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Forensic Mitochondrial DNA Analysis of 691 Casework Hairs*

ABSTRACT: A five year retrospective review of mitochondrial DNA (mtDNA) analysis on 691 casework hairs was carried out. A full or partial mtDNA profile was obtained for >92% of hairs. With increasing age of the hair, the likelihood of obtaining a full profile decreased, although "mini-primer sets" could often be used to capture a partial profile. With increasing color and diameter of the hair, the likelihood of obtaining a profile increased. Full or partial profiles were obtained on more than 80% of 114 hairs ≤1.0 cm. Mixtures were observed in 8.7% of hairs tested; mixtures increased with the age of the hair and were presumed to be due to exterior surface contamination that could not be sufficiently cleaned prior to extraction, since the overall level of laboratory contamination was low. The frequency of sequence heteroplasmy was 11.4%, and both hot-spot and novel sites were observed. In about one-third of these observations, another sample in the case showed either the same heteroplasmic site or a nucleotide substitution at that site.

KEYWORDS: forensic science, DNA typing, mitochondrial DNA, hair analysis, DNA sequencing, heteroplasmy

Mitochondrial DNA analysis is frequently applied to probative crime scene hairs. The science is no longer new, and the number of forensic cases in which it has been applied since its courtroom introduction in *Tennessee v. Ware* (1) in 1996 measures in the hundreds, if not thousands. Over nearly five years, we have amassed data to evaluate variables that may affect the outcome of mtDNA analysis such as hair age, size, condition, and type of submission on a slide or in a paper fold. These data will aid the forensic community in evaluating its current protocols to increase the frequency with which partial and full profiles are obtained in casework. Interest in the biological underpinnings of sequence heteroplasmy has led to our record-keeping of the location and frequency of heteroplasmic sites. Ultimately, a thorough characterization of mutation rates for specific sites may lead to an ability to place a numerical significance on a match where both questioned and known samples display the same heteroplasmic condition.

This laboratory has collected data on mtDNA analysis of 691 casework hairs that were analyzed from February 1999 through December 2003. Case folders were reviewed for questioned and known hairs that were submitted for mtDNA testing after a microscopic evaluation or an STR analysis of the hair root, if any, indicated that nuclear DNA analysis would be inappropriate.

Divisibility of a questioned hair can become a contested issue in forensic mtDNA testing. One goal in forensic mtDNA analysis, as in all forensic testing, is to optimize the probability of obtaining a profile while preserving as much sample as possible for re-analysis if necessary or requested. Each hair is evaluated independently, but in general, if a hair is 3–6 cm (cm) long, up to one-half of the hair is taken for testing leaving a similar sized portion for re-

testing. Hairs under 2 cm are generally consumed. A hair greater than 6 cm in length will have a 2–4 cm portion taken for testing; this determination is made based on the age, condition, and appearance of an individual hair.

The following parameters were of interest in reviewing data on 691 hairs tested using the above protocols: 1) the frequency of obtaining a full or partial mtDNA profile from a hair with respect to age, size, qualitative description, and form of submission (slide-mounted or loose in an envelope or paper fold), 2) the frequency that a submitted hair was consumed in testing, 3) the frequency of mixtures and their correlation with age, condition, and form of submission, 4) the frequency and location of sequence heteroplasmy, and 5) the frequency of cases where multiple samples within a case displayed the same heteroplasmic condition. All frequencies are displayed in the accompanying figures as frequency ±2 s.e., assuming a binomial distribution.

Definitions for some of the above variables are necessary. "Age" of the hair is defined as the date of mtDNA analysis of the hair minus the date of collection of the hair. A date of collection, or at least a crime date, is usually available in the documentation that accompanies the sample at submission. Of 691 hairs, a date of collection could not be estimated for 8 hairs (1.16%). A caveat accompanying this definition is that date of collection may not be the date the hair was shed from its donor. Therefore the age is a minimum estimate of time since the root of the hair became non-living biological material; obviously a shaft portion would be even older. A "full mtDNA profile" encompasses DNA sequence data for nucleotide positions 15998-16400 (hypervariable region 1; HV1) and 30–407 (hypervariable region 2; HV2). A "partial mtDNA profile" is defined as any portion of the above regions and may include all or part of HV1 and HV2 up to a full profile.

A "mixture" is defined as the presence of two or more nucleotide positions that display different nucleotides within a mtDNA profile. "Sequence heteroplasmy" is defined as the presence of a single nucleotide position that displays different nucleotides within a mtDNA profile (2). In both situations, the mixed bases are observed on both light and heavy strands or in multiple PCR products on a

background with low to no sequence artifact. Where a mixture occurs, the linkage phase of the mixed templates cannot be resolved, and interpretation is severely limited. Many caveats apply to the handling of mtDNA mixtures. A mixture of mtDNA templates from two different individuals cannot technically be distinguished from heteroplasmy, which is defined as the presence of two different mtDNA templates within a single individual. However, the average number of nucleotide differences between two randomly chosen individuals is much greater than one (3,4) and studies of heteroplasmy in freshly collected hair and blood samples using the most sensitive heteroplasmy detection methods have shown that sequence heteroplasmy is observed predominantly at a single position in the mtDNA control region (5,6). Therefore, these definitions are not arbitrary, but are based on published scientific observations.

Methods

All hairs undergo individual laboratory analysis. Laboratory protocols begin with a microscopic inspection for debris or stains adhering to the hair. Hairs mounted on glass slides are removed using xylene or xylene substitute. All hairs are cleaned in at least three 20-minute ultrasonic water bath cycles using freshly prepared 5% (w/v) Terg-a-zyme (Alconox, New York, NY) in each cycle. After thorough rinsing, the hairs are again inspected microscopically for adhering contaminants and then extracted by a mini glass grinder and phenyl/chloroform/isoamyl alcohol (PCIA) protocol (7). Single-use glass grinders (Kontes Glass, Vineland, NJ) are cleaned twice with sulfuric acid, rinsed thoroughly with tissue culture water, microwaved to dry, and exposed to ultraviolet light on all surfaces. Similar cleaning methods are applied in the preparation of all reagents and equipment, as is customary in mtDNA analysis (8). An extraction reagent blank negative control accompanies the hair extraction throughout the entire analysis.

PCR amplification is carried out using 10–15 μL of hair extraction product in a 50 μL reaction containing 1 μL of each primer (10 uM), 2 μL of dNTPs (10 mM) (Amersham Biosciences, Piscataway, NJ), 0.25 μL Amplitaq Gold, 5 μL Taq 10X buffer, 4 ul MgCl$_2$ (all from Applied Biosystems, Foster City, CA), and 2 ul BSA (bovine serum albumin, Sigma Aldrich, St. Louis, MO), brought to volume with sterile tissue culture water. Positive (HL-60; American Type Culture Collection, Manassas, VA) and negative PCR controls accompany the sample and its reagent blank extraction negative through sequencing. Thermal cycling conditions generally include a 12 min 95°C hot start followed by 36–40 cycles of 15 s at 95°C, 30 s at 56°C, and 45 s at 72°C, ending in a 4°C hold. Amplicon yield is evaluated by running 10 μL of PCR product on a 1–2% SeaKem agarose gel (Biowhittaker Molecular Applications, Rockland, ME). In general, primer pairs used on hairs follow those previously published (9) which amplify hypervariable regions 1 and 2 in two overlapping segments each. Additional primer pairs, called "mini-primer sets", are used to capture mtDNA in highly degraded samples when the standard approach yields no amplified product (10). PCR products are cleaned using Rapid PCR Purification Systems (Marligen Biosciences, Philadelphia, PA) according to the manufacturer's instructions.

Cycle sequencing of both strands is carried out according to the manufacturer's instructions using the Big Dye® Terminator Cycle Sequencing Kit (v1.0 or v1.1) (Applied Biosystems, Foster City, CA) with either the PCR primers or internal primers. Cycle sequencing products are purified using Performa DTR Gel Filtration Cartridges (Edge Biosystems, Gaithersburg, MD). Samples are electrophoresed on an Applied Biosystems 310 Genetic Analyzer (Applied Biosystems, Foster City, CA) and the resulting

DNA sequence data are analyzed by two qualified examiners using the LASERGENE SeqEd software package (DNASTAR, Madison, WI). Protocols may be obtained by contacting the authors.

Results

Between February 1999 and December 2003, 691 hair samples were extracted, amplified, and sequenced. Of these, 593 were questioned hairs (85.8%) and 98 were known reference hairs (14.2%). Reference hairs were generally submitted when known saliva, buccal, or blood samples were unobtainable, and within most cases, the reference hairs were the same age as the questioned hairs. Full mtDNA profiles were obtained on 570 hairs (82.5%), while partial profiles were obtained on 71 hairs (10.3%) and no profile was obtained on 50 hairs (7.2%). Overall, at least partial data was obtained on 92.8% of analyzed hairs. Figure 1 shows the distribution of profiles among hairs categorized by age (0–5 years old, 6–10 years old, 11–20 years old, 21+ years old). The oldest hairs tested were 37 years old. Of the 71 hairs for which partial profiles were obtained, 11 (15.5%) produced data on part of HV1, 45 (63.4%) produced data on all of HV1 and none of HV2, 13 (18.3%) produced data on HV1 and part of HV2, and 2 (2.8%) produced data on HV2 only. Most partial profiles were obtained using mini-primer sets because the extracted mtDNA was too degraded to amplify products 200–300 base pairs long.

Of 691 hairs, 262 were completely consumed in analysis (37.9%) while 425 (61.5%) were not consumed, leaving an additional portion of the hair for further testing. Data on consumption were not available for 4 hairs (0.6%). The length of the submitted hairs ranged from 0.2 cm to 40 cm, while the average length of the submitted hairs was 4.8 cm ± 4.8 cm; 23.1% were ≥6 cm. On review of the case folders, 22 hairs were simply described as "long", while the lengths of 12 hairs were unknown. The length of the portion of the hair used for testing ranged from 0.2 cm to 4.6 cm, while the average portion taken for testing was 1.9 cm ± 0.83 cm. A portion as long as 4.6 cm might be taken when difficulty in obtaining a profile was experienced with similar-appearing hairs in the same case.

Of interest was whether differences in the size of the analyzed hair fragment were correlated with obtaining a profile. Figure 2 shows the frequency of full, partial and no profiles in tested hair fragments sized either 1.0–1.9 cm long or 2.0–2.9 cm long. Of the total of 691 hairs, 216 (31.3%) analyzed hairs were 1.0 through 1.9 cm in length while 298 (43.1%) analyzed hairs were 2.0 through 2.9 cm in length. The frequency of full, partial, and no profiles is approximately equal across both categories. When examining the frequency of obtaining profiles from the oldest hairs (21+ years), however, Figure 3 shows that a full or partial profile may be more recoverable by using a larger portion of the hair.

Small probative hairs present a challenge to the hair microscopist and frequently are not evaluated microscopically, but may be amenable to mtDNA analysis. Hairs ≤1.0 cm in length were often submitted (N = 114, 16.5%), while submitted hairs within this category that were ≤0.5 cm comprised 2.9% of the total hairs tested (N = 20). Figure 4 shows the frequency of full, partial, and no profiles in these small hairs compared to all hairs. In general, the likelihood of obtaining a full profile from smaller hairs is lower than in larger hairs. However, partial profiles are readily recoverable from a large proportion of these hairs.

Slide-mounted hairs (Permount, nail polish, and other mounting media) comprised 366 (53.0%) of the samples, while hairs submitted in zip lock bags, paper or wax folds, coin envelopes, or on Post-it Notes comprised 325 (47.0%) of the samples. Figure 5 shows the frequency of full, partial, and no profiles depending on the type of
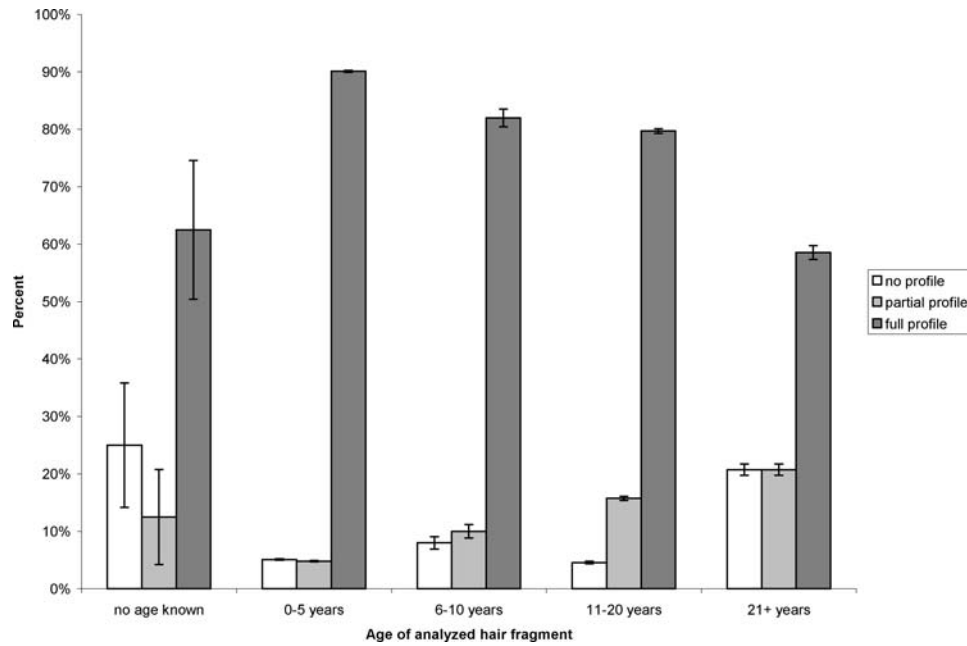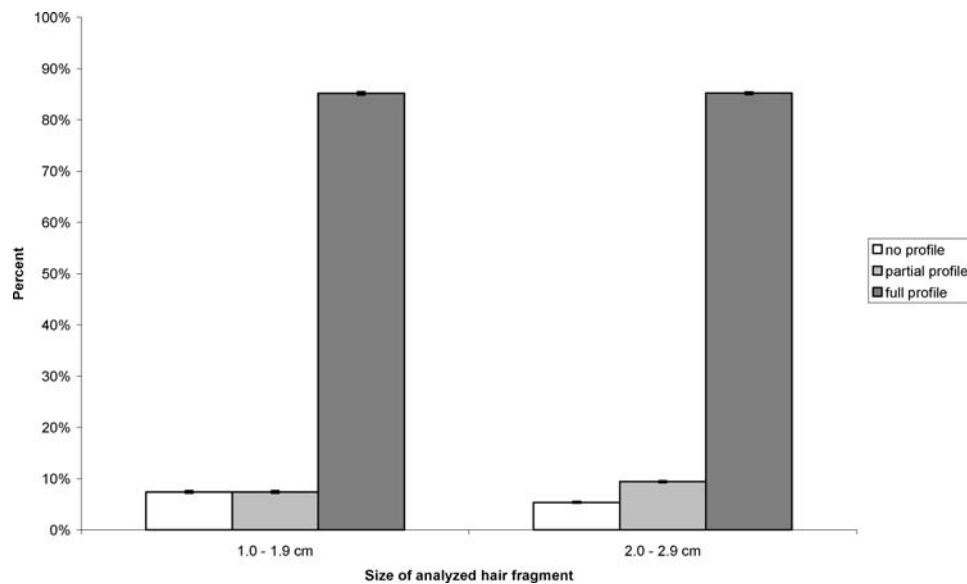
FIG. 1—*Profile obtained relative to age of hair.*

FIG. 2—*Profile obtained relative to size of hair.*

submission. These data indicate that the type of submission does not play a role in the ability to recover a profile.

Mixtures were observed in 60 hairs (8.7%). The presence of a mixture in a hair signals the presence of DNA that has been introduced from an exogenous source either prior to testing or during the testing process. Prior to testing, the source is likely to be DNA adhering to the external surface of the hair that cannot be adequately removed during the cleaning process. When the reagent blank negative control is free of DNA, an external surface contaminant is likely to be the explanation for a mixture. Although data from this laboratory is not specifically available for the frequency of reagent blank negative control contamination in hair testing, our frequency in all casework that does not involve mini-primer sets has been less than 1.8% annually while the frequency in casework involving mini-primer sets has been less than 3.8% annually. Therefore,

an explanation for mixtures in hairs due to reagent or equipment contamination is not highly supported. The frequency of mixtures increased with the age of the hair: mixtures were observed in 2.8% of the 0–5 year old hairs, 6.0% of the 6–10 year old hairs, 14.2% of the 11–20 year old hairs, and 20.7% of the 21+ year old hairs. Of the 60 hairs displaying mixtures, 28 (46.7%) were slide-mounted and 32 (53.3%) were loose in a bag, paper fold, envelope, or Post-it.

Appearance and condition of an analyzed hair can be recorded only subjectively. While occasionally hairs arrived for testing with descriptions provided by microscopists, such as "pubic hair", "head hair", or "body hair", in general these classifications were unknown. For each hair, therefore, a brief description was usually recorded by the laboratory technician prior to extraction. Of 691 hairs, 661 (95.7%) were described in the laboratory notes. These descriptions encompassed characteristics such as pigmentation and
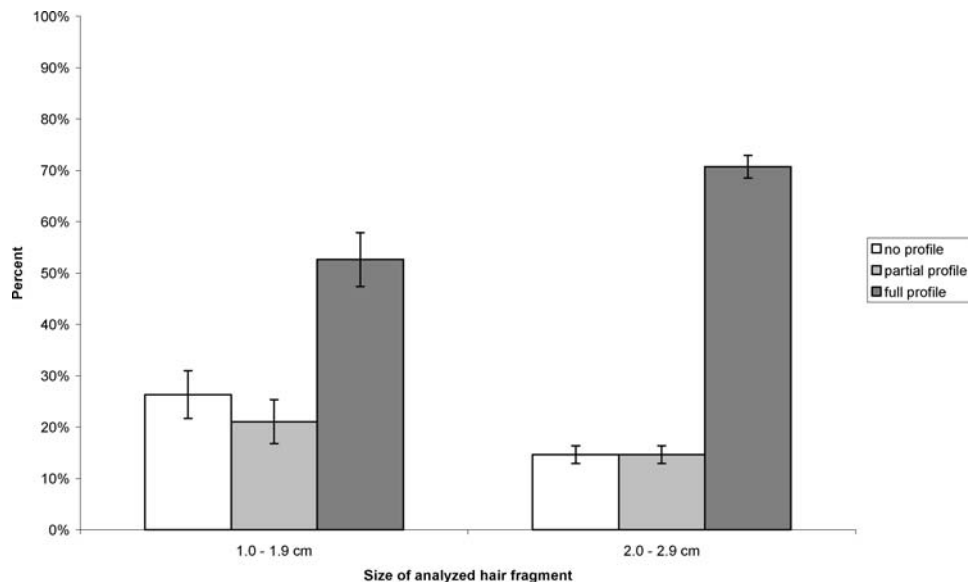
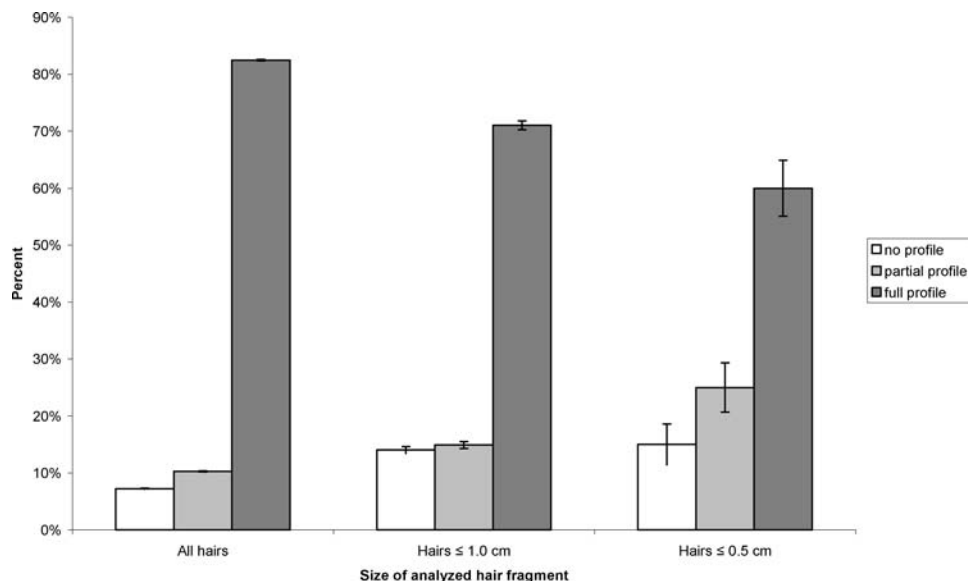FIG. 3—*Profile obtained relative to size of hair in 21+ year old hairs.*

FIG. 4—*Profile obtained in small hairs.*

diameter. While the descriptions varied widely, hairs could be assigned to one of five categories according to a quality we termed "robustness". Table 1 shows examples of this qualitative classification scheme that was used prior to assessing the frequency of obtaining profiles within each classification. Figure 6 shows the frequency of obtaining full, partial, or no mtDNA profiles with respect to the qualitative description that was recorded at the time of extraction.

The presence and location of sequence heteroplasmy was recorded for every hair. Length heteroplasmy, a common biological phenomenon (11,12), was also recorded and will be the topic of a separate paper. Sequence heteroplasmy was observed 85 times in 691 hair samples. However, an estimate of the frequency of hair heteroplasmy in casework must take into account the fact that some individuals have the same heteroplasmic condition throughout their tissues; the casework estimate must therefore rely on the frequency of independent observations in unrelated hairs. A frequency of

11.4% ($N = 78$; 78/684) of heteroplasmy in hairs was observed in casework after subtracting the number of hairs that displayed heteroplasmy at an identical position to another matched sample within a case ($N = 7$) since these samples cannot represent independent events. Table 2 shows the heteroplasmic sites observed in casework hairs from February 1999 through December 2003, and the relationships among samples within the specific case associated with each observation.

Sequence heteroplasmy was observed more than one time at nucleotide positions that are well-recognized mutational "hot spots" (13–15). Those sites occurring more than once were 152 ($N = 2$), 189 ($N = 2$), 215 ($N = 4$), 234 ($N = 3$), 16093 ($N = 14$), 16095 ($N = 2$), 16129 ($N = 2$), 16189 (T/C; $N = 2$), 16274 ($N = 2$), 16293 ($N = 2$), and 16327 ($N = 3$). Of the 78 hairs comprising the independent observations of heteroplasmy ("proband" hairs), 39 could be paired with matching mtDNA profiles observed within the case in another sample such as hair, blood, or buccal samples.
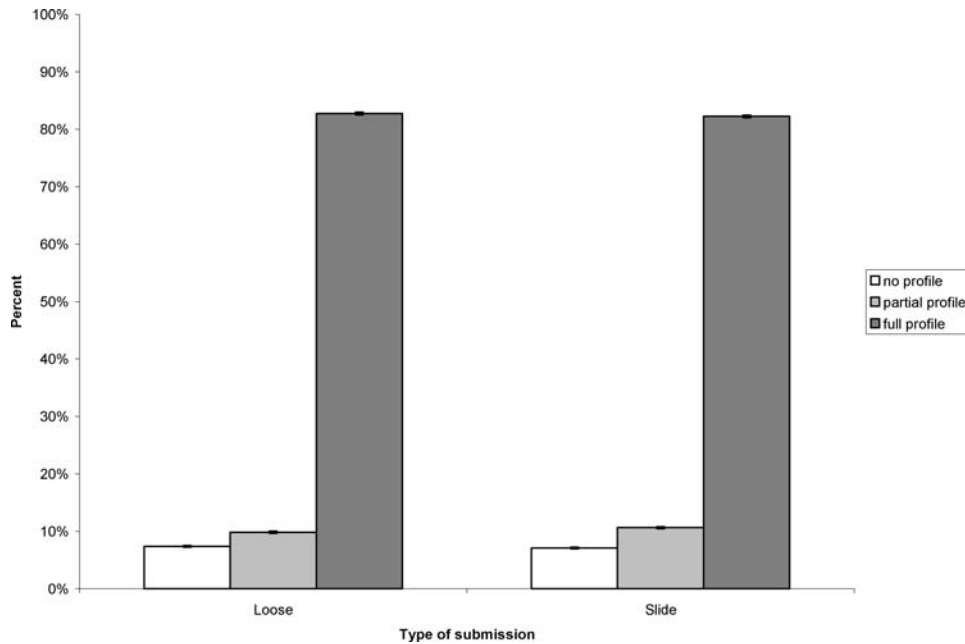
FIG. 5—*Profile relative to type of submission.*

TABLE 1—*Classification of hair by appearance and condition.*

	Size of analyzed fragments in category, mean ± s.d.	Examples of descriptors in five categories
Less Robust ↓ ↓ More Robust	2.11 ± 0.83 cm	Extremely pale, pale brown thin, very thin, thin blond, translucent, transparent, very fine, brittle, unpigmented, white
	2.35 ± 0.99 cm	Blond, light brown, thin, pale, reddish blond, reddish brown, light brown to blond, brown very thin, brown brittle
	1.96 ± 0.90 cm	Brown, wavy, amber, auburn, curved, medium brown, golden brown, dark blond, curly, light brown thick, light brown wavy
	1.88 ± 0.71 cm	Dark brown thin, dark brown flat, brown curly, black thin, glossy red, dark straight, dark tapering, dark brown flat
	1.63 ± 0.60 cm	Dark robust, very dark thick wiry, black flat thick, dark brown to black, red robust, dark brown curled, reddish brown thick, black curly wiry

For these 39 proband hair samples, the matched samples in 25 cases displayed the Cambridge Reference Sequence (CRS) at the site that was heteroplasmic in the proband sample, and the matched samples in 14 cases displayed either heteroplasmy or a substitution from the CRS at the site that was heteroplasmic in the proband sample.

Discussion

A high probability (92.8%) of obtaining a full or partial profile was observed using the protocols reported here. On reviewing our files, we determined that hairs from which no profile was obtained often had a history of extreme environmental exposure. Hair masses exposed to the environment in missing persons cases are rarely successful in analysis when the mass has been recovered after months or years. For this reason we recommend that skeletal material be used preferentially in a body identification even if hair is available. In some other failed analyses, a microscopic examination may have indicated that the hair might be non-human or even synthetic. Animal hairs, hair-weave hairs, and make-up brush hairs are examples of hairs that may be submitted with an erroneous microscopic evaluation. Although a microscopic evaluation and photography may be carried out prior to submission for DNA analysis, these processes may not occur.

Time takes its toll on mtDNA just as it does with nuclear DNA, as shown by the declining ability to obtain a profile with increasing hair age. Of interest, however, was that partial and full profiles could be obtained from hairs in the 21+ year old category almost 80% of the time. Among 12 hairs aged 30 years or greater, 4 gave full profiles, 3 gave partial profiles, and 5 gave no profiles. Of three 37-year-old hairs, one gave a full profile, one gave a partial profile, and one gave no profile. Eventually, a larger sample size for older hairs will reveal a possible age limit for successful analysis of these samples.

Early recommendations regarding an optimal size of hair for mtDNA analysis settled on approximately 2 cm (7). We were interested in whether this size was arbitrary or whether a different size fragment should be routinely used. This data review shows that not much difference in the ability to obtain a profile was observed when comparing hairs in the 1.0–1.9 cm category to those in the 2.0–2.9 cm category. We observed, however, that the oldest hairs may benefit from using a larger piece in testing. From a biological perspective, degradation over time reduces the total amount of mtDNA in a sample; this decline in older hairs can be counterbalanced by the extraction of more sample.

Hairs sized 1.0 cm or less are often forensically probative, such as when they are broken off under a victim's fingernails or shed from a suspect or victim with either extremely short hair or a shaved head. There is a high probability of obtaining full and partial profiles from these hairs (86.0%), including hairs as small as 0.2 cm. MtDNA analysis may be the only analysis available for these samples, since they are often too small to be examined microscopically. There was no increase in mixtures in these samples, with a frequency of mixtures in hairs sized ≤1.0 cm of 7.0% and in hairs sized ≤0.5 cm of 0%. No hair under 0.5 cm (N = 7) failed to give a mtDNA profile while 3 hairs sized 0.5 cm (N = 13) failed to give

FIG. 6—*Profile obtained relative to qualitative description.*

TABLE 2—*Seventy-eight independent observations of sequence heteroplasmy in 691 hairs.*

Heteroplasmy Position*	Other Matched K or Q Sample in Case for Comparison?	Nucleotide Present in Comparison Sample
70†	7 Q hairs	CRS in all 7
94	3 Q and 1 K hairs	CRS in all 4
120†	No	. . .
150	No	. . .
152	No	. . .
152	2 Q hairs and 1 K hair	All 3 hairs have T/C heteroplasmy
185	1 Q hair and 1 K blood	Both have A (substitution from CRS)
189	No	. . .
189	1 K blood	CRS
195	5 Q hairs	CRS in all 5
200	4 Q hairs, 1 K hair, 1 K blood	CRS in all 6
204	1 K blood	CRS
213†	1 K buccal	CRS
214	1 Q hair and 1 K blood	Both have A/G heteroplasmy
215	No	. . .
215	No	. . .
215	No	. . .
215	6 Q hairs	CRS in all 6
234	No	. . .
234	No	. . .
234	1 Q hair and 1 K blood	CRS in both
240	No	. . .
249 del	1 Q hair	CRS
279 T/G	No	. . .
313†	4 Q hairs	CRS in all 4
316	No	. . .
323†	No	. . .
16034†	7 Q hairs	CRS in all 7
16035†	No	. . .
16075	1 K blood	Blood has T/C heteroplasmy
16092	1 Q hair and 1 K blood	Hair has C (substitution from CRS) and blood has T/C heteroplasmy
16093	No	. . .
16093	No	. . .
16093	No	. . .
16093	No	. . .
16093	No	. . .
16093	No	. . .
16093	No	. . .

TABLE 2—*Continued.*

Heteroplasmy Position[*]	Other Matched K or Q Sample in Case for Comparison?	Nucleotide Present in Comparison Sample
16093	No	...
16093	No	...
16093	1 Q hair	Hair has T/C heteroplasmy
16093	1 K blood	Blood has C (substitution from CRS)
16093	1 K blood	CRS
16093	1 Q hair and 1 K blood	Hair has T/C heteroplasmy and blood has C (substitution from CRS)
16093	1 K blood	Blood has T/C heteroplasmy
16095	No	...
16095	6 Q hairs	CRS in all 6
16115[†]	3 Q hairs and 1 K hair	CRS in all 4
16129	No	...
16129	2 K bloods	CRS in both
16155	No	...
16166	1 K blood	CRS
16167	1 Q hair and 1 K blood	CRS in both
16189	No	...
16189	No	...
16189 T/A	No	...
16248 C/A	No	...
16250	No	...
16251[†]	1 Q hair	CRS
16266	1 K blood	CRS
16274	No	...
16274	1 K blood	Blood has G/A heteroplasmy
16290	No	...
16293	No	...
16293	4 Q hairs and 1 K blood	CRS in all 5
16294	1 Q hair and 1 K hair	Both have T (substitution from CRS)
16298	1 K blood	Blood has C (substitution from CRS)
16302	No	...
16303	No	...
16317	No	...
16320	1 Q hair	Hair has T (substitution from CRS)
16327	No	...
16327	No	...
16327	1 Q hair and 1 K blood	CRS in both
16344	1 K blood	CRS
16355	1 Q hair, 1 K hair, 1 K buccal	CRS in all 3
16390	3 Q hairs and 1 K blood	CRS in all 4
16399	3 Q hairs and 1 K hair	K hair has A/G heteroplasmy, 1 Q hair has G (substitution from CRS), other 2 Q hairs have CRS

[*]All heteroplasmic sites are transition types (T/C or A/G) unless otherwise noted.
[†]Novel sites with respect to MITOMAP (19) and SWGDAM database (20).
Abbreviations: T = thymine, C = cytosine, A = adenine, G = guanine, del = deletion, CRS = Cambridge Reference Sequence, Q = questioned, K = known.

a profile. Obviously, given a measurable failure rate in all hairs, longer hairs present identical challenges with respect to degradation, and based on our experience we expect the challenges to be the same regardless of size.

The likelihood of obtaining a mixture profile in hairs appears to increase with age. In spite of routinely applied and validated exhaustive cleaning methods, this unfortunate outcome is occasionally inevitable. Historically, many older hairs were handled with bare hands by laboratory personnel prior to the availability of DNA testing. Surprisingly, whether a hair is slide-mounted or loose does not seem to predict the likelihood of mixtures. One might suspect that mounting medium may render a hair more likely to collect epithelial, blood, or sperm cells and less likely to be cleaned sufficiently. We have not seen this to be the case. We have also observed that a repeat analysis of a hair from which a mixture has been obtained is rarely successful when more washes and rinses are applied to an additional fragment of the hair, and in fact another mixture (occasionally different from the first) will be obtained.

Of interest was the observation that hair "robustness" was positively associated with the likelihood of obtaining a profile. While this result has been observed empirically by mtDNA analysts for years, to our knowledge the data shown here are the first to place some numerical values on these likelihoods. Although subjective, the descriptions collected at the time of analysis were helpful in identifying some characteristics that might predict success, such as color and size. In general, darker and larger-diameter hairs, and hairs described by outside microscopists as pubic hairs, were more likely to give profiles. When inspecting the mean length of the fragment taken for testing in each category, we note that with the exception of the first two categories, the size fragment taken for testing decreased as the category became more robust, indicating that the analyst was predicting success *a priori* in more robust dark hairs and taking smaller pieces for extraction. Nevertheless, the success rate was still higher in these groups even though the size fragment was smaller on average. This information will assist mtDNA analysts in choosing fragments for testing and preserving

evidentiary hairs when consumption of an entire submitted hair may not be necessary. Noting that over one-third of submitted hairs were consumed in mtDNA analysis, any protocol modification that conserves evidence for re-testing if necessary is welcome.

Previous studies have reported that DNA from highly melaninized hairs is difficult to amplify due to PCR inhibition (16). On the contrary, we have observed that the most melaninized hairs routinely provide abundant mtDNA and do not show any PCR inhibition. If melanin is a factor, the PCIA extraction, which includes a column clean up, may successfully remove it.

Since a recently published report on hair microscopy showed that paired microscopic evaluations and mitochondrial DNA examinations are inconsistent approximately 10% of the time (17), the need to perform mtDNA analysis in conjunction with microscopic hair analysis has never been greater. We have observed cases where the microscopic evaluation was discordant with respect to the mtDNA analysis, however, we have observed many cases in which the microscopic evaluation was concordant with respect to the mtDNA analysis. In these cases, a microscopic evaluation performed by an experienced hair examiner was extremely useful in limiting the number of hairs which were then recommended for DNA testing. Therefore, we advocate hair microscopy as an adjunct to DNA testing, if the examiner is experienced and understands the limitations of this largely descriptive science. Because of the high cost of mtDNA analysis, it is likely that hair microscopy will long be a useful tool for screening of large numbers of hairs prior to submission and we urge the continued training and availability of hair examiners to aid the DNA testing community.

The frequency of sequence heteroplasmy in hairs in casework in this study is 11.4% and approximates that of 9.7% in a previous report from this laboratory (18). As reported elsewhere, our observation that sequence heteroplasmy at position 16093 is common supports a theory that this site is operating under forces of natural selection (6). It is possible that the high frequency of the 16093 mutation will lead to revised interpretational guidelines for cases where it is present as a substitution. For example, a case where two mtDNA profiles are identical with the exception of a difference at 16093 may no longer be considered an inconclusive result. Other "hot spots" were observed, but also a large number of less frequent heteroplasmic sites were recorded, including some never reported as variant positions in MITOMAP (19) or the SWGDAM database (20). These novel sites, which were clearly observed on either two or four strands in the absence of any notable sequence background, are 70, 120, 213, 313, and 323 in HV2, and 16034, 16035, 16115, and 16251 in HV1. By their presence on at least two sequenced strands, we may rule out sequencing artifact as an explanation for seven of these observations, and by their presence in at least two PCR products we may rule out a PCR artifact for the other two. A tenth substitution, 16317, has been reported in the SWGDAM database in three individuals but not MITOMAP. All other heteroplasmic sites have previously been reported.

The data presented here summarize five years of the mtDNA analysis of human hairs. This population of hairs is more highly representative of the range of conditions that may challenge a laboratory's protocols than any validation study created to simulate extremes of testing. Retrospectively, the ability to obtain profiles in the vast majority of cases shows that the protocols are valid and efficient. Combined with the application of quality controls, quality assurance, validation studies, and proficiency testing, we believe that a laboratory using these or similar protocols will be providing optimal services to the forensic community.

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