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PHENOTYPIC TRAITS, DIVERSITY LEVELS AND GENETIC RELATIONSHIPS OF CRETAN SHEEP BREEDS

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ABSTRACT

A comprehensive study of the indigenous Cretan sheep breeds was conducted involving both body measurements and genetic analyses. Body measurements of female sheep (Anogia (n=7); Asterousia (n=23); Sfakia (n=17) and Sitia (n=43)) included withers height, body length, ear length, tail length and girth, and body weight. Results indicated that the Sfakia breed had the highest withers height (62.7 ± 2.4 cm) while the Sitia breed had the lowest (59.1 ± 5.3 cm). In addition, the Sfakia breed showed the greatest body length (65.4 ± 5.5 cm), and the Sitia breed the smallest (60.8 ± 4.3 cm). In terms of body weight, the Sfakia breed was the heaviest (38 ± 4.2 kg) and the Anogia breed the lightest (21 ± 2.5 kg). For genetic identification, blood was sampled from 25 Anogia, 27 Asterousia, 31 Sfakia, 12 Sitia, 58 Kasos Island and 15 Karpathos Island sheep individuals. Genotypes of over 45,000 Single Nucleotide Polymorphisms (SNPs) were obtained for each breed, using the OvineSNP50 BeadChip (Illumina). Observed and expected heterozygosity, allelic richness, and the inbreeding coefficient were calculated. The highest observed heterozygosity based on SNP haplotype blocks was in the Anogia and Sitia breeds (0.78), while Anogia also had the highest expected heterozygosity (0.81) and allelic richness (7.53). The highest genomic inbreeding coefficient (0.175) was estimated in the Karpathos population and the lowest (0.062) in the Anogia breed. Genetic relatedness and distances among the breeds/populations were analyzed and depicted in phylogenetic trees, revealing significant insights into the genetic diversity and relationships of these sheep populations.

Keywords: *indigenous sheep, body measurements, genetic diversity, genetic distances.*

INTRODUCTION

Sheep and goats were among the first animals domesticated by humans, spreading from centers of domestication to wide regions. Sheep were introduced to Greece between 6,500 and 6,000 BC (Jarman, 1968). Sheep farming plays a pivotal role in the Greek economy, ranking third in Europe, following Spain, and Romania. It accounts for approximately 18% of the Greece's total agricultural income (Eurostat, 2022). According to ELSTAT (2022), Greece has a total sheep population of 7,378,357 animals. In particular, the Island of Crete, with a sheep population of 2,036,846 as reported by ELSTAT (2022), has the highest percentage of sheep among all regions in Greece, accounting for 27.6% of the total sheep population. Dairy sheep farming, significantly contributes to Europe's overall milk production. According to the latest data from ELSTAT (2022), Greek sheep's milk production in 2022 totaled 883,231 tons, accounting for approximately 29.4% of Europe's total production of 3,000,000 tons. Specifically, sheep's milk production in the Island of Crete reached 152,088 tons, representing about 5.1% of the overall European production. Greek soil and climate are ideal for sheep farming, utilizing less fertile areas like mountainous and semi-mountainous regions. Moreover, sheep farming plays a crucial role in the prevention of rural desertification and the maintenance of environmental balance (Scott & Robertson, 2008). The diverse indigenous Greek sheep breeds reflect a long history of domestication and evolution, showing unique traits due to natural and zootechnical selection pressures. These breeds developed characteristics such as decease resistance, adaptability to harsh conditions, and efficiency on poor vegetation (Bizelis and Koutsouli, 2021). Consequently, the genetic pools of these breeds contain valuable genes that contribute to advancements in genetic science and ensure the adaptability of sheep farming to future needs and preferences. Enhancing our understanding of the genetic diversity among and within local breeds is crucial. This knowledge is essential for maximizing their effective utilization in sustainable animal agriculture, especially in demanding and less intensive settings. It is also essential for successfully implementing conservation programs (Groeneveld et al., 2010). This study aims to comprehensively define the genetic landscape of indigenous Greek Cretan sheep breeds and contribute to their protection and conservation.

MATERIALS AND METHODS

Breeds and samples. 90 female sheep, representing four local Cretan breeds, were selected for the purpose of conducting body measurements. These included Asterousia (n=23), Anogia (n=7), Sfakia (n=17) and Sitia (n=43). In the Sitia breed, samples were obtained from two distinct flocks, 4 individuals were sourced from the Sitia region in Crete, while the remaining 39 were derived from a flock on Kasos Island. The body measurements included withers height, body length, body weight, ear length, tail length and girth. For the genetic analyses 168 animals were selected, representing the four indigenous breeds (Asterousia (n=27), Anogia (n=25), Sfakia (n=31) and Sitia (n=12)), plus the Kasos population (n=58); an

indigenous sheep population in Kasos Island that is considered to have originated from the Sitia breed, due to their similar phenotypic characteristics and reports of frequent population exchanges in the past, and Karpathos population (n=15), from Karpathos Island near Kasos (Figure 1).

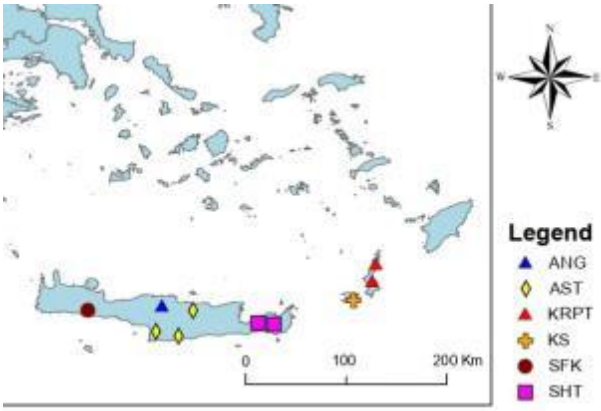

Figure 1. Map of sampling of the studied indigenous sheep breeds (ANG=Anogia, AST=Asterousia, KRPT=Karpathos, KS=Kasos, SFK=Sfakia, SHT=Sitia).

DNA Extraction, SNP Genotyping and Quality Control. Genetic material (DNA) was isolated from animal blood using a commercially available standard reagent kit (QIAamp DNA MiniKit, QIAGEN) in accordance with the manufacturer's instructions. The OvineSNP50 BeadChip from Illumina was employed for genotyping of single nucleotide polymorphisms (SNPs) in accordance with standard procedures as defined in the company protocol (http://www.illumina.com). The quality control of molecular data entails the removal of individuals and genetic markers with low information. In order to obtain high-quality data, a series of controls were applied as follows: (a) Only those individuals with a call rate exceeding 0.95 were included, (b) SNPs that mapped to unknown or sex chromosomes were removed from further analysis, (c) SNPs that were genotyped for less than 90% in all samples were excluded, (d) SNPs with a MAF (Minor Allele Frequency) lower than 0.02 were removed and (e) SNPs that deviated from Hardy-Weinberg equilibrium within the breed ($P \leq 0.01$) were removed. Finally, a database comprising 45,662 marker SNPs was generated. Genotyping with the OvineSNP50 BeadChip kit creates a bias in favour of artificially selected/improved sheep breeds at the expense of many local breeds. The bias is due to the construction of the kit, in the creation of which improved breeds from central and northern Europe were involved. To mitigate this 'bias', small haplotypes of four marker SNPs were used as multi-allelic blocks in many analyses. This approach has been utilized in other studies conducted on other animal species, such as goats and cattle (Pogorevc et al, 2021; Papachristou et al, 2020). Thus, 4,331 multi-allelic blocks were generated.

Genetic Diversity. The estimation of genetic diversity at the breed/population level was based on the multi-allelic blocks and the following parameters were calculated: the total number of alleles (n_A), the observed (H_o) and expected (H_e) heterozygosity (Nei, 1978), number of private alleles (np_A) and the allelic richness (AR) (El Mousadik & Petit, 1996). The calculation of genetic diversity estimators was performed using statistical packages in the *R* programming language. In particular, the estimators n_A, H_o, H_e, AR were calculated using "*HIERFSTAT*" (Goudet, 2005) and np_A using "*POPPR*" version 2.9.6 (Kamvar et al, 2015).

Genetic Relationships and Inbreeding Coefficient. Genetic relationships were calculated using the Unified Additive relationship (*UAR*) Matrix (Yang et al, 2010). The *UAR* was constructed using the *snpReady* package (Granato et al, 2018) in *R*, applied to genotype data comprising of 45,662 SNPs from 168 individuals. To calculate Runs of Homozygosity (ROH), a window-free approach for consecutive SNP-based detection was used, implemented in the *R* package "*detectRUNS*" (Biscarini et al, 2018). In this method, one SNP with a missing genotype and up to one heterozygous genotype was allowed within a run. The minimum ROH length was set at 1000 kb. ROH were determined for each animal and subsequently categorized into specific length classes: 1–2 Mb, 2–8 Mb, and >8 Mb. The total number of identified ROH within these length categories was calculated for every individual in each breed. To determine the mean sum of ROH, the lengths of all ROH for every individual in the sheep populations were summed, and these totals were then averaged for each breed or population. The genomic inbreeding coefficient based on ROH (F_{ROH}) was estimated by dividing the total length of all ROH per individual by the total autosomal SNP coverage, which is 2.44 Gb. The proportion of shared alleles (PS) among individuals of all indigenous populations was calculated using the *R* package "*Adegenet*" (Jombart, 2011). The table of genetic distances DPS was then constructed from the formula: DPS= -log (PS) based on the multi-allelic blocks. Multidimensional scaling (MDS) analysis was calculated using the DPS table. The results were visualized using the "*ggplot2*" package in *R* (Wickham, 2009). The Nei-Da distances (Nei et al, 1983) were calculated based on the multi-allelic blocks using "*HIERFSTAT*" in *R* package (Goudet, 2005) and then the Neighbor-Net graph was constructed using *SplitsTree* 4.14.5 software (Huson and Bryant, 2006).

RESULTS AND DISCUSSION

Body measurements. The body measurements of the female Cretan sheep breeds (Table 1) indicate that the Sfakia breed is the largest among the Cretan breeds, while the Sitia breed is the smallest, except for body weight, where the Anogia breed is the smallest. This variation could be attributed to the better vegetation in West Crete, where the Sfakia sheep are bred, in contrast to the poor vegetation and pasture in Central and East Crete, where the other three breeds are raised. Specifically, the Sitia sheep are reared in the easternmost region of Crete, which justifies the small size of the animals. This difference in vegetation is significant

due to the extensive productive system used for indigenous sheep in Crete. Tail length measurements were taken on animals whose tails were not cut. All four breeds exhibit thin, long tails (Table 1) and a small size, which is consistent with their classification as mountain-type sheep.

Table 1. Mean and standard deviation of body weight (kg), withers height (cm), body length (cm), ear length (cm), tail girth (cm) and tail length (cm) of the female Cretan sheep breeds

Breeds	Code	Phenotypic Traits					
		Body weight	Withers height	Body length	Ear length	Tail girth	Tail length
Anogia	ANG	21.0± 2.5	62.3± 3.1	63.5± 3.4	7.3± 1.8	7.8± 0.3	21.3± 12.0
Asterousia	AST	29.0± 5.8	60.6± 4.8	63.3± 5.4	9.3± 1.9	10.5± 4.5	19.3± 8.5
Sfakia	SFK	38.0± 4.2	62.7± 2.4	65.4± 5.5	9.0 ± 2.9	18.8± 6.2	18.8± 6.2
Sitia	SHT	26.2± 7.6	59.5± 5.5	60.8± 4.9	7.7 ± 2.0	9.2± 4.6	20.1± 10.4

Genetic Analysis. Table 2 shows the genetic diversity parameters of the indigenous Cretan sheep breeds in comparison with the sheep populations from Kasos and Karpathos Islands. Among them, the Anogia and Sitia breed have the highest observed heterozygosity (H_o), expected heterozygosity (H_e) and allelic richness (AR). In addition, the Anogia breed has the lowest genomic inbreeding coefficient (F_{ROH}), indicating minimal inbreeding within the breed.

Table 2. Estimates of genetic diversity, total number of alleles (n_A), number of private alleles (np_A), observed heterozygosity (H_o) and expected heterozygosity (H_e), allelic richness (AR), mean of genomic inbreeding coefficient (F_{ROH}) for the classes 1-2Mb, 2-8 Mb, >8Mb and for all the length of the genome (>1)

Breeds/ populations	n_A	np_A	H_o	H_e	AR	F_{ROH} (>1)	F_{ROH} (1-2)	F_{ROH} (2-8)	F_{ROH} (>8)
Anogia	39,818	1,881	0.78	0.81	7.53	0.062	0.033	0.012	0.016
Asterousia	36,465	1,124	0.74	0.78	6.88	0.115	0.032	0.022	0.061
Karpathos	29,224	526	0.69	0.77	6.37	0.175	0.032	0.040	0.102
Kasos	39,616	1,624	0.77	0.78	6.76	0.080	0.038	0.023	0.020
Sfakia	37,795	1,384	0.76	0.78	6.90	0.087	0.039	0.021	0.027
Sitia	29,212	541	0.78	0.77	6.74	0.069	0.031	0.010	0.028

This low $F_{ROH>1}$ value supports the high H_o and H_e values, suggesting that the Anogia breed has greater genetic diversity, contrasted with the others breeds. Conversely, the Karpathos population exhibits the lowest observed heterozygosity (H_o) and the highest genomic inbreeding coefficient (F_{ROH}), indicating significant inbreeding and reduced genetic diversity relative to the other breeds. Despite having, a higher expected heterozygosity (H_e), the Karpathos population shows the lowest observed heterozygosity (H_o) and allelic richness (AR). This discrepancy suggests a fragmentation of the Karpathos population, or a fragmentation of the sample used here, i.e. the population is divided into some highly inbred herds with low exchange rates between them. To summarize the actual observed genetic

diversity and allelic richness in the entire Karpathos population are limited. This is also supported by Figure 2 that illustrates the distribution of ROH segments across different length classes in the sheep breeds. Short ROH segments (1-2 Mb) were the most frequent across all studied breeds, with frequencies ranging from 61.67% in the Karpathos population to 85.86% in the Anogia breed.

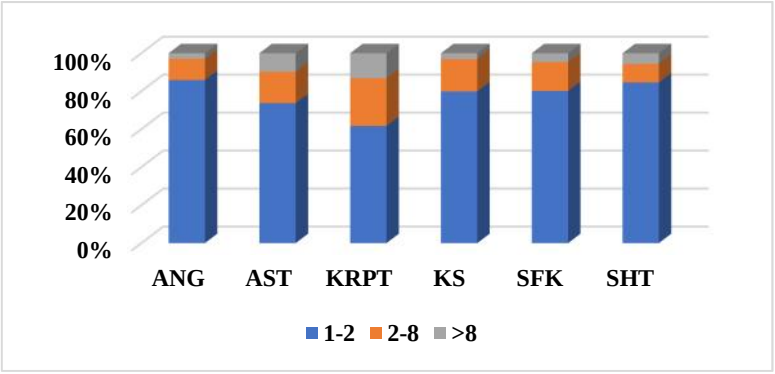

Figure 2. Distribution of ROH segments across different length classes (1–2 Mb, 2–8 Mb, >8 Mb) in indigenous Cretan sheep breeds, and Kasos and Karpathos population (ANG=Anogia, AST=Asterousia, KRPT=Karpathos, KS=Kasos, SFK=Sfakia, SHT=Sitia).

The Sitia breed exhibits the fewest ROH segments in the 2–8 Mb length class (9.79%), whereas the Karpathos population has the highest frequency (25.17%). The longest ROH segments (>8 Mb) were the least frequent, with proportions ranging from 2.99% in the Anogia breed to 13.16% in the Karpathos population. The Anogia breed has the highest frequency of short ROH segments and the lowest frequency of the longest segments, indicating a relatively high level of genetic diversity and minimal recent inbreeding, while the Karpathos breed has the highest frequency of long ROH segments and the lowest frequency of short segments, indicating significant recent inbreeding and reduced genetic diversity. The Sitia breed has the fewest ROH segments in the 2-8 Mb category, which may indicate a moderate level of inbreeding compared to other breeds.

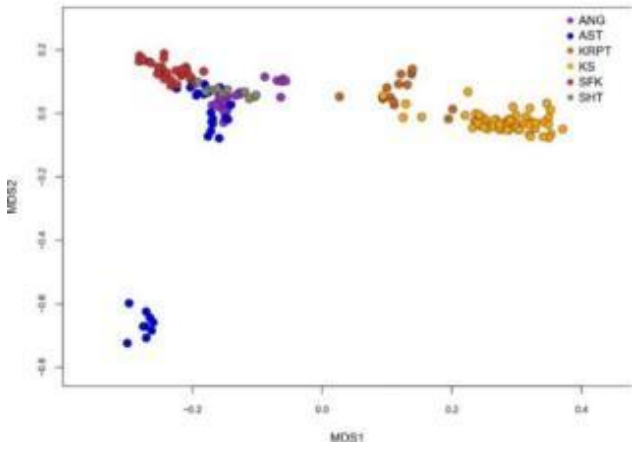

Figure 3. Multidimensional Scaling (MDS) of the estimated allele sharing distance matrix (DPS) at the individual level for the 6 studied breeds/populations (ANG=Anogia, AST=Asterousia, KRPT=Karpathos, KS=Kasos, SFK=Sfakia, SHT=Sitia).

The results of the Multidimensional Scaling (MDS) analysis in MDS1 axis, depicted in Figure 3, indicate that the six breeds form two distinct clusters. The Kasos and Karpathos populations are grouped together, forming one cluster, while the Cretan breeds, which include Anogia, Asterousia, Sfakia, and Sitia, form another distinct cluster. This pattern can be explained because the Karpathos population was mainly influenced by the Kasos population. In contrast, the Anogia, Asterousia, Sfakia and Sitia breeds, form their own cluster, and their genetic similarity reflects their geographic proximity. Additionally, the analysis reveals a significant clustering (MDS2 axis) of animals from a specific breeder within the Asterousia breed. These animals exhibit high levels of genetic affinity, as evidenced by their substantial genetic distance from other breeds in the Unified Additive Relationship (*UAR*) matrix. This pronounced genetic distance suggests that the breeder's animals are not crossbred with external sources but rather are exclusively inbred within the farm, maintaining a closed breeding system. Figure 4 presents a visual representation consistent with the MDS analysis, illustrating the formation of two distinct clusters. One cluster includes the Karpathos and Kasos populations, while the other includes the indigenous Cretan breeds: Anogia, Asterousia, Sfakia and Sitia.

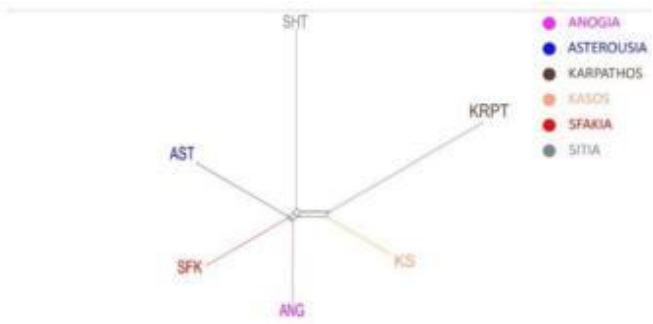

Figure 4. Neighbor-network based on pairwise Nei's D_A genetic distances among the 6 breeds (ANG=Anogia, AST=Asterousia, KRPT=Karpathos, KS=Kasos, SFK=Sfakia, SHT=Sitia).

CONCLUSION

Our results provide first evidence for genetic diversity and inbreeding status of indigenous sheep breeds of Crete. The above findings will be useful to plan conservation programs for these breeds.

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REFERENCES

Biscarini, F., Paolo Cozzi, P., Gaspa, G., & Marras, G. (2018). https://CRAN.Rproject.org/package=detectRUNS. *detectRUNS*: detect runs of homozygosity and runs of heterozygosity in diploid genomes. *R* package version 0.9.5.

Bizelis I. & Koutsouli P., 2021. Greek sheep breeds in "Greek Domestic Sheep Breeds, A Hidden Treasure" (1st ed.). AMALTHIA -Network for the protection of Greek indigenous farm animals. Athens, Greece

El Mousadik A, Petit RJ. (1996). High level of genetic differentiation for allelic richness among populations of the argan tree [Argania spinosa (L.) Skeels] endemic to Morocco. Theor Appl Genet.; 92: 832–839. doi: 10.1007/BF00221895 PMID: 24166548

ELSTAT (2022). Hellenic Statistical Authority: Agriculture, Livestock, Livestock farming. [online]: <https://www.statistics.gr/el/statistics/agr> [Accessed 18 June 2023]

EUROSTAT (2022). European Commission: Agriculture, forestry and fisheries. Number of Sheep. [online]: <https://ec.europa.eu/eurostat> [Accessed 18 June 2023]

Goudet, J. (2005), *hierfstat*, a package for *r* to compute and test hierarchical *F-statistics*. Molecular Ecology Notes, 5: 184-186. https://doi.org/10.1111/j.1471-8286.2004.00828.x

Granato, I.S.C., Galli, G., de Oliveira Couto, E.G. et al. *snpReady*: a tool to assist breeders in genomic analysis. Mol Breeding 38, 102(2018). https://doi.org/10.1007/s11032-018-0844-8

Groeneveld LF, Lenstra JA, Eding H, Toro MA, Scherf B, Pilling D, et al. (2010). Genetic diversity in farm animals—a review. Animal Genetics. 41:6–31.

Huson, D. H., and Bryant, D. (2006). Application of phylogenetic networks in evolutionary studies. Mol. Biol. Evol. 23, 254–267.

Jarman, M. R., and Jarman, H. N. (1968). Ann. Br. Schoo/ Arch. Athens, vol. 63, pp. 241-246

Jombart T. Ahmed I. (2011) *adegenet 1.3-1*: new tools for the analysis of genome-wide SNP data. Bioinformatics. 27:3070–1.

Kamvar ZN, Brooks JC & Grünwald NJ (2015). "Novel R tools for analysis of genome-wide population genetic data with emphasis on clonality." Front. Genet., 6,
208. doi:10.3389/fgene.2015.00208, https://doi.org/10.3389/fgene.2015.00208.

Nei M, Tajima F, Tateno Y. 1983. Accuracy of estimated phylogenetic trees from molecular data. J Mol Evol 19: 153-170.

Nei, M. (1987) Molecular Evolutionary Genetics. Columbia University Press, New York.

Papachristou, D., Koutsouli, P., Laliotis, G.P. et al (2020). Genomic diversity and population structure of the indigenous Greek and Cypriot cattle populations. Genet Sel Evol 52, 43. https://doi.org/10.1186/s12711-020-00560-8.

Pogorevc, N., Simčič, M., Khayatzadeh, N. *et al.* (2021). Post-genotyping optimization of dataset formation could affect genetic diversity parameters: an example of analyses with alpine goat breeds. *BMC Genomics* 22, 546 https://doi.org/10.1186/s12864-021-07802-z

Scott J.T. & Robertson F.A., (2008). Environmental effects of sheep farming. Research Gate, Environmental impacts of pasture-based farming, pp.144-186.

Wickham, H. (2009). *ggplot2*: Elegant Graphics for Data Analysis (New York: Springer-Verlag).

Yang, Jian et al. (2010). Common SNPs explain a large proportion of the heritability for human height. Nature Genetics 42. pp. 565-569.