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Cytogenetic study of the *Bison bonasus*; I: Identification of heterochromatic regions and NORs in European bison karyotype and comparison with domestic cattle (*Bos taurus*)

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Abstract. The karyotypes of European bison *Bison bonasus* and domestic cattle *Bos taurus* are characterized by a diploid number of chromosomes, 2n = 60. Here, we characterized the European bison karyotype in terms of size and distribution of constitutive heterochromatin blocks (C-bands) and the location and number of nucleolar organizer regions (NORs), results were compared with those obtained for domestic cattle. For this purpose, staining for C and NOR bands was performed. In the chromosomes of both species, C-bands were located in the centromeric region of all chromosomes analyzed, except for the X chromosome. Active NORs in European bison chromosomes were identified in the chromosomes from pairs 2, 3, 4, 25, and 28. In cattle, NORs were located in chromosomes from pairs 2, 3, 4, 11, and 25. The average number of NORs in the cell of European bison and cattle was 4.47 ± 1.74 and 4.56 ± 1.66, respectively. The obtained results shed new light on the European bison cytogenetics and confirmed the high similarity between studied species.

Keywords: *Bison bonasus*, *Bos taurus*, heterochromatin, nucleolar organizer regions, nor, c-banding.

INTRODUCTION

The identification of homologous chromosomes is carried out on the basis of different banding techniques that reveal specific regions of the chromosome, for example constitutive heterochromatin (Sumner 1972) or Nucleolar Organizer Regions (NOR) (Goodpasture and Bloom 1975). The differentiated NOR sizes within a homology pair and the variability of constitutive heterochromatin blocks are treated as a chromosomal polymorphism, and variants that differ in NOR or C-bands are considered as chromosomal markers.

Constitutive heterochromatin is visible in the form of C-bands, is a late replicating fraction and contains a small number of genes (Lawce 2017).

Moreover, it was found that the DNA found in the constitutive heterochromatin blocks is a region that does not express genetic expression (Brown 1966). Methylation within CpG islands affects the significant level of condensation of constitutive heterochromatin (Spector 2003). Heterochromatin is also involved in the transcription and segregation of chromosomes (Grewal and Jia 2007).

Sipko et al. (2004) published a study in which they compared the karyotypes of *Bison bonasus* and *Bos taurus* by using the Sumner (1972) staining method of C-banding and SCE (sister chromatid exchange). They found no difference between the chromosomes of both species. The C-band method used by the authors showed that heterochromatic blocks were identified in all chromosomes except the X chromosome in both species.

The nucleolar organizer regions usually form secondary constrictions and are the localization site for genes encoding ribosomal nucleic acids (rRNA). NORs are organized as blocks of tandem repeating units whose distribution, i.e. the number of chromosomal loci and the number of genes in each locus, is a constant and characteristic for a given species (Weisenberger and Scheer 1995). Due to the affinity of NOR for heavy metals, nucleolar organizer regions appear in the form of black silver stripes during the dyeing process. Silver staining does not allow revealing all areas containing rRNA genes, but only active areas for nucleus formation in interphase (Verma and Babu 1995).

Graphodatsky et al. (1990) using classic staining method identified NORs on 2nd, 3rd, 4th and 28th chromosome pairs, while Gallagher et al. (1999) used *in situ* hybridization to identify NORs in *Bison bonasus* chromosomes and observed signals on chromosomes from pairs 2, 3, 11, 25, and 28.

Because of the limited amount of available literature, there is still much scope to discover and describe the cytogenetics of the species *Bison bonasus* also from the standpoint of separately maintained two lines of European bison: Lowland and Lowland-Caucasian (Pucek et al. 2004).

The aim of the study was to characterize the European bison karyotype in terms of size and distribution of constitutive heterochromatin blocks as well as the location and number of nucleolar organizer regions. It was decided to compare the karyotypes of the two studied species and confirm their similarity. Furthermore, we decided to confirm the location of NORs in the European bison. This is the first study in which the size of the heterochromatin area was measured in European bison.

MATERIALS AND METHODS

The experimental material consisted of 12 blood samples collected from *Bison bonasus* males from four localities (Figure 1, Table 1). The samples were stored in 9 ml heparinized tubes (Medlab Products) to prevent blood clotting and stored in cold until laboratory analysis. Blood from domestic cattle bull (*Bos taurus*) obtained in abattoir was used as a comparative material.

Cell culture

The cultures were carried out in 15 ml falcon tubes containing 8.5 ml of the culture medium RPMI 1640

Figure 1. Collection sites of European bison (*Bison bonasus*) samples in Poland. The numbering of sampling localities corresponds to the data in the Table 1.

Table 1. Collection sites of European bison (*Bison bonasus*) samples in Poland. The numbering of sampling localities corresponds to the Figure 1. SM – submetacentric, A – acrocentric.

Locality	Latitude, longitude	No. of specimens	2n	X	Y
Niepołomice	50°02'29.6"N, 20°21'54.1"E	3	60	SM	A
Gołuchów	51°51'35.2"N, 17°55'28.6"E	3	60	SM	A
Białowieża	52°42'20.0"N, 23°47'46.0"E	3	60	SM	A
Muczne	49°08'32.1"N, 22°42'50.5"E	3	60	SM	A

(SIGMA) with addition of 10% fetal bovine serum (SIGMA), pokeweed mitogen (SIGMA), and antibiotic (penicillin 100 µg/mL and streptomycin 100 µg/mL)(SIGMA). A thoroughly mixed blood sample was added to the final volume of 10 ml. The cultures were carried out in duplicate for each individual. The tubes were incubated at 38.5 °C for 72 hours and regularly mixed twice a day. The cultures were treated for 1 hour by colchicine (0.01 µg/mL) (SIGMA) (added at 71 hours). Subsequently, the cultures were treated with hypotonic solution (0.05 M KCl (POCH)) for 20 minutes at 38.5 °C, and then three times fixed in freshly prepared freezing cold Carnoy's fixative (POCH).

Microscope slide preparation

Slides were prepared before the staining procedures. The mixture of the fixed precipitate was spotted on a microscope slide and then air dried.

C-banding and AgNOR staining

Initially, the slides were subjected to the standard staining procedure described by Sumner (1972). The results obtained in the form of poorly visible C-bands were unsatisfactory; hence, we decided to use the methodology described by Chaves et al. (2000). Silver staining was carried out according to the method described by Howell and Black (1980).

Analysis of the slide preparations

By using a Nikon Eclipse 90i microscope connected to the DS5-U1 digital camera (Nikon Corporation, Tokyo, Japan), and a Zeiss Axiophot fluorescence microscope and LUCIA software (Laboratory Imaging Ltd, Prague, Czech Republic), 25 clearly visible and well dispersed metaphase plates were photographed for each male of both species. The measurements were made in the IMAGEJ program by using the LEVAN plugin (Wayne Rasband, Research Services Branch, National Institute of Mental Health, Bethesda, Maryland, USA, 2018). The obtained results were characterized statistically.

RESULTS AND DISCUSSION

The constitutive heterochromatin is a fraction which is most often located near the centromere of the chromosome, but it is also located in the distal parts of the chromosome arms, sometimes between the centromere and the telomeres or occupies the entire chromosomal arms (Lawce 2017). In our own research we measured the block size of constitutive heterochromatin in relation to the entire length of the arms of all analyzed chromosomes. The used procedure allowed us to determine the position of C-bands on European bison and domestic cattle chromosomes, and thus to identify sex chromosomes of both species (Figure 2A and 3A, respectively). The C-bands were located in the centromeric regions of all autosomes in both species and on Y chromosomes. In both species, no positive band was identified on X chromosomes. A positive, small, and dark band was found at the end of the short arm of the smallest chromosome of *Bison bonasus*, the Y chromosome. This chromosome is largely heterochromatic. Further, on the Y chromosome of domestic cattle, a dark and distinct positive band located at the end of the shorter arm was visible. By comparing the image obtained for the Y chromosome of European bison and domestic cattle, the difference in their morphology can be seen. The Y chromosome of domestic cattle is submetacentric, while that of the European bison is a small acrocentric as reported (Graphodatsky et al. 1990). Originally, the European bison's Y chromosome was considered submetacentric (Fedyk and Sysa 1971). In both species, 59 blocks of constitutive heterochromatin were identified on the 30 pair of chromosomes (Figure 2B and 3B).

In the analyzed acrocentric autosomes of European bison (Table 2) and domestic cattle (Table 3), a clear differentiation in the extent of heterochromatin areas was observed. In the measurement of constitutive heterochromatin block size in relation to the entire length of the European bison chromosome, the first pair of chromosomes showed the smallest mean value (14.52%), and the twelfth pair of chromosomes (26.40%) showed the highest value. In cattle, the first pair of chromosomes showed the smallest mean value (14.06%), while the twenty-third pair showed the highest value (32.50%). There were no positive blocks of constitutive heterochromatin on the X chromosomes in both species. The heterochromatin content of the Y chromosome of European bison and cattle was 39.15% and 28.95%, respectively. This indicates that the European bison's Y chromosome is much more heterochromatic. The variable length of heterochromatin blocks does not affect the phenotype, however it differentiates the morphology of the chromosomes of a given species. It has been found that the variable width of positive C-banding blocks indicates the presence of non-identical heterochromatin size (McFeely 1990).

The number of active NORs and their location in chromosomes is a characteristic of each species. In the

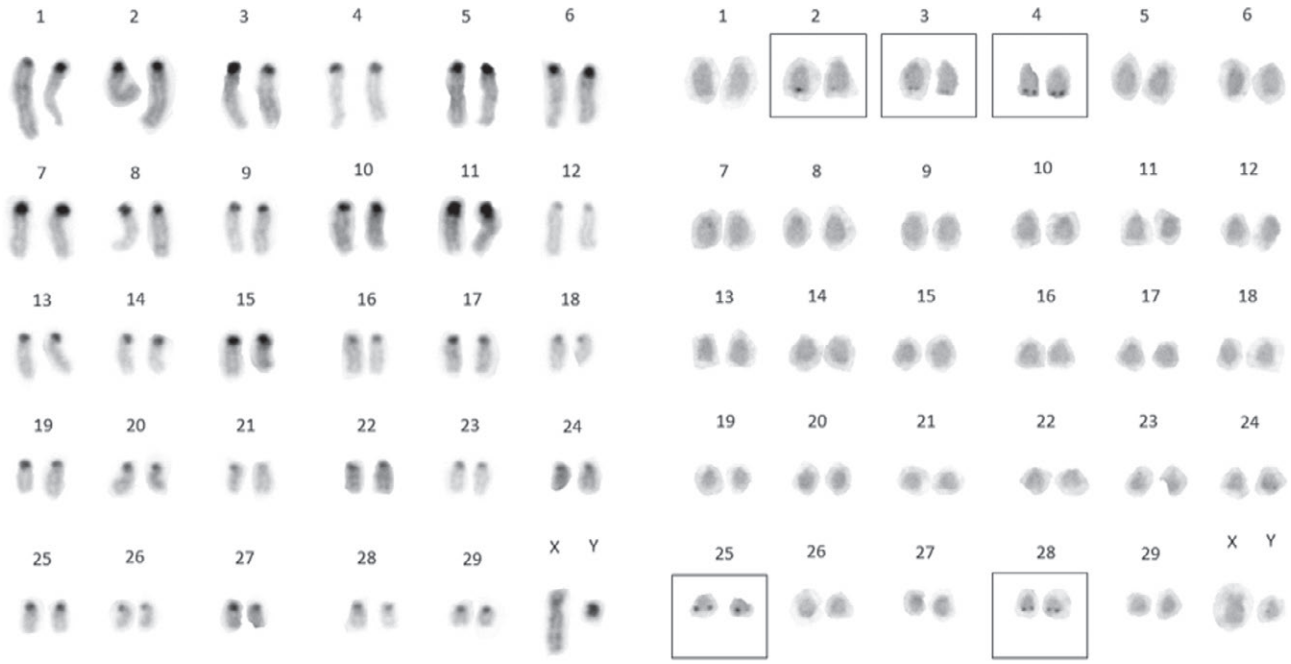

Figure 2. Karyogram of European bison male (*Bison bonasus*), (a) C-banding, (b) AgNOR-banding. Heterochromatic blocks are mainly located in centromeric positions of all autosomes and in sex chromosomes. NORs are located in 2, 3, 4, 25 and 28 chromosome pairs (marked in squares).

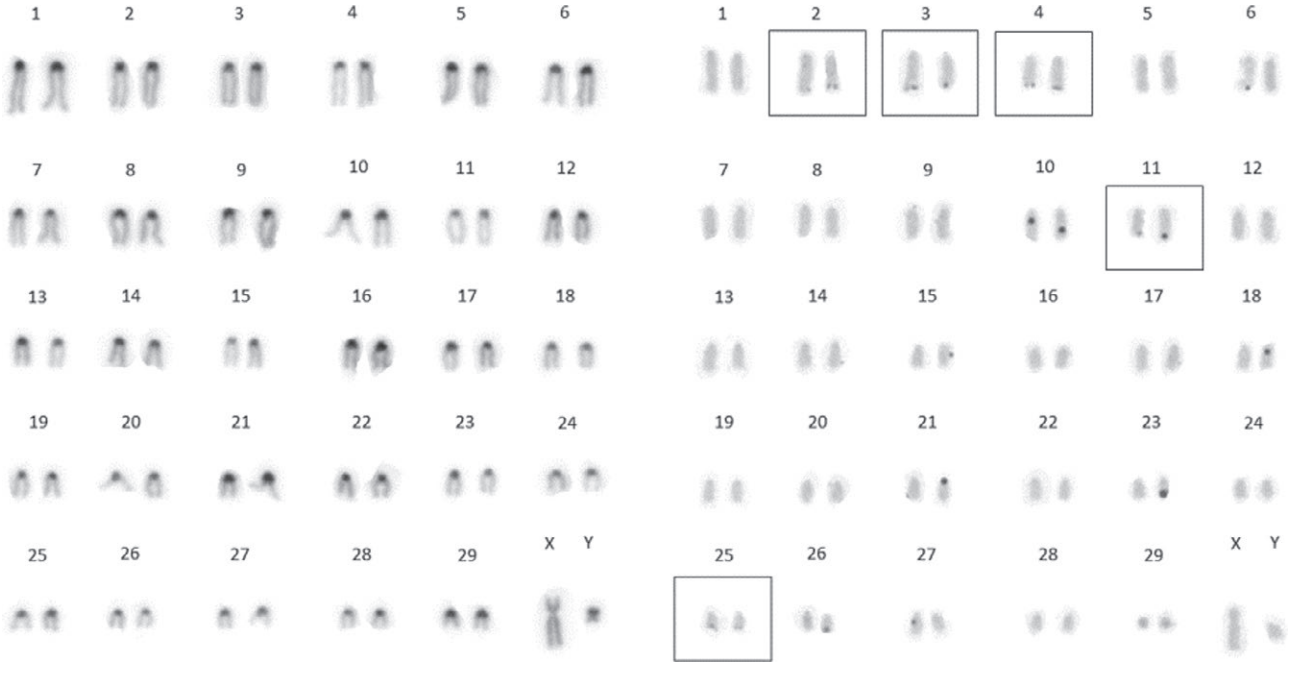

Figure 3. Karyogram of domestic cattle male (*Bos taurus*), (a) C-banding, (b) AgNOR-banding. Heterochromatic blocks are located in centromeric positions of all autosomes and in sex chromosomes. NORs are located in 2, 3, 4, 11 and 25 chromosome pairs (marked in squares).

metaphases of the same individual, there is an intercellular diversity of expression of NOR, which refers to the amount, size and intensity of silver staining (Weisenberger and Scheer 1995). The AgNOR staining enabled to identify active nucleating regions (NORs) found on the chromosomes of European bison and domestic cattle

Table 2. Size of constitutive heterochromatin blocks in European bison (*Bison bonasus*) chromosomes.

Chromosomes 1-14; XY	Average area of the constitutive heterochromatin $\bar{x} \pm S$	Chromosomes 15-29	Average area of the constitutive heterochromatin $\bar{x} \pm S$
1	14.52 ± 5.23	15	20.64 ± 2.24
2	16.49 ± 2.29	16	23.64 ± 12.46
3	17.78 ± 4.17	17	22.40 ± 0.89
4	16.05 ± 5.86	18	23.48 ± 1.68
5	25.06 ± 4.08	19	22.47 ± 0.70
6	23.95 ± 1.66	20	20.38 ± 1.84
7	16.79 ± 1.52	21	19.22 ± 1.77
8	18.80 ± 4.22	22	22.64 ± 3.04
9	21.33 ± 3.20	23	22.01 ± 7.13
10	23.68 ± 2.25	24	23.93 ± 2.16
11	18.12 ± 0.80	25	25.55 ± 3.41
12	26.40 ± 0.26	26	18.04 ± 6.04
13	17.18 ± 1.89	27	21.77 ± 3.64
14	21.14 ± 3.82	28	21.92 ± 2.21
X	-	29	21.11 ± 3.14
Y	39.15 ± 1.20		

Table 3. Size of constitutive heterochromatin blocks in cattle (*Bos taurus*) chromosomes.

Chromosomes 1-14; XY	Average area of the constitutive heterochromatin $\bar{x} \pm S$	Chromosomes 15-29	Average area of the constitutive heterochromatin $\bar{x} \pm S$
1	14.06 ± 1.60	15	19.95 ± 1.76
2	18.46 ± 3.15	16	18.39 ± 1.15
3	17.66 ± 1.40	17	23.11 ± 2.91
4	16.80 ± 2.51	18	25.19 ± 5.46
5	15.35 ± 1.87	19	25.51 ± 5.70
6	17.73 ± 3.63	20	26.69 ± 4.12
7	15.41 ± 4.47	21	32.15 ± 4.26
8	18.01 ± 1.13	22	30.98 ± 0.92
9	16.67 ± 2.53	23	32.50 ± 0.76
10	21.72 ± 4.86	24	21.47 ± 4.35
11	20.50 ± 3.35	25	27.57 ± 5.43
12	20.22 ± 2.44	26	25.54 ± 1.28
13	23.04 ± 3.21	27	25.77 ± 2.02
14	27.67 ± 2.80	28	24.56 ± 0.30
X	-	29	27.78 ± 6.29
Y	28.95 ± 9.34		

(Figure 2B and 3B, respectively). In European bison, the active nuclear regions were found in the terminal parts of chromosomes from pairs 2, 3, 4, 25, and 28 (Figure 2B). Similar conclusions were made by Gallagher et al. (1999) by using *in situ* hybridization for this purpose. In domestic cattle, visible silver grains were observed on chromosomes from pairs 2, 3, 4, 11, and 25 (Figure 3B). In a total of 325 metaphase plates analyzed (300 for European bison and 25 for domestic cattle) (Table 4), 1476 active nucleolar regions were observed (Table 5). There were visible differences in the intensity of NORs on chromosomes and the differences in the minimum and maximum number of active NORs in cells. At the cellular level, one to eight active NORs were observed in *Bison bonasus* species with an average value of 4.47 ± 1.75. In the *Bos taurus* species, it ranged from two to eight active nuclear regions with an average value of 4.56 ± 1.66. For comparison, in a similar experiment, the obtained active NORs average value for cattle was 6.06 (Mayr et al. 1987). The number of NORs in the cells of the animals varied and ranged from 107 to 115 within the individual. The most frequently observed cells had four active NORs, and the least frequently observed cells had eight.

Our research allowed us to characterize the European bison karyotype in terms of the C-band pattern and the number and location of active NORs. The following conclusions were made on the basis of the analyzes carried out. The number of active NORs per cell differed between the tested individuals and between the cells of the same individual. The method used in this study to identify C-bands can be successfully used to identify sex chromosomes. Constitutive heterochromatin has been identified in all European bison (*Bison bonasus*) and domestic cattle (*Bos taurus*) chromosomes, except for the X chromosome in both species. Chromosomes of both species can be compared on the basis of different classical staining methods, but a thorough analysis aimed at comparison of the Y chromosome requires more advanced analysis. The Y chromosome of European bison was smaller than domestic cattle Y chromosome, and classified as acrocentric. Studies on the comparison of the Y chromosome structure of *Bison* and *Bos* species can bring very interesting results. Potentially identified differences within the Y-chromosome-linked sequences will allow to design a quick and easy-to-use test that unambiguously indicates father's descent. In 1999, a genetic test was developed based on the sequence of the 16S rRNA subunit, which made it possible to detect the presence of cattle-specific mitochondrial sequences in individuals of the *Bison* genus (Ward et al. 1999). Unexpectedly, when performing the analysis

Table 4. Number of NORs in the chromosome pairs of examined animals. The number of active NORs analyzed in metaphases is given in brackets.

	Numbers of NOR Chromosome					
	2 $\bar{x} \pm S$	3 $\bar{x} \pm S$	4 $\bar{x} \pm S$	11 $\bar{x} \pm S$	25 $\bar{x} \pm S$	28 $\bar{x} \pm S$
Niepołomice	1.22 ± 0.43 (81)	1.17 ± 0.52 (72)	1.17 ± 0.39 (42)	-	1.59 ± 0.51 (81)	1.75 ± 0.45(84)
Gołuchów	1.47 ± 0.51 (75)	1.36 ± 0.50 (57)	1.40 ± 0.51 (63)	-	1.44 ± 0.51 (69)	1.47 ± 0.52 (66)
Białowieża	1.50 ± 0.51 (81)	1.51 ± 0.52 (63)	1.38 ± 0.50 (66)	-	1.63 ± 0.50 (78)	1.75 ± 0.45 (63)
Muczne	1.39 ± 0.50 (75)	1.46 ± 0.52 (57)	1.22 ± 0.43 (66)	-	1.40 ± 0.51 (63)	1.54 ± 0.52 (60)
Bos taurus	1.20 ±0.41 (24)	1.36 ± 0.50 (19)	1.24 ± 0.44 (26)	1.05 ± 0.22 (22)	1.21 ± 0.42 (23)	-
Total	1.36 ± 0.47 (336)	1.37 ± 0.51 (268)	1.28 ± 0.10 (263)	1.05 ± 0.22 (22)	1.45 ± 0.49 (314)	1.63 ± 0.49 (273)

Table 5. Number of NORs in the cells of the examined animals.

	Cell		
	n	$\bar{x} \pm S$	min-max
Niepołomice	360	4.60 ± 1.76	1-8
Gołuchów	330	4.40 ± 1.76	1-8
Białowieża	351	4.60 ± 1.83	1-8
Białowieża	321	4.28 ± 1.59	1-7
Bos taurus	114	4.56 ± 1.66	2-8
Total	1476	4.49 ± 1.72	1-8

using this test in our laboratory, it turned out that over 75% of the studied European bison population showed the presence of sequences characteristic of the *Bos* genus (Nowak et al. 2008). Examination of selected sequences of the Y chromosome would allow the scanning of the European bison population and show the potential differences, not only between domestic cattle and European bison, but also between the separately maintained European bison lines: Lowland and Lowland-Caucasian.

REFERENCES

Brown SW. 1966. Heterochromatin. Science, 151:417–425. doi: 10.1126/science.151.3709.417

Chaves R, Heslop-Harrison JS, Guedes-Pinto H. 2000. Centromeric heterochromatin in the cattle rob(1;29) translocation: α-satellite I sequences, in-situ MspI digestion patterns, chromomycin staining and c-bands. Chromosome Res. 8(7):621-626. doi: 10.1023/A:1009290125305

Fedyk S, Sysa P. 1971. Chromosomes of European bison, domestic cattle and their hybrids. Acta Theriol. 16(30):465-470.

Gallagher DS Jr, Davis SK, De Donato M, Burzlaff JD, Womack JE, Taylor JF, Kumamoto AT. 1999. A molecular cytogenetic analysis of the tribe Bovini (Artiodactyla: Bovidae: Bovinae) with an emphasis on sex chromosome morphology and NOR distribution. Chromosome Res. 7(6):481-492. doi: 10.1023/A:1009254014526

Goodpasture C, Bloom SE. 1975. Visualization of nucleolar organizer regions in mammalian chromosomes using silver staining. Chromosoma, 53(1): 37–50. doi: 10.1007/BF00329389

Graphodatsky AS, Sharshov AA, Biltueva LS, Popov VA. 1990. The karyotype of the bison (*Bison bonasus* L.). Tsitol Genet. 24(3):34-37.

Grewal SI, Jia S. 2007. Heterochromatin revisited. Nat. Rev. Genet. 8:35-46. doi: 10.1038/nrg2008

Howell WM, Black DA. 1980. Controlled silver-staining of nucleolus organizer regions with a protective colloidal developer: a 1-step method. Experientia, 36(8):1014-1015. doi: 10.1007/BF01953855

Lawce HJ. 2017. Chromosome stains. In: Arsham MS, Barch MJ, editors. The AGT Cytogenetics Laboratory Manual. 4th ed. Hoboken (NJ): The Association of Genetic Technologists. p. 213-300. doi: 10.1002/9781119061199

Mayr B, Schleger W, Auer H. 1987. Frequency of Ag-stained nucleolus organizer regions in the chromosomes of cattle. J. Hered. 78(3):206-207. doi: 10.1093/oxfordjournals.jhered.a110360

McFeely RA. 1990. Domestic Animal Cytogenetics. London, UK: Academic Press INC.

Nowak Z, Olech W. 2008. Verification of phylogenetic hypothesis concerning the evolution of genus Bison. Ann. Warsaw Univ. of Life Sci. - SGGW, Anim. Sci. 45:65-72.

Pucek ZP, Belousova IP, Krasińska M, Krasiński ZA, Olech W. 2004. European bison. Status survey and

conservation action plan. IUCN: Gland, Switzerland and Cambridge, UK.

Sipko TS. 2004. Comparative karyotypes of European bison and domestic cattle. In: Strelchenko NS, Andreeva LV, editors. Proceedings of the Conference "European Bison Conservation"; 30 September - 2 October; Białowieża, Poland: Mammal Research Institute PAS. p. 129-134.

Spector DL. 2003. The dynamics of chromosome organization and gene regulation. Annu. Rev. Biochem. 72:573-608. doi: 10.1146/annurev.biochem.72.121801.161724

Sumner AT. 1972. A simple technique for demonstrating centromeric heterochromatin. Exp. Cell Res. 75 (1):304-305. doi: 10.1016/0014-4827(72)90558-7

Verma RS, Babu A. 1995. Human Chromosomes: Principles and techniques. 2nd ed. New York (NY): McGraw-Hill, Inc. doi: 10.1002/mrd.1996.1080430105

Ward TJ, Bielawski JP, Davis SK, Templeton JW, Derr JN. 1999. Identification of domestic cattle hybrids in wild cattle and bison species: a general approach using mtDNA markers and the parametric bootstrap. Anim. Conserv. 2:51-57. doi: 10.1111/j.1469-1795.1999.tb00048.x

Weisenberger D, Scheer U. 1995. A possible mechanism for the inhibition of ribosomal RNA gene transcription during mitosis. J. Cell Biol. 129(3):561–575. doi: 10.1083/jcb.129.3.561