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Review Article

Advancements in Forensic DNA Analysis: Challenges and Future Directions in Molecular Biology

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Abstract

Forensic DNA analysis has revolutionized criminal investigations by providing valuable insights into identifying perpetrators, exonerating the innocent and unravelling mysteries of the past. Various molecular biology techniques, such as Short Tandem Repeat (STR) analysis, Single Nucleotide Polymorphism (SNP) profiling, mitochondrial DNA (mtDNA) sequencing, epigenetics and DNA methylation analysis, have played crucial roles in forensic investigations. While these techniques offer exceptional sensitivity and specificity, they also come with unique challenges. This review explores the current approaches of forensic DNA analysis, highlighting each technique. STR analysis, regarded as the gold standard in forensic DNA profiling, provides high discrimination power. However, it is limited in its ability to analyze degraded or mixed samples. On the other hand, SNP profiling offers advantages in analyzing highly degraded DNA samples but lacks the discriminatory power of STRs. Meanwhile, mtDNA analysis, particularly useful in cases involving compromised nuclear DNA, presents challenges due to its maternal inheritance pattern and lower discriminatory power. Furthermore, explore the exciting realm of epigenetics and the analysis of DNA methylation in forensic investigations. Epigenetic markers offer insights into gene expression patterns influenced by environmental factors, potentially aiding in the determination of tissue origin and chronological age estimation. DNA methylation analysis holds promise in forensic applications, providing additional layers of information for identity verification and tissue differentiation. Despite these advancements, several challenges persist in forensic DNA analysis, including the interpretation of complex DNA mixtures, standardization of methodologies, ethical considerations, and privacy concerns associated with the use of genetic information. Moreover, the integration of multi-omics data and machine learning approaches presents both opportunities and challenges in enhancing the accuracy and reliability of forensic DNA analysis. Looking ahead, future directions in molecular biology research for forensic DNA analysis involve the development of novel techniques with increased sensitivity, scalability, and robustness. By addressing these challenges and embracing emerging technologies, the field of forensic DNA analysis is poised for further advancements, offering enhanced capabilities in criminal justice and humanitarian efforts.

Keywords

DNA Analysis, Epigenetics, Forensics, STR and SNP

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1. Introduction

Background

Since being first used in a criminal case in 1987, the analysis of DNA from biological evidence has revolutionized forensic investigations [1]. Forensic DNA data plays a crucial role in supporting police detective work by helping to identify suspects or victims, eliminate individuals from criminal inquiries, and associate individuals with crimes [2]. The analysis of DNA from biological evidence has brought about a revolution in forensic investigations. Over the past thirty years, there have been considerable advancements in the discrimination power, speed, and sensitivity of DNA profiling methods. Additionally, there has been an improvement in the ability to type more difficult samples [1]. Forensic DNA analysis is a complex process that involves multiple steps in order to accurately identify and analyze DNA samples. These steps include sample collection, DNA extraction, quantification, amplification (PCR), DNA profiling, and the comparison and interpretation of results [3]. The first stage of forensic DNA analysis involves collecting samples. Various types of samples, such as blood, saliva, semen, genital or rectal swabs, skin or tissue cells, fingernails, and urine, can be collected from crime scenes and used analysis. The most commonly obtained sample types from suspects or individuals involved in paternity investigations or other genetic relationship inquiries are blood, oral swabs, and plucked hairs. Secondly, DNA is extracted from a sample. Friedrich Miescher conducted the initial DNA extraction in 1869. Since then, scientists have made significant advancements in developing extraction methods that are more efficient, cost-effective, and reliable. These methods also offer faster results and produce higher DNA yields. The increasing demand for reliable and efficient DNA isolation methods, particularly in the fields of gene-editing and personalized medicine, has led to the development of techniques that can produce substantial quantities of high-quality DNA with minimal impurities. Among the different extraction methods available, the Chelex-100 method, silica-based DNA extraction, and phenol-chloroform method are commonly used [4]. After extracting DNA, it is important to measure the quantity and quality of the DNA extract accurately. Adding the correct amount of DNA to the PCR process will yield the highest quality results in a shorter period of time. Adding too little or too much DNA will result in a profile that is difficult or even impossible to interpret [5]. DNA amplification is essential to ensure that there is enough DNA for downstream analysis, which is mainly carried out via polymerase chain reaction (PCR) methods. After amplifying the DNA for analysis, the DNA sample is passed through a capillary tube that contains a gel. This gel allows smaller STR sequences to move more quickly through its pores. At the end of the capillary, a laser beam and a detector record the fluorescent tag of each sequence. By measuring the time it takes for the sequences to travel and the strength of the fluorescent signal, the capillary gel electrophoresis system can determine the length of the STR sequences at each analyzed locus.

In addition to capillary gel electrophoresis, DNA analysis also utilizes next generation sequencing (NGS) techniques, which offer several advantages. For instance, NGS can differentiate between STRs of the same length but with different base pair sequences. Furthermore, NGS handles degraded DNA samples more effectively. However, NGS systems are only cost-effective when used with large numbers of samples. Therefore, both methods coexist in DNA fingerprinting [6]. The last step is to compare the genetic fingerprint obtained with that of another sample. The results of capillary gel electrophoresis, which are represented by electropherograms for each sample, can be easily compared to determine if the samples match or differ. This comparison process can be done manually or automated [7].

Human populations exhibit both length and sequence genetic variation. These types of variation are important for forensic DNA testing because noncoding regions of the genome can contain a wide range of different alleles. By combining information from multiple unlinked genetic markers, it becomes possible to achieve a high level of discrimination. In forensic DNA typing, the use of genetic markers to characterize biological samples is a key aspect of human identification. The initial approach to SNP-based typing for forensic analysis focused on the HLA-DQA1 locus (previously known as HLADQα) polymorphism. Another polymarker that was studied is Ampli®TypePM, an expanded version of HLA-DQA1 analysis. This polymarker includes five different markers: LDLR, GYPA, HBGG, D7S8, and GC, which have a high power of discrimination. However, the method has limitations when it comes to analyzing DNA samples with more than one contributor. This limitation has been overcome with the development of conventional STR typing [8]. Although short tandem repeat (STR) loci have long been established as the primary genetic marker for human identification, there has been exploration into the potential use of additional markers, such as single-nucleotide polymorphisms (SNPs), by genetic analysts [9, 10]. In recent years, short tandem repeat (STR) markers, which involve variations in the length of specific DNA sequences, have become the primary method used for forensic DNA profiling [3]. There are now large national DNA databases that contain millions of STR profiles. These profiles are based on a few core STR markers [11]. Due to having a specific set of Short Tandem Repeat loci in these massive databases, it is unlikely to see a new set of DNA markers to be introduced shortly [12]. Now a day, there has been an increase in the number of loci included in short tandem repeat typing kits. Additionally, there has been a standardization of core loci across different jurisdictions. These developments have enabled the enhanced sharing of

DNA profiling data across borders [13].

Forensic DNA analysis involves many barriers, from sample collection to the final interpretation. According to Butler [14], key challenges in the forensic science field are the subjectivity, inconsistency of the complex DNA mixture interpretations between different laboratories and analysts, and the need for training forensic analyst to enhance interpretation of DNA profiles.

2. Current Approaches in Forensic DNA Analysis

Forensic DNA analysis has made significant progress since its inception in the 1980s, with the development of various techniques. Currently, STR analysis is the primary method [15]. In addition to the adoption of STR approaches, other methods like Y chromosome analysis, mitochondrial DNA (mtDNA) analysis, single nucleotide polymorphism (SNP) typing, and mini STR analysis have also been developed.

2.1. Short Tandem Repeat (STR) Analysis

Short Tandem Repeats (STRs) are the main genetic tool used in forensic DNA examinations. These markers consist of short, repetitive DNA sequences that vary in both length and pattern of the core motif [16]. STR markers found on both autosomal and Y chromosomes are frequently used in human identity testing. Since approximately half of an individual's genetic material is inherited from each parent, sets of forensically relevant autosomal STRs represent a random assortment of alleles. The distinct combination of alleles observed across multiple loci can confidently link DNA collected from a crime scene to a known source [17]. Both auSTRs and Y-STRs have increased power of discrimination when more loci are examined. However, in certain casework scenarios, even interrogating a reasonably large number of loci may not be sufficient because the resulting profiles are limited by the size-based data generated [18].

STRs utilize polymerase chain reaction to amplify a set of short tandem repeats found in the DNA template. Capillary electrophoresis of short tandem repeats has been the route of method in DNA identification for more than 20 years. Enhance the accuracy and overlap with international databases, several commercial kits have been developed; include the Promega PowerPlex Fusion 6C System, the QIAGEN Investigator 24plex QS, and the Applied Biosystems GlobalFiler PCR amplification kit [19-21]. One crucial challenge that modern forensic DNA analysts face is the need to decrease the time it takes to analyze samples. Currently, there are various approaches to achieve this, such as reducing incubation times for DNA extraction [22] and some experts propose direct PCR as a solution, which completely eliminates the need for extraction and quantitation steps [23]. Additionally, efforts have been made to speed up the processes of amplification, separation, and detection [1].

There are various ways to increase the speed of the PCR amplification step. One of the common and significant for forensics to speed up the PCR amplification was successfully conducted by Gibson-Daw and his collages [24] a multiplex amplification in just 6 minutes using a 7-locus multiplex on a high-speed thermocycler and a rapid polymerase. It has been demonstrated that using lower total volumes can help to decrease the time required for heating and cooling of the sample, resulting in a 56-73% reduction in amplification time [25]. In a similar study, DuVall was achieved amplification in 15 minutes by using a 10-loci multiplex that included a subset of the CODIS loci, all of which were smaller than 350 base pairs [26]. The potential discriminatory power of both auSTRs and Y-STRs is not limited by a lack of variability within the regions themselves. Instead, it is limited by the fragment length approach currently used in forensic DNA examinations [27].

The Y chromosome, along with the X chromosome, is one of the pairs of human sex chromosomes found in the nucleus of human cells. It is accompanied by 22 pairs of autosomal chromosomes. Typically, individuals with an X and Y chromosome are male, while those with two X chromosomes are female. However, there are rare variations in the number of these chromosomes or other unique mutations that can impact this phenotype [28]. In situations such as sexual assault or when collecting fingernail scrapings, it is possible for the female victim's DNA to overshadow that of the male assailant. To address this issue, it is crucial to consider using data from the Y chromosome, as it is less likely to be influenced by the female victim's DNA. As a result, there have been ongoing efforts to expand and enhance the Y chromosome STR loci. Recently, a research group from China successfully developed and validated a typing system that involves the simultaneous amplification of 37 Y-STRs using capillary electrophoresis with 6 dye chemistry. The objective of this development was to enhance the discriminatory power when differentiating between male individuals [29]. Other scholars have investigated the use of Y-STR loci in anthropological and lineage studies to determine the reliability of Y-STR markers. They compared the commercial YFiler STR kit, which consists of 17 STR loci, with a new Y Filer plus kit that contains 27 loci. The findings showed that increasing the number of loci in the system enhances discrimination power and assists in confirming familial relationships [30].

Andersen and his colleague were conducted research to assess the reliability of Y-STR kits and reduce the impact of stochastic effects. They have developed custom software to enhance the determination of analytical thresholds used in system validation and application [31]. Some studies also concentrated on creating Y-based miniSTR loci to improve

the analysis of degraded and trace evidence [32] and massively parallel sequencing also employed to detect and classify variant Y-STR sequences [33]. In addition, studies have been conducted to develop X chromosome short tandem repeats (X-STRs). These X-STRs can be particularly valuable in paternity testing. The possibility of linkage between different X-STR loci was examined by testing a set of 15 X-STRs among 158 families [34]. More X-STR loci have been developed and validated to increase the discriminatory power and aid in the analysis of degraded samples [35]. In 2017, two separate papers focused on the discovery of novel X-STRs and the study of genetic linkage as a useful tool for kinship determination [36, 37].

Interpreting DNA profiles that include contributions from multiple donors is significantly more complex than single source profiles. This is not only due to the potential presence of a greater number of alleles in the profile, but also because these profiles often exhibit low-level characteristics with complicating factors like allele drop-out/drop-in and heterozygous imbalance [38]. Due to the advancement in STR profiling techniques, the chances of obtaining mixed DNA profiles have significantly increased. This can occur not only in samples where mixtures are anticipated, but also in low-quality or low-quantity samples recovered from handled items [23]. Such samples often result in complex mixtures with a large number of contributors and no individual who can be assumed to be present in the mixture [39].

The increasing complexity of mixed profiles has led to the development of more complex methods for interpreting mixtures. There has been a shift away from simple methods that determine whether an individual can be excluded as a potential contributor to a mixture, towards the use of likelihood ratio methods. These methods estimate the most likely genotype combinations of contributors to a mixture, with some of the more complex methods taking into account information from profile peak heights [40]. This has prompted the development of mixture interpretation methods that use probabilistic frameworks. These methods incorporate probabilities of allele drop-out and drop-in, which are model based on validation and empirical data [41]. Probabilistic genotyping methods can broadly be categorized as semi-continuous, which do not use peak height information or model artifacts such as stutter, and continuous, which do [42]. Generally STR analysis is a highly reliable and widely used technique in forensic science due to its high degree of polymorphism, sensitivity, and ability to analyze even highly degraded DNA samples.

2.2. Single Nucleotide Polymorphism (SNP) Analysis

The human genome is composed of approximately 3.2 billion base pairs. Within this genome, there are repeated DNA sequences that differ in size, number, and length of core repeat units [10, 43]. Polymorphism, or the variation in a sequence, is a significant characteristic of the human genome. Single-nucleotide polymorphism (SNP) specifically refers to sequence variants that occur when there is a single base pair change in the genome. This change in sequence can be caused by a substitution, insertion, or deletion of a base at a single site. The human genome contains approximately 10 million SNPs, of which around 1.4 million have been identified which are valuable for forensic identification. These SNPs are primarily located in noncoding regions, although they can also be found in coding regions of the genome. While the majority of SNPs are biallelic, there have been reports of tri- and tetra-allelic SNPs as well [44].

STR marker systems are widely used in forensic investigations for human identification. This is due to their high genetic instability and discriminatory power, as well as the ease of analysis using established CE-based typing workflows. Although STRs will likely remain the standard approach in forensic genetic analysis, certain types of cases may benefit from the use of relevant single base substitutions, insertions, and deletions [9]. These variants, known as SNPs, are common throughout the genome and account for about 85% of the observed inter-individual genetic variability in humans [45]. One advantage of SNPs is that they require shorter amplicons for analysis, making it possible to analyze samples with highly degraded or low template DNA. While it is unlikely that STRs will be completely replaced, SNPs provide additional genetic information that can enhance current forensic DNA analysis [9]. The use of SNPs also offers technical advantages. For example, by using an automated microarray technique, millions of SNPs can be analyzed rapidly. Second, the difficulties of allele calling (e.g., stutters and artifacts) associated with STR analysis are not encountered in SNP analysis. SNPs have a much lower mutation than STRs [46].

The high variability of STR markers is useful for human identity testing, but it doesn't tell us much about lineage, ancestry, and phenotype. On the other hand, lineage-informative SNPs found in the mtGenome and Y chromosome is valuable for understanding the evolutionary origins of human populations. These markers have a low mutation rate and do not undergo recombination, making them ideal for this purpose [9].

The use of single nucleotide polymorphism in sex chromosomes has increased with the development of better sequencing and genotyping techniques. Y-SNPs, in particular, have been recognized for their importance in determining ancestry and differentiating between individuals [47]. Ancestry and lineage markers are crucial in forensic casework, especially when dealing with unidentified suspects. The Ampliseq Identity Panel was utilized for haplogroup assignment and paternity testing [48, 49]. Additionally, Y-SNP variants have been identified in specific populations to enhance the size and discriminatory power of databases. A study conducted on the Flemish population

examined Y-SNPs and discovered variant alleles in 270 male samples [50]. Fifteen new SNPs were developed to subtype the haplogroup R1b-DF27, which is highly prevalent among Iberian and Iberian-influenced populations [51]. SNP analysis in forensic science typically involves methods such as polymerase chain reaction (PCR) to amplify specific SNP regions, followed by genotyping techniques to determine the genetic variants present at those SNP loci.

2.3. Mitochondrial DNA (mtDNA) Analysis

DNA profiling as a forensic technique has been in use for several decades. Specifically, the analysis of short tandem repeats found in nuclear DNA is the gold standard for comparing questioned samples to individuals [52]. However, there are situations nuclear DNA analysis may not be possible. In samples with little to no nuclear DNA, such as hair shaft samples and degraded or damaged human remains, mitochondrial DNA (mtDNA) is often able to provide useful information and important markers in forensic DNA analysis. The mitochondrial genome is generally considered to be maternally inherited [53]. Forensic samples, such as bone and hair, can benefit from the application of mtDNA. Unlike autosomal DNA, which only has a few copies per cell, there are hundreds of copies of mtDNA in each cell. This makes mtDNA analysis much more sensitive. Additionally, mtDNA can be used for lineage studies because male mtDNA is not transferred during fertilization. However, it is important to note that mtDNA is less probative than autosomal STRs and autosomal single nucleotide polymorphisms (SNPs) because it does not undergo sexual recombination. Typically, mtDNA is analyzed using sequencing methods. However, mtDNA SNPs can also be probed using other techniques, such as SNapShot. For instance, a SNaPshot procedure was developed to genotype a panel of 52 phylogenetic informative mtSNPs. This method proved to be efficient in classifying haplogroups and could be useful in forensic analysis [54]. An interesting paper by Strobl et al. analyzed mtDNA in hairs, bones, and teeth that had previously been analyzed using Sanger sequencing. Using massively parallel sequencing, the study demonstrated that full genome profiles can be obtained from samples stored over a period of years [55].

A similar procedure was used to assess the effectiveness of massively parallel sequencing for mixture analysis. The Precision ID mtDNA Whole Genome Panel, Ion Chef, and Ion PGM/S5 sequencer from Thermo Fisher were utilized in this study [56]. Another research study focused on deconvoluting mixtures by examining heteroplasmic sites in the sequence. Heteroplasmy refers to mutations in the mtDNA that lead to an individual having two different mtDNA sequences at the same location. To demonstrate the deconvolution of mixed sequences, artificial mixture samples were created and analyzed to identify heteroplasmy. The results revealed that mtDNA heteroplasmy with a peak height ratio [48] above 10% could be differentiated from sequencer noise [57]. In addition, a separate group analyzed very old skeletal samples using the latest next-generation sequencing (NGS) techniques to evaluate the impact of degradation and deamination. This project investigated degraded DNA and demonstrated that enzymatic repair might be an additional tool for forensic analysis of this nature [58].

2.4. Epigenetics and DNA Methylation

Epigenetics is the study of reversible, heritable changes that affect how genes are regulated without altering the underlying DNA sequence [59]. One of the well-studied epigenetic modifications is DNA methylation, which involves the addition of a methyl group to cytosine in DNA [60]. While most DNA methylation occurs in CpG dinucleotides [61], there have been documented cases of methylation occurring in other contexts such as CpT, CpA, and CpC [62, 63]. The human genome contains millions of CpGs, which can exist in different methylation states depending on factors like chromosomal location, alleles, cell type, or developmental phase [64]. Certain regions of DNA, such as introns, 3' untranslated regions (UTRs), and intergenic sequences, have a low density of CpGs, whereas exons tend to have a slightly higher density of CpGs [65].

Tests that rely on DNA methylation modifications are suggested for various forensic purposes, include estimating the chronological age of a DNA donor, distinguishing between identical (monozygotic) twins, and identifying body fluids [66].

Epigenome-wide association studies (EWAS) have identified numerous individual CpG sites and genomic regions that exhibit distinct methylation patterns between human tissue and body fluids. These sites are referred to as differentially methylated sites (DMS) and tissue-specific differentially methylated regions (tDMRs) respectively [67, 68]. tDMRs are primarily located at the edges of CpG islands, and they have lower CpG and G/C content compared to the surrounding regions. It is believed that tDMRs play a role in providing cells with an epigenetic memory by generating cell-type specific hypo- and hypermethylation patterns [69]. By comparing differential methylation profiles, DMSs and tDMRs can be used to differentiate between different tissues and fluids [70]. Forensic samples do not always exist in large quantities or high quality. It is crucial to limit the consumption and degradation of valuable evidence. DNA methylation-based assays are compatible with current short tandem repeat (STR) typing protocols. These assays also allow for multiplexing, which means multiple body fluids can be identified simultaneously [71, 72].

In generally the technologies that used to the detection and analysis of DNA methylation divides into three categories based on pre-treatment of the DNA prior to analysis: methylation sensitive restriction enzyme digestion, affinity

enrichment, and sodium bisulfite modification. The pattern of DNA fragmentation by restriction enzymes, whose cleavage ability is dependent on the methylation status of CpG sites in their recognition site, can be used to determine the DNA methylation status of target regions. This is a robust, simple approach that can be combined with capillary electrophoresis or sequencing-based techniques to produce quantitative methylation results. However, it relies on the presence of specific recognition sites flanking the CpG of interest [73], is prone to false positives due to incomplete digestion [74] and lacks sensitivity for tightly packed CpG sites in a genomic region [75].

Affinity binding involves the interaction between a protein or antibody and the methyl group [76]. This method is used to enrich and isolate methylated DNA for downstream PCR or array-based analysis [77]. While affinity-binding techniques preserve the integrity of the DNA sequence, they lack high specificity and require a high DNA input, which is impractical for forensic samples [78].

Bisulfite conversion is a chemical modification of cytosine residues using sodium bisulfite. Unmethylated cytosine residues are converted to uracil through hydrolytic deamination, while methylated cytosine residues remain unchanged. Bisulfite-treated DNA then undergoes PCR amplification, during which uracil residues are replaced by thymine. This conversion leads to different DNA sequences depending on the methylation status of a cytosine, which can be detected using various downstream methods. The proportion of DNA methylation at a specific CpG site can be determined by comparing the ratio of cytosine residues (previously methylated cytosines) to thymine residues (previously unmethylated cytosines). This method allows for the quantitative assessment of DNA methylation and offers specificity through primer design for target selection [78].

3. Challenges in Forensic DNA Analysis

Forensic DNA analysis is an incredibly powerful tool for solving crimes and identifying individuals. However, it also poses several challenges, including sample contamination, degradation, mixtures, low DNA quantity, complex DNA profiles, human error, and ethical legal concerns, and others.

One of the most significant challenges in forensic DNA analysis is the risk of DNA contamination. Contamination is when an individual's DNA is accidentally introduced into the evidence sample, either during or after collection. This can happen at the crime scene or in the laboratory [79]. When working with challenging samples, detecting contamination with exogenous DNA can be complex. Analyzing forensic DNA contamination presents two main challenges. The first challenge is identifying a minor contributor that cannot be assessed due to stochastic effects, making interpretation difficult. The second challenge lies in distinguishing between multiple contributors when contamination involves more than three profiles. The sources of DNA contamination can vary depending on the sample and analysis. Both endogenous and exogenous DNAs can be co-extracted or co-amplified, or only the contaminating DNA may be amplified and detected. The high sensitivity of PCR and its ability to amplify low copy number (LCN) DNA can create difficulties in managing challenging samples. Therefore, it is necessary to implement extraordinary measures and validation protocols in the laboratory to prevent sample contamination [80].

DNA can degrade over time due to environmental factors like heat, moisture, or sunlight. This degradation can result in incomplete or unreliable outcomes. The process of DNA degradation involves two mechanisms; first, nucleases break down DNA into fragments and then, microorganisms digest these fragments, causing random DNA fragmentation [81]. DNA fragmentation and modification occur simultaneously and randomly through the DNA bond cleavage [82, 83], Oxidative damage [84, 85], cross-links, hydrolysis-induced rupture [86] and others.

Sometimes, forensic samples contain very small amounts of DNA, which can be difficult to extract and amplify for analysis. Low DNA quantity can lead to incomplete profiles or increased risk of contamination, replicate analyses, and controls [87].

Crime scene samples often contain DNA from multiple individuals, making it challenging to isolate and analyze individual profiles accurately. Untangling mixed DNA samples requires sophisticated techniques and interpretation. Analyzing DNA mixtures poses a challenge for scientists, as it becomes increasingly difficult to distinguish individual profiles as the number of contributor’s increases. One common type of mixture involves a known source of DNA from a victim and an unknown source from a suspect [88].

4. Future Directions in Molecular Biology for Forensic DNA Analysis

Most forensic DNA laboratories primarily focus on the repeatability of autosomal STR markers, specifically 20 markers, to ensure a sufficient level of discrimination between two individuals [89]. STR markers are considered the most suitable markers for forensic DNA analysis due to their multi-allelic nature, high rate of mutation per generation (10−2), small size, and extensive diversity [90]. However, the analysis of STR markers has been mostly limited to 30 markers or fewer using the Capillary Electrophoresis (CE) approach due to the challenges associated with multiplexing PCR reactions [91]. Additionally, the amplification of STR markers often generates artifacts, complicating the analysis process. Moreover, identifying the DNA profile of a specific individual from a mixed sample, especially when the contributor's DNA is present in a low ratio, can be highly time-consuming [88]. However, there are several promising

directions in molecular biology that could further enhance its capabilities. These promising directions include next-generation sequencing, single-cell analysis, CRISPR-based technologies, nanopore sequencing, and machine learning and bioinformatics.

To address these issues, sequencing approaches, particularly the Next Generation Sequencing (NGS) technique, are being employed. However, most of the currently used NGS-based sequencing approaches for STR fragments rely on targeted sequencing, which reduces the fraction of informative reads containing a complete microsatellite to less than 6% due to the random fragmentation process [92]. Furthermore, as targeted sequencing is PCR-dependent and involves multiplexing, it also generates artifacts alongside the desired fragments. Whole Genome Sequencing (WGS) is not useful for sequencing STR fragments as it provides low sequencing coverage of intact STR fragments, typically around 30× or 60× [93].

Traditional DNA analysis requires a large amount of biological material, which can make it difficult to analyze trace samples. However, single-cell analysis techniques allow for DNA profiling from individual cells, making it possible to analyze cases where only a few cells are available, such as touch DNA samples. While analyzing mixed DNA samples is common in forensic investigations, it often presents challenges. Single cell analysis can help overcome these complexities by allowing targeted analysis of a single cell to obtain a single donor and source profile. Thanks to advancements in human DNA amplification kits and new methods like the DEPArray NxT system (Menarini Silicon Biosystems), single cell analysis is now possible in forensic science. Early methods of single cell recovery include laser capture microdissection (LCM) and fluorescence-activated cell sorting (FACS). The DEPArray system, which was introduced more recently, utilizes a dielectrophoretic grid on a microfluidic device. This system allows for the capture, identification, selection, and subsequent recovery of the cell of interest [94, 95]. Researchers have developed another method called STR-Seq that relies on CRISPR-Cas9 technology. This method allows for the generation of high-coverage, accurate genotypes by producing sequence reads that span microsatellite regions. In their study, Shin et al. used CRISPR–Cas9 guide RNAs (gRNAs) to selectively cut the genomic DNA near the targeted STR locus. They then prepared a single adaptor library and used 40-mer primer probes to facilitate STR targeting [92]. This technique offers several advantages over whole-genome sequencing (WGS), and it is consistent with traditional capillary electrophoresis (CE) results. Compared to other next-generation sequencing (NGS) methods, the CRISPR-dependent approach demonstrates superior efficiency in assay design and sequencing. Additionally, this technology can be applied to sequence STR-SNP conjugates, thereby expanding the range of genotypic information available. Consequently, the CRISPR-Cas9 system shows great potential for forensic DNA analysis, particularly in low copy number (LCN) samples. By cutting the DNA fragment at a specific location and performing PCR-independent sequencing of the target region, this method can greatly enhance the analysis of such samples.

An alternative method for rapid DNA profiling on-site is the pocket-sized MinION nano-pore sequencing device (Oxford Nano-pore Technologies, Oxford, UK). Nano-pore sequencing platforms enable real-time, long-read DNA sequencing, which can be particularly useful for analyzing degraded or challenging forensic samples. Weighing less than 100 g, the MinION has a maximum throughput exceeding 10 gigabase pairs (Gbp), which is theoretically enough to profile large numbers of samples. Furthermore, the device is portable, allowing for on-site analysis of samples. This feature could prove invaluable for tasks such as disaster victim identification or analyzing extremely time-sensitive crime scene samples [96]. However, using the MinION for forensic genetic analysis presents several challenges. Firstly, the MinION sequencer is not specifically designed for forensic analysis and relies on sample preparation and post-sequencing analysis. Secondly, nano-pore sequencing is more prone to errors compared to Sanger and massively parallel sequencing [97]. In pilot studies assessing the accuracy of STR profiling with the MinION, high error rates were observed in a limited number of samples [98-100]. When it comes to genotyping human SNPs, nanopore sequencing is more accurate than STR genotyping [101]. Identity-informative SNPs are considered supplementary markers to STRs in identity and kinship testing [102]. Nano-pore sequencing has been used to profile a 52-SNP panel developed by the SNP for ID consortium and a panel of 16 tri-allelic SNPs in a few DNA standards and individual samples, with characterization of several problematic SNP loci [103, 104]. By leveraging these emerging technologies and methodologies, forensic DNA analysis can become more robust, sensitive, and informative, ultimately enhancing its utility in criminal investigations and legal proceedings. However, it's important to ensure that any new techniques adhere to rigorous standards of reliability, accuracy, and ethical considerations within the criminal justice system.

5. Conclusion

In conclusion, forensic DNA analysis has become an essential tool in criminal investigations, providing unmatched precision in identifying individuals and linking them to crime scenes. Current approaches in forensic DNA analysis utilize techniques such as polymerase chain reaction (PCR), short tandem repeat (STR) analysis, and next-generation sequencing (NGS). These methods enable the extraction, amplification, and analysis of DNA from a wide range of forensic samples, including blood, saliva, and hair. Despite its remarkable success, the field faces challenges

such as DNA contamination during collection and processing, sample degradation, low DNA quantities, and the interpretation of complex mixtures.

However, advancements in sample collection and extraction techniques, sequencing technologies, and bioinformatics tools are expected to enhance the depth and accuracy of DNA analysis. Furthermore, the interpretation of complex DNA mixtures from multiple contributors presents a significant challenge. Future directions in molecular biology offer promising solutions to these challenges. Improved methods for sample collection, preservation, and extraction may enhance the recovery of DNA from challenging forensic samples. Advancements in sequencing technologies, such as next generation sequencing, RISPR-Cas9 and nanopore sequencing, hold potential for increasing the depth and accuracy of DNA analysis.

Additionally, the development of bioinformatics tools and statistical models aims to better interpret complex DNA profiles and handle mixture analysis.

Overall, ongoing research in molecular biology is poised to address current challenges in forensic DNA analysis and drive innovation in the field, ultimately improving the accuracy and reliability of forensic investigations.

Abbreviations

LCN	Low Copy Number
NGS	Next-Generation Sequencing
CE	Capillary Electrophoresis
WGS	Whole-Genome Sequencing
STR	Short Tandem Repeat

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