THE USE OF DENATURING GRADIENT GEL ELECTROPHORESIS IN EXAMINING THE SPECIES-SPECIFIC INFLUENCE OF ECTOMYCORRHIZAL FUNGI ON SELECTIVE BACTERIAL ENRICHMENT IN THE MYCORRHIZOSPHERE OF *Pinius rigida* GROWN IN A NATURAL PINE BARRENS HABITAT

by

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ABSTRACT OF THE THESIS

THE USE OF DENATURING GRADIENT GEL ELECTROPHORESIS IN EXAMINING THE SPECIES-SPECIFIC INFLUENCE OF ECTOMYCORRHIZAL FUNGI ON SELECTIVE BACTERIAL ENRICHMENT IN THE MYCORRHIZOSPHERE OF *Pinius rigida* GROWN IN A NATURAL PINE BARRENS HABITAT

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The study presented here describes the results of denaturing gradient gel electrophoresis (DGGE) used to detect bacterial diversity and selection in the ectomycorrhizasphere of *Pinius rigida* growing in a natural pine barrens habitat This study was the first to explore such a relationship in an environmental setting. Two study sites were chosen from the pine barrens in southern New Jersey, one from Rutgers Pinelands Field Station and one from Double Trouble State Park. Soils were from similar habitats and were similar in total phosphorous. The two sites were selected based on preliminary information that mercury content was high in the Double Trouble. However, upon further analysis, results showed that mercury was at a non-contaminant level in both sites. DGGE analysis of ectomycorrhiza fungi present on a root tip revealed that there were multiple species occurring where one morphotype was apparent from inspection through a dissecting microscope. DGGE analysis of ectomycorrhiza associated bacteria revealed a highly diverse community with little enrichment. While

there was some enrichment noted on the bacterial DGGE profiles, this did not correspond to a species-specific link between ectomycorrhiza fungi and their associated bacteria. It is the conclusion of this study that the natural ectomycorrhizasphere is a highly complex environment, and that this complexity diffuses the influence of any one ectomycorrhizal species on the enrichement on bacteria present in the ectomycorrhizasphere.

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DEDICATION

I dedicate my work to my mother, Martha Jane Ward, as it is her spirit that has guided me through trials which I did not know I could pass. Her memory has allowed me to see sunshine even in the grayest day, and to sing when I only thought of weeping.

To my father, Dr. Elliott Ward, for being an inspiration throughout my life. I thank him for searching for insects with me, catching lizards, and for showing me the wonder of the world.

To my husband, Dane Adam Orion Vick, who has proven stronger and more loving than I could have ever imagined. I thank him for this and for everything that he is, as it is he who keeps me steady in such a turbulent world.

Table of Contents

Page

List of Tables

List of Figures

1. Introduction

Mycorrhizal associations, the symbiotic relation between mycorrhizal plants and fungi, occur in virtually every ecosystem (Smith 1997). They are present in boreal, tropical, and temperate forests, mycorrhizal plants becoming colonized within days of emergence in the upper soil profiles. Ectomycorrhizal fungi are not only a relationship between one plant and its fungal symbionts, but ectomycorrhizal fungi can form a link from one tree to another. This, in essence, can link together a much larger community of plants (Martin et al 2007). Ectomycorrhizae are characterized by a mantle formed from fungal hyphae that encase the plant root and hyphal penetration between cortical cells to form a Hartig net. Fungal hyphae extend into the soil to procure nutrients for the plant host, virtually acting as extensions of the plant roots. Plants, in turn, supply the fungi with photoassimilites. In temperate forests, there is a broad range of ectomycorrhizal species that associate with a limited number of tree species. The number of ectomycorrhizal species present in a single stand of trees can reach into the hundreds (Buee et al. 2007)

The mycorrhizal ability to mobilize elements such as nitrogen, phosphorous, and carbon into a bioavailable form allows mycorrhizae to function as key players in global nutrient cycling. Extraction of nitrogen from ammonium, nitrate, amino acids, peptides and protein sources from the soil is common for both assimilation into their own biomass and for nutrient exchange to the host plant. Indeed, the symbiotic relationship contributes much of the nitrogen acquisition for plant hosts

(Brandes, 1998). Mycorrhizas likewise help the uptake of phosphorous, especially in soils where this nutrient is limited (Read 1996). The soil immediately surrounding mycorrhizal hyphae is rich in root exudates, providing a source of easily absorbed, low molecular weight carbon to microbial colonizers (Gadd 2004).

Although there are similarities between the functions of the symbiosis, overall function of individual mycorrhizae associations varies from fungal species to species. The amount or type of root exudates secreted from the host plant will vary according to the mycorrhizal fungi present (Sun 1992). This differential enrichment causes chemical alterations in the immediate microenvironment surrounding an ectomycorrhizal root tip (Frey-Klet 2005). The fungi themselves can also produce varying levels of organic acids or other compounds, producing differential antibacterial properties among fungal species. (Olssen et al. 1996). These and other capabilities are dependent upon the species of mycorrhiza present, and the variety of functional traits could have a significant effect on the immediate soil environment (Garbaye et al. 1994, Olssen et al. 1996, Paulitz et al. 1989).

There is evidence that ectomycorrhizas have an intimate association with bacterial colonizers in the mycorrhizosphere in the environment. The rhizosphere prokaryotic population is influenced by the changes in the microenvironment caused by ectomycorrhizal fungi, and in turn has profound effects on the

establishment, growth, and overall health of mycorrhizae. Not surprisingly, the number of bacterial colonizers which can be supported by a mycorrhiza is specific to fungal species (Paulitz et al. 1989), determined by such variables as hyphae surface area, root exudate production and chemical composition, and other species specific influences. In culture studies, the species composition of mycorrhiza colonizing bacteria vary among fungal species (Poole 2001, Mogge 2000), most likely due to specific modification of rhizospheric environment. In a reciprocal manner, evidence demonstrating bacterial selective inhibition or growth promotion of mycorrhiza is becoming increasingly common. Bacteria are able to selectively inhibit formation of certain ectomycorrhiza through the production of siderophores or anti-fungal compounds which favor one species over another (Paulitz et al. 1989). A general group of mycorrhiza helper bacteria has been described as those which promote the establishment of a preferential mycorrhiza (Duponnois et al. 1993, Garbaye at al. 1994). This group includes a wide range of bacterial genera including the fluorescent and non-fluorescent *Pseudomonads*, *Bacillus sp*. and *Paenibacillus*, and it is probable that this list will grow with further studies of the interactions of beneficial bacteria and mycorrhiza. If bacterial selectivity is present, it may enable the rhizospheric bacterial community to promote growth of fungal species favorable to their own growth. These interactions may lead to a feedback loop, with bacteria promoting growth of a preferred mycorrhiza species, and the mycorrhiza thereby enriching the growth of its beneficial bacteria.

The symbiosis between ectomycorrhiza and ectomycorrhiza colonizing bacteria could lead to increased plant growth and health, more so than the presence of mycorrhiza alone. As in other systems where bacteria are found to work in conjunction with mycorrhiza, the beneficial effects produced likely depend on the species specific abilities of the ectomycorrhiza and bacteria working together. However, the degree of selection in the natural environment is unclear. While culture studies point to some degree of enrichment of bacterial populations, the degree of enrichment could become masked by other processes in a natural environment. Other competing factors, such as soil microfauna, nutrient availability, or habitat disturbance, could have equal or greater effects on the enrichment of bacterial species, despite the influence that fungi have on the immediate soil environment.

The goal of the study was to elucidate any species specific link between the fungal hosts and the enrichment of bacterial colonizers within a natural habitat. This study was novel in its exploration of the selective environment of the ectomycorrhizosphere in a natural system. I hypothesized that the fungal hosts would cause a selective environment for ectomycorrhizal associated bacteria. I utilized denaturing gradient gel elecrophoresis (DGGE) to obtain visual profiles of both ectomycorrhizal fungi and the bacteria associated with ectomychorrhizal fungi from within two pine barrens habitats. These profiles were analyzed through principle component analysis to determine if there was any statistically significant link between the symbionts in a natural setting. Species richness of bacterial

populations was also analysed. This was done so as to determine if the species richness of the bacterial community was influenced the presences of specific ectomycorrhiza present.

2. Materials and Methods

2.1 Study sites

Two sampling sites were selected. The first site is within the forest adjacent to Rutgers Pinelands Field Station in New Lisbon, New Jersey. The second site is located approximately 30 miles east of the Rutgers Field Station in Double Trouble State Park. Both sites are characterized by the pine barrens ecosystem, with sandy soil and a thin organic horizon. The dominant vegetation consists of pitch pine, oak, huckleberry and blueberry. The Double Trouble site was initially chosen because preliminary results suggested a high mercury concentration (data not shown). However, upon further analysis, it was determined that mercury was below contamination levels in both of the sites.

2.2 Sample Collection

Samples from both sites were collected during the winter season: the Field station site was collected in December 2005 while samples from Double Trouble State Park were collected in January 2006.

Within both sites, a total of 18 samples were taken, or 9 soil samples from each

site. These samples consisted of the forest organic horizon, the first few centimeters of the soil profile composed of a humus layer. Within a site, all samples were located in close proximity to a trunk base of *Pinius rigida*. Each of the 9 samples were taken from points located at least 3 meters apart.

Samples of the organic soil horizon were collected by hand and trowel and stored in a sealed plastic bag. Samples were then stored at -20C until ready for further use.

2.3 Soil Characterization

In order to better compare the soil microbial environments, characterization of the soil physical and chemical characteristic was necessary. Characterization included determination of soil water content, organic matter content, percent carbon, percent nitrogen, and concentration of phosphorous. Prior to all analyses, soil was sieved with a 2mm seive to remove root biomass and large particulate matter.

2.3.1 Soil Water Content

A gravimetric analysis was undertaken to determine the percent soil moisture. This method determines the difference between soil wet weight and dry wieght. First, soil was placed in an aluminum pan to determine the wet weight. Each soil

sample was placed in an oven at 100°C and left to dry for 2 days. After soil was dried, the weight of the dry soil was recorded. The percent soil moisture was calculated by determining the difference between the wet weight and the dry weight, dividing the difference by the wet weight, and then multiplying this number by 100, or with the equation [(wet wt-dry wt)/wet wt]x100. The analysis was in triplicate for each site.

2.3.2 Soil Organic Content

The Loss on Ignition (LOI) method for determining soil organic content was utilized and performed in triplicate. Dry soil samples were weighed into crucibles and heated at 550°C for 5 hours. All crucibles were handled with metal tongs or gloved hands to reduce error introduced by contaminating organic material from hands or other material. After the soils had undergone combustion, the crucibles were removed from the oven, cooled, and re-weighed to obtain the ashed weight of the soil. Loss on ignition is expressed as a percentage and calculated by (dry wt- ashed wt)/ dry wt x 100.

2.3.3. Total Soil Nutrients

Soils were oven dried at 100°C prior to analysis for total nutrients. Following high temperature combustion, the dried soil samples were analyzed for total carbon by infrared CO_2 detection. Total nitrogen was measured by N_2 thermal

conductivity detection. Both were analyzed using a Leco TruSpec carbon/nitrogen analyzer (Leco Corp. St.Joseph MI).

A colorimetric analysis was utilized to assay total phosphorous. To assay for total phosphourous, samples were digested in a sulfuric acid-hydrogen peroxide solution, using a Tecator block digestor and Johns reagent according to the methods of Allen (1989). Phosphorous in the digested samples was measured colorimetrically, measuring light absorbance at 420 nm. The samples were calibrated against known standard concentrations of phosphate and subsequently analyzed colorimetrically according to Standard Methods protocols (American Public Health Association, 1998).

2.4. Ectomycorrhizal root tip isolation

Frozen soil samples were taken from -20°C and allowed to thaw completely before inspection. Once thawed, each sample was separated and segments of root were randomly chosen for visual inspection for ectomycorrhizal root tips. Sections of root were placed on a petri dish and viewed with a dissecting microscope using aseptic technique. Root tips were gently shaken to remove loose soil, thus maintaining rhizoplane integrity by not washing the root tips. Ectomycorrhizal root tips were selected based on morphotype, and an attempt was made to gather as many multiple unique morphotypes as could be collected cleanly. It is important to note that thawed morphotypes and fresh morphotypes

were compared. Freezing did not compromise the integrity of the appearance of a morphotype. When collecting, a single root tip was often deemed too small to yield sufficient extracted DNA, and more than one root tip was combined if the root tips were located along the same root (in close proximity) and of the same morphotypes. Each root tip composite was placed in either a sterile petri dish or sterile microcentrifuge tube and stored at -20°C. A total of 69 samples were taken: 37 samples were taken from the field station site and 32 were taken from the Double Trouble site.

2.5 DNA extraction

Total genomic DNA was extracted from all ECM root tips and using the Powersoil DNA kit (Mo Bio Laboratories, Inc, Carlsbad,CA) according to manufacturer's protocol.

Ectomycorrhizal root tips were removed from -20°C and placed in the Mo Bio beadbeating tube using sterile technique. The tube was vortexed to mix. Next, 60 µl of the manufacturer's solution C1 was added to the tube and briefly mixed. Tubes were then affixed to a flat-bed vortexer and vortexed at maximum speed for 10 minutes. After vortexing, the tubes were centrifuged at 10,000g for 30 seconds. 400 to 500 µl of supernatant was then transferred to a clean microcentrifuge tube. 250 µl of Solution C2 was added to the tube, which was then vortexed for 5 seconds. The tube was then incubated on ice for 5 minutes.

After being chilled, the tube was again centrifuged at 10,000g for one minute and a pellet was formed. Avoiding the pellet, up to 600 µl of the supernatant was transferred to a clean microcentrifuge tube. 200 µl of solution C3 was added and the tube was briefly vortexed. The tube was then placed on ice for 5 minutes. After this incubation, tubes were centrifuged for 1 minute at 10,000x g. Again avoiding the pellet formed during centrifugation, up to 750 µl of supernatant was transferred to a clean microcentrifuge tube. 1200 µl of solution C4 was added to the supernatant and the tube was vortexed for 5 seconds. Then, about 675 µl of the supernatant was added onto a spin filter and was centrifuged at 10,000x g for 1 minute. The flow through liquid was discarded and another 675 µl of the supernatant was placed onto the spinfilter, centrifuged for 1 minute, and flow through was discarded. Finally, any remaining supernatant was placed in the spinfilter and centrifuged at 10,000x g for one minute. The final flow through was then discarded. 500 µl of solution C5 was added and centrifuged for 30 seconds at 10,000 x g. The flowthrough was discarded and the tube was again centrifuged for 1 minute. After centrifugation, the spin filter was placed in a clean centrifuge tube. 100 µl of solution C6 was added to the center of the filter membrane and centrifuged for 30 seconds. The spin filter was removed from the tube and discarded.

Samples from the Field Station site were first labeled with a number to denote the site within the site from which it was taken. This number was followed by a letter to indicate separate ECM root tip isolations. Samples from Double Trouble were

first labeled with a number to denote the site within the site from which it was taken. This number was followed by another number to indicate separate ECM root tip isolations.

After extraction, the supernatant DNA was run on an agarose gel to visualize the extracted genomic DNA in order to determine success of the extraction. All samples were then placed at -20°C.

2.6 Casting an agarose gel

All agarose gels were prepared by adding an appropriate percent weight of agarose to volume of 1X TAE (Tris Acetic Acid) dependant upon the length of the product being visualized on the gel. The 1xTAE used for gel preparation is made from a 50X stock solution. This solution is prepared using 242g of Tris base, 57.1ml of Glacial Acetic acid and 18.6g of EDTA in a volume of 1L. After adding the desired amount of agarose to 1XTAE, the agarose was dissolved by heating. The dissolved solution was allowed to cool slightly before a 10mg/ml Ethidium Bromide solution was added. The volume of Ethidium bromide varied per gel, utilizing the standard that 5 µl of 10mg/ml concentration of ethidium bromide was to be added per every 100ml agarose/1XTAE solution. The mixture was then poured into a gel casting tray. Immediately, a gel comb was immersed to allow formation of wells and the gel was allowed to solidify. After solidification, the gel was removed from the casting stand and placed in an elecrophoresis chamber

filled with 1X TAE running buffer and the gel was loaded with samples. An electric current was passed through the chamber, causing migration of bands down the gel. After migration, all gels were visualzed using UV transilluminator and an electronic picture was taken.

2.7 PCR

The PCR reactions were initially carried out with all 69 samples to determine which ones would have most successful product. The sample size was reduced by a process of elimination as only those samples with a good product yield could be used for further analysis. A sample needed to have strong, clean band as seen on agarose gels from both steps of the nested PCR to be used for DGGE. PCR primers are listed in Table 2.1.

Nested PCR was used to increase the specificity and the quantity of PCR products. Nested PCR is the use of two primer pairs in conjunction with one another to obtain the desired DNA sequence. The first primer pair amplifies a longer DNA sequence, and the second primer pair utilizes priming sites within the first amplicon to obtain a shorter sequence. Nested PCR allows sufficient amplification of difficult products, or , in the case of the ectomycorrhizal fungal primer pairs in this study, to increase specificity of the target DNA. Figure 2.1 gives a schematic of nested PCR that was used to obtain the DGGE products.

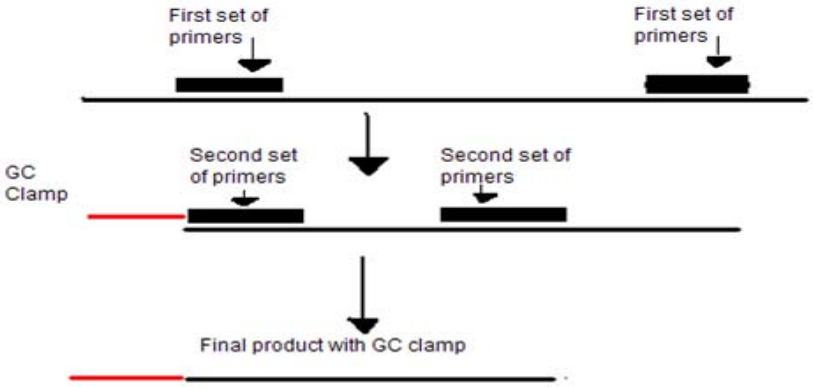


Figure 2.1. Nested PCR

Table 2.1. PCR Primers

Bacterial primers

Primer	Sequence	Target	Source
27f	5'-AGAGTTTGATCMTGGCTCAG-3'	Universal	Lane (1991)
548R	5'-ATTACCGCGGCTGCTGG-3'	Universal	Muyzer (1996)
341F (GC)	5'-CGCCCGCCGCGCCCCGCGCCCGTCCCGCCGCCCCCGCCCTCCTACGGGAGGCAGCAG-3'	Bacteria specific	Muyzer (1996)

Ectomycorrhizal primers

Primer	Sequence	Target	Source
ITS1	5'-TCCGTAGGTGAACCTGCGG-3'	ITS region	White et al (1990)
LR21	5'-ACTTCAAGCGTTTCCCTTT-3'	28s rDNA	Hopple and Vigalys (1999)
ITS1(GC)	5'-CGCCCGCCGCGCCCGCGCCAGCCGCCGCGCCCGCCGCGTCCGTAGGTGAACCTGCGG-3'	ITS region	White et al (1990), modified by Ward
ITS4	5'-TCCTCCGCTTATTGATATGC-3'	ITS region	White et al (1990)

2.7.1 PCR Master Mix

PCR reactions were carried out to produce a final volume necessary for the experiment, the volume varying dependent upon use. For all reactions, reagents were calculated in relation to the relative volumes contained in a 20ul reaction. A 20 µl master mix contains 2ul of 10X PCR buffer, 1.2 ul of 25 mM MgCl2, 0.4 µl of 10µM dNTPs, 0.4 µl of 20 µM forward primer, 0.4 µl of 20 µM reverse primer, 0.1 µl of taq polymerase, an appropriate amount of template (dependent on which primer pairs being used), and water to the final 20 µl volume.
All PCR reactions were carried out on the iCycler thermocycler (BioRad Laboratiries, Los Angelos, CA)

2.7.2 Bacterial PCR

For all genomic DNA environmental samples, nested PCR approach was utilized to amplify the desired final bacterial PCR product.

2.7.2.1. First Step PCR

PCR was performed with 5 µl from the total genomic DNA from each environmental sample in a 20 µl reaction. The forward primer was 27f and the reverse primer was 548R (table 2.1). The product was about 500 bp long. After amplification, all products were checked on a 1.5% w/v agarose gel at 80v for 30

min.

The PCR protocol was:

cycle 1(1x) step 1: 95˚C for 5 min

cycle 2 (20x) step 1: 94 ˚C for 10 sec

step 2: 65 ˚C for 30 sec

-- decrease temp after cycle 2 by 1˚C every 2 cycles

step 3: 72 ˚C for 1 min

cycle 3 (5x) step 1: 94˚C for 10 sec

step 2: 55 ˚C for 30 sec

step 3: 72˚C for 1 min

cycle 4 (1x) 72˚C for 5 min

cycle 5 (1x) Hold at 4˚C

2.7.2.2. PCR product clean up

The PCR products from all successful first step reactions were cleaned using the Iso Pure PCR Purification Kit (Denville Scientific, Metuchen, NJ). PCR products were stored at -20°C.

2.7.2.3. Second step PCR

Second step PCR were carried out using 1 µl of successful first step reactions in a 50 µl reaction. The reverse primer was 548r. The forward primer was 341f with an attached GC clamp on the 5' end (table 2.1). The product of this reaction was about 200 bp long. The touchdown PCR protocol was:

cycle 1(1x) step 1: 94˚C for 5 min

cycle 2 (20x) step 1: 94 ˚C for 30 sec

step 2: 65 ˚C for 30 sec

-- decrease temp after cycle 2 by 1˚C every 2 cycles

step 3: 72 ˚C for 30 sec

cycle 3 (5x) step 1: 94˚C for 30 sec

step 2: 55 ˚C for 30 sec

step 3: 72˚C for 30 sec

cycle 4 (1x) 72˚C for 7 min

cycle 5 (1x) Hold at 4˚C

2.7.2.4. Gel purification of the target band

Products from the second step PCR reaction were run on 2% agarose gels. These gels were visualized with UV transilluminator tray. The target 200 bp PCR product was cut out of the gel using sterile spatulas. The agarose containing the band was placed in a sterile 1.5 ml microcentrifuge tube. The PCR product was then extracted from the agarose using QIAquick Gel extraction kit (Quiagen, Valencia, CA). The extracted product was stored at -20°C

2.7.3. Ectomycorrhiza Fungi PCR

A nested PCR approach was utilized to amplify the final desired fungal PCR product. Primers targeted the Internal Transcribed Spacer (ITS) region. This is a highly variable region of the rDNA often used to molecularly identify fungi.

First step PCR was carried out using 1ul of template DNA in 20 ul reactions. A dilution series of 1, 10^{-1} , 10^{-2} was used to determine the appropriate concentration of template DNA to use in each 20ul reaction.
The primers used were Lr21 and ITS1 (table 2.1). These primers amplify the whole ITS region and both conserved and variable regions, with a product of about 800-900 bp long. The PCR protocol was designed by Rosling et all (2003).

It was:

cycle 1(1x) step 1: 94˚C for 3 min

cycle 2 (35x) step 1: 94 ˚C for 30 sec

step 2: 50 ˚C for 45 sec

step 3: 72 ˚C for 60 sec

cycle 4 (1x) 72˚C for 7 min

cycle 5 (1x) Hold at 4˚C

Products from this reaction were run on a 1% agarose gel.

2.7.3.1. PCR product clean up

The PCR products from the most successful first step reactions from each dilution series were cleaned using the Iso Pure PCR Purification Kit. The purified products were stored at -20°C.

2.7.3.2. Second step PCR reaction

Second step PCR reactions were carried out with 1ul of the product from first step reactions in a 20 µl master mix. Primers used were ITS4 and ITS1+GC, targeting the ITS region. The product was about 600-700 bp long.

A modified version of Rosling's PCR protocol was used for the second step of the nested PCR reaction. This final protocol utilized a touchdown annealing step to reduce non-specific binding of the primers.

The final protocol was:

cycle 1(1x) step 1: 94˚C for 3 min

cycle 2 (20x) step 1:94 ˚C for 30 sec

step 2: 64 ˚C for 45 sec

-- decrease temp after cycle 2 by 1˚C every 2 cycles

step 3: 72 ˚C for 1 min

cycle 3 (10x) step 1: 94˚C for 30 sec

step 2: 54 ˚C for 45 sec

step 3: 72˚C for 1 min

cycle 4 (1x) 72˚C for 10 min

cycle 5 (1x) Hold at 4˚C

2.7.4 Design of GC clamp

The primer ITS1 required a GC clamp before it could be used in DGGE. I

designed the clamp to be 20-40 base pairs long, to eliminate any repeats of 4 guanines or cytosines in a row close to where the primer sequence began, and to have an adenosine or thymine to the midregion of the clamp. The modified primer was called ITS1(GC).

2.7.5 Purification of band and sequencing

To determine the accuracy of the modified primer pair in targeting and amplification of the desired ectomycorrhizal PCR product, a band was extracted from a fungal DGGE gel. The gel was visualized on a UV transilluminator and a band selected from one fungal profile. A portion of this band was removed using sterile pipette tips and placed in a PCR tube filled with sterile PCR water. The band was then allowed to soak overnight. Afterwards, the water with extracted PCR product was used to perform PCR and amplify the product. This product was run on a second DGGE gel to ensure that the band was pure. The extraction, soaking, and PCR was repeated. After amplification, the product was run on a 1.5% w/v agarose gel to check success of PCR. Afterwards, aliquots of PCR product were placed into two PCR tubes. In one tube was placed ITS1 without the GC clamp and into the other primer ITS4 was added to the mixture. The PCR product from the extracted band was then sequenced using the Genewiz service (New Brunswick, New Jersey). The sequence was then blasted to determine the closest related species.

2.8 Denaturing Gradient Gel Electrophoresis (DGGE)

DGGE was run using the DeCode system (Biorad laboratories) and was used to visualize the molecular profile for ectomycorrhiza and ectomycorrhiza colonizing bacteria from each genomic DNA sample. To perform DGGE a denaturing gradient gel was cast. This was accomplished by first assembling the gel casting system, which consisted of the glass plate sandwich, a peristaltic pump, and a gradient former. Next, the gradient solutions were prepared on ice to reduce the rate of solution polymerization. For each DGGE gel, there were two gradient solutions, one with a high concentration of urea/formamide as the denaturant, and one with a low concentration.The solutions to create the denaturant gel contained 15 ml 40% Acrylamide/Bis37.5:1, 2 ml 50X TAE buffer, and, for every 10% of denaturant concentration desired, 4.2 g Urea and 4.0 ml of Formamide was added. The volume was then adjusted to 100 ml with Milli-Q water. Both the high and the low concentration solutions contained 11ml of the appropriate concentration urea/formamide solution containing acrylamide, 50ul of 10% ammonium persulphate, and 10 ul TEMED. The high denaturant solution was poured into the outflow chamber of the gradient maker and the low denaturant solution was poured into the other chamber. A stir bar was placed into the outflow chamber of the gradient maker and a magnetic plate was used to stir. The peristaltic pump was used to pump the solutions from the gradient former to the glass plate sandwich. When the first drops of the higher concentration denaturant solution reached the bottom of the glass plate sandwich, the gradient channel

was opened to allow mixing of the two urea/formamide solutions. When the gradient former was empty and the acrylamide gel cast, butanol was pipetted onto the top of the gel and the gel was allowed to polymerize for about 1.5 hours. After polymerization, the alcohol layer was poured off and the top of the gel was rinsed with Mili-Q water.

Next, an acrylamide stacking gel was mixed. This was a 0% denaturant gel containing no urea or formamide. The stacking gel was made with 15 ml 40% Acrylamide/Bis37.5:1, 2 ml 50X TAE buffer, and 83 ml of Milli-Q water.To prepare the stacking gel, 11ml of acrylamide solution, 50ul of 10% ammonium persulphate, and 25 µl TEMED were mixed together. This solution was pipetted onto the gradient gel, a plastic comb was placed in the stacking gel to form wells, and it then was allowed to polymerize for at least 3 hours. After the gel was polymerized, the gel comb was removed and the wells were filled with about 10ul of 1XTAE. The gel sandwich was attached to the central core of the DeCode system, and the core was placed in the buffer tank which had been pre-heated to 60°C. The PCR product was loaded into the wells and the samples were run.

2.8.1 DGGE Run Conditions

Bacterial DGGE were run using a 20% to 80% denaturant gel for 5 hours at 150 volts. 15 ul of PCR product was loaded into each lane.

Fungal DGGE were run for 8 hours at 100 volts using a 20% to 60% denaturing gradient. 15 µl of the second PCR product were run for each sample.

It is important to note that multiple attempts were made to increase the resolution of the bacterial banding patterns. Different run times, denaturing gradient concentrations, voltages, and sample volumes were used. However, the banding patterns consistently showed patterns similar to those in figures 3.13 and 3.14.

All gels were stained for 30 minutes in Gelstar nucleic acid stain (Cambrex) and visualized using UV light and an image was acquired.

2.8.2. Gel analysis

The DGGE banding patterns were visually inspected to determine presence or absence of ectomycorrhizal bands and ectomycorrhiza colonizing bacteria bands. Banding patterns were converted to binomial data, a 1 representing band presence and a 0 representing the absence of a band in each sample lane.

The profiles were analyzed using a principle component analysis in PCOrd. To compare the relative enrichment that a fungal component had on the bacterial banding patterns, the data was arranged so that each fungal band was considered a treatment,regardless of the sample in which it was found. Bands with similar migrations in more than one lane were considered replicate bands.

The program was run to view any influence that presence of a fungal band may or may not have on the presence of bacterial bands. To test the efficacy of this program, a false data set was engineered to have a known correlation between bacterial and fungal banding patterns. With the engineered data set, there was clear separation along axis 1 and axis 2 in the PCA analysis. Such a separation would be expected if bacterial banding pattern was influenced by fungal banding pattern.

The bacterial species richness was determined for each fungal band. This was done by counting the number of bacterial bands present for each fungal band and obtaining the mean number of bacterial bands. Species richness indicates the level of biodiversity that is associated with each fungal type. In this case, species richness is another indicator of the specific influence ectomycorrhizal fungi have on the mycorrhizosphere environment.

3. Results

3.1. Soil Characterization

Physical and chemical soil characteristics were determined to compare properties between the sites. Soils from the Double Trouble site had significantly higher soil moisture and Loss On Ignition (LOI). The total percent carbon and percent total nitrogen as measured with high temperature combustion were higher in the Double Trouble site, while the total phosphorous content was similar between sites. A summary of the data is given as averages of measurements with their standard errors. These results are presented in table 3.1.

Table 3.1. Summary of Soil Properties

	Double Trouble	**Field Station**			
	Mean+/-SE	Mean+/- SE	t	df	P value
%Soil Moisture	47.73+/-0.65	36.63+/-0.35	15.03	4	0.001
%LOI	36.2+/-1.2	26.1+/-1.6	5.058	4	0.0072
%C	38.64+/-2.68	22.99+/-3.72	3.505	16	0.0029
%N	1.045 +/- 0.07	0.62+/-0.12	3.178	16	0.0058
P(µg/g)	282.1+/-38.78	256.3+/-35.64	0.448	14	0.661

3.2. Bacterial PCR

The first set of primers, 27f and 548R used for bacterial PCR amplification resulted in products that were about 500bp long. The products of these reactions were used in the second step of the nested PCR protocol. The final products that resulted were about 241 base pairs long, including the GC clamp. The product, which was analyzed with DGGE after gel purification, is marked with an asterisk in the legend of the following figures. 15 samples for each site, or a total of 30 samples, were used in DGGE analysis. The samples were chosen by the appearance of a strong, clear band on agarose gels and were chosen to coordinate with the strong, clear bands of ectomycorrhizal amplifications from corresponding samples

.

Refer to section 2.5 regarding naming of samples in each lane.

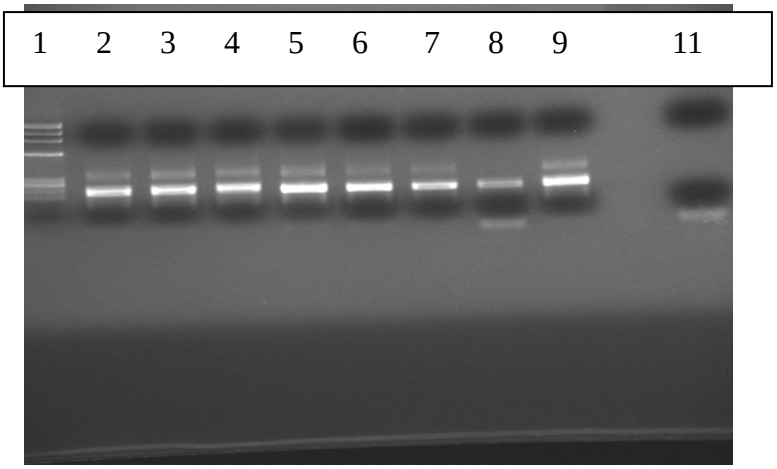


Figure 3.1 PCR products obtained after the second bacterial reaction using DNA targets from Field Station soil extracts. Lanes (left to right) contained:

Lane 1: Ladder

Lane 2: 1b*

Lane 3: 1c*

Lane 4: 1d*

Lane 5: 2a*

Lane 6: 2c*

Lane 7: 2d*

Lane 8: 2e*

Lane 9: 3a*

Lane 11: Blank

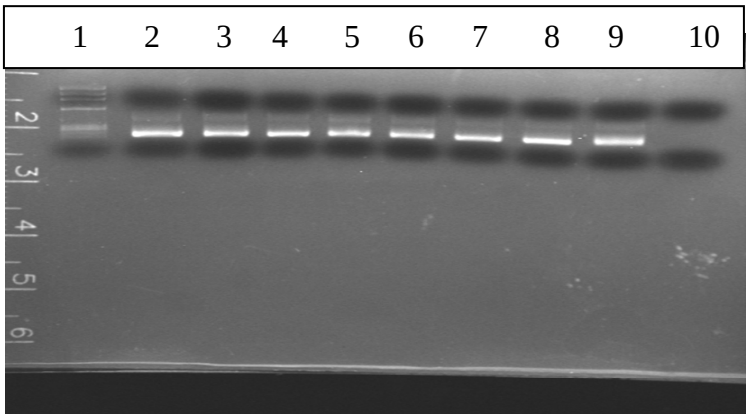


Figure 3.2. PCR products obtained after the second bacterial reaction using DNA targets from Field Station soil extracts. Lanes (left to right) contained:

Lane 1: ladder

Lane 2: 3b*

Lane 3: 4a*

Lane 4: 4b*

Lane 5: 4c*

Lane 6: 4d*

Lane 7: 5f*

Lane 8: 6f*

Lane 9: 7a*

Lane 10: Blank

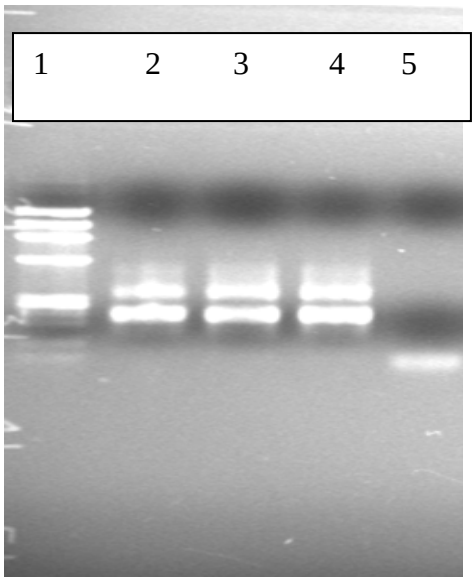


Figure 3.3.PCR products obtained after the second bacterial reaction using DNA targets from Field Station soil extracts. Lanes (left to right) contained:

Lane 1: Ladder

Lane 2: 7b*

Lane 3: 7c*

Lane 4: 8a

Lane 5: blank

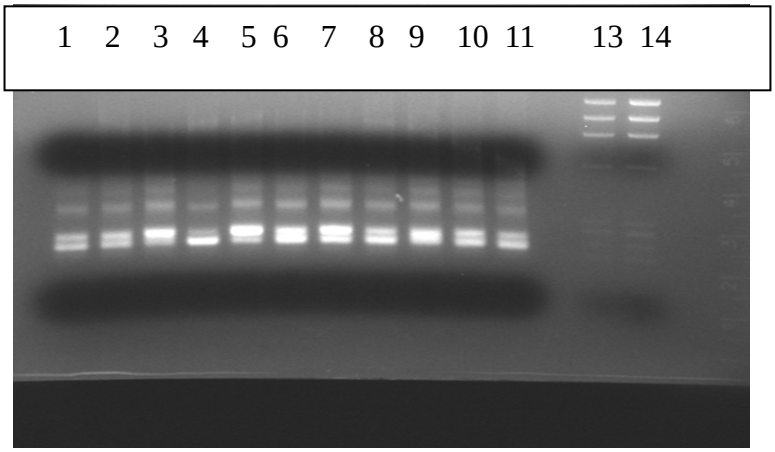


Figure 3.4.PCR products obtained after the second bacterial reaction using DNA targets from Double Trouble soil extracts. Lanes (left to right) contained:

Lane 1: 1.4*
Lane 2: 2.1*
Lane 3: 2.3*
Lane 4: 3.3*
Lane 5: 4.3*
Lane 6: 5.1*
Lane 7: 5.2*
Lane 8: 6.2*
Lane 9: 7.1*
Lane 10: 8.1*
Lane 11: 8.2*
Lane 13: Ladder
Lane 14: Ladder

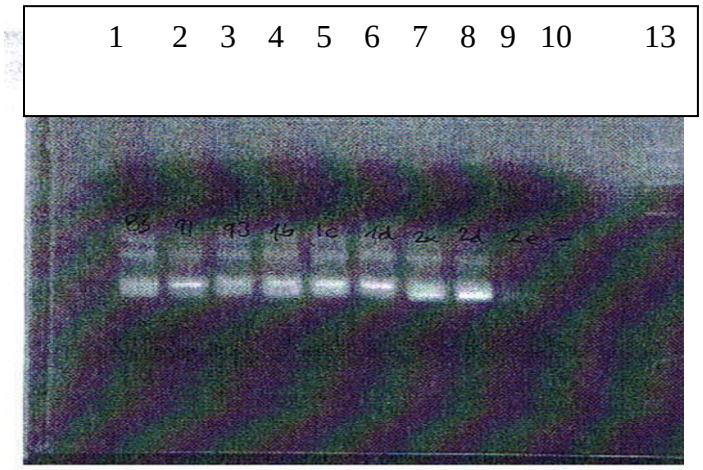


Figure 3.5.PCR products obtained after the second bacterial reaction using DNA targets from Double Trouble soil extracts. Lanes (left to right) contained:

Lane 1: 8.3*

Lane 2: 9.1*

Lane 3: 9.3*

Lane 4: 1b

Lane 5: 1c

Lane 6: 1d

Lane 7: 2a

Lane 8: 2d

Lane 9: 2e

Lane 10: blank

Lane 13: Ladder

3.3. Fungal PCR

The fungal primers used resulted in variable quality of PCR product. The first round of amplification using the ITS1/LR21 primer set had a 900 bp long. The second step of the nested pcr reaction used the primer set ITS1(GC) and ITS4. This yielded a product that was about 600 to 700 bp long. Products with the clearest band were used for DGGE analysis. The products that were used in DGGE are marked by an asterisk in the following figures. 15 samples were chosen from each site for a total of 30 samples between the two sites. Samples were chosen by the appearance of a strong, clean band as visualized on agarose gel.

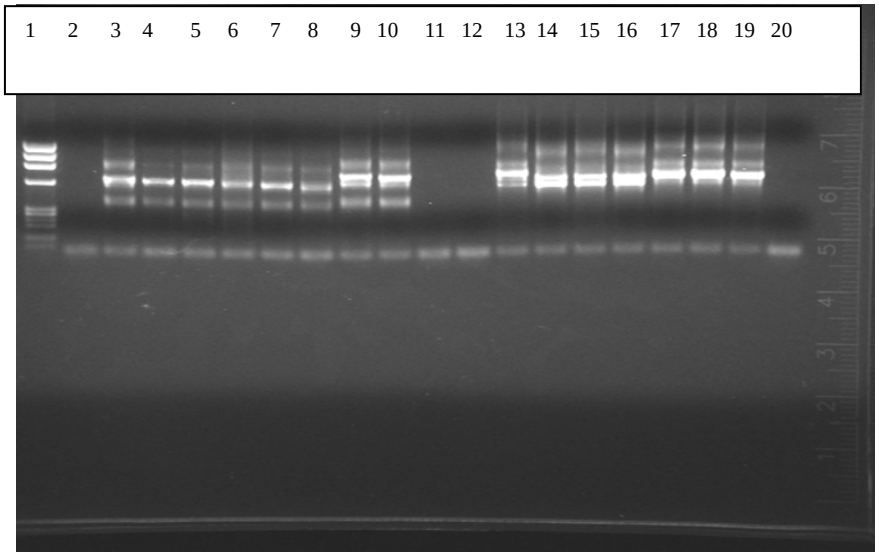


Figure 3.6. PCR products obtained after the second fungal reaction using DNA targets from Double Trouble soil extracts. Lanes (left to right) contained samples:

Lane 1: Ladder Lane 11: Blank

Lane 2: 4.1 Lane 12: 7.3

Lane 3: 4.2 Lane 13: 7.4

Lane 4: 4.3* Lane 14: 8.1*

Lane 5: 5.1* Lane 15: 8.2*

Lane 6: 5.2* Lane 16: 8.3*

Lane 7: 5.3* Lane 17:9.1*

Lane 8: 6.1 Lane 18:9.2

Lane 9: 6.4 Lane 19:9.3*

Lane 10:7.1* Lane 20: Blank

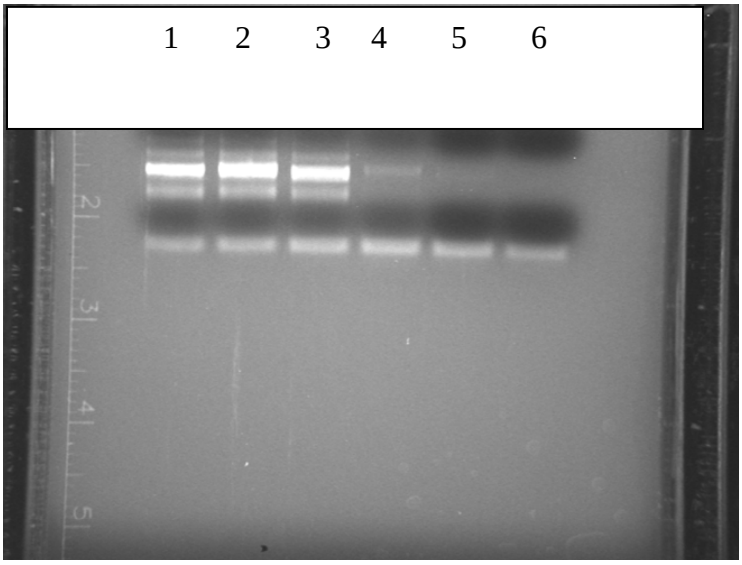


Figure 3.7. PCR products obtained after the second fungal reaction using DNA targets from Double Trouble soil extracts. Lanes (left to right) contained samples:

lane 1: 1.4*

Lane 2: 2.1*

Lane 3: 2.3*

Lane 4: 3.1

lane 5: 3.4

Lane 6: Blank

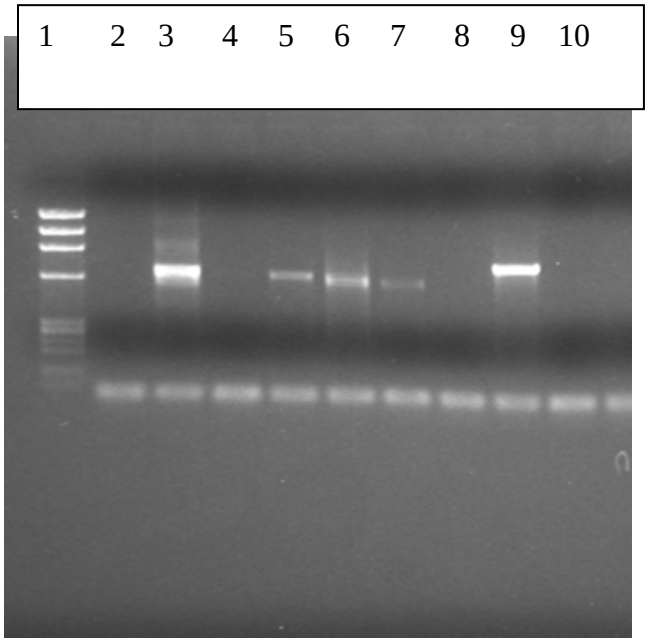


Figure 3.8. PCR products obtained after the second fungal reaction using DNA targets from Field Station soil extracts. Lanes (left to right) contained samples:

Lane 1: Ladder

Lane 2: 4.1

Lane 3: 4.2

Lane 3: 4.3

Lane 5: 5.1*

Lane 6: 5.2*

Lane 7: 5.3*

Lane 8: 6.1

Lane 9: 6.2

Lane 10: Blank

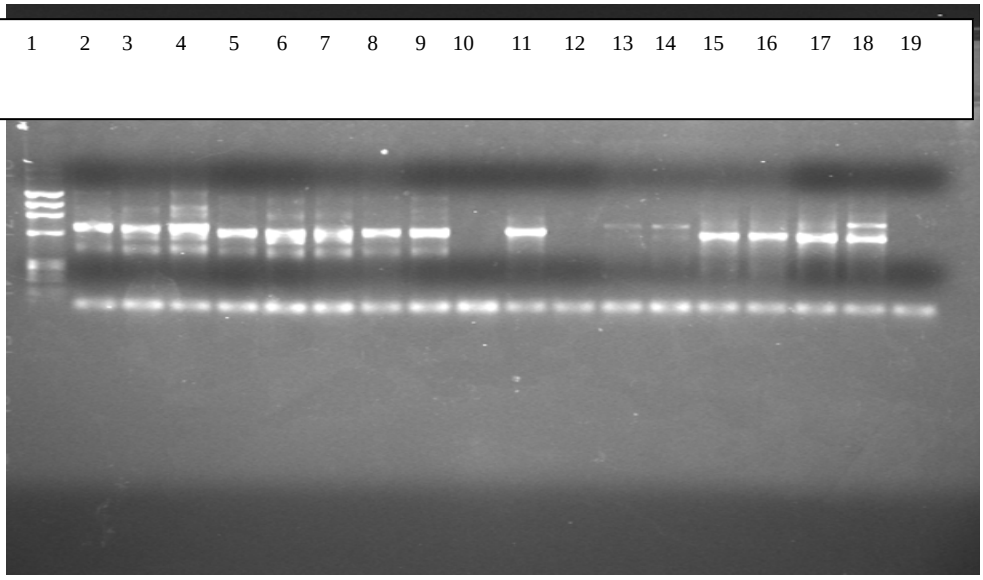


Figure 3.9. PCR products obtained after the second fungal reaction using DNA targets from Field Station soil extracts. Lanes (left to right) contained samples:

Lane 1: Ladder
Lane 2: 1b*
Lane 3: 1c*
Lane 4: 1d*
Lane 5: 2a*
Lane 6: 2b
Lane 7: 2c
Lane 8: 2d*
Lane 9: 2e*
Lane 11: 3a*
Lane 12: 3b*
Lane 13: 5f
Lane 14: 6f*
Lane 15: 7a*
Lane 16: 7b*
Lane 17: 7c*
Lane 18: 8a
Lane 19: Blank

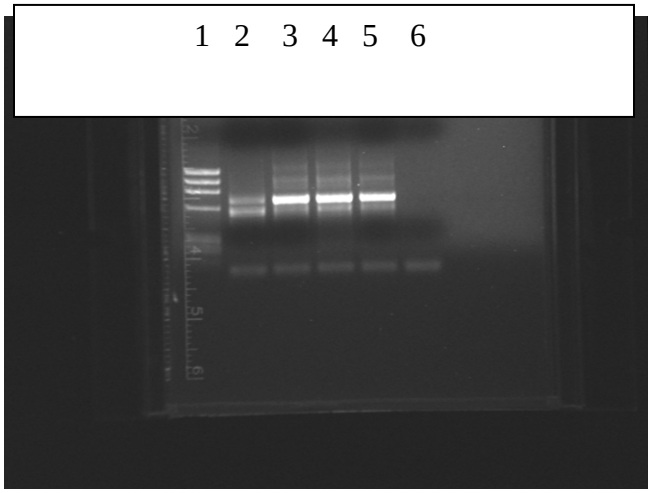


Figure 3.10. PCR products obtained after the second fungal reaction using DNA targets from Field Station soil extracts. Lanes (left to right) contained samples:

Lane 1: Ladder

Lane 2: 4a*

Lane 3: 4c*

Lane 4: 4d

Lane 5: 5b*

3.4. Success of DGGE primer pair

One band of a fungal DGGE gel was sequenced. The sequence had 98% similarity to *Cortninarius brunneus* with an E value of zero. *C. brunius* is an ectomycorrhizal fungus (Harrington 2002). The primers were deemed to target the appropriate DNA based on this information.

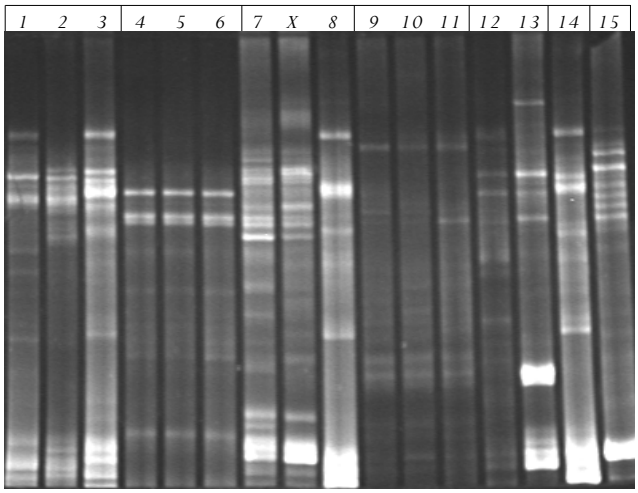
3.5. DGGE

Although an attempt was made to collect single ectomycorrhizal morphotypes, DGGE patterns clearly showed some diversity in every sample (Figures 3.11 and 3.12). Bacteria associated with ectomycorrhizae had a smeared profile with some banding patterns apparent (Figures 3.13 and 3.14).This likely reflects a high community diversity with minimal enrichment for individual species.

The banding patterns were converted to a binomial number chart, where the presence of a band in any lane was assigned a 1 and the absence of a band was denoted as a 0. These numbers are represented in figures 3.15 and 3.16 for bacteria and 3.17 and 3.18 for fungi. Inspection of the tables reveal that, although there seems to be little enrichment of bacteria, there are some common dominant bands present in each site. Bacterial band 16 shows up frequently in the field station site, while band 11 shows up frequently in the Double Trouble site. Bands can only be compared within a single gel and not between gels.

Bacterial band 8 on the Double Trouble gel is not the same as bacterial band 8 on the Field Station gel.

Figure 3.11. Field Station Ectomycorrhizal DGGE



Lane 1: 1b Lane 10: 7a

Lane 2: 1c Lane 11: 7b

Lane 3: 1d Lane 12: 7c

Lane 4: 2a Lane 13: 3a

Lane 5: 2d Lane 14: 3b

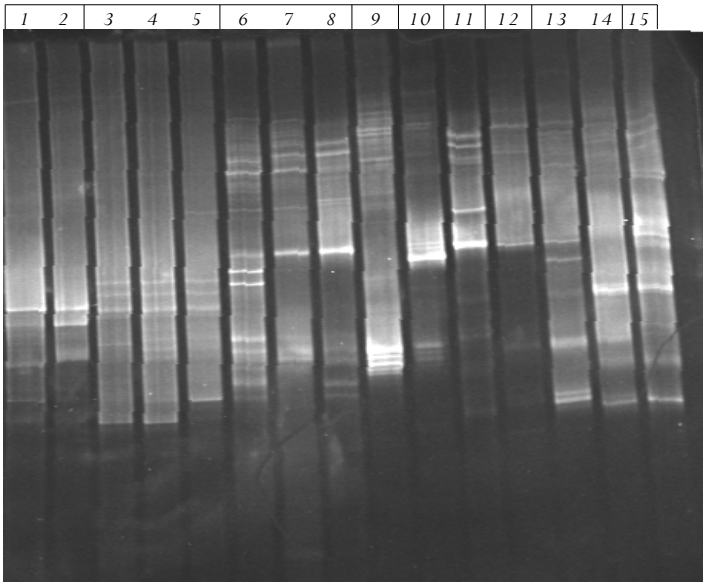
Lane 6: 2e Lane 15: 5b

Lane 7: 4a Lane 16: 6f

Lane 8: X= discarded sample lane

Lane 9: 4c

Figure 3.12. Double Trouble Ectomycorrhizal DGGE



Lane 1: 9.3

Lane 2: 9.1

Lane 3: 8.3

Lane 4: 8.2

Lane 5: 8.1

Lane 6: 5.3

Lane 7: 5.2

Lane 8: 5.1

Lane 9: 7.1

Lane 8: 5.1

Lane 9: 7.1

Lane 10: 6.2

Lane 11: 4.3

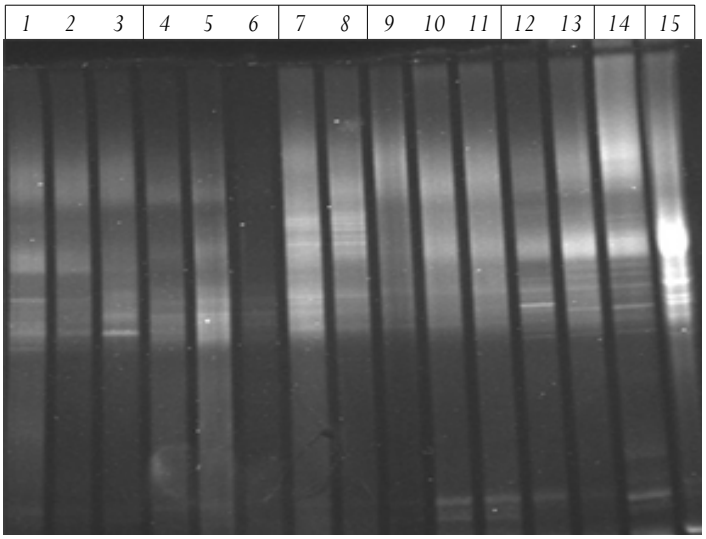
Lane 12: 3.3

Lane 13: 2.3

Lane 14: 2.1

Lane 15: 1.4

Figure 3.13. Field Station Ectomycorrhiza Associated Bacteria DGGE



Lane 1: 1b

Lane 2: 1c

Lane 3: 1d

Lane 4: 2a

Lane 5: 2d

Lane 6: 2e

Lane 7: 4a

Lane 8: 4c

Lane 9: 7a

Lane 10: 7b

Lane 11: 7c

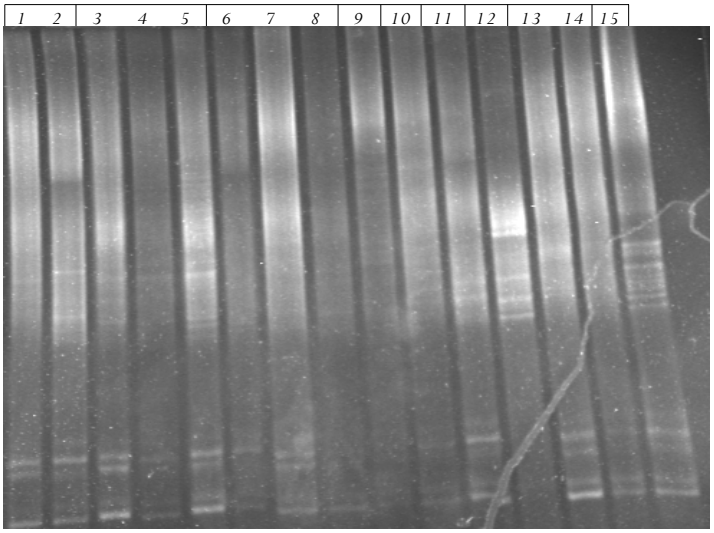
Lane 12: 3a

Lane 13: 3b

Lane 14: 5b

Lane 15: 6f

Figure 3.14. Double Trouble Ectomycorrhiza Associated Bacteria DGGE



Lane 1: 9.3

Lane 2: 9.1

Lane 3: 8.3

Lane 4: 8.2

Lane 5: 8.1

Lane 6: 5.3

Lane 7: 5.2

Lane 8: 5.

Lane 9: 7.1

Lane 10: 6.2

Lane 11: 4.3

Lane 12: 3.3

Lane 13: 2.3

Lane 14: 2.1

Lane 15: 1.4

Figure 3.15: Double Trouble Binomial Representation of Bacterial Banding Patterns Corresponding to Figure 3.11

Bacteria-Double Trouble site															
	Lane 1	Lane 2	Lane 3	Lane 4	Lane 5	Lane 6	Lane 7	Lane 8	Lane 9	Lane 10	Lane 11	Lane 12	Lane 13	Lane 14	Lane 15
Band 1	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0
Band 2	0	1	0	0	0	0	0	0	0	0	0	0	0	0	1
Band 3	1	1	1	1	1	0	0	0	0	0	0	0	0	0	1
Band 4	0	0	0	0	0	0	0	0	0	0	1	1	0	0	1
Band 5	0	1	1	0	1	0	0	0	0	0	1	1	0	0	1
Band 6	0	0	0	0	1	0	0	0	0	0	0	0	0	0	1
Band 7	0	0	0	0	0	0	0	0	0	0	0	1	0	0	1
Band 8	1	1	1	1	1	1	1	0	0	1	1	0	1	1	1
Band 9	1	0	1	0	1	0	1	0	0	0	0	0	1	0	0
Band 10	0	0	0	0	1	0	0	0	1	1	0	0	1	1	1
Band 11	1	1	1	1	1	0	1	1	0	1	1	0	1	1	1

Figure 3.16: Field Station Binomial Representation of Bacterial Banding Corresponding to Figure 3.12

Bacteria field station site															
	Lane 1	Lane 2	Lane 3	Lane 4	Lane 5	Lane 6	Lane 7	Lane 8	Lane 9	Lane 10	Lane 11	Lane 12	Lane 13	Lane 14	Lane 15
Band 1	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0
Band 2	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0
Band 3	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0
Band 4	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0
Band 5	0	0	0	0	0	0	1	1	0	0	0	0	0	0	0
Band 6	1	0	0	0	0	0	0	0	0	0	0	0	0	1	0
Band 7	0	0	0	0	1	0	1	1	0	0	0	1	1	1	0
Band 8	0	0	0	1	1	0	0	0	0	0	0	1	1	1	1
Band 9	1	1	1	0	0	0	0	0	0	0	0	1	0	0	1
Band 10	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1
Band 11	1	0	0	0	0	0	0	0	1	0	0	0	0	0	1
Band 12	0	0	0	0	0	0	0	0	0	1	0	1	0	1	0
Band 13	0	0	0	0	0	0	0	0	1	1	1	0	0	1	0
Band 14	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Band 15	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1
Band 16	1	1	1	1	1	0	1	1	1	1	1	1	1	1	0

Figure 3.17: Double Trouble Binomial Representation of Ectomycorrhizal Fungi Banding Pattern Corresponding to Figure 3.13

Fungi- Double Trouble site															
	Lane 1	Lane 2	Lane 3	Lane 4	Lane 5	Lane 6	Lane 7	Lane 8	Lane 9	Lane 10	Lane 11	Lane 12	Lane 13	Lane 14	Lane 15
Band 1	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0
Band 2	0	0	0	0	0	0	0	0	1	0	1	0	0	0	0
Band 3	0	0	0	0	0	0	0	1	0	0	1	0	0	0	0
Band 4	0	0	0	0	0	1	1	1	0	0	0	0	0	0	0
Band 5	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0
Band 6	0	0	0	0	0	1	1	1	1	0	0	0	0	0	1
Band 7	0	0	0	0	1	1	1	0	0	0	1	0	0	0	1
Band 8	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0
Band 9	0	0	0	0	1	1	1	0	0	0	0	0	0	0	0
Band 10	0	0	0	0	0	0	0	0	0	0	1	0	0	0	1
Band 11	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0
Band 12	0	0	0	0	0	1	1	1	0	1	1	1	1	0	1
Band 13	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0
Band 14	0	0	0	0	0	1	0	0	0	0	0	0	1	0	1
Band 15	0	0	1	1	1	0	0	0	0	0	0	0	0	0	0
Band 16	0	0	1	1	1	0	0	0	0	0	0	0	0	0	0
Band 17	1	1	1	1	1	0	0	0	0	0	0	0	0	1	0
Band 18	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0
Band 19	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0
Band 20	0	0	0	0	0	1	1	0	1	1	0	0	0	0	0
Band 21	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0
Band 22	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0
Band 23	0	0	0	0	1	0	0	0	0	0	0	0	1	0	0
Band 24	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0
Band 25	0	0	1	1	0	0	0	0	0	0	0	0	0	1	1

Figure 3.18. Field Station Binomial Representation of Ectomycorrhizal Fungi Banding Pattern Corresponding to Figure 3.14

Fungi- Field Station Site																
	Lane 1	Lane 2	Lane 3	Lane 4	Lane 5	Lane 6	Lane 7	Lane 8	Lane 9	Lane 10	Lane 11	Lane 12	Lane 13	Lane 14	Lane 15	
Band 1	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	
Band 2	1	0	1	0	0	0	0	1	0	0	0	0	0	1	0	
Band 3	0	0	0	0	0	0	0	0	1	1	1	0	0	0	0	
Band 4	0	0	0	0	0	0	1	0	0	0	0	0	0	0	1	
Band 5	0	1	1	0	0	0	1	0	0	0	0	0	0	0	1	
Band 6	1	1	1	0	0	0	1	1	0	0	0	0	0	1	0	
Band 7	0	0	1	1	1	1	0	0	0	0	0	0	0	1	1	
Band 8	1	1	0	0	0	0	0	0	0	0	0	0	0	0	1	
Band 9	0	0	0	0	0	0	1	0	0	0	0	0	0	0	1	
Band 10	0	0	0	1	1	1	1	0	0	0	1	1	1	0	1	
Band 11	0	0	0	1	1	1	1	0	0	0	0	0	0	0	0	
Band 12	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	
Band 13	0	0	0	0	0	0	0	1	0	0	0	0	0	1	0	
Band 14	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	
Band 15	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	
Band 16	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	
Band 17	0	1	1	0	0	0	0	0	0	0	0	0	0	1	1	
Band 18	0	0	1	0	0	0	0	1	0	0	0	0	1	0	0	
Band 19	1	1	0	0	0	0	0	0	0	0	0	0	0	1	0	
Band 20	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0	

3.6. Gel analysis

The Binomial Data were combined so that a principle component analysis would be able to detect a relationship between ectomycorrhiza fungi and ectomycorrhiza associated bacteria. The fungi were the independent variable and the bacteria were dependent upon the fungi present in a sample, the fungi acting as treatments in the symbiotic community (Figures 3.19 and 3.20). In the representation below, the rows represent the fungal bands present on a gel, while columns represent bacterial bands on the gel. Ones represent the presence of a bacterial band and zeros represent the absence of a bacterial band wherever the fungi was present on the fungal gels.

Figure 3.19. Field Station Bacteria to Fungi Banding Relationship

	BacB1	BacB2	BacB3	BacB4	BacB5	BacB6	BacB7	BacB8	BacB9	BacB10	BacB11	BacB12	BacB13	BacB14	BacB15	BacB16
FB1	0	0	0	0	0	0	1	1	0	1	0	0	0	0	0	1
FB2a	0	0	0	0	0	1	0	0	1	0	1	0	0	0	0	1
FB2B	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	1
FB2C	1	1	1	1	1	0	1	0	0	0	0	0	0	0	0	1
FB2D	0	0	0	0	0	1	1	1	0	1	0	1	1	0	0	1
FB3A	0	0	0	0	0	0	0	0	0	0	1	0	1	0	0	1
FB3B	0	0	0	0	0	0	0	0	0	0	0	1	1	0	0	1
FB3C	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	1
FB4A	0	0	0	0	1	0	1	0	0	0	0	0	0	0	0	1
FB4B	0	0	0	0	0	0	0	1	1	1	1	0	0	0	1	0
FB5A	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	1
FB5B	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	1
FB5C	0	0	0	0	1	0	1	0	0	0	0	0	0	0	0	1
FB5D	0	0	0	0	0	0	0	1	1	1	1	0	0	0	1	0
FB6A	0	0	0	0	0	1	0	0	1	0	1	0	0	0	0	1
FB6B	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	1
FB6C	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	1
FB6D	0	0	0	0	1	0	1	0	0	0	0	0	0	0	0	1
FB6E	1	1	1	1	1	0	1	0	0	0	0	0	0	0	0	1
FB6 F	0	0	0	0	0	1	1	1	0	1	0	1	1	0	0	1
FB7A	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	1
FB7B	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	1
FB7C	0	0	0	0	0	0	1	1	0	0	0	0	0	0	0	1
FB7D	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
FB7E	0	0	0	0	0	1	1	1	0	1	0	1	1	0	0	1
FB7F	0	0	0	0	0	0	0	1	1	1	1	0	0	0	1	0
FB8A	0	0	0	0	0	1	0	0	1	0	1	0	0	0	0	1
FB8B	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	1
FB8C	0	0	0	0	0	0	0	1	1	1	1	0	0	0	1	0
FB9A	0	0	0	0	1	0	1	0	0	0	0	0	0	0	0	1
FB9B	0	0	0	0	0	0	0	1	1	1	1	0	0	0	1	0
FB10A	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	1
FB10B	0	0	0	0	0	0	1	1	0	0	0	0	0	0	0	1
FB10C	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
FB10D	0	0	0	0	1	0	1	0	0	0	0	0	0	0	0	1
FB10E	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	1
FB10F	0	0	0	0	0	0	1	1	1	0	0	1	0	0	0	1
FB10G	0	0	0	0	0	0	1	1	0	1	0	0	0	0	0	1
FB10H	0	0	0	0	0	0	0	1	1	1	1	0	0	0	1	0
FB11A	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	1
FB11B	0	0	0	0	0	0	1	1	0	0	0	0	0	0	0	1
FB11C	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
FB11D	0	0	0	0	1	0	1	0	0	0	0	0	0	0	0	1
FB12A	0	0	0	0	1	0	1	0	0	0	0	0	0	0	0	1
FB13A	1	1	1	1	1	0	1	0	0	0	0	0	0	0	0	1
FB13B	0	0	0	0	0	1	1	1	0	1	0	1	1	0	0	1
FB14A	0	0	0	0	0	0	1	1	0	1	0	0	0	0	0	1
FB15A	0	0	0	0	1	0	1	0	0	0	0	0	0	0	0	1
FB16	0	0	0	0	1	0	1	0	0	0	0	0	0	0	0	1
FB17A	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	1
FB17B	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	1
FB17C	0	0	0	0	0	1	1	1	0	1	0	1	1	0	0	1
FB17D	0	0	0	0	0	0	0	1	1	1	1	0	0	0	1	0
FB18A	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	1
FB18B	1	1	1	1	1	0	1	0	0	0	0	0	0	0	0	1
FB18C	0	0	0	0	0	0	1	1	0	1	0	0	0	0	0	1

Figure 3.20. Double Trouble Bacteria to Fungi Banding Relationship

	BAC B1	BAC B2	BAC B3	BAC B4	BAC B5	BAC B6	BAC B7	BAC B8	BAC B9	BAC B10	BAC B11	
FB1		0	0	0	0	0	0	0	0	0	1	0
FB2 A		0	0	0	0	0	0	0	0	0	1	0
FB2 B		0	0	0	1	1	0	0	1	0	0	1
FB3 A		0	0	0	0	0	0	0	0	0	0	1
FB3 B		0	0	0	1	1	0	0	1	0	0	1
FB4 A		0	0	0	0	0	0	0	1	0	0	0
FB4 B		0	0	0	0	0	0	0	1	1	0	1
FB4 C		0	0	0	0	0	0	0	0	0	0	1
FB5		0	0	0	0	0	0	0	0	0	1	0
FB6 A		0	0	0	0	0	0	0	1	0	0	0
FB6 B		0	0	0	0	0	0	0	1	1	0	1
FB6 C		0	0	0	0	0	0	0	0	0	0	1
FB6 D		0	0	0	0	0	0	0	0	0	1	0
FB6 E		0	1	1	1	1	1	1	1	0	1	1
FB7 A		0	0	1	0	1	1	0	1	1	0	0
FB7 B		0	0	0	0	0	0	0	1	0	0	0
FB7 C		0	0	0	0	0	0	0	1	1	0	1
FB7 D		0	0	0	1	1	0	0	1	0	0	1
FB7 E		0	1	1	1	1	1	1	1	0	1	1
FB8		0	0	0	0	0	0	0	0	0	0	1
FB9 A		0	0	1	0	1	1	0	1	1	0	0
FB9 B		0	0	0	0	0	0	0	1	0	0	0
FB9 C		0	0	0	0	0	0	0	1	1	0	1
FB10 A		0	0	0	1	1	0	0	1	0	0	1
FB10 B		0	1	1	1	1	1	1	1	0	1	1
FB11		0	0	0	0	0	0	0	1	0	1	1
FB12 A		0	0	0	0	0	0	0	1	0	0	0
FB12 B		0	0	0	0	0	0	0	1	1	0	1
FB12 C		0	0	0	0	0	0	0	0	0	0	1
FB12 D		0	0	0	0	0	0	0	1	0	1	1
FB12 E		0	0	0	1	1	0	0	1	0	0	1
FB12 F		1	0	0	1	1	0	1	0	0	0	0
FB12 G		0	0	0	0	0	0	0	1	1	1	1
FB12 H		0	1	1	1	1	1	1	1	0	1	1
FB13		0	0	0	0	0	0	0	1	0	1	1
FB14 A		0	0	0	0	0	0	0	1	0	0	0
FB14 B		0	0	0	0	0	0	0	1	1	1	1
FB14 C		0	1	1	1	1	1	1	1	0	1	1
FB15 A		0	0	1	0	1	0	0	1	1	0	1
FB15 B		0	0	1	0	0	0	0	1	0	0	1
FB15 C		0	0	1	0	1	1	0	1	1	0	0
FB16 A		0	0	1	0	1	0	0	1	1	0	1
FB16 B		0	0	1	0	0	0	0	1	0	0	1
FB16 C		0	0	1	0	1	1	0	1	1	0	0
FB17 A		0	0	1	0	0	0	0	1	1	0	1
FB17 B		0	1	1	0	1	0	0	1	0	0	1
FB17 C		0	0	1	0	1	0	0	1	1	0	1
FB17 D		0	0	1	0	0	0	0	1	0	0	1
FB17 E		0	0	1	0	1	1	0	1	1	0	0
FB17 F		0	0	0	0	0	0	0	1	0	1	1
FB18		0	1	1	0	1	0	0	1	0	0	1
FB19		0	0	0	0	0	0	0	1	0	0	0
FB20 A		0	0	0	0	0	0	0	1	0	0	0
FB20 B		0	0	0	0	0	0	0	1	1	0	1
FB20 C		0	0	0	0	0	0	0	0	0	1	0
FB20 D		0	0	0	0	0	0	0	1	0	1	1
FB21		0	0	0	0	0	0	0	0	0	1	0
FB22		0	0	0	0	0	0	0	1	0	0	0
FB23 A		0	0	1	0	1	1	0	1	1	0	0
FB23 B		0	0	0	0	0	0	0	1	1	1	1
FB24		0	0	0	0	0	0	0	1	1	1	1
FB25 A		0	0	1	0	1	0	0	1	1	0	1
FB25 B		0	0	1	0	0	0	0	1	0	0	1
FB25 C		0	0	0	0	0	0	0	1	0	1	1
FB25 D		0	1	1	1	1	1	1	1	0	1	1

This information was input into a principle component analysis (PCA). The purpose of the PCA was determine if there was a link between the banding patterns of the bacteria and the banding patterns of the fungi. The PCA showed that there was not a strong correlation between the bacterial and fungal components sampled as there was no clear separation along axis 1 and 2 in the PCA (Tables 3.2 and 3.3)

Table 3.2. Double Trouble PCA, Axis 1 and 2

Double Trouble Coordinate Scores for fungi

	Axis 1	Axis 2			Axis 1	Axis 2
FB1	-0.82	-0.72		FB12	-0.41	0.05
FB2	-0.82	-0.72		FB12	1.82	-0.98
FB2	0.33	-0.21		FB13	-0.48	-0.54
FB3	-0.72	-0.25		FB14	-0.65	0.08
FB3	0.33	-0.21		FB14	-0.41	0.05
FB4	-0.65	0.08		FB14	1.82	-0.98
FB4	-0.44	0.59		FB15	0.57	0.94
FB4	-0.72	-0.25		FB15	-0.03	0.25
FB5	-0.82	-0.72		FB15	0.82	1.03
FB6	-0.65	0.08		FB16	0.57	0.94
FB6	-0.44	0.59		FB16	-0.03	0.25
FB6	-0.72	-0.25		FB16	0.82	1.03
FB6	-0.82	-0.72		FB17	0.04	0.84
FB6	1.82	-0.98		FB17	0.8	0.12
FB7	0.82	1.03		FB17	0.57	0.94
FB7	-0.65	0.08		FB17	-0.03	0.25
FB7	-0.44	0.59		FB17	0.82	1.03
FB7	0.33	-0.21		FB17	-0.48	-0.54
FB7	1.82	-0.98		FB18	0.8	0.12
FB8	-0.72	-0.25		FB19	-0.65	0.08
FB9	0.82	1.03		FB20	-0.65	0.08
FB9	-0.65	0.08		FB20	-0.44	0.59
FB9	-0.44	0.59		FB20	-0.82	-0.72
FB10	0.33	-0.21		FB20	-0.48	-0.54
FB10	1.82	-0.98		FB21	-0.82	-0.72
FB11	-0.48	-0.54		FB22	-0.65	0.08
FB12	-0.65	0.08		FB23	0.82	1.03
FB12	-0.44	0.59		FB23	-0.41	0.05
FB12	-0.72	-0.25		FB24	-0.41	0.05
FB12	-0.48	-0.54		FB25	0.57	0.94
FB12	0.33	-0.21		FB25	-0.03	0.25
FB12	0.27	-0.68		FB25	-0.48	-0.54
				FB25	1.82	-0.98

Table 3.3. Field Station PCA, Axis 1 and 2

Field Station Scores for Fungi

	Axis 1	Axis 2			Axis 1	Axis 2
FB1	0.19	-0.9		FB9	-1.62	-0.32
FB2	-0.69	0.56		FB10	-0.03	-0.07
FB2	-0.28	0.76		FB10	0.41	-0.42
FB2	1.58	0.43		FB10	-0.12	0.44
FB2	0.22	-1.74		FB10	0.97	0.23
FB3	-0.15	0.2		FB10	0.21	0.17
FB3	0.25	-0.15		FB10	0	-0.45
FB3	0.21	0.17		FB10	0.19	-0.9
FB4	0.97	0.23		FB10	-1.62	-0.32
FB4	-1.62	-0.32		FB11	-0.03	-0.07
FB5	-0.28	0.76		FB11	0.41	-0.42
FB5	-0.28	0.76		FB11	-0.12	0.44
FB5	0.97	0.23		FB11	0.97	0.23
FB5	-1.62	-0.32		FB12	0.97	0.23
FB6	-0.69	0.56		FB13	1.58	0.43
FB6	-0.28	0.76		FB13	0.22	-1.74
FB6	-0.28	0.76		FB14	0.19	-0.9
FB6	0.97	0.23		FB15	0.97	0.23
FB6	1.58	0.43		FB16	0.97	0.23
FB6	0.22	-1.74		FB17	-0.28	0.76
FB7	-0.28	0.76		FB17	-0.28	0.76
FB7	-0.03	-0.07		FB17	0.22	-1.74
FB7	0.41	-0.42		FB17	-1.62	-0.32
FB7	-0.12	0.44		FB18	-0.28	0.76
FB7	0.22	-1.74		FB18	1.58	0.43
FB7	-1.62	-0.32		FB18	0.19	-0.9
FB8	-0.69	0.56		FB19	-0.69	0.56
FB8	-0.28	0.76		FB19	-0.28	0.76
FB8	-1.62	-0.32		FB19	0.22	-1.74
FB9	0.97	0.23		FB20	-0.69	0.56
				FB20	-0.28	0.76
				FB20	-0.28	0.76

In addition to PCA analysis, the extent of species richness was also determined. Table 3.4 shows species richness for each fungal band present in the Field Station samples, while Table 3.5 shows the bacterial species richness for the Double Trouble samples. It can be seen that the richness varies between fungal bands, some indicating a lower bacterial diversity and some indicating a higher diversity. While both sites have variance, the Double Trouble site is somewhat more variable from fungal band to fungal band.

Table 3.4

Field Station Species Richness, Corresponding to Figure 3.19

Fungal Band	Mean Bacterial Species Richness
1	4
2	5
3	2.67
4	4
5	3
6	4.17
7	3.8
8	3.67
9	4
10	3.43
11	2.67
12	3
13	7
14	4
15	3
16	3
17	4
18	4.33
19	4.33
20	2.67

Table 3.5

Double Trouble Species Richness, Corresponding to Figure 3.20

Fungal Band	Mean Bacterial Species Richness
1	1
2	2.5
3	2.5
4	1.67
5	1
6	3
7	4.4
8	1
9	3
10	6.5
11	3
12	3.62
13	3
14	4.67
15	4.33
16	4.33
17	4.17
18	5
19	1
20	2
21	1
22	1
23	4.5
24	4
25	5

4. Discussion

This study used two different sampling sites located in the pine barrens habitat. Although differing in organic matter content (LOI), percent moisture content, total carbon and total nitrogen, the two samples were comparable in habitat, climate and total phosphorous.

Denaturing Gradient Gel Electrophoresis was utilized to observe both ectomycorrhiza fungi and ectomycorrhiza associated bacterial communities to determine the influence that the fungi may have on the bacterial community in the mycorrhizasphere. Molecular based techniques such as DGGE are useful tools in microbial ecology. DGGE can yield a fingerprint of a sample which reveals species diversity within a community. This method takes advantage of the fact that DNA has a variable melting point dependent upon its sequence (Muyzer et al. 1993). DGGE utilizes an electric current to facilitate the movement of DNA along a denaturing gradient. As the DNA strands denature, the DNA will stop in the gel once the melting point has been reached. A GC clamp is used so that complete denaturization is avoided, with the GC clamp acting as an anchor for the DNA in the gel, keeping the DNA in a double stranded form (Muyzer 1993; Muyzer, 1999).

Although DGGE is an important tool in microbial ecology, it is important to note limitations of the techniques. In order to obtain DGGE profiles, PCR was used to

amplify the target DNA. There is a bias in PCR amplification in that more dominant organisms will have a higher likelihood of representation. Indeed, organisms may also be better represented simply because their DNA sequence is more often amplified, regardless of relative abundance (Acinas et al. 2005). Also, DGGE is primarily useful for communities with limited diversity, as it is these communities which will reveal the clearest profile with a highly distinct banding pattern (Vallaeys et al.1997). Diverse communities, such as those present in this study, will result in a more smeared profile as there is less enrichment due to a greater number of species. However, because profiles obtained in DGGE gels are particularly useful when comparing the influence of different treatments in a specific community, this method was used to view the influence of ectomycorrhizal fungi as soil treatments.

The bacterial primer sets allowed amplification of the ectomycorrhiza associated bacterial communities where one round of amplification yielded no PCR product with a satisfactory yield. The second round of nested PCR was able to obtain the target DNA at a yield sufficient for DGGE analysis. Although nested PCR increases the PCR biases, it was necessary for obtaining a useful product in this experiment,

The fungal primer pair was used to both achieve amplification of a difficult product and to improve specificity. The final product used in DGGE was without contaminating plant DNA and increased the likelihood that all products were

ectomycorrhizal (Hopple and Vigalys, 1999). The primer pair ITS1(GC)/ITS4 yielded distinct banding patterns on the DGGE profile (Figure 3.11 and 3.12). This primer Pair is often used for amplification of ectomycorrhizal fungi (White et al, 1990)

Although every attempt was made to collect a single species of mycorrhiza (as judged by morphotyping), the DGGE profiles of ectomycorrhiza revealed that each sample contained more than one different species (Figures 3.11 and 3.12). This is likely due to DNA from multiple ectomycorrhiza fungi growing within very close proximity. The collection technique was engineered to retain the natural biota that grows in close proximity to the collected morphotype, so collection did not include a wash of the primary collected morphotype. This could possible allow multiple hyphae from the surrounding growth area to be included in the sample. Also morphotyping can be inaccurate, as many isolates or species can have similar morphotypes (Burke et al., 2005). Also, at different life stages, the same species can have different morphotypes. These confounding factors could lead to over or under estimation of fungal species, only detectable by molecular identification.

DGGE gel analysis of bacteria showed a very highly diverse community with minimal enrichment towards any dominant species (Figure 3.13 and 3.14). This was represented by a smeared profile in the lower end of the denaturing gradient. The enrichment that was noted, as shown by dominant bands, was

minimal. The weak banding pattern was most likely due to the highly diverse soil community present in the samples. There was some enrichment in the samples; however, these usually appeared random in relation to the presence or absence of ectomycorrhizal fungi within any given sample. Some bacterial bands were common among multiple samples. Nevertheless, the commonly enriched bacterial bands were not specific for any mycorrhizal type, as seen in the lack of separation along Axis 1 and 2 in the PCA analysis (Table 3.6 and 3.7).

The species richness present in the samples was determined for each ectomycorrhiza fungi band. Species richness is a measure of the biodiversity present among samples or ectomycorrhizal treatments. Double Trouble had greater variance between bacterial species richness. It is possible that within the study sites, the overall bacterial diversity is influenced by the presence or absence of ectomycorrhiza fungi, even though specific bacterial enrichment is more difficult to determine through DGGE.

Although it was hypothesized that specific ectomycorrhizal morphotypes would yield a strong influence on the microbial community structure in the ectomycorrhizosphere, these results suggest that the presence or absence of ectomycorrhiza fungal species yields no detectable selection for specific bacterial groups. While there was some selection noted, this did not correspond to the influence of any single ectomycorrhizal fungal component as noted by PCA analysis. It is interesting to note, however, that there were variances of bacterial

species richness among ectomycorrhizal fungi treatments. It is possible that fungal treatments act to enhance the biodiversity within the mycorrhizosphere, even if more specific selection is difficult to determine.

It is true that ectomycorrhizae are important players in the forest ecosystem, yet these results suggest that it is difficult to isolate the influence of a single fungal component in a natural setting. At the scale of this experiment, which worked with entire root tips, other factors, such as other microflora, microfauna, and abiotic factors may have a stronger influence on the mycorhizasphere. Inspection of the gels indicates a system that is highly complex. Lab studies indicated an enrichment for a specific microbial community (Mooge et al., 2000, Poole at al., 2001), yet it is apparent that this enrichment is difficult to translate into the natural environment at the detection level of the DGGE.

Future studies are needed to increase the resolution of bacterial species detection. This could be done with the use of bacterial clone libraries. A clone library would allow characterization of the ectomycorrhiza associated bacteria communities on a finer scale than the “fingerprint” level of DGGE which is better suited to low diversity communities.

5. Bibliography

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