

GENETIC POLYMORPHISM OF BLOOD PROTEINS IN YUGOSLAV SHEPHERD DOG

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The present study is aimed to provide the first insight into the genetic constitution of Yugoslav shepherd dog based upon analysis of blood proteins and enzyme polymorphism. A total of 60 Yugoslav shepherd dogs were included in the study and polymorphism of hemoglobin (Hb), acid phosphatase (Acp), superoxide dismutase (Sod), albumin (Al), and transferrine (Tf) was electrophoretically analysed. Hb system did not show polymorphism. FS and SS phenotypes of Acp were established with Acp^S allelic gene displaying significantly higher frequency (0.82) than Acp^F (0.18). The analysis of Sod polymorphism showed SodAA and SodAB phenotypes controlled by Sod^A (0.74) and Sod^B (0.26) with clear predominance of Sod^A allele. Both Al allelic genes, Al^F (0.30) and Al^S (0.70), and all three possible phenotypes (F, FS, and S) were detected. Seven Tf phenotypes controlled by five allelic genes Tf^A, Tf^B, Tf^C, Tf^D, and Tf^E were revealed which makes the Yugoslav shepherd dog the only dog breed displaying all five Tf genes. The associated gene frequencies were (0.13, 0.35, 0.06, 0.41 i 0.05), respectively. According to the calculated values of homogeneity index, Al and Tf were identified as highly polymorphic protein markers which is a result of importance for future genetic characterization of the Yugoslav shepherd dog. Significant deviations from Hardy-Weinberg equilibrium were detected at Sod and Tf loci, these being primarily attributed to genetic drift, although crossbreeding should not be disregarded as a factor of possible importance as well.

Key words: protein, polymorphism, genome, Yugoslav shepherd dog

INTRODUCTION

Studies focused on the genetic characterization of species and breeds of domestic animals have a wide range of possible applications. These studies have been carried out to investigate the origin and course of domestication of different species and breeds (Braend and Roed, 1987; Tanabe, 1990; Troy, 2001; Savolainen *et al*, 2002), to characterize the breeds and lines (by estimation of genetic drift and distance) (Pihkanen *et al*, 1996; Tsuda *et al*, 1997), to design adequate breeding programs based upon the identification of genes which

control economically relevant traits (Christensen *et al.*, 1985; Andersson *et al.*, 1987), for parentage control (Juneja *et al.*, 1987; Dostal and Stratil, 1994; Garcia *et al.*, 2002), etc. Genetic characterization of autochthonous breeds in different geographical localities is of particular importance. Some autochthonous breeds of sheep (Jovanović *et al.*, 1988), pigs (Jovanović, 1982), goats (Savić *et al.*, 1995), and horses (Trailović *et al.*, 1994) in our country have already been genetically characterized based upon analysis of protein polymorphism. To date, however, no analysis of the genetics of autochthonous dog breeds in the region has been undertaken.

The Yugoslav shepherd dog is believed to be between 1600 - 2200 years old and, thus, is considered as the oldest autochthonous dog breed in the Balkan peninsula (Dimitrijević, 1997). The breed was recognised by the F.C.I (Federation Cynologique Internationale) in 1939 under the name Illyrian Shepherd dog. In 1957, at the request of JKS (Yugoslav association), F.C.I. accepted to change the name into Yugoslav shepherd dog - Šarplaninac after the Šarplanina Mountain Range, where it is most common. Two countries, Serbia and Macedonia, are currently listed as countries of origin of this breed by the F.C.I. The exact ancestors and the origin of the Yugoslav shepherd dog still remain controversial. Great Tibetan Mastiff, ancient molosian dogs of Greece, ancient roman dogs, and the livestock guarding dogs of Turkey have all been suggested as possible ancestors for the Yugoslav shepherd dog. It seems likely that the dogs of this breed had come from Asia to Europe and the Balkans, alongside the cattle herded by the migrating people. Upon arrival to the Balkans, these dogs maintained their existence and developed as a breed in the specific settings of Šarplanina, Korab, Jablanica and Pelister. The original breed type has been maintained in these regions, where extensive sheep breeding is still prevalent, with this dog as an irreplaceable herding dog and protector of sheep herds against predatory animals.

The aim of this study was to provide the first insight into the genetic constitution of the Yugoslav shepherd dog based upon analysis of blood proteins and enzyme polymorphism.

MATERIAL AND METHODS

Study population

A total of 60 Yugoslav shepherd dogs were used in this study. All dogs were bred in the Center for Dog Breeding, Niš.

Blood samples collection

Blood samples (10 ml) with EDTA were collected by venipuncture of *v. cephalicae antebrachii*. Plasma and erythrocytes were separated by centrifugation at 3500 rpm over 10 minutes. Subsequently, the red cells were washed three times in physiological saline and hemolyzed by adding an equal volume of distilled water. All samples were stored at -20°C until electrophoretical investigation.

Analysis of protein polymorphism

For the purposes of genetic characterization of the Yugoslav shepherd dog, polymorphism of the following five blood proteins and enzymes was analyzed: hemoglobin (Hb), acid phosphatase (Acp), superoxide dismutase (Sod), albumin (Al), and transferrin (Tf). The Hb, Acp, and Sod types were analyzed by vertical polyacrylamide gel electrophoresis (PAGE) in discontinuous tris-citrate buffer system, while vertical PAGE in continuous tris-citrate buffer system was used for detection of Al and Tf polymorphism (Pasteur *et al.*, 1988). Considering that Hb is a stained protein, no specific staining technique was required for analysis of HB types. Detection of Sod types was achieved by nitroblue tetrazolium stain (Bio-Rad Laboratories, USA) in accordance to the method of Beakcham and Fridovich (1971). The Acp, Al, and Tf gels were stained by coomassie blue R-250 (Bio-Rad Laboratories, USA) (Pasteur *et al.*, 1988).

Statistical analyses

Gene frequencies were established by direct counting from the phenotypes and the homogeneity index was calculated. In order to assess the genetic equilibrium in the study population, Hardy-Weinberg equilibrium was performed on phenotypes deduced from the counted gene frequencies. The differences between phenotype frequencies expected in accordance with Hardy-Weinberg equilibrium and those observed in the study population were evaluated by χ^2 test. *p* values that were less than 0.05 were considered significant.

RESULTS AND DISCUSSION

Hb polymorphism

Evaluation of Hb polymorphism in blood of the Yugoslav shepherd dog revealed only one allelic product, namely phenotype B. The phenotype is characterized by the presence of two bands and presents the phenotypic expression of HbBB homozygous type. The obtained results are consistent with the results of previous studies since Hb locus did not show polymorphism in any of European breeds tested, nor in the majority of oriental dog breeds (Simonsen, 1976; Braend, 1987). Additional two Hb phenotypes, namely A and AB phenotypes, were established only in Japanese native dog breeds (Tanabe *et al.*, 1978). Quite understandably, the gene frequency established for the population of Yugoslav shepherd dog was identical to gene frequencies established in the previously examined European and oriental dog breeds. As already noted, Japanese native breeds did not correspond to this uniform distribution (Tanabe *et al.*, 1978) since frequency of allelic gene Hb^A in these breeds ranged from 0.000 (Shikoku) to 0.290 (San'in-Shiba). This discrepancy is primarily attributed to the specific genetic background of these breeds (Tanabe *et al.*, 1991), which most probably includes the influence of genes originating from an ancestral dog line brought from the Korean peninsula. It is noteworthy that similar inconsistencies have already been noted in Japanese native breeds for other loci, such as Acp^F and Al^S (Tanabe, 1990; Tanabe *et al.*, 1991). The overall data show that the results

of our survey are in agreement with the results of other studies investigating Hb polymorphism in various dog breeds and they provide support for the presumed Eurasian origin of the Yugoslav shepherd dog.

Acp polymorphism

The analysis of erythrocyte Acp in the Yugoslav shepherd dog detected only two of the expected three (FF, FS, and SS) phenotypes. Homozygous form Acp^{FF} was not established within the studied population. Acp^S allelic gene displayed a significantly higher frequency than Acp^F (Table 1) which has also been shown in other dog breeds (Braend and Austad, 1973; Elliot and Wong, 1973). Although the values of gene frequencies established in different studies were similar, there is a difference among phenotype distribution patterns. Two distinct phenotypes, FS and SS were found in the population of Yugoslav shepherd dog. All three theoretically possible phenotypes (FF, FS, and SS) were observed only in the breed Labrador retriever, while examination of German shepherd and German pointer revealed only one phenotype, namely SS (Braend and Austad, 1973). It is interesting to note that the study concerning biochemical genetic variability in race hounds did not establish any biochemical variation in Acp (Scherer and Kluge, 1993).

Table 1. Gene frequencies and observed vs. expected acid phosphatase phenotypes ratio in Yugoslav shepherd dog

Number of animals	Acp phenotypes			Gene frequency		
		FF	FS	SS	Acp ^F	Acp ^S
60	observed	0	22	38	0.18	0.82
	expected	2	17.94	40.06		
$\chi^2 = 3.0247$ ($p > 0.05$)						

Acp = acid phosphatase

Sod polymorphism

Analysis of Sod polymorphism in the Yugoslav shepherd dog showed two distinct phenotypes, homozygous Sod^{AA} and heterozygous Sod^{AB}, controlled by allelic genes Sod^A and Sod^B (Table 2). The Sod^{BB} phenotype was not found in the tested population. The obtained results support the findings of all previous studies which established a low Sod^B frequency (Muller *et al.*, 1987; Scherer and Kluge, 1993) since a clear predomination of Sod^A allele was seen in the studied population. It should be noted, however, that Sod^B allele frequency established in Yugoslav shepherd dog of 0.258 was higher than those of the allelic gene found in other tested dog breeds. Values of all Sod^B allele frequencies reported so far remained below 0.1 (Muller *et al.*, 1987; Scherer and Kluge, 1993). The most probable explanation for high level of Sod^B occurrence in the tested population is genetic drift. It seems reasonable to assume that intensive breeding within a

limited area and population resulted in unbalanced distribution of SodB allele originating from a relatively small number of males widely used as sires.

Table 2. Gene frequencies and observed vs. expected superoxide dismutase phenotypes ratio in Yugoslav shepherd dog

Number of animals	Sod phenotypes			Gene frequency		
		AA	AB	BB	Sod ^A	Sod ^B
60	observed	29	31	0	0.74	0.26
	expected	33	23	4		
$\chi^2 = 7.2674$ ($p < 0.05$)						

Sod = superoxide dismutase

Al polymorphism

As far as polymorphism of Al is concerned, both allelic genes described so far, (Al^F and Al^S) as well as all three theoretically possible phenotypes (F, FS, and S), were detected in Yugoslav shepherd dog (Table 3). Al has been established as a reliable marker in biochemical genetic variability studies due to its wide distribution and intermediate frequencies displayed by Al^F and Al^S allelic genes (Christensen *et al.*, 1985; Scherer and Kluge, 1993). The ratio between Al^F and Al^S frequencies established in the population of Yugoslav shepherd dog is consistent with results of the study which found a linkage between Al type and leg length (Christensen *et al.*, 1985). A higher frequency of the Al^F allele in short-legged breeds and predominance of the Al^S allele in long-legged breeds documented it. Al^S frequency ranged from 0.560 in German shepherd dog to 1.000 in the German Dane, Basenji and Siberian husky (Christensen *et al.*, 1985). In contrast to these findings, the study investigating protein polymorphism in race hounds did not establish the correlation between Al type and leg length (Scherer and Kluge, 1993). Given the Al^S frequency of 0.700 established in the Yugoslav shepherd dog, the results of the present study strongly support the hypothesis on the positive correlation between Al^S frequency and leg length.

Table 3. Gene frequencies and observed vs. expected albumin phenotypes ratio in Yugoslav shepherd dog

Number of animals	Al phenotypes			Gene frequency		
		FF	FS	SS	Al ^F	Al ^S
60	observed	7	22	31	0.30	0.70
	expected	5.4	25.2	29.4		
$\chi^2 = 0.9675$ ($p > 0.05$)						

Al = albumin

Tf polymorphism

The evaluation of the Tf locus revealed seven of 15 theoretically possible Tf phenotypes, which are controlled by five allelic genes: Tf^A, Tf^B, Tf^C, Tf^D, and Tf^E. The distribution of Tf phenotypes and calculated gene frequencies are shown in Table 4. Out of the 60 dogs tested, 35 (58.33%) had heterozygous BD phenotype, while a significantly lower number of animals, 12 (20%), displayed heterozygous AD phenotype. The proportion of BB homozygotes and CE heterozygotes of 1.66% was the lowest established within the population studied. Tf along with aryl esterase and C6 and C4 complement components belong to the category of most complex and polymorphic biochemical markers of genetic variability in dogs. The majority of earlier studies have reported the presence of three most frequent allelic genes, Tf^A, Tf^B, and Tf^C within the various populations tested (Komatsuzaki, 1983; Braend and Andersen, 1987). Only Juneja *et al* (1981) and Tanabe (1990) proved all five Tf allelic genes, but not within one dog breed. Yugoslav shepherd dog is the only dog breed which displayed all five Tf loci and thus exhibited the maximal polymorphism of this highly heterogeneous marker. In addition, a specific distribution of allelic gene frequencies was also established within the observed population. In the majority of other examined dog breeds, allelic genes Tf^B and Tf^C were found to appear most frequently (Juneja *et al*, 1981; Komatsuzaki, 1983; Braend and Andersen, 1987; Tanabe, 1990). In contrast to these studies, the highest gene frequencies were registered for allelic genes Tf^B and Tf^D within the studied population of the Yugoslav shepherd dog. We presume that this discrepancy is most probably related to genetic drift i.e. to specific breeding of Yugoslav shepherd dog within a restricted area where this breed has differentiated over the centuries.

Table 4. Gene frequencies and observed vs. expected transferrin phenotypes ratio in Yugoslav shepherd dog

Number of animals	Phenotypes	Observed	Expected	Gene frequency	
				Allele	Frequency
60	AA	0	1.06	Tf ^A	0.13
	BB	1	7.35	Tf ^B	0.35
	CC	0	0.21	Tf ^C	0.06
	DD	0	9.99	Tf ^D	0.41
	EE	0	0.15	Tf ^E	0.05
	AB	0	5.59		
	AC	4	0.94		
	AD	12	6.51		
	AE	0	0.8		
	BC	0	2.48		
	BD	35	17.14		
	BE	5	2.1		
	CD	2	2.88		
	CE	1	0.35		
DE	0	2.45			
$\chi^2 = 66.8981$ ($p < 0.01$)					

Tf = transferrin

Homogeneity index for Sod, Acp, Al, and Tf loci was calculated upon the number of established allelic genes for each locus and the corresponding gene frequencies (Table 5). Homogeneity index for Hb locus was not evaluated since the present study showed only one homozygous form of this protein in the Yugoslav shepherd dog. Out of four loci, which showed polymorphism in the studied population, the highest homogeneity index was established for Acp locus. Values of homogeneity indices for Al and Tf loci were significantly lower. Thus, protein markers displaying high polymorphism in the Yugoslav shepherd dog were identified, this provides guidelines for future biochemical genetic variability investigation in this breed.

Table 5. Homogeneity index for superoxide dismutase, acid phosphatase, albumin, and The homogeneity index (HI) was calculated transferrin loci in Yugoslav shepherd dog

Locus	Sod	Acp	Al	Tf
Number of alleles	2	2	2	5
Homogeneity index	0.2344	0.4020	0.1600	0.1409

Sod = superoxid dismutase; Acp = acid phosphatase; Al = albumin; Tf = transferrin

Upon the calculated allelic frequencies it was shown that the population of Yugoslav shepherd dog was in genetic equilibrium at Hb, Acp, and Al loci, while significant deviations from Hardy-Weinberg equilibrium were detected at Sod and Tf loci. The established imbalance to Hardy-Weinberg law at these two loci may be attributed to genetic drift, mutation, migration or crossbreeding. As already stated, we presume that the most prominent factor affecting genetic equilibrium was genetic drift. It is noteworthy, however, that crossbreeding of the Yugoslav shepherd dog with various dog breeds has become a common breeding practice over the last decades and, therefore, should also be considered as a factor of possible importance. Such mixing may lead to a serious disbalance in the genetic constitution of the breed, outbreed depression, and deprivation of major biological features of the breed.

Our study provided the first insight into the genetic structure of the Yugoslav shepherd dog and enabled comparison of the genetic constitution of this breed with dog breeds which have already been genetically characterized. In order to preserve the endogenous genetic pool and original characteristics of the Yugoslav shepherd dog, and to assess the possibly detrimental effects of crossbreeding which has already been undertaken, further investigation into genetic structure of this breed based upon molecular techniques is currently underway in our laboratory.

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GENETSKI POLIMORFIZAM PROTEINA U KRVI PASA RASE JUGOSLOVENSKI OVČARSKI PAS

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SADRŽAJ

Ova studija predstavlja prvu analizu genetske konstitucije pasa rase jugoslovenski ovčarski pas – šarplaninac na osnovu ispitivanja polimorfizma proteinskih i enzimskih sistema u krvi pasa ove rase. U studiju je bilo uključeno 60 pasa kod kojih je izvršena elektroforetska analiza polimorfizma hemoglobina (Hb), eritrocitne kisele fosfataze (Acp), superoksid-dismutaze (Sod), albumina (Al) i transferina (Tf). U sistemu Hb nije ustanovljen polimorfizam. Dokazano je prisustvo FS i SS fenotipova Acp pri čemu je učestalost Acp^S (0.82) alela u odnosu na Acp^F alel (0.18), bila značajno veća. Analizom polimorfizma Sod utvrđeni su Sod^{AA} i Sod^{AB} fenotipovi koji su kontrolisani Sod^A i Sod^B alelnim genima, sa frekvencama (0.74 i 0.26) prema redosledu, uz jasnu predominaciju Sod^A alela. U ispitivanoj populaciji dokazano je prisustvo oba do sada opisana Al alelna gena (Al^F i Al^S) sa frekvencama (0.30. i 0.70) kao i sva tri moguća fenotipa (F, FS i S). Utvrđeno je prisustvo sedam Tf fenotipova koje kontroliše pet alelnih gena, Tf^A, Tf^B, Tf^C, Tf^D i Tf^E sa frekvencama (0.13, 0.35, 0.06, 0.41 i 0.05) prema redosledu. Jugoslovenski ovčarski pas je jedina rasa pasa kod koje je ustanovljeno svih pet Tf alelnih gena pri čemu su najviše frekvence registrovane za alele Tf^B i Tf^D. Na osnovu izračunatih vrednosti indeksa homogenosti, ustanovljeni su izrazito polimorfni proteinski markeri što je od značaja za buduće studije genetske karakterizacije jugoslovenskog ovčarskog psa. Dokazano je da se ispitivana populacija pasa u odnosu na Sod i Tf lokus ne nalazi u genetičkoj ravnoteži. Faktor koji je primarno uticao na stanje genetičke ravnoteže je genetički drift, ali je i ukrštanje faktor od potencijalno velikog značaja.