Genetic approaches for characterizing soil microbial communities

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ABSTRACT

Soil microbial diversity is critically important to ecosystem functioning, but it can be very challenging to study. Microbial genomic DNA was extracted from forest soil samples using two chemical kits. From these extracts, the rRNA gene regions of prokaryotes and three types of fungi were amplified using four sets of primers. PCR was completely inhibited by the extract of one kit. The DNA could not be amplified until the extract was cleaned-up by an additional procedure and the PCR product was re-amplified. A second extraction kit produced a relatively clean extract, from which DNA was amplified without clean-up. These kits also varied dramatically in their ability to extract DNA from different organisms in the soil. The many unique sequences from a single PCR product were cloned in a DNA library. This library revealed that eleven unique organisms comprise the majority of the microbial community in that one sample. This technique circumvented the problems associated with using Sanger sequencing to read a diversity sequences in one PCR product.

INTRODUCTION

Microorganisms play a fundamental role in the transformation of energy and matter. For this reason, knowledge of microbial diversity is essential to understanding how ecosystems function (Torsvik et al. 1996, van der Heijden et al. 2008). Soils in particular can harbor some of the most diverse microorganism communities of any environment on earth (Roesch et al. 2007). This great diversity makes it possible for the community to perform a variety of functions. Not only do soil microbes play a role in biogeochemical cycling and

soil creation, they can also have direct and indirect effects on plant productivity (van der Heijden et al. 2008). Detritivores liberate nutrients locked-up in dead organic matter, increasing nutrient availability in the soil. Mutualistic symbionts, such as mycorrhizal fungi and rhizobia, give plants access to limiting nutrients, increasing plant productivity. Pathogenic microorganisms can reduce plant growth by directly removing carbon and nutrients from plant tissues (Wardle et al. 2004). The composition of the soil microbial community can be an indication of ecosystem health (Roose-Amsaleg et al. 2001).

In the past, studying soil microbial diversity has been problematic due to the fact that so few of the species can be cultured in the laboratory. In the case of fungi, the appearance of fruiting bodies is also an unreliable method for identifying the below-ground presence of root-associating mycorrhizal fungi (Dahlberg 1991). Molecular techniques offer more powerful tools for characterizing the diversity of soil microorganisms. In fact, advances in molecular microbial ecology in the past two decades have turned a spotlight on the tremendous genetic diversity of soil microorganisms that has escaped the notice of traditional methods of laboratory cultivation (Rondon et al. 2000). Instead of isolating and identifying individual species, molecular techniques allow us to characterize the entire soil community by analyzing the collection of genomes of the total microbiota in a soil sample – the soil metagenome (Rondon et al. 2000).

Genetic approaches for studying soil microbial communities come with their own unique challenges. Soils contain humic acids, which are known inhibitors of PCR (Wilson 2000). Soil bacteria and fungi also have variable cell wall structures, which hinders DNA extraction. Additionally, the wealth of metagenomic information that can be contained in a small soil sample requires special sequencing strategies. In this study two different chemical

kits were used to extract genomic DNA from forest soil samples. A methodology for amplifying short DNA sequences from four different types of soil microorganism was developed. In order to overcome the challenges associated with sequencing, a DNA library was built to isolate and identify the diversity of organisms amplified by one primer set. Finally, the relative extraction efficiencies of the two different soil DNA extraction kits were examined.

MATERIALS AND METHODS

Soil sampling and DNA extraction

Soil was collected from the forest at the Louis Calder Center on 4/3/11 using a 5 x 10cm soil core. The soil sample was filtered through a 4 mm sieve and stored at 4˚C before processing within 24 hours. An additional soil sample was collected from the old growth forest at the New York Botanical Garden (NYBG) on 4/12/11. This soil sample was also filtered through a sieve and stored at 4˚C before processing. This soil sample was processed within 24 hours and then frozen at -20˚C for future use. Both soil samples were collected at a distance of approximately one meter from the trunk of a red oak tree (*Quercus rubra*).

Three different extractions were performed using two different chemical extraction kits. The Calder Center soil sample was processed using the SoilMaster™ DNA extraction kit by EPICENTRE® Biotechnologies according to the manufacturer's instructions. After extraction the OD at 260nm of the first soil extract was measured to quantify the DNA, and the ratio of OD 260:280nm was measured as an estimate of DNA purity. The NYBG soil sample was also processed using the SoilMaster™ kit; however, during this extraction the lysis step was extended overnight at 65˚C instead of the 10 minutes recommended by the manufacturer. After use in some PCR reactions, this extract was purified using the

PowerClean® Soil DNA Clean-Up Kit by Mo Bio Laboratories, Inc. according to the manufacturer's instructions. The NYBG soil sample was used again for DNA extraction using the PowerSoil® DNA Isolation Kit by Mo Bio Laboratories, Inc. according to the manufacturer's instructions. The DNA in this extract was used without an additional clean-up procedure. The extract was later purified using the PowerClean® Soil DNA Isolation Kit for the purpose of comparison to the SoilMaster™ extract.

Primer selection and PCR amplification

Four primer sets were chosen to selectively amplify ectomycorrhizal fungi (phyla Ascomycota and Basidiomycota), arbuscular mycorrhizal fungi (phylum Glomeromycota), fungi of the phylum Basidiomycota, and prokaryotes. The Roche Adaptor A sequence was added to the 5' end of all of the forward primers and the Roche Adaptor B sequence was added to the 5' end of the reverse primers. A unique 8 base barcode – AGAGAGAG – was also added to the 5" end of the forward primers. These adapted primers are used in parallel-pyrosequencing by 454 Life Sciences. While they are not relevant to this study in particular, they were used to inform future research.

Primers ITS1-F and ITS4 bind to sequences on the 18S rRNA gene and the 28S rRNA gene, amplifying the internal transcribed spacer (ITS) region in higher fungi (Gardes and Bruns 1993). The ITS region contains two variable non-coding regions and the highly conserved 5.8S rRNA gene (Fig. 1). These primers produce an amplicon that is between 600 and 800 bp. The ITS1-F and ITS4 primers were used to amplify ectomycorrhizal fungi. A third primer, just downstream of ITS4, is ITS4-B. The ITS4-B primer can be used as a reverse primer to ITS1-F to bind specifically to fungi of the phylum Basidiomycota (Gardes and Bruns 1993). The ITS1-F/ITS4 primer set was chosen to examine the diversity of

ectomycorrhizal fungi. The ITS1-F/ITS4-B primer set was chosen for its increased specificity.

The ITS sequences in the phylum Glomeromycota exhibit a very high level of variation. Therefore, the primers AML1 and AML2 were used to amplify fungi of the phylum Glomeromycota - the arbuscular mycorrhizal fungus. These primers were designed by Lee et al. (2008) to optimally bind to the 18S rRNA gene in all known arbuscular mycorrhizal fungal groups (Fig 1.). These primers produce an amplicon that is about 795 bp.

The primers 27F and 338R target highly conserved regions of the 16S rRNA gene in bacteria. The 16S rRNA gene in prokaryotes is analogous to the 18S rRNA that is amplified in the fungi. These primers were selected because they have been found to be well-suited for amplification and analysis of bacteria in environmental samples (Hamady et al. 2008, Suzuki and Giovanni 1996, Fierer et al. 2008). These primers produce an amplicon that can be between 228 and 309 base pairs in length (Fierer et al. 2008, Suzuki and Giovani 1996).

For all four primer sets, PCR reactions consisted of 0.4μL of the forward primer, 0.4μL of the reverse primer, and 10 μL of GoTaq® Green Master Mix. While the volume of template DNA varied, the total reaction volume was always brought up to 20 μL with dH_2O. The PCR reactions for the ITS1-F/ITS4 primer set and ITS1-F/ITS4-B primer set had an initial 5 minute denaturing step at 95˚C, followed by 50 cycles of 35 seconds at 95˚C, 55 seconds at 55˚C, and 45 seconds at 72˚C and a final elongation step of 10 minutes at 72˚C (Gardes and Bruns 1993). PCR reactions for the AML1/AML2 primer set and the 27F/338R primer set had an initial 5 minute denaturing step at 94˚C, followed by 50 cycles of 60 seconds at 94˚C, 60 seconds at 50˚C, and 60 seconds at 72˚C and a final elongation step of 10 minutes at 72˚C (Lee et al. 2008).

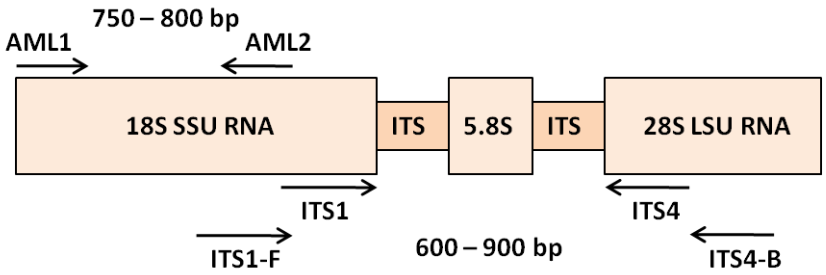

Figure 1. The rRNA gene region that is targeted by the fungal primers includes two highly variable internal transcribed spacer regions, and three rRNA subunits (18S, 5.8S, and 28L) which consist of both highly conserved and highly variable areas. The 16S rRNA subunit gene (not shown), which is targeted by the prokaryote primers, is analogous to the 18S rRNA subunit in fungus. These gene regions are often used in phylogenetic analysis because of their highly conserved regions suitable for primer binding and highly variable regions that can provide species-specific sequences (Clarridge 2004).

Electrophoresis

After amplification, 5 µL of all PCR products and 5µL of a 100 bp ladder were run on a 1% agarose gel made with TBE buffer. Small gels were run at 124 V while large gels were run at 170 V. Gels were visualized by ethidium bromide under UV light in a Biorad UV Trans-illuminator to determine band intensity and size.

Amplification of microorganisms in Soil DNA Extract 1

The first soil DNA extract using the SoilMaster™ kit (soil from Calder Center) was included in a PCR reaction with two primer sets: ITS1-F/ITS4 and 27F/338R. DNA extracted from a crimini mushroom (using the DNeasy Blood and Tissue Extraction Kit by QIAGEN according to the manufacturer's instructions) and *E. coli* genomic DNA were used as positive controls for the fungus and prokaryote primers. Each primer set had different dilutions of template and different dilutions of positive controls. Negative controls of water were included for each primer set. PCR was performed according to the cycles described above, and the products were separated by electrophoresis and visualized (Fig.2).

Amplification of microorganisms in Soil DNA Extract 2

The second soil DNA extract using the SoilMaster™ kit (soil from the NYBG) was tested for the presence of PCR inhibitors. Using the ITS1-F/ITS4 primer set, PCR reactions were set up with varying amounts of positive control diluted in either soil extract or in TE buffer (Fig.3). After the second soil DNA extract was cleaned up using the PowerClean® Soil DNA Clean-Up Kit, PCR was run with all four primer sets on different dilutions of the template and different dilutions of positive controls (Fig.4). There was no positive control for the AML1/AML2 primers. From the PCR reactions that were initiated with 9.2μL and 1.0μL of cleaned-up template with the ITS1-F/ITS4, AML1/AML2, and 27F/338R primer sets, 1 μL of PCR product was amplified using the same primer sets (Fig.5). Negative controls of dH_2O were included for every primer set in every PCR reaction. PCR was performed according to the cycles described above, and the products were separated by electrophoresis and visualized.

PCR Product Purification and Sequencing

From the PCR reaction that was initiated with 1.0μL of template with the ITS1-F/ITS4-B primer set, 10 μL of PCR product was purified using the Qiaquik® PCR Purification Kit by Qiagen according to the manufacturer's protocol. The purified PCR product was quantified by measuring the OD at 260, and 20 ng of product was sent to Genewiz for sequencing. The amplified positive controls were also purified and sent for sequencing. PCR products were sequenced in the forward and reverse directions using the Roche Adaptors A and B.

Amplification of microorganisms in Soil DNA Extract 3

The soil sample from NYBG was processed again, this time using the PowerSoil® DNA Isolation Kit according to the manufacturer's protocol. The concentration of DNA was

measured by reading the OD at 260. PCR was run with all four primer sets with varying amounts of template, along with positive and negative controls. PCR was performed according to the cycles described above for each primer set, and the products were separated by electrophoresis and visualized (Fig. 6). The PCR product that produced the strongest band for each primer set was purified and sent to Genewiz for sequencing.

Ligation and Transformation

The PCR product that produced the strongest band overall, the ITS1-F/ITS4-B primers with 9.2 µL of PowerSoil® template, was PCR purified and ligated into pGEM-T Easy Vector in a 3:1 (insert:vector) molar ratio. Four separate ligation reactions using the same PCR product were performed. The ligation reactions were incubated overnight at 4˚C before transformation. A volume of 38µL of JM109 High Efficiency Competent Cells was transformed with 2µL of each ligation reaction. Between incubations on ice, the cells were heat-shocked at 42˚C for 45 seconds to accept the vector. A volume of 460µL of LB medium was added to each of the four tubes of cells, and the solutions were shaken at 37˚C at a speed of 200 rpm for 1 hour. After shaking, 50µL of each cell solution was streaked onto three LB-Agar plates. The cells were grown up overnight in a 37˚C incubator. The next day, 25 white colonies from each transformation reaction were selected and transferred to a gridded LB-Agar plate. The presence of the insert was confirmed by insertional inactivation and Miniprep. After growing the cells overnight, the two gridded plates, each holding 50 distinct white colonies, were sent to Genewiz, Inc. for sequencing. The clones were sequenced in one direction from primer T7.

Interpreting sequencing results

The sequence results for the 91 clones that produced a sequence were aligned using EBI's Clustal W2 program. Those sequences that had 95% homology or greater were considered to be a single organism. These organisms were identified by looking for matches to the middle 480 bases of each sequence in the nucleotide collection database on GenBank via NCBI's BLAST.

Comparing extraction efficiencies of two chemical extraction kits

The cleaned-up extracts of both soil DNA extraction kits were amplified with PCR in the presence of α-^{33}P-dATP using the ITS1-F/ITS4-B primer set. These PCR reactions were run side by side on a urea poly-acrylimide sequencing gel. The sequencing gel was exposed on photographic film for two hours (Fig.7).

RESULTS

Extraction and amplification

The first PCR reaction successfully amplified the positive controls, but not the SoilMaster™ soil DNA extract (Fig. 2). The positive control result confirms that two primer sets are effective at amplifying DNA of the expected size. There are two possible explanations for the failure of the primers to amplify DNA in the soil DNA extract: either the extract contains no DNA, or the extract contains a contaminant that inhibits PCR.

The second PCR reaction was a test to see if the DNA extract contained PCR inhibitors (Fig.3). TE buffer was used for comparison to the soil DNA extract, because the extract is also diluted in TE buffer. Even though the same amount of positive control template DNA was amplified when diluted in TE buffer, no positive control template was amplified in any dilution in the soil DNA extract. These results

demonstrate clear inhibition of PCR in the reactions containing the soil DNA extract, but it does not confirm the presence of DNA in the extract.

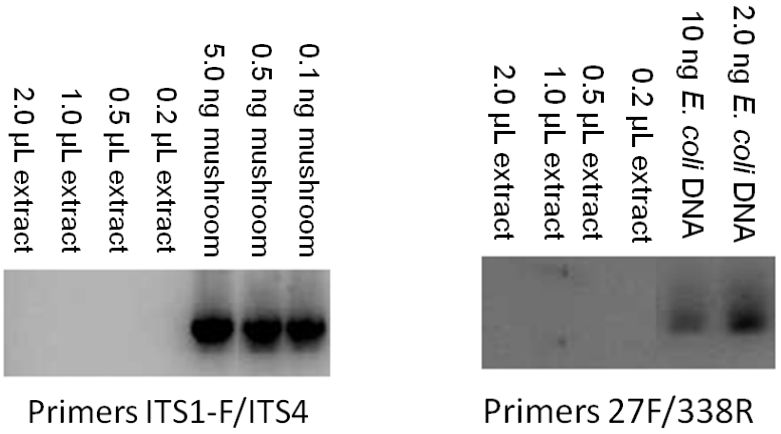

Figure 2. PCR results from Soil DNA Extract 1 without an overnight ligation using Primers ITS1-F/ITS4 and 27F/338R. The wells on the left side of each gel contain varying dilutions of the extract, while the wells on the right side of each gel contain varying dilutions of the appropriate positive control.

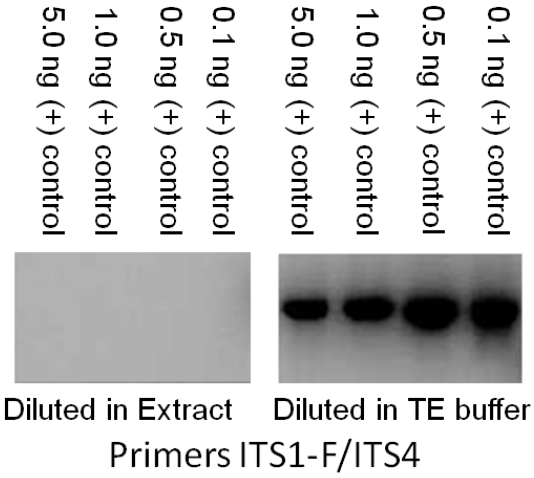

Figure 3. PCR results from Soil DNA Extract 2 with an overnight ligation using Primers ITS1-F/ITS4. In this reaction, the positive control was used as template in all wells. The positive control was run in varying dilutions as indicated by the amounts above each well, in either Soil DNA Extract 2 or in TE buffer.

The PowerClean® DNA Clean-Up Kit procedure was applied to the same soil DNA extract as above. The PCR reactions containing 9.2µL of extract failed to amplify with all primer sets, but some of the more dilute PCR reactions were successfully amplified (Fig.4).

These results suggest that even after the clean-up procedure, PCR inhibitors remain in the extract. However, they can be diluted down to a level where PCR can function as normal. This dilution effect is working in tandem with the fact that there is an optimal amount of DNA template for amplification. There may be little remaining inhibitor at the smallest volumes of extract, but there is also not enough DNA to produce a strong band. Still, the AML1/AML2 primers did not produce any product. The reason could be that the amount of Glomeromycota DNA in the soil sample is too low to be amplified by PCR, or the amount of DNA in the extract is too low to overcome the remaining PCR inhibitors. Due to the fact that there is no positive control for the AML1/AML2 primers, this result could also be a failure of the primers.

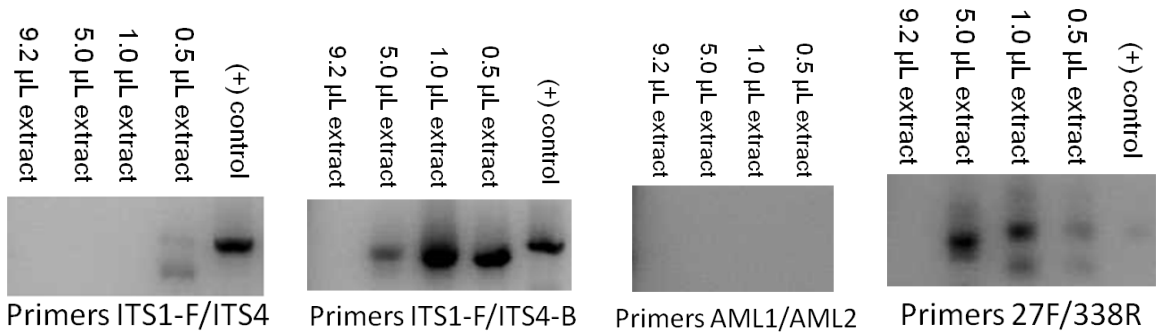

Figure 4. PCR results from Soil DNA Extract 2 after clean-up procedure using all four primer sets. PCR was performed on the cleaned-up extract in various dilutions as indicated by the volume above each well. The appropriate positive controls were also amplified.

A volume of 1µL of PCR reaction from those reactions initiated with 9.2µL and 1.0µL of cleaned-up extract was re-amplified (Fig.5). The results show that PCR completely failed in the previous 9.2µL reaction. However, in the previous 1µL reaction for the ITS1-F/ITS4 and 27F/338R primer sets, a small amount of DNA was amplified, which could be amplified again to an amount large enough to produce strong bands when the inhibitor is diluted out even further. This result is surprising for the ITS1-F/ITS4 primer set, because originally the 1µL reaction produced no band at all (Fig.4). In the re-amplification, the 1µL

reaction not only demonstrated the presence of product, it also clearly shows the presence of two pieces of different size. The two bands likely represent two different organisms. Again, the AML1/AML2 primers did not amplify any DNA.

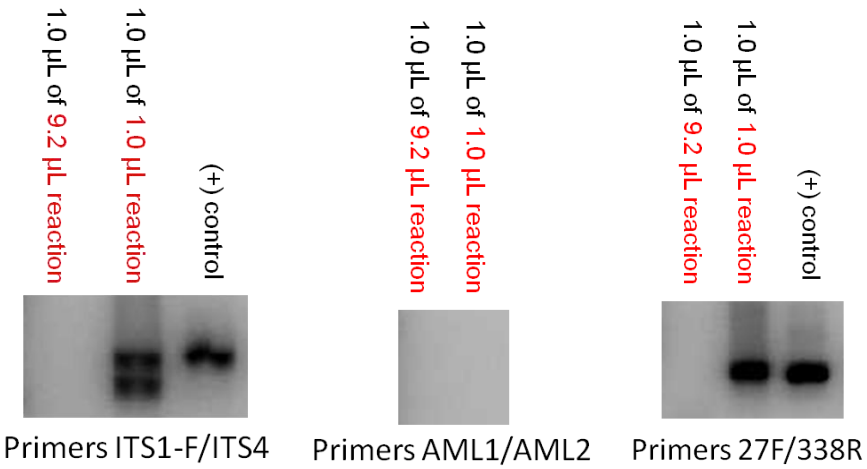

Figure 5. PCR results of the amplified PCR product from cleaned-up soil DNA extract 2. PCR was not able to amplify any DNA from the reaction initiated with 9.2µL of extract. These results demonstrate that even after clean-up, there remains enough inhibitor in 9.2µL of extract to completely inhibit PCR. The results of the re-amplification of the reaction initiated with 1.0µL shows that PCR was not inhibited completely in the previous reaction. It was still able to produce a small amount amplified DNA, which became a strong band in this reaction when the inhibitors were diluted out even further.

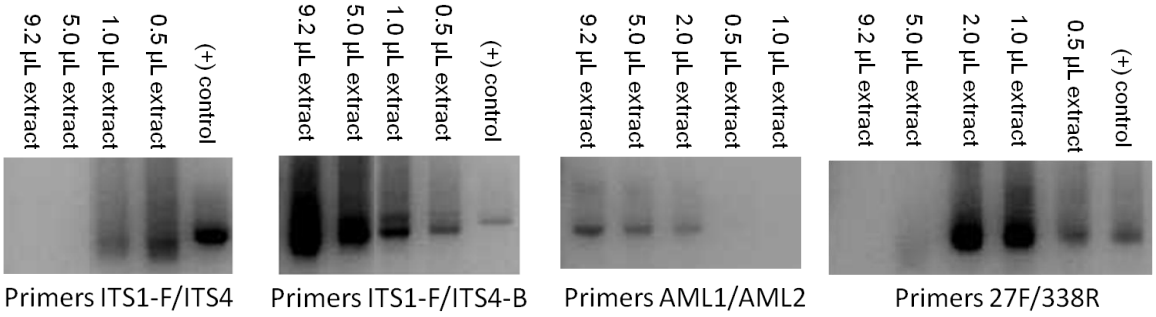

Figure 6. PCR results from DNA extracted from soil using the PowerSoil® kit. DNA was successfully amplified with all four primer sets. These results finally confirm the presence of Glomeromycota DNA in the soil sample and also confirm the effectiveness of primer set AML1/AML2.

DNA from the same soil sample was extracted with the PowerSoil® kit. Even without a clean-up step, the DNA in the soil was successfully amplified by all four

primer sets. PowerSoil® is either more effective at extracting DNA from cells, or it more effective at removing potential PCR inhibitors from the extract, or both.

Sequencing Results

Purified PCR products from each primer set and both positive controls were sent to Genewiz for sequencing. The sequences for the single-organism samples produced clear, readable trace files (Fig.7). Each peak is distinct, following the dominant sequence in the PCR product. The PCR products for both positive controls were a perfect match to the correct organism in the Nucleotide Collection Database on Genbank (via BLAST). However, the sequences of the PCR products from the soil extract were completely unreadable, with short peaks obscured by overlapping peaks.

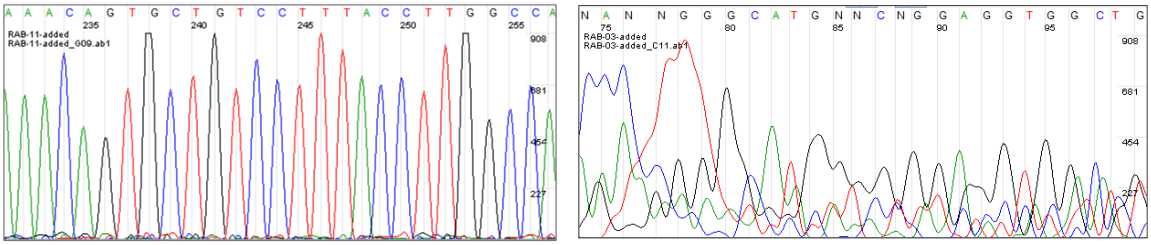

Figure 7. Left, the trace file following the sequence of the positive control, *Agaricus bosporus*. A clear sequence produces a single peak for one base. Right, the trace file following the sequence of the PCR product of soil DNA extract amplified by primer set ITS1-F/ITS4-B. Because of the many overlapping peaks , this file cannot be read as a single sequence, rather it is a collection of different sequences.

Individual sequence results were obtained from each clone in the DNA library. From the one hundred clones of the ITS1-F/ITS4-B PCR product, 91 had readable sequencing results. Eleven organisms represented 83% of Basidiomycota richness in the sample from the New York Botanical Garden (Fig.8). Three of those organisms could be identified to the species level. Five organisms were identified to genus level. Two distinct organisms were attributed to the same genus, but could not be identified to species. Two organisms, each comprising 2.1% of the basidiomycete community, could only be identified to the order

Trechisporales and the class Agaricomyetes. One organism, making up 5.4% of the basidiomycete community could not be identified using NCBI's BLAST.

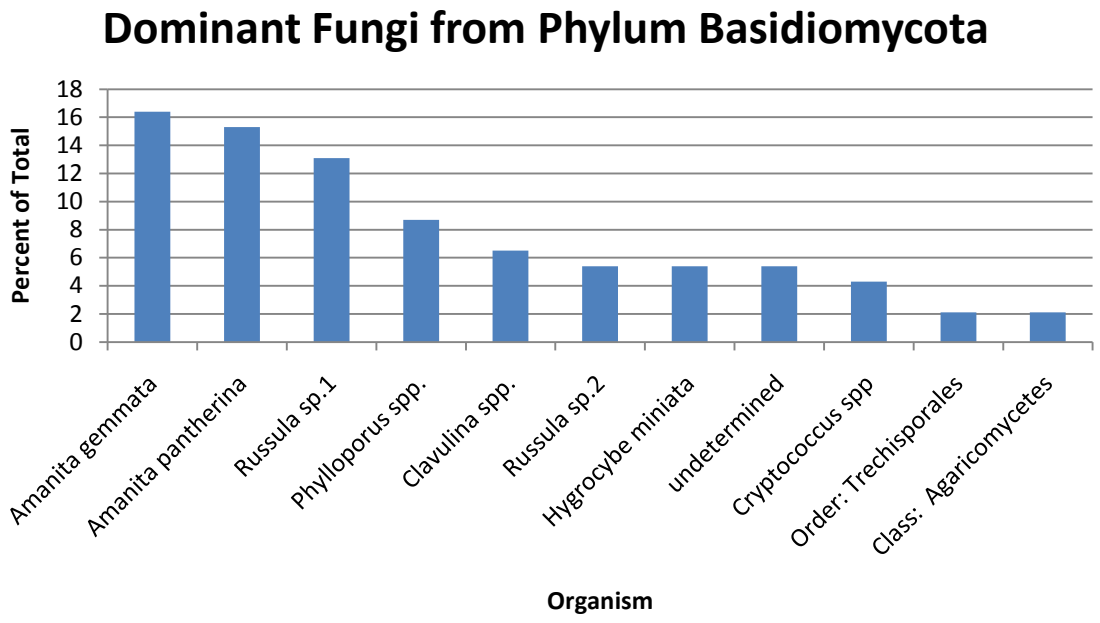

Figure 8. The dominant basidiomycetes observed identified from genomic DNA extracted from a soil sample from the New York Botanical Garden. Eleven distinct organisms comprise 83% of the soil microbial community.

Sequencing gel results

The urea poly-acrylimide gel separates DNA fragments that differ in size by one base. In this case, each band is a unique length of the amplified rRNA gene region, indicating a unique organism from the phylum Basidiomycota. If the two kits are equally efficient at extracting DNA from soil, we would expect them to capture the same organisms from the same soil sample – the banding patterns would be the same. While some of the bands are the same for both soil DNA extraction kits, there are many obvious differences (Fig.9). There are at least three bands that were resolved from the PowerSoil® extract that are either less intense or missing completely from the SoilMaster™ extract. However, the PowerSoil® kit does not produce all the bands that the SoilMaster™ kit can produce. There are two single

bands from the Soil Master™ extract that are either completely missing or very faint in the PowerSoil® extract. Additionally, there is a distinct double band in the SoilMaster™ extract that could not be resolved in the PowerSoil® extract.

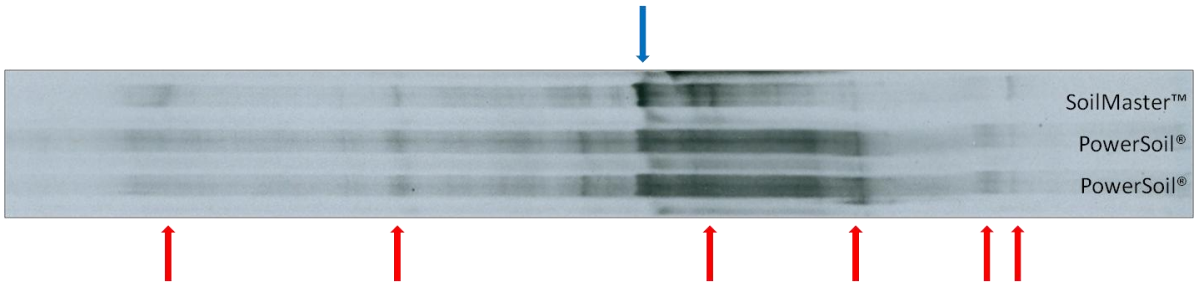

Figure 9. Sequencing gel containing PCR products from the SoilMaster™ kit and the PowerSoil® kit (in duplicate). Each band is a different sized piece of the rRNA gene region that was amplified by the primer set. Each band represents a unique organism. The blue arrow indicates a band that is the same for both kits. The red arrows indicate differences in the banding pattern between the two kits.

DISCUSSION

Understanding the composition of the soil microbial community can give us remarkable insight into the health and functioning of ecosystems. Unfortunately the complexity of the microbial community and the dearth of information to characterize individual species have hindered progress in this field. Molecular techniques offer a more powerful approach to study microbial diversity because they allow us to assess the entire soil metagenome rather than isolating individual species. However, soil introduces unique challenges to molecular research that must be overcome before we can access the vast amount of information that it contains.

Soils contain humic acids which are known PCR inhibitors (Wilson 1997). This study demonstrated that soil DNA extraction kits, particularly the SoilMaster™ kit, may leave behind humic acids or other inhibitors in the extraction process. These inhibitors were so potent that they effectively inhibited PCR at concentrations as low as 1% (0.2µL in a 20µL PCR reaction). Even after applying a clean-up methodology to the extract, PCR was still

inhibited at higher concentrations of template. In most cases this problem was solved by re-amplifying the PCR reaction, which increased the concentration of DNA while decreasing the concentration of inhibitor. For one primer set, which seemed to target a very small proportion of the total soil DNA, the only way to amplify the DNA was to use a different kit. The presence of PCR inhibitors in soil DNA extract present a serious problem that can obscure results. In the case of this study, PCR could not confirm the presence of Glomeromycota DNA, because the low concentration of DNA could not overcome the inhibitors. PCR inhibitors must be removed from soil DNA extracts to give an accurate picture of microbial diversity; otherwise PCR results may indicate that low density organisms are absent from the sample.

The DNA amplified from soil samples is difficult to sequence because it represents pieces of a metagenome. A single PCR product actually contains a variety of unique DNA sequences originating from an unknown diversity of organisms. Traditional Sanger DNA sequencing can identify one base at a time, and it is useful for identifying homogenous PCR products, such as the positive control *Agaricus bosporus*. When soil PCR products are sequenced by the Sanger method, multiple bases are detected at every step, which prevents the identification of one readable sequence. To overcome this problem, the individual pieces need to be separated from one another by ligation and transformation into cells, creating a DNA library. Each clone contains one sequence from the soil metagenome. These clones can be sequenced individually, providing a single, accurate read for every DNA piece that was amplified in the PCR reaction. When this procedure was utilized, more than eleven unique organisms were identified. Their relative abundances could be determined by the number of clones that contained that particular sequence.

Soil DNA extraction kits also vary in their extraction efficiency. These kits utilize different chemical means to lyse cells and extract the genomic DNA. To assess the differences in extraction efficiency between the SoilMaster™ and PowerSoil® kits, the kits were used to extract DNA from the same soil sample, and these extracts were amplified in the presence of radioactivity. The banding pattern of each kit, revealed on a sequencing gel, indicates the diversity of microorganisms that each kit is able to extract. The banding patterns were distinctly different. If this variability in extraction efficiency affects all kits, chemical DNA extraction alone is inadequate. There needs to be an additional step of physical extraction to ensure that all the cells are lysed. Using a chemical extraction kit alone introduces bias into research by selecting for a particular set of organisms.

CONCLUSION

Molecular techniques have allowed soil biologists to make stunning advances in their understanding of microbial diversity. However, these techniques are not perfect and this approach is still relatively new. As a result, the use of microbial techniques can sometimes produce flaws and introduce bias into a study. Still, the results show great promise. It is worthwhile to continue fine-tuning the molecular techniques we have at our disposal until we develop an effective methodology for characterizing soil microbial communities.

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