An Overview of the Forensic Analysis and Use of Non-Human DNA

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Abstract

DNA is present in every living organism on Earth. DNA is also present in a few non-living biological agents such as DNA-containing viruses, and is additionally unique to particular groups of organisms and often even to individuals. This characteristic allows DNA to be quite useful in forensic investigations. This study takes an in-depth look at the forensic use of non-human DNA. DNA analysis is a key component of many forensic investigations, but the majority of these analyses are focused on human DNA. This review examines the ways in which non-human DNA can be useful to forensic investigations. The various unique characteristics of non-human DNA will also be investigated. Finally, the protocols of various parts of the analysis process will be described. Non-human DNA requires alterations to be made in most steps in the DNA analysis process. Customizations in the areas of collection, handling, storage, extraction, and amplification will be assessed.

Keywords: non-human DNA, forensic DNA analysis

An Overview of the Forensic Analysis and Use of Non-Human DNA

Human DNA is a widely used and revered piece of evidence that is extremely useful in the field of forensic science. This is due to the fact that each human, except identical twins, possesses a unique DNA profile that is found in nearly all of the cells in the human body. This uniqueness enables DNA to serve as a genetic "fingerprint" with impressive discriminating power that is extremely useful in the field of forensic science. However, humans are not the only organisms that possess DNA. In fact, all living organisms and even some non-living biological agents, such as viruses, contain DNA. This DNA is similarly unique to a species or individual and has been shown to be similarly useful in forensic investigation. Non-human DNA does come with some particularities, however, and unique handling and analysis protocols must often be used. The ways in which non-human DNA can be useful make these additional protocols essential, as it is able to aid forensic investigations in a myriad of ways.

Sample Collection, Handling, and Storage

Proper sample handling is crucial in any forensic investigation but is especially important when dealing with biological evidence. Most types of biological evidence are susceptible to degradation, and extra precautions must be taken in order to best preserve the integrity of the sample. DNA-containing evidence requires particular consideration, as it is additionally sensitive to degradation. There are, however, many sets of guidelines and protocols for proper collection, handling, and storage, whether for DNA-containing evidence in general or for trace DNA.

General DNA Evidence

When it comes to DNA evidence, the proper protocols for collection, handling, and storage are crucial. Contamination of evidence is extremely important to avoid, and degradation

of the DNA sample can happen without effective storage and render the evidence useless. There are of course multiple types of DNA-containing evidence, and each of these types of evidence can be collected in a different way in order to best preserve that evidence's integrity (Lee et al., 1998).

The first precaution that should be taken with DNA-containing evidence involves storage. Because DNA can degrade rapidly in warm conditions, any DNA-containing samples must be stored either refrigerated or frozen, as the specific circumstances dictate. This is not the only precaution that must be taken with these types of evidence. As stated above, each unique type of DNA-containing evidence has special precautions that should be considered during the collection process. Liquid blood, for example, should be preserved in an anticoagulant so as to avoid clotting and increase the ease with which a sample can be collected for analysis. Hair is another type of evidence that requires specific care, as the majority of useful DNA will be contained within the hair's root. Special care must be taken, then, as to not disturb the hair during packaging lest that root tissue be lost or damaged (Lee et al., 1998).

Trace DNA

While the collection of most DNA evidence is relatively straightforward, trace DNA is another matter entirely. Trace DNA evidence exists in very small amounts, usually amounts smaller than recommended for accurate analysis. In general, these samples are difficult to identify on surfaces and assumptions have to be made about the areas from which to collect. Another way of identifying areas for collection makes use of an alternate light source (ALS) to examine the surface evidence is presumed to be on. Possible evidence can also be identified through the presence of fingerprints (Oorschot et al., 2010).

Identification of DNA evidence prior to collection could significantly improve obtained results from such evidence. When the area from which to collect is not strictly defined, collection methods risk collecting too little sample. One of the most popular methods of collection involves moistened swabs and swabbing an area that is either bigger or smaller than the area the sample to be collected is found on can result in inadequate collection (Oorschot et al., 2010).

The collection method itself could also be improved upon. A moistened cotton swab often collects little more than half of the available biological material. Often multiple swabs are used in order to maximize the sample that is processed. Different moistening solutions as well as alternative swab types have also been proposed to optimize the sample collection procedure, but little research has been conducted to determine these alternative methods' effectiveness when it comes to sample analysis (Oorschot et al., 2010).

Once collected, the sample must also be extracted from the collection device. In the case of a cotton swab collection, this can be done immediately afterwards while the swab is still moist, or it can be done at a later time when the swab has either dried or been frozen for preservation. Of course, these methods have different levels of effectiveness, and results have shown that the most DNA is obtained from the swab when it is either moist or has been frozen for storage (Oorschot et al., 2010).

Cotton swabs are not the only way to collect trace DNA. Especially when the DNA is suspected to be on clothing or other fabric, swabs have been found insufficient and tape is now commonly used. Vacuums with filters are one other collection method that has been tested (Oorschot et al., 2010).

Characteristics of DNA

The specific characteristics of DNA obtained from non-human sources must be taken into account in order to dictate how to best proceed with its analysis. DNA from one source may require an entirely different extraction or analysis method when compared to DNA from a myriad of other sources. Some of these major characteristics originate from the source organism's cellular (prokaryotic or eukaryotic) status, and viral DNA should of course be analyzed with an entirely different approach. The intra-organismal origin of the DNA must also be assessed in many circumstances, as nuclear and mitochondrial DNA should be treated quite distinctly (Brown, 2002).

Eukaryotes

The eukaryotic genome ranges from 10 to 100,000 Mb in length and is divided into those DNA molecules being contained in either the nucleus or the mitochondria. The differences between the DNA in either organelle will be examined in a later section of this review. The nuclear genome contains the bulk of the DNA and will be the focus of this section. One of the primary features of the eukaryotic nuclear genome is format. All eukaryotes possess at least two linear DNA molecules known as chromosomes (Brown, 2002).

These chromosomes rarely exist in a loose form, and the DNA is instead packed into a denser form. Specific DNA-binding proteins known as histones come together to form bead-like complexes called nucleosomes. These nucleosomes function to keep the DNA coiled around themselves, shortening the overall DNA string. This "beads-on-a-string" formation is then further wrapped into an arrangement around 30 nm in width, fittingly called the 30 nm fiber. This is the formation of DNA that exists for the majority of the cell's lifetime (Bassett et al., 2009).

During cell division, however, the chromosomes condense even more so as to be more easily transported. In this more condensed form, the chromosomes have been replicated and are connected to their copies at a region called the centromere. Each chromosome in this pairing is now called a 'sister chromatid.' This region serves as an attachment site for microtubules within the cell and allows the two sister chromatids to be separated from each other during cell division. The centromere regions of DNA are additionally unique because of the repeat sequences featured in the DNA. Repeating regions are found throughout DNA, but the majority is concentrated in the centromere and the telomeres (Brown, 2002).

The telomeres occur at the ends of the linear DNA molecules and function to protect the DNA from being lost during replication. Telomeres consist of large numbers of copies of a specific repeated sequence that are bound to specific proteins that serve to maintain the telomere region during various cellular activities (Brown, 2002).

Besides the repeating DNA found near the centromeres, which is classified as a type of satellite DNA due to the banding pattern that appears after fractionation, eukaryotic DNA features other types of repeating DNA. Minisatellites and microsatellites, also classed as satellite DNA, similarly form clusters of repeating units. Minisatellite DNA is featured in the telomeres, as well as in other areas in the chromosomes. Microsatellite DNA, though its biological function is unknown, has proven very useful. Microsatellites are variable, and the individuals of a species have different numbers of repeat units in specific microsatellites. This is the feature that allows DNA profiles to be compared and individuals to be identified (Richard et al., 2008).

Prokaryotes

Overall, the prokaryotic genome is quite small when compared to that of eukaryotes. While a few genomes reach up to 30 Mb in size, the majority remain less than 5 Mb. This

genome is distinct from that of eukaryotes in other ways as well. The first difference is how the prokaryotic genome is packaged. In most prokaryotes, the base form is that of a single circle, but the size is minimized through a process known as supercoiling. This is a method by which the number of turns in the double helix molecule are altered, introducing torsional stress to the whole molecule. The molecule then releases this stress as much as possible by twisting and winding around itself. This fully twisted molecule is finally attached to a protein-based nucleoid that serves to concentrate the genome. This method also serves to regulate prokaryotic transcription (Dorman & Dorman, 2016).

The content of the prokaryotic genome also differs from that of the eukaryotic genome. The proportion of the genome that is non-coding or does not code for any functional proteins. Prokaryotes have very compact genomes and only 11% of the total genome is non-coding. In addition, this non-coding DNA is scattered throughout the genome rather than existing in large sections (Brown, 2002).

Prokaryotic genomes also feature groups of genes known as operons. The genes inside these operons are linked together and are only expressed as a group. This feature is used in prokaryotes and some eukaryotes as a primary method of gene regulation. Most of the time the genes within an operon code for similar proteins or proteins that are involved in a single process, thus being related in more than just location (Brown, 2002).

Viruses

Viruses are unique in the area of DNA since these nucleic acid-containing “organisms” are not widely considered to be alive. Viruses are not able to reproduce on their own and rather perpetuate their lineage through parasitism. Viruses are obligate intracellular parasites and are unable to survive outside of a living host cell. Viruses are composed of proteins and nucleic

acids, and certain viruses possess a lipid envelope embedded with glycoproteins. The basic structure of a virus involves a symmetrical protein capsid containing the virus's genome. The capsid's symmetric and repeating nature allows the part of the genome that codes for these constituent proteins to be relatively small (Chaitanya, 2019).

The nucleic acid contained within the capsid is composed of double- or single-stranded DNA or RNA. Reproduction of this genome is carried out in most instances by using the host cell's resources. The host cell is parasitized for necessary energy, materials, and processes, with the only contribution of the virus being the genome delivery. In addition, the shapes and sizes of viruses are extremely diverse. Viruses can be as much as 400 nm in diameter and come in many different shapes. Specifically, viruses have three characteristic types of capsids: icosahedral, linear, and complex (Chaitanya, 2019).

Viral genomes also vary significantly. There are a multitude of ways in which viruses can be broadly categorized. The first division involves the classification of viruses as either DNA- or RNA-containing viruses. Secondly, either DNA or RNA viruses can possess various characteristics. The genome can be single- or double-stranded in addition to being circular, linear, or segmented. Additionally, viruses can be classified as monopartite or multipartite. Monopartite genomes contain all of the genes within a single molecule, while multipartite genomes are split into multiple separate molecules. Double-stranded DNA viruses are always monopartite, while single-stranded DNA viruses are infrequently multipartite. The majority of RNA viruses are multipartite, however. For single-stranded RNA genomes, another set of designations is available. These genomes can be classed as containing a positive sense strand, a negative sense strand, or an ambisense strand, a mix of the two. Positive sense genomes exist in the same polarity as mRNA, while negative sense genomes need to be copied prior to transcription. Finally, viral

genomes vary significantly in size. DNA viruses can reach up to 305,000 nucleotides in length, while RNA viruses can reach up to 31,000 nucleotides. The higher mutation rate and fragility of RNA may be the reason for this discrepancy. Each of these categories of viruses make up part of the Baltimore Classification Scheme. This scheme was developed and proposed in 1971 by David Baltimore and is still used some fifty years later (Koonin et al., 2021).

Nuclear and Mitochondrial DNA

Most organisms additionally possess two main types of DNA. There is DNA that is found in the nucleus, and that which is found in the mitochondria. Nuclear DNA (nDNA) is perhaps the more commonly known type of DNA, as it is what is commonly described as carrying all of the genetic information for an entire organism. Nuclear DNA is commonly used in forensic analyses because of its ability to discriminate between individuals. Some of the fundamental characteristics of nuclear DNA include form, copy number, and the recombination rate. Nuclear DNA can take many forms, but at its core it is simply a single length of double-stranded DNA. During cell division, the nDNA is packaged into its chromosomal form while maintaining its base characteristics, albeit in a tightly folded shape. Nuclear DNA is more or less rare in comparison with mitochondrial DNA, as no more than a single diploid copy is found in each cell. Finally, nDNA is unique to each individual, except in identical twins. This is perhaps the most forensically significant feature of nDNA, as this characteristic discriminates between individuals (Brown, 2002).

Mitochondrial DNA (mtDNA), on the other hand, possesses a few distinguishing characteristics. The DNA found in mitochondria is entirely distinct from nDNA. Mitochondrial DNA is a circular molecule made up of double-stranded DNA that codes for only a few specific proteins needed for oxidative phosphorylation. Another way in which mitochondrial DNA differs

from nuclear DNA is its copy number. Up to 10,000 copies of mitochondrial DNA can be present in a single cell. This is due to the fact that each mitochondrion has up to ten copies in itself, and a single cell can contain up to 1,000 mitochondria. Mitochondrial DNA is also unique in the fact that it is inherited from the maternal line and undergoes no recombination between relatives. Finally, mitochondrial DNA is haploid, and the majority of individuals are homoplasmic, meaning that sequencing results are relatively simple (Budowle et al., 2003).

Forensic Uses of Non-Human DNA

Non-human DNA can serve as important evidence in many different types of forensic cases. There are those cases in which a crime has been committed against the non-human organism, such as in wildlife crimes, and there are those cases in which the non-human organism represents a crucial component of the criminal act, such as in deforestation crimes, bioterrorism, drug-based crimes, illegal trade, or animal product falsification. Finally, non-human DNA can be crucial when it comes to linking the elements of a crime together or helping its investigation progress. Many non-human DNA samples can demonstrate that an individual or a piece of evidence was present in a specific region simply through the comparison of transferred DNA-containing materials.

Plants

Although not immediately apparent, plant DNA can be useful in a forensic capacity. There are primarily those situations where a plant is actually the main focus of the case, such as in illegal deforestation or plant toxicity cases. Plant materials have also been shown to be useful in terms of linking a person or object to the scene of the crime (Craft et al., 2006).

Illegal Deforestation

In environmentally protected areas such as the Amazon region in Brazil, illegal deforestation is an active issue. The Amazon region in particular houses an extremely large number of tree species, which makes this problem even more complex. Some tree species may be easier to identify based solely on phenotypic elements, but others are more difficult to visually identify. For this reason, DNA barcoding of at-risk tree species can be extremely useful for enforcing illegal deforestation policies (Paranaiba et al., 2020).

Poisonous Plants

There are several plants that are also important in the realm of forensic toxicology. Most exposures to these plants are accidental, but poisonous plants can also be used with criminal intent. Many cases of accidental toxicity come from the ingestion of household plants and the mixing of poisonous plants with medicinal herbs. However, not all plant toxicity cases are accidental and poisonous seeds and plant parts have been used for both self-poisoning and intentional poisoning of others with the intention of inflicting either harm or incapacitation (Nithaniyal et al., 2021).

With any of these cases, species identification through visual examination of fragmented or otherwise damaged samples is very difficult. This species data can be extremely useful in either determining treatment for those patients that are still living or as evidence in a criminal case. Forensic barcoding has been able to assist in the rapid and accurate identification of these poisonous species, however. No more than a small amount of plant tissue is required, and processing is inexpensive, rapid, and reliable (Nithaniyal et al., 2021).

Linking Evidence

Advancing research into plant DNA markers has increased the utility of plant samples as linking evidence in criminal cases. Plant parts such as pollen, seeds, and leaves, can be found associated with a suspect or with displaced evidence. DNA profiling can then be used to link that plant material to a crime scene and in turn link the suspect or evidence to that particular crime scene (Craft et al., 2006).

Fungi

Fungi are extremely prevalent in the world but are often overlooked. Prevalence can actually serve a myriad of purposes within a criminal investigation. Fungal growth patterns can be analyzed in order to determine a post-mortem interval with spores serving as valuable evidence to link victims or suspects with each other or with a particular location. Additionally, the hallucinogenic and toxic properties of fungi cause them to be at the center of many drug or bioterrorism-based cases, where DNA identification can be very useful (Hawksworth & Wiltshire, 2010; Nugent & Saville, 2004).

Time of Death

Certain species of fungi have also been shown to be able to assist in determining the time that has passed since death. Some fungal species can only be found on cadavers at specific post-mortem intervals. More extensive research in order to form a sizeable reference database would enable this information to provide robust forensic evidence (Schwarz et al., 2015).

Bioterrorism

Though fungal agents are not perhaps the most commonly used bioterrorism agents, they should not be overlooked. There are two main groups of mycotoxins that have been involved in historical bioterrorism events due to their potential as biothreats. Both trichothecenes and

aflatoxins, the two main mycotoxin groups, are particularly damaging and as such detection methods would be key in forensic cases involving various species (Venkataramana et al., 2021).

The first group of mycotoxins, trichothecenes, come from the *Fusarium* species of fungi that naturally occur in various common grains. Trichothecenes are classified into type A and type B classes, with the type A T-2 toxin being considered a potential biological weapon. Their potency comes partially from the component's ability to be absorbed through completely intact skin and subsequently cause toxicity throughout the system. DNA-based detection methods have demonstrated good success in identifying the presence of these toxins. The second group of mycotoxins is the aflatoxins, which are carcinogenic when ingested as well as being liver toxins. These products are made by fungus species in the *Aspergillus* genus and exist in six varieties. DNA-based methods for the detection of these mycotoxins have been successful as well and would be useful in forensic cases (Venkataramana et al., 2021).

Drugs

There are multiple genera of hallucinogenic fungi, most of which feature species that contain the illegal compounds psilocin and psilocybin. While there are a few cities and states in the United States that have decriminalized some aspect of either possession or cultivation of psilocybin, most areas continue to view psilocin and psilocybin as illegal or restricted. Because of these restrictions, it is important to confirm the presence of psilocin or psilocybin in a fungal sample in order for it to be robust evidence in court. However, as not all species of the multiple genera in question contain psilocin or psilocybin, species identification is additionally important (Nugent & Saville, 2004).

Linking Evidence

Fungi can actually provide quite a bit of linking evidence in forensic investigations. Fungal spores are very easily transmitted between surfaces, and so spores collected on a person's clothing can easily be linked to a fungi profile at a specific location. In these circumstances, DNA analysis of these spores and the originating fungi can serve to connect the two. Reference databases have been found lacking for concrete DNA identification of a sample, but comparison between two samples does not require reference sequences (Hawksworth & Wiltshire, 2010).

Animals

Animals are a large part in the lives of many people, and so it stands to reason that their DNA could play a significant part in the field of forensics. There is a variety of ways in which animals or animal products are central components to a given situation, such as in the areas of poaching, illegal trade, and animal product crime. In these situations, the animal or animal product is either the main victim of the crime or extremely central to whatever that crime is, and so any DNA that could be obtained from that animal or product would likely be able to significantly benefit the investigation. In other cases, however, animals may still be involved in a primarily unrelated matter. Many people live or work with animals, and materials that originate from these animals, such as hair or fur, can serve to link a person to a place due to the material's unique attributes (Coghlan et al., 2011; Kanthaswamy, 2015).

Poaching

Wildlife crime is, of course, one area that makes wide use of animal DNA. The area of poaching, especially, can make use of DNA profiling technologies. The greater one-horned rhinoceros (*Rhinoceros unicornis*) is one African animal that undergoes significant poaching centered around its horns. Chinese traditional medicine fosters the sale and use of these horns as

medicinal substances, which in turn drives the poachers' efforts. In tackling this problem, DNA profiling technologies prove to be quite promising. The ability to match poached rhinoceros horns to the original animal can provide very robust evidence in court, in turn improving the prosecution rate and hopefully reducing the overall poaching rate (Ghosh et al., 2021).

Illegal Trade

Animal trafficking is an issue that poses several difficulties such as disease transport or invasive species. One area that commonly undergoes illegal trade is the pet trade, specifically in reference to birds from the order Psittaciformes. These birds, represented by parrots and cockatoos, are very much in demand and thus quite profitable. The dangers with their trade, however, include disease vectoring and the reduction of endemic populations. For these reasons, the transport of avian eggs is closely monitored for suspected illegal trade (Coghlan et al., 2011).

Mitochondrial DNA sequencing technologies have made it possible to identify the species of smuggled eggs in order to remove them without spending time and resources to "rear them out" for later identification. This rapid identification technology allows for more timely enforcement of wildlife trading policies and prosecution of those parties involved (Coghlan et al., 2011).

Animal Product Falsification

Some businesses such as seafood restaurants make a profit by intentionally mislabeling seafood. Cheaper and less desirable fish species can be marketed as more expensive and desirable species in order to garner more business while turning a large profit. DNA barcoding can then be used to verify the species of confiscated seafood samples and in turn enforce penalties on the falsification of seafood products (Carvalho et al., 2015).

Linking Evidence

Domestic animals such as cats and dogs provide an abundant source of linking evidence. These household pets frequently shed hair, fur, skin, and dander as part of their natural growth cycles. Some of these materials cling extremely well to the clothing or belongings of a person who has had contact with a household pet long after the initial contact. These characteristics can be extremely useful in a forensic investigation, as the presence of a particular animal's hair, fur, skin, or dander on a suspect or victim can immediately link a suspect and victim to each other or to a specific location (Kanthaswamy, 2015).

Bacteria

Bacteria can be surprisingly useful in forensic investigations due to their impressive prevalence. Bacteria exist in and on every other living organism and play an integral role in the lives of those organisms. Some bacteria are pathogenic and as such are not uncommonly used as damaging agents such as in bioterrorism. Still other bacteria are discriminating enough in their habitat so as to serve as identifying features of specific organic substances. Finally, the immense variety among groups of bacteria enable different communities of these organisms to be uniquely identified and even serve as fingerprint-like markers for an individual person.

Bioterrorism

One of the main ways in which bacteria are involved in criminal activities is in the realm of bioterrorism. The scope of bioterrorism includes the use of pathogenic microbes or any of their pathogenic by-products in order to harm other organisms or the environment. In addition, these harmful bioterrorism attacks can be focused on either individual organisms or groups, and the attacks carry the purpose of causing societal instability or fear (Echeonwu et al., 2018).

Bioterrorism is not a new phenomenon. Bioterrorism attacks date back to the early-mid 14th century, when dead plague victims were thrown into the city of Caffa in order to spread the Bubonic plague via its causative agent, *Yersinia pestis*. More recently, 2001 saw the distribution of letters through the mail that contained spores of the bacteria *Bacillus anthracis*, the causative agent of the disease Anthrax (Echeonwu et al., 2018).

The forensic investigation of bioterrorism events includes three main areas. The identification of the toxic agent can inform both containment and prevention measures. Characterization then seeks to determine the intentions behind the release of the agent and can help in determining legal steps once the source has been identified. Finally, the attribution of the agent aims to identify the agent's source. This step attempts to determine both the originating strain of the agent and the event's perpetrator, and often provides valuable evidence (Echeonwu et al., 2018).

DNA analysis technologies may be useful in a number of these investigative areas, but is perhaps most applicable to source-tracing, part of the attribution step. Multiple polymorphic typing methods have been applied to various pathological agents, and these analyses have been able to assist in the characterization and identification of these biological agents in many ways. The analyses focus on comparing the evidentiary agent to other known strains and can trace the agent's source back to the original strain while distinguishing it from other similar strains. The known source of that main strain can then be ascertained, and this knowledge helps to connect the perpetrator to the attack (Echeonwu et al., 2018).

Body Fluid Testing

One of the important precursor steps to forensic DNA analysis of body fluids is testing in order to confirm their biological origin. Presumptive and confirmatory tests determine the type of

body fluid to be analyzed and inform the analyst as to the methods to be used in the extraction of DNA from that body fluid. Apart from blood, saliva is one of the common body fluids that is analyzed for a DNA profile. The usual presumptive and confirmatory tests for saliva involve the detection of α-amylase, an enzyme that is present in saliva and works to break down carbohydrates. This method can be extremely useful and is regularly used in tests such as the Rapid Stain Identification (RSID™-Saliva) test, but some results can be confusing. Other body fluids such as human breast milk can contain substantial amounts of α-amylase and yield weak false positives in the RSID™-Saliva test (Old et al., 2009). In these cases, bacteria can be used as a secondary test in order to confirm the identity of a body fluid sample.

One proposed method of saliva discrimination involves the identification of the presence of certain streptococcal bacteria. *Streptococcus salivarius* and *S. mutans* are two bacteria that are abundant in the human oral cavity, and as such are a reliable indicator of a saliva sample's identity. In order to identify the presence of these bacteria, PCR amplification and DNA analysis can be used. Using this method, species-specific DNA of the two aforementioned bacteria can be detected in saliva samples. At the same time, samples of other body fluids such as skin secretions, urine, semen, and vaginal secretions do not yield detectable levels of *S. salivarius* or *S. mutans*. This method could therefore be used to strengthen the presumptive testing protocols for saliva samples (Nakanishi et al., 2008).

Linking Evidence

Because microorganisms such as bacteria are so unique and prevalent on and in the human body, a person's individual microbiome can be used as a link between the person and certain items touched in their environment. Even just bacteria transferred from skin contact can remain on the contacted object for a long period of time. These characteristics allow individual

distributions of a person's microorganism population to provide important information in the context of a forensic investigation (Lee et al., 2016).

Viruses

Viral DNA analysis applications are mainly useful in disease-based applications. DNA analysis is able to be applied to various types of biological crimes such as bioterrorism and intentional disease transmission as well as to identification or location of a specific individual. These methods use various analytical aims that are generally centered on a specific viral sample's strain, which can be used to infer either origin or relatedness between two samples (Bernard et al., 2007; Espy et al., 2002).

Bioterrorism and Disease Crime

Due to the nature of infectious agents, viruses are prime candidates for bioterrorism and other infectious disease crimes. Many viral agents that have caused widespread disease in the past, such as smallpox, have morbidity and mortality rates that render them a decent bioterrorism threat (Espy et al., 2002). Other diseases such as human immunodeficiency virus (HIV) may be the center of personal cases, as individuals may purposely infect others with the virus (Bernard et al., 2007). Both smallpox and HIV, due to their infectious nature, have the potential to become the center of a forensic investigation.

For cases concerning either smallpox or HIV, analysis of the viral genome could prove quite informative. Information related to a particular viral sample's source strain can lead investigators to a potential source of a bioterrorism attack (Espy et al., 2002). In addition, though the HIV genome is not able to positively correlate two individuals involved in a complaint of intentional transmission, a person is able to be exonerated as a suspected individual by determining strain unrelatedness (Bernard et al., 2007).

Human Identification

There are a few viruses that possess two important characteristics that are used for estimating the geographical origin of unidentified individuals or cadavers. The first characteristic involves the prevalence of a virus in the human population. One virus that may come to mind is the Epstein-Barr virus, as it occurs in over 90% of the adult population, healthy or otherwise. This virus is commonly known to cause diseases such as several lymphomas, infectious mononucleosis, and nasopharyngeal carcinoma (Ikegaya et al., 2008).

The other characteristic of a virus that is used in the locating the geographical origin of an unidentified subject is its level of geographical variation. If the genome of a virus that was isolated from an unidentified individual can be compared to a specific strain with a known geographical origin, that individual's origin can be similarly narrowed to the reference strain's origin (Ikegaya et al., 2008).

Soil

Though it may not be immediately intuitive, soil samples contain small amounts of DNA from various organisms. Microorganisms contribute to the DNA that can be found within an environment's soil. Additionally, because of the wide variation in these organisms that can be found in any particular environment, the relative composition of the DNA isolated from a soil sample can provide information about the wider environment of its origin. This compositional data can be used not only for a simple match between soil evidence and an originating location, but also as information about that soil sample's origin if that location is not priorly known (Giampaoli et al., 2014).

Molecular Techniques

For non-human DNA samples, extraction and amplification techniques vary a great deal. Techniques for the extraction and amplification of human DNA may be partially adapted for some more closely related organisms, but the protocols for the majority of organisms must be specifically created. Extraction techniques differ widely due to the many forms of DNA evidence available, and amplification protocols must be tailored to the genetic organization of the source organism (Schiebelhut et al., 2017).

Extraction

Because the originating organisms of non-human DNA represent a very diverse group, the characteristics of various DNA-containing samples demand unique protocols. A few organisms require only a few changes to existing DNA extraction methods of human origin, but other samples necessitate different processing methods. Some samples, such as those from large plants, may require additional time for sample homogenization, while others simply need slightly different reagents due to unique cell chemistry.

Animals, Viruses, and Bacteria

The extraction of DNA from animals cells, in general, follows the protocols for the extraction of human DNA. Extraction protocols for viruses and bacteria also generally follow these methods. However, there are a few differences between human and non-human DNA, so a few modifications must be made to existing protocols.

A typical protocol for the extraction of DNA from human cells involves a few main steps. First, cell membranes are lysed in order to release the DNA. Second, nucleoproteins are separated from the DNA through enzymatic digestion and denaturation. Finally, DNA is separated from remaining cellular contents and components. Working from this basic outline,

multiple specialized extraction protocols have been developed for different types of DNA-containing evidence. Some of these more common methods involve organic extraction, extraction using Chelex beads, and a differential extraction technique for mixed samples (Chong et al., 2021). In addition to these base techniques, human DNA extraction often makes use of manufactured extraction kits such as the Qiagen DNeasy Blood & Tissue Kit (Qiagen, USA) and the Thermo Scientific KingFisher Cell and Tissue DNA Kit (Thermo Scientific, USA). These kits are highly specialized for human DNA and alternative protocols must be created for non-human DNA (Schiebelhut et al., 2017).

Overall, for animal DNA, an organic extraction method has yielded the best quality DNA. This particular method, the cetyltrimethylammonium bromide (CTAB)-phenol-chloroform method, has demonstrated high yield, purity, and efficacy in the extraction of non-human DNA. This method is fairly costly, however, so alternative methods may always be used for differing circumstances. (Schiebelhut et al., 2017)

Plants

The forensic use of plant DNA requires the ability to extract and analyze DNA from both fresh and dried samples. The extraction methods that have been created for plant material have thus been altered so that this is possible. One main method of extracting DNA from leaves uses liquid nitrogen to assist in grinding either fresh or dried material to a fine powder. The extraction process then proceeds by using the Qiagen DNA Mini Kit (Qiagen, USA) in order to extract the DNA (Craft et al., 2006).

The above extraction method has proven effective in more pliable plant samples such as leaves but extracting DNA from more robust materials is quite a bit more involved. One protocol for the extraction of DNA from woody tree trunk cross-sections requires many more steps. This

protocol begins by obtaining a sample of sawdust by drilling holes through the tree trunk samples. This sawdust is then put through a series of five mixing cycles in a mixer mill (MM400 Retsch®), each of which lasts three minutes and includes the addition of stainless-steel balls to the samples in order to assist in the grinding. Additionally, these rounds of mixing are separated from each other with storage at under -80 °C. Following this dry mixing process, high volumes of polyvinylpyrrolidone, proteinase K, and dithiothreitol are added to a lysis buffer and incubated before routine DNA extraction methods are carried out (Paranaiba et al., 2020).

Fungi

There are a few modifications that are made when extracting DNA from fungal samples. One specific protocol uses a modified form of the hexacetyltrimethylammonium bromide protocol (CTAB; Sigma). This modified protocol uses fungal mycelium samples specifically for DNA extraction. The samples are homogenized by vortexing using an extraction buffer and glass beads, then separated into two phases with chloroform. The DNA-containing phase is then precipitated with isopropanol and pelleted through centrifugation (Schwarz et al., 2015).

Amplification

The particular markers that are used for analyzing any given sample of DNA are very important. Markers are chosen so as to produce DNA banding patterns that are applicable to the analysis that needs to be performed. Different groups of organisms possess different overall DNA organization patterns, and so markers must be chosen specifically for each of these groups. In addition, markers must be chosen based on the objectives of the analysis. If the aim is to identify an organism’s species, the chosen set of markers will differ from that which is chosen to discriminate between individual organisms. In addition, a custom set of PCR primers must be generated for each set of markers. The specific primers chosen will in turn affect the

amplification process, as different PCR parameters must be set for each unique group of primers (Craft et al., 2006; Dawnay et al., 2007).

Plants

Plant DNA microsatellites are commonly used in the fields of ecology and population biology in order to study various topics. These topics include such areas as hybridization, seed dispersal, gene flow, and pollination patterns. These studies use particular microsatellite markers in order to discriminate between populations of plants as well as individual organisms. These technologies may also have the capability to have significant relevance in the area of forensic science.

Specific microsatellite markers have been shown to have the power to discriminate between plants and narrow down the origin of a sample of organic material to a single plant. In one case, which specifically examines a set of three *Quercus geminata*, or sand live oak, specimens, uses a set of four microsatellite loci. These loci were sourced from a set of closely related trees, specifically *Q. macrocarpa* and *Q. petraea*. The specific loci include MSQ4, MSQ13, QpZAG9, and QpZAG110. In this specific case, the allelic profiles at these four loci were able to securely exclude an individual tree as the origin of the evidentiary samples, suggesting that this approach would be similarly able to provide confirmatory evidence in other cases (Craft et al., 2006).

Fungi

The identification of fungi has not been widely researched, possibly due to the large number of species and high amount of morphological diversity between groups. Some of these morphological structures are not permanently occurring, which can make identification difficult. Complementary molecular methods have also been proposed in order to assist in identification. If

a universal DNA barcode could be determined, however, this information would greatly increase the accuracy of mycological species identification (Badotti et al., 2017).

The main genome section that has been proposed as a standard universal barcode for fungi is the nuclear ribosomal RNA internal transcribed spacer (ITS) region. However, this section is differentially variable between fungal groups. Some groups can be more accurately discriminated with this marker than others. These other regions may benefit from additional markers in order to better identify species. When using the ITS region for species identification, metabarcoding markers composed of ITS sub-regions may also be useful. Specifically, for the phylum Basidiomycota, the ITS_1 and ITS_2 sub-regions may prove helpful (Badotti et al., 2017).

Animals

For the majority of the animal kingdom, the same gene can be used to identify specimens to a species level. The genomes of many animal species are very similar, and most species display the same gene in their mitochondrial DNA that varies to a small degree between species. This gene is the cytochrome *c* oxidase I (COI) gene. Other genes such as the cytochrome *b* gene and the hyper-variable displacement loop are also widely used, but the COI gene is becoming more and more widely researched and used as a species indicator (Dawnay et al., 2007).

The COI gene is not a perfect choice, however, and not all species can be conclusively identified through the sole analysis of this gene. The Barcode of Life Database (BOLD) platform holds all of the species-unique COI barcodes reported so far, but not all of these barcodes can be tied to a single species. A collection of species from the genus *Tunnus*, representing a number of species of tuna, share the same barcode in respect to the COI gene. In these cases, additional research and analysis is often needed in order to determine if another gene is able to better

discriminate between the species and perhaps be used in tandem with the COI gene (Carvalho et al., 2015).

Viruses

Because viral DNA sequences vary so widely, no particular markers have been selected for general viral analysis. Instead, unique markers are chosen for each individual viral taxonomic group. As an example, the smallpox virus is in the genus *Orthopoxvirus* along with a number of other viruses such as the cowpox virus and monkeypox virus. Members of this particular genus possess a hemagglutinin gene that is unique enough for genus classification and also varies slightly between species, allowing for species classification (Espy et al., 2002). However, other virus genera would not necessarily be able to use this gene. Instead, a gene particular to that virus group would need to be identified and analyzed for its utility in discrimination.

Bacteria

Bacteria are also quite diverse, but their genomes show much more internal similarity than that of viruses. In fact, many different DNA markers that exist in the majority of bacteria have been suggested for bacterial speciation. One suggested marker is the *dnaK* gene, which codes for a heat shock protein that assists in protein folding and unfolding. Another, the *gyrB* gene, codes for a particular subunit of the DNA topoisomerase DNA gyrase, which helps to pack DNA molecules. A final third choice is the *recA* gene, which is important in DNA recombination. These choices, along with many unmentioned markers, all have their benefits and detriments, and selection would be highly dependent on the specific circumstances (Liu et al., 2012).

Conclusion

Non-human DNA is a powerful tool in the realm of forensic DNA analysis. The DNA typing of non-human DNA is able to yield information in many types of situations and can help

connect elements of a crime together. Non-human DNA that is found in a crime scene, on a victim, or on a suspect can be traced back to its origin in order to link these things together. Typing of non-human DNA is also able to discriminate between organisms of different species and even compare individuals of the same species. This information can be useful in situations where the species is important, such as in identification of the origin of a blood sample or verification of the identity of an animal product. Non-human DNA that is found at a crime scene can yield information about a sample's original location or how long a person has been dead through many ways. Fungi and insects display specific development patterns that can provide a timeline of death, and bacteria or animal fur can cling to evidence and serve to link that evidence to the scene of the crime. Non-human DNA evidence does come in a wide variety of forms, so the development of multiple unique processing and analysis protocols must be continued. Both extraction and amplification techniques must be tailored to the unique source of the non-human DNA. As these protocols are developed and reference data is accumulated, non-human DNA will become more and more often used in forensic cases, proving the impressive versatility of this novel type of evidence.

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