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Sexing of Mouse Preimplantation Embryos by Detection of Y Chromosome-Specific Sequences Using Polymerase Chain Reaction[1]

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ABSTRACT

Detection of genes known to be present on the mammalian Y chromosome was adapted for sexing mouse early embryos using the polymerase chain reaction (PCR) method.

Sry and *Zfy* genes located in the sex-determining region of the Y chromosome were chosen for Y-specific target sequences, and *DXNds3* sequence on the X chromosome was chosen for control. The two-step PCR method using two pairs of primers for each of the target sequences was employed for detecting the sequences. When DNAs of male and female mice were amplified with these primers, male-specific fragments were detected even in DNAs that were equivalent in amount to two cells.

Mouse embryos at the two-cell stage were separated into two individual blastomeres, and one blastomere was karyotyped at the second cleavage. The remaining blastomere was subjected to PCR amplification immediately or after having been cultured for 48 h up to the morula stage. The *Sry* and *Zfy* sequences were detected in about half the embryos; detection of the *Sry* and *Zfy* sequences corresponded exactly to the presence of the Y chromosome, except in one sample of male morula in which embryos may have been lost before the PCR amplification. It is concluded that the sex of mouse preimplantation embryos can be accurately determined through detection of the Y-specific sequences using the two-step PCR method, even with the single blastomeres separated at the two-cell stage.

INTRODUCTION

Sex identification of preimplantation embryos is important for controlling sex ratios of domestic animals as well as for preventing termination of pregnancy in cases of certain X-linked inherited disorders in humans; such sexing would be useful for investigating sexual differences in the developmental process. Several approaches have so far been developed for the sexing of preimplantation embryos, including karyotyping of blastomeres [1], detection of male-specific antigens [2], monitoring of X chromosome-linked enzyme activity [3], and hybridization of Y chromosome-specific probes [4]. Recently, amplification of Y chromosome-specific repetitive sequences by polymerase chain reaction (PCR) [5] has been employed for the sexing of embryos [6–9]. The application of the PCR method, which detects a particular DNA sequence in small amount of DNA [10, 11], may allow for accurate and rapid sexing of embryos. In the present study, detection of Y chromosome-specific single copy genes by the PCR method was employed instead of repetitive sequences for the sexing of embryos.

Some functional genes are known to be present in particular regions of the mammalian Y chromosome while a large portion of the chromosome is composed of non-functional repetitive sequences [12]. *SRY* gene has recently been found to be present in a particular sex-determining region of the human Y chromosome [13], and is the most potent candidate for testis determining factor (TDF) [14]. *ZFY* gene is also known to be present in this region [15], and is believed to be associated with male germ cell development [16, 17]. The *SRY* and *ZFY* genes are conserved among eutherian mammals, and mouse sequences homologous to these genes, *Sry* gene and *Zfy-1* and *Zfy-2* genes, have also been isolated [18, 19].

We have therefore investigated whether the sex of mouse preimplantation embryos can be determined through detection of the *Sry* and *Zfy* genes using the PCR method.

MATERIALS AND METHODS

Mice and In Vitro Fertilization

Male B10.BR-Y^{del} mice and female C57BL/6 mice were used. B10.BR-Y^{del} mice, which have partially deleted Y chromosome [20], have been maintained in the Institute of Medical Science, University of Tokyo. C57BL/6 mice were obtained from CLEA Japan Inc. (Tokyo, Japan).

Sperm suspension was prepared by releasing the content of the cauda epididymis into TYH medium [21] and incubating for 90–120 min prior to in vitro fertilization.

Four-week-old C57BL/6 female mice were superovulated by i.p. injection of 5 IU eCG followed by i.p. injection of 5 IU hCG 48 h later. Unfertilized, cumulus-intact eggs were recovered 14–16 h after the hCG injection, and re-

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leased directly into the TYH medium containing the spermatozoa in a concentration of 1–9 × 10^5 cells/ml. After incubating for 5–6 h at 37°C in a humidified atmosphere of 5% CO_2 in air, the eggs undergoing fertilization were cultured in Whitten's medium (WM) containing 0.1 mM EDTA [22].

Preparation of Blastomeres

About 30 h post-insemination, two-cell embryos were treated with 0.5% pronase in HEPES-WM for 3–5 min to remove zona pellucida, and then were washed with WM. Blastomere pairs were separated by gentle pipetting of the zona-free two-cell embryos using a micropipette with an internal diameter of 70–100 μm. One of the blastomeres was subjected to karyotyping, and the other was directly subjected to PCR amplification or transferred to WM droplet for further culturing.

Karyotyping

The blastomere was incubated overnight in Colcemid solution (0.06 μg/ml in WM) to stop mitosis at metaphase, treated with hypotonic solution of 40% fetal calf serum (FCS), and then placed on a clean slide. The slide was treated with fixative mixture of 3:1 methanol:acetic acid, and stained with 2% Giemsa solution by a conventional procedure [23]. Blastomeres bearing 40 chromosomes including one partially deleted Y chromosome, (which is approximately half the size of the normal Y chromosome) were classified as male, while those without a deleted Y chromosome were classified as female [24]. In the present study, 83% of the blastomeres separated at the two-cell stage could be classified as male or female by karyotyping.

Oligonucleotide Primers

The *Sry* gene [18] and *Zfy* genes [19, 25] were chosen for the Y-specific target sequences, and *DXNds3* locus, which is a polymorphic microsatellite locus located on mouse X chromosome [26, 27], was chosen for a control sequence. For each of the *Sry* and *Zfy* genes and *DXNds3* locus, two sets, an outer and an inner set, of oligonucleotide primers were synthesized with a Milligen model 7500 DNA synthesizer (Millipore Japan, Tokyo, Japan). Each of the inner pairs of the primers was located within the segments amplified by the corresponding outer pairs of the primers (shown in Fig. 1). Sequences of the primers are as follows:

SRY1, 5'GTGAGAGGCACAAGTTGGC3';
SRY2, 5'TCTTAAACTCTGAAGAAGAGAC3';
SRY3, 5'CTCTGTGTAGGATCTTCAATC3';
SRY4, 5'GTCTTGCCTGTATGTGATGG3';
ZFY3, 5'AAGATAAGCTTACATAATCACATGGA3';
ZFY4, 5'CCTATGAAATCCTTTGCTGCACATGT3';
ZFY11, 5'GTAGGAAGAATCTTTCTCATGCTGG3';
ZFY12, 5'TTTTTGAGTGCTGATGGGTGACGG3';
NDS1, 5'ATGCTTGGCCAGTGTACATAG3';

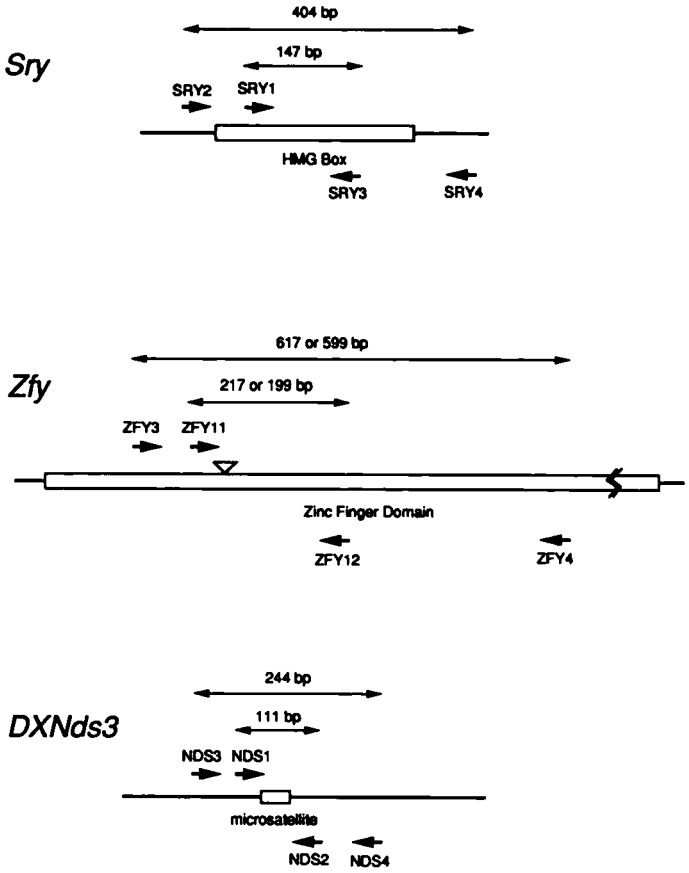

FIG. 1. Positions of various oligonucleotide primers on *Sry*, *Zfy*, and *DXNds3* sequences and sizes of fragments amplified with the primers. Single-headed arrows indicate positions of the primers and double-headed arrows indicate the regions amplified with the primers. Open boxes represent characteristic regions of the sequences. HMG box and Zinc finger domain are putative DNA-binding domains of the *Sry* and *Zfy* genes, respectively, and microsatellite is a polymorphic region consisting of a dinucleotide repetitive sequence. Open triangle on the *Zfy* gene indicates the region deleted in the *Zfy-2* gene.

NDS2, 5'TCCGGAAAGCAGCCATTGGAGA3';
NDS3, 5'GAGTGCCTCATCTATACTTACAG3';
NDS4, 5'TCTAGTTCATTGTTGATTAGTTGC3'

The sequences of ZFY3 and ZFY4, and NDS1 and NDS2 were the same as those used in Nagamine et al. [28] and Love et al. [26], respectively.

PCR Amplification and Gel Electrophoresis

The two-step PCR method using two pairs of primers for a target sequence [11] was employed in the present study. To each of the tubes containing the embryos, 100 μl of reaction mixture was added, and the sample was overlaid with 50 μl of mineral oil. Prior to addition of *Taq* polymerase (Boehringer Mannheim Yamanouchi, Tokyo, Japan), the sample was heated for 8 min at 94°C and then cooled for 2 min at 60°C, and this heating-cooling incubation cycle was repeated three times. The efficiency of the amplification may have been enhanced by this incubation

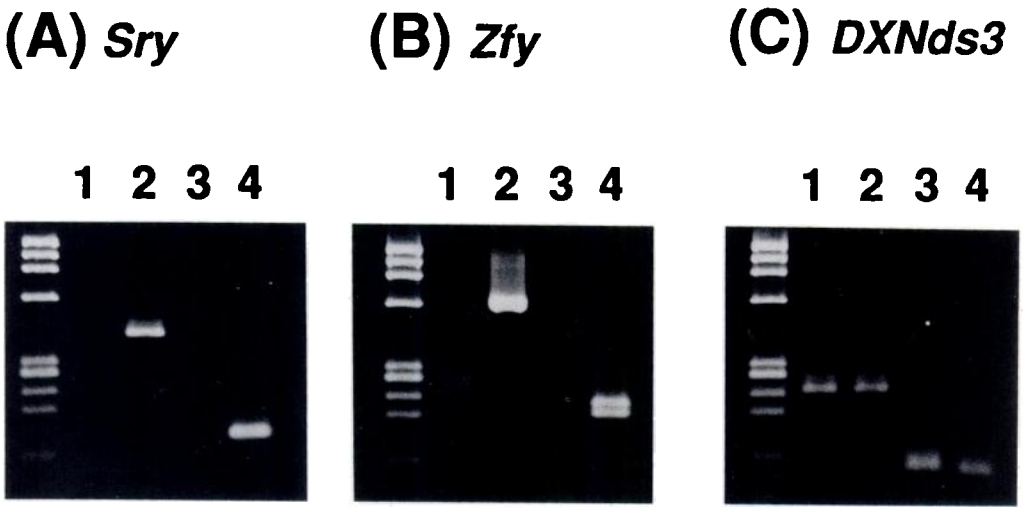

FIG. 2. Electrophoresed and stained PCR products amplified with various primers from purified mouse DNAs. (A) Products of PCR with pairs of *Sry*-specific primers: SRY2 and SRY4 (lanes 1 and 2), and SRY1 and SRY3 (lanes 3 and 4). (B) Products of PCR with pairs of *Zfy*-specific primers: ZFY3 and ZFY4 (lanes 1 and 2), and ZFY11 and ZFY12 (lanes 3 and 4). (C) Products of PCR with pairs of *DXNds3*-specific primers: NDS3 and NDS4 (lanes 1 and 2), and NDS1 and NDS2 (lanes 3 and 4). Lanes 1 and 3: 0.1 μg of purified DNA from female mouse; lanes 2 and 4: 0.1 μg of purified DNA from male mouse. Left lane contains DNA size marker of *Hae* III-digested ΦX174 DNA.

[29]. The first-step PCR was carried out using mixtures of each of the three outer pairs of primers, namely SRY2 and SRY4, ZFY3 and ZFY4, and NDS3 and NDS4. After 30 cycles of amplification, 10-μl portions of the products of the amplification reactions were dispensed respectively into three tubes and each was subjected to second-step amplification with 90 μl of a reaction mixture containing the corresponding inner pair of primers, namely SRY1 and SRY3, ZFY11 and ZFY12, and NDS1 and NDS2. Both steps of the PCR were carried out using a reaction mixture consisting of 10 mM Tris-HCl (pH 8.3); 50 mM KCl; 1.5 mM $MgCl_2$; 0.01% gelatin; 200 μM each dGTP, dATP, dTTP, and dCTP; 40 pmol of each of the oligonucleotide primers; and 2.5 U of *Taq* polymerase. PCR was performed for 30 cycles each consisting of denaturation for 60 sec at 94°C, annealing for 150 sec at 60°C, and extension at 72°C for 150 sec by Thermal Sequencer TSR-300 (Iwaki Glass, Tokyo, Japan). After the last cycle, the samples were kept at 72°C for a further 10 min. Positive and negative controls and a blank (including 0.1 ng of male DNA, 0.1 ng of female DNA, and no DNA, respectively) were also subjected to the two-step PCR procedure together with the embryo samples.

The products (10 μl of each) of the second amplification reactions were electrophoresed in 3% NuSieve 3:1 agarose (FMC) gels with Tris/borate/EDTA buffer. After electrophoresis at 50 V for 3 h, the amplified fragments were visualized directly by ethidium bromide staining and ultraviolet illumination.

Precaution Against Contamination

To avoid false positive detections, which result mainly from contamination of the sample with the final amplification product, we used the measures of Kwok and Higuchi [30] including physical isolation of the PCR preparation from the final products and use of positive displacement pipettes (Gilson, Villiers-le-Bel, France).

RESULTS

To determine whether pairs of primers could specifically amplify the Y-chromosomal and X-chromosomal sequences, 0.1 μg of genomic DNAs extracted from livers of adult male and female C57BL/6 mice were subjected to 40 cycles of PCR amplification. As shown in Figure 2, male-specific fragments were amplified by the pairs of the primers for the *Sry* and *Zfy* genes, while the pairs of the primers for the *DXNds3* amplified fragments from both male and female DNAs. Weak bands present in Figure 2B, lane 1, may be due to nonspecific amplification with the ZFY3 and ZFY4 primers; the bands disappeared when PCR amplification was carried out at an annealing temperature of 65°C. The sizes of the amplified fragments corresponded to those expected. No fragment was detected in female DNAs even when ten additional cycles of the amplification were carried out (data not shown). These findings indicate that each pair of primers could specifically amplify the target sequences. As shown in Figure 2B, two fragments of slightly different sizes were amplified by the primers for the *Zfy*. This may have been due to the presence of the two highly homologous sequence, *Zfy-1* and *Zfy-2* genes, on the mouse Y chromosome, because the sequences of regions chosen for the *Zfy* primers are identical between the *Zfy-1* and *Zfy-2* genes, and an 18-bp deletion is present in the segment of the *Zfy-2* gene defined between the primers (see Fig. 1) [17].

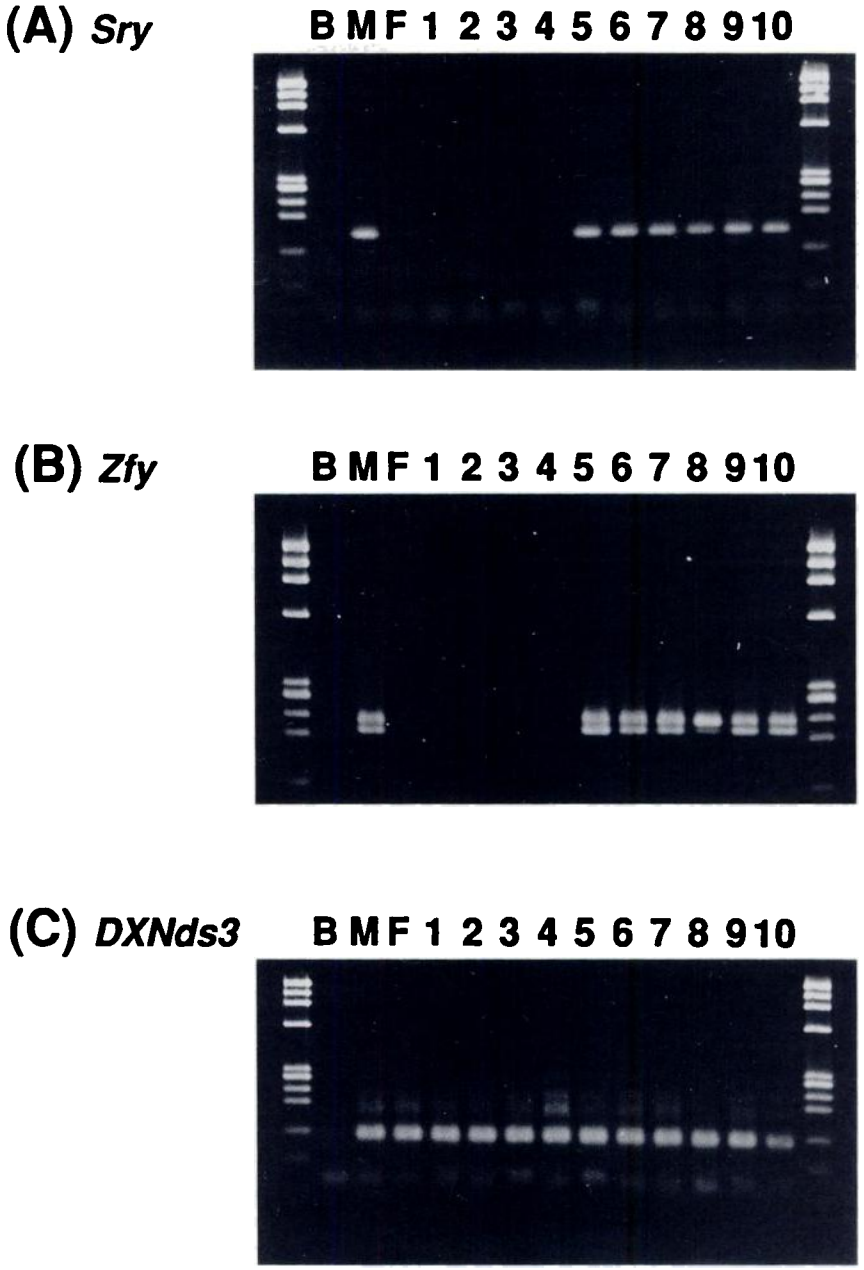

FIG. 3. Products of two-step PCR method amplified from single blastomere whose sex had been identified by karyotyping. (A) Products of the second-step PCR with *Sry*-specific primers. (B) Products of the second-step PCR with *Zfy*-specific primers. (C) Products of the second-step PCR with *DXNds3*-specific primers. Lane B: sample without DNA; lane M: 0.1 ng of purified DNA from male mouse; lane F: 0.1 ng of purified DNA from female mouse; lanes 1–4: blastomere predetermined as female; lanes 5–10: blastomere predetermined as male. Left and right lanes contain DNA size marker of *Hae* III-digested ΦX174 DNA.

To determine whether the two-step PCR method could detect the target sequences from DNAs in an amount as small as that of the mouse preimplantation embryo at an early stage, the mouse DNAs were serially diluted, and the DNAs in amounts equivalent to 200, 20, and two cells were amplified by the two-step PCR method. The results indicate that the two-step PCR method could detect the *Sry*, *Zfy*, and *DXNds3* sequences from an amount of DNA equivalent to two cells (0.01 ng) with no detectable background. On the other hand, the single-step PCR amplification with 40 cycles failed to detect the target sequences from 5 ng of the DNAs, although it could detect the sequences from 50 ng of the DNAs.

The method was then applied to the sex identification of preimplantation mouse embryos. Mouse embryos from their two-cell to blastocyst stage were subjected to the two-

TABLE 1. Data from two-step PCR of mouse embryos.

Stage of embryo	No. of embryos subjected to PCR	Male[b]	Female[b]
Two-cell[a]	15	7	8
Four-cell[a]	15	5	10
Eight-cell	10	3	7
Morula	12	5	7
Blastocyst	19	14	5
Total	71	34	37

[a]Zona-free embryos. Zona pellucida of these embryos were removed with pronase.
[b]Embryos showing amplified fragments of *Sry, Zfy,* and *DXNds3* sequences were identified as male; those showing only *DXNds3* sequence were identified as female.

step PCR method. The *Sry* and *Zfy* sequences were detected in about half of the embryos whereas the *DXNds3* sequence was detected in all embryos. The detection of the *Sry* sequence was perfectly consistent with that of the *Zfy* sequences. The positive controls of the male DNA gave amplified fragments of the *Sry*, *Zfy*, and *DXNds3* sequences while the female DNA gave only a fragment of the *DXNds3* sequence and the blank gave no fragment. Therefore, it was predicted that the embryos which gave fragments of *Sry*, *Zfy*, and *DXNds3* sequences would be male and that those giving only the *DXNds3* sequence would be female. As shown in Table 1, 71 embryos in total were examined and 34 and 37 were classified as male and female, respectively.

To further confirm the accuracy of the PCR method, the karyotyped half-embryos were used for PCR amplification to determine whether the detections of the *Sry* and *Zfy* sequences were actually due to the presence of the Y chromosome. The single blastomeres at the two-cell stage and the morulae developed from such single blastomeres were subjected to the two-step PCR method. Presence of the Y chromosome in the embryos was pre-examined by karyotypic analysis of the corresponding blastomeres at the second cleavage. As shown in Figure 3, the *Sry*, *Zfy*, and *DXNds3* sequences were amplified even from the single blastomeres. Correspondence between the detection of the *Sry* and *Zfy* sequences and the presence of the Y chromosome is shown in Table 2. The detection of the *Sry* and *Zfy* sequences perfectly corresponded to the presence of the Y chromosome except in one embryo in which all three sequences failed to be detected. Therefore, 59 of the 60 embryos were correctly sexed by the two-step PCR method. It is to be noted that one of the single blastomeres (Fig. 3, lane 8) gave only one distinct fragment upon amplification with the *Zfy* primers while all other embryos examined in the present study gave two distinct fragments, and also that on repetition of second-step amplification using the same products as in the first step, similar results were obtained. Therefore, it is likely that the *Zfy-2* gene failed to be effectively amplified in the first-step amplification of this sample.

DISCUSSION

In the present study, sexing of preimplantation mouse embryos was accomplished by the detection of the *Sry* and *Zfy* genes using the two-step PCR method.

Y chromosome-specific repetitive sequences have been used for the sexing of preimplantation embryos by PCR methods in humans [6, 7], mice [8], and cattle [9]. Due to the presence of a large number of copies of the sequence on the Y chromosome, the detection of the Y-specific repetitive sequence has proved to be efficient. However, presence of a small number of copies on some autosomal chromosomes has occasionally caused an amplification of the sequences in female DNAs to prevent an accurate sexing [8]. In the present study, instead of the Y-specific repetitive sequences, Y chromosome-specific single-copy genes were employed together with the two-step PCR.

Completely male-specific fragments were obtained by the pairs of the primers for the *Sry* and *Zfy* genes. Although some related sequences of considerable homology to the *Sry* and *Zfy* genes are known to be present on the autosomal and X chromosomes [28, 31, 32], no fragment was detected in female DNAs even when ten additional cycles of the amplification were carried out. This may have been due to a selection such that the sequences of the primers would be less homologous to the related sequences.

Since each target sequence is specified by four oligonucleotide primers, the two-step PCR method could greatly reduce the generation of nonspecific PCR products, which may characteristically result when a large number of amplification cycles are carried out. Thus, since the target sequences would be specifically detected from a small amount of DNA, this method may be effective for detecting a particular sequence from embryos. In fact, the target sequences were amplified by the two-step PCR method even from the single blastomeres in the present study.

There are some possibilities for failure of detection of the single sequence in the single blastomere. The *Zfy-2* gene could not be clearly detected in one blastomere. To prevent the false sexing resulting from such a failure in the detection of the target sequence, we used both *Sry* and *Zfy* genes as the Y chromosome-specific sequences. Furthermore, since the primers for the *Zfy* genes could individually detect both *Zfy-1* and *Zfy-2* genes, three Y-specific se-

TABLE 2. Detection of *Sry, Zfy,* and *DXNds3* sequences in karyotyped embryos.

Embryos	Karyotype	No. of embryos tested	*Sry*	*Zfy*	*DXNds3*
Single blastomere[a]	XY	6	6	6	6
	XX	4	0	0	4
Morula[b]	XY	34	33	33	33
	XX	16	0	0	16

[a]Single blastomere of two-cell stage embryos.
[b]Morulae developed from the single blastomeres of two-cell stage embryos.

quences were simultaneously detected in the present study. Although the *Zfy-2* gene was not clearly detected in one case, the sex of the embryo could be determined through detection of the *Sry* and *Zfy-1* genes. Therefore, accurate sexing of the embryos may require the detection of two or more Y-specific sequences.

In addition to detection of the Y-specific sequences, simultaneous detection of the X-chromosomal sequence, *DXNds3,* was carried out in the present study as an internal control to check the presence of the embryos in the sample. There was, in fact, one embryo that showed discrepancy between the presence of the Y chromosome and the detection of the *Sry* and *Zfy* genes. As this embryo gave no *DXNds3* fragment, this discrepancy may have been due to loss of the embryonal material from the sample before the sample was subjected to the PCR amplification. Simultaneous detection of the X-chromosomal sequence and the Y-specific sequences may ensure a reliable sexing.

Contamination, including the carryover through laboratory equipment of the amplified products to the samples, is a serious problem in the detection of specific sequences from a small amount of DNA, since the PCR procedure produces a very large number of molecules that could be amplified by subsequent PCR procedure to result in false positives. Because we took precautions against such contamination, no false positive result seems to have occurred in the present study, in contrast to preliminary experiments in which no such precautions were taken and some contamination was observed.

In our study, all embryos were typed either male or female with the exception of one sample, and the typed sex perfectly corresponded to the presence of the Y chromosomes. The detection of the target sequences by the two-step PCR method could be accomplished in less than 10 h, a period sufficient for transfer of the remaining half of the sexed embryos to recipients without freezing. It was thus concluded that the detection of Y chromosome-specific single-copy genes using two-step PCR is an accurate and rapid method for the sexing of preimplantation embryos.

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