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].

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].

[a]The 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.

[b]Editor's note: Comments on this article will appear in a forthcoming issue of this journal.

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

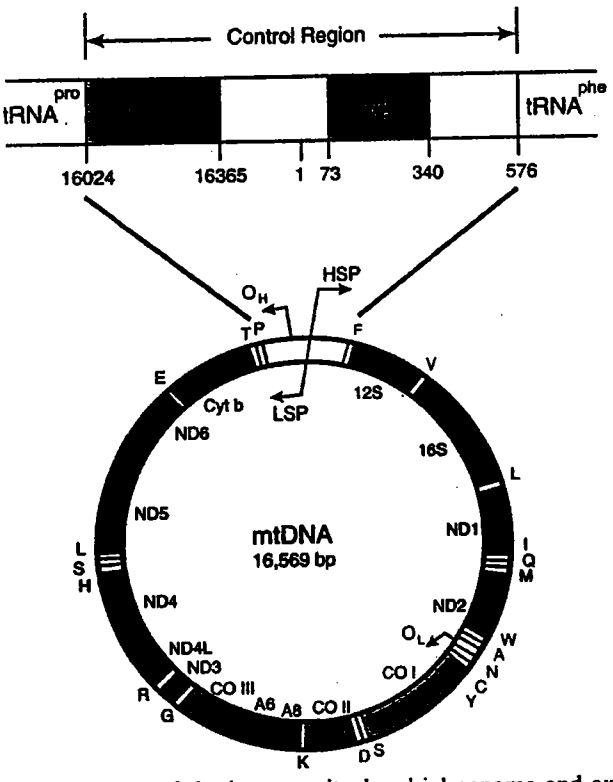

Figure 1. Map of the human mitochondrial genome and expanded diagram of non-coding control region. Listed are the genes for 12S and 16S ribosomal RNAs, subunits of the NADH-coenzyme Q oxidoreductase complex (ND), cytochrome *c* oxidase complex (CO), cytochrome *b* (cty b), ATP synthase (A), and 22 tRNAs (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, Gyllensten 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 Gyllensten 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

one in HV2 [12,140]). Within HV1, most people have a T at position 16,189 that interrupts a run of C's on each side. When a C is substituted for the T at 16,189 (a condition present in ~20% of the general population), this creates an unbroken run of C's that is apparently replicated with low fidelity by the mitochondrial DNA polymerase system. The result is a population of molecules within an individual that differ in the length of the C-stretch, producing an abrupt crash in the quality of direct sequencing data due to template molecules that are out-of-register with one another. When the HV1 C-stretch is present, it is virtually impossible to characterize the population of length variants by direct sequencing, so forensic labs often do not call a "type" for the HV1 C-stretch. The correct sequence outside of the C-stretch itself, however, can be determined by sequencing both strands and using alternative sequencing primers. Length heteroplasmy also occurs in the HV2 C-stretch (positions 303–315), but in that case there is often an identifiable predominant length variant, even when heteroplasmy is present [172].

Although there have been numerous reports of CR heteroplasmy, the sample size is too small to know with precision the frequency with which heteroplasmy occurs at most nucleotide positions. In addition to DNA sequence analysis, there are other, non-sequencing methods that can unambiguously indicate the presence of heteroplasmy: e.g., denaturing high performance liquid chromatography (DHPLC) [221], denaturing gradient gel electrophoresis (DGGE) [77,205], and single-strand conformation polymorphism (SSCP) [219] analysis. In work performed in the authors' laboratory [219], DGGE was used in a population study to assess the frequency and distribution of heteroplasmy within HV1. Blood samples from 253 individuals of various races (predominantly Caucasians) were assayed for heteroplasmy, with the result that 35 (~14%) showed some level of detectable heteroplasmy; two individuals were heteroplasmic at two positions. This high percentage is due in part to the high sensitivity of the DGGE method in detecting mixed sequences. Mixing experiments showed that DGGE is often capable of detecting a mixture even when the minority component is 1% of the total, and in this study was deemed capable of detecting mixtures at all positions in the region that had a minority component of 5% or greater. This is substantially more sensitive that direct sequencing, where the sensitivity threshold is ~10–15% at best [236]. In fact, only three of the individuals in the study had heteroplasmy that was detectable by direct sequencing. This underscores that when asking the question, "Is this individual heteroplasmic?" one also has to ask, "At what level of sensitivity?" Certainly, given the untold trillions of mtDNA molecules in an individual, we are all heteroplasmic at some trace level, but only occasionally at levels that are of functional significance to forensic identity testing.

A significant point that comes out of the DGGE heteroplasmy study cited above, as well as the published [13,14,38,98,101,161,172,208,219,220,235] and unpublished data (AFDIL) on heteroplasmy detected by sequencing, is that not all positions in the CR have an equal propensity for heteroplasmy. The DGGE study indicated two predominant hot spots for heteroplasmy, with 12 independent instances of heteroplasmy (34% of the total) occurring at position 16,093, and 6 instances occurring at 16,129. Four other sites had two instances of heteroplasmy, while heteroplasmy at all other sites occurred only once. A general correlation can be seen between the sites where heteroplasmy occurs and the sites where differences between individuals accumulate over evolutionary time in the human population [79,106,226]. This is to be expected as both heteroplasmy and population polymorphism have the same root cause, i.e., the substitution of nucleotides at particular base positions on mtDNA molecules. It has been well established that the rate of base substitutions between individuals in the population is quite heterogeneous among sites [79,226]. The sites where multiple instances of heteroplasmy have been observed, particularly the two HV1 hot spots 16,093 and 16,129, are all sites that are polymorphic for population variation as well. The same is true of the HV2 C-stretch, where individuals commonly show length heteroplasmy [140], and where the population is roughly evenly divided between having 7 Cs or 8 or more C's [26]. This general information on the distribution of sites where heteroplasmy occurs can guide interpretation when heteroplasmy becomes an issue in forensic casework (discussed below).

2. Intergenerational Substitution

Given that heteroplasmy occurs at quite tractable levels at only a site or two within individuals, it is not a factor that *a priori* confuses forensic identity testing. Indeed, it represents an additional level of variation that can increase the power of mtDNA testing [104]. If a reference sample and an unknown sample match each other by sharing a constellation of unusual polymorphisms, the co-occurrence of heteroplasmy at a particular site would provide additional evidence restricting the pool of potential donors, and increasing the odds that the reference is, in fact, the source of the unknown sample. However, this concept must be framed with reference to the manner with which heteroplasmic mtDNA variants segregate within an individual during development, and how they are passed between generations. As it turns out, these processes involve one or more genetic "bottlenecks." This creates the potential for different hetero-

plasmic ratios among different tissues of the same individual, as well as differences between maternal relatives. In extreme cases, the difference could be large enough to appear as a homoplasmic nucleotide substitution. While this feature of heteroplasmy can cause complications in forensic testing our current understanding of mtDNA biology is such that the complications can be dealt with appropriately in a great majority of situations that will be encountered.

The bottleneck theory was proposed in early studies to account for the rapid segregation of variant mtDNA sequences observed within pedigrees of Holstein cows [80,81,117]. Frequent transitions to heteroplasmy were observed, as well as shifts between generations from one apparent homoplasmic state to another, sometimes without a heteroplasmic intermediate. In the bottleneck theory, it is proposed that the number of mtDNA genomes is reduced to a relatively small number during some stage of oogenesis, or germ cell development. A subset of the mtDNA population is transmitted through the bottleneck and this founder population (or a portion thereof) is subsequently replicated to produce ~100,000 mtDNA copies in the mature ovum. The founder population, as a small subsample, may differ significantly in the proportions of variants from the original mtDNA pool, giving rise to differential segregation between different meioses.

It is quite apparent that an mtDNA bottleneck occurs during human oogenesis. Bendall et al. [13] studied 180 twin pairs and discovered CR heteroplasmy (detectable by direct sequencing) in four instances. Analysis of additional family members in these heteroplasmic lineages showed differential segregation of varying magnitude between generations. Applying a population drift model to the data, Bendall et al. estimated the size of the meiotic (intergenerational) bottleneck to range from 2 to >100 mtDNA molecules for particular mother:child transmissions, with an overall best estimate of 3–20 molecules. This is similar to the value Marchington et al. [141] calculate from studying individual ova from a single heteroplasmic woman. However, Howell et al. [97] present data from a particular heteroplasmy segregating in a lineage where the bottleneck does not always appear to be greatly restricted — as also appeared to be the case with certain transmissions in Bendall et al. Therefore, it may be that the size of the effective bottleneck can vary stochastically from transmission to transmission, or in response to other genetic factors [45].

The data of Parsons et al. [173] are also consistent with a narrow bottleneck. In that study, 327 mother:child events were surveyed (directly or indirectly) within 134 independent mtDNA lineages. Within these, there were ten instances of a substitution of one mtDNA type for another; i.e., generally one nucleotide substitution for another. In one lineage, two such apparent "substitutions" were observed. Additional such "intergenerational substitutions" were reported by Parsons et al. [174], indicating a higher rate than is predicted by evolutionary estimates of the mtDNA substitution rate. This higher rate was also reported by Howell et al. [98], but others studying single or restricted numbers of lineages failed to observe such a high rate of substitutions [107,202].

The explanation of why near-term empirical studies, when taken together, indicate a higher intergenerational substitution rate than predicted by evolutionary/phylogenetic studies has not been resolved. It has been suggested that it is because near-term substitutions occur at hot spots [107,137,171] providing an inflated estimate of the rate when applied to all CR sites. However, this would only be true if phylogenetic analyses had substantially underestimated the rate of reversion mutations at these hot spots [79,99,173]. Whatever the explanation, it is the empirical studies that are directly relevant to forensic identity testing, and it is apparent that maternal relatives can differ from one another in CR sequence with an appreciable frequency. Interpretation in mtDNA identity testing must take this into account when maternal relatives are compared, to avoid the potential for false exclusion. Further, when apparent differences are noted between individuals suspected of being maternal references, interpretation can be greatly abetted by careful analysis of both samples for low levels of heteroplasmy that would indicate the segregation of mtDNA variants within a lineage.

3. Heteroplasmic Segregation: Disease Studies and Development

A large body of work has investigated the segregation of disease-causing mtDNA mutations within individuals and between generations. While a detailed review is beyond the scope of this paper, we will provide a brief overview, focusing on how this information relates to issues of forensic significance. Common syndromes associated with tRNA or protein-coding point mutations are myoclonic epilepsy and ragged-red fiber (MERRF), mitochondrial encephalomyopathy, lactic acidosis, and stroke-like symptoms (MELAS), maternally inherited myopathy and cardiomyopathy (MCM), Leber's hereditary optic neuropathy (LHON), and neurogenic muscle weakness, ataxia, and retinitis pigmentosa (NARP). Common diseases associated with large-scale deletions of mtDNA are Kearns-Sayre syndrome and Pearson's syndrome. These diseases are sometimes due to homoplasmy for the mutant condition, but frequently are associated with varying levels of heteroplasmy. Pathological conditions become evident when the energy-producing capacity of mitochon-

dria falls below threshold levels in particular tissues with critical energy requirements, and the severity of symptomology is often correlated with the proportion of mutant mtDNAs (extensive reference lists relevant to this paragraph are in [9,227,228]). While there are abundant reports of heteroplasmy associated with mtDNA disease, these contribute little to our knowledge of the frequency of heteroplasmy in the CR because the pathogenic nature of these heteroplasmies ensures their detection even if the causal events are rare.

Many studies have investigated the segregation of disease-related mutations within pedigrees (e.g., [21,91,125,126,135,154,181,201,213,225]). Taken together, the information from intergenerational transmissions strongly reinforces the existence of a genetic bottleneck between generations, with different offspring of the same mother demonstrating widely different heteroplasmic proportions. While this appears to be the general rule, some disease studies have suggested that the bottleneck is not always tightly restricted [132,177,225]; as was the case in some instances with CR heteroplasmies [13], and neutral mutations in the coding region [97]. It is possible that these studies were influenced by the pedigrees evaluated and/or the limited number of pedigrees evaluated.

It is important to note that mutations involved in mitochondrial disease have functional consequences to cells and tissues, and the segregation and accumulation of these pathogenic mutations may be different than for non-coding CR variants. This is suggested by a general (but not absolute) trend for the progressive increase of deleterious mutants across generations. While this trend is clearly superimposed with a high degree of sporadic segregation consistent with a bottleneck (discussed above), it appears that in some cases there is a general replicative advantage for the mutant condition. Large-scale deletion mutations may have a replicative advantage due to their smaller size (e.g., [227]), but a progressive accumulation across generations occurs with some deleterious point mutations as well [201,246]. The cause of this is not understood, but may be related to the functional effects of the mutations. Therefore, one should use caution in using disease studies as a guide for how segregation may operate on forensically relevant CR variants.

Many studies of disease-associated heteroplasmies indicate an uneven distribution of mutant mtDNAs in various tissues of afflicted individuals [25,71,96,103,116, 125,130,135,144,167,213,245,248]. This phenomenon might reflect differential segregation of mtDNA variants during early development, but in general the studies do not discriminate this possibility from that of variable post-mitotic selection on, or accumulation of, mutant mtDNAs. The widespread occurrence of heteroplasmic mutations throughout multiple tissues strongly suggests that the individuals originally inherited heteroplasmy from their mother. In addition, a number of studies indicate that a relatively uniform distribution of heteroplasmic mixtures occurs throughout the establishment of the primary germ layers, as evidenced by a uniform distribution among fetal tissues, but subsequent differential accumulation or segregation can occur to result in different heteroplasmic mixtures among tissues in adults [138,144,219].

A common finding in inter-tissue disease studies is that levels of the mutant type are highest in tissues with high-energy demands such as brain, skeletal muscle, liver, and heart. The high-energy demands of particular cells may produce a more oxidative environment that leads to higher mutagenesis [71]. However, others have suggested the possibility that the functional nature of the disease mutations play a role in their differential accumulation in a tissue-specific fashion [144]. Once again, the dynamics of segregation and accumulation of deleterious mutations may be quite different than those of CR sequence variants.

There are a number of studies involving CR heteroplasmies in humans indicating that the primary bottleneck in mtDNA segregation occurs prior to maturation of the oocyte, and hence, prior to fertilization and cell division during embryogenesis [16,140,141]. This is also the picture that emerges from a detailed study of the segregation of neutral, non-functional variants in artificially heteroplasmic mice, where the bottleneck was demonstrated to occur during the expansion of the population of oogonia from primordial germs cells during oogenesis [110]. The implications of this are that the state of heteroplasmy or homoplasmy present within an oocyte determines to a large extent that a similar condition will be found throughout an individual after development [141,182]. However, there is evidence that post-zygotic differential segregation of mtDNA variants does occur to some extent in the CR. This was seen in a DGGE survey of CR heteroplasmy in 21 adults [220]. Examination of various tissues (blood, bone, brain, hair, liver, muscle) from these individuals indicated that the levels of heteroplasmy varied in some instances among the tested tissues. Some tissues were detectably heteroplasmic, while others were not. However, in almost all cases in this study, the heteroplasmy was quite low level (undetectable by sequencing) and the variability between tissues reflected rather minor fluctuations, some near the limit of detectability.

The issue of between tissue variation is relevant to forensic testing because it creates the potential for reference and questioned samples from the same individual to differ in heteroplasmic ratios, or in a more extreme case in an apparently homoplasmic manner. The data reviewed

above indicate that in general this is a rather restricted problem, by virtue of the primary bottleneck in mtDNA segregation occurring prior to embryogenesis. However, the data indicate that drift of heteroplasmic ratios can occur during development, and forensic scientists must be prepared to deal with this in both procedure and interpretation. Particularly, recent studies (primarily in the forensic context) have indicated the existence of another developmental bottleneck that exists in the generation of individual hairs from a single individual [14,208,235]. For example, in a particular woman, multiple extracts and amplifications from blood and buccal swabs gave consistent levels of CR heteroplasmy (the levels were consistent within each, but differed slightly between the blood and buccal swabs) [235]. However, extracts from different hairs of the same individual showed greatly skewed ratios; in five hairs thymine predominated at position 16,355, in three hairs cytosine predominated, and in two there was a roughly even mix.

Testing of shed hair recovered at crime scenes is a major application for mtDNA analysis, because hairs lacking roots generally possess insufficient amounts of nuclear DNA for testing. Therefore, the scenario of hair sequences differing from those of blood, buccal, or other hair samples of the same individual will be encountered. In such cases it is important to carefully scrutinize the reference samples (blood, buccal swab) for low-level heteroplasmy that would be expected to segregate differentially among individual hairs. Also of importance is to sample multiple hairs from the suspected individual, to test whether differential segregation of heteroplasmic variants is occurring in a manner consistent with the type observed in the questioned sample. Interpretation of mtDNA evidence in such a case will be discussed in a subsequent section.

D. Population Genetic Variation

Studies of the variation in human mitochondrial DNA have been pivotal in our understanding of the evolutionary origin and population genetic history of modern *Homo sapiens* (e.g., [31,43,79,150,175,186,224]). The maternal inheritance, lack of recombination, and high evolutionary mutation rate of mtDNA are properties that aid tremendously in the ability to draw inferences relating to genetic history. The control region is the most rapidly evolving segment of mtDNA, and sequence data from the CR has been characterized from a great many populations worldwide. A recent review lists 4079 HV1 and 969 HV2 sequences compiled from 38 separate primary publications [73]. It was this wealth of sequence information that revealed the high variability of control region sequences, and prompted the development of forensic analysis of the CR.

The large number of published sequences for the most part do not correspond exactly to the boundaries of the regions that are analyzed in forensic testing [153]. Additionally, it is difficult to evaluate the accuracy of the sequence data when compiled from such a wide variety of sources. For these reasons, forensic laboratories have assembled databases of their own, comprised of complete HV1 and HV2 sequences that have been determined in accordance with specified quality assurance criteria (e.g., [172,238]). The current database in use by the Armed Forces DNA Identification Laboratory and the FBI (and shared by other forensic laboratories worldwide) contains HV1 and HV2 sequences from 1657 individuals. The size of this database at the time of this writing, broken into component ethnic groups, is as follows: African American, 149; Afro-Caribbean 116; African, 115; U. S. Caucasian, 604; English Caucasian, 102; Austrian Caucasian, 101; French Caucasian, 109; American Hispanic, 99; Asian American, 58; Japanese, 162; Korean, 42 [26].

Sequence databases are our best source of information regarding the power of mtDNA for identity testing. Given that it is expected that maternal relatives will match each other (barring mutation or "substitution"), it is clear that mtDNA is not a unique identifier. Moreover, mtDNA lineages extend back into time to various depths of antiquity, with the result that one's mtDNA lineage generally encompasses many more individuals than would be included in one's known relatives. In addition to the potential for matching other individuals of the same lineage, matches will also occur to other mtDNA lineages that have mutated independently to consist of the same mtDNA sequence. The population genetic processes that govern the longevity and spread of mtDNA lineages, and the rate of change to and from a particular sequence, are complex. Therefore, we presently must turn to an empirical evaluation of the net result of these processes: the frequency with which particular sequences are detected within various populations.

Within the database of 604 U. S. Caucasian HV1 and HV2 sequences, there are 451 distinct mtDNA types. Among these, there are a small number of relatively common types, and a larger number of rare types. The most common mtDNA type occurs in 26 individuals, or 4.3% of database. There are 188 other mtDNA types that occur in more than one individual (ranging from one type that occurs 15 times to 31 types that occur twice), but 390 occur in only a single individual. Using Mitosearch, a program developed by the Federal Bureau of Investigation [26] one can perform a pairwise comparison of all sequences within the database. For U. S. Caucasians, this

results in 669 instances of a match out of 182,106 separate comparisons, for an empirically determined probability of 0.0037 for a random match. Thus, we can predict that two randomly chosen individuals from this population will match once out of ~270 times. However, given a known sequence of a particular type, the chance that another random individual will match depends greatly on the relative rarity of that particular type.

All other population databases show a pattern similar to that of the U. S. Caucasians in having many rarer types and fewer common types. However, it is known that different populations can have different levels of mtDNA diversity, with pools of mtDNA types that differ significantly [147–149,207]. For example, the African American database ($n = 149$) has a most common type occurring in 2.7% of individuals in the database, 27 additional types that occur more than once, and 118 types that occur only once. In the African American database, the average number of nucleotide differences between individuals is 14, higher than in Caucasians; the average number of nucleotide differences between individuals in the U.S. Caucasian database is eight. This is consistent with a higher mtDNA diversity known to occur in African populations, reflecting a greater evolutionary age of African versus non-African (and particularly, Caucasian) sequences. Moreover, if we compare all the sequences in the U.S. Caucasian database with those in the African American database, there is only a single instance of a sequence match, compared to pairwise match frequencies more than 200 times higher in each of the databases considered separately. This indicates the extent of the sequence differences between the databases and illustrates the necessity of maintaining separate databases, at least for the major population/racial groups.

It is apparent that, in general, the strength of mtDNA evidence is limited by the size of the current databases. Since there are many rare haplotypes, the "apparent frequency" of the unique haplotypes (1/n, where n is database size) is, in a great majority of cases, an overestimate of the true frequency. This can be illustrated by simulating the growth of a database. For the purposes of this example, the forensic databases of European-derived sequences (U.S. Caucasian, French Caucasian, Austrian Caucasian, English Caucasian) were pooled [there is evidence suggesting that there is little or no substructure within mtDNA of various native European populations, 149]. From this larger pool, 100 sequences were sampled at random to produce a smaller database, then sequences were added to this by further random addition in increments of 100. This resulted in a growing set of databases of size 100, 200, 300, 400, 500, 600, 700, and 800.

For these incremental databases, **Figure 2** plots the total number of mtDNA types and the total number of unique mtDNA types. Both of these values are still increasing steadily at the maximum database size of 800, even within Caucasians where diversity is lower than in some other major groups. We can see that when new sequences are added, a great majority of sequences that were unique in a smaller database remain unique, with a concomitant decrease in their "apparent frequency." For example, when 100 new sequences were added to the database of size 700, 66 new apparently unique sequences were added, while only six sequences were added that matched a sequence that was previously unique. Thus, in increasing the database size, the apparent frequency of six previously unique sequences increased, while the apparent frequency of 445 previously (and still) unique sequences decreased. It is not known when most of the mtDNA types in the population will have been sampled, but the limit has not yet been closely approached. Large-scale efforts that are underway to increase database size can be expected to increase the power of mtDNA testing for the many rare mtDNA types.

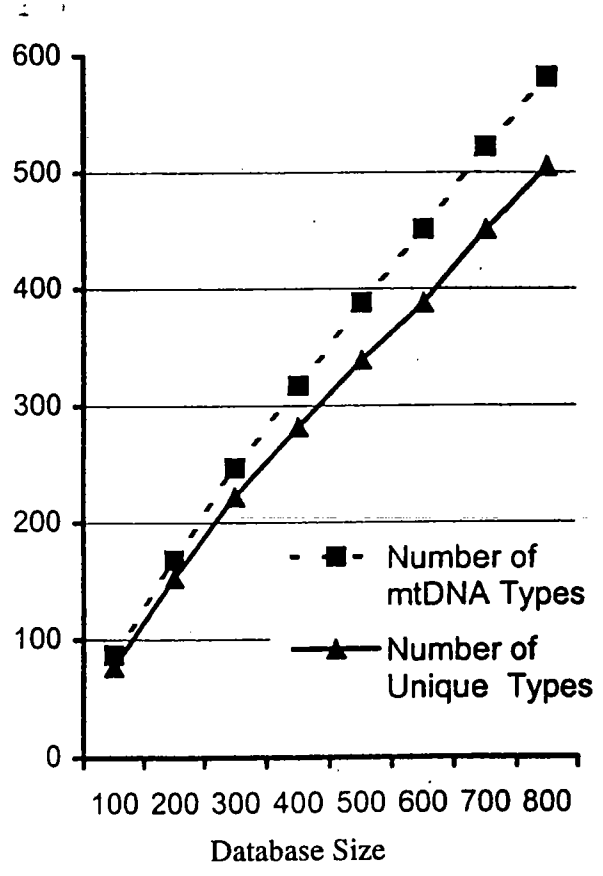

Figure 2. Graph showing effect of database size on the total number of mtDNA types within the database and the number of mtDNA types that are unique in the database. Databases increasing in size by increments of 100 were obtained by random subsampling of a large database of Caucasian sequences, as described in text.

II. INTERPRETATION OF MtDNA DATA IN THE FORENSIC CONTEXT

A. Match Significance

When one applies mtDNA sequence typing as a forensic test, it is clear that the purpose is to answer two related questions: (a) through sequence comparison, could an individual be excluded as a possible source of a questioned sample?, and (b) if not, what does this inclusion suggest regarding the chance that the individual is, in fact, the source of the questioned sample? If the answer to the first question is yes, the exclusion is an outcome of obvious significance (for which mtDNA testing has a clear and proven utility). However, if an individual is included as a potential source, one is immediately faced with the second question. Unless the discriminatory potential of a test can be objectively evaluated, an inclusion could mean anything. It is therefore incumbent on the forensic scientist to determine a means to evaluate and communicate the significance of an mtDNA inclusion or "match." In this section we will explore various alternative and/or complementary methods for communicating this significance, without intending to suggest that these definitively or exclusively constitute the manner in which mtDNA evidence should be presented in a court of law. In fact, mtDNA evidence has been introduced in only a small number of cases in the United States, and it seems likely that the presentation of mtDNA statistics in court will continue to evolve. What remains clear nonetheless is that there are many scientifically accurate statements that can be made concerning mtDNA match significance. As long as appropriately conservative approaches are taken, with clear statements of the premises and potential limitations of various statistics, forensic scientists have at their disposal a number of ways to aid investigators, lawyers, judges, and juries in a proper understanding of what the mtDNA evidence means.

In general, the significance of an mtDNA sequence match is dependent on the case in question, and the mtDNA type involved. In only a restricted set of circumstances can mtDNA matching be considered definitive evidence of identification. This can occur when the population of potential sources is "closed," that is, narrowed down to an identified set of individuals. An example would be an aircraft incident where recovered remains simply need to be associated with any of the crewmembers known to be aboard. If a reference sample (often a maternal relative in such a case) for a crewmember can be distinguished from those of all other crewmembers, a match to a set of recovered remains can be considered a positive identification. Here, the identification is not based on DNA statistics, but on a process of elimination that is, in turn, dependent on the surety that the sample could only have come from a known set of individuals. The identification of Michael J. Blassie, whose skeletal remains were interred for 14 years in Arlington Cemetery as the Vietnam Unknown Soldier, was an instance where mtDNA was used to distinguish among a restricted set of reference lineages.

More commonly, mtDNA testing involves cases where the potential source population is not closed or completely characterized. In these cases, mtDNA evidence alone cannot be considered a definitive identification, and we have to address the relative significance, or exclusion potential, of a match. Such assessments are based on some indication of the relative frequency of the mtDNA type in the population, or, relatedly, the chance that random individuals in the population will also match that mtDNA type. As a general indication of the power of mtDNA testing, one can present the frequency with which sequences in a database match one another when all pairwise comparison are made. For example, in a database of 604 U.S. Caucasian sequences, there are 669 instances of matching in 182,106 comparisons: on average, the chance that two randomly chosen individuals in this population would match is one in 272. While such average values can provide the framework for thinking about mtDNA match probabilities, this is not the best way to evaluate match significance in any particular case. A more accurate and useful guide for interpretation is based on the relative rarity of the particular sequence in question. The relative rarity (or relatedly, the frequency) of a sequence determines how likely it would be for a randomly selected individual to match that particular mtDNA sequence by chance.

The simplest factual statement that can be made concerning the relative rarity of an mtDNA sequence type is to report the number of times that the sequence has been observed in various databases. While this has been referred to as the "counting method," the manner in which it has been presented in mtDNA court cases differs from the "counting method" that the original NRC report defined as a means for estimating the frequency of a profile from the number of observations in the database [163]. In mtDNA cases, the number of observations in the database has been reported without any further interpretation precisely because the counting method is a poor method for estimating the frequency of rare mtDNA types. To date, the counting method (or "pseudo-counting method" as it has also been called) is the only method that has been reported in the United States. Despite some arguments, this presentation has been deemed acceptable by the courts in most instances. However, the counting method presentation of mtDNA evidence was in one instance found inadmissible under the Frye standard. The grounds

were that "the 'counting method' fails to provide meaningful comparison to assist, rather than confuse, the jury..." within the framework of "population frequency statistics based on principles of statistics and population genetics..." [53].

To go beyond the counting method, there are a number of valid statements that can be made regarding the frequency of an mtDNA type, based on the number of occurrences in the database. For common sequences in a population, we have an acceptably good idea of their actual frequency. For the most common type in the U.S. Caucasian population (263G, 315.1C), the best frequency estimate is 26/604 = 0.043. A 95% confidence interval can be placed on this value by accepted approaches such as a normal approximation of the binomial, or bootstrapping [49]. Using the former ($p \pm 1.96[pq/n]^{1/2}$; where $p = x/n$, $q = 1 - p$, n = database size, x = number of observations in the database), we calculate that the true frequency falls, with 95% certainty, between the values 0.027 and 0.059 (0.043 ± 0.016). Thus, the highest frequency that the true value could reasonably have would be 0.059, and for a sample with this type, we can (with 95% certainty) exclude 94.1% of the population as potential donors. Another way to express this is that for this mtDNA type there is a maximum match probability of 5.9%: at most, approximately one in 17 randomly chosen individuals would have this haplotype. We believe it is appropriately conservative to present the values for this maximum match probability. (We note that the normal approximation of the binomial applies to frequency estimates near 0.5, and the low estimated frequencies of even common mtDNA types approach the range where the normal approximation may be problematic. One proposed solution is to determine a confidence interval for the natural logarithm of the match probability, where the normal approximation is valid, then transform back to probability values [164].)

For rarer mtDNA types, including new sequences that have not been observed in the database, we have no good estimate of the true frequency in population. As discussed in the section on population genetic variation above, the apparent frequency of unique haplotypes in the database will generally underestimate the true population frequency. This situation results from a large number of rare mtDNA types in the population, so that even large databases are insufficient to estimate the true frequencies. In such cases, rather than report frequency estimates and associated confidence intervals, we can calculate a conservative upper bound on the maximum frequency that an mtDNA type could reasonably have in the population. For sequences observed more than once, this can be done using a computationally based bootstrapping approach [49], or by log transformation as noted above. However, neither of these approaches apply to sequences never before seen in a database. For such cases, one can calculate a very conservative "confidence limit from zero proportion:" $1 - \alpha^{-1/N}$ where α is set to 0.05 for a 95% confidence level. For N=604, this value is 0.005, meaning that we can, with 95% confidence, exclude 99.5% of the population as potential donors. This corresponds to a *maximum* match probability of one in 200 for types that are not represented in the database (604 U.S. Caucasians, in this example). This is not an estimate of the actual match probability, which in a great majority of cases would be much lower.

Another approach to evaluating the strength of mtDNA evidence uses "likelihood ratios" [49,164]. The likelihood ratio is calculated as the relative likelihood of the mtDNA evidence given various stated alternative hypotheses (a typical example would be a prosecution hypothesis that the suspect was the source of the sample, and a defense hypothesis that the sample came from some other unknown person). A likelihood ratio greater than one supports the prosecution hypothesis over the defense hypothesis, and the more the likelihood ratio exceeds one, the greater the support. In the straightforward situation of a homoplasmic mtDNA match, the probability of the evidence given that the suspect is the source is 1.0 (they are expected to match if the suspect is the source), and the probability of the evidence given that the source is an unknown, unrelated person is simply the match probability of the mtDNA type in question. Here, the likelihood ratio simply equates to the inverse of the match probability for that DNA type. To be conservative, one might best employ here the maximum match probability, determined as described above. Thus, for a previously unobserved mtDNA type in the U.S. Caucasian population, the likelihood ratio favoring an association between a suspect and an unknown sample would be 200, while for the most common U.S. Caucasian type the likelihood ratio would be 17. One strength of likelihood ratios is that the probability of the evidence given that the suspect is the source can actually be less than 1.0: this allows flexibility for considering the effects of mutation or heteroplasmy. Good examples of a likelihood ratio approach in an mtDNA identity case may be found in [58,104].

Again, the likelihood ratio concerns the relative likelihood of the DNA *evidence* given various stated alternative hypotheses. This is patently not the same thing as the likelihood that the suspect is actually the source of the sample (to confuse these two is known as a "transposed conditional" or "the prosecutor's fallacy"). Bayesian statistics provide a formal framework for attempting to determine the likelihood that the suspect is the actually the source. The Bayesian approach multiplies the DNA likelihood ratio by the "prior probability" (or, relatedly, the

prior odds), based on all independent non-mtDNA evidence, that the suspect may be the source of the sample [49,164]. A complication of the Bayesian approach is that it is generally quite difficult to ascribe a number to this prior probability (a detailed presentation of the strengths and complexities of Bayesian statistics and likelihood ratios may be found in [49], literature and issues relating to legal history and challenges of presenting this approach are reviewed in [118,164]. However, it would normally be the role of jury/court rather than the forensic mtDNA investigator to assess the prior probability, either numerically or qualitatively. What the mtDNA investigator can do is to clearly present the factor, given specific stated alternative hypotheses, by which the mtDNA likelihood ratio would multiply the prior probability — whatever it may be, known or unknown. In this framework, if independent non-DNA evidence provides some suspicion that an individual is the source of a sample, their sharing a previously unobserved mtDNA type would strengthen this belief by a factor of 200; or by a factor of 17 if the match involved the most common type (with reference to the U.S. Caucasian database, in this example). Either of these factors might be significant in helping to form a conclusion by one who might otherwise be wavering on the edge of reasonable doubt. On the other hand, if there were *no other reason* to suspect an individual, one might argue that a reasonable estimate of the prior probability would be the inverse of the population size (very low), and multiplying this by the mtDNA likelihood ratio would still result in a negligible likelihood of association. This takes us back to the point, with current information available in most case scenarios, unique identification cannot be made using mtDNA sequencing strictly by itself.

B. Consideration of Heteroplasmy and Mutation

How does the potential of mtDNA variation within an individual affect match significance? It was discussed in a previous section that heteroplasmy occurs at detectable levels at an appreciable rate, but in a manner that is generally tractable in the context of forensic casework. If a suspect and a sample match at all positions and share heteroplasmy at the same position(s), this represents the co-occurrence of additional unlikely events, and the mtDNA evidence is strengthened. A likelihood ratio treatment in such a case can be found in Ivanov et al. 1996 [104], where the co-occurrence of heteroplasmy was calculated to greatly increase the significance of a match between the bones of Russian Grand Duke Georgij Romanov and the bones presumed to be of Tsar Nicholas II. There, reasonable and conservative estimates were made of the frequency of heteroplasmy at a particular position. However, it is known that different positions have different rates of heteroplasmy. Therefore, until further data are available, we feel it is premature to attempt to present hard figures for increased significance of heteroplasmic matches in forensic cases. The simple and conservative solution for the time being is to proceed as if the heteroplasmy were not detected, while designating the heteroplasmic base position as an ambiguity, so that database searches would not exclude individuals with variants at that position.

Additional considerations can come into play under circumstances where there are pronounced differences observed in the mtDNA pools from a questioned sample and a reference that could be due to the action of a genetic bottleneck. As described in a previous section, bottlenecks in the transmission of mtDNA pools can occur between generations, or between tissues or hairs of a single individual. An example would be a missing person case, where the reference sample is a maternal relative and the questioned sample a bone suspected to be from the missing person. Here, the occurrence of one or more meiotic bottlenecks separating the sample from the reference could eliminate detectable heteroplasmy from one or both of the samples. An extreme case would be the appearance of homoplasmy in both, but for alternative variants. Similar differences can occur between individual hairs or between hair and blood samples from the same individual, due to the action of an apparent genetic bottleneck in the segregation to individual hairs.

When a heteroplasmic sample and a reference are separated by the action of a genetic bottleneck, it is expected that there will be fluctuations in the heteroplasmic ratios they manifest. Therefore, if heteroplasmy is clearly detected in both, evidence of association is not weakened by differing ratios (as before, the co-occurrence of heteroplasmy, if considered, would strengthen the mtDNA evidence). However, if heteroplasmy is not evident in both samples, it becomes apparent that we need to consider the likelihood of the bottleneck sampling event that is required to account for differences in the mtDNA populations of the samples in question. To exemplify, consider a hypothetical case where the ratio of heteroplasmy in blood from a reference subject is highly unbalanced, e.g., 90:10 C:T, whereas an evidentiary hair sample has apparent T homoplasmy at this position (and matches at all others). It is certainly possible that this heteroplasmic individual could produce a hair that appears homoplasmic for T; indeed, it is virtually certain that some hairs from this individual would appear homoplasmic for T. Therefore, this individual clearly cannot be excluded as a potential source of the evidentiary sample. However, simple "failure to exclude" does not provide any assessment of the

significance of the evidence. In this case, the significance of the evidence must logically be diminished. The hypothesis that the heteroplasmic individual is the source of the sample requires not only that the sample did not originate from some other non-heteroplasmic person who matches the sample, but also that there was a bottleneck segregation event of some moderate rarity.

At present, it has not been established what general treatment would best be applied to situations such as the example above. If one were to type multiple hairs from the heteroplasmic suspect, and determine that approximately one hair in ten appears homoplasmic for T, this could then provide the basis for a likelihood ratio approach. Here, the probability of the evidence given that the suspect is the source would be 0.1 rather than 1.0 (only $1/10^{th}$ of the time would a hair from this individual produce the mtDNA evidence in question), and the likelihood ratio would be $1/10^{th}$ as large as if there had been a homoplasmic match, or if the evidentiary sample also showed heteroplasmy. However, this level of additional information is not something that can be counted on in a general case. For the time being, we feel that the presentation of such mtDNA evidence is so dependent upon the specifics of the case that specification of general guidelines does not seem appropriate. Forensic mtDNA investigators must present and evaluate the evidence in relation to the details of a case and in light of relevant available information regarding mtDNA biology. In such cases, it would certainly be possible to suggest a conservative interpretation based on known properties of heteroplasmic segregation and the specifics of the case in question. However, a detailed consideration of the complexities of the general case will be the subject of a separate publication.

How can we deal with the fact that the action of a genetic bottleneck between a sample and reference can give rise to an apparent homoplasmic difference in their sequences? It is clear that we cannot definitively exclude based on a single difference [173], but once again it is difficult to specify for the general case how our assessment of the evidence should be modified. We can illustrate different extremes of "homoplasmic differences" by the following two examples: (a) a bone sample and a presumed maternal reference share a constellation of polymorphisms that is unique in the database, and differs by more than one polymorphism from any other database sequence, but these two samples have an apparent homoplasmic difference in the length of the HV2 C-stretch, and, (b) a crime scene hair sample and a suspect reference share the most common sequence within Caucasians, except that the suspect has an additional homoplasmic polymorphism at a site that has never before been seen to vary. Both these scenarios can be classified as "differing at a single position," but we feel that the first provides evidence that would strengthen an association between the subject and reference, while the second would be reasonable evidence of an exclusion (or, at least, the mtDNA evidence makes it substantially less likely that the suspect is the source of the sample). The difference in the two cases relates to the rarity of the sequences in question, and the variable propensity for heteroplasmy and mutation at various sites within mtDNA. Presentation of the significance of the mtDNA evidence in such cases would then hinge on plausible and conservative arguments relating to current knowledge of these aspects of mtDNA biology (we feel that reasonable presentations could be devised for both examples above, but a detailed analysis is beyond the present scope). We suspect that the likelihood ratio approach will emerge as an important tool for evaluating mtDNA evidence in cases involving complexities of heteroplasmic segregation and intergenerational mutation, but defer a detailed consideration to a separate publication.

III. VALIDATION OF COMPONENT METHODS INVOLVED IN mtDNA PROFILING

The word "validation", as defined by Webster's Ninth Collegiate Dictionary, means "an act, process, or instance of validating; esp. the determination of the degree of validity of a measuring device." In turn, "validity" means "well grounded or justifiable; esp. being at once relevant and meaningful." The charter of the Technical Working Group on DNA Analysis Methods (TWGDAM), sponsored by the Federal Bureau of Investigation (FBI) and established in 1988, has been to develop guidelines for the validation of DNA profiling systems [214,215], to evaluate the progress of validation studies, and in some instances, to conduct relevant studies [44,239]. Using these guidelines, the forensic community has properly validated a number of DNA typing systems [27,28,36,39,52,64,83, 89,207,209,229,236–238]. In addition to TWGDAM, groups of scientists in other parts of the world (e.g., EDNAP: European DNA Profiling group, and GDNAP: German DNA Profiling group) conduct similar studies [5,11,56,196]. Through these efforts, and the information being generated in the academic community, a DNA typing method can be properly evaluated prior to implementation into forensic casework. As stated in Section 4.1.1 of the TWGDAM guidelines from 1995 [214]:

> "Validation is the process used by the scientific community to acquire the necessary information to assess the ability of a procedure to reliably obtain a desired result, determine the conditions under which such results can be obtained, and determine the limitations of the procedure."

Most importantly,

> "The validation process identifies the critical aspects of a procedure which must be carefully controlled and monitored."

Once a method has been properly validated by the scientific community, "appropriate studies of limited scope" (Section 4.1.3) must be conducted to bring the typing method on line in the forensic laboratory. This "internal validation" should be performed to determine the "reliability of the procedure in-house" (Section 4.5), to include testing the system with known samples, comparing the procedure to the original procedure if significant changes have been made, ensuring that the testing procedure does not introduce contamination which would "lead to errors in typing", and requiring that the laboratory test the system through proficiency or "competency" testing. Given that this process is meant to supplement general validation studies, it can be limited in scope.

The general guidelines that were established by TWGDAM [214] can be used to evaluate whether mtDNA analysis of biological evidence has been properly validated. The Appendix lists the TWGDAM validation criteria and notes those sections of this review article that are relevant to each listed category. Although this is a comprehensive evaluation of mtDNA analysis in a forensic context, it helps to dissect the process into its individual components to gain a better appreciation for how well the analysis of mtDNA has been validated by the scientific community. The process can be broken down into three major components - DNA extraction, PCR amplification, and DNA sequencing.

The methods developed for extraction of DNA from biological material are numerous, well established, and transect multiple disciplines [3,10,32,33,52,62,63,74,82, 87–90,92,95,112,127,133,134,158,185,197,200,212, 231,233,237,243]. While many of the DNA extraction methods employed in forensic laboratories have been used to isolate DNA for both RFLP and nucDNA PCR-based profiling systems for years, these methods are universal in nature in that the DNA isolated from a sample can be analyzed by any genetic typing system, to include mtDNA. In addition, the range of specimens studied includes a number of those encountered in forensic mtDNA casework (e.g., blood, hair, skeletal material, teeth, nail material, saliva, urine, faeces, and even genital crab lice). As an illustration, Wasser et al. reported methods for the preservation, extraction and amplification of DNA isolated from the fecal material of Malayan sun bears and North American black bears. The authors illustrated that the DNA profiling results obtained from fecal material (both mtDNA and STR analysis) were identical to those obtained from blood and tissue of the same individual. The mtDNA data compiled were subsequently used to identify individuals within a species, or to provide a way to differentiate between the two species in order to solve cases of wildlife poaching.

PCR amplification was first discovered in the mid-1980's [159,160,190], and has since become one of the most important molecular tools ever developed for the analysis of DNA (e.g., [195]. As a testament of this, Kary Mullis won the Nobel Prize in Chemistry in 1993 for his discovery of PCR [8]. Since 1985, literally thousands of articles have been published in scientific journals that have used PCR to evaluate biological systems (a search of Medline, Grateful Med V2.6.1, for PCR resulted in greater than 88,000 citations in peer reviewed scientific journals). In addition, numerous books and protocol manuals have been written to provide the scientific community with useful reference sources on how to use PCR to study genetic systems [48,165]. As a result, the impact of PCR on the scientific community has been enormous. For example, the prenatal diagnosis of genetic disease has been one of the beneficiaries of PCR technology [18,131,217]. Once a method that took weeks to perform, placing the mother and child at potential risk, prenatal testing of genetic markers now takes only a handful of days to complete. In addition, pre-implantation diagnosis can be performed on a single cell, eliminating unwanted embryos [76], as several inherited diseases can be diagnosed for "at risk" parents, avoiding the possibility of terminating a pregnancy later in development. While PCR has impacted all of molecular genetics, ironically, one of the first applications of PCR was to study length variants of mtDNA by direct sequencing of PCR product in 1987 [242].

Sequencing DNA, including PCR product, has been well documented in the literature, and is a routine method in hundreds of laboratories (e.g., [24,52,70,74,78,88,89, 128,145,155,180, 184,204,207,209,210,223,230,232,237, 247,249]). In particular, the Sanger dideoxynucleotide or "terminator" method has been the gold standard for the last two decades [192,193]. The human genome project is a testament to the successes of DNA sequencing, and although the technique has become mundane, the ability to sequence DNA provides the basis for a host of other disciplines; e.g., the field of gene therapy [7,37,111]. Once a gene has been located and the primary sequence generated, gene-based therapy can be used to help cancer patients [60], or to simply assist with wound healing following coronary angioplasty [250].

IV. ANCIENT DNA

We have seen that the component molecular methods involved in forensic mtDNA testing are standard, universally accepted tools of molecular biology. While the reliability of these procedures as generally accepted scientific methods is beyond any reasonable doubt, one could further ask if the combined use of these techniques, as applied to samples like those encountered in mtDNA forensic testing, has also been adequately validated in the general scientific community. Indeed, one of the greatest challenges to the methodology comes from the context in which most mtDNA forensic identity testing occurs; cases where samples are badly degraded, and contain so little DNA that one must turn to high-copy-number mtDNA. The answer is that mtDNA identity testing can be related to the extremely rigorous parent field of "Ancient DNA" analysis. Within this field, the general (non-forensic) scientific community has extensively demonstrated that the methods used in forensic mtDNA analysis can reliably yield DNA sequences from a wide range of extremely old and degraded sources.

Specific fragments of DNA from ancient sources were first cloned in 1984 from an extinct equine, the quagga [85]. This was followed by the cloning of a segment of human DNA from an Egyptian mummy [169]. However, it was the successful application of PCR to ancient samples that truly gave life to the field of ancient DNA analysis [86,170]. Since then, ancient DNA has successfully been extracted, amplified, and sequenced from a great many sources, with a non-comprehensive list including an extinct marsupial wolf [120], extinct flightless moa birds [40], a 13,000 year old extinct ground sloth [170], a 14,000 year old saber-toothed cat [105], a 25,000 year old member of the horse family [94], ~150,000 year old wooly mammoths [66], and Pleistocene-era dung from an extinct ground sloth containing DNA from the sloth itself, and seven plant groups comprising its diet [179].

In addition to the non-human sources listed above, there has been extensive success with amplification and sequencing of ancient DNA from humans. This work is the most challenging of all because the PCR primers used for amplification of ancient human DNA also will amplify any modern DNA that contaminates the sample or any reagents. Such contaminating modern DNA is ubiquitous unless rigorous measures are employed to remove or avoid the contamination. Nonetheless, it has now been demonstrated in many laboratories that authentic ancient human DNA can be obtained, and a great majority of these instances have involved mtDNA. A non-comprehensive list includes a wide range of mummies [e.g., 166.191], the Tyrolean Ice Man [75], and ancient teeth from various sources (e.g., [151,240]). The success rate with ancient bone samples is such that researchers can now undertake ancient human population genetic analysis with meaningful sample sizes (e.g., [67,68,93,151,206]). Without doubt, the crowning achievement in ancient "human" mtDNA analysis was the recovery of CR sequences from a ~40,000 year old Neanderthal bone specimen [121].

Reviews of general practices and specific procedures to avoid the pitfalls of contamination in ancient DNA studies may be found in Handt et al. [74,75], Hummel and Herrmann [102], and Thomas and Paabo [216]. The most important general principles relating to forensic mtDNA testing are the following: 1) Laboratories where sample DNA is extracted and PCR is set up should be physically separated from laboratories where products of PCR amplification are handled; 2) Lab coats and laboratory equipment, etc, should be dedicated for use exclusively in the pre- and post-PCR laboratories; 3) Negative control experiments should be included at all phases. This must always include a mock extraction (or "reagent blank") performed with no sample. Also required are control PCR amplifications with no template DNA added. All PCR controls should be made from the same PCR master mix as used for the sample of interest, differing only in the template DNA; 4) Whenever possible, replicate testing should be performed to ensure consistent results, starting with new DNA extractions.

V. mtDNA IN CASEWORK

A. Further Forensic Validation Studies

The field of ancient DNA and the study of mtDNA biology have provided the foundation for the application of mtDNA analysis to the identification of human remains. In turn, this vast body of scientific knowledge, including remains identification casework, has provided the basis for validation of mtDNA analysis as applied to forensic cases involving criminal activities. The Appendix lists the TWGDAM guidelines for validation of a forensic DNA profiling system, identifies those sections of this paper that help to illustrate how each requirement has been addressed, and provides some additional information to support the validation of mtDNA for forensic criminalistic casework. In addition to the information provided in the Appendix, certain issues deserve particular attention.

One of the requirements for forensic validation is to identify the limitations of a DNA profiling system and develop controls to properly monitor the system. While PCR contamination is a limitation of mtDNA analysis that

has been identified and addressed (see Section IV), given the sensitivity of the mtDNA amplification process, additional controls and laboratory practices are necessary for mtDNA profiling that may not be necessary for other forensic PCR-based methods. While laboratory design can help to control contamination, numerous laboratory practices can help to minimize the risk of contamination. The universal cleaning agent for PCR contamination is 10% bleach (7 mM sodium hypochlorite), rendering PCR product un-amplifiable [183]; ultra-violet irradiation is also a good way to eliminate DNA contaminants [164]. A 10% solution of bleach is more effective than 2 N HCl in neutralizing PCR product. Consequently, the floors, benchtops, hoods, and any surface which comes in contact with the evidence should be washed weekly, daily, and in some instances per use. Control studies have been conducted by this laboratory and others to illustrate that PCR product can be cleaned from laboratory surfaces; i.e., swabbing a cleaned surface provides no template for subsequent PCR amplification.

Safety glasses, laboratory coats and disposable gloves are mandatory items in all laboratories. To reduce the risks of accumulating contamination, disposable laboratory coats can be used in the areas where mtDNA PCR product is being handled. Care should be taken when wearing disposable gloves not to touch any surface that may contain a contaminant; e.g., the surface of the skin, eyeglasses, or clothing. As a common practice, before handling evidence or items which come in contact with the evidence, always change gloves or wipe gloves with bleach, allowing the gloves to air dry. These practices have proven effective in reducing the level of analyst introduced contaminants.

Laboratories have developed ways to eliminate or control against contamination of the evidentiary material [88,109,200,227,233]. For hair samples, microscopic analysis followed by sonication may help to identify and remove cellular contaminants, respectively. Removing the outer surface of dried skeletal material with a Dremel tool fitted with a grinding stone will eliminate or reduce surface contaminants. Conservative sampling of DNA from dental remains can eliminate contamination by excluding surface material. Applying these methods will help to prepare the sample for DNA extraction and control for handling contaminants that may have been introduced prior to entering the laboratory.

Appropriate controls and measures to detect contamination have been identified as essential. Both extraction and PCR reagent controls should be run in every case to detect the occurrence of contamination. PCR reagent controls should normally give negative amplification results. However, despite all the precautionary measures taken to minimize contamination, extraction reagent controls occasionally show positive amplification results when performing mtDNA testing at high PCR cycle numbers (e.g., 38–40 cycles or more). Therefore, given the sensitive nature of mtDNA analysis, an analytical strategy should be developed for dealing with the occurrence of PCR amplification in reagent controls. These approaches should be aimed at preserving authentic information without sacrificing assurance of quality testing results.

Forensic scientists generally complete an extensive training program, many months in length, prior to performing mtDNA analysis in casework, and do not perform the analysis until they have demonstrated competency and/or proficiency. Nevertheless, expertise is supplemented by requiring that interpretation guidelines be rigorous and conservative [238]. For example, the reported "error rate" for DNA sequencing results generated on automated instrumentation (e.g., the Applied Biosystems DNA sequencers) is approximately 1–2%, with an increasing rate as the length of the sequence read is extended [51,120]. In addition, reported sequence data can have an even higher rate of inaccuracy due to data transcription errors. However, the most commonly reported error rates are based on the analysis of a single lane of DNA sequence, and transcription errors, while inaccuracies, do not reflect the actual data and are clerical mistakes. In many laboratories, but especially forensic laboratories, multiple sequencing runs are analyzed from the same PCR product (preferably from both the forward and reverse strands of the template), as well as from different PCR products (in many instances from multiple extractions of the same specimen), to generate an mtDNA sequence profile. In addition, the sequences generally do not extend beyond 500 base pairs in length. Thus, any suggestions that DNA sequencing error rates have an impact on the reliability of mtDNA sequence analysis in forensic casework are unfounded.

Forensic laboratories have demonstrated the ability to properly analyze mtDNA sequence data, illustrated in part by the sharing of sequence data between laboratories [172,173,238, unpublished correspondence]. Before a laboratory can contribute to the forensic mtDNA sequence database they must undergo an inter-laboratory exchange of samples [238]. To date, the seven contributing laboratories have all successfully completed this exercise. Furthermore, our laboratory has been involved in a quality assurance program with the National Institute of Standards and Technology (NIST) for the past five years to evaluate bloodstains being collected from U.S. military personnel and stored by the Department of Defense; the cards are used to assist in the identification of military personnel killed in the line of duty. One-eighth-inch punches are taken from duplicate bloodstain cards, and

analyzed independently by our laboratory and NIST. Although most of the analysis is performed using nucDNA markers (i.e., STRs, D1S80, PM+DQA1), duplicate mtDNA analysis has been performed on twelve samples thus far. In all cases, the analysis performed by the two laboratories was in concordance; including nucDNA results from greater than 5000 comparisons.

Finally, forensic laboratories undergo rigorous inspection by a variety of agencies that accredit and/or oversee the DNA typing methods being performed. In 1994, the Department of Defense (DoD) convened a panel of scientific experts to serve on a Defense Science Board (DSB) to evaluate the use of mtDNA for human remains identification in the U.S. military. The panel chairman, Nobel Laureate Joshua Lederberg, reported that "the DSB Task Force has found that mitochondrial DNA (mtDNA) sequencing currently offers the best means of identifying those skeletal remains that cannot be identified through traditional means. The Task Force finds that current DNA identification efforts are supported by sufficient scientific evidence to proceed, in particular with the application of mtDNA sequencing to ancient remains from the Korean conflict". In addition, a DoD Quality Assurance Oversight Committee was established to provide continued oversight, to include an annual inspection of our laboratory, and any laboratory that conducts mtDNA analysis for the DoD. While our laboratory is also accredited by the College of American Pathologists (CAP), the organization that accredits forensic laboratories in the U.S. is the American Society of Crime Laboratory Directors, Laboratory Accreditation Board (ASCLD-LAB). The two largest laboratories in the U.S. using mtDNA analysis, our laboratory and FBI Headquarters, are currently ASCLD-LAB accredited.

B. Review of Human Remains Identification Casework

There are hundreds of homicide, suicide and accident victims that remain unidentified worldwide each year. Partial skeletal remains, skeletal remains of children, partial torsos with unidentifiable features, and highly decomposed, desiccated and charred remains, are difficult and sometimes impossible to identify using conventional methods (e.g., anthropologic or dental comparison, fingerprint analysis, facial reconstruction). DNA testing can provide useful information for the identification of these types of remains. In particular, mtDNA analysis has played a key role in this capacity [20,41,58,59,69,88,89, 104,114,136,172,207,209,218,242, numerous unpublished correspondences]. Most cases have involved the analysis of skeletal material, although some have involved teeth, hair, and nail material. Two notable cases help to illustrate the utility, the strength, and the reliability of mtDNA analysis for remains identification: (a) The identification of the last Russian Tsar, Nicholas II, and the refuted identification of Anna Anderson Manahan as his daughter Anastasia; and (b) The identification of the soldier buried in the Tomb of the Vietnam Unknown Soldier for fourteen years, Air Force 1st Lt. Michael J. Blassie.

1. The Identification of the Russian Royal Family

Members of the Russian royal family, after being brutally assassinated by firing squad in 1918, were buried without ceremony and under a cloak of darkness outside the Siberian town of Ekaterinburg, where they remained unidentified for more than 70 years. A team of forensic scientists unearthed the bodies of nine individuals in 1991, and the quest to identify them as the royal family began [144]. Methods, to include anthropologic analysis and facial superimposition, were used to provide presumptive evidence that the remains were those of Nicholas II, his wife Alexandra, and three of his four daughters; remains representing individuals consistent with three servants and the family physician were also recovered. Missing were the Tsar's fourth daughter and his son, Alexis. While the forensic evidence was compelling, the information was insufficient for the Russian government to conclusively accept the identification of the remains as those of Nicholas II and his family.

In 1994, the Forensic Science Service (FSS) laboratory in the U.K. reported the use of mtDNA analysis to establish a maternal relation between the presumed remains of the Tsarina and a living relative, Prince Philip, the Duke of Edinburgh [58]. Unfortunately, when comparing the mtDNA profile of the putative remains of the Tsar to two maternal relatives (the Duke of Fife and the Countess Xenia Cheremeteff-Sfiri), an apparent sequence difference was observed. While the mtDNA match between the Tsarina's remains and Prince Philip was compelling, the apparent discrepancy between the Tsar and his maternal relatives was confounding. The FSS laboratory clarified much of the confusion by cloning the mtDNA isolated from the Tsar's remains, and showed that it was a mixture of two sequences (i.e., heteroplasmy), one which matched the maternal relatives, and one which was a single base pair mis-match. In support of this, the results were duplicated in a second laboratory in the U.K., verifying their authenticity. However, given that this was one of the first observations of heteroplasmy in a forensic case, the results, while scientifically clear, instead provided the necessary fuel for effective political resistance against the acceptance of the identification of the royal family.

In 1996, this laboratory, in conjunction with the lead forensic molecular biologist in Russia, Pavel Ivanov, reported that the remains of the Tsar's brother, Georgij Romanov, shared heteroplasmy with the Tsar at the same sequence position (i.e., [16,168], **Figure 3**) [104]. This was the first time heteroplasmy had been involved in a forensic identification; although since then a number of cases have involved heteroplasmic matches (unpublished data). Using the sequence databases available in 1995, and considering only a sequence match between the Tsar and Georgij, a likelihood ratio of 150 (i.e., the observed data was 150 times more likely if the remains were those of the Tsar than a random individual) was calculated. When a sequence match at the same heteroplasmic position, i.e., 16,169, was taken into consideration, the likelihood ratio jumped to more than 300,000, and when the sequence match between the Tsarina and HRH Prince Philip was taken into consideration, the total mtDNA data gave a likelihood ratio of more than 100,000,000 [104].

Of interest, George Romanov died in 1899 and was buried in St. Petersburg for almost 100 years in a casket that had been full of water for approximately the last 60 years before his remains were exhumed for this purpose. Nonetheless, mtDNA sequence analysis was successful, providing the final piece of evidence to conclusively identify the Ekaterinburg remains as those of Nicholas II. While the work done in this case conclusively identified the remains of the Tsar and Tsarina, they could not conclusively identify which of the three daughters were represented. This only perpetuated the long held belief that Anna Anderson Manahan was the Grand Duchess Anastasia. In 1995, our laboratory, the FSS, and Penn State University reported that the mtDNA sequence generated from Anna Anderson Manahan, obtained from two separate sources (i.e., a lock of hair and a tissue biopsy), did not match the mtDNA profile of the Tsarina [57]. Thus, Ms. Manahan, who died in 1984, could not have been the biological daughter of Alexandra Romanov. Instead, Ms. Manahan's mtDNA sequence matched that of a Polish farming family; the suspected ancestry of Ms. Manahan by many followers of this fantastical story.

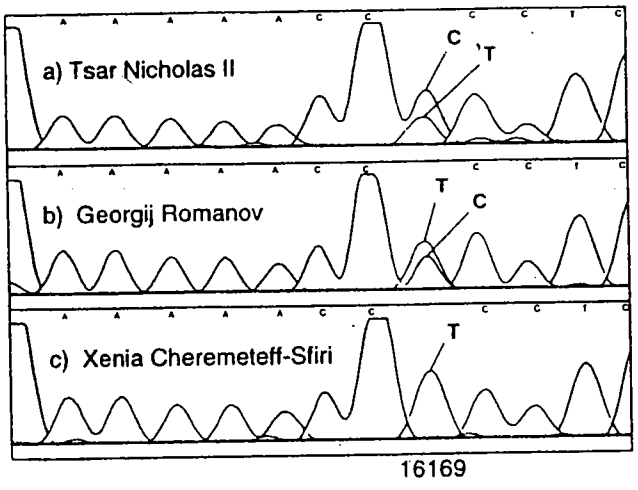

Figure 3. Automated sequence electropherograms comparing mtDNA sequences at position 16169: (a) sequence from Tsar Nicholas II, showing heteroplasmy with cytosine predominating thymine; (b) sequence from Grand Duke Georgij Romanov, showing heteroplasmy with thymine predominating cytosine; (c) sequence from living maternal relative of Tsar Nicholas, separated by five generational events, apparently homoplasmic for thymine.

The "Tsar" and "Anastasia" cases are described in some detail to represent routine cases encountered in forensic laboratories, i.e., they help to clarify some of the genetic characteristics of mtDNA (i.e., heteroplasmy), they illustrate that mtDNA results can be obtained from a variety of biological specimens (i.e., bone, hair, and biopsy specimens) and reproduced in four different laboratories (bone from the Tsar was analyzed in three different laboratories, while the hair of Anna Anderson Manahan was analyzed in one laboratory and the biopsy specimens in two other laboratories, with all results in concordance), and they illustrate that an mtDNA match can be compelling evidence for the identification of biological evidence, especially when the match involves heteroplamsic mtDNA sequences.

2. The Identification of the Vietnam Unknown Soldier

The Armed Forces DNA Identification Laboratory (AFDIL) was established in 1991 for the purpose of identification of U.S. military personnel using DNA techniques. This mission grew to include assistance in the identification of those military personnel killed in previous military conflicts (e.g., the wars in Vietnam and Korea, and World War II). There are more than 2000 individuals unaccounted for from the war in Southeast Asia, greater than 8000 from the war in Korea, and greater than 75,000 from World War II. While the responsibility for identification of these individuals lies with the Central Identification Laboratory in Hawaii (CILHI), this laboratory performs mtDNA analysis for CILHI on those cases that require additional support for identification. Testing is normally performed on skeletal material (i.e., bone or teeth), however, analysis of blood, saliva, hair, stamps and envelopes has also been conducted. As of February 1999, more than 125 mtDNA sequence matches had been made by this laboratory and reported to CILHI. In addition, ~200 additional matches are pending. While each of these cases is important to the families of the fallen soldier, the most notable case this laboratory has worked on is the identification of the soldier buried in the Tomb of the Vietnam Unknowns.

The casket containing the remains of the Vietnam Unknown Soldier was disinterred on 14 May 1998. The remains were thought to be those of 1st Lt. Michael J. Blassie, a highly decorated Air Force pilot, whose A-37 aircraft was shot down near An Loc, South Vietnam, in 1972. In 1984, CILHI had been unable to identify Mr. Blassie due to inconsistencies in the anthropologic evidence and personal effects, as well as blood typing results from human hairs. As a result, the remains were assigned to the Tomb of the Vietnam Unknowns. Late on the night of 14 May 1998, AFDIL took receipt of six skeletal fragments from the following skeletal elements — a humerus, pelvic bone and four ribs — for the purpose of performing mtDNA sequence analysis, and comparison of the results to references from seven families thought to be associated with the case.

This case helps to illustrate the strength of mtDNA analysis and the reliability of the testing results. When the relevant or target population has been reduced to a handful of candidates, mtDNA analysis can provide information that allows for identification of human remains. In the Vietnam Unknown Soldier case, the population of possible contributors of the skeletal remains in question had been reduced to two main candidates, and five marginal candidates by other means of forensic identification. When the mtDNA sequence profile of the skeletal remains from the Vietnam Unknown Soldier was compared to the mtDNA haplotypes of the seven families in question, the sequence matched the Blassie family and did not match the other six families. Thus, this information, along with all other circumstantial information, conclusively identified the remains as those of Mr. Blassie.

The relationship between CILHI and AFDIL is unique, given that a comparison to maternal relative(s) is not made until CILHI receives the results of analysis on the skeletal material, and a request is made to do so. As a result, duplicate analysis of a percentage of these cases can be performed, and this laboratory has been involved in a blind proficiency testing program with CILHI for the past three years; similar to the Partnership for Excellence program being conducted in the ancient DNA community (e.g., [244]). Approximately once every six months, CILHI submits cases to AFDIL involving samples for which AFDIL has previously reported results. In every blind proficiency case completed thus far, this laboratory has obtained the same results for the duplicate and original cases, highlighting the reliability of mtDNA sequence analysis. In addition, Mr. Blassie's case involved a type of blind analysis, in that AFDIL was given two cases to process with similar skeletal elements, and not told which of the two was the Vietnam Unknown Soldier. After completing the analysis of the skeletal material, the results were reviewed by an outside consultant and sent to CILHI, and only then were the references analyzed. Upon comparison, the Blassie family sequence matched only one of the two cases, the remains of the Vietnam Unknown Soldier, and did not match the mtDNA profile of the remains from the second case or the other six families. Once again, this illustrates the ability of mtDNA analysis to positively identify human remains, and the reliability that can be placed on the results.

C. Review of Forensic Casework

The use of mtDNA sequence analysis to identify human remains has led the way for the application of mtDNA analysis in forensic criminalistic casework. The first case where mtDNA results were introduced into a court of law in the U.S. was the State of Tennessee vs Paul William Ware in August of 1996. This case involved the association of a pubic hair, found in the throat of a four year old female child, to the suspect, Mr. Ware, who was subsequently convicted of rape and murder. Since then, mtDNA has been used in more than four hundred forensic cases, however, very few that have been published [2]. Based on the long list of citations for cases involving identification of human remains (see above), it is not surprising that publishers have little interest in continuing to publish material on this subject. Only those most notable and interesting cases, or cases with historical significance tend to make their way into scientific periodicals.

In the past three years, mtDNA results have been admitted into evidence in at least ten states (Tennessee, South Carolina, Michigan, North Carolina, Maryland, Pennsylvania, New Mexico, Indiana, Washington, and Texas), and has been found inadmissible in the State of Florida on a perceived lack of clarity when reporting mtDNA statistics (see Section II). Of the ten cases which have gone to court through the FBI laboratory, eight cases have resulted in convictions, in one case the suspect pled guilty after the mtDNA results were admitted, and the final case was awaiting a decision at the time this manuscript was in preparation. In addition, two of these cases involved admissibility hearings. Finally, the specimens analyzed in these ten cases were primarily hairs and skeletal remains, the types of biological material typically encountered in other scientific laboratories studying mtDNA.

Outside the U.S., mtDNA analysis has been applied to forensic casework for a number of years. The FSS laboratory in the U.K. took the lead and introduced mtDNA in case work in 1992. To date, they have completed more than 140 cases involving mtDNA analysis, the vast major-

ity of which are criminalistic cases. In addition, at least 40 other laboratories in ten other countries across Europe are performing mtDNA analysis, and there are laboratories in Asia, Australia, the Middle East, and South America that are developing or have developed mtDNA capabilities. Thus, mtDNA analysis has been well established in Europe, and is a generally accepted forensic DNA profiling method worldwide.

CONCLUSIONS

Our goal in this review has been to establish that the use of mitochondrial DNA sequence analysis for forensic identity testing is based on: (a) the straightforward application of universally accepted techniques of molecular biology, (b) an intensively studied and generally well-characterized mechanism of inheritance, (c) an extremely rich body of population genetic analysis, and (d) the rigorous parent field of ancient DNA analysis that has established the foundation for contamination avoidance and criteria for authenticity. While sometimes perceived as a "new" application, mtDNA identity testing has been performed routinely for at least seven years, with a proven track record of utility and reliability. As documented in this review, the forensic scientific community has amassed a vast base of experience in mtDNA identity testing, in many laboratories worldwide. As a result, mtDNA sequence analysis for forensic identity testing is robust and "validated."

Because of its demonstrated utility and unique advantages under particular circumstances, the demand for mtDNA testing is in a phase of explosive growth. This is, in turn, driving additional advances that are sure to enhance the power and efficiency of mtDNA testing in the near future. The size of mtDNA databases is growing dramatically due to specific large-scale efforts, and the establishment of additional regional laboratories generating local databases. This will directly increase the power of mtDNA testing in regards to the large number of rare mtDNA types. Limitations of mtDNA in discriminating common hypervariable region sequences will be reduced by targeting additional variation outside of the hypervariable regions, both in other sections of the control region, and in the ~14,600 bp mtDNA coding region [139,183]. In the future, specific informative sites can be targeted by current standard approaches, or with non-sequencing based assays of single nucleotide polymorphisms (e.g., using fluorogenic PCR assays that couple amplification and detection simultaneously) [129]. In addition, innovative microchip-based approaches to sequencing have also been devised that could at some point revolutionize the mtDNA sequencing [34,47]. Lastly, more extensive characterization of the rate and pattern of heteroplasmy and of the segregation of mtDNA variants will permit explicit utilization of this additional level of variation, adding greatly to the power of mtDNA discrimination.

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APPENDIX — TWGDAM VALIDATION GUIDELINES

1. Standard Specimens — "The typing procedure should have been evaluated using fresh body tissues and fluids obtained and stored in a controlled manner. DNA isolated from different tissues from the same individual should yield the same type".

Skeletal material from the Civil War, stored at the National Museum of Health and Medicine in Washington, DC, was used to help develop the methods for remains identification [52]. The methods for analysis of human hair were developed and tested with control hairs [236,237]. In addition, there is continual analysis of control specimens when performing mtDNA analysis. For example, this laboratory has run the same positive control more than 10,000 times, and has obtained the expected results from every analysis.

(*See* Sections I, IV, and V.)

2. Consistency — "Using specimens obtained from donors of known type, evaluate the reproducibility of the technique both within the laboratory and among different laboratories".

(*See* Sections IV and V.)

3. Population Studies — "Establish population distribution data in different racial and/or ethnic groups".

(*See* Sections I, II, and III.)

4. Reproducibility — "Prepare dried stains using body fluids from donors of known types and analyze to ensure that the stain specimens exhibit accurate, interpretable, and reproducible DNA types or profiles that match those obtained on liquid specimens".

(*See* Sections III and IV.)

5. Mixed Specimen Studies — "Investigate the ability of the system to detect the components of mixed specimens and define the limitations of the system".

Forensic laboratories should examine mixed stains from sexual assault evidence with caution when performing mtDNA analysis. Sperm contain only 50 copies of mtDNA, and the sperm head contains no mtDNA [35,211]. Thus, the female fraction of a differential extraction may have a mixture of the female and male nucDNA types, but will only have the female mtDNA type. Whereas, analysis of the male fraction, i.e., the sperm head fraction, will have one of three possible outcomes: no mtDNA sequence profile detected, only the female profile detected, or possibly the amplification of an insert of mtDNA in the nucDNA genome. Thus, while mtDNA analysis may not be useful for helping to identify the donor of the semen stain, if there is a need to determine the origin of the male fraction, i.e., whether it was initially associated with the female fraction, in many instances mtDNA analysis of the male fraction will yield the profile of the female.

(*See* Sections IV and V.)

6. Environmental Studies — "Evaluate the method using known or previously characterized samples exposed to a variety of environmental conditions. The samples should be selected to represent the types of specimens to be routinely analyzed by the method. They should resemble actual evidence materials as closely as possible so that the effects of factors such as matrix, age, and degradative environment (temperature, humidity, UV) on a sample are considered".

(*See* Sections IV and V. Additional references: [1,4,146, 198].)

7. Matrix Studies — "Examine prepared body fluids mixed with a variety of commonly encountered substances (e.g., dyes, soil) and deposited on commonly encountered substrates (e.g., leather, denim)".

(*See* Sections IV and V.)

8. Non-Probative Evidence — "Examine DNA profiles in nonprobative evidentiary stain materials. Compare the DNA profiles obtained for the known liquid blood versus questioned blood deposited on typical crime scene evidence".

(*See* Sections IV and V.)

9. Non-Human Studies — "Determine if DNA typing methods designed for use with human specimens detect profiles in nonhuman source stains".

Given that mtDNA analysis is based on DNA sequencing, it is not necessary to do a comprehensive evaluation of non-human sources of DNA. Even if human-specific primers amplify other sources, it will be obvious by the size of the PCR product, the sequence of the product, or both that the source is non-human (e.g., [194]).

(See Sections I and IV.)

10. Minimum Sample — "Where appropriate, establish quantity of DNA needed to obtain a reliable typing result".

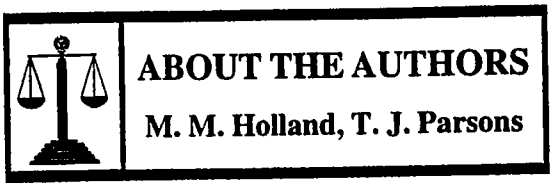
(*See* Sections I, II, IV, and V.)

11. On-Site Evaluation — "Set up newly developed typing methods in the case-working laboratory for on-site evaluation of the procedure".

(*See* Section V.)

12. Characterization of the Locus — "During the development of a DNA analysis system, basic characteristics of the loci must be determined and documented".

(*See* Sections I and IV.)



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