

Role of Mitochondrial DNA in Identification

by

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Abstract

The molecular marker that has revolutionized the discipline of human identification is the Mitochondrial DNA (mtDNA), which has revolutionarily redefined the sunset in the fields of forensic science, anthropological studies and genealogies in the last three decades. This overall overview is looking at the versatile use of the simple use of the method in identification processes with the current understanding of the methodology frameworks, technology development and their practical applications. The unique properties of the mitochondrial DNA, such as a large number of copies per cell (100-1,000 copies when compared to two copies of the nuclear DNA), strict patterns of maternal inheritance, high resistance to environmental disintegration and the circular structure of the genome makes the use of the mitochondrial DNA to be invaluable in analyzing degraded, old, or restricted biological samples in which the more conventional nuclear DNA technology would be inadequate.

The systematic review of the literature is based on an assessment of the efficacy of the various type of analyses of the mtDNA technique in various tasks of identifying crime, including forensic casework at the crime scene with damaged evidence, cold case investigations, victim identification after a mass disaster, and anthropological studies. Historical development since early 1990s methodologies up to modern Massively Parallel Sequencing technology is factored into the analysis of the development of quality assurance standards, statistical interpretation models, and population genetic issues that are a requirement to quality evaluation of evidence.

Comparative analysis shows that although the use of the program has proven itself to be very useful in problematic sample conditions and adult line following, it has lower discriminatory capability

than nuclear DNA markers and is unable to distinguish individuals that share common maternal genetic groups. The review focuses on such fundamental technical problems as contamination prevention, heteroplasmy interpretation, mixture analysis, and heterocellularity of the nuclear mitochondrial DNA segments. Statistical models on computing probability of matches, likelihood ratios and information on estimating a confidence interval is identified together with international standards of quality assurance on laboratory accreditation, staff qualification and on protocols of the validation of the analytical procedures.

The results emphasize the point that the properly used and carefully regarded quality-control doctor-level products of the mtDNA analysis can be used with superior identification features as a complement to the nuclear DNA techniques. The focus in future will be on technology integration, the overall expansion of databases, greater standardization, and multi-marker methods that incorporate both the nuclear coupled with the sexual chromosomal markers and markers of ancestry to maximize the accuracy of the identification in all the forensic, anthropological and archaeological.

Keywords: mitochondrial DNA identification, forensic genetics, maternal inheritance, degraded sample analysis, mtDNA sequencing, quality assurance standards, population genetics, mass disaster identification, molecular marker discrimination

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Table of Contents

Abstract.....	iii
Acknowledgement	v
Table of Contents	vi
List of Figures.....	x
Chapter 1: Introduction.....	1
1.1 Objectives of the Review.....	7
1.1.1 Primary Objectives	8
1.2 Scope and Purpose	8
1.2.1 Purpose of the Review.....	8
1.2.2 Scope of the Review	10
Chapter 2: Literature Review.....	15
2.1 Historical Development and Technological Evolution.....	15
2.2 Methodological Approaches and Technical Considerations	18
2.3 Applications in Forensic Science and Legal Contexts.....	21
2.4 Population Genetics And Statistical Interpretation	24
Chapter 3: Role and Application of Mitochondrial DNA in Identification.....	29

3.1 Characteristics of mtDNA Relevant to Identification	29
3.2 Maternal Inheritance and Lineage Tracking	32
3.3 mtDNA in Forensic Casework	35
3.4 Use of mtDNA in Anthropological and Archaeological Contexts	38
3.5 Advantages and Limitations	41
3.6 Case Studies and Real-World Applications	43
Chapter 4: Theoretical and Mathematical Framework.....	49
4.1 Models of mtDNA Mutation and Inheritance.....	49
4.2 Probability of Match Calculations.....	51
4.3 Statistical Measures in mtDNA Analysis	53
4.4 Equations Used in Identification Accuracy Estimation	55
Chapter 5: Comparative Analysis of mtDNA and Other Genetic Markers in Identification	65
5.1 Nuclear DNA STRs versus Mitochondrial DNA.....	66
5.2 Y-Chromosomal Markers and Paternal Lineage Tracking.....	67
5.3 Ancestry Informative Markers and Population Assignment	68
5.4 Single Nucleotide Polymorphisms (SNPs) in Identification.....	69

5.5 Degradation Resistance and Sample Suitability.....	70
5.6 Statistical Interpretation and Database Requirements.....	71
5.7 Future Directions and Technological Integration.....	72
Chapter 6: Quality Assurance, Standards, and Best Practices in mtDNA Analysis.....	73
6.2 International Standards and Guidelines	74
6.3 Laboratory Accreditation and Certification	76
6.4 Personnel Qualifications and Training	77
6.5 Facility Design and Environmental Controls	79
6.6 Equipment Validation and Maintenance	80
6.7 Analytical Validation and Method Development.....	81
6.8 Quality Control Procedures	83
6.9 Proficiency Testing Programs.....	84
6.10 Documentation and Record Management	85
6.11 Best Practices in Sample Handling and Processing.....	87
Conclusion	89
Fundamental Strengths and Unique Contributions.....	89

Significant Methodological Limitations	90
Technical and Analytical Challenges	91
Statistical and Interpretive Framework Concerns	92
Quality Assurance and Standardization Issues	92
Future Directions and Technological Integration.....	93
Critical Assessment and Recommendations.....	94
Reference list.....	95

List of Figures

Figure 1 Mitochondrial DNA (Wikipedia Contributors, 2019)	2
Figure 2 The human mitochondrial DNA genome with genes and control regions (Sahayasheela et al., 2022)	14
Figure 3 Mitochondria as the central environmental sensor (2016)	18
Figure 4 The two main types of selection, Negative and Positive, and their effects on patterns of mitochondrial genetic variability. (A) Different types of selection (negative or positive) affect the pattern of mitochondrial mutations in response to different environments (lower panels) or will undergo a selective sweep (negative selection to maintain function (upper panel). (B) The different levels in which selection may act on the mitochondria, i.e., (down-up) the single mitochondrion, cells and the entire organism. (Shtolz and Mishmar, 2019).....	21
Figure 5 Germline selection of human mitochondrial DNA is shaped by the nuclear genome. (Wei et al., 2019)	24
Figure 6 Nuclear vs. Mitochondrial DNA Inheritance Paths (TESTE DE ANCESTRALIDADE COMO FERRAMENTA GENEALÓGICA PARA HISTÓRIA DE FAMÍLIA, n.d.).....	28
Figure 7 Comparison of nuclear and mitochondrial genomes with mtDNA function. (Antón Vilasanzurjo et al., 2023).....	32

List of Tables

Table 1 Comparison of mtDNA and Nuclear DNA Characteristics for Identification	30
Table 2 Maternal Relationship Types and mtDNA Sharing Expectations	33
Table 3 Forensic Evidence Types and mtDNA Analysis Success Rates.....	36
Table 4 Archaeological mtDNA Applications and Time Periods.....	39
Table 5 Advantages and Limitations of mtDNA Analysis	41
Table 6 Notable mtDNA Identification Cases and Outcomes.....	44

Chapter 1: Introduction

Mitochondrial DNA (mtDNA), since its exploitation started, has become one of the strongest and most used molecular markers in the identification of human beings, transforming the practice of forensic science, human anthropology and genealogy within the last few decades. In contrast to nuclear DNA, which is passed down through both parents, and recombines, the maternally inherited mtDNA is present in greater numbers per cell (Amorim, Fernandes and Taveira, 2019), it is (relatively) constant throughout generations and thus makes an invaluable tool of identification in numerous scientific and legal applications.

Specific features of mitochondrial DNA are explained by its place of growth and evolution. Mitochondria, sometimes called the powerhouses of the cell, have their own separate circular and double-stranded DNA, unique in comparison to nuclear chromosomal DNA (Amorim, Fernandes and Taveira, 2019). The focus was made on the fact that every human cell carries between hundreds and thousands of mitochondria, each mitochondrion contains numerous copies of the mitochondrial genome and thus there are around 500 to 2,000 copies of the mitochondrial DNA per cell with only two copies of nuclear DNA. Such a high copy number has the major benefit in forensic work especially in cases where the biological material is deteriorated, old or low sample as nuclear DNA is underrepresented or entirely absent (Amorim, Fernandes and Taveira, 2019).

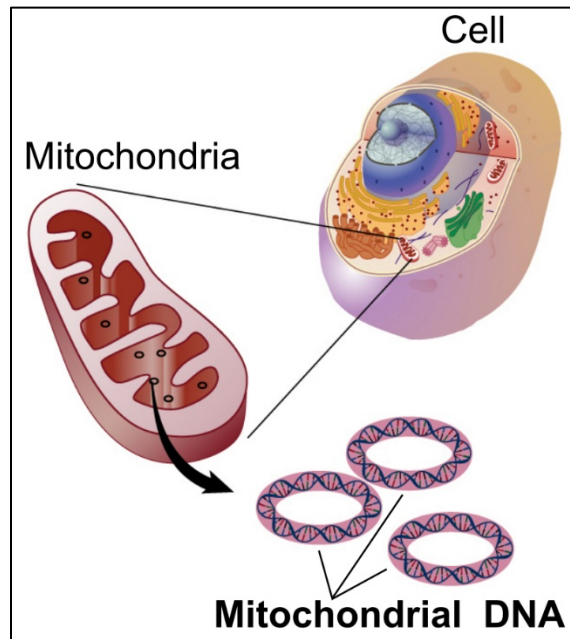


Figure 1 Mitochondrial DNA (Wikipedia Contributors, 2019)

The human mitochondrial has 16,569 base pairs with 37 genes where 13 protein- coding genes, 22 transfer RNA and 2 ribosomal RNA genes are included. To identify it, researchers majorly concentrate on a sufficient region of interest called the control region, or alternatively the displacement loop or D-loop, a length of around 1, 122 base pairs with the most sequence changes in the whole mitochondrion genome (Amorim, Fernandes and Taveira, 2019). There exists further subdivision in this region that will give hypervariable regions I and II (HVI and HVII) that have highest polymorphism and therefore the most discriminatory power in identification of individuals.

Mitochondrial DNA in human identification came to the limelight in the 1990s due to advancement in the techniques of polymerase chain reaction amplification of DNA and DNA sequencing techniques (Chinnery and Turnbull, 1999). The analysis of the remains of the Vietnam War and the Romanov family were early landmark cases which showcased the extraordinary

usefulness of mtDNA analysis when more conventional mediums of identification were inadequate. These breakthroughs placed the mitochondrial DNA to become an instrument that could not be ignored in any forensic laboratory in the world, and it initiated the process of being incorporated in everyday casework (Chinnery and Turnbull, 1999).

Among the greatest strengths of mitochondrial DNA in identification is that compared to nuclear DNA, it is resistant to degradation. The circular nature of the mtDNA and the keeping the structure in the safe shield of the protective mitochondrial matrix ensure its stability and preservation in harsh environmental circumstances (Chinnery and Turnbull, 1999). This attribute is especially useful in the examination of skeletal remains, hair shafts, teeth and other biological samples with the extreme hot temperatures and levels of humidity and exposure to chemicals or long-time decomposition. Mitochondrial DNA has frequently been used to represent the only means of identifying a victim in mass disaster situations where the more conventional forms of identification have failed due to damage imposed on the remains (Chinnery and Turnbull, 1999).

Mitochondrial DNA has a pattern of being passed from the mother, which leaves opportunity and limitations when it comes to identification applications. Because mtDNA is maternal in transmission and does not recombine, every member of a maternal ancestry will have the same or essentially the same mitochondrial sequences (Ding et al., 2013), and individuals may be identified in comparison with maternal relatives. This property has been proved worthy in the scenarios that involve lost persons, unknown remains and past cases where no direct comparisons could be employed (Ding et al., 2013). But on the other hand, the absence of the paternal component implies that mtDNA is inferior to nuclear DNA markers regarding their discriminatory ability by reason

that it cannot be used to discriminate between those who share a common ancestor in the female line.

Mitochondrial DNA analysis has proved to be thoroughly useful in a variety of forensic applications. Where there has been longer-term storage of evidence, associated with cold cases, often the analysis method is based on mtDNA (Ding et al., 2013), when nuclear DNA has itself degraded to unusable levels.

Mitochondrial DNA has been widely used in the field of anthropological genetics in population studies, studying evolution and the identification of ancient remains. Skeletal remains and other archaeological remains used in studying ancestry and migration patterns and in examining relationships among populations have been sampled successfully because of the limitation of the archaeological specimen used, such as mummified remains of certain time periods (Ding et al., 2013). Mitochondrial DNA is relatively stable and this has helped researchers to obtain the genetic information in specimens that are thousands of years old, revealing insights into the process of human evolution as well as prehistoric migration.

Another area of profound applications of the mitochondrial DNA analysis is mass disaster victim identification. Natural catastrophes, terrorist attacks, aviation accidents and others frequently lead to remains that are fragmented and burnt or otherwise damaged, which impair identification using more traditional methods (Ferreira and Rodriguez, 2024). The significance of the analysis using mtDNA during a mass casualty incident was demonstrated by the abundance of samples (thousands) that were examined through the analysis of the mitochondrial DNA during the World Trade Center attacks of 2001 in which the nuclear DNA analysis was not an option.

The last stage of technological development has seen much improvement of mitochondrial DNA analysis in addition to improving its utility. Techniques in massively parallel sequencing, or so-called next-generation sequencing, have transformed the study of mtDNA since they allow analysis of the whole mitochondrial genome instead of focusing on the control region only (Ferreira and Rodriguez, 2024). This whole-genome strategy offers greater discrimination and has the capability to contribute to solving those cases that cannot be solved using the control region method. Additionally, automated DNA extraction systems, real-time PCR amplification methods, and improved sequencing chemistries have reduced analysis times and increased throughput capabilities (Ferreira and Rodriguez, 2024).

Mitochondrial DNA results interpretation is a task that needs expert knowledge and should pay attention to numerous determining factors which are likely to affect the outcome of the analysis. The co-existence of the different sequences of the mtDNA in an individual is a particular phenomenon known as heteroplasmy (Ferreira and Rodriguez, 2024), which may complicate interpretation. Length Heteroplasmy and point Heteroplasmy on instances of nucleotide position should be recorded correctly and put into consideration when making comparisons. Also, the phenomenon of nuclear mitochondrial DNA segments, where during evolution some portions of the mtDNA have been transferred to the nuclear chromosomes may pose a potential problem to the analysis, unless it is corrected, by designing primers and strategies to prevent this effect (Ferreira and Rodriguez, 2024).

The work done on quality assurance and standardization has been important in making the mitochondrial DNA analysis acceptable and reliable as fitting in evidence used in courts. Various professional bodies such as International Society for Forensic Genetics and the Scientific Working

Group on DNA Analysis Methods have also put forward the guidelines and recommendations on the analysis of mtDNA and of course this covers all the aspects of sample collection, process (Kim et al., 2022), data interpretation and reporting. The proficiency testing programs are a guarantee that laboratories have sufficient guidelines and capacities in the analysis of mitochondrial DNA.

To be able to use the mitochondrial DNA in identification purposes, database development has been instrumental. Statistical evidence of the population databases consisting of the mtDNA sequence each of different ethnic and geographic populations is required to define the value of matching the DNA with the sample and compared to questioned DNA extracted by forensic assemblage (Kim et al., 2022). The FBI has a population database of mtDNA known as EMPOP (European DNA profiling group Mitochondrial DNA population database), and other regional databases that contain thousands of sequences that allow forensic scientists to get an estimate of the incidence of rare mtDNA types within pertinent populations.

While mtDNA analysis offers advantages and utility as forensic evidence, it has limitations and challenges that should be considered. Because mtDNA is maternally inherited, it is limited in its capacity to differentiate between maternal relatives, and mitochondrial DNA has less discriminatory power than nuclear DNA markers (Kim et al., 2022). The slow mutation rate of mtDNA can be an advantageous factor during the analysis of ancient DNA samples, but it is possible for siblings and even further distantly related individuals to possess the same mtDNA sequences potentially leading to adventitious matches. Other technical challenges include the amplification of nuclear pseudogenes, the interpretation of heteroplasmic mixtures, and the risks of contamination because multiple copies of mtDNA templates are held (Kowalczyk et al., 2021).

The future of mtDNA analysis is positive, with opportunities in emerging technologies and changes to methods to enhance the use of mtDNA in identification purposes. For instance, single-molecule sequencing technologies may help resolve heteroplasmic variants and aid in degraded DNA samples (Kowalczyk et al., 2021). Likely advances in bioinformatic interpretation and statistical methods could lead to advances in accuracy and reliability of mtDNA comparisons. The combination of mtDNA analysis with other genetic markers, including nuclear DNA analysis, Y-chromosomal markers, and ancestry informative markers could provide additional identification alternatives.

The purpose of this systematic review is to collate and examine the current knowledge of the role of mitochondrial DNA in identification applications, and as such provide a comprehensive overview of existing literature based on forensic casework, research studies, and developments in mitochondrial DNA technology (Kowalczyk et al., 2021). The systematic examination of the literature will allow us to identify strengths and weaknesses in existing methodologies and highlight emerging trends and new developments, with recommendations for future research directions. Our synthesis of the available evidence will assist others with the future applications and refinement of the use of mitochondrial DNA analysis and continue to advance its status as a foundational technology in human identification across the spectrum of scientific and legal applications (Kowalczyk et al., 2021).

1.1 Objectives of the Review

The increasing significance of mitochondrial DNA analysis in human identification for forensic, anthropological, and genealogical applications requires a thorough review of current practices, methodologies, and results. This systematic review will provide evidence-based

recommendations for practitioners, researchers, and policymakers to facilitate the utilization of mitochondrial DNA for identification purposes.

1.1.1 Primary Objectives

1. To evaluate the effectiveness of mitochondrial DNA analysis methods in different types of identification cases
2. To identify the main advantages and limitations of mitochondrial DNA compared to nuclear DNA in identification applications
3. To summarize current best practices and quality standards for mitochondrial DNA analysis in identification work

Through these three specific objectives, this systematic review will effectively summarize the available literature regarding mitochondrial DNA analysis in identification contexts in a manner that is both thorough and digestible. This summary will ultimately be a beneficial tool for forensic practitioners, anthropologists, lab managers and any professionals involved in human identification work, which will ultimately translate to better practices and outcomes in this area of work.

1.2 Scope and Purpose

1.2.1 Purpose of the Review

The main aim of this systematic review is to thoroughly review and synthesize existing scientific literature related to the use of mitochondrial DNA for human identification across multiple disciplines and contexts. This review intends to provide a comprehensive assessment of the methodology, technology, application, and interpretative scope that has shaped the field of mitochondrial DNA for identification purposes over the past thirty years (Lin et al., 2022).

The systematic review intends to establish a conceptual understanding of how mitochondrial DNA has developed from an area of research to one of the operational components of contemporary identification practices (Lin et al., 2022). In this systematic review of peer-reviewed literature, case series and technical publications we will demonstrate the evolution of mtDNA methodologies, document major developments and innovations, and outline ongoing problems and limitations that continue to impact the field.

An important specific aim of this review is to assess the accuracy, reliability, and validity of mitochondrial DNA results from a variety of sample types, degradation states, and analytical methods (Lin et al., 2022). In large part this will be included in the assessment of performance of a variety of different methods of mtDNA analysis - i.e., traditional Sanger sequencing, massively parallel sequencing, and newer and emerging technologies, with the intention of providing the best evidence-based practices recommendations across diverse scenarios for identification purposes.

Furthermore, this review aims to examine the statistical and interpretive frameworks used in mitochondrial DNA analysis, including population genetics considerations, database utilization, and the development of likelihood ratios for evidence evaluation (Lin et al., 2022). The synthesis will address how different populations, geographic regions, and ethnic groups are represented in current mtDNA databases and how this representation affects the interpretation and weight of evidence in identification cases.

The reviews also aim to highlight gaps in knowledge and practice, and to highlight where more research is needed to enhance the efficacy and reliability of mitochondrial DNA analysis. The review seeks to identify gaps via an understanding of the limitations and challenges outlined in the

literature (Lin et al., 2022). This will be used to establish priorities for future research, as well as areas for technological development.

1.2.2 Scope of the Review

This systematic review will also encompass multiple aspects of mitochondrial DNA analysis from an identification standpoint, from temporary, geographic, methodological and application based. The review will synthesize literature published from 1990 to the present, documenting the transition of mtDNA from its initial forensic applications, through the introduction of new automated sequencing, and onto next generation sequencing approaches (Lutz et al., 1996). The time frame will capture the foundational time when mtDNA was introduced in forensic laboratories, to the technological revolutions brought about by both automated sequencing and next generation sequencing platforms.

Geographically, the review will encompass work carried out at laboratories and research institutions globally (for example, the UK, Italy, Switzerland, Sweden, the Netherlands, New Zealand, Canada, Australia) as mitochondrial DNA analysis has become a global practice, with a variety of methodological practices, and quality standards applied to those methodologies (Lutz et al., 1996). The international scope of the review will serve to identify where best regional practices exist, and how populations' specific, regulatory, and legal frameworks have shaped mtDNA methodologies and analyze protocols when carried out on human and non-human materials.

The methodological scope will cover all the components of the mitochondrial DNA analysis considerations for identification, including sample collection and preservation, DNA extraction, amplification, sequencing, and data analysis (Lutz et al., 1996). The review will examine both

control region sequencing, which has been the traditional focus of forensic mtDNA analysis, and whole mitochondrial genome sequencing approaches that have gained prominence with the advent of massively parallel sequencing technologies.

In the review, multiple application areas where identification of mitochondrial DNA was critical to the case (Lutz et al., 1996) was considered. The review will focus on forensic applications of mitochondrial DNA in identification, to include regular casework involving degraded evidence, use in cold case investigations, and use in mass disaster victim identification and paternity testing where nuclear DNA is limited and/or degraded (Nurun, Sultana and Zakir, 2018). The scope will also include anthropological and archaeological applications where mtDNA has been used to identify ancient remains, identify past population movements, and answer historical questions.

Of particular interest are newer applications and innovative methods that will extend the utility of mitochondrial DNA in identification. This will include mtDNA for uses such as ancestry assignment, population affiliation studies, and the use of mtDNA in conjunction with other genetic markers in identification strategies (Nurun, Sultana and Zakir, 2018).

These applications and methods will also consider the different sample types typically found in identification scenarios and the scenario of different sample quality, ranging from biological samples to highly degraded samples. In addition to degraded samples, (e.g., hair evidence, skeletal remains, teeth, fingernails) other very degraded sample types will be the primary focus since evidence of this type will likely rely largely on mitochondrial DNA analysis for successful identification (Nurun, Sultana and Zakir, 2018).

Quality assurance and standardization will be within scope of the review, including, but not limited to laboratory accreditation, other testing proficiency programs, and increased adoption of international guidelines for best practices. The review will examine how different quality systems have influenced the reliability and acceptance of mtDNA analysis in legal proceedings.

The scope referred to the technical and interpretive aspects of mitochondrial DNA analysis, including the development and/or validation of analytical methods, the development of population databases, and the development of statistical frames for interpretation (Nurun, Sultana and Zakir, 2018). The review focused on how different statistical approaches have been developed and validated for the evaluation of mtDNA evidence.

The review also looked at the development and innovation of technology, which represented an important aspect of the review's scope. This technology includes the changes from manual sequencing methods to automated platforms, the introduction of real-time PCR, the adoption of capillary electrophoresis systems, and the recent implementation of massively parallel sequencing technologies (Nurun, Sultana and Zakir, 2018). The review evaluated connectivity that technology advances have had lead regarding sensitivity, specificity, throughput, and cost assumptions regarding mtDNA analyses.

The scope of this review included addressing challenges and limitations identified in mitochondrial DNA analysis, including contamination, heteroplasmy, nuclear pseudogenes, and mixture interpretation. This review will summarize approaches developed and address the current challenges and limitations in mtDNA analysis and assess the performance of these approaches (Nurun, Sultana and Zakir, 2018).

This review addressed legal and ethical considerations for mitochondrial DNA analysis, including issues such as the retention of samples, database storage and usage, the privacy issues surrounding the use of mtDNA by banks and law enforcement, and the way courts view mtDNA evidence. The review presented how different jurisdictions have dealt with each of these issues as well as how they might impact how mtDNA analysis is done routinely in practice (Newson, Wilkinson and Wrigley, 2016).

This review discussed educational and training needs, including the level of specialized knowledge and skills required for a valid interpretation of mtDNA analysis, certification programs in place to showcase qualifications and continuing education requirements for public servants (Nurun, Sultana and Zakir, 2018). The review considered how the field has instructional and training programs to accommodate the need for qualified people to perform mtDNA analysis.

The issues previously mentioned will also include consideration of future directions and emerging trends in mitochondrial DNA analysis, including the development of new technologies, novel applications, and the research priorities of the scientific community (Giordano et al., 2025). The forward-looking component will help to articulate future opportunities for advancement, and areas where additional research funding may be directed.

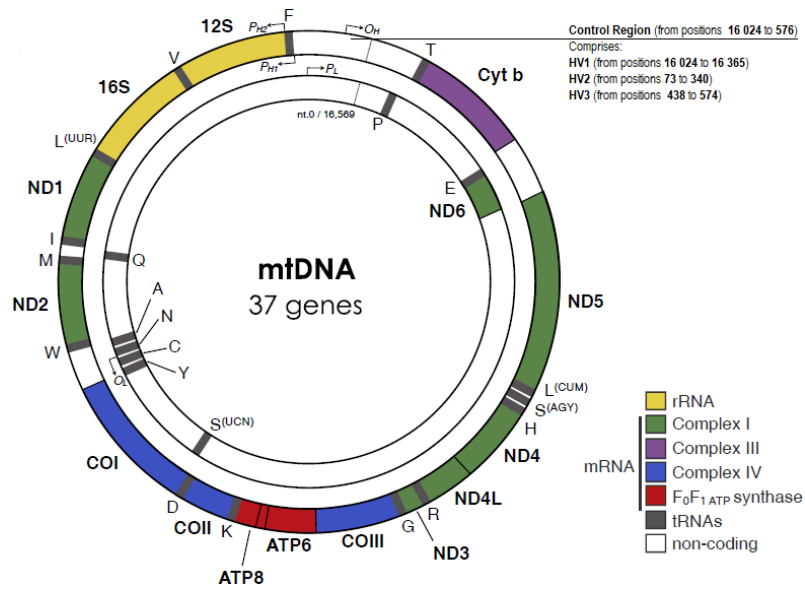


Figure 2 The human mitochondrial DNA genome with genes and control regions

(Sahayasheela et al., 2022)

Chapter 2: Literature Review

Mitochondrial DNA (mtDNA) has become an influential molecular biological marker in human identification, and it has changed the landscape of forensics, anthropology and in genealogy in the last 30 years. In contrast to nuclear DNA, the peculiarities of mtDNA, (i.e., copy numbers, maternal inheritance patterns and greater resistance of degradation) make it unique and more useful in elucidating hard-to-analyze biological objects. The paper is a literature review on the role of mtDNA in the identification applications and its methodological aspects, the development of technology, and the case studies. The review helps to understand the evidence-based best practices of the use of mtDNA by assessing efficacy in a wide range of sample types and analysis approaches. This way, the review contributes to the exploration of future options in human identification practice in terms of adopting new techniques and approaches.

2.1 Historical Development and Technological Evolution

The development of mitochondrial DNA as a form of human identification is recognized as one of the great advances in forensic genetics since the installation of DNA profiling. The earliest beginnings of mtDNA analysis as a type of gene-based forensic identification were in the late 1980s and early 1990s (Spano et al., 2022). Researchers began to understand the unique characteristics of mitochondrial DNA that would also allow it to be used in identification. Subsequently, they conducted studies, which demonstrated the use of mtDNA in identification because it exists in large copy numbers per cell, is circularly structured, and is located in a cellular compartment that has a high level of protection from degradation (Spano et al., 2022). While these early studies used restriction fragment length polymorphism analysis and had limited discriminatory power, they demonstrated the potential for mtDNA to be useful for identification.

With the creation of polymerase chain reaction amplification methods for specifically amplifying mtDNA, researchers had established an important step forward because it emphasized the ability to use very small or degraded samples that were not useful for any other type of genetic analysis, and it was important to have the ability to ascertain those results (Spano et al., 2022). While the early PCR amplification methods for mitochondrial DNA focused almost exclusively on amplifying short sections of the control region, it was primarily the hypervariable regions that related to mtDNA that also had the highest sequence variation between individuals.

The shift from manual sequencing technologies to automated DNA sequencing technologies represented a watershed moment in DNA analysis. Automated sequencing technologies improved the accuracy, reproducibility, and throughput of mtDNA analysis while simultaneously lowering the time and labor force needed to analyze mtDNA (Yu and Bennett, 2016). Automated sequencing technologies allowed forensic labs to incorporate mtDNA analysis as part of everyday casework, and improved the overall spectrum of casework samples available for mtDNA analysis.

Capillary electrophoresis systems were another advancement which added to the possibilities of mtDNA analysis. This technology improved the resolution with which the DNA could be separated and increased the sensitivity of detection methods (Yu and Bennett, 2016). Specifically, capillary electrophoresis systems provided laboratory personnel and investigators the opportunity to detect heteroplasmic variants, which were often difficult to observe using conventional sequencing technologies, and the ability to better characterize length polymorphisms in homopolymeric regions of the mitochondrial control region (Yu and Bennett, 2016).

Most recently, the advancements in the technology-related to mtDNA analysis have been related to the introduction of massively parallel sequencing technologies, or next-generation sequencing (NGS) (Kopinski et al., 2021). Next-generation sequencing has revolutionized traditional mtDNA analysis by capturing the entire mitochondrial genome, rather than the covering only mitochondrial control region. Whole mitochondrial genome sequence analysis provides enhanced discriminatory power that can provide researchers with critical information when a control region analysis isn't enough for a definitive identification, especially in high profile investigations (Kopinski et al., 2021).

As sequence platforms have developed technologically, so have the software and bioinformatics tools for data analysis. Software packages were dedicated, and designed for mtDNA sequence analysis developed that included algorithms for heteroplasmy calling, alignment, and quality control for data (Kopinski et al., 2021). These tools standardized the procedures for processing data and the perturbations to consistency across laboratories.

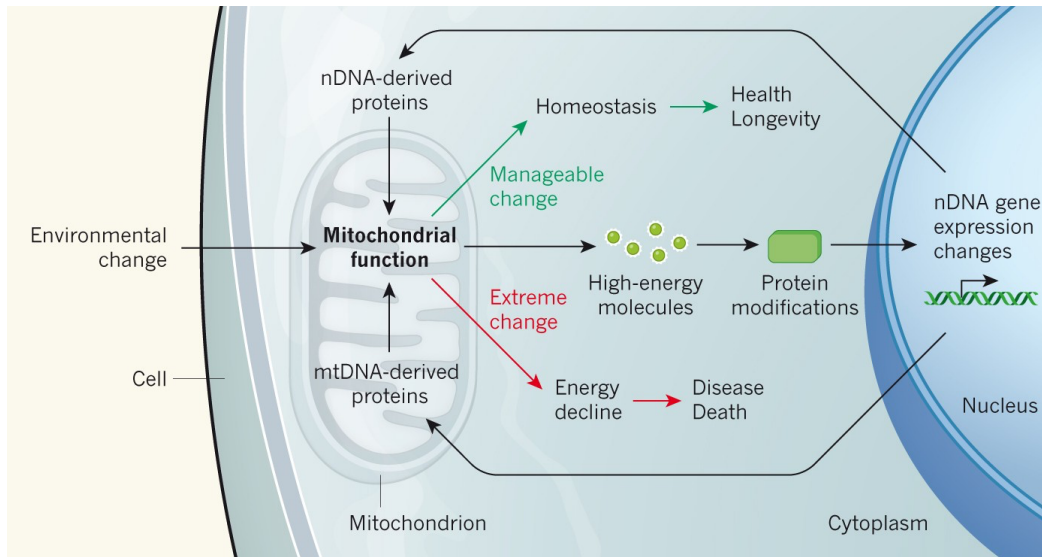


Figure 3 Mitochondria as the central environmental sensor (2016)

2.2 Methodological Approaches and Technical Considerations

The methodological framework for mitochondrial DNA analysis has many crucial steps, each of which presents different problems that have been given a great deal of research and optimization attention. Sample collection and retention is the first step and it is known that proper protocols are critical to the success of a lot of what happens later on (Newman and Shadel, 2023). There is compelling evidence that different sample types require different handling methods to maximize the recovery of viable DNA and minimize the degradation of the sample, before the mtDNA analysis can even begin (Newman and Shadel, 2023).

Hair evidence has received a lot of attention in the literature because it is quite different than other biological evidence and can be a quite common occurrence in forensic cases. It is established that hair shafts, lacking nuclear DNA, can be analyzed with mtDNA methods. However, analysis of hair samples is especially challenging because while you can extracting mtDNA (Newman and

Shadel, 2023), you have to deal with contamination and extraction efficiencies, and interpret DNA results from the weathered hair or hair that has been damaged with chemical treatment.

Skeletal remains also represent a difficult sample type and have been studied in the literature (Agbani et al., 2025). Research has documented the differences in qualitative and quantitative mtDNA specific to skeletal elements and how different skeletal elements vary in their ability to provide useful DNA, with teeth generally outperforming long bones or rib samples when it comes to DNA recovery (Newman and Shadel, 2023). The effect of the environment on the preservation of DNA in skeletal material has been examined – is there a literal reference for stared at? extensively with temperature, humidity, pH, and microbial activity being well studied and understood as impacting the recovery of mtDNA.

Methodological studies have proceeded further in providing guidelines for selecting samples, cleaning surfaces, preparing powders, and methods of extraction that are suited to skeletal materials (Newman and Shadel, 2023).

DNA extraction research has evolved based on technology and findings from research. Previous extraction methods typically used organic solvents coupled with a series of manual process steps that were time-consuming, and therefore increased the possibility of contamination (Lee et al., 2023). The advent of silica-based extraction methods provided enhanced DNA recovery and time savings. The introduction of automated extraction systems has improved the team capacity for processing DNA, allowing shared and benchmarked approaches to isolation, but less able to introduce contamination and human error (Lee et al., 2023).

Amplification strategies for mitochondrial DNA have evolved through the extensive characterization of primer design, reaction conditions, and quality control decisions. Researchers have evaluated the ability to sequence areas of the mitochondrial genome using various primer sets and amplification methods and under multiple conditions (Lee et al., 2023). The development and implementation of multiplex PCR amplification has improved efficiency, reduced the consumption of sample, and allowed for even better amplification of the mitochondrial genome by amplifying multiple regions of interest simultaneously.

Quality control strategies have also undergone extensive discussion and standardization inspired by the literature and experience. The literature demonstrates the importance of contamination prevention protocols - the studies have recommended separate work areas for pre- and post-PCR work, the use of disposable equipment, and personal protective equipment (PPE) (Lee et al., 2023).

The interpretation of mtDNA sequencing data has received considerable attention in the literature, mainly for heteroplasmy characterization and reporting. Guidelines have been developed to assist in the differentiation of true heteroplasmy from sequencing artefacts and standardized nomenclature for reporting heteroplasmic positions (Lee et al., 2023). The phenomenon of length heteroplasmy in homopolymeric regions has been characterized extensively in the literature, and normal variation ranges and reporting guidelines have been established for interpretation.

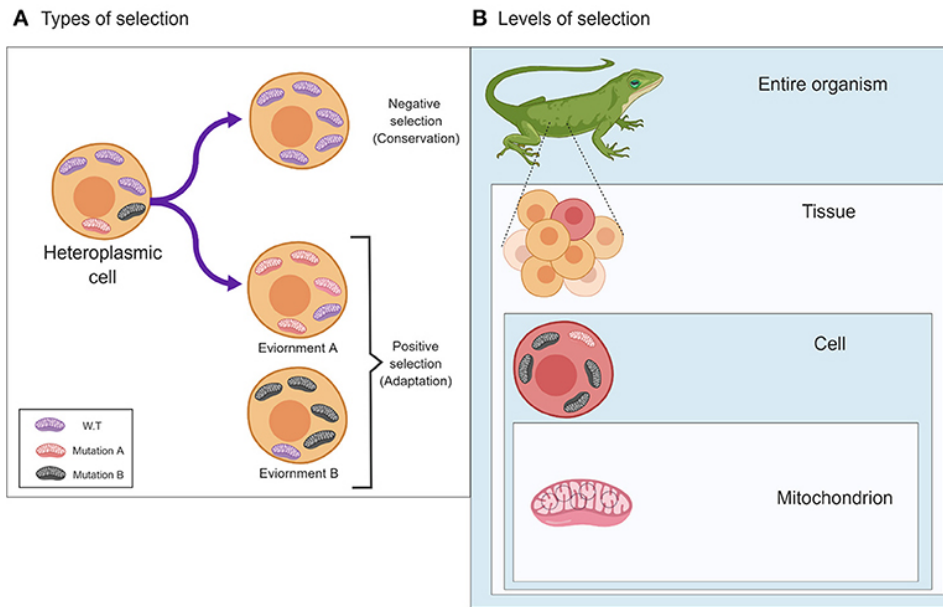


Figure 4 The two main types of selection, **Negative and Positive**, and their effects on patterns of mitochondrial genetic variability. **(A)** Different types of selection (negative or positive) affect the pattern of mitochondrial mutations in response to different environments (lower panels) or will undergo a selective sweep (negative selection to maintain function (upper panel)). **(B)** The different levels in which selection may act on the mitochondria, i.e., (down-up) the single mitochondrion, cells and the entire organism. (Shtolz and Mishmar, 2019)

2.3 Applications in Forensic Science and Legal Contexts

Since its early implementation, mitochondrial DNA analysis in forensic science has been applied significantly across many cases and contexts. There has been evidence documenting the effectiveness of the application of mtDNA analysis in cold case investigations (Liu et al., 2024). Cold case investigations are arguably the most critical context for mtDNA analysis - its use has resulted in the ability to investigate cases that have involved identified biological evidence that has become degraded and undetectable (Liu et al., 2024). Evidence from the literature supports the success rates for mtDNA analysis of aged evidence samples and documents explanatory factors that influence the possibility of obtaining useful results in cold case materials (Liu et al., 2024).

Mass disaster victim identification is an emerging and significant application area where mtDNA analysis has particular importance (Merdiatio Boedi et al., 2024). Evidence documenting the usefulness and effectiveness of mtDNA analysis in the aftermath of a major disaster is present but predated the introduction and development of the systematic application of mtDNA analysis for investigation into traditional identification methodology failure. Studies have produced methods for mass disaster response that include mtDNA analysis in the procedures for victim identification (Liu et al., 2024). Evidence from the literature establishes the ability of mtDNA analysis to be productive when faced with fragmented or cremated remains that cannot utilize alternative methods of identification.

Mitochondrial DNA (mtDNA) has been researched in the context of paternity testing. The use of mitochondrial DNA in paternity testing in the context of research examining cases in which the alleged father is passed away or unavailable for testing (Liu et al., 2024). Previous research established the potential value of mitochondrial DNA analysis to clarify maternal lineages' connections, and even established numbers and statistics for paternity testing based upon mtDNA evidence. Nevertheless, there have also been concerns in the literature regarding the limitations of mtDNA in paternity testing, like the exemptions for total maternal relationships and that the discernable results of mtDNA are less discriminatory than nuclear DNA markers (Shu and Shu, 2023). MtDNA has also been explored in relation to sexual assault cases, where it was noted in the literature as applicable to evidence when nuclear DNA is unavailable or too compromised. This has included articles that documented the application of mtDNA analysis to hair evidence taken from sexual assault cases, as well as how mtDNA analyses protocols and models could be applied in instances of evidence containing a mixture of samples, where contributors may include more

than the individual being tested (Shu and Shu, 2023). There have been many studies that have examined the challenges of mtDNA interpretation, in relation to sexual assault cases. Challenges in interpreting mtDNA evidence in cases of sexual assault include the determinations of the transfer mechanism (i.e., how the mtDNA bearing evidence came to be at the scene), and what alternative theoretical explanations could be offered (Shu and Shu, 2023).

Missing persons investigations have greatly benefited from mtDNA analysis. Many successful identifications have been reported through maternal relationships comparisons. The studies have established databases of mtDNA sequences from missing persons cases and have developed searching algorithms to compare unidentified remains to missing persons records (Ginther, Issel-Tarver and King, 1992). The literature is clear that mtDNA analysis can add great value to missing persons cases, especially when direct family reference samples are not available, but maternal relatives can provide comparison samples.

The attendance to mitochondrial DNA evidence has been studied and documented extensively. Research has reviewed the court decision involving mtDNA evidence and established factors that play a role in a judicial body's acceptance of this type of scientific evidence. Studies have noted that proper statistical interpretation and clear communication of the limitations of mtDNA analysis are important elements to convey for successful inclusion in court (Ginther, Issel-Tarver and King, 1992).

Quality assurance programs for forensic mtDNA analysis have also been studied and documented in the literature. The research has identified the critical components of an effective quality system and noted the importance of proficiency testing, validation studies, and continuing

statistical frameworks to evaluate evidence (Taylor and Turnbull, 2005). Population studies have identified major differences in mtDNA diversity between geographic areas and ethnic groups, emphasizing the importance of examining appropriate reference populations for statistical calculation(Taylor and Turnbull, 2005).

The development of reference databases for mtDNA has been a key focus of research, with studies compiling global comprehensive collections of mtDNA sequences. Research has assessed sampling methods for constructing databases, measures of quality control on sequences, and statistical methods for examining the database (Taylor and Turnbull, 2005). The literature has demonstrated that both size and population representation of databases impact the results of statistical calculations and the interpretations of mtDNA evidence.

Much research has been undertaken in the statistical understanding of mitochondrial DNA evidence, particularly with respect to random match probability (Taylor and Turnbull, 2005). Research has developed a mathematical framework for calculating the random match probability of observing any particular mtDNA profile within a population, as well as its special considerations with regard to markers that are maternally inherited (Wallace, 2010). Studies have demonstrated that traditional random match probability calculations may not be appropriate for mtDNA evidence and have developed alternative statistical approaches that better reflect the biological characteristics of mitochondrial inheritance.

There has been a lot of research on likelihood ratio computations for mtDNA evidence (Wallace, 2010). Also, there has been a lot of research developing mathematical models to include population genetic parameters and the information from databases into more nuanced arguments

in evidential evaluation (Wallace, 2010). Research has studied and reported on effects of database size, population structure and sequence variation on likelihood ratio computations and also made recommendations for the use of these metrics in contexts relevant to forensic applications (Wallace, 2010).

Evolutionary clustering in mtDNA sequences has been studied, which has implications for statistical inference. While studies have showed that mtDNA sequences are not random in a population and that they group together phylogenetically based on evolutionary relationships, earlier studies have developed statistical procedures to formally include phylogenetic structure; ignoring these relationships can lead to substantial errors in interpreting the evidence (Veeraragavan, Johansen and Johnston, 2024).

One of the most difficult aspects of mtDNA analysis is mixture interpretation. Research has examined mixture interpretation with respect to both technical and statistical perspectives. Studies have characterized the types of mixtures most commonly found in forensic samples and have proposed procedures for the detection and interpretation of mixed mtDNA profiles (Chatterjee, Mambo and Sidransky, 2006). In addition, research has provided statistics to assess evidence of mixtures, and has addressed the limitations and uncertainties regarding interpreting mixtures.

Research on the effect of heteroplasmy on statistical interpretation has been quite thorough as studies have outlined approaches for including heteroplasmic variants into evaluation of forensic evidence, studies have characterized the frequencies and distributions of heteroplasmy across a variety of populations, and studies have created statistical techniques to accommodate for any differences in heteroplasmy in mtDNA profiles (Chatterjee, Mambo and Sidransky, 2006).

Research studies on contamination evaluation and statistical assessment have classified the main sources of contamination and have created reliable means to evaluate seeming results from viable results (Chatterjee, Mambo and Sidransky, 2006). Studies have created statistical frameworks to evaluate the apparent traits of matches in situations where potential contamination exists and created directions on investigating contamination and reporting (Veeraragavan, Johansen and Johnston, 2024).

Research has also focused on the development of expert systems and automatic interpretation software. Research has investigated the state of the art for automatic systems to compare mtDNA sequences and calculate what course of action to take, and also studied the challenges to use such systems in forensic laboratories(Chatterjee, Mambo and Sidransky, 2006). And finally, research has shown the benefits and limitations of automatic interpretation and have defined the need for validation and quality control of expert systems(Chatterjee, Mambo and Sidransky, 2006).

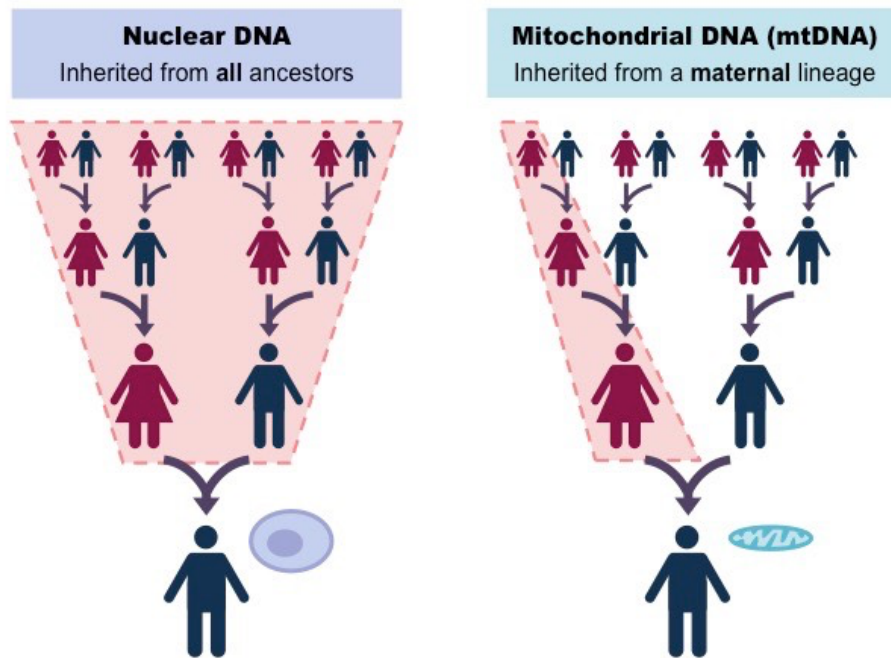


Figure 6 Nuclear vs. Mitochondrial DNA Inheritance Paths (TESTE DE ANCESTRALIDADE COMO FERRAMENTA GENEALÓGICA PARA HISTÓRIA DE FAMÍLIA, n.d.)

Chapter 3: Role and Application of Mitochondrial DNA in Identification

3.1 Characteristics of mtDNA Relevant to Identification

The mitochondrial DNA is a special genetic mechanism that has transformed the human identification discipline. In opposition to the nuclear DNA, the mtDNA is a small double-stranded, circular structure found in the eukaryotic cell mitochondria. The human mitochondrial genome has 16569 base pairs with 37 genes, 13 protein coding genes, 22 transfer RNA genes and 2 Ribosomal RNA genes (Elyasigorji et al., 2022). This is a small genotype structure which has a number of unique features that turn it into a genotype structure of particular value in terms of identification.

The greatest characteristic of the mtDNA in identification purposes is the high number of copies in cells. Whereas nuclear DNA exists in only two copies within a cell, phase and branches of the mitochondrial DNA may occur between hundreds and thousands of copies per tissue and metabolic activity. The abundance of this is what renders the mtDNA especially useful in the study of degraded, ancient, or otherwise limited biological sample where the material insufficient or absent nuclear DNA (Elyasigorji et al., 2022). The copy number is high, which adds chances of effective amplification and examination of even difficult forensic samples.

The other important feature is the maternal pattern of inheritance of mtDNA. When compared to the nuclear DNA, which has two parents as the people who passed that particular DNA to their children, the cycle of transmission is fundamentally different with the mtDNA, simply because only one parent, the mother, passes down the DNA to the children (Elyasigorji et al., 2022). This unilateral inheritance makes all the individuals of the same maternal stem to have identical profiles of mtDNA, mothers, children, maternal siblings, maternal grandmothers, and maternal aunts. The

property permits investigators to form maternal relationships and identify individuals based on the maternal relatives in the cases where direct comparison samples are not present.

The mtDNA structure also plays the role of its utility in identification. The molecule has segments of non-uniform evolutionary rates, the rapidly polymorphic control region (D-loop), and more conserved coding regions (Syndercombe Court, 2021). A somewhat 1,122 base pairs of control region is composed of two hypervariable segments (HVS-I and HVS-II) which have the most significantly different sequences in people. These areas represent the most effective regions of forensic examination because of the discriminating it carries with just enough conservation that it may amplify reliably.

Table 1 Comparison of mtDNA and Nuclear DNA Characteristics for Identification(Miller et al., 2019)

Characteristic	Mitochondrial DNA	Nuclear DNA
Copy number per cell	100-1,000 copies	2 copies
Inheritance pattern	Maternal only	Biparental
Genome size	16,569 bp	~3.2 billion bp
Mutation rate	5-10x higher	Lower
Recombination	None	Extensive

Degradation resistance	Higher	Lower
Individual discrimination	Moderate	High
Phylogenetic information	Excellent	Limited

The basic differences of mitochondrial and nuclear DNA and their subsequent implication in their use in identification settings are represented in Table 3.1. The genetic advantage that the higher copy number and a high degree of resistance to degradation give makes mtDNA of special use in difficult forensic cases and the maternal inheritance pattern presents unique genealogical information not accessible using nuclear DNA.

The mutation rate of mtDNA is about five to ten times higher than the mutation rate of nuclear DNA and this makes the variation of the sequences more diverse among unrelated people. The high mutation rate, together with non-recombination, makes these find and discernible maternal lines through evolutionary time (Syndercombe Court, 2021). Because there is no recombination, the sequences of the mtDNA are passed down intact, and as can be seen in the phylogenetic tree, they allow one to track the maternal line of descent.

generations and thus the investigators may derive contacts between the individuals who are separated by quite extended periods of time or distance.

The reliability of maternal lineages allows finding out individuals even in several generations. Historic examples have also established the triumphant identification of people with maternal relatives living some generations apart by the use of mtDNA (Antil et al., 2022). This is especially useful in situations of mass loses of victims of a disaster, where the immediate family members might not be present, but reference samples can be collected by more distant relatives on the maternal side of the family.

In maternal lineages, the events of mutation do take place but are however, to a small extent. (Andersen and Balding, 2018). With such mutations, they are passed to the next generation and this gives rise to new lineages of variants. Knowledge of mutation rates and patterns is vital in interpreting the results of mtDNA analysis quite well whenever minute differences in the sequences of the questioned and the reference samples are encountered (Antil et al., 2022). The most frequent type of variation is of the point type, but insertions and deletions also may take place in control region.

Table 2 Maternal Relationship Types and mtDNA Sharing Expectations (Andersen and Balding, 2018)

Relationship Type	Expected mtDNA Sharing	Generations Separated	Mutation Probability
Mother-Child	100% identical	1	<1%

Maternal Siblings	100% identical	0	<1%
Maternal Grandmother-Grandchild	99-100% identical	2	1-2%
Maternal Aunt-Nephew/Niece	99-100% identical	2	1-2%
Maternal Great-grandmother-Great-grandchild	98-99% identical	3	2-4%
Maternal Cousins (same grandmother)	99-100% identical	2	1-2%

Table 3.2 illustrates how the sharing of mtDNA between various types of maternal kinship would be anticipated and how probability of witnessing the mutations would grow as the generation gap widens. Such probabilities are important in calculating the importance of matches and exclusion of mtDNA in cases of identification.

Besides the identification of individuals, maternal inheritance patterns can be used in genetics of the population and evolution. Haplogroups of mtDNA, based on particular suites of mutations, are found to be important branches on the human maternal phylogeny (Antil et al., 2022). This information related to ancestral origins and migration patterns supports identification cases and anthropological cases and is identified in these haplogroups.

Patterns of geographic spread of mtDNA haplogroups therefore indicates past migration of human populations and movements. The information could be used as investigation leads in circumstances that the geographic origin of a person has to be identified (Antil et al., 2022). Yet, the interpretation of the haplogroup data should take precaution of population admixture, post-European migrations, and insufficiencies of genetic markers in ascertainment of geographic origins.

3.3 mtDNA in Forensic Casework

The mtDNA has reigned as an essential element in forensics studies with special focus being given to those cases displaying degraded, mixed owing to low amounts of biological data. Due to the peculiarities of possession, mtDNA can be used to examine problematic forensic specimens that cannot be used in the analysis of nuclear DNA. The real knowledge of the applications, methodologies, and interpretational frameworks of the forensic mtDNA analysis is critical towards effective resolution of a case (Wong and Boles, 2005).

Forensic studying often deals with the control region, i.e. the hypervariable locations HVS-I and HVS-II. These areas give adequate discrimination to the majority of forensic cases but are reliable enough to be presented in court. The analysis activity will consist of carrying out DNA extraction, amplification by use of polymerase chain reaction (PCR) (Wong and Boles, 2005), and sequencing to identify the profile of mtDNA of the questioned sample.

These kinds of biological evidence that could be used in the analysis of the mtDNA go much further than kinds that normally can be used in the nuclear DNA testing. Hair shafts, especially shots without roots are one of the most frequently used pieces of evidence to perform the analysis

of the mtDNA (Wong and Boles, 2005). Contrary to the nuclear one, whose DNA is concentrated mainly in the root of the hair, mtDNA is evenly dispersed along the hair shaft allowing to examine even the hair remains. Similar cases are often observed with bone and teeth samples, one of which might still hold its mtDNA even after the nuclear DNA has deteriorated.

DNA analysis in old and degraded biological samples poses special problems, but because of high copy number of mtDNA and its small size, it has a greater likelihood of standing up to the degrading forces. Nuclear DNA may be disastrously affected by environmental elements like heat, humidity as well as contact with chemicals whereas mtDNA is only fractionally ruined. This hardiness gives the mtDNA analysis usefulness in the investigations of cold cases, mass deaths, old remains (Wong and Boles, 2005).

Table 3 Forensic Evidence Types and mtDNA Analysis Success Rates (Burt et al., 2025)

Evidence Type	Success Rate (%)	Typical DNA Yield	Degradation Resistance	Analysis Challenges
Hair shafts	85-95%	Low	High	Length polymorphisms
Bone samples	70-85%	Variable	Very High	Inhibitors, contamination

Teeth	80-90%	Moderate	Very High	Hard tissue processing
Fingernails	75-85%	Low	Moderate	Mixed profiles
Degraded soft tissue	60-80%	Variable	Moderate	Bacterial contamination
Ancient remains	40-70%	Very Low	High	Contamination, inhibition

Table 3.3 shows different success rates of type of evidence that is typically sent in to be tested by mtDNA. These successful rates indicate not only the natural characteristics of the evidence types, but also the technical difficulty in the analysis of the picture.

Forensic mtDNA results can be interpreted only with attention paid to statistical significance and population genetics. Discrimination power of analysis of the nuclear DNA is tuned to very high between unrelated individuals whereas the discrimination power of analysis of the nuclear DNA is much lower. When carrying out the evaluation of the weight of the mtDNA evidence, the frequency of the occurrence of certain profiles used in relevant populations should be taken into consideration.

Forensic interpretation of the sphere of mtdna involves database comparison that is of high importance. In the U.S., The National DNA Index System (NDIS) that is the key to the DNA

database of the CODIS (Combined DNA Index System) program has a database based on the mtDNA that enables the FBI to compare the questioned samples with the reference profiles of both unsolved crimes and missing persons (Chinnery et al., 1999). Forensic analysis of the mtDNA requires quality assurance and its validation procedures. These technical difficulties in relation to the analysis of the mtDNA such as the possibility of contamination, length polymorphisms, and combined profiles necessitate a strong quality control system (Aggarwal et al., 2025). To get consistent results, laboratories should associate proper validation studies, testing proficiency, and monitoring of contamination of laboratory results.

3.4 Use of mtDNA in Anthropological and Archaeological Contexts

Analysis of mtDNA ushered in a new dawn in relation to anthropological and archeological studies because it has given way to molecular tools that can be used in the study of the evolutionary history of humans, their migration across the globe, and the tracing of their culture (Chinnery et al., 1999). The application of mtDNA analysis to both old and historical remains has led to further explorations in the sense of human populations and their interaction along time and space. The applications illustrate the versatility and the potential of the mtDNA analysis whether it is present-day criminal inquiries or otherwise.

Archeological use of mtDNA analysis dwells more on deriving genetic data of dead skeletal handlings. mtDNA could withstand the conditions of archaeological discovery and it is a factor of burial environment, climate conditions, soil chemistry, and the age of the remains. The best preservation conditions are dry cool climates with alkaline soil conditions although DNA is likely to be degraded due to warm humid temperatures with acidic soils.

The analysis of ancient DNA (aDNA) based on mtDNA has been used to understand population migration in the prehistoric period, genetic diversity in human population, and evolutionary correlation amongst populations. Such studies have formed our knowledge on some of the great demographic events in the history of humankind such as early settlement of continents, turnover of people, and cultural turnover (Chinnery et al., 1999). The maternal inheritance mode of mtDNA renders the type especially important to track movement of the populations and to examine female triumphant migration.

Table 4 Archaeological mtDNA Applications and Time Periods (Burt et al., 2025)

Application	Time Period	Success Rate	Key Findings
Paleolithic populations	40,000-10,000 years ago	10-30%	Early human migrations
Neolithic farmers	10,000-5,000 years ago	30-60%	Agricultural transitions
Bronze Age cultures	5,000-3,000 years ago	50-70%	Cultural continuity/change
Medieval populations	1,000-500 years ago	70-85%	Historical population structure
Colonial period	500-200 years ago	85-95%	European colonization impacts

Historical individuals	Various periods	Variable	Individual identification
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Table 3.4 shows how the success rate of extracting incipient mtDNA of archaeological remains has varied at different points of time. When judged by success rates they represent preservation situations as well as technological progress in both ancient DNA extraction and the ancient DNA analysis techniques used.

Searching into the population structure of the ancient human population, using the determination of mtDNA has unveiled elaborate patterns of population turnover and persistence across human history (Burt et al., 2025). As a case in point, genetic findings have been proven in European in the instances of major genetic shifts with the introduction of agriculture in the Neolithic era and movement back in the population. Such results are very significant in the context of mapping out the relationship between cultural and biological evolutions in human societies.

Some of the anthropological usage of the mtDNA analysis entail identification of persons of history and verify commonly purported lineages among renowned people. The use of mtDNA analysis has been of use in examining royal lineages, historical figures and those of cultural significance to either affirm or reject historical claims of identity or relationship (Merheb et al., 2019). Such studies demand vigilance in regard to contamination risks as well as authentication of ancient DNA findings.

Combining the evidence of mtDNA with that of archaeology, anthropology, and history brings forth the methodology of studying the population of human beings in multidisciplinary manner. Genetic information may supplement and in some cases qualify interpretations resting on material

culture, language evidence and historical documentation. The resulting increased complexity has enhanced our perceptions about human history in population dynamics and culture change.

3.5 Advantages and Limitations

The knowledge of benefits and limitations of analysis of the mtDNA is essential in the case of its proper application and interpretation in the situation of identification. Although mtDNA has distinct abilities when it comes to some kinds of investigations, it also has particular issues that should be approached with special attention (Forsythe, Melia and Harbison, 2020). A sober judgment of the same can help the practitioners to take informed decisions when and how is best to use the analysis of mtDNA with due diligence and care.

The main strengths of mtDNA analysis are connected with a high copy number and stability. The nature of these properties makes them viable in situations where it would not be possible to test the nuclear DNA product on a sample and this increases the scope of the classes of evidence that might yield successful analyses (Forsythe, Melia and Harbison, 2020). The maternal inheritance pattern offers some special genealogical data that supplements the evaluation of nuclear DNA and also helps in identification using maternal relatives due to lack of direct family members.

Table 5 Advantages and Limitations of mtDNA Analysis (Merheb et al., 2019)

Advantages	Limitations
High copy number enables analysis of degraded samples	Lower discrimination power than nuclear DNA

Maternal inheritance allows distant relative comparisons	Cannot distinguish maternal relatives
Resistance to environmental degradation	Higher cost and longer analysis time
Suitable for rootless hair analysis	Potential for heteroplasmy complications
Valuable for ancient DNA studies	Limited databases for frequency estimates
Phylogenetic information available	Contamination risks in laboratory
No recombination preserves lineage information	Requires specialized expertise
Small genome size facilitates complete analysis	Length polymorphisms in control region

Table 3.5 gives a clear idea about the key benefits and drawbacks of carrying out mtDNA analysis in identification work. These factors have to be understood in order to make proper case selection and interpretation of those results.

The discriminative ability of mtDNA is both a strength and narrows one down to the application at hand. Although mtDNA analysis will not give individual level discrimination found with nuclear DNA STR analysis, the fact that mtDNA has moderate discriminatory ability will generally exclude at an exclusion level and can give investigative information (Forsythe, Melia

and Harbison, 2020). In most investigative cases, the capability of ruling out people or groups of people may be valuable as the cases of positive identification.

Through cost considerations, a decision on conducting mtDNA analysis is majorly affected. That is because of the technical difficulty of sequencing mtDNA, dearth of special equipment, and the necessity of qualified machine operators makes it more expensive per sample than routine nuclear DNA analysis. Such costs have to be balanced with the possible value of the information that is received and availability of substitute testing solutions (Merheb et al., 2019).

mtDNA analysis involves special concern in quality control and contamination prevention since the techniques involved are very sensitive. The possibility of contaminating DNA is further increased by the high copy number which makes the analysis of mtDNA, which would lead to the presence of contaminant DNA caused by laboratory personnel, former samples, and environmental samples. Contamination precaution guidelines such as pre- and post-PCR separation, negative controls as well as staff elimination databases should be rigorous so as to ensure credible results (Forsythe, Melia and Harbison, 2020).

3.6 Case Studies and Real-World Applications

The reality of mtDNA analysis in real-life identification cases proves its performances as well as shortcomings. A closer look at the case studies gives a good idea about the decision-making processes, the manner of analytics and interpretational schemes underlying an effective intervention in the investigation of mtDNA. These examples explain that there are many settings where mtDNA analysis can be used to help in identification, as well as how valuable proper case management and understanding results interpretation may be.

The application of the mtDNA analysis has some of the strongest demonstrations in the historical cases of identification. The determination of the members of the Romanov family is a ground breaking investigation in forensic genetics where a family whose existence was a mystery decades ago was solved using mtDNA and nuclear DNA tests. This case was about the analysis of mtDNA fragments of the bones supposedly belonging to the members of the Russian royal family and comparing the results with reference samples that are the maternal relatives of Tsarina Alexandra and Tsar Nicholas II (Forsythe, Melia and Harbison, 2020).

Another area that is relevant in terms of mtDNA analysis is mass disaster victim identification. During the September 11, 2001, attacks on the World Trade Center identification of the victims involved unique problems in the task of identifying victims because of the high levels of biological data degradation and fragmentation. The use of mtDNA analysis was important when nuclear DNA was not able to identify victims, especially in case of highly degraded bones and bone fragments which are very tiny.

Table 6 Notable mtDNA Identification Cases and Outcomes(Forsythe, Melia and Harbison, 2020)

Case	Year	Sample Type	Challenge	Outcome	Significance
Romanov Family	1990s	Bone fragments	Historical remains	Positive ID confirmed	Royal lineage verification

World Trade Center	2001-ongoing	Degraded tissue	Mass disaster	300+ identifications	Largest mtDNA effort
Kennewick Man	1996-2015	Ancient bone	9,000-year-old remains	Native American ancestry	Archaeological controversy
Jesse James	1995	Tooth	Claimed outlaw remains	Exclusion of claimant	Historical hoax exposed
Unknown Child (Titanic)	2007	Tooth/bone	Disaster victim	Positive identification	Maritime disaster victim
Czar Nicholas II	2009	Bone/teeth	Authentication	Confirmed identity	Historical verification

Table 3.6 summarizes some of the most sensational cases in which the investigation involving the application of the use of mtDNA DNA analysis was central in the identification process. These examples reflect the flexibility of mtDNA analysis in various temporality and types of samples as well as the problems that it routinely faces in the field practice (Forsythe, Melia and Harbison, 2020).

The technology of the analysis and process of the mtDNA has helped greatly in cold case examination. Bodies have been identified as unidentified remains, whereby remains that had not been identified in years or decades were solved by the use of skeletal remains or other biological evidence degraded by the use of mtDNA. The success rate of these difficult cases has improved because new, even more sensitive, analytical techniques have been developed and reference databases expanded.

The inclusion of the outcome of the mtDNA analysis with other types of data proves the multidisciplinary character of identification research nowadays. To conclude on reasonable decisions, anthropological tests, dental comparisons (Vernot et al., 2021), radiological tests, and circumstantial evidence should form part of the genetic results. Such comprehensive strategy assists in ensuring that identifications can be improvised on numerous lines of evidence and chances of making errors are minimized.

International mtDNA identifications are also becoming more usual, especially where the case is cross-border or where the remains were of international interest. The efforts are promoted through standardized procedures, quality control procedures and data sharing conditions that allow collaborators with sufficient scientific basis of any identification(Vernot et al., 2021).

Technology development of the analysis of mtDNA extends its uses and success rates (Vernot et al., 2021). The next-generation sequencing technologies allow studying the mitochondrial genome in more detail that could provide higher discrimination power and give more data to phylogenetic research. Better DNA extraction procedures and purification processes have

enhanced success on hard samples of a particular nature and the process has gained shorter analysis times.

The certification and training programs in analyzing mtDNA make sure that all modifications put into the practice will establish successful results since practitioners will be adequately equipped with the knowledge and expertise demanded. Analysis of mtDNA poses challenges because it involves successes in molecular biology, population genetics, phylogenetics, and quality assurance above what one needs to do normal nuclear DNA analysis.

mtDNA analysis in identification applications in the future will probably include further developments in technology, the increased database and better ways to statistically interpret results. Combination with other molecular markers and methods of analysis can offer increased discrimination capability with merits of exceptionally retaining the exclusive characteristics that render mtDNA analysis applicable in cases where conventional methods of identification cannot be applied(Vernot et al., 2021).

As presented in case studies and applications in the present chapter, it can be regarded that analysis of the mtDNA is a great tool in human identification provided it is used properly and correctly interpreted. Knowing both the strength and the weakness of this technology allows practitioners to draw the best out of it without misapplication or exaggeration of findings. Thanks to the technological progress and growing databases (Vernot et al., 2021), it can be well imagined that although the use of mtDNA analysis would have been a part of the identification process within the field of forensics, anthropology and archaeology, it is going to become an important one in the future.

To achieve success in utilization of the analyses by using mtDNA, it is necessary to be attentive to the factors of the case, to select the sample, assure a high level of quality control and interpret the statistical findings correctly. Under these conditions, the analysis of the mtDNA can give significant identification information, unavailable otherwise, and that would help solve problematic cases, as well as give more understanding on the nature of human populations, including their history.

Chapter 4: Theoretical and Mathematical Framework

4.1 Models of mtDNA Mutation and Inheritance

Mitochondrial DNA analysis as a parameter used in identification is highly based on the interpretation of the mathematical models which explain the mutation mechanisms and inheritance schemes. The models give an interpretation of statistical significance to observed genetic variations and to determining the statistical significance of matches or exclusions of mtDNA to identification purposes (Laricchia et al., 2022). The establishment of powerful mathematical models is needed to make possible correct probability estimates and dependable interpretations of mtDNA evidence.

The mutation of mtDNA takes certain shapes that have predetermined mathematical models characterized by probability frameworks. The most frequent form of variations is a point mutation, which happens at reasonably predictable frequencies in various parts of the mitochondrial genome. The control region is more mutated than the coding region, as in these areas there is less pressure of selection and no functional restrictions (Laricchia et al., 2022). The depiction of such variation in rates is important in building effective mathematical models of evolution of the mtDNA.

The method behind mutation rate estimates and divergence time of the mtDNA is based on the molecular clock hypothesis (Laricchia et al., 2022). The assumption associated with this concept is that there exists a relatively steady (through time) schedule of accumulation of mutations, which enables researchers to calibrate evolutionary clocks and approximate the age of common ancestors. The rules of molecular clock, however, can be challenged with such factors as selection, demographic fluxes, and variation in generations; such elements must be carefully considered in mathematical modeling.

Randomness Stochastic modeling of mtDNA mutation takes account of the random occurrence and position of mutations. These models acknowledge that mutations are probabilistic events that has a Poisson distribution with time. Parameters that they have given in this mathematical framework in order to compute the likelihood of occurrence of certain numbers of mutations in specific time duration include: mutation rates, sequence length and time (Laricchia et al., 2022).

The infinite sites model is one of the models of the mutation of the mtDNA based on the assumption that there can be only one mutation on the site previously not mutated (Laricchia et al., 2022) . This model is very easy to calculate because it does not put the issue of multiple mutations at the same position, but this model might not fit the true state of situations in evolutionary scenario of mtDNA analysis. Other models exist, e.g. finite sites model (Bibi et al., 2023), which permit multiple mutations on the same site though those models necessitate greater mathematical formulations (Bibi et al., 2023).

Heteroplasmy brings further complication to the models in mtDNA inheritance. The existence of multiple mtDNA variants in a person needs mathematical models that can explain stochastic processes involved in transmission and tissue specificity of variants. Heteroplasmy models have to take into account problems like bottlenecks effects in the development of the oocyte, somatic mutations, and age-driven shifts in variant frequencies (Bibi et al., 2023).

The models of population genetics are used to model the behaviour of the variants of the mtDNA across populations. Such models include the size of the population, migration rates and history of a people in modeling patterns of genetic diversity and differentiation (Bibi et al., 2023).

The coalescent theory gives a mathematical framework to the genealogical relationship between the sequences of mtDNA and the estimation of population parameters with gene sequences.

4.2 Probability of Match Calculations

Match probability calculations are a basic part of the statistical analysis of mtDNA analyses, as they give the quantitative estimate of the evidential significance of genetic analyses. Such calculations are to be carefully considered on the basis of the principles of population genetics, the elements of the database and the particularities of the mtDNA inheritance (Bibi et al., 2023). The forensic and anthropological uses of the mtDNA analysis require the appropriate calculations of probability to be developed.

There are crucial differences between the calculations of the match probability of mtDNA analysis and in those of the nuclear DNA because of the special nature of the mitochondrial inheritance. The lack of recombination and the maternal pattern of inheritance provides some dependencies that also need to be factored into the calculations of probability (Bibi et al., 2023). As opposed to nuclear DNA markers, that can be analyzed as independent loci, mtDNA sequences are individual, closely linked, making special statistical methods necessary. (Bibi et al?)

Determination of the frequency of suitability of a given profile amongst particular population is the prerequisite calculation in the computation process of the basic match probability. This Minimum Number of Representatives (MNR) allows calculating using extensive databases that reflect the genetic foundations of populations in which the evidence or reference samples can be based (McCauley et al., 2023). The selection and referencing population can very much influence

the estimation of the probability of a match and so careful selection of the population is essential in the calculation techniques.

The size and the composition of the database influence precision and accuracy of estimates of match probabilities. Frequency estimates are biased because they may be based on intensive small databases which lack diversity of the populations represented. Population stratification may also result in poor aggregation across genetically heterogeneous groups and related people included in a reference database may cause artificial inflation of the presence of particular profiles (McCauley et al., 2023). Confidence intervals furnish valuable values of doubt linked to the estimations of probability of match. These are the confidence intervals that indicate the statistical variability of frequency values that rely on a finite sample size. The height of confidence intervals is determined by the observed frequency, size of the database and the confidence level. Narrow periods show an accurate estimation whereas broad periods show uncertainty.

The counting method is the simplest one used to estimate frequencies of the profiles of the mtDNA. This approach merely contends the frequency in which a particular profile is present in a reference database and then the result is divided by the total collection of profiles analyzed (McCauley et al., 2023). Nevertheless, such a method can be unsuitable in the case of rare profiles which are not presented in provided databases and different statistical methods need to be used.

Bayesian techniques provide advanced solutions to the task matching of probability calculation that can be characterized with prior data and uncertainty estimates. Such methods enable the researcher to pool information accessed from different sources and update the probability estimates

once gates of new information are obtained (McCauley et al., 2023). Bayesian methods are especially useful in rare profiles or where there is little information on a database.

This is because the theta correction factor takes into consideration the possibility of population substructure whereby there may appear homographic populations. It corrects on the fact that and people belonging to one population might be relatively closely related than what would apply to random mating models (McCauley et al., 2023). The theta parameter measures the amount of subdivision in the population and is used to make more conservative values of probability of a match.

4.3 Statistical Measures in mtDNA Analysis

Statistical tests in the analysis of the mtDNA include a wide variety of mathematical formulae and ideas used to measure the connections between genes, determine the strength of evidence, and give an estimate of uncertainty. These criteria are the basis of scientific interpretation of results of the mtDNA work and allow its practitioners to state results in quantitative form. It is necessary to understand how statistical measures must be used and interpreted to achieve reliable result of analysis of mtDNA (Irazoki et al., 2023).

The metrics used in genetic distances are used to quantify the extent of the deviations of the sequences between the profiles of the mtDNA. These actions present normalized metrics of comparing sequences of any size (age) with one another, as well as taking into consideration any type of mutations. The distance between two forms that is commonly used is the number of differences between them, Hamming distance and, in the case of multiple mutations at a single site, evolutionary distance measures(Irazoki et al., 2023).

Alignment of sequences is a basic process in comparison of mtDNA that directly affects the statistical analysis. The alignment process finds matching points between two sequences and takes into consideration insertion and deletions which can make direct matching difficult. Missing alignment may give wrong distance estimates and wrong statistical conclusions and hence close alignment is important. Phylogenetic statistics characterize the evolutionary relationships between sequences of information in the blending of a variety of genes and give some background to the understanding of genetic distance (Irazoki et al., 2023). These aspects involve an assessment like monophyly, paraphyly and polyphyly to fulfil the various forms of evolutionary relationships. Interpretation of the meaning of genetic similarities and differences in identification settings is significant because of understanding phylogenetic relations.

Diversity indices are measures of the genetic variation among the populations or databases. Ordinary measures of diversity are: nucleotide diversity; haplotype diversity; and indices of sequence diversity. The measures give valuable interpretive context to discriminatory power of the mtDNA analysis as well as evaluation of adequacy of reference databases.

Neutrality tests are checks to see whether visualised attempts of genetic variation can be due to the processes of natural selection or not (Irazoki et al., 2023). The tests are significant in testing the assumptions on which the models of population genetics and probability are based. Considerable departures from neutrality can imply the necessity of different analytical procedures or more caution about interpretation of results.

Population differentiation measures are the extent to which different population/groups of people are genetically different (Irazoki et al., 2023). Such measures constituted F_{ST} (Fixation

Index) and the additional statistics and are significant in choosing the correct reference populations and determining the value of genetic similarities between different populations.

Bootstrap and permutation methods offer non-parametric statistical inference techniques which do not consider any form of distributions. These bootstrap techniques may be used to produce confidence limits and significance tests on a wide range of statistics where standard parametric techniques are not appropriate or unreliable.

Quality metrics determine quality of reliability and accuracy of mtDNA sequence data. Measurements on quality of sequences, depth of coverage, and error rates are some of the measures that are crucial in the guaranteeing the statistical analysis. Any inference carried out using poor quality data results in poor statistical inferences and even inappropriate conclusions.

4.4 Equations Used in Identification Accuracy Estimation

The mathematical model of accuracy of the identification of the mtDNA is based on the chain of inter-related equations that measure numerous factors of genetic comparison and statistical indication. These formulae give the numerical basis to the interpretation of the mtDNA data and evaluation of reliability of the identification conclusion. (Wang et al., 2021). Each equation is concerned with certain parts of the identification process, starting with the simple comparison of the sequences to complicated probabilities usage.

Equation 1: Heteroplasmy Ratio Calculation

$$H = (A_1 / (A_1 + A_2)) \times 100$$

Where:

- H = Heteroplasmy ratio (percentage)
- A_1 = Peak area or intensity of major variant
- A_2 = Peak area or intensity of minor variant

Calculation Example: If the major variant peak has an area of 850 units and the minor variant peak has an area of 150 units:

$$H = (850 / (850 + 150)) \times 100 \quad H = (850 / 1000) \times 100 \quad H = 0.85 \times 100 = 85\%$$

This means the major variant represents 85% of the total mtDNA population, while the minor variant represents 15%.

The fraction of heteroplasmy is a measurement of relative loadings of the various mtDNA versions in one individual. The equation plays a pivoting role in forensic tests in which the heteroplasmy can make the comparison of profiles difficult. The ratio aids in the process of deciding on whether the observed as variations among the samples is a reflection of sequence variation or variation due to heteroplasmy. (Wang et al., 2021). Correct interpreting of the results of mtDNA analyses depends on the knowledge of heteroplasmy ratios, especially when minor variants can be on/or around the limits of detection. It presupposes the correct quantification of the peak and can be subjected to the adjustment of the amplification bias or any other technical component.

Equation 2: Sequence Divergence (D)

$$D = \left(\frac{S}{L}\right) \times 100$$

Where:

- D = Sequence divergence (percentage)
- S = Number of sequence differences
- L = Total sequence length compared

Calculation Example: Comparing two sequences of 400 base pairs with 6 differences:

$$D = (6/400) \times 100 \quad D = 0.015 \times 100 = 1.5\%$$

The sequences show 1.5% divergence, indicating relatively close evolutionary relationship.

Sequence divergence offers a normalized estimate of genetic distance between mtDNA profiles which takes into consideration the difference in the sequence length. The equation allows comparison of different length sequences and forms a framework of evolutionary and phylogenetic interpretation. The greater the value of divergence, the more distant is the relationship, and the lesser the value, the closer the genetic relationship. This estimation presupposes the equal weighting of all the differences, but, in some programs, it can be necessary to compensate transition/transversion bias or other mutational biases. The divergence in a sequence is basic in setting identification levels as well as determining the importance of similarity in genetic contents.

Equation 3: Match Probability (MP)

$$MP = p^3 + 2p(1 - p)\theta$$

Where:

- MP = Match probability
- p = Profile frequency in population
- θ = Population substructure coefficient (typically 0.01-0.03)

Calculation Example: For a profile with frequency $p = 0.02$ and $\theta = 0.01$:

$$MP = (0.02)^2 + 2(0.02)(1-0.02)(0.01) \quad MP = 0.0004 + 2(0.02)(0.98)(0.01) \quad MP = 0.0004 + 0.000392$$

$$MP = 0.000792 \approx 0.0008 \text{ or } 1 \text{ in } 1,250$$

The equation of the match probability is computing the probability that two subjects chosen at random in a population will have a common, identical profile in a particular strand of mtDNA. Theta parameter is included in the calculation, which considers population substructure, non-random mating and population stratification (Yousefi et al., 2022).. The equation offers more accurate estimation of probability more than direct calculation of probabilities of occurrences since it goes ahead to account the fact that there could be a close relationship between the members of the populations than would be assumed in random mating. In order to properly evaluate the evidential meaning of matching of mtDNA and to put identification conclusions into proper statistical context, match probability calculations are critical.

Equation 4: Likelihood Ratio (LR)

$$LR = \frac{P(E|H_1)}{P(E|H_2)}$$

Where:

- LR = Likelihood ratio
- $P(E|H_1)$ = Probability of evidence given hypothesis 1 (match)
- $P(E|H_2)$ = Probability of evidence given hypothesis 2 (coincidental match)

Calculation Example: If $P(E|H_1) = 1.0$ (evidence expected if true match) and $P(E|H_2) = 0.001$ (match probability):

$$LR = 1.0/0.001 = 1,000$$

The evidence is 1,000 times more likely if the samples come from the same maternal lineage than if they are coincidentally similar.

The likelihood ratio is a standardization of the evidential strength which compares the probabilities of a given item of evidence when faced with competing hypotheses. This formula is fundamental to forensic interpretation because it is used to measure the extent to which the evidence is more (or less) probable in one hypothesis than in the other one (Yousefi et al., 2022).. The likelihood ratios that will favour the first hypothesis are greater than one and those that will favor the second hypothesis will be less than one. Such structure allows evaluating the strength of evidence objectively and presents the findings to non-technical communities.

Equation 5: Mitochondrial Substitution Rate

$$\mu = \frac{D}{2t}$$

Where:

- μ = Substitution rate per site per year
- D = Sequence divergence between lineages
- t = Time since divergence (years)

Calculation Example: For sequences with 2% divergence that separated 10,000 years ago:

$$\mu = 0.02/(2 \times 10,000) \quad \mu = 0.02/20,000 \quad \mu = 1 \times 10^{-6} \text{ per site per year}$$

The equation of the rate of the substitution in the mitochondria gives an estimate of the rate at which the mutations grow in the mtDNA timeline evolution. This is the most basic calculation in molecular clock studies and dating of evolution events (Yousefi et al., 2022). The number 2 represents the mutations that have developed independently in the two lineages since they diverged in one common ancestor. The rate of substitution differs in various parts on mitochondrial genome and the substitution in control segments of genome are generally higher in comparison with coding segments. Such estimates of rates are needed in phylogenetic analyses, population genetic analyses, and are needed in archaeological use where timing of the problem is an important element of interpretation.

Equation 6: Genetic Distance Matrix

$$d(i, j) = \frac{-3}{4} \ln \left(1 - \frac{4S(i, j)}{3} \right)$$

Where:

- $d(i, j)$ = Genetic distance between sequences i and j
- $S(i, j)$ = Proportion of sites that differ between sequences i and j
- \ln = Natural logarithm

Calculation Example: For sequences with $S(i, j) = 0.05$ (5% of sites differ):

$$d(i, j) = -\frac{3}{4} \ln(1 - 4(0.05)/3) \quad d(i, j) = -\frac{3}{4} \ln(1 - 0.0667) \quad d(i, j) = -\frac{3}{4} \ln(0.9333) \quad d(i, j) = -\frac{3}{4}(-0.0690) = 0.0518$$

The equation of genetic distance matrix is founded on Kimura two parameter model (Amorim et al., 2019) which corrects both multiple substitutions within the same site which might administer false proximity in the calculation of evolutionary dimension between the sequences. This correction is especially important with increasing sequence divergence, because simple counting of differences leads to underestimation of real evolutionary distance in the event of multiple mutations at the same sites. The equation is under the assumption of equality of rate of transitions and transversion, but more complicated models can include an inequality in rates. Genetic distance matrices are crucial in phylogeny reconstruction, population genetic studies, and evaluation of affiliation among mtDNA in identification places.

Equation 7: Confidence Interval Estimation

$$CI = p \pm z \left(\frac{\alpha}{2} \right) \sqrt{\left(\frac{p(1-p)}{n} \right)}$$

Where:

- CI = Confidence interval
- p = Observed frequency
- $z_{\alpha/2}$ = Critical value for desired confidence level
- n = Sample size

Calculation Example: For $p = 0.02$, $n = 1000$, and 95% confidence ($z = 1.96$):

$$CI = 0.02 \pm 1.96\sqrt{(0.02(0.98)/1000)} \quad CI = 0.02 \pm 1.96\sqrt{(0.0000196)} \quad CI = 0.02 \pm 1.96(0.00443) \quad CI = 0.02 \pm 0.00869 \quad CI = (0.0113, 0.0287) \text{ or } 1.13\% \text{ to } 2.87\%$$

The equation is especially relevant to frequency estimates based on finite databases since this equation quantifies the accuracy of the estimates. Large confidence intervals are associated with less confidence and small interval relationships imply a more accurate estimation. The level of confidence (statistically perhaps, 95 percent) is the possibility of the true population parameter being inside the worked out interval. The confidence longitudes are essential in proper interpretation of frequency estimations and probability of a match in the identification setting.

Equation 8: Power Analysis

$$Power = 1 - \beta = 1 - \Phi\left(z\left(\frac{\alpha}{2}\right) - \frac{\delta}{\sigma}\right)$$

Where:

- Power = Statistical power (1 - Type II error rate)
- β = Type II error rate
- Φ = Cumulative standard normal distribution
- $z_{(\alpha/2)}$ = Critical value for Type I error rate
- δ = Effect size
- σ = Standard error

Calculation Example:

For $\alpha = 0.05$ ($z = 1.96$), $\delta = 0.5$, $\sigma = 0.2$:

$$Power = 1 - \Phi(1.96 - 0.5/0.2)$$

$$Power = 1 - \Phi(1.96 - 2.5)$$

$$Power = 1 - \Phi(-0.54)$$

$$Power = 1 - 0.295 = 0.705 \text{ or } 70.5\%$$

Power analysis determines the sensitivity of a statistical test to identify differences or relationship in their occurrence. Such an equation plays a definitive role in design of studies as researchers can then know how to take relevant sample sizes and study the value of the negative findings (Yousefi et al., 2022). The larger the power value, the greater is the potential to identify actual effects, and low power implies that there is an enhanced possibility that the outcome may turn out as false negative. Power analysis can be especially relevant in the study of mtDNA where samples can be restricted by either the availability of appropriate biological specimen or the expense of analysis. Statistics power gives researchers an idea on the proper design of studies so that the research question can be answered (Müller-Dott et al., 2023).

The basis of quantitative analysis in mtDNA identification is structuralized mathematical equations that offer the means by which objective measures of evidence strength, quantification of uncertainty, and statistical inference can be completed. The adequate use of these equations presupposes the comprehension of the basic premises, suitable data quality, and meticulous analysis of a particular situation of the identification process. Combination of several statistical measurements gives a reliable framework of mtDNA identification which would be able to pass the test of science and be supportive of the right conclusions in forensic, anthropological and archaeological analyses.

The further invention and optimization of the mathematical models of mtDNA analysis belongs to the developing insight into the field of mitochondrial genetics and the advance of the levels of analysis. Future developments can include more detailed models of mutation, better procedures to

deal with heteroplasmy, and better procedures to make population genetic inferences. Nevertheless, basic mathematical concepts described in these equations will remain the basis of quantitative application of mtDNA analysis and identification protocols.

Chapter 5: Comparative Analysis of mtDNA and Other Genetic Markers in Identification

Genetic identification is a sector that has tremendously been boosted with the creation of numerous molecular markers that have their own limit and upside in identifying human beings. Although traditionally used nuclear DNA markers, especially STRs, have dominated the field of forensic genetics (Camargo et al., 2023), the mitochondrial DNA (mtDNA) has become a powerful supplement to the field with its own distinct properties useful in certain identification cases (Tolstik et al., 2024). This comparative discussion contrasts the use of mtDNA with that of other genetic markers such as nuclear STRs, Y-chromosomal markers, and ancestry informative markers to get

a clear picture of each of these in relation to the way they are being used in modern identity practices.

5.1 Nuclear DNA STRs versus Mitochondrial DNA

Nuclear DNA STRs offer the best level of identification in forensic tests because they are due to outstanding discriminatory and innovative statistical models. These markers are present in two copies within an individual and their inheritance resembles that of Mendelian traits in the sense that they can yield personal-specific profiles (Tolstik et al., 2024), which have a high likelihood to differentiate two persons of no relation to a very high extent. The core loci on the Combined DNA Index System (CODIS), are highly discriminating, with unrelated individuals having a probability of the discrimination exceeding, and therefore quite useful in making direct identification (Tolstik et al., 2024).

On the contrary, mtDNA have different and fundamentally distinct characteristics which complement an analysis of nuclear DNA. The great abundance of mtDNA (100-1,000 copies per cell) has a great benefit and application in degraded or minimal biological samples where there would otherwise not be enough or any nuclear DNA (He et al., 2021). This copy number predisposes the mtDNA to be especially useful in difficult forensic instances, such as examination of rootless hair shafts, rudimentary degraded body parts and ancient samples.

These markers have quite different patterns of inheritance and produce different usages. Nuclear STRs use both parental contributions and are recombined, making them precise to identify people but not to track them over many generations (He et al., 2021). The strict maternal inheritance pattern of mtDNA makes clonal like lineages that will be minimally interrupted over

time, allowing identification of individuals when none of their families are available by the use of related maternal ones.

At the same time, however, the main limitation of mtDNA is because of this pattern of inheritance. Whereas STRs can be used to rule out maternal family members against each other, mtDNA cannot make any distinction between an individual and another individual carrying the same maternal ancestry (He et al., 2021). This diminished discriminatory power endows mtDNA with reduced utility in such contexts where discrimination at an individual level is desired and it is therefore better suited to the exclusion reagent or when it is used in a context where it is accompanied by some other forms of evidence.

5.2 Y-Chromosomal Markers and Paternal Lineage Tracking

Y-chromosomal markers can give paternally based genealogical information in parallel with maternal relics of the maternal DNA genealogy, resulting in complementary genealogical rebuilding systems. Similar to mtDNA, Y-chromosome markers are haploid and non-recombining (in non-pseudoautosomal regions) and thus show clonal inheritance on paternal lineages. This property gives Y-chromosomal analysis significant application in paternity analysis, name studies and population genetics research (Kharkov, 2021).

Comparative and contrastive analysis of the correlation of the two markers can be made with the help of the Y-chromosomal and the mtDNA. Both of marker systems offer the cases of lineage-specific, and the two human uniparental lineages have phylogenetic relationships, but they follow different parental lineages (Kharkov, 2021). Y-chromosomal markers are particularly convenient when trying to figure out male DNA in sexual assault cases with high quantities of female DNA,

and also when such testing is probable to be safe, like when that testing with the differentiation of the genome of a female would not be able to provide extra results (Kharkov, 2021)

Y-chromosomal Markers also possess the same weakness as mtDNA in terms of discriminatory yield in that only a substantial sample of all males who are related through the paternal line would have different or rather very close profiles. In addition, Y-chromosomes have regions with an elevated mutation rate, especially in so-called rapidly mutating Y-STRs (Kharkov, 2021), and are able to provide a discriminating power in paternal lineages that rises above that of the more slowly evolving mtDNA.

Y-chromosomal markers seem to be more stable in some areas of the chromosome than in others. Although these regions can be highly conserved, others can be very variable, hence the possibility to do long or short term phylogenetic works or even forensic DNA uses. This heterogeneity is in contrast to the control region of mtDNA in which the majority of forensically valuable variation is found whereas the coding regions are conserved.

5.3 Ancestry Informative Markers and Population Assignment

Another type of genetic markers is called ancestry informative markers (AIMs) which are planned to indicate the ancestry and geographic ancestry of a population. The selection of these nuclear DNA markers is based on the capacity to differentiate the major population groups and the capacity to give probabilistic ancestry assignments (Boudeau et al., 2023). AIMs can be used to supplement the mtDNA and Y-chromosomal markers by providing data on autosomal ancestry in which the data depicts the integrated genomes of ancestors overall that are not necessarily represented as individual parent groups (Boudeau et al., 2023).

When coupled with the analysis of the mtDNA, the presence of AIMs increases the investigative power. Although taxonomical haplogroups of mtDNA may indicate maternal lineage as well as migration patterns (Boudeau et al., 2023), AIMs may deliver a larger range of ancestry data that could indicate population admixture and could lead to more adequate ancestry statistics. This combination proves especially useful when it is possible that the probable population origin of unknown remains might be helpful in terms of providing investigative leads.

Nonetheless, AIMs have an issue with population databases and reference standards. Since ancestry assignments rely on the representativeness and comprehensive coverage of the reference populations, it shows similar problems to those encountered because of mtDNA frequency estimation (Boudeau et al., 2023). Also, ancestry assignments may not be simple with the current growing genetic admixture of modern populations, and may need advanced statistical models to capture the complex population history.

5.4 Single Nucleotide Polymorphisms (SNPs) in Identification

Another type of genetic marker, which is used in identification, occurs in the form of single nucleotide polymorphisms. Nuclear SNPs can be very high density in terms of genome coverage and they can generate very good discriminatory power depending upon the nature in which they are read in large numbers (Allemailem et al., 2021). SNP alleles are specifically well adapted to degraded DNA as shorter amplicon length can be amplified more effectively than longer products needed when using STR.

The use of massively parallel sequencing technologies has made it possible to examine the entire mitochondrial genome instead of only examining the control region; therefore, it has led to

an increase in mitochondrial SNPs (Allemailem et al., 2021). The whole genome strategy greatly increases the discriminatory capability of the mtDNA analysis and is able to distinguish when control region sequencing has weak discrimination capability.

The analogy of the nuclear and the mitochondrial SNPs shows that there is complimentary usage. Nuclear SNPs are great at individual identification, and can give the ancestry information; and mitochondrial SNPs are good in discriminating the maternal lineages, and can give the lineage information (Allemailem et al., 2021). The use of the above two types of markers in the next generation sequencing system offers rich genetic profiling capabilities.

5.5 Degradation Resistance and Sample Suitability

The fact that different genetic markers have different degradation resistance is another important element when it comes to their forensic applications and the circular structure of the mitochondrial DNA in conjunction with the protective matrix of the mitochondria cells makes mtDNA much more resistant to environmental breakdown than nuclear DNA (Yuan et al., 2024). This is the reason that makes the use of mtDNA analysis effective where nuclear DNA analysis is unsuccessful in situations like aging remains, fire victims and samples that have well endured bad weather, etc.

The male-specific chromosome markers are intermediate in degradation resistance, holding up better than the nuclear autosomal markers but doing worse than the mtDNA marker (Yuan et al., 2024). The bigger size of Y-chromosome and its position in the nucleus also exposes it to degradation in a greater way than the mitochondrial genome.

STR (Short Tandem Repeat)'s are most susceptible to decay because they have nuclear location and larger amplicons needed to conduct analysis are nuclear STRs, in spite of their forensic significance. By them a targeted DNA region has been engineered to produce DNA fragment. Actually they work as a markers which produces amplicons (DNA fragments). Success in degraded samples with mtDNA is, however, not repeated with mini-STR (Short Tandem Repeat) systems and reduced amplicon methods, though it is better compared with standard systems (Yuan et al., 2024).

5.6 Statistical Interpretation and Database Requirements

The statistics used to understand the implications of various genetic markers is also very different since they have very different inheritance patterns and understanding of population genetics. Nuclear STR analysis is supported by longstanding statistical frameworks, large population databases, and guidelines of interpretation standardization(Yuan et al., 2024). The independence of nuclear loci means that probability calculations can be easily performed via use of the product rule.

The statistical interpretation of mtDNA is different from that of nuclear STR analyses because of its non-recombining and matrilineal in inheritance. The population substructure and shared ancestry can invalidate the probability of identity and often necessitate conservative estimates of such probability as well as specialized databases. Creation of exhaustive datasets in the form of mtDNA databases is also an uphill and continuous undertaking especially concerning global populations.

Interpretation of Y-chromosomal markers presents the same type of problems as mtDNA in that they must be population-specific with patrilineal inheritance patterns considered. Evidence presented by Y-chromosomes should be treated with caution as mutation rates and null alleles are potential factors that complicate such evidence.

5.7 Future Directions and Technological Integration

The next step in genetic identification will be the combination of various marker systems as opposed to using an individual type. With the development of massively parallel sequencing methods, it is now feasible to obtain nuclear STR, mtDNA, Y-chromosome marker and ancillary ancestry informative marker data on single samples simultaneously to create the most comprehensive genetic profiles possible with the DNA to maximize the identification potential(Zhang et al., 2019).

This combination of marker systems counters the deficiency of relying on any one marker system, as well as taking advantage of the strength of each system. Those cases that cannot be solved using nuclear DNA alone may receive that extra bit of information on the lineage markers, whereas the ancestry informative markers can be used to come up with a lead in the investigation and the population perspectives.

The comparative approach to the results of analyses of mtDNA and other genetic markers suggests a multilayered map of complementing technologies, each of which has strengths and weaknesses. Although nuclear STRs remain the main technique of personal identification, mtDNA gives significant possibilities to work with problematic material and the maternal pedigree. Y-chromosomal markers can provide information about the paternal lineage and ancestry informative

markers can provide background information on the population. The best solution to genetic identification will be to comprehend these disparities and choosing ideal collection of markers with respect to the demands of each case, the circumstances of the samples and the purposes of the investigation. With further technology developments, the combination of several markers systems is soon to be available and allow to take maximum advantages of successful identification, as well as having detailed genetic data to use in forensic, anthropological and genealogical contexts(Zhang et al., 2019).

Chapter 6: Quality Assurance, Standards, and Best Practices in mtDNA Analysis

Analysis of mitochondrial DNA (mtDNA) has emerged as an invaluable resource in forensic identification, paternity testing and evolution. Nevertheless, the validity of the methods applied and the overall soundness of mtDNA data submitted into a case in a legal proceeding or used in a scientific study rely heavily on the observation of strict quality control, attainment of standards and the conformance to the best practices. This chapter discusses the wider methodology of quality control whereby the accuracy, reproducibility and legal defensibility of the results of the quality of mtDNA analyses are guaranteed.

These peculiarities of mtDNA analyses have their challenges and opportunities in front of the forensic laboratories. In contrast with nuclear DNA, mtDNA is present at high copies per cell (in the hundreds or thousands of copies per cell) and is hence of special value in dealing with degraded or underrepresented samples (Camargo et al., 2023). This richness, however, comes at the costs of the exposure to contamination, as well as the maternal manners of inheritance and quite a high mutation rate as such which necessitate special methods of analysis. Such requirements mean that

there must be an effective quality assurance framework to deal with the particular technical and interpretive issues present in mtDNA analysis (Camargo et al., 2023).

The quality assurance requirements in mtDNA testing are critical and interrelated to include laboratory accreditation, possession and expertise of personnel, testing equipment and validation, methods of analysis, proficiency testing and ongoing tracking systems (Camargo et al., 2023). The combination of these enables a holistic approach that not only supports technical exactness, but also up-holds the chain of custody, documentation standards and the consistency of interpretations necessary in applying a forensic context.

6.2 International Standards and Guidelines

The basic bedrock of quality assurance in mtDNA analysis is based on internationally accepted standards and guidelines authored by professional and accrediting bodies. The international organization of standardization (ISO) has developed ISO/IEC 17025 [C.17025-11] that lays down the general requirements over the competencies of a testing and calibration laboratories (Varillas-Delgado et al., 2022). This standard establishes the general framework which covers laboratory quality management systems and represents document control, management of records, corrective action and internal audits.

Particularly with regard to DNA analysis, a collection of standards referred to as ISO 21043 standards covers the competency requirements of DNA profiling laboratories. These criteria focus on the significance of validated procedures, qualified staff, proper places and sufficient quality controls. In the case of mtDNA, laboratories must prove compliance with these standards in the

face of any of the technical difficulties of mitochondrial genome analysis (Varillas-Delgado et al., 2022).

Guidelines on mtDNA analysis have been published in detail at the DNA Commission of the International Society for Forensic Genetics (ISFG). These guidelines address the most important issues of sample management, interrogation techniques, quality assurance practices, interpretation and reporting guidelines (Varillas-Delgado et al., 2022). The ISFG recommendations note the necessity of a protocol defining authenticity between mtDNA transfers and possible contaminants owing to the large copy number of the mitochondrial genomes together with its cross-contaminating liability (Varillas-Delgado et al., 2022).

In the United States, the requirements are set forth in the FBI Quality Assurance Standards for Forensic DNA Testing Laboratories (QAS) in order to engage in DNA testing to be included into the FBI Combined DNA Index System (CODIS). Although it is geared toward the analysis of nuclear DNA, these standards also provide coverage of the testing of mitochondrial DNA, covering the analytical process and the quality assurance/quality checks as well as personnel skills needed (Varillas-Delgado et al., 2022). The Scientific Working Group on DNA Analysis Methods (SWGDM) has also derived certain guidelines to be used during the exercise of mtDNA analysis which augments the FBI guidelines.

European Network of Forensic Science Institutes (ENFSI), in a bid to harmonize the practices of mtDNA analysis among the European laboratories, has stipulated guidelines. These norms include standardization of the processes of analysis, interpretation and reporting of data to coordinate international cooperation with the aim of exchanging information (Xu et al., 2023).

Included in the ENFSI guidelines is the issue of how to handle ancient or degraded samples, which are typical of forensic and archaeological cases.

6.3 Laboratory Accreditation and Certification

Laboratory accreditation can also be understood to be the major mechanism of ensuring that forensic laboratories have the technical proficiency and the quality management system that assure reliable DNA analysis of the mtDNA. The laboratories are placed under evaluation by accreditation bodies which compare them with the criteria set and assess them on a regular basis to determine that the same laboratories remain in compliance (Xu et al., 2023). The process of accreditation includes thorough examination of qualifications of personnel, facilities, equipment, methods of analysis, quality control, as well as managerial systems.

The American Society of Crime Laboratory Directors/Laboratory Accreditation Board (ASCLD/LAB) and American National Standards Institute-American Society of Quality National Accreditation Board (ANSI-ASQ NQAB) are some of the more prominent forensic DNA laboratory accreditation organizations in the United States that offer testing services to forensic DNA laboratories. These bodies have stringent on-site evaluations and laboratories have to prove that they are able to meet the set standards to be accorded the status of accreditation (Xu et al., 2023).

In Europe, national accreditation bodies that are members of the European Accreditation (EA) provide accreditation. These organizations have laboratories evaluated against the standards of ISO/IEC17025 but with additional requirements of forensic DNA testing (Sutherland et al., 2011).

The harmonization of accreditation standard in European countries encourages the recognition of test results across the European countries and also of consistency in the analytical practices.

The best practice of accreditation has various stages that include a thorough self-assessment of the laboratory against standards that apply to it. This is then followed by document review during which accreditors usually go through the quality manual, standard operating procedure, supporting documentation of the laboratory (Sutherland et al., 2011). On-site assessment phase includes personal observations of laboratory work, interviews of staff members and assessment of the facilities and equipment. After successful implementation of these phases, laboratories are granted the accreditation status which is sustained by visiting surveillance and eventual re-surveillance.

6.4 Personnel Qualifications and Training

The experience of the personnel performing the analysis of mtDNA is key to gaining reliable data and the public trust in forensic tests. The quality assurance standards define minimum qualification requirements to various positions in the ladder of laboratories such as the role of technical leaders, DNA analysts and support staff (Sutherland et al., 2011). The possible requirements are generally educational level, and special training, experience and continued professional development.

Technical expertise in DNA analysis using mtDNA is normally gained by a postgraduate degree in molecular biology, genetics, biochemistry or other related areas of science and followed by several years of experience in forensic DNA testing (Sutherland et al., 2011). They should have the role of supervising analytical activities, validation of new procedures, interpretation of complicated results and maintenance of quality. Technical leaders also need to show their

competency in and of mtDNA analysis via formal instruction, proficiency testing, and/or peer review approaches of practice (Zhang et al., 2019).

The analysts conducting DNA testing in the form of mtDNA should be of the right educational background, normally having studied a bachelor degree in natural sciences and having course work in genetics, molecular biology and statistics. They have to go through thorough training programs that include the theoretical basis of the mtDNA analysis, methods of work, quality control measures, analysis of the information, report-writing (Zhang et al., 2019). Training programs would usually have both classroom-based training and practical laboratory training under guidance of trained and certified personnel.

The competency assessments form the essential part of the personnel qualification systems. Such tests appraise the analytical skills of the analysts in carrying out certain analytical tasks, interpreting sophisticated data and writing proper reports (Zhang et al., 2019). Competency tests tend to consist of both written tests on theoretical knowledge and practical evaluation of performance in the laboratory. Before analysts are allowed to process casework samples independently they must be trained in every area of study concerning mtDNA analysis.

Personnel must keep up and develop their competency in this fast-changing mtDNA analysis arena. Quality standards require that the laboratories to give continuous training programs and to make sure that the members of personnel see any technological development, methods, and changes in regulation. This can be in the form of visiting professional conferences, taking part in special workshops, online trainings and also in collaborative research (Zhang et al., 2019).

6.5 Facility Design and Environmental Controls

The physical working conditions during the conduct of mtDNA analysis are also important as they dictate the quality of such data as well as contamination. Forensic laboratories should have facilities that are capable of ensuring proper segregation of various analysis procedures, control environments and effective fractionation interference (Tang et al., 2023). Because the copy number of mtDNA molecules is high, it is of critical importance to prevent contamination because small quantities of extraneous DNA could greatly affect the results.

The laboratory design must ensure that pre/post-amplification activities are spatially separated so as to reduce the risk of product contamination. Pre-amplification rooms (when internal extraction of the sample is done and PCR is set up) must be maintained at positive pressure (as compared to the post-amplification areas) to avoid contamination with amplified DNA products. Physical barriers, individual ventilation system and controlled access procedures provide additional defense against contamination (Tang et al., 2023).

Environmental monitoring systems also monitor important variables, including temperature, humidity, air pressure, and air quality, continuously in laboratory areas. These systems notify in real-time when conditions are outside of an acceptable range and keep detailed records of data used in the quality assurance documentation (Tang et al., 2023). Climate control systems should have stable conditions which prohibit degradation of reagents and samples and allow the maximum use of analytical equipment.

Lines of prevention pertaining to contamination include vigorous cleaning practices, proper way of disposing wastes, and frequent checking of the surrounding environment. Work surfaces

are decontaminated between samples with bleach solutions or by UV irradiation and our equipment is cleaned and maintained regularly to the manufacturer specifications (Tang et al., 2023). Air filtration systems filter out particulate and possible DNA contaminants in laboratories.

6.6 Equipment Validation and Maintenance

The analytical equipment and processes that are to be utilised in in mtDNA analysis has to undergo thorough validation processes to ensure that they are fit to purpose and uphold reliable analysis performance over their working life span. Equipment validation includes installation qualification, operational qualification (Linnet et al., 2012), and performance qualification processes that ensure that equipment is properly installed, that equipment operates properly, and that equipment performs analytically academically.

Qualification of installation confirms that equipment is installed correctly as recommended by the manufacturer and as stated by the laboratory (Linnet et al., 2012). These are verification of electrical connectivity, environmental, safety aspects and lab info system functional integration. The steps in installations and approvals are documented so as to achieve traceability and ease the future troubleshooting.

Operational qualification proves that equipment performs properly within its allowed range of operating parameters. This testing checks every functional operation, safety systems, alarms and control mechanisms to make sure they perform properly (Linnet et al., 2012). Operational qualification instead involves a stress test of characteristic operation at extreme limits, to assure adequate operation and response to failure.

Performance qualification is done to prove that equipment provides accurate, precise results when testing appropriate test materials consistently. Usually, this stage entails the examination of reference standards, control samples, and validation panels to prove analytical performance features e.g. sensitivity, specificity, and accuracy of precision (Linnet et al., 2012). Performance qualification sets out acceptance criteria and estimates of uncertainty of measurements that are used to drive the continuous quality control efforts.

Preventive maintenance programs will ensure that there is ongoing performance of equipment and therefore minimize any unforeseen malfunctions that may jeopardize the analytical results. Such programs involve routine periods of inspection, calibrations, and replacements of the parts according to the manufacturer specifications and the experience of the laboratory. Maintenance activities are well documented and performance of the equipment observed to discern emerging trends that may be an indication of problems to come.

6.7 Analytical Validation and Method Development

Design and QC of analytical processes used to analyze mtDNA should be systematically evaluated in terms of performance characteristics and shown to be fit-for-purpose. Validation of a discussion of the final product starts with its development and optimization (WHITE et al., 2008), followed by extensive testing of the performance of the product and subsequent incorporation in everyday use. This procedure is necessary to ensure that the procedures that are analytical provide reliable, accurate and legally defensible results.

Literature review and pilot experiments enable a partial validation of the development based on initial experiments to ascertain basic analytical parameters. Researchers compare various

extraction techniques, amplification methods, sequencing methods and data analysis algorithms to determine the best combination to use on a given sample type and to achieve the desired analytical goal. Early optimization is aimed at sensitivity maximisation whilst not compromising specificity and avoiding the possibility of artifacts and contaminants (WHITE et al., 2008).

Internal validation is the thorough testing of method performance with specimens that reflect the real situation on the variety of materials that may often be faced in the course of case work. At this phase, state-of-the-art performance characteristics such as sensitivity, specificity, precision, accuracy, robustness and measurement uncertainty is evaluated (WHITE et al., 2008). Assessment of validation samples has to incorporate difficult samples e.g. degraded DNA, mixed samples and low DNA concentrations to perceive the method performance under realistic situations.

Sensitivity studies establish detection limits of various sample type and determine the minimum quantity of DNA that is needed to achieve reliable results. Those routinely imply dilutions of reference materials over the series and determination of the success, pattern of allele dropout, and artifacts with various dilutions (WHITE et al., 2008). The sensitivity studies also determine the effects of PCR inhibitors and sample age to the performance.

Precision determinations determine how reproducible results are within and between analytical runs, across analysts and across time. This method of study usually requires the analysis of the same sample in various conditions in which the measurement can be in different conditions to measure the variance of the measurement. Statistically analysis of precision data gives estimates of the uncertainty to support the interpretation of results and compare.

6.8 Quality Control Procedures

Analytical performance quality control procedures offer continuous monitoring of quality control of any analysis and timely notification about possible flaws that might threaten the quality of the results. These procedures would involve examination of control samples, performance statistics and introduce correction actions in case of the performance not being within reasonable limits. A quality control system has to be built-in the entire analytical process, from receiving samples to reporting.

Positive controls consist of known quantities of target DNA strands and can assure that analytical procedures are working properly. These controls are intended to show various sequence variants and DNA concentrations to assess the performance of the method over the entire concentration range (Mishmar et al., 2002). Of each batch of analyses we include positive controls, whose results must satisfactorily compare against those expected before we report results on other samples of that analysis.

Negative controls are used to confirm that there is no contamination in processing and analyzing a sample. These would be extraction blanks that were run alongside of samples, amplification blanks that would be run into the PCR reactions, and sequencing blanks that would be run into the sequencing run (Mishmar et al., 2002). Negative controls are to be shown to have no signs of DNA amplification or sequencing artifacts prior to sample results being considered.

Reagent blanks will check the quality of reagents in analysis and indicate possible sources of contamination. These controls include processing blank samples with each lot of reagents to determine whether contaminating DNA or inhibitory substances are present. The testing of reagent

blanks expels specific concern in the case of mtDNA analysis due to the sensitivity of the amplification procedures and the possibility that low-level contamination can generate signals to be detected.

Statistical process control techniques can offer an objective assessment of the trends in analytical performance and early indications of problems. Control charts monitor performance measures, which could be the rate of success of amplification procedures, the quality score of sequences, and frequencies of contamination (Mishmar et al., 2002). Statistical analysis can help to reveal any trends, outliers, and changes in performance, which are to be investigated and corrected with the help of the results.

6.9 Proficiency Testing Programs

Proficiency testing programs enhance external assessment of laboratory performance, and give confidence that analysts remain capable of competent performance in the field of mtDNA analysis. These schemes involve the investigation of test samples issued by other entities, the results of which were compared with some reference values or with the consensus results of the leading laboratories. Proficiency testing can be used in various ways, including performance monitoring, comparison of methods, training and accreditation.

Some proficiency testing programs are external in nature and include such organizations as the College of American Pathologists (CAP) and Collaborative Testing Services (CTS). These programs will give out test samples at various intervals of the year, and these tests will be on a variety of aspects of mtDNA analysis such as sequence determination, mixture interpretation,

degraded samples analysis and kinship calculations. Participating laboratories process test samples with their normal laboratory protocols and send the results to be evaluated.

Internal proficiency testing supplements the external programs with more frequent testing of performance and the more focused evaluation of particular analytic difficulties. Internal programs can include reexamination of archived samples whose outcome is known, blind rereviews of past casework samples, or work with special test materials. Internal proficiency testing enables the laboratories to test performance more often and respond to care when certain areas of training or method improvement are in need.

Proficiency testing of the results is made through the comparison of the reported results relative to reference values or results reported by other laboratories. The evaluation criteria involve not only the technical accuracy of evaluation but also the reporting completeness, i.e., the accuracy of the sequence, the correctness of the interpretation, the correctness of the statistics, the correctness of the report layout. Laboratories that generate discrepant results need to carry out root cause analysis and put in place corrective measures to avoid reoccurrence.

6.10 Documentation and Record Management

Diligently detailed documentation and records management systems are paramount tools to quality assurance when using mtDNA. These systems provide tracking of samples and results, legality in the handling of evidence as dictated by the statutes and give the documentation required in the quality assessment and accreditation. Documentation needs to include case records, analytics data, quality control data, equipment upkeep records and training documentation(Zhang et al., 2019).

All records associated with sample receipt, chain of custody, method of analysis, quality control results, data interpretation and final reporting are contained in a case file documentation. These records are supposed to be complete, accurate, and up-to date, with every entry to be done during the process of doing the activities. The minimum detail required in the case files should enable independent evaluation and checking of results by appropriate individuals.

Raw data documentation and analytical data documentation consists of raw data recorded by all analytical instruments, data processing documentation and worksheets of interpretation. Electronic storage of data should be safeguarded and must ensure that they cannot be altered by unauthorized parties, and its records of all accesses and changes should always be recorded. The measures to the data integrity are routine backups, access controls, and checking of data transfer(Linnet et al., 2012).

Quality control documentation includes all quality control sample data, performance monitoring information, corrective action documentation and method validation studies. This documentation can show that a continuing quality assessment is being complied with and will show proof of the analytical dependability. The quality control records should be easily available for internal inspection and by an external audit.

Training documentation consists of personnel qualification, training completion records, and competency assessment, and continuing education records. These records can show that personnel have the knowledge and skills they require to conduct mtDNA test and have updated expertise. Training records should be well preserved during and after employees have left the organisation(Linnet et al., 2012).

6.11 Best Practices in Sample Handling and Processing

Appropriate sample handling and processing protocols are core in ensuring that the integrity of the samples is maintained, contamination is averted, and likelihood of obtaining meaningful results with regard to the isolation of mtDNA is enhanced. Best practices in this direction include the methods of sample collection, transportation, storage, and processing which considers the specifics of the mitochondrial DNA and what is required of the forensic examination.

Procedures of collecting samples should exclude possibilities of contamination with minimization of the samples numbers and maximization of DNA recovery potentialities. Collection staffs are to work with sterile collection supplies, they are not to cross contaminate samples and also to package samples in the right packaging materials that will not cause degradation of samples. Collection documentation, such as photographs and a description of the collection procedure, give valuable context to laboratory analysis and interpretation of the result (Ferreira and Rodriguez, 2024).

Chain of custody control measures guarantees sample integrity and admissibility in laboratories where the case might have to be drawn up, since the evidence might require documentation of all activities carried out on the sample, including collection, storage and analysis, as well as disposal. The records of chain of custody should indicate all the personnel involved in handling of the samples, reasons of such handling, times and dates of transfer, and storage conditions at various periods during custody. Samples cannot be accessed by the wrong individuals because security procedures have been taken into consideration to preserve the evidentiary properties of the sample.

Sample triaging may optimize the laboratory workflow by choosing analytical priorities to ensure samples with the highest probability of being useful are analyzed and approaches to each type of sample are chosen. Triage considerations would include sample age, storage conditions, type of tissue and apparent DNA content and case requirements. A preliminary screening procedure can be applied to evaluate the quality and the quantity of DNA prior to analysis of the entire mtDNA.

Samples of different types need to be extracted in an optimum way whereas the prevention of contamination should not be put at jeopardy. Specialised extraction procedures can be necessary difficult cases like degraded skeletal material, hair shafts or ancient material. The DNA extraction procedure must produce as high a yield as possible but filter out competing amplification inhibitory substances that may be present in the sample.

Conclusion

This extensive analysis of the contribution of the mitochondrial DNA in human identification shows that this exceptional scientific development is accompanied by substantial methodological issues. The history of mtDNA analysis, since its inception in the 1990s as an experimental method, to modern times where its use is well-established and in wide-spread practice, illustrates both the insurmountable potential of molecular genetics and the technological issues that arise when translating scientific discovery into mainstream forensics specimen identification.

Fundamental Strengths and Unique Contributions

The major advantage in the analysis of mtDNA is that it has exceptional use in cases of difficult biological materials where nuclear DNA analysis is not productive. Such extreme sensitivity of providing mitochondrial genome copies in comparison to only a few copies of nuclear DNA has an advantage of facilitation of use of highly degraded, aged or limited samples. This feature has been revolutionary in cold case investigations, victim identification of mass disaster victims, and archaeological work when standard identification processes do not suffice.

The maternal transmission of of mtDNA provides genealogical benefits that are not present in a nuclear DNA. With this form of clonal inheritance, identification can be through maternal relatives that can be multiple generations apart, which serves as investigative leads when direct family reference material is out of the question. Examples of historical crimes include the identification of the Romanov family and the victim identification work in the World Trade Center that illustrate the rich role of the use of mtDNA analysis to conclude important societal, legal cases(Ferreira and Rodriguez, 2024).

The double-stranded circularity and inherent protection within the mitochondrial matrix, provide data which make up the structure and content of the mtDNA endow a great aptitude to withstand environmental wear and tear. This reliability allows effective results of samples put through harsh conditions such as heat, moisture, chemicals, and long degradation times. That resilience has given the mtDNA analysis a great value especially in forensic cases where fire victims, skeletal remains and old archaeological specimens gave their value.

Significant Methodological Limitations

The mtDNA analysis, though limited in scope, has a number of strengths which overcome these limitations. The most important weakness is the diminished discriminatory power as compared to markers of nuclear DNA. Although nuclear STR analysis can discriminate probabilities greater than $1/10^{15}$ between un-related individuals, discrimination using mtDNA is significantly lower typically between 1/100s to 1/1000s (Reidla et al., 2003). This minimised the discrimination, as a result of smaller genome, and a lack of recombination, which limits the variety of sequences.

Failure to differentiate between maternal relatives is a major limitation in most identification cases. Every person with a common maternal ancestry will have the same or at least very similar sequences of the mtDNA such that no distinction can be made between mothers, daughters, maternal siblings and other related adults based on mtDNA alone. This drawback requires special case review, when the analysis of mtDNA can help investigators.

Heteroplasmy, which is the presence of more than one variant of mtDNA in an individual, is an added complication of interpretation. Although heteroplasmy may be used as a source of

increased discriminatory power, when properly characterized, it does make profile comparison and interpretation complex. The phenomenon poses special analysis considerations and documentation, especially when there are minor variants near the detectable scale. ((Amorim et al., 2019)

Technical and Analytical Challenges

Technicalities involved in mtDNA analysis have perennial issues that need to be attended to and enhanced. The levels of contamination risk are high because of large copy number of the mtDNA and the sensitivity of the analysis techniques. Cross-contamination of the samples, contamination due to laboratory staff and environmental contamination may give inaccurate results unless adequate controls are in place. This document shows that rigorous measures to prevent contamination such as physical separation between pre- and post-amplification processes are important but difficult to undertake consistently.

Mixtures of DNA sources such as those found in samples with several contributors pose an extremely difficult analysis problem. Contrary to nuclear DNA mixtures that can easily be resolved based on known statistical algorithms, separating mixtures of mtDNA is more problematic because of a lack of independent loci in addition to potential overlap among sequences changes(Antil et al., 2022). This weakness is used to analyze sexual assault material, samples of two or more people and degraded samples with background DNA.

The analytical difficulties of the phenomenon of nuclear mitochondrial DNA segments (NUMTs), in which segments of the DNA of the mitochondrion were transferred to chromosomes during evolution, are also present(Antil et al., 2022). Such nuclear copies may also be co-amplified

with authentic mitochondrial DNA, to give emergent apparent heteroplasmy or sequencing artifacts unless adjusted by careful design and analysis procedures.

Statistical and Interpretive Framework Concerns

Statistical interpretation of the evidence of the mtDNA is not a simple object of knowledge, it demands high levels of expertise as far as the positions in population genetics are concerned, and the possible restrictions of the databases should be taken into consideration. The review indicates that frequency estimates and calculations of match probabilities are quite sensitive as to the size and content of reference databases, which are incomplete in many world populations. This constraint has a special impact on the interpretation of evidence of people that have mixed heredity and are not of European descent (Reidla et al., 2003).

The mathematical models of mtDNA interpretation are scientifically strong, but then they require special skill that is unlikely to be readily available in all laboratories dealing with forensics. The estimation of likelihood ratios, confidence intervals, and specific frequency estimation requires the good knowledge of both molecular genetic knowledge and statistics. The misunderstanding of such calculations may result in misinterpretation about the weight of evidence of the mtDNA matches or exclusions.

Quality Assurance and Standardization Issues

The review also notes that quality assurance measures are practiced unevenly in different laboratories even though it is based on a series of international guidelines. There are some accreditation standards, but these standards are usually implemented and enforced differently across the jurisdictions (Reidla et al., 2003). This inconsistency impacts the reliability and legal

admissibility of the use of mtDNA in cases, especially international cases that involve cross-border collaboration.

Certification requirements, including training and competency testing, are demanding consequently more so than those of standard nuclear DNA testing, and there have not been standardized programs of certification(Reidla et al., 2003). The large amount of expert knowledge and skills necessary in particular to appropriately interpret heteroplasmy, mixtures analysis and population genetics calculations results in the need to design and implement rather lengthy training programs that cannot be easily made available universally.

Future Directions and Technological Integration

The new generation sequencing technologies promise useful solutions to some of the constraints. Mitochondrial genome sequencing improves resolution beyond that of the control region and has the potential to clarify cases in which there might otherwise be insufficient discrimination. Nonetheless, the adoption of the technologies is an expensive endeavor because of the significant investment in the form of equipment and training and the need to conduct validation studies.

Combination of the mtDNA analysis with other genetic markers such as nuclear STRs, Y-chromosomal markers, other ancestry informative markers is a logical extension in a direction of comprehensive genetic means of identification. The multi-marker method has the capability of optimizing the advantages of each system without losing self-insufficiency.

Critical Assessment and Recommendations

This critical review exposes the fact that the analysis of the mtDNA has been extremely successful in providing genetic identification beyond the boundaries of nuclear DNA analysis. Its use needs a case-by-case analysis to be properly used and interpreted. The technology can be used most effectively in selected cases of degraded samples and maternal lineage studies but its use in individual identity remains unacceptable compared to that of nuclear DNA.

It would be useful to strengthen the standardization of analysis procedures, broaden reference databases and to improve training of practitioners in the field. The reliability and utility of the pattern of analysis of mtDNA could be enhanced tremendously through international collaboration in development of databases and harmony in quality assurance.

The future evolution of the mtDNA test is predicated on the keeping realistic expectations about the test and capitalizing on the unique contribution it has to make to the discipline of human identification. The balancing of technological promotion with effective scientific scrutiny is what will make the future of D-Nails bright, because that is how this dynamic can be used to enhance justice and scientific discovery with the highest quality of precision, accuracy and validity.

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