Objective criteria to assess representativity of soil fungal community profiles

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Received 3 July 2006; received in revised form 18 September 2006; accepted 18 September 2006
Available online 3 November 2006

Abstract

Soil fungal community structures are often highly heterogeneous even among samples taken from small field plots. Sample pooling is widely used in order to overcome this heterogeneity, however, no objective criteria have yet been defined on how to determine the number of samples to be pooled for representatively profiling a field plot. In the present study PCR/RFLP and T-RFLP analysis of fungal 18S rDNA in ten soil samples obtained from a grassland plot of 400 m² also revealed this known heterogeneity in fungal community structures. Based on these data a three-step approach to assess representativity of fungal community profiles was established. First, soil DNA quantities needed for robust community profiling were determined. Second, profiles of single or multiple samples were theoretically averaged to test for statistically significant clustering in order to determine the minimal number of samples to be pooled to achieve representativity. Third, DNA extracts of single or multiple samples were pooled prior to profiling in order to test for statistically significant clustering. Analyses revealed robust profiles for 50 ng soil DNA but not for 5 ng. Averaged T-RFLP profiles from five or more soil samples and experimental T-RFLP profiles from pools of seven or more samples formed one significant branch. Theoretical averaging and experimental pooling revealed that five to seven samples have to be pooled for robustly representing the field plot. Our results demonstrate that representativity of soil fungal community profiles can objectively be determined for a field plot with only little deviation between theoretical and experimental approaches. This three-step approach will be of assistance for designing sampling and pooling strategies for comparative analyses of soil fungal communities in ecological studies.

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Keywords: Soil fungal community structure; Statistical analysis; Sample pooling; Ribosomal RNA genes; Representative genetic profiling

1. Introduction

Various methods to assess fungal communities in soil have been developed, which have great potential to contribute to a better understanding of the ecological role of fungi in soil habitats. Diversity described as total number and abundance of fungal species may not be determinable in soils due to limitations in taxonomic definitions and methods, thus composition of fungal communities may more generally be described by fungal community structures (Kirk et al., 2004). Ribosomal RNA (rRNA) genes have been shown to be suitable markers to study microbial community structures (Amann and Ludwig, 2000; Bundt et al., 2001; Woese, 1967) and analysis of their terminal restriction fragment length polymorphism (T-RFLP) (Liu et al., 1997) is considered a sensitive approach for comparative community profiling (Marsh, 1999) providing high-resolution profiles suitable for statistical analysis (Brodie et al., 2003; Hartmann et al., 2005). Although community-level T-RFLP profiling of rRNA genes has been reported to be highly robust for the analysis of bacterial communities (Hartmann et al., 2005; Osborn et al., 2000; Pesaro et al., 2004), robustness of fungal T-RFLP profiles appears to be much more affected by soil sample size (Ranjard et al., 2003), cell lysis efficiency (DeSantis et al., 2005; He et al., 2005; Kirk et al., 2004) or DNA quantity used for PCR (Brodie et al., 2003).

There might be several explanations for the reduced robustness of fungal community profiles. With approximately $10^5$ colony forming units (cfu) per gram soil, fungi are roughly 100 times less abundant than bacteria (Foster, 1988), with fungi–bacteria biomass ratios ranging between 0.22 in a litter rich prairie soil and 0.12 in a tilled soil (Allison et al., 2005). Distribution and density of fungi have been reported to be highly heterogeneous. For example, ectomycorrhizal species typically occur in 10 to 25% of soil samples with volumes of approximately 260 cm³, as estimated from several studies with...
an average of 30 samples per hectare (Horton and Bruns, 2001). Generally, the heterogeneity of fungal populations per area has been reported to be higher when compared to bacteria (Horner-Devine et al., 2004).

If fungal community structures of two or multiple sites are compared, within-site heterogeneity may reduce resolution of the analysis (Kasuga et al., 2002). Therefore robust field-representative fungal community profiles are needed. Mixed or composite samples have been used to increase the representativity of fungal community profiling (Anderson et al., 2003; Hagn et al., 2003; Klamer and Hedlund, 2004), but some studies still revealed high variability between replicated samples (Girvan et al., 2004; Klamer et al., 2002). This demonstrates the need to individually adjust sampling schemes for representative analyses of soil fungal community structures (Morris et al., 2002). Moreover, different schemes may be needed to either robustly analyze heterogeneities of fungal communities within a field or to generate a mean fungal community profile, representative for the entire field plot.

In the present study we applied PCR/RFLP and T-RFLP profiling of fungal 18S rRNA genes to analyze the fungal community structure of a grassland soil. Our objective was to develop a strategy to generate 1) fungal community profiles representative for individual samples in order to assess fungal heterogeneity and 2) fungal community profiles representative for an entire field.

2. Materials and methods

2.1. Experimental approach

Pooling of soil samples is often applied to obtain representative samples for a certain field plot or a specific experimental treatment (Anderson et al., 2003; Gomes et al., 2003; Milling et al., 2004). However, objective criteria for determination of the optimal number of samples to be pooled have not been available. We designed an experimental approach to address exactly this question. For these analyses we applied three different types of sample pooling, which were based on defined dress exactly this question. For these analyses we applied three, five, seven, nine or ten samples. For each level of DNA mixtures were obtained by mixing DNA extracts of ten replicates (a–f).

2.3. Soil DNA extraction, purification and quantification

Nucleic acids were extracted from fresh soil according to Hartmann et al. (2005): 0.6 g fresh soil and 0.75 g glass beads (0.10 mm diameter, B. Braun Biotech International, Melsungen, D) were suspended in 1 ml extraction buffer (0.2% hexadecyltrimethylammonium bromide (CTAB), 0.2 M sodium phosphate buffer pH 8, 0.1 M NaCl and 50 mM EDTA) and extracted using a bead beating procedure with a FastPrep FP120 (Savant Instruments, Farmingdale, NY) at 5.5 m s⁻¹ (approx. 6800 rpm) for 40 s. Supernatants were collected and pooled with corresponding supernatants of two further extractions of each soil sample. Extracted DNA was precipitated and resuspended in TE buffer (10 mM Tris–HCl, 1 mM EDTA; pH 8) at 1 ml g⁻¹ dry weight equivalent of extracted soil. Twenty-five microliters of each extract were purified using Extract-II DNA purification columns (Machery and Nagel, Easton, PA). DNA content was quantified fluorometrically using Pico Green (Molecular Probes, Eugene, OR) and a luminescence spectrometer LS30 (Perkin Elmer, Wellesley, MA). Herring sperm DNA (Promega, Madison, WI) was used as DNA concentration standard and soil DNA content was expressed as μg DNA g⁻¹ soil dry weight. All subsequent metagenomic analyses of single samples were processed in six replicates (a–f).

2.2. Soil sampling

The study site was a hay-meadow with an eutric cambisol (sandy loam) situated in Central Switzerland, at an elevation of 700 m, with an annual mean temperature of 7.2 °C and annual mean precipitation of 1491 mm (Meteo-Schweiz, Switzerland). In June 2003 an area of 400 m² (20 × 20 m) was sampled at 10 points, which were distributed across three longitudinal transects. At each point two adjacent soil cores were taken using a stainless-steel corer with an internal diameter of 5.5 cm. The 5 to 15 cm depth fractions of the two adjacent cores were pooled (Kessler et al., 2003). Fifty grams from each of the ten fresh samples were bulked and homogenized by sieving (5 mm) to form a ‘ten-soil-mix’ sample. Samples were stored in plastic bags at 4 °C and processed within 48 h. Dry weights were determined from 10 g fresh soil of each sample dried at 105 °C for 24 h.

2.4. Mixtures of metagenomic DNA

DNA mixtures were obtained by mixing DNA extracts of three, five, seven, nine or ten samples. For each level of complexity, three sample combinations were randomly selected and processed in six replicates, except for the ‘ten-DNA-mix’, containing DNA from all ten samples, which was processed in ten replicates.

2.5. PCR amplification

Partial fungal 18S rRNA genes were amplified from 50 ng or 5 ng template DNA respectively, according to Vainio and Hantula (2000) using 5′-6-FAM-(6-carboxyfluorescein) labeled forward primer NS1 (5′-GTAGCTCATGCGTTGCTTC-3′) and reverse primer FR1 (5′-AICCATTTAATCGGTANT-3′; 1 represents inosine) yielding a product of approximately 1650 bp (Vainio and Hantula, 2000). Samples were incubated in a volume of 11 μl in aqueous solution containing 0.6 μg BSA per ng DNA for 45 min at 37 °C to scavenge PCR inhibitory substances (Kreader, 1996; Watson and Blackwell, 2000). After chilling on ice, PCR mixture (containing 0.3 μg BSA) was added, yielding a final volume of 50 μl with concentrations of 1× PCR buffer (Qiagen, Hilden, Germany), 0.2 μM of each
primer, 3.5 mM MgCl₂, 0.4 mM dNTP (Invitrogen, Carlsbad, CA) and 2 U of HotStar Taq DNA polymerase (Qiagen). Amplifications were performed in a Bio-Rad iCycler (Bio-Rad Laboratories, Hercules, CA) either with 42 cycles for 50 ng template DNA or 46 cycles for 5 ng template DNA. After initial denaturation for 15 min at 95 °C, cycles with 25 s at 92 °C, 40 s at 53 °C and 3 min at 72 °C were performed, followed by a final elongation for 10 min at 72 °C. The quality of amplification products was verified by gel electrophoresis in 1.5% agarose gels and ethidium bromide staining.

2.6. Restriction fragment length polymorphism analysis

Twenty-five microliters of each PCR product were digested using restriction endonuclease HaeIII (Promega). Buffer conditions for restriction digests were adjusted directly in the PCR sample by adding 50 μl of concentration conversion buffer consisting of 10 mM Tris–HCl (pH 7.4), 50 mM NaCl and 13.25 mM MgCl₂ (Hartmann et al., 2005). Eight units of HaeIII in 5 μl 1× buffer C (Promega) were added. Digestion was performed for 10 h at 37 °C followed by inactivation of the enzyme at 65 °C for 20 min. Digestion products were purified using Montage SEQ96 Sequencing Reaction Cleanup Kit (Millipore, Bedford, MA) and adjusted to 20 μl with TE buffer. Three microliters of purified digests were separated by electrophoresis in a 12% polyacrylamide gel in 1× TAE buffer (40 mM Tris–EDTA, 1 mM EDTA, pH 8) for 4.5 h at 200 V and 35 °C (DCode system; Bio-Rad). Two microliters of 1 kb DNA size standard (Invitrogen) were used. Gels were stained for 30 min with SYBR Green (1:5000 in TAE (1×); Molecular Probes) and RFLP profiles were recorded with a GelDoc XRS system and the Quantity One software (Bio-Rad).

2.7. Terminal restriction fragment length polymorphism analysis

For T-RFLP analysis 2 μl purified restriction digest were mixed with 12 μl of formamide and 0.2 μl of X-Rhodamine labeled size standard MapMarker1000 (BioVentures, Murfreesboro, TN). Denatured DNA fragments were separated using an ABI Prism 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA) equipped with sixteen 36 cm capillaries filled with POP-4 polymer (Applied Biosystems). Sizes of terminal restriction fragments (T-RFs) were determined as relative migration units (amu) with the GeneScan 3.7 analysis software (Applied Biosystems). Signal intensities given as relative fluorescent units (rfu) of T-RFs were scored between 60 and 800 rfu using the Genotyper 3.7 NT software (Applied Biosystems).

Because sizing accuracy decreases with increasing sizes of the fragments, T-RFs below 300 rmu were categorized at ±0.5 rmu, between 300 to 400 rmu at ±1 rmu and fragments longer than 400 rmu at ±0.5% of the corresponding fragment length according to suggestions of ABI Switzerland. Because T-RF signal intensities can be important to distinguish samples (Blackwood et al., 2003) signal threshold was set at 20 rfu. However, if signal intensities of T-RFs belonging to the same length category were all below 100 rfu, they were excluded from the analysis, as differences in signal intensities were not interpretable. If unambiguous determination of signal intensities of a specific T-RF was not possible in all samples, this T-RF was excluded from further analysis.

2.8. Statistical data analysis

Relative signal intensities within a profile were calculated by dividing signal intensities of each individual T-RF by the sum of all signal intensities in a profile (Blackwood et al., 2003). This normalization procedure allowed to compare signal intensities among different samples. For multivariate analysis relative signal intensities were z-transformed to average 0 with a standard deviation of 1 in order to give each T-RF the same relative weight. Statistica software package version 6.1 (StatSoft, Tulsa, OK) was used for Ward cluster analysis based on squared Euclidean distances (Blackwood et al., 2003). Analysis of variance (ANOVA) and discriminant function analysis (DFA) (Statistica, version 6.1) were used to identify T-RFs that significantly discriminated among samples. To compare dendrograms generated by cluster analyses calculated from different numbers of T-RFs, similarities among distance matrices were determined with the NTSYS-pc 2.2 software (Rohlf, 2005) using Mantel test statistics (Mantel, 1967). Additionally, the proportion of means of linkage distances among samples to means of linkage distances among replicates was used to compare degrees of separation of samples and replicates. The larger this proportion, the better the distinction of sample-clusters. All further statistical analyses were exclusively calculated from T-RFs discriminating between samples.

Statistical significance of differences between samples was assessed with permutation testing applied on redundancy analysis (RDA) with the Canoco Software (Microcomputer Power, Ithaca, NY) according to ter Braak and Smilauer (2002) using 999 permutations. Classification of samples and mixes were tested using DFA (Statistica, version 6.1), an ordination technique that generates a set of discriminant functions separating a priori defined groups (Hastie et al., 2001). Groups were defined based on replicate T-RFLP data derived from specific groups, i.e. from individual soil samples as well as calculated T-RFLP averages across the soil samples.

3. Results

3.1. Reproducibility of RFLP and T-RFLP profiles

Metagenomic DNA yield for the ten soil samples from the 400 m² grass land plot was between 135 and 250 μg g⁻¹ soil dry weight with an average of 180 μg g⁻¹. Thus, 50 ng or 5 ng DNA used for PCR corresponded on the average to 0.27 mg and 0.027 mg soil, respectively. No inhibition of PCR was observed when 50 ng DNA was added to 50 μl reactions (data not shown). Three soil samples were used to test reproducibility and representativity of HaeIII RFLP patterns of fungal 18S rRNA genes. RFLP patterns of replicated PCR products derived from 50 ng template DNA consistently revealed sample-specific banding patterns (sample 7 shown in Fig. 1a), demonstrating
reproducibility of the method. However, RFLP patterns derived from 5 ng template DNA revealed variability among replicates with respect to inconsistent presence of bands, e.g. bands at 155 or 195 bp, or differences in intensities, e.g. bands at 600, 265, 165 or 95 bp (Fig. 1b).

T-RFLP analysis using 50 ng template DNA of three replicates of the same three samples yielded 22 to 30 T-RFs with a mean standard deviation of 0.58 fragments per sample. Fragment lengths ranged from 80 to 717 rmu. Cluster analysis revealed reproducible and significant \( p < 0.05 \) distinction of the three soil samples (Fig. 2a). T-RFLP profiling from 5 ng template DNA yielded between 15 and 30 T-RFs with a mean standard deviation of 4.55 fragments per sample. Cluster analysis revealed no distinct cluster for any of the three samples (Fig. 2b) and Monte Carlo permutation testing revealed only a significant \( p < 0.05 \) distinction of soil sample 2. Based on these results 50 ng soil metagenomic DNA were used for all further PCR analyses.

3.2. Selection of T-RFs significantly discriminating between samples

Cluster analysis of fungal community T-RFLP profiles from all ten samples, based on standardized rfu-values of 40 scorable

<table>
<thead>
<tr>
<th>T-RF length [rmu]</th>
<th>Relative signal intensities ( \times 100 )</th>
<th>ANOVA</th>
<th>DFA</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>( \bar{x} \pm SD )</td>
<td>( F )-value</td>
<td>( p )-class</td>
</tr>
<tr>
<td>302</td>
<td>0.81 ±2.46</td>
<td>2488.60</td>
<td>***</td>
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<tr>
<td>315</td>
<td>3.27 ±2.78</td>
<td>290.48</td>
<td>***</td>
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<tr>
<td>238</td>
<td>0.96 ±2.91</td>
<td>1083.15</td>
<td>***</td>
</tr>
<tr>
<td>688</td>
<td>0.12 ±0.20</td>
<td>69.88</td>
<td>***</td>
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<tr>
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<td>0.37 ±0.36</td>
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<tr>
<td>312</td>
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<tr>
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<td>13.91</td>
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<td>261</td>
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<td>624</td>
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<td>323</td>
<td>1.04 ±1.13</td>
<td>48.06</td>
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<td>717</td>
<td>1.01 ±0.19</td>
<td>3.77</td>
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<td>203</td>
<td>0.83 ±0.53</td>
<td>18.74</td>
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<td>215</td>
<td>2.13 ±0.98</td>
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<td>1.41 ±0.64</td>
<td>9.98</td>
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<td>0.30 ±0.40</td>
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<td>12.67</td>
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<tr>
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<td>0.55 ±0.28</td>
<td>11.74</td>
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<td>28.93 ±5.46</td>
<td>66.80</td>
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<tr>
<td>647</td>
<td>3.23 ±0.75</td>
<td>9.65</td>
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<td>357</td>
<td>1.15 ±0.57</td>
<td>57.97</td>
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<tr>
<td>158</td>
<td>2.41 ±0.86</td>
<td>5.26</td>
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Analyses were based on 6 replicate DNA extracts.

\*\*\* \( p < 0.001 \); \*\* \( p < 0.01 \); \* \( p < 0.05 \); n.s. not significant.

Fig. 1. PCR/RFLP patterns obtained from HaeIII digests of fungal 18S rRNA genes amplified from the soil metagenomic DNA of sample seven. PCR was performed on 50 ng a) or 5 ng b) template DNA in triplicates (7a, 7b, 7c). Bands revealing inconsistency or variability among replicated fingerprints are marked with arrows and fragment sizes are indicated. Lane M: 1 kb DNA size standard.

Fig. 2. Cluster analysis of T-RFLP data derived from soil fungal 18S rRNA genes using 50 ng a) or 5 ng b) template DNA of three samples (2, 7, 9) processed in three replicates (a, b, c). Dendrograms were inferred using Ward’s hierarchical cluster analysis based on squared Euclidean distances.
T-RFs (80–717 rmu) with relative signal intensities between $5 \times 10^{-4}$ (T-RF of 327 rmu) and $28.93 \times 10^{-2}$ (T-RF of 473 rmu), clustered the six profile replicates consistently into ten sample specific groups (data not shown). Distinction of samples may be increased if only the discriminating T-RFs are included in an analysis. ANOVA revealed that with exception of the T-RF at 83 rmu, all T-RFs significantly ($p < 0.05$) discriminated between samples (Table 1). DFA identified 20 T-RFs between 80 and 688 rmu with relative signal intensities between $6 \times 10^{-4}$ (T-RF of 353 rmu) and $9.05 \times 10^{-2}$ (T-RF of 624 rmu) that significantly ($p < 0.05$) discriminated the ten soil samples (Table 1). T-RFs not significantly discriminating soil samples were derived from the entire range of sizes and intensities (Table 1). Neither mean signal intensities nor their standard deviations were significantly different ($t$-test, $p > 0.05$) for the discriminating and non discriminating T-RFs. A dendrogram based on the 20 significantly discriminating T-RFs formed ten distinct clusters (Fig. 3), whereas a dendrogram calculated from the 20 not significantly discriminating T-RFs (Table 1) only clustered replicates of samples 1, 4, 6, 7 (data not shown). The Mantel test revealed a correlation of 0.54 ($p < 0.001$) between dendrogram matrices derived from 20 significant and 20 non-significant T-RFs. Mantel test analyses of dendrogram matrices derived from 40 T-RFs (data not shown) and 20 significantly discriminating T-RFs (Fig. 3) showed a correlation of 0.83 ($p < 0.001$), demonstrating consistency of tree topologies. Sample separation was increased when using only significantly discriminating T-RFs for cluster analysis, as seen by a decrease of mean squared Euclidean distances among replicates from 12.4 to 6.4 and an increase of mean distance among samples from 42.6 to 43.1. This increased the mean distance proportion from 3.4 to 6.7. In order to increase the stringency for assessing the field representativity of T-RFLP profiles, all further analyses were performed with the 20 significantly discriminating T-RFs.

3.3. Determination of averaged T-RFLP profiles

Mathematically averaged profiles were calculated from all six replicates (a to f) of each sample. These average profiles consistently clustered with corresponding replicates (Ø in Fig. 3). For a theoretical field-representative T-RFLP profile, average profiles of all six replicates were calculated across all ten soil samples and labeled ‘ten-T-RFLP-averages’. Cluster analysis revealed a separate cluster for the ‘ten-T-RFLP-averages’ with closest association to soil sample 3 (Fig. 3). These eleven groups were used in DFA as explaining variables while T-RFLP profiles were then tested as dependent variables in DFA. The resulting first three canonical axes explained 75% of total variation in the data and significantly discriminated the eleven groups identified by cluster analysis (data not shown).

3.4. Comparison of theoretical and experimental means of profiles

Cluster analysis of T-RFLP profiles of ‘ten-soil-mixes’ or of ‘ten-DNA-mixes’ together with the ten soil samples revealed that each mix clustered at the branch indicated with the arrow in Fig. 3 and dendrogram topology remained identical to the one in Fig. 3 (data not shown). When all three composite samples were combined with the ten soil samples in a cluster analysis they...
formed three closely associated clusters under the branch defined by the ‘ten-T-RFLP-averages’ and indicated with the arrow in Fig. 3 (Fig. 4, arrow). DFA of all three composite samples together with the ten soil samples resulted in ten independent sample groups and three overlapping groups of the pools (data not shown). The branch consistently formed by the three pool-types of all ten soil samples (arrows in Figs. 3 and 4) was therefore defined as representative for the field plot. For determining the minimal number of samples required for representing the field plot, the ‘ten-T-RFLP-averages’ were used as reference in the following analyses.

3.5. Determination of minimal pool sizes for representative profiling

T-RFLP profiles derived from pools of decreasing complexity, i.e. from nine, seven, five or three samples, were used to test whether subsets of the ten soil samples clustered with the ‘ten-T-RFLP-averages’, i.e. whether they were representative for the field plot. For each level of complexity, three randomized sample combinations were selected and each processed in six replicates. Replicates of nine, seven, or five ‘T-RFLP-averages’ consistently clustered with ‘ten-T-RFLP-averages’ at the branch indicated with the arrow in Fig. 3 (Table 2). For ‘T-RFLP-averages’ from three samples only 44% clustered with the ‘ten-T-RFLP-averages’. T-RFLP profiles from nine and seven ‘DNA-mixes’ consistently clustered with ‘ten-T-RFLP-averages’. Only 66% of the ‘five-DNA-mixes’ and 40% of the ‘three-DNA-mixes’ clustered with ‘ten-T-RFLP-averages’ profiles (Table 2). DFA assigned all replicates from nine, seven and five ‘T-RFLP-averages’ to the ‘ten-T-RFLP-averages’. Only 88% of ‘three-T-RFLP-averages’ were also assigned to ‘ten-T-RFLP-averages’ (Table 2). For DNA-mixes of seven or nine samples all replicates were assigned to ‘ten-T-RFLP-averages’ while only 73% of ‘five-DNA-mixes’ and 53% of ‘three-DNA-mixes’ were classified to the ‘ten-T-RFLP-averages’ (Table 2).

4. Discussion

A generally applicable three-step approach was developed to assess representativity of soil fungal community profiles. Fungal PCR/RFLP and T-RFLP were based on widely used fungi-specific PCR primers (Vainio and Hantula, 2000) and highly reproducible when derived from 50 ng soil metagenomic DNA. Fungal community structures were significantly different among ten soil samples from a 400 m² grassland field plot. Cluster analysis as well as discriminant function analysis consistently revealed that averages of five or pools of seven samples were sufficient to generate a representative profile for this field plot.

4.1. DNA quantity and reproducibility of community profiles

PCR inhibition caused by coextracted substances such as humic acids is one reason to use small quantities of template DNA. For this study we choose PCR template quantities of 5 ng and 50 ng soil DNA, which have been used for analyses of rhizosphere (Milling et al., 2004) and bulk soil metagenomic DNA (Anderson et al., 2003; Ranjard et al., 2003; Viaud et al., 2000). T-RFLP analyses resulted in 15 to 30 T-RFs per sample. These figures were in the same range as reported for comparable studies on fungal community structures of grasslands (Brodie et al., 2003; Edel-Hermann et al., 2004; Klammer and Hedlund, 2004). RFLP as well as T-RFLP derived from 50 ng template DNA revealed high reproducibility as illustrated by sample characteristic RFLP banding patterns and consistent clustering of T-RFLP replicates resulting in significant separation of samples. In contrast, RFLP patterns derived from 5 ng template DNA were not reproducible and corresponding T-RFLP profiles revealed inconsistent clustering (Fig. 2). This high reproducibility of restriction fragment analyses derived from 50 ng template DNA indicated that neither primer binding biases (Suzuki and Giovannoni, 1996) or stochastic PCR artifacts like nucleotide misincorporation (Eckert and Kunkel, 1991) nor chimera formation (Kanagawa, 2003) were limiting factors in these analyses. Variability in T-RFLP profiles derived from 5 ng template DNA may have originated from stochastic dilution of specific target sequences in individual PCR reactions or from PCR artifacts related to the large cycle numbers required. Required template DNA quantities may also depend on the profiling method. For example, Brodie et al. (2003) have found 10 ng of template DNA to be sufficient for reproducible T-RFLP profiling, but to be insufficient for denaturing gradient gel electrophoresis (DGGE). Improvements in DNA purification have allowed to increase template DNA quantities (Watson and Blackwell, 2000), e.g. up to 200 ng (Kennedy et al., 2005). Reproducibility of fungal genetic profiling may also depend on the quantity of extracted soil (Ranjard et al., 2003) and on the DNA extraction method applied as these strongly influence both quality and quantity of extracted soil metagenomic DNA (Bürgmann et al., 2001; Martin-Laurent et al., 2001). Whether 50 ng template DNA is generally sufficient for soil fungal community profiling is unknown and needs to be tested on a case by case base.

4.2. Discriminative T-RFs for improved sample separation

T-RFs common to and evenly occurring in all samples may mask differences between samples. In an earlier study, ANOVA
has been used for the detection of indicator T-RFs for bacteria associated with heavy metal contaminated soils (Hartmann et al., 2005). In the present study ANOVA revealed all but the T-RF of 83 rmu as significantly \( p < 0.05 \) discriminating between ten soil samples taken across a 400 m\(^2\) area. While ANOVA calculates the partial contribution of all T-RFs on the overall separation in one analysis, DFA stepwise adds, or likewise, removes variables from the dataset and calculates the separation of groups until no further improvement is achieved (Gill, 2001; Leotta, 2004). This process excludes common T-RFs, reduces the number of variables under analysis and thus improves the validity of multivariate analyses (Kourtev et al., 2002). This is illustrated by our results where DFA revealed only 20 significantly discriminating T-RFs (Table 1). The analyses of profiles, which exclusively contain T-RFs discriminating between samples emphasize differences between samples and thus represent a more stringent approach for defining representative profiles. Therefore, in this study we discarded 20 of the measured T-RFs and calculated reference profiles exclusively from the 20 T-RFs, which discriminated significantly between the ten soil samples.

4.3. Representative reference profiles

Pooling of PCR products has been applied to increase representativity of molecular genetic profiles (Blackwood et al., 2003; Hunt et al., 2004; Von Wintzingerode et al., 1997). Instead of mixing PCR products, multiple T-RFLP profiles were averaged in the present study. When the average was calculated from replicated T-RFLP profiles of a sample, it was shown representative for this sample. In analogy, we averaged profiles of all ten soil samples to generate a profile representing the entire field plot (Fig. 3, arrow). T-RFLP profiles derived from mixes of the ten soil samples or of the ten DNA extracts, associated in distinct clusters under the same branch (Fig. 4, arrow). The small differences among these three clusters may be explained with differences in sample preparation. However, data demonstrate that effects of different preparations of these ‘ten-mixes’ were minor if compared to differences among individual soil samples. These results also demonstrated comparability of DNA extracts obtained from soil samples mixed prior to extraction to DNA pools prepared from extracts of individual soil samples. This information was used to define the branch that was considered representative for the field plot (indicated by the arrows in Figs. 3 and 4). There have been several studies, which indicated reduced resolution caused by within-site variability of fungal communities. Klamer et al. (2002) analyzed fungal communities based on internal transcribed spacer (ITS) analysis of DNA extracted from one sample of 0.25 g soil per replicate plot. Heterogeneity among 8 replicated plots has been found to be as high as heterogeneity between controls and treatments. Girvan et al. (2004) assessed both fungal and bacterial community structures by DGGE profiling from mixed samples derived from 20 soil cores collected over an area of 1.2 ha. They detected effects of a pesticide treatment on bacterial communities, but heterogeneity of fungal communities interfered with the detection of differences between treated and control plots. The approach presented here will be of assistance to assess profiling robustness and to define the sample numbers required for representative profiles.

4.4. Optimized sample pool size for representative fungal community profiling

The initial collection of ten soil samples allowed for efficient assessment of spatial heterogeneity of fungal community structures within the field plot of 400 m\(^2\) and to define its representative fungal community profile. If the averages of T-RFLP profiles derived from individual samples were calculated, averages of five were sufficient to yield a representative profile of the community structure. If pooling was performed at the DNA level, it was necessary to combine at least seven DNA extracts to obtain a representative profile. As all averages of five T-RFLP profiles or mixes of seven DNA extracts consistently clustered at the same branch (Table 2), differences in T-RF composition among the ten samples were considered quantitative and stochastic. However, in cases of non-stochastic distributions, e.g. due to gradient forming influences, or restriction of T-RFs to particular samples, the number of samples in a pool might not be reduced without loss of representativity. In such cases, a representative sample pool may not be obtained and analyses of changes in community structures may be difficult. Even though not performed in this study, we assume that the same strategy will be applicable to soil mixes of different complexities in order to determine the number of soil samples to be pooled for representative genetic profiling. However, this would be laborious and possibly not feasibly performed for many studies. The use of ‘T-RFLP-averages’ and ‘DNA-mixes’ as performed in the present study represents a feasible approach in order to assess representativity of soil fungal community profiles.

4.5. Conclusions

In this study we established a three-step approach to assess representativity of soil fungal community profiles. Optimal template DNA quantities for robust profiling, theoretical reference profiles, and experimental sample pools were determined for assessing representativity. For many studies determination of theoretical reference profiles based on ‘T-RFLP-averages’, may be sufficient to feasibly assess representativity. If required also ‘DNA-mixes’ or even ‘soil-mixes’ could be used. Our strategy will be advantageous to reliably separate stochastic within-site heterogeneity of fungal T-RF genotypes from between-site heterogeneity and will allow to improve resolution in effect studies.

Acknowledgements

We are grateful to Martin Hartmann, Roland Kölliker, Johannes Sarnthein and Fritz Schwarzenbach for their valuable comments on the manuscript. This research project was supported by the Swiss Federal Office for the Environment within its research program on “Biosafety in non-human genetic engineering”.

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