3 Environmental DNA Analysis and the Expansion of the Fungal Tree of Life

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CONTENTS

I. Introduction

Fungi play pivotal roles in global environments; they form mycorrhizal associations with land plants, symbioses with algae as lichens, are comensal microbes of animals, are the causal agents of numerous diseases of animal and plant species, and perform vital functions as decomposers to enable carbon and nutrient cycling. As such, the Fungi represent a significant proportion of the microbial diversity (Hawksworth 2001; Hibbett et al. 2007; Mueller et al. 2007), biomass and genomic variations found on Earth and include a number of very useful organisms. For example, fungi are the primary source for a number of leading pharmaceuticals (e.g., cholesterol-lowering statins, the immunosuppressant cyclosporinA, several antibiotics including penicillin and cephalosporin). The evolutionary diversification of the Fungi has included several radical changes in cell biology, including loss of true mitochondria (Embley et al. 2003; van der Giezen et al. 2002), loss of phagotrophy and gain of osmotrophic mechanisms, loss or losses of a cilium (flagellum) (James et al. 2006a, b), gain of a rigid chitin rich cell wall (Bartnicki-Garcia 1987), development of filamentous structures, and the acquisition of saprotrophic and pathogenic lifestyles (James et al. 2006a). However, it is currently unclear when these major cellular changes occurred on the fungal phylogeny, and furthermore, the true extent and complexity of the fungal tree of life (James et al. 2006a; Liu et al. 2009).

Previously, the diversity of the kingdom Fungi was loosely classified as four major groups: (i) the ascomycetes of the subkingdom Dikarya (filamentous or yeast forms that reproduce sexually with internal spore maturation within an ascus), (ii) basidiomycetes also of the subkingdom Dikarya (filamentous or yeast forms that reproduce sexually with external spore maturation on a basidium), (iii) zygomycetes (multinucleated cells which produce filaments and lack complex fruiting bodies), and (iv) chytrids (an informal name given to species which produce motile flagellated spores during their lifecycle) (Hibbett et al. 2007). However, this simplistic model of fungal taxonomy has been challenged at a number of levels. These revisions include the placement of the microsporidia with (Hirt et al. 1997; Hirt et al. 1999) and potentially within the Fungi (Adl et al. 2005; James et al. 2006a; Keeling 2003), division of the 'chytrids' and zygomycetes into multiple paraphyletic clades and subsequent taxonomic reclassifications (Hibbett et al. 2007; James et al. 2006b; Liu et al. 2009). All progress made in understanding the fungal tree of life and its taxonomy has, in

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the most part, been made by studying fungi that have been isolated and cultured. This, however, represents only a narrow perspective on the true evolutionary complexity of the Fungi.

The number of fungal species present on Earth is unknown, however one estimate suggests a minimum of 1.5 million species (Hawksworth 1991). This estimate was primarily based on the diversity of fungi associated with land plants in specimens from Western Europe, which was then extrapolated to a global number using estimates of global plant diversity (Hawksworth 1991, 2001). This figure is controversial because it makes several risky assumptions and is primarily based only upon observations of fungal diversity associated with vascular plants in temperate regions. Such assumptions include the premise that the diversity of plant and fungal species are positively correlated and that fungal and plant species have comparable range sizes (May 1991, 1994; Muller and Schmit 2007). This has called some authors to suggest this figure may represent an overestimation of fungal diversity (May 1991, 1994). Yet in contrast, this figure is also suggested to be an underestimate of fungal diversity, because it is based on morphological identification and therefore does not account for cryptic species diversity (Hawksworth 2001). Furthermore, this estimate does not include the numerous alternative ecosystems where fungi are both abundant and diverse, suggesting that global fungal diversity may in fact be much greater than this estimate suggests. For example, a study by Suh et al. (2005) isolated 650 yeast species from a variety of beetles from different geographical regions.

Sequencing of a 600 bp fragment of the large subunit (LSU) ribosomal RNA (rRNA) gene (D1/D2 loop) showed a dominance of ascomycete yeast among the isolated strains, 68% of which differed by >5 bp from all previously known genotypes. Phylogenetic analyses demonstrated 200 previously undescribed phylotypes, clustering into 45 clades throughout the fungal phylogeny.

This one publication increased the number of described yeasts by 30%, and it demonstrates the importance of exploring differing environments to enable a greater understanding of fungal diversity (Suh et al. 2005).

Our understanding of fungal evolutionary complexity is expanding as researchers use increasingly direct environmental DNA methods to investigate microbial diversity. Recent work using environmental ITS sequencing of soil samples has suggested a further upward revision of global fungal diversity from 1.5 to 3.5–5.1 million species, based on conservative 97% ITS similarity clustering (O'brien et al. 2005). Independent of the accuracy of these estimates it suggests that less than 2–6% of fungal species are currently described. Furthermore, of the 77269 species described (Hawksworth 2001) to date 478 fungal genome sequence projects are listed as complete or in progress (Gold database http:www.genomesonline.org). This demonstrates the effort required to narrow the huge diversity gap between the fungi isolated, cultured, and studied, and fungal communities present in natural environments. In this chapter we will review the progress made in identifying fungal diversity using direct molecular approaches from a range of different environments and begin to determine what these data mean for the fungal tree of life (see Chapter 1 in this volume).

II. Environmental DNA Analysis, the Clone Library Approach

Traditionally researchers have investigated fungal diversity by collecting macroscopic reproductive organs or by using culture based isolation techniques. However, such approaches have several limitations, they preferentially sample the few species that are easily propagated into culture and which therefore grow and reproduce at high rates in rich growth media, or species which have larger body sizes and possess distinctive morphologies. This raises a number of difficulties for the study of fungi, primarily because a large proportion of fungal diversity resides in soil and sedimentary environments, highly complex and heterogeneous environments which limiting direct microscopic observations (Arnold et al. 2000; O'brien et al. 2005). An additional problem arises because the majority of fungi that remain uncultured are likely to include huge amounts of cryptic diversity indistinguishable using microscopy of environmental samples. Further complications occur because similar morphotypes can often branch in distant and paraphyletic positions on the fungal tree of life (James et al. 2006a). This

makes classifications based on environmental sample observations extremely difficult and therefore encourages the increasing use of molecular approaches (e.g., Anderson and Cairney 2004; Brock et al. 2009; Seifert et al. 2007; White et al. 1990).

Molecular analyses of microbial diversity from environmental samples began with the sequencing of 5S and 16S rRNA gene markers from environmental DNA samples (e.g., Giovannoni et al. 1990; Hugenholtz et al. 1998; Pace 1997). This work borrowed many components from earlier research by Woese and colleagues who developed the use of ribosomal RNA encoding genes to reconstruct phylogeny (e.g Olsen and Woese 1993; Winker and Woese 1991). They followed the logic that all cellular life possessed a ribosome with both small and large subunit ribosomal RNA components and that phylogeny of these rDNA sequences can be used to reconstruct a tree of life (Olsen and Woese 1993; Winker and Woese 1991; see Chapters 1, 2 in this volume). Woese and colleagues were encouraged to focus on the ribosomal RNA encoding genes because the rRNA molecules fold to form a complex secondary structure with loops and helix regions (e.g., Olsen and Woese 1993; Van de Peer et al. 1997; Winker and Woese 1991; Wuyts et al. 2000) which means these gene sequences were composed of regions with a mixture of fast and slow rates of character variation. Consequently, these molecules seemed particularly suited to defining evolutionary relationships, among both relatively ancient and recently derived branches. A second advantage of this molecular characteristic was that polymerase chain reaction (PCR) primers could be developed which were either specific to narrow taxonomic groups, or were comprehensive for a wide diversity of species. This inspired the early use of rDNA sequences for investigating evolutionary relationships among microbes and rDNA sequences were soon among the best-sampled gene databases for identifying the placement of microbial lineages in molecular phylogenies (Cole et al. 2003; Sogin et al. 1986; Maidak et al. 1996; Woese et al. 1990). This approach was adapted for the investigation of microbial diversity present in environmental samples by using PCR amplification of ribosomal RNA encoding genes from environmental DNA samples (see Olsen et al. 1986; Pace 1997). This method is now commonly called environmental clone library analysis or environmental gene library analysis, and has been widely applied to a range of environments and evolutionary groups (e.g., Bass et al. 2007; Giovannoni et al. 1990; Moon-van der Staay et al. 2001; O'brien et al. 2005; Richards et al. 2005; Stoeck and Epstein 2003). This approach follows a six-step methodology with numerous variants (Fig. 3.1, left column):

Step 1 involves the extraction of DNA from an environmental sample either as a filtrate from an aquatic sample or directly from sediment or soil samples. We note standard approaches to environmental DNA extraction in many published environmental clone library analysis often do not encompass chemical or physical preparations specifically tailored to the preparation of fungal cells, especially among general eukaryotic molecular diversity studies. Consequently, it is possible that many of these studies have failed to sample true fungal diversity because the DNA preparation protocol lacks chemical or physical cell lysis steps to release DNA from cells enclosed within robust chitin-rich fungal cell walls. As such, future adaptation of this technology for the study of fungal microbes should consider experimentation with additional chemical and physical cell lysis steps.

Step 2 involves PCR amplification of environmental DNA using primers designed to target a taxonomic unit of interest. A great body of work is now available on the universal amplification of small subunit (SSU) ribosomal RNA (rRNA) genes of both prokaryotes and eukaryotes, enabling environmental clone library analyses from a range of different environments (e.g., Giovannoni et al. 1990; Moon-van der Staay et al. 2001; Olsen et al. 1986; Pace 1997; Richards et al. 2005; Stoeck and Epstein 2003). A large number of additional studies have also demonstrated amplification conditions specific for fungal SSU rDNA sequences (e.g., Bass et al. 2007; Anderson and Cairney 2004; Anderson et al. 2003; Jebaraj et al. 2009; O'brien et al. 2005; Vandenkoornhuyse et al. 2002) and a selection of these approaches are discussed in more detail below.

Step 3 involves the use of a standard cloning approach and involves the ligation of the PCR product into a plasmid vector. During step 4 the population of vectors carrying a selection of different inserts is transformed into host *Escherichia coli* cells and grown on selective media (step 5). For step 6, subsets of the transformed cells are then picked and the plasmid insert is sequenced

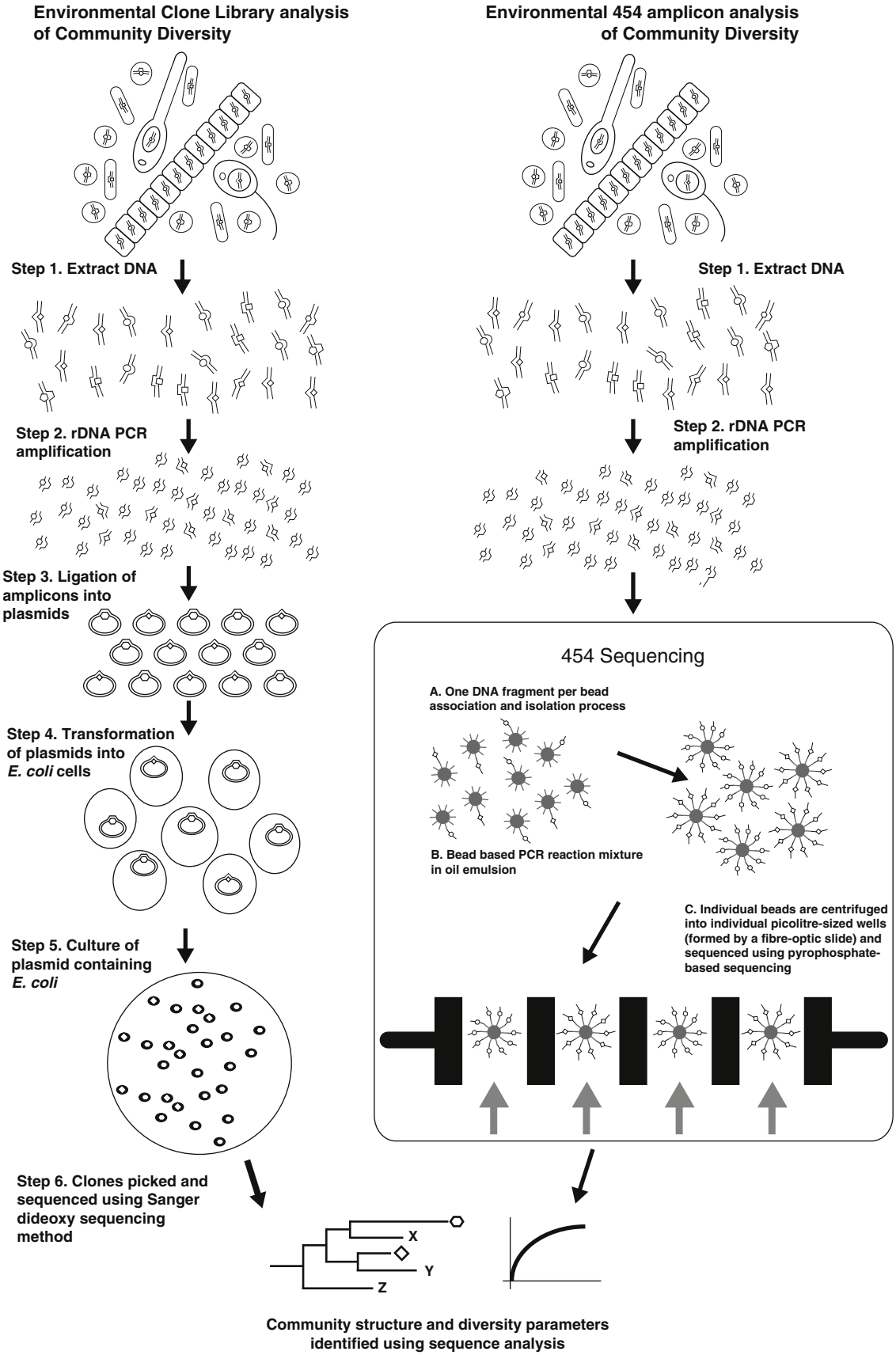

Fig. 3.1. Schematic showing comparison of environmental gene library and 454 amplicon diversity analyses; two methods used for investigating microbial diversity in environmental samples. 454 amplicon analysis allows large-scale sequencing of an environmental sample without the need of a cloning step minimising some sources of sampling error and radically increasing the magnitude of sequences retrieved

using a standard Sanger dideoxy sequencing approach. Steps 3–6 can all convey considerable sampling biases, which can mean that the results of the clone library sequencing profiles are not equivalent to the true community profile.

Finally the resulting sequences are checked for evidence of chimaeras formed during the multi-template PCR using manual alignment checks (e.g., Berney et al. 2004; DeSantis et al. 2006; Hugenholtz and Huber 2003), partial treeing analyses (Robison-Cox et al. 1995) and/or an increasing number of bioinformatic tools (e.g., Cole et al. 2003; Huber et al. 2004). The remaining sequences are then identified in an alignment of sequences from known taxa and phylogenies are calculated to identify the branching position of the environmental sequences.

This approach has been used for both prokaryotes and eukaryotes to place many previously unrecognised branches on the tree of life, in many cases redefining our understanding of the evolutionary complexity of eukaryotic groups among the highest taxonomic grades (Bass and Cavalier-Smith 2004; Dawson and Pace 2002; Edgcomb et al. 2002; López-García et al. 2001; Moon-van der Staay et al. 2001; Richards et al. 2005; Stoeck et al. 2006), although these results have been the subject of much debate and revision (Berney et al. 2004; Cavalier-Smith 2004; Richards and Bass 2005). Furthermore, such techniques have demonstrated that poorly recognised groups are important ecosystem components (Bass and Cavalier-Smith 2004; Massana et al. 2004; Moreira and López-Garcia 2002; Chambouvet et al. 2008). For example, early applications of environmental clone library analysis focusing on eukaryotic microbial diversity has demonstrated that protist diversity in marine environments encompasses numerous hitherto unsampled groups of marine stramenopiles (MAST) and alveolates (López-García et al. 2001; Massana et al. 2002, 2004; Moon-van der Staay et al. 2001) radically expanding both groups.

Follow up work suggested the novel stramenopile groups consisted of at least 12 separate clades, now labelled as MAST groups 1 to 12 (Massana et al. 2004). While the novel alveolate sequences fall into five extremely diverse clades, which branch close to the mainly photosynthetic dinoflagellate protists (e.g., Guillou et al. 2008). The bulk of these microbial forms remain uncultured, and therefore many aspects of their biology and ecology remain relatively unknown.

But where does this leave our understanding of fungal biodiversity and how it fits on to the fungal tree of life? Environmental fungal clone library analyses have generally focused on regions within the rRNA gene array, with a mixture of approaches and sequence targets, with some researchers focusing on the SSU rDNA sequence whilst others focus on the internal transcribed spacer (ITS) regions. The two ITS regions are sections of DNA located between the 18S and 5.8S rRNA genes and between the 5.8S and 28S rRNA genes, the variable nature of these regions relative to the flanking rRNA genes enables increased accuracy when assigning sequences to genus and species level classifications within well-sampled groups (Bruns and Gardes 1993; Gardes and Bruns 1993; Horton and Bruns 2001). Assignment is further facilitated by an increasingly well-sampled database of sequences (Buchan et al. 2002; James et al. 2006a; O'brien et al. 2005), although it is important to note that some database sequence classifications may be erroneous and rates of ITS variation can vary between taxonomic groups hindering classifications using these data (Nilsson et al. 2006, 2008; Vilgalys 2003).

The ITS regions are commonly amplified using either the universal ITS1 and ITS4 primers, fungal specific primers ITS1f and ITS4f, or increasingly taxon-specific variants, e.g., ITS1b and ITS4b that are basidiomycete-specific (Gardes and Bruns 1993; Horton and Bruns 2001; Anderson et al. 2003).

Molecular studies based around the amplification of the ITS marker from environmental DNA have reported much fungal diversity (e.g., Buchan et al. 2002; O'brien et al. 2005). However, with much of this research focused on ITS1-5.8S-ITS2 comparisons, results are often biased towards previously sampled groups (Anderson and Cairney 2004). Whilst this approach is useful for determining species diversity and can be used for ecosystem comparisons when targeting well defined taxonomic groups, it is of limited value for inferring higher-level phylogenetic relationships and identifying novel groups. This is because phylogenies based on ITS sequences demonstrate weak resolution among phyla level relationships (Horton and Bruns 2001). Therefore, some researchers have taken a different approach and have instead focused on sampling across the SSU rDNA region in order to investigate novel fungal diversity

among higher taxonomic groups (e.g., Anderson et al. 2003; Bass et al. 2007; Jebaraj et al. 2009; Vandenkoornhuyse et al. 2002). However, it is important to stress both approaches have benefits and they are not mutually exclusive, with some authors calling for a combination of the ITS and the SSU approaches (O'brien et al. 2005). This is achievable by using a forward primer in the SSU region and a reverse primer in the LSU region, enabling amplification from the SSU, through the ITS1, 5.8S, ITS2, and LSU regions. This approach enables multi gene phylogenetic analyses, which can improve tree support and assignment of environmental sequences to taxonomic classifications (e.g., Porter et al. 2008; Jones et al. 2011).

We shall now briefly review how the application of environmental clone library techniques has altered our understanding of the fungal tree of life. We will focus mainly on studies that have sampled the SSU region to evaluate the complexity of the fungal phylogeny at higher taxonomic levels. Eukaryotic SSU ribosomal DNA environmental gene sampling methods have consistently demonstrated a broad range of novel fungal lineages indicating that many potentially key evolutionary branches and environmentally important fungal microbes remain unexplored. This review is not an exhaustive account of all the literature in this area but focuses on key studies which have provided progress towards a fungal phylogeny that accounts for the large complexity of fungal forms present in natural environments that have currently not been isolated and cultured.

A. Fungal Molecular Diversity in Aquatic Environments using a Clone Library Approach

1. Marine Fungi

In terrestrial ecosystems fungi perform critical roles in breaking down complex biomolecules and recycling nutrients. This process is very important because it underpins the wider ecosystem, but it is much less clear which organisms perform equivalent roles in marine environments. Culture-based fungal isolation studies of marine environments (e.g., Burgaud et al. 2009; Damare and Raghukumar 2008) have suggested the diversity of fungi present is limited, with only 467 described marine fungal species, belonging to 244 genera, equivalent to ~0.6% of the described fungal species (Kis-Papo 2005). Culture based studies of marine fungi have demonstrated that 'pink basidiomycete yeasts' are the most common fungal isolate. Marine yeasts are generally associated with accumulations of nutrient concentrations, e.g., pollution, plankton blooms, and macro algae (Kohlmeyer and Kohlmeyer 1979). Fungi are therefore suggested to play a critical role in detritus processing in marine environments (Mann 1988; Raghukumar 2004) providing essential nutrients to the wider food web such as lysine and methionine amino acids, various vitamins, and sterols (Mann 1988). For example, it is now clear that Crustacea require the polyunsaturated fatty acid, docosahexaenoic acid, for growth (Harrison 1990) which is made accessible to benthic food webs by actions of detritus microbes (Raghukumar 2004).

In 2005, during the early days of eukaryotic SSU rDNA clone library analysis, Richards and Bass conducted a meta-analysis of 13 environmental gene library studies encompassing 49 separate SSU rDNA environmental clone libraries and including a total of 1077 environmental sequences from soils, freshwater and marine samples. Of these sequences 124 (11.5%) clustered within, or close to, the Fungi (Richards and Bass 2005). Interestingly, this meta-analysis suggested that although fungi were present in aquatic sediments, low-oxygen aquatic environments, freshwater samples, and soils; the fungi appeared to be almost entirely absent from surface marine waters from both coastal sites and the open ocean. This pattern was confirmed by a separate marine specific meta-analysis which included 23 coastal water libraries (1349 clones) and 12 open sea libraries (826 clones) where only 16 fungal clones, equivalent to 0.8% of marine SSU rDNA sequences, were recovered (Massana and Pedrós-Alió 2008). This pattern is a little surprising as fungi are thought to be a major contributor to the decomposition of woody and herbaceous substrates and animal remains in coastal and surface marine environments (Kohlmeyer and Kohlmeyer 1979; Mann 1988; Newell 1996) raising questions of the significance of fungal communities in these marine ecosystems.

A study by Jebaraj et al. (2009) examined the diversity of fungi in oxygen-depleted regions of the Arabian Sea using clone library construction.

Multiple primer sets, two fungal specific and one set routinely used for eukaryotic diversity studies, were used to amplify the SSU from samples collected at 25 m depth (Jebaraj et al. 2009). Comparisons between fungal diversity detected using the fungal specific primer sets compared with the universal eukaryotic primers revealed a greater diversity detected within the fungal specific clone library. This result highlights the importance of using multiple primer sets to negate PCR biases and demonstrates that a proportion of fungal diversity is often missed when using 'universal' primer sets, potentially caused by the preferential detection of alternative taxonomic groups (Jebaraj et al. 2009; Stoeck et al. 2006). Phylogenetic analysis of SSU clones identified 48 distinct fungal phylotypes (clustered at 99% sequence similarity): 27 branching within the ascomycete radiation, 20 branching within the basidiomycete, while only a single highly unique phylotype was detected among the 'lower fungi' branching as a sister to mortierellales taxa (traditionally classified as a zygomycete). A number of the ascomycete and basidiomycete sequences formed highly novel branching positions in the phylogeny and in several cases clustered with additional environmental sequences recovered from oxygen-depleted habitats (Jebaraj et al. 2009). For example, several sequences branched within a clade previously termed the 'hydrothermal and/or anaerobic fungal group' identified as part of a study of deep-sea eukaryotic environmental clone libraries (López-Garcia et al. 2007). No 'chytrid' sequences were identified from this study, suggesting the possibility these taxonomic groups have a low diversity in these marine environments or alternatively the PCR primers or DNA sampling methodology used for this study were collectively biased towards the ascomycetes and basidiomycetes.

2. Deep-Sea Environments

The majority of the planet is covered by ocean with an average depth of ~3200 m (Gage and Tyler 1991) deep-sea environments therefore represent the largest and most underexplored habitats on Earth. The deep-sea environment is comprised of a variety of habitats such as low-nutrient water, sediments, and in some areas hydrothermal vents associated with high temperature and extreme pH. Preliminary exploration of these environments is redefining our understanding of both marine biodiversity and the tree of life, with an average of two new species discovered within deep-sea waters each month (Fisher et al. 2007), yet we are far from understanding many of the ecological processes occurring in deep-sea environments. Using SSU rDNA environmental PCR, followed by clone library analyses, Bass et al. (2007) investigated the composition of fungal communities within deep-sea sediments and water column samples ranging from depths of 500–4200 m and including several hydrothermal vent samples. The sequences recovered showed deep-sea fungal communities to have low diversity, dominated by ascomycete and baidiomycete forms that branched closest to yeast taxonomic groups on phylogenetic trees. Many of these phylotypes were also shown to branch close to known pathogens, perhaps indicative of the presence of fungal pathogens of deep-sea animals and consistent with additional observations suggesting fungi constitute important pathogens in deep-sea ecosystems (Van Dover et al. 2007). The phylogenies reported by Bass et al. (2007) included additional marine environmental SSU rDNA sequences and showed seven clusters of highly unique environmental sequences branching within the opithokonts, six specifically within the fungal radiation, indicating the presence of unknown fungal forms in marine environments.

A further study by Le Calvez et al. (2009) using an environmental SSU rDNA clone library approach to study deep-sea hydrothermal vent ecosystems also demonstrated the recovery of several novel fungal lineages. These lineages included three 'unknown phylotypes' branching within the basidiomycete radiation, and a further two 'unknown phylotypes' branching with 'chytrid' taxa (Le Calvez et al. 2009). The primer set used in this study was different from that chosen by Bass et al. (AU2–AU4; Vandenkoornhuyse et al. 2002) but both studies demonstrate the identification of several novel fungal lineages and that deep-sea fungal communities, although relatively non-diverse, are dominated by a large proportion of species branching among the ascomycete and basidiomycete yeast species. This observation is consistent with molecular analysis of eukaryotic diversity in marine sediments, which has demonstrated yeast species can dominate deep-sea microbial eukaryotic communities (Takishita et al. 2006).

3. Freshwater Fungi

A number of papers report a SSU rDNA clone library approach to investigate the eukaryotic microbial community of freshwater environments, from either direct environmental DNA extractions (Amaral Zettler et al. 2002; Berney et al. 2004; Lefèvre et al. 2007; Lefranc et al. 2005; Slapeta et al. 2005) or environmental DNA extracted from experimental detritus ecosystems, created from dead algae which have been inoculated with lake water microbial communities (van Hannen et al. 1999). In contrast to many of the equivalent marine studies (Massana and Pedrós-Alió 2008), freshwater studies have reported a high proportion of fungal sequences among the eukaryotic clones recovered (e.g., 19%, Lefèvre et al. 2008; 23%, Lefèvre et al. 2007; 33%, Berney et al. 2004; or 25%, Lepère et al. 2006). Furthermore, in contrast to the majority of sequences recovered from marine environments (e.g., Bass et al. 2007; Jebaraj et al. 2009), a large proportion of the fungal freshwater sequences branch among 'lower fungi' and/or 'chytrid' clades, where the majority of sequences recovered from marine environments are Dikarya (Fig. 3.2). These results suggest a large and underexplored diversity of 'lower fungal' groups in freshwater environments (Lefèvre et al. 2007, 2008; Lefranc et al. 2005; Slapeta et al. 2005) and suggests a contrasting pattern of fungal diversity between freshwater and marine environments although further and equivalent sampling is required to investigate this pattern.

4. Novel Basal Branches in the Fungal Radiation

Interestingly, freshwater studies have also identified a large and complex clade of environmental

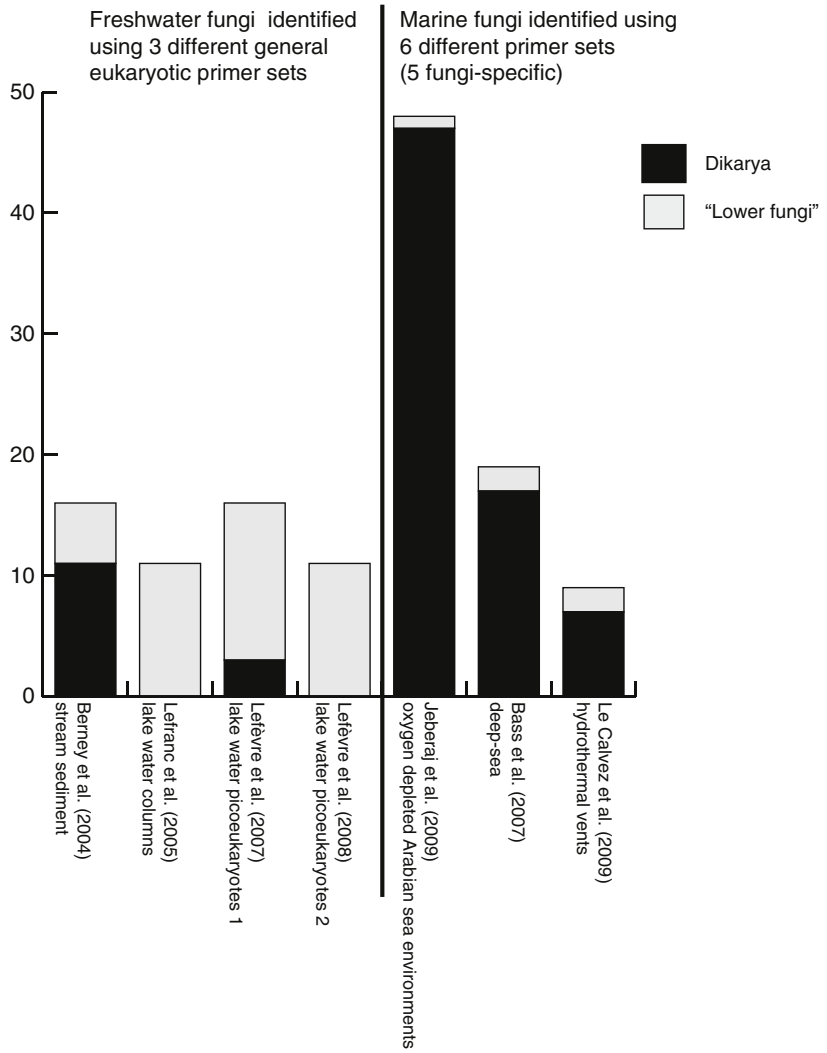

Fig. 3.2. Bar chart showing comparisons of the numbers of fungal phylotypes recovered during multiple studies of freshwater and marine samples. Whilst the differing clone library sizes, methodologies, and primers mean that comparisons cannot be directly drawn between the two environment types. Dikarya appear to dominate the marine environment, whilst 'lower fungi' appear dominant in the freshwater samples

sequences that form one of the deepest branches in the Fungi (e.g., Berney et al. 2004; Lefèvre et al. 2007, 2008). van Hannen et al. (1999) first identified this group from sequencing DNA recovered from an experimental detritus system composed of dead cultured algae seeded with lake water microbial communities. Of the four fungal-like sequences recovered in this experiment, three formed this deep-branching clade, often named LKM11 after the first clone sequenced from this phylogenetic group (van Hannen et al. 1999). Follow up sequencing of freshwater environments have considerably expanded the diversity of this clade (Lefèvre et al. 2007, 2008; Lefranc et al. 2005) while Lepère et al. (2006) have used terminal restriction fragment length polymorphism to demonstrate that the LKM11 group are highly abundant in the lake water column and linked to differential algal population abundances. Further phylogenetic analysis, with additional sampling, suggests that the LKM11 group is extremely diverse and branches with the intracellular parasitic genus *Rozella*, suggested to be the first branch in the fungal radiation (James et al. 2006a; Jones et al. 2011; Lara et al. 2010; see Chapter 1 in this volume). This is an interesting relationship because the genus *Rozella* comprises of intracellular parasites which do not form a standard fungal cell wall and are also capable of phagotrophy (Held 1981). These characteristics represent distinct differences to standard fungal phenotypic characteristics which have a rigid chitin wall, feed by osmotrophy, and cannot perform phagotrophy, thus calling into question whether the *Rozella* genus and perhaps the LKM11 clade of environmental sequences should be classified within the true Fungi, or whether they represent an intermediate form (Lara et al. 2010; Liu et al. 2009). Jones et al. (2011) used a FISH strategy to reveal that cells of this group have cyst, flagellated zoospore, and epibiotic lifecycle stages. Furthermore, co-staining with cell wall specific markers revealed that these cells lack chitin/cellulose-rich walls during the observed stages of their life-cycle, a characteristic previously considered one of the defining features of the fungal kingdom. Jones et al. name this group the cryptomycota (hidden-fungi), in anticipation of formal classification.

Interestingly, both the cryptomycota and the chytrid-like sequences recovered from freshwater environments all group most closely to fungi known to parasitise algae, other fungi, or protists (Held 1981; Lefèvre et al. 2007, 2008; Lefranc et al. 2005). These observations have led some authors to re-evaluate the functional role of microbial eukaryotes, and especially fungal parasites in freshwater environments, suggesting they play a very significant role in the microbial loop of freshwater ecosystems (Lefèvre et al. 2008).

B. Fungal Diversity in Soil and Plant Environments using the Clone Library Approach

A number of studies have used environmental clone library analyses to investigate the presence and diversity of fungal taxa in soils and plant-associated environments (Buchan et al. 2002; Horton and Bruns 2001; Vandenkoornhuyse et al. 2002), with the aim to identify important ecological agents associated with plant derived ecosystems. Other studies have focused on analysing the diversity of eukaryotes (Lawley et al. 2004; Lesaulnier et al. 2008) and fungi (O'brien et al. 2005; Schadt et al. 2003) from a range of soil habitats in order to investigate the complexity of these communities. Together this work has demonstrated that fungi represent a significant fraction of soil communities as identified using general eukaryotic environmental gene library studies (e.g., 20%, Lawley et al. 2004; 28–30%, Lesaulnier et al. 2008).

Schadt et al. (2003) used environmental clone library analysis to reveal microbial activity in snow-covered tundra soil, an environment which until recently was thought to harbour only inactive forms (Schadt et al. 2003). Clone libraries generated from total DNA revealed this community comprised largely of ascomycetes, of which 40% clustered into novel and distinct branches, indicating a large diversity of previously unidentified ascomycete groups. These newly identified phylotypes split into two major clades, branching separately within the ascomycete radiation. Using comparisons of clone abundance, Schadt et al. (2003) demonstrated the abundances of these two groups appeared to be coupled to opposite seasons, confirming an active and dynamic fungal community to be present in snow covered soils.

Porter et al. (2008) further examined group I of these novel organisms by investigating soil samples using group specific nested PCR primers to amplify the SSU-ITS1-5.8S-ITS2-LSU regions of the rRNA gene array. This approach allowed the construction of soil clone group 1 libraries

from a broad range of geographic and environmental locations, and demonstrated the presence of the group within alpine and forest tundra, tropical, and forest soils from a variety of locations around America, Europe, and Australia. Phylogenetic analyses of the recovered 2.4-kb sequences firmly rooted the group within the ascomycetes (Porter et al. 2008). It is also of note that two thirds of the taxa detected in this study were in low abundance; indicating saturation of the library was not achieved. Therefore, it is likely the diversity of the ascomycete soil clone group 1 is far greater than currently sampled.

Fungi are commonly associated with plant roots where they form mutualistic mycorrhizal associations. Vandenkoornhuyse et al. (2002) used SSU clone library generation to reveal novel fungal forms present on the roots of the grass *Arrhenatherum elatius* (Vandenkoornhuyse et al. 2002). From 200 clones, 49 different sequences were obtained distributed across all traditionally classified fungal phyla (1 'chytrid', 8 zygomycetes, 16 basidiomycetes, 25 ascomycetes). Interestingly, Vandenkoornhuyse et al. (2002) note this diversity pattern is in stark contrast to culture isolation based protocols that recover only ascomycete taxa (Arnold et al. 2000), suggesting fast growth and a preference for nutrient-rich media is a bias for these ascomycetes. Of these sequences, only seven showed greater than 99% identity to known species, while a large proportion of the remaining sequences formed four novel phylogenetic clusters that branched within the basidiomycete and ascomycete radiation. These results again demonstrate numerous additional branches within the fungal phylogeny raising questions regarding the ecological implications of these novel species and the role of these novel groups in the mycorrhizae and general soil habitats.

C. Limitations of the Clone Library Approach

Whilst environmental gene library techniques have proved to be very useful, revealing numerous previously undetected fungal groups, the generation of clone libraries is known to result in biases and to suffer from a number of experimental limitations. The majority of the studies discussed use single primer sets and sample $<$500 clones. Consequently, to our knowledge, no eukaryote-wide study, or fungus-specific study, has demonstrated sampling saturation (e.g., Edgcomb et al. 2002; Jebaraj et al. 2009; Porter et al. 2008; Stoeck et al. 2006). This implies further sampling from the same environments would reveal additional diversity (Moreira and López-Garcia 2002; Sogin et al. 2006; Stoeck et al. 2006). For example, a recent study of the Cariaco Basin, Venezuela, demonstrated that, in a comparison of three clone libraries, each derived from the same environmental DNA but using different primer combinations, and with a sampling of 241, 119, and 137 clones respectively, only three operational taxonomic units were present in all three clone libraries (Stoeck et al. 2006). This result demonstrates a very low overlap between the clone libraries and suggests that molecular diversity remains largely undersampled using these approaches.

These problems mean that lineages which are locally rare or patchily distributed are likely to remain undetected; consequently, we currently know very little about organisms belonging to either category. Artefacts produced by incomplete sampling are further compounded, as generally only a single set of primers are chosen to amplify the targeted gene region, or indeed the DNA preparation methodology may favour certain species and exclude other groups. Furthermore, standard practice is to amplify as much of the SSU and/or ITS regions in order to acquire as many characters as possible for molecular phylogeny. However, it is known that the targeting of longer amplicons can reduce the recovery of diversity profiles in clone libraries (Huber et al. 2009). Additionally, ligation of amplicons and transformation protocols often result in the exclusion of rare amplicon types. The net effect of these limitations is that most environments remain under-sampled and meaningful comparisons of species diversity across differing environment samples are not possible. Environmental gene libraries generated from multiple group-specific primers provide a possible means of detecting low-abundance microbes, improving sampling efficiency and enabling systematic comparisons of microbial diversity between and within environments (e.g., Bass and Cavalier-Smith 2004; von der Heyden et al. 2004). However, it is important to note that environmental clone library methods are limited when trying to understand microbial community dynamics including activity, habitat distribution, and complete diversity profiles.

III. Alternative Environmental DNA Methods

A. 454 Sequencing of Taxon Identification Sequence Tags from Environmental DNA

Recently, Sogin and co-workers (Sogin et al. 2006) employed DNA sequencing using micro-fabricated high-density picolitre reactors (454 sequencing) to investigate the molecular diversity of prokaryotes in marine environments. This uses a relatively new technology where millions of nucleotide base pairs are sequenced with '99.5%' accuracy in 100–500 bp fragments (Margulies et al. 2005) depending on which version of the technology is applied (e.g., GS-FLX or GS-FLX titanium). Consequently, the level of sequence sampling achieved is much greater than can be contemplated for standard environmental clone library analysis. The method begins as with clone library generation, by the amplification of a gene sequence of interest from environmental DNA (Fig. 3.1, right column) but steps 3–6 of the environmental clone library analysis are eliminated and replaced by the 454 protocol. Briefly: adapter sequences are incorporated on the 5' region of the primer to enable the attachment of the PCR products to DNA capture beads. The adapters can be ligated to the PCR products or incorporated as part of the PCR primer. We recommend the latter to minimise sampling artefacts. The 454 method incorporates a one fragment per bead association and isolation process, followed by a PCR reaction mixture in oil emulsion, which in effect isolates single templates on each bead and then generates millions of copies per bead. The DNA strands are denatured and the beads, now carrying millions of identical single-stranded DNA molecules, are centrifuged into individual picolitre-sized wells (formed by a fibre-optic slide) and sequenced using pyrophosphate-based sequencing (Margulies et al. 2005). This method avoids the need to use a cloning step (Fig. 3.1), enabling PCR amplicons to be effectively sequenced directly, without cloning and transformation biases. This factor, combined with the very high number of sequence reads generated, increases the probability that low-abundance amplicons can be recovered, thus removing a strong potential source of artefact between environment sampling and sequence sampling.

In the first experiment of this kind Sogin and co-workers (Huber et al. 2007; Sogin et al. 2006) recovered 118778 prokaryote V6 SSU rDNA sequences demonstrating an 'unprecedented level of bacterial complexity within marine samples'. This method has now been adapted for the analysis of eukaryotic microbial communities (Amaral-Zettler et al. 2009; Stoeck et al. 2009, 2010). For example, Stoeck et al. (2010) used a multiple marker approach targeting both the V9 and V4 variable regions of the SSU rDNA sequence in order to investigate the complexity of microbial eukaryotic communities in anoxic water from a Norwegian coastal fjord. This experiment demonstrated that eukaryote DNA diversity profiles, like prokaryotes, appear to be composed of a small pool of highly abundant genotypes, while the majority of the molecular diversity detected is present in very low numbers. This suggests the greater part of the microbial diversity present in these environments is at low abundance (Huber et al. 2007; Sogin et al. 2006). The idea that microbial communities are arranged in this manner is a popular concept in microbial ecology and is often termed the 'rare biosphere' (e.g., Pedrós-Alió 2006). The pattern of diversity observed for both prokaryote and eukaryote 454 datasets has been compared with this ecological concept (e.g., Sogin et al. 2006; Stoeck et al. 2010). The significance of the large diversity of sequence tags both in eukaryotes (Stoeck et al. 2010) and prokaryotes (Huber et al. 2007; Sogin et al. 2006) awaits further investigation and correction for factors that potentially artificially inflate the diversity profile detected (e.g., PCR and sequencing errors, levels of intragenomic SSU rDNA polymorphism, PCR chimaera formation).

The eukaryotic V4 and V9 SSU 454 sequencing approach on the Norwegian coastal fjord samples recovered a number of sequences which cluster with or within the fungi (Stoeck et al. 2010). When the dataset was clustered at 98% similarity values, 0.71% of 3993 unique V4 sequence clusters and 3.8% of 2633 V9 sequence clusters were classified as fungi. As part of the study Stoeck et al. (2010) also compare this data with 1000 environmental SSU rDNA clones sequenced from the same environment. The clone library approach indicated an absence of the fungi within the costal marine water columns, consistent with the pattern identified from the review of

marine SSU rDNA literature discussed earlier (Massana and Pedrós-Alió 2008). However, the 454 approach revealed the presence of a significant fungal community and suggests that deep sequencing initiatives, potentially in combination with fungal specific primers, will demonstrate an increasingly complex fungal community in marine environments. This hidden diversity may prove important for understanding the fungal tree of life.

Similar methods have also been applied to investigate the diversity of fungal communities in soil and phyllosphere environments using fungal specific ITS PCR amplicon 454 sequencing. These studies used conservative sequence clustering of 95% (Jumpponen and Jones 2009) and 97% (Buée et al. 2009), but still demonstrated that both environment types harbour complex fungal communities. Both analyses note that accurate taxonomic classification was dominated by the Dikarya, with the phyloplane environments dominated by ascomycetes (93.4%; Jumpponen and Jones 2009), while the soil environment was dominated by basidiomycetes (44%; Buée et al. 2009). Interestingly, the analysis of soil environments using a filtered database containing only identified fungal strains and excluding uncultured environmental sequences enabled improved taxonomic classification and identified that 20% of the 454 ITS sequence tags recovered grouped as 'unclassified Dikarya', while 11% were grouped as 'unclassified fungi', with many of these novel sequences clustering with the soil clone group 1 investigated by Schadt et al. (2003) and Porter et al. (2008). This data again demonstrated that relatively well-sampled environments like forest soils harbour numerous unexplored fungal forms.

Similar 454 methods have also been applied to target arbuscular mycorrhizal glomeromycete communities of forests where the method again proved itself as an effective technique for sampling increasing levels of fungal diversity. However, the vast majority of sequences detected could be classified as known taxa using 97% clustering thresholds. The majority of the remaining sequences were putatively novel sequences belonging to *Glomus* group A, however the authors suggest the novelty of these sequences could be the product of putative errors mainly in single nucleotide repeat tracks (homopolymer 454 errors) of the recovered sequences (Öpik et al. 2009). The authors could identify this source of artefact because they were sampling within the confines of a relatively well-sampled group and were using strict sequences clustering criteria. This approach is not always possible at wider taxonomic sampling scales but these results demonstrate an important concern relating to 454 data (Öpik et al. 2009).

While the 454 approach enables us to better understand the breath of fungal diversity, and hints at the existence of a vast number of undetected species, classification of much of this novel diversity remains elusive due to the small size of the DNA sequence regions amplified and a lack of understanding of how this relates to wider genome/phenotype variation, intra-genomic rDNA variation, and inconsistent understanding of how measures of rDNA variation relate to species boundaries (Nilsson et al. 2006, 2008). Whilst such studies greatly enhance the discovery of novel species, drawbacks have been found. The generation of sequencing artefacts including homo-polymer read errors (Öpik et al. 2009) has been reported as a problem for pyrosequencing and stringent sequence analyses have to be performed using conservative sequence clustering approaches (Kunin et al. 2009). Sequencing both strands and the incorporation of only sequences containing both primer sites is one approach that increases the reliability of the sequence data and can help with correction of homo-polymer read errors. The application of this technique to reveal the same novel sequences from multiple samples and multiple locations, and combining this technique with fluorescence *in situ* hybridisation (FISH) represent important additional experiments for the validation of novel phylogenetic groups.

B. Fluorescence *In Situ* Hybridisation of Uncultured Fungi

This method works through the illumination of target cells by the use of a fluorophore-labelled DNA oligonucleotide probe designed to be specific to the target group. As such the FISH method requires the prior discovery of rDNA sequences of the target group in the environment sampled, and has been successfully combined with SSU rDNA environmental clone library analysis. The FISH approach has been used to reveal the cellular forms represented by novel environmental sequences and has proved successful in identifying cells from the previously unknown MAST groups (Massana et al. 2002) discussed earlier, a marine

picoplanktonic group – the 'picobiliphytes' (Not et al. 2007), and a highly diverse group of alveolate parasites present in marine environments (Chambouvet et al. 2008). This method is especially effective on aquatic environments where microbial communities of specific size ranges can be isolated using filtration. Briefly a unique 15–20 bp sequence motif is identified for the target group using comprehensive DNA alignment. These are often compared to rRNA accessibility models (e.g., Behrens et al. 2003) to identify a region with greater accessibility in the ribosome. A reverse complement ~18 bp oligonucleotide is synthesised with a 5' fluorophore attached. The target microbial community is usually fixed using formaldehyde (although other fixative approaches can be used and can minimise some autofluorescence signals), filtered using specific size selection onto filters (often polycarbonate filters), dried, cut into small sections, placed on glass slides, hybridised with FISH probes under a range of differing chemical or thermal stringency conditions, washed to remove unbound probes, mounted, and then imaged using epifluorescence microscopy (as described by Massana et al. 2002; Moter and Göbel 2000; Pernthaler et al. 2001). We note that the robust chitin cell wall often associated with the fungi can block FISH probes from entry into the cell and therefore prevent detection, therefore some researchers when looking at fungi often add a chitinase incubation wash step prior to probe hybridisation (Baschien et al. 2001). It is important also to add a range of control experiments, especially when targeting uncultured microbes. These can include testing for cross reactivity with non-target cultured microbes that branch near the target group on phylogenetic trees, and repeating the hybridisation experiments using a range of stringency conditions.

The FISH method is primarily useful for identifying the abundance of target communities within given environments, but it can also be combined with a range of additional cell stains to identify morphological and cellular characteristics. These include standard DNA stains such as 4',6-diamidino-2-phenylindole (DAPI) and probidium iodide which are useful to confirm the target cells possess a nucleus and therefore demonstrate the probe is illuminating an eukaryotic cell rather than cross reacting with a prokaryote, or trapped probe within environmental debris. Furthermore, cell wall stains can be used to identify the presence of chitin or other cell wall components (Jones et al. 2011). Subsequent hybridisation of probes to environmental samples enables visualisation of target cells under fluorescent light and reveals cell morphology. The approach has also been used to identify the ecological aspects of a target group. For example, FISH can be used to identify if the target group is an intracellular parasite of other organisms (Chambouvet et al. 2008). Furthermore, incubation of samples with fluorescently labelled bacteria, followed by fixation and FISH has been used to identify targeted cells as phagotrophic bacterial grazers (Massana et al. 2002).

Whilst the presence of the chitin rich cell wall found surrounding most fungal cells might be a hindrance (Baschien et al. 2001), FISH has proven successful at targeting fungi from natural environments (Baschien et al. 2001; Kosse et al. 1997; Li et al. 1996). Excitingly these methods have been used to demonstrate subgroups of cryptomycota (discussed above) represented 4.5% of the eukaryotic community in a lake water environment, suggesting this novel group represents a significant component of this aquatic ecosystems. However, this technique is rarely applied when investigating novel fungal lineages identified using environmental sequencing and therefore these techniques await exploitation for investigating the ecology and cell biology of novel uncultured fungal forms.

IV. Conclusions and Future Perspectives

Our knowledge of fungal diversity is expanding, facilitated by the use of environmental DNA methods. The detection of novel forms from habitats such as freshwater, marine, and soil environments has been discussed in this chapter. However, in many cases the detection of novel fungal diversity was part of a larger eukaryotic diversity study. There are of course exceptions, such as the recovery of novel yeasts from beetle guts (Suh et al. 2005), and the identification of novel ascomycete groups from soils (e.g., Porter et al. 2008), but such directed studies appear to be in the minority. These studies greatly enhance our knowledge of both diversity and fungal evolution. Of particular note is the cryptomycota clade (Jones et al. 2011), the putative primary branch in the fungal radiation. The discovery of such a diverse group is rare, and its exciting phylogenetic placement could

yield much information regarding the evolutionary history of the Fungi.

The development of new sequencing technologies, such as 454 sequencing combined with environmental DNA PCR, enables sequencing of community diversity to a far greater depth than was previously possible. This methodology has enabled detection of large sequence diversity within both prokaryotic and eukaryotic communities (e.g., Sogin et al. 2006; Stoeck et al. 2010) and has revealed greater fungal diversity in marine environments than detected using standard clone library approaches. These results suggest fungal diversity is much greater than previously identified in many environments; hinting at the possibility of a large diversity of low abundance fungal forms which are yet to be explored (e.g., Buée et al. 2009; Jumpponen and Jones 2009; Stoeck et al. 2010). With the decrease in cost, large-scale sequencing efforts are becoming more financially viable and more readily implemented. Numerous primers for the direct amplification of fungal DNA from the environment have been developed and successfully applied; yet, to the best of our knowledge no large scale sequencing efforts based purely upon determining fungal diversity from multiple environments using multiple primer strategies have been undertaken. With the advancement in sequencing technologies this approach is now viable. Furthermore, until this approach has been applied, our understanding of the evolutionary complexity of the Fungi remains primarily based on cultured representatives, and therefore is likely to remain both biased and incomplete.

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