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Neeta Raj Sharma *Editors*

Crime Scene Management within Forensic Science

Forensic Techniques for Criminal Investigations

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Forensic DNA Analysis: A Powerful Investigative Tool

1

Lovepreet Kaur and Shiwani Guleria Sharma

1.1 Introduction

The use of deoxyribonucleic acid, i.e. DNA, for the testing in criminal justice explains the term forensic DNA analysis in simple words. It was first introduced in 1981. The term forensis which is a Latin word has given birth to the forensic science where forensic means pertaining to; thus, the term forensic sciences means the use of various applications for the resolution of criminal disputes either criminal or civil. The DNA analysis has become an indispensable part of the modern forensic science; with the use of PCR techniques, it has become a major tool for the analysis of the biological material. The method of DNA analysis used in forensic science is also known by the popular term DNA profiling [1]. The main focus of this science is on the use of the genetic material in case of the criminal justice for solving the cases and answering the concerns related to the cases. It has become a very significant source for establishment and expansion of the databases of DNA collected from the suspected criminals. The technique of DNA analysis involves the use of genetic material in this process; the sample in the form of hair, skin and blood is collected from the suspects that are linked to a particular or various crime scenes [2]. Then the large numbers of isolated DNA sample are interrogated with the DNA databases in order to match the profiles of DNA at the scene of the crime. A DNA database comprises of the profiles of different DNA that are used for the analysis of genetic diseases, genetic fingerprinting or genealogy purposes.

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DNA carries genetic instructions in form of molecules of nucleotides in all living organisms. It is organized into chromosomes in a very structural manner. The nucleotide molecule of DNA comprises of phosphate backbone sugar moieties and nitrogen bases, namely, adenine (A), guanine (G), thymine (T) and cytosine (C). A double-helix structure of the DNA is formed by the attachment of the nucleotides in such a manner that they form two long strands [3]. Each individual DNA contains the specific information related to their heritage. The information gathered from the molecules of the DNA can help to find out certain diseases occurred due to mutations. Only 0.1% of the DNA is unique to each individual which is what the investigator looks for; the rest 99.9% of human DNA sequences are same in every person [4].

Every year, various new technologies are invented to bring out the best approach under the field of DNA profiling. The process of the DNA analysis is not one step; various steps are followed for the analysis of any sample collected. Some of the steps are preparation of sample and extraction of the DNA from the collected sample which is followed by amplifications of the isolated DNA [5]. After amplification, the DNA quantification in which different sizes of DNA present in form of fragments is separated, and at last, the DNA profile matching is done. Various techniques have been updated for the use of the genetic material in investigation. Some of them are restriction fragment length polymorphism (RFLP), polymerase chain reaction, variable number tandem repeat, Y-chromosome analysis, short tandem repeat (STR) analysis, low copy number analysis, single-cell DNA fingerprinting, touch DNA, mitochondrial DNA analysis, etc. [6]. From the past three decades, the development of all these techniques divided the process of analysis under different time periods and frames such as that during first decade only, the method of exploration was used for the analysis in which the utilization of the restriction fragment length polymorphism was done but at that time the analysis through PCR was not specific. Moving further by the second decade, the method of STR was on top for the DNA profiling in criminal cases as these genetic markers provide specific results from the distinct STR loci. Moreover, they have high degree of sensitivity through the PCR amplification. Then the rapid growth of the DNA databases lead to the use of the new Y-STR and use of STR kits on demand in the process of DNA analysis [7]. There are four different types of DNA databases, namely, forensic, genealogical, medical and national; each type of DNA databases has a main role of storing data, but under categories, they are divided. A forensic DNA database comprises of the data that is collected from the crime scene. The collected sample from the sites are sent to the forensic labs where the DNA profiles are generated. These profiles are stored in forensic DNA databases in order to use them for future investigations. They are widely used for the criminal investigations. They are also known as national databases as they are governed by the government. The first national database was developed in the United Kingdom in 1995 [8].

Genealogical DNA databases store the genome sequences which are first submitted by genealogists or geneticist. They are not used for cases that are not registered under police station; they are used more specifically for storage of the genealogical DNA test results. GenBank is one of the most widely used genealogical databases for

the storage of the genome sequences. In GenBank, the data is stored under different categories as per their species specificity. The information related to the genetic variations is stored in medical DNA databases. Such databases comprise of all the medical information about an individual related to their health, disease, variations in genome or other genetic variations. This information stored in medical DNA databases is very helpful in drawing a correlation between various diseases and the environmental factors or a correlation between diseases and the lifestyle of an individual. By this information, the generation of new drugs can be achieved [9]. Medical DNA database is used in forensics in cases where the relation between diseases and the victim is need to be found in order to clarify the case in a better manner. Each database plays a great role in the forensic science as without history of profiles of DNA the comparison of new profiles generated from the suspect would be difficult to analyse to generate end results. Moreover, with the storage of different DNA profiles, we can figure out even if the same culprit or one single person is responsible for two or more crime, and also it will also save time for solving cases. Various techniques have been implemented so that DNA profiling can be used in forensic science effectively. Even the degraded and decomposed samples can be collected, and isolation of the DNA can be done from them. With advancements in science and technology, now the use of nanotechnology has been introduced in combination to forensic science so that new portable devices can be manufactured and the cases can be solved in less time at the crime scene [10]. Nanoparticle such as gold nanoparticle has a quality to attract the charge present on the DNA towards itself so generation of biosensors in combination with the nanoparticle will be a great idea to be utilized in forensic studies to generate portable products. Currently, various techniques of the DNA fingerprinting that are RFLP, VNTR, STR, SNP, low copy number and Y-chromosome analysis are used to solve cases.

1.2 Developments in Forensic DNA Analysis

In early 1990s, the only method used involves the utilization of the blood groups and serum proteins isolated from the crime scenes; in addition to this, the use of various electrophoretic techniques was the main focus for the study in forensic science for solving criminal and rape cases. Various techniques were used under the blood grouping such as ABO typing, MNS system and Rh factor of a person, but due to the high probability of similar blood groups in a population globally, it led to decrease in efficacy of these conventional methods [11]. In addition to this, the markers used in this method of analysis were isoenzyme markers or proteins, but in these techniques, the problem was that the DNA isolation was not successful from that of the highly degraded or decomposed samples collected from the site of the crime. The advancements in DNA profiling increased extensively after 1985; the modern techniques of the forensic science evoke from the first application taken from the work of Alec Jeffrey. In 1985, while working on the myoglobin gene, Sir Alec Jeffrey from the University of Leicester, UK, introduced the new modern technique of the DNA fingerprinting that can be used for solving various cases in a short time

period. He named the repeated sequence of the nucleotides as variable number tandem repeat after his observation that he made in regard to them. He observed the repeated sequences in a specific combination in a nucleotide. Further, he found out that these repeated sequences differ from person to person, thus being helpful to determine clearly about the various DNA sequences and their human link. He termed this method as multilocus testing. He used restriction fragment length polymorphism to solve a rape case. RFLP is used for the analysis of the distinct fragments of the DNA of different sizes. In this method, a restriction endonuclease is used to cut the DNA fragments, and further, with the help of Southern blotting, the location of the repeat sequences is established [12]. Later in 1990, another effective technique for DNA analysis was developed, namely, STR, i.e. short tandem repeat. It is also known as microsatellites and simple sequence repeats (SSRs). They comprise of 2–6 base pairs, short sequences. They are very helpful in representing the discrete alleles that are not identical to each other [13]. But these approaches fail to deal with the problem of the small samples of the DNA as small samples give poor quality of the fingerprints, thus leading to negative results. This issue was resolved by the low copy number analysis which was developed by the UK forensic science service in 1999. Earlier to this, in 1997 at Australian Genome Research Facility, another successful development of the single-cell DNA fingerprinting method was invented by Dr. Lan Findlay. This technique was less time-consuming as compared to the others [14]. Currently, analysis through restriction fragment length polymorphism (RFLP), short tandem repeat (STR), variable number tandem repeat (VNTR), dot blots of allelic sequence information and mitochondrial sequence determination are widely used, and results are acceptable. A broad range of specialists work under the one field that is forensic science; some of them are criminalistics. They use logical and critical thinking for the investigation of cases, digital evidence analysis, expertise for fingerprint, dentistry, odontology, nursing, pathology, and toxicology under which various substances used by criminals to attack on victim are studied and questioned documents are maintained by the investigators to keep a record of every individual (Fig. 1.1).

Currently, introduction of nanotechnology to the forensic science is under process, and various methodologies are adopted in order to save time. Moreover, a single integrated platform for the extraction, amplification and sequencing of the DNA has already been developed with the help of microfabrication of capillary electrophoresis, but validation of such techniques is still under the process in order to utilize them freely in forensic sciences for investigation purposes [15], such as the development of Sci-Fi, a handheld device that can be taken to the crime scene. It is defined as a lab on the chip; the chip would be enough to test the samples at the crime scene in order to generate their sequences of the DNA. This method will provide great advantage as the number of samples can be tested at a single time and place; moreover, it is less time-consuming. Morphological analysis of the skull using three-dimensional computer automated techniques is under study in field of the forensic biology. In addition to this, determination of the colour of skin, hair and eyes with the help of the various techniques of gene sequencing is under the next-generation technologies of the DNA fingerprinting. Use of virtual autopsy, that is, virtopsy, is in

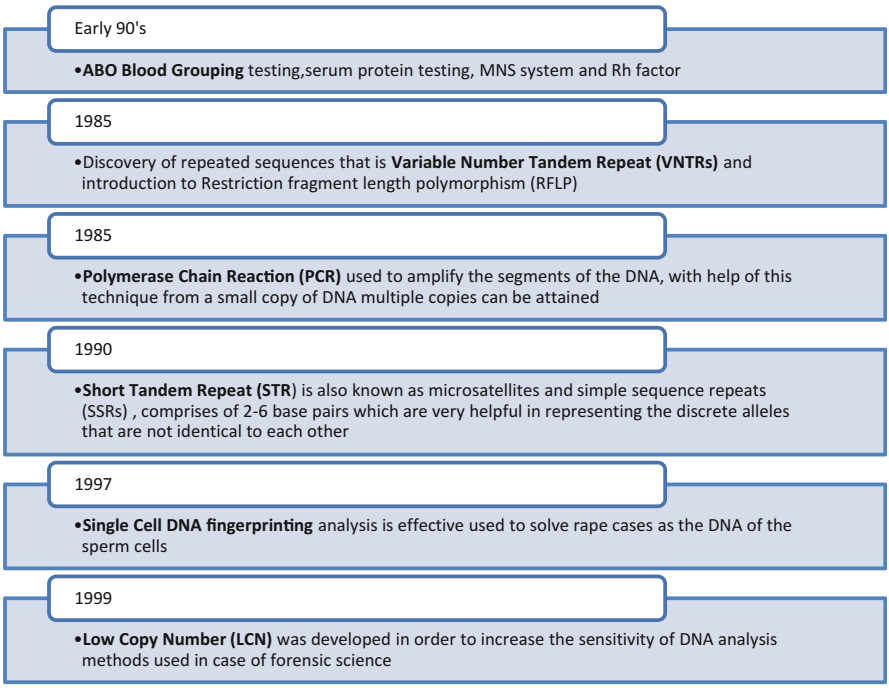

Fig. 1.1 Developments in various techniques of DNA analysis used in forensic science

the near future in coordination to the forensic biology. In this method, the collection of the images will be done [16].

1.3 Steps in Forensic DNA Analysis

The entire process of the DNA analysis is divided into four major parts, namely, serology, which further comprises of collection of sample followed by storage and its characterization. After serology is biology in which the isolation of the DNA is carried out; this involves extraction, quantification and amplification of fragments of the DNA and STR markers. The next step requires the technological aspects in which separation or detection of the DNA is done. After this, interpretation of the data is carried out to determine the characteristic of the isolated DNA from the sample [17]. At last, the role of genetics is there in which the statistical interpretation is done. This helps in the final analysis of the sequence of the DNA isolated from various samples. The genome sequence is compared with that of database DNA or with the suspect's DNA extracted so as to find out the actual culprit behind the crime. The detail process is explained as follows:

1. **Serology**: This is the initial step of any DNA analysis. In this step, firstly, the investigators report the crime site registered by police administration. Under this process, further collection of items, photography of crime scene, storage of samples like semen, hair, skin patches, blood or saliva, is done [18]. Further, after sample collection, the storage of them is mandatory in order to carry out any future investigations if required.
2. **Biology**: The next is based on the biotechnological aspects in which the collected samples are taken transported to forensic labs where the extraction of the DNA is done from both the victim and the suspected samples collected. After extraction, the quantification of the DNA is done in which the average concentration as well as the purity of the DNA are estimated. For the process of the quantification, spectrophotometer is used in which a fluorescent dye is used such as ethidium bromide or SYBR green dye is added to the samples; then, the samples are run on an electrophoretic chamber. The separated bands are visualized on transilluminator or on the gel documentation system [19]. The desired fragments of the DNA are amplified with the help of polymerase chain reaction in which from a small copy of DNA, multiple copies can be attained by using various enzymes such as DNA polymerases. DNA polymerases can be isolated from various organisms such as from bacteria. *Thermus acquaticus* generates Taq enzyme, similarly Pfu enzyme, from *Pyrococcus furiosus* and vent from *Thermococcus litoralis*. This process of amplification of DNA is done under controlled conditions. In the case of forensic science, PCR plays an important role for the identification of the repetitive DNA region [20]. After amplification, with the help of the STR markers, the regions are located, and the final analysis between the two sequences is done.
3. **Technology**: In this process, the separation and the detection of the isolated DNA sequence are done. The human and non-human DNA is separated so that the comparison of the other sequences from database can also be done to find out the person responsible for conducting a crime [21]. With the help of the bioinformatics tools and new technologies, the genome sequences are compared. GenBank is one of the most widely used genealogical databases for the storage of the genome sequences. In GenBank, the data is stored under different categories as per their species specificity. Thus, it helps to distinguish between various sequences of the DNA.
4. **Genetics**: In this step, the statistical interpretation of the collected data is done, and a final report is generated. The report contains all the information starting from the registered date of the case to the final report analysis. The information in a report consists of the photographs from the crime scene taken as evidence, list of samples collected as evidence, reason why the a specific person is taken as suspect and analysis of the DNA genome sequence that shows the matching of the two sequences giving clear information about the accused [22] (Fig. 1.2).

All the above-mentioned steps are carried out in forensic DNA analysis in order to solve the cases related to the criminal, paternity, and mass disaster and rape cases. The three possible outcomes are expected from the results obtained that include

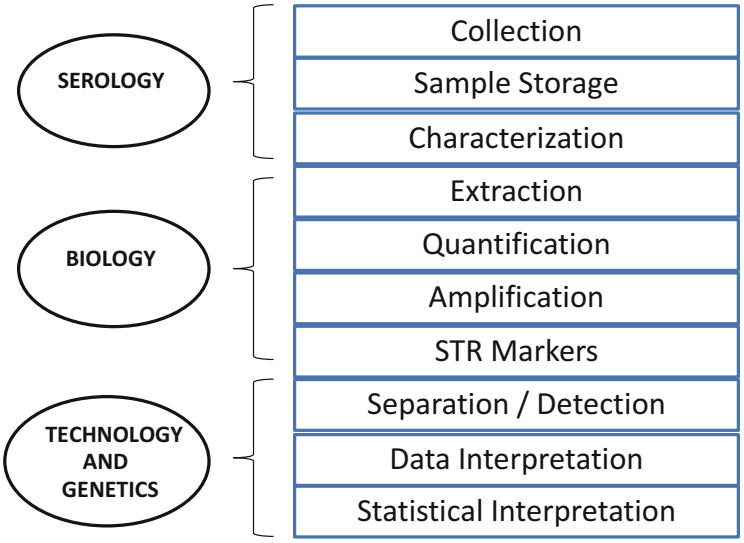

Fig. 1.2 Overview of steps involved in DNA testing

exclusion, inclusion and inconclusive results. Various techniques are discovered for the proper determination of the samples; some of them are low copy number, restriction fragment length polymorphism (RFLP), short tandem repeat (STR), variable number tandem repeat (VNTR) and mitochondrial DNA. Some of them are discussed as follows.

1.4 Various Techniques of DNA Profiling

- Restriction fragment length polymorphism (RFLP)
- Polymerase chain reaction (PCR)
- Short tandem repeats (STR)
- Low copy number (LCN) analysis
- Variable number tandem repeat (VNTR)
- Y-chromosome analysis
- Single-cell DNA fingerprinting
- Mitochondrial DNA (mtDNA) analysis
- Single nucleotide polymorphism

1.4.1 Restriction Fragment Length Polymorphism (RFLP)

This technique is used for the analysis of the different sizes of the DNA that are present in the form of small fragments. These fragments are formed by a digestion enzyme which is restriction endonuclease. It cuts the sequence of the DNA at a

specific site. The fragments obtained after the enzymatic reaction are separated using agarose gel electrophoresis. Furthermore, the fragments are separated by the technique of Southern blotting. Then with the help of multiple probes, the repeated DNA sequences are labelled with radioactive isotopes such as P^{32}. In place of radioactive isotope, a chemiluminescent dye can also be used. The probes can be either multilocus or single-locus probes as per the analysis requirements [23]. The difference in the DNA sequences present in a homologous manner can be detected with the help of RFLP. Most of the RFLP markers are highly specific in nature and work well under codominance conditions. Moreover, the method of development of the RFLP probes is quite simple. This technique was first used by Alec Jeffrey in 1985, when he was approached by the police; they convinced him to help them in the investigation of a rape-homicide case. This technique is effectively used in studying the evolutionary relationships, wildlife migration and breeding pattern in case of animals and for the detection as well as diagnosis of certain diseases [24]. As for the detection of various disease, the researcher can collect the DNA of family members so as to draw a comparison and find out the location of the affected gene and similar patterns of inheritance if occurred.

1.4.1.1 Advantages of RFLP

- A wide range of detection and diagnosis of diseases can be done.
- Specific for locus, thus helping in detecting the gene responsible for a particular disease.
- The evolutionary relationships can be studied.
- Highly reproducible in nature.
- Codominant markers making them species-specific.
- Utilize in genome mapping.
- No prior information on DNA sequence is required.

1.4.1.2 Despite of the Advantages of this Technique, Some of the Demerits Are

- Slow and tedious.
- Requirement of large amount of DNA sample.
- The quality of DNA should be good.
- Expensive technique.
- Radiolabelled probes are required.
- Probes are not available for every species.

1.4.2 Polymerase Chain Reaction (PCR)

This technique was developed in 1985 by Kary Mullis. It is a molecular biology approach that is used to amplify the segments of the DNA. With the help of this technique from a small copy of DNA, multiple copies can be attained by using various enzymes such as DNA polymerases. DNA polymerases can be isolated from various organisms such as from bacteria. *Thermus acquaticus* generates Taq

enzyme, similarly Pfu enzyme, from *Pyrococcus furiosus* and vent from *Thermococcus litoralis*. This process of amplification of DNA is done under controlled conditions [25]. This technique has a wide range of applications in the field of biotechnology; it can be used in genetic engineering, protein cloning methods, forensic DNA fingerprinting and paternity testing. In addition to this, it is effectively used in the analysis of various environmental samples. In the case of forensic science, PCR plays an important role for the identification of the repetitive DNA region [26]. These repetitive DNA are present outside the coding regions of the DNA. They are different from individual to individual. The process of the amplification requires two oligonucleotide primers; these primers are designed in such a manner that they are able to hybridize the opposite strands of the target sequence.

1.4.2.1 Polymerase Chain Reaction Method Is Carried Out as Follows

1. **Initialization:** This is the very first step in which only DNA polymerases are required, as they are needed to get activation by heat. For this purpose, the heating chamber is raised to 94–96 °C. Sometimes, in the case of thermostable polymerases, the temperature is increased to 98 °C for 2–10 min [27].
2. **Denaturation:** In this step, the hydrogen bonds break which are present between the two complementary bases of the DNA molecules, thus leading to melting or denaturation of the DNA. Temperature 90–98 °C is maintained for 10–20 s [27, 28].
3. **Annealing:** In this step, temperature is lowered so as the primers can anneal to the single stranded templates of the DNA. For 20–40 s, the temperature is maintained at 50–65 °C. The temperature should be specifically maintained as it should not be too low or too high; a moderation is required so that hybridization of the primer can occur on the specific target of complementary strand. In this step, the DNA formation begins when polymerase binds to the primer hybrid template [28].
4. **Extension and elongation:** In this step, the addition of the free deoxynucleotide triphosphates (dNTPs) from the reaction mixture; these are complementary to the template in the 5′-to-3′ direction. An optimum temperature is used for the thermostable Taq DNA polymerase enzyme which is 72 °C for 3–15 min for the last cycle of the PCR. After this step, the temperature of the chamber is decreased to 4–15 °C. This stage is termed as final hold; the main purpose of lowering the temperature is to cool down the reaction chamber [27].

This technique is highly preferred for the forensic analysis when the sample is minute or damaged as the amplification of DNA can be achieved by using this method and further analysis can easily be carried out. For instance, a rape case was registered in police under Indian panel code, the victim was brutally gang-raped, the sample was collected to confirm the criminal, and a real-time PCR was performed for the quantification of the DNA samples at State Forensic Science Laborartory. It can also be used for the detection and diagnosis of various diseases in such a manner that we can find out whether the disease has occurred due to mutations or some sort of inheritance [28]. Preimplantation diagnosis is widely used in case of in vitro fertilization approach as this helps in the prevention of defective births of neonatal.

Table 1.1 Various advantages and disadvantages of PCR in DNA analysis

Advantages	Disadvantages
Replication of specific nucleotide sequences from low levels of DNA or degraded DNA	Requirement of special markers that are specific for locus
Creation of large amount of DNA from a very small sample	Lower specificity towards culture or staining
Detection of diseases	Costly protocol
Require small sample for analysis	Chances of contamination
Less time-consuming	Possibility of amplification of unknown flora

Two widely used techniques for the process of DNA profiling under PCR are allele specific oligonucleotide and amplified fragment length polymorphism.

- **Allele specific oligonucleotide (ASO)**: It is short sequence oligonucleotide of 15–21 bases of nucleotides which is synthetic in nature and complementary to the sequence of the variable target DNA. In case of molecular techniques such as Southern blotting or dot blot which are effectively used in forensic science investigations, it acts as a source of probe. It is used for the diagnosis or detection of diseases such as sickle cell anaemia, which is caused by an altered mutation in the codon region [29]. In ASO, a complementary region is prepared to the test region in order to diagnose the disease.
- **Amplified fragment length polymorphism (AFLP)**: It is a technique used to detect various polymorphism among the different genomic regions. It was demonstrated in 1993 by Vos and Zabeau. It is used for the identification of various variations in genetics in same or distinct strains. In a single time frame, AFLP has a great capacity to amplify 50–100 fragments in one go. In addition to this, it is highly preferable technique for the analysis in criminal, paternity testing and generating linkage maps for the process of further quantitative trait analysis [30]. The process of amplified fragment length polymorphism involves the cellular DNA digestion with the help of some restriction enzymes followed by ligation of site specific adapters to those restriction fragments. The next step is the amplification of the fragments by the use of primers which are corresponding to adapters and restriction sites. At last, gel is run over an electrophoretic chamber to obtain bands which can further be visualized [31]. This method of DNA analysis is widely used in the study of various taxa; the main advantage of this is if the genomic makeup is not known, still one can do analysis and study of taxa by using this approach. Some of the demerits include the development of the locus – specific markers for the individual fragments are difficult (Table 1.1).

1.4.3 Short Tandem Repeats (STR)

They are also known as microsatellite and simple sequence repeats (SSRs). Just as variable number tandem repeats, the STR are short sequences of 2–6 base pairs long.

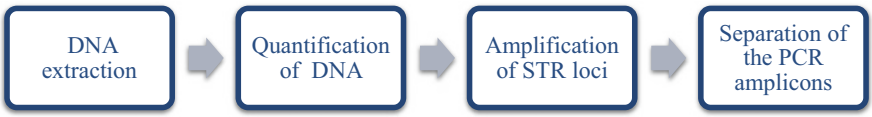

Fig. 1.3 Steps involving in DNA profiling through the process of STR are explained

In 1990, this technique was successfully used in forensic DNA analysis for the investigation purpose as they represent those alleles which are distinguishable from each other. In case of evidence, loci is stable, and even small amount of sample can be used as a short length of fragments is required [32]. This technique of DNA analysis in forensic science requires the use of polymerase chain reaction (PCR) for the process of amplification of short tandem fragments. STR is widely used in genetics for the construction of the linkage maps through linkage maps; diagnosis of genetic disorders can be done. STRs are divided on the basis of the length of the repeats as mono-, di-, tri-, tetra-, penta- and hexanucleotides. Due to the polymorphic nature and loci specific of the STRs, they are considered by the manufacturers to be in kit (330). They vary in size from person to person; such repetitive sequence does not affect the genetic health of the individuals. Mostly, they are found in non-coding regions, but in case of coding regions, they are even less than 10%. Special codes are used for the representation of the STRs, for example, D13S317; in this, D means DNA, 13 is the chromosome number on which the STR is located, and S stands for STR while the unique identifier is 317 (Fig. 1.3).

The very first step is the isolation of DNA by a process called DNA extraction, which is followed by the quantification of the DNA in the sample and at last the separation of the PCR amplicons [33]. The separation of amplicons is done on a genetic analyser by the utilization of bioinformatics tools that help to analyse the resulting data and compare the data from one specimen to databases which has the housing previously generated STR sets, thus helping in the final determination of the criminal among the suspects under study from the crime scene.

For investigation purposes, the samples in the form of bloodstains, semen or some biological traces from the victim's body are collected from the crime scene. This collected sample is investigated by forensic scientist in order to use such evidences for tracing the criminal by comparing the DNA profiling reports with databases of the DNA; after this analysis, the criminal can be found easily if the profile matches [34]. For instance, in Fig. 1.1, the sample from the crime scene was collected and then compared with the two suspects. From the analysis, it has been observed that the suspect 2 DNA profile shows repeat sequences of the STR loci which were identical to the evidence (Fig. 1.4).

The above diagram clearly explains that the repeated sequences were observed in case of sample 2; they are matching with evidence collected. Thus, this is how the process of short tandem repeat helps to solve the forensic science cases in order to trace the main criminal of the scene [36].

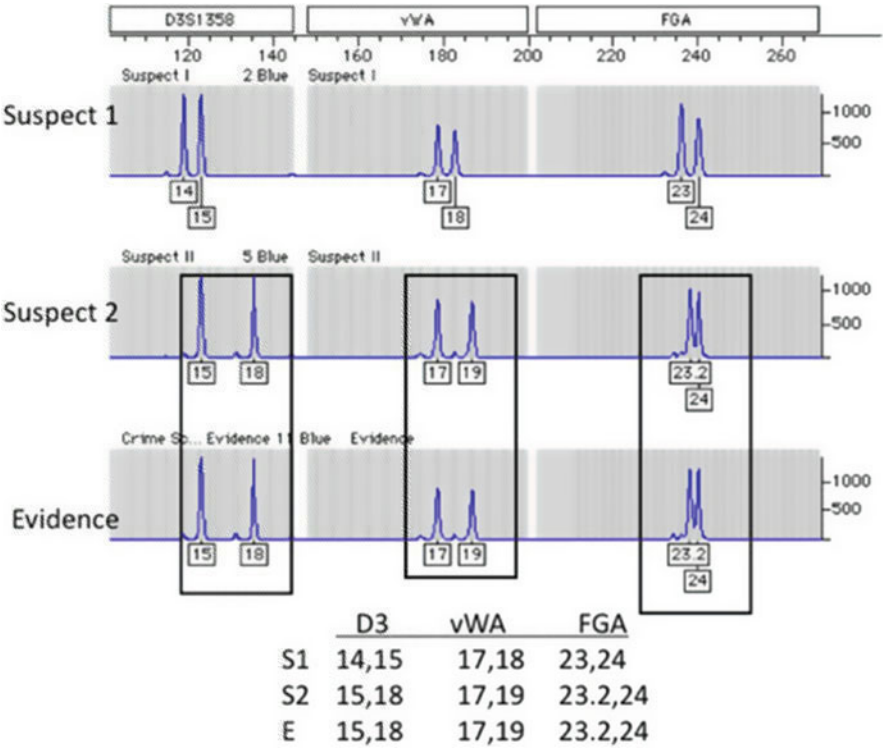

Fig. 1.4 The repeat sequence of Samples collected and the evidence [35]

On the Basis of the Pattern of Repeats, the STR is Divided into Following Categories:

1. **Simple repeats**—they contain similar length and sequence of the units.
2. **Compound repeats**—they are formed by combining two or more simple repeats.
3. **Complex repeats**—multiple repeat blocks of variable length of the units with intervening sequences are present.
4. **Complex hyper variable repeats**—due to allelic nomenclature issues, they are not widely used in forensic studies. The alleles differ in size and sequence, thus making it difficult for genotype reproducibly [37].

Various Applications of Short Tandem Repeats are as Follows:

1. They allow multiplexing due to narrow size of alleles.
2. From degraded DNA samples, the information can also be recovered by using STR method as they only require short repetitive sequence to analyse information.
3. The chances of mutation are low in this method.

4. Use of separate chromosomes in STR markers makes the technique more simple and unique, thus preventing any problem related to linkage between the markers.
5. They are highly preferred in DNA profiling as they can easily amplified by using PCR techniques without any complications.

1.4.4 Low Copy Number (LCN) Analysis

This technique was developed in 1999 by the UK Forensic Science Service. It was developed in order to increase the sensitivity of DNA analysis methods used in the case of forensic science. Low copy number refers to the process of analysis of template DNA which in amount is less than 200 pg in a sample. In case of standard techniques, the PCR cycles are only kept up to 28, but in case of LCN, for the better quality of results, the cycle's number are increased to 38 PCR cycles [38]. In order to increase better sensitivity to results in case of LCN, various approaches are used in which the amendments are made in case of pre- or post-PCR cycles. Other methods that can help in achieving better results via this method include the use of nested PCR, reducing the volume of the PCR, and the use of pure formamide in case of sample preparation that need to be used in capillary electrophoresis.

There are Various Disadvantages of this Technique as:

- The chances of the error are more as compared to other techniques.
- These profiles generated by LCN are not much reproducible.
- Major problem occurs when different profiles mix during LCN typing; then the results are not reliable.
- In case of LCN, the assay is very sensitive so the samples that need to be analysed require effective handling.
- Some reagents and chemicals used may contain extraneous DNA in very low amounts; these can interpret the results.
- High chances of contamination.

This technique is used for the identification of the cases where biological evidences are compromised and other method for DNA analysis cannot be used. An example of this technique is used in DNA analysis where the results were not reliable due to inappropriate measure used by the technicians in forensic laboratory [38, 39]. In 1998, terrorist attack happened at Ireland in which 200 people severely wounded and 29 people died; the police administration suspected an electrician behind the attack. He was 38 year old. Evidence was collected from the site, and that to sample was collected from the suspect and LCN analysis was done, but unfortunately, the results did not show any matching between the suspect and the evidences collected. At the courtroom, the judge declared that due to the ineffective handling, not using of appropriate measures for handling the evidence and not using precaution while doing LCN analysis, the results are inappropriate [39].

1.4.5 Variable Number Tandem Repeat

In 1985, this technique of DNA analysis was discovered by Alec J. Jaffreys. They are comprising of 7–10 base pairs and are also known as minisatellites. In human genome, either one copy of the variable number tandem repeat locus or multiple copies are present. These VNTRs are inherited from parents to offspring. As per their number, they are divided into two categories, i.e. unique loci and multiple loci [40]. An example of the use of VNTR in forensic DNA analysis is as follows:

Dr. Jeffrey conducted DNA analysis on various cases to know the real culprit. He conducted an analysis by taking samples from Leicestershire area crime scene, along with the police. The saliva and blood samples were collected from 4000 men, but none of the sample matched the evidence collected. Later, a person named Colin Pitchfork confessed that he was paid money to give false sample. After this, he was prisoned, and a sample of him was taken, and DNA analysis was done. The results obtained showed matching of the VNTR to the evidence collected from crime scene. Pitchfork was the first person who was sentenced to jail as he convicted the murder. With the help of VNTR technique, various other cases were solved. In 1987, with the help of this technique, a rape case was solved in the United States. The semen sample collected matched to the traces recovered from the body of the victim [41].

1.4.6 Mitochondrial DNA (mtDNA) Analysis

Mitochondrial DNA is isolated from the mitochondria of the cells. Mitochondrion is also known as the power house of the cells. Mitochondrial DNA is present in a circular form. It comprises of 16,569 base pairs. A minute variation is present between the sequences of two individual as this sequence of base pairs is highly conserved but entirely functional in nature. Out of this 16,569, the 1000 base pair long sequence which is known as the control region consists of non-coding D-loop. The non-coding regions contain hyper variable regions which further undergoes variations [42]. The variations are termed as single nucleotide polymorphic (SNP) regions; they are the main regions of this sequence and are more focussed by the forensic investigators. This technique of mitochondrial DNA analysis is highly effective in forensic investigations in cases where nuclear DNA analysis cannot be done. Such possibility occurs in case DNA damage is high due to either burn samples or hair without root. Moreover, this method is highly used in case of lineage studies as mtDNA is inherited only from mother to offspring, so to form an analysis on lineages, this method can effectively be used. Various steps involved in the process of DNA profiling via the use of mtDNA include primary visual analysis, preparation of the sample followed by DNA extraction and then steps of PCR, i.e. amplification and post-amplification. It is important to carry out PCR step carefully as the area of interest is a hypervariable region [43]. After the PCR, the isolated product is quantified, purified and automated; DNA sequencing and data analysis are done. It is advised to handle the samples carefully starting from the collection to analysis in order to prevent any mixing of the samples and to prevent

cross-contamination between samples. This technique is highly used in case of the missing individual. A reference sample is not available or in case of mass disaster [44].

The first case solved in forensic science by using this methodology was related to a 3-year-old child who disappeared from her own residence. After 2 years of this incident, the remains of a human child were found approximately 3 km away from their residence. From that site, the skeletal remains were collected from the desert. Using techniques of DNA profiling, the control regions of mtDNA were recovered. Those recovered regions were matching to that of her mother mitochondrial DNA [44]. It was observed that mtDNA typing can be used in case of missing individual cases as well as in sexual assault cases.

1.4.7 Single-Cell DNA Finger Printing

In 1997, another great technique of DNA fingerprinting was developed by Dr. Lan Findlay; along with few of his colleagues, he developed the method of doing DNA profiling from a single cell. They discovered this new approach at the Australian Genome Research Facility. In this method, a single cell is isolated by using the technique of microscopy prior to final analysis, the cells obtained for identification are collected by swabbing the material, and then, with the help of microscope, initially, the identification of the cells is done [45]. This method of DNA analysis is effectively used to solve rape cases as the DNA of the sperm cells is highly conserved and they are compacted in a protein head. Moreover, this technique is quite fast solving cases in hours, thus making it easy to find out the criminal at the same point. Single cells can be obtained from the fingerprints or marks on pens and car keys, but the only limitation to this method is the requirement of the DNA. More than 1 ng of DNA is required which is equal to 200 cells.

1.4.8 Y-Chromosome Analysis

In this technique of DNA analysis, the more focus is given on the different types of marker, i.e. amelogenin marker. These markers are only present on the sex chromosomes. A specific part of the Y chromosome of the males is used in forensic DNA analysis. This technique is widely used in case of paternity disputes of male child, in case of sexual assault and traces the donors of the missing persons. Various new systems have been developed in order to analyse the short tandem repeats present on the Y chromosome; one such system is applied biosystems [46]. Y-DNA analysis involves the analysis of short tandem repeat segments on Y chromosome. These STRs are first recognised as genetic markers. These repetitions vary from person to person, and STR present on the Y chromosome contains a unique DYS number. In case of this method, the test usually involves the examination and analysis of the 10–100 short tandem repeats that are present on the Y chromosome.

The sample from the crime scene and suspect are isolated and looked for these repeated sequences.

1.4.9 Single Nucleotide Polymorphism

In cases where DNA is badly degraded, the technique of DNA analysis used is Single nucleotide polymorphism (SNPs). Single nucleotide polymorphisms are present in abundance in human genome. They particularly have low rate of mutation, and the size of the amplicon is also small. Basically, SNPs are caused due to point mutations. They are present in the noncoding regions of the genome sequence. For the sequencing under this technique, some basic steps involved include the development and identification of SNP with the use of shotgun sequencing, PCR amplicon targeted sequencing and RNA sequencing. The short fragments can be amplified by using SNP technique for DNA analysis in forensic science; thus, this make easy to solve the cases where DNA sample is degraded or low quantity of DNA template is available. Moreover, due to their low mutation rate, they are regarded as more stable in nature so they are effectively used in the reconstruction of the pedigree and lineage. They can also be used in the identification of the individuals and phenotypic inference studies [47].

In the Case of Forensic DNA Analysis, These SNP are Divided into Four Categories:

1. **Identity testing single nucleotide polymorphism**—in case of individualization where low inbreeding coefficient and high heterozygosity is required. They provide the genetic information in order to distinguish between the different individuals and also help to exclude those suspects or samples that are not part of the putative family member [47].
2. **Lineage informative single nucleotide polymorphism**—in this, the markers are used for the identification of the missing persons through the process of the kinship analysis. Tightly linked SNPs are used that function as haplotype markers (47, 48).
3. **Phenotype informative single nucleotide polymorphism**—they are used to establish the high link of the probability regarding the phenotypic characteristics of an individual such as skin, hair and eye colour as an investigation [48].
4. **Ancestry informative single nucleotide polymorphism**—they are used to establish the high link of the probability regarding the biogeographically characteristics of an individual in link to the phenotypic relationships [48].

Single nucleotide polymorphisms (SNPs) have great advantages in forensic DNA analyses because of the presence of abundant potential markers and amenability to automation. In addition to this, they can be used for the phenotypic identification of the suspect as the physical description of a person can help to portray the individual, thus making it easy for the bureaucrats to solve some cases. Despite of all these advantages, some limitations are that SNPs are biallelic in nature so they are less

informative for the identification testing as compared to the other methods such as STR [49]. Moreover, some ethical and legal concerns arise in the use of single nucleotide polymorphism because the noncoding DNA regions are used and some rules and regulations are set by the higher authorities that need to be followed as the privacy is the main concern in any case of the DNA profiling. Gene-gene interaction acts as a hurdle for solving the cases of phenotypic informative SNPs.

1.5 Challenges in Forensic Science for DNA Profiling

- Various challenges are faced by the investigators at the site or even with the direct contact with victim as in some cases like rape or sexual assault when the victim is not ready to give evidences or even sample for the DNA profiling to match with the suspected person. This could be due to family or social issues that some people do not want to disclose the right information; thus, it's a challenge in such cases to catch the culprit [50].
- The sample collection needs a great focus as in some cases when the requirement is of blood sample but the authorities related to case fail to provide it and as in some cases the law enforcement people give a number of items collected from the crime scene to the forensic laboratory to solve the case while in actual the requirement is not so. It requires a critical thinking to choose the item which should be sent for further investigation [51].
- Chances of error are high in case of handling the sample of DNA. Sometimes the sample is collected from more than two to three individuals. This can even lead to mixing of samples. Mixing of samples led to challenge for analysis for the DNA in order to produce desired results [52].
- Requirement of developed new bioinformatics tools in laboratory for the handling of large number of samples in case of mass disaster. In such cases, there is requirement of handling, managing and analysis of the collected samples in a huge number; thus, the trained technical staff is required. Moreover, the laboratories are not prepared to handle the complex mixtures [50, 51].
- Conflict of interest between the bureaucrats and forensic investigators as they tend to look more in the history but the scientist shows their interest towards the future [51].
- Sometimes, even the bureaucrats do not want to disclose the right criminal due to social conflicts and corrupt officers, so they try to misinterpret with the data in order to hide the truth, and there is a possibility that DNA can be replaced by the non-criminals sample even at the crime scene [50].
- In case of DNA analysis for solving criminal cases, the degradation of the samples is the major issue which occurs due to mishandling of samples and inappropriate labelling issues. DNA degradation starts with contact in sunlight or heat so proper handling by technicians is required [51].
- In order to mislead the investigators, sometimes, the culprits try to use synthetic or fake marks of DNA. They left fake marks of DNA at the site which creates complications to analyse the different sample. One such case was reported in

1922; John Schneeberger who was a Canadian physician uses fake DNA sample. He raped one of his patients and left someone else semen sample, so at the time of investigation, the blood sample collected from John and the semen collected did not match; thus, it led to confusion among the investigators to solve the case [52].

- Trouble with various instruments that are used, old instruments and biological contamination of the tools led to unreliable results.
- Hacking of the DNA databases is the main concern. The DNA databases comprises of all the information regarding DNA profiles; thus, hacking by the past culprits with the use of technological innovations is the main cause and the challenge that the forensic sciences has to face.
- Various ethical norms act as a major hurdle in case of solving sensitive cases like rape or acid attacks as the privacy of the individual with regard to community and religion affects the process of solving cases. Sometimes people are unwilling to disclose what actually happen. Such scenarios act as a challenge for forensic science investigators for solving a case. Moreover, the privacy concern is related to DNA because it contains a lot of information of an individual such as family relationships and diseases related to a person [51].
- Another major challenges faced by the investigators is at the times when the damage to the body is more, in cases related to burn, sometimes, the criminal in order to hide any evidences burns the body of the victim or the surrounding area; this led to great challenge for the extraction of the DNA from such sites [50].
- A large number of cases are solved per day globally using various techniques of DNA profiling, so numerous data is stored regarding profiles of the DNA of either suspect or victim. For the storage of this data, an expansion of the DNA databases will lead to overburden of crime laboratories which is another major challenge that requires scientific experts to maintain the privacy of this data in order to prevent it from hacking [52] (Fig. 1.5).

1.6 Cases Resolved Using Various Techniques of DNA Analysis in Forensic Science

1.6.1 Case 1

A married couple was found dead in the city of Kicevo. Their bodies were corded and hanged. The samples were collected from the crime scene; blood was extracted from the body of the victims, and the nail debris was taken into consideration as a sample. The collected samples were transported to the forensic laboratory for the process of the DNA analysis. Another sample from rope was isolated as suspect to find actual criminal. The method of DNA profiling was continued with extraction of DNA and amplification of it, and with STR typing, the DNA profile was generated which was sent to forensic DNA database of Macedonian. It was observed that the results showed matching of DNA profile to an unknown male to that of sample collected from the legs of male victim. At the same time, another case was reported of burglary in church. During the investigation, they found bloodstains near the

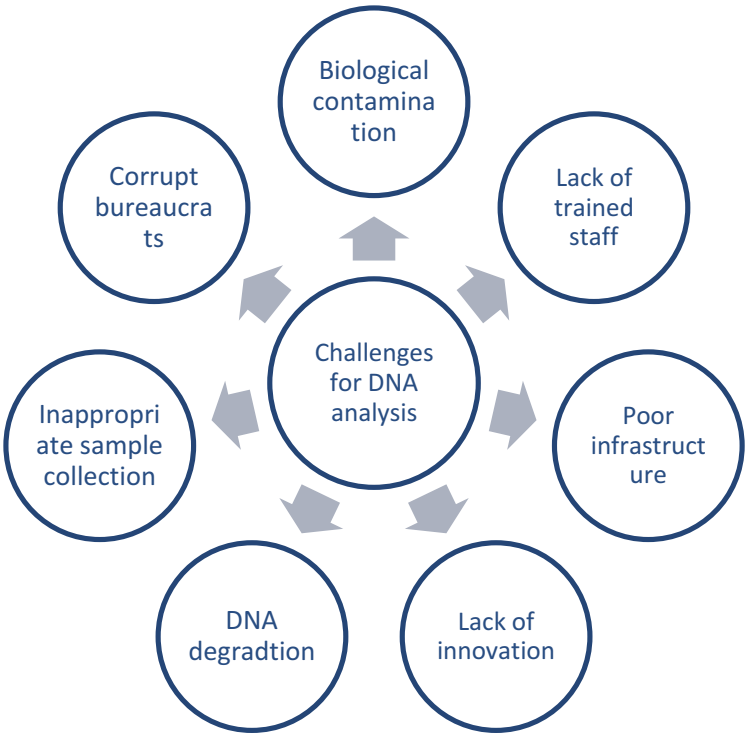

Fig. 1.5 Challenges in forensic DNA analysis

window of the church. The sample was collected from the suspected person, and the DNA analysis was done. The results matched to that of the suspect. The same DNA profile of the culprit was used to run analysis with earlier two profiles of couple murder, and it showed positive results [53]. Thus, the same person was responsible for the murder of married couple and the burglary of the church. This was concluded from the reports analysed by performing DNA analysis.

1.6.2 Case 2

Another case was solved using DNA analysis techniques in forensic sciences. This case was reported regarding the murder of a young man of 35 years old. He was found shot dead in his backyard. Policemen and a team from forensic science reached the crime scene, and as per the witness, several men were observed at the scene. Just after the death, all left. The team started their research and collected three evidences: a handgun, pair of gloves and sleeves of two shirts. The DNA was isolated from the collected evidences, and by using VNTR method, the case was resolved [54]. The results showed matching of profile to an unknown person; this profile was then matched with the DNA data profile stored database of CODIS. The

hit was matched, and shooter was sentenced to in prison for 65 years for murder and robbery.

1.6.3 Case 3

In Bronx, by using low copy number, a case was solved which was related to a gunshot. In 2008, a guy (victim) and his brother had some argument with a group of teenagers. By one of the member of that group, the victim died who was shot with a gun by two bullets. Days after the defendant went to New York, later, he was arrested there as a gun was found in the cavity of the wall in his apartment. From the evidence of the video shoot, an eyewitness to bullet shot case identified that the same man as the shooter. The sample was collected from the person and sent to forensic laboratory to conduct and matched the evidences collected from earlier crime scene. With the technique of low copy number, it was clarified that he was the shooter [53]. He was sentenced to jail for 20 years for the case against weapon charge and manslaughter.

1.6.4 Case 4

A female body was found degraded in an open field during the month of July in 1990. The identification of the body was near to impossible for analysis by individual clothes and fingerprints. The investigators collected fragments of some bones, specifically heel bone and fibula. The samples collected were sent to forensic laboratory for the further DNA isolation and analysis. They tried to amplify the hyper variable regions within HV1 and HV2 present in mitochondrial DNA. On the other side, blood sample was collected from the putative sister of deceased. After analysis, the profile showed matched sequence between the sample collected from decomposed body and her sister. The technique of mtDNA of DNA profiling was used to solve this case as the mtDNA methodology is highly effective to provide reliable results in case of totally decomposed bodies and missing cases [54].

1.6.5 Case 5

In 2012, from the last known place of King Richard III in Leicester, a skeleton was found at Grey Friars. In order to find out if the remains excavated belong to that of King Richard III, the sample was collected in the form of bones, and sequencing was done. The HV1 and HV2, the two hyper variable regions of the mtDNA, were sequenced from the collected sample and collected. This sequence was compared with the sequence obtained from the sample collected from living relatives of King Richard III. The results showed perfect match between the two sequences, and thus, it was confirmed that the remains are of King Richard III. The technique of mtDNA

was widely used for the DNA profiling in cases where mass graves were found in order to determine their lineages to human [53].

1.6.6 Case 6

A 16-year-old girl's body was found in a dense forest on July 6, 2017. She went to school on 4 July and didn't come back, so her parents filed an FIR in nearby police station. Later, her body was found. The case was handed over to Central Bureau of Investigation as numerous marks were found over her body; the team of CBI examined the case and found that the little girl was gang-raped and murdered. The samples from the crime scene were collected which included blood and semen samples; for more evidence, a bottle of liquor and clay were also collected from the crime scene. Later, from approximately 250 people from nearby areas, the blood sample was taken in order to match to the DNA isolated from semen sample, but initially, the results came negative. A percentage and lineage test was conducted by the investigators, and fortunately, they found some match which was with a family from Kangra. Then the samples were collected from that family member to carry out further research and find the true victim. After narrowing down their analysis, they found a person named Anil whose sample was showing matched to that of the DNA sample collected from the crime scene. Anil was arrested by the police, and from the details given by him, the other five suspects were also arrested [53]. By using DNA profiling technique, their samples showed match to that of the semen sample collected from the site. Thus, the case was solved, and culprits were punished.

1.6.7 Case 7

In 1995, a murder case was reported in Utah. A female named Beslanowitch was 17 years old and found dead near Provo River. Her skull was crushed with a hard stone which was later collected from the crime scene for investigation. At that time, the techniques of DNA analysis for forensic science were not fully developed, but the officer investigating this case was curious and dedicated to know what has actually happened. So, later in 2013, when new DNA technologies were introduced, he decided to forward the case to forensic team along with the stone collected from crime scene [54]. By using the technique of touch DNA and with the help of forensic vacuum for the extraction of the DNA, an analysis was made. From the results obtained, the DNA matched to a bus driver who was working in 1995 in a resort near to crime scene. The criminal was arrested and sent to prison.

1.6.8 Case 8

A lady reported a case that she was raped and filed a case against the person to be responsible father of her child. The police started the investigation, and they decided

to carry out the method of DNA fingerprinting to solve this parental issue case. The blood samples were collected from the women, child, and the person he filed a case against. Following the initial step of the isolation, the DNA was extracted by organic extraction method. Further, the quantification of the DNA was done. In order to increase the quantity of the DNA sample, PCR technique was used for the amplification, and at last, the sequence was generated. With the help of the STR technique of the DNA fingerprinting, a 16 loci STR sequence was generated and analysed [53]. The results obtained were quite shocking as the alleles obtained showed matching of the maternal alleles to that of the child, but the suspected person was not showing any matching of alleles, so he was not supposed to be the biological father of the child. Thus, the techniques of DNA profiling in forensic science help to secure the innocent suspects and to punish the real culprits.

1.6.9 Case 9

In woody mountains of the Italy, two corpses covered with very thick vegetation were found. The investigation on them was carried out by the Bresic Forensic Institute; they collected the remains from the area and carried out various investigations on it to determine their age, sex and morphological characteristics from the collected samples. The necroscopic investigators concluded that both were males and their cause of the death was injuries due to gunshot or stabbing. In addition to it, by using mtDNA technology, they were able to analyse the DNA profiles which later showed matched to the three suspects that were arrested for this crime [53].

1.7 Applications of DNA Analysis in Forensic Science

- **Generation of DNA data banks**—Presently, the technique of the DNA fingerprinting is widely used in forensic science for solving various cases, and a lot of DNA is isolated to generate a sequence. Each individual has a specific sequence, and each sequence plays an important role for generating a DNA database. In order to preserve the DNA fingerprints of all the collected samples on daily basis, the Federal Bureau of Investigation (FBI) has created data banks [55]. These data banks are maintained and handled by potential team of expertise, thus providing a number of resources to the people working and maintaining them. Moreover, these databanks are maintained in order to solve the criminal or any other cases by the use of techniques of DNA fingerprinting.
- **Paternity determination**—The technique of the DNA analysis plays an important role to solve the paternity dispute cases of various offsprings. Moreover, it can also be used to identify the dead person. In the cases in which the bodies of the victim are completely burnt, the bones can be used to determine the DNA of the body in order to find out the true identity of the person. Earlier, the ABO blood typing was used, but now with the advancement in science and development of