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Population structure of four indigenous chicken breeds undergoing *in situ* conservation*

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The latest advances in molecular biology techniques, such as microsatellite markers, have provided new opportunities to evaluate the genetic variability among animals at the genetic level. Numerous studies have comprised microsatellite analyses of genetic diversity among chicken breeds, including analysis of local chicken breeds, although very few publications referred to genetic differentiation among indigenous chicken breeds carried out over-time. The main goal of this study was to analyse the genetic diversity and population structure of local native chicken breeds undergoing *in situ* **conservation. The genetic variability of four populations: the Green-legged Partridgelike chicken (GP), the Transylvanian Naked Neck Black (TNB), Transylvanian Naked Neck White (TNW) and the Hungarian Speckled (HS) was evaluated based on the presence of eight microsatellite markers (on average 27 individuals per breed). Our study presents an average genetic variation between the TNB and HS breeds (Fst=0.20) and a high genetic variation between the TNB and TNW breeds (Fst=0.35). Measures of genetic variability show no statistically significant differences between expected and observed heterozygosity. Comparing these results to the ones obtained in 2009 and 2010 we can state that the structure of these populations has not changed after 10 years of *in situ***

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conservation. These results indicate that this type of animal management had no negative effect on these populations. Our study identified 19 private alleles for the analysed populations. The results of our experiment emphasise the abundance of genetic diversity among these indigenous chicken breeds.

KEY WORDS: chicken / native breed / population structure / microsatellite / *in situ*

The chicken plays an important role in the poultry industry as a source of human nutrition. After centuries of rearing and adaptation to different environmental conditions several chicken breeds have been developed. The animal genetic resources, such as indigenous chickens, constitute an important part of biodiversity programmes and are of particular interest (economically, scientifically and culturally) to humans in terms of agricultural and specifically food production. Biodiversity conservation is vital in the genetic management of chicken breeds, to minimize inbreeding and loss of genetic variability.

The term "biodiversity" refers to diversity among all the organisms living on Earth, including the diversity at the genetic level [Primack 2006]. Biological diversity is the basis for maintaining a balanced life on Earth, and therefore is essential for the proper functioning of life activities across the whole planet. However, suburbanization has resulted in fragmentation of all animal populations, leading to the extinction of several species. This problem causes a huge and irreversible loss of biodiversity on a global scale. As a consequence, not only species, but also alleles, genes, and gene combinations disappear from the population, leading to the loss of some traits which have not been recognised yet.

Many strategies have been devised to preserve and renew biodiversity [Kearns 2010]. The first strategy involves conservation by the *in situ* method, which is based on preserving the species in their natural habitat. For this purpose, national parks, nature reserves, biospheres, protected areas, etc., are formed.

The *ex situ* conservation approach includes two strategies: *in vivo* and *in vitro*. The *in vivo* strategy involves maintenance of biodiversity outside the natural environment under controlled (partially or totally) conditions. The in vitro strategy is based on the storage of genetic material /sperm, cells, embryoin liquid nitrogen under deep freezing conditions. *Ex situ* methods include important strategies that aid in restoring endangered species [Sawicka *et al*. 2015].

In the cases where comprehensive data concerning breed characteristics and the origin of breeding populations are lacking, molecular marker information may provide reliable estimates of genetic diversity within and between populations. These markers are appropriate tools to investigate the genetic relationships and population structure among farm animals, while they also provide information regarding evolutionary relationships and parentage within populations. Variability is a consequence of genetic differences between individuals, families and populations within a species.

Recent developments in molecular technology have provided new opportunities for assessing genetic variability at the DNA level [Beugin *et al*. 2017, Kévin *et al.* 2019]. Worldwide, numerous studies have been based on microsatellite analyses to

explain the diversity among chicken breeds [Berthouly *et al.* 2008, Roh *et al*. 2018, Karsli *et al*. 2019], including the European research project AVIANDIV (https://aviandiv.fli.de) and subsequent studies [Hillel *et al*. 2003, Granevitze *et al*. 2007, Bodzsar *et al*. 2009]. Microsatellite polymorphism analysis is the most commonly used valuable tool for assessing genetic diversity and livestock relationships in chickens, since microsatellites show a high degree of polymorphism compared to other molecular markers such as allozyme or random amplified polymorphic DNA [Zhang *et al*. 2002a]. In addition, they are easy to identify and show low mutation rates [Milligan *et al*. 1994, Zhang *et al*. 2002b].

A reduction in the number of local poultry breeds as a consequence of their replacement with commercial stocks suggests the need for the conservation of local breeds. Molecular analysis provides valuable insight to support conservation and maintenance of various breeds [Zanetti *et al*. 2011]. Nevertheless, relatively few publications refer to the genetic diversity of local chicken breeds [Bodzsar *et al*. 2009, Fathi *et al*. 2018, Mwambene *et al.* 2019], including assessment of genetic diversity and the relationship between indigenous populations of red jungle fowl [Zhang *et al.* 2002b; Hillel *et al*. 2003, Kaya and Yıldız 2008].

The Green-legged Partridgelike chicken, commonly known as the "Galician hen", has been preserved for generations in the present south-eastern Poland, a region occupied by the Austro-Hungarian Empire during the entire 19th century. Trade exchange between Vienna, Budapest and Lviv, the largest cities of Galicia, also included trade in farm animals, such as e.g. poultry. Therefore, it seems reasonable to compare native Hungarian hens with the oldest Polish local breed of chickens.

In the last decades there has been an increasing demand for breeding techniques ensuring low-cost production of increased amounts of meat, therefore local chicken breeds were not bred much frequently. This is one of the reasons why some of these breeds are lost or have become endangered. Moreover, for many years indigenous chicken populations were maintained by *in situ* methods. This state of affairs entails some consequences, for example the risk of inbreeding and gene flow. The aim of this study was to analyse the genetic diversity and population structure of four local native chicken breeds managed under *in situ* conservation programmes. The analyses were performed using eight microsatellite markers. The following breeds were studied: one Polish (the GP chicken) and three Hungarian populations (the TNB, TNW, and HS). The Hungarian breeds were obtained from the ASSOCIATION OF HUNGARIAN SMALL ANIMAL BREEDERS FOR GENE CONSERVATION in Godolo, Hungary. The Polish breed, GP, was obtained from the Teaching and Research Station in Felin – the University of Life Sciences in Lublin.

The exact origin of Hungarian and Transylvanian chicken breeds (such as e.g. TNB, TNW, HS) is unknown. It is suspected that at the end of the 9th century ancestors of these birds were brought into these regions from Asia by Hungarian conquerors. Nowadays, since the expansion of commercial poultry breeding in the early 1960s, except for some small stock breeders keeping them because of their

unusual appearance and excellent meat quality, these breeds have been kept primarily as a gene pool reserve. HS is kept in 8 populations with about 300 males and 1800 females, while TNB and TNW are kept in 4 populations with a total population of approximately 100 males and 700 females.

GP, an old native Polish chicken, is derived from Galician chickens, which at the end of the 19th century were found in Galicia. This breed was first described in 1879 [Wójcik 2011]. Presently, the GP chicken has been protected from extinction by means of *in situ* preservation. However, there still exists a small unregistered population of these birds, thanks to the interest of small stock breeders and those who keep them as fancy animals. There are two major GP populations comprising approximately 600 females and 70 males each. The animals in the conservation programme were kept in a flock for more than 50 generations with no selection process for more than 50 generations.

To date very few authors have reported on genetic differentiation patterns among local chicken breeds over time [Zanetti *et al*. 2011]. The second goal of this study was to determine whether microsatellite markers are unique to each individual and can be used in the identification of separate individuals.

Material and methods

The research specimens used for this study included hens and roosters: TNB, TNW, HS, and GP breeds. The birds were kept in separate cages under controlled environmental conditions (temperature 20±5°C, relative humidity 58%, 14L: 10D hours light-dark cycle). The animals were fed with standard commercial poultry feed and received tap water *ad libitum*.

A total of 109 female birds (about 27 individuals per breed) were selected for the study at random from among all the four breeds. Blood samples were collected to sterile test tubes (2ml Vacutest) containing K_2EDTA anticoagulant. Genomic DNA was isolated using the MasterPure ™ DNA Purification Kit for Blood Version II (Epicentre Technologies) according to the manufacturer's instructions. The microsatellite markers selected for genotyping were those recommended by the FAO/MoDAD Advisory Group (http://www.fao.org/dad-is) for the study of polymorphism in chicken (FAO, 1998) and are summarized in Table 1. Reverse primers (R) were labeled with fluorescent 6-FAM, VIC, and NED dyes. The genes of interest were amplified by the polymerase chain reaction (PCR). The PCR reaction was performed as single (ADL136), triplex (ADL158, ADL176, ADL267) and quadriplex (ADL268, LEI094, MCW216, MCW248) reactions in a 10 µl of reaction mixture, which contained 160-180 ng of the DNA sample and 0.275 U of Taq polymerase. A thermostable polymerase AmpliTaq GOLD® 360 DNA Polymerase (Applied Biosystems) was used. PCR products (0.5 µl), formamide (9.5 µl) (Thermo Scientific) and the GeneScanTM-350 ROX TM size marker (0.5 µl) (Applied Biosystems) were applied to each of the 96 wells of the MicroAmp™ plate (Applied Biosystems).

Table 1. Characteristics of microsatellite *loci* used

Locus	Sequence of primer (5' to 3')	Dye	Allele sizes (bp)	Ta
ADL0136	F – TGTCAAGCCCATCGTATCAC R – CCACCTCCTTCTCCTGTTCA	6FAM	127-159	54
ADL0158	F – TGGCATGGTTGAGGAATACA R – TAGGTGCTGCACTGGAAATC	NED	183-191	58
ADL0176	F – TTGTGGATTCTGGTGGTAGC R – TTCTCCCGTAACACTCGTCA	VIC	181-201	58
ADL0267	F - AAACCTCGATCAGGAAGCAT R - GTTATTCAAAGCCCCACCAC	NED	97-115	58
ADL0268	F - CTCCACCCCTCTCAGAACTA R - CAACTTCCCATCTACCTACT	VIC	97-113	58
LEI0094	F - GATCTCACCAGTATGAGCTGC R - TCTCACACTGTAACACAGTGC	VIC	247-287	58
MCW0216	F - GGGTTTTACAGGATGGGACG R - AGTTTCACTCCCAGGGCTCG	6FAM	138-150	58
MCW0248	F - GTTGTTCAAAAGAAGATGCATG R - TTGCATTAACTGGGCACTTTC	NED	205-225	58

Ta – hybridisation temperature of the primers (annealing temperature); F – forward; R – reverse; 5' end of the reverse primer was labeled with fluorescent dye.

Genetic analyses were performed using an ABI Prism 3100-*Avant* Genetic Analyzer (Applied Biosystems). The 3100-*Avant* ABI Prism Data Collection and Gene Mapper v. 3.5 (Applied Biosystems) software were used to analyse the results and interpret the length of the fragments. Allele sizes were determined using an internal standard (GeneScan 350 ROX Size Standard, Applied Biosystems).

To determine whether analysis of microsatellite alleles, among other alleles, is a good tool for the identification of separate individuals, we assessed the occurrence of eight microsatellite loci. The results demonstrated the presence of private and common alleles in all the studied populations. Private alleles are described as those found exclusively in only one population [Szpiech and Rosenberg 2011], whereas common alleles are those that occur in at least two populations.

Statistical parameters, such as the number of alleles, expected and observed heterozygosity, inbreeding coefficient (Fis), and Weir and Cockerham estimates (1984), genetic correlation coefficient (Fst) (GenePop), polymorphism information content (PIC; Microsatellite ToolKit), as well as genetic distance between populations (DAS) and the phylogenetic tree (UPGMA) (POPULATIONS 1.2.32) were determined for each population [Raymond and Rousset 1995, Langella 2002, Kim and Sappington 2013].

Results and discusion

The birds (n=109) were screened using eight microsatellite markers. The total number of alleles amounted to 51, which indicates the chromosomal position of each

Table 2. Private and common alleles for all populations and Polymorphism Information Content values for each *locus*

Locus	Common alleles (bp)	Private alleles (bp)				PIC
		TNB	TNW	HS	GP	
ADL136	-, 127, 143, 145,149	137	125, 159	151	155	0.48
ADL158	185, 191		189			0.39
ADL176	-, 183, 189, 195, 199, 201	191	181			0.58
ADL267	101, 111		103	-*, 99	109, 113	0.51
ADL268	101, 107, 109, 111, 113	103		-*	97	0.57
LEI094	-, 263, 265, 267, 279			283	269, 273	0.47
MCW216	138, 140, 142					0.35
MCW248	214, 218, 220, 222					0.32

PIC – Polymorphism Information Content, TNB – Black Transylvanian Naked Neck, TNW – White Transylvanian Naked Neck, HS – Speckled Hungarian, GP – Green-legged Partridgelike.
*Lack of allele which is exclusive only for that population in this *locus*.

Table 3. Private alleles for separate individuals

Breed	Number of individuals	*Locus*	Allele
TNB	1	ADL268	103
TNW	4	ADL136	125, 159
		ADL176	181
		ADL267	103
HS	3	ADL136	151
		ADL267	-
		ADL268	-
GP	2	ADL267	113
		LEI094	273

TNB – Black Transylvanian Naked Neck, TNW – White Transylvanian Naked Neck, HS – Speckled Hungarian, GP – Green-legged Partridgelike.

loci. The highest number of alleles (34) was obtained for the HS breed, while 27 alleles were detected for the TNB and TNW breeds and 29 alleles for the GP breed.

The results of microsatellite marker analysis revealed the occurrence of 19 private and 32 common alleles among all the studied populations (Tab. 2). Private alleles were detected for different individuals, also either they are specific for only them or have specific set of alleles by which they can be identified. Ten birds showed the presence of private alleles, represented by at least one animal from each breed, whereas four animals from the TNW stock were identified with private alleles (Tab. 3).

In this study the presence of private alleles in the analysed populations indicates significant genetic diversity in these breeds, showing that the studied material possesses the genetic structure suitable for the current research. Hillel *et al*. [2003] found one private allele in the GP breed, which was observed in more than 10% of the

population. No private alleles were detected in the birds of Transylvanian Naked Neck breeds. Siwek *et al*. [2010] reported the presence of nine microsatellite markers in two chicken breeds (White Leghorn and GP). They identified 19 private alleles that were specific for the GP breed and 29 common alleles shared with the White Leghorn breed. The highest number of private alleles (5) was reported for the ADL176 microsatellite.

Table 2 presents the PIC estimates, confirming informativeness of the microsatellite markers used (Microsatellite ToolKit) [Kim and Sappington, 2013]. As reported by Botstein *et al*. [1980], PIC >0.50 indicates a highly informative *locus*, PIC in the range of 0.25-0.50 shows a reasonably informative locus, while PIC <0.25 – a slightly informative *locus*, respectively. The findings of the present study show a high degree of polymorphism for three microsatellite *loci* analysed. The PIC values for the ADL176, ADL267, and ADL268 loci were greater than 0.5, which indicates that they are highly informative *loci*. For the other markers their PIC values were found to be between 0.32 and 0.48, which represents reasonably informative *loci*. Kaya and Yildiz [(2008] analysed five populations of two Turkish breeds using 10 microsatellite markers. The PIC value was the highest for the ADL176 and ADL136 *loci* (0.835 and 0.830, respectively), while the mean values were found to be 0.546, 0.687, and 0.5790 for ADL157, ADL267, and ADL268, respectively. This result also indicates that these loci are highly informative.

In the current study, genetic diversity (Fst) values were calculated between the populations. These indexes specify a decrease of heterozygosity in the population in relation to all stocks as a result of selection or genetic drift. The Fst value indicates the intensity of gene flow and evaluates the genetic distance between the populations. The lowest value corresponds to the greatest genetic similarity and in this study it was represented by the TNB and HS breeds (Fst=0.20) (average genetic variation). On the other hand, the highest Fst value corresponds to the most genetically diverse populations, in this study represented by the TNB and TNW breeds (Fst=0.35) (high genetic variation) (Tab. 4). These two breeds are phenotypically similar and carry the same naked neck gene, but the breeds might differ at the genetic level. The obtained results indicate the presence of high (0.15-0.25) and very high (>0.25) genetic differentiation between all the analysed populations (GenePop) [Raymond and Rousset 1995].

Table 4. Genetic relationships (Fst) between studied populations based on 8 microsatellite *loci*

Population	TNB	TNW	HS
TNW	0.35		
HS	0.20	0.27	
GP	0.27	0.34	0.31

TNB - Black Transylvanian Naked Neck, TNW – White Transylvanian Naked Neck, HS – Speckled Hungarian, GP – Green-legged Partridgelike

Bodzsar *et al*. [2009] presented similar results in their study. They investigated populations of 3 breeds of the same origin. For genetic correlations (Fst) between the TNB, TNW and HS breeds the highest value was estimated between the TNB and TNW breeds (0.258).The Fst estimate between TNB and HS was 0.214, while between the TNW and HS breeds it was found to be 0.237. Figure 2 shows the network tree of 27 populations constructed based on the Marker estimated kinship (MEK) values obtained by Bodzsar *et al*. [2009], with the TNB, TNW and HS breeds marked.

The high Fst values between the GP and TNW breeds, and the GP and HS breeds (0.34 and 0.31, respectively) are confirmed in Table 5, which shows the highest genetic distance (DAS) value between these populations (using POPULATIONS 1.2.32 software) [Langella, 2002]. The greatest genetic distance (DAS) value indicates the lowest genetic similarity between the breeds. The genetic distance values between the studied populations (presented in Tab. 5) are shown in a phylogenetic tree (UPGMA) (Fig. 1) (POPULATIONS 1.2.32) [Langella 2002], which shows a clear distinction between GP and the other populations. This result could have been expected, as GP is a native Polish breed and the others are Hungarian populations. Moreover, the PIC estimates for the TNB and HS breeds show that these populations exhibit the closest genetic relation compared to the other populations.

Table 5. Shared allele distances between 4 studied populations

Population	TNB	TNW	HS
TNW	0.48		
HS	0.26	0.36	
GP	0.39	0.51	0.52

TNB – Black Transylvanian Naked Neck, TNW – White Transylvanian Naked Neck, HS – Speckled Hungarian, GP – Green-legged Partridgelike.

We also need to stress a smaller genetic distance between the HS and TNB and between the HS and TNW breeds compared to the TNB and TNW breeds (Fig. 1). Bodzsar *et al.* [2009] in their analysis of 27 Hungarian populations of chickens also reported similar findings. They used the MEK method to present the network tree between these populations (TNB, TNW and HS). The results show that the TNB and TNW breeds were more distant from each other than from the HS breed. The branch of the HS breed lies between the branches of the TNB and TNW breeds (Fig. 2). Bodzsar *et al.* [2006, 2009] also investigated TNB, which genetic structure was always found to be unique and showed a distant relationship with the other investigated breeds. They also studied the relationship between the TNW and White Hungarian breeds, showing a close association with each other, as opposed to the association with TNB. This may have been caused by the fact that in the 1960s the TNW and White Hungarian chickens were kept together because of their color. Animal owners believed that both these populations were of same breed, but differed in the presence or absence of feathers. This may be one of the reasons for the lack of a close relationship between the TNB and TNW breeds.

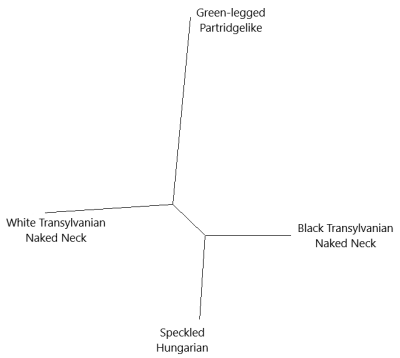

Fig. 1. A phylogenetic tree (UPGMA) showing genetic distance between examined populations (POPULATIONS 1.2.32) [Langella 2002].

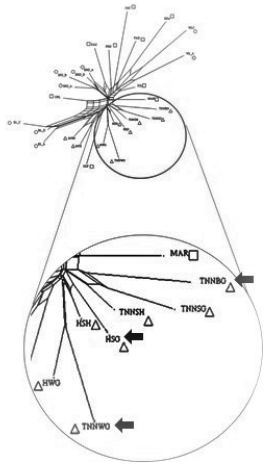

Fig. 2. A network tree of 27 populations based on MEK distances [after Bodzsar et al. [2009]; TNNBG – Transylvanian Naked Neck Black Godöllo, TNNWG – Transylvanian Naked Neck White Godöllo, HSG – Hungarian Speckled Godöllo].

Measures of genetic variability within the studied populations are summarized in Table 6 and were determined by the expected (He) and observed (Ho) heterozygosity and inbreeding coefficient (Fis) of a breed. All of the measures were non-significantly different from zero. Heterozygosity within the populations was estimated based on the set of markers showing substantial heterozygosity in the number of alleles identified. Expected heterozygosity is the estimated participation of heterozygotes for allele frequency of a locus in a population according to the Hardy-Weinberg equilibrium, whereas observed heterozygosity is the actual frequency of heterozygotes in the stock. When the population is in a balanced state, then according to the Hardy-Weinberg equilibrium the frequency of mutations, selection, migration and genetic drift is low.

Table 6. Basic diversity measures within each studied population

Population	H_e (SD)	H_o (SD)	F_{is}
TNB	0.09 (0.04)***	0.07 (0.05)**	0.19
TNW	0.10 (0.05)***	0.10 (0.07)***	0.14
HS	0.08 (0.19)***	0.06 (0.05)**	0.27
GP	0.08 (0.03)***	0.09 (0.04)***	0.04

H_e – expected heterozygosity, H_o – observed heterozygosity; F_{is} – inbreeding coefficient, SD – standard deviation; **P <0.01 and ***P <0.005 – result significantly different from "0", TNB – Black Transylvanian Naked Neck, TNW – White Transylvanian Naked Neck, HS – Speckled Hungarian, GP – Green-legged Partridgelike.

The condition, which leads to this state of balance is random mating and a sufficient number of individuals.

The TNW population showed the highest expected (He=0.10) and observed heterozygosity values (Ho=0.10), whereas the lowest expected (He=0.08; He=0.08) and observed heterozygosity values (Ho=0.06; Ho=0.09) were obtained for the HS and GP stocks, respectively. The level of heterozygosity was mirrored by the inbreeding coefficient, being the lowest in the GP breed (0.04) (GenePop) [Raymond and Rousset 1995]. The differences between expected and observed heterozygosity values in the studied populations were small and statistically non-significant, indicating a strong correlation to the Hardy-Weinberg equilibrium. The genetic diversity within each studied population was low, as evidenced by the low calculated estimates of heterozygosity.

Bodzsar *et al.* [2009] obtained higher expected and observed heterozygosity values for the TNB, TNW, and HS breeds. The highest values were recorded for the HS breed (0.55 and 0.54, respectively). For the TNB breed the values were found to be 0.44 and 0.44, while for the TNW breed it was 0.49 and 0.51, respectively. Siwek et al. [2010] in their microsatellite analysis for the GP breed obtained the Ho value of 0.53. The highest He and Ho values for the GP breed (0.44 and 0.51, respectively) were also estimated by Granevitze *et al*. [2007], who analysed 65 populations of chickens using 29 microsatellite markers. These differences might have resulted from the different microsatellite loci used during analysis.

The results obtained in this study and those reported by Bodzsar *et al*. [2009] and Siwek *et al*. [2010] provide information concerning the structure of populations and how it has changed after 10 years of *in situ* conservation. In both studies the expected and obtained heterozygosities were similar, indicating that these populations exhibited a close match to the Hardy-Weinberg equilibrium and that *in situ* conservation exerted no significant effect on these populations. Moreover, which is probably the most important inference, that animal breeding system had no negative effect on these populations.

The inbreeding coefficient is a measure of inbreeding in a population and its value depends on the expected and observed heterozygosity ratio. The Fis value less

than "0," which was not obtained in the current study, indicates an overabundance of heterozygotes in the population. This may be due to the selection for heterozygotes or the bottleneck effect (colonisation with a small number of individuals). The Fis values were greater than "0" for all the populations in our study. These results were statistically significant and were probably the outcomes of inbreeding or genetic drift (random fixation or elimination of a portion of alleles and a change in their frequency), sexual selection or loci coupling, caused by an overabundance of homozygotes in the populations. All the populations investigated in this study are indigenous breeds and the expected results were obtained for Fis values in these populations.

Another study conducted by Bodzsar *et al*. [2009] provided different findings. Those researchers analysed the TNB, TNW, and HS breeds, among other Hungarian breeds, with the Fis estimates for these breeds amounting to 0.015-0.039 and 0.031, respectively. The Fis estimate for the TNW breed was found to be less than "0," which was significant and indicated an overabundance of heterozygotes in the population. The Fis values for the TNB and HS breeds in this experiment were statistically significant and might have been related to an overabundance of homozygotes in the populations.

Microsatellite analysis is considered to be the most appropriate tool for studies on the population structure among animals. Results of this study proved applicability of microsatellite analysis to identify separate individuals, which may be used for further experiments.

Our findings also confirmed the importance of genetic diversity in indigenous chicken breeds. We proved that *in situ* preservation exerted no negative effect on the population structure of the analysed breeds. There was no statistically significant difference between the expected and observed heterozygosity values reported in a study of Bodzsar *et al*. [2009] and the current study.

Conserving the genetic structure of local chicken breeds is crucial for preserving chicken biodiversity at local and global levels. To obtain the best preservation results the *in situ* conservation techniques should be supported by *ex situ* methods, such as cryopreservation, especially in the case of endangered species.

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