Forensic Science International 240 (2014) 41–47

Contents lists available at ScienceDirect

Forensic Science International

journal homepage: www.elsevier.com/locate/forsciint

The environmental biological signature: NGS profiling for forensic comparison of soils

S. Giampaoli [a], A. Berti [b], R.M. Di Maggio [c], E. Pilli [d], A. Valentini [e], F. Valeriani [a], G. Gianfranceschi [a], F. Barni [b], L. Ripani [b], V. Romano Spica [a,f,*]

[a] University of Rome "Foro Italico", Department of Movement, Human and Health Sciences, Public Health Unit, Piazza Lauro De Bosis, 6, 00135 Rome, Italy
[b] Carabinieri, Reparto Investigazioni Scientifiche di Roma, Viale di Tor di Quinto, 119, 00191 Roma, Italy
[c] Geoscienze Forensi Italia®, Viale Mediterraneo 77, 00122 Rome, Italy
[d] Department of Biology, University of Florence, Via del Proconsolo 12, 50122 Florence, Italy
[e] Spygen, Savoie Technolac, Bâtiment Koala, 17, rue du Lac Saint-André, BP 274, 73375 Le Bourget-du-Lac Cedex, France
[f] MDD University Spin off, Viterbo, Italy

ARTICLE INFO

Article history:
Received 12 November 2013
Received in revised form 11 February 2014
Accepted 25 February 2014
Available online 3 April 2014

Keywords:
Soil
mFDNA
Metabarcoding

ABSTRACT

The identification of the source of a specific soil sample is a crucial step in forensic investigations. Rapid advances in next generation sequencing (NGS) technology and the strong reduction of the cost of sequencing have recently opened new perspectives. In the present work a metabarcoding approach has been successfully applied to forensic and environmental soil samples, allowing the accurate and sensitive analysis of microflora (mfDNA), plants, metazoa, and protozoa DNA. The identification of the biological component by DNA metabarcoding is a strong element for the discrimination of samples geologically very similar but coming for distinct environments.

1. Introduction

Soil is the top layer that covers the Earth's crust, resulting from the alteration of the bedrock. A variety of living being belonging to different kingdoms characterizes the biological component of a soil. This biological component plays principal roles in several ecosystem functions, making each ecosystem unique [1–3]. Current molecular methodologies allow researchers to directly analyze the biodiversity of a specific environment by the simultaneous identification of genomic DNA of bacteria, fungi, plants, protozoa, and metazoa. In particular, with the implementation of standard sequence libraries of small, species-specific portions of the genome called DNA barcodes, scientists developed protocols for the automated identification of multiple taxa from a single bulk sample containing entire organisms or from a single environmental sample containing degraded DNA (soil, water, feces, etc.) [4,5]. While the DNA metabarcoding has been already applied to ecological and environmental studies, the relevance in the forensic field has not been yet tested. Currently, the application of soil evidence in forensic science is mainly based on pedological and geological analysis only in part complemented with biological studies [6–8]. Although the bacterial DNA fingerprint have been proposed as an useful approach to prove the similarity between soil samples, only the fine characterization of the large majority of living beings at a taxonomical level can allow the description of original environment of a specific sample [9–12]. A molecular analysis of the whole DNA component of a soil can effectively links the benefits of palynology, micology, microbiology, botany and zoology applied to forensic investigations. The analysis of the organic signature can lead to an in-depth description of an environmental sample allowing also the discrimination between soil sample sharing similar mineral composition, but different ecological provenance. Starting from these considerations, the present work describes the identification of the biological component of different soils by DNA metabarcoding, opening interesting perspectives for forensic soil analysis.

2. Experimental

2.1. Sampling

In order to investigate the potentiality of the DNA metabarcoding in forensic identification of soils, six samples were collected in

* Corresponding author at: University of Rome "Foro Italico", Piazza Lauro De Bosis, 6, 00135 Roma, Italy. Tel.: +39 06 36733 247; fax: +39 06 36733 247.
E-mail address: vincenzo.romanospica@uniroma4.it (V. Romano Spica).

http://dx.doi.org/10.1016/j.forsciint.2014.02.028
0379-0738/

Table 1
Identification of collected samples.

Sample ID	Sampling location	Type of sample
A1	Farm	Soil naturally fertilized
A2	Farm	Natural fertilizer
A3	Farm	Soil not fertilized
F1	Lake	Soil from country road
F2	Lake	Soil close to country houses
F3	Lake	Lake sand

central Italy between September and November 2012. Table 1 summarizes samples characteristics. Three specimens (A1, A2 and A3) came from a farm. A1 and A3 were geologically and mineralogically similar to each other but they were differentially exposed to organic matter, a natural fertilizer (A2) used on A1 but not on A3. Specimens F1, F2 and F3 were collected near a lake during a forensic investigation: sample F1 was collected from country roads few kilometers far from the lake, sample F2 was collected near the house of a person suspected of being involved in a crime, sample F3 was collected on the lake beach. The two sampling locations (farm and lake) were approximately 150 km away from each other.

2.2. Geological analysis

Soil samples were dried at 60 °C, weighed into tubes, and disaggregated in an ultrasonic bath. The soils were wet-sieved with sieve mesh sizes spaced at one-phi intervals between 2000 μm and 4 μm [13,14]. Samples were viewed with stereo-binocular microscope (within a range of 10–50×), and color of clay fractions assessed with Munsell Color Charts [15]. Samples were embedded in resin, and thin sections of the sand particles were examined by polarizing light microscopy. To determine the volume fraction percentage of identifiable constituents in the thin sections, semi-quantitative analysis was carried out by point counting and size measurement. This was performed using a manual, mechanical stage with mm-graduated *x–y* stage translation controls for moving the thin section.

The whole of each soil sample was subjected to X-ray diffraction (XRD analysis) by means of a Philips PW 1800 diffractometer, with radiation Cu-Kα generated at 40 kV and 40 mA [16,17]. Each of the XRD analysis charts was drawn within an angular value range of 5–80°, at a step size of 0.01, and at a time per step of 0.9 s (Fig. 1).

2.3. DNA extraction, next generation sequencing and bioinformatic analysis

Samples were extracted and analyzed following the protocol proposed by [5] slightly modified. For the extraction, after the mixing step using the phosphate buffer, two aliquots of 1400 μL were recovered and extracted separately. The DNA of the two separate extractions was recovered in 50 μL and pooled together. The DNA extraction and its preparation before the amplification were done in a dedicated room.

DNA amplifications were carried out in a final volume of 25 μL, using 3 μL of the diluted DNA extract. The DNA amplification per samples was carried out in duplicate. The amplification mixture contained 1 U of AmpliTaq_Gold DNA Polymerase (Applied Biosystems, Foster City, CA, USA), 10 mM Tris–HCl, 50 mM KCl, 2 mM of $MgCl_2$, 0.2 mM of each dNTP, 0.25 μM of each primer and 0.005 mg of bovine serum albumin (BSA; Roche Diagnostic, Basel, Switzerland). The mixture was denatured at 95 °C for 10 min, followed by 45 cycles of 30 s at 95 °C, 30 s at T_m and 1 min at 72 °C. The T_m was 45–50 °C for eukaryota and 61.5 °C for bacteria [18]. All the primers were modified by the addition of specific 7 bp tags on the 5′ end to allow the assignment of sequence reads to the relevant sample [19]. All the PCR products from the different samples were first titrated using capillary electrophoresis (QIAxcel, Qiagen GmbH, Hilden, Germany) and then mixed together, in equimolar concentrations. This mix underwent sequencing using MiSeq sequencer (Illumina). Negative controls were analyzed at each step of the protocol in order to control the purity of the reagents and to detect potential cross contaminations during the experiment. The sequence reads were analyzed using the OBITools software (http://www.grenoble.prabi.fr/trac/OBITools), as described in Taberlet et al. [5].

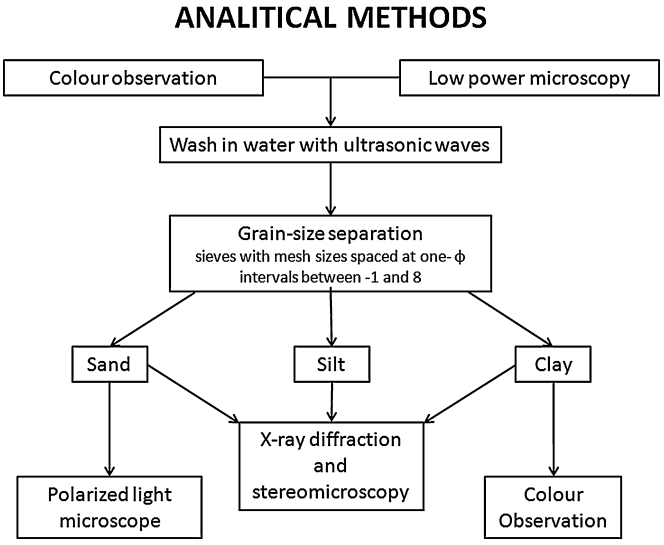

Fig. 1. Analytical methods applied for the soil samples. Modified from Di Maggio et al. [31].

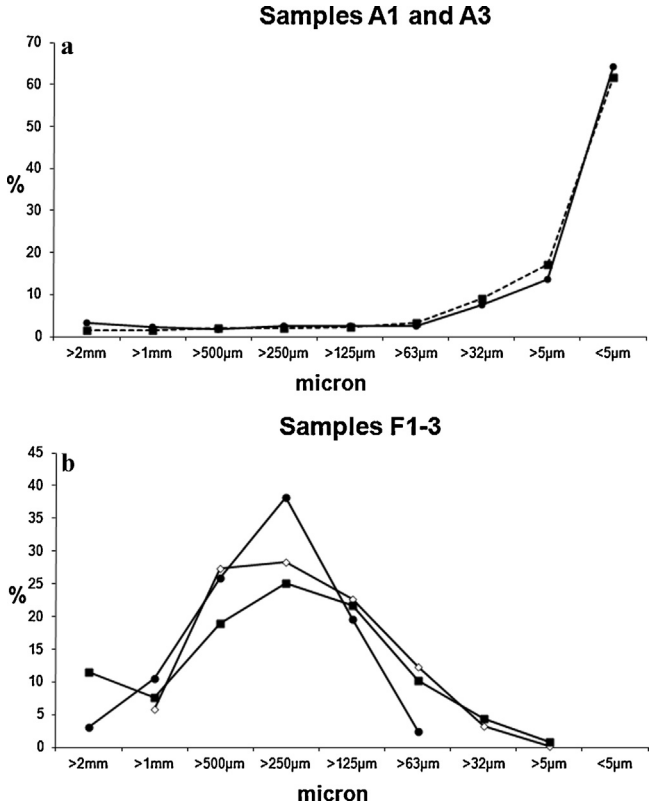

Fig. 2. Grain distribution of soils. (a) Sample A1 (circles) and sample A3 (squares). (b) Sample F1 (open diamonds), F2 (squares), F3 (circles).

Table 2
Color of the soil samples.

Sample	Color
A1	10YR 3/4 dark yellowish brown
A2	2,5Y 3/2 dusky red
A3	10YR 4/3 brown
F3	10YR 2/1 black
F2	10YR 7/2 light brownish gray
F1	10YR 5/2 grayish brown

Table 3
Crystalline phases in soils.

Sample	Crystalline phases
A1	Kaolinite, quartz, feldspar
A2	Quartz, calcite, plagioclase
A3	Kaolinite, quartz, feldspar
F1	Quartz, feldspar, augite, magnesian calcite
F2	Quartz, feldspar, augite, magnesian calcite
F3	Feldspar, augite, magnesian calcite

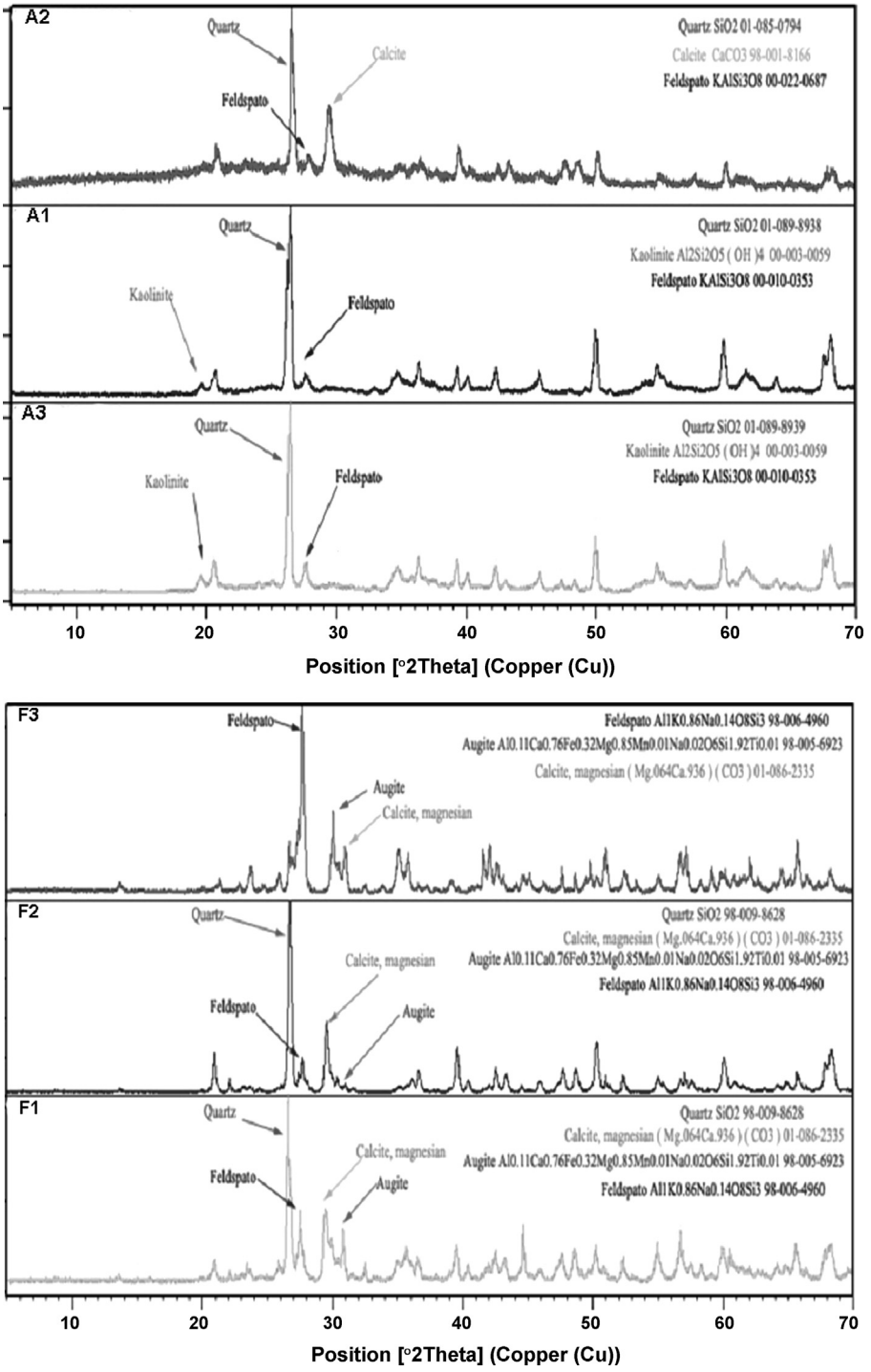

Fig. 3. XRD spectra of the soil A1–3 (a) and F1–3 (b).

Table 4
Minerals and rock fragments in sample F1–3 and their abundance.

	F3	F2	F1
Minerals			
Quartz	R	R	R
Sanidine	P	P	P
Microcline		P	
Plagioclase	R	R	R
Augite	A	A	A
Ortopyroxene	R		
Calcite	R	P	P
Leucite	R	R	R
Biotite	R		
Muscovite		R	
Zircone		R	
Iron oxide	P	P	P
Rocks			
Leucitite		R	R
Tuff		R	R
Tephrite	R	R	R
Quartzites	R	R	R
Quartz–arenite	R	P	P
Limestone		A	P

A, abundant; P, present; R, rare.

Table 5
Minerals and rock fragments in samples A1 and A3.

	A3	A1
Minerals		
Quartz	A	A
Sanidine	P	P
Microcline	R	R
Plagioclase	P	P
Calcite	R	R
Dolomite	R	R
Serpentine	R	R
Muscovite	R	R
Iron oxide	R	R
Rocks		
Quartz–arenite	P	P
Lithic arenite	R	R
Micaschists	R	R
Phyllade	R	R
Quartzites	A	A
Flint	R	R

A, abundant; P, present; R, rare.

3. Results and discussion

3.1. Grain size analysis

The two sampling locations are environments rather different from a point of view of the geological and pedological characteristics. For this reason the two sample sets are analyzed separately.

Samples A1 and A3 show a very similar grain size distribution and their texture is mainly clayey (Fig. 2a) (sample A2 was not included in the comparison due to the fact that was clearly different, characterized by particles with diameter <63 μm). The other three soils have a sandy texture but they show substantial differences: the sample F3 mainly contains the sand fraction between 1 mm and 125 μm, and totally lacks silt and clay fraction; although the samples F1 and F2 have more similar distribution they differ for the presence of gravel in the second one (particles with diameter greater than 2 mm) and for the different distributions of sand fraction with diameter greater than 125 μm (Fig. 2b).

3.2. Color

Each soil sample is characterized by a different color pattern. In Table 2 number references of colors and the relative qualitative terms are shown. The difference between A3 (Soil not fertilized) and A1 (soil naturally fertilized) was expected, since the natural fertilizer shifts dyes toward dark colors.

3.3. X-ray diffraction

The X-ray diffraction reveals the main crystalline phases in soil samples. Soils A1–3 share the same crystalline phases as shown in Table 3. Sample A2 shows a reduced intensity of signal, due to the exiguous mineralogical component. Samples A1 and A3 show an identical crystalline signature. Similarly, F1 and F2 cannot be distinguished by X-ray diffraction (Fig. 3).

3.4. Polarized microscopy

The observation under a polarizing microscope allows the detailed identification of the minerals and rock fragments in the

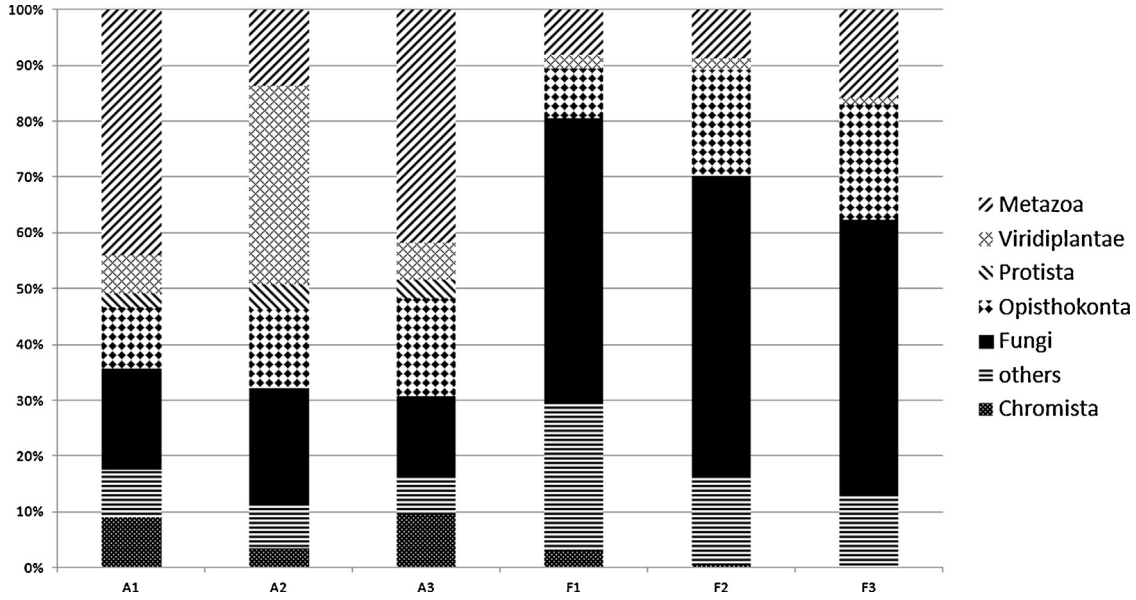

Fig. 4. Proportion of DNA sequences of different taxa found in the six soil samples.

soil samples and the determination of the abundances, by semi-quantitative analysis. The collected information can be used to better discriminate sample A1 and A3 (Table 4) and samples F1–3 (Table 5). The analysis is not performed on sample A2, due to the very small size of particles.

3.5. NGS

All soil samples are used in next generation sequencing with the metabarcoding approach. Three matrices of data, Bacteria, Eukaryota and Plants, are obtained for each sample and used in comparative analysis.

Fig. 4 shows the distribution of eukaryotic DNA sequences found within samples. The data are classified into different taxonomical groups of the domain. At first sight, the presence of two patterns is obvious: one characterizing the farm samples, and the other one characterizing the lake samples. In addition in the soil labeled A2 one third of the sequences identified belong to the group of Viridiplantae, a group representing less of 10% in samples A1 and A3. This result is in line with the origin of the sample, a kind of organic fertilizer containing manure of herbivores. The Viridiplantae component is not so abundant on sample A1, probably due to a dilution effect of the fertilizer that normally represents a small fraction of a soil. Samples F1–3 show a pattern with the general predominance of fungi over the other taxonomical groups.

The principal component analysis performed on the data grouped in Viridiplantae, Eukaryota and bacteria confirms the pattern separation between the six samples (Fig. 5). In particular, the analysis of all prokaryotic sequences allows a good discrimination between these soils, with the exception of samples A1 and A2. It is possible that the natural fertilizer has contributed largely to the remodeling of bacteria population in the soil with the introduction of many species. This is in agreement with previous observation reporting that bacterial abundance is significantly greater in manured soil than in untreated ones [20]. The biological signature of samples F1–3 looks very similar when considering the sequences of all eukaryotic taxa. This uniformity disappears when the analyses is restricted only to the group of Viridiplantae. In particular, sample F3 on a total of 81,262 DNA shows 78,427 sequences belonging to taxa not represented in F1 and F2 (see Table 6). These taxa describe a woodland close to a lake or a river, an environment full of organisms, such as aquatic plants (Ceratophyllum), ferns (Dryopteridaceae), oaks (Quercus), water-milfoil (Myriophyllum), while are completely devoid of elements, such as plants more diffused in farms, country landscapes, and houses (like for example Geranium). This description corresponds to the sampling area where the sand has been collected.

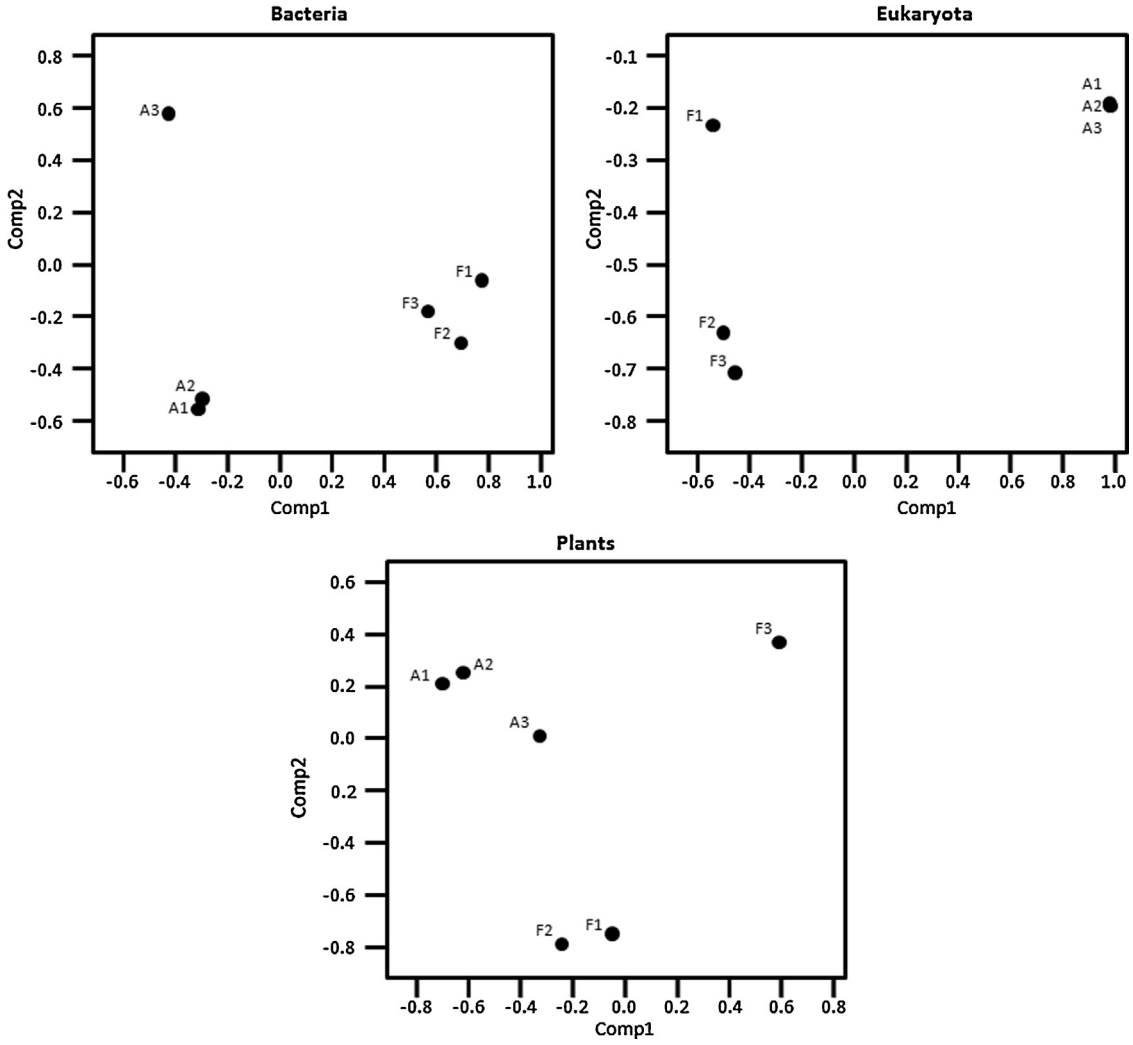

Fig. 5. Principal component analysis performed on the data grouped in Viridiplantae, Eukaryota and bacteria.

Table 6

Taxon	F1	F2	F3
Aizoaceae	44		
Chenopodium		229	
Apiaceae		106	
Araliaceae	63		2124
Asteraceae	102	1385	
Calendula	17	57	
Alnus			2956
Capsella rubella	178	96	
Caryophyllaceae	53		
Polycarpon tetraphyllum	848		
Ceratophyllum demersum			601
Convolvulaceae	128	71	
Cupressaceae	236	92	
Dryopteridaceae			825
Euphorbia maculata	10	69	
Medicago sativa		74	
Trifolieae		10	
Robinia	4347	34	
Trifolium		10	
Trifolium squarrosum		13	
Quercus	99	61	597
Geranium		20	
Myriophyllum			73,240
Clinopodium		15	
Lamiaceae		28	
Abutilon theophrasti	31	50	
Cedrus	32,686	26,905	
Pinus		61	
Plantago	26		
Platanus occidentalis			71
Avena	63	559	
Bromus		124	
Dactylis glomerata		44	
PACMAD clade	45	42	
Pooideae	49	53	
Triticeae		66	
Paspalum	73		
Polygonum	147	2106	
Rumex		24	
Potamogeton			114
Maleae		10	
Rosoideae	184	79	
Saliceae			734
Ulmaceae	26	79	
Vitaceae		216	
	39,455	32,788	81,262

4. Conclusions

The identification of the source of a specific soil sample is a crucial step in forensic investigations. Geological analyses are useful tools, but in some specific situation they are not discriminative for samples collected where the landscape geology and mineralogy are relatively homogeneous and very similar over large areas. This is for example the case described in the present work, with samples A1 and A3 that can be differentiate only for color tone.

On the other side, the analysis of the biological component of environmental matrices can give a huge quantity of information related to the activity and biodiversity of a specific soil. This kind of investigation has been for a long time performed by morphological classification of the living being present in the collected samples. This procedure is time consuming and requires personnel specialized in several biological fields like palynology, dendrochronology, micology, limnology, systematics, ecology, protistology, entomology, microbiology, etc. [21–23].

With the rapid diffusion of molecular biology the approach for taxonomical classification shifted from morphology (phenotype) to nucleic acids (genotype). Protocols based on polymerase chain reaction (PCR) amplification were proposed as useful tools for the identification of a specific genetic signature for each sample. Specific forensic applications for human body fluids identification have been developed starting from microflora DNA (mfDNA) extraction [24,25]. In addition, terminal restriction fragment length polymorphism (t-RFLP) analysis has been suggested in microbial DNA profiling for forensic comparison of soil [26,27–30]. While these protocols are very useful in the identification of a the matrix fingerprint leading to an easy and rapid comparison of more samples, they are not informative on the true biodiversity and quality of the living being present in the specific environment. In other words, they do not allow the identification of the species present at the collection site.

Rapid advances in next generation sequencing (NGS) technology and the strong reduction of the cost of sequencing are opening new opportunity also in forensic sciences [28]. In particular the DNA barcoding is already changing entomological and botanical analysis [29,30]. The present work represents a first attempt to apply the DNA metabarcoding to typical soil samples collected in forensic application. The approach allows to infer the species composition of an environmental sample, giving a true-color picture to the scientist. The data collected between the taxonomical group of plants has clearly depicted the environment of F3, suggesting a woodland close to fresh water. At the same time, sample F2 was easily speculated as collected in a country landscape typical of Central Italy. But the analysis of also other eukaryota and of prokaryota allowed the identification of three sample collected from a farm: in particular the microbiological component was useful in discriminating soils A1 and A3, geologically indistinguishable, but characterized by differences in manuring.

In conclusion NGS and DNA barcoding are promising tools, able to rapidly extrapolate environmental data from soils collected for forensic investigations.

Acknowledgment

A special thank to Prof. Alessio Valentini (MDD University spin off) for kind collaboration.

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