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ORIGINAL PAPER

# Rationalizing molecular analysis of field-collected roots for assessing diversity of arbuscular mycorrhizal fungi: to pool, or not to pool, that is the question

**C. Renker · K. Weißhuhn · H. Kellner · F. Buscot**

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**Abstract** For rationalizing molecular analysis of field-collected roots in diversity studies on arbuscular mycorrhiza, we compared three different approaches. After DNA extraction from 50 root samples of *Plantago lanceolata* grown on monoculture plots at a former arable field site, (1) DNAs were amplified separately by nested PCR and each amplicon was cloned separately; (2) DNAs were amplified separately by nested PCR, 1 μl of each amplicon was pooled, and a single cloning was made from the resulting amplicons mix; and (3) DNAs were pooled and the single amplicon derived from the nested PCR was cloned. Based on these three different methods, 109 nuclear ribosomal internal transcribed spacer sequences were obtained. Methods 1 and 2 enabled the detection of almost similar levels of arbuscular mycorrhizal fungal diversity. However, method 1 was expensive and time-consuming as much more cloning had to be done. Method 3 was completely biased by preferential amplification of nontarget organisms, which were only detected in low frequencies by the other methods.

**Keywords** Arbuscular mycorrhizal fungi · DNA extraction · PCR bias · Preferential amplification

C. Renker · K. Weißhuhn · H. Kellner · F. Buscot
Terrestrial Ecology, Institute of Biology I, University of Leipzig,
Johannisallee 21,
D-04103 Leipzig, Germany

*Present address:*
C. Renker (✉) · H. Kellner · F. Buscot
Department of Soil Ecology, UFZ Centre for Environmental Research Leipzig-Halle Ltd.,
Theodor-Lieser-Str. 4,
D-06120 Halle, Germany
e-mail: carsten.renker@ufz.de

*Present address:*
K. Weißhuhn
Department of Community Ecology, UFZ Centre for Environmental Research Leipzig-Halle Ltd.,
Theodor-Lieser-Str. 4,
D-06120 Halle, Germany

## Introduction

Development of molecular technologies to explore microbial diversity in soils has been a challenge in the last two decades (Torsvik and Øvreås 2002). However, these methods present inherent drawbacks. DNA extracts from complex media such as soils or plant roots contain phenolic compounds that may hamper PCR amplification (Koide 2005). Artifacts might be introduced by polymerase errors, mutational hot spots, and cloning of heteroduplexes or chimeras (Patel et al. 1996; Speksnijder et al. 2001). Microbial community analysis is also biased by the differential amplification (or nonamplification) of target organisms, which can distort the obtained biodiversity picture (Reysenbach et al. 1992; Schmalenberger et al. 2001).

How to process mycorrhizal field samples to address ecological issues is still a controversially debated question, bearing all of the above-mentioned flaws in mind. For arbuscular mycorrhizal fungi (AMF), recent diversity studies analyzed DNA extracts from roots (Vandenkoornhuyse et al. 2002, 2003; Renker et al. 2005). Fulfilling the requirements of ecological samplings implies analyzing a high number of extracts. It is tempting to pool the extracts to reduce the costs and analysis time, but this practice bears the risk of introducing artifacts and bias. Handling each root extract

separately increases time and finance budgets without totally eliminating the flaws of molecular techniques. In this article, we try to find a rational compromise between both strategies.

## Materials and methods

### Field site and soil sampling

The investigated *Plantago lanceolata* monoculture, a plot of 20×20 m established in May 2002, is part of the Jena Experiment (http://www.the-jena-experiment.de), a long-term experiment to study interactions between biodiversity and ecosystem processes. It is located on the floodplain of the Saale river, 130 m above sea level in Jena (Thuringia, Germany; 11°37′27″ E/50°57′04″ N). Mean annual air temperature is 9.3°C (1961–1990) and mean annual precipitation amounts to 587 mm. The soil is an Eutric Fluvisol developed from up to 2-m-thick loamy fluvial sediments that are almost free of stones (for further details, see Roscher et al. 2004).

On August 7, 2004, five soil cores from the first 20 cm of top soil were taken randomly from the plot with a soil column cylinder auger (Eijkelkamp, Giesbeek, The Netherlands) and transported on ice to the laboratory, where they were stored at −20°C for 2 days prior to further analysis.

### DNA extraction

Ten *P. lanceolata* roots from each of the five soil cores were isolated and rinsed in tap water, resulting in 50 roots (Fig. 1). DNA was extracted from single 2-cm-long root fragments of all 50 root samples; fragments were chopped with a razor blade and transferred into a 0.5-ml Eppendorf tube. After the addition of NaOH (20 µl of 0.25 M), the roots were incubated at 90°C for 10 min. Afterwards, Tris–HCl (10 µl 0.5 M pH 7.3) and HCl (20 µl 0.25 N) were added and the extraction mix was incubated for another 10 min. The supernatant was separated from root fragments by centrifugation, diluted 1:100 in Tris–EDTA buffer, and used as PCR template. The 50 DNA root extracts were processed according to three distinct methods.

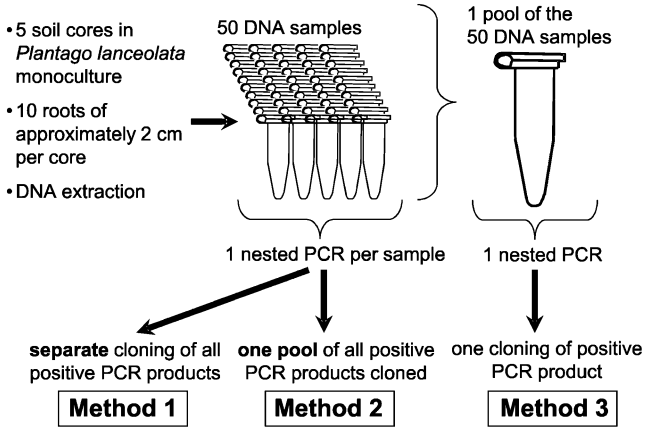


**Fig. 1** Visualized description of three different methods used for PCR amplification and cloning of AMF rDNA from field-collected root samples

### PCR conditions

In methods 1 and 2, each of the 50 DNA root extracts was processed separately in PCR (Fig. 1). In method 3, 1 µl of each DNA extract was pooled in a new tube and processed as bulk sample in PCR (Fig. 1).

Amplification of the internal transcribed spacer (ITS) region by PCR was performed on a Hybaid OmniGene TR3 CM220 Thermo Cycler (MWG-Biotech, Ebersberg, Germany) in a total volume of 50 µl containing 2 U *Taq* DNA polymerase (Promega, Heidelberg, Germany), 5 µl of 10× *Taq* polymerase reaction buffer (Promega), 4 µl 25 mM $MgCl_2$, 10 nmol of each deoxyribonucleotide triphosphate (MBI-Fermentas, St. Leon-Rot, Germany), 50 pmol of each of the two primers, and 100–500 ng of the genomic DNA. The reactions were performed as hot start PCR with a 10-min initial denaturation at 94°C before adding the *Taq* polymerase at 80°C. The PCR program comprised 32 cycles (40 s at 94°C, 30 s at 54°C, and 40 s at 72°C). A final elongation of 10 min at 72°C followed the last cycle.

In the first reaction of nested PCR, the primer pair SSU-Glom1/LSU-Glom1 (Renker et al. 2003) was used while the second step of the PCR with 1 µl of PCR template from the previous PCR was performed with the primers ITS5/ITS4 (White et al. 1990).

### Cloning, sequencing, and sequence analyses

PCR yielded 18 amplicons (36%) altogether from the 50 root DNA extracts. This is in a range detected in previous studies (cf. Stukenbrock and Rosendahl 2005a: 24.7–37.4%). In method 1, each amplicon was cloned separately. In method 2, 1 µl of each amplicon was pooled in a new tube and cloning was based on this bulk sample. In method 3, the single amplicon derived from the PCR done with the bulk sample of all 50 DNA extracts was cloned (Fig. 1).

PCR products were cloned into the pCR4-Topo Vector following the manufacturer's protocol of the TOPO TA Cloning Kit (Invitrogen Life Technologies, Karlsruhe, Germany) and transformed into TOP10 chemically competent *Escherichia coli*. The plasmid DNA, containing the PCR product, was extracted from 1.5 ml of *E. coli* TOP10 chemically competent cells using the Perfectprep plasmid mini kit (Eppendorf, Hamburg, Germany). Sequencing was done on an ABI PRISM 3100 Genetic Analyser (Applied

Biosystems, Warrington, United Kingdom) using the Big Dye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) following the manufacturer's instructions.

DNA sequences of the full ITS region were submitted to the European Molecular Biology Laboratory database under the accession numbers given in Fig. 2. 5.8S-sequences, all gathered in this study, were aligned manually with BioEdit version 5.0.9 (Hall 1999), resulting in an alignment including 156 putatively homologous sites.

A phylogenetic tree (Fig. 2) was performed using MrBayes 3.0b4 (Huelsenbeck and Ronquist 2001), a program for Bayesian inference of phylogeny. A Markov chain Monte Carlo run with four simultaneous chains and 5,000,000 generations was performed. As substitution model, the general time reversible model with invariable

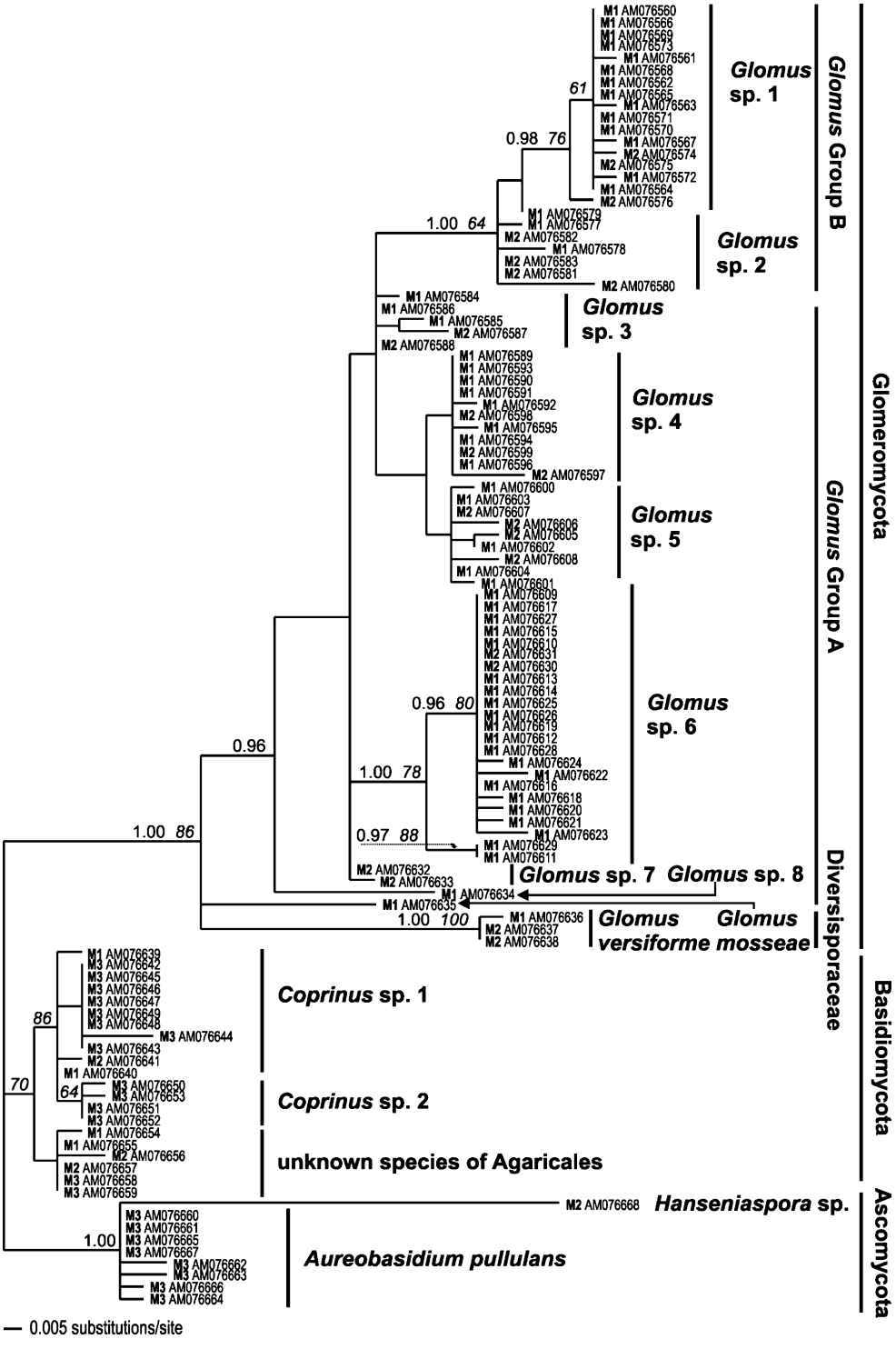


**Fig. 2** Consensus tree derived from Bayesian phylogenetic inference analysis of 109 5.8S nuclear ribosomal sequences. Regular numbers above the branches are a posteriori probabilities derived from 9,000 Markov chain Monte Carlo sampled trees giving an estimate that the respective groups are monophyletic. *Italic numbers* are neighbor-joining bootstrap values from 1,000 replications using Kimura two-parameter genetic distances

sites and gamma shape distributed substitution rates (GTR+I+G) was chosen. Every 500th generation, the tree with the best likelihood score was saved, resulting in 10,000 trees. The first 1,000 trees without reaching a stable likelihood score were deleted. The remaining trees were condensed in a majority rule consensus tree using PAUP* 4.0b10 (Swofford 2003). Branch supports were assigned as posterior probabilities on the consensus trees. Only high support values above 0.94 are shown. Following Larget and Simon (1999), branch supports less then 0.95 using Bayesian posterior probabilities are not significant. Additionally, a neighbor-joining analysis was conducted based on the Kimura-2-Parameter model. The confidence of branching was assessed using 1,000 bootstrap resamplings (Fig. 2). Species separation found in the 5.8S analysis was also confirmed regarding alignments of whole ITS regions (data not shown). A full ITS alignment of all species was impossible due to the high variability within the ITS1 and ITS2 spacer regions.

### Statistical analysis

Diversity of the clones was analyzed by rarefaction analysis (Simberloff 1978; Fig. 3). For methods 1 and 2, rarefaction curves were produced with the analytical approximation algorithm (Hurlbert 1971). Calculations used the Analytic Rarefaction freeware program (http://www.uga.edu/strata/software/Software.html). Based on the assumption that rarefaction curves generally show an exponential rise to a maximum, the results were fitted for the formula $y=a(1-e^{-bx})$.

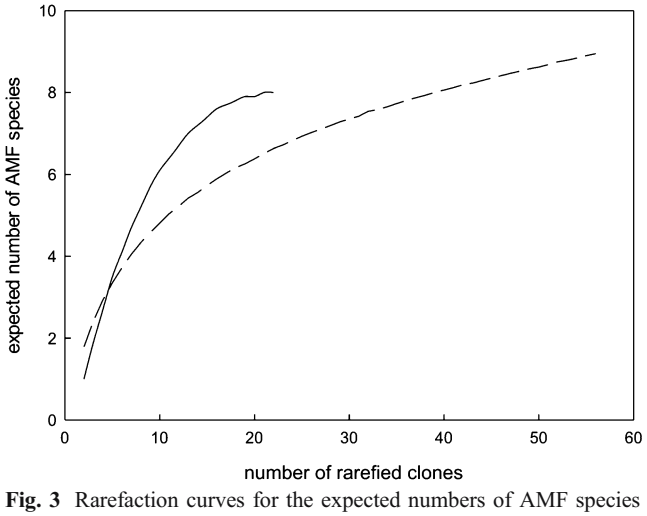


**Fig. 3** Rarefaction curves for the expected numbers of AMF species (*y-axis*) against the number of sequenced clones (*x-axis*). The *dashed line* refers to method 1 (separate DNA extraction from 50 root fragments, PCR amplification, separate cloning of all positive PCR products), the *solid line* to method 2 (separate DNA extraction from 50 root fragments, PCR amplification, pooling of all amplicons, one cloning from this pool)

## Results

Altogether, 109 ITS sequences were obtained from the 50 DNA extracts of *P. lanceolata* roots, of which 79 (72%) corresponded to Glomeromycota and 30 (28%) to other fungi (Table 1). Glomeromycota sequences could be attributed to 10 different species, representing members of all *Glomus* groups (Schwarzott et al. 2001; Fig. 2). Non-Glomeromycota sequences belonged to three species of Basidiomycota and to two species of Ascomycota (Fig. 2).

Following method 1, we did 18 cloning reactions of all PCR amplicons derived from the 50 root DNA extracts after separate nested PCR of all 50 DNA samples (Fig. 1). An average of three *E. coli* colonies per cloning reaction was checked, which yielded in 57 Glomeromycota sequences (93%) representing nine AMF species against four non-Glomeromycota sequences (7%) (Table 1). Method 2, for which the 18 positive amplicons were pooled prior to a unique cloning, resulted in 22 Glomeromycota sequences (85%) representing eight AMF species against four non-Glomeromycota sequences (15%) (Table 1). One of these AMFs (*Glomus* sp. 7) had not been detected with method 1, while two AMFs found with method 1 (*Glomus* sp. 8 and *Glomus mosseae*) were not detected by method 2. Method 3 exclusively detected four species of non-Glomeromycota (Fig. 2), based on 22 sequences.

Diversity rarefaction curves displayed a fit of $y=8.5474(1-e^{-0.0757x})$ ($R^2=0.9667$) for method 1 and $y=8.7307(1-e^{-0.1304x})$ ($R^2=0.9986$) for method 2 (Fig. 3). Both formulas indicate that methods 1 and 2 enabled approaching a diversity plateau lying by approximately nine species.

## Discussion

Using method 1 we checked three clones of each cloning reaction because previous studies have indicated that different species of Glomeromycota might coexist even in small root fragments (Rosendahl and Stukenbrock 2004; Börstler et al. 2006). In our case, mycorrhizal identity in most clones of one cloning was the same, which is in accordance with the observations of Rosendahl and Stukenbrock (2004) for roots colonized by *Glomus intraradices*. However, "singletons" (i.e., *Glomus* sp. 8, *G. mosseae* and *Glomus versiforme*) especially would not have been detected, as these fungi co-occurred in root fragments also inhabited by other members of the Glomeromycota. The overall bad success in sampling diversity by checking three clones of each cloning reaction can also be seen in the shallower slope of this sampling procedure in the rarefaction analyses (Fig. 3), as most clones of the overall 18 clonings represent the same species.

**Table 1** Comparison of three different methods used for the amplification of AMF from field-collected *Plantago lanceolata* roots

| | | Method 1[a] | Method 2[b] | Method 3[c] | Total numbers |
|---|---|---|---|---|---|
| Number of sequenced clones | | 61 | 26 | 22 | 109 |
| Species number of all soil fungi | | 11 | 11 | 4 | 15 |
| Species number of Glomeromycota | | 9 | 8 | 0 | 10 |
| Number of clones from Glomeromycota | *Glomus* sp. 1 (602–606 bp) | 14 [25%] | 3 [14%] | 0 [0%] | 17 [22%] |
| | *Glomus* sp. 2 (611–614 bp) | 3 [5%] | 4 [9%] | 0 [0%] | 7 [9%] |
| | *Glomus* sp. 3 (567–568 bp) | 3 [5%] | 2 [9%] | 0 [0%] | 5 [6%] |
| | *Glomus* sp. 4 (555–563 bp) | 8 [14%] | 3 [14%] | 0 [0%] | 11 [14%] |
| | *Glomus* sp. 5 (622–629 bp) | 5 [9%] | 4 [18%] | 0 [0%] | 9 [11%] |
| | *Glomus* sp. 6 (567–571 bp) | 21 [37%] | 2 [9%] | 0 [0%] | 23 [29%] |
| | *Glomus* sp. 7 (565–566 bp) | 0 [0%] | 2 [9%] | 0 [0%] | 2 [3%] |
| | *Glomus* sp. 8 (575 bp) | 1 [2%] | 0 [0%] | 0 [0%] | 1 [1%] |
| | *Glomus mosseae* (593 bp) | 1 [2%] | 0 [0%] | 0 [0%] | 1 [1%] |
| | *Glomus versiforme* (568–569 bp) | 1 [2%] | 2 [9%] | 0 [0%] | 3 [4%] |
| | ∑ clones | 57 [93%] | 22 [85%] | 0 [0%] | 79 [72%] |
| Number of clones from non-Glomeromycota | | 4 [7%] | 4 [15%] | 22 [100%] | 30 [28%] |

Following each member of detected Glomeromycota, the respective size range of its PCR products is given. The relative abundance of the detected Glomeromycota is based on the number of clones containing Glomeromycota (i.e., 57 and 22 clones), whereas relative abundances in the last two rows of the table do refer to the absolute number of investigated clones with each method (i.e., 61, 26, and 22 clones).

[a] Separate DNA extraction from 50 root fragments, PCR amplification, separate cloning of all positive PCR products

[b] Separate DNA extraction from 50 root fragments, PCR amplification, pooling of all amplicons, one cloning from this pool

[c] Separate DNA extraction from 50 root fragments, pooling of all 50 DNA extracts, PCR amplification, one cloning of the PCR product

Comparing the three methods to analyze AMF biodiversity on plant roots showed that method 1 revealed the largest Glomeromycota diversity (i.e., nine species). However, regarding the number of sequences (i.e., 61) and the cost for 18 clonings (approximately $18.35 per cloning based on a price of $734 for the TOPO TA Cloning Kit with 40 reactions), method 2 was more rational. Pooling all amplicons of the 50 nested PCRs for one single cloning reduced the costs to $18.35, whereby an almost similar diversity level (eight species) was reached after sequencing only 26 clones (Fig. 3). This result is in accordance with studies on bacteria, in which pooling a number of PCR products prior to cloning enabled the detection of diversity levels of 16S rRNA genes similar to those in the template DNA (e.g., Chandler et al. 1997; Reed et al. 2002; Webster et al. 2003). One should keep in mind that the absolute number of clones detected in this survey (Table 1) cannot be used as a quantitative measure of biodiversity. Therefore, data are compared based on qualitative differences. The only method that could allow a quantitative measure of mycorrhizal presence or absence is method 1. However, checking three clones of each cloning reaction in method 1 has to be interpreted as pseudoreplication in quantitative analyses. Cloning a pool of PCR products in method 2 might prefer AMF species with shorter ITS fragments compared to those with larger ITS fragments and, therefore, distorts the original ratio (cf. Suzuki et al. 1998). However, in our case, we did not observe this phenomenon, maybe due to the similar size ranges of most PCR products ranging from 555–629 bp (Table 1). In contrast, Glomeromycota with larger PCR products (i.e., *Glomus* sp. 5 and *Glomus* sp. 2) were detected in larger proportions with method 2. The size range of PCR products, given for each species in Table 1, indicates the clonal diversity, which is obvious in Glomeromycota (Stukenbrock and Rosendahl 2005b). Noteworthy, however, is the fact that neither method 1 nor method 2 revealed all 10 species detected in the plot.

The shortcoming of being unable to detect our target organisms (Glomeromycota) in large mixes of root DNA extracts (method 3) reflects difficulties encountered in biodiversity assessments of bacteria (Reysenbach et al. 1992; Chandler et al. 1997) or in studies on allele

frequencies of single-nucleotide polymorphisms (Yang et al. 2005). DNA pooling prior to PCR has been recommended in domains such as medicinal studies (Barratt et al. 2002; Sham et al. 2002). However, this procedure would ideally necessitate balancing the DNA concentration in the mixed extracts and using primers strictly specific for the target organisms. Both conditions are impossible to fulfill when analyzing environmental samples as in most molecular biodiversity studies in soils (e.g., Douhan et al. 2005; Renker et al. 2003, 2004). Method 3 showed that, if there is an unfavorable DNA ratio towards nontarget fungi, the primer set might even amplify the DNA of fungi that have one or two mismatches in the primer-binding region. Additionally, the DNA amount of nontarget fungi might be large enough so that they can be amplified by a single PCR with the second primer pair of the nested PCR.

In this study, we show that pooling amplicons after separated PCR on extracts is a practicable way to save time and money. As shown in our survey, pooling DNA extracts from soils or roots bears the risk of estimating diversity levels including nontarget organisms if the assessment is not based on systematic sequencing, like in this study, but on fingerprint techniques such as terminal-RFLP and denaturing gradient gel electrophoresis. Nevertheless, to address ecological questions in diverse grassland habitats, studies have to be based on a sophisticated root sampling design where DNA from a lot of roots from different plant species have to be extracted (e.g., Stukenbrock and Rosendahl 2005a,b) before these extracts can be amplified. Reaching this point, it will be possible to succeed with method 2 to save time in the subsequent sample processing.

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