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Cytogenetic identification of *locus*-specific unstable trinucleotide repeats in selected *Bovidae* species and *Sus scrofa domestica*

Cytogenetyczna identyfikacja *locus* specyficznych niestabilnych powtórzeń trzynukleotydowych u wybranych gatunków *Bovidae* i *Sus scrofa domestica*

Summary. Applying *in situ* the PCR method and GTG/QFQ/DAPI banding techniques, the cytogenetic location of CCG-repeats of the human *FRM1* gene was assigned to structurally unstable heterosome X regions of cattle – BTA Xp13, sheep – OAR Xq22, goats – CHI Xq22 and domestic pig – SSC Xq26. The results obtained confirmed homology and conservation of regulatory sequence in 5'UTR region of mammalian *FRM1* gene, which can be a basis for further comparative, evolutionary and phylogenetic studies.

Key words: *Bovidae*, *Sus scrofa domestica*, chromosome X fragility, *FRM1* gene, trinucleotide tandem repeats

INTRODUCTION

Tandem repeat DNA tracts are ubiquitous feature of mammalian genomes however their evolution and functional properties need interspecies comparative studies. In humans, long, hyper-variable repeats are considered to be associated with genetic disorders affecting many biological processes. Highly polymorphic repeats in the coding sequence may be the cause of toxic proteins production, while non-coding repeats can have significant effects on chromosome fragility, gene silencing, transcription, translation, splicing modulation and cell architecture [Usdin 2008]. One of the examples may be the phenomenon of CGG repeat expansion within 5'UTR region of the human *FRM1* (frag-

ile X mental retardation 1) gene, followed by Xq27.3 chromosomal fragile site expression (fragile X syndrome), which leads to severe phenotypic changes related to neurodegeneration and ovarian insufficiency [Pearson *et al.* 2005, Hagerman 2006, Wittenberger *et al.* 2007]. *FRM1* gene codes protein most commonly found in the brain and is essential for normal cognitive development and female reproductive function. In humans, the CGG segment of the *FMR1* gene promoter region is repeated approximately 5–44 times and increased expression of expanded trinucleotide repeats is associated with developmental delays and other cognitive deficits. Expansions in the range of 55 to 200 repeats result in the premutation, while the full mutation ranges from 200 to several thousand repeats and results in the fragile X syndrome [Auer *et al*. 2006, Hagerman, 2006, Wittenberger *et al.* 2007]. Sequence analysis of the *FMR1* CGG repeats in mammals showed a high degree of length polymorphisms (in cattle and pigs 5-15 CGG alleles). In some species, including domestic pigs, CGG length exceeds the minimal length present in human gene that is prone to expand, which indicates that trinucleotide repeats may have a functional role in the harbouring genes (regulate gene expression and directly influence protein interaction properties or transcriptional level) [Pearson *et al.* 2005, Madsen *et al.* 2007, Usdin, 2008]. Homologous CGG region of this gene was identified in genomes of several farm animals but its chromosomal location need to be precisely assigned in further experiments [Deelen *et. al.* 1994].

The aim of this study was cytogenetic identification of *locus*-specific trinucleotide repeats in domestic *Bovids* and pig karyotypes basing on CCG-repeats within 5'UTR of the human *FRM1* gene.

MATERIAL AND METHODS

Cattle, sheep, goat and pig metaphase chromosome slides (from 10 animals of each species studied – totally 80 slides for hybridization experiments) were prepared following classical cytogenetic protocols of lymphocyte culture and banding techniques (GTG/QFQ/ DAPI). Karyotypes were arranged according to the international karyotype standards of the species under study [Gustavsson 1988, Di Berardino *et al.* 2001].

Primers complementary to the sequences flanking CCG-repeats within 5'UTR region of human *FRM1* gene (1.03 kb): 5' GGTTTCACTTCCGGTGGAGG (forward) and 5**'** CCATCTTCTCTTCAGCCCTGC (reverse) [Kremer *et al*. 1991] were used for amplifying and biotin-16 dUTP labeling of the homologous sequence directly on microscopic slides with metaphase spreads, according to the protocols published by Troyer *et al.* [1994] and Auer *et al.* [2006]. Briefly, PCR reactions were carried out in 10 ml volumes containing: 50 ng genomic DNA, 1 mmol/l of primers, 1.5 mmol/l MgCl2, 250 mmol/l of dATP, dCTP, dTTP and deazadGTP, 5% dimethyl sulfoxide and 2.5 U *Taq* DNA polymerase. *In situ* PCR cycling was performed in MJR PTC-100 thermocycler with metal heating block for glass slides as follows: 94°C for 5 min followed by 35 cycles 94°C for 30 s, 55°C for 30 s; 72°C for 30 s and final elongation at 72°C for 10 min.

The amplified and labeled CCG-repeat gene fragment was detected by avidin-conjugated FITC, and hybridization signals were analyzed in fluorescence microscope equipped with CDD camera and LUCIA software.

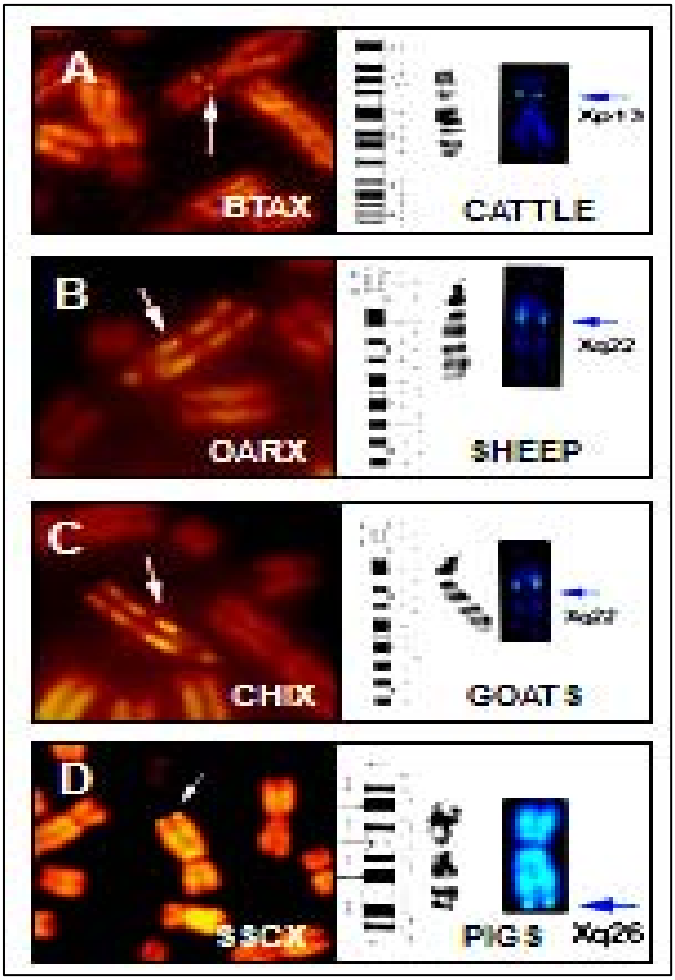

Fig. 1. Cytogenetic localization of CCG-repeats within 5'UTR region of *FRM1* gene in GTG/QFQ/DAPI banded karyotypes of selected *Bovidae* species and domestic pig: corresponding fluorescence signals in structurally unstable heterosome X regions of cattle – BTA Xp13 (A), sheep – OAR Xq22 (B), goats – CHI Xq22 (C) and pigs – SSC Xq26 (D)

Rys. 1. Cytogenetyczna lokalizacja powtórzeń CGG w obrębie regionu 5'UTR genu *FRM1* w barwionych metodą GTG/QFQ/DAPI kariotypach wybranych gatunków *Bovidae* oraz świni domowej: specyficzne sygnały fluorescencyjne w stukturalnie niestabilnych regionach heterosomu X bydła – BTA Xp13 (A), owiec – OAR Xq22 (B), kóz – CHI Xq22 (C) i świń – SSC Xq26 (D)

RESULTS AND DISCUSSION

Trinucleotide repeats in the form of (CCG)n occur relatively frequently within the genome and are inherently unstable, particularly prone to expansion during gametogenesis and/or embryogenesis. Mutations (depending on repeat compositions and flanking

sequence) affect chromosomal replication and gene expression or protein function. In humans, *locus*-specific polymorphic CCG-repeats (inserted/deleted) e.g. within *FRM1* gene sequence predispose to sever disease changes (neurological or leukemic) and reproduction inefficiency [Auer *et al.* 2006; Hagerman 2006, Wittenberger *et al.* 2007]. Taking into account the common phenomenon of mammalian CCG-repeats mutability, the cytogenetic localization of these repeats in genomes of domestic animals need to be strictly ascertained in order to extend comparative studies. Therefore, basing on combined GTG/QFQ/DAPI banding techniques and *in situ PCR* method with primers complementary to the sequences flanking CGG-repeats within 5'UTR region of human *FRM1* gene, corresponding *locus*-specific repeats were mapped to the structurally unstable chromosome X regions of cattle – BTA Xp13, sheep – OAR Xq22, goats – CHI Xq22 and pigs – Xq26 (Fig. 1A, 1B, 1C, 1D, respectively).

The results obtained supported the preliminary cytogenetic mapping of bovid X chromosome by FISH or in situ PCR methods which revealed homologies and divergences between the subfamilies Bovinae and Caprinae, suggesting transposition of common chromosome X segments during their karyotypic evolution [Iannuzzi *et al.* 2000, Kozubska-Sobocińska *et al.* 2002, 2007, Słota *et al.*, 2007a, Kozubska-Sobocińska 2009]. The experiments carried out confirmed provisional predictions about conservation of the FRM1 gene sequence and locus as well as equivalent unstable chromosome X regions in Bovids and pigs as well as more distantly related mammals, suggested by previous interspecies comparative analyses (including our preliminary studies by in situ PCR with oligonucleotide probes detecting slighter gene fragment due to short CGG-repeats flanking sequences) [Auer *et al.* 2006, Danielak-Czech and Słota 2006, Słota *et al.* 2007b, Kaczor *et al.* 2009, Danielak-Czech *et al.* 2010, 2011, 2012]. The findings reported in this paper proved especially strong sequence homology and evolutionary conservation of CCG tandem repeats within 5'UTR region of FRM1 gene in mammalian species, which can be evidence of their important regulatory role [Kremer *et al.* 1991, Deelen *et al.* 1994, Madsen *et al.* 2007].

Disclosure of specific trinucleotide repeat loci in farm animals' genomes (with tract lengths similar to that present in humans like FRM1) motivates future exploration of domestic Bovids or pigs as natural animal models to study tandem repeat variability and function.

CONCLUSIONS

1. The results obtained can be applied to ascertain the relationships between tandem repeat *loci* mutability, region-specific chromosome X instability and phenotypic effects in farm animals.

2. Our findings can be useful as a basis for further interspecies comparative analyses and broaden evolutionary and phylogenetic studies.

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S**treszczenie.** Stosując metodę *in situ* PCR oraz techniki prążkowe GTG/QFQ/DAPI, określono cytogenetyczną lokalizację powtórzeń CCG ludzkiego genu *FRM1* w strukturalnie niestabilnych regionach heterosomu X bydła – BTA Xp13, owiec – OAR Xq22, kóz – CHI Xq22 i świni domowej – SSC Xq26. Uzyskane wyniki potwierdziły homologię i konserwatyzm sekwencji regulatorowej w regionie 5'UTR genu *FRM1* u ssaków, co może stanowić podstawę do dalszych badań porównawczych, ewolucyjnych i filogenetycznych.

Słowa kluczowe: *Bovidae*, świnia domowa, łamliwy chromosom X, gen *FRM1*, tandemowe powtórzenia trzynukleotydowe