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Full Paper

Correlation between characteristics of soil at crime scene and on suspect's sole

Jong-Tar Kuo [1], Cheng-Lung Lee [2, 3] and Ying-Chien Chung [1, *]

[1] Department of Biological Science and Technology, China University of Science and Technology, Taipei City 11581, Taiwan
[2] Department of Criminal Investigation, Taiwan Police College, Taipei City 11696, Taiwan
[3] Crime Scene Theater, Qatar Police College, PO.BOX:7157 Doha, Qatar
* Corresponding author, e-mail: ycchung@cc.cust.edu.tw

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Abstract: In addition to shoe prints, forensic personnel use the chemical and biological characteristics of soil attached to a suspect's sole as critical evidence to connect the suspect to a crime scene. However, the current forensic applications of soil analysis are limited. In this study 15 types of riverside soil and soils attached to mock suspect's sole were collected. The characteristics of these soils were analysed to establish a correlation between the soil on the suspect's sole and that at the mock crime scene. Results indicated a correlation between the soil conductivity and nitrate concentration of the riverside soils and those attached to the suspect's sole. Excluding the special soil texture, this could be used as auxiliary evidence. To enhance the discrimination of soil source identification, soils were first classified according to their texture, and next-generation sequencing was then used to analyse the bacterial community in the soil. The results indicated that the bacterial communities in the soils attached to the suspect's sole were similar to those in the riverside soils. In conclusion, by analysing the soil texture, conductivity, nitrate concentration and bacterial community, we identified the origin of soil samples for forensic comparison after 48 hr.

Keywords: soil discrimination, next-generation sequencing, molecular bioassay, forensic science

INTRODUCTION

Traditional soil forensic techniques mainly involve soil-class examination and geological analysis to establish soil characteristics such as grain size, pH and moisture content in tested soils [1]. Soils are heterogeneous, complex and transferable and have great potential for forensic

investigation. By using soil characteristics, we can obtain clues to identify the origin of an unknown sample or find the correlation between soil samples collected from a suspect and at a crime scene [2]. In other words, soil adhering to clothing, footwear or tools of the suspect is a particularly significant material in criminal investigation, which can provide clues to connect the suspect, victims and crime scene and even aid conviction in the court of law [3, 4]. However, geological analyses such as the primary comparison of soil inorganic and organic components that differentiate among soil samples generally lack sensitivity and sufficient discriminatory potential unless soils possess special features [2].

Soil, the top layer over the Earth's crust, results from the alteration of the bedrock. One gram of soil contains 4×10^7–2×10^9 bacteria, which vary widely in species diversity and abundance among different soils [5]. In other words, soil's heterogeneity favours its colonisation by a huge diversity of microorganisms, allowing soil samples to be differentiated by the specificity of their microbiota [6]. Typical culture techniques for quantifying bacteria measure only viable cells under suitable growth conditions. This causes a steep underestimation of diversity, further limiting the applicability of these techniques for forensic purposes [7]. Using non-culturable techniques such as molecular methodologies including analysis of DNA fingerprinting or sequencing characteristics can resolve the aforementioned technical problems [8]. Modern DNA methodologies aid direct analysis of the biodiversity or biological community of a specific environment by identifying microbial DNA. The establishment of DNA barcode libraries enables scientists to develop reliable protocols for taxa identification from samples containing entire or partial DNA and helps them judge the possible source of the tested soil or water sample [9]. Moreover, 16S ribosomal RNA (rRNA) gene analysis to construct bacterial phylogenies is a potential molecular identification method [10]. Although 16S rRNA phylogenetic analysis has already been applied to the ecological and environmental field, its relevance in the forensic field has not been determined thus far. Thus, the establishment of an overarching and objective technique for soil identification and comparison is urgently required. In addition, bacterial fingerprint analysis is widely used to understand the similarity between soil samples [1], which has potential for the individualisation of soil for forensic purposes. However, the current analysis techniques only speculate on the original environment of a tested sample under specific conditions [11].

Next-generation sequencing (NGS) is an effective, high-throughput and low-cost technology for forensic investigation. In the past the application of soil evidence in forensic science was mainly based on pedological and geological analyses. In 2014 Giampaoli et al. [12] were the first to complement biological studies by using NGS profiling for forensic comparison of soils. Soil samples from farms and lakes (i.e. distinct but geologically similar environments) could be preliminarily identified; however, further discrimination of these samples was difficult. Hopkins [13] applied NGS technology to soil bacterial profile analysis for forensic application and found it to be promising. Bacterial abundance charts, non-metric multidimensional scaling, and supervised classification based on NGS technology potentially provide an expert witness with a useful visualisation tool for the jury, thereby fulfilling a primary requirement of forensics. However, the statistical measure of relative similarities among bacterial community profiles in various soil samples has not been well established. Jesmok et al. [14] analysed the bacterial community profiles of nine habitats including beach, coniferous forest, marsh, corn agricultural field and fallow agricultural field, and found them to share 75% of the same major bacterial classes. Bacterial community profiling in soil samples could be achieved using NGS analysis; however, the relationship between the bacterial communities of soils in different woodlots has not been

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established. To establish these correlations or improve the identification proficiency, further classification of soil textures in similar habitats is necessary.

In this study we analysed the textures and chemical characteristics of soils and established correlations between riverside soils and soils attached to a suspect's sole. We subsequently determined the bacterial profiles of these soils and differentiated them through NGS and similarity analysis. To the best of our knowledge, such a strategy for adopting this method for forensic soil analysis has not been employed for soil identification.

MATERIALS AND METHODS

Sampling Location

A total of 15 sampling sites in Taiwan were selected for investigation. These sampling sites were distributed across upstream, midstream and downstream sites of Danshuei River, Jhuoshuei River, Erren River, Gaoping River and Donggang River, geographically located in that order from north to south. The sampling sites were positioned using GPS. Soil samples were obtained at a distance of 1 m from the river bank. The GPS sites and soil textures of these sampling sites as well as the neighbouring water quality are listed in Table 1.

Table 1. Sampling locations and environmental conditions of five tested territorial waters

	Danshuei River			**Jhuoshuei River**			**Erren River**		
Sampling site	Ganyuan Bridge (up-)*	Dahan Bridge (mid-)	Kuandu Bridge (down-)	Mingzhu Bridge (up-)	Tzuchiang Bridge (mid-)	Xibin Bridge (down-)	Guting Bridge (up-)	Nansyong Bridge (mid-)	Nandin Bridge (down-)
Location	25°07'N	25°04'N	24°96'N	23°49'N	23°49'N	23°50'N	22°53'N	22°53'N	22°55'N
	121°27'E	121°46'E	121°39'E	120°42'E	120°23'E	120°17'E	120°23'E	120°20'E	120°11'E
Water quality	Moderately polluted	Severely polluted	Moderately polluted	Moderately polluted	Unpolluted	Unpolluted	Moderately polluted	Moderately polluted	Negligibly polluted
Soil texture	Loam	Silty loam	Clay loam	Loam	Silt	Sandy clay	Loam	Loam	Sandy clay loam

Table 1. (continued)

	Gaoping River			**Donggang River**		
Sampling site	Sandimen Bridge (up-)	Kaoping Bridge (mid-)	Suanyen Bridge (down-)	Longdon Bridge (up-)	Cinsheda Bridge (mid-)	Donggang Bridge (down-)
Location	22°42'N	22°37'N	22°29'N	22°36'N	22°32'N	22°28'N
	120°38'E	120°26'E	120°25'E	120°35'E	120°30'E	120°27'E
Water quality	Unpolluted	Moderately polluted	Severely polluted	Moderately polluted	Severely polluted	Moderately polluted
Soil texture	Loamy sand	Silty loam	Clay loam	Sandy loam	Loam	Silty clay

* up-, mid-, down- represent upstream, midstream and downstream

Soil Collection from Suspects' Soles

The suspects (testers) wore sneakers to walk around mock crime scenes (sampling sites) for 20 min. before leaving the scenes by car. The soils attached to their sneakers' soles were partially

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removed by scraping after 2 hr. Subsequently, the shoes were placed in a cabinet (28°C) for 24 hr and 48 hr before the attached soils were collected by scraping. All tests were performed in triplicate at least.

Environmental Conditions and Chemical Analysis of Soils

Soil texture was identified according to a soil survey manual [15]. Water quality based on the river pollution index was obtained from official data of Taiwan's Environmental Protection Administration [16].

To measure soil conductivity, 10 g of air-dried soil was placed in a beaker and mixed with distilled water so that no excess water covered the soil surface, forming a saturated soil paste. After standing for 15 min. the soil paste was poured into a Buchner funnel, and the filtrate was collected using suction. The conductivities of the filtrate and distilled water were measured using a portable multiparameter instrument (Multi 350i, WTW, Germany). The conductivity of the filtrate minus that of the distilled water equaled the soil conductivity. Because nitrate (NO_3^-) concentration may reflect or be associated with the soil type, NO_3^- concentration in the soil was determined [17]. To measure this, 5 g of air-dried soil was mixed with 50 mL of 2 M KCl solution. After shaking for 1 hr, the solution was filtered through Whatman Grade 42 filter paper, and the nitrate in the filtrate was measured using a water quality analyser (photoLab 7000, WTW, Germany) [18]. All tests were performed in triplicate.

Biological Analysis of Soils

To measure bacterial cell numbers in the soil, 1.5 g of soil was mixed with 15 mL of sterile distilled water and enumerated by traditional plate-counting methods. Bacteria were cultured at 35°C for 48 hr and plate count agar was used. Experiments were conducted in triplicate at least.

To analyse the soil bacterial community profile, cell lysis, DNA extraction, polymerase chain reaction (PCR), and Illumina sequencing were conducted according to the processes described by Zielińska [19]. A FastDNA SPIN kit (MP Biomedicals, USA) was used for DNA extraction. The V3-V4 hypervariable regions of bacterial 16S rDNA were amplified using the following primer set: 341F-CCTACGGGNGGCWGCAG and 785R-GACTACHVGGGTATCTAATCC. The PCR conditions were as follows: 94°C for 3 min., followed by 35 cycles of 95°C for 30 sec., 54°C for 30 sec., 72°C for 45 sec. and a final elongation step of 72°C for 8 min. The Illumina MiSeq platform and a V3 600-cycle kit were used to sequence the PCR products. Chemicals, their concentrations and steps for the sequencing reaction followed the manufacturer protocol (Illumina Inc., USA). Sequences were quality filtered, allowing a maximum of one expected error per merged read; the sequences containing ambiguous bases were discarded. The resulting data were clustered using USEARCH (version 8.1.1861) at a 97% identity level to form operational taxonomic units [20], which were assigned to taxa by using the Greengenes release 13.08 as a reference, with the taxonomy assignment tool PyNAST [21].

The NGS of 16S rDNA gene was conducted by Yourgene Bioscience Company (Taiwan). Correlation between soil samples collected from a suspect and at a mock crime scene was subjected to group analysis using the Analysis Toolpak add-in in MS Excel. For all experiments, two separate soil samples were collected, and all sample analyses were conducted at least in duplicate.

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RESULTS AND DISCUSSION

Background Conditions of Sampling Sites

Table 1 lists the GPS sampling locations and environmental conditions in the five tested territorial waters. These sampling sites were distributed across northern, central and southern Taiwan. Because riverside soils are usually infiltrated by river water, the quality of the river water affects the chemical and biological characteristics of the soils. Several upstream, midstream and downstream sites along these rivers are polluted stretches. The downstream reaches of their tributaries are also influenced by the tide and subject to seawater intrusion. The extent of river pollution is usually classified into four levels, namely unpolluted, negligibly polluted, moderately polluted and severely polluted, according to the river pollution index in Taiwan [22]. As shown in Table 1, the waters of three sampling sites are unpolluted, one is negligibly polluted, eight are moderately polluted, and the remaining three are severely polluted.

Regarding soil texture, soils at three (Sandimen, Longdon and Nandin Bridges), four (Donggang, Xibin, Kuandu and Shuanyen Bridges) and three (Dahan, Tzuchiang and Kaoping Bridges) sampling sites had relatively high proportions of sand, clay and silt respectively. The other soils were classified as loam, composed mostly of sand and silt and a smaller amount of clay. The soil samples selected for this study were composed of most soil textures, thereby meeting the standard of representativeness.

Crucial Chemical and Biological Characteristics of Soils at Sampling Sites

Figure 1 (A–C) indicates the conductivity, NO_3^- concentration and bacterial cell count of soils from the five rivers (upstream, midstream and downstream), which showed great variations. The conductivity, NO_3^- concentration and bacterial cell number in the soil at different sampling sites changed from 0.37 to 18.80 ms/cm, 0.46 to 22.05 mg/L, and 3.30×10^5 to 9.04×10^6 cfu/g-soil respectively. The culturable bacteria count range was similar to that of 16 soil samples from Heihe River in north-western China [23]. In addition, the variation of soil characteristics among the upstream, midstream and downstream sites was quite large, implying that different soil characteristics may be used as clues to identifying the possible origin of an unknown soil sample in forensic investigations [2].

Correlation between Soil Characteristics at Sampling Site and on Suspect's Sole

Based on the aforementioned results, to understand or establish the relationship between the soil characteristics at sampling site (mock crime scene) and on the suspect's sole, the conductivity, NO_3^- concentration and bacterial cell number in the soils were analysed. Figure 2 shows the correlation between conductivity of soil at the mock crime scene and that on the tester's (suspect's) sole during the experimental periods (2, 24 and 48 hr). The results show that the soil conductivities on the suspect's sole and at mock crime scene are highly correlated ($R^2 = 0.9993$) after 2 hr, even if the suspect has passed over various road surfaces. When the suspect goes indoors and places the shoes in the cabinet for 24 hr, the coefficient of determination (R^2) between the soil conductivity on the suspect's sole and at the sampling site reaches 0.9977. However, the coefficient between both decreases to 0.9651 after 48 hr of storage as some volatile substances in the soil escaped or were loosened [24]. Because loamy sand, sandy loam and sandy clay loam all contain high proportions of sand that is not easily adhered to the sole [25], the soil conductivity becomes distorted after 48 hr of

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storage. Therefore, when sandy soil samples were excluded, a high correlation coefficient (y_{48h} = 0.9712x − 0.0152, R = 0.9956) was obtained.

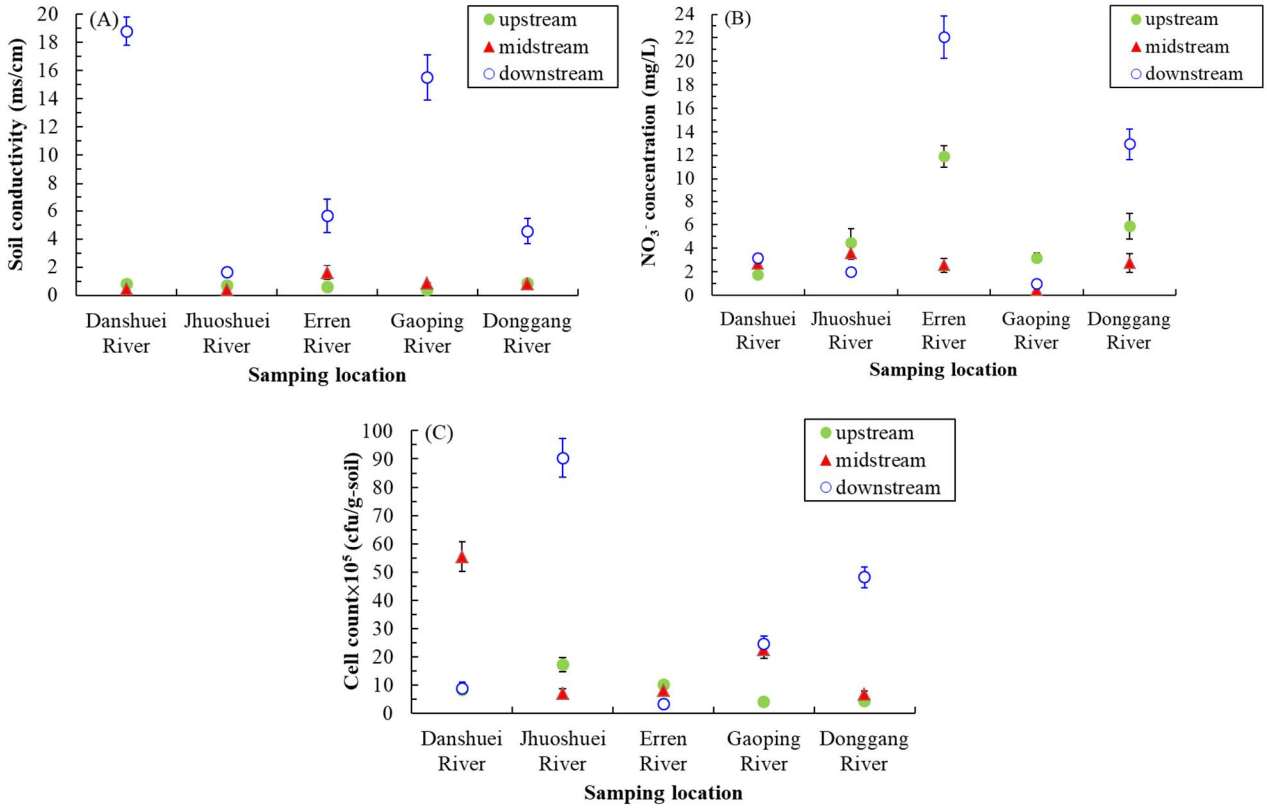

Figure 1. Conductivity (A), NO_3^- concentration (B), and bacterial cell number (C) of soils near five rivers

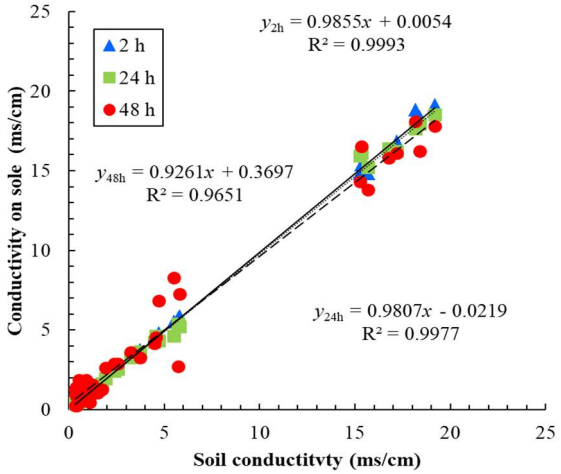

Figure 2. Correlation between soil conductivity at mock crime scenes and that on the suspect's sole during the experimental periods (2, 24 and 48 hr)

Figure 3 shows the correlation between soil NO_3^- concentration at mock crime scenes and that on the suspect's sole during 2, 24 and 48 hr. The results reveal a high correlation of R^2 (= 0.9956), even when the suspect had left the scene 2 hr previously. When the suspect went to the laboratory and placed the shoes in the cabinet for 24 hr, the coefficient of determination remained

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high (0.9924). However, after 48 hr of storage, the correlation ($R^2 = 0.9397$) significantly decreased as the NO_3^- might have been metabolised by soil microbes on the sole [26]. Soil with a high proportion of sand is easily loosened from soles, and moisture is not homogeneously dispersed in soils with a high proportion of clay; thus, NO_3^- measurement for these soil types would result in a deviation. Therefore, when five soil samples classified as sandy soils (loamy sand, sandy loam and sandy clay loam) and clay soils (sand clay and silty clay) were excluded, an improved correlation coefficient ($y_{48h} = 0.8263x + 0.0516$, $R^2 = 0.9954$) was obtained for loamy soils (loam, silty loam and clay loam) and silty soil (silt).

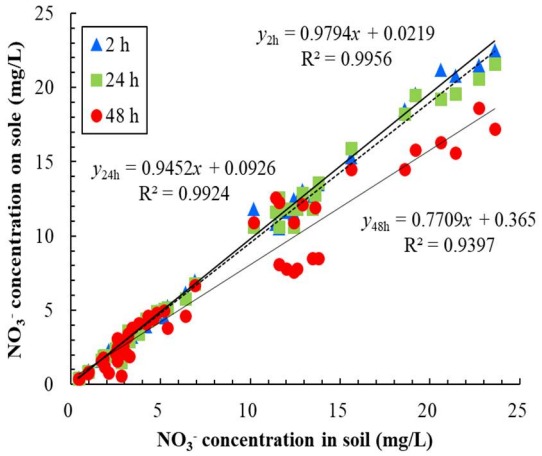

Figure 3. Correlation between NO_3^- concentration of soil at mock crime scenes and that on the suspect's sole during the experimental periods (2, 24 and 48 hr)

Figure 4 shows the correlation between bacterial cell count in the soil at the mock crime scenes and that on the suspect's sole during the experimental periods (2, 24 and 48 hr). The results indicate a high correlation ($R^2 = 0.9968$) even if the suspect left the scene 2 hr previously. A reliable regression equation was obtained ($y_{24h} = 0.9953x - 1.0415$) and could be applied to forensic investigation. However, when the suspect placed the shoes in the cabinet for 24 or 48 hr, the degree of correlation considerably decreased to 0.7968-0.8516. Furthermore, exclusion of any soil samples or characteristics did not give the desired correlation for forensic application.

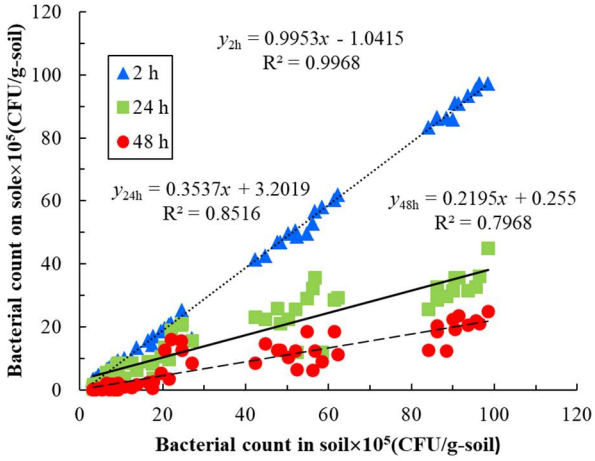

Figure 4. Correlation between bacterial cell count in the soil at the mock crime scenes and that on the suspect's sole during the experimental periods (2, 24 and 48 hr)

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Correlation between Bacterial Community in Soils at Sampling Sites and on Suspect's Sole

By analysing the soil conductivity and NO_3^- concentration in the soils on suspects' soles, we could obtain partial clues to the possible origin of the soil sample after the suspect had left the crime scene 48 hr previously. However, discrimination based on a single mineralogical characteristic was not enough for a conviction or even as evidence [2]. Also, a reliable correlation of bacterial numbers in the soils was only found 2 hr after the crime occurred. Therefore, obtaining more accurate biometric evidence is necessary.

Bacterial DNA, which is retained in the environment for a longer time than viable bacterial cells, may be used to identify individuals [27, 28]. To achieve this, the collected soil samples were classified into four main types according to the soil fractions (i.e. sand, silt and clay): loamy soils (loam, silty loam, clay loam and silty clay loam), sandy soils (sand, loamy sand, sandy loam and sandy clay loam), clay soils (clay, sandy clay and silty clay), and silty soils (silt) [29]. Among the classifications, loamy soils constituted the largest class—nine soil samples from the 15 sampling sites were classified as loamy soils (Table 1).

Bacterial soil communities in these samples were analysed using NGS techniques and group analysis to establish a correlation between nucleic acid profiles in soils at the mock crime scenes and on the suspects' soles. Figure 5 shows a correlation between the structure of bacterial soil communities at the mock crime scenes and on the suspects' soles after 48 hr of storage. Figure 5A indicates that the bacterial soil communities in the soil at the Sandimen Bridge site featured at least 11 phyla. A similar structure of bacterial communities (type and proportion) was found in the soil on the suspects' soles. Using numerical correlation analysis of the two soil groups, a high similarity (0.9859) was found even when the suspects had left the crime scene 48 hr previously. Among the bacterial soil communities, Proteobacteria, Actinobacteria, Acidobacteria, Cyanobacteria, Bacteroidetes, Verrucomicrobia, Firmicutes, Planctomycetes, Chloroflexi and Gemmatimonadetes in the two soil groups accounted for 45.0-47.2%, 16.0-13.5%, 8.0-5.1%, 5.0-3.2%, 6.0-4.4%, 2.0-1.8%, 5.0-8.4%, 1.5-1.1%, 2.0-0.9% and 6.0-8.6% respectively.

The bacterial soil community in the clay soils (silty clay) at the Donggang Bridge site featured seven phyla; loamy soils (loam) at the Cinsheda Bridge site featured eight phyla; and silty soils at the Tzuchiang Bridge site featured at least seven phyla (Figures 5B–5D). Their similarities or correlation levels between the structure of the bacterial communities at the mock crime scenes and on the suspects' soles when the suspects had the left crime scene 48 hr previously for clay, loamy and silty soils were 0.9883, 0.9754 and 0.9852 respectively. For soil samples not shown in Figure 5, their correlation levels were more than 0.9754. The results suggest the presence of unique combinations of bacterial taxa (phylum and family taxonomic classification levels) that can enable discrimination between individual soils.

Similar soil differences were observed using any of the following: 18S rRNA profiles for six soil samples [30]; a combined analysis of mfDNA, plant, metazoal and protozoal DNA for six soil samples [12]; 16S rRNA profiles for 10 diverse soils [14]; or a combined analysis of ribosomal intergenic spacer and 16S rRNA gene sequencing for six soil samples [2]. However, a relatively low number of tested samples, complicated analysis techniques, and failure to evaluate the effect of time decrease the feasibility of these methods.

Figure 5 indicates that some relative abundance of bacterial soil communities increases with time (48 hr) on shoe sole, for example Proteobacteria, Firmicutes and Gemmatimonadetes at the Sandimen Bridge; Proteobacteria and Firmicutes at the Donggang Bridge; Proteobacteria and

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Bacteroidetes at the Cinsheda Bridge; Acidobacteria and Firmicutes at the Tzuchiang Bridge. The dynamic change in bacterial community may be attributed both to their characteristics and soil properties [31]. Among these bacterial communities, Proteobacteria and Firmicutes were identified to have good adaptability to the environment in the past study [32].

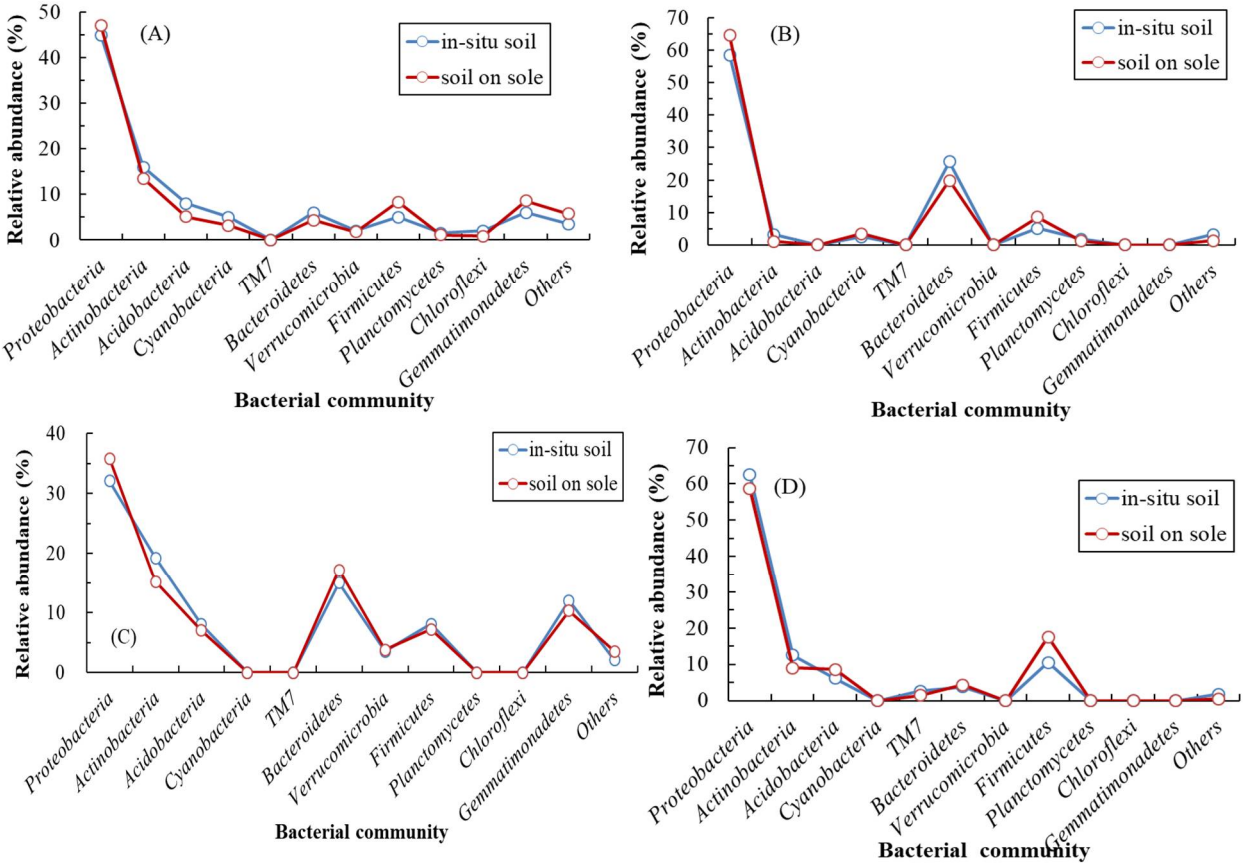

Figure 5. Correlation between structure of bacterial soil communities at mock crime scenes and on suspects' soles after 48 hr: (A) Loamy sand (Sandimen Bridge, upstream site of the Danshuei River); (B) silty clay (Donggang Bridge, downstream site of the Donggang River); (C) loam (Cinsheda Bridge, midstream site of the Donggang River); (D) silt (Tzuchiang Bridge, midstream site of the Jhuoshuei River)

In addition, specific indicator phyla exist in the soils of different habitats that can respond to the original living environment or provide a site-specific clue in forensic soil discrimination [14, 30]. For instance, Chloroflexi was found in soil samples from the Sandimen Bridge site, but it was not detected at other sites. The proportion of Bacteroidetes in soil samples at the Donggang Bridge site was relatively high (25.6%), and so was the proportion of Gemmatimonadetes in soil samples at the Cinsheda Bridge site (12%). The TM7 phylum was detected only at the Tzuchiang Bridge site. Thus, these features (bacterial phyla, bacterial community proportion or specific indicator phylum) can effectively aid in detecting the possible original source of a soil sample found on the suspect, even after the suspect has left the crime scene long before.

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CONCLUSIONS

The conductivity and NO_3^- concentration of soils attached to the suspect's sole can be linked with those in the soil at the mock crime scene; however, these parameters are non-discriminative and therefore each can only be used as auxiliary evidence or a tool for primary screening. A strong correlation between bacterial cell numbers in soil on the suspect's sole and at the mock crime scene was only observed within 2 hr of the incident. A significant similarity or correlation in the structure of bacterial communities in the suspect's sole and on mock crime scene was successfully obtained by performing soil texture classification and applying NGS technology even if the suspect had left the crime scene for 48 hr. Among the four representative soils tested, namely loamy, sandy, clay and silty soils, the bacterial composition and proportion of the soil attached to the suspect's sole within 48 hr were similar to those of the original soil samples, with a correlation level of more than 0.9754. Moreover, soils from different habitats were found to have their own specific indicator (bacterial phylum). Overall, this forensic technology can be applied to various soil types by first analysing soil conductivity and NO_3^- concentration, classifying the soil texture, applying NGS technology, and finally evaluating the correlation of all factors. This developed analysis method may therefore be a potential tool for presenting strong soil forensic evidence.

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