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GENETIC DIVERSITY MEASURES OF THE CROATIAN SPOTTED GOAT

RAMLJAK JELENA*, MIOČ B*, ĆURKOVIĆ M**, PAVIĆ VESNA*, IVANKOVIĆ A* and MEĐUGORAC I***

*Faculty of Agriculture, Zagreb, Croatia; **Faculty of Agriculture, Mostar, Bosnia and Herzegovina; ***The Ludwig-Maximilians-University Munich, Faculty of Veterinary Medicine, Germany

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In the present study, microsatellite data of 20 loci were generated and utilized to evaluate genetic variability of the Croatian Spotted goat. Genetic variability was high, with means for expected gene diversity of 0.771, observed heterozygosity of 0.759, and 8.1 for the total number of alleles per locus. There are no indications for deviations from random breeding within the population. Level of inbreeding was only 2% and non-significant. The population was found to deviate significantly under infinitive allele model (IAM) and two phase model (TPM), while stepwise mutation model (SMM) and qualitative mode-shift test of allele frequencies indicate the absence of genetic bottleneck in the recent past in the population of the Croatian Spotted goat. High level of genetic diversity, as it is presented in this study, may be seen as an initial guide for conservation decisions in the future.

Key words: Croatian Spotted goat, genetic diversity, microsatellite markers

INTRODUCTION

Goat breeds contribute with 8.5% to the total number of recorded animal breeds in the world, mainly breed in Asia (China), Near and Middle East (India and Pakistan; FAO DADIS, 2011). They are recognized as valuable components of world diversity and play an important role in sustainable agriculture, especially in climatic regions where other livestock species can not be adjusted (i.e. Africa and India). In Europe they are of limited importance mainly as a result of the increasing importance of cattle (milk and meat production) and sheep (wool production of Merino sheep instead of mohair), although some high-yielding dairy (Saanen, Alpine) and meat breeds (Boer) have been developed. As in most countries of the Mediterranean area, native goat breeds in Croatia play a major role in utilizing resources available under extensive production systems and marginal areas, where other domestic animals cannot easily be kept, thus contributing to environmental and socio-economic stability.

The Croatian Spotted goat is a numerous autochthonous breed, widespread in areas of Dalmatinska Zagora, Bukovica, mountains Velebit, Dinara,

Kamešnica and Biokovo (Figure 1 and Figure 2). Today the population size is approximately 25000 animals of which 616 are under selection (26 bucks, 590 does) with a status of not endangered breed and a stabile population trend (CAA, 2010). Mainly is used for meat production, especially young kids and "kastradina" (dry goat meat), but a minor number of smallholders produce milk. Natural service is the method of breeding, where a buck is maintained for every 15 to 20 does and usually replaced after 2 to 3 years of service. Body (except the legs) is overgrown with colorful, lengthy and dense hair, but white (5.1%), black (4.9%), brown (0.8%) and gray (0.5%) individuals can be found (Mioč et al., 2008). The average body weight of the male and female is 51.3 kg and 44.0 kg respectively. Hight at the withers is 65.3 cm and 61.3 cm (Mioč et al., 2008). All investigated animals were bearded and horned, and this can be considered as breed characteristics. Horns are dark colored, wrinkled, two-edged, grown backward and look like a sword. The udder is small, poorly developed, pigmented or spotted covered with dense hair. During lactation of 150 to 220 days the Croatian Spotted goat can produce 100 to 250 kg of milk. Hoofs are well developed, mostly black (75%) and to a smaller extent white (13%). An average fertility index is 100% to 110%, but in well fed herds 20-30% twins is usual. For meat production, kids old 3 to 6 months and body mass from 15 to 27 kg are slaughtered, and the dressing percentage is in the range from 46 to 52% (Prpic et al., 2010).

Information of genetic structure and variability of autochthonous goat breeds (Spotted goat and White goat) does not exist, and this is the first study of the kind. Microsatellite markers have been used as tools to analyze genetic variation in goats, as well as conservation decisions for genetic resources (Canon *et al.*, 2006; Agha *et al.*, 2008; Glowatzki-Mullis *et al.*, 2008; Mahmoudi *et al.*, 2009; Ramamoorthi *et al.*, 2009; Sadeghi *et al.*, 2010; Bruno-de-Sousa *et al.*, 2011). In order to design rational breeding strategies for optimal utilization and conservation of available genetic variability in Croatian Spotted goat, it is essential to reveal their genetic architecture, which was the main goal of this research.

MATERIALS AND METHODS

Animals and molecular analysis

Blood samples were collected from a total 46 female animals of Croatian Spotted goat in the area of Dalmatinska Zagora (Figure 1). Since no parentage records were available, to ensure unrelatedness, animals were selected from two different villages (Krč and Gljev) after interviewing farmers in detail. Blood samples were collected from the jugular vein using EDTA vacutainer tubes and stored at -20°C until isolation.

From a total of 30 microsatellites, 20 were included in the analysis (Table 1), while 10 of them were excluded for various reasons (see Results and Discussion). Details for 30 microsatellites genomic DNA was isolated from whole blood using QIAmp ®DNA Blood Mini Kit according to the manufacturer protocol (www.qiagen.com).

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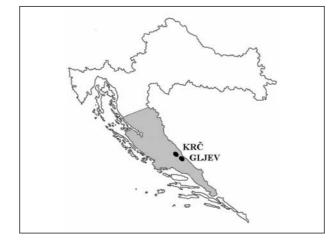


Figure 1. Breeding area of Croatian Spotted goat (gray colored) and sampling locations (black dots)



Figure 2. An image of Croatian Spotted goat

The PCR reaction was performed in a final volume of 15 μ L containg 4 μ L H₂O bidest, 5 μ L diluted primers, 5 μ L *Taq* Polymerase and 1 μ L isolated DNA. Amplification was carried out in a thermal cycler (PTC-100TM DNA Programmable Thermal Controller) using an amplification protocol consisting of denaturation at 95°C for 2 min, followed by 35 cycles of 95°C for 45 s, precise annealing temperature of primer for 58°C for 60 s, 72°C for 90 s, and finally extension at 72°C for 7 min. The PCR products were separated by capillary electrophoresis in an automated sequencer ABI Prism 310 (Applied Biosystems) and results were read directly with GeneScan® software and interpreted using Genotyper® software.

[1		
Locus/	Chr	Allele size (bp)	Primer sequence forward and reverse	Position
BL1038	6 (sheep)	99-127	5' GGCAAGCTAGAGTCAGACACG 3' 3' GCAAAAGTCTAGGTGAAATGCC 5'	132.10
BM1577	9 (sheep)	166-188	5' AGGAGCAGAACCACTAGGAGG 3' 3' TGGACTTTAGGCTTGTTTAGCC 5'	89.70
BM2830	3 (sheep)	98-118	5' AATGGGCGTATAAACACAGATG 3' 3' TGAGTCCTGTCACCATCAGC 5'	284.70
BM3501	3 (sheep)	162-186	5' CCAACGGGTTAAAAGCACTG 3' 3' TTCCTGTTCCTTCCTCATCTG 5'	48.90
BM6506	1 (sheep)	254-271	5' GCACGTGGTAAAGAGATGGC 3' 3' AGCAACTTGAGCATGGCAC 5'	222.60
BMC1009	3 (sheep)	272-298	5' GCACCAGCAGAGAGGACATT 3' 3' ACCGGCTATTGTCCATCTTG 5'	196.50
BMS0887	2 (sheep)	133-155	5' AAGCTAACTGATATTCTGCCACA 3' 3' TTCCCTCTCTCCCTCTCC 5'	66.80
BMS1678	9 (sheep)	198-228	5' TCTTCTCTGCACTTTGGTTGC 3' 3' ATAGCTGACATCCACTGGGC 5'	38.50
ILSTS005	10 (cow)	197-209	5' GGAAGCAATGAAATCTATAGCC 3' 3' TGTTCTGTGAGTTTGTAAGC 5'	97.40
ILSTS008	14 (cow)	165-179	5' GAATCATGGATTTTCTGGGG 3' 3' TAGCAGTGAGTGAGGTTGGC 5'	339.00
ILSTS011	11 (cow)	266-282	5' GCTTGCTACATGGAAAGTGC 3' 3' CTAAAATGCAGAGCCCTACC 5'	4.00
ILSTS022	5 (cow)	225-239	5' GTCTGAAGGCCTGAGAACC 3' 3' CTTACAGTCCTTGGGGTTGC 5'	147.90
ILSTS049	11 (cow)	204-220	5' CAATTTTCTTGTCTCTCCCC 3' 3' GCTGAATCTTGTCAAACAGG 5'	413.05
INRA063	18 (cow)	179-187	5' ATTTGCACAAGCTAAATCTAACC 3' 3' AAACCACAGAAATGCTTGGAAG 5'	595.97
MAF214	16 (sheep)	248-362	Ann	49.00
MCM527	5 (sheep)	183-209	Ann	127.40
OARCP34	11 (cow)	76-96	Ann	116.10
OARFCB20	2 (cow)	86-100	5' AAATGTGTTTAAGATTCCATACAGTG 3' 3' GGAAAACCCCCCATATATACCTATAC 5'	62.00
SPS113	Ann	137-157	Ann	Ann
TGLA53	12 (sheep	136-160	Ann	10.50

Table 1. Twenty microsatellite markers, chromosome (Chr), allele size displayed in base pairs (bp), primer sequences foward and reverse, position on chromosome on which they are located

Ann – unknown

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Statistical analysis

Observed number of alleles with observed (H_0) and expected heterozygosity (H_E) frequencies and Factorial Correspondence Analysis (FCA) were calculated using GENETIX v4.02 (Belkhir *et al.*, 2001). Fixation index (F_{IS}) of Weir and Cockerham (1984) were estimated using the same software. Polymorphism information content (PIC) was calculated according to the formula given by Botstein *et al.* (1980). GENEPOP v3.4 (Raymond and Rousset, 1995) was used to estimate Hardy-Weinberg equilibrium (HWE) over loci. The Wilcoxon signrank test (Luikart *et al.*, 1998) for heterozygosity excess was applied to detect recent bottlenecks, under three models i.e. infinite allele model (IAM), two phase model (TPM) and stepwise mutation model (SMM) using the program BOOTLENECK (Cornuet and Luikart, 1996). A qualitative descriptor of the allele frequency distribution (mode-shift indicator) which discriminates bottlenecked populations from stable populations was also used.

RESULTS

From 30 microsatellite loci, 20 of them were used for estimation of genetic variability of Croatian Spotted goat. According to Barker (1994) microsatellite is considered polymorphic if poses minimum 4 alleles, what was the reason for excluding 10 loci from further analysis. Two loci were monomorphic, OARFCB226 (allele 88) and BL0004 (allele 140), four loci have 2 alleles (BM6465, allele 102,108; BMS1636, allele 132, 134; BMS2626, allele 162, 164; OARCP43, allele 58, 60) and SPS115 with alleles 246, 248, 250. Microsatellites with three alleles were: BMS2321 (allele 134, 126, 138), ETH225 (allele 148, 150, 152), MGTG4B (allele 110, 112, 114). The remaining loci were highly informative with average PIC value of 0.743 ranging from 0.581 (ILSTS005) to 0.882 (BL1038), and thus supported the suitability of markers for genetic diversity analysis.

The estimated values of different measures of genetic variation in Croatia Spotted goat across 20 loci are presented in Table 2. Numbers of genotyped individuals were higher than 95% in 19 loci, only on locus MCM527 number of genotyped individuals was 31% (Table 2). A total of 162 alleles were detected. The number of alleles observed across the microsatellite loci varied from 4 (ILSTS005) to 14 (BL1038) with an overall mean of 8.1. The observed number of alleles for all 20 loci exceeded the effective number of alleles which varied from 2.6 (ILSTS005) to 8.9 (BL1038) with mean 4.63. The observed and expected heterozygosity varied between 0.500 (MCM527) to 0.956 (ILSTS049) and 0.629 (ILSTS005) to 0.897 (BL1038) as presented in Table 2. The overall mean observed and expected heterozygosity were found to be 0.759 and 0.771, respectively. Out of the total 20 studied loci, only three loci (ILSTS049, MCM527 and TGLA53) showed significant (p<0.05) deviations from Hardy-Weinberg equilibrium (HWE). Coefficient of inbreeding indicate nonsignificant deficit of heterozygotes of 2% (F_{IS}=0.019, Table 2).

The SMM, IAM and TPM mutation models under Wilcoxon sign-rank test because of its relatively high statistical power and it can be used with as few as four polymorphic loci and any number of individuals. Thus, 45 individuals and 20 loci of this research easily fulfilled these conditions. The heterozygosity was significant under IAM and TPM (p<0.01), but not under SMM (p>0.05). The allele

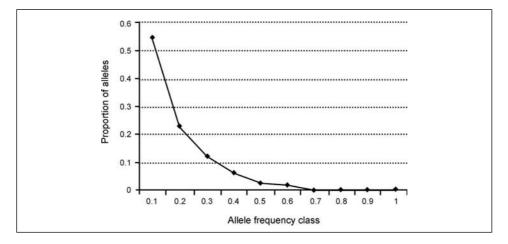
frequency spectrum visualized by the quantitative graphical method is presented in Figure 3 which distribution followed the normal L-shaped curve, since alleles with low frequency (0.01–0.1) are the most abundant.

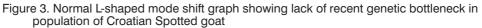
Table 2. Measures of genetic variations across 20 microsatellite loci in Croatian	
Spotted goat	

Locus	N	Allele size (bp)	PIC	nA	EnA	Ho	H _E	F _{IS}	HWE
DBL1038	45	99-127	0.882	14	8.88	0.889	0.897	0.010	ns
BM1577	44	166-188	0.828	10	6.20	0.932	0.848	-0.100	ns
BM2830	45	98-118	0.684	9	3.30	0.756	0.705	-0.072	ns
BM3501	45	162-168	0.827	11	6.06	0.844	0.844	0.000	ns
BM6506	44	254-271	0.803	8	5.45	0.841	0.826	-0.019	ns
BMC1009	44	272-298	0.782	10	4.94	0.841	0.807	-0.043	ns
BMS0887	45	133-155	0.777	8	4.84	0.778	0.802	0.031	ns
BMS1678	44	198-228	0.761	7	4.62	0.659	0.793	0.170	ns
ILSTS005	44	197-209	0.581	4	2.64	0.545	0.629	0.134	ns
ILSTS008	45	165-179	0.667	6	3.15	0.711	0.690	-0.030	ns
ILSTS011	43	266-282	0.701	7	3.73	0.744	0.741	-0.005	ns
ILSTS022	45	225-239	0.685	7	3.43	0.756	0.716	-0.056	ns
ILSTS049	45	204-220	0.847	8	6.96	0.956	0.866	-0.105	*
INRA063	45	179-187	0.620	5	2.97	0.644	0.671	0.040	ns
MAF214	45	248-362	0.789	11	5.10	0.778	0.813	0.044	ns
MCM527	14	183-209	0.718	5	3.53	0.500	0.743	0.336	*
OARCP34	45	76-96	0.824	9	6.08	0.822	0.845	0.027	ns
OARFCB20	45	86-100	0.681	7	3.52	0.778	0.724	-0.076	ns
SPS113	45	137-157	0.717	9	3.77	0.711	0.743	0.044	ns
TGLA53	45	136-160	0.683	7	3.52	0.689	0.724	0.049	*
Average			0.743	8.1	4.63	0.759	0.771	0.019	ns

N-number of analyzed individuals; bp – base pairs; PIC – Polimorphic Information Content; nA – mean number of alleles; EnA – effective number of alleles; H_O – observed heterozigosity; H_E – expected heterozygosity; F_{IS} – within population inbreeding estimate; HWE – Hardy-Weinberg equilibrium; *p<0.05; ns – not significant

Factorial correspondence analysis (FCA) suggests that first axis accounted for 5.57%; and second 4.75% of the total variance as presented in Figure 4. Four individuals (rounded in Figure 4) show higher genetic distance from the rest of the populations, and thus greater genetic diversity. When we split the here examined samples of Croatian Spotted goats on two subpopulations according to the sampling location (POP1 was from Krč area, POP2 was from Gljev area, Figure 1), those four individuals originated from the Gljev breeding area. Although the whole population showed a non-significant deficit of heterozygotes of 2%, FCA indicate a close genetic relationship between few sampled individuals in both subpopulations (here conditionally named POP1 and POP2 and marked with rectangle; Figure 4).





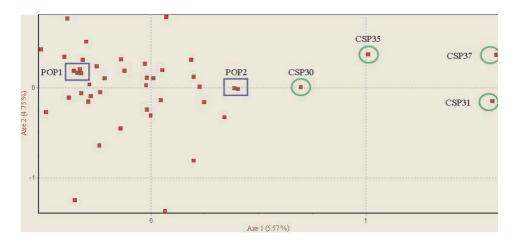


Figure 4. Factorial Correspondence Analysis (FCA) between individuals of the Croatian Spotted goat); CSP – Croatian Spotted goat; POP1 – individuals belongig to Krč area; POP2 – individuals belongig to Gljev area

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DISCUSSION

The result of this study suggests that there is essential genetic variation and polymorphism in the population of Croatian Spotted goat. The same observed number of alleles of 8.1 was recorded for Markhoz goat from Iran (Mahmoudi et. al., 2009) and lower values was reported for Barbari goats from India (nA=6.3; Ramamoorthi et al., 2009), for Egyptian and Italian goat breeds (nA=6.5; Agha et al. 2008) or Raeini goat from Iran (nA=7.8; Sadeghi et al., 2010). However, this average values were lower than nA=9.60 obtained for 11 Swiss goat breeds (Glowatzki-Mullis et al., 2008) and six Portuguese goat breeds (Bruno-de-Sousa et al., 2011). Average values of H_O and H_E in Croatia Spotted goat were higher than those reported by Agha et al. (Ho=0.654, HE=0.722; 2008), Glowatzki-Mullis et al. (H_O=0.594, H_E=0.601; 2008), Bruno-de-Sousa et al. (H_O=0.636, H_E=0.702; 2011). Also, gene diversity of Croatian Spotted Goat was higher than gene diversity of each of 44 European and Middle Eastern goat breeds, except in Abaza breed from Turkey, with an almost similar value of 0.772 (Canon et al., 2006). Higher values for gene diversity was found in Raeini goats ($H_{F}=0.805$, Sadeghi et al., 2010). In assessing diversity estimates from different studies, it should be mentioned that the values are not directly comparable, because different microsatellites have been used. The high genetic diversity could be explained by mixing populations from different geographical locations or natural selection favoring heterozygosity. Croatian Spotted goat is widely distributed across Dalmatia for centuries, thus increasing the likelihood for the enrichment of different alleles in geographically widely scattered populations. As already proven for cattle (Medugorac et al., 2009; Ramljak et al., 2011), the Croatian Spotted Goat is close to the domestic center of goats in the Near East and is expected to possess higher genetic diversity. Higher level of genetic variability in 15 Middle Eastern breeds (Canon et al., 2006), Egyptian goats (Zeder and Hesse, 2000) and Agha et al. (2008) explained by the proximity to the domestication centre. In a lesser extent, to the observed high genetic diversity contributed crossing Croatian Spooted goat firstly with Alpine and Saanen goat breeds (increasing milk yield), and at the end of the twentieth century with Boer goat for improving meat production. In the population of Croatian Spotted goat exists random mating, since only three loci showed departure from HWE what was confirmed with low nonsignificant values of inbreeding coefficient of 2%. This value emphasizes the low rate of inbreeding within the population as compared to F_{IS}=0.10 of Canon et al. (2006) for 45 goat breeds, Agha et al. (2008) for three Egyptian and two Italian breeds (FIS=0.096), Traore et al. (2009) for five breeds (Fng1033IS=0.05), Brunode-Sousa et al. (2011) for six Portugeese breeds (FIS=0.07), but similar to the one found for 11 Swiss breeds (FIS=0.014) of Glowatzki-Mullis et al. (2008). Only two microsatellite markers contribute to this heterozygote shortage, MCM527 with 33.6% and BMS1678 with 17%. Main reason for the deficit of heterozygotes at MCM527 is due to a small number of only 14 sampled individuals. Since there is no pedigree record for sampled individuals all information considering nonrelatedness are provided entirely by comunication with breeders, so sampling related individuals are not excluded entirely and can contribute to the noticed

deficit of heterozygotes. The observed distribution of allele frequencies suggests that the breed did not encounter a genetic bottleneck in the recent past. It is supported by the high average number of alleles, as well as high gene diversity and distribution of individuals in FCA analysis. Higher distance of four individuals from the rest of the population can be interpreted in two opposite ways: firstly, with controlled and systematic mating and secondly, with greater presence of a foreign genome in the genome of Croatian Spotted goats, thus it should be taken with caution. For more detailed results, comparative analyses with other autochthonous (Croatian White goat) and allochtnonous goat populations should be done. The reason for the proximity of individuals within subpopulation (POP1 and POP2) is probably the product of sampling related individuals due to the lack of pedigree records and in the future should be avoided.

Generally speaking, a large amount of genetic variability maintained in breeds is important since it contributes to the animals' adaptation to the challenging environment and their integration in the rural economy. By preserving a high level of genetic diversity, especially in native breeds, they contribute to long-term preservation strategies and leave an open option to use alternative traits and to develop new ones in the future.

Address for correspondence: Prof. dr. Boro Mioč Department of Animal Science and Technology Faculty of Agriculture Svetošimunska cesta 25 10000 Zagreb, Croatia E-mail: bmioc@agr.hr

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PARAMETRI GENETSKOG DIVERZITETA HRVATSKE ŠARENE KOZE

RAMLJAK JELENA, MIOČ B, ĆURKOVIĆ M, PAVIĆ VESNA, IVANKOVIĆ A i MEĐUGORAC I

SADRŽAJ

Istraživanje genetske strukture hrvatske šarene koze procijenjeno je temeljem analize 20 mikrosatelitnih biljega. Utvrđena je visoka očuvana genetska raznolikost, s prosječnim brojem alela po lokusu (8,1), visokom razinom uočene (0,759) i očekivane (0,771) heterozigotnosti. Unutar populacije nema naznaka odstupanja od nasumičnog parenja. Zabilježeni nizak stupanj uzgoja u srodstvu unutar populacije (F_{IS} =0.019) nije bio statistički značajan. Uporaba beskonačnog (IAM) i dvofaznog (TPM) modela mutacije alela ukazivala je na prisustvo genetskog uskog grla, dok stepenasti mutacijski model (SMM) i metoda kvalitativne promjene L-oblika krivulje frekvencije alela nisu ukazivali na genetsko usko grlo u nedavnoj prošlosti populacije hrvatske šarene koze. Uočena visoka razina genetske raznolikosti hrvatske šarene koze ovog istraživanja pomoći će pri donošenju strategije daljnjeg korištenja i očuvanja ove izvorne pasmine koza.