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Improvement of PCR Amplification Bias for Community Structure Analysis of Soil Bacteria by Denaturing Gradient Gel Electrophoresis

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Abstract Denaturing gradient gel electrophoresis (DGGE) is one of the most frequently used methods for analysis of soil microbial community structure. Unbiased PCR amplification of target DNA templates is crucial for efficient detection of multiple microbial populations mixed in soil. In this study, DGGE profiles were compared using different pairs of primers targeting different hypervariable regions of thirteen representative soil bacteria and clones. The primer set (1070f-1392r) for the *E. coli* numbering 1,071–1,391 region could not resolve all the 16S rDNA fragments of the representative bacteria and clones, and moreover, yielded spurious bands in DGGE profiles. For the *E. coli* numbering 353–514 region, various forward primers were designed to investigate the efficiency of PCR amplification. A degenerate forward primer (F357IW) often yielded multiple bands for a certain single 16S rDNA fragment in DGGE analysis, whereas nondegenerate primers (338f, F338T2, F338I2) differentially amplified each of the fragments in the mixture according to the position and the number of primer-template mismatches. A forward primer (F352T) designed to have one internal mismatch commonly with all the thirteen 16S rDNA fragments efficiently produced and separated all the target DNA bands with similar intensities in the DGGE profiles. This primer set F352T-519r consistently yielded the best DGGE banding profiles when tested with various soil samples. Touchdown PCR intensified the uneven amplification, and lowering the annealing temperature had no significant effect on the DGGE profiles. These results showed that PCR amplification bias could be much improved by properly designing primers for use in fingerprinting soil bacterial communities with the DGGE technique.

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One of the main research objectives in the study of microbial ecology is to investigate the genetic and functional diversities of microbial communities in various environments. Traditionally, microbiological experiments depended on cultivation of individual species, which can be used for further phylogenetic and physiological characterizations [16, 34]. However, cultivation has limited usefulness, since most of the microorganisms in the environments are known to be unculturable on laboratory media [3, 9].

Several cultivation-independent molecular methods have recently emerged to overcome the limitation of culture-based techniques [10–12, 19, 24–26, 30, 32, 39]. Among them, denaturing gradient gel electrophoresis analysis of 16S rDNA fragments has become the most popular and culture-independent method to investigate the genetic diversity of microbial communities in nature. Since DGGE also allows the phylogenetic identification of community members through cloning and sequencing of the DGGE band, this technique is very useful for studies of individual dominant microbial populations as well as spatiotemporal variations of whole microbial communities.

In DGGE fingerprinting interpretation, an individual band is presumed as a discrete bacterial sequence type and the relative band intensity is considered to be related to the relative density of the corresponding sequence type within the sample [13]. This is based on two assumptions: no bias is obtained during the whole extraction-amplification procedure of the bacterial genomes and each sequence

type is separated from each other in the DGGE profile. However, many studies showed that these are not the cases: PCR bias to 1:1 ratio [31], PCR bias due to difference in primer binding energy [28] and primer mismatch [17], similar migration behaviors of nonrelated sequences [33], and formation of artificial bands by heteroduplex molecules in DGGE profiles [11] have been reported. However, some of these problems can be alleviated through the use of improved primers [35], the use of alternative PCR regimens [18], and the use of different variable regions in 16S rDNA [40].

In this work, we compared the PCR-DGGE banding profiles arising from a phylogenetically representative set of soil bacterial isolates and clones to obtain information on improvement of PCR amplification bias in molecular studies of microbial community structures. Two different regions of 16S rDNA (*E. coli* numbering 353–514 and 1,071–1,391) were amplified with different primer sets using the mixture of the thirteen representative 16S rDNA fragments of soil bacterial community as a template. The effects of a touchdown PCR program and different annealing temperatures on PCR amplification bias were also analyzed. Finally, how the choice of primer sets influences DGGE profiles was demonstrated using various soil bacterial community DNAs as PCR templates.

Materials and Methods

Preparation of DNA Templates

The 16S rDNA fragments used in this study were prepared in the previous studies [1, 19] and are presented in Table 1. Eleven of the thirteen 16S rDNA fragments were affiliated with ten different phyla, and two were affiliated with candidate phyla, TM7 and OP11, respectively [29]. The similarities between these sequences range within 66–88% when calculated using the DNADIST program in the PHYLIP package [8]. All the fragments, except I1, were cloned into the pGEM-T Easy Vector (Promega, Madison, U.S.A.) and PCR-amplified with the vector-specific primers T7 (5'-TAATACGACTCACTATAGGG-3') and SP6 (5'-TATTTAGGTGACA CTATAG-3') to eliminate the possible contamination with genomic DNA from *E. coli*. The PCR products were gel-purified with the QIAquick Gel Extraction kit (QIAGEN, Hilden, Germany) and re-amplified with the primers 27mf and 1492r as previously described [19]. The 16S rDNA fragment of I1 was amplified from the cell lysate with the primers 27mf and 1492r. The cell lysate was obtained using Lyse-N-Go PCR reagent (Pierce, Rockford, U.S.A.). Each of the thirteen PCR products was gel-purified and spectrophotometrically adjusted to the same copy number ($3\pm0.04\times10^{12}$ copies/ml). The mixture of the thirteen 16S rDNA fragments was obtained by equally combining all the fragments ($2.30\pm0.03\times10^{11}$ copies/ml for each).

PCR Primers

The PCR primers were selected from the previously published primers that had been used to amplify the 16S rDNA of the domain *Bacteria* for DGGE analysis. The primers were selected based on % match with the 16S rDNA sequences in the RDP database using the probe match function [5]. The *E. coli* numbering 1,071–1,391 region of the 16S rDNA was amplified using the primers 1070f and 1392r [10]. The forward primers used to amplify the *E. coli* numbering 353–514 region and the corresponding sequences of the thirteen 16S rDNA fragments are presented in Table 2. 519r (5'-ACCGCGGCTGCTGG-

Table 1. 16S rDNA fragments used in this study.

Sequence ID[a]	GenBank Accession no.	Size (bp)[b]	Phylogenetic affiliation[c]	Closest relative (% Identity)	Reference
C1	DQ136096	1,457	β-*Proteobacteria*	*Acidovorax* sp. R-25075 (99)	[1]
C2	AY921546	1,480	δ-*Proteobacteria*	*Myxobacterium* KC (97)	[19]
I1	DQ136074	1,474	*Firmicutes*	*Bacillus cereus* strain G8639 (99)	[1]
I2	DQ136068	1,439	*Bacteroidetes*	Uncultured *Bacteroidetes* bacterium clone CrystalBog5D8 (98)	[1]
I3	DQ136063	1,440	*Actinobacteria*	*Rhodococcus erythropolis* CV71b (99)	[1]
C3	AY930443	1,411	*Cyanobacteria*	*Synechococcus* sp. NAN (99)	[19]
C4	AY930447	1,450	*Planctomycetes*	Uncultured bacterium clone: RB522 (94)	[19]
C5	DQ136104	1,422	*Acidobacteria*	Uncultured *Acidobacterium* clone JG36-TzT-31 (98)	[1]
C6	DQ136121	1,489	*Verrucomicrobia*	Uncultured soil bacterium clone 27-1 (95)	[1]
C7	AY921494	1,462	*Nitrospira*	Candidatus *Nitrospira defluvii* (98)	[19]
C8	AY921524	1,402	*Chloroflexi*	Uncultured soil bacterium clone S095 (92)	[19]
C9	AY930458	1,490	TM7	Uncultured bacterium clone B1_21BS (95)	[19]
C10	AY921508	1,394	OP11	Uncultured candidate division OP11 bacterium clone WSA68 (91)	[19]

[a]C, the fragment obtained by cloning the 16S rDNA product amplified from soil DNA; I, the fragment amplified from the genomic DNA of an isolate.
[b]The primer-annealing sites were removed.
[c]Assigned using the RDP classifier program [5].

Table 2. Forward primers used to amplify the 353–514 region of 16S rDNA and the corresponding sequences of the 16S rDNA fragments used in this study.

Primer or template	Sequence[a] (5'→3')																				Reference
Primer	338[b]																			357	
F357IW[c]				C	C	T	A	C	G	G	G	I	G	G	C	W	G	C	A	G	Modified from [24]
338f[d]	A	C	T	C	C	T	A	C	G	G	G	A	G	G	C	A	G	C			[36]
F338T2[d]	A	C	T	C	C	T	A	C	G	G	G	T	G	G	C	T	G	C			This study
F338I2[d]	A	C	I	C	C	T	A	C	G	G	G	I	G	G	C						This study
F352T[e]	A	C	T	C	C	T	A	C	G	G	G	T	G	G	C						This study
Template																					
C1	-	-	T	-	-	-	-	-	-	-	-	A	-	-	-	A	-	-	-	G	
C2	-	-	T	-	-	-	-	-	-	-	-	A	-	-	-	A	-	-	-	G	
I1	-	-	T	-	-	-	-	-	-	-	-	A	-	-	-	A	-	-	-	G	
I2	-	-	T	-	-	-	-	-	-	-	-	A	-	-	-	A	-	-	-	G	
I3	-	-	T	-	-	-	-	-	-	-	-	A	-	-	-	A	-	-	-	G	
C3	-	-	T	-	-	-	-	-	-	-	-	A	-	-	-	A	-	-	-	G	
C4	-	-	A	-	-	-	-	-	-	-	-	T	-	-	-	T	-	-	-	G	
C5	-	-	T	-	-	-	-	-	-	-	-	A	-	-	-	A	-	-	-	G	
C6	-	-	A	-	-	-	-	-	-	-	-	T	-	-	-	A	-	-	-	G	
C7	-	-	T	-	-	-	-	-	-	-	-	A	-	-	-	A	-	-	-	G	
C8	-	-	T	-	-	-	-	-	-	-	-	A	-	-	-	A	-	-	-	G	
C9	-	-	T	-	-	-	-	-	-	-	-	A	-	-	-	A	-	-	-	G	
C10	-	-	A	-	-	-	-	-	-	-	-	T	-	-	-	A	-	-	-	A	

[a]I, deoxyinosine; W, A+T; -, nucleotide identical to that in the primers.
[b]*E. coli* numbering.
[c]GC clamp (CGCCCGCCGCGCCCCGCGCCCGGCCCGCCGCCCCCGCCCC) connected to the 5' end
[d]GC clamp (CGCCCGCCGCGCCCCCGCCCCGGCCCGCCGCCCCCGCCC) connected to the 5' end
[e]GC clamp (CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCACGGGGGG) connected to the 5' end

CAC-3') [6] was used as a reverse primer to amplify the 353–514 region and showed perfect match with the corresponding sequences of all the fragments used.

PCR Conditions

PCR amplification was performed in 25 μl of reaction mixture containing 1×PCR buffer, 2.5 mM $MgCl_2$ (GeneCraft, Munster, Germany), 1 μl of the template DNA, 12.5 pmol each of the forward and reverse primers, 200 μM each of dNTP (GeneCraft, Munster, Germany), 25 μg of bovine serum albumin (Q-BIO Gene, Irvine, CA, U.S.A.), and 1 U of *Taq* polymerase (GeneCraft, Munster, Germany). The initial PCR program for all the primer pairs was fixed as follows: 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. In the case of the 353–514 region, touchdown PCR and conventional PCR at various annealing temperatures were also performed. The touchdown PCR program consisted of an initial denaturation at 95°C for 5 min, followed by 10 cycles of 95°C for 1 min, 65°C for 1 min, and 72°C for 1 min, in which the annealing temperature was reduced by 1°C/cycle from the preceding cycle, and then 20 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. To examine the effect of annealing temperature on the DGGE profile, various annealing temperatures (40–55°C) were tested, with the other steps being identical to those of the initial PCR program.

Denaturing Gradient Gel Electrophoresis (DGGE) Analysis

DGGE analysis was performed with a Dcode Mutation Detection System (Bio-Rad, Hercules, CA, U.S.A.). Gels of 8% acrylamide (37:1 acrylamide-bisacrylamide) were formed between 40 and 70% denaturant, with 100% denaturant defined as 7 M urea and 40% (vol/vol) formamide. The gels polymerized for about 2 h were run at 60 V for 16.5 h and maintained at a constant temperature of 60°C in 0.5×TAE buffer. After electrophoresis, the gels were stained in 1×TAE buffer containing ethidium bromide (0.1 mg/l) and photographed with UV transillumination (302 nm). During this study, repeated PCR and DGGE analyses consistently produced the same DNA banding profiles and nearly identical band intensities.

PCR Amplification and DGGE Analysis of Environmental Samples

Soil samples were taken from three microbial ecological research sites; *i.e.*, a rice field soil, a hydrocarbon-contaminated

forest soil, and a pristine forest soil. Total soil microbial community DNAs were extracted using the FastDNA Spin Kit for soil (Q-BIO Gene, Irvine, CA, U.S.A.) according to the manufacturer's instructions. PCR amplification was performed with the primer pairs F357IW-519r, 338f-519r, F352T-519r, and 1070f-1392r. 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 (52°C for the primer pair F352T-519r), and 72°C for 1 min and a final extension at 72°C for 7 min. DGGE analysis was performed as before, except that the gels were stained in 1×TAE buffer containing 1×SYBR Green I (Cambrex Bio Science, Rockland, ME, U.S.A.) for 15 min and rinsed in 1×TAE buffer for 20 min.

RESULTS

DGGE Profiles Generated by Using Different Pairs of Primers

The DGGE profiles generated by using the primer pairs 1070f-1392r and F357IW-519r are presented in Figs. 1 and 2, respectively. The PCR products amplified from each of the thirteen 16S rDNA fragments and their mixture were loaded together. When the primer pair 1070f-1392r was used for PCR amplification of the 1,071–1,391 region of the 16S rDNA fragments, the mixture yielded two spurious bands that corresponded to the faint bands produced by each of the 16S rDNA fragment templates (Fig. 1, arrowheads). Moreover, the band corresponding to I1 did not appear in the profile of the mixture (Fig. 1, dotted arrow). The two pairs of bands, C4-C2 and C6-C7, migrated to the same positions, respectively, and were not resolved in the profile of the mixture under the condition used (Fig. 1, solid arrows).

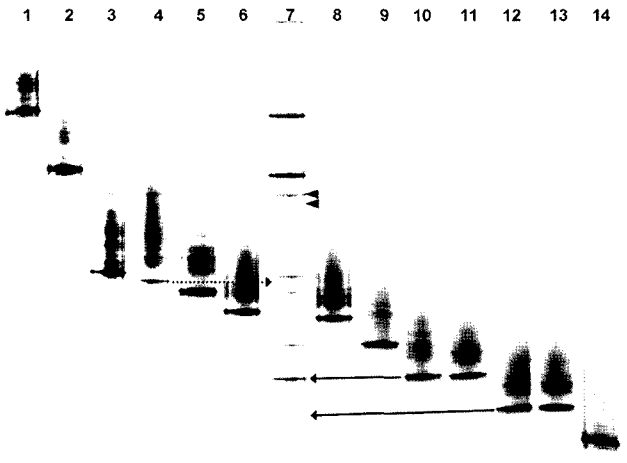

Fig. 1. DGGE profiles generated using the primer pair 1070f and 1392r.
Lanes 1, C9; 2, C10; 3, I2; 4, I1; 5, C1; 6, C3; 7, the mixture of thirteen 16S rDNA fragments; 8, C5; 9, I3; 10, C4; 11, C2; 12, C6; 13, C7; 14, C8. The dotted arrow points to the position of the band of I1 in the profile of the mixture and the arrowheads indicate spurious bands. Two solid arrows point to each position of the two pairs of bands, C4-C2 and C6-C7, respectively, in the profile of the mixture.

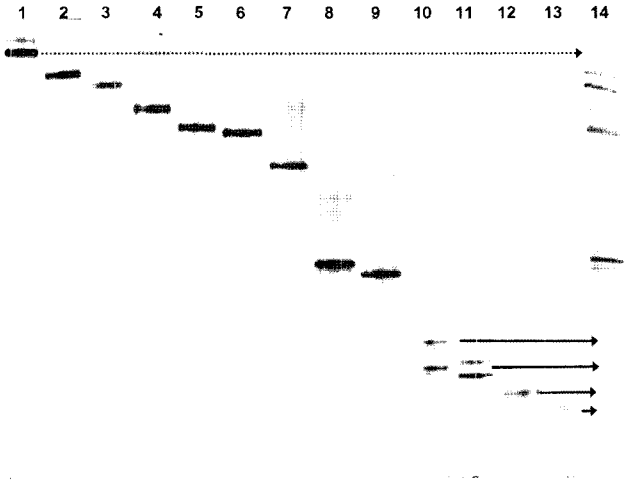

Fig. 2. The DGGE profiles generated using the primer pair F357IW and 519r.
Lanes 1, C10; 2, C9; 3, C6; 4, I2; 5, C1; 6, C3; 7, I1; 8, C4; 9, C5; 10, C7; 11, C8; 12, C2; 13, I3; 14, the mixture of thirteen 16S rDNA fragments. The dotted arrow points to the position of C10 and solid arrows point to the position of C7, C8, C2, and I3 in the profile of the mixture.

For PCR amplification of the 353–514 region, the degenerate primer F357IW was modified from the primer 1 [24] to accommodate the sequence divergence more thoroughly. When the primer pair F357IW-519r was used for PCR amplification of the 16S rDNA fragments (Fig. 2), the band of C10, which has a mismatch with the 3' end of the primer F357IW (Table 2), did not appear in the profile of the mixture (Fig. 2, dotted arrow). Each of C7, C8, and I3 yielded multiple bands for this primer pair (Fig. 2, lanes 10, 11, and 13) and C7, C8, C2, and I3 did not appear as distinctive bands in the profile of the mixture (Fig. 2, solid arrows).

When different forward primers were used to amplify the 353–514 region of the 16S rRNA gene, different DGGE banding profiles were generated (Fig. 3, lanes 1, 3, 5, and 7). The forward primer 338f has 3' end match with all the fragments and no deoxyinosine or degenerate residue (Table 2). With this nondegenerate primer, C10, which was not detected with the degenerate primer F357IW (Fig. 2, lane 14 and Fig. 3, lane 1), produced a clear DGGE band in the profile of the mixture (Fig. 3, lane 3, band no. 1). In addition, C7, C8, C2, and I3 appeared as stronger bands (Fig. 3, lane 3, bands no. 10, 11, 12, and 13) than those generated by using the degenerate primer F357IW (Fig. 3, lane 1, bands no. 10, 11, 12, and 13). In contrast, C4, which has two A-A mismatches at the positions 3 and 7 from the 3' end of the primer 338f (Table 2), did not

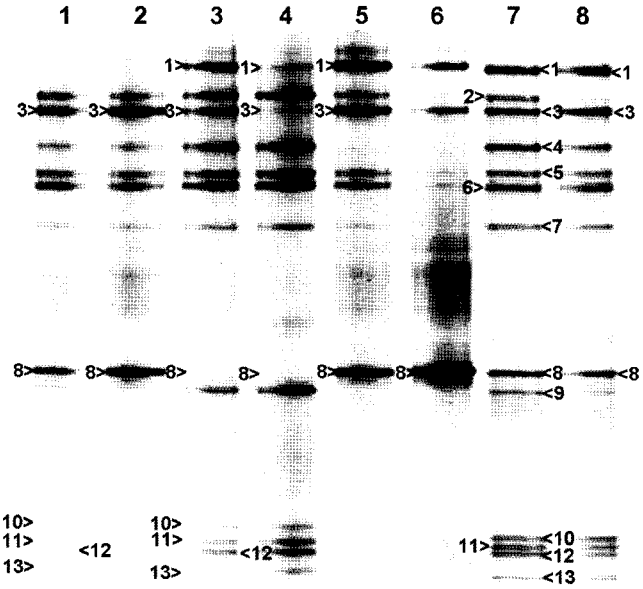

Fig. 3. The DGGE profiles generated from the mixture of thirteen 16S rDNA fragments using various primer pairs, in which a conventional PCR program (lanes 1, 3, 5, and 7) or a touchdown PCR program (lanes 2, 4, 6, and 8) was used.
Lanes 1 and 2, F357IW-519r; 3 and 4, 338f-519r; 5 and 6, F338T2-519r; 7 and 8, F352T-519r. Band numbers: 1, C10; 2, C9; 3, C6; 4, I2; 5, C1; 6, C3; 7, I1; 8, C4; 9, C5; 10, C7; 11, C8; 12, C2; 13, I3.

appear in the profile of the mixture with this primer (Fig. 3, lane 3, band no. 8). Although C6 and C10 have two mismatches with the primer 338f at the positions 7 and 16 from the 3' end of the primer (Table 2), they appeared as distinctive bands in the profile (Fig. 3, lane 3, bands no. 1 and 3).

The forward primer F338T2 has two thymidine residues instead of deoxyadenosine residues at the positions 3 and 7 from the 3' end of the primer (Table 2). When this primer was used for PCR amplification of the 16S rDNA fragments (Fig. 3, lane 5), the 16S rDNA fragment templates having two T-T mismatches at these positions (Table 2, those except C4, C6, and C10) produced relatively weak or invisible bands in the profile of the mixture (Fig. 3, lane 5, all bands except bands no. 1, 3, and 8) compared with those generated by using the primer 338f (Fig. 3, lane 3, all bands except bands no. 1, 3, and 8). In the case of the forward primer F338I2, which was designed to have only one mismatch at the 3' region of the primer and had two deoxyinosine residues at the two variable sites (Table 2), the PCR yield was very low under the PCR condition used, and this could not be improved significantly by lowering the annealing temperature to 40 (results not shown). Therefore, the primer F338I2 was not included in subsequent experiments.

The forward primer F352T, which had two thymidine residues instead of deoxyinosine residues in the primer

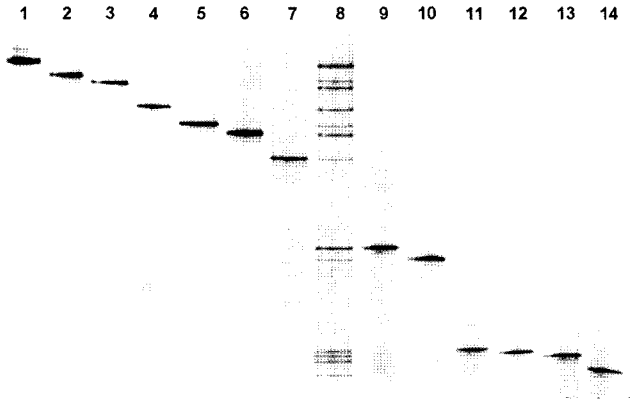

Fig. 4. The DGGE profiles generated using the primer pair F352T and 519r.
Lanes 1, C10; 2, C9; 3, C6; 4, I2; 5, C1; 6, C3; 7, I1; 8, the mixture of thirteen 16S rDNA fragments; 9, C4; 10, C5; 11, C7; 12, C8; 13, C2; 14, I3.

F338I2, was designed to have one T-T mismatch with each of the 16S rDNA fragment templates at either position 4 or 13 from the 3' end of the primer, and supposed to have the same binding efficiency for all of the 16S rDNA fragment templates used. When the primer F352T was used for PCR amplification, each of the thirteen representative templates produced a distinct and clear band with relatively similar intensity to each other (Fig. 3, lane 7, and Fig. 4). Overall, the DGGE banding profiles obtained with the primer set F352T-519r was the best when compared with the profiles generated with the other primer sets tested.

The Effect of Touchdown PCR on the DGGE Profiles

When the touchdown PCR program was used for amplification of the 16S rDNA fragment templates with different forward primers, different DGGE profiles were generated according to the degree of primer-template mismatch (Fig. 3, lanes 2, 4, 6, and 8).

In the case of the primer F357IW, the bands of C6 and C4 showed relatively strong intensities (Fig. 3, lane 2, bands no. 3 and 8) compared with those generated by the conventional PCR (Fig. 3, lane 1, bands no. 3 and 8). These two templates have an I-A pair whereas the others have an I-T pair at the position of deoxyinosine of the primer. This is consistent with the previous report that the order of stabilities is I:C>I:A>I:T=I:G [23]. When the primer 338f was used for PCR amplification, the band intensities of C10 and C6, which have an A-A mismatch at the position 7 from the 3' end of the primer, became weaker (Fig. 3, lane 4, bands no. 1 and 3) than those generated by the conventional PCR (Fig. 3, lane 3, bands no. 1 and 3).

When the primer F338T2 was used, the band of C4, which is the only fragment having perfect match with the primer at the 3' region, showed the strongest intensity (Fig. 3, lane 6, band no. 8), whereas the other templates, which have one or two mismatches at the positions 3 and

7 from the 3' end of the primer, showed weaker band intensities (Fig. 3, lane 6, bands except band no. 8) than those generated by the conventional PCR (Fig. 3, lanes 5, bands except band no. 8). For the primer F352T, PCR band intensities of the templates that have a mismatch at the position 4 from the 3' end of the primer were generally weaker (Fig. 3, lane 8, all bands except bands no. 1, 3, and 8) than those of the templates having a match at this position and a mismatch at position 13 from the 3' end of the primer (Fig. 3, lane 8, bands no. 1, 3, and 8).

The Effect of Annealing Temperature on the DGGE Profiles

When the mixture of the thirteen 16S rDNA templates was amplified with the primer pairs F357IW-519r, 338f-519r, and F338T2-519r at annealing temperatures of 40, 45.1, and 50.4°C (Fig. 5), the unevenness of band intensities by primer-template mismatch was not alleviated significantly. For the primer pair F352T-519r, which produced the best DGGE profiles (Fig. 3), the PCR efficiencies of the thirteen templates were compared at different annealing temperatures (40, 43.1, 45.9, 49.2, 52.0, and 55.0°C) (Fig. 6). Although C9 appeared to produce slightly improved band intensity with the decrease of temperature from 55 to 52 (Fig. 6, arrowhead), there was no significant difference between the DGGE profiles at the lower annealing temperatures.

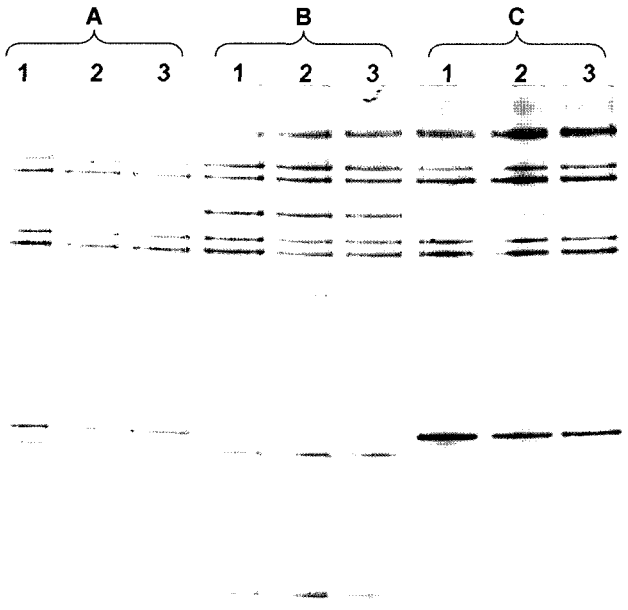

Fig. 5. The DGGE profiles generated using the primer pairs F357IW-519r (**A**), 338f-519r (**B**), and F338T2-519r (**C**) with the mixture of the thirteen 16S rDNA fragments as a template at various annealing temperatures.
Lanes 1, 40°C; 2, 45.1°C; 3, 50.4°C.

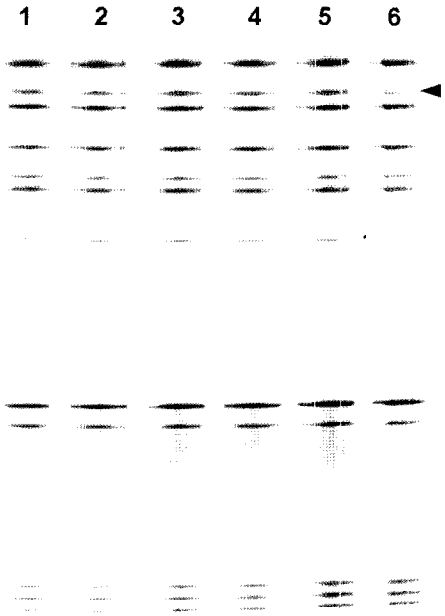

Fig. 6. The DGGE profiles generated using the primer pair F352T-519r with the mixture of the thirteen 16S rDNA fragments as a template at various annealing temperatures.
Lanes 1, 40°C; 2, 43.1°C; 3, 45.9°C; 4, 49.2°C; 5, 52.0°C; 6, 55.0°C. The arrowhead indicates the band of C9.

The DGGE Profiles of Environmental Samples

When the bacterial communities from three different types of soils were subjected to DGGE analysis using the different pairs of primers (Fig. 7), the profiles from the 353–514 region yielded generally more discernible bands than those from the 1,071–1,391 region did, and these bands were distributed more broadly in the gel than those from the 1,070–1,391 region were. Of the primer pairs amplifying the 353–514 region, the F352T-519r pair produced the best DGGE banding profiles (Fig. 7, lane 3), which is consistent with the above results obtained from the thirteen 16S rDNA fragment templates.

DISCUSSION

We analyzed the effects of different hypervariable regions of 16S rDNA and primer pairs on PCR amplification bias, which has been a potential problem for accurate analyses of microbial community structures, by denaturing gradient gel electrophoresis. For this study, thirteen different 16S rDNA fragments representing a typical soil bacterial community were chosen as templates in PCR-DGGE analyses. Of two different hypervariable regions that have frequently been used for diversity study of soil microbial communities [1, 4, 9, 10, 18, 23, 32, 35], the 353–514 region of the 16S rRNA gene yielded more complex and representative

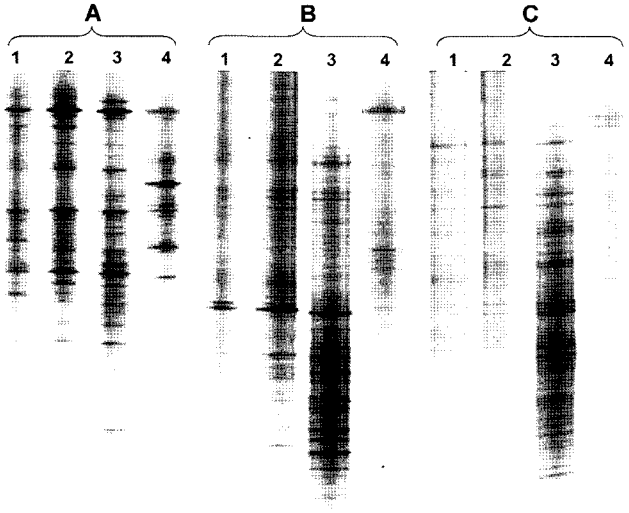

Fig. 7. The DGGE profiles generated by various primer pairs using soil DNA samples from a hydrocarbon-contaminated soil (**A**), a pristine forest soil (**B**), and a rice field soil (**C**) as templates.
Lane 1, F357IW-519r; 2, 338f-519r; 3, F352T-519r; 4, 1070f-1392r.

DGGE profiles than the 1,071–1,391 region, in concordance with the previous report of Yu and Morrison [40]. To compare the efficiencies of PCR amplifications of the 353–514 region, four combinations of primer pairs were selected for this region based on % match with the 16S rDNA sequences. The results revealed that the forward primer F352T, which was designed to have one mismatch with each of the 16S rDNA fragments at either position 4 or 13 from the 3' end of the primer, produced the best DGGE profiles among the forward primers, amplifying the 353–514 region in combination with the reverse primer 519r.

When the primer pair 1070f-1392r was used for PCR amplification, the band of I1 was not detected in the DGGE banding profile of the mixture of 16S rDNA fragments (Fig. 1). It was reported that different binding energies, resulting from primer degeneracy and the GC content of template DNA, could bias PCR amplification [31]. Since I1 has only one mismatch at position 12 from the 3' end of the primer 1070f and has relatively low GC content (54%) in both the whole 16S rDNA and the amplified region, neither of these mechanisms appeared to account for the missing I1 band. Other possible causes of bias could also be eliminated, such as rrn operon heterogeneity [37] and the formation of heteroduplex molecules [11], the latter being not expected to occur in our experiments, because of low similarities among the 16S rDNA fragments used. Template folding might be an alternative explanation, but further investigations are needed to verify the observed phenomenon. The spurious bands obtained by the primer pair 1070f-1392r with the mixed templates (Fig. 1) are probably due to aggregation of their nonspecific weak products amplified from each of the 16S rDNA fragments. Touchdown PCR has been frequently used in the 16S rDNA PCR for DGGE [10, 14, 25, 27] to increase the specificity of the amplification and to reduce the formation of spurious by-products. However, this PCR procedure is not recommended in the case where primer-template mismatches are expected [35], because it amplifies the templates differentially according to the degree of primer-template mismatches, which leads to PCR amplification bias for analysis of microbial community structure. The results in the present study revealed that touchdown PCR intensified the unevenness of amplification for the mixture of 16S rDNA fragments by primer-template mismatch (Fig. 3), suggesting that this method does not efficiently detect complex bacterial community structure in environmental samples.

C10 was not detected in the DGGE profile obtained from the mixture of the 16S rDNA fragments with the primer set F357IW-519r (Fig. 2). It was reported that extension of primer by DNA polymerases was prevented by a single mismatched base at the 3' end of the primer sequence [2], and a sequence having a 3' end mismatch with a primer was not detected in the clone library, whereas it was detected when primers having no 3' end mismatch were used [17]. Hence, the 3' end mismatch with the primer F357IW appears to cause the band of C10 missing in the mixture. C10 is affiliated with a candidate phylum OP11. Although the sequences having the 3' end mismatch to the primer F357IW constitutes only about 0.2% of the 16S rDNA sequences in the RDP database, these sequences occupy a relatively high proportion (28%) in the phylum OP11 when calculated with the Probe match function for the sequences >1,200 bp [5]. The OP11 group is one of the phyla most widely detected in environmental molecular surveys [15] and has been reported as a dominant group in an underground hydrocarbon-contaminated aquifer [7].

Kowalchuk *et al.* [21] showed that a degenerate primer produces a mixture of PCR products from a single sequence, and these products can be separated by DGGE. The multiple bands of C7, C8, and I3 generated by the primer F357IW (Fig. 2) appeared to be caused by the degeneracy of the primer F357IW, because these sequences yielded a single band with the nondegenerate primer F352T (Fig. 4). Whether a single sequence yields multiple bands or not can be partially explained by the melting profile of that sequence. When the melting profiles of the 16S rDNA fragments used in this study were constructed using the MELT94 program [22], C7 and I3 showed one melting domain in the amplified region, whereas the other fragments showed at least two melting domains, the difference between which being more than 1°C (result not shown). Because the annealing region of the primer F357IW is located in the higher melting domain, the sequence variation in this region cannot be detected in the DGGE profile and thus the fragments will appear as a single band [38].

Ishii and Fukui [18] found that PCR bias caused by one internal primer-template mismatch was reduced by lowering the annealing temperature in multitemplate PCR. However, Hongoh *et al.* [17] reported that the sequences having 5–8 internal mismatches in a 21-bp length primer were not detected in the clone library, even though the annealing temperature was lowered to 45°C. In our study, both the 3' end mismatch and two internal mismatches within the 3' region of the primer induced significant bias in the multitemplate PCR (Fig. 3), and this bias was hardly improved at lower annealing temperatures (Fig. 5).

It is unlikely that the images generated by DGGE or related techniques represent all the richness of the 16S rDNA sequence types in a complex environment such as soil. However, these images provide the structure of the bacterial community; *i.e.*, the relative abundance of the main bacterial populations [26]. Therefore, it is necessary to examine whether the PCR condition used can reflect the distribution of various bacterial groups in the original sample. In our results, the design of the PCR primer appeared to be the most important to achieve this unbiased PCR. The primer F352T used in this study, however, has some limitations. In order to increase sequence match with the templates, the length of the primer F352T (15 bp) was reduced from those of the published primers (17–18 bp), possibly leading to the decrease of specificity. Furthermore, the T_m of the primer F352T decreased, so that the difference in T_m between the forward and reverse primers was more than 10°C when calculated using various T_m calculators. Nevertheless, these problems did not appear to have significant effects on the PCR amplification of the templates used in this study. Because the primer pair F352T-519r showed relatively good performance on the 16S rDNA fragments used, and the nucleotide sequences of the fragments corresponding to F352T and 519r represent 95 and 96% of the 16S rDNA sequences in the RDP database, respectively [5], this pair of primers is expected to provide a more realistic structure of soil bacterial communities than the other primers tested in this study.

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