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# Mitochondrial DNA Amplification Success Rate as a Function of Hair Morphology

**ABSTRACT:** This study examines the amplification success rate of mitochondrial DNA from human head hair with respect to their potential for forensic application. Mitochondrial DNA was isolated using a Chelex-based extraction method and amplified using the LINEAR ARRAY™ duplex PCR system. The particular focus of this study was to characterize the morphological features of human head hair in order to further the understanding of the factors that influence amplification success rate in hair tissue using the LINEAR ARRAY™ duplex PCR system. 2554 head hairs from 132 individuals representing four population groups were amplified. The hair samples were characterized as follows: 1251 were identified microscopically as telogen hairs and 1303 were classified as hairs without roots (removed before extraction). Amplification success was assessed as a function of several independent variables: morphological characteristics; telogen root versus no root; donor age; scalp origin; use of cosmetic hair treatments; and race of the donor. The results show that a positive correlation exists between amplification success and the presence of a telogen root. Combining the amplification success with either the original or optimized protocol, telogen hairs result in an overall success rate of 77.5% compared with 65% for hairs with no roots. Controlling for telogen hairs, the findings indicate that the overall success rate is independent of cosmetic hair treatments; medulla structure; shaft length, diameter, and volume; and scalp origin. Conversely, the age of the donor, the race of the donor, and hair pigmentation all contribute to a variation in amplification success rate.

**KEYWORDS:** forensic science, mitochondrial DNA, amplification success rate, hair morphology

The human scalp contains an estimated 100,000–150,000 hair follicles in three stages of growth. In the anagen phase (hair actively growing), the germinal cells within the bulb matrix undergo rapid self-proliferation, requiring high energy. Thus, it is not surprising that this area, particularly in the region proximal to the follicles, has an abundance of mitochondria; each germinal cell within the bulb matrix contains *c*. 1000 mitochondrial DNA (mtDNA) molecules. Plucked hairs are mostly in the anagen phase and they generally have follicular material adhering to their surface. Both the root bulb and the follicular tissue provide good sources of mtDNA and nuclear DNA (nuDNA) in anagen hairs (1–6).

In addition, the hair bulb contains melanocytes, the cells that impart color to the hair shaft. Melanocytes themselves remain within the root bulb, although, as they mature, they form cytoplasmic "dendritic" processes that contain mitochondria and melanosomes (contain the hair pigments). Hair color is due to a combination of two biochemical pigments: eumelanins contribute dark brown/black pigments whereas pheomelanins are associated with red/yellow pigments (6). Precortical cells are believed to pick up the mitochondria and melanosomes as they pass from the root bulb to the hair shaft, undergoing differentiation to cortical cells in the process (7). These cortical cells begin synthesizing keratin shortly afterward, which requires mitochondria. Thus, as reported by Linch, cortical cells in the hair shaft may contain two sources of mtDNA: one originating from the germinal bulb matrix cells and one derived from the melanocytes (8). Once the hair enters the catagen phase, hair growth gradually ceases as mitosis activity in the matrix cells terminates. This process can last several weeks before making a transition to the dormant telogen phase.

Hair analysis is an integral part of many forensic investigations, mainly as associative evidence. Primarily, the microscopic characteristics of a representative sample of a known source of hairs are compared with the questioned hair(s) (9). In particular, the following characteristics are recorded: the color, size, distribution, and pattern of the pigment granules within the cortical cells, the appearance of the medulla, hair shaft diameter, indications of chemical treatments, and the physical condition of the shaft (10). These observations are generally preceded by a macroscopical examination to record the three-dimensional configuration of the hair, overall length, texture, and the existence of trace material adhering to the hair shaft.

This type of morphological evaluation is not amenable to the "one-to-one" comparisons typically performed with other types of forensic evidence due to the intra-individual variation exhibited by head hairs. Further, many of the characteristics are distributed as a range rather than as discrete variables. Intra-sample variation makes it more difficult for the hair examiner to assign an association between known and unknown hairs, and, as a result, the approach is often criticized because of the inherent subjectivity involved. While there is general acceptance that inter-individual variation of microscopic characteristics greatly exceeds that of intra-individual variation, the possibility exists that hairs from two different individuals may be morphologically indistinguishable. Compounding this problem is the fact that hair examiners are unable to predict the expected frequency of this coincidental match. Further, it could be argued that the greater the intra-individual variation, the greater the potential for a coincidental match. Conversely, an individual's hair may change between samplings,

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which may lead to a false exclusion based on a morphological hair examination. Smith and Linch provide a review of the major factors contributing to errors in human hair association by microscopy (11).

PCR-based mtDNA technology provides a more objective method for performing hair comparisons. mtDNA analysis is particularly applicable to telogen hairs or hair fragments, which generally lack a nuclear genome (12,13). In addition, the application of microscopy and mtDNA to hair comparisons may provide complementary information. For example, maternally related individuals share the same mtDNA haplotype. Further, given the lower discrimination power of mtDNA relative to nuDNA, this possibility also exists for unrelated individuals; however, hairs from these individuals may be distinguished based on morphological features. Conversely, two hairs may be morphologically indistinguishable but may exhibit different haplotypes. Houck et al. (14) examined 170 hairs; 41.8% of the hairs were either unsuitable for microscopic analysis or gave inconclusive results, whereas 5.3% were unsuitable for mtDNA analysis or gave inconclusive results. The authors report that microscopic and mtDNA analysis of head hair exhibited concordance in 69 of 80 associations; of the remaining microscopic associations, nine were excluded by mtDNA analysis and two gave inconclusive or insufficient mtDNA results.

mtDNA analysis is valuable in a forensic context in the identification of human remains (15). Given the high copy number per cell, the mitochondrial genome is particularly suited to the genetic analysis of samples containing limited amounts of biological material. Insufficient sample may be attributed to small sample size, DNA degradation, or due to the absence of a nuclear genome. For example, mtDNA sequence information has been obtained from the ancient human remains of a 7000-year-old brain (16) and a 5000-year-old mummified body (7), teeth (18–21), skeletal fragments (22,23), dried tissue (24), and human feces (25). Several studies of mtDNA from human hair have also been performed (26–28). The aforementioned have all proven to be difficult samples when attempting to yield nuDNA markers. MtDNA was first introduced as evidence in *Tennessee v. Ware* (29) in 1996; it has now been applied in hundreds of cases. Several techniques for mtDNA analysis are currently in use, including DGGE, sequencing, pyrosequencing, and LINEAR ARRAY™ analysis. The latter technique, which uses a duplex amplification and a probe-based detection system, was used in the study reported here.

The present study was conducted in order to better understand whether hair morphology, donor demographics, or hair cosmetic treatments have any bearing on the potential of human head hair to amplify successfully. The following independent variables were examined as a function of amplification success rate: morphological characteristics; donor age and race; telogen root versus no root, location on scalp, cosmetic treatments (dyed, permanent chemical waving, semi-permanent waving, highlighting, straightening, etc.), and hair from living versus deceased donors. The research also explored several experimental parameters including variation in the PCR protocol and cycle number, and modification of the extraction procedure.

## Methods and Materials

### *Subjects*

Participants in this study were asked to self-collect 50 head hairs in order to ensure a sample that represented the natural morphological variations that can exist within individuals. Specifically, each donor was asked to collect 10 hairs from each of five regions on the scalp designated: front, center, rear, left, and right side. Of these 10 hairs, five were plucked (anagen) and the other five were obtained by combing (telogen) the respective areas. Participants were also asked to answer three demographic questions related to their age, gender, and race, and one relating to chemical treatment of their hair. (Collection of samples was in accordance with City University of New York IRB-approved protocol; all living subjects provided informed consent.)

In addition, postmortem hair samples were collected from decedents. Although information relating to chemical treatments was not available for the decedents, morphological observations of the hair using light microscopy provided a limited amount of information with regard to certain potential treatments.

A total of 2554 head hairs (132 individuals) were analyzed over a period of *c*. 8 months. Of these, 2433 hairs (95.3%) measured 2 cm in length and 121 hairs (4.7%) measured $<2$ cm in length (ranging from 0.8 to 1.5 cm, mean = 1.28 cm). Initially, subjects were accepted one at a time and assigned to a subgroup based on age range, gender, and race. In all, 64 males and 68 females were assigned. The age of the subjects ranged from 18 to 88 years, each falling into one of four age ranges. A summary of the distribution of population groups collected for this study indicates 44 Caucasian, 35 African American, 25 Asian, and 28 Hispanic individuals (Table 1). Three of the Caucasian individuals who participated in this study are natives of Canada, while one Caucasian is a native of the United Kingdom; the remaining living subjects were obtained by sampling individuals who resided in the southwest region of the United States at the time of collection. Further, the population group of all decedents was obtained directly from the autopsy report (Los Angeles County Coroner Office), although the national origin of the decedents was not known.

## Experimental Methods of Analysis

### *Photomicrography*

Representative hairs from each donor were observed microscopically. Hairs that exceeded 2 cm in length were preferentially

TABLE 1—*Sub-groups of sample demographics.*

| AGE | C Male | C Female | AA Male | AA* Female | A Male | A Female | H Male | H Female | Total |
|---|---|---|---|---|---|---|---|---|---|
| 18–29 | 5 | 10 | 5 | 5 | 9 | 6 | 6 | 8 | 54 |
| 30–49 | 7 | 5 | 5 | 8 | 5 | 5 | 6 | 5 | 46 |
| 50–69 | 7 | 5 | 3 | 5 | 0 | 0 | 3 | 0 | 23 |
| 70+ | 2 | 3 | 1 | 3 | 0 | 0 | 0 | 0 | 9 |
| TOTAL | 21 | 23 | 14 | 21 | 14 | 11 | 15 | 13 | 132 |

*Includes one individual who reported African American/other.
C, Caucasian; AA, African American; A, Asian; H, Hispanic.

selected as this was considered to be the minimal length required to obtain sufficient mtDNA as determined in an earlier study (12). Each hair was mounted (Cargille mineral oil $N_D$ at 23°C = 1.5150 ± 0.0002) and examined microscopically (X100, Olympus Model POS, Center Valley, PA). Photomicrographs were taken of each hair to record the root end in addition to representative regions along the shaft (35 mm Fugichrome Provia 100F RDP III 135 film, with 80B filter; Honeywell Pentax 35 mm SLR SP 500 with Asahi Pentax microscope adapter II). A 2 cm portion was cut from the proximal end of each telogen hair selected. In the case of anagen hairs, the root tissue was initially removed before cutting a 2 cm portion from the proximal end (henceforth referred to as hairs with no roots).

### *MtDNA Chelex Extraction of Hair*

In order to isolate the total genomic DNA from the hair shaft, each hair was washed to remove the slide mounting material (95% ethanol, followed by distilled/deionized water and dried). The hair was then cleaned by sonication in 500 µL 2% SDS (10 min). Each hair was rinsed (distilled/deionized water), inspected, and placed in a micro-tissue grinder. The grinders were pre-sterilized with 95% ethanol, then washed with a hot water detergent. They were rinsed with distilled/deionized water, soaked with 4 N $H_2SO_4$, rinsed again, and dried in a microwave oven before storage under UV light. TE buffer (150 µL) was added to each grinder and the hair was homogenized (Kontes Glass Company, Vineland, NJ). The homogenate was transferred to 50 µL 20% Chelex (BioRad, Hercules, CA). A preliminary study was performed to compare the efficiency of the Chelex extraction method (30) with and without the inclusion of an incubation step with Proteinase K digestion. The samples were vortexed (10 sec), centrifuged (10 sec at 10–15 k × *g*), and then boiled in a water bath (8 min). All samples were again vortexed (10 sec) and centrifuged (3 min at 10–15 K × g) before amplification.

## PCR Amplification Protocol

### *Procedure for Amplification of mtDNA*

A study of the amplification parameters was performed using the amplification reagents from the LINEAR ARRAY™ mtDNA HVI/HVII Region-Sequence Typing Kit. This research included modifications of the cycle parameters (34 vs. 38 cycles), the amount of primer incorporated (0.2 µM vs. 0.3 µM), and a comparison of the amplification success rates obtained with an optimized protocol. The volume of template mtDNA added to the reaction mixture was dependent on the tissue source (20 µL for hair extracts and 5 µL for the blood-positive control). The sequences for the biotinylated primers used to amplify the mtDNA products are: HVI: F15975-93B (5′-XCTCCACCATTAGCAC CCAA) R16418-01B (5′-XATTTCACGGAGGATGGTG) and HVII: F15-34B (5′-XCACCCTATTAACCACTCACG) R429-10B (5′-XCTGTTAAAAGTGCATACCGC) (31).

The amplification reaction was conducted using a Perkin-Elmer 2400 DNA Thermal Cycler (programmed with the PCR profile times and temperatures provided in Table 2). Either 34 or 38 cycles were performed for each PCR reaction, depending on the final PCR product yield (samples that failed to amplify or showed a low yield were amplified for an additional 4 cycles). A positive control (extracted blood sample), an extraction blank, and a PCR-negative control were run in the Thermal Cycler simultaneously with the research samples.

### *PCR Amplification Reaction: Original Protocol*

Template mtDNA was amplified in 60 µL reaction volumes containing 0.2 µM of each primer, 12 mM Tris-HCl, pH 8.3, 60 mM KCl, 2.4 mM $MgCl_2$, 0.2 mM each dinucleotide triphosphate (dNTP), and 0.25 U/µL Ampli*Taq* Gold® DNA Polymerase.

### *PCR Amplification Reaction: Optimized Protocol*

Template mtDNA was amplified in 50 µL reaction volumes containing 0.3 µM of each primer, 12 mM Tris-HCl, pH 8.3, 60 mM KCl, 2.4 mM $MgCl_2$, 0.2 mM each dNTP, and 0.25 U/µL Ampli*Taq* Gold® DNA Polymerase.

## Qualitative Analysis of the PCR Product

The amplified mtDNA product was analyzed by agarose gel electrophoresis on either a 2% product gel (2.0 g NuSieve GTG agarose in 100 mL 1 × TBE gel running buffer, run at 100 V for 30 min; stained with 0.5 µg/mL ethidium bromide) or a 3%/1% product gel (3.0 g NuSieve GTG agarose and 1 g SeaKem GTG agarose in 1 × TAE gel running buffer, run at 140 V for 60 min; stained with 0.5 µg/mL ethidium bromide). A qualitative assessment of the PCR product fragment size was performed by comparison with a DNA Mass/M Wt Ladder (Apex™ DNA Quantladder; 100–1000 bp). For analysis using a 2% product gel, 5 µL of the amplified DNA was added to 1 µL of gel loading buffer (6 × ). In addition, 5 µL of a DNA Mass Ladder was mixed with 1 µL of gel loading buffer (6 × ). Under optimal conditions using a 3%/1% product gel, the analysis was repeated using 4 µL volumes of each sample/control. It is recognized that electrophoretic agarose gel separation provides limited quantitative data. However, the method was useful in this study, given that the amplification results were only evaluated qualitatively.

## Results

For the purpose of this particular study, amplification success with the LINEAR ARRAY™ duplex is evaluated based on a qualitative assessment of the final PCR product obtained

TABLE 2—*Perkin-Elmer GeneAmp® > 2400 PCR profile time and temperature parameters for amplification (32).*

| Step | Temperature (°C) | | Interval | | Cycle Number |
|---|---|---|---|---|---|
| | Original | Optimized | Original | Optimized | |
| Activation | 92 | 94 | 12 min | 14 min | 1 |
| Denaturation | 92 | 92 | 30 sec | 15 sec | 34–38* |
| Annealing | 60 | 59 | 30 sec | 30 sec | 34–38* |
| Extension | 72 | 72 | 30 sec | 30 sec | 34–38* |
| Final Extension | 72 | 72 | 10 min | 10 min | 1 |
| Hold | 4 | 4–10 | ∞ | ∞ | |

*Determined by the quality and the quantity of the PCR yield.

following the extraction of mtDNA from a hair sample of fixed length. It should be noted that the recovery is relative to unknown quantities of DNA. Therefore, based on this approach, it is not possible to evaluate the actual recovery of mtDNA. The authors recognize that several other factors may influence amplification success, including incomplete DNA template recovery, failure to yield a clean template, unusual or variable amounts of protein, and potential co-extraction of PCR inhibitors. While real-time assays to quantify mtDNA may improve amplification success as the technique provides a more accurate means of estimating the volume of DNA template to add to the PCR reaction, the present study predates the availability of real-time PCR assays for quantifying mtDNA.

The data pertaining to the amplification success rate were collected and organized into three groups according to PCR amplification conditions, hair morphology characteristics, and demographic factors.

### *PCR Conditions*

Based on the original protocol at 34 cycles, the results show that telogen hairs correlate with a higher success rate: 74% when compared with hairs with no roots: 51.6% (Table 3a). Modification of the PCR reaction to include 38 cycles resulted in an additional 200 successful amplifications (34 telogen hairs and 166 hairs without roots). This gave a total amplification success rate of 76% for telogen hairs and 64% for hairs with no roots (original protocol: 34 or 38 cycles combined). This difference is also statistically significant (Table 3a). Further, a subset of the hair samples ($N = 1246$) were amplified using an optimized protocol. It should be noted that a large proportion of these hairs successfully amplified under the original protocol. Combining the amplification success with either the original or optimized protocol, telogen hairs result in an overall success rate of 77.5% compared with 65% for hairs with no roots.

### *Hair Morphology Characteristics*

The percentage of hairs that amplified per individual was assessed by considering only the data obtained from the original protocol (either 34 or 38 cycles). For telogen hairs, closer analysis of the amplification success rate reveals that the majority of hairs (91–100%) amplify successfully for 68 individuals; only three hairs failed to amplify for these individuals combined (1%). An intermediate number of hairs (50–90%) successfully amplify from 39 individuals, with 90 hairs failing to amplify (30.5%). A lower number of hairs (1–50%) successfully amplify from 25 individuals, who, when combined, account for 141 of the hairs that did not amplify (47.8%). In addition, all of the telogen hairs failed to amplify for six individuals, which accounts for the remaining 53 hairs (18%).

For hairs with no roots, the majority of hairs (91–100%) amplify successfully for 45 individuals; no unsuccessful amplifications were observed for these 45 individuals combined. An intermediate number of hairs (50–90%) successfully amplify from 43 individuals, with 97 hairs (20.5%) failing to amplify. A lower number of hairs (1–50%) successfully amplify from 25 individuals, who, when combined, account for 177 of the hairs without roots that did not amplify (37.4%). In addition, in the case of 23 individuals, none of the hairs without roots successfully amplified, which represent the remaining 219 hairs (46.3%). These data are summarized in Table 4.

In general, the data reflect similar patterns for telogen hairs and hairs without roots in terms of the number of cases falling within a particular success range. However, the number of hairs with no roots that did not amplify appears to be clustered within a smaller group of individuals, whereas the same number of unsuccessful telogen hairs tends to be more evenly distributed over a larger group of individuals. Further, no hairs amplified (telogen hairs and cut hairs combined) for two individuals.

In order to determine whether the amplification success rate is correlated to other hair characteristics, the data were analyzed to test for statistical significance using the $\chi^2$ distribution. The analysis, comparing the success rate of all hairs (original protocol: 34 or 38 cycles) that measured 2 cm in length with those that were less than 2 cm, showed that the results were statistically significant. Shorter hairs correlate with lower amplification success (Table 3b). This analysis was also statistically significant when considering the data for 34 cycles only (Table 3b). Further, this analysis was compared controlling for the telogen hairs versus hairs with no root. For hairs that lack a root, shorter hairs correlate with lower amplification success (Table 3b). However, for telogen hairs, the data indicate that there is no correlation between hair length and amplification success (Table 3b).

The following statistical analyses are based on the data obtained from the original protocol for 34 PCR cycles controlling for telogen hairs. For this study, amplification success is based on a qualitative assessment of the PCR yield. No attempt was made to

TABLE 3—*Hair amplification success rate (original protocol).*

| | Hair Roots (34 Cycles) | | No Hair Roots (34 Cycles) | | Hair Roots (38 Cycles) | | No Hair Roots (38 Cycles) | |
|---|---|---|---|---|---|---|---|---|
| (a)* | | | | | | | | |
| Total number | 1245 | | 1302 | | 303 | | 231 | |
| % amplified | 74 | | 51.6 | | 12.6 | | 71.9 | |
| | Combined Hairs (34 or 38 Cycles) (cm) | | Combined Hairs (34 Cycles) (cm) | | Telogen Hairs (34 Cycles) | | Hairs With No Roots (34 Cycles) (cm) | |
| Length of Hair Shaft | 2 | <2 | 2 | <2 | 2 | <2 | 2 | <2 |
| (b)† | | | | | | | | |
| Total number | 2433 | 121 | 2426 | 121 | 1169 | 76 | 1257 | 45 |
| % amplified | 70.8 | 52.1 | 63.1 | 51.2 | 74.1 | 72.4 | 52.9 | 15.6 |

*Thirty-four cycles only: (Pearson's $\chi^2$ $\Sigma = 135.864$; df = 1; continuity correction $\Sigma = 134.911$; likelihood ratio = 137.713). Significant correlation.
Thirty-four or 38 cycles combined: (Pearson's $\chi^2$ $\Sigma = 46.536$; df = 1; continuity correction $\Sigma = 45.946$; likelihood ratio = 46.882). Significant correlation.
†Combined hairs 34 or 38 cycles: (Pearson's $\chi^2$ $\Sigma = 19.176$; df = 1; continuity correction $\Sigma = 18.297$; likelihood ratio = 17.755). Significant correlation.
Combined hairs 34 cycles: (Pearson's $\chi^2$ $\Sigma = 6.930$; df = 1; continuity correction $\Sigma = 6.432$; likelihood ratio = 6.732). Significant correlation.
Telogen hairs 34 cycles: (Pearson's $\chi^2$ $\Sigma = 0.109$; df = 1; continuity correction $\Sigma = 0.038$; likelihood ratio = 0.107). Significant correlation.
Hairs with no roots 34 cycles: (Pearson's $\chi^2$ $\Sigma = 24.265$; df = 1; continuity correction $\Sigma = 22.793$; likelihood ratio = 26.370. Significant correlation.

TABLE 4—*Percentage of hairs that amplified for each individual (original protocol for 34 or 38 cycles).*

| % Hairs Amplified Per Individual | # Individuals (Telogen Hairs Amplified) | Total # Telogen Hairs Did Not Amplify | # Individuals (Hairs Without Roots Amplified) | Total # Hairs Without Roots Did Not Amplify |
|---|---|---|---|---|
| 91–100 | 68 | 3 | 45 | 0 |
| 81–90 | 14 | 15 | 17 | 17 |
| 71–80 | 7 | 15 | 11 | 22 |
| 61–70 | 8 | 23 | 7 | 21 |
| 51–60 | 5 | 20 | 4 | 17 |
| 41–50 | 5 | 25 | 4 | 20 |
| 31–40 | 5 | 30 | 3 | 18 |
| 21–30 | 2 | 13 | 9 | 64 |
| 11–20 | 8 | 63 | 5 | 39 |
| 1–10 | 4 | 36 | 4 | 36 |
| 0 | 6 | 53 | 23 | 219 |
| Total | 132 | 296 | 132 | 473 |

correlate the amount of PCR yield with a particular morphological feature. The data indicate that there is no correlation ($N = 1018$. Pearson's $\chi^2\ \Sigma = 1.450$; df = 1. Likelihood ratio = 1434) between the use of cosmetic treatments (69.3% of treated vs. 73.0% of untreated hair amplified) and amplification success rate. In terms of hair morphology, the data indicate that medulla structure (absent: 73.4%, fragmented: 79.0%, discontinuous: 75.6%, continuous: 73.1%) has no statistically significant affect on the amplification success rate ($N = 1228$. Pearson's $\chi^2\ \Sigma = 2.504$; df = 3. Likelihood ratio = 2.589).

The results also indicate that there is no correlation between the diameter of the hair shaft (10–47 μm; 73.1%, 48–85 μm; 73.9%, 86+μm; 76.2%) and amplification success rate. In this study, 41 hairs fell into more than one diameter range. A $\chi^2$ analysis was performed after placing these hairs in the lower and higher range. Regardless of the placement, no correlation was evident between the diameter of the shaft and amplification success ($N = 1245$. Smaller range: Pearson's $\chi^2\ \Sigma = 0.359$; df = 2. Likelihood ratio = 0.364. Larger range: Pearson's $\chi^2\ \Sigma = 2.361$; df = 2. Likelihood ratio = 2.471). The data obtained for the diameter and length of each hair were combined in order to calculate the volume of the hair shaft (note that the volume was calculated based on the assumption that each hair represents a uniform and circular cross-section and, therefore, a cylindrical volume). Based on diameter variation, some hairs fell into more than one volume range and were placed in the lower and higher range for statistical analysis. Regardless of the placement, no correlation was observed between hair volume (0.0012–0.0475 $mm^3$; 74.9%, 0.0476–0.0940 $mm^3$: 73.7%, 0.0941–0.1400 $mm^3$: 70.6%, 0.1401 $mm^3$+: 84.6%) and amplification success ($N = 1245$. Smaller range: Pearson $\chi^2\ \Sigma = 3.847$; df = 2. Likelihood ratio = 4.083. Larger range: Pearson's $\chi^2\ \Sigma = 3.088$; df = 2. Likelihood ratio = 3.405).

The data indicate that there is no statistically significant correlation ($N = 1245$. Pearson's $\chi^2\ \Sigma = 0.418$; df = 4. Likelihood ratio = 0.421) between amplification success rate and the region of the scalp that the hairs originated from. An average of 249 hairs from five regions of the scalp were collected. The present research indicates that scalp origin has no significant effect on the amplification success rate of hairs obtained from the front (72.8%), back (73.8%), left side (73.4%), right side (75%), and center (74.9%) of the scalp. However, hair pigmentation does appear to correlate; two pigmentation categories (light red, light-medium red brown, and medium-dark red, dark red brown) correlate with lower success rates relative to other pigmentation categories (Table 5).

### *Demographic Factors*

The amplification success rate was also measured as a function of two demographic factors: the racial group and age group of an individual. Analysis of the amplification success rate by racial group indicates a statistically significant correlation (Table 6a). This is attributed to the positive crrelation exhibited by Caucasians and the negative correlation exhibited by African Americans. The data in this study also indicate that there is a correlation between the age group and the success rate. Amplification success increases for the two oldest age groups (Table 6b). Although the data indicate a correlation for the amplification success rate of

TABLE 5—*Hair amplification success by pigmentation range for all telogen hairs (original protocol for 34 cycles).*

| Pigmentation of Hair Shaft | Total Number* | % Amplified |
|---|---|---|
| Light–medium blonde | 101 | 74.3 |
| Dark blonde, light golden brown, light brown, light gray brown | 182 | 83.5 |
| Medium–dark golden brown to medium brown | 314 | 72.3 |
| Dark brown, medium–dark gray brown | 399 | 76.9 |
| Light red, light–medium red brown | 101 | 58.4 |
| Medium–dark red, dark red brown | 50 | 40 |
| No pigment | 98 | 82.7 |

*Excludes hairs where no color was recorded but includes dyed hairs and deceased individuals.

(Pearson's $\chi^2\ \Sigma = 57.411$; df = 6; likelihood ratio = 53.015). Significant correlation.

TABLE 6—*Hair amplification success for all telogen hairs (original protocol for 34 cycles).*

| Racial Group | Caucasian | African American | Hispanic | Asian |
|---|---|---|---|---|
| (a)* | | | | |
| Total number | 411 | 320 | 275 | 229 |
| % amplified | 89.6 | 52.5 | 78.2 | 74.7 |
| Age range | 18–29 | 30–49 | 50–69 | 70 and older |
| (b)† | | | | |
| Total number | 511 | 471 | 201 | 83 |
| % amplified | 71.6 | 67.3 | 83.6 | 84.3 |

*Excludes individual 52 (African American/other = 3 hairs) (Pearson's $\chi^2\ \Sigma = 114.070$; df = 3; likelihood ratio = 111.185). Significant correlation.

†(Pearson's $\chi^2\ \Sigma = 24.892$; df = 3; likelihood ratio = 26.600). Significant correlation.

telogen hairs when comparing living versus decedent subjects (Pearson's $\chi^2$ $\Sigma = 12429$; df = 1. Likelihood ratio = 13.387), this appears to be attributed to the uneven distribution of hairs from African American and Caucasian individuals within the decedent group (see discussion).

## Discussion

The goal of this research study was to investigate the factors that influence the amplification success rate in hair tissue. The particular focus was to characterize morphological features such as medulla structure, pigmentation, and diameter. The hair samples were cut at the proximal (root) end to generate a 2 cm portion of hair shaft, which was extracted and amplified with the duplex primer set. For some individuals, the length of an entire hair was less than 2 cm. In this situation, the length was measured and the entire hair was consumed in the extraction. The results indicate that there is a significant difference in the success rate when comparing hairs with no root versus hairs that possess a telogen root using the LINEAR ARRAY™ duplex primer set. A positive correlation exists for telogen hairs and amplification success. One explanation for this observation is that in removing the root, a rich source of mtDNA may also be removed from within the hair bulb matrix. Presumably, there are more mitochondria within the root and cutting this region reduces the amount of mtDNA in an equivalent portion of the shaft (adjacent 2 cm fragment). The implication for casework analysis is that hair fragments (lacking a root), and in particular, fragments that originate closer to the distal (tip) end that are submitted as evidence may show a reduced amplification success rate. The greater number of cells in the latter may reflect an increase in mtDNA copy number.

When considering the entire sample in this study (telogen hairs and cut hairs), the results indicate that one of the mitigating factors for amplification success of hairs is length, with shorter hairs ($<2$ cm) showing significantly lower success. However, although the length of the shed hair sample may be important, the section of the hair (distal vs. proximal to the root) and the presence of a root bulb might also be a factor. The results of this study indicate that length may only be an issue for cut hairs. For example, the data show that longer hairs that lack a root exhibit a significantly lower amplification success rate (52.9%) relative to telogen hairs of comparable length (74.1%). In comparison, the success rate of shorter telogen hairs is 72.4%. However, short hairs that lack a root exhibit a particularly low success rate (15.6%). The most likely explanation for this result is that the amount of template DNA extracted from short hair fragments is too low or dilute for amplification. Presumably, with a root and/or a larger length of hair, there is a greater amount of template mtDNA.

Another explanation for the lower success rate of hair fragments is that a PCR inhibitor is co-extracted and a significant amount is introduced into the PCR. The results of the present study suggest that inhibitory activity alone does not explain the lower amplification success observed for hair fragments. For example, when larger hair fragments are extracted, the amplification success improves (52.9%) relative to shorter hair fragments (15.6%). As indicated above, this is most likely attributable to the greater amount of template mtDNA associated with longer fragments. However, given the lower mtDNA copy number, inhibitory activity may also have more of an impact on the amplification success for shorter fragments. The presence of a sufficient level of PCR inhibitor can reduce the activity of the polymerase, which may significantly decrease product yield. Examples of inhibitors that relate to this study are melanin and extraneous sources such as chemical dyes incorporated into the hair or possibly substances adhering to a hair shaft.

The study also assesses whether selection of the hair within a representative sampling of the casework and control samples is important. Here, the question was whether hair morphology is a reliable predictor of amplification success. This information is valuable in terms of selection of hairs for subsequent mtDNA typing. Controlling for telogen hairs, the results indicate that there is no difference in the success rate observed across the shaft diameter range. While the majority of the hairs fell within the 40–80 μm range, given that no correlation exists between amplification success and hair length (0.8–2 cm) for telogen hairs in this study, it seems reasonable that variation in shaft diameter (10–141 μm) likewise shows no correlation. The data for length and shaft diameter were combined to determine hair volume (0.0012–0.3079 $mm^3$); again, no correlation exists between amplification success and hair volume. Further, the medulla structure is not correlated to amplification success. Although a hair with a continuous medulla might be expected to contain less template mtDNA relative to a hair where the medulla is classified as absent (8), the results of the present study indicate that there is no statistical difference between the success rates of hairs where the medulla is classified as either absent, fragmented, discontinuous, or continuous. There may be quantitative differences in the amount of template mtDNA for each of these medulla classifications but this was not evaluated.

Another experiment involved collecting and comparing hairs that had been subjected to different cosmetic treatments. The question is whether chemical hair treatments degrade or otherwise reduce the quantity of typable mtDNA in the shaft. Evidence from the present research indicates that the use of cosmetic treatments has no effect on the amplification success rate. Potentially, chemical treatments may be a factor contributing to some degradation in mtDNA copy number. It is also possible that chemical treatments may impart inhibitory effects that may be contributing to a reduced PCR yield. However, the yield was not quantified in the present study.

Also at issue is whether the demographic variables of age and race of the donor impact hair amplification success rate. The natural pigmentation of an individual's hair changes over time; the number of melanosomes that are present in the hair shaft decline with age, although it is not known whether activity related to proliferation of the germinal cells is also reduced. Further, the degree of natural pigmentation varies across different population groups. This research suggests that amplification success rate is correlated to the race of the donor. The findings indicate that Caucasian hairs demonstrate a significantly higher success rate (86.9%) when compared with African Americans (52.5%). This lower success rate may be attributed to the degree of pigmentation (melanin density) causing potential inhibition (33). The results obtained from a comparison of the success rate as a function of hair pigmentation also demonstrate a correlation. In general, darker-pigmented hairs exhibit lower success rates relative to lighter-pigmented hairs. The data in this study also indicate that success rate is dependent on the age of the hair donor. There is a positive correlation between older age groups and amplification success. Given that melanin production declines with age, one explanation for this result is that there may be a reduction in the inhibitory effects manifested by melanin during the PCR.

It has been reported that postmortem tissues undergo decompositional changes that are generally attributed to microbial and autolytic action (34). Studies indicate that various postmortem morphological changes occur in anagen and catagen hair roots; however, there are no reports indicating that these changes are

observed in telogen hairs. Postmortem microscopic changes include root banding and the development of brush-like roots (34,35). One report further distinguishes root banding into two categories: distal and proximal root banding and describes a fourth type as the formation of a hard keratin point (35). In the present study, the amplification success rate of hairs from living and deceased donors was compared. Although postmortem microscopic changes have only been reported in anagen and catagen roots, the possibility that postmortem biochemical activity may degrade mtDNA in telogen hair roots was considered. Given that the root was removed from each anagen hair before analysis, it was not possible to evaluate the impact of decomposition on the recovery of mtDNA from actively growing hairs.

The data indicate a statistically significant difference in the success rate of telogen hairs from decedents (83.3%) and living subjects (71.9%). However, no correlation exists when a similar comparison is performed for cut hairs (decedents—50% and living subjects—52%). One explanation for the higher success observed with telogen hair from decedents compared with living subjects is the possibility that scalp tissue was inadvertently collected with the decedent "telogen" hairs. However, this was not evident from a review of the photomicrographs. Another consideration is the race distribution within living and decedent subjects. Controlling for African American telogen hairs, there is no correlation between the amplification success of living (150/286 = 52.4%) and decedent subjects (18/34 = 52.9%). Similarly, controlling for Caucasian telogen hairs, there is no correlation between the amplification success of living (267/305 = 87.5%) and decedent subjects (90/106 = 84.9%). This finding supports the suggestion that telogen hairs may not be susceptible to decompositional changes in the hair root (35). It is possible that decompositional changes may be contributing to a reduced PCR yield. However, the yield was not quantified in the present study.

It should be noted that the ratio of Caucasian:African American hairs is $\sim$3:1 for decedents compared with $\sim$1:1 for living subjects. Therefore, the combination of underrepresentation of African American hairs (15%) and overrepresentation of Caucasian hairs (46.7%) may account for the overall higher success rate observed for telogen hairs from decedents.

Several experimental parameters were varied during the course of this study in order to optimize the amplification success rate. The Chelex extraction method was compared with an incubation step that both included and excluded the addition of a proteinase K digestion. Initially, a small subset of samples ($N = 32$) was incubated without proteinase K. These samples all gave a negative PCR yield. The addition of 10 μL proteinase K to the homogenate, followed by an incubation step at 56°C for at least 6–8 h (or overnight), improved the mtDNA yield (30 of 32 samples amplified). After modifying the extraction procedure by incorporating Proteinase K digestion for all of the hair samples, the yield was assessed by running a product gel. Samples generating low yields (or, potentially, high yields but with co-extracted inhibitors) were subjected to an additional four PCR cycles. A sub-set of the samples in this study was subjected to optimized amplification parameters while the amount of primer incorporated into the PCR reaction mix was increased from 0.2 to 0.3 μM. All of these factors contributed to an increased amplification success rate.

Melton et al. (27) performed mtDNA analysis on 691 casework hairs. The authors report obtaining a full or partial mtDNA profile in >92% of the casework hairs analyzed. Further, the percentage of casework hairs analyzed giving a full profile is reported as 82.5%. The percentage of casework hairs successfully amplified using the four amplicon system mtDNA primers sets (without requiring additional amplifications with the mini primer sets) is not reported. Also of note, the present study only analyzed freshly collected hairs; none of these hairs were <0.5 cm in length. The Melton study, on the other hand, included hairs aged 0–31+ years old and 20 of these hairs were $\leq$ 0.5 cm in length. Therefore, it is difficult to compare the two studies directly. However, there are some notable differences between the studies that may contribute to the differences in success rates.

In the present study, a 76% success rate (original protocol, telogen hairs only) and 77.5% success rate (original+optimized protocol, telogen hairs only) were observed for the simultaneous amplification of two $\sim$400 bp fragments. The Melton study targets shorter mtDNA fragments ($\sim$280 bp, including primers) using four separate reactions and degraded samples were subjected to mini-primer sets when the initial amplification failed; both of these factors may contribute to the higher success rate observed in Melton's study. Also, a total of 36–40 amplification cycles were applied in Melton's study compared to 34–38 in the present study, which may also be contributing to the greater amplification success.

In Melton's study, hairs exhibiting certain morphological characteristics were assigned to one of five categories based on their "robustness." Generally, darker-pigmented and larger diameter hairs were described as more robust, indicating that the likelihood of obtaining a full profile increased with increasing color and diameter. These results contrast with those obtained in the present study, where a lower amplification success is obtained for heavily pigmented hairs relative to the success of the lighter-pigmented hairs. For example, controlling for telogen hairs only, hairs in the dark blonde to light gray brown category gave an 83.5% success rate, whereas medium-dark red/dark red brown gave a 40% success rate. Considering the diameter results obtained for all hairs in the present study, when controlling for telogen hairs only, no differences were observed in the success rate across the diameter ranges.

One possible explanation that may account for the lower success obtained for heavily pigmented hairs in the present study (which, in turn, contributes to the overall success rate observed) is that different methods were applied during sample processing. In the Melton study, samples were subjected to a phenyl/chloroform/isoamyl alcohol (PCIA) extraction protocol. When coupled with the fact that Bovine Serum Albumin (BSA) was incorporated into the amplification protocol and the inclusion of a Microcon™ filter unit to clean up the extraction product, it seems plausible that these variations may reduce the effects of amplification inhibitors (including melanins), resulting in increased amplification of more "robust," darker-pigmented hairs.

In conclusion, based on the results of the present study, lower amplification success is associated with hair pigmentation, coupled with donor race and age. It is most likely that this lower success is attributed to the extraction method used (e.g., ineffective removal of inhibitors such as melanin) as opposed to the LINEAR ARRAY™ duplex amplification system. Considering only those samples that gave a full profile, the amplification success observed for hairs in the present study using the duplex primers ranged from 71.3% (overall success) to 77.5% (telogen hairs only) compared with 82.5% with the 280 bp four-singleplex system used in Melton's study. It is not known how many of the casework hairs that Melton analyzed were identified as telogen hairs versus hair fragments; therefore, the most appropriate comparison of success falls within the 71.3–77.5% range. Of course, the larger amplicon associated with the LINEAR ARRAY™ duplex amplification system may also be contributing to this reduced success. Therefore, in addition to exploring modifications to the extraction procedure, an initial approach might be to amplify the hair extract

with the duplex primers; samples that do not amplify with the duplex could then be subjected to either the 280 bp singleplex system and/or the mini primer sets.

This study evaluated amplification success as a function of hair morphology. We did not consider, *a priori*, the underlying rationale (low mtDNA template versus inhibition versus incomplete recovery, etc.) for the variation in amplification success rate. However, based on the results obtained, several follow-up studies are being investigated in order to increase mtDNA yield in human head hair using LINEAR ARRAY™ analysis. Inhibition activity will be evaluated by including known quantities of control DNA with the extract during PCR. Other studies include dilution of the extract in order to reduce the concentration of inhibitors that affect the final PCR product, and treating the extract with BSA. Alternative extraction methods are also being explored (for example, incorporating a Microcon™ clean-up step with the organic PCIA procedure to remove melanin).

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