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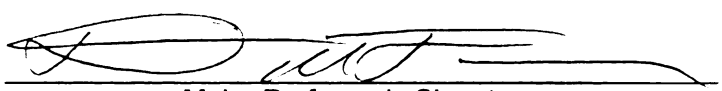

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THE UTILITY OF MICROBIAL DNA AND TERMINAL RESTRICTION FRAGMENT LENGTH POLYMORPHISM ANALYSIS IN THE FORENSIC EXAMINATION OF SOIL

By

Melissa Sue Meyers

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ABSTRACT

THE UTILITY OF MICROBIAL DNA AND TERMINAL RESTRICTION FRAGMENT LENGTH POLYMORPHISM ANALYSIS IN THE FORENSIC EXAMINATION OF SOIL

By

Melissa Sue Meyers

Soil can be of evidentiary value in that a soil sample collected from a shoe, tire, or other material may help associate a victim or suspect with a crime scene. Traditional soil analyses focus on physical and chemical characteristics and while these can help differentiate soils, there are few objective methods that can trace a questioned soil to a specific location. Analysis of microorganisms to trace soils has been briefly mentioned in the forensic literature. Differences in bacterial DNA among species has the potential to help delineate a soil, and thus act as a biological 'signature' that may be useful for forensic purposes. The use of Terminal Restriction Fragment Length Polymorphism (TRFLP) as a viable forensic tool for identifying soils depends on several factors including changes in microbial communities over time, the uniqueness of soils from different locations, and the extent of local heterogeneity. Soils from five diverse locations were sampled each month for a one year period. Soils were collected at a primary site, as well as 10 feet in all directions. The greatest similarity occurred among sites within a location. The similarity at a site from month to month, as well as one month compared to a random month, fluctuated substantially over the course of the year. Variability was on average greatest when comparing different locations to each other. Even though soils were distinguishable on average, there was substantial overlap in similarity indices for different comparisons which may limit the forensic usefulness of TRFLP.

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INTRODUCTION

Overview

Soil can play a crucial role in a forensic investigation, acting as trace evidence that may link a victim or suspect to a crime scene. Soil is not a specific item however; instead it is a complex mixture of crystalline and amorphous minerals, inert and decomposing organic material, animals, plant material, pollens, microbial residues in different stages of decay, and a living microbiota that includes bacteria, fungi, algae, and yeast. Soil is of wide evidentiary value owing to its variable makeup, its prevalence in the environment, and because it is easily transferred from a crime scene to a suspect or victim. It can also be carried away from the crime scene on shoes and clothing, and has the potential to be transferred to other objects such as a shovel used by a suspect or the tire of a suspect's vehicle. In all cases, the soil from the suspect or victim and the crime scene can be compared to see if both samples are so similar that the crime scene cannot be excluded as the source of the questioned soil.

Traditional Analysis of Forensic Soil Samples

The analysis of forensically related soil samples relies heavily on physical appearance and chemical composition, encompassing a wide range of methods to differentiate various features. A visual comparison alone is often adequate for distinguishing soil samples that originate from different locations by determining physical characteristics including color, soil type, and particle size. Soil color can vary greatly depending on features such as moisture and clay content (Murray and Solebello

2002; Janssen *et al.* 1993). Also, organic material and grain type (e.g. coarse or round) can influence the color of the soil (Murray and Solebello 2002).

Another physical characteristic examined in forensic soil analysis is particle size distribution. Particles of soil in the sample are separated into defined size categories and the weights or volumes in each category are compared among samples using microscopy, sieving, or laser diffraction (Pye and Blott 2004; Wonogho *et al.* 1989; Wonogho *et al.* 1987). Light microscopy is utilized to examine the soil for the presence of plant material, animal material, or other debris which can act as additional points of comparison for the questioned and exemplar samples (Saferstein 2001). A density gradient test is also commonly employed in forensic soil analysis to see if a questioned soil sample shares the same properties as the exemplar (Chaperlin and Howarth 1983). Both elements and minerals in the soil are characterized by their physical properties using scanning electron microscopy (SEM) (Cengiz 2004; McVicar and Graves 1997). Chemical characteristics provide further information about soil. High Performance Liquid Chromatography (HPLC) (Siegel and Precord 1985) and Fourier Transform Infrared Spectroscopy (FTIR) (Cox *et al.* 2000) measure the organic content in soil.

Though it is possible to differentiate soil samples using one or more of these techniques, there are several limitations when analyzing soils in a forensic context. One disadvantage is that there is the potential for soils from completely different locations to share the same physical and chemical characteristics. Even though two soils may appear to be from the same location, it is difficult to individualize soil without the presence of unusual debris, rocks, or minerals (Saferstein 2001). Also, the value of forensic soil analysis is dependent on the variation of the soil around the crime scene. If physical and

chemical characteristics are not very diverse, it is difficult for the analyst to associate the soil with the crime scene. In contrast, extensive variation around the crime scene may make it difficult to link questioned and exemplar samples unless they came from the exact same location.

Another limitation with traditional methods is that they require an individual who has specialized experience in forensic geology. Junger (1996) stated that soil analysis is often excluded from forensic laboratories since there is the perception that expenses for equipment and training are too high for the "limited" value of the information gained. Further, many of the visual comparisons rely on the subjectivity of the analyst and statistical analysis is only used for particle size distribution to determine if there is a difference in the amount of soil found in each size class for different soil samples (Charzottes *et al.* 2004; Pye and Blott 2004; Wonogho *et al.* 1985). Finally, the amount of soil recovered from the suspect is often too small to carry out all the necessary analyses (Murray and Solebello 2002).

Microbial Community Analysis

Even though there are limitations using traditional soil analyses, few efforts have been made to develop alternative methods that might provide an easier, more objective way of differentiating soils from different locations. Some authors have described special cases where unique pollen found in a questioned soil sample was able to be linked to a crime scene (Brown 2002). Also, the analysis of microbial communities has been briefly mentioned in the forensic literature. Thornton (1986) suggested that microorganisms in the soil could be an additional method of classification for forensic soil samples based on

a previous environmental microbiology study that identified bacteria based on their enzymatic activity. Van Dijck and van de Voorde (1984) found that cultures of soil microorganisms from two gardens contained colonies that differed in morphology and color but did have some of the same fungi present. Fungal and bacterial cultures taken 5 meters apart in each garden had colonies of different colors and morphologies and some of the fungal species identified differed between these samples as well. Soil microbes from a suspected crime scene have been compared to soil collected from a suspect on two occasions, and both times colony color and morphology differed (Van Dijck and van de Voorde 1984).

A preliminary study by Horswell *et al.* (2002) showed the potential of microbial community analysis for forensic samples using Terminal Restriction Fragment Length Polymorphism (TRFLP; outlined below). The authors found that soil collected from a shoe or clothing and a simulated crime scene shared 90% of the same bacterial species and that samples collected from different locations were less similar than soil collected at the simulated crime scene. In addition to these promising studies, microbial community analysis is a potentially useful tool because it can be performed with equipment that is commonly used for standard DNA analysis in forensic laboratories. There is little additional training and expertise involved and the analyses can be performed quickly in comparison to traditional techniques. However, even though there are several advantages to microbial community analysis, the technique has yet to be utilized in a forensic laboratory setting and has gone virtually unnoticed in the field.

The 16S rRNA Gene in Microbial Community Analysis

A key feature of microbial community analysis is identifying a genetic marker or set of markers shared by the different bacterial species present, but which differs enough among them to distinguish each. Carl Woese and George Fox (1977) sequenced the 16S ribosomal ribonucleic acid (rRNA) gene from a variety of organisms and proposed that there were three domains of life, Bacteria, Archaea, and Eukaryote which led to the subsequent use of the 16S rRNA gene as a phylogenetic marker. The gene codes for a ribosomal RNA that is approximately 1500 nucleotides in length and makes up the majority of the small subunit of the ribosome in prokaryotes (Madigan *et al.* 2000). It is a functionally constant gene that is universally distributed in bacteria and has a well conserved sequence across distant species. However, there are enough sequence differences to allow identification of species (Madigan *et al.* 2000; Woese 1987). The characteristics that make the 16S rRNA gene a good marker for examining relationships among bacteria also allow it to be analyzed using common molecular biology and microbiology techniques. Molecular methods such as the polymerase chain reaction (PCR), widely exercised in forensic laboratories, make it possible for small amounts of ribosomal gene DNA (rDNA) from bacteria in the soil to be amplified. More importantly, rDNA from many bacterial species can be selectively amplified with the use of universal PCR primers that target conserved regions of the 16S rRNA gene (Liu *et al.* 1997).

Several techniques are available to examine variation in the 16S rRNA gene as well as other regions in the bacterial genome (Kitts 2001). Denaturing Gradient Gel and Temperature Gradient Gel Electrophoresis detect differences in base composition in the 16S rRNA gene and have been used to identify bacteria present in agricultural and zinc

contaminated soil (Brim *et al.* 1999; Ovreas *et al.* 1998). Single Strand Conformation Polymorphism also identifies differences in base composition in the 16S rRNA gene and has been used to examine microorganisms from soil surrounding plants in an agricultural field (Schwieger and Tebbe 1998). However, the major downfall of these techniques is that they have low resolving power and cannot necessarily detect single base pair differences among species. Another technique, Restriction Analysis, takes advantage of sequence differences in 16S rDNA by using restriction enzymes, which cut DNA at specific sites in the sequence. This results in a unique group of different sized pieces of DNA, or restriction fragments, for different bacterial species.

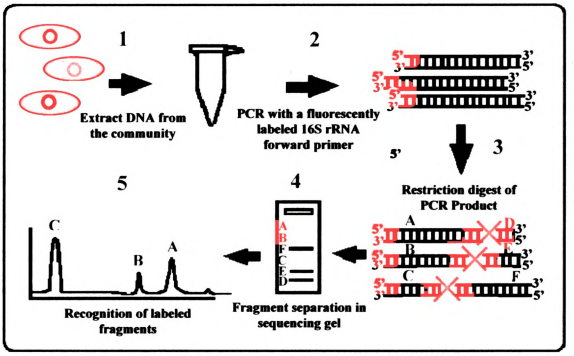
Terminal Restriction Fragment Length Polymorphism (TRFLP) Analysis

TRFLP (Figure 1) is an extension of Restriction Analysis except that only the terminal restriction fragments are compared among samples, allowing the rapid analysis of complex microbial communities. In recent years, TRFLP has become an increasingly popular technique for microbial analysis and has been employed in a wide range of applications (Kitts 2001). The utilization of TRFLP on soil samples is well documented and microbial community structure has been examined in a variety of environmental samples including sediment, sludge, sand, rice field soil, agricultural soil, and grassland soil (Kuske *et al.* 2002; Buckley and Schmidt 2001; Chin *et al.*1999; Clemet *et al.* 1998; Liu *et al.* 1997). The first step in obtaining a TRFLP profile is to extract bacterial genomic DNA. Soil bacteria have been cultured on selective media and then the DNA extracted (Avaniss-Aghajani *et al.* 1996), however, the use of universal PCR primers allows bacterial DNA to be amplified directly without the bacteria being cultured on

selective media (Kitts 2001). DNA from the soil can be isolated by homogenizing the soil to lyse bacterial cell walls and then performing an organic extraction of the genomic DNA (Liu *et al.* 1997). Commercial kits have also been developed to extract bacterial DNA from soil and remove inhibitors such as humic acid (http://www.mobio.com/products/productdetail.php?pid=159).

Once isolated from the soil, the 16S rRNA gene is PCR amplified (Step 2 of Figure 1). TRFLP requires that one of the primers is fluorescently labeled at the 5' end (Liu *et al.* 1997). After amplification, the 16S rDNAs are digested with a restriction enzyme producing different sized fragments for different bacterial species (Step 3 of Figure 1). Since the fluorescently labeled primer is at the 5' terminus of the 16S rDNA amplicons, only the 5' terminal restriction fragment is detected by the capillary electrophoresis machine (Step 4 of Figure 1). This results in a chart of fragments separated by size, or an electropherogram, and each terminal restriction fragment represents a different bacterial species (Step 5 of Figure 1) (Liu *et al.* 1997; Avaniss-Aghajani *et al.* 1996). The combination of peaks in a TRFLP profile shows the microbial community structure of the soil.

Figure 1. Schematic Illustrating the Terminal Restriction Fragment Length Polymorphism Procedure



First, genomic DNA from bacteria is extracted from the soil. Bacterial DNAs are amplified using PCR with a fluorescently labeled primer which results in many amplicons with a fluorescent label at one end. Amplicons are digested with a restriction enzyme, which generates fragments of different sizes. These fragments are separated using gel or capillary electrophoresis which detects the labeled fragments and generates a TRFLP profile (Image modified from the Ribosomal Database Project at Michigan State University, http://rdp8.cme.msu.edu/html/t-rflp_jul02.html) **This image is presented in color.**

Data obtained using TRFLP can be analyzed in several ways, including web-based analysis programs such as Michigan State University's Ribosomal Database Project (Cole *et al.* 2003) and the University of Idaho's Microbial Community Analysis Website (http://mica.ibest.uidaho.edu). One way TRFLP data can be compared among samples is to compute the degree of similarity between a pair of samples. A similarity index between zero and one is calculated by determining the number of terminal

restriction fragments that two samples have in common, with zero indicating that the samples share no peaks and one meaning they share all peaks.

Problems Associated with Microbial Community Analysis of Soil

Humic Acid Content

Though microbial community analysis can be a useful tool, the very nature of soil can introduce problems into the analysis. Soil contains humic acid which is made up of organic matter, humus, and humic substances (http://www.agconcepts.com/humicacid.htm), which are easily co-extracted with nucleic acids (Ogram *et al.*1987). Tebbe and Vahjen (1993) showed the activity of Taq polymerase was inhibited when they added commercially prepared humic acid to PCR reactions. Tsai and Olsen (1992) found that the sensitivity of PCR was lower for environmental samples compared to purified genomic DNA. Taq polymerase was also inhibited when humic extract was added to a PCR reaction regardless of the amount of DNA in the reaction. In addition, 10ng of commercially available humic acid inhibited a 100µl PCR reaction. The addition of proteins such as bovine serum albumin (BSA) or gp32 decreased inhibition from humic acids (Kreader 1996), and it was found that inhibition was removed if the humic extract added to the PCR reaction was highly diluted (Tsai and Olsen 1992). Additional purification steps can also be performed to reduce the levels of humic acid present in the DNA extracts (Smalla *et al.* 1993).

The presence of humic acid has also been shown to inhibit the activity of restriction enzymes. Smalla *et al.* (1993) isolated DNA from soil using an organic extraction/ethanol precipitation. The DNA underwent a series of purification steps

including a Cesium chloride (CsCl) precipitation followed by a potassium acetate (KAc) precipitation to remove proteins, RNA, and humic acid, and restriction analysis was performed before and after each step. The original DNA extract and the DNA that had undergone only the CsCl precipitation could not be digested. After the KAc precipitation, the DNA was partially digested. A final purification was done using either HCl-spermine or glass milk purifications and both resulted in complete digestion. LaMontagne *et al.* (2000) found that DNA extracted from compost using a method that resulted in higher humic acid content caused a decrease in the number of terminal restriction fragments and the intensity of the terminal restriction fragments compared to samples with less humic acid. TRFLP profiles from soils with high levels of humic acid also contained peaks that were not reproducible in replicate runs of a single sample.

Limitations of TRFLP Analysis

Analysis of TRFLP profiles is potentially difficult because there are often irreproducible peaks in replicate samples due to slight variations in the amount of DNA loaded. Dunbar *et al.* (2001) found that only 12% of peaks found in profiles from nine replicates of a single digestion were reproducible. Peak heights of all the terminal restriction fragments were added together to determine the total fluorescence for each replicate profile, ranging from 15000 to 35000 fluorescence units. The authors hypothesized that small pipetting errors were responsible for the variation seen. However, Osborn *et al.* (2000) found that profiles varied by a maximum of only 11% when three replicates of a single digestion were tested. The difference in the level of reproducibility between the two studies arises from the minimum threshold for peak heights that were included in the analysis of the data. Dunbar *et al.* (2001) included peaks that had a height

greater than 25 fluorescence units while Osborn *et al.* (2000) used a much higher threshold of 100 fluorescence units. When Dunbar *et al.* (2001) reanalyzed their data using 100 fluorescence units as the minimum threshold, the number of irreproducible peaks decreased by 75%.

Another limitation with TRFLP is that data analysis is still in the developmental stage. Often times TRFLP profiles are normalized to account for differences in DNA quantity among samples. In order to normalize profiles, those that have larger quantities of DNA are standardized to those with less DNA by proportionally reducing peak heights. The heights of all peaks are summed for each sample and the total fluorescence is divided by the smallest total fluorescence to obtain a correction factor. The height of each peak in a sample is then divided by the sample's correction factor and those that fall below the minimum height threshold are removed from the analysis. One must be careful when comparing normalized TRFLP profiles because standardizing a group of samples which includes a profile that is an outlier can remove important data. However, comparing data that have not been normalized can include irreproducible peaks in the analyses (Dunbar *et al.* 2001).

The Utility of TRFLP in Forensic Soil Analysis

The introduction of TRFLP as a viable forensic tool for identifying soils depends on several factors including changes in microbial community structure over time, the uniqueness of soil from different locations, and the extent of heterogeneity within a location. Temporal variability is important because soil from a crime scene will most likely be collected days, weeks, or even months after the crime has occurred. The

bacterial composition of soil may change over time due to climate and may make it difficult to link soil from a crime scene to soil collected from a suspect or victim. Large changes in microbial community structure at a single location over time may make the soil appear as though it is more similar to soil from a completely different location than from the original location. Little is known about the effects of time on microbial community structure and TRFLP profiles. Horswell *et al.* (2002) found that soil collected from a shoe and soil from the shoeprint had a similarity index of 0.910. When soil was collected from the original location of the shoeprint eight months later, the similarity index had decreased to 0.700. The abundance of certain microbes in soil containing potato plants have been shown to vary temporally (Lukow *et al.* 2000). Nineteen of 40 terminal restriction fragments had peak areas that varied significantly over time ($P<0.001$). Changes in the first three months were minimal with the most significant change occurring four months after the initial collection ($P<0.001$).

Variation in soils from different locations is another factor that needs to be taken into consideration. There are few studies that have simultaneously looked at the similarity or uniqueness of soils from different locations and heterogeneity within a location. Authors have shown that soils from different locations are different (Hackl *et al.* 2004; Horswell *et al.* 2002; Dunbar *et al.* 2000) but it is not known if soil in close proximity is more or less similar. Also, there are only a few studies examining soils from different locations that have not been contaminated with pollutants, contain similar vegetation, or are void of other factors that may influence microbial communities. Horswell *et al.* (2002) found that there were differences when soil from a simulated crime scene was compared to reference soils from different locations, with similarity indices among all

locations less than 0.600. Hackl *et al.* (2004) examined the diversity and structure of soils originating from different types of forests in Austria, including two pine forests, two oak-hornbeam forests, and two spruce-fir-beech forests. When terminal restriction fragments were compared among the three forest types, there were many bacterial species present in the two pine forests that were not seen in the oak-hornbeam and spruce-fir-beech samples. Cluster analysis of terminal restriction fragments showed that the two pine forests clustered separately from the other forests. Within the second cluster, the oak-hornbeam forests clustered together while the spruce-fir-beech samples did not. Also, the intensity of particular peaks was higher for the pine forest while other peaks were of higher intensity in the oak-hornbeam and spruce-fir-beech forests.

Finally, the level of heterogeneity within a location is important because if variation is large within a single location, it may be difficult to link two soil samples that originated from the same area. Previous research has shown that local heterogeneity does exist in soil. For example, spatial variation can be dependent on the plant material that grows at a location. Mummey and Stahl (2003) found differences in small and large scale spatial samples for two Wyoming grasslands harboring different grass species. Soils collected in close proximity and up to 100 meters away from the grassland containing *Boutelous gracilis* showed an average similarity of 0.730 while the other grassland (*Artemisia tridentate)* had an average similarity of 0.410 for soils that were located very close to each other.

Determination of the Utility of TRFLP in Forensic Analyses

The purpose of the research presented here was to consider the utility of TRFLP for the analysis of forensic soil samples by examining the site uniqueness, spatial variation, and temporal variation of microbial communities. Soil was systematically collected from five locations in central Michigan including an agricultural field, a marsh, a yard, a woodlot, and a sandy woodlot, monthly over a one year period. TRFLP profiles from the five locations were then compared to each other and similarity indices were calculated to examine the uniqueness of profiles at each location. Every third month, additional samples were collected 10 feet in each direction (N, S, E, and W) which were compared to determine the level of local heterogeneity. By examining these three issues collectively, the utility of TRFLP for forensic soil analysis was tested.

MATERIALS AND METHODS

Sample Collection

Soil samples were collected at a main (central) site at the beginning of each month from September 2004 through August 2005 from five locations in central Michigan: an agricultural field (A), a marsh (M), a yard (R), a woodlot (W), and a sandy woodlot (S) (Figures 2 – 4). In addition, soils were collected 10 feet from the main site in four directions (north (N), south (S), east (E), and west (W)) every three months; the north site could not be accessed at the marsh. For labeling purposes, soils were characterized by the month and year of collection, location, and site. For example, 904AM was collected from the agricultural field's main site in September 2004. Several scoops of soil were taken from the soil surface (approximately 0 to 5cm depth) and were placed in a plastic ziploc bag and mixed thoroughly. Soil samples were stored at -20°C within an hour of the time of collection.

Figure 2. Agricultural Field and Marsh Collection Locations

Left: Photograph of the agricultural field located in East Lansing, MI. Soybean was planted in the field during the summer of 2004 and corn was planted in the summer of 2005. Soil was tilled and fertilized at the beginning of May 2005.
Right: Photograph of marsh located in Williamston, MI. This location was undisturbed by human activity during the collection period.
These images are presented in color.

Figure 3. Yard and Woodlot Collection Locations.

Left: Photograph of the yard located in Holt, MI. The yard was mowed on a weekly basis during the summer months.
Right: Photograph of woodlot located in Mason, MI. The woodlot was undisturbed by human activity during the collection period.
These images are presented in color.

Figure 4. Sandy Woodlot Collection Location.

Photograph of the sandy woodlot located in Harrison, MI. This location was undisturbed by human activity during the collection period. Note that this location was approximately 100 miles away from the other locations. **This image is presented in color.**

DNA Extractions

DNA extraction and purification was performed using an UltraClean™ Soil DNA Kit (MO BIO Laboratories). One gram of soil was used for each extraction and the manufacturer's instructions were followed. Bacterial DNA was suspended in 50µl of Solution S5 and was stored at –20°C. When a TRFLP profile could not be obtained from a particular soil sample, DNA was isolated using a PowerSoil™ DNA Kit (MO BIO Laboratories). Each extraction required 0.25g of soil and the manufacture's instructions were followed. DNA isolated using the PowerSoil™ DNA Kit was suspended in 100µl of Solution S6. Five microliters of genomic DNA was separated on a 1% agarose gel to ensure the extraction was successful.

DNA Amplification of Genomic DNA from Soil

PCR amplification was performed using universal primers for the 16S ribosomal RNA gene (Amann *et al.* 1995; Giovannoni 1991; Lane 1991). DNA from *Escherichia coli* served as a positive control; no-DNA negative controls were also included. Initially, 8F (5'-AGAGTTTGATCCTGGCTC-3') was chosen as the forward primer and 1392R (5'-ACGGGCGGTGTGTACA-3') as the reverse primer. As the experiments proceeded, the reverse primer was changed to 1492R (5'-GGTTACCTTGTTACGACTT-3'), eliminating non-specific bands found with 1392R. 8F and 1492R amplify an approximately 1.4kb region of the 16S rRNA gene. In preliminary studies, the 8F primer was labeled with a 5' D4 phosphoramidite dye (Proligo) for detection on a Beckman-Coulter CEQ 8000 Genetic Analysis System. Subsequently, samples were electrophoresed on an ABI Prism 310, with the 8F primer 5'labeled with the blue dye 6-FAM (6-carboxyfluorescein).

Preliminary PCR reactions consisted of 10X PCR buffer (Promega), 10X $MgCl_2$ (Promega), 0.2mM dNTP (Promega), 1μM of each primer, 2μl of 1 μg/μl BSA, 1 unit Taq DNA polymerase (Promega), and 2μl of template DNA in a final reaction volume of 20μl. Primer concentrations were optimized at 10μM and 20μM. Both concentrations produced bands with similar intensity so a primer concentration of 10μM was used in subsequent PCR reactions. As experiments proceeded, 1 unit HotMaster Taq (Eppendorf), along with the provided 10X buffer, was used instead of standard Taq. The amplification reaction consisted of denaturation at 94°C for 2 minutes, followed by 20 cycles of denaturation at 94°C for 30 seconds, annealing at 58°C for 45 seconds, and extension at 72°C for 1 minute and 30 seconds and ended with an additional extension

step at 72°C for 4 minutes. If no band was present after 20 cycles, cycles were increased to 30. Amplicons were visualized by running 2µl of the PCR product on a 1% agarose gel followed by staining with ethidium bromide. DNA quantity for each sample was estimated from the gel by running 1µl of 1kb DNA ladder (New England Biolabs), comparing the amplicon to the 3kb fragment which contained approximately 250ng of DNA.

Restriction Digestion of Amplified 16S rDNA

The remaining 18µl of PCR product was purified using a Montage PCR Centrifugal Filter Device (Millipore) with one rinse of 400µl of TE buffer (10 mM Tris, 1 mM EDTA) , and centrifugation for 15 minutes at 1000x g. The samples were eluted in 18µl of TE. Restriction digest reactions consisted of 1 unit MspI (New England Biolabs), 1X NEBuffer 2 (New England Biolabs) and approximately 250ng of purified PCR product in a total volume of 10µl. Samples were incubated at 37°C for 4 – 6 hours. The digestion was terminated by deactivating the restriction enzyme at 70 – 75°C for 20 minutes. Digested DNAs were purified using a Microcon YM-30 column (Millipore) with 300µl of TE buffer and were centrifuged for 12 minutes at 14000x g. A total of two washes (300µl of TE buffer per wash) were performed and the final volume was returned to 10µl.

Capillary Electrophoresis of Restriction Digests

The restriction fragments were first separated on a CEQ 8000, which was later switched to the ABI Prism 310 genetic analyzer format. For the latter, 3µl of purified 16S

rDNA digest, 21.5µl of formamide and 0.5µl of ABI GeneScan 500 Liz size standard were heat denatured (95°C for 3 minutes), then chilled on ice. If samples were overloaded using 3µl of the 16S rDNA digest, 1µl of digest was used. TRFLP profiles were generated using ABI 310 Genetic Analyzer Data Collection Software version 3.0.0 (GS STR POP4 (1ml) G5.md5 module, 5 second injection, 15kV injection, 15kV run voltage, 28 minute run time). However, the GS STR POP4 (1ml) G5.md5 module was modified to include a 60 second injection and a 35 minute run time.

Analysis of TRFLP Profiles

Data analysis was done with ABI GeneMapper ID, version 3.1 software. A similarity index was calculated using the Ribosomal Database Project's TRFLP analysis program (http://rdp8.cme.msu.edu/cgis/trflp.cgi?su=SSU) by multiplying the number of terminal restriction fragments (± 1 base) shared between two samples by two and dividing by the total number of peaks present in both.

Terminal restriction fragments from 50 – 500 bases with heights over 50 fluorescence units were included in the data analysis. A TRFLP profile was considered successful when there were approximately 40 to 70 peaks within the analysis parameters and had a total fluorescence greater than 20,000 RFU. However profiles with a total fluorescence under 20,000 RFU were included in data analysis if their total fluorescence was no less than one third of the profiles they were being compared to; this prevented the removal of informative peaks from profiles that were normalized.

Four techniques were used to determine what peaks would be included when calculating similarity indices. First, all peaks that fell within the specified parameters (50

– 500 bases with a height greater than 50 fluorescence units) were used to calculate similarity indices. Second, data were normalized by summing the peak heights of all samples being compared. The combined peak height of each sample was divided by the combined peak height of the smallest sample to obtain a correction factor. The height of each peak in a sample was then divided by the sample's correction factor, and any peaks whose new height was below 50 fluorescence units were excluded from the analysis. In order to remove small and potentially irreproducible peaks, similarity indices were calculated using the top 20 and top 40 peaks by height. Similarity indices produced using each technique were compared to determine if a particular technique was best suited for TRFLP analysis.

Reproducibility of TRFLP profiles was examined in two ways. Similarity indices were calculated for triplicate PCR reactions of samples from each location's main site in September and March. In addition, similarity indices were also determined for five injections of a single restriction digest.

Similarity indices were calculated to examine temporal variability, site uniqueness, and within location heterogeneity. Monthly samples from the main site were compared to the other 11 months to determine the change in similarity between two consecutive months, and the similarity of one month compared to the other months. Main site samples from each location collected in January were compared to determine among location similarity, which was repeated for each month. Local heterogeneity was examined by comparing the five sites within a location. Also, each site was compared to itself during the months of March, June, September, and December.

Statistical Methods

Statistical analyses were performed using R statistical package version 1.9.1 (R Development Core Team 2004). Single factor Analysis of Variance (ANOVA) was chosen to examine the following: temporal variability for a single site within a location, the influence of time on among location variability, and the effect of time on local heterogeneity. ANOVA compares the difference among the means of two or more sample sets, while considering the variance around each mean. Results were considered significant at $p<0.05$.

Multivariate Analysis of Variance (MANOVA) was also used because soil was collected and analyzed over time. MANOVA, like ANOVA, compares the difference among the means of two or more sample sets but includes all dependent variables in a single analysis. With repeated measures data the independent variable for each level of the within subject factor, location and month in this study, was treated as a different dependent variable. Results for MANOVA were significant at $p<0.05$.

RESULTS

Determination of Soil Type and Organic Content

Soil type was determined for each location. Soils are classified based on the percent of sand, silt, and clay found in the soil as well as soil particle size and shape. The percentage of organic material was also determined for each location. Though the yard and woodlot were both sandy loams, the two locations differed in how much organic matter was present. Soil composition and percent organic material can be seen in Table 1.

Table 1. Soil Classification and Organic Material for All Locations.

Results for the mechanical analysis of soil samples from each location. The soil type for each location is given, which includes the percent sand, silt, and clay. The percent organic material is also shown.

Location	Soil Type	Sand (%)	Silt (%)	Clay (%)	Organic Material (%)
Agricultural	Loam	46.4	33.2	20.4	3.0
Marsh	Silt Loam	30.0	52.6	17.4	8.8
Yard	Sandy Loam	70.4	26.2	3.4	3.0
Woodlot	Sandy Loam	56.4	24.8	18.8	12.1
Sandy Woodlot	Loamy Sand	84.4	12.9	2.7	6.8

DNA Isolation and Purification

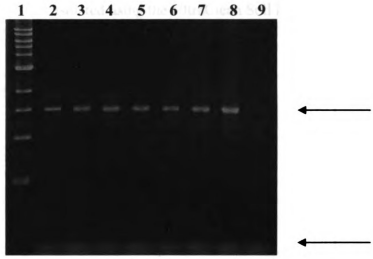
During the DNA isolation and purification process using the UltraClean Soil DNA Kit, the color of the products varied. DNA from the agricultural field was clear while DNAs from the marsh, yard, and woodlot were brown in color. Those from the sandy woodlot were dark red through much of the isolation and purification process. The discoloration was often removed during the filtration steps of the purification process.

When the PowerSoil DNA Kit was used, most of the discoloration was removed during earlier steps of the isolation and purification process. Remaining discoloration did not seem to affect downstream applications in a predicable manner, as DNA with any coloration experienced similar problems with 16S rDNA amplification and TRFLP profiling success.

16S rDNA Amplification

Amplification of 16S rDNA was successful for all 136 soil samples. Typical PCR results can be seen in Figure 5. A result was positive when the 1.4kb target amplicon was present (upper arrow) and negative when no band was observed but there were still primer dimers (small amplicons formed by the interaction of two primers). This product can be seen as the light bands at the bottom of Figure 5 (lower arrow) and should be present even if no bacterial DNA is added to the amplification reaction.

Figure 5. 16S rDNA Amplification Results.



Lane 1 is a 1kb DNA ladder. Lane 2 is the positive control (*E.Coli*). Lanes 3 – 8 are different soil samples from the agricultural field that had positive amplification results. Lane 9 is a negative control. The light bands near the bottom of the photograph are primer dimers.

Initially, DNA isolated using the UltraClean kit was tested. All 29 DNAs amplified using the labeled 8F primer for the CEQ 8000 after 20 cycles. When the labeled 8F primer for the ABI 310 was used 114 of the UltraClean isolates amplified after 20 cycles while the target band was absent for the remainder of the samples (Table 2). Primer dimers were still present indicating that PCR inhibition was not occurring. DNAs with negative results were then amplified using 30 cycles and all produced the 1.4kb amplicon. Even though increasing the number of cycles to 30 produced a positive result, there were often problems in obtaining TRFLP profiles for many of the DNA isolates (detailed below).

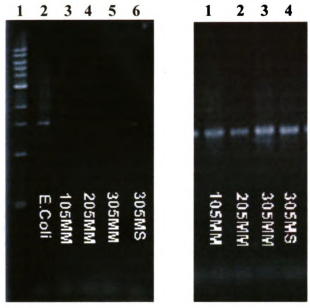
DNAs that did not produce complete or high intensity TRFLP profiles were re-extracted using the PowerSoil kit, all of which amplified after 20 cycles (Table 2). Figure 6 shows amplification results after 20 cycles which were negative for UltraClean extractions and positive for PowerSoil extractions.

Table 2. Amplification Results for UltraClean DNA Kit versus PowerSoil DNA Kit.

Amplification results for DNA isolated using the UltraClean Soil DNA Kit and the PowerSoil DNA Kit. Both sets of DNA were amplified using 20 cycles. – indicates a negative amplification result. + indicates a positive amplification result.

Sample	Amplification Results	
	UltraClean Kit + 20 Cycles PCR	PowerSoil Kit + 20 Cycles PCR
904MS	–	+
904ME	–	+
904MW	–	+
1204ME	–	+
1204MW	–	+
105MM	–	+
205MM	–	+
305MM	–	+
305MS	–	+
305ME	–	+
305MW	–	+
605MS	–	+
1204RM	–	+
1204RN	–	+
205RM	–	+
305RM	–	+
305RN	–	+
305RE	–	+
305RW	–	+
904WE	–	+
1004SM	–	+
1204SS	–	+

Figure 6. Amplification Results after 20 cycles of PCR for UltraClean and PowerSoil Isolations.



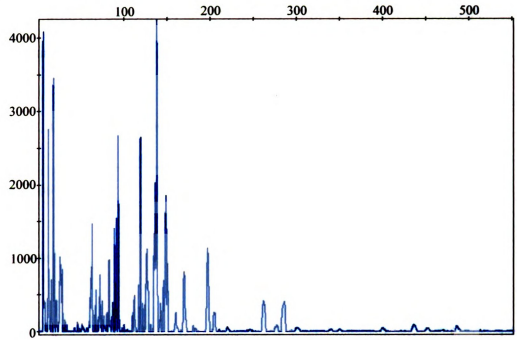
Left: An example of UltraClean isolated DNAs that did not amplify after 20 cycles of PCR. Lane 1 is a 1kb DNA ladder. Lane 2 is the positive control *(E.coli)*. Lanes 3 – 5 are DNAs with negative amplification results. Lane 6 had a very faint band.
Right: PowerSoil DNA samples that amplified after 20 cycles of PCR. Lanes 1 – 4 contained the target 1.4 kb fragment. Notice that the 305MS PowerSoil DNA has a much more intense band that the 305MS UltraClean DNA.

TRFLP Profiles

Terminal restriction fragments were initially analyzed using a CEQ 8000. Of the 29 samples tested, only seven produced a profile. The intensity of the peaks in these profiles was very low and some profiles contained very few peaks compared to profiles in other studies (Osborn *et al.* 2000; Liu *et al.* 1997). Since profiles were obtained from only 25% of the samples, TRFLP analysis was not continued on the CEQ 8000.

Profiles were obtained for all 136 samples using an ABI Prism 310. An example of a TRFLP profile that was considered successful (40 – 70 peaks and a total fluorescence over 20,000 RFU) can be seen in Figure 7.

Figure 7. Typical TRFLP Profile.

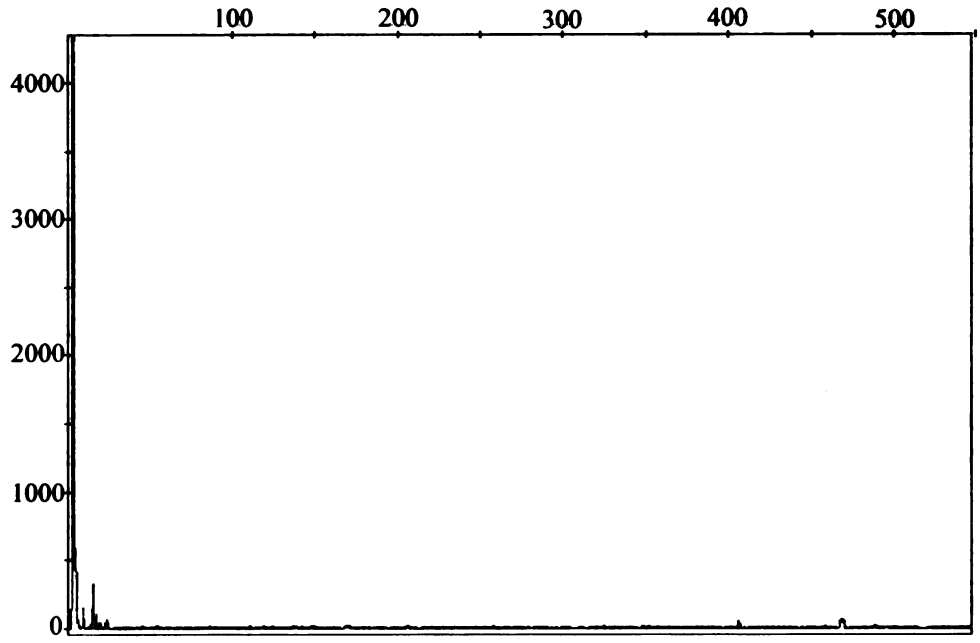


An example of a successful TRFLP profile. Peak size in bases is along the x axis while relative fluorescence units are on the y axis. Peaks less than 50 relative fluorescence units were excluded from analysis, as were peaks less than 50 bases or greater than 500 bases. **This image is presented in color.**

There was a difference between the UltraClean and PowerSoil DNA isolations in terms of obtaining a TRFLP profile. DNAs isolated using the UltraClean kit were analyzed using an ABI 310 and profiles were not produced for 46 of the samples. TRFLP profiles were negative or unsuccessful for one sample from the agricultural field, 22 samples from the marsh, five samples from the yard, seven samples from the woodlot, and 11 samples from the sandy woodlot. An example of a negative TRFLP profile can be seen in Figure 8. An additional 21 samples produced TRFLP profiles that were deemed unusable because their intensity was below 20,000 RFU and may have caused informative peaks to be removed from profiles of other samples during normalization. Seven of these were from the agricultural field, eight were from the yard, three were from

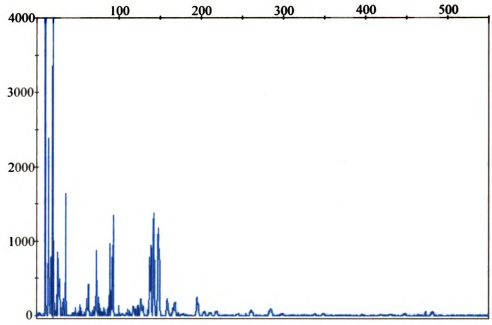
the woodlot, and three were from the sandy woodlot. An example of a low intensity TRFLP profile can be seen in Figure 9. In total, 67 of the 136 DNAs isolated using the UltraClean kit did not produce a useable TRFLP profile.

Figure 8. TRFLP Profile with No Signal.



An example of a TRFLP profile with no peaks present. Peak size in bases is on the x axis while relative fluorescence is on the y axis. Peaks were not detected within the analysis parameters. A few small peaks are present in the profile but were less than 50 bases. **This image is presented in color.**

Figure 9. TRFLP Profile with Low Intensity.



An example of a TRFLP profile where peak heights were too low to compare to other samples. Peak size in bases is on the x axis while relative fluorescence is on the y axis. The intensity of the peaks in this profile was nearly 75% lower than the peaks in Figure 7. **This image is presented in color.**

The 67 samples that did not produce a useable TRFLP profile were analyzed using extracts from the PowerSoil kit and all produced a profile. However, six of these were excluded from similarity index analyses because their total fluorescence was below 20,000 RFU. Samples 605RM and 904MW were excluded from normalization analyses because they had a total fluorescence of 6500 RFU and 8300 RFU respectively. 605AM was excluded from within location analysis and 904RW, 904SS, and 605SN were excluded from analysis of a single site within a location over time because their total fluorescence was one third or less the value of the other samples; when normalized to these at least half of the peaks were removed from the other profiles resulting in the loss of potentially informative data.

Similarity Index Calculations

An overall average similarity was calculated for each data analysis technique, no normalization (all peaks), normalized peaks, top 20 peaks by height, and top 40 peaks by height (Table 3). Twenty-three of the 25 temporal variability and within location heterogeneity comparisons had average similarity indices using the four methods that were within 0.1 of each other. However, there were 11 instances where top 20 average similarities were the lowest. For nine of these, the averages for the other techniques were more similar to each other than they were to the top 20 average similarities. For example, the average similarities for all peaks, normalized peaks, and top 40 peaks were within 0.003 of each other when the main site for a given month was compared to every other month for the sandy woodlot. In contrast, the average similarity of the top 20 peaks was at least 0.050 lower than the other techniques. When among location average similarities (by month) were examined, all 12 indices were lowest using the top 20 peaks while the average similarities obtained for the other methods were more similar to each other. Since the top 20 similarity indices were lower than the other analyses over 50% of the time, they were excluded from further analyses. Also, similarity indices calculated using all peaks within the analysis parameters were not included because these profiles may have contained small irreproducible peaks. Further analyses were done with normalized data since it is a common method and the differences in normalized and top 40 indices were minimal. The top 40 peak similarity indices were used for 605RM (removed above owing to low RFUs) when examining temporal variability.

Table 3. Average Similarity Indices for All Peaks, Normalized Peaks, Top 20 Peaks by Height, and Top 40 Peaks by Height.

Similarity indices were calculated using each of the data analysis techniques, with an average similarity value determined. The different comparisons in which similarity indices were calculated are displayed in the analysis column. "Annual" analyses compared each main site for a given month to every other month. "Within location" analysis compared the five sites within a location to each other for a given collection period. "Among locations" compared the main sites of all locations to each other for every month.

Table 3. Average Similarity Indices for All Peaks, Normalized Peaks, Top 20 Peaks by Height, and Top 40 Peaks by Height.

Analysis	All Peaks	Normalized	Top 20	Top 40
Annual Agriculture	0.563	0.557	0.521	0.546
Annual Marsh	0.604	0.626	0.613	0.603
Annual Yard	0.591	0.571	0.551	0.628
Annual Woodlot	0.600	0.633	0.619	0.653
Annual Sandy Woodlot	0.606	0.608	0.551	0.605
Within Agriculture March	0.670	0.685	0.678	0.711
Within Agriculture June	0.770	0.773	0.749	0.770
Within Agriculture September	0.711	0.716	0.617	0.709
Within Agriculture December	0.658	0.676	0.680	0.703
Within Marsh March	0.619	0.635	0.649	0.648
Within Marsh June	0.684	0.681	0.575	0.744
Within Marsh September	0.496	0.518	0.566	0.511
Within Marsh December	0.573	0.582	0.571	0.564
Within Yard March	0.644	0.707	0.669	0.700
Within Yard June	0.738	0.740	0.668	0.729
Within Yard September	0.636	0.645	0.645	0.690
Within Yard December	0.607	0.624	0.513	0.612
Within Woodlot March	0.528	0.562	0.532	0.610
Within Woodlot June	0.520	0.519	0.430	0.515
Within Woodlot September	0.574	0.612	0.627	0.617
Within Woodlot December	0.623	0.661	0.577	0.676
Within Sandy Woodlot March	0.580	0.578	0.587	0.578
Within Sandy Woodlot June	0.594	0.614	0.550	0.620
Within Sandy Woodlot September	0.516	0.550	0.560	0.560
Within Sandy Woodlot December	0.624	0.621	0.597	0.640
Among Locations January	0.477	0.492	0.425	0.463
Among Locations February	0.492	0.473	0.382	0.455
Among Locations March	0.435	0.418	0.400	0.500
Among Locations April	0.506	0.528	0.427	0.510
Among Locations May	0.472	0.490	0.362	0.481
Among Locations June	0.448	0.441	0.390	0.455
Among Locations July	0.502	0.488	0.357	0.477
Among Locations August	0.480	0.487	0.385	0.469
Among Locations September	0.534	0.540	0.390	0.515
Among Locations October	0.564	0.576	0.485	0.556
Among Locations November	0.541	0.564	0.435	0.555
Among Locations December	0.512	0.501	0.415	0.504

Reproducibility of TRFLP Profiles

The similarity among triplicate PCR reactions was determined using September and March samples from the main sites at all locations (Table 4). The highest similarity between two replicate reactions was 0.879 (305SM) while the lowest was 0.504 (305WM). The highest average similarity of 0.797 was seen for 904AM and the lowest similarity among PCR reactions was 0.610 for 305WM.

Table 4. Average Similarity Indices for Triplicate PCR Reactions.

Average similarity among three PCR reactions for several different soil samples. Triplicate PCR reactions were performed on September and March samples from all locations.

Sample	Average Similarity Index
904AM	0.797
904MM	0.657
904RM	0.755
904WM	0.640
904SM	0.772
305AM	0.707
305MM	0.748
305RM	0.712
305WM	0.610
305SM	0.643

The similarity of five different ABI 310 injections of the same restriction digest was then determined for two samples, 904WM and 305AM. The highest similarity between two replicate injections was 0.969 for 904WM and 0.948 for 305AM, while the lowest similarities were 0.610 and 0.609 respectively. It is notable that the first three injections had similarity indices between 0.863 and 0.969 for 904WM and the first four injections ranged from 0.826 and 0.948 for 305AM. Similarity indices for both samples

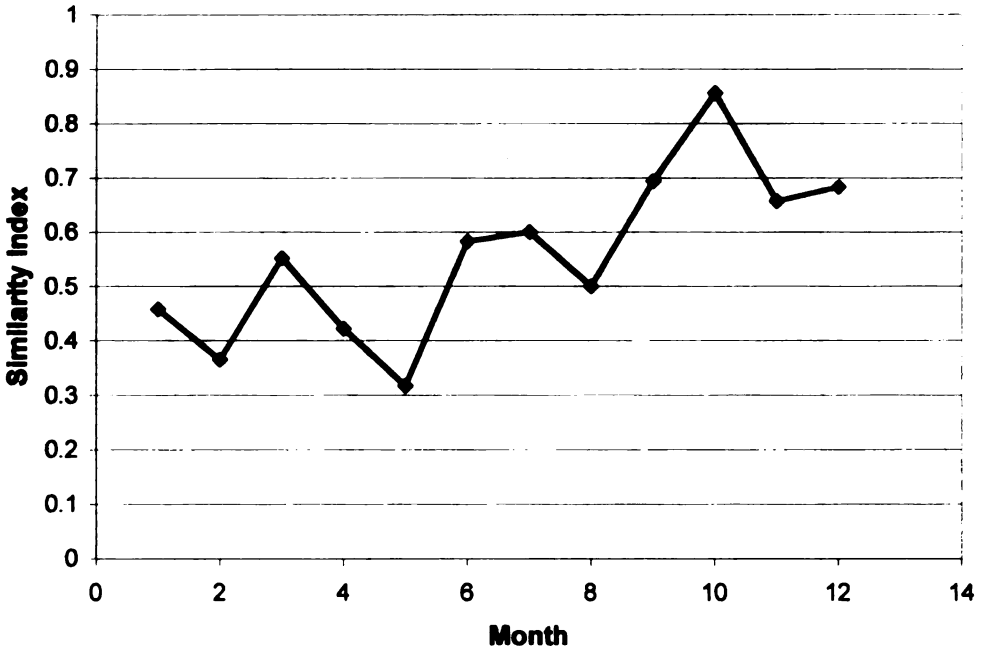
decreased during subsequent injections, ranging from 0.600 to 0.720. For example, the similarity between the first and second injection for 305AM was 0.948 while the similarity between the first and fifth injections was 0.644. The average similarity index for the five replicate injections was 0.780 for 904WM and 0.781 for 305AM.

Temporal Variability within a Location

Similarity indices from each month were compared to every other month for the five main sites at each location, to examine the similarity between any two given months. The ranges of similarity were 0.362 to 0.856 for the agricultural field, 0.417 to 0.841 for the marsh, 0.325 to 0.783 for the yard, 0.406 to 0.926 for the woodlot, and 0.409 to 0.785 for the sandy woodlot (Appendix A).

Temporal variability was examined by determining the change in similarity between consecutive months over the entire year (January through December). An example (agricultural field) is shown in Figure 10, where similarity indices fluctuated temporally, and there did not seem to be a pattern in the direction of the change. However, similarity indices were higher in the fall months (September through December) in general. The greatest change in similarity from one month to the next was 0.266 (May:June) while the smallest change in similarity was 0.017 (June:July).

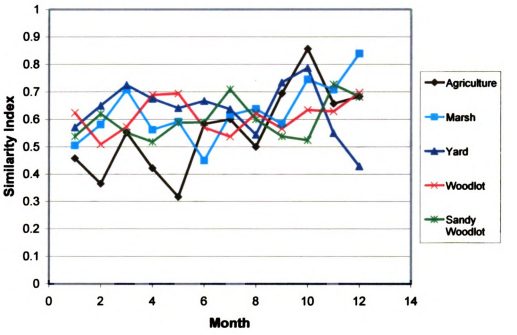
Figure 10. Monthly Variation in Similarity Indices for the Agricultural Field.



Change in similarity index from month to month for the agricultural field. Month is found on the x axis and similarity index is on the y axis. Data begin at month 1 (January) and continue through month 12 (December). Note that September through December soils were collected in 2004 and January through August soils were collected in 2005.

As seen with the agricultural field, similarity indices for all locations fluctuated between consecutive months, and the amount and direction of change varied from month to month (Figure 11). The greatest change was 0.266 which occurred from May to June in the agricultural field. In contrast, the change in similarity from May to June in the sandy woodlot was only 0.001. Though similarity indices varied widely, there was an overall increase in similarity during the fall months (September through December) for all locations except the yard. The greatest average change per month was seen for the agricultural field at 0.142, followed by the marsh (0.119), yard (0.094), sandy woodlot (0.079), and woodlot (0.068).

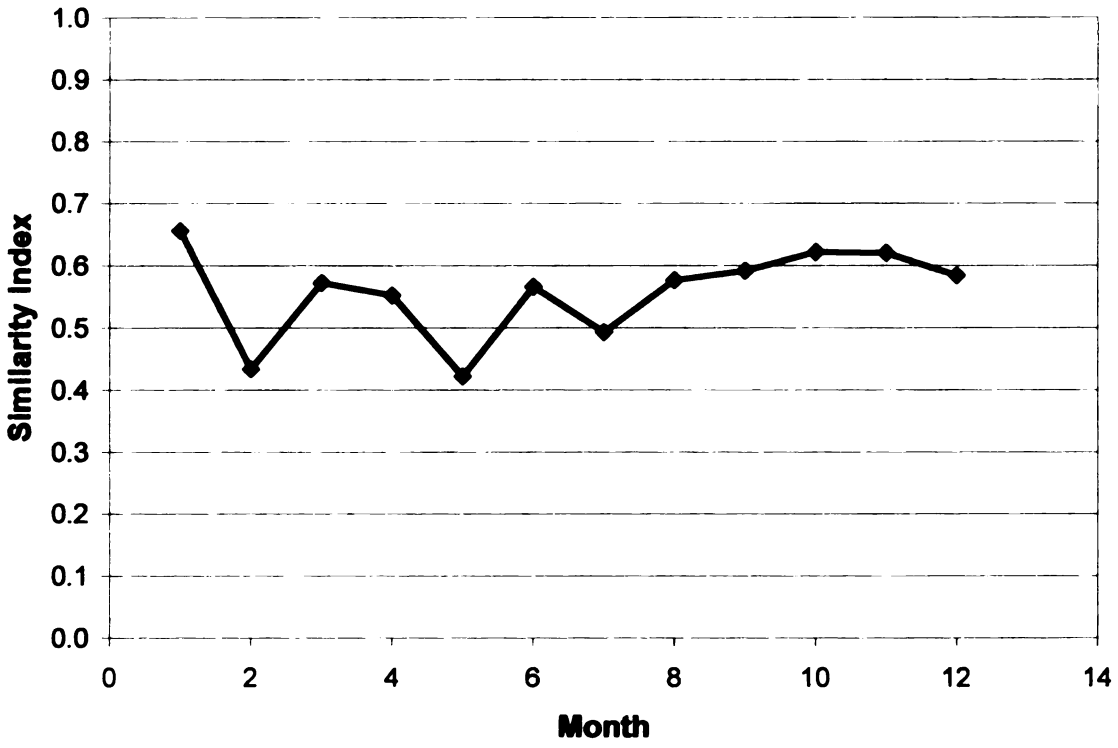
Figure 11. Monthly Variation in Similarity Indices for All Locations.



Change in similarity index from month to month for all locations. Month can be found on the x axis while similarity index is on the y axis. Similarity indices began in January and continued through December. Data for the yard in June were calculated using the top 40 peaks. The greatest change of 0.266 was seen from May to June in the agricultural field while the lowest change was 0.001 which was from May to June in the sandy woodlot. Similarity indices also increased during the fall months. Note that September through December soils were collected in 2004 and January through August soils were collected in 2005. **This image is presented in color.**

An average similarity was calculated for any given month compared to the other 11 months for the agricultural field (Figure 12), which fluctuated from January through July and began to level off during the fall months. The highest similarity of one month compared to the others was seen in January at 0.656 while the lowest was in May at 0.422 (Figure 12).

Figure 12. Average Similarity of a Single Month Compared to "Random" Month for the Agricultural Field.

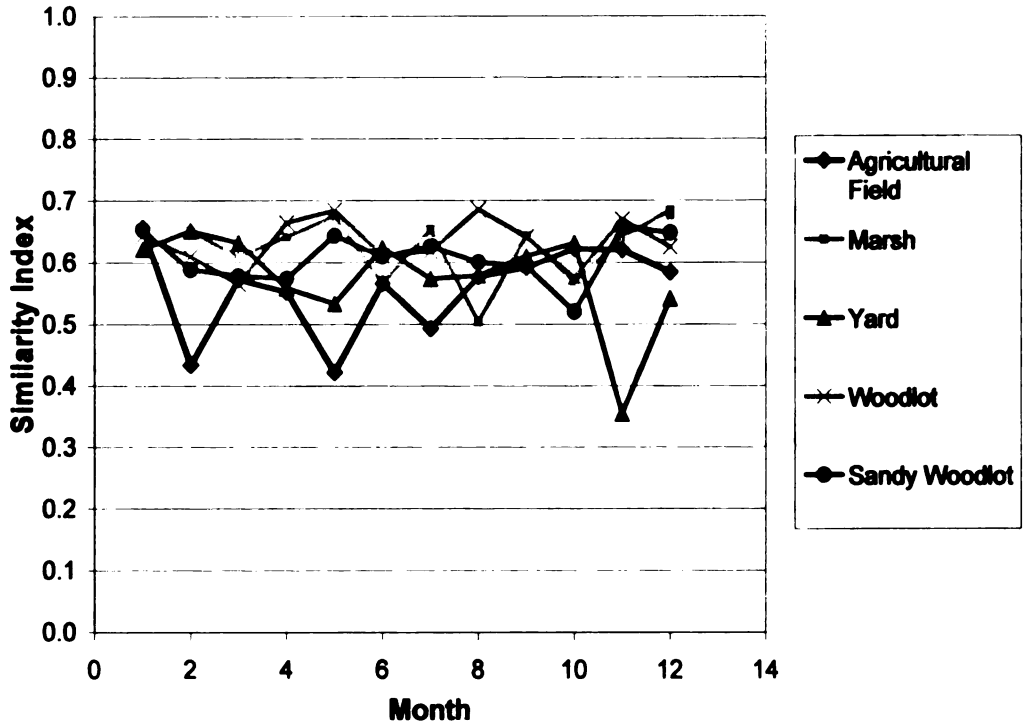


Average similarity of a given month compared to the other 11 months for the agricultural field. Month can be found on the x axis starting with January and continuing through December. Similarity index is found on the y-axis. Note that September through December soils were collected in 2004 and January through August soils were collected in 2005.

The average similarity for any given month compared to the other 11 months was calculated for all locations (Figure 13), which fluctuated as well. The range of average similarities was 0.504 to 0.684 for the marsh, 0.355 to 0.651 for the yard, 0.565 to 0.669 for the woodlot, and 0.520 to 0.657 for the sandy woodlot. During the first half of the year the agricultural field had the lowest average similarities of the five locations, however during the latter part of the year similarity levels were closer to the other locations. The similarity of the yard was much lower than the other locations in

November. There was no particular month where all locations had increased or decreased similarity compared to the other 11 months.

Figure 13. Average Similarity of a Single Month Compared to "Random" Month for All Locations.



Average similarity of a given month compared to the other 11 months for all locations. Month can be found on the x axis starting with January and continuing through December. Similarity index is found on the y-axis. Data for the yard in June were calculated using the top 40 peaks. Note that September through December soils were collected in 2004 and January through August soils were collected in 2005. **This image is presented in color.**

When month to all other month data were analyzed using MANOVA, there was a significant difference in similarity indices among locations ($p = 8.577*10^{-6}$) and a weak but significant difference in similarity indices by month ($p = 0.04561$). When each location was analyzed individually using ANOVA, only the agricultural field showed a statistical difference in similarity indices by month ($p = 0.04554$). There was no

significant difference in similarity indices by month for the marsh ($p = 0.5213$), yard ($p = 0.3552$), woodlot ($p = 0.654$), or sandy woodlot (0.8176).

Among Location Variability

Similarity indices were calculated to determine how similar or unique the five locations were to each other during any given month. The lowest among location value was 0.235 when the woodlot was compared to the sandy woodlot in March. The highest similarity index was 0.713, which occurred in November between the marsh and the woodlot (Appendix A). These values were then averaged for each location (e.g., the agricultural field was compared to the other locations for January, and these values were averaged; Table 5). The trend in fall similarity was borne out in that this value was highest in September for the agricultural field, in October for the marsh and sandy woodlot, and in November for the yard and woodlot. Most locations were least similar to the others in March, including the marsh, woodlot, and sandy woodlot. The agricultural field had the lowest similarity in March and June, while the yard was least similar in February. Location was not a significant factor in similarity indices comparing each location to the others ($p = 0.6445$). However, there was a significant difference in the similarity indices based on month ($p = 6.34*10^{-5}$). Month was significant in how similar a location was to the other locations for the marsh ($p = 0.03347$) and sandy woodlot ($p = 0.01646$) using ANOVA. There was no statistical difference in similarity index by month for the agricultural field ($p = 0.2209$), yard ($p = 0.1367$), or woodlot ($p = 0.07421$).

Table 5. Monthly Average Similarity Indices Comparing One Location to Other Locations.

Samples from the main site of each location were compared to the other four locations for a given month; these four values were then averaged. In addition, overall averages were calculated for each location and month. The similarity index was not available (N/A) for the yard location in June because the intensity of the profile was too low.

	Agriculture	Marsh	Yard	Woodlot	Sandy Woodlot	Average
January	0.535	0.485	0.518	0.524	0.399	**0.492**
February	0.443	0.473	0.467	0.509	0.475	**0.473**
March	0.434	0.398	0.506	0.372	0.340	**0.410**
April	0.525	0.562	0.590	0.544	0.418	**0.528**
May	0.478	0.437	0.523	0.520	0.494	**0.490**
June	0.434	0.454	N/A	0.472	0.401	**0.440**
July	0.464	0.506	0.547	0.536	0.358	**0.482**
August	0.494	0.492	0.507	0.524	0.420	**0.487**
September	0.570	0.544	0.545	0.528	0.511	**0.540**
October	0.557	0.584	0.578	0.519	0.642	**0.576**
November	0.535	0.562	0.591	0.602	0.475	**0.553**
December	0.490	0.500	0.519	0.554	0.442	**0.501**
Average	**0.497**	**0.500**	**0.536**	**0.517**	**0.448**	

The locations were most different from each other in March and most similar in October. The decreased similarity in March resulted from very low values for the marsh and both woodlots. The highest similarity in October was due to the sandy woodlot, which had the highest average seen for any location (0.642), although all locations had relatively high values in October, and through much of the fall. There was also an increase in similarity among locations in April in comparison to the other winter and spring months.

The woodlot and sandy woodlot were compared to each other (excluding the other locations) to see if two locations with potentially similar environments had similar microbial community structures. The average for the two locations over the entire year was a relatively low 0.450.

Within Location Heterogeneity

The similarity among the five sites (main, north, south, east, and west) at each location was determined for the four collection periods. The ranges for similarity indices between any two given sites were 0.560 (March) to 0.926 (September) for the agricultural field, 0.333 (September) to 0.784 (June) for the marsh, 0.487 (December) to 0.864 (June) for the yard, 0.391 (June) to 0.798 (March) for the woodlot, and 0.474 (September) to 0.704 (June) for the sandy woodlot (Appendix A).

Similarity results between all pairs of sites (e.g., N. to S.) within a location were then averaged for each of the four collection periods (Table 6). The agricultural field, marsh, and yard showed the highest within location similarity during the month of June while the highest similarity for the woodlot and sandy woodlot was seen in December. The sites within the agricultural field and yard were most different in December. Within location similarity was lowest for the marsh and sandy woodlot in September while the woodlot had the lowest similarity in June. Differences among locations were highly significant ($p = 2.288 * 10^{-11}$), while there was not a significant difference in overall similarity indices based on month ($p = 0.8576$). When each location was examined independently there was a significant difference in similarity indices based on month for the marsh, yard, and woodlot ($p = 0.002773$, $p = 0.001223$, and $p = 0.0001376$ respectively), but not for the agricultural field ($p = 0.6123$) or the sandy woodlot ($p = 0.1674$).

Table 6. Average Similarity Indices for Each Month of Within Location Collection.

Average within location similarity for each location. Similarity indices for all pairs of sites were averaged during the four months when soil was collected from all five sites within a location.

	Agriculture	Marsh	Yard	Woodlot	Sandy Woodlot
March	0.685	0.635	0.707	0.562	0.578
June	0.773	0.681	0.740	0.519	0.614
September	0.716	0.518	0.645	0.612	0.550
December	0.676	0.582	0.624	0.661	0.621
Average	**0.712**	**0.604**	**0.679**	**0.589**	**0.591**

In addition, each site at a location was compared to itself over the course of the year, and an average similarity was calculated (Table 7) producing similarities from 0.550 to 0.811. The two highest average similarities occurred in the yard (north and west) while the north site at the sandy woodlot had the lowest. The effect of a site was not a significant factor when measuring among site similarity ($p = 0.8633$). There was no significant difference in among site data based on site for the agricultural field ($p = 0.6526$), marsh ($p = 0.682$), yard ($p = 0.08277$), woodlot ($p = 0.7366$), and sandy woodlot ($p = 0.9573$) using ANOVA.

Table 7. Average Similarity Indices for Individual Sites Over the Year

Averages were calculated using similarity indices for a single site during within location collection periods. The similarity index was not available (N/A) for the north site at the marsh since it could not be accessed.

	Agriculture	Marsh	Yard	Woodlot	Sandy Woodlot
Main	0.636	0.645	0.63	0.555	0.637
North	0.619	N/A	0.783	0.58	0.417
South	0.685	0.647	0.637	0.597	0.612
East	0.702	0.552	0.64	0.577	0.521
West	0.632	0.591	0.811	0.561	0.609
Average	**0.655**	**0.609**	**0.574**	**0.574**	**0.559**

Comparison of Similarity Indices Using Minimum Thresholds of 50 and 100 RFU

Similarity indices comparing each month to every other month for the agricultural field were recalculated using a minimum threshold of 100 RFU. June was excluded from this analysis because its intensity was less than 20,000 RFU when peaks less than 100 RFU were removed from the profile. The resulting similarity indices were then compared to those using a minimum threshold of 50 RFU (Appendix B). One hundred and ten indices were compared, and of these 79 decreased in similarity from 50 RFU to 100 RFU, while 29 indices increased, and two did not change. The average change resulting from increasing the minimum threshold to 100 RFU was 0.036; there was a change greater than 0.100 in five instances.

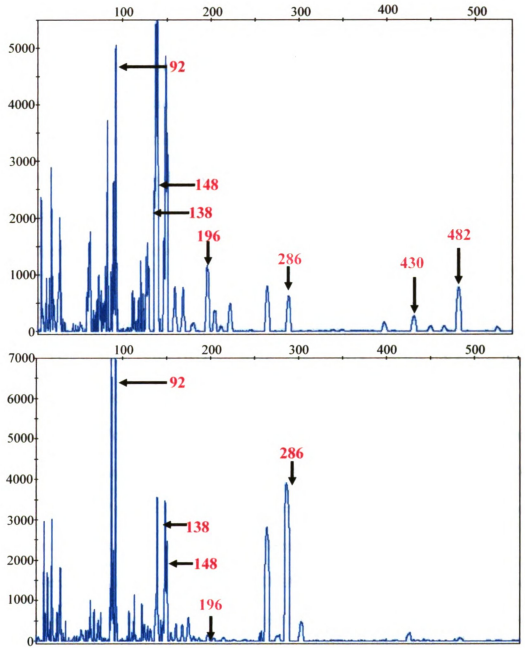
Unique and Shared Peaks Among Samples

Profiles from October and March were compared to see if specific peaks (bacterial species) were present, absent, or at highly different levels during certain times of the year, as well as if they were shared among locations. Some peaks were found to be common among profiles. For instance, a 92 base peak was present at all locations throughout the entire year and was always one of the highest peaks (Figure 14). Some other high intensity peaks shared by all locations were those at 138 and 148 bases. In contrast, some peaks were large and stable (occurring throughout the year) in some samples while being absent from others. The 197 base peak had a high intensity only in the woodlot profiles while the 170 base peak was large only in the yard profiles.

Peak intensities were found to change during different months at the various locations. For example, a 286 base peak was the same intensity in October and March in

the woodlot profiles, decreased from October to March in the agricultural field and marsh, and increased from October to March in the yard and sandy woodlot. On the other hand some peaks were present only during certain times of the year within a location including large 430 and 482 base peaks in the sandy woodlot profiles that were present in October but not in March (Figure 14). The 196 base peak present in the sandy woodlot profiles had a height of 1100 RFU in October and then decreased to 141 RFU in March (Figure 14).

Figure 14. TRFLP Profiles from the Main Site at the Sandy Woodlot in October and March.



The top profile is from the sandy woodlot in October and the bottom profile is from the same location in March. Peak size in bases is on the x axis while relative fluorescence is on the y axis. The 92, 138, and 148 base peaks were high intensity during both months. The 196 base peak decreased in intensity from October to March while the 286 base peak increased during this time. The 430 and 482 base peaks were present in October but not in March. **This image is presented in color.**

DISCUSSION

The goal of this study was to determine the utility of TRFLP in the analysis of forensic soil samples. Though analysis of microbial communities has been suggested in the forensic literature, very few studies have addressed this issue in a methodical and scientific way. This research was designed to examine if TRFLP could be used to match a questioned soil sample to a crime scene while taking three factors into consideration: whether samples collected at two different times can be linked to each other, if soils from different locations can be distinguished from one another, and whether local heterogeneity affects the ability to identify soil.

Discoloration during DNA Isolation and Purification

One purpose of the DNA purification process is to remove substances that may inhibit subsequent analyses. Though discoloration of DNA was present during both the UltraClean and PowerSoil isolations, the latter removed all discoloration before the DNA was filtered through a spin column. There was no apparent trend in residual DNA color and PCR/TRFLP profile success for the UltraClean kit. DNAs from the agricultural field were much clearer than those from other locations and always amplified after 20 cycles. However, the degree of discoloration of DNAs from the other locations did not seem to influence whether the DNA could be amplified after 20 or 30 cycles. For example, both the marsh and woodlot DNAs were brown when using the UltraClean kit, with the discoloration removed during the spin column step. However, even though the DNAs behaved in a similar fashion during purification, nearly 50% of the marsh samples did not amplify after 20 cycles compared to only 4% of the woodlot samples. Similarly, the

amount of discoloration did not seem to influence the quality of TRFLP profiles as DNAs of all colors produced profiles with no or low intensity peaks. DNA isolated using the PowerSoil kit amplified and produced TRFLP profiles no matter how much discoloration was present before the samples were filtered. Therefore, the color of the DNA extract could not be used as an indicator of amplification or TRFLP profile success using either kit.

UltraClean Soil DNA Kit versus PowerSoil DNA Kit

Bacterial DNA was amplified using universal 16S rRNA gene primers. DNA could not be amplified after 20 cycles for some samples (Table 2) and approximately 50% did not produce useable TRFLP profiles. DNAs from samples that did not produce a TRFLP profile were purified with the PowerSoil DNA kit, which was developed to handle a wider variety of soils than the UltraClean kit. The PowerSoil kit includes a "humic substance/brown color removal procedure" that claims to decrease inhibitors of PCR and produce better amplification results compared to kits from other suppliers (http://www.mobio.com/products/productdetail.php?pid=159). The DNAs that were extracted using the PowerSoil kit all amplified after 20 cycles and produced TRFLP profiles. Only one TRFLP profile could not be used for further analysis because its total fluorescence was too low. One possible reason for the difference in amplification and TRFLP profile success between the two DNA isolation procedures is that the PowerSoil kit was able to effectively remove humic acids that cause problems with DNA amplification and TRFLP profiling. DNAs isolated using the UltraClean kit likely retained impurities that reduced the activity of Taq polymerase and prevented the

restriction enzyme from working properly during the restriction digest. These results are in line with previous studies (LaMontagne *et al.* 2000; Smalla *et al.* 1993; Tsai and Olsen 1992) that found that humic acids interfere with PCR, restriction digests, and the intensity of TRFLP profiles.

Similarity Index Calculations

Similarity indices were calculated using four techniques: all peaks, normalized peaks, and the top 20 and 40 peaks by height. TRFLP profiles are typically normalized to account for differences in DNA quantities among samples and to remove small peaks that are irreproducible (Dunbar *et al.* 2001). The top 20 and top 40 peaks by height were used to calculate similarity indices because peaks with larger heights are often reproducible among samples (Osborn *et al.* 2002). Similarity indices calculated using the top 20 peaks were the lowest of the four techniques for over half of the comparisons. One possible reason this occurred is that the top 20 technique did not include all of the informative peaks in a TRFLP profile. The profile shown in Figure 7 had approximately 40 peaks over 500 RFU. This means that the top 20 technique excluded over 20 major peaks. The three other techniques produced similarity indices that were closer to each other than to the top 20 peak technique as they contained informative peaks that were reproducible among samples. In this study, similarity indices calculated using all peaks within the analysis parameters were not used because they could easily include a small number of irreproducible peaks. For example, the sample in Figure 7 contained almost 20 peaks that were below 200 RFU, which could easily drop out if less DNA was injected. In general, using either the normalized or the top 40 techniques would be appropriate for analyzing

the similarity among samples. Both techniques include informative peaks and exclude irreproducible peaks, however if a profile contains less than 40 peaks, or if many peaks in the profile have a low intensity, similarity indices should not be calculated using the top 40 peaks.

Reproducibility of TRFLP Profiles

One factor to take into account when performing any type of microbial analysis is how much of the variation among samples can be contributed to the method itself. Reproducibility was first examined by comparing triplicate PCR reactions from each location's main site in September and March. Substantial variability was observed among triplicate PCR reactions as most had an average similarity between 0.600 and 0.800 (Table 4). Osborn *et al.* (2002) found that triplicate PCR reactions varied more than triplicate digests as all peaks except three were present in the replicates. Dunbar *et al.* (2001) noted that before normalization of profiles, 24 peaks were reproducible and 169 peaks were irreproducible using a threshold of 25 fluorescence units. When the threshold was increased to 100 fluorescence units, the number of reproducible and irreproducible peaks decreased to 20 and 13 respectively. Given the impact on previous studies, similarity indices comparing one month to every other month for the agricultural field were calculated using both 50 and 100 RFU as minimum thresholds. In this study, increasing from 50 to 100 RFU had very little effect on similarity indices; the average change was 0.036 for 110 comparisons. Using a threshold of 100 RFU rather than 50 RFU caused 70% of the similarity indices to decrease, indicating that a threshold of 100 RFU appears to have excluded some informative peaks. However, it is still possible that

triplicate PCR reactions contained varying amounts of DNA and samples with less DNA contained low intensity peaks that dropped out.

Technical reproducibility was also examined by determining the variability among five injections of a single restriction digest, using two soil samples. Similarity indices ranged from 0.600 to 0.970, with an average similarity of 0.780. These were higher for the first injections and decreased with subsequent injections. Osborn *et al.* (2002) found that a single restriction digest showed no more than 11% variation, however, these were aliquots of a single digest and not replicate injections. A potential reason for the differences among replicate injections is that the amount of DNA in the sample decreased after every injection. Fragments that were just above the minimum threshold initially may have decreased below the threshold in later injections. This would explain why similarity indices were lower for subsequent injections and would also account for the difference in similarity indices between Osborn *et al.* (2002) and this study. Another possibility for the variability seen among replicate injections is the 60 second injection time utilized. Size standard fragments greater than 300 bases were found to become broadened with the longer injection, potentially leading to sizing miscalls.

Temporal Variability in Microbial Communities

A major goal of this study was to determine how microbial communities change temporally, by comparing soils collected monthly during a one year period. The difference in similarity indices between consecutive months showed that microbial communities do change over short periods of time (Figures 11 and 12). Similarity levels fluctuated from month to month; there did not seem to be a pattern in the direction or

amount of change. The likely reason for fluctuations between two consecutive months, and throughout the year, is changes in climate. In Michigan, extended periods of dry weather are limited and temperatures can vary greatly during a single month. Most bacteria found in terrestrial environments, including soil, are in the mesophile temperature class, which has optimal temperatures for growth ranging from 25 – 45°C. These bacteria do not grow well below 10°C (Madigan *et al.* 2000), meaning that any species that grows well at cooler temperatures will be at a selective advantage during much of the year.

Other environmental factors have been shown to cause competition among bacteria as well. Different bacterial species and strains can out compete each other on soybean roots in soil (George *et al.* 1987) and certain bacteria that degrade ammonia can be at a selective advantage (Verhagen *et al.* 1992). Month to month similarity may have been low in the summer because temperature and resources are optimal for the growth of many bacteria, putting none at an advantage. Likewise, selection is most likely minimal in winter as temperatures are too cold for the bacteria to grow. Similarity indices were generally higher in the fall for all locations with the exception of the yard. It is possible that there were certain species that grew well below the optimal temperatures and specialized on decaying vegetation such as leaves as an energy source. Such species would out compete others and become predominate at all locations, leading to higher similarity profiles. When samples from different seasons were compared there were peaks that were only present during certain months and the intensity of some peaks changed temporally (Figure 14). These changes show that bacteria grow differently over

the course of a year, potentially allowing certain species in certain habitats to out compete others during specific times of the year.

Additionally, average similarity indices for any given month compared to the other 11 months were calculated to determine how similar a particular month was to a random month (Figures 13 and 14). Forensically this is important in that reference soils could be collected days, weeks, or months after a crime occurred. The average random month similarity varied from 0.504 to 0.684 over the entire year for all locations, except for the yard and agricultural field (Figure 13). The agricultural field generally had lower averages during the first half of the year, while the yard had the lowest similarity of all locations (0.355) in November. The yard value was specific to that month, as its results were on par with other locations during the rest of the year.

Overall, average similarity for random month comparisons fluctuated much as they did for month to month similarity. Similarity indices did not appear to increase or decrease in one month compared to a random month, and there was no particular month(s) where microbial communities were more similar to those in other months for all locations. In fact, the five locations behaved differently temporally. When month to all other month similarity indices were analyzed using MANOVA, there was a significant difference among locations, which was heavily influenced by the agricultural field. This was the only location in which similarity indices were significantly influenced by month. They also had the greatest change in similarity index per month, and the lowest similarity among random months during the first part of the year. The high month to month variation in the agricultural field is likely due to the regular soil disturbance that took place there. Crops were planted during the summers of 2004 and 2005, the field was

tilled and fertilized in May, and crops were harvested in October. New plantings and fertilizers would be expected to influence the microbial community structure, and tilling would redistribute any microbes that were originally found in specific parts of the field. The yard also did not follow the pattern of having higher similarity indices in the fall, particularly for the month of November. The reason for this is unclear, and may have resulted from a simple outlier, although a yard is somewhat unique in being a long lasting monoculture that goes dormant in the fall. The role this unique habitat type played in the November data is not known.

Comparison of Microbial Communities Among the Five Locations

A critical factor addressed in this project was to see if soils from different locations could be distinguished using TRFLP. Soils from the main sites at the five locations were compared monthly (e.g., agriculture to yard for January; Table 5). The monthly similarity indices for a location were then averaged for the year (Table 5), producing values ranging from 0.448 for the sandy woodlot to 0.536 for the yard. These TRFLP results were in line with those obtained by Horswell *et al.* (2002) where similarity indices ranged from 0.480 to 0.590 when reference samples from different locations were compared to each other. There was a statistical difference in among location similarity based on month. However, when the similarity indices of one location compared to the other locations were analyzed, a statistical difference was only present for the marsh and sandy woodlot. The average similarities of one location compared to the other locations were highest in October or November and lowest in February or March.

Though the overall averages for each location were within 0.100 of each other, the sandy woodlot was most different when compared to the other locations. The similarity did not increase when the two woodlots were compared alone showing the two locations that represent the most similar ecosystems did not share a more similar microbial community structure. One possible explanation for the difference in average similarity indices is that the sandy woodlot was located nearly 100 miles from the other locations. It seems plausible that distance has as much or more of an effect on the microbial makeup of soil as does habitat.

The soil type of each location did not appear to greatly influence how similar a location was to other locations. Both the yard and woodlot were classified as sandy loam and their average similarities were not any closer than locations with different soil types. Additionally, the average similarities of the agricultural field and the marsh differed by 0.030 even though they had different soil types.

The influence of time of year was examined by combining values from all locations for each month (Table 5). Overall similarity was highest in October (0.576) and the fall in general, and lowest in March (0.410). There was also an increase in similarity among locations in April in comparison to the other winter and spring months. It is interesting to consider why this was the case. March to April carries a strong change in climatic conditions in Michigan. It seems plausible that certain common bacterial species begin to 'takeoff' as the weather warms, before a more diverse flora can form. In the fall, this diversity may begin to disappear when cooler temperatures, moisture, and presence of organic material make conditions optimal for growth of certain bacterial species. The statistical difference in similarity indices by month for the marsh and sandy woodlot

could result from some common bacteria being present less often in these locations, differences in the way the locations responded to weather, or simply distance—with one of the sites being distant to the others.

Heterogeneity of Microbial Communities Within a Location

The final issue that was addressed in this study was heterogeneity within a location. Average similarities comparing the sites within locations ranged from 0.518 (Marsh in December) and 0.773 (Agricultural Field in June; Table 6). This indicates that microbial makeup varies within a location, even at a distance of only 10 feet. This is comparable to what Mummey and Stahl (2002) found when they examined two grasslands that were approximately 90 miles apart. The grassland containing *Boutelous gracilis* had an average similarity of 0.730 while the *Artemisia tridentate* grassland had an average similarity of 0.410. One possible cause for the higher similarity within the agricultural field is that the soil was mixed during tilling, acting to homogenize the samples. A second explanation is that the same vegetation was present throughout the agricultural field, decreasing the environmental variation that could affect microbial communities. The marsh, woodlot, and sandy woodlot may have had increased local heterogeneity because the sites themselves were more heterogeneous, perhaps in the amount of decaying vegetation, sunlight exposure, etc. In contrast, it appears locations that are moderately or heavily modified by humans, such as the yard or agricultural field, have an increased similarity among sites, which also makes sense given the monocultures that existed in them.

The agricultural field, marsh, and yard showed their highest within location similarity during the month of June while the highest similarity for the woodlot and sandy woodlot was seen in December. Higher similarity indices in June may be due to increased temperatures causing similar bacteria at the different sites to grow while the increased similarity in December for the two woodlots could have been due to the presence of decaying leaves on the ground or more sunlight reaching the ground because leaves had fallen from the trees. As previously mentioned, all of these factors could help explain why some bacteria may out compete other bacteria within a location. There was a significant difference among sites based on location. However, a significant difference in within location similarity indices based on month was only detected for the marsh, yard, and woodlot, demonstrating that the sites within locations had more variability during particular months.

Individual sites within a location were also examined over the course of a year (Table 7). All but three sites had average similarities between 0.550 and 0.700 ensuring that most sites were behaving similarly over the year. The yard's north and west sites had higher average similarities over time (0.783 and 0.811 respectively) than other sites, perhaps in part because they were found near the roots of a black walnut tree (*Juglans nigra)*. Black walnuts produce juglone (5 hydroxy-1, 4-napthoquinone), a chemical which can travel into the soil and is toxic or growth stunting to many different types of plants. Bacteria that break down juglone have been shown to grow at a faster rate than those that cannot (Schmidt 1988) and these species may have been dominant in the soil at the two yard sites. It could not be determined why the sandy woodlot had an average

similarity that was lower than the other sites, although this diversity is consistent with that seen at the main site of the sandy woodlot.

The average similarity of each site over the year was compared to the average similarity among the five sites over the year. For all locations except the yard, similarity was higher among the five sites during a collection period than was similarity at a given site over time; in other words, time resulted in more heterogeneity than position. The exception to this in the yard may again have resulted from a very different microflora around the walnut tree.

Utility of TRFLP in Forensic Soil Analysis

This study shed light on several factors that could influence the ability of TRFLP to link a questioned soil to a crime scene. When determining the utility of TRFLP in forensic soil analysis it is important to examine temporal variability, among location similarity, and within location heterogeneity collectively to see if similarity indices for one sample can be distinguished from others. In the data presented there was substantial overlap in similarity indices for all of these factors. The heterogeneity at a location, assayed by examining the main site and four sites 10 feet away in all four directions (Table 6) generated similarity indices that were usually higher than the other comparisons (Table 5), meaning that in general the five locations could be differentiated from each other. However, among location averages during April and the fall months (Tables 5) overlapped with within location averages for the marsh, woodlot, and sandy woodlot (Table 6), making soil differentiation difficult or impossible during these times. The large amount of month to month variation observed (Figure 10) also made soil differentiation

uncertain. For example, during the months of January, February, and May, the similarity indices in the agricultural field were as low or lower than many among location results.

A potential problem with soil identification is that testing a single sample can generate a similarity value that could be found among unrelated soils. For instance, a similarity index close to 0.500 was obtained between June and July at the agricultural site, as well as the east and west sites within the marsh, and between the woodlot and sandy woodlot in September. However, each location was more similar to itself on average, thus including multiple samples in a forensic soil analysis may have some utility. Certainly this should not be a problem for most crime scenes, but may be much more difficult for the soil accompanying the victim or suspect.

The reproducibility of TRFLP profiles also introduces complications into TRFLP analysis. Similarity indices for replicate injections and PCR reactions were on average higher than other comparisons, as expected. However, there were instances where the similarity of two replicate PCR reactions, or even replicate injections, was lower than soils from two different locations. If replicate PCR reactions from a single DNA extraction show this level of variation, there is the risk that differences between questioned and exemplar soils from the same source may arise from the methods itself. Therefore, a single comparison between two samples may be misleading. This problem could be remedied by performing replicate PCR reactions for both the questioned and exemplar samples to get a better picture of the bacteria present. It may also be useful to run multiple digests of the DNA, producing a set of profiles for each sample. These should on average result in a more thorough examination of the similarity or lack thereof between two samples.

The change in microbial communities over time adds another confounding factor to a forensic analysis. Exemplar soils from the crime scene are often collected weeks or even months after the crime has occurred. The amount and unpredictable nature of fluctuation in similarity indices over time could make a soil sample collected from a crime scene appear to have come from a completely different location. A portion of this is caused by normal climatic change temporally, however human disturbances also appear to affect microbial communities. The agricultural field experienced the most disturbances over the year and was the only location where month was a significant factor in similarity indices. The passage of time can only increase the chance of disturbance at a crime scene, resulting in a decrease in similarity between a questioned and reference sample. These differences could lead to the false exclusion of soils that came from the same location and would be detrimental to the forensic investigation. Likewise, at certain times of the year unrelated soil samples can look quite similar and a false inclusion would occur.

This is not to say that TRFLP will never be useful in forensic soil analyses, and the research presented give insight into certain steps that should be taken when collecting soil. First, it is undoubtedly better if soil is collected from the crime scene as soon as possible, ideally within the first month or earlier. If collection occurs shortly after the crime, there is less time for climatic change and other factors to occur that can affect microbial community structure. However, further research should be performed on soil collected daily, biweekly, or weekly to determine how similarity changes over these shorter time spans. This should be done during different times of year to establish what factors may influence microbial communities over time. In addition, soil should be

collected from several places around the crime scene to get an idea of the local variation that exists. Knowledge of local heterogeneity could help to exclude a questioned soil if it is compared to many different exemplar samples.

TRFLP could have increased utility if a more specific assay is employed, since the 16S rRNA gene is ubiquitous among bacterial species in soil. The use of the 16S rRNA gene may have resulted in TRFLP profiles that were too detailed and included high levels of noise. The inclusion of too much information about all bacteria species in soil could allow one to overlook certain bacterial clades that differ among soils. In this case it would be helpful to target genes that are unique to bacterial groups not found in all soil types or environments. For example, methanogens (bacteria that produce methane as a byproduct) are commonly present in wetlands or marshes. Lueders *et al.* (2001) analyzed the methyl-coenzyme M reductase gene in methanogens isolated from rice field soil and found there were group specific terminal restriction fragments. Some genera of methanogens had a single TRFLP fragment, while others, such as *Methanosaetecae* and *Methanobacteriaceae*, had two or three characteristic terminal restriction fragments. None of these distinct peaks were shared among groups, allowing separation of methanogenic lineages. Additionally, species from the genus *Bacillus* are often found in soils with organic material while those from the genus *Streptomyces* are present in aerobic conditions, including sandy loams (Madigan *et al.* 2000). Using a set of genetic markers that is more specific to certain clades rather than an all-inclusive maker has the potential to differentiate soils that originated from diverse locations. It would also help link soils that came from the same location if they share genetic markers present in bacteria that are unique to certain soil types or environments.

If these steps are taken, a TRFLP profile may contain information that is useful to the forensic investigation. Combined with results from traditional forensic soil analyses, microbial similarity between the questioned sample and the suspected crime scene could add more power to the soil evidence. However, the addition of these steps does not guarantee that TRFLP will become a useful tool in linking questioned and exemplar soil samples. Using the technique presented here, the overlap in similarity indices and changes in microbial communities over time would make it almost impossible to identify soil samples, and thus it is unlikely that the technique will ever stand alone in the forensic laboratory setting.

Conclusions

Based on the results obtained from the current study TRFLP is a relatively easy and quick method that can be used to monitor microbial communities found in soil. The greatest similarity in microbial communities occurs among adjacent sites within a given location, collected at the same time. However, the similarity at a site from month to month, as well as one month compared to a random month, fluctuates substantially over the course of the year. There is no apparent trend in how much or in which direction similarity fluctuates, but similarity from month to month at a location does seem to increase in the fall in general. Similarity indices were on average lowest when comparing different locations to each other, but here again similarity among locations increased during particular times of the year, specifically during the fall, and covered a wide range of values.

The overlap in similarity indices for different comparisons indicates that the forensic usefulness of TRFLP may be limited. Also, the similarity among TRFLP profiles from replicate PCR reactions and injections overlapped with similarity indices from other comparisons. When examining all factors together, it would be unlikely to successfully link a single soil sample from a victim or suspect to a crime scene, especially if soil is collected long after the crime has occurred. It appears that substantially more research will be required if TRFLP is to become commonplace in the forensic laboratory.

APPENDIX A:

Similarity Indices Calculated to Determine Temporal Variability, Among Location Similarity, and Within Location Heterogeneity.

Table 8. Annual Similarity Indices for Each Location.

Each month was compared to every other month for the five main sites at each location to determine the similarity between any two given months during the year. The number of peaks in each profile is displayed in the first column. Month can be found in the second column and along the top row.

Agricultural Field													
Number of Peaks		Sept	Oct	Nov	Dec	Jan	Feb	Mar	Apr	May	Jun	Jul	Aug
48	September		0.694	0.689	0.705	0.775	0.433	0.725	0.478	0.404	0.604	0.500	0.500
60	October	0.694		0.856	0.645	0.754	0.461	0.650	0.606	0.462	0.602	0.500	0.608
58	November	0.689	0.856		0.657	0.759	0.380	0.634	0.667	0.385	0.664	0.469	0.660
47	December	0.705	0.645	0.657		0.683	0.404	0.578	0.571	0.419	0.638	0.471	0.652
54	January	0.775	0.754	0.759	0.683		0.458	0.701	0.663	0.460	0.625	0.609	0.729
42	February	0.433	0.461	0.380	0.404	0.458		0.365	0.395	0.602	0.460	0.362	0.452
43	March	0.725	0.650	0.634	0.578	0.701	0.365		0.552	0.416	0.604	0.494	0.576
44	April	0.478	0.606	0.667	0.571	0.663	0.395	0.552		0.422	0.598	0.549	0.570
46	May	0.404	0.462	0.385	0.419	0.460	0.602	0.416	0.422		0.317	0.286	0.466
58	June	0.604	0.602	0.664	0.638	0.625	0.460	0.604	0.598	0.317		0.583	0.530
38	July	0.500	0.500	0.469	0.471	0.609	0.362	0.494	0.549	0.286	0.583		0.600
42	August	0.500	0.608	0.660	0.652	0.729	0.452	0.576	0.570	0.466	0.530	0.600	

Table 8 (cont'd)

Marsh

Number of Peaks		Sept	Oct	Nov	Dec	Jan	Feb	Mar	Apr	May	Jun	Jul	Aug
51	September		0.583	0.752	0.649	0.541	0.568	0.452	0.582	0.474	0.594	0.417	0.638
52	October	0.583		0.745	0.776	0.657	0.646	0.670	0.636	0.513	0.794	0.433	0.585
50	November	0.752	0.745		0.708	0.598	0.691	0.685	0.773	0.626	0.686	0.547	0.712
46	December	0.649	0.776	0.708		0.839	0.589	0.841	0.753	0.550	0.683	0.429	0.610
47	January	0.541	0.657	0.598	0.839		0.505	0.820	0.702	0.562	0.676	0.565	0.693
44	February	0.568	0.646	0.691	0.589	0.505		0.581	0.703	0.578	0.657	0.528	0.663
42	March	0.452	0.670	0.685	0.841	0.820	0.581		0.708	0.579	0.649	0.414	0.656
47	April	0.582	0.636	0.773	0.753	0.702	0.703	0.708		0.562	0.775	0.554	0.673
65	May	0.474	0.513	0.626	0.550	0.562	0.578	0.579	0.562		0.592	0.591	0.597
55	June	0.594	0.794	0.686	0.683	0.676	0.657	0.649	0.775	0.592		0.450	0.624
45	July	0.417	0.433	0.547	0.429	0.565	0.528	0.414	0.554	0.591	0.450		0.616
54	August	0.638	0.585	0.712	0.610	0.693	0.663	0.656	0.673	0.597	0.624	0.616	

Yard

Number of Peaks		Sept	Oct	Nov	Dec	Jan	Feb	Mar	Apr	May	Jun	Jul	Aug
50	September		0.745	0.663	0.604	0.554	0.625	0.664	0.704	0.704	0.558	0.366	0.589
44	October	0.745		0.798	0.629	0.623	0.579	0.598	0.761	0.783	0.641	0.355	0.640
45	November	0.663	0.798		0.604	0.551	0.635	0.611	0.667	0.774	0.615	0.364	0.656
61	December	0.604	0.629	0.604		0.426	0.634	0.581	0.606	0.578	0.590	0.376	0.547
33	January	0.554	0.623	0.551	0.426		0.563	0.531	0.580	0.667	0.667	0.338	0.500
70	February	0.625	0.579	0.635	0.634	0.563		0.692	0.551	0.568	0.667	0.402	0.487
63	March	0.664	0.598	0.611	0.581	0.531	0.692		0.649	0.586	0.577	0.358	0.519
48	April	0.704	0.761	0.667	0.606	0.580	0.551	0.649		0.708	0.641	0.338	0.538
48	May	0.704	0.783	0.774	0.578	0.667	0.568	0.586	0.708		0.641	0.325	0.613
38	June	0.558	0.641	0.615	0.590	0.667	0.667	0.577	0.641	0.641		0.667	0.603
32	July	0.366	0.355	0.364	0.376	0.338	0.402	0.358	0.338	0.325	0.667		0.325
45	August	0.589	0.640	0.656	0.547	0.500	0.487	0.519	0.538	0.613	0.603	0.325	

Table 8 (cont'd)

Woodlot

Number of Peaks		Sept	Oct	Nov	Dec	Jan	Feb	Mar	Apr	May	Jun	Jul	Aug
51	September		0.566	0.583	0.674	0.667	0.727	0.546	0.720	0.663	0.483	0.798	0.622
48	October	0.566		0.634	0.609	0.645	0.448	0.552	0.519	0.621	0.674	0.406	0.663
45	November	0.583	0.634		0.629	0.633	0.548	0.664	0.733	0.761	0.735	0.613	0.826
44	December	0.674	0.609	0.629		0.697	0.554	0.545	0.680	0.692	0.549	0.565	0.681
45	January	0.667	0.645	0.633	0.697		0.624	0.566	0.574	0.685	0.614	0.656	0.717
48	February	0.727	0.448	0.548	0.554	0.624		0.509	0.760	0.642	0.558	0.792	0.547
68	March	0.546	0.552	0.664	0.545	0.566	0.509		0.573	0.565	0.538	0.560	0.600
56	April	0.720	0.519	0.733	0.680	0.574	0.760	0.573		0.689	0.617	0.712	0.728
47	May	0.663	0.621	0.761	0.692	0.685	0.642	0.565	0.689		0.694	0.589	0.926
38	June	0.483	0.674	0.735	0.549	0.614	0.558	0.538	0.617	0.694		0.570	0.694
48	July	0.798	0.406	0.613	0.565	0.656	0.792	0.560	0.712	0.589	0.570		0.537
47	August	0.622	0.663	0.826	0.681	0.717	0.547	0.600	0.728	0.926	0.694	0.537	

Sandy Woodlot

Number of Peaks		Sept	Oct	Nov	Dec	Jan	Feb	Mar	Apr	May	Jun	Jul	Aug
53	September		0.538	0.638	0.700	0.606	0.638	0.535	0.545	0.611	0.547	0.575	0.600
53	October	0.538		0.524	0.530	0.500	0.571	0.505	0.409	0.593	0.442	0.547	0.564
52	November	0.638	0.524		0.727	0.720	0.635	0.680	0.642	0.785	0.617	0.676	0.587
47	December	0.700	0.530	0.727		0.682	0.626	0.621	0.587	0.725	0.652	0.680	0.596
41	January	0.606	0.500	0.720	0.682		0.538	0.573	0.704	0.708	0.699	0.702	0.755
52	February	0.638	0.571	0.635	0.626	0.538		0.620	0.578	0.579	0.617	0.543	0.532
48	March	0.535	0.505	0.680	0.621	0.573	0.620		0.552	0.544	0.667	0.545	0.524
57	April	0.545	0.409	0.642	0.587	0.704	0.578	0.552		0.518	0.717	0.536	0.526
55	May	0.611	0.593	0.785	0.725	0.708	0.579	0.544	0.518		0.588	0.787	0.643
42	June	0.547	0.442	0.617	0.652	0.699	0.617	0.667	0.717	0.588		0.589	0.566
53	July	0.575	0.547	0.676	0.680	0.702	0.543	0.545	0.536	0.787	0.589		0.709
57	August	0.600	0.564	0.587	0.596	0.755	0.532	0.524	0.526	0.643	0.566	0.709	

Table 9. Among Location Similarity Indices

Samples from the main site of each location were compared to the other four locations for a given month. Month can be found in the first column while number of peaks in each profile can be found in the second column. Location is in the third column and along the top row. The yard in June was excluded from similarity index calculations because its intensity was below 20,000 RFU.

January							
	Number of Peaks		Agriculture	Marsh	Yard	Woodlot	Sandy Woodlot
	67	Agriculture		0.603	0.567	0.580	0.389
	49	Marsh	0.603		0.442	0.585	0.311
	37	Yard	0.567	0.442		0.549	0.513
	45	Woodlot	0.580	0.585	0.549		0.384
	41	Sandy Woodlot	0.389	0.311	0.513	0.384	
February							
	Number of Peaks		Agriculture	Marsh	Yard	Woodlot	Sandy Woodlot
	41	Agriculture		0.395	0.387	0.356	0.636
	45	Marsh	0.395		0.470	0.626	0.402
	70	Yard	0.387	0.470		0.603	0.410
	46	Woodlot	0.356	0.626	0.603		0.452
	47	Sandy Woodlot	0.636	0.402	0.410	0.452	
March							
	Number of Peaks		Agriculture	Marsh	Yard	Woodlot	Sandy Woodlot
	52	Agriculture		0.583	0.500	0.392	0.420
	44	Marsh	0.583		0.483	0.245	0.283
	72	Yard	0.500	0.483		0.615	0.425
	50	Woodlot	0.392	0.245	0.615		0.235
	48	Sandy Woodlot	0.420	0.283	0.425	0.235	
April							
	Number of Peaks		Agriculture	Marsh	Yard	Woodlot	Sandy Woodlot
	53	Agriculture		0.505	0.627	0.569	0.400
	48	Marsh	0.505		0.670	0.654	0.419
	49	Yard	0.627	0.670		0.581	0.481
	56	Woodlot	0.569	0.654	0.581		0.372
	57	Sandy Woodlot	0.400	0.419	0.481	0.372	

Table 9 (cont'd)

May							
	Number of Peaks		Agriculture	Marsh	Yard	Woodlot	Sandy Woodlot
	66	Agriculture		0.418	0.483	0.491	0.520
	75	Marsh	0.418		0.472	0.496	0.364
	52	Yard	0.483	0.472		0.569	0.569
	50	Woodlot	0.491	0.496	0.569		0.523
	57	Sandy Woodlot	0.520	0.364	0.569	0.523	
June							
	Number of Peaks		Agriculture	Marsh	Woodlot	Sandy Woodlot	
	58	Agriculture		0.468	0.418	0.417	
	51	Marsh	0.468		0.560	0.348	
	33	Woodlot	0.418	0.560		0.437	
	38	Sandy Woodlot	0.417	0.348	0.437		
July							
	Number of Peaks		Agriculture	Marsh	Yard	Woodlot	Sandy Woodlot
	50	Agriculture		0.452	0.515	0.491	0.400
	54	Marsh	0.452		0.607	0.643	0.321
	53	Yard	0.515	0.607		0.622	0.444
	58	Woodlot	0.491	0.643	0.622		0.389
	55	Sandy Woodlot	0.400	0.321	0.444	0.389	
August							
	Number of Peaks		Agriculture	Marsh	Yard	Woodlot	Sandy Woodlot
	46	Agriculture		0.510	0.429	0.600	0.439
	54	Marsh	0.510		0.566	0.551	0.340
	45	Yard	0.429	0.566		0.539	0.495
	44	Woodlot	0.600	0.551	0.539		0.406
	52	Sandy Woodlot	0.439	0.340	0.495	0.406	
September							
	Number of Peaks		Agriculture	Marsh	Yard	Woodlot	Sandy Woodlot
	55	Agriculture		0.579	0.556	0.651	0.495
	52	Marsh	0.579		0.610	0.437	0.550
	53	Yard	0.556	0.610		0.519	0.495
	51	Woodlot	0.651	0.437	0.519		0.505
	48	Sandy Woodlot	0.495	0.550	0.495	0.505	

Table 9 (cont'd)

October								
	Number of Peaks		Agriculture	Marsh	Yard	Woodlot	Sandy Woodlot
	71	Agriculture		0.606	0.508	0.553	0.563
	56	Marsh	0.606		0.600	0.472	0.658
	49	Yard	0.508	0.600		0.455	0.750
	52	Woodlot	0.553	0.472	0.455		0.598
	55	Sandy Woodlot	0.563	0.658	0.750	0.598	

November							
	Number of Peaks		Agriculture	Marsh	Yard	Woodlot	Sandy Woodlot
	62	Agriculture		0.625	0.566	0.566	0.383
	50	Marsh	0.625		0.670	0.713	0.463
	44	Yard	0.566	0.670		0.602	0.528
	44	Woodlot	0.566	0.713	0.602		0.528
	45	Sandy Woodlot	0.383	0.463	0.528	0.528	

December							
	Number of Peaks		Agriculture	Marsh	Yard	Woodlot	Sandy Woodlot
	53	Agriculture		0.500	0.535	0.539	0.388
	47	Marsh	0.500		0.537	0.552	0.413
	61	Yard	0.535	0.537		0.582	0.425
	49	Woodlot	0.539	0.552	0.582		0.543
	45	Sandy Woodlot	0.388	0.413	0.425	0.543	

Table 10. Within Location Similarity Indices.

The five sites (main, north, south, east, and west) were compared at each location for the four collection periods. Month can be found in the first column while number of peaks in each profile can be found in the second column. Site is in the third column and along the top row. The agricultural field and yard's main sites in June were excluded from similarity index calculations because their intensity was below 20,000 RFU.

Agricultural Field							
March	Number of Peaks		Main	North	South	East	West
	52	Main		0.626	0.608	0.703	0.765
	63	North	0.626		0.717	0.836	0.611
	50	South	0.608	0.717		0.734	0.560
	59	East	0.703	0.836	0.734		0.688
	50	West	0.765	0.611	0.560	0.688	
June			North	South	East	West	
	71	North		0.912	0.616	0.878	
	65	South	0.912		0.672	0.887	
	54	East	0.616	0.672		0.672	
	68	West	0.878	0.887	0.672		
September			Main	North	South	East	West
	44	Main		0.598	0.697	0.760	0.711
	48	North	0.598		0.563	0.600	0.644
	55	South	0.697	0.563		0.822	0.926
	52	East	0.760	0.600	0.822		0.838
	53	West	0.711	0.644	0.926	0.838	
December			Main	North	South	East	West
	53	Main		0.642	0.675	0.650	0.613
	56	North	0.642		0.802	0.660	0.688
	70	South	0.675	0.802		0.592	0.585
	50	East	0.650	0.660	0.592		0.864
	53	West	0.613	0.688	0.585	0.864	
Marsh							
March	Number of peaks		Main	South	East	West	
	44	Main		0.725	0.613	0.667	
	47	South	0.725		0.615	0.667	
	49	East	0.613	0.615		0.527	
	61	West	0.667	0.667	0.527		

Table 10 (cont'd)

June			Main	South	East	West	
	75	Main		0.632	0.674	0.755	
	61	South	0.632		0.591	0.651	
	66	East	0.674	0.591		0.784	
	68	West	0.755	0.651	0.784		
September			Main	South	East	West	
	30	Main		0.515	0.571	0.617	
	36	South	0.515		0.565	0.333	
	33	East	0.571	0.565		0.508	
	30	West	0.617	0.333	0.508		
December			Main	South	East	West	
	56	Main		0.679	0.532	0.559	
	56	South	0.679		0.495	0.486	
	55	East	0.532	0.495		0.655	
	55	West	0.559	0.486	0.655		
Yard							
March	Number of Peaks		Main	North	South	East	West
	54	Main		0.683	0.602	0.663	0.564
	47	North	0.683		0.791	0.780	0.713
	44	South	0.602	0.791		0.761	0.747
	44	East	0.663	0.780	0.761		0.769
	47	West	0.564	0.713	0.747	0.769	
June			North	South	East	West	
	-61	North		0.642	0.740	0.823	
	-62	South	0.642		0.637	0.736	
	-62	East	0.740	0.637		0.864	
	-63	West	0.823	0.736	0.864		
September			Main	North	South	East	West
	55	Main		0.598	0.673	0.692	0.712
	57	North	0.598		0.688	0.604	0.758
	52	South	0.673	0.688		0.594	0.696
	49	East	0.692	0.604	0.594		0.509
	63	West	0.712	0.758	0.696	0.509	
December			Main	North	South	East	West
	61	Main		0.619	0.555	0.513	0.487
	52	North	0.619		0.584	0.773	0.798
	49	South	0.555	0.584		0.533	0.564
	58	East	0.513	0.773	0.533		0.818
	52	West	0.487	0.798	0.564	0.818	

Table 10 (cont'd)

Woodlot							
March	Number of Peaks		Main	North	South	East	West
	59	Main		0.476	0.798	0.642	0.542
	46	North	0.476		0.526	0.531	0.553
	70	South	0.798	0.526		0.575	0.559
	50	East	0.642	0.531	0.575		0.418
	48	West	0.542	0.553	0.559	0.418	
June			Main	North	South	East	West
	40	Main		0.517	0.437	0.391	0.467
	49	North	0.517		0.625	0.656	0.446
	47	South	0.437	0.625		0.713	0.404
	47	East	0.391	0.656	0.713		0.525
	52	West	0.467	0.446	0.404	0.525	
September			Main	North	South	East	West
	51	Main		0.659	0.520	0.649	0.713
	40	North	0.659		0.368	0.616	0.578
	47	South	0.520	0.368		0.645	0.670
	46	East	0.649	0.616	0.645		0.708
	50	West	0.713	0.578	0.670	0.708	
December			Main	North	South	East	West
	61	Main		0.592	0.646	0.720	0.719
	52	North	0.592		0.557	0.612	0.679
	49	South	0.646	0.557		0.703	0.644
	58	East	0.720	0.612	0.703		0.743
	52	West	0.719	0.679	0.644	0.743	
Sandy Woodlot							
March	Number of Peaks		Main	North	South	East	West
	-48	Main		0.598	0.547	0.593	0.521
	-44	North	0.598		0.549	0.683	0.489
	-47	South	0.547	0.549		0.636	0.642
	-60	East	0.593	0.683	0.636		0.519
	-48	West	0.521	0.489	0.642	0.519	
June			Main	North	South	East	West
	39	Main		0.573	0.595	0.642	0.654
	43	North	0.573		0.557	0.682	0.549
	45	South	0.595	0.557		0.621	0.560
	42	East	0.642	0.682	0.621		0.704
	39	West	0.654	0.549	0.560	0.704	

Table 10 (cont'd)

September			Main	South	East	West	
	46	Main		0.701	0.539	0.610	
	41	South	0.701		0.476	0.547	
	43	East	0.539	0.476		0.474	
	54	West	0.610	0.547	0.474		
December			Main	North	South	East	West
	54	Main		0.495	0.648	0.583	0.685
	45	North	0.495		0.596	0.670	0.606
	54	South	0.648	0.596		0.641	0.685
	49	East	0.583	0.670	0.641		0.602
	54	West	0.685	0.606	0.685	0.602	

APPENDIX B:

Annual Agricultural Field Similarity Indices Calculated using Different Minimum Thresholds

Table 11. Similarity Indices for the Agricultural Field using Minimum Thresholds of 50 and 100 RFU.

Similarity indices were calculated comparing each month to every other month for the main site at the agricultural field. The number of peaks in each profile is displayed in the first column. Month can be found in the second column and along the top row. The first set of similarity indices were calculated using a minimum threshold of 50 RFU while the second set of similarity indices were calculated using a threshold of 100 RFU. June was excluded from the 100 RFU calculations because the intensity of the profile was too low to compare to the other profiles.

50 RFU Threshold													
Number of Peaks		Sept	Oct	Nov	Dec	Jan	Feb	Mar	Apr	May	Jun	Jul	Aug
48	September		0.694	0.689	0.705	0.775	0.433	0.725	0.478	0.404	0.604	0.500	0.500
60	October	0.694		0.856	0.645	0.754	0.461	0.650	0.606	0.462	0.602	0.500	0.608
58	November	0.689	0.856		0.657	0.759	0.380	0.634	0.667	0.385	0.664	0.469	0.660
47	December	0.705	0.645	0.657		0.683	0.404	0.578	0.571	0.419	0.638	0.471	0.652
54	January	0.775	0.754	0.759	0.683		0.458	0.701	0.663	0.460	0.625	0.609	0.729
42	February	0.433	0.461	0.380	0.404	0.458		0.365	0.395	0.602	0.460	0.362	0.452
43	March	0.725	0.650	0.634	0.578	0.701	0.365		0.552	0.416	0.604	0.494	0.576
44	April	0.478	0.606	0.667	0.571	0.663	0.395	0.552		0.422	0.598	0.549	0.570
46	May	0.404	0.462	0.385	0.419	0.460	0.602	0.416	0.422		0.317	0.286	0.466
58	June	0.604	0.602	0.664	0.638	0.625	0.460	0.604	0.598	0.317		0.583	0.530
38	July	0.500	0.500	0.469	0.471	0.609	0.362	0.494	0.549	0.286	0.583		0.600
42	August	0.500	0.608	0.660	0.652	0.729	0.452	0.576	0.570	0.466	0.530	0.600	
100 RFU Threshold													
Number of Peaks		Sept	Oct	Nov	Dec	Jan	Feb	Mar	Apr	May	Jun	Jul	Aug
40	September		0.628	0.716	0.658	0.741	0.368	0.711	0.475	0.395		0.514	0.533
46	October	0.628		0.809	0.683	0.725	0.392	0.585	0.581	0.354		0.500	0.531

Table 11 (cont'd)

48	November	0.716	0.809		0.680	0.720	0.368	0.595	0.659	0.357		0.512	0.602
80	December	0.658	0.683	0.680		0.704	0.417	0.526	0.550	0.405		0.455	0.670
45	January	0.741	0.725	0.720	0.704		0.384	0.704	0.600	0.346		0.610	0.662
28	February	0.368	0.392	0.368	0.417	0.384		0.359	0.353	0.516		0.350	0.444
36	March	0.711	0.585	0.595	0.526	0.704	0.359		0.526	0.361		0.500	0.549
40	April	0.475	0.581	0.659	0.550	0.600	0.353	0.526		0.395		0.625	0.493
36	May	0.395	0.354	0.357	0.405	0.346	0.516	0.361	0.395			0.294	0.338
0	June												
32	July	0.514	0.500	0.512	0.455	0.610	0.350	0.500	0.625	0.294			0.687
35	August	0.533	0.531	0.602	0.670	0.662	0.444	0.549	0.493	0.338		0.687	

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