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Genomic stability and adaptation in Kazakh Tobet dogs: a cytogenetic analysis

Abstract: There is an urgent need to preserve the ancient and culturally significant Kazakh dog breed Tobet, which is threatened with extinction. The evaluation of genomic instability and adaptive potential of Tobet dogs in comparison with outbred dogs as a model of genetic diversity and adaptability seems to be an interesting study and could shed light on the necessary efforts to improve the Tobet breed. In this context, the aim of this study was to analyze the cytological and cytogenetic (micronucleus test) parameters of peripheral blood cells of Tobet dogs and outbred dogs. The analysis revealed no significant differences in micronucleus frequency between Tobet and outbred dogs, indicating robust genomic stability. However, the observed cytological abnormalities, such as poikilocytosis, suggest potential health issues in the Tobet breed and highlight the need for continued health monitoring. This research underlines the importance of combining traditional breeding methods with modern genetic analyses to ensure the survival of the breed.
Key words: Tobet, purebred dog, outbred dog, genomic instability, adaptive potential, cytogenetic analysis.

Introduction

The Kazakh national dog breed Tobet is an ancient national, cultural and historical heritage of our country. For centuries, Tobet dogs guarded thousands of nomadic cattle, protected livestock from wolves, hunted foxes together with their owners and were even used as labourers. This breed got its name since Tobet dogs choose the highest point to have a better view of the protected area ("tobe" – a hill). Unfortunately, this unique breed has almost disappeared today. The reasons for this are the reduction in grazing areas and the decline in sheep farming, the use of other breeds as priority dogs and the use of the Tobet for dog fighting, spontaneous breeding and finally the lack of a scientific basis for breeding work. The current situation requires urgent action to preserve the Tobet breed [1].

Genetic and other modern methods have revolutionized breeding practice and provide tools for improving breed characteristics. For example, the evaluation of genomic instability and adaptive potential may be a tool that can provide valuable information to breeders. Genomic instability manifests itself as damage to genetic structures, including chromosomes (chromosomal aberrations), under the influence of stress factors. This analysis makes it possible to favor animals with a more stable genome in breeding. This leads to more predictable and stable results in breeding recovery and selection. The purpose of evaluating the adaptive potential is to determine the genetic traits that ensure the adaptability of the population to environmental conditions. When the values of the factor exceed the optimal limits, the genetic structures of the cells are activated. Such a process is the basis for the organism's ability to make functional changes in response to environmental influences. The evaluation of adaptive potential enables breeders to select and breed animals that are better adapted to current and future conditions [2, 3].

It should be noted that the breed often has a higher genetic homogenecity due to selective breeding practices, which can predispose it to hereditary diseases and lower adaptability. In contrast, outbred dogs generally benefit from a broader genetic pool, which can lead to greater genetic stability and resilience to certain genetic disorders. It has already been shown that, unlike purebred dogs whose genotype is controlled by strict standards, the genome of outbred dogs is the result of random crossbreeding, leading to a higher degree of genetic diversity and adaptation [4–6]. In addition, while

outbred dogs are more likely to carry deleterious mutations, these mutations are usually in a harmless heterozygous state, presumably because two carriers of the same mutation are less likely to produce offspring. In contrast, purebred dogs are more likely to be genetically affected by one of these disorders due to homozygosity for the mutation [7]. In addition, the breed often descends from a limited number of founder animals, leading to potential founder effects and a higher risk of inherited diseases as the gene pool is more limited [8]. It has been shown that analyzing dogs of a specific breed in comparison to outbred dogs can be an interesting and valuable approach for a more comprehensive evaluation of the breed [9]. Against this background and in the absence of studies on genetic instability in dogs [9–14], the evaluation of genomic instability and adaptive potential of dogs of one breed compared to outbred dogs as a model for genetic diversity and adaptability seems to be an interesting study.

In this context, the aim of this study was to analyze cytological and cytogenetic parameters of peripheral blood cells of Tobet dogs and outbred dogs. The micronucleus test was chosen for these studies because of its informative value, simplicity, and accessibility. Another advantage of the micronucleus test is its independence from karyotype. As is well known, the karyotype of dogs contains a large number (2n=78) of small, poorly distinguishable chromosomes [15]. Its low invasiveness and the possibility of conducting ante-mortem screening to determine the dynamics of changes in this indicator over time are also important [16].

Materials and methods

The main object of the study was Tobet dogs. The collection of biomaterials from Tobet dogs was carried out during expeditions, exhibitions, and special events. Testing for compliance with the breed characteristics of the Kazakh Tobet breed was carried out by cynologists from "KANSONAR", who met the qualification requirements and were experts in Kazakh national breeds and in the group such as the Central Asian Shepherd Dog (CAS), to which the Tobet belongs. The main document for conducting the test and establishing the breed standard that guided the experts was the breed standard for the Kazakh Tobet, which was approved by the Order of the Ministry of ecology and natural resources of the Republic of Kazakhstan dated 30 March 2023 No. 101 "On the Approval of Standards for Kazakh Dog Breeds".

As a control group for the study also included free-breeding, or outbred dogs, which represent the model for genetic diversity and have a high potential for adaptation. The biomaterial from outbred dogs was collected from Tailed Paradise PF, one of the largest animal shelters in Almaty, where more than 500 dogs live.

Peripheral blood to prepare a blood smear on a glass was collected from the leg vein of Tobet and mixed breed dogs by an experienced veterinarian of the research group using a vacuum tube. All sterility criteria were met.

The collection of the biomaterial was accompanied by the photography of the dogs and the questioning of their owners. The questionnaire included information about the owner, age, sex, origin, place of residence of the dog, its description, and measurements. As studies on unrelated animals are necessary to capture the maximum range of genetic diversity, detailed pedigree information was requested for all dogs. For those dogs for which pedigree information was not available, non-relatedness and/or relatedness was confirmed by owners and handlers. The data from the questionnaire was processed and entered an electronic database. In addition to the questionnaire, the owner's informed consent to carry out a genetic study on their dog was also completed.

The study was approved by the bioethics committee of the RSE at REM Institute of Molecular Biology and Biochemistry named after M.A. Aitkhozhin CS MSHE RK (Protocol No. 1, August 18, 2023). The study is based on the "Bioethical rules for conducting research on humans and animals" and is in accordance with the legislation of the Republic of Kazakhstan and the European Convention on Bioethics. In the study, no experiments are conducted on the animals themselves, i.e., dogs; only biomaterials collected from dogs are used. The collection of all types of biomaterials mentioned in the study is a minimally invasive procedure that causes no harm to the dog.

Peripheral blood was used to prepare and analyze cytogenetic smear preparations. The smears were prepared according to the generally recognized method.

The cytogenetic analysis was carried out on 88 dogs of the Tobet breed, 72 of which are kept in the Almaty region and 16 in Ust-Kamenogorsk, as well as on 36 mixed-breed dogs kept in a kennel in the Almaty region. The sex and age composition of the Tobet dogs is shown in Table 1. The mixed-breed dogs were mainly represented in the third age category.

Table 1 – Age and gender composition of dogs of the Tobet breed

Age group	total	female	male
0-9m	10	3	7
10-15m	24	14	10
16m-8y	48	23	25
9y and older	6	3	3
Total	88	43	45

The preparations were processed under laboratory conditions using a camera. The peripheral blood smears were fixed in 96% ethyl alcohol for 30 minutes, dried and stained with 4% Romanowsky-Giemsa solution for 20 minutes [17].

The frequency of micronuclei and cytological disorders was examined in normochromic erythrocytes of peripheral blood using a AxioLab A.1 microscope (Zeiss, Germany) under oil immersion and a magnification of 10x100. During the cytogenetic examination, all changes in the structure of the erythrocytes that deviated from the normal morphology characteristic of this species were recorded. Up to 10,000 erythrocytes were analysed from each dog. The most characteristic abnormalities of the erythrocytes in the peripheral blood were documented photographically.

Reticulocyte analysis was performed to assess the extent of erythropoiesis [17]. Two reticulocyte stains were used: In the first, 50 µl of brilliant cresyl blue solution was mixed with 50 µl of blood, incubated for 25-30 minutes at 37°C or 1.5 hours at room temperature (18-25)°C and smears were prepared. With this staining, the erythrocytes are greenish-grey, the granular-filamentous substance (reticulum) is blue. In the second variant, similarly prepared smears were stained with May-Grunwald fixative. In this staining variant, the erythrocytes are pink-red, the granular-filamentous substance is light blue. The number of reticulocytes per 1000 erythrocytes was counted and expressed as a percentage.

For the statistical calculations, the arithmetic mean and its deviation (M±SE) were calculated as a percentage per 100 cells. The significance of the differences between the means was determined using Student's t-test. The threshold for statistical significance was set at $p \leq 0.05$. Statistical data analysis was performed using Microsoft Excel (Microsoft Corporation, Redmond, Washington, DC, USA).

Results and discussion

One of the indicators of the health, stability of the genome and adaptability of populations is cytogenetic homeostasis. It can be characterised by a micronucleus test and the analysis of other cytological disorders of the blood cells.

The red blood cells of mammals represent the final stage of erythropoiesis and are the product of several blast cell divisions. After the last mitosis, the main nucleus is expelled, and the daughter cells enter the stage of anucleated polychromatophilic (immature) and then normochromic (mature) erythrocytes. After the main nucleus has been expelled, the micronuclei, which are a consequence of the occurrence of chromosomal abnormalities in erythroblast cells, remain in the cytoplasm and are visible under the microscope as round or oval, differently sized, densely coloured bodies with a clear outline [18].

In addition, under the influence of unfavorable factors or diseases, other disorders can occur in the red blood cells, among which regenerative and degenerative changes can be distinguished [18]. The former are characterized by reversible changes, such as echinocytes in the early stages, immature forms of red blood cells or nucleated red blood cells. In degenerative (ageing) red blood cells, the elasticity of the cell membrane decreases over time, leading to irreversible changes such as anisocytosis (change in size), anisochromia (change in color), the appearance of various inclusions and poikilocytosis (change in shape) of the red blood cells.

The main advantage of analyzing such cytological and cytogenetic disorders lies in the simplicity and availability of blood. Their application in the context of endangered species and breeds provides an effective tool for monitoring the health of the population and understanding the impact of various factors on its genetic structure, which can be useful in the development and implementation of conservation and recovery strategies.

In this study, the cytological and cytogenetic (micronuclear assay) parameters of peripheral blood cells from Tobet dogs were analyzed. The study included mixed or outbred dogs as a control group. The results of the cytogenetic analysis of the erythrocytes and cytological abnormalities of these dogs are shown in Table 2.

Table 2 – Results of the analysis of cytological and cytogenetic parameters of peripheral blood cells from Tobet dogs and outbred dogs

Sample	Micronuclei, ‰	Cytological abnormalities %
Tobet		
Age group		
0-9m	0.30	0.98
10-15m	0.31	0.81
16m-8y	0.20	1.57
9y and older	0.20	1.65
Gender		
Female	0.24	1.47
Male	0.26	1.54
Total	0.25±0.01	1.50±0.01*
Outbred (control)		
Female	0.21	1,2
Male	0.32	0,9
Total	0.26±0.03	1,05±0,01
Note: * p≤0,01		

It was shown that the frequency of micronuclei in the erythrocytes of the peripheral blood of Tobets dogs was not statistically significantly different from the results obtained when analysing outbred dogs. This result is in contrast to the available literature data, which showed a significant increase in the frequency of micronuclei, nuclear buds and total number of nuclear abnormalities in purebred dogs compared to mongrels [19]. Our results could indicate either a low level of inbreeding in Tobets or a low "pedigree" of these dogs. In addition, the sensitivity of the methods used may make its own adjustments. We have analysed micronuclei in erythrocytes from peripheral blood and Santovito (2024) from cheek epithelial cells. This publication also reports that endogenous factors such as sex and age do not significantly contribute to the increase in genomic damage observed in purebred dogs. In our study, Tobets of age groups 1 and 2 had a higher frequency of micronuclei (0.3‰) than adult animals, and in about 30% of cases the micronuclei were quite large. In Tobets, the differences in the incidence of micronuclei by sex are insignificant, with a slight increase in males. In bred dogs, however, these differences are statistically significant.

In an individual analysis, 10% of the Tobets and 5% of the outbred dogs had a high number of micronuclei in the erythrocytes, which was 3-6 times higher than the average. It is possible that 10% of the Tobets had a high level of inbreeding.

The degree of cytogenetic abnormalities in the body (genetic instability) is not only an indicator of genetic status and health, but also an important indicator for environmental monitoring. Since we studied Tobets from two regions of Kazakhstan, an analysis of the frequency of micronuclei in the studied animals was carried out depending on their place of residence. In Tobets living in Ust-Kamenogorsk, the frequency of erythrocytes with micronuclei (0.25‰) is statistically significantly ($p \leq 0.01$) higher than the indicators in dogs from the Almaty region. It is known that Ust-Kamenogorsk is one of the most industrially polluted cities in Kazakhstan [20].

Cytological abnormalities are usually an indication of the health status of the animal. The presence of an increased level of cytological abnormalities in the erythrocytes of the examined animals indicates the development of degenerative processes in the body caused by various reasons – physiological, medical, environmental [16]. According to the type and extent of cytological abnormalities in the anuclear erythrocytes, two groups of erythrocytes are distinguished: regenerative and degenerative. Regenerative forms are erythrocytes that have undergone reversible changes to the cell membrane. These include – the early stage of echinocytosis as well as immature forms of erythrocytes or nucleated erythrocytes (basophilic, polychromatophilic and oxyphilic erythrocytes, erythrocytes with basophilic granularity and reticulocytes of varying degrees of maturity). There are many classifications of degenerative changes in erythrocytes. These pathological disorders of red blood cells are associated with changes in size (anisocytosis), red blood cell shape (poikilocytosis), changes in normal colour (anisochromia), inclusions in the red blood cells (Jolly-Howell bodies, Pappenheim bodies, Cabot rings, Heinz bodies, basophilic granularity of the cytoplasm, etc.) [16]

Compared to outbred dogs, Tobet has a higher level of various cytological abnormalities, in particular red blood cells of irregular size, especially small (microcytes) or large (macrocytes). In some cases, poikilocytosis was observed. These were mainly echinocytes (tooth-shaped cells), acanthocytes (red blood cells with different sized protrusions) and dacryocytes (tear-like cells) (Fig. 1).

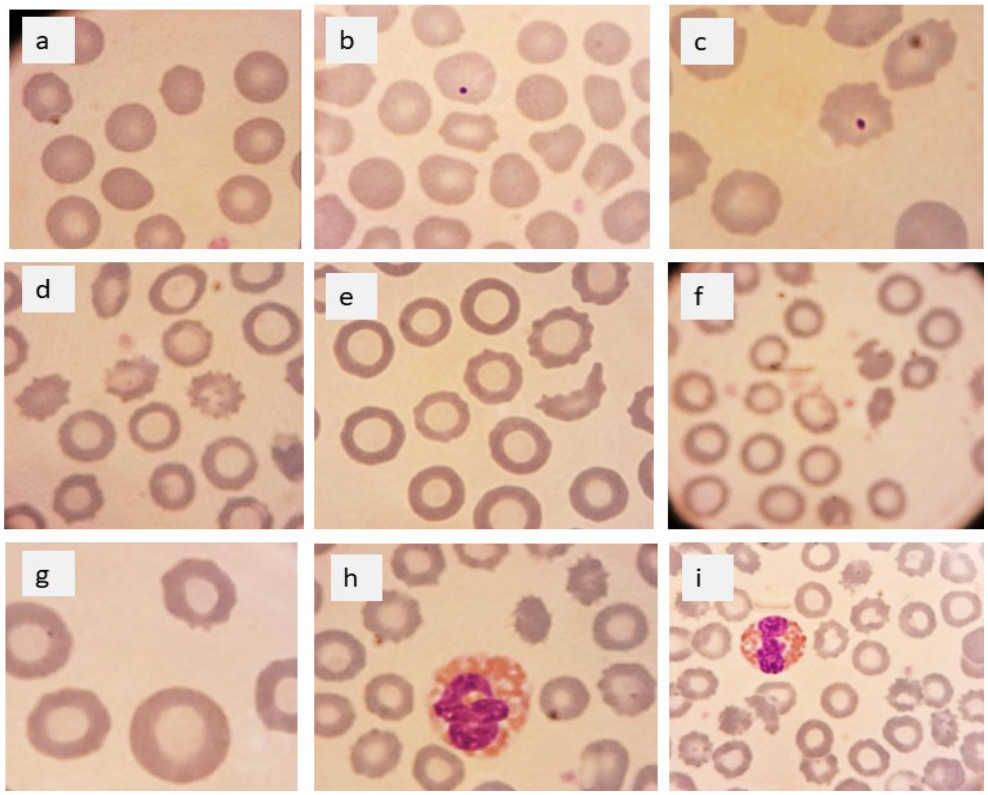

Figure 1 – Preparations of the peripheral blood of Tobet dogs (a) normal red blood cells; (b,c) micronuclei in erythrocytes; d-f – poikilocytosis of erythrocytes with hypochromia; (g) macrocyte; (h,i) toxic granularity of neutrophils, magnification 16 x 100

Given the high percentage of poikilocytosis in some Tobet dogs (10-30%) compared to 3-10% in outbred dogs, it can be assumed that these animals suffer from health problems. It is known from the literature that poikilocytosis, which is characterised by the presence of abnormally shaped red blood cells, may indicate underlying health problems in dogs, including chronic kidney disease, ventricular outflow tract obstruction, severe babesia infestation and congenital heart defects [21-24]. In addition, hypochromia, a low haemoglobin level due to iron deficiency and intoxication, has been reported in some Tobet's dogs. A toxic granularity of neutrophils resembling azurophilic granules has also been observed (Figure 1). Their formation occurs inside the cell due to physicochemical changes in the protein structure of the cytoplasm under the influence of intoxication products, which is observed in infectious or inflammatory processes.

In some dogs of the Tobet breed, the formation of "coins columns" occurs – this is the gluing of red blood cells into chains resembling the shape of coin columns (Figure 2). This usually occurs when the acid-base balance of the blood changes towards increased acidity (from 7.43 to 7.33) and can be associated with chronic liver inflammation [16]. This phenomenon is related to the aggregation of red blood cells, which has a negative effect on blood microcirculation. Normally, the blood of dogs contains a single number of "coins columns".

Figure 2 – Preparations of the peripheral blood of Tobet dogs – the formation of "coin columns", 10x100

Another important indicator of health is the degree of erythropoiesis, which can be assessed by the level of reticulocytes in the blood (Fig. 3-4). In dogs, the reference values are 0.5 – 1.2% [25]. On average, the level of erythropoiesis is at the lower limit of the norm in both Tobets and outbred dogs. There are practically no differences between the sexes. As can be seen from the data presented, the reticulocyte value is only included in the reference values for young dogs, which is of course explained by the higher erythropoiesis in young animals. With increasing age, this indicator decreases significantly and is below the normal limit, which most likely indicates the presence of physiological and veterinary problems. This, in turn, reduces dogs' adaptive potential and their working qualities [2, 3, 26, 27].

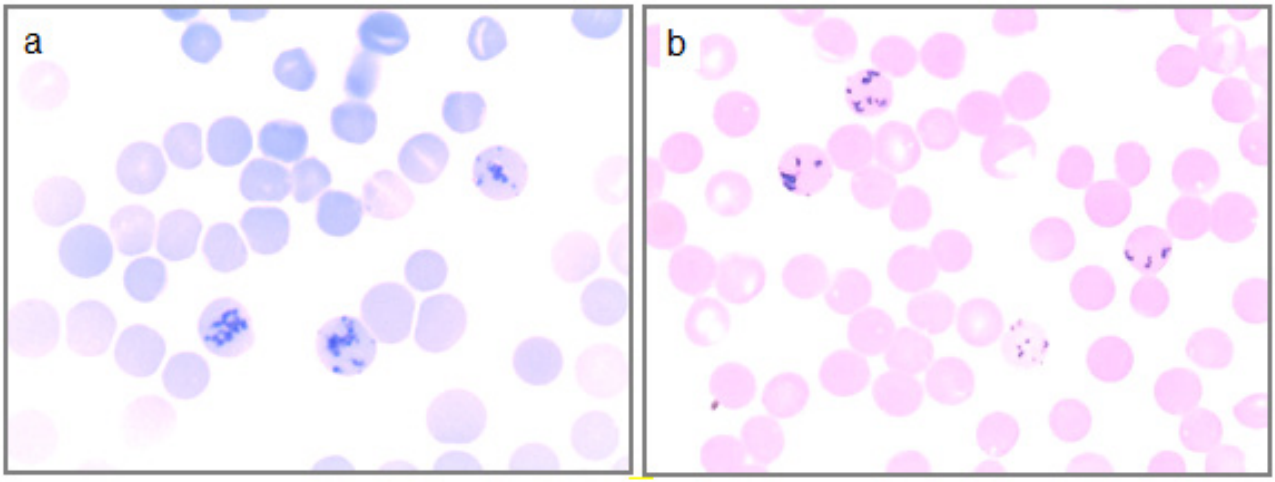

Figure 3 – Reticulocytes in the peripheral blood of dogs (a) first staining method (BCS only); (b) second staining method (BCS+May-Grunwald), 10x100

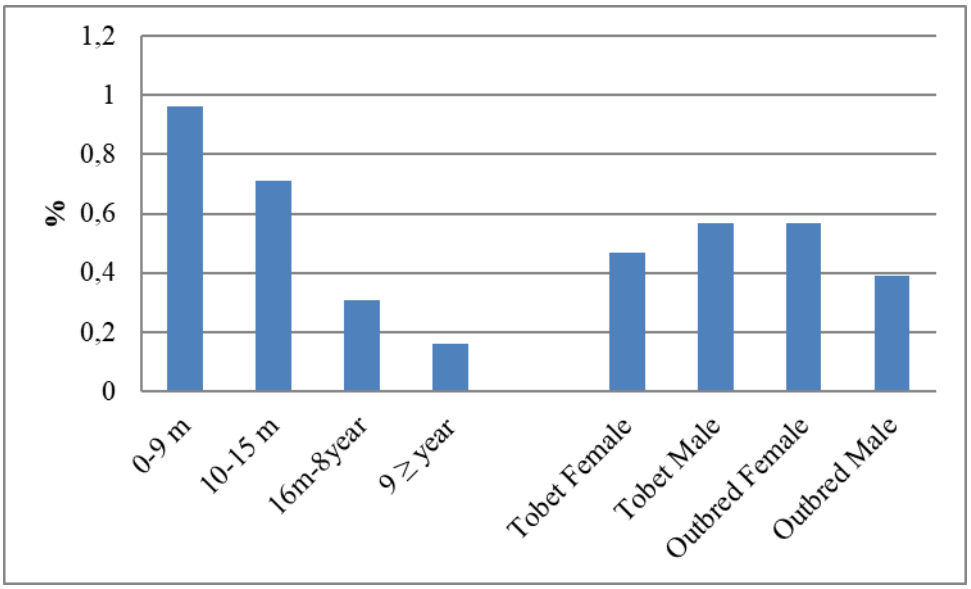

Figure 4 – Reticulocyte content in the blood of the Reticulocyte content in the blood of Tobet dogs and outbred dogs

Thus, preliminary cytological analysis has revealed health problems in some Tobet dogs that may not yet have clinical manifestations. The owners of these dogs have been informed of the need for additional biochemical testing to determine the causes of these problems. The micronucleus test did not reveal any differences in the stability of the genetic apparatus in Tobet dogs and outbred dogs. Unlike Santovito (2024) [19], however, a more thorough study is continuing by analysing the cytogenetic parameters in relation to the age and sex of the animals tested.

Conclusion

The study, which compared the cytological and cytogenetic parameters of Tobet dogs with those of outbred dogs, showed no significant differences in the frequency of micronuclei, indicating comparable genomic stability in both groups. At this stage of the study, it can be assumed that the genetic integrity of Tobet dogs remains intact despite the challenges facing the breed, such as the decline in grazing areas, the decline in traditional breeding methods and the lack of scientific breeding programmes.

However, the discovery of cytological abnormalities, including poikilocytosis – a condition characterized by the presence of abnormally shaped red blood cells – points to potential health problems in the breed. These abnormalities need to be further investigated to understand their impact on the health and viability of the Tobet breed. Overall, the results of the study argue in favor of integrating traditional and modern breeding approaches to improve the breed's resilience and adaptability. This integrated strategy could be crucial for the survival of the Tobet breed.

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Conflict of interest

All authors are aware of the article's content and declare no conflict of interest.

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