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NOTE

Theriogenology

X monosomy in the endangered Kiso horse breed detected by a parentage test using sex chromosome linked genes and microsatellites

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ABSTRACT. A routine parentage test as part of a conservation program for Kiso horses identified a possible sex chromosome anomaly in a 7 months-old filly because of an aberrant result using *LEX3*, an X-linked marker. We then analyzed X-linked markers (*LEX26*, *TKY38*, and *TKY270*), Y-linked markers (*Eca.YH12*, *Eca.YM2*, *Eca.YA16*, and the sex-determining region Y gene), and an X/Y marker (Amelogenin gene). This analysis demonstrated that the filly had not inherited an X chromosome from her sire. A karyotyping analysis confirmed that the filly was 63,XO. As it was suspected that the horse would be sterile, we avoided using the horse as a broodmare; the information should also serve to prevent unnecessary conflict between owners transferring and receiving the horse.

KEY WORDS: Kiso horse, parentage test, XO monosomy

The Kiso horse is a native Japanese breed and traditionally was reared in the Kiso area spanning from southern Nagano to eastern Gifu prefectures. However, the number of Kiso horses rapidly decreased after World War II, and dropped to 32 in the 1970s [12, 13]. At present, the number of Kiso horses has increased to approximately 150, due to the efforts of enthusiasts dedicated to this horse breed. Still, the Kiso horse population is small, and a conservation program has been established to maintain the breed as a genetic resource and also as a living cultural asset of the area [12, 13].

To ensure the effective conservation of the Kiso horse breed, the biological management of the population is conducted by the Kiso Horse Conservation Association and others. As part of this management, parentage tests using microsatellites, which are widely employed in racehorses, are routinely performed on newborn Kiso foals to confirm their parentage and ensure reliability of the studbook. In addition, genotyping helps in the identification of individuals.

Detection of abnormalities in reproduction is an important aspect of the conservation of rare animals [6]. Chromosomal anomalies are one of common causes of reproductive impairment in horses. In particular, X monosomy is important as it results in an XO syndrome, which is manifested as showing infertility, small stature, irregular or absent estrus cycle, and hypoplasia of the ovaries and uteri. This syndrome has been found in a range of mammalian species including horses [1, 7–10]. A cytogenetic survey of 272 mares identified 10 with a chromosomal abnormality: one mare showed non-mosaic X monosomy, seven others had a mosaic form of X monosomy, i.e., 63,XO/64,XX [2]. Thus, non-mosaic and mosaic forms of X monosomy are a major cause of disorders of sex development involving in chromosomal abnormality in horses [2].

In this note, we report a Kiso foal that was found to have X monosomy in a routine parentage test using 31 microsatellites; this is the first diagnosed case of X monosomy in a Japanese native horse breed. The diagnostic procedure was carried out according to the Regulation for Animal Experiments in Gifu University and approved by Committee for Animal Research and Welfare of Gifu University (17159).

In autumn 2017, we carried out a routine collection of blood samples from new born foals and performed a parentage test using 31 microsatellites, including those in the recommended panel of microsatellites for parentage verification of the International Society for Animal Genetics (ISAG) [4, 12, 15]. Then, DNA sample was obtained from whole blood using an automated genome

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Fig. 1. Appearance of the 63,XO Kiso horse at 7 months of age. No abnormality was observed in her appearance.

Table 1. Parentage analyses using X-linked, Y-linked, and X/Y markers

Samples	Y-linked loci				X-linked loci				X- and Y-linked loci
	Eca.YH12	*Eca.YM2*	*Eca.YA16*	*SRY*	*LEX3*	*LEX26*	*TKY20*	*TKY38*	*AMEL*
Sire	Amplify	Amplify	Amplify	Amplify	F	M	M	F	AMELY AMELX
Dam	Non	Non	Non	Non	FL	MM	NN	GP	AMELX AMELX
Filly	Non	Non	Non	Non	F	M	N	P	AMELX

*Allele codes of *LEX3* are expressed using an ISAG code, but others are the originals applied in our institute. AMEL: Amelogenin.

DNA extractor, MagExtractor-MFX2000 (TOYOBO, Tokyo, Japan), and multiplex PCR was conducted according to Kakoi *et al.* [4] and Tozaki *et al.* [15]. PCR products were mixed, and 2 μl of this mixture was added to 20 μl of loading buffer. The mixture was heated to 95°C for 2 min and cooled at 4°C before loading. Electrophoresis was conducted for 30 min by 3130xl Genetic Analyzer (Applied Biosystems, Carlsbad, CA, U.S.A.). Finally, we genotyped each marker using the GeneMapper Software® (Applied Biosystems). Among the tested foals, we found a filly with an aberrant result for the *LEX3* probe, the microsatellite only located on a sex chromosome. Since the results of the other microsatellites were consistent with the expected parentage, the filly was suspected to have an X chromosome anomaly (Fig. 1).

To confirm the presumptive sex chromosomal anomaly, a PCR analysis was carried out on sex chromosome microsatellite markers in the filly, her dam, and her sire. The genotype analysis was performed in accordance with a published method [5] with minor modifications, and utilized *LEX3, LEX26*, *TKY38*, and *TKY270* as X-linked markers, *Eca.YH12*, *Eca.YM2*, *Eca.YA16*, and the sex-determining region Y (*SRY*) gene as Y-linked markers, and Amelogenin (*AMEL*) as a marker of the X/Y homologous region.

The Y chromosome microsatellites were not amplified from the DNA of the filly. With regard to the X-linked markers, all of those amplified from the filly were monoallelic and matched the alleles of the dam; no alleles derived from the sire were detected (Table 1). Consequently, the X chromosome from the sire was not inherited, and the filly was therefore diagnosed as having X monosomy.

To confirm the diagnosis of X monosomy, we carried out a karyotype analysis on a R-banded technique. One m*l* of whole blood was cultured in 10 m*l* RPMI 1640 (Sigma-Aldrich Japan, Tokyo, Japan) containing 20% FCS (Thermo Fisher Scientific K.K., Tokyo, Japan) and 0.2% phytohemagglutinin (Thermo Fisher Scientific K.K.). After 48 hr of culture, thymidine (Tokyo Chemical industry Co., Tokyo, Japan) was added at a final concentration of 300 μg/m*l* and the culture was continued for 16 hr. The cells were then washed twice in PBS, and cultured for 5 hr in 10 m*l* medium containing 25 μg/m*l* BrdU. Metaphase chromosome spreads were prepared from the lymphocytes in the standard manner; the spreads were stained with Hoechst 33258 [14], and the spreads were analyzed under a fluorescence microscope. Fifty metaphase spreads were analyzed: all contained 63 chromosomes. Karyotyping of 10 cells showed they lacked an X chromosome (Fig. 2). Consequently, the karyotype was confirmed as 63,XO, and

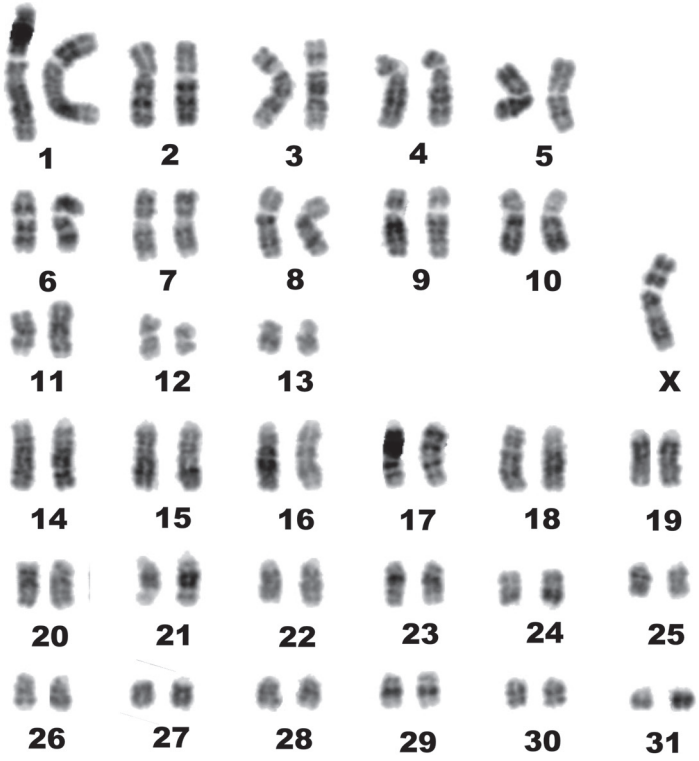

Fig. 2. R-banded karyotype of the filly. The analysis revealed the presence of a single X chromosome.

the filly was diagnosed as an X monosomy.

Although the analysis of the filly confirmed X monosomy, no abnormalities was observed in her appearance, including external genitalia, at 7 months of age. Moreover, no abnormalities were observed in complete blood cell counts or serum biochemistry according to reference values for the Kiso horse [11].

Our routine parentage test fortuitously contained a sex-linked chromosome marker, *Lex3*, and thereby allowed identification of the sex chromosome anomaly. The Kiso filly was confirmed to have a 63,XO karyotype, suspecting that the horse would be sterile, before clinical symptoms appeared. The information acquired from the parentage allowed us to avoid selecting the horse as a broodmare and should also help to prevent any conflict between owners transferring and receiving the horse.

This experience indicates the benefit of parentage tests using microsatellites to screen for chromosomal abnormalities in native horse breeds that may impair conservation programs. Microsatellite markers are reliable for detection of sex chromosomal abnormalities in the horse; routine microsatellites analyses in fact detected 63,XO, 65,XXY, 65,XXX, and 63,XO/64XY in light-breed foals [3, 5]. Consequently, we recommend that other sex chromosome linked markers should be used for the parentage tests despite the additional cost, and that such expanded parentage tests should be used in conservation of other endangered horse breeds.

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