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FUTURE ASPECTS OF FORENSIC BIOLOGICAL EVIDENCE: USE OF SEQUENCING IN FORENSIC DNA ANALYSIS

Riya Kaushik[1], Postgraduate Student, Amity Institute of Forensic Sciences, Amity University

Dr. Biswa Prakash Nayak[2], Faculty Guide, Amity Institute of Forensic Sciences, Amity University

ABSTRACT

A major step forward with far-reaching consequences for forensic biology's future is the use of sequencing technology to forensic DNA analysis. The potential, threats, and current tendencies of using sequencing in forensic investigations are discussed in this article. This research takes a multi-disciplinary approach by reviewing the literature, conducting case studies, interviewing experts, and analysing data to look at how sequencing might improve accuracy, grow forensic databases, and solve problems in complicated situations. Important topics include the need for strong regulatory frameworks, ethical concerns around privacy and permission, and the revolutionary power of sequencing to overcome the shortcomings of conventional approaches. If forensic scientists - stakeholders approach these obstacles with caution and planning, sequencing has the potential to transform forensic DNA analysis while bringing about digital justice.

Keywords:

Forensic DNA Analysis, Next-Generation Sequencing, Rapid DNA Sequencing, Forensic Biology

INTRODUCTION

The use of cutting-edge technology has completely altered the course of criminal case investigations and prosecutions in the dynamic field of forensic science. One of the most important technical developments with far-reaching consequences for forensic investigations is the use of sequencing in forensic DNA analysis. In this introductory piece, we will look at the current state of forensic sequencing and its potential applications in improving accuracy, growing forensic databases, and solving difficult cases. When it comes to solving crimes, identifying suspects, and clearing innocent people's names, forensic DNA analysis remains an indispensable tool. Forensic DNA profiling relies heavily on time-honored techniques like polymerase chain reaction (PCR) as well as short tandem repeat (STR) analysis, which allow for very precise comparisons of biological evidence to known persons. The need to investigate more sophisticated technologies arises from the fact that current procedures have their limits, especially when dealing with DNA samples that are damaged or mixed. Here we come to sequencing-based techniques, which include a range of technologies including Rapid DNA sequencing and Next-Generation Sequencing (NGS). Sequencing allows for the unprecedented breadth and depth of analysis of whole genomes or particular genetic loci, in contrast to conventional approaches that focus on specific areas of DNA. Improved discriminating power, resolution of complicated mixes, and extraction of useful genetic information beyond conventional markers are all made possible by this capacity, which opens up new forensic investigative options.

The expansion of forensic DNA databases—which are vital in connecting crimes to suspects including finding missing persons—holds tremendous promise in the realm of sequencing. Aside from the usual set of genetic markers used in conventional forensic databases, sequencing allows for the inclusion of other markers by producing more comprehensive genetic profiles. Not only does this development improve forensic analysis' discriminatory power, but it also makes it easier to solve cold cases and find missing persons. Automation, standardisation, and process streamlining are all possible outcomes of sequencing technology's use into forensic operations. For example, on-site analysis using rapid DNA sequencing systems might provide useful information to law enforcement in real-time. Especially in high-stakes situations when prompt identification is crucial, this quick turnaround time may greatly speed up investigations. The broad use of sequencing in forensic DNA analysis raises ethical concerns and poses obstacles, albeit its enormous promise. For sequencing technologies to be used ethically and responsibly in forensic settings, concerns about data privacy, abuse, and the lack of strong validating frameworks must be addressed. Ultimately, the integration of sequencing methods signifies a sea change within forensic DNA analysis, providing hitherto unseen skills for tackling current problems and opening up new avenues for criminal investigations. The incorporation of these technologies into forensic procedures has the potential to improve justice administration in terms of accuracy, efficiency, & fairness as they become more available and continue to advance.

Traditional STR profiling has its uses, but it may be useless when no profile matching is identified, when DNA amounts are too low, or when DNA is too damaged to be examined. New procedures are being developed to allow the analysis of these hard samples and to provide knowledge about the source with a biological sample. The judiciary system is sluggish to adopt innovative techniques and technology. As seen by the increasing number of publications over the area over the last twenty years, forensic genetics (as well as now forensic genomics) has had a remarkable increase in recent years. This study aims to present a synopsis of key developments in DNA forensic analysis as a comprehensive examination of the subject would be too large for a single article.

With an emphasis on the revolutionary possibilities of sequence in forensic DNA analysis, this introductory section lays the groundwork for future investigations into forensic biological evidence.

1.1 BACKGROUND OF THE STUDY

Due to rising expectations for more precise and trustworthy criminal investigative techniques and technological developments, forensic biology has seen profound changes throughout the last few decades. The rise of sequencing technology as a potent instrument for forensic analysis of biological evidence is one of the most remarkable advancements in this field. Forensic DNA analysis has long depended on PCR and STR profiling, two methods with great discriminating power but limited capacity to work with DNA samples that were degraded or combined. There was a need for new methods that could get above these restrictions since instances with such difficult samples started popping up. Forensic DNA analysis has taken a giant step forward with sequencing-based approaches like Rapid DNA sequencing and Next-Generation Sequencing (NGS). Sequencing enables the thorough examination of DNA sequences, in contrast to PCR-based methods; this provides insights into the whole genome or particular genetic loci with unmatched depth and precision. With this capacity, forensic analysis becomes much more selective, and significant genetic information may be extracted from sources other than standard markers. Quick technical developments and an increasing amount of research showing sequencing's effectiveness in several forensic applications have driven its acceptance in forensic biology. In situations when there is little or damaged biological material, sequencing-based techniques improve the likelihood of accurate DNA profiling & identification compared to conventional procedures. Law enforcement organisations throughout the globe rely on forensic DNA databases, and sequencing offers hope for their expansion. Enhancing the precision and reliability in forensic matching, sequencing technologies include more genetic markers and provide more complete genetic profiles. This helps with solving cold cases and identifying missing individuals.

Despite these improvements, there are still a number of factors to think about before sequencing becomes standard practice in forensic DNA analysis. This research aims to provide significant insights into the changing field of forensic science and how sequencing will shape its future by thoroughly analysing current literature, case studies, & expert opinions.

1.2 RECENT IMPROVEMENTS IN DNA ANALYSIS

There is a mountain of forensic evidence due to the exponential growth in the use of DNA swabs in recent decades, as the technology has become more accessible, affordable, and rapid, leading to their widespread use in even the most trivial of crimes. The skills and capacity of forensic analysis are expected to grow in the next decades, with improvements in speed, sensitivity, and cost. There are now entirely computerised DNA profiling devices available that can separate, amplify, & genotype samples in about 90 minutes; nevertheless, the high operational expenses make their widespread use rather unlikely. Police stations might be equipped with these gadgets to expedite the process of charging persons during the legally permitted brief time of detention. At now, PCR amplification—which needs a series of cooling and heating cycles to generate enough DNA for analysis—is often the most laborious step in DNA analysis. Many new approaches to PCR amplification are now under research and development. One such approach is the micro-PCR machine, which uses smaller amounts of liquid and can heat or cool them more rapidly.

Stochastic effects, which occur during PCR amplification, cause certain alleles to be underrepresented or even excluded if they are overrepresented early on. Forensic DNA analysis might become more sensitive in the future thanks to more well-established methods; computer simulations can help estimate the impact of different factors on this. Although next-generation sequencing (NGS) is more often used in academic settings, it is capable of whole-exome sequencing of mitochondrial and chromosomal DNA. To enable very precise identification & comparison of DNA sequences, future forensic DNA analyses will include an enormously increased number of recorded loci. But more data means more data interpretation, and in a forensic context, it may not be required to gather data beyond the threshold of likely positive identification.

Improved recovery of difficult samples—those with damaged DNA that do not provide an adequate genetic profile after standard PCR amplification—is one of the main goals of forensic DNA sequencing. Because the complementary sequences constitute more likely to identify the proper partner without adding erroneous bases, smaller PCR amplicons may be utilised to better reproduce damaged DNA. These amplicons are a product of the sample's DNA or RNA and serve as a template for amplifying complementary DNA. With the use of known phenotypic traits like height, eye and hair colour, ethnicity, and other traits impacted by genes, it may one day be able to estimate an individual's genetic profile even in the absence of a reference sample. Furthermore, in the future, microbial DNA analysis might help identify people at crime scenes. This is because microbiomes are very unique, bacteria can shed their cells in many ways, one of which is via breath, and evidence such as this could be easily disseminated. Additionally, unlike conventional DNA analysis, the microbiome may provide a person's previous geolocation, cause of death, and the amount of time since death.

1.3 SANGER (DIDEOXY) DNA SEQUENCING

For the first time, DNA sequencing in the lab was done systematically using Sanger's dideoxy technique. For Sanger sequencing to work, you'll need:

- The DNA sequencer's blueprint
- A 32P-labeled oligonucleotide primer (5'-end).
- A polymerase for DNA sequencing
- The four deoxynucleoside triphosphates are dATP, dGTP, dCTP, and dTTP.
- The four nucleoside triphosphates that are dideoxy are those that do not include
- ddATP, ddGTP, ddCTP, and ddTTP (containing both 2'- and 3'-hydroxy groups)

One kind of DNA replication is Sanger sequencing. A particular locus of the template is hybridised with the primer, and a reverse mirrored copy to the template is assembled by binding and incorporating nucleotides by the polymerase. Four sequencing processes are performed in separate tubes to ensure that this technique does not reveal any information about the template's sequence. A little amount of the active substance, a 2',3'-dideoxy nucleoside triphosphate, is added to each tube. In tubes 1, 2, 3, and 4, the dideoxy triphosphate nucleoside (ddATP), ddGTP, ddCTP, and ddTTP, respectively, are introduced.

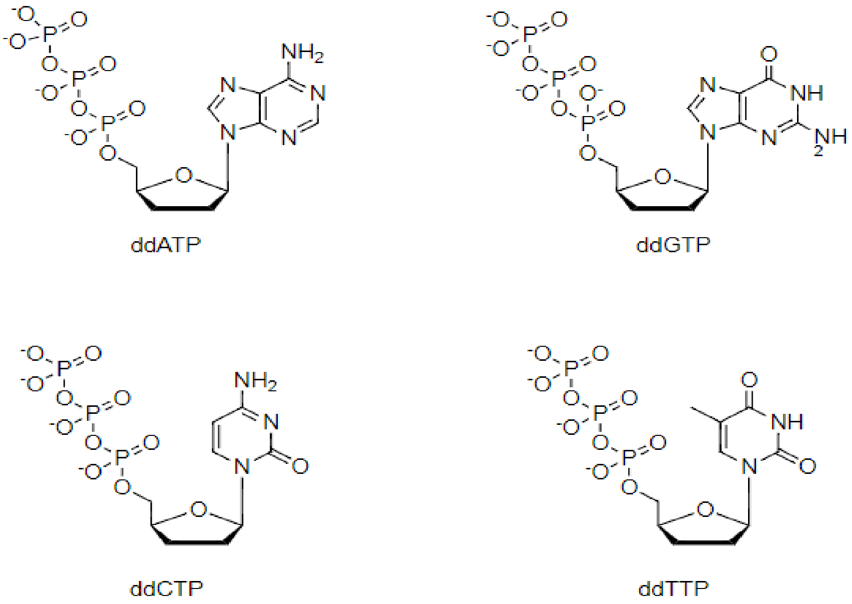

ddATP
ddGTP
ddCTP
ddTTP

Fig.1 Structures of dideoxy nucleoside triphosphoates (ddNTPs)

At each stage, either deoxynucleoside triphosphates (dNTPs) or dideoxynucleoside triphosphates (ddNTPs) may be added because the polymerase enzyme does not differentiate between the two. The addition of dNTPs causes the DNA chain to keep going, while the addition of ddNTPs causes it to stop growing because it lacks the 3'-hydroxyl group necessary to react with incoming nucleoside triphosphates. An assortment of oligonucleotides with varying lengths and specific ddNTP terminations is produced in each of the four tubes; in tube 1, this assortment is at A, in tube 2, it is at G, in tube 3, it is at C, and in tube 4, it is at T. The next step is to use electrophoresis to sort the oligos by size. In order to determine the DNA sequence, which is the reverse-complement portion of the template, a photographic film may be used to expose a polyacrylamide gel that has all four ladders running parallel to it. This will reveal the 32P-labeled fragments. This approach can really sequence approximately 300 bases in a DNA sequence in practice.

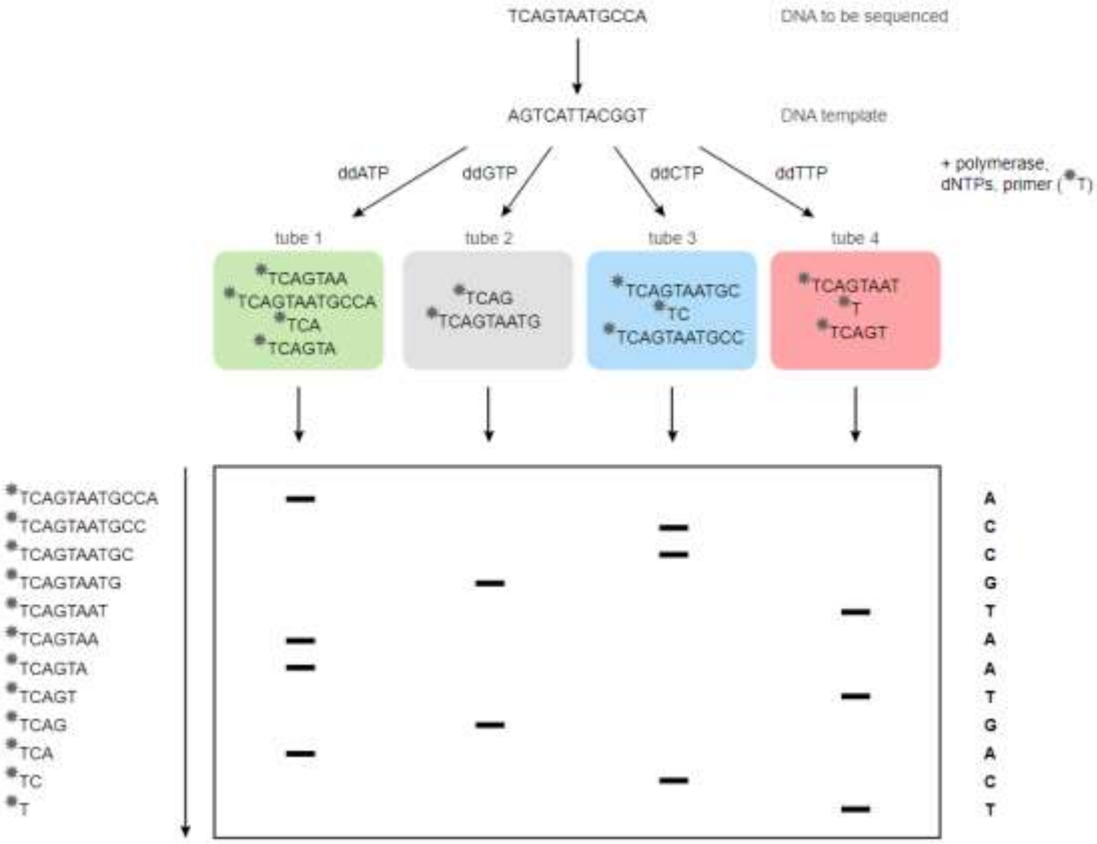
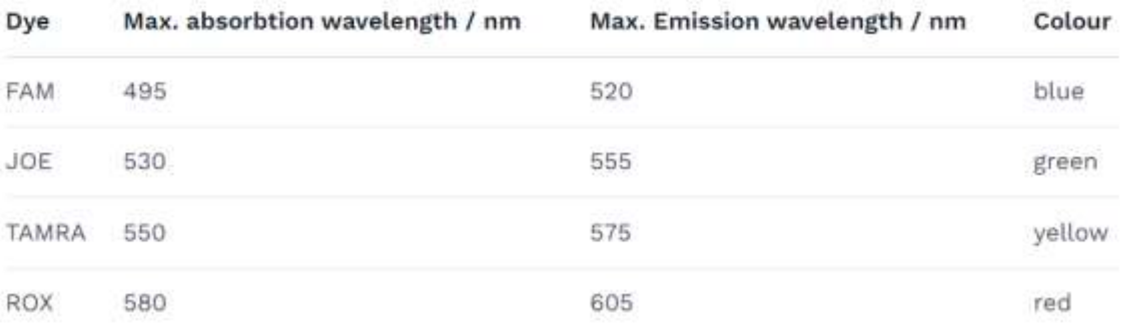


Fig.2 Sanger (dideoxy) sequencing

1.4 FLUORESCENCE-BASED DIDEOXY-DNA SEQUENCING

Thermostable DNA polymerase, four standard deoxynucleoside triphosphates, & 4 dideoxy nucleoside triphosphates with varying fluorescent labels are all components of the automated high-throughput fluorescence Sanger sequencing method. An unlabeled oligonucleotide primer is also used. Since ddA termination provides the DNA fragments a certain fluorescent colour, ddG another hue, the DDC a third colour, and ddT an additional colour, just one sequencing process is needed. Fluorescent dyes with well-resolved emission spectra are essential, yet the specific kind of dyes utilised will vary with each DNA sequencer. The four dyes often used in this technique are FAM, JOE, TAMRA, and ROX.



Dye	Max. absorbtion wavelength / nm	Max. Emission wavelength / nm	Colour
FAM	495	520	blue
JOE	530	555	green
TAMRA	550	575	yellow
ROX	580	605	red

Fig.3

Electrophoresis separates the pieces, and a laser excites the fluorescent pigments. A DNA sequence may be generated by analysing the gel picture using a computer. It is possible to read 800 bases in only one gel lane. With three gels per day and the ability to analyse 96 lanes in a single gel, automated DNA sequencing devices can analyse over 50,000 bases per year, or around 230,400 bases per day. It is now possible to analyse 384 sequencing reactions all at once using newly built machines. Dideoxy nucleoside triphosphates are labelled using two fluorescent dyes; this is an example of a more recent breakthrough ("Big Dye chemistry"). The first dye, often fluorescein, is excited at 495 nm, which causes emission at 520 nm. This emission is then transmitted to the second dye, which has a λmax at 520 nm, using FRET. The second dye has a much stronger fluorescence at longer wavelengths. If the second fluorescent pigment were excited directly at 495 nm, the resulting signal would be weaker than what is achieved here. Also, gel technology has come a long way. Automating sample loading and evaluation using capillary gels instead of flat bed gels has allowed for even greater throughput.

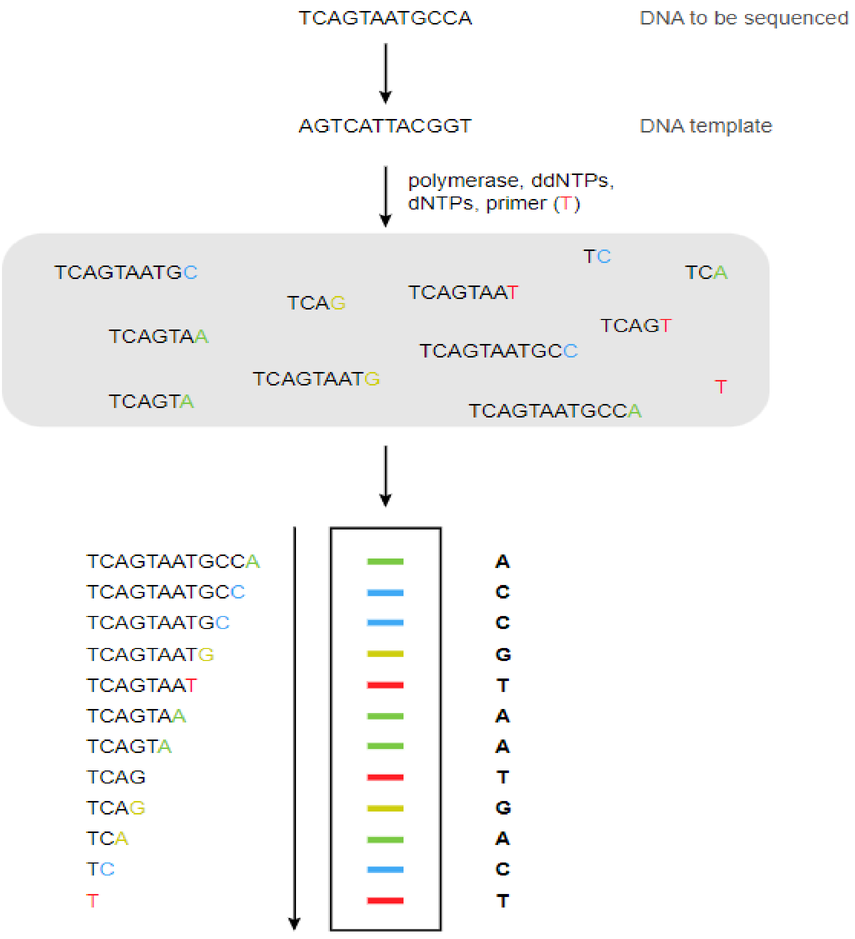


Fig.4 Fluorescent Sanger (dideoxy) sequencing

1.5 NEXT-GENERATION SEQUENCING

Between 1990 and 2003, a massive international effort was known as the Human Genome Project, and it succeeded in sequencing all three billion base pairs comprising the human genome. Despite its optimisation and automation, Sanger sequencing was the backbone of the Human Genome Project. New sequencing methods, generally referred to as next-generation DNA sequencing, have made it feasible to sequence a whole human genome in a few days.

- **Sequencing modified DNA:** Even with next-generation sequencing, current DNA sequencing techniques cannot identify altered nucleotides. Considering the current wave of interest in epigenetics, one major limitation of sequencing methods is their inability to differentiate between cytosine and 5-methylcytosine, two bases that form Watson-Crick base combinations with guanine.
- **Bisulfite sequencing:** Bisulfite (HSO3-) cannot interact with methylcytosine, but it breaks down unmethylated cytosine to uracil. A technique for sequencing DNA with 5-methylcytosine bases is thus presented. Prior to and during bisulfite treatment, DNA is sequenced; any transition from cytosine t uracil is attributed to unmethylated cytosine, and any cytosine bases that persist following bisulfite treatment are presumed to have been methylated in the initial sample.

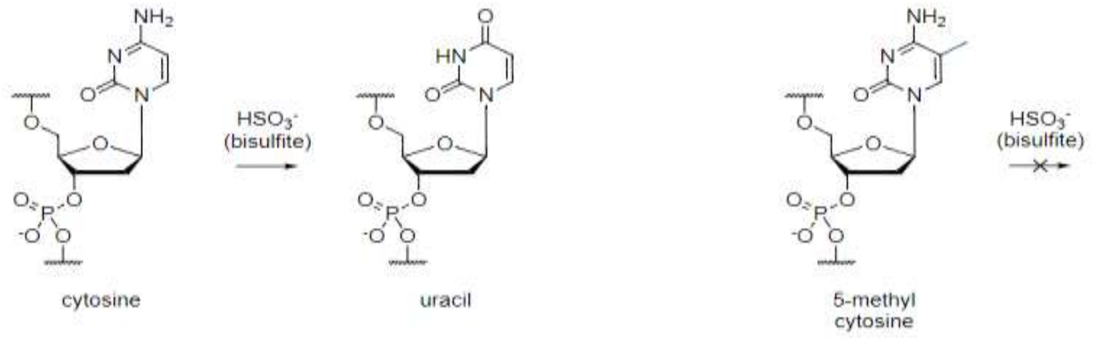


Fig.5 Bisulfite conversion of cytosine to uracilBisulfite (HSO3-) converts unmethylated cytosine to uracil, but does not convert methylcytosine to thymine.

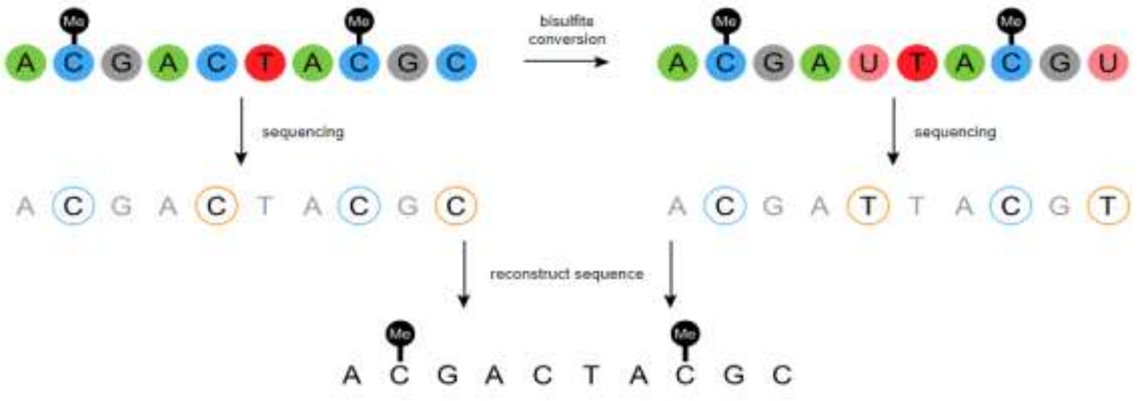


Fig.6 Bisulfite sequencingSequencing of DNA before and after treatment with bisulfite (HSO3-), which deaminates unmethylated cytosine bases to uracil, allows the methylation state of a DNA sample to be determined.

LITERATURE REVIEW

After reviewing the progress made in forensic DNA testing over the last three decades, the author offers his or her predictions for the next decade. In the same way that the Olympic slogan encourages athletes to "go faster, jump higher, and stronger," forensic DNA methods are poised to enhance their sensitivity, speed, and investigative power. The basic collection of DNA markers utilised for identifying people in the United States and Europe has been increased by the addition of fresh short tandem repeat (STR) loci. New possibilities are about to open up as a result of rapid DNA testing. Greater information depth on STR alleles may be possible with next-generation sequencing. The capabilities of genetic databases have been increased in regions where it is legal to conduct familial DNA searches. There is a need for training and education to enhance the interpretation of complicated DNA profiles, among other possibilities and threats that will affect the future in forensic DNA. **(Butler, J. M. 2015)**

When a DNA profile obtained during an inquiry matches one on a database or produced from a suspect, the results of the testing of DNA via biological evidence may be very persuasive. Traditional STR profiling has its uses, but it may be useless when a single match is identified, when DNA amounts are too low, or when DNA is too damaged to be examined. In order to analyse difficult forensic samples and learn more about a sample's source, the fast developing discipline of genetics in forensics has developed a number of new approaches. This article outlines some of the most significant recent developments in the field. These include using massively parallel sequencing to analyse STRs and other markers, improving DNA mixture interpretation through probabilistic genotyping methods, identifying body fluids using RNA profiling, using SNP markers to predict phenotypes relevant to forensics, determining tissue type and age through epigenetics and DNA methylation analysis, and the new field from forensic genetic genealogy. To make the most of these advances' potential advantages, researchers will need to carefully evaluate how to incorporate them into forensic practice. **(Haddrill, P. R. 2021)**

Three types of markers will be crucial in forensic DNA typing going forward: short repeat repeats, polymorphisms of one nucleotide, & entire mitochondrial analysis. Forensic research has benefited greatly from the advent on massively parallel sequencing systems, which have made possible the discovery of hitherto overlooked details, such as the complexity and variety of the markers, and the analysis of data sets beyond large for human analysis. Bioinformatic approaches are necessary for processing and interpreting this massive amount of new data, in addition to the sequencing chemicals used. Learning more about these technologies' potential forensic uses has led to the creation and standardisation of efficient, beneficial tools for every step of data processing, as well as the improvement of methodologies in terms of speed and accuracy. To facilitate studies, sequencer makers have integrated pipelines into their software while forensic labs seek the best combination of instruments. Analyses on forensic markers sequenced on the most popular massively parallel sequencing (MPS) systems are discussed in this paper, which also examines the present status of bioinformatic approaches and tools in this area. **(Rittner, C. 2000)**

Next generation sequencing (NGS) techniques revolutionised genetic research shortly after their introduction over ten years ago. Full genomes are now being mapped and released almost weekly, with prices going down and speeds going up. Over the last decade, next-generation sequencing (NGS) technologies and methodologies have advanced to the point that NGS can be utilised for human clinical diagnostics due to the high quality of the sequences. Additionally, forensic genetic labs have investigated NGS technology; moreover, there has been a little upsurge in the quantity of scholarly publications and conference presentations addressing forensic NGS features in the last year. These advancements prove that next-generation sequencing (NGS) opens up new avenues for genetic forensic investigation. Combinations comprising indicators (STRs that are, SNPs, insertion/deletions, mRNA) which cannot be analysed concurrently with the current conventional PCR-CE procedures allow for more information to be retrieved from distinct samples in a single experiment. New STR alleles were identified, and the actual variance at important forensic STR loci was revealed. The statistical significance of the evidence will be enhanced by the specific sequence information, which may assist in mixture interpretation. This paper will provide an overview of next-generation sequencing (NGS) and single-molecule sequencing (SMS), as well as talk about how NGS may be used in forensic genetics. **(Morling, N. 2015)**

Having a system in place to name sequence-based alleles and artefacts is essential for forensic DNA analysis on casework samples conducted using enormously parallel sequencing (MPS) technology. In light of the need for distinct sequence tagging, the ISFG DNA Commission has released some thoughts on a nomenclature format. Although this format is suitable for databases and conveying sequence kinds, it is too long to be utilised in software interfaces. To fill this need, the sequence identifier (SID) nomenclature generates concise labels that can distinguish between all sequences in single-source or casework profiles, whether they are allelic or artifactual. Casework profile sequences may be uniquely identified using a maximum of three SID characters, resulting in a compact format. Algorithms for artefact detection and filtering, as well as for expressing relationships between artefacts and their probable parent alleles, may make use of SID labels. This naming scheme works with any downstream mixture analysis programme that can read character values instead of numeric ones. An overview of the SID nomenclature is provided, along with examples of its use in forensic mixture analysis and its capacity to distinguish between sequence-based alleles and artefacts. **(Armogida, L. 2019)**

The use of mitochondrial DNA (mtDNA) analysis has grown in forensic medicine in recent years due to its usefulness in circumstances when nuclear DNA analysis is not an option. Genomic sequencing of the D-loop's 2 hypervariable regions, HVI & HVII, has long been the gold standard for mtDNA analysis. Traditional Sanger sequencing for DNA sequence analysis is labour- and time-intensive yet extremely robust. In contrast, pyrosequencing-based mtDNA analysis yields rapid and precise findings using the human mtDNA found in a wide variety of forensic evidence materials. The purpose of developing this test is to enhance the discriminatory power of mitochondrial DNA analysis by identifying polymorphic sites within the coding region and the mitochondrial D-loop. Using both control samples and real casework materials, the sensitivity, repeatability, and success rate of the pyrosequencing method for mtDNA polymorphism analysis have been evaluated. There is an elevated rate of success for casework samples, and the findings are clearly interpretable, demonstrating that the procedure is very accurate & sensitive. For the mtDNA polymorphisms, the appropriate panel of pyrosequencing procedures was selected based on the discrimination power relative to the number of bases identified. **(Allen, M. 2002)**

One feasible approach for forensic DNA studies is amplicon (targeted) sequencing using massively parallel sequencing (PCR-MPS). Capillary electrophoresis for STR & Sanger sequencing for mitochondrial DNA type are two examples of alternative instrumental analytical techniques that PCR-MPS might complement or even replace in this context. PCR-MPS has the potential to open up new avenues for forensic DNA analysis by allowing the incorporation of marker systems that are now assayable utilising techniques like quantitative PCR and microarrays, such as insertion/deletions (indels) and single nucleotide polymorphisms (SNPs). For PCR-MPS to gain traction as a forensic tool, standards and guidelines outlining the method's bounds, such as a defendable analytical threshold or detection limit, are necessary. In this study, we detail a procedure for developing multiplexed PCR-MPS-compatible objective analytical thresholds. We provide an analytical threshold that is based upon background noise and offer a specification for PCR-MPS technique background noise. **(Armogida, L. 2017)**

Human mitochondrial DNA (mtDNA) isolated from different tissues and amplified by polymerase chain reaction (PCR) was the subject of two sets of investigations designed to assess its forensic value. A Perkin-Elmer/Applied Biosystems Division (PE/ABD) model 373A automated DNA sequencer was used for the sequencing process. Typical validation tests onto forensic DNA markers were included in the initial set of investigations. These covered things like the effects of chemical contaminants on blood and semen DNA and the effects of typing DNA obtained from bodily fluid samples placed on different substrates. Human hair shafts were the exclusive subjects of a separate series of investigations. In these investigations, mtDNA was typed from hairs that had been: (1) extracted from various parts of the body; (2) subjected to chemical treatment; (3) taken from people who had passed away; and (4) purposefully polluted with different bodily fluids. The results prove that mtDNA typing by PCR with direct automatic sequencing is an accurate and trustworthy forensic identification method. **(Budowle, B. 1995)**

Sequencing technology has come a long way in the previous few decades. The ability to quickly generate millions of sequence reads and to generate huge DNA sequences from fragments of any size are two of Next Generation Sequencing's (NGS) most significant accomplishments. It is not necessary to know the sequence of the DNA or RNA area of interest in order to construct libraries; complete genomes or regions of interest may be used. Because of this, it is possible to search for variants and make genetic identification easier. An examination of the present state of next-generation sequencing (NGS) technology and its uses, with a focus on forensics, outlining the benefits and drawbacks of this technology as it pertains to genetic identification. From 2012 to 2015, we systematically searched the electronic databases PubMed, Science Direct, and Scopus for relevant articles. Both the scarcity and deterioration of accessible samples pose significant challenges in the forensics industry. With the ongoing reduction in the quantity of DNA needed to prepare NGS libraries, it will be possible to sequence almost any material, allowing for the extraction of maximal information in all biological remains. Furthermore, crime scene characterization using microbiome typification might be an intriguing area to investigate. **(Gonzalez, L. J. 2017)**

Applications in biotechnology, virology, & medical diagnostics have made DNA sequencing—which began with Sanger's termination of chains method in 1977 & has now progressed to next generation sequencing (NGS) methods that use massively parallel sequencing (MPS)—essential. These methods have also received more attention in the forensics sector, as seen by the increasing number of studies published in the last 2-3 years. New advances is human DNA analysis with forensic applications are the subject of this paper, which provides a concise overview of all three previous generations sequencing methods. The ability to sequence DNA repeats in search of polymorphisms is relevant to forensic investigation, in addition to the development of typical STR-profiles. To learn more about a person's genealogy, paternity, or phenotypic, more SNPs may be sequenced. When DNA samples taken from crime scenes are either extremely little or very degraded, the present MPS techniques are nevertheless very useful. Sequencing mitochondrial DNA may be used for maternal ancestry analysis in cases when there is insufficient autosomal DNA. All signs point to next-generation sequencing (NGS) being an integral part of forensic research. **(Gardeniers, H. 2018)**

There has been tremendous progress in DNA sequencing technology recently. From molecular diagnostics across cancer research to spore identification in bioterrorism, several platforms have shown usefulness in all facets of health and scientific inquiry. One single-molecule sequencing platform in the forensics field is showing signs of becoming a practical option for small to medium forensic labs. An on-site Zika and Ebola virus detector, a portable nanopore-based sequencing instrument created by Oxford Nanopore Technologies (ONT) has already found use in full genome sequencing, the assessment of DNA and RNA base modifications, along with enrichment-free mitochondrial DNA analysis, among other applications. Standard sequencing of DNA, direct examination of forensic samples (such as blood, semen, and buccal swabs), DNA from mitochondria, analysis, SNP as well as STR analysis, familial identification, and microbial identification for bioterrorism along with geolocation are all made possible by the fast development of this technology. This platform is ideal for smaller labs looking to start building competence in sequence-based forensic studies due to its compact size, inexpensive cost, and minimal equipment requirements. We discuss the possible use of nanopore sequencing methods for forensic investigation, as well as their recent advancements and current uses. Here we discuss methods that have been developed and others that are still in the works for identifying microbes, mitochondrial DNA, and SNPs and STRs. Further refining the sequencing procedure that integrates workfl ows in labs or distant field settings, we also address new improvements in library preparation and data processing tools. **(Mills, D. 2021)**

The outwardly visible features (EVCs) and biogeographical ancestry (BGA) of an evidential sample donor may be better understood with the use of forensic phenotyping. The most popular method for inferring BGA and EVCs based on single nucleotide polymorphisms (SNPs) at the moment is the single base extension (SBE) test known as SNaPshot®. But only 30–40 SNPs can be tested with one SNaPshot multiplex PCR. Genotyping hundreds or even thousands of single nucleotide polymorphisms (SNPs) from different samples all at once may be possible using next generation sequencing (NGS). Using three distinct DNA template levels (0.1, 0.2, and 0.3 ng) in three samples (9947A & 007 control DNAs and a male donor), the PCR multiplexes from five SNaPshot tests were applied: SNPforID 52plex, a, SNPforID 34plex, Eurasiaplex, IrisPlex, and an unpublished BGA assay. The Ion TorrentTM PGM system from Life Technologies was used to sequence the 136 unique SNPs included in the pooled PCR amplicons. Our two 10-MB Ion 314TM v1 chips were able to produce a sequence of around 72 Mb. All three template quantities were easily used to get accurate genotypes. Out of 408 genes, 395 (or 97% of the total) were in perfect agreement with SNaPshot for all three template concentrations. Sanger sequencing was completely consistent with six Ion Torrent genomes (1.5%) out of those genotypes that were incongruous with SNaPshot across all three template concentrations. There was either a discrepancy between the template quantities and seven SNPs (1.7%) or between the template amounts and Sanger sequencing. It is necessary to set a threshold in background amounts of sequence output & heterozygous balance based on the sequence coverage measured in the negative control and the allele coverage variance for heterozygous genotypes. Based on the results of this pilot investigation, several Ion Torrent PGM techniques system shows great promise as a low- to medium-throughput next-generation sequencing (NGS) platform for forensic DNA studies using the well-established SNaPshot tests. **(McNevin, D. 2015)**

In the latter part of the 17th century, Antonie van Leeuwenhoek noticed, among other tiny things, germs from the mouth cavity while experimenting with his microscopes. The field of microbiology was established by van Leeuwenhoek, who provided extensive descriptions for bacteria, spermatozoa, & cells. Robert Koch & Louis Pasteur, who succeeded him, further piqued interest in the microbiome by revealing the function of bacteria in fermentation and illness. **(Kuiper, I. 2016)**

Mineralogy, geophysics, texture, and colour analysis are often used in forensic casework to establish a connection between a suspect and a crime scene. On the other hand, the enormous variety of species found in soils may also be characterised using DNA analysis. By identifying particular taxa and studying non-culturable microbial species, DNA metabarcoding & high-throughput DNA sequencing (HTS) now provide a way to better discriminating across forensic soil samples. The ITS & 18S markers both demonstrate dependable amplification and a high degree of discriminating strength even when dealing with modest amounts of background DNA. After filtering, the 16S rRNA flag still displayed a significant amount of background DNA, but it had equivalent discriminating strength. **(Cooper, A. 2014)**

METHODOLOGY

OBJECTIVE OF THE STUDY

- To Conduct a comprehensive review of existing literature on sequencing technologies in forensic DNA analysis. This includes academic journals, conference proceedings, books, government reports, and other relevant sources.
- To Identify and analyze relevant case studies showcasing the use of sequencing in forensic DNA analysis.
- To Evaluate the current landscape of sequencing technologies available for forensic DNA analysis, including Next-Generation Sequencing (NGS), Rapid DNA sequencing, and emerging platforms.

METHODOLOGY

When evaluating the data that was readily accessible on the topic, the method of content analysis was taken into consideration. There was a long list of materials provided, which included a variety of websites and research papers, to which it aims to provide a thorough examination of the role of sequencing in the future of forensic DNA analysis.

DISCUSSION

The Sanger sequencing technique was introduced in the 1970s , and since then, the technology for sequencing DNA has allowed for tremendous advancements in genetics and molecular biology. Using this technique, several large-scale projects have been accomplished, including the Human Genome Project, the Rice Genome Project, the Swine Genome Project, and genome studies of numerous other species. However, traditional Sanger sequencing has limited use in more in-depth and complicated genomic investigations due to its poor throughput, high cost, and operational challenges. Thanks to next-generation sequencing (NGS) technology's low cost and high throughput capacity, these issues have been mostly resolved. NGS has found applications in many areas of the life sciences, such as forensics, diagnosing illnesses, agrigenomics, and ancient DNA analysis. With the hope of serving as a point of reference for cutting-edge forensic science research and applications in the future, this paper reviews the current state of NGS technology in this field.

4.1 OVERVIEW OF NGS TECHNOLOGY

"NGS technology" means high-throughput DNA sequencing that isn't reliant on Sanger sequencing. Throughput may be greatly increased and the fragment-cloning approach, which is often utilised in Sanger sequencing, can be minimised by sequencing billions or millions of DNA molecules in parallel. This method encompasses both third-generation sequencing that can identify the base makeup of individual DNA molecules and second-generation sequencing that uses loop array sequencing to analyse several samples at once.

One of the first high-throughput sequencing systems to use pyrosequencing was the 454 Genome Sequence System, which was developed by Roche in 2005. While the current max read length was 1000 bp, the initial 454 Genome Sequencer could generate over 200,000 reads that were 110 bp in length. While Illumina debuted their Solexa sequencing technology in 2007, Applied Biosystems (ABI) unveiled their SOLiD second-generation sequencing system, which relies on the oligonucleotide ligation process and two-base encoding mechanism. Despite producing reads that were just 35 bp long, the Illumina & SOLiD sequencers outpaced the 454 system in terms of read generation, with 30 million and a hundred million reads, respectively. Introduced in 2010, Ion Torrent is a low-cost, quicker sequencer that relies on semiconductor technology. Neither chemiluminescence nor enzyme cascades nor fluorescence are used to detect sequencing signals by this sequencer. The current limit for this system is a maximum reading length of 400 bp.

Both real-time sequencing and the identification of individual molecules are made possible by third-generation sequencing technologies. Using single-molecule, real-time (SMRT) sequencing of DNA technology, Pacific Biosciences' PacBio RS system is now the market leader. The sequencing-by-synthesis method is the foundation of SMRT sequencing. The polymerase DNA molecules used to synthesise the target DNA fragments are connected to thousands of zero-mode wave guides on an SMRT chip. Modern SMRT technology may accomplish average read lengths ranging from 5500 to 8500 bp, which is an improvement over second-generation sequencing. Additional epigenetic alterations that may be directly detected include 4-methylctosine (mC), 5-methylctosine, and 6-methyladenine (mA).

4.2 FORENSIC APPLICATION PROSPECTS OF NGS TECHNOLOGY

Because of its usefulness in forensic investigations, DNA analysis has become an integral part of forensic science. The challenges faced by forensic DNA analysis are unique when compared to other areas of the life sciences. These include working with samples that are extremely deteriorated or polluted, requiring very accurate and reproducible results, and dealing with time and expense constraints. Detecting length variation among short tandem repeat (STR) indicators is done by most forensic DNA testing nowadays using PCR and fragment analysis techniques based on capillary electrophoresis (CE).

Certain sections of mtDNA, or mitochondrial DNA, have been analysed using the CE-based Sanger sequencing. The Sanger technique became the accepted norm for DNA sequencing with the advent of miniaturised gel electrophoresis & the automation both reaction gel loading & signal detection. Some limitations of CE-based analysis include: low-resolution genotyping for current markers; the loss of useful genomic data to degraded DNA samples; low-resolution mtDNA or mixture analysis; and the inability to analyse multiple genetic polymorphisms within one response using a single workflow. Due to these drawbacks of FGS, forensic specialists all around the globe are investigating the potential of NGS for forensic investigations.

4.3 DNA MIXTURE INTERPRETATION

As shown in Figure 3, DNA profiles that incorporate contribution from multiple donors are significantly more difficult to interpret than single source profiles. This is due to a combination of factors, including the fact that these profiles can contain a large number of alleles and the fact that they are frequently low-level, with complicating features like heterozygous imbalance and allele drop-out/drop-in. The recovery using mixed DNA profiles is becoming increasingly prevalent due to the increased sensitivity using STR profiling methods. This is true not just for samples where mixes can be anticipated, such samples from sexual offences, but also for poor quantity and quality samples recovered from touched things. We can't always tell which individuals were in these complicated mixes because of the sheer amount of variables introduced by these samples.

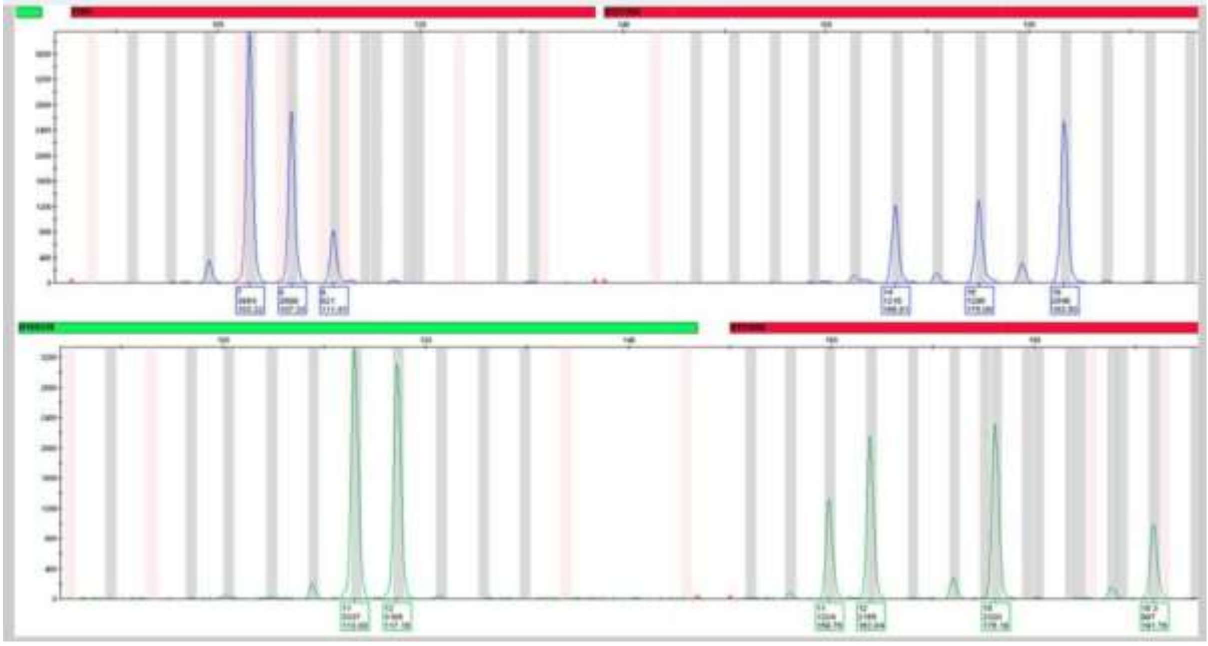


Fig.7 Electropherogram showing part of a mixed DNA profile at four STR loci

Due to the growing complexity of mixed profiles, various methods for mixture interpretation have been developed. These methods have evolved from simpler ones, such as checking if an individual might not qualify as a possible mixture contributor, to more complex ones that use information from profile peak heights to estimate the likely genotype combinations among mixture contributors. As a result, probabilistic frameworks that include allele drop-out & drop-in probabilities predicted from validation & empirical data have been developed as mixture interpretation approaches. You may classify these probabilistic genotyping approaches into two main groups: continuous and semi-continuous. The former group doesn't use peak height information and model artefacts like stutter, while the latter group does. Due to the intricate nature of the statistical computations involved, these approaches need the use of specialist software; fortunately, a number of programmes are already accessible to meet this need. Forensic laboratories have jumped on these programmes because of their capacity to analyse mixtures that were previously thought to be too complex for interpretation. Several regulatory bodies have also issued guidelines for the use of probabilistic genotyping software and published studies documenting the development and internal validation of various programmes.

4.4 STR ANALYSIS

Forensic scientists will continue to rely on STR analysis as the gold standard genetic method for the time being. There are a lot of benefits to this method, including accurate and quick allele identification, digital findings, making use of an abundant genomic element, minimal DNA template need, multiplex amplification, and detection based on fluorescence. There are forensic DNA databases built around STRs in over 60 nations already, and their numbers are continually increasing. As an example, China's forensic database now has over 27 million records. "If statistical analyses were conducted solely using the 13 commonly used Combined DNA Index System (CODIS) STR markers (i.e., CSF1PO, FGA, THO1, TPOX, VWA, D3S1358, D5S818, D7S820, D8S1179, D13S317, D16S539, D18S51, and D21S11) or 15 markers (13 CODIS loci plus D2S1338 and D19S433), the likelihood of a random match between unrelated individuals would increase." The current standard forensic typing test has extra STR markers added to it in an effort to circumvent this. However, existing fluorescent-based CE sequencers have technological constraints that make simultaneous detection of multiple STR markers exceedingly challenging. Conventional CE-based STR typing relies on DNA fragment size detection. Thus, it is not possible to differentiate between alleles that have the same length but have distinct sequences. Therefore, conventional CE-based STR analysis is generally unable to determine STR mutations in complicated paternity instances. Complex DNA combinations including DNA from several individuals further complicate forensic DNA testing. These days, criminal investigations seldom benefit from studies of DNA samples that are mixed because of the poor detection rates they provide.

Read lengths were often too short while the method of NGS was first introduced to genomics, making it unsuitable for STR testing. The typical duration of a read has been steadily rising with technical developments. Several researchers have begun to use NGS methods for STR testing due to the ease with which alleles of identical length may be separated and the potential benefits of digital read count in identifying mixed DNA samples & analysing difficult paternity situations. For instance, Zajac and colleagues conducted a groundbreaking work that used the 454 Genome Sequencing System's trinucleotide threading (TnT) method to examine three CODIS STR loci: TPOX, CSF1PO, and D18S51. Then, using multiplex identifier technology to single source samples, Irwin et al. used the 454 GS Junior system to examine thirteen CODIS STR loci. Additionally, Bornman et al. demonstrated that the 13 CODIS STR loci and the AMEL gene can be correctly identified using high-throughput sequencing techniques for both mixed and single-source data. The programme STRait Razor, created by Warshauer et al., can handle forensic NGS information for 44 STRs, includes 23 autosomal & 21 Y chromosomal STRs. Furthermore, Van Neste et al. found that the majority of locus genotyping findings were consistent and dependable when they used Illumina's MiSeq technology to create a reference allele databases for detecting single source or mixed DNA samples.

4.5 MITOCHONDRIAL GENOME ANALYSIS

The properties of mitochondrial DNA (mtDNA)—its tiny size, many copies, maternal lineage, high mutation rate, and absence of recombination—make it an effective forensic tool in instances involving low DNA levels or where investigating the maternal lineage is necessary. At now, forensic mtDNA tests often only find variations in a hypervariable area. Nevertheless, in order to enhance the discriminatory strength of identification using mtDNA for a genetic sequence marker, extra polymorphic loci are necessary. Thus, full mitochondrial sequence analysis may benefit substantially from NGS technology.

Equipment and reagent costs have dropped significantly due to the widespread use of NGS technology. Saving money is another benefit of parallel sequencing, a method which enables the examination of several samples all at once. For example, the GS-FLX instrument now uses sixteen picotiter plates instead of two, and each channel can do the analysis of 192 samples at once with the use of multiplex identification (MID) technology. In addition, Binladen et al. utilised a primer coding approach to generate 256 tagged primers. These primers were then utilised for multiple parallel sequencing, enabling the simultaneous sequencing of

256 samples. In addition, by using NGS technology, Gunnarsdóttir and colleagues simultaneously sequenced the mitochondrial genomes pf 109 Filipino people, achieving an average of almost 55 times coverage per sequence and less than 1% of data missing per sequence.

Heteroplasmy in cells from various organs within a single person has also been seen, in addition to the usual occurrence of human mtDNA heteroplasmy. Forensic mitochondrial analysis may be impacted by mtDNA heteroplasmy, among other causes. Reports of whole-genome heteroplasmy detection provide credence to the claims that next-generation sequencing (NGS) may identify this condition with great sensitivity and accuracy while also offering high throughput, cheap cost, and easy operation. In an independent investigation, the 454 GS Junior technology was used to investigate several mitochondrial hypervariable areas, an inherited STR locus (D18S51), and a Y chromosomal STR site (DYS389I/II). The findings proved that a DNA mixing ratio of 1:250 is detectable, and the scientists came to the conclusion that a mixture ratio if 1:1000 may also be visible if sequencing coverage was increased. By analysing 64 entire mitochondrial genome sequences, we were able to compare the haplotypes established by NGS technology on the whole mitochondria genome level with traditional Sanger sequencing. The findings revealed that these two approaches differed in their treatment of ~0.02% of nucleotides. Moreover, nearly two-thirds of these discrepancies were found in or near homopolymeric stretches, which are known to be locations where sequencing mistakes are more likely to occur. Using the 454 NGS technology, Mikkelsen et al. found that 95% of the reads were sequenced properly in homopolymers spanning up to 6 bases when the data were visually examined closely, allowing them to assess the reproducibility across samples that had been sequenced twice using NGS. The GM9947A portion of the NIST humans mtDNA SRM-2392 standards reference was found to include an unrecognised heteroplasmy in this investigation.

4.6 Y CHROMOSOME ANALYSIS

In the field of forensic molecular biology, Y-chromosome genetic markers have become more important. The most typical applications of Y-STRs include reconstructing paternal ties between male individuals or clearly identifying the male component in DNA mixes with a substantial feminine background. Two males who traced their lineage back thirteen generations used next-generation sequencing technology to compare over ten million Y chromosomal nucleotides. Y chromosome sequencing may be the answer to the conundrum of how to differentiate between male samples that are genetically similar to one another, as four distinct variations were found. More specifically, 118 unrelated males from 109 different places were able to have their differences confirmed by Van Geystelen et al.'s AMY-tree, which they created utilising the Y chromosome. single nucleotide polymorphisms (SNPs). Finding undiscovered Y-SNPs from various places and Y chromosome pedigrees were both accomplished via AMY-tree in this work.

4.7 MICRORNA ANALYSIS

The use of microRNAs (miRNAs) is relatively new to the field of forensic research, in contrast to the widespread use of messenger RNA (mRNA) analysis. endogenous microRNAs (miRNAs) are tiny RNA molecules that range in length from 18 to 24 nucleotides. Their tiny size, resistance to degradation, and ability to express particular or diverse tissues make them ideal for forensic purposes, such as identifying species, post-mortem interval (PMI) inferences analysis, and forensic body fluid identification. At now, the majority of miRNA analyses are conducted using biochip technology and real-time PCR, which limit the study to known miRNA sequences. Hanson et al. brought miRNA profiling to the field of forensic research in 2009 and demonstrated the genotyping of 452 miRNAs from forensic material using the quantitative PCR approach. Another research used a microarray to characterise the expression of a total of 718 miRNAs in various biological fluids, including vaginal secretions, menstrual blood, saliva, venous blood, and semen. A total of fourteen microRNAs (miRNAs) were found to be differently expressed; these could be possibilities for use in identifying bodily fluids. A potent tool for forensic investigation, next-generation sequencing (NGS) technology allows for the fast examination of millions of miRNA sequences, revealing disease-specific miRNA expression, organ-and stage-specific expression, and more.

CONCLUSION

Forensic DNA analysis with sequencing technology ushers in a new age of opportunities and threats for forensic biology. The importance of thoroughly examining the ethical, technical, as well as regulatory implications of this technology, as well as its revolutionary potential, have been emphasised throughout this examination of the future for forensic biological evidence with a focus on sequencing. By allowing for the thorough evaluation of DNA sequences at hitherto unseen depth and precision, sequencing provides analytical skills in the field of biological evidence that are second to none. Sequencing is a powerful tool for improving the precision and dependability of forensic investigations since it can handle materials that are deteriorated or contaminated, among other problems that classic PCR-based approaches have.

Sequencing also has the potential to help with cold case closure, missing person identification, and forensic DNA database expansion. The discriminating capacity of forensic analysis is strengthened by sequencing technologies, which use new genetic markers to provide more complete genetic profiles. This strengthens the relationship between crimes & suspects. Concerns about privacy, challenges with permission, and the possible abuse of genetic information are among the major ethical dilemmas brought up by the extensive use of sequencing within forensic biology. In order to promote public faith within the criminal justice system and protect individual rights, strong regulatory frameworks & ethical criteria must be put in place to govern the use of sequencing technology in forensic situations. The incorporation of sequencing into forensic processes has the potential to revolutionise forensic science, impact criminal investigation, and reform justice administration as sequencing technology improves and becomes more widely available. Given the limitations of DNA samples in practical forensic research, it is frequently not possible to analyse several loci on various chromosomes in the mitochondrial genome all at once. This might make it harder to provide enough information and restrict their use as evidence in court. Moreover, conventional STR genotyping methods are unable to resolve complicated paternity situations or mixed stain identification. DNA database development, ancestry with phenotypic deduction, MZ twin studies, bodily fluid etc species identification, forensic microbiological analysis, and many more fields of study might possibly benefit from NGS technology, which not only fits these criteria but also has the ability to apply it.

Even more crucially, massive volumes of data have been produced by high-throughput screening methods, allowing for a methodical comprehension of connections between molecular parts. Thus, new understandings in applied forensics will be revealed by combining genomics, proteomics, transcriptomics, and epigenomics with comprehensive genome-wide analysis.

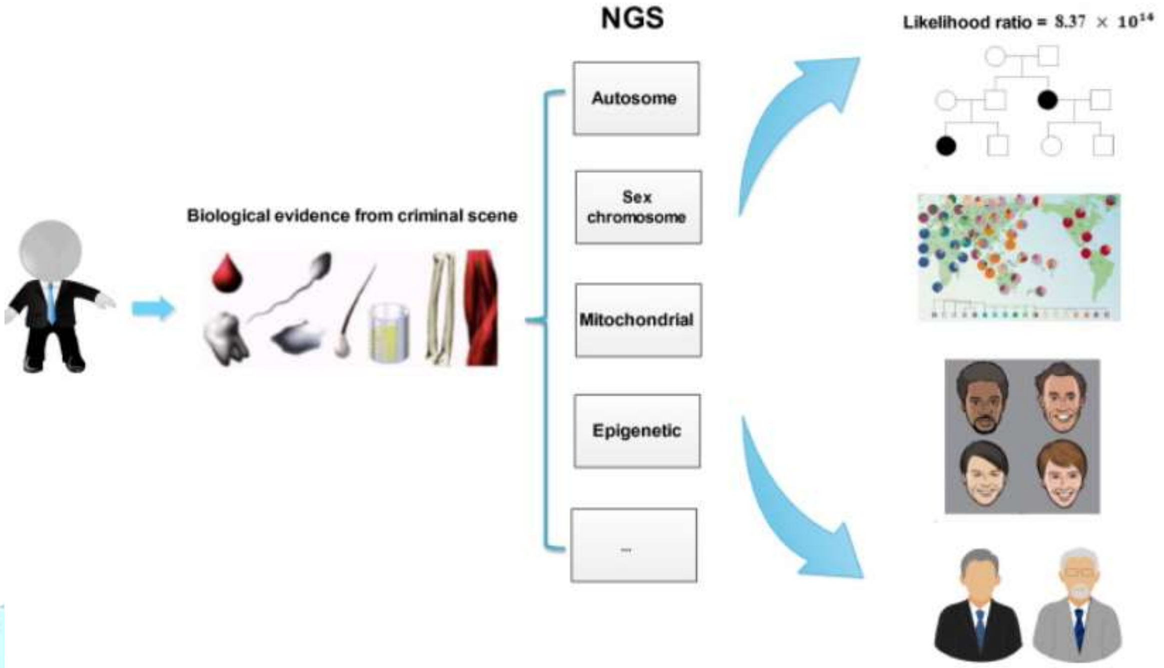


FIG.8 Diverse range of information can be obtained by NGS of biological evidence samples collected from crime scenes

Most nations have now set up massive forensic DNA databases to help solve crimes using STR technology, and normal STR typing has more than enough discriminating power for most purposes in forensic research. Despite the potential benefits of whole-exome and whole-genome sequencing for forensic studies, the high cost and lack of compatibility make NGS technology unlikely to replace traditional STR typing anytime soon. In order to examine STR loci, forensic experts used target-enrichment panels that were specifically created for the purpose.

REFERENCE

1. Sanger, F., Nicklen, S., & Coulson, A. R. (1977). DNA sequencing with chain-terminating inhibitors. *Proceedings of the National Academy of Sciences of the United States of America*, 74(12), 5463–5467.
2. Fullwood, M. J., Wei, C. L., Liu, E. T., & Ruan, Y. (2009). Next-generation DNA sequencing of paired-end tags (PET) for transcriptome and genome analyses. *Genome Research,* 19(3), 521–532.
3. Weber-Lehmann, J., Schilling, E., Gradl, G., Richter, D. C., Wiehler, J., & Rolf, B. (2014). Finding the needle in the haystack: Differentiating "identical" twins in paternity testing and forensics by ultra-deep next generation sequencing. *Forensic Science International.* Genetics, 9, 42–46.
4. McCarthy, J. J., McLeod, H. L., & Ginsburg, G. S. (2013). Genomic medicine: A decade of successes, challenges, and opportunities. *Science Translational Medicine*, 5(189), 189sr4.

5. Goddard, M. E., & Hayes, B. J. (2009). Mapping genes for complex traits in domestic animals and their use in breeding programmes. *Nature Reviews Genetics*, 10(6), 381–391.
6. Poinar, H. N., Schwarz, C., Qi, J., Shapiro, B., Macphee, R. D., Buigues, B., et al. (2006). Metagenomics to paleogenomics: Large-scale sequencing of mammoth DNA. *Science*, 311(5759), 392–394.
7. Margulies, M., Egholm, M., Altman, W. E., Attiya, S., Bader, J. S., Bemben, L. A., et al. (2005). Genome sequencing in microfabricated high-density picolitre reactors. *Nature*, 437(7057), 376–380.
8. Van Dijk, E. L., Auger, H., Jaszczyszyn, Y., & Thermes, C. (2014). Ten years of next-generation sequencing technology. *Trends in Genetics*, 30(9), 418–426.
9. Karn, T. (2013). High-throughput gene expression and mutation profiling: Current methods and future perspectives. *Breast Care (Basel),* 8(6), 401–406.
10. Derrington, I. M., Butler, T. Z., Collins, M. D., Manrao, E., Pavlenok, M., Niederweis, M., et al. (2010). Nanopore DNA sequencing with MspA. *Proceedings of the National Academy of Sciences of the United States of America*, 107(37), 16060–16065.

11. Luan, B., Peng, H., Polonsky, S., Rossnagel, S., Stolovitzky, G., & Martyna, G. (2010). Base-by-base ratcheting of single-stranded DNA through a solid-state nanopore. *Physical Review Letters*, 104, 238103.
12. Eid, J., Fehr, A., Gray, J., Luong, K., Lyle, J., Otto, G., et al. (2009). Real-time DNA sequencing from single polymerase molecules. *Science,* 323(5910), 133–138.
13. Murray, I. A., Clark, T. A., Morgan, R. D., Boitano, M., Anton, B. P., Luong, K., et al. (2012). The methylomes of six bacteria. *Nucleic Acids Research,* 40(22), 11450–11462.
14. Rizzi, E., Lari, M., Gigli, E., De Bellis, G., & Caramelli, D. (2012). Ancient DNA studies: New perspectives on old samples. *Genetics Selection Evolution*, 44, 21.
15. *Proceedings of the 4th national symposium on forensic DNA inspection technology & 2012 international symposium on new advances in forensic genetics.* (2012, December 9–12). Fuzhou, China.
16. Zajac, P., Oberg, C., & Ahmadian, A. (2009). Analysis of short tandem repeats by parallel DNA threading. *PLoS One,* 4(11), e7823.
17. Irwin, J., Just, R., Scheible, M., & Loreille, O. (2011). Assessing the potential of next-generation sequencing technologies for missing persons identification efforts. Forensic Science International. Genetics Supplement Series, 3, 447–448.
18. Bornman, D. M., Hester, M. E., Schuetter, J. M., Kasoji, M. D., Minard-Smith, A., Barden, C. A., et al. (2012). Short-read, high-throughput sequencing technology for STR genotyping. Biotechniques. *Advance online publication*.

19. Warshauer, D. H., Lin, D., Hari, K., Jain, R., Davis, C., LaRue, B., et al. (2013). STRait Razor: A length-based forensic STR allele-calling tool for use with second-generation sequencing data. *Forensic Science International. Genetics*, 7(4), 409–417.
20. Van Neste, C., Vandewoestyne, M., Van Criekinge, W., Deforce, D., & Van Nieuwerburgh, F. (2014). My-Forensic-Loci-queries (MyFLq) framework for analysis of forensic STR data generated by massive parallel sequencing. *Forensic Science International*. Genetics, 9, 1–8.
21. Binladen, J., Gilbert, M. T., Bollback, J. P., Panitz, F., Bendixen, C., Nielsen, R., et al. (2007). The use of coded PCR primers enables high-throughput sequencing of multiple homolog amplification products by 454 parallel sequencing. *PLoS One*, 2(2), e197.
22. Gunnarsdottir, E. D., Li, M., Bauchet, M., Finstermeier, K., & Stoneking, M. (2011). High-throughput sequencing of complete human mtDNA genomes from the Philippines. *Genome Research,* 21(1), 1–11.
23. Cao, Y., Wan, L. H., Gu, L. G., Huang, Y. X., Xiu, C. X., & Hu, S. H. (2006). Heteroplasmy in human mtDNA control region. Fa Yi Xue Za Zhi, 22(3), 190–192.
24. Li, M., Schonberg, A., Schaefer, M., Schroeder, R., Nasidze, I., & Stoneking, M. (2010). Detecting heteroplasmy from high-throughput sequencing of complete human mitochondrial DNA genomes. *American Journal of Human Genetics*, 87(2), 237–249.
25. Tang, S., & Huang, T. (2010). Characterization of mitochondrial DNA heteroplasmy using a parallel sequencing system. *Biotechniques,* 48(4), 287–296.
26. Holland, M. M., McQuillan, M. R., & O'Hanlon, K. A. (2011). Second generation sequencing allows for mtDNA mixture deconvolution and high-resolution detection of heteroplasmy. *Croatian Medical Journa*l, 52(3), 299–313.
27. Parson, W., Strobl, C., Huber, G., Zimmermann, B., Gomes, S. M., Souto, L., et al. (2013). Evaluation of next-generation mtGenome sequencing using the Ion Torrent Personal Genome Machine (PGM). *Forensic Science International.* Genetics, 7(5), 543–549.
28. Walsh, S., Liu, F., Wollstein, A., Kovatsi, L., Ralf, A., Kosiniak-Kamysz, A., et al. (2013). The HIrisPlex system for simultaneous prediction of hair and eye colour from DNA. *Forensic Science International: Genetics,* 7, 98–115. https://doi.org/10.1016/j.fsigen.2012.07.005
29. Walsh, S., Chaitanya, L., Clarisse, L., Wirken, L., Draus-Barini, J., Kovatsi, L., et al. (2014). Developmental validation of the HIrisPlex system: DNA-based eye and hair colour prediction for forensic and anthropological usage. *Forensic Science International: Genetics,* 9, 150–161. https://doi.org/10.1016/j.fsigen.2013.12.006
30. Walsh, S., Chaitanya, L., Breslin, K., Muralidharan, C., Bronikowska, A., Pospiech, E., et al. (2017). *Global skin colour prediction from DNA. Human Genetics,* 136, 847–863. https://doi.org/10.1007/s00439-017-1808-5