A modified mini-primer set for analyzing mitochondrial DNA control region sequences from highly degraded forensic samples

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To facilitate the analysis of mitochondrial DNA (mtDNA) control region sequences from highly degraded skeletal remains, a modified mini-primer set was designed to overcome the limitations of the Armed Forces DNA Identification Laboratory (AFDIL) mini-primer set. This modified mini-primer set is less affected by nucleotide variability and PCR amplification conditions than the AFDIL mini-primer set, and was able to amplify the mtDNA sequences of 55-year-old skeletal remains with high efficiency, indicating that it is a useful tool for analyzing mtDNA control region sequences from highly degraded forensic samples.

Analysis of the human mitochondrial DNA (mtDNA) has become a powerful tool for testing the forensic identity of highly degraded skeletal remains due to its stability and the large number of genome copies per cell. Since highly degraded samples contain populations of intact DNA molecules that are severely restricted in size (1–4), attempts have been made to target and preferentially amplify mtDNA sequences with small products (1,2,5,6). The mini-primer set amplification strategy, originally proposed by the Armed Forces DNA Identification Laboratory (AFDIL) (5,6), can recover reliable mtDNA sequences from highly degraded skeletal remains and dramatically increase the PCR amplification success rate when compared to using products of approximately 250 bp (5).

However, the AFDIL mini-primer set includes a few primers whose 3′ ends are located at polymorphic nucleotide positions (n.p.): the first and second nucleotides from the 3′ ends of F16190 at n.p. 16209, and R16322 at n.p. 16304, respectively. These polymorphisms can reduce PCR yields and are found in substantial frequencies in various racial groups (7). According to the FBI mtDNA database, the polymorphism frequencies of n.p. 16209 are 1.33%, 3.19%, and 6.27% in Caucasians, Asians, and African Americans, respectively; those of n.p. 16304 are of 8.58%, 14.74%, and 0.87% (7). The 3′ ends of the R16158, R16251, F16268, and F220 primers are located at n.p. 16140, 16235, 16286, and 239, respectively; the polymorphism frequencies are 5.05% at n.p. 16140 and 1.33% at n.p. 16235 in Asians, 5.75% at n.p. 16286 in African Americans, and 1.93% at n.p. 239 in Caucasians (7). In addition, when eight products were simultaneously amplified using the same annealing temperature, the Mps4a fragment was not amplified. This failure appears to be due to the unusually low T_m value of the Mps4a primer pair, and using a different annealing temperature for each primer pair would make amplification of the mtDNA control region cumbersome and time consuming.

We have developed a modified mini-primer set designed with consideration of HV1 length heteroplasmy, nucleotide variability within the control region, and primer T_m value for simultaneous amplification (Table 1). Some primers were identical to those in the AFDIL mini-primer set (6), while others were redesigned using the Primer3 program (http://www-genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi) (8) (Figure 1A). Through screening PCR amplifications, the final set of primers was selected from several candidate primer pairs based on high amplification efficiency, relatively short primer length, and high T_m.

To evaluate the final modified set of primers, DNA samples were simultaneously amplified with the modified set and with the original AFDIL mini-primer set (6). DNA from Koreans belonging to the mtDNA haplogroup D4 (a typical polymorphism of the HV1-HV2 region: 16223–16362–73–263–315.1C) and DNA with mutations located at the AFDIL mini-primer annealing sites were analyzed. To assess the performance of primers in highly degraded samples, 40 long bones obtained from 55-year-old skeletal remains of Korean War (1950–1953) victims were tested, and 9 samples associated with PCR failure when using larger products (235–263 bp) were also used to compare the PCR performance. DNA from buccal swabs and blood samples was extracted using the QIAamp DNA Mini kit (Qiagen, Hilden, Germany), as were DNA from skeletal remains as previously reported (9).

PCR was performed in a 25 µL reaction mixture containing 2 µL of template DNA, 1.0 U of AmpliTaq Gold DNA Polymerase (Applied Biosystems, Foster City, CA, USA), 2.5 µL of Gold ST*R 10 × buffer (Promega, Madison, WI, USA), and each forward and reverse amplification primer at 0.4 µM. Thermal cycling was performed in a PTC-200 DNA engine (MJ Research, Waltham, MA, USA) using the following conditions: 95°C for 11 min; 35 cycles of 95°C for 20 s, 56°C for 20 s, and 72°C for 30 s; and 72°C for 7 min. The DNA from skeletal remains was amplified using 2.5 U of AmpliTaq Gold DNA Polymerase and 42 cycles of PCR amplification with an annealing temperature of 50°C.

The amplification success for the 55-year-old skeletal remains was at least as high for the modified mini-primer set as for the AFDIL mini-primer set (Figure 1B). Successful mtDNA analysis results from nine samples in which PCR amplification failed with four larger products (>235 bp) also confirmed the utility of the modified mini-primer set for highly degraded forensic samples (Figure 1C). Also, the entire HV2 region sequence could be completely determined using

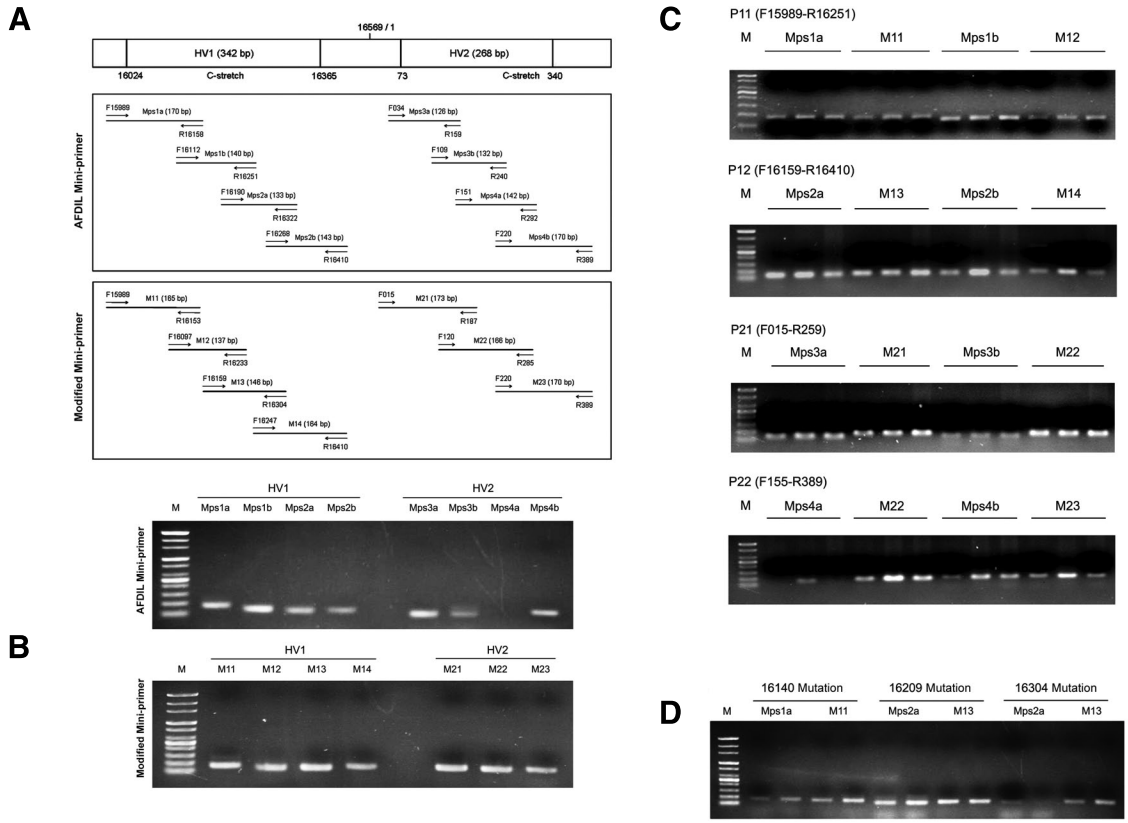

Figure 1. Comparison of the modified mini-primer set with the Armed Forces DNA Identification Laboratory (AFDIL) mini-primer set on highly degraded samples. (A) Mini-primer set amplification strategies for highly degraded specimens from the AFDIL and the present study. Primer nomenclature designates the 5′ end nucleotide for each primer. PCR product sizes for each set of primers are indicated in parentheses. (B) The amplification success for the 55-year-old skeletal remains was at least as high for the modified mini-primer set as for the AFDIL mini-primer set. A sample was amplified using either the AFDIL mini-primer set or the modified mini-primer set under the same PCR conditions as described in the present study, and equal volumes of each PCR were analyzed on an agarose gel. Mps4b and M23 were amplified with the same primer pair in the two mini-primer sets. (C) The modified mini-primer set worked on highly degraded samples with better performance than the AFDIL mini-primer set. For each indicated larger PCR product fragment (P11, P12, P21, and P22: 263 bp, 252 bp, 245 bp, and 235 bp, respectively), three samples that showed PCR failure with the abovementioned larger products were tested using either the AFDIL mini-primer set or the modified mini-primer set under the same conditions except for annealing temperature. The annealing temperature for the AFDIL primer pair was used as previously reported (5,6). (D) The modified mini-primer set was less affected by frequent mutations than those of the AFDIL. For each indicated mutation, two samples with the given mutation were amplified using either the AFDIL mini-primer set or the modified mini-primer set under the same conditions.

Table 1. Modified Mini-primer Set for Analyzing Mitochondrial DNA Control Region Sequences

Region	Product	Primer Sequence (5′ to 3′)		Product Size
HV1	M11	F15989	CCC AAA GCT AAG ATT CTA AT	165 bp
		R16153*	CAG GTG GTC AAG TAT TTA TGG	
	M12	F16097*	TAC ATT ACT GCC AGC CAC CA	137 bp
		R16233*	TGA TAG TTG AAG GTT GAT TGC TGT	
	M13	F16159 *	CAT AAA AAC CCA ATC CAC AT	146 bp
		R16304*	ACT GTT AAG GGT GGG TAG GT	
	M14	F16247*	ACT CCA AAG CCA CCC CTC A	164 bp
		R16410	GAG GAT GGT GGT CAA GGG AC	
HV2	M21	F015	CAC CCT ATT AAC CAC TCA CG	173 bp
		R187*	CGC CTG TAA TAT TGA ACG TA	
	M22	F120*	CGC AGT ATC TGT CTT TGA TTC C	166 bp
		R285	GTT ATG ATG TCT GTG TGG AA	
	M23	F220	TGC TTG TAG GAC ATA ATA AT	170 bp
		R389	CTG GTT AGG CTG GTG TTA GG	

Primers that were newly designed in the present study were indicated with an asterisk (*). To facilitate the PCR amplification, the R16233 primer sequence has the nucleotide A instead of nucleotide G at n.p. 16223. The nucleotide that is complementary to the 16223T is underlined.

only three products without any PCR amplification failure or extra sequence overlaps. However, reducing the number of products in HV2 resulted in increased product sizes of ~170 bp for each, which could in theory decrease success with some particularly degraded samples. This has not been extensively examined, but we had better success with these primer sets than with the AFDIL mini-primer set on some samples that failed to amplify for larger products (>235 bp). In addition, since the first to third nucleotides from the 3′ end of each primer were designed to be located at nucleotide positions with a polymorphism frequency of 1.0% or less, the seven products were expected to be less affected by frequent mutations than those of the AFDIL mini-primer set, and this was confirmed by PCR amplification comparison of samples containing polymorphisms (Figure 1D).

Moreover, since high sequence variability over the entire control region hinders the design of primers that are universally applicable to every population, alternative primers are suggested for use in specific racial groups, and need to be further evaluated for future use. In African Americans, n.p. 16265 displays a high polymorphism frequency (6.54%) (7), and accordingly, F16255 (5′-GCCACCCCTCACCCACTAG) is suggested as a replacement for F16247 in the M14 product, along with the replacement of R16304 by R16322 from the AFDIL mini-primer set in the M13 product. The polymorphism frequency of n.p. 239 is 1.93% in Caucasians (7), therefore F220* (5′-TG CTTGTAGGACATAATAATAACAA) is suggested as a replacement for F220 in the mini-primer sets of both the AFDIL and the present study.

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COMPETING INTERESTS STATEMENT

The authors declare no competing interests.

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