1 Detection and Diversity of Fungi from Environmental Samples: Traditional Versus Molecular Approaches

R. Jeewon and K.D. Hyde

1.1 Introduction

Microbial life within the soil ecosystem is a fascinating aspect of soil biology, and has recently caught the attention of microbiologists. Many fungi grow in the soil and some have evolved to thrive in harsh conditions, such as those found in acidic or alkaline soils. These microorganisms can be considered as "highly developed" as they flourish and reproduce in these ecological niches and unusual habitats and have successfully made use of soil and its nutrients for their energy sources. Fungi are an important component of the soil microbiota, they mediate important ecological processes such as nutrient recycling, and they maintain important symbiotic relationships with plants and bacteria (Garrett 1981; Parkinson 1983; Yu et al. 2005). Many fungi are pathogenic (e.g Jaworski et al. 1978; Cahill and Mohr 2004) and some may be useful in bio-exploitation (e.g Vinokurova et al. 2003). The realms of soil mycota are possibly the largest on the planet.

A diverse range of fungi are present in soil ecosystems and include ascomycetes, basidiomycetes, some being ectomycorrhizal fungi, anamorphic fungi and arbuscular mycorrhizal fungi (AMF). At present, there is no clear morphological, phylogenetic or ecological definition of soil fungi. Any definitions based on these concepts are very difficult to implement because the soil ecosystem harbours a plethora of fungi with great morphological, genetic and functional diversity and lacks geographic boundaries. Perhaps the best definition of soil fungi should be encapsulated in the word itself (fungi from soil!). Most of our current knowledge of soil mycota is based on traditional systematics, which does not reflect any real sense of evolutionary relationships. The interaction between

Department of Ecology and Biodiversity, University of Hong Kong,
Pok Fu Lam Road, Hong Kong, email: rjeewon@hku.hk; rjeewon@graduate.hku.hk

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these fungi with plant roots and other biotic or abiotic factors within the soil constitutes a challenge to soil microbiologists. Obviously there must have been a long evolutionary history of adaptation and competition that permitted fungi to evolve in diverse forms and interact with other organisms.

In this chapter we explore the limits of conventional and molecular techniques used to assess and detect soil microfungal diversity and provide insights into their feasibility. In particular we address the problems associated with the dilution plating technique, importance of the rDNA gene in fungal systematics, the reliability of other molecular approaches (especially denaturing gradient gel electrophoresis; DGGE) and their drawbacks.

1.2 Microscopy and Culture-Based Methods

Traditional methods to assess fungal diversity in soil environment rely mainly on the dilution-plating technique (coupled with the use of selective media) and microscopy to identify sporulating fungal bodies. Davet and Rouxel (1997) have already detailed all the experimental procedures commonly used in the dilution plate method and direct comparison. Both methods are direct isolation techniques; and the dilution-plating method involves a combination of gentle dispersion, soil dilution and serial dilution, small amounts of which are ultimately plated on artificial media and incubated. The direct comparison method involves sprinkling of a known amount of soil onto a medium, which is then incubated (Davet and Rouxel 1997). Both methods provide a reasonably sensitive recognition of soil fungi and have been widely used in diversity studies in different habitats (e.g. Elmholt et al. 1999; Cho et al. 2001; Cabello and Arambarri 2002). Cultural methods, coupled with morphological details from microscopy, are among the earliest techniques used and allow one to detect exactly which taxon is present (identification). They have also commonly been used because of their simplicity, low cost and the fact that they are easy to conduct. Williams et al. (1965) has already detailed the efficiency of the soil washing technique, its applicability and potential for studying soil microhabitats and these are not detailed here. While these methodologies are easy, fast and reliable in finding the dominant culturable fungal taxa, they have a number of limitations which impede a proper diversity assessment.

Davet and Rouxel (1997) mentioned that the traditional methods outlined above tend to overestimate species that sporulate in soil, while those in mycelial state or those that have slow growth in culture are largely overlooked. In addition, most of these methods result in isolation of only the most common and abundant fungi (often referred to as "generalists"), such as the asexual ascomycetes *Fusarium, Penicillium* and *Trichoderma* and oomycetes (*Pythium*). These cultivated organisms are those that can utilise the energy source under

the physical and chemical limitations of the growth medium. The continuous isolation of similar fungi following these traditional approaches clearly indicates that many others do not respond readily to cultural techniques. Therefore, the diversity data cannot be considered as accurate (Bridge and Spooner 2003). Although these unculturable fungi play a vital role in the soil ecosystem, they were not previously thought to be central part of any biological processes in soil. Altered and optimised growth medium, coupled with 16S rRNA gene comparative analysis, has demonstrated that a larger proportion of uncultured bacteria (above the 5% level postulated) and belonging to novel bacterial lineages could be isolated and identified (Janssen et al. 2002). Similar strategies are required for fungi. However, there is insufficient knowledge on the nutritional and environmental demands of soil fungi and these present methodological drawbacks in providing a clear assessment of fungal communities associated with soil.

Another major complication with cultural studies is that a large number of other fungi existing as mycelial (vegetative) propagules or dormant spores can be numerically dominant populations in their natural environment but never grow in culture. These organisms will escape normal isolation-based detection procedures and therefore provide bias data regarding fungal diversity. Even for fungi that sporulate and can be cultured, it is not always easy to correctly identify them with certainty. Our knowledge regarding the taxonomy and classification of these fungi are still limited. In addition, there are being many species that appear to be similar under cultural conditions and exhibit similar morphology, but are in fact different species. It is thought that only a small fraction (0.1% to 10.0%) of microorganisms existing in the nature can be cultured artificially (e.g. Muyzer et al. 1993; Torsvik and Øvreås 2002). Hawskworth and Rossman (1997) suggested that commonly used methods have probably only recovered 17% of known fungal population and the majority of them await discovery. Even if morphological assessment of some taxa is possible, nothing conclusive regarding the viability, percentage occurrence, physiologic and phylogenetic information can be accrued.

Processing of cultures can be time-consuming and laborious when a large number of isolates has to be handled. During these processes, the risk of culture contamination is always high and in most cases the fast-growing fungi will overgrow others and occupy the whole medium (even when Rose Bengal solution is used). Many fungi assume different life forms (e.g. existence as vegetative hyphae or dormant spores) depending upon environmental or seasonal factors. Therefore it is highly probably that many fungi are only either collected in forms that: (1) do not allow them grow in artificial media or (2) preclude their identification via microscopy. Given that fungal diversity may be quite high in soil and each population or species may occupy a specific niche, there is no single method that is appropriate to target all of them efficiently.

Garbeva et al. (2004) and Buckley and Schmidt (2002) have reviewed the effects of factors, such as plant type, soil type, soil management regime, microenvironment and disturbance, on soil microbial diversity, from single soil ag-

gregates to entire landscapes. These are not detailed here. Generally it appears that both cultural and direct morphological methods have specific bias, as data generated is largely dependent upon the methodologies involved.

1.3 Molecular-Based Methods

The drawbacks associated with culture-dependent methods for the detection and identification of fungi in soil samples prompted the development of alternative methods which largely circumvent cultivation of target organisms. Molecular techniques have been employed, basically involving the application of hybridisation probes, PCR amplification of rDNA genes and other DNA fingerprinting techniques. These include terminal restriction fragment length polymorphism (T-RFLP), amplified rDNA restriction analysis (ARDRA), amplified random intergeneric spacer analysis (ARISA), denaturing gradient gel electrophoresis (DGGE), temperature gradient gel electrophoresis (TGGE), oligonucleotide fingerprinting of rRNA genes or single-stranded conformation polymorphism (SSCP) and have been used frequently in combination with traditional techniques to analyse fungal community composition (e.g. Egger 1995; van Elsas et al. 2000; Lowell and Klein 2001; Maarit-Niemi et al. 2001; Ranjard et al. 2001; Kirk et al. 2004). Several freshwater fungi have successfully been identified with fluorescence in situ oligonucleotide hybridisation (FISH) (Baschien et al. 2001). Another important PCR-based fingerprinting technique recently applied to assess fungal diversity is oligonucleotide fingerprinting of ribosomal RNA genes (ORFG), a new method which sorts arrayed ribosomal RNA gene clones into taxonomic clusters through a series of hybridisation experiments (Valinsky et al. 2002). These DNA-based techniques can provide a comprehensive measure of the diversity and composition of fungal communities, since they survey both the cultured and often-predominant non-culturable members of a community (Muyzer et al. 1993; van Elsas et al. 2000; Borneman and Hartin 2000; Landeweert et al. 2001; May et al. 2001; Kirk et al. 2004).

The implications of PCR-based methodologies have altered our views about the way we used to think about soil fungal diversity. For instance, Baek and Kenerley (1998) assessed the feasibility of quantitative competitive PCR in the detection and quantification of a genetically modified strain of *Trichoderma virens*. They found that the detection limit of PCR was 10–1000 times lower when compared with traditional dilution plating. By using a combination of culture-dependent and culture-independent approaches (PCR-RFLP), Viaud et al. (2000) found that the latter was an efficient molecular tool for ecological studies and for assessing unexplored fungal diversity. These methods have also been extremely useful in assessing the diversity of fungi that are difficult to isolate from soil, such as basidiomycete and arbuscular mycorrhizal fungi (AMF: Bougoure

and Cairney 2005; Kouichi et al. 2005). Other methods relevant to these aspects are outlined by Akkermans et al. (1995).

1.4 The Nuclear-Encoded Ribosomal DNA Gene: Phylogenetic and Systematic Value

Morphological characters provide the basis of current fungal systematics. They provide a wealth of information to distinguish taxa and have been used extensively at different hierarchies. In some cases, however, morphological criteria present some problems and fail to resolve taxonomic relationships. This is true in cases where morphological characters are inadequate, convergent, reduced, missing or overlapping. As a consequence, many taxonomists have combined available morphological characters with biochemical or molecular characters to clarify taxonomic relationships, as well as to infer phylogenies among fungal species. Various molecular techniques that have been applied successfully in fungal systematics and the application of DNA sequencing coupled with phylogenetic analysis have greatly expanded, owing to the ever-increasing amount of sequence data available from a myriad of organisms. Molecular characters offer considerable potential, as they not only close the gap between the traditional and molecular methods, but also may determine relationships between uncultured and cultured fungi.

For several decades, the nuclear-encoded ribosomal DNA (rDNA) gene has been the gene of choice to assess phylogenetic relationships and resolve taxonomic questions at different taxonomic levels (Gouy and Li 1989; Bruns et al. 1991; Spatafora 1995; Liew et al. 2000; Jeewon et al. 2002, 2003a, b, 2004; Duong et al. 2004; Cai et al. 2005). Genes of eukaryotic rDNA are organised in a cluster that includes a small subunit gene (18S), a large subunit gene (28S) and the 5.8S gene that lies in between two internal transcribed spacers (ITS; White et al. 1990). The region that separates the cluster of three genes along the chromosome is called the non-transcribed spacer (NTS) and prior to where the 18S gene is transcribed, there is another small spacer region called the externally transcribed spacer (ETS). Together the ETS and NTS regions comprise the intergeneric spacer region (IGS; Fig. 1.1). These components are repeated in a tandem array but they evolve as a single unit and vary in length around 3000–4500 base pairs (Mitchell et al. 1995).

The ribosomal DNA has attracted increased attention among fungal systematists, especially those interested in applying DNA sequencing analysis to study taxonomic relationships and genetic variation in fungi. The most remarkable feature of the rDNA is the overall sequence homogeneity among repeat units of the gene family (Hillis and Dixon 1991, Dixon and Hillis 1993). This gene shares the same function in all organisms and evolves at approximately the same

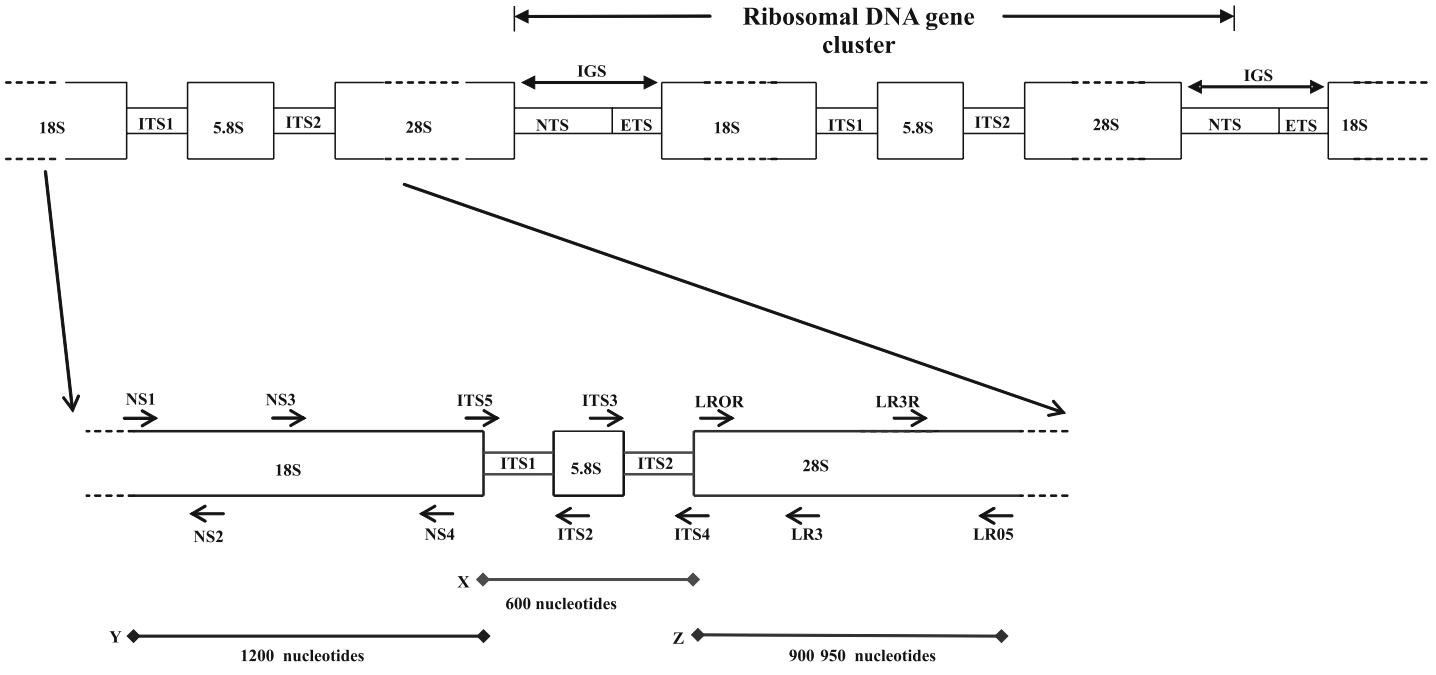

Fig. 1.1 Diagrammatic representation of the rDNA gene cluster, showing the positions of the PCR and sequencing primers. The gene is split into coding regions (*18S*, *5.8S*, *28S* genes) and non-coding regions (*IGS*, *ITS*). Positions of the primers and their direction of replication are indicated by *arrows*. *X* Product amplified with primers ITS4 and ITS5, *Y* product amplified with primers NS1 and NS4, *Z* product amplified with primers LROR and LR05. Sizes of products are approximate. Modified from Mitchell et al. (1995)

rate. However, the three different regions (structural genes, transcribed spacers, NTS) evolve at different rates, thus yielding informative data to reconstruct the phylogeny at different taxonomic levels. The 18S rDNA (small subunit; SSU), which evolves relatively slowly and is quite conserved, has been used to provide insights into the phylogeny of distantly related organisms, particularly at the ordinal and family level. The 28S (large subunit; LSU) is moderately conserved but provides sufficient variation to study relationships at the generic as well as species level. The ITS and IGS regions evolve faster and are highly variable and therefore valuable for comparing fungal species at the intraspecific level. Sequence comparisons of selected regions within the rDNA have been useful for inferring phylogenetic relationships among fungi for several reasons. Universal single primers that are complementary to several regions within this gene are ready available (Vilgalys and Hester 1990; White et al. 1990). The region is short and its multicopy nature makes it easy to amplify. It is easily accessible and a large number of sequences are available for comparison. It has a high nucleotide variability, which makes it feasible to estimate genetic distances as well as investigating systematics.

1.5 Denaturing Gradient Gel Electrophoresis: Applicability, Usefulness and Bias

While rDNA has been the most widely used gene for systematics studies, DGGE has been the most useful genetic fingerprinting technique to investigate complex microbial communities from a diversity of environmental samples. Basically this method involves separation of individual sequences (with different base composition and melting properties) from a mixture. DNA extracted from environmental samples is amplified with a primer pair (specific to the groups of organisms under investigation and one of them attached to a GC clamp) and then purified PCR samples are separated electrophoretically through a gradient of increasing chemical gradient (urea: formamide). Based on the melting behaviour, different sequences migrate at different positions, producing different banding patterns where each presumably represents a microbial taxon. The bands can then be excised from the gel and processed (either by construction of clone libraries and screening clones, or reamplified and sequenced) to obtain phylogenetic sequence information on individual microbial members of the microbial community. DGGE has been used to profile fungal microbial communities from many diverse environments (Kowalchuk et al. 1997; Smit et al. 1999; Omar and Ampe 2000; Gurtner et al. 2001; May et al. 2001; Möhlenhoff et al. 2001; Nikolcheva et al. 2003; Nikolcheva and Bärlocher 2005).

In view of the fact that so little is known about the distribution and abundance of fungi in soil environments, DGGE coupled with phylogenetics has

been successfully applied to assess fungal diversity in soil samples and, in most cases, it has been reported that soil possibly consists of a much more diverse micromycota than that observed. van Elsas et al. (2000) assessed the efficiency of two DNA extraction protocols from soil microcosms, the applicability of the NS2f/Fung5r primer pair, and the persistence of *Trichoderma harzianum* and *Arthrobotrys oligospora* in response to petrol treatment. DGGE fingerprints of total DNA from tropical soil and rhizosphere revealed that there was a relationship between fungal community composition and rhizosphere development (Gomes et al. 2003). In the same study, phylogenies revealed that fungal taxa from the order Pleosporales (Ascomycetes) and basidiomycetons yeast were the most dominant phylotypes. Fungal community diversity from organic soil was investigated by PCR-DGGE followed by sequence analyses of ITS fragments (Anderson et al. 2003a). DGGE profiles revealed a clear shift in fungal community composition along a moorland pine forest environment gradient. In addition, phylogenies indicated that the majority of phylotypes (sequence types) were ascomycetes, especially Helotiales, and that the fungal communities were different from those derived using cultural methods.

DGGE is the preferred environmental fingerprinting approach as it: (1) enables large and multiple samples to be analysed simultaneously, (2) overcomes diversity bias from traditional approaches (e.g. cultural methods), (3) can successfully monitor community shifts and succession over time, (4) allows the profiling of communities under different environmental conditions (especially in degraded/polluted ecosystems), (5) makes it possible to acquire taxonomic information via phylogenetic analyses, and (6) gives an indication about the possible biological role of specific microorganisms in the sample (e.g. those that can be involved in the decomposition of organic matter or degradation of pollutants).

Nevertheless there are limitations. The lysis of cells to release DNA in the external environment is the most crucial step. Given that soil is a heterogeneous environment, there can be abundant fungi that are free-living and not localised and are therefore easily extracted. In contrast, those that are less abundant and localised in microhabitats (e.g. inside soil particles, in water-filled spaces) are difficult to extract (van Elsas and van Overbeck 1993). There is always a possibility that fungi that do not release their DNA will not contribute to diversity or that vigorous extraction procedures can result in highly fragmented DNA, producing chimeric PCR products (Wintzingerode et al. 1997). In addition, different fungal structures (spores, mycelia) have different lysing efficiency; and an inappropriate extraction method can potentially give a biased estimate of diversity (Prosser 2002). There are no specific protocols for soil fungi, although there has been considerable improvement in the procedures involved, for instance the addition of PVPP to precipitate PCR inhibitors (Wintzingerode et al. 1997; Prosser 2002; Anderson and Cairney 2004; Kirk et al. 2004). Caution is required because, in bacterial diversity studies, it has been shown that different DNA protocols and purification methods yield different DGGE profiles (Maarit-Niemi et al. 2001). The efficiency of different DNA extraction protocols

and the effect of different soil types have partially been dealt with (Laurent et al. 2001; Ranjard et al. 2001; Anderson and Cairney 2004).

PCR is the basis of most molecular methods involved in diversity estimates. However, DNA from environmental samples contains PCR inhibitors and contaminants that interfere with PCR reactions (e.g. humic acid from soil). In many cases, there can be differential amplification, loss of DNA following purification, production of PCR artefacts, and contamination (Wintzingerode et al. 1997). PCR amplification of chimeric sequences is not uncommon. Sequence analyses of these usually indicate that they are not phylogenetically related to other known fungi, as they occupy unique position in the phylogenetic tree. In these cases, one will erroneously assume that these sequences represent novel taxa that escape microscopic or cultural detection. Most of the gene regions targeted in community analyses are from the conserved 18S rDNA gene and are less than 600 base pairs, so that a reasonable DGGE resolution can be achieved. This is, however, to the detriment of accurate systematics and phylogeny. In many cases, the primer pairs used are specific to a group of fungi, while some at the same time can amplify DNA from totally unrelated organisms. Our laboratory has undertaken diversity studies on leaves of *Magnolia Liliifera* (Duong et al. 2006) and pine needles using NS1 and GCfung primers as described by May et al. (2001). In both studies based on DGGE, we recovered only ascomycetous fungi, especially those from Dothideales, Helotiales, Hypocreales, Pleosporales, Rhystimatales and Xylariales, but no basidiomycetous taxa. Anderson et al. (2003b) and Anderson and Cairney (2004) have already demonstrated the potential bias of rDNA in estimating fungal diversity in soil and aspects pertaining to primer design and these are not discussed here.

Although DGGE is a promising tool, it can still underestimate fungal diversity (Nikolcheva et al. 2003, 2005). The number of bands depends on the resolution of the gels; this takes time to optimise and is difficult to reproduce (Fromin et al. 2002). The quality of sequence data recovered can be highly variable due to contaminating background sequences. We have repeatedly encountered this phenomenon when sequencing purified PCR-DGGE bands. It is not necessarily true that one "phylotype" or "operational taxonomic unit" or "sequence type" generated from an environmental sample is representative of an individual organism. As the amount of nucleic acid extracted does not necessarily reflect all the species/populations within one sample, interpretation of bands can be difficult. Often, dominant bands might mask more than one species, resulting in an underestimation of diversity. Another ambiguity we have noticed with leaf and pine needle samples is that co-migrating bands (similar melting behaviour) can actually represent taxa that are phylogenetically unrelated. The reverse also holds true. This is not surprising as it has already been demonstrated in previous studies that phylogenetically distant taxa can have co-migrating bands and that one band does not necessarily mean one unique phylotype (Rosado et al. 1998; MacNaughton et al. 1999; Sekiguchi et al. 2001). Therefore careful interpretation is essential.

Sequences obtained from DGGE bands are quite difficult to analyse as they are usually from different orders and classes. Our taxonomic knowledge is still poor and, phylogenetically, most of the sequence types do not fit clearly within any known family/genera or species, although their ordinal classification seems to be reliable. Definitive species identification is very difficult unless a large number of representatives are available from databases and a sufficiently variable gene region is analysed. Another important question is: which genes and what features of that genetic sequence are crucial, useful and reliable to identify uncultured fungi? Most of the available sequences and phylogenies are derived from the rDNA gene, but classification and taxonomic schemes based on this gene alone are inadequate, subject to debate and need to be re-evaluated. Although rDNA provides sufficient variability for evolutionary and phylogenetic inferences, should more genes be sampled?

The degree of similarities/differences of sequence types obtained from environmental samples also poses a problem. It is commonly assumed that, for bacteria, >97% sequence identity can be regarded as different species (Stackebrandt and Goebel 1994). However, there is no report for such concepts in fungal taxonomy. Another important concern is that the number of novel phylogenetic lineages and new phylotypes is on the rise. In a recent paper published in *Science*, a combination of microbiological and molecular techniques revealed three novel phylogenetic clades that constitute three major new groups of fungi (Schadt et al. 2003). As mentioned before, many sequence types cannot be confidently assigned to any particular genus or family and these have been referred to as novel taxa or lineages. Berney et al. (2004) analysed 484 environmental 18S rRNA gene sequences, including 81 new sequences, to test the potential technical and analytical pitfalls and limitations of eukaryotic environmental DNA surveys. Based on phylogenetic analyses, they suggested that the number of novel higher-level taxa revealed by previously published environmental DNA surveys was overestimated possibly due to: (1) the presence of undetected chimeric sequences, (2) the misplacement of several fast-evolving sequences, and (3) the incomplete sampling of described, but yet unsequenced eukaryotes. It is highly possible that a similar situation exist in fungal studies.

In addition, a number of studies involving the use of DNA fingerprinting techniques did not address the evolutionary history and affinities of fungal taxa based on phylogenetic analyses. This is partly because DNA fingerprinting techniques do not provide any real quantitative data regarding community function; it is time-consuming and requires expertise. It is also far easier to generate a putative uncultured sequence than to understand its biological significance from a practical standpoint. Most of the molecular techniques involved do not discriminate between active and inactive stages. This hampers a proper interpretation of the genetic/phylogenetic diversity with respect to ecology and function. For instance, DGGE analyses from pine needles in our laboratory revealed several dominant phylotypes associated with decay stages, but it is still speculative which ones are actively involved in decomposition.

1.6 Conclusions and Future Directions

Current knowledge pertaining to the *diversity*, *detection* and *distribution* of soil fungi and the *dynamics* of soil ecosystem is still rudimentary. Obviously improvement in traditional approaches combined with other biochemical/serological methods and incorporation of various molecular techniques (DNA-based) has provided new data on these aspects but, for a clearer picture and a better understanding, a combination of all approaches (polyphasic) is essential. There is a need to unravel the taxonomic diversity of speciose groups. Diversity of nematode-trapping fungi from soil (either terrestrial, estuarine or marine) is purely based on morphology and cultural studies and the most common species isolated are from *Arthrobotrys, Dactylaria* and *Monacrosporium*. To date, there are no reports on the feasibility of specific primers targeting other nematode-trapping fungi (most importantly those that are possibly unculturable). Given their relative pathological and biotechnological importance, molecular tools should be employed to assess their genotypic diversity in soil. Fungal diversity studies in soil have previously been carried out mainly in terrestrial habitats, especially those around plant roots. Future studies should target different habitats such as freshwater, estuarine or marine environments.

Our knowledge is extremely limited and we are a long way from realising the components of the soil mycota.

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