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GENETIC STRUCTURE OF THE POPULATION OF PRZEWALSKI'S HORSE (*EQUUS PRZEWALSKII*) ACCORDING TO CYTOGENETIC AND ISSR MARKERS

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Background. Przewalski's horse is included in the Red List of the International Union for Conservation of Nature and the Red Data Book of Ukraine as an endangered species. To confirm the uniqueness and consolidation of rare animal species, cytogenetic and molecular genetic monitoring is necessary. Obtaining biological material (blood) for genetic research is preceded by immobilization of wild ungulates. The successful selection of drugs for the purpose of sedation and analgesia helps to preserve the life and health of the animal.

Materials and Methods. Przewalski's wild horse population (10 heads) of the F. E. Falz-Fein "Askania-Nova" biosphere reserve, immobilization of animals with the Madison drug and the Reverson antidote, cytogenetic and molecular genetic (ISSR-fingerprinting) analysis.

Results. The effectiveness indicators of doses of Madison and Reverson were: in horses m = 200 kg – a dose of Madison 20 mL/head, immobilization after 22 min, in horses m = 300 kg, a dose of Madison – 25 mL/head, immobilization in 20–22 min. The Reverson antidote was applied in the following doses: animal m = 200 kg – a dose of Reverson 5–15 mL/goal, cessation of the sedative effect – 12 min; animal m = 300 kg –

a dose of Reverson 5–15 mL/goal, cessation of the sedative effect – 18 min. Observation of the effect of the drugs did not reveal any negative side effects. Cytogenetic analysis determined the karyotype norm of somatic cells with 2n = 66 chromosomes. Genomic disorders, aneuploidy, accounted for 6.7%, polyploidy – 1.3 %. Structural violations (chromosomal and chromatid breaks) were not detected. The results of the micronucleus test: the share of lymphocytes with a micronucleus – 3.0 ‰, binuclear lymphocytes – 2.3 ‰, mitotic index – 7.7 ‰. Genetic indicators of the population of Przewalski's horses according to ISSR markers: when using $(GA)_9C$ as a primer, microsatellite repeats of polymorphic loci were not found, and according to primer $(GAG)_6C$ – 50 % of polymorphic loci. The main indicators of genetic diversity, with the help of ISSR markers: the share of polymorphic loci was 25 %, the average gene diversity per locus – 0.39, the Shannon–Wiener information index – 2.5.

Conclusions. No negative side effects occur when Madison and Reverson drugs are used to immobilize Przewalski's horses. According to the results of cytogenetic analysis, the stability of the karyotype of the studied animals was established. The study of genetic polymorphism of the horse population by ISSR markers (AG)9C and (GAG)6C indicates a high degree of genetic consolidation. All tested animals are relatively safe according to the revealed intra-population genetic diversity.

Keywords: Przewalski's horse, immobilization of horses, cytogenetic analysis, ISSR markers, intra-population diversity

INTRODUCTION

The last species of wild horses has survived to this day in the Dhungar desert of Central Asia. Here, in 1879, a wild horse was found and described by the famous traveler Mykola Przewalskii, and because of that, the Dzungarian wild horse has the scientific name of Przewalski's horse (*Equus przewalskii*) (Vasyliuk *et al.,* 2020a).

In Ukraine, the first refuge of Przewalski's horse was the Askania-Nova Biosphere Reserve. These animals occupy a special place in the history of the formation of the animal collection in the Zoological Park "Askania-Nova". The former owner of "Chapel" – the old Ukrainian name of the state reserve of geographical origin (Vasyliuk *et al.,* 2020b), "Faltz-Fein organized special expeditions to Dzungaria, spent a lot of money on this, used all kinds of measures until he was lucky enough to get live Dzungarians horses and settle them in Chaply" (Vasyliuk *et al.,* 2020b). It was for the first time in the world that these wild horses were introduced to Europe, namely to Askania-Nova. The Przewalski's horse is included in the Red List of the International Union for Conservation of Nature (IUCN) as an endangered species, category: IUCN Red List of Endangered Species (EN) (King *et al.,* 2015). At the beginning of the 90s of the previous century, Przewalski's horses were released into the Ukrainian exclusion zone of the Chernobyl nuclear power plant as an experiment, where they began to actively reproduce. Now, there are about 120–140 of these animals, specifically three herds, in this area. The core of these herds consists of 28 horses taken from the Askania-Nova reserve and released into the wild as part of the "Fauna" program conducted by the Institute of Zoology of the National Academy of Sciences of Ukraine (Akimov, 2009; Gashchak, 2018). Due to this, in 2009, the wild horse was included in the Red Data Book of Ukraine. Preservation of the only wild horse species on our planet, the Przewalski's horse, requires scientists to conduct further studies and restore the gene pool of these animals.

The purpose of our work was to study the effect of Madison drugs and the Reverson antidote on the body of horses when used to immobilize them during the collection of biological material and to study the genetic variability of the population of wild Przewalski's horses using molecular genetic and cytogenetic methods.

MATERIALS AND METHODS

Immobilization of horses. To study the genetic variability of the population of wild horses of Przewalski (10 heads) of the biosphere reserve "Askania-Nova" named after F. E. Falz-Fein, for the first time immobilization of wild ungulates was carried out with the help of Madison drugs and the Reverson antidote, which is a type of live trapping method (**Fig. 1**). Horses from the steppe selected for research and manipulation were driven into a large enclosure and kept without food for 12 hours. A few hours before the start of immobilization, the water supply was also stopped. One animal at a time was driven from a large enclosure into a small enclosure. To immobilize the horses, a solution for injections was applied – Madison (Medison, 2017), using a plastic syringe that resembles an arrow, 15 cm long, with a volume of 2 mL. The syringe was filled with a solution from the side of the needle, and air was injected from the side of the shank through the valve. So, the piston was under pressure. The filled syringe was inserted into a plastic tube. For an aimed shot, the tube was brought to the lips and during an energetic exhalation, the syringe hit the thigh of the animal. The liquid got into the muscle tissue, the injection was gentle, the animal was not injured. After the administration of the drug, horses were initially partially immobilized. To prevent a sharp fall of the animal, which could lead to injury, a string was put around the horse's neck and the animal was brought to a sufficiently soft litter prepared in advance so that it could lie down there. A completely immobilized animal was approached quietly, without noise, then its head was raised and covered with a dark blanket to reduce the effect of external stimuli. During the complete immobilization of the animal, which lasted 20–30 min, marking, biometric studies, cleaning of hooves and collection of biological material for genetic studies (blood from the jugular vein, hair from the mane and tail) were carried out. After all planned work was carried out promptly, the Reverson antidote was administered to the animal (Reverson, 2020) to stop the sedative effect of Madison. The owner of the registration certificate and the manufacturer of the finished products of the Madison drug and the Reverson antidote is LLC "Brovafarma" of Brovary, Kyiv region.

To carry out cytogenetic and molecular genetic studies of Przewalski's horses, peripheral blood was collected from the jugular vein.

Cytogenetic studies. Somatic cell culture samples (blood) were used to analyze karyotype disorders. The culture was carried out in sterile vials, packed with RPMI-1640 medium (Sigma, USA) of approximately 5 mL per vial, 1 mL of bovine blood serum (preferably embryonic), an antibiotic at the rate of 0.001 mL of gentamicin per 1 mL of medium, 0,5 ml of whole blood, mitogen phytohemagglutinin – a substance that stimulates the mitotic division of lymphocytes in culture, type P in a dose of 0.02 mL per 10 mL of culture mixture. The mixture was cultivated in a thermostat at a temperature of +37 °C for 48 hours, the vials being periodically shaken. Two hours before fixation, a colchicine solution heated to 37 °C was injected into the culture at a final concentration of 0.3–0.5 μg/mL of the culture medium. A freshly prepared 0.55 % potassium chloride solution was used for hypotonization. Hypotonization was carried out for 20 min in a thermostat at a temperature of +37 °C. After the end of hypotonization, the culture was

centrifuged, the supernatant liquid was poured off, and a fixing liquid cooled to +4 °C was added to the sediment, mixing one part of glacial acetic acid with three parts of methyl alcohol. After that, the sediment was resuspended and centrifuged, this operation was repeated 2–3 times. The cell suspension was applied to clean, cooled glass slides using an automatic dispenser. The glass was dried in the air. The obtained preparations, after being stained with a ready-made Giemsa dye, were analyzed for chromosomal variability under the immersion magnification of an Axiostar plus microscope (Carl Zeiss, Germany) by 1000 times and photographed (Burkat, 2005).

Fig. 1. Immobilization of Przewalski's horses: ***A*** – population of wild horses of Przewalski Biosphere Reserve "Askania-Nova" named after F. E. Falz-Fein; ***B*** – administration of the Madison drug to immobilize the animal; ***C*** – prevention of a sharp fall of a horse; ***D*** – complete immobilization of the animal

The number of binuclear lymphocytes, mononuclear lymphocytes with micronuclei, and the mitotic index were counted on the same preparations. The frequency of binucleated lymphocytes, lymphocytes with a micronucleus, and the mitotic index were calculated in parts per million (‰), the number per 1000 cells.

Molecular genetic studies. To confirm the uniqueness and consolidation of the population of Przewalski's horses, the genetic polymorphism of the studied horses was evaluated using molecular diagnostics (ISSR-fingerprinting). ISSR-PCR labeling is effectively used for interspecies genetic variability, for taxonomic and phylogenetic comparisons and as a means of mapping a wide range of organisms.

To study DNA polymorphism of horses using ISSR markers, optimization of the temperature regimes for the polymerase chain reaction was carried out (**Table 1**).

Table 1. **Primer sequences and annealing temperature regimes used in the study**

Primer	Nucleotide sequence (5′–3′)	Amplification conditions
$(AG)_9C$	AGAGAGAGAGAGAGAGAGC	94 °C – 7min; 94 °C – 30 sec, 58 °C, 72 °C – 2 min, 32 cycles; 72 °C – 7 min
$(GAG)_6C$	GAGGAGGAGGAGGAGGAGC	94 °C – 7min; 94 °C – 30 sec, 60 °C, 72 °C – 2 min, 32 cycles; 72 °C – 7 min

Polymerase chain reaction (PCR) was run in a Thermal Cycler amplifier (Applied Biosystems, USA). A PCR cocktail consisted of 2 μL of buffer for DNA polymerase, 1 μL of triphosphate mixture (Amplisens), 0.8 μL of the appropriate primer, 0.2 μL of DNA polymerase (Fermentas, Lithuania). Genomic DNA was added in the amount of 1.2 μL (25 ng). The total volume of the PCR mixture was adjusted to 10 μL of ddH_2O (Zietkiewicz *et al.*, 1994; Mokhnachova, 2022).

After that PCR fragments were analyzed by electrophoresis on 2 % agarose/1X TBE gel stained with ethidium bromide. Differentiation of amplicons by size was carried out with the help of a molecular size marker of Gene Ruler 1 kb Plus DNA Ladder (Thermo Scientific™). The bands were visualized under UV light and the gels were photographed using Camera.

Each spectrum amplicon was considered as one DNA locus. The polymorphism of such a locus (P) was assessed by the presence or absence of an amplicon of the corresponding length in the spectra. Parameters of genetic diversity were determined using the Microsoft Excel computer program.

Bioethical norms. Experiments on animals were carried out in compliance with the requirements of the Law of Ukraine “On the Protection of Animals from Cruelty Treatment” (Article 230 of 2006), “General Ethical Experiments on Animals”, approved by the National Congress on Bioethics and agreed with the provisions of the “European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes” (Strasbourg, 1986), permission for the study was obtained from the Commission on the treatment of animals in scientific research Institute of Animal Breeding and Genetics nd. a. M. V. Zubtsya NAAS (protocol No. 2 of 28 February 2024).

RESULTS AND DISCUSSION

Immobilization of horses. In order to conduct scientific research, especially in large animals without killing or injuring them, it is important to improve the method of their immobilization. Therefore, for a more effective implementation of the immobilization process, the Przewalski’s horses under study were divided into two groups, depending on the animal’s body weight (200 and 300 kg) (**Table 2**). For sedation and analgesia during diagnostic manipulations and the collection of biological material (blood) for scientific research of these animals, for the first time in the biosphere reserve “Askania-Nova” named after F. E. Falz-Fein, the Madison drug, article number 000014808, and the Reverson antidote, article number 000016832 were used. Madison is a relatively new drug characterized by a prolonged action.

Table 2. **Effectiveness indicators of the actual doses of the "Madison" and "Reverson" drugs applied to Przewalski's horses using a remote method of administration**

Animal group	Actual body weight (kg)	Number of injections/time interval	Dose applied (mL/head)	Time to visible effect (min) complete immobilization/ceasing of sedation
Madison				
1	within 200 kg	2/5 min	20 mL	22 min
2	within 300 kg	2/5 min	25 mL	20–22 min
Reverson				
1	within 200 kg	1	20 mL	12 min
2	within 300 kg	1	25 mL	18 min

During the use of Madison and Reverson, no side effects, such as impaired coordination of movements, increased sweating, vomiting, slight tremor of individual muscles or muscle groups, or uncontrolled urination were observed in animals. The expediency of using Madison and Reverson drugs to immobilize horses is that when using them, the veterinarian can control the duration of the sedation and analgesia process, and the effect of this drug is prolonged.

Analysis of the karyotype of the studied horses. One of the principles of establishing the qualitative uniqueness of rare species of animals and confirming the uniqueness and consolidation of animal populations as a basis for their inclusion in national programs for the conservation of biological resources is genetic monitoring. To determine the genetic specificity at the chromosomal level, the karyotype of animals is analyzed.

An analysis of the karyotype of Przewalski's horses (n = 10 heads) of the Munich line, in which there is no admixture of domestic horse blood, was carried out. According to the results of the study, it was established that the norm of the karyotype of the somatic cells of these animals is 2n = 66 chromosomes, while domestic horses have 2n = 64 chromosomes. Cytogenetic screening of the studied horses showed the absence of constitutive abnormalities, in particular, Robertsonian and other autosomal translocations (Chosh *et al.,* 2020). (**Table 3**, **Fig. 2**).

Table 3. **Analysis of karyotype variability of the Przewalski horse population**

Cytogenetic indicators	Aneuploidy (%)	Polyploidy (%)	Chromosomal breaks (%)
M±m	6.7±1.60	1.3±1.00	–

Genomic disorders of chromosomes, aneuploidy, was expressed mainly by hypoploid cells (2n = 56–65), with the range of this variability from 0 to 10 % (Shilton *et al.,* 2020). A multiple increase in the number of chromosomes, polyploidy, was within 0–5 % and did not exceed the level of spontaneous chromosomal variability characteristic of domestic horses (Starodub, 2023). Structural violations of chromosomes (chromosome and chromatid breaks) were not detected. The absence of these violations indicates the stability of the genotype of the studied animals and the absence of genotoxic effects.

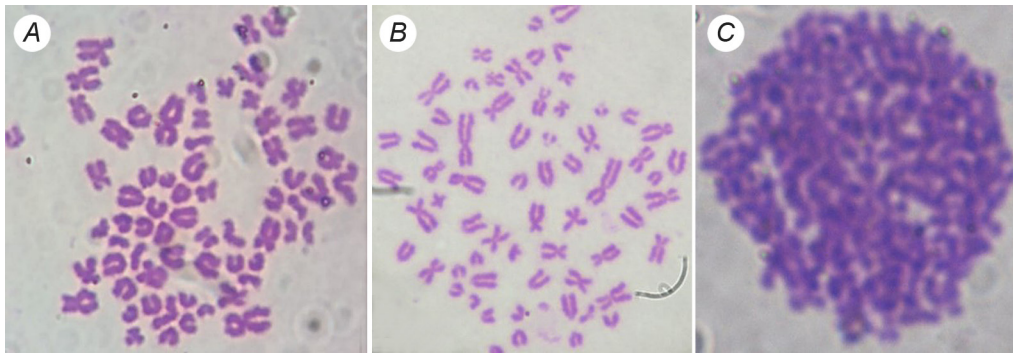

Fig. 2. Karyotypic variability of the Przewalski horse population: ***A*** – karyotype norm-2n = 66; ***B*** – aneuploidy-2n = 62; ***C*** – polyploidy-2n = 132; magnification×1000 times

The micronucleus test is an effective biomarker of diseases and processes associated with the induction of DNA damage. To establish the course of these processes in the studied horses, we conducted a micronucleus test. The results obtained showed that the proportion of lymphocytes with a micronucleus, binucleate lymphocytes, and the mitotic index did not exceed the spontaneous level of cytogenetic parameters of mammalian cells (**Table 4**, **Fig. 3**) (Lanovenko, 2020).

Table 4. **Results of the micronucleus test of the Przewalskii horse population, Biosphere Reserve "Askania-Nova" named after F. E. Falz-Fein, for spontaneous mutagenesis**

Cytogenetic parameters of cells	Lymphocyte with a micronucleus (‰)	Binuclear lymphocyte (‰)	Mitotic index, (‰)
	3.0±0.82	2.3±1.12	7.7±3.12

The ratio between the number of binucleated lymphocytes (2.3 ‰) and the mitotic index (7.7 ‰) indicates the absence of cytokinetic blocking during cell division. It is known that there is a high correlation between the state of the immune system and cytogenetic status of cells (r = 0.95–0.96) (Lanovenko, 2020; Bonassi & Fenech, 2021). So, our research confirms the high stability of the immune system of Przewalski's horses.

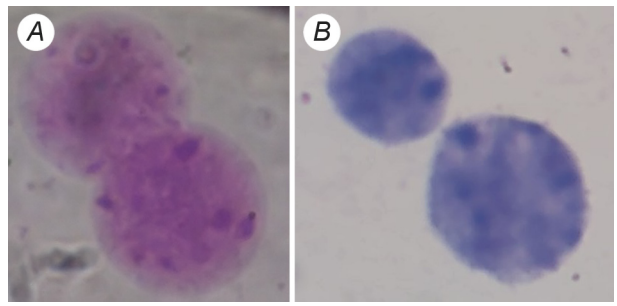

Fig. 3. ***A*** – binuclear lymphocyte; ***B*** – a lymphocyte with a micronucleus collected ×1000 times

Genetic polymorphism of the Przewalski horse population by ISSR markers. In order to reveal the variability of polymorphism of DNA fragments flanked by inverted

repeats of microsatellite loci in Przewalski's horses, studies of polylocus spectra were conducted (**Table 5**, **Fig. 4**).

Table 5. **Genetic parameters of the population of Przewalski's horses according to ISSR markers**

Primers	Number of loci	Range of nucleotide pairs	Polymorphic loci	Index of polymorphic information content	Percentage of polymorphic loci (%)
$(GA)_9C$	4	300–590	0	0	0
$(GAG)_6C$	6	300–1050	3	0.19	50
∑	10	300–1050	3	0.09	25

In the spectrum of amplification products using dinucleotide microsatellite repeats with anchor nucleotides $(GA)_9C$ as a primer, the following results were obtained: 4 loci were detected with complete absence of polymorphism of amplification products. The length limits of these loci were 300–310, 380–400, 500–520, 0–590 nucleotide pairs (bp).

When using the trinucleotide $(GAG)_6C$ ISSR-PCR marker, 6 loci were found, three of which were polymorphic. The length limits of conservative loci were 380–400, 500–520, 680–710 bp; of polymorphic ones – 300–310, 820–870, 1000–1050 bp. The polymorphic information content (PIC) index was 0.19, and the percentage of polymorphic loci was 50 %.

Therefore, when microsatellite repeats were used as a primer $(GA)_9C$, no polymorphic loci were found, while with $(GAG)_6C$ primer – 50 % of polymorphic loci were detected.

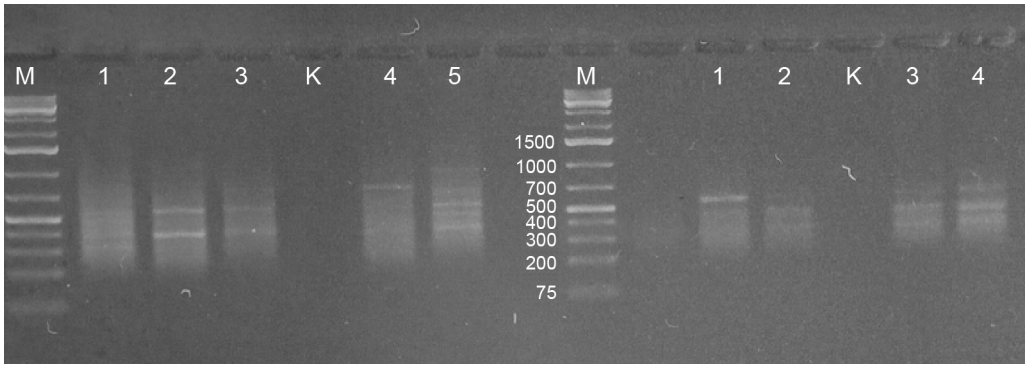

Fig. 4. Spectra of fragments of DNA amplification of Przewalski's horses using $(GAG)_6C$ and $(GA)_9C$ as primers. Notations: M – marker of molecular masses; 1–5 DNA amplification products; K is a control sample

To identify the main indicators of genetic diversity, the proportion of polymorphic loci was calculated using ISSR markers, average gene diversity per locus, which determines heterozygosity at all loci, and Shannon's information index, which characterizes the chaotic distribution of the frequency spectrum of loci (**Table 6**).

Table 6. **Main indicators of genetic diversity of the Przewalski horse population**

Subspecies	Percentage of polymorphic loci (%)	Average gene diversity per locus	Shannon–Wiener information index
Przhevalsky's horse	25	0.39	2.50

Indicators such as the percentage of polymorphic loci (less than 30 %), the average genetic diversity per locus (less than 0.044), the Shannon information index (less than 0.063) may imply inbreeding depression in the population or its strict isolation (Nei, 1972, 1987). Analysis of the parameters of genetic diversity according to the ISSR markers of Przewalski's horses indicates that all the tested animals are relatively healthy in terms of the intra-population genetic diversity, with the exception of the value of the parameters of the percentage of polymorphic loci (25 %) (Suprun & Kurylenko, 2014).

CONCLUSION

According to the results of cytogenetic analysis, it was established that the studied Przewalski's horses were in ecologically clean conditions and were characterized by karyotype stability.

The study of genetic polymorphism of the studied horse populations by ISSR markers $(AG)_9C$ and $(GAG)_6C$ showed a low value of the share of polymorphic loci (P = 0.25) and the index PIC = 0.09, which indicates a high degree of genetic consolidation. All tested animals are relatively safe according to the revealed intra-population genetic diversity.

COMPLIANCE WITH ETHICAL STANDARDS

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Human Rights: this article does not contain any research involving human subjects by any of the authors.

All authors have read and approved the published version of the manuscript.

AUTHOR CONTRIBUTIONS

Conceptualization, [L.S.]; methodology, [L.S.; N.M.; A.B.]; formal analysis, [S.L.]; investigation, [L.S.; N.M.; N.Y.]; resources, [M.P.; N.Y.]; data curation, [M.P.; N.Y.; K.K.]; writing – original draft preparation, [L.S.; N.M.; A.B.; N.Y.]; writing – review and editing, [M.P.; K.K.] ; visualization,[L.S.; N.M.; A.B.; N.Y.]; project administration, [M.P.; N.Y.]; attracting financing [-]

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ГЕНЕТИЧНА СТРУКТУРА ПОПУЛЯЦІЇ КОНЯ ПРЖЕВАЛЬСЬКОГО (*EQUUS PRZEWALSKII*) ЗА ЦИТОГЕНЕТИЧНИМИ ТА ISSR-МАРКЕРАМИ

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Обґрунтування. Кінь Пржевальського внесений до Червоної Книги Міжнародного союзу охорони природи та до Червоної книги України як вид, що перебуває під загрозою зникнення. Для підтвердження унікальності й консолідованості рідкісних видів тварин необхідне проведення цитогенетичного та молекулярно-генетичного моніторингу. Одержанню біологічного матеріалу (крові) для генетичних досліджень передує знерухомлення диких копитних. Вдалий підбір лікарських препаратів з метою седації та анальгезії сприяє збереженню життя і здоров'я тварини.

Методи та матеріали. Популяція диких коней Пржевальського (10 гол.) біосферного заповідника "Асканія-Нова" імені Ф. Е. Фальц-Фейна, іммобілізація тварин препаратом Медісон і антидотом Реверсон, цитогенетичний та молекулярно-генетичний (ISSR–фінгерпринтинг) аналіз.

Результати досліджень. Показники ефективності доз препарату Медісон та Реверсон становили: у коней m = 200 кг доза Медісону 20 мл/гол., знерухомлення через 22 хв, у коней m = 300 кг доза Медісону – 25 мл/гол., знерухомлення через 20–22 хв. Застосування антидоту Реверсон у таких дозах: тварина m = 200 кг доза Реверсону 5–15 мл/гол, припинення седативної дії – 12 хв; тварина m = 300 кг доза Реверсону 5–15 мл/гол, припинення седативної дії – 18 хв. Спостереження за дією препаратів не виявило побічних негативних ефектів. Цитогенетичним аналізом визначено норму каріотипу соматичних клітин 2n = 66 хромосом. Геномні порушення, анеуплоїдія становила 6,7 %, поліплоїдія – 1,3 %. Структурні порушення (хромосомні та хроматидні розриви) виявлені не були. Результати мікроядерного

тесту: частка лімфоцитів із мікроядром – 3,0 ‰, двоядерних лімфоцитів – 2,3 ‰, мітотичний індекс – 7,7 ‰. Генетичні показники популяції коней Пржевальського за ISSR-маркерами: у разі використання як праймера $(GA)_9C$ мікросателітних повторів поліморфних локусів не знайдено, а за праймером $(GAG)_6C$ – 50 % поліморфних локусів. Основні показники генетичного різноманіття, за допомогою ISSR-маркерів: частка поліморфних локусів становила 25 %, середнє на локус генне різноманіття – 0,39, інформаційний індекс Шеннона–Вінера – 2,5.

Висновки. Під час використання препаратів Медісон і Реверсон для іммобілізації коней Пржевальського не виникає негативних побічних дій. За результатами цитогенетичного аналізу встановлено стабільність каріотипу досліджуваних тварин. Вивчення генетичного поліморфізму популяції коней за ISSR-маркерами $(AG)_9C$ та $(GAG)_6C$ свідчить про високий ступінь генетичної консолідації. Усі протестовані тварини відносно благополучні за виявленим внутрішньопопуляційним генетичним різноманіттям.

Ключові слова: кінь Пржевальського, іммобілізація коней, цитогенетичний аналіз, ISSR-маркери, внутрішньопопуляційне різноманіття

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