

A Comparative Study of Fungal Communities in Vineyard Soils Using Terminal Restriction Fragment (TRF) Analysis

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Abstract

Studies performed have indicated the possibility of identifying fungal communities using terminal restriction fragment (TRF) analysis (N.S. Lord et al., 2002). The purpose of this study was to compare and contrast the fungal communities of organic and non-organic vineyard soils using the molecular method of Terminal Restriction Fragment (TRF) analysis. We hypothesized that TRF patterns would indicate the presence of more fungi in the organic soil. Fifty grams of soil was collected using a composite sampling method from two vineyards located in the same region of north San Luis Obispo county wine country. One vineyard uses organic practices and the other does not. Community genomic DNA was extracted and used for PCR amplification. Primers designed to the fungal ribosomal ITS region were used to amplify community genomic DNA, one of which was fluorescently labeled. Amplicons were then restricted separately using *HpaII* and *HaeIII* restriction enzymes prior to detection of fluorescently labeled TRFs using capillary gel electrophoresis. Patterns from the two soil samples show differences for peak number, size, and height. These data suggest that the non-organic soil samples contain a greater abundance of fungi compared to the organic soil. In this study, we determined that TRF analysis is a powerful technique for fingerprinting fungi in soil samples, but its use for phylogenetic classification would be tedious and time consuming without an adequate database.

1. Introduction

In recent years, novel molecular research techniques have allowed researchers to more extensively document the presence of fungi in different soil types. Previously, the task of enumerating and differentiating soil fungi has been a laborious and time consuming process, and has been less than accurate due to the limitations of culture and sequencing techniques. A new technique, terminal restriction fragment (TRF) or terminal restriction fragment length polymorphism (T-RFLP) analysis, has given researchers a new diagnostic tool to detect the fungal community members present in soil samples. The method involves amplifying the conserved internal transcribed spacer (ITS) region of rDNA using primers, one of which is

fluorescently labeled. PCR products are then digested separately with restriction enzymes prior to fluorescence detection with capillary gel electrophoresis. The usefulness of this technique alone in identifying the members of a fungal community requires an extensive, not yet available, eukaryotic TRF pattern database. However, other studies have been mildly successful in species identification when used in conjunction with DNA sequencing and culture methods (Lord et al., 2002).

The use of TRF analysis in detecting microbial communities has been successful in such applications as bioremediation in oil spills (van Elsas et al., 1997 and Sylvia et al., 1988) and sewer sludge (Lord et al., 2002), and in the analysis of other beneficial microbial roles. The importance of fungal communities and their roles in

agriculture has also become increasingly apparent as further research is conducted, particularly in the wine industry where growers must contend with the presence of deleterious fungi, such as mildews. Much attention has also recently been given to the use of “biocontrol,” which utilizes competitive microorganisms to limit the growth of unwanted soil flora (van Elsas et al., 1997). Organic farming methods (sans chemical pesticides) have increased in recent years and drawn new attention to the roles of native fungi in the health of plants and the subsequent crop yield. It has also become an economic necessity to have a more rapid method of detection, such as TRF, to further study the metabolic role of microbes and to eventually determine the viability of soils.

The primary goals of this study were to (1) determine the utility of terminal restriction fragment analysis in detecting the presence and quantification of fungi in soil samples taken from vineyards, and (2) to compare and contrast the fungal communities of organic and non-organic vineyard soils.

2. Materials and Methods

2.1. Soil sample collection and storage

Fifty grams of soil was collected using a composite sampling method from two vineyards located in the same region of north San Luis Obispo county wine country. Samples were stored at -80°C. All samples were taken 9 inches below the surface. The non-organic soil had been treated with Round-Up™ six months before samples were taken.

2.2. Community DNA extraction

Fungal Community DNA was extracted from the soil samples in five replicates using a modified Ultra Clean MoBio Soil DNA kit (MoBio, Solana Beach) protocol. 1 g of soil was added to each bead solution tube with 60 µl of S1 Solution and 200 µl of Solution IRS. The tubes were then vortexed on the Fast Prep bead beater (Bio 101, Vista CA) at 5 m s⁻¹ for 45 seconds. Samples were spun down at 10,000 X g for 5 min and approximately 450 µl of supernatant was transferred to a clean tube with 250 µl of S2 Solution. After a brief vortex, samples were incubated at -20°C for 5 min. Samples were then centrifuged at 10,000 X g for 1 min. Avoiding the pellet, the entire volume of supernatant (~700 µl) was transferred to a clean tube with 900 µl of S3 solution, and then vortexed for 5 sec. Approximately 600 µl of S3 solution and DNA mix was loaded onto a spin filter and spun at 10,000 X g for 1 min. The flow through was discarded and the process was repeated until all of the S3 solution and DNA mix passed through the spin filter. 300 µl of S4 solution was then added to the spin filter and spun for 30 sec at 10,000 X g. The flow through was loaded onto the spin filter and then spun again for 30 sec at 10,000 X g. The flow through was then discarded and the tube was centrifuged for 1 min at 10,000 X g. The spin filter was placed in a new tube and DNA was eluted in 50 µl of S5 solution or sterile distilled water; samples were then stored at 4°C until PCR.

2.3. ITS primers and community PCR

Primers designed to amplify conserved fungal ITS rDNA region, ITS1F 5'-CTTGGTCATTTAG-AGGAAGTAA-3' and ITS4 5'-TCCT-

CCGCTTATTGATATGC-3' were used for PCR (Clapp, 1996). The forward primer, ITS1F, was 5'-labeled with D4, a fluorescent dye for analyzing samples using the CEQ 8000 DNA Analysis System (Beckman-Coulter, Fullerton, CA, USA). Reactions were performed in 50 μ l with 1X *AmpliTaq* Gold buffer (Roche), 0.6 μ M dNTP, 0.8 μ g ml⁻¹ bovine serum albumin, 2 μ M MgCl₂, 1.5 U of *AmpliTaq* Gold DNA polymerase (Roche), 0.1 μ M of each primer and ~20 ng template DNA. Reaction temperatures and cycling were: 94°C for 10 min, 13 cycles of 95°C for 35 sec, 55°C for 55 sec, 72°C for 45 sec, 13 cycles of 95°C for 35 sec, 55°C for 55 sec, 72°C for 2 min, and 9 cycles of 95°C for 35 sec, 55°C for 55sec, 72°C for 3 min, followed by 72°C for 10 min. PCR products were detected by agarose gel electrophoresis.

2.4. Primer removal and PCR product concentration

For the two samples, five replicate PCR reactions were cleaned up and combined using a MoBio PCR Clean Up kit according to the manufacturer's instructions.

2.5. PCR product digestion

Prior to digestion, PCR product was quantified using a Microplate Fluorescence Reader (FLX800, Bio-Tek Instruments, Inc.) according to the manufacturer's instructions. Digestion reactions consisted of ~100 ng PCR product and 5 U enzyme in the manufacturer's suggested reaction buffer. The reactions were incubated for 4 h at 37°C followed by enzyme denaturation at 80°C for 20 min. Samples were ethanol-precipitated by

incubating at -20°C in 100 μ l of 95% ethanol, 2 μ l 3M sodium acetate (pH 5.2), and 1 μ l 20 mg ml⁻¹ glycerol (for smooth capillary run) for 30 min and then centrifuged at 13000 X g for 20 min. Ethanol was removed and samples were centrifuged with 125 μ l of 70% ethanol at 13000 Xg for 15 min. The remaining ethanol was removed and samples were air dried for 30 min in a fume hood.

2.6. Capillary gel electrophoresis

TRFs from soil community DNA were obtained using the CEQ 8000 DNA analysis system (Beckman-Coulter, Fullerton, CA, USA) as follows. Digested PCR product DNA was resuspended in 20 μ l of CEQ Sample loading solution (formamide) and 0.25 μ l of DNA size standard-600 (Beckman-Coulter, Fullerton, CA, USA). Samples were injected for 15 s at 2 kV, and separated for 85 min at 4.2 kV with a capillary temperature of 50°C. TRFs were analyzed by the Beckman-Coulter DNA analysis system software version 5.0.345.

3. Results

3.1. PCR from community genomic DNA

Community genomic DNA was successfully extracted and amplified. Both the organic and non-organic DNA samples yielded a 600bp PCR band, while the non-organic soil yielded an additional 700bp band (Fig. 1).

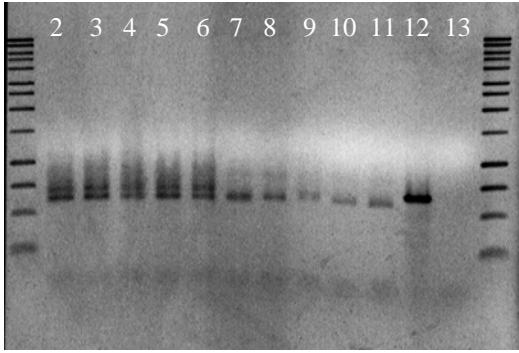


Fig. 1. Gel showing PCR product. Lanes 2-6 non-organic (600bp and 700bp bands), lanes 7-11 organic (600bp band), lane 12 positive control, lane 13 negative control. Sizes determined using 1Kb DNA ladder.

3.2. TRF analysis of soil samples

The Resulting amplicons were digested with *HpaII* and *HaeIII* separately and then fluorescently labeled TRFs were detected using capillary gel electrophoresis. The Non-organic sample produced nearly twice the number of TRFs than the organic sample for both *HpaII* and *HaeIII* (Figs. 2 and 3). The most concentrated area of the non-organic patterns is in the 100-200-bp range. The organic patterns show fewer peaks but higher fluorescence intensity. Both of the organic patterns show high fluorescence intensity around 475-bp.

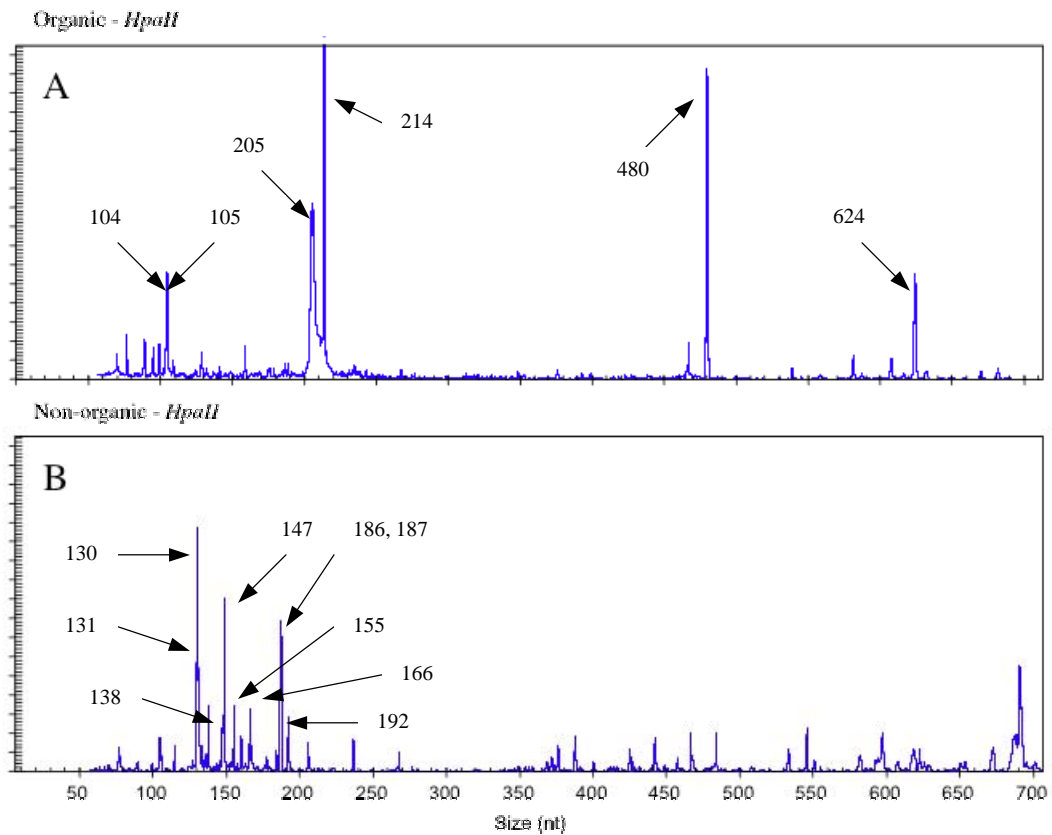


Fig. 2. TRF data from soil samples. TRF patterns generated using *HpaII* for both organic samples (A) and non-organic samples (B). Peak sizes are shown in base pairs (across bottom) and heights are shown in relative fluorescence. Fluorescence scales for both electropherograms are the same.

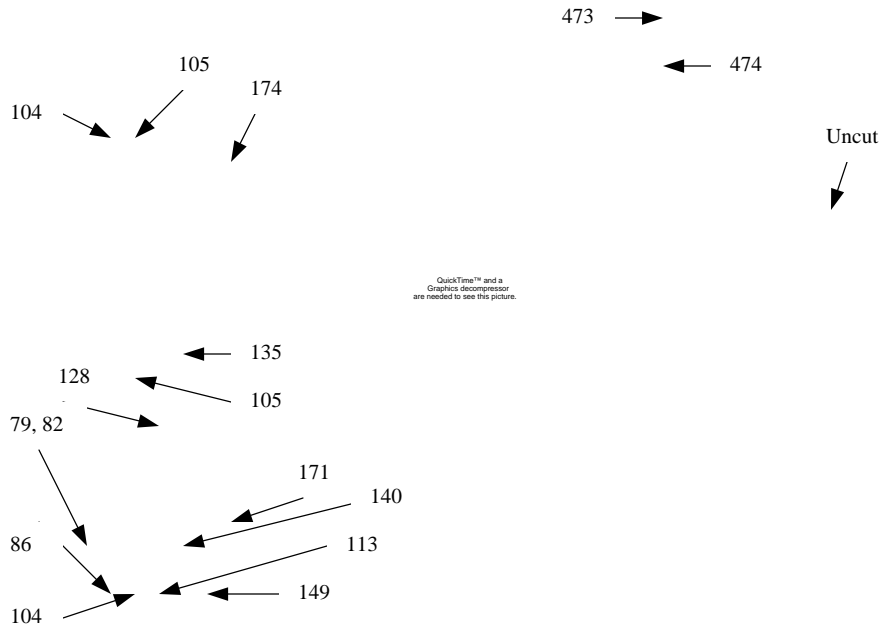


Fig. 3. TRF data from soil samples. TRF patterns generated using *HaeIII* for both organic samples (A) and non-organic samples (B). Peak sizes are shown in base pairs (across bottom) and heights are shown in relative fluorescence. Fluorescence scales for both electropherograms are the same.

Gel images of extracted community genomic DNA showed that the non-organic soil samples contained more DNA than the organic samples. From the gDNA images, eyeball estimation was used to control for the amount of gDNA used for PCR. To further control for amounts of DNA used, fluorescence was measured and used to determine the amount of PCR product prior to restriction digest. Once determined, digests were performed with approximately the same concentration of DNA. GelDoc images of the PCR products showed solid bands at 600 bp for both the organic and non-organic

4. Discussion

samples, and an additional 700 bp band for the non-organic sample. The fluorescently labeled PCR amplicons were digested using *HaeIII* and *HpaII* enzymes to produce terminal restriction fragments. The TRF pattern resulting from the *HpaII* digest revealed six major peaks for the organic soil and nine major peaks for the non-organic soil (see Fig. 2). The *HaeIII* TRF pattern revealed five major peaks for the organic soil; and eleven major peaks for the non-organic soil (see Fig. 3).

Successful PCR and TRF patterns showed a greater and more diverse amount of fungal rDNA present in the non-organic soil than the organic soil. PCR amplification of the ITS region of the isolated DNA produced two amplicons for the non-organic soil and only one amplicon for the organic soil. One possibility for the difference in amplicon numbers between the samples could be due to the differences in the amount of DNA isolated from each sample and used for PCR. Even though we attempted to control for differences in the amount of DNA between the samples, we did so only by eyeball estimation, which may have still resulted in major differences. TRF analysis also produced results consistent with greater fungal diversity in the non-organic soil. Once again, the possibility exists that these results are due to differences in the amount of DNA from each sample used for PCR.

The internal transcribed spacer (ITS) in the 18S and 28S small subunit ribosomal RNA region produces an appropriately sized DNA fragment for TRF analysis (Lord et al., 2002). Accordingly, a particular fungus in a soil sample will have one corresponding TRF peak for each enzyme used, and the size of the peak will reveal its relative population size in the sample. Using two or more restriction enzymes allows a more discrete profile of the fungus to be made based upon the fragment sizes produced. Choosing the appropriate enzyme is important since the wrong enzyme could produce the same TRF pattern for two different fungi, or it may exclude too many fungi that lack the corresponding restriction cut sites (B.G. Clements et al., 1998).

Intuitively, we'd hypothesized that the organic soil samples will contain

more fungal DNA per volume than the non-organic soil considering that the use of pesticides/fungicides would keep fungal survival and growth to a minimum. In our study, however, this was not the case. In fact, approximately three times as much DNA was isolated from the non-organic soil per gram as from the organic soil. This may have been due to the troubles experienced trying to extract DNA from the organic soil. DNA isolation was much more difficult for the organic sample because the soil was very dry and coarse, making it difficult to produce supernatant during the initial stages of the extraction process. PCR amplification and TRF analysis also produced more diverse results for the non-organic soil than for the organic soil. The non-organic soil sample produced nearly twice as many TRFs for both restriction enzymes. Taken together, the two enzymes produced results consistent with DNA quantification and PCR amplification. Therefore, it can be reasoned that the non-organic soil contained a greater number of fungi per volume than the organic soil. These results, however, are inconclusive simply due to the sampling bias introduced by using only two vineyards. Though region and time of day were controlled in this study, other factors, such as soil types, surrounding topography, watering, and nutrients could not be controlled, and therefore, may have contributed to the abundance of fungi in one soil sample over another.

Alternatively, the use of pesticides may have contributed to the abundance of fungi in the non-organic soil due to competitive inhibition (van Elsas, 1997). The spraying of Round-Up™ prior to planting may have lowered the number

of microorganisms and native flora competing for limited soil nutrients, allowing certain species of fungi to flourish under these conditions. Future research could possibly examine the effect of Round-Up™ and other pesticides on fungal communities. One explanation for the discrepancy in fungal concentration and diversity between the two soil samples is the small sampling population and environmental factors that were not controlled. But another possible explanation is that maybe a healthy soil fungal vineyard community (being the organic soil) has only a few dominant fungi. Discrepancies in the comparative study of the organic and non-organic vineyards in the future may be resolved with a greater sampling population (more vineyards), a longitudinal study of the fungal communities, and the use of different restrictive enzymes. We determined that utility of TRF analysis is sufficient to produce a snapshot of the current diversity and size of the fungal population; however, it would give a more accurate view of the fungi present if used in conjunction with statistical analysis of the soil over time as in Clement et al. (1998).

References

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