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# Sequences, the environment and fungi

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ABSTRACT

In this article we review briefly the retrieval and analysis of molecular signals from the environment in relation to fungal diversity. Such molecular diversities have been assessed for terrestrial and aquatic systems, at various study levels, using PCR-based and nucleic acid hybridization-based techniques. The approaches adopted in some of these studies will be compared, including mention of the problems encountered, and discussed in broad terms detailing the identification of sequences representing fungal groups and their activities.



A central problem in microbial ecology is the accurate determination of diversity that can be related to environmental processes. Without this knowledge it is difficult to define ecologically active groups or the factors that affect ecosystem community stability. The adoption of molecular techniques in ecology provides a framework to link organisms to the processes they control, by the identification of their molecular signals present in the environment (Kennedy & Clipson 2003). This article explores briefly how these techniques are used to identify fungi, and their activities, in the natural world, including some cautionary comments on their application.

## 1. Molecular techniques used to studying fungal diversity

A variety of molecular techniques exists that can be used to assess diversity and follow the fate of fungi in natural systems (Anderson & Cairney 2004). These fall into two main categories, excluding immunological methods: (1) polymerase chain reaction (PCR) based approaches and (2) DNA/RNA probe hybridisation technology. Techniques in these categories rely upon the design and application of short DNA fragments that can selectively identify nucleic acids from mixed sources. Hybridisation techniques (such as macro- and microarrays, and fluorescence *in situ* hybridisation - FISH) use DNA probes (radioactively or chemiluminescence tagged, short single-stranded DNA molecules that specifically anneal to their complement) to find identical, or near identical, sequences amongst complex mixtures, and they result in a hybridisation signal that is detected via autoradiography, immunology or chemiluminescence methods. PCR-based methods use primers (short oligonucleotides that bind at the beginning and end of a particular sequence) to initiate enzymatic replication of a target sequence *in vitro*. The PCR product can be recovered for further analysis. Techniques in both categories can incorporate fluorescence reporter dyes in the nucleotides so that they may be easily visualised. These fluor-based detection methods can help increase the speed of sample processing

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and improve the sensitivity of an assay. Although both types of methods can be used to study complex nucleic acid mixtures from environments without culturing (Amann *et al.* 1995), it is the PCR-based approaches that have dominated molecular diversity assessments.

## 2. PCR-approaches to assess molecular diversity

A scheme highlighting the various stages of a PCR-based analysis is shown in Fig. 1. In brief, these studies start with the extraction of nucleic acids from the environment followed by PCR amplification of a target molecule and its *separation* into individual entities for characterisation and identification. It is important to include an ecological hypothesis to be tested, reflected in a realistic sampling regime, into this strategy (Horton & Bruns 2001).

### *Environmental nucleic acid extraction*

DNA or RNA can be extracted from terrestrial and aquatic environments (soils, sediments, roots, leaves, stones and water) using a variety of purpose-designed kits (e.g. Mo Bio Laboratories Soil DNA and RNA isolation kits or Qiagen DNeasy™ Plant mini kit), as well as other protocols (Yeats *et al.* 1998; Griffiths *et al.* 2000). These extracted nucleic acids are of mixed origin, comprising DNA or RNA from bacteria, animals, plants, fungi and other microeukaryotes, and they are complex. The efficiency with which the nucleic acids are extracted depends upon the species present, the environmental substrata sampled, as well as the method used. It is wise, therefore, to assess a number of extraction techniques for a particular system before embarking on a full analysis. This is because the extraction efficiencies for yield and purity can vary enormously, even between replicate samples. Such variation can dramatically skew molecular diversity assessments if this is not taken into account (Kline & Paschke 2004).

It is often claimed that environmental DNA detects the 'footprints' of living, dead or resting cells, whereas RNA records the active ones, but care should be taken when interpreting this statement. Fungal spores, for instance, contain transcripts for a range of housekeeping genes (coding for general metabolism products) including ribosomal RNA molecules (Osherov *et al.* 2002). Detecting these sequences after PCR amplification of environmental RNA certainly denotes the recent presence of a population but not, necessarily, its activity.

Obtaining PCR amplifications from environmental sequences often depends upon the purity of the environmental nucleic acids. During extraction PCR-inhibitory components, such as humic acids, polysaccharides and tannins, can be co-precipitated with the DNA and RNA. Removal of these impurities may be achieved by dilution or the inclusion of selective detergents, such as cetyltrimethylammonium bromide (CTAB) for the removal of polysaccharides, or polyvinylpolypyrolidone (PVPP), which binds polyphenols, in the extraction buffer. Clean-up columns, such as silica-based Sephadex G-200 or hydroxyapatite examples, can also be used to separate DNA from inhibitory contaminants. The best method of purification, however, is by caesium chloride density centrifugation if the amount of environmental material is plentiful.

### *Choosing a target sequence for PCR amplification*

The next stages of analysis require careful consideration. Choosing the target sequence and a molecular separation technique requires some prior knowledge of fungal sequence variation, and of the environment to be assessed. The choice of both will depend upon the ecological hypothesis to be tested, and the level of sequence discrimination needed to identify the environmental signals.

Broad-based diversity studies can be performed using the nuclear large (the 25S or 28S rRNA subunit - LSU) or the small (the 18S rRNA subunit - SSU) ribosomal RNA gene. These genes form part of a tandemly repeated cluster that also contains the 5.8S rRNA gene interspersed with transcribed and non-transcribed spacer regions (Fig. 2). They have been used predominantly in phylogenetic studies to determine evolutionary relationships between taxa, and these sequences provide the 'backbone' for identifying environmentally amplified rDNA signals.

Different levels of sequence variation are observed for these genetic regions. The nuclear small subunit rRNA gene is the most conserved and resolves little phylogenetically beyond the family level (Horton & Bruns 2001). The nuclear large subunit rRNA gene is more variable, particularly in domains 2 and 8 (Hopple & Vilgalys 1999), and contains sufficient variation to discriminate sequences at the genus level. Greater sequence variation is needed to identify environmental signals representing species, isolates, strains or biovars. The internal transcribed spacer regions (ITS regions 1 and 2, Fig. 2) display the greatest sequence and size variation for this gene cluster. These regions can be used to identify sequences (via BLAST homology searches) at the species, and sometimes strain, level; it should be realised, however, that the variation here is due to the presence of indels (inserted or deleted regions) and repeated sections as well as nucleotide substitutions. The frequent occurrence of indels and repeated sequences can make broad-based alignments, and hence phylogenetic analysis, difficult (Bruns 2001). Furthermore, recently evolved species might not have sufficient variation within the ITS regions to allow strain or biovar identification.

Other genes, or their products, can be used to make assessments of fungal diversity. Once again the level of sequence divergence observed for a particular gene will determine the possible level of fungal group identification. Broad-based diversity studies have been performed using laccase gene sequences (Lyons *et al.* 2003; Luis *et al.* 2004), but faster evolving regions are needed to define the boundaries at the species and population level (Carbone & Kohn 2004). The nucleotide substitution rate for the ITS spacers is comparable to that observed for exons (coding regions of eukaryotic genes), whereas spliceosomal introns (non-coding regions of genes that are removed from transcripts by the spliceosome – a RNA-editing complex) and repetitive DNA (mini- and microsatellites) accumulate mutations at a higher rate (Kasuga *et al.* 2002). It is these regions that are traditionally targeted

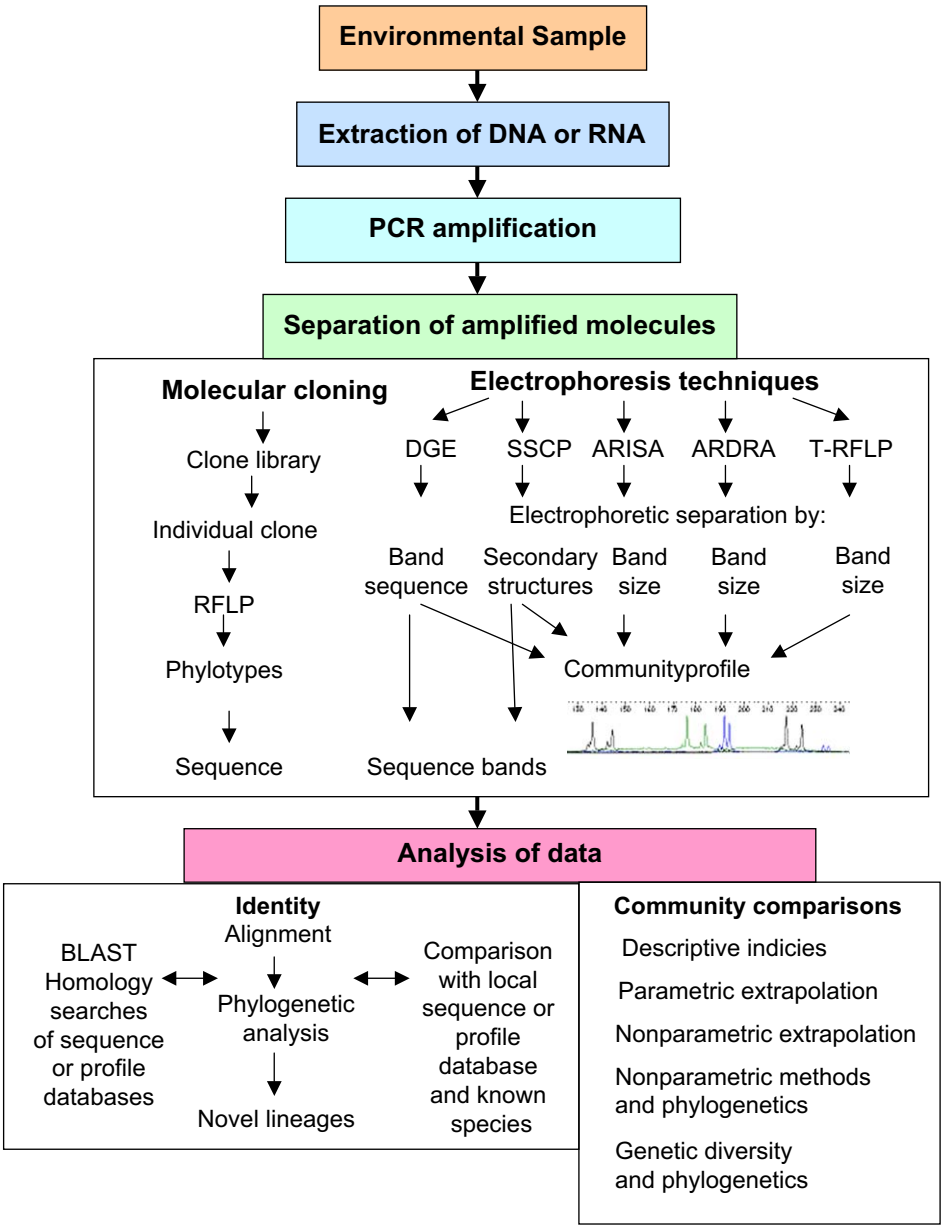


**Fig. 1 – PCR-approaches to environmental nucleic acid analysis. DNA or RNA is extracted from the environmental source and is subjected to PCR amplification to produce a heterogeneous mixture of sequences. These are separated into individual molecules by cloning or electrophoresis techniques (DGE – denaturant gradient electrophoresis; SSCP – single stranded conformational polymorphism; ARISA - amplified ribosomal intergenic spacer analysis; ARDRA - amplified rDNA restriction analysis; T-RFLP – terminal restriction fragment length polymorphism). The electrophoresis techniques give banding patterns that represent the individually separated sequences, and these profiles can be used to characterise the PCR-amplified DNA from the environment. It is these profiles that are used to make diversity assessments after the molecules have been identified by sequencing or comparing the fragments electrophoretic mobility. Alternatively, the number of unique sequence groups from a clone library can be counted and used to estimate diversity indices.**

for population studies where morphotypes can be easily distinguished.

Deciding which genes should be targeted for strain identification requires a different approach; one that allows the effects of recombination and heterogeneous nucleotide substitution rates to be taken into account (Taylor *et al.* 1999). Multi-locus sequencing typing (MLST) is a PCR-based technique that makes use of rapid sequencing technology to uncover allelic variants for a variety of conserved genes present in isolates (Tavanti *et al.* 2003). This information identifies polymorphisms that can be used to classify, subtype and characterise fungal populations (Taylor & Fisher 2003). It also provides a basis to discover which protein-coding genes might be useful as molecular probes for environmental studies.

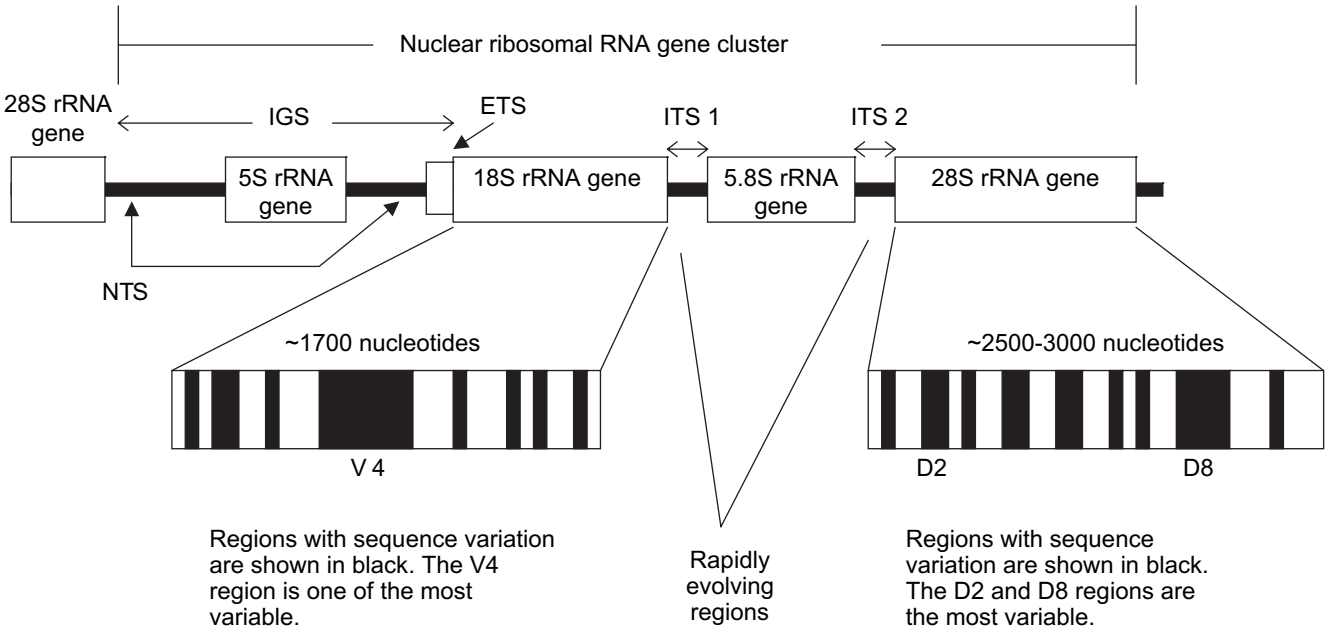


**Fig. 2 – The ribosomal RNA gene cluster. The cluster comprises three main genes (5.8S, 18S and 25S or 28S rRNA molecules) interspersed between spacer regions (IGS – intergenic spacer, NTS – non-transcribed spacer, ETS – externally transcribed spacer, ITS – internally transcribed spacer). The degree of sequence conservation varies between these genetic regions and within the genes. The spacer regions are the least conserved and evolve at a faster rate than the genes. The IGS region comprises the NTS regions and the 5S rRNA gene. The NTS and ETS contain repeated sequences that are important in gene expression. The ITS regions contains variable sequences that also have a fast rate of evolution. It is these spacers that are useful in determining the evolutionary relationships at the species and below level. The genes comprise variable (the V and D regions) and conserved sections. The least conserved of these are the V4 region in the 18S rRNA gene, and the D2 and D8 regions in the 28S rRNA gene. The PCR primers used in environmental studies are designed using sequences from the conserved regions (shown in white) so that the amplified product spans these variable sections. The 18S rRNA molecules are used in evolutionary studies to delineate at the kingdom to family level, whereas the variation in the 28S rRNA molecule can separate sequences representing groups from the family to genus level.**

### *PCR primers for molecular diversity studies*

A variety of primers exist that can amplify regions of the rRNA gene cluster from single templates (http://mollie.berkeley.edu/~bruns/primers.html; http://www.biology.duke.edu/fungi/mycolab/primers.htm). Not all of these, however, are suitable for working with environmental DNA or RNA. The amplification or detection of fungal sequences, particularly rare ones, from mixed-origin samples requires that the primers exhibit fungal specificity. Many primer combinations have not been fully validated against other organisms, and some, particularly the nuclear SSU primers, can amplify sequences from a variety of plants, animals and other microeukaryotes (Lord *et al.* 2003; Anderson *et al.* 2003a; Zuccaro *et al.* 2003). The nuclear LSU and ITS primers appear to be more fungal specific, although, as these have not been fully validated against other organisms, it is possible that they might allow cross-kingdom amplifications. Table 1 lists some primer combinations that have been used successfully in diversity studies.

A popular choice of primer sets include those that amplify the nuclear SSU rRNA gene or the ITS regions. Phyla-favouring primers have been designed for the ITS region allowing selective amplification from species of the Basidiomycota or Ascomycota (Gardes & Bruns 1993; Larena *et al.* 1999). The advantage of restricting the analysis to specific groups is that it simplifies the subsequent molecular separation and characterization of molecules, improving the resolution of molecular signals from complex environments. Taxon-specific primers might, however, be biased towards certain targeted groups, skewing the distribution of recovered sequences. Other ITS-5.8S rDNA primers with improved fungal specificity have been described that co-amplify sequences from the Ascomycota and Basidiomycota (Martin & Rygiewicz 2005).

### *Molecular separation and community profiling techniques*

The separation of mixed PCR-products, obtained after amplification of environmental DNA or RNA, can be achieved in a number of ways (Fig. 1). Gene libraries can be constructed by cloning individual molecules into a DNA vector that can be amplified independently. This method is time consuming but it does give a full record of what has been amplified from the environment. In this type of analysis it is usual to choose a number of environmental clones, characterise them by restriction fragment length polymorphism (RFLP) analysis, and group them into genetic types. A representative of each group can then be sequenced for further analysis.

**Table 1 – Published primer combinations used to PCR-amplify fungal sequences from the environmental Sources**

| Primer pair | Sequence (5′-3′) | Product (bp) | Reference and comment |
|---|---|---|---|
| **Nuclear 18S (SSU) rRNA gene** | | | |
| nu-ssu-0817 | TTAGCATGGAATAATRRAATAGGA | 420 | Borneman and Hartin 2000 (E, P) |
| nu-ssu-1196 | TCTGGACCTGGTGAGTTTCC | | |
| nu-ssu-0817 | TTAGCATGGAATAATRRAATAGGA | 760 | Borneman and Hartin 2000 (E, P) |
| nu-ssu-1536 | ATTGCAATGCYCTATCCCCA | | |
| EF4 | GGAAGGGRTGTATTTATTAG | 1500 | Smit *et al.* 1999 (DGE, E, P) |
| EF3 | TCCTCTAAATGACCAAGTTTG | | |
| EF4 | GGAAGGGRTGTATTTATTAG | 550 | Smit *et al.* 1999 (DGE, E, P) |
| Fung5 | GTAAAAGTCCTGGTTCCCC | | |
| NS1 | CCAGTAGTCATATGCTTGTC | ~1500 | White *et al.* 1990 (G, P) |
| FR1* [VH] | AICCATTCAATCGGTAIT | | Vainio and Hantula 2000 (DGE, E, P) |
| NS1 | CCAGTAGTCATATGCTTGTC | ~1600 | White *et al.* 1990 (G, P) |
| EF3 | TCCTCTAAATGACCAAGTTTG | | Gomes *et al.* 2003 (DGE, E) |
| NS2 | GGCTGCTGGCACCAGACTTGC | — | White *et al.* 1990 (G, P) |
| Fung5 | GTAAAAGTCCTGGTTCCCC | | Elas van *et al.* 2000 (DGE, E, P) |
| EF4 | GGAAGGGRTGTATTTATTAG | 500 | Brodie *et al.* 2003 (DGE, E) |
| NS3 | GGCTGCTGGCACCAGACTTGC | | White *et al.* 1990 (P) |
| EF390 | CGATAACGAACGAGACCT | — | Vainio and Hantula 2000 (E, P) |
| FR1* [VH] | AICCATTCAATCGGTAIT | | |
| NS1 | GTAGTCATATGCTTGTCTC | — | May *et al.* 2001 (DGE, E) |
| GC-fung | ATTCCCCGTTACCCGTTG | | |
| F1300 | GATAACGAACGAGACCTTAAC | — | Nikolcheva *et al.* 2003 (E, P) |
| Primer D† | CYGCAGGTTCACCTAC | | |
| NS5 | AACTTAAAGGAATTGACGGAAG | — | Nikolcheva *et al.* 2003 (DGE, E), |
| Primer D† | CYGCAGGTTCACCTAC | | White *et al.* 1990 (G, P) |
| **Nuclear 28S (LSU) rRNA gene** | | | |
| NL209 | AAGCGCAGGAAAAGAAACCAACAG | 700 | Zuccaro *et al.* 2003 (E, P) |
| NL912 | TCAAATCCATCCGAGAACATCAG | | Zuccaro *et al.* 2003 (E, P) |
| NL359 | GGACGCCATAGAGGGTGAGAGC | 559 | Zuccaro *et al.* 2003 (DGE, E, P) |
| NL912 | TCAAATCCATCCGAGAACATCAG | | |
| ITS1-F | CTTGGTCATTTAGAGGAAGTAA | — | Gardes and Bruns 1993 (P) |
| TW14 | GCTATCCTGAGGGAAACTTC | | Taylor and Bruns 1999 (E, P) |
| Ctb6 | GCATATCAATAAGCGGAGG | — | Taylor and Bruns 1999 (E, P) |
| TW13 | GGTCCGTGTTTCAAGACG | | |
| **ITS regions and 5.8S rRNA gene** | | | |
| EF3 | TCCTCTAAATGACCAAGTTTG | — | Anderson *et al.* 2003b (E) |
| ITS4 | TCCTCCGCTTATTGATATGC | | White *et al.* 1990 (P) (G) |
| ITS1-F | CTTGGTCATTTAGAGGAAGTAA | — | Gardes and Bruns 1993 (P), |
| ITS2 | GCTGCGTTCTTCATCGATGC | | Anderson *et al.* 2003b (E) |
| | | | White *et al.* 1990 (P) |

**Table 1 – (*continued*)**

| Primer pair | Sequence (5′-3′) | Product (bp) | Reference and comment |
|---|---|---|---|
| ITS1-F<br>ITS4-B | CTTGGTCATTTAGAGGAAGTAA<br>CAGGAGACTTGTACACGGTCCAG | — | Gardes and Bruns 1993 (P),<br>Klamer *et al.* 2002 (E)<br>Gardes and Bruns 1993 (P) |
| ITS1-F<br>ITS4-A | CTTGGTCATTTAGAGGAAGTAA<br>CGCCGTTACTGGGGCAATCCCTG | — | Klamer *et al.* 2002 (E)<br>Larena *et al.* 1999 (P) |
| NSA3<br>NLC2 | AAACTCTGTCGTGCTGGGGATA<br>GAGCTGCATTCCCAAACAACTC | | Martin and Rygiewicz 2005 (P) |
| NSI1<br>NLB4 | GATTGAATGGCTTAGTGAGG<br>GGATTCTCACCCTCTATGAC | | Martin and Rygiewicz 2005 (P) |
| NSI1<br>58A2R | GATTGAATGGCTTAGTGAGG<br>CTGCGTTCTTCATCGAT | ITS1 | Martin and Rygiewicz 2005 (P) |
| 58A2F<br>NLB4 | ATCGATGAAGAACGCAG<br>GGATTCTCACCCTCTATGAC | ITS2 | Martin and Rygiewicz 2005 (P) |

A more extensive list of primers can be found at:http://ocid.nacse.org/research/aftol/primers.php and http://plantbio.berkeley.edu/~bruns/.
P- paper describing the primer.
E- paper where the primer combination has been used in an environmental study.
G- universal.
DGE- where the product of the primer combination amplification has been separated using denaturing gradient electrophoresis.
T-RFLP- where the product of the primer combination amplification has been separated using terminal restriction fragment length polymorphism analysis.
* - a number of primers named FR1 exist in the literature.
† - originally described as primer 1860 by Elwood *et al.* (1985).

The other separation methods are based upon gel or capillary electrophoresis. The basis of the molecular separation is due either to differences in fragment size [such as in terminal restriction fragment length polymorphism (T-RFLP) analysis (Clement *et al.* 1998), amplified ribosomal intergenic spacer analysis (ARISA), and amplified rDNA restriction analysis (ARDRA) (Gich *et al.* 2000)], sequence [as in denaturant gradient gel electrophoresis - DGGE and temperature gradient gel electrophoresis – TGGE (Muyzer & Smalla 1998)], or secondary structure formation (single-strand conformation polymorphism-SSCP (Peters *et al.* 2000)). The electrophoresis-banding techniques are popular amongst ecologists for evaluating community structure, because the banding patterns from different samples can be compared in the same gel (Osborn *et al.* 2000; Fromin *et al.* 2002; Kennedy & Clipson 2003). Changes in community structure can be followed molecularly and correlated to environmental factors or host substrates (Smit *et al.* 1999; Elas van *et al.* 2000; Anderson *et al.* 2003b; Brodie *et al.* 2003; Gomes *et al.* 2003; Kennedy *et al.* 2005).

Choosing a particular technique, or primer combinations, depends upon the ecological questions to be answered, the nature and number of the samples and the community complexity. All of the techniques can be used to genetically characterise isolates, which is valuable when the environmental sequences are to be identified. The profiling techniques do not measure diversity *per se*, unless the community is simple, because only a fraction of the sequences present in an environment can be visualised on a gel or through a capillary column. Popular profiling techniques include T-RFLP and DGGE/TGGE analyses. The T-RFLP technique requires access to an automatic sequencer but it does allow quick sample processing. This is advantageous when species-rich environments are to be studied, or during investigations of species' spatial and temporal distributions where many samples need to be compared. DGGE and TGGE techniques are more appropriate for the analysis of less species-rich environments, but they appear to be less sensitive at detecting rare sequences. DNA fragments can be recovered for sequencing after DGGE or T-RFLP analyses.

The ecological application of molecular separation and community profiling techniques is not without problems. Some of these are related to band resolution and the detection of PCR artefacts, while others are associated with the subsequent analysis. PCR artefacts include: non-specific (the presence of spurious products), preferential (where replication of one template is favoured) and hybrid amplifications (where novel molecules are created – these are often referred to as chimeras). Many of these artefacts can be eliminated by careful optimisation of the replication reaction prior to the environmental analysis. The detection of hybrid sequences is more problematic. Although it is assumed that the frequency of their production is rare, they appear in databases at worrying proportions (Hugenholtz & Huber 2003). Demonstrating that an environmental sequence is authentic is best achieved by matching it unambiguously to a known species or isolate from the same environment. If this is not possible, the

sequence should be checked for mixed signals (Robinson-Cox *et al.* 1995; Komatsoulis & Waterman 1997; Roose-Amsaleg *et al.* 2004) and its frequency of recovery monitored. Chimeric molecules will contained mixed, conflicting signals and be localised in their distribution.

Unequal binding of the primers to different templates and size heterogeneity of the targeted molecules can lead to biased replication reactions. These biases can lead to differential PCR amplifications where some molecules are replicated preferentially. Most of the primer combinations listed in Table 1 have not been assessed for this type of error. Anderson *et al.* (2003a), however, reported little bias associated with four primer pairs amplifying nuSSU rRNA and ITS-5.8S rRNA gene sequences, although fungal specificity was problematic for one nuSSU rDNA pair.

With the band profiling techniques it is often assumed that a single band represents a single molecular or taxonomic entity, but this is not always true. The reason for this may be biological or physical. Some fungi may contain multiple genetically distinct nuclei or have a heterogeneous rDNA cluster (O'Donnell & Cigelnik 1997; Kuln *et al.* 2001). The amplification of rDNA sequences from such isolates will generate multiple bands for the same species. The electrophoresis techniques can also fail to resolve different sequences, so that the observed band contains mixed sequence types. It is important, therefore, to obtain accurate identities for each band so that the PCR artefacts and unresolved bands do not complicate the analysis. In general, molecular cloning should accompany DGGE and T-RFLP analysis to provide templates for comparison.

### *Analysis of environmental sequences*

The identity of an environmental sequence is obtained after a homology search of genetic databases, such as Genbank at the National Centre for Biotechnology Information (NCBI, http://ncbi.nlm.nih.gov/BLAST/) and ones held at the European Bioinformatics Institute (EMBL-Bank http://www.ebi.ac.uk/embl/), the Ribosomal Database Project (http://rdp.cme.msu.edu/; http://www.psb.ugent.be/rRNA/blastrrna.html), and the AFTOL project (http://aftol.biology.duke.edu/pub/blastUpload). This involves using a computer algorithm to search the database for similar sequences by aligning the unknown sequence with those held in the database. Searches performed by the Basic Local Alignment Search Tool (BLAST) at the NCBI return results listing the probable matches to the unknown with sequence similarity values.

One problem with fungal sequence identification is that the genetic databases are under-represented for many fungal groups. Genbank at the NCBI is the largest database containing a taxonomy section and it houses sequences representing 16 421 fungal species, although this figure is likely to be an overestimate (Hawksworth 2004). This database currently holds rDNA sequences mainly from the Ascomycota, representing approximately 63 % of all deposits with 34 % for the Basidiomycota and 1 % for the Zygomycota. This deposit deficiency can make the identification of environmental sequences difficult without access to other sources of information, such as that derived from a local culture collection. Furthermore, the taxonomic accuracy of many sequences in databases may be questionable. In a study of three major taxonomic groups, Bridge *et al.* (2003) recorded a sequence miss-identification level of 20 %. Although some flaws exist in the calculation of this figure, it is generally agreed that it could be true (Hawksworth 2004). Descriptions of molecular diversity based solely upon homology matches from current databases may therefore be misleading (Vilgalys 2003).

Creating sequence alignments from local databases, representing isolates and voucher species obtained from the study environment, provides one way by which unknown sequences can be checked. This needs to be done from a systematic base, and requires making extra efforts to compare environmentally obtained sequences with those from taxonomically defined isolates (Agerer *et al.* 2000). Sequence alignments for taxonomically defined species are available for some groups (mycorrhizal fungi – Bruns *et al.* 1998; yeasts - Scorzetti *et al.* 2002; and agarics - Moncalvo *et al.* 2002), and others may be obtained from databases such as TREEBASE (http://treebase.bio.buffalo.edu/treebase/) or through the DEEP HYPHA research coordination network (http://ocid.nacse.org/research/deephyphae/) and the Tree of Life programme (http://tolweb.org/tree/phylogeny.html). Search engines can also be found at some culture depositories. The Centraalbureau voor Schimmelculture (CBS), in collaboration with BioAware SA, has developed BioloMICS for polyphasic fungal identification (http://www.cbs.knaw.nl/yeast/wcbc.asp); this data handling software allows identification based on similarity using molecular, physiological and morphological data. It is linked to external databases (Genbank and PubMed) and external data-acquiring equipment (such as microtitre plates and electrophoretic gel readers) via BioGalaxy (ACQUI Vision, Liege, Belgium) software. At present this database contains information on 850 species (5500 strains) of yeasts, but similar ones will go on-line later for filamentous fungi. Another search machine can be found at ISTH (International Subcommission on *Trichoderma* and *Hypocrea* Taxonomy) at http:isth.info/. Without adopting this rigorous approach the value of sequence-based assessments of diversity will be less meaningful.

Alignments of environmental sequences with those from voucher and type species can be subjected to phylogenetic analyses that reveal the presence of novel environmental lineages. This approach may mean working closely with institutions that house fungal culture collections but it can be rewarding. By working with scientists from the Centraalbureau voor Schimmelcultures (CBS), Zuccaro *et al.* (2004) traced novel isolates of *Acremonium* from *Fucus* species to a marine clade of *Emericellopsis* – a genus comprising mainly terrestrial members. Nuclear large rDNA subunit sequences from this group matched those detected associated with *Fucus serratus*, and potentially identified a novel group of marine fungi.

Once the environmental sequences have an identity, however loose, it is possible to compare electrophoretic community profiles or gene libraries. This type of analysis is important because it allows ecological and biodiversity questions to be answered. Choosing an appropriate statistical analysis for the molecular data remains a challenge because of the inherent difficulties associated with evaluating small sample sizes. Most methods rely upon multivariate statistical analyses, as discussed by Fromin *et al.* (2002) and Hughes *et al.* (2001). Three basic statistical approaches to studying microbial diversity

have been adopted: parametric methods using species-abundance models to estimate diversity, non-parametric methods using detection probabilities to estimate diversity, and community phylogenetic analysis (Bohannan & Hughes 2003).

One limitation of the parametric and non-parametric tests used to assess diversity is that the bands or sequences representing the species are counted equivalently. This assumption can lead to an underestimation of diversity or the omission of genetic disparity measurements when comparing communities. For instance, some sequences can be highly divergent and phylogenetically unique, whereas others form parts of closely related species groups. Communities with similar species richness and rarefraction profiles but which differ in the quantity of these sequence types are not equally diverse; ones with a greater proportion of phylogenetically unique sequences are genetically more diverse. This part of diversity can be taken into account by using phylogenetic tests (Martin 2002), and this is important when demonstrating associations between community composition and ecosystem function. It is possible to link environmental sequences or profiles directly into a phylogenetic context using the search algorithm in ARB (Ludwig *et al.* 2004).

## 3. DNA/RNA hybridisation probe technology

Nucleic acid hybridisation techniques, using fluorescence-tagged probes, provide high-throughput screening methods that can increase rates of sample processing, and avoid some of the problems associated with the PCR-based approaches. In particular high-density macro- and microarrays allow the rapid screening of mixed sequences to detect large numbers of specific genes, or their transcripts (Hardman 2004). These techniques were originally developed from genome sequencing projects to study gene expression (Luo *et al.* 2005), but they can be adopted for use in ecology.

Array-based technology has been used to identify fungal ribosomal RNA genes from soil-extracted and seawater-extracted DNA (Valinsky *et al.* 2002; Kiesling *et al.* 2002). Environmental DNA or RNA can be screened against several thousand probes fixed to a solid support (Fig. 3). The target probes can comprise ribosomal RNA sequences, or functional genes such as those coding for metabolic enzymes, pathogenicity factors and resistance determinants. The power of these techniques is that all of these genes can be processed in one single step. Using these systems, environmental nucleic acids can be rapidly screened and quantified for a range of functions. This 'multiple-probe' approach has already been adopted for use in bacteriology, where *Phylochips* and databases are available to identify sulphur-reducing bacteria and members of the Rhodocyclales (Loy *et al.* 2002; Loy *et al.* 2005). Although the application of these techniques has great promise for environmental work, several problems exist in their development, such as reducing the level of false hybridisation signals. Standardising the hybridisation conditions for many gene probes, particularly the conserved regions of rDNA sequences, is problematic and it requires using probes of similar length and G + C content. As with all hybridization techniques, prior sequence knowledge is required to design the probes, but this information is becoming available via genome sequencing projects and the technique promises to be an excellent tool in recording functional diversity.

The attachment of fluorescent tags to probes allows the direct microscopic visualization of fungi *in situ*. The FISH technique was originally developed to identify the location of genes on chromosomes, but it can be used to study fungal interaction, as well as to identify fungal mycelium present in the environment (Li *et al.* 1997; Sterflinger *et al.* 1998; Schroder *et al.* 2000; Tsuchiya & Taga 2001). The development of fungal probes for this technique is behind that in bacteriology, but some phyla, genus and species specific probes exist based upon nuLSU rRNA sequences important in secondary structure formation (Baschien *et al.* 2001). Two development-limiting factors here are the high conservation observed in the nuclear ribosomal RNA molecules and the unsuitability of the ITS regions for use, which are absent in ribosomes – the target organelle for this type of analysis.

## 4. Molecular rRNA diversity of fungi

The fungal diversity of natural environments described by nuSSU rRNA and ITS sequences appears to be extensive. Vandenkoornhuyse *et al.* (2002a,b) reported novel nuSSU rRNA gene lineages of arbuscular mycorrhizal (AM) fungi (non-culturable obligate symbionts) associated with the roots of *Arrhenatherum elatius*, and concluded that the non-culturable forms dominated this environment. The diversity of AM fungi recorded molecularly suggests that there is a non-random association with host species in temperate and tropical zones; and that the community structure can change through space and time (Helguson *et al.* 2002; Daniell *et al.* 2001; Husband *et al.* 2002).

Novel nuLSU rDNA lineages of ascomycetes have been detected in tundra soils, appearing during the winter and early spring (Schadt *et al.* 2003). The nature of these lineages remains unknown. Sequence identification is only possible once its characteristics have been matched to a known, and preferably voucher, species. It is possible that some environmentally amplified sequences represent fungi that are known morphologically but not molecularly. Nevertheless, nuLSU rDNA sequence information, followed by phylogenetic analysis of voucher species, certainly helps to define potential novel lineages or groups (Zuccaro *et al.* 2003).

A greater diversity of phylotypes is detected using ITS sequences (Lord *et al.* 2003). Obtaining identities for each environmental variant from databases can be difficult however, because of the molecular under representation of fungal groups (Bruns 2001). For example, Guo *et al.* (2001) managed to obtain identities to the family or genera level for ITS sequences amplified from *Livistonia chinesis* tissues, but many were left unmatched. Similar observations were made by Roose-Amsaleg *et al.* (2004) when assessing fungal diversity associated with termite mounds. Obviously, better identity matches will be obtained using ITS regions as the number of sequences in databases increases.

The absence of taxonomic molecular identities does not necessarily hamper the initial descriptions of community structure using molecularly defined mycelium. Undifferentiated mycelium, clones or sporocarps may be characterised by

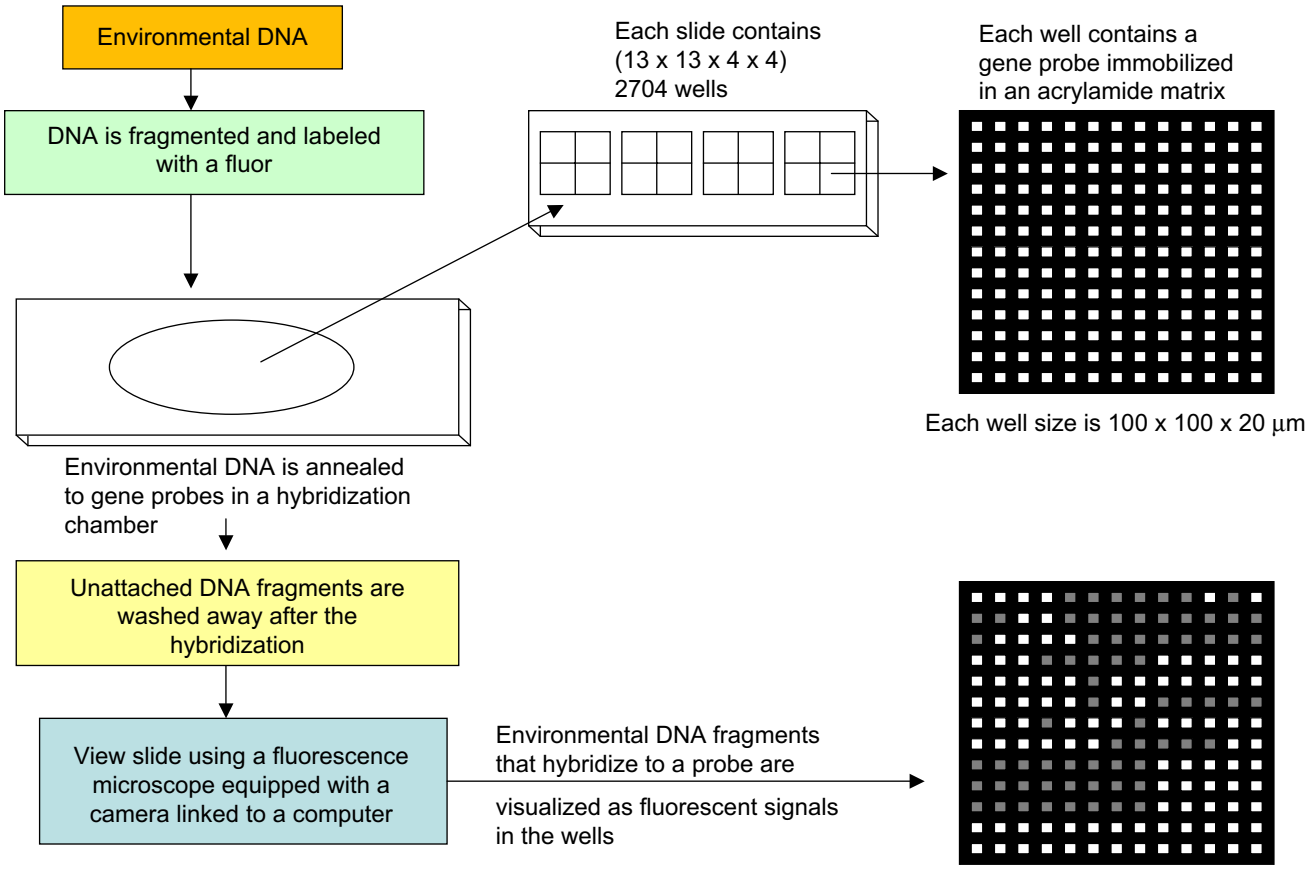


**Fig. 3 – Analysis of environmental DNA using microarray hybridization displays. The environmentally extracted DNA is fragmented, denatured into its component strands and labelled with a fluorescence dye. After it has been labelled it is mixed with many DNA probes located on a chip in a hybridisation chamber on a microscope slide. Each chip contains many wells that house individual probes. During the hybridisation reaction, complementary strands combine and this allows the environmental DNA to become fixed to the chip. DNA that has not been fixed in this way is washed away after the reaction. The slide containing the chip is viewed under a microscope and the fluorescence of each well recorded. If a gene is present in the environmental DNA it will bind to the appropriate gene probe and fluoresce. Different coloured dyes can be incorporated into DNA so that an array of coloured signals can be recorded that represent the gene activity or species presence in a given environment.**

identifying unique restriction enzyme banding profiles (RFLP or T-RFLP analysis) and/or sequencing. These types may be matched with those obtained from the environment or those represented in a clone library, as well as those present in sequence depositories. For example, ectomycorrhizal fungi (EM) comprise mainly vegetative forms with few sporulating types. These morphotypes can be characterized molecularly, with ITS RFLPs, giving an identity that can be used to map community structure. The molecular distribution of EM fungi recorded in this way differs according to the soil horizon with little correspondence between the dominant fruiting species and the vegetative forms (Horton & Bruns 2001).

In a large-scale environmental sequencing project, O'Brien *et al.* (2005) assessed fungal soil diversity at two temperate forest sites. Using ITS sequences they identified 412 types with the predominant groups found within the Basidiomycota and Ascomycota. Many of these sequences closely matched mycorrhizal, plant pathogenic and saprophytic fungi, although the high rate of novel sequence detection suggested that fungal diversity was much greater than expected. Other fungal phyla were recovered at much lower proportions. Compositional differences were also observed according to soil depth. Greater richness was observed in deeper soil samples, which comprised mainly mycorrhizal fungi.

Detailing the molecular diversity associated with particular substrata can provide information that is otherwise unobtainable. Bjelland and Ekman (2005) examined the diversity in a rock beneath a crustose lichen. They observed that the hyphae of *Ophioparma ventosa*, a lichen fungus, penetrated 10-12 mm into the rock and was thicker than that of the symbiotic thallus on the surface. Molecular signals for 13 non-lichen fungal groups were also observed; 5 of which could not be matched to known species.

Buchan *et al.* (2002, 2003) reported on the fungal diversity associated with *Spartina alterniflora*. The approach adopted in this survey was to match ITS profiles and sequences with those from fungal isolates allowing the identification of a new species and the presence of cryptic sub-groups. The

availability of a local collection of isolates, cultured from environmental samples used to assess the molecular diversity, is one way to overcome the immediate problems associated with obtaining accurate sequence identity matches from genetic databases. It also gives an opportunity to compare sequence diversity with that obtained culturally.

In a broad-based study of ascomycetes associated with *Fucus serratus*, Zuccaro *et al.* (2003), Zuccaro and Mitchell (2005) amplified nuLSU rRNA sequences from the same algal tissue used to culture isolates. Few sequences matched those from the isolates, although the same broad taxonomic groups were recovered culturally and molecularly. Interestingly, only one unambiguously matched sequence came from the genetic database; the rest were matched by comparing the molecular profiles from the isolates with the retrieved sequences and taxonomically defined, or identified, species. Other authors have noted the importance of combining molecular and traditional mycological methods. Nikolcheva *et al.* (2003) united nuSSU rDNA-PCR T-RFLP and DGE analyses with culturing techniques to examine freshwater hyphomycete leaf succession. They observed, however, inconsistent differences in species richness and evenness with both approaches. Allen *et al.* (2003) also noticed a culturing bias when comparing the distributions of cloned environmental ITS sequences from the roots of salal (*Gaultheria shallon*) with isolates cultured from the same material.

It is difficult to comment on the reliability of molecular diversity assessments at present. This, however, will become apparent as ecosystems are probed for different environmental signals using a variety of primer systems. Nevertheless, the use of molecular techniques has highlighted the presence of a large number of fungal groups not previously characterized molecularly. In this respect, fungi are no longer hidden in environments but are starting to be revealed. The next question to be answered about these fungi concerns their role in natural environments.

## 5. Beyond ribosomal RNA gene diversity

Descriptions of diversity based upon ribosomal RNA sequences do not necessarily account for biochemical or physiological groups that affect ecological processes. One way of relating mycological activity to diversity is to target genes (or their products) that are directly involved in an ecological process. Many protein-coding genes display more variation in their sequence than the slower evolving ribosomal RNA genes (Kasuga *et al.* 2002). Using this variation, it is possible to discern ecologically distinct fungal groups, species or populations using phylogenetics provided that the sequences are related by descent (Taylor *et al.* 1999). In assessing the diversity associated with *Spartina alterniflora* present in a salt marsh, Lyons *et al.* (2003) amplified laccase sequences for analysis. This enzyme is involved in the breakdown of plant material, and the diversity of its sequences revealed the presence of cryptic fungi previously undetected. Luis *et al.* (2004) investigated the diversity of this gene from basidiomycetes in forest soil horizons. They observed greater diversity amongst the $O_h$ horizons with reduced levels from the lower $A_h$ and $B_V$ horizons. Both of these studies produced results in agreement with the rDNA studies, but they also indicated the presence of potential functional groups. This approach allows for more detailed studies on these potential groups and plant decay processes.

Evaluating the activity of fungi directly from the environment is possible by extracting mRNA transcripts, produced by expressed genes, from the sample. Transcripts for lignin peroxidases, cellobiohydrolyases and Beta-tubulin have been detected from fungal-colonized soils and wood (Lamar *et al.* 1995; Bogan *et al.* 1996; Vallim *et al.* 1998). Monitoring these in natural environments promises to provide valuable information on fungal colonization, substrate choice and the mechanisms of cell signalling important in fungal associations (Harrier 2001). Incorporating molecular quantitative techniques, such as hybridisation methods and real-time PCR (Wittwer *et al.* 1997; Atkins *et al.* 2003), is of great importance in this area so that environmental gene expression levels may be assessed (Read & Perez-Moreno 2003).

The extent of microbial diversity is great, although much of it might be functionally redundant at any given instance of time (Nannipieri *et al.* 2003). The accurate identification of functional groups and their roles in community maintenance are needed to help explain why diversity is so extensive. Obtaining a direct link between an ecological process and the organisms responsible is difficult, however, without the development of new techniques. One such synergetic technique, stable isotope probing (SIP), has already demonstrated fungal (*Fusarium* sequences) activity for methanol breakdown in soil (Leuders *et al.* 2004). This technique provides a direct link between the degradation of a labelled compound and the organisms responsible by following the fate of heavy density isotopes into DNA and RNA. Separation and characterization of the heavy density nucleic acids can subsequently be used to identity the fungal group involved in the process.

New insights into fungal biology have been obtained by incorporating molecular techniques into synecology. These include, amongst others: identifying the extensive genetic diversity of the arbuscular mycorrhiza, their plant specificities and extended roles in carbon, nitrogen, as well as phosphorus nutrient cycling (Fitter 2005); and the relationships that exist between ectomycorrhizal fungi, forest trees and orchids (Bidartondo *et al.* 2004). Questions relating to the mechanisms involved in maintaining and stabilising biodiversity, however, have yet to be addressed. Identifying such mechanisms will involve building models, which can be tested, on how ecosystems function. Such models are particularly important where biological systems are managed, as in forestry, conservation and agriculture, where little is known about functional fungal diversity.

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