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Preface

DNA fingerprinting technique is the most trusted gift of science to the mankind, as it is helpful in the field of the criminal justice system, deciphering the genetics of living organisms, diagnosis of the genetic disorder, neonatal diagnosis, cracking of ancestral belongingness, wildlife forensics, and many more. The technique has undergone much advancement with time since its inception. With the advent of time, the technology has spread its tentacles in various other fields which have resulted in the introduction of new academic courses along the globe to fulfill the demand of expertise in diverse aspects of DNA fingerprinting. This demand also resulted in the demand of available literature. However, most of the books available in this field concentrate either on forensic applications or disease diagnosis or legal issues. However, a consensus reference book in this field describing the basics, various applications and use of the technology in real case studies are lacking. Hence the present volume is planned to touch the fields of genetics, tools, and techniques, description of real-time case studies, wildlife forensics, molecular diagnosis of human diseases, legal aspects, and microbial forensics.

The current volume includes four parts: *Part 1*: Basics of DNA Fingerprinting: Tools and Techniques, *Part 2*: Applications of DNA Fingerprinting, *Part 3*: DNA Fingerprinting: Case Studies, and *Part 4*: Future of DNA Fingerprinting. Part 1 consists of four chapters describing the discovery and advancements of DNA technology as well as the involvement of various tools and technology for DNA fingerprinting application. Part 2 covers various applications of DNA fingerprinting including wildlife forensics, identification of mutilated remains, molecular diagnosis of human diseases, human trafficking, and from judicial point of view. Application of various types of currently practiced DNA fingerprinting techniques using Autosomal and Y chromosome STR typing as well as mitochondrial DNA sequencing for criminal justice system has been described in Part 3. Finally, Part 4 harbors some futuristic approach of current day DNA fingerprinting such as whole genome sequencing and microbial forensics.

The current volume has been written in simple English that may require basic biological science background to understand. It will be helpful for the students' from the fields of Zoology, Wildlife, Medicine, Anthropology, Microbiology,

Forensic Science, Genetics, and Law at graduate, postgraduate, and research level. For scientific fraternity, it will be a handy reference to quickly summarize the technological advancements in the field of DNA fingerprinting, to understand the problems faced by this field of science and possible updated solutions to these problems. As nowadays, DNA fingerprinting is used in solving most of the criminal cases, this book will be helpful among the law practicing friends as well. Investigating agencies can also gather a sound knowledge from this book as real case studies have been included here.

We have tried our best to share the available knowledge around the globe in the field of DNA fingerprinting with the aim to provide an important and rationalized resource material in the form of this edited volume. Throughout the editing process of the book, we have faced many problems and hurdles and all have been overcome due to God's grace, self-belief, and nice people surrounding to us. We are highly thankful to each and every one for their support and encouragement during this process. Wishing a good luck to all the readers.

Sagar, Madhya Pradesh, India — Hirak Ranjan Dash
Sagar, Madhya Pradesh, India — Pankaj Shrivastava
New Delhi, Delhi, India — Braja Kishore Mohapatra
Rourkela, Odisha, India — Surajit Das

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Dr. Hirak Ranjan Dash completed his Ph.D. from the Department of Life Science, National Institute of Technology, Rourkela, India, and is currently working as a Scientific Officer (DNA) at the Forensic Science Laboratory, Madhya Pradesh, India. He received his M.Sc. in Microbiology from Orissa University of Agriculture and Technology, Odisha, India. His research interests include forensic microbiology, thanatomicrobiome analysis, molecular microbiology, environmental microbiology, DNA fingerprinting, microbial phylogeny, genetic manipulation of bacterial systems, and microbial diversity. He has developed a number of microbial techniques for the assessment of mercury pollution in marine environments and has successfully constructed a transgenic marine bacterium for enhanced utilization in mercury removal by simultaneous mercury volatilization and sequestration. He has written 3 books and published 28 research papers, 11 book chapters, and 12 conference proceedings.

Dr. Pankaj Shrivastava received his Ph.D. in Microbiology from Rani Durgavati University, Jabalpur. He is presently serving as a Scientific Officer at the DNA Fingerprinting Unit, Forensic Science Laboratory, Madhya Pradesh, India. He has more than 10 years of experience in examining a variety of criminal cases using DNA fingerprinting. The central theme of his research is the DNA analysis of caste and tribal populations of different parts of India, along with the development of new methodologies for improved forensic DNA typing. Till date, he has published 11 books and 61 scientific articles in reputed international journals. He is a visiting faculty of National Police Academy, Hyderabad; National Institute of Criminology and Forensic Science, Government of India, Delhi; and the Central Police Academy, Bhopal, along with many central and state universities of India. He is a recipient of the Pt. Govind Vallabh Pant Samman Award from the Ministry of Home, Government of India; the Anusrijan Samman Award from AISECT University, Bhopal; the Dr. Lalji Singh Memorial Award; and the FICCI Smart Policing Award for the development of a direct protocol in forensic DNA typing.

Dr. Braja K. Mohapatra completed his Ph.D. from Utkal University, Bhubaneswar. He is presently serving as a Senior Scientific Officer and Head of the Department of Biology and DNA Profiling Unit, Central Forensic Science Laboratory (CBI), New Delhi, India. He has more than 10 years of experience in examining various criminal cases using DNA fingerprinting. His research interests include the interpretation of DNA profiles in mixed samples, touch DNA, and population genetics. He has 13 peer-reviewed publications in reputed national and international journals to his credit. He is a recipient of the meritorious service award in forensic science. He has solved various high-profile cases through DNA fingerprinting, both in India and The Republic of Seychelles.

Dr. Surajit Das is an Associate Professor at the Department of Life Science, National Institute of Technology, Rourkela, India. He received his Ph.D. in Marine Biology from the Centre of Advanced Study in Marine Biology, Annamalai University, Tamil Nadu, India, for his research work on marine microbiology. He has been awarded an Endeavour Research Fellowship by the Australian Government to carry out postdoctoral research at the University of Tasmania. As group leader of the Laboratory of Environmental Microbiology and Ecology (LEnME), he is currently conducting research on the biofilm-based bioremediation of PAHs and heavy metals using marine bacteria; nanoparticle-based drug delivery and nanobioremediation; and metagenomic approaches for exploring the diversity of immunoglobulins in the Indian Major Carps, supported by research grants from the Ministry of Science and Technology; Indian Council of Agricultural Research; Ministry of Environment, Forest and Climate Change; and the Government of India. He is an Academic Editor for *PLOS One* and an Associate Editor (Ecological and Evolutionary Microbiology) for *BMC Microbiology*.

Part I
Basics of DNA Fingerprinting: Tools and Techniques

Chapter 1
DNA Fingerprinting: Discovery, Advancements, and Milestones

Jahangir Imam, Romana Reyaz, Ajay Kumar Rana, and Vrijesh Kumar Yadav

Abstract The discovery of DNA fingerprinting is one of the most fascinating scientific discoveries till date. It is not only limited to the laboratory research but also showed a huge potential in forensic science and criminal justice system. It was one of the milestones in resolving crimes by exploring the polymorphism of human DNA in noncoding regions. Since its inception, DNA fingerprinting has taken a great leap in terms of advancements in technology, accuracy, and reliability of the results as well as rapidity of the process for its more efficient application in justice delivery systems. This has become the most valuable armory of the judiciary system to aid in the conviction of guilty as well as exoneration of the innocent. Advancement of DNA fingerprinting technique from RFLP to STR and now NGS has sped up the process of DNA profiling with better discriminating power among individuals with greater efficacy. In this prospect, the current chapter elaborately recapitulates the process of advancement in DNA fingerprinting describing the use of different STR kits, i.e., autosomal STRs, Y-STRs, X-STRs, miniSTRs, etc., for forensic applications. We have also highlighted the importance of SNPs and amalgamation of NGS kits in forensic application. Notably, the importance of wildlife forensic has been discussed for the identification of species as well as its geographic origin. Another important budding aspect of RNA-based identification of forensically relevant biological fluids has also been discussed in much detail.

Keywords DNA fingerprinting · Criminal justice system · STRs · Forensic analysis

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1.1 Introduction

DNA or deoxyribonucleic acid is the hereditary polymer molecule made up of four different nucleotides, i.e., adenine (A), thymine (T), guanine (G), and cytosine (C). These nucleotides arranged in a definite sequence represent the genetic makeup of all known living organisms and some viruses. In humans, the DNA is present within the nucleus of every cell (except mature red blood cells) and is organized into 46 different chromosomes (22 pairs of autosomes and one pair of sex chromosomes). A haploid DNA is approximately three billion base pair in length. Additionally, 99.9% DNA sequences are similar between two individuals [70]. The difference of 0.1% of the DNA sequence between two individuals is usually present along the noncoding regions of the DNA. This difference of sequences is targeted by the forensic scientists to discriminate between individuals by matching DNA isolated from varied samples, i.e., blood, tissue, body fluid, or hair/nails, with high level of accuracy. The difference in DNA sequence basically occurs in terms of around 2–9 base pair repetitions called as microsatellites or of 10–100 bps called as minisatellites [39, 57]. In earlier times, Sir Alec Jeffreys analyzed minisatellites or variable number tandem repeats (VNTR) using Southern blotting to visualize DNA markers [73]. However, currently, microsatellites of around 4 bp repeats are being analyzed along with fluorescent tags of respective markers using multiplex amplification of at least 20 core loci [46, 71] as defined by the Federal Bureau of Investigation (FBI), USA [12, 75]. These loci along with their respective repeat patterns are inherited from parents to their offspring by a random process during meiosis, and every individual can be discriminated from another (as calculated by FBI among Caucasian population) by a probability factor of 1.683×10^{-15} [20], i.e., one individual can have the same DNA pattern with another only in a population of 594 trillion. The exception is monozygotic twins who carry the same loci with the same repeat pattern.

1.2 Discovery

DNA fingerprinting was initially used to find human genetic diseases by linking particular DNA sequences with the help of segregating markers which were present in close proximity within a chromosome [12, 15]. Eventually, it was also used for criminal investigations and forensic science, when an undaunting Ph.D. scholar Alec J. Jeffreys from Leicester University, United Kingdom, took a scientific responsibility to nab the culprit of famous twin girl's rape and murder case from Narborough using classical DNA fingerprinting assay using VNTR method [12, 15, 73]. The geneticist Alec J. Jeffreys discovered that short repetitive DNA sequences are almost unique to every individual, and he called them "minisatellites."

In 1984, at the Leicester University, UK, Dr. Jeffreys was studying hereditary diseases in families and was also focusing on developing methods to resolve

paternity and immigration disputes by linking genetic elements between individuals, and he published his results in *Nature* journal [28, 29]. He discovered that variable number tandem repeats (VNTR) is a part of junk DNA and these repeats vary from one individual to another. He employed various restriction enzymes in his technique to cut these variable DNA sequences and generate length polymorphism. He then run these digested sequences in a very long agarose gel and demarcated the variations from individual to individual. He named his technique "genetic fingerprinting" and demonstrated that this restriction fragment length polymorphism (RFLP) of DNA was unique to each individual and cannot match on earth except for identical twins. However, his technique was launched into the world of forensic science when two murders were committed in a little village of Narborough in the city of Leicestershire, east of Birmingham, UK. In 1983, 15-year-old lady Lynda Mann was found raped and murdered, and in the same village after 3 years in 1986, Dawn Ashworth, also 15, was also raped and murdered in similar manner. Dr. Jeffreys was asked to do DNA analysis of the semen samples recovered from the two victims with the blood sample of a suspect named Richard Buckland, aged 17. Some of the detectives of this case were in suspicion as Buckland was below 14 years of age when the first crime occurred. From the DNA analysis of the samples, Dr. Jeffreys demonstrated and proved that the same killer's semen is present in both the crime scenes and this does not match with Buckland blood sample's DNA. After this, the law enforcement took an exhaustive task to match the blood types and then DNA of total 4582 men from the three towns. Dr Jeffrey did genetic fingerprinting of the 10% men who were tested same for blood group (Group A) and isoenzyme, i.e., phosphoglycerate mutase (PGM). However the DNA could not match with any one of them. After several months, a resident heard a conversation in a pub, where a person named Ian Kelly confessed that he had taken bribe for replacing his photo with a photo of a local baker named Colin Pitchfork in his passport and had submitted his own blood instead of Pitchfork [32]. Twenty-seven-year-old Colin Pitchfork was arrested on September 19, 1987, and when his DNA was compared with the semen samples, it was indistinguishable, i.e., identical. Pitchfork was found guilty in both rapes and murders, and in 1987, Pitchfork became the first person in the world to be identified and convicted as a result of the DNA fingerprinting. He was sentenced to life imprisonment on January 22, 1988 for both murders and is currently in jail. In 1994, Professor Jeffreys was knighted by The Queen of England, and today he is still a professor in the Department of Genetics at the University of Leicester in Great Britain.

1.3 Advancement and Milestones in DNA Fingerprinting

DNA fingerprinting, since its discovery around two and half decades back, has taken a great leap in its advancement and made the justice delivery system more efficient and accurate in the investigation of criminal and civil cases [28–30]. This is much like a valuable armory in the hands of judiciary which aids in the conviction

of the guilty as well as exoneration of the innocent [10]. It has also been proven helpful in linking relationship of reference samples to dead remains of missing person and in mass disasters like plane crash, vehicle collision, earthquakes, etc. [10, 51]. With the discovery and innovation of new techniques for DNA extraction and genotyping, the generation of DNA profiles is becoming more and more accurate and easy, even for challenged and trace DNA samples. The known fact about DNA fingerprinting is its uniqueness among human populations, and this attribute makes it the method of choice [19]. DNA profiling generally involves the five basic steps from sample preparation, DNA extraction, DNA quantitation, DNA amplification to capillary electrophoresis, and profile generation [60]. With the advancement in different fields of science, new technologies are regularly introduced and validated in forensic laboratories to aid the process of DNA fingerprinting with improved sensitivity and informativeness. Day by day, crimes are increasing which poses a paramount pressure on judiciary system. In this regard, the use of automation techniques for sample preparation and data interpretation by the forensic laboratories will be useful to meet this increasing throughput demands on the laboratories [10].

DNA fingerprinting requires good scientific skills and data interpretation ability which is essential in result outcome. Earlier, the methodology used for DNA extraction to profile generation had lots of limitations for the type and quality of biological samples available for forensic investigation. The advancements in genomic and post-genomic era have put the forensic science one step ahead, and now it is much faster, higher, and stronger in crime investigation and judiciary system [10], *faster* in terms of rapid DNA instrumentation, recovering *higher* and good quality of data from biological evidences and *stronger* conclusion on complex evidences [10]. The development in forensic DNA analysis is possible because the pioneer work, progression, and innovation transpire over the past three decades (Table 1.1) [9, 60].

1.3.1 Current Status of DNA Fingerprinting Technique

The discovery of DNA fingerprinting is nothing less than bliss, not only to the scientific world but also to the judiciary system. Alec Jeffreys laboratory was the only one to work on DNA fingerprinting during 1985–1987, and his work and contribution in solving civil and criminal cases with the help of DNA fingerprinting is pioneer in establishment and adoption of this technique in judicial investigations worldwide. The last three decades are the golden periods in the field of forensic science with the advent and implementation of new techniques, the use of advanced commercial DNA typing kits with various genetic marker systems, and NGS in forensic science.

Since the discovery of DNA fingerprinting and its application in forensic judiciary system, the procedure for biological sample collection from the crime scene and DNA extraction methodology from different biological specimens are well established [24, 25, 43, 58, 60, 65, 69, 74]. Here we will discuss the advancement and emerging techniques and methodologies of DNA fingerprinting. Forensic sci-

Table 1.1 Timelines and milestones for forensic DNA analysis

Timeline	Major area	Activities and milestones
1985–1995	Development and exploration	1. Discovery of DNA fingerprinting and first publication by Alec Jeffreys
		2. RFLP using VNTRs
		3. Rapid and sensitive PCR assays
		4. Formation of DNA profiling groups
1995–2005	Stabilization and standardization	1. Improvement in PCR technology
		2. Development of sensitive, fast, and genotypic precision STR-based DNA analysis
		3. National DNA database formation by the United Kingdom with six STR markers
		4. 13 core STR markers designated by the United States for FBI CODIS software
		5. Multiplexing and capillary electrophoresis
		6. Autosomal and Y-STR kit released
		7. Expansion of forensic research in different laboratories
		8. Importance of Human Genome Project (HGP) in forensic DNA technology development
2005–2015	Augmentation	1. Faster and efficient DNA extraction methods adopted
		2. Automation
		3. Accumulation of DNA database
		4. Advancement in different STR kits
		5. Development and validation of new STR kits
		6. Encroachment of DNA forensic in wild life
		7. Forensic DNA phenotyping (FDP)
		8. Implementation of microbial DNA fingerprinting of human fingerprints
		9. Advancement in forensic RNA typing
		10. Introduction and exploitation of NGS in forensic science
2015–2025	Sophistication	1. Development of tools for rapid DNA testing outside of laboratories or at crime scene
		2. Huge population database formation
		3. Development of cost effective forensic DNA analysis
		4. More better multiplexing system and DNA typing
		5. More research in the field of wild life forensic, microbial DNA fingerprinting and forensic RNA typing
		6. Development of cheaper and more robust STR kits
		7. Development of skills and ability to interpret forensic evidence results
		8. Higher sensitive methodologies applied to casework and probabilistic software approached to complex evidence

ence has gone through several stages of development since its discovery and application in the 1980s [19]. The first generation of DNA analysis RFLP-based profiling is obsolete now from the forensic point of view, because it was not suitable for degraded and challenging biological forensic samples as it was not able to analyze the samples with accuracy. The PCR-based second generation of DNA analysis based on dot-blot methods was then developed but could not be fruitful enough as it was not helpful with DNA fragments which were longer in length. The third generation is STR (short tandem repeats) based which is easy, suitable, and most widely accepted for DNA analysis, but, sometimes, for highly degraded DNA samples, getting DNA profile becomes difficult. The fourth generation of DNA analysis which is introduced in forensic science is NGS (next-generation sequencing) which has attracted the forensic community with its high-throughput capacity and low cost [4]. There is a continuous effort to develop more effective, cheap, and fast DNA profiling techniques with more discriminatory power to address the application of forensic science in different fields (Table 1.2). Here, we have highlighted some of the recent progresses made in the analysis of STRs, SNPs, low-template DNA, mitochondrial DNA, DNA methylation, microbial forensic, and NGS in forensic and illustrate how different technologies can be integrated for new-generation forensic science.

1.3.1.1 Evolution of Capillary Electrophoresis as a Tool for Forensic DNA Analysis

Capillary electrophoresis (CE) is one of most important instrumental advancements to be implemented for forensic DNA typing. After PCR invention, scientists consider it as the second most needed development. Application of capillary electrophoresis in forensic DNA analysis not only makes the work easier but also makes it more accurate and authentic, which is of paramount importance in forensic DNA analysis [63]. The application of capillary electrophoresis is not only limited for biological samples but also has a huge importance in the analysis of gunshot residues, explosive residues, and drugs. For forensic DNA analysis, STR profiling (highly polymorphic markers) which is based on fragment analysis is of great value for human identification (HID) due to the single-base resolution capability of CE [63]. Introduction of capillary electrophoresis in STR typing circumvents the tedious and expensive approach of DNA sequencing for STR typing. The approach of CE like precise sizing, its sensitivity for the detection of fluorescence emitted by different dyes, automatic electrophoresis, and data collection software are key factors in the worldwide adoption of CE as the preferred platform for forensic DNA analysis. The most common CE systems used in forensic DNA analysis include the ABI PRISM® 310, 3100, 3100 Avant, 3130, 3130xL, 3500, and 3500xL Genetic Analyzers (GAs). The advanced CE automated machines are developed with advanced features which are useful for forensic scientists. It has many advantages like normalization of peak height, accurate sizing of fragments, sample injection, single-base resolution, high run-to-run precision, good temperature control and

Table 1.2 Different commercial STR kits for forensic application produced after the year 2000

S.No.	STR kit name	No. of markers	Application and advantages	Make
1	Identifiler™, IdentifilerPlus™ and IdentifilerDirect™	Amelogenin Plus 15 marker (13 CODIS + D2S1338 and D19S433)	Greater sensitivity and improved performance on inhibited forensic samples. More robust master mix and modified thermal cycling parameters	Life Technologies
2	PowerPlex® 16 and PowerPlex® 16HS	Amelogenin Plus 13 CODIS marker	Enhanced buffer system with improved robust performance	Promega Corporation
3	PowerPlex® 18D	Amelogenin Plus 15 marker (13 CODIS + D2S1338 and D19S433 + Penta E and Penta D)	Improved allelic ladder featuring many additional alleles not found in original PowerPlex® 16 PowerPlex® 16HS kit. It is also compatible with direct amplification from FTA card punches as well as non-treated paper	Promega Corporation
4	NGM™	Amelogenin Plus 15 marker (ENFSI loci -mini- and midi-STRs)	It enables simultaneous, multicolor fluorescence detection in a single PCR by using five-dye chemistry	Life Technologies
5	NGM SElect™ and NGM SElect™ express	Amelogenin Plus 16 marker (ENFSI loci- mini- and midi-STRs)	It enables simultaneous, multicolor fluorescence detection in a single PCR by using five-dye chemistry	Life Technologies
6	PowerPlex® ESX-16 and PowerPlex® ESI-16	Amelogenin Plus 15 marker (ENFSI loci – mini- and midi-STRs)	It enables simultaneous, multicolor fluorescence detection in a single PCR by using five-dye chemistry	Promega Corporation
7	PowerPlex® ESX-17 and PowerPlex® ESI-17	Amelogenin Plus 16 marker (ENFSI loci – mini- and midi-STRs)	It enables simultaneous, multicolor fluorescence detection in a single PCR by using five-dye chemistry	Promega Corporation
8	ESSplex and ESSplex SE	Amelogenin Plus 15 marker (ENFSI loci – mini- and midi-STRs)	It enables simultaneous, multicolor fluorescence detection in a single PCR by using five-dye chemistry	Qiagen, Hilden, Germany
9	PowerPlex® ES	Amelogenin Plus 8 marker (SE33 locus)	Addition of highly polymorphic SE33 locus. It has limited use in relationship testing due to highest mutation rate	Promega Corporation

(continued)

Table 1.2 (continued)

S.No.	STR kit name	No. of markers	Application and advantages	Make
10	SEfiler™ and SEfilerPlus™	Amelogenin Plus 10 marker (SE33 locus)	Addition of highly polymorphic SE33 locus. It has limited use in relationship testing due to highest mutation rate. Improved synthesis and purification processes, enhanced sensitivity for inhibited samples	Qiagen, Hilden, Germany
11	PowerPlex® 21	Amelogenin Plus 20 marker (13 CODIS + D2S1338 and D19S433 + Penta E and Penta D + 3 marker)	It can work with a variety of sample types, including casework samples. It is also compatible with direct amplification from FTA card punches as well as non-treated paper	Promega Corporation
12	GlobalFiler™ Express	Amelogenin Plus 23 marker (13 CODIS + D2S1338 and D19S433 + 7 marker + Y Indel gender loci)	Additional loci included (MiniFiler loci) which are designed to meet the expanded US core loci requirements. It is optimized for efficient amplification of low level DNA and to overcome common inhibitors of the PCR. Optimized for the amplification of single-source samples	Life Technologies
13	PowerPlex® Fusion	Amelogenin Plus 23 marker (13 CODIS + D2S1338 and D19S433 + Penta E and Penta D + 6 marker)	Additional loci included (MiniFiler loci) which are designed to meet the expanded US core loci requirements. It is a dual-purpose kit in that it can be used for common casework samples as well as direct amplification of reference samples stored on paper with only minor changes to the PCR amplification conditions	Promega Corporation
14	PowerPlex® CS7 System (nonstandard STR marker system)	Seven STR loci	It is used as a confirmatory kit in paternity applications	Promega Corporation
15	Investigator HDplex kit (nonstandard STR marker system)	Amelogenin Plus 13 marker (highly polymorphic markers)	It is developed specifically to discriminate closely related individuals. It is designed for difficult forensic and paternity cases	Qiagen, Hilden, Germany
16	MiniFiler ™	Amelogenin Plus 8 CODIS marker	For genotyping degraded DNA samples. First commercial kit designed to amplify miniSTRs	Life Technologies
17	PowerPlex Y	12 Y-STR loci	First sex-chromosome STR kit developed to identify male lineages	Promega Corporation

18	Yfiler ™	17 Y-STR loci	Most commonly used sex-chromosome STR kit to identify male lineages as it works well in most outbred populations	Applied Biosystems
19	Argus Y-12 QS	12 Y-STR loci + internal control	Sex-chromosome STR kit developed to identify male lineages. The internal control system provides helpful information about PCR efficiency and about the presence of inhibitors in tested samples	Qiagen, Hilden, Germany
20	PowerPlex® Y23	12 Y-STR loci of Yfiler Kit plus six additional new informative loci for male-lineage differentiation	It allows Y-STR analysis of both human forensic samples and database samples. It features fast amplification time and better tolerance of inhibitors of the PCR when compared to previous generations of Y-STR multiplexes	Promega Corporation
21	Argus X-12 kit	12 X-STR loci	Simultaneous amplification of 12 X-chromosomal markers for kinship and paternity testing, as well as population genetics and anthropological studies. Also suited for forensic stains, such as female traces in male background	Qiagen, Hilden, Germany

automation, better sensitivity, high throughput, user-friendly, and easy software features to analyze the raw data to the level of precise accuracy [63]. Definitely the incorporation of CE in forensic application must be considered as a milestone for the service of the mankind.

1.3.1.2 STR and Next-Generation STR Genotyping Kits for Forensic Application

Forensic DNA typing has been constantly evolving driven by innovations from academic laboratories as well as kit manufacturers [47]. Much technological advancement took place during the last 30 years, but the PCR-based STR genotyping is central to all. STRs are now the markers of choice for various human identification (HID) applications as the STR loci are considered polymorphic as they are unique to each individual [8]. The basis of individual identification by STRs is the measurement of length of different alleles which exhibit the highest variability among individuals [21]. Mono-, di-, tri-, tetra-, penta-, and hexanucleotide repeats of STRs are available, but tetranucleotides are commonly used and preferred in STR analysis because the chance of stutter production is minimal and it can analyze the amplicons that are one repeat less than the true allele [60]. Now, PCR-based multiplexing of STRs and capillary electrophoresis enables the analysis of several different loci at the same time with better accuracy and ready to available data for interpretation [44]. In 1997, the United States established a core set of 13 STR loci known as the Combined DNA Index System (CODIS) loci. The 13 STR loci set had strong distinguishing power with average random match probability of one in a quadrillion (1×10^{-15}). After the establishment of CODIS loci, Promega Corporation (Madison, Wisconsin) and Life Technologies developed the commercial kits to meet the demand of the forensic community [47].

1.3.1.3 Multiplex STR Kits

After 2000, Promega designed the PowerPlex 16 System, and Life Technologies came up with AmpFLSTR Identifiler PCR Reaction kits which were capable of amplifying all 13 CODIS markers in a single PCR reaction along with the amelogenin sex identification marker [14, 38]. The AmpFLSTR Identifiler PCR Reaction Kit and the PowerPlex 16 System have been widely used for forensic database generation and casework analysis, both. This helps in the generation and addition of millions of STR profiles to the DNA databases helping the criminal judiciary system. With huge criminal cases happening frequently, forensic laboratories are continuously seeking and adopting enhanced technologies which help them to process database and casework samples more efficiently and effectively. The AmpFLSTR

Identifiler Plus PCR Amplification Kit and PowerPlex HS System were developed for various challenging forensic samples for greater sensitivity and improved performance. Various autosomal STR kits have been produced after the year 2000 for challenging and inhibited biological samples which are listed in Table 1.2. Later on in 2011, CODIS announced an increase in the number of CODIS loci to reduce the likelihood of adventitious matches, to increase international compatibility, and to increase the discrimination power (Mulero and Hennessy). Life Technologies developed GlobalFiler™ and GlobalFiler™ Express PCR Amplification kits, and Promega Corporation developed PowerPlex Fusion System as a next-generation STR kit to meet this challenge by incorporating extra loci in the kits, hence making it more robust (Table 1.2). For genotyping degraded DNA samples, the first commercial STR kit, "MiniFiler Kit," was developed to amplify "miniSTRs." The improvement was done to amplify degraded samples by repositioning the primers as close to STR repeat region, as possible (Table 1.2) [11, 13, 66]. New STR kits were developed for human identification by incorporating highly polymorphic STR locus, SE33 (a tetranucleotide STR). Inclusion of SE33 in multiplex has shown a huge advantage due to its structural variations and polymorphism with differentiation of around 1 bp [55, 59]. This marker also possesses the highest mutation rate, thus limiting its application in forensic use such as relationship testing [16, 49]. PowerPlex® CS7 System (Promega Corporation) and Investigator HDplex Kit (Qiagen), a nonstandard STR marker system, were also developed for very special cases which involved in kinship analysis and testing with samples deficient of close relatives. It has been developed specifically to discriminate closely related individuals for difficult forensic and paternity cases (Table 1.2). Many sex-chromosome STR kits were also developed to identify male and female lineages (Table 1.2).

The next-generation commercial kits listed in the Table 1.2 have been developed by improving the performance with inhibited samples, increased sensitivity, high throughput with direct amplification, increased CODIS loci, and paternity- and lineage-specific STRs which provide a "driving force" for the progress of forensic science.

1.3.1.4 ABO Typing with Multiplexes STRs

Few years back, Jiang et al. [31] developed a successful technique, in which both ABO genotyping and STR analysis were combined in a single reaction, where a forensic scientist could get both the information from a biological sample in a single reaction. The developed technique has a combination of all the 15 autosomal STR loci, gender-determining locus amelogenin, and markers for six ABO genotypes. This was an important development as it is more accurate and has better confirmatory power than normal ABO blood group typing.

1.3.1.5 Autosomal SNPs and Indels in Forensic Analysis

Many STRs have been developed (autosomal, MiniFiler, and sex chromosome STRs) in lieu to overcome the challenges of generating DNA profiles from forensic samples. This is only possible because of the greater variability of DNA polymorphisms. But forensic science always faces tough challenges in terms of low-level DNA or degraded DNA, to obtain complete STR profiles. Degraded DNA or low template (LT) causes problems in STR typing either with allele dropouts or allele drop-ins which in many times are unable to get even with increased sensitivity and miniSTR typing [1, 2]. The possible alternatives to increased PCR sensitivity of STRs for degraded DNA or low template (LT) are to use SNPs and insertion/deletions (indels). SNPs used in LT DNA can result in fewer allele drop-ins [5]. SNPs (single-nucleotide polymorphisms) and indels (insertion/deletion polymorphisms) are the most common short binary markers of the human genomic variations. SNPs allow allele detection of comparatively small amplicon size ranges (about 41 bp) and therefore can be a better option with highly degraded samples [6]. But still, SNPs have not been whole heartedly adopted in forensic science as the marker of choice, for highly degraded samples, as new and advanced STR technologies have come up which can enhance the profiling performance even for highly degraded DNA [7]. Therefore, SNPs are not very much suitable for normal forensic casework and database entries when working with DNA mixtures. It is most suitable for identification of missing persons and relationship establishment. STRs present better information than single SNPs, but as we increase the number of SNP markers, they could provide better discriminating power. One of the valuable characteristics of SNPs is variation at heterozygosity level of the genome. Other important advantages of SNPs is that it does not require separations on the basis of size which makes multiplexing and automation easier compared to the STR analysis and low mutability rate making it more stable genetic marker [18].

Two SNP multiplexes have been developed for forensic identifications: a 52-SNP assay developed by the SNPforID consortium, comprising a 52-plex PCR followed by tandem 21- and 29-plex primer extension reactions [50, 61] and a 44-plex PCR followed by tandem 18- and 26-plex extensions [41] based on the Kidd Lab forensic identification marker panels, consisting of a list with almost twice as many ID-SNPs than the 44 collated in this assay [53]. The 52-plex multiplex is best suited for highly degraded DNA samples, i.e., for very old skeletal remains or body recovered from the river or sea. This shows its importance in identification of missing person. Here, it is also important to note that the number of SNPs required to match the informativeness of STRs is higher in relationship testing than in identification applications. Table 1.3 listed a few techniques and variations of SNP analysis.

Indels (insertion/deletions) comprise about 5% of known polymorphisms in human genome and are thus considered as potential markers in forensic identification as they have combined the application of both SNPs and STRs [48]. The advantages of indels over SNPs are as follows: first, ease of analyzing indels from very short amplicon size as compared to SNPs, and, second, the ease of doing indel analysis by combining the advantage of direct PCR-to-capillary electrophoresis

Table 1.3 List of few techniques and variants of SNP analysis

Sl. No.	SNP techniques	Details	Application	References
1	SNaPshot assay	1. Capable of multiplex SNP analysis in small amount of template DNA samples	SNPs analysis for degraded DNA samples	
		2. Highly sensitive, can analyze samples shorter than 70 bp amplicons		
2	TaqMan assay	1. Capable of multiplex SNP analysis in small amount of template DNA samples	SNPs analysis for degraded DNA samples	
		2. Highly sensitive, can analyze samples shorter than 70 bp amplicons		
3	Y-SNPs multiplex system	1. It is sensitive, human specific	Applicable for Chinese Han population	
		2. Informative in cases of degraded DNA and male-male mixture		
		3. Resistant to high background of female DNA in male-female mixture		
4	Mitochondrial DNA SNPs (mtSNPs)	1. Mt DNA SNPs are lineage markers	Lineage-specific SNP analysis for degraded teeth and bone DNA samples	
		2. Not capable of genetic individualization		
		3. Can be employed for degraded bone and teeth samples		
5	Nucleosome SNP assay (18-plex single-base extension assay)	1. Based on the principle that histone-DNA complexes found in nucleosomes offer protection from DNA degradation process	Best suited for highly degraded DNA samples. It provides a new marker set that can be used to supplement existing SNP assays	Freire-Aradas et al. [18]
		2. It offers better results than other existing forensic SNP assays		
6	21 SNP multiplex system	1. Target amplicon length for 21 SNPs is from 63 bp to 192 bp	This system provides a reliable technique for individual identification of nondegraded and degraded DNA samples	
		2. More efficient in the analysis of degraded DNA compared with standard STR typing		

typing, as this is not possible with SNP typing using SNaPshot assay. Different indel typing kits were developed like Investigator DIPplex Kit (Qiagen, Hilden, Germany) and indel-plex. Both have shown successful application for typing highly degraded DNA samples in forensic science.

1.4 Next-Generation Sequencing and Its Application in Forensic Sciences

Next-generation sequencing (NGS), the technology which has overcome the limitations of conventional Sanger sequencing, has grown rapidly in recent years in the field of genomics research because of its high-throughput capacity and low cost and ancient DNA analysis [54, 62]. Over the last 10 years, NGS methods and platforms have evolved, and sequencing quality has now reached a level where NGS can be launched in forensic science, and in the last 2 years, there has been an explosion in scientific articles, with forensic applications of NGS. Since the number of casework samples which require DNA processing is increasing day by day, the CE-based methods which have fixed capabilities are sometimes unable to stand. Therefore, NGS technology and platforms show promising results in DNA testing and identification of missing persons, kinship testing, ancestry investigation, and other human identification applications. In NGS technology, simultaneous amplification of multiple STR marker types and SNPs can be achieved in a single run for large number of samples.

1.4.1 Next-Generation Sequencing Kits or Systems in Forensic

1.4.1.1 HID-Ion AmpliSeq Ancestry Panel

HID-Ion AmpliSeq ancestry panel (Life Technologies) enables simple and fast target selection of hundreds of SNPs using multiplex PCR. Thousands of primer pairs can be used in a single tube for target amplification followed by next-generation sequencing (NGS) on the Ion PGM™ System. This ready-to-use panel consists of 165 autosomal markers that provide biogeographic ancestry information. Fifty-five of these markers were selected based on a poster by Dr. Kenneth Kidd [35, 36], and 123 markers were selected based on a publication by Dr. Michael Seldin [37]. Ion AmpliSeq technology makes it possible to multiplex 165 PCR reactions in one tube with only 1 ng of input DNA. With small amplicon sizes, the panel is optimized for degraded DNA samples that provide the biogeographic ancestry information and guide the investigation process.

1.4.1.2 Precision ID NGS System for Human Identification

Precision ID NGS System for human identification (Applied Biosystem) for human identification can help in solving tough cases by getting more information from the challenging samples. It is the combination of Ion Chef System and Ion S5 or Ion S5 XL Systems with forensically relevant Precision ID panels that utilize Ion AmpliSeq technology. It includes the same 21 autosomal STRs, along with Y indel and amelogenin sex marker found in the GlobalFiler DNA amplification kit. Instead of using SE33, this panel includes nine additional multiallelic STR markers (for a total of 33 targets) to aid in mixture interpretation of complex casework samples.

1.4.1.3 ForenSeq™ DNA Signature Prep Kit

ForenSeq™ DNA Signature Prep Kit (Illumina) is developed by incorporating multiple STRs kits which include over 200 forensically relevant genetic markers in a single, streamlined workflow, eliminating the need for multiple STR kits [64]. This kit includes global autosomal STRs, Y-STRs, X-STRs, identity-informative SNPs (iiSNPs), phenotypic-informative SNPs (piSNPs) (eye and hair color), and biogeographical ancestry SNPs (aiSNPs) in a single platform, which is not available with traditional CE-based methods. This kit overcomes the limitations of other CE-based kits for degraded, mixed, or PCR-inhibited samples.

1.4.1.4 PowerSeq™ Systems

PowerSeq™ systems (Promega) for forensic identification include three systems: (a) PowerSeq™ Auto includes 23 STR loci and amelogenin, (b) the PowerSeq™ Mito system generates ten small amplicons (adapted from Eichmann and Parson) covering the control region of the mitochondrial genome, and (c) PowerSeq™ Auto/Mito/Y combines both sets of amplicons in one multiplex plus 23 Y-STR loci. PowerSeq™ Auto system is a 24-plex kit for analyzing autosomal STRs, amelogenin, and DYS391. PowerSeq™ Mito system is based on sequencing of the mtDNA control region (HV1 and HV2). PowerSeq™ Auto/Mito/Y system has been configured for the simultaneous analysis of 22 autosomal STRs, amelogenin, 23 Y STRs, and the control region of the mitochondrial genome [3, 17, 72].

1.4.2 Forensic Application Prospects of NGS Technology

Forensic science technology has embraced DNA technology as the main weapon to address various crimes and help the judiciary. Today, PCR- and CE-based DNA typing is the backbone of forensic science and criminalistics. Various STRs

Table 1.4 Forensic application prospects of NGS technology

Sl. No.	Application	Advantages
1	STR typing	High throughput, low cost, simultaneous detection of large numbers of STR loci (autosomal and sex chromosome STRs), and the ability to distinguish alleles with similar length facilitate the identification of mixed DNA samples and analysis of complex paternity cases
2	Mitochondrial genome analysis	Important in maternal lineage identification; whole mitochondrial sequence for identification with high discrimination power
3	Y-chromosome analysis	Establishes paternal relationship between male individuals
4	Forensic microbiological analysis	NGS is suitable for whole-genome typing of microbial pathogens during forensic and epidemiological investigations which can detect even the rare polymorphisms and thus give forensic data higher resolution and greater accuracy for accurate identification of criminals and biological terrorists
5	Animal and plant DNA analysis	NGS technology has allowed the DNA typing in plant and animal species identification
6	Ancestry studies and phenotypic inferences	NGS technology for whole-genome sequencing will be helpful in determination of ancestry and personal characteristics like ethnicity, physical and physiological characteristics, and age
7	Epigenetic analysis	Epigenetic changes like methylation pattern can be easily studied and employed in forensic genetics with the aid of NGS technology
8	MicroRNA analysis	NGS has proved to be very critical in analysis of millions of miRNA sequences for rapid identification of organ and developmental stage-specific expression and expression in different diseases which is a powerful tool in forensic analysis

(autosomal, miniSTRs, sex chromosome, phenotypic STRs) are CE based, and CE still is the method of choice for forensic analysis because of its accuracy, specificity, discriminatory power, and easy handling. The use of NGS in forensic science as in human identification (HID) and determination of phenotypic traits leads to its larger application in forensic analysis. Definitely NGS technology has a lot of advantages over tradition CE-based typing, and there is little doubt that NGS will be implemented and used in forensic laboratories in the future. Table 1.4 presents the overview of NGS application prospects in forensic application.

1.5 RNA Profiling and Its Application in Forensic Science

The presence of biological evidences at the crime scene and its correct screening for the possible source of DNA have always been the challenges for the forensic expert. Many conventional biochemical and immunological assays are there for the screening of biological fluids, but they are time-consuming and laborious and even consume the important evidentiary material. Because of this problem, many forensic scientists bypass these preliminary screening processes and directly proceed for

DNA analysis which many times lead to failure to provide the information regarding the nature of crime [23]. In recent times, molecular approaches for the identification of body fluids have been developed which have significantly improved the sensitivity. The use of RNA profiling strategies is considered the better option for the identification of forensically relevant biological fluids and tissues such as saliva, vaginal secretions, menstrual blood, and skin [23].

The forensic identification of human body fluids and tissues by means of messenger RNA (mRNA) profiling is a long-studied methodology that is being increasingly applied to casework samples [68]. From a singleplex PCR technique to multiplex RT-PCR platform, mRNA profiling has evolved in a big way in providing expression of data on multiple genes simultaneously [40]. A single mRNA-based system, 19-plex system, has been developed for the discrimination of common forensic body fluids as well as skin cells [40]. This 19-plex system is able to establish both the donor and the cell type of the samples. This 19-plex mRNA assay showed good results with body fluids, with high sensitivity and specificity. The 19-plex mRNA assay targets six different cellular origins to provide better assessment, which is important in forensic casework.

1.6 Wildlife Forensics

Soon after the discovery, the forensic DNA profiling has revolutionized criminal investigation process in humans. Today, forensic DNA analysis has become an indispensable tool for different criminal cases and helps in the arrest of many perpetrators as well as exonerations of many innocent individuals who were wrongly convicted. As the forensic DNA analysis is growing day by day and its applications are also being introduced in other fields, its need is also felt in the investigation of wildlife-related crimes and wildlife conservation. Wildlife and their products constitute the third most illegally traded commodity worldwide, after arms and drugs [34, 42, 45]. An important difference between crimes against humans and wildlife is that an animal becomes a “silent witness” to a human crime scene and it’s like no “victim” to provide information regarding the investigation. Another important issue with wildlife forensic science is that various species of animals or plants have to be analyzed as against single species in human identification cases [33]. Various sample types can be there in wildlife forensic science like whole animals (dead or alive), skins or skeletal of animals, exoskeleton and shells, and animal body parts such as the leg, wings, head, fur, scales, teeth, beak, claws, skin, carcass, horns, organ parts, blood samples, and many more [33]. Therefore, good and improved preservation techniques are required to support the prosecution in smuggling, poaching, and laundering of wildlife and their products [45]. In wildlife forensics, species identification is more important than individual identification. In addition, geographical region of the samples can also be analyzed.

Table 1.5 List of currently available techniques in wildlife forensic analysis

Sl. No.	Techniques used	Application	References
1	STR loci	1. Individual identification, i.e., investigation to determine if two samples are from the same individual	Johnson et al. [33] and van de Goor et al. [67]
		2. Pedigree analysis of a particular individual	
		3. Dinucleotide repeat STRs are common in identification of domesticated species	
2	Mitochondrial DNA (mtDNA) typing	1. Most commonly used in forensic DNA typing because:	Johnson et al. [33], Ivanova et al. [27], Guha and Kashyap [22], Imaizumi et al. [26], Pun et al. [56] and Osborne et al. [52]
		A. Mitochondrial loci are used for molecular taxonomy and phylogenetic	
		B. The presence of universal primers can be applied to unknown samples	
		C. It works well with highly degraded samples	
		2. Commonly used mitochondrial loci are cytochrome *b* (cyt*b*) and cytochrome oxidase 1 (COI)	
		3. Other loci are ribosomal RNA genes, D-loop region, and subunits of mitochondrial encoded NADH dehydrogenase	
		4. MtDNA sequencing for wildlife forensic identification	
3	Pyrosequencing techniques	1. Direct sequencing of thousands of small DNA fragments (degraded DNA) in a single run	Karlsson and Holmlund [34]
		2. Individual DNA typing of previously unknown species	
		3. It is rapid, accurate, and flexible and allows parallel processing	
4	NGS or high-throughput sequencing	1. Mass parallel sequencing for the identification of repetitive DNA sequences, for the identification of new STRs and SNPs	Johnson et al. [33] and Kidd et al. [35], [36]

DNA-based analysis is now frequently used and is most commonly applied in species identification cases. Now it's also being introduced for the analysis of geographic location of the species captured/claimed. The DNA markers applicable for the wildlife forensic science are different from human identification (HID) markers. As stated earlier, this is because of the complexities of species identification. STRs and SNPs are used for individual identification, pedigree analysis, and assignment of an unknown sample to a population [33]. Table 1.5 lists some of the currently available techniques of wildlife forensic analysis.

1.7 Conclusion

In this review, a brief overview of discovery, advancements, and milestones in the field of forensic DNA analysis has been outlined. Many new discoveries took place after the discovery of DNA fingerprinting, and these new approaches continue to be explored for more effectiveness. DNA fingerprinting relies most basically on the quality of DNA, DNA isolation from the biological samples has improved a lot, and good-quality DNA can be extracted even from highly degraded samples. The discovery of PCR and then advancements in real-time PCR have put the forensic science on the road of successful DNA fingerprinting. The advancement in STR technology and kits further improved the ability to decipher and interpret DNA results from challenging samples which provided the opportunity for future advances in forensic DNA analysis. The various forms of STR kits have revolutionized the field of forensic DNA profiling. The evolution in capillary electrophoresis technology and tough competition among the firms lead to cheaper and better kits, and this is the reason why forensic DNA fingerprinting has advanced so much, and now, it is helping the judiciary to solve the ever-increasing criminal and civilian cases. NGS has also knocked the door of forensics, and the day is not very far when it will be validated and incorporated as a conclusive technique to serve the judiciary system. RNA profiling is also proving to be a helping hand of forensic science in many relevant cases. The development in various molecular tools to investigate wildlife-related crimes has advanced the wildlife forensic analysis, and very soon, a well-placed technique will be available for individual and species identifications. Although various new techniques and scientific improvements are coming up, the current methods of STR typing are reliable, valid, and widely applicable.

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