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Assay Development for Stachybotry's Identification

Michelle L. Davis

ASSAY DEVELOPMENT FOR STACHYBOTRYS IDENTIFICATION

THESIS

BY

MICHELLE L. DAVIS

2005

ASSAY DEVELOPMENT

FOR

STACHYBOTRYS IDENTIFICATION

THESIS

Presented in Partial Fulfillment of the Requirements for

The Degree Master of Science in the Graduate School

Of Texas Southern University

By

Michelle L. Davis, B.S.

Texas Southern University

2005

Approved by

Chairperson, Thesis Committee

Dean, The Graduate School

ASSAY DEVELOPMENT FOR

STACHYBOTRYS IDENTIFICATION

By

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Texas Southern University, 2005

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Pyrosequencing is a new DNA sequencing method relying on the sequencing-by-synthesis principle and bioluminometric detection of nucleotide incorporation events. The objective of this thesis is to design a protocol for the improvement of the identification of fungi and mold samples in the outdoors and in enclosed environments. Fungi (mold) and bacteria are naturally present in both indoor and outdoor environments. Pyrosequencing method has been introduced as an alternative detection. Efforts are currently shifting from structural to functional genomics, in which the related functions of genomic sequences are being studied. An application of this is in determination of potential toxicity of organisms based on the presence or absence of particular genes, which produce enzymes that are necessary in the biosynthetic pathways of the toxins in question. This study will be used as a model to accurately identify *Stachybotrys* and other fungi using short DNA-sequencing. To test multiple species and subspecies with conventional methods are very labor intensive and time consuming.

Approved By

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Committee Member	10-25-2005 Date
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Committee Member	Date

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ABBREVIATIONS

AMP	adenosine monophosphate
APS	adenosine phosphosulfate
ATP	adenosine triphosphate
bp	base pair
CCD	charge-coupled device
dATP	deoxyadenosine triphosphate
dCTP	deoxycytidine triphosphate
dGTP	dideoxyguanine triphosphate
DNA	deoxyribonucleic acid
DNTP	deoxynucleoside triphosphate
DsDNA	double-stranded DNA
dTTP	deoxythymidine triphosphate
kb	kilo base
PCR	polymerase chain reaction
PPi	inorganic pyrophosphate
RNA	ribonucleic acid
SSB	single-strand DNA-binding protein
SsDNA	single-strand DNA
Tm	melting temperature
ITS	Internal Transcribed Spacer Region

VITA

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I Can Do All Things Through Christ That Strengthens Me.

CHAPTER 1

INTRODUCTION

Microorganism and Toxins

Fungi (mold) and bacteria are naturally present in both indoor and outdoor environments. Fungi have the ability to grow in any environment where available moisture (Aw) and organic materials (substrate) are present. The organisms of the fungal lineage include mushrooms, rusts, smuts, puffballs, truffles, morels, molds, and yeasts, as well as many less well-known organisms (Alexopoulos, et al., 1996). About 70,000 species of fungi have been described; however, some estimates of total numbers suggest that 1.5 million species may exist (Hawksworth, 1991; Hawksworth, et al., 1995).

Fungi also share some common characteristics, which are summarized as follows: (1) fungi are eukaryotic organisms; (2) fungi live heterotrophically as saprophytes, parasites or mutualists; (3) fungal cells have cell walls, which are composed mostly of chitin and (4) Most fungi consist of hyphae, which combine to make up the fungal mycelium. Fungi can reproduce sexually or asexually by spores that grow from hyphae. A key factor in the growth and reproduction of fungi is the combination of moisture and any organic material - dirt, dust, wood, etc (Cohen, 2003). Essentially, fungi grow by digesting the organic material. Consequently fungi gradually destroy the organic material, which grow through

chemical digestion. Fungi have the unique ability to grow and reproduce on any given surface as long as it's suitable organic (carbon) materials and moisture are present (Cohen, 2003). Most biologists have seen dense filamentous fungal colonies growing on rich nutrient agar plates, but in nature the filaments can be much longer and the colonies less dense. In general fungi can be identified visually usually in the form of black, green, gray, and brown surface growth. Fungal spores (conidiaspores and other spore types) and mycelium (microfungi parts) have the ability to become airborne and consequently affect indoor air quality levels and health. Fungi include molds, yeasts and higher fungi. All fungi are eukaryotic and have sterols but not peptidoglycan in their cell membrane. They are chemoheterotrophs (requiring organic nutrition) and most are aerobic. Many fungi are also saprophytes (living off dead organic matter) in soil and water and acquire their food by absorption. Characteristically they also produce sexual and asexual spores. There are over 100,000 species recognized, with 100 infectious agents of man (Roberts and Mackenzie, 1984). The Fungi kingdom has become an integral part of the natural prospective. Fungi have been used for recycling of dead organic matter into useful nutrients. Fungi can produce toxins that make people or animals sick. Although some toxins can be inhaled, they are most often introduced into the person or animal by ingestion of mold-contaminated foods. Some of the toxins are very powerful (Sterin, 2002).

Molecular Genomic Techniques

Molecular genomic techniques have become a very important tool in contemporary taxonomy studies. It has proved to be very efficient, accurate and non-laborious. Efforts are currently shifting from structural to functional genomics, in which the related functions of genomic sequences are being studied. An application of this is in the determination of potential toxicity of organisms based on the presence or absence of particular genes, which produce enzymes that are necessary in the biosynthetic pathways of the toxins in question. This study will be used as a model to accurately identify *Stachybotrys* species and other fungi using short DNA-sequencing. Some Stachybotrys species are known to produce a toxin, which has been identified in illness in humans and animals Conventional methods used for the detection and identification of fungal species are very labor intensive and time consuming.

CHAPTER 2

HISTORY AND LITERARY REVIEW

Stachybotrys

Stachybotrys is a member of the Deuteromycetes, order Moniliales, family Dematiaceae and is a common inhabitant of plant debris and soil. *Stachybotrys* is said to have first been discovered by August Corda in 1837.

The genus *Stachybotrys* produces a mass of dark sticky, single-celled ornamented conidia from phialides on each conidiophore. *Stachybotrys* spores grow in clusters at the end of stem-like structures known as hyphae.

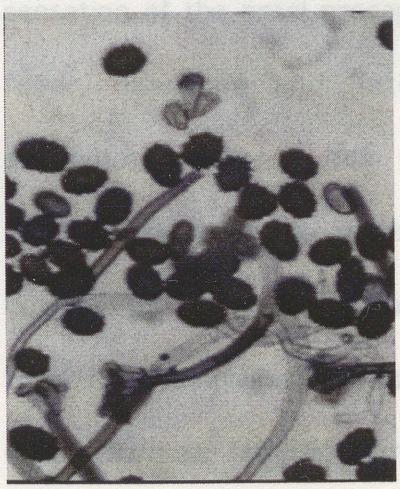


Figure 1. Stachybotry Spores. *Stachybotrys* spores cultured for 5 to 7 days on MEA obtained from www.emslabs.com.

Health Problems

Health problems associated with *Stachybotrys* were first noted in the 1930's and 1940's in Russian and Eastern European farm animals, in 1938 Russian scientists determined the disease was associated with *Stachybotrys* (then known as S. alternans) growing on the straw and grain fed to the animals (Sterin, 2002). The first reported human health effects were seen in agricultural workers who handled the moldy straw or hay. Russians reported stachybotryotoxicosis in humans in early 1940's. People affected were those who handled or were in close contact with hay or feed grain infested with *Stachybotrys*. Some of these individuals had burned the straw or slept on straw-filled mattresses. Common symptoms in humans were dermatitis, pain and inflammation of the mucous membranes of the mouth and throat, a burning sensation of the nasal passages, tightness of the chest, cough, bloody rhinitis, fever, headache and fatigue. There were continued publications on stachybotryotoxicosis but few that indicated a potential problem with *Stachybotrys* in homes and buildings until 1986, when Croft et al. reported an outbreak of trichothecene toxicosis in a Chicago home. Over a 5-year period, the family complained of headaches, sore throats, flu-like symptoms, recurring colds, diarrhea, fatigue, dermatitis and general malaise. Air sampling of this home revealed spores of *Stachybotrys*. The fungus was found growing on moist organic debris in an uninsulated cold air duct and on some wood fiber ceiling material. The home had a chronic moisture problem that favored mold

growth. Extracts from the duct debris and contaminated building materials were toxic to test animals and several macrocyclic trichothecenes were identified in the extracts. When the mold problem was corrected, these symptoms, associated with trichothecene toxicosis, disappeared.

Figure2. ***Stachybotrys*** **Chartarum** *Stachybotrys* chartarum growing behind a wall obtained from www.e-moldandmildew.com.

Since the collapse of the Twin Towers, many surrounding buildings in NYC have shown contamination with "heavy amounts of *Stachybotrys*." Most of these buildings were old and water damaged. With more public awareness, there will be more and more reports of *Stachybotrys* appearances.

Identification Methods

Identification of molds is very difficult and takes a great deal of training, experience, and practice. The personnel of most "Environmental" or "Indoor Air

Quality" companies have little training in Mycology. There are many false negative and positive diagnoses being made, there is no regulation of the industry causing difficulty in training an environmental mold specialist (Volk, 2002).

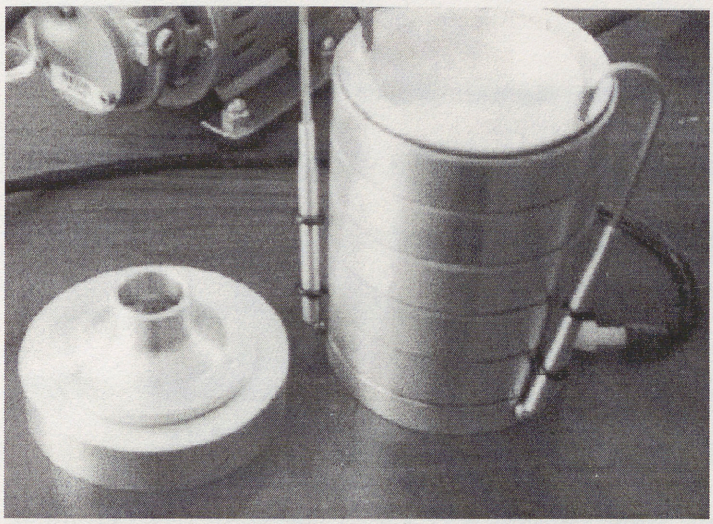


Figure 3. Six Stage Anderson Sampler obtained from www.cihrental.com/iaq.htm.

A generic type of fungal identification is the Anderson sampler, which has been used for identification. Samples are selectively trapped by the size of fungi particles according specially to their momentum. This sampler consists of a stack of 6 to 8 metal sections that fit together with ring seals to form an air-tight cylinder. Each metal section has a perforated base, the size of these perforations is progressively reduced from the top of the column to the bottom. To use this sampler, open agar plates are placed between each metal section, resting on three studs. Samplings with exposed agar plates are not exceptionally accurate for measuring airborne or sedentary organisms. The technique has to

be in a controlled environment with a single air sample that sometimes can be very difficult to achieve accuracy. Fungal identification requires expertise of medical mycologists to have an accurate identification. Even with such skilled personnel, there can be misidentification of organisms that produce toxins because of the close similarities (Kuhn, et al., 2003).

Biological Weapon

Some toxins, particularly aflatoxins and trichothecenes that have been produced by *Stachybotrys*, have the potential to be used as weapons. There is evidence that several countries are currently involved in mycotoxin weapons research (Kuhn, et al., 2003).

Region of Interest

Ribosomal RNA genes are organized in a ribosomal operon (rrn) and normally located in the order 16S-23S-5S rRNA. There are internal transcribed spacer regions (ITS) between the genes of 16S rRNA-23S rRNA and 23S rRNA-5S rRNA, designated ITS1 and ITS2, respectively. ITS1 contains tRNA genes and the regions required for coordinated transcription of rrn and processing of the transcript. Multiple rrn operons are found in most bacterial genomes. Their number can vary from 1 to 10. For some bacterial groups, variations in length and sequence between intragenomic copies of rrn (intercistronic heterogeneity) were found both in the RNA coding and intergenic regions (Irina, et al., 2004). Research previously done has identified (ITS) regions of fungal rRNA gene suitable targets for molecular analysis of fungal

communities (Buchan, et al., 2002). The internal transcribed spacer (ITS) region is now perhaps the most widely sequenced DNA region in fungi. It has typically been most useful for molecular systematics at the species level, and even within species. ITS1 is immediately upstream of the 5.8S gene and ITS2 is immediately downstream of the 5.8S gene. In addition to the standard ITS1+ITS4 primers used by most labs, several taxon-specific primers have been described that allow selective amplification of fungal sequences as described by Gardes & Bruns 1993 analysis of the amplification of basidiomycete ITS sequences from mycorrhiza samples.

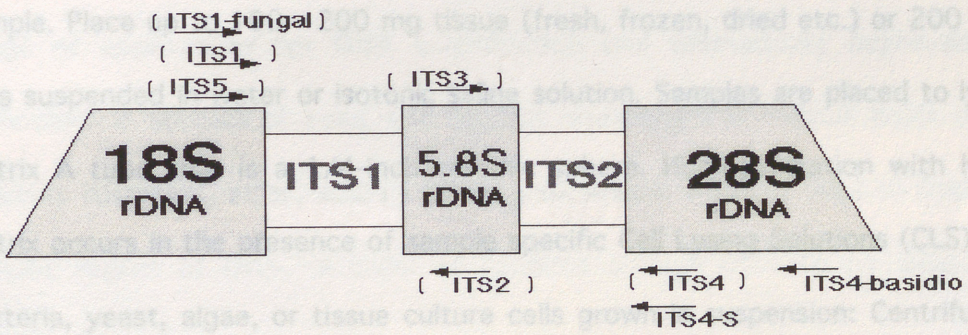


Figure 4. ITS Region Ribosomal operon in order of 18S-5.8S-28S showing the local of the ITS region, also where ITS primers would bind, obtained from http://plantbio.berkeley.edu/~bruns/.

Forward Primer	Reverse Primer	Region Amplified
ITS1	ITS4	Entire ITS
ITS1	ITS2	ITS1
ITS3	ITS3	ITS2
ITS1-fungal	ITS4	Entire ITS
ITS1-fungal	ITS2	ITS1
ITS5	ITS4	Entire ITS
ITS5	ITS2	ITS
ITS3B	ITS4	ITS2

Table 1. ITS General Primer and Amplification Regions.

Genomic DNA Extraction

Qbiogene FastDNA® Kit protocol was used for extraction of the fungal isolates. The kit can be used to lyse and isolate DNA from 200mg of almost any sample. Place up to 100 - 200 mg tissue (fresh, frozen, dried etc.) or 200 µl of cells suspended in water or isotonic saline solution. Samples are placed to lysing matrix A tube that is a 1/4-inch ceramic sphere. Homogenization with lysing matrix occurs in the presence of sample specific Cell Lysing Solutions (CLS). For bacteria, yeast, algae, or tissue culture cells grown in suspension: Centrifuge a sufficient volume of culture to provide a pellet size of 50-100 mg wet weight or up to 109 bacteria, 108 yeast/algae, or 107 mammalian cells. Resuspend pellets in water or isotonic saline to give a maximum suspension volume of 200 µl Eluted DNA is know ready for application including digestion, electrophoresis and PCR (http://qbiogene.com).

Polymerase Chain Reaction

The Polymerase chain reaction (PCR) is one of the revolutionizing scientific developments in the field of biotechnology. Kary Mullis developed PCR

technology in 1983and for that was awarded the Nobel Prize in chemistry 1994. PCR is the cornerstone of modern biotechnology and it is also a key procedure in numerous basic studies involving DNA and other biomolecules. The method is especially useful for the detection and identification of disease organisms that are difficult or impossible to culture, including many kinds of bacteria, fungi, and viruses, PCR is capable of generating analyzable quantities of the organism's genetic material for identification. PCR is still the most popular amplification method, however alternatives to PCR have successfully invaded the scientific arena. The emergence of such methodologies has significantly widened the range of approaches for DNA amplification and dramatically improved the technological abilities of basic and applied researchers in various fields of life sciences (Demidov, et al., 2004). PCR is a technique, which is used to amplify a specific region of DNA, in order to produce enough DNA to be adequately tested.

This technique can be used to identify with a very high-probability, disease-causing viruses and/or bacteria. In order to use PCR, one must already know the exact sequences which flank (lie on either side of) both ends of a given region of interest in DNA (may be a gene or any sequence). One need not know the DNA sequence in-between.

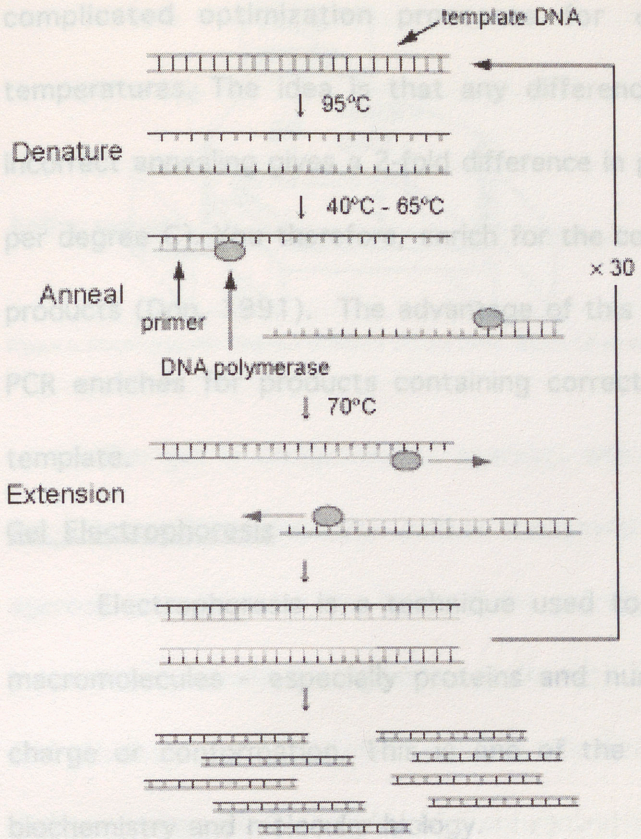


Figure 5. PCR Amplification Shown above A diagram of PCR amplification that involves denaturizing, primer annealing and synthesis. In general the mixture is first heated to apprx. 95°C to denature the dsDNA, then the chosen DNA polymerase is activated by the high temperature. The temperature for the mixture is then lowered to apprx. 65°C depending on the base composition of the primer, this step forms the bond of the primer and the ssDNA. This is followed by another increase to apprx. 72°C for the elongation of the complementary strand. This process repeats for a specified number of cycles, each doubling the number of target DNA fragments.

Touchdown PCR

Touchdown PCR has also become an increasingly popular technique for optimization of amplification of DNA. The temperature at which the primers anneal during a PCR cycle determines the specificity of the amplification region. Touchdown PCR involves decreasing the annealing temperature by half a degree Celsius to a degree Celsius every second cycle to a 'touchdown' annealing temp which is then used for 10 or so cycles. It was originally intended to bypass more

complicated optimization processes for determining optimal annealing temperatures. The idea is that any differences in Tm between correct and incorrect annealing gives a 2-fold difference in product amount per cycle (4-fold per degree C). You therefore, enrich for the correct product over any incorrect products (Don, 1991). The advantage of this technique is that the touchdown PCR enriches for products containing correct matches between primers and template.

Gel Electrophoresis

Electrophoresis is a technique used to separate and sometimes purify macromolecules - especially proteins and nucleic acids - that differ in size, charge or conformation. This is one of the most widely used techniques in biochemistry and molecular biology.

When charged molecules are placed in an electric field, they migrate toward either the positive (anode) or negative (cathode) pole according to their charge. In contrast to proteins, which can have either a net positive or net negative charge, nucleic acids have a consistent negative charge imparted by their phosphate backbone, and migrate toward the anode. Nucleic acids are electrophoresed within a matrix or "gel". The gel with the loading buffer is immersed within an electrophoresis buffer that provides ions to carry a current and some type of buffer to maintain the pH at a relatively constant value.

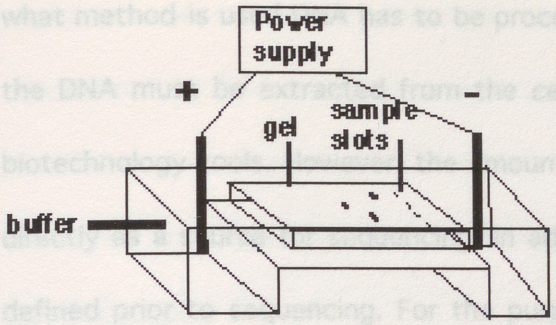


Figure 6. Electrophoretic Diagram of Sample Gel and Power Supply Schematic.

The gel is composed of agarose, which is a polysaccharide, extracted from seaweed. It is typically used at concentrations of 0.5 to 2%. The higher the agarose concentration the "stiffer" the gel. Agarose gels are extremely easy to prepare: you simply mix agarose powder with buffer solution, melt it by heating, add ethidium bromide is make possible visualization of DNA after electrophoresis and pour the gel. By varying the concentration of agarose, fragments of DNA from about 200 to 50,000 bp can be separated using standard electrophoretic techniques.

Principles of DNA sequencing

The DNA sequencing methods that so far have been developed are based on two fundamental different strategies namely direct or indirect sequencing. Direct sequencing techniques involve a variety of synthesis, degradation, and/or separation techniques, and include two techniques the traditional dideoxy method by Sanger (Sanger, et al., 1977) and the Pyrosequencing method (Nyrén, 2001; Ronaghi, et al., 1998b). Among the indirect sequencing methods sequencing-by-hybridization dominate (Drmanac, et al., 1989). Regardless of

what method is used DNA has to be processed before it can be sequenced. First the DNA must be extracted from the cell, which is easily achieved by modern biotechnology tools. However, the amount of DNA in a cell is to small to be used directly as a source for sequencing. In addition, the region of interest has to be defined prior to sequencing. For the purpose of amplification of a specific DNA region, PCR is often utilized. In most DNA sequencing methods the DNA template must be processed under or after the amplification step, simply for visualization, detection or capture reasons. In the original gel based dideoxy method by Sanger (see description of the method below) the terminated fragments were labeled by modified radioactive nucleotides. In the Pyrosequencing method one of the primers in the PCR is biotinylated to allow preparation of single-stranded DNA after capturing of the DNA on streptavidin-coated magnetic beads.

Sanger Dideoxy Method

In 1977, Sanger and colleagues developed an elegant DNA sequencing method that has become known as the dideoxy or enzymatic method (Sanger, et al., 1977). The method capitalizes on the ability of the DNA polymerase to use 2',3'-dideoxynucleoside triphosphates (ddNTP). Four reactions are set up, which included a primed ssDNA template, DNA polymerase, dNTP's and one of the four ddNTPs in each reaction. When a ddNMP is incorporated at the 3' end of the growing primer chain, chain elongation is terminated due to the lack of a free 3'-hydroxyl group. Each of the four elongation reactions contains a

population of extended primer chains, all of which have a fixed 5' end determined by the annealed primer and a variable 3' end terminating at a specific nucleotide position. The chains can be visualized after electrophoretic separation on a high resolution denaturing polyacrylamide gel.

Pyrosequencing

Pyrosequencing is based on a sequencing-by-synthesis method. The background work started in 1985 (Melamede and Wallace, 1985; Nyren and Lundin, 1985) and based on these theories the work had started towards the development of a working bioluminescence based technique. In 1993 came the first design for a solid phase-sequencing assay (Nyren, et al., 1993) a single stranded DNA template using a sequencing primer (5'-3' direction) and a primer-directed polymerase extension to initiate incorporation. Pyrosequencing is a DNA sequencing technique that is based on the detection of released pyrophosphate (PPi) during DNA synthesis. In a cascade of enzymatic reactions, visible light is generated that is proportional to the number of incorporated nucleotides. The cascade starts with a nucleic acid polymerization reaction in which inorganic PPi is released as a result of nucleotide incorporation by polymerase. The released PPi is subsequently converted to ATP-by-ATP sulfurylase, which provides the energy to luciferase to oxidize luciferin and generate light. Because the added nucleotide is known, the sequence of the template can be determined. The nucleic acid molecule can be either RNA or DNA. However, because DNA polymerases show higher catalytic activity than RNA polymerases for limited

nucleotide extension, efforts have been focused on the use of a primed DNA template for pyrosequencing (Ronaghi, 2001).

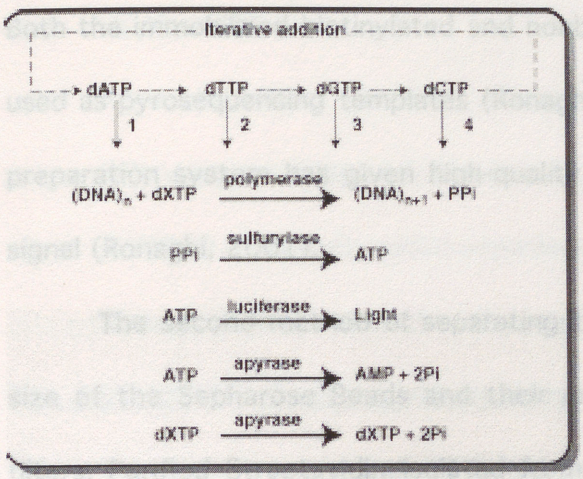


Figure 7. Pyrosequence In Pyrosequencing sequencing method, four nucleotides are added stepwise to the template hybridized to a primer. The PPi released in the DNA polymerase-catalyzed reaction is detected by the ATP sulfurylase and luciferase in a coupled reaction. A nucleotide-degrading enzyme continuously degrades the added nucleotides. After the first added nucleotide has been degraded, the next nucleotide can be added. As this procedure is repeated, longer stretches of the template sequence are deduced. dXTP, one of the four nucleotides.(Ronaghi, et al 1998).

Preparation for Pyrosequencing

Template preparation for pyrosequencing is straightforward. After generation of the template by PCR, the product should be purified prior to pyrosequencing. Unincorporated nucleotides and PCR primers in PCR reaction perturb the pyrosequencing reaction. The salt in the PCR reaction slightly inhibits the enzyme system and should be removed or diluted. Two strategies are currently available for generation of a primed DNA template for pyrosequencing (Ronaghi, 2001). Streptavidin-coated magnetic beads have been used to prepare a primed DNA template for pyrosequencing. This technology enables biotinylated PCR product to be captured onto magnetic beads. Through a

complex it forms with the streptavidin-coating the beads after sedimentation The remaining components of the PCR reaction can be removed by washing to obtain pure double-stranded DNA followed by alkali denaturation to yield ssDNA. Both the immobilized biotinylated and nonbiotinylated strands in solution can be used as pyrosequencing templates (Ronaghi, et al. 1998a, 1999). This template preparation system has given high-quality sequence data with low background signal (Ronaghi, 2001).

The second method of separating the two DNA strands is based on the size of the Sepharose Beads and their inability to pass through certain sized filters. Purified Streptavidin isolated from Streptomyces avidinii (according to instructions manual, Streptavidin Sepharose High performance beads, Amersham Biosciences, Piscataway, NJ, USA) is immobilized on the beads. The immobilized Streptavidin will bind to the biotinylated PCR-fragments. The mean particle size is 34 μm and the beads are constructed from highly cross-linked spherical agarose and stored in 20% ethanol. The reaction tube containing the attached dsDNA to the Sepharose beads is completed with the vacuum preparation protocol.

<u>The PSQ MA96 (multi application machine)</u>

Pyrosequencing PSQ MA96 system is the first and standard model. It is originally designed for both SNP-analysis (single-nucleotide-polymorphism) and de novo sequencing. The machine is loaded with a PSQ 96 SQA reagent cartridge and is loaded with the following amounts of reagents and nucleotides:

- Enzyme (**E**) (DNA polymerase, ATP sulfurylase, Luciferase and Apyrase), Volume/ well: 6 μl Cartridge dead volume: 100-μl
- Substrate (**S**) (APS and D-luciferin), Volume/ well: 6 μl Cartridge dead volume: 100 μl
- Nucleotides (dATP_S (**A**), dCTP (**C**), dGTP (**G**) and dTTP (**T**)),Volume/addition: 0.2 μl Cartridge dead volume: 100 μl
- SSB (2.2 mg/ml), added separately to each well, Volume/well: 1.5 μl

Biotage provides all nucleotides, substrates and enzymes.

<u>The PSQ HS96A (high sensitivity machine)</u>

This is the more advanced model. It is originally designed for SNP-analysis and can also be used for de novo sequencing, by making some changes to the programming set-up. The machine is loaded with either a CDT (capillary dispensation tip) reagent cartridge (Biotage) or a NDT (nucleotide dispensing tips) reagent cartridge (Biotage). The use of NDT cartridge allows for more advanced dispensation through a gold-plated micro dispensator. It manages to dispense smaller volumes of nucleotides, however, the tips clogs very easilyand are then unusable. Both cartridge types use RDT (reagent dispensation tip) (Biotage) for dispensing the enzyme and substrate, because of the lesser volume dispensation requirements. The NDTs are more suited for longer sequencing runs and was not used in this study. The CDT cartridge is loaded in a similar fashion as the one used for the MA96 machine. The CDT cartridges are loaded with the following ingredients:

- Enzyme (E) (DNA polymerase, ATP sulfyrulase, Luciferase and Apyrase), Volume/ well: 2 μl Cartridge dead volume: 50 μl
- Substrate (S) (APS and D-luciferin), Volume/ well: 2 μl Cartridge dead volume: 50 μl
- Nucleotides (1/3 dATP_S (A), 1/2 dCTP (C), 2/3 dGTP (G) and 2/3 dTTP (T)), Volume/addition: 0.2 μl Cartridge dead volume: 50 μl
- SSB (2.2 mg/ml), added separately to each well, Volume/well: 0.2 μl

Pyrogram

Pyrogram™ is a quantitative representation of the samples' nucleotide base sequence. Every Pyrogram shows determined polymorphic position and is ideal for quantitative analysis (Biotage). The light is detected by a charge coupled device (CCD) camera and displayed as a peak in a pyrogram™. Each peak height is proportional to the number of nucleotides incorporated. Unincorporated dNTP and excess ATP are continuously degraded by Apyrase (Ramon, et al., 2003).

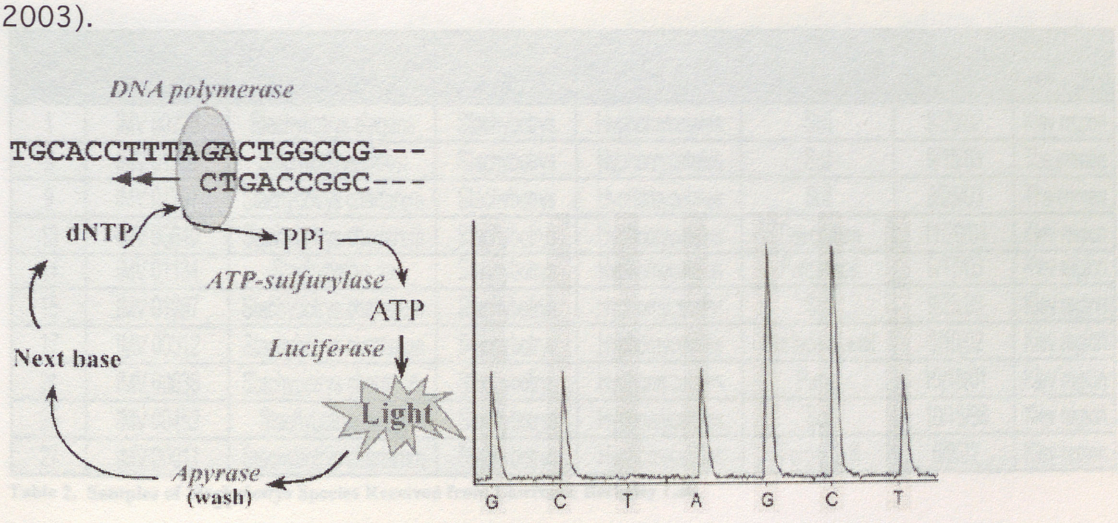


Figure 8. Pyrosequencing Cycle Diagram of Pyrosequencing cyle and Pyrogram of the Incorporation of dNTP's.

CHAPTER 3

DESIGN OF STUDY

Genomic Material

The samples of *Stachybotrys* isolates where obtained from Lawrence Berkley Lab which where collected from various locations. *Stachybotrys* isolates where spread on pre made agar plates for 72hrs, at 37°C. *Stachybotrys* colony was taken from plate and grown in Sabouraud Dextrose for 48-72hrs at 37°C. Sabouraud Dextrose Broth is a liquid medium used for cultivation of yeasts and molds from liquid, which are normally sterile. Acid pH of the medium inhibits bacterial growth and enhances yeasts and molds recovery for the extraction of the *Stachybotrys* fungi.

No.	SAMPLE ID	SPECIES NAME	GENUS	ORDER	ISOLATED FROM	DATE FOUND	REGION
1	IMV 00220	Stachybotrys elegans	Stachybotrys	Hyphomycetales	Soil	8/29/02	Kiev region
3	IMV 01338	Stachybotrys bisbyi	Stachybotrys	Hyphomycetales	Soil	9/18/03	The crimea
9	IMV 01297	Stachybotrys chartarum	Stachybotrys	Hyphomycetales	Soil	9/25/03	The crimea
13	IMV 00640	Stachybotrys chartarum	Stachybotrys	Hyphomycetales	The crimea	11/20/01	Kiev region
14	IMV 01134	Stachybotrys sp	Stachybotrys	Hyphomycetales	Forest soil	8/17/03	Kiev region
15	IMV 01297	Stachybotrys chartarum	Stachybotrys	Hyphomycetales	Soil	9/25/03	Kiev region
17	IMV 00012	Stachybotrys chartarum	Stachybotrys	Hyphomycetales	Surface of wall	6/10/02	Kiev region
21	IMV 00638	Stachybotrys chartarum	Stachybotrys	Hyphomycetales	Paper	10/18/01	Kiev region
25	IMV 00452	Stachybotrys bisbyi	Stachybotrys	Hyphomycetales	Soil	10/18/98	Kiev region
27	IMV 00011	Stachybotrys chartarum	Stachybotrys	Hyphomycetales	Forest Soil	6/9/02	Kiev region

Table 2. Samples of *Stachybotrys* Species Received from Lawrence Berkeley Lab.

The Qbiogene kit was used, implementing minor changes to the manufacturers protocol for optimization. The DNA extraction was done at the Lawrence Berkeley Lab because pathogenic samples where not allowed at the Stanford Genome Technology Center. What was done first was to choose the appropriate lysing matrix for the sample to be processed. The fast DNA kit comes with a matrix appropriate for fungi DNA extraction. For a better homogenizing process, a _ ceramic sphere is included/ added to each tube. Next, the appropriate Sample-specific lysing solution (CLS) in the case of fungi, CLS-Y is used. Add 1ml of CLS-Y to the tube containing the lysing matrix. To this solution of Lysing matrix-CLS complex, add Biomass of the sample to be lysed. Make sure there is about .25cc space left at the top of the tube to avoid sample loss. Homogenize the sample in a Fast Prep Instrument for about 80secs or 40 twice at a speed of 4.5-5. The tube is transferred a shaker and shake for about 30mins at 100rpm. the centrifuge forming a pellet , which will be discarded. The centrifugation will continue at 14,000rpm for 5mins. Once the centrifugation is complete the transfer of about 600ul of supernatant are put into a clean micro centrifuge tube. To the tube then 600ul of binding matrix is added mixing gently and incubated for about 5mins at room temp. Another pulse spin is done for 5 seconds to pellet binding matrix and discard supernatant. Gently resuspend the pellet with 500ul of SEWS-M and transfer suspension to a Spin filter. After resuspending the pellet centrifuge for 1min and

discard content of catch tube. Centrifuge for another 1 minute to dry binding matrix/DNA complex, transfer Spin filter to a new catch tube. Resuspend Matrix/DNA complex in 100ul DES to elute DNA. Wait for 2-3mins and centrifuge for 1min at 14,000rpm to transfer DNA containing DES to catch tube. DNA is now ready for use without further manipulation (Obtained from Qbiogene w/modification).

Bioinformatics

The use of computers to characterize the molecular components of living things has become very popular in molecular biology. Bioinformatics describes any use of computers to handle biological information, additional nucleotide information of *Stachybotrys* where located on NCBI and DNA Data Bank of Japan websites. The BLAST search, using the spacer sequence as query, identified complete matches perfect alignment of sequences of *Stachybotrys* species with variable and conserved regions. This information was used to develop primers for the multiple sequencing primer technique as well as select general primers for PCR. Once the sequences where developed, for better optimization the Oligo®4.05 primer analysis program was used. To elevate the potential for formation of any unwanted unstable dimers or hairpin loops of sequencing primers.

DNA Amplification

DNA amplification was performed in 50μl mixtures containing 3μl of prepared DNA sample, 5μl of PCR buffer, 5μl $MgCl_2$, 5μl dNTP 10x, 1μl of the

forward and reverse ITS primers and 0.3 μl of TITANIUM™ Taq DNA Polymerase for touchdown PCR.

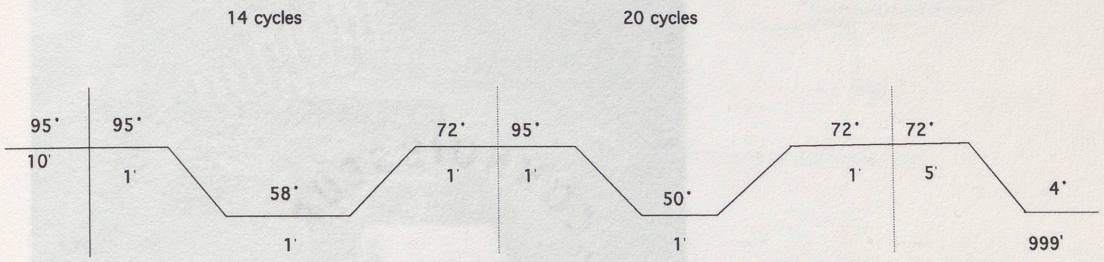


Figure 9. The Thermocycler Temperature for PCR Amplification of the *Stachybotrys* DNA Samples.

Template Preparation for Pyrosequencing

This method of separating the two DNA strands is based on the size of the Sepharose Beads and their inability to pass through certain sized filters. Purified Streptavidin isolated from Streptomyces avidinii (according to instructions manual, Streptavidin Sepharose High performance beads, Amersham Biosciences, Piscataway, NJ, USA) is immobilized on the beads. The immobilized Streptavidin will bind to the biotinylated PCR-fragments. The mean particle size is 34 μm and the beads are constructed from highly cross-linked spherical agarose and stored in 20% ethanol. The reaction tube containing the attached dsDNA to the Sepharose beads is vacuumed through a filtered metal tip (which the beads can not pass through). The beads are cleaned and the dsDNA is

denatured through a vacuum preparation method according to the protocols suited for use of the Vacuum Prep Worktable (Biotage, Uppsala, Sweden).

Figure10. Vacuum Preparation Worktable (Biotage, Uppsala, Sweden). Picture taken at Stanford Genome Technology Center Stanford, CA. 2004.

The Vacuum Prep Tool is consists of a holder with 96 metal reusable filtered tips. The inside of the Vacuum Prep Tool is air tight and contains a liquid chamber and an outlet connected to an applied source of vacuum. The vacuum force is obtained by a vacuum pump (Laboport, KNF Neuberger, Freiburg, Germany) connected to a plastic container for capture of the vacuumed liquid.

Single-Strand DNA-Binding Protein (SSB)

SSB has several positive effects in Pyrosequencing (Ronaghi, 2000). Improvements were observed as increased efficiency of the enzymes, reduced mis-priming (primer dimers), as measured by nonspecific signals, increased signal intensity during the reaction, higher accuracy in reading the number of identical adjacent nucleotides in difficult templates and longer reads. SSB Binds to

separated strands and prevents secondary DNA structures proven to cause problems for the Pyrosequencing technology.

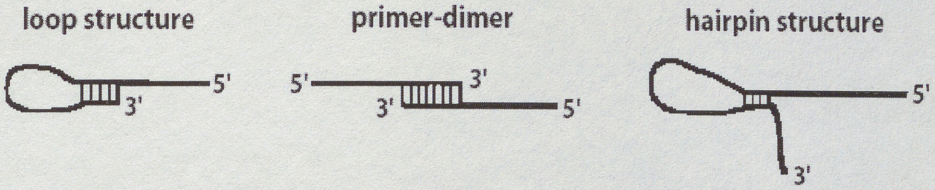


Figure11. Secondary Structure. Shows the secondary structure types of looping and primer dimer associated with not using SSB.

Pyrosequencing

PSQ HS96A

The PSQ HS96A (high-sensitivity machine) was used because of the sensitivity of HS light detection system, which enables small amounts of sample DNA to be analyzed (typically 5-10 μl of PCR product). This also reduces the running cost of the experiment, having to only minimal amounts of the PCR product.

Picture 1. PSQ HS96A (Biotage, Uppsala, Sweden) picture taken by Michelle Davis at the Stanford Genome Technology Center Stanford, CA. 2004

The following is an schematic drawing of the automated system for liquid-phase pyrosequencing. Four dispensers move on an X-Y robotics arm over the microtiter plate and add four different nucleotides, according to the prespecified order. The microtiter plate is agitated continuously to mix the added nucleotide. Generated light is directed to the CCD camera using a lens array located exactly below the microtiter plate (Ronaghi, 2001).

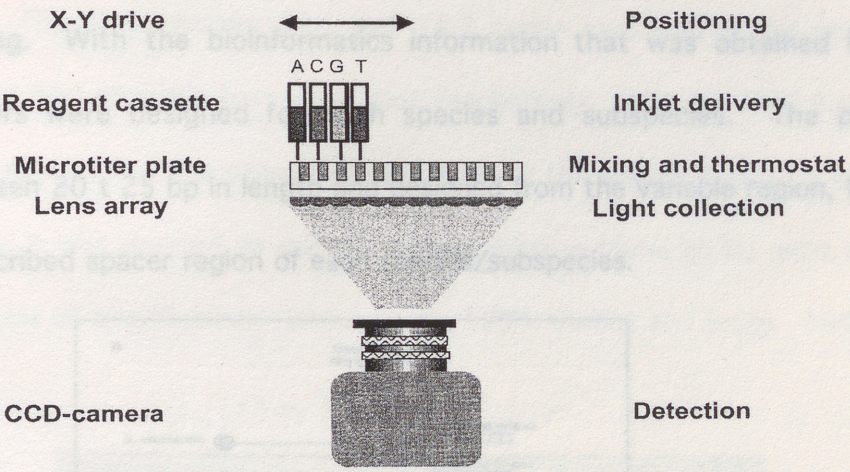


Figure 12. CCD Light Schematic drawing of the CCD Light Emission Process Used During Pyrosequencing.

Pyrosequencing Based Fungal Detection

DNA sequencing is a useful technique for detection and typing of many different organisms. However, if several species/genotypes are present in a sample, hence comprising different nucleotide patterns at a variable site, the risk that the sequence information is difficult or impossible to interpret is high, since signals from all the available types will be produced. The sequencing primer

binds to all types present in the sample. Introducing a mixed set of target-specific sequencing primers to a sample containing multiple infections or unspecific amplification products can solve this problem. This strategy is suited for selective detection and genotyping of relevant microorganisms and samples harboring different DNA targets such as multiple variant/infected samples as well as unspecific amplification products (Gharizadeh, et a.l, 2003). This method can eliminate the need for nested PCR, re-performing unsuccessful PCR and cloning. With the bioinformatics information that was obtained from NCBI, primers were designed for each species and subspecies. The primers are between 20 t 25 bp in length and designed from the variable region, the internal transcribed spacer region of each species/subspecies.

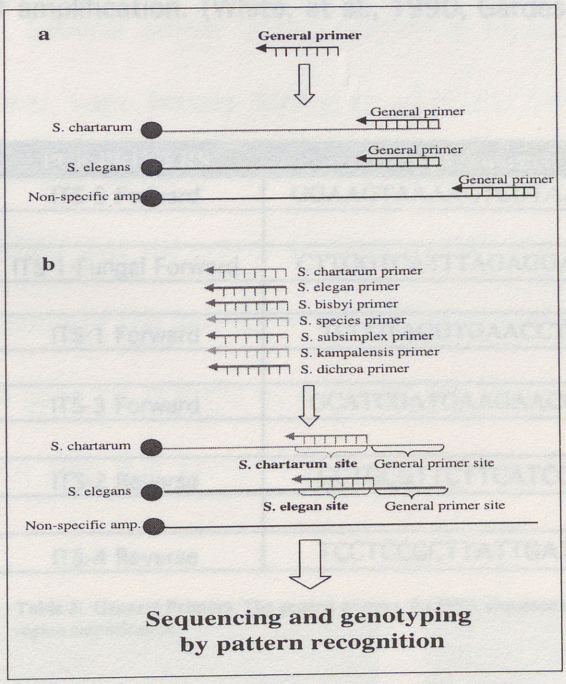


Figure 13. Pattern Recognition Shows a schematic of the pattern recognition for sequencing and genotyping using multiple sequencing primers.

CHAPTER 4

RESULTS AND DISCUSSION

The *Stachybotrys* ITS-1 and ITS-2 region examined in this study contain 570 to 620 alignable base pairs (bp), respectively. With the NCBI Blast program I was able to find the conserved region for all the species types. From previous papers it was decided to use the genera primers found to be most common in this type of amplification. (White, et al., 1990, Gardes and Bruns , 1993).

PCR PRIMERS	DNA sequence
ITS-5 Forward	GGAAGTAAAAGTCGTAACAAGG
ITS-1-Fungal Forward	CTTGGTCATTTAGAGGAAGTAA
ITS-1 Forward	TCCGTAGGTGAACCTGCGG
ITS-3 Forward	GCATCGATGAAGAACGCAGC
ITS-2 Reverse	GCTGCGTTCTTCATCGATGC
ITS-4 Reverse	TCCTCCGCTTATTGATATGC

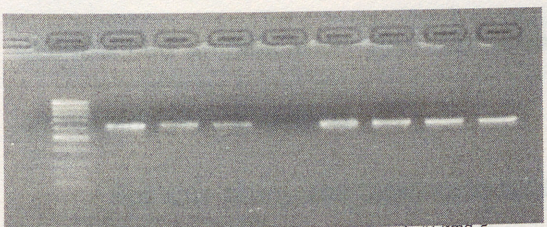
Table 3. General Primers. The general primers for DNA sequence used in ITS region amplification.

Primer Mix No.	Forward Primer	Reverse Primer	Region Amplified
1	ITS5	ITS4	Entire ITS
2	ITS5	ITS2	ITS1
3	ITS3	ITS4	ITS2
4	ITS1-fungal	ITS4	Entire ITS
5	ITS1-fungal	ITS2	ITS1
6	ITS1	ITS2	ITS1
7	ITS1	ITS2	ITS
8	ITS3B	ITS4	ITS2

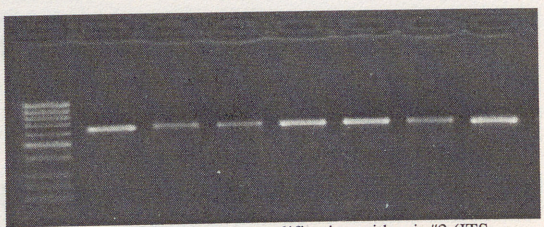
Table 4. Primer Pairs Indicates primers used for primer pairs and region amplified. The 3'-end of 18S to the 5'-end of 28S; ITS1 and ITS2 are regions within this complete region.

Touchdown PCR

Touchdown PCR using the general primers showed good results; Using of touchdown protocol produced more specific amplification and increased PCR product. . Both regions have been amplified, currently only the ITS 1 region was sequenced. Several primer pairs, contain a forward primer and biotinylated reverse primer, were initially applied to all of the Stachybotrys as to determine which primer pair would be ideal for all amplification experiments/reactions. It was established that the primer containing ITSFF and ITS 4 would be most useful for amplification of the species of interest. Mixes of the forward and reverse ITS region were used for PCR the best mix for my samples where the ITS FF and the ITS 4R, with this PCR primer mix I was able to amplify the majority of my samples.



Picture. 2 Gel Run showing amplification with mix#1(ITS 5 Forward/ITS4R)



Picture. 2a. Gel Run showing amplification with mix#2 (ITS Fungal Forward/ITS4R)

Primer Mix No.	Forward Primer	Reverse Primer	Region Amplified
1	ITS5	ITS4	Entire ITS
2	ITS5	ITS2	ITS1
3	ITS3	ITS4	ITS2
4	ITS1-fungal	ITS4	Entire ITS
5	ITS1-fungal	ITS2	ITS1
6	ITS1	ITS2	ITS1
7	ITS1	ITS2	ITS
8	ITS3B	ITS4	ITS2

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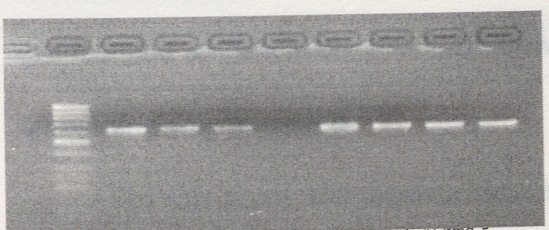
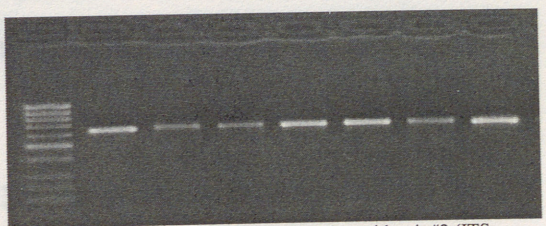


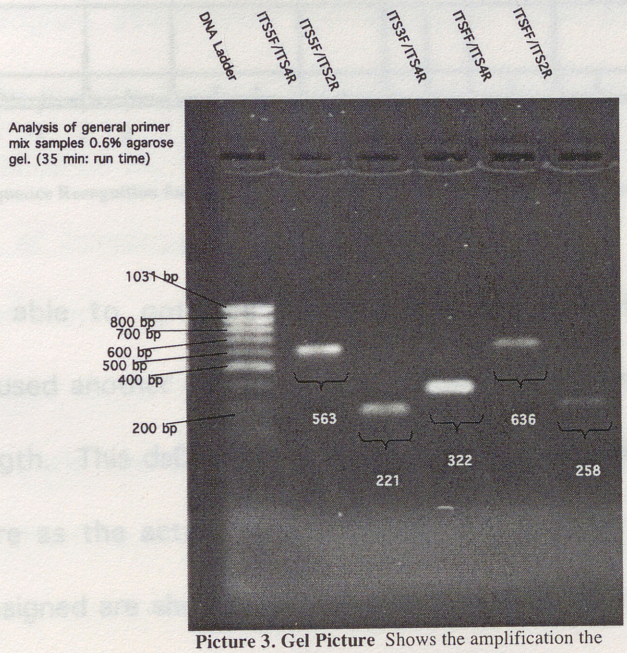
Picture. 2 Gel Run showing amplification with mix#1(ITS 5 Forward/ITS4R)



Picture. 2a. Gel Run showing amplification with mix#2 (ITS Fungal Forward/ITS4R)

	Primer Mix	ITS 5F/ ITS-4R	ITS-FF/ ITS-4R
LBL stock	Stachybotrys Species	#1	#2
IMV 00220	(1)Stachybotrys elegans	+	+
IMV 01338	(3)Stachybotrys bisbyi	+	+
IMV 01297	(9)Stachybotrys chartarum	+	+
IMV 00640	(13)Stachybotrys chartarum	-	+
IMV 01298	(15)Stachybotrys chartarum	+	+
IMV 00012	(17)Stachybotrys chartarum	+	+
IMV 00638	(21)Stachybotrys chartarum	+	+
IMV 00011	(27)Stachybotrys chartarum	+	+

Table 5. Results Shows the results of the gel run, showing the species that where amplified by the primer mixes 1 and 4.



Picture 3. Gel Picture Shows the amplification the Stachybotrys chartarum using 5 different primers mixes.

The species of greatest concern was the *Stachybotrys* chartarum because of their production of the trichothecene toxin that causes illness in humans and animals. The gel picture of the two mixes shows the very pronounced amplification with touchdown PCR and showing clearing that something was amplified.

For this study the oligonucleotide E3PN was used as positive-control for

the pyrosequencing machine, to make sure that the reagents and nucleotides were correctly loaded and that they are working. The E3PN sequence takes about 20 minutes to sequence, but it is enough to sequence it for 5 minutes, because the oligonucleotide is smartly designed to be able to test all the nucleotides at an early stage of the sequencing reaction (Akhras, 2004).

E3PN sequence pyrogram

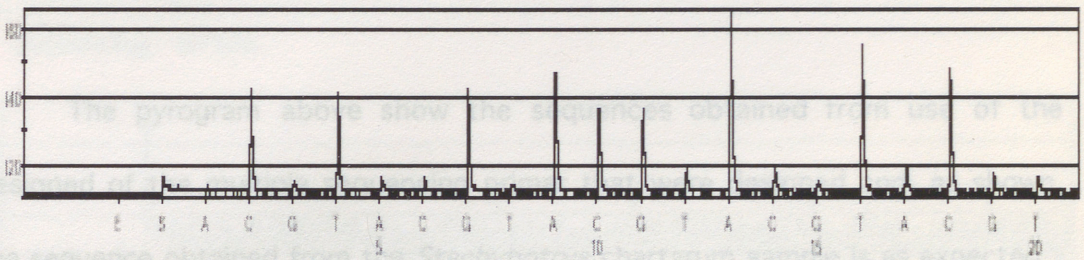


Figure 14. Sequence Recognition for the E3PN sequence used for control during Pyrosequenceing.

To be able to optimize all the parameters of the Pyrosequencing protocols we used another dsDNA template called LS (long sequence) template 303 bp in length. This dsDNA template that will go through the same sample prep procedure as the actual samples would. The multiple sequencing primer that where designed are shown with the after sequence for identification of the organism.

Organism	Multiple Sequencing Primer	Sequence Determination
Stachybotrys chartarum	GCGCCCTGCGCCCGGATCCAG	GCGCCCGCCGGAGACCCCAAACTC
Stachybotrys elegans	CGCGCCCTCCGACCCTCCCG	TCCGCGGGGGGATCGGGGAGCC
Stachybotrys bisbyi	TTCAGTATATTTCTCTGAGTGG	CAAACGCAAAATAAATCA
Stachybotrys subsimplex	TAACAAACTCCCAACCCACTG	TGGACATACCCATTCGTTG

Table 6. Multiple Sequencing Primer used for the determination of the *Stachybotrys* organism for the study.

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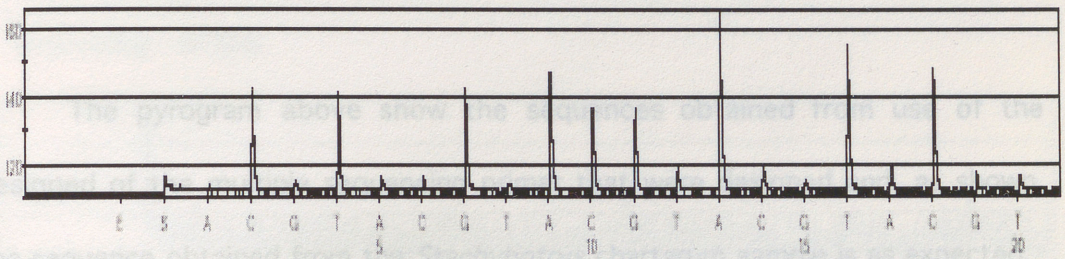
E3PN sequence pyrogram



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Organism	Multiple Sequencing Primer	Sequence Determination
Stachybotrys chartarum	GCGCCCTGCGCCCGGATCCAG	GCGCCCGCCGGAGACCCCAAACTC
Stachybotrys elegans	CGCGCCCTCCGACCCTCCCG	TCCGCGGGGGGATCGGGGAGCC
Stachybotrys bisbyi	TTCAGTATATTTCTCTGAGTGG	CAAACGCAAAATAAATCA
Stachybotrys subsimplex	TAACAAACTCCCAACCCACTG	TGGACATACCCATTCGTTG

Table 6. Multiple Sequencing Primer used for the determination of the *Stachybotrys* organism for the study.

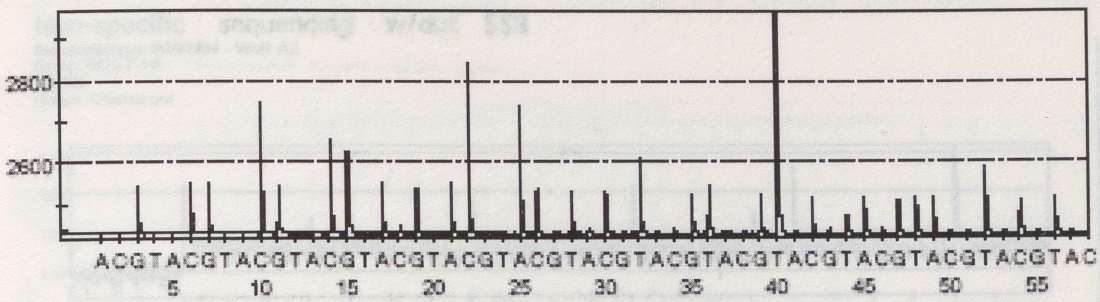

Figure 15. Sequence Determination. Shows the sequence determination for Stachybotrys chartarum sequenced with the multiple sequencing primer.

The pyrogram above show the sequences obtained from use of the designed of the multiple sequencing primer that were designed and, as shown, the sequence obtained from the *Stachybotrys* chartarum sample is as expected.

Optimization of Pyrosequencing using SSB

SSB was used in an attempt to improve sequence quality by preventing secondary (primer-dimer/hairpin) structure formations that can contribute to non-specific signals and inaccurate sequence reads. Use of the SSB also increases the efficiency of the enzyme used in a Pyrosequencing run. With this optimization less interference and background is observed, as indicated by the following pyrograms.

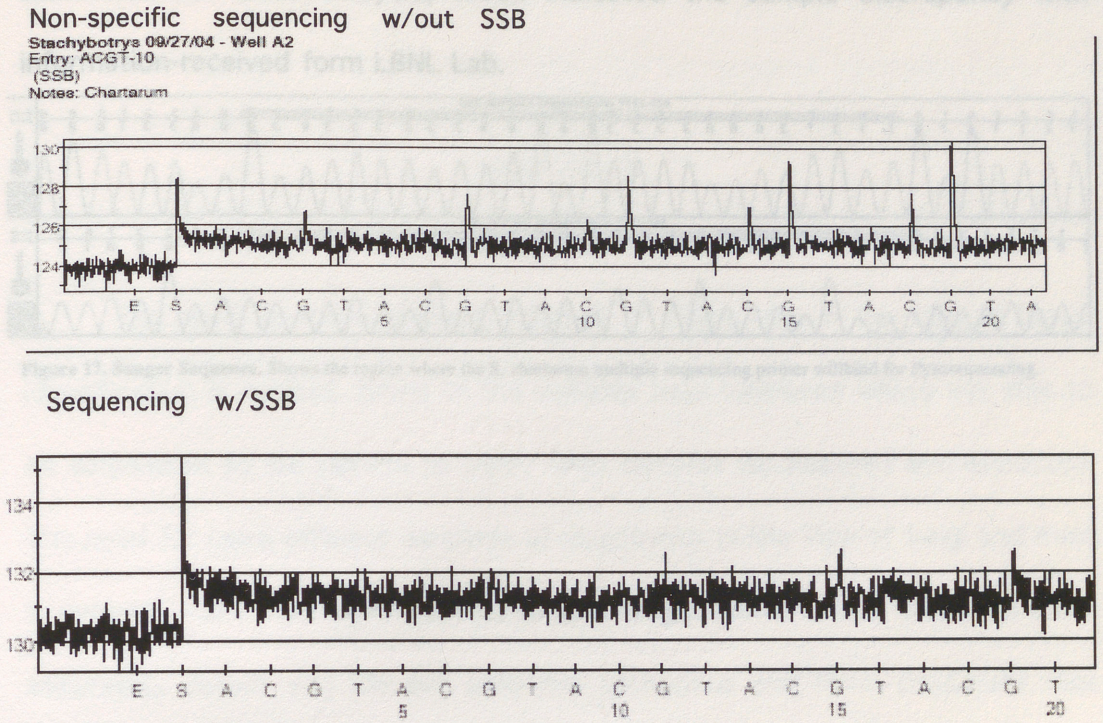

Figure 16. Pyrogram. Shows the pyrogram of the multiple sequencing primer for S. chartarum without SSB and with SSB.

The same samples used for Pyrosequencing were also to perform Sanger sequencing. 1.6pmol of the ITSFF and ITS4R and 2.2μl buffer was used for Sanger. Three of the PCR samples where diluted by 1:2 because of the intensity of the bands, in Sanger for better results the intensity of the PCR should be equal. Two standard 96-well plates prepared with the following: Plate one contained 2.2ml buffer/enzyme mix, and 10ml of PCR product. Second plate contained 10ml (1:6pm/ml conc.) primer. Sanger findings suggest that the S. elegans sample was in fact Penicillium Janczewskii. The sequence obtained was

submitted for Blast analysis, which indicated the sample discrepancy with information-received form LBNL Lab.

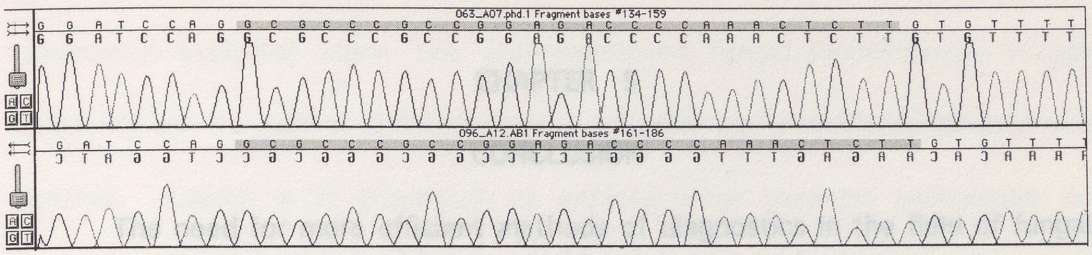

Figure 17. Sanger Sequence. Shows the region where the S. chartarum multiple sequencing primer willbind for Pyrosequencing.

CHAPTER 5

CONCLUSION

The need for more efficient methods of diagnostics in the field of fungal identification is evident. Some of the samples from Lawrence where not able to be sequenced so we wanted to verify what samples we cultured and extracted. The need for more efficient methods of diagnostics in the field of fungi and mold is evident. With the pyrosequencing technology, there is the ability to more accurately detect and identify potential pathogens and toxin producers that could affect humans and animals. There was however the question of the integrity of the samples, results from Sanger sequencing. The touchdown PCR amplification shows improved product yield and quality with the help of TITANIUM™ Taq DNA Polymerase, which was used to further optimize the protocol. The single-strand DNA binding protein (SSB) (Ronaghi, 2000) has been highly used for optimizing the Pyrosequencing system.

Future objective would include designing detection assays according to the geographic prevalence of microbes, as well as designing specific primers for all types of *Stachybotrys* that exist in environments of interest (i.e., buildings, homes and agricultural products). Future plans include using ATCC samples as to limit contamination possibilities. There is also a possibility of identifying other genes (e.g. Cox1 gene and Tri5 gene) for assay development, and comparing

between LBNL samples and also compare similarities with wild type isolates possible mutations in the genetic make-up of samples obtained from the Chernobyl exclusion zone. The aforementioned fungal identification models currently can be used as detection model for other relevant microorganisms and viruses. Lastly, it is important to explore other genomic techniques for detection and identification of relevant fungal species.

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