# Denaturing gradient gel electrophoresis as a fingerprinting method for the analysis of soil microbial communities

**V. Valášková, P. Baldrian**

*Laboratory of Environmental Microbiology, Institute of Microbiology of the Academy of Sciences of the Czech Republic, Prague, Czech Republic*

**ABSTRACT**

In soil microbial ecology, the effects of environmental factors and their gradients, temporal changes or the response to specific experimental treatments of microbial communities can only be effectively analyzed using methods that address the structural differences among whole communities. Fingerprinting methods are the most appropriate technique for this task when multiple samples must be analyzed. Among the methods currently used to compare microbial communities based on nucleic acid sequences, the techniques based on differences in the melting properties of double-stranded molecules, denaturing gradient gel electrophoresis (DGGE) or temperature gradient gel electrophoresis (TGGE), are the most widely used. Their main advantage is that they provide the possibility to further analyze whole sequences contained in fingerprints using molecular methods. In addition to the analysis of microbial communities based on DNA extracted from soils, DGGE/TGGE can also be used for the assessment of the active part of the community based on the analysis of RNA-derived sequences or for the analysis of sequences of functional genes encoding for proteins involved in important soil processes.

**Keywords**: DGGE; bacteria; fungi; microbial community; soil ecology; TGGE

In the last two decades, the methods used to describe the diversity of microbial communities in soils have undergone a shift from cultivation-based approaches to more comprehensive culture-independent methods. This is of critical importance since only a minor fraction of a soil microbial community can be analyzed using cultivation-dependent techniques. Most recent molecular methods are based on the analysis of nucleic acids extracted from environmental samples. Compared to cloning and sequencing, which is very labor-intensive, time consuming and expensive if several samples are analyzed at a time, the molecular fingerprinting method provides a rapid, simultaneous and reproducible analysis of samples, although with limited resolution (Kowalchuk and Smit 2004, Muyzer et al. 2004, Oros-Sichler et al. 2007). In modern soil ecology, denaturing gradient gel electrophoresis (DGGE) and temperature gradient gel electrophoresis (TGGE) are among the most commonly used methods and they provide several advantages over other common techniques. Importantly, they allow for the simple follow-up identification of at least the most prominent members of the microbial community. The aim of this paper is to compare the DGGE and TGGE techniques to other fingerprinting methods and to offer recommendations for their use in the analysis of soil bacterial and fungal communities.

## Fingerprinting methods to assess the microbial biodiversity in soils

In order to determine how microbial communities change due to external factors, the rapid, comparative analysis of multiple samples is usually required. Fingerprinting methods are designed to allow for the rapid comparison of samples, identifying any similarities or differences in composition or diversity. None of these techniques, however, are able to identify individual members of the microbial com-

Supported by the Czech Science Foundation, Grant No. 526/08/0751, by the Ministry of Education, Youth and Sports of the Czech Republic, Project No. LC06066 and by the Institutional Research Concept of the Institute of Microbiology of the Academy of Sciences of the Czech Republic, Project No. AV0Z50200510.

munity. Most fingerprinting methods are based on molecular biology techniques using isolated DNA or RNA and several employ a polymerase chain reaction (PCR) amplification step followed by electrophoretic separation. One exception is the analysis of phospholipid fatty acids (PLFA), which is based on the molecular fingerprinting of lipid molecules of the microbial membranes. DGGE and TGGE are based on the electrophoretic separation of double stranded DNA molecules based on the differences in their melting behavior in a gradient of either a denaturing agent or temperature. Single-strand conformation polymorphism analysis (SSCP) separates single-stranded DNA molecules based on differences in their secondary structures, while terminal restriction length polymorphism (T-RFLP) detects differences in the localization of restriction sites in DNA sequences. Both length heterogeneity PCR (LH-PCR) and automated ribosomal intergenic spacer analysis (ARISA) separate whole sequences that differ in length. Microarray analysis uses the specific hybridization of environmental nucleic acids to an array of probes to detect targeted sequences in the sample. For more detailed information on the background of individual methods and their use, see Kowalchuk et al. (2004) and Oros-Sichler et al. (2007).

The most important advantages and disadvantages of individual methods are summarized in Table 1. The main advantage of the PLFA analysis is the ability to assess the diversity of both bacterial and fungal (and with some modifications *Archaea*) communities simultaneously. However, this high coverage is unfortunately contrasted by a very low level of taxonomic discrimination. While an advantage of microarrays is that they can overcome the potential PCR bias, only the part of the community defined by the test probes can be examined. T-RFLP has the highest resolution of the PCR-based methods and can reliably analyze a large number of samples, but a significant portion of the microbial community is inaccessible due to technical exclusion of some of the molecules from the analysis.

Table 1. Common fingerprinting methods used in the analysis of soil microbial communities. See text for the explanation of abbreviations

| Method | Advantages | Weaknesses | Reference |
|---|---|---|---|
| DGGE/ TGGE | provides full sequences that can be subject to further analysis | gel-to-gel variation<br>PCR primer design (GC clamp)<br>only short sequences < 400 bp can be analyzed using TGGE | Riesner et al. (1990), Muyzer et al. (1993) |
| SSCP | provides full sequences that can be subject to further analysis<br>technically simple gel preparation | complicated DNA preparation (two purification steps)<br>only short sequences < 200 bp can be analyzed<br>variant folding of single strand molecules | Lee et al. (1996), Dohrmann and Tebbe (2004) |
| T-RFLP | technically simple<br>reproducible<br>high discrimination power (number of types/analysis) | loss of some variability (sequences not cleaved or cleaved near to primer)<br>low phylogenetic specificity of terminal restriction sites | Liu et al. (1997) |
| LH-PCR/ ARISA | technically simple | low discrimination power | Fisher and Triplett (1999) |
| Microarrays | no bias due to PCR | detects only sequences corresponding to probes<br>detection limit lower than in PCR-based methods | Shalon et al. (1996) |
| PLFA analysis | can cover whole communities across kingdoms<br>quantitative description of the community<br>no bias due to PCR | low taxonomic separation<br>limited to community composition analysis | Findlay et al. (1989) |

## Specifities of DGGE and TGGE analyses

DGGE was originally developed in the 1980s for the identification of point mutations and was first used for the analysis of microbial communities in the early 1990s (Muyzer et al. 1993). The technique separates DNA fragments of the same or similar length but of different sequences by electrophoresis in a gradient of a denaturant. Similar separation principles underlie TGGE, only temperature gradient is used as the denaturant in addition to the chemical compounds in the gel. In the denaturing gradient, DNA fragments migrate under the influence of an electric field. When the fragment reaches a position in the gradient in which it 'melts' (the strands separate), the mobility of the fragment decreases rapidly. In order to prevent complete denaturation of the fragment, a GC clamp – a GC-rich sequence that does not melt – is attached to the 5'-end of one of the primers used in PCR. The GC clamps usually consists of 20–40 bases. The most frequently used GC clamp is the one designed by Muyzer et al. (1993): 5'-CGC CCG CCG CGC CCC GCG CCC GTC CCG CCG CCC CCG CCC G-3'. This clamp is suitable for most applications; however there are several more GC clamps currently in use that differ in their melting properties and are thus suitable for specific conditions (Muyzer et al. 2004).

DGGE analysis provides a picture composed of an array of bands with different intensities. Band intensities correspond to the frequency of individual PCR products in the reaction mixture. Importantly, the excision of selected bands followed by direct reamplification and sequencing can, in some cases, yield a taxonomic identity of the bands. A cloning step before sequencing is usually required in particularly diverse communities where bands can be composed of several sequences or contain background DNA molecules. Alternatively, DGGE gels can be hybridized with taxon-specific probes that can identify one or more bands. In addition to community analysis, DGGE was also successfully used for the analysis of populations of functional genes (Gremion et al. 2004, Sakurai et al. 2007, Wartiainen et al. 2008). There is a resolution limit with this method as only sequences with intensity higher than 0.1–1% of the total intensity can be technically assessed. Depending on band intensities, up to approximately one hundred of bands can be distinguished on a gel. However, this limitation is relative since a comparable limit of detection can be reached using cloning only if several hundreds to thousands of clones are analyzed. Additionally, in DGGE, the preparation of denaturing gels can result in significant gel-to-gel variations that can make the comparison of large sample sets difficult.

As stated above, TGGE is based on a similar principle as DGGE (Riesner et al. 1990). Temperature gradients are usually generated by a Peltier-based heating/cooling system that can be regulated as required. As in DGGE, the resolution of TGGE is also mostly dependent upon gel size. The advantage of TGGE is that the gels are chemically homogeneous and ready-made gels can be purchased for specific types of equipment, which decreases the variation between gels. Moreover, TGGE analyses are faster, with an electrophoresis step less than 6 h compared to more than 14 h for DGGE. On the other hand, it is often reported that only relatively short fragments (< 400 bp) are generally separated well. Unfortunately, there are no detailed comparisons of DGGE and TGGE currently available. In our experience, however, it is more difficult to achieve a sharp banding pattern with TGGE than with DGGE. This problem may be due to a limited choice of available instrumentation for TGGE compared to DGGE, resulting in lower use. Therefore, DGGE is better suited for the analysis of more diverse populations.

## Methodological considerations

Although perhaps the most critical step in the process, the optimization of the initial soil sampling strategy for fingerprinting methods has attracted little attention. Given the possible gel-to-gel variations in DGGE, it is better to limit the quantity of samples to a number that can be analyzed on a single gel or a few gels. This, however, requires a good sample collection strategy, pooling of samples and their size to obtain nucleic acids from representative soil samples. In the soil environment, especially in the top layers of highly stratified soils, the composition of microbial communities is vertically structured (Kandeler et al. 1999, Baldrian et al. 2008). Changes in microbial community composition occur, even at the centimeter scale within individual soil horizons or among litters of different ages (Fioretto et al. 2000, Šnajdr et al. 2008). There is also a considerable horizontal component of the spatial variability in microbial soil communities at scales of several centimeters to meters (Saetre and Baath 2000, Šnajdr et al. 2008).

The recovery of nucleic acids from soil or litter samples and their quality are of great importance in all fingerprinting methods. Although there are methods proposed to work well with a relatively

wide range of soils, it is difficult to find one method that is the most suitable for all types of soils (Zhou et al. 1996, Griffiths et al. 2004, Korkama-Rajala et al. 2008, Sagova-Mareckova et al. 2008). If sufficient amounts of nucleic acids are available, it is better to limit the number of PCR cycles or to run the PCR in multiple separate reactions to limit the PCR bias.

The sequences of the genes encoding ribosomal RNA or the sequences between these genes attract the most attention. Alternative phylogenic markers, such as the gene encoding the elongation factor Tu (EFTu) and the gene for the RNA polymerase β subunit (rpoB), have not been commonly used (Oros-Sichler et al. 2007). The preference for rRNA-based markers is mainly due to the fact that these sequences are present in all members of microbial communities and contain both conserved regions suitable for primer design and variable regions allowing the discrimination between individual microbial taxa (Oros-Sichler et al. 2007). However, there are some difficulties with using the rRNA-based markers that need to be taken into account. Organisms may vary in the copy number of these sequences or multiple different sequences can be present within a single strain, which can result in multiple DGGE bands. In the case of bacteria, the 16S rRNA gene is by far the most frequently used marker and its hypervariable regions V3 (primers 341f-gc/518) and V6-V8 (primers 968gc-1378) of 16S ribosomal DNA (rDNA) are the most commonly used (Table 2).

The primers used for the analysis of fungal communities are more variable and target the 18S or 28S rDNA or the internal transcribed spacers (ITS) 1 or 2 between the rDNA genes (Table 2). The ratio of sequences belonging to Ascomycota, Basidiomycota, Chytridiomycota and Zygomycota is essentially the

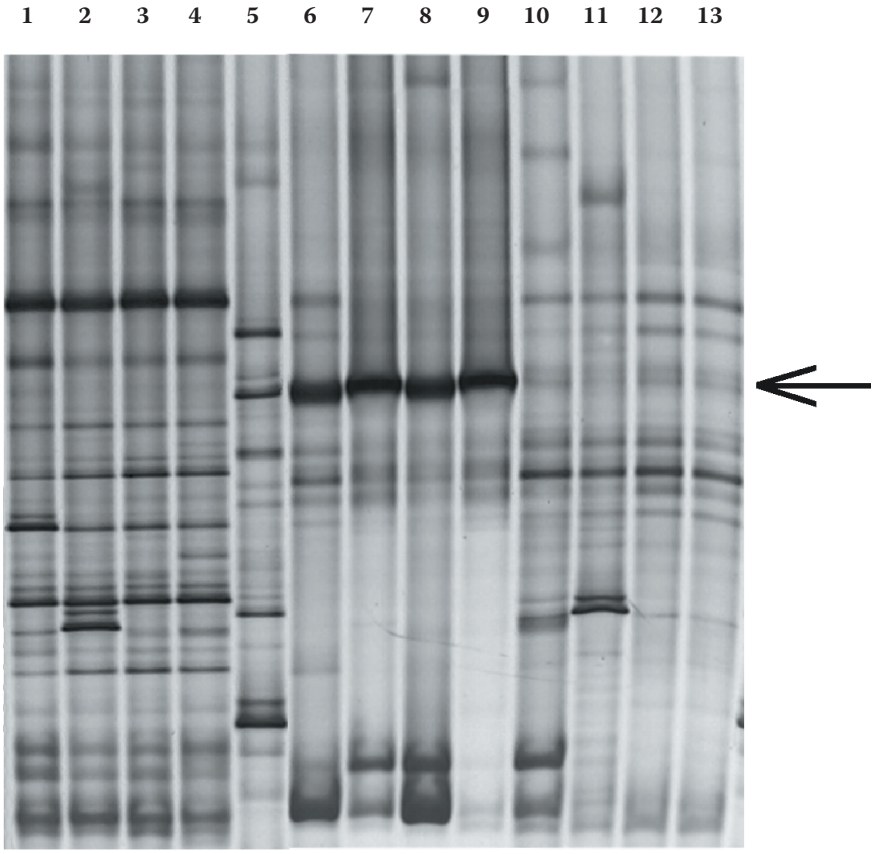


Figure 1. DGGE pattern of ITS1 region of fungal and basidiomycete community in *Quercus* sp. forest litter (Xaverov Natural Reserve near Prague, Czech Republic; Šnajdr et al. (2008)) developed during a 15-week laboratory cultivation in the presence or absence of the mycelia of a saprotrophic basidiomycete *Hypholoma fasciculare* isolated from the same site (Valášková et al. 2007). The DGGE analysis was run using the procedure described in this paper. In order to specifically analyze the fungi belonging to Basidiomycota, the reverse primer ITS4B 5'- CAG GAG ACT TGT ACA CGG TCC AG-3' was used instead of ITS4 in the first round of the nested PCR. From left: lanes 1–4 fungal community in nonsterile leaf litter unaffected by colonization by *H. fasciculare*, lane 5 marker, lanes 6–9 community of basidiomycetes in nonsterile litter colonized by *H. fasciculare*, lanes 10–13 control community of basidiomycetes in nonsterile litter unaffected by colonization of *H. fasiculare*. The thick band in lanes 6–9 indicated by the arrow indicates the band of *H. fasciculare*

Table 2. Overview of the common PCR-DGGE systems used for microbial community analysis in soils

| Target group | PCR target sequence | Primer pair | Reference |
|---|---|---|---|
| Bacteria, general | bacterial 16S, V3 | 341f-gc/518r | Muyzer et al. (1993) |
| | bacterial 16S, V3-V4 | 341f-gc/U758 | Phillips et al. (2008) |
| | bacterial 16S, V3-V5 | 341f/907r-gc | Muyzer et al. (2004) |
| | bacterial 16S, V6-V8 | 968-gc/1378 | Kozdrój and van Elsas (2001), this paper |
| | bacterial 16S, V6-V8 | 968-gc/1401 | Heuer and Smala (1997) |
| Bacteria, alpha-proteobacteria | bacterial 16S, V6-V8 | 203a f/1492r (or 1494) nested 968-gc/1378 | Gelsomino and Cacco (2006) |
| Bacteria, beta-proteobacteria | bacterial 16S, V6-V8 | 948b f/1492r (or 1494) nested 968-gc/1378 | Gelsomino and Cacco (2006) |
| | bacterial 16S, V6-V8 | 968 / 1492r (or 1494) nested 968-gc/1492 | Gremion et al. (2004) |
| Bacteria, Actinobacteria | bacterial 16S, V3 | 243f/513-gc | Heuer et al. (1997) |
| | bacterial 16S, V3-V5 | S-C-Act-0235-a-S-20-gc/ S-C-Act-0878-a-A-19 | Jaatinen et al. (2008) |
| | bacterial 16S, V6-V8 | 243f/1492r (or 1494) nested 968-gc/1378 | Gelsomino and Cacco (2006) |
| Bacteria, Pseudomonads | bacterial 16S, V3 | Psf/1378 nested 341-gc/518r | Yao et al. (2006) |
| Bacteria, ammonia oxidizers | bacterial 16S, V2-V4 | 63f/1378r nested (CTO189fA/B-gc and CTO189fC-gc)/CTO654r | Ros et al. (2006) |
| | bacterial 16S, V2-V4 | (CTO189fA/B-GC and CTO189fC-GC)/ CTO654r | Kowalchuk et al. (1998) |
| Fungi, general | fungal 18S | NS1/Fung-gc | Möhlenhoff et al. (2001) |
| | fungal 18S | EF390/FR1-gc | Vainio and Hantula (2000) |
| | fungal 18S | NS1/FR1-gc | Vainio and Hantula (2000) |
| | fungal 18S | NS1/EF3 nested NS1/FR1-gc | Oros-Sichler et al. (2006) |
| | fungal 18S | EF4/EF3 nested EF4/Fung5-gc | van Elsas et al. (2000) |
| | fungal 18S | EF4/EF3 nested NS2/Fung5-gc | van Elsas et al. (2000) |
| | fungal 18S | EF4/EF3 nested EF4/NS2-gc | van Elsas et al. (2000) |
| | fungal 18S | EF4/EF3 nested nu-ssu0817/NS4-gc | Bastias et al. (2007) |
| | fungal 18S | EF4/EF3 nested EF4/NS3-gc | Smit et al. (1999) |
| | fungal 18S | NS1-gc/NS2 | Kowalchuk et al. (1997) |
| | fungal 18S | EF4/Fung5 nested NS2/Fung5-gc | Clegg (2006) |
| | fungal 28S | 403f/662r-gc | Diouf et al. (2006) |
| | fungal 28S | NL359/NL912-gc | Zuccaro et al. (2003) |
| | fungal ITS | ITS1f/ITS4 nested ITS1f-gc/ITS2 | Anderson et al. (2003) |
| | fungal ITS | ITS1f-gc/ITS2 | Bougoure and Cairney (2005) |
| | fungal ITS | ITS1/ITS2-gc | Yao et al. (2006) |
| | fungal ITS | ITS3/ITS4-gc | Arenz et al. (2006) |
| Fungi, Ascomycota | fungal ITS | ITS1/ITS4A nested ITS1/ITS2-gc | Larena et al. (1999), Yao et al. (2006) |
| Fungi, Basidiomycota | fungal ITS | ITS1f/ITS4B-gc | Kowalchuk and Smit (2004) |
| | fungal ITS | ITS1/ITS4B nested ITS1/ITS2-gc | White et al. (1990), Yao et al. (2006) |
| Fungi, Basidiomycota | fungal ITS | ITS1/ITS4B nested ITS1f-gc/ITS2 | this paper |
| Fungi, arbuscular mycorrhiza | fungal 18S | AM1/NS31-gc | Kowalchuk et al. (2002) |

same for the 18S- and ITS-based primers (Anderson et al. 2003). However, it is generally accepted that the ITS region is preferred due to its higher variability. The identification of fungi using the 18S rRNA sequence is usually limited to genus or family in Ascomycota and Basidiomycota, although it can be useful for the discrimination of Glomeromycota. The ITS sequences are species-specific for most fungi (Anderson and Cairney 2004, Kowalchuk and Smit 2004). However, even the ITS regions are not variable enough to discriminate some fungal species, like *Penicillium* spp., where beta-tubulin or cytochrome oxidase genes are used for taxonomy instead (Seifert et al. 2007).

Previous studies on soil microbial diversity demonstrated that soil can contain tens of thousands of bacterial species per gram of soil, with different levels of community evenness (Roesch et al. 2007). Thus, this is not surprising that the molecular fingerprints of such communities may result in a very complex fingerprint patterns. Using DGGE, this is represented by many equally intense bands and sometimes even by a smear with no clear resolution of individual bands. To overcome these difficulties, individual subgroups of bacterial or fungal communities can be targeted specifically (Tables 2 and 3). In this paper, we demonstrate a nested PCR procedure that is able to show on a single gel both the whole fungal community and the subcommunity of fungi belonging to Basidiomycota, where the localization of individual bands corresponds between the two communities (Figure 1).

DNA is the most generally used molecule in molecular fingerprinting methods. However, after extraction from soil or litter and reverse transcription, RNA-based communities can also be analyzed. This led to the idea that ribosomal RNA (rRNA) molecules extracted from the environment could be a good tool for studying active members of microbial communities due to the fact that each molecule represents one ribosome copy and cell activity (growth rate) is coupled to an increase in ribosomes (Rosset et al. 1966). Several studies on bacteria and fungi in soil or litter demonstrated that active populations are formed by a subset of species present in the total residential community (Duineveld et al. 2001, Aneja et al. 2004, Pennanen et al. 2004). While this seems to be a useful approach for functional studies, the presence of copious ribosomes in some resting spores of fungi and bacteria can make it less exact. Currently, the analysis of the whole ribosomal RNA transcripts containing both the rRNA genes and ITS in soil and litter was shown to work well for the analysis of fungal communities (Korkama-Rajala et al. 2008). Since the turnover of these molecules is rapid, this is probably the best method to analyze the active fungal community.

Once the DGGE fingerprint is obtained, there are several statistical methods available to analyze the community variability or similarity of samples. The most important decision in the analysis is whether just the presence or absence of bands or their intensities should be used. The latter approach should be selected with great caution and only in the case that all bands on a gel contain the same amount of DNA. For more information on classical fingerprint processing techniques, please refer to Oros-Sichler et al. (2007). Alternatively, DGGE can be followed by cutting and reamplification of individual (usually dominant) bands, which can be later cloned and sequenced to reveal the identity of the most prominent members of the community (Muyzer et al. 2004). This approach was found to work well in several cases, but can be only used for a relatively limited number of bands per sample.

## Recommended procedures

The following procedures were used to successfully characterize bacterial and fungal communities in soils (Heuer and Smala 1997, Kozdrój and van Elsas 2001, Anderson et al. 2003, Marschner et al. 2003, Hernesmaa et al. 2005, Artz et al. 2007). The procedures target particularly variable parts of the respective rDNA regions (see above) and result in rich banding patterns that are suitable for subsequent sequence analysis and taxonomic identification of corresponding bacteria or fungi.

**Bacterial community analysis**. The forward primer 968-gc 5'-CGC CCG GGG CGC GCC CCG GGC GGG GCG GGG GCA CGG GGG GAA CGC GAA GAA CCT TAC-3' and the reverse primer 1378 5'-CGG TGT GTA CAA GGC CCG GGA ACG-3' are used. These primers target the V6-V8 hypervariable region of bacterial 16S rDNA (Table 3).

The PCR reaction consists of 1 × PCR buffer with $MgCl_2$, 100μM dNTPs, 20 pmol forward primer, 20 pmol reverse primer, 3 U DNA polymerase (e.g. Dynazyme II, Finnzymes), 1–2 μl DNA (50 ng/μl) template and $H_2O$ to bring the volume to 50 μl. The amplification yield of soil samples can be improved by the addition of 30 μg of bovine serum albumin per 50 μl reaction. Cycling conditions: 94°C for 2 min, 35 cycles (92°C for 30 s, 55°C for 1 min, 72°C for 45 s + 1 s/cycle) and 72°C for 5 min. The PCR product quality is tested on 1.5% (w/v) agarose gels and the size of PCR products is

Table 3. PCR primers used for DGGE/TGGE microbial community analysis in soils

| PCR target | Primer | Sequence 5' to 3' | Primer position | Reference |
|---|---|---|---|---|
| Bacterial 16S | 513 | CGGCCGCGGCTGCTGGCACGTA | 16S-513 | Heuer et al. (1997) |
| | 1378r | CGGTGTGTACAAGGCCCGGGAACG | 16S-1378 | Heuer and Smala (1997) |
| | 1401r | CGGTGTGTACAAGACCC | 16S-1401 | Heuer and Smala (1997) |
| | 1492r | TACGGYTACCTTGTTACGACTT | 16S-1492 | Heuer and Smala (1997) |
| | 1494r | CTACGGYTACCTTGTTACGAC | 16S-1494 | Gremion et al. (2004) |
| | 203a f | CCGCATACGCCCTACGGGGGAAAGA TTTAT | 16S-203 | Gelsomino and Cacco (2006) |
| | 243f | GGATGAGCCCGCGGCCTA | 16S-243 | Heuer et al. (1997) |
| | 341f | CCTACGGGAGGCAGCAG | 16S-341 | Muyzer et al. (1993) |
| | 518r | ATTACCGCGGCTGCTGG | 16S-518 | Muyzer et al. (1993) |
| | 63f | CAGGCCTAACACATGCAAGTC | 16S-63 | Ros et al. (2006) |
| | 907r | CCGTCAATTCCTTTGAGTTT | 16S-907 | Muyzer et al. (1995) |
| | 948b f | CGCACAAGCGGTGGATGA | 16S-948 | Gelsomino and Cacco (2006) |
| | 968f | AACGCGAAGAACCTTAC | 16S-968 | Felske et al. (1996) |
| | CTO189fA/B | GGAGRAAAGCAGGGGATCG | 16S-189 | Kowalchuk et al. (1998) |
| | CTO189fC | GGAGGAAAGTAGGGGATCG | 16S-189 | Kowalchuk et al. (1998) |
| | CTO654r | CTAGCYTTGTAGTTTCAAACGC | 16S-654 | Kowalchuk et al. (1998) |
| | P27f | GAGTTTGATCCTGGCTCAG | 16S-27 | Heuer and Smala (1997) |
| | Psf | GGTCTGAGAGGATGATCAGT | | Yao et al. (2006) |
| | S-C-Act-0235-a-S-20 | CGCGGCCTATCAGCTTGTTG | 16S-235 | Jaatinen et al. (2008) |
| | S-C-Act-878-a-A-19 | CCGTACTCCCCAGGCGGGG | 16S-878 | Jaatinen et al. (2008) |
| | U758 | CTACCAGGGTATCTAATCC | 16S-758 | Phillips et al. (2008) |
| Fungal 18S | AM1 | GTTTCCCGTAAGGCGCCGAA | | Santos et al. (2006) |
| | EF3 | TCCTCTAAATGACCAGTTTG | 18S-195 | Smit et al. (1999) |
| | EF4 | GGAAGGGRTGTATTTATTAG | 18S-573 | Smit et al. (1999) |
| | EF390 | CGATAACGAACGAGACCT | 18S-1317 | Vainio and Hantula (2000) |
| | FR1 | AICCATTCAATCGGTAIT | 18S-1664 | Vainio and Hantula (2000) |
| | Fung | ATTCCCCGTTACCCGTTG | 18S-368 | May et al. (2001) |
| | Fung5 | GTAAAAGTCCTGGTTCCCC | 18S-747 | Smit et al. (1999) |
| | NS1 | GTAGTCATATGCTTGTCTC | 18S-17 | White et al. (1990) |
| | NS2 | GGCTGCTGGCACCAGACTTGC | 18S-337 | White et al. (1990) |
| | NS3 | GCAAGTCTGGTGCCAGCAGCC | 18S-573 | White et al. (1990) |
| | NS31 | TTGGAGGGCAAGTCTGGTGCC | | Santos et al. (2006) |
| | NS4 | CTTCCGTCAATTCCTTTAAG | 18S-1131 | White et al. (1990) |
| | nu-ssu0817 | TTAGCATGGAATAATRRAATAGGA | 18S-817 | Bastias et al. (2007) |
| Fungal 28S | 403f | GTGAAATTGTTGAAAGGGAA | 28S-403 | Diouf et al. (2006) |
| | 662r | GACTCCTTGGTCCGTGTT | 28S-662 | Diouf et al. (2006) |
| | NL359 | GGACGCCATAGAGGGTGAGAGC | 28S-359 | Zuccaro et al. (2003) |
| | NL912 | TCAAATCCATCCGAGAACATCAG | 28S-912 | Zuccaro et al. (2003) |
| Fungal ITS | ITS1 | TCCGTAGGTGAACCTGCGG | 18S-1787 | White et al. (1990) |
| | ITS1f | CTTGGTCATTTAGAGGAAGTAA | | Gardes and Bruns (1993) |
| | ITS2 | GCTGCGTTCTTCATCGATGC | | White et al. (1990) |
| | ITS3 | GCATCGATGAAGAACGCAGC | | White et al. (1990) |
| | ITS4 | TCCTCCGCTTATTGATATGC | 28S-41 | White et al. (1990) |
| | ITS4A | CGCCGTTACTGGGGCAATCCCTG | | Larena et al. (1999) |
| | ITS4B | CAGGAGACTTGTACACGGTCCAG | | Gardes and Bruns (1993) |

approximately 500 bp. The DNA samples (0.5 µg DNA/lane for a rich bacterial community) are mixed with a loading dye (e.g., the DNA loading dye from Fermentas) 4:1 prior to application.

The gel solution consists of 6% (w/v) acrylamide/bisacrylamide (37.5:1), in 0.5 × TAE buffer, pH 8.3, containing 55% to 61% of the denaturant (100% denaturant consists of 7M urea and 40% formamide). The stacking gel consists of 6% acrylamide/bisacrylamide (37.5:1) and 0.5 × TAE buffer, pH 8.3. The gels are prepared, loaded and run according to the instructions of the manufacturers of individual DGGE systems (e.g., D-Code, BioRad or PhorU 2 × 2, Ingeny). The typical time of separation proceeds for 17 h at 200 V in 0.5 × TAE buffer (60°C). The gels can be stained with ethidium bromide (EtBr), Gel Star, SYBR Green or silver stained. For EtBr staining, the gels should be immersed for 15 min in an EtBr bath (50 µl of 1% EtBr per l of $H_2O$) and the background reduced by destaining for 15 min in water.

**Fungal community analysis**. The forward primers ITS1f 5'-CTT GGT CAT TTA GAG GAA GTA A-3' and ITS1f-gc 5'-CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGG GCT TGG TCA TTT AGA GGA AGT AA-3' and the reverse primers ITS2 5'-GCT GCG TTC TTC ATC GAT GC-3' and ITS4 5'-TCC TCC GCT TAT TGA TAT GC-3' are used (Table 3).

Nested PCR is used for the amplification of the ITS1 region of fungal rDNA. A fragment comprising both ITS1 and ITS2 is amplified in the first PCR reaction using the primer pair ITS1f/ITS4. After the purification of the PCR product, the ITS1 region is specifically amplified in the second PCR reaction using the ITS1f-gc/ITS2 primers.

The first PCR reaction consists of 1 × PCR buffer with $MgCl_2$, 100µM dNTPs, 10 pmol ITS1f primer, 10 pmol ITS4 primer, 1.5 U DNA polymerase, 1 µl DNA template (50 ng/µl) and $H_2O$ to bring the volume to 25 µl. The amplification yield of soil samples can be improved by the addition of 15 µg of bovine serum albumin per 30 µl reaction. Cycling conditions: 94°C for 5 min, 35 cycles (94°C for 1 min, 50°C for 1 min, 72°C for 1 min) and 72°C for 10 min. The size of PCR products is approximately 700–900 bp.

The second PCR reaction consists of 1 × PCR buffer with $MgCl_2$, 200µM dNTPs, 20 pmol ITS1f-gc primer, 20 pmol ITS2 primer, 3 U DNA polymerase, 1 µl of PCR product from the first PCR as a template and $H_2O$ to bring the volume to 50 µl. Cycling conditions: 94°C for 5 min, 35 cycles (94°C for 30 s, 55°C for 30 s, 72°C for 30 s) and 72°C for 10 min. The size of the PCR product is approximately 400 bp. The DNA samples (0.5 µg DNA/lane for a rich bacterial community) are mixed with a loading dye (e.g., the DNA loading dye from Fermentas) 4:1 prior to application. The DGGE gel is prepared and run as described for analysis of bacterial 16S rDNA with two differences: 9% (w/v) of acrylamide/bisacrylamide is used with a linear gradient of denaturant between 36% and 44%.

**General recommendations for DGGE analysis**. The amount of DNA should reflect the number of expected bands in the fingerprint: the more bands expected the higher amount of DNA should be used. When numbers of bands differ widely between samples, always use the same amount of DNA per electrophoresis lane. Markers should always be used to reduce the effect of minor gel defects (e.g., the 'smiling' effect) or to allow between-gel comparisons. The use of at least three marker lanes per gel is recommended. Markers can easily be derived from environmental samples by cutting and reamplification of 7–10 selected bands. These should preferably be evenly spaced and cover the whole separation area of the gel. If possible, the outer lanes of the gel are better left empty, as the smiling effect may distort the banding pattern. When establishing a DGGE analysis protocol in an experiment with newly designed primers, it is wise to test a broad denaturant gradient in the first trial and to estimate a suitable focused gradient after analyzing the separation results.

The main advantage of DGGE or TGGE is the fact that it offers the possibility to further analyze fingerprints by molecular methods and DGGE is today probably the most commonly used method for typing and comparing microbial communities. In the future, the value of this method can be further increased when suitable primers are developed to address underexplored microbial taxa or functional genes.

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Received on July 1, 2009

*Corresponding author:*

Dr. Petr Baldrian, Mikrobiologický ústav Akademie věd České Republiky, Vídeňská 1083, 14220 Praha 4, Česká Republika
e-mail: baldrian@biomed.cas.cz