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STR DIVERSITY OF A HISTORICAL SHEEP BREED BOTTLENECKED, THE CIKTA

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ABSTRACT

The population structure of the endangered Cikta sheep breed was evaluated by means of nine microsatellite polymorphisms. Seventy-two individuals from three flocks were sampled to determine genetic indices in the Hungarian population. Overall, average observed and effective allele numbers were 5.63 and 3.76, respectively. Discriminant analysis based on genotype frequencies revealed moderate genetic diversity among Cikta flocks, since only three loci (*OarCP49*, *CSSM47* and *OarHH41*) contributed significantly ($P<0.05$) to differences between subpopulations. Low squared Mahalanobis distances from group centroids also confirmed that the breed is almost equally represented by the three flocks. Moderate level of diversity between flocks was attributed to the long-term effects of a population bottleneck dating back to the 1970s. Negative average F_{IS} value (-0.18) indicated heterozygote excess. Chi-squared tests identified significant ($P<0.05$) deviation from HWE in the case of *BM8125*, *CSSM47*, and *MAF214* markers. Continuous microsatellite information is required for the preservation of rare alleles and diversity in Cikta sheep.

Key words: Cikta sheep; microsatellite; indigenous; population structure.

INTRODUCTION

Long-established historical farm animal breeds represent valuable genetic resources required to overcome future challenges of animal breeding such as climate change and severe loss of biodiversity. Therefore, all efforts should be made to carefully maintain and describe existing populations of local autochthonous breeds. The Cikta is one of several indigenous sheep breeds registered in Hungary. According to breed history, Swabians (an ethnic German population) introduced and propagated the Cikta sheep in Hungary when they settled in the region in the 17th century. Originally used as a triple-purpose (meat, milk, and wool) breed, Cikta have become very well adapted to the different geographic and climatic conditions of Hungary during the last centuries (Korth, 1825; Bohm, 1878). The Cikta population was fluctuating but has been a consistent part of the Hungarian sheep stock; however, Cikta never became the leading or the most populous breed of Hungary. After World War II this valuable fallow sheep was almost completely excluded from production (Koppány, 2000). In 1974, the National Animal Husbandry Inspectorate started preservation of Cikta by collecting 40 ewes and 3 rams in the country. The population grew to 200 animals in the following decade. This pool has been the root of today's living seed stocks of about 600 individuals. Therefore, the national Cikta stock is now considered to be the descendant of that single homogeneous population. The Cikta as a protected indigenous breed exists nowadays in some small-sized flocks with state support. The goal is to preserve the triple purpose type of the breed. Cikta historically belongs to the Zaupel type of sheep, along with the living representatives of the so-called Steinschaf group such as the Alpines Steinschaf, the Waldschaf, the Tiroler Steinschaf, and the Krainer Steinschaf (Baumung *et al.*, 2006). The examination of Neubauer *et al.* (2015) confirmed that the Cikta is genetically well separated from the breeds of Zackel type (e.g. Hungarian Racka and Transylvanian Racka) and other more numerous breeds in Hungary. Based on pairwise Nei's genetic distances. Pichler *et al.* (2017) have also undoubtedly distinguished the Zaupel type (Western European; samples analysed from the Krainer Steinschaf) from the Zackel type of sheep (Eastern European origin).

The aim of the present study is to expand the population genetic analysis in Cikta breed based on the microsatellite polymorphisms recommended by the FAO. Additionally, flock-discriminating power of the microsatellites was also tested to determine the level of genetic identity between subpopulations of the breed.

MATERIALS AND METHODS

Sampling and DNA isolation: The seventy-two individuals selected for the present examination were representatives of the oldest families. The experimental animals with 6-5-4 ancestral generations belonged to 36 families (maternal lineages). Two living representatives from each ancient family were chosen. Blood samples were collected from three farms in October 2015. One of

the chosen farms was the state-owned Duna-Dráva National Park (station Nagydorog, with 20 families and 40 specimens), whereas the other two were private farms owned by Mr. T. Nagy (Pénzesgyőr, with 11 families and 22 specimens) and Mr. J. Jánosi (Szécsénke, with 5 families and 10 specimens). Collection tubes containing EDTA as anticoagulant were stored at -20°C pending processing. After thawing, DNA was extracted from the blood samples using Wizard Genomic DNA Purification Kit (Promega, USA). The DNA concentration of isolates was determined by NanoDrop 2000 spectrophotometer (Thermo Scientific, USA), followed by the dilution of each sample to 100 ng/µL final DNA concentration. Only samples with 260/280 and 260/230 absorbance ratios over 1.8 were subjected to later PCR steps.

PCR and fragment analysis: Amplification of DNA was done by means of a programmable PCR machine (DNA Thermal Cycler, Perkin Elmer, USA). Nine microsatellites were analysed in two sets of multiplex reactions, where the first set contained primers for loci *BM0757*, *BM8125*, *OarCP49*, *BM0827*, and *OarHH47*, whereas the second set contained primers for loci *CSSM47*, *MAF214*, *OarHH41*, and *OarVH72*. The 5'-ends of forward primers were labelled with fluorescent dyes as indicated in Table 1.

Table 1. Selected microsatellites and primers used for the analysis of Cikta sheep populations.

Microsatellite	Primer sequence (5'-3')	Dye	Allele size range (bp)	Chromosome
BM0757[*]	F: TGGAAACAATGTAAACCTGGG R: TTGAGCCACCAAGGAACC	FAM	178-198	9
BM8125[*]	F: CTCTATCTGTGGAAAAGGTGGG R: GGGGGTTAGACTTCAACATACG	FAM	109-119	17
OarCP49	F: CAGACACGGCTTAGCAACTAAACGC R: GTGGGGATGAATATTCCTTCATAAGG	HEX	82-102	17
BM0827[*]	F: GGGCTGGTCGTATGCTGAG R: GTTGGACTTGCTGAAGTGACC	HEX	214-227	3
OarHH47[*]	F: TTTATTGACAAACTCTCTTCCTAACTCCACC R: GTAGTTATTTAAAAAAATATCATACCTCTTAAGG	ROX	132-138	18
CSSM47[*]	F: TCTCTGTCTCTATCACTATATGGC R: CTGGGCACCTGAAACTATCATCAT	FAM	129-132	2
MAF214	F: AATGCAGGAGATCTGAGGCAGGGACG R: GGGTGATCTTAGGGAGGTTTTGGAGG	FAM	188-264	16
OarHH41[*]	F: TCCACAGGCTTAAATCTATATAGCAACC R: CCAGCTAAAGATAAAAGATGATGTGGGAG	HEX	120-140	10
OarVH72[*]	F: CTCTAGAGGATCTGGAATGCAAAGCTC R: GGCCTCTCAAGGGGCAAGAGCAGG	ROX	124-142	25

[*] FAO-recommended microsatellite loci in sheep (FAO, 2011)

Touchdown PCR was applied for the amplification of microsatellite loci as follows: denaturation at 95°C for 1 min, then annealing temperatures were lowered by 2°C in every 3 cycle from 60 to 50°C for 1 min, followed by 15 cycles at 50°C annealing, whereas elongation step throughout the reaction was at 72°C for 1 min in a total of 30 cycles. Reaction mixtures contained 100 ng genomic DNA, 0.6 µL *Taq* DNA polymerase, 200-200 µM dNTPs, 50-50 pM primers, $MgCl_2$ at concentration 1.5 mM, and nuclease free water up to 25 µL total reaction volume (Fermentas products, Thermo Fisher Scientific, USA). PCR product detection, microsatellite allele determination and analysis were carried out using an ABI PRISM 3130XL Genetic Analyzer controlled by ABI PRISM 310 Data Collection Software (Applied Biosystems, USA). For the identification of microsatellite alleles, the 310 GeneScan Analysis Software 3.1 was applied, that uses internal standards for length determination.

Data analysis: Genotyper Software (Applied Biosystems, USA) was used for data interpretation and microsatellite length verification. Basic population genetics parameters – such as number of different alleles (N_a), number of effective alleles (N_e), observed, expected, and unbiased expected heterozygosity (H_o and H_e), inbreeding coefficients (F_{IS}, F_{ST}, F_{IT}) – were calculated for Cikta populations by means of the Microsatellite Analyser (Dieringer and Schrötterer, 2003) and the GenAlEx 6.503 (Peakall and Smouse, 2012) population genetics softwares. Hardy–Weinberg equilibrium (HWE)

was tested by Chi-square analyses of observed and expected microsatellite allele frequencies.

The comparison of the flocks was done by discriminant analysis. In the analysis, individual observations are grouped under predetermined conditions. In present study the observations were microsatellites, whereas the groups were the flocks. The identity/dissimilarity of groups (flocks, dependent variables) was evaluated taking more quantitative traits (microsatellite length, independent variables) together. Categorization was done with Bayes classification; the comparison of the groups was based on the centroids for the groups. The deviation of the given observation from the centroid was measured by the Mahalanobis distance (Dell Inc., 2015).

RESULTS AND DISCUSSION

Population genetics indices: The number of alleles was 5.63 on average. In all loci the number of alleles was over four by microsatellites (with the only exception of *CSSM47*) which satisfies the FAO (1998) recommendation for genetic diversity studies. An overview on genetic diversity parameters is presented in Table 2.

Table 2. Summary statistics for microsatellites in Cikta sheep breed.

Microsatellites	N_a mean ± SD	N_e mean ± SD	I mean ± SD	I_{rel} %	H_o	H_e
BM0757	5.33 ± 0.58	3.43 ± 0.49	1.41 ± 0.05	54.5	0.72	0.79
BM8125	4.67 ± 0.58	3.35 ± 0.84	1.31 ± 0.26	56.4	0.80	0.73
OarCP49	5.67 ± 0.58	4.12 ± 0.18	1.53 ± 0.05	59.2	0.88	0.78
BM0827	6.00 ± 1.00	4.59 ± 0.74	1.63 ± 0.13	58.1	0.92	0.81
OarHH47	4.00 ± 0.00	2.66 ± 0.62	1.07 ± 0.17	46.1	0.52	0.60
CSSM47	3.33 ± 1.53	2.54 ± 0.55	0.98 ± 0.28	42.2	0.77	0.65
MAF214	6.00 ± 0.00	3.60 ± 0.26	1.46 ± 0.09	52.0	0.84	0.74
OarHH41	9.33 ± 1.53	6.15 ± 0.58	1.97 ± 0.10	56.9	0.82	0.87
OarVH72	6.33 ± 1.53	3.44 ± 0.31	1.42 ± 0.05	47.3	0.81	0.71
Overall mean ± SD	5.63 ± 1.71	3.76 ± 1.10	1.42 ± 0.29	52.5 ± 6.01	0.78 ± 0.11	0.74 ± 0.08

N_a = number of alleles; N_e = number of effective alleles; I = Shannon's Information Index; I_{rel} = relative Shannon's Information Index; H_o = observed heterozygosity; H_e = expected heterozygosity

Comparing our effective allele numbers to results of Radha *et al*. (2011) in Kilikarsal sheep breed of India remarkable deviations in both directions have arisen in the following microsatellites: *BM8125* (3.35 vs. 1.53), *OarHH41* (6.15 vs. 3.46) as well as *OarHH47* (2.66 vs. 4.52) and *OarVH72* (3.44 vs. 5.17).

The values of relative Shannon's Information Index are placed in a larger range (from cca. 40 until 60%). The I_{rel} is the proportion of the actual Shannon's value and the theoretically highest Shannon's value (under equal allele frequency condition). This figure is advantageous when microsatellite loci of different allele (haplotype) numbers are to be compared (Gáspárdy *et al*., 2018).

The averages of observed and expected heterozygosity were always over 0.50 and lied in a broader range of 0.52-0.92 and 0.65-0.87, respectively. The values of unbiased expected heterozygosity (figures are here not presented) completely corresponded with the values of expected heterozygosity.

Naqvi *et al.* (2017) investigated five Pakistani sheep breeds by use of microsatellites, three of which were common to ours (*OarHH47*, *BM8125*, *MAF214*) and found the expected heterozygosity to be lower than 70% on average, and at the same time, positive F_{IS} values in all the breeds. For *MAF214* Crispim *et al.* (2014) published a much lower H_e (58%) also together with positive F_{IS} value in Pantaneiro sheep of Brazil.

Fixation indices: Fixation indices are presented in Table 3. Negative F_{IS} is an indicator of heterozygous excess, the advantageous phenomenon in an outbred population. Cikta population F_{IS} with an average value of -0.18, reflecting an elevated level of genetic differentiation within the all individuals of the breed. The highest F_{IS} value was exactly 0.00 for *OarHH41*, whereas the lowest was -0.40 for *CSSM47*. Similarly, in the ancient Gute sheep breed from the Swedish island Gotland Rochus and Johansson (2017) observed that the flocks had excess heterozygosity (average F_{IS} = -0.11; here, *MAF214* was the only common microsatellite). In Barbarine, the most important native mutton breed of Tunisia Sassi-Zaidy *et al.* (2016) described positive F_{IS}, an involuntary sign of genetic erosion (sharing *OarCP49* and *MAF214*). Also in Tsigai and several Zackel type breeds, positive F_{IS} values were determined indicating heterozygote deficiency and risks of inbreeding (Kusza *et al.*, 2008; 2010).

Table 3. Fixation indices (F_{IS}, F_{ST}, F_{IT}) for microsatellites in the Cikta sheep breed.

Microsatellites	F_{IS}	F_{ST}	F_{IT}	HWE (Chi^2 values)	PIC
BM0757	-0.15	0.10	-0.04	18.7	0.74
BM8125	-0.24	0.05	-0.18	25.4*	0.69
OarCP49	-0.20	0.02	-0.17	21.3	0.73
BM0827	-0.20	0.02	-0.17	21.7	0.78
OarHH47	-0.02	0.06	0.04	9.5	0.53
CSSM47	-0.40	0.08	-0.30	148.3*	0.57
MAF214	-0.23	0.02	-0.20	93.1*	0.69
OarHH41	0.00	0.02	0.02	59.1	0.85
OarVH72	-0.23	0.02	-0.20	24.7	0.67
Overall mean ± SD	-0.18 ± 0.12	0.04 ± 0.03	-0.13 ± 0.11	46.86 ± 46.11	0.70 ± 0.10

HWE = Hardy–Weinberg equilibrium; PIC = polymorphism information content
* significant deviation from zero with $P < 0.05$

F_{ST}-values varied between 0.02 and 0.10. Its overall value was 0.04 which shows that approximately 4% of genetic diversity is explainable by genetic variation among flocks. Significant deviation ($P < 0.05$) from Hardy–Weinberg equilibrium occurred for three microsatellites, namely *BM8125*, *CSSM47* and *MAF214*. The polymorphism information content (PIC) values were always less than pair values of the expected heterozygosity. With regards to PIC, Agaviezor *et al.* (2012) received larger figures on average compared to the present study for *OarHH47* (0.83 vs. 0.53) and *OarVH72* (0.85 vs. 0.67) in the merged pool of analysed Nigerian indigenous sheep breeds.

Allele size range: An overview on microsatellite lengths for each flock is given in Table 4. Differences were found in three cases (*OarCP49*, *CSSM47* and *OarHH41*) only which mean that the flocks have a high degree of identity, and the families of Cikta breed are genetically close to each other.

Table 4. Microsatellite sizes by Cikta flock presented in base pairs (mean ± SD; n = sample size).

Microsatellite	Nagydorog n = 80	Pénzesgyőr n = 44	Szécsénke n = 20	All n = 144	P-value
BM0757	182.4 ± 4.6	183.2 ± 10.0	183.8 ± 5.3	182.8 ± 6.5	0.645
BM8125	114.4 ± 2.3	114.8 ± 2.8	114.2 ± 2.1	114.5 ± 2.4	0.596
OarCP49	91.0^{b} ± 8.4	88.5^{ab} ± 7.9	86.3^{a} ± 6.3	89.5 ± 8.1	0.020
BM0827	220.6 ± 3.5	220.0 ± 2.9	219.9 ± 2.7	220.3 ± 3.2	0.513
OarHH47	133.8 ± 3.0	134.1 ± 3.4	134.7 ± 2.5	134.0 ± 3.0	0.283
CSSM47	129.5^{a} ± 1.3	130.0^{b} ± 1.5	131.0^{b} ± 1.0	129.9 ± 1.4	0.005
MAF214	206.6 ± 21.3	202.4 ± 19.0	202.4 ± 22.0	204.8 ± 20.8	0.334
OarHH41	129.8^{b} ± 8.0	125.9^{a} ± 6.1	131.8^{b} ± 7.5	129.2 ± 7.7	0.015
OarVH72	131.1 ± 5.7	132.8 ± 6.4	129.3 ± 5.2	131.2 ± 5.9	0.073

^{a,b} different letters indicate significant ($P < 0.05$) differences

Four common microsatellites (*BM8125*, *OarHH47*, *MAF214* and *OarVH72*) from three Albanian sheep breeds (Bardhoka, Ruda and Shkodrane) shown similar allelic range (size) to our observations. furthermore, the breeds displayed heterozygote deficiency (Hoda and Marsan, 2012). Yilmaz *et al.* (2014) also investigated *OarCP49* and found on average a greater allelic size (82-141) in nine local sheep breeds of Turkey than we did (mean cca. 90). However, their negative F_{IS} values for *OarCP49* indicated heterozygote excess (outbreeding) similar to our results. Serious deviation from our results seems to be characteristic of *BM8125* and *MAF214* (with lengthening cca. 100 bps and shortening cca. 80 bps, respectively) according to the data processing of four Tunisian sheep breeds made by Kdidi *et al.* (2015). In the investigation of Sharma *et al.* (2016) carried out in the endangered Tibetan sheep of India which contains each of our microsatellites there was an unexpected identity revealed in the allelic range (size) in all cases.

Discriminant analysis: The common Wilks' Lambda of the discriminant functions was 0.606. This higher value indicates the significant ($F = 18.2$ and $P < 0.001$) discriminating ability of the functions. Not all of the independent variables were significant ($P > 0.05$),

consequently not each microsatellite plays role in isolating the flocks and families per flocks. There were only three microsatellites (*OarCP49*, *CSSM47* and *OarHH41*) which significantly increased the difference between the flocks compared to the others as seen by their relative effects and P-values from Table 5.

Table 5. Summary of discriminant analysis.

Microsatellite	Wilks' Lambda	F-value	P-value	R^2	Relative effect (%)
BM0757	0.607	0.048	0.953	0.220	0.18
BM8125	0.607	0.036	0.965	0.303	0.14
OarCP49	0.665	4.752	0.011	0.232	18.23
BM0827	0.612	0.497	0.610	0.382	1.91
OarHH47	0.607	0.038	0.963	0.192	0.15
CSSM47	0.778	13.904	<0.001	0.351	53.33
MAF214	0.614	0.604	0.549	0.261	2.32
OarHH41	0.652	3.703	0.028	0.263	14.21
OarVH72	0.637	2.488	0.088	0.338	9.54

As classification result the overall accuracy was 64.2%. The flocks' accuracies were as it follows: Nagydorog 85.2%, Pénzesgyőr 21.4%, and Szécsénke 60.0%. Low precision values indicate that the flocks (and individuals representing families) are similar, and the breed is almost equally represented by the three flocks.

The squared Mahalanobis distances from group centroids were as follow: 9.6 (P = 0.56), 9.7 (P = 0.257), and 11.1 (P = 0.184). These lower and non-significant values confirm that there is no pairwise separation of the centroids of the flocks being compared. This condition is well illustrated in Figure 1, where the individuals of the smaller populations of Pénzesgyőr and Szécsénke are placed among the individuals of the most populous Nagydorog flock, even if function (Root) 1 is to discriminate mostly between Szécsénke and the others (Nagydorog and Pénzesgyőr; P for all roots was < 0.001). In the vertical direction (Root 2), no trend of points to fall below or above the centre line (0) is apparent (the significance test gave for all remaining roots after removing the first root P = 0.102). In endangered Tsigai breed, the flocks (5 herds, 48 ewes in each) differed strongly from each other according to all eight microsatellites (Wilk's $\lambda = 0.059$, $p<0.001$). The average proportion of correct classification (83.7%) was highly satisfactory (Gáspárdy *et al*., 2013).

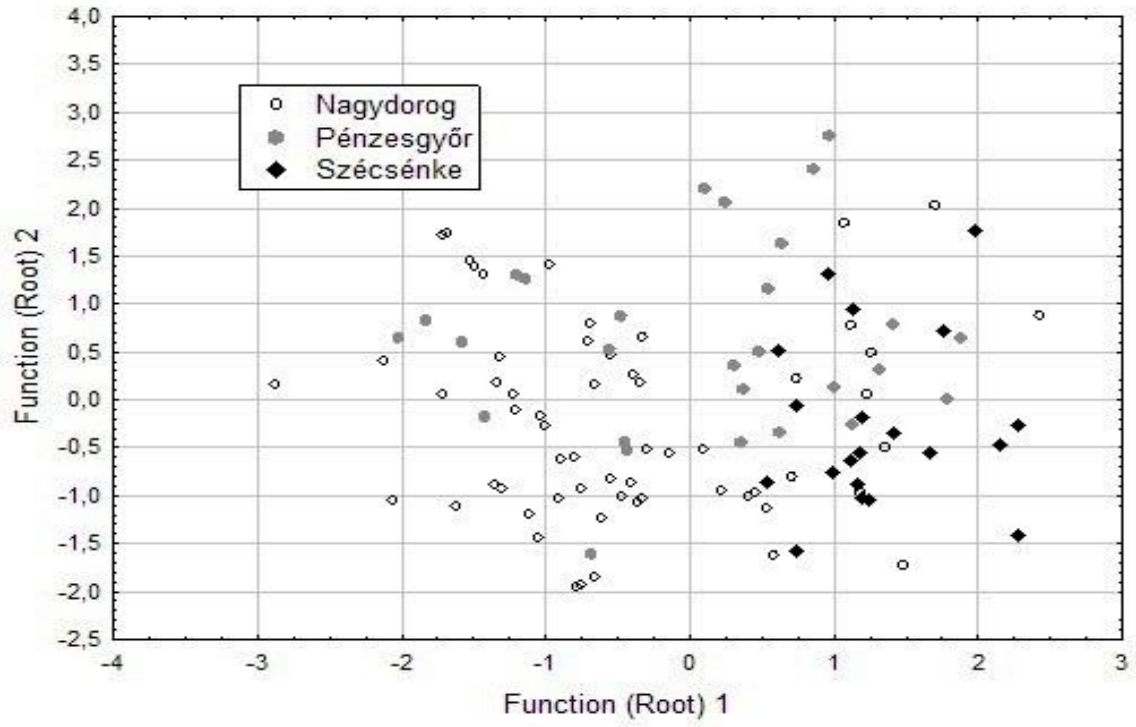

Figure 1. Scatterplot of canonical scores of the three Cikta flocks investigated according to nine microsatellites

Conclusions: Our results based on microsatellite loci information have confirmed the fact that the Cikta stock of the early 21st century is actually made up of genetically closely related individuals, families or flocks. The similarity is attributed to the shrinking national stock to a small population by the 1970s and the consequence of bottleneck effect. Since the farms share and breed maternal lineages more than those in this study, the difference between the flocks can be even smaller. Despite this situation large allelic diversity, heterozygote excess, elevated level of genetic differentiation, and genetic equilibrium condition were reflected in most of the microsatellites. The degree of heterozygosity which is considered to be high (cca. 75-80%) can be attributed to rams used in larger proportion than usual. From 2011, the newly introduced centralized raising program contributes to a well-organized use of breeding rams among flocks. An interesting proposition to preserve diversity within the breed is the regular replacement of females as well among the flocks. This can be implemented; however, the movement of animals also involves more and more prudent animal health measures. The insistence on genetic unchangingness and the doubt about the acceptable level of genetic change occupy a central place in conservation of rare domestic animals. However, the flocks are constantly changing in their genetic composition as well despite all the will to maintain permanence and immutability.

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