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A novel variant of the amelogenin gene (*AMEL-X*) in cattle and its implications for sex determination

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The amelogenin gene (*AMEL*) is often used in the research requiring sex determination of humans and animals. In *Bos taurus* the most striking difference between the X- and Y-chromosomal *AMEL* genes is the 63 bp deletion in exon 6 of the Y-linked copy. Size differences in the gene *AMEL* between the sex chromosomes are used for embryo sexing as well as in forensic casework, prenatal diagnosis, *etc.* The present paper shows that sex determination of cattle samples based on DNA sizing of gene *AMEL* is probably more complex than it was hitherto supposed.
Used were 363 cows and 262 bulls of different breeds. According to a new polymorphism revealed in gene *AMEL* in chromosome X in both the homo- and heterogametic sex, the novel PCR fragments of atypical size can be detected.

KEYWORDS: cattle / amelogenin gene / chromosome X

Simple and precise methods for sex determination in animals are a pre-requisite for a number of applications in animal production and forensic casework. For example, manipulation of the live-born sex ratio of cattle would give rise to a more flexible breeding and production strategy [Grzybowski and Dymnicki 1997]. Genetic difference between male and female is the presence or absence of chromosome Y. Therefore, many of existing methods for sex identification are based exclusively on the detection of sequences specific for chromosome Y [Bondioli *et al.* 1989, Herr *et al.* 1990, Schröder *et al.* 1990, Wayda *et al.* 1995]. However, the absence of a signal does not necessarily mean that the sample is of female origin, as negative results may also be generated by experimental errors. Thus, the detection of Y- and X-chromosome specific sequences is advantageous. Amelogenin gene (*AMEL*), the origin of which

has been traced back to the Precambrium period [Delgado *et al.* 2001] has been used as a model in the field of molecular phylogenetics [Delgado *et al.* 2005, Toyosava *et al.* 1998] as well as in the analyses of biological traces aiming at determining the sex in humans and animals [Sullivan *et al*. 1993, Ennis and Gallagher 1994, Reklewski *et al.* 1996, Lechniak and Cumming 1997, Miścicka-Śliwka *et al.* 1997, Pfeiffer and Brenig 2005]. The genes for enamel matrix proteins (*i.e*. amelogenin, enamelin and ameloblastin – EMPs) belong to a family comprising also milk caseins and salivary proteins and descend from a common ancestor by tandem duplication. *EMP* genes remain linked, with the except for amelogenin. In eutherians (e.g. cattle, human, horse, black bear, monkeys), a copy of *AMEL* is present on each sex chromosome, while the gene is autosomal in *monotremata*, *marsupialia* and non-mammalian species [Sire *et al.* 2005]. The gene *AMEL* is generally known to be composed of seven exons [Delgado *et al.* 2005]. The hydrophobic, central region of exon 6 (approx. 300 bp), is the most variable compared to the other exons and to the 5'and 3' regions, which are hydrophilic and show a high sequence similarity [Sire *et al.* 2005]. The X- and Y-linked amelogenin *loci* do not evolve in concert, and several differences (substitutions and indels) have arisen between the copies. In *Bos taurus* the most striking difference between the X- and Y-chromosomal *AMEL* genes is the 63 bp deletion in exon 6 of the Y-linked copy [Gibson *et al.* 1991, 1992]. Size differences in *AMEL* between these chromosomes have been used for embryo sexing, in forensic casework, prenatal diagnosis, *etc*.

The aim of this paper was to demonstrate that determination of sex of cattle samples based on DNA sizing technology of gene *AMEL* is presumably more complex than is usually assumed. Basing on a new deletion in gene *AMEL* discovered in this study in chromosome X it is anticipated that the novel PCR fragments of atypical size can be detected. Such fragments can alter the results of sample identification.

Material and methods

The material covered a total of 363 cows and 262 bulls with typical karyotypes. The cows were Polish Red (PR, n=150), Polish Whiteback (PWb, n=72), Red-and-White (RW, n=72), and Black-and-White with Holstein-Friesian blood share (BW, n=69). The male material consisted of 12 PR, 30 RW, 125 BW with Holstein-Friesian blood share, 67 purebred Holstein-Friesian and 7 bulls of various beef breeds. Moreover, used were 6 young PWb and 15 young PR bull-calves.

Genomic DNA was extracted from blood samples using Wizard® Genomic DNA Purification Kit (PROMEGA, USA). The polymerase chain reaction (PCR) primers were those described by Ennis and Gallagher [1994]. PCR reaction was performed in a volume of 10 µl using 1 µl of template DNA (approximately 50 ng), 1 unit of AmpliTaqGold™ (APPLIED BIOSYSTEMS) with reaction buffer consisting of 50 mM KCl, 10mM tris-HCl pH 8.3, 1.5mM $MgCl_2$, 200 µM each dNTP and 0.05-0.18 µM of each primer. The PCR products were separated in 5% Long Ranger gel (FMC

BIOPRODUCTS) on an ABI PRISM 377 DNA sequencer (APPLIED BIOSYSTEMS) using GeneScan-500 internal size standard. Fragment sizes were determined using GeneScan v.3.1 software (APPLIED BIOSYSTEMS).

Results and disscussion

Primer pairs used for amplification of exon 6 of the bovine gene *AMEL* produced the 215 bp fragment specific for chromosome Y and 278 bp fragment specific for chromosome X. Typical pattern for the heterogametic sex (XY karyotype) comprised one fragment of 215 bp and one of 278 bp (Fig. 1-a), while that for homogametic sex (XX karyotype) consisted of two fragments of 278 bp generating single detection signal (Fig. 1-b). According to preasumption presented, the testing result for homogametic sex should always be "homozygous", whereas "heterozygous" phenotype should always appear for heterogametic sex. Additionally, the PCR products specific for chromosome X and Y must have the constant position in a separating medium, corresponding exactly to the fragment size of 278 bp and 215 bp.

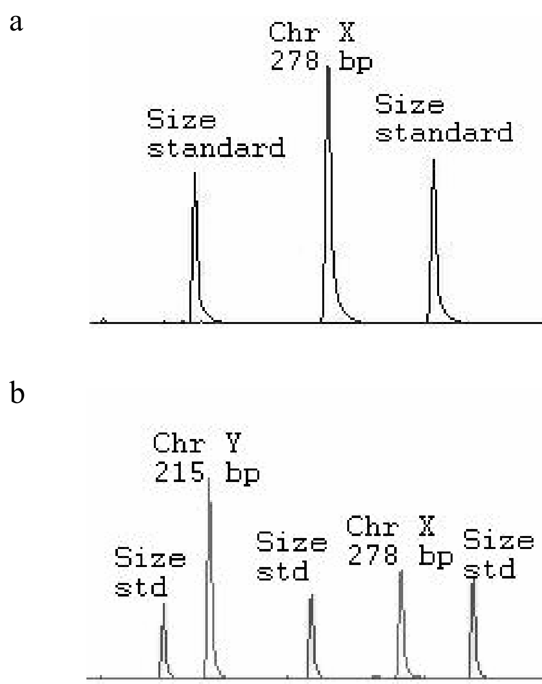

Fig. 1. Electrophoregrams showing the amelogenin gene amplicon from a blood sample withdrawn from: a – cow of genotype 278/278; b – bull of genotype 215/278.

The typical size for the PCR product for chromosome X was observed for the majority of animals tested. Only for 24 PR cows and two PR bulls a novel variant for amelogenin gene in chromosome X was found, non hitherto reported. In addition to typical PCR product of 278 bp length a fragment of a smaller size, *i.e.* 269 bp appeared (Fig. 2-a and Fig. 2-b).

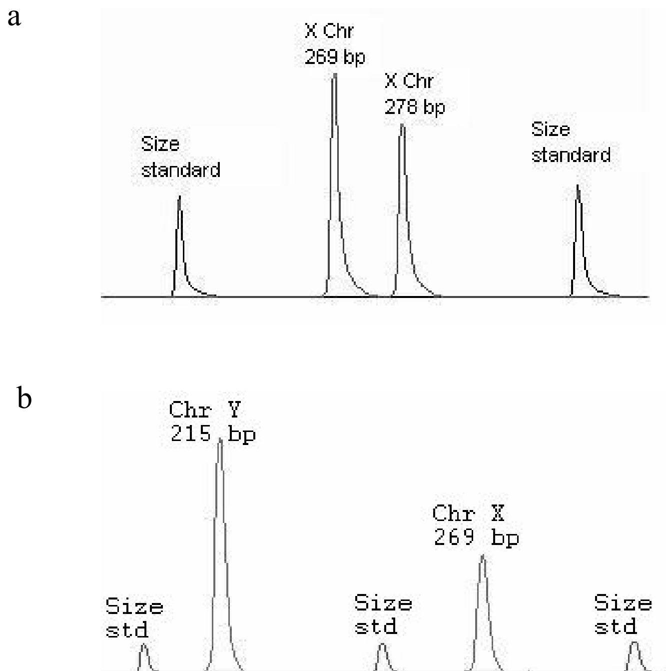

Fig. 2. Electrophoregrams showing the amelogenin gene amplicon from a blood sample withdrawn from: a – cow of genotype 269/278; b – bull ofgenotype 215/269

Review of relevant literature indicates that polymorphism in gene *AMEL* on chromosome X has not been hitherto detected, while in the sequence of *AMEL* in chromosome Y many differences have been reported. In addition to the characteristic deletion of 63 bp in exon 6, there are 41 single-nucleotide differences between the X and Y gene-coding regions. There are 13 amino acid differences including six conservative and five non-conservative changes in the secreted protein plus two differences in the signal sequence [Gibson *et al.* 1992]. Since in placental mammals both the gene and protein sequences of amelogenin are highly conservative [Sire *et al.* 2005], detection of novel sequence variant in cattle *AMEL* is surprising. The sequence was introduced into GenBank (accession nos. Q99004 and M63499) and is considered

as the reference sequence for cattle gene *AMEL-X*. The only possible explanation for the phenomenon of detection of two different PCR products for homogametic sex (278 bp and 269 bp) could be a deletion within the annealing region of the primers.

Variant *AMEL-X(269)* was identified exclusively in PR cattle, indicating that the detected deletion is breed-specific. It seems that information about existence of *AMEL-X(269)* in cattle genome could be useful for appropriate managing of PR cattle preservation programme. An analysis of the DNA microsatellite alleles confirmed that 80% of PR animals included in the Polish National Rare Livestock Breeds Preservation Programme (NRLBPP) comprised a separate genetic group with high level of genetic diversity, differing from the other European cattle populations [Lubieniecka *et al.* 2001]. Identification of hitherto unknown variant of gene *AMEL* occurring only in PR cattle confirms this opinion.

Comparison of bovine *AMEL* sequence (GenBank accession nos. Q99004 and M63499) with those identified in 26 species representing main taxonomic groups of mammals [Toyosava *et al.* 1998, Delgado *et al.* 2005] leads to the conclusion that detected deletion of 9 bp is located in the region of exon 6 which, in turn, is considered as a mutational hot spot in mammalian *AMEL* gene. Many differences in this region have been detected between taxonomic groups, but on the species level both the gene and protein sequence are highly conservative [Toyosava *et al.* 1998, Delgado *et al.* 2005] presumably due to selection pressure. The number of variations observed in *AMEL-Y vs AMEL-X* sequences indicate that both genes were not subjected to the same evolutionary constraints [Delgado *et al.* 2005]. Thus, it seems particularly important to verify whether amelogenin protein coded by the new gene variant *AMEL-X(269)* maintains its normal biological efficiency. Given the predominance of amelogenin in forming enamel matrix, one can easily understand that defects in the AMEL structure and/or organization have a detrimental effect on enamel formation. Twelve mutations (either deletions or substitutions of nucleotides) in the human amelogenin gene sequence have been reported to be responsible for various types of *amelogenesis imperfecta* [Hart *et al.* 2002]. In humans, the structural defects that lead to several cases of the disease concern only *AMEL-X* (X-linked effects). In light of important consequences for enamel structure, such defects would probably be lethal in wild animals [Delgado *et al.* 2005]. It remains to be determined whether both genes on the X and Y chromosomes are functional. So far, the function of amelogenin proteins coded by *AMEL-Y* remains unknown [Delgado *et al.* 2005]. The sequence of the Y-linked gene is probably not under strong functional constraint as possible inactivating mutations are masked by the X-linked gene. The eutherian Y-linked copy may, therefore, be tending towards pseudogene status [Toyosava 1998, Gibson *et al.* 1992]. In forensic cases it has been shown that some mutations identified in human gene *AMEL-Y* can lead to serious mistakes in sex determination analyses. Several PCR primer sets have been developed, and the most commonly used PCR-based sex test was described by Sullivan *et al.* [1993], flanking a 6 bp deletion on the X homologue, resulting in 106 bp and 112 bp PCR products from the human X and Y chromosomes,

respectively. Amelogenin-based sex tests are part of various PCR multiplex reaction kits from different manufacturers and are videly used for DNA typing of both reference and casework samples in the forensic field, especially for DNA databasing purposes. Reported cases of erroneous samples sexing based on gene *AMEL* [Steinlechner *et al.* 2002, Thangaraj *et al.* 2002] resulted from deletion in the sequence of *AMEL-Y* gene copy and in consequence from the lack of amplification of gene fragment specific for chromosome Y. In these cases the samples analysed were erroneously assigned to homogametic sex, *i.e.* women. The frequency of so-called „deleted-amelogenin males" was estimated to 0.018% [Steinlechner *et al.* 2002]. In cattle, any changes in sequence of *AMEL-Y* gene copy influencing sexing results have not hitherto been reported. In the present paper we discuss other aspects of possible problems that could arise in connection with new polymorphism discovered in gene *AMEL* in chromosome X. Namely, the phenotypic pattern of sex could be more complex since the presence of two variants for *AMEL* on chromosome X: *AMEL-X* (278 bp) and *AMEL-X* (269 bp) results in five genotypes possibly existing in cattle. Three concern cows: 278 bp/278 bp, 269 bp/269 bp and 269 bp/278 bp whilst two presumably exist in bulls: 215 bp/278 bp and 215 bp/269 bp. In this study we identified all possible genotypes with the exception of the genotype homozygous for 269 bp variant. Results presented are contrary to widely hitherto spread conviction that homogametic sex is always detected as "homozygous" phenotype. However, in heterogametic sex PCR products for X and Y chromosomes are of different length and thus the phenotype is "heterozygous", but besides normal pattern the occurrence of pattern with a new shorter variant is also possible. The problems with correct sex determination can arise particularly during analysing the old, degraded or contaminated samples when the risk of detection of unspecific products of length similar to specific PCR products is higher. Therefore, in questionable cases of testing unknown samples the need of result verification by direct sequencing of amplified PCR products could be necessary.

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Nowy wariant genu *AMEL-X* u bydła oraz jego implikacje w seksowaniu próbek biologicznych

S t r e s z c z e n i e

W genomie bydła występują dwie kopie genu amelogeniny, umiejscowione w chromosomach płci (*AMEL-X* i *AMEL-Y*). Obecność charakterystycznej delecji 63 pz w szóstym eksonie kopii *AMEL-Y* sprawia, że amplifikując wspomniany obszar sekwencji w reakcji PCR, można odróżnić płeć homogametyczną od heterogametycznej (odpowiednio jeden lub dwa sygnały detekcji dla produktów PCR).

Przebadano 625 kariotypowo normalnych krów (XX) i buhajów (XY) różnych ras z hodowli krajowej. Materiał żeński obejmował 150 krów rasy polskiej czerwonej, 72 krowy rasy białogrzbietej, 72 rasy czerwono-białej i 69 rasy czarno-białej z udziałem genów bydła hf. W grupie osobników męskich analizami objęto 12 buhajów rasy polskiej czerwonej, 30 rasy czerwono-białej, 125 rasy czarno-białej z udziałem genów hf, 67 czystorasowych buhajów hf, 7 buhajów różnych ras mięsnych, 6 buhajków białogrzbietych oraz 15 buhajków rasy polskiej czerwonej.

W kopii genu *AMEL-X*, u 24 krów i 2 buhajków rasy polskiej czerwonej zidentyfikowano obecność nowego wariantu charakteryzującego się delecją 9 pz. Wyniki dowodzą, że seksowanie próbek bydlęcych z wykorzystaniem polimorfizmu genu *AMEL* jest bardziej złożone niż dotychczas przyjmowano, gdyż u płci homogametycznej (XX) i heterogametycznej (XY) występować mogą odpowiednio – trzy i dwa różne fenotypy płci.