A Novel Repeated Sequence Located on the Bovine Y Chromosome: Its Application to Rapid and Precise Embryo Sexing by PCR

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ABSTRACT. A novel repeated sequence specific to male cattle was identified and named S4. S4 is a highly repetitive sequence and is a 1.5 kb repeating unit that contains various internal repeated sequences. FISH analysis showed that S4 is localized on the whole long arm and the proximal region of the short arm of the Y chromosome. We found that a PCR primer set for S4 amplified a male-specific 178 bp product in addition to a 145 bp product common to both male and female cells. Although the origin of the 145 bp product is unknown, it acts as a positive internal control in practical embryo sexing. Due to the high copy number of S4, PCR required only 0.5 pg purified DNA for accurate amplification. This made it possible to reduce the amount of biopsy sample required for embryo sexing and thus result in less damage to embryos manipulated. These studies indicate that embryo sexing based on the S4 sequence is accurate and sensitive.

KEY WORDS: bovine Y chromosome, embryo sexing, FISH, PCR, repeated sequence.

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Simple and precise methods for embryo sexing are important for the management and improvement of bovine strains. Polymerase chain reaction (PCR) is mainly used for sexing bovine embryos [6, 22] with various target sequences, including male-specific repetitive sequences [3, 20] and single copy genes on the Y chromosome, such as amelogenine [4, 5] and ZFY genes [1, 9, 17]. When embryo sexing is performed with male-specific target sequences, one cannot distinguish the false-negative amplification caused by sampling errors from a lack of amplification in female samples. To solve this problem, various combinations of two different PCR primer sets, one for a male-specific sequence and another for a sequence common to both sexes, have been examined for PCR-based embryo sexing [2, 7, 10, 12, 15, 16, 18, 23, 25]. But this kind of PCR occasionally results in incorrect diagnoses due to the complexity of using two different primer sets in the PCR reaction. Here we report an improved embryo sexing method with conventional PCR. We found a novel repeated sequence, named S4, on the bovine Y chromosome. One of the PCR primer sets specific to the S4 sequence amplified a male-specific product in addition to a product common to both sexes. Furthermore, it was found that S4-based PCR is accurate and sensitive for practical embryo sexing.

MATERIALS AND METHODS

Isolation and analysis of a novel male-specific repetitive sequence: High molecular weight DNA was isolated from male or female bovine (Holstein) liver cells as previously described [19]. To isolate bovine repetitive sequences, 10 µg DNA from male or female liver cells was digested with *EcoRI*, separated by electrophoresis on a 1% agarose gel and stained with ethidium bromide. A visible DNA band of

approximately 1.5 kb was extracted from the agarose gel, purified and cloned into the PUC118 plasmid vector (Takara, Japan). Plasmid DNA extracted from 50 recombinant clones was labeled with Digoxigenin (Boehringer, Germany) and used as DNA probes. For Southern blotting, 10 µg of DNA was digested with various restriction enzymes (*BamHI*, *BglII*, *EcoRI*, *HindIII*, *MspI*, *PstI*, *RsaI*, or *TaqI*), separated by electrophoresis, transferred to a nylon membrane (Hybond-N, Amersham, England) and hybridized as described previously [19]. For dot blot analysis, a dilution series of DNA (1 pg to 500 ng) was applied to the nylon membrane and hybridized with digoxigenin-labeled pS4 and *SRY* as a control single copy gene [24]. Nucleotide sequences of plasmids or PCR products were determined automatically with an Applied Biosystems 373S fluorescence sequencer according to the manufacturer's instructions.

PCR: One ng of DNA was used for a PCR reaction with 15 pmol primers, Taq DNA polymerase (Boehringer, Germany) for 45 cycles (95°C, 30 sec; 52°C, 45 sec; 72°C, 45 sec). Primers used in the PCR study are shown in Table 1. After amplification, a 10 µl aliquot was subjected to electrophoresis in a 3% agarose gel and 1x TAE buffer (40 mM Tris-acetate, pH 8.0, 1 mM EDTA) and stained with 0.5 µg/ml ethidium bromide. To examine the sensitivity of the PCR assay, various amounts of purified DNA (0.1 pg to 100 pg) were subjected to PCR. Each PCR was run in triplicate to control for PCR variation.

FISH: For fluorescence *in situ* hybridization (FISH), a 250 ng pS4 DNA was labeled with Biotin-16-dUTP (Boehringer, Germany) with a nick translation kit (Boehringer, Germany). The probe mixture consisted of 50% formamide, 10% dextran sulfate and 2 mg/ml BSA in 2x SSC. For metaphase chromosome preparations, male peripheral blood

Table 1. Oligonucleotide primers used in the present study

Name of the primer	Nucleotide sequence of primer set	Based spanned
S4AF	CCATGGACTGTCGCCTATCAGGCTCCTC	522-549
S4AR	CTCCACATCCTCTGCAGCACTTG	770-792
S4BF	CAAGTGCTGCAGAGGATGTGGAG	770-792
S4BR	GAGTGAGATTTCTGGATCATATGGCTACT	919-947
S4CF	CCATGATAGTTCAGAGGTTAGGAC	6-19
S4CR	GTCCATGGGGTCGCAAAGAGTCGG	506-529

cells were cultured for 72 hr in RPMI 1640 medium supplemented with 10% fetal bovine serum (Gibco BRL Life Technologies, U.S.A.) containing 2% phytohemagglutinin (Murex, England). Actively growing cells were harvested after a 1 hr treatment with Colcemid (Gibco BRL Life Technologies, U.S.A.), incubated in 75 mM KCl for 15 min, fixed with 3:1 (v/v) methanol:acetic acid and dried on a slide. Hybridization and signal detection were performed as previously described [30].

Embryo sexing: For practical embryo sexing, 525 uterine-stage embryos were collected from superovulated donor cattle 7 days after estrus. Approximately 10% of the trophoderm of the embryo was excised with a microblade (Micro Feather Blade, K-715, Feather Safety Razor Co., Japan) attached to the micromanipulator. The excised cell mass was transferred by a capillary glass pipette into a microfuge tube (0.5 ml) containing 5 μ l of distilled water, then denatured by heating in boiling water for 1 min to extract DNA followed by rapid chilling on ice. To assess the accuracy of PCR results, the remaining part of the dissected embryos were subjected to chromosomal analysis as described by Hishinuma *et al.* [8]. In this comparative experiment a total of 36 embryos were analyzed. For the sequence analysis, both the male-specific DNA band and the DNA band common to male and female embryos were excised from the gel.

Embryo transfer: To confirm the results of PCR, we also performed embryo transfer. Sexes of blastocyst embryos obtained from Holstein donors were determined by PCR, and 71 embryos judged to be female were transferred non-surgically to synchronized heifers 6 to 8 days after estrus. Pregnancy diagnosis was performed with ultrasound between 39 and 50 days after estrus. The accuracy of the procedure was confirmed based on the sex of calves at birth.

RESULTS

To isolate a novel-repetitive sequence specific to male cattle, DNA derived from male liver cells was digested completely with *EcoRI* and subjected to electrophoresis. As shown in Fig. 1a, a single band (approximately 1.5 kb in size) was detected after ethidium bromide staining, suggesting that this band contained highly repeated sequences. Because a similar result was obtained with female DNA (Fig. 1a), the great majority of these repeated sequences

Fig. 1. Highly repeated sequences in the bovine genome. (a) Male and female genomic DNA was completely digested and subjected to electrophoresis on a 1% agarose gel and stained with Ethidium bromide. A 1.5 kb DNA band from male DNA was used for random cloning (▶), and a similar band was observed in female genomic DNA. (b) Southern blot analysis of *EcoRI* digested bovine DNA hybridized with clone pS4. A 1.5 kb male specific band (→) was observed. A common band (2.3 kb) was observed in both samples (*). M: λ /*HindIII* digested molecular size markers.

seemed to be common to both sexes. The 1.5 kb band derived from the male liver cells was extracted from the agarose gel, purified and cloned into the pUC118 plasmid. Of 90 recombinants obtained, 50 clones were screened as hybridization probes for Southern blotting. In female and male DNA digested with *EcoRI*, one of these clones, pS4, detected a male-specific 1.5 kb band in addition to a 2.3 kb band common to both male and female cells (Fig. 1b). Various sex-dependent polymorphisms were also observed in DNA digested with *BamHI*, *BglII*, *HindIII*, *MspI*, *PstI*, *RsaI*, or *TaqI* (data not shown). The copy number of S4, the insert of plasmid pS4, was estimated by dot blot assay. The signal intensity of S4 in the male liver cells was about 100 times

that of a control signal copy gene, *SRY*, and 2–3 times that of S4 in the female liver cells (data not shown), suggesting that S4 was a highly repetitive sequence in the male bovine genome. Consistent with these results, FISH analysis

Fig. 2. Chromosomal localization of the S4-repeated sequence revealed by FISH. Strong signals were detected throughout the long arm, and weak signals in the proximal region of the short arm of the Y chromosome were also observed. X: X chromosome, Y: Y chromosome.

showed that S4 was localized throughout the long arm of the Y chromosome (Fig. 2). We also observed weak signals on the proximal region of the short arm of the Y chromosome. No consistent signals were observed on autosomes or the X chromosome, although very faint signals were occasionally found in the telomere region of autosomes.

As shown in Fig. 3, S4 was 1542 bp long and contained various internally repeated sequences. Long internally repeated sequences found in S4 were 120 bp in length (designated as R1 and R2). The R1 and R2 nucleotide sequences were 80.5% identical. We also identified short 27 bp tandem repeated sequences (R3 and R4), and these were 81.4% identical. Four-nucleotide units (5'-GAAA-3') distributed throughout the S4 sequence and a (GpT)₂₆ repeat sequence were found in the S4 sequence (Fig. 3). Based on a homology search analysis, it was found that the bovine short interspersed repetitive sequences (SINE), BovA (115 bp) and Bov-tA (188 bp) were closely related to the 120 bp-internal repeats R1 (80% identity) and R2 (87% identity). A part of S4 (the region between bases 1 and 453) showed close similarity to a *Bos taurus* microsatellite sequence, INRA149 (bases 27 and 427) (83.4% identity; Fig. 3). Other than these sequences, we did not find significant similarity with the DNA sequences deposited in the EMBL database, including the *B. taurus* repeating sequence family BRY, which occurs throughout the Y chromosome [13,14,21,27], or the bovine repeated sequence FBNY [29], which is located on the Y chromosome (Y_q12-Y_qter) [29]. Analysis of the protein-coding capacity revealed that there are no extensive open reading frames in the S4 sequence.

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S4      1 GAATTCATG ATAGTTCAGA GGTAGGACT CCACGCTTTG CTATCAGGGG CTTGGGTAGG GTTCTCAACC AGGAAAGTGT CATTCAAGCT GCATAGCACA GCGAAACTA TGACAACAAC
INRA149 27 .....T.....AA.....T A.....T.....A.....T.....AA.....

S4     121 AATAAAACA CACTGTAAC GTGTGTGTGT GTGTGTGTGT GTGTGTGTGT GTGTGTGTGT GTATTAGTGT CTCAGTCATG TATGAGTCTT TGTCTTCACA TGGACTGTGG
INRA149 147 ---C...A.....AC...AC C.....ACT...G G.....G.....C...A...TG-C-C.....A...

S4     241 CCCATCAGGC TCCTTTGTCC ATGGGGTTCT CCAGGCCAAA ATACTGGAGT GGGTGGCCAT TTCTTTGTC AAAAGGCTG ATGAAAGTAT GTGAATACT TAGCATGTA AAATGGTTAA
INRA149 254 ---C.....G...A.....G.....C.....A.....T G.....C.....

S4     361 AAT-GGTGAA ATTAAAGTT AGA---TTTT TTTTITTTAC AAC---AAT TAAAACTTT TAACAACGAG GAAAGAATT TGAATAGACA TTTCACAAA AATACTGCTT AACATGATTG AAGGTGA
INRA149 374 ---G A.....TG--GG.....AAGG A A...AA C.....C G---AT...G G G C.....C...

S4     481 AAGAAAT TAA GTTGCTCAGT CCTTGCCGAC TCTTTGGGAC CCCATGGACT GTGGCTATC AGGCTCTCC ATCCATGGGA TTTCAGGC AGAGTGCTG GGGTGGATTG TCATTCTCTT
601 CTCAGGGGGA TCTTCCTGAC CCAGGAATCA AACCAGGTC TCCTGCATTG CAGGCAGACA CTTTACCTTC TGAGCCACCA GGAAGCCCT TAGCAAAATG TAAATCAGTT TCATTGAAA
721 ACTACTTCAC ACACATTAGC TTGGCTATAA TCAAAACGAG GAAATAAC AAGTGCTGCA GAGGATGTGG AAGAACGAA TCCTCATATA TTGTGAAA ATGTTGAAA TATACTGCTG
841 AAATATATTG TTGAAATAT ATTGCTCTAA AATGGTACAA CCACCTTGGT TTGTCAGTAC CTCAAAAATT AAATATATAG TAGCCATATG ATCCGAAAT CTCACTCTTT TGTATATATA
961 TATAAAAAAA AAAATCCCTG AAATATTAA GTCCATTCAA AAACAGTGT CCCAACAGCA TTACTCACA GAGCCACAAA GAATACACCA TCAATATATT ATCTATTAAT TGATGAATGG
1081 AATAAAATC GTGGTAATG TTCACAATGG AGTTCATGTA AGCCATATAC CATATTGATG TACCATATAC AGCCATTAAA AAAATGCATT GCTATACAGC CAAGAAGTGG ATGAATCTTG
1201 AAATACACA AACTAGGGGA CTTCCCTGGA AATGTTTAAG TGTTTAAGAC TATGCACAGT TTGCATCCCT GGGCAGGGAA CTAAGATCCC ACATGCCACA TAGCTAATA AATAATATT
1321 TTAAGAAATG ATGTGACATC TGTATAAAGC CAGACACAGA GGTACACATC TGATGTGGA AGAAGTGTAG GTAGAAAGA AGCAGGAAAG AGAAGCATTG TGAAGCCAA ATGAATATT
1441 TTTCAGG GAGGAGGAA TGCAGTCTC TGATAAAGG TGTCAATAAA TTAATAAAA TAACAATAAA AAATTGACCA CTGAGTTAAA CATTTGAAT TC

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Fig. 3. Nucleotide sequence of S4. S4 was 1542 bp long and contained various internal repeats: R1 and R2 [underscored with a single line (—)]; R3 and R4 (=); (GT)_n (----); (5'-GAAA-3') repeat units are boxed. Sequence comparisons were performed between S4 and the consensus sequence INRA149 by means of the homology search program BLAST in the EMBL DNA database. Sequence information was deposited into the DDBJ sequence database. The accession number is D16357.

Fig. 4. PCR with three different primer sets specific to S4. M: size marker, ♂: bovine male genomic DNA (50 ng); ♀: bovine female genomic DNA (50 ng); P: plasmid DNA (p-S4) (250 pg).

Fig. 5. Sensitivity of PCR with S4BF/BR primers. M: DNA size marker (ϕ X174/*Hinf* I digest); Lane 1: 50 pg, Lane 2: 12.5 pg, Lane 3: 6 pg, Lane 4: 3 pg, Lane 5: 1 pg, Lane 6: 0.5 pg, Lane 7: (-) control.

Fig. 6. Embryo sexing by the S4-based PCR method. M: DNA size marker (ϕ X174/*Hinf* I digest); Lanes 1, 4 and 5, male embryo biopsies. Lanes 2 and 3, female embryo biopsies; Lanes 6 and 9, negative control; Lanes 7 and 8, male and female genomic DNA (200 pg).

We designed three different PCR primer sets specific to the S4 sequence (Table 1). One of these primer sets, S4AF and S4AR, gave rise to a male-specific PCR product with the male DNA (Fig. 4). This PCR product migrated on the agarose gel at its expected molecular weight (271 bp). Amplification was not detected with the female genomic DNA. Similarly, S4CF and S4CR gave a PCR product (524 bp) only in the male DNA. To our surprise, a third primer set, S4BF and S4BR, consistently amplified an unexpected PCR product (145 bp) in addition to a product (178 bp) predicted from the S4 sequence. The former product was also detected in the female DNA, and therefore assumed to be common to both male and female cells. The amplification efficiency of the 145 bp product was almost identical to that of the male-specific 178 bp product. This suggests that the 145 bp product can be used as an internal positive control in embryo sexing.

To estimate the minimum amount of DNA necessary for proper amplification in the PCR reaction, a dilution series of purified male DNA was used for PCR with the S4BF/BR

primer set (Fig. 5). After 45 cycles of amplification, PCR products were detected in reactions containing as little as 0.5 pg of DNA, much less than the amount of DNA in a single mammalian cell. PCR products could not be reproducibly amplified with 0.1 pg of DNA (data not shown).

To test the accuracy of our sexing method, the sex of embryos was determined by conventional karyotyping, followed by our PCR-based sexing. Biopsy samples were collected from 36 embryos and subjected to PCR. Chromosome analysis was then performed with the remaining embryos. The sexes predicted by the PCR method agreed with those determined by conventional karyotyping (26 male, 10 female).

Next we applied the S4-based PCR to practical embryo sexing (Fig. 6). Of 525 uterine-stage embryos tested, 481 (91.6%) gave rise to male- or female-specific PCR products with the S4BF/BR primers (Table 2). The sex ratio predicted by the PCR method was almost 50:50. A PCR product was not detected in the remaining 44 embryos (8.4%). Embryos judged to be female by the PCR method were

Table 2. Embryo-sexing by PCR with S4BF/BR primer set

No. of embryos tested	No. (%) of successfully sexed		
	Male	Female	Total
525	253 (52.6)	228 (47.4)	481 (91.6)

Table 3. Embryo transfer of sexed embryos

embryos transferred	No. (%) of		Sex predicted by PCR (M/F)	Phenotypic sex after calving (M/F)	Accuracy
	Pregnancies	Births			
71	40 (56.3%)	25	0/25	1/24	96.0%

M: Male, F: Female.

transferred into synchronized heifers. Out of 71 transfers, 40 were diagnosed as pregnant by rectal palpation, and, from those, 25 calves were born and grew normally (Table 3). All but one of these calves was female.

DISCUSSION

We isolated a male-specific repeated sequence S4. Although S4 contained various internal repeating units and some of them were similar to short repeating sequences such as bovine SINEs [11,28], database analysis showed that S4 is a novel repeated sequence. FISH analysis showed that S4 is localized along the entire length of the long arm as well as the proximal region of the short arm of the Y chromosome. The PCR primers specific for S4, S4BF/BR, gave rise to a male specific 178 bp product along with a 145 bp product common to both males and females. Consistent with the fact that S4 was highly repetitive in the male bovine genome, we detected these two PCR products with as little as 0.5 pg template DNA. Weikard *et al.* [29] reported that one primer pair set simultaneously amplifies male-specific FBNY and microsatellite FBN17 sequences. A PCR product derived from FBN17 that was present in both sexes served as a positive internal control. But in contrast to our findings, their PCR method required at least 10 pg DNA for successful embryo sexing. Thus, the sensitivity of the S4-based sexing method is about 20 times higher than that of their PCR method. It will reduce the amount of biopsy sample needed and will result in less damage to the transferred embryos.

We applied this PCR method to the bovine embryo sexing and found that the sexes predicted by the PCR method agreed with the actual sexes of newborn individuals in 24 out of 25 cases, so that the accuracy of the S4-based embryo sexing method is 96%. The commercial PCR-sexing protocols for bovine embryos utilize Y-specific primers in conjunction with bovine control primers, and provide 90–95% efficiency and 93–98% accuracy [18, 20, 26]. Therefore, the PCR sexing procedure developed here appears to be sufficiently accurate for bovine embryo sexing. In one excep-

tional case, the predicted sex was female but the actual sex was male. This may have been due to an error in embryo handling or during the biopsy. Further, the high conception rate of biopsied embryos indicated that biopsy had little or no effect on pregnancy rates when fresh embryos were used.

The origin of the 145 bp PCR product is unknown. The S4 sequence itself does not contain the 145 bp sequence, because the 145 bp product was not amplified in the control PCR mixture which contained the pS4 plasmid as a DNA template (see Fig. 4, lane 9). We failed to determine the DNA sequence of the 145 bp product, probably due to heterogeneity of the PCR product extracted from the agarose gel (data not shown). Therefore it is likely that multiple target sequences of S4BF/BR (145 bp long) are dispersed throughout the bovine genome. This may explain why consistent signals were not identified on the X chromosome or on autosomes in FISH analysis with the pS4 probe, even though the same DNA probe gives rise to the 2.3 kb band in Southern blots indicating that it is common to both male and female samples. Further analysis is necessary to identify the origin of the 2.3 kb band found in the Southern blots.

In conclusion, with the novel repeated S4 sequence, we improved the conventional PCR-based bovine embryo sexing method. With a single primer set, two PCR products, a male-specific PCR product and a PCR product present in both sexes, can be detected simultaneously. Furthermore, a high copy number of the target S4 sequences ensures sensitive and accurate embryo sexing.

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