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Microbial Metagenome Profiling Using Amplicon Length Heterogeneity-Polymerase Chain Reaction Proves More Effective Than Elemental Analysis in Discriminating Soil Specimens*

ABSTRACT: The combination of soil's ubiquity and its intrinsic abiotic and biotic information can contribute greatly to the forensic field. Although there are physical and chemical characterization methods of soil comparison for forensic purposes, these require a level of expertise not always encountered in crime laboratories. We hypothesized that soil microbial community profiling could be used to discriminate between soil types by providing biological fingerprints that confer uniqueness. Three of the six Miami-Dade soil types were randomly selected and sampled. We compared the microbial metagenome profiles generated using amplicon length heterogeneity-polymerase chain reaction analysis of the 16S rRNA genes with inductively coupled plasma optical emission spectroscopy analysis of 13 elements (Al, B, Ca, Cu, Fe, K, Mg, Mn, Na, P, S, Si, and Zn) that are commonly encountered in soils. Bray–Curtis similarity index and analysis of similarity were performed on all data to establish differences within sites, among sites, and across two seasons. These data matrices were used to group samples that shared similar community patterns using nonmetric multidimensional scaling analysis. We concluded that while chemical characterization could provide some differentiation between soils, microbial metagenome profiling was better able to discriminate between the soil types and had a high degree of reproducibility, therefore proving to be a potential tool for forensic soil comparisons.

KEYWORDS: forensic science, soil forensics, microbial forensics, microbial profiling, amplicon length heterogeneity (ALH), soil metagenome, inductively coupled plasma optical emission spectroscopy, elemental analysis

Soil is an ubiquitous material that can provide valuable clues in forensic investigations due to the vast array of information contained therein. Crime scenes in which soil evidence was found have established soils' geological ability to provide critical findings and ultimately aid in the conviction or exoneration of individuals (1–4). However, soil analysis has been limited for the most part to physical analyses of the material (5–7) that are conducted by experts in the geological field. In addition, the type of analyses performed usually requires large sample sizes that are seldom encountered in crime scenes (8). These analyses include, but are not limited to, color, particle size distribution, microscopic comparisons, rock and mineral identification as well as chemical methodologies to identify fragments of glass and other trace materials that might be found in the soil (9). We have limited this investigation to compare two methods, elemental and microbial analyses, that will require little or no extra expertise from the analyst side. These two methods exploit an aspect of soil that, although vastly used in the ecological field, has not been embraced in the forensics field. Although it is very difficult and highly unlikely to individualize soils because they are constantly in a state of flux, soil comparisons for forensic purposes, given a known and an evidence sample, can provide very useful, exclusionary information.

Elemental analyses of soils have been performed using sensitive techniques such as atomic absorption spectroscopy (AAS) and inductively coupled plasma mass spectrometry (ICP-MS) to identify the elements in environmental samples (10–13). In this study, inductively coupled plasma optical emission spectrometry (ICP-OES) was used to measure elemental concentration of the sampled soils. This technique, for example, has been used to identify heavy metal contamination by providing information regarding the forms of metals present in soil and has aided in establishing preventive and remediation techniques (14–17). These analytical techniques have been frequently used with soils to obtain information that would otherwise be impossible to detect.

Except for the work of Horswell et al. (18), the biological aspect of soils for forensic purposes has been ignored. In that study, they concluded that a given soil could be traced back to its origin based on the microbial community profile obtained by terminal restriction fragment length polymorphism (T-RFLP) analysis of the 16S rRNA genes. This molecular profiling method has been extensively used by ecologists to establish differences between microbial communities (19–22). While it provides good discrimination, T-RFLP analysis requires a large amount of time, and the output can be compromised by incomplete enzymatic DNA digestion (23). With the growing interest in microbial forensics and the development of new techniques that have proven useful in the forensic field, soil biotic characterization has been gaining import and the wealth of information contained therein is being decoded.

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We proposed that physical analysis, other than the aforementioned, and routine chemical analyses cannot impart enough discrimination between soils for them to be useful assays in soil differentiation. However, if soil type drives the microbial community inherent to it (24–26), then microbial metagenome profiling can produce a unique soil fingerprint that could potentially be used as collaborative evidence when trying to establish an evidentiary relationship between a suspect and a crime scene. In this study, standard methods for ICP-OES were used to measure the elemental concentration of three different soil types. For the microbial investigation, amplicon length heterogeneity-polymerase chain reaction (ALH-PCR), a technique that has often been used in ecological settings to characterize microbial communities, was performed (27,28). The technique uses universal primers to amplify hypervariable domains within the 16S rRNA gene. The PCR amplicons are separated on high-resolution genetic analyzers (29) and provide a unique pattern for specific samples. This robust and highly reproducible method, unlike T-RFLP, does not depend on restriction enzyme recognition sites, but is based on the natural variation in sequence lengths of specific domains within the gene (19,27).

Methods

Soil Sampling

Three of the six Miami-Dade soil types recognized by the U.S. Department of Agriculture (USDA) (30) were sampled in February and August 2004 in order to account for seasonal variations. February, with an average rainfall of 2.75 in., was considered the dry season, whereas, August, which averaged 9.17 in., was named the wet season (31). The sampled soil sites were: Urban Land Udorthents (ULU), which are moderately well-drained soils consisting of fill material, usually stony, that is 20 cm to $>$203 cm deep over limestone bedrock, and the vegetation consisted mainly of wild grasses; Perrine–Biscayne–Pennsuco (PBP), with poorly drained soils that consist of marl and are 20 cm to $>$203 cm deep over hard limestone, whose dominant vegetation was sawgrass; and Perrine–Terra–Ceia–Pennsuco (PTCP), which are poorly drained soils consisting of marl that is 102–203 cm deep over limestone, and the vegetation consisted mainly of black mangrove. Nine samples from each soil type were collected at each site. Triplicate 10-cm deep subsamples were randomly taken within the three 2 m^2 quadrats. Each quadrat was located 30.5 m apart along a linear transect for each of the sites. Two different sites were sampled within the ULU soil type: ULU-1 and ULU-2. The collection sites were 32 km apart and were used to determine whether our method would be able to differentiate soil samples within the same soil type.

Moisture Content and pH

Percent moisture by weight was calculated after drying the samples in a 104°C oven for 24 h. The pH of each soil was determined using an AB15+ pH meter (Fisher Scientific, Suwanee, GA).

Elemental Analysis Using ICP-OES

Two hundred and fifty milligrams of sieved and homogenized soil sample aliquots were digested according to the Environmental Protection Agency (EPA) specifications (32). Trace analysis was performed on the digested samples with a Perkin-Elmer Optima 300 ICP-OES (Perkin-Elmer, Boston, MA) at the USDA facilities (Kimberly, ID) following the guidelines stipulated by the EPA (33). Each of the soil types was tested for the following suite of elements: aluminum (Al), boron (B), calcium (Ca), copper (Cu), iron (Fe), potassium (K), magnesium (Mg), manganese (Mn), sodium (Na), phosphorus (P), sulfur (S), silicon (Si), and zinc (Zn). Concentration data were normalized to Al using the Fingerprinting Analysis of Leachate Contaminants (FALCON) protocol (34).

Soil DNA Extraction

Each soil sample was extracted in duplicate using the FastDNA™ Spin Kit for Soil (Qbiogene, Irvine, CA) and 500 mg of soil, following the manufacturer's specifications.

High-molecular-weight DNA was quantified using a Versafluor™ Fluorometer (BioRad, Hercules, CA) and diluted to a 10 ng/μL working stock.

ALH-PCR of Microbial Metagenome

Three 16S rRNA hypervariable domains were amplified for each sample using paired universal eubacterial primers (29,35). The hypervariable domains amplified are (i) from the beginning of V1 to the end of V2 (V1_V2), (ii) V1 alone, and (iii) V3 alone. The V1_V2 region combination is used to assess the V2 region whose length is too short to be analyzed on its own. Even though the V1 region is being technically assayed twice, it is being done with different primer sets, thus likely to pick up different microorganisms. Each domain can be considered a different marker as it provides with different length amplicons. The V1_V2 region was amplified using the fluorescently labeled 27F-6FAM forward and the 355 reverse primers (Table 1). The first (V1) and third (V3) hypervariable domains were multiplexed using the fluorescently labeled forward primers, P1F-6FAM and 338F-6FAM, and unlabeled reverse primers, P2R and 518R, respectively (Table 1).

The PCR reaction was performed using 1 × PCR Buffer, 2.5 mM $MgCl_2$, 0.5 U AmpliTaq Gold LD DNA Polymerase™ (Applied Biosystems, Foster City, CA), 250 μM dNTPs (Promega, Madison, WI), 0.5 μM forward and reverse primers, 1.0% bovine serum albumin (BSA) fraction V (Fisher Scientific), 10 ng of metagenomic DNA and diethylpyrocarbonate-treated water to a final volume of 20 μL. For the multiplex reactions, the water volume was adjusted to account for the additional primer volume. Amplification was performed using a 9700 thermocycler (Applied Biosystems) and the following parameters: initial denaturation at

TABLE 1—*Primer selection.*

Primer Name	Region Amplified	Primer Sequence	Reference
27F	V1_V2	5′-6FAM-AGAGTTTGATCMTGGCTCAG-3′	(29)
355R	V1_V2	5′-GCTGCCTCCCGTAGGAGT-3′	(29)
P1F	V1	5′-6FAM-GCGGCGTGCCTAATACATGC-3′	(35)
P2R	V1	5′-TTCCCCACGCGTTACTCACC-3′	(35)
338F	V3	5′-6FAM-ACTCCTACGGGAGGCAGCAG-3′	(35)
518R	V3	5′-ATTACCGCGGCTGCTGG-3′	(35)

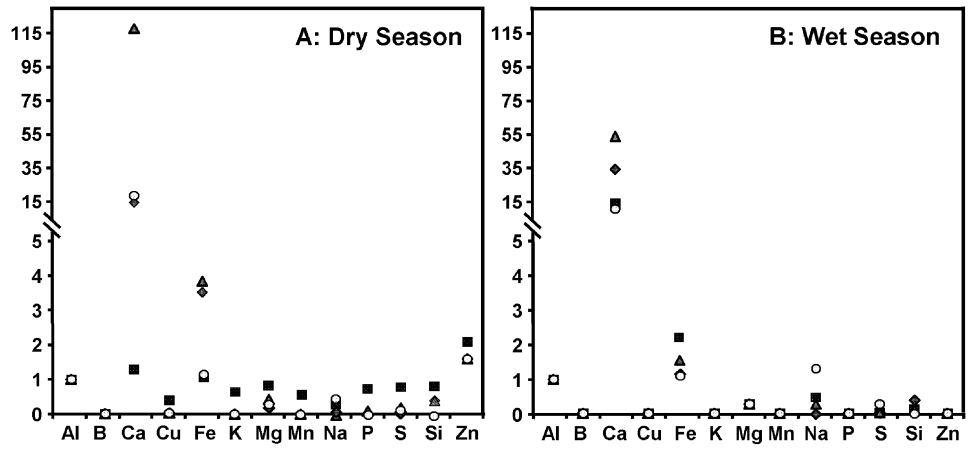

FIG. 1—*Elemental "fingerprint" of soil types. Soil types are represented by:* ■, *Urban Land Udorthents Site 1 (ULU-1);* ◆, *Urban Land Udorthents Site 2 (ULU-2);* ▲, *Perrine–Biscayne–Pennsuco (PBP); and* ○, *Perrine–Terra–Ceia–Pennsuco (PTCP). Each marker represents the abundance of each element per site relative to the Al concentration representative of each soil type.*

95°C for 11 min, 25 cycles of denaturation at 94°C, annealing at 55°C and extension at 72°C (each for 1 min), and a final elongation at 72°C for 10 min.

Microbial Profiling

Fragment analysis was performed by adding 0.5 μL of the PCR product to 9.5 μL of a 96:1 concentration mixture of Hi-Di™ formamide and GeneScan™ 500 ROX™ size standard (Applied Biosystems). Samples were heated for 2 min and snap cooled for 5 min before running for 28 min on an ABI™ 310 Genetic Analyzer (Applied Biosystems). Capillary electrophoresis separation used the POP-4 polymer (Applied Biosystems), matrix DS-30_6FAM_HEX_NED_ROX, and filter D.

Electropherogram Analysis

Output data were analyzed using GeneMapper™ ID, version 3.2 software (Applied Biosystems). Analysis parameters were set to the local Southern size calling, and the minimum noise threshold was set to 50 fluorescent units (27,36). Markers were created for the V1 and V3 and V1_V2 domains, ranging from 50–290 bp and 300–400 bp, respectively. Length separation was set to be recorded every one base pair using bins.

Statistical Analyses

All data were imported into MS Excel (Microsoft, Redmond, WA), interleaved, ratios calculated using the relative abundance of the observed peaks, and then exported and analyzed using PRIMER 5 statistical software (PRIMER E Ltd., Plymouth Marine Laboratory, Plymouth, U.K.). Bray–Curtis's similarity index and analysis of similarity (ANOSIM) (37) were performed on all data to establish differences within sites, among sites, and across two seasons. In addition, nonmetric multi-dimensional scaling (MDS) cluster analysis was used to compare site data (38,39).

Results

Moisture Content and pH Determination

Percent moisture was calculated to determine whether differences in moisture could be a factor in microbial community variations within a site during the wet and dry seasons. It was observed that only the PTCP site showed significant differences ($p < 0.05$) in percent moisture content between the sampled seasons (data not shown). Sampled soils exhibited a pH range of 6.4–7.6, which is considered neutral in terrestrial environments (40).

Elemental Analyses

Elemental concentration data showed that the contribution of most of the elements tested was relatively low in both seasons, except for Ca and Fe (Fig. 1). While for most of the elements the concentration decreased during the wet season, the Ca and Fe concentrations increased for both ULU sites, decreased for the PBP site, and remained approximately the same for the PTCP site. Although the ULU-1 and ULU-2 sites are geologically similar, the concentrations of Ca varied significantly between them.

The similarities between the different sites were statistically evaluated to a significant p-value of < 0.01 using ANOSIM (Table 2) (41). This method produced a test statistic (R) that ranged between -1 and 1 depending on whether the average rank similarity between samples within a soil site was more similar than the average rank similarity between samples from different soil sites (37). The ANOSIM Global R value is based on the difference of mean ranks between and within soil sites. A value close to 0 indicates little or no separation between the tested sites, whereas a value close to 1 indicates complete separation (37,42). The PBP site exhibited significant differences in its elemental composition as compared with all other soil sites for both seasons. In addition, wet season analyses showed that sites ULU-2 and PTCP had significant differences between them ($p < 0.01$; Table 2).

Microbial DNA Analyses

Three of the nine hypervariable domains contained in the 16S rRNA gene were used to evaluate the differences in DNA of the microbes inhabiting the four distinct locations with three different soil types in Miami-Dade County. The differences were statistically compared to determine the site discrimination based on this data. The variations in the microbial profiles between the four sites were obvious even in the raw data electropherograms that visualize the length of the amplicons and the relative abundance of the

TABLE 2—*ANOSIM for possible site combinations in both dry and wet seasons using elemental composition data.*

Soil Site*		Dry Season		Wet Season	
I	II	*R* Value†	Significance Level	*R* Value	Significance Level
ULU-1	ULU-2	−0.043‡	0.715	0.061	0.157
	PBP	0.409	0.006§	0.251	0.008§
	PTCP	0.044	0.191	0.066	0.159
ULU-2	PBP	0.523	0.001§	0.673	0.001§
	PTCP	0.063	0.145	0.273	0.003§
PBP	PTCP	0.598	0.001§	0.551	0.001§
Global *R*¶		0.25	—	0.31	—

*Soil types are represented by Urban Land Udorthents (ULU-1), Urban Land Udorthents Site 2 (ULU-2), Perrine–Biscayne–Pennsuco (PBP), and Perrine–Terra–Ceia–Pennsuco (PTCP).
†An *R* value close to 1 indicates statistical differences between the given sites.
‡Negative values are indicative of higher differences within a soil site than between soil sites.
¶Global *R* values indicate the level of similarity between all sampled sites, with $R = 0$ indicating strong similarity and $R = 1$ indicative of strong dissimilarity.
§Significant differences at the $p < 0.01$ level.
Column I is compared with Column II to account for all possible combinations.
ANOSIM, analysis of similarity.

individual peaks (Fig. 2). Similarly, seasonal variation was captured in the raw data output.

Nonmetric MDS Analysis of the Chemical Profile to Determine the Similarity Between Samples

Although some overlapping from the other sites was observed, statistical analyses suggest that soil site PBP is significantly different from the other three sampled sites (Fig. 3). Analysis without Ca was performed to determine whether the overwhelming concentration of this element (Fig. 1) was biasing the results. However, complete soil site differentiation was not achieved in the absence of Ca (Fig. 3).

Nonmetric MDS Analysis of the Microbial Metagenome Profile to Determine the Similarity Between Samples

The dissimilarities in amplicon presence and abundances within each sampled site for the two collection periods were confirmed by both ANOSIM (data not shown) and the nonmetric MDS analysis. Two clearly defined clusters were observed for dry and wet seasons in all the four soil sites sampled (Fig. 4).

All three hypervariable domains used for the microbial fingerprinting analyses were analyzed using ANOSIM (Table 3) and Bray–Curtis to determine the extent of similarity between sampled soils collected from the same site (e.g., PBP sample 1 compared

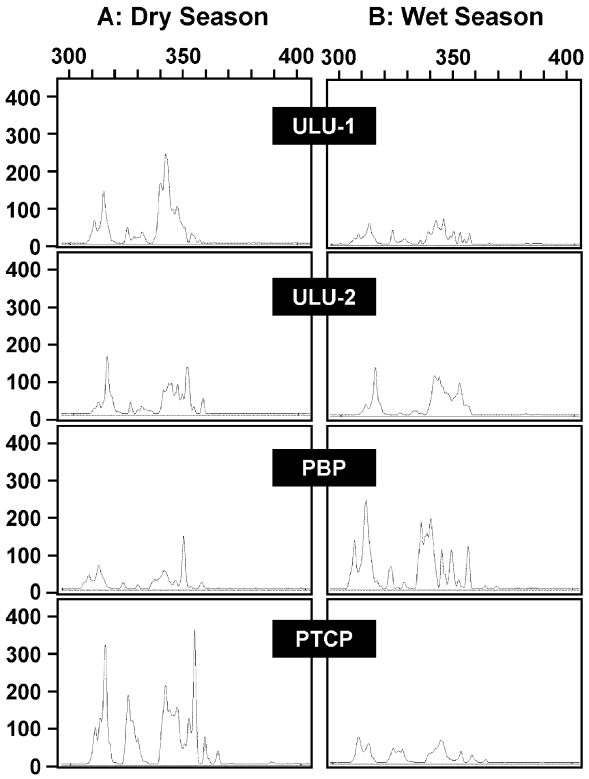

FIG. 2—*Sample electropherogram output. Sites are represented by Urban Land Udorthents Site 1 (ULU-1), Urban Land Udorthents Site 2 (ULU-2), Perrine–Biscayne–Pennsuco (PBP), and Perrine–Terra–Ceia–Pennsuco (PTCP). The panel shown represents the 16S rRNA V1_V2 hypervariable region output for both the dry and wet seasons.*

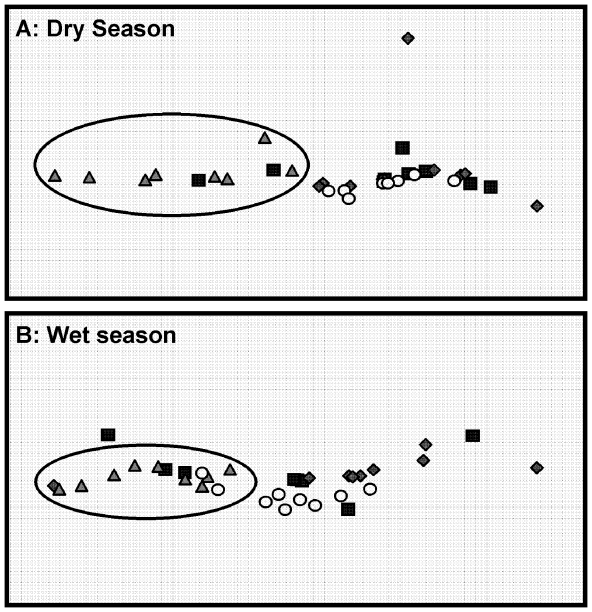

FIG. 3—*Nonmetric multidimensional scaling analysis of chemical data based on Bray–Curtis similarity coefficient. Sites are represented as follows:* ■, *Urban Land Udorthents Site 1(ULU-1);* ◆, *Urban Land Udorthents Site 2 (ULU-2);* ▲, *Perrine–Biscayne–Pennsuco (PBP); and* ○, *Perrine–Terra–Ceia–Pennsuco (PTCP).*

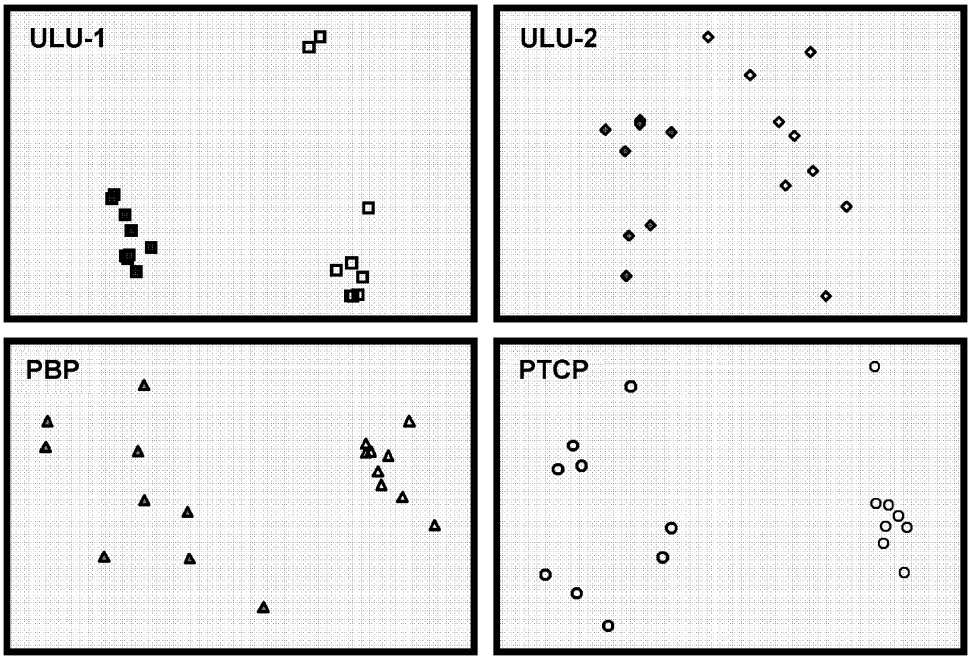

FIG. 4—*Seasonal nonmetric multidimensional scaling (MDS) analysis within sites. Sites are represented as follows: ■, Urban Land Udorthents Site 1 (ULU-1); ◆, Urban Land Udorthents Site 2 (ULU-2); ▲, Perrine–Biscayne–Pennsuco (PBP); and ○, Perrine–Terra–Ceia–Pennsuco (PTCP). The dry season is depicted by shaded marks and the wet season is represented by open markers.*

with PBP sample 2) as well as among sites (PBP compared with PTCP). The Global *R* value for V1_V2 domains was the lowest: 0.535 and 0.542 for the dry and wet seasons, respectively. Thus, V1_V2 represents the hypervariable domain least likely to discriminate among sites. The Global *R* value was the highest (0.874 and 0.777 for the dry and wet seasons, respectively) when using V3 alone. The combined V1 and V3 domains provided the highest discriminatory power with a Global R value of 0.973 in the dry season and 0.823 in the wet season (Table 3). A combination of three domains did not increase the discriminatory power (Global *R* value of 0.972 and 0.828 for the dry and wet seasons, respectively). Visual representation of the Bray–Curtis analysis obtained using MDS shows clear clustering of the samples from the four sites in both seasons (Fig. 5).

Discussion

Miami-Dade Soils Show No Variation in Moisture Content and pH

Percent moisture content is not based on the amount of water infiltrating the soil during a rain event; it is the amount of water that a specific type of soil can absorb until all soil pores are saturated and water accumulates on the soil surface. Because of this phenomenon, no variations were observed in percent moisture

TABLE 3—*ANOSIM R coefficient results for the different 16S rRNA regions examined and their possible combinations.*

Soil Sites		Single			Combination of Two			
I	II	V1	V3	V1_V2	V1-V3	V1, V1_V2	V3, V1_V2	All Regions
(A) Dry season								
ULU-1	ULU-2	0.877	0.886	0.540	0.968	0.859	0.951	0.960
	PBP	0.713	0.999	0.436	0.984	0.721	0.997	0.974
	PTCP	0.894	0.997	0.711	1.0	0.948	0.977	1.0
ULU-2	PBP	0.910	0.542	0.397	0.894	0.886	0.573	0.911
	PTCP	0.917	0.980	0.841	1.0	0.970	0.999	1.0
PBP	PTCP	0.832	1.0	0.436	1.0	0.893	0.999	1.0
Global *R**		0.803	0.874	0.535	0.973	0.830	0.911	0.972
(B) Wet Season								
ULU-1	ULU-2	0.538	0.340	0.350	0.517	0.569	0.410	0.547
	PBP	0.555	1.0	0.502	0.719	0.570	0.978	0.715
	PTCP	0.749	0.887	0.547	0.792	0.752	0.859	0.797
ULU-2	PBP	0.772	0.787	0.324	0.869	0.801	0.787	0.873
	PTCP	0.998	0.556	0.685	0.961	1.0	0.634	0.970
PBP	PTCP	1.0	1.0	0.902	1.0	1.0	0.999	1.0
Global *R*		0.772	0.777	0.542	0.823	0.789	0.788	0.828

ULU-1, Urban Land Udorthents; ULU-2, Urban Land Udorthents Site 2; PBP, Perrine–Biscayne–Pennsuco; PTCP, Perrine–Terra–Ceia–Pennsuco; ANOSIM, analysis of similarity.

*Global *R* values close to 1 indicate high dissimilarity between sampled sites.

Column I is compared with Column II to account for all possible combinations.

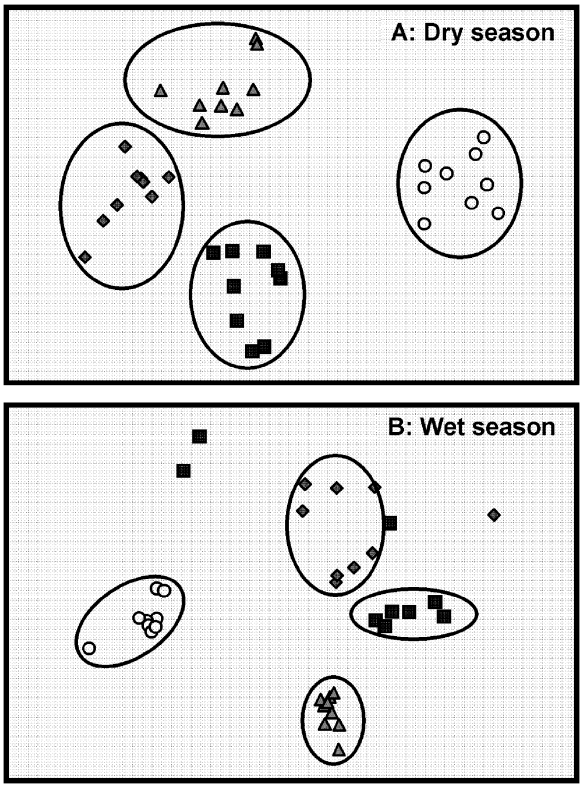

FIG. 5—*Multidimensional scaling analysis of microbial community data based on Bray–Curtis similarity coefficient using a combination of markers V1 and V3. Sites are represented as follows:* ■*, Urban Land Udorthents Site 1 (ULU-1);* ◆*, Urban Land Udorthents Site 2 (ULU-2);* ▲*, Perrine–Biscayne–Pennsuco (PBP); and* ○*, Perrine–Terra–Ceia–Pennsuco (PTCP).*

content within sites for the Miami-Dade soils in the different seasons. This indicated that any change in the microbial community observed within a soil type during a particular season was not significantly influenced by moisture content. However, we cannot rule out that moisture may affect dissolved macronutrient bioavailability that, in turn, could positively (or negatively) impact the microbial communities. It is not possible to infer, based on the results of this investigation, what is the direct factor shifting the microbes in a particular soil type. Perhaps, the amount of rainfall that affects the vegetation may drive the changes in microbial community composition. For soils that are exposed to extreme and extended stresses such as snow and flooding, these factors have to be included when assessing the soil microbial structure.

The fact that soil pH remained constant and at a neutral value in all sites and seasons was not surprising. Low soil pH is usually not a problem in south Florida because the natural soil bedrock is composed of limestone, an efficient buffering agent (30).

Chemical Analyses of Common Soil Elements Are Not a Good Discriminatory Tool

Although elemental analyses of a wide array of materials are performed in the forensic sciences field, soil has yet to become a popular forensic tool among chemists. Having only been tested in a limited number of investigations (43,44), soil chemistry has not established a method that embodies the reliability and reproducibility that characterizes any good forensic technique. For instance, polarized light microscopy (PLM), along with other types of microscopy, has been used in the past to compare trace evidence that can be embedded in soil evidence in order to establish a match. In this investigation, we attempted to use soil chemistry as a marker to discriminate between soils for forensic purposes. We used a technique that, although somewhat more complex and time consuming than others, had the potential to produce reliable and reproducible results. ICP-OES has been used successfully in forensic scenarios (45,46) as well as in other aspects of soil monitoring (17,47).

When working with natural ecosystems in which variability can be induced by a number of factors, a normalization method that takes into account possible induced alterations to the system has been found to be critical. The method must help in the reduction of background "noise" contributed by water infiltration, leaching, or by the formation of aluminosilicates. This approach is needed to obtain the basal concentration of elements (48–50). In soil, normalization is usually performed against a conserved element such as Al or Fe (49). Al, however, has proven to reduce the confounding effects produced by external addition of chemicals (51) and in contrast to other conserved elements, has the capacity of taking into consideration the formation of aluminosilicates, the common carrier phase for adsorbed elements found in soil (52,53).

Our analysis revealed that the overall elemental composition of the sampled Florida soil was relatively low as compared with the amount of Ca present, regardless of the season (Fig. 1). Florida's bedrock is essentially the same throughout most of the state and it is composed of the mineral limestone that is constituted by calcium carbonate and sediment. We argued that the elemental composition of Florida soils would be masked by the overpowering concentration of this mineral within similar soils. Thus, the analysis with the inclusion of Ca was deemed not to be a good marker for forensic comparisons. However, analysis performed without this abundant element produced a similar result (data not shown).

Despite the increase/decrease in elemental concentration during the wet season, it was observed that the clustering pattern of the soils remained the same for both seasons, indicating a similar mineral composition regardless of season. This pattern, however, failed to discriminate between the sampled soil sites, making this chemical differentiation an unsuitable forensic tool. Despite the lack of success in separating the soils, chemical characterization cannot be ruled out as a potential tool in forensics, as "blind" determination of chemical concentrations could provide a totally different pattern.

Microbial Metagenome Profiling Using the 16S rRNA Genes Proves to be a Useful Discriminatory Tool in Soil Forensics

Initial electropherogram data obtained from ALH-PCR analysis showed perceptible differences by visual inspection (Fig. 2); however, it cannot be inferred that these differences are significant enough to be used as conclusive results. Bray–Curtis similarity matrices are nonparametric tests best suited for continuous data sets such as ALH-PCR profiles, when normal distribution of data cannot be assumed. It bases its performance on the fact that identical samples have 100% similarity and samples with no species in common have 0% similarity, and negligible bias is introduced into the calculations due to the shared absences of amplicons (37,54). The similarity measures are then statistically tested for significant differences using one of many ordination methods, in this case nonmetric MDS analysis. MDS refers to a group of methods to analyze pairwise dissimilarities of entities. MDS analysis produces a configuration where each entity is represented by a point, and relative interpoint distances reflect the relative dissimilarities between pairs of entities. This method works with both linear and nonlinear data and it is relatively simple to interpret (37).

Several previous studies have investigated the effectiveness of the different hypervariable regions of 16S rRNA in discriminating soil samples based on their microbial profiles (54,55). In particular, Yang et al. (55) designed machine learning tools that could learn from a training set of profiles, which could then help to discriminate soil samples. In the current work, the relationship between samples was confirmed using ANOSIM (Table 3). Soil discrimination based on data obtained from a single marker showed that both V1 (R values ranging from 0.713 to 0.917) and V3 (R values ranging from 0.542 to 1.0) regions produced sufficient variations to be able to distinguish between the given soils during the dry season (Table 3). The V3 marker was overall the most consistent in discriminating between soil types. This is partly due to the fact that this marker is the least variable of the ones tested. While the relative abundance and number of peaks obtained with the V1 and V1_V2 varied greatly, the V3 marker showed consistent dominant peaks, giving more statistical weight to rare peaks that was critical for greater discrimination between sites. It should be noted that the number of peaks does not necessarily correlate with the degree of discrimination of a particular marker. In the two previous studies, conducted with the same set of primers and the same hypervariable domains, it was concluded that the V1_V2 domain provided with the highest discrimination among sampled soils (54,55). We suggest that the particular technique, statistical analysis, and interpretation applied to samples and regions studied can provide with discrepancies regarding the significance of any particular domain within the 16S rRNA.

The combination of two domains considerably increased the power of differentiation, with the V1–V3 combination being the most effective (Table 3). During the wet season, the pattern was repeated. The combination of all three domains tested did not increase the discrimination attained with just the V1–V3 markers. As stated earlier, the V1_V2 provided more amplicons but did not exhibit constancy in the number of samples containing the amplicons; therefore, its degree of discrimination was lower than that of the other analyzed markers. Again, previous studies showed that a combination of V1 and V1_V2 regions improved the discrimination ability of an automatic classifier (55). In that study, comparable results were also obtained when a combination of V1, V1_V2, and V3 regions was used for the analysis.

The fact that the Global R values decreased significantly during the wet season was probably attributed, in part, to leaching. As with the chemical data, the amount of water infiltrating a particular soil provides a means of particulate leaching. The leaching process could be responsible for microbial population movement in and out of a site, thus limiting the possibility of reproducing the same results in dry and wet seasons. This indicates that, although good biotic discrimination can be achieved, the interpretations of differences observed within a site or among soil types are dependent on the season in which the samples are collected.

We concluded that Miami-Dade soil microbial communities provided a higher degree of discrimination as compared with elemental chemical soil analyses. Although a greater number of samples, as well as soil-type variety, must be assessed in order to be able to attempt to individualize soils, ALH-PCR proved to be a robust, reliable comparison technique that requires equipment already found in crime laboratories, allows a relatively fast turnaround time, and entails less sample handling, and therefore less technical difficulties, as compared with similar methodologies. Thus, ALH-PCR microbial metagenome profiling can provide a novel application of this established ecological tool for forensic soil discrimination and most importantly could have exclusionary value in criminal cases that contain soil as evidence samples.

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