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Optimization of molecular methods and statistical procedures for forensic fingerprinting of microbial soil communities

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ABSTRACT

Soil may constitute physical evidence that connects people to geographic locations. We studied several parameters of molecular fingerprinting of microbes from arid soils, revealing that (i) for extraction of soil DNA, the 'total nucleic acid' method provides higher spatial and temporal resolution than the PowerSoil® method; (ii) denaturing gradient gel electrophoresis (DGGE) and terminal restriction fragment length polymorphism (TRFLP) were equally powerful at discriminating between soils; and (iii) the most effective restriction enzymes for TRFLP were MspI (for Archaea), HhaI (for Actinobacteria), AciI (for Fungi), TaqI (for Bacteria) and HaeIII (for α-proteobacteria). In addition, we optimized TRFLP data analysis procedures, including outlier and rare-terminal restriction fragments' deletion, relativization and transformation. Overall, Bacterial fingerprints proved better than Actinobacteria and α-proteobacteria, providing higher spatial resolution (i.e. distinction between geographical sites) combined with lower temporal resolution (i.e. stable fingerprints regardless of sampling season).

Keywords: Forensic science, molecular fingerprinting, microbial communities, soil DNA, terminal restriction fragment length polymorphism.

INTRODUCTION

Soil may constitute physical evidence that connects a suspect or a victim to a particular crime scene (Hopkins et al., 2000). The value of soil as evidence stems from its prevalence and transferability: soil might be found on a suspect's shoe, clothes, vehicle etc., and might be transferred from or into a crime scene. Traditionally, forensic analysis of soil samples includes analysis of

various parameters such as grain type, size, color and organic matter content (Saferstein 2001), using optical light microscopy or X-ray diffraction (Brown et al., 2002). However, soil mineralogy is not currently widely used in forensic investigations, due to (i) operator dependence (i.e. lack of repeatability) for microscopy sample analysis and (ii) time and cost of detailed quantitative analysis (Pirrie et al., 2004). Additionally, visual identification of soil particles requires great expertise, whose absence can lead to erroneous court rulings (Rahn, 2007). High-tech methods such as chemical spectrometric analysis and/or scanning electron microscopy provide far better results (Pirrie et al., 2004); however, their high price is

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often prohibitive, leaving the forensic investigator to use the traditional methods (e.g. in the Russian Federation; Gradusova and Nesterina 2009) or disregard soil evidence altogether (e.g. in Israel; Gafny, personal communication).

Biological techniques have also been used in an attempt to 'fingerprint' soil samples, including pollen (Horrocks 2004) or plant wax (Dawson et al. 2003). In the past decade, with the increase in reliability of microbial community analyses, microorganisms were proposed as a tool for the identification of a soil's origin (e.g. Horswell et al. 2002; Meyers and Foran 2008). Among the technologies and approaches that have been developed to study microbial soil diversity, the most popular in recent years is terminal restriction fragment length polymorphism (TRFLP). This method uses fluorescently marked fragments of the 16S ribosomal RNA (rRNA) encoding gene to create a fingerprint of microbial diversity in a sample. However, very few studies have been performed to assess the potential of soil bacterial community analyses in a forensic context (Lerner et al. 2006; Moreno et al. 2006). In these studies, DNA extraction protocols, sample sizes, soil types and different profiling methods were evaluated. So far, applying bacterial TRFLP to soil analysis in a forensic context has met with limited success: on the whole, soil profiles collected within a given site are more similar than are profiles between sites (e.g. Heath and Saunders 2006), but it was claimed that this system is still not mature enough to be a reliable forensic tool (Lenz and Foran 2010). For molecular soil fingerprinting to be useful forensically, optimization and standardization of basic techniques and procedures must first take place. In an effort to address this issue, we compared (I) two popular soil-fingerprinting molecular techniques, TRFLP (Liu et al. 1997) and denaturing gradient gel electrophoresis (DGGE; Fischer and Lerman 1979); (II) two popular soil-DNA extraction techniques, total nucleic acid (TNA; Angel et al. 2011) and MoBio PowerSoil®; (III) five microbial markers: archaea, fungi, total bacteria, actinobacteria, and α-proteobacteria; and (IV) three restriction enzymes for each microbial marker. In addition, we aimed to optimize and standardize the statistical analysis procedures for TRFLP.

MATERIALS AND METHODS

Soil characteristics and sampling: three sites from the LTER network (long-term ecological research; http://lter.bgu.ac.il) were sampled. The first site (31 38N, 34 56E) was in a dry Mediterranean climate (400 mm rain/year) and the soil contained 13% sand, 60% silt and 27% clay. The second site (31 25N, 34 48E) was in a semi-arid climate (300 mm rain/year) and the soil contained 26% sand, 56% silt and 18% clay. The third site (30 47N, 34 45E) was in an arid climate (100 mm rain/year) and the soil contained 32% sand, 55% silt and 12% clay. Soil samples were taken from undisturbed, barren plots that were fenced so that no livestock grazing or human activity took place there for at least five years prior to sampling. At each of the sites, two sub-sites were chosen, ca. 500 m apart; at each sub-site, eight sampling points (ca. 25 m apart) were randomly selected using a spatially stratified, random sampling grid. Each soil sample was collected aseptically from the nearest shrub inter-space patch by removing ca. 200 g from the top five cm of soil (after carefully brushing aside any loose litter) using ethanol-cleaned tools. The samples were placed in individual sterile plastic bags, stored at 4°C and transported to the laboratory where they were homogenized within 24 h. The bulk part of the sample was then stored at ambient temperature for physical and chemical analysis and a small portion intended for microbial analysis (50 g) was stored at -80°C until use. Sampling took place during late winter (March) and summer (August) of 2009, yielding a total of 2 seasons × 3 sites × 2 sub-sites = 12 soil samples. For comparing the TRFLP and DGGE techniques, 12 soil samples, six in spring (April-May) and six in autumn (October), were taken from a Mediterranean site (32 43N, 34 58E) cultivated with a persimmon (*Diospyros kaki*) orchard. The soil composition at this site was 58% clay, 28% silt and 14% sand. The sampling and handling procedures were as described above.

Nucleic acid extraction: total nucleic acids (TNA) were extracted from each homogenized soil sample according to Angel et al. (2011). Some of the extracted DNA samples had heavy coloring indicating high concentration of contaminants that might inhibit the subsequent PCR reaction, thus these samples were further purified using MicroSpin S-200 HR columns (GE Healthcare, Waukesha, WI). The extracted nucleic acids (50 µL) was incubated at 37°C for 30 min with 1µL of RNAse and then the mixture was cleaned using DNA extraction Kit (Bioneer, Seoul, S. Korea) according to the manufacturer's protocol. The same soils were DNA extracted using the PowerSoil DNA Isolation Kit (MoBio, Solana Beach, CA), used according to the manufacturer's protocol.

Denaturing gradient gel electrophoresis (DGGE): PCR reaction for DGGE was performed as described previously (Green et al. 2004) using the general bacterial primers 341F-GC, containing a 40-bp GC-clamp to enhance separation in DGGE, and 907R (Muyzer et al. 1996). The densitometric curves of the samples were produced using Fingerprint II software (Bio-Rad, Hemel Hempstead, UK).

TRFLP: Table 1 describes the primers used for amplifying the 16S rDNA (for prokaryotes) and the Internal Transcribed Spacer (ITS for Fungi). The PCR reaction was carried out in a thermocycler (Bio-Rad, Munich, Germany) in triplicate using a composite of three DNA extractions from each sample as a template, to

minimize extraction bias. PCR runs were conducted in triplicates of 50 μL each, to minimize reaction bias. The PCR mixture contained 200 nM of each PCR primer, 2.5 mM $MgCl_2$, 0.8 μL of DreamTaq DNA polymerase (Fermentas, Burlington, Canada), 5 μL DreamTaq buffer, 5 μL of bovine serum albumin solution (New England Biolabs, Beverly, MA) and 0.2 mM of dNTPs (TaKaRa, Otsu, Japan). 5 μL of the PCR product were visualized on 1% agarose gel (Sigma, St. Louis, MO) to ensure successful amplification. Prior to TRFLP analysis, the PCR amplicons were treated with Mung bean exonuclease (TaKaRa, Otsu, Japan) according to the manufacturer's instructions in order to eliminate the single strand DNA samples that might result in pseudo-terminal restriction fragments (TRFs) (Egert and Friedrich 2003). The resulting dsDNA fragments were purified immediately using a PCR purification kit (Bioneer, Seoul, S. Korea). The DNA concentration of each run was estimated using spectrophotometric analysis (NanoDrop, Wilmington, DE) and visualized on an agarose gel. Three different restriction enzymes (REs) per taxonomic group were used in order to increase the reliability of the TRFs' emerging patterns (Moyer et al. 1996). The REs were chosen using MiCA (Shyu et al. 2007) and a local installation of www.restrictionmapper.org; files and procedures for automating RestrictionMapper are freely available at our website, http://departments.agri.huji.ac.il/plantpath/jurkevitch/RestrictionMapper_for_remote_use.zip (for remote usage) and http://departments.agri.huji.ac.il/plantpath/jurkevitch/RestrictionMapper_local_installation.pdf (for local usage). The sequences used were good-quality, long (>1200 bp) sequences of the 16S rRNA gene of bacteria and archaea, downloaded from the RDP-II (Cole et al. 2009), and long, good-quality sequences of the fungi ITS, downloaded from Emerencia (Nilsson et al. 2005). The selected REs were: Bacteria – HhaI, TaqI, $HpyCH_4IV$; Archaea – MseI, MspI, AciI; Fungi – MnlI, MseI, AciI; Actinobacteria – HhaI, HapII, AciI; α-proteobacteria – HhaI, HapII, HaeIII. Cutting was performed according to the specification of the manufacturers. Digestion was followed by purification using SigmaPrep™ spin column (Sigma, St. Louis, MO) and resuspension in double-distilled water. These samples were analyzed with an ABI Prism® 3100 genetic analyzer (Applied Biosystems, Foster City, CA). The peaks in each profile were related to specific fragment lengths based on a size marker (Liz500, Applied Biosystems, Foster City, CA). The TRFs were visualized using Peak Scanner V.1.0 (Applied Biosystems, Foster City, CA). TRFs with base pair size below 40 and above 600 were truncated (restricted by the size standard used). 'Noise' peaks were filtered according to Angel et al. (2010), and the true TRFs were aligned to the nearest integer. After alignment, matrixes were created for the peak height that indicated the relative abundance of each peak using T-REX (http://trex.biohpc.org; Culman et al. 2009). The above

analysis was repeated for each restriction enzyme separately and matrixes composed of all the true peaks of TRFs were formed with numbers indicating each peak abundance and position for all the sites.

Statistical analysis: the following analyses were performed using PC-ORD 5.32 (MjM, Gleneden Beach, OR). Outliers were checked based on the average dissimilarity of each sample from all other samples in its group. We calculated the average Bray-Curtis distance of each sample from every other sample in its group, constructed a frequency distribution of these distances, and flagged outliers as samples which lie >2 standard deviations away from the mean. Rare TRFs were flagged as TRFs, which occur in less than a certain percentage of the soil samples. Relativization was by sample total, i.e. each TRF abundance was relativized according to the total abundance of TRFs in that specific sample: $b=X/(\sqrt{\Sigma X_{1..n}})$, where b is the relativized TRF abundance value, X is the original value, and n is the number of TRFs in that specific sample. Transformation was by square root: $b=\sqrt{X}$, where b is the transformed TRF abundance value and X is the original value. Community structures were compared using Bray-Curtis distances (BCD) as the effect-size between 0 and 1: zero if the communities at the different sites were identical and one if the communities were completely different (i.e. did not share any TRFs). The statistical differences between soil fingerprints were measured using multi-response permutation procedure (MRPP; Mielke 1984) on the Bray-Curtis distance matrices. Ordinations were created using non-metric multidimensional scaling (NMDS; Mather 1976) on the Bray-Curtis distance matrices. Cluster analyses were performed with flexible beta (β=-0.25) using the Bray-Curtis distances.

RESULTS

Comparing molecular fingerprinting technique

For comparing the two popular molecular fingerprinting techniques, we studied the bacterial community structure of a cultivated site (1 km^2) at two seasons: six samples collected in April-May 2007 when freshwater was used for irrigation, and six samples collected in October 2007 when treated wastewater was used for irrigation. DGGE and TRFLP showed an identical differentiation power, both significantly separating freshwater-irrigated soil from treated wastewater-irrigated soil (MRPP tests, P=0.04 for both DGGE and TRFLP).

TRFLP restriction enzyme

For each of the five microbial markers, we tested three restriction enzymes (REs) in TRFLP analysis using identical procedures (as outlined above and in Table 1).

Table 1. PCR primers and procedures used in this study for TRFLP and DGGE.

Marker	Temperature cycling	Primer sequences (5'->3')
Bacteria (TRFLP)	30 cycles of 45 S @ 94℃, 1 min @ 45℃, 3 min @ 72℃.	341F: CCTACGGGAGGCAGCAG
		907R: CCGTAATCMTTTGAGTT
Actinobacteria (TRFLP)	30 cycles of 45 S @ 94℃, 1 min @ 51℃, 3 min @ 72℃.	S-C-Act235-a-S-20: CGCGGCCTATCAGCTTGTTG
		16Sact1114r: GAGTTGACCCCGGCRGT
α-proteobacteria (TRFLP)	30 cycles of 45 S @ 94℃, 1 min @ 45℃, 3 min @ 72℃.	ADF-681F: AGTGTAGAGGTGAAATT
		ADF-685R: TCTACGRATTTCACCYCTAC
Archaea (TRFLP)	30 cycles of 60 S @ 94℃, 60 S @56℃, 60 S @72℃.	344F: ACGGGGCGCAGCAGGCGCGA
		915R: GTGCTCCCCCGCCAATTCCT
Fungi (TRFLP)	35 cycles of 60 S @ 95℃, 2 min @ 56℃, 2 min @ 72℃.	ITS1: CTTGGTCATTTAGAGGGAAGTA
		ITS86: TTCAAAGATTCGATGATTCAG
Bacteria (DGGE)	27 cycles of 30 S @ 94℃, 30 S @ 64℃, 30 S @ 72℃.	341F-GC: CGCCCGCCGCGCCCCGCGCCCGTCCCGCCGCC CCCGCCCGCCTACGGGAGGCAGCAG
		907R: CCGTCAATTCMTTTGAGT TT

For each RE, the power to differentiate between sites was analyzed as the A-statistic of the MRPP test; the best RE of the three (i.e. with highest A) was set as 100%, and the differentiation power of the other two RE was calculated as percentage of the best RE (called A%). Table 2 shows the best RE for each marker, which was used in all subsequent analyses: TaqI for Bacteria, HhaI for Actinobacteria, HaeIII for α-proteobacteria, MspI for Archaea and AciI for Fungi.

Soil DNA extraction

We compared two DNA extraction methods for soils, TNA (total nucleic acid) and PowerSoil® DNA extraction kit, in order to choose the method most effective for forensic analysis. The fingerprints from bacterial and actinobacterial samples were used, and both of them exhibited site segregation, i.e. the communities significantly differed according to sites both when the PowerSoil kit (MRPP tests, P<0.01 and P=0.01, respectively) and TNA extraction (MRPP tests, P<0.01 for both taxonomic groups) methods were used. In addition to this spatial profiling (discrimination between soil locations), we also tested both DNA extraction methods in temporal profiling (discrimination between summer and winter). In testing the entire bacterial community, the PowerSoil-extracted DNA did not reflect differences in bacterial communities between seasons (P=0.60), whereas the TNA-extracted DNA detected a marginal seasonal difference (P=0.06). Analyzing the actinobacterial community yielded pronounced distinction between the two DNA extraction methods: the PowerSoil-based analysis found no significant difference between seasons (P=0.13) whereas the TNA-based analysis found that each season supported a different actinobacterial community (P<0.01). These results might be attributed to the marked increase in Actinobacterial TRF richness when soil DNA was extracted by TNA compared to the yield of the PowerSoil kit (107±33 and 9±3 TRFs, respectively).

Microbial markers

In order to optimize the forensic analysis procedure, we studied the forensic value of five different taxonomic groups as markers for distinguishing soil samples from

Table 2. Comparison of three restriction enzymes (RE) for TRFLP analysis using each of the five microbial markers. For each RE, the power to differentiate between sites was analyzed as the A-statistic of the MRPP test. For each marker, the best RE (i.e. highest A) was set as 100%, and the differentiation power of the other two RE was calculated as percentage of the best RE (called A%). Asterisk denotes significant differentiation between sites (MRPP test, P<0.05).

Marker	RE	A%
Bacteria	TaqI	100.0*
	HhaI	92.5*
	HaeIII	78.3*
Actinobacteria	HhaI	100.0*
	HapII	25.7
	AciI	6.6
α-proteobacteria	HaeIII	100.0*
	HhaI	46.7*
	HapII	15.9*
Archaea	MspI	100.0*
	MseI	97.7*
	AciI	32.3
Fungi	AciI	100.0*
	MnlI	25.1*
	MseI	16.9*

different geographic locations. Bacteria, α-proteobacteria and actinobacteria were studied in the three geographic sites; in addition, all five taxonomic groups (Bacteria, α-proteobacteria, actinobacteria, Archaea and fungi) were tested for their ability to distinguish between the two sub-sites located 500 m apart at the Semi-arid site. Using the bacterial domain as a forensic marker provided the best differentiation between the three soils, as evidenced from both NMDS ordination and multivariate MRPP analysis (Figure 1); α-proteobacteria were less powerful, and actinobacteria the least successful in distinguishing the three sites (Figure 1). When comparing the five microbial markers at the semi-arid site, two resolutions were considered: spatial (between the two sub-sites located 500 m apart) and temporal (between two seasons, summer and winter). All the taxonomic groups were able to significantly distinguish between the sub-sites (MRPP tests, P<0.03); however, neither bacterial nor α-proteobacterial communities showed a significant temporal difference (P=0.32 and P=0.09, respectively), while the other three taxonomic groups significantly differentiated between seasons (P<0.05). In other words, the bacterial and α-proteobacterial fingerprints were temporally stable.

Statistical analysis

We tested five datasets, each composed of the TRFs

from different soil samples. In each case, we measured the resolution – the power of the TRFLP assay to differentiate between samples taken from two sites. Resolution was presented by the MRPP tests' A-statistic, ranging from A=0 (the two groups are completely different and share no TRFs) to A=1 (the two groups are composed of exactly the same TRFs in exactly the same abundances). Our analysis showed that in each case, outlier deletion, followed by rare-TRF deletion and relativization, increased the resolution by at least 150% of the raw data. In some cases, a final step of square-root transformation further improved the resolution, but in others cases, the transformation decreased the resolution. To quantify this effect, we tested seven 'site couples' from our TRFLP dataset: arid vs. semi-arid, arid vs. shifting sand, semi-arid vs. dry Mediterranean, and so on. For each couple, we measured the difference between the sites (MRPP A statistic) with and without square-root transformation of the data. The results show that square-root transformation was beneficial (i.e. increased the resolution) when the effective number of TRFs (Jost 2006) was low, and detrimental (i.e. decreased the resolution) when the effective number of TRFs was high (Figure 2). Finally, to quantify the effect of deletion of rare TRFs, i.e. TRFs that appear only in a small percentage of the samples, we again tested the seven 'site couples' mentioned above. We found that rare-TRF deletion improves the resolution (i.e. increases the MRPP A statistic) no matter which other data-adjust-
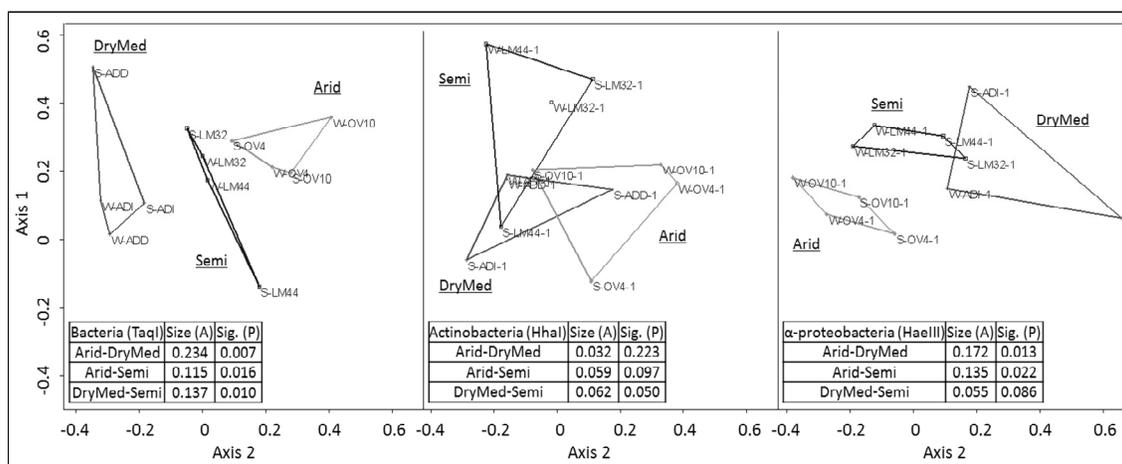
Figure 1. Differences in community structure (measured by Bray-Curtis distance) of three taxonomic groups between soils from three sites. Left, bacteria using TaqI as restriction enzyme; center, actinobacteria using HhaI; right, α-proteobacteria using HaeIII. Samples from each of the sites (Arid, semi-arid [denoted 'Semi'] and dry-Mediterranean [denoted 'dryMed') are bound by convex hulls to illustrate the variance within and between sites. Individual sample names begin with S (summer) or W (winter). Inset tables detail the results of MRPP tests between each two sites, with the size and significance of the differences between communities given as the A and P statistic, respectively.
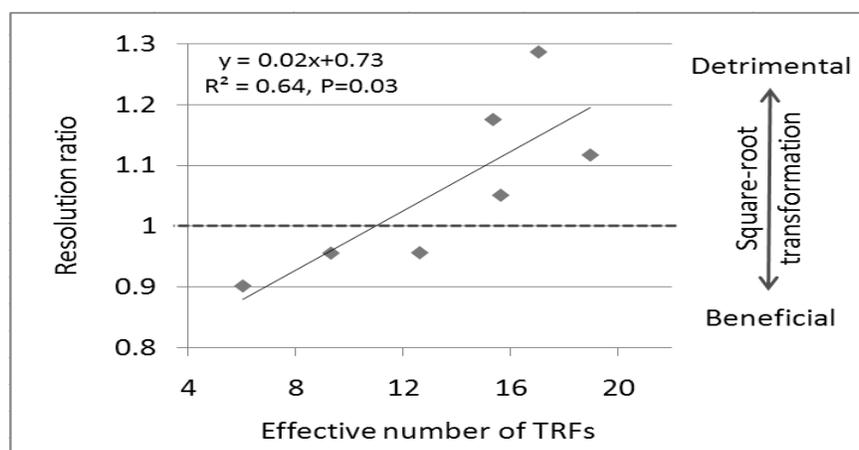
Figure 2. The effect of square-root transformation on the resolution is determined by the effective number of TRFs. Resolution ratio is the MRPP test's A statistic without square-root transformation, divided by the A statistic with square root transformation. Effective number of TRFs is the exponential of the Shannon diversity index. Each data point denotes a different restriction enzyme.

ments are used; however, the optimal percentage of samples below which a TRF should be deleted must be carefully determined in each case. As a typical example, comparing semi-arid vs. dry-Mediterranean soils using TaqI restriction of bacterial DNA, optimal results were obtained when deleting TRFs which appeared in no more than 15% of the sample units (Figure 3).

DISCUSSION

Community analysis

DGGE, TRFLP and 454 pyrosequencing are discussed and compared at length elsewhere (Prosser et al. 2010 and references therein). In short, DGGE employs the
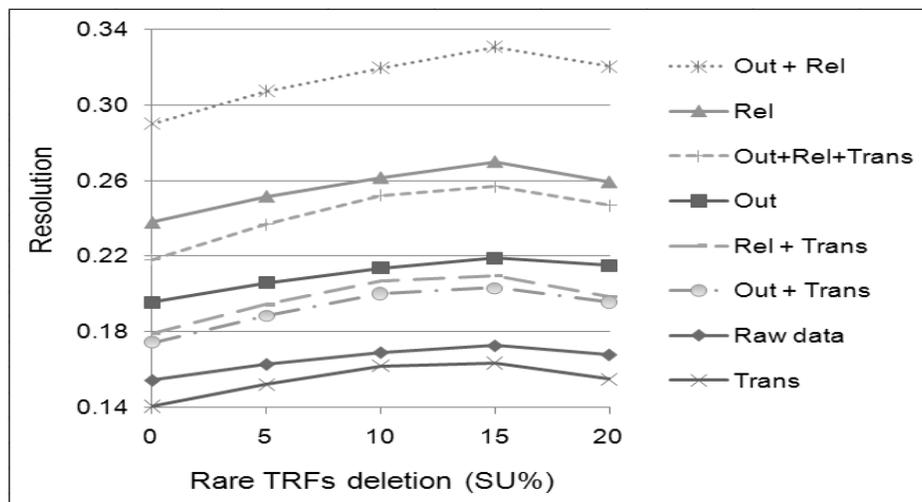
Figure 3. The effect of deleting rare TRFs on the resolution. Resolution is the A-statistic of the MRPP test. SU%, the percentage of sample units in which the TRF appeared; out, outlier deletion; rel, relativization; trans, square-root transformation.

separation of PCR products based on their GC content, whereas TRFLP relies on the fragments' restriction sites. In both these methods, the detection levels are low: only the most dominant members of the community are detected. The main advantage of DGGE is that bands of interest can be identified while the main advantage of TRFLP is its robustness. Community analysis via 454 pyrosequencing of the gene coding for 16S rRNA can generate deep coverage of a community but at this point in time is not part of the standard equipment of forensic labs. In the current study, DGGE and TRFLP both successfully distinguished between soils/treatments. Previous studies also found that DGGE and TRFLP provide similar fingerprinting capabilities (Smalla et al. 2007; Enwall and Hallin 2008); however, TRFLP might be more suited for forensic purposes for technical reasons since most forensic laboratories can perform TRFLP analyses with their existing equipment.

DNA extraction method

Previous studies showed that the method of DNA extraction from soil is crucial for both DNA yield and the consequent diversity of the microbial community (Feinstein et al. 2009; Hu et al. 2010; Inceoglu et al. 2010). We compared the two soil extraction methods that seem to be the most popular in forensic studies - the PowerSoil DNA extraction kit (MoBio) and the phenol-chloroform bead-beating DNA extraction method (TNA). Both methods were successful and after analysis we could discriminate between soils (arid, semi-arid and Mediterranean); however, the TNA method more readily identified the seasonal distinctions. Moreover, the

actinobacterial TRFs richness depicted in DNA extracted using the TNA method was considerably higher. These results suggest that the PowerSoil method might be less effective in extracting actinobacterial DNA from arid soil. Actinobacteria are a diverse and extremely successful group of microorganisms that consist over 45% of the phyla diversity in the examined soils (Bachar et al. 2010). One possible explanation to our results may be related to spores: the bead-beating step of the TNA method may increase the efficiency of breaking the spores and extracting their DNA, unlike the PowerSoil method that involves a vortexing step. Since some Actinobacteria may form spores in harsh conditions (Goodfellow and Williams 1983), it seems plausible that actinobacterial DNA would be under-represented in dry soils when extracted without the bead-beating step. The extraction bias may be minimized by pooling DNA from successive extractions of the same soil sample; three successive extractions seem to be sufficient (Feinstein et al., 2009).

From a forensic investigation point of view, PowerSoil and TNA each have their own merits. If less seasonal 'noise' in microbial community structure is reported with the PowerSoil method, then this method may be more robust regarding time of collection of comparator samples (post-crime) versus removal of soil from the source at time of crime. In other words, by being less sensitive to temporal changes in the microbial community, PowerSoil may actually be more beneficial in a forensic context, successfully matching two samples which were taken from the same place at different times. Conversely, the TNA method may be more useful in cases where the investigator wishes to determine the season in which the soil evidence was removed from the scene. Since it is more sensitive to temporal variation, the evidence soil

can be compared to several post-crime samples, each taken at different seasons, and a crude timeline can be estimated. Whatever the forensic goal, it is important to assess and be aware of the specific traits, advantages and shortcomings of each DNA extraction method, if we are to robustly and reliably use them in forensic settings.

Microbial markers

Soil constitutes an extremely heterogeneous medium. Accordingly, microbial community structure differs between soils, but also within the same soil, depending on the presence of a root (rhizosphere effect), agricultural practices, as well as temporal and spatial heterogeneity (Lenz and Foran 2010; MacDonald et al. 2011). Although this certainly renders matching of soil samples for forensic purposes more difficult, the resolution at which the marker gene analysis is performed is important. In our study, the bacterial domain provided the highest ability to differentiate between the sites, followed by the α-proteobacteria; these two were also less temporally sensitive, thus providing stable fingerprints no matter when the soil sample was taken. Contrarily, the other markers (Archaea, fungi and actinobacteria) may be more prone to temporal instability, i.e. providing different fingerprints at different seasons. TRFLP studies of community structure are typically performed using primers targeting the bacterial domain, thus providing information on the most abundant populations at this level. Lenz and Foran (2010) suggested that due to the high level of variability within and among habitats, assaying heterogeneous soil microorganisms (i.e. the bacteria) might not be a useful tactic for forensic soil comparisons. Contrarily, our results suggest that bacterial soil communities from dry and arid soils are relatively homogeneous and are very well suited for forensic purposes. Differences between microbial communities in dry soils can likely not be solely explained by geographical distance (see Fierer and Jackson, 2006). Alternatively, they could be attributed to the differences found in climate, mineralogy (e.g. soil microhabitats) and organic matter. Thus, any choice of target microbial group(s) for forensic investigations needs to take all these factors into account.

Statistical analysis

Most datasets benefit from one or more data adjustments prior to statistical analyses. The reasons may be statistical, such as improving assumptions of normality, homogeneity of variance etc., or ecological, for instance equalizing the relative importance of common and rare species. There are a great variety of possible data adjustments, but in the context of TRFLP we examined only the most important four: outlier deletion, rare-TRF deletion, relativization and transformation. Outliers (scores that are extreme relative to the rest of the sample) can cause non-normality and skewness the data, thus profoundly influencing multivariate analyses (McCune and Grace 2002). Yet, they are seldom discussed or dealt with explicitly. There are a number of ways of dealing with outliers (Osborne 2002), and we found that removal of samples, whose Bray-Curtis distance is above two standard deviations from the mean, significantly improves the MRPP test resolution, thus improving our ability to detect differences between the TRF patterns of soil samples from different sites. Rare-TRF deletion is another useful way of reducing the bulk and noise in the dataset without losing much information. In fact, it often enhances the detection of relationships between community composition and environmental factors, and is also useful for extracting patterns with multivariate analysis (McCune and Grace 2002). However, we must walk a fine line - when including too many TRFs in the analysis, the noise from the rare ones may weaken the structure, whereas deleting too many may reduce the resolution. We found that removal of TRFs, which appear in less than 5-15% of the samples, increases the resolution and reduces the skewness of the data (Figure 3). Relativization adjusts matrix elements by a row or column standard (e.g. maximum, mean or sum); in other words, it re-scales individual soil samples in relationship to some criterion based on the other soil samples. TRFLP data benefits from relativization because we are interested in the proportions of the different TRFs, not their total amount. There are many possible relativization procedures, appropriate at different occasions; we relativized by sample totals, which is appropriate when using analytical tools based on city-block distance measures such as the Bray-Curtis distance (McCune and Grace 2002). We found that using relativization always increased the resolution (Figures 2 and 3). Relativization is also important as a preparatory step before square-root transformation (Osborne 2002). Transformation may be needed since many statistical procedures assume normal distribution of variables; true normality is rare in nature, so data transformations are often used for improving normality (McCune and Grace 2002). Moreover, non-parametric tests (where no explicit assumption of normality is made) can suffer as much as parametric tests when normality assumptions are violated (Zimmerman 1998). Square-root transformation changes the values of the data points but not their rank. This transformation fits TRFLP data very well because the data sets are non-negative, and unlike the log transformation, special treatment of zeros is not needed. Additionally, TRFLP data often exhibits a few abundant TRFs and many rare ones. A multivariate analysis of the raw data might emphasize only the abundant TRFs and ignore the rare ones; a square-root transformation of the data usually moderates this imbalance (McCune and Grace 2002). We found that transformation should only

be used if TRFs diversity is low (and skewness is high). To conclude, simple statistical manipulations can greatly reduce the amount of noise and increase the significant signals within the microbial community dataset. Such treatment is valid and routine in other areas of forensic science, e.g. human fingerprints which are often enhanced, both chemically and digitally (Bond 2011).

The forensic context

Unlike human DNA fingerprinting, which can provide absolute error rates for sample-matching, soil fingerprinting cannot provide definite proof linking suspects or victims to crime scenes. The statistical strength of matching two microbial communities inevitably depends upon the quantity and quality of the samples at hand, and an obtained match between a scene and a sample obtained from a suspect cannot rule out a match between the latter and untested samples. Furthermore, in order for any analytical method to provide a robust tool for forensic application, there must be an understanding of the uncertainty associated with any comparison. The uncertainties are especially high in soil microbial fingerprinting, and we have attempted to address some of the most obvious ones in this study; yet more data and tests, especially in forensically-relevant settings, are required if this method is to offer a reliable supporting tool to forensic investigators. For example, soil samples recovered as forensic evidence are often very small and/or are desiccated on an item of evidence, introducing more uncertainties to the microbial analysis process. This study attempted to further the introduction of soil fingerprinting into forensic context, building on previous "proofs of concept" which showed that forensic soil evidence can indeed be discriminated and used in a court of law (e.g. Concheri et al. 2011). Continuation of this research may eventually provide the ability to routinely use soil microbial community analysis as associative evidence in forensic investigations.

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