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Variation in the *ASIP* and *DUN* Genes Responsible for Coat Colour in Bosnian Mountain Horse

Key words

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dun dilution;
DNA polymorphism;
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Abstract: Accurate determination of coat colours in Bosnian Mountain Horse (BMH) can be challenging as there are variations in coat colour shades and several dun dilution variants occur. In other studies found single nucleotide polymorphisms (SNPs) within two colour loci *T-box 3* (*TBX3*) and 11-bp indel polymorphism within *Agouti Signalling Protein* gene (*ASIP*), were genotyped in 313 BMH individuals. The obtained genotypes were then compared to the identified phenotypes by using the observed coat colour types from the International Association of Bosnian Mountain Horse Breeders (IABMHB) database. It was found that the dark bay and black were the most representative coat colours in BMH. The frequency of the dominant *Dun* (*D*) dilution allele in the study is higher (0.09) than the previously predicted frequency recorded in the available BMH register. Among the identified alleles, there was a discrepancy or inconsistency between the predicted coat colour based on genotypes and the observed coat colour in 73 horses (23%). The most frequent error concerned the misclassification of horses with genotypes *aa* and *Aa* at the *ASIP* gene, *non-dun1/non-dun1 (nd1/nd1)* and *non-dun2/non-dun1 (nd1/nd2)* at the *TBX3* gene, which can be associated with the occurrence of slight dilution phenotypes in these individuals. In contrast to the Konik and Hucul breeds, no homozygosity of the *D* allele was found in the BMH. The *D* allele can be easily overlooked or not recognised in different phenotypic groups, such as dark bay and black horses. Therefore, the hypothesis that Dun dilution effects itself is not as strongly epistatic in the BMH as described in other horse breeds. The results of the study confirm the importance of molecular testing in accurately determining the coat colour of horses. This would help to avoid errors in coat colour descriptions in official breeding records and provide valuable information for selective breeding programmes aimed at producing specific and desired coat colours.

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Introduction

Coat colour as a trait was favoured in the domestication of horses. In recent years, rapid progress has been made in understanding the genetics of coat colour in horses (1). Tradition and practise are guided by certain principles and breeding programmes, which may be directed either towards the promotion of colour diversity or towards the pursuit of colour uniformity. In particular patterns associated with white are deliberately avoided because their occurrence is believed to be a sign of crossbreeding (2, 3). This deliberate avoidance can be attributed to concerns arising from potential negative influences, such as pleiotropism, which could compromise the safety and integrity of breeding initiatives (4). Long-standing selection for colour has resulted in the allele frequencies responsible for the different coat colours in horses changing under the influence of specific breeding practises, reproduction methods and breeding standards. Breed-specific segregation of alleles is related to the breeding history of breeds (5-7).

BMH is a breed with a long history, native to the region and adapted to semi-wild rearing conditions over a long period of time. BMH originate from the Alpine/Dinaric region, which is known for its difficult environmental conditions such as high altitude, rugged terrain, cold temperatures and lack of food, especially in winter (8). Similar to other indigenous small mountain horse breeds, the BHM has experienced a decline in its role as a working horse. This decline can be attributed to factors such as the migration of the population from rural areas to the cities, changes in the lifestyle of the Balkan inhabitants and the modernisation of agriculture. The number of purebred and registered animals includes 340 animals in the studbook (8). The majority of the BMH population consists of brown and black horses, while only a few individuals have white markings. In the breeding programme for the BMH, all colours except grey, pinto, chestnut and spotted, without white markings, are allowed (9, 10).

The analysis of official breeding documentation conducted by Mesarič et al. (2015) revealed that BMH registered in the IABMHB studbook are mainly dark bay, black, bay dun or blue dun. The presence of Dun colours in the BMH population is a clear indication of the influence of earlier horse types that contributed to the development of the native BMH. These horses often exhibit primitive markings such as horizontal leg stripes and distinctive eel stripes. The phenotypic similarity of Dun horses to the Tarpan and other related breeds such as the Hucul, Konik and BMH provide additional evidence of their common ancestry (8,11-13). The BMH mare lines Una, Medina, Lasta and Zorka in particular have retained a share of Dun horses. Historical records indicate that Dun horses accounted for about 3.2% of the BMH population in 1944 (9).

The *Dun* dilution gene, which affects the pigmentation of both red and black coat colour, is considered to represent the ancestral or wild colouration of horses (14). Dun horses have a dark dorsal stripe and many of them bear other "primitive markings" (leg stripes, shoulder shadow/stripes, face mask) (15). The presence of Dun horses can be seen on prehistoric cave paintings, such as at Chauvet Cave, suggesting a long-standing association between this colouration and equids (16). Several closely related Equus species, including the Przewalski's horse, the onager, the kiang, the African wild ass, an extinct subspecies of plains zebra known as the quagga, and an extinct subspecies of horse, the tarpan, have characteristics associated with the dun phenotype.

According to a study by Imsland et al. (2016), the Dun dilution effect was attributed to the presence of the *G* allele in SNP 18,227,267+1,066G > T at a 1.6 kb insert in the downstream region of the *TBX3* gene on ECA8. This insertion is known as the dominant *Dun* allele (*D*). On the other hand, the presence of the *T* allele is associated with the recessive allele *non-dun 1* (*nd1*) or the absence of a 1.6 kb fragment is associated with a recessive allele *non-dun 2* (*nd2*).

In countries where horse breeding plays an important role, breeders have found patterns and coat colours extremely interesting, as they can significantly increase the market value of horses. As a result, different colours and patterns have developed in many horse breeds. Accurate identification of the desired coat colour is of great importance to breeders as it helps in the selection of specific colours and facilitates future breeding plans. The development of a simple and efficient method to identify mutations responsible for dun dilution is of great interest to horse breeders as it allows for a better understanding and control of coat colour genetics in their breeding programmes (17).

The aim of this study was to characterize the variations in the base colour of the endangered BMH by revealing the genetic basis of coat colour. Furthermore, we want to investigate the influence of selection on coat colour and examine the relationship between genotype combinations of the *ASIP* and *DUN* genes and the variation in coat colour within the existing population.

Material and methods

A total of 313 genomic DNA samples from BMH individuals of both genders, representing different shades of coat colour, were used for this comprehensive study. These samples were obtained from the Laboratory of Molecular Biology and Genetics at the Faculty of Veterinary Medicine, University of Ljubljana, Slovenia. To compare, phenotypic data was taken from the IABMHB database, matching the coat colour descriptions in the official breeding documents. The validity of the method was confirmed by examining 28 dun and 78 non-dun horses. In addition, 10 samples of horses with known coat colour genotypes from the International Society for Animal Genetics (ISAG) Comparison Equine Test 2018/2019 were used as reference. First, a cohort of 80 individuals was randomly selected from the original sample group to calculate the frequencies of *TBX3* and *ASIP* genotypes. This selection was made to ensure a representative sample encompassing the aberrant traits observed in the closely related animals in a population of horses in this study. Due to the rare occurrence of chestnut coat colour in the BMH population, as chestnut horses are usually excluded from breeding, our focus was directed towards the two dominant alleles *TBX* and *ASIP*. These loci play an important role in determining the different coat colours observed in this breed. In addition, we carried out a comprehensive photographic documentation of all animals involved in the studies.

Genotyping of Dun / non-dun1 / non-dun2 alleles

Genomic DNA from hair roots was extracted according to the Chelex extraction protocol.

Genotype for the G > T SNP (*D* v. *nd1* alleles) on the *TBX3* gene was analysed using a dual-fluorescent multiprobe

assay. Analysis of the 1.6 kb indel of the *TBX3* gene (*D* v. *nd2* alleles) was developed using quantitative polymerase chain reaction (q-PCR). The oligonucleotide primers and probes for discrimination between *D* and *nd1* alleles were outsourced to a commercial service (Assay by Design Service, Applied Biosystems). These primers and probes were designed for the equine *TBX3* gene with the corresponding GenBank accession number KT 896509.1 and KT896508.1 (15). On the other hand, the oligonucleotide primers and probes for the detection of the *nd2* allele (1.6 kb deletion) were developed using Primer3 software v.4.1.0 (http://bioinfo.ut.ee/primer3/), targeting the regions upstream and downstream of the 1.6 kb deletion (18). The probes are labelled with different fluorescent dyes: VIC for *nd1*, FAM for *D* and Cy5 dye for *nd2*.

Genotyping of *TBX3* alleles was performed using two different reactions: an allele discrimination assay for *D/nd1* alleles and q-PCR for detection of the *nd2* allele (1.6 kb deletion). A standard PCR programme on a q-PCR reaction system (Quantstudio 5, Applied Biosystems) was used. The results were analysed using QuantStudioTM Design&Analysis software v1.5.2. (Applied Biosystems).

Sequencing of TBX3

To assess the reliability of the assay, direct sequencing of a 240 bp fragment of the *TBX3* gene was performed. The sequence of the *TBX3* gene was amplified by PCR. The sequence reaction of PCR product was performed on a thermal cycler (SimplyAmp, Applied Biosystems) according to the manufacturer's instructions (BigDye Terminator v1.1 cycle sequencing kit, Thermo Fisher Scientific). Sequencing was performed using SeqStudio (Applied Biosystems) and subsequently analysed (Chromas sequencing software, Technelysium, Brisbane, Australia).

Genotyping of the ASIP insertion-deletion

To determine the *ASIP* genotypes, the coat colour gene loci were genotyped for *ASIP* using polymerase chain reaction (PCR) according to procedures described by Rieder et al. (2001). The PCR products were analysed by capillary electrophoresis QIAxcel ScreenGel 1.5.0

Statistical analyses were performed in IBM SPSS Statistics 28.0.0.0. A group of 80 horses was selected to assess the correlation between *TBX3* and the *ASIP* genotypes. This correlation was assessed using the chi-square test and a p-value was determined. Comparisons of genotype frequency between groups were made using Fisher's exact test. Bonferroni's p-value correction was applied to account for multiple comparisons. Subsequently, the recorded coat colour of each horse in the database was compared to the predicted coat colour based on the genotypes for the *TBX3* and *ASIP* loci. All discrepancies between the recorded genotypes and coat colours were carefully noted. In addition, the percentage error rate for each phenotypic group (bay, dark bay, dun, and black) was calculated. This rate was determined by dividing the number of animals with misclassified coat colours by the total number of horses in the respective group.

Results

Genotyping of *TBX3* gene variants, including the 1.6 kb indel polymorphism and the *D/nd1*-related SNP, was carefully performed in a population of BMH. The result showed the

Table 1: The genotype frequencies of *TBX3* and *ASIP* in the Bosnian Mountain Horse population

	Genotype and allele frequencies												
	TBX3								***ASIP***				
Coat colour (classified by IABMHB)	***D/nd1***	***D/nd2***	***nd1/nd1***	***nd1/nd2***	***nd2/nd2***	***D***	***nd1***	***nd2***	***AA***	***Aa***	***aa***	***A***	***a***
Randomly sampled (n=80)	0.02	0.13	0.13	0.34	0.39	0.07	0.31	0.62	0.09	0.31	0.60	0.24	0.76
	All horses in this study (n=313)												
Bay (n=34)	0.03	0.00	0.09	0.18	0.71	0.02	0.19	0.80	0.29	0.71	0.00	0.64	0.36
Dark bay (n=138)	0.01	0.02	0.09	0.33	0.54	0.02	0.26	0.72	0.08	0.54	0.38	0.36	0.64
Black (n=115)	0.00	0.02	0.08	0.28	0.62	0.01	0.22	0.77	0.00	0.07	0.93	0.03	0.97
Dun (n=26)	0.08	0.73	0.04	0.15	0.00	0.41	0.15	0.46	0.04	0.15	0.81	0.12	0.88

IABMHB = International Association Of Bosnian Mountain Horse Breeders; *TBX3* = the T-box 3 gene; *ASIP* = the agouti signalling protein gene; *D* = dominant *Dun* allele (*TBX3*); *nd1* = recessive *non-dun 1* allele (*TBX3*); *nd2* = recessive *non-dun 2* allele (*TBX3*); *A* = dominant allele (*ASIP*); *a* = recessive allele (*ASIP*).

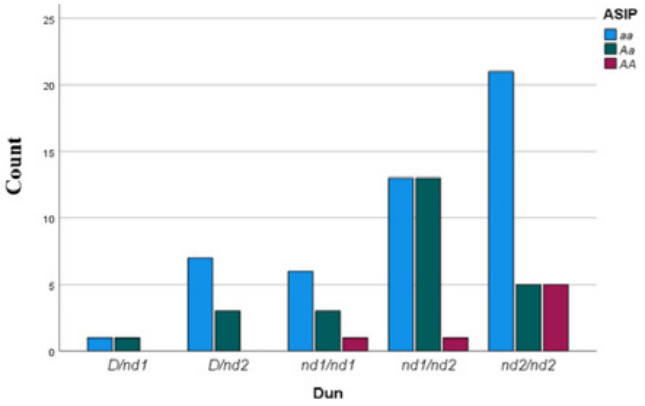

Figure 1: Comparison between *TBX3* and *ASIP* genotypes in a randomly selected population of the Bosnian Mountain Horse (BMH)

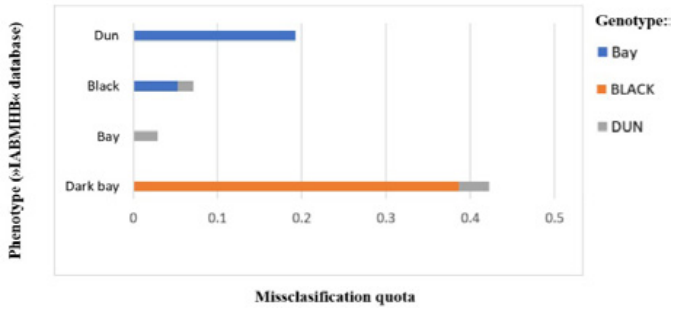

Figure 2: Misidentified coat colours within the phenotypic classifications in the BMH (IABMHB = International Association of Bosnian Mountain Horse Breeders)

presence of five of the six possible genotypes, with *nd2/nd2* having the highest frequency (0.39), while *D/nd1* had the lowest frequency (0.02) (Table 1).

Of note, the frequency of the *D* was determined to be 0.07, while the frequencies of *nd1* and *nd2* were 0.31 and 0.62, respectively. As for *ASIP* genotype frequencies, the distribution was as follows: *AA* - 0.09; *Aa* - 0.31 and *aa* - 0.60. Of the total data set of 313 genotyped horses, 29 animals (9.2%) were heterozygous for the *D* locus, while none of the horse's showed homozygosity. Interestingly, a higher frequency of the heterozygous *D/nd2* genotype was observed within the group of Dun horses, with a sixfold higher compared to the *D/nd1* genotype.

Genotyping of the 11 bp *ASIP* indel polymorphism, which is responsible for the bay base coat colour (Rieder et al. 2001), within the above-mentioned group of 80 randomly selected BMH individuals revealed that 40 % of the horses possessed the genotype *AA* or *Aa*, indicating that their genetic base colour is bay (Figure 1).

Comparison between *ASIP* and the *TBX3* gene yielded several interesting results regarding the *TBX3* genotype in a randomly selected group of BMH. When the combined genotypes of *TBX3* and *ASIP* were considered together, beneficial genotype combinations associated with different coat colours were identified (as shown in Figure 1).

Horses with dun-coloured coats had two genotype combinations: *D/nd1 - D/nd2*. Within this group, *ASIP* genotype combinations were observed: *aa* (1 and 7 horses) and *Aa* (1 and 3 horses), respectively. In contrast, three genotype combinations were observed in the non-coloured horses: *nd1/nd1*, *nd1/nd2* and *nd2/nd2*. These genotype combinations were associated with the three *ASIP* genotype combinations *aa* (6 and 13 horses), *Aa* (3 and 13 horses) and *AA* (1 and 1 horse, respectively). In particular, horses with the *nd2/nd2* genotype had the following *ASIP* genotype combinations: *aa* (21 horses), *Aa* (5 horses) and *AA* (5 horses) (Figure 1).

The population of BMH included individuals with different coat colours. The distribution of coat colours within the population was as follows: dark bay (138 horses, representing 44% of the population), black (115 horses, 36%), bay (34 horses, 11%) and dun (26 horses, 8%). When comparing the obtained genotypes with the recorded coat colour descriptions from the IABMHB database, remarkable inconsistencies were found, especially with regard to the dun dilution effect. About 2.9 % of the horses originally described as bay (including all shades) turned out to be genetically bay dun (Figure 2). Similarly, about 1.8 % of black horses were genetically identified as blue dun possessing at least one copy of the dominant *D* allele. However, within this relatively small sample, the majority of dun horses (85%) were correctly classified (Figure 2). It was also found that in 54 cases horses that were genetically black (with genotype *aa* in the *ASIP* gene and without the dominant allele *D*) were incorrectly classified as dark bay.

Discussion

In the breeding of BMH, coat colour is of great importance as a breeding objective. Breeding practises within the BMH population have led to a strong trend towards the use of dark stallions. As a result, approximately 81% of the BMH population consists of horses with black or dark bay coat colours. This preference for dark coat colour has influenced by the higher frequency of the recessive a allele at the Agouti locus. Similar high estimated frequencies for the recessive a allele at the Agouti locus have also been observed in several other horse breeds (19-23). The high frequency of the A/a genotype observed in the BMH horses for bay coat colour may reflect the breed's preference for a darker coat colour over lighter shades such as light bay, which are rare. In a recent association study, Corbin et al. (2020) confirmed the findings of a correlation between genotype *A/a* at the Agouti locus and genotype *E/E* at the Extension locus, especially in relation to the presence of dark shades of bay (24-27). The BMH breed is believed to have a similar origin as it is probably descended from the Tarpan, which was found in Europe and Asia until its extinction at the end of the 18th century (6, 9). We genotyped a large number of

Figure 3: Dun phenotype classification categories (IABMHB; first line: bay dun, dark blue dun; second line: dark bay, dark bay) and *TBX3* and *ASIP* genotypes (Fotos Matjaž Mesarič)

BMH for the *TBX3* gene Dun variant and found that it was not homozygous in any of the 313 horses. The low frequency of the *D* allele is a result of the long history of selection for the dark base coat colour in BMH. Through analysis of successive studbook volumes and previous studies using pedigree information, it has been documented that the incidence of BMH with coat colour diluted by the *Dun* gene has been consistently low (8, 9, 28, 29). Despite the ancestral nature of dun pigmentation, the overall frequency of the *D* allele in BMH has declined over time due to the preference for undiluted coat colours in the BMH studbook. However, our study has shown that the actual frequency of the dominant *D* allele in the *TBX3* gene is higher than predicted by Mesarič et al. (2015) based on the information recorded in the studbooks (0.09 and 0.03, respectively). One of the results of our study was the absence of the *AA* genotype at the agouti locus in individuals from the reference group with *D* allele at the dun locus, while an increased *AA* frequency was observed in the *nd2*/*nd2* genotype.

In the group of dark-coloured Dun horses, the frequency of individuals with a bay or light bay base coat is relatively low. During the emergence of BMH breeding, there was a strong preference for the use of the dark non-dun stallions, as darker horses were preferred by the Yugoslav army. The *D* allele has been maintained only among specific mare families, predominantly in the genotype *D/nd2*, which represents a darker shade of colour as in the Hucul and Polish primitive horse breed (11, 13). This trend in BMH breeding confirms the study by Cieslak et al. (2021), which indicates that the majority of the original dark Polish horses were *D/nd2* heterozygous and that mating dark individuals increased the probability of producing black offspring.

A comparison of *Dun/non-dun* allele frequencies between our study and previously published studies on Huculs (11,23) and Konik horses (13) shows clear differences. The population of BMH has a relatively high frequency of the *nd1* allele, reaching a value of 0.31, and a high frequency of the *nd2* allele (0.62). In comparison, the overall frequency of the *nd1* allele calculated in the study by Imsland et al. (2016) for 1.841 horses of different breeds was twice as low (0.18) as that determined in our study. This suggests that in the BMH breed, the *nd2* allele is subject to strong positive selection, while the *nd1* allele contributes to the presence of a diverse range of coat colours and primitive markings. Similar to Ezoe et al. (2019), who genotyped *TBX3* gene variants in four horse populations from Kazakhstan, Laos, Nepal and Vietnam, we also observed the presence of five possible genotypes, with the exception of the *D/D*. The *nd1*

allele was found in high frequency in Iberian horses (0.97 - PRE, 0.87 - Andalusian, 0.57 - Lusitano) as well as in the Arabian (0.68) (20).

Interestingly, the observed high frequency of the *nd1* allele in the BMH population might be similar to the genetic composition found in the ancestral population of wild horses that contributed to the process of domestication or might be influenced by Arabian horses brought to Bosnia with the Turkish invasion and later by the Austro-Hungarian Empire (8, 15). It should be noted, however, that the original allele distribution in the BMH breed was probably greatly affected by the bottleneck event during World War II and later during the Yugoslav Wars, as only a very limited number of founders survived (6).

Within the population of animals evaluated, we encountered several cases where genetically black or bay individuals were misclassified by the inspectors of the IABMHBA. Surprisingly, the number of discrepancies between molecular and phenotypic data exceeded our expectations and was comparable to previous records for Hucul breeds maintained in Poland (11). These discrepancies can be attributed to various genetic factors, such as the high frequency of *nd1*/*nd1* and *nd1*/*nd2* genotypes in the BMH breed. Therefore, we assume that carriers of the *nd1*/*nd1* and *nd1*/*nd2* genotypes have an 'intermediate' phenotype, showing traits that lie between fully diluted and non-diluted coat (Figure 3). The influence of *nd1*, along with individual factors such as age, season, *ASIP* and *MC1R* genotype, can be a challenge in accurately determining a horse's coat colour. An additional complication in visually distinguishing phenotypes may arise from an independent locus upstream of the *ASIP* gene, which has recently been identified as a factor that can subtly alter the pigmentation shade of dark colours (22). This can lead to the common nomenclatural problems in coat colour classification, as animals with such traits can be visually classified as 'non-dun' even though they show subtle signs of dilution in their coats. In our study, we found that some individuals with *nd1*/*nd1* or *nd1*/*nd2* genotypes were incorrectly assigned to the dun group.

Conclusions

We have observed and confirmed selection for dark basic coat colours in the BMH, as evidenced by the low frequency of the dominant *A* allele in the *ASIP* gene and the relatively higher frequency of the recessive *a* allele. In this study, we have shown that the *D* allele segregates at low frequency in the BMH population, while the *nd1* allele is present at high frequency. We confirm that due to the complex molecular background found within the coat colour genes in the BMH the correct assignation of particular coat colour can be challenging. Our study represents the first comprehensive investigation of the genetic background underlying coat colour in BMH and provides valuable insights into the phenotypic effects of Dun dilution. The results underline the importance of studying indigenous horse breeds as they contribute significantly to our understanding of the genetic basis of coat colour variation in horses.

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Author's contribution: Cotman Marko, DNA extraction and genotyping, writing, fundraising; Mesarič Matjaž, conceptualisation, sampling, data collection, data analysis, writing - original draft, Kotiščak Jelena, data analysis; all authors: reading, commenting and reviewing the final draft manuscript.

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Variabilnost genov *ASIP* in *DUN*, odgovornih za barvo dlake pri bosanskem planinskem konju

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Izvleček: Določanje barv dlake pri bosanskem planinskem konju (BPK) je lahko izziv, ker obstajajo razlike v barvnih odtenkih dlake in več oblik redčenja barve dlake (plavci). Pri 313 konjih pasme BPK smo genotipizirali polimorfizme posameznih nukleotidov (SNP) znotraj dveh lokusov za barvo dlake T-box 3 (*TBX3*) in polimorfizma 11-bp indel znotraj Agouti signalnega proteina (*ASIP*). Posamezne genotipe smo nato primerjali z vpisanimi fenotipi barv dlake iz baze podatkov MZRBPK (Mednarodno združenje rejcev bosanskega planinskega konja). Ugotovljeno je bilo, da sta bili temna rjava in črna najbolj reprezentativni barvi dlake pri BPK. Pogostnost prevladujočega alela za redčenje *Dun* (*D*) v študiji je višja (0,09) od predhodno zabeležene v razpoložljivem registru BPK. Med ugotovljenimi aleli je prišlo do neskladja ali nedoslednosti med predvideno barvo dlake na podlagi genotipov in opazovano barvo dlake pri 73 konjih (23%). Najpogostejša napaka se je nanašala na napačno razvrstitev konjev z genotipoma *aa* in *Aa* v genu *ASIP*, *non-dun1/non-dun1* (*nd1/nd1*) in *non-dun1/non-dun2* (*nd1/nd2*) pri genu *TBX3*, kar je lahko povezano s pojavom redčenja barve dlake pri teh fenotipih.V nasprotju s pasmami Konik in Hucul pri BPK ni bila ugotovljena homozigotnost alela *D*. Alel *D* je mogoče zlahka spregledati ali ga ne prepoznati v različnih fenotipskih skupinah, kot so temni rjavci in vranci. Zato hipoteza, da učinki redčenja Dun (plavci) pri BPK sami po sebi niso tako močno epistatični, kot je to opisano pri drugih pasmah konj. Rezultati študije potrjujejo pomen molekularnega testiranja pri natančnem določanju barve dlake konj. To bi pripomoglo k preprečevanju napak pri opisih barve dlak v uradnih rejskih evidencah in pomembna informacija pri selekciji na posebne in želene barve dlake pri konjih.

Ključne besede: barva dlake konj; dun redčenje barve dlake; polimorfizem DNK; frekvenca alelov; frekvenca genotipa; bosanski planinski konj