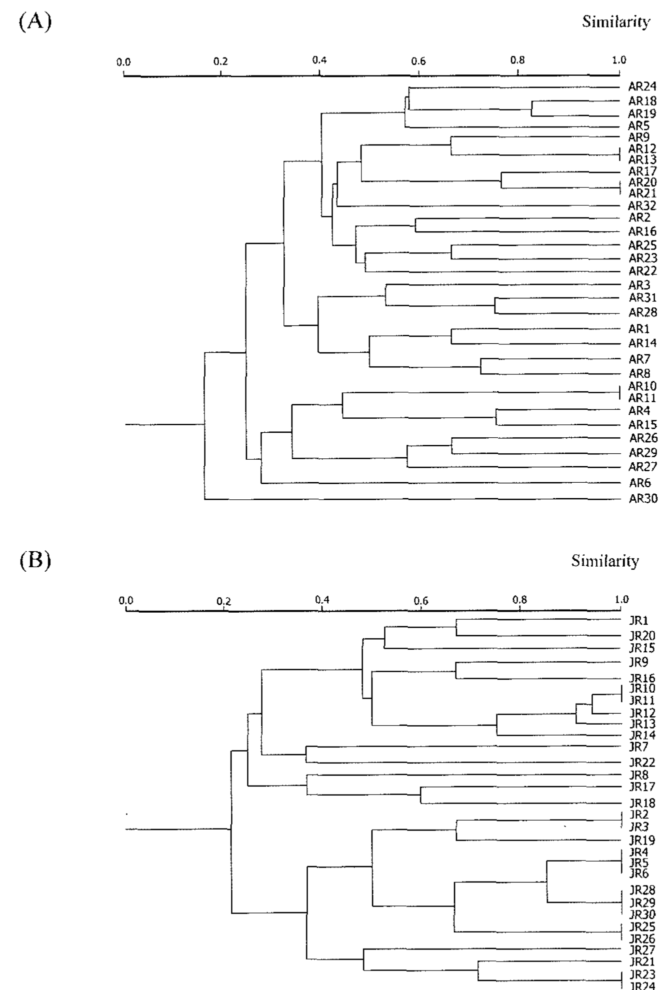

Fig. 3. REP-PCR-based dendrogram, showing chromosomal genetic relatedness of the bacterial strains isolated from April (A) and June (B) rice field soils.

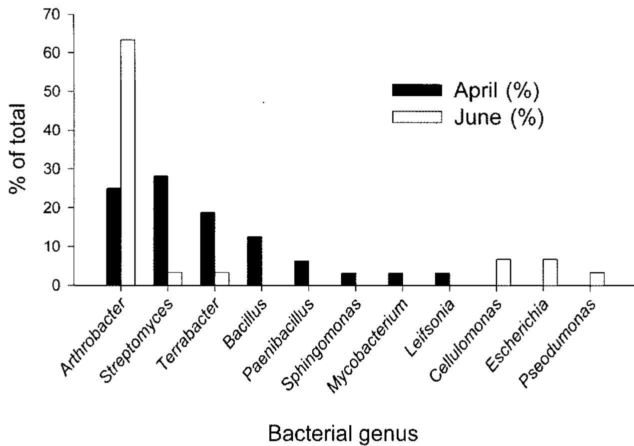

Fig. 4. Genus distribution of the dominant bacterial isolates obtained from April and June rice paddy soils.

many of the dominant isolates of June after flooding exhibited the same REP-PCR patterns; therefore, the thirty strains of June soil produced twenty two distinct groups (Fig. 3B). It is known that flooding of rice soil changes the chemical and physical properties of soil, resulting in changes of the microbial community structure of the soil [30]. Our result indicated that the bacterial community of April soil was significantly affected by sudden flooding, leading to selection of several strains in accordance with the decline of species diversity in June after flooding.

Analysis of 16S rDNA sequences revealed that the isolates of April soil belonged to *Streptomyces*, *Arthrobacter*, *Terrabacter*, *Bacillus*, *Paenibacillus*, *Sphingomonas*, *Mycobacterium*, and *Leifsonia* species, while those of June soil were strains of *Arthrobacter*, *Cellulomonas*, *Escherichia*, *Pseudomonas*, *Streptomyces*, and *Terrabacter* species. Figure 4 shows a comprehensive view on the distribution of the isolates over the major bacterial genera in April and June. It is of note that the isolates of April soil are dominated by several genera, whereas those of June soil are predominated by a genus, *Arthrobacter*, of the *Actinobacteria* division. This genus is often found in various soils, such as those of wheat fields and grasslands [23, 32]. The results indicated that flooding of rice soil, which could act as a selection pressure, gave a significant impact on bacterial community structure, resulting in predominance of *Arthrobacter* species in accordance with a decline of species diversity.

DNA-Based Characterization of the Dominant Bacterial Community

Since most of the bacterial populations in soil are known to be unculturable on laboratory media [2, 8], the phylogenetic information obtained from culturable bacteria may not properly represent the total bacterial community in rice soil. In order to investigate the change and diversity of the bacterial community using the molecular method, the dominant 16S rDNA sequences were cloned from rice soil DNA after PCR amplification. Phylogenetic analysis of these 16S rDNA sequences revealed that they were closely affiliated with a wide range of bacterial species or clones of various divisions (Fig. 5). The majority of the clone sequences identified appeared to cluster in bacterial taxonomic groups that are generally found in soil. In particular, members

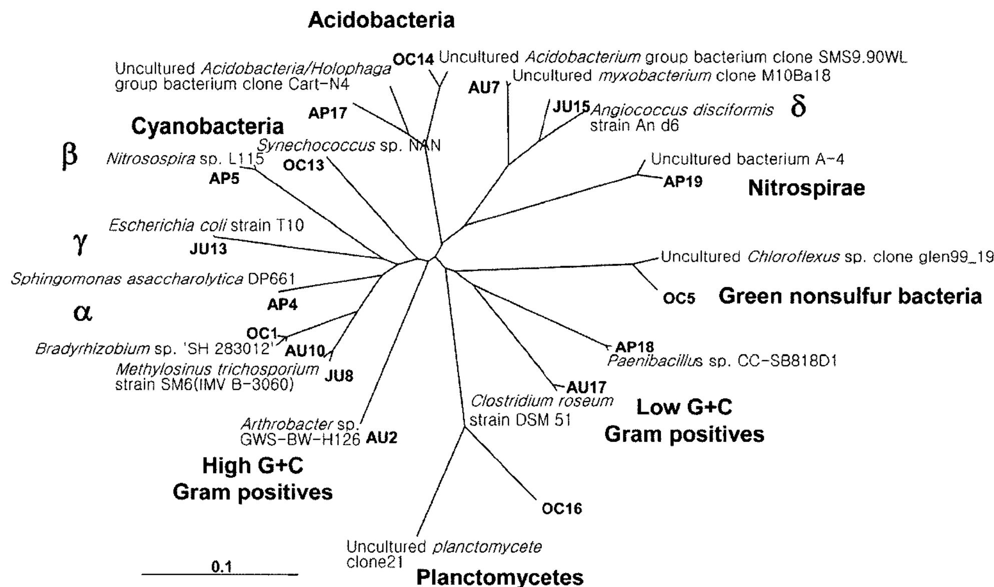

Fig. 5. Unrooted tree, showing the phylogenetic relationship of 16S rDNA clones (in bold) obtained from soil DNAs and reference strain rDNA sequences.

Analyses were based on approximately 400-bp rDNA fragments. The scale bar represents 0.1 estimated change per nucleotide.

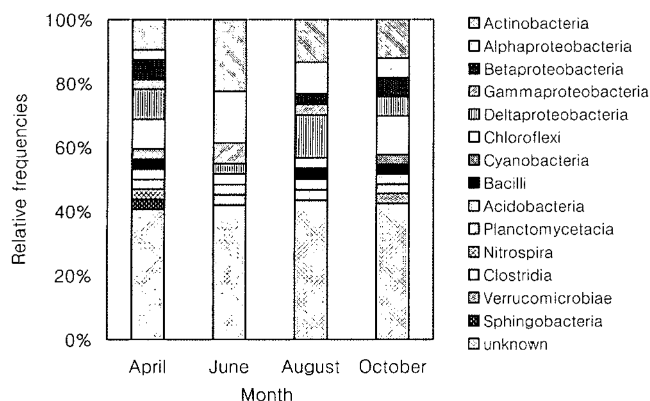

Fig. 6. Distribution of the 16S rDNA sequences obtained from rice paddy soils over various bacterial divisions.

belonging to the *Actinobacteria* division dominated the clone library of June after flooding (Fig. 6), which was consistent with the result obtained from the cultivation method. The next most abundant group of June, which consisted of five clones, had close relationship with members of the *Alphaproteobacteria* division. Regarding the distribution of the isolates over the bacterial divisions, April, August, and October showed high values, having twelve, ten, and ten divisions, respectively, whereas June had members in seven divisions (Fig. 6). The relatively low species diversity in June, which coincided with the result of the cultivation method, suggested that a sudden flooding changed the physicochemical conditions of the rice soil, thus giving a selection pressure on specific microorganisms. The recovery of the species diversity in August and October, which is consistent with the result of DGGE, reflects that soil microorganisms can adapt quickly to the flooded environment, recovering their diverse, stable community over the rest of the period. It has been suggested that the ratio between the *Proteobacteria* and the *Acidobacteria* division of the clone library might be indicative for the nutrient status of the soil [32]. McCaig *et al.* [24] found that 50% of their clones belonged to the *Proteobacteria* and 7% to the *Acidobacteria* division in an intensively fertilized agricultural soil. In our study with agricultural rice soil, approximately 26% of the clones belonged to the *Proteobacteria* and 3% to the *Acidobacteria* division; therefore, the ratio between the *Proteobacteria* and the *Acidobacteria* division was similar to that of McCaig *et al.* [24].

There was a large difference in the species diversity between the results obtained by the cultivation-based method and the DNA-based method. The majority of the isolates detected by the culture-based method were affiliated with the *Actinobacteria*, whereas the 16S rDNA clones detected with the molecularly based method were more evenly distributed among the *Actinobacteria, Alphaproteobacteria, Betaproteobacteria, Gammaproteobacteria, Deltaproteobacteria, Cyanobacteria, Chloroflexi, Bacilli, Acidobacteria,* and *Planctomycetacia*. Moreover, unlike the culture-based method, a majority of the clone sequences obtained by the molecular method could not be matched with known clones or bacterial species. The results suggested that the culturable fraction of the soil bacterial community is only a small part of the total community, and that the DNA-based method is able to detect more diverse bacterial species than the cultivation-based method.

This work shows that a sudden flooding gave a substantial impact on the indigenous microbial community of the rice paddy soil, leading to dramatic changes of DGGE banding patterns in June after flooding. The results of both the cultivation method and molecular method revealed that the bacterial diversity in June was greatly decreased ,possibly due to the selection pressure imposed by flooding. However, the observation that the initial microbial community structure was gradually recovered over the rest of the period indicates that the soil microbial community is quite stable to overcome sudden environmental changes. The results also indicated that there were no significant differences in the structure of the soil microbial populations between GM rice and non-GM rice over the experiment.

Acknowledgment

This study was supported by the BioGreen 21 Project.

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