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**TECHNICAL NOTE**

**CRIMINALISTICS**

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# Alterations of Length Heteroplasmy in Mitochondrial DNA Under Various Amplification Conditions*

**ABSTRACT:** There are several areas within mitochondrial DNA that show length heteroplasmy. If the heteroplasmy pattern is unique and consistent for each person, it may be used to support an interpretation of exclusion in identity testing. We investigated whether the length heteroplasmy pattern would be consistent under different amplification conditions. We also determined whether various amplification parameters would affect the homopolymeric cytosine stretches (C-stretch) in HV1. Monoclonal samples tended to be heteroplasmic after amplification. After several repetitions, C-stretch patterns of all samples were inconsistent even under the same amplification conditions. Increased PCR cycles and high template concentrations resulted in a more frequent heteroplasmic tendency. These amplification parameters seem to have little effect if samples are not long enough in C-stretch or total length of the segment from nt 16180 to nt 16193. It is suggested that the pattern of length heteroplasmy cannot be used as an additional polymorphic marker.

**KEYWORDS:** forensic science, DNA typing, mitochondrial DNA, amplification, length heteroplasmy, cytosine stretch

In forensic casework, mitochondrial DNA (mtDNA) testing is used to provide circumstantial evidence for association (1,2). It is also used in many phylogenetic works because mtDNA is transmitted as a single genetic element without recombination (3,4). Another peculiar characteristic of mtDNA is heteroplasmy (5–8). There may be different mtDNA types within a person, a cell, or even a mitochondrion. A specific point or length of an area within mtDNA may be variable, which is point heteroplasmy or length heteroplasmy. Length heteroplasmy is related to homopolymeric cytosine stretch (C-stretch). Segments of nt 16184–16193 and nt 303–315 in hypervariable regions 1 and 2, respectively, which are the most polymorphic regions are commonly referred to as C-stretches. The heteroplasmy pattern is highly variable, and there have been several attempts to classify or utilize the length heteroplasmy pattern (9–13). If the pattern is reproducible under different conditions at a practical level, this can be used as an additional polymorphic marker in forensic mtDNA typing.

This study was attempted to determine whether various amplification parameters such as DNA concentration, the number of PCR cycles, and DNA Taq polymerases would affect the length heteroplasmy pattern.

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## Materials and Methods

### *Preparation of DNA Samples*

Monoclonal samples of different sequences and C-stretch numbers were prepared to investigate how various amplification parameters affect the length heteroplasmy pattern.

Fifteen unrelated Korean DNA samples, which are known to be heteroplasmic around the nt16189 position with T/C transition, were amplified for the HV1 region using F15900 and R16493 primers (Table 1). The PCR products were purified and then cloned using the pCR2.1 TOPO-vector (Invitrogen, Carlsbad, CA) following the manufacturer's recommendations. Positive clones were sequenced without amplification from the primer F15900 using the BigDye Terminator Cycle Sequencing Ready Reaction kit Version 1.1 (Applied Biosystems, Foster City, CA). After confirmation of its monoclonality and the number of C-stretches, 26 monoclonal DNA samples that had different numbers of adenines and cytosines were selected (Table 2). The samples were classified as AxCy (*x* and *y*, the number of repeated nucleotides). If a sample showed the sequence AACCCCCCCCCCCC from nt 16180 to nt 16193, for example, we abbreviated these sequences as A2C12.

### *Sequencing of the Clones with Amplification*

Considering the number of adenine before C-stretch and the total number of cytosine, we selected 10 representative samples (nos. 3, 5, 7, 8, 11, 15, 16, 18, 21, and 24) from the above 26 monoclones. To investigate whether the amplification process reproduces length heteroplasmy, we amplified the C-stretch area and sequenced it.

TABLE 1—*Sequences of primers used for mitochondrial DNA PCR and sequencing.*

| Primer | Primer Sequence (5′ → 3′) |
|---|---|
| F15900 | AAT GGG CCT GTC CTT GTA GTA |
| R16493 | GAA GTA GGA ACC AGA TGT CGG |
| F15989 | FAM-CCC AAA GCT AAG ATT CTA AT |
| R16258 | TGG CTT TGG AGT TGC AGT TGG C |

TABLE 2—*Samples classified by AxCy in HV1 poly C-stretch.*

| AxCy | Sample Number |
|---|---|
| A2C12 (AACCCCCCCCCCCC) | 1, 2, 3 |
| A2C13 (AACCCCCCCCCCCCC) | 4, 5 |
| A2C14 (AACCCCCCCCCCCCCC) | 6, 7 |
| A3C10 (AAACCCCCCCCC) | 8 |
| A3C11 (AAACCCCCCCCCC) | 9, 10, 11 |
| A3C12 (AAACCCCCCCCCCC) | 12, 13 |
| A3C13 (AAACCCCCCCCCCCC) | 14, 15 |
| A4C9 (AAAACCCCCCCCC) | 16, 17 |
| A4C10 (AAAACCCCCCCCCC) | 18, 19, 20 |
| A4C11 (AAAACCCCCCCCCCC) | 21, 22, 23 |
| A4C12 (AAAACCCCCCCCCCCC) | 24, 25, 26 |

AxCy (*x, y:* the number of repeated nucleotides) was classified by nucleotide number from nt 16180 to nt 16193.

### *Variation of C-Stretches with Changes in Amplification Conditions*

To check for length heteroplasmy variation depending on several amplification parameters, we amplified the C-stretch area in HV1 as we changed amplification conditions. DNA concentrations, the number of PCR cycles, and different Taq polymerases were altered. To compare results under different amplification conditions, we set up a standard condition. The condition was as follows. All 26 samples were amplified in a total of 20 μL of a PCR mixture containing 0.01 ng/μL DNA template, 1.0 U AmpliTaq Gold polymerase (Applied Biosystems), 10× PCR buffer (15 mM $MgCl_2$; Applied Biosystems), 2.5 mM dNTP, and 10 pM of the primers, F15989 and R16258 (Table 1). Thermal cycling was performed using the GeneAmp®PCR system 9700 (Applied Biosystems) under the following conditions: 95°C for 12 min, 30 cycles at 94°C for 1 min, 54°C for 1 min, 72°C for 1 min, and a final extension at 72°C for 60 min. The PCR products were separated by capillary electrophoresis using an ABI 3130 Genetic Analyzer (Applied Biosystems). The resulting data were analyzed using Genemapper ID v3.2 (Applied Biosystems). The experiments were repeated four times for reproducibility. Then, we changed the conditions as follows. First, the template concentration of all samples was increased to 0.1 ng/μL. Second, the number of PCR amplification cycles was changed to 26, 28, 35, and 40 cycles. Third, all samples were amplified using different Taq polymerases including rTaq DNA polymerase (Takara, Shiga, Japan) and recombinant Taq DNA polymerase (Invitrogen). These experiments were repeated at least twice under the same conditions.

## Results and Discussion

### *Occurrence of Length Heteroplasmy by PCR Amplification*

When 26 monoclonal samples were sequenced without amplification, none of the samples showed patterns of length heteroplasmy on electropherograms. After amplification, 9 of the 10 samples showed length heteroplasmy (Fig. 1). Only sample no. 16 (A4/C9), which was one of the shortest in total length, did not show this change. After amplification, the patterns of length heteroplasmy were variable for different samples. Based on this result, we assumed that heteroplasmy is usually produced during the manipulation of the PCR product. As slippage is known to be one of the popular mechanisms related to the production of length heteroplasmy, and it happens with both *in vivo* and *in vitro* processes, the earlier phenomenon may not be surprising (14,15).

### *Inconsistent C-stretch Patterns with the Same Amplification Condition*

In cases of heteroplasmy, the peaks are presented as a mixture of various peaks. With the number and height of peaks, we could interpret the status of the mixture. This method using GeneScan analysis is similar to that reported by Lee et al. (9) and Shin et al. (10). After repetition four times, none of the samples showed the same peak patterns. The inconsistent pattern of length heteroplasmy is shown in Fig. 2*a*, and these patterns normally showed one or two peak patterns in most samples. However, sample nos. 6, 7, 12, 14, 15, 23, 24, and 25 showed a high grade of heteroplasmy representing height alteration and an increase in the number of minor peaks compared to the other samples (Fig. 2*b*). From the results, we verified that the appearance of heteroplasmy on amplification is not consistent, thus restricting the usefulness of length heteroplasmy in identity testing. Our finding is consistent with that of Stewart et al.'s report (16), although the approach was not the same. They reported that differences in the number of cytosines at the C-stretch region or a variation in the relative amounts of heteroplasmic length variants could not be used to support an interpretation of exclusion.

### *Alterations of Length Heteroplasmy by Different Amplification Conditions*

The heteroplasmy pattern also changed according to changes in PCR parameters. These changes were not the same in different trials, but we observed certain tendencies. As in the standard condition, it was difficult to verify the effect of DNA concentration on heteroplasmy. However, some samples (nos. 6, 7, 14, 15, 24, and 25) showed different patterns representing an increase in height and/or the number of minor peaks compared to the pattern shown in 0.01 ng/μL (Fig. 2*c*). According to the number of PCR cycles, we divided our trials into two groups: the group with 26 and 28 PCR cycles and the group with 35 and 40 PCR cycles. In the group with a

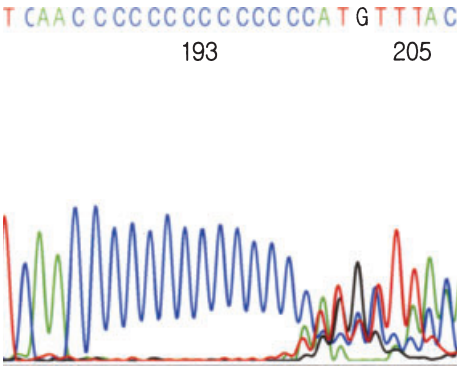


FIG. 1—*Electropherogram obtained by sequencing after PCR amplification of sample no. 7.*

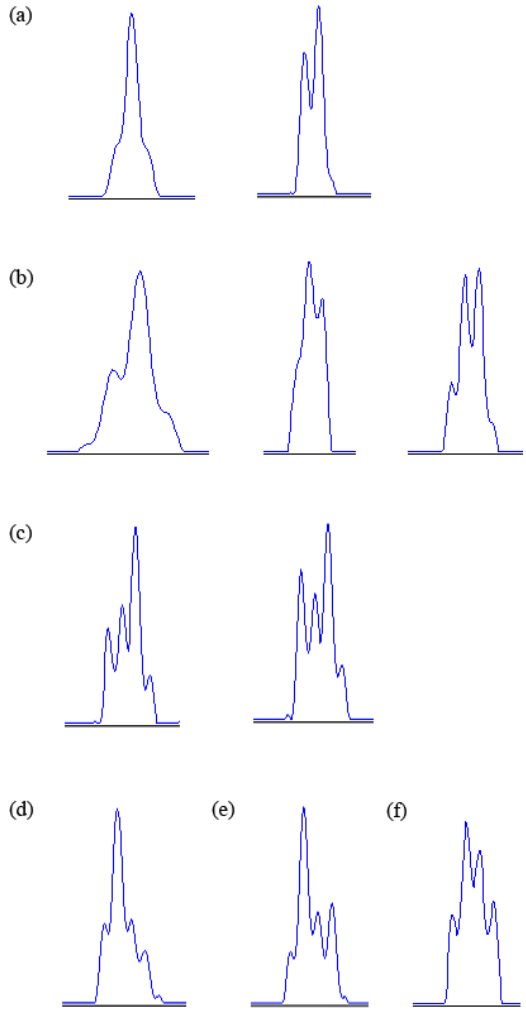


FIG. 2—*Electropherograms showing mitochondrial DNA peak patterns after PCR amplification. (a) This is the representative result, which shows one or two peaks (sample no. 2) in four repeated tests. (b) The peak results of sample no. 7 show an increased length heteroplasmy pattern. (c) With an increase in DNA concentration from 0.01 to 0.1 ng/2μL, sample no. 7 showed an increased heteroplasmy pattern. (d and e) Each electropherogram showed representative results when samples were amplified at 35 PCR cycles (sample no. 7) and 40 PCR cycles (sample no. 15). In the 35 and 40 cycle trials for sample no. 7, the patterns were similar. (f) This is the peak result of sample no. 7 using Taq DNA polymerase recombinants.*

high number of cycles, the heteroplasmy pattern was more complex, and thus the number of minor peaks increased (Fig. 2*d*,*e*). Especially, in sample nos. 25 and 26 with 26 PCR cycles and sample nos. 6, 7, 14, 15, 24, and 25 with 35 and 40 PCR cycles, the length heteroplasmy pattern was rather complex. These results suggest that as the cycle number increases, length heteroplasmy may occur more frequently. It was hard to observe any definite effect of the use of different DNA Taq polymerases from different companies. Sample nos. 6, 7, 14, and 15, which used Invitrogen Taq polymerase and sample nos. 5, 6, 7, and 14, which used Takara polymerase showed increased heteroplasmy patterns. Their peak shape was rather similar, which showed two major peaks and two minor peaks (Fig. 2*f*).

### *An Increase in Length Heteroplasmy*

From various amplification experiments, it was found that some samples tended to show more variations. Ten samples (nos. 5, 6, 7, 12, 14, 15, 23, 24, 25, and 26) showed at least one more complex heteroplasmy pattern. To find what differences between the samples induced this phenomenon, these samples were analyzed after being classified according to the base sequences of AxCy (*x* and *y*, the number of repeated nucleotides) from nt 16180 to nt 16193. Through this analysis, it was found that these samples had at least 11 cytosines (Cy) and 15 nucleotides (AxCy) in common. The samples without the aforementioned nucleotide composition did not show patterns of clearly increased heteroplasmy. It is conceivable that heteroplasmy occurs more frequently as the number of cytosines increases. Heteroplasmy did not occur in any samples of A2C12, but occurred in one sample (no. 5) of A2C13 and two samples (nos. 6 and 7) of A2C14. This tendency was also observed in the samples of A3C10, A3C11, A3C12, A3C13 (by increasing cytosine length), and A4C9–A4C12. The results of this study correspond with those of earlier studies, which have reported that a homopolymeric cytosine stretch of ≥8 residues usually results in an increase in heteroplasmy at positions 303–309 although the positions are different from those of previous studies (9,16–18). We also found that the full length of AxCy appeared to be an important factor for increasing unstable length heteroplasmy. Increased heteroplasmy was not observed in the samples of A2C12, whereas it was observed in one sample (no. 12) of A3C12 and three samples (nos. 24, 25, and 26) of A4C12 by increasing adenine length. Among the samples, increased heteroplasmy was more prominent in sample nos. 6, 7, 14, 15, 24, and 25. These samples have at least 12 cytosines (Cy) and 16 nucleotides (AxCy) in common.

There have been few studies on factors affecting length heteroplasmy except for the length of C-stretch. Mechanisms other than common replication slippage may affect length heteroplasmy. Further studies are needed to confirm these mechanisms.

## Conclusion

In this study, when samples with homopolymeric cytosine stretches were amplified under various conditions, their patterns of length heteroplasmy showed inconsistent results. This result suggests that these sequences may not be useful in forensic science. Furthermore, this study showed that a greater number of PCR cycles and higher DNA concentrations were associated with a greater frequency of increased length heteroplasmy in some samples. Therefore, the nucleotide composition of samples may be an important factor for the occurrence of length heteroplasmy.

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