Chapter 4

Genetic Identification of Fungi Involved in Wood Decay

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Wood decay is a complex process that involves contributions from molds, bacteria, decay fungi, and often insects. The first step in the accurate diagnosis of decay is identification of the causal agents, but wood decay in the strictest sense (white and brown rot) is caused by cryptic fungal species that are very difficult to identify using traditional methods. Genetic methods offer fast, reliable, and accurate means to identify microbes from infected woody material. The purpose of this chapter is to summarize the available first generation DNA based techniques for identification of microorganisms, primarily fungi, involved in the decay process and to discuss their strengths and limitations.

Introduction

In the 2003 ACS text (*Wood Deterioration and Preservation*), Jellison, Jasalavich, and Ostrofsky gave an overview of the past and current DNA-based technologies that have been used to study fungi involved in the decay process. The intent of this chapter is to build on their overview by providing additional background about several of the first generation molecular techniques that have been used over the past two decades, discuss advantages and limitations specific to the given methodologies, and attempt to simplify the often confusing terminology associated with molecular analysis of micro-organisms.

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There have been significant advances made in sequencing technology, including accuracy and efficiency. Throughput and maximum read lengths are being pushed to new limits. The use of next-generation sequencing platforms, such as pyrosequencing, sequencing by synthesis, and semiconductor sequencing, will be covered in another chapter by Tang and Diehl.

Traditional Methods for Identification of Wood Decay Micro-Organisms

Morphological

Fungal morphology has been the classical means to identify decay fungi obtained from wood. There are several available keys for identification (*1–3*), which contain most of the brown and white rot fungi. However, the morphological keys require some familiarization with specialized fungal structures and hyphal morphology that are not always readily visible when decay fungi are propagated on artificial media. In addition, isolation of decay fungi from woody material is challenging due to the additional non-decaying stains, molds, and yeasts also present on the surface and interior of the wood.

Cultural

Nobles (*4*) developed a key for identification of wood decay fungi using cultural characteristics. The key was based on enzyme chemistry, oxidation, culture growth rates on select media, and some morphological considerations. A second edition was published in 1968 that expanded the key to 252 species from the original 149 (*5*). The key used a diagnostic species code where numerical values were assigned at each diagnostic step and the ID was determined by the unique species code obtained at the end. This method was also used (*6*) in a punch card analysis of the Aphyllophorales. The Nobles key remains an important contribution to our understanding of decay fungi and how they react to different cultural conditions. In its prime, the Nobles Laboratory in Ottawa was identifying 3000 isolates per year using this method and was regarded as a worldwide authority in this field. The descriptions are detailed and reliable, but this method requires the time involved with using specialized media as well as specialized expertise in the diagnosis of the many different tests involved.

Phospholipid Analysis

Analysis of fatty acids from the phospholipid bilayers of microbial cells can be a useful tool for soil microbial communities and identification of individual organisms. Fatty acids are extracted, saponified, separated, and often derivatized before analysis using GC-MS or HPLC. The main premise behind phospholipid lipid analysis is that an individual bacterial or fungal species has a unique make-up of fatty acids in their phospholipid bilayer of the cell membrane. There are two types of phospholipid analyses typically used for soil microbial characterization,

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[1] Phospholipid Fatty Acid Analysis (PLFA) or [2] Total Soil Fatty Acid Methyl Esters (TSFAMES). TSFAMES have been shown to provide better yield than PLFAs, but are more complex to analyze (*7*). The PLFA technique has been used to characterize and quantify bacterial communities in soils (*8*), as well as fungi in decaying wood (*9*). One potential limitation of this approach is that the identification requires standardization of unknown cultures on artificial media prior to analysis, which is not suitable for organisms that do not grow well on traditional media. This method also involves a considerable amount of preparatory steps and reagents.

Immunological Assays

There are several available methods that use antibodies derived from wood decay fungi to identify early stages of decay, and include techniques such as particle agglutination, immunofluorescence assays, dot blots, ELISA tests, "dipstick assays" and chromatographic assays (*10*). Immunological detection relies on the presence of an antigen which previously required specialized cultivation techniques to produce sufficient antigens and antibodies for the tests. Biological supply companies now produce a wide range of antibodies commercially, but cost can be a limiting factor. Immunological based tests can also be confounded by the presence of inhibitory compounds that occur in later stages of decay (*11*).

Genetic Methods

General Considerations

The Central Dogma

The basis for all genetic analyses revolves around the central dogma of molecular biology (*12*). It simplistically shows the proper flow of genetic information in all biological systems beginning with deoxyribonucleic acid (DNA). DNA is transcribed into ribonucleic acid (RNA) which is the active messenger that in turn translates into proteins. This is important to keep in mind when interpreting molecular data. DNA based analyses should always be considered for the potential microbial inhabitants of a given system since DNA is a very stable inactive state of nucleic acids and can persist in the environment in resting structures, whereas RNA is the active messenger involved in metabolic processes and gives a better representation of what is actively causing degradation. The organisms detected using a DNA based assay aren't necessarily all metabolically active and contributing to active deteriorative processes; some may simply exist as fragments of mycelia, spores, latent resting structures, and other inactive forms. There are additional methodologies developed that attempt to address these but require additional techniques (*13*).

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Polymerase Chain Reaction

A major development in molecular biology that serves as the basis for most of the analyses discussed in this chapter is the polymerase chain reaction (PCR). PCR enabled scientists to multiply DNA on an exponential scale (*14*) so that a specific gene or region of DNA can be isolated, differentially amplified and studied. The premise of PCR involves three basic steps: denaturing the template DNA, annealing of the primers, and extension of the DNA. Denaturing is the melting or loosening of the DNA helix that allows the primers access to a strand of DNA. Annealing is the process of attaching nucleotide specific primers that flank the areas of the DNA to be copied. Extension involves the actual reading of the original strand and attaching the matching bases on a new strand of DNA based on the genetic code. Taq DNA polymerase is a thermally stable enzyme that is used to copy and build the strands of the DNA region of interest. PCR is the underlying principle that drives all of these emergent technologies for molecular ID and which prompted the development of numerous technologies that can be used to identify and characterize organisms based on their genetic information.

PCR Primer Selection

An important consideration when applying molecular approaches to characterizing fungi is selection of amplification targets, which will determine what regions of DNA will be copied in the subsequent PCR. The aforementioned DNA based procedures can also be used as useful tools in characterization of fungal population genetics (mating systems, mutant detection, and countless other possibilities), but in this review they are only discussed in the context of fungal identification and how they have previously been used for wood associated fungi. There are several commonly used priming targets used in fungal genetics for identification, with the most common of these being the internal transcribed spacer (ITS) region. ITS is a conserved region of ribosomal DNA that can be used to differentiate between species of fungi. There are two commonly used primers for amplifying ITS, the general fungal primer ITS1-ITS 4 primer pair (*15*) and the ITS1-F and ITS4-B primer sets (*16*). These are commonly used for sequencing, community analysis, and are a reliable target for routine amplification of fungal DNA from wood. The general primer amplifies for all fungi (includes mold, stains, yeast, etc.) while the basidiomycete specific primer amplifies only fungi that belong in the basidiomycota. Basidiomycete fungi also include those fungi that are key components of the wood decay cycle. There are also Ascomycete specific ITS primer sets that only amplify DNA from members of the phylum Ascomycota (*17*). The use of selective primers is one way to exclude some of the generalist micro-organisms that commonly predominate environmental samples. Additionally, large subunit (LSU) ribosomal DNA, small subunit (SSU) ribosomal, intergenic spacer regions (IGS) and beta-tubulin have all been used to amplify and differentiate species. There have been efforts to standardize and develop universal loci for DNA barcoding of fungi and perspective targets include

cytochrome oxidase, translation elongation factors, and ribosomal polymerase B2 (*18*). At this time the ITS region the most widely used DNA region for routine molecular analysis.

Methods Based on DNA Sequence Information

Cloning And Sequencing

The most straight forward and common method of molecular identification of decay fungi is through direct sequencing of conserved regions (*15*) and subsequent Basic Local Alignment Search Tool (BLAST) search through the NCBI database which compares the sequences to known sequences in the database. There is an exponentially growing amount of genbank entries that can be matched with sequenced data. Sequencing of PCR products has been used by many research labs to study wood decay fungi (*19–29*).

Sequencing may be difficult from severely decayed samples due to the presence of inhibitory compounds (i.e. humic acids polyphenols). The main downside to direct sequencing is that it requires a pure culture. To address this, many researchers use cloning to propagate their PCR products. In cloning, the PCR products are inserted into vectors, such as plasmid or other circular DNA form, and that way can be stabilized for future study and manipulation. Cloning requires some specialized equipment and incubator space, but does make retaining reference material and downstream applications much simpler.

Species-Specific Probes

A more targeted approach for detection is through the use of species-specific oligonucleotide probes (SSOP). These can be incorporated onto an array set-up where multiple species may be screened for presence/absence. Moreth and Schmidt (*30*) developed species specific probes for *Serpula lacrymans* and were able to detect the fungus in wood samples. Oh et al. (*31*) developed species specific probes for 11 wood decay fungi based on sequence specific probes from sequence data from the ITS 1 and ITS2 region of several wood rotting basidiomycetes and developed a highly sensitive “reverse southern blot” procedure that could successfully identify the target fungi in both laboratory samples and naturally decayed wood. SSOPS are a highly targeted approach to detection of wood decay fungi, but probes have to be first made and this requires sequence data and successful incorporation onto the SSOP filters.

Quantitative PCR (Q-PCR)

Q-PCR was made possible by developments in real-time PCR technology, and is simply an adaptation of conventional PCR that incorporates fluorescent dyes or probes that gives earlier, more sensitive quantification of target copies produced in a sample. Q-PCR has numerous applications and several formats are currently available. Horisawa et al (*32*) developed a species specific real-time PCR assay

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for detection and identification of five different wood decay fungi and was able to quantify these fungi in mixed samples from as little as 0.01ng of genomic DNA. Eikenes et al. (*33*) used a qPCR assay to monitor colonization of birch blocks through the course of an EN 113 decay test and compared them to other routinely used methods for quantifying fungal biomass (ergosterol and chitin assays) and found excellent correlations in early stages of decay but concluded that the qPCR assay was not suited for late stage decay, possibly due to inhibitory compounds, extraction efficiency, and high background from highly decayed samples.

Multiplex PCR Methods

Multiplex PCR allows for simultaneous amplification of multiple targets within a single PCR reaction. Guglielmo et al. (*34*) developed a multiplex PCR detection method for 11 specific taxa using ribosomal DNA, which included two variable domains (D1, D2), the conserved ITS1, 5.8S, and ITSII regions, and mitochondrial DNA, for fungi that that occur on hardwoods. This method could detect fungi with as low as 1 picogram of fungal material and had an 82% success rate for identification. An extensive validation of the method was first performed using spiked wood samples and finally on increment cores. Interestingly, the method also identified additional fungi not visually confirmed in 35% of the samples. One potential drawback to multiplex-PCR is that each component of the PCR reaction has to be optimized and often different PCR products will be incompatible based on their PCR settings (PCR settings are highly dependent on nucleotide composition of the template and primer composition).

Restriction Fragment Length Polymorphisms (RFLP)

RFLPs were the earliest technology developed for DNA fragment analysis, and PCR-RFLP is derived from this earlier procedure and is now more widely used. The basis of most of these fingerprinting methods rely on polymorphisms to yield information on the genetic make-up. Polymorphisms are different forms of the genotype of an organism that exist in a natural population. Polymorphisms are used by evolutionary biologists to observe speciation and natural selection, but can also yield information about the relatedness of individuals within a population (closely related individuals will share more polymorphisms than un-related ones). These cuts create fragments of smaller DNA pieces which create a characteristic DNA fingerprint based on the differences in nucleotide composition of the fungi in the sample. These are PCR products that are digested using multiple restriction enzymes, and banding patterns are visualized on high resolution agarose gels. The patterns are usually characteristic to a certain species or strain and are used for comparison and characterization. Jellison and Jasalvich (*35*) have used RFLP for identification and characterization of wood decay fungi in spruce and were able to detect and identify both white and brown rots in both early and late stages of decay. Some difficulties were noted with later stage decayed samples due to the presence of inhibitory compounds produced in the decay process. Prewitt et al. (*36*) used the fragment pattern from digests of the ITS region to construct phylogenetic trees based on multiple enzyme

digests. They concluded that phylogenies based on multiple digests of the ITS region was not sufficient to properly resolve species identifications as the RFLP phylogenetic trees did not agree with trees generated using sequence data. They concluded that while RFLP was useful for distinguishing species, it is not well suited for phylogenetic analysis. Adair et al. (*37*) used PCR-RFLP using an ITS1-F-2NL primer combination to detect fungi in chip piles of hemlock and lodgepole pine, and reported being able to detect and identify fungi in chip piles 4 days after inoculation. Their method was able to differentiate ascomycetes from basidiomycetes at early stages of decay. A potential drawback to using RFLP is that pure cultures are required and it cannot be used for characterizing mixed cultures or environmental samples without additional preparatory steps (i.e. cloning). RFLP can also have difficult in resolving closely related species and may require additional restriction digests to differentiate them.

- **Amplified Ribosomal DNA Restriction Analysis (ARDRA)** –ARDRA is an extension of the RFLP procedure molecular technique that was specifically developed for polymorphsims encoded in the small (16S) ribosomal subunit of bacteria for distinguishing species of bacteria (*38*). This method has also been used to study changes in microbial communities in contaminated soils (*39*) and efforts have been made to bridge prokaryote and eukaryote domains using a Universal Amplified Ribosomal Region (UARR) (*40*). Schmidt and Moreth (*41*) used ARDRA for detection of *Serpula lacrymans* in indoor environments in order to differentiate it from *Serpula himantoides* and found that ARDRA was successful using specific enzyme combinations even though the ITS fragments were of identical size. Potential drawbacks to using ITS-ARDRA are that there are few restriction sites contained in the ITS1 and ITS 2 regions, and several fungi produce closely sized fragments that can't be resolved on the output gels. Also, certain fungi have been found to undergo DNA methylation as they age and this methylation can cause difficulties with certain restriction enzymes (*31*).

Amplified Fragment Length Polymorphisms (AFLP)

AFLP was developed in the 1990's by Keygene (*42*) and is a PCR based analysis that relies on selective PCR amplification of restriction fragments from a total digest of genomic DNA. AFLP uses primers that correspond to the restriction digest recognition sites so that the fragments are selectively amplified. The banding pattern indicates presence or absence of restriction sites, and individual species or mutants present unique banding patterns. These patterns can be used to compare closely related genera to discriminate and to observe genetic changes over different gradients. The number of fragments can be increased or decreased based on the selectivity of the primers. AFLP has been used to study species compatibility among *Armillaria* (*43*), long distance dispersal of *Serpula himatoides* (*44*), and hybridization in Coniophora (*45*).

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Terminal Restriction Fragment Length Polymorphisms (T-RFLP)

T-RFLP combines the RFLP methodology with fluorescent tags on the ends of the PCR products. The tagged PCR products are digested with restriction enzymes and the terminal fragments are detected using capillary electrophoresis. The resultant fragments can be used to identify species (*46*, *47*) or characterize changes in microbial community structure (*48*). An important advantage of T-RFLP is that it can analyze environmental samples with multiple species and can also be multiplexed to include multiple PCR targets (*49*). Data can be exported as either binary data representing presence/absence, or relative intensity can be used to determine relative species abundance in a sample. Analysis and interpretation of T-RFLP data requires careful interpretation, but yields informative results. There are several software packages that can be used to process T-RFLP data that can also be exported for additional community or statistical analysis. Potential drawbacks of T-RFLP is the possibility of overlapping fragments leading to underestimation of total diversity or potentially missing data when looking at peak-profile data (*50*, *51*). In order for T-RFLP to be used as an identification tool, prior fragments must be generated and stored in a database for reference matching. Also, careful attention must be made during interpretation to avoid polymerase errors, intraspecific ITS variation, and extra peaks due to restriction enzyme ineffecicency (*52*).

Gradient Gel Electrophoresis

Although these are not DNA based methods for identification and characterization of fungi, Gradient gel electrophoresis is included in this review because it allows for better separation between closely spaced PCR products that would normally overlap in traditional electrophoresis (*53*). These are in fact imaging techniques that are used to size and confirm DNA fragments resulting from PCR amplification, but still provide a useful tool for separation of highly diverse or mixed sample matrices. The two most common means of gradient gel electrophoresis techniques are:

- **Denaturing Gradient Gel Electrophoresis-**uses a chemical denaturant incorporated into the gel that breaks apart the DNA as it migrates through the gel and increases the separation of visualized DNA fragments on the gel. DGGE has been used to study wood decay fungi on Norway spruce stumps (*54*). Five primer pairs were investigated and final tests were performed on spruce stumps showing varying levels of decay. Highly dissimilar populations were noted when comparing the samples obtained through direct extraction of DNA compared to those obtained by culturing followed by DNA analysis, presumably due to the selective nature of artificial media. DGGE can be used to characterize complex sample matrices, but differential migration and overlapping fragments can be difficult to resolve. It is also possible to excise fragments from DGGE gels for further sequencing to obtain species information, but the techniques are challenging. PCR-DGGE, which is the direct

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amplification of DNA from decayed wood samples, was also used to identify *Phlebiopsis gigantea* and several other wood decay fungi in decayed conifer stumps that were pre-treated with *P. gigantea*. This method also effectively detected six species from reference samples (*55*). Subsequent sequencing from excised bands did not yield additional identifications.

- **Thermal Gradient Gel Electrophoresis** or TGGE relies on a similar process as DGGE, but uses a thermal gradient to change the structure of the samples to improve separation. Kulkabnova (*56*) used TGGE to analyze communities of decomposer fungi from different forest stands and found that tree species composition did not have an effect on species richness, but it did have a strong effect on species composition of both fungi and bacteria. TGGE has many of the same limitations as DGGE (detection limits of rare species, co-migration of similar fragments, and inability to image overlapping species). It has been suggested that the two techniques can be combined to improve the resolution of the technique as well as the incorporation of fluorescently labeled probes (*54*), but newer metagenomic methods may provide more informative results with less time and effort.

Conclusions

Genetic identification and characterization of wood decay fungi has undergone drastic changes in the last two decades and will likely undergo even more changes as new sequencing technologies become more cost effective. As a result of the reduced cost structure associated with these technologies, they are now more readily available to smaller laboratories, independent investigators, and researchers in developing countries. These molecular methods provide excellent tools for sensitive characterization of complex environments and have greatly expanded our knowledge of the fungal communities that contribute to the decay process.

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