Semi-Nested PCR Approach to Amplify Large 18S rRNA Gene Fragments for PCR-DGGE Analysis of Soil Fungal Communities

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Abstract

Denaturing gradient gel electrophoresis (DGGE) of 18S rRNA gene fragments PCR-amplified from total community DNA is a powerful tool for the parallel comparative analysis of environmental fungal communities. The 18S rRNA gene has the advantages of universality, high phylogenetic information content, and a large number of sequences in the data banks. The comparative analysis of soil fungal communities from large numbers of samples by PCR-DGGE requires consistent amplification and separation efficiency, as achieved by the following semi-nested PCR-DGGE protocol based on two-step PCR of 1,650 bp rRNA gene fragments from bulk soil DNA and their separation in DGGE.

Introduction

Along with bacteria, fungi are involved in soil functionality, comprising physical, chemical, and biological aspects [1]. As components of the complex interactive soil food web, fungal assemblages in soil respond to changes of the different soil trophic levels with community changes, which, in turn, affect the soil properties. Therefore, fungal community shifts may serve as indicators for soil food web modifications and their analysis is of crucial importance for the understanding of soil ecosystems [2].

For a long time the soil fungal community composition was studied only by methods based

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on isolation of fungi directly from environmental samples plated onto nutrient media. However, the isolation techniques are very fastidious and confined by the boundaries set by unculturability of many fungi. The analysis of the community structure and dynamics of fungal communities from soils achieved important advances in the past two decades thanks to the molecular techniques [3]. Analysis methods based on PCR amplification of marker gene fragments from total DNA extracted from environmental samples brought forth suitable approaches to analyze comparatively high numbers of samples in a rapid and efficient manner.

The genes of the ribosomal gene complex, consisting of the small subunit (SSU) 18S rRNA gene, the large subunit (LSU) 28S rRNA gene, the internal transcribed spacer (ITS), and the intergenic spacer (IGS), are frequently used in fungal community profiling [4]. These marker genes comprise both highly conserved domains and variable regions [5, 6], allowing the design of suitable primer systems and the high resolution analysis of fungal communities at taxonomical levels ranging from phylum to strain [7, 8]. The fungal sequence data bases are considerably informative, especially for intensively studied taxonomic groups (e.g., arbuscular mycorrhizal fungi) [9].

Several primer systems, group specific or fungal universal, were designed to amplify either SSU, LSU rRNA gene fragments or the ITS/IGS regions and used for the characterization of fungal diversity in soils [10]. Theoretical and practical evaluation of primers targeting the 18S rRNA gene or the ITS region revealed that some of them amplify also nonfungal sequences, or other primers exclude major fungal taxa. Four different primer pairs were tested for their specificity toward fungal rRNA genes and their suitability for assessing the diversity of fungal communities in grassland soils [11]. Based on cloning and sequencing of amplicons obtained with each primer system from soil DNA, the authors concluded that primer biases might be less significant than previously expected.

Subsequent to the PCR amplification, the amplicon pools can be identified taxonomically by sequence analysis or separated by means of molecular profiling methods that exploit the differences in their DNA sequence or conformation and result in taxonomically anonymous fingerprints that allow comparisons between different sample types.

Primer systems targeting fungal rRNA genes, coupled with molecular fingerprinting techniques such as denaturing gradient gel electrophoresis (DGGE) to analyze PCR products obtained from total community DNA, provide appropriate strategies for descriptive and comparative analysis of soil fungal community structure [12]. Due to the fact that the fungal rRNA genes are more conserved than bacterial 16S rRNA genes, the molecular fingerprints obtained for 18S rRNA gene fragments are less complex and, thus, easier to evaluate than the bacterial profiles. If different taxa contribute to the same band in the DGGE fingerprints [13, 14], the analysis resolution can be improved by using taxon-specific primers (e.g., for *Trichoderma* community composition) [15].

The specificity of the primer system and the phylogenetic information contained within the amplified fragments are decisive factors for the degree of resolution with which the community structure is revealed. The fungal universal primer NS1 [7], combined with the fungus-specific reverse primer FR1 [8], amplifies about 1,650 bp of the 18S fungal rRNA gene and thus allows the use of the most phylogenetic information contained by this gene. Initially designed and used for the study of wood-inhabiting fungi [8], this primer system was used later in only a few studies—for example, to investigate the fungal communities associated with the bulk and rhizosphere soil of maize from tropical climate [14] and to compare the fungal community structure under different agricultural practices in soil mini ecosystems [16] or in the endorhiza of different potato lines at two different sites [17]. These studies asserted the reproducibility of this system and suitability even for a soil with high contents of humics [16]. However, when analyzing different soils originating from 36 sites with known properties, we encountered difficulties in obtaining PCR amplicons from total community

DNA of a broad sample range using directly the primer combination NS1-FR1-GC [18].

The literature mentioned several different factors affecting the efficiency of DNA amplification from soil samples. For example, insufficient amount or low priming availability of the template DNA is known as a limitation for successful amplification [19, 20]. Also, co-extracted humic substances were often reported to inhibit the yield of amplicons from soil samples [19, 21]. Both the proportion in which the fungal DNA contributes to the total community DNA extracted, and the amount of co-extracted contaminants (e.g., humic acids), cannot be assessed by agarose gel electrophoresis and might reduce the amplification efficiency [19]. In addition to that, it was already speculated in the literature that the GC-clamp necessary for the DGGE analysis [22] might bias direct PCR amplification [23, 24].

Nested PCR approaches have the advantage of enhanced sensitivity, allowing the detection of problematic DNA (e.g., low target amount, high contaminant amount) and the reduction of nonspecific amplification [25]. Moreover, the GC-clamp necessary for DGGE analysis can be included without difficulties in the second PCR step, after the specific templates reached a sufficient amount in the first PCR step.

Therefore, we designed the semi-nested PCR protocol presented in this chapter, which consists of a first amplification with the novel primer combination NS1-EF3, followed by a second amplification with the primers NS-FR1-GC [8, 18]. EF3 was designed and used formerly in other primer combinations [26–28]. The semi-nested PCR protocol presented in this chapter was used successfully for the comparative analysis of soil fungal communities from 36 different sites [18], as well as for the study on the impact of the site, the sugar beet cultivar, the seasonal dynamics, and the rhizosphere effect on the fungal community structure at three different sites planted with sugar beet [29].

However, the semi-nested procedure presented in this chapter is far from free of limitations.

For example, inconsistent PCR amplification was reported when using the protocol presented in this chapter, resulting in a high variability of the replicate DGGE patterns when investigating fungi of the rhizosphere of strawberry and oil seed rape at different sites [30]. The problem was partially solved by these authors by switching to a nested PCR version, replacing in the first PCR step the primer NS1 with NS0, which is located upstream from NS1.

Furthermore, two different studies reported on the retrieval of nonfungal sequences when working with the PCR protocol presented in this chapter. Firstly, sequences of ubiquitous soil flagellates were retrieved in the analysis of fungal communities from bulk soils of three different sites [14]. Secondly, it was impossible to compare fungal communities from the gut of *Diabrotica virgifera* feeding on maize roots in different soils with the protocol in this chapter, as the DGGE patterns generated were dominated by a band of *D. virgifera* [31].

Last but not least, because of the relatively high conservation of the 18S rRNA gene within the fungi, some of the fragments might not contain enough variation to allow DGGE separation and thus migrate with similar electrophoretic mobility, as previously observed [13, 14].

Therefore, the ITS should be mentioned here as a valuable alternative marker for the analysis of soil fungal communities. The use of ITS fragments, complementary or independently to the analysis of 18S rRNA gene fragments, might allow DGGE separation up to an intraspecific level [32]. Thus, additional insights might be gained for comparative studies (e.g., when analyzing potential effects of transgenic crops on the microbial communities in comparison with nontransgenic lines) [33]. The number of ITS entries in the GenBank (32,050) exceeded already by June 2005 the number of submitted 18S rRNA gene fragment sequences (30,651) [34] and attained 50,956 fully identified and 27,364 insufficiently identified ITS sequences as of February 2008 [35]. Several PCR-DGGE protocols based on ITS fragments are available for the analysis of soil fungal communities, of which a semi-nested protocol with the primers ITS1 and ITS4 in the first PCR and the primers ITS1-GC and ITS2 in the second PCR was described recently in detail [36].

Equipment and Materials

Equipment

1. FastPrep® Instrument FP 120 for bead beating (BIO101, Carlsbad, California).
2. Microcentrifuge.
3. Vortex.
4. Magnetic stirrer.
5. Electrophoresis chamber with power supply and accessories.
6. Thermocycler.
7. DGGE DCode™ Universal Mutation Detection System (Biorad, München, Germany) and accessories.
8. Gradient maker with peristaltic pump.
9. Gel documentation system with UV transilluminator and camera (e.g., UV System, INTAS®, Mitsubushi Electric Corporation).
10. Fast DNA®Spin®Kit for Soil (BIO101, Carlsbad, California).
11. GENECLEAN®SPIN® Kit (BIO101, Carlsbad, California).
12. 1 kb molecular weight DNA marker (e.g., Invitrogen).
13. Ethidium bromide.
14. Agarose
15. AmpliTaq DNA Polymerase Stoffel Fragment (Applied Biosystems, Foster City, California).
16. Deoxynucleotide Triphosphate Set (Roche Diagnostics, Germany).
17. Primers (Table 23.1).
18. 2% dimethyl sulfoxide (DMSO).
19. 0.5 M EDTA pH 8.
20. Deionized formamide (Stir slowly for about 30 min 10 g Serdolit MB-1 and 1 L formamide. Filter through Whatman paper to remove ionic exchange resin. Store at -20 ° C as 50 mL Falcon tube aliquots).
21. 18% denaturant 7.5% acrylamide stock solution (see Notes 1, 2).
22. 38% denaturant 7.5% acrylamide stock solution (see Notes 1, 3).
23. 10% Ammonium peroxodisulfate (APS) (w/v) in MilliQ water (stored as aliquots at -20 ° C).
24. Tetramethylethylendiamine (TEMED).
25. MilliQ (deionized) water.
26. 5× TBE Buffer (27.5 g boric acid, 54 g Tris base, 20 mL 0.5 M EDTA pH 8.0 in 1 L distilled water).
27. 50× TAE Buffer: (242.2 g Tris base, 18.6 g EDTA, 57.1 mL acetic acid in 1 L distilled water; diluted 1:50 for DGGE run).
28. 6× DGGE loading buffer (25 mg bromophenol blue, 25 mg xylene cyanole, and 3 mL glycerol in 10 mL distilled water, stored at 4 ° C).
29. DGGE standard (see Note 4).
30. GelBond film (Lonza, Switzerland).
31. Reaction vials (1.5 and 2.0 mL).
32. Pipette tips (10, 20, 100, 200, 1,000 μL) and capillary pipette tips.
33. Syringe needles.
34. 15 mL polypropylene Falcon tubes**.**
35. DGGE fixative solution (10 mL acetic acid and 200 mL ethanol in 1,790 mL MilliQ water).
36. DGGE staining solution (0.2 g silver nitrate in 100 mL MilliQ water, made freshly for each gel).
37. DGGE developing solution (400 μL 37% formaldehyde in 100 mL 1.5% sodium hydroxide, made freshly for each gel).
38. DGGE stopping solution (7.5 g of sodium carbonate in 1 L MilliQ water).
39. DGGE conservation solution (250 mL ethanol and 100 mL glycerol in 650 mL MilliQ water).

Methods

Initial Material

Total community DNA extracted directly from replicate composite bulk soil samples taken from random plots at different sites (see Notes 5–8).

Semi-Nested PCR

The First Amplification Step

The primer combination NS1-EF3 (see Table 23.1) is used, amplifying almost the entire

Table 23.1 Primers used for the semi-nested PCR amplification of 18S rRNA gene fragments from bulk soil total community DNA (GC-clamp underlined)

Primer	Sequence	References
NS1	5′-GTA GTC ATA TGC TTG TCT C-3′	White et al. [7]
EF3	5′-TCC TCT AAA TGA CCA AGTTTG-3′	Smit et al. [26]
FR1-GC	5′-<u>CCC CCG CCG CGC GCGGCG GGCGGG GCG GGG GCA CGG GCC G</u>-AIC CAT TCA ATC GGT AIT-3′	Vainio and Hantula [8]
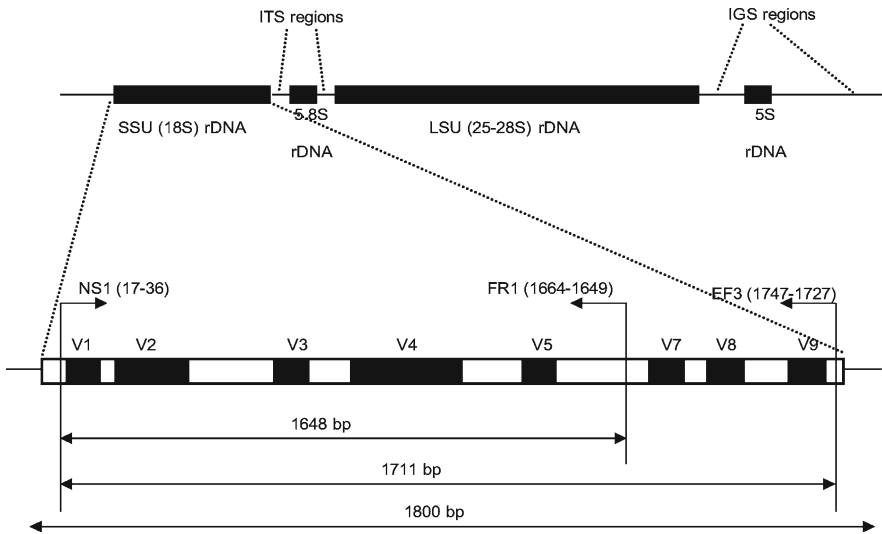
Fig. 23.1 Position of the annealing sites of the primers used in this chapter (NS1, FR1, EF3) on the 18S rRNA gene of *Saccharomyces cerevisiae* and length of the amplicons generated with different primer combinations

18S rRNA gene (Fig. 23.1). Perform the reaction with ca. 15–20 ng DNA extract in 25 μl volume containing: Stoffel buffer (10 mM KCl, 10 mM Tris–HCl pH 8.3), 0.2 mM deoxynucleoside triphosphates, 3.75 mM $MgCl_2$, 2% (w/v) dimethyl sulfoxide (see Note 9), 0.2 μM of each primer, and 2 U/μl of *Taq* DNA polymerase Stoffel fragment. PCR cycling program: 5 min denaturation at 94 ° C, followed by 25 thermal cycles of 30 s at 94 °C, 45 s at 47 °C, 3 min at 72 °C, and final extension at 72 °C for 10 min.

The Second Amplification Step

The primer combination NS1-FR1-GC (see Table 23.1) is used, amplifying 1,650 bp of the 18S rRNA gene (see Fig. 23.1). Perform the reaction with optimized dilutions of the amplicons from the first PCR step in 25 μL volume containing Stoffel buffer (10 mM KCl, 10 mM Tris–HCl pH 8.3),

0.2 mM deoxynucleoside triphosphates, 3.75 mM $MgCl_2$, 2% (w/v) dimethyl sulfoxide, 0.2 μM of each primer and 2 U/μl of *Taq* DNA polymerase Stoffel fragment). PCR cycling program: 5 min denaturation at 94 ° C, followed by 20 thermal cycles of 30 s at 94 ° C, 45 s at 48 ° C, 3 min at 72 ° C, and final extension at 72 °C for 10 min.

DGGE Fingerprinting of Soil Fungal Communities

See Figs. 23.2a,b and Note 10.

Gel Casting

Assembly of the gel sandwich:
1. Place the glass plates on a plane table. Carefully clean the surface of the glass plates with 97% ethanol.
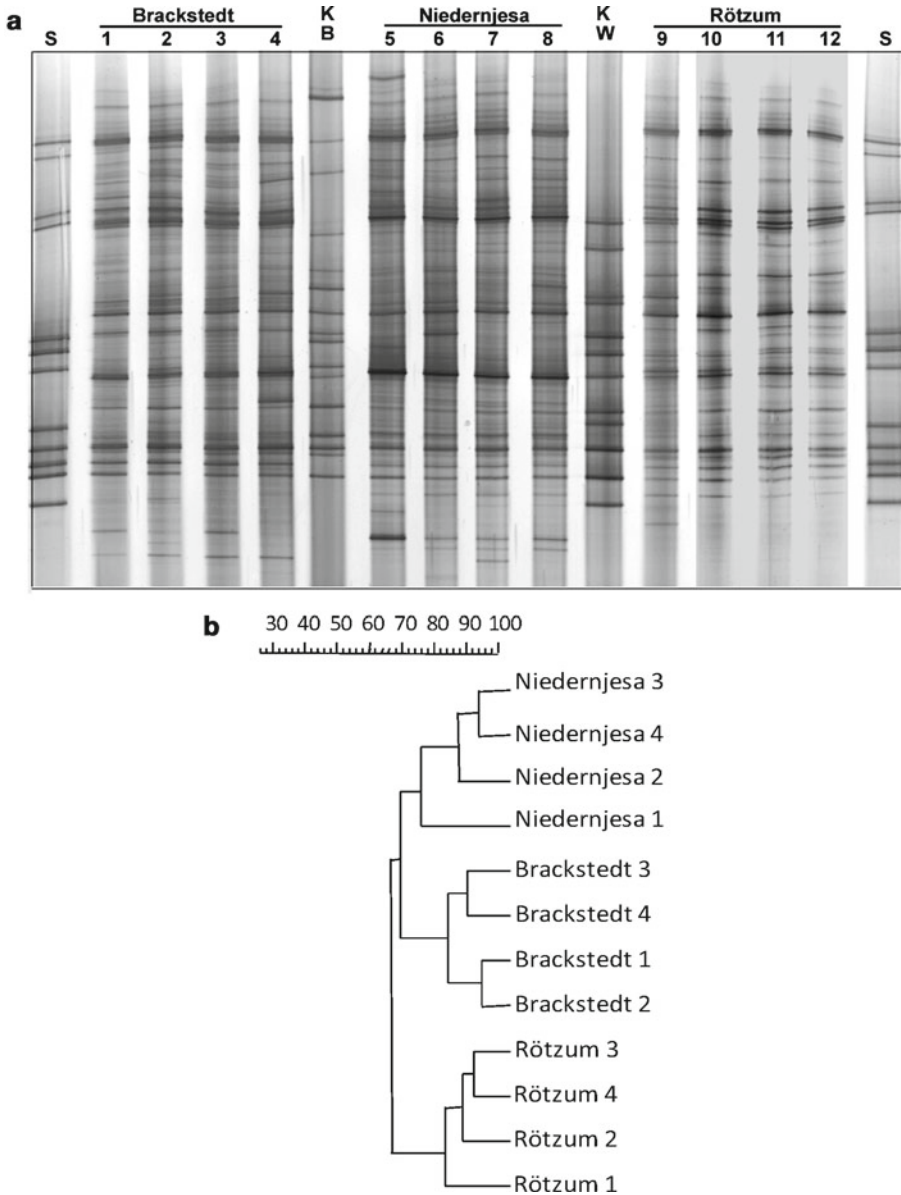
Fig. 23.2 (**a**) DGGE fungal community fingerprints of 18S rRNA gene fragments amplified from bulk soil DNA from three sites with different soil types: Lanes *1–4*, replicates from Brackstedt (sandy soil); lanes *5–8*, replicates from Niedernjesa (alluvial silt); lanes *9–12*, replicates Rötzum (clay-rich blacksoil) loess loam; lane *S*, standard mixture of PCR-amplified 18S rRNA gene fragments of fungal isolates; lanes *KB* and *KW*, standard mixtures of PCR-amplified 18S rRNA gene fragments cloned from total soil DNA of similar soils. (**b**) Dendrogram based on the Pearson correlation indices and UPGMA cluster analysis of the fungal community fingerprints of 18S rRNA gene fragments amplified from bulk soil DNA from Brackstedt, Niedernjesa, and Rötzum. The differences between the profiles are indicated by percentage of similarity. Patterns of soil samples originating from different site clusters separately

2. Spread a couple of tap-water drops on the large glass plate.

3. Place the GelBond film with the hydrophobic side in direct contact with the large glass plate, ensuring the perfect alignment of the film to the bottom of the glass.

4. Fix the film to the glass free of air bubbles (e.g., with a Drigalski spatula). Remove excess tap water.

5. Place two spacers at the outer sides of the large glass plate and the small glass on the top.

6. Insert the glass plate assembly within sandwich clamps ensuring that the bottoms correspond perfectly.

7. Place the sandwich assembly in a casting stand with a rubber strip at the bottom to prevent leakage. Ensure tight contact of the sandwich assembly bottom with the rubber strip and a stable position of the casting assembly. Close both clamps with equal pressure using the alignment card. Do not over-tighten clamps to avoid usage of plates.

8. Insert the comb in the glass plate sandwich.
Casting of denaturing gradient gels and polymerization:

1. Thaw and keep denaturing stock solutions on ice.

2. Add 45 μL 10% APS and 26 μl TEMED to each 15 mL of 18% respectively 38% denaturing solution and mix by inverting the vials. Work on ice to prevent premature polymerization.

3. Place the gradient maker on a stir plate at speed 300 rounds/min with a magnet stirrer in the outlet port chamber.

4. Connect the gradient maker to the peristaltic pump (the pump is off and the gradient maker channel is closed). Provide the pump tube with a syringe needle. Insert the needle centrally between comb and small glass plate.

5. Pour the 38% denaturing solution into the outlet port chamber of the gradient maker. Briefly open and close the valve to remove air between chambers. Pour the 18% denaturing solution in the remaining chamber.

6. Turn on the peristaltic pump and open simultaneously the valve between chambers.

An optimal flow of 5 mL/min is recommended. Ensure the solutions flow without leaking from the sandwich until air bubbles reach the syringe needle.

7. After gel casting, remove the needle and water-flush the gradient maker and tubing to discard solution rests.

8. Let the gel polymerize unmoved for at least 1 h. Use the same day or keep at 4 ° C wrapped in wet towels.

Pre-Run

1. Insert two gel sandwiches into the electrophoresis core. If only one gel is used, replace second gel with a glass plate sandwich without spacers.

2. Place the core assembly into the buffer tank filled with 1× TAE buffer. Renew 50% of the buffer between runs. Check buffer level, set up the temperature for 58 °C and start the pump.

3. When the buffer reaches the run temperature, turn off the system and remove the comb from the gel.

Sample Loading and Electrophoresis

1. Adjust the volume of PCR products to load at similar DNA concentrations. The different concentrations of the samples can interfere with software analysis of the gel.

2. Mix PCR products 1:1 with DGGE loading buffer and load with microcapillary pipette tips. Ensure that a maximum of 20 μL of sample is loaded to prevent well overflowing.

3. Load a DGGE standard in the outside lanes to assess the gradient formation and the band positions and to normalize the gel in further software analysis.

4. Close the system; check buffer, temperature, and pump; and start electrophoresis.

Gel Staining, Drying, and Scanning

1. Work on a switch rocker.

2. Transfer the gel in a recipient with 100 mL fixative solution for 10 min. or unmoved overnight.

3. Discard the fixative solution and add 100 mL 0.2% silver nitrate fresh solution for 15 min.

4. Discard the silver nitrate solution in a specific waste. Wash the gel at least twice for 1 min with MilliQ water.

5. Change the gel in a new recipient and add 100 mL fresh developing solution.

6. Discard the developing solution as pale bands appear. Add 100 mL stopping solution for ca. 10 min.

7. Discard the stopping solution. Add 100 mL conservation solution for at least 7 min.

8. Transfer the gel on a rigid frame. Cover the gel without air bubbles with a cellophane film soaked with conservation solution. Fix with clamps. Air-dry at room temperature for 2 days.

9. Transform the gel image in a digital picture using any transparency scanning system available.

10. Analyze the digitalized gel by means of software—e.g., GelCompar 4.0 (Applied Maths, Ghent, Belgium)—with unweighted pair group method using arithmetic averages (UPGMA) cluster analysis.

11. Apply any statistical method available to ensure statistical significance of results (see reference [37]).

Notes

1. Consider that 100% denaturant solution contains 40% deionized formamide and 7 M urea.

2. 18% denaturant 7.5% acrylamide stock solution: Dissolve 18.93 g urea in 100 mL MilliQ water. Add 5 mL 50× TAE, 18 mL deionized formamide, and 62.5 mL acrylamide Rotiphorese Gel 30 (37.5:1) (Roth, Germany). Adjust the volume to 250 mL in a volumetric flask and filter. Aliquot 15 mL solution in Falcon tubes. Store at -20 °C.

3. 38% denaturant 7.5% acrylamide stock solution: Dissolve 39.94 g urea in 100 mL MilliQ water. Add 5 mL of 50× TAE, 38 mL deionized formamide and 62.5 mL acrylamide Rotiphorese Gel 30 (37.5:1) (Roth, Germany). Adjust the volume to 250 mL in a volumetric flask and filter. Aliquot 15 mL solution in Falcon tubes. Store at -20 ° C.

4. The DGGE standard is an artificial mixture of 18S rRNA gene fragments PCR-amplified from single fungal isolates or clones known to migrate with different electrophoretic mobilities in DGGE protocol used.

5. Ensure at least four replicate samples per site for representative statistical results.

6. Dig a number of bulk soil cores per plot representative for the plot dimensions; e.g., 8–10 cores (15–20 cm of top soil) per 10 m². Avoid root material if bulk soil analysis is intended. Mix well by sieving.

7. Ensure an amount of 0.3–1 g dry weight of soil per replicate for DNA extraction to minimize eventual heterogeneous distribution of fungal DNA targets and to ensuring representative results.

8. For total DNA extraction, use one of the commercial kits for soil, preferably BIO101 Fast DNA®Spin®Kit for Soil, combined with a harsh cell lysis to break fungal cell walls, e.g., with the FastPrep® Instrument for 1 min at 4,000 rpm. Purify the crude DNA from eventual humic contaminants, e.g., with the GENECLEAN®SPIN® Kit. Store DNA extracts at -20 °C until further procedures.

9. Dimethyl sulfoxide in the reaction mixture is known to enhance PCR by eliminating nonspecific amplification and to improve the primer annealing efficiency by destabilizing secondary structures within the template.

10. All DGGE materials, gel casting procedures, and running conditions presented in this chapter are strictly referred to the DGGE DCode™ Universal Mutation Detection System (Biorad, München, Germany). Use an 18–38% denaturing gradient.

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