J Forensic Sci, May 2008, Vol. 53, No. 3
doi: 10.1111/j.1556-4029.2008.00728.x
Available online at: www.blackwell-synergy.com

Melissa S. Meyers,[1] *M.S. and David R. Foran,*[2] *Ph.D.*

Spatial and Temporal Influences on Bacterial Profiling of Forensic Soil Samples*

ABSTRACT: Bacterial content may be helpful in differentiating forensic soil samples; however, the effectiveness of bacterial profiling depends on several factors, including uniqueness among different habitat types, the level of heterogeneity within a habitat, and changes in bacterial communities over time. To examine these, soils from five diverse habitats were tested over a 1 year period using terminal restriction fragment length polymorphism (TRFLP) analysis. Soil samples were collected at central locations monthly, and 10 feet in cardinal directions quarterly. Similarity indices were found to be least related among habitats, while the greatest bacterial similarities existed among collection locations within a habitat. Temporally, however, bacterial content varied considerably, and there was substantial overlap in similarity indices among habitats during different parts of the year. Taken together, the results indicate that while bacterial DNA profiling may be useful for forensic soil analysis, certain variables, particularly time, must be considered.

KEYWORDS: forensic science, terminal restriction fragment length polymorphism, 16S ribosomal RNA gene, bacterial DNA fingerprint

Soil can play a crucial role in legal investigations, acting as trace evidence that may link a victim or suspect to a crime scene. The evidentiary value of soil results from its complex composition, its prevalence in the environment, and because it is easily transferred to individuals or objects, including shoes and clothing, vehicles, and tools. In all cases, soil from a suspect and/or victim and the crime scene are compared to see if the samples are so similar that the crime scene cannot be excluded as the source of the questioned material.

Traditional forensic analysis of soil relies on detailing its physical appearance and chemical composition—examining features such as soil type, color, and particle size, as well as its elemental, mineral, and organic content (1–6). Of course, soils from different locations may or may not share characteristics. The value of forensic analysis is dependent on the presence of variation among soils; if physical and chemical characteristics do not vary, it is difficult for an analyst to associate a soil with a specific scene. Conversely, if extensive soil heterogeneity exists at a crime scene, it may be equally difficult or even impossible to link questioned and exemplar materials.

Given the limitations of traditional forensic soil analyses, surprisingly few efforts have been made to develop independent, alternative methods that might provide easier and perhaps more objective ways to differentiate or match soil samples. The characterization of a soil's indigenous microbiota has been proposed as a technique that could fulfill this goal, if indeed bacterial content varies substantively among unrelated soils and is relatively stable within a location or habitat (7–10). Current molecular and microbiological methodologies allow researchers to directly analyze the variability in the genomes of co-existing bacterial species, which functions as a proxy survey of the organisms present in a sample. Although several techniques are in use, terminal restriction fragment length polymorphism (TRFLP) analysis has become increasingly popular for microbial studies (11). TRFLP is based on amplifying a region of DNA common to multiple species using conserved PCR primers, one of which is 5′ labeled (12). The pool of amplicons is then digested with a restriction enzyme, and based on sequence heterogeneity among species found between the primer sites, a collection of labeled terminal fragments is produced. These are separated via capillary electrophoresis, resulting in a series of peaks that constitutes a bacterial profile for the soil sample. A standard target for TRFLP is the 16S ribosomal RNA gene, and rDNA "fingerprints" have been readily obtained from samples (12–17), generally to characterize soils for agricultural and ecological purposes, or to assay bacteria in the medical/clinical fields.

Because TRFLP analysis uses technologies familiar to forensic DNA analysts, it could easily be introduced into a forensic laboratory setting; however, its viability as a tool for identifying soils rests on several assumptions. First, measurable differences among soils from diverse habitats must exist. To some extent this has been established (8,9,18,19), although its relevance depends on a second supposition—that soil from a given location is itself relatively homogeneous. If the bacterial composition of soil a small distance away from a specific location differs as much as that from soil from much farther away, the utility of bacterial fingerprinting may be minimal. There is some evidence that bacterial heterogeneity in proximal soils might be common (9,20), although the generality of this has not been examined. Lastly, a thorough understanding of temporal variability in species content is requisite, as soil from a crime scene will most likely be collected days, weeks, or even months after the crime has occurred. If the bacterial composition of soil changes substantially over time (e.g., due to climate) it may be impossible to link soil from a suspect or victim back to a specific location. Unfortunately, little is known about temporal change in microbial community structure, although it appears that such communities can vary, at least over large time spans (8,21).

[1]Forensic Science Program, School of Criminal Justice, Michigan State University, East Lansing, MI 48824.
[2]Forensic Science Program, School of Criminal Justice and Department of Zoology, Michigan State University, East Lansing, MI 48824.
*A portion of this work was presented at the American Academy of Forensic Sciences 58th Annual Meeting in Seattle, WA, February 23, 2006.
Present address: Melissa S. Meyers, Indiana State Police Laboratory, 550 West 16th Street, Suite C, Indianapolis, IN 46202.
Received 8 April 2007; and in revised form 10 July 2007; accepted 28 July 2007.

The goal of the research presented here was to systematically investigate the utility of TRFLP analysis for the typing of forensic soil samples, by exploring the habitat uniqueness, local heterogeneity, and temporal variation of bacterial communities. Soils from five diverse habitats in central Michigan were collected, TRFLP profiles were produced, and similarity indices were examined. Samples were collected from a central location at each habitat monthly for a 1 year period, and were compared to evaluate similarity among habitats. Additionally, every third month, further samples were collected 10 feet in each cardinal direction from the central location, to determine levels of local heterogeneity. Finally, soils collected from each sampling location within a habitat were compared over the year to evaluate temporal variability. By examining all of these factors collectively, the overall utility of bacterial TRFLP profiling for forensic soil analysis was investigated.

Materials and Methods

Sample Collection

Soil samples were collected from a central (C) location at the beginning of each month from September 2004 through August 2005 from five diverse habitats in central Michigan: an agricultural field (A), a marsh edge (M), a yard (Y), a woodlot (W), and a sandy woodlot (S); soil properties can be seen in Table 1. In addition, soils were collected 10 feet from the central location in the cardinal directions (north [N], south [S], east [E], and west [W]) every 3 months, with the exception of the inaccessible north location of the marsh edge. For labeling purposes, soils were identified by the month and year of collection, habitat, and location; for example, 904AC was collected in September 2004 from the agricultural field's central location. Several scoops of soil were taken from the soil surface (*c.* 0–5 cm in depth), placed in a plastic zip-loc style bag, and mixed thoroughly. Soil samples were stored at −20°C within an hour of the time of collection.

DNA Extraction

DNA isolation and purification from 1 g of soil was performed using an UltraClean™ Soil DNA Kit (MO BIO Laboratories, Carlsbad, CA). When a TRFLP profile could not be obtained from a sample, DNA was extracted from 0.25 g of soil using a PowerSoil™ DNA Kit (MO BIO Laboratories). Both procedures utilized the manufacturer's instructions. Five microliters of DNA was separated on a 1% agarose gel to ensure that the extraction was successful.

TABLE 1—*Soil classification and habitat information.*

Habitat	Soil Type*	Organic Material (%)	Primary Vegetation†
Agricultural	Loam	3.0	Summer 2004—Soybean Summer 2005—Corn
Marsh	Silt loam	8.8	Cattail, Joe-Pye-weed‡
Yard	Sandy loam	3.0	Walnut, Grass
Woodlot	Sandy loam	12.1	Oak, Maple
Sandy woodlot	Loamy sand	6.8	Oak, Maple

*Results for the mechanical analysis of soil samples from each habitat. Soil type was based on the levels of sand, silt, and clay.
†The dominant plant species identified are shown.
‡*Eupatorium maculatum.*

Amplification of Genomic DNA from Soil

PCR amplification was conducted using universal primers for the 16S ribosomal RNA gene (8F [6-FAM-5′-AGAGTTTGATCCTGGCTC-3′] and 1492R [5′-GGTTACCTTGTTACGACTT-3′]; 22, 23), producing *c.* 1.4 kb amplicon. DNA from *Escherichia coli* served as a positive control; negative controls had DNA replaced with sterile water. PCR reactions included 1X HotMaster Taq PCR buffer (Eppendorf, Westbury, NY), 0.2 mM of each dNTP (Promega, Madison WI), 1 μM of each primer, 2 μg bovine serum albumin, 1 unit HotMaster Taq DNA polymerase (Eppendorf), and 2 μL of template DNA, in a final volume of 20 μL. The amplification reaction consisted of denaturation at 94°C for 2 min followed by 20 cycles of denaturation at 94°C for 30 sec, primer annealing at 58°C for 45 sec, extension at 72°C for 1 min and 30 sec, and ended with an additional extension step at 72°C for 4 min. In some instances samples purified using the UltraClean Soil DNA Kit required increased cycle number (25 or 30 cycles) for amplicon production—however, all but four of these were reanalyzed following PowerSoil DNA extraction. Amplicons were visualized by electrophoresing 2 μL of the PCR product on a 1% agarose gel. DNA quantity was estimated through comparison of 1 μL of 1 kb DNA ladder (New England Biolabs, Beverly, MA), comparing the amplicon to the 3 kb fragment which contained 250 ng of DNA. A single trial was conducted for each soil sample.

Restriction Digestion of Amplified 16S rDNA

The remaining 18 μL of PCR product was purified using a Montage PCR Centrifugal Filter Device (Millipore, Bedford, MA). Restriction digestions contained one unit *MspI* (New England Biolabs), 1X NEBuffer 2 (New England Biolabs), and an estimated 250 ng of purified PCR product, in a final volume of 10 μL. Samples were incubated at 37°C for 4–6 h. The digestion was terminated by deactivating the restriction enzyme at 70–75°C for 20 min. Digested DNAs were purified using a Microcon YM-30 column (Millipore). DNAs were washed twice using 300 μL of 10 mM Tris pH 7.5, 1 mM EDTA (TE), and the final volume was returned to 10 μL.

Capillary Electrophoresis of Restriction Digests

Restriction fragments were separated on an ABI Prism 310 genetic analyzer (Applied Biosystems, Foster City, CA) using 3 μL of the purified 16S rDNA digest, 21.5 μL of formamide, and 0.5 μL of ABI GeneScan 500 Liz size standard, which was heat-denatured at 95°C for 3 min then chilled on ice. If peaks were broad, the amount of DNA was reduced to 1 μL. TRFLP profiles were generated using ABI 310 Genetic Analyzer Data Collection Software version 3.0.0 (Applied Biosystems) (GS STR POP4 [1 mL] G5.md5 module, 5 sec injection, 15 kV injection, 15 kV run voltage, 28-min run time). However, the module was modified to include a 60-sec injection and a 35-min run time.

Analysis of TRFLP Profiles

Data analysis was conducted with ABI GeneMapper ID version 3.1 software (Applied Biosystems). Terminal restriction fragments from 50 to 500 bases with heights over 50 or 100 relative fluorescence units (RFU) were included in the data analysis (50 RFU was used for final analyses based upon preliminary findings; see Results). A TRFLP profile was eliminated from the study if its

total RFUs fell below 20,000 and was less than one-third as intense as the profiles to which it was being compared following normalization (see below).

A similarity index was calculated using the Ribosomal Database Project's TRFLP analysis program (24) 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. This generated values ranging from 0.00 (no peaks shared) to 1.00 (all peaks shared). Four techniques were used for peak inclusion when calculating similarity indices. First, all peaks that fell within the specified parameters (50–500 bases with a height greater than 50 RFU) were considered. Second, data were normalized by summing the peak heights of each sample being compared, then dividing 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 correction factor, and any peak whose new height was below 50 RFU was excluded from the analysis. The last two techniques generated similarity indices by comparing the highest 20 or 40 peaks from each sample. Similarity indices using the different techniques were examined to determine if one in particular was best suited for TRFLP analysis.

Among soil samples, 5412 comparisons were made, examining habitat uniqueness, within-habitat heterogeneity, and temporal variability. Based on the findings (see Results), 1531 normalized indices were used for further analyses. Five types of comparisons were made: (1) central location profiles from each habitat were compared to the other habitats during the same month, to determine among-habitat similarity throughout the year, (2) local heterogeneity was examined by comparing the five locations within a habitat during the four collection periods, (3) each habitat's central location was compared to itself on a month-to-month basis over the course of the year, assaying sequential fluctuations in bacterial composition, (4) a central location's profile for a given month was compared to the other 11 months *in toto*, examining how a monthly profile compared to the rest of the year as a whole, and (5) each of the five collection locations within a habitat was compared to itself during the year.

Statistical Methods

Statistical analyses were performed using R statistical package version 1.9.1 (25). Single factor Analysis of Variance (ANOVA) was used to test temporal variability among habitats, within habitats, and at each location within a habitat. Results were considered significant at $p < 0.05$.

Multivariate Analysis of Variance (MANOVA) was conducted on among-habitat comparisons, within-habitat comparisons, and each location's monthly/quarterly comparisons. MANOVA, like ANOVA, tests 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 variables—habitat and month in this study—for each type of comparison (i.e., among habitat, within habitat, and temporal) were treated as different dependent variables. Results for MANOVA were considered significant at $p < 0.05$.

Results

DNA Isolation, 16S rDNA Amplification, and TRFLP Profiles

DNAs isolated using a MO BIO UltraClean kit were tested initially. Of the isolates, 114 were amplified after 20 cycles, while an additional 22 required 25 or 30 cycles. Of these, 46 did not generate viable TRFLP profiles, and 21 produced profiles deemed unusable because their intensity was below the requisite 20,000 RFU. DNAs were re-extracted from soils that failed to produce analyzable TRFLP profiles using a MO BIO PowerSoil kit. All of the isolates amplified after 20 cycles and produced a TRFLP profile. Four (UltraClean kit) profiles were obtained using 25 PCR cycles, and six samples were excluded from similarity comparisons because their total fluorescence was below 20,000 RFU and more than half of the peaks were removed from other samples when normalized to them.

Similarity Index Calculations

An 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. The "top 20 peak" method produced the lowest similarity indices more than half the time, with the other three methods generally producing indices similar to one-another. The "all peak" method resulted in small, irreproducible peaks being included in some instances; thus it and the "top 20 peak" method were excluded from further analyses. The "normalized peak" and "top 40 peak" methods produced very similar results overall, but because the "normalized peak" method is widely used (19,20,26), it was employed for subsequent analyses.

Similarity Indices Using Minimum Thresholds of 50 and 100 RFU

The utility of using a minimum peak threshold of 50 versus 100 RFU was examined by comparing similarity indices obtained from the agricultural field for a given month to every other month. June was excluded from the analysis because its total fluorescence was far below 20,000 RFU when peaks less than 100 RFU were removed from the profile. One hundred and ten indices were compared; of these 79 decreased in similarity when using a minimum peak threshold of 100 RFU instead of 50 RFU, 29 increased, and two did not change. The average decrease in similarity levels resulting from raising the threshold to 100 RFU was 0.036; there was a decrease greater than 0.100 in five instances. Given this, 50 RFU was used for the analyses detailed below.

Bacterial Variability Among Habitats

The ability to differentiate habitats based on bacterial composition was tested by comparing a habitat to the other four during each month. A habitat's resulting four similarity indices were then averaged for each month (Table 2). Interestingly, the sandy woodlot produced both the highest and lowest level of among-habitat monthly similarity, with an average high value of 0.642 during the month of October, and a low value of 0.340 in March. The yearly calculation (Table 2) showed that the yard had the highest overall similarity to the other habitats (0.536), followed by the woodlot (0.517), the marsh (0.500), the agricultural field (0.497), and the sandy woodlot (0.448). There was no statistical difference among habitats when the entire year was considered ($p = 0.64$); however, when comparisons were broken down by month, a statistical difference was seen ($p = 6.34 \times 10^{-5}$). By habitat type, month was a significant factor for the marsh ($p = 0.033$) and sandy woodlot ($p = 0.016$), but not for the agricultural field ($p = 0.22$), yard ($p = 0.14$), or woodlot ($p = 0.074$).

Overall, the habitats were most similar to one-another in the fall (Table 2). The agricultural field produced its highest average similarity in September, the marsh and sandy woodlot in October, and

TABLE 2—*Monthly average similarity indices comparing one habitat to other habitats.**

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

*Samples from the central location of each habitat were compared to the central location of the other four habitats monthly; these four values were then averaged. In addition, overall averages were calculated for each habitat. A similarity index was not available (N/A) for the yard in June because the intensity of the profile was too low.

the yard and woodlot in November. There was also an increase in similarity among habitats in April when compared to the winter and spring months. Three habitats, the marsh, woodlot, and sandy woodlot, were least similar to the others in March, while the agricultural field had the lowest similarity in March and June, and the yard in February.

Finally, the two woodlots were evaluated to see if the presumably most similar habitats had comparable bacterial community structures. The average similarity for the habitats over the entire year was a relatively low 0.450.

Within-Habitat Heterogeneity

The central and four cardinal locations were sampled at each habitat quarterly. Average pair-wise similarities among the five locations ranged from 0.518 to 0.773 (Table 3), with the agricultural field and yard displaying less overall heterogeneity than the others. The agricultural field, marsh, and yard had their highest within-habitat similarity in June, while the woodlot and sandy woodlot were highest in December. Similarities were lowest in December for the agricultural field and yard, in September for the marsh and sandy woodlot, and in June for the woodlot. Overall differences in heterogeneity among habitats were highly significant ($p = 2.3 \times 10^{-11}$), while there was not a significant difference in heterogeneity among all habitats based on collection period ($p = 0.86$). The former result is explained by examining each habitat independently, wherein a significant difference based on sampling period was seen for the marsh, yard, and woodlot ($p = 0.0028$, $p = 0.0012$, and $p = 0.00014$, respectively), although not for the agricultural field or the sandy woodlot.

TABLE 3—*Average similarity indices for the five collection locations within each habitat.**

	Agricultural	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

*Similarity indices for all locations at a habitat (central, north, south, east, and west) were averaged for the quarterly soil collections.

Temporal Variability Within a Habitat

The influence of time on a habitat's bacterial composition was first examined by comparing each central location's TRFLP profile to sequential months. Similarity levels fluctuated widely between consecutive months (Fig. 1). The greatest observed change was 0.266, which occurred between May and June in the agricultural field, while the smallest was 0.001 from May to June in the sandy woodlot. Fluctuations in bacterial similarity indices varied throughout the year; however, the highest similarity was again observed through the fall. The yard behaved a bit differently in that its similarity increased earlier in the fall, but then decreased substantially (see Discussion). The greatest average month-to-month change was seen in the agricultural field (0.142), followed by the marsh (0.119), yard (0.094), sandy woodlot (0.079), and woodlot (0.068).

The similarity of a habitat during a given month was next compared to the other 11 months, to assay the overall level of fluctuation in a habitat (Fig. 2). The highest month-to-month similarity (0.926) was observed in the woodlot, comparing the months of May and August, while the lowest (0.268) was between May and July in the agricultural field. In general, July showed the most dissimilarity with the other months.

When averaging each month to all other months within a habitat, the range of similarities was 0.422–0.656 for the agricultural field, 0.504–0.684 for the marsh, 0.355–0.651 for the yard, 0.565–0.669 for the woodlot, and 0.520–0.657 for the sandy woodlot. The extremely low result for the yard (November, 0.355) may have been an outlier (see Discussion). During the first half of the year, the agricultural field had the lowest average similarity, which then increased bringing it in line with the other habitats; it was the only habitat that showed a statistical difference in monthly similarity indices when habitats were examined individually ($p = 0.045$). There was no apparent month or set of months where habitats shared exceptionally high or low average similarity levels, and indeed, temporal change among habitats was significantly different from one another ($p = 8.577 \times 10^{-6}$).

Finally, each of the five sampling locations within a habitat was compared to itself over the course of the year, and an average similarity was calculated (Table 4). These produced values ranging from 0.417 to 0.811, with the highest similarities occurring in the yard while the sandy woodlot had the lowest overall similarity. There was no significant difference among sampling locations within any of the five habitats, with only the yard showing an indication of location dissimilarity ($p = 0.0828$; see Discussion).

Unique and Shared Peaks Among Samples

A screening of profiles from two randomly chosen months (October and March) was performed to see if specific peaks/bacterial species were present, absent, or at very different levels within or among habitats during certain times of the year. Some peaks were found to be common among profiles, for instance, a substantial 92 base peak was found in all habitats (Fig. 3). Likewise, 138 and 148 base peaks were seen in every sample. In contrast, there were instances where large peaks were consistently observed in some samples while being absent or at a much lower

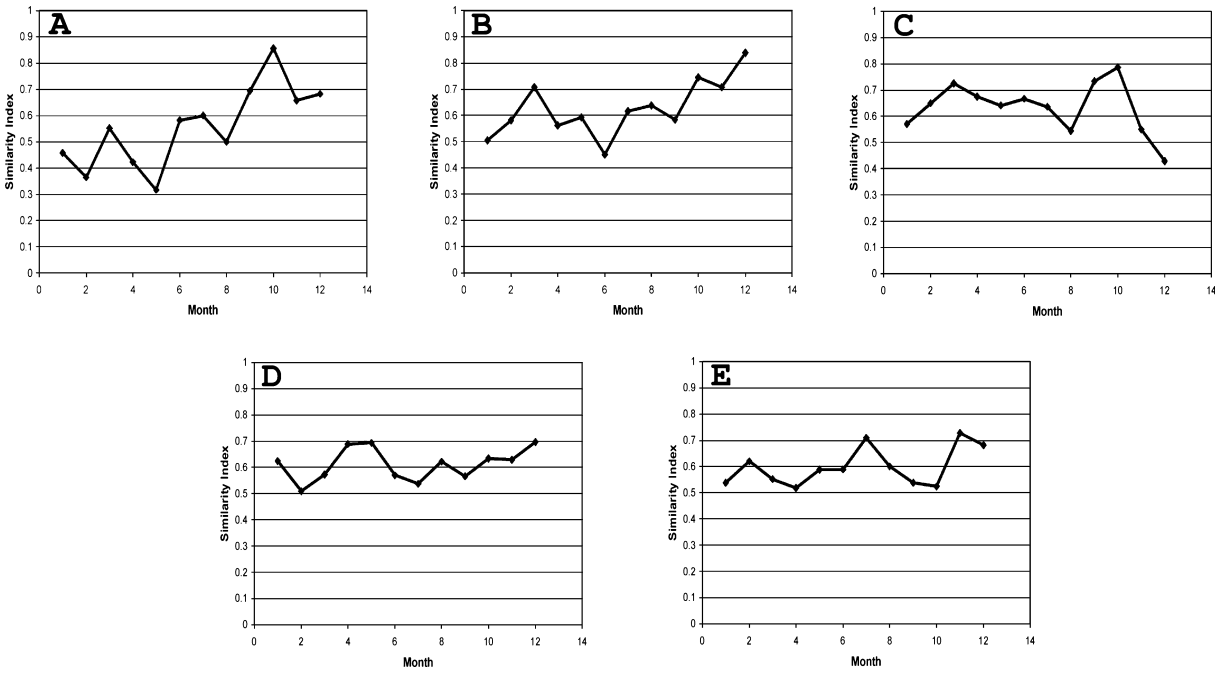

FIG. 1—*Consecutive month-to-month similarity for each habitat. (A) refers to the agricultural field, (B) to the marsh, (C) to the yard, (D) to the woodlot, and (E) to the sandy woodlot. Month can be found on the x-axis while similarity index is on the y-axis. Data for the yard in June were calculated using the "top 40 peak" method, all others were normalized. For simplicity, similarity indices are displayed from January through December; however, the collection regimen began in the fall. September through December soils were collected in 2004 and January through August soils were collected in 2005.*

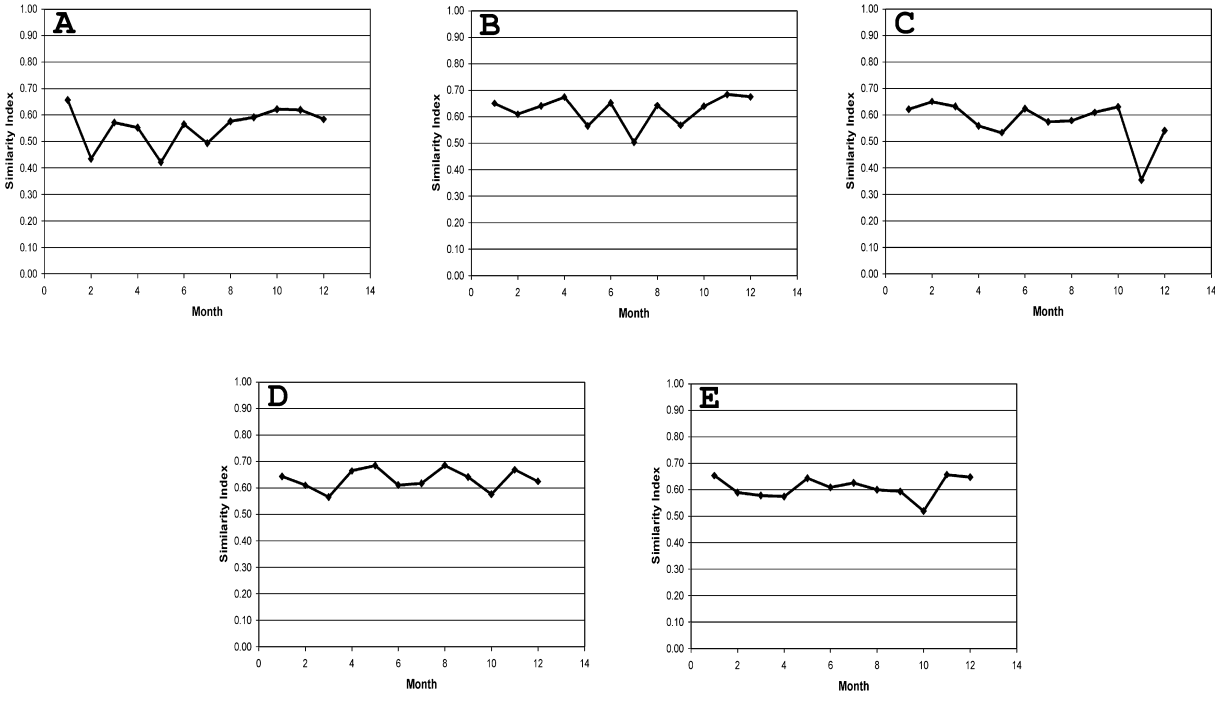

FIG. 2—*Average similarity of a given month compared to the other 11 months for each habitat examined. (A) refers to the agricultural field, (B) to the marsh, (C) to the yard, (D) to the woodlot, and (E) to the sandy woodlot. Month can be found on the x-axis while similarity index is on the y-axis. Data for the yard in June were calculated using the "top 40 peak" method, all others were normalized. For simplicity, similarity indices are displayed from January through December; however, the collection regimen began in the fall. September through December soils were collected in 2004 and January through August soils were collected in 2005.*

intensity in others, including large 145 and 195 base peaks in the marsh profiles and a large 170 base peak seen only in the yard profiles.

Peak intensities also changed temporally at the various habitats. For example, the 286 base peak showed similar intensity in October and March in the woodlot profiles (Fig. 3), was higher in

TABLE 4—*Similarity indices for locations within a habitat averaged over the year.**

	Agricultural	Marsh	Yard	Woodlot	Sandy Woodlot
Central	0.636	0.645	0.630	0.555	0.637
North	0.619	N/A	0.783	0.580	0.417
South	0.685	0.647	0.637	0.597	0.612
East	0.702	0.552	0.640	0.577	0.521
West	0.632	0.591	0.811	0.561	0.609
Average	0.655	0.609	0.700	0.574	0.559

*Average similarities were calculated for each location during the four within-habitat collection periods. A similarity index was not available (N/A) for the marsh north location.

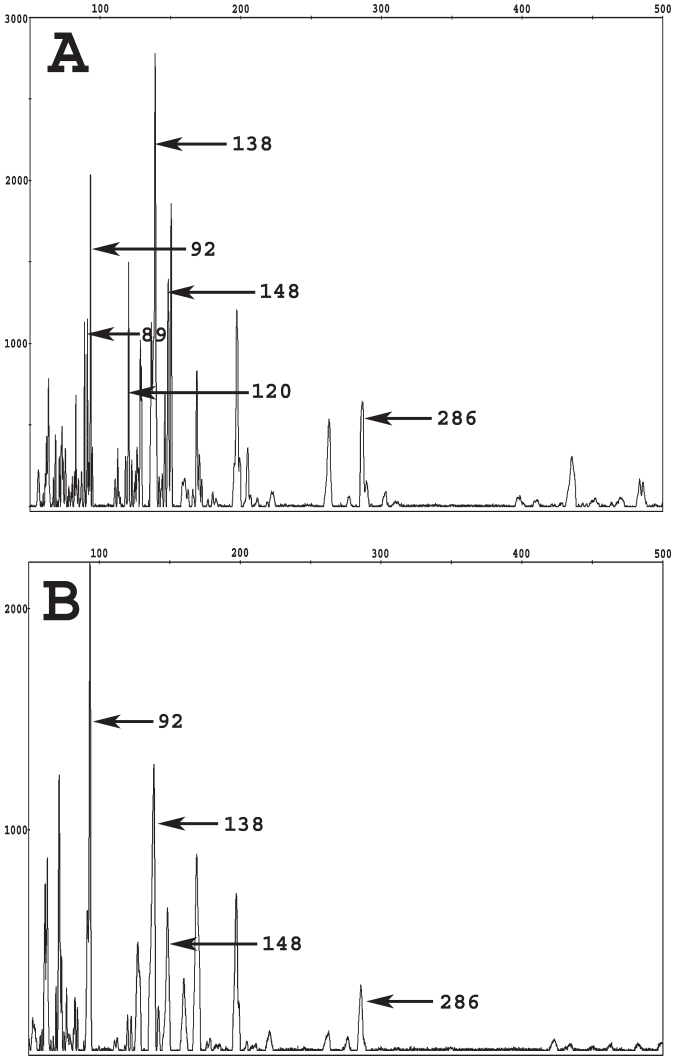

FIG. 3—*TRFLP profiles from the central location of the woodlot—panel A shows the profile generated in October of 2004, while panel B shows the profile generated in March of 2005. Peak size in bases starting at 50 bp is shown on the x-axis while relative fluorescence is on the y-axis.*

October than in March in the agricultural field and marsh, and was lower in October than in March in the yard and sandy woodlot. Further, some peaks were substantially larger during certain times of the year within a habitat, including 89 and 120 base pair peaks in the woodlot profiles (Fig. 3) which were very large in October but barely above the 50 RFU threshold in March.

Discussion

TRFLP Methods and Data Analysis

The goal of the research presented here was to examine the utility of TRFLP in the forensic analysis of soil samples, reliably matching a questioned sample back to a specific location based on the similarity of the microbiota present. Three main questions were considered: if soils from different habitats can be distinguished from one another, if local heterogeneity affects the ability to identify soil, and if soils collected at two different times from a single location can be reliably related to one another.

Soil is known to be a difficult substrate from which to obtain amplifiable DNA; thus, the method of DNA isolation/purification is critical for successful analysis. When using a MO BIO UltraClean DNA kit, PCR products were not obtained from many samples after 20 PCR cycles, and almost half did not produce viable TRFLP profiles. These samples were re-extracted using a MO BIO PowerSoil DNA kit, which was developed to handle a wider variety of soils, and includes a "humic substance/brown color removal procedure" that is claimed to reduce PCR inhibition and increase DNA quality (27). DNAs extracted with the PowerSoil kit all amplified after 20 cycles and produced usable TRFLP profiles in virtually all instances. It appears that DNAs isolated using the UltraClean kit often retained impurities that inhibited Taq and/or the restriction enzyme. Previous studies have shown that humic acids in soil interfere with PCR, restriction digests, and TRFLP profile intensity (28–30). The results obtained in this research using the two soil DNA isolation kits accentuate the importance of removing inhibitors during the DNA extraction process, and how different protocols may affect this.

Similarity indices used for comparing the bacterial composition of soil samples were originally calculated using four techniques: the "all peak" method, the "normalized peak" method, the "top 20 peak" method, and the "top 40 peak" method. TRFLP profiles are generally normalized to account for differences in DNA quantities among samples and to remove small peaks that are irreproducible or fluctuate above and below the RFU cutoff (26). However, it is not clear that this is the best method for analysis, and it is plausible that examining a fixed number of peaks might generate a more "honest" comparison. For instance, two soil samples could be functionally identical, but if one profile contains a small number of peaks due to the amount of DNA analyzed while the other has far more peaks of the requisite RFU, then they would type as quite different. By limiting the analysis to the largest 20 or 40 peaks, with larger peaks tending to be more reproducible (31), peak number would not be a confounder. However, it became clear that including only 20 peaks resulted in a loss of resolution, as similarity indices were generally lower than when the other analysis methods were employed. In contrast, the "top 40 peak" method produced similarity indices much more in line with the other two analysis methods. Because inclusion of all peaks has the potential to consider small peaks that are either nonreproducible or may vary based on slight differences in the amount of DNA injected into the capillary, the results presented herein were normalized; however, it appears that using a set number of peaks, given that this number is substantial (e.g., the 40 tested here or more), is equally effective.

TRFLP Profile Similarity Among Habitat Types

It is crucial that different habitats produce diverse TRFLP profiles, if the technique is to be used as a forensic tool. Soils from the central locations of the five habitats were compared across

habitats monthly, and the results for each habitat were averaged for the year. As would be predicted, among-habitat comparisons produced low average values, ranging from 0.448 for the sandy woodlot to 0.536 for the yard (Table 2). These figures are in line with those obtained by Horswell et al. (8), wherein similarity indices ranged from 0.480 to 0.590 among five soils analyzed.

Soil type itself did not appear to influence bacterial similarity. When the yard and woodlot (both sandy loams) were compared to each other over the year, their average similarity was no higher than other habitat comparisons (data not shown). Likewise, habitats that visually seemed most similar, the two woodlots, produced profiles as different as any other of the habitats. Interestingly, the sandy woodlot, with the lowest similarity among habitats, was located far from the others (approximately 100 miles); thus, distance may represent one of the most important factors in bacterial diversity.

Heterogeneity of Bacterial Communities Within a Habitat

The second critical factor if TRFLP profiles are to be used for investigative purposes is that local heterogeneity in bacterial composition be minimal. It is unlikely that exemplar soil from a site will be collected from the exact same location where soil associated with a victim or suspect was attained; thus, slightly different locations within a habitat must generate comparable similarity indices. Mummey and Stahl (20) found that two grasslands approximately 90 miles apart had differing levels of intra-habitat bacterial variability, with the site containing *Boutelous gracilis* having a within-habitat average similarity of 0.730 while an *Artemisia tridentate* grassland had an average similarity of 0.410. Comparable results were reported by Heath and Saunders (9). In the current research, variability, sometimes substantial, existed among soil samples collected as little as 10 feet away, emphasizing the importance of recognizing local heterogeneity during forensic soil analyses.

The highest level of habitat similarity was seen in the two human-manipulated locations, the agricultural field and the yard. Both habitats were largely represented by monocultures (crops or grass), and both underwent substantial human management over time (see below). The marsh, woodlot, and sandy woodlot, being more heterogeneous in regard to plant species, amount of decaying vegetation, quantity of sunlight reaching the soil, and countless other factors, would seem to possess all the variables required for larger levels of bacterial heterogeneity. Intuitively it makes sense that a more diverse habitat would lead to a more diverse microbiota; this seems to have been the case in the habitats examined here. Such findings point to the care that will need to be taken when using bacteria for identification, as specific and localized variables could have a dramatic influence on the species present.

Temporal Changes in Bacterial Composition

Finally, understanding the manner and level to which bacterial communities change temporally is critical if they are to be used as markers of specific soils. Time was found to influence soil comparisons in all factors examined—similarity among habitats, similarity within a habitat, and similarity at specific locations within a habitat. As seen in Fig. 1, month-to-month comparisons within each habitat often showed broad fluctuations in bacterial similarity. Autumn months tended to show the least fluctuation (with the exception of the yard, although the November result may have been an outlier; see Fig. 2*C*), perhaps resulting from the cooler weather and/or increased organic matter in the soil, either of which might prove advantageous to a smaller set of species, thereby decreasing heterogeneity. Counter-intuitively, the winter months showed greater heterogeneity than autumn, even though the ground was generally frozen and bacterial growth was presumably curtailed. This may mean that dormant bacterial forms are differentially affected by freezing, with species prevalent in the autumn dying back while others stay relatively stable. It is also interesting to note the increase in similarity among habitats in April (Table 2) in comparison to the other winter and spring months. March to April carries a strong change in climatic conditions in Michigan, and certain common bacterial species might begin to "take off" as the weather warms, before a more diverse flora can form.

Among habitats, the marsh and sandy woodlot had statistical differences in similarity indices when compared to the other habitats. The marsh site was wet year-round, representing a clear difference from the other habitats, while the sandy woodlot was located approximately 100 miles distant to the other habitats; thus, this too could differentially influence changes over time. Likewise, the five locations within each habitat, sampled quarterly, fluctuated over the year (Table 3), and varied significantly for the marsh, yard, and woodlot. The reason for this is unclear, although the marsh and woodlot would be expected to have substantial site-heterogeneity (e.g., in penetrating sunlight and decaying plant material) compared to the agricultural field for instance; however, the same should be true of the sandy woodlot. Taken together, it is clear that time is a critical factor in the feasibility of using TRFLP profiling for the forensic analysis of soil.

The Effects of Human Disturbances on Bacterial Composition

Any human activity occurring at a crime venue has the potential to alter the scene to some extent. In the habitats examined here, extensive human activity appeared to homogenize bacterial content, in that, for each collection period there were higher levels of similarity among the five collection locations for both the agricultural field and the yard than for the undisturbed habitats. As mentioned, both habitats generally contained a plant monoculture; thus, a lack of biodiversity could, as might be expected, result in fewer bacterial species. Likewise, human activity acted to directly homogenize the agricultural field, in that it was tilled annually. The yard was mowed weekly during the spring and summer months, raked during the fall months, and underwent heavy foot traffic, which might also influence bacterial content.

Interestingly, while the agricultural field showed high levels of within-habitat similarity during the four quarterly collection periods, it showed the greatest, and statistically significant, month-to-month variation at its central location. This was 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. Soybeans, being a legume, harbor symbiotic, nitrogen fixing bacteria in their roots, which would be expected to greatly increase in number following planting, and subside after harvest. Further, through their introduction of nitrogen into the surrounding soil, other bacteria may have been able to take advantage of the new nutrients. Such factors introduce the caveat of examining human disturbances when considering soil TRFLP profiles.

The Overall Utility of TRFLP in Forensic Soil Analyses

The research presented here revealed several factors that influence the utility of using TRFLP profiling to link a questioned soil to a crime scene. Profile similarity must necessarily be much higher

within a habitat than among habitats. Further, habitats cannot be highly heterogeneous over small spatial distances; it is unlikely that a reference sample will be collected from the exact same spot from which the questioned soil originated. Finally, similarity cannot change substantially over time, as reference sample collection will always take place at some point after the crime occurred. Overlap in similarity indices among and within habitats will also make utilization of TRFLP profiling for forensic soil analyses difficult or impossible. In the current study, habitats were on average distinguishable from each other during the majority of the year; however, the higher among-habitat averages during April and the fall months overlapped with within-habitat averages for the marsh, woodlot, and sandy woodlot (Table 3). Further, during several months in the agricultural field (Fig. 1*A*), similarity indices were as low or lower as many among-habitat results. These types of problems could potentially be remedied by analyzing multiple reference samples, as is commonly done in forensic hair analysis, since, much like hairs, two soils from the same source (habitat) can be markedly different; however, they are unlikely to be highly similar by chance.

It is also possible that the TRFLP technique may need to be "tweaked" for it to be useful in forensic soil analysis. The method used in these experiments targeted the 16S ribosomal gene via universal primers; thus, "all" bacteria in a soil sample were assayed. This "shotgun" approach might produce far too much noise to be useful in the long run. Perhaps it would be beneficial to target specific groups of microorganisms, which in theory could go beyond the bacteria examined in this study, incorporating fungi and the like. A cocktail of microbes could potentially be assayed using far more specific PCR primers, producing a much finer bacterial fingerprint of a soil sample. Advances in molecular biology and microbiology may eventually allow all bacteria in a soil sample to be identified via microarrays. Obviously this will require more research, but has the potential to make DNA-based soil comparisons even more objective, possibly introducing a level of precision, and allowing estimates of error, that meet important components of Daubert. Naturally these data will not supplant standard forensic analyses of soil, but could certainly add to them. Genetic data have the potential to introduce a distinctive facet to forensic soil analysis, advancing the field in a unique way.

Acknowledgments

We thank Dr. Darryl Warncke of Michigan State University for providing access to one of the habitats used in this study. The assistance of Aaron Tarone for statistical analyses and the input of Heather Wood and Ken Eilert were greatly appreciated. The Soil and Plant Nutrient Laboratory at Michigan State University performed the standard soil characterization.

References

1. Murray RC, Solelbello LP. Forensic examination of soil. In: Saferstein R, editor. Forensic science handbook Volume 1, 2nd ed. Upper Saddle River, NJ: Prentice Hall, 2002;615–33.
2. Janssen DW, Ruhf WA, Prichard WW. The use of clay for soil color comparisons. J Forensic Sci 1993;28(3):773–6.
3. Cengiz S, Karaca AC, Cakir I, Uner HB, Sevindik A. SEM-EDS analysis and discrimination of forensic soil. Forensic Sci Int 2004;141(1):33–7.
4. McVicar MJ, Graves WJ. The forensic comparison of soils by automated scanning electron microscopy. Canadian Soc Forensic Sci 1997;30(4):241–61.
5. Siegel JA, Precord C. The analysis of soil samples by reverse phase-high performance liquid chromatography using wavelength ratioing. J Forensic Sci 1985;30(2):511–25.
6. Cox RJ, Peterson HL, Young J, Cusik C, Espinoza EO. The forensic analysis of soil organic by FTIR. Forensic Sci Int 2000;108(2):107–16.
7. Van Dijck PJ, van de Voorde H. Evaluation of microbial soil identity in forensic science. J Leg Med 1984;93:71–7.
8. Horswell J, Cordiner SJ, Maas EW, Martin TM, Sutherland KBW, Speir TW, et al. Forensic comparison of soils by bacterial community DNA profiling. J Forensic Sci 2002;47(2):350–3.
9. Heath LE, Saunders VA. Assessing the potential of bacterial DNA profiling of forensic soil comparisons. J Forensic Sci 2006;51(5):1062–8.
10. Moreno LI, Mills DK, Entry J, Sautler R, Mathee K. Microbial metagenome profiling using amplicon length heterogeneity-polymerase chain reaction proves more effective than elemental analysis in discriminating soil samples. J Forensic Sci 2006;51(6):1315–22.
11. Kitts CL. Terminal restriction fragment patterns: a tool for comparing microbial communities and assessing community dynamics. Curr Issues Intest Microbiol 2001;2(1):17–25.
12. Liu W, Marsh TL, Cheng H, Forney LJ. Characterization of microbial diversity by determining terminal restriction fragment length polymorphisms of genes encoding 16S rRNA. Appl Environ Microbiol 1997;63(11):4516–22.
13. Kuske CR, Ticknor LO, Miller ME, Dunbar JM, Davis JA, Barns SM, et al. Comparison of soil bacteria communities in rhizospheres of three plant species and the interspaces in an arid grassland. Appl Environ Microbiol 2002;68(4):1854–63.
14. Buckley DH, Schmidt TM. The structure of microbial communities in soil and the lasting impact of cultivation. Microbial Ecol 2001;42(1):11–21.
15. Chin K, Lukow T, Conrad R. Effect of temperature of structure and function of the methanogenic archaeal community in an anoxic rice field soil. Appl Environ Microbiol 1999;65(5):2341–9.
16. Clemet BG, Kehl LE, DeBord KL, Kitts CL. Terminal restriction fragment patterns (TRFPs), a rapid, PCR-based method for the comparison of complex bacterial communities. J Microbiol Methods 1998;31:135–42.
17. Avaniss-Aghajani E, Jones K, Holtzman A, Aronson T, Glover N, Boian M, et al. Molecular technique for rapid identification of mycobacteria. J Clin Microbiol 1996;34(1):98–102.
18. Hackl E, Zechmeister-Boltenstern S, Bodrossy L, Sessitsch A. Comparison of diversities and compositions of bacterial populations inhabiting natural forest soils. Appl Environ Microbiol 2004;70(9):5057–65.
19. Dunbar J, Ticknor LO, Kuske CR. Assessment of microbial diversity in four southwestern United States soils by 16S rRNA gene terminal restriction fragment analysis. Appl Environ Microbiol 2000;66(7):2943–50.
20. Mummey DL, Stahl PD. Spatial and temporal variability of bacterial 16S rDNA-based T-RFLP patterns derived from soil of two Wyoming grassland ecosystems. FEMS Microbiol Ecol 2003;46:113–20.
21. Lukow T, Dunfield PF, Liesack W. Use of the T-RFLP technique to assess spatial and temporal changes in the bacterial community structure within an agricultural soil planted with transgenic and non-transgenic potato plants. FEMS Microbiol Ecol 2000;32:241–7.
22. Giovannoni SJ. The polymerase chain reaction. In: Stackebrandt E, Goodfellow M, editors. Nucleic acid techniques in bacterial systematics. New York, NY: John Wiley & Sons, 1991;177–203.
23. Lane DJ. 16S/23S rRNA sequencing. In: Stackebrandt E, Goodfellow M, editors. Nucleic acid techniques in bacterial systematics. New York, NY: John Wiley & Sons, 1991;115–75.
24. Cole JR, Chai B, Marsh TL, Farris RJ, Wang Q, Kulam SA, et al. The Ribosomal Database Project (RDP-II): previewing a new autoaligner that allows regular updates and the new prokaryotic taxonomy. Nucleic Acids Res 2003;31(1):442–3.
25. R Development Core Team. R: a language and environment for statistical computing. Vienna, Austria: R Foundation for Statistical Computing, 2004. ISBN 3-900051-00-3, http://www.R-project.org. Accessed on March 15, 2008.
26. Dunbar J, Ticknor LO, Kuske CR. Phylogenetic specificity and reproducibility and new method for analysis of terminal restriction fragment profiles of 16S rRNA genes from bacterial communities. Appl Environ Microbiol 2001;67(1):190–7.
27. http://www.mobio.com/products/productdetail.php?pid=159.
28. LaMontagne MG, Michel FC, Holden PA, Reddy CA. Evaluation of extraction and purification methods for obtaining PCR-amplifiable DNA from compost for microbial community analysis. J Microbiol Methods 2000;49:255–64.
29. Smalla K, Cresswell N, Medonca-Hagler LC, Wolters A, van Elsas JD. Rapid DNA extraction protocol from soil for polymerase chain reaction mediated amplification. J Appl Bacteriol 1993;74:78–85.

30. Tsai Y, Olson BH. Detection of low numbers of bacterial cells in soils and sediments by polymerase chain reaction. Appl Environ Microbiol 1992;58(2):754–7.
31. Osborn AM, Moore ERB, Timmis KN. An evaluation of terminal restriction fragment length polymorphism (T-RFLP) analysis for the study of microbial community structure and dynamics. Environ Microbiol 2000;2(1):39–50.

Additional information and reprint requests:
David R. Foran, Ph.D.
School of Criminal Justice and Department of Zoology
560 Baker Hall
Michigan State University
East Lansing, MI 48824
E-mail: foran@msu.edu