Current and Future Developments in Forensic DNA Typing

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SUMMARY

DNA analysis has become one of the most definitive methods for human identification in forensic science. Major developments in the field of genetics including the discovery of restriction enzymes, Southern Blot hybridization and PCR contributed to the rapid development of forensic DNA typing. The human genome was first explored by using restriction fragment length polymorphism (RFLP) analysis. In 1985 it was discovered that DNA contains highly polymorphic regions of repeated DNA that could be visualized by "Jeffreys probes". These probes reveal a minisatellite pattern on unknown loci throughout the genome and are called multilocus probes (MLPs). Scientists soon applied this discovery to human identification and paternity determination. Lack of discrete alleles made it impossible to set up a reference database to calculate allele frequencies rendering a major drawback of MLPs for legal purposes, since random match probabilities could not be generated. Then in 1988 the single locus probes (SLPs) were introduced to the court system. This method is based on known loci that vary in number of tandem repeats (VNTRs). Combinations of SLPs are used to increase discriminating power and population databases could be generated. SLPs were used up to 1993 when a new PCR-based technique became popular. This is based on short tandem repeats (STRs) of 2-6 bp. Whereas RFLP analysis required microgram amounts of intact DNA, PCR allowed the amplification and detection of subnanogram amounts of lower molecular weight DNA in hours rather than days. Commonly used sets of STR markers can reach likelihood ratios (the chance this result was obtained randomly) to one in a billion. Several countries standardize marker sets and create DNA databases. These databases can be searched periodically against unsolved cases; matches that occur are called "cold hits". Certain DNA sources, such as hairs, teeth and ancient remains, have too little or too degraded DNA for nuclear DNA analysis, mtDNA is an alternative to genomic DNA due to its high copy number. Polymorphic regions on the Y chromosome are particularly useful to distinguish the male component within a mixture of samples (e.g. rape cases). Future developments include the development and application of markers for commonplace characteristics, allowing a profile of the suspect to be created that includes eye and hair color and ethnicity. On a technological perspective mini-thermal cyclers and chip-based mini-electrophoresis instruments will allow faster and more efficient STR typing. Portable devices are being developed to help with human identification in mass disaster areas. Single nucleotide polymorphisms (SNPs) have advantages such as better amplification due to the shorter fragments and the enormous amount of them in the human genome. However, complete transition to a new marker system shall not be easy. Other developments can be

seen within legislature, allowing more (types of) offenders to be added to the DNA databases. The American state of Virginia celebrated its 1000th cold hit in 2002. This event illustrates not only how in these cases the perpetrator was found by the click of a button, saving the community money and time, but also how this event potentially prevented new crimes from occurring since a large portion of felons is known to re-offend. Therefore, the combination of technological advances within forensic biology and expansion of DNA databases could be the formula for success in the future justice system.

Chapter 1 GENERAL INTRODUCTION

Introduction

In the present times, it is common that media present us sensational stories, such as court cases, that were solved through DNA evidence. An example of an extraordinary story is given by a case in the United States where a 200-year old politically question was answered through DNA typing: President Thomas Jefferson was said to have fathered a son by one of his slaves, Miss Sally Hemings. A study published in *Nature* thought to have solved this case once and for all, Thomas and Sally had indeed created a son, a remarkable discovery that changed American history (Foster *et al*, 1998). When two years later additional research was done, it became clear Thomas Jefferson had fathered all of Sally's six children (Monticello, 2000).

What seemed impossible hundreds of years ago is now possible: the genetic material a person leaves behind can now be used to identify the owner, and in the case of President Jefferson, elucidate disputed kinship two centuries later. The applications of DNA based human identification go beyond crime solving and paternity testing; another application is identification of wartime casualties, even long after the war. With help of bloodstained cards from living relatives and a family tree, a so-called "reverse paternity analysis" can be performed. The International Criminal Tribunal for the former Yugoslavia (ICTY) in The Hague uses the expertise of the Netherlands' Forensic institute (NFI) to identify victims from the Balkan war. Also, when in 1992 an Israeli cargo aircraft crashed into a block of flats in Amsterdam, the NFI aided in identification process though molecular techniques. DNA typing has also become a useful tool for the Dutch Bureau of Immigration and Naturalisation (IND) to verify family relations. More recently, a large team of forensic biologist was needed to help identify the 2,801 victims of the September 11th 2001 terrorist attacks on the World Trade Center in New York. The meticulous approach to this elaborate task will be discussed in box 3.

Today, the general public is familiar with the fact DNA techniques are being used. But what may sound like "a simple test" is in fact the result of great efforts by a relatively new branch within the Forensic Sciences, one that has combined technological advancement, molecular genetics, statistics, and epidemiology into one: Forensic Biology. This paper gives an overview of technologic developments over the past two decades that have enabled forensic

detectives to make a giant leap forwards through DNA based-human identification and it will highlight upcoming new techniques. First, a chronological review of the past developments will be given, followed by a description of the most popular current method, the STR marker-system. Chapter 4 gives a glance at possible future technologies and summarizes what principles and demands they are based on. The final chapter highlights the legal and ethical aspects that accompany the use of a persons DNA profile and the consequences of creating a suspect-, as well as a national / international database.

History and principles of human identification

In the light of crime, there has been a need for human identification methods throughout the ages. In order to rightfully include an individual having been present on a crime scene, an ideal method would give a guaranteed perfect match in which there is no chance anyone else on this planet could have given the same results (that would be a false positive). Likewise, to exclude an individual, one must look at a trait so unique that the chance for a random match of the suspect and the biological specimen (false positive) does not exist. To eliminate false positive results, the occurrence, or frequency of a certain trait must be as small as possible. An example of a relatively unique trait is given by dermatoglyphic fingerprints, developed by law enforcement a hundred years ago (Butler, 2001). Figure 1 represents a time line in which developments in forensic biology are indicated alongside the foregoing scientific progress. The first genetic tool for human identification was the use of ABO blood groups after its discovery at the beginning of the twentieth century. There are only four different blood groups (A,B, AB and O) and roughly forty percent of the population is type A and another forty percent is type O (Janeway *et al*, 1997; Olsen *et al*, 1997). This method is most useful for excluding an individual from being the source of a crime-scene-specimen, or when it can be used in combination with other markers. For inclusions this method is not very useful, the chance for a wrongful inclusion of an innocent suspect is high due to the limited variability within the blood group system and the high frequency of the A and the O allele. Blood group systems, polymorphic protein and enzyme systems were used in combination to decrease the frequency and thereby adding more confidence to the results. The quality and amount of body fluid available however, sometimes limited this. Despite its imperfections, this was the conventional typing that was used before a major breakthrough in 1985, when the first DNA based human identification method was introduced to the forensic world: multilocus profiling (chapter 2) (Butler, 2001; Kloosterman, 2002). A few years later, multilocus profiling was replaced by single locus profiling (chapter 2) which was the method of choice until 1993 when the first fluorescent STR-marker kit became available

(chapter 3), bearing numerable advantages as illustrated by its worldwide use today. The main advantage that a DNA based method of identification has over others, is that DNA is more resistant to environmental decay than proteins or other biochemical molecules and is likely to be present at a crime scene. Also, conventional fingerprints are generally sparse compared with the DNA in blood, semen, hair, epithelial cells, or other tissues.

Figure 1. Overview of scientific progress and its implementation in forensics

Scientific Progress	Year	Forensic Implementation
Gregor Mendel developed **theory of inheritance**	**1850**	
Uniqueness of dermatoglyphic fingerprints discovered Blood group system discovered by Karl Landsteiner	**1900**	Use of **dermatoglyphic fingerprints** for identification
Inheritance of **ABO blood group system** confirmed by von Dungern and Hirzfeld	**1910**	
Oswald Avery describes Deoxyribonucleic acid **(DNA)**	**1944**	
Double helix structure of DNA elucidated by James D. Watson and Francis H.C. Crick	**1953**	
Isolation of the 1st **restriction enzyme**, from *E.Coli* by Werner Arber's group	**1962**	
Edward M. Southern develops **Southern Blotting,** Alec Jeffreys synthesizes radioactive DNA probe	**1970**	Difference among **ABO blood group** types between people used for human identification
Southern describes restriction fragment length polymorphism **(RFLP)** analysis	**1975**	
Wyman and White report one of the first highly polymorphic DNA regions. This locus has at least 8 alleles, making it useful for genetic identification.	**1980**	
Jeffreys develops multi-locus RFLPs probe **(MLP)**	**1985**	Start of the **DNA fingerprinting-era** using MLPs
Kary Mullis describes Polymerase Chain Reaction **(PCR)**	**1986**	
Nakamura et al. describe new highly polymorphic genetic loci with variable numbers of tandem repeat **(VNTR loci)** and develop a series of new VNTR probes for use in RFLP analysis	**1987**	DNA testing solves "Black Pad" murders in the UK. This is the **first case** in which DNA evidence is used to determine the identity of a murderer and in which a prime suspect was exonerated due to DNA evidence
Jeffreys shows that PCR can amplify VNTR loci requiring much less DNA	**1988**	Use of **single locus probes** by the FBI
Short tandem repeats **(STRs)** are found to be highly polymorphic and easily amplified by PCR.	**1989**	Use of **single locus probes** in The Netherlands
	1991	
Promega releases its first STR detection systems	**1993**	
	1994	1st multiplex STR typing system applied in The Netherlands and other European countries, known as **Quadruplex**
	1995	FSS starts UK DNA database
	1996	**Mitochondrial DNA evidence** is first used to solve the rape and murder of a 4 yr. old Tennessee girl: mtDNA from hairs found on the child's body matched that of Paul Ware's.
	1997	Netherlands Forensic Institute **(NFI)** operates DNA database
	1998	FBI launches **CODIS** database, linking all 50 states and allowing federal, state and local forensic agencies to share DNA profiles of convicted offenders and crime scene evidence
The PowerPlex® 16 System,which allows amplification of all 13 CODIS loci in a single reaction, is made available by Promega.	**2000**	
AMPFℓSR SGM® Plus™ 10 STR, becomes available	**2002**	The Division of Forensic Science Laboratory in Virginia becomes the first state laboratory to mark **1,000 "cold hits"** from its DNA database.
	2003	
	2004	Parliamentary discussion in the Netherlands in favor of a **convicted offender database**

The basis of human identification: DNA Polymorphisms

If we compare any two genomes, they will differ from one another roughly once every 500-1000 base pairs. This is usually expressed as 99.9% of the human genome sequence is identical between individuals. Knowing the human genome consists of approximately 3.5 billion basepairs, the 0.1% difference can add up to three million basepairs, enough to account for the individual and racial variations in the human species (Alcamo, 2001). In the context of human identification, we need a way to map out and utilize these differences. A more appropriate term for these differences in DNA is polymorphisms, literally "many forms". There are two types of polymorphisms within DNA: Sequence polymorphisms and length polymorphisms.

Sequence polymorphisms

Sequence polymorphisms are variations in a single base pair, and are called Single Nucleotide Polymorphisms (SNPs). The discovery of restriction enzymes in 1962 was key to be able to visualize DNA sequence polymorphisms (Alcamo, 2001). Restriction enzymes are endonucleases isolated from bacteria that cleave DNA at specific, restricted sites spanning 4-8 base pairs. A variation on the DNA that alters the recognition site of a restriction enzyme is called a Restriction Site Polymorphism (RSP). If such a base sequence variation changes the recognition site of a restriction enzyme, the product length will vary after digestion of the DNA; the restriction site may or may not be present with this specific polymorphism and the DNA will or will not be cleaved at that point. Because of the differences in product size, this type of sequence polymorphism is called Restriction Fragment Length Polymorphism (RFLPs). visualization of these fragments was made possible with a technique called Southern Blot-hybridization, developed by Edward Southern in 1970 and with radioactive DNA probes, which were synthesized by the British geneticist Alec Jeffreys in the same year. Southern Blot-hybridization is commonly used to visualize RFLPs. Purified DNA is digested with a specific restriction enzyme, after which the obtained fragments are separated by size through electrophoresis; smaller fragments will move faster through the pores of the gel than larger ones. Next, the DNA in the gel is denatured to obtain single strands and transferred to a positively charged nylon membrane, this transfer step is the "blotting". After hybridization with a radioactively labeled specific probe, the matching complementary fragments will emit radioactivity, visualized with X-ray film (Alberts, 1994). The process of utilizing restriction site polymorphisms to identify individuals is called restriction typing. Sequence polymorphisms have a low variability within the polymorphic sites (there are only four bases) and were not

used for forensic DNA identification. However, chapter 4 will discuss a revival in interest for these single nucleotide polymorphisms because of their short size and abundance throughout the genome, predicting the use of these sequence polymorphisms in forensic applications.

Length Polymorphisms

The second type of DNA polymorphism is length polymorphism. Eukaryotic genomes are full of repeated DNA sequences called satellite DNA. These repeated stretches of DNA sequences come in variable types and sizes, consisting of a core repeat unit that is tandemly repeated. Figure 2 lists definitions and origins of the two types of satellite DNA, the mini- and microsatellites. A repeat of approximately 10-100 base pair is called a minisatellite or a VNTR (variant number of tandem repeats) and was first described by Dr. Alec Jeffreys (Jeffreys *et al*, 1985). VNTRs are somewhat similar to RFLPs, except that VNTRs arise from the number of repeated base sequences between two known points on the DNA. The fragment-sizes of RFLPs depend solely on restriction site polymorphisms. The core repeat unit of a VNTR is in the range of 10-100 bases in length. The DNA can be cleaved at restriction sites outside the tandemly repeated sequence. An example of a VNTR is the forensic DNA marker D1S80, a minisatellite with a 16 base pair repeat unit containing alleles consisting of 16-41 repeat units (Butler, 2001). These variants in repeat number lead to variants in fragment-length after digestion with restriction enzymes. Each homologous chromosome will give a band after electrophoresis and Southern blot hybridization. The pattern will reveal two bands for a heterozygous individual and one for a homozygous individual since both fragments have the same length. Not only are these VNTRs abundant in the genome, they are also highly variable among individuals. As will be described in chapter 2, these VNTRs that differ in length between individuals can be analyzed using single locus probes.

Table 1. Types of repeated DNA sequences

Type of DNA	Description	Length	Reference
Satellite DNA	• Regions of long stretches of repeated DNA, mostly found along the centromeres.	< 1000 bp	Alberts, 1994
Minisatellite DNA	• Type of satellite DNA consisting of medium length repeat units	10-100 bp	Jeffreys, 1985
VNTR (variant number of tandem repeats)	• Term reserved for moderately large repeat, also called "Hypervariable minisatellite DNA"	5-64 bp	
Microsatellite DNA	• Type of satellite DNA consisting of smaller repeat units the so-called Simple Tandem Repeat, or Short Tandem Repeats (STR)	2-6 bp	Edwards, 1991

Minisatellite patterns form the basis of the multi-and single locus probes that were used in the forensic community between 1985 and 2000 and will be discussed in the next chapter. The other type of repeated DNA is called microsatellite DNA. Microsatellites are tandemly repeated sequences consisting of bi-, tri-, tetra-, penta-, hexanucleotide repeats. An example of a tetranucleotide repeat is the STR marker TH01 consisting of $(TCAT)_n$ repeat units. These highly variable repeats have been widely used in DNA typing since their discovery in 1991 up until today and will be discussed in chapter 3.

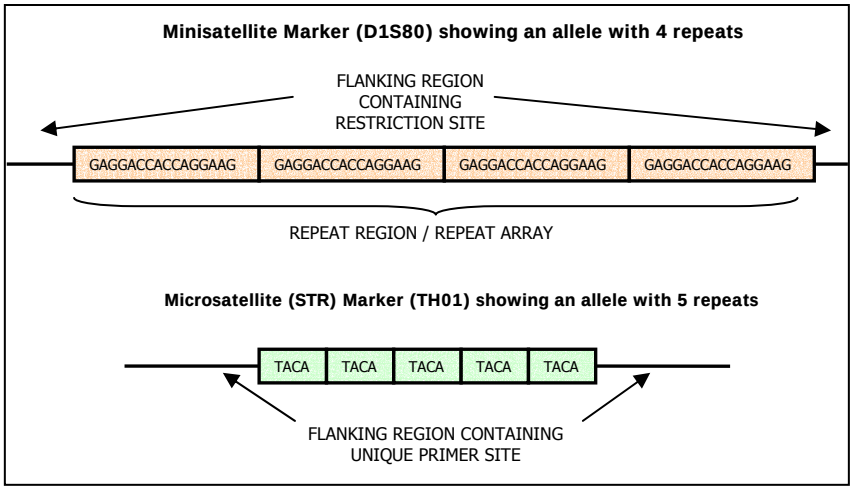
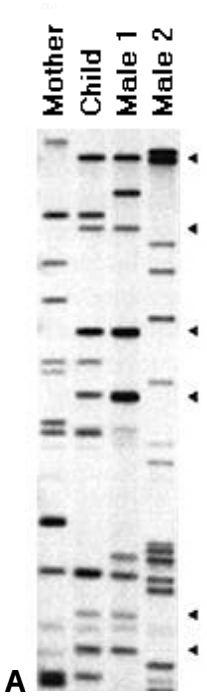


Figure 2. Schematic of minisatellite and microsatellite DNA markers. The minisatellite marker D1S80 is based on variant numbers of tandem repeats and invariant* flanking regions with specific restriction sites. For the microsatellite marker PCR primers are designed to target the invariant* flanking regions. The length of the repeat array varies among individuals and results in a high number of alleles, making this polymorphism a useful tool in human identification.* *Sometimes there is polymorphism in these restriction or primer sites, rendering a so-called null allele, where no band or peak representing the allele will be present.*

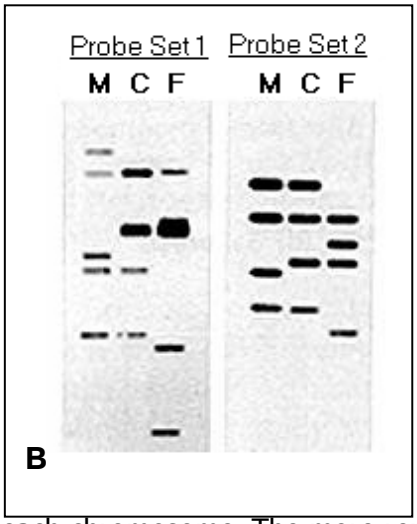
Chapter 2 DNA FINGERPRINTING

The DNA fingerprinting-era started in 1985 when the English geneticist Alec Jeffreys developed a method called multilocus DNA fingerprinting. In fact, the term "DNA fingerprinting" originally refers to this specific technique, but is now used to describe all procedures for characterizing VNTRs, RFLPs and other sequence polymorphisms. The classical picture of a DNA fingerprint is still often depicted as an array of 15-25 black bands obtained by using a radioactive multilocus probe (MLP). Box 1 gives an example of what a multilocus DNA fingerprint looks like. The name multilocus indicates that the specific repeat used as a probe has multiple loci on the genome and the term fingerprint indicates that the MLP typing system has high discriminating power. Refinement of this system due to several drawbacks lead to the next generation of forensic DNA-tests: the Single Locus Probes (SLP) in which only two bands can be seen in a heterozygous individual, representing one specific locus.

Box 1. Fingerprint Patterns using MLP, SLP and STR-markers



EXAMPLE A
Paternity Case using Multi-Locus Probe
The probe $(CAG)_5$, recognizes a large number of loci. While the probe itself is a microsatellite probe due to its small size, it reveals a minisatellite pattern of repeated DNA within the genome. Arrows indicate the informative alleles from the child that exclude male#2 as being the father and with a certain likelihood include male#1 as the father of the child.

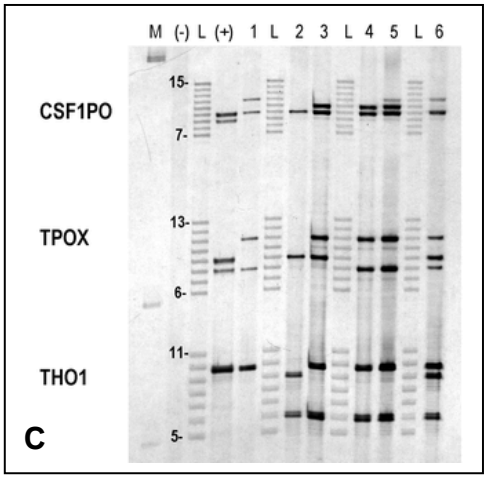


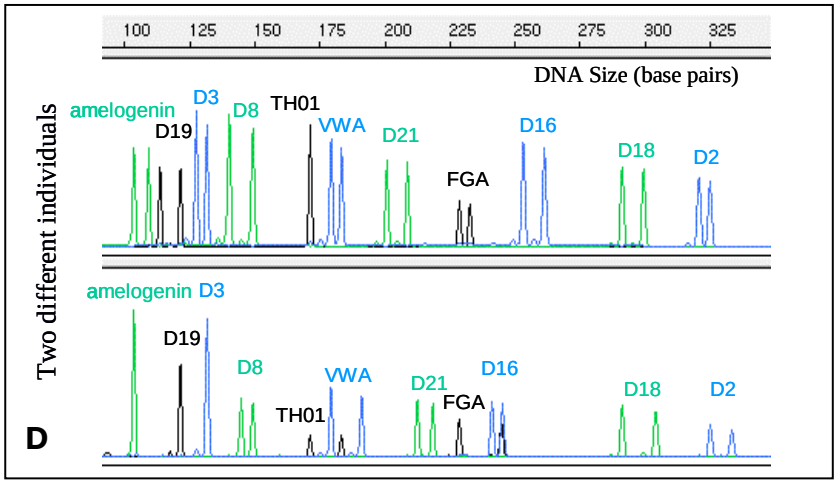
EXAMPLE B:
Paternity Case using 2 Single Locus Probes
A mixture of three and two single locus probes (set 1 and 2) reveals polymorphic alleles for VNTR minisatellite markers. Each probe will reveal two bands for a heterozygous individual, one from each chromosome. The more repeat units, the larger the band. Bands are separated by size and hybridized with the minisatellite probe. The loci detected in the child (C) are an exact composite of those present in the mother (M) and father (F).

EXAMPLES C & D

Short Tandem Repeat markers visualized by silver staining (C) Examples of allelic patterns for 3 multiplex STR loci from amplified DNA visualized by silver staining. Lane 1-4 contains samples from 4 different people. Lane 5 contains a mixed sample containing DNA from individuals 1 and 4. Lane 6 contains DNA from individuals 1 and 2. (M) size marker; (−), negative control; L, allelic ladder; and (+), positive control. Each allele is represented by a band of a different size, so there will be 2 bands in a person heterozygous for that marker.

Short Tandem Repeat markers visualized by fluorescent primers (D) Examples of allelic patterns for 2 individuals using 10 multiplex STR loci and a gender ID. Sample DNA is amplified using fluorescent primers with three different colors distributed over the 10 markers. The amplified DNA is separated by size through capillary electrophoresis. Each allele is represented by a peak, so there will be two peaks in a heterozygous individual, one for each homologous chromosome.





When multiple SLPs are combined ("multiplexed") the number of possible bands will increase. An example of a DNA fingerprint pattern obtained by mixtures of single locus probes is given in box 1. Both techniques will be discussed in the next paragraphs even though they are not used in forensic cases anymore. MLPs and SLPs served the forensic community from the mid-eighties to the year 2000 when they were replaced by the STR marker system. PCR-based techniques have several advantages over Southern blotting based techniques - for example, much less DNA is required and in many cases, typing can be done using partially degraded DNA.

Multi-locus probes

Multi-locus fingerprint patterns make use of the fact that many tandem repeat arrays occur in various parts of the genome and in variable numbers of tandem repeat elements. The repeat arrays themselves are used as a probe, which is applied to a Southern blot of genomic DNA cut by restriction enzymes. The probe will bind to unknown loci that reveal a highly polymorphic pattern, resulting in a DNA phenotype rather than a genotype. The variability of multilocus probes is thought to be due to three factors: (1) the number of repeat elements in one array and (2) the distance between the repeat array and the nearest recognition site for the restriction enzymes used for digestion of the DNA. Another level of variation occurs through (3) variability in probe binding. This promiscuity of probes added another level of complexity to multi-locus probe patterns, generating darker bands where the probe matched the sequence on the DNA and lighter bands where it matched only partially (Epplen et al.1999). The "classic" multilocus probe developed by Jeffreys is derived from a minisatellite, containing the core sequence of GGGCAGGANG. The "N" stands for any of the four bases. The number of repeats in one array differs from 3-40 and generates a multitude of different bands that are hard to interpret. The multilocus probe was soon replaced by a microsatellite probe (bi-, tri-, tetranucleotide probes), used to detect minisatellite patterns. Despite the two different probes used, the multilocus profiling principle is still based on minisatellite patterns obtained after digestion of genomic DNA and hybridizing with a probe to visualize these minisatellite sites. The MLP-blot shown in box 1 (Example A) is generated by hybridizing with a radioactive probe consisting of the $(CAG)_5$ repeat unit. The multiple (unknown) loci of this minisatellite DNA are characterized for a child; it's mother and two putative fathers. The MLP profiling method was used in forensic casework by the American Federal Bureau of Investigation (FBI) from 1988 on and from 1989 on in The Netherlands.

Shortcomings of MLP DNA fingerprinting

The main disadvantage is that with MLP typing it is not possible to tell which pairs of bands in a fingerprint represents true alleles. The final fingerprint pattern, that usually consists of 15-25 bands, is generated by a combination of factors, such as the number of repeat elements, the distance between the repeat array, the nearest recognition sites of the enzymes used for digesting the genomic DNA sample and the binding of the probe, a factor which depends on the promiscuity of the probe and on the DNA sequence. Some probes will hybridize with sequences that are not their exact match. It is impossible to decide whether a band shared between two individuals in terms of position, but exhibiting different intensity, reflects variability in the number of tandem repeat elements, is a result from comigration of nonallelic fragments, or reflects differences in probe binding. Another disadvantage of MLPs is that large amounts of undegraded are DNA needed, up to 1µg and profiles from contaminated (mixed) DNA samples could not be adequately separated from the reference sample. Samples from the suspect, victim and the references must be run at the same time and in the same gel in order to accurately determine the size of the alleles. The need of creating a DNA database could not be fulfilled with MLPs because digitizing of MLP data is not possible. Moreover, since no discrete alleles could be defined, no reference database with allele frequencies could be set up, giving the legal system too few grounds to stand on. Taking all these drawbacks into account it was obvious the MLP DNA typing method was in need for a drastic change (Kloosterman, 2002).

Single-locus probes

By 1990 the MLP system was replaced by single locus probe (SLP) profiling. SLPs are based on VNTR loci with core repeats that range from 5-64 base pairs (Jeffreys, 1985). For example, the minisatellite marker D1S80 depicted in figure 2 is based on the 16 base pair core sequence GAGGACCACCAGGAAG that occurs in variant numbers of tandem repeats elements. In this case the single locus probe will address length polymorphisms on that specific locus, which is done the following way: Each SLP is defined for a specific VNTR locus and specific restriction enzymes will cleave the DNA flanking the hypervariable region, resulting in fragments that vary in length due to their variations in repeat number. After separation by gel-electrophoresis, Southern blot hybridization with a probe specific for the sequence of interest will reveal the alleles for the polymorphic sites. The more repeats, and thus the larger the allele size, the larger the fragment on the gel and the slower it will move through the gel. Using a highly informative size marker, genotypes can be generated and compared. This was one of the greater advantages over MLPs, digitizing of the data was

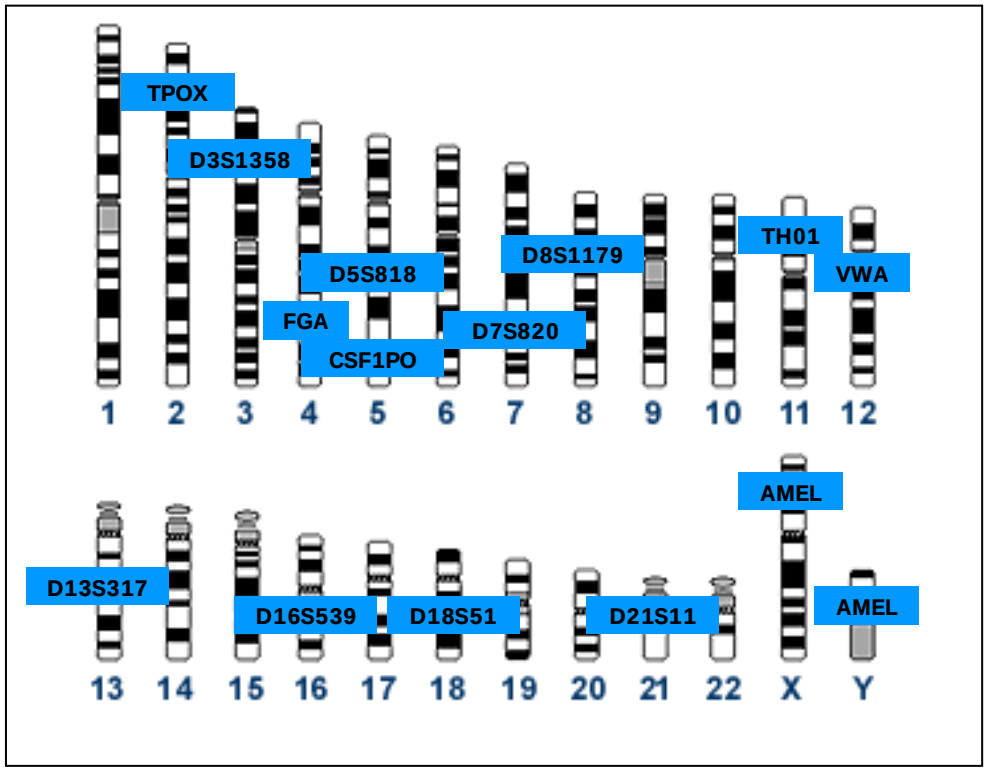
possible and therefore the possibility of generating a DNA database was created. An initial drawback was the lack of evidential value when looking at only one locus. When using one single locus probe, the SLP blot will reveal one to two bands, for a homozygous and heterozygous individual respectively. So, multiple SLPs were analyzed sequentially and multiplexed, decreasing probabilities for a random match to less than one in 10^6, leading to careful acceptance in the justice system. As numbers in the population database used as a reference grew, the match frequencies decreased up to 1 in 10^9. The four different SLPs used in The Netherlands are YNH24 and the three probes developed by Jeffreys MS-1, MS43a, g3 (Kloosterman, 2002). Unfortunately, a relatively large amount of undegraded DNA was needed to create a full profile, so still not all forensic cases were eligible for this method. An example of two multiplexed SLP-blots is shown in box 1 (Example B).

Chapter 3 STR BASED DNA TYPING

DNA regions with repeat units between 2-6 base pairs are called microsatellites, simple sequence repeats, or short tandem repeats (STRs). An example of a microsatellite repeat array is given in figure 2, where the allele shown consists of 5 tetranucleotide core repeats with sequence TACA. The electrophoresis method used for the larger VNTRs, was not sensitive enough to adequately distinguish between repeat-lengths of the smaller STRs. VNTR arrays can be up to 10,000-20,000 where the most commonly used tetranucleotide STR arrays (including flanking regions) are under 400 base pairs. STRs are visualized by a groundbreaking technique invented in 1986, called the polymerase chain reaction (PCR) that made DNA amplification possible for the first time. In brief the polymerase chain reaction allows the DNA from a selected region of the DNA to be amplified up to a billion fold, provided that at least some part of its nucleotide sequence is known. The DNA undergoes multiple cycles of heat-initiated denaturing followed by polymerization by DNA polymerase. This process cannot take place without the presence of specific oligonucleotides that serve as primers for the DNA polymerase and deoxynucleotide triphosphates, the building blocks of DNA (Alberts, 1994). STR markers consist of alleles that vary in repeat-number, but are flanked by a unique and highly conserved region, the so-called flanking region. The primers used in the polymerase chain reaction are designed for this region and carry a fluorescent flag. After PCR has taken place, fragments are sorted by size by either slab gel electrophoresis or capillary electrophoresis (Butler, 2001). Size markers are added to the reaction mix to be able to determine the discrete alleles. Results are obtained digitally in the form of an electropherogram. Today, visualization is done by using fluorescent dye labeled primers, but it has also been described via other staining methods such as silver staining and

autoradiography (Hammond et al., 1994). See box 1 for examples of STR profiles using silver staining and fluorescence (examples C & D). There are literally hundred thousands of STR systems, which have been mapped throughout the human genome; examples are given in figure 3, where chromosomal distribution of commonly used STR markers is shown**.** An additional sex determination marker is added, amelogenin. This marker is based on a 6 base pair deletion in a region on the X-chromosome. The Y chromosome does not have this deletion, so its allele is 6 base pairs longer than that of the X-chromosome. A female profile therefore has only one peak for the amelogenin marker and a male has two, because of the 6 base pair difference between his X and Y chromosome. Several dozen have been investigated for application to human identity testing. These STR loci are found on almost every chromosome. They may be amplified using a variety of PCR primers. Tetranucleotide repeats have been most popular among forensic scientists due to their fidelity in PCR amplification although some tri- and pentanucleotide repeats are also in use. To increase discriminating power, STR-markers are multiplexed.

Figure 3. 13 commonly used STR markers and their distributions over the human genome. The Amelogenin marker is added for gender determination (see text). Adapted from: http://www.cstl.nist.gov/biotech/strbase/chrom.htm



First Generation Multiplex STR typing

The first multiplex STR typing system applied in The Netherlands and other European countries is known as Quadruplex, developed by the Forensic Science Service in 1994, involving multiplex amplification of four tetrameric STR loci. These loci include TH01 (seen in example C & D in box 1) vWA, HUMFES and HUMF13A1. After a large population study (Sjerps et al. 1995) was conducted, Dutch Forensic Science Laboratory received accreditation for the use of the so-called "quad-mix" in forensic casework. The 4 Quad loci started to replace the SLP typing system. Match probabilities with the Quad mix were at best in the range of less than one in five thousand. For this reason a combination of the typing results of the Quad-mix and results of two other typing results (HLADQA1 and Polymarker system; not discussed) were compared to gain match probabilities of less than one in a million (Kloosterman, 2002). Table 2 displays the composition of each of the historically and currently used STR systems.

Second generation of multiplex STR typing

The Forensic Science Service (FSS) introduced a new set of multiplex STR markers in 1996, consisting of two of the Quad minisatellite loci (TH01 and vWA) combined with the STR microsatellite loci D8S1179, D18S51, D21S11 and FGA. Of course one of the greater advantages was the fact that only one multiplexed PCR reaction was necessary, to yield match probabilities of less than one in a million. This set of 6 loci became known as the second-generation multiplex (SGM) system and was implemented in The Netherlands in 1997 (Kloosterman, 2002).

Expansion of the SGM-system

In 1999 four additional loci (D3S1358, D16S539, D2S1338 and D19S433) were added to the six SGM-markers, a system now known as AMPFISRT,SGM Plus™. A full SGM plus profile could deliver match probabilities of less than one in a million. The SGM plus system was fully accredited for use in forensic cases in The Netherlands in 2000. The uniformly used European core loci, include 4 of the FSS loci (D21S11, FGA, TH01, and vWA) and the gender-specific amelogenin loci, and was expanded by three more core loci that were added in 2000 (D3S1358, D8S1179 and D18S51) leaving a total of 7 core STR loci (Kloosterman, 2002). Due to international harmonization, these 7 core loci used by the European Network of Forensic Science Institute (ENFSI) are also found within the core 13 loci used by the North American counterpart Combined DNA Index System (CODIS) (Butler,2001).

Table 2. Comprehensive overview of composition of different multiplex-loci

Locus	Quadruplex	FFS-SGM system	AMPFISRT. SGM Plus™	CODIS core	ENFSI core
CSF1PO				✓	
D2S1338			✓		
D3S1358			✓	✓	✓
D5S818				✓	
D7S820				✓	
D8S1179		✓	✓	✓	✓
D13S317				✓	
D16S539			✓	✓	
D18S51		✓	✓	✓	✓
D19S433			✓		
D21S11		✓	✓	✓	✓
FGA	✓	✓	✓	✓	✓
TH01	✓	✓	✓	✓	✓
TPOX				✓	
vWA		✓	✓	✓	✓
HUMFES	✓				
HUMF13A1	✓				
amelogenin		✓	✓	✓	✓

Advantages of STRs over traditional RFLP techniques

PCR-based STRs have several advantages over conventional Southern blotting techniques of the larger variable number tandem repeats (VNTRs). Determination of discrete alleles allows results to be compared easily between laboratories because actual genotypes for specific markers are generated. In addition, smaller quantities of DNA (0.1-1 ng), including degraded DNA, may be typed using STRs. Due to the smaller size of the repeat arrays, degraded DNA is less of a problem with STR-markers than for the larger VNTR arrays. Thus, the quantity and integrity of the DNA sample is less of an issue with PCR-based typing methods than with

conventional RFLP methods. Other advantages are that the method is much faster than RFLP analysis, which can take up to 6-8 weeks using radioactive probes and one week with fluorescent probes. Results of STR typing can be obtained within a day and are also better suitable for high-throughput analysis and automation. The discriminating power of multiplexed STR markers in very high compared to RFLP methods, and with proper use of the population databases, estimates of match probability approach 1 in 1 billion.

Additional techniques

Polymorphic regions on the Y chromosome are particularly useful to distinguish the male component within a mixture of samples (e.g. rape cases) and will be discussed in the next paragraph. Certain DNA sources, such as hairs, teeth and ancient remains, have too little or too degraded DNA for nuclear DNA analysis. The use of mitochondrial DNA (mtDNA) is an alternative to genomic DNA due to its high copy number within the cell, and will be discussed in the next paragraph also.

Y-chromosomal markers

In cases of contaminated stains, as in a rape case, when male and female DNA is mixed, Y-chromosomal marker STR systems can be used to analyze male DNA without having the interference of the victim's female DNA. Also the amelogenin sex-typing locus is not a 100% guaranteed to give the right gender; therefore, the Y-chromosomal markers would be a great addition. The Y chromosome is haploid and the genetic material on the Y-chromosome is referred to as "haplotype". What makes the Y-chromosome useful is that it contains short tandem repeats with a unique flanking region, not found on genomic DNA. Also, the Y-chromosome holds information on geographical origin, as the Y-chromosome is only inherited through males, from father to son. Despite its promising character, presently it is not feasible to test the majority of the crime-samples on Y-chromosomal markers, simply because too much DNA is needed. According to an infamous Dutch geneticist Dr. P.de Knijff there certainly is a future for Y-chromosomal markers to be used in cases to elucidate the unknown suspect's ethnicity (de Knijff, 2003).

Mitochondrial DNA

During the 1960s it was discovered that mitochondria possess their own self-replicating DNA, called mtDNA (Alcamo, 2001). Since then, several genes have been discovered within the mtDNA, which is shaped as a double stranded circular unit. A paper published in Nature in 1987 asserted that the mtDNA from all modern humans is derived from a common ancestor

of 200,000 years ago (Wilson et al, 1987). Both egg and sperm contain mitochondria, but the sperm contributes no mitochondria, as only the sperm nucleus enters the oocyte. Our mitochondria are solely inherited through our mothers and our mothers from their mothers and so on. Human diversity depends much on the recombination occurring during meiosis. Mitochondrial genes on the other hand, do not undergo this mixing or division, thereby representing an unbroken line of genetic information back to the first woman (or population of women), the mythical Eve (Alcamo, 2001). mtDNA however is subjected to mutations, with a mutation rate of approximately 2-4% every million years. In this very fact lies the weakness of using mitochondrial DNA; the high frequency of the mtDNA alleles results in low evidential value in comparative forensic casework. The reason mtDNA typing has and will be further explored is because of its high sensitivity due to the high number of copies present per cell and the highly stable nature of mtDNA, enabling to type ancient samples with only minute amounts of DNA needed (Kloosterman, 2002). Previously mtDNA typing has been used to identify the bones of ancient remains, such as those of soldiers from the Vietnam War and in the highly publicized case of the identification of the remains of Tzar Nicholas II of Russia. mtDNA has also been used in helping identify a number of the September 11th victims of the World Trade Center terrorist attacks in New York City. Not only were the victims in and around the building pulverized by the collapse of the buildings, the jet fuel caused the debris to burn for months, thus degrading most of the DNA. Box 3 explains how mitotyping was used after the 2001 mass disaster in conjunction with STR analysis and SNP analysis in a major collaborative effort to identify every victim.

Box 2. Notorious Cold hits

United States

- The first case in the USA solved by a cold hit was the rape and murder of a 23-year-old secretary in her apartment on November 17th 1991 in Lowry Hill, Minneapolis. No physical evidence was found. However, the killer had left behind enough genetic evidence to raise a DNA profile. Matched against a database of known profiles, the sample revealed a cold hit; convicted rapist Martin Perez, who was later found guilty of the murder and sentenced to life in prison without parole(1).
- In Virginia, a suspect linked to a 1994 slaying through a cold hit in 2001 was indicted on first-degree murder charges(1).
- Louisiana police recently had their first out-of-state cold hit when authorities linked an inmate at a state penitentiary to an unsolved 1987 rape-murder in Texas (1).
- A Washington state man was charged in the 1999 kidnapping and rape of a 14-year-old girl abducted from her school bus stop after a cold hit linked the convicted felon to the attack (1).
- In El Dorado County, California, investigators reported a break in a 1971 murder after DNA from the crime scene was linked to the DNA profile of an inmate in a California prison. It is believed to be the oldest case in the state re-examined as a result of a cold hit (1).
- The rape and murder of 27-year-old rising punk-rock star Mia Zapata, lead singer of "The Gits," remained unsolved for nine years. Investigators found their suspect thanks to well-preserved swabs of saliva taken from the victim's body, a knowledgeable Florida parole officer and a DNA match. This led to the arrest of Jesus Mezquia, a 48-year-old Florida fisherman with a history of sexual assaults, who police say sexually assaulted and strangled Zapata then left her dead on a deserted Seattle street in the early morning of July 7th 1993 (2).
- Hoping for a cold hit… Recently, DNA evidence has been submitted to help solve the murder of JonBenet Ramsey, a former Little Miss Colorado. The six year old girl was found beaten and strangled in the basement of her parents' home in Boulder, Colorado, on December 26th 1996. A sample of male DNA found on JonBenet's underwear has been submitted to FBI investigators seven years after her violent death. Earlier DNA tests on the blood indicated it was from a male who was not a member of the Ramsey family. At the time, the DNA sample wasn't of a high enough quality to compare against a national databank of DNA. A high quality sample of DNA from the garment was sent to the FBI in 2003 (3).

United Kingdom

- DNA testing solves "Black Pad" murders in the UK. This is the first case in which DNA evidence is used to determine the identity of a murderer and in which a prime suspect was exonerated due to DNA evidence
- A DNA sample taken from a man caught urinating on the street in Britain linked him to an unsolved rape (1).
- In another recent case, DNA taken in conjunction with a drunk and disorderly charge linked a man to 1999 burglary (1).

The Netherlands

- In 2003 the murder of a 21 year old woman working in a clothing store near Amsterdam (Zaandam) would be solved nearly 20 years after she was murdered. Improved examination of DNA on the knife confiscated 20 years ago, lead to a match in the DNA database. The killer, who was addicted to controlled substances died in 1992 (4).

Sources:
(1) http://www.courttv.com/news/forensics/dna_anniv/databases.htm
(2) http://www.courttv.com/news/forensics/dna_anniv/zapata_ctv.html
(3) http://www.usatoday.com/news/nation/2003-12-27-jonbenet-murder_x.htm
(4) http://www.nrc.nl/dossiers/genetischerevolutie/ 988647180248.html

Chapter 4 FUTURE DEVELOPMENTS

This chapter will give an overview of future developments. A number of future technological advances will be discussed briefly. Many of these would facilitate and improve the current method of the multiplex STR marker system (e.g. improving low copy number DNA). The second part of this chapter will focus on applications of already existing techniques that are most likely to be implemented in the near future (e.g. SNPs) or that would complement the current methodology (e.g. ethnicity markers).

Part I: IMPROVING CURRENT TECHNIQUES

Scaling Up STR typing

An inevitable direction that forensic biology will follow is a trend seen in many other industries: scaling up. This enables a higher volume throughput, saves reagents, energy, time, and thus decreases the overall costs. Levels at which this can be achieved are at the level of the PCR by using miniature thermal cyclers, which are currently developed. A smaller thermal cycler uses fewer reagents but also performs its cycles quicker due to faster heating and cooling. Another way to increase throughput is with capillary array electrophoresis instruments (CAE). Capillary electrophoresis is now performed sequentially rather than parallel. The use of the recently developed 96-array capillary electrophoresis systems for STR typing has already been reported to be successful and it is likely that this CAE will be used increasingly. Another way to speed up DNA separations using microchip CE devises. In these chips the regular 30 cm capillary is replaced by one of several centimeters long, resulting in a 10-100 faster separation. When these chips are parallelized in a 96-channel microplate an even higher throughput can be achieved (Butler, 2000). In fact, the Virginia and Florida police have begun field-testing a chip based system that can perform STR analysis in two minutes and can process thirty times the number of samples per hour of some existing protocols. The system consists of a mini-electrophoresis device capable of holding 16 samples at once and is not only time saving, it is also portable making it suitable for use in war zones (Miller, 2002).

Improving low copy number DNA profiling

The minimum amount of DNA required for a full profile using a commercial multiplex DNA kit is 100 pg, which is contained by no more than 18 diploid nuclei. Often times traces left at a crime scene contain fewer cells, for instance the epithelial cells left on a strangulation victim

or the palm marks on a firearm. It would be a major contribution to solving many cases if profiles could be created from these few, and sometimes even single cells. One approach involves hyperamplification of low copy number (LCN) DNA samples (Kloosterman, 2002). The standard set of 28 cycles of PCR is extended with 6 more cycles (28+6). Not always does this result in a full profile, but when multiple cells are subjected to the same analysis better results are obtained. The major problem is that of allelic dropout (ADO) of the larger loci and the occurrence other amplification artifacts. There is a newly developed method to amplify DNA from a few cells other than by altering the number of PCR cycles, this is by a new technique called multiple displacement amplification (MDA) and will be discussed in the next paragraph. When the ability of PCR has reached is lower limit, the way evidence is collected becomes crucial, the more nucleated cells one can retrieve, the better. Besides hyperamplification shorter STR loci could improve results for degraded and LCN DNA. This would be useful when a reference sample is available from a potential suspect, but becomes a problem when wanting to compare the profile with current databases. In the line of improving profiles from LCN DNA is the goal to create a full profile from a single hair. There are cases in which a hair is the only specimen available for DNA typing. A recent study optimized DNA typing of a single hair and found enough copies of nuclear and mitochondrial DNA in the root and even in the shaft of the hair to be detected using PCR (Miscicka-Sliwka et al, 1997). As little as 500 pg DNA isolated from a single hair root and 1.5 ng DNA from the shaft was successfully amplified and the correct genotype was generated using multiplex STR markers, with the results confirmed by mitochondrial DNA analysis. Two studies on extracting DNA from fingerprints on various substrates gave differential results. The first showed successful profiling of a latent fingerprint on paper (Balogh et al, 2003) and the second found that in a significant number of experiments no DNA could be obtained from glass, wood or metal (Alessandrini et al, 2003). The latter study did point out that there are individual differences in "shedder-status" meaning individuals can differ in the amount of DNA they leave behind with their fingerprint. The substrate the fingerprint is left on also seems to play an important roll.

Multiple Displacement Amplification

The multiple displacement amplification (MDA) method is based on opening the DNA and amplifying pieces of it by adding abundant random hexamer primers to the DNA, without any DNA purification and with only a few cells. Crucial to this process is the presence of highly processive strand-displacing DNA polymerase that extends up to 70 kb from the primer. Once initial priming has occurred, an exponential cascade of branched amplification

generates highly accurate copies of the genomic template, due to the proofreading ability of the DNA polymerase. The polymerase is able to copy, prime, and recopy any DNA target without the need to repeatedly denature the template as required by PCR. The exponential nature of the amplification process is powerful enough to amplify DNA from even a single cell up to a million-fold in only a few hours. The DNA generated is in excess of 10kb in length enabling DNA typing through RFLP or STR methods (Molecular Staging, 2003). In one recent application study, amplified DNA was indistinguishable from the original genomic DNA template in 5 SNP and 10 microsatellite DNA assays on three different clinical sample types from 20 different individuals. The minimum amount of blood they used was 0.05 µL, which contains about 300 nucleated cells. Amplification of genomic DNA also eliminates the need for DNA purification; only the use of a mild lysis buffer is required. In the previous example 0.05 µL of blood was added to a reaction volume of 100 µL, giving a 2,000-fold dilution of the blood in the MDA reaction. A 10,000 or greater amplification through MDA enriches the DNA relative to the contaminants (Hosono et al, 2003). MDA represents a dramatic technical improvement in the ability to amplify a whole genome compared to older PCR-based method that has drawbacks like inconsistent amplification (uneven sequence amplification) and short fragments (generally less than 1 kb). Unlike PCR, MDA is isothermal, requiring no heating or cooling steps.

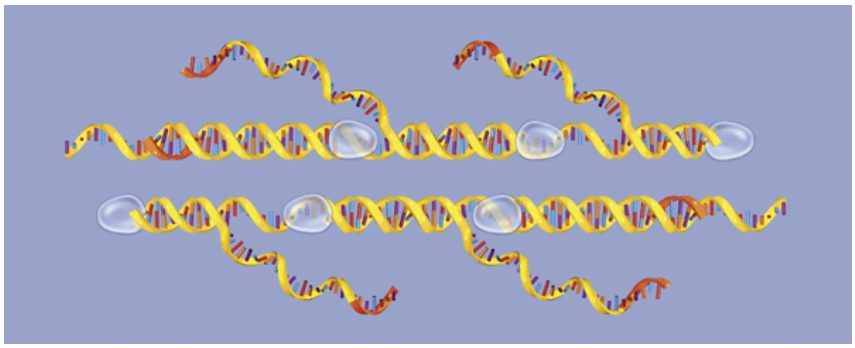


Figure 4. MDA in action. Highly processive DNA polymerase opens the DNA strand, primes, copies and recopies target DNA without the need to repeatedly denature the template as required by PCR. DNA from even a single cell can be amplified up to a million-fold in only a few hours. The "branched DNA" generated is in excess of 10kb in length. Adapted from Molecular Staging, 2003.

Database Expansion

"The most important factor affecting the success of a DNA databank is:
What the law allows you to do with it."

This citation comes from Dr. C. Asplen, a former Assistant United States Attorney and summarizes how in theory much more cases could be solved if the law would allow expansion of DNA database entries. Legislature around DNA typing differs greatly between

countries. As a result, some countries have larger DNA databases than other. For example, the United Kingdom has 2 million profiles in its DNA database. They allow DNA samples to be taken from all suspects that come into contact with police an also all convicted offenders have their profile entered in the database. As a result their crime solving rate is much higher than that of many other countries. This legislation results in a dramatic expansion of the number of profiles in the database which lead to the increase in the number of crimes solved. Some States in the USA have similar legislation (e.g. Virginia and Florida), with a correlating high rate of cases solved (Asplen, 2003). In the USA each state is free to decide which offenders to profile. Virginia decided to profile all felons and later added juvenile offenders of 14 years or older (Hollon, 2001). On November 13, 2002, the Division of Forensic Science in the Commonwealth of Virginia became the first state to mark 1000 DNA matches, or cold hits, from its DNA database. These 1000 cold hits are broken up into 109 homicides, 241 rapes, 12 rape/homicides, 9 malicious woundings, 14 carjackings, 57 robberies, 465 burglaries or larcenies, and more than 80 other crimes (Promega, 2002). Legally, cold hits can identify offenders in cases without an eyewitness or suspect. A cold hit can also match DNA from a number of crime scenes, linking multiple crimes to a single perpetrator. For more on cold hits, please see box 2 for a list of notorious cold hits. The dramatic success in solving old and new cases comes mainly from the observation that a large number of US felons on probation re-offend and therefore have their profiles in the system. As of 2003, 26 states require DNA sampling of all convicted felons. In the same year, Virginia began collecting DNA samples from anyone arrested for a violent felony, making it the first state to begin mandatory testing so early in the legal process. Last year, Texas began testing certain repeat offenders once they are indicted, a step just beyond arrest. Louisiana has similar arrestee legislation on the books, but staffing and financial concerns have kept the process on hold. Regardless of state laws, however, the national CODIS system currently only includes profiles of those convicted of a crime. The White House wants to open the database to a wider set of offenders (Irsay, 2003). DNA profiles from those who were found not guilty are naturally removed from the CODIS database. In the Netherlands suspects can be legally (and physically if necessary) forced to undergo DNA-testing if they are suspected of a crime for which preventative custody is allowed. For every DNA-test (and subsequent addition to the DNA-database) requested by the police, authorization is needed of a prosecutor or an investigating judge, who has to consider if the test is expected to contribute to the resolution of the case which is investigated. All DNA-profiles of suspects, crime-scene stains and deceased victims are added to the Dutch DNA-database and compared to all DNA-profiles already present. DNA-profiles of suspects only

remain in the DNA database if they are convicted. If not they have to be removed, just like in the American system. After conviction, DNA-profiles of crime-scene stains have to be removed from the DNA-database. The Dutch DNA-database is operated by the Netherlands Forensic Institute (NFI) FBI-CODIS-software is used to store and compare DNA-profiles. The program also includes stain-to-stain-hit information resulting in DNA-clusters: series of crime-scenes at which the same DNA-profile has been found. Up for discussion in the Dutch parliament in 2004 is a proposal for DNA-testing of all persons convicted of crimes for which the law allows a maximum imprisonment of 4 years or more, to create a so-called "convicted offender database". Because in the Netherlands DNA-testing is the responsibility of the judiciary, permanent exchange of DNA-profiles with other countries or Interpol is legally not possible. International comparison of Dutch DNA-profiles is only possible by means of an official international legal request The Dutch police region Limburg-Zuid, which is surrounded by Belgium and Germany, therefore cannot efficiently use DNA-testing as an investigative tool. Official international legal requests to compare all unidentified DNA-profiles from crime scene stains from the Dutch police region Limburg-Zuid, will be explored as an alternative for the permanent exchange of DNA profiles with Belgium and Germany (www.DNAsporen.nl). The ethical considerations at the basis of legislature on DNA databases will be discussed in chapter 5.

Catching up on backlog

Many forensic cases, especially in a country as large as the United States, get stuck in legal limbo because authorities have to wait weeks, or even months for DNA test results. The seemingly insurmountable backlog means re-offending criminals may continue to walk free (Miller, K.A., 2002). A report written in 2001 showed that the US has a backlog of more than a million unprocessed crime samples, including 100,000-plus unprocessed rape kits. Unfortunately, many states' DNA profile databases exist only as unfunded legislative mandates. It would take billions of dollars to get these samples processed and added to the DNA database in a timely manner (Hollon, 2001). Recently, the White House has promised $1 billion over five years to help bolster DNA analysis in the United States, including the enormous backlog of rape kits waiting to be tested (Irsay, 2003). In comparison, the Netherlands Forensic Institute (NFI) has a turnaround of approximately 3 weeks for samples of cases from high volume crime (HVC), thanks to an efficient standardized system. Regional police departments have a fixed number of samples they're allowed to submit for HVCs, accepted in only four forms; swabs containing blood (1), swabs containing saliva (2), cigarette butts (3), or chewing gum (4). More complicated cases that are not HVCs often

have similar turnaround times. Efforts are being made to analyze the multitude of evidence that has been stored in freezers from cold cases. This is evidence that has been collected before the DNA-era, and has already proven to be successful in solving a number of cold cases in the Netherlands (Kloosterman et al., 1997). Obviously, new faster and cheaper technologies would be of great benefit to all the unsolved cold cases.

Part II: NEW APPLICATIONS WITHIN FORENSIC BIOLOGY

MALTI-TOF mass spectrometry

Matrix-assisted laser ionization time-of-flight mass spectrometry (MALDI-TOF MS) has emerged as a rapid and efficient analytical tool in DNA sequencing and genotyping, but could also be applied to STR analysis. Compared with gel electrophoresis-based sequencing systems, MALDI-TOF MS produces high-resolution short DNA sequencing fragments of <100 base pairs (bp), rapid fragment separation on microsecond timescales and the complete elimination of the compressions that are associated with gel electrophoresis. Figure 5 illustrates the principle of MALDI-TOF mass spectrometry. In this method DNA sequencing fragments and matrix molecules (typically ultraviolet (UV) or infrared (IR) light-absorbing small organic molecules) are mixed in solution. They are then co-crystallized on a flat sample plate, which is subsequently loaded into the vacuum chamber of the mass spectrometer. DNA molecules are gently desorbed and ionized along with the matrix molecules by UV laser irradiation and the resulting charged ions are accelerated under a constant electric voltage, which causes them to fly towards the ion detector. The charged molecules arrive at the detector at different times on the basis of their masses: lighter molecules fly faster and therefore arrive at the detector sooner than heavier molecules. The masses of the charged ions are determined from their time of flight to the detector, which allows not only separation based on molecular weight of individual nucleotides, but also allows the different lengths of amplified STR fragments (Kim et al, 2003; Kloosterman, 2002)

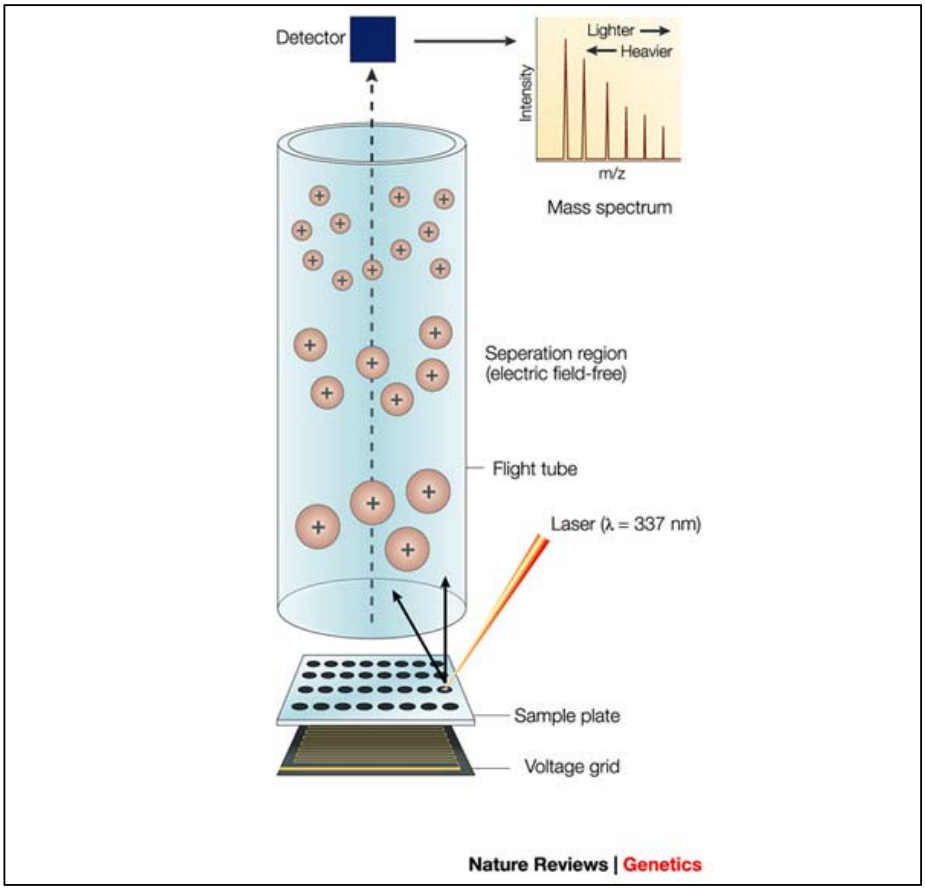


Figure 5 Principle of Matrix-assisted laser desorption/ ionization time-of-flight mass spectrometry (MALDI-TOF MS).
This technique is widely used for detecting the masses of high-molecular weight molecules such as DNA and proteins. In this method, DNA sequencing fragments) and matrix molecules (typically ultraviolet (UV) or infrared (IR) light-absorbing small organic molecules) are mixed in solution. They are then co-crystallized on a flat sample plate, which is loaded into the vacuum chamber of the mass spectrometer. DNA molecules are gently desorbed and ionized along with the matrix molecules by UV laser irradiation and the resulting charged ions are accelerated under a constant electric voltage, which causes them to fly towards the ion detector. The charged molecules arrive at the detector at different times on the basis of their masses: lighter molecules fly faster and therefore arrive at the detector sooner than heavier molecules. The masses of the charged ions are determined from their time of flight to the detector. m/z, mass per charge ratio Adapted from Kim et al., 2003.

Ethnicity markers

Large population studies sometimes empirically discover that certain (STR) markers are more distinctive to certain ethnic populations. For example, a recent study of 1056 individuals from 52 populations using hundreds of STR markers, found 5 genetic clusters that corresponded to major geographic regions. It was possible to pinpoint the ancestral continent of virtually every individual in the study from Africa, East Asia, Oceania and the Americas. However, a proportion of the marker-genotypes were found in people from several continents, suggesting that only a tiny fraction of genetic traits are distinctive to specific populations. This means that visible differences between human groups - such as skin colour and skull shape - result from differences in a very small proportion of genetic traits, making it a great challenge to pinpoint these polymorphisms (Rosenberg et al. 2002). The research company of the American Dr. Frudakis (DNAPrint genomics, Inc.) specializes in special kind of single nucleotide polymorphisms; the so called "ancestry informative markers" (AIMs). AIMs are genetic loci showing alleles with high frequency differences between populations. Mutations are the result of millions of years of genetic drift and random selection. Dr. Frudakis has pinpointed four major continental groups that can measure a persons ancestry by using a large set of SNPs throughout the genome. An estimate can be given of the ancestral proportions of that individual, the so-called admix ratio. For example, a person who has self reported himself as Puerto Rican had 62% European ancestry and 33% Native American. A self-reported Mexican American had 36% Native American, 28% East-Asian and 36% European as a result of his ancestry (Frudakis, 2003). Techniques such as these could aid in describing an unknown suspect, for instance by examining his admix ratio. The technology helped Louisiana police capture serial murderer Derek Todd Lee. Police there had been looking for a white man, until they learned the DNA was that of a black man. The company, DNAPrint genomics in Sarasota, Florida, says it has been used in about 30 cases so far nationwide. Two arrests have resulted from their work. Their DNA test has a 3.8 percent margin of error. As of January 2004, police have new information about the person who killed Susannah Chase in Boulder Colorado. The 23 year old student was found dead in December 1997 after being beaten with an aluminum bat, raped and then dumped in an alley. Until now, Boulder police had no witnesses, no leads and no description of the killer. One key piece of evidence was the DNA left behind by the killer. No matches turned in a national databank. Now, 6 years later, investigators may be one step closer. The DNA sample from the crime scene has been sent to DNAPrint Genomics with the result that the race of the suspect is most likely to be Hispanic or Native American (CBS4, 2004).

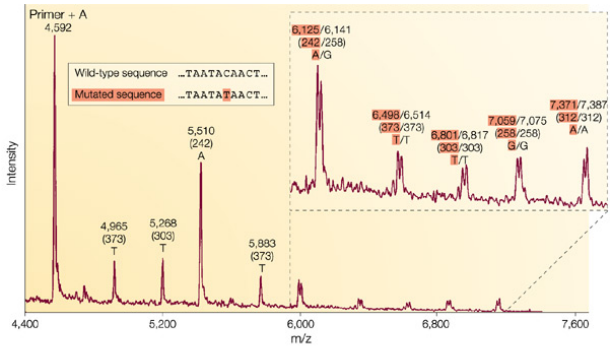
Markers for Commonplace Characteristics

In two countries it is already legally possible to use DNA investigation to help determine external personal characteristics of sex and race. As of September 2003 the British and Dutch judiciary and police are allowed to use visible external personal characteristics to aid in the search for unidentified perpetrators. Currently there are no "ready-made" kits for distinguishing hair, eye and skin color, but with the new bill that passed, usage thereof would be allowed under regulated circumstances, as they become available. Research groups in different countries, of which the Netherlands, are working on validating these specific markers (www.DNAsporen.nl). There is a research group in the United Kingdom working on the "red hair gene". Variation in human hair and skin color is caused by differences in the distribution, level and type of the pigment melanin which is produced in specialized cells known as melanocytes. Melanin exists in two forms: eumelanin which is brown/black in color and phaeomelanin which is red/yellow. Although human pigmentation shows a wide variety of phenotypes it is thought that thought to be under control of several loci, recent work has shown that in many cases the red hair phenotype can be associated with variants of a single gene, the melanocortin-1 receptor (MC1R). Twelve mutations on the MC1R gene have been found, of which eight are associated with the red hair phenotype. When a random group of individuals was tested for these mutations, 96% of the individuals with two red hair causing mutations had in fact red hair. Another finding of this study was that a certain type of mutation was found with high frequency in the Chinese population (Grimes et al., 2001). Likelihood ratios on the possible phenotypes can be estimated concordantly. Also, Dr. Frudakis' research company discussed earlier is developing markers for intraocular distance and iris pigmentation. The Florida based company DNAPrint Genomics has completed the screening of an additional 11,000 polymorphisms across the human genome to identify and validate a number of additional human iris color markers. From its screen of a total of 13,000 polymorphisms in over 1,200 samples, 40 markers from a number of human chromosomes (notably chromosomes 1 and 15) have been identified as associated with iris color in individuals of majority European BioGeographical Ancestry (BGA). Iris color classification was calculated at 97% considering a two-group classification scheme: dark and light (DNAPrint Genomics, 2003). According to the director of the company, Dr. Frudakis, he is already engaged in the next phase: to introduce a more specific eye color determination, like blue or brown, from a crime scene sample. His credo is that DNA is more credible than the subjective description by an eyewitness (Frudakis, 2003).

SNPs will make a comeback

Single nucleotide polymorphisms are highly abundant in the human genome, containing millions of SNPs, and are not only present in the non-coding stretches of the DNA such as STRs, but also in the coding regions of the DNA. The pharmaceutical industry uses these SNPs to identify differential drug related responses of patients, such as its differential adverse effects. The prediction is that SNP analysis will be become the major source of DNA markers in science and clinical genetics. A way of rather fast SNP analysis can be done by multiplex target sequence amplification, after fragments will be sequenced. A much faster way could be by simultaneously hybridizing the PCR fragments onto a microarray to detect large numbers of allele specific fragments. Also, MALTI-TOF MS offers fast and efficient sequencing of DNA in order to find SNPs. Figure 6 illustrates the application of MS for SNP analysis, an example of a SNP detected by differences in mass is shown. If forensic biology is going to follow this trend like it followed other scientific developments over the past decades, it would mean transitioning to a completely new system, and that old genotypes cannot readily be compared. A prerequisite is that there be international laws for uniformity of the newly formed database system. Advantages of SNPs over STRs in the forensic setting are numerous: (1) degraded DNA less of a problem if size of PCR fragments is designed to only be 50-60 base pairs, (2) equivalent PCR lengths prevent formation of preferentially amplified fragments (3) there is room for multiplexing (4) abundance of markers to create indefinite match probabilities (5) applicable to Y-chromosome polymorphisms and mtDNA. Box 3 illustrates the use of SNP analysis in helping identify victims of the September 11th victims of the World Trade Center terrorist attacks in New York City. .

Figure 6 MS spectrum from a DNA template that contains a SNP. Each peak is labeled with the absolute mass value that is unique for each nucleotide. At the polymorphic site, double peaks are detected each corresponding to a mutant (A) or wild type (G) allele. The downstream peaks also appear as a doublet, owing to the mass shift (16 daltons for A/G) that occurs at the polymorphic site. The inset shows a magnified view of the last 5 peaks. The signal of each DNA fragment is distinct and the heterozygote (A/G) can be easily recognized. Adapted from Kim et al, 2003



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Box 3. Identifying Those Remembered

INTRODUCTION

A total of 2,801 people were killed that day of September 11th 2001 when two hijacked commercial air busses loaded with enough fuel to fly from New York to Los Angeles flew into the Twin Towers of the World Trade Center in New York. The attacks presented a complex challenge for forensic DNA matching and handling, since human remains were crushed and co-mingled by the falling towers. With so many casualties, "spread out" in hundreds of pieces in millions of tons of debris it was a gigantic task to identify every piece of human remains so family could bury their loved ones. Relatively intact bodies could be identified by classical methods such as fingerprinting, dental records etc. Unfortunately, most of them depended on DNA based human identification. Not only were the victims in and around the building pulverized by the collapse of the buildings, the jet fuel caused the debris to burn for months, thus degrading most of the DNA. It was necessary to combine DNA typing results from STR and SNP with mitotyping. Also a record of all the donated reference samples had to be kept, not only reference samples from the reported missing (RM), but also from the living relatives. The New York City Office of Chief Medical Examiner (OCME) put together a team of forensic scientists, programmers, system engineers and many other who developed a program called M-FISys (pronounce: emphasis). This program is able to match profiles from different human remains gathered through different typing methods and to aggregate those samples, then knowing they all come from one owner. A program like CODIS would not suffice because too many hits would be obtained and they would have to be sorted manually.

STR ANALYSIS

The most widely used forms of DNA-based human identification involve STR analysis at 13-15 nuclear loci. Some samples are so severely burnt that full STR profiles will not be available for either direct or kinship analysis. When samples are badly compromised through harsh environmental degradation, many of the loci may be blank and the likelihood of observation can drop to levels where one would expect many instances of shared "partial profiles" in a population of 2,801 victims. An alternative technique is mitochondrial DNA analysis.

MtDNA TYPING

Mitochondrial typing involves direct sequencing of the highly variable regions of the genome adjacent to the origin of replication. The standard in forensic communities is to report results a compact format that only shows the difference between the experimental results and an established and internationally recognized standard known as *The Anderson Sequence*g. If the sequence being typed is identical to the Anderson reference, the difference report will be null. Point mutations are described as a base position, plus the base that differs from the reference sequence. Deletions are represented as a "D" character (not to be confused with the IUB code for "A, G or T, but not C"). This presentation is called the "delta representation." A typical mitotype might look like this:

16093: C or 16224: D or 6311: C or 195: C or 263: G or 315.1: C

The first lines indicate that this sample has a C at position 16093 where a T is usually found, and a deletion of the base at position 16224. To maintain the integrity of numbering, an insert is indicated as a decimal point position on the base that the insert follows. In this case, "315.1: C" indicated that there is a C insert after position 315 in the reference sequence.

SNP TECHNOLOGY

Even though SNP technology is still under development at Orchid Cellmark, the OCME asked if the technology could be used on WTC specimens. Orchid developed a technique to examine mere 100-base DNA fragments, rather than the standard 100- to 400-base lengths, which helped with such degraded samples. A panel of 71 SNPs provides more powerful identification on average than a full profile of 13 STR loci. Many samples recovered at the WTC gave a partial STR profile, but typically four to five markers are not enough to make identification. If you couple a partial STR profile with 20 to 30 SNP markers, identification is feasible. So far, Orchid has tested 2,500 tissue specimens and continues to study the remaining samples. The SNP data are sent to Gene Codes to be incorporated into M-FISys so OCME analysts can plug gaps in the STR profiles if necessary.

BONE TISSUE

Tissue such as bone is difficult for several reasons. One issue is that the dust created when a sample is extracted must not contaminate the next sample from the same bench. More significantly, calcium is a PCR inhibitor. Therefore, in many cases bone samples were sent to a specialized laboratory to perform that analysis.

STATISTICS

The likelihood value for a given profile will differ depending on ethnic population; however, access to that information is not always available or reliable where badly damaged remains are involved. The "worst case scenario" was used, taking the likelihood values across four races (Asian, Black, Caucasian & Hispanic) and take the lowest value as the final likelihood. By using this lowest value, false positives were prevented.

CONCLUSION

The result of this great collaborative effort is that slightly more than half of the 2,801 victims have been identified, but it remains an ongoing process.

Sources:

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Chapter 5 DISCUSSION

In this paper various techniques used for human identification have been presented. From the multilocus probes developed by Alec Jeffreys in 1985, to the currently used multiplex STR markers. This paper also reviewed future developments, of which SNPs are the most promising technological change, especially in combination with the ultra fast sequencing method of MALTI-TOF MS. However, switching to a different marker system means that the current information is no longer useful. If you can only analyze your minute amount of DNA once, then you have to choose one or the other. A long buffer-period in which both methods will be used side-by-side will be necessary to overcome this gigantic problem. Therefore, the most likely improvements we can expect in the near future are simply faster and cheaper ways to do STR profiling. This can be done by using mini-thermal cyclers and micro-chip based mini-electrophoresis devices.

The amount of DNA needed to create a reliable profile has dropped dramatically from 50-500 ng to 0.1-1 ng DNA and the random match probabilities have increased to chances of one in a billion when a minimum of 8 loci are used. Sufficient profiles can often be created despite mixed samples or samples with slightly degraded DNA. This spectacular increase in sensitivity requires increased caution when collecting specimens, the slightest contamination with DNA from a different source can have serious consequences. Especially in cases with LCN DNA-evidence, one must be aware of the possibility of secondary transfer (e.g. the true perpetrator and the innocent suspect had some form of physical contact, or both touched the same item). In these cases the combinations of all other DNA and non-DNA evidence have to be evaluated to create a better picture. These great developments have also raised new issues, such as the ethical aspects concerning creating (inter-) national DNA intelligence databases. Naturally, DNA-use is accompanied by ethical issues regarding privacy legislation.

A law adopted in the Netherlands per 2001 states that suspects can no longer refuse to donate a DNA sample if this may potentially link him to a specified crime. In contrast, the state of Virginia expanded its DNA testing law in January 2003: samples for DNA testing are now taken whenever a person is arrested and charged with a violent felony or a burglary. The samples are then entered into a national law enforcement database. Before the law was changed in January, DNA samples could only be taken after a conviction, including all felons and juvenile delinquents over 14 years of age. Some legal experts think the new law can streamline the legal process, by exonerating people through the legal process very quickly. If

the DNA doesn't match the suspect can go home, if it does give some clue, then keep him in the loop. The new law in Virginia benefits investigators but is not a silver bullet for solving all crimes. For instance just because someone's DNA is present at the crime scene doesn't mean that person committed a crime. More often than not that person is guilty, but the whole range of evidence will be necessary to convict the suspect. If the individual is acquitted in court, or the charges are dismissed, any DNA samples taken in accordance with the law must be destroyed and records deleted from the database. The court system must find the right balance between the protection of civil liberties and the protection of society. Some would argue that if a DNA sample be taken from every individual, there would be no more unsolved crimes, which is hypothetically true. However, like said before, DNA could be present through secondary transfer, and what about crimes in public places where hundreds of people have left their genetic material behind. It would simply give the investigators more information they could handle, not to mention the insurmountable amount of work and financing it requires. Creating a convicted offender database is very useful since many criminals re-offend: a lot of criminals are "professional" like high volume thieves or mentally ill, like serial rapists etc. Unfortunately The Netherlands does not have a convicted offender database yet. It will be proposed in (the Dutch) parliament in 2004. The other extreme is the United Kingdom, where every arrestee donates a DNA sample that stays on file. It will be interesting to see if besides the enormous crime solving success rate, the total number of crimes will drop, as if it would scare off anyone to re-offend and work as a preventive manner.

Another "privacy" issue arises when from the crime-scene and the suspects' reference sample match very closely, but is still a mismatch, but the sample is indicating relatedness to the "real" suspect. It would be ignorant to ignore such valuable information and not to use this as a clue for further investigation. Then again, is using such information a violation of the privacy law? A set of rules to determining the odds for relatedness has been proposed to answer this question (Sjerps, 1999). Another consideration can be thought of in the event of pursuing SNPs in forensic biology. Whereas the pharmaceutical industry studies the human genome in search of disease-related SNPs, in forensics the opposite is desired; finding SNPs in the noncoding regions of the DNA. If this is not pursued careful enough, there may be a possibility of creating informative SNPs that harbor medical information, something that has major ethical implications.

Another place where there is room for improvement concerns the DNA-kits supplied to police departments. If the officer at the crime-scene is adequately trained in how and where to look for DNA evidence, and submits his samples promptly, a lot more crimes would be solved. Especially now that for instance a profile can be created from even a bit of saliva on e.g. an assault victim, like the case described in box 2, of Mia Zapata. One does not *see* the saliva, but with proper reconstruction of the crime, the possibility of the perpetrators DNA on the crime scene must be assumed so that the power of DNA can be used to the fullest. Crime solving is one of the most important assets of DNA, yet preventing another (violent) crime is just as valuable of an asset of DNA, only made possible with the appropriate legislation.

Forensic biology is the fruit of several disciplines such as molecular biology, epidemiology and jurisdiction, but most importantly, it is the result of great efforts made by molecular geneticists over the last 50 years. Human identification based on DNA typing has proven to be invaluable to modern life in societies with high crime rates, enduring events such as mass tragedies. In addition to helping identify suspects of crimes, DNA evidence has also been used in numerable cases to prove an accused, or even convicted person's innocence.

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- Box 1 Example A and B: http://arbl.cvmbs.colostate.edu/hbooks/genetics/medgen/dnatesting/dnatest_tech.html
- Box 1 Example C: Chow-Shaffer, E. ,Sina B., Hawley W.A. De Benedicts J., Scott T.W. Laboratory and Field Evaluation of Polymerase Chain Reaction-Based Forensic DNA Profiling for Use in Identification of Human Blood Meal Sources of*Aedes aegypti*(Diptera: Culicidae) *Journal of Medical Entomology* 37 (4) 492–502 (2000)
- Box 1 Example D: http://www.cstl.nist.gov/biotech/strbase/
- Figure 4: www.molecularstaging.com

AUTHOR'S ENDNOTE

My interest in forensic biology started somewhere in 1998 when I choose to interview Dr. P. de Knijff for an assignment in my freshman year of Biology in Groningen (The Netherlands). Then, after two more years of my biology studies, I was offered an internship at the Department of Human Genetics at the Academic Medical Center (AMC) in Amsterdam, where I worked for 6 months. There, the terms Southern blotting, PCR and DNA sequencing really came to life, as they were my daily duties. Then I was fortunate to come to work for Dr. David Heber in Los Angeles to revive a dormant project, and two years later, I am still there. It was there actually that I got my fascination back for forensic sciences. Not only is Los Angeles the city of O.J. Simpson and Kobe Bryant, there is also a desperate need for better forensic DNA facilities in the city itself. Then one day a Qiagen rep presents our group a piece of equipment that has fully automated the DNA isolation process. Then after I ask if these are used currently in DNA crime labs I end up with a contact at the San Diego Police Department. One phone call later a criminalist from the SDPD, Mr. S. Montpetit gives me a very illustrative tour through the SDPD's crime lab. At that point, midway August 2003 I knew for sure that I would want to finish my studies with a paper about this fascinating field.

Some may not know that this paper was written under somewhat unique circumstances. The writing commenced in The Netherlands and halfway through, it was continued in The United States of America. It was my wish to obtain most pivotal information from my supervisor Dr. Kloosterman and his colleagues in The Netherlands and then to finish the project in The USA, which is currently the place where I live and work. I look back on a very successful time in The Netherlands, where I obtained full co-operation from the Netherlands Forensic Institute (NFI) and the University of Amsterdam for this project. Also, my employer in the USA (UCLA) supported my wish to start this project even though it meant leaving my job for quite a while. I was fortunate to have the chance to attend a National DNA symposium, a place where the forensic community gathered to exchange thoughts and scientific knowledge.

I would like to credit the following persons for their support and guidance: First of all I want to thank my supervisor Dr. A.D. Kloosterman (NFI) for his warm welcome into the world of forensic biology and for the critical feedback as this paper progressed. I would also like to mention the contribution of Dr. W.de Leeuw (head of R&D, NFI), Dr. H. Hardy (NFI), Mr. M. Hoenstok (documentalist NFI). I must also thank my employer and supervisor in the USA,

Dr. David Heber and Dr. Diane Harris for their support of my studies. Also I would like to thank Dr. Hans van der Spek (UvA) for being my examinator. Last but not least my friend Tim Sjollema for creating the picture on the front cover. I'm very thankful for everyone's co-operation, especially because of my unique situation, where I live in two countries and sometimes feel very remote from home due to the busy (American) life that I choose to live.

Emily Besselink,
Los Angeles, California 2003