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## Abstract

In this study, sex determination using polymerase chain reaction (PCR) on tooth material was evaluated from the viewpoint of forensic medicine. The sensitivity of PCR for detection of the Y chromosome-specific alphoid repeat sequence and the X chromosome-specific alphoid repeat sequence was 0.5 pg of genomic DNA. Sex could be determined by PCR of DNA extracted from the pulp of 16 freshly extracted permanent teeth and dentine including the surface of the pulp cavity of 6 freshly extracted milk teeth. Sex could be determined using the pulp in all 20 teeth (10 male and 10 female) preserved at room temperature for 22 years. For the pulp of teeth stored in sea water, the sex could be determined in all 8 teeth immersed for 1 week and in 5 of 6 teeth immersed for 4 weeks. In the remaining 1 tooth, in which sex determination based on the pulp failed, the sex could be determined correctly when DNA extracted from the tooth hard tissue was examined. For teeth stored in soil, the sex could be determined accurately in all 8 teeth buried for 1 week, 7 of 8 teeth buried for 4 weeks, and in all 6 teeth buried for 8 weeks. When teeth were heated for 30 min, sex determination from the pulp was possible in all teeth heated to 100, 150, and 200 degrees C, and even in some teeth heated to 250 degrees C. When this method was applied to actual forensic cases, the sex of a mummified body estimated to have been discovered half a year to 1 year after death could be determined readily by examination of the dental pulp. In the skeletons of 2 bodies placed under water for approximately 1 year and approximately 11 years and 7 months, pulp tissues had been dissolved and lost, but sex determination was possible using DNA extracted from hard dental tissues. These results indicate that this method is useful in forensic practices for sex determination based on teeth samples.

**KEYWORDS:** personal identification, sex determination, tooth, deoxyribonucleic acid (DNA). polymerase chain reaction

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# Forensic Study of Sex Determination Using PCR on Teeth Samples

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In this study, sex determination using polymerase chain reaction (PCR) on tooth material was evaluated from the viewpoint of forensic medicine. The sensitivity of PCR for detection of the Y chromosome-specific alphoid repeat sequence and the X chromosome-specific alphoid repeat sequence was 0.5 pg of genomic DNA. Sex could be determined by PCR of DNA extracted from the pulp of 16 freshly extracted permanent teeth and dentine including the surface of the pulp cavity of 6 freshly extracted milk teeth. Sex could be determined using the pulp in all 20 teeth (10 male and 10 female) preserved at room temperature for 22 years. For the pulp of teeth stored in sea water, the sex could be determined in all 8 teeth immersed for 1 week and in 5 of 6 teeth immersed for 4 weeks. In the remaining 1 tooth, in which sex determination based on the pulp failed, the sex could be determined correctly when DNA extracted from the tooth hard tissue was examined. For teeth stored in soil, the sex could be determined accurately in all 8 teeth buried for 1 week, 7 of 8 teeth buried for 4 weeks, and in all 6 teeth buried for 8 weeks. When teeth were heated for 30 min, sex determination from the pulp was possible in all teeth heated to 100, 150, and 200 °C, and even in some teeth heated to 250 °C. When this method was applied to actual forensic cases, the sex of a mummified body estimated to have been discovered half a year to 1 year after death could be determined readily by examination of the dental pulp. In the skeletons of 2 bodies placed under water for approximately 1 year and approximately 11 years and 7 months, pulp tissues had been dissolved and lost, but sex determination was possible using DNA extracted from hard dental tissues. These results indicate that this method is useful in forensic practices for sex determination based on teeth samples.

**Key words:** personal identification, sex determination, tooth, deoxyribonucleic acid (DNA), polymerase chain reaction

Sex determination is the first step of personal identification in forensic medicine. In general, the sex of an unidentified body can be determined based on anatomical characteristics of the external genitalia or whether the gonads are ovaries or testes. However, bones and teeth are the only available materials for sex determination in markedly decayed or skeletonized bodies. The sex of skeletonized adult bodies used to be determined on the basis of anatomical characteristics of the pelvis and cranium and anthropometric data. By this method, however, sex determination was considered to be difficult in pre-adolescent bodies, in which sex characteristics of the skeleton have not been sufficiently developed. Cytological sex determination using X and Y chromatin has been attempted (1, 2), but these methods based on the assumption that the cell nucleus has not been disintegrated were limitedly effective for sex determination using forensic materials recovered from diverse environmental conditions.

Recently, with advances in gene analysis techniques, methods for sex determination using X and Y chromosome DNA analysis have been developed, and investigations regarding their forensic application have been conducted (3-10). Among these methods, the method to amplify the Y chromosome-specific alphoid centromeric repeat sequence (DYZ3) by polymerase chain reaction (PCR) reported in 1989 by Witt and Erickson (11), which can detect X chromosome-specific alphoid centromeric repeat sequence (DXZ1) as well as DYZ3, is

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considered to provide a more accurate sex determination based on the results of examination of the 2 sequences. We previously performed verification studies of this method and, by partial modification of the primers, could improve its sensitivity to Y and X chromosome-specific sequences to a practical level for forensic use (9, 12).

In the present study, we examined Y and X chromosome-specific alphoid centromeric repeat sequences using this modified method on single teeth and experimentally evaluated its usefulness for sex determination in forensic medicine. We also confirmed its usefulness by applying it to actual forensic cases.

## Materials and Methods

***Materials.*** Normal teeth and slightly decayed teeth (65 male and 65 female teeth) extracted from 64 male and 65 female subjects for dental treatments were used as materials.

DNA which had been extracted from teeth preserved in a dry state at room temperature from 3 months to 22 years, DNA extracted from the pulp or the dentin including the surface of pulp cavity of teeth immersed in sea water in a plastic bucket for a maximum of 4 weeks, and of teeth burid in soil at a depth of 30 cm for a maximum of 8 weeks, and DNA extracted from the pulp of teeth heated at 100 °C to 300 °C for 30 min were examined by PCR, following which sex determination was attempted. Sex determination was also attempted using DNA extracted from the pulp or hard tissue of teeth in a few actual cases.

***Extraction of DNA from teeth.*** DNA was extracted from the dental pulp by fragmenting the tooth using a dental cutter or a hammer, collecting the pulp from the exposed pulp cavity, treating it with SDS-proteinase K, extracting DNA with phenol-chloroform, and precipitating it with isopropanol. DNA was extracted from the hard tooth tissue by fragmenting it and then pulverizing it in a mortar, decalcifying the pulverized tooth with 0.5 M EDTA (pH 8.0) for 2–4 days, treating it with SDS-proteinase K, extracting DNA with phenol-chloroform, and precipitating it with isopropanol. The DNA collected was dissolved in TE buffer (10 mM Tris-HCl, 1 mM EDTA pH 8.0) and stored at 4 °C or − 20 °C until use.

***PCR.*** Amplification of the 172-base-pair Y chromosome-specific alphoid repeat sequence and the 131-base-pair X chromosome-specific alphoid repeat sequence by PCR was performed basically as described previously (9). Briefly, PCR was performed in a 500-$\mu$l tube using 30 $\mu$l of a reaction mixture volume. The composition of the PCR buffer was 10 mM Tris-HCl pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 100 $\mu$M each of dNTP, 200 $\mu$g/ml bovine serum albumin, 1 unit AmpliTaq Gold DNA polymerase (Perkin Elmer, Foster City, CA, USA), 0.5 $\mu$M each of Y11 (ATGATAGAAACGGAAATATG) and Y22 (AGTAGAATGCAAAGGGCTC) primers or X1 (AATCATCAAATGGAGATTTG) and X2 (GTTCAGCTCTGTGAGTGAAA) primers, and 1 $\mu$l of a sample, layered by about 50 $\mu$l of mineral oil in each tube. The heating cycles of PCR were preheating at 95 °C for 3 min and 35 heating cycles (94 °C for 40 sec, 55 °C for 40 sec, and 72 °C for 40 sec) using a PC-700 (Astec, Fukuoka, Japan).

***Electrophoresis and sex determination.*** The PCR products were electrophoresed in 5% polyacrylamide gel at 200 V for 1 h, ethidium bromide staining was performed, and amplified bands of Y- and X-specific sequences were examined under UV irradiation. The sex of the subject was considered to be male when both Y- and X-specific sequences were detected, but female when only the X-specific sequence was detected.

***Sensitivity of detection of Y and X specific sequences by PCR.*** The concentrations of DNA extracted from lymphocytes of 2 males and 2 females were determined using a GeneQuant II (Amersham Pharmacia Biotech, Cambridge, England) by absorption spectrophotometry, these samples were diluted to a DNA concentration of 10 ng/$\mu$l to 0.1 pg/$\mu$l, and Y- and X-specific sequences were detected by the PCR method.

## Results

### *Basic Evaluation of Sex Determination by PCR of DNA from Teeth.*

Table 1 and Fig. 1 show the results of evaluation of the minimum detection levels of Y and X chromosome-specific sequences from a trace amount of DNA. Y and X chromosome-specific sequences in male samples, and X chromosome-specific sequence in female samples could be detected in a DNA quantity of 0.5 pg.

Table 2 shows the results of sex determination by PCR in DNA extracted from the dental pulp collected from freshly extracted permanent teeth and that extracted

**Table 1** Detection of sex chromosome-specific sequences from minute DNA samples extracted from male and female lymphocytes by polymerase chain reaction

| Subject | Sex | Specific sequence | Amount of DNA | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | 10 ng | 1 ng | 100 pg | 10 pg | 5 pg | 1 pg | 0.5 pg | 0.1 pg | NC |
| 1 | ♀ | X | + | + | + | + | + | + | + | − | − |
| | | Y | − | − | − | − | − | − | − | − | − |
| 2 | ♀ | X | + | + | + | + | + | + | + | − | − |
| | | Y | − | − | − | − | − | − | − | − | − |
| 3 | ♂ | X | + | + | + | + | + | + | + | − | − |
| | | Y | + | + | + | + | + | + | + | − | − |
| 4 | ♂ | X | + | + | + | + | + | + | + | − | − |
| | | Y | + | + | + | + | + | + | + | − | − |

NC, negative control.

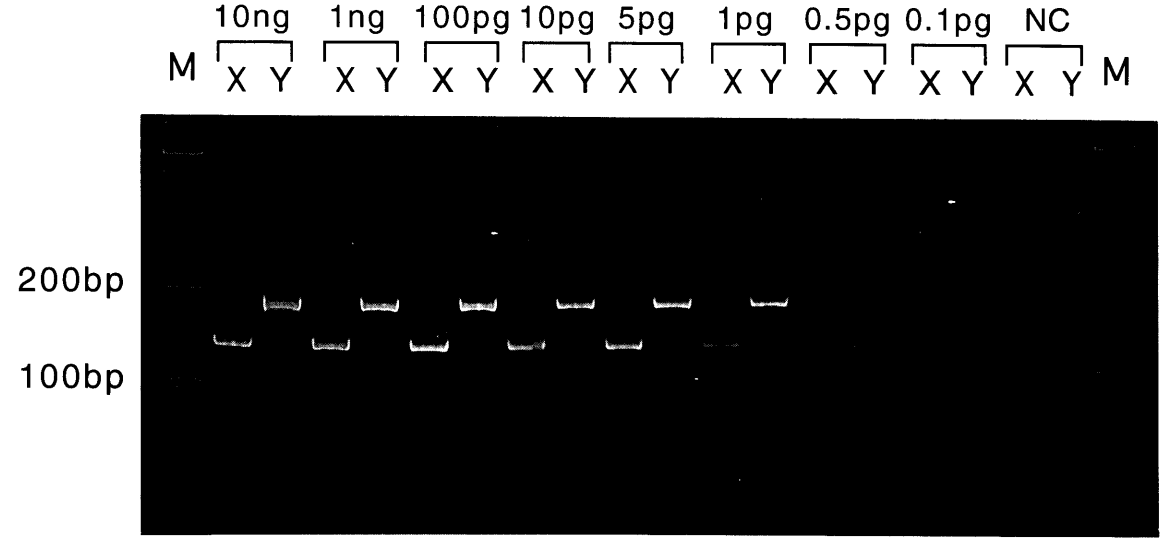


**Fig. 1** Detection of sex chromosome-specific sequences from male lymphocyte DNA by polymerase chain reaction. NC, negative control; M, DNA size marker; bp, base pair.

from dentin including the surface of the pulp cavity of freshly extracted milk teeth. Sex determination was possible in all 22 teeth examined (16 permanent and 6 milk teeth).

Table 3 shows the results of sex determination using the dental pulp collected from teeth stored in a dry state at room temperature. The sex could be determined correctly in all teeth preserved at room temperature for 3 months, 6 months, 1 year, and 5 years (4 male and 4 female teeth at each time point). The sex could also be determined in 20 teeth (10 male and 10 female teeth) stored at room temperature for 22 years. Fig. 2 shows electropherograms of 2 male and 2 female teeth stored at room temperature for 22 years.

When sex determination was attempted using the dental pulp of teeth immersed in sea water, the sex could be determined in all 8 teeth immersed for 1 week and 5 of 6 teeth immersed for 4 weeks (Table 4). In the 1 tooth in which sex determination was incorrect, the amplified band of the Y chromosome-specific sequence was not detected (sample No. 4 in Fig. 3) although it was a male sample. However, the Y and X chromosome-specific sequences could be detected using the same method on DNA extracted from hard tissue of the tooth and DNA extract-

**Table 2** Sex determination from extracted permanent and milk teeth

| Tooth | Position of the tooth | Sex | Specific sequence | PCR amplified product | Rate of correct result (%) |
|---|---|---|---|---|---|
| Incisor | $\overline{1}$ | ♀ | X | + | 4/4 (100) |
| | | | Y | − | |
| | $\underline{1}$ | ♀ | X | + | |
| | | | Y | − | |
| | $\overline{2}$ | ♂ | X | + | |
| | | | Y | + | |
| | $\underline{1}$ | ♂ | X | + | |
| | | | Y | + | |
| Canine | $\overline{3}$ | ♀ | X | + | 4/4 (100) |
| | | | Y | − | |
| | $\underline{3}$ | ♀ | X | + | |
| | | | Y | − | |
| | $\overline{3}$ | ♂ | X | + | |
| | | | Y | + | |
| | $\underline{3}$ | ♂ | X | + | |
| | | | Y | + | |
| False molar | $\overline{5}$ | ♀ | X | + | 4/4 (100) |
| | | | Y | − | |
| | $\overline{4}$ | ♀ | X | + | |
| | | | Y | − | |
| | $\overline{5}$ | ♂ | X | + | |
| | | | Y | + | |
| | $\underline{5}$ | ♂ | X | + | |
| | | | Y | + | |
| True molar | $\underline{8}$ | ♀ | X | + | 4/4 (100) |
| | | | Y | − | |
| | $\underline{8}$ | ♀ | X | + | |
| | | | Y | − | |
| | $\overline{8}$ | ♂ | X | + | |
| | | | Y | + | |
| | $\overline{8}$ | ♂ | X | + | |
| | | | Y | + | |
| Milk incisor | $\overline{A}$ | ♀ | X | + | 2/2 (100) |
| | | | Y | − | |
| | $\overline{A}$ | ♂ | X | + | |
| | | | Y | + | |
| Milk molar | $\overline{D}$ | ♀ | X | + | 4/4 (100) |
| | | | Y | − | |
| | $\underline{E}$ | ♀ | X | + | |
| | | | Y | − | |
| | $\overline{D}$ | ♂ | X | + | |
| | | | Y | + | |
| | $\overline{E}$ | ♂ | X | + | |
| | | | Y | + | |

+, a distinct band was observed; −, no band was observed.

**Table 3** Sex determination of teeth stored at room temperature

| Sex | Number of correct results/number of samples | | | | |
|---|---|---|---|---|---|
| | 3 months | 6 months | 1 year | 5 years | 22 years |
| ♀ | 4/4 | 4/4 | 4/4 | 4/4 | 10/10 |
| ♂ | 4/4 | 4/4 | 4/4 | 4/4 | 10/10 |

**Table 4** Sex determination of teeth immersed in sea water

| Sex | Number of correct results/number of samples | |
|---|---|---|
| | 1 week | 4 weeks |
| ♀ | 4/4 | 3/3 |
| ♂ | 4/4 | 2/3* |

*The Y-specific band was very weak and a nonspecific band appeared in 1 of 3 male teeth.

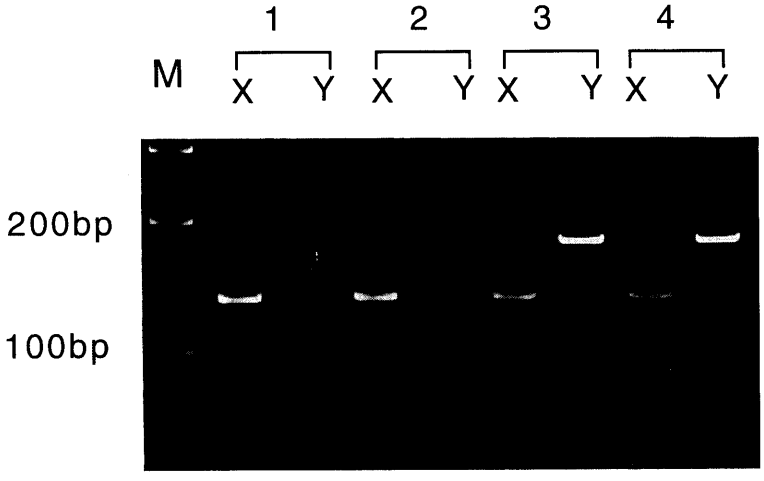


**Fig. 2** Sex identification from teeth stored at room temperature for 22 years.
1 and 2, female teeth; 3 and 4, male teeth; M, DNA size marker; bp, base pair.

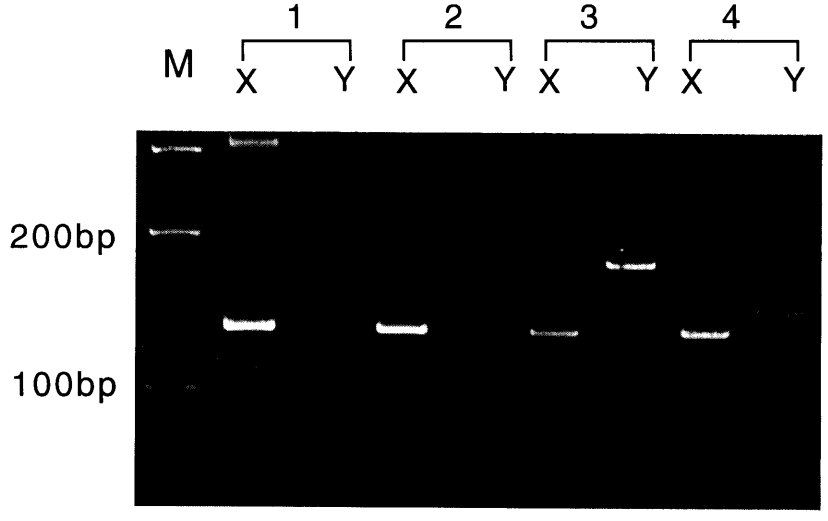


**Fig. 3** Sex identification from teeth soaked in sea water for 4 weeks.
1 and 2, female teeth; 3 and 4, male teeth; M, DNA size marker; bp, base pair.

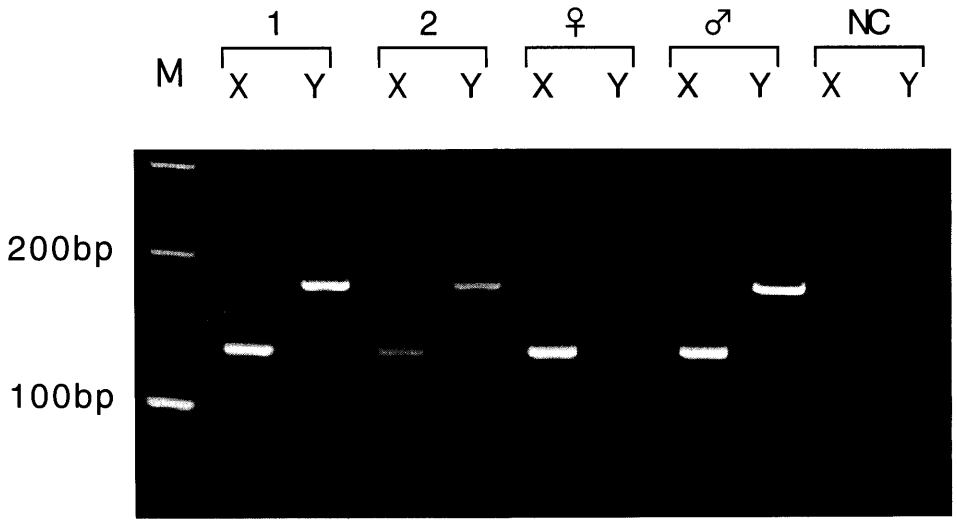


**Fig. 4** Detection of sex chromosome-specific sequences from blood of the donor of tooth No. 4 and hard tissue of tooth No. 4 soaked in sea water for 4 weeks.
1, blood from the donor of tooth No. 4; 2, hard tissue of tooth No. 4; ♀, female control (blood); ♂, male control (blood); NC, negative control; M, DNA size marker; bp base pair.

ed from blood from the same donor (Fig. 4), allowing correct sex determination.

For teeth buried in soil at a depth of 30 cm, the sex could be determined in all 8 teeth buried for 1 week, 7 of 8 teeth buried for 4 weeks, and all 6 teeth buried for 8 weeks (Table 5). Fig. 5 shows electropherograms of 2 male and 2 female teeth. Very clear bands were demonstrated by this method even in samples stored under these conditions. In the 1 male tooth buried in soil for 4 weeks in which sex determination was incorrect, sex determination was possible using DNA extracted from hard tissue.

For teeth heated in an electric furnace for 30 min, the sex could be determined in the 2 male and 2 female teeth each heated at 100, 150, and 200 °C, and sex determination was possible in some teeth heated at 250 °C for 30 min, although the amplified band was considerably faint (Table 6).

### *Sex Determination in Actual Cases.*

*Case 1: Sex determination of a mummified and skeletonized body.* Fig. 6 shows a skeletonized body discovered in a house in a city of Okayama Prefecture, western part of Japan, in May 1992. The body was estimated to have been discovered between half a year to 1 year after death and was nearly totally skeletonized with decomposed mummified soft tissue being only partially left in the back. The anatomical features of both external and internal sex organs were completely obliterated.

**Table 5** Sex determination of teeth buried in soil

| Sex | Number of correct results/number of samples | | |
|---|---|---|---|
| | 1 week | 4 weeks | 8 weeks |
| ♀ | 4/4 | 3/4* | 3/3 |
| ♂ | 4/4 | 4/4 | 3/3 |

*The X-specific band was very weak in 1 of 4 female teeth.

At autopsy, the sex of the body was estimated to be female from the anatomical features of the cranium and pelvis and the anthropometric values, and its age to be 40–50 years, but sex determination was attempted using the PCR method on the dental pulp of 2 teeth for confirmation. As shown in Fig. 7, only the X chromosome-specific sequence was amplified in both teeth, and the sex of the body was judged to be female.

According to police investigation, a 46-year-old brother and a 39-year-old sister, both having psychiatric disorders, lived in the house in which the body was found, but the sister had been missing for about 10 months. Although confirmation by the brother was impossible because of his psychiatric disorders, the police were confident that the body was that of his sister. The autopsy findings and the results of sex determination by PCR were in agreement with this opinion.

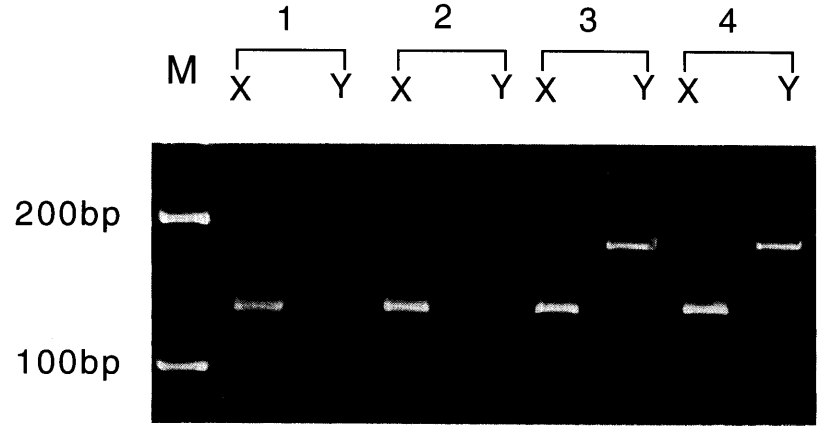


**Fig. 5** Sex identification from teeth buried in soil for 8 weeks.
1 and 2, female teeth; 3 and 4, male teeth; M, DNA size marker; bp, base pair.

**Table 6** Sex determination of teeth heated for 30 min at various temperature

| Sex | Number of correct results/number of samples | | | | |
|---|---|---|---|---|---|
| | 100 °C | 150 °C | 200 °C | 250 °C | 300 °C |
| ♀ | 2/2 | 2/2 | 2/2 | 1/2 | 0/2 |
| ♂ | 2/2 | 2/2 | 2/2 | 1/2 | 0/2 |

**Fig. 6** A skeletonized body with decomposed mummified soft tissue in its back found in a house.

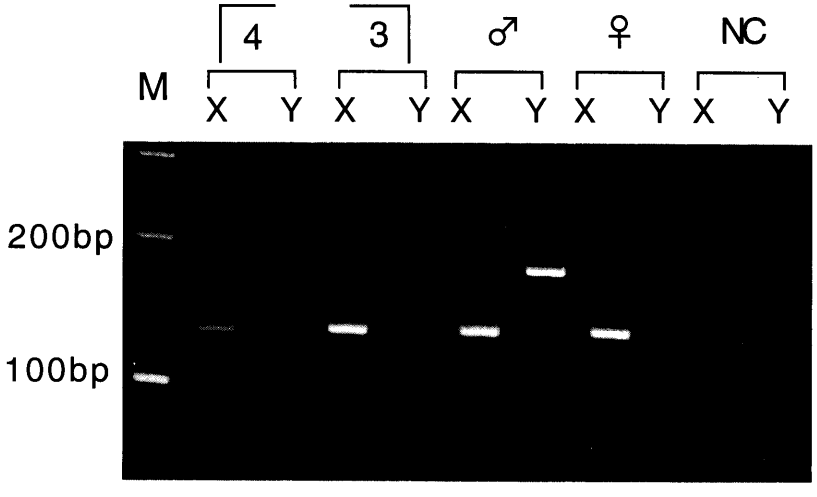


**Fig. 7** Sex identification based on the teeth of the skeletonized body found in a house.
♂, male control (blood); ♀, female control (blood); NC, negative control; M, DNA size marker; bp, base pair.

*Case 2: Sex determination of a skeletonized cranium immersed in water for 1 year after death.* Fig. 8 shows a cranium discovered in an irrigation canal in Okayama Prefecture in August 1997. This cranium was estimated to have been in the canal for about 1 year after death, and soft tissues were completely lost except that a mud-like material considered to be decayed brain was observed in the cranium. Anatomical examination suggested that the body was likely to have been a male aged 30–50 years.

Sex determination by PCR was attempted using 2 molars among the teeth remaining in the maxilla of the cranium. Since the pulp of these teeth was already decomposed, DNA extracted from hard tissue of the teeth was examined. As shown in Fig. 9, both X and Y chromosome-specific sequences were amplified from 2 teeth, and the sex of the body was judged to have been male.

*Case 3: Sex determination of an adipoceratous skeletonized body immersed in water for 11 years and 7 months after death.* Sex determination was attempted in an adipoceratous skeletonized body immersed in water for 11 years and 7 months after death using teeth and adipoceratous soft tissue. This body was skeletonized in the driver's seat of a car discovered at the bottom of a pond in western Okayama Prefecture during a drought in 1994. At discovery, most of the body was reported to have been buried in the mud at the bottom of the pond. As shown in Fig. 10, the body was nearly skeletonized with adipoceratous soft tissues remaining only in the abdomen, hips, and bilateral knees. Necropsy findings indicated that the body was a male aged 50–55 years. Also, based on the car in which the body was found, the pajamas worn by the body, and its belongings including a gas station credit card, the body was estimated to be a 55-year-old male who left his house in his car in pajamas in January 1983 and had since been missing.

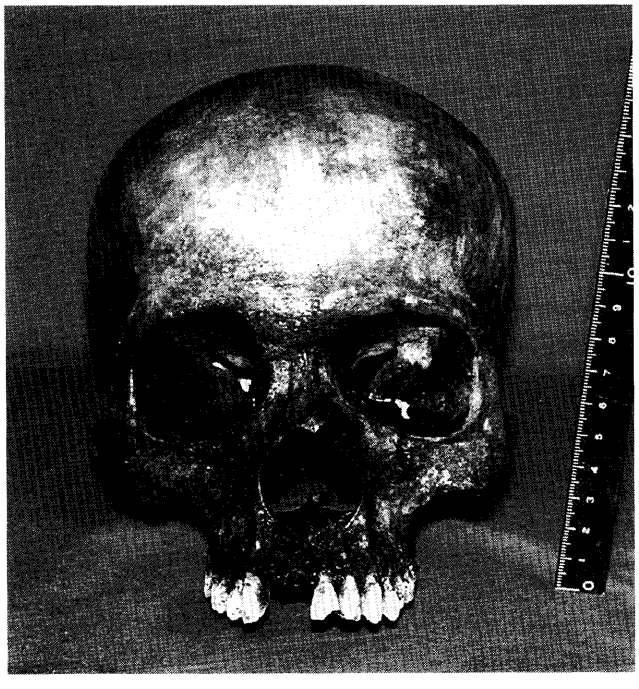

**Fig. 8** A skull which had been immersed in water of an irrigation canal.

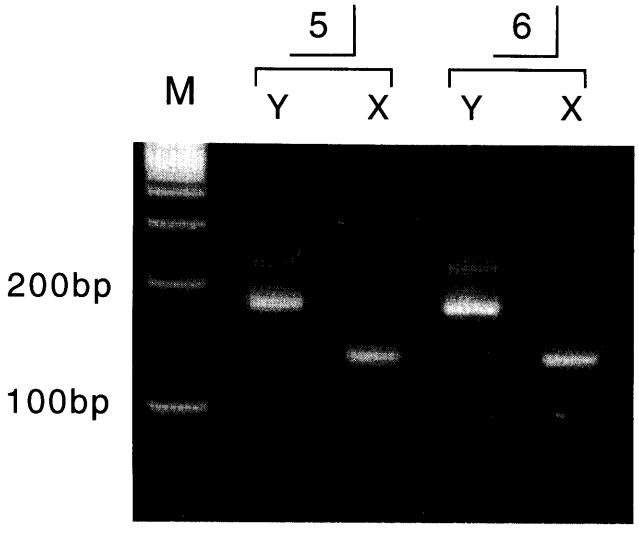


**Fig. 9** Sex identification based on the teeth of the skull found in an irrigation canal.
M, DNA size marker; bp, base pair.

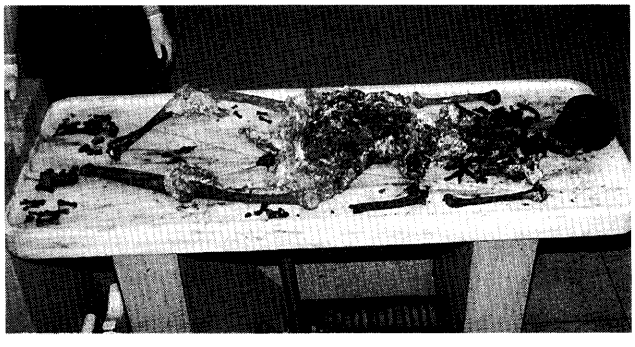

**Fig. 10** An adipoceratous skeletonized body which had been immersed in pond water for 11 years and 7 months.

Sex determination of this skeletonized body was attempted by PCR. As shown in Fig. 11, both the X and Y chromosome-specific sequences were amplified in samples including hard tissue of teeth, and the body was judged to be male.

## Discussion

Sex determination using teeth is important in markedly decayed or skeletonized bodies. Teeth enamel is the hardest tissue of the human body, and teeth remain long after death. For this reason, many studies to determine an individual's sex according to sexual differences in the anatomical morphometry of teeth (male > female) have been carried out (13-20). However, sex differences in dental morphometric values are not distinct except in the canine teeth, and determination of the sex from a random single tooth is extremely difficult. There has been no method to distinguish the sex particularly based on milk teeth. In forensic medicine, it is necessary to develop a method for reliable determination of sex in a single tooth regardless of whether it is permanent or milk or whether it is an incisor, canine, or molar.

Recently, with the development of DNA analysis, sex determination based on the analysis of Y chromosome DNA has become possible, but the test using Southern blot analysis requires a relatively large amount of high-molecular weight (HMW) DNA so that it was not highly applicable to sex determination from the teeth of rotten or skeletonized bodies. The method for sex determination using PCR was then developed, and its application to forensic medicine has been evaluated (8-10).

In 1989, Witt and Erickson (11) reported a method to amplify the specific alphoid centromeric repeat sequence by PCR and to determine sex based on blood stains. This method was expected to allow more accurate sex determination since the X chromosome-specific alphoid repeat sequence can be detected along with the Y chromosome-specific alphoid repeat sequence. However, when we performed PCR according to the original method and detected Y and X chromosome-specific sequences, the X chromosome-specific sequence was amplified to such a degree as to allow its detection in less than 100 pg of DNA. However, several hundred nanograms of DNA was needed for detection of the Y chromosome-specific sequence, showing a wide difference in the sensitivity of this method for the 2 sequences, and the sensitivity for the Y chromosome-specific sequence was insufficient for practical use. We further evaluated the method and succeeded in improving its sensitivity to both Y and X chromosome-specific sequences to about 5 pg DNA (about 0.0002 $\mu$l as whole blood) by partially modifying oligonucleotide sequences of the primers for the amplification of the Y-specific sequence (9). We attempted sex determination using the method using various forensic materials with good results (9, 12).

Before we applied the method to sex determination from teeth, we examined the detection limits of Y and X chromosome-specific sequences by the present method with slight modifications of a previous protocol (9) in

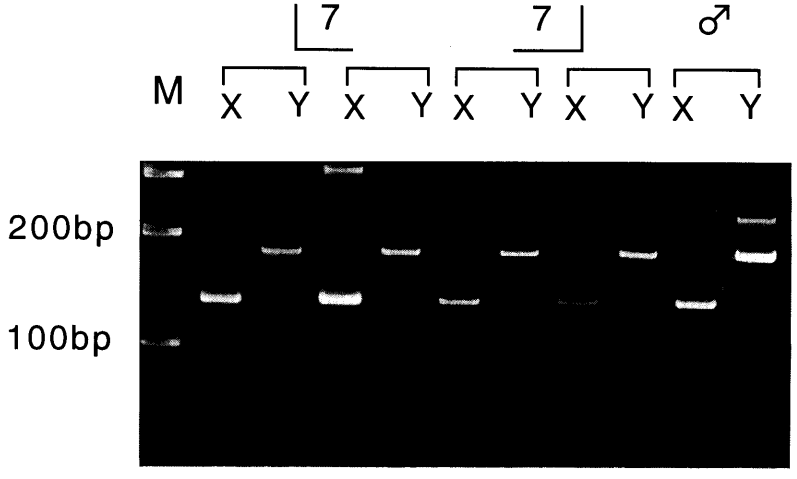


**Fig. 11** Sex identification based on the teeth of the skeletonized body which had been immersed in pond water for 11 years and 7 months. ♂, male control (hard tissue of a male tooth stored for 24 years at room temperature); M, DNA size marker; bp, base pair.

DNA extracted from lymphocytes. As a result, this method's extremely high sensitivity was demonstrated, with detection limits of both sequences being 0.5 pg DNA. Hanaoka *et al.* (21) has also reported sex determination based on teeth using the method of Witt and Erickson (11). However, they used the same primers described in the original method (11), so that the Tm values of primers for amplification of the Y-specific sequence were discrepant to each other and its sensitivity was not as high as our method. Thus, when minute and/or degraded DNA samples were tested, their method was thought apt to give false negative results regarding the Y-specific sequence even in male specimens. DNA analysis of teeth for sex determination must be able to be carried out in bodies such as markedly decayed or skeletonized bodies that provide no other materials. In such bodies, dental DNA is considered to be considerably decomposed and fragmented. Therefore, a highly sensitive method such as our present method is considered to be suited for sex determination using DNA from teeth. The Y and X chromosome-specific alphoid repeat sequences, which we examined in this study, are repeated 100 and several thousand times per genome (22, 23), respectively, and can be amplified by PCR if part of these copies remain intact. Therefore, this method is more advantageous with regard to sensitivity than methods using a single copy base sequence (10, 24, 25). Among single copy base sequences, X-Y homologous amelogenin gene sequences have been detected for sex determination from forensic specimens because the X-specific product may act as an internal control for PCR (26, 27). However, the amelogenin gene methods were not as sensitive as our method to detect multicopy alpha-satellite sequences. Further, a single copy sequence may occasionally not be amplified from such minute DNA templates as in a few cells because the template dispensed into a reaction tube may not by chance contain the sequence. While in our method, the target sequences could be detected readily from such a minute amount of DNA templates, and because the detection sensitivity of the Y-specific sequence was adjusted to fit or superior to that of the X-specific sequence on purpose, our method could give reliable results even when minute DNA samples close to the detection limit, about 1 pg of DNA, were examined.

Sex determination was possible using this method on DNA extracted from the pulp of freshly extracted permanent teeth regardless of the tooth type. Since the pulp cannot be obtained from freshly extracted milk teeth, sex determination was performed by PCR method using DNA extracted from dentin including the surface of the pulp cavity, but sex could be determined correctly in all teeth. Sex determination using milk teeth has been practically impossible using the conventional morphometric method. By DNA analysis, however, sex determination could be made readily in milk teeth as well as in permanent teeth.

It has been reported that DNA of the dental pulp of extracted teeth preserved at room temperature was stable and that recovery of HMW DNA was possible even after 19 years (28). In this study, also, sex determination was possible in the pulp of all 20 teeth preserved for 22 years in a dry state at room temperature.

Next, sex determination was attempted using teeth placed in a wet state, *i.e.*, under water or in the soil. Due to decay or DNA degradation caused by autolytic processes in a wet state, DNA analysis in aged samples is considered to be difficult (28).

In the teeth immersed in sea water, sex could be determined accurately in 5 of 6 teeth even after 4 weeks. For the remaining 1 tooth in which the sex could not be correctly determined, no amplified band of the Y chromosome-specific sequence was observed, although the sample was obtained from a male, and an amplification product of nearly 150 bp was detected. Clear amplified bands of the X and Y chromosome-specific sequences were detected in DNA extracted from hard tissue of this tooth and that extracted from blood of the same donor. In this tooth, therefore, decay due to bacteria is considered to have progressed markedly by infiltration of sea water into the pulp cavity, and DNA of the pulp tissue is considered to have been degraded to such a degree that amplification of the Y chromosome-specific sequence by PCR became impossible, and a small amount of amplification product derived from the DNA of bacteria may have been generated at 150 bp. In this sample, the amplified band of the X chromosome-specific sequence was detected although that of the Y chromosome-specific sequence was not, possibly because the amplified fragment of the X chromosome-specific sequence, which is shorter than that of the Y chromosome-specific sequence, was less liable to be effected by DNA degradation due to decay and because the number of repetitions of the X chromosome-specific sequence in a single genome is several tens of times greater than that of the Y chromosome-specific sequence.

Although sex determination was possible in all 6 males

and females 8 weeks after the samples were buried in the soil, in 1 of the 4 female samples buried for 4 weeks in the soil the amplified band of the X chromosome-specific sequence was not detected by PCR and sex determination was impossible. Decay in the pulp cavity is considered to have progressed faster, and DNA to have been degraded to a greater degree, in this tooth than in the other teeth because of the immaturity of this tooth's roots.

In a wet state, pulp tissue gradually lyses due to autolysis and decay, and its sampling becomes difficult. Furthermore, DNA contained in the pulp tissue is degraded and fragmented so that detection of sex chromosome-specific DNA by PCR becomes impossible. In such a case, sex determination can be performed by collecting DNA from the hard tooth tissue, which remains after the progression of decay. In this study, also, sex chromosome-specific DNA could be detected by PCR in the extract from the hard tooth tissue, allowing sex determination, even when sex determination using pulp tissue was difficult.

The sex could be determined in all 4 teeth heated at 200 °C for 30 min and 2 of the 4 teeth heated at 250 °C. Previously, we reported that sex determination was possible when blood stains were heated at 150 °C or less for 30 min but that no Y or X chromosome-specific sequence was detected when they were heated at 200 °C for 30 min (12). Compared with blood stains, DNA in dental pulp was more stable against heating, probably because the pulp cavity is protected by the hard tooth tissue mitigating the effect of heating.

When this method was applied to actual sex determination of skeletonized bodies, the sex of a skeletonized mummified body in a dry state could be determined by extracting DNA from the remaining dry pulp tissue. The other 2 cases were skeletons discovered in water after being submerged for approximately 1 year and approximately 11 years and 7 months, respectively. Since the pulp tissue was completely lysed and lost, sex determination was attempted using DNA extracted from the hard tooth tissue. Unlike extracted teeth, the roots of teeth in actual skeletonized bodies were located in the alveoli, so that they are considered to be less apt to allow the entry or proliferation of bacteria and physicochemical changes even in water or soil. Also, in the skeletonized body immersed in water for about 11 years and 7 months after death, the upper body including the head is considered to have been buried in the mud at the bottom of the pond and to have been in an anaerobic condition. This is considered to have protected the teeth from the above effects and to have delayed progression of DNA fragmentation.

From these results, sex determination of teeth by means of PCR is considered to be extremely useful for identification of markedly decayed or skeletonized bodies, which has been difficult using the conventional morphological methods.

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