



## Mitochondrial DNA Analysis

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### ABSTRACT

The aim of this study is to write a literature review on Mitochondrial DNA analysis. The article reviews the various techniques and applications of mitochondrial DNA analysis. Mitochondrial DNA, present in the mitochondria, can be assessed as the smallest chromosome coding for 37 genes and containing approximately 16,600 base pairs. Human mitochondrial DNA was the first significant part of the human genome to be sequenced. In most species, including humans, mtDNA is inherited solely from the mother. Mitochondrial DNA Analysis is defined as the extraction of genomic DNA from biological material such as tooth, hair or blood sample. The genetic material obtained is amplified using Polymerase Chain Reaction (PCR) and sequenced using various techniques. mtDNA provides a valuable locus for forensic DNA typing and studying genetic variations. The comparison of mtDNA sequences represents a mainstay of phylogenetics, where it allows biologists to elucidate the evolutionary relationships among species. The high no. of nucleotide polymorphisms in hypervariable portions in the non-coding sequences allows discrimination among individuals. mtDNA analysis has acquired significance due to its greater stability attributed to its high copy number as compared to nuclear DNA and has become an important tool in scientific research.

**Keywords:** mitochondrial DNA, forensic analysis, heteroplasmy, PCR.

### INTRODUCTION

Mitochondria are semiautonomous, eukaryotic, double membraned intracellular organelles majorly functioning as the site for oxidative phosphorylation and ATP synthesis<sup>1</sup>. Popularly known as the “powerhouse of the cell”, its other metabolic functions include breakdown of carbohydrates and fatty acids, interconversion of ATP and ADP and synthesis of steroids and lipids. The replication of mtDNA and translation of mitochondrial protein also occurs in the mitochondrion<sup>2</sup>. The origin of the mitochondrion has been explained by the endosymbiosis theory.

According to this theory, purple photosynthetic bacterium was assimilated by eukaryotic cells. Over a period of time, these bacterial populations lost their ability to function as an independent organism and were incorporated as the cell organelle, the mitochondrion<sup>3</sup>. By evolutionary processes, the eukaryotic host cell was equipped with the eubacterium derived mitochondrial genome. This means that the mtDNA closely resembles the eubacterial genome and differs from the nuclear DNA.

Human mtDNA is a 16,569 bp, double stranded, circular molecule encoding 37 densely packed genes. 22 of the genes encode transfer RNAs (tRNAs), 2 encode ribosomal RNAs (12S and 16S rRNAs) and 13 encode protein enzymes involved in electron transport chain of oxidative phosphorylation and ATP production<sup>4</sup>.

The well conserved function of the mtDNA contrasts with its remarkable genetic variation in terms of conformation, size, gene content and arrangement<sup>5</sup>. In terms of genome

size, plant mtDNA ranges in size from 180 to 700kbp whereas animal mtDNA falls in the range of 14 to 20 kbp with extremely low population of noncoding DNA<sup>6</sup>.

The human mtDNA differs from nuclear DNA in having thousands of copy numbers per cell which attributes to its growing significance in forensic casework<sup>7</sup>. This feature increases the probability of obtaining results from degraded, low copy DNA samples for which nuclear DNA fails to give a result (eg. Bones, hair, faces, processed wood, food products, etc.)<sup>8</sup>. It is possible to obtain results from critical samples because the circular shape of the mtDNA molecule prevents its degradation by exonucleases. In animals, mtDNA is typically transmitted to offspring exclusively by one parent (strict uniparental inheritance)<sup>9</sup> which enables the identification of putative samples by comparison between closely related individuals belonging to the same maternal lineage<sup>10</sup>. Certain portions of the control regions of mtDNA are highly variable among individuals due to the high mutation rate and are examined in forensic analysis. These characteristic features of the mtDNA have led to its increased application in forensic analysis.

mtDNA profiling using techniques such as DNA extraction, PCR amplification and sequencing of DNA is done in laboratories around the world to study the mtDNA recovered from a range of forensically relevant biological sources<sup>11</sup>. Once the mtDNA has been recovered from the biological material, segments of the mtDNA control region are amplified using and subjected to Sanger-based sequencing and data interpretation is performed using available softwares. Heteroplasmy, which is the possibility of two or more different mtDNA sequences



existing in the same cell or organism also, has an important consequence in the profiling. mtDNA replicates in the somatic cells by a process called relaxed replication which occurs independently of the cell cycle<sup>12</sup>.

Somatic mutations in the mtDNA accumulate and may result in expression of different mtDNA haplotypes in different tissues from the same individual which can be used to identify the individual. Thus, mtDNA analysis has become a significant tool in the field of forensics by providing accurate and reliable results even for highly degraded samples.

### Structural Aspects of Human mtDNA

The 16,569 bp, double stranded, circular molecule represents the human mtDNA, a representative of the mammalian mitochondrial genome<sup>13</sup>. It encodes 37 densely packed genes-22 tRNAs, 2rRNAs and 13 polypeptides. These polypeptides are involved in the electron transport chain of oxidative phosphorylation and are subunits of cytochrome *c* oxidase, cytochrome *b* and ATPase<sup>14</sup>. Determination of the primary sequence of the mitochondrial genome was done by scientists working under Fredrick Sanger at Cambridge University using the Cambridge Reference Sequence (CRS) in 1981. The mtDNA can be separated into purine-rich "heavy" (H-strand) and pyrimidine-rich "light" (L-strand) in alkaline density gradient centrifugation due to the assymetric distribution of guanine and cytosine<sup>15</sup>. These strands together constitute the coding regions of the mtDNA. Apart from this, a single non coding region called the Control Region (CR) or D-loop is present. It contains approximately 1125 bp and contains promoters for transcription of polycistronic RNAs of genes on H and L strands. The site for the origin of replication of the H strand is also present in the D-loop<sup>16</sup>.

Some portions of the CR, relative to the coding regions are highly variable among individuals due to the decreased selective constraint of the non-coding DNA. Hypervariable region 1 (HV1) extends from position 16,024 to 16,365 while hypervariable region 2 (HV2) extends from 73 to 340<sup>17</sup>. These regions mutate at 5-10 times higher rate than nuclear genome and are employed for forensic analysis.<sup>18</sup>

### Inheritance and Copy Number

The two characteristic features of mtDNA, the high copy number per cell and maternal inheritance have attributed to its increased application in forensic analysis.

A mature oocyte has more than 1,00,000 copies of mtDNA and approximately 200-1700 mtDNA copies are found in somatic cells of various tissue types<sup>19</sup>.

Nuclear DNA has only 2 copies per diploid cell; as a result, mtDNA testing is more prevalent for degraded samples.

Inheritance of mtDNA is unique in the sense that it is maternally inherited and does not undergo chromosomal recombination.<sup>20</sup> It is presumed that our ancestors living

in Africa around A.D 2, 00,000 years ago are the source of all mtDNA types.<sup>21</sup>

During fertilization in humans, the mitochondria of the sperms undergo "selective destruction, inactivation or simple dilution" by the action of the mitochondria of the ovum<sup>22</sup>. The mitochondria of the sperms are mainly involved in providing energy for the motility of the sperm. Therefore, the zygote only inherits the mitochondrial genome from the ovum and the sperm does not contribute to it.

However, some cases of paternal inheritance of mtDNA have been reported in mice and this variation could possibly be due to mutation.

### Replication of Mitochondrial DNA

The replication of mammalian mtDNA is asymmetrical and begins by the initiation of synthesis of H strand synthesis at a specific site resulting in the formation of a displacement (D) loop with a newly synthesized H strand containing 680 bases known as 7S DNA<sup>23</sup>. Promoters in the D loop region are primarily involved in the transcription process. The mapping of the RNA and DNA species in the D loop region showed that RNA derived from the L strand promoter serves as a primer for H strand DNA replication<sup>24</sup>.

A small, 30 bp long, non coding region flanked by tRNA genes serves as the site for origin of light (L) strand replication which is located two thirds of the way around the genome from the region of H strand synthesis<sup>25,26</sup>. Short RNA molecules generated from DNA primase are required for the initiation of L strand replication. Processing of the transcripts gives rise to the 12S and 16S rRNAs, 22 tRNAs and polyadenylated mRNAs<sup>27</sup>.

Many factors such as subunits A and B of DNA polymerase  $\gamma$ , mitochondrial RNA polymerase, mitochondrial single stranded binding protein (mtSSB), mitochondrial transcription factor A (mtTFA) and RNA processing enzymes play a major role in regulating the replication process.<sup>28</sup>

### Post Transcriptional Modification

Intron sequences are absent in vertebral mtDNA. A unique feature of the mitochondrial genome is that the genes encoding the tRNAs flank the genes encoding the rRNAs. Maturation of mitochondrial tRNAs occurs. It involves three enzymatic activities:

1. Cleavage at 5' end by mitochondrial RNase P (mtRNase P).
2. Cleavage of 3' end by endonuclease.
3. Maturation of excised tRNA by addition of the sequence CCA at 3' end by expenditure of ATP<sup>29</sup>.

### Overview of Laboratory Procedures for mtDNA Typing

The basic requirement for implementing such procedures in forensic casework is that all DNA profiling systems



must be approved by the forensic community and the laboratory conducting the analysis. This process includes: characterisation of the genetic marker, species specificity, reproducibility, precision, accuracy and sensitive studies<sup>30</sup>. A wide range of techniques are available for typing mtDNA.

Criteria such as quantity and quality of sample, availability of resources in the laboratory, time constraints help in choosing the best possible method for partial or complete sequencing of this genetic marker. Some of the methods used are PCR-RFLP<sup>31</sup>, microchip electrophoresis<sup>32</sup>, sequence specific oligonucleotide dot blot assay, minisequencing<sup>33</sup>, and pyrosequencing.

Extraction of mtDNA should be done in such a way that the DNA structure is preserved and the possible PCR inhibitors are removed from the sample. Organic-phenol chloroform method followed by a purification step can be employed<sup>34</sup>.

In order to obtain high quality forensic samples, Chelex extraction is done where the DNA is separated from the cellular environment but not from the residual proteolytic components which inhibit PCR<sup>35</sup>.

In order to produce a meaningful result, the next step is to determine whether a sample will contain enough DNA for PCR amplification. A total DNA quantification can help estimate the quantity of mtDNA only if the ratio between nuclear and mitochondrial DNA is well known. Otherwise, a direct determination of mtDNA molecules can be done using real time PCR<sup>36</sup>.

The next important thing is the right choice of primers for PCR. These oligonucleotides should be designed against evolutionarily conserved regions of the target sequence in order to avoid mutations in the primer binding regions. Also, the primers should be designed from sequences of the same species<sup>37</sup>.

In forensic analysis, the samples obtained are often highly degraded and the probability of obtaining a successful PCR amplification decreases with the increase in the size of the target DNA region. Thus, PCR should be designed for amplification for short portions (less than 300 bp)<sup>38</sup>. The optimum conditions for performing PCR depend on the type of polymerase being used and the characteristics of the template DNA available.

Reducing the annealing temperature by a few degrees facilitates the binding of primers which are not entirely complementary to the template DNA or have been derived from a different species. However, for forensic situations, slight changes from the optimum conditions need to be made for amplification of highly degraded or low copy number (LCN) samples. A problem which arises here is that these changes to increase the sensitivity of the reaction often increase the chances of contamination<sup>39</sup>.

After this, sequencing of the fragments is done using Sanger's Sequencing Method.

## Application in Forensic Casework

### Identification of the Russian Royal Romanov Family

The members of the Russian royal family were assassinated by a firing squad in 1918 and buried without a ceremony and remained unidentified for more than 70 years. The 9 bodies were unearthed by a team of forensic scientists in 1991. Anthropological analysis and facial superimposition proved that the remains belonged to Nicholas II, his wife Alexandra, three of his four daughters, three servants and a family physician. The bodies of his fourth daughter and his son, Alexis were missing<sup>40</sup>. The Forensic Science Service (FSS) laboratory in the U.K established a maternal relation between the presumed remains of the daughter and a living relative, Prince Philip, the Duke of Edinburgh, using mtDNA profiling<sup>41</sup>.

An interesting finding was that the remains of Nicholas II showed that it was a mixture of two sequences (i.e. heteroplasm), one which matched the maternal relatives and one which was a single base pair mismatch.

Anna Anderson Manahan who claimed to be the fourth daughter could not have been the biological daughter of Alexandra Romanov as her mtDNA obtained from two sources; (a lock of hair and tissue biopsy) did not match the mtDNA profile of the princess, Anastasia<sup>42</sup>.

### Criminal Casework

Mitochondrial DNA has been used for criminalistic casework as the mtDNA can be used to identify individuals. The application of this characteristic was first done in the court of law in the U.S for the case State of Tennessee vs. Paul William Ware in August 1996 by the analysis of mtDNA obtained from pubic hair which led to his conviction for rape and murder<sup>43</sup>.

mtDNA results have been used as evidence in at least 10 states around the globe. Both private and public agencies like FBI, AFDIL, etc have incorporated the use of mtDNA in their casework.

mtDNA population databases have been formulated which serve as a means to approximate the frequency of haplotypes observed when an individual's mtDNA sequence matches that of a particular sample. High quality information can be obtained from these databases created by compiling mtDNA samples from thousand of maternally unrelated samples.<sup>44</sup>

## CONCLUSION

This review has successfully covered all aspects of the mtDNA, from its origin, evolution, structure, inheritance, copy number, replication, analysis and its application in forensic casework.

The use of mtDNA has led to a "golden age" in the field of genetics. However, there is still scope for improvement in the quality of the results and research is still being done to make full use of this genetic material. All the



procedures done should be scientifically validated to ensure that it is accepted by the community. Safety protocols should be given utmost importance as we cannot afford to contaminate any sample obtained. mtDNA databases are growing around the world due to the sincere efforts taken by individuals.

Thus, the mtDNA has proved to be the hidden treasure where it's up to the researchers to unfold its true potential.

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