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A novel method for sex determination by detecting the number of X chromosomes

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Abstract A novel method for sex determination, based on the detection of the number of X chromosomes, was established. Current methods, based on the detection of the Y chromosome, can directly identify an unknown sample as male, but female gender is determined indirectly, by *not* detecting the Y chromosome. Thus, a direct determination of female gender is important because the quality (e.g., fragmentation and amelogenin-Y null allele) of the Y chromosome DNA may lead to a false result. Thus, we developed a novel sex determination method by analyzing the number of X chromosomes using a copy number variation (CNV) detection technique (the comparative Ct method). In this study, we designed a primer set using the amelogenin-X gene without the CNV region as the target to determine the X chromosome copy number, to exclude the influence of the CNV region from the comparative Ct value. The number of X chromosomes was determined statistically using the CopyCaller software with real-time PCR. All DNA samples from participants (20 males, 20 females) were evaluated correctly using this method with 1-ng template DNA. A minimum of 0.2-ng template DNA was found to be necessary for accurate sex determination with this method. When using ultraviolet-irradiated template DNA, as mock forensic samples, the sex of the samples could not be determined by short tandem repeat (STR) analysis but was correctly determined using our method. Thus, we successfully developed a method of sex determination based on the number of X chromosomes. Our novel method will be useful in forensic practice for sex determination.

Keywords X chromosome · Amelogenin gene · Copy number variation · Real-time polymerase chain reaction · Sex determination

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Introduction

Sex determination in forensic samples is an important aspect of investigations and is performed primarily with a commercially available short tandem repeat (STR) typing kit. Sex determination using the STR typing system is based on detection of the amelogenin gene on the X and Y chromosomes. Briefly, an unknown sample can be determined to be male when the amelogenin gene is detected on both the X and Y chromosomes. However, when the amelogenin gene is detected on the X chromosome only, an unknown sample would be deemed to be female. Many sex determination methods using detection of the amelogenin gene have been reported previously [1–5]. Recently, detection of the amelogenin gene was made easier and quicker using the real-time polymerase chain reaction (PCR) [4] and loop-mediated isothermal amplification [5]. Other than detecting the amelogenin gene, sex determination methods based on detecting the X and Y centromeric regions [6], detecting the "sex-determining region Y" (SRY), and steroid sulfatase (STS) gene [7] have been reported. However, these methods identify a sample as male by detecting the

Y chromosome. Thus, current methods determine the sex of a sample based on the presence or absence of the Y chromosome. Consequently, female gender is determined *indirectly*, because the decision is based on *non*-detection of the Y chromosome.

Methods based on the detection of the Y chromosome can lead to false results depending on the quality of the template DNA. Forensic samples are frequently degraded. In such samples, when only the X chromosome is detected, there are two possibilities for sex determination. Either the Y chromosome is truly not there, indicating a female sample, or the Y-chromosome cannot be detected due to the presence of highly fragmented DNA in a male sample. However, distinguishing between these possibilities is difficult because current methods are unable to *directly* demonstrate the female gender. However, it is also well known that the Y chromosome is not detected in STR analyses due to the amelogenin-Y null allele despite the sample being of male origin [8]. In such cases, even if the quality of the template DNA is sufficient, sex determination may lead to false results due to deletion and variation in the Y chromosome [9, 10]. If it is possible to make a sex determination by analyzing the X chromosome, it will not be necessary to consider the above possibility in forensic cases. Thus, there is a continuing need for direct methods to determine the female gender.

Human females and males have two and one X chromosome(s), respectively. If a sample is confirmed to have two X chromosomes, then sex can be confirmed positively as female. Recently, the copy number variation (CNV) detection method was developed [11–13]. CNV is caused by deletion, duplication, or multiplication of a certain gene section, and shows one or more copies of the section in an individual [14]. In humans, CNV is observed in various regions over ~12 % of the genome [15] and includes genes associated with disease and drug susceptibility [16, 17]. The copy number of these genes has been detected using the comparative Ct method and real-time PCR [18]. Thus, if X chromosome amelogenin genes in forensic samples can be detected by the CNV detection technique and correspond to the copy number of X chromosomes (one and two copies found in male and female samples, respectively), the female gender can be identified directly. However, the X chromosome amelogenin gene includes a CNV region [19]. Thus, in this study, we developed a novel sex determination method involving enumeration of X chromosomes using the X chromosome amelogenin gene without the CNV region by the comparative Ct method. Moreover, we evaluated the forensic utility of this method using mock forensic samples.

Materials and methods

Sample preparation

Intraoral epithelial cells were collected from 40 healthy Japanese adults with no known diseases (20 males, 20 females) to gain intact DNA. All samples were collected with a Whatman FTA card using EasiCollect (GE Healthcare, Piscataway, NJ, USA). DNA extraction and purification were performed using an EZ1 Investigator Kit (Qiagen, Valencia, CA, USA), according to the manufacturer's protocol, and elution was performed using 50 μL of water. DNA was quantified using the Quantifiler Duo DNA Quantitation Kit (Life Technologies, Carlsbad, CA, USA) according to the manufacturer's protocol. Human Genomic DNA Male and Human Genomic DNA Female (Promega, Madison, WI, USA) were used as standards of known DNA concentrations.

This study was approved by the Ethics Committee of Saitama Medical University (No. 674). Informed consent was obtained from all participants who provided samples.

Real-time PCR

The amelogenin gene region (GenBank accession number M55418.1) without a CNV region was determined to be the target region of the X chromosome gene. Primers were designed using the GeneAssist Copy Number Assay Workflow Builder software (Life Technologies) from the manufacturer's website (http://www5.appliedbiosystems.com/tools/cnv/). The selected primer target sites were compared with all available sequences using the BLAST software (http://www.ncbi.nlm.nih.gov/BLAST), and were confirmed to be complementary to the target region, not to other regions. Primer sequences are shown in Table 1.

PCR was performed in 20-μL reaction mixtures containing 2× TaqMan Genotyping PCR Master Mix, 20× working stock of TaqMan Copy Number Assay (oligonucleotide primers set for target region), 20× TaqMan Copy Number Reference Assay (oligonucleotide primers set for ribonuclease P (RNase P) region), and template DNA (all reagents were from Life Technologies). PCR amplification was performed using the 7500 Real-Time PCR System (Life Technologies) programmed for 10 min at 95 °C, followed by 40 cycles of 15 s at 95 °C and 1 min at 60 °C. Assays were performed in quadruplicate.

Counting X chromosomes

The copy number of the target sequence in a sample was determined by relative quantitation using the comparative Ct

Table 1 Primer sequences

Primer	Sequence (5′-3′)	Label
Forward	GCTTGCCTCTGCTGAAATATTAGTG	FAM
Reverse	CTCATGCATTCCGCTGTTCTG	–
Prove	ACTAAGTGGTATAGGAGAGACTCC	NFQ-MGB

($\Delta\Delta$Ct) method. Briefly, the method measures the Ct difference (ΔCt) between a target (X chromosome) and a reference sequence (RNase P), and then compares the ΔCt values of test samples to a calibrator sample (20 ng of female DNA) known to have two copies of the target sequence. The copy number of the target is calculated to be two times the relative quantity. The equations are as follows:

(1) ΔCt=(Ct value of target sequence)−(Ct value of reference sequence),
(2) $\Delta\Delta$Ct=(ΔCt value of test sample)−(ΔCt value of calibrator sample), and
(3) the copy number of sample=$2^{-\Delta\Delta Ct}$.

X chromosomes were counted automatically using the CopyCaller software (ver. 2.0; Life Technologies) [17]. Briefly, this software can automatically calculate the start and end of the baseline for the amplification plot. In an analytical setting, the threshold value (0.2) is entered and an automatic baseline is selected according to the manufacturer's protocol (http://tools.lifetechnologies.com/content/sfs/manuals/cms_062369.pdf). The threshold is the line where the intersection with the amplification curve defines the threshold cycle (Ct). We empirically determined Ct to be 37 in the analytical setting because the copy number of X chromosomes was determined accurately when the Ct value was below this threshold.

Statistical analyses

Copy numbers were compared statistically, with one and two, because males and females have one and two X chromosome(s), respectively. The numbers were evaluated using two-way analysis of variance by t tests, with a significance level of 0.01. Briefly, when the means of the copy numbers were significantly one and not two ($p<0.01$), the samples were considered to be males. In contrast, when the means of the copy numbers were significantly two and not one ($p<0.01$), the samples were considered to be females. The numbers are expressed as means±standard deviation (SD) from quadruplicate assays.

STR typing

STR typing was performed using the AmpFlSTR Identifiler PCR Amplification Kit (Life Technologies) according to the manufacturer's protocol. STR alleles were detected using an ABI 3130xl Genetic Analyzer (Life Technologies), following analysis using the GeneMapper ID software (Life Technologies). Values greater than 150 relative fluorescent units (RFU) were deemed to be positive.

Forensic applications

DNA degraded by exposure to ultraviolet radiation was used as a mock forensic sample. DNA (1 ng) degradation was simulated by ultraviolet irradiation at 0.2 or 0.4 J. Degraded DNA was compared with normal DNA in terms of the number of X chromosomes and STR typing.

The quality of DNA was assessed by the ratios of fragment DNA (129/41 bp and 305/41 bp amplicon ratios) using a KAPA Human Genomic DNA Quantification and QC Kit (Kapa Biosystems, Inc., Woburn, MA, USA) and the StepOne-Plus Real-Time PCR System (Life Technologies) [20]. Degraded DNA was increased in an ultraviolet irradiation-dependent manner (Table S1).

Results

Sex determination based on analysis of the number of X chromosomes

To investigate the PCR amplification efficiency of the amelogenin-X primer and RNase P primer, real-time PCR was performed using various amounts of female DNA as the template. The standard curves were generated automatically by the software using a real-time PCR system. In the plot of Ct value against template DNA quantity in each primer, the slope and intercept values were almost equal (data not shown).

The results of sex determination by this method using 1 ng template DNA with four replicates for each sample are shown in Fig. 1. The sex of the 20 male and 20 female participants was determined accurately by this method using intraoral epithelial cell samples.

Subsequently, to assess the minimum amount of template DNA required for correct sex determination, we performed three independent experiments in quadruplicate using male and female standard DNA of known concentrations (Fig. 2). The results showed that more than 0.2 ng of template DNA was needed for correct sex determination (t test, $p<0.01$).

Forensic application

The results of sex determination by STR analysis and our novel method using ultraviolet-irradiated template DNA (1 ng) are shown in Figs. 3 and 4, respectively. Male DNA amelogenin-Y was not detected by STR analysis after ultraviolet irradiation at 0.2 J, and neither amelogenin-X nor -Y was detected after ultraviolet irradiation at 0.4 J. In female DNA, amelogenin-X was not detected using the STR typing kit after ultraviolet irradiation at 0.4 J. In contrast, accurate sex determination with both male and female DNA was possible using
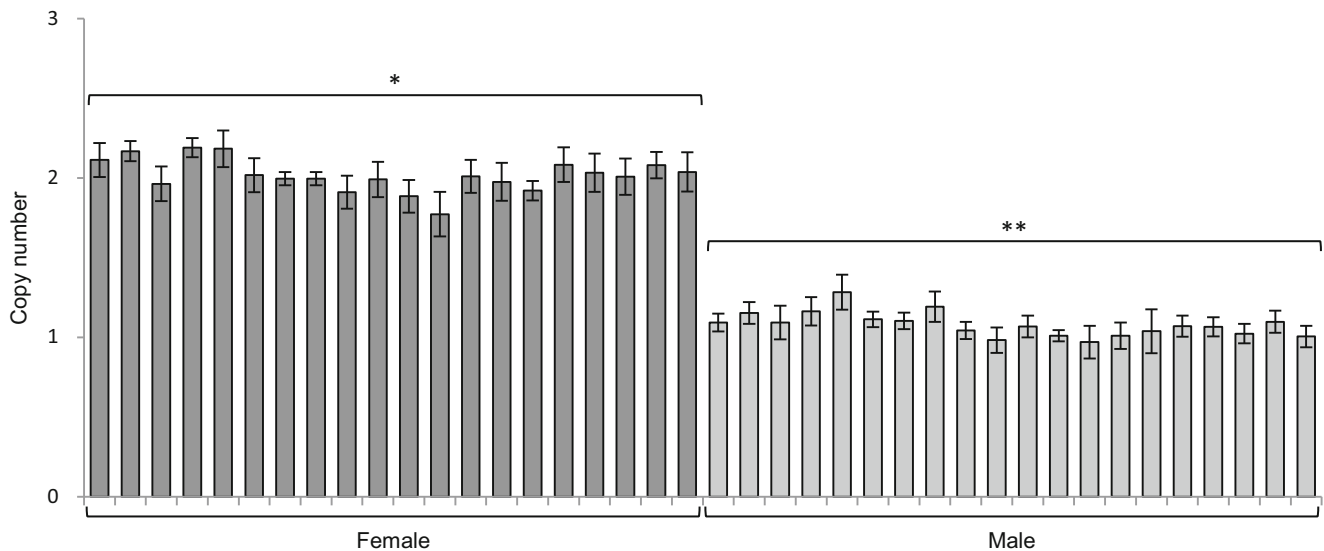
Fig. 1 Results of sex determination by the method described using 1-ng template DNA in quadruplicate for each sample. The *gray bars to the left* indicate the 20 female samples and the *light gray bars to the right* indicate the 20 male samples. Mean values±SD are shown for quadruplicate assays. *$p<0.01$ (the means of the copy numbers were two and not one). **$p<0.01$ (the means of the copy numbers were one and not two). The statistical analyses are described in the "Materials and methods"

the novel method after ultraviolet irradiation at 0.4 J. Moreover, the amplification size of these amplicons was confirmed by agarose gel electrophoresis, and the specificity of these amplicons was confirmed by direct sequencing using the BigDye Direct Cycle Sequencing kit (Life Technologies; data not shown). However, the X chromosome copy numbers calculated increased slightly with increasing ultraviolet irradiation (Fig. 4).

Discussion

In this study, we developed a new method for sex determination based on the number of X chromosomes. Forensic sex determination is usually performed simultaneously with personal identification by STR analysis. Additionally, many current sex determination methods depend on detection of the Y chromosome. However, because the quality of template DNA
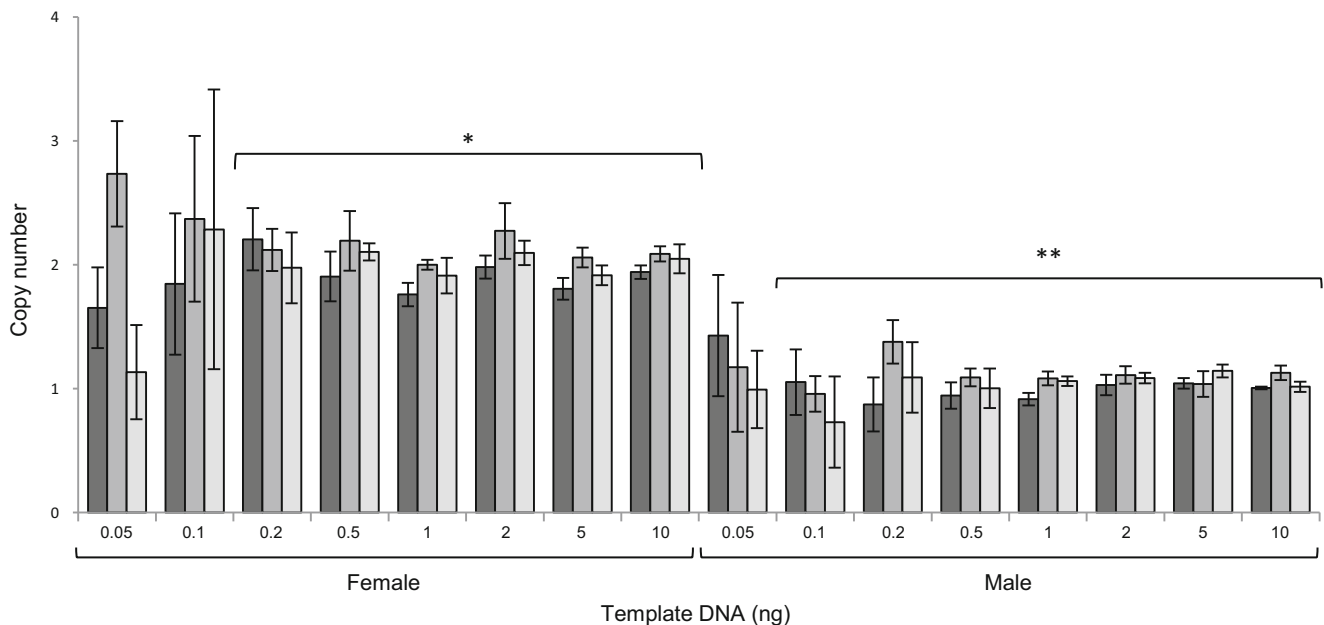

Fig. 2 Assessing the number of X chromosome using various amounts of template DNA. The results of three independent experiments with quadruplicate analysis for each sample are shown. Mean values ± SD are shown for quadruplicate assays. *$p<0.01$ (the means of the copy numbers were two and not one). **$p<0.01$ (the means of the copy numbers were one and not two). The statistical analyses are shown in the "Materials and methods"

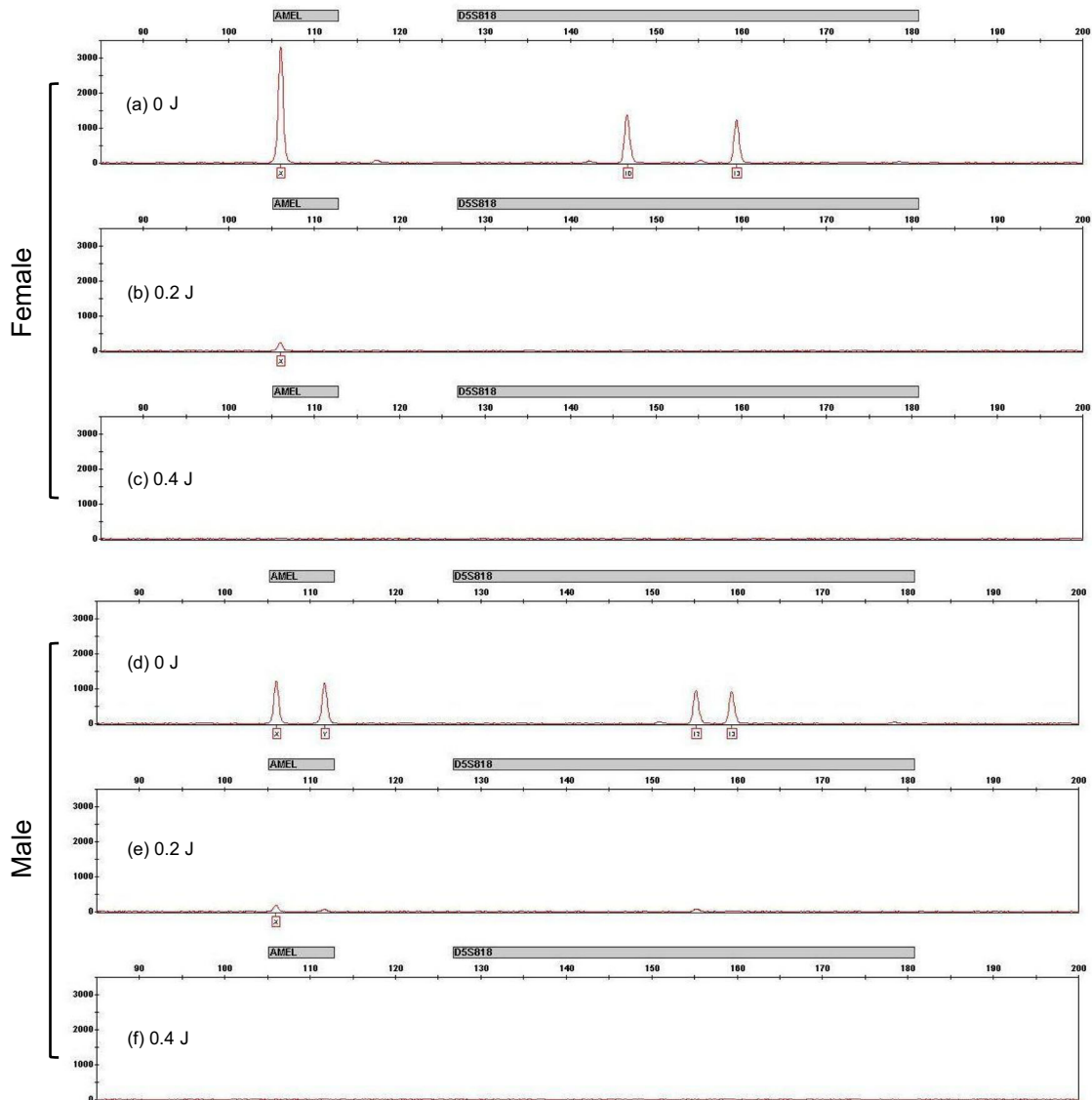**Fig. 3** Results of sex determination using 1-ng ultraviolet-irradiated DNA template by the STR typing kit. *(a)* Female DNA UV-irradiated at 0 J, *(b)* female DNA UV-irradiated at 0.2 J, *(c)* female DNA UV-irradiated at 0.4 J *(d)*, male DNA UV-irradiated at 0 J, *(e)* male DNA UV-irradiated at 0.2 J, and *(f)* male DNA UV-irradiated at 0.4 J. Type labels are attached to peaks greater than 150 RFU, the positive threshold

may lead to false results, direct determination of female gender is important. Thus, for forensic sex determination, we determined the X chromosome copy number using the CNV detection technique (comparative Ct method). First, we targeted the amelogenin-X gene lacking a CNV region to determine the X chromosome copy number to exclude the influence of the CNV region from the comparative Ct value. In the comparative Ct method, the PCR amplification effect of each primer must be equal. The PCR amplification efficiencies of the amelogenin-X and RNase P primers were almost equal. Thus, X chromosomal amelogenin genes in samples could be enumerated by the CNV detection technique and corresponded to the copy number of X chromosomes. Thus, the number of X

chromosomes could be determined accurately with this method using the amelogenin-X primer sets we designed.

We developed a sex determination method involving counting the X chromosomes. All DNA samples from participants (20 males, 20 females) were evaluated correctly using this method with 1 ng template DNA. Moreover, by statistical analyses, we concluded that >0.2-ng template DNA was needed for accurate sex determination in the quadruplicate assays (total >0.8 ng). The comparative Ct method might not reflect accurately low levels of template DNA because copy number was determined by calculating from the relative quantity (see "Materials and methods"). When the quality of template DNA is sufficient, the sensitivity of STR analysis should be better than
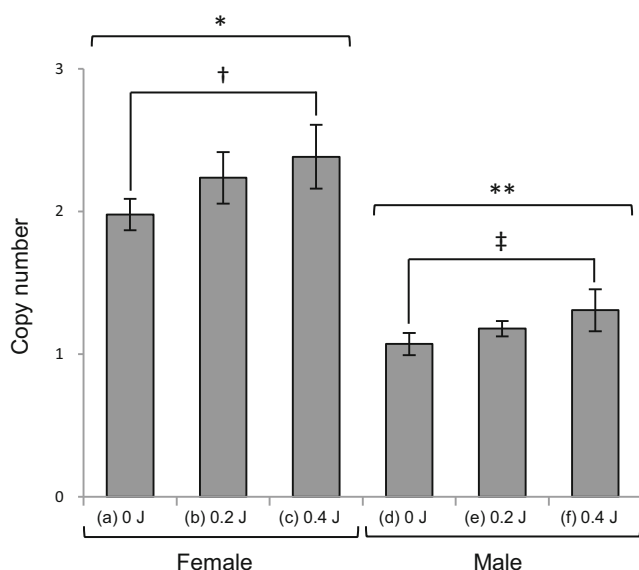
Fig. 4 Results of sex determination by the method described using 1-ng ultraviolet-irradiated DNA template in quadruplicate for each sample. *(a)* Female DNA UV-irradiated at 0 J, *(b)* female DNA UV-irradiated at 0.2 J, *(c)* female DNA UV-irradiated at 0.4 J, *(d)* male DNA UV-irradiated at 0 J, *(e)* male DNA UV-irradiated at 0.2 J, and *(f)* male DNA UV-irradiated at 0.4 J. Mean values±SD are shown for quadruplicate assays. *$p<0.01$ (the means of the copy numbers were two and not one). **$p<0.01$ (the means of the copy numbers were one and not two). The statistical analyses are shown in the "Materials and methods". Statistical analyses between UV-irradiated groups were performed using a *t* test. †$p<0.05$, compared with *(a)*. ‡$p<0.05$, compared with *(d)*

our method. However, because forensic samples have often deteriorated, our method, based on the detection of X chromosomes, would be useful as a forensic sex determination tool.

Because forensic samples are often highly degraded, STR analysis cannot identify female gender, when some other STR alleles are not observed even if only the X chromosome is detected in the amelogenin allele. In mock forensic male DNA samples subjected to ultraviolet irradiation (0.2 J), amelogenin-X, but not amelogenin-Y, was detected by STR analysis using the AmpFlSTR Identifiler kit. Moreover, in male and female DNA samples subjected to ultraviolet irradiation (0.4 J), neither amelogenin-Y nor amelogenin-X could be detected. In contrast, our novel sex determination method could accurately identify the male and female genders in mock forensic samples. Thus, this method facilitates sex determination using even highly fragmented DNA forensic samples for which STR analysis is unsuitable. In forensic samples, the primer sets used for PCR are generally designed to produce short amplicons to increase the robustness of the assay [21]. Because the amelogenin-X primer sets we designed resulted in considerably shorter amplicons (71 bp) than the STR analysis (AmpFlSTR Identifiler kit: ~100 bp), this method could detect the X chromosome in fragmented DNA, which was not possible by STR analysis using the AmpFlSTR Identifiler kit.

Commercial STR typing kits for degraded DNA, such as the PowerPlex ESX and ESI kit (Promega), were developed.

The amelogenin gene primer sets are also included in these kits. By STR typing using the ESX 17 kit, amelogenin-Y could not be detected in the male DNA samples subjected to 0.2- and 0.4-J ultraviolet irradiation (data not shown). In mock forensic female (0.2- and 0.4-J ultraviolet irradiation) and male (0.2-J ultraviolet irradiation) DNA samples, amelogenin-X was detected using this kit. Because the amplicon (93 bp) produced using amelogenin-Y primer sets is longer than that using amelogenin-X primer sets (PowerPlex, 87 bp; novel method, 71 bp), the detection of amelogenin-Y would be more difficult in more highly degraded samples. In contrast, because our novel method for sex determination does not depend on detecting amelogenin-Y, the method will be more useful for highly fragmented forensic DNA samples. However, as shown in Fig. 4, the X chromosome copy numbers in the mock forensic samples were increased slightly in an ultraviolet-irradiation-dependent manner. This could be due to a difference in the degradation ratio to ultraviolet irradiation between a target (amelogenin-X) and a reference sequence (RNase P), because the copy number was determined by calculating the relative quantities of the sequences. In future, we will design primer sets specific for a more stable region as a reference sequence, as well as the target sequence.

Furthermore, in some forensic cases, amelogenin-Y is not detected by STR analysis even when the sample is of male origin. We found previously that amelogenin-Y was not detected by STR analysis in a DYS458-deletion sample. The frequencies of amelogenin-Y-negative males in global populations have been reported [8, 22, 23]. For example, the frequencies of the amelogenin-Y null allele were 0.023 % (18/79,304) [8], 1.852 % (5/270) [21], and 8.333 % (2/24) [23], in China, India, and Sri Lanka, respectively. Thus, amelogenin-Y dropout, which results mainly from amplification failure due to deletion, translocation, and other mutations in the primer-binding site, may lead to sex misidentification [9, 10] and affect Y chromosome STR analysis [8]. Because the amplification region is located on the X chromosome, our method allows accurate sex determination even in the presence of a deletion on the Y chromosome. We used our method for sex determination in an actual forensic case in which the amelogenin-Y could not be determined by STR analysis due to a DYS458 deletion (data not shown). Amelogenin-X gene null alleles have also been reported in the Chinese population at a frequency of 0.037 % (3/8,087) [24]. The gene deletion in the above-mentioned sample was due to accidental variation at the primer-binding site used in the PowerPlex 16 system (Promega Corporation, Madison, WI, USA) [24]. The binding site of the primer sets that we designed differs from this variable site. However, in the future, if the primer-binding site proves to be included within other deletion sites in the amelogenin-X gene, incorrect sex determination may also result. In such a case, the primer location in this method

should be revised again. Furthermore, this method would not be able to determine the sex of patients with Turner and Klinefelter syndromes, although it may be used to support diagnoses of these syndromes. Additionally, from mixed samples of female and male origin, this method would not be able to determine sex, because the copy numbers of the X chromosome are indicated as integers.

In the present study, we determined the X chromosome copy number by real-time PCR using the CNV technique. Real-time PCR is widely used for various quantitative applications. However, the copy number may not reflect accurately low levels of template DNA, as mentioned above. However, a digital PCR method that can detect CNV with higher sensitivity and accuracy than real-time PCR was developed recently [25, 26]. This method allows quantification of DNA in any sample, effectively eliminating the errors inherent in other quantitative techniques. Application of digital PCR to our technique will reduce the errors associated with the comparative Ct method, and facilitate sex determination with greater sensitivity and accuracy.

In conclusion, we developed a method of sex determination based on the number of X chromosomes, which can determine female gender directly. Our novel method should be useful as a forensic sex determination tool for samples in which sex determination is difficult by STR analysis.

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