Molecular approaches to assessing fungal diversity in the natural environment

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This paper explores the possibility of using molecular techniques to assess fungal diversity in natural environments. The use of molecular tools for the isolation and analysis of fungal DNA from different environmental substrates and matrices is examined. A comparison of techniques employed in related fields is also presented. This paper also discusses some of the limitations in using molecular tools to assess fungal diversity, as well as highlighting several distinctive advantages.

Introduction

"Fungi in the family tree" (Blaney, 1995) and "Bamboozled again! Inadvertent isolation of fungal rDNA sequences from bamboos" (Zhang, Wendel and Clark, 1997) are two recent eye-catching titles in the literature which appeared to be of cursory interest to mycologists. Both are reports cautioning plant phylogeneticists to be aware of the risk involved in using universal rDNA primers in amplifying plant DNA. In tracing phylogenies within the plant kingdom, plant molecular phylogeneticists have been inadvertently isolating fungal DNA concomitantly with plant DNA. In the above examples, this occurred in spruce and bamboo. The true identity of these DNA molecules was not realised until alignment of the DNA sequences was made with various reference fungal sequences. For mycologists, however, these have important implications in developing novel techniques to measure and monitor fungal diversity in natural environments. The fortuitous phenomenon of identifying fungal entities by direct extraction and analysis of DNA molecules must be considered ground breaking and important in the light of the destruction and disappearance of some of the ecosystems in which fungi are found.

The number of fungi presently known is staggeringly low in proportion to the number that actually exists (Hawksworth, 1991). Only 5 to 20 % of fungal species have been discovered and described out of the estimated 1.5 million

extant species. This is especially true of the tropics (Hyde, 1995). The 1.5 million figure may even be conservative (Hyde and Hawksworth, 1997). However, eventhough mycologists are actively collecting and constantly describing numerous new taxa particularly in the tropics, no new methodologies solving age-old problems, such as the presence of non-culturable taxa and non-sporulating isolates (mycelia sterilia) have been developed. In current mycological literature, one cannot help but be bombarded by a plethora of reports on the use of molecular tools. These include the specific detection of fungal species or subspecies, either in their natural habitat or *in vitro*; genetic diversity of species; and phylogenetic relationships of fungal taxa at all taxonomic levels. However, there has been little development in the area of molecular detection and identification of unknown fungal taxa in natural environments. Bacteriologists, on the other hand, have wasted no time in developing new methodology and, armed with ribosomal DNA primers and probes, have proceeded to investigate microbial diversity in soil matrices and aquatic environments. This has resulted in the discovery of new bacterial taxa, many of which are not amenable to *in vitro* culturing (Liesack and Stackebrandt, 1992; Lee *et al.*, 1996; Wise, McArthur and Shimkets, 1997).

Molecular techniques

The fundamental concept of using DNA based techniques in detecting the presence of a particular DNA molecule lies in the specific hybridisation of the double stranded DNA as facilitated by complementary nucleotides within the respective strands of DNA. When rendered single stranded, these molecules will only hybridise with other DNA molecules with complementary nucleotide sequences under specific conditions. In practice, only short DNA sequences (approx. 50-2000 bp) from the entire fungal genome of 0.1-10 $\times$ 10^8 bp (Cavalier-Smith, 1985) are used in the detection and identification of fungal taxa. This seemingly untenable approach is justified by the presence of sequences from which phylogenetic information can be inferred. These sequences have the same function in all taxa, evolve at a consistent rate, and are present in the genome either as a single copy or several copies having evolved together (for further details, see Bruns, White and Taylor, 1991; Palumbi, 1996). In the fungal kingdom, examples of DNA sequences fulfilling the above criteria are regions of the ribosomal RNA genes (rDNA) from the nuclear and mitochondrial genomes, the cytochrome oxidase genes, the actin genes, and certain ribosomal protein elongation factors. These DNA sequences, particularly the nuclear encoded rDNA, will be discussed in detail later. Short DNA sequences can be detected

and multiplied several million times by the polymerase chain reaction (PCR) (Mullis and Faloona, 1987), a molecular tool of unrivaled potential.

This paper explores the different ways in which molecular techniques can be used in assessing fungal diversity in natural environments. A critical assessment of the criteria from a practicable and applicable viewpoint will be made, and a review and comparison of the techniques employed in the fields of mycology, bacteriology, medical microbiology, and molecular ecology will be presented. The term diversity will generally refer to species diversity and less emphasis will be placed on intra-specific diversity. For the sake of defining a more feasible scope, the molecular techniques discussed herein will only refer to techniques involving nucleic acids, in particular DNA, and thus exclude those involving proteins.

Isolation of environmental nucleic acids

Hyde and Hawksworth (1997) listed 31 microhabitats categorised into eight principal niches in which fungi are found in a tropical forest. These are also applicable in most other ecosystems and can generally be grouped into four types of matrices or substrates for the purpose of DNA isolation: soil, water, and animal and plant hosts.

Isolation of community DNA from soil and sediment

Steffan and Atlas (1991) reviewed the different techniques employed in the extraction of nucleic acids from soil and sediment samples. Two basic approaches have been used. The cell extraction approach involves a prior separation of microbial cells from an environmental sample before cell lysis, followed by extraction and purification of nucleic acids (Faegri, Torsvik and Goksøyr, 1977). The direct extraction approach bypasses the need for cell separation from an environmental matrix and involves the direct lysis of microbial cells in the matrix, followed by extraction and purification of nucleic acids (Ogram, Sayler and Barkay, 1987). The latter approach generally results in a higher DNA yield, but of lower purity (Leff *et al.*, 1995). Protocols for these two approaches have been compiled and outlined by Atlas (1993), Van Elsas and Smalla (1995) and Holben (1997). Procedures for the isolation of DNA from sewage and manure slurries have also been outlined by Smalla (1995). It is worth noting that the two basic approaches were initially designed for the isolation of prokaryotic DNA. Since most fungi have a vegetative filamentous stage and are not easily extracted from the soil matrix, the cell extraction method is not suitable for isolation of fungal DNA. A combination of the two approaches, in which all organic matter is first separated by washing and sieving,

followed by DNA isolation, has also been used to specifically detect the presence of *Rhizoctonia solani* in soil samples (Whisson, Herdina and Francis, 1995).

The isolation of DNA from filamentous organisms in soil using the direct extraction approach was first performed by Hilger and Myrold (1991). They successfully isolated DNA from the actinomycete *Frankia* in soil. Although no study has been conducted to date on the diversity of soil fungi *per se* based on direct isolation of DNA from soil or sediment matrices, the direct isolation of fungal DNA from soil has been reported (Johnston and Aust, 1994; Volossiouk, Robb and Nazar, 1995; Borneman *et al.*, 1996). Borneman *et al.* (1996) have identified several rDNA sequences of eukaryotic nuclear origin, one of which was fungal, from an agricultural soil sample. This was in spite of the fact that they were primarily interested in isolating DNA from currently nonculturable bacteria. The adsorption of DNA to soil particulates has been identified as a problem in extracting community DNA from soil in general (Ogram *et al.*, 1988, 1994). Volossiuok *et al.* (1995) isolated total DNA from soil samples and specifically detected the presence of *Verticillium dahliae*. In their isolation, skim milk was added to the initial cell lysate mix in order to eliminate losses of nucleic acids due to degradation by and adsorption to soil particles. Johnston and Aust (1994) extracted DNA from *Phanerochaete chrysosporium* in soil samples but the samples had been artificially inoculated with the fungus and enriched with an external nutrient source. Porteus and Armstrong (1993) also isolated fungal DNA directly from soil samples. However, whether the origin of these DNA sequences was truly fungal was unclear since the primers used were not conserved for fungi only but applicable for algae and protists as well. The Porteous and Armstrong (1993) method has been successfully adopted to extract bulk soil DNA containing endomycorrhizal fungal DNA (Classen, Zasoski and Tyler, 1996).

Variations in the direct extraction approach, primarily in the initial cell lysis step, have been developed. Ogram *et al.* (1987) developed a method which involved treatment with SDS followed by physical disruption with a bead beater. Alternative methods using lysozymes coupled with freeze-thaw disruption (Tsai and Olson, 1991), incorporation of bead-mill homogenisation (Moré *et al.*, 1994), sonication, microwave heating and thermal shocks (Picard *et al.*, 1992) have also been used. Isolation of bulk DNA from environmental soil and sediment samples is often compounded by the co-isolation of impurities, in particular humic material which can inhibit enzymatic manipulation (Steffan *et al.*, 1988; Tebbe and Vahjen, 1993). The addition of polyvinyl polypyrrolidone (PVPP) is commonly used in order to separate DNA from humic acids (Sayler

and Layton, 1990; Atlas, 1993). Another successful approach to overcoming the co-isolation of soil impurities involved the separation of the impurities from DNA molecules by electrophoresis in low-melting-temperature agarose (Porteous and Armstrong, 1993). A magnetic capture-hybridisation PCR (MCH-PCR) technique was also developed to separate DNA molecules from humic material and other soil contaminants (Jacobsen, 1995). This method involves coating magnetic beads with specific DNA strands complementary to a region of the target DNA, followed by hybridisation in a suspension of soil DNA. This facilitates the separation of the target DNA from contaminants by magnetic extraction.

Isolation of community DNA from aquatic environments

The isolation of nucleic acids from organisms in water samples is generally easier than that from soil samples. Basically, it involves filtering and concentrating the organic component, followed by lysis of cells, and extraction and purification of nucleic acids. DNA extraction from the organic component is similar to that from pure cultures. However, additional steps of DNA purification are required as large amounts of organic impurities are still present, albeit not to the extent as those in soil and sediment matrices. A simple method for isolating nucleic acids from aquatic environments that allows filtration of relatively large quantities of water was demonstrated by Sommerville *et al.* (1989). Studies of microbial diversity in aquatic environments based on the isolation of nucleic acids has mainly concentrated on marine planktonic microorganisms, in particular bacteria, cyanobacteria and protists (e.g. Giovannoni *et al.*, 1990; Knauber, Berry and Fawley, 1996; Lim, 1996; Suzuki *et al.*, 1997; Wise, 1997). To date there has only been one brief report on the identification of fungal DNA isolated from an aquatic substrate. Ma *et al.* (1997) isolated total DNA from glacial ice from ice strata, up to 12,000 years old, and analysed the rDNA regions of these DNA molecules. Their results revealed DNA sequences that were closely related to extant fungi, while several exhibited little similarity.

Isolation of community DNA from animal hosts

Fungi occur in association with both vertebrates and invertebrates. They occur as dermatophytes on the skin or surfaces of mammals and birds, in ruminant guts, on fish scales and in fish guts; as biotrophs or necrotrophs of insects, in invertebrate guts and on arthropod scales (Hyde and Hawksworth, 1997). Techniques for the isolation of total DNA from invertebrates are well-established (Skibinski, 1994; Louis, 1997). Protocols for the isolation of DNA

from larger animals, e.g. fish, birds and mammals, involve only a small portion of a particular tissue type of the animal (e.g. Sambrook, Fritsch and Maniatis, 1989; Hillis *et al.*, 1996). However, this does not present a problem as most studies of microbial diversity associated with large animal hosts are only concerned with a particular niche on or within a particular host, e.g. skin surface, hair and gut. Most of the studies reported on the diversity of microbes within vertebrates have mainly concentrated in the bacterial composition (Amann, Ludwig and Schleifer, 1995). Stahl *et al.* (1988) extracted total DNA from a bovine rumen for the assessment of ruminal microbiota. The extraction protocol used was similar to that for DNA isolation from sediment samples, but without the necessity to separate persistent impurities, such as humic substances which are co-isolated with the nucleic acids. Bock *et al.* (1994) extracted DNA from human skin samples and identified DNA of fungal dermatophyte origin on the basis of rDNA sequences. Detection of fungal diversity on animal hosts has scarcely been investigated despite the availability of both traditional and modern techniques.

Isolation of community DNA from plant hosts

There is no shortage of methods developed for the isolation of DNA from plants (e.g. Zimmer, Rivin and Walbot, 1981; Saghai-Moroof *et al.*, 1984; Rogers and Bendick, 1985; Doyle and Dickson, 1987). These methods differ mainly in their requirements for starting material (e.g. fresh, frozen or lyophilised; small or large quantities) and the use of ultracentrifugation steps. Reports on the isolation of total community DNA from plant tissue for the detection of fungi can be primarily grouped into those concerned with the detection of plant pathogens, mycorrhizae and endophytes. Most research has been carried out on the detection of pathogens in plant tissue (Miller and Martin, 1988; Henson and French, 1993; Miller and Joaquim, 1993). Recent reports on the direct detection of fungal pathogens within plant tissue include those of Beck and Ligon (1995), Érsek, Schoelz and English (1994), Johanson and Jeger (1993), Liew, Maclean and Irwin (1998), Moukhamedov *et al.* (1994), Tooley *et al.* (1997) and Wigglesworth *et al.* (1994). Protocols for DNA isolation cover a whole range of plant taxa as well as plant parts. However, in all cases they are concerned with the detection of specific fungal genera or species.

Most reports on the detection of mycorrhizal fungi by direct isolation of community DNA from plant tissue have also targeted one or two fungal species (Erland *et al.*, 1994; Di Bonito, Elliot and Des Jardin, 1995; Abbas, Hetrick and Jurgenson, 1996; Mello *et al.*, 1996). Several research groups (e.g. Simon, Lalonde and Bruns, 1992; Clapp *et al.*, 1995; Harney, Edwards and Allen, 1997)

have extracted DNA from plant roots and identified the presence of a multi-generic community of mycorrhizal fungi. The method used for DNA isolation can be very simple as demonstrated by Di Bonito *et al.* (1995). Crushed root samples were subjected to boiling in an extraction buffer followed by the addition of a commercial DNA chelating resin to separate the DNA from the mixture. The DNA obtained was of sufficient purity for PCR.

There are very few reports on the detection of fungal endophytes by direct isolation of community plant host DNA. Doss and Welty (1995) isolated DNA from plants and specifically identified the DNA of *Acremonium coenophialum*, while Groppe and Boller (1997) identified DNA of two fungal genera (*Epichloë* and *Acremonium*) within the total community DNA isolated from grass tissues. Of particular interest is the report of the identification of fungal DNA from the grass clothing of the Iceman (a mummified human body of neolithic origin found in 1991 at 3270m above sea level in the Ötztaler Alps near the Austro-Italian border; Rollo, Sassaroli and Ubaldi, 1995). A fragment of a woven grass cloak found nearby was subjected to DNA analysis and found to be a mixture of grass, algal and fungal DNA. The fungal component was identified to have originated from a psychrophilic basidiomycetous yeast, and from two ascomycetes possibly related to the Eurotiales.

The above data indicate that DNA from a whole range of fungal taxa can be extracted from different substrates and matrices in natural environments and are amenable to further analysis including taxonomic identification. How the isolated DNA is processed and analysed is crucial for the assessment of fungal diversity. It is important to be comprehensive and inclusive of all fungal taxa, yet exclusive of taxa from other kingdoms. As mentioned earlier, only a small part of the entire genome is assessed and the choice of DNA region analysed is therefore a primary consideration.

Processing of fungal DNA Isolated from natural substrates

Two general approaches have been used to detect fungal DNA from a pool of environmental DNA isolated from the natural substrate or matrix. The first involves the use of specific DNA probes which are short sequences of DNA and, when rendered single-stranded, used to hybridise specifically to DNA molecules with complementary nucleotides. The second involves the use of specific primers which are short sequences of nucleotides used in pairs to anneal (hybridise) to the two specific ends flanking a particular region of a DNA molecule.

Detection using specific DNA probes

On its own, the approach involving specific DNA probes is less sensitive primarily due to the involvement of the PCR amplification in the approach using specific primers. Moreover, specific probes have only been used to detect DNA molecules that are species-specific (Rollo *et al.*, 1987; Goodwin, Kirkpatrick and Duniway, 1989; Goodwin *et al.*, 1990; Tisserat, Hulbert and Nus, 1991; Marmeiss, Debaud and Casselton, 1992). Using this approach, the assessment of species diversity within a particular sample of environmental DNA would necessitate an impractical suite of probes, even if these were specific to higher taxonomic ranks, e.g. family-specific probes. On the other hand, DNA probes have been used to enrich the amount of fungal DNA in a plant-fungal DNA mixture by subtractive hybridisation (Clapp *et al.*, 1995). This procedure was coined Selective Enrichment of Amplified DNA (SEAD) by Clapp *et al.* (1993). Briefly, SEAD involves the combination of biotinylated plant DNA in excess into the plant-fungal DNA mixture, followed by denaturing and reannealing the whole mixture. This results in the formation of double stranded molecules of plant-derived DNA with at least one strand biotinylated. The biotinylated DNA is easily extracted with streptavidin. The remaining mixture with an enriched proportion of fungal DNA is then subjected to the second approach, i.e. PCR amplification of the fungal DNA using specific primers.

Detection using specific primers

While this technique is well-established in studies of bacterial diversity in the natural environment (e.g. Stackebrandt, Liesack and Goebel, 1993; Ueda, Suga and Matsuguchi, 1995; Borneman *et al.*, 1996; Lee *et al.*, 1996; Wise *et al.*, 1997), it is still incipient in studies of fungal diversity. Detection using specific primers basically involves the amplification, using specific primers, of a specific region of the genome that contains phylogenetic information. The amplified DNA fragments are subjected to size fractionation, denaturing gradient gel electrophoresis (DGGE), restriction enzyme analysis, or nucleotide analysis. For nucleotide analysis, the fragments are either directly sequenced or cloned prior to sequencing. The region of DNA that has proven to be very useful, particularly in bacterial diversity studies, is the nuclear encoded ribosomal RNA genes. The characteristics and usefulness of this region has been well-reviewed (Bruns *et al.*, 1991; Hillis and Dixon, 1991; Hibbett, 1992). Key features of this region which make it attractive for use in biodiversity analysis include: the presence of a mosaic of conserved and variable regions, the occurrence of the region in multiple copies per genome which evolve together, the ease of PCR amplification, and the availability of a vast amount of sequence data from

various taxa which are readily accessible in databases, such as GenBank and EMBL for comparison. In bacterial studies, primers specific for the amplification of the 16S subunit particular to prokaryotes are most commonly used. These primers exclude the amplification of eukaryotic DNA and are therefore ideal for diversity analysis of bacterial communities. Primers frequently used for the amplification of the 18S subunit, 5.8S gene and the internal transcribed spacers (ITS 1 and 2) in fungal systems (e.g. White *et al.*, 1990) are highly conserved. These conserved primer sequences are common among eukaryotes, including plants and animals (e.g. Mello *et al.*, 1996; Palumbi, 1996; Zhang *et al.*, 1997). While most reports on specific primers have been concerned with targeting only a small group of taxa, there are several reports on the use of more non-specific primers (Bock *et al.*, 1994; Makimura, Murayama and Yamaguchi, 1994; Rollo *et al.*, 1995; Ma *et al.*, 1997). Makimura *et al.* (1994) and Bock *et al.* (1994) used primers to amplify specific regions within the 18S subunit. The former group used primers designed for a wide range of medically important fungi, 25 species of ascomycetes and basidiomycetes, while the latter group designed a pair of fungal specific primers that were used to detect both pathogenic and non-pathogenic fungi. A more encompassing approach is the use of more than one pair of primers, separately, to amplify regions from the 18S subunit and the ITS. This approach was used by Rollo *et al.* (1995) and Ma *et al.* (1997) with further cloning and sequencing of the amplified fragments to facilitate taxonomic placement of taxa detected in the Iceman's grass clothing and glacial ice, respectively. A diverse range of fungal taxa were detected. Using primers to amplify only the ITS regions, Zhang *et al.* (1997) were able to detect DNA of basidiomycete, ascomycete and zygomycete origins in community DNA extracted from bamboo.

Analysis of DNA data

Data obtained from the processing of the isolated DNA are essentially taxonomic characters that can be analysed to facilitate the taxonomic placement of organisms. As in conventional fungal taxonomy which uses morphological characters, the availability of reference taxa for comparison is a prerequisite for appropriate classification of a particular fungus. Whereas comparison of morphological data in the conventional approach is performed with the aid of keys, molecular data are usually compared directly with the characters alongside each other. Where data are obtained from size fractionation, DGGE or restriction analysis, the size and pattern of the DNA fragments, either separately or simultaneously, are compared with those of known taxa (Simon *et al.*, 1992; Clapp *et al.*, 1995; Mello *et al.*, 1996; Groppe and Boller, 1997; Harney *et al.*,

1997; Tooley *et al.*, 1997). These approaches have primarily been used to compare the presence or absence of specific fragment or fragments, but not in conferring relationships between taxa, particular those without the nature of fragment pattern or size established *a priori*.

DNA sequence data have the distinct advantage of allowing sequences of unknown taxa to be given a probable taxonomic status. Data generated from DNA sequencing are subjected to sequence alignment with reference sequences, the results of which are in turn subjected to cladistic analysis. These processes are routinely performed with the aid of computer programmes, such as Clustal V (Higgins, Bleasby and Fuchs, 1992), PAUP (Swofford, 1993) and PHYLIP (Felsenstein, 1993). The outcome is usually in the form of phylogenetic trees showing inter-relationships of taxa. The construction of such trees has been well-reviewed (e.g. Miyamoto and Cracraft, 1991; Swofford *et al.*, 1996) and is beyond the scope of this paper.

With the appropriate reference sequences, a given sequence of interest (representing a taxon) could fall within a clade consisting only of known taxa from a particular taxonomic group. For example if the clade consists of representatives of a family, the given taxon would be given a placement within that particular family. This approach enabled Rollo *et al.* (1995) to identify a fungal taxon closely related to *Leucosporidium scottii* in the Basidiomycota, and others in the Ascomycota. Using a similar approach, Zhang *et al.* (1997) were able to identify DNA sequences separated from a bamboo community DNA sample to be of fungal origin, which were related to *Glomus mosseae*, *Rhizoctonia solani*, *Volvariella volvacea*, and *Lentinula lateritia*. The extent of taxonomic resolution derived from such a phylogenetic tree relies on the availability of known sequence data. In addition, taxonomic resolution also depends on the DNA sequence chosen for the analysis since a highly conserved region would only allow placement of higher taxonomic rankings. The use of "framework trees" of different taxonomic rankings and DNA regions facilitating taxonomic placement with varying resolutions, not unlike keys currently used in classification on the basis of morphological characters, is a distinct future possibility. Clearly, the availability of a good database of key sequences from well-established taxa of various taxonomic rankings is essential.

Perspectives

It is apparent that molecular tools are available for assessing fungal biodiversity in the natural environment, and that these should be exploited by fungal taxonomists and ecologists. Even though the techniques involved are constantly being refined or modified for greater efficiency in general, it is only

when mycologists get "hands-on" experience in using these techniques that they can be further improved to meet the needs of mycologists.

There are several fundamental problems with regard to using molecular approaches in monitoring fungal diversity. The measure of biodiversity conventionally considers the total number of species and the relative abundance of particular species in an environmental niche, with the species as the unit of measure (Hurlbert, 1971; Hill, 1973). While it is not necessary to define a universal species concept for fungi for this purpose, the placement of a DNA sequence from an unknown taxon to species level requires well-defined boundaries for that particular species, e.g. with established levels of intra-specific genetic variation based on a specified DNA region. Currently, sequence data available in database centres are far from sufficient for the construction of "framework trees" appropriate for all taxa at the species level.

The very small amounts of DNA present in hyphae of certain fungi may also present a problem. Bornemann *et al.* (1996) noted that some fungal hyphae may contain very little cytoplasm and few nuclei, hence very small amounts of DNA may be present. However, several reports have shown that only minute amounts (picograms) of DNA are required for the detection and amplification of specific DNA (Groppe and Boller, 1997; Liew *et al.*, 1998). A further problem is that the presence of DNA from a particular fungus is not necessarily indicative of the viability of that fungus. Fungal DNA can be detected in dead mycelium or in non-viable dormant spores (Lee and Taylor, 1990). It is also noteworthy that current molecular tools do not provide a reliable quantitative measure of the abundance of organisms in the natural environment, despite the numerous reports on quantitative PCR procedures.

Although the use of morphological characters in fungal taxonomy is fundamental, convenient and indispensable, it should be noted that the advantages of using molecular tools far outweigh their inherent problems and limitations. Molecular technology provides a great potential for assessing fungal diversity which is unrestricted by the limits of pure-culture techniques. While molecular technology is constantly being refined and new concepts being introduced, mycologists should have an active involvement in the development of molecular techniques specifically to measure fungal diversity. In this way the specific needs and problems addressed by mycologists and fungal ecologists will be taken into consideration.

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