Forensic Mitochondrial DNA Analysis: Current Practice and Future Potential

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TABLE OF CONTENTS

INTRODUCTION 102
I. CURRENT PRACTICE 103
A. Demand, Customers, and Sample Type 103
B. Evolution of Protocols 104
C. Interpretation Guidelines 105
D. Mixture Interpretation 106
E. Statistics and Databases 109
F. Courtroom Experience 110
G. Regulation and Accreditation 110
II. ALTERNATIVE METHODS AND FUTURE POTENTIAL 111
A. Screening Methods 111
B. Expanded Sequence Analysis 112
C. Mixtures and Heteroplasmy Investigation: DGGE and dHPLC 113
D. Mass Spectrometry 114
E. Pyrosequencing and Deep Sequencing 115
CONCLUSIONS 117
ACKNOWLEDGMENTS 118
REFERENCES 118
ABOUT THE AUTHORS 121

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ABSTRACT: Current practices for performing forensic mitochondrial DNA (mtDNA) sequence analysis, as employed in public and private laboratories across the United States, have changed remarkably little over the past 20 years. Alternative approaches have been developed and proposed, and new technologies have emerged, but the core methods have remained relatively unchanged. Once DNA has been recovered from biological material (for example, from older skeletal remains and hair shafts), segments of the mtDNA control region are amplified using a variety of approaches, dictated by the quality of the sample being tested. The amplified mtDNA products are subjected to Sanger-based sequencing and data interpretation is performed using one of many available software packages. These relatively simple methods, at least in retrospect, have remained robust, and have stood the test of time. However, alternative methods for mtDNA analysis remain viable options (for example, linear array assays and dHPLC), and should be revisited as the desire to streamline the testing process, interpret heteroplasmy, and deconvolute mixed mtDNA profiles intensifies. Therefore, it is important to periodically reassess the alternative methods available to the mtDNA practitioner, and to evaluate newer technologies being put forth by the scientific community, for example, next-generation sequencing. Although the basic mitochondrial DNA protocols and practices of public and private laboratories are similar, an overview of the current practices of forensic mtDNA analysis is provided, helping to frame the path forward.

KEY WORDS: Deep sequencing, DGGE, dHPLC, DNA damage, mass spectrometry, mtDNA mixtures, screening.

INTRODUCTION

Refinement of methods for forensic mitochondrial DNA (mtDNA) analysis that were introduced in the early 1990s has led to the present "golden age" of mtDNA testing in public or government laboratories such as the Federal Bureau of Investigation (FBI) and the Armed Forces DNA Identification Laboratory (AFDIL), as well as in private-sector laboratories such as Mitotyping Technologies, the Bode Technology Group, and Orchid Cellmark. The success of mtDNA in forensic DNA analysis can be gauged from the fact that a laboratory such as Mitotyping has completed over 1,000 mtDNA cases since its inception in 1998; hundreds have resulted in resolution of criminal cases, contributory and relevant trial testimonies, and postconviction exonerations. Although it is not possible to retroactively review the history or present the workings of the entire forensic mtDNA community of test providers, a retrospective analysis of the operation at Mitotyping provides a comprehensive overview of the testing process. In this review we will describe how the "state of the art" has evolved since 1996 when mtDNA testing was introduced to the criminal justice system in the case of *Tennessee v. Ware* [19], review alternative methods for mtDNA analysis, and describe forthcoming new methods with the potential to change the ways in which casework is carried out.

Certain portions of the control region of mtDNA are highly variable among individuals. Forensic analysis typically involves examination of the sequence variation within two hypervariable (HV) regions, HV1 and HV2. While laboratories may work with slightly different ranges, HV1 spans at least from position ~16024 to ~16365 and HV2 from position ~73 to ~340. Mitochondrial DNA analysis is employed when degraded skeletal remains or hairs without roots are encountered in forensic casework or human identification cases. Mitochondrial DNA offers two primary advantages over nuclear DNA analysis: (1) thousands of copies of mtDNA are present in a cell compared to two copies of nuclear DNA, leading to higher sensitivity and (2) mtDNA is maternally inherited, enabling distant maternal relatives to be compared to the analyzed samples for relationship hypothesis testing or when the original depositor of the sample is not available. However, the discrimination power of mtDNA analysis is limited compared to that of short tandem repeat (STR) analysis. Readers are advised to refer to earlier reviews for a detailed description of molecular biology, genetics, sequence determination procedures, interpretation practices, and utility of mtDNA sequence analysis in forensic casework/human identification [47,67].

I. CURRENT PRACTICE

A. Demand, Customers, and Sample Type

Mitochondrial DNA forensic laboratories, regardless of whether they are private or public, share many attributes, particularly the application of common scientific approaches to typical samples requiring mtDNA testing. Federal and state laboratories such as the FBI or AFDIL, and academic providers such as the University of North Texas may have specific or unique mandates for testing, such as current criminal cases, military identifications, or missing persons cases. In general, the clientele served by private service providers include state and defense agencies, more or less evenly divided. Prosecutors and law enforcement agencies with time-critical cases proceeding quickly to investigative conclusions or trial dates are in need of a faster alternative to public labs such as the FBI that carry backlogs, even though those services are free of charge. Coroners and medical examiners, especially those who need supporting documentation for body identifications and family notifications, may also need a quick turnaround time. Defense and postconviction testing clients are often legally unable to access the public sector labs, and this population is always at risk of being underserved by the forensic crime laboratory system. Some countries do not have mtDNA laboratories and hence depend solely on private service providers. Custom mtDNA analysis remains costly and time-consuming because of hands-on analysis of individual samples, some of which are degraded due to age and environmental challenges. Cost is a significant consideration, and can be an obstacle for the agencies or individuals submitting samples to the private laboratory.

A large number of cases analyzed by our laboratory involve mtDNA analysis of samples from old or cold crimes, small crime scene hairs less than 10 mm, nonhuman samples, and canine mtDNA analysis [66,70,76]. Analysis of hairs less than 1cm is routine [in press, *Investigative Genetics*]; some public laboratories have minimal hair size limits that prohibit acceptance of affected cases. Regardless of the case-specific approach, the methods for extraction, amplification, and sequencing are largely shared among all mitochondrial DNA testing laboratories, with minor differences. These minor differences permit ready comparison between results from different laboratories when necessary.

Over a period of years, and largely due to educational efforts by the laboratory and the public sector, clients have adapted to the realities that (a) mtDNA analysis cannot usually be used effectively on samples that are impossible to clean prior to testing, such as stains, swabs, and swatches, due to the likelihood of complex mixtures occurring, and (b) there is limited statistical power to a failure to exclude with mtDNA compared to that of STRs. Even now, frequent requests or inquiries are made about testing of stains that were unsuccessfully tested for STRs. Conversely, laboratories have learned that there is a continuum of cuttings, swabs, and swatches that may successfully, and rarely, undergo mtDNA testing if the original surface was pristine prior to deposit of a sample. For example, a bloodstain on a UV-exposed surface such as the hood of a vehicle may be too degraded for STRs but suitable for mtDNA analysis, and a single type may be easily obtained. Stains on clothing almost always result in an uninterpretable complex mtDNA mixture, with some, such as stains on shoes, being the most complex given their extensive environmental exposure.

The limited statistical power of mtDNA analysis compared to STR analysis results in the submission of many fewer samples than in the early days of testing. Interested parties now inquire about testing for only the most highly probative samples, as opposed to early submissions of many crime scene hairs. Directing the choice of samples to be submitted for testing is a frequent service. In our longstanding experience, the number of samples within a case averages about four, albeit we have encountered several cases with 30–60 samples and one case with approximately 200 samples. Microscopic preliminary evaluation of hairs is recommended in the interest of collecting the most information about a sample, such as size, color, diameter, and hair structure, prior to destructive testing. Since the late 1990s it is no longer customary for probative hair evidence to go to trial without confirmatory mtDNA analysis, based on one published study of the relative value of hair microscopy and its potential to give erroneous results [50].

The cost of testing in the private sector limits the number of samples submitted but also forces the client to carefully consider the relative value of any single piece of evidence. Costs of doing mtDNA casework have increased for public and private sector laboratories. For example, the accredited fee-for-service laboratory is required to adhere to all standards and guidelines promulgated by overseeing accrediting bodies, and the number of these rules and regulations is increasing. Fee structures take into account the nearly 30% of resources that are expended each year to accommodate all these requirements in areas such as training, quality control, quality assurance, accreditation, and proficiency testing in addition to the actual costs of doing casework. Public laboratories also contend with funding challenges, and often receive external funding from federal agencies such as the National Institute of Justice for projects such as cold case

investigation, missing persons identification, and postconviction testing, all areas in which mtDNA analysis is frequently required.

B. Evolution of Protocols

Laboratory methods for all mitochondrial DNA test providers have functionally changed very little since the original Sanger sequencing protocols were applied in mtDNA testing by both the FBI and AFDIL in the early to mid-1990s [47,53]. Preextraction sample prep is a critical part of the analysis. Hairs are washed multiple times in an ultrasonic water bath and rinsed with sterile water and ethanol. Bones are prepped with sanding of the exterior surface before cutting or powdering, and 10% bleach washes can be applied to teeth and bone fragments. An organic in-house or kit extraction protocol is applied to samples such as hair, bone, buccal, or blood samples, followed by PCR amplification using strategies designed to capture extracted control-region mtDNA template from within the two hypervariable regions [71]. As an example, a preliminary amplification of region 16160–16400 can be carried out and a yield gel run to determine if any product has been obtained, and if so, how robust a product it is. The amount of DNA extraction product then drives the required expenditure of remaining template for the additional three amplifications on a questioned sample. Other laboratories carry out a microarray-based quantification step at this point, such as that provided by Agilent Technologies [75]. Regardless of quantitation method, if a product is obtained, the input of PCR product to a cycle sequencing reaction is titered to obtain the best quality sequence via electrophoresis in a genetic analyzer such as an Applied Biosystems 310, 3130, or 3130xl Genetic Analyzer (Life Technologies, Foster City, CA) after cycle-sequencing with Big Dyes v. 1.1 (Life Technologies). In general, the profile obtained from multiple PCR reactions that capture overlapping segments of the hypervariable regions are reconstructed during sequence editing into the "mitochondrial DNA profile" that characterizes that particular sample. Sequence editing is typically carried out by two qualified forensic examiners using DNA editing software such as Lasergene (DNAStar, Madison, WI), Sequencher (Genecodes, Ann Arbor, MI), or Mutation Surveyor (SoftGenetics, State College, PA).

Methods to quantitate mtDNA after DNA extraction and prior to PCR amplification via use of probes and real-time PCR are available [1]. In the case of skeletal remains, nuclear DNA quantitation protocols are applied (Quantifiler®, Life Technologies, Foster City, CA) because these samples are often eligible for STR analysis. Postextraction mtDNA quantification methods are found most often in laboratories where both mtDNA and STR profiles are desired for skeletal remains that will be entered into missing persons databases, and can save time and money in determining the best triage approach when the amount of nuclear DNA in a sample is unknown and mtDNA analysis might be required as well.

A significant protocol change for the handling of skeletal remains was the introduction in 2008 of an EDTA demineralization protocol described by AFDIL [62]. This approach has been adopted by a number of mtDNA test providers. Skeletal material is cleaned with rotary tools, cut, and powdered via blender and then incubated at 56 °C overnight in 0.5 M EDTA with rotation in a hybridizing oven. Full dissolution of the bone powder occurs, releasing DNA from the bony matrix. An organic extraction and silica cleanup yields significantly larger quantities of DNA template than previous methods where the bone powder was not completely dissolved. Early work with the PrepFiler™ BTA kit (Life Technologies, Foster City, CA) seems to indicate that mtDNA results can be obtained from as little as 50 mg of bone, even those that are hundreds of years old (unpublished data).

For almost all laboratories, a standard examination on questioned samples captures HV1 and HV2 in four overlapping amplification products, whereas a standard examination on known samples captures these regions in two longer amplification products. The four amplification products are approximately 300 base pairs long, and all questioned samples are presumed to have somewhat degraded DNA, defining this approach. While not used in many public or private laboratories, "miniprimers" were developed to capture template from degraded samples [29]. With this approach, a set of eight primer pairs, four for each hypervariable region, target amplification products less than 200 base pairs in size; the eight amplification products together cover nearly all of hypervariable regions 1 and 2. The miniprimer approach was pioneered at Penn State University in the early 1990s and an early adopter of this method was AFDIL. Additional primers were developed to cover difficult sequencing regions, capture degraded DNA, and add more control data to the regions normally tested for further discrimination of common types. Further, the additional primers provide replicate overlapping sequence coverage in cases where there are deletions (e.g., at positions 249, 290, 291) or homopolymeric C-stretches (16189, 309.1 etc.), because these phenomena typically result in either a failure to amplify any product with standard primer pairs or generation of only single-stranded data unless secondary coverage is obtained via the use of internal primers.

Two additional primer pairs cover regions designated as "VR1" and "VR2" (Variable Regions, nucleotide po-

sitions 16471–16561 and 424–548, sometimes called "HV3", respectively) [64]. In a rare case, these regions can aid in discriminating between two samples with the same common profile, particularly the H1 haplogroup "263G, 315.1C" haplotype that is observed in about 7% of individuals with European/Caucasian maternal origins. There are other "common" types observed in other ethnic groups. Although minisequencing and single nucleotide polymorphism (SNP) assays have been developed for discriminating between common types by examining mtDNA coding regions [16,74,77], there appears to be relatively little cost benefit to using these assays for the low-throughput laboratory that may have only a rare case needing the application. The method would require costly multiplex amplification protocols, additional instrumentation, validation when no kit is available, and annual proficiency testing. In addition, for a single hair, there is often insufficient DNA template to set up the required multiplex reactions. A simple linear array assay for SNPs in the coding region has been developed and may fulfill this need in future [57]. A more comprehensive review of these methods is provided in a subsequent section of this paper.

Amplifying and sequencing a ~150 base pair (bp) fragment of mtDNA that codes for 12S ribosomal RNA was developed to identify the species origin of nonhuman casework samples, particularly mammalian hair [68]. The ~100-bp sequence product is searched at http://www.ncbi.nlm.nih.gov/BLAST and the species match is reported. The use of this assay has halved the number of samples for which no mtDNA results are obtained and is useful on all mammalian hairs, especially because preliminary hair microscopy is applied less frequently by submitting clients with each passing year. The size of the 12S amplification product is in line with those of the mtDNA miniprimer sets used for degraded samples, meaning that the assay is successful even on highly degraded samples. Species determination aids forensic investigators in opening or closing off lines of inquiry where a highly probative hair is submitted. The assay is frequently required in casework, and crime-scene hairs have yielded a range of species including sheep, pig, mouse, and raccoon. While, to our knowledge, only one mtDNA testing laboratory provides this assay, it is utilized in other biological disciplines.

A frequent crime-scene sample is hair from domestic dogs and cats. Pet hairs have the potential to connect victims, suspects, and crime scenes. However, while STR analyses for canine and feline blood or saliva samples provide near individualization of a domesticated pet much like in human STR typing [24], mtDNA analysis must be used on rootless or naturally shed fur just as for human hair. Mitochondrial DNA diversity in both these species is very restricted compared to that in humans due to the short history of domestication [37,43,94]. However, using precisely the same extraction, amplification, and sequencing methods as those used for human-specific samples, three canine hypervariable regions can be analyzed (HV1: nucleotide position [np] 15431–15782; HV2: np 15739–16092; HV3: np 16451–00014). A reference dog sequence is available [56], and matches are searched from within *C. familiaris* mtDNA control region data compiled by us from GenBank for a frequency statistic much as is computed in human mtDNA testing. There are two providers of canine mtDNA analysis in the United States: Mitotyping Technologies and the University of California Davis Veterinary Genetics Laboratory.

C. Interpretation Guidelines

Interpretation guidelines for the mtDNA forensic arena were published by SWGDAM (Scientific Working Group on DNA Analysis Methods) and the European agency EDNAP in 2003 and 2001, respectively, but no formal guidelines have been promulgated since then [92,95]. The SWGDAM guidelines primarily addressed the interpretation of sequence data, including basic nomenclature of base-calling for polymorphisms, homopolymeric C-stretches, and insertions/deletions (indels). Ensuing discussions in the forensic literature have revolved around more complex treatments of length variation in homopolymeric stretches [7,101,102] as well as consistent nomenclature for difficult-to-assign base-number calls when indels occur [11,85].

In the early years of forensic mtDNA analysis, mixtures and heteroplasmy were not discussed, likely due to the poorer quality of sequence data obtained from early reagents and instruments. With early genetic analyzers such as the ABI 373, or early dye chemistries used for the ABI 310, noisy sequence baselines did not always permit easy recognition of either phenomenon. As chemistry and sensitivity improved, heteroplasmy captured a great deal of attention beginning around 1994, with a glut of publications debating the actual and relative effects on forensic application of mtDNA analysis (see for example, [17,35,36,67,91]). Since that time, with research indicating that low-level heteroplasmy is widespread in all tissues of the body yet is manageably interpreted in forensic applications due to the common major variant that most individuals display [83], forensic laboratories have validated their own interpretational protocols that cover reporting of sequence mixtures and heteroplasmy. For example, based on internal validation studies, heteroplasmy can be defined as the presence of a single

mixed-nucleotide position within the region reported and a mixture from two or more individuals as the presence of two or more mixed positions for this region [67]. There is now substantial literature on both length and site heteroplasmy in human mtDNA [5,20,27,60,83,88,100].

D. Mixture Interpretation

Interpreting mtDNA mixtures continues to present itself as one of the major challenges in forensic DNA analysis. A typical mixture in forensic casework samples may be defined as DNA originating from more than one individual. Possible explanations for a DNA mixture result can range from contamination of a sample during collection or DNA testing, to a "naturally occurring" mixture such as an intimate sample or a biological sample taken from an article of clothing worn or stained by more than one person. In STR analysis it is often possible and appropriate to deconvolute mixtures in order to determine the number of contributors and sometimes also determine the major and minor components of the mixture. With mtDNA mixtures, such deconvolution can prove to be difficult if not impossible using current methods. In addition, because mtDNA analysis is inherently more susceptible to contamination, many laboratories choose not to interpret mixtures at all, and simply categorize the result as inconclusive.

To date, there has been very little published in the literature about interpreting mtDNA mixtures. In fact, the only published information specific to forensic casework involves two cases where STR analysis failed to detect a minor component of the mixed sample and therefore mtDNA analysis was performed using cloning techniques [41], or analysis of mismatch primer-induced restriction sites [93]. Another study on resolving mtDNA mixtures using cloning methods identified potential pitfalls with this approach, including the possibilities of overestimating the number of contributors due to naturally occurring heteroplasmies or underestimating the number due to individuals with identical haplotypes [99]. Cloning methods are also generally labor-intensive and low-throughput, and therefore not practical for forensic casework. To the best of our knowledge, no published research exists on interpreting mtDNA mixtures derived from Sanger sequencing data, which is the current method utilized by most forensic mtDNA laboratories. There is also no mention of interpretation of mtDNA mixtures in the SWGDAM guidelines [92].

Using decades of experience and caution to ensure that any conclusions drawn from a mixture are conservative and not overstated, it is possible to interpret mtDNA mixtures. Mixtures in hair and bone analyses can be cautiously interpreted for the purposes of exclusions, using a validated concept that if the mixture profiles that can be generated from all possible combinations of the mixed sites exclude an individual, especially within individual amplicons, this is fair evidence of exclusion. However, each polymorphism must also be considered individually. In general, Sanger sequencing and the current instruments and chemistry associated with that analytical approach allow for detection of a secondary minor nucleotide where the minor variant is at least 5–10% of the total component of the mixture, whether due to heteroplasmy or a mixture of mtDNAs from two or more individuals. Below that level, there is a possibility of mixed-base dropout (akin to allelic dropout in STR analysis) for the minor templates in one or more amplification products that constitute a full analysis. Therefore, interpretations of hair and bone mixtures must be applied judiciously.

Disregarding an mtDNA mixture profile may result in discarding useful information. The following is an excerpt from an SOP regarding interpretation of mtDNA mixtures: "The clear presence of mixed nucleotide bases at two or more positions will be assumed to represent a mixture of two or more mitochondrial DNA types, which probably originate from two or more individuals. If the known sample cannot be excluded as one of the many possible contributors to the mixture, the report will clearly reflect that multiple contributors to the mixture are possible. The report will state that when a mixture profile is obtained, the number of potential mtDNA types that may be derived from that mixture is equal to 2^n, where n is equal to the number of nucleotide positions at which two different nucleotides have been observed, and that all of these types are not equally probable. The report will state how many possible types there are for the mixture observed. Mixtures may be used with care to exclude an individual as contributor of a sample. Because of the phenomenon of base dropout, mixtures containing any of the substitutions characterizing the known comparison sample must be interpreted with extreme caution in order to conclude that there is an exclusion" (Mitotyping Technologies). Mixed sites can be included in a case report, and in the event that a known sample is included as a contributor to the mixture, no database search or statistical analysis would be provided. Therefore, this result is more for investigative purposes, since court testimony cannot be provided regarding an inclusion in a mixture because no statistics are provided to put the inclusion into context or add appropriate weight to the evidence.

A more progressive approach to mtDNA mixture interpretation has recently been proposed, including a possible method for providing mixture statistics [22].

This statistical approach uses phylogenetic knowledge to deconvolute mtDNA mixtures, noting that not all combinations of variants are equally possible because many of them do not fit into the accepted human evolutionary phylogenetic tree. Two different statistical methods are recommended, one using categorized or qualitative data and the other using quantitative data. The method using categorized qualitative data only takes into account the nucleotide positions where the mixed sites occur, assumes a given number of contributors, and uses a likelihood ratio approach with the following two hypotheses: (1) the mixture comes from the haplotypes of two known individuals (e.g., suspect and victim), or (2) the mixture comes from two random individuals (or victim and an unknown donor). Because the qualitative approach has limitations in cases where there may be several different haplotype combinations or an unknown number of contributors, the quantitative method uses peak heights or areas and regression models to estimate the contributor fractions to the mixture. More specifically, the challenge of mixture deconvolution is determining which haplotypes may have contributed to forming the mixed mtDNA profile. If several haplotype combinations are possible, further quantitative data is needed. By quantitatively measuring the contribution of different nucleotide variants to the same position using signal strength and peak height estimates, it may be possible to deconvolute the mixture into its individual haplotypes. This can be achieved by using standard linear regression analysis to distinguish between two competing hypotheses (as in the example above) based on how well the data fits each hypothesis. As the authors note, however, Sanger sequencing is not a pure quantitative method. Therefore, it is suggested that by replicating the PCR and subsequent sequencing reactions, the information pertaining to the relative proportion of contributors may improve. Also, the use of next-generation sequencing or other methods that allow the sequencing of single copies of DNA would facilitate a more precise determination of the donor contributions. In recent years, researchers have been pursuing efforts to quantify mtDNA mixtures using pyrosequencing [3] and to resolve mtDNA mixtures by denaturing HPLC (dHPLC) [18]. Also, newer methods such as next-generation sequencing and SNPs may allow for better methods of mtDNA mixture deconvolution in the future [46,48].

A significant component of the experience necessary to interpret mtDNA mixtures is the ability to recognize and contend with postmortem DNA damage. DNA decays rapidly after death in biological samples and chemical damage begins to accumulate in the DNA [61]. This damage can take many different forms, including strand breakage or fragmentation, oxidative damage that may inhibit PCR, and the generation of miscoding lesions [49,61,79]. These miscoding lesions can be manifested as base modifications, which can in turn lead to erroneous substitutions (and/or mixed sites in Sanger sequencing). The mechanism causing these base modifications is deamination, which is one of the most common forms of DNA damage. Deamination is particularly rapid for cytosine [44], which results in the conversion of cytosine to uracil, an analog of thymine. Deamination of adenine to hypoxanthine (HX), an analog of guanine, has also been documented as a common form of DNA damage [40]. These deamination conversions result in two complementary groups of transitions, termed "type 1" (A-G/T-C) and "type 2" (C-T/G-A) [40]. It is generally reported that the "type 2" transitions resulting from the deamination of cytosine occur more frequently than the "type 1" transitions [32,40,61]. Most importantly, these deamination events cannot be properly repaired postmortem, and are therefore something that must be considered during mtDNA sequence analysis.

DNA extraction of an old or degraded sample may yield a low number of template molecules, some of which may have base modifications due to postmortem damage. Depending on the distribution of molecules that are incorporated into the PCR reaction, the sequencing of this amplified product may result in three possible scenarios: (1) the number of original templates in the reaction is significantly outnumbered by the modified templates, yielding the incorrect sequence; (2) the number of original templates in the reaction significantly outnumbers the modified templates, yielding the correct sequence; and (3) a mixture of the two is observed [30]. Most, if not all, published data on postmortem DNA damage relates to "ancient" DNA from paleontological and archeological remains. It is hypothesized that if one starts with a DNA sample that is not "ancient" but still "old" (~20–50 years), as is commonly seen in forensic casework samples, there may be fewer damaged DNA molecules that would be starting templates for PCR amplification as compared to ancient DNA samples. This would hypothetically result in mtDNA sequence data that is most often the "correct" undamaged sequence or a mixture sequence of damaged and undamaged DNA. Therefore, mtDNA damage resulting in sequence misidentification should in theory be a rarity in forensic casework. Indeed, it is generally considered that when the initial template number is >1,000 copies, postmortem damage rates are unlikely to bias results [39,58]. However, when few DNA templates initiate a PCR, the resulting sequences are likely to contain base modifications. Several instances of damaged mtDNA have been observed in older hair samples and skeletal remains that were manifested as a mixed

mtDNA sequence (unpublished data). Postmortem DNA damage manifesting itself as a mixture has not been previously discussed as an mtDNA interpretation issue in ancient DNA studies, most likely because cloning methods (resulting in single-molecule profiles) are typically utilized in ancient DNA research whereas Sanger cycle sequencing (resulting in a pooled molecule profile), is most often used in forensic mtDNA laboratories.

There are obviously many forensic implications associated with interpreting mtDNA mixtures, especially with regard to postmortem DNA damage. Because there are currently no guidelines set forth on this subject, up until recently mtDNA damage mixed sites have been treated no differently than typical mixed sites. All mixed sites have been included in case reports and regular mixture guidelines were typically followed according to protocol. Over time, however, experience has allowed for the recognition of key differences between DNA damage and typical mixtures. Three core observations have emerged that have become the foundation for our enhanced mixture guidelines relating to damaged DNA: (a) noting at which base positions the mixed sites are occurring; (b) noting whether or not the mixed sites are reproducible through repeat extractions or amplifications; and (c) noting other substitutions that show no signs of a mixture.

Evidentiary samples received by laboratories for mtDNA testing typically consist of single hair shafts and skeletal remains. Skeletal samples usually provide ample opportunity for replicate testing, both in reamplification(s) and reextraction(s). Conversely, with hair shaft evidence, there is generally very limited opportunity for duplication of results. Because of this, mixed sites from potential damage in hair shaft samples are conservatively reported as a regular mixture. However, due to the ability to duplicate results from bones and teeth, mixture interpretation policies can be changed with respect to skeletal remains samples that may have damaged DNA. For example (taken from a Mitotyping Technologies protocol):

a. The full profile will be developed using regular primers and/or miniprimers.
b. The full profile will be edited in a first pass, noting the locations of unmixed polymorphisms and mixed sites, if any.
c. If there are no mixed sites, the profile will be edited by both examiners and reported.
d. If more than one mixed site is noted, there will be an attempt to determine if there are any unmixed polymorphic sites present.
e. If all polymorphic sites or most polymorphic sites are mixed, the mixture will be assumed to be a true mixture of DNA from two or more individuals and reported as a mixture.
f. If there are unmixed polymorphic sites, the region(s) containing the mixture will be reamplified.
g. The products of these reamplifications will be sequenced and then edited in the original project along with the previously amplified and sequenced amplification products.
h. If the mixed sites disappear in the second-round amplification and/or if new mixed sites appear in the second-round amplification, the mixed sites will be assumed to have resulted from damaged mtDNA template being captured in early rounds of amplification.
i. The layout (printout of analyzed sequence data) containing all data will note the unmixed polymorphisms or persistent mixed polymorphisms as highlighted and labeled sites.
j. If more than one mixed site persists as mixed, along with the unmixed polymorphic sites, this sample will be reported as a mixture.
k. The layout containing all data will have an asterisk below mixed sites that are not reproducible in any subsequent amplification. This asterisk will reference an accompanying case note.
l. The accompanying case note will show a table of the nonreproducible mixed sites and which amplifications they were observed in. This table will be created by one examiner and co-signed by a second examiner.
m. The final report will not need to show these nonreproducible mixed sites.
n. Three conditions are required to report this kind of sample as an unmixed profile:
    - The mixed sites are not reproducible in any subsequent amplification.
    - The unmixed polymorphisms remain unmixed in all amplifications.
    - A single mixed site is permitted due to the possibility of heteroplasmy.
o. The appropriate call as to whether a sample is composed of a mixture or not will be left up to the discretion of both examiners, with both examiners making and agreeing on the determination.
p. In general, the inability to reproduce mixed sites will lead to a conclusion that these sites are due to damage and not to additional DNA templates from a second individual.

Nine skeletal remains cases with sample mtDNA sequence mixtures were interpreted in the past three years following these guidelines (unpublished data). Six of these cases resulted from DNA damage, one was a true mixture, and two were likely DNA damage but there was not enough template DNA to replicate the data. The six cases with DNA damaged samples each had unmixed, duplicated substitution sites that were reported as the true mtDNA profile. Five of these cases resulted in inclusions with a known sample (known samples were tested after the skeletal remains in each case), and the sixth case was a historical case consisting of Late Prehistoric Native

American skeletal remains that had no known reference samples for comparison. Presumed mtDNA damage has also occasionally been observed in hair samples. Although these hairs have been reported as regular mixtures, the following specific mtDNA casework examples involving aged hair samples clearly exhibited DNA damage:

Case #1. A questioned hair (~25 years old) with the following mtDNA profile: 16188 Y(C/T), 16218 Y(C/T), 152 C, 214 R(A/G), 263 G, and 315.1 C. The three mixed sites were in overlapping regions where one PCR product resulted in a mixture and the other PCR product resulted in the rCRS base. Suspecting that DNA damage was causing at least two of the three mixed sites (with potential heteroplasmy at 214), another piece of this same hair was reextracted. The two mixed sites in HV1 disappeared, while the three clean substitutions along with the 214 potential heteroplasmic site remained (in both PCR products). The known sample from the suspect buccal swab gave the same three substitutions as the questioned hair and showed no heteroplasmy at position 214. Knowing that heteroplasmy is more prolific in hairs than in body fluids [67,70], a known hair from the same suspect was tested in order to validate that position 214 was a true heteroplasmic site in the questioned hair. When the known suspect hair was typed, heteroplasmy was observed at position 214 and interestingly, another heteroplasmy was seen at position 16222.

Case #2. Eight questioned hairs (~15 years old) were tested. Three hairs collected from the same item of evidence gave the same profile (unmixed substitutions at positions 16126, 152, and 263). One of the three hairs also showed five mixed damaged sites (at positions 16234, 100, 140, 269, and 307). The suspect and victim were both excluded as contributors of these three hairs.

There have been several trends observed with regard to DNA damage. First, in agreement with other studies [32,40,61], most, if not all of the damaged sites in casework samples have been observed at either cytosine or guanine rCRS positions, resulting in C/T or G/A mixed sites (Type 2 damage). Another noteworthy observation is in regard to identifying postmortem damage "hotspots". Previous studies on this topic have reported conflicting results. For example, Hofreiter et al. [44] reported that "there is no evidence for 'hotspots' for mis-incorporation in the resulting sequences." However, Gilbert et al. [31,33] suggested that there are postmortem damage hotspots and that they correspond with sites of elevated *in vivo* mutation rates. Other data suggest that most damaged sites occur at random sites that are not associated with *in vivo* mutational hotspots (unpublished data). A possible explanation for these conflicting results could be the differing definitions of which sites constitute mutational hotspots [25,72,89].

Two instances have been observed where DNA damage resulted in complete substitutions, rather than just mixed sites. Both cases involved old skeletal remains, one of which was approximately 25 years old and showed two C to T substitutions at sites 16107 and 16112. As there was not enough template DNA to replicate this result, these two substitutions were included in the case report with an asterisk, indicating that they were not duplicated and therefore might not be true substitutions. The submitted bone sample from the second case was approximately 15 years old and gave several G to A substitutions at random (nonmutational) sites. Replicated amplification products gave the same profile, but without the G to A substitutions observed in the first PCR, further indicating that they were a result of damaged DNA. This highlights the importance of duplicating results whenever possible—a recommendation also noted in ancient DNA research. For example, Hofreiter et al. [44] recommended that when extracts of ancient specimens contain only a few template molecules for PCR that DNA sequences are determined from at least two independent amplification products. It is interesting to note that an increase in DNA damage has been observed from skeletal remains soon after the incorporation of a new bone demineralization extraction procedure in 2008. Two possible reasons for this may be: (1) an elongated incubation period (overnight) subjects the bone samples to additional heat, and (2) the increased efficiency of the protocol results in more DNA recovered overall, including more damaged DNA. A more detailed study examining damaged DNA is forthcoming (manuscript in preparation).

E. Statistics and Databases

In the United States, the criminal justice community including both Combined DNA Index System (CODIS) and private laboratories have relied on the SWGDAM database of human mtDNA sequences to derive the statistical weight of a failure to exclude with mtDNA results [73]. Using common statistical equations [47] to estimate the upper-bound proportion of a population that cannot be excluded as having the casework profile in question with 95% or 99% confidence has been the method of choice in courtroom presentations since the mid- to late 1990s. In most cases, this approach permits the exclusion of well over 99% of individuals in a population as donors of a sample, due to the large number of mtDNA types present

in the world population, but it is also conservative enough to counteract normally small effects of mtDNA population substructure. With current technologies emerging to rapidly type whole mtDNA genomes, a good understanding of the phylogenetic structure and saturation levels of human mtDNA haplotypes has been gained [87], indicating that databases can be more accurately planned to establish frequency estimates of haplotypes within different populations [23]. Sequencing assays, at least on pristine samples, are faster and less expensive than ever, and an estimated 6,700 whole human genomes were reported to be present in GenBank and the scientific literature as of 2010 [87].

The FBI and others are currently evaluating whether a slightly different calculation may be applied for haploid lineage markers like mtDNA and Y-STRs [12]. The Clopper and Pearson method provides a two-tailed upper 95% confidence limit, and can be equally applied to cases where the profile observed is one not previously observed, as well as to cases where the profile has been observed before [14]. The resulting number is slightly more beneficial to a defendant, especially when database sizes are small. Other methods of presenting the weight of mtDNA "matches" have been proposed, including match probabilities and likelihood ratios [10].

As of early 2012, the SWGDAM CODIS database that had been available online at FBI.gov for use in the public sector is no longer available, although there are plans to make it available via the National Institute of Standards and Technology (NIST) after review of the data therein at the FBI (Eric Pokorak, FBI DNAU2, personal communication). CODIS laboratories continue to have use of this database, called CODIS 7.0, for criminal casework as well as for missing persons cases. Alternatives exist for database searching, most notably the online searchable database EMPOP (empop.org), which contains at present over 15,000 human profiles from highly vetted forensic datasets as well as separate datasets from the published anthropology literature [80]. Searching via DNA text strings or lists of polymorphisms in a sample is possible. The database contains samples primarily from Europe, but is expected to grow with the addition of more than 12,000 sequences from North Americans during 2012 (Jodi Irwin, AFDIL, personal communication). Outputs classifying sequence match results with respect to geographic origins, such as "European", "West Asian", "Sub-Saharan", etc., as well as for metapopulations such as African, Asian, European, etc., provide a very helpful approach for forensic applications. For example, EMPOP may be searched for African-American samples collected within North America or within Africa. Recent funding of Lakehead University to produce "MitoNorth, a forensic mtDNA database for Canada, will provide a resource for North American cases outside the United States. In addition, a Korean website (www.mtmanager.yonsei.ac.kr) is available for searching 9,294 human sequences, although there is overlap with data found in other databases.

F. Courtroom Experiences

After a flood of admissibility hearings under the Kelly-Frye and Daubert scientific evidence rules between 1996 and about 2005, mtDNA testing appears to be well accepted in the criminal justice system, although many jurisdictions have not yet tried an mtDNA court case. Many of the written decisions on notable and early cases may be found at www.denverda.org. The only federal appellate decision on mtDNA to date is *United States v. Beverly* in the 6th Circuit Court of Appeals in 2004. This case was tried in Columbus, OH, in 2000. Beverly was convicted in part based on mtDNA comparison of a hair recovered from a hat in a getaway vehicle after bank surveillance cameras captured Beverly wearing the hat during commission of a bank robbery. The court's primary finding was that the maternal inheritance and nonunique characteristic of mtDNA can be suitably explained by both cross-examination and well-accepted statistical analysis, guaranteeing that the result is not more prejudicial than probative.

Presentation to juries remains a critical feature of forensic mtDNA usage. Emphasis on the nonunique status of the marker is important so that juries are not confused about the differences between the powerful statistics used for nuclear DNA and the more modest statistics possible with mtDNA results [55]. To date, no court decision has been overturned due to any misrepresentation during testimony about the strength of the statistical conclusion or a failure to represent the nonunique haploid mode of mtDNA inheritance.

G. Regulation and Accreditation

Although accreditation remains optional for non-CODIS laboratories in the United States, most if not all laboratories performing mtDNA testing have chosen to become accredited under the ISO 17025:2005 Standards titled "General requirements for the competence of testing and calibration labs". Various accrediting bodies exist such as the American Society of Crime Laboratory Directors/Laboratory Accreditation Board (ASCLD/LAB) and Forensic Quality Services (FQS), and laboratories have a choice of which agency will provide their accreditation. During the accrediting body inspections under

ISO, external auditors also audit laboratories under the FBI's Quality Assurance Standards for Forensic DNA Testing Laboratories which are required operating standards to be a CODIS provider. In particular, most private laboratories are undergoing annual or semiannual visits that cover a range of standards and guidelines, internal audits, management reviews, proficiency testing, and numerous other required activities. The single most striking change in this area over the last 14 years has been the rapid increase in standards and guidelines, and the increased rigor of these programs.

Private laboratories that serve multiple states also are required to conform to those states' individual forensic testing standards. For example, the New York State Department of Health has separate Forensic Identity Standards that a laboratory must follow to perform casework shipped from New York, and laboratories must undergo audits biannually by this agency as well as participate in their mandated proficiency tests. In contrast, other states such as Texas simply require proof of current accreditation via ASCLD/LAB or another entity for cases to be sent to the private sector. Private laboratories are ineligible to use CODIS 7.0, although there are steps that could be taken to allow a CODIS laboratory to take ownership of a private contractor laboratory's data for a search in a current criminal case or missing person case.

II. ALTERNATIVE METHODS AND FUTURE POTENTIAL

It is clear that conventional mtDNA sequence analysis is robust and reliable for routine forensic investigations [47]. However, there are a number of alternative methods that can be employed to enhance or advance current practices. Some of these methods are currently available to the practitioner, while others are still in development and have the potential to significantly impact the testing process in the future. The alternative methods presented here include screening techniques to identify potential mtDNA matches prior to full-scale sequence analysis, expanded analysis of the mtDNA genome within and outside of the control region, technologies that provide for a deeper assessment of mtDNA mixtures and heteroplasmy, and a second-generation sequencing approach that provides a more sensitive means for detecting and quantifying heteroplasmic variants and mixture components, and thus may allow for the deconvolution of mixtures.

A. Screening Methods

Conventional mtDNA sequence analysis is often considered relatively time-consuming and expensive, but remains the most comprehensive approach to developing a forensic mtDNA profile. A quick assessment of work performed by population geneticists to classify population structure through the clustering of mtDNA sequences into haplogroups illustrates the value of obtaining complete sequence information (see, for example, [89] for a recent worldwide mtDNA phylogeny). While haplogroup designations can clarify the relationship between and within population groups, private polymorphisms found within haplotypes that typically do not contribute to haplogroup assignment can significantly increase discrimination potential and make conventional mtDNA sequence analysis a highly informative typing system.

Although potential obstacles of time and expense for conventional sequence analysis can be mitigated by using a variety of screening approaches [13], only the Roche mtDNA Linear Array has been adopted in a limited way within the forensic community, especially within the community of laboratories doing extensive missing persons projects. The value and usefulness of linear arrays has been the focus of investigation [21,57]. However, the proposed extent of their use has also spurred debate [69]. The Roche Applied Science LINEAR ARRAY Mitochondrial DNA HVI/HVII Region-Sequence Typing Kit examines 18 polymorphic sequence positions along the length of HV1 and HV2. Ten short sequences are targeted, encompassing the 18 polymorphic sites. Each of the 10 targets has multiple sequence combinations, or alleles, for a total of 30 possible alleles. The individual alleles are interrogated using 33 sequence-specific oligonucleotide probes immobilized as 31 lines or strips on a nylon membrane. The HV1 and HV2 segments are co-amplified from input DNA using PCR primers with biotin moieties attached to the 5'-end of each of the four primers. Products of PCR amplification bind to the probe-bound strips on the nylon membrane through allele-specific hybridization and are detected using an enzyme-conjugate-based development process similar to the HLA DQA1 and Polymarker systems [86]. The subsequent interpretation of linear array results is relatively simple, and the result takes the form of a barcode-like profile.

The specific application of the linear array assay has been debated. While some believe that the assay is useful as a routine screening tool to eliminate samples from needing full sequence analysis, others contend that the potential uses should be limited. For example, the assay was used in 16 adjudicated forensic cases containing 57 evidence samples and 33 references to exclude 56% of the

samples as potential matches; thus, less than half of the samples required further sequencing [21]. Of the samples that were originally excluded through sequence analysis, 79% could be omitted using the array system alone. These results were the impetus for suggesting the use of linear arrays to decrease sequencing efforts and turnaround time, and thereby reduce the cost of analysis in routine casework. Therefore, laboratories with limited capabilities or instrumentation may consider using linear arrays to conduct mtDNA analysis. However, practitioners working in the field have questioned the wisdom of using the array system for routine forensic casework, or whether the system is substantially more cost-effective or efficient [69].

The time and expense required for extraction of DNA from hair shafts (or any biological specimen) will be similar for each of the two typing methods (conventional sequencing versus linear arrays). The amplification process, including post-PCR product gel analysis, will also be the same, and the reagent costs for DNA sequencing are basically offset by the kit costs for linear array analysis. Therefore, one must look further to pinpoint the efficiencies and limitations with each method. For example, the current linear array system is more susceptible to cross-hybridization anomalies or null results, potentially complicating the interpretation process. To minimize cross-hybridization artifacts, a reasonably precise post-PCR quantification system is required to estimate the amount of product for hybridization. At least 2 cm of hair shaft are ideally required for linear array analysis to obtain a full profile. In contrast, conventional mtDNA sequencing is relatively insensitive to the amount of input DNA added to the sequencing reaction, and typing results can routinely be obtained from less than 1 cm of hair shaft (in press, *Investigative Genetics*). In a study of more than 2,500 freshly collected head hairs, the success rate of developing a linear array profile never exceeded 75% [84], whereas when using conventional DNA sequencing the success rate routinely exceeds 92%, including 80% of casework hairs less than 1 cm in length [70]. Much of this difference in rates can be attributed to the size of the target amplicons, which varies between the two systems: approximately 400 base pairs for the linear array assay and 100–300 base pairs for sequencing. Of course, a limitation of conventional sequence analysis is the significantly longer time to complete the analysis (instrument and interpretation time), especially when considering high-volume scenarios. Therefore, the linear array method may be a valuable tool in missing persons or human rights investigations that require rapid reassociation of thousands of commingled remains where there is the potential for multiple reextractions [28]. However, for routine mtDNA testing of crime scene hairs, where DNA template is limited and complete profiles are desirable for courtroom presentation, conventional sequence analysis may be the appropriate method of choice.

As an alternative to the linear array approach, fractional mtDNA sequencing can also be used as a screening tool, given judicious selection of appropriate samples. Assuming the full mtDNA profiles of reference samples are known and the screening region displays a low-frequency profile, informative polymorphic sites can be targeted to identify which evidence samples are potential matches or exclusions. For example, given the HV1 profile of the following victim reference sample as 16093C, 16189C, 16278T, and 16311C, the practitioner can amplify and sequence nucleotide positions 16160–16400 in evidence samples for comparison to the reference profile. Evidence samples that share the 16189C, 16278T, and 16311C polymorphisms can be assumed to be from the victim and discounted if not probative (for example, victim hairs on victim clothing). Although use of this system relies heavily on the relative rarity of a particular profile in the screening region utilized (so as not to falsely assume the profile belongs to the known individual), an approach like this will significantly reduce the workload of a full-length sequence analysis, while maintaining the discrimination potential of the system. In addition, when this partial profile is not exclusionary and is probative, continuing quickly on to develop the remaining portion of the full-sequence profile is possible.

B. Expanded Sequence Analysis

In the early 1990s, it became clear that a handful of common mtDNA sequence profiles within HV1 and HV2 were being encountered when performing mtDNA analysis on population groups of European Caucasian descent that were collected in the United States (unpublished observations). When these common profiles are encountered today, whether revealed by the linear array assay [57] or through conventional DNA sequencing (see [15] for a list of common mtDNA sequence types), they can impede the practitioner's ability to differentiate between two individuals, or two samples, if sequence analysis is limited to HV1 and HV2. Therefore, an expanded investigation of mtDNA sequence information is required to resolve these identical profiles. There are two principal approaches that can be employed, neither of which must be performed to the exclusion of others: expand the range of sequence being analyzed in the control region [63,64], and query SNP sites in the coding region of the mitochondrial genome [15,81].

As an example, an early population study of 200 unrelated individuals from Germany revealed 88 variable nucleotide positions in HV1 (26% of the total sites) and 65 variable sites in HV2 (24% of the total) [63]. A third segment of the control region, HV3 (encompassing positions 438–574, and sometimes referred to as variable region two, VR2), exhibited lower variability, with 25 polymorphic sites (18% of the total), but in contrast to other segments of the control region, was quite informative; only 7% of polymorphic sites occurred between positions 16,366–16,569 and 1–72 (sometimes referred to as variable region one, VR1), and 3% of polymorphic sites occurred between positions 341–437 (included in VR2). Approximately 20% of the identical HV1/HV2 sequences could be resolved through HV3 analysis. In addition, while VR1 has lower overall variability (7%), 19 polymorphic sites were identified in the dataset, revealing a high degree of discrimination potential primarily due to a highly polymorphic site at position 16519. This site is one of two in the entire control region that has a frequency approaching 50% in the population, and thus is highly informative.

If DNA sequence in the control region is not sufficient to resolve identical HV1/HV2 profiles, practitioners can look to the coding region for help. For example, mtDNA genomes from 241 individuals who matched common European HV1/HV2 profiles have been sequenced to identify polymorphisms that enhance forensic discrimination [15]. Individuals with the same HV1/HV2 profile rarely matched across the entire genome. The 13 protein-coding genes in the mitochondrial DNA genome are composed of more than 11,000 nucleotides. When datasets on the mtDB website are queried (www.genpat.uu.se/mtDB), approximately 40% of the codon wobble positions show variable sequence [16]. Therefore, when attention is placed on these neutral positions, eight panels with 7–11 multiplexed SNPs per panel can be designed to provide additional levels of discrimination. The appropriate panel can then be chosen because of its direct association with one or more of the common HV1/HV2 profiles, which helps to conserve sample extracts while providing for maximum discrimination. This added level of separation reduced the frequency of the most common European profiles in the Coble et al. 2004 study [15] from ~7% down to ~2%, and the 18 common profiles were resolved into 105 different haplotypes, 55 of which were seen only once. When including key nonsynonymous SNPs, the total number of haplotypes increased to 127 [16]. Therefore, it is clear that expanding the range of mtDNA sequence to the coding region has a dramatic impact on resolving identical HV1/HV2 profiles. Of course, the ability to perform coding-region assays is dependent on the quantity of DNA extract available for analysis; said quantity is quite limited in the case of small fragments of hair shaft. In addition, because the majority of the mtDNA genome contains coding information vital to the survival of the cell and may therefore be medically relevant, this tactic for increasing discrimination may need to be assessed for use in forensic analysis. One of the challenges of performing new analytical procedures on forensic casework samples is the availability of commercial kits and technologies. Historically, forensic mtDNA analysis has not relied on commercialization of coupled amplification and sequencing kits, but instead has involved the development of in-house amplification reagent systems [26,45,47]. The same holds true for the development of assays that query DNA sequence within the mtDNA coding region. Although effective strategies have been developed by practitioners for the analysis of coding region SNPs using primer extension or SNaPshot approaches [52,97], a major concern remains, as each in-house assay contains different target SNPs, and may not include the most informative loci. In addition, a forensic context requires a database upon which the statistical weight of the profiles is based, and while there are large forensic databases for the control region, no comparable searchable database has been prepared for SNP datasets. While many whole genomes are available for databasing, there has not been an effort to collect this information for forensic purposes. Until a kit-based system is commercially available that includes the most informative target loci and a frequency database of profiles is developed from those loci, it is doubtful that coding-region SNP assays will have widespread appeal in the forensic community.

C. Mixtures and Heteroplasmy Investigation: DGGE and dHPLC

When using the Sanger method of DNA sequence analysis, it is difficult to identify low-level heteroplasmic variants, and it is nearly impossible to resolve mixtures. Each of these phenomena has been the subject of assay development and has resulted in a number of potential solutions. However, no one technique has emerged with widespread acceptance. Other than the laborious yet effective technique of cloning [34], the verification and identification of heteroplasmic variants can be accomplished in a variety of ways, but not always by addressing both interests — i.e., verification and identification. In the late 1990s, a denaturing gradient gel electrophoresis (DGGE) approach was developed to verify the presence of heteroplasmic variants in HV1, and to provide a means for isolating each variant for subsequent sequence analy-

sis [90]. The DGGE technique allows for verification of heteroplasmy through the resolution of mismatch-containing heteroduplices from fully base-paired homoduplices in an increasingly denaturing environment. Detection of heteroplasmy was accomplished down to a minor component proportion of 1%. This level of detection is approximately onefold better than Sanger sequence analysis provides, where variants as low as 5–10% of the total can sometimes be reported. As an illustration of the effectiveness and reliability of the DGGE technique, mixtures of 49 pairs of HV1 sequences, each pair differing by a single polymorphism, were successfully verified as exhibiting heteroplasmy-like characteristics; i.e., heteroduplex and homoduplex bands on a DGGE gel. In addition, heteroplasmy was successfully verified in 13 samples known to have heteroplasmic sites. Variant nucleotide positions were identified (confirmed) by reamplifying physically excised homoduplex bands of DNA from DGGE gels and performing sequence analysis on the products.

The DGGE assay has also been used effectively to determine the rate of control-region heteroplasmy in the population [96]. Heteroplasmy in HV1 was observed in 35 of 253 randomly chosen individuals (sample source was whole blood), or 13.8% of those tested. Given the greater detection level of DGGE, it is not surprising that this is a higher rate of heteroplasmy than the reported rate of 11.4% for the hypervariable regions when using Sanger sequencing on casework samples involving hairs [70]. The identified heteroplasmic sequences revealed single-nucleotide differences in 33 of the 35 individuals tested, whereas two individuals exhibited heteroplasmic sites at two different positions (triplasmy). Heteroplasmy occurred at 16 different nucleotide positions throughout HV1, with the most frequent observations at positions 16093 and 16129, consistent with prior and recently published studies [46,53]. In addition, the majority of heteroplasmic variants occurred at low frequency and could not be detected by conventional sequencing. The study from Tully et al. in 2000 [96] was the first to indicate that low-level heteroplasmy in HV1 was more common than was previously believed, and that it occurred across the entire control region, a finding that continues to have importance in evolutionary studies and forensic applications.

It became apparent that the gel-based DGGE system was too arduous for routine mtDNA analysis, so an advanced column-base system was developed using a dHPLC approach [59]. A total of 920 pairwise combinations of HV1/HV2 amplicons from 95 individuals were assessed for sequence concordance. For combinations of amplicons from individuals who shared identical HV1/HV2 sequences, dHPLC verified sequence concordance. However, for 849 combinations with different sequences, dHPLC was able to detect the presence of sequence nonconcordance in all but 13 samples (98.5%), including the detection of transitions, transversions, insertions, and deletions. This study clearly illustrated the utility of the dHPLC assay as an indicator of mtDNA sequence heteroplasmy, and by extension, the presence of a mixture. In addition, the dHPLC system provided a means for relatively simple fractionation of the individual components of a simple mixture from two individuals by enriching the homoduplices for one variant or another and allowing for subsequent sequence analysis of isolated DNA fragments representing the separate contributions of the two individuals. Given these capabilities, the dHPLC system could in theory be used for screening purposes to determine if two samples are concordant prior to sequence analysis. A dHPLC system, the Transgenomic Wave System 3500 or 4500, is commercially available, making it accessible to practitioners. However, some challenges have been encountered with the interpretation of dHPLC results. Length heteroplasmy, a common mtDNA phenomenon, broadens heteroduplex peaks and is often observed with shoulders representing the multiple variants. This can make the detection of neighboring-point heteroplasmy more difficult to interpret. In addition, the detection level for heteroplasmy using the dHPLC system is only marginally better than Sanger sequencing. Nonetheless, the ability of the dHPLC system to fractionate individual variants is significant because the only other option, historically, has been cloning of heteroplasmic variants or excision of variants from DGGE gels for sequencing.

D. Mass Spectrometry

The use of mass spectrometry to identify variants of mtDNA sequence has been investigated by forensic laboratories to analyze simple mixtures and heteroplasmy, as well as to develop single-source profiles [78]. More recently, an automated system for high-resolution analysis has been developed [38]. The nucleotide base composition of DNA fragments is determined after multiplex PCR amplification by electrospray ionization mass spectrometry (ESI-MS), and is commercially available using Abbott's PLEX-ID™ System. The ESI-MS method targets 1,051 nucleotides of DNA sequence within the control region, including HV1 and HV2. Twenty-four overlapping segments of DNA are amplified in eight triplex reactions with a sensitivity of less than 25 pg of genomic DNA per reaction. Automated PCR product purification occurs prior to injection onto the ESI-MS.

Mass calculations of individual DNA fragments are converted into base composition values for each amplicon; the full profile is assembled with computer algorithms that recognize and link overlapping end-point homologies for the 24 fragments. The profile can be compared to population databases of composition profiles derived from sequence information, and therefore, can be subjected to the same statistical approach used for assessing the significance of matching mtDNA sequences. Although only 94% of the information obtained by direct sequencing of HV1 and HV2 is detected with the ESI-MS assay, ESI-MS is more informative overall because it covers more than 400 additional base pairs of the control region. The reduced discrimination potential within HV1/HV2 is due to reciprocal nucleotide changes that cause fragment masses to appear unchanged (e.g., C150T, T152C). More importantly, while the ESI-MS system can quantitatively deconvolute heteroplasmic sites, the precise nucleotide differences between samples cannot always be elucidated, as changes in sequence can happen across the length of the DNA fragment being analyzed. On the contrary, the ESI-MS system can effectively resolve length variants in homopolymeric stretches—an attractive feature, as a large percentage of mtDNA profiles include length variants. Therefore, while the assay is not hindered by length heteroplasmy, identifying the location of point heteroplasmy can be a challenge because mass weights are determined rather than the precise order of bases.

The robustness of the ESI-MS method has been tested on more challenging sample types [51]. In 2009, a project was launched by the Commonwealth War Graves Commission to identify the remains of 250 World War I soldiers recovered from a mass grave in Fromelles, France. A comparative assessment of the performance of Sanger sequencing and the ESI-MS method was conducted on 225 of those skeletal remains. Assessment included the ability to amplify extracted DNA, to develop an mtDNA profile (sequence or base composition), and the ease-of-use associated with each method. The ESI-MS approach fared well during this comparative analysis. The smaller amplicon lengths when using the ESI-MS method are an advantage with degraded DNA (40–100 bp). More than 99% of the 225 skeletal samples produced at least partial results using the ESI-MS method, generating data for at least 75% of the target amplicons. Almost 60% of the samples produced full base-composition profiles. This was as good as or better than the Sanger sequencing results, and is even more compelling given that a miniprimer-set approach was used for the Sanger method (amplicon sizes of 150–225 bp) [29]. It is quite possible that the ESI-MS method would have been superior to the routine primer-set approach using amplicon sizes of approximately 250 bp.

Given the amplification strategies of each method (sequencing or base-composition profiling), a more applicable comparison is the respective coverage rates of generated sequence information. The Sanger and ESI-MS methods produced equivalent levels of DNA sequence: coverage of approximately 98% of the respective ranges of sequence. The only exception was when a small stretch of sequence, which is not covered with the ESI-MS method, was considered (nucleotide positions 16251–16253). Overall, the ESI-MS method was easy to use, and was highly automated. However, the instrument is relatively complex from an engineering perspective, and is quite expensive to procure, so it is unclear how these factors will impact laboratory operations. In addition, while the ESI-MS method has a higher overall discrimination potential than Sanger sequencing of HV1 and HV2 alone, databases of base composition profiles do not currently exist that can be used for comparison purposes. Fortunately, work is progressing forward to address this deficiency. In the short term it has been recommended that the ESI-MS method be used as a rapid high-throughput screening tool prior to conventional sequence analysis [38].

E. Pyrosequencing and Deep Sequencing

Pyrosequencing techniques have been used to quantify SNP profiles in the mtDNA coding region and to detect mixture or heteroplasmic variants in both the coding and control regions [3,4]. The pyrosequencing method involves sequential introduction of the four nucleotides (dATP, dCTP, dTTP, and dGTP), followed by a cascading series of events that will lead to the emission of light when a nucleotide is incorporated into the newly synthesized strand of DNA. With the incorporation of a nucleotide, through the action of DNA polymerase, pyrophosphate is released as a byproduct that is fed into a coupled enzymatic pathway. The route was initially a three-enzyme system, but in some applications moved to a two-enzyme approach to increase read lengths [65]. The three original enzymes included *sulfurylase* to convert the pyrophosphate into ATP in the presence of ASP (adenosine-5'-phosphosulfate), then *luciferase* to convert luciferin to oxyluciferin in the presence of ATP with the release of photons of light, and finally, *apyrase* to digest unincorporated nucleotides between sequential steps in the sequencing process. However, it turns out that apyrase is an inefficient enzyme, so read lengths of the early pyrosequencing method were limited to approximately 100 bp. Replacing the use of apyrase with a wash

step between nucleotide additions greatly increased read lengths, although the wash step reduced overall yield due to loss of template. As discussed below, the advent of next-generation sequencing instruments has helped to resolve this problem.

A pyrosequencing method for coding region analysis, comprising 17 sequencing reactions performed on 15 PCR fragments, was used to increase the potential for separating similar HV1/HV2 profiles [4]. The assay was performed on 135 samples, 60 of which had zero to one difference from the HV1/HV2 reference sequence [2,6], while the other 75 samples had two differences from the reference. An average read length of 81 of 165 nucleotides was obtained from each sample, with a range of 20–120 bases. A total of 52 SNP sites were identified, of which 18 had a single SNP variant. This is a significant increase in discrimination potential when compared to methods that employ primer-extension assays, but it remains to be seen whether the target sites are well suited for a wide range of populations. Most importantly, for the 60 samples with zero or one sequence differences in the control region, only 12 samples (20%) could not be resolved through the addition of at least one coding-region difference. Therefore, the use of this pyrosequencing-based coding region approach may effectively enable the differentiation of samples with similar HV1/HV2 profiles.

An easy-to-use and rapid pyrosequencing method has also been developed to assess the linear relationship between incorporated nucleotides and released light, allowing for quantification of variants in mtDNA mixtures or samples with heteroplasmy [3]. The assay was designed for five PCR amplified targets, ranging in size from 200–310 bp, to query seven variable positions in the control and coding regions. For all detected SNPs, the measured mixture ratios were consistent with the expected, providing reliable quantification data. However, a significant drawback of this method is the relatively low level of mixture detection, similar to that of Sanger sequencing. Minor mixture components less than 10% of the total DNA content are not well resolved, reducing the value of the pyrosequencing system.

When investigating mtDNA mixtures or heteroplasmy, the practitioner would like access to methods that are sensitive enough to detect low-level variants, and are precise enough to identify the variants. It is quite possible that deep-DNA-sequencing approaches will address both of these interests in the future. However, before the forensic community can rely on the next-generation sequencing platforms for routine mtDNA analysis, forensic standards must be applied, and forensic concerns addressed. For example, in a *Nature* paper from 2010 it was reported that intraindividual heteroplasmic variation was frequently observed at levels of around 1–2% when employing the Genome Analyzer from Illumina (San Diego, CA) to sequence the mtDNA genome of CEPH families and soft-tissue samples from the same person [42]. While this claim was consistent with previous studies using DGGE analysis (unpublished data from M. Holland), later phylogenetic assessment of the reported data revealed that, on average, at least five polymorphic sites were missed in the reported sequences [8]. To address the higher error rates commonly observed with deep-DNA-sequencing approaches, a second study reported an assessment of the accuracy of mtDNA-sequencing results generated on the Genome Analyzer II from Illumina [60]. It was determined that minor components down to 1–2% could be reliably reported. Therefore, it is possible that the samples from the previous study were contaminated [42], or that errors in interpretation of the data had occurred. Regardless of the reasons for the discrepancies, neither of these studies suitably addressed forensic standards or concerns.

In a more recent study, the HV1 segment of the mtDNA control region was sequenced using the pyrosequencing-based 454 GS Junior instrument from Roche Applied Science (Indianapolis, IN) [46]. Mock mixtures were employed to evaluate the ability of the 454 method to deconvolute variant components and to reliably detect heteroplasmy. Amplicon sequencing was performed on full-length HV1 amplicons (approximately 400 bp). The amplification primers included multiplex identifier (MID) sequences to allow for multiplexing, and adaptor sequences for the three-enzyme pyrosequencing process. The 454 method uses a wash step to enhance removal of residual dNTPs and ATP, to assure longer read lengths. Sensitivity levels of the 454 instrument are maintained by reducing loss of template during the washing step between each addition of a new nucleotide, a process that is accomplished by confining the DNA template to a fixed micro-bead. A lens array is used to focus the generated light or luminescence from each well of the picotiter plate onto the chip of a CCD (charge-coupled device) camera. The CCD camera captures light and records it in a raw data output file, resulting in a series of pyrograms. Intensity of peaks in the pyrograms is proportional to the number of nucleotides incorporated during the sequencing events. Using this method on mock mixtures, the different contributors were detected down to a minor component ratio of 1:250, or 0.4% (40 minor variant copies with a coverage rate of 10,000 sequences), and could be identified down to a 1:1,000 ratio (0.1%) with expanded coverage.

The 454 method was also used to analyze 30 individuals from 25 different maternal lineages [46]. Low-level heteroplasmy was detected for 11 of the 25 lineages, a 44% rate of observed heteroplasmy. Minor component variants ranged from 0.33% to 20% of the total sequencing reads. When using the Sanger method on these same samples, only one sample exhibited heteroplasmy at a detectable level (the one sample with 20% heteroplasmy), equating to a 4% rate of observed heteroplasmy. The nucleotide positions where heteroplasmy was observed were consistent with mutational hotspots or sites where forensic polymorphisms and heteroplasmy have been observed in past studies [82,96]. Concerns regarding the reliability of the sequence data were at least partially addressed through reproducibility studies. Multiple samples were run in either duplicate or triplicate, with results confirming the positions of heteroplasmy, and at very similar minor component percentages. Therefore, polymerase-driven artifacts were ruled out as the source of the relatively high frequency of observed heteroplasmy. In addition, the coverage rates and total number of reads for all reported positions of heteroplasmy were high. In part, this was due to the fact that all reported instances of low-level heteroplasmy resulted from at least 40 reads of sequence (most with more than 100 reads), and with a balanced ratio of forward and reverse reads. These data and observations allowed for the development of initial standards for reporting low-level heteroplasmic variants in a forensic setting. Recommended reporting criteria include a requirement that low-level variants be reported only when at least 40 reads are generated, and when the ratio of forward to reverse reads is consistent with the total read ratio. For example, mixtures of 1:100, 1:250, 1:500, and 1:1,000 would require total coverage of at least 4,000, 10,000, 20,000, and 40,000 reads, respectively. This level of coverage is well within the capability of the 454 instruments.

The concept of resolving mtDNA mixtures is quite different than identifying low-level heteroplasmic variants. Depending on the mixture ratio of the two or more components, it may be difficult to identify which component goes with which contributor, especially when the ratios are closer to 1:1. Therefore, a phylogenetic approach may be necessary to identify which sequences from which amplicons are associated with the same individual. Algorithms can be developed to perform this type of assessment, although it will become increasing more difficult to tease apart the components when the individuals involved originate from the same related population group. As a precautionary step, it is currently prudent to restrict the reporting of mixture data using the 454 method to minor component ratios above 1:100, and when the ratio stays below 1:5. In addition, more work is necessary to address sequencing error rates for the 454 system, and how the components of mixtures can be effectively resolved. For example, the pyrosequencing approach results in poor resolution of homopolymeric sequences, and both PCR and sequencing artifacts require a filtering mechanism similar to that for STR stutter and spectral bleedthrough. In addition, chimeric sequences from jumping PCR are quite commonly observed and must be addressed before making the 454 method an operational system in forensic laboratories.

CONCLUSIONS

The most significant challenge for mtDNA analysis remains the high cost and low throughput for evidentiary samples. Many samples require some special handling of one form or another, whether it is accommodation for degradation via miniprimer sets, additional internal sequence replication to cover indels or length heteroplasmy on duplicate strands, extra scrutiny of site heteroplasmy, or management of minimal contamination that is detected in reagent blanks. The system is robust when a sample contains good-quality abundant DNA, but also works well for difficult samples when all possible extra steps for recovering a profile are applied. Cleaning and DNA extraction of samples are the most critical steps, and it is difficult to automate these steps without risking crosscontamination due to the high number of manipulations required for hairs and skeletal samples. Postextraction improvements in PCR amplification and sequencing, or the introduction of new methods that do not require abundant template for detection of variation at the level of fine discrimination, are desirable. Several methods that confront these challenges have been proposed, such as PCR multiplexing of additional hypervariable-region fragments either for mtDNA alone or in conjunction with STRs [9,98]. The advantage of these methods is that they recognize the level of challenge presented by the worst samples (the least common denominator) and could in theory be applied to all samples. Overall, the limitations are more profound for single shed hairs than for skeletal remains, because skeletal material is often virtually unlimited in any single case.

Significant growth of forensic mtDNA databases to serve as foundations for population statistics continues to be a priority. These datasets, such as the one exemplified by EMPOP, should be freely available online for all users and highly vetted for quality data, and they will continue to be newly developed around the world for the growing forensics community. There is need for a statistical method that appropriately weights a failure to exclude when a

forensic case contains both questioned and known samples with the same site heteroplasmy. A very good understanding of the mutation rates of most polymorphic human hypervariable sites has been gained over the last 20 years since the original *Forensic Science Review* article on the validation of forensic mtDNA [47]; simply rank-ordering polymorphic sites by their rate of change could be easily accomplished from published data [89] to determine an effective, realistic, and conservative multiplier or additive for current statistical applications.

Newly developed and forensically validated methods such as ESI-MS have great potential for high-throughput applications where samples are abundant, such as skeletal remains from mass graves, missing persons projects, mass disasters, and other situations where rapid reassociation of thousands of skeletal remains is desired. Of course, all such applications should be firmly grounded in an understanding of the mtDNA variation that is present in the populations to which they are applied; for example, a description and frequency estimates of common mtDNA types and any population substructuring. Next-generation sequencing is promising for the elucidation of mixture characteristics and heteroplasmy, as the high throughput with this system allows scrutiny of DNA in, effectively, thousands of single strands. Using appropriate computer algorithms, overlapping amplicons, and phylogenetic contexts, mixture deconvolution may for the first time become routine, expanding the pool of samples with degraded and insufficient nuclear DNA for mitochondrial DNA analysis.

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Her prior positions at the Armed Forces DNA Identification Laboratory (AFDIL) and The Bode Technology Group gave her extensive forensic DNA casework experience, especially with difficult and challenged biological samples. Ms. Holland has been involved with noteworthy cases such as the identification of remains from several plane crash disasters (US Air Flight 427, Alaska Air Flight 261, and American Airlines Flight 587) and the identification of the Vietnam Tomb of the Unknown Soldier. She has participated in DNA casework for the International Commission on Missing Persons, National Center for Missing and Exploited Children, and the Innocence Project. She was also a project leader for the identification of the victims of the 9/11 World Trade Center terrorist attacks.

Mitchell Holland received a B.S. in chemistry from Hobart College (Geneva, NY) and a Ph.D. in biochemistry from the University of Maryland (College Park, MD), and did postdoctoral work in human genetics at Johns Hopkins Hospital (Baltimore, MD). Dr. Holland is the director of the Forensic Science Program at The Pennsylvania State University located in State College, Pennsylvania.

Dr. Holland teaches courses in forensic molecular biology and leads a research group focused on detection and reporting of mtDNA heteroplasmy using next-generation DNA sequencing platforms. Prior to being asked to help develop the Forensic Science Program at Penn State in 2005, Dr. Holland was the senior vice president for operations and laboratory director of the Bode Technology Group (Springfield, VA). From 1991–2000, he held various positions at the Armed Forces DNA Identification Laboratory (Rockville, MD), including scientific laboratory director from 1993–2000. A recent publication in the *Croatia Medical Journal* highlighted his work on the use of deep pyrosequencing for the detection of low-level mtDNA heteroplasmy.