

MICROSATELLITE ANALYSIS OF THE GENETIC DIVERSITY IN THE BLACK SLAVONIAN PIG

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This research is focused on the genetic diversity and population structure of the Black Slavonian pig, with the aim of assessing the situation regarding the endangerment of genetic diversity, and defining the further role of this breed in the preservation of its genetic diversity. Genetic population parameters were assessed in 42 animals at 8 microsatellite loci. On three of the observed loci there was a deviation from the Hardy-Weinberg (HW) equilibrium with $P < 0.01$, one locus showed $P < 0.05$, and in all of them the stated deviation resulted from a deficiency of heterozygous animals. The entire population's deviation from the HW equilibrium, with $\chi^2 = 92.8$, $Df = 16$, and $P < 0.0001$ was also significant. Average PIC value for all 8 loci was 0.368. The average number of detected allelic variants was 2.50, with a standard deviation of 0.76 alleles. Such a small number of detected allelic variants, as well as the significant deviation from the HW equilibrium in four loci caused by a deficiency of heterozygotes, can be taken as one of the indicators of the reduced genetic diversity of the breed.

Key words: molecular marker, pig breed, protection of genetic diversity

INTRODUCTION

The loss of breeds can result in huge social and economic losses for humanity. The long-term preservation of species and breeds, as well as of genetic diversity within them, is the imperative duty of the human species (Fanklin, 1980). Loss of genetic diversity, resulting from small populations (caused by breeding methods), poses a threat to long-term variability of both the populations and genetic improvement within them (Coulson *et al.*, 1998). Numerous breeds of pigs, out of which today's highly productive breeds have emerged, have already disappeared. The main reason for this is the preference for high yield breeds and neglect of those that yield less but which are highly resistant. Driven by the aim of achieving a profit as high as possible, breeding procedures which were used created breeds and genotypes of pigs with similar production properties. In order to ensure genetic diversity out of which further genetic improvement is going to

depend on, the preservation of the remaining indigenous breeds is of great significance. One among such indigenous breeds is the Black Slavonian pig, which emerged in the second half of the 19th century. The creation of the breed, which was a dominant one on the territory of Croatia up to the mid-20th century, resulted from crossing the following breeds: Mangulica, Berkshire, Poland China and Cornwall. The second half of the century witnessed the import of the Landrace and Yorkshire (thoroughbred breeds), and the slow decline of the Black Slavonian pig. Today, it is bred, with almost unchanged properties only in small numbers in extensive production conditions. In 1996 only 46 sows and 6 boars in 10 farms were registered (Croatian Livestock Center, 2005). Those remaining animals were marked and registered and financial support was approved with the purpose of preserving the breed. Those steps led to an increase in the number of animals, and in 2004 there were 388 sows and 53 boars in 56 husbandries (Croatian Livestock Center, 2005).

Major steps have recently been made throughout the world in order to preserve genetic diversity. In addition to the registration of the remaining breeds in the FAO data bank, efforts are being made to establish the state of genetic diversity, that is, genetic profitability. In that respect a significant role is played by molecular techniques which make it possible to carry our analyses of neutral variability, particularly of the microsatellite markers (Goldstein and Schlötterer, 1999; Luikart and England, 1999; Rousset, 2001).

The aim of this research was to assess the condition of the genetic variability of the Black Slavonian pig, and to provide answers to the following questions: What is the current level of genetic diversity? Is there proof of losing allelic variants? Should the preservation programme be focused on this population from the genetic standpoint?

MATERIALS AND METHODS

The Black Slavonian pig is reared mostly on the territory of Slavonia in east Croatia. The research involved 42 animals from 6 husbandries located in different villages.

Blood samples were taken in the year 2003. A blood sample of about 10 mL was taken from each animal and stored in test-tubes previously treated with EDTA anticoagulant preservative. The samples were kept at temperature of -20°C until subsequent analysis. Isolation of DNA from about 200 µL was performed according to the standard phenol-chloroform protocol (Ausubel *et al.*, 1987). Eight microsatellite markers of pigs (Table 1) were multiplied. They were selected for their pronounced polymorphism from the list of 50 loci recommended by FAO for research of genetic diversity and relation between breeds of pigs. This list can be found at: <http://www.fao.org/dad-is/> or: <http://www.projects.roslin.ac.uk/pigbiodiv/markers.html> (FAO, 2004).

PCR reaction was induced in a volume of 25 µL in which the amount of DNA was about 50 ng, 0.2 µL Taq DNA polymerase (Eppendorf), µL of each of the oligonucleotide primers (Qiagen) (10 pmol/µL), 2.5 µL dNTP (Eppendorf) (2 mM),

2.5 μ L 10*PCR buffer and 2.5 μ L MgCl₂ (2.5 mM). The reaction was performed in a thermocycler (Eppendorf) according to the following programme: 95°C/5 min., 30 cycles of 1 min. at 95°C (denaturation), 30 sec. at 53-63°C annealing temperature characteristic for microsatellites (Table 1) and 1 min./72°C. In the final cycle the extension was lengthened to 10 min. PCR products were then analysed by gel electrophoresis. Genotyping of microsatellite loci was conducted by means of horizontal electrophoresis (Elchrom electrophoretic system SEA 2000) using Spreadex EL 400 Wide Mini S-50 gel (Elchrome) in 1 x TAE buffer (2M Tris-acetate, 0.05M EDTA, pH=8.3) at a constant temperature of 57°C, at the power of 92V and in duration of about 4h for shorter fragments, and 6h for longer fragments (c.180 or more bp). The length of alleles at each individual microsatellite locus was determined through the use of a standard M3 marker (Elchrome).

Table 1. Microsatellite loci, related chromosomes (Ch), annealing temperatures (AT), range of allele lengths (Min-Max)

Loci	Ch	(AT)	Min - Max
SW2406	6	58	220-256
SW1632	11	58	259-270
SW940	9	58	103-109
SW1023	18	63-53 TD*	84-117
SO355	15	55	243-277
SO277	4	55	231-240
SW936	15	58	80-89
SWR1941	13	62	234-242

TD* – touch down protocol

Variability of individual loci was assessed on the basis of frequencies of alleles, the expected (H_e) and the observed (H_o) heterozygosity, and the fixation index (F_{is}) per single locus, for all loci (Weir and Cockerham, 1984), for all animals individually. The expected heterozygosity was corrected using the Leven correction method. Deviations from the HW equilibrium were assessed and tested for each of the loci using the exact test (Raymond and Rousset, 1995). Deviation of all the loci of the analysed sample ($n=42$) was assessed and tested using the Fisher method. The given parameters were analysed using the Genepop 3.4 computer program (Raymond and Rousset, 1995). Polymorphism index (PIC) was evaluated using the Cervus 2.0 computer program (Marshall *et al.*, 1998). Unbiased genetic diversity was calculated according to Nei (Nei, 1987).

RESULTS

Genotypic data from 8 microsatellites were used to assess the genetic structure of the Black Slavonian pig. All the loci were polymorphic (≥ 2 alleles) (Craford *et al.*, 1995). With respect to PIC, two loci (SW936, SWR1941) may be considered as highly informative. In the case of other microsatellite markers the PIC values were < 0.5 (Botstein *et al.*, 1980). As expected, a fairly low level of genetic heterogeneity was further reflected within this breed by a mean PIC value of 0.368.

Table 2 shows values of parameters evaluating the genetic variability and the probability (P) of random mating within the population. The average observed heterozygosity across all loci was 0.357 ± 0.026 and ranged from 0.000 (SW2406) to 0.738 (SW936) (Table 2). Genetic diversity according to Nei (H_z) amounted to 0.441 ± 0.075 and ranged from 0.155 (SW1632) to 0.703 (SWR1941) (Table 2). A significant deviation from HW equilibrium was observed for three loci (SW2406, SO277, SWR1941) at $P < 0.01$, while locus SW940 showed a deviation at $P < 0.05$ (Table 2). In all of these, deviation was caused by lack of heterozygotes. Results obtained with Fisher's method confirm that the entire population deviates significantly from the HW law, with $\chi^2 = 92.8$, Df = 16 and $P < 0.0001$.

Table 2. Average number of alleles (Na), number of heterozygotes (Het), number of homozygotes (Hom), Ho, He, PIC and test of the Hardy-Weinberg equilibrium for eight loci on the overall sample

Loci	Na	Het	Hom	Ho	He	PIC	P (HW _{test})
SW2406	2	0	42	0.000	0.174	0.157	.0000**
SW1023	2	17	25	0.405	0.503	0.374	.2275 ^{NS}
SO355	2	14	28	0.333	0.281	0.239	.5719 ^{NS}
SW1632	2	7	35	0.167	0.155	0.141	1.0000 ^{NS}
SW940	2	12	30	0.286	0.469	0.370	.0103*
SO277	3	10	32	0.238	0.579	0.478	.0000**
SW936	3	31	11	0.738	0.638	0.552	.3745 ^{NS}
SWR1941	4	29	13	0.690	0.703	0.632	.0000**

Significance of deviation from Hardy-Weinberg equilibrium

^{NS} non-significant; * significant ($P < 0.05$), ** significant ($P < 0.01$)

Fixation index (F_{is}) values vary from very high, which is characteristic for the high level of inbreeding (SW2406, SW1023, SW940, SO277, SWR1941), to negative values indicating excess of heterozygotes (SO355, SW1632, SW936). Average values for all the loci included in the research proves the presence of inbreeding for the entire population ($F_{is} = 0.216$) in all ten loci. The number of observed alleles (Na) varied between two and four with an average of 2.50 ± 0.76 . Low mean number of observed alleles (allelic diversity) per locus displays low

genetic variation in the Black Slavonian pig and the breed is therefore not under mutation drift equilibrium.

DISCUSSION

The selected loci demonstrated the effectiveness for estimation of genetic variation within different pig breeds (FAO, 2004). Harcet *et al.* (*in press*) used similar loci (at least six out of ten) on the Croatian autocht honous breed of Turopolje pig and obtained results which verified the endangered condition of this breed. Numerical data which resulted from neglected breeding of the only two remaining autochthonous breeds of pig in the Republic of Croatia form the basis for comparison of results within these studies. There is a relatively low number of detected alleles (2.50) and a low level of heterozygosity (0.657) present in the Black Slavonian pig. This can be compared to microsatellite allele numbers of 2.40 in Turopolje pig breed (Harcet *et al.*, *in press*). Other studies of pig diversity show microsatellite allele numbers to be in the range from 3.220 to 5.840 in European breeds (Martinez *et al.*, 2000; Laval *et al.*, 2000), 4.351 to 6.108, in Chinese breeds (Fan *et al.*, 2003; Li *et al.*, 2000), 7.00 to 7.74 in Indian breeds (Behl *et al.*, 2002).

As expected, our study shows a fairly low level of genetic heterogeneity, further reflected within this breed by a mean PIC value of 0.368, which was higher than the value in Turopolje pig (0.222) (Harcet *et al.*, *in pess*). Average expected heterozygosity in the Black Slavonian pig was $H_e=0.657$, while the expected heterozygosity in Turopolje pig breed was slightly lower, i.e., $H_e=0.306$ (Harcet *et al.*, *in press*) despite the greater number of investigated loci. Thus, the observed differences in gene diversity (H_o) values might be ascribed to the choice of microsatellite loci types (hypervariable/exhibiting wide range of variation), as well as to the existing population structure (Kim *et al.*, 1999). The low mean heterozygosity values could be attributed to the high level of inbreeding, high selection pressure and small number of alleles present in the respective population. Demographic structure of the Black Slavonian pig revealed that farmers allowed their animals to be only naturally bred. Since boars and sows are housed and grazed together there was no rotation of boars and no controlled mating. The population fixation index (F_{IS}) for the investigated population (0.216) further showed a very high rate of inbreeding, which could be caused due to the absence of directional selection. The reason for inbreeding could also come from the circumstance that re-establishment of this breed began in 1996 with only 46 sows and 6 boars (Uremović, 2004).

Results of this research underline the limited number and the extensive method of breeding as the main problems of this population. The purpose of this study was to determine a range of genetic variation in the investigated breed and maintain their gene pool for further generations. The results of this study contribute to the knowledge of the genetic structure of the Black Slavonian pig under constant risk due to uncontrolled breeding. Conservation of genetic diversity should be considered by pig breeders, in the interest of the long-term future of this breed. The need of suitable genetic management for retaining

founder alleles to the possible extent is clearly pinpointed. Black Slavonian pig Development Scheme needs to be undertaken so that good breeding boars are made available to the pig farmers for genetic improvement. These boars should be rotated in various villages of the breeding area in a manner to avoid inbreeding. It is to be expected that measures taken over the last few years will improve the condition of this breed.

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MIKROSATELITNA ANALIZA GENETSKE RAZNOLIKOSTI CRNE SLAVONSKE SVINJE

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SADRŽAJ

Proučavana je genetska raznolikost i struktura populacije Crne slavonske svinje u svrhu procene stanja ugroženosti genetske raznolikosti i definisanja dalje uloge ove pasmine u održanju genetske različitosti. Kako bi se utvrdila genetska raznolikost pasmine procenjeni su populacijsko-genetski parametri na 42 jedinice na osam mikrosatelitnih lokusa. Tri posmatrana lokusa pokazala su odstupanje od Hardy-Weinbergove (HW) ravnoteže uz $P < 0.01$, a jedan lokus uz $P < 0.05$. Kod svih je odstupanje posledica manjka heterozigotnih jedinki. Značajno je i odstupanje čitave populacije od Hardy-Weinbergove ravnoteže, uz $\chi^2 = 92.8$, Df = 16, i $P < 0.0001$. Prosečna PIC vrednost za svih 8 lokusa iznosi 0.368. Prosečan broj detektovanih alelnih varijanata je 2.50, uz standardnu devijaciju 0.76 alela. Ovako mali broj detektovanih alelnih varijanata, kao i značajno odstupanje od HW za 4 lokusa uzrokovano manjkom heterozigota može biti jedan od pokazatelja smanjene genetske varijabilnosti pasmine.