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Pyrosequencing Analysis of the Trichodiene Synthase 5 (TRI 5) Gene from the Members of the Genus Stachybotrys

Tineace Taylor Farrow

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THESIS

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PYROSEQUENCING ANALYSIS OF THE TRICHODIENE SYNTHASE 5 (TRI 5) GENE FROM THE MEMBERS OF THE GENUS *STACHYBOTRYS*

THESIS

Presented in Partial Fulfillment of the Requirements for

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By

Tineace Taylor Farrow, B.S.

Texas Southern University

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Approved By

Chairperson, Thesis Committee

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By

Tineace Taylor Farrow, M.S.

Texas Southern University, 2008

Professor Olufisayo Jejelowo, Ph.D., Advisor

This research will focus on multiple isolates of *Stachybotrys* species from culture collection companies which will be studied in an attempt to determine the presence of the Trichodiene synthase 5 (Tri5) gene, an enzyme involved in the early stages of the biosynthesis of trichothecenes. Using general primers, the Tri5 gene will be amplified by customized PCR technique. The amplicons will be sequenced using a new multiple sequence technique personally designed.

Stachybotrys is a genus of filamentous fungi belonging to the class of anamorphic ascomycetes (Kirk et al, 2001). The genus has a single well-known species, *Stachybotrys* chartarum. *S. chartarum* was initially associated with stachybotryotoxicosis, a disease in horses and other animals in the Ukraine and other parts of Eastern Europe during the 1930s. At the same time, it was also reported in humans who handled the infested hay or feed grain or who were exposed to the aerosols of dust and debris from the contaminated materials (Drobotko, 1945 and Gajdusek, 1953).

Outbreaks of stachybotryotoxicosis have since been reported among farm workers in Hungary (Andrassy et al., 1980) and Germany (Dill et al., 1997). During the past two decades *Stachybotrys*-associated health effects from inhalation exposure in humans have been reported in the United States (Croft et al., 1986; Johanning et al., 1993 and 1996; Montana et al., 1997; Etzel et al., 1998; Dearborn et al., 1999 and Flappan et al., 1999; Elidemir et al., 1999). The organism has been implicated in sick building syndrome and pulmonary hemosiderosis. In a report by Li et al., (2001) S. chartarum was associated with soybean root rot.

The goal of my study is to test the isolates for trichodiene synthase 5 (tri5) gene to determine if they have the potential to produce trichothecenes or any of its metabolites by using specific multiple sequencing primers that will be designed.

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CHAPTER 1

INTRODUCTION

Pyrosequencing analysis of the Trichodiene synthase 5 (Tri 5) gene from the members of the Genus *Stachybotrys*

Occurring worldwide, most fungi are largely invisible to the naked eye, living for the most part in soil, dead matter, and as symbionts of plants, animals, or other fungi. They perform an essential role in all ecosystems in decomposing matter and are indispensable in nutrient cycling and exchange. Some fungi become noticeable when fruiting, either as mushrooms or molds.

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.

Fungi are a class of organisms that includes yeasts, molds, mildews, and mushrooms. Fungi, other than mushrooms, live as single cells or as threadlike structures known as hyphae. Fungi reproduce through the production of spores. Spores can enter the air (be aerosolized); therefore, humans can come in contact with spores through skin and respiratory exposure. Fungi can produce secondary metabolites which include antibiotics

(penicillin) and mycotoxins. These toxins may adversely impact human health. Some other metabolites are volatile organic compounds that cause musty, moldy smells. Fungi require water to grow and can tolerate a wide range of temperatures.

The past twenty years have brought the recognition that an important factor in the health of people in indoor environments is the moisture and humidity of the buildings in which they live and work. Furthermore, it is now appreciated that the principal biology responsible for the health problems in such building are fungi rather than bacteria or viruses. Although fungi in this context have been traditionally viewed as allergens (and, in unusual circumstances, pathogens), data have accumulated to show that the adverse health effects resulting from inhalation of fungal spores are due to multiple factors. One factor produced by these fungi is small molecular toxins or mycotoxins. Traditionally, mycotoxins are held to be important in human and animal health because of their production by toxigenic-fungi-associated food and feed. However, mycotoxins tend to concentrate in fungal spores, and thus present a potential hazard to those inhaling airborne spores. Toxigenic spores strongly affect alveolar macrophage function and pose a threat to those exposed. Reports have indicated that *Stachybotrys chartarum*, *Aspergillus versicolor*, and several toxigenic species of *Penicillium* are potentially hazardous, especially when the air-handling systems have become heavily contaminated.

Perhaps the most hazardous of the toxigenic fungi found in wet buildings is *S. chartarum*, a fungus known to produce the very potent cytotoxic macrocyclic trichothenes along with a variety of immunosuppressants and endothelin receptor

antagonists mycotoxins. There are over 100,000 fungal species recognized, with 100 infectious agents of man (Roberts and Mackenzie, 1984).

Molecular genomic techniques have become very important tools in modern taxonomic studies. They have proven 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 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 research focused on multiple species of *Stachybotrys* from culture collection companies which will be studied in an attempt to determine the presence of the Trichodiene synthase 5 (Tri5) gene, which codes for an enzyme (trichodiene synthase) involved in the early stages of the biosynthesis of trichothecenes. Using general primers, the Tri5 gene was amplified by customized PCR technique. The amplicons were sequenced using Pyrsequencing.

CHAPTER 2

LITERATURE REVIEW

Stachybotrys is a genus of filamentous fungi belonging to the class of anamorphic ascomycetes (Kirk et al, 2001). The phylum Ascomycota consists of a large and diverse group of fungi that have one characteristic in common, they contain their sexual spores inside a sac-like structure called an ascus. This phylum contains only one Class, the Ascomycetes, which is divided into three broad groups, Archiascomycetes, Saccharomycetales, and Filamentous Ascomycetes (Taylor, *et. Al.*, 1996).

Fungi a genus of mold that grows on water damaged materials such as ceiling tiles, insulation, wallpaper, wood, sheet rock, and can be found in dust from contaminated building materials. It is a greenish-black fungus found worldwide that colonizes particularly well in high-cellulose material, such as straw, hay, paper, dust, lint, and cellulose-containing building material such as fiber board, and gypsum board that becomes chronically moist or water damage due to excessive humidity, water leaks, condensation or flooding . The genus has a single well-known species, *Stachybotrys chartarum*. *S. chartarum* was initially associated with stachybotryotoxicosis, a disease in horses and other animals in the Ukraine and other parts of Eastern Europe during the 1930s. At the same time, it was also reported in humans who handled the infested hay or feed grain or who were exposed to the aerosols of dust and debris from the contaminated materials (Drobotko, 1945 and Gajdusek, 1953). *Stachybotrys chartarum* grows and

produces spores in the temperature range of 2°-40°C (36°-104°F). It is also capable of producing several toxins. It has been postulated that moist high-cellulose and low-nitrogen materials at a temperature range of 0-40°C can provide sufficient condition for production of *Stachybotrys* toxins. Surfaces exposed to air with a relative humidity above 55% and subjected to temperature fluctuations are ideal for toxin production. Individuals with chronic exposure to the toxin produced by this fungus reported cold and flu symptoms, sore throats, diarrhea, headaches, fatigue, and dermatitis (Health and Energy).

Outbreaks of stachybotryotoxicosis have since been reported among farm workers in Hungary (Andrassy et al., 1980) and Germany (Dill et al., 1997). During the past two decades *Stachybotrys*-associated health effects from inhalation exposure in humans have been reported in the United States (Croft et al., 1986; Johanning et al., 1993 and 1996; Montana et al., 1997; Etzel et al., 1998; Dearborn et al., 1999 and Flappan et al., 1999; Elidemir et al., 1999). The organism has been implicated in sick building syndrome and pulmonary hemosiderosis. In a report by Li et al., (2001) S. chartarum was associated with soybean root rot.

As a result of these health complications, various authors have attempted to unravel the toxins from these organisms. The toxins that have been found belong mainly to the macrocyclic trichothecenes, non-trichothecenes (spirocyclic drimanes and triprenyl phenols), and various other compounds. The macrocyclic trichothenes are known to be highly cytotoxic. They are protein and DNA synthesis inhibitors, and are known to have antibiotic activity. The spirocyclic drimanes and triprenyl phenolics are immunosuppressants, hormone receptor antagonists, and inhibitors of various functions.

They are also known to have antiviral properties as well as being cytotoxins. The trichothecenes constitute a family of more than sixty sesquiterpenoid metabolites produced by a number of fungal genera, including *Fusarium, Myrothecium*, *Phomopsis, Stachybotrys*, *Trichoderma*, *Trichothecium*, and others (6, 30, 32).

Trichothecenes are classified as macrocylic or nonmacrocyclic, depending on the presence of a macrocylic ester or an ester-ether bridge between C-4 and C-15 (5). The macrocyclic trichothecenes are produced largely by *Myrothecium, Stachybotrys*, and *Trichothecium* species. The trichothecenes produced by *Stachybotrys atra* (*Stachybotrys chartarum*) have received the most attention.

The other compounds that have been found in members of the genus *Stachybotrys* include atranones, which are not known to have any toxic biological activity, Beta-1,3-glucan, which are inflammatory agents, and stachyflin compounds. These have antiviral activities. It has been found though that not all the members of this genus are toxic, and also that differences may be present within species, especially *S. chartarum*. Increase in pathogenic evidence and public concern about this mold in buildings, led the American Academy of Pediatrics Committee on Environmental Health to recommend that until more information is available, pediatricians should try to ensure that infants under 1 year of age are not exposed to moldy, water-damaged environments (AAP, 1998). In addition, Congressman John Conyers, Jr. of the 14th congressional district of Michigan introduced "The Melina Bill" H.R. 5040: The United States Toxic Mold Safety and Protection Act" in the U.S. House of Representatives (Conyers, 2002).

Since the storm known as Hurricane Katrina hit New Orleans (the Gulf Coast), extremely high mold levels indoors and outdoors, particularly in areas that were flooded after Hurricane Katrina were found. Forty-five different species of mold were identified, including *Stachybotrys* ("toxic mold"), *Cladosporum,* and *Aspergillus/Penicillium*. These types of molds cause symptoms such as major allergic and asthmatic attacks, pneumonia-like illnesses, and reactions to mold toxins (mycotoxins).

Polymerase Chain Reaction

Polymerase chain reaction (PCR), a process of *in vitro* targeted DNA replication, is the most important tool in our molecular ecology toolbox. PCR allows us to make multiple copies of specific loci, which can then be studied in greater detail. PCR allows us to take small, non-lethal samples from living organisms. It allows us to study ancient DNA from museum specimens and to carry out forensic analysis.

First, just like in a cell during DNA replication, DNA is denatured, i.e. the hydrogen bonds between strands of DNA are broken with heat. Next, synthetic DNA strands (primers) attach (anneal) to complimentary sequences of now single-stranded DNA. DNA polymerase attaches at the 3' end of the primer and extends the DNA fragment, i.e. reads the next base pair on the single strand and attaches the complimentary nucleotide (dNTPs) to it, thus creating double-stranded DNA.

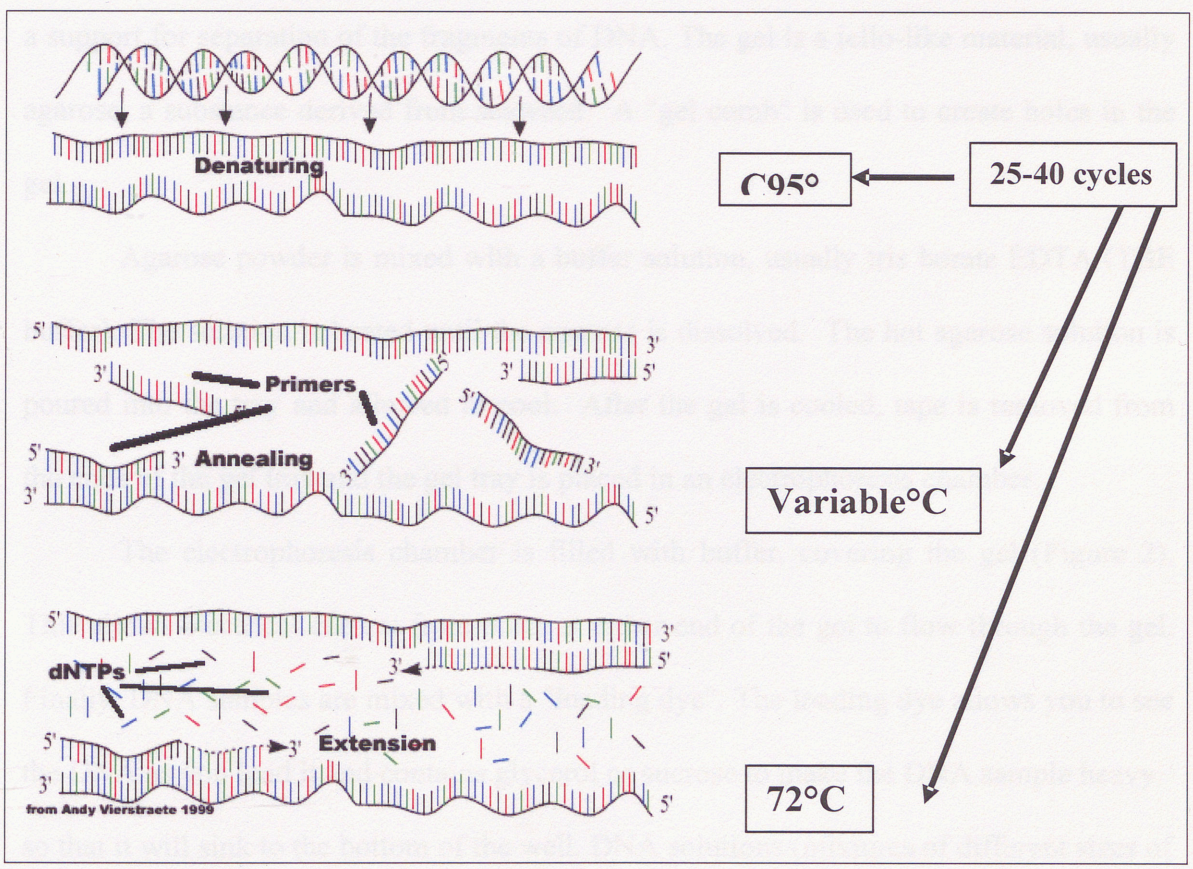
Primers anneal to complimentary strands of DNA when the solution is cooled. *Taq* DNA polymerase attaches and extends the new DNA when the temperature is increased again, but not so much that the DNA denatures again. This heating and cooling of solutions was once done in heated waterbaths like the one used in DNA extraction.

Now it is done much more efficiently in a machine called a thermal cycler (sometimes thermocycler).

Thermal cyclers heat and cool solutions based on a program entered into the computer running the machine. This program is called the thermal cycler profile. A typical PCR profile consists of a denaturation step (95°C), an annealing step (temperature depends on the primer sequence), and an extension step (72°C). Going through each of these steps in order once is one cycle. A typical PCR profile consists of 25-40 cycles (Figure 1).

FIGURE 1

PCR



With each cycle you double the amount of target DNA that is in the solution. Assuming you had just a single copy of DNA to begin with, after 30 cycles you would have over a BILLION copies of the target locus.

Gel Electrophoresis

In the early days of DNA manipulation, DNA fragments were laboriously separated by gravity. In the 1970s, the powerful tool of DNA gel electrophoresis was developed. This process uses electricity to separate DNA fragments by size as they migrate through a gel matrix. Organic molecules such as DNA are charged. DNA is negatively charged because the phosphates (red circles) that form the sugar-phosphate backbone of a DNA molecule have a negative charge. A gel is prepared which will act as a support for separation of the fragments of DNA. The gel is a jello-like material, usually agarose, a substance derived from seaweed. A "gel comb" is used to create holes in the gel.

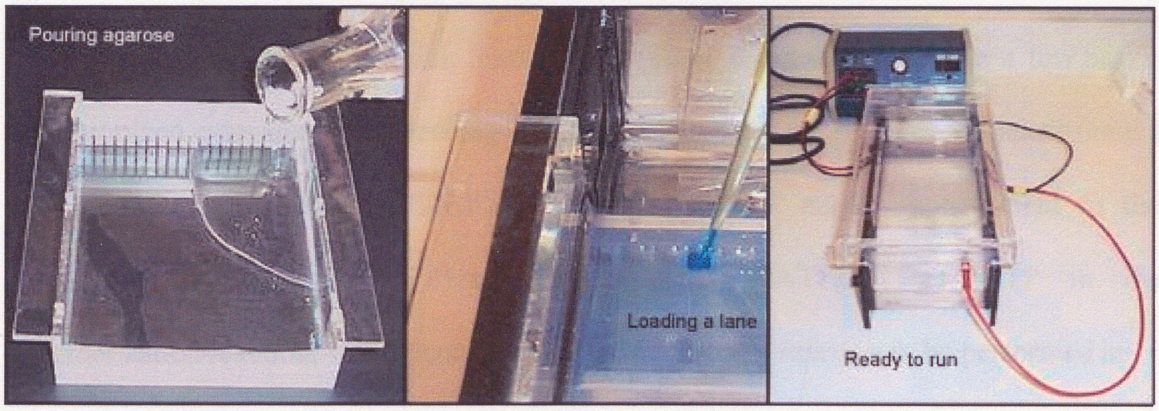
Agarose powder is mixed with a buffer solution, usually tris borate EDTA (TBE buffer). The solution is heated until the agarose is dissolved. The hot agarose solution is poured into the tray and allowed to cool. After the gel is cooled, tape is removed from the ends of the gel tray and the gel tray is placed in an electrophoresis chamber.

The electrophoresis chamber is filled with buffer, covering the gel (Figure 2). This allows electrical current from poles at either end of the gel to flow through the gel. Finally, DNA samples are mixed with a "loading dye". The loading dye allows you to see the DNA as you load it and contains glycerol or sucrose to make the DNA sample heavy so that it will sink to the bottom of the well. DNA solutions (mixtures of different sizes of

DNA fragments) are loaded in a well in the gel. A safety cover is placed over the gel (to keep you from frying yourself) and electrodes are attached to a power supply. Electrical current is applied. DNA fragments will migrate through the gel at various rates, depending on their size. The gel matrix acts as a sieve for DNA molecules. Large molecules have difficulty getting through the holes in the matrix. Small molecules move easily through the holes. Because of this, large fragments will lag behind small fragments as DNAs migrate through the gel. As the separation process continues the separation between the larger and smaller fragments increases. Molecular weight markers are often electrophoresed with DNAs. Molecular weight markers are usually a mixture of DNAs with known molecular weights. Molecular weight markers are used to estimate the sizes of DNA fragments in your DNA sample. When the dye marker indicates that DNA fragments have moved through the gel, the current is turned off and the gel is removed from the tray.

FIGURE 2

Electrophoresis Chamber



Pyrosequencing

Pyrosequencing is a bioluminometric sequence-by-synthesis approach that is based essentially on the detection of pyrophosphates (PPi) released during synthesis (Ronaghi, 1998). After the subsequent enzymatic reactions, visible light is produced. This light is proportional to the number of nucleotides incorporated during synthesis. The technology involves the sequential incorporation of four deoxynucleotides into a single stranded DNA template. A sequencing primer is used and is extended through the polymerase catalyzed incorporation of the nucleotides. After a complementary nucleotide is incorporated, a pyrophosphate molecule (PPi) is released. The Pyrosequencing enzyme cascade involves four enzymes, DNA polymerase, ATP sulfurylase, luciferase, and apyrase, and two substrates, adenosine 5'-phosphosulfate (APS) and luciferine.

The theory behind sequencing-by-synthesis was described in 1985 (Melamede, 1985) and based on this principle, detection of pyrophosphate was used in DNA sequencing (Hyman 1988). Efforts were also put into the development of this principle for sequence determination using labeled nucleotides (Canard and Sarfati, 1994; Cheesman, 1994; Metzker, 1994; Rosenthal, 1989; Tsien et al. 1991). However, Metzker (1994) showed that the incorporation efficiency of labeled nucleotides is low, causing nonsynchronized extension, which made it difficult to sequence more than a few bases. Synchronized extension in sequencing-by-synthesis requires exonuclease-deficient (exo^-) DNA polymerase and unmodified nucleotides. The first major improvement to the Pyrosequencing Methodology was substitution of dATPαS for dATP in the polymerization reaction, which enabled the pyrosequencing reaction to be performed in

homogeneous phase in real time (Ronaghi, 1996). It was later shown that the nonspecific signals were attributed to the fact that dATP is a substrate for luciferase. Conversely, dATPαS was found to be inert for luciferase, yet could be incorporated efficiently by all DNA polymerases tested (Ronaghi, 1996).

The second improvement was the introduction of apyrase to the reaction to make a four-enzyme system (Ronaghi, 1998b). The addition of apyrase allowed nucleotides to be added sequentially without any intermediate washing step. This enzyme shows high catalytic activity and low amounts of this enzyme in the pyrosequencing reaction system efficiently degrade the unincorporated nucleoside triphosphates to nucleoside diphosphates and subsequently to nucleoside monophosphate. Apyrase is less inhibited by its products as compared to other nucleotide-degrading enzymes.

Most recently, the addition of ssDNA-binding protein to the pyrosequencing reaction system has simplified the optimization of different parameters in pyrosequencing. This protein has proven to be useful for long read sequencing and sequencing of difficult templates, as well as providing flexibility in primer design (Ronaghi 2000).

Template 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 currently available for generation of a primed DNA template for pyrosequencing are described below.

Solid-Phase Template Preparation

Streptavidin-coated magnetic beads have been used to prepare primed DNA template for pyrosequencing. This technology enables biotinylated PCR product to be captured onto magnetic 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 single stranded DNA. Both the immobilized biotinylated and nonbiotinylated strands in solution can be used as pyrosequencing templates (Ronaghi 1998a, 1999). This template preparation system has given high-quality sequence data with low background signals.

Enzymatic Template Preparation

Recently, enzymatic template preparation was developed for sequencing on double-stranded DNA template (Nordstrom 2000). This template preparation method employs a nucleotide-degrading enzyme and exonuclease I. The enzymes are added to the PCR product and the mixture is incubated at room temperature or 35°C. During this step, the nucleotide-degrading enzyme removes the nucleotides and exonuclease I degrades the PCR primers remaining from the amplification step. The sequencing primer is dispensed into the treated mixture and the temperature of the solution is increased to heat-inactivate the enzymes. Template/primer complexes are formed by rapid cooling of the solution. Two different enzyme systems can be used. The use of alkaline phosphatase from shrimp or calf intestine together with exonuclease I allows the template to be prepared within

20 min, whereas a combination of a low amount of apyrase, inorganic PPi, and exonuclease I enables the template to be prepared in three min. High quality pyrosequencing data has been obtained by enzymatic template preparation using a prototype pyrosequencing system that employs a very sensitive light detector. However, this template preparation method needs to be further optimized for use with the standard system that uses microtiter plates, because the dilution that is required to compensate for incompatible buffer systems results in low amounts of primed DNA template. Improvements may be obtained by running PCR in a compatible buffer with pyrosequencing reaction or by using a more sensitive CCD camera in the pyrosequencing machine.

Pyrosequencing Enzyme Systems

Pyrosequencing takes advantage of the cooperativity of several enzymes to monitor DNA synthesis. Parameters such as stability, fidelity, specificity, sensitivity, K_M, and k_{cat} are of utmost importance for the optimal performance of the enzymes used in the reaction (Ronaghi 1998). The kinetics of the enzymes can be studied in real time by following the pyrosequencing signals (a pyrogram). The slope of the ascending curve in a pyrogram is determined mainly by the activities of polymerase and ATP sulfurylase; the height of the signal is determined by the activity of luciferase, and the slope of the descending curve by the efficiency of nucleotide removal. In the solid-phase system using microfluidics, which employs the three-enzyme system, the descending curve is determined by the washing efficiency. In the four-enzyme system of liquid-phase pyrosequencing, the accumulation of inhibitory substances decreases the efficiency of

luciferase and apyrase. In both systems, the activity of ATP sulfurylase is relatively constant during the sequencing reaction. In pyrosequencing, the most critical reactions are DNA polymerization and nucleotide removal by either washing or enzymatic degradation. Nucleotide removal (descending curve) competes with the polymerization reaction (ascending curve). Therefore, slight changes in the kinetics of these reactions directly influence the performance of the sequencing reaction. The Pyrosequencing enzyme cascade involves four enzymes, DNA polymerase, ATP sulfurylase, luciferase, and apyrase, and two substrates, adenosine 5'-phosphosulfate (APS) and luciferine.

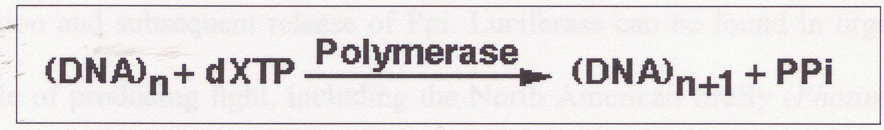
DNA Polymerase

DNA polymerase is an enzyme derived from *Escherichia coli* that assists in DNA replication/synthesis. It does so by catalyzing the polymerization of deoxynucleotides to a DNA strand that is read and used as a template. The newly polymerized molecule is complementary to the template strand. DNA polymerases synthesize DNA in the 5' to 3' direction. A primer is required for synthesis, and the polymerase catalyzed incorporation of nucleotides begins at the 3' end of the primer.

Once a nucleotide is incorporated, the two terminal phosphates (PPi) are spliced, allowing for incorporation of the next nucleotide (Figure 3). The PPi molecule is used in the subsequent reaction.

FIGURE 3

DNA Polymerase



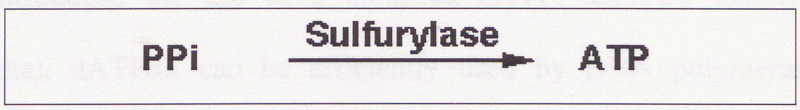
The enzyme DNA polymerase catalyzes the incorporation complementary nucleotides; upon incorporation, a pyrophosphate is released for each nucleotide incorporated.

ATP Sulfurylase

Assisting in the second reaction of the Pyrosequencing enzyme cascade is ATP sulfurylase. ATP sulfurylases are ubiquitous enzymes that catalyze the primary step of intracellular sulfate activation which involves ATP reacting with inorganic sulfate to form adenosine 5'-phosphosulphate (APS) and pyrophosphate (PPi). ATP sulfurylase is common in organisms such as *Saccharomyces cerevisiae*, also known as baker's yeast (Ullrich, et al., 2001). The Ppis released in the previous polymerase catalyzed reaction are converted by sulfurylase to ATP in the presence of the substrate adenosine 5'-phosphosulphate (APS) (Figure 4).

FIGURE 4

ATP Sulfurylase



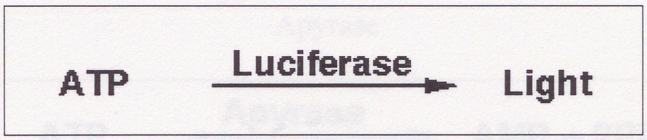
Pyrophospates (Ppi) in the presence of adenosine 5'-phosphsulfate (APS) is converted to ATP, a reaction catalyzed by sulfurylase.

Luciferase

Luciferase provides for a bioluminescent means by which to detect nucleotide incorporation and subsequent release of Ppi. Luciferase can be found in organisms that are capable of producing light, including the North American firefly (*Photinus pyralis*), from which the luciferase used in Pyrosequencing is derived. The ATP produced from the previous reaction is used to drive the luciferase catalyzed reaction of the substrate luciferine to oxyluciferine (Figure 5). This conversion generates the light that is detected by a charge coupled device camera (CCD) and indicated as a peak in a pyrogram. The light produced is proportional to the number of nucleotides incorporated.

FIGURE 5

Luciferase



ATP is used as energy to drive the luciferase catalyzed conversion of luciferine to oxyluciferine, producing visible light.

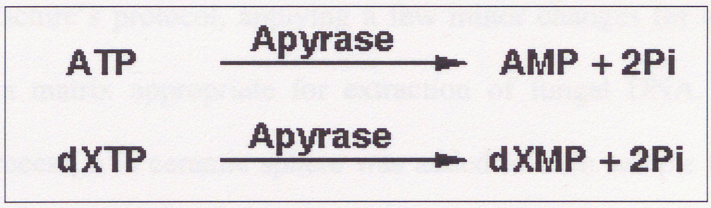
Because luciferase is known to recognize and react with dATP as well, Ronaghi et al., (1996) introduced the use of a modified dATP, dATPαS (deoxyadenosine α-thiotriphosphate). dATPαS can be efficiently used by DNA polymerase but is not recognized by luciferase.

Apyrase

ATP-diphosphohydrolase, also known as ATPDase or apyrase, is a nucleotide degrading enzyme involved in the last reactions of the Pyrosequencing enzyme cascade. Apyrase catalyzes hydrolysis of phosphoanhydride bonds of nucleotide tri- and di-phosphates in the presence of divalent cations (Nourizad et al., 2002). Its broad specificity for nucleotides makes it an ideal enzyme for DNA sequencing. A naturally occurring enzyme, apyrase can be found in a number of different organisms. Apyrase from *Solanum tuberosum* (potato) is most commonly studied and is used in Pyrosequencing as the last enzyme in the enzyme-catalyzed reaction cascade. Apyrase is responsible for degrading unincorporated nucleotides as well as excess ATP (Figure 6).

FIGURE 6

Apyrase



The enzyme apyrase degrades unincorporated nucleotides and excess ATP within the reaction

CHAPTER 3

DESIGN OF STUDY

Culturing of Isolates

A total of 12 fungal isolate samples were studied. The fungal isolates to be cultured for genomic DNA extraction were obtained from the American Type Culture Collection (ATCC) purified stock. The isolates obtained from ATCC were cultivated on potato dextrose agar plates at 37°C for 72 hours. A single colony was isolated from the plate and transferred to tubes containing 50 mL of Sabaroud dextrose broth and cultivated for 48 to 72 hours at 37°C

Isolation of Genomic DNA

Extraction of genomic material was done using Q-BIOgene's FastDNA® Kit following manufacture's protocol, applying a few minor changes for optimization. The kit comes with a matrix appropriate for extraction of fungal DNA. To improve the homogenizing process, a ¼ ceramic sphere was added to each sample tube. The sample specific lysing solution, CLS-Y is used for the fungal samples. One mL of CLS-Y is added to each tube, which contains the lysing matrix. The samples were homogenized in a Fast Prep Instrument for about 80 seconds at a speed of 4.5-5. The tubes were then transferred to a shaker and shaken for approximately 30 minutes at 100 rpm. The samples were centrifuged at 14,000 rpm for 5 minutes, transferring the supernatant to a clean

micro centrifuge tube and discarding the pellet. To the tube, 600 µL of binding matrix was added, mixed gently and incubated for 5 minutes at room temperature. The tubes were pulse spun for 5 seconds, and the supernatant was discarded. The pellet was gently resuspended in 500 µL of SEWS-M and transferred to a spin filter. The spin column is centrifuged for 1 minute and contents of the catch tube were discarded. The column is centrifuged again for 1 minute to dry the binding matrix/DNA complex. The spin filter was transferred to a new catch tube. The matrix/DNA complex was re-suspended in 100 µL DES to elute the DNA. After 2 to 3 minutes, the tube was centrifuged for 1 minute at 14,000 rpm to transfer DNA containing DES to catch tube. The DNA is ready for use without further manipulation.

Sequencing Primer Design

Sequence data collected from reliable DNA sequence databases (NCBI, EBI, DDBJ) was used for to develop one sequencing primer to work for all of the species isolated. Reference sequences were aligned using the ClustalX 1.43 sequence alignment program to establish variable regions within the amplified region of interest. The sequences were also subjected to BLAST analysis/alignment to further establish regions of variability. The variable region is a stretch of nucleotides showing variability in sequence between different species/types relative to a given gene (Gharizadeh, et al., 2003).

PCR for TRI 5

The reaction mixture consisted of 30.7µL H2O, 5µL 10X Buffer, 2µL to sample, 0.3µL Taq Gold, 1µL each of forward and reverse primers, 5µL 10X dNTP mix, and 5µL MgCl2. PCR was performed in 50µL reactions with the following cycling parameters: i) 10 min at 95°C; ii) 14 cycles of 1 min at 95°C, 1 min at 58°C, 1 min at 72°C; iii) 20 cycles of 1min at 95°C, 1 min at 50°C, 1 min at 72°C; iv) 5 min at 72°C; v) reaction mixture held at 4°C.

PCR Product Analysis/Confirmation

Amplification products were confirmed and analyzed on a 2.5% agarose gel [100 mL volume: 2.5 g of agarose melted in 100mL of TBE (Tris-Borate-EDTA- buffer, 8.3 pH + .1, electrophoresis grade)]. 8µL of EtBr was added to the gel before cooling. 7Ml of each PCR product was combined with 3µL of loading buffer and then loaded in appropriate well. The gel was electrophoresed at 100 V for approximately 1 hour. The gel then viewed and photographed with a UV imager.

Template Preparation

In order to perform a Pyrosequencing reaction, a primed single-stranded DNA (ssDNA) templated is necessary (Ronaghi, 2001). One strand of the PCR template used for Pyrosequencing is biotinylated as the result of a biotinylated reverse primer used for amplification. Biotin, a water soluble vitamin, has a strong affinity for streptavidin. The biotin-streptavidin complex is very stable over a wide range of temperatures and pH. After denaturation of the dsDNA template and washing, there will remain the biotinylated ssDNA bound to a streptavidin-coated agent (paramagnetic or sepharose beads). The now single stranded template can be primed and used for sequencing.

Sepharose Beads Protocol

This protocol uses a vacuum preparation tool that allows for high throughput, parallel sample preparation. Pyrosequencing's Vacuum Prep Workstation consists of a hand-held Vacuum Prep Tool and a Vacuum Prep Worktable (Figure 7). The hand-held tool holds 96 replaceable metal filter tips and is connected to a vacuum source. The filters capture the streptavidin coated beads, to which the biotinylated PCR product has attached, when suction is applied. The worktable contains five troughs for the various solutions necessary for sample preparation (www.pyrosequencing.com).

FIGURE 7

Pyrosequencing Sample Preparation Tools

At left, Vacuum Prep Workstation (www.biotage.com); on right, picture of Vacuum Prep Tool used in this study.

Step (1) Preparation of Sample Plates

- Two standard 96-well PCR plates were prepared and used with the vacuum prep tool.

Plate 1

Prepared with the following ingredients per sample/well:

- 37 µL 1X Binding Buffer
- 15 µL PCR Product
- 25 µL H2O
- 3 µL Streptavidin coated sepharose beads

Plate 2

Prepared with the following ingredients per sample/well:

- 34.8 µL 1X Annealing Buffer
- 1.2 µL 10 pM Sequencing primer

Step (2) Immobilization of PCR Product

In PCR Plate 1, the biotinylated PCR product attaches to the steptavidin-coated sepharose beads.

Step (3) Denaturation of dsDNA

a. The vacuum prep tool is used to vacuum up the liquid in the prepared PCR Plate 1, capturing the sepharose beads in the filtered tips.

b. With the vacuum still applied, the tips of the vacuum prep tool are submerged in the section of the Vacuum Prep Station containing 70% ethanol, allowing it to suction for at least 5 seconds.

c. The vacuum prep tool is then placed in the NaOH container and allowed to suction for at least 5 seconds.

d. Lastly, the vacuum prep tool in placed in the section containing tris-EDTA (TE) buffer for approximately 15 seconds to wash away all unattached ssDNA.

e. The vacuum prep tool is removed from solution and the vacuum is turned off. The tips of the vacuum prep tool are carefully placed into PCR Plate 2, allowing the beads to diffuse into the solution.

f. The vacuum prep tool tips are cleaned by vacuuming up at least 150 mL of purified water.

Step (4) Sequence Primer Annealing

PCR Plate 2 is covered with plastic tape and placed in a thermal cycler and heated to 90°C for 2 minutes. It is cooled to 60°C for 5 minutes and cooled to 4°C for 20 minutes.

Step (5) PSQ Reaction Plate Preparation

The PSQ HS96 plate is prepared with 12 μL of annealing buffer per well.

Step (6) Sequence Primer Washing

a. The vacuum prep tool is used to vacuum up the liquid in PCR Plate, capturing the sepharose beads in the filtered tips.

b. With the vacuum still applied, the tips of the vacuum prep tool are submerged in the section of the Vacuum Prep Station containing 1X annealing buffer and allowed to suction for at least 5 seconds, washing away excess primer.

c. The vacuum prep tool is removed from solution and the vacuum is turned off. The tips of the vacuum prep tool are carefully placed into the prepared PSQ plate, allowing the beads to diffuse into the solution.

d. The vacuum prep tool tips are cleaned by vacuuming up at least 150 mL of purified water.

PSQ HS 96A

Pyrosequencing's PSQ HS96A (high-sensitivity) model was designed originally for SNP-analysis but can be quite efficiently used for short de novo sequencing through a few changes to the programming. This system uses capillary dispensation tip (CDT) reagent cartridges or nucleotide dispensing tip (NDT) reagent cartridge (Biotage) along with reagent dispensation tips (RDT) that can be used in either cartridge. To each sample well of the Pyrosequencing plate, 1.5 µL of SSB (2.2 mg/ml) is added. The Pyrosequencing CDT reagent cartridge is loaded with the following:

a. Enzyme (E) solution consisting of DNA polymerase, ATP sulfurylase, Luciferase, and Apyrase (2µL/well plus 50µL dead volume)

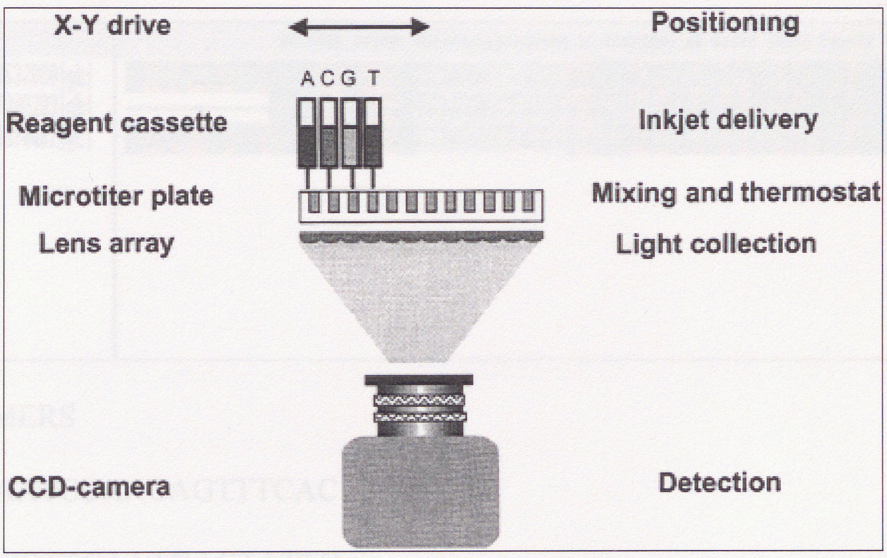
b. Substrate (S) solution consisting of APS and D-luciferine (2µL/well plus 50µL dead volume).

c. Nucleotides mix consisting of dATP, dCTP, dGTP, and dTTP (0.2µL/well plus 50µL dead volume).

The Pyrosequencing systems were programmed to dispense nucleotides in the order A-C-G-T for 10-15 cycles (one dispensation of each of the four nucleotides in the specified order constitutes one cycle).

FIGURE 8

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.

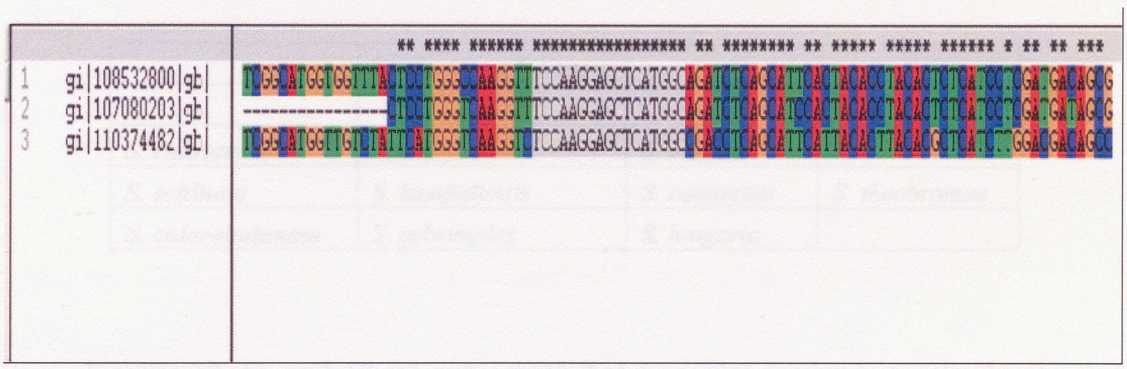
CHAPTER 4

RESULTS AND DISCUSSION

Consensus primers were selected for PCR based on conserved regions within Stachybotrys Tri 5 gene. With the Clustal X program I was able to align data base sequences as to establish the conserved region for all species types. Three complete tri 5 gene sequences for three *Stachybotrys* were found.

FIGURE 9

Clustal Sequence Alignment



PCR PRIMERS

5'-CATCAATCCAACAGTTTCAC

3'-GCAACCTTCAAAGACTATTG

Sequence Primer

5'-TCCAAGGAGCTCATGG-3'

There ten isolates that were used (Table 1). Of the ten isolates used three isolates were proven to have the Tri 5 gene, and therefore they have the potential to have trichothecene mycotoxin production. The three isolates that have the tri 5 gene were *S. chartarum, S. echinata,* and *S. chlorohalonata.* The sequencing data also proves that the Tri 5 gene is present. Pyrosequencing was subsequently performed on the 10 samples that either produced no sequence or produced sequence not consistent with sequence data as obtained from sequence databases. Three of the samples produced interpretable sequences.

TABLE 1

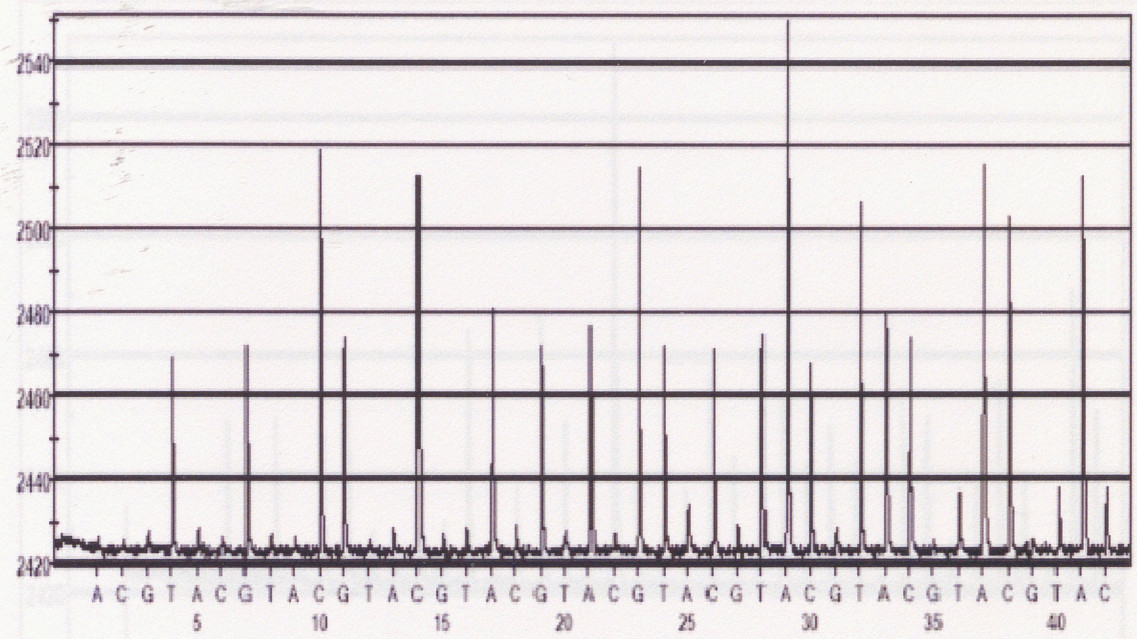
Isolates

	Stachybotrys Isolates		
S. chartarum	*S. bisbyi*	*S. elegans*	
S. echinata	*S. kampalensis*	*S. oenanthes*	*S. theobromae*
S. chlorohalonata	*S. subsimplex*	*S. longoria*	

Figures 10, 11, and 12 are pyrograms that represent sequence results for the three *Stachybotrys* species proven to contain the Tri 5 gene.

FIGURE 10

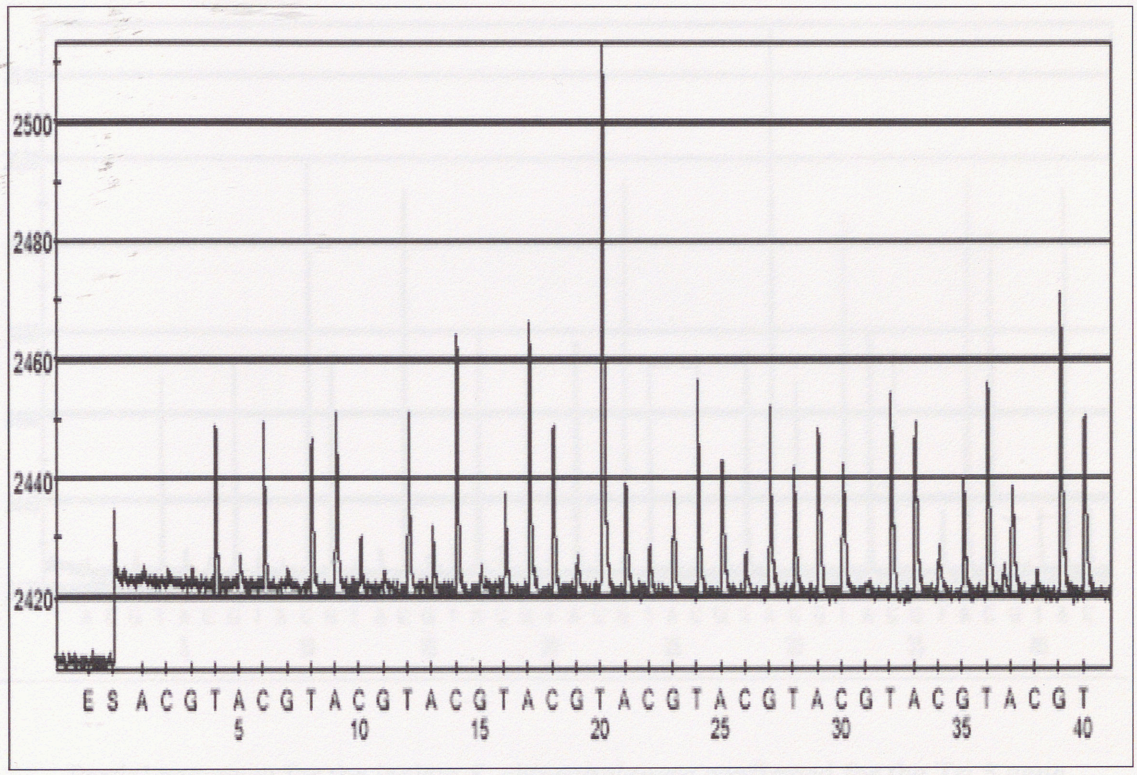
S. chartarum



Partial sequence for the isolate *S. chartarum* confirmed for the Tri 5 gene.

FIGURE 11

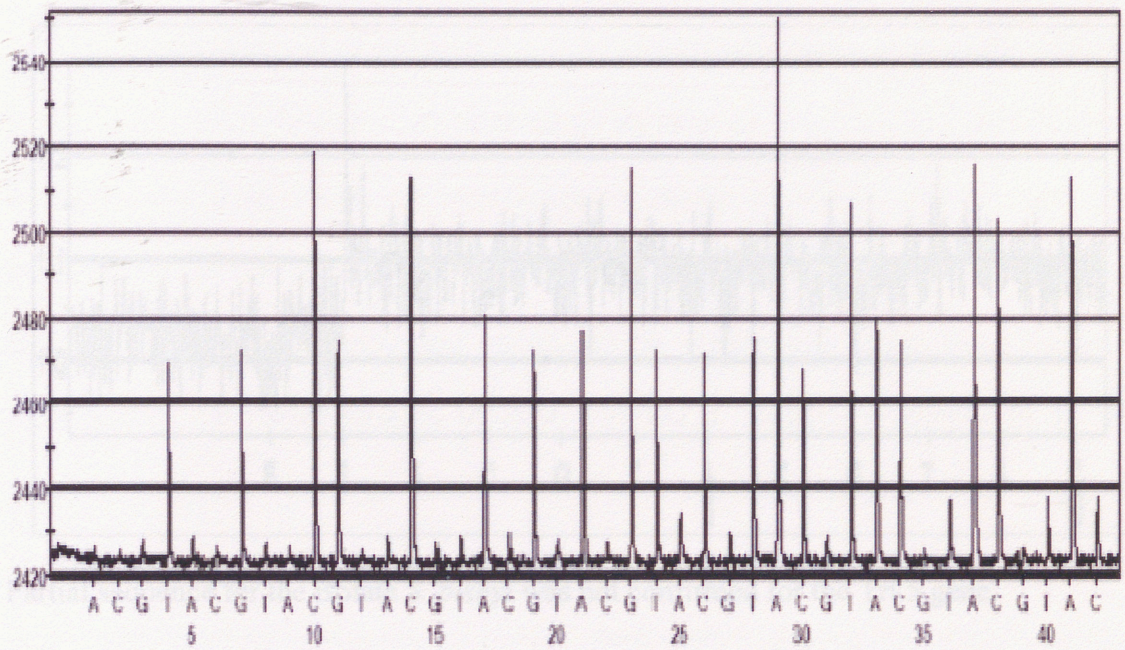
S. echinata



Partial sequence for the isolate *S. echinata* confirmed for the Tri 5 gene.

FIGURE 12

S. chlorophalonata

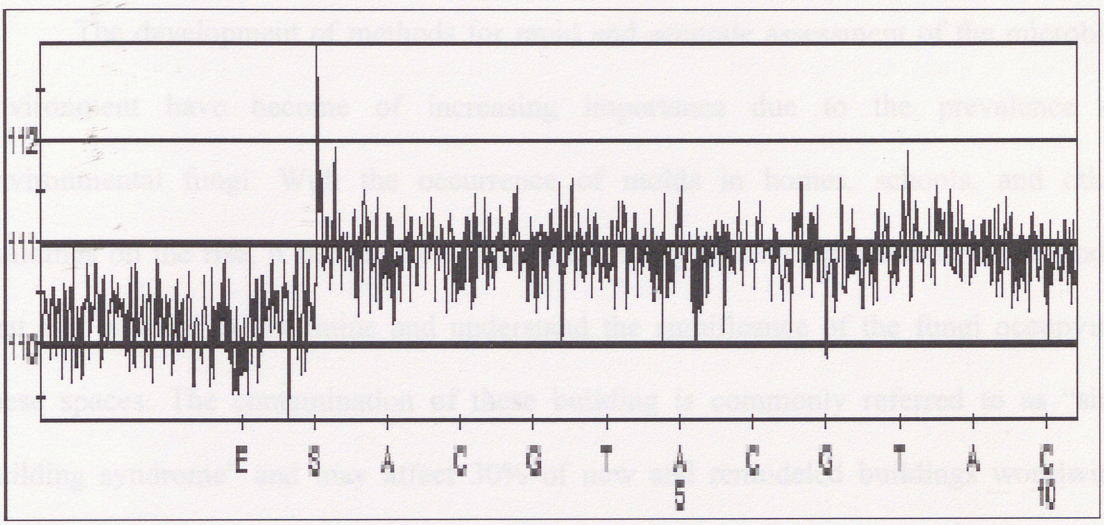


Partial sequence for the isolate *S. chlorohalonata* confirmed for the Tri 5 gene.

The pyrogram, representing *S. bisbyi* is one example of the seven isolates that were proven not to contain the Tri 5 gene (Figure 13). There is no sequence data and what is seen is just background. The addition of the substrate gives false signal which why a peek is seen above the S.

FIGURE 13

S. bisbyi



Partial sequence for the isolate *S. bisbyi* was not confirmed for the Tri 5 gene.

The presence of Tri5 gene was identified in 3 species of *Stachybotrys* Amplifed Tri5 using general primers. A single primer was designed that was specific for all *Stachybotrys* for Pyrosequencing analysis

CHAPTER 5

SUMMARY, CONCLUSIONS, AND RECOMMENDATIONS

The development of methods for rapid and accurate assessment of the microbial environment have become of increasing importance due to the prevalence of environmental fungi. With the occurrence of molds in homes, schools, and other buildings on the rise, it becomes even more necessary to develop a standardized model that can be used to determine and understand the significance of the fungi occupying these spaces. The contamination of these building is commonly referred to as "sick building syndrome" and may affect 30% of new and remodeled buildings worldwide (Pierson et al., 2002). The most commonly occurring of the problematic fungi present in these "sick building" cases are species of *Stachybotrys, Aspergillus, and Penicillium*. *Stachybotrys chartarum* is the most hazardous of this group of fungi, and it, along with a few others belong to the genus, has been proven to be toxigenic. *S. chartarum* has been the focus of much research due to its potential to produce the very toxic macrocylic trichothecenes. Studies have long since proven that the production of these mycotoxins is due to the presence and subsequent expression of the trichodiene synthase (Tri5) gene. Because of the many detrimental effects of these toxigenic fungi, or rather the toxins they produce, it is imperative to understand their potential for toxicity as determined by the presence or absence of functional genes, i.e. Tri5.

Identification methodologies that eliminate the need for plating and culturing, which can be quite time consuming and laborious, or microscopic/morphological analysis, which requires a very skilled, highly experienced technician/mycologist, are problematic. Nucleic acid based assays, as used in this study, allow for rapid as well as accurate identification. Pyrosequencing can potentially eliminate the need to perform many of the conventional identification techniques that can take days to weeks to complete. Moreover, the Pyrosequencing technology can be automated.

Sequence analysis based on sequence data obtained from Pyrosequencing proved that three of the *Stachbotrys* species studied contained the TRI5 gene and therefore have the potential to produce the very toxic trichothecene mycotoxins. Analysis of the remaining seven isolates indicates the absence of the TRI5 gene as confirmed via PCR and Pyrosequencing. This information supports the use of nucleic acid-based assays such as Pyrosequencing for accurate identification.

Future objectives will include developing a nucleic-based model for identification of environmental fungal species and exploring other genomic methods for detection and identification. Additionally, other regions of the genome can be explored for identification, including the Cox1 and Cox2 genes, which are part of mitochondrial genome. The developed approach can also be used as a detection and identification model for other organisms.

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