Chapter 14
Applications of the Mitochondrion in Forensic DNA Typing

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Abstract Prior to the coronary stent implantation (CSI) era, a typical biology course would introduce the mitochondrion simply as the energy-producing organelle of the cell. Little, if any, discussion was provided about the mitochondrial genome and its participation in human molecular inheritance and evolutionary biology. Now that human identification, via DNA typing, is the driving force behind several forms of television entertainment, the traditional role of the mitochondrion has taken a backseat. In this chapter you will learn how and why mitochondria have been targeted by scientists for use in forensic analysis, human molecular genetics, evolutionary biology, human migration studies, and recovery operations in identifying deceased persons, both ancient and modern. You will also learn how mitochondrial DNA (mtDNA) has provided forensic scientists with a valuable tool for determining the source of DNA recovered from damaged, degraded, or very small biological samples. This chapter explains how mtDNA analysis offers a unique maternal ancestral view of an individual's molecular pin code, through examination of a very specific region of the mitochondrial genome. This chapter will also evaluate both the pros and the cons of mtDNA utility in forensic analysis. Though data have proven increased utility of mtDNA in both historical and modern cases, it is still discounted by many and considered an unreliable forensic tool. An ongoing source of controversy in mtDNA analysis is centered on both data acquisition and data analysis (i.e., how differences in mtDNA sequences are reported). Before the forensic community can approve a DNA typing or classification technique, extensive research on its accuracy, reliability, and discriminatory power must be validated.

Keywords DNA typing · Mitochondria · Sequencing · Forensics · Matrilineal · Ancestor

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14.1 Human Inheritance: Nuclear and Mitochondrial DNA

14.1.1 Nuclear DNA

Multicellular organisms develop by a slow process of progressive change which begins with the fusion of two specialized cells, i.e., the egg and sperm. This results in the formation of a zygote, which contains the genetic information carried out from both the contributing parents which regulates the development of the fetus [11]. Zygote divides mitotically and produces all cells of the body. In most of the cases, the nuclear genes are neither lost nor mutated; hence, the genetic nature of each cell is equivalent to that of the other cells except the lymphocytes. In case of lymphocytes, the cells tend to rearrange their DNA through differentiation for the formation of new immunoglobulin and antigen receptor genes [28]. Nuclear DNA is packaged into 23 pairs of chromosomes in normal cells. Each chromosome consists of one very long, linear DNA molecule concomitant with many protein and RNA molecules. A subunit of the DNA molecule which carries the organism's inherited traits is called as gene (Fig. 14.1). The DNA molecule carries hundreds to thousands of genes, the units of information that specify an organism's inherited traits.

Briefly, generalized eukaryotic cells have many different ways to partition off different functions to various locations of the cell. Thus, a typical eukaryotic cell is housed to different specialized compartments called as cell organelles to impart many different designated functions. These organelles play a pivotal role in cellular development as well as function of the organism.

Most of the knowledge regarding the inheritance of human genes have come from the careful observation of human traits over generations and the analysis of family history. Improved analysis of inheritance pattern of specific genes within the families has led to the discovery of major genetic traits within the families. However, the contribution of the great scientist Gregor Mendel cannot be ignored when the knowledge and research on genetics is being talked about. Mendel's early experiments with pea plants have led to the development of two thumb rules of genetics, i.e., equal segregation and independent assortment [43]. According to the first law of Mendel, the two members of a gene pair, the alleles, are separated into gametes, and half of the gametes carry one allele, and another half carries the second allele. Thus, half of

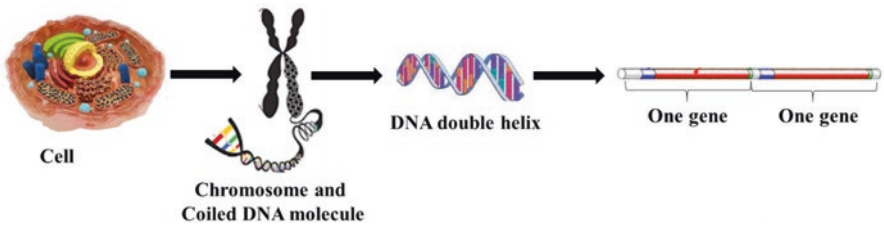

Fig. 14.1 Organization of nuclear DNA and genes in a generalized mammalian nucleated cell

our nucleated genes are inherited from our father and another half from our mother that have been reunited through fertilization in a randomly occurred event. Additionally, according to the second law of Mendel, the independent assortment, one gene pair, is segregated independent to the segregation of the other gene pairs. Thus, the segregation of one allele occurs independent to the other unlinked loci. Additionally, the law of independent assortment is considered to be the fundamental principle which allows the high discrimination power by applying the product rule to the unlinked core loci in forensic typing [17]. Thus, Mendel's law of independent assortment is the source of tremendous genetic variation that allows the exploitation in the field of medical, anthropological, and forensic applications.

Most of the studies so far have exploited the nuclear DNA markers for population genetic studies. The major cause of this is the polymorphicity of these nuclear markers with highest level of discrimination power among individuals except the homozygous twins [20]. In this regard, the population statistics is considered to be essential for statistical evaluation of a match in interpretation of a DNA profile. However, this chapter describes the inheritance of mitochondrial DNA and its suitability in forensic applications.

14.2 The Evolution of the Mitochondrion

Most of the studies on mitochondria deal with the energy-producing ability and metabolism of the cell involving mitochondrion [9]. However, little have been studied and explored regarding the mitochondrial genome and its participation in human genetic inheritance and phenotype determination. Currently human identification via DNA typing has become the driving force for many technological involvements; the traditional role of mitochondrion has taken a backseat. Still, it is ignorant to many people regarding the utility of mitochondrion at genetic level as most of them are aware on the pivotal role of this organelle in the energy house of the cell which is the location of the final phase of aerobic respiration (Fig. 14.2).

Mitochondrial genome (mtgenome) imparts a large forensic advantage due to its stability and high copy number which is the consequence of its function and evolutionary history. In this regard, it is highly essential to understand the necessary functions of the encoded genome and its appreciation to molecular genetics via mitochondrial gene variation [14]. Understanding the necessary functions encoded in mtgenome enhances our appreciation of the molecular genetics of mitochondrial gene variation. All living cells require energy from external sources. Many studies have deciphered the process of energy consumption, its conversion, and transformation by living cells through the process of cellular respiration (Fig. 14.3). Many stages of cellular respiration such as citric acid cycle and oxidative phosphorylation take place inside the matrix of mitochondria. Through the process of glycolysis, glucose is broken down into pyruvate followed by the complete breakdown of glucose by citric acid cycle and ATP synthesis by oxidative phosphorylation [27]. Following the generation of pyruvate during glycolysis, the oxidation of glucose is

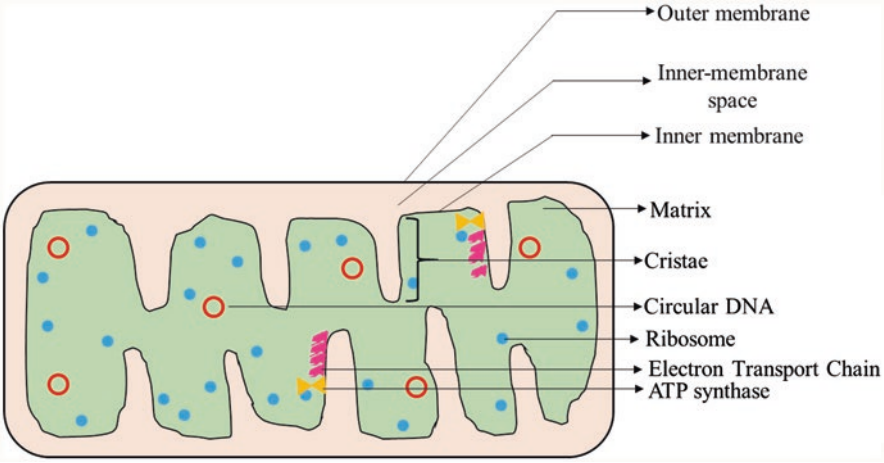

Fig. 14.2 A generalized ultrastructure of a typical mitochondrion

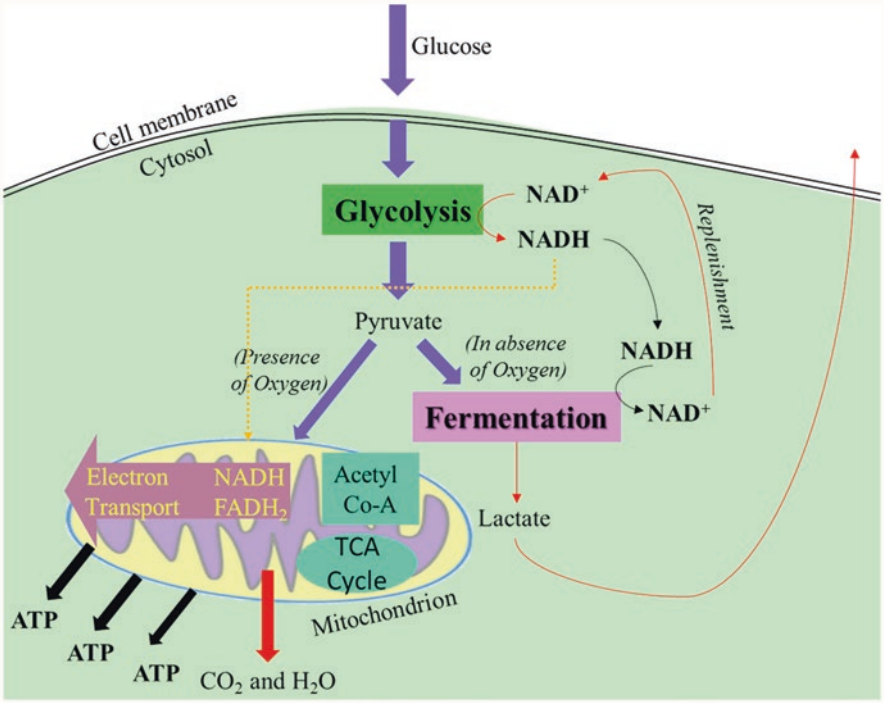

Fig. 14.3 Within mitochondria there are a series of metabolic processes involved in cellular respiration

completed in the mitochondrion in the presence of oxygen (O_2). Enzymes within the outer and inner membranes of the mitochondria assist in converting cellular materials into adenosine triphosphate (ATP), which fuels the metabolic activities of the cell [10].

The separation of biochemical processes such as the citric acid cycle, which occurs in the mitochondrial matrix, and oxidative phosphorylation, which traverses an electrochemical gradient of the tightly folded inner membrane, enables cells to use aerobic respiration to produce approximately 15 times more ATP than anaerobic respiration [10, 24] (Fig. 14.4). Five protein complexes in the mitochondrial inner membrane involved in the electron transport and oxidative phosphorylation pathways have been identified. Complexes I, II, III, and IV are part of the electron transport chain. Complex V is the enzyme complex that carries out oxidative phosphorylation reaction [5].

The dynamic function of a mitochondrion in energy production is reflected from its complex internal compartmental structure. A typical mitochondrion consists of two membranes separated into four distinct compartments, and each membrane-bound compartment can function cohesively for the efficient generation of ATP [3]. The double-membrane structure of a mitochondrion generates a narrow intermembrane space and a large internal matrix. The channel proteins and porins are present in the outer membrane of the mitochondrion which play a major role in selective filtration of cellular components. Additionally, the inner membrane of the mitochondrion harbors structures with extensive folding called as cristae [10]. Major catastrophes during the process of oxidative phosphorylation can lead to severe damage to the mitochondrion and to the organism possessing these organelles. Mitochondria produce reactive oxygen species (ROS) as by-products of inefficient electron transfer across the electron transport chain (ETC) [33]. These superoxide radicals further react to form other ROS, which may lead to mitochondrial trauma.

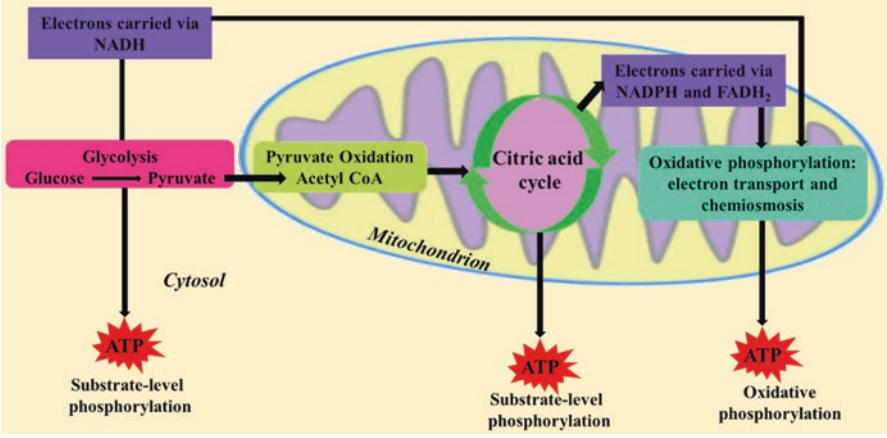

Fig. 14.4 Separation of biochemical processes within the mitochondrion enables cells to produce ATP more efficiently

Additionally, decrease in efficiency of mitochondrial ETC increases oxidative damage directly. Many studies have linked this accumulation of mitochondrial oxidative damage with age and have proposed that increased ROS production may shorten one's life span [44]. Several factors are believed to be responsible for elevated mtDNA mutation rates including inefficient DNA repair mechanisms, omission of DNA protective proteins, and continuous exposure to the mutagenic effects of reactive oxidative species generated by oxidative phosphorylation.

Mitochondria are found in nucleated cells of most eukaryotes which include plants, fungi, animals, and multicellular protists. The number of mitochondria per cell is largely dependent on the cell metabolic requirements. Typically the copy number of mitochondrial DNA (mtDNA) is 100–10,000 copies per cell [22] depending on cell type. Morphologically, mitochondria are diverse and fluctuate in size and shape regularly. They range in shape from long interconnected tubules to small separate spheres [29].

14.3 The Mitochondrial Genome

Evolution and the origin of eukaryotes are important topic in the field of population genetics. Decades of debates and studies have resulted in the rarely unquestioned bacterial ancestry of the mitochondrion. The endosymbiont theory is attributed to the origin and evolution of the mitochondria. The endosymbiont theory states that the mitochondrion evolved from a bacterial progenitor via symbiosis within the eukaryotic host cell. This theory provides an explanation for the mitochondrion having its own genome, separate from that of the nucleus it surrounds. The concept of symbiosis (Latin for *living together*) was first described by a Swiss botanist Simon Schwendener who discovered that lichens consist of a fungus and a photosynthesizer [23]. Endosymbiotic theories attest that cells unite, one inside the other, during evolution to give rise to novel lineages at the highest taxonomic levels via combination. This theory is in direct contrast with Darwin's description of evolution described as gradualism. Theorists still take issue with this explanation of endosymbiosis and rely more heavily on the origin of eukaryotes as the product of gene duplication, point mutation, and micromutational processes [38].

Most of the mitochondrial genome (mtgenome) codes for proteins and enzymes required for their function. The 13 proteins that are encoded by the compact, circular, double-stranded mtgenome are the subunits of the electron transport chain. The mtgenome also encodes two (02) ribosomal RNAs (rRNAs) and twenty-two (22) transfer RNAs (tRNAs) (Fig. 14.5). However, this coding region of mitochondrial genome makes up only about 3% of human genomic DNA.

Mitochondria are semiautonomously functioning organelles that contain an inherent genome that undergoes replication, translation, and transcription of their own DNA [13]. Mammalian mitochondrial DNAs (mtDNA) have two separate origins of replication. The origin of the heavy strand (guanine-rich) is located within a region termed the displacement loop (D-loop), and the light-strand (cytosine-rich)

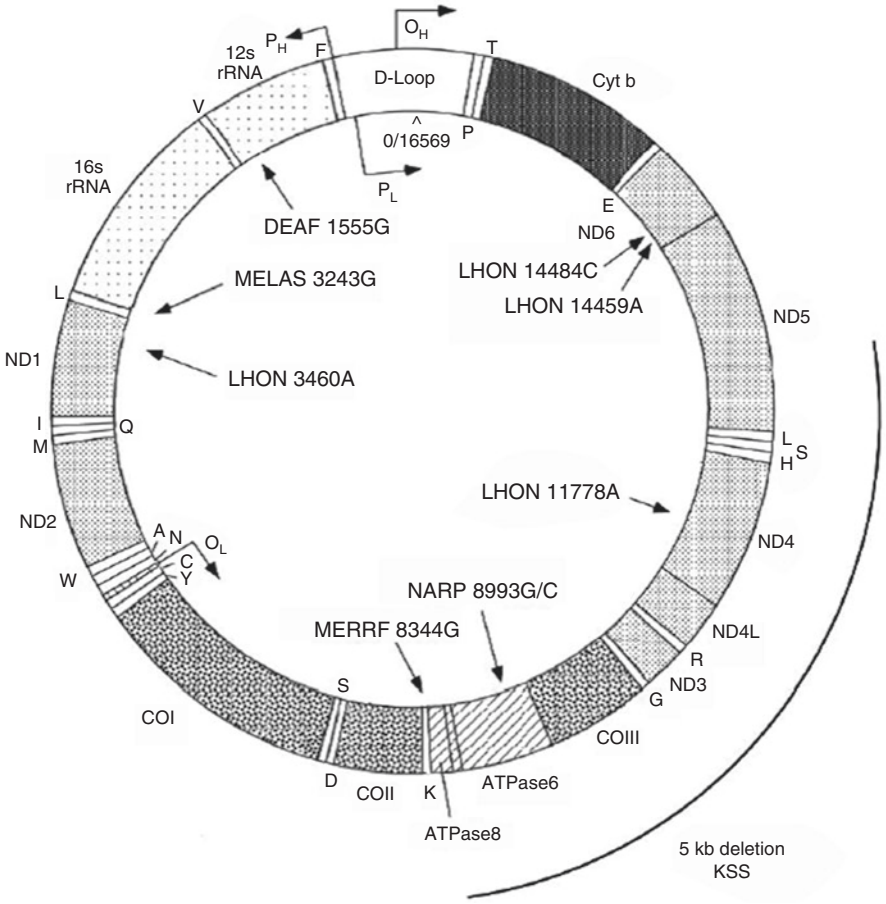

Fig. 14.5 Mitochondrial DNA map

synthesis originates within a cluster of five tRNA genes nearly opposite of the D-loop. The single focus of current forensic typing is the D-loop [30]. The D-loop consists of approximately 1100 base pairs of "noncoding" DNA and is commonly referred to as the hypervariable region due to an increased frequency of mutation as compared to the remaining portion of the mtgenome. The hypervariable region is further divided into three segments. Hypervariable region I (HV1) spans nucleotide positions 16,024–16,365; hypervariable region II (HV2) spans nucleotide positions 73–340; and hypervariable region III (HV3) spans nucleotide positions 438–574. HVI and HV2 are traditionally targeted, whereas HV3 is rarely examined in forensic settings. The hypervariable region has been reported to mutate at a rate of 10 to 17 times more frequently than marked areas of the nuclear genome, namely, single-copy nuclear polymorphic sequences (scnp's) [34].

Mtgenome population-specific variation is widely reported in human evolutionary studies. Many studies have found the usability of variation studies of mitochondrial genome to study the high-frequency haplotype variations among Northwest Africans [35]. Another study reveals the suitability of testing HV1 and HV2 regions in resolving the genetic relationship among Fulani nomads and neighboring sedentary populations in sub-Saharan Africa [7]. Additional studies on HV1 and HV2 regions have found that 70% of the 700 Hispanic individuals residing in United States belong to "Hispanic haplogroups" [1]. Though a typical mitochondrion contains 0.25% of a cell's total DNA, the presence of large numbers of mitochondria in the cytosol of physiologically active cells makes them a suitable source for the DNA to be used in forensic analysis [19].

Current studies suggest the utilization of mtDNA from hair and calcified tissues of forensic samples for utilization in ancient samples, mass disasters, and identification of missing persons with environmentally compromised samples [16]. The odds of mtDNA forensic markers surviving cellular damage are greater than that of the nuclear genome since there are hundreds to thousands of mtgenomes in each nucleated cell. MtDNA is also beneficial in cases with scanty extracted DNA samples [16]. The advantage of mtDNA analysis as an exclusionary tool is often overlooked due to its mode of inheritance. Unlike the nuclear genome, mtgenome does not undergo chromosomal recombination, Mendelian inheritance, or replication repair as only the mother passes clonal copies of her mtgenome to her progeny through the egg (Fig. 14.6). Thus, barring mutation, progeny inherits an identical mtDNA signature that is shared between maternally related individuals. MtDNA is passed through generations independent of male influence as the fertilizing sperm only contributes cellular components directly to the nucleus [39].

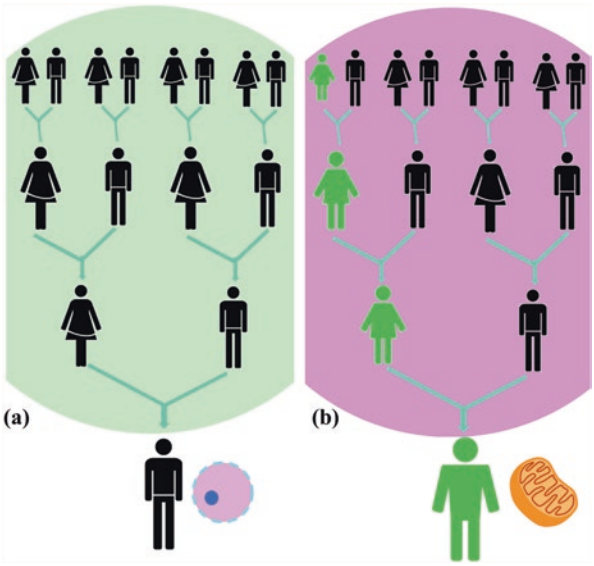

Fig. 14.6 Inheritance of (**a**) nuclear DNA from all ancestors and (**b**) mitochondrial DNA from a single, maternal lineage

14.3.1 Heteroplasmy

Specific genetic markers and procedures used for DNA testing in forensics depend largely upon the quality and quantity of DNA present. Additionally availability of control samples for comparison is also a prerequisite in choosing the methodology. Mitochondrial DNA (mtDNA) has long offered advantages for certain forensic genetic analyses because of its abundance. In comparison to nuclear DNA, each human cell contains hundreds to thousands of copies of mtgenome [26]. Thus, analysis of mtDNA becomes more relevant for ancient and forensic samples with limited sample size and contains highly fragmented and damaged DNA. In this regard, the high copy number of the mtgenome can produce a good profile even with several attempts to generate a profile from nuclear DNA. This is quite evident from the fact of generation of DNA profile from mtDNA in the case of charred remains collected after World Trade Center terrorist attacks in September 2001 in the United States [6]. MtDNA is mostly present in samples that may contain little or no intact nuclear DNA, such as hair shafts and aged fingernails [2]. As a result, mtDNA has been the historical marker of choice for these sample types [25]. MtDNA in forensic casework is of great use in identification cases. Maternal relatives can be used as references for unknown sample, in such cases. This is extremely valuable in a number of scenarios but particularly when the direct references or close relatives required for kinship analyses based on autosomal markers are unavailable.

Forensic comparisons using mtDNA are generally consistent. Evidence interpretation typically involves a direct comparison of the sequences of unknown origin to the sequences of known origin. When the mtDNA sequences of both unknown and known samples are consistent across all nucleotide positions considered for interpretation, the samples are designated as "cannot be excluded as originating from the same source or same lineage" [4]. Nonmatching mtDNA sequences between unknown and known samples are considered "exclusion." Routine mtDNA testing tends to be relatively uncomplicated and easy to interpret. However, there are scenarios that make mtDNA analysis interpretation challenging. A common challenge involves the analysis of heteroplasmy. Heteroplasmy refers to the presence of different mtDNA haplotypes within an individual or tissue [4]. Individuals may possess mtDNA molecules that differ in length, i.e., length heteroplasmy (LHP), or at single nucleotide positions, i.e., point heteroplasmy (PHP). Heteroplasmic variation of either type is not used for exclusionary purposes in forensic mtDNA analysis. This is because of the high mutation rate of mtDNA and the variation that has been observed between different tissue types. Shared PHP between maternal relatives can provide further support for non-exclusion and has proven increased utility of the mitochondrial genome in a plethora of historical cases. Though germ line and somatic mtDNA mutations occur with relative frequency and are often observed in mtDNA profiles as heteroplasmy, the phenomenon of heteroplasmy is believed to be rare by many [32].

Increased frequency of observed heteroplasmy in the general population has been demonstrated by Sanger technology and must be considered in the interpretation of forensic evidence. These Sanger-based studies have formed the basis of our understanding and led to the development of appropriate interpretation guidelines. However, the sensitivity and robustness of newer sequencing technologies have refined our knowledge of heteroplasmy.

Heteroplasmy generally generates mtDNA sequence data in the form of mixture. This distinguishes authentic mtDNA, heteroplasmy, and mixed data from other causes. Thus, the appearance of heteroplasmy adds an added level of complexity to data interpretation regardless of sequencing technology used [15]. Other factors contributing to the problems in analysis of mitochondrial DNA sequences include the mixtures of mtDNA from distinct individuals, contamination by nuclear mitochondrial pseudogene sequences, and chemistry-based sequencing errors [12].

14.3.2 Haplogroup Analysis

MtDNA hypervariable region-based haplogroups have recently garnered more interest in medical genetics, anthropology, and population genetics irrespective of the unique rationale of each field. However, forensic biology is yet to introduce the use of haplogroup classifications as an exclusionary tool for lead in investigation of cold cases. Though few studies have been carried out in the field of mitochondrial DNA and ethnicity [18], instances are there with the use of mtDNA haplogroup analysis in solving criminal investigations.

Mourad Topalian, an Armenian nationalist, was convicted in 1996 of conspiracy acts and possession of illegal weapons and explosives. In this case, "ancient" mtDNA analysis linked the suspect to the hair fragments found on an abandoned storage locker in the 1970s leading to the conviction process [42]. Additionally, molecular evolutionary studies involving human mitochondrial genome reveal the root of modern human origin to be Africa [37]. Most of these comprehensive studies have been carried out by the analysis of single nucleotide polymorphism (SNP) in mtDNA determined by RFLP technique followed by sequencing of HV1 region. It is believed that mtDNA is structured geographically which can be classified into many groups of related haplotypes. Formation of this haplotype is based on the migration of the people across the globe which has been accumulated in the form of genetic signature over time [41]. In this regard, a haplotype is a combination of alleles or genes that are located on the same mtgenome and are therefore inherited as a group. A haplogroup is a genetic signature comprised of a group of similar haplotypes that share a common ancestor (Fig. 14.7). As per current report, two major haplogroups (M and N) along with their derivatives are present in non-African region post migration of modern human out of Africa. Macrohaplogroup L is geographically limited to sub-Saharan Africa which has been subdivided into four major haplogroups, i.e., L0, L1, L2, and L3 [36, 41].

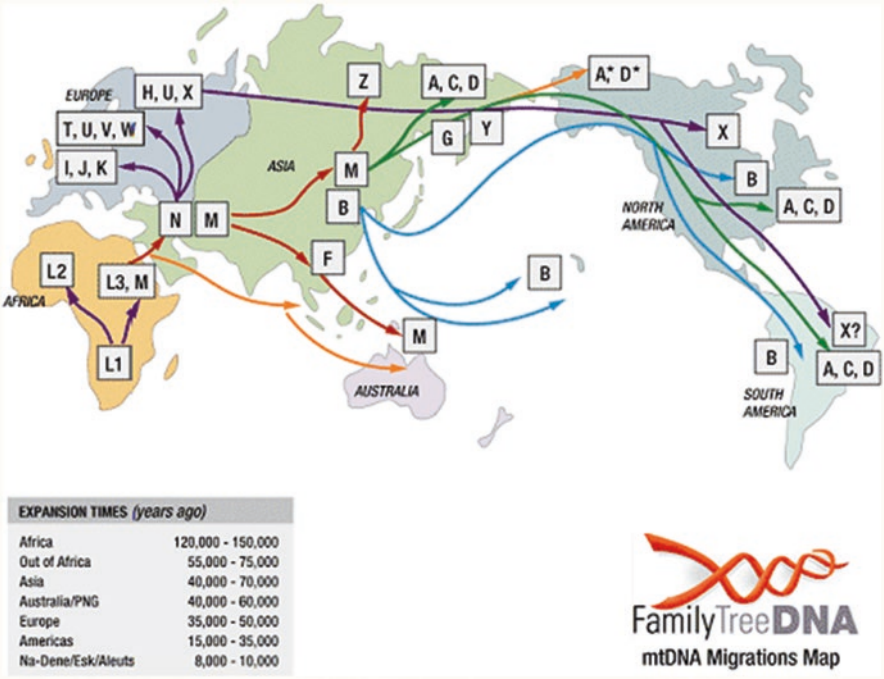

Fig. 14.7 Migrations of human populations and major haplogroups based on the analysis of human mitochondrial DNA. (Original author: https://www.FamilyTreeDNA.com)

14.4 Future Perspectives

There have been tremendous advances in the field of forensic science, and the technology has grown and perfected over centuries. Still there exist many rooms for growth. Forensic science has been criticized for a lack of standards and coordination: complaints about the unreliability of some scientific evidence used in courts are long-standing and also widespread [8]. A 2009 report on the facts of strengthening forensic science in the United States by the US National Research Council called for major reforms to the US forensic science system as well as throughout the globe. It advocates for better standardization of protocols and more research inputs for reliability of techniques used [21]. In this regard, the utility of Innocence Project cannot be ignored and should be conducted in other states as well [40].

Guidelines are published by the DNA Commission of the International Society of Forensic Genetics (ISFG) frequently concerning the application, analysis, and interpretation of mitochondrial DNA (mtDNA) in forensic casework. Though nuclear DNA guidelines for forensic casework have been widely established, the need for similar guidelines for mtDNA analysis is now required for the mtDNA reference population data used to assess the statistical weight of the evidence [31]. In this context, a total of 16 recommendations have been published by ISFG DNA Commission (Table 14.1).

Table 14.1 Recommendations for the use of mtDNA in forensic casework

Sl. No.	Guidelines
1	Good laboratory practice and standard operating protocols for mtDNA analysis must be followed
2	Controls (positive, negative, and extraction blanks) must be carried through the entire process
3	Minimum of 1 reverse sequence and 1 forward sequence (total of 2X coverage) for all reported consensus sequences
4	Avoid manual transcription of data, and independent confirmation of reported data by two scientists must be performed
5	Regular participation in proficiency testing programs for all laboratories using mtDNA typing in forensic casework
6	The full mitochondrial DNA control region should be sequenced for all population genetic studies
7	The revised Cambridge Reference Sequence (rCRS, NC001807) must be used for alignment
8	IUPAC conventions shall be used to describe differences to the rCRS and (point heteroplasmic) mixtures
9	The alignment and notation of mtDNA sequences should be performed in agreement with the mitochondrial phylogeny (established patterns of mutations)
10	Laboratories must establish their own interpretation and reporting guidelines for observed length and point heteroplasmy
11	For population database samples, length heteroplasmy in homopolymeric sequence stretches should be interpreted by calling the dominant variant, which can be determined by identifying the position with the highest representation of a non-repetitive peak downstream of the affected stretch
12	mtDNA population data should be subjected data quality control
13	The entire database of available sequences should be searched
14	Laboratories must justify database(s) and statistical approaches used in reporting
15	Laboratories must establish statistical guidelines for use in reporting an mtDNA match between two samples
16	Length variants in homopolymeric stretches should be omitted from calculations of frequency estimates

14.5 Conclusion

MtDNA's high diversity (the likelihood that two persons selected at random will be different) and low heterogeneity (signatures of the same type rarely occur in populations) yield an informative forensic marker. In many cases, at least 99% of the population will be excluded as contributors, and the pool of random individuals who could have contributed the same is less than 1%. Another advantage of mtDNA typing is in the instance of a "no-body homicide." Because mtDNA is maternally inherited, any maternal relative may donate the mtDNA reference sample to compare to suspected crime scene victim tissues. The utility of the mtgenome should not be discounted, as mtDNA typing is a reliable forensic tool.

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