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Mitochondrial DNA Heteroplasmy Among Hairs from Single Individuals*

ABSTRACT: A denaturing gradient gel electrophoresis (DGGE) assay was used to detect mitochondrial DNA (mtDNA) sequence heteroplasmy in 160 hairs from each of three individuals. The HV1 and HV2 heteroplasmic positions were then identified by sequencing. In several hairs, the heteroplasmic position was not evident by sequencing and dHPLC separation of the homoduplex/heteroduplex species was carried out with subsequent reamplification and sequencing to identify the site. The overall detection frequency of sequence heteroplasmy in these hairs was 5.8% (28/480) with DGGE and 4.4% (21/280) with sequencing. Sequence heteroplasmy of hair was observed even when the reference blood sample of the individual was homoplasmic. The heteroplasmic positions were not necessarily observed at sites where high rates of substitution have been reported. In two hairs, a complete single base change from the reference blood sample was observed with sequencing, while the heteroplasmic condition at that site in the hair was observed using DGGE. The DGGE results in such samples would serve as an aid in considering the possibility of match significance. In a forensic case, this situation would lead to the possibility of a failure to exclude rather than to be inconclusive.

KEYWORDS: forensic science, DNA typing, mitochondrial DNA, hair DNA, heteroplasmy, denaturing gradient gel electrophoresis

MtDNA analysis is a useful tool for the identification of old or degraded biological evidentiary samples and is also important in the forensic comparison of hair samples. Heteroplasmy, the presence of two or more types of mtDNA in a single individual, was first observed in humans in association with a mitochondrial disorder (1). However, heteroplasmy in mtDNA has been reported, not only in association with mitochondrial disorders, but in the non-coding control region as well. Heteroplasmy in the control region was initially not considered to be significant (2), but it now appears to be a more frequent phenomenon than had originally been thought (3–6). Inter- and intra-generational heteroplasmy has been reported and the proportion of heteroplasmy appears to vary among different family members (5,7,8). Intra-individual heteroplasmy has also been reported in several studies (9,10). Length heteroplasmy in the HV1 and HV2 region, near nucleotide positions 16189 and 309–315, was found among hairs within a single individual (11,12) and the mechanism involved in this length heteroplasmy has been investigated (13,14). Site heteroplasmy, at a single nucleotide position in the control region of mtDNA in hairs, has also been reported and its proportions varied among hairs within a single individual (5,15). The frequency of site heteroplasmy among hairs from an individual with a homoplasmic blood sample has also been reported (16,17). Hühne et al. reported that no heteroplasmy was detected in an analysis of 15 hairs from each of 10 individuals, whereas Grzybowski reported numerous heteroplasmic positions in an analysis of 2–6 hairs each from each of 13 individuals. Because of the high level of heteroplasmy, the latter report has been questioned and can be regarded as controversial (18,19) and reanalysis of Grzybowski's study showed fewer heteroplasmic positions in hairs without using nested PCR (20). Alonso et al. also found various levels of heteroplasmy and several mutations in hairs from a single individual during an interlaboratory study (21). A denaturing gradient-gel electrophoresis (DGGE) assay has been demonstrated to be a sensitive tool for detecting heteroplasmy (6,22). The use of a DGGE assay for the detection of mutations or heteroplasmic positions in the HV1 region has been reported (22). The detection limit was investigated and the DGGE assay was found to be more sensitive in the detection of heteroplasmy than sequencing analysis (6). In the present study, we designed new primer pairs for the HV1 and HV2 regions so that the PCR products would not contain the C-stretch region. The rationale for this is that the PCR region used in former studies contains the C-stretch region and this length heteroplasmy led to a complex DGGE pattern (6). The purpose of this study was to screen a number of hairs using the DGGE assay in order to investigate the level of heteroplasmy in hair samples within a single individual.

Materials and Methods

DNA Extraction

Human head hair shafts were collected from three Japanese male individuals (M1, M2, and M3). One hundred and sixty hairs without roots were collected from each individual by cutting with scissors about 0.5–1 cm above the scalp. Hairs were collected from seven head areas; 16 hairs from the left/right frontal and left/right occipital regions and 32 from the left/right temporal and parietal regions. Hairs were cut in 2 cm sections in length proximal to a hair root. Prior to DNA extraction, the hairs were successively washed with 5% (w/v) Terg-A-Zyme® (Alconox, New York, NY), rinsed in sterilized distilled water, washed in 100% ethanol and dried. DNA was extracted using a QIAamp DNA mini kit (QIAGEN, Valencia, CA) with the following modifications: 40 mM dithiothreitol (DTT) (Wako Pure Chemical Industries, Osaka) was added during the proteinase K digestion and the reaction was performed until the complete digestion of hair shafts was achieved (approximately 1 h). The eluted DNA solutions were concentrated to about 40 μL

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on a vacuum-centrifugal evaporator. DNA was also extracted from blood samples obtained from the corresponding individuals by using a QIAamp DNA mini kit following the manufacturer's instructions. The extracted DNA was immediately used for PCR or DNA samples were stored at –30°C until immediately before the PCR. All plastic ware used for the extractions was irradiated by UV light just prior to its use. All participants provided appropriate informed consent.

PCR Amplification

For detecting heteroplasmy by the DGGE assay, the mtDNA HV1 and HV2 regions were analyzed as three fragments by use of the following PCR primer pairs: DGGE-B [L15997-GC (GC-ACCATTAGCACCCAAAGCT)/H16174 (GGGGGTTTTGATG-TG)], DGGE-C [L16208-GC (GC-CATGCTTACAAGCAAG)/H16401 (TGATTTCACGGAGGATGGTG)], and DGGE-E [L29-GC (GC-GGTCTATCACCCTATTAACCAC)/H290 (GGGGG-GGTTTGGTGGAAATT)]. A GC-clamp (5′-CGCCCGCCGCGC-CCCGCGCCCGTCCCGCCGCCCCCGCCC-3′), which was added to the 5′ end of one primer (GC-) in each pair, resulted in PCR products suitable for DGGE analysis (6). PCR was conducted using a 5 μL aliquot of the DNA extracted from hair or 1 ng of the DNA extracted from blood in a 50 μL volume containing 2.5 U of AmpliTaq GOLD DNA polymerase (Applied Biosystems, Foster City, CA), 1 × PCR GOLD Buffer (Applied Biosystems), 200 μM of dNTPs (Applied Biosystems), 0.02% of BSA (Amersham Pharmacia Biotech, Piscataway, NJ) and 0.2 μM of each primer pair. Thermal cycling was performed using a GeneAmp PCR system 9700 thermal cycler (Applied Biosystems) starting with 9 min at 95°C, and followed by 35 cycles of 15 sec at 95°C, 10 sec at 60°C (ramping value was set at 50%) and 20 sec at 72°C. A 5 μL aliquot of the PCR product was analyzed on a 2% agarose gel and visualized by ethidium bromide staining to confirm the quality and quantity of the product. PCR tubes were exposed to UV light for a minimum of 10 min immediately prior to use. Negative controls were included in the extraction and PCR reactions to detect any possible contamination. All the negative controls indicated no contamination.

Denaturing Gradient Gel Electrophoresis (DGGE)

DGGE was performed using a DCode Universal Mutation Detection System (Bio-Rad Laboratories, Hercules, CA) for 16 h at 75 V and 60°C. The denaturing gradient gel was an 8% polyacrylamide gel (37.5:1 acrylamide/bisacrylamide) with a 30%–50% denaturant for the DGGE-B, a 35%–55% denaturant for the DGGE-C and a 25%–45% denaturant for the DGGE-E (100% denaturant is defined as 7M Urea and 40% formamide). After the electrophoresis, the gels were stained with 1 × SYBR Green I (Molecular Probes, Eugene, OR) for 20 min and visualized using Fluorimager 595 (Amersham-Pharmacia Biotech). The relative proportion of each band in the heteroplasmic samples was determined by calculating the peak intensities from the scanned gel image using the NIH image software (developed at the United States National Institutes of Health) (22).

If multiple bands were observed in the DGGE or if the mobility of the bands from hair samples were different from those from the blood sample, they were suspected of being heteroplasmic. PCR amplification and DGGE analysis of such heteroplasmic samples were performed at least twice to confirm heteroplasmy, and sequencing analysis was then performed in order to locate the heteroplasmic position.

Sequencing Analysis

If the samples were suspected of heteroplasmy, PCR was performed for a sequencing analysis (23). The resulting PCR products were purified using a QuickStep 2 PCR Purification Kit (Edge BioSystems, Gaithersburg, MD). Sequencing analysis was performed using BigDye Terminator Cycle Sequencing Ready Reaction Kit V2.0 (Applied Biosystems) and a PRISM 310 Genetic Analyzer (Applied Biosystems). Each template was sequenced in both directions and the sequence reactions were carried out at least twice in order to confirm the sequencing results.

When the proportion of the minor heteroplasmic species was too low for detection by sequencing analysis, the corresponding heteroduplex bands were separated and collected from the PCR products by using denaturing high-performance liquid chromatography (WAVE DNA Fragment Analysis System; Transgenomic Inc., Omaha, NE) and a Fragment Collector (Transgenomic Inc.). The DNA recovered from the heteroduplex bands was dried under a vacuum concentrator to remove organic solvents in preparation for the subsequent PCR. The collected DNA were re-amplified and the heteroplasmic positions were confirmed by DGGE. The samples were then sequenced to determine the heteroplasmic position, as described above.

Results

We successfully obtained PCR products in all three regions from a total of 480 hair samples from three male individuals. DGGE analysis was conducted on all samples and the number and mobility of the bands were observed in each sample and compared with the bands from blood samples. Two individuals (M2 and M3) showed no heteroplasmy in the blood samples by either sequencing analysis or the DGGE assay. One individual (M1) also showed no heteroplasmy by sequencing analysis but showed heteroplasmy by the DGGE assay, probably because his mother showed heteroplasmy at position 16291 (7). In the DGGE assay, in a comparison of the samples, when the PCR products of each sample are identical, each band showed the same mobility on the gel and when the PCR products are not identical, each band could show a different mobility on the gel. If heteroplasmic positions were present in PCR products that contained two different sequences, two homoduplex bands and two heteroduplex bands were normally observed in the gel (22). Most of the 480 hair samples showed a single homoduplex band with the same mobility as the blood samples, indicating no heteroplasmy, but 28 samples among three individuals and three regions (DGGE-B, DGGE-C and DGGE-E) showed bands that were different from the blood samples, indicating either a mutation or heteroplasmy. Eleven hairs and a blood sample from individual M1 showed two heteroduplex bands and two homoduplex bands in the DGGE-C regions (Fig. 1*a*). The amount of the other polymorphism in some samples was too small to permit the detection of heteroduplex bands (Fig. 1*a*, lane 3). Three hairs from M1 showed two heteroduplex bands and two homoduplex bands in the DGGE-E region (Fig. 1*b*). One hair from individual M2 showed two heteroduplex bands and one homoduplex band in the DGGE-B region, indicating that the mobility of another polymorphism was nearly the same as that in the blood samples (Fig. 1*c*). One hair from M2 showed two heteroduplex bands and two homoduplex bands and two hairs from M2 showed one heteroduplex band and two homoduplex bands in the DGGE-C region (Fig. 1*d*). Four hairs from M2 showed two heteroduplex bands and two homoduplex bands in the DGGE-E region (Fig. 1*e*). One hair from M3 showed two homoduplex bands, respectively, in the DGGE-B and DGGE-C regions

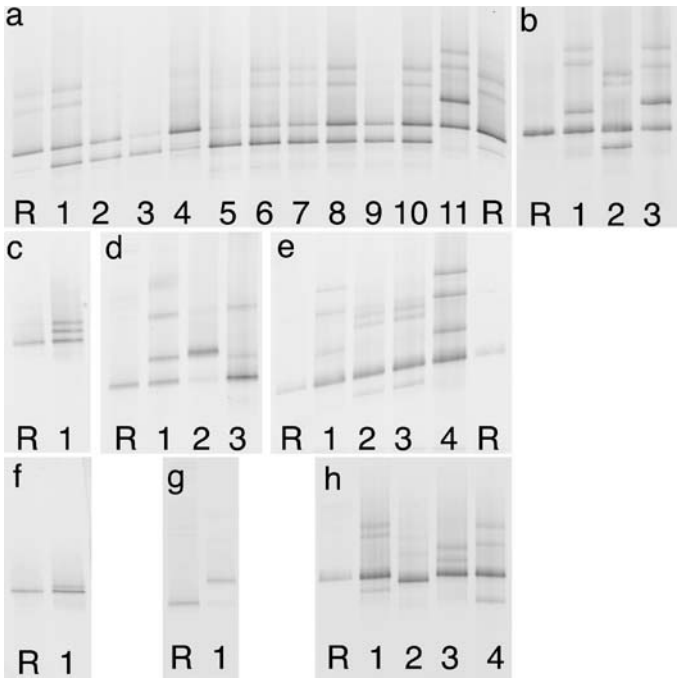

FIG. 1—*DGGE gel images of heteroplasmies found in hairs from each individual with a comparison to a blood sample from each individual (lane R). Lane assignments for each gel are denoted in Table 1. Individual and regions of each gel are as follows: a: individual M1, DGGE-C region; b: individual M1, DGGE-E region; c: individual M2, DGGE-B region; d: individual M2, DGGE-C region; e: individual M2, DGGE-E region; f: individual M3, DGGE-B region; g: individual M3, DGGE-C region; h: individual M3, DGGE-E region.*

(Fig. 1*f* and 1*g*), indicating that none of the heteroduplex bands were too small to be observed. Four hairs from M3 showed two heteroduplex bands and two homoduplex bands in the DGGE-E region (Fig. 1*h*).

The position at which heteroplasmy occurred in each sample was determined by performing a second amplification of the same hair DNA, followed by sequencing. The position was confirmed by sequencing both strands (heavy strand and light strand of mtDNA). We classified the sample as having heteroplasmy only when the position was detected as an apparent heteroplasmy in both strands in order to distinguish heteroplasmy from the noise of the electropherogram (24). According to this criterion, heteroplasmic positions of five samples could not be found by the usual sequencing method (indicated in Table 1 as parenthesized nucleotide). Heteroduplex bands or minor homoduplex bands of those five samples were then separated and collected from each PCR product of these samples by a WAVE system. We were able to confirm that the correct bands were collected by the WAVE system by performing DGGE analysis of fractionated samples. The heteroplasmic position and its proportion are described in Table 1.

Discussion

In this study, individual M1 showed a relatively high proportion of heteroplasmy (14 hairs out of 160), but ten were in the same position at 16291. Heteroplasmy at this position was not detected in a sample of his blood by sequencing analysis although it was detected by DGGE analysis in both blood and saliva samples and in a sample from his mother (7). Our previous study indicated that variable proportions of heteroplasmy were present in hairs especially if the person had an apparent heteroplasmy, that is, nearly equal proportions of heteroplasmic species (7). Another study also reported variable proportions of heteroplasmy in hairs where heteroplasmy was found in a buccal swab sample (5). High levels of heteroplasmy are most likely to be observed where the heteroplasmy is inherited by the offspring of a heteroplasmic individual. For other heteroplasmic positions found in hairs in this study, except for the nucleotide position 16291 of individual M1, no indication of heteroplasmy was found either by sequencing analysis or by a DGGE assay of a blood sample. Therefore, we conclude that the other instances of heteroplasmy are likely to be sporadic, arising during single hair development including the initial formation of the hair bulb or the growth of the hair. Four hairs out of 160 contained heteroplasmy in individual M1 except position 16291 and this degree of heteroplasmy was not significantly different for the other individuals in our study. The total level of DGGE detection of heteroplasmy was 5.8% overall (28/480), and ranged from 3.75% (M3) to 5% (M2) to 8.75% (M1), while that of sequencing detection was 4.4% overall (21/480), and ranged from 1.25% (M3) to 2.5% (M2) to 8.75% (M1). No multiple positional heteroplasmy was detected, i.e., only a single positional heteroplasmy was present. Melton and Nelson examined 144 hairs in their commercial casework and found 9.7% of hairs to be heteroplasmic with sequencing (25). The number of heteroplasmic positions found in a single hair within the HV1 and HV2 regions except the C-stretch region was reported as one in studies from other laboratories (26). Their results are in relatively good agreement with our results. Heteroplasmy probably exists in some hairs of all individuals but may be undetectable. The possibility of the detection of heteroplasmy depends on the detection techniques used, and heteroplasmy must be present at a level approaching 20% to be distinguished from the background for a mutation to be detected by sequencing (24). Because the DGGE assay is more sensitive in detecting heteroplasmy than sequencing, the ratio of heteroplasmy in hairs would be lower in the case where heteroplasmy was detected only by sequencing. However, even if the threshold of detection of heteroplasmy is used at a level of 20%, heteroplasmy or mutations could be detected in 14 hairs (M1), 5 hairs (M2) and 3 hairs (M3), respectively, out of 160 hairs (average: 4.6%, standard deviation: 3.7%) per single individual (Table 1), and these could be detected by sequencing analysis.

This study demonstrates that heteroplasmy is found not only in positions (e.g., nucleotide position: 16291, 146, 152, 189, and 207) where high levels of evolutionary substitution have been reported (27–29), but also in positions (e.g., 16321, 229, and 273) where no mutation exists in the population databases (30–32) (Table 2). The heteroplasmic positions were varied within a single individual and two hairs at most were obtained with heteroplasmy in the same position (nucleotide position 189 and 251 in individual M2) except position 16291 in individual M1 which was most likely inherited from the mother (7). Most sites, where heteroplasmic mutations have already been observed among individuals, were in hypervariable sites (33). Some of the heteroplasmic positions (16321, 229, and 251), where this study was reported, do not appear in the Mitomap database (34). Although extraction controls and reagent blanks were performed, the possibilities of contamination, PCR error, or sequencing noise cannot be excluded, if these heteroplasmic positions did not arise from a mutational event in hairs. However, the possibility of contamination is unlikely because no result was observed from negative and extraction controls, none of our laboratory personnel had mutations at these positions and no multiple heteroplasmic positions were observed in a single sample. The possibilities of PCR error and sequencing noise are also unlikely because heteroplasmy was observed at least twice in the DGGE assay and the sequences were confirmed by analyzing both strands.

TABLE 1—*Sequencing results of individual hairs and the proportion of heteroplasmy.*

Individual	Position	Blood Sample Reference Sequence*	Sequence Analysis		DGGE Analysis (Estimated Proportion, %)		Lane in Figure 1	Hair Location‡
			Major Sequence	Minor Sequence†	Major Sequence	Minor Sequence		
M1	16291	T	T	-	T (87)	C (13)	a-R	(Blood)
	16291	T	T	C	T (56)	C (44)	a-1	RF
	16291	T	T	C	T (54)	C (46)	a-2	RF
	16291	T	**C**	T	**C** (75)	T (25)	a-3	RF
	16291	T	T	C	T (80)	C (20)	a-4	RF
	16291	T	**C**	T	**C** (86)	T (14)	a-5	RF
	16291	T	**C**	T	**C** (70)	T (30)	a-6	LT
	16291	T	**C**	T	**C** (69)	T (31)	a-7	RT
	16291	T	T	C	T (54)	C (46)	a-8	RT
	16291	T	**C**	T	**C** (62)	T (38)	a-9	RO
	16291	T	T	C	T (55)	C (45)	a-10	P
	16301	C	C	T	C (50)	T (50)	a-11	P
	229	G	G	A	G (66)	A (34)	b-1	LF
	189	A	A	G	A (64)	G (36)	b-2	RO
	273	C	**T**	C	**T** (55)	C (45)	b-3	P
M2	16170	G	G	A	G (59)	A (41)	c-1	RO
	16390	G	G	A	G (53)	A (47)	d-1	LT
	16321	C	**T**	-	**T** (84)	C (16)	d-2	LO
	16391§	G	G	(A)	G (83)	A (17)	d-3	LF
	251§	G	G	(A)	G (81)	A (19)	e-1	P
	189§	A	A	(G)	A (82)	G (18)	e-2	LT
	189	A	A	G	A (80)	G (20)	e-3	LT
	251	G	G	A	G (67)	A (33)	e-4	LF
M3	16168	C	C	T	C (77)	T (23)	f-1	RT
	16360	C	**T**	C	**T** (85)	C (15)	g-1	LT
	189§	A	A	(G)	A (83)	G (17)	h-1	LT
	152	T	**C**	-	**C** (98)	T (2)	h-2	LT
	146	T	T	C	T (74)	C (26)	h-3	RT
	207§	A	A	(G)	A (87)	G (13)	h-4	RO

Bold type indicates that major sequence was different from a reference sequence.

* Sequence from blood samples in each individual.

† Hyphen (-) indicates that no trace of minor sequence exists. Parenthesized nucleotide (N) indicates that the minor sequence cannot be recognized as heteroplasmy only by the sequencing method.

‡ LF: Left frontal area, RF: Right frontal area, LT: Left temporal area, RT: Right temporal area, LO: Left occipital area, RO: Right occipital area, P: Parietal area.

§ Nucleotide position of heteroplasmy was determined by the WAVE system.

TABLE 2—*The position of HV1 and HV2 heteroplasmy from three individuals, compared with population studies.*

Position (Type)*	Individual	Relative Rate of Evolutionary Substitutions per Site, as Estimated by			Population Frequency of Minority Variant		
		No. of Hairs	Excoffier et al. (28)	Meyer et al. (29)	Caucasian (30) ($N = 1655$)	Korean (31) ($N = 306$)	Japanese (32)† ($N = 450$)
16168 (C/T)	M3	1	0.6	0.8	0.002	0.01	0.01
16170 (G/A)	M2	1	0.6	0.7	0.002	0.003	0.004
16291 (C/T)	M1	10	4.4	1.8	0.02	0.03	0.04
16301 (C/T)	M1	1	1.2	0.3	0.005	0.003	0.000
16321 (C/T)	M2	1	0	0	0.00	0.00	0.00
16360 (C/T)	M3	1	0.4	1.4	0.005	0.00	0.002
16390 (G/A)	M2	1	-	-	0.002	0.01	0.01
16391 (G/A)	M2	1	-	-	0.00	0.003	0.00
146 (T/C)	M3	1	-	6.2	0.10	0.15	0.07
152 (T/C)	M3	1	-	6.2	0.20	0.24	0.23
	M1	1					
189 (A/G)	M2	2	-	6.2	0.03	0.02	0.02
	M3	1	-				
207 (A/G)	M3	1	-	3.2	0.03	0.06	0.08
229 (G/A)	M1	1	-	0	0.00	0.00	0.00
251 (G/A)	M2	2	-	0	0.001	0.00	0.00
273 (C/T)	M1	1	-	0	0.00	0.00	0.00

* Positions where heteroplasmy was observed. Numbering and sequence are according to the revised Cambridge Reference Sequence (27).

† Unpublished population data for Japanese were combined with that of Imaizumi et al. (32).

Therefore, the heteroplasmic positions observed in this study were likely derived as a result of mutation during the development of hairs. These heteroplasmic positions may also exist in blood or buccal cells in very low proportions, but are undetectable both by sequencing and the DGGE method because a large number of the dominant mtDNA species also exist in blood or buccal cells which would conceal low levels of heteroplasmic mtDNA. However, if such minute mutations occurred in hairs, they could be focused because hair is produced from a scalp follicle by the mitotic division of a small number of cells that comprise the matrix or germination center of the follicle (35,36).

The heteroplasmy in hairs containing at least 20% of the minor variant could be detected by sequencing as well as by DGGE (Table 1). This percentage is in good agreement with previous studies (24). At levels of below 20% of heteroplasmy in DGGE, in most cases, no band corresponding to a minor sequence could be detected or the minor sequence was not distinguished from the noise peak in sequence method. In two samples in our study (e.g., M1: a–5, M3: g–1), however, a minor sequence could be detected by the sequencing method even when its proportion was below 20% in DGGE. Because the peak height of a sequencing electropherogram is not always an even peak height, the efficiency of incorporation of dye-labeled dNTP in DNA polymerase may be slightly different in every nucleotide (37). As the result of different efficiencies in incorporation, the minor sequence, which was below 20%, could be detected in these heteroplasmic positions. In three samples (e.g., M1: a–R, M2: d–2, M3: h–2), heteroplasmy could be detected only by DGGE. For two of these samples (M2: d–2, M3: h–2) a one base difference was observed between hair and the reference blood sample in the sequencing method. In these two samples, the DGGE results indicate that the blood reference type was always present in the hair samples although its proportions were 16% and 2%, respectively. This indicates that when the sequence of unknown sample contains one base difference from the reference sample in the sequencing method, the reference sequence might be detected in the unknown sample by using a sensitive detection method such as DGGE, if the unknown sample was really derived from the reference individual.

Heteroplasmy was observed in 3.75% to 8.75% of the hairs from a single individual and their nucleotide positions were varied regardless of mutational hot spots, even if the individual did not have an apparent heteroplasmy in his/her blood. The DGGE results in such samples would serve as an aid in considering the possibility of match significance. In a forensic case, if there is one nucleotide difference between the questioned (e.g., hairs) and known (e.g., blood) samples by sequencing, the interpretation will be inconclusive. However, if the existence of heteroplasmy at such nucleotide position in either the questioned or known samples or both is proved by DGGE or other sensitive detection methods, the interpretation would lead to the possibility of a failure to exclude rather than to be inconclusive.

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