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PAPER

CRIMINALISTICS

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Characterization of Mitochondrial DNA Sequence Heteroplasmy in Blood Tissue and Hair as a Function of Hair Morphology*,†,‡

ABSTRACT: This study characterizes mitochondrial DNA (mtDNA) sequence heteroplasmy in blood tissue and hair as a function of hair morphology. Bloodstains (127 individuals) and head hairs (128 individuals) were typed using the mtDNA LINEAR ARRAY™ assay. A total of 1589 hairs were interpreted: 1478 (93%) were homoplasmic and 111 (7%) exhibited heteroplasmy at one or more positions. Seventy-one percent (82/116) of individuals were homoplasmic, whereas 29% (34/116) exhibited heteroplasmy in at least one hair. The results demonstrate intra- and inter-tissue differences in heteroplasmy within individuals. Sequence heteroplasmy among hairs from each individual varied from 0 to 90%; the frequency does not differ significantly with population group, cosmetic treatment, age, gender, medulla morphology, region of the scalp, hair growth phase, or, when comparing living and deceased donors. However, the results support a correlation between heteroplasmy and hair pigmentation; typically, lighter-pigmented hairs exhibit a higher incidence of sequence heteroplasmy compared to darker hairs.

KEYWORDS: forensic science, human mitochondrial DNA, sequence heteroplasmy, hair morphology, blood, intra- and inter-tissue differences

Studies have demonstrated that mitochondrial DNA (mtDNA) is susceptible to genetic mutation (1–6). In particular, a high degree of polymorphism is observed within the hypervariable regions (referred to as HVI and HVII) of the noncoding control region (1–3). Evidence suggests that the mitochondrial genome mutates at a rate that is an order of magnitude greater than that of nuclear DNA (4–6). Generally, only a single mtDNA type is detected within an individual, a condition referred to as homoplasmy. When a mutation arises, but only reflected in some copies of an individual's mitochondrial genomes, the result is a mixture of mtDNA sequences that is referred to as heteroplasmy (7). The mutations may be somatic (mitotic) or may originate in the female germ line (meiotic).

If the heteroplasmic state is somatic in origin, the mutant and normal mtDNAs can be randomly distributed into the daughter cells during mitotic division (8). As a result, the percentage of mutant and wild-type molecules can drift within the cell, toward either pure mutant or pure wild type, approaching homoplasmy. This process, referred to as replicative segregation, causes the phenotype to be largely influenced by the proportion of mtDNA molecules harboring the mutation in the different tissues. In germ line heteroplasmy, a random distribution of wild and mutant types into the daughter cells occurs during meiotic division and can also drift toward either pure mutant or pure wild type. If the mixture does persist in the oocyte, it can be transmitted to the progeny. Several inter-and intra-generational studies are reported in the literature (9–11). These studies indicate that mitotic replicative segregation usually requires multiple cell divisions for a mutant sequence to accumulate to a detectable level and approach homoplasmy, whereas meiotic replicative segregation can be quite rapid (12–14).

Generally, two forms of heteroplasmy are observed, length and sequence heteroplasmy. Length heteroplasmy is associated with mutations involving nucleotide insertions or deletions, which is most commonly observed in the HVI and HVII poly-cytosine regions (C-stretches) (15,16). Stewart et al. (17) demonstrated that hairs could exhibit intra-individual variation in length polymorphism within the HVII region.

Sequence heteroplasmy is attributed to point mutations in mtDNA (18). Although the incidence of point heteroplasmy was originally thought to be quite low, subsequent studies indicate otherwise (19–22). One explanation for the increase in reported observations may be the fact that heteroplasmy can now be more readily detected given the improved sensitivity of currently available sequencing techniques. Another factor to consider is the source of tissue analyzed because inter-tissue variation has been observed within individuals. One study indicated that higher levels of mutated mtDNA are found in skeletal muscle and brain tissue from a single individual, compared to lower levels present in the corresponding blood sample (23).

Studies also report intra-individual differences in hairs. Sullivan et al. reported a heteroplasmic point mutation in hair shafts from

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an individual whose blood was homoplasmic (24). Bendall et al. (25) also found highly variable levels of heteroplasmy in single hair roots within an individual, despite the fact that heteroplasmy was consistent in samples obtained from peripheral blood and buccal cells. Similarly, Alonso et al. (26) found varying levels of heteroplasmy in hairs from the same donor. Further, Sekiguchi et al. detected sequence heteroplasmy in hairs from three individuals using a denaturing gradient gel electrophoresis (DGGE) assay (27). Melton et al. report a number of independent observations of heteroplasmy in casework hairs using cycle sequencing (28). However, Huhne et al. (29) compared the mtDNA sequences of head hair shafts of individuals with blood and saliva samples and found no evidence of heteroplasmy. Pfeiffer et al. (30) conducted a similar study of mtDNA sequence comparisons from human axillary, pubic, and head hair shafts, again, reporting no incidence of heteroplasmy. The sampling differences (number of hairs and/or number of individuals) in addition to detection methodologies may be contributing to these different findings.

This study expands on the current understanding of mtDNA sequence heteroplasmy in human head hair and bloodstains. We analyzed 1589 telogen and cut hairs in addition to bloodstains from 128 individuals using a sensitive assay. This approach utilizes a duplex amplification that is applied to a sequence-specific oligonucleotide probe (SSOP) assay to detect sequence variation within 10 highly polymorphic regions (31–37). DNA-sequencing analysis of the HVI and HVII regions was also performed on a selected number of hairs and blood samples (38–42). The particular focus of this study was to further the understanding of the factors that influence both the condition and detection of sequence heteroplasmy in human head hair using the LINEAR ARRAY™ mtDNA HVI/HVII Region-Sequence Typing Kit (Roche Applied Science, Indianapolis, IN). A number of hairs were analyzed from the same individual to determine whether the hairs exhibit different haplotypes. To demonstrate differences in heteroplasmic expression (nature and frequency of occurrence) between blood and hair tissue, parallel experiments were conducted to compare the mtDNA type of peripheral blood with hair samples from the same donor. A comparison of the tissues permits both the identification and the extent of sequence mosaicism that exist between these tissues in the same individual. Given that each scalp hair grows independently of any other hair, it is possible that mutations may accumulate in discrete areas of the scalp, or are distributed randomly. This selective mutation has important ramifications because it impacts hair collection procedures in attempting to obtain a representative sample intended for mtDNA analysis. To investigate spatial mosaicism, hair samples were collected from five regions of the scalp: front, back, left side, right side, and center.

This study also addressed whether exposing a hair to different chemical treatments affects the ability to obtain an mtDNA haplotype (mitotype). Indirectly, this addresses whether or not chemical treatments degrade or otherwise reduce the quantity of mtDNA in the shaft. The present study also evaluated whether the heteroplasmic condition correlates to those hairs exhibiting distinct morphologies. The morphological features evaluated were microscopic characteristics, including color, medulla structure, and diameter of the hair shaft. The study also considered whether or not the age of an individual is a basis for predicting a heteroplasmic condition. Studies have indicated that somatic deletions *and* point mutations accrue with age (23,43). Most are common deletions that have been identified primarily in clinical medicine research on mtDNA diseases in the coding region of the genome. Studies have shown that some diseased states manifest when the mutated gene reaches a certain threshold level within the heteroplasmic mixture and shown to be more pronounced with age. This is an important consideration from a forensic standpoint because different haplotypes may be observed when comparing contemporaneous and historical samples from the same individual because of an accumulation of mutations with age. Likewise, sequence differences may be observed when comparing contemporaneous samples with a reference sample from a maternal relative. To investigate whether expression of the mutant form of the genome accumulates with age, blood and hair samples were collected from living and deceased individuals ranging from 18 to 88 years in age. Finally, hairs possessing a telogen root were compared to anagen hair shafts (root removed) to investigate whether the presence of a root impacts the level or frequency of heteroplasmy.

Methods and Materials

Subjects

Blood and hair samples were collected from 127 and 128 individuals, respectively (59 men and 69 women), in accordance with City University of New York IRB-approved protocol; all living subjects provided informed consent. Participants in this study were asked to self-collect a minimum of 50 head hairs to ensure a sample that represented the natural morphological variations that can exist within individuals. Specifically, donors were 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 to obtain anagen hairs, while the other five were obtained by combing the respective areas for telogen hairs.

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. The age of the subjects ranged from 18 to 88 years, each falling into one of four age ranges: 51 individuals in the 18–29 years category; 45 in the 30–49 years category; 23 in the 50–69 years category; and nine in the 70 years and above category. The distribution of population groups collected for this study can be summarized as follows: 44 U.S. Caucasian, 31 U.S. African American, 25 U.S. Asian, 27 U.S. Hispanic individuals, and one U.S. African American/Other. In addition, postmortem hair samples were collected from 24 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.

Photomicrography

A minimum of 10 hairs from each donor were observed microscopically to determine growth phase, color characteristics, and chemical treatment, as previously described (44). Each hair was mounted (Cargille mineral oil N_D at 23°C = 1.5150 ± 0.0002) and examined microscopically (×100; Olympus Model POS, Center Valley, PA). Photomicrographs recorded the root end of each hair in addition to representative regions along the shaft (35 mm Fugichrome Provia100F RDP III 135 film, with Kodak 80B filter; Honeywell Pentax 35 mm SLR SP 500 with Asahi Pentax microscope adapter II; Honeywell International Inc., Morristown, NJ).

Mitochondrial DNA Duplex HVI/HVII PCR Amplification and LINEAR ARRAY™ Typing

Following photomicrography, 10 hairs from each donor were extracted and amplified as previously described (44). The polymerase chain reaction (PCR) product yields were estimated by

comparison to a DNA Mass Ladder (100–1000 bp; Apex™ DNA Quantladder; Genesee Scientific, San Diego, CA). In each case, the target concentration of the PCR product was in the 50–100 ng range to attain optimal probe signal intensity. The PCR product was denatured by adding the appropriate volume of PCR product based on the estimated concentration to 15 μL of denaturation solution (1.6% 0.5 M sodium hydroxide (w/w), Amplicor®; Roche Diagnostics, Indianapolis, IN).

Blood samples from 127 individuals and head hairs from 128 individuals (1854 hairs) were typed using the LINEAR ARRAY™ mtDNA HVI/HVII Region-Sequence Typing Kit following the manufacturer's recommended procedure. The technique involves the immobilization of SSOPs to a nylon membrane and the hybridization of biotin-labeled amplified DNA to the membranes. The hybridization product is detected colorimetrically: the pattern of colored bands produced determines the individual's mtDNA haplotype.

Interpretation of LINEAR ARRAY™ mtDNA HVI/HVII Results

The LINEAR ARRAY™ probe panel used in this study consisted of 33 probes striped in 31 lines targeting 18 polymorphic sites within 10 regions of the hypervariable control region of the mitochondrial genome. Each of the 10 regions targets sequence polymorphism at 1–4 nucleotide positions that differs from the revised Cambridge Reference Sequence (rCRS) (45). Probes designated by a "1" correspond to sequence motifs found in the rCRS. The sequences that are distinguished by the probes in the HVI and HVII regions are summarized in Table 1.

Following typing, the wet probe panels were aligned to an mtDNA HVI/HVII reference guide to determine the mtDNA haplotype. The pattern of blue lines was recorded on an mtDNA Interpretation Sheet for each of the 10 regions. In a given region, one of four categories of probe signals is expected: a single probe is positive; a single probe gives a weak signal (scored as a 'w' followed by the probe designation); no probe signals are visible (scored as '0'); and two probes are visible either with equal or uneven intensity (both probes are noted and intensity).

If the PCR product hybridizes to two probes within the same region, this is an indication that the sample may be heteroplasmic when contamination or cross-hybridization is excluded. Multiple probe signals observed in two or more regions within the probe panel suggests the possibility of a DNA mixture resulting from contamination. For the purposes of this study, samples with mixtures in two or more regions were excluded. Also, suboptimal typing conditions, such as a low water bath temperature (below 54°C), can lead to cross-hybridization for certain mtDNA sequences and probes (i.e., IIB4 sequence with the IIB2 probe). To rule out cross-hybridization, samples were retyped ensuring optimal typing conditions.

A permanent record of the mitotyping results was obtained by photographing the LINEAR ARRAY™s with the interpretation template. Kodak 35-mm black-and-white print film was selected as the photographic medium (ASA 400 F/5.6, shutter speed 1/30–1/60″). An SLR camera was used (Honeywell Pentax SP 500/Super-Takumar 50 mm lens/Marumi 49 mm + 3 macro lens) with tungsten floodlights (even illumination at 45°).

TABLE 1—*Probe designation of LINEAR ARRAY™ mtDNA HVI/HVII Region-Sequence Typing Kit (46).*

HVI/HVII Probe Designation	Sequence Variation Detected
	16093
16093(1)	T
16093(2)	C
	16126 16129
IA1	T G
IA2	C G
IA3	T A
	16304 16309 16311
IC1	T A T
IC2	C A T
IC3	T A C
IC4	T G T
	16362
ID1	T
ID2	C
	16270 16278
IE1	C C
IE2	C T
IE3	T C
	73
IIA1	A
IIA2	G
	146 150 152
IIB1	T C T
IIB2	C C T
IIB3	T C C
IIB4	C C C
IIB5	T T T
IIB6	T T C
IIB7	C T C
	189 195 198 200
IIC1	A T C A
IIC2	A C C A
IIC4	A C T A
IIC5	G T C G
	247
IID1	G
IID2	A
	189 195
189(1)	A T
	A C
189(2)	G T
	G C

Cycling Sequencing

A subset of the samples indicating site heteroplasmy were sequenced to confirm the LINEAR ARRAY™ typing results. The unincorporated deoxyribonucleotide triphosphates and single stranded primers were removed from the PCR products by adding 2 μL of ExoSAP-IT™ (USB, Cleveland, OH) to 5 μL of amplified product. The reactions were activated at 37°C for 15 min, followed by a deactivation of ExoSAP-IT™ at 80°C for 15 min. The ABI Prism® BigDye™ Terminator v1.1 cycle sequencing kit was used (Applied Biosystems, Foster City, CA). Twenty-microliter sequencing reactions were performed as follows: 2 μL of 5× BigDye™ sequencing buffer, 4 μL of BigDye™ Terminator mix, 3.2 pmol of HVI or HVII primer (forward [HVI F15975-93B/HVII F15-34B] and reverse [HVI R16418-01B/HVII R429-410B] strands

were analyzed in separate sequencing reactions) and 10 ng of purified PCR product. The following cycle sequencing parameters were used: initial activation at 96°C for 1 min, followed by a 25-cycle reaction at 96°C for 15 sec, 50°C for 5 sec, and 60°C for 4 min.

Removal of unincorporated dye-labeled terminators from the extension products was accomplished by ethanol/sodium acetate precipitation as follows: 80 μL of ethanol/sodium acetate solution (3 μL of 3 M sodium acetate, pH 4.6; 62.5 μL of nondenatured 95% ethanol; 14.5 μL of deionized water) was added to 20 μL of reaction product and mixed by inversion. The samples were incubated at room temperature for 15 min, followed by a centrifugation at 3000 × *g* for 30 min. The supernatant was discarded by inversion, and the precipitated products were centrifuged (with sample plate inverted) at 50 × *g* for 1 min. To the sample pellet, 150 μL of 70% ethanol was added, which was mixed by inversion and then centrifuged at 3000 × *g* for 10 min. Again, the supernatant was discarded by inversion, and the pellets were centrifuged (with sample plate inverted) at 50 × *g* for 1 min.

Samples were reconstituted in Formamide (Applied Biosystems) and denatured by heating at 96°C. The samples were electrophoresed on the ABI Prism® 3730xl Genetic Analyzer using a 96-capillary array and POP6™ polymer (Applied Biosystems). Sequence data analysis was performed using Sequencher software (Gene Codes Corporation, Ann Arbor, MI).

Results

Typeable results were obtained for a total of 127 blood samples and 1854 hairs from 128 individuals. Further evaluation of the hair mitotypes resulted in the exclusion of 84 contaminated samples (4.5%), where two or more regions display multiple signals; and 11 discordant samples, where the blood and hair mitotype did not correspond (0.6%). Additionally, the LINEAR ARRAY™ typing results of 170 samples fell below the minimum probe signal threshold set for this study and were excluded from the heteroplasmy analysis. Based on these exclusions, a qualifying mitotype was obtained for a total of 1589 hairs.

The mtDNA haplotypes for each sample were determined as described previously and are reported in Table 2. For the 127 individuals, a total of 78 different haplotypes were observed (excluding heteroplasmy). The haplotypes are presented in the standard format for LINEAR ARRAY™s and represent differences with respect to the rCRS sequence. The reader may refer to Table 1 to convert the sequence variation detected.

Sequence Heteroplasmy in Blood and Hair Characteristics

Sequence heteroplasmy was detected using the LINEAR ARRAY™ panels in approximately 2% (3/127) of the blood samples analyzed in this study. All three heteroplasmic blood samples were subsequently confirmed by cycle sequencing. Heteroplasmy was detected in 111 (7%) of the hair samples analyzed, and 1478 (93%) were reported as homoplasmic.

Approximately 71% (82/116) of individuals in this study exhibited a single mtDNA haplotype within all of their hair samples typed, whereas 29% (34/116) of individuals exhibited a heteroplasmic state in at least one hair. For the purpose of evaluating the results of this study, an individual was categorized as exhibiting homoplasmy if a single mitotype of normal intensity was obtained for a minimum of four hairs. This gave an indication of the frequency of homoplasmy *versus* heteroplasmy within an individual based on the random selection of a few hairs across all regions of the scalp. Of the 34 heteroplasmy cases, heteroplasmy was observed exclusively in cut hairs of nine individuals (26.5%), 14 individuals were exclusively telogen hairs (41.2%), and 11 individuals (32.3%) overlapped in that both cut and telogen hairs showed evidence of heteroplasmy. The frequency of heteroplasmic hairs observed for individuals in this study is illustrated in Fig. 1. This figure shows a similar distribution for both the telogen and cut hairs; for example, 13 and 10 individuals exhibited a single mtDNA haplotype for telogen (11.2%) and cut (8.6%) hairs, respectively. Within individuals, heteroplasmy most often manifests itself in one hair from a modest size sampling of between 4 and 29 hairs. Fifteen individuals fall into this category; nine individuals exhibited the condition in one telogen hair and six individuals displayed the condition in one cut hair. Further, six individuals in this study were observed to have two hairs displaying the condition, four of which were exclusively telogen hairs, while two individuals expressed a single heteroplasmic hair in both cut and telogen hairs. There were also 13 individuals who exhibited heteroplasmy in more than two hairs. Of these 13 individuals, 10 expressed heteroplasmy exclusively within their hairs, while the remaining three individuals were heteroplasmic in both their blood and hair tissue. Twenty-three hairs did not possess a root and could not be classified as cut anagen hairs or telogen hairs. Considering the entire sample pool of 1566 hairs, 8% of cut anagen hairs and 6.3% of telogen hairs were heteroplasmic. Chi-square analysis indicates that there is no correlation between heteroplasmy and cut anagen versus telogen hairs ($N = 1566$; Pearson chi-square, $\Sigma\chi^2 = 1.817$; df = 1; Likelihood ratio = 1.811).

Comparing the use of cosmetic treatments to the incidence of heteroplasmy, the results indicate that there is no correlation at the 95% significance level. Sequence heteroplasmy is detected in 6.7% and 6.5% of treated and untreated hairs, respectively ($N = 1169$; Pearson chi-square, $\Sigma\chi^2 = 0.017$; df = 1; Likelihood ratio = 0.017). Similarly, the results reflect that there is no statistically significant correlation between the occurrence of heteroplasmy for living and deceased individuals (95% significance level). Sequence heteroplasmy is observed in 28.6% of decedents when compared to 29.5% living individuals ($N = 116$; Pearson chi-square, $\Sigma\chi^2 = 0.007$; df = 1; Likelihood ratio = 0.007). Also, there is no statistical correlation between the incidence of heteroplasmy and the region of the scalp where the hair was collected; the following is a summary of percentage of sequence heteroplasmy by region of the scalp: front, 5.4%; back, 10.5%; center, 6.4%; right side, 6.5%; left side, 5.8% ($N = 1589$; Pearson chi-square, $\Sigma\chi^2 = 8.551$; df = 4; Likelihood ratio = 7.871). Similarly, there is no correlation between medulla structure and heteroplasmy at the 95% significance level. The following is a summary of the percentage of sequence heteroplasmy as a function of medulla structure: absent medulla, 8.6%; fragmented medulla, 6.5%; discontinuous medulla, 4.7%; continuous medulla, 1.8% ($N = 1425$; Pearson chi-square, $\Sigma\chi^2 = 8.671$; df = 3; Likelihood ratio = 10.856). However, there does appear to be a correlation between the occurrence of heteroplasmy and hair pigmentation when considering hair from all individuals represented in the study. Chi-square analysis supports that this is true when considering naturally pigmented and dyed hairs collectively and when considering only naturally pigmented hairs (Table 3). Generally, this correlation is attributed to the higher than expected number of heteroplasmic cases associated with lighter-pigmented hairs and the lower than expected number of heteroplasmic cases associated with darker-pigmented hairs across individuals.

Demographic Data and Heteroplasmy

The demographic variables of the subjects were also analyzed. The data indicate that there is no significant correlation (95%

TABLE 2—*Distribution of mtDNA SSOP haplotype profiles for hairs in study.*

Sample #	Age	Race	# Hairs Obs.	% Hairs Het.	Hair and Blood SSOP Haplotype									
					16093	IA	IC	ID	IE	IIA	IIB	IIC	IID	189
01 blood	26	A			1	1	1	1	1	2	1	1	1	1
hairs			19	0	1	1	1	1	1	2	1	1	1	1
02 blood	29	A			1	3	1	1	1	2	5	0	1	1
hairs			15	0	1	3	1	1	1	2	5	0	1	1
03 blood	25	A			1	1	1	1	1	2	1	2	1	1
hairs			18	0	1	1	1	1	1	2	1	2	1	1
04 blood	26	A			1	1	0	1	1	2	1	1	1	1
hairs			15	0	1	1	0	1	1	2	1	1	1	1
05 blood	26	A			1	3	2	1	1	2	3	1	w1	1
hairs			9		1	3	2	1	1	2	3	1	w1	1
hairs			3	25	**1**	**3**	**2**	**1**	**1**	**2**	**1/3**	**1**	**w1**	**1**
06 blood	26	H			1	1	0	2	1	2	0	1	1	1
hairs			16	0	1	1	0	2	1	2	0	1	1	1
07 blood	34	C			1	2	1	1	1	2	6	1	1	1
hairs			18	0	1	2	1	1	1	2	6	1	1	1
08 blood	28	A			1	1	1	1	1	2	1	1	1	1
hairs			14		1	1	1	1	1	2	1	1	1	1
hairs			1	6.67	**1**	**1/3**	**1**	**1**	**1**	**2**	**1**	**1**	**1**	**1**
09 blood	25	A			1	1	1	1	1	2	2	1	1	1
hairs			12		1	1	1	1	1	2	2	1	1	1
hairs			2	14.29	**1**	**1**	**1**	**1**	**1**	**2**	**1/2**	**1**	**1**	**1**
10 blood	27	C			1	1	1	1	1	2	3	1	1	1
hairs			16		1	1	1	1	1	2	3	1	1	1
hairs			1	5.88	**1**	**1**	**1**	**1**	**1**	**2**	**1/3**	**1**	**1**	**1**
11 blood	26	C			1	1	1	1	1	2	1	1	1	1
hairs			16	0	1	1	1	1	1	2	1	1	1	1
12 blood	32	C			1	1	2	1	1	1	1	1	1	1
hairs			19	0	1	1	2	1	1	1	1	1	1	1
13 blood	27	A			1	1	1	2	1	2	1	0	1	0
hairs			14		1	1	1	2	1	2	1	0	1	0
hairs			1	6.67	**1**	**1**	**1**	**2**	**1**	**2**	**1/3**	**0**	**1**	**0**
14 blood	28	A			1	1	0	1	1	2	2	0	1	0
hairs			21	0	1	1	0	1	1	2	2	0	1	0
15 blood	28	A			1	1	1	2	1	2	1	1	1	1
hairs			16	0	1	1	1	2	1	2	1	1	1	1
16 blood	26	A			1	3	3	1	1	2	5	1	1	0
hairs			11		1	3	3	1	1	2	5	1	1	0
hairs			1	8.33	**1**	**3**	**3**	**1**	**1**	**2**	**5**	**1/2**	**1**	**0**
17 blood	19	H			1	1	1	1	1	2	1	1	0	1
hairs			14	0	1	1	1	1	1	2	1	1	0	1
18 blood	22	H			1	1	0	2	1	2	0	1	1	1
hairs			14	0	1	1	0	2	1	2	0	1	1	1
19 blood	26	H			1	1	0	2	1	2	0	1	1	1
hairs			11	0	1	1	0	2	1	2	0	1	1	1
20 blood	25	A			1	1	3	1	1	2	5	1	1	1
hairs			12		1	1	3	1	1	2	5	1	1	1
hairs			2	14.29	**1**	**1**	**3**	**1**	**1**	**2**	**5**	**1**	**1**	**1/2**
21 blood	21	H			1	1	1	2	1	2	1	1	1	1
hairs			18	0	1	1	1	2	1	2	1	1	1	1
22 blood	27	C			1	2	1	1	1	2	1	1	1	1
hairs			12		1	2	1	1	1	2	1	1	1	1
hairs			5	29.40	**1**	**2**	**1/2**	**1**	**1**	**2**	**1**	**1**	**1**	**1**
23 blood	27	A			1	3	1	1	1	2	5	0	1	w1
hairs			17		1	3	1	1	1	2	5	0	1	w1
hairs			2	10.5	**1**	**1/3**	**1**	**1**	**1**	**2**	**5**	**0**	**1**	**w1**
24 blood	27	H			1	1	0	1	1	2	1	1	1	1
hairs			19	0	1	1	0	1	1	2	1	1	1	1
25 blood	39	H			1	1	1	1	2	2	w7	4	1	0
hairs			2	0	1	1	1	1	2	2	w7	4	1	0
26 blood	24	AA			1	0	1	1	1	2	3	0	1	2
hairs			12		1	0	1	1	1	2	3	0	1	2
hairs			2	14.29	**1**	**0**	**1**	**1**	**1**	**2**	**1/3**	**0**	**1**	**2**
27 blood	26	H			1	1	0	2	1	2	0	1	1	1
hairs			17	0	1	1	0	2	1	2	0	1	1	1
28 blood	23	A			1	1	1	2	1	2	1	1	1	1
hairs			13	0	1	1	1	2	1	2	1	1	1	1
29 blood	29	C			1	1	1	1	1	2	1	1	1	1
hairs			15		1	1	1	1	1	2	1	1	1	1
hairs			1	6.25	**1**	**1/2**	**1**	**1**	**1**	**2**	**1**	**1**	**1**	**1**

TABLE 2—*Continued*.

Sample #	Age	Race	# Hairs Obs.	% Hairs Het.	Hair and Blood SSOP Haplotype									
					16093	IA	IC	ID	IE	IIA	IIB	IIC	IID	189
30 blood	28	C			1	1	1	1	1	1	5	1	1	1
hairs			16	0	1	1	1	1	1	1	5	1	1	1
31 blood	24	C			1	1	3	1	1	2	1	1	1	1
hairs			5	0	1	1	3	1	1	2	1	1	1	1
32 blood	24	C			**1/2**	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**1**
hairs			5		1	1	1	1	1	1	1	1	1	1
hairs			3	37.5	**1/2**	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**1**
33 blood	33	C			1	2	1	1	1	2	1	1	1	0
hairs			15	0	1	2	1	1	1	2	1	1	1	0
34 blood	80	C			1	1	1	1	1	2	1	0	1	2
hairs			15		1	1	1	1	1	2	1	0	1	2
hairs			1	6.25	**1**	**1**	**1**	**1**	**1**	**2**	**1**	**0**	**1**	**1/2**
35 blood	38	H			1	1	1	1	1	2	1	1	1	1
hairs			16		1	1	1	1	1	2	1	1	1	1
hairs			1	5.88	**1**	**1**	**1**	**1**	**1**	**2**	**1**	**1/2**	**1**	**1**
36 blood	56	C			1	2	1	1	1	2	1	1	1	1
hairs			16	0	1	2	1	1	1	2	1	1	1	1
37 blood	41	AA		Not Available										
hairs			20	0	1	3	3	0	0	2	3	0	2	0
38 blood	45	H			1	1	0	2	1	2	0	1	1	1
hairs			20	0	1	1	0	2	1	2	0	1	1	1
39 blood	83	C			1	1	1	1	1	1	0	w1	1	w1
hairs			15	0	1	1	1	1	1	1	0	w1	1	w1
40 blood	30	C			**2**	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**1**
hairs			9		**1/2**	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**1**
hairs			1	90	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**1**
41 blood	50	C			2	1	1	1	1	2	3	1	w1	1
hairs			3		2	1	1	1	1	2	3	1	w1	1
hairs			7	70	**1/2**	**1**	**1**	**1**	**1**	**2**	**3**	**1**	**w1**	**1**
42 blood	39	AA			1	1	3	2	1	2	7	2	1	1
hairs			11	0	1	1	3	2	1	2	7	2	1	1
43 blood	41	H			1	1	1	1	1	1	1	2	1	1
hairs			11		1	1	1	1	1	1	1	2	1	1
hairs			4	26.67	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**1/w2**	**1**	**1**
44 blood	23	C	6	58.3	**1**	**1**	**1**	**1**	**1**	**1**	**1/w3**	**1**	**1**	**1**
hairs			1		**1**	**1**	**1**	**1**	**1**	**1**	**w1/3**	**1**	**1**	**1**
hairs			4		1	1	1	1	1	1	3	1	1	1
45 blood	22	H			1	1	0	2	1	2	0	1	1	1
hairs			13	0	1	1	0	2	1	2	0	1	1	1
46 blood	44	C			1	1	1	1	1	1	1	2	1	1
hairs			7		1	1	1	1	1	1	1	2	1	1
hairs			3	30	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**1/2**	**1**	**1**
47 blood	26	AA			1	1	0	1	1	2	5	4	1	0
hairs			20	0	1	1	0	1	1	2	5	4	1	0
48 blood	49	C			1	1	1	1	1	1	1	1	1	1
hairs			16		1	1	1	1	1	1	1	1	1	1
hairs			1	5.88	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**1/2**	**1**	**1**
49 blood	38	C			1	1	1	1	w1	2	1	1	1	1
hairs			15		1	1	1	1	w1	2	1	1	1	1
hairs			2	11.76	**1**	**1**	**1**	**1**	**w1**	**2**	**1**	**1**	**1**	**1/2**
50 blood	59	C			1	1	3	1	1	1	1	1	1	1
hairs			18	0	1	1	3	1	1	1	1	1	1	1
51 blood	47	C			1	2	1	1	1	2	1	1	1	0
hairs			13	0	1	2	1	1	1	2	1	1	1	0
52 blood	41	AA/Other			1	1	1	1	1	2	1	1	0	1
hairs			7	0	1	1	1	1	1	2	1	1	0	1
53 blood	29	A			1	1	1	1	1	2	1	1	1	1
hairs			9	0	1	1	1	1	1	2	1	1	1	1
54 blood	46	C			1	1	3	1	1	2	1	1	1	1
hairs			15	0	1	1	3	1	1	2	1	1	1	1
55 blood	45	C			1	1	1	1	1	2	3	1	1	1
hairs			15	0	1	1	1	1	1	2	3	1	1	1
56 blood	62	H			1	1	1	1	1	2	1	1	1	1
hairs			16	0	1	1	1	1	1	2	1	1	1	1
57 blood	28	H			1	3	3	1	1	2	4	1	0	1
hairs			12	0	1	3	3	1	1	2	4	1	0	1
58 blood	41	C			1	1	2	1	1	1	1	0	1	0
hairs			17		1	1	2	1	1	1	1	0	1	0
hairs			1	5.56	**1**	**1**	**1/2**	**1**	**1**	**1**	**1**	**0**	**1**	**0**

TABLE 2—*Continued*.

Sample #	Age	Race	# Hairs Obs.	% Hairs Het.	Hair and Blood SSOP Haplotype									
					16093	IA	IC	ID	IE	IIA	IIB	IIC	IID	189
59 blood	65	H			1	1	0	2	1	2	0	1	1	1
hairs			16	0	1	1	0	2	1	2	0	1	1	1
60 blood	66	C			1	1	1	1	1	1	1	1	1	1
hairs			15	0	1	1	1	1	1	1	1	1	1	1
61 blood	23	C			1	2	1	1	1	2	1	1	1	1
hairs			16		1	2	1	1	1	2	1	1	1	1
hairs			1	5.88	**1**	**2**	**1**	**1**	**1**	**2**	**1**	**1**	**1**	**1/2**
62 blood	79	C			**1/2**	**1**	**3**	**1**	**1**	**2**	**1**	**1**	**1**	**1**
hairs			3		1	1	3	1	1	2	1	1	1	1
hairs			14	82.35	**1/2**	**1**	**3**	**1**	**1**	**2**	**1**	**1**	**1**	**1**
63 blood	36	H			2	1	0	2	1	2	0	1	1	1
hairs			6	42.86	**1/2**	**1**	**0**	**2**	**1**	**2**	**0**	**1**	**1**	**1**
hairs			6		2	1	0	2	1	2	0	1	1	1
hairs			2		1	1	0	2	1	2	0	1	1	1
64 blood	63	AA			1	3	3	0	2	2	0	0	2	0
hairs			16	0	1	3	3	0	2	2	0	0	2	0
65 blood	63	AA			1	0	1	1	1	2	3	1	1	1
hairs			17	0	1	0	1	1	1	2	3	1	1	1
66 blood	44	A			1	1	3	1	1	2	1	1	1	1
hairs			13		1	1	3	1	1	2	1	1	1	1
hairs			1	7.14	**1**	**1**	**3**	**1**	**1**	**2**	**w1/3**	**1**	**1**	**1**
67 blood	49	H			1	1	1	1	1	2	1	1	1	1
hairs			9		1	1	1	1	1	2	1	1	1	1
hairs			2	18.18	**1**	**1**	**1**	**1**	**1**	**2**	**1/3**	**1**	**1**	**1**
68 blood	44	AA			1	3	3	0	2	2	0	0	2	0
hairs			2	0	1	3	3	0	2	2	0	0	2	0
69 blood	26	H			0	1	0	2	1	2	0	1	1	1
hairs			9	0	0	1	0	2	1	2	0	1	1	1
70 blood	74	C			1	3	1	1	1	2	3	0	0	w1
hairs			2	0	1	3	1	1	1	2	3	0	0	w1
71 blood	59	C			1	1	1	1	1	1	1	1	1	1
hairs			15	0	1	1	1	1	1	1	1	1	1	1
72 blood	60	C			1	2	1	1	1	2	1	0	1	0
hairs			15	0	1	2	1	1	1	2	1	0	1	0
73 blood	50	C			1	1	1	1	1	1	1	1	1	1
hairs			15	0	1	1	1	1	1	1	1	1	1	1
74 blood	62	C			1	1	3	1	3	2	5	1	1	1
hairs			11		1	1	3	1	3	2	5	1	1	1
hairs			5	31.25	**1**	**1**	**3**	**1**	**1/3**	**2**	**5**	**1**	**1**	**1**
75 blood	61	C			1	1	0	2	1	2	5	0	1	2
hair			8	0	1	1	0	2	1	2	5	0	1	2
76 blood	47	AA			1	1	1	1	2	2	7	4	1	0
hair			29	0	1	1	1	1	2	2	7	4	1	0
77 blood	24	C			2	1	3	1	1	2	3	2	1	1
hair			7		2	1	3	1	1	2	3	2	1	1
hair			12	63.16	**1/2**	**1**	**3**	**1**	**1**	**2**	**3**	**2**	**1**	**1**
78 blood	66	C			1	3	3	1	1	2	1	0	0	w1
hair			16	0	1	3	3	1	1	2	1	0	0	w1
79 blood	54	C			1	1	0	2	1	2	0	1	1	1
hair			17	0	1	1	0	2	1	2	0	1	1	1
80 blood	18	AA			0	1	0	1	2	2	5	0	1	w1
hair			9	0	0	1	0	1	2	2	5	0	1	w1
81 blood	22	AA			1	1	0	2	1	2	0	1	1	1
hair			13	0	1	1	0	2	1	2	0	1	1	1
82 blood	23	C			1	2	2	1	1	2	0	1	1	1
hair			18		1	2	2	1	1	2	0	1	1	1
hair			1	5.26	**1**	**2**	**1/2**	**1**	**1**	**2**	**0**	**1**	**1**	**1**
83 blood	23	A			1	1	1	1	1	2	0	0	1	w1
hair			15	0	1	1	1	1	1	2	0	0	1	w1
84 blood	53	H			1	1	1	1	1	2	0	1	1	1
hair			11	0	1	1	1	1	1	2	0	1	1	1
85 blood	37	H			1	1	1	1	1	2	1	1	1	1
hair			3		1	1	1	1	1	2	1	1	1	1
hair			6	66.67	**1**	**1**	**1**	**1**	**1**	**2**	**1/3**	**1**	**1**	**1**
86 blood	35	C			1	3	w1	1	1	1	1	1	1	1
hair			5	0	1	3	w1	1	1	1	1	1	1	1
87 blood	25	C			1	1	1	1	2	2	7	4	1	0
hair			3	0	1	1	1	1	2	2	7	4	1	0
88 blood	40	H			1	1	1	1	1	2	1	1	0	1
hair			18	0	1	1	1	1	1	2	1	1	0	1

TABLE 2—*Continued*.

Sample #	Age	Race	# Hairs Obs.	% Hairs Het.	Hair and Blood SSOP Haplotype									
					16093	IA	IC	ID	IE	IIA	IIB	IIC	IID	189
89 blood	88	AA			1	0	3	2	2	2	1	1	1	1
hair			8	0	1	0	3	2	2	2	1	1	1	1
90 blood	67	C			1	1	3	1	1	2	1	1	1	1
hair			11	0	1	1	3	1	1	2	1	1	1	1
91 blood	38	H			1	2	0	1	1	2	0	1	1	1
hair			18	0	1	2	0	1	1	2	0	1	1	1
92 blood	77	C			1	2	3	1	1	2	1	1	0	1
hair			8	0	1	2	3	1	1	2	1	1	0	1
93 blood	42	AA			1	1	3	1	2	2	7	0	1	0
hair			11	0	1	1	3	1	2	2	7	0	1	0
94 blood	48	A			1	1	1	2	2	2	2	1	1	1
hair			1	0	1	1	1	2	2	2	2	1	1	1
95 blood	20	H			1	1	0	1	1	2	0	1	1	1
hair			10	0	1	1	0	1	1	2	0	1	1	1
96 blood	62	AA			1	3	3	1	2	2	0	0	2	0
hair			3	0	1	3	3	1	2	2	0	0	2	0
97 blood	38	H			1	1	0	1	2	2	7	4	1	0
hair			12	0	1	1	0	1	2	2	7	4	1	0
98 blood	36	AA			1	0	0	1	1	2	3	1	1	1
hair			17	0	1	0	0	1	1	2	3	1	1	1
99 blood	48	A			1	1	1	2	1	2	1	0	1	0
hair			15	0	1	1	1	2	1	2	1	0	1	0
100 blood	43	A			1	1	1	2	2	2	1	1	1	1
hair			16		1	1	1	2	2	2	1	1	1	1
hair			1	5.88	**1**	**1**	**1**	**2**	**2**	**2**	**1**	**1**	**1**	**1/2**
101 blood	21	AA			1	1	1	2	2	2	w4	2	1	1
hair			1	0	1	1	1	2	2	2	w4	2	1	1
102 blood	33	A			1	1	1	1	1	2	5/w6	1	1	1
hair			11	0	1	1	1	1	1	2	5/w6	1	1	1
104 blood	31	A			1	3	1	1	1	2	1	1	1	1
hair			10	0	1	3	1	1	1	2	1	1	1	1
105 blood	31	A			1	1	1	1	1	2	1	1	1	1
hair			2	0	1	1	1	1	1	2	1	1	1	1
106 blood	55	AA			0	3	3	0	2	2	0	0	2	0
hair			3	0	0	3	3	0	2	2	0	0	2	0
108 blood	34	H			1	1	0	2	1	2	0	1	1	1
hair			9	0	1	1	0	2	1	2	0	1	1	1
109 blood	34	A			1	3	1	1	0	2	3	1	0	w1
hair			4	0	1	3	1	1	0	2	3	1	0	w1
110 blood	62	AA			1	2	3	1	0	2	3	0	2	0
hair			2		1	2	3	1	0	2	3	0	2	0
hair			1	33.33	**1/2**	**2**	**3**	**1**	**0**	**2**	**3**	**0**	**2**	**0**
111 blood	27	AA			1	1	1	2	2	2	4	2	1	1
hair			1	0	1	1	1	2	2	2	4	2	1	1
112 blood	59	AA			1	1	4	1	2	2	4	2	1	1
hair			12	0	1	1	4	1	2	2	4	2	1	1
113 blood	30	AA			1	3	3	1	2	2	w6	0	2	0
hair			11	0	1	3	3	1	2	2	w6	0	2	0
114 blood	82	AA			1	0	1	1	1	2	3	0	1	0
hair			15	0	1	0	1	1	1	2	3	0	1	0
115 blood	45	AA			1	2	3	1	0	2	3	2	2	0
hair			7	0	1	2	3	1	0	2	3	2	2	0
117 blood	71	AA			1	1	1	2	2	2	w4	2	1	1
hair			17	0	1	1	1	2	2	2	w4	2	1	1
118 blood	47	AA			1	1	1	1	2	2	7	4	1	0
hair			11	0	1	1	1	1	2	2	7	4	1	0
119 blood	23	AA			0	3	1	0	2	2	0	0	2	0
hair			5	0	0	3	1	0	2	2	0	0	2	0
120 blood	33	A			1	1	1	2	2	2	1	1	1	1
hair			7	0	1	1	1	2	2	2	1	1	1	1
121 blood	24	AA			1	1	1	1	2	2	7	4	1	0
hair			2	0	1	1	1	1	2	2	7	4	1	0
123 blood	29	C			1	1	3	1	1	1	1	1	1	1
hair			8		1	1	3	1	1	1	1	1	1	1
hair			1	11.11	**1**	**1**	**3**	**1**	**1**	**1**	**1**	**1/2**	**1**	**1**
124 blood	48	AA			1	3	3	w1	1	1	1	w5	2	0
hair			7	0	1	3	3	w1	1	1	1	w5	2	0
125 blood	57	AA			1	3	3	0	2	2	0	0	2	0
hair			6	0	1	3	3	0	2	2	0	0	2	0

TABLE 2—*Continued.*

Sample #	Age	Race	# Hairs Obs.	% Hairs Het.	Hair and Blood SSOP Haplotype									
					16093	IA	IC	ID	IE	IIA	IIB	IIC	IID	189
126 blood	66	AA			1	2	3	1	0	2	3	0	2	0
hair			15	0	1	2	3	1	0	2	3	0	2	0
127 blood	73	AA			1	1	3	1	1	2	5	w5	1	0
hair			18	0	1	1	3	1	1	2	5	w5	1	0
128 blood	19	H			1	1	0	2	1	2	0	1	1	0
hair			9	0	1	1	0	2	1	2	0	1	1	0
129 blood	23	C			1	1	1	1	1	1	1	1	1	1
hair			4	0	1	1	1	1	1	1	1	1	1	1
130 blood	21	H			1	1	0	2	1	2	0	1	1	1
hair			4	0	1	1	0	2	1	2	0	1	1	1
131 blood	27	C			1	3	3	1	1	2	1	0	0	2
hair			4	0	1	3	3	1	1	2	1	0	0	2
132 blood	44	AA			1	3	3	0	2	2	0	0	2	0
hair			2	0	1	3	3	0	2	2	0	0	2	0

A, U.S. Asian; AA, U.S. African American; C, U.S. Caucasian; H, U.S. Hispanic. Values in bold represent samples where sequence heteroplasmy was detected with the LINEAR ARRAY™ assay.

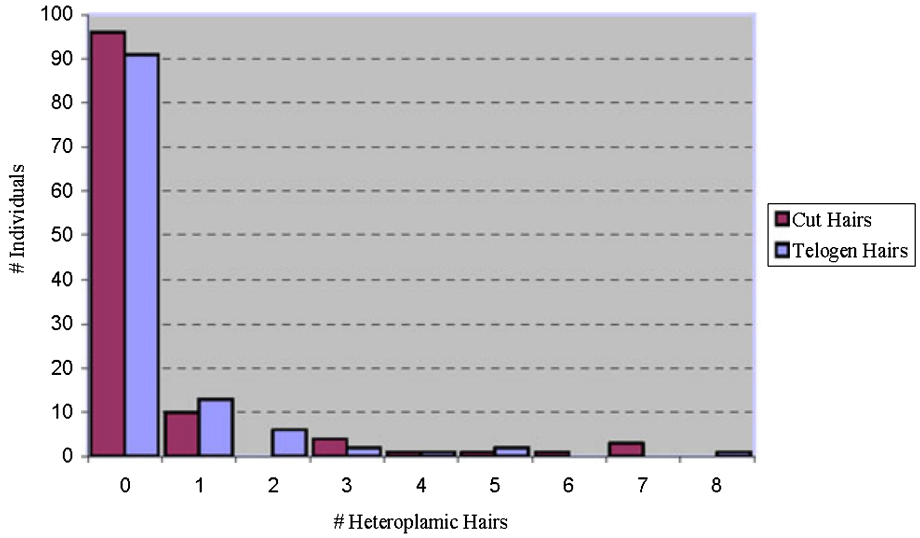

FIG. 1—*Frequency distribution of heteroplasmy in hairs.*

TABLE 3—*Pigmentation range of hair shaft (all hairs/excluding dyed hairs).*

Pigmentation of Hair Shaft	Homoplasmy All hairs*/excl. dyed**	Heteroplasmy All hairs*/excl. dyed**	Total All hairs*/excl. dyed**	% Sequence Heteroplasmy All hairs*/excl. dyed**
No Pigment	127/79	14/12	141/91	9.9/13.2
Light–Medium Blonde, Dark Blonde, Light Golden Brown, Light Brown	254/134	37/25	291/159	12.7/15.7
Light Gray Brown, Medium–Dark Golden Brown to Medium Brown	333/225	28/7	361/232	7.8/3.0
Dark Brown, Medium–Dark Gray Brown	521/349	21/14	542/363	3.9/3.9
Light Red, Light–Medium Red Brown, Medium–Dark Red, Dark Red Brown	117/31	5/1	122/32	4.1/3.1

*Excludes hairs where no color was listed. $N = 1457$; Pearson chi-square, $\Sigma\chi^2 = 25.690$; df = 4; Likelihood ratio = 25.259. Statistically significant at 95% level.

**Excludes hairs where no color was recorded and hairs of individuals we reported dying their hair. $N = 877$; Pearson chi-square, $\Sigma\chi^2 = 37.075$; df = 4; Likelihood ratio = 32.804. Statistically significant at 95% level.

significance level) between heteroplasmy and the race of an individual when comparing across all four of the population groups ($N = 115$; Pearson chi-square, $\Sigma\chi^2 = 11.103$; df = 3; Likelihood ratio = 12.263). However, a higher proportion of heteroplasmic cases are observed within the U.S. Caucasian population group relative to the U.S. African American population group: 18 U.S. Caucasian individuals (43%) versus 2 U.S. African American individuals (8%) exhibit the heteroplasmic condition. Further, the data indicate that there is no significant correlation (95% significance level) between heteroplasmy and the age-group of the donor;

TABLE 4—*Observed heteroplasmic and homoplasmic individuals across age and racial group.*

Age Range (Years)	Homoplasmy				Heteroplasmy				
Racial Group	C	AA	H	A	C	AA	H	A	Total
18–29	5	4	13	8	9	1	0	7	47
30–49	7	9	5	6	5	0	5	2	39
50–69	10	5	3	0	2	1	0	0	21
70 and above	2	4	0	0	2	0	0	0	8

however, sequence heteroplasmy shows a tendency to decrease as the age-group of the donor increases ($N = 116$; Pearson chi-square, $\Sigma\chi^2 = 3.436$; df = 3; Likelihood ratio = 3.734). This data is delineated as a function of age and racial group (Table 4). Analysis of gender as a function of heteroplasmy revealed that there is no statistically significant difference between men (25.5% sequence heteroplasmy) and women (32.8% sequence heteroplasmy) subjects and the heteroplasmic condition ($N = 116$; Pearson chi-square, $\Sigma\chi^2 = 0.750$; df = 1; Likelihood ratio = 0.438).

Detection of Sequence Heteroplasmy Summary

Given that the mtDNA genome is haploid, the observation of two probe signals within a probe region may be due to heteroplasmy. Of the 127 samples of mtDNA extracted from blood and typed with the mtDNA LINEAR ARRAY™ assay, three (2.36%) appear to have two sequences present. These are summarized as follows: sample #32 indicates a 1/2 mixture at 16093 (cycling sequencing 16093 T > C); sample #44 (Fig. 3*C*) indicates a 1/w3 mixture at HVIIB (cycle sequencing 152 T > C); and sample #62 indicates a 1/2 mixture at 16093 (cycling sequencing 16093 T = C). All of the three individuals who show a mixture of haplotypes in their blood samples also exhibited varying degrees of heteroplasmy in their hair samples. Sample #32 indicates a 1/2 mixture at 16093 in three hairs; sample #44 indicates a 1/3 mixture at HVIIB in seven hairs; and sample #62 indicates a 1/2 mixture at 16093 in 14 hairs. In addition, 31 individuals reveal heteroplasmy exclusively in their hair samples; the percentage of hairs per individual exhibiting heteroplasmy ranges from 5.88 to 90%. These results are summarized in Table 2 and Fig. 2. For the 34 individuals exhibiting heteroplasmy, seven are observed in the HVI region and 27 are observed in HVII. One common heteroplasmic site was 16093 (e.g., individual 63, in Fig. 3*D*); seven of the 34 individuals exhibited a mixture in their hairs at this site. Further analysis of these seven individuals reveals that one individual also exhibits the mixture in their blood, four individuals exhibit a "2" in their blood, and two individuals exhibit a "1" in their blood. The point mutation sites observed for hairs in this study is illustrated in Table 5. A summary of mtDNA sequence heteroplasmy per site is categorization by race in Table 6.

Discussion

LINEAR ARRAY™ assay typing is a simple technique. However, the fact that it is a hybridization technique means that stringency conditions are greatly influenced by fluctuating incubation temperatures. The temperature must be maintained at 55 ± 1°C to avoid nonspecific probe hybridization (reduced temperatures) or weak signal intensity/signal dropout (elevated temperatures). Two other factors may contribute to probe cross-hybridization or signal

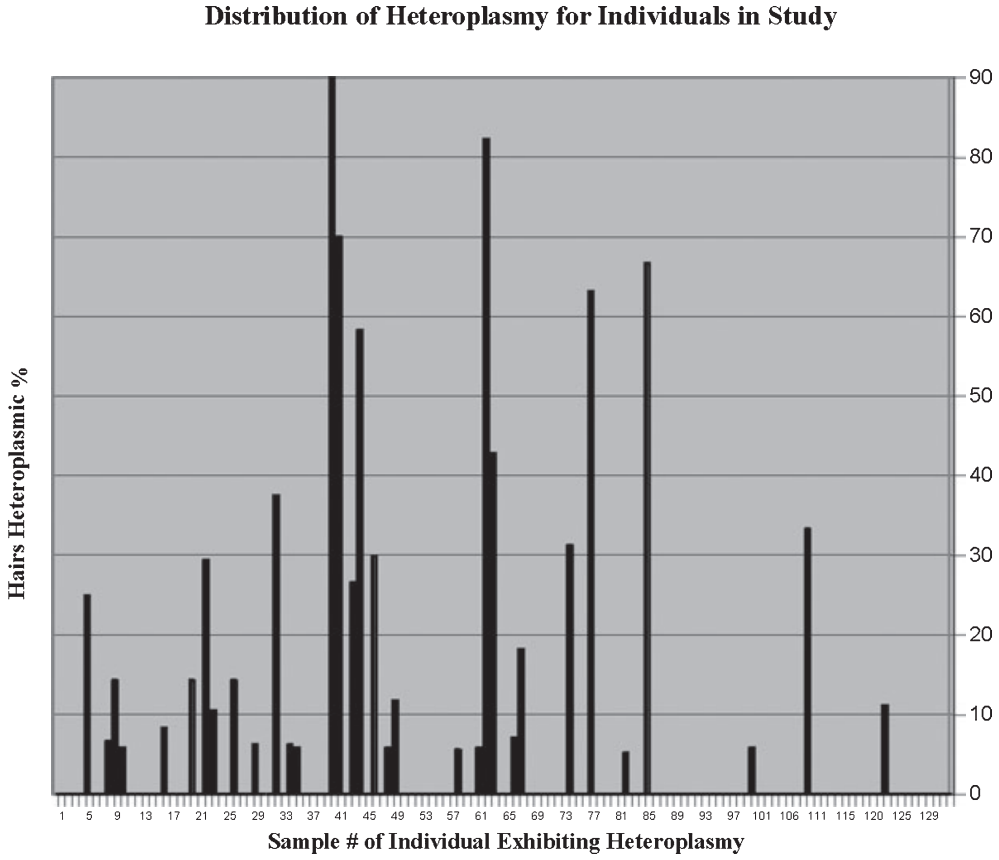

FIG. 2—*Distribution of heteroplasmy across all individuals.*

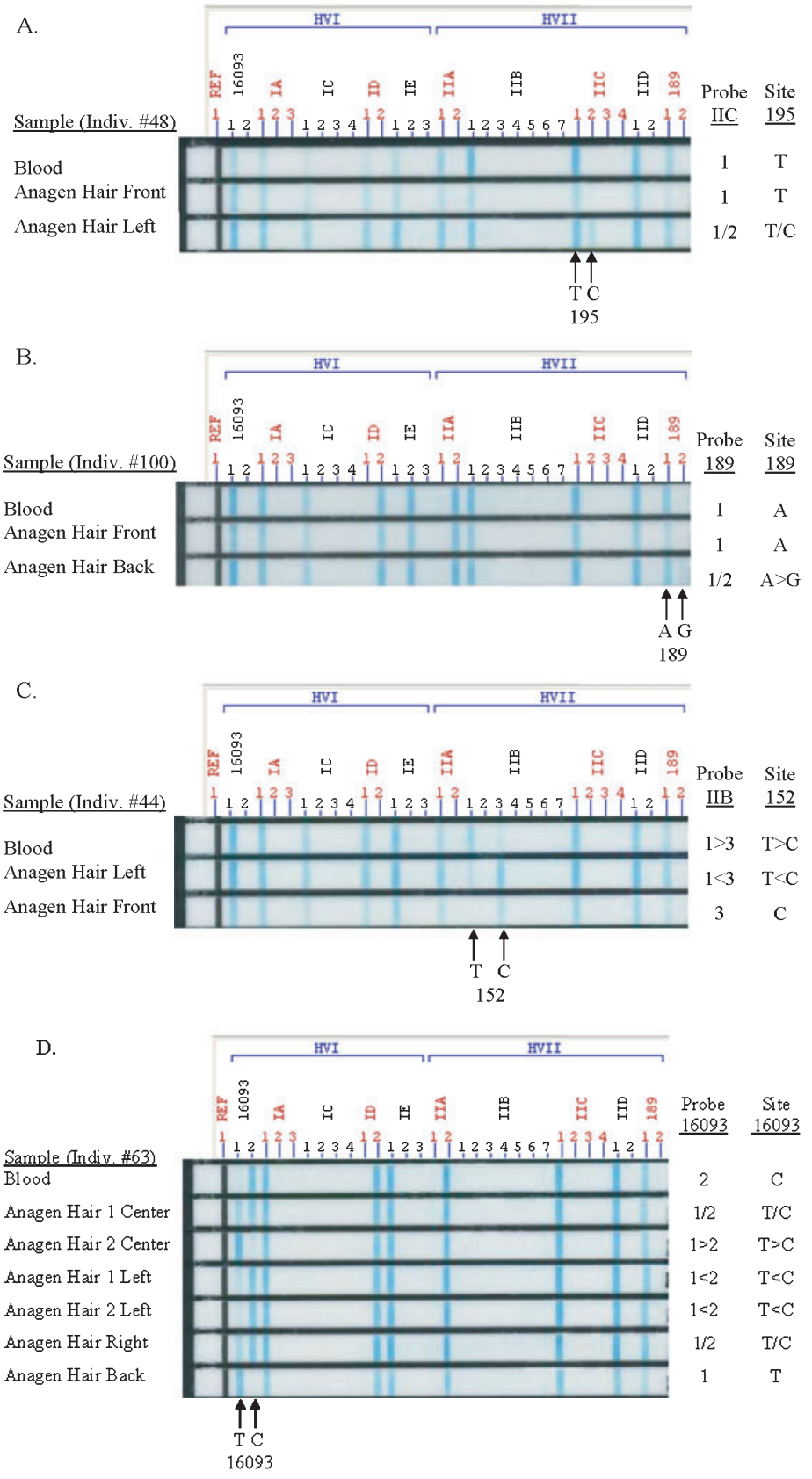

FIG. 3—*LINEAR ARRAY™ assay typing data for selected blood and hair samples: individual 48 (A), individual 100 (B), individual 44 (C), and individual 63 (D).*

TABLE 5—*Summary of mtDNA sequence heteroplasmy.*

Probe Designation	Probe Signal Observed	Corresponding Position	# Individuals	% Heteroplasmic Individuals
HVII 16093	1/2	16093 T/C	7	20.6
HVII B	1/3	152 T/C	8	23.5
HVII C	1/2	195 T/C	6	17.7
HVII 189	1/2	189 A/G	5	14.7
HVI C	1/2	16304 T/C	3	8.8
HVI A	1/2	16126 T/C	2	5.9
HVI A	1/3	16129 G/A	1	5.9
HVI E	1/3	16270 C/T	1	2.9
HVII B	1/2	145 T/C	1	2.9

dropout: the amount of sample input into the typing reaction and the development time of the LINEAR ARRAY™s. As indicated previously, sample input should be monitored to avoid probe saturation (excess sample) or weak signal intensities (limited sample). Similarly, shorter development times generate weak signal intensities, whereas over-development may result in the formation of weak bands at multiple locations (cross-hybridization).

Controlling for the above experimental parameters, one of four probe signal patterns may be detected on the LINEAR ARRAY™ assay. A single probe generates a positive signal; a single probe gives a relatively weak signal; no probe signals are detected within a region; or two probe signals are visible within a region (either with equal or uneven intensity). A weak or absent signal at a particular region is attributed to a sequence variant at the probe-binding site that causes a destabilization of the probe–template interaction. When the destabilization is extreme, the template fails to hybridize and the region is assigned a "0." If the destabilization reduces the template binding, the signal intensity is likewise reduced and the region is assigned the prefix "w." If two probes signals are detected in a region both probe designations are noted. For example, the designation 1/w3 at IIB indicates that two probe signals are visible: variant "1" is of normal intensity, whereas variant "3" gives a relatively weak signal. It should be noted that the proportion of each variant is not quantitative and is based on a visual estimation of intensity. The genetic diversity of the assay has been widely reported (31,32).

When two probes within a region hybridize to the PCR product, this may indicate heteroplasmy. However, it may also be an indication of a mixture of more than one contributor. To eliminate contamination as a source of heteroplasmy, pre-PCR and PCR controls should be performed and extraction/amplification reactions should be run in duplicate. The type of the analysts should also be determined to rule them out as the source of contamination. Finally, cycle sequencing of the HVI/II region can be performed on all probative samples to confirm the LINEAR ARRAY™ results. Additional precautions are necessary when analyzing hair. For example, prescreening samples microscopically provides a means of assessing hairs to determine the species, somatic origin, root morphology, possible racial origin, and whether cosmetic treatments have been used. In this study, for example, if an individual reported using a chemical dye, this was confirmed by microscopical examination. It also allows the examiner to identify outlier hairs that are not representative of the sample (i.e., hairs that may have been collected as a result of a secondary transfer). Further, it permits an examination of potential extraneous biological material coating the surface of the hair that may contribute to a mixture (for example, traces of blood or semen). This study further demonstrates that detectable levels of sequence heteroplasmy can vary between and within an individual. For blood, the condition was observed in three individuals (2.4%) compared to 34 individuals (29.3%) who expressed the condition in at least one hair. Within individuals, the proportion of hairs with detectable levels of sequence heteroplasmy varied from 0 to 90%. Sequence heteroplasmy was observed in 7% of all hairs in the present study (N = 111; 111/1589). Although not a direct comparison, a study of 691 casework hairs resulted in a sequence heteroplasmy frequency of 11.4%, when considering independent observations (28).

A further casework study reports observing site heteroplasmy in 9.7% (N = 14; 14/144) of casework hairs (47). A number of factors may be contributing to the lower incidence of sequence heteroplasmy in the present study compared to the two casework studies. First, the variation in mutation sites across the studies may be contributing to the differences in sequence heteroplasmy. The difference may also be attributed to detection methodology; both casework studies employed direct sequencing to detect site heteroplasmy, while the present study utilized the LINEAR ARRAY™ assay. Given that the assay only detects select sites within the control region, it may be underestimating site heteroplasmy; however, the increased sensitivity of the LINEAR ARRAY™ assay in detecting the minor component may have a counteracting effect. It should also be noted that in the present study, an individual was categorized as exhibiting heteroplasmy or homoplasmy if a mitotype of normal intensity was obtained for a minimum of four hairs. This criterion excluded 12 individuals who would otherwise have been classified as homoplasmic, and this exclusion influenced the frequency of sequence heteroplasmy. Finally, the sample size (number of individuals and number of hairs) examined for each individual may also account for the differences observed. Perhaps a better comparison would be the incidence of site heteroplasmy across and within individuals rather than as a function of the totality of hairs evaluated. However, this comparison is not possible because although the number of cases manifesting site heteroplasmy in at least one hair is provided, the number of cases with exclusively homoplasmic hairs is unknown. Another study of 480 hairs detected sequence heteroplasmy in all three of the individuals evaluated; the frequency of sequence heteroplasmy among hairs from each individual varied from 1.25 to 8.75% with direct sequencing and 3.75 to 8.75% with DGGE (27). In comparison, the frequency of sequence heteroplasmy among hairs from each individual in the

TABLE 6—*mtDNA sequence heteroplasmy per site for all hairs (hairs) and individuals (ind) across racial groups.*

	Heteroplasmy Site 16093 (1/2)		Heteroplasmy Site HVIIB (1/3)		Heteroplasmy Site HVIIC (1/2)		Heteroplasmy Site 189 (1/2)	
Race	Homo* ind/hairs	Het ind/hairs	Homo* ind/hairs	Het ind/hairs	Homo* ind/hairs	Het ind/hairs	Homo* ind/hairs	Het ind/hairs
U.S. Caucasian	37/540	5/45	40/577	2/8	39/580	3/5	39/581	3/4
U.S. Asian	23/322	0/0	20/317	3/5	22/321	1/1	21/319	2/3
U.S. Hispanic	25/345	1/6	24/373	2/8	24/346	2/5	26/351	0/0
U.S. African Amercian	23/323	1/1	23/322	1/2	24/324	0/0	24/324	0/0

*Homoplasmic at site reported; may be heteroplasmic at another site.

present study varied from 0 to 90% with the LINEAR ARRAY™ assay. This difference may be attributed to the sample size obtained from each individual in the study reported here. Again, differences in mutation site and sensitivity method may also be contributing factors.

When heteroplasmy was observed in the donor's blood sample, typically, multiple hairs were heteroplasmic for these individuals. Individuals with homoplasmic blood manifested heteroplasmy in one or two hairs (21 individuals), three to four hairs (three individuals), and five or more hairs (seven individuals). When five or more hairs exhibit heteroplasmy when the blood is homoplasmic, we observed that the mutated type is most commonly because of a mutation at the C16093T base pair position. One individual (#110) whose blood exhibited a 1 at 16093 had a single hair with a 1/2 mixture at this position; however, this analysis was based on a small sample size of three hairs. In contrast, all individuals whose blood exhibited a 2 at 16093 had multiple hairs with a 1/2 mixture at this position. As inter-tissue sequence variation is a possibility, blood may not be the best reference for comparing other tissues. Therefore, it is better to compare corresponding tissues, e.g., multiple hairs with each other rather than with the blood sample.

The most common positions for site heteroplasmy in biological samples reported in the literature are 16093 and 189. However, heteroplasmy has also been observed at positions 73, 189, 152, and 195. Therefore, the results of this study are consistent with previously reported heteroplasmic "hotspots."

The results of this study show no correlation between the detection of heteroplasmy and the use of cosmetic treatments. This is true regardless of the type of treatment reported. This suggests that provided the sample amplifies, chemical treatments do not reduce the level of the secondary sequence below the detection threshold. For example, four individuals (three reported no treatment and one reported using a chemical dye) exhibit a "2" at base pair position 16093 for their blood type and all four exhibit a 1/2 mixture at this position for multiple hairs. Also, one individual who reported using a chemical dye exhibited heteroplasmy in donated blood and in multiple hairs.

The data also indicate that the frequency of heteroplasmy does not differ in living compared to deceased individuals. The three individuals (two living, one deceased) who show heteroplasmy in blood exhibited similar patterns of heteroplasmy in their hair. This suggests that postmortem changes that occur in hair do not necessarily influence the detection of heteroplasmy; however, the limited sample size precludes further statistical analysis. In addition, the data from this study suggest that the frequency of heteroplasmy in human hair does not differ significantly with age. This contrasts with the findings of an earlier study that the frequency of heteroplasmy in muscle and brain tissue increases with age (23). We have identified three possible explanations that may account for this difference. First, the sampling is skewed toward the younger individuals, and under representation of individuals in the older age range may be contributing to the statistical data. Second, the mutation may be tissue specific. Finally, germ line mutation may be contributing to the sequence heteroplasmy detected in hair, particularly when the condition is detected in both the blood and the hair tissue within an individual or when the condition is detected in multiple hairs within an individual. Mutations that increase with age indicate a somatic origin, whereas heteroplasmy across multiple tissues is presumably because of a germ line mutation, with replicative segregation (12) possibly accounting for differences in the level of heteroplasmy across tissues. They may also be because of somatic mutations occurring very early on in the development of the zygote prior to tissue differentiation. In the current study, the origin of the mutation cannot be confirmed because maternal reference samples are not available; however, we are currently engaged in a study that is designed to characterize the origin of sequence heteroplasmy.

Based on the results of the present study, there is no significant difference in the incidence of heteroplasmy across different regions of the scalp. This alludes to the possibility that heteroplasmic hairs are randomly distributed in the scalp rather than forming a mosaicism in one particular region. The implication is that if current hair collection procedures are followed, a representative sample of heteroplasmic hairs will also be collected. As expected, there was no difference in the frequency of heteroplasmy observed in comparing male and female subjects.

Additionally, the findings show that the frequency of heteroplasmy was higher in hairs from the U.S. Asian (39%) and U.S. Caucasian (43%) population groups than the U.S. African American (8%) and U.S. Hispanic (19%) groups. Conversely, when considering all four groups, there was no statistically significant correlation between race and the incidence of heteroplasmy in hair. Linch et al. propose that hairs with continuous, broad medullas would be expected to have less mtDNA debris, and therefore less mtDNA molecules, than hairs without a visible medulla (48). However, the present study supports that there was also no correlation between the medulla structure of a hair and observation of sequence heteroplasmy. Linch et al. also propose that the hair shaft may comprise at least two populations of mitochondria: one derived from the germinal bulb matrix cells and the other from melanocytes (48). This would suggest that the incidence of observed heteroplasmy might be higher for naturally heavily pigmented hair. Hair pigmentation does appear to correlate with heteroplasmy in the present study. However, a higher than expected incidence of heteroplasmy was observed for lighter-pigmented hairs and a lower than expect incidence of heteroplasmy was observed for darker-pigmented hairs. One potential explanation for this finding is that it may be attributed to the disproportionate representation of U.S. Caucasians, particularly in relation to the 16093 base pair mutation site.

One limitation of the version of the arrays used in the present study is that probes were not included that identify length heteroplasmy. A further limitation of LINEAR ARRAY™ assay analysis is that the panel was limited to detection of sequence polymorphism at 18 positions. Although the panel targets several heteroplasmic hotspots in addition to the most polymorphic regions of the control region, this method may underestimate the level of heteroplasmy in the HVI/HVII region because not all sites are analyzed. However, because of the increased sensitivity of the LINEAR ARRAY™ compared to Sanger sequencing, there is a greater chance of detecting sequence heteroplasmy at these sites. Studies indicate that heteroplasmic mixtures in peripheral blood are detected by sequence analysis if the minor component is >10–20% of the overall mixture. Thus, sequence analysis can usually detect major and minor variants if the ratio is in the order of 10:1 to 5:1 (49). In comparison, the results of the LINEAR ARRAY™ sensitivity indicate that the minor component is detectable down to a 20:1 ratio (31,49). However, although the LINEAR ARRAY™ assay is considered more sensitive that direct sequencing, heteroplasmy below this level may not be detected. This may account for the observation of intra-tissue variation in blood and hair for an individual.

This research presents a clearer understanding of some of the issues associated with the phenomenon of heteroplasmy and its detection in human hair and bloodstains. It also highlights the

potential of the analysis—absent precautionary measures—to generate both false exclusions and false inclusions. Current interpretation guidelines take into account the inter- and intra-individual differences that may be observed (50). Present guidelines dictate that when the mtDNA sequences of the reference and known heteroplasmic samples are being compared, both demonstrate heteroplasmy at the same site, the interpretation is cannot exclude (or concordance). If the condition is identified in either the evidence or the reference sample and both samples are otherwise concordant, the guidelines indicate that this is also reported as a failure to exclude. Given the potential for inter-tissue (blood vs. hairs) and intra-tissue variation (among a random sampling of hairs), the current study supports this conclusion. Further, an inconclusive result is reported if both samples are homoplasmic and differ at only a single base pair position. Given the potential for a genetic switch, two samples originating from a single donor may be homoplasmic and differ at only a single base pair position. This scenario was observed in samples analyzed in the present study. Finally, two homoplasmic samples must exhibit at least two base differences before rendering exclusions.

The study reported here represents one of the largest studies to evaluate hair morphology versus heteroplasmy. In conclusion, our research demonstrates differences in heteroplasmic expression between hair and blood tissue. Further, differences in expression were also observed within each respective tissue. The frequency of sequence heteroplasmy among hairs from each individual in the present study varied from 0 to 90% with the LINEAR ARRAY™ assay. This suggests that a sufficient number of hair samples may need to be analyzed to gain a representative sampling. The frequency of heteroplasmy across racial population groups is higher in the U.S. Asian and U.S. Caucasian population; however, there is no statistical correlation across all population groups. Further, there is no statistical correlation between the heteroplasmic condition and each of the following independent variables: cosmetic treatment, when comparing living and deceased donors, medulla morphology, hair growth phase, the region of the scalp, age of donor, or gender. This suggests that the heteroplasmic condition is successfully detected in hairs regardless of exposure to potentially compromising factors, such as the presence of cosmetic chemicals or when hairs are exposed to the effects of postmortem decomposition. Further, there is no indication that morphological characteristics, such as medulla structure or the presence/absence of a root may be predictive of the heteroplasmic condition. It is possible that the detection of heteroplasmy may vary based on the region sampled along the length of the hair, but this was not tested in the present study. It should also be noted that when hair morphology examinations precede mtDNA analysis, this might contribute to reducing the number of false inclusions. Further, the finding that hairs exhibiting heteroplasmy is not dependent on the region recovered from the scalp clarifies hair collection issues: provided current hair collection procedures are followed, a representative sample of heteroplasmic hairs will also be collected. Although the findings of this study do not support that heteroplasmy is correlated with the age of a donor, it is possible that somatic versus germ line heteroplasmy is site specific. However, the results do support a statistically significant correlation between heteroplasmy and hair pigmentation; typically, lighter-pigmented hairs exhibit a higher incidence of sequence heteroplasmy when compared to darker hairs. Finally, the percentage of hairs within an individual that exhibit heteroplasmy varies from 0 to 90%. The actual proportion observed may be related to the mutation site; in the present study, a higher percentage of heteroplasmic hairs were observed for individuals exhibiting the 16093 (1/2) and HVIIB (1/3) mutation.

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