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Sex Typing of Forensic DNA Samples Using Male- and Female-Specific Probes

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ABSTRACT: Forensic DNA samples have been examined to ascertain the feasibility of a sex-typing procedure that we have recently developed. This uses two sets of primers complementary to the DXZ4 and SRY genes for polymerase chain reaction (PCR). PCR target in the DXZ4, an 80-bp sequence within the 130-bp fragment specific to females, is generated from inactive chromosome X by the DNA digestion with a methylation-sensitive restriction enzyme, HpaII. Therefore, the DXZ4 amplification and subsequent agarose gel electrophoresis detect the 80-bp fragment from female DNA. On the other hand, the SRY probe identifies a male-specific sequence on chromosome Y. Testing DNAs from fresh Turner's blood and from postmortem tissues exhibited band-signals confirming the sex identification. Degraded DNAs isolated from severely decomposed specimens were also identifiable when high-molecular-weight DNA was isolated before the assay. This demonstrates the usefulness of this method in forensic identification.

KEYWORDS: pathology and biology, female-typing, inactive chromosome X, methylation, SRY gene, polymerase chain reaction (PCR)

Diagnosing the sex of biological evidence can provide important information in a forensic investigation. Many methods for determining the human male have been developed basically with the use of chromosome Y-specific sequences [*1–8*]. In this study we also used such an assay, that is, detecting the SRY gene sequence on chromosome Y [*4*] with polymerase chain reaction (PCR) [*9*]. On the other hand, there are few methods for the identification of females due to the lack of female-specific sequence. We have recently developed a novel method for positively determining female-sex by PCR [*10*]. This female-typing is based on the sex difference of methylation pattern at a repetitive sequence, DXZ4, on chromosome X; males have an highly methylated DXZ4 whereas females have two DXZ4's, one of which is hypomethylated on the inactive X [*11*].

We reported that the combined use of the two DXZ4 and SRY assays accurately determines the sex of blood samples. In this study we have tested the applicability of this protocol to forensic samples. The results demonstrate that DNAs obtained from postmortem tissues of

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autopsy cases give the sex identification with this method. Cases of Turner syndrome are also presented.

Materials and Methods

Isolation of DNA from Blood and Tissues

Blood samples were obtained from normal males and females, and patients with Turner syndrome (TS). Three to five mL of heparinized fresh blood was diluted with ten volumes of 0.2% sodium chloride (NaCl) for hemolysis. Nucleated cells were collected by centrifugation at 3000 rpm for 10 min and rinsed twice with 0.2% NaCl solution. The cells were suspended in TNE buffer [10 mM tris (hydroxymethyl) aminomethane (Tris)/hydrochloric acid (HCl), 100 mM NaCl, and 1 mM disodium edetate (EDTA), at pH 7.5], containing 0.5% sodium dodecyl sulfate (SDS) and digested with 100 μg/mL of proteinase K(Merck Co.) at 56°C overnight. The DNA was extracted twice with phenol, once with phenol/chloroform (1:1), followed by chloroform extraction and then precipitated with two volumes of ethanol. The precipitation was rinsed with 70% ethanol, dissolved in TE buffer(10 mM Tris-HCl and 1 mM EDTA, at pH 7.5) and stored at 4°C until use. The amount of isolated DNA was quantitated at 260 nm in an ultraviolet spectrophotometer, and the DNA size was determined with 1% agarose minigel electrophoresis.

Several kinds of postmortem tissue specimens, such as skin, psoas muscle, liver, spleen, and gallbladder, were taken at autopsy from cadavers. Samples used in this study, skin, psoas muscle and gallbladder, were obtained from different individuals. About 0.3 g of tissue sample was rinsed with 0.9% NaCl, cut into pieces as small as possible in 5 volumes of TNM buffer [20 mM Tris-HCl, 100 mM NaCl and 1.5 mM magnesium chloride ($MgCl_2$), at pH 7.5], and pelleted by centrifugation at 2000 rpm for 5 min. The pellet was washed with TNE buffer and resuspended in 15 volumes of TNE buffer containing 0.3% SDS and 150 μg/mL of proteinase K. After digestion, DNA was extracted as described.

Recovery of High-Molecular-Weight (HMW) DNA

Approximately 10 μg of degraded DNA samples obtained from putrefied tissues was electrophoresed in a 1% low melting (LM) agarose (Sea Plaque GTG agarose, FMC BioProduct) minigel in TBE buffer (50 mM Tris-HCl, 48 mM borate, and 2 mM EDTA, at pH 8.4) and visualized by ethidium bromide (EtBr) staining. A gel slice containing approximately 10~ 23-kb DNA fragment was excised by comparing with size markers and incubated for remelting the gel at 65°C for 5 min in 10 volumes of TE buffer (10 mM Tris-HCl and 0.1 mM EDTA). After cooling to 50°C, an equal amount of phenol/chloroform solution was added and gently mixed. DNA was extracted, precipitated with ethanol, and then dissolved in a small volume (10μ 1) of distilled water. Recovered HMW DNA was subjected to the DXZ4 testing.

Sex Typing by Amplification of DXZ4 Region

DNA isolated from somatic cells was digested with HpaII at 37°C 4 h, and 250 to 350 ng of the digests were electrophoresed on 4% NuSieve GTG agarose (FMC BioProducts) minigel in TBE buffer. The gel was cut into three fractions: fraction 1, > 520-bp; fraction 2, 520~ 220-bp; fraction 3, 220~ 75-bp. Each of the fractions was subjected to centrifugation in a spin column (0.45 μm, Millipore Co.) for 20 min at 10,000 rpm. About 50μ 1 of filtrate was extracted with phenol/chloroform followed by chloroform, and an aliquote (5μ 1) was used for PCR assay as a template. The primers used are 5′-AAGGCCAGCTTGACC-AAGAG-3′ (F) (forward) and 5′-GTTCACCTGAAGTTTGAGGCC-3′ (R) (reverse), which yield a 80-bp product. Samples were mixed with 10 pmol of each primer and 1 unit of

Taq polymerase (Perkin-Elmer Cetus) in 30μ 1 of PCR buffer [10 mM Tris-HCl at pH 8.4, 50 mM potassium chloride (KCl), 1.5 mM $MgCl_2$ and 200μ M of each deoxy nucleoside triphosphates (dNTPs), and the solution was covered with 20μ 1 of mineral oil (Aldrich Chemical). The amplification was carried out in a temperature controller with 25 to 30 cycles of reaction at 94, 60 and 72°C for 1, 0.5 and 0.5 min, respectively. Amplified products were electrophoresed on 15% polyacrylamide gel (19:1 acrylamide:bis) containing 3.8% glycerol and visualized by silver staining (Bio-Rad). As an internal reference template, 10^{-5} to 10^{-6} ng of 68-bp DNA fragment was added to each reaction mixture. The DNA template was obtained by amplification of the 80-bp PCR product from DXZ4 locus using another set of primers: 5'-CAGCTTGACCAAGAG GCGACTGGCGTCC-3' (forward) and primer R, which was described above. Underlined sequence of the forward primer is same with a part of F primer. Therefore, the reaction produced an internally short fragment of 68-bp as positive control.

Male Typing by Amplification of SRY Gene

The primers used for PCR were 5'-ATGAACGCATTCATCGTGTGGT-3' (forward) and 5'-GGTCGATACTTATAATTCGGGT-3' (reverse). These SRY primers yield a 203-bp product. Amplification was carried out in a 20μ 1 vol containing 100 to 200 ng of genomic DNA, 0.5μ M each primer, 10 mM Tris-HCl at pH 8.4, 50 mM KCl, 2.5 mM $MgCl_2$, 1 unit of Taq polymerase and 200μ M of each dNTPs. After denaturation for 5 min at 94°C, 25 to 30 cycles were performed as follows: denaturation at 94°C for 1 min, annealing at 55°C for 30s and extention at 72°C for 30s, followed by a final extention of 10 min at 72°C. The PCR products were electrophoresed on a 12% polyacrylamide gel and the gel was then silver-stained.

Results and Discussion

The Experimental Protocol

A PCR primer set used in the female-sex typing is illustrated in Fig. 1, which amplifies a 80-bp fragment from the DXZ4 macrosatellite with PCR. The DXZ4 is a repetitive sequence on chromosome X and has 28 HpaII sites within the 3-kb repeat unit. Since the DXZ4 shows the methylation difference between male and female, the typing method

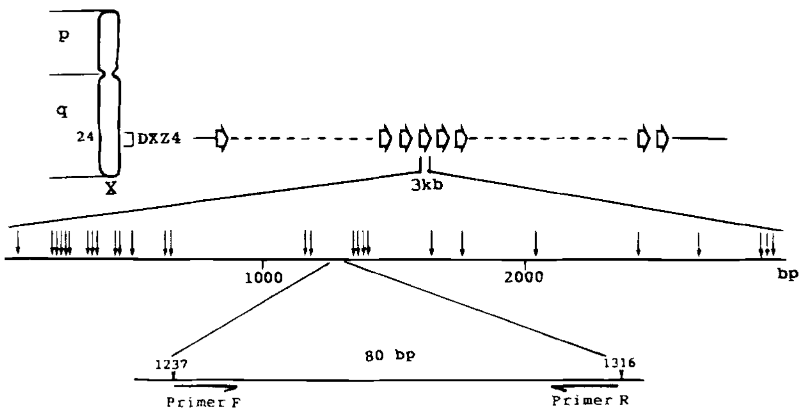

FIG. 1—*A strategy for DXZ4 amplification for sex identification. The diagram illustrates HpaII sites (marked by arrows) and the location of the primers F and R in DXZ4. Primer F, 5'-AAGGCCAGCTTGACCAAGAG-3'; and Primer R, 5'-GTTCACCTGAAGTTTGAGGCC-3'.*

detects the difference with the use of the methylation-sensitive enzyme, HpaII, as follows. HpaII-digested DNA fragments are size-fractionated by agarose gel electrophoresis, and three fractions containing fragments longer than 520-bp (fraction 1), fragments between 520-bp and 220-bp (fraction 2), and fragments between 220-bp and 75-bp (fraction 3) are obtained. The DXZ4 on inactive chromosome X specific to female is rarely methylated, and hence DNA of the region is cut into small fragments of approximately 130-bp that are recovered in fraction 3. In contrast, the DNAs on active chromosome X are yielded in fraction 1, because they are resistant to the enzyme. The female typing is thus performed by the examination of which fraction exhibits the 80-bp DNA.

Determination of Inactive Chromosome X in Turner

DNA was prepared from blood of healthy donors and Turner's patients and digested with HpaII. An aliquot of the digests was electrophoresed on 4% NuSieve GTG agarose minigel and the three fractions were obtained as described in the above section. Then, each fraction was subjected to the DXZ4 amplification and the products were analyzed by polyacrylamide gel electrophoresis (Fig. 2). Male DNA (panel A) showed an 80 bp band mainly in fraction 1 that contained large DNA fragments. This is consistent with male active X being highly methylated at HpaII sites in the repeat units [*11*]. A 68-bp band in the gel was fragments used as an internal standard that were amplified with the same primers (see **Materials and Methods**). A faint 80-bp band observed in fractions 2 and 3 is probably due to incomplete hypermethylation of the DXZ4 on active chromosome X. On the other hand, female DNA (B) gave the band in every three fractions. This is interpreted as follows; the band in fraction 1 is derived from the female active X and the band in fraction 2 and 3 comes from the inactive X that are hypomethylated. These results indicated

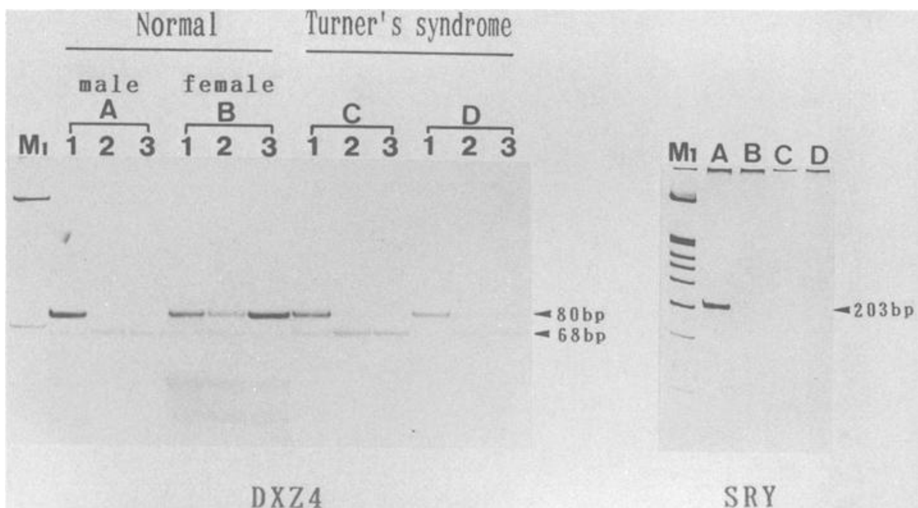

FIG. 2—*Sex determination by amplification of DXZ4 locus* (left panel) *and SRY gene* (right panel). *DNAs from normal male* (A), *female* (B) *and patients with Turner syndrome* (C, D) *were used for PCR analysis. In the protocol using intra-DXZ4 sequence, HpaII digests were size-fractionated into three (1: >520-bp, 2: 520 ~ 220-bp, 3: 200 ~ 75-bp). Each fraction was amplified with primers F and R. In the left panel, arrowheads point to 80-bp (target) and 68-bp (positive control). As shown in the right panel, a male-specific band (203-bp) was obtained only in A. Lane M_1: pBR322/HinfI size markers.*

that comparison of the band-intensity in fraction 3 (and fraction 2) and fraction 1 provides identification of the sex of DNA samples.

One Turner sample (C) gave the band only in fraction 1, which pattern was identical to that of the male. This indicated that this patient's cells contained only one active chromosome X. This is consistent with a result obtained from the karyotype analysis; the karyotype is 45, X in most cases. The other sample (D), however, exhibited a weak but clear band in fraction 3, suggesting that some of the cells carried an inactive X. This may reflect the mosaicism of the chromosome X, which is frequent in Turner syndrome [*12*].

To confirm these results, we carried out a male typing that detected a SRY gene specific to chromosome Y. A SRY-specific 203-bp band was observed in sample A but not in either sample B, C or D (Fig. 1, the right panel). This result was compatible with that of the female-sex typing described above. These results indicated that the combined use of the two PCR assays provides a useful means for the identification of human sex.

Application to Degraded DNA Samples

To examine whether the sex can be typed with the present protocol of degraded DNAs in forensic samples, we examined DNAs obtained from postmortem tissues. Four forensic samples obtained from skins were first analyzed of their DNA sizes. Figure 3 (the left panel) shows patterns stained with EtBr after agarose gel electrophoresis. Comparison of the positions between the pattern and the size markers showed that all samples contained HMW DNA and some degraded DNA fragments. The samples were subjected to the female- and male-sex typing without any other treatment.

The middle panel of Fig. 3 shows the female-typing. The two males gave the 80-bp band only in fraction 1, and the two females in all the three fractions. The SRY amplification of the four samples exhibited a clear band only for male samples E and F (the right panel). These results suggest that this typing is applicable to postmortem tissues if they contain HMW DNA.

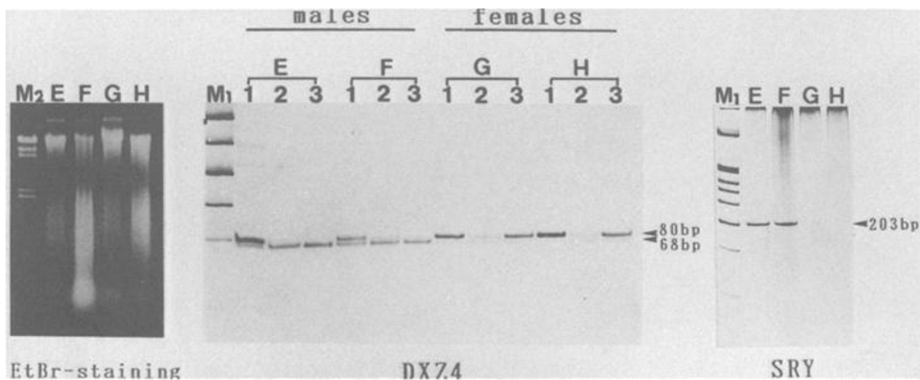

FIG. 3—*Amplification of male* (E, F) *and female* (G, H) *DNAs from postmortem skin tissues. The samples were taken from dead bodies, 39h* (E), *9h* (F), *2.5d* (G), *6h* (H) *after death respectively. These DNAs were electrophoresed on a 1% agarose minigel and visualized with EtBr to assess their size and amount* (left panel). *Samples* E *and* F *showed the male band-pattern in DXZ4 test and male-specific band in SRY test, while samples* G *and* H *gave the female band-pattern in DXZ4 test and no signals in SRY test. Lane* M_1*: pBR322/HinfI size markers, and Lane* M_2*: λ DNA/HindIII size markers.*

Application to more degraded DNA specimens was also investigated. The left panel of Fig. 4 shows the DNA sizes of samples I and J that were isolated from male muscle and female gallbladder, respectively. These samples mostly consisted of dedraded DNA less than 10-kb. Without any other treatment, they gave the DXZ4 80-bp band at almost same intensity in all three fractions and did not provide any information of the sex typing (see lanes marked "before").

Hence, we isolated the HMW DNA of 10-kb to 23-kb from the degraded samples by agarose gel electrophoresis (see **Materials and Methods**). The female typing for them exhibited the 80-bp band only in fraction 1 for the male sample and that in all fractions for the female ("after" lanes in Fig. 4). This suggested that highly degraded DNA samples as I and J are applicable to this typing. Consistently, the SRY 203-bp fragment was detected for I but not for J (right panel).

Application to a Putrefied Hand

Figure 6 shows an example of application of this combined method to a putrefied human hand of undetermined sex. A right hand was found on seashore in early spring, 1993, and the time elapsed after death was unknown (Fig. 5). DNA was extracted from bone marrow in metacarpal bone of the hand. The sample contained a little HMW DNA as shown in the left panel. The HMW DNA was isolated and subjected to the typing. The DXZ4 band was seen in fraction 1 more intense than in fraction 3 (the middle panel), suggesting that it was a female hand in origin.

The SRY typing was done in parallel. A first amplification with 30 cycles failed to give the 203-bp band (lane 1 in the right panel). Then, a second amplification was carried out. Several weak and broad bands were detected, but they were judged as non-specific products (lane 2); that is, the sample DNA did not contain the SRY gene. This is consistent with the result of the DXZ4 typing.

The SRY male-typing is easier to be performed than the DXZ4 typing is, but has a drawback due to the high sensitivity, that is, if samples derived from females are contami-

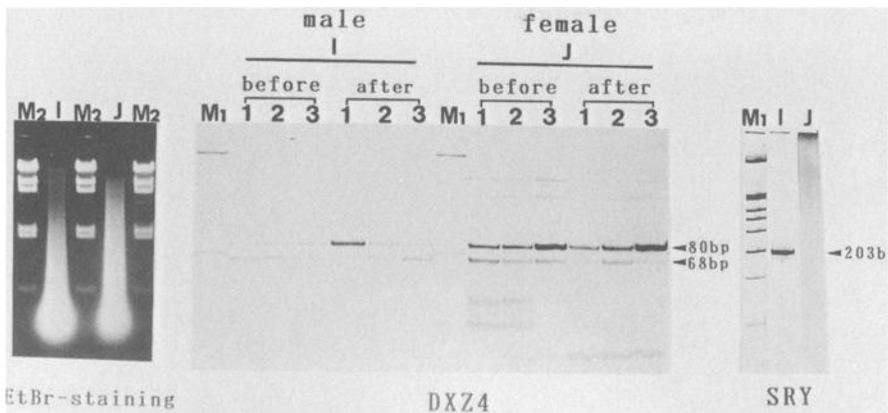

FIG. 4—*DXZ4 amplification of highly degraded DNA from forensic specimens before and after isolating HMW DNA. DNA samples were obtained from psoas muscle of male corpse(I), 10 h after death, and gallbladder of female corpse* (J), *9 h after death. EtBr-staining gel shows degradation status of the DNA. HMW DNA (10 ~ 23-kb) was recovered as described in materials and methods. HMW DNA of male and female gave each sex band-pattern in DXZ4 testing* (middle panel). *SRY band from male degraded DNA was visible in second PCR* (right panel). *Lane M_1: pBR322/HinfI size markers, and Lane M_2: λ DNA/HindIII size markers.*

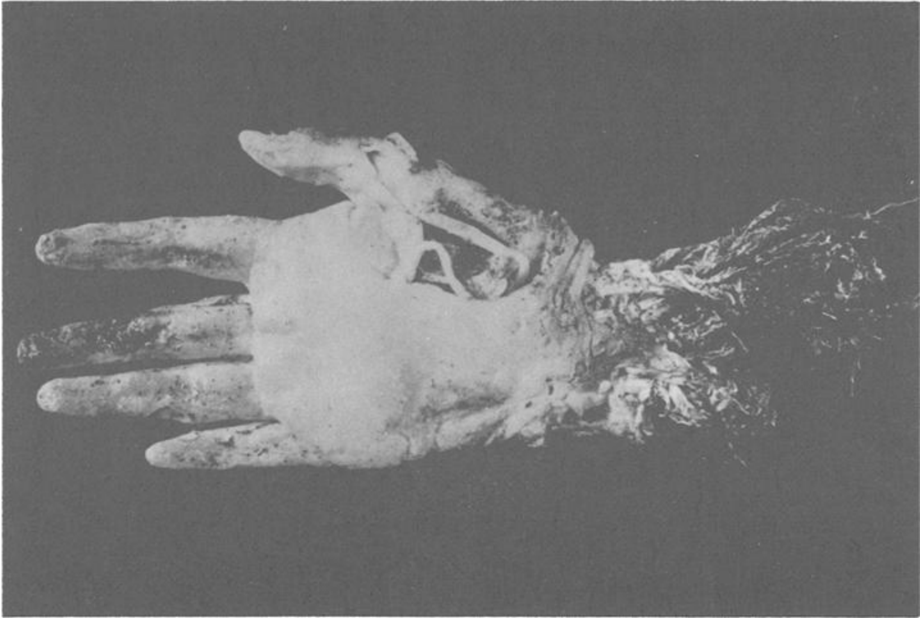

FIG. 5—*A putrefied human hand of undetermined sex.*

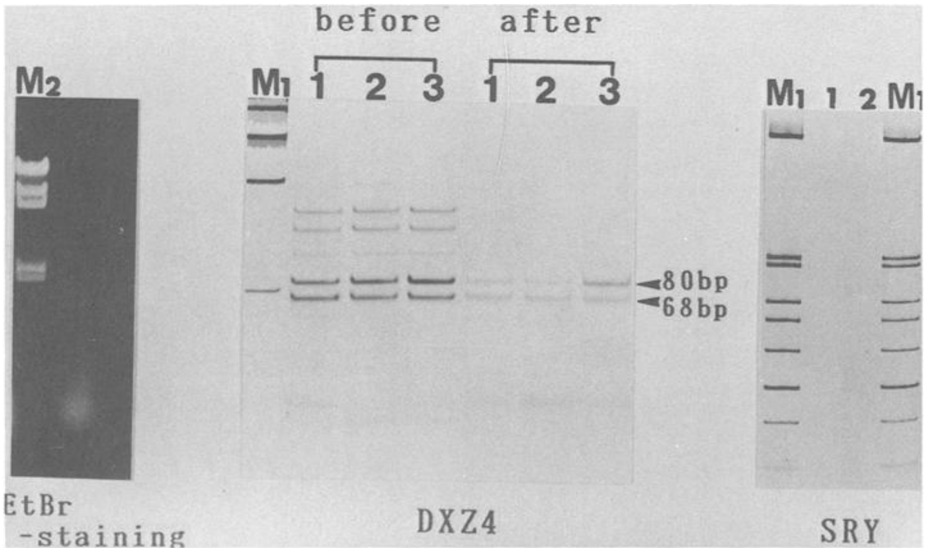

FIG. 6—*A sex diagnosis of the putrefied human hand (Fig. 5) whose gender was unknown. In the* left panel, *it is indicated that the sample DNA was severely degraded. Male specific-band from SRY gene was not detected in first(1) and second(2) PCR* (right panel). *And middle panel shows the results of DXZ4 amplification of its HMW DNA. Lane M_1: pBR322/HinfI size markers, and Lane M_2: λ DNA/HindIII size markers.*

nated with small amount of male DNA, they exhibit a male-specific band, which leads to a "false positive" diagnosis. It should be, therefore, recommended that the two complementary assays are done for the accurate sex-typing of forensic samples.

Conclusion

Sex determination of forensic samples is important, especially in those obtained from dismembered or putrefied corpse. Recently, we have reported a novel method for female-sex typing. In this study we show that the combined use of this assay and the SRY male-sex typing provides a useful information for not only determining human sex of forensic samples but also diagnosing Turner patients. We have also demonstrated applicability of the present protocol to degraded DNA samples of forensic importance. Isolation of high-molecular-weight DNA before assay overcomes the drawback of this female-sex typing that requires high-molecular-weight DNA. A successful sex-typing of putrefied tissue was exemplified in this study.

Acknowledgments

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