Animal (2014), **8:2**, pp 200–207 © The Animal Consortium 2014
doi:10.1017/S1751731113002243

animal

Genetic diversity and differentiation of 12 eastern Adriatic and western Dinaric native sheep breeds using microsatellites

D. Salamon[1], B. Gutierrez-Gil[2], J. J. Arranz[2], J. Barreta[2], V. Batinic[3] and A. Dzidic[1†]

[1]*Department of Animal Science I, Faculty of Agriculture, University of Zagreb, Svetošimunska cesta 25, 10000 Zagreb, Croatia;* [2]*Departamento de Producción Animal, Universidad de León, Campus de Vegazana s/n, 24071 León, Spain;* [3]*Faculty of Agriculture and Food Technology, University of Mostar, Biskupa Čule b.b., 88000 Mostar, Bosnia and Herzegovina*

(Received 30 April 2013; Accepted 14 November 2013)

Nuclear genetic diversity and differentiation of 341 sheep belonging to 12 sheep breeds from Croatia and Bosnia and Herzegovina were examined. The aim of the study was to provide the understanding of the genetic structure and variability of the analysed pramenka sheep populations, and to give indications for conservation strategies based on the population diversity and structure information. The genetic variation of the sheep populations, examined at the nuclear level using 27 microsatellite loci, revealed considerable levels of genetic diversity, similar to the diversity found in other European indigenous low-production sheep breeds. Population-specific alleles were detected at most loci and in breeds analysed. The observed heterozygosity ranged from 0.643 (in Lika pramenka) to 0.743 (in Vlasic pramenka), and the expected heterozygosity ranged from 0.646 (in Lika pramenka) to 0.756 (in Dalmatian pramenka). Significant inbreeding coefficients were found for half of the populations studied and ranged from 0.040 (Pag island sheep) to 0.091 (Kupres pramenka). Moderate genetic differentiation was found between the studied sheep populations. The total genetic variability observed between different populations was 5.29%, whereas 94.71% of the variation was found within populations. Cres island sheep, Lika pramenka and Istrian sheep were identified as the most distinct populations, which was confirmed by the factorial analysis of correspondence and supported through a bootstrapping adjustment to correct for the difference in the sample sizes. The population structure analysis distinguished 12 clusters for the 12 sheep breeds analysed. However, the cluster differentiation was low for Dalmatian, Vlasic, Stolac and Krk pramenka. This systematic study identified Lika pramenka and Rab island sheep as those with the lowest diversity, whereas Istrian sheep and Pag island sheep had the highest. Conservation actions are proposed for Istrian, Rab and Cres island sheep, Lika and Kupres pramenka because of high estimated coefficients of inbreeding.

Keywords: sheep breeds, genetic diversity, population structure, microsatellites

Implications

Loss of farm animal genetic diversity was on the rise during the last 50 years, as the spread of a few highly developed breeds started to threaten the existence of well-adapted local breeds either by cross-breeding or by substitution. For more than 30% of the livestock breeds the situation is unknown, and 36% of known sheep breeds are endangered or extinct. Marginal and transitional areas with harsh environment, often used for low-input sheep farming, are predominantly the ones affected. Genetic variability is important for the sustainable use and development of native sheep populations.

† E-mail: adzidic@agr.hr

Introduction

In addition to natural geographical isolation at the indented eastern Adriatic coastline and karst of Bosnia and Herzegovina, the history of the autochthonous sheep populations of this region includes important political and economical changes that have taken place in this dynamic area: changes of the borders, management practices such as horizontal and vertical transhumance, and controlled and uncontrolled crossbreeding. The sheep chosen for this study are the local populations from Croatia and from Bosnia and Herzegovina. They are crucial for the preservation of the genetic diversity in this geographic region, as they are either numerous in the current overall livestock production, or predominant in the sheep production of a specific micro region. The studied populations are of pramenka

type, which is the Bosnian, Croatian and Serbian name descriptive for open fleece of sheep breeds with mixed wool, included in the Zackel/Valachian phyletic sheep group (Draganescu and Grosu, 2010). In Croatia, about 80% of the 630 000 registered breeding sheep are individuals representing one of the nine autochthonous breeds, predominately reared in low-input production systems. We chose eight breeds representing almost exclusively the sheep production of the Mediterranean part of Croatia: Istrian sheep (IST), Krk island sheep (KRK), Rab island sheep (RAB), Cres island sheep (CRE), Lika pramenka sheep (LIK), Pag island sheep (PAG), Dalmatian pramenka (DAL) and Dubrovnik ruda sheep population (RUD). Additional four multipurpose pramenka breeds included in this study graze more than 50% of the total agricultural areas in low-input highland systems of Bosnia and Herzegovina, and are currently stable at about one million sheep: Kupres pramenka (KUP), Vlasic/Travnik/Dubska pramenka (VLA), Privor pramenka (PRI) and Hum/Stolac pramenka (STO) sheep.

The high level of phenotypic diversity within the studied breeds (Böhm, 2004) and the large phenotypic differences among different breeds studied here (Posavi *et al.*, 2003; Brka *et al.*, 2007) suggest high levels of genetic diversity within populations and high levels of genetic differentiation among them. Differentiation and neutral nuclear diversity studies are scarce and have been reported only in a limited number of these pramenka populations (Bradic *et al.*, 2003; Lawson Handley *et al.*, 2007; Ćinkulov *et al.*, 2008) using different sets of markers, which makes comparison of results between breeds and with other breeds difficult (Food and Agriculture Organization of the United Nations, 2011). Ancestral origin using mtDNA and Y chromosome data has also been investigated in Pramenka sheep (Bradic *et al.*, 2005; Ivanković *et al.*, 2005; Ferencakovic *et al.*, 2013). Assessment of genetic diversity using nuclear data for setting the conservation priorities is a standardized method for estimating the genetic diversity of different ruminant populations in many countries (Baumung *et al.*, 2004; Ligda *et al.*, 2009; Barreta *et al.*, 2012). Therefore, in this study, we report on the pramenka breeds from Croatian and from Bosnia and Herzegovina breeds using nuclear microsatellite markers, with the aim of assessing the current status of the genetic diversity and differentiation of the considered breeds, revealing the genetic variability that exists within and among them, while also comprehensively exploring the genetic structure of the studied sample.

Material and methods

Sampling and DNA extraction

A total of 341 blood samples were collected for the 12 breeds (Supplementary Figure S1), with 20 to 33 unrelated animals sampled from each breed (Supplementary Table S1). One of the 12 sample groups was obtained from a reproductively isolated population of Istrian sheep in Slovenia. Blood Genomic DNA Kit was used to extract DNA from the whole blood samples (GenEltue™; Sigma-Aldrich®, St. Louis, MO, USA). For the initial selection of 30 markers, four different PCR-multiplex reactions were optimized using fluorescent-labelled primers and hot-start polymerase (JumpStart™ REDTaq® ReadyMix™; Sigma-Aldrich®, St. Louis, MO, USA). Twenty of the markers had been selected from the sheep diversity list recommended by Food and Agriculture Organization of the United Nations (FAO, 2011). The remaining 10 markers had previously shown good features for multiplexing. Diluted PCR products were processed in a 16-capillary electrophoresis ABI3130XL Genetic Analyser, with two of the PCR-multiplex reactions being combined into a single multi-loading mix (Supplementary Table S2). Electropherograms from the loading panels were analysed automatically using GeneMapper® software (AB, CA). Two of the markers had more than 5% of missing genotypes and were excluded from further analysis. Genetic diversity parameters were estimated for the remaining 28 microsatellite loci.

Data analysis

Allele frequency, the number of alleles (A), observed heterozygosity (Ho) and heterozygosity expected (He) under the Hardy-Weinberg (HW) equilibrium assumption across the populations and the markers were calculated using the GENETIX 4.04 software (Belkhir *et al.*, 2002). Locus-wise deviations of the markers from HW equilibrium across the populations were tested by means of the GENEPOP 4.1.3 software package (Raymond and Rousset, 1995) and the method of Guo and Thompson (1992). The same software was used to determine the possibility of null-alleles and gametic disequilibrium test. Statistical significance of the values obtained in all cases was estimated by bootstrapping, using 1000 replications. Markers showing deviation from the HW equilibrium were excluded from further analysis if the deviation was significant in more than half of the 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 in MOLKIN 3.0 (Gutierrez *et al.*, 2005), using bootstrapping to standardize among different sample size populations. Hulbert's rarefaction correction and sample size correction were based on 50 diploid individuals. Pair-wise genetic distances (F_{st}), coefficients of inbreeding (F_{is}) and gene flow estimates were obtained using ARLEQUIN 3.1 (Excoffier *et al.*, 2005) and GENETIX 4.04 (Belkhir *et al.*, 2002). GENETIX 4.04 was also used to evaluate the significance of the F_{is} by permuting the alleles within populations over all loci in each breed, and under the assumption of heterozygosis deficit, as well as for the factorial correspondence analysis. Genetic variation and the distribution of genetic diversity among and within the groups were determined by the analysis of molecular variance (AMOVA) using the ARLEQUIN 3.1 software. Several groupings of populations in nested AMOVA were tested to find the grouping that best explains the variance in the genotype data. Individual multi-locus genotypes were used in clustering methods to study the population differentiation. Individual assignment in the populations was investigated using the STRUCTURE software 2.3.1 (Pritchard *et al.*, 2000). We performed 10 runs to choose the appropriate number of inferred clusters (K), fitting K from 2 to 20. Burn-in period for all runs

was 20 000 iterations, and data were collected during the period of 10 000 iterations. To choose the optimal *K*, the posterior probability *L*(*K*) was calculated using the mean log-likelihood of *K* for each value of Evanos' Δ*K*. *L*''(*K*) with respect to *K* was also calculated. Graphic representations of these statistics were obtained from Structure Harvester 0.6.8 (Dent and VonHoldt, 2012).

Results

We identified a high level of genetic diversity based on the analysis of the 28 loci (Table 1). A total of 392 different alleles were identified in the 341 genotyped individuals. The average number of alleles per locus was 14. The highest number of detected alleles recorded was 26 for marker *HUJ616*, whereas *ETH10* showed only three alleles (Table 1). The PIC values per marker varied from 0.142 for *ETH10*, to 0.943, for *OarCP49*. The highest Ho was recorded for locus *HSC* (0.854). The highest He was estimated for locus *INRA132* (0.889). In the global population, accounting for multiple tests (28 loci, 12 populations), 13 loci were found to be in HW disequilibrium, with the average number of 2.5 populations in disequilibrium per marker. The maximum of six populations in HW disequilibrium was recorded for marker *OarFCB128*. Non-amplifying null alleles showed frequency estimates ranging from 0.0030 (*BM8125*) to 0.3634 (*BM1824*) (Table 1). *BM1824* was excluded from subsequent analysis of genetic differentiation because of the high estimated frequency of null allele. Hence, the results of genetic variability for the 12 studied populations are given based on the remaining 27 microsatellite markers analysed.

Considering detected alleles per population per locus, one marker was found to be fixed in three populations (*ETH10* in RUD, PAG, CRE), whereas the highest number of alleles was 15 (*TCRGC4B* in STO). In total, 61 private alleles were sampled (total N of alleles for 27 marker was 387) and were distributed across all populations. The highest numbers of private alleles were noted in PAG (12) and IST (10) breeds (Table 2). The highest frequencies of private alleles were observed in CRE for *TB6* (0.18), RAB for *HSC* (0.16) and CRE for *ILST5* (0.14). The largest rarefacted mean number of alleles per locus (MNA), when all of the markers are considered jointly, was found in STO (8.63). Similar MNA values

Table 1 *Genetic diversity parameters estimated for the 28 microsatellite loci analysed in the 12 sheep populations*

Markers	A	Ho	He	HWE	F (null)	F_{is}	PIC
HUJ616	26	0.678	0.727	**	0.033	0.047	0.706
MAF214	10	0.458	0.603	***	0.122	0.188	0.559
MCM140	12	0.801	0.825	ns	0.049	−0.001	0.804
OarHH47	16	0.752	0.857	***	0.058	0.079	0.842
TCRVB6	16	0.824	0.821	ns	0.035	−0.047	0.804
TCRGC4B	20	0.702	0.836	***	0.070	0.132	0.823
SPS115	11	0.632	0.749	**	0.064	0.107	0.711
SPS113	13	0.759	0.748	ns	0.009	−0.038	0.713
FCB304	15	0.691	0.681	ns	0.010	−0.050	0.642
OarFCB128	12	0.615	0.828	***	0.121	0.227	0.838
OarCP49	25	0.767	0.876	***	0.060	0.073	0.943
MCM527	12	0.695	0.756	ns	0.033	0.029	0.726
MAF65	12	0.761	0.799	ns	0.012	−0.00019	0.770
MAF209	13	0.684	0.818	***	0.071	0.114	0.797
INRA132	16	0.831	0.889	*	0.035	0.036	0.878
INRA063	20	0.729	0.789	ns	0.037	−0.00048	0.765
ILSTS011	8	0.712	0.792	*	0.052	0.060	0.761
ILSTS005	11	0.542	0.672	***	0.091	0.148	0.630
HSC	17	0.855	0.887	ns	0.010	−0.015	0.876
ETH10	3	0.158	0.152	ns	0.051	−0.099	0.142
CSRD247	20	0.710	0.821	***	0.053	0.075	0.801
BM1824	4	0.589	0.684	**	0.363	0.096	0.626
BM8125	9	0.706	0.711	ns	0.003	−0.049	0.677
DYMS1	16	0.709	0.730	ns	0.011	0.002	0.711
JMP29	22	0.836	0.844	ns	0.022	−0.040	0.827
OarCP34	7	0.694	0.755	ns	0.044	0.069	0.719
OarJMP58	17	0.750	0.800	ns	0.027	−0.010	0.780
OarVH72	9	0.732	0.796	ns	0.103	0.036	0.774
Overall	392	0.692	0.759				

A = number of alleles per locus; Ho = average observed heterozygosity; He = average expected heterozygosity; HWE = deviation from the HW equilibrium; F (null) = frequency of null alleles estimated for each locus, F_{is} = coefficient of inbreeding; PIC = polymorphic information content; ns = non-significant.
*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

Table 2 *Genetic variability parameters estimated for the 12 populations of sheep studied, based on the analysis of the 27 microsatellite markers*

Groups	*n*	Ho	He	MNA	P_A	F_{is}
CRE	25	0.699 ± 0.225	0.664 ± 0.192	6.51	2	−0.033
DAL	25	0.718 ± 0.146	0.757 ± 0.120	8.53	8	0.072***
IST	69	0.684 ± 0.149	0.722 ± 0.148	7.18	10	0.061***
KRK	23	0.699 ± 0.166	0.726 ± 0.130	7.93	2	0.060**
KUP	25	0.700 ± 0.169	0.752 ± 0.125	8.61	3	0.091***
LIK	25	0.643 ± 0.145	0.643 ± 0.142	6.03	4	0.021
PAG	25	0.693 ± 0.188	0.707 ± 0.166	8.06	12	0.040*
PRI	25	0.720 ± 0.184	0.711 ± 0.158	7.77	2	0.008
RAB	25	0.652 ± 0.187	0.701 ± 0.155	7.04	2	0.091***
RUD	25	0.698 ± 0.218	0.691 ± 0.187	7.71	5	0.010
STO	25	0.723 ± 0.157	0.730 ± 0.149	8.63	6	0.031
VLA	24	0.743 ± 0.129	0.749 ± 0.121	8.62	5	0.030
Overall	341	0.707	0.765	8.92	61	

n = sample size; Ho = average observed heterozygosity (± s.d.); He = average expected heterozygosity (± s.d.); MNA = mean number of alleles (rarefacted); P_A = number of private alleles; F_{is} = coefficient of inbreeding.
F_{is} estimates and significance of the deviation of HW equilibrium per population across the 27 loci.
*P<0.05, **P<0.01, ***P<0.001.

Table 3 *Genetic differentiation parameters estimated for the 12 populations of sheep studied using of 27 microsatellite markers*

Groups	CRE	DAL	IST	KRK	KUP	LIK	PAG	PRI	RAB	RUD	STO	VLA
CRE	–	0.074***	0.066***	0.058***	0.063***	0.149***	0.047***	0.072***	0.091***	0.078***	0.063***	0.063***
DAL	3.13	–	0.039***	0.033***	0.015***	0.082***	0.033***	0.025***	0.043***	0.054***	0.023***	0.018***
IST	3.54	6.09	–	0.026***	0.028***	0.104***	0.029***	0.042***	0.055***	0.056***	0.038***	0.028***
KRK	4.04	7.28	9.35	–	0.022***	0.106***	0.020***	0.035***	0.040***	0.062***	0.032***	0.027***
KUP	3.75	15.99	8.52	11.11	–	0.071***	0.030***	0.024***	0.047***	0.040***	0.015***	0.007*
LIK	1.43	2.79	2.16	2.12	3.27	–	0.106***	0.108***	0.114***	0.120***	0.097***	0.074***
PAG	5.04	7.32	8.27	12.22	8.04	2.10	–	0.035***	0.034***	0.058***	0.032***	0.028***
PRI	3.23	9.91	5.68	6.98	10.33	2.05	6.90	–	0.055***	0.058***	0.034***	0.013**
RAB	2.51	5.52	4.29	6.06	5.11	1.94	7.15	4.28	–	0.088***	0.054***	0.050***
RUD	2.94	4.40	4.20	3.77	6.05	1.84	4.02	4.10	2.59	–	0.040***	0.042***
STO	3.69	10.60	6.30	7.50	16.46	2.33	7.57	7.12	4.37	5.97	–	0.021***
VLA	3.75	13.63	8.71	8.90	33.84	3.14	8.74	18.68	4.72	5.64	11.42	–

Pairwise genetic distances (F_{st}) with their significance levels, and number of effective migrants per generation (N_m) are presented above and below the diagonal, respectively.
*P<0.05, **P<0.01, ***P<0.001.

were estimated for VLA, KUP and DAL (Table 2). Average Ho values among all the populations were high and resembling, ranging from 0.643 ± 0.145 (LIK) to 0.743 ± 0.129 (VLA). Similarly, the average He values varied from 0.643 ± 0.142 (LIK) to 0.757 ± 0.120 (DAL) (Table 2). Possible artefacts owing to the different sample sizes can be ruled out, as the values obtained after the sample size correction did not show remarkable differences when compared with the diversity estimates reported above. F_{is} was estimated for each locus in the global population and for each population across loci. Estimated F_{is} values for the markers ranged from −0.099 (*ETH10*) to 0.227 (*OarFCB128*) (Table 1), and were positive and significant (P < 0.05) for 13 markers, whereas for six of them the values were high. High F_{is} value for *OarFCB128* was evident in 10 of the breeds, ranging from 0.026 (DAL) to 0.484 (KRK). Considering the individual populations, half of them showed significant (P < 0.05), positive and low F_{is} values (Table 2). The highest significant F_{is} values were estimated for RAB and KUP (F_{is} = 0.091, P < 0.001).

For the 12 considered groups, the genetic differentiation estimates of pairwise Wright's fixation index (F_{st}) were low (0.007 for VLA–KUP pair) to considerable (0.149 for LIK–CRE pair) (Table 3). Largest genetic differentiation was found for the LIK population and the estimated F_{st} coefficients ranged between 0.071 and 0.149. Even for the substantial genetic differentiation identified for LIK, the estimates for the number of effective migrants (N_m) were very low (1.43 to 3.27). In contrast, KUP showed a low level of differentiation, with F_{st} coefficients reaching maximally 0.063 (KUP–CRE). The highest gene flow was estimated for the KUP-VLA pair (33.84), and both of these groups had the highest estimates for the gene flow compared with other populations (Table 3).

Table 4 *Global AMOVA results for the 12 population and results of the nested AMOVA performed by grouping the sheep geographically[a] and utility-wise[b]*

Source of variation	Degrees of freedom	Sum of squares	Variance components	Percentage of variation	F_{st} estimate
Among populations	11	417.335	0.514	5.29	
Within populations	670	6172.428	0.213	94.71	0.053***
Among groups geographically[a]	1	64.16	0.074	0.76	0.008**
Among populations within groups	10	352.17	0.474	4.85	0.050***
Within populations	670	6172.43	9.213	94.39	0.056***
Among groups utility-wise[b]	1	60.00	0.060	0.61	0.006*
Among populations within groups	10	357.33	0.481	4.94	0.050***
Within populations	670	6172.43	9.213	94.45	0.056***

AMOVA = analysis of molecular variance.
Genetic distances (F_{st}) with their significance levels are given.
[a]Geographically: islands and peninsula (IST, KRK, CRE, PAG, RAB, RUD); mainland (DAL, LIK, KUP, VLA, PRI, STO).
[b]Utility-wise: group used predominately for milk (IST, KRK, CRE, PAG, RAB, VLA); group used predominately for meat (DAL, LIK, KUP, PRI, STO, RUD).
*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

Table 5 *Proportion of membership for the 12 sheep populations across the clusters indentified in the assignment analysis (the highest contribution shown in bold)*

	Cluster											
Group	1	2	3	4	5	6	7	8	9	10	11	12
CRE	0.009	0.017	0.022	0.012	**0.807**	0.012	0.010	0.007	0.017	0.029	0.016	0.042
DAL	**0.338**	0.053	0.046	0.015	0.016	0.160	0.013	0.021	0.032	0.053	0.163	0.091
IST	0.019	0.013	0.133	**0.650**	0.017	0.024	0.014	0.011	0.016	0.018	0.043	0.039
KRK	0.048	0.021	**0.417**	0.030	0.014	0.053	0.009	0.010	0.016	0.026	0.279	0.077
KUP	0.120	0.073	0.029	0.033	0.015	0.224	0.030	0.032	0.013	0.032	**0.236**	0.163
LIK	0.033	0.008	0.010	0.013	0.007	0.018	0.020	**0.822**	0.011	0.028	0.012	0.018
PAG	0.014	0.011	0.078	0.027	0.047	0.060	0.014	0.012	0.043	**0.539**	0.128	0.026
PRI	0.045	**0.604**	0.017	0.010	0.007	0.053	0.014	0.006	0.009	0.040	0.018	0.178
RAB	0.038	0.009	0.013	0.012	0.012	0.017	0.010	0.021	**0.725**	0.038	0.081	0.022
RUD	0.019	0.037	0.012	0.016	0.013	0.061	**0.698**	0.011	0.018	0.034	0.046	0.035
STO	0.064	0.087	0.031	0.023	0.022	**0.404**	0.046	0.013	0.010	0.048	0.090	0.161
VLA	0.079	0.125	0.042	0.024	0.017	0.109	0.028	0.043	0.014	0.094	0.075	**0.350**

AMOVA showed a significant ($P < 0.001$) and higher source of variation within 94.79% than among 5.21% populations (Table 4). The F_{st} value (0.052) obtained by this analysis suggested a moderate genetic differentiation for the global population. Variance components among populations were highly significant ($P < 0.001$) for all of the studied loci, and markers *OarJMP58* and *INRA063* contributed to explain 8.05% and 8.57% of the variability, respectively. Utility-wise and geography-wise nested AMOVA showed similar results (Table 4), with more variability among populations than between geographical or utility groups. In the factorial correspondence analysis, the first three components together accounted for 43.42% of the variation, and explained 16.73%, 13.78% and 12.91% of the total variation, respectively. The first component separates the LIK and CRE groups (Supplementary Figure S2). Addition of the second component confirms the differentiation of CRE and IST, whereas the third component separated IST and demonstrated the isolation of LIK from all the other populations.

The most appropriate number of clusters for the 12 populations according to Delta *K* (7.92) was ascertained to be 12, and the best value of ln Pr(*X*|*K*) for $K = 12$ was −30 947.2 (Supplementary Figure S3). Graphical representation of the clustering outcomes suggested for $K = 12$ is shown in Supplementary Figure S4, and the proportion of membership for the indentified clusters is provided in Table 5. Populations CRE, LIK and RAB were each associated with their own cluster, for which the corresponding estimated membership was higher than 0.725. RUD, IST, PRI and PAG populations were also assigned to their own clusters, but because of more admixture their highest estimated membership was moderate and higher than 0.539. The admixtures were low and homogeneously distributed, except in PRI, which was influenced by VLA-related cluster 12, and PAG, which showed influence of cluster 11. For populations VLA, DAL, KRK, STO and KUP, the higher proportion of membership was lower than 0.417 and showed influence of many of the identified clusters. Although there are 12 clusters for

12 populations, cluster 11 does not seem to correspond to any of the sampled populations, in particular. It influences most of the populations to some extent, except for CRE, LIK and PRI. The second most heterogeneous cluster is the VLA-related cluster 12, influenced by the STO, PRI and KUP populations. All of the 12 analysed pramenka breeds showed, to some degree, a 'background' influence from the two rustic populations, DAL and VLA.

Discussion

A large number of markers was covered in this study and, with the exception of *ETH10*, all were highly polymorphic. We observed a clear deficit of heterozygotes, as reported in other sheep breeds (Lawson Handley *et al.*, 2007). In the overall population, 13 of the 28 markers analysed had a significant deviation from HW equilibrium (Table 1). Except for *OarFCB128*, these markers showed no correlation to the occurrence of null alleles and can be explained by a reduced effective size of the flocks in the studied populations.

Although a comparison of the genetic diversity parameters described here with those reported in other populations is difficult because of different markers and sample sizes used, our results indicate values similar to those reported for the Balkan pramenka breeds (Ćinkulov *et al.*, 2008). In general, our analyses revealed higher within-breed variability than reported for selected breeds such as Sarda sheep, as expected for sheep populations that have not been highly pressured by selection (Arranz *et al.*, 2001; Pariset *et al.*, 2003). Moreover, we found that sheep genetic diversity in this south European region was not lost, with values fitting the pattern of radiation of genetic variation from the Near East hot-spot (Tapio *et al.*, 2010). For example, we found the MNA (range 6.03 to 8.6) to be within the range reported for Balkan pramenka breeds (Ćinkulov *et al.*, 2008) and alpine sheep (Dalvit *et al.*, 2008). However, it was lower than that reported for Greek (Ligda *et al.*, 2009) and Turkish sheep breeds (Gutierrez-Gil *et al.*, 2006).

Moderate genetic variation component estimated between the studied populations (5.29%) was similar to values reported for other Balkan sheep (Ćinkulov *et al.*, 2008), and somewhat higher than those reported for Greek breeds (Ligda *et al.*, 2009). The low percentages of the variance explained by utility-wise (0.008, $P < 0.01$) and geography-wise (0.006, $P < 0.05$) grouping, compared with results reported in Indian sheep (Arora *et al.*, 2011), suggest a low influence of specific selection strategies, but also a poor influence of the geographical isolation in the overall sampling area. The genetic differentiation estimates of pairwise F_{st} index, complemented with the gene flow estimates, are in accordance with the results of the factorial correspondence and structure analyses, and consistent with the geographical distribution of the breeds, their history and their breeding practices. The most recent threats to genetic variability of sheep populations include the Croatian War of Independence and the War in Bosnia and Herzegovina. The total number of sheep was almost halved. However, the island and peninsula breeds were not affected directly by war activities, as was the case with LIK, RUD, DAL, and the breeds from Bosnia and Herzegovina, which could have been exposed also to isolation breakage. Re-establishing of the studbooks after the war was performed by assigning the breed membership according to phenotypic appearance, similar as with other indigenous breeds in this area (Galov *et al.*, 2013).

As reported in Ćinkulov *et al.* (2008), IST group can be considered as a distinct breed, but VLA is clustering with other Balkan pramenka populations. Factorial correspondence and structure analysis showed that LIK, CRE and IST are the most distinct groups. From the genetic point of view, the rest of the populations are closer together, with RUD, KRK and DAL showing certain differences. In Croatia, most of the breeds are isolated on the Mediterranean islands, and the one showing the largest genetic distance (LIK) is located in the mountain region. Although the factorial correspondence analysis shows IST as the third most distinct population, it is close to KRK, and its distinction is only identified when considering the third component. The number of migrant individuals estimated for the KRK–IST pair (9.35) is also visible in the structure results. However, we found that the KRK admixture was not widely spread, but limited to some of the IST samples. In addition, IST had one of the higher numbers of private alleles, indicating that, owing to samples with a lot of KRK admixture, distinctiveness of IST was probably underestimated. RAB and RUD showed firm clusters and distinction from the other studied sheep populations because of their isolation. Namely, RAB is an island population, whereas RUD population is considered endangered (712 animals), isolated in the area on the furthest south of Croatia and bred for different purpose (wool). The most poorly defined population among the eight groups from Croatia was DAL and was also the only population in Croatia that showed connections to all four populations from Bosnia and Herzegovina. This by far is the largest population, covers areal along the Adriatic coast with some of the islands, and is sympatric with the other studied groups. The difference of results regarding the distinctiveness between populations sampled in Bosnia and Herzegovina and in Croatia could be a result of systematic recording and selection programme implemented by the Croatian Agriculture Agency during several decades, complemented with clear geographical boundaries between the populations/breeds in Croatia. As reported for Baltic sheep (Tapio *et al.*, 2005), four populations, sampled as traditional breeds in Bosnia and Herzegovina, do not equate to genetically distinct populations. According to the structure analysis and the N_m and F_{st} estimates reported here, the largest influence on other Bosnian populations comes from VLA, with status and distribution similar to DAL in Croatia. KUP was influenced by the largest number of the identified clusters and it is questionable how much of the initial diversity and specific adaptations to mountain enviroment remains in the KUP population proposed for 'status nascendi' conservation efforts. Although to have better milk and wool production, 'hybridization programmes' were established with PRI rams in KUP flocks, a higher influence is recorded from VLA (Palian *et al.*, 1960).

The most distinct among the Bosnia and Herzegovina populations was PRI, with a well-defined cluster showing minor admixtures and obvious influence from the sympatric VLA population.

According to Lawson Handley *et al.* (2007), Weitzmans' conservation approach, which favours the groups that clearly stand out in the factorial correspondence analysis (LIK, CRE and IST), or are differentiated in clusters showing unique genotypes (LIK, CRE and RAB), should be treated with caution. Similarly, we have noted that it does not account for the within-group genetic diversity levels and the geographical structure that can be found in some breeds. Namely, LIK had the lowest diversity indicators, as was also reported by Ferencakovic *et al.* (2013) for mtDNA and chromosome Y diversity. The second most distinct population, CRE, had Ho somewhat higher than that observed for LIK, but other diversity indicators were quite low as well. In addition, the same as in RUD, Ho being higher than He indicates suspicion regarding an isolate braking effect. Interestingly, Pavić *et al.* (2006) found lower He (0.6575) and higher F_{is} (0.094) in RUD, using 10 microsatellite markers on 44 animals, indicating possible recovery of the population in recent 5 years. Contrary to LIK and CRE, the IST population showed reasonable distinctiveness and favourable levels of diversity parameters. Nonetheless, the significant and relatively high estimated F_{is}, higher than reported for IST by Ćinkulov *et al.* (2008) (0.011), indicates heterozygote deficiency, which might be caused by a population subdivision effect because of the sampling of animals in different locations. When the sampled IST subpopulations are analysed separately (Salamon *et al.*, 2012), F_{is} values are lower. The F_{is} values for other sheep are similar to ones reported for Greek breeds (Ligda *et al.*, 2009), and lower than those reported for Portuguese sheep (Santos-Silva *et al.*, 2008). Unlike in IST, estimated F_{is} values in other populations are most likely caused by breeding practices carried out without knowledge regarding the genetic variants available in flocks of these populations. Artificial insemination is not used and, depending on the population, one ram is used per 17 to 33 ewes. The results of avoidance of mating the rams with their offspring are questionable, as there is no parentage assessment in any of the investigated breeds. As Pariset *et al.* (2003) noted, ram exchange policy provides gain of a very few genetic variants when the rams are exchanged between flocks with a similar genetic pool. In the Supplementary Table S1, we presented small estimated sizes for the studied geographical groups, especially for RAB, IST and KRK. Although Ćinkulov *et al.* (2008) estimated the highest F_{is} value among the analysed western Balkan pramenka breeds in Dubska sheep (aka VLA), we did not estimate significant F_{is} in this population.

Conclusions

This study detected high levels of genetic diversity in all studied breeds, except for LIK and RAB. The less distinct populations (VLA, STO, PRI, DAL and KUP) showed the highest heterozygosity levels and mean number of alleles. The two distinct breeds that showed the highest diversity were dairy breeds: IST and PAG. The 12 populations showed moderate differentiation with low influence of specific selection and geographical isolation. The Bosnia and Herzegovina populations were the least differentiated; however, PRI was the most distinct among them.

Additional studies based on more detailed sampling would be desirable to identify structure of the genetic pools within the breeds, especially to address the significant and substantial F_{is} values for KUP, RAB, IST, LIK and CRE. It would be recommendable to revise the strategy of the breeding practices to amplify the gain of genetic variants through ram exchange practice by identifying structure of the genetic pools of the farms within the breeds and to organize parentage testing.

Acknowledgements

The samples and analysis were funded by National research grants from the Ministry of Science, Education and Sports of the Republic of Croatia 178-1780460-0407. The research stay at the University of Leon was supported by the Trust fund of the Faculty of Agriculture at University of Zagreb. The samples of the Istrian sheep from Croatia were provided by the Croatian Agricultural Agency, and the samples of the Istrian sheep from Slovenia by the Zootechnical Department of Biotechnical Faculty, University of Ljubljana. The authors would also like to express their gratitude to dr. sc. Antun Kostelić for his efforts in obtaining samples from the Adriatic sheep breeds.

Supplementary material

To view supplementary material for this article, please visit http://dx.doi.org/10.1017/S1751731113002243.

References

Arora R, Bhatia S, Mishra BP and Joshi BK 2011. Population structure in Indian sheep ascertained using microsatellite information. Animal Genetics 42, 242–250.

Arranz JJ, Bayon Y and 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 AM, Echalar J, Gutiérrez-Gil B and Arranz JJ 2012. Genetic diversity and population structure of Bolivian alpacas. Small Ruminant Research 105, 97–104.

Baumung R, Simianer H and Hoffmann I 2004. Genetic diversity studies in farm animals – a survey. Journal of Animal Breeding and Genetics 121, 361–373.

Belkhir K, Borsa P, Chikhi L, Raufaste N and Bonhomme F 2002. GENETIX 4.04, logiciel sous Windows TM pour la génétique des populations. Laboratoire Génome, Populations, Interactions: CNRS UMR 5000, Université de Montpellier II, Montpellier, France.

Böhm O 2004. Sheep and goat husbandry in Primorje (the Slovenian litoral) between 1946 and 1952. Slovenian Veterinary Research 41, 95–123.

Bradic M, Pavic V and Mioc B 2003. Genetic difference of some sheep breeds in the Republic of Croatia. In Book of Abstracts of the 54th Annual Meeeting of the European Association for Animal Production (ed. Y van der Honing), pp. 15. Wageningen Academic Publishers, Wageningen, The Netherlands.

Bradic M, Mioc B, Pavic V and Barac Z 2005. Pairwise comparison of mtDNA sequences in two Croatian sheep populations. In Book of abstracts of the 56th Annual Meeting of the European Association for Animal Production

(ed. Y van der Honing), pp. 97. Wageningen Academic Publishers, Wageningen, The Netherlands.

Brka M, Muhamedagić S, Ivanković S, Vegara M, Rahmanović A, Dokso A and Zečević E 2007. Autochthones breeds of domestic animals in Bosnia and Herzegovina. In Proceedings of 1st Conference on Native Breeds and Plant Varieties as part of natural and cultural heritage,13 to 16 November 2007, Šibenik, Croatia, pp. 35–36.

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 and 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 and Piasentier E 2008. Genetic diversity and variability in Alpine sheep breeds. Small Ruminant Research 80, 45–51.

Dent AE and VonHoldt BM 2012. STRUCTURE HARVESTER: a website and program for visualizing STRUCTURE output and implementing the Evanno method. Conservation Genetic Resources 4, 359–361.

Draganescu C and Grosu H 2010. Valachian (Zackel) heritage philetic sheep group – a taxonomic problem. DAGENE. Retrieved May 24, 2012, from http://ebookbrowse.com/draganescu-and-grosu-dagene-2010-pdf-d58690637

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

Ferencakovic M, Curik I, Royo LJ, Pérez-Pardal L, Cubric-Curik V, Fernández I, Álvarez I, Kostelic A, Sprem N, Krapinec K and Goyache F 2013. Mitochondrial DNA and Y-chromosome diversity in East Adriatic sheep. Animal Genetics 44, 184–192.

Food and Agriculture Organization of the United Nations (FAO) 2011. Molecular genetic characterization of animal genetic resources. FAO Animal production and health guidelines. No. 9. Commission on genetic resources for food and agriculture. FAO, Rome, Italy.

Galov A, Byrne K, Gomercic T, Duras L, Arbanasic H, Sindicic M, Mihelic D, Kovacic A and Funk SM 2013. Genetic structure and admixture between the Posavina and Croatian Coldblood in contrast to Lipizzan horse from Croatia. Czech Journal of Animal Science 58, 71–78.

Guo SW and Thompson EA 1992. Performing the exact test of Hardy–Weinberg proportion for multiple alleles. Biometrics 48, 361–372.

Gutierrez JP, Royo LJ, Álvarez I and 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 JJ, San Primitivo F, Yildiz S, Cenesiz M and Bayon Y 2006. Genetic diversity in Turkish sheep. Acta Agriculturae Scandinavica A 56, 1–7.

Ivanković A, Dovč P, Kavar T, Caput P, Mioč B, Pavić V, Štuhec V and Leto J 2005. Genetic characterisation of the Pag island sheep breed based on microsatellite and mtDNA data. Small Ruminant Research 57, 167–174.

Lawson Handley LJ, Byrne K, Santucci F, Townsend S, Taylor M, Bruford MW and Hewitt GM 2007. Genetic structure of European sheep breeds. Heredity 99, 620–631.

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

Lewis PO and Zaykin D 2001. Genetic data analysis: computer program for the analysis of allelic data, version 1.0 (d16c): 45pp. Retrieval January 24, 2012, from http://www.eeb.uconn.edu/people/plewis/software.php

Palian B, Nikolic T and Bagaric D 1960. Results of commercial crossing with sheep under extensive conditions of nutrition. Stocarstvo 14, 426–433.

Pariset L, Savarese MC, Cappuccio I and Valentini A 2003. Use of microsatellites for genetic variation and inbreeding analysis in Sarda sheep flocks of central Italy. Journal of Animal Breeding and Genetics 120, 425–432.

Pavić V, Bradić M, Mioč B, Ivanković A, Barać Z and Safner T 2006. Use of molecular markers in sheep genotyping. In Proceedings of 41st Croatian and 1st International Symposium on Agriculture (ed. S Jovanovac and V Kovačevič), pp. 639–640. Faculty of Agriculture in Osijek, Osijek, Croatia.

Posavi M, Ernoić M, Ozimec R and Poljak F 2003. Enciklopedija hrvatskih domaćih životinja. Katarina Zrinski, Varaždin, Croatia.

Pritchard JK, Steephens M and Donnelly P 2000. Inference of population structure using multilocus genotype data. Genetics 155, 945–959.

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

Salamon D, Gutierrez-Gil B, Kostelic A, Gorjanc G, Kompan D and Dzidic A 2012. Preliminary study on the genetic diversity of the Istrian sheep, Lika and Krk pramenka sheep populations using microsatellite markers. Acta Agriculturae Slovenica 100, 125–129.

Santos-Silva F, Ivo RS, Suosa MCO, Carolino MI, Ginja C and Gama LT 2008. Assessing genetic diversity and differentiation in Portugese coarse-wool sheep breeds with microsatellite markers. Small Ruminant Research 78, 32–40.

Tapio I, Tapio M, Grislis Z, Holm LE, Jeppsson S, Kantanen J, Miceikiene I, Olsaker I, Viinalass H and Eythórsdóttir E 2005. Unfolding of population structure in Baltic sheep breeds using microsatellite analysis. Heredity 2005, 1–9.

Tapio M, Ozerov M, Tapio I, Toro MA, Marzanov N, Ćinkulov M, Goncharenko G, Kiselyova T, Murawski M and Kantanen J 2010. Microsatellite-based genetic diversity and population structure of domestic sheep in northern Eurasia. BMC Genetics 11, 76.