



BIOMEDICAL SCIENCES

Forensic use of human mitochondrial DNA: A review

PABLO CAVALCANTI, TATIANA LÚCIA S. NOGUEIRA, ELIZEU F. DE CARVALHO & DAYSE A. DA SILVA

Abstract: In forensics, genetic human identification is generally achieved by nuclear STR DNA typing. However, forensic samples often yield DNA in exiguous quantity and low quality, impairing the generation of conclusive DNA profiles by STR typing. In such cases, mitochondrial DNA (mtDNA) can be used as an alternative solution in forensic human identification. The high copy number, small circular DNA, high mutation rate, maternal inheritance, and absence of recombination are mtDNA's key features in forensics. In this work, we review mtDNA characteristics, forensic applications, sequencing methodologies and present some relevant examples in the forensic science literature.

Key words: mtDNA, NGS, NUMTs, Heteroplasmy, Human Identification, Forensic biology

INTRODUCTION

Since the advent of DNA fingerprinting, derived from the study of hypervariable regions in human DNA by Jeffreys et al. (1985a), the human identification field has experienced great advances. The emergence of DNA typing provided invaluable recovery of genetic information with the ability to generate human identification (Jeffreys et al. 1985b). The forensic applications of that improvement go from mass disaster victim's identification (Montelius & Lindblom 2012) to kinship and missing person identification (Zietkiewicz et al. 2012).

Although nuclear short tandem repeats (STR) are the gold standards for human DNA typing, in some situations STRs may fail to generate full or informative DNA profiles (Cavalcanti et al. 2015). Due to the *post-mortem* degradation process, in general, the available samples are bones, teeth, or hairs, oftentimes recovered from a water environment, a burial site, or burned, resulting in small quantities and insufficient quality for

nuclear DNA typing (Alvarez-Cubero et al. 2012). It is known that the genetic material generally presents a high degradation level due to significant *post-mortem* time and environmental conditions such as elevated temperatures, high moisture, acidic pH, soil composition, microorganisms' activity, and ultraviolet light exposure (Latham & Miller 2018, Alaeddini et al. 2010).

In such cases, the use of mitochondrial DNA (mtDNA) can be considered as an alternative to nuclear genomic DNA identification (Cavalcanti et al. 2017). The mtDNA has proven to be a valuable resource for human identification (Holland & Huffine 2001). The high copy number per cell, the protective small circular structure, elevated mutation rate, and the non-mendelian maternal inheritance without recombination are key features to mtDNA forensic use (Melton 2014, dos Reis et al. 2019).

MITOCHONDRIAL DNA IN FORENSIC GENETICS: A HISTORICAL PERSPECTIVE

The first study using mtDNA for human identification was published by Stoneking et al. (1991). In this work, 23 sequence-specific oligonucleotide (SSO) probes targeting the mtDNA hypervariable regions HVI and HVII on the control region were employed for sequencing the skeletal remains of a human child, discovered in 1986. The results showed no differences between the skeletal remains questioned sample and the mother reference sample, leading to the conclusion that those skeletal remains were the missing child remains.

Sullivan et al. (1992) published a study in which they aimed to identify the body of a female found in an advanced state of decomposition. They used Sanger sequencing for HVI and HVII mtDNA sequence determination from human bone fragments and necrotic skin samples with degraded DNA. The mtDNA sequences matched those found in a blood sample from a presumptive sister, confirming a familial relationship. No differences were found between the questioned sample and the supposed sister's reference sample, leading to the conclusion that they were sisters.

The probably most famous study involving mtDNA sequencing for human identification was published by Gill et al. (1994), wherein a mitochondrial DNA analysis was conducted on nine skeletons found in a grave in Ekaterinburg, Russia, which were provisionally identified as the remains of the last Tsar Nicholas II, Tsarina, three of their five children, the Royal Physician, and three servants. The HVI and HVII mtDNA Sanger sequencing attested to the existence of a family group at the burial site. A perfect sequence alignment was discovered between the supposed Tsarina and her three offspring with a living maternal kin. The mtDNA obtained

from the alleged Tsar exhibited heteroplasmy and corresponded with two living maternal kin of the Tsar. So, the hypothesis that the discovered remains belong to the Romanov family was confirmed, solving a historical enigma through the application of forensic science.

Boles et al. (1995) published a study in which the mtDNA HVI region was sequenced to identify 12 human teeth/tooth remains. The samples were exhumed in 1992, from two mass graves of alleged Indian villagers murdered, in 1982, by the Guatemalan dictatorship government. The mtDNA Sanger sequencing contributed to the identification of most of the human remains, leading to the conclusion that they were the missing victims of the massacre.

The first use of mtDNA analysis in court was in 1996 when Paul William Ware was convicted of the rape and murder of a four-year-old child in the state of Tennessee, United States (Melton 2009). During the necroscopic examination, a hair was found in the throat of the victim, whose sequencing of mitochondrial DNA showed coincidence with the accused.

Corach et al. (1997) published a study detailing their efforts to identify 340 skeletons found in two mass graves that have been related to the Argentine dictatorship victims. The mtDNA Sanger sequencing of HVI and HVII was performed to identify one victim. The comparison of the questioned sample and the mother reference sample found no sequence differences, identifying the sample as the son of a living mother.

Deng et al. (2005) conducted a study in the aftermath of the 2004 Southeast Asia Tsunami, a disaster that claimed nearly 5,400 lives in Southern Thailand, including both foreign tourists and residents. The researchers' objective was to extract as much DNA evidence as possible from the severely decomposed bodies, employing both bone and tooth

samples and utilizing both mitochondrial and nuclear markers for DNA identification. Despite the difficult conditions, the team successfully recovered and analyzed DNA samples, resulting in the identification of 51% of the samples. This research represented a substantial contribution to forensic genetics, showcasing the potential of mtDNA analysis in disaster victim identification and carrying significant implications for future disaster response and forensic investigations.

In 2012, skeletal remains were excavated at Grey Friars Friary, in Leicester, England, known as the last resting place of King Richard III. King et al. (2014) published a study aiming to identify those remains. To investigate if the remains belonged to King Richard III, mtDNA analyses of the skeletal remains and living relatives of Richard III were performed. Sanger sequencing of the HVI, HVII, and HVIII fragments, as well as the entire mitochondrial genome sequencing by ION Torrent PGM and 100 SE Illumina HiSeq 2000, were employed in mtDNA analyses. The results showed a perfect match between the remains and one living relative and a single-base substitution when compared to the other living relative, suggesting that the remains belong to King Richard III.

The Spanish Civil War (1936-1939) and the repression that took place in that country until the 1970s resulted in more than two hundred thousand deaths, with many victims buried improperly and unidentified (Preston 2012). Baeta et al. (2015) published a study in which they aimed to contribute to the identification of 252 *post-mortem* human remains. The use of HVI and HVII Sanger sequencing succeeded in the recovery of supplemental information for the identification of 27% of the 252 samples analyzed.

In 1998, a Viking age mass grave with at least 19 individuals was discovered in Sweden. Aiming to investigate the possibility of maternal

kinship among the individuals, Bus et al. (2019) conducted a study. Bones and teeth samples were submitted to mtDNA analysis by Sanger sequencing of the HVI and HVII segments. A whole mtDNA genome was also performed in a subset of samples by MPS using the Precision ID mtDNA Whole Genome Panel. The results showed that at least 20 individuals were buried in the mass grave and that only two pairs were related, as siblings or mother-child.

When Loreille et al. (2022) used an optimized DNA extraction protocol based on hybridization DNA capture coupled with NGS Illumina NextSeq 500 platform to analyze single rootless hairs found in relics associated with the Romanov family, it was revealed that the hair belonged to a woman who shared Tsar Nicholas' maternal lineage, including the well-known point heteroplasmy at position 16169. Previously published mtDNA sequences of Tsar Nicholas II and his wife, Tsarina Alexandra, contributed to this study. In addition, autosomal SNPs were analyzed to assess relatedness. The results showed that the hairs came from the same individual. The profile was compared to the control region (CR) of two living relatives used as maternal references: James George Alexander Bannerman Carnegie, 3rd Duke of Fife (1929–2015), the Tsar's first cousin twice removed and Countess Xenia Cheremeteva Sfiris (born in 1942), his great grandniece. However, the Tsar's C/T point heteroplasmy at position 16169 was absent in the two references CR profiles that only had the 16169T variant. Following the heteroplasmy inheritance pattern through six generations, it was observed that they endured between the two generations that separate the Tsar from his grandmother but rapidly evolved to homoplasmy in the three different branches of the family that were studied. The results are then consistent with the hairs belonging to Louise of Hesse-Kassel, Queen of Denmark

or another person maternally related to Tsar Nicholas II.

Biology and features of the mitochondrial DNA

Although the nucleus is known as the cell region that concentrates most of the human genome, approximately 3.2 billion base-pairs, there are 0.25% of the genome located outside it (Butler 2012). These 16,569 remaining base pairs are located inside cell organelles called mitochondria, which are in the cytoplasm of eukaryotic cells (Goodwin 2016). Yet discovered, identified and isolated by Nass & Nass (1963), mtDNA was first completely sequenced in 1981, in the laboratory of Frederick Sanger, generating the Cambridge Reference Sequence (CRS), also known as “Anderson” sequence, with 16,569 base-pairs arbitrarily numbered from 1 to 16,569, by restriction enzyme sites in the L-strand generated by the MboI (Anderson et al. 1981). In 1999, the CRS was resequenced, then generating the revised Cambridge Reference Sequence (rCRS), the accepted standard for comparison (Andrews et al. 1999). Unlike nuclear DNA, which holds two copies per cell (one paternal and the other maternal), mitochondrial DNA presents hundreds to thousands of copies of a tiny circular genome per cell, whose amount may vary according to biological tissue (Chapman et al. 2020).

The mitochondrial DNA, as the bacterial genome, has a circular structure with thirty-seven genes compactly disposed of codifying products necessary to the oxidative phosphorylation process, responsible for the cellular energy production (Nicholls & Gustafsson 2018). Thirteen of these genes encode proteins, two encode ribosomal RNAs (rRNA) and twenty-two encode transfer RNAs (tRNA) (Asin-Cayuela & Gustafsson 2007).

Mitochondrial genes are compactly arranged in the coding region, with no introns

and only fifty-five nucleotides not being used in the transcription of rRNA, tRNA, and proteins (Butler 2012). The remaining 1,122 base pairs constitute the control region, where the origin of mitochondrial DNA replication is located and where no gene is present, which is therefore also known as the non-coding region or displacement loop region (D-loop region) (Taanman 1999, Samehsalari & Reddy 2018).

Curiously, there is a bias in the distribution of nucleotides between complementary strands of the mitogenome, generating a “light” strand with large amounts of adenine and thymine and another “heavy” strand rich in guanines and cytosines (Druzhyna et al. 2008, Yasukawa & Kang 2018).

Since the process of endosymbiosis - a crucial step in the evolution of life on Earth - occurred, wherein a larger prokaryotic cell engulfed a smaller alpha-proteobacterium, the latter survived and formed mitochondria and chloroplasts (Baum & Baum 2014). This event established a mutually beneficial relationship with the host, and the transfer of genetic material to the host's nucleus led to the emergence of complex eukaryotic cells, complete with a nucleus and organelles (Archibald 2015). This marked a significant transition from simple prokaryotic cells to the diverse array of eukaryotic organisms present today. Over the course of evolution, most of the bacterial ancestral genome was lost or transferred to the nucleus, resulting in a highly compact mitochondrial DNA (Gustafsson et al. 2016). Notably, there is a strong correlation between the presence and expression of mtDNA and the existence of a functional respiratory chain in the mitochondria (Stewart & Larsson 2014). In a total of 37 genes present in the mitochondrial genome, 13 encode polypeptides related to oxidative phosphorylation. Except for Complex II, which is fully encoded by nuclear DNA, all the

others have a nuclear and mitochondrial genetic origin (Nicholls & Gustafsson 2018).

The mitochondrial DNA is transmitted without recombination and matrilineally, in other words, only by the mother, in a non-Mendelian pattern (Bermisheva et al. 2003, Amorim et al. 2019). There are two main processes responsible for this kind of inheritance: (1) strong negative regulation (i.e., downregulation) of the number of copies of mtDNA during spermatogenesis and (2) the existence of a mechanism that actively degrades the mitochondria of sperm after fertilization by ubiquitination followed by proteolysis (Sutovsky et al. 2000, Gustafsson et al. 2016).

This inheritance pattern is especially useful in forensic approaches such as identification of missing persons, mass disaster, and terrorist attack victims, where reference samples can be provided by maternal relatives for direct comparison to the questioned sample (Cavalcanti et al. 2017). In addition, due to the lack of recombination, even distant maternal relatives can be potential sources of reference samples, which is useful when there is no other reference sample available. This is a common scenario in disaster victim identification and missing persons cases (Budowle et al. 2003). These characteristics differentiate mtDNA from autosomal genetic markers. On the other hand, it also explains why mtDNA yields lower discriminatory power when compared to those nuclear DNA markers.

Nevertheless, despite overwhelming evidence to the contrary, a few questionable studies suggest paternal inheritance and recombination of mitochondrial DNA. Hagelberg et al. (1999) observed a rare variant in three unrelated human mtDNA lineages in an isolated population in the Vanuatu archipelago and assumed it as evidence of paternal leakage and recombination. Later, however, the study was

retracted, and the assumptions revealed as due to an alignment error (Hagelberg et al. 2000). Another study led by Luo et al. (2018) raised the hypothesis of biparental mtDNA inheritance based on three families with supposed high heteroplasmy. However, it was highly criticized due to methodological inconsistencies and wrong deductions. Those findings were more likely the result of multi-copy full nuclear mitogenome inserts (Mega-NUMTs) co-amplification contamination (Marshall & Parson 2021).

The mitochondrial genome, unlike the nuclear DNA, is not associated with histone proteins, leading to the hypothesis that the high rate of mutation observed, when compared to the nuclear DNA, could be explained by the absence of these proteins, assumed to have a protective effect against damages and mutations (Brown et al. 1979). However, such a statement is not supported by any evidence (Alexeyev et al. 2013). In turn, similarly to the bacterial genome, mitochondrial DNA is packaged in a nucleoprotein complex called nucleoid (Falkenberg & Gustafsson 2020). The main structural protein component of the nucleoid is the mitochondrial transcription factor A (TFAM), which has as much protective action against damage and mutations as histones (Guliaeva et al. 2006, Gustafsson et al. 2016).

The evolution rate of human mitochondrial DNA is quite high when compared to nuclear DNA, approximately between ten and twenty times higher (Brown et al. 1982, Neckelmann et al. 1987). This difference is the result of the mutation rate, between one hundred and one thousand-fold higher than the nuclear DNA, multiplied by the fixation rate of the mitochondrial DNA mutations (Wallace & Chalkia 2013). For a certain period, this high mutation rate of the mitochondrial DNA was attributed to the oxidative stress caused by the reactive oxygen species (ROS)

produced by the mitochondria during cellular respiration and to the supposed absence of mtDNA repair mechanisms (Brown et al. 1979). Nevertheless, although some damage is caused by the production of ROS, most mutations are caused by spontaneous polymerase γ replication errors (Zheng et al. 2006). On the other hand, mtDNA also has repair mechanisms such as base excision repair (BER), mismatch repair (MMR), homologous recombination (HR), and non-homologous end joining (NHEJ) (Akbari et al. 2008, de Souza-Pinto et al. 2009, Bacman et al. 2009, García-Lepe & Bermúdez-Cruz 2019). In addition to repair mechanisms, the mitochondrial DNA can also undergo a degradation process in response to damage, eliminating the damaged DNA, something possible due to the redundancy of this genome, with hundreds to thousands of copies per cell (García-Lepe & Bermúdez-Cruz 2019).

When mtDNA control mechanisms are overcome and a mutation appears, a heteroplasmic mixture of this genome is created (Wallace & Chalkia 2013). These heteroplasmic mutations can segregate during cell division, since the mitochondrial DNA reproduces independently of the cell cycle, generating a mosaic distribution of mutated mitochondrial genome (Clayton 1982, Nissanka & Moraes 2020). Such mosaicism has both clinical relevance, once some organs or tissues may be randomly affected by physiological alterations, and forensic, since individuals may present heteroplasmy in some samples, depending on the organ or tissue analyzed (Falkenberg et al. 2007). Furthermore, it is important to highlight that although somatic tissues may contain high levels of mutations in the mitochondrial DNA (Larsson 2010), to be transmitted, mutations must, besides being present in germ cells, overcome maternal control mechanisms of mutations transmission in the mitochondrial genome such as the bottleneck

effect during oogenesis, in which few copies of maternal mitochondrial DNA are present in the precursor cell (Hauswirth & Laipis 1982, Stewart & Chinnery 2015); the existence of a purifying selection mechanism against mutations in the maternal germline that may cause alterations in amino acids within codified proteins or in tRNAs (Stewart et al. 2008); and fertility reduction in women with high levels of mtDNA mutations in germ cells (Ross et al. 2013).

MITOCHONDRIAL DNA VARIANTS, HAPLOTYPES AND HAPLOGROUPS

Mitochondrial genome mutations often originate variant sequences that can be classified into three relevant categories: deleterious mutations, ancestral adaptive mutations, and somatic mutations (Wallace & Chalkia 2013).

The class of variants of mitochondrial DNA with greater clinical relevance is that of deleterious mutations (Li et al. 2019). A high mtDNA mutation rate has allowed hundreds of pathogenic mutations to be introduced in human populations (Wallace et al. 2013). The G11778A, G3460A, and T14484C mutations, for instance, were correlated with Leber's Hereditary Optic Neuropathy (LHON) (Nissanka & Moraes 2020). Diseases such as diabetes mellitus and Alzheimer's were also associated with mtDNA mutations - A3243G and G5460A, respectively (Wallace et al. 2013, Li et al. 2019).

Somatic mutations accumulate in cells, organs, and tissues over time, progressively impairing mitochondrial function, being associated with aging-related processes such as the emergence of neurodegenerative diseases, cancer, and allowing the establishment of an aging clock (Wallace & Chalkia 2013, Nissanka & Moraes 2020).

When an mtDNA mutation appears and is transmitted in each population, it originates a

new haplotype, since mitochondrial genomes are inherited together from a single parent (Yamamoto et al. 2020). If that haplotype is perpetuated in a population, it originates a haplogroup. Generally, mtDNA adaptive mutations generate evolutionary advantages in each environment and thus allow the growth of their respective haplogroups, which tend to predominate in that region (Wallace et al. 2013). Thus, continents and geographic regions are associated with specific haplogroups, which has a great impact on population study and forensics (Wallace 2015). Haplogroup assignment provides valuable information about an individual's matrilineal geographic origin to assist forensic investigations, potentially allowing a match on globally established mtDNA databases, such as EMPOP, that store thousands of quality-controlled reference samples obtained worldwide. It is also useful for quality assurance purposes since the observation of unexpected variants may reveal sequencing or interpretation errors (Parson et al. 2014).

Heteroplasmy

The occurrence of heteroplasmy is considered when the presence of more than one type of mitochondrial DNA is verified in an individual, in a tissue, cell, or mitochondria (Melton 2004). It is probable that all individuals present heteroplasmy at some level, although often below the detection limit of current sequencing techniques (Steighner et al. 1999, Tagliabracci & Turchi 2020).

Heteroplasmy occurs due to a combination of mtDNA characteristics, such as elevated mutation rate, autonomous replication, and high copy number (Melton 2004). Although mutations that originate heteroplasmic mixtures at the somatic level are not transmitted through generations, those originated in the germ line, once able to overcome controlling mechanisms such as

the bottleneck effect during development, can be inherited from mother to child (Nicholls & Gustafsson 2018). In such cases, it will result in two mtDNA populations, i.e., heteroplasmy.

Two different types of heteroplasmy are known: one derived by InDels (length heteroplasmy: LHP) and the other by SNPs (point heteroplasmy: PHP) (Budowle et al. 2003; Bhatti et al. 2017).

Length heteroplasmy usually occurs due to insertions/deletions of one or more nucleotides. Examples of mtDNA regions prone to the occurrence of that phenomenon are the homopolymeric extensions formed in cytosine extension regions (C-stretches) which, in the revised Cambridge reference sequence and several samples, extend between positions 16184 and 16193 of the HVI region, with one thymine (T) in position 16189 (Lee et al. 2004). When, in some samples, thymine in position 16189 is replaced by cytosine (C), a homopolymer extension with ten or more cytosines appears. Another known extension of cytosines extends between positions 303 and 315 of the HVII region, with one thymine (T) in position 310 (Andrews et al. 1999). Similarly, when this thymine is replaced by one cytosine, a homopolymeric extension of cytosine is originated (Lutz-Bonegel et al. 2004). Likewise, HVIII AC residues, which usually extend between positions 514 and 524, also generate length heteroplasmy due to insertions of one or more pairs of AC dinucleotides (Bhatti et al. 2017). In these cases, there is an increased probability of the occurrence of a phenomenon called polymerase slippage, causing a deletion/insertion that can result in length heteroplasmy, in which there is a mixture of mitochondrial DNA sequences with different sizes in the same cell, commonly observed in Sanger type sequencing (STS) by the sudden loss of quality of the sequence right after the homopolymer region. (Butler 2012, Lee et al. 2016).

On other hand, sequence heteroplasmy, caused by a single mutation that generates two populations of mtDNA in a single individual, a tissue, organ, or cell, is easy to detect by observation of two overlapping nucleotides at the same position in an mtDNA sequencing analysis (Butler 2012).

Although its presence represents a challenge, heteroplasmic variants are also a useful and valuable element of mtDNA analysis in forensic casework, as they potentially provide additional discrimination power (van der Gaag et al. 2020, Gallimore et al. 2018, Holland et al. 2011, Just et al. 2015, Melton 2004, Melton et al. 2005). Heteroplasmy may contribute to distinguishing between maternally related individuals as well as supporting the identification of human remains. One well-known example was the identity confirmation of the human remains attributed to Tsar Nicholas II of Russia, achieved by a comparison with the remains of his brother Georgij Romanov, who shared a heteroplasmy at position 16,169 of the mtDNA control region with his brother (Ivanov et al. 1996).

Following the guidelines and recommendations on the generation and interpretation of mtDNA heteroplasmy in forensic casework is usually enough to overcome issues related to heteroplasmy (Kim et al. 2018, Parson et al. 2014, SWGDAM Guidelines 2019). Artifacts that occur, such as damage, system noise, and error are not reproducible (Marshall & Parson 2021). Being able to distinguish minor variants from noise is essential to proper reporting of heteroplasmy and that is achievable by setting correct thresholds and choosing the most appropriate sequencing methodologies. In this sense, massive parallel sequencing (MPS) can detect and resolve heteroplasmy at threshold levels as low as 1-2%, much more sensitive than STS, which in some cases must be set above 20%

(Gonzalez et al. 2020, Holland et al. 2011, Just et al. 2015, Melton 2004).

Nuclear mitochondrial insertions

Nuclear mitochondrial insertions (NUMTs) correspond to segments of DNA highly homologous to mitochondrial DNA that are present throughout the human nuclear DNA (nuDNA) (Bücking et al. 2019). NUMTs arise from the transposition of mtDNA into nuDNA in a process not fully understood but driven by mitochondrial insertion events. This phenomenon is explained by the evolution of mitochondrial endosymbiosis, in which segments of mtDNA were transferred to the nucleus of the eukaryotic cell (Ramos et al. 2009). Because of that process, most mitochondrial genes and many pseudogenes are present in the nuclear DNA (Puertas & González-Sánchez 2020). Several studies showed that NUMTs are present throughout the human genome and although the majority are unique, some exist in multiple copies that emerged due to duplication events (Mishmar et al. 2004, Parr et al. 2006). The NUMT duplication rate in humans is quite like the substitution rate on SNPs, once it was estimated to be $2.2 \times 10^{-9}/\text{year}/\text{NUMT}$ (Bensasson et al. 2003).

Woerner et al. (2020) compiled a database of all the NUMTs already discovered, revealing an impressive total of 1090, which range from 13 to 18649 bp long. Some NUMTs are also present as multi-copy full mitogenome inserts, a phenomenon known as mega-NUMT (Balciuniene & Balciunas 2019).

Although when using a long-range amplification approach, NUMTs are usually not co-amplified as they are generally shorter in length than the mtDNA sequence, with a short amplicon approach chances are higher (Chaitanya et al. 2015). NUMTs can potentially generate analysis difficulties in forensic casework if co-amplified

with mtDNA target sequences by convoluting mixtures or heteroplasmy interpretation. To master this, researchers have filled databases of known NUMTs since they are likely observed at defined locations and developed bioinformatic techniques for identifying and filtering them (Duan et al. 2019, Maude et al. 2019, Ring et al. 2018, Santibanez-Koref et al. 2019, Smart et al. 2019, Woerner et al. 2018). Another effective approach to generate NUMTs-free mtDNA sequences is to simply dilute the DNA (Calvignac et al. 2011).

Applications of mitochondrial DNA in forensic genetics

Mitochondrial DNA main characteristics that make it an interesting tool in forensics are the presence of hundreds of copies per cell, high mutation rate, maternal inheritance, lack of recombination, the circular DNA resistance to the degradation processes, and the high incidence of polymorphisms (Zietkiewicz et al. 2012, dos Reis et al. 2019).

Since mitochondrial DNA is maternally inherited without recombination and has a high mutation rate, it is a good candidate to search for matrilineal lineages. Additionally, mutations clustering related to environmental/geographic factors make it relevant to geographical genetic ancestry research. However, what expands the range of mtDNA forensic applications and justifies its choice over other markers, such as nuclear ones, is its abundance and resistance to degradation. Due to this combination of characteristics, mitochondrial DNA is a useful tool in forensic approaches such as disaster victim identification, missing person cases, terrorist attack victim identification, criminal cases, and identification of historical/ancient human remains (Cavalcanti et al. 2017).

Concerning the high incidence of polymorphisms, since the control region (CR)

does not codify products necessary to the cellular function, it has fewer restrictions to nucleotide variability and, in this way, concentrates most polymorphisms in the mitochondrial DNA (Tagliabracci & Turchi 2020).

In the mtDNA, CR has located regions of great nucleotide variability called Hypervariable Region I (HVI), II (HVII), and III (HVIII), which have been targets of several studies in forensic genetics (Lutz et al. 2000, Nagai et al. 2004, Fridman & Gonzales 2009, Melton et al. 2012, Imad et al. 2015). However, the most recent recommendation from the International Society for Forensic Genetics suggests the sequencing of at least the entire mitochondrial DNA control region (Parson et al. 2014).

Despite the high interindividual variability in the control region, there are cases where this approach fails to discriminate between distinct maternal lineages (Coble et al. 2004). Additionally, some haplogroup defining mutations lie outside the CR (Van Oven & Kayser 2009). To overcome this limitation, an alternative is to submit the entire mitogenome to massive parallel sequencing (MPS) approaches, also known as next-generation sequencing (NGS) or second-generation sequencing.

Mitochondrial DNA Sequencing techniques and interpretation of results

Since the advent of the first mtDNA sequencing technique by Frederik Sanger, in 1977, known as sanger-type-sequencing (STS), scientific and technological improvements have allowed the development of automated, less time-consuming, and high throughput techniques labeled as second and third-generation sequencing (Bruijns et al. 2018). Although still being used by a relevant number of forensic laboratories, the first generation and pioneer Sanger sequencing technique has lost space in the market to second and third-generation

sequencing techniques since the mid-2000s (Bruijns et al. 2018).

Regarding the workflows for forensic analysis, all the existing sequencing techniques, either STS or MPS based, share similar overall steps; DNA extraction, quantification, enrichment of mitogenome segments through amplification or DNA capture, preparation of the mtDNA regions of interest (ROI) for sequencing, sequencing of the DNA templates, and analysis of the generated data through specific software. The most significant differences arise from the possibility to generate gigabases (GBs) of sequence information from a single run on an automated machine.

With respect to the target enrichment for mtDNA sequences, it is characterized by a focused amplification of the ROIs thousands of folds from the genomic initial background, ensuring that they correspond to most of the sequenced DNA (Singh 2022). This step is important to ensure a sufficient sequencing level or depth for targeted ROIs further reliable analysis. There are two major enrichment approaches: namely PCR-based amplicon and DNA capture methodologies (Mertes et al. 2011).

Due to practical restrictions, STS mtDNA analysis has been generally limited to the mitochondrial DNA control region or, at least, its hypervariable regions (Melton et al. 2012). Nonetheless, when the mtDNA samples are highly degraded, such amplification strategy may not work (Cavalcanti et al. 2017). So, mini-primer sets were developed and used on “mini-midi-mito” methods, addressed to allow amplification of mtDNA fragments as small as 140bp (Berger & Parson 2009). In this approach a series of overlapping amplicons are generated by two multiplex PCRs to cover the entire control region, overcoming the issues previously described (Cavalcanti et al. 2017). The suitability of such mini-primer approaches to MPS

techniques expanded the enrichment options and the commercialization of NGS kits designed to cover the entire control region (PowerSeq® CRM Nested System from Promega, Precision ID mtDNA Control Region Panel from Thermo Fisher, or ForenSeq™ mtDNA Control Region Solution from Verogen) allowed many forensic laboratories to adopt mtMPS approaches (Holt et al. 2019). The analysis of the entire mitogenome is also possible since the availability of commercial kits such as PowerSeq® WGM, Precision ID Whole mtDNA Genome Panel, or ForenSeq™ mtDNA Whole Genome Kit, increasing the discriminate power and allowing the analysis of degraded samples.

Target enrichment (capture) techniques

However, in forensic casework, some circumstances can result in sequencing failure when using PCR-based approaches. That is the case of harsh environmental conditions such as extreme heat, insolation, moisture, water submersion for long periods, as well as the advanced age of ancient remains (Eduardoff et al. 2017, Loreille et al. 2018). Those samples are candidates for DNA capture methods once they were developed for analyzing highly degraded DNA samples, with fragments of 50bp or less (Eduardoff et al. 2017, Marshall et al. 2020). Combined with NGS approaches it has the potential to generate successful results from samples too degraded for typical sequencing methods. There are two main DNA capture methods used in forensics: Prime Extension Capture (PEC) and Hybridization Capture.

PEC involves the production of an adapter-ligated library from low copy number DNA extracts and sequence-specific designed biotinylated primers addressed to capture and isolate targeted segments from the library for analysis (Briggs et al. 2009). This technique is effective for capturing mtDNA fragments

approximately 70 bp in length, requires less time, and has been refined for forensic contexts to work with minimal DNA input. It is useful for analyzing ancient teeth and bone samples, as well as modern hair shaft samples (Eduardoff et al. 2017).

Hybridization capture, in turn, does not require specifically designed primers but instead uses biotinylated DNA or RNA probes (Marshall et al. 2017). On the other hand, it involves generating a shotgun adaptor-ligated library that is hybridized with the probes, enabling the capture and isolation of mtDNA fragments smaller than 100 bp in length (Marshall et al. 2017). When coupled with MPS, it provides a method for obtaining whole mitochondrial genomes from highly fragmented samples. However, hybridization capture is also characterized as low throughput, labor-intensive, and involving multiple tube transfer steps (Templeton et al. 2013). Marshall et al. (2020) used a hybridization capture method combined with NGS to identify the skeletal remains of a Croatian nun known as Sister Marija Crucifiska Kozulic, then considered for Sainthood by the Vatican authority.

Sanger type sequencing

The Sanger method, first described more than forty years ago, continues to be widely used for mitochondrial DNA sequencing, with the incorporation of new technologies though (Tagliabracci & Turchi 2020). The process consists basically of the enzymatic incorporation of deoxyribonucleotide triphosphates (dNTPs) by the DNA polymerase enzyme, forming the mtDNA sequence, interrupted by the incorporation of dideoxyribonucleotide triphosphate (ddNTPs) as terminators, and followed by a process of separation and detection with sufficient resolution to discriminate each nucleotide (Sanger et al. 1977). The reaction mixture contains only one mtDNA primer and both dNTPs and

ddNTPs, so that some portions with different sizes of mitochondrial DNA are extended and, at the end of the sequencing reaction, several sequences are present that differ for a single base. Since Smith et al. (1986) made some modifications in this method, each dNTP is marked with a fluorophore of a different color for visual identification.

Over the years, Sanger's method has evolved and become more sensitive and efficient. Instead of a simple Taq polymerase, better balanced and efficient reagents such as those of the Big Dye® kit (Applied Biosystems) were used; the ratio between signal and pollution was also improved with the incorporation of new fluorophores (Lee et al. 1997); which together, allowed conclusive results with DNA quantities up to 1ng (Stewart et al. 2003).

Nowadays, mitochondrial DNA sequencing protocols, are often composed of the following steps: (1) DNA extraction, (2) quantitation, (3) amplification of the control region by the PCR reaction; (4) enzymatic purification of PCR products with a mixture of Exonuclease I and Shrimp Alkaline Phosphatase (EXO-SAP) for the removal of dNTPs and remaining primers; (5) visualization of PCR products in gel submitted to electrophoresis; (6) performance of mtDNA sequencing reaction; (7) removal of remaining ddNTPs and dNTPs by filtration, usually in QIAamp spin columns; (8) dilution of the products of the sequencing in formamide; (9) separation and detection by capillary electrophoresis in a sequencing device; and (10) analysis of the results (Ballard 2016).

Second generation (short read) sequencing

The search for simplification, automation, time optimization, and high throughput sequencing culminated with the development of new sequencing methods previously known as Next Generation Sequencing (NGS), in 2005 (Calabrese

et al. 2020). Although there are differences between NGS technologies, they share some common principles such as improved yield in sequencing due to massive parallel reactions, similar workflows, the requirement of libraries preparation before the sequencing, and the generation of only small DNA fragments (Bruijns et al. 2018). The most used NGS approaches are based on sequencing-by-synthesis, which entail a serial extension of a primed DNA template by a polymerase or ligase enzyme (Bruijns et al. 2018). This results in cycles of nucleotide incorporation that are followed by a synthesis reporting method, which is either based on fluorescence/light emission capture or pH changes measurement. The most relevant NGS techniques to mention are the ones developed by Roche, Illumina, and Applied Biosystems.

Roche's 454 system, the first NGS technology developed and made available, played a pivotal role in the evolution of NGS technologies (Ansorge 2009). However, this platform has not been routinely used since the mid-2010s. More accurate methods have since been developed and are currently favored in the field of human forensic genetics. The principles of the 454 system were based on the pyrosequencing technique, a sequencing-by-synthesis (SBS) method. In this method, a light signal emitted from the incorporation of a nucleotide in the new DNA strand being constructed is detected by a camera (Goodwin et al. 2016). With the nucleotide incorporation in the synthesized DNA strand, the enzyme sulfurylase converts the pyrophosphate into adenosine triphosphate (ATP) (Margulies et al. 2005). Therefore, luciferase converts luciferin with ATP to oxyluciferin, emitting a light signal (Metzeker 2010). Nonetheless, before sequencing, a library must be prepared by an emulsion PCR (em-PCR) method, in which small beads with 44 bases primer sequences, called "adapters" A and B, are added to the ends of

each DNA target sequence (Liu et al. 2012). At the end of the em-PCR process, each bead is loaded in a well located in a fiber-optic slide (Bruijns et al. 2018). When developing an experimental design to test Roche's 454 system capability to sequence the entire control region, Bekaert et al. (2013) observed 100% concordance with STS method, pinpointing the suitability of this technique for forensic casework.

Illumina's Solexa. HiSeq and MiSeq systems are also based on the SBS approach and require a library preparation with "adapters" A and B placed at the ends of the DNA sequences (Bruijns et al. 2018). Differing from the 454 system, no emulsion PCR technique is used, but a bridge amplification in planar solid glass support (Glenn 2011). Oligos complementary to the adapter's sequences are located on the planar support and hybridizes with the DNA target sequence, creating a bridge structure attached to the support for amplification (Buermans & Den Dunnen 2014). Thus, the amplicons-clusters are then sequenced with fluorescently labeled ddNTPs (Stranneheim & Lundberg 2012). At last, a charge-coupled device (CCD) camera detects the different emitted fluorescent signals and determines the DNA sequences (Kumar 2012). Zavala et al. (2022) performed successful sequencing of disinterred skeletal remains from the Korean War and World War II using the Miseq System, thus showing its adequacy to sequencing analysis of even highly degraded forensic samples.

The Ion Torrent's Personal Genome Machine employs em-PCR with small magnetic beads for library preparation. However, the detection method is based on pH change due to nucleotide addition in each DNA sequence (Quail et al. 2012). The proton release, following the pyrophosphate cleavage necessary to the nucleotide ligation, is detected by a complementary metal-oxide-semiconductor (CMOS) sensor array chip that

monitors the potential with a sensor surface located at the bottom of the well plate (Heather & Chain 2016). When evaluating the Precision ID mtDNA Whole Genome Panel from Applied Biosystems™, specially developed for forensic casework, sequenced with the Ion S5™ system from Ion Torrent™, Gouveia et al. (2017) observed concordance between the 162 haplotypes obtained and previous results from STS.

Third generation (long read) sequencing

At the beginning of the last decade, as an evolution of the NGS technologies, third-generation sequencing technologies were developed, enabling the reading of single molecules in real-time (Goodwin et al. 2016). Furthermore, no PCR-library preparation before sequencing is required, increasing the accuracy by avoiding PCR-related errors (Calabrese et al. 2020). Also, the ability to sequencing the entire mitogenome at once, in a single reaction, offers an invaluable alternative to NGS short fragments, diminishing errors and strand bias (Kchouk et al. 2017). The most relevant third-generation sequencing techniques are PacBio and Oxford Nanopore Technologies.

PacBio was the first third-generation sequencing technology developed and released in 2010 (Schadt et al. 2010). It is based on a sequencing-by-synthesis approach called single-molecule real-time (SMRT) sequencing, in which a single DNA molecule is detected while the DNA polymerase is synthesizing the DNA with fluorescently labeled nucleotides (Eid et al. 2009). Small glass wells coated with a metal film forming a photonic nanostructure known as zero-mode waveguides (ZMW) allow a single-molecule-resolution (Korlach et al. 2010). At the bottom of that structure, a high-resolution camera detects in real-time the fluorescence emission of each nucleotide incorporated (Lundquist et al. 2008). Using a long-read single

molecule real-time sequencing (SMRT) strategy on the PacBio Sequel platform, Chen et al. (2020) decoded the mtGenome of 32 monozygotic twin individuals accurately distinguishing them and revealing 785 low-level variants within all the 16 twin pairs with threshold of 2% and high-quality control. Although not largely implemented, those results show the remarkable potential of that approach in forensics.

Like PacBio, the MinION sequencer by Oxford Nanopore Technologies is also based on the SBS method and does not require a PCR library preparation (Bruijns et al. 2018). Rather than rely on expensive fluorescent labels, MinION sequencing is based on the detection of ion current differences generated by nucleotides passing through a protein nanopore in a membrane that divides two chambers filled with a conductive electrolyte (Branton et al. 2008). In a study to verify the capability of the MinION to deconvolute mixtures from individuals belonging to the same haplogroup and correctly identify the donor of a DNA sample, performed by Lindberg et al. (2016), accurate identifications were made with relatively high precision and recall for single-source samples. Moreover, the proof-of-concept phasing of the long reads in a 1:1 mixture showed that it can provide SNPs information inaccessible with second-generation sequencing, resulting in differentiation between two individuals of the same haplogroup. A drawback is the inability to reliably use this technique when dealing with degraded mtDNA.

Mitochondrial DNA sequencing interpretation

Following the mtDNA sequencing and analysis of the reference or known (K) sample and the evidence or question sample (Q), K and Q are aligned with the revised Cambridge Reference Sequence (rCRS) and compared to interpret the results (Holland 2012). Generally, the

interpretation process follows the ensuing SWGDAM recommendations (SWGDAM 2019):

■ *Exclusion*: if there is no length heteroplasmy and the questioned and known samples show two or more differences at nucleotide positions, they can be excluded as belonging to the same individual or maternal lineage.

■ *Inconclusive*: if the questioned and known samples differ at one nucleotide position, the result must be reported as inconclusive.

■ *Cannot exclude*: if both samples have the same sequence at each nucleotide position under comparison, they cannot be excluded as originated from the same individual or maternal lineage.

The reason why the result must be considered inconclusive when a single base difference is observed is that mutations have been reported between individuals belonging to the same maternal lineage (Parsons et al. 1997). If possible, more reference samples should be sequenced to enable a better interpretation in such a scenario.

When a cannot exclude result is obtained, a mtDNA database must be searched to verify the frequency of the sequence in the population, by counting the number of times the obtained sequence (or haplotype) is observed and then estimate the statistical significance of a match (Melton 2014). This approach is known as the counting method.

mtDNA SEQUENCING CHALLENGES: TISSUE-SPECIFIC VARIATIONS AND FORENSIC IMPLICATIONS

Mitochondrial DNA sequencing often presents special challenges due to tissue-specific issues related to the presence of heteroplasmy, NUMTs, and the potential for cytosine deamination (Marshall & Parson 2021). Each of these factors

must be carefully considered to ensure accurate analysis and interpretation of mtDNA.

Studies have shown that heteroplasmy levels can be highly tissue-specific (Wachsmuth et al. 2016). For instance, certain mtDNA mutations may be present in one tissue but not in others, or they may occur at different frequencies across tissues. This tissue-specific distribution of heteroplasmy is thought to arise due to the varying replicative demands and mitochondrial turnover rates in different cell types (Melton 2004). Tissues with high energy demands, such as muscle and brain, may exhibit different patterns of heteroplasmy compared to those with lower energy requirements.

In this regard, Naue et al. (2014) discovered that the relative number of heteroplasmies was highest in muscle and liver, detected at 79% and 69%, respectively. This was followed by the brain, hair, and heart, which had a heteroplasmy range of 36.7% to 30.2%. Lower percentages of heteroplasmies were observed in bone, blood, lung, and buccal cells, ranging from 19.8% to 16.2%.

Although heteroplasmic variants may contribute to forensic analysis casework, potentially providing extra discrimination power, distinguishing between true heteroplasmic variants and sequencing errors becomes challenging when sequencing mtDNA from a tissue with high heteroplasmy levels (Holland et al. 2018). In forensic genetics, this poses a challenge as the questioned mtDNA profile may not match the reference sample if they originate from different tissues. Moreover, the degree of heteroplasmy can affect the interpretation of mtDNA evidence, potentially leading to false exclusions or inclusions if not properly accounted for.

Cytosine deamination is an issue often observed in specific tissues, such as teeth and bones (Hansen et al. 2017). This process

tends to be exacerbated in aged or degraded samples due to environmental factors such as age, temperature, pH levels, and the presence of water (Gorden et al. 2018). Cytosine deamination in mtDNA is a form of DNA damage where cytosine bases are converted to uracil. During PCR amplification and library preparations, this can lead to mutations, typically observed as C to T transitions in the DNA sequence (Marshall & Parson 2021). The presence of deaminated cytosines can complicate the analysis and interpretation of mtDNA data, as it may introduce artifacts that mimic true genetic variations.

In forensic genetics, the correlation between NUMTs and tissue types is an important consideration. The prevalence of NUMTs can vary between different tissues due to differences in mtDNA copy number and the processes of DNA repair and replication (Wachsmuth et al. 2016). Tissues with a higher mtDNA copy number, like buccal samples, tend to yield fewer NUMTs compared to those with a lower mtDNA copy number, such as blood (Zhou et al. 2023). This is due to the constant number of nuclear DNA (nDNA) copies in cells. Semen samples can pose difficulties for DNA analysis because the mtDNA to nDNA ratio may be greatly diminished. This issue is especially relevant for samples containing concentrated sperm heads, such as those obtained from the pellet of a differential lysis fraction, or for older and decomposed stains where the tails and connecting pieces of the flagellum are absent (Marshall & Parson 2021). NUMTs can be problematic in mtDNA sequencing because they may be amplified and sequenced along with genuine mtDNA, leading to mixed or contaminated sequence data. This is particularly challenging when dealing with low-level heteroplasmies, as NUMTs might be mistaken for true mtDNA variants.

In the context of mitochondrial DNA (mtDNA) analysis, nuclear mitochondrial DNA segments

(NUMTs) are typically found at established NUMT locations, while point heteroplasmy and stochastic errors appear randomly throughout the mitochondrial genome (Smart et al., 2019). Point heteroplasmy, especially in the coding region, is not commonly detected in most mitochondrial haplotypes under forensic variant detection thresholds, which are usually set between 5 and 10% (Just et al. 2015). Both NUMTs, including larger NUMTs known as mega-NUMTs, and point heteroplasmy can be consistently reproduced in PCR amplifications from the same DNA sample, unlike stochastic errors, which are not consistent (Marshall & Parson 2021).

Cytosine deamination, which leads to cytosine (C) to thymine (T) and guanine (G) to adenine (A) substitutions, is more readily identified than point heteroplasmy. This is due to its occurrence at the ends of degraded DNA fragments (Marshall & Parson 2021). Such deamination-induced variants, observed in forensic samples, do not replicate across different PCR or library preparations (Gorden et al. 2018). Conversely, mixtures of DNA from different individuals can be reproducible and are identifiable when they result in low-frequency variants at sites diagnostic of different haplogroups.

Common NUMTs, as opposed to mega-NUMTs, can be differentiated from mixtures based on the location of the variants, which typically align with known NUMT-prone regions of the reference mitochondrial genome (rCRS) (Marshall & Parson 2021). Sequencing errors, another source of low-frequency variants, can be indicated by a strand imbalance where a variant appears in only one direction of sequencing. While sequencing errors may lead to lower base quality, this is not an indicator of NUMTs. A careful consideration of these factors is crucial for accurate mtDNA forensic analysis.

Mitochondrial DNA nomenclature

Recommendations for naming mitochondrial DNA (mtDNA) types are designed to align with the codes of the International Union of Pure and Applied Chemistry (IUPAC). For convenience, the Revised Cambridge Reference Sequence (rCRS) serves as a standard for mtDNA nomenclature (Parson et al. 2014).

When comparing an individual's mtDNA sequence to the rCRS, only differences at specific sites and nucleotides, designated by numbers and letters, are recorded. For instance, if the rCRS has an adenine (A) at site 73 (in HVII), but a significant portion of the population carries a guanine (G) at that site, an individual's mtDNA sequence would be described as 73G, using a capital letter. If no other variations are noted, it is assumed that the analyzed mtDNA sequence is identical to the rCRS, except for the difference at site 73.

As a difference is observed compared to the rCRS, the observed variant is indicated as a suffix to the position (e.g., 73G). In contrast, the rCRS variant is denoted as a prefix (e.g., A73). In cases of unresolved ambiguity at any site, the base number is followed by an 'N' (e.g., 16125N). Insertions are described by indicating the site immediately before the insertion, followed by a decimal point and a numeric identifier (e.g., 315.1C for the first insertion after site 315).

When dealing with homopolymeric tracts, where the exact insertion position is unknown, the assumption is that the insertion occurs at the highest-numbered end of the homopolymeric region (Carracedo et al. 2000). For example, if a common homopolymeric region spans nucleotide positions 311 to 315 (inclusive) and a C insertion occurs after site 315, the nomenclature used is 315.1C. Deletions are recorded by listing the missing site followed by 'DEL', 'del', or '-' (e.g., 220del, 220DEL, or 220-).

If heteroplasmy is observed, it is recommended to use the letter R to denote a mixture of A and G, and Y for C/T heteroplasmy. The IUPAC code employs capital letters, allowing for an extension of the existing nomenclature to include lowercase letters (Bandelt & Dür 2007). This extension is particularly useful for describing heteroplasmic mixtures involving both deleted or undeleted and inserted or non-inserted bases. For example, 152c corresponds to a heteroplasmic mixture of a transition and a deletion at nucleotide position T152. On the other hand, 315.1c describes a mixture of an insertion at position 315.1C with another sequence that lacks this insertion.

To prevent ambiguities arising from erratic alignments and to assist in determining haplotype representation, guidelines for phylogenetic nomenclature have been proposed (Parson et al. 2014). These guidelines stipulate that the alignment of difference-coded haplotypes should be based on the recognized mutation patterns of mitochondrial phylogeny.

Therefore, it is recommended to align sequences in accordance with the current understanding of phylogeny. The most comprehensive repository is Phylotree (www.phylotree.org), where the rCRS-oriented version is advised (van Oven & Kayser 2009). In situations with multiple equally plausible solutions, the maximum (weighted) parsimony approach must be considered. However, variants adjacent to long C tracts should follow sequence-specific conventions. For example, the long C tracts of HVS-I and HVS-II should always be scored with 16189C and 310C, respectively. Length variations of the short A tract before 16184 should be annotated favoring transversions unless the phylogeny indicates otherwise. Correspondingly, indels should be placed 3' with respect to the light strand unless the phylogeny indicates otherwise.

While no nomenclature scheme can seamlessly address the full complexity of mtDNA diversity, the phylogenetic approach provides an evolutionary perspective, which is highly recommended for several reasons. The phylogenetic alignment conventions align with practices common to other genetic fields within the forensic community, with which data are regularly exchanged (Parson et al. 2014). Furthermore, the phylogenetic notation of haplotypes seeks to elucidate the actual biological mutations, enabling the estimation of positional mutation rates and laying the groundwork for a more detailed, scientifically and biologically informed interpretation of forensic evidence. In this regard, the EMPOP database, in addition to storing the largest quality-controlled number of haplotypes, includes the necessary tools for annotating mtDNA sequences (Rock et al. 2011).

Human mitochondrial DNA databases

Human mitochondrial DNA population databases have great importance in the forensic area, since the mtDNA sequence obtained from a questioned sample (a suspect in a crime, for example) corresponds to the profile of an evidential sample (Tagliabracci & Turchi 2020). When a mitogenome from a questioned and another from a known reference sample cannot be excluded as originating from the same source, it's recommended to search some information about the mitochondrial DNA profile's rarity. The attainability of reference sequences is important as the weight of an mtDNA match comparison is determined by estimating the haplotype frequency in relevant population groups (Just et al. 2015, Taylor et al. 2020). In this sense, it is convenient to count the number of times a specific sequence is observed in a population database. As such, great international efforts have been made to develop databases with

population profiles of thousands of matrilineally related individuals (Butler 2012).

In this way, the FBI has created a population database of mitochondrial DNA known as CODISmt (Combined DNA Index System - mitochondrial) to establish an estimate of frequencies for legal application. CODISmt has approximately five thousand mtDNA profiles from fourteen different populations (Butler 2012).

The European DNA Profiling Group (EDNAP) has developed the largest international database with mitochondrial DNA population profiles: the EMPOP, with more than forty-eight thousand quality-controlled mtDNA sequences from several populations in the globe (Huber et al. 2018).

Another database with population profiles of mitochondrial DNA that should be highlighted is the Human Mitochondrial Genome Database (mtDB), developed and maintained by researchers at the University of Uppsala (Sweden), which has over two thousand sequences of mitochondrial DNA divided into ten geographical regions: Africa, North America, South America, Asia, Australia, Europe, Melanesia, Middle East, Polynesia, and Southeast Asia (Ingman & Gyllensten 2006).

Nonetheless, the reachability of mitogenomes on public databases raises some potential ethical issues, such as genetic privacy concerns. Although genes related to predisposition to diseases are generally not the focus of forensic laboratories, a growing number of forensic research makes use of full mitogenome approaches to increase discrimination potential. Information about them is easily accessed on mtDNA databases. To deal with this, forensic laboratories must develop stringent genetic privacy policies to protect the unintended discovery of medical information (Scudder et al. 2018).

CONCLUSIONS

Over the last decades, mtDNA analysis has been instrumental in a wide range of forensic applications. Mitochondrial DNA has proven to be a valuable resource for human identification due to its unique characteristics, such as a high copy number per cell, a protective circular structure, a high mutation rate, non-mendelian maternal inheritance without recombination and resistance to degradation. It has been used to assist in the identification of victims of mass disasters, resolve historical mysteries, and provide critical evidence in criminal cases. Remarkable examples include the identification of Tsar Nicholas II's remains, the victims of the Argentine dictatorship, and the Romanov family's rootless hairs.

Mitochondrial DNA sequencing techniques have evolved significantly, from the pioneering Sanger sequencing to the emergence of next-generation sequencing (NGS) technologies. Second-generation sequencing methods from companies like Roche, Illumina, and Applied Biosystems have improved speed and accuracy; in turn, third-generation sequencing technologies like PacBio and Oxford Nanopore Technologies offer the advantage of reading single molecules in real-time.

In summary, mitochondrial DNA analysis has significantly expanded the capabilities of forensic science by providing a valuable tool for human identification in cases where nuclear DNA may fail due to scarcity or degradation. Advances in sequencing technologies and the development of comprehensive mtDNA databases continue to enhance its effectiveness in solving complex forensic cases.

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PABLO CAVALCANTI^{1,2}

<https://orcid.org/0000-0003-3445-3828>

TATIANA LÚCIA S. NOGUEIRA³

<https://orcid.org/0000-0002-1608-9500>

ELIZEU F. DE CARVALHO^{1,2}

<https://orcid.org/0000-0003-4620-7253>

DAYSE A. DA SILVA^{1,2}

<https://orcid.org/0000-0003-3805-2837>

¹State University of Rio de Janeiro (UERJ), DNA Diagnostic Laboratory (LDD), São Francisco Xavier St., 524, Maracanã, 20550-013 Rio de Janeiro, RJ, Brazil

²State University of Rio de Janeiro (UERJ), Postgraduate Program in Biosciences, 28 de Setembro Ave., 87, 4th Floor, Vila Isabel, 20551-030 Rio de Janeiro, RJ, Brazil

³Brazilian Army Institute of Biology, Francisco Manuel St., 102, Triagem, 20911-270 Rio de Janeiro, RJ, Brazil

Correspondence to: **Pablo Cavalcanti**

E-mail: cavalcanti.p.gen@gmail.com

Author contributions

PC: Conceptualization (Lead), Data curation (Lead), Formal analysis (Lead), Investigation (Lead), Methodology (Lead), Writing - original draft (Lead), Writing - review & editing (Lead). TLSN: Investigation (Supporting), Writing - review & editing (Supporting). EFC: Conceptualization (Supporting), Funding acquisition (Supporting), Project administration (Supporting), Supervision (Lead), Writing - review & editing (Supporting). DAS: Conceptualization (Equal), Formal analysis (Supporting), Funding acquisition (Supporting), Investigation (Supporting), Project administration (Supporting), Supervision (Equal), Writing - review & editing (Equal).

