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DNA Profiling Technologies in Forensic Analysis

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ABSTRACT The remarkable advances in DNA technologies over the past two decades have had an enormous impact on human identification, medical diagnosis, population genetics, understanding of evolution of species, wildlife management, characterization and unfolding the mysteries of antiquity of archaic specimens. This article presents an overview of the current technologies in the field of forensic genetics, their evolution and the emerging trends. DNA techniques have opened a new frontier in forensic analysis. Until 1985 all polymorphic immunological and biochemical markers employed to identify biological samples in criminal cases had limited applicability. The way to a new course of events was first paved by the introduction of minisatellites using multilocus probes (MLPs), providing incomparably higher discriminatory power. However, the procedures for multilocus typing were too arduous and required very large amounts of intact DNA which were usually not recovered from the difficult forensic biological specimens. This paved way for initiation of single locus probes (SLPs) in VNTR (variable number of tandem repeats) detection analysis. The advent of the polymerase chain reaction (PCR) was the turning point in the crucial issue of analytical efficiency of the DNA variants in the genome. Since then the field of molecular identification seems to have acquired a virtual unlimited power of analysis allowing experts to address even the most inaccessible sources of DNA. The first PCR amplification based DNA technique used in human identification was the reverse dot blot assay (HLADQA1 and PM typing), which was followed by short tandem repeat (STR) PCR multiplex analysis. Ease of amplification and automation are the salient features of STR analysis. Other contemporary DNA markers and techniques in vogue in forensic investigations include Y-STRs, mtDNA polymorphisms, RAPD and analysis of sequence variation in conserved mitochondrial genes for species identification. Single nucleotide polymorphisms (SNPs) analysis using single stranded conformational polymorphisms (SSCP) and micro array techniques are innovative additions in DNA field, throwing new light into the field of human identification and population studies. A brief discussion of these technologies, overview of their applications, their merits and demerits have been provided in the paper. National and international scenario on application of DNA methods, information on quality assurance programs and DNA databases have been dealt with for better comprehension of the advantages and limitations of DNA based technologies.

INTRODUCTION

Investigation of violent crime has always been the decisive issue for law enforcement agencies in any country and more so in societies of developing countries like India. Biological specimens recovered from the scene of crime provide remarkably valuable information about the crime and involved personnel. DNA technology has gained wide acceptance in crime investigations involving biological evidence, such as murder (blood evidence), sexual assault (semen evidence), murder with sexual assault (blood and semen evidence) or more so, in cases of mass disasters like the World Trade Center attacks (saliva evidence and body tissues) and in identification of mutilated bodies and exhumed skeletons (tissues and bones).

Variability in biomolecules has been exploited for identification of the source of the biospecimen. Earlier, identification procedures employed for the characterization of biological specimens were protein or "classical" markers such as the ABO blood group antigens, serum proteins and RBC enzymes. They, however, suffered from low polymorphism, poor stability and restricted activity of the molecules and limited resolution of the detection methods. The advent of DNA markers rendered greater precision and higher discriminatory power to forensic testing.

Deoxyribonucleic acid (DNA) is the vehicle for generational transfer of heritable traits. It encodes the genetic information in most organisms and is identical in every cell of an

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individual. Chemically, the DNA molecule is a highly stable polymer composed of subunits known as nucleotides and in humans makes up the 22 pairs of autosomal and single pair of sex chromosomes. Each parent contributes a chromosome to the pair an individual inherits. The information content of DNA resides in the sequence of bases and although DNA sequence in different individuals is more similar than different, many regions of human chromosomes exhibit a great deal of diversity. Such variable sequences are termed "polymorphic" (meaning many forms) and are used for human identification, paternity testing and diagnosis of genetic diseases. Most of the polymorphisms are located in the estimated 95% of the human genome that does not encode for proteins.

CLASSES OF DNA POLYMORPHISMS

DNA molecules demonstrate various types of polymorphisms (Figure 1), which can be categorized into the following classes:

A. Polymorphisms in the coding region
B. Polymorphisms in the noncoding regions comprising of:
1. Variable Number of Tandem Repeats
1.1 Minisatellites
1.2 Microsatellites
2. Single Nucleotide Polymorphisms

Minisatellites

VNTRs are regions of DNA comprising hundreds to several thousand base pairs and are arranged as tandem repeat units. Loci with long motifs (e.g., 8-80 bp) are referred to as minisatellites (Jeffreys et al. 1985) or variable number of tandem repeats (VNTRs) (Nakamura et al. 1987). The number of repeats varies greatly from person to person (Wyman and White 1980). Restriction enzymes were used for cleaving VNTR fragments varying in lengths, which were detected by the restriction fragment length polymorphism (RFLP) technique. The initial probes used for the detection of minisatellites were multilocus probes (MLPs), which could identify polymorphisms of consensus sequences at multiple loci simultaneously. These probes consisted of the "core" sequence of tandem repeats and when hybridized to Southern blots under conditions of low stringency, detect a family of minisatellites that all share the same "core" sequence, generating individual-specific DNA "fingerprints". Nevertheless, the patterns generated were complex and these probes could not be used to identify the alleles at different loci. This marked the advent of single locus probes (SLPs), which comprised unique DNA sequences flanking the VNTR under study rather than containing the core repeat sequence. Thus, SLPs were used to characterize polymorphism at a single locus with individual alleles being detected. Several such SLPs used in a series would yield individual-specific profiles. The application of VNTRs for gene mapping, population genetics and personnel identification has, however, been limited by the low stability, low frequency (Armour et al. 1990), asymmetric distribution (Royle et al. 1988) of these repeats in the genome, requirement of high molecular weight DNA template, poor amenability to PCR and inability to determine the alleles precisely with Southern hybridization-based detection methods.

Polymorphisms in the Coding Region

In 1985, Kary Mullis developed a process of invitro DNA amplification called the polymerase chain reaction (PCR), a simplified procedure that generates millions of copies of template DNA in a short duration, thus rendering genotyping an easier exercise. This technique has revolutionized not only forensic DNA science, also other fields of biology including medical and agricultural sciences. PCR is a method which mimics the cell's normal machinery for replicating DNA and amplifies DNA within a sample, which may contain even as little as a single molecule of DNA.

DNA sample from a biospecimen at a crime scene can be used as a template for amplification by PCR resulting in numerous copies of the DNA, which otherwise would have been undetectable thus enabling the investigators to further manipulate or analyze this region of DNA. Lower cost, greater sensitivity, and enhanced speed of the PCR offers advantage over Southern technology in personnel identification (Table 1). The first PCR-based tests involved reverse dot blots, where the test depended on the development of a colour reaction from probe hybridization on a filter membrane. Such tests analyze sequence-based rather than length-based polymorphisms (HLA DQA1, LDLR, GYPA, HBGG, D7S8 and GC analysis).

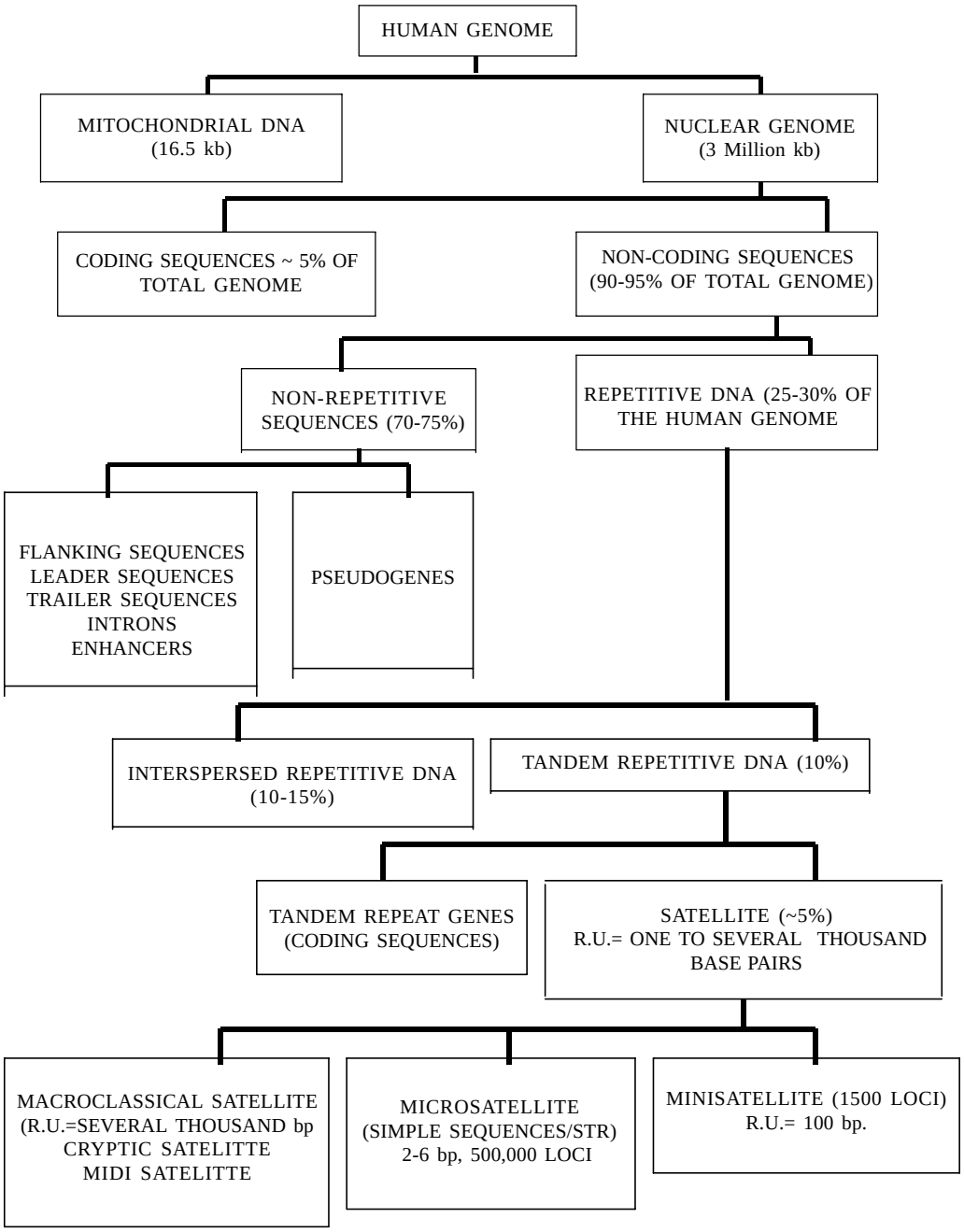


Fig. 1. Polymorphic regions in the human genome (R.U.: Repeat unit)

Table 1: Informativeness and legal admissibility of different DNA technologies in human identification.

	RFLP (SLPs)	*Reverse Dot Blot Assay (HLADQA1+PM)*	*STR Detection Technologies*	*DNA Sequencing/SNPs*
Quantity of DNA required for testing	50 x 10^{-6} mg	50 x 10^{-6} mg	1 x 10^{-6} mg	1 x 10^{-7} mg
Scoring of Alleles	Bins treated as alleles	Sequence based alleles	Real alleles	Sequence based alleles-Highly Precise
Nature of Loci	Defined	Defined	Defined	Defined
Test Time	4-8 Days	1 Day	1 Day to Few Hours	Few Hours
Probability of Match	1 in 10^{23} (Depends on the number of markers used)	1 in 10^{4}	1 in 10^{23} (Depends on the number of markers used)	1 in 10^{23} (Depends on the number of markers used)
Legal Admissibility	Accepted		Accepted	Accepted

Microsatellites

Short tandem repeats or microsatellites (Litt and Luty 1989) are tandem repeat units of 2 to 7 nucleotides and are abundant in the human genome. Trimeric and tetrameric STRs occur every 300 to 500 kb on the human chromosome and appear to be interspersed at this frequency (approximately 400 million loci) throughout the genome. These repeats can be amplified faithfully with the polymerase chain reaction (PCR), enabling precise allele designations in population surveys on the basis of their DNA sequence (Edwards et al. 1991). Approximately 50% of STRs studied by human identification groups have been polymorphic. Tetrameric STRs have features such as negligible or no slippage effects with easy resolution of consecutive alleles making them suitable for the physical and genetic mapping of the human genome, disease diagnosis, and personal identification in medical and forensic sciences. Although amplification of VNTRs with PCR enables more precise allele determinations (Boerwinkle et al. 1989; Jeffreys et al. 1990), the large size of the products makes them less suitable for general application than tandem repeats with amplification products of 100 to 500 bp. The small size of dimeric, trimeric, and tetrameric STRs facilitates their simultaneous study in a multiplex PCR, in which nine to sixteen or more loci are amplified in one reaction from a single DNA sample (Chamberlain et al. 1988), offering both higher throughput and greatly increased sensitivity over conventional single and multilocus DNA probe techniques. The STR loci that have been selected for forensic use generally have 7 to 30 different alleles. As in VNTR systems, the size (in number of DNA bases) of STR fragments detected is used to characterize the sample DNA. The allele designation of each locus is generally the number of times a repeated unit is present within identified fragments. STRs were first used in forensic casework for the identification of human remains in Persian Gulf War in 1991. Most forensic STRs contain tetranucleotide repeat units. CSF1PO, THO1, TPOX and vWA (CTT, CTTv) were used most extensively in the early STR systems. Recently, some interest has been generated in larger STRs (pentameric repeats) to eliminate stutter and thus resolve issues of mixtures. Large multiplexed sets of STRs permit greater discriminatory power than RFLP. The applicability of STR systems for automation may be among their most important attributes. The evolution of various DNA profiling technologies is presented in Figure 2.

Single Nucleotide Polymorphisms

Single nucleotide polymorphisms (SNPs) are single base-pair changes in the DNA sequence, which can be detected by sequencing, RFLP-PCR or single-strand conformational polymorphism (SSCP) techniques. A set of SNPs decoding identification of an individual demands only a short stretch of DNA (<100 bp) for analysis. This is of great advantage over the conventional methods in genotyping highly degraded forensic and archaic samples. The presence of ~1.8 million SNPs in the human genome makes it imperative to include SNPs in forensic investigations. The forensic DNA community already has experience in applying SNP markers for a screening process. Due to amenability to automation, SNPs can prove very helpful for excluding the innocent from prosecution.

Mitochondrial DNA Sequencing

Although STR markers are very precise in determining human identity, they might fail to

amplify when the template DNA is insufficient, extensively degraded and has undergone modifications and are uninformative if appropriate control samples are not accessible for comparison. In such cases, mtDNA analysis can help in identification of a person through maternal lineage. Mitochondria have inherent DNA, which differs from nuclear DNA in several ways: it is smaller than nuclear DNA and consists of a single circular, double-stranded molecule that is 16,569 base pairs in length. MtDNA is present in a high copy number/cell and can be recovered from skeletal remains, hair shaft, etc.: biospecimens, which are poor sources of nuclear DNA. In addition, the circular nature of the mtDNA makes it less susceptible to exonuclease degradation. MtDNA is inherited exclusively from the mother, hence it is essentially a haploid sequence, whereas nuclear DNA (except for X and Y chromosomes) is inherited equally from both parents. The target sequence in mtDNA is a hypervariable region that has a five to ten times greater mutation rate than nuclear DNA, so the variation arises extensively from mutation. The discriminatory power of the technique arises from the polymorphic characteristics of the hypervariable regions I and II located within the mitochondrial displacement loop (D-loop) (Aquadro and Greenberg 1983; Greenberg et al. 1983). The designation of the dominant sequence is usually displayed as variations compared with the Anderson sequence standard (Anderson et al. 1981). Biospecimens are analyzed by comparing the polymorphisms found in HVI and HVII with those of a matrilineal reference in forensics, or from known population databases in evolutionary biology or anthropology. Usually there is 1-2% variance in the mtDNA sequence between unrelated individuals, or 1-2 in 100 bases. Mutations are random and preserved through maternal inheritance, so there is only a remote chance that any two individuals will show exactly similar mutations, unless they are of recent maternal lineage from the same ancestor. Amplification and automated sequencing of hypervariable region I (HVI) and hypervariable region II (HVII) of the mitochondrial D-loop region complements the autosomal STR approach practiced in a large number of DNA typing laboratories.

Y Chromosomal Polymorphism

Like mtDNA testing, analysis of STRs on the Y chromosome is also a robust technique and is widely used in human identification for resolving paternity disputes, male gene flow, etc. Microsatellite loci on the Y chromosome are helpful in identification of a person through paternal lineage as well as in cases of sexual assaults where mixtures of vaginal secretions with semen are found.

Since the HVI and HVII regions of mtDNA and STRs of Y chromosome are excellent markers for identification as well as for automation, they have emerged as additional tools for the contemporary autosomal markers in human identification.

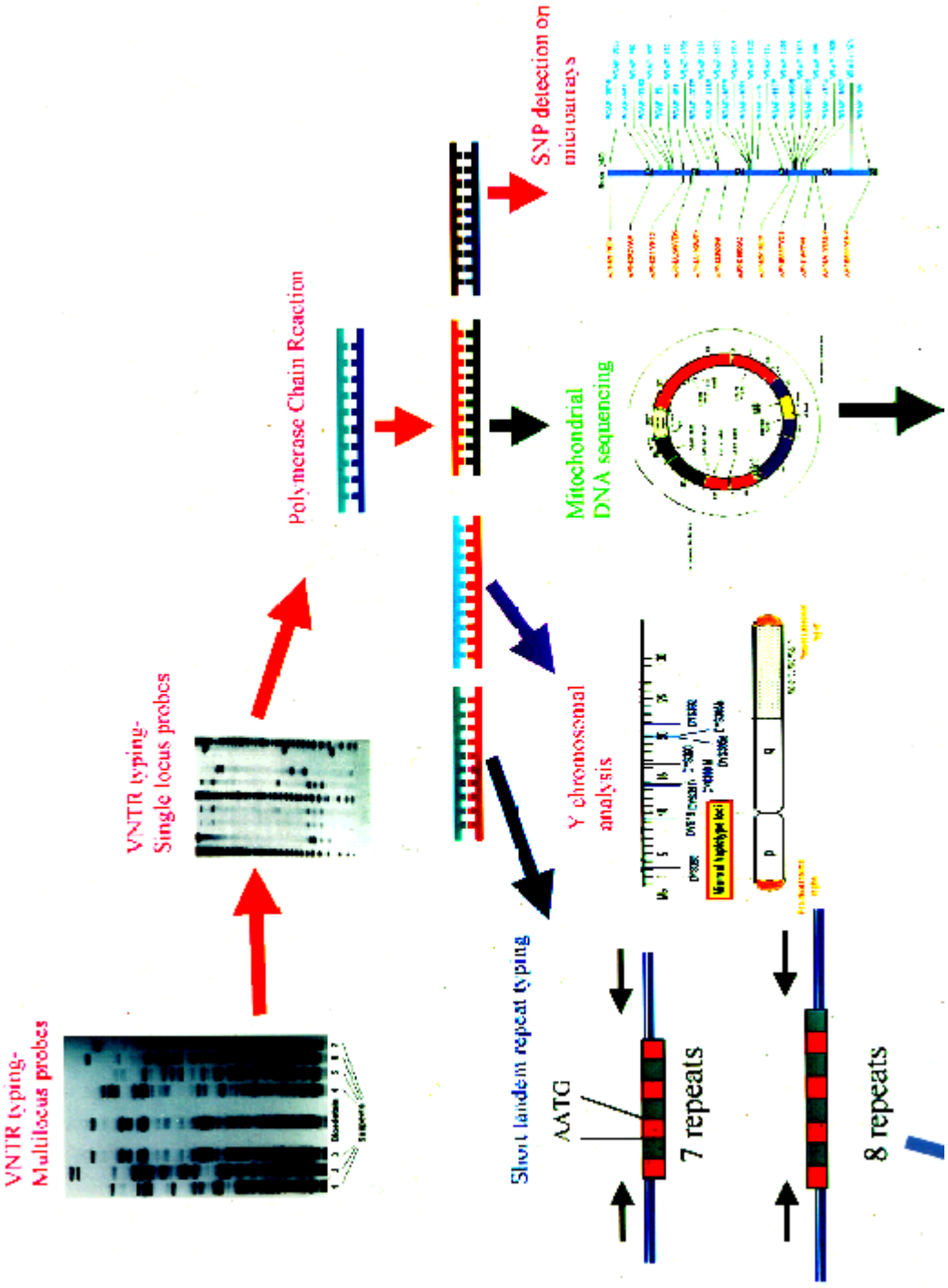
Species Identification

Species identification, in many instances, is essential for establishing the biological source of the questioned sample. Random amplification of polymorphic DNA (RAPD) has been widely used for species identification and can be carried out on those organisms where there is very little or no information available on their genomic sequences and organization. In addition, sequencing of genes from conserved regions of questioned DNA samples is being currently employed for species identification.

Other modern DNA technologies being validated by forensic DNA communities in human identification include microarray systems and time-of-flight mass spectrometry (TOF-MS). These modern DNA techniques have been dealt with brevity.

The strength of the legal power of forensic DNA profiling lies in its genetic concordance. These techniques cannot typically prove that a person committed a particular crime, but they can prove the person's presence at the crime scene from the biological evidence left behind. Higher discriminatory power of DNA evidence can be used to exonerate innocents wrongly prosecuted on the basis of traditional genetic markers. Based on statistical results from allele frequency databases, a DNA investigator can generate the likelihood that a suspect's DNA profile will randomly match an evidentiary sample.

The entire gamut of DNA typing technologies used over the past 15 years for identification is compared in Figure 3. The various DNA markers used in DNA profiling have been divided into four quadrants based on their power of discrimination, i.e. precision to discern the difference between individuals, and the speed of analysis. New and improved methods have been developed over the years; those having a high degree of discriminatory power and an analysis period of a few hours. Locus information and commercial sources of commonly employed markers are provided in Table 2.


VNTR typing-
Multilocus probes
VNTR typing-
Single locus probes
Polymerase Chain Reaction
SNP detection on
microarrays
Mitochondrial
DNA sequencing
Y chromosomal
analysis
Short tandem repeat typing
AATG
7 repeats
8 repeats

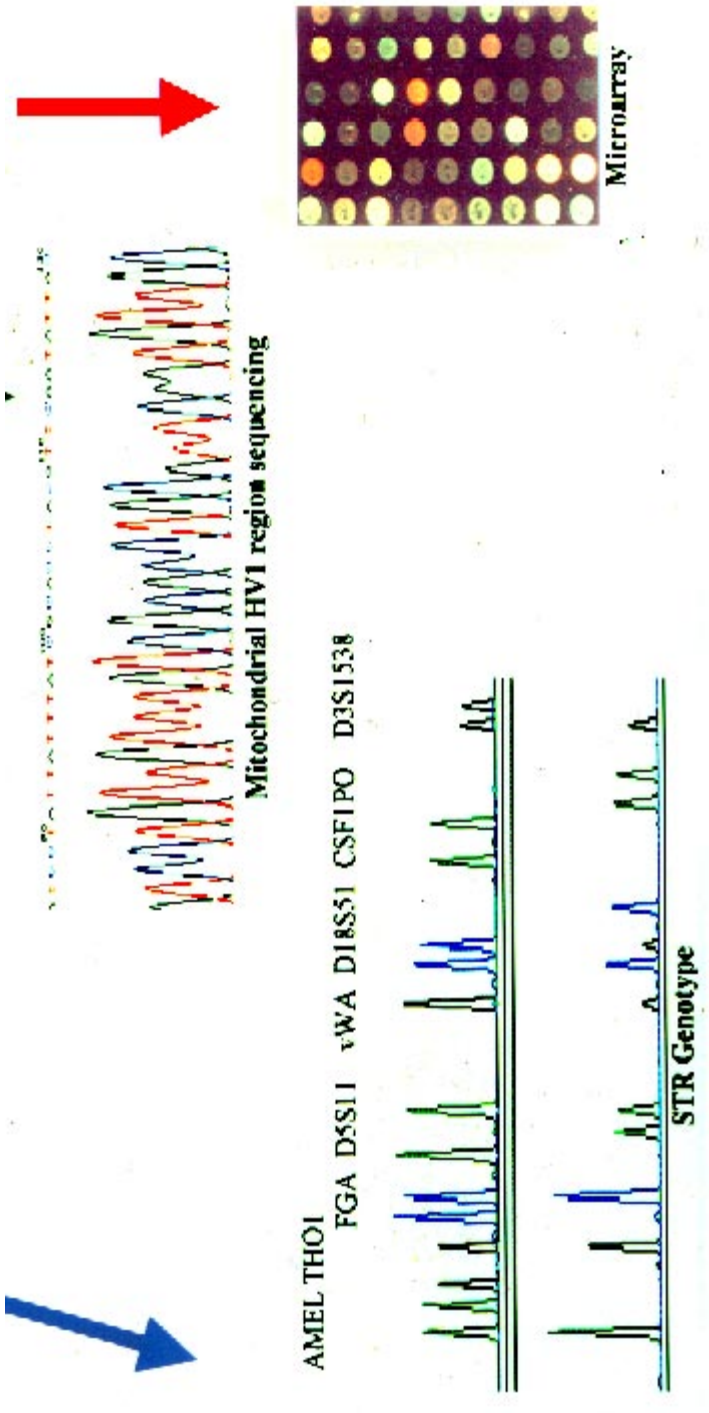


Fig. 2. DNA profiling technology: From Minisatellities to SNPs

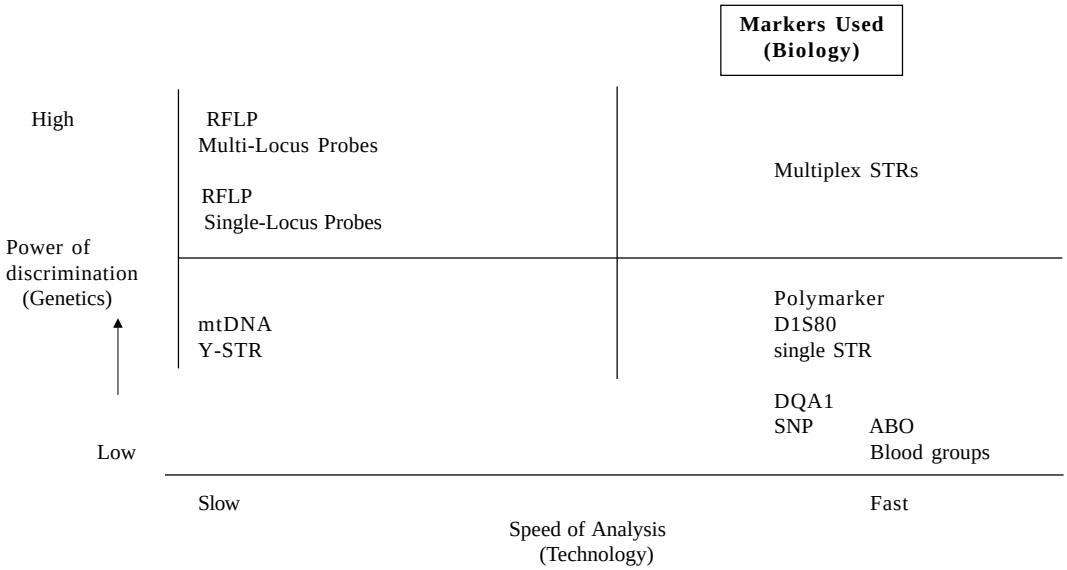


Fig 3. Comparison of DNA Typing Technologies. Forensic DNA markers are arbitrarily plotted in relationship to four quadrants defined by the power of discrimination for the genetic system used and the speed at which the analysis for that marker may be performed. Note that this diagram does not reflect the usefulness of these markers in terms of forensic cases.

Table 2: Locus details and genomic coordinates of commercial markers routinely employed in forensic analysis.

Marker	*Chromosomal Location*	*Repeat Sequence 5′-3′*	*Trade name and source*
*D3S1358	3p	TCTA complex	AmpFlSTR® Profiler Plus™
*VWA	12p12-p ter	TCTA complex	kit(PE Applied Biosystems)
*FGA	4q28	TTTC complex	
*D5S818	5q23.3-32	AGAT	
*D13S317	13q22-q31	TATC	
*D7S820	7q11.21-22	GATA	
*D8S1179	8q	TCTA complex	
*D21S11	21q11-21q21	TCTA complex	
*D18S51	18q21.3	AGAA	
*Amelogenin	Xp22.1-22.3 Yp11.2	NA†	
D16S539	16q24-qter	GATA	PowerPlex® 16 System
THO1	11p15.5	AATG	(Promega Corporation)
TPOX	2p23-2pter	AATG	
CSF1PO	5q33.3-34	AGAT	
Penta D	21q	AAAGA	
Penta E	15q	AAAGA	
DYS19	Yp	TAGA	Reliagene Technologies
DYS389I	Yq	[TCTG] [TCTA]	
DYS389II	Yq	[TCTG] [TCTA]	
DYS390	Yq	[TCTG] [TCTA]	
DYS391	Yq	TCTA	
DYS392	Yq	TAT	
DYS393	Yp	AGAT	
DYS385	Yq	GAAA	
HLADQA1	6p21.3	NA†	Perkin Elmer
LDLR	19p13.1-13.3	NA†	[Roche Molecular Systems]
GYPA	4q28-31	NA†	
HBGG	11p15.5	NA†	
D7S8	7q22-31.1	NA†	
GC	4q11-13	NA†	

* Included in the Powerplex® 16 system † Not applicable

ANALYSIS OF HYPERVARIABLE REGIONS

DNA profiling tests are of different kinds, based on the nature of variable region to be analyzed, whether minisatellites, microsatellites, or the sequence of nucleotides.

The methods of analyzing different types of polymorphisms are:

1.0 Hybridization-based method
Restriction fragment length polymorphism (RFLP)
2.0 Polymerase chain reaction (PCR) based assays
2.10 Fluorescent nonanchored inter simple sequence repeat (FISSR) PCR technology
2.20 Short tandem repeat (STR) typing by monoplexing and multiplexing methods
2.21 Manual genotyping and detection by staining
2.22 Automated STR genotyping
2.23 Time-of-flight mass spectrometry in STR analysis
2.30 Random amplification of polymorphic DNA (RAPD) in species identification
3.0 Sequence-based technology - detection of single nucleotide polymorphisms (SNPs)
3.10 Reverse dot blot assay in detection of HLADQA1 and PM loci
3.20 mtDNA sequence analysis
3.30 Single strand conformational polymorphisms (SSCP)
4.0 Microchip assays
Micro array-based analysis

1.0 Analysis of Minisatellite Regions – [Restriction Fragment Length Polymorphisms (RFLPS)]

The basic principle of RFLP (Botstein et al. 1980) is separation of the desired repetitive sequences by cleaving them out from the genome using an appropriate restriction endonuclease enzyme, electrophoresis of the digested DNA and thereafter their detection by DNA probes. According to the nature of core sequence of the minisatellite, restriction enzyme and probe are selected. RFLP was the first technique that was adapted for forensic DNA analysis in human identification. This kind of analysis determines variation in the length of a defined DNA fragment. The method consists of (i) isolation of DNA from specimen (ii) breaking the DNA into small fragments with a restriction enzyme such as *Hae*III (iii) separation of fragments according to size by electrophoresis (iv) transfer of restricted DNA onto nitrocellulose solid surface (v) location of region of interest after hybridization with labeled MLP/SLPs and (vi) autoradiography. Sir Alec Jeffreys deserves credit for introducing the use of hypervariable loci for individual identification. Jeffreys' multi locus probes, derived from a 33 bp core sequence of an intron of the human myoglobin gene, developed a set of bands in autoradiography after hybridization to create a bar code like pattern termed 'DNA fingerprinting' or DNA profiling (Jeffreys et al. 1985). However, it was difficult to interpret statistically the multibanded pattern produced from multilocus probes and from samples containing more than one source, as in samples from most of the sexual assault cases. Furthermore, multilocus probes required a large amount of intact DNA. This led to the advent of SLPs. The general characteristics of these two classes are as follows:

i) Detection with multilocus probes (MLPs): In this technique, a probe consisting of the "core" sequence that is in consensus with a number of minisatellites is used to detect polymorphisms in a class of HVRs in one reaction. The DNA profile reveals a set of band patterns (similar to a product bar code), which are unique to an individual. The most commonly used multilocus probes were λ33.6, λ33.15, BKm2 (8), MZ1.3, AC365, Alpha-globin HVR, C-Ha-ras-J, F10 and 'per' gene used for estimating polymorphism among human populations. Restriction enzymes commonly used in cleaving minisatellite sequences include HaeIII, HinfI and BstNI. This is the classical DNA fingerprinting method used in forensic testing, which is not practiced anymore due to its technical limitations.

ii) Detection with locus specific probes (SLPs): In this system, only alleles at one locus are detected with a probe derived from unique flanking sequences of the region to be analyzed. In one reaction, either one or two bands develop, depending on whether the locus is homozygous or heterozygous. Sets of reactions for identifying variants in different regions are conducted using different probes to obtain meaningful individual specific patterns. Most commonly used SLPs include MS1 (D1S7), YNH24

(D2S44), pH30 (D4S139), LH1 (D5S110), TBQ7 (D10S28) and V1 (D17S79). Restriction enzymes commonly employed include AluI, HaeIII, BamHI, HindIII and HinfI. This method has been extensively employed worldwide (1989-98) for DNA typing due to its precision, fidelity and technical robustness.

In hybridization-based methods, two types of labeling procedures are generally used for detecting coupling of the probe with template DNA: radioactive detection using p^{32} labeled probes and chemiluminescence detection using ACES probes. Although the sensitivity of radioactive probes is much higher, their limited half-life and hazardous properties have resulted more in the usage of chemiluminescent probes.

Although minisatellites yield a higher degree of discrimination per locus than the newer PCR-based STR methods, they require large amounts (~10-50 ng) of high molecular weight DNA. Since, forensic evidence is often old, degraded and of limited quantity, minisatellite analysis is sometimes not possible. The technique is also laborious and difficult to automate, limiting its utility for building and using large DNA databases.

2.0 Amplification Based Technology (PCR)

The polymerase chain reaction (PCR) (Mullis and Faloona 1987; Saiki et al. 1985), technique has revolutionized the entire field of DNA study. The process mimics the biological process of DNA replication but confines it to specific DNA sequences of interest. Instruments that perform thermal cycling are now easily available from commercial sources. This process can produce a million-fold or greater amplification of the desired region in two hours or less. Two alleles from a single locus may have been amplified by PCR, but not separated, during the amplification process. The amplified DNA has to be separated to allow follow-up analysis. Agarose gel electrophoresis, reverse dot blot, PAGE and automated electrophoresis are few commonly employed methods for separating the amplified allele segments based either on their size or sequence. Accuracy, precision and rapidness are the hallmarks of PCR based technology, rendering it the most informative status, also owing to its simultaneous amplification and typing of template sequence. It is also indispensable in situations where the specimen is very little or the DNA is degraded.

2.10 FIISR-PCR Technology

Nonanchored inter simple sequence repeats (ISSRs) are arbitrary multiloci markers profiled by PCR amplification with a microsatellite primer. No prior genomic information is required for their use. It is a stable technology across a wide range of PCR parameters; hence these markers are used in DNA fingerprinting (Bornet and Branchard 2001).

The sensitivity, speed and informativeness of the existing ISSR-PCR method can be enhanced substantially by using fluorescent dye labeled nucleotides in the ISSR-PCR reaction, which is termed FISSR-PCR (Nagaraju et al. 2002), followed by separation of PCR products on an automated sequencer.

2.20 Analysis of STR Loci

The most convenient approach of analysis of variations in length of the repeat sequence of microsatellites is to amplify the region of interest and determine the size of the amplified product. A set of microsatellites can be profiled by multiplex PCR reactions followed by manual or automated genotyping. The use of PCR in STR analysis permits very tiny amounts of DNA, as found on a postage stamp, cigarette butt, or coffee cup, to be amplified to produce large amounts of DNA sufficient for analysis. In the case of VNTRs, the entire genomic DNA is on the gel, while in STRs, only the region of interest is amplified. By the use of more discriminating separation systems even the smaller range of fragments of STRs can be separated allowing identification of all alleles at a locus. In forensic applications, amplified and separated STR fragments are generally detected using one of the two methods. One method uses the propensity of silver or SYBR green to bind to DNA. The entire gel is stained with silver nitrate, greater amount of DNA exists in amplified fragments, and hence they stand out prominently against the more dilute background. A second prevalent method requires that some of the primers used during the amplification contain fluorescent tags, which are incorporated into the STR fragments generated during amplification, and detected by automated platforms. Fortunately, it is possible to analyze many STR loci

simultaneously in a DNA sample. Such systems (multiplexes) have been developed that allow amplification of 3 to 16 loci in a single instance. Many forensic laboratories now have instruments that discriminate various fluorescent dyes that are utilized for tagging and individuali-zing specific loci.

The Federal Bureau of Investigation (FBI), USA has designated 13 specific, extremely informative STR loci which are highly polymorphic across various ethnic groups, as a core set to be used in the combined DNA index system (CODIS) for matching crime scene materials to previously genotyped convicts.

The advantages of STR based DNA profiling over other methods are:

- Even highly degraded samples yield results (since shorter fragments of DNA can be analyzed)
- Small amounts of DNA (1ng) can also be analyzed because of amplification-based protocols
- STR based DNA profiling involves a large number of loci, providing greater discriminatory power
- It is a rapid, speedy procedure and can be completed in a day or two
- Multiplexing and automation make it more effective
- Commercially available kits and inexpensive silver staining detection method for some of the multiplexes without the requirement of expensive equipment make it a desirable option

2.21 Detection of Cmplified Product by Staining

Silver nitrate and dyes like SYBR green that show affinity to the DNA molecule, are found highly suitable for amplicon detection. Silver staining of polyacrylamide gels has been extensively used in detecting miniscule amounts of proteins and visualizing nucleic acids. In DNA profiling, silver staining procedures were highly useful for manual genotyping platforms that employed the first commercially available STR kits from Promega Corporation (CTT, CTTv).

The procedure involves transferring the gel containing the electrophoresed amplicons into tanks filled with various solutions that expose the DNA bands to a series of chemicals for staining (Bassam et al. 1991). First, the gel is submerged in a tank of 0.2% silver nitrate solution. The silver binds to the DNA and is reduced with formaldehyde to form a deposit of metallic silver on the DNA molecules in the gel. A photograph is then taken of the gel to capture images of the silver-stained DNA bands and to maintain a permanent record of the gel. Alternatively, the gels themselves may be sealed and preserved in dried condition.

2.22 Automated DNA Genotyping for Fluorescence Based Multiplex STR

Presently, amongst the known DNA tests in human identification, this technique is most informative, precise, robust, rapid and hence most sought after. Nine to fifteen STR loci are simultaneously amplified and subsequently analyzed by an automated DNA sequencer. In recent times, more than 15 STR loci have been analyzed concurrently. The typing results are highly individual specific and have worldwide acceptance in the courts.

DNA is extracted and subjected to multiplex amplification of the different STR loci. PCR products of STR loci can be easily resolved by size using gel electrophoresis with high resolution (Edwards et al. 1991; Fregeau and Fourney 1993) or polymer-loaded capillaries (Butler et al. 1994; Wang et al. 1995). In utilizing fluorescent dye-labeling technology, STR loci with overlapping size ranges can be co-amplified in a single tube and yet be detected individually due to the different characteristic emission spectrum of each dye (Mayrand et al. 1992). The fluorescent dyes commonly employed in the automated fluorescent STR analysis include 5-FAM (blue), 6-FAM (blue), JOE (green), VIC (green), NED (yellow), PET (red), fluorescein (blue) and TMR (yellow), and an additional dye for internal size standards such as ROX-350/500 (red) for Profiler Plus™ system, CXR (Carboxy-X-rhodamine) (red) for PowerPlex®16 markers system and LIZ (orange) for Identifiler™ system. Dye-labeling technology facilitates the use of an in-lane size standard, which can greatly improve the sizing precision of alleles by automatic sizing of fragments tagged with the coloured dye. The use of an internal standard circumvents the problems of band shift artifacts, provides an internal control for lane-to-lane comparison of data, and also offers a means to automate the analysis.

In addition to the STR markers, a segment of the X-Y homologous gene, amelogenin is also

amplified with all the multiplex systems. Amplifying a segment of the amelogenin gene with a single primer pair can be used for gender identification because different length products from the X and Y-chromosomes are generated. One primer, of each locus-specific pair is labeled with either the 5-FAM, JOE or NED NHS-ester dye, which is detected on sequencer systems like ABI PRISM 373 DNA sequencer, ABI PRISM 377 DNA sequencer, NEN Global IR^2 DNA Sequencer/Analyzer of LI-COR Inc., USA; flat bed scanners Hitachi FMBIO III or capillary based automated DNA sequencers like ABI PRISM 310, ABI PRISM 3100/3730xl DNA Sequencer systems, MegaBase 1000/500 of Amersham Pharmacia Biotech, USA and Beckman Coultier CEQ 2000XL DNA Sequencer.

The laser based technology for STR analysis provides higher detection sensitivity than standard methods. Typically, between 0.5 and 2 nanograms of DNA is adequate for genotyping. Only 1% to 2% of the PCR products of a 28 PCR cycle (ProfilerPlus™ STR amplification) reaction are desired for allele typing. These fluorescence-based methods are approximately 200 times more sensitive than any other standard staining technique.

Y-STR Polymorphism

Several highly polymorphic STRs have also been identified on the human Y chromosome, like DYS389 (I and II), DYS390, DYS19, DYS385, DYS391, DYS392, etc. The Y chromosome is nuclear DNA, present in one copy per cell and only in males. It displays paternal inheritance. Like autosomal STR markers, Y-STRs are amenable to typing small or degraded samples of DNA and can be analyzed on the same instrumentation platforms.

DNA polymorphism on the human Y-chromosomes is a prized tool for identity testing as well as for evolution and migration studies. Y chromosome loci are not independent and hence the product rule cannot be used to estimate population frequencies at multiple loci. The Y chromosome markers are collectively inherited as a single locus having a large number of alleles; the population frequency of a given haplotype is determined by counting in a population database. In forensics, Y chromosome markers are particularly useful in multiple rape cases, and in identification of mutilated bodies, if control samples of a few genetically related individuals from the paternal side are only available for comparison.

METHOD

Analysis is carried out following the guidelines and recommendations of the DNA Commission of the International Society of Forensic Genetics (ISFG) (Gill et al. 2001) and the European DNA profiling (EDNAP) group. Genomic DNA is extracted and amplified with marker specific primers. Primers are employed as described by Kayser et al. 1997 or Schneider et al. 1998.

Capillary electrophoresis of the amplified products can be carried out on ABI 310/ 3100 genetic analyzer (PE), using the internal standard. The allele attribution can be made by comparison with allelic ladders constructed from reference samples. Correct allele calling is additionally assured by successful participation in the quality control tests of the Y chromosome short tandem repeat (STR) haplotype reference database: http://ystr.charite.de. Commercial kits for Y-STR typing have recently become available from ReliaGene Tecnologies, Inc. A particularly useful feature of these kits is the allelic ladder that allows allele calls in samples by direct comparison. The Y-PLEX™6 and Y-PLEX™5 kits type six and five different Y-STR loci respectively. The National Institute of Standards and Technology (NIST), U.S.A. has also developed a 20-plex system for typing Y-STRs that permits simultaneous amplification of 20 STRs in a single reaction (Butler et al. 2002).

ADVANTAGES

- The Y chromosome is transmitted en bloc to all male descendents, hence it is particularly useful for tracing family relationships and male lineage
- Paternal transmissions and the lack of recombination renders the Y chromosome markers useful for measuring relatedness of individuals originating from a common geographic region
- Y chromosomal analysis can be helpful for analyzing non-sperm containing samples comprising both male and female contributions such as a mixture of blood or male saliva deposited on a female victim where differential extraction cannot be employed to separate male-derived DNA

2.23 Improved Analysis of STRs with Time-of-Flight Mass Spectrometry

Before the advent of mass spectrometry DNA analysis technology, in automated DNA genotyping, instruments could handle only a few dozen samples per day. Gene Trace Systems, USA, has developed high-throughput DNA analysis capabilities using time-of-flight mass spectrometry coupled with parallel sample preparation on a robotic workstation. This technology allows several thousand samples to be processed daily in seconds, rather than minutes or hours, and with improved accuracy compared with conventional electrophoresis methods. Mass spectrometry is a versatile analytical technique involving detection of ions and measurement of their mass-to-charge ratio. Because these ions are separated in a vacuum environment, analysis times can be extremely rapid, often within microseconds. The new ionization technique for measuring biomolecules known as matrix-assisted laser desorption-ionization (MALDI) coupled with time-of-flight mass spectrometry (MALDI-TOF-MS) (Ross et al. 1998) overcomes molecular photo-dissociation of the sample ions induced by direct laser irradiation. This technology focuses on STR loci that have been developed by commercial manufacturers and studied extensively by forensic scientists (Becker et al. 1996; Butler et al. 1998). Positive features of mass spectrometry for STR analysis include:

- Rapid results: STR typing at the rate of seconds per sample
- Accuracy: no allelic ladders
- Direct DNA measurement: no fluorescent or radioactive labels
- Automated sample preparation and data collection
- High-throughput capabilities of thousands of samples every day per system
- Flexibility: single nucleotide polymorphism (SNP) assays can be run on the same platform

2.30 RAPD Analysis in Species Identification

This test employs primers based on conserved sequences among different species and generates amplicons variable between species and even individuals. RAPD (Welsh and McClelland 1990; Williams et al. 1990) requires fewer steps to identify and map markers when compared to RFLP analysis. The steps for RAPD consist of DNA extraction, PCR, separation of amplified fragments on an agarose gel, and detection using ethidium bromide-stained gel. RAPD analysis has been used for many studies of animals and plants, and is a very useful technique for species identification.

The significance of the method is that no prior information of template sequence is required and universal primers can be used.

3.0 Sequencing Based Technology

Single nucleotide polymorphisms represent alterations in DNA sequence at a single nucleotide position, either due to base changes, insertion or deletion of one or a few bases. The means to detect these alterations and the number of such known polymorphisms in the human genome have changed dramatically in recent years. While DNA sequence analysis is still relatively cumbersome today, SNP analysis can be performed by a range of simpler and more rapid methods such as the dot-blot format using labeled sequence-specific oligonucleotide (SSO) hybridization, reverse dot blot analysis (HLA DQA1+PM typing), or hybridization array technologies (chip technologies). SNPs are the most frequent form of DNA sequence variation in the human genome and are also becoming increasingly popular genetic markers for genome mapping studies and medical diagnostics. The most important attribute of SNPs is their suitability for new automated instrumentation platforms, particularly mass spectrometry and microchip instrumentation, as well as in-solution techniques such as molecular beacon and fluorescence polarization.

Methods for SNP Detection

There are at least 11 methods for detecting and analyzing single nucleotide polymorphism (SNPs) that include:

Genetic bit analysis (Nikiforov et al. 1994), direct sequencing (Kwok et al. 1994), denaturing high performance liquid chromatography (HPLC) (Hecker et al. 1999), real-time PCR employing molecular beacons (Giesendorf et al. 1998) and Taq Man 5′–nuclease assay (Livak 1999), fluorescence polarization (Chen et al. 1999), mass spectrometry (Haff and Smirnov 1997), high-density arrays (Affymetrix chip) (Wang et al 1998; Sapolsky et al. 1999), electronic dot-blot (nanogen

chip) (Sosnowski et al. 1997), oligonucleotide ligation assay (OLA) (Delahunty et al. 1996) and T_m – shift genotyping (Germer and Higuchi 1999).

The advantages of SNPs are:

- These markers are numerous in mammalian genomes
- Multiple methods of SNP detection are available
- The amplification of alleles is not prone to preferential amplification and robust multiplex amplification is relatively easy to achieve
- Amenable to digitalization on the basis of presence or absence of SNP thus simplifying the identification technique

3.10 Reverse Dot Blot Assay in Detection of HLA DQA1 and PM (LDLR, GYPA, HBGG, D7S8 and GC Loci)

Other than determining the extent of allelic diversity by sequence analysis, one can detect the presence of specific alleles in a PCR-amplified sample by dot blot hybridization with labeled oligo-nucleotide probes, exploiting variations in the DNA sequence, e.g. HLADQA1 (Bugawan et al. 1989) and PM systems. One can use the procedure either with p^{32}, biotin or horseradish peroxidase labeled oligonucleotide probes for signal detection. The AmpliType® PM + DQA1 PCR amplification and typing kit was extensively employed in early traditional forensic testing.

3.20 mtDNA Sequence Analysis

A novel approach in DNA analysis is the application of mitochondrial DNA (mtDNA) sequencing and haplotype frequency calculations in human identification (Stoneking et al. 1991; Sullivan et al. 1992), population studies, evolutionary biology (Avise and Saunders 1984) and anthropology (Chakraborty and Weiss 1991).

The current forensic procedure consists of amplification and sequencing of both the HVI and HVII regions from the sample and a reference standard before any comparison may be made (Orrego and King 1990; Holland et al. 1995). If the sequences are different, exclusion is reported while identical sequences support a match. Several population specific databases for HVI and HVII sequences exist so that one can determine the frequency of a particular sequence or mtDNA type in a population. Once the sample is sequenced, it is compared to the Anderson reference sequence and the differences are reported according to the recommendations laid down by ISFG (Carracedo et al. 2000) and EDNAP (Tully et al. 2001). This technique, which takes into account even very small differences in the mtDNA amongst individuals, is especially very useful for minute samples, or ancient or degraded samples. Sequencing is becoming far more rapid, less expensive and facile, creating the potential for complete sequencing by service laboratories.

A completely different approach in the analysis of the mtDNA hypervariable regions involves the identification of mutational hot spots and the creation of a set of defined probes for these sites. This type of test is similar to the reverse dot blot technique and has been developed by Roche Molecular Systems. MtDNA probe strips contain a total of 31 immobilized probes that distinguish variants in 10 segments that span the HVI and HVII regions, resulting in hundreds of possible types. Since the test is presented in a digital format, this setup is ideal for computerized detection, analysis, storage and comparison. Although information obtained by this method is relatively limited when compared to direct sequencing, the ease of use and lack of necessity to purchase additional expensive equipment make it an attractive alternative.

Due to length variation of regions of mtDNA among species, the most popular method for obtaining phylogenetic data from different species is RAPD analysis. Recently, sequencing of conserved regions using universal primers from cytochrome b, 16s rRNA, 12s rRNA and 3' UTR sequences have been extensively employed for species discrimination.

3.30 Single Stranded Conformational Polymorphism (SSCP)

Another technology for analysis of genomic variation is single stranded conformational polymorphism (SSCP), based on the tendency of single-stranded DNA of different sequences to fold back upon themselves to form different conformational structures. An SSCP is a polymorphism within the fragment amplified from different individuals in a population that causes the single strands to differ in electrophoretic mobility. SSCP is therefore one method of detecting single-nucleotide polymorphisms in any region of the genome without the need for sequencing the homologous DNA fragment isolated from a large

number of individuals. The alleles underlying SSCP polymorphisms are codominant, so heterozygous genotypes can be precisely scored. Denaturing gradient gel electrophoresis (DGGE), temperature gradient gel electrophoresis, and the constant denaturant derivatives of those procedures including constant denaturing gel electrophoresis, and more recently constant denaturing and temperature-programmed capillary electro-phoresis (Khrapko et al. 1994) are all important methods for resolving DNA sequences differing by a single nucleotide.

4.0 Microchip Technology

Akin to the microchip technology which has been the backbone in the area of personal computers, micro-fluidic systems are the very basis of microelectromechanical system (MEMS) devices in the domain of 'laboratories on a chip'. The basic molecular biology techniques of electrophoresis, thermal cycling and hybridization can be accomplished by microchip formats. Microchannel capillary electrophoresis is a miniature version of the currently practised capillary electrophoresis. Exceedingly small channel lengths are sufficient for detectable separation of DNA fragments. STR determinations have been reliably performed in millisecond electrophoretic runs. Multiple channels can be placed on a single microchip. PCR and capillary electrophoresis can be integrated on a single chip. PE Biosystems, Nanogen technologies and Caliper, as well as a number of institutes in USA and Europe such as the Whitehead Institute are vigorously pursuing R&D to develop a microchip-based capillary electrophoresis suitable for human identification. Forensic analysis is bound to be immensely benefited by the success of these efforts. DNA typing of biological material in the field will then be a matter of a few minutes and could be performed without any academic expertise in molecular biology.

APPLICATIONS

DNA profiling is a highly conclusive, informative procedure in identification of biological specimens, genomic diversity in population studies, characterization and tracing antiquity of ancient DNA and for diagnosis of a plethora of diseases. The important forensic applications are in:

Medico-legal Issues

- *Murder:* Assailant, victim or the crime scene can be positively identified from very small amounts of the tissue left behind with the victim on the scene of crime or on the personal belongings of the accused
- *Sexual Assault:* Rapist can be positively identified from semen on the body or apparel of the victim or from the scene of crime
- *Accidents, Mass Disasters:* Identification of mutilated bodies can be done by comparing the DNA profile of biological remnants with blood relatives
- *Parentage Disputes:* Parentage of a child can be established by comparing the DNA of the child with putative mother and/or father. When one of the parents is not available for the testing, his/her DNA make up can be reconstituted from the DNA of close relatives
- For identifying potential suspects whose DNA may match with the evidence left at crime scenes
- To exonerate innocent persons wrongly accused of crime
- Identification of victims of catastrophes, abandoned bodies, mutilated bodies, riot victims, victims of terrorism or any instance where human identification is required.

Other than in human identification, DNA technologies are also applied in:

Diagnostics

- Detection of bacteria and other organisms that may pollute air, water, soil, and food
- Detection of organisms of bioterrorism (Anthrax, etc)
- Matching organs for transplantion and monitoring the acceptance of grafts
- Detection of mislabeled slides and tissues

Animal and Plant Genomics

- Pedigree determination of seed or livestock breeds
- Identification of plant and animal species under intellectual property rights
- Seed certification and quarantine programmes

Anthropology and Evolutionary Studies

- Ancient DNA analysis for addressing the origin of modern humans, migration and

evolution.
- Characterization of archaic fossil remains for identifying animal and plant species
- Understanding genetic structure, variation and affinities of populations

Wildlife Forensics

- Identification of protected flora and fauna thus aiding wildlife forensics for conservation and management of endangered species (could be used for prosecuting poachers)

Food Technology

- Authentication of consumables such as caviar and wine, thus maintaining quality assurance and control

NATIONAL AND INTERNATIONAL STATUS OF DNA PROFILING IN CRIME INVESTIGATIONS

In 1985, Sir Alec Jeffreys first applied DNA profiling in UK in a much publicized immigration case (Jeffreys et al. 1985) and a sexual assault and murder case of two teenage girls in Leicester, which have since then tremendously helped the British court to form its opinion on accepting DNA evidence. In USA, the first DNA case was reported in 1992.Till date, over two million cases of murder, rape and parentage disputes have been presented before courts globally. The maximum number of such cases has been successfully tried in USA followed by UK. At present DNA profiling is carried out in about 60 laboratories of USA and 15 laboratories of UK. DNA profiling is the method of choice in about 45 advanced countries for crime investigation including India.

In India, RFLP-based DNA profiling technology was invented, validated and employed in crime investigation in 1987. The main DNA service providers are Central Forensic Science Laboratory (CFSL), Kolkata and Centre for DNA Fingerprinting and Diagnostics (CDFD), Hyderabad, an autonomous institute under the Department of Biotechnology (DBT). These laboratories have the facilities of automated multiplex autosomal STR analysis using 9 to15 markers (ProfilerPlus™, Identifiler™ and PowerPlex®16 systems), Y STR markers and FISSR. In addition, sequencing of forensically important regions such as HVI and HVII regions of mtDNA, RAPD analysis and sequencing of cytochrome b and 16S rRNA regions for species identification are routinely performed. Recently, state forensic laboratories (FSLs) of Tamil Nadu, Gujarat, Maharashtra and Delhi have initiated automated STR analyses. FSL, Madhya Pradesh and WII, Dehradun are in the process of setting up DNA profiling facilities.

QUALITY ASSURANCE AND CONTROL

Since DNA profiling has far-reaching implications on the outcome of legal trials, courts insist on observance of stringent quality assurance and control for conducting DNA tests and interpretation of the results. The DNA Typing Unit of CFSL, Kolkata, follows international standards, guidelines and norms in DNA profiling as laid down by the National Research Council (NRC) 1992; 1996 and the Scientific Working Group on DNA Analysis Methods (SWGDAM) 2000. QA/QC system is in place and the analysts of the laboratory are asked to undertake open and blind proficiency tests frequently. Applications for accreditation of the Unit by National Accreditation Board for Calibrating and Testing Laboratories (NABL), New Delhi, India and by the American Society of Crime Laboratory Directors (ASCLD), USA, are in process. Proficiency tests from the Collaborative Testing Services (UK) and American College of Pathologists are being arranged.

CODIS (Combined DNA Index System) DATABASE

CODIS is an electronic 4 tier database of DNA profiles that is maintained by the FBI, developed under provisions of legislation. This DNA database comprises the following categories of DNA records:
- Convicted Offenders - DNA identification records of persons convicted of crimes
- Forensic - Analyses of DNA samples recovered from crime scenes
- Unidentified Human Remains - Analyses of DNA samples recovered from unidentified human remains
- Relatives of Missing Persons - Analyses of DNA samples voluntarily contributed from relatives of missing persons

The Federal Bureau of Investigation (FBI), USA, is one of the leaders in implementing DNA

typing technology in the identification of perpetrators of violent crime. In 1997, after extensive collaborative testing, the FBI announc-ed the selection of 13 STR loci (D3S1358, vWA, FGA, D8S1179, D21S11, D18S51, D5S818, D13S317, D7S820, D16S539, THO1, TPOX, CSF 1PO) to constitute the core loci for human identi-fication in the United States national database, CODIS. All CODIS STRs are tetrameric repeat sequences. The Combined DNA Index System (CODIS) blends forensic science and computer technology into effective tools for solving violent crimes. CODIS enables central, state, and local crime laboratories to exchange and compare DNA profiles electronically, thereby linking crimes to each other to convict offenders. All forensic laboratories that use the CODIS system can contribute to a national database, once they have a recognized system of QA/QC testing.

ALLELE FREQUENCY (GENOTYPE) DATABASE

The Central Forensic Science Laboratory of Kolkata has ventured into an ambitious program of developing the "Allele frequency data of 15 forensically important STR loci for human population groups of India" consisting of 480 predominant population groups. It has already developed a database of about 8000 individuals belonging to 120 subpopulations which also includes data on 8 polymorphic Y STR loci and mtDNA sequence variations in the HVI and HVII regions (Ashma and Kashyap 2002a, b; Gaikwad and Kashyap 2002; Kashyap et al. 2002a, b; Rajkumar and Kashyap 2002; Sahoo and Kashyap 2002a, b; Sitalaximi et al. 2002; Tandon et al. 2002; Trivedi et al. 2002; Sitalaximi et al. 2003). In India, the Centre for DNA Fingerprinting and Diagnostics, Hyderabad along with like-minded institutes, has taken the initiative for D-PAC (DNA Project Advisory Committee) and legislation for creating a national database of convicted people and standards in DNA analysis. The draft proposal is likely to be enacted by the Indian Parliament in near future.

CONCLUSION

DNA profiling technologies are extensively applied in resolving civil and criminal cases and in supporting various types of investigations. As techniques for manipulating and analyzing DNA become more efficient and more robust, forensic DNA testing will improve further. Currently, using a wide array of techniques, DNA can be used very specifically for discriminating individuals. Depending on the quantity of sample and extent of degradation, several techniques can be applied to narrow the likelihood that a particular individual was present at a crime scene. An additional advantage of DNA testing is the ability to review previous cases that were decided primarily using classical tests. In these instances, DNA techniques can be used to reanalyze material that may provide previously convicted individuals an opportunity for acquittal. Till date, 111 post-convicted exonerations of individuals are recorded in the USA. It is clear that DNA technology will advance as will the database for analyzing forensic data. Thus, the power of DNA technology will be immensely useful for criminal justice delivery systems in the years to come. However, considering the limitations of these technologies it should always be kept in mind that DNA can never be used to prove that an individual committed a crime; it can only establish mere presence at the crime scene. The rapid application of DNA techniques in diverse fields such as medical genetics, wildlife forensics, evolutionary anthropology, plant and animal genomics has yielded significant inferences with remarkable resolution unseen in earlier systems, thus revolutionizing scientific approaches comprehensively.

Internet resources:
http://www.cstl.nist.gov/div831/strbase (contains details of published VNTR and STR studies)
http://www.ystr.charite.de (for comprehensive Y STR information)
http://www.mitomap.org (human mitochondrial genome database)
http://www.fbi.gov/hq/lab/codis/index1.htm (CODIS)
http://hgvbase.cgb.ki.se (summarizes all known variations in the human genome,
allele frequency database of global populations)
http://www.ncbi.nlm.nih.gov (resource for molecular biology information)
http://www.promega.com (Promega Corporation)
http://www.perkinelmer.com (Perkin Elmer)

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