Genetic distances between horse breeds in Poland estimated according to blood protein polymorphism

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ABSTRACT. The objective of the study was to estimate the heterozygosity and phylogenetic relationship between horse breeds in Poland, according to erythrocyte antigens and protein polymorphisms. The study included 15 434 horses: Polish Coldblood, Małopolski, Wielkopolski, Hucul, Polish Konik, Biłgorajski, Felin Pony, and ponies of Shetland origin. A total of 14 loci were studied: seven blood groups and seven protein polymorphism systems. Phylogenetic trees obtained for the erythrocyte antigens and protein variants were mostly alike which suggests that both kinds of markers may be equally used in estimating the similarity of animal populations. The lower polymorphism of the structural and enzymatic proteins, as compared with the erythrocyte antigen, resulted in a lower number of alleles per locus, lower heterozygosity, and closer genetic distances. The level of heterozygosity and phylogenetic trees of the breeds turned out to be mostly concordant with the known history of the populations. Małopolski and Wielkopolski horses are the most homozygous, the Huculs, Polish Koniks, and Biłgorajskis have a middle position, while the Polish Coldbloods and the ponies are the most heterozygous. The Polish Koniks are the most related to other breeds which shows that all the breeds studied, Polish Coldbloods included, have many indigenous ancestors. The Huculs, Polish Koniks, and Biłgorajskis are closely related. In spite of different histories, the Małopolski and Wielkopolski horses have the closest relationship. The Felin Ponies cluster together with the Shetlands. According to the blood groups and protein variation, the genetic diversity of the studied horse breeds is low and mainly due to individual differences. The low genetic variability of the breeds suggests reconsidering the long-term strategies of horse breeding in Poland, particularly of the conserved breeds.

Keywords: erythrocyte antigen; heterozygosity; horse population; protein marker; relationship

INTRODUCTION

Genetic distance is a measure of genetic diversity in taxonomy units or breeds. Genetic distance may be estimated using genetic markers which code antigen proteins as well as structural and enzymatic proteins (Jiskrová et al., 2002; Lippi and Mortari, 2003; Nogaj et al. 2003a). Nowadays, analyses are more often conducted on non-coding sequences of nuclear DNA, e.g. microsatellites (Cañon et al., 2000; Silva et al., 2012; Xu et al., 2012) as well as on mitochondrial DNA (Vilà et al., 2001; Mirol et al., 2002; Kavar and Dovč, 2008).

Different genetic markers have been used in breeding: firstly, for identification and parentage testing, secondly, for detection of genetic diseases, and thirdly, for identifying QTLs. In cattle, genome scanning by SNP microarrays has already been introduced. For equine parentage testing, the microsatellite sequences are preferable, however,

until recently, blood protein polymorphism has routinely been detected. Most of the sires and broodmares in Poland were analyzed using blood groups and protein variants. Although those markers are less polymorphic than the DNA markers, the blood groups and protein variation are still studied in horses when the parents were identified only by the blood protein polymorphism. Direct transition to DNA markers based on mutation detection, without re-testing, has not yet been available. Rosenberg et al. (2002) demonstrated a surprisingly high correlation between blood protein polymorphism and non-coding DNA sequences in human populations. However, a number of studies show discrepancies in the estimation of the heterozygosity and in the relations between horse breeds according to different markers (Bjørnstad et al., 1998; Luís et al., 2007). The differences may result both from the kind of marker and the method used. The use of various kinds of markers allows for valuable documentation of the genetic relationships between horse breeds.

Analyses of the genetic markers and phylogenetic trees can be used to find the biodiversity within populations and to catalogue the animal gene pool (Bjørnstad et al., 2001; Šveistienė and Jatkauskienė, 2006; Semik and Ząbek, 2013). Recent studies on the origin of the domestic horse have looked at the inter-population distances (Jansen et al. 2002; Aberle and Distl, 2004; McGahern et al., 2006). The genetic monitoring of endangered populations is an important issue (Ząbek et al., 2005; Àlvarez et al., 2011). Analyses of population structure performed on the genetic level contribute to the conservation of the gene pool and can act as supplementary information to be used in traditional breeding programmes.

Up till now, various markers have been used to study the genetic variation in chosen horse populations bred in Poland. The blood group loci and/or protein loci were used by Nogaj (1995), Pikuła et al. (1997), Gralak et al. (1998), Sasimowski et al. (2000), Nogaj and Nogaj (2000, 2001), and Nogaj et al. (2003a, b). Blood group and protein loci as well as microsatellite DNA sequences were used by Niemczewski and Żurkowski (2000). Studies based solely on DNA microsatellite polymorphism were conducted by Ząbek et al. (2003, 2005, 2006, 2008) and Semik and Ząbek (2013). In some of the mentioned investigations, the relations between breeds were depicted in phylogenetic trees.

The Polish Coldblood (PC) horse was formed in the second half of the 20th century by merging regional types of heavy horses, and by introducing mainly Swedish and French Ardennes sires into Poland. The regional types originally derived from warmblood and primitive dams covered by imported coldblood sires. The Małopolski (MP) and Wielkopolski (WP) horse breeds are warmbloods. They were created throughout the ages by crossings of indigenous horses living in the east and north-west Polish territories, respectively, with Thoroughbred, Purebred Arabian, Anglo-Arabian, and other foreign breeds. The Hucul (HC) horses are an old primitive breed created in the East Carpathian Mountains by crossing many types of indigenous and eastern primitive horses. The HC are also bred in the Czech Republic, Hungary, Slovakia, Romania, and Austria. The Polish Konik (PK) is a native primitive horse breed which derives from the wild Tarpans. The Biłgorajski (BK) horses are a very small population whose origin is similar to the PK, but they were recreated from farm stock half a century later than the PK. The Felin Ponies (FP) are a breed created by the planned crossing of ten breeds of ponies and small horses: the PK, HC, MP, Shetland-type ponies (SH), and Purebred Arabians included. The FP have been bred since the 1980s, and are the only Polish pony breed. The SH are registered in the Polish studbook as ponies of Shetland origin whose ancestors of other breeds than Shetlands may not exceed 50%. In spite of sometimes sharing a similar origin with the primitive horses, the pony populations (FP and SH) are assumed to be a cultural type, like the warmbloods and coldbloods.

Programmes for the conservation of animal diversity are conducted for the above-mentioned first five breeds, according to the Global Strategy for the Management of Farm Animal Genetic Resources established by the Food and Agricultural Organization in 1999. Those individuals who conform to specific, mainly pedigree criteria, can be admitted to the programme. Of the unified PC population, only Sztumski and Sokólski indigenous types were reidentified and they are conserved at present. As to the MP and WP breeds, approximately 30% and less than 10% of dams, respectively, are included in the conservation programme. The dams cannot be offspring of Thoroughbred, Purebred Arabian or Anglo-Arabian sires, nor can the dams be covered by sires of those breeds. The status of the HC, and particularly PK, is different since the programmes for the conservation of these horses were created earlier. The PK was the first farm animal breed

conserved long before the Global Strategy was established. At present, the HC and PK programmes include almost the entire breeding populations, except for a few dams.

The aim of the study was to estimate the heterozygosity and phylogenetic relationship between the above-mentioned horse populations bred in Poland, according to erythrocyte antigens and protein polymorphisms.

MATERIAL AND METHODS

Horses. Blood samples were collected randomly from 15 434 horses destined for breeding: 6100 PC, 2290 MP, 200 WP, 5180 HC, 1054 PK, 60 BK horses as well as 400 FP and 150 SH. The percentage of these breeds in the effective horse population size in Poland is the following (%): 49.7, 7.8, 7.7, 7.4, 5.4, 0.3, 1.8, and 0.7, respectively. In the study, both horses entered in the programmes for conservation of breeds of the Global Strategy and horses bred independent of the programmes, were included. For simplicity, the word "breeds" is used in the study, although the BK and SH are small populations not assumed to be breeds.

Analytical methods. The laboratory analysis was conducted in the Chorzelów Experimental Station of the National Research Institute of Animal Production in Cracow-Balice, Poland. A total of 14 loci were studied: seven blood groups (EAA, EAC, EAD, EAK, EAP, EAQ, EAU) and seven protein polymorphism systems: albumin (AL), vitamin D-binding protein (GC), serum carboxylesterase (ES), A1B-glycoprotein (A1B), transferrin (TF), 6-phosphogluconate dehydrogenase (PGD), and glucosephosphate isomerase (GPI). The erythrocyte antigens were identified by serological tests according to Stormont and Suzuki (1964). Erythrocyte enzymes (PGD, GPI) were detected in accordance with the Gahne and Juneja (1985) method, whereas other proteins (AL, GC, ES, A1B, TF) were identified according to Juneja et al. (1978). The latter method was completed with a reagent set which enabled easier ES detection.

Statistical methods. The erythrocyte antigens, the proteins, and both kinds of biochemical markers were analyzed separately. The mean number of alleles detected in the loci was calculated. The average heterozygosity was determined according to Nei and Roychoudhury (1974). The formulae according to Hartl and Clark (2007) were used to calculate total heterozygosity (H_T), mean heterozygosity in population divided into subpopulations (breeds) (H_S), fixation index (F_{ST}), and the Wahlund effect. The genetic distances were estimated using Nei's (1972) method which was the method used in most studies on various domestic animals (e.g. Nogaj et al., 2003b; Ząbek et al., 2005; Čítek et al., 2006). Thus, it was possible to directly compare the results of different studies. The genetic distance matrices were transformed into phylogenetic trees by Neighbour Joining (NJ), using PHYLIP software (Version 3.65, 2005) (Sokal and Michener, 1958; Saitou and Nei, 1987; Felsenstein, 2005). For visualizing the phylogenetic trees MEGA software (Version 4.0, 2007) was used.

RESULTS

A total of 57 erythrocyte antigen alleles and 27 structure and enzymatic blood protein alleles were found in the studied horses. The mean numbers of alleles per locus identified in the horse breeds are presented in Table 1. The numbers of alleles coding erythrocyte antigens were double the numbers of alleles coding protein variants. In the PC, the numbers of alleles producing the erythrocyte

Table 1. Mean number of alleles in erythrocyte antigen loci and protein loci

Loci	Breeds							
	MP	WP	HC	PK	BK	FP	SH	PC
Erythrocyte antigens	6.571	5.429	5.429	6.143	3.286	6.714	6.286	7.286
SE	0.790	0.985	0.688	0.772	0.548	0.925	0.875	0.995
Proteins	3.571	3.286	3.000	3.000	2.286	3.143	3.143	3.857
SE	0.580	0.651	0.609	0.667	0.416	0.564	0.698	0.725
Total	5.071	4.357	4.214	4.571	2.786	4.929	4.714	5.571
SE	1.097	1.087	0.827	1.031	0.395	1.273	1.160	1.489

MP = Małopolski, WP = Wielkopolski, HC = Hucul, PK = Polish Konik, BK = Biłgorajski, FP = Felin Ponies, SH = Shetland-type ponies, PC = Polish Coldblood, SE = standard error

Table 2. Total heterozygosity (H_T), mean heterozygosity in population divided into subpopulations (breeds) (H_S), fixation index (F_{ST}), and Wahlund effect in erythrocyte antigen loci and protein loci

Loci	H_T	H_S	F_{st}	Wahlund effect
Erythrocyte antigen loci				
EAA	0.708	0.653	0.077	0.055
EAC	0.498	0.430	0.137	0.068
EAD	0.887	0.823	0.072	0.064
EAK	0.074	0.067	0.089	0.007
EAP	0.627	0.595	0.051	0.032
EAQ	0.675	0.594	0.120	0.081
EAU	0.550	0.519	0.056	0.031
Mean	0.574	0.526	0.086	0.048
Protein loci				
AL	0.471	0.455	0.033	0.016
GC	0.222	0.210	0.054	0.012
ES	0.473	0.423	0.107	0.051
A1B	0.209	0.183	0.121	0.025
TF	0.751	0.699	0.070	0.052
PGD	0.314	0.288	0.082	0.026
GPI	0.075	0.070	0.065	0.005
Mean	0.359	0.333	0.076	0.027
Total mean	**0.467**	**0.429**	**0.081**	**0.037**

EAA, EAC, EAD, EAK, EAP, EAQ, EAU = blood groups, AL = albumin, GC = vitamin D-binding protein, ES = serum carboxylesterase, A1B = A1B-glycoprotein, TF = transferrin, PGD = 6-phosphogluconate dehydrogenase, GPI = glucose-phosphate isomerase

antigens and protein variants were the highest (7.286 ± 0.995 and 3.857 ± 0.725, respectively), whereas in the BK both numbers were the lowest. Consequently, the total mean allele number was the highest in the PC (5.571 ± 1.489) and the lowest in the BK (2.786 ± 0.395). A high total mean allele number was also detected in the MP and FP breeds.

In particular loci, H_T and H_S values showed a high compatibility: EAD, TF, and EAA loci were the most heterozygous, while GPI and EAK loci were the least heterozygous (Table 2). The H_T and H_S means in loci controlling the erythrocyte antigens equaled 0.574 and 0.526, respectively. The H_T and H_S means in protein polymorphism loci amounted to 0.359 and 0.333. The F_{ST} index ranged from 0.051 to 0.137 in blood group loci and from 0.033 to 0.121 in protein polymorphism loci. In turn, the Wahlund effect ranged from 0.007 to 0.081 in blood group loci and from 0.005 to 0.052 in protein polymorphism loci.

The mean heterozygosity values per locus in various breeds are presented in Table 3. The heterozygosity estimated using erythrocyte antigens (0.454–0.593) was, on average, 1.5 times higher than that obtained from protein polymorphisms (0.288–0.404). When taking all the analyzed markers into consideration, the lowest heterozygosity was found in the MP (0.371 ± 0.005) and WP (0.402 ± 0.006) horses. In the HC, PK, and BK populations the heterozygosity was approximately 0.41. The FP and PC horses showed heterozygosity over 0.45 and in the SH the heterozygosity reached almost 0.50.

According to the erythrocyte antigens, the mean genetic distance values ranged from 0.114 (PK and SH) to 0.152 (MP) (Table 4). The least genetic distance occurred between the MP and WP breeds (0.020). Shorter distance was also detected between the PC and PK, the FP and HC, as well as the FP and SH horses. The largest genetic distance was noted between the MP and PK as well as between the MP and PC horses. For protein polymorphisms, the distances were lesser than the distances obtained for the erythrocyte antigens. The mean distance values according to protein polymorphism ranged from 0.037 (PK) to 0.062 (BK). The shortest distance occurred between the MP and WP (0.011). The genetic relationships were

Table 3. Heterozygosity ($\bar{H}$) in erythrocyte antigen loci and protein loci

Loci	Breeds							
	MP	WP	HC	PK	BK	FP	SH	PC
SE	0.009	0.008	0.007	0.009	0.005	0.009	0.005	0.009
Proteins ($\bar{H}$)	0.288	0.322	0.292	0.352	0.327	0.394	0.404	0.309
SE	0.008	0.008	0.012	0.007	0.004	0.005	0.008	0.009
Total ($\bar{H}$)	0.371	0.402	0.412	0.419	0.415	0.476	0.499	0.452
SE	0.005	0.006	0.007	0.005	0.006	0.006	0.007	0.007

MP = Małopolski, WP = Wielkopolski, HC = Hucul, PK = Polish Konik, BK = Biłgorajski, FP = Felin Ponies, SH = Shetland-type ponies, PC = Polish Coldblood, SE = standard error

Table 4. Genetic distances between horse breeds with regard to erythrocyte antigen loci (above diagonal) and protein loci (below diagonal)

	Breeds	Erythrocyte antigens								
		MP	WP	HC	PK	BK	FP	SH	PC	Mean*
Proteins	MP		0.020	0.211	0.231	0.139	0.150	0.095	0.221	0.152
	WP	0.011		0.165	0.123	0.186	0.107	0.163	0.161	0.132
	HC	0.048	0.065		0.097	0.087	0.053	0.089	0.125	0.118
	PK	0.039	0.058	0.023		0.092	0.073	0.120	0.061	0.114
	BK	0.071	0.065	0.056	0.023		0.101	0.152	0.178	0.134
	FP	0.021	0.021	0.059	0.037	0.058		0.062	0.096	0.092
	SH	0.044	0.066	0.075	0.047	0.090	0.039		0.120	0.114
	PC	0.036	0.052	0.028	0.030	0.069	0.034	0.047		0.137
Mean*		0.039	0.054	0.051	0.037	0.062	0.038	0.058	0.042	

MP = Małopolski, WP = Wielkopolski, HC = Hucul, PK = Polish Konik, BK = Biłgorajski, FP = Felin Ponies, SH = Shetland-type ponies, PC = Polish Coldblood

*mean genetic distance for particular breeds; total means 0.124 (erythrocyte antigens) and 0.048 (proteins)

also relatively weak between the PK and HC, the PK and BK, the FP and MP, as well as the FP and WP. A greater divergence was found between BK and SH. When taking into consideration all of the examined markers, the mean genetic distance value was the lowest in the PK (0.061) and FP (0.066) and the greatest in the case of the BK (0.093) and WP (0.090) (Table 5). The strongest connection was found between the MP and WP (0.015) and the weakest relationship was between the MP and BK (0.127) (Table 5).

Figure 1 illustrates phylogenetic trees constructed using erythrocyte antigens, protein variants, and all of the considered markers. Regarding erythrocyte antigens, the MP clustered with WP, PK with PC, and HC with BK. The HC and BK clustered together with the PK and PC. The distance between the FP and SH was also relatively short. As for the protein polymorphisms, the links were the closest between the MP and WP as well as between the BK and PK. The FP and SH were also related, forming one cluster with the MP and WP breeds. Another cluster was formed by the BK, PK, and HC horses. The position of the PC was in the middle of the two clusters; a little bit closer to the MP-WP-FP-SH group. According to all the considered markers, the relationships were almost the same as those based exclusively on the protein variants. Only the PC showed a slightly closer link with the BK-PK-HC group.

DISCUSSION

Until now, studies done on the heterozygosity and genetic distances between horse populations

Table 5. Genetic distances between horse breeds with regard to erythrocyte antigen loci and protein loci (overall)

Breeds	MP	WP	HC	PK	BK	FP	SH	PC
WP	0.015							
HC	0.112	0.105						
PK	0.074	0.085	0.053					
BK	0.127	0.115	0.067	0.051				
FP	0.062	0.115	0.055	0.050	0.075			
SH	0.101	0.102	0.078	0.074	0.113	0.046		
PC	0.082	0.095	0.062	0.040	0.106	0.057	0.075	
Mean*	0.082	0.090	0.076	0.061	0.093	0.066	0.084	0.074

MP = Małopolski, WP = Wielkopolski, HC = Hucul, PK = Polish Konik, BK = Biłgorajski, FP = Felin Ponies, SH = Shetland-type ponies, PC = Polish Coldblood

*mean genetic distance for particular breeds; total mean 0.078

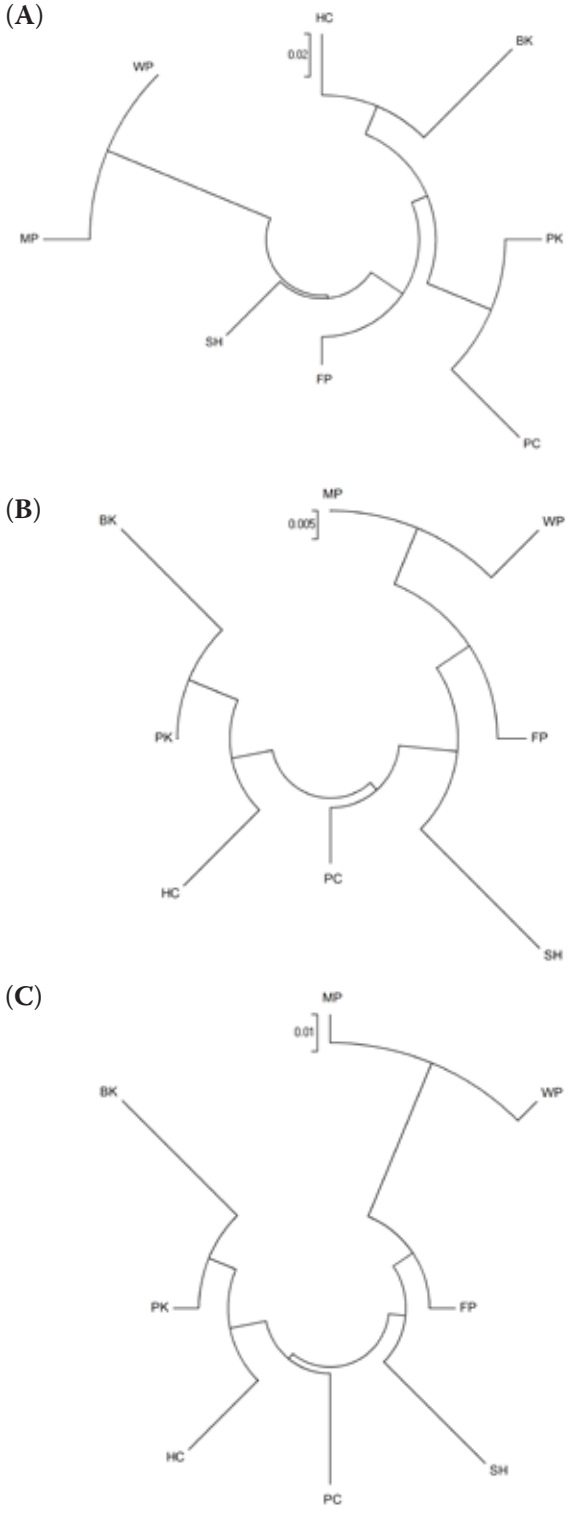

Figure 1. Dendrogram constructed by Neighbour Joining method according to (**A**) erythrocyte antigens, (**B**) protein polymorphisms, and (**C**) erythrocyte antigens and protein polymorphisms (overall)

MP = Małopolski, WP = Wielkopolski, HC = Hucul, PK = Polish Konik, BK = Biłgorajski, FP = Felin Ponies, SH = Shetland-type ponies, PC = Polish Coldblood

bred in Poland neither have included so many horses, nor have so many breeds been included in the phylogenetic trees (e.g. Nogaj et al., 2003b; Semik and Ząbek, 2013). In our study, based on over 15 thousand horses, the dendrograms show the links among eight populations of three horse types bred in Poland. The adopted methods estimating the genetic variation of populations by the blood protein polymorphisms enabled to investigate the real identity of genes. Hence, the methods were more relevant than pedigree analyses. As known, the pedigree analyses show the probability of identity of genes only according to the origin of the gene, although the genes can be not identical *sensu stricto*.

The mean number of alleles per protein locus was twice as low as per blood group locus and the heterozygosity in protein loci was 1.5 times as low as in blood group loci. Such findings reflect the relation between the allele polymorphism and the heterozygosity: the higher the number of alleles per locus the higher the maximum expected heterozygosity (MacHugh et al., 1997). Therefore, the levels of H_T were higher for the more polymorphic loci controlling blood groups and lower in the less polymorphic loci responsible for protein variants. The results agree with earlier studies conducted on various types of markers (Gralak et al., 1998; Luís et al., 2007). The lower allelic polymorphism of the biochemical markers is the reason for the lower heterozygosity obtained for those loci compared to the non-coding DNA sequences.

As known, the effective population size may influence the level of inbreeding: the lower the effective population size, the higher the inbreeding level (Filistowicz, 2011). The higher inbreeding level is associated with the lower heterozygosity. We found similar heterozygosity values in populations of completely different sizes. For instance, the FP and the PC showed similar levels of heterozygosity, although the FP effective population size is incomparably smaller than that of the PC. The similar heterozygosity values may indicate that even the small populations, like the FP, are simultaneously big enough so that inbreeding is not a risk.

The F_{ST} levels for both kinds of loci turned out to be moderate (0.05–0.15) or low (below 0.05), which shows that populations were not very differentiated. The diversity mostly resulted from individual variability and less from inter-breed differences. Lippi and Mortari (2003) studying biochemical polymorphism markers in two Brazilian horse breeds found slightly lower F_{ST} estimates

in GC, A1B, TF, and PGD loci and slightly higher F_{ST} estimates in AL and ES loci. Our results are similar to the F_{ST} found by Ząbek et al. (2005) for microsatellite loci in BK, MP, and Thoroughbred horses (0.053–0.151). MacHugh et al. (1998) discuss the phenomenon of microsatellite allele frequency drift which plays a more important role in genetic differentiation among closely related populations than mutation. The authors consider among others the F_{ST} as an appropriate drift-based classical measure of such relationships. In seven European cattle breeds the mean F_{ST} for 20 microsatellite loci amounted to 0.112. The similarity of the H_T and H_S values in particular loci found in the present study resulted in a low Wahlund effect. The low F_{ST} values and Wahlund effect show that the division into subpopulations (breeds) did not cause a significant heterozygosity reduction in the total population and that the breeds are not considerably differentiated (Gillespie, 2004).

The mean genetic distances calculated using the proteins were 2.5 times as low as those based on erythrocyte antigens, which is also associated with the differentiated level of marker polymorphism. The mean distance estimated using the biochemical loci (0.078) was larger than that calculated using the data presented by Moazami-Goudarzi et al. (1997) in 10 European cattle breeds for 17 microsatellite loci (0.050). The authors suggest that the genetic distance measurements might be underestimated.

The relatively lower heterozygosity of the MP and WP reflects the common opinion that those warmblood breeds are more homozygous than other Polish horses. It is important to note, that it was only in the 1960s that the MP and WP breeds were officially approved. Maintaining the constant pedigree selection criteria for several generations, however, could have resulted in the lower heterozygosity compared to other breeds. From four Polish halfbred warmblood populations (MP, WP, Silesian, and Polish Warmblood), the MP breed is assumed to have been crossed the least with foreign breeds. According to the results, the MP heterozygosity is indeed slightly lower than that of the WP. On the other hand, according to Ząbek et al. (2006), the MP breed is under the strong influence of other breeds, especially Thoroughbreds. The expected MP heterozygosity for the microsatellites was similar to that reported in other crossbred horse populations.

Three primitive populations, the HC, PK, and BK, showed a medium level of heterozygosity, which is somewhat surprising. It seems the HC and PK populations accepted as pure breeds in the 1980s, when the studbooks were closed, should be more homozygous. The level of inbreeding in these two breeds was investigated to exclude a possible depression effect on the reproduction traits. Jezierski (1993) revealed that the inbreeding level in 14.9% of PK dam matings exceeded 10%. According to Kwiecińska and Olech (2008), the inbreeding in 7.6% of the HC dams was over 8%. The heterozygosity analyzed in the present study shows that inbreeding depression is not a threat. The ancestors of BK were more often crossed with warmblood horses than the PK. In consequence, the BK heterozygosity could have been higher than the one found. The population, however, is so small that presumably the inbreeding and genetic drift caused the opposite effect: a decrease in the heterozygosity.

The relatively higher heterozygosity in the SH and FP was expected since as mentioned, the populations have been created by crossbreeding many types or breeds of horses. The diversity of ancestors accepted in the SH group causes certain traits to show up, e.g. some ponies display a leopard pattern that is unacceptable in pure Shetlands. The results of the study show the two pony populations as still being less homozygous compared to other breeds.

The PC population comes from crossing different types of Polish coldblood horses, in accordance with the studbook regulation implemented in 1972. The types bred in various regions were phenotypically differentiated, hence, genetic differences were probably also significant. The differentiation could be the reason for the higher heterozygosity in the PC breed. Iwańczyk et al. (2006), on the basis of blood protein polymorphisms, microsatellites, and mtDNA evaluated both the heterozygosity and diversity levels in the PC horses as fairly high.

The heterozygosity analyzed using blood groups is consistent with that found by Nogaj et al. (2003b) in the SH, FP, HC, BK, and MP horses. Such a consistency occurred in spite of the fact that there were fewer samples of those breeds in the cited study (639). Only the heterozygosity of the PK was different: it was the lowest in the cited study, and at a medium level in our results.

According to protein variants, the genetic distances were lower than when erythrocyte antigens were used. That dependence is associated with the above-mentioned level of marker polymorphism (MacHugh et al., 1997). The phylogenetic dendrograms obtained for the blood group and protein

polymorphism markers are mostly concordant. Slight differences in the clusters may have resulted from the generally low genetic distances between the breeds which is shown by the low F_{ST} index. The similarity of the trees suggests that both kinds of markers may be used in estimating the similarity of animal populations.

The dendrograms show the close relation between the MP and WP horses, according to the erythrocyte antigens, protein variants, and both kinds of biochemical polymorphism markers. Such a relationship is difficult to interpret since the breeds are considered to be historically different. Many ancestors of the MP were oriental horses and in the 20th century, Thoroughbreds, which is why the breed is often still called halfbred Anglo-Arabian. The WP horses partly derive from German breeds and have a good deal of Thoroughbred blood as well as some Purebred Arabian and Anglo-Arabian blood. Crosses between the MP and WP were not frequent in the past and nowadays such offspring are registered exclusively in the Polish Warmblood studbook. Our results are consistent with findings by Pikuła et al. (1997), who analyzed the protein polymorphisms. The authors showed a closer genetic distance between WP and MP stallions (0.009) than that between Polish Warmblood and the WP (0.012) or the Polish Warmblood and the MP (0.016). The more distant relationships of the Polish Warmbloods with the WP and MP were expected, since the Polish Warmbloods are crosses of many breeds, not only the WP and MP. Similar results were found by Nogaj and Nogaj (2001) using antigen erythrocytes (0.009, 0.012, and 0,021, respectively). It seems that the close relationship between the MP and WP may be partly due to the extensive past use of Thoroughbred, Purebred Arabian, and Anglo-Arabian sires meant to improve the populations. Phylogenetic analysis of microsatellite sequences performed by Ząbek et al. (2006) revealed that Thoroughbreds made the greatest contribution to the gene pool of the current MP population. Semik and Ząbek (2013) also used microsatellites and found that Thoroughbred and WP horses were closely related. Simultaneously, both breeds were more distantly related to the MP. The authors interpreted the latter result as the effect of a greater contribution of Purebred Arabians to the creation of the MP. We suggest that different markers, blood protein polymorphisms and microsatellites, may have been the reason for the difference in the results. The total number of WP and MP horses included in the cited study was only 65, which probably also caused the disagreement in the results. The close genetic relation between the MP and WP found in the present study, among others, could have been caused by the selection towards similar performance traits in both breeds. Recent studies have shown a number of protein loci that may have an impact on the horse athletic performance (Schröder et al., 2011).

The mean genetic distance according to the considered markers shows that of the populations studied, the PK are the most related to the other breeds. Since the PK is the indigenous breed, its relation with other breeds indicates that all the breeds have many native ancestors. The distinctness of the PK is often pointed out: their relationship with wild horses and their characteristic blue-dun colour without white markings. According to the present results, the PK are derived from the same stem as other populations bred in Poland. In turn, the low FP mean genetic distance to other breeds seems to be the consequence of the recent crossbreeding.

The relationships between the PK, BK, and HC are noticeable. The three primitive breeds cluster together, although the BK according to the erythrocythe antigens, are more closely related to the HC, whereas when considering the protein variants, the BKs are closer to the PK. The PK and BK were expected to be related populations, because as mentioned, their indigenous ancestors were similar. However, in the case of the PK, the national programme of restoration and conservation started in 1930s. The BK, on the other hand, were kept on small farms sometimes being crossed with warmblood or indigenous primitive horses for more than 50 years longer. It was only in the 1980s that BK breeding was founded. Those facts may have caused the differentiation of the two populations. The closer connection of the BK with HC than the BK with PK according to the blood groups may indicate that the BK and HC populations were created in a similar way. When the protein variants and studied markers together are considered, the genetic divergence between the BK and HC is more noticeable. It seems that the difference may have resulted mainly from the different territories of the breeds. Interestingly, Georgescu et al. (2011) classified the HC horse from Romania separately from all other primitive breeds, inferred from mitochondrial D-loop variation. Presumably the Romanian HC genetically differ from the HC bred in Poland.

Simultaneously, the mean BK distance to other breeds, particularly the distances to MP and WP breeds, turned out to be relatively large. Those results are not entirely concordant with common opinions concerning the numerous crossings between the BK and MP ancestors. Instead, the large distance may be because of the stronger influence of the indigenous and various local crossbred sires used in BK breeding (Sasimowski, 1964). According to Ząbek et al. (2005), who used microsatellites, the genetic distance between MP and BK was shorter than that between the BK and Thoroughbreds. The Thoroughbreds have always been assumed to be a sophisticated saddle breed, used mainly for racing. It seems obvious that the Thoroughbreds were not crossed with BK ancestors which were mainly draught animals. Instead, the MP and their ancestors were a multi-purpose type and, presumably, were sometimes mated with the BK ancestors. Our results are fairly consistent with earlier findings by Nogaj et al. (2003b) who used erythrocyte antigens and found that PK, BK, and HC populations were close to each other. To sum up, the trees suggest the three breeds had common ancestors in the past.

The PC, according to blood groups clusters with the primitive breeds (HC, PK, BK) and with regard to protein variants, is in a middle position between the primitive and cultural types (MP, WP, FP, SH). As for the blood groups, the clustering between the PC and the PK shows that the PC are derived not only from imported horses but also from some indigenous horses. It seems that similar indigenous horses could have been the ancestors of the PK. In the 1930s, the PK ancestors were selected for breeding to renew the extinct Tarpan, so they did not carry the coldblood phenotypic traits and were not influenced by the coldbloods. On the other hand, the PC were not influenced by the PK. Hence, the PC–PK relationship may have resulted from some common ancestors. The dendrogram constructed using the protein variants does not show a strict relationship between the PC and PK. Maybe in the selection, some phenotypic traits associated with the protein variants were indirectly considered, whereas the blood groups were not taken into account. The erythrocyte antigens are more indifferent to the organism, hence the loci which produce them show greater polymorphism and heterozygosity. The difference in the role of the markers may be partly the reason for the differences in the dendrograms. Interestingly, studies by Iwańczyk et al. (2006) revealed that PC horses formed a cluster that paired with pony breeds.

Low mean FP genetic distance to other breeds reflects the numerous crossings which were made when creating the FP population. The close relationship between FP and SH pony populations is in accordance with the earlier finding by Nogaj et al. (2003b). As mentioned, the FP was formed with extensive use of SH sires. The FP and SH relationships with the PC and with the MP-WP cluster are probably due to some native ancestors.

Summing up, it is the PK which are most related to the other breeds. This result shows that all the studied breeds, PC included, have many indigenous ancestors. The HC, PK, and BK are closely related. In spite of different histories, the MP and WP horses have the closest relationship which is presumably due to the Thoroughbred, Purebred Arabian, and Anglo-Arabian contribution. The FP cluster together with the SH.

The results of the study may be used in developing strategies of horse breeding in Poland. The low genetic diversity leads to a risk of loss of the distinctiveness of the breeds. From the genetic point of view, separate breeding of closely related populations does not seem to be justified, whereas a high individual diversity within a breed would suggest a possibility of division of such a population. In that regard, there were good reasons for the distinction of the old Sztumski and Sokólski indigenous types from the PC breed into separate programmes for conservation.

CONCLUSION

Phylogenetic trees obtained for the erythrocyte antigens and protein variants were mostly alike which suggests that both kinds of markers may be used in estimating the similarity of animal populations. The lower polymorphism of the structural and enzymatic proteins, as compared with the erythrocyte antigen, resulted in a lower number of alleles per locus, lower heterozygosity, and lower genetic distances. The level of heterozygosity and phylogenetic trees of the breeds turned out to be mostly concordant with the known history of the populations. According to the blood groups and protein variation, the genetic diversity of the studied horse breeds is low and mainly due to individual differences. The low genetic variability of the breeds suggests reconsidering the long-term

strategies of horse breeding in Poland, particularly of the conserved breeds.

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