Length difference between equine *ZFX* and *ZFY* genes and its application for molecular sex determination

Sang-Hyun Han · Byoung-Chul Yang · Moon-Suck Ko ·
Hong-Shik Oh · Sung-Soo Lee

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Abstract

Purpose We analyzed the sex chromosome-encoding *ZFX*-*ZFY* genes and tested molecular sexing using the amplification patterns of intron 9 of *ZFX*-*ZFY* in the horse.

Methods and results The amplification of the *ZFX*-*ZFY* produced two distinct patterns, reflecting sexual dimorphism based on a length difference between the X and Y chromosomes. The amplification products from foals showed two distinct bands: one was common to all foals and mares, indicating that this band was amplified from *ZFX*, while the other was specific to some foals, indicating that it was from *ZFY*. The result based on the PCR assay was identical to the results of amplification of the Y chromosome-specific *SRY* gene and those of investiga-

tions of the phenotypic gender in three different horse populations.

Conclusion We suggest that this PCR strategy for determining sexes by comparing the amplification patterns of *ZFX*-*ZFY* genes is a convenient and precise method for discriminating sexes in horses.

Keywords Sexual dimorphism · Horse · *SRY* · *ZFX* · *ZFY*

Capsule Length difference was found in PCR amplification products between equine *ZFX* and *ZFY* genes. This sexual dimorphism may be a diagnostic molecular marker for determining the sexes in the horse.

S.-H. Han
Educational Science Research Institute, Jeju National University,
Jeju 690-756, South Korea

S.-H. Han
Mirae Biotech. Co./Jeju National University
Stem Cell Research Center,
Seoul 143-854, South Korea

B.-C. Yang
National Institute of Animal Science, RDA,
Suwon 441-350, South Korea

M.-S. Ko · S.-S. Lee (✉)
National Institute of Animal Science, RDA,
Jeju Sub-station,
Jeju 690-150, South Korea
e-mail: lee6470@rda.go.kr

H.-S. Oh
Department of Science Education, Jeju National University,
Jeju 690-756, South Korea

Most mammals have an X-Y chromosome sex determination system. Sexual differentiation is determined primarily by the presence or absence of the Y chromosome, and the *sex determining region Y (SRY)* gene plays a key role in inducing male development, while inhibiting female differentiation [1–3].

Control of the sex ratio of animals is desirable in the livestock industry. In mammals, the X-Y homologous sequences for the X- and Y-linked genes *zinc finger*, *amelogenin-X* and *-Y (AMELX-AMELY)*, and *SMCX/SMCY* have been compared [4–6]. PCR assays for molecular sexing are often more useful because they provide sensitive, precise, rapid, and reliable results. To date, PCR-based sex typing has been used for embryonic sexing in cattle, mice, and pigs [7–12]. To establish a rapid and precise molecular sexing method using PCR amplification for horse breeding, we analyzed the sequence characteristics and sexual dimorphism of the *ZFX* and *ZFY* genes and compared the results to those obtained from Y chromosome-specific *SRY* gene amplification and phenotypic investigation.

Total of 128 genomic DNA samples were collected from three horse populations (61 Thoroughbreds, 20 Tsushima native horses from Japan, and 47 Jeju native horses from South Korea). To amplify the intron 9 of *ZFX* and *ZFY* genes, the primer pair was designed from exon 8 and 10 of the sequences of human and cattle [13]. The primer

Fig. 1 PCR amplification patterns of intron 9 flanking region of equine *ZFX* and *ZFY* genes **a** and *SRY* gene **b**. Arrows indicate the lengths of each PCR product

sequences for the amplification of *ZFX* and *ZFY* genes were as follows: eqZF9iF, 5'-AAA TCA AAA CCT TCA TGC CAA T-3' and eqZF9iR, 5'- TTC CGG TTT TCA ATT CCA TC-3'. To compare the *ZFX-ZFY* amplification patterns to those from the Y chromosome specific *SRY*, which was amplified with *SRY*-specific primers [14] eqSRYf, 5'- TGC TAT GTC CAG AGT ATC CAA CA -3' and eqSRYr 5'-TGA GAA AGT CCG GAG GGT AA-3'. PCR reaction was carried out in 25-μl volumes, containing distilled water, 2.5 μl of reaction buffer, 200 μM of dNTPs, 2.0 units of *Taq* DNA polymerase (Promega, USA), 10 mM of each primer, and about 100 ng of total DNA. Amplification was performed using the PTC-200 thermal cycler (MJ Research, USA) under the following conditions: an initial 2-min denaturation at 94°C was followed by 35 cycles of 45 s at 94°C, 60 s at 58°C, and 60 s at 72°C. The PCR products for *ZFX-ZFY* and *SRY* gene were separated on

2.0% agarose gels containing ethidium bromide, and purified each band using a QIAEX II Gel Extraction Kit (Qiagen, USA). After purification of PCR products, for each homozygous genotype the PCR amplified fragments from three individuals were selected and sequenced using ET Dye-Terminator Sequencing kit (Amersham Pharmacia, USA). To compare the intron 9 sequences of equine *ZFX* and *ZFY* with those from cattle, we also determined the intron 9 sequences of *ZFX* and *ZFY* from cattle. The nucleotide sequences for intron 9 and flanking regions obtained in this study were deposited in GenBank database under accession numbers DQ179227 (bovine *ZFY*), DQ179228 (bovine *ZFX*), DQ179229 (equine *ZFY*), and DQ179230 (equine *ZFX*). To scan the repeat DNA elements, all sequences were analyzed using web programs CENSOR (<http://www.girinst.org/censor>) and RepeatMasker (<http://www.repeatmasker.org/cgi-bin/WEBRepeatMasker>).

Fig. 2 Comparison of the sequences of intron 9 flanking region of equine *ZFX* and *ZFY* genes with those of bovine genome. Asterisks and dots indicate the identical sequence comparing to that of bovine *ZFY*. Dashes indicate nucleotide deletion. Rectangles in exon 9 are nucleotide

substitution mutations between equine *ZFX* and *ZFY*. Underlined sequences are SINE-like element Bov-tA found in intron 9 of bovine *ZFY* using RepeatMasker and CENSOR program comparing to that previously reported (GenBank Accession no. of X46124)

The amplified PCR product for *ZFX-ZFY* genes showed two distinct band patterns. Those from some foals had heteroduplex bands (553- and 604-bp), while those from mares had a single 604-bp band, respectively (Fig. 1a). The band that foals shared with mares is likely that amplified from the X chromosome, while the foal-specific band could be amplified from the Y chromosome. The results of amplifications of the intron 9 flanking region of the *ZFX-ZFY* genes were identical to those based on investigations of phenotypic gender and a PCR assay of the Y chromosome-specific *SRY* gene. Figure 1b shows that the foals had an *SRY* band, while the mares did not. Since the *SRY* gene is specific to the Y chromosome, the presence of an *SRY* gene means that the DNA was isolated from animals possessing a Y chromosome. In other words, without exception, all individuals in three horse populations (Thoroughbred, Tsushina native horse, and Jeju native horse), that possessed the 553-bp *ZFY* PCR product also had the *SRY* PCR product. The DNA sequences of the purified PCR products, which included the complete intron 9 and flanking exons 9 and 10, were determined. Two nucleotide substitutions were found on exon 9 that did not involve an amino acid substitution or length difference. No mutations were found on the exon 10 partial sequences in *ZFX* and *ZFY*. However, the intron 9 sequence of *ZFX* was 51-bp longer than that of *ZFY*, making it readily distinguishable on agarose gels, and various nucleotide substitutions were also identified. The nucleotide alignment shows the nucleotide substitutions and indels of the sequences of the two partial exons and intron 9 of *ZFX* and *ZFY* (Fig. 2). The sequence characteristics of the *ZFX* and *ZFY* genes in cattle and pigs, while the horse sequence of intron 9 of *ZFX* has not been reported [13]. To our knowledge, ours is the first study to determine the sequence of intron 9 of *ZFX* and compare it to that of *ZFY*. The length variation is due to multiple indels between the two sequences. The presence of interspersed repeat elements on the intronic regions of the *ZFX-ZFY* genes causes marked length variation among mammalian species [13, 15]. Using the programs Repeat-Masker and CENSOR, we identified fragments of the interspersed repeat sequence. A short interspersed nuclear element (SINE)-like sequence was found in cattle *ZFY* intron 9 and two Alu-like sequences are present in the human *ZFY* intron 9. However, no retroelement-derived sequence was found in intron 9 flanking regions of the equine *ZFX-ZFY* genes. This result is similar to previous reports documenting the absence of a SINE-like sequence on intron 7 of the equine *ZFX* and *ZFY* gene, in contrast to the human and bovine *ZFY* intron 7 [13].

The molecular sexing of horses had been described using the *SRY* and *AMELX-AMELY* genes [16]. Stallions have a Y-specific *SRY* band and *AMELX-AMELY* heteroduplex

bands, while mares only have homoduplex *AMELX*. The detectable dimorphic patterns had been also documented in horses in the *ZFX* and *ZFY* genes using *HaeIII* PCR-RFLP and subsequent DNA sequencing [5]. However in this study, we found a length difference, despite the relationship of the insertion/deletion of the SINE element, more rapidly and easily using a simple amplification and electrophoresis. Moreover, the result using this molecular approach for sex determination of horses was identical to the results of phenotypic investigations and another PCR assay based on the amplification of the Y chromosome-specific *SRY* gene (Fig. 1b). All horse populations tested had no XY females; therefore, phenotypic males and females were distinguished using both PCR amplification tests using the *SRY* and *ZFX-ZFY* genes. Our analysis of the intronic organization showing sexual dimorphism on the *ZFX* and *ZFY* genes will contribute to molecular sexing as a useful method for selective breeding and for understanding the evolution of the horse genome.

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