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Study of genetic differences among Slovak Tsigai populations using microsatellite markers

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ABSTRACT: In this study genetic diversity, population structure and genetic relationships of Tsigai populations in Slovakia were investigated using microsatellite markers. Altogether 195 animals from 12 populations were genotyped for 16 microsatellites. 212 alleles were detected on the loci. The number of identified alleles per locus ranged from 11 to 35. In the majority of the populations heterozygosity deficiency and potential risks of inbreeding could be determined. High values of F_{ST} (0.133) across all the loci revealed a substantial degree of population differentiation. The estimation of genetic distance value showed that the Slovak Vojin population was the most different from the other populations. The 12 examined populations were able to group into 4 clusters. With this result our aim is to help the Slovak sheep breeders to establish their own mating system, to avoid genetic loss and to prevent diversity of Tsigai breed in Slovakia.

Keywords: Slovak Tsigai sheep; microsatellite markers; genetic relationship

Tsigai is an old, independent sheep breed, a remnant of the ancient Small-Asian domain of breeds where the majority of the domesticated breeds originate from. Indirectly, the breed descends from the oriental wild sheep called the arkal (*Ovis ammon orientalis*) (Kukovics and Jávor, 2002). Within the sheep species (*Ovis domesticus*) the breed belongs to the mountain sheep group where it creates a separate subgroup as Tsigai sheep breeds.

The ancient Tsigai migrated from its original region to the Balkan Peninsula, through "Olah and Moldva" countries and Transylvania, as far as the northern mountain chains of the Carpathians and reached the Hungarian Plain. The breed reached the northern part of Hungary and Bohemia during the 18th century. Others moved along the eastern coast of the Black Sea (West Caucasus) and found their niche in the Crimea and on the northern coastal area of the Black Sea, by reaching the breeding area of the Tsigai from the Balkan in Southern Ukraine (Schandl, 1955).

Over the last two hundred years, the Tsigai breed, although in fluctuating ratio (1–10%), has composed a constant part of Hungarian sheep stock, but in Slovakia the Tsigai sheep has become the second most important breed. Some 185–200 thousand Tsigai sheep (120 thousand ewes within it) were kept in Slovakia at the beginning of the present decade (Gyarmathy, 2000), but its number significantly decreased in subsequent years. The breed has been kept mainly in the central and eastern parts of Slovakia. After the accession of Slovakia to the EU the number of Tsigai sheep has increased a little.

During the decades between 1950 and 2000, especially over the period from 1980 to 2000, se-

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veral kinds of exotic breeds were used (in various crossing programs) in order to improve the meat and milk production abilities of Tsigai sheep in the Slovak part of Czechoslovakia. As the consequences of these crossing programs different variants of Slovak Tsigai sheep were developed (Gyarmathy, 2000).

In this breed the weight of a mature ewe is 40 to 45 kg with a height at withers of 60–65 cm, while the weight of a ram is 65–75 kg with a height at withers of 70–75 cm (Kukovics and Jávor, 2002). The examined Tsigai variants have darker colour on the face and legs and their lambs have dominantly uniform bright black colour all over the body.

In this study we have used microsatellites because they are a powerful tool for the evaluation of paternity, to maintain pedigree records, for tracking alleles through a population and to estimate genetic variability and inbreeding (Rooney et al., 1999). Microsatellites are excellent genetic markers because of their locus identity, high *PIC* value, widespread distribution in the genome, multi-allelism, co-dominant inheritance and PCR basis. In the last decades microsatellites have been used in many studies for determination of genetic difference and genetic variation among sheep breeds (Farid et al., 2000; Li et al., 2004; Gladyr et al., 2005; Gizaw et al., 2007; Ozerov et al., 2008; Glowatzki-Mullis et al., 2009).

In order to preserve and maintain the Tsigai in Slovakia the first step is to know the genetic relationship among the Tsigai populations or variants. In this study our aim was to start the preservation work of Tsigai sheep in Slovakia.

MATERIAL AND METHODS

Altogether 195 animals belonging to twelve Tsigai populations from different parts of Slovakia were studied. The number of examined individuals per studied populations is documented in Table 3. Hair samples were taken from all individuals by picking up. Sampling of close relatives was avoided.

Genomic DNA was extracted from the hair bulb as previously described (FAO, 2004). Microsatellite markers were selected on the basis of their location in several chromosomes and according to the suggestion of the Food and Agriculture Organisation (FAO) and the International Society for Animal Genetics (ISAG). Forward primers were labelled on their 5' end by one of the fluorescent compounds

FAM, JOE or NED to enable analysis on automated sequencers. The following sixteen microsatellites were studied: *BM6506, OarFCB20, MAF70, MCM527, INRA127, ILSTS11, TGLA53, TGLA357, MAF65, OarCP49, OarAE119, OarCP20, BM1314, MAF35, MCMA7, CSSM43*.

PCR amplifications were carried out in 25 µl reactions with 10–50 ng genomic DNA as template. Reaction mixtures contained 10mM Tris-HCl (pH 8.3), 50mM KCl, 2.5mM MgCl$_2$, 0.5 U *Taq* polymerase, 160nM of the fluorescently labelled forward primer, 200nM from unlabelled reverse primer and 0.2mM of each deoxynucleotide. The initial denaturation step at 94°C for 3 min was applied that was typically followed by 35 cycles with the following steps: 92°C for 1 min, 60°C for 1 min and 72°C for 1 min. The reactions were terminated by the final extension step at 72°C for 7 min. Multiplex PCR reactions using 2 or 3 primer pairs simultaneously were performed whenever it was possible. For multiplex capillary runs 0.5–0.5 µl PCR reactions of FAM, JOE and NED fragments were added to 10 µl formamide containing ROX labelled internal size standards. The mixtures were denatured for 3 min at 85°C, immediately chilled on ice and loaded onto ABI3100 capillary sequencers using 36 cm capillaries filled with POP4 polymer (Applied Biosystems).

Running data were collected by the GeneScan software (Applied Biosystems). Genescan data were imported and converted to pseudogel images by the Genographer software (Benham, 2001). Sized fragments were scored manually and fragment length data were written into spreadsheet tables. The number of alleles per locus and population mean observed (H_{obs}) and mean expected (H_{exp}) heterozygosities were calculated by the Genepop program

Table 1. Population specific alleles

Locus	Allele	Population
OarFCB20	107	Handel
TGLA357	130	Jurbis
INRA127	209	Vancover
BM1314	175	Jurbis
	174	Rybar
OarAE119	156	Sirig
	125	Jurbis
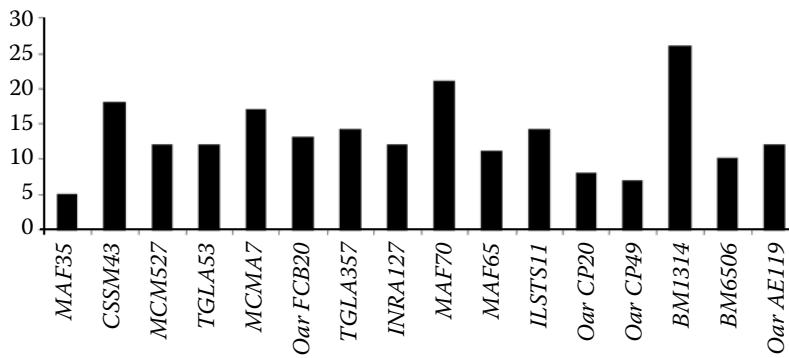
Figure 1. Number of alleles at the examined loci

(http://wbiomed.curtin.edu.au/genepop). Single locus *F*-statistics (Fwc) were estimated according to as in Weir and Cockerham (1984). Population data were evaluated by the Arlequin (Version 2.0; Schneider et al., 2000) and Populations (Version 1.2.28, Langella; 1999) programs. Genetic distances from diploid population data were calculated by the Populations program using Nei's minimum genetic distance formulas (D_A) (Nei, 1987). Phylogenetic tree was constructed using the UPGMA algorithm of the Neighbour program of the Phylip software package (Version 3.57c; Felsenstein, 1995). Graphical rendering of the phylogenetic tree was done by the Drawgram program of the Phylip package.

Table 2. Range of allele sizes, mean number of alleles, observed (H_{obs}) and expected (H_{exp}) heterozygosities and result of *F*-statistic

Locus	Range of allele sizes (bp)	Mean number of alleles per locus	*Hobs*	*Hexp*	*Fis*	*Fst*	*Fit*
MAF35	104–122	3.9	0.503	0.625	0.195	0.101	0.276
CSSM43	237–273	8.5	0.523	0.828	0.368	0.092	0.426
MCM527	150–185	7.0	0.535	0.753	0.289	0.108	0.366
TGLA53	114–143	7.3	0.641	0.754	0.151	0.141	0.271
MCMA7	228–270	6.8	0.659	0.756	0.127	0.144	0.253
OarFCB20	92–118	5.3	0.400	0.658	0.393	0.118	0.465
TGLA357	113–154	7.8	0.662	0.814	0.186	0.076	0.248
INRA127	181–215	5.1	0.534	0.634	0.157	0.129	0.266
MAF70	128–175	7.3	0.369	0.795	0.536	0.146	0.604
MAF65	116–140	5.6	0.338	0.706	0.522	0.142	0.589
ILSTS11	180–296	5.4	0.517	0.720	0.282	0.170	0.404
OarCP20	88–195	5.1	0.636	0.692	0.081	0.175	0.242
OarCP49	88–140	4.0	0.651	0.677	0.038	0.132	0.165
BM1314	136–176	7.4	0.701	0.791	0.114	0.117	0.217
BM6506	184–212	4.5	0.590	0.639	0.076	0.166	0.229
OarAE119	98–160	5.5	0.340	0.697	0.512	0.166	0.593
Mean			0.537	0.721	0.252	0.133	0.351

RESULTS AND DISCUSSION

Microsatellite loci

The total number of alleles was 212 at the 16 studied loci. According to our results all loci were polymorphic with the number of alleles per locus ranging from 5 at *MAF35* to 26 at *BM1314* (Figure 1). Ozerov et al. (2008) detected more alleles at the *OarCP20* locus (10), while less at the *BM1314* (14), *BM6506* (8), *MAF65* (9), *McM527* (10) locus in Kazakh sheep breeds compared to our result in Tsigai populations. Dalvit et al. (2008) detected 17 alleles at *McM527*, 18 alleles at *OarCP20* in Alpine sheep breeds.

The average number of alleles per locus varied between 3.9 (*MAF35*) and 8.5 (*CSSM43*) (Table 2). Allele frequencies are available from the authors on request.

Seven population specific alleles were detected in 12 populations (Table 1). *MAF35*, *CSSM43*, *MCM527*, *TGLA53*, *MCMA7*, *MAF70*, *ILSTS11*, *OarCP20*, *OarCP49*, *BM6506* and *MAF65* loci were without any private allele.

Mean observed and expected heterozygosities are presented in Table 3. For all 16 loci the mean expected heterozygosities were 0.721 while mean observed heterozygosities reached the level of 0.537. In each marker the mean expected heterozygosity was higher than the observed heterozygosity. The mean observed and expected heterozygosities varied from 0.338 (*MAF65*) to 0.701 (*BM1314*) and from 0.625 (*MAF35*) to 0.828 (*CSSM43*).

Genetic structure and differentiation of the populations were examined by Wright's *F*-statistics for each locus. Mean estimated values for F_{IP}, F_{ST} and F_{IS} were 0.351, 0.133 and 0.252, respectively. It means that the deficit of heterozygotes across populations was about 35% and inbreeding within populations was about 25%. The F_{IS} index among loci varied from 0.038 (*OarCP49*) to 0.536 (*MAF70*). Multilocus F_{ST} indicated that around 13.3% of the total genetic variation was explained by a population difference, the remaining 86.7% corresponding to differences among individuals. This value was higher than those from other genetic diversity studies, e.g. 5.7% for Alpine sheep and European and Middle-Eastern breeds (Peter et al., 2007; Dalvit et al., 2008), 4.6% for Ethiopian sheep (Gizaw et al., 2007), 5% for Pramenka types (Cinkulov et al., 2008), 3.7% for Manchega sheep (Calvo et al., 2006) while it was lower than for Indian sheep (18.3%; Mukesh et al., 2006).

Population

The average number of alleles per populations varied between 3.125 (Olymp, Vojin) and 7.937

Table 3. Number of studied animals, observed and expected heterozygosities, *Fis* values in the Slovak populations

Population	Number of animals	Mean number of alleles per population	*Hobs*	*Hexp*	*Fis*
Ondrej	16	7.0	0.574	0.835	0.313
Kamendin	16	6.5	0.552	0.806	0.316
Kamo	19	6.9	0.461	0.751	0.386
Sirig	22	7.9	0.487	0.805	0.395
Vancover	15	4.9	0.513	0.780	0.342
Handel	25	7.7	0.537	0.812	0.339
Jurbis	24	6.9	0.561	0.761	0.263
Jugat	22	7.5	0.582	0.804	0.276
Olymp	5	3.1	0.439	0.843	0.480
Rybar	16	5.3	0.560	0.741	0.244
Brend	10	5.6	0.583	0.781	0.253
Vojin	5	3.1	0.613	0.812	0.245

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Table 4. Genetic distances (below diagonal) among the examined Slovak Tsigai populations

Populations	Ondrej	Kamendin	Kamo	Sirig	Vancover	Handel	Jurbis	Jugat	Olymp	Rybar	Brend	Vojin
Ondrej	0.000											
Kamendin	0.267	0.000										
Kamo	0.326	0.352	0.000									
Sirig	0.409	0.433	0.298	0.000								
Vancover	0.599	0.500	0.919	0.572	0.000							
Handel	0.526	0.475	0.662	0.270	0.287	0.000						
Jurbis	0.512	0.421	0.826	0.508	0.165	0.234	0.000					
Jugat	0.473	0.431	0.638	0.272	0.258	0.140	0.173	0.000				
Olymp	0.520	0.583	0.826	0.462	0.372	0.367	0.261	0.297	0.000			
Rybar	0.850	0.832	0.591	0.254	0.744	0.382	0.725	0.332	0.735	0.000		
Brend	0.744	0.796	0.667	0.317	0.530	0.262	0.597	0.384	0.514	0.241	0.000	
Vojin	1.044	1.051	0.693	0.542	1.334	0.581	1.053	0.623	1.160	0.523	0.520	0.000

(Sirig); however, it was lower compared to the values of European and Spanish sheep breeds (Arranz et al., 1998; Dalvit et al., 2008). Tapio et al. (2005) found similar allelic richness studying European sheep (2.62–6.26). In Romanian Tsigai 7.52 alleles per breed were detected (Peter et al., 2007). Since genetic drift has an impact on genetic differentiation among populations, therefore the least variable populations, breeds are usually the most distinct (Hedrick, 1999), as presented with the Olymp and Vojin populations in our study.

The mean observed and expected heterozygosities per population were between 0.439 (Olymp) and 0.613 (Vojin), and between 0.741 (Rybar) and 0.843 (Olymp), respectively. All examined populations were less heterozygous than it was expected. The values of genetic diversity measurements suggested that the studied populations did not contain a high level of genetic variability.

The F_{IS} value ranged between 0.244 (Rybar) and 0.480 (Olymp). The heterozygosity deficit was the highest in Olymp and the lowest in Rybar among the examined populations (Table 3). However, there was no negative F_{IS} value, which would have indicated an excess of heterozygotes in some populations.

Nei's minimum genetic distance (D_A) values were calculated from microsatellite data on each population pair (Table 4). Genetic distances between the examined Slovak Tsigai populations were large. The values varied between 0.140 (Jugat-Handel pairs) and 1.334 (Vancover-Vojin pairs). The UPGMA tree constructed from D_A is shown in Figure 2. The studied populations clustered into 4 groups on the tree. Vojin was in an individual group and far from the others. However, this fact might be the result of a low number of studied animals. Jugat and Handel were the closest to each other, but they were close to Jurbis, Vancover and Olymp. Brend, Rybar and Sirig were presented in one group. Ondrej, Kamendin and Kamo were in another branch.

CONCLUSION

Genetic difference was studied among 12 Tsigai populations originated from different parts of Slovakia using microsatellite markers. Tsigai populations analysed in this study have never been characterised before, therefore it could be of special interest, but the results cannot be compared with literature. This study did not show high variation
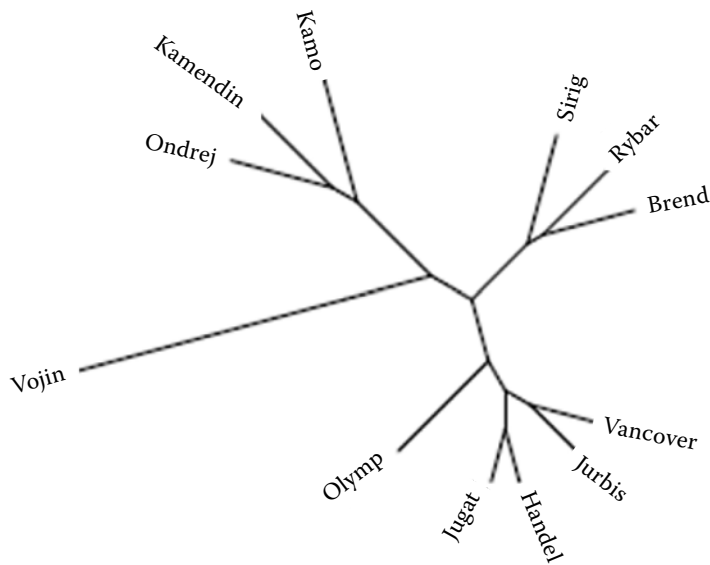
Figure 2. UPGMA tree constructed from D_A distances, showing the relationship among twelve Slovak populations

within and between studied Slovak Tsigai populations. In all populations expected heterozygosity was much higher than observed heterozygosity. It could mean that these populations were developed as a result of different crossbreeding programs and over the time of the program uniformity was also in target of the given program, which resulted in severe losses in the genetic bases. While the original Tsigai population was more or less the same, the use of different exotic breeds resulted in differences in the terminal products. The new Tsigai variants also have black to dark brown hair covering the head and the legs, and there are not any large differences among them in other phenotypic traits, like body measurements, but their genetic bases have become different.

The cooperation among different groups might result in the loss of improvements from previous crossbreeding programs, however, the reconstruction of original Slovak Tsigai sheep could be the imperative duty. In future our plan is to complete the genetic characterisation of the studied populations by mtDNA.

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