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POINT OF VIEW

# CRACKING THE CODE: CAN FORENSIC GENETICS DISTINGUISH IDENTICAL TWINS? A TECHNICAL PERSPECTIVE

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## ABSTRACT

DNA fingerprinting has revolutionized the field of forensic investigation in recent times. The utility of DNA fingerprinting technology in differentiating between monozygotic twins has always been an area of academic interest for researchers. While traditional chiral fingerprinting effectively differentiates monozygotic twins, situations may arise where the absence of conventional fingerprints at a crime scene necessitates DNA analysis for conclusive identification. Although Short Tandem Repeats (STR) profiling is of not much help in that case, monozygotic twins can still be differentiated using current advancements in the field of forensic genetics. The methods that can help discriminate between identical twins are mitochondrial genome (mtGenome) analysis, Single Nucleotide Polymorphism (SNP) profiling, Epigenetic profiling (DNA methylation profiling), Copy Number Variants (CNV) profiling, and studying Single Nucleotide Variants (SNV) in the genetic material by traditional sequencing methods like sanger sequencing or advanced methods like Whole Genome Sequencing (WGS). In conclusion, these alternative methods may be able to distinguish between monozygotic twins, but they also have drawbacks that must be considered when using them in real-world crime investigations. To establish their accuracy, dependability, and application in forensic situations, more study, validation, and standardization are required. A combination strategy will strengthen the case and lessen the fallacy of individual profiling methods.

**Keywords:** *Copy number variants; mitochondrial DNA; monozygotic identical twins; single nucleotide polymorphism; single nucleotide variants.*

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## INTRODUCTION

Identification is one of the cardinal objectives to be fulfilled in day-to-day forensic casework as and when a need arises. 'Dactylography' or fingerprint evidence remains a cornerstone of forensic practice due to its unique and reliable nature for individual identification. Across the globe, within the context of distinguishing monozygotic twins, forensic medicine experts often highlight the practicality and cost-effectiveness of traditional dactylography, while occasionally not fully exploring the capabilities of modern approaches like DNA fingerprinting. Despite the ease and familiarity of traditional methods, it is important to recognize the potential of advanced techniques for medico-legal identification in cases involving monozygotic twin differentiation. These techniques are of immense help when only trace evidence is recovered at the crime scene and traditional fingerprints are not recovered.

The history of crime is replete with infamous cases involving identical twins that have been documented both in the pre-DNA and post-DNA eras. The use of DNA evidence in criminal investigations marked a revolutionary breakthrough in forensic science[1]. In 1986, in the landmark Enderby murders case, geneticist Sir Alec Jeffreys

employed DNA fingerprinting to link a suspect to the crime scenes, establishing an unprecedented level of certainty in identification. In the modern era of DNA technology, instances like that of Hassan and Abbas O., accused of a bank robbery in Germany, have spotlighted the complexities of distinguishing between identical twins in criminal investigations. Being familiar with current trends in Monozygotic Twin (MZ) discrimination from a forensic angle is advisable due to its potential relevance in our practice.

Traditionally, STR (Short Tandem Repeat) DNA profiling is a widely employed method for identifying individuals based on their distinct genetic makeup. STR sequencing involves profiling multiple polymorphic variations within different individuals, specifically DNA fragments ranging in size from 10 to 500 base pairs (bp) that belong to the microsatellite region of the genome. The Taq Polymerase enzyme is utilized for multiplex PCR in this profiling technique, generating unique genetic profiles distinguished by varying repeat numbers (differences in the number of repeated DNA sequences), thereby ensuring a prominent level of identification precision. This method is characterized by its rapid execution and robust discriminatory capability.

Nevertheless, the STR method has limitations when it comes to identifying identical twins, who share the same DNA. Identical twins, also known as monozygotic twins, originate from a single fertilized egg that splits into two embryos, resulting in individuals with nearly identical genetic profiles. As a result, traditional STR DNA profiling, which relies on identifying variations in repetitive DNA sequences in the microsatellite region, fails to distinguish between the DNA of monozygotic (MZ) twins[2]. However, the following techniques have been used with some success in this area. Technically speaking mitochondrial genome (mtgenome) profiling, epigenetic profiling, single nucleotide variant (SNV) profiling, and copy number variant (CNV) profiling methods offer discrimination capabilities that can overcome the limitations of STR profiling in differentiating monozygotic twins.
The mitochondrial genome, inherited solely from the mother, can exhibit sequence variations due to random segregation during early development, resulting in divergent mtDNA profiles. Epigenetic marks like DNA methylation can vary due to environmental influences and stochastic processes, accumulating differences over time even in monozygotic twins. SNV profiling identifies rare genetic variants acquired through somatic mutations or environmental exposures, while CNV profiling detects structural genetic differences, allowing for comprehensive genome-wide discrimination that STR profiling, focused on specific repetitive regions, may not achieve.

**Mitochondrial DNA (mtDNA) Profiling**

Mitochondrial DNA (mtDNA) profiling is one alternative approach to discriminate between identical twins. Mitochondria are cellular organelles that contain their DNA, separate from the nuclear DNA found in the cell's nucleus. Unlike nuclear DNA, which is inherited from both parents, mtDNA is matrilineal in origin. MtDNA can potentially be used to differentiate between identical twins as their mtDNA can differ due to random mutations over time, known as mtDNA polymorphisms. The mitochondrial DNA variations compared to the nuclear DNA variations are a potential marker owing to higher mutation rates. So, studying the variation due to random segregation of mtDNA during early development or accumulated random mutations in mtDNA over time may be used for differentiating monozygotic twins with some success. The mtDNA can be sequenced using the traditional Sanger sequencing, which is easier, cost-effective, and with less turnaround time. However, much more advanced methods like massively parallel sequencing (Next Generation Sequencing) may be attempted for research purposes if feasible as has been done in some centers[3]. Moreover, it has been demonstrated that ultra-deep mtGenome sequencing could be used to differentiate between MZ twins[4]. Nevertheless, it is essential to note that mtDNA profiling's comparative discriminatory power is somewhat limited in contrast to routine nuclear DNA profiling and is never used alone as a tool for differentiation between monozygotic twins. This stems from the lower diversity of mtDNA and its distinct slow evolutionary pattern, which, although useful, warrants careful consideration during application.

**Single Nucleotide Polymorphism (SNP) Profiling**

SNP profiling is another approach that can provide greater discrimination between individuals, including identical twins. SNPs are variations in single nucleotides (building blocks of DNA) that occur in the genome. SNP profiling can be used to identify unique genetic variations between identical twins, as well as other individuals, by examining specific SNPs in their DNA. Some rare mutations may occur as soon as the human blastocyst splits into two (the origin of twins) and such mutations pass on into the somatic tissue and germlines. Hence, traditional genetic sequencing and Whole Genome

sequencing (WGS) to identify SNPs are good methods to discern between identical twins[5]. In one real-world case involving MZ twins, the use of SNP profiling from mtDNA has helped in identifying the perpetrator[6]. MZ twins were differentiated using WGS, allele-specific PCR, and deep-amplicon sequencing (a targeted high throughput DNA sequencing method, that involves PCR amplification and next-generation sequencing of specific regions). Mitochondrial DNA, due to its unique heterogeneous SNPs (both common SNPs and rare/private SNPs), proved superior in discerning twins, aiding in criminal identification and exoneration across cases. A major limitation of SNP profiling includes higher costs and more complex analysis methods, which may cause some difficulties (owing to the extreme similarity of the SNPs between all individuals including MZ twins) in its practical application in forensic cases. Additionally, the presence of certain commonly occurring SNPs may limit their discriminatory power for identifying unique genetic differences between identical twins and is never supposed to be used in isolation. This method also complements other methods in differentiating between MZ twins.

**Epigenetic Profiling (DNA Methylation Profiling)**

Epigenetic profiling is an emerging technique that holds promise for identifying identical twins. Epigenetics refers to changes in gene activity that are not caused by changes in the DNA sequence itself, but rather by modifications to the DNA molecule or to the proteins with which DNA interacts. Epigenetic profiling can provide information about an individual's unique epigenetic signature, which can differ between identical twins due to various environmental factors.

Many differentially methylated regions (tDMRs) with varying amounts of methylation in different cell types and tissues have been found through genome-wide methylation studies employing high throughput DNA technologies which can aid in differentiating between individuals including MZ twins[7,8]. The magnitude of the intra-pair or longitudinal methylation discordance of the CpG sites inside the CpG islands is more than those outside the CpG islands having the potential to discriminate MZ twins. CpG islands are regions of DNA where a cytosine (C) nucleotide is followed by a guanine (G) nucleotide in the linear sequence of the DNA strand, and they are often associated with gene regulatory regions. The "p" in CpG stands for the phosphate group that links the two nucleotides[9]. LINE 1 DNA methylation was also suggested to be a potential marker for discriminating MZ twins[10]. Interestingly, stochastic processes cause variations in methylomes which occur post-twinning during embryonic development and later life that can become a basis for discrimination. A new epigenetic fingerprinting method was also developed based on stochastic methylation variation[11]. However, the role of whole genome sequencing in this regard and its limitations have been described in detail here[12].
The limitations of epigenetic profiling include the ongoing establishment of its reliability and accuracy for forensic discrimination of MZ twins, as well as challenges in the standardization and interpretation of results for the same. The complexity and variability of epigenetic marks may affect accuracy and reproducibility, and further research is needed to determine their practical applicability in differentiating MZ twins. While several stable epigenetic variations have shown promise for applications in identification, it's crucial to recognize that epigenetics is a highly dynamic field within molecular biology, and it is extremely difficult to standardize it as an identification tool. Moreover, the evidence derived from epigenetic profiling should not only be indicative but must also possess probative value, meaning that it should provide substantial and conclusive support for identification purposes, which is still elusive.

**Copy Number Variation (CNV) Profiling**

CNV profiling is another approach that can potentially identify differences in CNVs between MZ twins. CNVs are structural changes in DNA that involve the deletion or duplication of DNA segments, resulting in changes in the number of copies of certain DNA regions. CNVs can occur naturally in the human genome and can vary between individuals, including identical twins. Analysing CNV profiles using techniques like array comparative genomic hybridization (aCGH), quantitative polymerase chain reaction (qPCR), or next-generation sequencing (NGS) can potentially identify differences in CNVs between MZ twins, allowing for discrimination due to genetic differences arising from early embryonic development or environmental factors. The potential application of CNVs in the field of forensic medicine has been explained in detail here[13].

The limitations of CNV profiling techniques include potential issues with accuracy and reliability due to sample quality, technique used, and presence of technical artifacts. CNVs can also occur naturally in the genome and may not always indicate genetic differences between identical twins, yet the variability shown between MZ twins can be of use for differentiating. These limitations should be

considered in forensic applications of CNV profiling for MZ twin differentiation.

This research question of distinguishing MZ twins has been given adequate importance by the scientific community across the globe. Some interesting methods like using Immune Repertoire (IR) as a potential biological marker for this purpose have been suggested[14]. Similarly, the differences in microRNA expressions between individuals can also be used to solve this question according to a published paper[15]. One can also explore the possibilities of metabolome and proteome level variations which can be useful to solve this question. Some researchers have proposed studying Single Nucleotide Variations (SNVs) for discriminating between MZ twins[16]. SNPs are a subset of SNVs that are commonly used as markers in population genetics and can be stably inherited by the offspring. Whereas, SNVs encompass a broader range of genetic changes, including any single-letter change in DNA, regardless of its frequency or relevance to a population. SNVs can include both common and rare variations and can result from various mechanisms and not solely by inheritance. They may or may not have functional consequences and cover a wider spectrum of genetic diversity beyond the specific characteristics of SNPs. However, not all SNVs are usable for the proposed purpose of differentiating MZ twins. SNVs can be either germline mutations, which are inherited from parents and present in every cell of an individual's body, or somatic mutations, which occur after fertilization and are only present in specific cells or tissues. Hence one must be cautious in choosing an SNP/SNV for identification purposes bearing in mind the common nature of presence in the population and the frequency of incidence. The variations which occur after the blastocyst formation are of much use for our purpose.

All these complex methods can be done after the successful extraction of DNA from the autopsy or clinical medicolegal samples in forensic casework apart from trace evidence collected at the crime scene. Nowadays, DNA extraction process is routinely conducted using kit-based methods and is easy to do with less training. However, each molecular genetics laboratory has its own customized protocol for further analysis of extracted DNA (sequencing and identification), which is the domain of a forensic molecular geneticist[17-22]. It is agreed that identification is police business and DNA casework is done in forensic science laboratories by scientists in most parts of the Eastern world, but as a forensic pathologist, one has to be aware of the new developments in the field of forensic genetics/ DNA profiling methods.

In summary, while these alternative techniques may have the potential for discriminating between MZ twins apart from the traditional chiral fingerprinting, they also have limitations that need to be considered in their practical application for forensic purposes. Further research, validation, and standardization are needed to establish their accuracy, reliability, and applicability in forensic settings. A combined approach will increase the weight of evidence and reduce the fallacy of individual profiling techniques. It would be of significant help to the scientific community if a consortium of forensic genetics experts came up with a workable standard operating procedure to be followed in cases involving discrimination between MZ twins by using a combination of all these methods. There is also a need to assess the costs involved in the application of several new technologists to solve this question *vis a vis* other method to solve the crime.

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The author declared no conflicts of interest.

## ETHICAL ISSUES

Not applicable.

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## AUTHOR CONTRIBUTIONS

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