MOLECULAR CHARACTERIZATION OF FUNGAL SPECIES FROM PURE CULTURES AND ENVIRONMENTAL SAMPLES

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Abstract

Traditionally, the species composition of fungal communities has been determined through a combination of media culturing and identification based on macro or microscopic features. These techniques have several drawbacks that can lead to a misrepresentation of the fungal community being studied including the over or underestimation of fungal species within a community based on the presence or absence of fruiting bodies, the inability of certain species to be grown in culture, and the difficulty in identifying fungi to the species level based on microscopic features. Molecular methods for determining the species of a fungus based on amplification and sequencing of the internal transcribed spacer (ITS) region of the fungal rRNA operon using PCR with universal primers has proven a reliable alternative to traditional methods. In this study, the species identity of nine unidentified fungal samples was determined using this method. This technique also allowed for the identification of three fungal species from three different soil samples. A second objective of this study was to test the ability of single stranded conformational polymorphism (SSCP) to identify the presence of multiple fungal species in a mixed sample. The analysis showed that individual fungal species produced a distinct banding pattern and that separation of bands generated in a mixed reaction adequately showed the presence of multiple species.

Introduction

A common goal in the field of fungal ecology is to determine the structure of fungal communities with regards to their species composition. Traditionally, the species present within a fungal community were determined through visual aspects of their morphology (Kendrick, 2000). Surveys of macroscopic structures such as fruiting bodies or ectomycorrhizas have commonly been used as assessments of the fungal community in a number of systems (Allen, 1991; Dix and Webster, 1995). The drawback of this technique is that not all fungi present within the community will produce fruiting bodies simultaneously, some fruiting bodies are inconspicuous and easily overlooked, and some may not produce fruiting bodies altogether. Any of these situations could lead to an over or underestimation of the presence or abundance of particular species, thus generating an inaccurate portrayal of the community. Other methods commonly used to identify fungi include a combination of media culturing from environmental samples followed by classification based on microscopic features such as spore producing structures. Again, there are several drawbacks associated with these techniques, including the inability of certain fungi to be grown in culture and the difficulty in identifying fungi to the species level based on microscopic features. Once more, inaccurate identification or interpretation of these results can lead to a misrepresentation of community structure.

Quick and simple methods for determining the species composition of fungal communities based on sequencing of particular regions of the fungal genome have proven a reliable alternative to traditional methods. Through targeted amplification of specific regions of the fungal genome via the polymerase chain reaction (PCR), researchers can

now quickly and accurately identify all fungal species present within a community (Horton and Bruns, 2001).

Ribosomal genes and spacers regions within the fungal genome have proven good candidates for amplification via PCR because they are comprised of highly conserved tracts with heterogeneous regions in between (Grades et al. 1991). The conserved tracts are ideal for universal primer design that can allow for the amplification of the separating heterogeneous regions. Most molecular fungal species identification relies on the amplification and sequencing of the internal transcribed spacer (ITS) region of the fungal genome, which is highly variable among species or even populations of the same species (Hibbett, 1992; Horton and Bruns, 2001). This region lies between the 18S small subunit (SSU) and the 28S large subunit (LSU) ribosomal RNA (rRNA) genes and contains two noncoding spacer regions (ITS-A and ITS-B) separated by the 5.8S rRNA gene (Kendrick, 2000). In fungi, the ITS region is typically 650–900 bp in size, including the 5.8S gene and is usually amplified by the universal primer pair ITS 1 and ITS 4 designed by White *et al*. (1990). Once the region is amplified, it can be sequenced and that sequence can be compared to those of known species.

The primary objective of this study was to assess the ability of PCR to amplify the ITS regions of fungal samples for the purposes of sequencing and species identification. The method was tested using DNA extracted from nine unidentified fungal cultures, as well as from three soil samples. Because the ITS regions of different fungal species vary in nucleotide, sequence but may be extremely similar in size, the second objective of this study was to test the ability of single stranded conformational polymorphism (SSCP) to generate a unique banding pattern for each fungal species present in mixed sample. This

technique looks at variations in migration rates of single stranded DNA fragments with different folded conformations based on sequence and not on size. This technique was first developed to detect polymorphisms in human genes (Orita et al., 1989), but may prove useful in the detection of multiple fungal species in a mixed sample

Materials and Methods

Fungal Cultures and Soil Samples

Nine unidentified fungal cultures isolated from environmental samples collected at the Louis Calder Center in Armonk, NY were obtained from the collection of Dr. Amy Tuininga. The cultures were grown on potato dextrose agar (PDA), incubated at room temperature for one week to allow for colony growth, and then stored at 4^{o} C until this study was performed. All cultures were removed from cold storage (4^{o} C) 24 hours prior to DNA extraction and stored at room temperature to restore fungal metabolic activities and growth. One soil core (~10 cm deep) was taken from below the litter layer at the base of three different tree species, red oak (*Quercus rubra*), black birch (*Betula lenta*), and shagbark hickory (*Carya ovata*), growing in the Calder Forest in Armonk, NY. Samples were homogenized then stored prior to DNA extraction as stated above.

DNA Extraction and Purification

Hyphae and spores of the unknown fungal cultures were scraped from the surface of the culture media using a heat sterilized needle. Approximately 0.1 g of tissue from the cultured fungi or 0.25 g of soil sample was transferred to a 2 ml tube and the total fungal DNA was isolated following the protocol of the UltraClean™ Soil DNA Isolation Kit

(Mo Bio Laboratories, Solana Beach, CA, USA). The DNA was eluted with 30 µl of nuclease free H_2O and its concentration was quantified using a spectrophotometer. The DNA from each culture was then diluted to 2 ng DNA ml/H_2O. To make DNA template for PCR reaction containing multiple fungal species, 2 µl of DNA extract from each species was mixed together in a microcentrifuge tube.

PCR

The forward (ITS-1) and reverse (ITS-4) primers used in the PCR reactions performed in this study were developed by White et al. (1990) and are designed to amplify the ITS region of the rRNA operon. Sequence information, melting temperature, and primer location on the rRNA operon can be seen in figure 1.

Each PCR reaction was comprised of 12.5 µl Go Taq Green Master Mix 2X (Promega Corporation, Madison, WI, USA), 0.5 µl of both the forward and reverse primers (10 pmol), 2 µl DNA template diluted to 2 ng (4.0 ng DNA total) for each individual species or 2 µl of the mixed DNA sample extracted from soils, and 9.5 µl nuclease free H_2O for a total reaction volume of 25 µl. The DNA template was replaced with an equal amount of nuclease free H_2O in negative controls. The thermocycler program for each PCR reaction began with an initial denaturation step at 94^o C for 5 minutes followed by 50 cycles of a 30 second denaturation step at 94^o C, a 30 second annealing step at 56^o C, and a 30 second elongation step at 72^o C, then one final 7 minute elongation step at 72^o C. Post-PRC products were held at 4^o C until analysis could be performed. To examine the amplified DNA and to assess any potential size differences

among PCR products, 4 µl of each reaction was run on a 2% agarose gel containing ethidium bromide and visualized using ultraviolet light.

PCR Product Purification and Sequencing

PCR products were purified following the protocols of the QIAquick® PCR Purification Kit (Qiagen, Valencia, CA, USA). The purified DNA was eluted from spin columns with 30 µl of nuclease free H_2O and DNA concentrations were determined with a spectrophotometer. Samples were prepared in sequencing tubes with 1 µl (10 pmol) of either the ITS-1 or ITS-4 primer added to 3 µl (300 ng) of DNA template and brought up to a final volume of 12 µl with nuclease free H_2O. The samples were then sent to Genewiz for sequencing. Sequences were then blasted against known sequences in the NCBI database to provide species identification.

SSCP

Prior to PCR, ITS-1 and ITS-4 primers were radiolabeled in two separate tubes containing 2 µl (10pmol/µl) of either primer, 1.5 µl 10X kinase buffer, 5 µl γ-^{33}P-ATP, 5 units T4 kinase, and 5.5 µl nuclease free H_2O. The tubes were incubated at 37^{o} C for 10 minutes then incubated at 90^{o} C for 5 minutes to stop the reaction.

Two separate PCR reactions were required to perform SSCP analysis of each individual or mixed species sample, one containing radiolabeled ITS-1 primers and non-radiolabeled ITS-4 primers and one containing non-radiolabeled ITS-1 primers and radiolabeled ITS-4 primers. Each reaction contained 4.8 µl Go Taq Green Master Mix 2X, 0.25 µl of the radiolabeled primer solution, 0.1 µl of cold primer, 1 µl DNA template,

and 3.85 µl nuclease free H_2O. The thermocycler program for each PCR reaction was as described above.

Three fungal species (*Penicillium expansum*, *Cladosporium uredinicola*, and *Mucor hiemalis*) as well as a mixture of these three species underwent PCR as described and processed for SSCP analysis. The samples were prepared by mixing 2 µl of PCR product with 4 µl of 0.1% SDS/10 mM EDTA and 6 µl of SSCP sample buffer (95% formamide, 20 mM EDTA, 0.05% bromophenol blue, and 0.05 xylene cyanol). The samples were denatured at 95^o C for 5 minutes, then placed immediately on ice for 5 minutes. 2 µl of each sample was loaded onto a SSCP gel comprised of 11.9 ml of acrylamide stock (30% acrylamide, 0.8% bis-acrylamide), 7 ml 10X TBE buffer, 50.6 ml dH_2O, 24 µl Temed, and 10% APS that had been stored at 4^o C for 30 minutes. The gel was run at 1100 volts for 4.5 hours at 4^o C to prevent overheating.

Results

DNA from all nine unknown cultures was successfully extracted and amplified using the ITS primer pair. After sequencing, blast results revealed the species identity of the unknown fungi to be 1: *Penecillium expansum*, 2: *Cladosporium uredinicola*, 3: *Mucor hiemalis*, 4: *Beauveria bassiana*, 5: *Beauveria bassiana*, 6: *Beauveria bassiana*, 7: *Metarhizium flavoviride*, 8: *Beauveria bassiana*, 9: *Paecilomyces fumosarous*. The banding pattern produced by each species on a 2% agarose gel can be seen in figure 2.

DNA from soil samples was successfully extracted and amplified in the same manner. Although there was most likely multiple fungal species present in each soil sample, only a single species from each could be identified through sequence analysis. A

Russula species was identified from soil sample 1 taken from the base of red oak, *Russula velenovskyi* was identified from soil sample 2 taken from the base of black birch, and *Rhodotorula mucilaginosa* was identified from soil sample 3 taken from the base of shagbark hickory. The banding pattern produced by these species on a 2% agarose gel can be seen in figure 3.

Fractionation of mixed samples on a 2% agaros gel did not yield enough resolution to separate PCR products of similar sizes. SSCP analysis showed that individual fungal species produced a distinct banding pattern that was not based on PCR product size. Separation of bands generated by a mixed reaction adequately showed the presence of multiple species as seen in figure 4.

Discussion

Traditional methods of identifying fungi can be extremely difficult and may result in a species list that misrepresents the fungal community. However, molecular techniques that allow for the comparison of DNA sequence information between known and unknown fungal species is much simpler and more reliable then traditional methods and has revolutionized the field of fungal ecology.

A major objective of this study was to determine the effectiveness of using PCR to amplify the ITS region of fungal DNA for the purposes of sequencing and eventual species identification. The technique proved successful as its application led to the identification of all nine unknown fungi in culture to the species level. The technique was also successful in the identification of fungi from soil samples. Each of the soil samples showed the presence of three different fungal species. In the case of soil sample 2, taken

from the base of black birch, three distinct bands were fractionated on a 2% agarose gel after PCR amplification of the soil extract (data not shown). This clearly indicated the amplification of three separate fungi, yet sequencing of the mixed reaction was only sensitive enough to parse out the most abundant sequence.

Agarose gels were successful in providing enough band separation to identify the presence of multiple fungal species with largely different sized ITS regions, such as *P. expansum*, *C. uredinicola,* and *M. Hiemalis* (see figure 2) in a mixed sample. However, agarose did not provide sufficient resolution to successfully separate bands produced by fungi with very similarly sized ITS regions, such as *B. bassiana, M. flavoviride*, and *P. fumosarous*. In these situations, all three products migrate to the same region of the gel when run in the same well. Therefore, one can say with no confidence that single bands produced from a mixed sample of unknown fungi contain a single species.

SSCP proved a possible alternative to fractionation using agarose. The analysis showed each individual fungal species produced a distinct banding pattern based on folded conformations of single stranded DNA fragments with different sequences and not on PCR product size. This technique was originally developed to identify genes with single nucleotide polymorphisms. Therefore, very small sequence differences can produce a unique and identifiable result. Because each fungal species has some variation in the nucleotide sequence of their ITS regions, each should produce their own unique banding pattern using SSCP analysis. This experiment showed each species that was tested produced a very different banding pattern and that each could be identified in a mixed reaction containing all three. This technique could prove extremely useful in the rapid identification of fungi from environmental samples as bands could simply be

excised from the gel and sequenced. More intensive screening of fungal species using this technique could eventually lead to the compilation of a fungal species barcode in which sequencing the bands would no longer be necessary.

Acknowledgments

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A.

Primer	Sequence 5' → 3'
ITS1 (forward)	TCCGTAGGTGAACCTGCGG
ITS4 (reverse)	TCCTCCGCTTATTGATATGC

B.

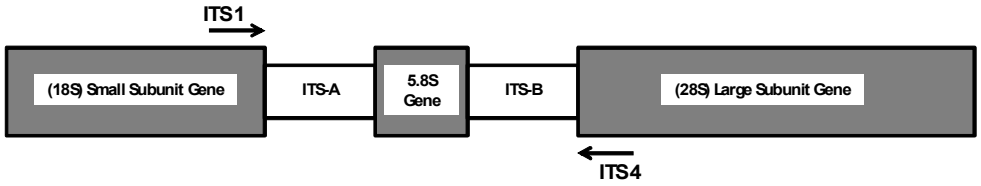

Figure 1 – (A) The ITS primer pair used in this study. (B) The rRNA operon showing the location of primers ITS-1 and ITS-4.

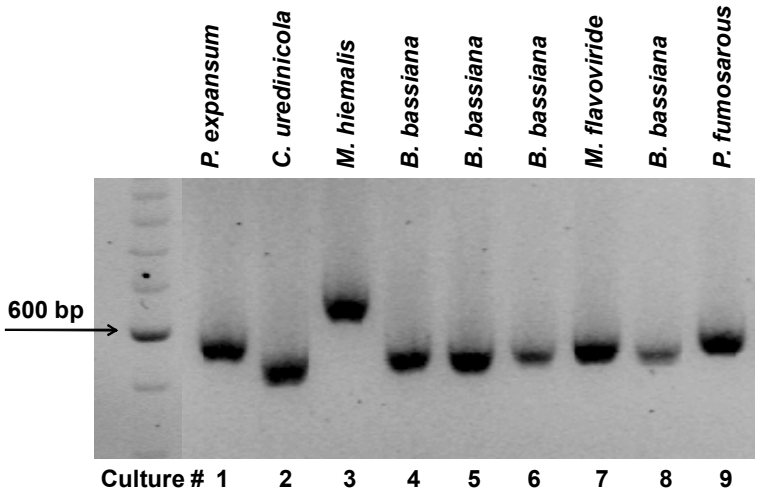

Figure 2 – PCR products generated from culture samples using the ITS primer pair. Numbers below each lane indicate the culture number from which tissue samples were taken. Black labels above each lane indicate the identity of each fungus based on the alignment of their sequenced ITS regions with published sequences in the NCBI database. Samples were run on a 2% agarose gel

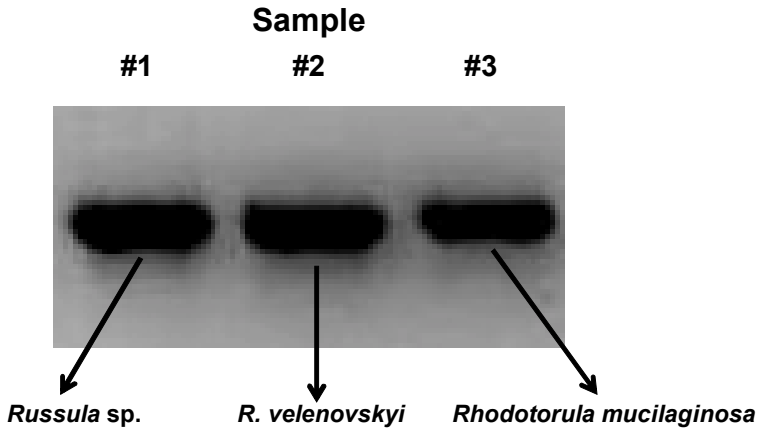

Figure 3 – PCR products generated from three soil samples using the ITS primer pair. The species identity of each fungus was determined based on the alignment of their sequenced ITS regions with published sequences in the NCBI database. Samples were run on a 2% agarose gel.

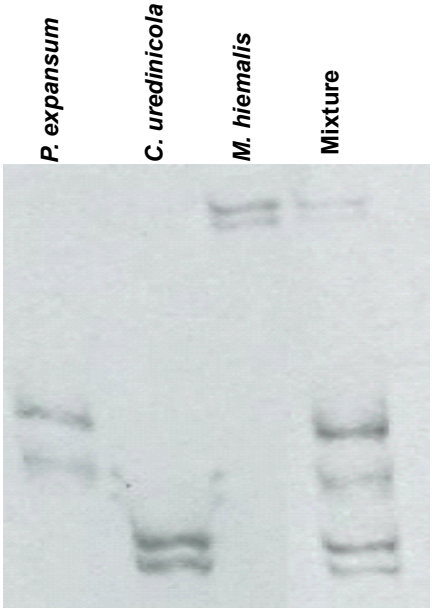

Figure 4 – SSCP products generated through amplification of the ITS region of *P. expansum, C. uredinicola,* and *M. hiemalis*. Lanes 1-3 show SSCP products generated by the three species individually. Lane 4 shows SSCP products generated by a reaction containing the DNA of all three species.