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Prediction of X and Y chromosome content in bovine sperm by using DNA pools through capillary electrophoresis

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Abstract

Livestock resource management through gender offspring preselection is an efficient tool in terms of genetic improvement and farm management and additionally provides the opportunity to adjust offspring to market demands. In this study bull ejaculates were tested using PCR amplification of a segment of the X–Y homologous *amelogenin* gene in order to estimate the X and Y chromosome frequencies by capillary electrophoresis. Results were quantified against a regression function constructed with pools prepared with DNA from bulls and cows with known X and Y ratios. An average of $50.02 \pm 2.79\%$ X chromosome content was found with normal distribution ranging from 38.7 to 58.2%. Bull effect was significant in the analysis of variance representing 8.5% of the total variance. This simple analysis provides a low-cost and quick method of evaluating an X–Y ratio in a high number of ejaculates, particularly when external factors can be manipulated to alter it.

Keywords: Sex ratio; DNA Pools; *amelogenin* gene; Bovine sperm

1. Introduction

Knowledge of the gender of bovine offspring before performing fertilization procedures is a desirable objective with considerable economic, health, and ecological interests. The importance of this information is seen in some of its applications, e.g. obtaining more males or females according to a particular production system in animal breeding, minimizing

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gender-linked genetic diseases, and restoring a balanced male–female ratio in endangered species in order to maximize the effective number in the population [1].

Although the expected male to female offspring ratio in a population is 1:1, several situations exist which can, and frequently do, lead to discrepancies in the ratio. The first is based on the primary sex ratio stated as the coefficient between X and Y chromosomes in the ejaculate with a theoretical value of 1:1; the second is related to the time of insemination or mating during estrus [2,3]; and the third is based on the influence of environmental or physiological conditions on the survival of the fetus according to its gender [4–6]. Since the role of these chromosomes in mammalian primary sex determination and their function in gametogenesis remains unclear, the origin of a possible imbalance between the sex chromosomes in ejaculates is not well known. However, analysis of evidence found in other species, e.g. *Drosophila*, where both sex chromosomes and the way meiotic recombination occurs have been studied thoroughly, indicates that an increase in the proportion of X-bearing gametes consistently leads to an imbalance (towards an excess of females) in the gender ratio [7,8]. Thus, the aim of this study is to analyze the natural variability of the X and Y chromosome frequencies in bovine semen as estimated by capillary electrophoresis of DNA pools.

2. Materials and methods

2.1. Quantifying the proportion of X chromosome bearing sperm

In order to predict the response variable, i.e. the X and Y chromosome ratio in the ejaculates, we first needed to construct a prediction function, e.g. a regression equation, which gives us an acceptable prediction of the X or Y chromosome content by using some predictor variables. Thus, to assess the accuracy and sensitivity of the technique and to estimate the parameters of the regression function, several pools with different known X and Y chromosome content were prepared using DNA extracted from bull and cow blood samples. With this aim, different PCR conditions, e.g. DNA concentration, number of cycles, $MgCl_2$ concentration, and final PCR volume, were tested on the pools with the objective of attaining optimal results. In addition, both electropherogram traces (peak height and peak area) from the amplicon capillary electrophoresis were measured and used as predictor variables to estimate the chromosome content. The DNA pool results were used to estimate the regression parameters and the corresponding regression function was used to predict the relative X chromosome frequencies in the ejaculates sampled.

2.2. Biological samples

A total of 204 samples, 120 of which were the result of two consecutive ejaculates (later mixed and analyzed as a single sample (10 samples per bull)), of bulls semen were obtained following procedures used for commercially available semen. The remaining 84 ejaculates were collected and analyzed individually in order to detect variation between chromosomes. No other considerations, such as time of collection, that could alter the ratio, were

taken into account. Using a standard procedure, samples were diluted in a preservative buffer solution and cooled until their analysis. DNA extracted from blood taken from different bulls and cows was used for control samples.

2.3. Semen processing

Two independent DNA extracts were taken from each semen sample using a standard phenol–chloroform protocol [9], diluted and quantified through the detection of a DNA-specific dye (Picogreen) with a fluorimeter TD-360 Mini-Fluorometer (Turner Designs). The final DNA sample concentration was 2 ng/μl.

2.4. Quantitative PCR

Amplification and quantification of X and Y chromosomes were performed using primers AM-a and AM-b and co-amplifying homologous X and Y chromosome alleles of the *amelogenin* gene. In cattle, two different *amelogenin* transcripts (Classes I and II) issued from the same gene and located on the X and Y chromosomes respectively were amplified [10,11]. A 63 bp deletion in the fifth exon of the Class II *amelogenin* gene distinguishes the Y from the X chromosome [11]. Primers AM-a labeled with TET-fluorocrome (5′-CAGC-CAAACCTCCCTCTGC) and AM-b (5′-CCCGCTTGGTCTTGTCTGTTGC) amplified a 280 bp fragment from the X chromosome (Class I) and a 217 bp Y chromosome (Class II) *amelogenin* sequence respectively [12]. All PCR reactions were carried out in a PTC 200 thermocycler (MJ Research, Waltham, MA, USA). The PCR product was visualized in a capillary-sequencer ABI-310 (Applied Biosystems, 850 Lincoln Centre Drive, Foster City, CA, USA) following the manufacturers recommended electrophoresis conditions. An electropherogram of the *amelogenin* gene amplicon obtained from a bull is shown in Fig. 1.

2.5. Statistical analysis

A repeated measures analysis was carried out in order to evaluate any differences between two consecutive ejaculates from the same bull on the same day. The influence of bull, ejaculate nested within bull, replicate (DNA extraction and PCR amplification), and the interaction of bull and replicate were tested using ANOVA. Both analyses were performed using SAS v8 [13].

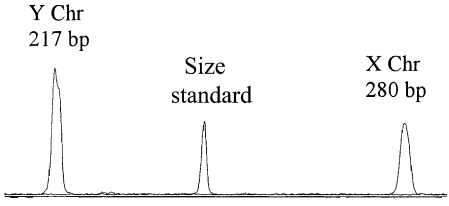

Fig. 1. Electropherogram showing the *amelogenin* gene amplicon from a blood sample taken from a bull and electrophoresed on a capillary automatic sequencer (ABI310).

3. Results

3.1. Estimating the proportion of X chromosome bearing sperm

The best amplification conditions in bovine DNA pools were found to be a final reaction volume of 25 μl, 6 ng of total DNA, 1.5 mM Cl_2Mg, 5 pmol of each primer, and 25 PCR cycles. This limited number of cycles was used in order to avoid both, the preferential amplification because it allows for better amplification of the Y chromosome fragment, and the plateau phase, as these are factors capable of modifying the interpretation of the original X–Y ratio present in the ejaculates. Although previous studies varied in choosing peak height or peak area (PA), the most consistent results, were found when peak area as opposed to peak height was used as the predictor variable.

The linear regression coefficient estimated from the data obtained from the pools amplified under these conditions is shown in Fig. 2. The slope was significantly different from 0 ($P < 0.0001$) and a value of 0.93 was obtained for R. The amount of X chromosome DNA present in each semen sample was scored by using the sample relative X chromosome PA value: $100 \times [(\text{X Chr PA})/(\text{X Chr PA} + \text{Y Chr PA})]$, in the prediction equation

$$\%\,\text{X Chr} = 23.3 + 0.8\,\text{relative X Chr PA}.$$

For instance, a relative X Chr PA of 28.23 corresponds to 72% of X Chr content.

Fig. 3 illustrates this linear increase in the relative X Chr PA which corresponds to the linear increase in amounts of DNA from cows within the pools.

3.2. Sources of variation

The repeated measures analysis used to examine differences between two consecutive same-day ejaculates of a bull demonstrated no significant difference. Furthermore, the absence of interaction between the effect of bull and replicate with ejaculate justifies the use of both ejaculates in a single sample.

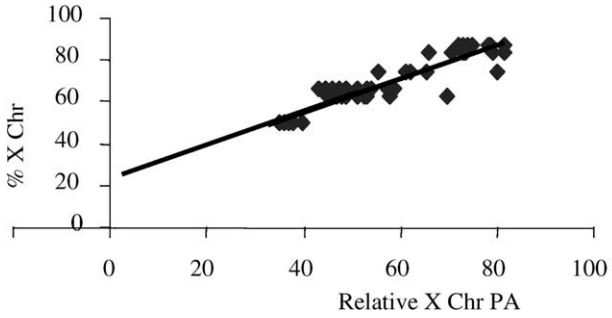

Fig. 2. Linear regression showing the relationship between the frequency of X chromosome bearing sperm (% X Chr), the dependent variable, and the relative X chromosome peak area (relative X Chr PA) which is the measurement obtained through capillary electrophoresis when amplifying the *amelogenin* gene from bull and cow DNA pools. The parameter estimation values for this linear regression are 23.3 for the intercept and 0.8 for the slope.

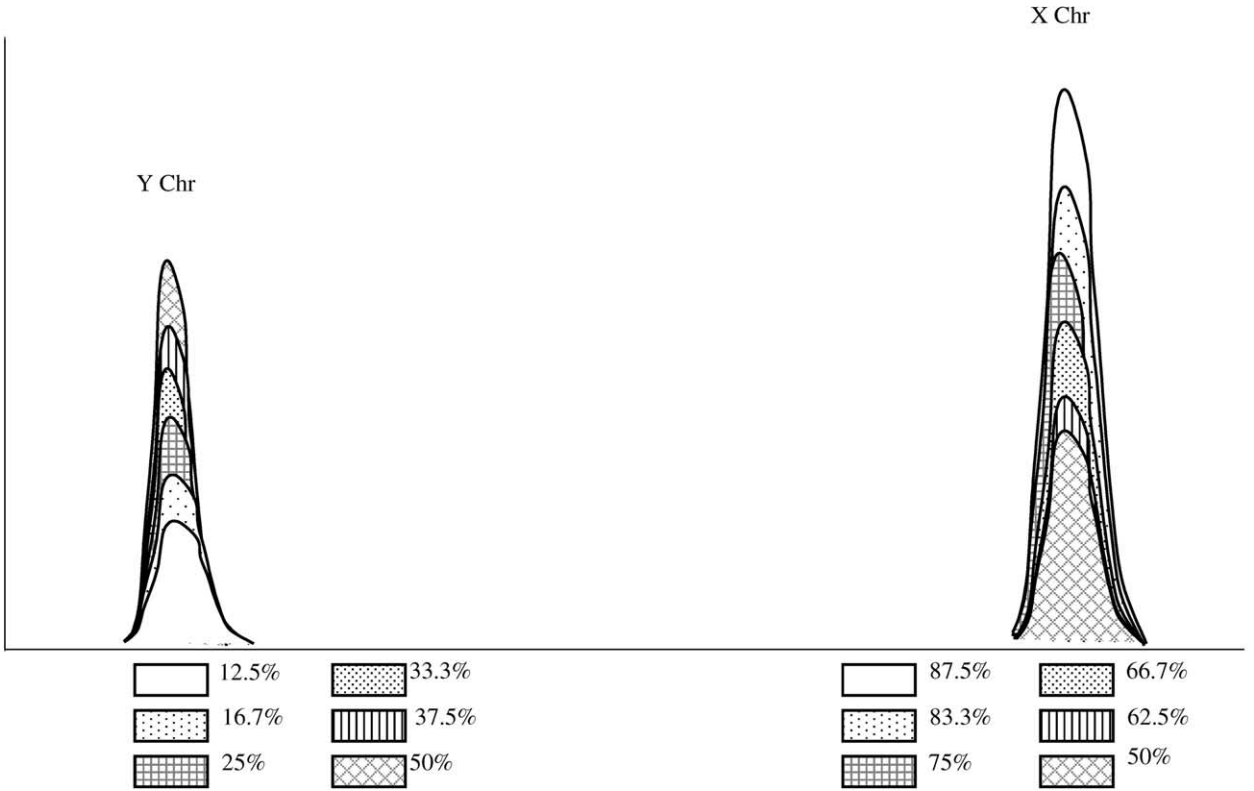

Fig. 3. Overlapped electropherograms from six different bull and dam DNA pools for the *amelogenin* gene amplicon and electrophoresed on a capillary automatic sequencer (ABI310). Peaks (X or Y Chr) belonging to the same pool are shown with the same background shading.

Table 1
Analysis of sources of variance between two consecutive same-day ejaculates from the same bull

Source of variation	Degree of freedom	*F* value
Bull	41	3.80*
Ejaculate (bull)	121	1.05[a]
Replicates[b]	5	8.40*
Bull × replicates	26	2.86*

[a] Non-significant.
[b] Replicates include DNA extraction and PCR amplification treatments.
* $P < 0.01$.

Results of the analysis of variance are shown in Table 1. Total variance explained by the model was 0.79 observing no differences between distinct same-bull ejaculates; however, factors showing significant influence on X chromosome content were bull, replicate, and the interaction of both. Bull effect represents 8.5% of the total variance and the replicate effect 24%.

3.3. Gender chromosome frequencies

The batch of semen samples was found to contain an average of 50.02% X chromosome content. Individual samples were assembled in a normal distribution (Fig. 4) ranging from 38.7 to 58.2% X chromosome. The mean standard error was 2.79 which means that the probability of finding a sample with an X chromosome content higher than 80% or lower than 20% is nearly null.

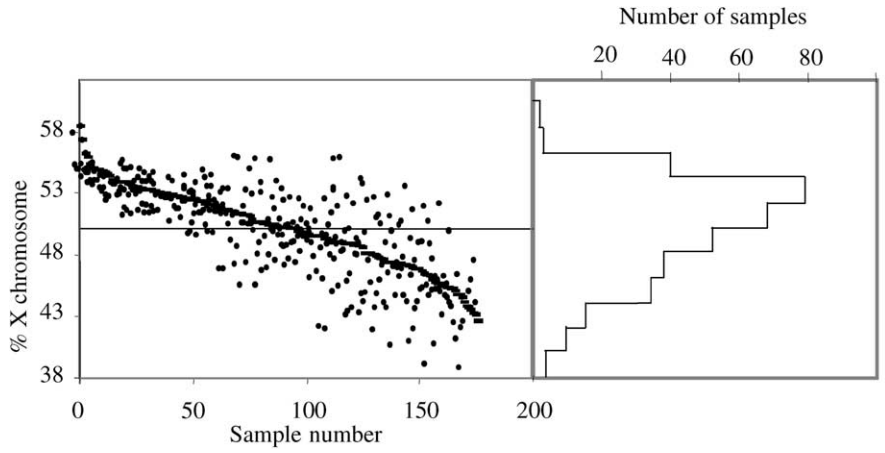

Fig. 4. Gender chromosome composition of ejaculates. Left panel: duplicate determinations for semen samples are recorded as dots and the mean of each replicate set is indicated by a short horizontal line. Right panel: frequency histogram plots the sample number included in each range.

4. Discussion

The *R* value obtained (0.93) and the regression equation showed the validity of the technique in detecting variations in the amount of DNA issued from X or Y chromosomes in an ejaculate.

The fact that bull effect is significant could be due, as hypothesized by Szyda et al. [14], to genetic causes based on a possible mechanism relating sperm survival to the genetic recombination between X and Y bearing homologues during gametogenesis. This is the way these authors explain an excess of X bearing sperm in the majority of bulls and also a high distortion in individuals belonging to a same family. Another explanation supported by Szyda et al. [14] is that X-specific genes located closed to the bovine pseudoautosomal region and expressed in spermatids could affect sperm viability. Chandler et al. [15], on the other hand, found significant variation in levels of Y chromosome bearing spermatozoa between different ejaculates within bulls. Nevertheless, neither the effect of bull nested within PCR nor the effect of unit or lane of electrophoresis contributed to that variation in Chandler's study. This lack of alternative hypothesis acceptance for these effects is probably a consequence of the high residual noise in the analysis.

The gender chromosome frequencies obtained here are very similar to those reported by Lobel et al. [16] in their study of 98 human semen samples in which they found an average of 50.3% Y chromosome with a coefficient of variation among samples of 6.5%. These researchers used a similar PCR technique but amplified ZFX and ZFY genes, located on the X and Y chromosomes respectively, using radioactivity to visualize DNA on acrylamide gel. Szyda et al. [14], using the same PCR technique and same sequences, found a significantly higher proportion of X bearing (53.5%) gametes in bovine. This value was observed in a test over all families studied. Their results showed that the gender ratio significantly correlates with changes in the recombination rate among X bearing, though not Y bearing sperm.

In contrast, clearly different results were described by Chandler et al. [15] who found a wider range (24–84%) of the percentage of the Y chromosome bearing sperm when using PCR techniques and a Y chromosome-specific sequence (BRY1). They found that 20% of the ejaculates differed from the overall mean. These disparities could be due to the way DNA was quantified by these researchers who used a spectrophotometer which does not provide as precise a measurement as a fluorimeter. Breen et al. [17] found that measurements of DNA concentration were substantially more accurate when using fluorimetry as opposed to a UV absorbance method with an average coefficient of variation of 1.2 and 28.3%, respectively. Also, it should be noted that, although these researchers used several controls, they did not use X–Y homologous genes which offer the advantage, as in our case, of an internal positive control, since both X- and Y- specific sequences are amplified in a single reaction. On the other hand, the amplification of a second locus is useful for verifying that the absence of a signal is not the result of PCR failure.

In summary, the present study shows the accuracy and sensitivity of DNA pooling to quantify X and Y chromosome present in an ejaculate by amplifying the *amelogenin* gene using PCR and posterior capillary electrophoresis. Not only is the method quick and simple but it also requires a unique PCR reaction and can be used whenever it is necessary to check sexual chromosome variation.

Although no ejaculate with an altered ratio of X and Y bearing sperm out of the 38–60% X chromosome range was found in this study, the use of this technique is of great interest when external factors must be identified in order to obtain ejaculates with extreme frequencies of X or Y chromosomes.

Acknowledgements

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References

[1] Seidel Jr GE, Johnson LA. Sexing mammalian sperm—overview. Theriogenology 1999;52:1395–9.
[2] Ballinger HJ. The effect of inseminations carried aout early or late in oestrus on the sex ratio of calves born. Vet Rec 1970;86:631.
[3] Foote RH. Sex ratios in dairy cattle under various conditions. Theriogenology 1977;8:349–56.
[4] CluttonBrock TH, Lason GR. Sex ratio variation in mammals. Q Rev Biol 1986;61:339–74.
[5] Hardy ICW. Possible factors influencing vertebrate sex ratios: an introductory overview. Appl Anim Behav Sci 1997;51:217–41.
[6] Ostrowsji JEB. Pasture breeding of oestrus synchroized beef heifers and its apparent effect on sex-ratio of offspring. Dtsch Tierarztl Wochenschr 1988;95:365–8.
[7] Cazemajor M, Landre C, MontchampMoreau C. The sex-ratio trait in *Drosophila simulans*: genetic analysis of distortion and suppression. Genetics 1995;147:635–42.
[8] Lyttle TW. Segregation distorters. Annu Rev Genet 1991;21:511–57.
[9] Sambrook J, Fritsch EF, Maniatis T. Molecular cloning: a laboratory manual. New York: Cold Spring Harbor Laboratory Press, 1999.
[10] Gibson C, Golub E, Abrams WR, Shen G, Ding W, Rosenbloom J. Bovine amelogenin message heterogenety: alternative splicing and Y-chromosomal gene transcription. Biochemistry 1992;31:8384–8.
[11] Gibson C, Golub E, Herold R, Risser M, Ding W, Shimokawa H, et al. Structure and expression of the bovine amelogenin gene. Biochemistry 1991;30:1075–9.
[12] Ennis S, Gallagher TF. A PCR-based sex-determination assay in cattle based on the bovine amelogenin locus. Anim Genet 1994;25:425–7.
[13] Statistical Analysis Systems Institute Inc. SAS/STAT users guide, version 8. Cary, NC: Statistical Analysis Systems Institute Inc., 1999.
[14] Szyda J, Simianer H, Lien S. Sex ratio distortion in bovine sperm correlates to recombination in the pseudoautosomal region. Genet Res 2000;75:53–9.
[15] Chandler JE, Steinholt-Chenevert HC, Adkinson RW, Moser EB. Sex ratio variation between ejaculates within sire evaluated by polymerase chain reaction, calving, and farrowing records. J Dairy Sci 1998;81:1855–67.
[16] Lobel SM, Pomponio RJ, Mutter GL. The sex ratio of normal and manipulated human sperm quantitated by the polymerase chain reaction. Fertil Steril 1993;59:387–92.
[17] Breen G, Sham P, Li T, Shaw D, Collier D, St Clair D. Accuracy and sensitivity of DNA pooling with microsatellite repeats using capillary electrophoresis. Mol Cell Probes 1999;13:359–65.