

Mitochondrial DNA Sequence Analysis — Validation and Use for Forensic Casework

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ABSTRACT: With the discovery of the polymerase chain reaction (PCR) in the mid-1980's, the last in a series of critical molecular biology techniques (to include the isolation of DNA from human and non-human biological material, and primary sequence analysis of DNA) had been developed to rapidly analyze minute quantities of mitochondrial DNA (mtDNA). This was especially true for mtDNA isolated from challenged sources, such as ancient or aged skeletal material and hair shafts. One of the beneficiaries of this work has been the forensic community. Over the last decade, a significant amount of research has been conducted to develop PCR-based sequencing assays for the mtDNA control region (CR), which have subsequently been used to further characterize the CR. As a result, the reliability of these assays has been investigated, the limitations of the procedures have been determined, and critical aspects of the analysis process have been identified, so that careful control and monitoring will provide the basis for reliable testing. With the application of these assays to forensic identification casework, mtDNA sequence analysis has been properly validated, and is a reliable procedure for the examination of biological evidence encountered in forensic criminalistic cases.

KEY WORDS: DNA sequencing, forensic science, mtDNA, PCR.

INTRODUCTION

The role of DNA profiling in forensic investigations has become increasingly important as the analysis techniques have evolved. Cases such as the Nicole Brown Simpson murder trial, the identification of Nicolas Romanov II, the last Russian Tsar, and the identification of the Tomb of the Vietnam Unknown Soldier have brought DNA profiling to the forefront of public awareness. The expanded use of DNA profiling has significantly assisted the investigation of crimes, and has allowed for the identification of human remains where other methods have failed. As a result, the DNA recovered from a minute bloodstain found at the scene of a crime can be associated with a suspect through DNA profiling. Biological specimens (e.g., stains or hair) found at a suspect's residence can be associated with a victim. In many cases, DNA testing can play a critical role in establishing the identity of the victim. Although alternative biological-based identification methods exist, such as blood group markers and polymorphic protein variants [46,55,234], DNA profiling remains the most powerful and robust method of identification since the discovery of the human fingerprint [222].

^aThe opinions and assertions contained herein are solely those of the authors and are not to be construed as official or as views of the U.S. Department of Defense or the U.S. Department of the Army.

^bEditor's note: Comments on this article will appear in a forthcoming issue of this journal.

The majority of DNA profiling methods currently performed in forensic laboratories analyze chromosomal DNA found in the cell nucleus (i.e., nucDNA). Variations in the length of specific nucDNA fragments, generated using either restriction digestion (RFLP: restriction fragment length polymorphism) [15,22,29,108,162,203] or polymerase chain reaction (PCR) amplification (AmpFLP or AFLP: amplified fragment length polymorphism) [27,30,54,72,190], are the most commonly used methods to differentiate between individuals. The most commonly used AmpFLP's are the short tandem repeats (STRs). The length of a DNA fragment is dependent on the composition of tandemly repeated sequences (i.e., a variable number of tandem repeats or VNTRs). The more "repeat units", the larger the DNA fragment. Individuals will have two fragments or "alleles", representing one inherited allele from the mother and one from the father. The inherited alleles can vary in length, resulting in a heterozygous banding pattern (i.e., "profile"), or the alleles can be of the same length, resulting in a homozygous profile. A marker, or forensic DNA "locus", is considered "discriminating" if there are a number of different alleles observed in the population (e.g., 5-10 or more), and if a large percentage of individuals in the population have heterozygous profiles (e.g., 70-95%). In turn, the more loci analyzed to generate a DNA profile, the more likely the biological specimen originated from a single source. Thus, the interpretation of a DNA profile "match" between the evidence and an individual has great significance, as most multi-locus RFLP or AmpFLP DNA profiles will conclusively identify the origin of the specimen, making nucDNA markers the gold standard or method of choice in the forensic community [42,50,72,84].

RFLP and PCR-based DNA profiling systems have survived the scrutiny of both the academic and legal communities, and although the reliability and acceptability of the procedures are still attacked in courts of law on a routine basis, there is now a general acceptance of both the methodologies used to generate a nucDNA profile, as well as the statistical weight placed on a profile [123,164]. In particular, AmpFLP analysis has routinely been admitted as evidence, given that PCR-based testing and length-based fragment analysis have already been generally accepted in the courts; in addition to length-based differences, sequence specific polymorphisms can be detected with PCR in conjunction with the reverse dot blot formats (i.e., AmpliType™ PM and HLA DQA1), a PCR-based method widely accepted in the U.S. legal system [39,83,176,189]. Given these efforts, crime laboratories currently have the ability to evaluate biological evidence with great accuracy, precision, and reliability.

Recent years have seen the advent of mitochondrial DNA (mtDNA) profiling, with a greatly increasing use and emphasis. In addition to nucDNA, mitochondria found in the cytoplasm of a cell contain a second human genome, the mtDNA genome. Although nucDNA profiling is highly informative and the method of choice if available, the mtDNA genome contains useful information which can be used to help establish identity or the source of a biological specimen. mtDNA has two primary advantages over nucDNA. First, on average there are thousands of copies of mtDNA in each cell compared to two copies of nucDNA, making mtDNA analysis a more sensitive assay, and thus, more successful on highly degraded specimens (e.g., old skeletal material and hair shafts). Second, mtDNA is maternally inherited, increasing the range of references available for the identification of human remains, e.g., distant maternal relatives become potential sources for comparison. As a result, a great body of mtDNA forensic profiling has already been performed, with a growing number of laboratories performing the analysis, and with the presentation of mtDNA in the courtroom both in the U.S. and around the world.

Although much is known about mtDNA and the techniques used to analyze the locus, forensic mtDNA profiling is still relatively new, and has some unique features when compared to nucDNA, so it is still being challenged in admissibility hearings. Thus, it is the purpose of this paper to review the characteristics and biology of mtDNA, and based on these characteristics, to describe how mtDNA data is being interpreted forensically. We will demonstrate that the component methods used to perform mtDNA analysis (i.e., DNA extraction, PCR amplification, and sequencing of DNA) are well established and validated, and that combined, these methods have been used to study mtDNA recovered from a wide

range of forensically relevant biological sources, including ancient specimens (e.g., mummified soft tissue, bone, and hair). In turn, mtDNA analysis has been used successfully in a variety of forensic identification cases. As a result, mtDNA analysis has become a validated, robust, reliable, and well established forensic DNA profiling system.

I. MITOCHONDRIAL DNA (mtDNA) BIOLOGY AND BACKGROUND

A. Characterization of the Locus

In addition to the nucDNA genome, human cells contain additional genetic elements within mitochondria. Mitochondria are double-membraned organelles present in the cytoplasm, and are the site of many crucial metabolic processes such as oxidative phosphorylation. For this reason, the mitochondrion is often referred to as the energy powerhouse of the cell. It is now established that present-day mitochondria are derived evolutionarily from an ancient bacterial ancestor that at one time formed an intracellular symbiosis with early eukaryotic (or pre-eukaryotic) cells [61]. In the ensuing hundreds of millions of years, this ancestor lost the ability to function as an independent organism. The ancestral genome is now greatly attenuated, so that most of the functional proteins of mitochondria are coded for by genes present in the nucleus [124]. What remains within the human mitochondrion is a ~16,569 bp circular genome that encodes 37 densely packed genes (Figure 1). Twenty-two of the genes encode transfer RNAs (tRNA), 2 encode ribosomal RNAs (12S and 16S rRNAs), and 13 encode protein enzymes involved in the electron transport chain of oxidative phosphorylation and ATP production [228]. The mitochondrial genome has been extensively characterized from the standpoint of function, population variation, and genetic disease. Deletions, duplications and numerous point mutations have been identified within the coding region that are cause to various pathological syndromes, and the progressive accumulation of some of these in normal adults is thought to contribute significantly to degeneration with aging (extensive reference lists are in [9,227,228]).

The primary sequence of the mitochondrial genome was determined in 1981 [6]. The two strands of mtDNA have significantly different base compositions, with a pyrimidine-rich "light strand," and a purine-rich "heavy strand." In addition to the coding regions of mtDNA, there is only one significant non-coding section, the control region (CR). The CR is also sometimes called the D-loop, so named for structures visible by electron microscopy that are formed during mtDNA replication. The CR is

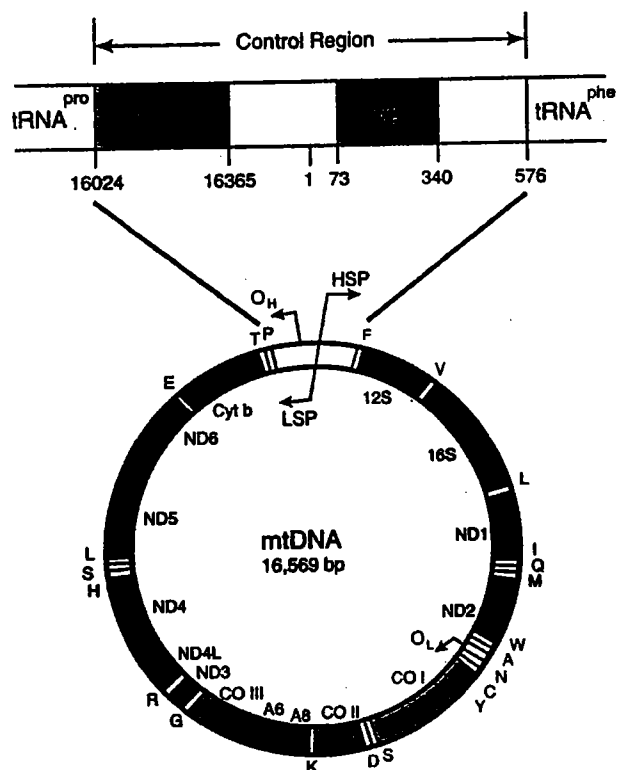


Figure 1. Map of the human mitochondrial genome and expanded diagram of non-coding control region. Listed are the genes for 12S and 16S ribosomal RNAs, subunits of the NADH-coenzyme Q oxidoreductase complex (ND), cytochrome *c* oxidase complex (CO), cytochrome *b* (cty *b*), ATP synthase (A), and 22 tRNAs (labeled with single letter amino acid code). The light strand (O_L) and heavy strand (O_H) origins of replication, and the light strand (LSP) and heavy strand (HSP) transcriptional promoters are shown by arrows. Control region diagram shows flanking tRNAs and location of hypervariable region 1 (HV1) and 2 (HV2); numbering system follows that of the standard reference sequence [6].

approximately 1125 base pairs in length, and contains promoters for polycistronic RNA transcription of genes on both the light and heavy strands, as well as the origin of DNA replication for the heavy strand. The base numbering system of the standard reference sequence (or Cambridge Reference Sequence, CRS [6]) begins arbitrarily near the middle of the control region, so that the control region spans positions 16,024 to 16,569, then continues from base position 1 to 576 (Figure 1). Relative to the coding regions, portions of the CR are highly variable among individuals, presumably due to decreased selective constraint of the non-coding DNA. For this reason, forensic identity testing has so far focused on sequence variation within two hypervariable regions of the CR (e.g., [89,172,238]). Hypervariable region 1 (HV1) extends from position 16024 to ~16365, while hypervariable region 2 (HV2) extends from ~73 to ~340 (the boundaries are not rigidly defined, and vary among particular studies or laboratories).

B. Copy Number and Inheritance

A characteristic of mtDNA that is of great advantage in forensic testing is the high copy number per cell. Whereas nuclear DNA is present in two copies per diploid cell, a mature oocyte is estimated to have thousands of mitochondria and more than 100,000 copies of mtDNA [152,178]. Somatic cells range from having ~200-1700 mtDNA copies depending on tissue type [19,188]. This relative abundance of mtDNA imparts a correspondingly higher likelihood of its recovery from samples where DNA is generally highly degraded, and is one of the principle reasons why mtDNA testing is used.

A second characteristic that lends great power to mtDNA analysis as a forensic tool is that mtDNA is maternally inherited. A primary reason for this may be simply numerical: sperm heads contain only a few copies of mitochondrial DNA compared to the many thousands of copies in the ovum [35]. However, there also appears to be a specific recognition mechanism that can eliminate even the few paternal mitochondria that may be introduced into the ovum. For example, when mitochondria from human sperm cells were introduced into somatic culture cells devoid of mtDNA, 10–20% of the cells contained functioning sperm mitochondria immediately following introduction, while only a very small fraction of the cells ($1/10^5$) survived more than 48 hours [139]. However, when mitochondria from somatic cells were introduced into cultured cells, there was rapid replacement of endogenous mtDNA [115]. This points to the existence of mechanisms that specifically eliminate sperm-derived mitochondria, but not mitochondria derived from somatic cells.

Additional information regarding mtDNA inheritance comes from studies of other mammals. Further supporting a mechanism for the elimination of sperm-derived mitochondria, Sutovsky et al. [211] found that sperm mitochondria were undetectable by the late four-cell stage of *in vitro*-fertilized bovine embryos. However, Gyllenstein et al. [65] studied mouse species whose mtDNA are readily distinguishable, and performed repeated hybridizations between *M. spretus* females to C57BL males. Using highly sensitive PCR techniques designed to specifically amplify paternal DNA, low levels of paternal sequences (0.01–0.1% relative to maternal contributions) were detected. Similar results were obtained by Kaneda et al. [113], also using interspecific mouse hybrids, with paternal mtDNA detectable throughout development and birth. However, when Kaneda et al. performed crosses using mice of the same species, paternal mtDNA was detected only through the early pronucleus stage. These latter results suggest that the mechanism for elimination of paternally derived mitochondria (or mtDNA) is based on

a self:non-self recognition system that does not function with mitochondria derived from a different species. This would account for the persistence of paternal mtDNA that Gyllenstein et al. [65] observed with inter-specific mouse hybridization.

While the mechanism for elimination of paternal mtDNA is not fully elucidated, nor known to be absolute in terms of extremely low level persistence, it is clear that from the practical standpoint of mtDNA forensic testing, mtDNA behaves as maternally inherited. Parsons et al. [173] report comparison of mtDNA sequences of 69 father:child pairs, and in no case was any trace of the paternal sequence detected by direct sequencing of PCR-amplified mtDNA (this being the same methodology in use for forensic testing). While many instances have now been observed of mixtures of more than a single mtDNA type within an individual (a condition known as heteroplasmy, reviewed in more detail below), in no case have the mixtures involved more than a small number of base positions (i.e., usually 1 or 2). If such heteroplasmic mixtures were the result of paternal inheritance, the expectation would be for mixtures at many more positions, as there are on average eight differences in control region sequences between two randomly selected Caucasian individuals.

C. Heteroplasmy

1. Overview

During development, mtDNA molecules are replicated independently of one another, are not strictly tied to mitotic or meiotic cell division, and are thought to be essentially non-recombining [reviewed in 100]. Further, mitochondrial DNA replication is associated with a much higher error rate than is nuclear DNA (e.g., [23,122]). These factors create the possibility that the population of mtDNA molecules found within an individual could be diverse, with many variants replicating and segregating independently. Indeed, if all the mtDNA variants present in a mother were passed to her offspring, one would predict that over population genetic history the accumulation of variants would be so extensive as to invalidate the concept of a mitochondrial DNA "type" that would be useful for identity testing. However, we know from vast experience in human population genetic studies and forensic testing that this is not the case: i.e., when PCR-amplified mtDNA is sequenced, individuals typically harbor a single mitochondrial DNA type that is distinguishable from that of other maternal lineages.

While mechanisms clearly exist that restrict the level of mtDNA variation that is passed between generations (genetic bottlenecks in mtDNA transmission, discussed below), it is now known that mixtures of two or more

subpopulations of mitochondrial DNA — a condition known as heteroplasmy — can occur within individuals. Heteroplasmy has the potential to both complicate and strengthen forensic identity testing, and must be taken into account. Fortunately, the frequency of heteroplasmy in the population (when analyzed by DNA sequence analysis) is relatively low (i.e., 2–8% of the population), so it is not an issue in a majority of cases, and our current knowledge is sufficient to appropriately deal with it in a great majority of cases where it does occur.

The expectation of readily detectable sequence variation within the mtDNA population of single individuals motivated early studies that compared the sequences of multiple fragments of cloned mtDNA from single individuals, as well as from different tissues within an individual [156,157], and from retinal tissue suspected of being highly susceptible to DNA damage [17]. The then-surprising result of these studies was that remarkably little variation was detected. As a result, an expectation was established that variation within individuals was minimal, and that individuals could be considered to be essentially homoplasmic. This assumption of homoplasmy was not violated by a vast body of work performed in the field of human population genetics and molecular evolution. Many population studies of human CR sequence variation were performed using PCR amplification and direct sequencing (reviewed in a subsequent section), using both manual and automated fluorescence sequencing techniques. These studies were conducted under the assumption of homoplasmy, and none reported other than a single mtDNA type within thousands of individuals. The explanation for this is that heteroplasmy can be difficult to distinguish from "background" in sequencing data, where apparent signal from alternative nucleotides can be present at variable levels due to artifacts of the sequencing chemistry and/or detection methods. However, newer and "cleaner" sequencing technologies are now being used, and as a result, it is not particularly difficult to distinguish heteroplasmy from background. However, the data from population genetic studies were not analyzed with heteroplasmy in mind, and the positions where heteroplasmy occurred in those samples were undoubtedly either called as the predominant nucleotide, or denoted as isolated "ambiguities" of unknown cause.

The first documented instance of point mutation heteroplasmy within the human CR occurred in a forensic case involving the skeletal remains of Tsar Nicholas II [58,104]. Since then, heightened scrutiny has resulted in multiple reports of point mutation heteroplasmy in the CR, indicating that it is not an extremely rare occurrence [13,14,38,98,101,161,172,208,219,220,235]. In addition, it has been known for some time that length heteroplasmy is common in two polycytosine stretches (one in HV1 and

