Optimizing direct polymerase chain reaction (PCR) with germinated smut fungus (*Sporisorium ellisii*) teliospores for use in ecological studies

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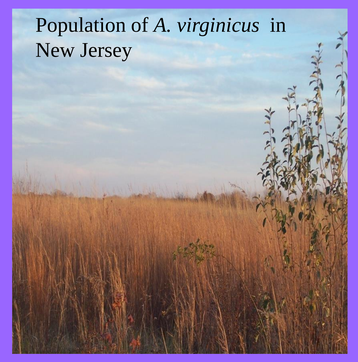
Abstract

Plant pathogens can strongly affect plant populations by influencing the structure and dynamics of natural populations and by decreasing crop yield and quality, yet they have received far less attention from ecologists than have herbivores. In many cases, little is known about even the most fundamental aspects of fungal pathogen ecology, such as the in situ life cycle and basic population genetic features. Our lab is focused on studying the grass *Andropogon virginicus*, and its fungal pathogen, *Sporisorium ellisii*. Infection can cause host sterility and mortality. A goal of our laboratory group is to document the population genetic structure of *S. ellisii* within and among individual host plants, populations, and regions. Currently, nothing is known about genetic variation in this ecologically important fungal pathogen. Identification of DNA markers in fungi traditionally requires costly and time consuming DNA extraction from slow-growing fungal cultures or scarce teliospores, prior to PCR. We are optimizing a new technique to perform PCR directly on newly germinated teliospores, without first culturing the fungus and extracting DNA. The direct PCR method is most successful when three germinated, one-day-old teliospores are used per reaction. Single germlings produce unacceptably inconsistent results. If all teliospores within an individual host are genetically identical, the three-spore direct PCR method will enable very efficient and inexpensive genetic data collection at the multiple-population scale, making this technique very useful for fungal pathogen population genetics.

Germinated *S. ellisii* teliospores

Population of *A. virginicus* in New Jersey



Healthy tiller (left) and infected tiller (right)

Old-field population of *A. virginicus*

Introduction

Sporisorium ellisii is a smut fungus (Phylum Basidiomycota, Order Ustilaginales) that infects populations of the common, old-field perennial grass *Andropogon virginicus* in its native range within the eastern United States. Preliminary studies have shown genetic variation of *S. ellisii* within one plant population, but it is likely that spores within a plant are identical. We would like to test this hypothesis as well as to use molecular markers that can differentiate genetic differences of this fungus within sori, individual plants, plants within a population, and among populations. Current methods for DNA extraction require fungal subculture techniques prior to PCR, which are highly susceptible to contamination and have low growth success rates. Even if growth is successful, the entire process for just one spore takes approximately two weeks.

With a "direct PCR" method, one-day-old germinated teliospores can be added directly to a PCR reaction. For single spores, past success rates have been about 50%, while over 80% of three spore direct PCR runs have been successful. This approach, while still being perfected, will provide a much more time- and cost-efficient technique for large-scale genetic studies of wild fungi.

Methods

Sample collection

- *Sporisorium ellisii* teliospores collected from infected *Andropogon virginicus* in Mercer County Park Northwest, NJ, March 2008.
- Spores removed by hand from sorus and stored dry in airtight tubes at 4C.

The success of direct-spore PCR relies on a carefully developed and executed stepwise technique:

1. Inoculate potato dextrose agar Petri plates with sterilized teliospores.
2. Incubate 24 hr at 25ºC.
3. Pluck germinated teliospores from the plate, under an inverted microscope, using a *platinum tipped* glass tool and carefully transfer to a 15.0 µl droplet of 0.1% bacto or potato dextrose agar.
4. Pipette 5.0 µl of the agar, along with the transferred spore, to a 25.0 µl PCR reaction tubes.
5. Add 1.0 µl each forward and reverse primers (designed by our lab for *S. ellisii* from universal ITS primers) to each tube, along with molecular grade water to bring up the volume to 25.0 µl.
6. Run negative controls of water and plain bacto agar (5.0 µl), and positive control of *S. ellisii* (SPEL) DNA (2.0 µl), to test for contamination and accuracy of results.
7. Run PCR reactions in thermocycler, with initiation at 95ºC for 3 minutes; cycle two at 95ºC for 1 minute; cycle three (the annealing cycle) at 50ºC for 30 seconds; and cycle four at 72ºC for 30 seconds. Repeat cycles two through four 34 times. The final extension period is 10 minutes at 70ºC.
8. Detect amplified PCR products by electrophoresis on 1.5% agarose gel in 1X TAE buffer solution, with 2.5-5.0 µl of ethidium bromide to enhance band appearance. PCR products were mixed with 2.0 µl of loading dye and added to the appropriate lanes, as was 3.0 µl of DNA ladder for base pair length comparison, and run at 100 V for ca. 45 minutes.
9. Isolate DNA from each band with Qiagen QiaQuick kits.
10. Sequence isolated DNA with CEQ automated sequencing reactions.

Results

These results show that we can successfully perform PCR directly on germinated fungal spores, and obtain good quality DNA sequence data.

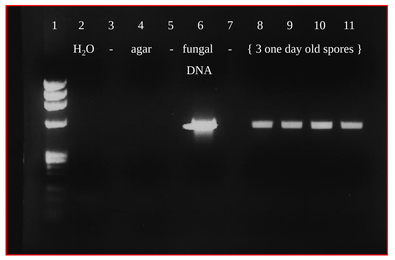
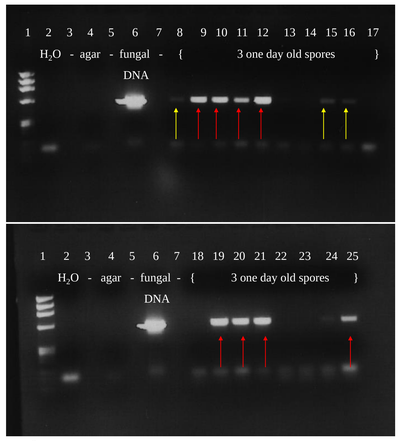


Figure 1. Four replicates of 3 one-day-old spores (lanes 8-11). Water, 0.1% agar, and fungal DNA (lanes 2, 4 and 6, respectively) are used as controls.



Figures 2 (top) and **3** (bottom). Eighteen replicates total (lanes 8-17 in Figure 2 and lanes 18-25 in Figure 3) of PCR with three one-day-old spores per reaction. DNA from lanes with yellow arrows was gel-extracted, and was gel-extracted and sequenced for lanes with red arrows.

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CAAAACCCGGCAGGGAAGAAAGAGCGAAAGAGTGAGCTTT  40
CGTCCGTTCTTGCCTATCAAATGGATGCGCTAATGTATTT  80
CGAGGGAGCCACGGTTAATGGCAAAAGAAACCCTCACTAC 120
CGATCCGTCCCTTTTTTAATTAAAAGAAGGGTCGTTCGAA 160
ACAATTCGCGGCCCTCAAACAGGCATGCTCCCCAGATTAG 200
ATCTGCAGGGAGCGCAAGGTGCGTTCAAAGATTCGATGAT 240
TCACTTCTGCAATTCACATTACTTATCGCAATTCGCTGCG 280
TTCTTCATCGATGGGAGAACCAAGAGATCCGTTGCCAAAA 320
GTTGTTTTTAAATTTAAACGACCGAATTACCAGTCATAAA 360
TTGTCATTCAAATCCTAGAGAATCAAAAGTGTTTGTGTAA 400
AAAGTGTCGAGGGG                            414
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Figure 4. Sequence of DNA extracted from 3 spore PCR (lanes with red arrows in figures 2 and 3 on the right. Spores were all from the same plant, and all 8 sequences were found to be identical.

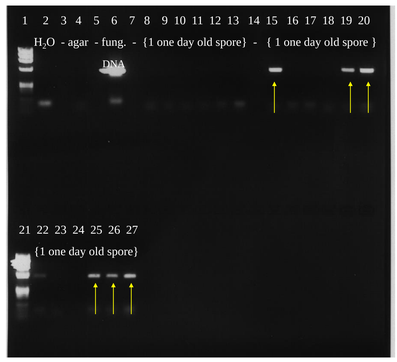

Figure 5. Six replicates of a single one-day-old spore from 3 different plants within a population: plant 8S for lanes 8-13, plant 9S for lanes 15-20, and plant 29S for lanes 22-27. DNA from lanes with yellow arrows was gel-extracted.

Discussion

Andropogon virginicus is an ecologically important plant species; it is a dominant community member during the early phase of old field succession. Approximately 50% of populations are infected, and past studies have found disease prevalence to range from 10-30% of total plants in a population. Very little is known about the genetic structure of wild fungal species generally; even for pathogenic species such as *S. ellisii* that have potentially important consequences for host plants (the fungus decreases seed production and increases mortality). One reason for this lack of knowledge is the tedious and painstaking process involved in obtaining genetic data from fungal spores. Previous methods that require fungal culture and DNA extraction prior to PCR involve much time and materials, and yield very low success rates. The new method addresses these problems.

The direct PCR method we have developed allows us simply to collect spores, germinate them, and obtain DNA data. This provides a highly efficient and much less expensive procedure for future genetic studies of *S. ellisii*, with potential for other fungal species. In the future, we plan on sampling spores within the same sorus and within plants (to find out if individual plants are infected with just one strain of the fungus); among plants within a population (to find out whether or not there is genetic variation, and if so, whether this genetic difference influences infection rates); and among populations (to find out if there is genetic isolation by distance, and if pathogen populations are adapted to different plant populations). This requires many samples and will be much more efficient with direct PCR.

The first step we are taking is to use the method on single spores from individual plants within a population (as shown in figure 5). The resulting sequence data for those spores that had successful PCR will allow us to make comparisons within individual plants and between different plants in a single population.

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