

Minireview

Diversity and ecology of soil fungal communities: increased understanding through the application of molecular techniques

Ian C. Anderson^{1*} and John W. G. Cairney²

¹The Macaulay Institute, Craigiebuckler, Aberdeen AB15 8QH, Scotland, UK.

²Mycorrhiza Research Group, Centre for Horticulture and Plant Sciences, Parramatta Campus, University of Western Sydney, Locked Bag 1797, Penrith South DC, NSW 1797, Australia.

Summary

Fungi fulfil a range of important ecological functions, yet current understanding of fungal biodiversity in soil is limited. Direct DNA extraction from soil, coupled with polymerase chain reaction amplification and community profiling techniques, has proved successful in investigations of bacterial ecology and shows great promise for elucidating the taxonomic and functional characteristics of soil fungal communities. These community profiling techniques include denaturing gradient gel electrophoresis (DGGE), temperature gradient gel electrophoresis (TGGE), single-strand conformation polymorphism (SSCP), terminal restriction fragment length polymorphism (T-RFLP), amplified rDNA restriction analysis (ARDRA), amplified ribosomal intergenic spacer analysis (ARISA) and cloning, and are generally coupled with DNA sequencing. The techniques and their potential limitations are discussed, along with recent advances that have been made possible through their application in soil fungal ecology. It is unlikely that a single approach will be universally applicable for assessing fungal diversity in all soils or circumstances. However, judicious selection of the methodology, keeping the experimental aims in mind, and the exploitation of emerging technologies will undoubtedly increase our understanding of soil fungal communities in the future.

Received 2 April, 2004; revised 25 May, 2004; accepted 25 May, 2004. *For correspondence. E-mail i.anderson@macaulay.ac.uk; Tel. (+44) 1224 498200; Fax (+44) 1224 498207.

Introduction

'True' fungi are ubiquitous in the environment and fulfil a range of important ecological functions, particularly those associated with nutrient and carbon cycling processes in soil (Christensen, 1989). Despite this, our understanding of soil fungal community diversity and functioning remains poor relative to that of soil bacterial communities, and it is not uncommon for articles that purport to review aspects of 'soil microbial ecology' to consider only bacteria (e.g. Hattori *et al.*, 1997; Ogram, 2000; Kent and Triplett, 2002). A major contributing factor has been the tendency of mycologists to rely upon culture-based methods in ecological investigations of soil fungi. The limitations of these approaches have frequently been highlighted (e.g. Zak and Visser, 1996; Bridge and Spooner, 2001), and the data provide only a selective, and invariably biased, window on diversity. A critical factor in advancing bacterial ecology has been the widespread adoption of culture-independent methods such as analyses of DNA and/or RNA extracted directly from soil (for review, see Ranjard *et al.*, 2000). Exploitation of the variation within 16S rRNA gene sequences of different bacterial species, in combination with the application of molecular techniques, has driven a bacterial ecology revolution over the last decade or so, significantly increasing our understanding of the diversity and functioning of bacteria in a range of environments. While molecular methods have been used in many investigations of soil fungi, for the most part, these have been used to aid the identification of isolated fungi or to investigate fungi in discrete units such as ectomycorrhizal root tips or Glomalean spores (reviewed by Horton and Bruns, 2001; Clapp *et al.*, 2002). As such, they provide little information regarding the distribution, diversity and activities of fungal mycelia in soil. Similar limitations are encountered in the use of phospholipid fatty acids (PLFAs) as, although they have been widely used (for review, see Zelles, 1999), there are a limited number of fungal-specific markers, and they only provide an estimate of total fungal biomass in soil.

In recent years, direct nucleic acid extraction approaches have become more widely applied in investi-

gations of soil fungal ecology and are beginning to provide significant advances in our understanding of genetic diversity in soil fungal communities. Direct nucleic acid extraction coupled with polymerase chain reaction (PCR) amplification has further facilitated the development of techniques that are providing novel insights into the genetic–functional diversity nexus in soil fungal communities. In this review, we discuss the methods for the analysis of soil fungal diversity and functioning by direct nucleic acid extraction, consider the limitations associated with the methodologies and data analysis, and highlight some of the key findings from recent investigations.

Direct soil DNA extraction and PCR amplification

Several approaches have been used to extract fungal genomic DNA from soil, including commercially available kits that have been designed for the extraction of microbial DNA from soil (e.g. Dickie *et al.*, 2002; Guidot *et al.*, 2003; Ranjard *et al.*, 2003) and techniques that have been optimized for the extraction of nucleic acids from specific soil types (e.g. Yeates and Gillings, 1998; Anderson *et al.*, 2003a,b; Ranjard *et al.*, 2003). The quality and purity of the extracted nucleic acid pool is vital for successful PCR amplification of target genomic DNA/RNA. The presence of humic acids or humic substances that are co-extracted with nucleic acids from soil can inhibit DNA-modifying enzymes such as *Taq* DNA polymerase (e.g. Tebbe and Vahjen, 1993). To attempt to circumvent this problem, and to avoid extensive DNA purification procedures that often result in the loss of template, PCR additives including BSA and other commercially available substances are commonly used in the PCR amplification reactions of soil DNA. Estimates of soil microbial diversity can be markedly influenced by the extraction technique used, with different protocols providing conflicting views on diversity (Martin-Laurent *et al.*, 2001). This highlights a major difficulty in comparing data generated using different DNA extraction protocols. A further confounding effect is the size of the soil sample used for DNA extraction. It has been shown, for example, that although DNA extracted from soil with a sample size of 0.125–4 g had no effect on the assessment of bacterial diversity from a single homogenized soil sample, changes in the fungal community structure in the same soil sample were observed when sample sizes <1 g were used for DNA extraction (Ranjard *et al.*, 2003). There is evidence that certain template–primer combinations used in bacterial ecology possess a bias resulting in 1:1 mixtures of genes in final PCR products, regardless of the initial starting concentrations of each individual template (Suzuki and Giovannoni, 1996; Polz and Cavanaugh, 1998). This bias has been shown to be reduced by performing fewer PCR cycles, using high template concentrations and by mixing replicate reactions (Polz and

Cavanaugh, 1998). At present, it is unclear how the choice of PCR primers and the relative abundance of certain fungal templates in a community DNA sample influences PCR amplification, and whether the most abundant fungal templates are preferentially amplified at the expense of templates at lower concentrations (Dickie *et al.*, 2002). In addition, the multicopy nature of the genomic regions targeted in fungal ecology assumes that the number of copies in different fungal species is similar. The number of copies of the rRNA operon is known to vary between different fungal species (Hibbett, 1992); however, the exact number of copies contained within the genome of any single species remains unknown for most species at present. This variation between species complicates the quantification of different fungal species in a mixed DNA pool if the rRNA operon is the target region.

A further complicating factor is the potential presence of both spores and mycelium in a single soil sample, which are likely to be co-extracted during the DNA extraction process (Dickie *et al.*, 2002; Guidot *et al.*, 2003; Landeweert *et al.*, 2004). Guidot and colleagues (2003) investigated the spatial distribution and temporal persistence of extraradical mycelia of a basidiomycete fungus, *Hebeloma cylindrosporum*, and found that the presence of 100 basidiospores in 0.5 g of soil could be detected for this species in community DNA. Other authors investigating soil mycelial communities have attempted to avoid the extraction and amplification of basidiospores by collecting soil samples either before (Landeweert *et al.*, 2004) or after (Dickie *et al.*, 2002) peak sporocarp production for most fungal species at the field sites. In addition, as the majority of spores are likely to be found in the upper layers of soil, they are less likely to be of concern in studies that sample deeper soil horizons (Dickie *et al.*, 2002; Landeweert *et al.*, 2004). Nonetheless, the longevity of fungal spores in soil remains unclear, and this must be borne in mind when considering soil mycelial communities.

Fungal PCR primers

Variation within 16S rRNA gene sequences of different bacterial species has significantly increased our understanding of the diversity and ecology of soil bacterial communities. In contrast to bacteria, taxonomic identification of fungi based on sequences of the eukaryotic ribosomal small subunit, the 18S rRNA, is more problematic, with identification commonly limited to genus or family level. This is primarily due to the relative lack of variation within 18S rRNA genes between closely related fungal species as a result of the relatively short period of evolution of the kingdom fungi compared with bacteria (Hugenholtz and Pace, 1996). This is compounded by the lack of an exhaustive database of fungal reference sequences. However, while this is particularly true for most Ascomycota

and Basidiomycota, early radiation of the Glomeromycota within the kingdom fungi (see Schübler *et al.*, 2001) has resulted in greater 18S rRNA sequence variation between species belonging to this phylum. 18S rDNA primers are thus used more commonly in the context of symbiotic arbuscular mycorrhizal (AM) fungi as there is sufficient variation in 18S rRNA gene sequences of different species to allow discrimination between isolates to species and sometimes below species level (Vandenkoornhuysen and Leyval, 1998).

One of the major limitations in investigating fungal diversity in soil, in which the extracted DNA pool constitutes DNA from a diverse range of eukaryotic and prokaryotic organisms, has been the suitability of available PCR primers. The challenge has been to design PCR primers that amplify as broad a taxonomic range of fungi as possible, but at the same time to prevent co-amplification of closely related eukaryotic DNA. The primary target for the development of PCR primers for assessing fungal diversity in soil has been the rRNA gene cluster and, despite its limitations, the 18S rRNA gene has been the most widely used, exploiting both the conserved and the variable regions contained within it (e.g. White *et al.*, 1990; Smit *et al.*, 1999; Borneman and Hartin, 2000; Vainio and Hantula, 2000). In addition, the internal transcribed spacer (ITS) region located between the 18S rRNA and 28S rRNA genes, and incorporating the 5.8S rRNA gene, has also been targeted (e.g. White *et al.*, 1990; Gardes and Bruns, 1993; Larena *et al.*, 1999) (see Table 1). Non-

coding rDNA spacer regions, such as the ITS, benefit from a fast rate of evolution, resulting in greater sequence variation between closely related species compared with the more conserved coding regions of the rRNA gene cluster. Thus, fungal ITS sequences generally provide greater taxonomic resolution than sequences generated from coding regions (e.g. Lord *et al.*, 2002; Anderson *et al.*, 2003b).

White and colleagues (1990) designed the first PCR primers for amplification of fungal 18S rDNA and ITS regions from extracted DNA. Although these primers were designed with limited reference sequence information, they proved to be very powerful tools and are still widely used today. The primers were, however, designed to amplify as broad a taxonomic range as possible and, as such, some of them also amplify plant DNA from mixed plant–fungal DNA samples (Gardes and Bruns, 1993) and other eukaryotic DNA (White *et al.*, 1990). This lack of specificity for fungal template limits their usefulness in mixed DNA samples, particularly where the ratio of fungal DNA to non-fungal DNA is low. This led Gardes and Bruns (1993) to design the primers ITS1F and ITS4B for the specific amplification of basidiomycete DNA from mixed DNA samples extracted from colonized ectomycorrhizal (ECM) plant root tips. These primers have subsequently been widely used in ECM research and have revolutionized our understanding of ECM fungal communities (see Horton and Bruns, 2001). Furthermore, the ITS1F primer has been used in conjunction with ITS4 (White *et al.*,

Table 1. Summary of PCR primers that have been used for assessing fungal diversity in soil DNA extracts.^a

PCR primer	Primer sequence (5'–3')	Genomic target	Primer reference
NS1	GTAGTCATATGCTTGTCTC	18S rDNA	White <i>et al.</i> (1990)
NS2	GGCTGCTGGCACCAGACTTGC	18S rDNA	
NS3	GCAAGTCTGGTGCCAGCAGCC	18S rDNA	
NS8	TCCGCAGGTTACCTACGGA	18S rDNA	
ITS1	TCCGTAGGTGAACCTGCGG	ITS	
ITS2	GCTGCGTTCTTCATCGATGC	ITS	
ITS4	TCCTCCGCTTATTGATATGC	ITS	
ITS1F	CTTGGTCATTTAGAGGAAGTAA	ITS	Gardes and Bruns (1993)
ITS4B	CAGGAGACTTGACACGGTCCAG	ITS	
2234C	GTTTCCGTAGGTGAACCTGC	ITS	Sequerra <i>et al.</i> (1997)
3126T	ATATGCTTAAGTTCAGCGGGT	ITS	
ITS4A	CGCCGTTACTGGGGCAATCCCTG	ITS	Larena <i>et al.</i> (1999)
EF3	TCCTCTAAATGACCAAGTTTG	18S rDNA	Smit <i>et al.</i> (1999)
EF4	GGAAGGGRTGTATTATTAG	18S rDNA	
Fung5	GTAAAAGTCCTGGTTCCCC	18S rDNA	
nu-SSU-0817	TTAGCATGGAATAATRRRAATAGGA	18S rDNA	Borneman and Hartin (2000)
nu-SSU-1196	TCTGGACCTGGTGAGTTTCC	18S rDNA	
nu-SSU-1536	ATTGCAATGCYCTATCCCCA	18S rDNA	
FR1	AICCATTCAATCGGTAIT	18S rDNA	Vainio and Hantula (2000)
FF390	CGATAACGAACGAGACCT	18S rDNA	
PN3	CCGTTGGTGAACCAGCGGAGGGATC	ITS	Viaud <i>et al.</i> (2000)
PN34	TTGCCGCTTCACTCGCCGTT	ITS	
Fun18S1	CCATGCATGTCTAAGTWTAA	18S rDNA	Lord <i>et al.</i> (2002)
Fun18S2	GCTGGCACCAGACTTGCCCTCC	18S rDNA	

a. Caution should be taken when selecting PCR primers for assessing fungal diversity in soil as several of these primers have been shown to amplify other eukaryotic DNA in certain circumstances. Primers that lack specificity for fungal template can sometimes be used in conjunction with a primer that has higher specificity for fungal DNA, thus eliminating amplification of DNA from non-fungal sources.

1990) specifically to amplify fungal templates from mixed community DNA samples (e.g. Chen and Cairney, 2002; Dickie *et al.*, 2002; Lord *et al.*, 2002; Anderson *et al.*, 2003a,b), and with the ITS reverse primer ITS4A, which was designed to have increased specificity for ascomycete DNA (Larena *et al.*, 1999).

PCR primer bias and specificity

The limitations of the original 18S rDNA primers (White *et al.*, 1990) restricted their application in the molecular analysis of soil fungal communities and resulted in the design and development of several new 18S rDNA primer pairs (see Table 1). Although several of these primer pairs have been developed to amplify a broad taxonomic range of fungi (White *et al.*, 1990; Smit *et al.*, 1999; Borneman and Hartin, 2000; Vainio and Hantula, 2000; Vandenkoornhuysen *et al.*, 2002a), some have been designed preferentially to amplify specific groups of fungi such as AM fungi (e.g. Helgason *et al.*, 1998). Two key criteria that require consideration when designing primers for the analysis of soil communities are: (i) the specificity of primers to the target template DNA; and (ii) whether the primers preferentially amplify a certain template in a mixed community DNA sample, thus biasing the view of diversity obtained. The high level of sequence similarity between 18S rDNA of fungi and some closely related eukaryotes has meant that the design of new primers has not been straightforward, as increasing the specificity of primers for fungal template invariably increases the potential bias associated with the primers and vice versa (Smit *et al.*, 1999). The problem of specificity and bias of fungal PCR primers has been highlighted several times in the literature (Smit *et al.*, 1999; Borneman and Hartin, 2000; Vainio and Hantula, 2000; Anderson *et al.*, 2003b).

There are conflicting reports in the literature regarding the specificity of fungal primers for the analysis of soil fungal communities. For example, although the 18S rDNA PCR primer pairs EF4/EF3 and EF4/fung5 only amplified fungal template from DNA extracted from a wheat rhizosphere soil (Smit *et al.*, 1999), they were also shown to amplify some non-fungal template from cultured organisms and avocado grove soil (Borneman and Hartin, 2000). Similarly, while the 18S rDNA PCR primer pair nu-SSU-0817 and nu-SSU-1196 only amplified fungal template from DNA extracted from an avocado grove soil (Borneman and Hartin, 2000), they were also shown to amplify invertebrate 18S rDNA sequences from genomic DNA extracted from an agricultural soil (Anderson *et al.*, 2003b). These conflicting reports may indicate that the specificity of some 18S rDNA PCR primers can vary depending on the origin of the substrate being analysed and the diversity of eukaryotic DNA contained within the extracted DNA pool. Nonethe-

less, specificity of PCR primers for the analysis of soil fungal communities in mixed DNA samples is critical, particularly when they are used in conjunction with community profiling techniques where each band in a community profile is assumed to be of fungal origin (e.g. Edel-Hermann *et al.*, 2004).

Preferential amplification of particular taxonomic groups of fungi from mixed community DNA samples may lead to a biased assessment of diversity rather than a true reflection of the taxonomic diversity contained within a sample. The primers EF4/EF3 appeared to be slightly biased towards amplification of Basidiomycota and Zygomycota based on comparison of the primer sequences with fungal 18S rDNA sequences in the Ribosomal Database Project (RDP; Maidak *et al.*, 1999; Smit *et al.*, 1999). However, Anderson and colleagues (2003b) used the same primers in conjunction with three further sets of 18S rDNA and ITS primers and found that the relative proportion of sequences representing the four main fungal phyla obtained from DNA extracted from an agricultural soil were similar. They concluded that bias associated with the primers may not be as significant as previously thought and that it is possible that the data obtained may truly reflect the community composition in the DNA sample (Anderson *et al.*, 2003b). Even 18S rDNA primers that have been designed to be specific for AM fungi have been shown to have some limitations under certain conditions (Clapp *et al.*, 2002).

Although primer bias is an acknowledged problem (e.g. Smit *et al.*, 1999; Anderson *et al.*, 2003b), we remain relatively uncertain about the bias associated with each primer set and whether bias is a major problem or not. This is primarily because of the lack of a targeted analysis of bias associated with the primers using defined template mixtures or synthetic fungal communities. The potential for primer bias thus needs to be taken into consideration when making conclusions about fungal diversity in environmental samples.

Community profiling techniques and limitations

Community fingerprinting techniques have commonly been applied to studies of bacterial ecology and have significantly increased our understanding of the role and diversity of bacteria in soil (for review, see Ranjard *et al.*, 2000; Johnsen *et al.*, 2001; Kozdrój and van Elsas, 2001). However, these techniques, including denaturing gradient gel electrophoresis (DGGE), temperature gradient gel electrophoresis (TGGE), single-strand conformation polymorphism (SSCP), terminal restriction fragment length polymorphism (T-RFLP), amplified rDNA restriction analysis (ARDRA), amplified ribosomal intergenic spacer analysis (ARISA) and cloning, have only recently been adopted and applied to studies of soil fungi.

Although each of the community profiling approaches is different, several of the techniques exploit the same properties of DNA for the separation process. For example, DGGE and TGGE separate DNA fragments of the same size but of different sequence based on the melting behaviour of DNA, with DNA of different sequences (i.e. different G+C content) melting at different positions in a polyacrylamide gel containing either a linear gradient of denaturants (DGGE) or temperature (TGGE). This limits the use of degenerate primers in DGGE/TGGE as their use may result in multiple bands leading to an overestimation of diversity (Muyzer, 1999). These techniques have been the most widely adopted community fingerprinting techniques in bacterial ecology since the introduction of DGGE by Muyzer and colleagues (1993), hence the potential and pitfalls of the techniques for the analysis of soil microbial communities are well documented (for review, see Muyzer, 1999). DGGE/TGGE provides a rapid means of investigating soil fungal communities, particularly where the aim is to investigate shifts or changes in community composition (e.g. van Elsas *et al.*, 2000; Anderson *et al.*, 2003a). The techniques benefit from the ability to analyse and compare numerous samples on a single gel and allow a rapid, simultaneous comparison between samples. The development of specific software packages has substantially improved the way in which community fingerprints can be analysed by allowing the comparison of both the position and the relative intensity of different bands within gels. This enables statistical analyses to be performed on the data and subsequent interpretation of ecological inference (Fromin *et al.*, 2002). The accuracy of the comparison, however, is heavily dependent upon the inclusion of suitable internal standards and assumes that the resolution and quality of gels has been standardized. This is particularly crucial where comparison between several different gels is required as a result of large sample numbers. The reproducibility between gels has been highlighted as one of the main pitfalls of DGGE (Fromin *et al.*, 2002), although the way in which gels are prepared can be optimized, for example by using a constant flow rate of denaturant solutions and standardized equipment for each gel that is prepared.

One of the main advantages of gel-based community profiling techniques is the ability to excise and sequence bands of interest, thereby obtaining taxonomic information for interesting members of the community via database searches and/or phylogenetic analysis. In addition, the techniques can be used in conjunction with DNA oligonucleotide probes to increase the specificity of the analysis (e.g. Stephen *et al.*, 1998). Although this approach has not been used for soil fungi, probing has been used in conjunction with cloning to group clones into taxonomic clusters [oligonucleotide fingerprinting of rRNA genes (OFRG); Valinsky *et al.*, 2002]. Despite the advantages of

DGGE/TGGE, as is the case for all community profiling techniques, there are also disadvantages. In general, shorter fragments of DNA result in better resolution between bands in a profile (<500 bp), thereby limiting the taxonomic information that can be obtained by sequencing excised bands, although some larger products have also been used successfully in a few cases (Ranjard *et al.*, 2000; Landeweert *et al.*, 2004). In addition, even the most sensitive staining methods are often not sensitive enough to detect all the diversity present within a sample, particularly for the less dominant members of the community. Furthermore, in some cases, single bands on a gel have been shown to comprise more than a single sequence type (e.g. Schmalenberger and Tebbe, 2003).

Through application of automated DNA sequencer technology, T-RFLP has enabled significantly increased throughput compared with gel-based community profiling techniques (for review, see Marsh, 1999). T-RFLP is modified from the conventional RFLP approach using fluorescently labelled PCR primers (forward, reverse or both primers) before restriction digestion and size detection of fluorescently labelled terminal restriction fragments using a DNA sequencer. T-RFLP is also an improvement of the community profiling technique ARDRA, as it only detects the terminal fragment of each 18S rDNA or ITS sequence within a sample, thus potentially reducing the complexity of the community profile without reducing the diversity detected (Kozdrój and van Elsas, 2001). ARISA is a similar technique, with the exception that whole amplicons are analysed rather than terminal fragments produced as a result of restriction digestion (e.g. Ranjard *et al.*, 2001; Leckie *et al.*, 2004). The use of an automated DNA sequencer for ARISA and T-RFLP not only increases the potential throughput, it also increases the accuracy in sizing the generated fragments through the inclusion of an internal standard in each sample provided running conditions remain constant. T-RFLP has been used for assessing the diversity of soil fungal communities (e.g. Klamer *et al.*, 2002; Lord *et al.*, 2002) along with the identification of particular ECM fungal species in soil samples (e.g. Dickie *et al.*, 2002); however, the identification of particular fungal species requires the development of a robust T-RFLP database. The inability to generate sequence information from T-RFLP peaks makes the identification of unknown species in a sample difficult. Although identification has sometimes been artificially determined by performing virtual restriction digests on public database sequences, this approach is potentially dangerous as it assumes that only a single species or operational taxonomic unit (OTU) can have a peak of that size in a sample and that the database sequence is accurately identified and is of good quality. This problem is highlighted, for example, by the fact that different species of the ECM genus *Cortinarius* can have the same RFLP

profiles with two restriction enzymes (Kâren *et al.*, 1997) and that isolates of the same species of *Pisolithus* can have different RFLP profiles (e.g. Hitchcock *et al.*, 2003). Therefore, judicious selection of restriction endonucleases is an important criterion for T-RFLP analyses, particularly as it has been shown that the use of two different restriction enzymes on a single sample results in a contrasting view of the level of diversity contained within it (e.g. Klamer *et al.*, 2002).

The detection of diversity in a sample using T-RFLP is limited by the detection threshold of the sequencer being used for the analysis, similar to the way in which gel-based techniques are limited by the sensitivity of the stains used. Therefore, the absence of a peak does not necessarily confirm the absence of a species (Dickie *et al.*, 2002). Equally, the presence of a peak does not necessarily guarantee the presence of an individual species or OTU in a sample, as it has been shown that pseudo-terminal restriction fragments resulting from the presence of single-stranded amplicons are also sometimes formed (Egert and Friedrich, 2003). Limitations aside, T-RFLP is a very powerful technique particularly for the identification of target organisms within a sample. However, the inability to be able to generate sequence data from T-RFLP peaks makes the taxonomic identification of unknown OTUs difficult and the application to studies on 'total' fungal diversity more problematic.

Cloning PCR amplicons that have been generated from environmental DNA has also been used for assessing soil fungal diversity (Chen and Cairney, 2002; Anderson *et al.*, 2003b; Jumpponen, 2003). Although clones can be screened using RFLP to group clones into OTUs before DNA sequencing, thus reducing the number of clones requiring sequencing, it is difficult to determine how many clones require analysis to have fully sampled the diversity contained within a single sample. The production of collectors or species abundance curves has shown that, in an agricultural soil, the number of 18S rDNA clones that require analysing to have covered the diversity of fungi is substantially less than that required for bacterial 16S rDNA clone libraries (Anderson *et al.*, 2003b). However, these authors also found that more ITS clones than 18S rDNA clones from the same sample need to be screened to achieve coverage of fungal diversity in the sample. It is also difficult to determine common versus uncommon sequence types in a sample unless a large number of clones are analysed. Despite being laborious and potentially costly, the analysis of clone libraries produced from environmental DNA is a useful technique and is complementary to the other community profiling techniques mentioned above. Indeed, it is now frequently used in conjunction with other community profiling techniques in bacterial ecology (e.g. Freitag and Prosser, 2003).

While community profiling techniques are now com-

monly used in conjunction with DNA sequencing and phylogenetic analysis for the taxonomic identification of species present within a sample, the possible presence of chimeric DNA sequences generated from environmental DNA samples must not be overlooked (e.g. see Wintzingerode *et al.*, 1997). Indeed, the presence of chimeric sequences in a data set has been shown substantially to influence the level of fungal diversity detected in a single sample (e.g. Jumpponen, 2003). Although identification of 'possibly' chimeric sequences can be guided by software packages [e.g. CHIMERA CHECK program of the RDP, version 2.7 (Maidak *et al.*, 1999)], their detection and elimination from a data set is often difficult and requires personal judgement.

The aforementioned techniques provide a suite of powerful tools for the analysis of soil fungal communities. Their extensive application to bacterial ecology over the last decade has resulted in their refinement over time, although each still has its own technical limitations. The taxonomic resolution of each community profiling technique is not only limited by the technique itself, but also by the PCR primers chosen for the initial PCR amplification of the community DNA and by lack of exhaustive sequence information in the public databases. However, with continued effort by fungal ecologists over the coming years, it is likely that these limitations will gradually become less of a constraint.

Recent advances in molecular ecology of soil fungi

Recent investigations of soil fungal ecology by PCR amplification from total extracted DNA have significantly increased our understanding of fungal diversity in plant roots or the rhizosphere and/or bulk soil, along with spatio-temporal dynamics of the extraradical mycelia of certain root-dwelling fungi.

Cloning and sequencing both 18S and ITS rDNA PCR products from total soil DNA has been useful in confirming the long-held belief that culturing fungi from soil provides only a selective view of diversity (Borneman and Hartin, 2000; Viaud *et al.*, 2000; Hunt *et al.*, 2004). Amplification of 18S rDNA coupled with TGGE and DGGE has also effectively demonstrated reduced fungal diversity in the rhizosphere compared with that in bulk soil, along with a plant age effect, in microcosms and field soil (Smit *et al.*, 1999; Gomes *et al.*, 2003). Analysis of 18S rDNA clones has also revealed differences in fungal assemblages in unvegetated glacier terminus and terminal moraine soils (Jumpponen, 2003). Shifts in soil fungal community structure in response to treatments such as petrochemical pollution (van Elsas *et al.*, 2000), nitrogen addition (Lowell and Klein, 2001), controlled vegetation burning (Chen and Cairney, 2002), elevated atmospheric CO₂ concentration (Klamer *et al.*, 2002) and compost or manure addition

(Edel-Hermann *et al.*, 2004) have been reported from studies that have used 18S or ITS rDNA amplification coupled with cloning, DGGE, SSCP or T-RFLP analysis. Anderson and colleagues (2003a) observed a sharp demarcation between ITS1 rDNA DGGE profiles for fungal communities in forest and adjacent moorland vegetation that were consistent with changes in soil chemical, physical and other biological properties. Similarly, Brodie and colleagues (2003) found changes in soil fungal community structure associated with an upland grassland floristic gradient using 18S rDNA PCR along with DGGE and T-RFLP. Communities identified by DGGE appeared to be less taxonomically rich than those observed by T-RFLP, although the extent to which this indicates differences in the relative resolution of the techniques is unclear as different DNA extraction procedures and primers were used in the analyses.

Kowalchuk and colleagues (1997) used 18S rDNA PCR and DGGE to investigate the diversity of fungi in roots of marram grass (*Ammophila arenaria*) in a sand dune soil. Taxonomic richness was found to be broadly similar to that of an isolated fungal assemblage from the same host, although some sequences from the DGGE profiles did not match those of the isolates. Taxa such as *Phoma* and *Microdochium*, which, on the basis of isolated assemblages, were regarded as important components of the *A. arenaria* root fungal community, were not identified by DGGE. Similarly, Allen and colleagues (2003) reported that ITS2 PCR and cloning of DNA from roots of *Gaultheria shallon* identified an unculturable *Sebacina*-like basidiomycete as being an important component of the ericoid mycorrhizal fungal community. Moreover, Vandenkoornhuyse and colleagues (2002a) found evidence for the possible presence of previously unknown fungal groups after phylogenetic analysis of sequenced 18S rDNA clones from grass (*Arrhenatherum elatius*) root DNA.

Subsequent 18S rDNA PCR and DGGE work on AM endophytes of *A. arenaria* (Kowalchuk *et al.*, 2002) provided clear evidence for lack of correlation between the taxonomic structure of AM fungal assemblages in roots and those inferred from soil-borne spore diversity. Although direct amplification of AM fungi from total root DNA has been used for some time (e.g. Simon *et al.*, 1993; Clapp *et al.*, 1995) and the viability of competitive PCR for quantification of AM infection in roots has been demonstrated (Edwards *et al.*, 1997), it is only relatively recently that the methods have been applied to address significant ecological questions. Using partial 18S rDNA PCR from total root DNA and cloning, Helgason and colleagues (1998) found lower AM fungal diversity in roots of monoculture arable crop plant hosts than in woodland plant taxa. As many AM fungi can infect a broad range of host plants, this was thought to reflect an influence of agronomic management practices on diversity rather an

effect of monoculture planting. Subsequent work using the same methods along with T-RFLP has, however, provided evidence that roots of different plant taxa at the same site house different AM communities (Vandenkoornhuyse *et al.*, 2002b; 2003). Indeed, using a partial 18S rDNA PCR and T-RFLP approach to investigate the AM fungal assemblages in roots of *Plantago lanceolata* bait plants from microcosms that contained monoculture and mixed plant communities, Johnson and colleagues (2003) provided further convincing evidence that floristic diversity may strongly influence diversity of the endophytes.

Studies of below-ground communities of ECM fungi have previously relied upon analysis of infected root tips and provided little insight into the structure and dynamics of the extraradical mycelia of the fungi in soil (Horton and Bruns, 2001). ITS rDNA PCR, along with cloning, DGGE or T-RFLP, has now been used successfully in the ECM context. In particular, these studies have been important in revealing vertical stratification of ECM mycelia in soil that may reflect differential resource utilization by the fungi (Dickie *et al.*, 2002; Landeweert *et al.*, 2003a; 2004), along with the influence of certain management practices on ECM fungal diversity (Smit *et al.*, 2003; Edwards *et al.*, 2004). Spatial distribution and temporal persistence of mycelia of the ECM basidiomycete *Hebeloma cylindrosporum* in soil has also been investigated recently using competitive quantitative PCR. Using this approach, Guidot and colleagues (2002; 2003) found that mycelia of this fungus in soil occurred exclusively under, or close to, basidiomes and that such mycelia disappeared within 12 months.

Future perspectives

Although community profiling techniques have only recently been applied to studies of soil fungal ecology, their influence on the field has been considerable. The techniques are now being widely adopted in laboratories around the world, and it seems likely that our perceptions and understanding of the structure and dynamics of soil fungal communities will be enhanced significantly in the near future. It must be emphasized that no single set of primers or community profiling technique will be optimal for the assessment of fungal diversity in all instances, but rather they should be chosen with the aims of the research, the target group of fungi and the data analysis in mind. Moreover, as we have highlighted in this review, the methods have inherent limitations that must be taken into consideration in the context of individual studies and the hypotheses being tested. Some of these limitations have been addressed in investigations of soil bacterial communities, resulting in the optimization of the techniques, while others are specific to fungal ecology. Thus, for example, although analysis of taxonomic richness of

soil fungi is relatively straightforward, consideration of diversity on the basis of richness and relative abundance, along with community dynamics, is complicated by both the mycelial nature of fungi and the potential co-occurrence of spores in a given sample.

Stable isotope probing (SIP), a method that separates nucleic acids of different organisms according to their abilities to use particular substrates labelled with stable isotopes, has proved useful in separating different functional groups of soil bacteria (e.g. Radajewski *et al.*, 2000; Morris *et al.*, 2002). Significantly, it has also recently been applied to soil fungi (Lueders *et al.*, 2004). These authors clearly demonstrate that the technique can be used for the analysis of fungal DNA in soil. More importantly, as incorporation of carbon isotopes into fungal DNA is slow relative to bacteria, they also demonstrated that SIP can be used for the analysis of fungal RNA, which incorporates ¹³C more rapidly than DNA. Indeed, the successful analysis of fungal RNA extracted from soil is, in itself, a substantial step forward for fungal ecology as the analysis of RNA allows the detection and identification of those members of the community that are active compared with those that are present in the community but are dormant. Quantitative real-time PCR has also been used for the quantification of fungal DNA and RNA in soil (Filion *et al.*, 2003; Landeweert *et al.*, 2003b; Lueders *et al.*, 2004) and offers great promise for the future, as do advances being made in the development and application of metagenomics approaches (Rodriguez-Valera, 2004; Tyson *et al.*, 2004) and microarray technology (e.g. Ye *et al.*, 2001; Taroncher-Oldenburg *et al.*, 2003) for studying the diversity and functioning of microbial communities. These methods, used in conjunction with the community profiling techniques, have the potential to enhance further our understanding of fungal communities and their functional roles in soil ecological processes.

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