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PAPER

CRIMINALISTICS

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Discrimination of Soils at Regional and Local Levels Using Bacterial and Fungal T-RFLP Profiling*

ABSTRACT: DNA profiling of microbial communities has been proposed as a tool for forensic comparison of soils, but its potential to discriminate between soils from similar land use and/or geographic location has been largely unexplored. We tested the ability of terminal restriction fragment length polymorphism (T-RFLP) to discriminate between soils from 10 sites within the Greater Wellington region, New Zealand, based on their bacterial and fungal DNA profiles. Significant differences in bacterial and fungal communities between soils collected from all but one pair of sites were demonstrated. In some instances, specific terminal restriction fragments were associated with particular sites. Patch discrimination was evident within several sites, which could prove useful for site-specific matching (e.g., matching shoe/car tire print to an object). These results support the need for further understanding of the spatial distribution of soil microbial communities before DNA profiling of soil microbial communities can be applied to the forensic context.

KEYWORDS: forensic science, soil, microbial community structure, bacteria, fungi, terminal restriction fragment length polymorphism

Soils are a valuable resource in forensic investigations as they contain mineral and organic signatures relating to provenance that can provide investigative intelligence and/or evidential value. To date, soil evidence has largely been utilized through analysis of soil color, particle size, and mineral examination (1). However, it is not always possible to effectively discriminate samples using these approaches (2), and they may benefit from analyses that provide complementary information. Horswell et al. (3) first proposed DNA profiling of soil bacterial communities using terminal restriction fragment length polymorphism (T-RFLP) as a method for forensic comparison/discrimination of soils because it is capable of using small sample sizes, utilizes equipment already established in forensic laboratories for human DNA analysis, lends itself to automation, and is cost-effective. Since, a handful of studies have discussed, in the forensic context, utilizing differences in microbial community structure between different soils (4–7). Using a small number of soil comparisons, these investigations have demonstrated that differences in microbial community structure can be detected between soils from different locations, while those studies that have used crime scene scenarios (3,4) have demonstrated that soils collected from the same location, or taken from an item of clothing worn at that location, generally shows a higher degree of similarity between each other in terms of microbial community structure. Thus, such information could be used in an evaluative mode whereby evidential samples (e.g., soil from a shoe, clothing, and car tire) could be compared/evaluated against soil from a crime scene or alibi site. Equally, if differences in microbial community structure exist between different land management and/or vegetation type as is supported in ecological literature (e.g., [8–12]), the potential exists for microbial community profiling to be used as an intelligence tool to help determine providence of an unknown sample. A further potential of utilizing microbial profiling methods in forensic comparison of soils may lie in the sensitivity of this approach to detect differences in microbial communities of soils from similar habitats or local scales where methods based solely on mineralogy may provide limited information. Conventional mineralogical approaches may be unable to discriminate soils from different locations within a small locality (e.g., different parks within a city, or different locations within a park) because this technique is driven largely by underlying geology which generally differs across regional scales. Variability in the soil microbial community driven by local differences in abiotic and biotic factors may provide a further level of discrimination between soils collected from similar locations. Few studies have investigated the variability between microbial communities of different sites of similar vegetative composition and/or land use (9,12,13). Further, the fungal community, which is also influenced by abiotic and biotic factors (e.g., [14,15]), has received little attention in the forensic context but has recently been highlighted as a potential target for forensic comparison of soils (16). Before microbial profiling methods can be applied to forensic analysis of soils, such comparisons are needed to assess whether microbial communities "vary in such a way as to allow samples from a particular location (patch) to be differentiated from samples deriving from other places" (17, pp. 49).

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The purpose of this study was to determine whether profiling of both bacterial and fungal community structures could be used to discriminate between soils collected from 10 locations (sites) across a 25 km^2 area in the Greater Wellington region of New Zealand that had similar underlying geology throughout (18). Variations in microbial (bacterial and fungal) community structure were assessed both between (site) and within (patch) each of the 10 sites to test the hypothesis that profiling of soil microbial communities could discriminate between soil sources at regional (site) and local (patch) levels. Further, we assessed the use of bacterial and fungal community profiles independently and combined with the potential of applying these methods to forensic comparison of soil evidence to provide clues as to the likely landscape characteristics or potential source sites.

Materials and Methods

Sampling Sites and Sample Collection

Ten sites were identified within the Greater Wellington region (Fig. 1) of the North Island of New Zealand. The sites identified covered a range of recreational activities, such as woodland and parkland, across the Greater Wellington region (25 km^2) and included a range of soils and broad vegetation types as described in Table 1. Within each site, three distinct patches were identified ranging from 5 to 60 m apart. At each patch, triplicate samples were taken from within a 20 cm^2 area. Where relevant, vegetation was cleared from the 20 cm^2 area, defined in this study as a patch, and samples taken to a depth of 2 cm using a 1.5 cm diameter corer. Samples were returned to the laboratory and air-dried for 3 days before homogenization with a mortar and pestle. Air drying represents a realistic and practical method for storage of soils and evidence items containing soils evidence in forensic case work (19). Viable microbial DNA has been recovered from archived soils that have been air-dried for several years (20,21). Samples were stored in the dark in airtight containers until analysis.

DNA Extraction and Polymerase Chain Reaction (PCR) Amplification

DNA Extraction and Quantitation—DNA was extracted from 0.5 g of soil using MoBio PowerSoil™ DNA isolation kit (MoBio Laboratories, Inc., Carlsbad, CA) as per manufacturer's instructions

FIG. 1—*Map of the Greater Wellington region showing the location of the 10 sites sampled in the study. Sites are as follows: 1. Kaitoke; 2. Judgeford; 3. Walker Estate; 4. Whiteria; 5. Belmont; 6. Taita; 7. Rimutaka; 8. East Harbour; 9. Wellington; and 10. Otari.*

TABLE 1—*Location, description, and main vegetation cover of the 10 sites samples within the Greater Wellington region.*

Site #	Location	Land Use	Soil Type	Sub-site	Vegetation
1	Kaitoke	Open Parkland	Yellow Brown Earth: moderately clay alluvial	Site 1A	Grass/clover
				Site 1B	Tall grass
				Site 1C	Trodden path/moss
2	Judgeford	Open Parkland	Yellow Brown Earth: weakly clay alluvial	Site 2A	Bare soil-river bank
				Site 2B	Native Woodland—leaf litter
				Site 2C	Native Woodland—shrub
3	Walker Estate	Open Parkland	Yellow-Gray Earth	Site 3A	Grass/clover
				Site 3B	Mud track
				Site 3C	Gravel track
4	Whiteria	Coastal Parkland	Yellow-Gray Earth	Site 4A	Grass
				Site 4B	Grass
				Site 4C	Grass
5	Belmont	Open Parkland	Yellow Brown Earth: weakly clay alluvial	Site 5A	Grass
				Site 5B	Grass
				Site 5C	Grass
6	Taita	Cemetery	Recent Soils from Alluvium	Site 6A	Grass
				Site 6B	Shrubs
				Site 6C	Grass
7	Rimutaka	Wooded Parkland	Yellow Brown Earth: moderately clay alluvial	Site 7A	Mud path
				Site 7B	Moss covered path
				Site 7C	Native Woodland
8	East Harbour	Wooded Parkland	Yellow Brown Earth: moderately clay alluvial	Site 8A	Native Woodland—bare soil
				Site 8B	Native Woodland/Liverwort
				Site 8C	Native Woodland—leaf litter
9	Wellington	Open Parkland	Yellow Brown Earth: moderately clay alluvial	Site 9A	Mud path
				Site 9B	Mud path
				Site 9C	Grass/clover
10	Otari	Wooded Parkland	Yellow Brown Earth: weakly clay alluvial	Site 10A	Gravel path
				Site 10B	Grass/clover
				Site 10C	Native Woodland—leaf litter

and quantified using Quant-iT™ Pico Green® dsDNA Assay Kit (Invitrogen, Auckland, NZ). Samples were stored at −20°C prior to amplification.

PCR Amplification—All PCRs were performed in a final volume of 50 μL containing: 1 × $MgCl_2$ reaction buffer, 0.5 mM $MgCl_2$, 200 μM of each deoxynucleoside triphosphate, and 2.5 U of Taq DNA polymerase (all reagents from Qiagen, Doncaster, Victoria, Australia), 20 μg bovine serum albumin (BSA; Roche Diagnostics, Auckland, NZ), and 20 ng of template DNA. Bacterial primers 63F (22) and 1087R (23), targeting the variable region of the 16S rRNA gene, were used at a concentration of 200 nM while fungal primers ITS 1F (24) and ITS 4R (25), targeting the ITS region between the 18S and 23S regions, were used at a concentration of 400 nM. For both bacteria and fungi, the forward primer was labeled with FAM (6-carboxyfluorescein). All PCR amplifications were performed on a Palm-Cycler (Corbett Research, Sydney, Australia). For bacteria, PCR conditions consisted of 5 min at 95°C, followed by 30 cycles of denaturing at 95°C for 30 sec, annealing at 59°C for 30 sec, and elongation at 72°C for 1 min, followed by a final 10-min extension period at 72°C. For fungi, the PCR conditions consisted of 5 min at 95°C, followed by 30 cycles of denaturing at 95°C for 30 sec, annealing at 55°C for 30 sec, and elongation at 72°C for 1 min and a final 10-min extension at 72°C. PCR products were visualized with ethidium bromide staining on a 1% (w/v) agarose gel using UV radiation.

Amplicon Digestion and Terminal Restriction Fragment (TRF) Detection—Prior to digestion, PCR products were purified using UltraClean™ PCR clean-up kit (MoBio Laboratories, Inc.) according to the manufacturer's instructions and quantified using Quant-iT™ Pico Green® dsDNA Assay Kit. Five hundred nanograms of PCR product was digested with 20 U of Msp I in a final volume of 20 μL containing 0.1 μg/μL of acetylated BSA (all reagents from Promega, Sydney, Australia). Samples were incubated at 37°C for 3 h followed by a 15-min enzyme inactivation period at 95°C. After digestion, samples were cleaned using MinElute reaction cleanup kit (Qiagen) following the manufacturer's instructions. Samples were quantified using Quant-iT™ Pico Green® dsDNA Assay Kit and each sample was diluted to 10 ng/μL. LIZ-labeled GS500(−250) was used as an internal size standard (Applied Biosystems , Melbourne, Australia) and fragment size analysis was carried out using 1 μL of cleaned digest on an ABI PRISM 3730 genetic analyzer (Applied Biosystems).

Data Analysis

T-RFLP profiles were analyzed using GeneMapper software (version 3.7; Applied Biosystems) and fragments quantified using the advanced mode and second-order algorithm. Fragment analysis was performed between 50 and 500 bp with a detection limit of 50 fluorescence units. A matrix was created representing the presence or absence of each peak within a profile, which was used for further comparative analysis.

Statistical Analysis

Analysis of variance was used to test for differences in total number of TRFs per site (Gentstat v11; VSN International, Hemel Hempsted, UK). Bray–Curtis similarity matrices were generated on binary (presence/absence) data that had been square root transformed prior to analysis. Analysis of similarity (ANOSIM) was used to determine significant effect of site and significant effect of patch within a site. The ANOSIM *R*-statistic indicates the level of discrimination between groups (site or patch), with a value close to unity indicating complete group discrimination, and a value close to zero

implying little or no discrimination. The associated significance level (p) is analogous to the univariate p-value (26) where 0.1%, 1%, and 5% equate to a conventional p-statistic of $p < 0.001$, $p < 0.01$, and $p < 0.05$, respectively. Nonmetric multidimensional scaling (MDS) was used to display the relative relationship between samples for each of the profiling approaches. All analyses were performed using Primer-E v.6 (Plymouth Marine Laboratory, Plymouth, U.K.). SIMPER analysis was used to identify individual TRFs contributing to dissimilarity between sites.

Results

Effect of Site on Bacterial and Fungal Community Structure

The total number of bacterial TRFs per sample ranged between 16 and 56 (data not shown), but there was no significant difference in the number of bacterial TRFs between site ($p > 0.05$). ANOSIM demonstrated that the bacterial community was significantly influenced by site ($R = 0.40$, $p = 0.1\%$). Pairwise comparisons between sites are presented in Table 2a. There were three of 45 site comparisons that could not be discriminated between using bacterial T-RFLP profiles; Kaitoke and Wellington; Taita and Walker Estate; and Taita and Otari. Whiteria discriminated at a high level ($p = 0.1\%$) from all other sites. MDS was used to display the relative relationship between samples. Points closer together are more similar to each other than those further apart. Although the two-dimensional representation of a multidimensional data set is problematic in terms of masking some differences that were observed in the ANOSIM, it can be used to visualize that there were clear differences in bacterial community structure between some sites (e.g., East Harbour and Whiteria; Whiteria and Rimutaka; Otari and Taita; Judgeford and Rimutaka) while other sites shared a more

TABLE 2—*Pairwise comparisons (*R*-value) between different sites for bacteria, fungal, and combined bacteria and fungi within the 10 sites sampled within the Greater Wellington region.*

(a) Bacteria	Global $R = 0.43$ $p = 0.1\%$									
	Park Pairwise Comparison									
	Kaitoke	Judgeford	Walker Estate	Whiteria	Belmont	Taita	Rimutaka	East Harbour	Wellington	Otari
Kaitoke										
Judgeford	0.40***									
Walker Estate	0.24**	0.28**								
Whiteria	0.72***	0.33***	0.38**							
Belmont	0.43***	0.62***	0.27***	0.73***						
Taita	0.24*	0.16*	NS	0.40***	0.29**					
Rimutaka	0.69***	0.55***	0.54***	0.84***	0.66***	0.29***				
East Harbour	0.47**	0.69***	0.45***	0.89***	0.52***	0.39**	0.39**			
Wellington	NS	0.42***	0.43**	0.51***	0.57***	0.32***	0.71***	0.68***		
Otari	0.22*	0.22*	0.21*	0.49***	0.45**	NS	0.42***	0.58***	0.21*	
(b) Fungi	Global $R = 0.51$ $p = 0.1\%$									
	Park Pairwise Comparison									
	Kaitoke	Judgeford	Walker Estate	Whiteria	Belmont	Taita	Rimutaka	East Harbour	Wellington	Otari
Kaitoke										
Judgeford	0.80***									
Walker Estate	0.59***	0.57***								
Whiteria	0.97***	0.52***	0.70***							
Belmont	0.69***	0.73***	0.28***	0.95***						
Taita	0.49***	0.44***	0.34***	0.55***	0.45***					
Rimutaka	0.86***	0.62***	0.75***	0.98***	0.79***	0.54***				
East Harbour	0.33**	0.64***	0.37***	0.65***	0.41***	0.29**	0.55***			
Wellington	0.62**	0.58***	0.26**	0.56***	0.48**	0.23**	0.66***	0.55***		
Otari	0.45**	0.28**	0.33*	0.57***	0.46**	NS	0.52***	0.30***	0.22*	
(c) Bacteria and Fungi Combined	Global $R = 0.49$ $p = 0.1\%$									
	Park Pairwise Comparison									
	Kaitoke	Judgeford	Walker Estate	Whiteria	Belmont	Taita	Rimutaka	East Harbour	Wellington	Otari
Kaitoke										
Judgeford	0.68***									
Walker Estate	0.44***	0.46***								
Whiteria	0.97***	0.48***	0.62***							
Belmont	0.62***	0.75***	0.27***	0.94***						
Taita	0.36***	0.33***	0.26**	0.56***	0.41***					
Rimutaka	0.87***	0.68***	0.74***	0.98***	0.80***	0.50***				
East Harbour	0.36**	0.73***	0.37***	0.76***	0.42***	0.32**	0.54***			
Wellington	0.52**	0.53***	0.32**	0.61***	0.52**	0.28**	0.77***	0.62***		
Otari	0.39**	0.23**	0.26*	0.60***	0.44**	NS	0.51***	0.38***	0.20*	

Significance levels are *p = 5%; **p = 1%; ***p = 0.1%.

common/similar community structure (e.g., Wellington and Otari; Walker Estate and Whiteria) (Fig. 2*a*). SIMPER analysis suggested the absence of two TRFs (12 and 28) from Whiteria contributed somewhat to this difference (Table 3). Rimutaka and East Harbour also showed a good discrimination from all other sites (Table 2a) which may in part be attributed to the absence of TRFs 30 and 2, respectively (Table 3). Despite the high percentage (93%) of sites that could be discriminated between using ANOSIM, several of the discriminations had a low *R*-statistic reflecting the patch scatter seen in Fig. 2*a*. Twenty-seven sites could be discriminated at the highest significance level ($p \leq 0.1\%$), nine at $p \leq 1\%$, and six at $p \leq 5\%$ (Table 4a).

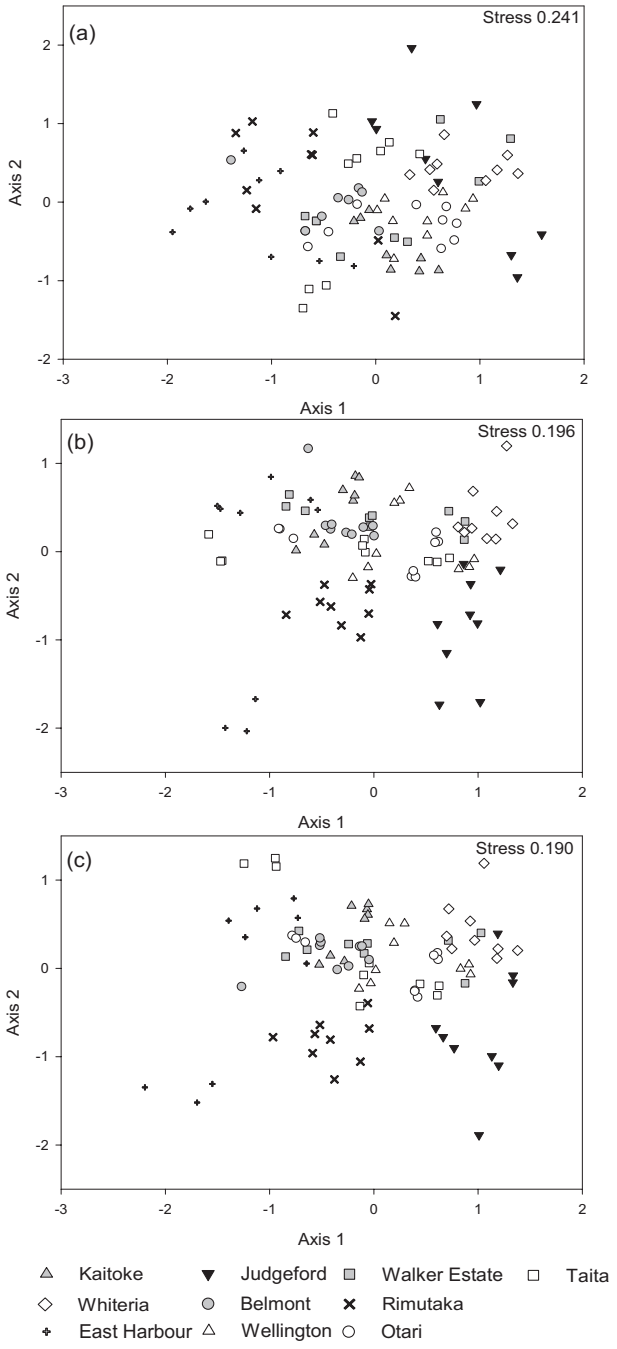

FIG. 2—*Multidimensional scaling biplot representing the (a) bacterial, (b) fungal, and (c) combined bacterial plus fungal populations representing the relative ordination between soils collected from 10 sites different recreational sites across the Wellington region.*

For the fungal community, the mean number of TRFs within a site ranged from 17 to 31, and there was no significant difference in the number of TRFs between sites ($p > 0.05$, data not shown). As had been observed for the bacterial community, the structure of the fungal community was significantly influenced by site ($R = 0.51$, $p = 0.1\%$). Figure 2*b* demonstrates that some sites had

TABLE 3—*SIMPER analysis demonstrating the average dissimilarity between sites and identifying the presence/absence of individual bacterial (1–67) and fungal (68–160) TRFs that contribute to the dissimilarity. Where no TRFs are shown, all TRFs within the site comparisons are common in at least one of the samples in each site.*

		TRF number	
	% Dissimilarity	Present	Absent
Kaitoke			
Judgeford	46	98	74,97,160
Walker Estate	35		
Whiteria	43	86,111,151	12,28,33,108,109,114,
Belmont	31	89	160
Taita	38		74,160
Rimutaka	42		49,90,160
East Harbour	41	89	26
Wellington	34	124	74,160
Otari	35	89	
Judgeford			
Walker Estate	45	93	
Whiteria	42		82,145,146,141
Belmont	48	98	74,93,95,97
Taita	46		97
Rimutaka	46		
East Harbour	56	143	98
Wellington	43	124	
Otari	41		
Walker Estate			
Whiteria	40	109,114	82,151
Belmont	31		
Taita	40		
Rimutaka	43		30,90,158
East Harbour	45		2
Wellington	37		158
Otari	38		
Whiteria			
Belmont	42	12,13,28,96, 108,109	82,86,146,151
Taita	43	109	111,146,151
Rimutaka	48	12,28,108, 109,112	30,82,86,104,111, 145,146,151
East Harbour	53	12,15,28,143	2,26,86,104,111, 145,146,151
Wellington	37	28,109	111,151
Otari	40	12,28,109	82,111,145,146,151
Belmont			
Taita	37	74,119	
Rimutaka	39	94	95
East Harbour	41	15	2
Wellington	35	74	71,124
Otari	35		
Taita			
Rimutaka	42		158
East Harbour	47		2
Wellington	38		158
Otari	38		
Rimutaka			
East Harbour	46		
Wellington	41		
Otari	40		112
East Harbour			
Wellington	48	26	71,124
Otari	45		15
Wellington			
Otari	34		

TRF, terminal restriction fragment.

TABLE 4—*The number of site comparisons that showed significant level of discrimination between based on bacterial, fungal, and bacterial plus fungal T-RFLP profiles.*

Significance Level p (%)	Bacteria (%)	Fungi (%)	Combined (%)
<0.1	27 (60)	33 (73)	32 (71)
<1	9 (20)	9 (20)	10 (22)
<5	6 (13)	2 (4)	2 (4)
>5	3 (7)	1 (2)	1 (2)

clear differences in fungal community structure (e.g., Whiteria and Rimutaka), while others shared a more common fungal community (e.g., Otari and Taita). Pairwise comparisons between sites are presented in Table 2b. Forty-four of the 45 comparisons could be discriminated between using fungal T-RFLP profiles; Taita and Otari were the only two parks that could not be discriminated between. A high number of parks (33/45) discriminated from each other at the highest level of significance ($p \leq 0.1\%$) with nine discriminating at $p \leq 1\%$ and two at $p \leq 5\%$ (Table 4). Generally, the *R*-statistic within pairwise comparisons was higher for the fungal community than the bacterial community (Table 4). The presence/absence of several TRFs at the sites could in part attribute to some of the discrimination between sites. For example, TRF 160 was absent in five parks, but present in Kaitoke while TRF 109 was absent from Whiteria but present in six other sites, while TRF 146 was present at Whiteria, but absent from six sites (Table 3).

Data from both the bacterial and fungal community profiles were combined, and analysis was performed treating the data set as one. Ordination plot of this analysis is presented in Fig. 2*c* and reflects a similar pattern that was observed for both the bacterial (Fig. 2*a*) and fungal (Fig. 2*b*) communities whereby Whiteria and Rimutaka separated well on the ordination plot, while Otari and Taita did not. Pairwise comparisons between sites are presented in Table 2c. Forty-four of the 45 site comparisons showed some significant level of discrimination, with Taita and Otari being the only two sites that could not be discriminated between. Thirty-two sites could be discriminated at the highest significance level ($p \leq 0.1\%$), ten at $p \leq 1\%$, and 2 at $p \leq 5\%$ (Table 2).

Effect of Broad Vegetation Class on Bacterial and Fungal Community Structure

Figure 3 illustrates the distribution of different broadscale vegetation classes within the ordination plot across the 10 sites. There was no apparent discrimination between different vegetation classes for either the bacteria (Fig. 3*a*) or the fungi (Fig. 3*b*).

Within-Park (Patch) Variation

It was clear from Fig. 2 that both the bacterial and the fungal communities within each site were subject to spatial variability, as there was generally clustering of the replicate samples taken from each individual patch but some scattering between patches from the same site. Pairwise comparisons between the different patches (A, B, and C) within each site are presented in Table 5. For both bacteria and fungi, there was a significant effect of patch within each site, with the exception of Taita, in which there were no significant differences in either the bacterial or fungal community structure (Table 5a,b). In Kaitoke, Judgeford, Walker Estate, East Harbour, Wellington City, and Otari sites, patches A, B, and C all showed significant differences in the fungal and to a lesser extent the bacterial community structure (Table 5a,b, respectively). Because there was only a possible 10 permutations within each pairwise

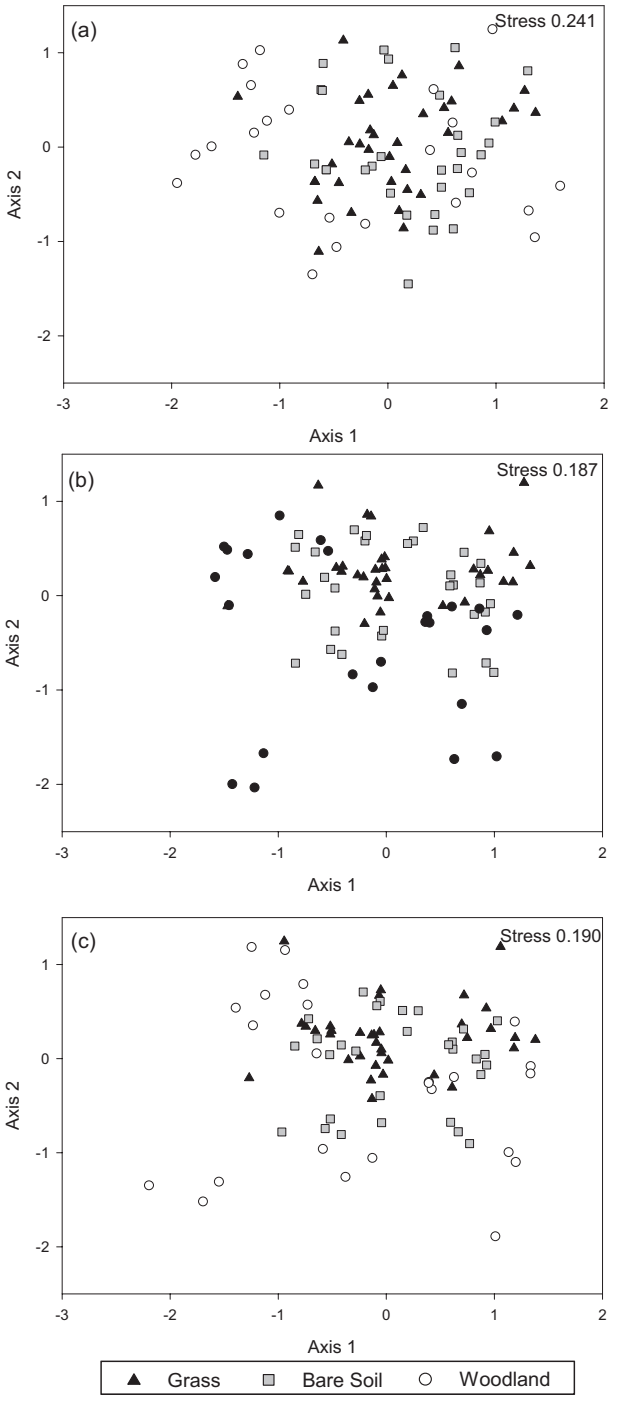

FIG. 3—*Multidimensional scaling biplot representing the (a) bacterial, (b) fungal, and (c) combined bacterial plus fungal populations in 10 sites within the Wellington region. The plots represent the relative ordination between soils collected from grass (▲) bare soil (■) or woodland (○) across the 10 sites.*

comparison, significant levels are restricted to 10% (i.e., $p < 0.1$) but *R*-values of close to 1 indicate a high level of discrimination between patches. Within Whiteria, patches A and C showed a high level of similarity in microbial community structure (Table 5), and there was no discrimination between these two patches. Within Belmont, the fungi showed a higher level of patch discrimination than the bacteria (as indicated by the higher *R*-values) whereas in Rimutaka, the bacteria could discriminate between patches A and B when the fungi could not (Table 5a,b). Generally, combining

TABLE 5—*Pairwise comparisons (*R*-value) between different patches within each site for bacteria, fungal, and combined bacteria and fungi within the 10 sites sampled within the Greater Wellington region.*

	Bacteria R = 0.786***					Fungi R = 0.896***					Bacteri and Fungi R = 0.787***			
(a)	Global R		A	B	(b)	Global R		A	B	(c)	Global R		A	B
Kaitoke		A			Kaitoke		A			Kaitoke		A		
	1**	B	1			1**	B	1			1**	B	1	
		C	1	1			C	1	1			C	1	1
			A	B				A	B				A	B
Judgeford		A			Judgeford		A			Judgeford		A		
	0.951**	B	1			1**	B	1			1**	B	1	
		C	1	0.963			C	1	1			C	1	1
			A	B				A	B				A	B
Walker Estate		A			Walker Estate		A			Walker Estate		A		
	1**	B	1			1**	B	1			1**	B	1	
		C	1	1			C	1	1			C	1	1
			A	B				A	B				A	B
Whiteria		A			Whiteria		A			Whiteria		A		
	0.564*	B	1			0.284*	B	0.630			0.399*	B	1	
		C	NS	0.630			C	NS	0.370			C	NS	0.407
			A	B				A	B				A	B
Belmont		A			Belmont		A			Belmont		A		
	0.572**	B	0.407			0.761**	B	0.667			0.737**	B	0.667	
		C	0.537	1			C	0.704	1			C	0.667	1
			A	B				A	B				A	B
Taita		A			Taita		A			Taita		A		
	0.305	B	0.704			0.148	B	NS			0.173	B	NS	
		C	0.481	NS			C	0.370	NS			C	0.426	NS
			A	B				A	B				A	B
Rimutaka		A			Rimutaka		A			Rimutaka		A		
	0.642**	B	0.667			0.667*	B	NS			0.605*	B	0.556	
		C	1	0.444			C	1	0.426			C	1	0.333
			A	B				A	B				A	B
East Harbour		A			East Harbour		A			East Harbour		A		
	1**	B	1			1**	B	1			1**	B	1	
		C	1	1			C	1	1			C	1	1
			A	B				A	B				A	B
Wellington		A			Wellington		A			Wellington		A		
	0.909**	B	0.963			1**	B	1			1**	B	1	
		C	1	0.778			C	1	1			C	1	1
			A	B				A	B				A	B
Otari		A			Otari		A			Otari		A		
	1**	B	1			1**	B	1			1**	B	1	
		C	0.852	1			C	1	1			C	1	1

Significance levels are *p = 5%; **p = 1%; ***p = 0.1%.

bacterial and fungal profiles did little to discriminate further between patches than was provided by fungi alone.

Discussion

Currently, there is a lack of data relating to variation across soils at forensically relevant scales. To the best of our knowledge, this is the first study to demonstrate variation in bacterial and fungal communities across soils from defined geographic location at forensically relevant scales.

Discrimination Between Parks and Vegetation Category

Drawing on findings from numerous ecological studies, it is apparent that microbial communities can differ between different land uses (27,28) and vegetation types (7,29,30). Ecological studies are often designed to test the hypothesis that changes or perturbations to a particular ecosystem will result in changes in the microbial community structure. However, from a forensic perspective, comparison of soils from similar locations (in terms of land use, plant cover, and mineralogy but geographically distinct) may be of greater interest/relevance, and thus we focused our study on assessing microbial community structure across parkland within the Greater Wellington region. Currently, our knowledge of how microbial communities vary across similar land use and/or locations is lacking. Our results demonstrate that soils from different sites within the Greater Wellington region that were of a similar type/series and that had developed on similar underlying geology could be discriminated between to differing degrees based on microbial T-RFLP profiles (although the degree of discrimination varied between sites and it was not possible to discriminate between soils collected from two of the sites, Taita and Otari).

A recent study suggests fungi as a potentially more robust target for the application to soil forensic studies (16). In this investigation, generally fungal community structure showed a stronger level of discriminator between sites than the bacteria. The reasons for this could be several fold. First, many fungi form specific associations (both symbiotic and pathogenic) with plants and therefore the presence of particular plant species at a site may be reflected, to some extent in fungal community composition. Indeed, SIMPER analysis highlighted a greater number of fungal TRFs that were absent from some sites compared to bacteria. Whiteria was of particular interest as it had a relatively high number of TRFs that were absent/of lower frequency than at other sites as well as several TRFs that were absent. This site was located close to the shore line and further investigations are needed to determine whether these TRFs

could be signature of soils located close to the shore line, and if so whether the presence of particular TRF markers could be used in an intelligence-based mode to determine provenance of an unknown sample. Further research identifying key microbes with specific roles in soil, such as mycorrhizal fungi and nitrogen-fixing bacteria could eventually lead to development of an indicator species system, where the presence, absence, or combination of specific bacterial species in a soil sample could predict the geographic location with more certainty than looking at the entire microbial community.

Combining data from the bacterial and fungal T-RFLP profiles provided minimal additional information than was provided by fungi alone. The variability in community structure within a park, at the patch level was higher for the bacterial community then was observed for the fungal. Such an observation is not unexpected as the fungal community is likely to be less affected by small-scale fluctuations in soil water and resource availability because of their ability to bridge air-filled pore space and their sporulating tendencies, and these results add weight to the suggestion that the fungal community may be a more robust target for soil forensic comparison (16) than the bacterial community. The use of additional specific taxonomic groups may provide further discriminatory power and requires further investigation.

In contrast to many ecological studies (e.g., [7,12]), there was little evidence within our data set to suggest that either bacterial or fungal community structure differed significantly between vegetation category. The vegetation categories described here were broad, with grass species and tree species differing between sites. A more in-depth study would be required where vegetation class was more rigidly defined and replicated before the potential for such an approach as an intelligence-based tool to provide evidence on potential origin of soils could be evaluated.

Discriminating Across Different Spatial Scales

Variation in the structure of soil microbial communities within a location is an inevitable reality because the factors (abiotic and biotic) that drive community structure are not confined within defined boundaries. Thus, community structure is likely to change along environmental gradients even at local scales as was demonstrated here for both the bacteria and the fungi at the patch level. Here, we only investigated three patches within each site, and three replicate soil samples within each patch, but clearly demonstrated that significant variation in both bacterial and fungal community structure could be detected at the patch level (i.e., between patches). The level of similarity/dissimilarity between patches within a site varied greatly between sites. Variations in the level of intra-habitat bacterial variability have been previously reported (5,7,31). The patch variation within a site reported here, highlights the need for careful consideration as to the number of samples collected and the distance they are sampled across if profiling of microbial communities from environmental samples is to be used as an evaluative or intelligence-based tool in forensic investigation. This is likely to be guided by specific case circumstances, for example how heterogeneous the vegetation is within a site. For example, Meyers and Foran (7) suggest human manipulated locations (e.g., agricultural field) are likely to have a higher degree of habitat similarity. Schwarzenbach et al. (32) suggest that when a high degree of variation of 18S rRNA fungal T-RFLP profiles was observed over 400 m^2 grassland, representative profiles could be obtained by pooling seven replicates within this area. Pooling of PCR products has previously been applied to increase representatively of molecular genetic profiles (33–35). In this instance, microbial community profiling could be useful when trying to pin-point an unknown location from forensic soil evidence found on a spade or clothing. This type of application could be useful in soil database searching and pin pointing geographic locations for police to search, although clearly a more intensive analysis would be required to generate such a database.

The inherent resource variability associated with heterogeneous soil environments inevitably leads to spatial variability in the structure of soil microbial communities (e.g., [36,37]). Such variability may be perceived as a limitation to the application of microbial profiling for forensic comparison of soil (7), but equally, could prove advantageous in cases where exact matches are required (e.g., between a shoe print and soil on a shoe). Several studies have been undertaken to determine the spatial variability of microbial communities across a range of soil types and land uses (37–39). Most studies report variability in the structure of soil microbial communities in a single soil type or land use (e.g., agricultural field or pine plantation) and while variability at small spatial scales (centimeters) may be high, similarity between samples at small spatial scales could be as similar or as dissimilar as those at larger scales (meters or kilometers) (13,31,40,41). Indeed, previous crime scene scenario–based research has demonstrated potential for site-specific discrimination (3), but undoubtedly further research is needed on a larger number of samples across a range of different habitats and soils.

The results presented here demonstrate that differences in microbial community structure can be seen between different park locations within a relatively confined regional area, highlighting the potential of microbial profiling to be used in forensic comparison of soils. Further, the potential to discriminate between soils taken from within a park location could prove useful for site-specific matching in an evaluative mode but requires further research to gain a better understanding of variation across different spatial scales. The presence/absence of particular TRFs in specific sites merits further investigation to determine the potential for an intelligence-based approach using microbial DNA profiling. Further validation of this method is needed before it can be used in forensic investigation and its potential when combined with soils evidence, such as soil color, mineralogy, or palynology studies, is needed. Nevertheless, utilizing differences in soil microbial communities presents a potentially powerful yet simple forensic tool, providing the ability to routinely use soil as associative evidence.

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