Harnessing the potential of the environmental microbiome in forensic science

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Summary

A scientific consortium led by the Central Forensic Laboratory of the Police has undertaken to develop a method for DNA analysis of the soil microbiome to be used in forensic investigations. The aim of the project entitled Soil Microbiome Analysis Forensic Tool – SMAFT (http://smaft.eu/), financed by the National Center for Research and Development (DOB-BIO10/03/01/2019), is to develop a new tool that enables the association of a trace in the form of a soil sample with a specific geographical location. The first part of the paper introduces the concept of the microbiome and presents the possibilities of using microbiome DNA analysis in forensic science. In the second part, the stages of the SMAFT project are described in detail, beginning from the collection of soil samples from different sites in Poland across all seasons and isolation of microbiome DNA through massively parallel sequencing (MPS) technology-based analysis of isolates and the development of a genetic test containing a set of metagenomic markers allowing for effective individualization of soil samples, up to the creation of an IT system enabling analysis and interpretation of the obtained results, which includes a database of soil microbiome DNA profiles from various locations in Poland.

Key words: microbiome, soil, forensics, metagenomics, metataxonomic analysis, MPS sequencing, bioinformatics, NCBiR project

Introduction

The Central Forensic Laboratory of the Police (CFLP), as a research institute, conducts scientific research in the field of forensic science. The projects carried out at the CFLP are aimed at developing methods and technologies to support the prevention, detection and combating of crime. One of the major issues of interest to forensic genetics in recent years is the use of possibilities offered by forensic genomics (genomics of individual differences), epigenomics, metagenomics, and metataxonomic analysis to design and develop innovative tools intended for use in forensic police laboratories. This is in line with the priorities set by the member states of the North Atlantic Treaty Organization (NATO), particularly by the Allied Command Transformation, the NATO Science and Technology Organization and the European Defence Agency, recognizing genomics as one of twenty breakthrough technologies whose development is critical to ensuring security.

Unlike genetics, which mainly involves the study of genes and the mechanisms of their inheritance, genomics is a relatively new field of biology that addresses the analysis of the genome (complete genetic information of an organism). Genomics has emerged due to the intensive development of computer science and molecular biology techniques, especially modern sequencing technologies such as massively parallel sequencing (MPS), which represents one of the most important advances in biological sciences in the last two decades (Heather, Chain, 2017). Structural genomics involves determining the sequence of an organism's genome and its organization, whilst identifying the regions corresponding to individual genes. The role of functional genomics, together with epigenomics (DNA modifications), transcriptomics (mRNA), proteomics (proteins), and metabolomics (metabolites), is to determine the functions of genes and non-coding regions, the mechanisms of their regulation, and to study the gene-gene and gene-environment

interactions, including the analysis of the effects of gene expression products on the functioning of cells, tissues, organs, and whole organisms (Khodadadian et al., 2020). Theoretical genomics, comparative genomics and genomics of individual differences analyze general rules applicable to genes, the evolution of genes and the individual variation of genomes, respectively (Ślósarek, 2012). The last is the most interesting from a forensic point of view.

The use of genomics combined with MPS technology in forensic investigations allows analyses that until recently were too expensive or even unfeasible due to technical limitations. Nowadays it is possible to simultaneously test short tandem repeat (STR) and single nucleotide polymorphism (SNP) markers of autosomal DNA, X and Y sex chromosome markers as well as mitochondrial DNA (mtDNA).These methods, in addition to the forensic identification data of a person, allow to obtain information on phenotypic traits and biogeographical origin, even in the case of small amounts or poor quality DNA found at the crime scene (Kayser, 2015; Ambers et al., 2016; Bruijns, Tiggelaar, Gardeniers, 2018). Additionally, epigenetic markers can be used in the analysis of methylation profiles of human genomes and may contribute to a relatively accurate determination of the age of an unknown individual, distinguish between monozygotic twins, and identify tissues and body fluids (Zbieć-Piekarska et al., 2015; Vidaki, Kayser, 2018).

The potential of the microbiome in forensic science

Regardless of ongoing improvements in the area of identification methods, a stand-alone application of genomics is not always sufficient to solve a crime. A review of recent research papers indicates that in such situations certain questions can be answered with the aid of metagenomics, a field of science that involves the study of microbiome DNA, i.e. DNA recovered directly from environmental samples, without the need for establishing laboratory cultures.

Although scientists have been studying microbiomes for several decades, until recently no clear, universal definition of the microbiome was available. For this reason, in March 2019, the Microbiome Support project (https://www.microbiomesupport.eu/) hosted a combined workshop meeting of a large international group of microbiome experts to discuss redefinition of this term. In the following year, Berg et al. (2020) presented a proposal developed at that time in a comprehensive commentary published as a summary of the meeting. It is based on the definition from 1988 (Whipps, Lewis, Cooke, 1988), which describes the microbiome as a community of microorganisms (microbiota) inhabiting a well-defined environment with characteristic physicochemical features. As noted by Berg et al. (2020), this definition takes into account, in addition to a microbial community with distinct characteristic and functions, its interactions with the environment resulting in the formation of specific ecological niches. The microbiome, which is a dynamic and interactive microecosystem that undergoes changes in time and space, remains integrated with macroecosystems, such as the eukaryotic host, affecting its functioning and condition. It should be emphasized that the microbiome comprises the microbiota (i.e. living microorganisms of different taxa) and their numerous activities (their "theatre of activity") encompassing the whole spectrum of molecules produced by the microorganisms, including their structural elements (nucleic acids, proteins, lipids, polysaccharides), metabolites (signaling molecules, toxins, organic and inorganic molecules) and molecules formed by various environmental conditions in the surrounding environment, including those produced by coexisting hosts, as well as any mobile genetic elements, e.g. phages, viruses, plasmids, transposons, integrons and extracellular DNA, including relic DNA from dead cells (Berg et al., 2020).

Every human being, like every other living organism or every particular place, such as a meadow and its soil or a lake and its water, represent unique habitats determining to a large extent the diversity and quantitative proportions of the indigenous microorganisms. Sequencing and/or metataxonomic analysis of the microbiome DNA from a specific site allows the identification of its unique profile, which can be compared to microbiome profiles from other habitats. The results of research performed over the past few decades indicate that in the future the use of human microbiome DNA from various parts of the human body will be possible for forensic purposes (Fierer et al., 2008; Ravel et al., 2011; Tridico et al., 2014; Schmedes et al., 2018). Humans leave their microbial "footprint" in places they visit, such as crime scenes (Hampton--Marcell et al., 2020), on objects they touch (Lax et al., 2014), such as cell phones (Fierer et al., 2010), clothes (Lax et al., 2015), and fabrics (Lee et al., 2016), and on other individuals they come in contact with (Neckovic et al., 2020). It has also been proven that humans are accompanied by specific microbial clouds whose composition can be potentially utilized for the purpose of identification (Meadow et al., 2015). Futhermore, as noted by Clarke et al. (2017), sequencing the microbiome of an individual can provide data sufficient not only for identification, but also to obtain information about the individual's gender, health, and lifestyle, which is very important from a law enforcement perspective.

In parallel to the research conducted on the human microbiome in a forensic context, many research centers are developing methods and tools that enable the use of the soil microbiome for investigative and forensic DNA intelligence purposes. For example, between 2013 and 2015, a Microbial Soil Analysis (MiSAFE) project was implemented within the framework of the European Union's action for security in Europe, dedicated to the development of tools and procedures for routine testing of soil samples in forensic laboratories

(https://forensicmisafe.wixsite.com/misafe/project). It is well known that soil represents particularly valuable evidence due to its omnipresence in the environment, its diversity as well as the ability to adhere to shoes, tires, tools or clothes. It is also often overlooked by suspects attempting to obliterate their traces (Young, Austin, Weyrich, 2017). Therefore, the analysis of soil samples can provide sufficient information to link a suspect, victim, or object to a crime scene (Johll, 2009; Dawson, Hillier, 2010; Concheri et al., 2011), as well as to infer the likely geographic location of origin of the soil sample. (Pirrie, Dawson, Graham, 2017). Soil is a complex mixture of minerals, organic matter including living organisms, gases, and water (Needelman, 2013). Currently, forensic soil analysis is based on determining physical characteristics and chemical composition. The analysis includes soil color, texture, particle size, pH, elemental composition, mineral content, and sometimes organic compounds such as plant waxes (Habtom et al., 2017; Murray, 2012; Woods et al., 2016), which provide information relevant to the investigation (Fitzpatrick, Raven, Self, 2017; Petraco, Kubic, Petraco, 2008). However, in particular cases, e.g. when the soil samples come from a geologically homogeneous area, from adjacent sites, or have low inorganic content (e.g., peat soils), routine analysis is unfeasible for discrimination between samples (Giampaoli et al., 2014; Young, Austin, Weyrich, 2017; Young, Higgins, Austin, 2019). The limitations of differentiating soil samples at the local scale can be overcome by performing analyses of biological material. It is estimated that 1 gram of dry soil contains on the average: 10^{10} viruses, 10^{10} bacteria and archaeons (including 10^{8} actinomycetes), 10^{6} each of fungi and algae, 10^{5} protozoa, and 10^{2} nematodes (Trevors, 2010). Additionally, soil may also contain plant fragments (e.g. roots, pollen, spores, seeds, leaves) and invertebrates other than nematodes (Young, Austin, Weyrich, 2017) as well as extracellular DNA. Because all soil-dwelling organisms have specific habitat requirements, environmental conditions, such as soil type and texture, pH, moisture, temperature and organic carbon levels significantly influence the composition of the microbiome, i.e. the community of microorganisms living at a specific site (Maron, Mougel, Ranjard, 2011; Pasternak et al., 2013). Due to a variable spatial structure of the soil, it does not contain a “typical”, uniform microbiome (Fierer, 2017). Studies of the relative abundances of major bacterial taxa and archaeons in soil samples have demonstrated diversity not only for different soil types, but also for soils collected from sites only several or even a few centimeters away (Habtom et al., 2019; O’Brien et al., 2016; Pasternak et al., 2013; Sensabaugh, 2009). DNA analysis of the soil microbiome may provide data to enable its effective individualization and, in the future, prove as useful for comparing soil traces or determining the soil origin, as human DNA profiles for establishing a link between biological traces and the offender (Damaso et al., 2018).

It is important to note that currently the analysis of microbiome DNA is practically absent in the investigation, although the potential of this type of data as evidence or aid in conventional forensic testing methods has been increasingly recommended (Robinson et al., 2021). In order for the potential of the microbiome to be utilized effectively by law enforcement, further research is needed to demonstrate that statistical inference based on such analyses is stringent enough to be considered by courts as scientific evidence as being characterized, for instance, by a known and accepted error rate (Velsko, 2020; Robinson et al., 2021). Expanding the set of samples to be analyzed in ongoing studies, creating databases of microbiomes from diverse environments, based on clearly defined and well-documented procedures, improving bioinformatics tools, including machine learning techniques for interpreting results, or studying the dynamics of temporal and spatial changes in the microbiomes, are examples of research goals that, if achieved, may contribute to including microbiome analysis in the toolbox used in forensic laboratories (Robinson et al., 2021).

SMAFT project

Bearing in mind the challenges outlined above, the Central Forensic Laboratory of the Police (CFLP) as a leader representing a consortium consisting of: Medical University of Warsaw, Jagiellonian University, Pomeranian Medical University and ARDIGEN company, has received funding from the National Center for Research and Development to implement the **S**oil **M**icrobiome **A**nalysis **F**orensic **T**ool – **SMAFT** (http://smaft.eu/) project aimed at using the potential of the soil microbiome in forensic science (DOB-BIO10/03/01/2019). The main objective of the project is to develop a predictive tool for forensic DNA analysis of the soil microbiome, which will allow profiling the geographical location of soil samples of unknown origin in Poland. In other words, the system under development will seek to determine the possibility of establishing the link between a soil trace recovered from a shoe, tire or shovel and a specific geographic location. This type of evidence is likely to link the suspect with a particular location with a higher degree of probability, or it can allow to trace the movement of the criminal offender. In the future, the information from the SMAFT system will aid to direct and speed up both criminal and terrorist investigations. The system under development also has the potential to be used to prosecute environmental crimes or, more broadly, to conduct biodiversity research.

The research planned under the SMAFT project is divided into several stages, beginning with the collection of soil samples. The project involves collecting close to 1,000 soil samples, 250 each in the fall, winter, spring and summer, from 80 different locations across Poland. According to the authors, such an approach allows to detect possible seasonally independent differences in

topsoil microbiome composition. The selection of soil sampling sites was based on computer analysis of data from measurements taken at all hydrological and meteorological stations located throughout Poland, covering the period of the last 20 years. The map of Poland has been divided into five areas with different climatic conditions. Twelve main sampling locations were eventually selected, and several (five to eight) sampling sites were designated around each location. Three soil samples would be collected from each specific sampling site. In addition, the physicochemical characteristics of soils in Poland, using data collected under the EU Land Use/Cover Area frame statistical Survey – LUCAS project (https://ec.europa.eu/eurostat/web/lucas/) were taken into account while selecting the sampling sites. This stage also includes developing a detailed methodology for collecting, preserving and labeling soil samples, designating and documenting the collection sites, as well as transporting samples to the laboratory under appropriate conditions. In the next stage of the project, microbiome DNA will be extracted from the collected samples. As soil is a difficult, heterogeneous material, containing a lot of substances potentially inhibiting the enzymes used in subsequent project activities, finding an isolation method that will yield soil microbiome DNA of sufficient quantity, purity and quality will determine the success of subsequent stages. The efficiency of DNA extraction from gram-positive, gram-negative, spore-forming, or envelope-producing bacteria varies depending on the procedure used, hence obtaining DNA from soil that is representative of the entire bacterial community of a given microbiome is not straightforward. In the third stage of the project, the obtained DNA isolates will be used to prepare libraries containing DNA fragments of microorganisms extracted from soil samples. In order to obtain the best quality libraries with the desired fragment lengths, optimization of the library construction procedure is planned, at both the DNA fragmentation and amplification stages. In the process of preparing the libraries, all DNA fragments from each sample will be assigned a unique barcode allowing – after obtaining sequencing data – their identification and assignment to a specific soil sample. The fragment lengths of the prepared libraries will be verified by capillary electrophoresis and the concentration of DNA within the libraries will be determined by a fluorometric assay. After calculating the molar concentration all the libraries will be normalized and pooled, taking into account unique index combinations. The prepared library pools will be sequenced in the next (fourth) stage using Illumina® SBS technology and the latest generation Illumina® NovaSeq 6000 sequencer. Between 80–100 million 150 bp paired-end reads per single soil sample will be generated. The resulting raw data will be converted to a format that allows bioinformatics analysis. The fifth stage will involve data analysis, in order to identify the optimal set of markers to evaluate the microbiome composition of a sample of unknown origin and assign the sample to a specific location. The authors plan to design a soil DNA identification panel, containing a unique set of highly informative markers that enable comparison of soil samples. The sixth stage of the project provides for the development, optimization, and validation of a targeted NGS method to analyze the soil microbiome or, more specifically, the development of a genetic test based on next-generation sequencing (NGS). Sample testing will be performed using the selected genomic sequences defined in stage five. The compatibility of the soil sample sequencing results obtained in stage four (deep sequencing) with the sequencing results of the genetic test developed in stage five (targeted sequencing) will also be determined using several medium throughput sequencing methods, ultimately leading to selection of the optimal technology. The selected method will be validated, with particular requirements and limitations of forensic analyses. In the next (seventh) stage, the authors aim to create an IT system for analysis and interpretation of the results obtained by genetic tests and selected NGS technology. The data gathered from DNA sequencing of soil microbiomes will be uploaded to a database included in the IT system under development, thus creating a "map" of the soil microbiomes in Poland. Additionally, a tool for efficient database searching and interpreting analysis results will be implemented as part of the system. The results of sequencing DNA isolated from a soil sample of unknown origin obtained by the test will be compared with the database and assigned to the most probable location on the map of Poland. Eventually, a complete predictive system will be developed that includes a test for identification of bacterial communities of the soil microbiome and the software for interpretation of test data. The last stage of the project will involve testing the effectiveness of the predictive system in conditions mimicking real-life situations and preparation of Standard Operating Procedures (SOPs) enabling its implementation into practice. An evaluation of the system's parameters and performance will also be conducted. Additionally, guidelines, procedures, and instructions necessary to conduct predictive testing and comparative analysis of DNA isolated from soil samples, performed using the system created under the SMAFT project will be developed.

As a result of the SMAFT project a complete predictive system designed to identify and determine the site of origin of soil samples based on the composition of the microbiome will be created.

It should be noted that, to the best of the authors' knowledge, no scientific paper published to date on soil microbiome research has reported such deep sequencing of so many DNA isolates from different soil samples. The conducted research will additionally contribute to broadening the knowledge on soil biodiversity in various regions of Poland.

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