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MICROBIAL PROFILING OF SOIL: A NOVEL TOOL IN FORENSIC INVESTIGATIONS

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ABSTRACT

Soil is one of the most common forms of trace evidence in forensic investigations. Traditional forensic analyses rely on physical and chemical soil characteristics, which often lack sufficient discriminatory power in complex environments. Advances in molecular biology and high-throughput sequencing now enable microbial profiling of soil, revealing unique microbial community structures that vary spatially and temporally. This review examines methodological approaches for forensic soil microbial profiling, including sampling, preservation, DNA extraction, marker selection, and sequencing strategies (metagenomics and next-generation sequencing). Applications in forensic science are explored, including crime scene association, geographic provenance determination, activity reconstruction, and integration with other trace evidence. Limitations, such as environmental variability, sample heterogeneity, and lack of standardized databases, are discussed alongside future directions, including multi-omics integration, AI and machine learning applications, and portable field-based sequencing technologies. Overall, microbial profiling of soil represents a novel and powerful tool to complement traditional forensic methods, providing biological signatures that enhance the evidentiary value of soil in criminal investigations.

Keywords: Soil microbiome, Microbial profiling, DNA sequencing, Crime scene investigation, Metagenomics, Trace evidence

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INTRODUCTION

Soil is one of the most commonly encountered forms of trace evidence in forensic investigations due to its omni-presence in outdoor environments and its ability to adhere to objects, footwear, clothing, and tools that might be linked to a crime scene [**1**]. Traditional forensic soil analyses have predominantly relied on physical and chemical properties such as color, texture, mineral composition, and organic content. While these methods can be useful, they often lack sufficient discriminatory power, especially when comparing soils from geographically similar or adjacent locations [**2**]. This limitation has driven the scientific community to explore biological dimensions of soil, most notably the composition and structure of soil microbial communities [**3**]. Advances in molecular biology have enabled researchers to characterize the immense diversity of microorganisms in soil collectively termed the soil microbiome which includes bacteria, archaea, fungi, and other microscopic life. These microbial communities are shaped by environmental factors such as climate, vegetation, geochemistry, and land use, resulting in distinct community patterns across landscapes [**4**]. Because microbial community structures vary spatially and are sensitive to environmental influences, they have emerged as potential biological signatures that can improve the discriminative resolution of forensic soil comparisons beyond traditional methods .In forensic applications, microbial profiling typically involves sequencing conserved genetic markers such as the 16S ribosomal RNA gene for bacteria and the internal transcribed spacer (ITS) region for fungi to generate community profiles that can be statistically compared between samples from known and

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questioned sources [**5**]. Empirical studies indicate that microbial community profiles can successfully differentiate soils collected from distinct locations, even when only small amounts of soil are available suggesting significant forensic potential. Despite this promise, challenges such as environmental variability, sampling artifacts, and the absence of standardized databases or protocols limit practical implementation in forensic casework, and ongoing research aims to address these obstacles [**6**].

Soil as Biological Evidence

The conceptual framework for using soil microbial profiles as forensic evidence is rooted in the ecological principle of microbial biogeography, which suggests that microbial community composition reflects the local environment and can vary predictably across spatial gradients [**7**]. Soil is not a static physical substrate but a dynamic ecosystem inhabited by an extraordinarily rich diversity of microorganisms on the order of 10^7 to 10^9 microbial cells per gram of soil, which respond to soil chemistry, moisture, vegetation, and climatic conditions. This diversity and spatial variability are central to the idea that soil microbiomes can serve as biological fingerprints indicative of soil origin [**8**]. Microbial profiling approaches leverage high to examine the composition of microbial communities and generate profiles that can be statistically analyzed for similarity or dissimilarity. Methods such as beta diversity metrics (differences between communities) allow researchers to quantify the degree of microbial community variation between soil samples, potentially linking evidence samples to specific locations [**7**]. Early experimental studies have shown that combining genetic profiling approaches such as ribosomal intergenic spacer analysis (RISA) and 16S rRNA sequencing can differentiate between soil samples and even detect mixed soil origins [**9**].

METHODOLOGICAL APPROACHES IN SOIL MICROBIAL PROFILING

Microbial profiling of soil for forensic purposes requires a sequence of carefully designed procedures that together produce reliable community profiles from complex environmental matrices. These procedures must account for the heterogeneous nature of soil, contamination risks, inhibitory compounds, and sequencing biases [**10**]. The following subsections describe the current best practice and scientific evidence in sampling and preservation, DNA extraction and marker selection, and sequencing approaches including metagenomics.

Sampling and Preservation

Soil is inherently variable within very small spatial scales, and microbial communities can change significantly over distances as little as a few centimeters or with depth changes. Therefore, sampling design must strive to capture representative community structure while minimizing contamination or degradation [**11**]. In forensic contexts, soil samples are often collected using sterile tools (e.g spatulas, cores) and placed into clean, sealed tubes, with field blanks to monitor contamination. Sub samples collected from the same site help quantify microbes. Preservation is equally critical, because microbial DNA can degrade rapidly after collection due to enzymatic activity or temperature fluctuations. Ideally, samples are kept cold or frozen (e.g −20 °C) soon after field collection to slow microbial metabolism and preserve nucleic acids until laboratory processing [**12**]. Preservation buffers or immediate freezing has been shown to influence downstream DNA extraction efficiency and community profiles; delays or improper storage can alter relative abundances, especially for rare taxa. Because forensic analyses often deal with trace amounts of soil, best practice involves rapid transport under cold conditions and clear documentation of storage methods [**13-15**].

DNA Extraction and Marker Selection

The DNA extraction step from soil is one of the most technically demanding in microbial profiling because soil contains numerous inhibitors, notably humic acids, which can co-effective extraction protocols must maximize DNA yield while removing inhibitors and preserving a broad representation of microbial taxa [**16**]. Currently, many forensic and ecological studies use commercial soil DNA extraction kits optimized for environmental samples, which often rely on physical disruption (bead beating) and chemical lysis combined with inhibitor removal steps. The choice of extraction method influences both the quantity and quality of DNA recovered. Significant differences in microbial richness can result from different extraction strategies, although later steps such as sequencing depth often have a stronger effect on observed diversity than extraction method alone. Regardless, protocols must be validated for specific soil types to ensure reproducibility [**17-20**]. Once DNA is extracted, the selection of appropriate genetic markers determines which components of the soil microbiome can be profiled. For bacteria, the 16S ribosomal RNA (rRNA) gene remains the most widely used target because of its presence in all bacteria and the existence of extensive reference databases. For fungal communities, the internal transcribed spacer (ITS) region is typically targeted, while eukaryotic groups may be profiled using 18S rRNA markers or chloroplast regions for plant DNA [**21, 22**]. Marker selection influences both taxonomic resolution and discriminatory power, and some studies recommend including multiple markers to capture a broader picture of soil biodiversity for forensic comparisons. The careful choice of primers and amplification regions is essential, since mismatches can bias representation of community members [**23, 24**].

SEQUENCING APPROACHES

After DNA extraction and marker amplification (when applicable), sequencing is used to generate the data that form the basis of forensic comparisons. Two primary strategies are used in soil microbiome research: metagenomics and NGS.

Metagenomics

Metagenomics refers to sequencing all DNA present in a soil sample without prior marker amplification, also called shotgun sequencing. This approach enables comprehensive capture of microbial taxa including bacteria, archaea, fungi, viruses, and even trace eukaryotic DNA and may provide both taxonomic and functional insights into the soil community. Because metagenomics does not rely on PCR amplicons, it avoids some of the bases associated with marker gene amplification and can detect organisms that may be missed by targeted approaches [**25, 26**]. However, metagenomic sequencing requires higher DNA input, greater sequencing depth, and more computational resources to assemble and interpret the large volume of data generated. In forensic applications, where only small amounts of soil may be available, these requirements can limit the feasibility of full metagenomic workflows unless pre-metagenomics remains a powerful approach because it can reveal not just who is present in the community but also what genetic capabilities those organisms possess, potentially offering additional discriminative features [**27-29**].

Next Generation Sequencing

Next-amplicon sequencing of marker genes such as 16S rRNA for bacteria or ITS for fungi, which is sometimes called metabarcoding. These reads are compared against reference databases to determine the presence and relative abundance of taxa in the soil sample. An early feasibility study demonstrated that profiles generated by sequencing the 16S rRNA gene via NGS could classify soil samples to their site of origin with high accuracy (95.4 %) when combined with multivariate statistical techniques, suggesting forensic utility of this approach [National Institute of Justice report [**22, 30, 31**]. NGS platforms such as Illumina MiSeq are now commonly used because they provide high coverage, enabling detection of rare taxa and fine. Depth of sequencing (i.e., total number of reads) has been shown to influence observed bacterial richness and diversity metrics more strongly than DNA extraction method in some comparison studies, emphasizing the importance of adequate sequencing depth in soil profiling analyses (e.g16S V3–V4 region studies) [**22, 32**]. NGS based on amplicon sequencing is currently the most accessible and widely applied method in soil microbial forensics due to well-metagenomics.

FORENSIC APPLICATIONS OF SOIL MICROBIAL PROFILING

Microbial profiling of soil has shown increasing potential to complement traditional forensic methods by providing biological signatures unique to specific soil environments [**33**]. The applications can be grouped into four main categories: crime scene association, geographic provenance determination, activity reconstruction, and integration with other trace evidence types.

Crime Scene Association

Linking a soil sample from an evidentiary item to a particular location is a primary forensic goal. Microbial community profiles can serve as biological fingerprints to assess similarity between soil collected at a crime scene and soil adhered to objects, clothing, or vehicles**[7,34]** .For example, studies using 16S rRNA sequencing demonstrated that even small soil samples (<0.5 g) retained enough microbial diversity to differentiate sites separated by as little as 100 meters .Statistical analyses such as principal coordinate analysis (PCoA) and UniFrac distances allow researchers to quantify similarity and assess the probability that questioned and reference samples originate from the same location [**5, 35**]. In practical terms, soil microbial profiling has been used in mock crime scene studies to test the feasibility of matching soils on shoes to the location where the shoes were worn. Results indicate high discriminatory power, even in urban and semi-rural environments, demonstrating that microbial data can support associative evidence when traditional physical soil characteristics alone may be insufficient [**7, 36**].

Geographic Provenance

Beyond linking evidence to a specific scene, microbial profiling can help determine the geographic origin of a soil sample. Microbial biogeography principles indicate that environmental variables such as soil type, vegetation, and climate produce location-specific microbial communities. Using high-throughput sequencing, researchers have successfully classified soils to regions, even across broad geographic scales, with accuracies exceeding 80% in validation studies [**37, 38**]. Machine learning algorithms like random forest classifiers have been particularly effective for geographic provenance prediction, integrating microbial community composition data to predict the probable source region of an unknown soil sample. The ability to predict provenance has implications for criminal investigations involving illicit transport of soil, illegal dumping, or locating clandestine sites. While still an emerging application, it provides a quantitative framework for linking evidence to environmental sources with a measurable degree of confidence [**39, 40**].

Activity Reconstruction

Microbial profiles can also provide insights into past activity at a crime scene, such as identifying whether objects were moved across different environments or whether soil deposition occurred recently or historically. For example, differences in microbial taxa that respond rapidly to environmental changes, like moisture or organic matter input, can indicate temporal patterns. Additionally, the presence of human-associated microbial taxa or plant DNA in soil can help reconstruct human activity patterns or distinguish anthropogenic influence from natural soil background [**36, 38**]. These applications are enhanced when microbial profiles are integrated with environmental metadata, such as soil chemistry, land use, or vegetation cover, which provide contextual information to interpret microbial data accurately. Combining these sources allows investigators to reconstruct sequences of activity, such as tracking movement of suspects or objects between different microenvironments [**7, 41**].

Integration with Trace Evidence

Soil rarely exists in isolation in forensic contexts; it often co-occurs with other trace evidence like pollen, plant fragments, dust or human derived DNA. Microbial profiling can complement these analyses by providing additional discriminatory features, increasing the evidentiary value of combined datasets. Multi-modal analyses, integrating soil microbiome, pollen DNA, and chemical fingerprints, have been shown to improve site attribution and reduce false positives when compared to single-method analyses. This integrated approach aligns with the modern forensic paradigm of evidence convergence, where multiple independent lines of evidence are used to strengthen conclusions, especially in cases with limited or degraded samples. Microbial data can also serve as cross-validation, confirming associations suggested by physical or chemical evidence, and improving robustness in forensic reporting [**6, 34, 37**].

LIMITATIONS AND CHALLENGES

Despite its promise, microbial profiling of soil for forensic investigations faces several methodological, environmental, and interpretive limitations that must be considered for reliable application.

Environmental Variability

Soil microbial communities are highly dynamic, influenced by seasonal changes, weather, moisture, pH, vegetation cover, and anthropogenic activity. These factors introduce temporal variability, which can complicate the comparison between a questioned sample and a reference collected at a different time. For example, rainfall or agricultural activity can alter community composition significantly, potentially leading to false negatives if reference soils are outdated [**42, 43**].

Sample Heterogeneity and Small Quantities

Soil is inherently heterogeneous, with microbial composition varying across centimeters and with depth. Trace quantities of soil often recovered in forensic cases may not capture this variability, leading to incomplete representation of the source community. This limitation is especially important when dealing with minimal soil transferred to clothing, shoes, or tools [**34**].

Technical Limitations

DNA extraction efficiency can vary between soil types due to humic acids and other PCR inhibitors, potentially biasing microbial profiles. Similarly, marker choice and amplification strategies (16S rRNA, ITS, etc.) influence detection of rare taxa, which may be critical for discrimination .Sequencing depth and platform choice further affect the observed diversity and reproducibility of microbial profiles [**22, 44**].

Database and Standardization Gaps

Unlike human DNA forensics, forensic soil microbiome research lacks standardized reference databases, which complicates reproducibility and comparison across studies. Currently, most studies rely on regionally collected reference soils, limiting generalizability. Additionally, standardized protocols for sampling, storage, DNA extraction, and bioinformatic analysis remain in development, which could impede routine forensic adoption [**34, 37, 45**].

FUTURE DIRECTIONS

Ongoing advances in technology, bioinformatics, and environmental microbiology promise to enhance the forensic utility of soil microbial profiling. Key areas for development include multi-omics integration, AI-driven data analysis, database creation, and field-based technologies.

Multi-omics Integration

Combining multiple molecular layers including metagenomics, meta-transcriptomics, metabolomics, and proteomics can provide a multi-dimensional view of soil communities. Multi-omics approaches can detect not only microbial composition but also functional potential and activity patterns, which may improve discriminatory power for both crime scene association and geographic provenance prediction. Integrating chemical profiles with microbial data may also strengthen trace evidence interpretation [**6, 45, 46**].

AI and Machine Learning

Machine learning (ML) and AI algorithms are increasingly applied to pattern recognition in complex microbial datasets. Random forests, support vector machines, and neural networks have shown promise in predicting soil origin and classifying samples with high accuracy. AI can also integrate environmental metadata, microbial abundance, and functional information to improve forensic conclusions. Future research should focus on developing robust, validated AI pipelines capable

of handling the noisy and variable data characteristic of soil microbiomes [**47, 48**].

Development of Databases

The creation of centralized, curated microbial soil databases is critical for forensic application. Such databases would enable the comparison of questioned soil samples to reference profiles across broader geographic regions, facilitating location inference and provenance prediction. Databases should include raw sequencing data, metadata on soil type, land use, seasonality, and extraction methods to maximize usability. Global initiatives like the Earth Microbiome Project provide a foundation, but forensic-specific, validated datasets are still required [**6, 38**].

Field Based Technologies

Current microbial profiling is laboratory-intensive, but field-deployable technologies are emerging. Portable sequencing devices such as Oxford Nanopore allow for on-site DNA sequencing, enabling near real-time microbial profiling. Such tools can reduce sample degradation risks, allow rapid decision-making during investigations, and facilitate rapid presumptive matching of soil evidence. Field-based technologies will likely be combined with cloud-based bioinformatics pipelines for on-site analyses with validated forensic reliability [**49, 51**].

CONCLUSION

Microbial profiling of soil offers unprecedented opportunities for forensic investigations, providing a biological dimension to trace evidence analysis. The soil microbiome exhibits spatially distinct and reproducible community patterns that can link evidence to specific locations, determine geographic provenance, and reconstruct activity patterns at crime scenes. Advances in high-throughput sequencing, metagenomics, and next-generation sequencing have enabled detailed characterization of these microbial communities, while integration with statistical and machine learning approaches enhances interpretation. Despite its promise, limitations remain, including environmental variability, soil heterogeneity, low biomass in forensic samples, and the absence of standardized databases and protocols. Addressing these challenges through method validation, multi-omics integration, AI-driven analysis, and field-deployable sequencing technologies will be critical to realizing the full forensic potential of soil microbial profiling. Future research should focus on creating robust, reproducible, and globally applicable frameworks to integrate microbial evidence into routine forensic practice. As the field advances, soil microbial profiling is poised to become a powerful complement to traditional forensic methods, strengthening evidentiary interpretation and supporting the pursuit of justice.

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