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Allele Frequencies in Loci Controlling Coat Colour in Polish Coldblood Horses

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ABSTRACT

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The objective of this study was to estimate the frequencies of alleles which produce coat colour in Polish Coldblood horse population, and to verify the hypothesis that coat colour is not considered in its selection. The analysis included 35 928 horses and their parents having been registered in the studbook over a half-century. Allele frequencies in Agouti (*A*), Extension (*E*), Dun (*D*), Roan (*Rn*), and Grey (*G*) loci, in parental and offspring generations, were estimated according to test matings and the square root of recessive phenotype frequency. The population structure is in Hardy–Weinberg equilibrium only at *E* locus and coat colour is regarded by breeders. Black horses are favoured. Higher *E* locus homozygosity in blacks than in bays makes it easier to obtain black foals. Dun-diluted, roan and grey coat colours are undesirable and the population has come to consist almost uniformly of basic coat colours. These results show the importance of studies on population genetic structure, which despite no formal criteria for breeding for colour, can considerably change through generations.

Keywords: generation; population genetic structure; test mating

Horse breeders often have different preferences for coat colours, not only for aesthetic reasons, but also because they believe that colour may be associated with the horses' performance and temperament. Racing scores of differently coloured Thoroughbreds did not show such a relationship (Stachurska et al. 2007). A recent study on horses, genotyped in two loci and their behaviour, has shown that black mares *(aa)* were more independent than bay mares *(A_)* (Jacobs et al. 2016). A study by Finn et al. (2016) also revealed some behavioural differences between bay and chestnut horses. Several diseases associated with coat colour genes are known, e.g. lethal white foal syndrome found in frame overo horses (Metallinos et al. 1998), lavender foal syndrome (Brooks et al. 2010), congenital night blindness in leopard-spotted horses (Bellone et al. 2013), and multiple congenital ocular anomalies in silver horses (Andersson et al. 2013). The coat colour is often a primary source for identification and also a first indicator of incorrectly assigned parentage (Thiruvenkadan et al. 2008). In view of breeding, it is also interesting whether a population is selected for coat colour and what is the effect of such a selection (Stachurska and Brodacki 2008). Regarding the coat colour in the selection may help maintain genetic variability of a breed (Druml et

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al. 2009) or, inversely, may lead to important and undesirable changes in the genetic population structure (Stachurska et al. 2012).

The first cold-blooded horses, such as Ardennes, Bretons, Belgians and less often Percherons, were imported to Poland as late as the second half of the 19th century (Golebiewska et al. 2013). Some of them were crossed to indigenous horses. Genetic distances show a relationship between Polish Coldblood horses and primordial Polish Konik horses. Such a relationship could result from some common ancestors of both breeds (Stachurska et al. 2014). Nowadays, Polish Coldblood horses constitute half of the entire Polish horse stock. The studbook is not closed, hence among others some foreign sires and dams are still being included. To date, the colour structure of the Polish Coldblood breed has not been analysed. The objective of the present study was to estimate the frequency of alleles which produce coat colours in the Polish Coldblood horse population and to verify the hypothesis that coat colour is not regarded in the selection of this breed. The analysis is an example of studies on changes in allele frequency in a large population.

MATERIAL AND METHODS

The material included Polish Coldblood horses and their parents (sires, dams) which were registered in the studbook database since the foundation of the breed in 1964 till 2015, i.e. for over 52 years (Table 1). The material was divided into nine consecutive six-year periods (subpopulations) according to a horse's year of birth. The horses were registered in the studbook as adult stallions and mares after selection. Formally, the coat colour was not regarded in the breeding. Only in Sztumski and Sokólski horses, which belong to Polish Coldblood breed and are preserved as animal genetic resources, the conservation programme defined that horses of grey, blue dun, tobiano and leopard-spotted colours were not permitted. However, this fact did not concern the material studied, because the programme has worked in latest years. Since the foundation of the studbook, the horses were recorded as chestnut, bay, black, bay dun, blue dun, palomino, roan, grey and tobiano. In the study, horses by parents of unknown colours (registered mainly in the first subpopulation) and

Table 1. Division of Polish Coldblood horses registered in the studbook into subpopulations

Subpopulations	1	2	3	4	5	6	7	8	9	Mean/total
Years of registered horses' birth	till 1967	1968–1973	1974–1979	1980–1985	1986–1991	1992–1997	1998–2003	2004–2009	2010–2015	
Horses discarded (%)*	6.6	4.8	3.3	1.9	1.1	1.2	1.7	1.0	0.5	1.6
No. of horses studied	1 017	841	2 159	3 238	1 669	3 694	7 962	9 499	5 849	35 928

*parents of unknown colours and coat colours genetically not compatible with parents' colours

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horses of coat colours genetically not compatible with parents' colours were excluded from the material. Thus, 35 458 horses were analysed while considering Agouti (*A*), Extension (*E*), Dun (*D*), and Roan (*Rn*) loci, whereas 35 928 horses in the case of Grey (*G*) locus because of 470 grey horses. In greys, only the *G* locus could be regarded, since only the main colour in them was recorded in the studbook. The *C* and *To* loci, responsible for cream dilution and tobiano pattern, were not taken into account in the study since the number of horses of these colours was very low (5 and 13 animals, i.e. 0.014% and 0.036%, respectively).

Chestnut, bay, and black coat colours are controlled by *ASIP* (*A*) and *MC1R* (*E*) loci (Marklund et al. 1996; Rieder et al. 2001). The genotype of chestnut horses is *_ _ee*, that of bays is *A_E_*, and that of blacks is *aaE_* (Munckel 1929). We did not regard colour shades because hypotheses on bay and chestnut shades (Sponenberg 2003), as well as two kinds of blacks (Sponenberg and Weise 1997), have not been proven with molecular methods. According to current knowledge on coat colour inheritance, the *TBX3* (*D*) gene controls the presence of dun-diluted colours: red dun (*_ _eeD_*), bay dun (*A_E_D_*), and blue dun (*aaE_D_*) (Adalsteinsson 1978). Hence, appearance of bay dun and blue dun horses implicates the appearance of red duns which, however, were not registered in the studbook. Similarly, when the *MATP* (C^{cr}) gene occurs, not only palomino horses (*_ _ee*$C^{cr}C$) should be present, but also buckskins (*A_E_*$C^{cr}C$), smokies (*aaE_*$C^{cr}C$), and various kinds of cremellos (*_ _ _ _*$C^{cr}C^{cr}$) (Adalsteinsson 1974; Mariat et al. 2003). We suspect that possible red duns were classified as chestnuts, buckskins as bay duns, and smokies as blacks, whereas cremellos, if they appeared at all, were not registered. The *PMEL17* (S) gene responsible for silver colour was also ignored in the studbook, hence silver horses, which could occur in the population, were probably classified in the studbook as chestnut horses (Brunberg et al. 2006; Cieslak et al. 2013). No sufficiently exact tools to consider these facts were available; hence we did not interfere in the data downloaded from the studbook. The roan pattern is inherited as a dominant trait produced by *Rn* allele. The mutation responsible for the pattern was assigned to equine chromosome 3 (ECA3), in the *KIT* sequence, and was found to be lethal in the homozygous condition (Hintz and Van Vleck 1979). A dominant *G* allele in the *STX17* locus produces the grey pattern (Swinburne et al. 2002; Rosengren Pielberg et al. 2008). The tobiano pattern is controlled by a dominant *To* gene (*KIT*) (Haase et al. 2008). The patterns may appear on any basic or diluted colour (Sponenberg 2003).

SAS/Genetics software (Version 13.1, 2013) was used to estimate the allele frequencies and to compare the significance of differences between the distributions of alleles, phenotypes and genotypes with the χ^2 test. Frequencies of dominant homozygous and heterozygous parents were calculated according to the frequency of recessive homozygous offspring obtained in test matings (TM). The proportion of heterozygous to recessive homozygous offspring (e.g. *Aa* : *aa*) obtained from TM of dominant parents (*A_*) * recessive parents (*aa*) showed the genotype frequency (*AA* and *Aa*) within the tested dominant parents (*A_*). On this basis, dominant allele frequencies in sires and dams, as well as mean frequencies in parents and in the progeny were calculated. The frequency of recessive alleles in the offspring was also estimated in successive subpopulations, as well as in the entire parental and offspring generations from the square root of the recessive phenotype frequency (RF). The following phenotypes were considered: black, *aaee* chestnut, blue dun, black-roan, and black-tobiano horses in *A* locus; chestnut, palomino, and chestnut-roan horses in *E* locus; non-dun horses in *D* locus; non-roan horses in *Rn* locus; and non-grey horses in *G* locus. To estimate the frequency of *aaee* chestnuts, it was assumed that the ratio of phenotype frequencies produced by *A_E_* genotypes to *aaE_* genotypes should conform with that of *A_ee* chestnuts to *aaee* chestnuts (Stachurska et al. 2012). In the study, dominant allele frequency was considered because in most cases, the dominant genes produce the coat colours. The heterozygosity reduction resulting from the division into subpopulations was calculated with a fixation index (F_{st}) (Hartl and Clark 2007). According to gamete frequency in sires and dams estimated from TM, genotype distribution in the progeny was anticipated. Then, the expected and observed genotype distributions in the progeny were compared.

RESULTS

The total Polish Coldblood population was mainly chestnut (48.4%) and bay (42.0%), however in suc-

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cessive periods the coat colour structure changed (Figure 1). The percentage of chestnuts had increased until the 5th subpopulation, and since the 6th subpopulation, it has decreased to 41.4% in the latest subpopulation. Considering bays, the percentage had lowered in the first three periods and afterwards it grew to 46.4%. The percentage of blacks had decreased until the 5th subpopulation, but since then it has increased to 9.9%. Dun-diluted and roan horses were almost eliminated. The share of dun-diluted horses (bay dun and blue dun) reached 6.3% in the 3rd period, but since the 6th period, it has decreased to less than 1%. The percentage of roan horses started out at 16.3%, but since the 6th period, it has also been less than 1%. Horses with the grey pattern fluctuated around 1% and in the 9th subpopulation reached 1.6%. Thus, there have been 97.7% horses of basic colours in the latest period.

The frequency of dominant alleles producing coat colour in Polish Coldblood subpopulations is presented in Figure 2. The *A* and *E* allele frequencies changed the most, however in both cases, they returned almost to initial levels in the 9th period: 0.5842 and 0.3492, respectively. The tendency of the *A* allele to lessen, and the *E* allele to increase could be seen since the 5th subpopulation. The *Rn* allele frequency distinctly decreased from the initial 0.0842 to below 0.01 in the 5th period and remained at this level until the latest period. The frequency of *D* was below 0.01, except for 2nd and 3rd subpopulations. The *G* allele frequency has always been low and nowadays remains below 0.01.

The TM constituted 60.5% of all matings (Table 2). Considering the *A* locus, the frequency of homozygous *AAE_* bay parents calculated according to TM was higher in sires (0.4420) than in dams (0.3500). The frequency of chestnut *AAee* sires (0.3438) was similar to that of dams (0.3462). Bay homozygous *AAE_* parents were more frequent than chestnut homozygous *AAee* parents (0.4152 and 0.3456, respectively). More *aaE_* black stallions than mares born from bay * black and chestnut * black TM were registered in the studbook, regardless of whether their sires or dams were bay, chestnut or black.

As for the *E* locus, the frequency of dominant homozygous *_ _EE* parents, calculated according to chestnut dams mated to black and bay sires (0.3830 and 0.2101), was higher compared to black and bay dams mated to chestnut sires (0.2600 and 0.1580). In addition, the homozygosity of black sires in this locus (0.3830) exceeded the homozygosity of bay sires (0.2101). The total homozygosity of black parents (0.3400) was higher than in bay parents (0.1860). Fewer homozygous recessive *_ _ee* progeny, born from chestnut dams mated to black and bay sires (0.3083 and 0.3950), were registered in the studbook than when sires were chestnut and dams black or bay (0.3700 and 0.4207). The TM of both black and bay sires with chestnut dams resulted in the chestnut mare frequencies (0.3186 and 0.4060) higher than those of chestnut

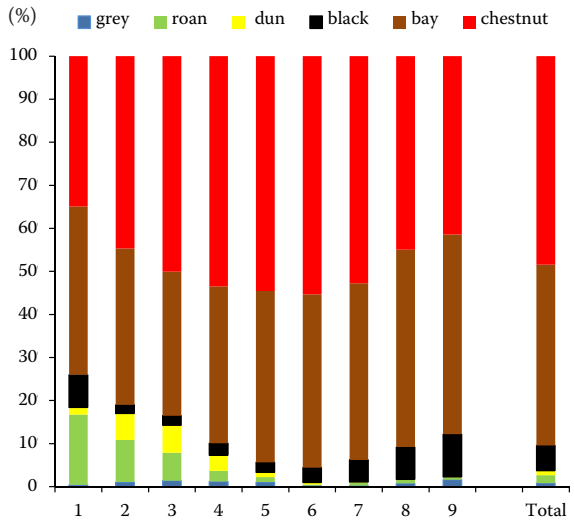

Figure 1. Percentage of variously coloured Polish Coldblood horses in successive subpopulations registered in the studbook

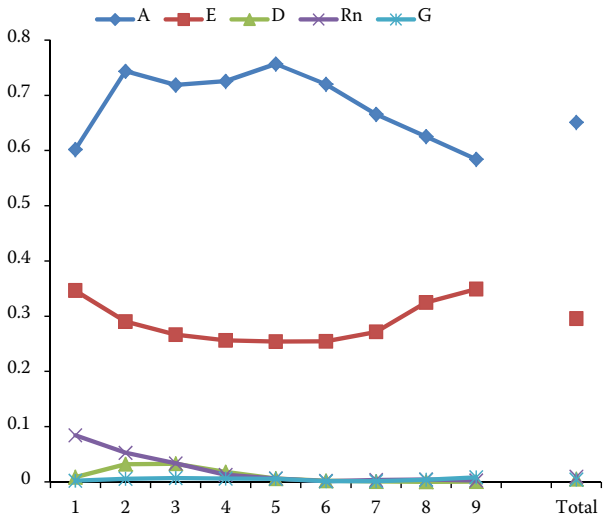

Figure 2. Frequency of dominant alleles in Polish Coldblood horses in successive subpopulations registered in the studbook

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Table 2. Frequency of genotypes producing coat colours obtained in test matings and registered in Polish Coldblood horse studbook (frequencies of dominant homozygous and heterozygous parents calculated according to the frequency of recessive homozygous progeny)

Locus	Parents				*n*	Parents		Recessive homozygous progeny			
	sires		dams			dominant homozygous	heterozygous	stallions	mares	horses in total	*P*
	colour	genotype	colour	genotype							
						AA	*Aa*	*aa*	*aa*	*aa*	
	bay	*A_E_*	black	*aaE_*	756	0.4420	0.5580	0.3307 M	0.2590 M	0.2790	A
	black	*aaE_*	bay	*A_E_*	1 048	0.3500	0.6500	0.3955 N	0.2913 N	0.3250	A
A	subtotal				1 804	0.4152	0.5848	0.3666 O	0.2780 O	0.2924	B
	chestnut	*_ _ ee*	black	*aaE_*	605	0.3438	0.6562	0.3478 Q	0.3218 Q	0.3281	
	black	*aaE_*	chestnut	*_ _ee*	1 087	0.3462	0.6534	0.4416 R	0.2860 R	0.3267	
	subtotal				1 692	0.3456	0.6544	0.4118 S	0.2972 S	0.3270	B
						EE	*Ee*	*ee*	*ee*	*ee*	
	black	*aaE_*	chestnut	*_ _ee*	1 087	0.3830	0.6170	0.2731 T	0.3186 T	0.3083	C
	chestnut	*_ _ee*	black	*aaE_*	605	0.2600	0.7400	0.3655	0.3717	0.3700	C
E	subtotal				1 692	0.3400	0.6600	0.3053 U	0.3378 U	0.3298	D
	bay	*A_E_*	chestnut	*_ _ee*	7 609	0.2101	0.7899	0.3690 V	0.4060 V	0.3950	E
	chestnut	*_ _ee*	bay	*A_E_*	6 410	0.1580	0.8420	0.4125	0.4238	0.4207	E
	subtotal				14 019	0.1860	0.8140	0.3877 X	0.4142 X	0.4070	D
						DD	*Dd*	*dd*	*dd*	*dd*	
D	dun	*D_*	non-dun	*dd*	340	0.0000	> 1.0000	0.5649	0.5376	0.5500*	
	non-dun	*dd*	dun *(D_)*	*D_*	357	0.0000	> 1.0000	0.5508	0.5816	0.5714*	
	subtotal				697	0.0000	> 1.0000	0.5588	0.5623	0.5610*	
						RnRn	*Rnrn*	*rnrn*	*rnrn*	*rnrn*	
Rn	roan	*Rn_*	non-roan	*rnrn*	522	0.0000	> 1.0000	0.5845	0.5905	0.5881*	
	non-roan	*rnrn*	roan	*Rn_*	859	0.0000	> 1.0000	0.6454 Y	0.5659 Y	0.5950*	
	subtotal				1 381	0.0000	> 1.0000	0.6212 Z	0.5763 Z	0.5923*	
						GG	*Gg*	*gg*	*gg*	*gg*	
G	grey	*G_*	non-grey	*gg*	118	0.4237	0.5763	0.2857	0.2892	0.2880	F
	non-grey	*gg*	grey	*G_*	324	0.1420	0.8580	0.4253	0.4304	0.4290	F
	subtotal				442	0.1903	0.8097	0.3876	0.4118	0.4050	
Total					21 727						

genotype frequencies marked with the same capitals (A…F in columns and M…Z in rows) differ at $P < 0.01$

*difference between the observed and expected frequency (0.5000) significant at $P < 0.01$; P – significant differences

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stallions (0.2731 and 0.3690). Similarly, a higher frequency of chestnut mares (0.3378 and 0.4142) than chestnut stallions (0.3053 and 0.3877) was found considering mating of blacks with chestnuts or bays with chestnuts, in total.

The TM showed that all dun parents were heterozygous for the *D* locus. The frequencies of homozygous recessive *dd* progeny in stallions and mares were similar. In addition, there was no dependence between the total frequencies of homozygous recessive *dd* progeny and TM direction (whether sires or dams were *D*_). The total *dd* frequencies in the offspring significantly exceeded 0.5, i.e. the highest frequency possibly obtainable in such matings.

The total frequency of homozygous recessive non-roan *rnrn* stallions and mares born from either TM direction (whether sires or dams were *Rn*_) and registered in the studbook was significantly higher (0.5923) than expected (0.5000), in the case when only heterozygotes would be mated to recessive homozygotes. Hence, all roan parents were heterozygous. The frequency of registered homozygous recessive progeny of both sexes from the two TM directions did not significantly differ.

According to the TM results, the registered non-grey *gg* progeny from non-grey dams mated to grey sires was almost 1.5 times fewer (0.2880) than from grey mares mated to non-grey sires (0.4290). The frequency of homozygous dominant sires (0.4237) was almost three times as high as that in dams (0.1420). The frequency of registered non-grey stallions and mares born from both TM directions (*G*_ sires or *G*_ dams) did not significantly differ.

Frequencies of the dominant *A* allele, calculated according to TM as well as estimated according to RF, were higher in sires (0.6210 and 0.6342, respectively) than in dams (0.6123 and 0.6267, respectively) (Table 3). Using both estimation methods, the *A* allele frequency was lower in the parental generation (0.6167 and 0.6304) than in the offspring (0.6582 and 0.6503). The frequency in dams and in parents in total was higher according to RF compared to TM.

Both estimation methods showed that the dominant *E* allele frequency was higher in sires (0.3093 and 0.3086) than dams (0.2733 using both methods). A comparison between the parental and progeny generations did not show a significant difference.

The dominant *D* allele frequency was similar in sires and dams. It was higher in parents than in offspring (0.0062 and 0.0053) according to both methods.

Table 3. Total statistical characteristics in loci producing coat colours in horses registered in Polish Coldblood studbook

Locus:		*A*		*E*		*D*		*Rn*		*G*	
Characteristics:		TM	RF	TM	RF	TM	RF	TM	RF	TM	RF
Sires	dominant allele frequency	0.6210 A	0.6342 B	0.3093 C	0.3086 D	0.0061	0.0061	0.0092	0.0092	0.0097 E	0.0069 F
Dams	dominant allele frequency	0.6123 AX	0.6267 B X	0.2733 C	0.2733 D	0.0063	0.0063	0.0139	0.0139	0.0056 E	0.0049 F
Parents	mean dominant allele frequency	0.6167 GY	0.6304 HY	0.2913	0.2896	0.0062 i	0.0062 j	0.0116 K	0.0116 L	0.0077 M	0.0059 N
Progeny	*n*	35 458	35 458	35 458	35 458	35 458	35 458	35 458	35 458	35 928	35 928
	mean dominant allele frequency	0.6582 G	0.6503 H	0.2946	0.2954	0.0053 i	0.0053 j	0.0092 K	0.0092 L	0.0043 M	0.0041 N
	SE	0.0025	0.0025	0.0024	0.0024	0.0004	0.0004	0.0005	0.0005	0.0003	0.0003
	F_{ST}		0.0166		0.0100		0.0181		0.0326		0.0088

TM = allele frequency expected according to test matings, RF = allele frequency expected according to recessive phenotype frequency, SE = standard error, F_{ST} = fixation index
genotype frequencies marked within a locus with the same capitals (A…N in columns and X, Y in rows) differ at $P < 0.01$; marked with the same letters (i, j) in columns differ at $P < 0.05$

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Table 4. Frequency of different main coat colours in 660 roan horses registered in Polish Coldblood studbook

Coat colour	Frequency	
	observed	expected
Bay-roan	0.5199	0.4370
Black-roan	0.0682	0.0620
Chestnut-roan	0.4119	0.5000

difference in observed and expected frequency in different roan horses significant at $P < 0.01$

Considering the dominant *Rn* allele frequency, sires and dams were similar, and the two estimation methods showed identical results. In the offspring, the frequency was lower (0.0092) than in parents (0.0116). The roan pattern occurred 19% more often with bay colour than was expected (Table 4). It occurred 18% less often than expected with chestnut colour.

The frequency of the dominant *G* allele, estimated according to both TM and RF, was higher in sires (0.0097 and 0.0069) than dams (0.0056 and 0.0049) (Table 3). Additionally, it was higher in the parents (0.0077 and 0.0059) than in progeny (0.0043 and 0.0041).

F_{st} estimated according to RF was low (0.0088–0.0326).

Expected and observed genotype frequency in the population calculated according to TM did not differ in horses of basic coat colours (Table 5). The observed frequencies of *D_* dun diluted, *Rn_* roan, and *G_* grey genotypes were lower than expected.

DISCUSSION

Horse breeding programmes specify which coat colours are admissible in a population, in order to maintain a model of a breed, particularly in breeds which have been preserved as animal genetic resources (Blackburn 2004). The genetic structure of a population can, importantly, change through generations, resulting in an altered frequency of coat colour phenotypes, i.e., an altered look of a breed (Stachurska and Brodacki 2008; Stachurska et al. 2012). It is important to analyse the source of such processes, and to monitor them, which requires studying a population across many years. This may be achieved on the basis of data included in studbooks. Today's molecular studies usually focus on samples of populations and thus, they cannot show trends underway in a whole population, since its foundation.

The material in the study was large, including over 35 000 horses registered in the studbook, over a half-century. The small coat colour loci based F_{ST} shows that the genetic differentiation between the successive subpopulations in the Polish Coldblood population was low. Thus, the population could be analysed in totality, without division. The genetic drift and migration effect were weak, since the standard error (SE) of allele frequency was low. Two methods of allele frequency estimation were used to make the analysis as accurate as possible: TM and RF. The TM, in principle, shows an observed allele frequency enabling us to determine homozygous genotype frequencies. However, it should be recalled that TM results do not include all horses born from those matings, because the horses are selected before registration in studbooks. Despite this, the SE was low as mentioned, both estimation methods showed comparable results and a difference was found only in the case of the *A* locus, where the dominant *A* allele frequency was particularly high.

The fact that more black stallions than black mares were registered in the studbook is not surprising and shows that the beautiful and rare black coat colour is desired. Probably, a downward trend in the dominant *A* allele frequency, visible in

Table 5. Expected and observed phenotype and genotype frequency in horses registered in Polish Coldblood studbook calculated according to gamete distribution in 21 727 test matings

Phenotypes:	Bay	Chestnut	Black	Dun	Roan	Grey
Genotypes:	*A_E_ dd rnrn gg*	*__ee dd rnrn gg*	*aaE_ dd rnrn gg*	*____D_ rnrn gg*	*____ dd Rn_ gg*	*_____ G_*
Expected frequency	0.4380	0.5039	0.0619	0.0123 A	0.0230 B	0.0152 C
Observed frequency	0.4376	0.5006	0.0613	0.0105 A	0.0184 B	0.0082 C

expected and observed genotype frequencies marked with the same capitals (A, B, C in columns) differ at $P < 0.01$

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four latest subpopulations, despite an increase of the dominant *A* allele frequency in total progeny compared with the parents, was also connected with favouring black horses. The *A* locus homozygosity in bay sires was higher than in bay dams. These results indicate that the breeders favour homozygous sires for their uniform offspring. Homozygosity is an important issue in population genetics. It is particularly valuable in sires because of their larger number of progeny. The genotype of a sire is usually determined according to the coat colours of its progeny after first breeding seasons, although presently, molecular tests for the presence of recessive alleles are also available. Breeders use homozygous sires more extensively in the breeding, hence such sires have a greater influence on the offspring allele frequencies. In turn, the similar *AA* dominant homozygote frequency in chestnut sires and dams shows that chestnut horse matings are random with regard to *A* locus. The recessive epistasis of *ee* genotype in chestnuts completely masks expression of the *A* locus (Sponenberg 2003). In the *E* locus, homozygosity was higher in black parents than in bay parents. The preferred black coat colour produced by recessive *aa* homozygotes is associated with a higher homozygosity of the *E* locus, because of e.g. frequent matings of like with like, in order to breed black foals. Similarly to the *A* locus, black and bay sires were more homozygous at the *E* locus than dams of respective colours, which is the consequence of the above-mentioned higher attention paid to males in breeding. In turn, weaker selection of females could be the reason of registering more chestnut mares than stallions in the studbook. Evidently, the breeders of Polish Coldblood horses were indifferent to chestnut coat colour. A lack of significant differences in allele frequencies between parents in total and progeny, as well as between allele frequencies estimated according to TM and RF, shows that the population is in Hardy–Weinberg equilibrium (HWE) at the *E* locus.

The fact that 16% more recessive *dd* homozygous stallions and mares were registered in the studbook than expected, and the *D* allele frequency was lower in the progeny compared to the parents, indicates that the dun-diluted colours are undesirable and breeders conduct a selection against this gene. Dominant *DD* homozygous sires and dams do not occur, whereas e.g. in blue dun Polish Konik horses *DD* homozygotes prevail (Stachurska and Brodacki 2003). The latest molecular findings have demonstrated more variants occurring at the *D* locus (Imsland et al. 2016; Stefaniuk-Szmukier et al. 2017). The homozygosity is lower, in the case of a multiple allelic series, hence the allelic series can be a reason of the lack of *DD* homozygotes in our study. The lower occurrence of dun genotypes than expected infers that the *D* locus is not in HWE. Dun-diluted colours frequently occur in so called "primitive" breeds, although even in primordial Huculs, they are not well liked (Stachurska et al. 2012). Only the mentioned primitive Polish Koniks are strictly selected for the blue dun coat colour (Stachurska and Brodacki 2003).

Over 50% of recessive *rnrn* homozygotes in the offspring from TM, indicating more than 100% of heterozygotes in parents (which is impossible), demonstrate that mainly non-roan horses are registered in the studbook, whereas roans are registered reluctantly. It would not be possible to obtain such results in any other case. The lack of dominant *RnRn* homozygotes in parents may confirm the earlier reported homozygous embryonic lethality (Sponenberg et al. 1984). However, evidence from the Quarter horse breed indicated otherwise (Sponenberg 2003). Production records documented the existence of roan horses that produce exclusively roan foals. In addition, DNA tests confirmed homozygosity in such horses in the genomic region that contains the *Rn* allele (www.vgl.ucdavis.edu/services/Roan.php). The higher bay-roan and lower chestnut-roan horse frequency than we expected in our study, confirms the linkage of the dominant *E* and *Rn* genes which are located closely in ECA3 (Marklund et al. 1999). Polish Coldblood horse breeders, unlike e.g. Austrian Noriker Draught horse breeders, have always regarded the roan pattern to be unattractive. Roan Noriker Draught horses are mated rotationally to black horses which enables on the one hand to avoid the possible lethal homozygous combination and on the other hand to maintain the roan horse percentage at 5–10% (Druml et al. 2009). However, this breed is under stabilising selection and breeding for colour makes it possible to retain the genetic diversity in the population. The limited registration of roan Polish Coldbloods in the studbook, the decreased *Rn* allele frequency in the progeny compared to the parental generation, as well as a lower observed roan genotype frequency than what was expected, concordantly document that the roan pattern is

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not desired, as it is in the case of the dun-diluted coat colours. The common opinion that the roan pattern is typical and usual in Polish Coldbloods can be disproved by our results.

A lower recessive *gg* homozygote frequency in progeny of grey sires than from grey dams shows a higher *GG* frequency in these sires. In consequence, more grey progeny from grey sires * non-grey dams were registered than from non-grey sires * grey dams. In addition, breeders preferred the progeny of coat colours similar to sires, not dams. The high homozygosity in grey sires was probably connected with the fact that many grey sires of foreign breeds were introduced in Polish Coldblood breeding. Frequent matings of greys *inter se* could be another reason for the relatively high frequency of homozygous grey sires.

It should be stressed that in spite of a very low *G* allele frequency, TM showed the presence of dominant *GG* homozygotes, both in sires and dams. Dominant *D* and *Rn* alleles were of a slightly higher frequency than *G*, however neither *DD* nor *RnRn* parents were found. Regardless of possible *RnRn* lethality, and the importation of grey homozygous sires, the argument that the lack of dominant homozygotes may be due to a low dominant allele frequency seems to be not always true. It is true when matings are random, whereas in the case of assortative mating of like with like, dominant homozygotes may occur even when the dominant alleles are rare.

On the other hand, the dominant *G* allele frequency was higher in parents than in progeny. This indicates a selection against grey coat colour. The number of grey-patterned Polish Coldblood horses has been small for many years, despite that such a selection criterion has not been formally established in the breeding programme. In addition, many Polish Coldblood horses are bred for meat purposes. The lower *G* allele frequency in the registered progeny than in parents, and fewer grey genotypes than expected, have been associated with a reluctance of slaughterhouses to purchase grey horses. This reluctance results from frequent melanomas which occur in these horses (Rosenberg Pielberg et al. 2008).

Summing up, the hypothesis that the coat colour was disregarded in the selection of Polish Coldblood horses turned out not to be entirely true. Of five loci analysed, only the *E* locus remains in *status quo*. The selection resulted in changes in the population genetic structure and the current Polish Coldblood horses are mainly bay and chestnut, sometimes black, and rarely grey. To further disseminate the black colour, an extensive use of homozygous black parents and conducting black * black matings should be continued. In turn, elimination or limitation of *Rn*, *D,* and *G* allele frequency may be considered risky. These alleles increase the diversity of the breed and, in addition, they were not studied with regard to possible pleiotropic effects on e.g. body growth and development.

CONCLUSION

The genetic structure in the Polish Coldblood population is not constant for loci controlling coat colours and coat colour is regarded by breeders in the selection. The population is in HWE only at the *E* locus. In compliance with breeding rules, sires are more homozygous than dams, with regard to loci controlling desired coat colours. Although black horses are not numerous, the black coat colour is favoured and higher *E* locus homozygosity in blacks than in bays makes it easier to breed black foals. Inversely, the lack of the *A* locus expression in chestnut coloured horses, which in addition are not preferred by breeders, results in random matings with regard to this locus. Dun-diluted, roan, and grey coat colours are undesirable and the population has come to consist, almost uniformly, of basic coat colours. These results show the importance of studies on the population genetic structure, which despite no formal criteria for breeding for colour, can change through generations, leading to an altered look of a breed.

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