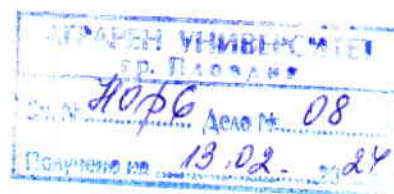


Review



on a dissertation for obtaining the educational and scientific degree "doctor" in: field of higher education 6. Agricultural sciences and veterinary medicine, professional direction 6.3. Animal husbandry, the scientific specialty "Breeding of agricultural animals, biology and biotechnology of reproduction"

Author of the dissertation: Georgi Todorov Yordanov

Dissertation topic: Genealogical structure of the Danube horse breed, its place in the vernier structure and direction of development, in the context of an overall concept for the development of the breed.

Reviewer: Prof. PhD Svetlana Yordanova Georgieva,;
Trakia University, Faculty of Agriculture;
Scientific specialty: GENETICS:

Reason for presenting the review: participation in the composition of the scientific jury for the defense of the dissertation according to Order No РД -16-129918.12.2023/ of the Rector of AU, Plovdiv.

1. Brief biographical reference

Georgi Todorov Yordanov was born on 16.05. 1966. He graduated from secondary school Kiro Konarov in 1984, at Trakia University, AF, obtained a bachelor's degree (1996) and a master's degree (2006), Agricultural University 2017 - doctoral studies. In 1987, he started working at the "Khan Asparuh" horse stud as a jockey. Since 1992, he has been deputy director at the "Khan Asparuh" horse stud, in 1996 he was appointed chief specialist at the "Khan Asparukh" horse stud, and since 1998 he has been working as an expert at the NaSSR. In the period 2000-2009, he was the head of the department at the Executive Agency for Selection and Reproduction in Livestock Breeding, and since 2009 he has been the executive director of the National Horse Breeding Association. In 2016, he was elected chairman of the National Union of Zooengineers in Bulgaria. Currently, he is the executive director of the IASRZ. It is clear from the presented brief biographical reference of the PhD student that his entire conscious life was dedicated to animal husbandry and, in particular, to the development of horse breeding in Bulgaria. In his work, Georgi Todorov Yordanov shows high competence, shows very good communication skills and shows leadership qualities and responsibility towards the team of over 100 people he manages.

2. Relevance of the problem.

The Danube horse is a valuable domestic breed that is in danger of extinction. The preservation and development of the breed, as a national and world treasure, is extremely important and requires a complex evaluation and maintenance of genetic diversity, prevention of unwanted inbreeding, reduction of the genetic load and development of the breed.

3. Purpose, tasks, hypotheses and research methods.

The aim of the scientific study is to assess the current state of the Danube horse breed, its genealogical structures and their relationship with the structure of the Nonius breed, as a prerequisite for the development of a concept for the breeding work and future development of the breed. The goal is clearly justified and to achieve it, and the task is set to conduct experiments and analyzes that are described within 2 pages

To achieve the goal, classical and modern methods and analyzes were used: phylogenetic, genealogical, molecular-genetic and population-genetic. For the statistical analyses, extremely suitable programs, such as GENEALX 6.5., PHYLIP 3.69, STRUCTURE 2.3.4, were applied. DnaSP6.0., Network 10.0, etc.

4. Transparency and presentation of the obtained results.

The results and discussion section occupies 199 pages. Followed by a summary - 3 pages, conclusions and recommendations - 2 pages. The list of used literature includes 427 authors, of which 87 are in Cyrillic and 340 are in Latin.

The results and discussion section begins with a phylogenetic analysis of the Danube horse breed and its genealogical structure from the end of the 1980s. The conditions for the development of the breed after 1990 are described, and the author points out that the considered period is characterized by the regression of Bulgarian horse breeding and livestock breeding. He notes that this is due to the lack of experience in the selection activity, which until then was carried out by state offices in the public sector, work under new economic conditions. loss of valuable genetic material. These and other factors have brought the Danube horse breed to its lowest point of development creation. The situation changed in 1999, when the National Horse Breeding Association was established. The modern state of the Danube horse and its relationship with the Nonius breed after 1990 is analyzed. It has been established that the scheme for breeding the Danube horse, from the creation of the breed to the present, has not been changed, and the philosophy set nearly a hundred years ago - to create a Bulgarian breed draft horses, based on the Nonius breed, has been preserved and is still applied today.

Genealogical analysis of the lineal and family structure of the Danube horse breed and their phylogenetic development was carried out. In Fig. 1. the lineal affiliation of the stallions of the Nonius breed, which participated in the creation of the Danube horse breed, is presented. The development of the lines of stallion N XXIX "A", stallion Zdravko /Nonius XXIX-6/, Leader /Nonius XXX-1/, stallion Erno /Nonius XVII-30/, Line of stallion N XXXI "B", stallion Hrabar /E. Nonius XI-26/, N XXXIV "C" Line, Torpedo Stallion Line, Calif. Stallion Line is described.

The main parameters of the exterior, the dynamics of the exterior indicators and the body composition indices of the line extenders, etc., were calculated. The pedigrees of the stallions are presented in attractive figures. In Fig. 28. Family structure of the Danube horse breed, 2022, is presented, which includes 6 families with origins from the Nonius breed and 6 families with origins other than the Nonius breed.

According to the author, in our modern times the family structure of the Danube horse has preserved the approach established during the creation of the breed, that the founders of families should only be mares of the Nonius breed, Bulgarian local and local improved mares. The development of the families has been duly described and an analysis of the breeding activity of the mares - progenitors of the families has been carried out, the main parameters of the exterior have been calculated, the generational dynamics of the exterior measurements of the mares from the families, the dynamics of the constitution indices, the blood of the individual lines of the breed in the living mares of the families, a forecast made for their development, as well as for the maintenance of genetic diversity by insemination with suitable stallions to achieve the goals of selection. The results are illustrated with color schemes of the progenitor mares.

The genetic structure and genetic diversity of the Danube horse breed and its genealogical lines were studied by means of microsatellite analysis. For this study, 15 microsatellite loci approved by the International Society and International Foundation of Animal Genetics (ISAG) were used: AHT4, AHT5, ASB2, ASB17, ASB23, HMS1, HMS2, HMS3, HMS6, HMS7, HTG4, HTG6, HTG7, HTG10 and VHL20. Microsatellite markers are widely used for paternity testing and for studying the genetic structure of populations. 166 animals were studied and a total of 184 alleles were identified. The average number of alleles (N_a) for all investigated microsatellite markers was 12.29 ± 0.44 . The smallest number of alleles, 10.0, was found in the ASB17 locus, and the largest, 13.33, in the HMS3 and HMS7 loci. The number of alleles found significantly exceeds the recommendations of the FAO, for the study of genetic diversity, according to which the microsatellite markers used must have no less than 4 alleles. (16ISAG/FAO, 2022). The overall mean number of alleles found in the Danube horse was higher than that of the Nonius and the Bulgarian population of the Thoroughbred English horse. The mean effective number of alleles (N_e) was 9.48 ± 0.42 with a range from 4.8 at the ASB17 locus to 11.28 at locus HMS7. The polymorphic information content (PIC) index ranged from 0.65 for the HMS1 locus to 0.81 for the AHT4 locus. The average PIC for the 15 studied loci was 0.73 ± 0.15 . These results together with the high average number of alleles per locus indicate that the panel of 15 microsatellite markers used is suitable for the study of genetic diversity in the Danube horse breed. A comparative analysis with other breeds shows that the average value of PIC in the Danube horse is higher than that observed in the Chinese Guanzhong horse (0.51), the Italian population of the Thoroughbred English horse (0.62) and the Konik Polish (0.67). but it is lower than that of the population of Turkmen horses (0.77). The information index of (I) (Shannon, 1948) - an indicator of the genetic diversity of the population at the studied loci has an average value for the population of 2.22 ± 0.07 , which is an indicator of "abundance" of alleles. In 13 of the 15 loci examined, H_o values were higher than those of H_e and lacked heterozygous deficiency. These results show that the Danube breed has a high genetic diversity (0.84) at the studied loci. The established total coefficient of inbreeding (FIT) has an average value of 0.037 ($P = 0.004$), i.e. The Danube breed is not threatened by inbreeding depression.

At the studied 15 microsatellite loci, the average value of the Intrapopulation Inbreeding Coefficient (FIS) was negative - 0.043. For all microsatellite markers, the FIS value is not higher than 0.1, which confirms the low level of inbreeding and the absence of heterozygous deficiency in the studied six lines of the Danube breed. The FIS index is lower than those found in the breeds Nonius, Polish Konik, Czech Haflinger, etc. To establish the closeness and differentiation of the lines in the Danube breed, the Ney index of genetic differentiation (F_{ST}) was calculated, which shows how

much of the total heterozygosity in the populations is due to the difference between the intra-population structures. The average value of the coefficient for the studied loci is 0.078 ± 0.009 , i.e. genetic diversity between lineages was significantly lower than within-population (FIS). At F_{ST} values of 0.05 to 0.15, the populations are assumed to be very similar, i.e. the 6 studied lines of the Danube horse are "very similar" to each other. The overall mean value of the G_{ST} coefficient accounting for genetic differentiation was 0.061, meaning that only 6.1% of the total genetic diversity was due to differences between lineages. The value of the Nei coefficient for total genetic diversity confirms that the population has a high level of heterozygosity. In all lines, a Hardy-Weinberg population equilibrium test (HWS tests) was performed for each studied locus (Table 26.). In Table. 27. Basic parameters characterizing the structure and genetic diversity of the examined loci in the Danube horse lines are reflected, as follows N_a - number of alleles; N_e - number of effective alleles; H_o - observed and H_e - expected heterozygosity; I - information index of Shannon; number of alleles unique to the lineage (NPA) and N_{DA} average number of alleles for which the frequency is equal to or lower than 5% For the six lines of the Danube breed, genetic distances and similarity were compared using Nei's matrix and F_{ST} (table 28). The results of the analysis showed that the closest lines are Lider and Kalifa and Lider and Torpedo ($DA=0.188$), and the greatest genetic distance is between Zdravko and Erno. The Zdravko line is the furthest from the other lines. Hraber's line is as distant as Zdravko's line and the other lines, being closer to the latter.

The genetic structure of the Danube breed was determined by the program STRUCTURE software v 0.6.94, based on the level of "admixture" in each individual, using the correlated allele frequency model. The results of the Delta K analysis show that the optimal number of genetic clusters demonstrating maximum similarity is at $K = 3$ (Fig. 63 a, b). In fig. 64. the lines and each individual in them are represented graphically. The figure clearly shows that, based on the studied marker loci, the lines are differentiated into three genetic groups. Individuals mostly from the Zdravko line fall into one cluster, and according to the author, the probable reason for the Zdravko line to form a separate cluster is its greatly reduced numbers. Most individuals from the second cluster belong to the lines of Erno, Torpedo, Leader and Calif. In this cluster there is a presence of individuals that could be assigned to the third cluster - the Hraber line). According to the author, the grouping of individuals in this cluster is more likely due to the influence of the genetic profile of the families involved in the breeding process in creating the Danube horse. The shared number of gene pools in the second cluster can be explained with the common origin of the representatives. The third cluster is formed by the Hraber line, and there are very few individuals with a share of the second cluster. The reason for the formation of this independent cluster, according to the author, is not clear. The grouping into 3 clusters made by the STRUCTURE program was also confirmed by principal coordinate analysis (PCoA) (Fig. 65.).

By means of mitochondrial analysis, genetic similarity and distances of the Danube horse breed with populations of the Nonius breed, Bulgarian breeds and horse populations were studied. Based on the mitochondrial types, the author analyzes and compares the breeds in order to trace the origin of local and modern horse breeds, as well as the connections and differences between them. As a result of the Sequencing analysis of the D-loop mtDNA region, it has been proven that the modern Danube horse is a unique national breed, with a specific mitochondrial profile and high haplotype diversity $H_d=0.748 \pm 0.047$. Haplogroup G (0.450) has the highest frequency, followed by haplogroup M (0.218), haplogroup L (0.210), haplogroups Q (0.053), O'P (0.035), A and C with (0.017). The analysis established the relationship of the Danube

horse with the modern populations of the Serbian (haplotype A) and Hungarian (haplogroup M) Nonius, as well as the differences between them. Haplogroups L and C are not found in Hungarian and Serbian Nonius. Haplogroup D found in the Serbian Nonius (0.667) and E - in the Hungarian Nonius (0.130) are not found in the Danube horse.

According to the author, the uniqueness of the Danube breed is related to the breeding activity, which is confirmed by the fact that - haplotype O'P occurs in the Danube horse, the two studied populations of Nonius and the local national populations, and is not found in the Pleven and Eastern Bulgarian horses. Haplotype A is found in the Danube horse, the Eastern Bulgarian horse, the local populations and the Serbian Nonius, but was not found in the Hungarian Nonius and the Pleven horse. Haplotype M is found in Danube horse, Hungarian Nonius, national local populations, but not found in Pleven, Eastern Bulgarian horse and Serbian Nonius. Haplotype G, which occurs in ancient horses, was found in all analyzed breeds, with a very high frequency (0.45) in the Danube horse. On the basis of the reported haplotype diversity in the different horse breeds, the author confirms the uniqueness of the Danube horse, the probability that it is related to the preservation of the gene pool of the local mares that participated in its creation. The presence of haplogroup L in all local populations and the newly created Bulgarian breeds, but not in the Noniuses, is proof of the uniqueness of the newly created Bulgarian breeds, as well as the importance of the local populations in their formation. The stability of the gene pool of the local populations over time is also emphasized, which is probably related to ensuring the adaptability of the created breeds to the local ecological and economic conditions. On the basis of the obtained results, a thorough analysis of the modern state of the Danube horse breed was carried out and the guidelines for the breed's breeding activity were outlined. The author formulates a concept for short-term and long-term development of the breed as follows: In the short-term, work with the Danube horse must continue in the direction of preserving the available gene pool. The concept of long-term development of the Danube horse should be oriented towards sustainable development of the breed, based on three components - "genetic sustainability", social and economic effect. I highly appreciate the results presented by the author from the Sequencing analysis of the D-loop mtDNA region, through which it was proven that the modern Danube horse is a unique national breed, with a specific mitochondrial profile and high haplotype diversity, as well as the parallel interpretation of the results of the genetic (microsatellite) and genealogical analyses, on the basis of which he proposes the application of a complex approach in the development of breeding strategies for the Danube breed. At the end of the dissertation, the doctoral student Georgi Yordanov presents a synthesized summary of the conducted in-depth and large-scale studies of the Danube horse breed, which is an excellent conclusion of the scientific development.

5. Discussion of results and used literature.

On the basis of the obtained results and the used literary sources, a thorough analysis and discussion of the modern state of the Danube horse breed was carried out, and the guidelines of the breed's breeding activity were outlined.

The list of used literature includes 427 authors, of which 87 in Cyrillic and 340 in Latin.

6. Evaluation of scientific and scientific-applied contributions

The dissertation student presents 15 original contributions of a scientific and scientific-applied nature, which I fully accept and highly appreciate.

1. For the first time in our country, a complex genealogical, microsatellite and mitochondrial analysis was conducted to characterize the genetic structure, condition and phylogenetic aspects of the development of populations in animal husbandry. - An original contribution of scientific nature.
2. It has been established that the data from the genealogical and DNA analyzes used alone do not provide accurate information about the state and genetic processes in the population during its phylogeny and must be used in a complex way, when developing breeding strategies. An original contribution of scientific nature.
3. A genealogical structure and the genetic condition of the Danube horse breed, at the modern stage, have been established, as prerequisites for developing a strategy for the development of the breed in the short-term and long-term aspects. It is shown that, although small in number, the Danube breed has a well-established genealogical structure that guarantees sustainable development of the breed. - An original contribution of applied nature.
4. On the basis of a complete genealogical analysis of the lineal and family structures of the Danube horse and an analysis of the generational dynamics of the exterior parameters, the trends in the change of the exterior of the breed were demonstrated for the first time, in a phylogenetic aspect, and recommendations were made for future work. It was found that the selection was aimed at creating animals of the "medium" type, intermediate between the "small" and the "large" Nonius, but closer to the latter. As a result, a breed consolidated in exterior was created. - An original contribution of applied nature.
5. On the basis of microsatellite analysis of 15 marker loci, the relationship between the Danube horse breed and the populations involved in its formation - Serbian Nonius and Hungarian Nonius - was analyzed for the first time at the genetic level. The three populations were found to have a specific genetic structure, with the Danube horse being closer to the Hungarian Nonius. An original contribution of scientific nature.
6. Based on a microsatellite analysis of 15 marker loci, it was established that the Danube breed has a high genetic diversity and is not at risk of inbreeding depression at this stage. - An original contribution of scientific and applied nature.
7. On the basis of a microsatellite analysis of 15 marker loci, it was established that the lines of the Danube breed have a high intralineal diversity, but with an extremely high genetic similarity to each other, which requires maximum precision when developing the selection schemes. The distances between the 6 lines of the breed were established. - An original contribution of scientific and applied nature.
8. In order to clarify the phylogenetic relationships of the Danube horse with breeds that participated in its creation, a unique large-scale study of the D-loop region of mtDNA in prehistoric wild horses that inhabited our lands, modern representatives of local autochthonous horse populations from Stara - Planina, was carried out, the Rilo-Rhodope massif and the Karakachan horse, of the newly created Bulgarian breeds - Danube horse, Pleven horse and Eastern Bulgarian horse, of the Serbian and Hungarian population of Nonius. - An original contribution of scientific nature.
9. On the basis of mtDNA analysis, it was established that the Danube horse is a unique national breed, with a high haplotype diversity and a specific mitochondrial profile, demonstrating its relationship both with the modern populations of the Serbian

and Hungarian Nonius, and with the gene pool of the local national populations. - Original contribution scientific in nature.

10. On the basis of mtDNA analysis, it was established that the newly created Bulgarian breeds - the Danube horse, the Pleven horse and the Eastern Bulgarian horse - are genetically closely related to each other, to the modern local populations and to prehistoric wild horses that inhabited today's territories of the country. - An original contribution of scientific nature.

11. On the basis of mtDNA analysis, a loss of genetic diversity of local populations was clearly demonstrated for the first time when creating cultural breeds by crossing with introduced ones. - An original contribution of a scientific nature.

12. The results obtained from the conducted research contribute to clarifying the origin, domestication and biogeography of the species *Equus ferus caballus*. - An original contribution of scientific nature.

13. On the basis of mtDNA analysis of prehistoric wild horses and modern national breeds and populations, phylogenetic and historical analysis, it is assumed that the present-day territories of Bulgaria were a place of domestication of the horse, as part of the western periphery of the Pontic -The Caspian Center. - An original contribution of scientific nature.

14. The 120 sequences obtained for the first time, during the sequence analysis of populations of modern Bulgarian horse breeds, have been published in the genetic database (GenBank) under the numbers: Danube horse (GenBank Acc. no. MG420898-MG420955); Pleven horse (GenBank Acc. no. MK465427-MK465437) and East Bulgarian horse (GenBank Acc. no. MK465388-MK465426) - Original contribution of scientific nature.

15. The molecular data obtained for the first time on the genetic profile of Nonius and Serbian Nonius have been published in the genetic database (GenBank) under the numbers: Nonius (GenBank Acc. no. MG420956-MG420978) and Serbian Nonius (GenBank Acc. no. MG420979- MG420990). - An original contribution of scientific nature.

7. Criticisms, recommendations and questions

I don't have any.

8. Published articles and citations.

The PhD student submits 9 publications in connection with the dissertation work, of which two are in scientific journals with Q2, two with Q3 and one with Q4. The publications were cited 21 times, which reflects the interest of the scientific community in the published results.

The presented abstract reflects objectively the structure and content of the dissertation work.

CONCLUSION:

Based on the various research methods learned and applied by the PhD student, the correctly performed experiments, the generalizations and conclusions made, I believe that the presented dissertation meets the requirements of the ŽARSRB and the

Regulations of the Agrarian University for its application, which gives me a reason to evaluate it POSITIVE.

I take the liberty of proposing to the honorable Scientific Jury to also vote positively and award Georgi Todorov Yordanov, the educational and scientific degree "Doctor" in field of higher education 6. Agricultural sciences and veterinary medicine, professional direction 6.3. Animal husbandry, the scientific specialty "Breeding of agricultural animals, biology and biotechnology of reproduction".

Date: 06.02.2024.

Stara Zagora

REVIEWER:

Prof. Svetlana Georgieva

