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Heteroplasmies Detected in an Amplified Mitochondrial DNA Control Region from a Small Amount of Template

ABSTRACT: When mitochondrial DNA (mtDNA) heteroplasmies are detected, they often confound forensic identification, especially if they are the result of poor biological sampling. In this study, we determined the ratio of heteroplasmy in samples that were amplified from a very small amount of template mtDNA or a few cells using a highly sensitive nested polymerase chain reaction (PCR) procedure and a direct sequencing analysis. As a result, more than half of the detected sequences (i.e., 17/20, 15/20, and 14/20) showed homoplasmy derived from a variation in the heteroplasmy proportion when only 10 copies of template mtDNA samples were amplified and analyzed. Additionally, with products amplified from one or several white blood cells (WBCs), several previously undetected heteroplasmies were detected. These results indicate the risks associated with using highly sensitive mtDNA techniques in forensic investigations because of the variable proportions of heteroplasmy or nucleotide substitutions that can possibly be detected from a very small biological sample.

KEYWORDS: forensic science, mitochondrial DNA, hypervariable region, heteroplasmy, nested PCR

Sequence analysis of the mitochondrial DNA (mtDNA) control region is a valuable tool for identifying human samples. The hypervariable region 1 (HV1) and hypervariable region 2 (HV2) in the mitochondrial control region, which are known as the noncoding portion of mtDNA, have high levels of polymorphism (1). Forensic identification using mtDNA analysis is performed by the determination and comparison of the HV1 and HV2 sequences in biological samples. The advantages of the analysis are its higher detection sensitivity than nuclear DNA because thousands of copies of mtDNA exist in each cell (2). Because of its high sensitivity and polymorphism, the mtDNA analysis has been utilized in many cases of forensic identification (3–5). However, several aspects of the analysis need to be considered to evaluate the validity of the results. One of these aspects is related to heteroplasmy which is a state in which two or more haplotypes of mtDNA coexist in a single individual. In rare cases, heteroplasmy could be a decisive factor in an identification (6), but in many cases, it can lead to confusion when the sequences are compared. For example, in some cases of maternal-individual identification, the ratio of heteroplasmy is sometimes significantly different between generations and the haplotypes of each sequence are apparently mismatched (7–9). The "bottleneck" at the stage of oocyte development is sometimes cited to explain the difference in the ratio between a mother and her offspring (10,11). In another example, it is known that discrete hairs from a single individual often contain apparently different mtDNA haplotypes (9,12,13) and a comparison of such forensic hairs could be inconclusive. It has been suggested that the low level of heteroplasmy generated by mutations will be fixed as obvious heteroplasmy or substituted homoplasmy through the bottleneck at the development stage of a hair germ (4,14). The ratio of heteroplasmy in the small amount of mtDNA at the "bottleneck" in oocytes and hair germs probably vary (14). Similar to the biological bottleneck within an organism, the ratio obtained in polymerase chain reaction (PCR) products that are amplified from very small amounts of template mtDNA could vary.

In this study, we examined the relationship between the copy number of mtDNA as a template in PCR amplification and the ratio of heteroplasmy detected in the subsequent sequence analysis. MtDNA samples from which obvious heteroplasmy was detected in our previous report (15) were used in this study.

In another experiment, we attempted to examine potential heteroplasmy in each cell. MtDNA included in each cell may have variable minor heteroplasmy which would not be detected because of the large amount of nonmutated mtDNA in each cell. To clarify this phenomenon, white blood cells (WBCs) were collected and used as cell samples from five individuals from whom no obvious heteroplasmy had been detected in our previous study (15).

Materials and Methods

DNA Extraction from Bloodstain Samples

DNA samples were extracted from bloodstains of three individuals who consented to participate in the studies using their mtDNA control region. The control regions of these three individuals had been sequenced previously and obvious heteroplasmy was observed in the HV1 region as shown in Table 1. DNA was extracted from the bloodstains using protainase K digestion following extraction by an automated DNA extraction instrument BioRobot EZ1 (Qiagen, Hilden, Germany) according to the manufacturer's manual and are numbered as numbers 21, 61, and 76.

Quantification of mtDNA

The mtDNA molecules included in the extracted solution were quantified using a quantitative PCR method in a real-time PCR instrument LightCycler (Roche Diagnostics, Mannheim, Germany).

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TABLE 1—*Sequence of HV1 in the mitochondrial control region of each sample as the difference from the reference sequence.*

Nucleotide position	CRS	Heteroplasmy samples			White blood cell samples				
		21	61	76	a	b	c	d	e
16,209	T				C				
16,223	C		T	T	T	T	T	T	
16,227	A			G					
16,234	C	**T/C**							T
16,243	T	C							C
16,261	C			**C/T**					
16,278	C			T					
16,291	C	T							T
16,294	C		**C/T**						
16,299	A	**A/G**							
16,324	T				C				
16,362	T		C	C	C		C	C	

CRS, the Cambridge Reference Sequence (25). Bold letters indicate the heteroplasmies existing in the sequence of each sample.

The primer set for the mtDNA-specific amplification is the same as that used for nested second PCR described below. Reactions and analyses were carried out using the LightCycler Software Version 3 (Roche Diagnostics) program in accordance with the operator's manual. As a control DNA, the amplified products of the mtDNA control region of commercially available human DNA K562 were inserted into plasmid vector pT7Blue and cloned by the Takara Bio Custom Cloning Service (Takara Bio, Shiga pref., Japan). The absorbance of the extracted plasmid solution was determined and the concentration (copies/μL) was calculated. In addition, a dilution series of copy number 10^6, 10^5, 10^4, 10^3, and 10^2/μL of plasmid solutions was prepared as a set of standard DNA for each reaction. Amplifications were carried out using 30 cycles of denaturing at 94°C for 0 sec, annealing at 55°C for 10 sec and extension at 72°C for 20 sec in a reaction mixture containing 1x of LightCycler™ DNA Master SYBR GreenI (Roche Diagnostics), 25 mM $MgCl_2$, and 1 μM of each primer.

Quantified sample DNA solutions 21, 61, and 76 were diluted into 1000, 100, and 10 molecules of mtDNA per microliter with EASY Dilution for real-time PCR (Takara Bio) for templates for amplification.

WBC Samples

Fresh blood was collected using heparinized vacuum tubes VENOJECT®II VP-H100 (Terumo, Tokyo, Japan) from five individuals who consented to participate in the studies using their mtDNA control region. Their mtDNA control region had been sequenced previously, and no heteroplasmy was detected in these individuals (Table 1).

Red blood cells were washed out with Red Blood Cell Lysis Buffer (Roche Diagnostics) following the manufacturer's instruction manual and the concentrated WBCs were then re-suspended in autoclaved saline (0.85% NaCl) and numbered as a, b, c, d, and e. The concentration of WBC in each suspension was measured on a Thoma hemocytometer. Samples were prepared by diluting the suspensions with saline into a theoretical concentration equal to one cell per microliter. These WBC suspensions were used in the following analysis as direct-PCR templates.

Nested PCR

In this study, we used a newly designed tandem nested PCR procedure to amplify very small amounts of template mtDNA or mtDNA from a few WBCs. The PCR amplification was carried out in a real-time PCR instrument LightCycler (Roche Diagnostics) controlled by the LightCycler Software version 3 (Roche Diagnostics) program. The real-time PCR system can easily confirm whether amplification can occur or not by the real-time monitoring of fluorescence. The LightCycler system also allows very rapid PCR because of its air heating system and the high surface-to-volume ratio of glass capillary reaction tubes (16).

The primer set for the first PCR, mtA0-F: 5′-CA-AGGACAAATCAGAGAAAAAGT-3′ and mtA0-R: 5′-GGAGCGAGGAGAGTAGCAC-3′ was used to amplify HV1 region (nucleotide 15,946–16,454). One microliter of sample solutions including 1000, 100, or 10 copies of sample 21, 61, and 76 and one microliter of WBC suspension a, b, c, d, and e were added to the reaction mixture as a template. Amplification was carried out with 40 cycles of denaturing at 94°C for 0 sec, annealing at 50°C for 10 sec, and extension at 72°C for 20 sec in the 10 μL of reaction mixture containing 1x of LightCycler™ DNA Master SYBR GreenI (Roche Diagnostics), 25 mM $MgCl_2$, and 1 μM of each primer. After the first PCR, reaction solutions were collected from capillary tubes as "first products." The lids of capillary tubes were removed and the tubes were placed in 1.5-mL microcentrifuge tubes upside down. The microcentrifuge tubes with capillary tubes were centrifuged for a few seconds and the PCR solutions were collected in the bottom of the microcentrifuge tubes.

The inner primer set for the second PCR, L16208F: 5′-tgtaaaacgacggccagCCCCATGCTTACAAG-3′ and H16401R: 5′-caggaaacagctatgaccTGATTTCACGGAGGATGGT-3′ was used to amplify the sequence-tagged (lower-case letters) 16,190–16,420 of HV1 region. One microliter aliquots of the "first products" were added to the reaction mixture as a template. Amplification was carried out with 20 cycles of denaturing at 94°C for 0 sec, annealing at 60°C for 10 sec, and extension at 72°C for 20 sec in 20 μL of a reaction mixture containing 1 μL of the first product, 1x of LightCycler™ DNA Master SYBR GreenI (Roche Diagnostics), 25 mM $MgCl_2$, and 1 μM of each primer. The success or failure of the amplification in the second PCR was determined by monitoring the real-time fluorescence and only solutions containing amplified products were retrieved as "second products" using a previously described procedure. The "first" and "second products" from which no fluorescence increase were detected while the second PCR was excluded. The set of first and second PCR were repeated until 20 "second products" from each sample were prepared for use in the following sequencing analysis. The negative control reaction mixture containing no DNA was amplified with each reaction to confirm the absence of a fluorescence increase, i.e., possible contamination.

Sequencing Analysis

The recovered "second products" were purified using QuickStep™ 2 PCR Purification Kit (Edge BioSystems, Gaithersburg, MD) following the manufacturer's manual and 2 μL of collected products were applied for each sequencing reaction mixture. The sequencing analysis was performed using a BigDye Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA) following the manufacturer's manual. DNA sequences were determined in both directions using forward M13Fwd primer and reverse M13Rev primer to confirm the sequence. The sequence products were purified using a QuickStep™ 2 PCR Purification Kit and analyzed using an ABI PRISM™ 310 Genetic Analyzer system and then using ABI PRISM™ Sequencing Analysis Version 3.3 (Applied Biosystems) program. The sequence of the HV1

region (16,209–16,401) of each product was confirmed using an ABI Sequence Navigator™ v1.1 (Applied Biosystems) program.

Measurement of Proportion of Each Heteroplasmy

On the electropherogram, the peak heights of co-existing nucleotides at each heteroplasmic position of sample 21, 61, and 76 were measured. The peak heights for both directions were averaged and the ratio of the peak height of each nucleotide was recorded.

Screening of Heteroplasmy

To determine unknown heteroplasmies in WBC samples a, b, c, d, and e, the obtained sequencing electropherograms were investigated. When suspected heteroplasmy was detected on an electropherogram of both sequences, the "first products" of the nested PCR in which heteroplasmy was detected were reamplified and sequenced again to confirm the heteroplasmy. Uncertain heteroplasmy which included less than 10% of minor nucleotides were excluded from data because extremely low peaks were difficult to distinguish from noise peaks.

Results and Discussion

In this study, we observed the ratio of heteroplasmy at the heteroplasmic position of each sequence shown in Table 1 analyzed on products amplified from very low copy numbers of template DNA.

As a result of the amplification from 10-fold diluted mtDNA (1000, 100, and 10 copies) of samples 21, 61, and 76, the correlation between the amount of template DNA and the ratio of heteroplasmy could clearly be observed. The observed proportion of the heteroplasmic positions on each sample is shown in Figs. 1–3. In each figure, chart (a) indicates results obtained from products amplified from 1000 copies of mtDNA, while charts (b) and (c) show the results for 100 and 10 copies respectively. The bar graphs in each chart were sorted in descending order of T% and renumbered from 1 to 20. The average and standard deviation (SD) for each chart are shown.

As shown in Figs. 1–3, the greater variation in heteroplasmy was observed when a lower copy number of template mtDNA was amplified. These results are consistent with our above preliminary prediction. For example, the ratio of every product from 1000

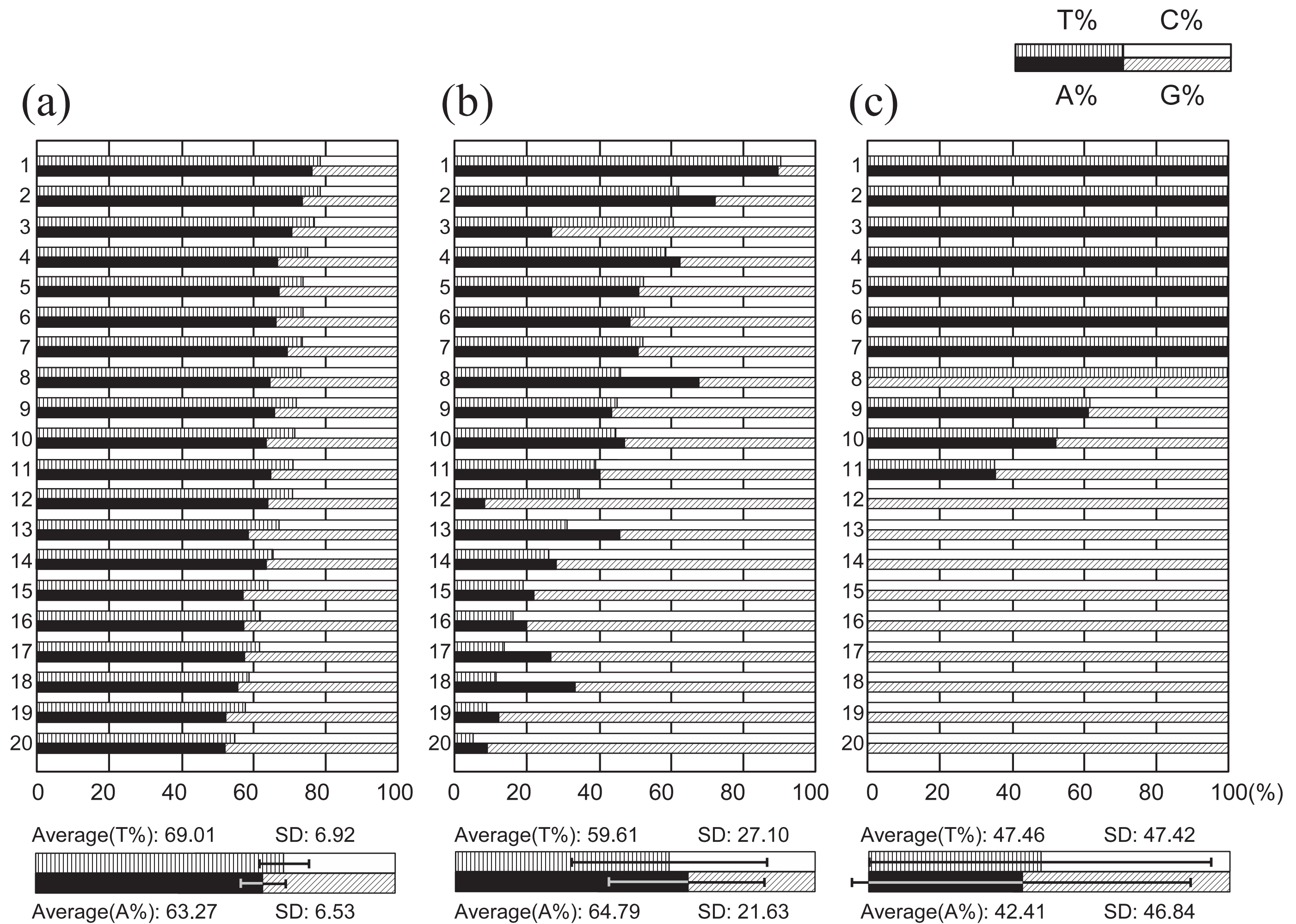

FIG. 1—*Comparisons of heteroplasmic proportions at nucleotide 16,234(T/C) and 16,299(A/G) of sample 21 measured on 20 sequence electropherograms analyzed from 1000 copies (a), 100 copies (b), and 10 copies (c) of mtDNA. The results were sorted in descending order of T% and are numbered from 1 to 20. The average and standard deviation in T% and A% from each of the 20 samples are shown. Eight products from 10 copies (c; 1–8) show homoplasmy at T, and nine products (c; 12–20) show homoplasmy at C at nucleotide 16,234, whereas seven products (c; 1–7) and 10 other products (c; 8, 12–20) show homoplasmy at A and G, respectively, at nucleotide 16,299. All 17 products without heteroplasmy have the haplotype of 16,234T/16,299A or 16,234C/16,299G except for one (c; 8), which has the type of 16,234T and 16,299G.*

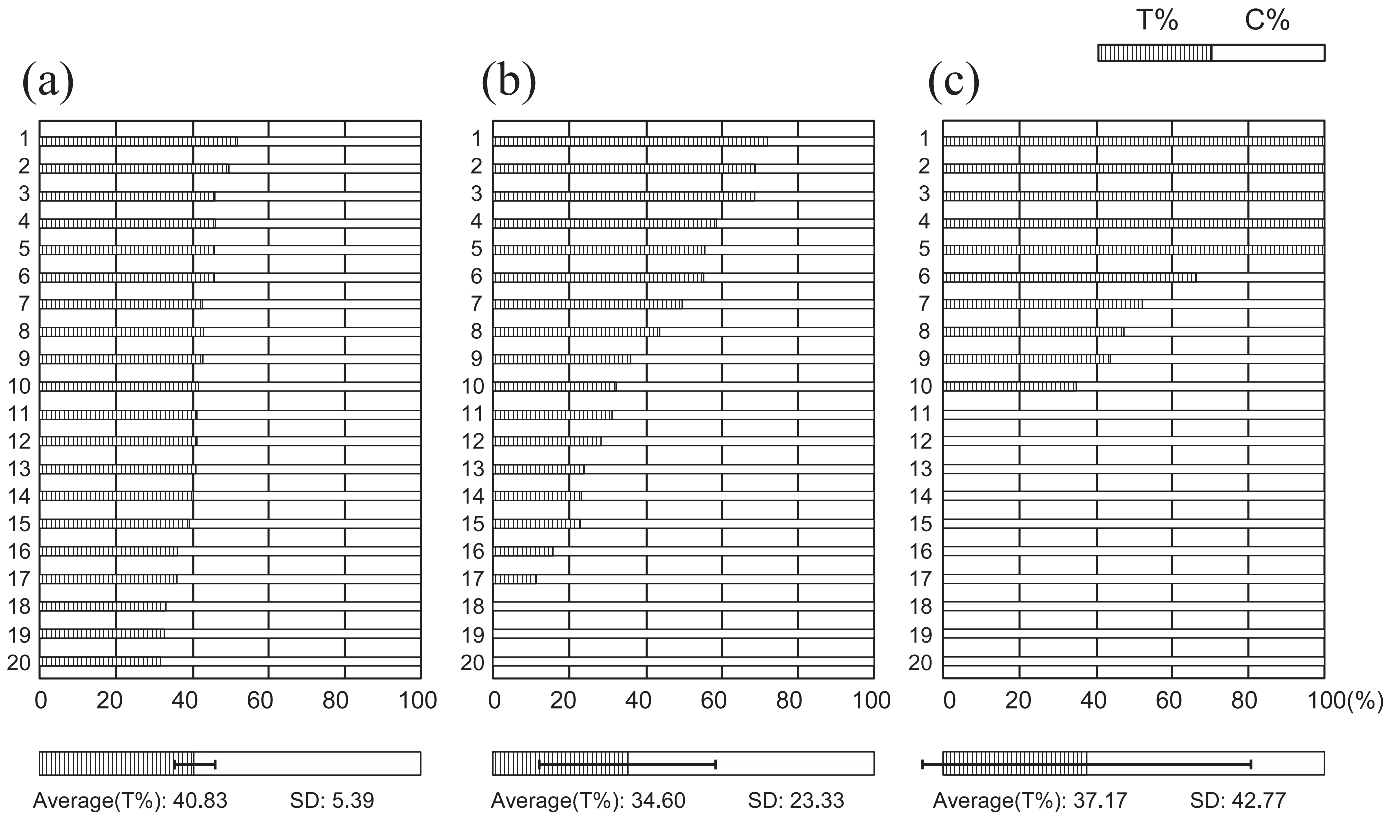

FIG. 2—*Comparisons of heteroplasmic proportions at nucleotide 16,294(C/T) of sample 61 measured on 20 sequence electropherograms analyzed from 1000 copies (a), 100 copies (b), and 10 copies (c) of mtDNA. The average and standard deviation of T% obtained for each of the 20 samples are shown. Three products from 100 copies (b; 18–20) and 10 products from 10 copies (c; 11–20) show homoplasmy at C, and five products (c; 1–5) show homoplasmy at T.*

copies of each sample did not vary greatly and the SD for 20 products from each sample was less than 10% (5.39–6.92%). The SD for 20 products from 100 copies was more than 20% (20.82–27.10%), much higher than the value for 1000 copies.

When 100 copies of each sample were amplified, three products of sample 61 (Fig. 2b; 18–20) were determined as homoplasmy but not heteroplasmy. When 10 copies of the samples were amplified, more than half of the products from each sample, 17 of sample 21 (Fig. 1c; 1–8, 12–20), 15 of sample 61 (Fig. 2c 1–5, 11–20), and 14 of sample 76 (Fig. 3c; 1–4, 11–20), showed a homoplasmic sequence. Each type of nucleotide present in the heteroplasmic position was detected as homoplasmy, respectively.

Sample 21 has two heteroplasmic positions at nucleotides 16,234 (T/C) and 16,299 (A/G). In the results of the amplification from 10 copies of the sample (Fig. 1c), 17 products showed a homoplasmic sequence (Fig. 1c; 1–7, 12–20) at each position. In addition, 16 of 17 products could be classified into two haplotypes of 16,234 T/16,299 A and 16,234 C/16,299 G. This result indicates that the original mtDNA of sample 21 contains mainly these two haplotypes. But only one of 17 homoplasmic products (Fig. 1c; 8) showed the exceptive haplotype of 16,234 T/16,299 G. This unique haplotype seems to be produced by back mutation or crossover occurring in mtDNA. The result and the possibility are consistent with the previous theory which says that minor haplotypes are produced by ongoing mutation of mtDNA in individuals (17,18).

It is generally thought that each cell contains many types of minor heteroplasmies, because there are large numbers of mtDNA in one cell and the rate of mutation of mtDNA is known to be very high (11–13). As a result, an unknown heteroplasmy may be detected when a very low amount of a biological sample is analyzed. In this study, PCR amplification was carried out using one WBC. In the sequence results, 13 sequences of 100 products (20 products amplified, respectively, from five individuals' WBCs) showed 15 heteroplasmies and a nucleotide substitution (Table 2). None of these heteroplasmies and a substitution were detected from the same individuals when large amounts of template DNA, equal to 1 ng of total genomic DNA (theoretically including several hundred thousand copies of mtDNA) was amplified in our previous analysis. No heteroplasmy was detected from 20 products derived from individual c. The products possibly had underlying heteroplasmies which had an insufficient peak height to permit their detection.

In our routine analysis of mtDNA, when two or more positions of heteroplasmies are found within one product, contamination by other DNA samples is usually suspected. However, in this study, products were unlikely to be contaminated because no products were obtained from negative controls in each PCR and the detected positions of heteroplasmy were completely random.

There is a possibility that the detected heteroplasmy was the result of polymerase misincorporation during the PCR amplification. Some reports concerning the fidelity of Taq polymerase show variable values for the error rate and the rate is lower than 1×10^{-4} (19,20). Less than 1 bp of misincorporation is theoretically caused in every 20 products in each amplification cycle because the fragment length of the first PCR product in this study is about 500 bp. If the copy number of template DNA is too small, the misincorporation produced during the initial cycles of PCR amplification would influence the final interpretation. For example,

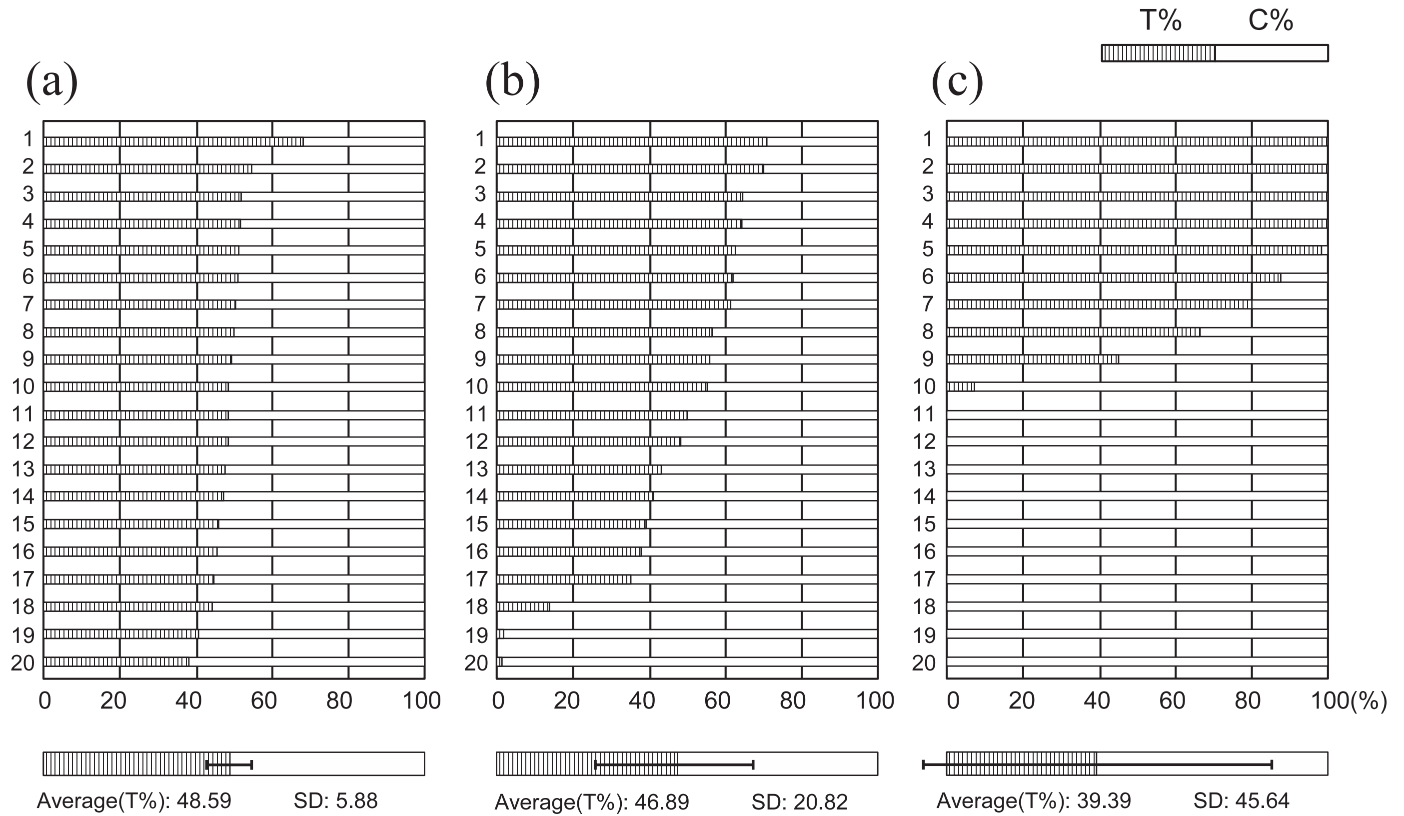

FIG. 3—*Comparisons of heteroplasmic proportions at nucleotide 16,261(C/T) of sample 76 measured on 20 sequence electropherograms analyzed from 1000 copies (a), 100 copies (b), and 10 copies (c) of mtDNA. The average and standard deviation of T% obtained from each of the 20 samples are shown. Four products from 10 copies (c; 1–4) show homoplasmy at T, and 10 products (c; 11–20) show homoplasmy at C. (Although it is not clear whether product "b; 19, 20 and c; 5" is heteroplasmy or not in this figure, a low rate of "T" or "C" existed.)*

TABLE 2—*Detected heteroplasmies or nucleotide substitution from one or a few WBCs of samples a, b, d, and e.*

		Sample number																
Nucleotide position	CRS	a*	a09	a14	a24	a27	b*	b18	b32	d*	d15	d22	d32	e*	e089	e108	e109	e232
16,209	T	**C**	**C**	**C**	**C**	**C**												
16,218	C										**C/T**							
16,223	C	**T**	**T**	**T**	**T**	**T**	**T**	**C/T**	**T**	**T**	**T**	**T**	**T**					
16,225	C														**T/C**			
16,234	C													**T**	**T**	**T**	**T**	**T**
16,236	C				**C/T**													
16,243	T											**T/C**		**C**	**C**	**C**	**C**	**C**
16,270	C							**T/C**										
16,285	A			**A/G**														
16,291	C													**T**	**T**	**T**	**T**	**T**
16,293	A					G												
16,298	T								**T/C**									
16,311	T								**T/C**									
16,319	G								**G/A**									
16,320	C															**C/A**		
16,324	T	**C**	**C**	**C**	**C**	**C**												
16,351	A																	**A/C**
16,362	T	**C**	**C**	**C**	**C**	**C**				**C**	**C**	**C**	**C**					
16,374	A		**A/–**															
16,376	C																**C/T**	
16,380	C												**–/C**					

CRS, the Cambridge Reference Sequence (25). Bold letters indicate sequence difference from CRS. Underlined letters indicate the detected heteroplasmies or substitution. Asterisked sequences indicate the results of our previous analysis using 1 ng of each template DNA (shown in Table 1).

if the reaction mixture contains only a few dozen template DNA molecules, misincorporation could occur in the initial cycles of amplification. It has been reported that the copy number of mtDNA included in one WBC is $1 \times 10^{(2.99 \pm 0.52)}$ (21). It is unlikely that a random misincorporation would affect the detection of unknown heteroplasmy because the copy number of mtDNA in WBCs seems to be sufficient to negate this effect. Nonetheless, it is actually difficult to determine whether the heteroplasmies detected in this study

existed in each cell or were a PCR artifact produced by misincorporation by the DNA polymerase because the actual error rate of the polymerase in each amplification is unpredictable. In either case, it can be concluded that an mtDNA analysis starting with a small amount of sample would have risks associated with it, in terms of detecting unknown heteroplasmies or substitutions derived from varying the ratio of heteroplasmy. Not only obvious heteroplasmy, but also potential heteroplasmy in each cell can influence the result of an analysis when a small amount of mtDNA is amplified.

Heteroplasmy is not a rare case as believed previously (8,22,23). Some reports proposed careful interpretation when an analysis of mtDNA containing heteroplasmies is performed (24). We additionally suggest that a sufficient amount of mtDNA should be used as a template for PCR and the subsequent sequence analysis, because a very low amount of template DNA can lead to the misinterpretation of a sample. We need to know how much template DNA should be used for amplification to obtain a reproducible ratio of heteroplasmy. In this study, the ratio determined from the products of 1000 copies of template mtDNA did not vary significantly and did not show homoplasmy in either sequence. As a result, more than 1000 copies of template DNA are recommended in each reaction. PCR conditions which cannot amplify a very small amount (less than thousands) of template DNA should be regulated. Increasing the cycle number of PCR or the nested PCR for the purpose of improving the detection sensitivity is not recommended for forensic DNA analysis. MtDNA-specific quantitative PCR will help to confirm the amount of template DNA. Comparing mtDNA sequences between hairs, old bones, or other poor samples containing very small amounts of mtDNA is unreliable. For reliable comparison, samples containing a relatively large amount of DNA, such as blood or buccal swabs of the same individuals, should be prepared as reference samples to take potential heteroplasmies into consideration and to confirm the sequence.

In conclusion, forensic scientists analyzing mtDNA for identification should recognize the potential risks; different haplotype of mtDNA sequences derived from potential heteroplasmy (or from polymerase misincorporation) can be potential problems, when a very small amount of template DNA is amplified using high-sensitive PCR.

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