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Preliminary research to determine spontaneous instability in the Reeves's muntjac genome*

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Summary

Reeves's muntjac is one of the species of the Muntjac deer taxonomic group of Ruminantia which has a high karyotype variability in terms of structure, but above all in the number of chromosomes. The aim of the study was to determine the level of instability of cellular nucleus chromatin in this species using cytogenetic tests: comet assay (SCGE), sister chromatid exchange assay (SCE), micronucleus assay (MN) and fragile site assay (FS). The study was performed on a group of 7 individuals (Reeves's muntjac). All animals were 2-5 years of age and were in a good health condition. The study material consisted of peripheral whole blood lymphocytes (2.5 mL each animal). The analysis exhibited considerable diversification between levels of spontaneous damage to chromosomes and DNA in individual tests. The largest differences in the level of chromosome instability among individuals of Reeves's muntjac were noted in the comet test. The mean percentage of DNA in comet tail was 3.31 ± 0.43%. The comet test is the only method among the applied techniques of genome instability assessment which allows for exact determination of the degree of breaks directly in DNA strands. Other three tests used (SCE, CBMN and FS) also enable identification of damage to DNA strands but at a higher level of its organisation in the genome.

Keywords: Reeves's muntjac, spontaneous chromosome instability, DNA integrity, biomonitoring

Muntjacs (*Muntiacinae, Cervidae*) are a taxonomically diverse group of small ruminants comprising a range of nine species and characterised by variation in the number of diploid chromosomes ranging from 2n = 6 in female Indian muntjac (*Muntiacus muntjak vaginalis*) to 2n = 46 chromosomes in the Reeves's muntjac (*M. reevesi reevesi*) (30).

Reeves's muntjac has a wide distribution range, which is associated with a high level of tolerance and adaptation of these animals to variation of their habitat (31). This capacity for rapid environmental adaptation may be related to the high stability of the genome of muntjacs.

The karyotype of Reeves's muntjac consists of 22 pairs of acrocentric autosomal chromosomes and a pair of sex chromosomes: acrocentric X chromosome and a small metacentric Y chromosome. Cytogenetic studies on different muntjac deer species and their karyotype characterization focused primarily on karyotypic description using GTG, CBG, and Ag-NOR banding techniques in order to identify karyotype homology between the species (34). However, the data on the application of chromosomal instability tests, which describe the parameters of muntjac deer genome stability, are limited.

The chromatin nucleus and its integrity are among the most important parameters of genome stability and are crucial for its proper functioning. Broadly understood mutagenesis may negatively influence fundamental processes related to genome functions, e.g. gene expression or activity of DNA repair system (35).

Such disorders may take the form of single spontaneous damages, but under the influence of a toxic factor

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their number increases and they become mutagenic (16). Mutagenesis may be generated by chemical or physical factors, the source of which is, for example, pollution of the environment in which animals live.

One of the tools used to determine the genome instability level is cytogenetic analysis, in which the type and number of damages in nucleus chromatin at different organisation levels are observed. The parameters characteristic of genome instability are chromosome damages with two most important types of chromatid breaks: single-strand breaks (SSB) and double-strand breaks (DSB), as well as micronuclei occurrence. Damages at the DNA level are assessed based on the degree of integrity in interphase nuclei. The most important cytogenetic tests that allow for evaluation of genome instability are comet test, sister chromatid exchange, fragile sites, and micronucleus test (16).

In the presented research the hypothesis assumed applicability of cytogenetic tests in the first assessment of spontaneous level of genome instability in a selected species of *Muntiacinae*, *Cervidae*, namely Reeve's muntjac, using cytogenetic tests. The obtained results of preliminary research will be used in the future to expand knowledge on Reeve's muntjac genome and its link to health disorders of these animals.

The preliminary research was aimed at determining spontaneous genomic instability in the karyotype of Reeves's muntjac lymphocytes using cytogenetic methods, including comet assays, SCE, FS, and MN.

Material and methods

Material. Blood samples used for the study were collected from six male Reeves's muntjac (*Muntiacus reevesi*). All animals originated from a private breeding facility located in Southern Poland.

They all reached somatic maturity, they were 2-5 years of age and were in a good health condition. 2.5 mL of whole peripheral blood was collected from each animal as waste biological material obtained during slaughter performed as part of the research project approved by the Local Ethics Committee in Krakow, resolution No. 150/2018, in accordance with the legal regulations of the European Union.

Methods. For sister chromatid exchange (SCE) and fragile site (FS), full blood was used, whereas for micronuclei (MN) and comet studies, the lymphocyte fraction that was isolated on Histopaque 1077 (Sigma Aldrich).

Comet assay (SCGE, Single Cell Gel Electrophoresis). The comet test was carried out in an alkaline form according to the protocol of Singh et al. (26). The unfixed lymphocytes were mixed with 1% low melting point agarose (Sigma Aldrich) and distributed on 1% normal melting point agarose (Sigma Aldrich). The samples were then treated with a lysis buffer containing 2.5 M NaCl, 0.1 M $EDTANa_2$, 10 mM TRIS and 1% Triton X-100, pH = 10 (all from Sigma Aldrich) for 1 h at a temperature of 4°C. In the next step, samples were incubated for 20 minutes in a cold (4°C) electrophoresis buffer containing 30 mM NaOH (Sigma Aldrich), 2 mM $EDTANa_2$, pH = 12.5 (Sigma Aldrich) for traceless DNA, then electrophoresis was performed for 20 minutes at 0.6 V/cm. For the analysis, the microscopic slides were stained with 2.5 µg/ml of propidium iodide (Sigma Aldrich). For each animal, 100 comets were analysed. The percentage of DNA in the tail (% of DNA in the tail, TD%) and tail moment were used as end points of DNA damage. Tail moment was calculated as the percentage of DNA in the tail multiplied by the length of the tail-migration distance of DNA in the tail (26).

Cell cultures. Lymphocytes were cultured in Lympho Grow medium (Cytogen) with Pokeweed Mitogen (Sigma Aldrich) for 72 hours at 37.5°C and with constant humidity. To obtain mitotic chromosomes in SCE and FS tests, 1 µg/ml of colchicine was added in the 70th hour of culture. For SCE and FS tests, with the exception of the MN test and the comet test (SCGE), the cells were treated with a hypotonic solution (0.075% sodium citrate). Finally, all tests were fixed in Carnoy's solution (3 : 1 mixture of methanol and acetic acid, POCH). Next, the cell suspension was spotted on the slides and treated with different staining techniques (depending on the test).

Sister chromatid exchange assay (SCE). The sister chromatid exchange test procedure was performed according to the protocol proposed by Wolff and Perry (33), but with the modification proposed by Kuchta-Gładysz et al. (16). The modified procedure was as follows. After 24 hours of incubation, 1 µg/ml BrdU was added to the cells. Differential staining of sister chromatids was performed using FPG (Fluorescence plus Giemsa) technique (14). Briefly assuming: RNAs digestion, incubation in 0.5 × SSC solution (0.75 M sodium chloride with 0.075 sodium citrate; pH = 7.0) with Hoechst's solution, UV irradiation, incubation at 4°C in the dark, re-exposure to UV, incubation at 58°C in 0.5 × SSC solution and 3% Giemsa staining. For each individual, 20 differentially stained metaphases were evaluated and then the number of SCEs in the chromosomes and their location were determined.

Cytokinesis-Block Micronucleus assay (CBMN). The micronucleus test was carried out according to the method described by Słonina and Gasińska (27). In brief, in the 44th hour of lymphocyte culture, cytochalazine B at 5 µg/ml (Sigma Aldrich) was added to block cytokinesis. This protocol helps to recognize cells that have completed one nuclear division as binuclear cells (BN) and indicate micronuclei only in these BN cells (9). The lymphocytes were stained with Giemsa phosphate buffer, pH 6.8 (Sigma Aldrich). For each animal, the percentage share of lymphocytes binuclear (BN) in 1,000 lymphocytes and micronucleus (MN) in 500-900 BN lymphocytes were evaluated. Micronuclei were identified as morphologically identical to nuclei but smaller than them and separated from them, and the percentage of BN cells with at least one micronucleus (% BNC+MN) and the number of micronuclei per BN cell (MN/BNC) were determined as the evaluation parameter.

Fragile site assay (FS). The Wolff and Perry (33) protocol was used to determine the fragile sites in chromosomes. After 65 hours of cell incubation, the culture medium was supplemented with 50 µg/ml BrdU. Finally, microscopic slides were prepared and 4% Giemsa staining (Sigma Aldrich) was performed for 30 minutes. The analysis included 20 complete and well-dispersed metaphase plates

per animal. The identified chromosome damages had the form of chromatid breaks, gaps or deletions.

Microscopic analysis. The evaluation of slides and photographic documentation was performed under the ZEISS Image A2 microscope connected to the ZEISS AxioCam MRc5 digital camera. The tests were evaluated in the following programmes: Comet test in CASP 1.2.0 and MN, SCE and FRA tests in NIS-Elements ver. F2.31.

Statistical analysis. Due to the specificity of the present study in which a single Reeves's muntjac study group was monitored cytogenetically for the first time on a pilot basis, a statistical analysis was carried out based on the analogous work of Kuchta-Gładysz et al. (16). The results were statistically verified by one-way ANOVA where the significance of differences was tested using the Tukey test or non-parametric Kruskal-Wallis ANOVA test with Dunn's multiple comparisons. The significance of differences was tested using the Tukey test. Differences showing $p < 0.05$ were considered to be statistically significant. All results are expressed as means + standard error. The data were analysed using Statistica 13.0 software (TIBCO Software Inc., Palo Alto, USA).

Results and discussion

The presented study involved cytogenetic analyses performed in scheduled spontaneous chromosomal instability tests on material collected from six individuals, marked as M1 to M6. Not all test results were obtained for all the animals studied. The exact number of samples that were analysed using each test is presented in the latter part of the paper.

Tab. 1. Spontaneous chromosome instability – results of comet, SCE, MN and FS assays

Animals	Comet assay		SCE assay	MN assay	FS assay
	% Tail DNA	TM	Number of SCE	% BNC + 1 MN	Number of FS
	Mean ± SE	Mean ± SE	Mean ± SE	Mean ± SE	Mean ± SE
M1	8.23 ± 1.45^{A}	0.08 ± 0.01^{A}	2.91 ± 0.34^{A}	2.18 ± 0.81^{A}	0.86 ± 0.27^{A}
M2	0.42 ± 0.04^{BC}	0.07 ± 0.01^{AB}	2.03 ± 0.31^{AB}	1.63 ± 0.50^{A}	0.47 ± 0.12^{AB}
M3	10.13 ± 1.92^{AB}	0.06 ± 0.01^{BCDE}	–	4.56 ± 1.54^{A}	0.14 ± 0.08^{B}
M4	0.34 ± 0.04^{BC}	0.03 ± 0.00^{C}	4.00 ± 1.15^{AC}	2.18 ± 0.83^{A}	0.25 ± 0.16^{AB}
M5	0.27 ± 0.02^{C}	0.04 ± 0.00^{BCD}	7.73 ± 0.29^{BC}	2.88 ± 1.04^{A}	0.19 ± 0.15^{B}
M6	0.48 ± 0.05^{BC}	0.11 ± 0.01^{AE}	1.30 ± 0.42^{B}	4.81 ± 1.75^{A}	0.10 ± 0.08^{B}
Mean	3.31 ± 0.43	0.07 ± 0.01	2.07 ± 0.18	3.18 ± 0.53	0.35 ± 0.06

Explanations: A, B, C, D, E – mean values in the same column with a different letter differ significantly at $p < 0.05$

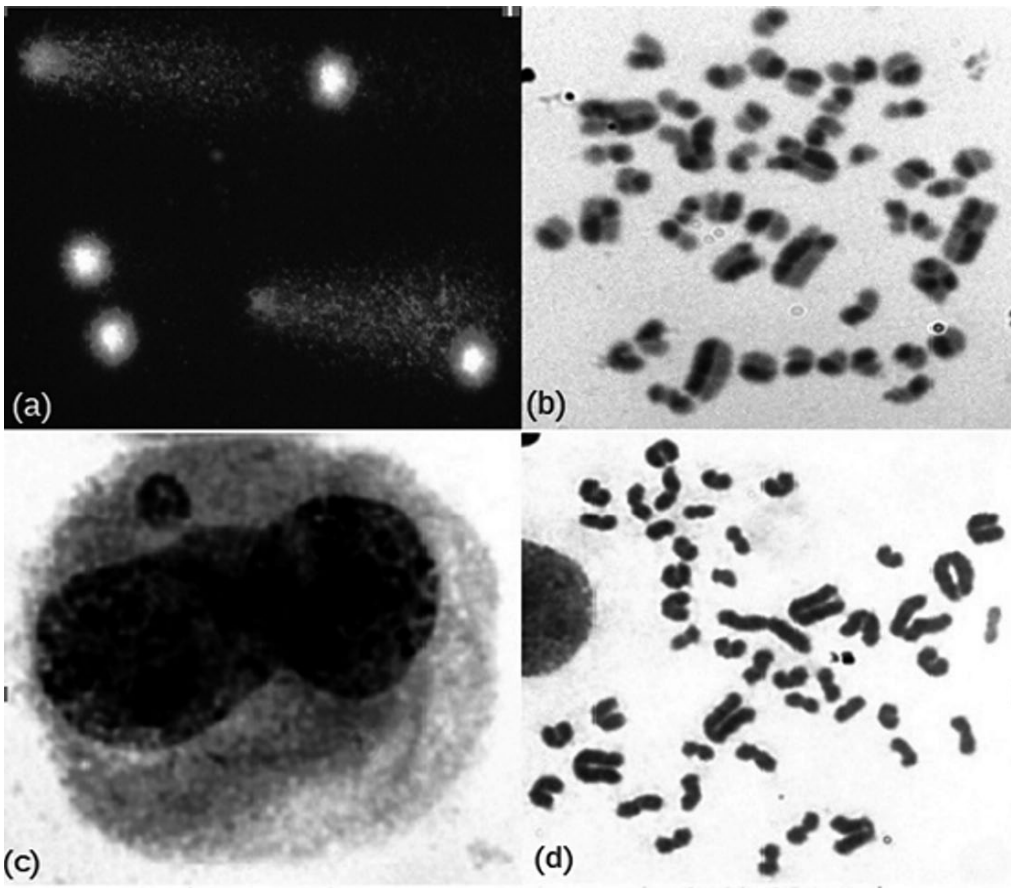

Fig. 1. Images of identified spontaneous damage and of the Reeveses's muntjac: (a) Comet assay, (b) SCE assay, (c) MN assay, (d) FS assay. Scale bar 10 µm

Comet assay, SCGE. DNA integrity disturbances were determined for the whole experimental group (M1-M6) of muntjac. The mean percentage of DNA in comet tail was 3.31 ± 0.43%. Based on measurements of the % DNA in tail, it was found that the large spontaneous damage occurred for M3 individual and it was 10.13 ± 1.92% Tail DNA (Tab. 1). The least spontaneously disturbed DNA integrity was found for M5 individual, with 0.27 ± 0.02% Tail DNA. Statistically significant differences of % Tail DNA were found for M2 and M3, M4 and M6 individuals. Additionally, the value of an auxiliary parameter in a comet test, i.e. tail moment (TM). It is calculated as a product of percentage DNA content in the comet tail and tail length as a unitless parameter. In the tested group of individuals, an average value of the parameter is defined as 0.07 ± 0.01 and in specific individuals, TM values were similar. Detailed data are presented in Table 1 and Figure 1a.

Sister chromatid exchange, SCE. The analysis of spontaneous chromosome instability based on SCE test was performed for five out of six examined individuals (without M3). It was shown that the SCE number was on average 2.07 ± 0.18, with the greatest number of sister chromatid exchanges observed for M5 individual (7.73 ± 0.29), and the lowest number for M6 individual (1.30 ± 0.42) (Tab. 1). Moreover, large statistical differences in the number of observed SCEs were found between the studied individuals. These differences were observed between M1 and M5 and M6, as well as between M4 and M6. Detailed results are presented in Table 1 and Figure 1b.

During the analysis of metaphases, qualitative assessment of SCE in terms of the place of their occurrence on chromosomes was carried out. The process of sister chromatid exchange was mainly terminal, with the mean SCE number equal to 1.46 ± 1.42, mainly at pair 1 chromosome in the area of secondary constriction. Most frequently, the exchange included the whole satellite segment below the secondary constriction in ch1, and only in some cases, its fragment was exchanged. SCE did not occur on both ch1 chromosomes each time. The largest number of terminal SCEs was found for M5 individual (2.86 ± 0.79), and the lowest for M6 (0.87 ± 1.29). Additionally, exchanges were located in the centromeric region (0.31 ± 0.73) and interstitially (0.21 ± 0.52).

Cytokinesis Block Micronucleus assay, CBMN. To expand the results with other forms of chromosome damage indicating increased chromatin spontaneous instability, a micronucleus test was performed (Tab. 1). The percentage of binuclear cells (BNCs) with micronuclei (MN) % BNC + MN was chosen as a parameter for defining material sensitivity. Generally, in BNC, single MN were observed most frequently, and their mean number was 3.18 ± 0.53% BNC + MN. The highest number was found for M3 individual (4.56 ± 1.54% BNC + MN) and the lowest for M2 individual (1.63 ± 0.50% BNC + MN). Statistical differences between the individuals were not found, details are presented in Table 1 and Figure 1c. Moreover, a percentage of nucleoplasmatic bridges (NPB) was observed with the mean occurrence rate of 2.63 ± 0.43, and with the highest number in M6 individual (4.27 ± 1.31). Moreover, the single presence of nuclear buds (BUDs) in BNCs was found in samples from two individuals.

Fragile site assay, FS. The test was performed using material from all six individuals. Chromosomes with breaks or chromosomes with torn p or q arms were mainly observed (Tab. 1). The analysis showed a slight involvement of these damages in the general spontaneous chromosome instability in the studied muntjacs, and the mean number of FS was 0.35 ± 0.06 per cell, with the largest number found for M1 individual (0.86 ± 0.27) and the lowest for M6 (0.10 ± 0.08) (Tab. 1). Statistical differences in FS numbers were observed between M1 and M3, M5, M6 (Tab. 1). Detailed data is presented in Table 1 and Figure 1d.

Cytogenetic tests are a useful and effective tool to define spontaneous genome instability and to monitor its growth as well as relation to health disorders in domesticated animals. An elevated level of chromosome instabilities identified using these methods implies various modifications of chromatin as a result of exogenous or endogenous factors which are genotoxic and which destabilise its structure. This instability generates further mutations, which may lead to developmental disorders, death of embryos, reproductive disorders, or may induce neoplastic transformations (4). Cytogenetic analysis allows for the determination of genome instability level by observation of types and number of nuclear chromatin damages at different organisational levels, such as disturbances of DNA integrity, sister chromatid exchange, micronucleus or fragile site (29).

A comet test is a sensitive method used to measure DNA damage in single cells (10). SCGE is most frequently used in humans, mainly to assess the impact of environmental factors, to diagnose patients with cancers and diseases resulting from disorders in DNA repair mechanisms (5). In animals, it is mainly used to examine the toxicity of selected factors of the breeding environment or to assess the quality of semen (11). The use of SCGE test in the study of spontaneous genome instability in various animal species has shown that the percentage of tail DNA is correlated with the degree of cellular nucleus DNA integrity disturbances, e.g. in sheep 2.72 ± 0.12% Tail DNA (1) and in young goats 0.75 ± 6.23 (3). Our research has shown that this parameter was at the level of 3.31 ± 0.43% Tail DNA in muntjacs. Our study enabled observation based on two parameters: percentage of tail DNA and tail moment. However, in general, % Tail DNA for all the animals was below 6%, which indicates genomic stability of this species.

The analysis of chromosome instability based on sister chromatid exchange (SCE) assay is used most often in research on mutagenic factors, including physical ones (X or UV radiation) or chemical ones (heavy metals, pharmaceuticals, crop protection chemicals, cosmetics, toxins of biological origin, nanoparticles or smog) influencing an animal genome. For example, by using SCE assay, it was possible to determine the mutagenic effect of chloramphenicol on chromosomes of cattle, swine, rat, buffalo, and also human (5). Moreover, an increase in chromosome instability can be related to development of cancers, Bloom syndrome or Down syndrome. The mean number of spontaneous and non-induced SCEs per cell was analysed in different animal species and it was at a level of 5.28 ± 2.51 to 7.9 ± 3.4 in cattle, 6.63 to 7.86 SCE in goats, 4.30 ± 1.28 to 5.96 ± 0.89 SCE in sheep and for buffalos 8.3 ± 1.21 to 13 ± 0.45 SCE (2, 12, 32). The frequency

of SCE on chromosomes of the Indian muntjac was studied on the basis of individual autosome pairs. The mean number of SCEs for pair 3 ranged from 1.43 to 2.6, while in the sex chromosomes the mean number was 1.16 SCE (chromosome X), and 0.67 SCE (chromosome Y) (25). Our research has shown that this parameter is approximately 2.11 ± 1.81 SCE per cell in the karyotype of Reeves's muntjac.

The authors have not come across any literature reports addressing the effects of BrdU on the Reeves's muntjac genome. The concentration of BrdU used as a marker in the SCE test has a measurable and sometimes additionally toxic effect on the number of SCEs in different species. In view of interspecific differences, the dose of the reagent should be determined individually for a given species, without adopting a universal standard. In studies conducted on primates, BrdU doses ranged from 10 to 500 μM BrdU/ml (17). In their study of domestic cattle, Leibenguht and Thiel (18) used BrdU at a concentration of 14-120 μg BrdU/ml of culture. Using sister chromatid exchange (SCE) analysis in crossbred (*Bos taurus* x *Bos indicus*) cattle, Murali and Panneerselvam (20) found that 10 μg BrdU/ml – a BrdU concentration selected from four different values (5, 10, 15 and 20 μg BrdU/ml) – was the best option for the study of spontaneous SCE. This value is recognised as a standard by most researchers, though it needs to be noted that the BrdU concentrations listed above are not the lowest levels allowing for the observation of SCEs (21, 22). Wilson and Thompson (31) emphasise that measurable spontaneous SCEs are those that occur only at very low BrdU concentrations, which should be used as an indicator in SCE tests for a given mutagenic factor. Among the available studies on spontaneous sister chromatid exchanges, the lowest concentrations were selected by Di Berardino et al. (7). Adopting the concentrations of 0.1, 0.25, 0.5, 1.0, and 5.0 μg BrdU/ml, they demonstrated that a positive SCE test result can be obtained in cattle at a BrdU concentration of 0.1-0.25 μg/ml of culture. In the authors' own studies conducted to determine the level of genome instability as a trait, the concentration of 1 μg/ml was used, in conformance with our own study modification. This BrdU level was sufficient to observe the positive effects of FPG staining and obtain SCE on chromosomes in the studies of Reeves's muntjac. Excessively low concentrations typically prevent SCE detection in chromosomes, while excessively high levels induce additional SCEs, thus falsifying the test result.

An alternative (with respect to SCE) method of analysing chromosome instability is the micronucleus (MN) assay and, similarly to SCE, it is used to study the influence of physical or chemical mutagens on genetic stability of karyotype in an animal cell. The CBMN assay is most often applied for the purposes of controlling acute mutagen doses or simply biomonitoring of various professional groups exposed to long-term radiation of chemical environmental pollution. This method is also broadly used in the pharmaceutical or cosmetic industry to determine toxicity of a given product. However, the MN assay is used most often in oncology with chemotherapy or in veterinary medicine (24). Mean spontaneous MN frequency in cells of healthy animal lymphocytes is usually 0.98 for goats (15), 2.4 for bovines or 3.5 for sheep (13). However, for MN frequency, the relative sensitivity of peripheral blood lymphocytes in cattle and swines was 0.86 and 0.41, respectively (15).

When evaluating the effect of bisphenol A on bovine lymphocytes, Šutiaková et al. (28) found that micronucleus frequency in the control group was at the level of 36.0 ± 5.66 MN/1000 BNC cells. The content of spontaneous micronuclei (MNs) as a form of chromosomal instability in the genome of these Reeves's muntjacs has not been previously investigated using the CBMN assay. In our research, % BNC + MN was 3.18 ± 0.53. It can be seen that for muntjac lymphocytes, the mean MN number obtained in this study was higher than the one indicated by other authors for various mammals, but for the same cell type. These data indicate differences in the sensitivity of cytogenetic tests analysing the same cell type even at the same level of chromatin organisation but in different species. This is why a species-specific determination of the spontaneity of chromosomal instability is so important.

The analysis of chromosome changes in number and structure due to breaking and the assessment of chromatin proneness to exo- or endogenous factor-induced damage can be achieved by the use of Fragile Site (FS) assay. This assay (FS) allows for the assessment of chromosome sensitivity in the breaking points by forming breaks or fractures in chromatids on chromosomes. This method is applied not only in medicine, but also in animal breeding. Such research was conducted by Danielak-Czech and Słota (6) on four farm animal species (including cattle and sheep) and by Perucatti et al. (23) on sheep. Also, in studies of four breeds of sheep, the number of fragile sites on chromosomes was determined to vary from 2.13 ± 0.78 to 4.34 ± 0.94/cell (32), and in goats it was shown to be equal to 1.5 ± 0.4% cells with FS (19). In their studies evaluating the effects of dioxins on blood cells in cattle, Di Meo et al. (8) obtained a total mean number of FS equal to 0.3 ± 10.91/cell in the control group. In contrast, a diagnostic study conducted by Peretti et al. (22) on cattle revealed the total mean number of FS in the control group to be 1.82 ± 2.03/cell. Spontaneous chromosomal FS levels have not been investigated in Reeves' muntjac earlier. In the presented studies, an FS test was used and it showed that the average number of FS/cells was 0.35 ± 0.06 for this species, which indicated the genetic stability of chromatin in this species.

In the presented research, the level of spontaneous chromosome instability in lymphocytes of Reeves's muntjac was assessed using cytogenetic methods such as comet test, SCE, MN and FS. When testing

the karyotype of Reeves's muntjac, the above cytogenetic tests were used for the first time. The analysis exhibited considerable diversification between levels of spontaneous damage to chromosomes and DNA in individual tests. The greatest differences in the level of chromosome instability among individuals of Reeves's muntjac were noted in the comet test. Diversified values of % Tail DNA in the examined group might have resulted from high sensitivity of the method and specific characteristics of detected damage. The comet test is the only method among the applied techniques of genome instability assessment which allows for exact determination of the degree of breaks directly in DNA strands. Other three tests used (SCE, CBMN and FS) also enable identification of damage to DNA strands but at a higher level of its organisation in the genome. The above analysis of spontaneous chromosome instability is a pilot study and requires verification on a larger group of animals, taking into account diversified research groups. But the use of the foregoing methods in environmental biomonitoring may be a useful tool in the assessment of genome damage.

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