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Microsatellite based genetic structure of regional transboundary Istrian sheep breed populations in Croatia and Slovenia

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Abstract

Istrian dairy sheep is a local breed essential for the identity and development of the Northern-Adriatic karstic region through high-quality products, primarily the hard sheep artisanal cheese. Border changes fragmented the initial Istrian dairy sheep population in three genetically isolated subpopulations in Italy (1000 animals), Slovenia (1150 animals) and Croatia (2500 animals). Due to the drastic reduction of their population sizes and fragmentation, the populations in Croatia and Slovenia are included in governmentally supported conservation programs. The initial subpopulation in Italy was restored after near extinction with stock from Slovenia, and is used today in meat production. The aim of this study was to provide an initial understanding of the current genetic structure and distribution of the genetic variability that exists in Istrian sheep by analysing individuals sampled in two regional groups of Istrian sheep from Croatia and Slovenia. Cres island sheep and Lika pramenka sheep were used as out-groups for comparison. Genetic differentiation was analysed using factorial correspondence analysis and structure clustering over 26 microsatellite loci for a total of 104 sheep belonging to three breeds from Croatia and Slovenia. Factorial correspondence analysis and clustering-based structure analysis both showed three distinct populations: Lika pramenka sheep, Cres island sheep and Istrian sheep. We did not find a marked genetic divergence of the regional groups of Istrian sheep. Istrian sheep regional group from Slovenia showed lower genetic variability compared to the one from Croatia. Variability and structure information obtained in this study considered alongside with socio-cultural-contexts and economic goals for the Istrian sheep reared in Croatia and Slovenia indicate that the cross-border exchange of genetic material of animals carrying private alleles among populations would maintain these alleles at low frequencies and minimize the inbreeding rate.

Key words: population structure, genetic differentiation, microsatellites

Introduction

The definition of a breed is based on the homogeneous external characteristics or on a generally accepted identity of animals of a geographically or culturally separated group (FAO 1998), and in practice this breed population can be broader or narrower than a genetic population (Galov et al., 2013). Sometimes closely related populations may be defined as separate breeds due to administrative borders. However, it is helpful to use the information on genetic structure together with phenotypic or demographic data to guide management efforts and to define management units (Tapio et al., 2005), especially in indigenous sheep populations of small sizes

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that are reared in extensive conditions. In small animal populations the increase of inbreeding, which is likely to occur due to the limited number of parents, causes genetic fragility (Pariset et al., 2003). Since indigenous breeds are often reared in extensive conditions, breeding strategies applied to local breeds are usually constrained by poor pedigree records which leads to limited genetic progress for selected traits, and suboptimal inbreeding control (Serrano et al., 2009). Significant inbreeding was estimated in Istrian sheep in previous studies (Ćinkulov et al., 2008, Salamon et al., 2012, 2014). Implementation of breeding strategies that increase effective population size and minimize genetic drift effects, such as increased ram to ewe ratio, maintains genetic diversity of small indigenous populations especially where no artificial insemination is used. The breed is the management unit for which factors such as inbreeding are controlled. Where local breeds are regional transboundary breeds, they can benefit from cross-border cooperation. Additionally, the cooperation is usually encouraged to avoid duplicates in long-term cryopreserve of these breeds and to make optimal use of funds as seen in regional and international transboundary breeds of cattle (Hiemstra et al., 2010).

Istrian dairy sheep is an indigenous breed of the Northern Adriatic area important for the identity and development of the region through high-quality products, primarily the hard artisanal sheep cheese. Today, the initially transhumant Istrian breed population (Bohm, 2004) is fragmented in separate reproductively isolated populations in Slovenia (about 1150 animals in Coastal-Karst Statistical Region) and Croatia (about 2500 animals, mainly in Istrian County). A smaller number is present in Italy (about 1000 animals) where they have been imported almost exclusively from Slovenian herds, and with a less pronounced role in local milk and cheese production. Lika pramenka sheep (8500 animals under selection control in Croatia) is reared predominately for lamb production on free range pastures of mountain and highland areas of Lika and Gorski Kotar. Cres island sheep (1000 animals under selection control in Croatia) is a dual purpose breed used mostly for milk production.

Assessment of genetic diversity using microsatellite marker data for setting the conservation priorities in sheep (FAO, 1998) is a standardised method for estimating the genetic diversity of different ruminant populations in many countries (Baumung et al., 2004; Ligda et al., 2009; Salamon et al., 2014). Bayesian model-based clustering methods that allow for the inference of population structure and assignment of individuals to populations (Pritchard et al., 2000) are proven to be useful for the definition of management units. Additionally, the multivariate approach is simple, powerful and robust regarding the admixture and mutation models and is a good validation of Bayesian clustering outputs particularly those using admixture models. Together with other studies of genetic variation, relationships and diversity, the results of the present study can provide a useful tool for cross-border cooperation in mating and ram exchange designs, as well as management plans for both regional populations of Istrian sheep. Hence, the aim of this study was to investigate whether geographical and reproductive isolation of Istrian sheep populations has contributed to increase genetic differences between these populations, and whether there are biological grounds for cross-border cooperation. Cres island sheep and Lika pramenka sheep were used as out-groups for comparison.

Material and methods

Animal samples and molecular analyses

Blood samples were collected from 104 randomly chosen unrelated animals from three local breeds from Croatia: Istrian sheep (35 samples, ISTc), Cres island sheep (25, CRE) and Lika pramenka sheep (25, LIK), and Istrian sheep from Slovenia (19 samples, ISTs). The number of sampled flocks per population ranged from 2 (CRE) to 18 (IST). Blood Genomic DNA Kit (GenEltue™, Sigma-Aldrich®, St. Louis, MO, USA) was used to extract the DNA. Nineteen markers were chosen from the FAO list of markers recommended for sheep diversity (*OarVH72*, *OarJMP58*, *OarCP34*, *JMP29*, *DYMS1*, *BM8125*, *BM1824*, *ILSTS005*, *ILSTS011*, *INRA063*, *MAF209*, *MAF65*, *McM527*, *OarFCB128*, *FCB304*, *OarHH47*, *MCM140*, *MAF214*, *HUJ616*), and the remaining markers (*CSRD247*, *ETH10*, *HSC*, *INRA132*, *OarCP49*, *SPS113*, *SPS115*, *TCRGC4B*, *TCRVB6*) were chosen due to their good multiplexing abilities. PCR-multiplexing, processing of the PCR products and the analysis of the electropherograms was performed as reported in Salamon et al. (2014).

Statistical methods

Marker informativeness and diversity parameters were calculated as reported in Salamon et al. (2012). Because of null-allele estimates higher than 0.15, markers *BM1824* and *FC128* were excluded from the further analysis. Additionally, marker *BM1824* deviated from Hardy-Weinberg equilibrium significantly in more than half of the populations studied. Hence, the information from the remaining 26 markers was used to perform the subsequent genetic analyses. Polymorphic information content (PIC) and the rarefacted allelic richness were estimated with the Molkin 3.0 software (Gutierrez et al., 2005) using bootstrapping and the rarefaction correction based on 50 diploid individuals to standardize among different sample size populations. Pairwise genetic distances (F_{st}), inbreeding coefficients (F_{is}) and gene flow estimates were obtained using Genetix 4.04 (Belkhir et al., 2002) and Arlequin v.3.5 (Excoffier et al., 2005). Arlequin v.3.5 was used also to determine the genetic variation and the distribution of genetic diversity among and within the groups by an analysis of molecular variance (AMOVA). Population structure was explored using a factorial correspondence analysis (FCA) performed with the Genetix 4.04 software. Since admixture is possible between the sheep populations included in this study, assignment of the individuals to populations was investigated using the admixture model and with the breed prior information option of the Structure software 2.3.4 (Pritchard et al., 2000). A total of 20000 iterations were used as burn-in period for all runs. Data was collected during 10000 iterations, while K value was fitted from 1 to 8 with 10 runs each. To choose the optimal K value the posterior probability, L(K), was calculated using the mean log-likelihood of K for each value of Evannos' ΔK (Evanno et al., 2005). Graphic presentation of these statistics was obtained with the web-based Structure Harvester v0.6.8 (Dent and vonHoldt, 2012).

Results and discussion

Through the genetic analysis presented here, this study provides the first detailed analysis of the genetic structure of regional Istrian sheep populations from Croatia and Slovenia, which may be of great value for cross border cooperation in effective conservation of this endangered breed (Barać et al., 2011). A previous study has analysed microsatellite based genetic diversity of a global Istrian population including samples from Croatia and Slovenia, as a whole, and in comparison with other autochthonous sheep breeds of Croatia (Salamon et al., 2014). In this study we present a more detailed analysis focused on the two different Istrian sheep populations to provide a scientific assessment of their current genetic structure.

Except for marker *ETH10* (PIC 0.562), the great majority of markers were highly informative and polymorphic. The highest PIC was observed for marker *INRA132* (0.884). Overall, 291 different alleles were found. On average there were 10.39 alleles per locus observed. *HUJ616* had the largest number of alleles (20), and *ETH10* showed only two alleles in the studied populations. In the global population, and accounting for the multiple tests performed (28 loci, 4 populations), 8 loci were found to be in Hardy-Weinberg (HW) disequilibrium. However, only for *BM1824* deviation was observed in three out of four studied populations. Additionally, null-allele estimates for *BM1824*, as well as for *FC128* were higher than 0.15. Consequently, both markers were excluded from subsequent analyses of genetic diversity and differentiation.

Based on the analysis of the 26 remaining loci, the local sheep populations revealed a high level of genetic diversity, with LIK showing the lowest diversity estimates (Table 1). The values were similar to other European and Turkish indigenous sheep breeds, and higher than that reported for selected breeds (Arranz et al., 2001; Gutierrez-Gil et al., 2006). ISTc showed the highest number of private alleles, while the lowest was observed in ISTs. Mean rarefacted numbers of alleles (MNA) were high and similar in both ISTs and ISTc regional groups, while in LIK population this estimate was the lowest (Table 1). The range of MNA was in the low levels of the range reported for Balkan pramenka type populations, Alpine and Greek sheep (Ćinkulov et al., 2008; Dalvit et al., 2008; Ligda et al., 2009), and higher compared to Italian sheep (Bozzi et al., 2009), but lower than in Xalda sheep (Alvarez et al., 2008).

Since the values obtained after the sample size correction did not change remarkably, possible arte-

Table 1. Genetic variability parameters estimated for ISTc, ISTs, CRE and LIK populations, based on the analysis of the 26 microsatellite markers

Group	n	Ho	He	MNA	pA	Fis
ISTc	35	0.695±0.157	0.713±0.144	5.82	23	0.041
ISTs	19	0.680±0.176	0.707±0.144	5.99	16	0.067
CRE	25	0.698±0.229	0.660±0.195	5.53	19	-0.037
LIK	25	0.643±0.148	0.637±0.141	5.13	20	0.012
Overall	104	0.668	0.745	6.58	78	

CRE - Cres Island Sheep, LIK - Lika Pramenka Sheep, ISTs - Istrian sheep population from Slovenia, ISTc - Istrian sheep population from Croatia, n - sample size, Ho - average observed heterozygosity (± SD), He - average expected heterozygosity (± SD), MNA - mean number of alleles (rarefacted), pA - number of private alleles. Fis estimates and significance of the deviation from HWE per population across the 26 loci analysed

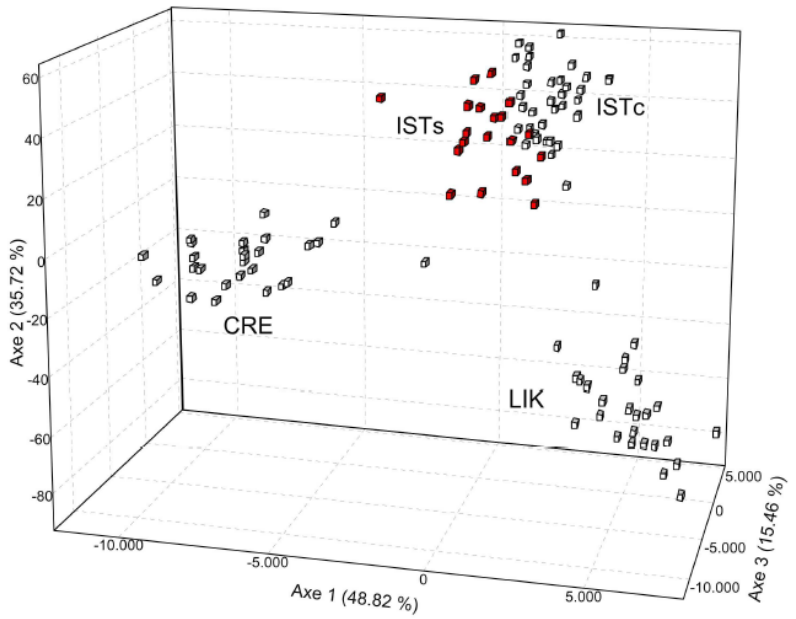
Table 2. Genetic differentiation parameters estimated for ISTc, ISTs, CRE and LIK, based on the analyses of the 26 microsatellite markers

Group	ISTc	ISTs	CRE	LIK
ISTc	-	0.014	0.075	0.115
ISTs	34.55	-	0.078	0.095
CRE	6.20	5.91	-	0.156
LIK	2.71	4.75	2.71	-

Significant (P<0.001) pair-wise genetic distances (Fst) (above diagonal), and number of effective migrants per generation (Nm) (below the diagonal)
CRE - Cres Island Sheep, LIK - Lika Pramenka Sheep, ISTs - Istrian sheep population from Slovenia, ISTc - Istrian sheep population from Croatia

facts due to the different sample sizes can be ruled out. Observed heterozygosities were high and resembling in ISTc, ISTs and CRE. Observed heterozygosity in LIK and CRE populations was higher than the expected one. Herdbooks for the Croatian breeds were re-established after the Croatian War of Independence, and while LIK was affected directly by the war activities, island and peninsula breeds, such as ISTc and CRE were influenced only indirectly through the decrease of the population sizes. General genetic parameters, such as low diversity in LIK, or suspicion regarding an isolate braking effect in LIK and CRE indicated by heterozygosity estimates, are in accordance with these events (Salamon et al., 2014). ISTs has been found to be less variable at the genetic level than ISTc confirming the preliminary results (Salamon et al., 2012). Although ISTs had the highest estimated inbreeding level among the four analysed populations, the estimate was not found to be significant in this analysis (Table 1). However, statistically significant F_{is} values were reported for Istrian sheep breed in other studies (0.011, Ćinkulov et al., 2008; 0.042 and 0.052 for ISTc and ISTs, respectively, Salamon et al., 2012) and show higher estimates in ISTs than in ISTc.

Genetic differentiation estimates of pair-wise Wright's fixation index (F_{st}) were low (0.014 for ISTc-ISTs pair) to considerable (0.156 for LIK-CRE pair) (Table 2). Geographical neighbours were found to have the lowest pair-wise genetic distances and the highest numbers of effective migrants per generation. The global AMOVA analysis showed a significant and higher source of variation within (90.98 %) than among (9.02 %) populations, which was higher than values reported for other pramenka type sheep breeds (Ćinkulov et al., 2008; Salamon et al., 2014) or Greek sheep breeds (Ligda et al., 2009). Even though the sampling area is small (approximately 100 km radius) this result could suggest the influence of geographical isolation. The F_{st} value (0.090, P<0.001) suggested a moderate genetic differentiation for the global population. Variance components among populations were significant (P<0.05) for all loci except for *CP34*. Loci *CSRD247* and *INRA063* contributed to explain 19.47 % and 16.15 % of the variability, respectively. In the factorial correspondence analysis, the first three components explained 48.81 %, 35.72 % and 15.46 % of the total variation, respectively (Figure 1). The first component separates the mountain LIK and island CRE population. The second component confirms differentiation of both ISTc and ISTs from CRE and LIK populations. Finally, the third component, showed a certain separation of the two Istrian sheep populations from Croatia and Slovenia, although they showed a close genetic relationship. Similar to results of multivariate analysis of sheep morphology data by Legaz et al. (2011), ISTs and



CRE - Cres Island Sheep, LIK - Lika Pramenka Sheep, ISTs - Istrian sheep population from Slovenia, ISTc - Istrian sheep population from Croatia

Figure 1. Spatial presentation of the four sheep populations based on the results of the factorial correspondence analysis performed on 104 samples and 26-locus genotypes. The percentage of inertia explained by each component is indicated next to the names of the axes

ISTc can be seen as the same population having some differences due to the short time (less than 20 years) of separating process.

The Evannos' method implemented in the Structure Harvester software v0.6.8 (Dent and vonHoldt, 2012) showed that the plateau shape of ln Pr(x/K) values was started at k=3, and the best value at that point was -8326.290 (Figure 2A). The most appropriate number of clusters according to estimates of ΔK (Figure 2B) was also k=3 (ΔK = 177977). Based on this, graphical presentation of the clustering outcomes suggested for k=3 is shown in Figure 3, and the proportion of membership for the identified clusters is shown in Table 3. The clustering-based structure analyses supported the results from the factorial correspondence analysis, with three genetically distinct populations: CRE, LIK and Istrian sheep were identified, with ISTc and ISTs regional populations grouping together.

Estimated membership coefficients were high (>0.920) for the four analysed populations. Cluster 1 was found to be LIK-related (0.921), and Cluster 2 was CRE-related (0.951). Cluster 3 was found to be related to Istrian sheep, although it showed a stronger influence on ISTc (0.979) than on ISTs samples (0.952). The admixture was the highest in the LIK samples, and was similar in CRE-related Cluster 2 and IST-related Cluster 3 (0.032-0.042). The lowest admixtures were in ISTc samples, and were similar in CRE-related Cluster 2 and LIK-related Cluster 1 (0.007-0.014). ISTs samples showed more admixture with LIK-related Cluster 1 (0.030) than with CRE-related cluster 2 (0.017). While in ISTc and ISTs regional groups the influences of other clusters were mostly due to low genetic admixture in most of the individual samples, in LIK and CRE populations few of the samples clustered in population-unrelated clusters (Figure 3).

Although genetic distinctiveness is not the only criterion that should be used for conservation decisions, the use of other isolated criteria such as the geographical location or a distinctive phenotype should not also be applied. For example, in the Spanish Manchega sheep separating sheep populations based on colour phenotype only was not a good criterion for the conservation purpose because of

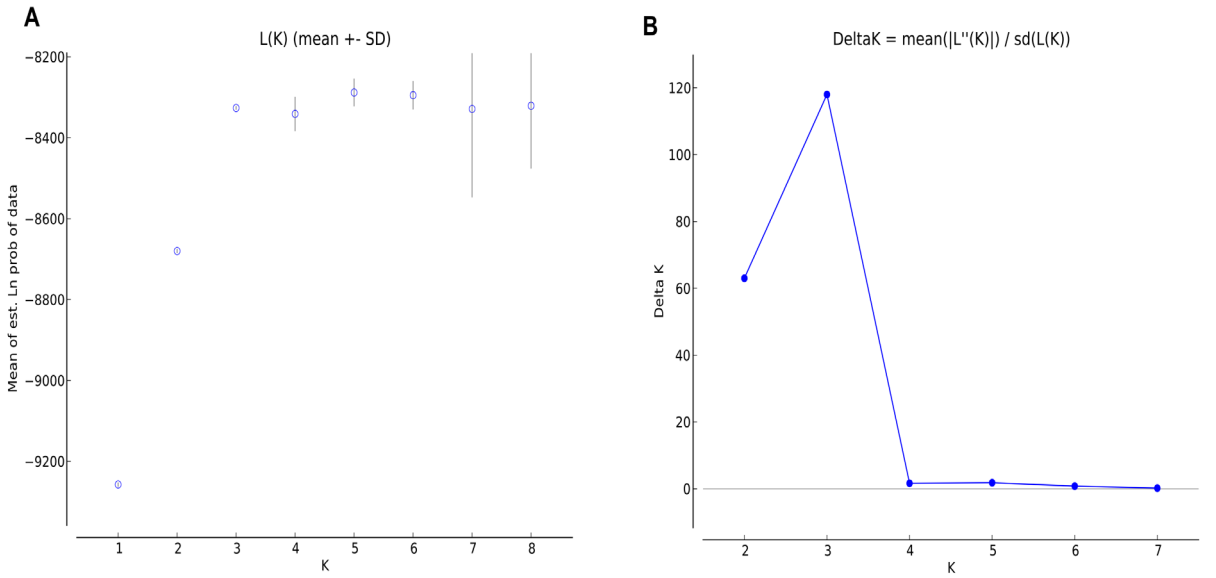
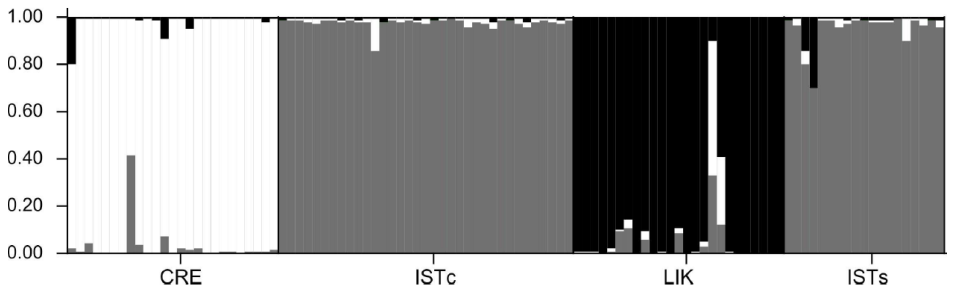


Figure 2. Graphical presentation of the results of the structure population analysis used to determine the true number of clusters (K) of the analysed sheep populations. A: Mean log likelihood L (K) (±SD) over 10 runs for each K value tested. B: Delta K curve estimated according to Evanno et al. (2005). Graphics obtained with the STRUCTURE HARVESTER software v0.6.92 (Dent and vonHoldt, 2012)



CRE - Cres Island Sheep, LIK - Lika Pramenka Sheep, ISTs - Istrian sheep population from Slovenia, ISTc - Istrian sheep population from Croatia

Figure 3. Graphical presentation of the clustering outcome suggested by the Bayesian analysis performed to assess the structure of the studied populations at K = 3. Each colour represents one cluster, and the length of the vertical coloured bars are the individuals' estimated proportions of membership in that cluster

Table 3. Proportion of membership for each of the four sheep populations studied across the three clusters identified in the assignment analysis (the highest contribution for each population is indicated in bold font)

Group	Cluster		
	1	2	3
ISTc	0.007	0.014	**0.979**
ISTs	0.030	0.018	**0.952**
CRE	0.017	**0.951**	0.032
LIK	**0.921**	0.042	0.037

low genetic variability in the black fleece sub-population and high genetic similarity of the white and black fleece phenotype sub-populations as shown by structure analysis (Calvo et al., 2006). Hence, the global genetic variability should be considered in order to optimize investment of governmental support for conservation. According to Rege and Gibson (2003), socio-cultural contexts in which the breed exists and future economic goals rooted in functional diversity are important considerations as well. Since both ISTs and ISTc regional groups are

used for milk production and are reared in similar predominately extensive conditions and there is no marked genetic divergence, the recommendations based on the results presented herein would suggest joining the current conservation efforts. The exchange of the genetic material for current or possible future inbreeding management would be necessary.

Conclusion

Taking into account the context of chosen outgroup breeds, the population separation observed between ISTc and ISTs populations was not large enough for the artificial selection to impact on the genetic divergence of the populations pronouncedly, nor was the drift severe enough to influence marked differentiation between the two populations. The two regional groups show valuable genotype differences as indicated by the identified private alleles and marked genetic distinctiveness from other breeds with low levels of admixture. However, ISTs regional group showed lower genetic variability than ISTc. Taking into account the diversity and structure information reported here for Istrian sheep from Croatia and Slovenia, complemented with socio-cultural-contexts and economic goals, it is recommended to exchange genetic material, in order to maintain private alleles present at low frequencies in such small populations and minimize the inbreeding rate. Among the three clearly separated breeds, LIK was found to be the most distinct, while the Adriatic populations (CRE, ISTc and ISTs) are more similar.

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Genetska struktura regionalnih prekograničnih populacija istarske ovce u Hrvatskoj i Sloveniji utvrđena mikrosatelitskim markerima

Sažetak

Istarska ovca je autohtona pasmina ključna za identitet i razvoj sjevernojadranske regije zbog visokokvalitetnih mliječnih proizvoda. Promjenama granica inicijalna populacija istarske ovce je fragmentirana u tri genetski izolirane sub-populacije u Italiji (1000 životinja), Sloveniji (1150 životinja) i Hrvatskoj (2500 životinja). Zbog fragmentacije i smanjenja veličine populacija, istarske ovce su u Sloveniji i Hrvatskoj uključene u konzervacijske programe državnih potpora. Talijanska populacija je nakon inicijalnog drastičnog smanjenja broja danas obnovljena isključivo iz slovenskih stada. Cilj ovog istraživanja bio je omogućiti razumijevanje današnje genetske strukture i raspodijele genetske raznolikosti istarske ovce istraživanjem slovenske i hrvatske sub-populacije. Creska i lička pramenka korištene su kao grupe za usporedbu. Genetska diferencijacija istražena je faktorijalnom analizom korespondencije i strukturnim klasteriranjem na temelju 26 mikrosatelitskih lokusa genotipiziranih kod 104 ovce. Obje analize pokazale su tri distinktne populacije: lička i creska pramenka, te istarska ovca. Genetička divergencija istarske ovce iz Slovenije i Hrvatske nije izražena. Istarska ovca iz Slovenije pokazala je nižu genetsku varijabilnost od sub-populacije iz Hrvatske. Temeljem informacija o varijabilnosti i strukturi iz ovog istraživanja, kao i društveno-kulturalnog konteksta te ekonomskih ciljeva za slovensku i hrvatsku sub-populaciju istarskih ovaca, može se reći da bi izmjena genetskog materijala životinja s privatnim alelima omogućila održavanje tih alela u niskoj učestalosti, te smanjila genetske posljedice parenja u srodstvu.

Ključne riječi: populacijska struktura, genetska diferencijacija, mikrosateliti

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