FINAL PROJECT REPORT
WTFRC Project Number: CP-07-703

Project Title: DNA arrays for monitoring orchard soil microbial communities

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Notes:

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Budget History:

Item	Year 1: 2007	Year 2:	Year 3:
Salaries	20,000		
Benefits	6,000		
Wages			
Benefits			
Equipment			
Supplies	9,000		
Travel			
Miscellaneous			
Total	**35,000**		

The goal of these studies was to develop a DNA array-based method that could be used in the analysis of fungal community composition and function in orchard soils, for rapid identification of prominent taxa in mixed samples, and that is also more accessible and flexible than current options. As a more easily accessible and cost-effective technology relative to slide-based DNA arrays, we utilized nylon membranes as the printing medium with a colorimetric labeling and detection system. To avoid the time and costs needed to design and validate oligonucleotide probes, we employed the entire **Internal Transcribed Spacer** (ITS) region (ITS1/5.8S/ITS2) of the fungal ribosomal DNA as both the target on the array and the probe. The fungal ITS regions are well known to be particularly useful for species-level distinctions in fungi. While the 5.8s region is generally conserved, the ITS1 and ITS2 regions are quite variable and collectively should allow maximal potential for distinguishing fungal taxa.

OBJECTIVES

1) Develop DNA microarrays containing a comprehensive collection of DNA sequences from fungi found in orchard soil and apple roots in central Washington.

2) Evaluate the capacity of the DNA microarray to measure community structure and function for predictive purposes.

SIGNIFICANT FINDINGS

- The method developed allowed for distinguishing fungi that differed by approximately 5-10% in ITS DNA sequence
- Using an artificially constructed fungal community, the DNA array identified greater than 80% of the members in the community
- The array effectively described an orchard soil fungal community of known composition and identified previously known and unknown changes in composition induced by seed meal amendments
- The array identified the majority of fungal lineages in a previously undescribed and geographically distant (Quebec, Canada) orchard soil
- Based on these findings the method developed appears to be practical for the rapid detection of multiple genus-level lineages of fungi in complex orchard root and soil samples

RESULTS AND DISCUSSION

Fungal samples used in array construction came primarily from the rhizosphere of apple or soils from the Columbia View Research and Experimental (CV) Orchard (Orondo, WA). The fungal community was sampled in a number of ways to maximize the taxonomic diversity obtained. Fungi were sampled from soils directly or 5 days following amendment with various Brassicaceae seed meals in the greenhouse as described previously (Mazzola et al. 2007). Fungi were recovered from soils by manual isolation of visibly apparent hyphal growth, as well as by plating serial dilutions of a soil suspension onto 1/5 strength potato dextrose agar amended with ampicillin (100 $\mu g\ ml^{-1}$). DNA was isolated from these individual isolates and the ITS region was amplified.

Alternatively, mass recovery of fungal ITS products were obtained by PCR amplification of DNA extracted from Gala/M26 roots collected at 24 plots across the CV orchard that had been planted either in native soils or soils previously amended with the same Brassicaceae seed meal amendments. The resulting DNA products were cloned and unique individuals were identified by sequence analysis. Sequence types were assigned a unique name based on their coarse affinities to taxa based on BLAST searches of Genbank.

Arrays were constructed by linking the unique ITS products onto Hybond+ membrane in triplicate. Various probe construction methods were used to test the utility of the array. These included:

1.) Probe consisting of PCR product from three different *Pythium* species.

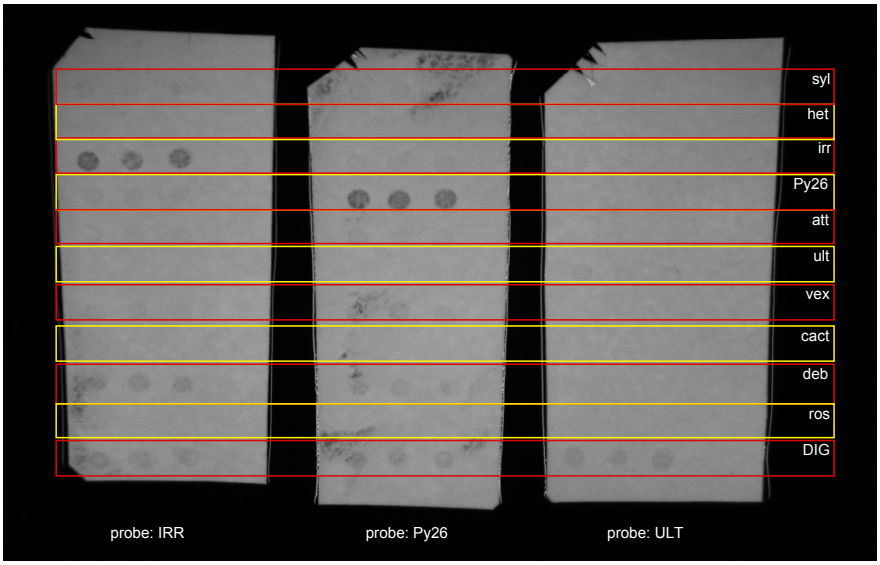
2.) Probe consisting of a mixed community of known composition constructed by amplifying 10 different environmental clones

3.) DNA was isolated from brassica seed meal amended soil at the Columbia View orchard and the ITS region was amplified to generate the probe.

4.) DNA was isolated from soil and roots obtained from an orchard in Quebec Canada and the ITS region was amplified to generate the probe

Experiment 1.

Under high stringency hybridization conditions, probes composed of the ITS region from *Pythium. irregulare* and an unidentified *Pythium sp*. ("Py26") each hybridized to targets on the array corresponding to the closest related sequences, with the strongest signal detected for the perfect match to itself (Figures 1 and 2). For example, when the *P. irregulare* probe was used, substantial hybridization signal was only detected for *P. irregulare*, *P. sylvaticum*, and *P. debaryanum* all of which possessed ITS sequence similarity greater than 90%, and the array targets corresponding to *P. irregulare* yielded the strongest signal. Similar results were obtained with the *Pythium sp.* "Py26" probe, which prominently detected *Pythium sp* "Py26" and showed lower signal for the two other most closely related species (*P. sylvaticum* and *P. debaryanum,* both possessing sequence similarities between 80-90%). The *P. ultimum* probe detected *P. ultimum,* however the signal strength was considerably lower. Under low stringency DNA hybridization conditions, the array signal remained limited to species of *Pythium* most closely related to the probe with the identical target sequence showing the strongest signal (data not shown). However, the lower stringency conditions also led to signal being detected from *Pythium* species that possessed sequence similarity of only 75-80% of the probe utilized. Even under low stringency conditions certain *Pythium* species targets were not detected with each probe, and these generally had the lowest sequence similarity to the probe species. *Phytophthora cactorum* – a representative of the genus highly related to *Pythium* - was not detected with any of the *Pythium* probes.



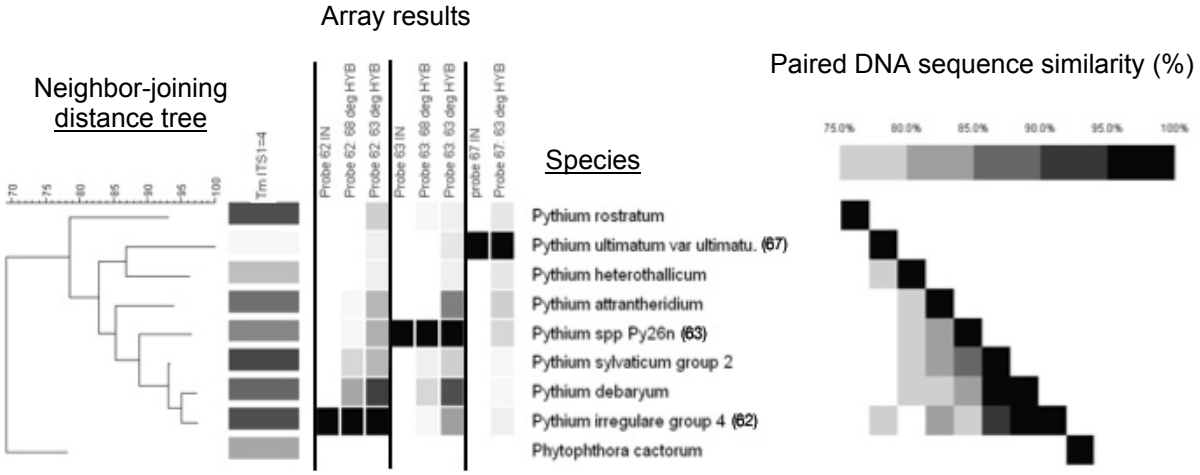


Figure 1. Identification of lineages within the genus *Pythium* using ITS PCR amplicon probes. Top: Developed miniarray blot of hybridizations performed at high temperature (68°C). Bottom: Relative hybridization signal obtained when using either "high" (68°C) or "low" (63°C) stringency temperatures with single species probes relative to DNA relatedness of species and target melting temperatures. Array signal is colored on a relative scale of 0-1.0 (white-black). Melting temperatures (Tm) of the PCR product resulting from amplification using the primers ITS1 and ITS4 are colored on a scale from 84-95°C (white-black). The *Pythium* species used to generate the probe is noted by black box under the "probe" heading.

Experiment 2 and 3.

Analysis of artificially constructed fungal communities

A mixed community of known composition was constructed by amplifying 10 different environmental clones using the primer pair ITS1f and ITS4a. The majority (8/10) of the probe taxa that were used to construct the known community probe were successfully detected on the array under high stringency conditions (Figure 2). Additionally, targets that were less than 90% similar (based on DNA sequence distance) to samples contained in the probe typically produced weak or no signal. The two samples that were not detected were both from the *Mortierella*-related lineage which previously was found to have lower melting temperatures for the ITS target region. Thus, at the hybridization temperature (68^{o}C) used in these studies, the probe would dissociate from the corresponding target on the array. Lowering the stringency condition (63^{o}C) allowed detection of more of the known community (9/10 samples), however this resulted in false detection of many other array targets that were not in the probe (Figure 2B).

Analysis of seed meal amended soils

Array analysis detected a range of fungi from CV orchard soils amended with *B. napus* (Figure 2C). Following hybridization with the fungal probe generated from the *B. napus* treated soil, 18 of the 95 taxonomic array spots were visually positive. Based on DNA analysis, these 18 spots represented at least 8 unique lineages (>10% different from each other). Genera represented in array spots with signal included *Fusarium* (2 lineages), *Trichoderma*, *Acremonium*/*Nectria*, *Arthrobotrys*, *Cylindrocarpon* (2 lineages), *Mariannae*, and *Neonectria*. No signal was detected on array targets with affinities to *Mortierella.* Notable differences were observed in the fungal community detected in *B. juncea* amended soils via array analysis. Specifically, fewer taxa were identified and neither *Cylindrocarpon destructans* nor *Cylindrocarpon olidum*, both important pathogens of apple, were detected. This corresponds to results based upon the plating of Gala/M26 root samples on agar media, where infection by Cylindrocarpon spp. was not modified by *B. napus* soil amendment but was virtually eliminated in *B. juncea* amended soils (Table 1).

Table 1. Impact of soil treatments on Gala/M26 root infection by *Cylindrocarpon* spp.

Treatment	*Cylindrocarpon* root infection (%)
Control	19.5a
Brassica juncea PG	1.9b
Brassica napus DE	18.0a
Sinapis alba IG	7.1b

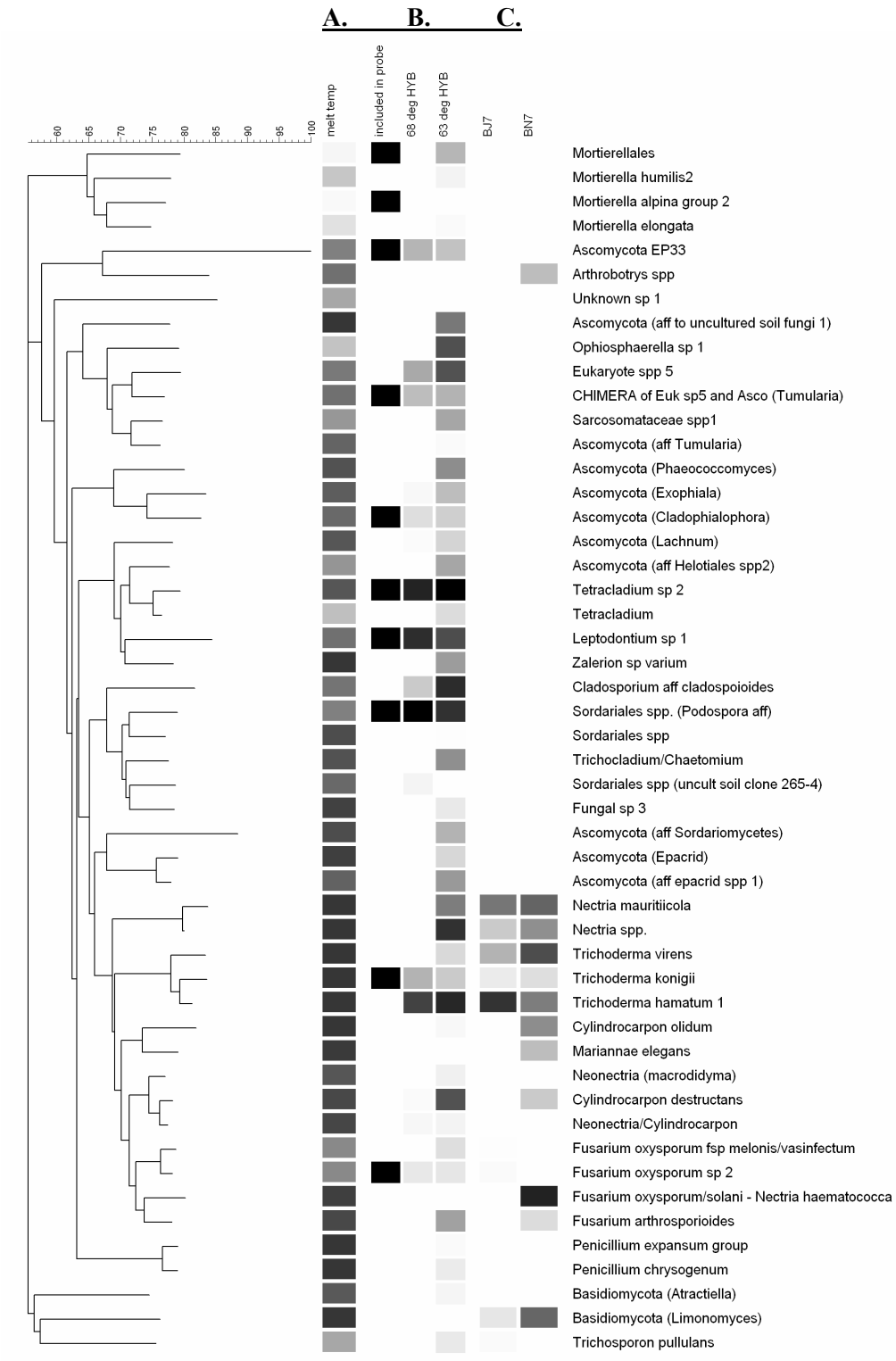


Figure 2. Results of three tests are presented in context of DNA sequence similarity. A) melting temperature of array targets; B) composition of an artificially constructed community probe and resulting array relative target signal in assays conducted under high or low stringency conditions; C) array results for probe composed of soil fungal community generated using DNA isolated from soil amended with *Brassica napus* or *Brassica juncea* seed meal. Scales are the same as Fig. 1.

Experiment 4.

Array analysis and cloning of uncharacterized environmental sample

Community composition based upon DNA sequence analysis

DNA extracted from an orchard soil in Quebec was used in amplification reactions using ITS primers to generate the probe used in experiment 4. Resulting product was also cloned and transformed into *E. coli* prior to screening by RFLP analysis to identify unique sequences. The clone screening revealed 50 unique RFLP types. DNA sequence data was obtained from 44 of the unique RFLP types and these accounted for 138 of the 144 clones screened. Approximately 40% (55/138) of the sequences contained in the probe were 90-100% similar to at least one sequence known to exist among the array targets. This portion of the probe community was composed of taxa with affinities to the genera *Leptodontium*, *Geomyces*, *Nectria*, *Tetracladium*, *Zalerion*, and *Mortierella*. A number of these probe constituents were 90% or higher similar to more than one target spot on the array. Two clone RFLP types with affinity to *Leptosphaerulina* dominated the probe community with (32% of total), but were only 80% similar to the closest DNA sequence match among array targets. Of the 17 RFLP types that were predicted to be detected on the array based on DNA sequence similarity to an array target, 14 were detected based on signal shown in the corresponding array target. Of the three that were not detected, two had affinities to the *Mortierella* group.

Community composition based upon DNA array analysis

Hybridization of the 190 taxon array with the probe generated from Quebec orchard soil fungal community DNA resulted in the detection of 26 array targets (Figure 3). Target spots with affinities to the genera *Leptodontium*, *Geomyces*, *Zalerion*, *Cadophora* were among those yielding the strongest array signals. Targets generating 0.50 or higher relative signal could be accounted for by the presence of cloned probe sequences that were 90-100% similar and that made up a large portion of the detectable clone sequences. Of the array targets yielding relative signals within the 25-50% range, three of the five had close sequence matches in the probe community. Although there were a few exceptions, most of the target spots below a relative signal of 0.20 possessed matches in the probe community however the similarities were much lower.

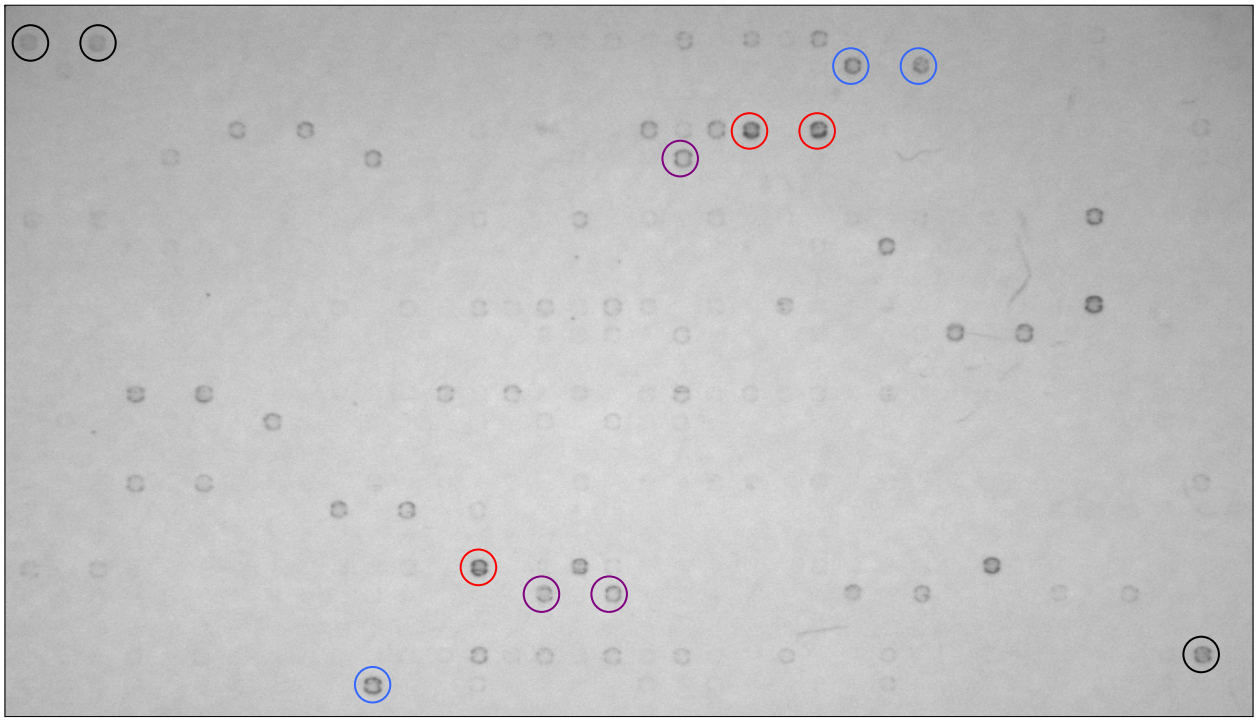


Figure 3. Fungal ITS array probed with environmental fungal community PCR product (primers ITS1 and ITS4) amplified from a Quebec orchard soil DNA sample. A DIG-labeled positive control and three prominent samples are noted by circles. Each array sample is replicated with three spots, one of which is in the mirror opposite position. ○ Positive internal DIG control; ○ *Leptodontium*; ○ Unknown cultured fungus; ○ *Geomyces*.

A DNA array approach utilizing fungal ITS PCR product as both the probe and target proved adequate for the identification of fungal genera present in orchard soil samples. Given the genus-level resolution this method is suitable to assess broad-scale patterns and to attain initial views into fungal community composition.

The general utility of the DNA array technique was demonstrated in studies which successfully described composition of the fungal community from a Quebec orchard soil that was geographically distant from that (CV orchard soil) which was utilized to generate the array targets. By cloning and sequencing the same amplicon community used to generate the probe for the Quebec orchard soil, we were able to confirm that a) probe constituents possessing sequences within 90% similarity to at least one array target were detected, and b) array targets exhibiting strong relative signal corresponded with an element of the probe population possessing sequence similarity at these same levels. As with all array-based approaches, the utility of a given array is limited to the degree to which the overall population is represented on the array. One fungus in particular, *Leptosphaerulina*, was prominent in the Quebec orchard soil but it possessed an ITS sequence that was not within 90-95% similar to any array target. As a result, the fungus was detected in the cloning screen but not by array hybridization. This problem can be addressed in the construction of future arrays by improving the breadth of taxonomic sampling within genera such that all sequences known to exist, based on larger databases like Genbank, are 90% or more similar to at least one target spot. Despite the potential to overlook such

samples, the ability to account for even 200 lineages should provide a considerable advantage when compared to culture-based analyses or molecular techniques that require continued screening, such as the cloning and sequencing of DNA.

Signficance to industry:

Most of our understanding of root disease in orchard systems has been generated through culture-based studies. As is often seen when contrasting approaches are used, revisiting root disease with molecular-based approaches may potentially reveal other species not previously appreciated to be associated with root health. The approach we have developed offers that opportunity to obtain a unique view into these systems and can be utilized to revisit previously addressed questions that will further our understanding of fungal ecology in orchard soils and the rhizosphere of apple. The method has also revealed its capacity to identify prominent changes in fungal community composition in response to disease management strategies. For instance, the eradication of *Cylindrocarpon* spp. from *B. juncea* seed meal amended soil, which was predicted by DNA array analysis, was documented to be valied based upon the recovery of this root pathogen from trees grown in the treated soil. A previously unrecognized functional group of potential importance to disease management was also documented. *Arthrobotrys* was a significant component of the fungal community in *B. napus* as determined by DNA array analysis. Members of this fungal genus are known to be significant predators of plant parasitic nematodes and may contribute to the suppression of these parasites, including the lesion nematode. Finally, such a methodology would enhance the capability to conduct more powerful long-term research at sites such as the newly purchased WSU-Sunrise orchard at Quincy