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Mitochondrial DNA in Fornescis: Principles, Applications, and Limitations

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Abstract

Forensic science involves the scientific analysis and interpretation of evidence that is used in legal proceedings to establish guilt or innocence. The use of mitochondrial DNA (mtDNA) in forensics is crucial as it has several advantages over other types of DNA. For instance, mtDNA is more resistant to degradation than nuclear DNA, making it suitable for analysis even in small or degraded samples. Additionally, mtDNA is maternally inherited, and this makes it useful in tracing an individual's maternal lineage or identifying relationships between individuals across multiple generations. Another advantage of mtDNA analysis is its reliability and accuracy since it is less prone to errors or contamination. This is because mtDNA is found in the mitochondria, which are separate from the cell nucleus and are less likely to be affected by extraneous DNA or contamination. Overall, the use of mtDNA in forensic science has significantly improved the accuracy and reliability of criminal investigations, making it a vital tool in the criminal justice system. In mitochondrial DNA (mtDNA) analysis, the two regions of the mtDNA genome that are typically analyzed are hypervariable regions 1 and 2 (HV1 and HV2). HV1 and HV2 are non-coding regions of the mtDNA genome, meaning that they do not contain information for the production of proteins. Instead, they contain a high degree of variation in their nucleotide sequences, which makes them useful for identifying individuals and their maternal lineages. The interpretation of mtDNA analysis results from HV1 and HV2 involves comparing the nucleotide sequences obtained from the evidence sample to a reference database of mtDNA sequences. This comparison is done to identify any matches or differences between the sample and the reference sequences. If there are no differences or only a few, the sample is considered to be a match to the reference sequence. The more differences there are between the sample and the reference sequences, the less likely it is that the sample and reference sequences came from the same individual or maternal lineage.The interpretation of mtDNA analysis results from HV1 and HV2 can be used to provide information about the identity and maternal lineage of an individual. For example, if an evidence sample matches a reference sequence from a known maternal relative, this can be used to support the identification of the individual.

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1. Introduction

Forensic science, abbreviated as 'forensics,' is a set of theories and methods that are used across a wide range of fields. In general, forensics' goal is to address questions of importance to the legal system, in both criminal and civil cases [1]." DNA fingerprinting" also known as DNA typing (profiling), was initially described in 1985 by Alec

Jeffreys, an English biologist used in forensic science. Dr. Jeffreys discovered that certain DNA areas included DNA sequences that were repeated repeatedly adjacent to one another[2]. The typing of Variable Number Tandem Repeat (VNTR) loci through Restriction Fragment Length Polymorphism (RFLP) analysis is a valuable technique employed in genotyping and molecular epidemiology research. VNTRs serve as crucial genetic markers in RFLP-based genome mapping and have become indispensable in forensic investigations involving criminal cases. [3,4], Analysis of single nucleotide polymorphisms (SNPs) using polymerase chain reaction (PCR)-based techniques [5, 6], VNTR loci, mitochondrial DNA direct sequencing and short tandem repeat (STR) loci (mtDNA) [7, 8]. Identity cases in violent crimes, lesser crimes, acts of terrorism, mass disasters for more than 30 years around the world and cases of missing persons have all been addressed using DNA typing analytical technologies [9]. Traditional short tandem repeat (STR) type schemes may not always function. Nuclear DNA typing techniques frequently fail to yield findings with DNA samples from the distant past or those that have significantly deteriorated. Mitochondrial DNA (mtDNA), on the other hand, can sometimes restore information from DNA that has been damaged by the environment. While a nuclear DNA analysis is normally preferred, an mtDNA result is preferable to no result at all. [10]. mtDNA has been widely used to enhance our understanding of the worldwide and geographical structure of global migration from our latest maternal season [11]. It was toward the present. Human phylogenetic ancestors are roughly 150 to 200 thousand years ago [12]. Mitochondrial DNA (mtDNA) has proven to be a potent tool in unraveling the enigmas surrounding numerous historical murder cases. The utilization of mtDNA typing, which involves examining sequences from the control region or conducting a comprehensive analysis of the full genomic sequences, has become indispensable in the scrutiny of various forensic specimens, including aged bones, teeth, hair, and other biological samples with scant DNA content. MtDNA boasts several advantageous features for forensic investigations, particularly its lack of recombination, high copy number, and inheritance through the maternal line. The remarkable sensitivity of mtDNA analysis empowers forensic experts to extract valuable insights from aged pieces of evidence linked to cold cases and minute traces of biological material. In criminal proceedings, mtDNA can furnish valuable supplementary data when autosomal information is scarce or negative, and it can be especially beneficial in scrutinizing evidence associated with sexual assault cases. Techniques developed for the analysis of ancient DNA offer valuable approaches for sequencing severely degraded materials discovered at crime scenes. [13, 14].

2. Mitochondria and Mitochondrial DNA Characteristics

Mitochondria are eukaryotic cells' vital organelles that play a key role in energy production. An organelle of mitochondrial origin can be found in all eukaryotic species. Mitochondria are found in all nucleated cells in mammals [15]. Mitochondria are considered essential organelles that conduct a wide range of essential tasks, including ATP generation and being linked to programmed cell death. The outer membrane, inner border membrane, intermembrane space, cristal membranes, intracristal space, and matrix are the six compartments of mitochondria. The internal structure of mitochondria is complicated and changing. Mitochondrial pleomorphy and motility reflect this intrinsic dynamism. Mitochondria have their genetic material (mtDNA) [16]. The number of mitochondria per cell is mostly determined by the metabolic needs of the cell, while mitochondrial DNA copy numbers normally range from 100 to 10,000 copies per cell [17]. Mitochondria are morphologically diverse, varying in size (1-10μm) and shape regularly. They have a variety of shapes, from long interconnected tubules to small separated spheres as shown in (figure. 1) [18].

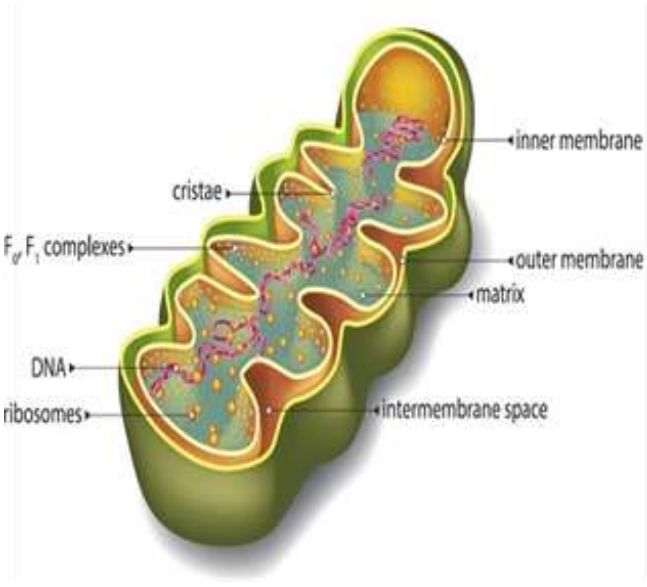

Figure 1. Structure of Mitochondria [18].

Mitochondria are crucial for cellular energy production. The organelles have their genome, which has been altered genetically. The female germ line is the only way for the mammalian mitochondrial genome to be passed along. [19]. This non-Mendelian form of transmission means that an individual acquires their mitochondrial DNA from just one of their sixteen great-great-grandparents, although this maternal ancestor contributed only one-sixteenth of the individual's nuclear DNA. The contribution of mitochondria to the zygote nearly entirely by the egg cytoplasm, with limited transmission of paternal mitochondria from the sperm, is the reason for clonal inheritance through the female lineage [20]. Mitochondrial DNA (mtDNA) analysis has been employed to investigate human origins and evolutionary processes by examining dispersal patterns and determining divergence and coalescence dates within and among species. MtDNA possesses distinct attributes that differentiate it from nuclear DNA. [21, 22]. The human mitochondrial genome is a 16.6-kb closed-circular double-stranded DNA molecule. In humans, the mitochondrial genome is 16,569 base pair (bp) long and comprises 37 genes that code for two rRNAs, 22 tRNAs and 13 polypeptides. mtDNA has different characters from Nuclear DNA shown in Table(1) [23].

Table 1. The difference between Mitochondrial DNA and nuclear DNA[23].

Feature	Nuclear DNA	Mitochondrial DNA
Size (in bp)	~3 x 10^9	16,569
Shape	Linear double helix	Circular double helix
Inheritance	Both parents	Maternal
DNA copies/cell	2	~10-50,000
Number of genes	~20,000 protein coding	13 protein-coding + 24 non-protein coding
Gene density	~1 in 40,000 bp	1 in 450 bp
Introns	Found in almost every gene	Absent
% coding DNA	~3%	~93%
Histones	Associated with the DNA	Not associated with the DNA
CpG islands	24,000-27,000	None
CpG density	1%	2.6.%
Methylation	Present (3-4% of all Cs (~70-80% of all CpGs)) (mainly CpG)	Present (~1.5.-5% of all Cs) (both CpG and CnonG)
Hydroxymethylation	Present (0.0.3-0.6.9% of all Cs)	Present (6-8)

Other Interesting Dissimilarities mtDNA and nuclear DNA are two types of DNA. Nuclear DNA has a distinct genetic coding from mitochondrial DNA. In mitochondria, the codon UGA is used as a chain terminator, but it can also encode for the amino acid selenocysteine. However, UGA can also be reassigned to encode for tryptophan in some organisms, including Phytoplasmas and an alpha-proteobacterial symbiont of cicadas. UGA is considered a stop codon in its universal (nuclear) code of genetics. Mitochondrial DNA has fewer DNA repair systems than nuclear DNA, resulting in greater mutation rates. Furthermore, the mtDNA polymerase's lack of proofreading capability amplifies during replication, and mutations occur. The mutation rate is ten times higher (in comparison to nuclear DNA) aids in the introduction of Variation in samples from maternal lineages that would otherwise be unvarying. While mutations can pose challenges in establishing clear familial connections, their increased unpredictability can be advantageous for most applications in human identification testing. The circular shape of mtDNA renders it less sensitive to exonucleases, which cause DNA molecules to break down, than genomic DNA. The mtDNA survival rate is also improved by encapsulating it in a two-walled organelle [24]. Mitochondrial DNA comprises a guanine-rich heavy (H) strand and a cytosine-rich light (L) strand shown in (Figure 2). 12 of 13 polypeptide-encoding genes, 14 of 22 tRNA-encoding genes, and both rRNA-encoding genes are found on the heavy strand. In mtDNA, introns do not exist, and all of the coding sequences

are continuous [25,26]. The displacement loop (D-loop) is an 1121 bp segment of mitochondrial DNA (mtDNA) that includes the origin of replication for the H-strand (OH) and the transcription promoters for both the L and H strands. This region represents the only non-coding portion of mtDNA. Displacement loop (D-loop) is a non-coding region consisting of 1200 base pairs which is not involved in the production of gene products. The D-loop contains three hypervariable regions (HVI, HVII, and HVIII), which are relevant and studied in a variety of fields, including population genetics, medical genetics, evolutionary studies and forensic investigation. The D-loop is a non-coding triple-stranded length of mtDNA with the highest mutation rate [27, 28].

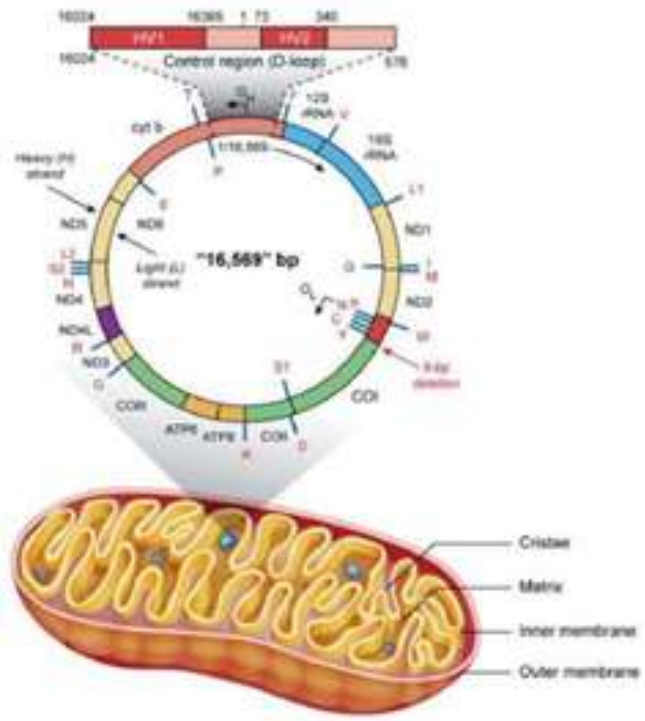

Figure 2. The circular human mitochondrial genome (16569 bp) long. Two highly variable areas, HVI and HVII, are found in the regulatory region (D-loop), and are commonly used in population genetic investigations[27].

Since its introduction, the examination of the short variable control region within mitochondrial DNA (mtDNA) has demonstrated its reliability and widespread adoption. Mitochondrial DNA can be analyzed using methods such as restriction site polymorphisms, SNP typing, pyrosequencing, or Sanger sequencing. The mitochondrial genome has a high mutation rate, however, it is not uniform throughout the molecule. Because there are no genes in the noncoding regions, mutations accumulate at a 10-fold greater rate than in the coding regions. [29, 30] As a result, they are the most varied regions among persons. On average, the D-loop reveals 1-3 base changes across non-related people each hundred bp. [31]. The HVI and HVII regions, which span 16024-16365 and 73-340, respectively, are the most comprehensively studied regions to date. HVIII is a less variable region, although it is increasingly being studied, as comprehensive control region analysis is now suggested. HVIII spans 438-574. Single nucleotide polymorphisms are tracked in the mtDNA genome (SNPs), Single base alterations, insertions, and deletions are all possible. The revised Cambridge reference sequence (rCRS), the first published mtDNA genome in revised form is still used to identify changes between individuals [32]. Other sequences under scrutiny are subsequently compared to this reference sequence, with any variations meticulously documented to establish an individualized mtDNA profile. Certain principles must be adhered to establish a shared basis for the analysis[33]. The C-stretch length heteroplasmy has been noticed in both Hypervariable regions HVI, HVII, and HVIII, and the heteroplasmic length variation rates showed considerable variations between populations. Individuals might have two or sometimes more different C-stretch lengths in several tissues. Furthermore, heteroplasmy in C-stretch length was seen in various hair shafts and even different regions of the same tissue [34]. In comparison to other sections of mtDNA, the C-stretch is difficult to detect as an 'out-of-phase' nucleotide pattern, and the C-stretch length heteroplasmy enhances the difficulty in DNA sequencing [35, 36, and 37]. The presence of a C-stretch within the central portion of mtDNA HVII is crucial to acknowledge, as neglecting to interpret these sequence alterations could impede the effective utilization of the mtDNA control region in both forensic and population genetics contexts. Consequently, the C-stretch may hold significant relevance in the realm of identification within forensic and population genetics investigations [38]. Mitochondrial DNA is a circular molecule that exists in multiple copies, typically numbering from 100 to 1000 per cell. This characteristic renders it advantageous when dealing with a limited number of target sequences, a feature particularly pertinent in fossil research (figure 3).

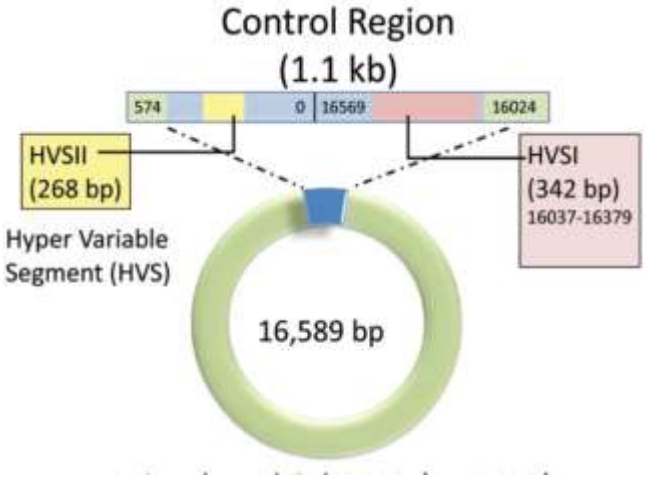

Figure 3. Mitochondrial DNA genome containing hypervariable regions I and II

3. The Use of mtDNA in Evolutionary Studies

i. High Copy Number: MtDNA is present in large quantities within cells, with an average of about 10,000 mitochondria per cell, each containing approximately 5 to 10 mtDNA molecules. Its abundance simplifies isolation and purification due to the small size and the location of mtDNA within a cytoplasmic organelle [23].

ii. The complete mtDNA nucleotide sequence of one individual has been determined [23]. This is referred to as the reference sequence. All new mutations may be mapped to a specific position on the reference sequence either by restriction enzyme mapping or DNA sequencing. In this way the exact nature of the mutation and often its consequence (if in a coding region) can be established, and this makes it possible to examine the evolutionary history of different regions of the mtDNA genome [39].

iii. Perhaps the most useful property of mtDNA is its strict maternal inheritance and the absence of recombination [40, 41]. This means that phylogenetic trees that show the relationship of all mtDNA types, may be interpreted as genealogies that reflect the maternal history of the species.

iv. mtDNA accumulates mutations at a rate approximately 5 to 10 times faster than nuclear DNA. It has been estimated that the average rate of mtDNA sequence divergence is about 2 to 4% per million years compared with 0.5% per million years for nuclear DNA [42, 43]. In this way, population differentiation is accentuated facilitating the identification of subtle differences both within and between groups, making mtDNA extremely useful in evolutionary studies [44].

4. mtDNA Applications

Mitochondrial DNA variation is examined extensively in a variety of fields other than forensic science. A variety of disorders have been related to mtDNA mutations by medical researchers [45]. To discover relationships, Human mtDNA sequence variants are compared to those of other species by evolutionary scientists. The findings derived from the analysis of control region sequences in ancient bones demonstrate that Neanderthals are not the immediate progenitors of contemporary humans [46]. With the use of mtDNA testing, many interesting historical identifications have been made in recent years. Michael Blassie's remains have been identified from the Unknown Soldier Tomb during the Vietnam War [47]. Tsar Nicholas II's bones were unearthed in Russia in 1991 and proved to be his [48, 49, and 50]. Anna Anderson Manahan's claims to be the Russian princess Anastasia were debunked [51]. Assessing small quantities of DNA poses a challenge in both forensic genetics and ancient DNA analysis. This difficulty arises from limited sample availability, either due to age or the source being microscopic stains or trace DNA (commonly referred to as touch DNA). Furthermore, DNA can be impacted by unusual events like mass disasters, floods, fires, or natural environmental conditions, leading to DNA degradation. DNA degradation implies that the DNA segments within the sample are fragmented and challenging to work with. However, mitochondrial DNA (mtDNA) analysis offers greater sensitivity and enables investigations into kinship due to its maternal inheritance pattern in certain samples. Depending on the range of mtDNA genomes examined in the study, some geographical information may be recovered. Consequently, mtDNA proves to be a valuable identifier for very ancient samples and is capable of addressing historical inquiries [52,53]. In forensic investigations, human mitochondrial DNA has proven to be a useful tool. Investigators have been able to use sequencing information to identify war dead, missing people, and people embroiled in mass tragedies and criminal cases due to its polymorphism nature and maternal inheritance [54]. When biological evidence is deteriorated (charred remains) or in limited quantities, mtDNA will be employed. In this case, the evidence consisted of (hairs, bones, teeth). Cases of missing persons (use skeletal remains). Individuals are being identified as suspects (hair evidence) [55].

5. Mitochondrial Sequencing in Forensic Casework

There are numerous methods for determining the sequence of mitochondrial DNA [56, 57, and 58]. Several steps used in the process of comparing mitochondrial DNA sequences . Because of the large number of copies per cell, mtDNA has a higher risk of contamination than nuclear DNA and must be extracted in a very clean laboratory setting. To avoid any potential contamination, The reference samples should be analyzed after the evidence samples have been thoroughly processed. Sanger sequencing chemistry is extensively used to analyze mitochondrial DNA. [59, 60]. DNA sequencing is a procedure where the nucleotide bases of a DNA molecule are read. To maintain precision, sequencing is carried out in both the forward and backward directions, and then the complementary strands are compared. In lab reports, results are typically presented as a percentage of variation from the rCRS reference sequence. For example, if a C nucleotide is identified at position 16126, as opposed to the T in the reference sequence, it would be recorded as 16126C. In cases where no further nucleotide mutations are observed, it is assumed that the remaining portion of the sequence corresponds to the rCRS (revised Cambridge Reference Sequence) figure (4).

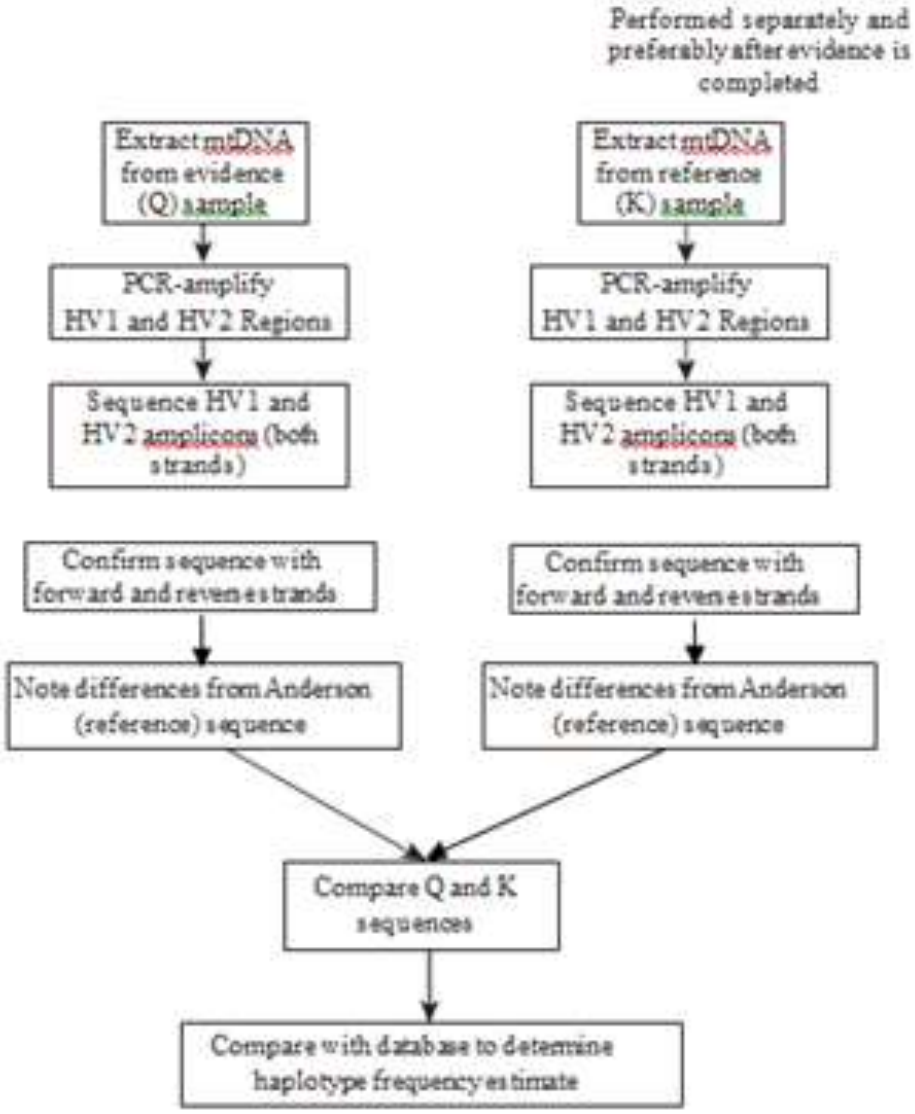

Figure 4. The procedure for analyzing mtDNA samples. [60].

6. Sequencing of DNA

Over 30 years ago, the Sanger method for DNA sequencing was originally disclosed. Once the polymerase incorporates the ddNTPs as chain terminators, the next step involves a resolution process that allows for the determination of the DNA sequence with single-nucleotide precision. With a ddNTP[59]. In the Sanger sequencing method, individual DNA strands are sequenced in distinct reactions, with each using a single primer. Typically, PCR primers, either the forward or reverse ones, are employed for this purpose. To distinguish between the four different ddNTPs (dideoxynucleotide triphosphates), they are each tagged with fluorescent dyes of distinct colors. For instance, ddTTP (thymine) is associated with a red dye, ddCTP (cytosine) with a blue dye, ddATP (adenine) with a green dye, and ddGTP (guanine) with a yellow dye, although the latter is often represented in black for enhanced visibility. Over the last decade, DNA sequencing chemistries have developed from using a simple Taq polymerase to today's well-balanced Big Dye. Brighter dyes have enhanced signal-to-noise ratios, allowing for more accurate findings with less material. Each DNA sequencing procedure can now require as little as 1 ng of the mtDNA PCR product [62]. MtDNA sequencing has also been done using next-generation DNA sequencing. The apparatus, chemicals, and data analysis software needed for the method are currently cost-effective, and also the method is unstable and time-consuming. However, next-generation sequencing technology is fast evolving and could someday supplement, or may completely replace, Sanger sequencing techniques [63].

7. mtDNA Results Interpretation

To compare the altered and examined mitochondrial DNA (mtDNA) sequences between a question (Q) sample and a known (K) sample, you can utilize the features available in the Revision History report provided by NCBI. When viewing a nucleotide or protein display page, click on "Display Settings" located in the top left corner. This will bring up a menu where you can find 'Revision History. This page offers links to view each update, including minor ones that do not alter the sequence. You can employ the provided tools to compare any two versions either by highlighting the differences in text details for each update or by conducting a sequence alignment, (Figure.5). A section of the HV1. Between samples being compared, all 610 nucleotides (positions 16024–16365 and 73–340) are generally analyzed.

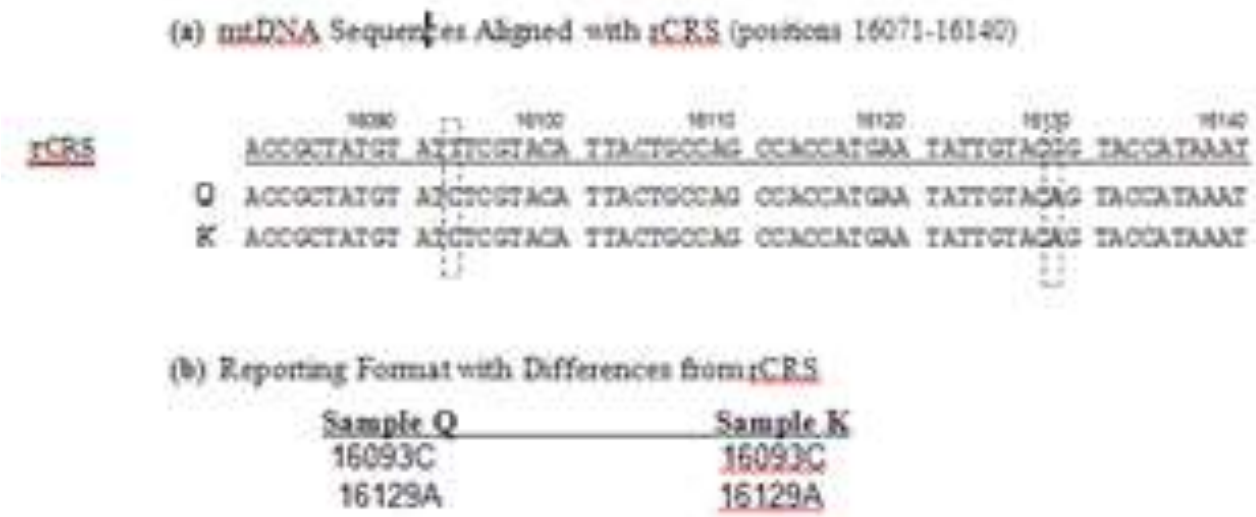

Figure 5. (a) A sequence alignment comparisons for question Q and K samples, with (b) discrepancies converted to revised Cambridge Reference Sequence (rCRS) of reporting[64].

Based on the Q-K comparison, mtDNA sequencing results can be divided into three groups: exclusion, inconclusive and failure to exclude. SWGDAM mtDNA interpretation guidelines make the following recommendations [64]. Exclusion: If the questioned and known samples have two or more nucleotide discrepancies, samples may be ruled out as coming from maternal lineage or the same

individual. Inconclusive: The result will be inconclusive if a single nucleotide variation between the questioned and the known samples exists.
Failure to Exclude, Cannot Exclude if the sequences from the questioned and the known sample exhibit a shared base at each position or common length variance in HV2 C-stretch, the samples may not be ruled out as being from the same individual or from maternal lineage. In case of uncertainty (e.g. heteroplasmy) in sequence, a common base is also known as the shared base [58]. For example, if there is a sequence that has heteroplasmy at a certain location while the other does not (Figure 6), they cannot be separated. Length variance alone, particularly in the homopolymeric C-stretch of HV2, may not be used to establish an exclusion interpretation [62,64]. A number of instances are given, along with their relative interpretations based on SWGDAM standards. Because mutations have been identified between mothers and offspring, a single base difference is regarded as an "inconclusive finding." [56]. If a maternal relative is chosen as a reference sample, for example, there could be a single base variance between two maternally related samples. If a result is equivocal, further samples, usually to clarify the interpretation, more reference samples are frequently conducted [65]. Hairs from a single person may be combined to determine heteroplasmy. When multiple hairs from the same individual are combined, it is possible to detect heteroplasmic variants in the mtDNA. In cases where heteroplasmy is present, different hair strands may carry distinct variations in the mtDNA sequence. Combining multiple hair samples increases the likelihood of detecting these variations, which can be useful in establishing the uniqueness of an individual's DNA profile. [58]. More recently [66] have provided some additional mtDNA analysis and interpretation recommendations.

8. Heteroplasmy

Heteroplasmy is defined as the detection of more than one mtDNA type in a single person. (Heteroplasmy is divided into two categories: point substitution or sequence and length substitution; there is a focus on heteroplasmy at specific places.) Heteroplasmy can appear in several ways: 1) In a single tissue, an individual can have multiple mtDNA types; 2) an individual may be heteroplasmic in one tissue in one sample and in another tissue is homoplasmic, and 3) In one tissue, an individual may have one form of mtDNA, while in another, they may have a different type [13, 67]. The last of these three probable heteroplasmic scenarios is the least probable to happen. Heteroplasmy is not found in most forensic investigations using the control region HV1 and HV2 of the mtDNA genome. Nevertheless, in cases where heteroplasmy is identified, the mtDNA variants within an individual typically exhibit a single-base difference (as determined by HV1 and HV2 typing). While heteroplasmy at two or even three sites is feasible, it is observed at considerably lower frequencies. In the literature, point substitution and length heteroplasmy have also been documented [68, 69, and 70]. HV1 length heteroplasmies at position 16184 to position 16193 and HV2 length heteroplasmies at position 303 to position 310 are common [69]. In a sequence electropherogram, the presence of two nucleotides at a single site appears as overlapping peaks, which is a common indicator of sequence heteroplasmy (Figure 6).

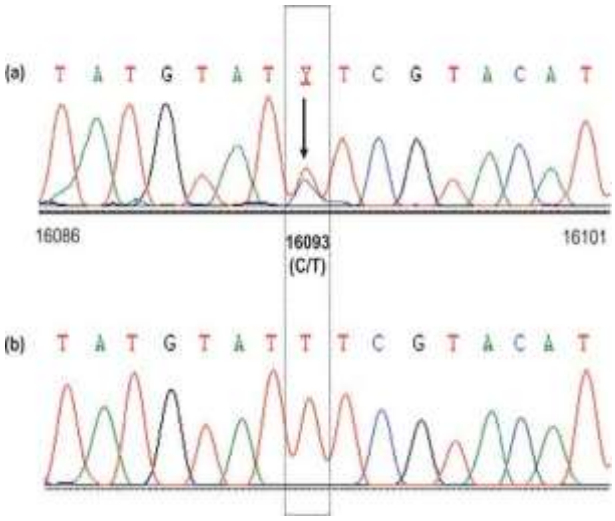

Figure 6. (a) Sequence heteroplasmy with C and T nucleotide at position 16093 is compared to (b) identical area (position 16086 to 16101) in a separate sample with just T at position 16093 [69].

One of the most challenging elements of heteroplasmic samples is the base ratio might differ between different tissues, such as blood and hair, or even between multiple hairs. [71, 72, and 73]. To validate heteroplasmy, some mtDNA techniques currently advocate sequencing many hairs from one individual. Positions 16093, 16129, 16153, 16189, 16192, 16293, 16309, and 16337 74 and 75 in HV1 and 72, 152, 189, 207, and 279 in HV2 are hotspots for heteroplasmy. According to one study, the frequency of heteroplasmy varies by tissue type, with muscle tissue having the highest frequency, and heteroplasmy increases with age, according to

statistical significance across age groups [76]. Heteroplasmy has also been observed to be hereditary rather than age-related, as it has remained steady over time in the same individuals [77]. While heteroplasmy can make interpreting mtDNA results more difficult, According to the Romanov study, heteroplasmy at identical loci can improve the likelihood of a match [49]. Most individuals were assumed to be homoplasmic a decade ago [78, 79, and 80]. Researchers have discovered several incidences of heteroplasmy in people, thanks to improved technology for detecting tiny components in a sample and more samples being typed. Heteroplasmy was detected at site 16169 in the mtDNA regulatory region in the alleged remains of Tsar Nicholas II of Russia, for example [81, 82]. The bones of the Tsar's brother, Grand Duke of Russia Georgij Romanov, Heteroplasmy were identified at position 16169 in the analysis of mtDNA sequencing. In the mtDNA from an unidentified donor's plucked hair, heteroplasmy was observed at two sites, specifically 16293 and 16311. A family was found to have a heteroplasmic condition at position 16355 in their mtDNA. Blood and buccal swab samples taken from this family, which included a mother and two children, exhibited heteroplasmy, with individual hairs carrying either a C or a T at position 16355. Consequently, some hairs from the same individual appeared to have consistent mitochondrial DNA (mtDNA) sequences, while others exhibited variability at just one nucleotide location. In legal challenges aiming to exclude the use of mtDNA evidence, it is common to question the increased frequency of heteroplasmy compared to prior observations, as well as the uncertainty surrounding the mechanisms and rate of heteroplasmy. Nevertheless, by conducting a thorough assessment of the circumstances, it is possible to avoid drawing inaccurate conclusions.

9. Using mtDNA in Tracing Human Migration

The only way to trace human migration and gain insight into evolution a few decades ago was to expose and discover skeletal remains. However, advances in the study of molecular biology have provided anthropologists with significant successes in the recent two decades. Research until the late 19th century, albeit with less precision. However, the discovery and adoption of new genetic tools and molecular techniques have empowered anthropologists to achieve a more profound comprehension of intra and inter-population variations. This, in turn, has facilitated the formulation of new theories related to evolution and migration. Whether it's nuclear or extranuclear DNA, it can record a series of mutations in its sequences that accumulate over time. Anthropological geneticists leverage DNA sequences to deduce the evolutionary history of both humans and their primate relatives. These DNA sequences allow for inferences about the relationships among different organisms and the timing of their divergence. The genetic material, known as deoxyribonucleic acid (DNA), contains crucial information regarding the evolutionary history of life itself. Additionally, cultural factors and processes can exert an influence on migration patterns and the genetic isolation of populations. These cultural factors can contribute to patterns of genetic variation, stemming from the co-inheritance of genes and culture. Acting as a biological calendar. Although mitochondria have long been known to have a function in a variety of biological and physiological processes, their involvement in monitoring evolutionary history has just been investigated in the last three decades. The rate of evolution is defined by the accumulation of rapid changes/mutations through time, and it can be used to determine the time and age of divergence. Consider the following example: Humans and Chimpanzees share a shared lineage, according to genetic and paleontological evidence, and their divergence occurred about 5 million years ago [83]. The whole mitochondrial genome doesn't evolve simultaneously at the same rate [84]. The D-loop or displacement loop [85], a non-coding triple-stranded stretch of mtDNA with three hyper variable sections, HVI, HVII, and HVIII [28], has the highest rate of mutation. This region, which makes up around 7% of mtDNA, has been extensively examined and scrutinized [83]. The mutation rate in the D-loop region is ten times that of the coding area [30]. In their D-loop sequences, two unrelated individuals differ by 1-3 bp per 100 bp on average [31]. To identify population variances in mitochondrial DNA (mtDNA) sequences, they are aligned with the Revised Cambridge Reference Sequence (rCRS). The sequencing of the complete human mitochondrial genome has yielded numerous polymorphic sites for comparative purposes. The HVI region covers nucleotide positions 16024-16365, while the HVII region encompasses positions 73-340, and the HVIII region includes positions 438-574. However, comparing these sequences can be challenging due to the presence of other types of mutations, such as back mutations and parallel mutations. Moreover, the existence of mutation hotspots can further complicate the comparison process. Nevertheless,

the rCRS serves as the benchmark reference sequence for annotating mtDNA in the fields of molecular anthropology, forensic science, and medical genetics [32]. Mitochondrial DNA (mtDNA) analysis has played a pivotal role in uncovering the evolutionary history of humans and their primate relatives. Researchers have utilized mtDNA polymorphisms like HVR1 and HVR2 to trace human migrations and deduce population origins. Based on mtDNA analysis, it's estimated that the split between Homo sapiens and Neanderthals occurred approximately 500,000 years ago[46]. The first phylogenetic tree for mitochondrial DNA was constructed by Cann using Restriction Site Polymorphism. Mitochondrial Eve, our most recent common maternal ancestor, is believed to have lived around 200,000 years ago in Africa. Haplotypes, which consist of maternally inherited genetic variations, form the basis of haplogroups, defined by specific SNPs (Single Nucleotide Polymorphisms) [83]. In Africa, the oldest haplogroups are L0 through L7, with haplogroup L3 thought to be the source of the M and N haplogroups. These later diversified into various forms during and after human migration out of Africa. While sequencing the complete human mitochondrial genome has provided numerous polymorphic sites for comparison, the process can be challenging due to the presence of other mutation types like back mutations and parallel mutations, as well as mutation hotspots[86]. Nevertheless, the Revised Cambridge Reference Sequence (rCRS) has been widely employed as the standard reference sequence for annotating mtDNA in the fields of molecular anthropology, forensic science, and medical genetics (figure 7) [87].

Figure 7. Human migration out of Africa [86].

10. Conclusion

The utilization of mitochondrial DNA (mtDNA) in forensic science has proven to be a crucial and reliable tool in establishing the guilt or innocence of individuals in legal proceedings. Its resistance to degradation, ability to trace maternal lineage, and reduced susceptibility to contamination make it a valuable asset in the field of criminal investigations. The analysis of hypervariable regions 1 and 2 (HV1 and HV2) within the mtDNA genome aids in the identification of individuals and their maternal lineages, offering vital information for forensic analysis. Overall, the application of mtDNA analysis has significantly enhanced the accuracy and effectiveness of criminal investigations, providing substantial support to the criminal justice system.

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