

## Summary

### **INVESTIGATION OF THE GENETIC DIVERSITY OF SHEEP BREEDS AND THEIR EVALUATION FOR DESIGNING APPROPRIATE CONSERVATION SCHEMES**

DNA of 31 animals per population, of ten Greek sheep breeds was analysed using 31 polymorphic microsatellite markers. In total, DNA from 310 animals was analysed, in order to investigate the genetic diversity and genetic relationships among these breeds. The breeds under study were Anogeiano, Kalarritiko, Karagouniko, Kefallenias, Kymi, Lesvos, Orino, Piliou, Sfakia and Skopelos.

The number of alleles observed in each population ranged from 3-15 (Anogeiano 4-14; Kalarritiko 5-15; Karagouniko 4-14; Kefallenias 4-13; Kymi 5-13; Lesvos 5-13; Orino 5-15; Piliou 5-12; Sfakia 4-14; Skopelos 3-10). Skopelos showed the lowest values concerning the number of observed alleles (3) and mean number of alleles (6.36). The maximum number of observed alleles was shown in Kalarritiko and Orino (15). This indicates that diversity of alleles was shared by the breeds as a whole.

Gene diversity in each breed over all microsatellite loci ranged as following: Anogeiano 0.428-0.886; Kalarritiko 0.576-0.899; Karagouniko 0.485-0.894; Kefallenias 0.513-0.868; Kymi 0.508-0.893; Lesvos 0.482-0.895; Orino 0.481-0.863; Piliou 0.415-0.873; Sfakia 0.363-0.886 and Skopelos 0.257-0.847. The lowest value of gene diversity (0.257 of OARFCB193 locus) over all populations and over all loci was observed in Skopelos breed, while the highest (0.899 of MAF70 locus) in Kalarritiko, indicating that MAF70 locus showed the greatest variability.

The observed heterozygosity ranged between 0.161 (OARCP38 in Kymi) to 1.000 (OARFCB20 in Kalarritiko). The values in the breeds examined overall loci were: Anogeiano 0.290-0.935; Kalarritiko 0.387-1.000; Karagouniko 0.322-0.903; Kefallenias 0.419-0.903; Kymi 0.161-0.871; Lesvos 0.451-0.935; Orino 0.387-0.903; Piliou 0.354-0.838; Sfakia 0.387-0.903 and Skopelos 0.225-0.838.

Nei's F-statistics were calculated. The range of the values of  $H_o$ ,  $H_s$ ,  $H_t$ ,  $G_{st}$ ,  $G_{st}'$  and  $G_{is}$  were as follows:  $H_o$ : 0.481-0.835;  $H_s$  0.515-0.859;  $H_t$  0.524-0.873;  $G_{st}$  0.007-0.054;  $G_{st}'$  0.007-0.059 and  $G_{is}$  -0.031-0.250. The Nei's coefficient of differentiation  $G_{st}$ , equivalent to  $F_{st}$  of Wright, was generally low for all loci and also when averaged over all breeds and loci (0.031). This may indicate that these breeds are not differentiated enough and they may have common history of breeding practices. Thus, a small part of the variability at the 28 microsatellite loci analyzed was ascribed to the between-breed variability, while most of the variability was due to within breeds, since 0.969 was the variation related to within breeds or populations. Therefore, it can be recommended that within-breed diversity is actively maintained to enable these extensively managed stocks to adapt to future demands and conditions.

Average expected and average observed heterozygosity were estimated averaging over all loci in each breed. Non biased average expected heterozygosity ranged from  $0.68 \pm 0.134$  (in Skopelos) to  $0.76 \pm 0.103$  (in Karagouniko) and averaged 0.74, while the average observed heterozygosity ranged from  $0.626 \pm 0.1320$  (in Skopelos) to  $0.74 \pm 0.135$  (in Kefallenias) and averaged 0.696. It was noted, that heterozygosity estimates found were generally higher than those of goats and cattle and thus, indicating the higher genetic diversity of sheep over those species. However, it was noted that all the average observed heterozygosity were lower than the expected in all populations, which might indicate the presence of heterozygosity deficit to different levels the different breeds except in Kefallenias and Lesvos which showed no deficit.

A t-test was done to test for a significant difference between the different populations according to average expected and observed non biased heterozygosity. It is believed that crossbreeding played an important role in raising heterozygosity estimates in the breeds under study, in addition to a probable dispersion between populations, which can not be excluded. Similarly, the observed genetic uniformity of some populations might be due to elevated gene flow among these subpopulations, a finding corroborated by the high, similar levels of heterozygosity.

Hardy-Weinberg equilibrium was tested in order to evaluate the significance of inbreeding occurred in each locus in each population and overall loci in each population. Coefficient of inbreeding,  $F_{is}$ , was estimated for each locus in each population and overall loci in each population according to Wright.  $F_{is}$  values per microsatellite marker in each population ranged from: -0.207-0.322 in Anogeaino; -0.175-0.399 in Kalarritiko; -0.146-0.599 in Karagouniko; -0.155-0.238 in Kefallenias; -0.017-0.728 in Kymi; -0.094-0.238 in Lesvos; -0.165-0.501 in Orino; -0.195-0.283 in Piliou; -0.125-0.374 in Sfakia; and from -0.102-0.268 in Skopelos. The highest values were found in Karagouniko (0.599 in OARFCB128) and Kymi (0.728 in OARCP38).

In order to evaluate the significance of the  $F_{is}$  values, permutation tests were carried out by permuting the alleles within samples over all loci in each breed using the FSTAT software. The hypothesis of heterozygosity deficit was assumed in the tests. It was found that the estimates of inbreeding coefficient of each population were significant ( $P < 0.05$ ) except in Kefallenias and Lesvos populations ( $F_{is} = 0.003$ ,  $P < 0.435$ ; and  $F_{is} = 0.021$ ,  $P < 0.107$ , respectively). This supports the fact of having some heterozygote deficit in eight of the breeds that were under study, although  $F_{is}$  estimates in some breeds were small, the values were significant ( $P < 0.05$ ) ( $F_{is} = 0.03$  in Anogeiano;  $F_{is} = 0.086$  in Kalarritiko;  $F_{is} = 0.046$  in Karagouniko;  $F_{is} = 0.123$  in Kymi;  $F_{is} = 0.063$  in Orino;  $F_{is} = 0.091$  in Piliou;  $F_{is} = 0.054$  in Sfakia; and  $F_{is} = 0.081$  in Skopelos). The highest inbreeding coefficient was found in Kymi breed, which might be a result of a bottleneck effect, since its population size decreased drastically during the last twenty years from several thousands to recently few hundreds.

Fst estimates ranged from 0.007 between Orino and Karagouniko, to 0.078 between Skopelos and Sfakia. The estimated number of migrants after correction for size, using private allele method averaged 9.78. The gene flow between populations ranged from 2.95 (between Skopelos and Sfakia) to 23.61 (between Anogeiano and Sfakia) with an average of 9.78. The Skopelos breed showed the highest values of pair wise Fst and the lowest values of Nem with other breeds. Fst and gene flow estimates of Skopelos breed indicated that this population has maintained an important genetic isolation from all the other breeds and only limited exchange of breeding animals with all the other breeds occurred.

Reynold's genetic distance, Cavalli-Sforza's chord measure and Nei's standard genetic distance were calculated. Highest estimates were found for the three distances between Skopelos and all the other breeds (Reynold's values ranged from 0.073 to 0.098; Cavalli-Sforza's ranged from 0.045 to 0.057; Nei's ranged from 0.210 to 0.303).

A phylogenetic tree was built based on Reynold's genetic distance. The tree showed three groupings according to the high bootstrapping values. The first group was between Anogeiano and Sfakia with a high bootstrapping value of 998 out of 1000. The second group was Skopelos alone even with a low bootstrap value of 415 (its topology on the tree). The third group included the rest of the breeds (Kefallenias, Orino, Karagouniko, Lesvos, Kymi, Piliou and Kalarritiko) with low bootstrap values (lower than 400 out of 1000, except the clustre containing Piliorritiko and Kalarritiko with a bootsrap value of 443). The close relationships between the seven breeds of the third group cluster could be mainly the result of processes of admixture.

The building of a phylogenetic tree with the use of genetic distances does not take into account the effects of admixtures between the branches of the tree. Alternatively, the representation of the genetic relationships between the different populations can be obtained by the use of multivariate techniques which are able to condense the information from a large number of variables (alleles and loci on the one hand, and the populations on the other hand) into a few synthetic variables. Thus, correspondence analysis was used to furthermore, investigate the differentiation of the breeds. The results showed, three clusters of breeds: Skopelos alone in a group; Anogeiano and Sfakia in another group; and the rest of the breeds (Kefallenias, Orino, Karagouniko, Lesvos, Kymi, Piliou and Kalarritiko). These results indicated the occurring of admixture especially between the breeds-members of the third group.

Combining the objectives of conserving the most of the genetic diversity and uniqueness from the total of breeds that were examined, as well as conserving the breeds that are in danger of extinction, we propose that: Kymi breed has a direct priority for conservation, because of the small population size, below 1000 with tendency of reduction. The Skopelos breed with a population size of 1800 animals can also be considered of having direct priority for conservation. Piliou and Kalarritiko which according to the criteria of European Union are threatened, can be included in the priorities of conservation,

especially as they are characterized by the high number of private alleles and high heterozygosity, but also because these are breeds that are raised in mountainous regions and consequently the maintenance of livestock farming in these regions will contribute in the sustainable management and development of the regions. Anogeiano, with a population size of 4500 animals, can be included for priorities of conservation. Nevertheless, it should be pointed out that by means of all the analyses Anogeiano and Sfakia were separated together and having the maximum values of gene flow. Orino breed showed the highest mean number of alleles and of private alleles, as well as high heterozygosity, which may indicate that this breed and despite of the respectful amount of crossbreeding presented the highest genetics diversity, as well as probable particular characteristics. Consequently, even if it is not in danger immediately, it can be included in a conservation program. Moreover, Kefallenias breed, which showed high heterozygosity and number of private alleles should be maintained as although the total population size of this breed is high (roughly 40000 individuals), only about 2800 are raised in purebred flocks.

This study was mainly focused on breeds raised in marginal agricultural areas in order to assist in situ conservation. The results presented add important information on the puzzle of sheep genetic diversity and conservation in Greece, where it is of crucial economic relevance to increasingly marginalized rural communities.