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Molecular Approaches in Soil Microbial Analysis: Forensic Perspective

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The growing field of soil microbial forensics provides the legal answer to how microorganisms play a crucial role in criminal investigations. It is an advanced cross-disciplinary science capable of offering significant physical evidence with considerable interest in criminal investigations, environmental crime, and counterterrorism. Microbial forensics of soil consists of different techniques to identify and evaluate microbial abundance, their complexity, and their interaction with soil and surrounding objects. The present review highlights various microbial analysis techniques such as Terminal restriction fragment length polymorphism, Temperature/Denaturing Gradient Gel Electrophoresis, Amplified Ribosomal DNA Restriction Analysis, Length Heterogeneity PCR, Phospholipid-derived fatty acids, Fluorescence in situ hybridization, Stable-isotope probing and metagenomics using next-generation sequencing. This article also summarises the challenges faced in soil microbial forensics, various statistical approaches, reference databases commonly used in forensic soil studies, and different methodological approaches used in forensic laboratories. Literature was studied using various online bibliographic databases like Google Scholar, Web of Science, Pub Med, Scopus, and several other search engines. Conclusive evidence generated by this paper signifies the importance of soil microbes to assist forensic scientists and researchers in selecting adequate methods to differentiate soil samples. The present comparative study concluded that targeted molecular analysis of microbes as a forensic soil typing tool has a lot of potential and should be investigated further.

Keywords: DNA; Forensic; Metagenomics; Microbes; Soil.

Forensic Pedology is the science that uses soil information to solve officially approved questions, problems, or hypotheses [1]. Soil is more likely to transfer and retained as it is typically found on clothes, footwear, and vehicles following a crime. It could be a potent method of contact as physical evidence, especially when criminals tend to forget soil evidence while tempering other pieces of evidence [2].

During the early 1990s, soil or sediment as physical evidence gained great attention [3-5]. The soil and sediment analysis was used in forensic investigations by Prof. Ehrenberg in the 19th century to solve the missing silver coin mystery and effectively settle crime [2]. In 1887, Sir Arthur Connan Doyle published many fictional novels involving soil comparison to solve the murder by Sherlock Holmes. In 1904, George Popp, a forensic

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scientist, skilfully studied soil, mineral contents, dirt, and debris from clothing to determine criminal cases [6]. The soil analysis is also effectively utilised as evidence in legal proceedings in Australia, the United Kingdom, and the USA [7].

Soil forensics is an emerging discipline that needs the standardization of novel methods to construct a database so that soil can be utilised as physical evidence in criminal investigations. Forensic scientists frequently employ a systematic methodology for screening purposes based on soil colour, soil texture, consistency, particle size, pH, and density [8]. After this, spectroscopic techniques were used for the elemental analysis of the soil [9-12]. After the initial screening and spectroscopic analysis of soil, more robust and statistically reliable methods are required for microbial profiling. This research presents a more detailed picture of microbial methods, including DNA fingerprinting methods and metagenomics, to identify a specific microbial community in the soil.

Soil microbial forensics is the science that can define how microbial communities can be used in criminal enquiries [13]. Microbes are clearly a substantial soil constituent, with one tablespoon of soil having roughly 10^9 microorganisms. Merely 1% of soil microbes can be cultured by traditional methods, which makes soil one of the most complex ecosystems requiring molecular techniques to assess them [14].

Soil microbial communities

The (micro) bio-composition of soil significantly enlightens the new field of soil microbial forensics [15]. Microbes can convey information about the specific ecological environment that sustains them. The authors claimed that soil might be distinct due to the presence of specialised microbes present in the soil [16].

Each community may be present at a given density based on the soil type. Physical, chemical, biological, environmental, and anthropogenic factors can significantly impact the variety and diversity of soil microorganisms, which are unique for the particular soil type to be sampled [17].

Materials and methodology

In this review, attempts have been made to document the importance of soil microbes to assist forensic scientists and researchers in selecting adequate methods to differentiate soil samples. Recent literature was cited by conducting a thorough search of electronic databases, such as PubMed, ScienceDirect, Web of Science, Scopus, and Google Scholar, by using appropriate/ specific combinations of words.

Molecular approach for the soil's microbial diversity

The two primary categories of approaches used to explore soil microbial diversity are molecular and biochemical methods. However, current molecular tools are gaining more attention for crime investigations than biochemical methods due to their precision, sensitivity, feasibility and early results [18]. In the present review, we emphasised the utilisation of molecular technologies for forensic investigations.

DNA-based analysis of soil using DNA fingerprinting techniques

The forensic community uses several analysis methods to generate soil DNA profiling, but none are specifically designed for forensic use. Therefore, the forensic community must pick an approach that best meets its specific needs. DNA fingerprinting techniques that evaluate fragment length variation comprise Terminal Restriction Fragment Length Polymorphism (T-RFLP) [19, 20], Denaturing Gradient Gel Electrophoresis (DGGE) [21, 22] and Temperature Gradient Gel Electrophoresis (TGGE) analysis, Amplified Ribosomal DNA Restriction Analysis (ARDRA) [23] and Length Heterogeneity- Polymerase Chain Reaction (LH-PCR) [18, 24].

Terminal Restriction Fragment Length Polymorphism (T-RFLP)

The T-RFLP approach, first introduced by Liu et al. [25] in 1997, has been acknowledged as a quick and effective way to create or monitor modified changes in the "structure and composition" of microbial communities [26]. This approach is based on various fragment length that provides a unique pattern (fingerprint) liable bio-composition of the species present in the sample. T-RFLP fingerprinting method could be used to generate DNA profiles from the small amount of soil used to differentiate the samples [19].

Macdonald et al. [27] explored the application of multiplex T-RFLP as a tool for soil microbes' comparison from a forensic point of view. This technique employed the combined benefits of multiple taxa with the T-RFLP approach, which offers a fast and cost-effective study of a microbial population at high-resolution. Macdonald et al. [28] investigated the ability of T-RFLP for microorganisms to discriminate between soils of different sites and exhibited clear differentiation, which may be helpful for location identification. Various forensic investigation of soil microbiota profiling has employed T-RFLP because it has high reproducibility and automatic nature [19, 29, 30]

T-RFLP is a valuable tool for the basic evaluation of soil microbes' population. Resolution and taxonomic identification are limited by the co-migration of many taxa during electrophoresis, resulting in displays as a single band [31]. Because of this, additional analysis through complex methods like next-generation sequencing, which has enough discriminatory power to identify soil microbes, is required for the sample that seems to possess the same T-RFLP profile but may not necessarily originate from a common source [32]. Another drawback of this approach is library dependency, which necessitates the design of a library database for bacteria, archaea, and fungi.

Denaturing gradient gel electrophoresis (DGGE) and temperature gradient gel electrophoresis (TGGE) analysis

DGGE and TGGE are gel-based methods, employing either chemical or temperature gradients that denature DNA samples while passing across an acrylamide gel. This method can be utilised with DNA and RNA samples and proteins but is less widely used. DGGE distinguishes genes of the same size based on their various denaturing potentials, which are defined by the arrangement of their base pairs.

A study published by Lerner et al. [21] described the use of DGGE to conduct criminal investigations and has enough discriminating potential to discriminate between variations in soil microbiota's temporal and spatial variability. In one example, a young woman's corpse was found near the river bank. After some days, the primary suspect was apprehended and claimed to be with the victim in the parking lot. After committing the crime, the suspect washed his clothes and shoes. There was no proof except a small amount of soil inside the suspect's shoes. DGGE method was conducted on the samples taken from the crime scene, alibi scene, the accused's house, and the surrounding areas. With the help of cluster analysis, banding characteristics were assessed. The samples gathered from the crime scene, and its environs were grouped, whereas those from the alibi scene and its surrounding could easily be differentiated from the crime scene. Furthermore, Sanachai et al.[33] elucidated that the origin of soil, obtained from the sole of a shoe, could be identified by a similarity comparison of soil bacterial 16S rDNA profiles separated by the DGGE method.

Amplified Ribosomal DNA Restriction Analysis (ARDRA)

This fingerprinting technique is a robust tool for bacterium identification [34] and for investigating bacterial diversity in soil microorganisms [35]. Horswell et al. [36] investigated a case of forensic soil identification in which they employed ARDRA to analyse 16S rDNA of soil bacteria by detecting the fluorescent labelled terminal fragment of RFLP (T-RFLP). In an investigation into a murder case in northern Italy, Concheri et al. [23] used the ARDRA method to compare or match the similarity of soil samples taken from the crime scene and the carpet of a suspect's car. The results showed that the soil found in the car matched the soil taken from the crime scene. The results of this research had a significant role in the court's decision. The ARDRA method was also used by Naknim et al. [37] to compare soil evidence from shoes collected from a mock crime scene with soil from the mock crime scene as well as irrelevant areas, and results showed that ARDRA is a reliable method for identifying the origin of soil by comparing soil bacterial community structure by clustering of 16S rDNA restriction profiles.

Length Heterogeneity- Polymerase Chain Reaction (LH-PCR)

Length Heterogeneity PCR (LH-PCR), a modified version of the PCR method, is used extensively in several microbiology fields and is gaining prominence in the field of soil microbial analysis. LH- PCR differentiates different microorganisms based on naturally occurring sequence length of DNA [24, 38]. Moreno et al. [18] compared the microbial metagenome profiles generated using LH-PCR analysis of 16S rRNA genes with Inductive Coupled Plasma- Optical Emission Spectroscopy (ICP-OES) analysis of 13 elements commonly found in soils. The findings revealed that microbial metagenome profiling

Table 1. Some case studies solved using soil microbial community profiling

Types of cases	Evidence	Country	Method of analysis	References
Murder case	Suspect's shoes	Israel	DGGE	21
Murder case	Car carpet and tyres	Italy	ICP-MS, ICP-OES and ARDRA	23
Mock case study	Shoes, shovel, car tyres	Australia	HTS and MIR spectroscopy	50
Mock crime scene	shoes	Thailand	ARDRA	37
Looting of a burial vault	A pair of green and brown boots	Spain	Colour, particle size distribution, elemental analysis, anion concentration, pH, rDNA 16S sequencing	51
Rape case	Shoes, shovel	Not specified	tRFLP profiling and pollen analysis	7
Mock crime scene (drug burial case)	Spade	Not specified	RISA and 16S rRNA gene sequencing with Illumina MiSeq	52
Missing and murder case	Socks	Arizona	Illumina MiSeq	53
Simulated crime scene	Shoe and sampling tool	China	HTS of 16S rRNA gene	54

was better than chemical characterisation could discriminate between various soil types and had a high reproducibility, proving a potential tool for soil comparisons in the criminal investigation.

This fingerprinting method helps predict geographical locations and provides an investigative tool that the suspect spent time at a specific site. A brilliant example of a study design for the prediction of location is provided by Damaso et al. [39]. The Authors studied soil DNA profiles using LH-PCR method to test the biogeographical patterns of soils to determine whether soil microbial community is spatially correlated with a geographic location or not. Moreover, the results found that soil microbial communities have unique patterns and are spatially auto-correlated.

Phospholipid Fatty Acids in Soils (PLFAs)

PLFAs are the key component present in the soil microbe cell membrane. PLF analysis is the strategic biotechnological tool that shows significant differences in cellular membrane compositions of 2 different microbial communities for forensic soil discrimination application purposes [40]. PLFA analysis collected from the various soil sample provides efficient structural information on the microbial community. PLFA also provide information about the pattern of fatty acids present and the total microbial biomass of the soil microbial community [41]. It is relatively inexpensive, reproducible, highly precise, lowest error rate and rapid methods employ certain advantages viz. community-level physiological profiling [42] over DNA-based (DNA fingerprinting, Electrophoresis) methods [43].

Fluorescence *in situ* hybridization (FISH)

Individual soil microbial cells can be simultaneously recognized, counted, localized and discriminate by using Fluorescence In Situ Hybridization (FISH) advanced analytical technique [44]. FISH is a non-fingerprinting and highly sensitive technique due to the specificity to work on the low amount of rRNA [45, 46]. It is a frequently used, strong and effective technique in differentiating the microbial profiles of several soil samples with related geological properties [47].

Stable Isotope Probing (SIP)

SIP is a biomedical technique used for the tracking of the movement of nutrients from isotopically tagged substrates to particular microbes in microbial communities. Stable-

Table 3. Advantages and disadvantages of various molecular-based methods used in studying soil microbial community

Approach	Molecular methods	Advantages	Disadvantages	References
DNA Fingerprint techniques	T-RFLP	• High sensitivity. • Effective at analysing the relationship of bacterial communities in diverse ecological samples • The capability of high throughput and microbial community quantification • Short run time.	• Overestimation of diversity due to incomplete restriction digestion. • Complex profiles make phylogenetic assignments very difficult. • Required a diverse range of restriction enzymes to explore microbial diversity.	68-70
	DGGE/TGGE	• For amplification and sequencing, bands of interest can be excised from the gel.	• Limited sensitivity. • Handling gels need experience. • DNA sequences of different bacterial species can exhibit the same separation as a result of equivalent GC contents.	71, 72
	ARDRA	• Fast, accurate and straightforward molecular tool to determine environmental population profile. • No special equipment is needed.	• Low discriminatory power as compared to other fingerprinting methods. • Needs multiple restrictions for adequate genotypic resolution. • Difficulty in locating a particular phylogenetic group within a community fingerprint.	73, 74
	LH-PCR	• Monitor bacterial populations in diverse settings. • Fast and allow simultaneous analyses of multiple and complex samples.	• Shows the high number of secondary peaks.	75
DNA sequencing techniques	454 Pyrosequencing	• Estimation of soil microbial diversity at a vast geographical scale. • The maximum number of unique sequences can be identified.	• Highly conserved primers are used to amplify hypervariable regions. • Expensive equipment. • High background signal after every cycle. • Difficulties in the sequencing of GC-rich templates.	76, 77
	Illumina (Mi Seq model)	• Simple, scalable and high yield. • Have a much faster turnaround time.	• Low throughput molecular techniques. • Expensive equipment.	78
	Ion Torrent	• Generate and read sequences from both ends of a fragment. • Low cost and fast run.	• High rate of sequencing errors.	77

Isotope Probing, or SIP, continues to be one of the most comprehensive methods for in situ microbial community analysis [48]. To reconstruct the metagenome-assembled genomes (MAGs) of the microorganisms that produce tagged proteins, proteomic SIP and targeted metagenomic binning were used [49]. Using proteomic SIP, active complex microbial communities from different soil samples collected from different places are functionally characterised in forensic prospectives.

Cases studies of soil microbial evidence

Around the world, several cases have been solved utilising soil evidence by using microbial DNA analysis techniques (Table 2).

Metagenomics

The term metagenomics was invented in 1998 by Handelsman et al. Metagenomics (also called environmental genomics, eco-genomics, or community genomics) is the study of genetic material derived from a diverse community of organisms that are used to provide taxonomic and functional profiles of soil microbes [55]. It is a molecular tool used to analyse DNA obtained from soil samples to study the soil microbes' community without obtaining pure culture. Metagenomics allows an understanding of the different characteristics of a sample, characterises microbes, and describes the functional roles

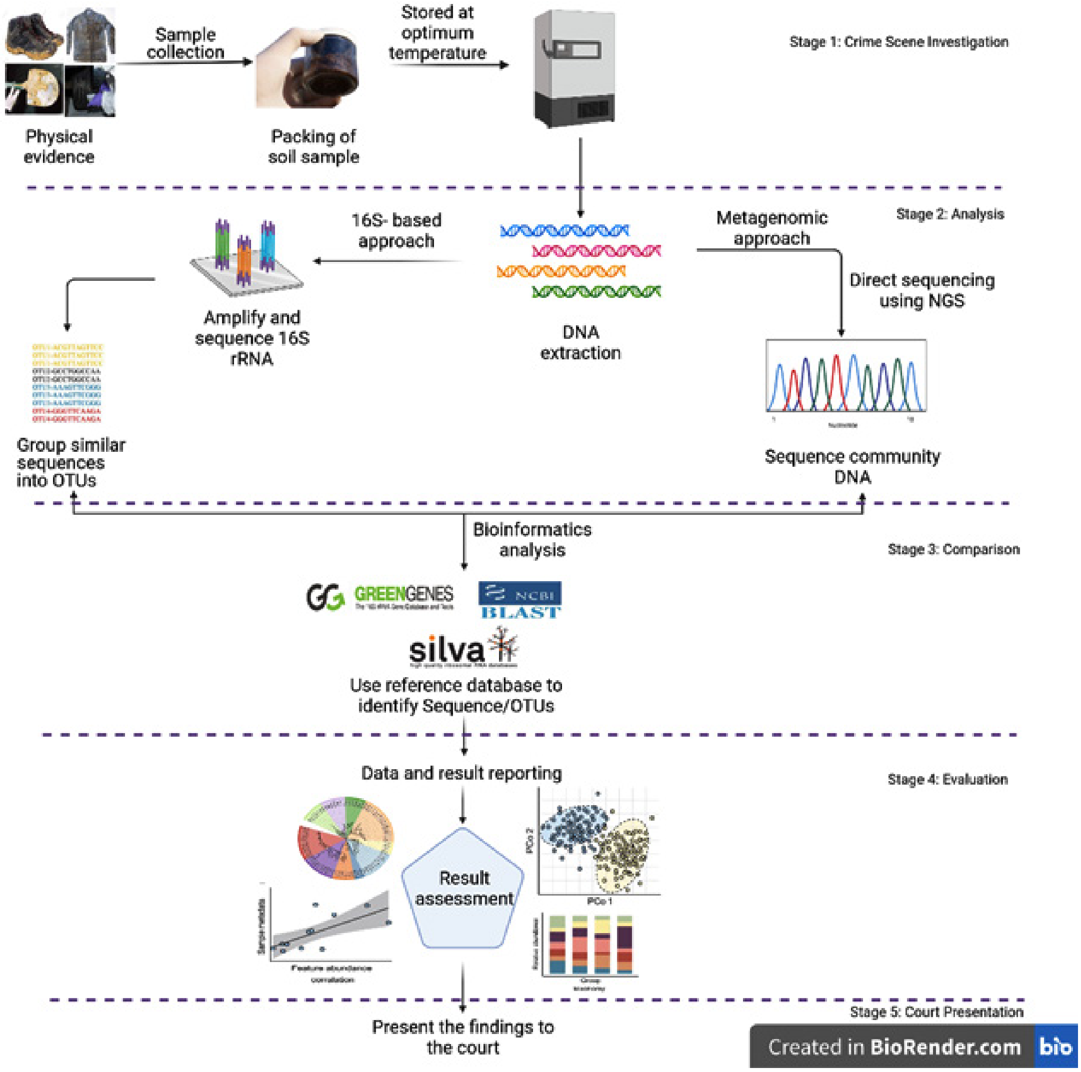

Fig. 1. Diagrammatic representation of microbial analysis of soil evidence from the crime scene to the court. (Although the author made the figure, the inspiration for it came from the source ref.[67])

of environmental soil microbes present in the samples. The use of metagenomics has steadily increased and provided new forensic identification opportunities [56, 57].

The primary aim of these techniques is to rebuild large-scale genomic data or functional processes of a selection of their genes. Several newer culture-independent metagenomics approaches do not use DNA, such as amplicon-based, whole metagenome-based, and functional-based metagenomic analysis.

16S and 18S rRNA are the most common genes sequenced and amplified in the soil microbe's community. Both genes offer valuable information about the diversity and abundance of bacteria and archaea (16S rRNA) and eukaryotes as fungi (18S rRNA). Above mentioned sequences are the most conserved and represent microbial genetic dissimilarity. Therefore, it indicates fundamental differences in phyla, genera, and species. This is the primary tool for identifying and characterising the soil microbial community, which may be valuable for soil microbial forensics in criminal investigations [58]. It is a reliable and cost-effective way to differentiate microbial communities from thousands of samples.

Metagenomics using Next Generation Sequencing (NGS)

NGS is a high-throughput sequencing method used for metagenomics analysis of the diverse microbial population, including their metabolic potential, structure, and effects on ecosystem function. Before NGS technology, forensic experts could not work with microbes because sequencing methods were too slow and expensive or dependent on culture-based methods. But with the advent of NGS technology, experts can identify DNA sequences of every microorganism present in a sample accurately, rapidly and comprehensively [59], and avoid experimental contamination caused by microbial cultures, which has proven helpful in forensics [60].

The use of NGS tools has increased in such studies over the past decade as technologies have evolved from the 454 Roche and MiSeq Illumina to Nanopore and SMRT PacBio. [61] compared non-culturing dependent tools and concluded that NGS can be combined with PLFA (PhosphoLipid Fatty-acid Analysis) to get a structural and functional picture of the entire microbial community in soil.

Numerous studies have been carried out by microbiologists to explore soil microbes using a next-generation sequence, but there is little research from a forensic point of view. Researchers surveyed soil microbes' diversity and solved a fictional case study using a next-generation sequencing-based on 16s and 18sRNA genes, plant chloroplast leucine tRNA gene and fungal spacer region between rRNA genes [62, 63]. The authors evaluated the effect of the development of fungal profiles through next-generation sequencing. The findings suggested that the development of the fungal profile was unaffected by the quantity of soil. Even tiny traces of soil, typically encountered in forensic case studies, provided valid genetic details [64]. The researchers distinguished very similar and dissimilar habitat types over time and space and soil on evidence items by assessing bacterial 16s rRNA gene through next-generation sequencing. Forensic studies have also examined statistical approaches to accurately evaluate the large sequencing datasets to classify and differentiate soil samples [65, 66]. Finley et al. [67] provided comprehensive literature about potential soil microbes ecology and NGS applications for forensic purposes.

Advantages and disadvantages of molecular-based methods

Each molecular method has its advantages and disadvantages. An overview is given in Table 3.

Statistical analysis tools for multivariate data

Although various methods for routine investigations have been developed and characterised, the findings must be evaluated with suitable and reliable statistical methods. Molecular techniques such as T-RFLP, DGGE, FAME, FISH, DNA sequencing, etc., provide complex data interpreted through specific statistical methods. The statistical tools *viz.*, SIMPER [79], ANOSIM [80], PCA [81-83], and Cluster analysis [84] are used to identify and individualise the component from multivariate data.

Reference databases for soil microbial community analysis

Soils have high microbial diversity and variability, which challenges studying microbial communities. To improve the understanding of the soil microbial community, we require a reference database or tools. Various tools and software are used for the soil microbial analysis for evaluation and interpretation such as Greengenes [85], SILVA [86, 87], RDP (Ribosomal Database Project) [88], NCBI (National Center for Biotechnology Information) [89], MG-RAST (Metagenomic Rapid Annotations using Subsystems Technology) [90, 91], IMG/M (Integrated Microbial Genomes and Metagenomes) [92], CAMERA [93], GOLD (Genomes OnLine Database) [94], MEGAN (Metagenomic Analyzer) [95] and RefSoil [96].

Sequential examination of soil evidence in forensic laboratories

Soils are extremely diverse, complex, and external disturbance and pollution can alter physicochemical and microbial content; therefore, it is necessary to manage, store and transport to the forensic laboratory properly. The microbial analysis of soil in forensic laboratories has five steps: (1) collection, storage, and transportation of soil samples from the crime scene; (2) analysis by using the molecular tool, metagenomic sequencing, and classification of taxa; (3) comparison of the findings with the database; (4) evaluation and interpretation of the results and last (5) presenting the findings to the court (Fig. 1).

Challenges faced in soil microbial forensics

There are several challenges faced in soil microbial forensics, some of which include:

1. Sample collection and preservation: Soil samples need to be collected and preserved correctly to prevent contamination or degradation of the microbial community. Improper handling of the soil samples can lead to false results, making it challenging to determine the source of the microorganisms.
2. Microbial diversity: Soil contains a vase and a diverse microbial community that can vary depending on the location, time of year, and other factors. Therefore, it can be challenging to identify and differentiate microbial populations, making it difficult to trace the source of the microorganisms [97].
3. Limited reference databases: There is limited availability of microbial reference databases that can be used to compare and identify microbial populations. This makes it difficult to match soil samples to a specific location or source [98].
4. Environmental factors: Soil microbial populations can be affected by various environmental factors, such as temperature, moisture, and pH levels. These factors can cause variations in the microbial community and make it challenging to determine the origin of the microorganisms [99].
5. Legal challenges: Soil microbial forensics is a relatively new field, and there is limited legal precedence for the use of microbial evidence in court cases. Therefore, there may be challenges in presenting microbial evidence in a court of law.

Overall, soil microbial forensics is a complex field that requires specialised knowledge and techniques to overcome the challenges. Advances in technology and collaboration among experts can help address some of these challenges and improve the accuracy and reliability of soil microbial forensics.

Conclusive findings and discussion

Soil microbial forensics, an emerging field that exploits the diversity and dynamics of soil microorganisms, has great potential for various applications, such as environmental monitoring, agriculture, and forensics[100]. Through application of advanced techniques, the article summarizes the novel strategies related to the composition, spatial patterns, and ecological roles of soil microbiomes in forensic prospectives. Diverse group of techniques focused on identifying microbial signatures that can be used to differentiate between soils from different geographic regions or environments[101]. These signatures may include specific microbial taxa, functional genes, or metabolic pathways.

Nowadays significant advancements in the methods used to study soil microbial communities, including the development of high-throughput sequencing techniques and bioinformatics tools for analysing microbial data[99]. Despite the potential of soil microbial forensics, some significant challenges and limitations need to be addressed. These include issues related to reproducibility and standardization of methods, as well as the need for a better understanding of the factors that influence microbial community composition in soil. Cited literature highlights the development of new methods for analysing soil microbial communities, as well as further investigations into the potential applications of soil microbial forensics in different fields. The review summarizes the current state-of-the-art molecular methods for soil microbial profiling and characterization and discusses the challenges and future directions of this field.

CONCLUSION

The diverse, complex, and heterogeneous nature of soil makes it a reliable resource for linking culprit-victim relations in the criminal field. Forensic science and legal courts' proceedings are progressively relying on the study of DNA for crime resolution. Soil offers a wealth of DNA data that needs to be implemented to the full extent and value. The use of molecular methods for forensic purposes is novel and holds excellent potential by offering forensic scientists an additional 'tool in the toolbox'. This molecular approach proves to be a powerful and differentiating toolbox for the molecular exploration of different soil microbial samples with identical geographical features. The purpose of this study is to explore the geopolitical location of the crime site, help in intelligence work, locate clandestine burials, estimate Post-mortem Interval (PMI), reduce the search area and comparative analysis of microbial community that supports the evidence when used in court.

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Conflict of Interest

Authors declare no conflict of interest.

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