TECHNICAL NOTE

Yoichi Hanaoka,[1] *Ph.D. and Kiyoshi Minaguchi,*[1] *Ph.D.*

Sex Determination from Blood and Teeth by PCR Amplification of the Alphoid Satellite Family

REFERENCE: Hanaoka Y, Minaguchi K. Sex determination from blood and teeth by PCR amplification of the alphoid satellite family. J Forensic Sci 1996;41(5):855–858.

ABSTRACT: Sex determination from blood and teeth by PCR amplification of the alphoid satellite family according to the method of Witt and Erickson was investigated. Amplification of the X-(131 bp) and Y-(172 bp) specific sequences in males and that of the X-specific sequence in females was satisfactory in almost all samples. However, of the 22 samples (blood: 5, dental pulp: 12, tooth hard tissue: 5), a false positive fragment was detected at 172 bp among the female samples (blood: 17, dental pulp: 30, tooth hard tissue: 23) as a result of silver staining. These bands were not amplified by the dual PCR method and were different from the Y-specific band. Subsequent restriction enzyme digestion (DdeI, HinfI, and NciI) of these bands resulted in different patterns from the typical Y-specific band. Furthermore, in five male samples of hard tooth tissue specimens (n = 14), a Y-specific band was detected as a weak band, and in two of these five samples, this weak specific band was not amplified by dual PCR in spite of the male origin of the samples. These results showed that this method is useful for sex determination, but that dual PCR and restriction analysis should be used together in practical applications.

KEYWORDS: forensic science, odontology, human identification, sex determination, alphoid satellite, DNA, repetitive sequences, blood, dentition, X chromosome, Y chromosome

Sex determination from teeth can provide an important means of personal identification in the event of a mass disaster such as an air plane crash or fire. Formerly, older cytological methods using the X and Y chromatins (*1–3*) of dental pulp tissue was widely used for sex determination from teeth. However, in recent years, with the progress of gene analysis, sex determination methods using DNA analysis techniques have been developed and applied to forensic scientific samples including teeth (4–20).

Witt and Erickson (5) performed a sex-diagnosis test in which dried blood in quantities as small as 5 to 10 μL on filter paper were used as the source of a DNA template for PCR of the alphoid satellite family (ASF). They reported that this method provided a very simple, rapid, and reliable means for sex determination because ASF is located in the pericentromeric regions of human chromosomes and has a higher repeat organization.

Received for publication 28 Aug. 1995; revised manuscript received 4 Dec. 1995 and 16 Jan. 1996; accepted for publication 22 Jan. 1996.

[1]Assistant professor and professor, respectively, Department of Forensic Odontology, Tokyo Dental College, Chiba, Japan.

We investigated Witt and Erickson's methods (5) for sex determination using blood, dental pulp tissue, and hard tooth tissue as DNA sources and obtained good results. However, we found several matters that demand special attention when applying this method to the practical judgment of sex determination.

Materials and Methods

Sample Preparation

DNA was isolated from blood cells (n = 54, males: 37, females: 17), from intact dental pulp tissue obtained from extracted teeth (n = 43, males: 13, females: 30), and from hard tooth tissue (n = 37, males: 14, females: 23) according to the proteinase K digestion and phenol-chloroform extraction methods, which have previously been described as extraction methods for teeth (20). Isolated DNA was diluted with tris-ethylenediaminetetraacetic acid (TE) buffer and quantitated spectrophotometrically (1 OD_{260} = 50 μg/mL of DNA) before PCR amplification. Ten nanograms of DNA were used for amplification of DNA samples from blood and dental pulp tissue, and 20 ng was used for that from hard tooth tissue.

PCR Amplification

Two sets of oligonucleotide primers were used: Y11, Y22 flanking the 172-bp fragment of the alphoid repeats of the human Y chromosome (Y11: 5′-ATGATAGAAACGGAAATATG; Y22: 5′-AGTAGAATGCAAAGGGCTCC) (21) and X1, X2 flanking the 131-bp fragment of alphoid repeats of the human X chromosome (X1: 5′-AATCATCAAATGGAGATTTG; X2, 5′-GTTCAGCTC-TGTGAGTGAAA) (22). PCR was performed in Amplitaq DNA polymerase buffer (Perkin-Elmer, New Jersey), 200μM each deoxynucleoside triphosphate (dNTP), 1 unit (U) of Amplitaq (Perkin-Elmer, New Jersey), and 0.2μM each of the primer. The reactions were conducted in a final volume of 20 μL. The thermal cycle profiles using the DNA thermal cycler (Model PJ2000, Perkin-Elmer) were 36 cycles at 94°C for 1 min, 55°C for 1 min, and 72°C for 2 min, followed by preheating at 94°C for 5 min, then extension of the last cycle to 7 min. In amplification, the annealing temperature of some samples was modified to 37°C, and in other samples, 5 μL of amplified sample was reamplified under the preceding condition (dual PCR).

Comparison of PCR Products

The PCR reaction products were analyzed by ultraviolet (UV) visualization of ethidium bromide (EB)-stained and silver-stained (23) 8% polyacrylamide gel after electrophoresis in Tris-borate-EDTA (TBE) buffer (100m*M* Tris, 100m*M* boric acid, 2m*M* ethylenediaminetetraacetic acid [EDTA] at pH 8.3). In electrophoresis, two gel lanes were needed per sample because X1, X2 and Y11, Y22 primers were used in separate reactions.

For restriction analysis, the product was purified from contaminating primers using Centricon-100 miniconcentrators (Amicon, Beverly, Massachusetts), then digested with DdeI, NciI, and HinfI (New England BioLabs, Beverly, Massachusetts). Each restriction enzyme digestion was carried out at 37°C for 3 h, then analyzed by 12% polyacrylamide electrophoresis (PAGE).

Results

Detection of X- and Y-Specific Sequences from Blood and Dental Pulp Tissue

Figure 1 shows the results of amplification of blood specimens in accordance with Witt and Erickson's method (5). Witt and Erickson reported that the X-specific fragment size was 130 bp and the Y-specific was 170 bp. However, when calculated, the estimated fragment sizes of the two sequences predicted from the report of Wolfe et al. (21) were that the former was 131 bp and the latter was 172 bp. Therefore, we use these values for the following interpretation. Satisfactory amplification of X-(131 bp) and Y-specific sequences (172 bp) in the males and that of the X-specific sequence in the females was observed on the EB-stained gel (Fig. 1*a*) and the silver-stained gel (Fig. 1*b*) in all blood samples (n = 54). In all of the dental pulp tissue samples (n = 43), the X- and Y-specific sequences in the males and the X-specific sequence in the females were also amplified similarly to the blood samples (Fig. 2*a*). However, in five cases of the female blood samples (n = 17) and 12 of the female samples of dental pulp tissue (n = 30), a weak band was detected by silver staining at 172 bp that was in the same position as the Y-specific sequence (Lanes 2 and 5 in Fig. 2*b* and Lane 1 in Fig. 3).

Detection of X- and Y-Specific Sequences from Hard Tooth Tissue

In all samples of hard tooth tissue (n = 37), the X-specific sequence was amplified distinctly, and in nine of the male samples (n = 14), the Y-specific sequence was also equally amplified. However, in five of the male samples, the Y-specific band was faint (Lane 4 in Fig 4). In five of the female samples (n = 23), as with the samples of blood and dental pulp tissue, a 172-bp unexpected fragment appeared (Lanes 2 and 5 in Fig. 5), and

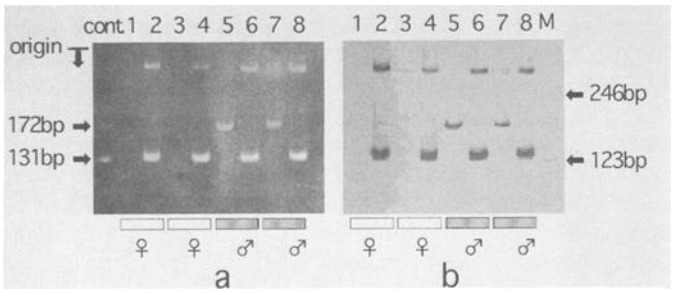

FIG. 1—*Results of the detection of X- and Y-specific sequences from blood specimens in accordance with Witt and Ericson's method. DNA from two different females (Lanes 1, 2, 3, and 4) and two different males (Lanes 5, 6, 7, and 8) were used as a template; Lanes 1, 3, 5, and 7 with Primers Y11,Y22; Lanes 2, 4, 6, and 8 with Primers X1 and X2. (a) EB-stained gel; (b) silver-stained gel; M: 123 ladder marker. Same samples were represented in each of the figures.*

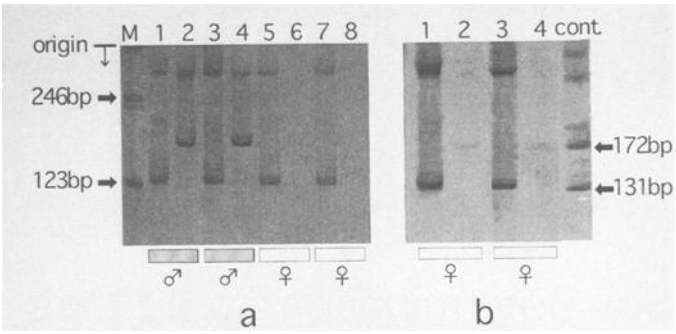

FIG. 2—*Results of the detection of X- and Y-specific sequences from dental pulp specimens by silver staining. (a) DNA from two different males (Lanes 1, 2, 3, and 4) and two different females (Lanes 5, 6, 7, and 8) were used as a template. The X- and Y-specific sequences in the males and the X-specific sequence in the females were amplified. (b) DNA from two different females (Lanes 1, 2, 3, and 4) were used as a template. A false positive fragment was detected in Lanes 2 and 4.*

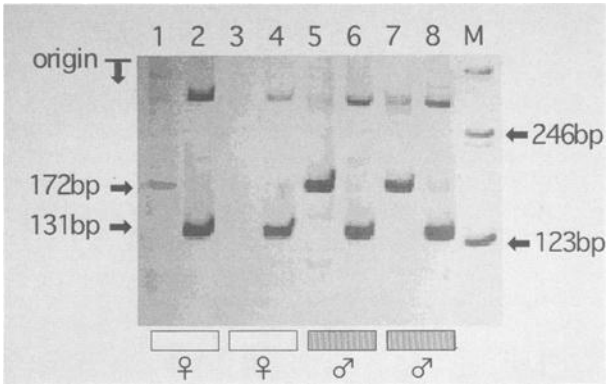

FIG. 3—*In five cases of the female blood samples, a false positive fragment was detected by silver staining (Lane 1). DNA from two different females (Lanes 1, 2, 3, and 4) and two different males (Lanes 5, 6, 7, and 8) were used as a template.*

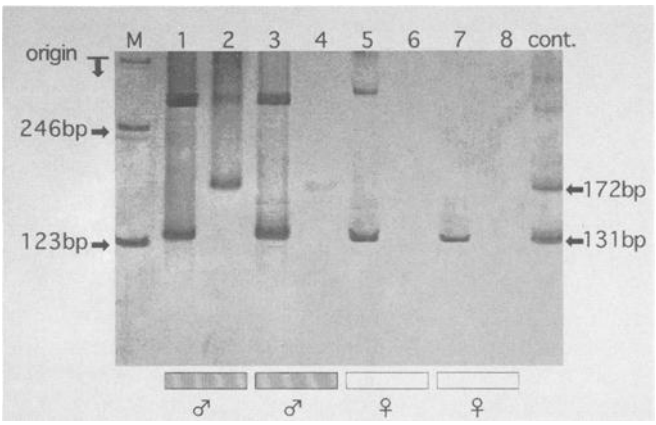

FIG. 4—*Results of the detection of X- and Y-specific sequences from hard tooth tissue. DNA from two different males (Lanes 1, 2, 3, and 4) and two different females (Lanes 5, 6, 7, and 8) were used as a template. In five of the male samples, the Y-specific sequence was not amplified satisfactorily (Lane 4).*

especially in two of these five cases, the band at 172 bp was evident (Lane 2 in Fig. 5). Note that it is highly likely that the female samples in these two cases were misjudged to be male without following confirmatory experiments.

The above-mentioned results are represented in Tables 1 and 2.

Investigation of the 172-bp False Fragments with Dual PCR

The five samples of blood, twelve samples of dental pulp tissue, and five samples of hard tooth tissue in which the band at 172 bp appeared in spite of the sample being female were investigated with dual PCR amplification. Consequently, in all investigated samples, the 172-bp fragment was not amplified at all (Lanes 3 and 6 in Fig. 2*b*, Lanes 3 and 6 in Fig. 5). The five male samples of hard tooth tissue that had only a weak band for the Y-specific sequence were also investigated with dual PCR. As a result, the Y-specific sequence was amplified clearly in three samples (Lane 8 in Fig. 5) but were not amplified in the other two samples (Lane 10 in Fig. 5). Accordingly, these two samples could not be determined to be male.

Identification of Restriction Sites of the 172-bp Fragment

Restriction enzyme digestion of the 172-bp Y-chromosomal fragment was performed. Figure 6 shows the restriction analysis of the Y-chromosomal fragment with HinfI, DdeI, and NciI on Lanes 2 through 4. HinfI digestion resulted in 133- and 39-bp fragments (Lane 2), DdeI digestion showed 124- and 48-bp fragments (Lane 3), and NciI digestion gave 90- and 82-bp fragments (Lane 4) as described by Witt and Erickson (5).

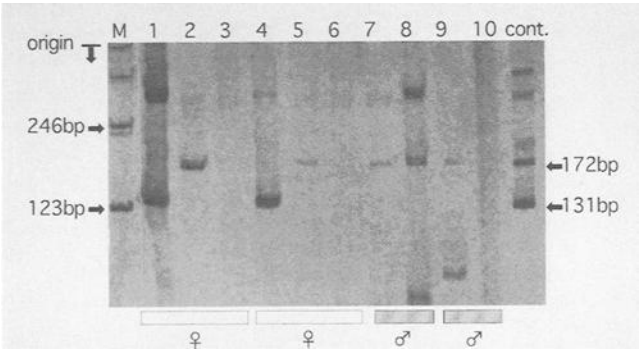

FIG. 5—*Results of the detection of X- and Y-specific sequences from hard tooth tissue. Lanes 1, 2, and 3—in this female sample, a false positive fragment was detected clearly by first PCR (Lane 2), but this false positive fragment was not amplified by dual PCR (Lane 3). Lanes 4, 5, and 6—in this female sample, as with the samples of blood and dental pulp tissue, a weak band at 172 bp appeared (Lane 5), but this band was not amplified by dual PCR (Lane 6). Lanes 7 and 8—in this male sample, the Y-specific sequence was not amplified satisfactorily by first PCR (Lane 7), but amplified satisfactorily by dual PCR (Lane 8). Lanes 9 and 10—in this male sample, the Y-specific sequence was not amplified satisfactorily by first PCR (Lane 9) and also was not amplified by dual PCR (Lane 10).*

TABLE 1—*The number of female samples in which the false positive fragments appeared.*

Materials	Total	False Positive Fragments/Total Number of Female Samples
Blood	54 (♂37, ♀17l)	5/17 (29.4%)
Dental pulp tissues	43 (♂13, ♀30)	12/30 (40.0%)
Hard tooth tissues	37 (♂14, ♀23)	5/23 (21.7%)

TABLE 2—*Results of PCR analysis of DNA obtained from hard tooth tissue.*

Sex	Total	First PCR		No.		Dual PCR	No.
♀	23	false positive, fragments	weak	3	→	not amplified	
			clear	2			
♂	14	Y-specific band	weak	5	↗	amplified	3
					↘	not amplified	2

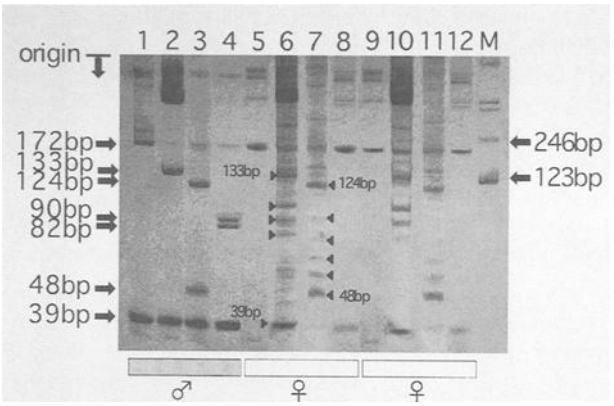

FIG. 6—*Restriction analysis by restriction enzyme digestion. Lanes 1, 5, and 9—no digestion; Lanes 2, 6, and 10—HinfI digestion; Lanes 3, 7, and 11—DdeI digestion; Lanes 4, 8, and 12—NciI digestion. Restriction enzyme digestion of false positive fragments of female samples (Lanes 5 and 9) resulted in different patterns from the Y-specific fragment (Lane 1).*

All the false positive fragments at 172 bp of the female samples were also analyzed by restriction enzyme digestion (Lanes 5 through 12 in Fig. 6). Previously, the weak bands at 172 bp in the female samples were amplified by PCR at a modified annealing temperature of 37°C to get enough materials for enzyme digestion. Consequently, HinfI and DdeI digestion gave the same sized fragments as digestion of the 172-bp Y-chromosomal fragment but also showed other fragments—three fragments between 133 and 39 bp at Lane 6 and four fragments between 124 and 48 bp at Lane 7. These fragments did not always appear distinctly, as shown in Lanes 10 and 11 in Fig. 6 in which a female sample had not all the counterparts of these. NciI did not digest the 172-bp fragment of the female samples in contrast to the predicted male Y-chromosomal fragment, and this enzyme is suitable to confirm false positive fragment of the female samples.

Discussion

There have been a number of reports on sex determination using demonstration of the Y-chromosome by means of DNA analysis techniques. Y-chromosome sequences have also been identified on Southern blots (4,6) following hybridization of restricted and unrestricted genomic DNA with specific Y probes. Y-chromosome sequences of DYZ1 (7,8), SRY (9), TDF (10), DYS19 (11), and amelogenin genes (12–14) have been amplified by PCR amplification. In comparison with these methods, it was reported that Witt

and Erickson's method (5) that we used in this study, has a higher sensitivity and reliability (15,16).

The present method has been applied to many kinds of forensic materials including teeth for sex determination (15–19). However, in these reports, the PCR products were not assessed by silver-stained gel but by ultraviolet visualization of EB-stained gel. In this study, although we confirmed that their method has a higher sensitivity because the X-specific fragment was amplified in all samples and the Y-specific fragment was also amplified in almost all the samples except for two hard tooth tissue samples, we also demonstrated that in some female samples the false positive fragment was detected in the same position as the Y-specific fragment (172 bp) by silver staining, which has a higher sensitivity than EB staining. This band was not amplified by dual PCR but amplified clearly by PCR annealed at 37°C.

Further investigation by restriction enzyme digestion analysis with HinfI, DdeI, and NciI confirmed that this band was not Y specific because NciI digestion of this band resulted in differences from the Y-chromosomal fragment. In both the HinfI and DdeI digestion, the same size fragments that were obtained from the restriction analysis of the Y-specific band and some other fragments were observed simultaneously. That is, another three fragments were observed between 133 and 39 bp in HinfI digestion and another four fragments between 124 and 48 bp in DdeI digestion. The appearance of these fragments probably means that the 172-bp fragment in the female specimen had different restriction sites from the Y-specific fragment that were digested by HinfI and DdeI. However, these fragments were not observed in some samples, so we presume that the false positive fragments at 172 bp detected in the female specimens were PCR products with more than two kinds of fragments that have a similar sequence with the Y-specific product that was amplified. We referred to restriction sites for HinfI, DdeI, and NciI within the human alphoid sequences of Y chromosome reported by Wolfe et al. (21); however, restriction sites that produced these fragments were not found. In addition, cross hybridization of the Y-centromeric repeat is stronger to the centromeres of Autosomes 13, 14, and 15 than it is to the X (21), while DXZ1 hybridizes more strongly to the centromeres of Chromosome 22 than it does to the Y (24). Accordingly, these fragments may have come from autosomes.

Male samples that have only a weak band of the Y-specific fragment have been already reported (15–19). In this investigation, we found that in the five male samples of hard tooth tissue, the Y-specific sequence was not amplified efficiently. In addition, we found that in two of these samples, the 172-bp fragment was not amplified at all by dual PCR. Consequently, this method requires very careful examination when applying it to hard tooth tissue as source of degraded DNA (20).

This method is very useful for sex identification but the high risk of misinterpretation demonstrated in this study should also be remembered. Accordingly, when this method is applied to real situations, the dual PCR method and restriction analysis by restriction enzyme digestion should be used together.

References

(1) Dixon AD, Torr JBD. Sex chromatin as an aid to the identification of sex in forensic medicine. Nature 1956;178:797.

(2) Seno M. Sex identification of the human tooth by Y chromatin in the nucleus of dental pulp cell. Nippon Hoigaku Zasshi 1977;31:172–9.

(3) Toi M. A study on sex differences of human dental pulp by sex chromosome identification. The Shikwa Gakuho 1977;77:1623–36.

(4) Tyler MG, Kirby LT, Wood S, Vernon S, Ferris JA. Human blood stain identification and sex determination in dried blood stains using recombinant DNA techniques. Forensic Sci Int 1986;31:267–72.

(5) Witt M, Erickson RP. A rapid method for detection of Y-chromosomal DNA from dried blood specimens by the polymerase chain reaction. Hum Genet 1989;82:271–4.

(6) Yokoi T, Sagisaka K. Sex determination of bloodstains using recombinant DNA probe, comparison with radio active and nonradioactive labelling method. Forensic Sci Int 1989;41:117–24.

(7) Nakahori Y, Mitani K, Yamada M, Nakagome Y. Human Y-chromosome specific repeated DNA family (DYZ1) consists of a tandem array of pentanucleotide. Nucleic Acids Res 1986;14:7569–80.

(8) Kogan SC, Doherty M, Gitschier J. An improved method for prenatal diagnosis of genetic disease by analysis of amplifies DNA sequences. N Engl J Med 1987;317:985–90.

(9) Sinclair AH, Berta P, Palmer MS, Hawkins JR, Griffiths BL, Matthijs J S, et al. A gene from the human sex determining region encodes a protein with homology to a conserved DNA-binding motif. Nature 1990;346:240–4.

(10) Nakagome Y, Seki S, Fukutani K, Nagafuchi S, Nakahori Y, Tamura T. PCR detection of distal Yp sequences in an XX true hermaphrodite. Am J Med Genet 1991;41:112–4.

(11) Santos FR, Pena SDJ, Epplen JT. Genetic and population study of a Y-linked tetranucleotide repeat DNA polymorphism with a simple nonisotopic technique. Hum Genet 1993;90:655–6.

(12) Akane A, Shiono H, Matsubara K, Nakahori Y, Seki S, Nagafuchi S, et al. Sex identification of forensic specimens by polymerase chain reaction (PCR): two alternative methods. Forensic Sci Int 1991;49:81–8.

(13) Akane A, Seki S, Shiono H, Nakamura H, Hasegawa M, Kagawa M, et al. Sex determination of forensic samples by dual PCR amplification of an X-Y homologous gene. Forensic Sci Int 1991;52:143–8.

(14) Hanaoka Y, Minaguchi K. Sex determination from blood and teeth by PCR amplification of segment of the amelogenin gene. DNA Polymorphism 1995;3:276–9.

(15) Yamamoto Y, Nakanishi A, Semba S, Ishizu H. Sex determination of blood stains by polymerase chain reaction (PCR) of Y-chromosome-specific alphoid repeat sequences. Acta Crim med leg jap 1991;57:187–91.

(16) Gaensslen RE, Berka KM, Grosso DA, Ruano G, Pagliaro EM, Messina D, et al. A polymerase chain reaction (PCR) method for sex and species determination with novel controls for deoxyribonucleic acid (DNA) template length. J Forensic Sci 1992;37:6–20.

(17) Honda K, Harihara S, Nakamura T, Hirai M, Misawa S. Sex identification by analysis of DNA extracted from hard tissues. Nippon Hoigaku Zasshi 1990;44:293–301.

(18) Ishizu H. Sex identification of forensic biological materials. Nippon Hoigaku Zasshi 1993;47:423–34.

(19) Semba S, Yamamoto Y, Ishizu H. Sex determination from blood and bloodstains by polymerase chain reaction (PCR). Nippon Hoigaku Zasshi 1994;48:7–18.

(20) Hanaoka Y, Inoue M, Tsai TH, Minaguchi K. Fundamental and practical study for DNA analysis using tooth as a source of DNA. Nippon Hoigaku Zasshi 1995;49:1–10.

(21) Wolfe J, Darling SM, Erickson RP, Craig DW, Buckle VJ, Rigby PWJ, et al. Isolation and characterization of an alphoid centromeric repeat family from the human Y chromosome. J Mol Biol 1985;182:477–85.

(22) Waye JS, Willard HF. Chromosome-specific alpha satellite DNA: nucleotide sequence of the 2.0 kilobasepair repeat from the human X chromosome. Nucleic Acids Res 1985;13:2731–43.

(23) Budowle B, Chakraborty R, Giusti AM, Eisenberg AJ, Allen RC. Analysis of the VNTR locus D1S80 by the PCR followed by resolution PAGE. Am J Hum Genet 1991;48:137–44.

(24) Yang TP, Hansen SK, Oishi KK, Ryder OA, Hamkalo BA. Characterization of a cloned repetitive DNA sequence concentrated on the human X chromosome. Proc Nat Acad of Sci 1982;79:6593–7.

Address requests for reprints or additional information to
Yoichi Hanaoka, Ph.D.
Department of Forensic Odontology, Tokyo Dental College,
1-2-2 Masago, Mihama-ku, Chiba-shi, Chiba-ken, 261 Japan