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PAPER

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Erin J. Lenz,[1,†] *M.S. and David R. Foran,*[2] *Ph.D.*

Bacterial Profiling of Soil Using Genus-Specific Markers and Multidimensional Scaling*

ABSTRACT: Forensic identification of soil based on microbial DNA fingerprinting has met with mixed success, with research efforts rarely considering temporal variability or local heterogeneity in soil's microbial makeup. In the research presented, the nitrogen fixing bacteria rhizobia were specifically examined. Soils were collected monthly from five habitats for 1 year, and quarterly in each cardinal direction from the main collection site. When all habitats were compared simultaneously using Terminal Restriction Fragment Length Polymorphism analysis of the rhizobial *recA* gene and multidimensional scaling, only two were differentiated over a year's time, however pairwise comparisons allowed four of five soils to be effectively differentiated. Adding in 10-foot distant soils as "questioned" samples correctly grouped them in 40–70% of cases, depending on restriction enzyme used. The results indicate that the technique has potential for forensic soil identification, although extensive anthropogenic manipulation of a soil makes such identification much more tentative.

KEYWORDS: forensic science, terminal restriction fragment length polymorphism, rhizobia, bacterial DNA fingerprint, soil microbiology, recombination protein A

Soil has long been considered to be of broad evidentiary value, potentially linking a victim or suspect to a crime scene (1,2). Traditional forensic soil analysis includes observation of color and particle size, assaying chemical features such as pH and organic content, and evaluation of extraneous materials (2). Biological techniques have also been used in an attempt to "fingerprint" soil samples, including surveying pollen (3) or plant wax (4). More recently, the highly variable microbial makeup of soil has been targeted as a tool for its fingerprinting, potentially leading to the identification of a soil's origin (5–11).

Terminal restriction fragment length polymorphism (T-RFLP) analysis has become one of the primary molecular techniques for identifying and differentiating the bacterial content of biological or physical specimens (5,12–15). In this method, a specific segment of DNA is amplified based on conserved primer binding sites for the polymerase chain reaction (PCR), which allows all bacterial strains or species of interest to be scrutinized. One primer carries a 5′ fluorescent tag for subsequent detection (13,14). The amplicons are digested with a restriction enzyme that, based upon sequence variability between the primer sites, produces different-sized products. Digested amplicons are separated and detected via capillary electrophoresis, resulting in a bacterial profile. Specific bacterial strains or species may be identified, or more general overall comparisons between or among samples can be made.

Applying bacterial T-RFLP analysis to soil in a forensic context has met with some, although limited, success. Different soils do generate diverse T-RFLP profiles (11,13,16) and as a rule soil profiles collected within a given site are more similar than are profiles between sites (5,11,15). However, for this technique to be useful forensically, the bacterial content of soil must be relatively homogeneous both spatially and temporally. For instance, it is unlikely that a known soil sample will be collected from the exact same location from which evidentiary material originated. Therefore, if microbial content is heterogeneous over small distances, a DNA profile could erroneously exclude soil from the questioned site. Likewise, known soil samples will rarely be collected immediately after a crime occurs; if the bacterial makeup of soil changes substantially over time, once again the origin of evidentiary soil may be excluded in error.

In an effort to address all of these factors, we recently undertook a spatial and temporal study of soils collected from five different habitats in central Michigan (woodlot, yard, marsh, agricultural field, and a sandy woodlot *c*. 100 miles distant) (11). Soil samples were collected from the same location monthly for 1 year, as well as 10 feet in the cardinal directions every 3 months. The widely utilized 16S ribosomal RNA (rRNA) gene, present in all bacteria, was assayed, and T-RFLP profiles were compared pairwise, based on the similarity of peaks produced.

The results showed that bacterial profiles within a habitat were on average more similar than among habitats. However, there were large levels of variability within habitats both spatially and temporally. Indeed, it was not unusual to find month-to-month similarity values that were lower within a habitat than between habitats, suggesting that the forensic utility of the methodology was limited at best, and might even be misleading. Specific reasons for this are unclear and likely were affected by a variety of factors. Two of the habitats, the yard and agricultural field, were heavily manipulated

[1]Forensic Science Program, School of Criminal Justice, 560 Baker Hall, Michigan State University, East Lansing, MI 48824.
[2]Forensic Science Program, School of Criminal Justice and Department of Zoology, 560 Baker Hall, Michigan State University, East Lansing, MI 48824.

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†Present address: Indiana State Police Laboratory, 550 West 16th Street, Suite C, Indianapolis, IN 46202.

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anthropogenically (including plantings and/or fertilization), which could easily influence microbial content. The distant sandy woodlot was least similar to the other habitats, which may have resulted directly from remoteness, but could also have been a consequence of geological or meteorological variables. Most importantly, the T-RFLP profiles were extremely "complex," resulting from the very large number of species assayed. The high level of variability within and among habitats indicated that assaying all bacteria present in soil would likely not prove to be a useful tactic for forensic soil comparisons.

Given this, a new strategy for soil bacteria profiling was investigated, in which far fewer species were considered. In this research, the recombination protein A (*recA*) gene specific to the nitrogen-fixing bacteria (rhizobia) was assayed in an attempt to simplify T-RFLP profiles. Rhizobia form root nodules in leguminous plants and vary by habitat and plants species present (17–19). Approximately 75 rhizobial species have been described (20), a subset of which would be expected in any one habitat. The same soil DNAs used in (11) were assayed, again examining bacterial profiles within and among habitats, both spatially and temporally. Profiles were analyzed using multidimensional scaling, which allowed more data from each habitat to be included than did pairwise similarity indices.

Materials and Methods

Soil DNA Isolation and Amplification

Soils were collected from the above-mentioned habitats from September 2004 to August 2005, with DNA extracted thereafter, as detailed in (11). The rhizobial *recA* gene was amplified using forward primer 5′-CATGCRCTGGATCCGGTCTATGC-3′ and reverse labeled primer 5′-[6FAM]CTTGTTCTTGTCGACCTTGACGCG-3′) (18). Twenty microliter amplification reactions included 1 unit of AmpliTaq Gold® DNA Polymerase (Applied Biosystems, Foster City, CA), 1× of the included buffer, 2.5 mM $MgCl_2$, 2 μL of 1 μg/μL bovine serum albumin, 0.2 mM of each dNTP (Promega, Madison, WI), and 2 μM of each primer. Cycling parameters were an enzyme activation step of 10 min at 94°C, followed by 32 cycles of 30 sec denaturation at 94°C, primer annealing at 55°C for 30 sec, and a 45 sec extension at 72°C, with a final extension of 5 min at 72°C. Amplicons were visualized by separating 2 μL of the PCR product on a 2% agarose gel along with a 100 base pair (bp) DNA ladder (New England Biolabs, Beverly, MA). The gel was stained with ethidium bromide, and DNA yields were estimated by comparing the amplicon to the 400-bp size marker that contained *c*. 38 ng of DNA.

T-RFLP Profiling

The remaining PCR product was purified as described in (11). Amplified products were digested with *RsaI*, *MspI*, or *DpnII* (New England Biolabs), using 1 unit enzyme, 1× supplied buffer, 150 ng of PCR product as estimated by gel electrophoresis, and water to 10 μL. Digests were incubated at 37°C overnight, terminated by heating at 75°C for 20 min, purified, and electrophoresed as in (11), with a 10 sec injection time and 35 min separation time.

Analysis of T-RFLP Profiles

T-RFLP profiles were generated with ABI GeneMapper® ID, version 3.1 software (Applied Biosystems, Foster City, CA). Terminal restriction fragments from 40–450 bases with a peak height of at least 50 relative fluorescent units were included. Profiles were aligned and a matrix produced using T-Align (21), based on the presence/absence of shared peaks, with a confidence interval of 0.5 bases. The matrix was exported to Excel (Microsoft, Redmond, WA) as a binary file. Nonmetric multidimensional scaling (MDS) was performed in SPSS® version 15.0 (SPSS Inc., Chicago, IL), examining site heterogeneity, the effect of time, and the ability to differentiate among habitats. MDS is used to create a spatial representation of the data being compared. The program interprets the matrix data based on weighted Euclidean measurements, comparing how similar the samples are, with those having a large number of shared peaks being more similar. A spatial configuration of the samples in two-dimensional space (in this case) is then created. The dimensions have no specific value but simply plot similarities of the samples. This allows complex data sets to be easily visualized, with more similar samples grouping together to form clusters, and dissimilar samples being located further away in the two-dimensional plot.

The 12 monthly main collection sites of all five habitats were first analyzed using MDS as a group, to determine the extent to which the habitats could be differentiated in general. The same data were then analyzed as habitat pairs, generating 30 random comparisons for each of the three restriction enzymes. Next, one of the cardinal direction soils, 10 feet distant from the main collection site, was added into pairwise habitat analyses, acting as a "questioned" sample that should group with the known soils from a habitat if the technique is successful. A questioned soil was rated as grouping correctly if it plotted within 0.5 units of soils from the same habitat, incorrectly if plotted within 0.5 units of soils from a different habitat, and inconclusive if located approximately equidistant between the two.

Results

Amplification of the RecA Gene and T-RFLP Results

RecA was successfully amplified from all but one DNA sample, producing 135 T-RFLP profiles (exemplified in Fig. 1). Comparison of the 12 monthly collected soil DNAs from all habitats produced some generalizable results, in that the sandy woodlot and woodlot tended to plot separately, while the remaining three habitats (agricultural field, marsh, and yard) were not well differentiated (Fig. 2). The restriction enzyme used did not impact the discrimination of habitats. The *DpnII* digested DNAs (Fig. 2*a*) from the

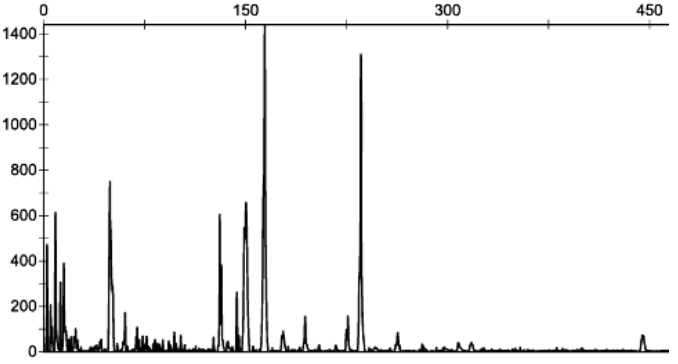

FIG. 1—*An exemplary terminal restriction fragment length polymorphism profile. Fragment size in number of bases is on the* x *axis and relative fluorescence units are on the* y *axis. Fragments between 40 and 450 bases with peaks heights 50 relative fluorescent units and greater were included in the analysis.*

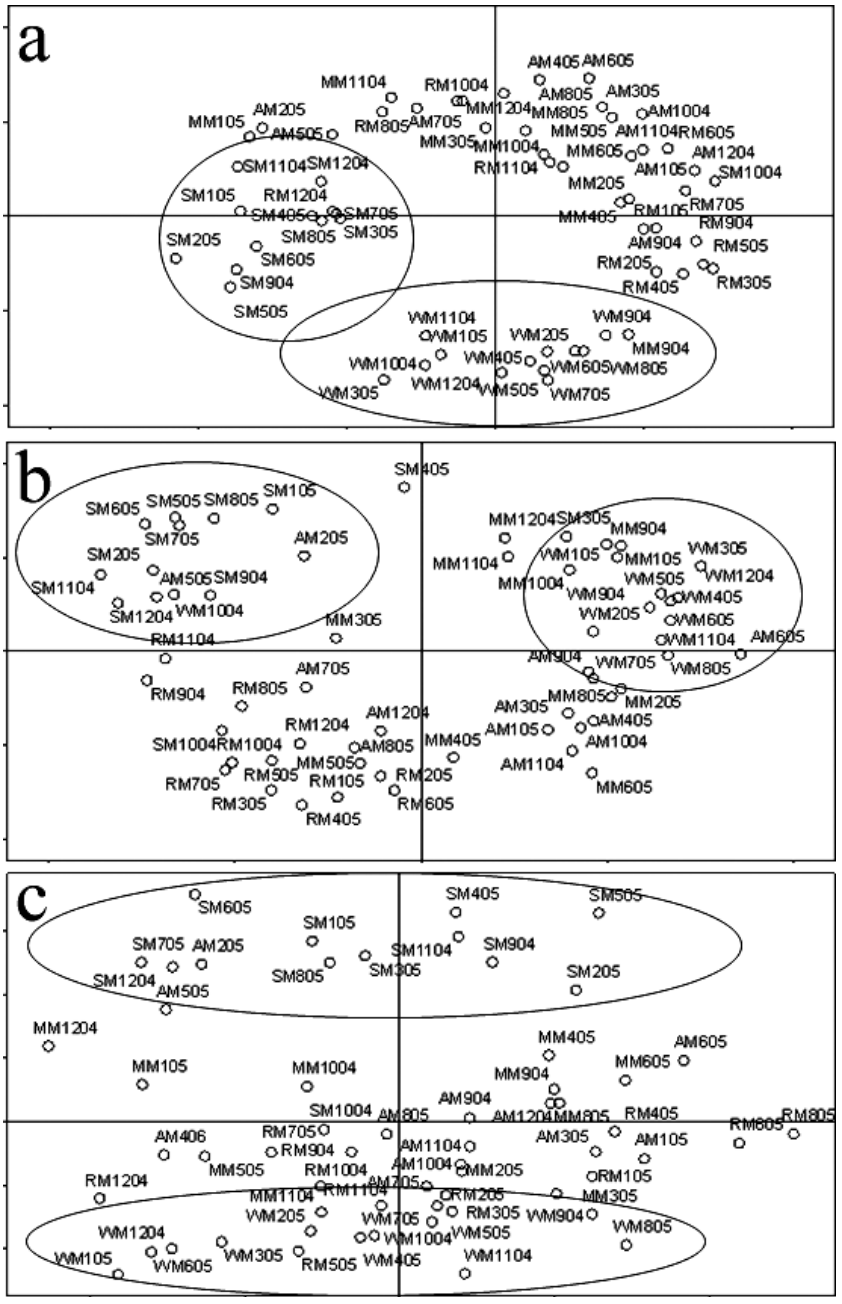

FIG. 2—*Multidimensional scaling plots produced when comparing the main sampling sites on a monthly basis from all habitats. Soils from the two woodlots (S; sandy woodlot, W; woodlot) frequently plotted separately and are circled, while the other habitats (A; agricultural field, M; marsh, R; yard) did not. (a) depicts a DpnII digestion, (b) an RsaI digestion, and (c) an MspI digestion. Month and year of collection are indicated with each plotted point.*

sandy woodlot and woodlot were distinguishable with little overlap from other habitats (the exceptions being soil collected from the marsh in September plotting similarly to woodlot samples, and soil from the yard in December plotting with the sandy woodlot). In contrast, the three other habitats were indistinguishable from each other. DNAs from the sandy woodlot and woodlot digested with *RsaI* (Fig. 2*b*) were also differentiated, with only February and May agricultural field samples and the October woodlot sample grouping with the sandy woodlot, while January, September, and October marsh soils and the June agricultural field sample plotted with the woodlot. Yard soils tended to plot in the lower left quadrant, with the marsh and agricultural field not being differentiated. Plots produced from *MspI* digestions (Fig. 2*c*) generated similar results, again generally differentiating the two woodlots, although agricultural field soils from February and May plotted with the sandy woodlot and woodlot, respectively. However, only one dimension distinguished the habitats, resulting in a wide distribution of plotted points for each habitat along the *x* axis.

Habitats were correctly differentiated for all enzymes when pairwise comparisons were conducted (e.g., Fig. 3*a–c*). The sole exception was the agricultural field, which was indistinguishable from the habitat to which it was compared in ten of twelve plots (e.g., Fig. 3*d*), the exceptions being comparisons with *DpnII* and *RsaI*-digested woodlot soils. Owing to this, the agricultural field was excluded from subsequent pairwise analyses detailed below.

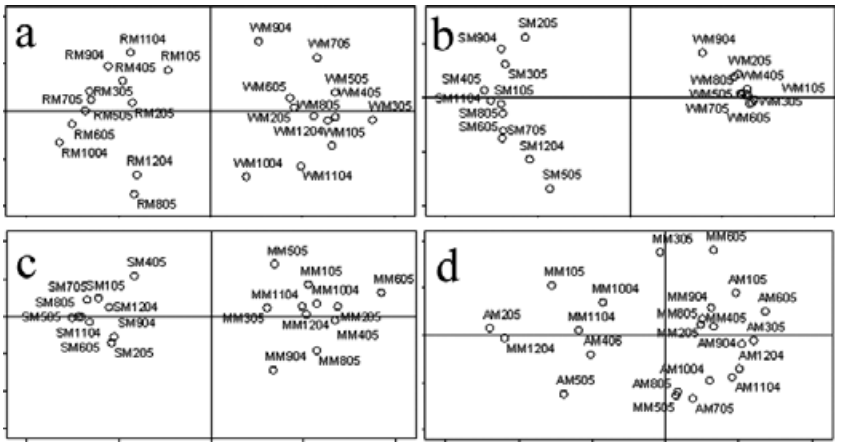

FIG. 3—*Multidimensional scaling plots produced when performing pairwise comparisons of the main sampling sites. (a) shows a DpnII digestion of yard (R) and woodlot (W) soils, (b) an MspI digestion of sandy woodlot (S) and woodlot (W) soils, (c) an RsaI digestion of sandy woodlot (S) and marsh (M) soils, and (d) soils collected from the agricultural field (A) and marsh (M) digested with MspI. Due to the inability to differentiate between habitats, the agricultural field was excluded from further pairwise comparison analysis.*

Inclusion of a Questioned Soil in Pairwise Habitat Comparisons

When 10-foot distant soils were added to the pairwise habitat comparisons described directly above, they grouped with soils originating from the same habitat 40% to 70% of the time depending on the enzyme used (e.g., Fig. 4*a*,*b*). Inconclusive samples (those plotting approximately equidistant between the two habitats) varied from 17% to 47% (Fig. 4*c*), and incorrect grouping ranged from 13% to 23% (Fig. 4*d*). Specifically, the "questioned" soil clustered with the same habitat 70% of the time using *DpnII*, while 17% grouped incorrectly and 13% were inconclusive. *MspI* "questioned" digestions plotted correctly 50% and incorrectly 23% of the time, with the remaining 27% inconclusive. Finally, plots from *RsaI* digestions grouped the "questioned" soil with soils from the same habitat with 40% accuracy, while 13% grouped incorrectly and 47% were inconclusive.

Discussion

Previous research examining the feasibility of using T-RFLP or similar molecular techniques to produce microbial fingerprints of soils has met with, at best, mixed success. Given the very large number of bacterial species/strains that exist in any given soil sample, this may stem, at least in part, from the generation of an exceedingly complicated picture of a soil's bacterial makeup. We thus decided to focus on a more limited bacterial community, with the goal of simplifying and clarifying soil bacterial fingerprints. In addition, multidimensional scaling analysis allowed for a deeper inclusion of available data than did similarity indices, the latter of which only examine profile pairs, multiplying shared peaks by two and dividing by the total number of peaks present in both (22). This may be a major drawback in a forensic context, when the precise origin of a soil sample is unknown. In contrast, MDS can be used to generate a matrix of similarities that are then weighted, with peaks found in multiple habitats accentuated and background "noise" eliminated. The projected similarities are plotted in two-dimensional space, resulting in easy to interpret profiles depicting which soils group similarly.

T-RFLP profiles produced using the rhizobium-specific *recA* gene were less complex than those produced via the bacterial 16S rRNA gene (11), while still generating an adequate number of peaks for comparison. Examining all five habitats at once generally

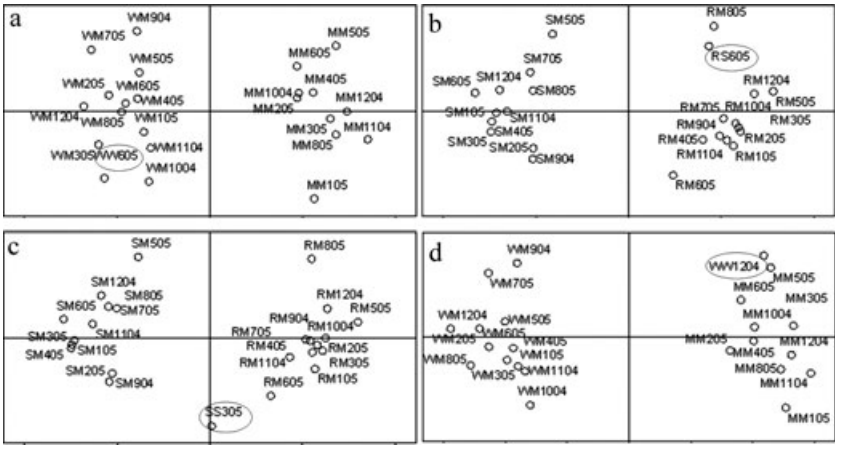

FIG. 4—*Multidimensional scaling plots produced when adding "questioned" soils (circled) collected from the four cardinal directions to pairwise comparisons of the main sampling sites. (a) shows a DpnII digestion of marsh (M) and woodlot (W) soils, (b) an MspI digestion of sandy woodlot (S) and yard (R) soils, (c) an MspI digestion of sandy woodlot (S) and yard (R) soils, and (d) soils collected from the woodlot (W) and marsh (M) digested with DpnII. (a) and (b) represent correct groupings of the questioned soil, (c) portrays an inconclusive result, and (d) an incorrect grouping of the questioned soil with the contrasting habitat.*

produced ambiguous (overlapping) results, although this is perhaps not surprising given a two-dimensional scale with so many habitats and data points. Interestingly, the woodlot and sandy woodlot were effectively differentiated even in the five-way plots, utilizing any of the three restriction enzymes. Only the yard was likewise differentiated, in one instance (*RsaI* digests); the remaining habitats producing broad, overlapping groupings. The specific reason(s) for the differentiation of only the woodlots is unknown, although it is notable that the sandy woodlot was also the most unique habitat based on bacterial 16S rRNA T-RFLP analysis (11). The fact that the sandy woodlot was 100 miles distant from the other habitats indicates that distance might play a large role in bacterial diversity. This has clear forensic implications, wherein large differences in bacterial makeup between questioned and known soil samples may indicate not just habitat variability, but spatial divergence as well. The woodlots were also the least disturbed of the habitats, with the agricultural field being highly manipulated (detailed below), the yard generally being a monoculture that was mowed regularly during the growing season, and the marsh, although much less perturbed, being adjacent to several dwellings.

Overall, it appears that comparing five habitats at a time, each with a dozen soil collection time points, overwhelms the ability to differentiate them in unison. Given this, pairwise habitat analyses were performed, which represent a more realistic forensic scenario as well—where the prosecution claims soil originated from one source while the defense identifies a contrasting location. Pairwise comparison proved much more successful in differentiating soils, again distinguishing both woodlots, but also separating the marsh and yard as well, for all restriction enzymes tested. A major implication of these findings is that known soil samples can potentially be collected well after a crime occurred without detrimental outcomes, given that time/season did not have a substantial negative influence on the ability to group soils from a habitat, even though samples were collected throughout a 1 year period.

In contrast to these four habitats however, the agricultural field was not distinguishable from other habitats in 10 of 12 pairwise comparisons, nor did its soil samples group among themselves. The likely reason for this anomaly is the extreme amount of human manipulation the field experienced, having undergone rounds of tilling, plantings, and fertilization. Soybean (a legume that relies on rhizobia) was sown during the first summer, which was replaced by corn the second summer. The latter crop requires extensive nitrogen fertilization, which took place in the spring prior to its planting. Clearly, all of these could drastically influence the bacterial makeup of the soil. Tilling would also act to mix and homogenize the soil, further altering its bacterial content near the surface where soil collection took place. Such anthropogenic manipulation may explain not only the failure of agricultural field data points to cluster in pairwise comparisons, but also the lack of data clustering in rhizobial profiles seen when all five habitats were compared simultaneously.

The ultimate test of a technique designed for soil identification is determining whether a questioned soil sample correctly groups with known soil from a site, versus soil from an incorrect site. For this, we utilized a subset of the soils obtained 10 feet distant from the main soil collection location and applied them back into 90 pairwise habitat comparisons of main collection sites. These distant soils (acting as the "questioned" samples) were employed as it is possible that known samples will not be collected from the exact spot where a questioned soil originated. It should be noted that the nature of MDS is that all data are utilized, thus these tests did not simply compare the distant soil to the others, but instead reanalyzed the data with the unknown soil's profile incorporated as part of the overall pairwise comparison.

The 10-foot distant questioned soils grouped back with the correct habitat soils 40–70% of the time (dependant on restriction enzyme); however they also grouped with the incorrect soil 13–23% of the time. Inconclusive results (i.e., the questioned sample grouped with neither known soil) were observed 13–47% of the time. There was no clear pattern as to which questioned soil grouped with its respective habitat, and only the marsh samples never incorrectly clustered with the compared habitat. This was surprising given how well four of the habitats were clearly differentiated in pairwise comparisons, or even in five-way comparisons when considering the woodlots. The fact that the marsh did not produce any erroneous results indicates it was more spatially homogeneous than were the other habitats, while still containing enough unique strains of rhizobia to differentiate it. Most importantly, the results clearly indicate that spatial heterogeneity is an extremely important factor in microbial DNA profiling and relatively small distances between soil sampling can have a critical influence on such assays. In contrast, if a questioned soil originated from the same location as the knowns, it seems likely to group with them, even if they were collected at a different time.

Local heterogeneity could stem from a wide range of environmental factors, such as unique plant species, the amount of sunlight reaching the soil, and differing moisture levels. Mummey and Stahl (23) obtained samples from two different types of grasslands in Wyoming and found that similarities within a grassland decreased rapidly at distances >3.6 m. The authors proposed that differences in plant distribution, rooting patterns, or soil organic content could induce different species of bacteria to flourish across such a small area. The results presented here are consistent with this, and local heterogeneity may end up playing a large role in the utility of microbial fingerprinting of soils.

Conclusions

T-RFLP analysis has become a valuable technique in microbial ecology and can be performed quickly and easily with a resolution that allows even related strains of bacteria to be differentiated. Likewise, soils show the potential to be successfully differentiated via T-RFLP, given the right circumstances. The markers utilized (in the current research, rhizobia-specific) must be considered, and the soil cannot have been heavily modified and manipulated between the time when a questioned soil originates and known samples are obtained. If these variables are controlled for, T-RFLP examination appears to have forensic potential. This is not to say, however, that bacterial T-RFLP analysis is ready to be implemented in a forensic setting. Before this can happen, spatial heterogeneity of soil bacteria will need to be better understood, so that known samples, most likely collected some distance from a soil of questioned origin, reliably group together. Further, while the T-RFLP technique is well documented in the peer-reviewed literature, other tenants of Daubert, particularly error rates, will need to be addressed. As bacterial profiling of soil matures in general, genetic identification of soils seems plausible and may offer an objective and reliable method of forensic soil analysis.

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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