A Simple Polymerase Chain Reaction/Restriction Fragment Length Polymorphism Assay Capable of Identifying Medically Relevant Filamentous Fungi

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Abstract

Due to the accumulating evidence that suggests that numerous unhealthy conditions in the indoor environment are the result of abnormal growth of the filamentous fungi (mold) in and on building surfaces it is necessary to accurately reflect the organisms responsible for these maladies and to identify them in an accurate and timely manner. To this end, we have developed a method that is cost effective, easy to perform, and accurate. We performed a simple polymerase chain reaction/restriction fragment length polymorphism (PCR/RFLP) analysis on multiple members of species known to negatively influence the indoor environment. The genera analyzed were *Stachybotrys, Penicillium, Aspergillus,* and *Cladosporium*. Each organism underwent PCR with universal primers that amplified ribosomal sequences followed by enzymatic digestion with *Eco*RI, *Hae*III, *Msp*I, and *Hinf*I. Our results show that using this combination of restriction enzymes enables the identification of these fungal organisms at the species level.

1. Introduction

The identification, control, and removal of filamentous fungi (mold) from the indoor environment has become an important area of research in recent years. This is because the unwanted growth and dissemination of fungal spores and mycelial fragments negatively contribute to the quality of indoor air. It has been estimated that around 40% of all the homes in North America contain mold growth, while the proportion is between 20% and 40% in Northern Europe.(*1*, *2*) Of the mold species that have been identified in homes, currently only a small fraction have actually been implicated in adverse health effects. It is probable that, with increased awareness and additional research, the number of fungi that contribute to an unhealthy indoor environment will increase.

Fungal genera such as *Stachybotrys, Penicillium, Aspergillus,* and *Cladosporium* are organisms that have been isolated from unhealthy buildings and are potentially associated with negative health effects in humans (*3*, *4*). These health effects may include itchy eyes, stuffy nose, headache, fatigue, and—in severe cases—idiopathic pulmonary hemosiderosis (IPH) in infants (*5, 6, 7, 8, 9, 10, 11*). In order to provide building owners, building occupants, and remediators accurate information, necessity dictates that methods of fungal screening and identification be developed that are rapid, unambiguous, highly specific, and cost effective.

From a historical perspective, fungal isolates have traditionally been identified based on macroscopic and microscopic examination, analyzing the distinctions present in their colonies such as color and texture along with conidial size, shape, and structure (*12, 13, 14, 15, 16*). These methods are time consuming (possibly taking weeks for an identification to be made), and identification of fungi based on morphology can also be highly inaccurate. Contributing to the inaccuracies of morphological identification are fungi that visually appear to be very similar, while at the same time not all of the organisms present in a sample will be culturable (*3*). These conditions allow for misidentification and understatement of the complexity of the fungal community (*17*). New methods of fungal identification are being developed that circumvent these difficulties, allowing for rapid, specific, and cost effective identification of indoor fungal contaminants.

Recent research has developed new molecular methodologies capable of highly specific identification and quantification of the filamentous fungi. These techniques include random amplified polymorphic DNA (RAPD) analysis, quantitative polymerase chain reaction (qPCR), restriction fragment length polymorphism (RFLP), and image analysis and can be used to identify different fungal organisms from numerous diverse environmental settings. These methods enable rapid, sensitive, and specific identification of fungal organisms (*18, 19, 20, 21, 22*).

Velegraki et al. recently developed an RFLP screening technique that is capable of identification of medically relevant fungal genera (*19*). However, at this time, RFLP has not been used to identify indoor fungal contaminants. Here we describe a RFLP screen that we have developed and optimized, which is capable of identifying individual members of four fungal genera by monitoring DNA band migration following restriction digestion.

1. Materials and Method

2.1. Fungal Isolates

Aspergillus tamarii, purchased from the American Type Culture Collection (ATCC) as Aspergillus *flavus* ATCC# 11498, was reidentified by M. Kohan based on ribosomal sequence analysis. *A. fumigatus* ATCC# 1022 and *C. elatum* ATCC# 11280 were obtained from the American Type Culture Collection. *A. versicolor, A. oryzae, A. proliferans, A. awamorii, A. sydowii, A. niger, P. glabrum, P. chrysogenum, P. variabile, P. oxalicum, C. sphaerospermum, C. cladosporioides, C. oxysporium, S. chartarum* 3441, *S. chartarum* 3559, *S. chartarum* 3657, S. *chartarum* 3454, and *S. chartarum* 3514 were all donated by Research Triangle Institute (RTI). Numbers following the organism name are our laboratory identification numbers. All of the organisms provided by RTI were environmental isolates obtained from environmental dust samples from houses in Cleveland, Ohio. *Penicillium purpurogenum* was donated by Steve Vesper from EPA/ORD/NERL. It was also isolated from environmental dust samples from houses in Cleveland, Ohio.

2.2. Growth and Harvest of Spores

All fungal organisms were grown on Sabouraud Dextrose Agar (SDA) plates, which were prepared according to the suppliers instructions. Each organism was plated and grown to confluence on three different SDA plates in preparation for spore harvest. Organisms were allowed to grow for at least 10 days prior to spore harvest. Spores were harvested as previously reported (*23, 24*). Basically, spores were harvested from plates with 3 mL of 0.01 M phosphate buffer with 0.05% (v/v) Tween 20 (Sigma Chemical, St. Louis, MO, USA) by gently disrupting the plate surface with a bent glass rod. The supernatant from the three plates was combined and the spore suspension centrifuged at 12,000 X g for 5 min. The supernatant was then decanted leaving the spore pellet intact. The pellet was washed three times with 10 mL of phosphate buffer and stored at 4 °C until needed. The total spore counts were enumerated by direct microscopic counting on a hemacytometer as described by Roe et al. (*25*).

2.3. Fungal DNA Purification

The spore DNA was purified as previously reported (*23*). The spores were mechanically lysed using a bead milling method followed by a phenol:$CHCl_3$ -ethanol precipitation step. For bead milling, 0.25 g of acid-washed glass beads (212-311 μm) were placed in a 2 mL screw cap conical tube. 200 μL, or approximately 10^7 spores, were added to the glass beads. The tube was then shaken in a mini bead beater (Biospec

Products, Bartlesville, OK) for 50 seconds at the maximal rate. The tube was then placed on ice for 1 minute to cool the sample and then shaken a second time. The supernatant was removed from the beads and subjected to a phenol:$CHCl_3$ extraction and an ethanol precipitation (*26*). Following precipitation, the samples reconstituted in 200 μL dH_2O and were stored at minus 80 °C until needed.

2.4. Primers and PCR Conditions

Primers were chosen based on their ability to amplify ribosomal sequences from numerous and different fungal organisms. Reactions were carried out using forward primer ITS1 (5' TCCGTAGGTGAACCTGCGG 3') and reverse primer ITS4 (5' TCCTCCGCTTATTGATATGC 3') (*27*). These primers are considered universal fungal primers and have been shown to amplify the organisms used in this study (*17*). These primers amplify from the 18S ribosomal RNA gene, through the internal transcribed spacer 1, 5.8S ribosomal RNA gene, internal transcribed spacer 2, and into the 28S ribosomal RNA gene. Each polymerase chain reaction (PCR) contained: 0.2 mM each dNTP, 1.5 mM $MgCl_2$, 0.5 U Platinum Taq DNA polymerase, buffer (50 mM KCl, 10 mM Tris-HCl, pH 9.0 at 25 °C, 0.1% Triton X-100), and 10^5 spores. PCR was performed for 35 cycles of 96 °C 30 sec; 50 °C 15 sec; and 68 °C 2 min. PCR products were separated by electrophoresis in 2% low melting point agarose, and visualized by ethidium bromide staining. PCR products were clean and did not require subsequent purification. To confirm that the proper ribosomal sequences were being amplified, each PCR product was sequenced using an ABI 3100 Genetic Analyzer with the output sequences analyzed for accuracy. Output sequences were also used as a comparison between identification based on restriction digestion pattern and the identification based on the sequence information.

2.5. Restriction Digestion

Initial sequence data was used to generate restriction maps with BioEdit software (*28*). These restriction maps were then used to choose restriction enzymes that would generate different banding patterns. Combining this search with results from Velegraki et al., we chose *Eco*RI, *Hae*III, *Msp*I, and *Hinf*I based on their ability to distinguish between *Cladosporium*, *Stachybotrys*, *Aspergillus*, and *Penicillium* spp.

Reactions consisted of 10% or 5 μL of PCR amplified DNA, 2 μL of 10X reaction buffer (50 mM NaCl, 10 mM Tris-HCl, 10 mM $MgCl_2$ 1 mM DTT, pH 7.9), 12 μL dH_2O, and 10, 20, 10, and 20 units of *Hae*III, *Msp*I, *Hinf*I, and *Eco*RI respectively. Total reaction volume was 20 μL. Each reaction was incubated at 37 °C overnight, or a

minimum of 16 hours, and then heat inactivated at 80 °C for 20 minutes. Following digestion, 10 μL of each reaction was run on a 2% agarose gel, or in the case of *Msp*I and *Hae*III, digestions a 4% agarose gel (NuSieve GTG Agarose, Cambrex Bio Science Rockland Inc., Rockland, ME), to resolve the digestion pattern.

3. Results and Discussion

All of the fungal strains that were used amplified successfully, producing a single PCR product of the desired length, approximately 550-600 base pairs (data not shown). The resultant PCR products were very clean and did not require additional purification prior to restriction digestion. Prior to restriction analysis, each PCR product was sequenced and compared to published sequences to confirm that ribosomal genes were indeed being amplified and that proper identification of each organism was obtained.

All *Cladosporium* analyzed could be distinguished, at the species level, based on the differing patterns following digestion (figure 1). All of the *Cladosporium* produced the same banding pattern following digestion with *Eco*RI. When the analysis included *Hae*III, *C. elatum, C. cladosporoides*, *C. oxysporium* and *C. sphaeropsermum* produced individual banding patterns that differentiated each of them from the other members of the genus. *C. elatum* also produced significantly different patterns following digestion with *Hinf*I and *Msp*I. It appears, at least for the organisms chosen in this study, that *Hae*III is best suited for identification; however, *Hinf*I and *Msp*I are also capable of producing identifying bands.

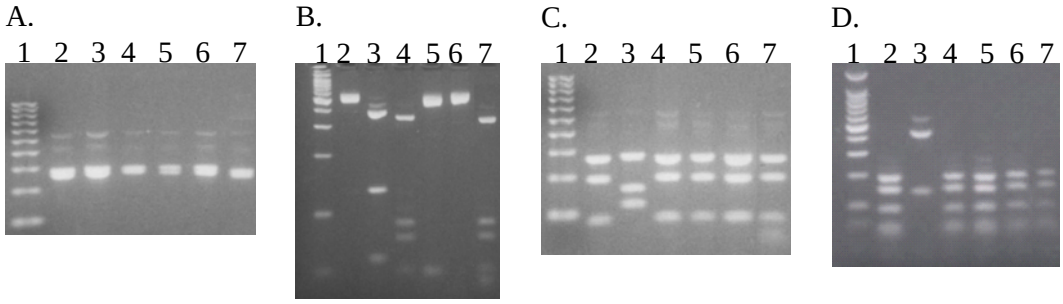
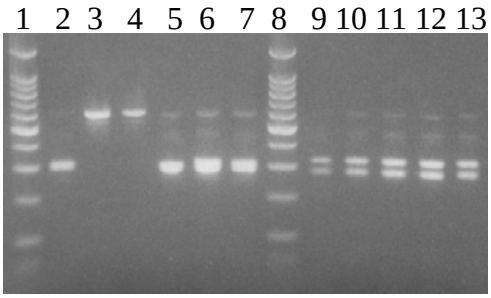
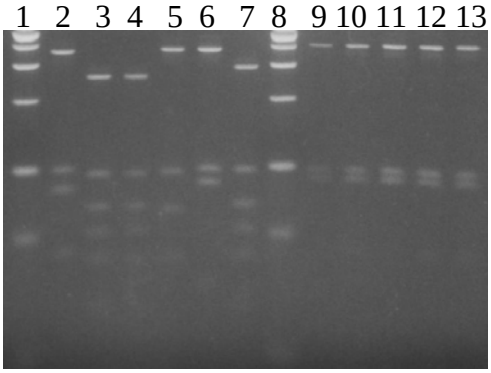


Figure 1. Restriction analysis showing digestion patterns of *Cladosporium* species. Lane 1, contains size markers; lane 2, *C. oxysporium*; lane 3, *C. elatum*; lane 4, *C. sphaerospermum*; lane 5, *C. cladosporoides*; lane 6, *C. oxysporium*; lane 7, *C. sphaerospermum*. Panel A shows digestions using *Eco*RI, Panel B shows digestions with *Hae*III, Panel C shows digestions using *Hinf*I, and Panel D shows digestions using *Msp*I.

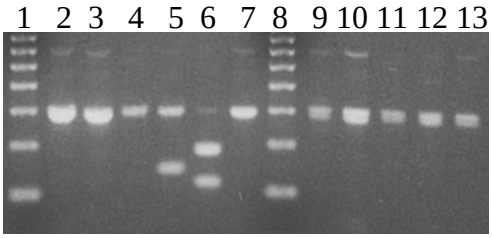
A.



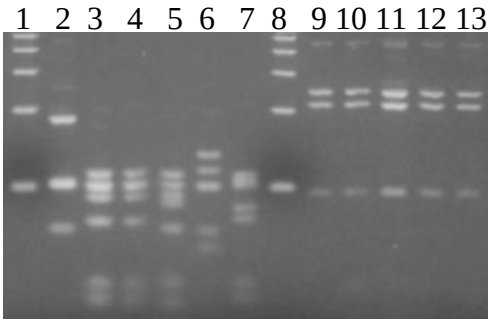
B.



C.

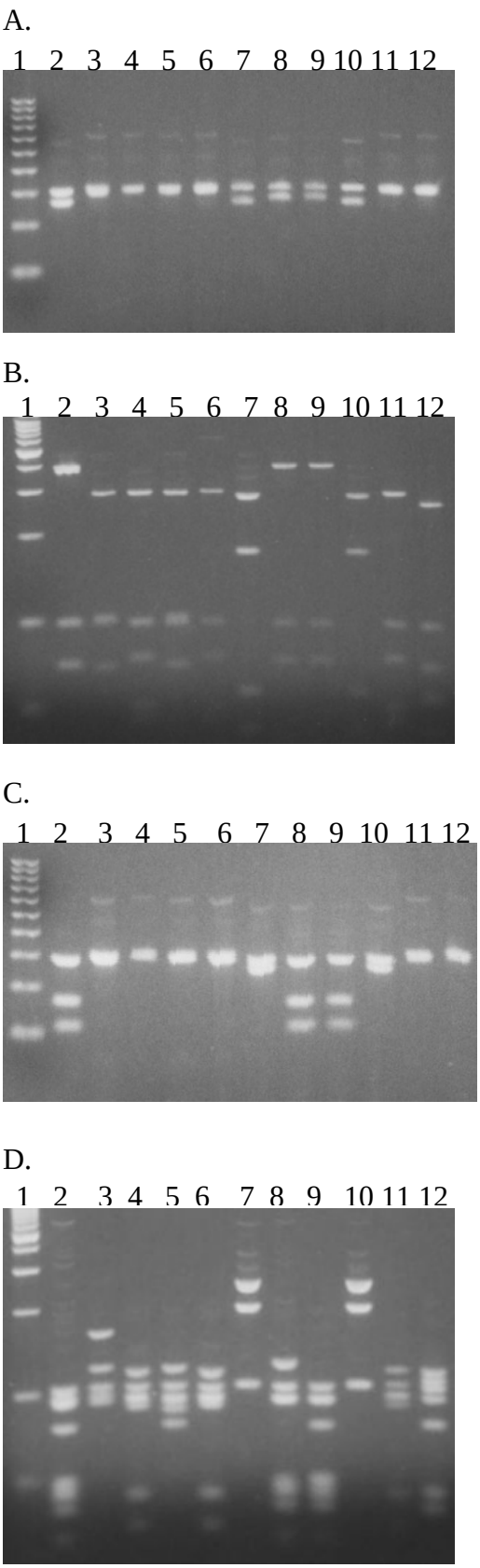


D.



The results following amplification and digestion of *Penicillium* species showed that the choice of *Hae*III, *Msp*I, *Hinf*I, and *Eco*RI were sufficient for positive identifications to be made at the species level (figure 2). *Eco*RI and *Hae*III both produced banding patterns that distinguished *P. chrysogenum* from the other members of the genus used in this study. *Hae*III was also able to distinguish *P. glabrum* and *P. oxylicum*. Analysis involving *Hinf*I allowed for the identification of *P. glabrum* and *P. variabile*. *Msp*I was also a highly useful enzyme showing the ability to identify *P. purpurogenum*, *P. variabile*, and *P. oxylicum*.

Figure 2. Restriction analysis showing digestion patterns of *Penicillium* and *Stachybotrys* isolates. Lane 1, contains size markers; lane 2, *P. purpurogenum*; lane 3, *P. chrysogenum*; lane 4, *P. chrysogenum*; lane 5, *P. glabrum*; lane 6, *P. variabile*; lane 7, *P. oxylicum*; 8, size markers; lanes 9 – 13, *Stachybotrys chartarum*. Panel A shows digestions using *Eco*RI, Panel B shows digestions with *Hae*III, Panel C shows digestions using *Hinf*I, and Panel D shows digestions using *Msp*I.



Restriction analysis of numerous *Aspergillus* species was favorable with most capable of being identified based on their resulting restriction patterns (figure 3). Included in this analysis were two samples previously identified as *Aspergillus* sp. that we subsequently sequenced and reidentified as *Eurotium repens*. Both sequencing and restriction analysis showed that they were indeed a different genus altogether. *Hae*III, *Msp*I, *Hinf*I all show banding patterns inconsistent with the *Aspergillus* group while identifying them as *E. repens* (figure 3).

Sequence analysis showed that *A. flavus* and *A. oryzae* have the same ribosomal sequence within the section that was amplified. *A. niger* and *A. awamori* were also shown to posses identical sequences. Therefore, identification based on sequence information or restriction analysis of this region is not possible between *A. flavus* and *A. oryzae* or

Figure 3. Restriction analysis showing digestion patterns of *Aspergillus* species. This analysis also contains previously identified *Aspergillus* species reidentified as *Eurotium repens*. Lane 1, contains size markers; lane 2, *A. sydowii*; lane 3, *A. tamarii*; lane 4, *A. awamori*; lane 5, *A. oryzae*; lane 6, *A. niger*; lane 7, *E. repens*; 8, *A. versicolor*; lane 9, *A. versicolor*; lane 10, *E. repens*; lane 11, *A. niger*; lane 12, *A. fumigatus*. Panel A shows digestions using *Eco*RI, Panel B shows digestions with *Hae*III, Panel C shows digestions using *Hinf*I, and Panel D shows digestions using *Msp*I.

between *A. niger* and *A. awamori*. However, the *A. flavus*/*A. oryzae* pair does produce an identifying pattern when digested with *Msp*I. *Msp*I is also capable of identifying the *A. niger*/*A. awamori* pair based on a unique restriction pattern. *Msp*I was also successful at producing a distinguishing restriction pattern with *A. tamarii*.

Restriction analysis involving two different isolates of *A. versicolor* and *A. sydowii* produced interesting results. This analysis was unable to distinguish between one of the isolates of *A. versicolor* and *A. sydowii* (figure 3 lanes 2 and 9). Sequence analysis showed that there are 7 base differences in the amplified sequences; however, none of the differences are in the restriction sites. Another interesting finding was that digestion with *Msp*I was able to distinguish between the two *A. versicolor* isolates that were used. Sequence analysis showed that there was a single C $\rightarrow$ T base change falling within a restriction site which accounts for the difference in the banding pattern.

Analysis of five different isolates of *Stachybotrys chartarum* produced the same banding pattern with each of the four restriction enzymes that were used (figure 2; lanes 9-13). When these data are taken collectively, *Hae*III, *Msp*I, *Hinf*I, and *Eco*RI provide the ability to positively identify *S. chartarum* from *Penicillium*, *Aspergillus*, and *Cladosporium*.

Analysis with *Hae*III, *Msp*I, *Hinf*I, and *Eco*RI was not only sufficient to distinguish between organisms at the genus level, but also able to identify most of the species that were tested. The only exceptions were in the *Aspergillus* group where some of the organisms contained the same sequences throughout the amplified region. This situation could be remedied by amplifying different genes that have undergone more evolutionary divergence. This analysis clearly shows the ability of a PCR/RFLP methodology in identifying different fungal organisms found in unhealthy indoor living environments.

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