COBISS: 1.08
Agris category code: L10

PRELIMINARY STUDY ON THE GENETIC DIVERSITY OF THE ISTRIAN SHEEP, LIKA AND KRK PRAMENKA SHEEP POPULATIONS USING MICROSATELLITE MARKERS

Dragica SALAMON [1], Beatriz GUTIERREZ-GIL [2], Antun KOSTELIC [1], Gregor GORJANC [3], Dragomir KOMPAN [3], Alen DZIDIC [1, 4]

ABSTRACT

Genetic diversity and genetic differentiation were analysed, in a total of 103 sheep from four different populations from Croatia and Slovenia using 24 microsatellite loci. The aim of the study was to provide an initial understanding on the genetic diversity and structure of Istrian dairy sheep by analysing individuals sampled from reproductively isolated populations of Croatia (IST) and Slovenia (ISTs), while Krk island sheep (KRK) and Lika pramenka (LIK) were used as outgroups. Results revealed considerable levels of genetic diversity in the studied samples, similar to results reported in other indigenous sheep breeds related to low production systems. The genetic parameters estimated showed the highest diversity in KRK, and the lowest in LIK sheep population. Istrian breed populations were in between with expected and observed heterozygosity, and the number of private alleles identified, being higher in IST than in ISTs. In the four populations analysed, 67 private alleles were identified. KRK had the highest number of loci with population-specific alleles (12). On the contrary, LIK and ISTs showed the lowest number of private alleles (8). The observed and expected heterozygosity ranged from 0.648 and 0.634 (in LIK), respectively, to 0.723 and 0.732 (KRK), respectively. KRK had the lowest Fis value (0.034), while ISTs showed the highest Fis estimate (0.052). In conclusion, the results presented here show high level of conserved genetic diversity in the Istrian dairy sheep breed. The smaller and reproductively isolated Istrian sheep population from Slovenia shows less diversity and a higher inbreeding level.

Key words: sheep / breeds / genetic diversity / microsatellites

1 INTRODUCTION

The Coastal-Karst statistical region in Slovenia and Istrian County in Croatia with their recognizable Northern-Adriatic karstic landscape, offer a habitat of high ecological value for the rearing of the autochthonous regional Istrian dairy sheep. Physiology and phenotype of Istrian sheep show good adaptation to these habitat conditions. Besides natural occurring geography and isolation, important aspects of the history of the Istrian sheep breed include diverse political and economical changes, which influenced the borders, management practices, such as horizontal and vertical transhumance, and the controlled and uncontrolled crossbreeding (Böhm, 2004). Today, the initial breed population is fragmented in reproductively isolated sub-populations in Italy (1.000 animals), Slovenia (1.500 animals) and Croatia (4.600 animals). Istrian dairy sheep is the predominant breed in the sheep production of Istrian region (Mulc *et al.*, 2012) and essential for the identity and development of the region through high-quality products, primarily the hard sheep artisanal cheese. The knowledge about the genetic diversity of such a breed is of high importance for the future of the region. Non-coding part of the genome as represented by microsatellites is not the only criterion that should be considered when conservation strategies are discussed; however, it can give valuable insight and indications necessary to complement the information on

1 Dept. of Animal Science, Fac. of Agriculture, Univ. of Zagreb, Svetošimunska cesta 25, 10000 Zagreb, Croatia
2 Departamento de Produccion Animal, Universidad de Leon, Campus de Vegazana s/n, 24071 Leon, Spain
3 Depatment of Animal Science, Biotechnical Fac., Univ. of Ljubljana, Domžale, Slovenia
4 Corresponding author, email: adzidic@agr.hr

adaptive traits and socio-economic aspect. Numerous studies have been described in different ruminant species using microsatellite markers to assess genetic diversity (Glowatzki-Mullis *et al.*, 2008; Ligda *et al.*, 2009; Barreta *et al.*, 2012). Therefore, the aim of the study was to provide an initial understanding on the genetic diversity and structure of Istrian sheep in Croatia and Slovenia.

2 MATERIALS AND METHODS

Blood samples from 103 randomly chosen unrelated animals were obtained from four sheep populations: Istrian sheep from Croatia (35 samples, IST), Istrian sheep from Slovenia (20 samples, ISTs), the Krk island sheep (23, KRK) and the Lika pramenka sheep (25, LIK) (see Fig. 1). The number of flocks per population ranged from 2 (KRK) to 18 (IST).

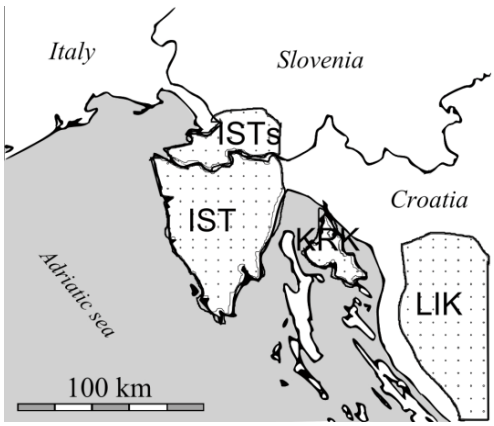

Figure 1: *Geographical locations of the four populations sampled in this study. IST – Istrian sheep in Croatia; ISTs – Istrian sheep in Slovenia; KRK – Krk island sheep; LIK – Lika pramenka sheep.*

The DNA was extracted from the whole blood samples using the Blood Genomic DNA Kit (GenEltue™, Sigma). Using fluorescent-labelled primers and a hot-start polymerase (JumpStart™ REDTaq® ReadyMix™), a total of 28 microsatellite markers were amplified (Table 1) through the optimization of 4 PCR multiplex reactions. Samples were processed in the 16-capillary electrophoresis ABI3130XL Genetic Analyser using Genescan-500 LIZ as size standard (Applied Biosystems, AB, CA). Electropherograms were genotyped automatically with the GeneMapper® software (AB, CA). Markers with 5% and more of missing genotypes were excluded from further analysis.

Marker informativeness and diversity statistics were calculated using the Genetix 4.04 software (Belkhir *et al.,* 2002). Locus-wise deviations from Hardy-Weinberg equilibrium (HWE) across the populations were tested by an exact test (Guo and Thompson, 1992) in Genepop 3.3 (Raymond and Rousset, 1995). Possibility of null-alleles was determined using the same software. Bootstrapping using 1000 replications was used to estimate the statistical significance of the obtained values in all cases. Markers showing deviation from the HWE were excluded from the subsequent analysis if the deviation was significant in more than half populations studied. Private alleles were accounted for utilizing the GDA software (Lewis and Zaykin, 2001). 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. Pair-wise genetic distances (Fst), inbreeding coefficients (Fis) and gene flow estimates were obtained using Genetix 4.04 and Arlequin v.3.1 (Excoffier *et al.*, 2005). Arlequin v.3.1 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).

3 RESULTS AND DISCUSION

The great majority of markers were highly informative and polymorphic. A total of 291 different alleles were found in 103 genotyped individuals. The average number of alleles per locus was 10.39. The highest number of detected alleles recorded was 18 for marker HUJ616. The PIC values per marker varied from 0.142, for ETH10, to 0.943 for OarCP49 (Table 1). In the global population, and accounting for the multiple tests performed (28 loci, 4 populations), 11 loci were found to be in Hardy-Weinberg (HW) disequilibrium (Table 1). Markers MAF214 and OarFCB128 were excluded from further analysis since the HWE deviation was recorded in more than half of the populations. Frequencies of non-amplifying null alleles inferred from the heterozygote deficiency for the complete set of makers analyzed showed estimates ranging from 0.000 (ETH10 and FCB304) to 0.365, for ILSTS011, and 0.372, for BM1824 (Table 1). The last two markers were excluded from subsequent analyses of genetic diversity and differentiation.

With the exception of LIK, the local sheep populations (IST, ISTs and KRK) revealed a high level of genetic diversity, based on the analysis of the 24 loci (Table 2). The high number of markers covered indicates a representative result. Although comparison of diversity with other studies on the object populations is difficult because of different markers and sample sizes used, our results indicate values for Istrian sheep to be similar to the results obtained for this breed in Lawson Handley *et al.* (2007).

Table 1: *Genetic diversity parameters estimated for the 28 microsatellite loci (more than 95% genotyping success) analyzed in the four sheep populations used in this study*

Marker	Multiplex[a]	A[b]	Ho[c]	He[d]	HWE[e]	F(null)[f]	Fis[g]	PIC[h]
OarVH72[i]	PET, 56 °C	8	0.775	0.797	n.s.	0.083	0.011	0.771
OarJMP58[i]	6-FAM, 56 °C	13	0.706	0.771	n.s.	0.068	0.026	0.747
OarCP34[i]	6-FAM, 56 °C	6	0.677	0.751	n.s.	0.074	0.097	0.713
JMP29[i]	VIC, 56 °C	14	0.825	0.820	n.s.	0.022	−0.049	0.797
DYMS1[i]	NED, 56 °C	12	0.689	0.687	n.s.	0.004	−0.033	0.665
BM8125[i]	NED, 56 °C	8	0.673	0.716	n.s.	0.032	−0.003	0.678
BM1824[i]	VIC, 56 °C	4	0.427	0.648	**	0.372	0.286	0.594
CSRD247	PET, 55 °C	14	0.743	0.814	n.s.	0.039	0.052	0.791
ETH10	VIC, 55 °C	3	0.214	0.192	n.s.	0.000	−0.119	0.175
HSC	6-FAM, 55 °C	10	0.842	0.848	n.s.	0.041	−0.072	0.832
ILSTS005[i]	NED, 55 °C	8	0.549	0.655	***	0.081	0.155	0.604
ILSTS011[i]	PET, 55 °C	7	0.696	0.787	*	0.365	0.097	0.756
INRA063[i]	6-FAM, 55 °C	12	0.657	0.713	n.s.	0.009	−0.014	0.670
INRA132	VIC, 55 °C	14	0.804	0.900	*	0.093	0.081	0.892
MAF209[i]	PET, 55 °C	11	0.677	0.808	**	0.074	0.096	0.784
MAF65[i]	VIC, 55 °C	11	0.657	0.758	n.s.	0.048	0.081	0.727
McM527[i]	NED, 55 °C	6	0.608	0.630	n.s.	0.026	−0.013	0.595
OarCP49	VIC, 55 °C	15	0.711	0.873	**	0.095	0.129	0.935
OarFCB128[i]	6-FAM, 55 °C	9	0.505	0.802	***	0.289	0.347	0.791
FCB304[i]	PET, 55 °C	11	0.784	0.742	n.s.	0.000	−0.094	0.708
SPS113	NED, 55 °C	10	0.777	0.758	n.s.	0.004	−0.033	0.728
SPS115	VIC, 55 °C	8	0.598	0.725	*	0.084	0.078	0.678
TCRGC4B	NED, 55 °C	15	0.695	0.807	**	0.068	0.107	0.790
TCRVB6	NED, 55 °C	11	0.767	0.762	n.s.	0.035	−0.030	0.737
OarHH47[i]	6-FAM, 58 °C	15	0.778	0.858	n.s.	0.052	0.018	0.842
MCM140[i]	6-FAM, 58 °C	10	0.753	0.767	n.s.	0.058	−0.022	0.734
MAF214[i]	VIC, 58 °C	8	0.485	0.644	***	0.143	0.203	0.596
HUJ616[i]	VIC, 58 °C	18	0.592	0.741	**	0.087	0.192	0.713
Overall		291	0.667	0.742	***			

[a] The three multiplexes are indicated by the fluorochorme used for the marker and the annealing temperature of the PCR. [b] A – number of alleles per locus. [c] Ho – average observed heteozygosity. [d] He – average expected heterozygosity. [e] HWE – signifficant deviation from the Hardy-Weinberg equilibrium (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, n.s. – not significant). [f] F(null) – frequency of null alleles estimated for each locus. [g] Fis – coefficient of inbreeding. [h] PIC – polymorphic information content. [i] FAO recomended marker for sheep diversity.

Genetic variability revealed in studied populations was similar to sheep breeds that have not been subjected to a high selection pressure and higher than that reported for selected breeds (Arranz *et al.*, 2001). Although the ranges of observed and expected heterozygosity were similar to other European local sheep breeds, and the Turkish sheep breeds (Gutierrez-Gil *et al.*, 2006), the range of mean number of rarefacted alleles (Table 2) was in the low levels of the range reported for Balkan pramenka type populations (Ćinkulov *et al.*, 2008) and lower than in Alpine (Dalvit *et al.*, 2008), Spanish (Rendo *et al.*, 2004), and Greek sheep (Ligda *et al.*, 2009), but higher than in Italian sheep (Bozzi *et al.*, 2009). Significant ($P < 0.05$) inbreeding coefficients were found in all the populations except LIK (Table 2). Estimated inbreeding coefficients (Fis) for populations across loci are within the literature ranges with the estimates being similar to values found in Greek breeds (Ligda *et al.,* 2009), and lower than in Por-

Table 2: *Genetic variability parameters estimated for IST, ISTs, KRK and LIK populations, based on the analysis of the 24 microsatellite markers*

Group	n[a]	Ho[b]	He[c]	MNA[d]	pA[e]	Fis[f]
IST	35	0.695 ± 0.163	0.714 ± 0.148	5.88	20	0.042*
ISTs	20	0.694 ± 0.160	0.710 ± 0.148	6.08	12	0.052*
KRK	23	0.723 ± 0.153	0.732 ± 0.133	6.73	24	0.035*
LIK	25	0.648 ± 0.150	0.634 ± 0.147	5.22	11	−0.001
Overall	103	0.668	0.745	6.71	67	

[a] n – sample size. [b] Ho – average observed heterozygosity (± SD). [c] He average expected heterozygosity (± SD). [d] MNA – mean number of alleles (rarefacted). [e] pA – number of private alleles. [f] Fis estimates and significance of the deviation of HWE per population across the 24 loci analysed (* $P < 0.05$).

tuguese sheep (Santos-Silva *et al.*, 2008). The AMOVA analysis showed a significant and higher source of variation within (93.75%) than among (6.25%) populations. The Fst value (0.062, $P < 0.001$) suggested a moderate genetic differentiation for the global population, similar as previously reported for west Balkan sheep (Ćinkulov *et al.*, 2008) and somewhat higher than in Greek sheep breeds (Ligda *et al.*, 2009).

For the IST, ISTs, KRK and LIK groups, the genetic differentiation estimates of pair-wise Wright's fixation index (Fst) were low (0.015 for IST-ISTs pair) to considerable (0.111 for LIK-IST pair) (Table 3). The largest genetic differentiation was found for the LIK group and was associated with restricted gene flow to and from other populations. On contrary, ISTs showed little differentiation paired with IST and KRK populations. The highest gene flow was estimated for the IST-ISTs pair (16.96) and both of these groups showed a considerable estimate for the gene flow with the KRK sheep population (Table 3).

4 CONCLUSIONS

IST and ISTs sheep populations are closer to KRK than LIK population when their intra-population diversity estimates are concerned. The estimated inbreeding coefficient (0.052) indicates that inbreeding exist in ISTs.

Table 3: *Genetic differentiation parameters estimated for IST, ISTs, KRK and LIK, on the basis of 24 microsatellite markers*

Group	IST	KRK	LIK	ISTs
IST	-	0.027	0.111	0.015
KRK	8.99	-	0.108	0.025
LIK	2.01	2.08	-	0.102
ISTs	16.96	9.86	2.21	-

Significant ($P < 0.001$) pair-wise genetic distances (Fst) (above diagonal), and number of effective migrants per generation (Nm) (below the diagonal).

Results for diversity parameters in LIK and KRK prove these populations to be good out-groups. Although the Fis estimate was not shown to be significant for LIK, in comparison with the other studied breeds, it showed far less diversity and variability. Additionally, observed heterozygosity being higher than the expected indicates suspicion regarding an isolate-breaking effect in LIK population. On the contrary, KRK showed remarkably favourable diversity statistics' values.

Although further studies would be recommended to determine the pattern of the diversity of Istrian sheep more precisely, our results suggest that the reproductively isolated population from Slovenia shows less diversity than that from Croatia. Obtained diversity values, following the knowledge on the history of populations, show possible genetic drift due to the founder effect. To answer this question, further studies which would provide a deeper insight into the structure of these populations are required.

5 ACKNOWLEDGEMENTS

The samples of the Istrian sheep from Croatia were provided wit the help of the Croatian Agricultural Agency.

6 REFERENCES

Arranz J.J., Bayon Y., San Primitivo F. 2001. Differentiation among Spanish sheep breeds using microsatellites. Genetics Selection Evolution, 33: 529–542

Barreta J., Iñiguez V., Saavedra V., Romero F., Callisaya A.M., Echalar J., Gutiérrez-Gil B., Arranz J.J. 2012. Genetic diversity and population structure of Bolivian alpacas. Small Ruminant Research, 105: 97–104

Belkhir K., Borsa P., Chikhi L., Raufaste N., Bonhomme F. 2002. GENETIX 4.04, logiciel sous Windows TM pour la génétique des populations. Laboratoire Génome, Populations,

Interactions: CNRS UMR 5000. Montpellier, France, Université de Montpellier II

Bozzi R., Degl'Innocenti P., Rivera Diaz P., Nardi L., Crovetti A., Sargentini C., Giorgetti A. 2009. Genetic characterization and breed assignment in five Italian sheep breeds using microsatellite markers. Small Ruminant Research, 85: 50–57

Böhm O. 2004. Sheep and goat husbandry in Primorje (the Slovenian litoral) between 1946 and 1952 (studied in the course of erradication of brucellosis, caused by B. melitensis, in order to better understand its epidemiology). Slovenian Veterinary Research, 41, 9: 95–123

Ćinkulov M., Popovski Z., Porcu K., Tanaskovska B., Hodžić A., Bytyqi H., Mehmeti H., Margeta V., Djedović R., Hoda A., Trailović B., Brka M., Marković B., Važić B., Vegara M., Olsaker I., Kantanen J. 2008. Genetic diversity and structure of the West Balkan Pramenka sheep types as revealed by microsatellite and mitochondrial DNA analysis. Animal Genetics, 125: 417–426

Dalvit C., Sacca E., Cassandro M., Gervaso M., Pastore E., Piasentier E. 2008. Genetic diversity and variability in Alpine sheep breeds. Small Ruminant Research, 80: 45–51

Excoffier L., Laval G., Schneider S. 2005. Arlequin ver.3.0: an integrated software package for population genetics analysis. Evolutionary Bioinformatics Online, 1: 47–50

Glowatzki-Mullis M.L., Muntwyler J., Bäumle E., Gaillard C. 2008. Genetic diversity measures of Swiss goat breeds as decision-making support for conservation policy. Small Ruminant Research, 74: 202–211

Guo S.W., Thompson E.A. 1992. Performing the exact test of Hardy-Weinberg proportion for multiple alleles. Biometrics, 48: 361–372

Gutierrez J.P., Royo L.J., Álvarez I., Goyache F. 2005. MolKin v2.0: A Computer Program for Genetic Analysis of Populations Using Molecular Coancestry Information. Journal of Heredity, 96: 718–721

Gutierrez-Gil B., Uzun M., Arranz J.J., San Primitivo F., Yildiz S., Cenesiz M., Bayon Y. 2006. Genetic diversity in Turkish sheep. Acta Agriculturae Scandinavica, A, 56: 1–7

Lawson Handley L.-J., Byrne K., Santucci F., Townsend S., Taylor M., Bruford M.W., Hewitt G. M. 2007. Genetic structure of European sheep breeds. Heredity, 99: 620–631

Lewis P.O., Zaykin D. 2001. Genetic Data Analysis: Computer Program for the Analysis of Allelic Data, Version 1.0 (d16c): 45 p.

Ligda Ch., Altarayrah J., Georgoudis A., ECONOGENE 2009. Genetic analysis of Greek sheep breeds using microsatellite markers for setting conservation priorities. Small Ruminant Research, 83: 42–48

Mulc D., Jurković D., Duvnjak G., Sinković T., Daud J., Lješić N., Špehar M., Dražić M. 2012. Annual report 2011: Breeding of sheep, goats and small animals. Križevci, Croatian Agricultural Agency,: 80 p.

Raymond M., Rousset F. 1995. GENEPOP (version 1.2): population genetics software for exact tests and ecumenicism. Journal of Heredity, 86: 248–249

Rendo F., Iriondo M., Jugo B.M., Mazon L.I., Aguirre A., Vicario A., Estonba A. 2004. Tracking genetic diversity and differentiation in six sheep breeds from the North Iberian Peninsula through DNA variation. Small Ruminant Research, 52: 195–202

Santos-Silva F., Ivo R.S., Sousa M.C.O., Carolino M.I., Ginja C., Gama L.T. 2008. Assessing genetic diversity and differentiation in Portuguese coarse-wool sheep breeds with microsatellite markers. Small Ruminant Research, 78: 32–40