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Objective criteria to assess representativity of soil fungal community profiles

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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 m^2 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.

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 cm^3, as estimated from several studies with

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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 mixes of soil samples ('soil-mixes'), mixes of soil DNA extracts ('DNA-mixes'), or calculated averages of T-RFLP data ('T-RFLP-averages'). This approach aimed at the establishment of a strategy for efficient determination of the optimal number of soil samples to be pooled in order to obtain representative genetic soil fungal community profiles.

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 m^2 (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.

2.3. 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 (Sandaa et al., 1998) 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).

2.4. 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′-GTAGTCATATGCTTGTCTC-3′) and reverse primer FR1 (5′-AICCATTCAATCGGTANT-3′; I 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