AGRICULTURAL UNIVERSITY PLOVDIV
DEPARTMENT OF LIVESTOCK SCIENCES

YANKA DENEVA IVANOVA-MIHAILOVA

MOLECULAR MARKERS FOR GENOTYPING AND EVALUATION OF GENETIC RESOURCES FROM LOCAL SHEEP BREEDS IN BULGARIA

ABSTRACT

OF A DISSERTATION FOR AWARDING AN EDUCATIONAL AND SCIENTIFIC DEGREE "DOCTOR"

SCIENTIFIC SPECIALTY: "BREEDING OF AGRICULTURAL ANIMALS, BIOLOGY AND BIOTECHNICS OF REPRODUCTION"

SCIENTIFIC SUPERVISORS

PROFESSOR PHD VASIL NIKOLOV
PROFESSOR PHD ELENA TODOROVSKA

PLOVDIV

2022

The dissertation is written on 187 pages, of which 33 pages are literature review, 1 page Objectives and Tasks, 11 pages “Material and Methods”, 79 pages “Results and Discussion”, 3 pages “Conclusion”, 3 pages “Conclusions”, 2 pages “Recommendations and Contributions”, 30 pages “References” and 14 "Applications" pages. 296 sources are cited, of which 22 in Cyrillic and 274 in Latin. The material is illustrated with - 29 figures and 22 tables.

The work was discussed at a meeting of the Extended Department Council at the Department of Animal Sciences of the Faculty of Agriculture of the Agricultural University - Plovdiv, № ....., and aimed at protection.

The defense of the dissertation will take place on …… ..year from …… hours in …… of the Agricultural University - Plovdiv.

At a meeting of the Specialized Scientific Jury, appointed by the Rector of the Agricultural University - Plovdiv with Order №RD- …….

Reviewers:

Opinions from:

The materials on the defense are available on the website of the Agricultural University - Plovdiv, www.au-plovdiv.bg and in the library of the Agricultural University - Plovdiv, Plovdiv, 12 Mendeleev Blvd.

Note: The numbering of the tables and figures used in the abstract does not correspond to that in the dissertation.

INTRODUCTION

Bulgarian native breeds are the basis of sheep breeding in Bulgaria. They are of great importance both in the breed-forming process and as an element of genetic diversity in farm animals. These are breeds with high resilience, adaptability to local conditions, and with important historical and cultural significance for our country.

Systematic molecular studies covering a larger number of breeds in Bulgaria have not been performed. Separate research in this direction is a prerequisite for conducting a more in-depth study of local breeds at the genomic level, which is in line with European (EAAR) and global (FAO) directives for the conservation of global genetic diversity. The study will not only give a clearer picture of the genetic diversity of local breeds, but will also allow the establishment of their genetic identity.

1. PURPOSE AND TASKS

Objective: Application of microsatellite markers for assesment of the population structure and characterisation of the genetic diversity of local Bulgarian sheep breeds.

For the achievement of this goal the following tasks were performed:

1. Study of the current state of the controlled populations of the local autochthonous sheep breeds.
2. Optimisation of the conditions for conducting multiplex PCR amplification of microsatellite loci in sheep.
3. Genotyping of local sheep breeds with microsatellite markers from the recommended list of ISAG / FAO.
4. Study of polymorphism in microsatellite loci, genetic diversity, level of inbreeding and variation in the studied autochthonous sheep population.
5. Study of polymorphism in microsatellite loci, genetic diversity, level of inbreeding and variation in the studied breeds.
6. Determining the genetic distances between the local autochthonous sheep breeds.
7. Study of genetic diversity and the level of inbreeding in the studied herds.
8. Determining the genetic distance between herds.
9. Determining the genetic structure of breeds.
10. Determining the phenotypic distances between breeds, and the relationship of phenotypic traits with the studied genetic markers.

2. MATERIAL AND METHODS

To analyze the trends and the current state of the populations of local sheep breeds, the dynamics of the controlled populations for the 13-year period - 2009-2021 was studied. Public data from the reports of the breeding

organizations, analyzes and reports of the EAFRD, annual reports of the Ministry of Agriculture, the information systems EFABIS and DAD-IS, etc. were used.

Based on the analysis of the current state and trends of change, degree of research, geographical principle and degree of threat, 12 local indigenous breeds were selected for genotyping: Local Stara Zagora / SZ /; Central stara planina / SSP /; Duben / DAB Central rodope / SR /; Teteven / TET /; Koprivshtitsa / KOPR /; Karakachan / KARA /; Local Karnobat / MK /; Replyan / Rep /; Sakar / SAK /; Breznik / BREZ /; Kotel / KOT /.

A total of 600 animals from 50 herds were genotyped.

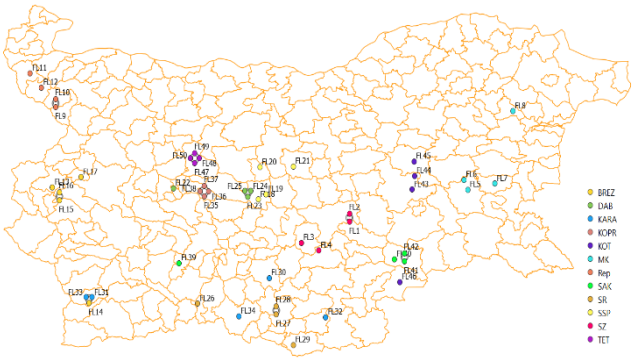


Figure 2. Geographical location of the herds of the studied breeds.

On Fig. 2 is shown the geographical location of the herds.

Genotyping was performed with 15 microsatellite markers (D5S2, INRA5, MAF65, OarAE129, OarFCB11, INRA23, OarFCB20, McM527, CSRD247, HSC, MAF214, OarCP49, INRA63), recommended by the Food and Agriculture Organization (FAO) and International Society for Animal Genetics (ISAG)), based on their level of allelic diversity, according to the recommendations for use for genotyping and paternity tests. In order to provide the widest possible range, markers were selected to cover 20 of the 54 chromosomes (Ovis aries, 2n = 54), resulting in genomic coverage of about 37% of the total chromosomes.

All PCR reactions were performed in a volume of 20 µl containing 20 ng DNA, 10 µl 2x MyTaq HS mix (Bioline), 10 pmol of each primer (F and R) and type I ultrapure water to the final volume of the reaction mixture.

For the amplification of the 15 loci, 4 multiplexes were used as follows:

Multiplex A: D5S2 (FAM), INRA5 (VIC), MAF65 (PET);

Multiplex B: OarAE129 (PET), OarFCB0304 (VIC), OarFCB11 (FAM);

Multiplex C: INRA23 (PET), OarFCB20 (FAM), SPS113 (FAM), McM527 (VIC);

Multiplex D: CSRD247 (VIC), HSC (FAM), MAF214 (PET), OarCP49 (VIC).

The INRA63 locus was amplified on its own using a FAM F-labeled primer.

The analysis of the amplified DNA fragments corresponding to different microsatellite loci was performed on a capillary sequencer ABI 3130 Genetic Analyzer (Applied Biosystem, USA). LIZ 500 (Applied Biosystem, USA) was used as an internal standard to determine the length of amplified microsatellite alleles. Electropherograms were analyzed with GeneMapper ™ v4.0 software.

Determination of allelic frequencies, expected (He) and observed heterozygosity (Ho), Nei genetic distance between breeds, identification of rare alleles, fixation indices (Fis, Fit and Fst), genetic flux (Nm), molecular variance analysis AMOVA) as well as Principle Coordinate Analysis (PCoA) were performed using GenAlEx v 6.50 software.

The polymorphic information content (PIC) for each SSR markerwas determined using PowerMarker v 3.25 software.

The Structure v 2.3.4 software was used to analyze the genetic structure, where a population mixing model (Admixture) was chosen for the pedigree.

The FAMD 1.31 program was used for cluster analysis based on phenotypic data.

The Mantel test using GenAlEx v 6.50 was applied to analyze the correlation between the genetic distance data of the studied breeds and the distance calculated on the basis of phenotypic data.

3. RESULTS AND DISCUSSION

3.1. CURRENT STATE OF CONTROLLED POPULATIONS OF LOCAL INDIGENOUS SHEEP BREEDS

The main goal of the analysis of the current state of the local autochthonous sheep breeds is the selection of breeds for genotyping.

The analysis shows that in 2021, ten breeding organizations in Bulgaria carried out breeding activities with 119,586 sheep of 17 indigenous breeds.

From 17 breeds presented in table 3.1., 14 continued to be endangered, according to the current methodology in our country, which determines the thresholds of endangerment of breeds by species, and for sheep the threshold is 11,000 female animals under the control of breeding organizations.

From table 3.1., in which the data for the controlled populations of the autochthonous breeds by categories are presented, it is evident that three breeds - Duben, Karakachan and Cooper red shumen are above the threshold of endangerment. Others, such as the Central Stara Planina, the Central Rhodope and the Kotel, are close to endangering.

Table 3.1. Distribution of animals from the controlled populations of the local autochthonous breeds by categories, for 2021 and their number.

№	Breed	Flocks	Total animals	Male	female	
					Total	including mothers
1	Local Stara Zagora sheep	13	888	21	867	865
2	White Maritza sheep	12	836	26	810	807
3	Patch faced Maritza sheep	92	7721	298	7423	7392
4	Central stara planina sheep	67	11268	319	10949	10899
5	Duben sheep	73	15266	436	14830	14791
6	Central rhodope sheep	44	9389	234	9155	9144
7	Teteven breed	34	4564	153	4411	4399
8	Koprivshtitsa sheep	35	5063	120	4943	4941
9	Karakachan sheep	101	16614	480	16134	15977
10	West stara planina sheep	41	4656	166	4490	4459
11	Replyan sheep	30	4964	112	4852	4852
12	Sakar sheep	15	2467	99	2368	2347
13	Sofia sheep	75	6911	264	6647	6591
14	Breznik sheep	11	2365	92	2273	2273
15	Cooper Red Shumen sheep	102	15764	345	15419	15292
16	Kotel sheep	50	9238	217	9021	8979
17	Local karnobat sheep	8	1612	56	1556	1552
	Total	**910**	**138 895**	**3 907**	**134 988**	**134 204**

3.2. OPTIMIZATION OF THE CONDITIONS FOR CONDUCTING MULTIPLEX PCR AMPLIFICATION OF MICROSATELITE LOCUSES IN SHEEP

To optimize PCR conditions, all primer pairs were initially amplified individually and analyzed on a capillary sequencer. The optimal hybridization temperatures for each primer pair were determined and based on the obtained results, including hybridization temperature, size of the obtained fragments and the type of fluorescent dye, 4 groups of primer pairs for multiplex reactions were determined, including:

Multiplex A: D5S2 (FAM), INRA5 (VIC), MAF65 (PET);

Multiplex B: OarAE129 (PET), OarFCB0304 (VIC), OarFCB11 (FAM);

Multiplex C: INRA23 (PET), OarFCB20 (FAM), SPS113 (FAM), McM527 (VIC);

Multiplex D: CSRD247 (VIC), HSC (FAM), MAF214 (PET), OarCP49 (VIC)

The temperature programme for multiplexes B and D includes: 95 ^{o}C in 12 min, 31 cycles of 95 ^{o}C in 20 sec, 63 ^{o}C in 1 min, 72 ^{o}C in 1 min, final extension 72 ^{o}C in 5 min. The temperature programme for multiplexes A and C includes: 95 ^{o}C for 10 min, 31 cycles of 95 ^{o}C for 30 sec, 55 ^{o}C for 30 sec, 72 ^{o}C for 1 min, final extension 72 ^{o}C for 5 min. INRA 63 marker was analysed

independently using the following temperature programme: 95 ^{o}C for 12 min, 31 cycles of 95 ^{o}C for 20 sec, 58 ^{o}C for 1 min, 72 ^{o}C for 1 min, final extension 72 ^{o}C for 5 min.

Subsequent analyses of a larger number of animals from the analysed populations confirmed the good repeatability of the results and the possibility for successful application of the developed multiplexes for analysis of the genetic diversity and structure of the studied populations. Exceptions were markers OarFCB0304 and SPS0113, which showed unsatisfactory results in terms of the quality of the profile of PCR fragments or the lengths of the alleles detected in the analysis of a large number of samples, and therefore were not included in subsequent analyzes.

3.3. POLYMORPHISM IN MICROSATILITE LOCUSES, GENETIC DIVERSITY, INBREEDING AND VARIATION IN THE SHEEP POPULATION STUDY

Based on the fragment analysis of the amplified PCR products in 600 sheep of 12 indigenous breeds, polymorphism was found in all 13 autosomal microsatellite loci (Table 3.3.1). A total of 228 alleles were identified, ranging in number (Na) from 8 at the D5S2 locus to 32 at the OarCP49 locus with an average of 17.54 alleles / locus.

Table 3.3.1. Allele range, total (Na) and effective number of alleles (Ne), average number of alleles (Mn), heterozygosity - observed (Ho) and expected (He), polymorphic information content (PIC).

Locus	Allele range	Na	Mn	Ne	Ho	He	PIC
D5S2	188-202	8	6.083	3.410	0.664	0.700	0.689
INRA5	116-152	15	10.333	6.148	0.745	0.818	0.855
MAF65	121-147	13	7.750	3.980	0.744	0.744	0.737
OarAE129	139-319	9	5.000	2.819	0.516	0.636	0.618
OarFCB11	118-146	15	8.917	5.518	0.805	0.811	0.827
INRA23	196-224	15	10.583	6.429	0.815	0.822	0.858
OarFCB20	88-118	16	11.000	6.611	0.824	0.841	0.858
McM527	161-248	12	8.167	5.170	0.802	0.798	0.808
CSRD247	209-265	24	11.500	5.586	0.807	0.819	0.837
HSC	181-303	20	11.667	6.531	0.771	0.842	0.870
MAF214	165-275	24	11.167	3.979	0.722	0.714	0.732
OarCP49	72-140	32	15.333	6.438	0.837	0.833	0.864
INRA63	157-213	25	12.417	5.632	0.810	0.809	0.832
Mean		**17,54**	**9.994**	**5.250**	**0.759**	**0.784**	**0.799**
Total		**228**					

The average number of alleles in the locus (Mn) varies from 5 in the OarAE129 locus to 15.333 in the OarCP49, and in our study the average number of alleles in the 13 loci was 9.994.

The average effective number of alleles (Ne) obtained in our study is 5.25. The effective number of alleles / loci is considered to be another important indicator of intra-breed genetic diversity. The effective number of alleles is the number of alleles in the respective loci that are represented with equal frequency in the individual populations. Comparing the number of identified alleles in each locus with the effective number of alleles provides information on the predominance of certain alleles in each breed or population. In the present study, the frequencies of alleles in the same locus vary widely, so the effective number of alleles is lower than the found.

The expected heterozygosity (He), which is considered the best criterion for the level of genetic diversity in the population, ranges from 0.636 at the OarAE129 locus to 0.842 at the HSC locus with an average of 0.784 for the 13 loci analyzed. He exceeds the value of Ho, which is an indication of heterozygous deficiency. Absence of heterozygous deficiency was observed in 5 loci, where the values of He and No were the same (MAF65, INRA63, McM527) or No was lower than No (MAF214 and OarCP49).

Presented in table 3.3.1 results show that all studied loci are highly polymorphic, which confirms the effectiveness of the set of SSR markers used to identify the analyzed genotypes of sheep included in the study. The polymorphic information content (PIC) ranges from 0.618 for the OarAE129 marker to 0.87 for the HSC. The PIC is also high for INRA23 and OarFCB20 and OarCP49 markers with values of 0.858, 0.858 and 0.864, respectively. The average PIC for the 13 microsatellite markers was 0.799, and there were no markers PIC value lower than 0.618.

The average values of the fixation indices in the studied loci - Fis, Fit and Fst were 0.034, 0.078 and 0.046, respectively (Table 3.3.2).

The inbreeding coefficient (Fis) is an indicator of a tendency to kinship between individuals in a population and is considered to be the main reason for the deviation from the Hardy-Weinberg equilibrium. The average value of Fis in the analyzed 13 loci is 0.034, which shows a low level of inbreeding in the studied population of 12 sheep breeds. The highest level of heterozygous deficiency was observed in the OarAE129 locus (Fis = 0.189 or 18.9%), and the lowest in the McM527 locus, where a negative value was found (Fis = -0.005).

Table 3.3.2. F statistics in the studied SSR loci (Fis - intrapopulation coefficient of inbreeding, Fit - interpopulation coefficient of inbreeding, Fst - coefficient of genetic differentiation) and gene flow (Nm) in the studied loci.

Locus	Allele range	Fis	Fit	Fst	Nm
D5S2	188-202	0.052	0.097	0.047	5.064
INRA5	116-152	0.089	0.142	0.058	4.076
MAF65	121-147	0.000	0.034	0.034	7.047
OarAE129	139-319	0.189	0.237	0.058	4.042
OarFCB11	118-146	0.008	0.050	0.042	5.747
INRA23	196-224	0.008	0.064	0.056	4.205
OarFCB20	88-118	0.021	0.055	0.035	6.931
McM527	161-248	-0.005	0.034	0.038	6.245
CSRD247	209-265	0.014	0.053	0.040	6.058
HSC	181-303	0.083	0.125	0.045	5.274
MAF214	165-275	-0.011	0.043	0.054	4.406
OarCP49	72-140	-0.004	0.044	0.048	4.951
INRA63	157-213	-0.001	0.044	0.045	5.292
Mean		**0.034**	**0.078**	**0.046**	**5.334**

The Fit fixation index, which measures the loss of heterozygosity of individuals relative to the general population, is 0.078, indicating an 8% overall deficit of heterozygous individuals in the sheep population. The mean value of Fis in the present study is generally lower than that of Fst (0.034 <0.046), and is an indicator of the absence of heterozygous deficiency in the studied loci.

The coefficient Fst characterizes the level of genetic differentiation between populations based on the frequency of alleles in the respective microsatellite loci and varies from 0 to 1. In the present study, the average value of the coefficient of genetic differentiation between populations (Fst) was 0.046, which is an indicator for low level of genetic differentiation. It is obvious that the general genetic variation is mainly due to differences between individuals (95.4%) of the studied populations (breeds), i.e. intra-breed genetic diversity and only 4.6% is the result of differences between breeds, the so-called. interbreeding variation (Fig. 3.3.). These results are a clear indication of a low level of genetic differentiation between the studied breeds.

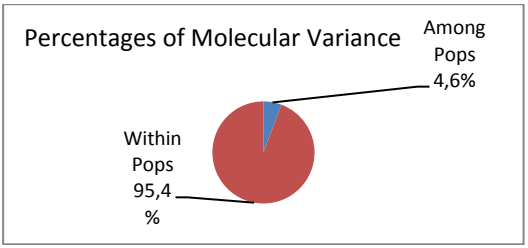


Figure 3.3. Genetic variation between and within the analyzed sheep population in Bulgaria, represented by 12 breeds based on AMOVA analysis.

Among the main reasons for the lack of clear differentiation between the studied national indigenous breeds are the geographical proximity between the analysed populations, similar ecological conditions and applied breeding practices, as well as the flow of genes between them in the past and probably now.

The Nm gene flow (Number of migrants per population or gene flow) in the present study ranged from 4.076 at the INRA5 locus to 6.931 at the OarFCB20 locus with a mean of 5.334. Gene flow is an indicator of the level of genetic differentiation of breeds and reflects the process of migration of genes from the gene pool of one population to another as a result of migration and crossbreeding.

3.4. POLYMORPHISM IN MICROSATELLITE LOCUSES, GENETIC DIVERSITY, INBREEDING AND VARIATION IN THE STUDY BREEDS

In the table. 3.4. the results for the number of identified alleles in the studied SSR loci, the average values of total (Na) and effective number of alleles (Ne), the observed (Ho) and expected heterozygosity (He), the inbreeding coefficient (Fis) in the studied breeds are presented.

Table 3.4. Total and effective number of alleles (Na and Ne), observed and expected heterozygosity (Ho and He) and inbreeding coefficient (Fis) in the studied SSR loci in the studied breeds.

Breed	Number of alleles	Na	Ne	Ho	He	Fis
SZ	106	8.15	3.85	0.66	0.70	0.04
MK	82	6.31	3.80	0.74	0.72	-0.03
REP	140	10.77	5.65	0.78	0.81	0.04
BREZ	139	10.69	5.01	0.76	0.78	0.04
SSP	140	10.77	5.68	0.76	0.80	0.05
DAB	136	10.46	5.97	0.81	0.82	0.01
SR	135	10.38	5.76	0.81	0.81	0.00
KARA	125	9.62	5.00	0.71	0.78	0.10
KOPR	138	10.62	5.20	0.77	0.79	0.03
SAK	137	10.54	5.88	0.79	0.81	0.02
KOT	149	11.46	5.86	0.79	0.81	0.04
TET	132	10.15	5.34	0.73	0.78	0.07
Mean	**129,92**	**9.99**	**5.25**	**0.76**	**0.78**	**0.03**

The largest number of identified alleles - 149 were found in the Kotel breed, and the smallest in the local Karnobat breed. The average number of identified alleles in the studied breeds is 129.92. High values of this parameter suggest a large allelic diversity, which is most likely the result of crossbreeding or mixing of populations.

The total number of alleles (Na) varies from 6.31 in the Local Karnobat sheep to 11.46 in Kotel. This is understandable, having in mind the homogeneity and small number of sheep at the beginning and the significant increase in population size for a short period of time afterward, which does not preclude the control of atypical animals or crosses. The average number of alleles / breed is 9.99, which is an indicator of significant genetic diversity in the breed. The effective number of alleles (Ne) varies from 3.80 (Local Karnobat) to 5.97 (Duben). The average effective number of alleles in breeds (Ne) is 5.25. In our study, the effective number of alleles in all herds was lower than found.

The observed heterozygosity (Ho) varies from 0.66 (Local Stara Zagora) to 0.81 (Duben and Central Rhodope), and the expected (He) - from 0.70 (Stara Zagora) to 0.82 (Duben). The mean values of Ho and He are 0.76 and 0.78, respectively, which indicates a high level of heterozygosity in the breeds studied. In most of them, with the exception of Local Karnobat, the average values of He do not exceed Ho, which is an indicator of heterozygous deficiency, but its levels are very low. The coefficient of inbreeding (Fis) varies from - 0.03 (Local Karnobat) to 0.1 (Karakachan). Only in 2 breeds the

inbreeding coefficient is higher than 0.05 - Teteven and Karakachan, while in all others, except the Central Stara Planina, it is lower than 0.05. The values obtained are an indication that there is a risk of increased inbreeding only in the first two breeds. If for the Teteven breed such a finding can be considered logical, as all herds are from the Teteven region, for the Karakachan breed, with a significant range and size of the population, it is unlikely. The reason, rather, can be found in the longer and more focused team, rather in terms of the typicality of the exterior in the studied herds.

3.5. HOMEOSTASIS OF POPULATIONS (HARDI-WEINBERG BALANCE TEST)

Hardy-Weinberg's equilibrium test was applied for all 13 microsatellite loci in the twelve breeds studied here. In 5 of the loci, some of the breeds are out of balance (p = 0) - OarAE129 (Replyan); McM527 (Teteven); CSRD247 (Kotel) OarCP49 (Breznik) and HSC (Teteven). In some of the loci, deviations are observed in all breeds (Table 3.5). The largest deviation (p <0.001) was observed in 3 loci: D5S2 (Replyan and Duben); INRA5 (Koprivshtitsa); CSRD247 (Teteven), due to heterozygous deficiency. In three of the loci, as a result of very low levels of heterozygous deficiency, some breeds were in equilibrium (p = 1) - McM527 (Kotel); MAF214 (Teteven, Stara Zagora); OarCP49 (Teteven).

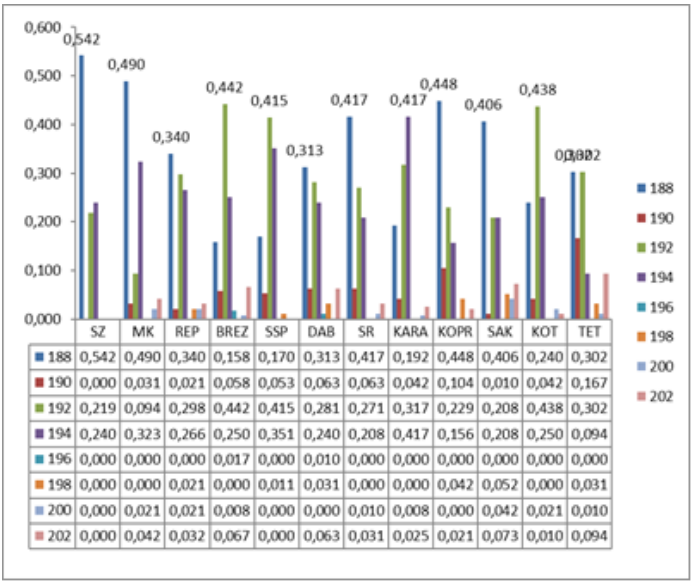
Table 3.5. Hardy - Weinberg (HW) equilibrium test in analyzed loci by breed.

Locus / Breed	D5S2	INRA5	MAF65	Oar AE129	Oar FCB11	INRA 23	Oar FCB20	McM527	CSRD 247	HSC	MAF 214	Oar CP49	INRA 63
SZ	0.118	0.026*	0.55	0.37	0.862	0.001***	0.017*	0.604	0.355	0.003**	1	0.759	0.522
MK	0.537	0.257	0.424	0.633	0.769	0.872	0.009**	0.105	0.859	0.988	0.195	0.177	0.392
REP	0.001***	0.032*	0.848	0***	0.302	0.739	0.564	0.657	0.789	0.009**	0.65	0.956	0.314
BREZ	0.427	0.157	0.488	0.555	0.035*	0.077	0.057	0.002**	0.446	0.971	0.227	0***	0.95
SSP	0.4	0.684	0.008**	0.999	0.756	0.015*	0.474	0.145	0.187	0.203	0.264	0.128	0.216
DAB	0.001***	0.819	0.952	0.109	0.9	0.634	0.343	0.635	0.985	0.011*	0.2	0.98	0.992
SR	0.598	0.83	0.215	0.002**	0.911	0.032*	0.086	0.393	0.898	0.433	0.966	0.521	0.341
KARA	0.052	0.004**	0.015*	0.005**	0.108	0.015*	0.346	0.071	0.979	0.855	0.248	0.723	0.267
KOPR	0.711	0.001***	0.002**	0.256	0.91	0.255	0.01**	0.741	0.703	0.078	0.177	0.013*	0.947
SAK	0.755	0.441	012*	0.615	0.522	0.397	0.871	0.947	0.825	0.946	0.017*	0.598	0.993
KOT	0.432	0.456	0.998	0.847	0.876	0.865	0.437	1	0***	0.427	0.834	0.999	0,393
TET	0.025*	0.618	0.982	0.424	0.625	0.667	0.731	0***	0.001***	0***	1	1	0,037

*** P<0.05, ** P<0.01, *** P<0.001**

3.6. FREQUENCY OF ALLELES IN THE LOCI STUDIED

Determining the frequency of alleles at each locus is the basis for estimation of genetic diversity (He). This indicator can be used to compare populations in which the number and distribution of alleles differ, and the variation in the frequency of individual alleles allows their use to assess the genetic diversity between individual populations. In addition, the frequency of alleles in the studied microsatellite loci gives an idea of the level of informativeness of the markers used.



	SZ	MK	REP	BREZ	SSP	DAB	SR	KARA	KOPR	SAK	KOT	TET
188	0,542	0,490	0,340	0,158	0,170	0,313	0,417	0,192	0,448	0,406	0,240	0,302
190	0,000	0,031	0,021	0,058	0,053	0,063	0,063	0,042	0,104	0,010	0,042	0,167
192	0,219	0,094	0,298	0,442	0,415	0,281	0,271	0,317	0,229	0,208	0,438	0,302
194	0,240	0,323	0,266	0,250	0,351	0,240	0,208	0,417	0,156	0,208	0,250	0,094
196	0,000	0,000	0,000	0,017	0,000	0,010	0,000	0,000	0,000	0,000	0,000	0,000
198	0,000	0,000	0,021	0,000	0,011	0,031	0,000	0,000	0,042	0,052	0,000	0,031
200	0,000	0,021	0,021	0,008	0,000	0,000	0,010	0,008	0,000	0,042	0,021	0,010
202	0,000	0,042	0,032	0,067	0,000	0,063	0,031	0,025	0,021	0,073	0,010	0,094

Figure 3.6. Frequencies of alleles in the D5S2 locus by breed

The figure shows that the frequencies of alleles in the D5S2 locus in different breeds are very different. In the majority of the studied breeds with the highest frequency is the allele 188 bp, and in the Stara Zagora breed the value is the highest (0.542). The frequency of this allele is similar in the Local Karnobat breed (0.49), Koprivshtitsa (0.448), Central Rhodope (0.417) and Sakar (0.406), and the lowest value is reported in the Karakachan breed (0.192).

The frequency of alleles 192 bp and 194 bp is also high, the former being dominant in the Breznik, Central Stara Planina and Kotel breeds, and the latter in the Karakachan breeds. The other 5 alleles are with relatively low frequency. They are most often found in a heterozygous state, in one or in a minimal group of individuals, in some of the analysed breeds. The indicated 5 alleles have not been found in the Stara Zagora breed, and in the Central Stara Planina

breed 3 of them are absent (196-, 200- and 202 bp). Alleles 196- and 198 bp were not found in the local Karnobat, Central rhodope and Kotel breeds. Of these alleles, 190 bp is more common in Koprivshtitsa and Teteven sheep, which are bred in two neighboring areas on both sides of the Balkan Mountains.

In most of the breeds, although with a lower frequency (0.021-0.094), allele 202 bp is observed, which was not found in the studied populations of Stara Zagora and Central Stara Planina sheep. In these breeds, as well as in Duben, allele 200 bp has not been identified.

Allele 198 bp was found in half of the studied breeds. The least common allele wasis 196 bp, which is found only in Breznik and Duben breeds, with low frequency, respectively 0.017 and 0.010.

In this locus, the lowest level of polymorphism is observed in the Stara Zagora sheep, in which only 3 of the 8 alleles are found. In 5 breeds - Replyan, Breznik, Duben, Sakar and Teteven 7 of the 8 alleles were identified in this locus.

Data on the number, distribution and frequency of alleles were obtained and analysed for each of the 13 loci, indicating that they vary from breed to breed.

3.7. POPULATION-SPECIFIC ALLELES IN THE STUDIED BREEDS

All alleles with a frequency above 3% (0.03), which occur in only one breed, are accepted as population-specific alleles.

As such, the following alleles can be differentiated:

- allele 147 bp in locus MAF65 with a frequency of 0.031 (3.1%) in the Replyan breed, which is observed and represented with different frequency in 2 of the herds;
- 146 allele 146 bp in locus OarFCB11 with a frequency of 0.083 (8.3%) in the Replyan breed, represented with different frequency in 3 of the herds;
- allele 243 bp in locus MAF214 with frequency 0.033 (3.3%) in Breznik breed, found with different frequency in 3 of the herds;
- allele 275 bp in locus MAF214 with frequency 0.031 (3.1%) in Sakar breed;
- allele 140 bp in locus OarSR49 with frequency 0.031 (3.1%) in the Central Stara Planina breed.

In this study , alleles specific for each breed were found with a frequency between 1% and 3% (Table 3.7.), referred to as rare alleles. These alleles also contribute to the differentiation of breeds, although to a very small extent and are the reason for the high allelic diversity observed in them. These were found in the Central Rhodope sheep in 3 loci (OarFCB20, CSRD247 and INRA63), as in the OarFCB20 locus, allele 88 bp was found in 2 flocks with equal frequency, and the other rare alleles were found in only one flock. In the

Breznik breed 1 allele with a frequency of 0.025 was found in locus INRA63, and in the Sakar breed - 1 allele with a frequency of 0.011 in locus INRA23.

Alleles with a frequency equal to or less than 1%, which belong to the group of unique alleles, have been reported. From table 3.7. it can be seen that in the Stara Zagora sheep 5 alleles unique for the breed were found in 3 loci (OarAE129, MAF214 and OarCP49), each observed in only one of the 4 flocks studied. One unique allele in 1 locus was found in the Replyan breed. In Breznik breed 2 rare alleles were found in 2 loci, and the allele in locus MAF65 was found in 2 herds with different frequency. Such are also two of the alleles in loci OarFCB11 and HSC in Breznik sheep, with a frequency below 1%, and in the breed Central Stara planina sheep found 1 unique allele in 1 locus (MAF214), represented in only one flock. The situation is the same with the Duben breed, in which 1 unique allele was found in 1 locus in one herd. In the Central Rhodope breed 1 unique allele was found in 1 locus (INRA5), and in the Karakachan breed 2 unique alleles were found in 2 loci in two herds, which are presented with the same frequency. In Koprivshtitsa sheep 1 unique allele was found in 1 locus in one of the herds, similar to the one observed for the Sakar breed. In the breed Kotel sheep 4 unique alleles were found in 4 loci, each of which was found in only one flock and all alleles have the same frequency. In the Teteven sheep breed, 2 unique alleles were found in 2 loci, each of which was found in only one flock.

Table. 3.7. Rare and unique alleles with frequency <3% and <1%, respectively identified specific loci in the analyzed breeds

Breed	Locus	Allele	
		bp	frequency
SZ	OarAE129	174	0.010
	MAF214	165	0.010
	MAF214	251	0.010
	OarCP49	105	0.010
		138	0.010
REP	INRA63	181	0.010
BREZ	MAF65	121	0.025
	OarFCB11	118	0.008
	HSC	181	0.008
	INRA63	197	0.025
SSP	MAF214	241	0.010
KOPR	OarAE129	319	0.009
	OarCP49	72	0.008
		114	0.010
SR	INRA5	122	0.010
	OarFCB20	88	0.021
	CSRD247	251	0.021
	INRA63	199	0.011
SAK	INRA23	196	0.011
	INRA63	175	0.010
KOT	INRA5	116	0.010
	McM527	248	0.010
	CSRD247	265	0.010
	INRA63	209	0.010
TET	CSRD247	223	0.010
	MAF214	205	0.010
DAB	INRA63	213	0.010

It is noteworthy that none of the rare or unique alleles are found in all analyzed herds of a given breed, so they cannot be a starting point for identifying the breed, but are the main reservoir for increasing allelic (genetic) diversity.

In addition, the figures presented in the previous subsection showed that the most common alleles (i.e. evolutionarily the oldest) are represented with different frequencies in different breeds. For example, allele 187 bp in locus MAF214 occurs with the highest frequency (0.677) in the Stara Zagora breed. In 6 breeds, alleles with frequencies higher than 0.5 were found in individual loci. In the Stara Zagora breed alleles with such frequency were found in 5 loci - MAF214 (allele 187 bp, 0.677), OarAE129 (allele 139 bp, 0.615), INRA5 (allele 220 bp, 0.594), D5S2 (allele 188 bp, 0.542) and OarFCB11 (allele 136 bp, 0.531). In some of these loci, as noted, the frequency of these alleles in other breeds is up to 14 times lower than in Stara Zagora.

In the local Karnobat sheep, alleles with a frequency above 0.5 were identified in two loci - OarAE129 (allele 151 bp, 0.585) and MAF214 (allele 187 bp, 0.583). In other breeds such values were found only in one locus at Teteven in MAF214 (allele 187 bp, 0.635), Breznik in OarAE129 (allele 151 bp, 0.508), Karakachan in OarAE129 (allele 151 bp, 0.578) and Central Stara planina in MAF214 allele 187 bp, 0.510).

There are other major alleles, with a frequency lower than 0.5, but whose values also vary widely in the breeds we studied, and although they do not belong to population-specific alleles, differences in their frequency between breeds are related to their differentiation.

Among the reasons for the emergence of new alleles in certain breeds and the presence of alleles represented with varying frequency in them are genetic drift and gene flow between populations. Undoubtedly, with so many breeds located in such a small area with overlapping regions of distributions that are not only not isolated, but also geographically poorly differentiated over the centuries there has been and still continued the process of gene exchange. Moreover, all our breeds belong to two types - Tsakel, Tsigay or are their crosses. More importantly, after penetration into new populations, some alleles are fixed or eliminated, their number increases or decreases in breeds as a result of adaptation to new specific ecological and geographical conditions, guidelines of national selection, specific approaches to the team, the exchange and use of rams, etc.

In the context of the above, despite the low level of genetic differentiation between the studied indigenous breeds (5%), the analysis of the heterozygosity of the studied loci, the presence and absence of individual alleles and allelic combinations, differences in allele frequencies are evidence of uniqueness. of each of the studied breeds and the need for its preservation and breeding in pure condition.

The large number of low-frequency alleles found is extremely suitable for tracking the dynamics of the genetic structure of the population and the direction of the team - to eliminate or fix the unique alleles, increase or decrease genetic diversity. This possibility is confirmed by the comparative analysis of inbreeding and heterozygosity of the breeds studied by us and the same, studied by other authors. The analysis shows that in the last 10 years the level of inbreeding in the local national breeds has not increased, taking into account a certain increase in heterozygosity. This is probably due to the increase in the size of the populations and the inclusion of new herds under selection control, which allows for more effective implementation of inbreeding avoidance schemes.

3.8. GENETIC DIFFERENCE BETWEEN LOCAL INDIGENOUS SHEEP BREEDS

3.8.1. Comparison of Fst between breeds

In order to analyze the degree of differentiation between the studied breeds, the values of Fst between each pair of breeds were calculated. The values of Fst and their significance are presented in table 3.8.1.

The highest values of Fst was found between Stara Zagora and Karakachan (0.065), Stara Zagora and Local Karnobat (0.063), Stara Zagora and Replyan (0.059), as well as between Stara Zagora and Karakachan (0.056) breeds. Values of Fst above 0.5 were also observed between the Stara Zagora and Kotel (0.053), Stara Zagora and Central Stara Planina (0.052), Stara Zagora and Koprivshtitsa (0.051) breeds. Close to this value are those between the Stara Zagora breed and the remaining 4 studied breeds - 0.048 with Sakar and 0.049 - with the others.

Considerable high values of the indicator, over 0.4, were also obtained with regard to the Local Karnobat breed, when comparing it with the Breznik, Karakachan, Koprivshtitsa and Teteven breeds.

Fst among the other breeds is lower than 0.2, except for values between the Teteven, Breznik, Duben, Karakachan and Sakar breeds. The lowest values of the coefficient were reported betweenKotel and Karakachan (0.010), Central Stara Planina (0.011) and Central Rhodope breeds (0.011).

Table 3.8.1. Comparison of Fst (coefficient of differentiation) between breeds.

Breed	SZ	MK	REP	BREZ	SSP	DAB	SR	KARA	KOPR	SAK	KOT
MK	0.063										
REP	0.059	0.037									
BREZ	0.049	0.040	0.017								
SSP	0.052	0.039	0.013	0.013							
DAB	0.049	0.036	0.012	0.014	0.013						
SR	0.049	0.034	0.012	0.015	0.012	0.012					
KARA	0.065	0.046	0.018	0.024	0.018	0.020	0.016				
KOPR	0.051	0.042	0.016	0.018	0.015	0.014	0.014	0.021			
SAK	0.048	0.034	0.016	0.020	0.016	0.015	0.013	0.023	0.018		
KOT	0.053	0.039	0.012	0.015	0.011	0.012	0.011	0.010	0.015	0.016	
TET	0.056	0.043	0.018	0.021	0.015	0.020	0.018	0.027	0.018	0.020	0,018

3.8.2. Genetic distances between breeds

The calculation of the genetic distances between breeds was used to establish the phylogenetic relationships between them. The most appropriate method for calculating genetic distances is the Nei method, as it takes into account the action of the main evolutionary events - mutations and genetic drift.

In the present study, the minimum Nei genetic distances were calculated based on the results obtained for allele frequencies in the 13 microsatellite loci studied. The obtained values of genetic distances are presented in table 3.8.2.

The reported high value of genetic distances between Stara Zagora breed and Karakachan (0.437), Stara Zagora and Replyan (0.412), Stara Zagora and Local Karnobat (0.362), and Stara Zagora and Teteven (0.362), is a reflection of differences at the genome level in the studied microsatellite loci, expressed as differences in the lengths of the corresponding alleles, respectively in their frequencies.

In locus INRA63, the main differences between the Stara Zagora and the other four breeds like Local Karnobat, Karakachan, Replyan and Teteven are found with respect to allele 173 bp, whose frequency in Stara Zagora (0.427) is significantly higher than in the Local Karnobat (0.052), Karakachan (0.121), Teteven (0.146) and Replyan (0.042) breeds. A similar trend was observed with respect to allele 139 bp in the OarAE129 locus, where the frequencies in these breeds are 0.615, 0.223, 0.147, 0.042, 0.136, respectively. In the INRA23 locus, the frequency of allele 220 bp is 0.625 in the Stara Zagora breed, 0.294 in the Local Karnobat breed, 0.200 in the Karakachan breed, 0.146 in the Teteven breed and 0.083 in the Replyan breed.

The genetic distances between the Stara Zagora breed and the other breeds are also significant: Central Stara Planina (0.325), Koprivshtitsa (0.316), Duben (0.316), Central Rhodope (0.308), Sakar (0.300) and Breznik (0.294).

Table 3.8.2. Genetic distances between breeds according to Nei.

Breed	SZ	MK	REP	BREZ	SSP	DAB	SR	KARA	KOPR	SAK	KOT
MK	0.362										
REP	0.412	0.233									
BREZ	0.294	0.257	0.108								
SSP	0.325	0.243	0.067	0.063							
DAB	0.316	0.229	0.068	0.078	0.071						
SR	0.308	0.209	0.066	0.090	0.062	0.065					
KARA	0.437	0.312	0.109	0.167	0.108	0.133	0.090				
KOPR	0.316	0.278	0.095	0.106	0.086	0.079	0.083	0.139			
SAK	0.300	0.202	0.102	0.130	0.094	0.098	0.075	0.162	0.114		
KOT	0.349	0.248	0.061	0.088	0.057	0.063	0.052	0.044	0.083	0.099	
TET	0.362	0.282	0.107	0.131	0.079	0.130	0.107	0.181	0.106	0.130	0.113

High values of genetic distances were observed between the Local Karnobat sheep and Karakachan (0.312), Karakachan and Teteven (0.282), Karakachan and Koprivshtitsa (0.278), while the distance between the Local Karnobat and other breeds ranges between 0.257 and 0.202.

The values of the genetic distances between the other breeds were significantly lower (<0.200), as the lowest were between the Kotel and Central stara planina (0.057), Kotel and Central rhodope (0.052) and Kotel and Karakachan (0.044).

The basis for the relatively high values of the reported genetic distances between Stara Zagora and these four breeds may be different reasons - origin, selection guidelines, the peculiarities of agro-ecological conditions in the breeding areas, etc.

3.8.3. Phylogenetic relationships between breeds

The kinship between the 12 studied breeds can be visualized graphically by constructing a phylogenetic tree - dendrogram. In it, the individual populations are grouped hierarchically into phenons or so-called clusters.

In the present study, the dendrogram was constructed by the Neighbor-Joining (NJ) method, based on the matrix representing the calculated genetic distances (Fig. 3.8.3).

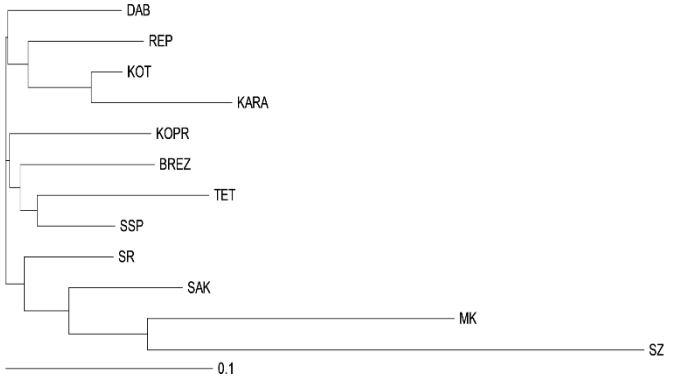


Figure 3.8.3. Dendrogram reflecting the phylogenetic relationships between the 12 breeds

The dendrogram consists of 3 main clusters. The first includes 4 breeds - Duben, Replyan, Kotel and Karakachan breeds, and the second - Koprivshtitsa, Breznik, Teteven and Central Stara planina breeds. The last includes the Central Rhodope, Sakar, Local Karnobat and Stara Zagora breeds. Genetically the most distant are the breeds Stara Zagora and Local Karnobat, and the most similar are Kotel and Karakachan, as well as Teteven and Central Stara Planina breeds.

The distribution of the breeds in certain clusters in the phylogenetic tree obtained by us is to a large extent logical and in accordance to a certain extent with the geographical distribution of their habitats. In the previous sections we have already commented on the proximity of the habitats of the breeds of the "middle" cluster. The areas of the Central Stara Planina and Teteven sheep practically overlap. The areas of the Teteven and Koprivshtitsa sheep are of equal geographical length, on both sides of the Balkan Mountains, and between and around them is the Central Stara Planina sheep breed. Between the area of the Koprivshtitsa and Breznik sheep is the area of the Elinpelin sheep, as the last two breeds are described as one and were separated at a later historical stage.

The areas of the breeds from the last cluster are also adjacent. To the northwest the Sakar region borders the area of the Karnobat and Stara Zagora breeds. There are no other local breeds in the southern region between the Sakar and Rhodope breeds.

It is more difficult to explain the genetic similarity between the breeds of the first cluster, although based on craniometric studies, Balevska and Petrov (1972) identify the Karakachan, Karnobat, Replyan, Kul, Panagyurishte sheep and the Copper Red Shumen sheep as offspring of common origin from *Ovis ammon musiimon* and that they are typical Bulgarian Tsakel.

In three of the breeds in the first cluster - Duben, Karakachanska and Kotel, the number of animals has increased many times over the last decade, and the distribution areas of the breeds have changed. It is not excluded that atypical animals as well as crosses are included under selection control.

3.9. MAIN COMPONENT ANALYSIS (Principle Coordinate Analysis, PcoA).

Based on the calculated genetic distances, an "Analysis of the main components" was performed, which confirms the clear demarcation of individuals from the Stara Zagora and Local Karnobat breeds. The observed differentiation of these breeds corresponds to the constructed dendrogram. In other breeds there is more or less overlap, and individuals in the coordinate system. It is noteworthy that the individuals of the Karakachan breed, although insignificant, stand out in their distribution in relation to the "mixing area". From the data obtained from the PcoA analysis, the most informative are the first 2 components (factors), which correspond to 8.59% of the total genetic variation. The horizontal axis (Axe1) corresponds to 4.52% genetic variation, thus distinguishing the herds of the Stara Zagora and Local Karnobat breeds from other populations and especially from the Karnobat breed. The vertical axis (Axe2) corresponds to 4.07% variation and differentiates the Local Karnobat breed from the Stara Zagora breed.

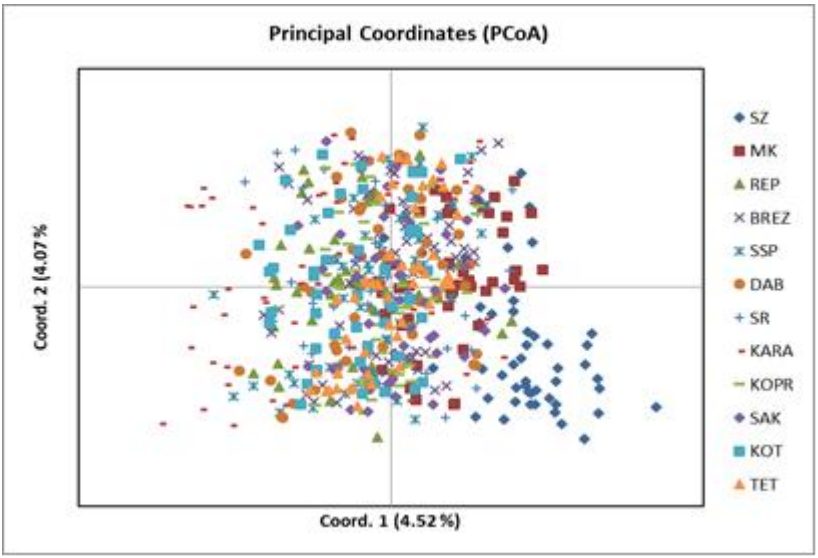


Figure 3.9.1. Analysis of the main components (PcoA), performed on the basis of the genetic distances of Nei between 600 animals from 12 local sheep breeds in Bulgaria.

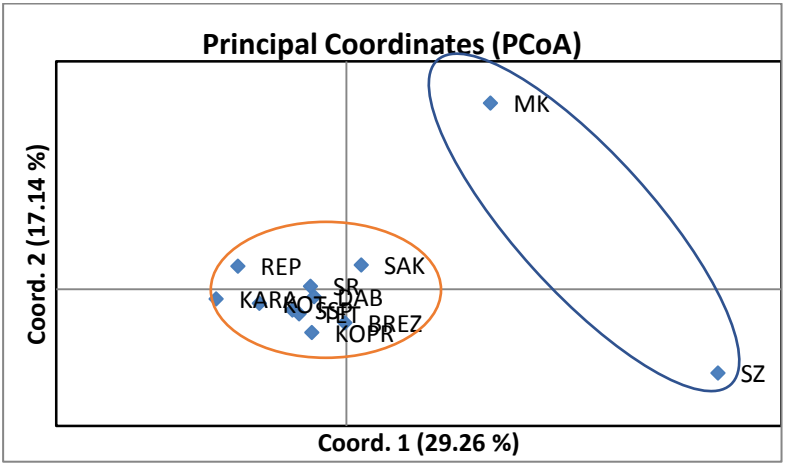


Figure 3.9.2. Principal Component Analysis (PcoA) based on Nei genetic distances in 12 breeds.

The analysis of the main components (PcoA) based on the genetic distance according to Nei between the breeds shown explains 46.40% of the total genetic variation (Fig. 3.9.2.). The first axis explains 29.26% of the total variation and separates the Stara Zagora and Local Karnobat breeds from the rest. The second axis, representing 17.14% of the total variation, shows the demarcation of the Local Karnobat from the Stara Zagora breed. The other 10 breeds are grouped together in a separate cluster, as they are not clearly separated from each other.

3.10. GENETIC DIVERSITY AND INBREEDING AND GENETIC DISTANCE BETWEEN STUDIES RESEARCHED

In the table 3.10., data on the total (Na) and effective number of alleles (Ne), the observed (Ho) and expected heterozygosity (He), the inbreeding coefficient (Fis) in the studied SSR loci by herds are shown.

Table 3.10. Total (Na) and effective number of alleles (Ne), - observed (Ho) and expected (He), heterozygosity, inbreeding coefficient (Fis) in the studied herds of local indigenous breeds.

Flock/Breed	Na	Ne	Ho	He	Fis
FL1 - SZ	5.538	3.478	0.712	0.663	-0.088
FL2 - SZ	5.769	3.452	0.660	0.649	-0.026
FL3 - SZ	5.077	3.361	0.705	0.665	-0.047
FL4 - SZ	4.923	2.948	0.577	0.601	0.058
FL5 - MK	4.846	3.403	0.763	0.677	-0.131
FL6 - MK	5.462	3.791	0.776	0.719	-0.073
FL7 - MK	5.154	3.490	0.684	0.686	0.002
FL8 - MK	5.231	3.468	0.737	0.692	-0.070
FL9 - REP	6.385	3.789	0.730	0.707	-0.035
FL10 - REP	7.692	5.225	0.841	0.791	-0.060
FL11 - REP	6.308	3.986	0.755	0.720	-0.055
FL12 - REP	6.615	4.645	0.776	0.764	-0.011
FL13 - BREZ	5.846	3.778	0.763	0.699	-0.089
FL14 - BREZ	5.846	3.551	0.731	0.697	-0.042
FL15 - BREZ	6.538	3.877	0.788	0.711	-0.114
FL16 - BREZ	5.692	3.797	0.731	0.695	-0.049
FL17 - BREZ	7.385	4.809	0.771	0.777	0.009
FL18 - SSP	5.846	3.486	0.744	0.685	-0.076
FL19 - SSP	6.692	4.191	0.782	0.716	-0.102
FL20 - SSP	5.385	3.409	0.722	0.693	-0.035
FL21 - SSP	7.308	5.118	0.790	0.774	-0.014
FL22 - DAB	7.308	4.811	0.819	0.771	-0.057
FL23 - DAB	6.692	4.645	0.776	0.764	-0.008
FL24 - DAB	6.923	4.627	0.808	0.764	-0.059
FL25 - DAB	6.846	4.763	0.827	0.775	-0.067
FL26 - SR	6.846	4.576	0.829	0.755	-0.091
FL27 - SR	6.462	4.544	0.805	0.769	-0.044
FL28 - SR	6.077	4.117	0.814	0.734	-0.106
FL29 - SR	6.769	4.519	0.782	0.756	-0.033
FL30 - KARA	7.000	4.983	0.788	0.772	-0.020
FL31 - KARA	6.077	3.826	0.724	0.716	-0.010
FL32 - KARA	5.462	3.961	0.693	0.709	0.038
FL33 - KARA	3.692	2.452	0.552	0.522	-0.058
FL34 - KARA	6.000	4.244	0.786	0.739	-0.064
FL35 - KOPR	5.846	3.699	0.716	0.693	-0.025
FL36 - KOPR	6.692	4.640	0.764	0.766	0.002
FL37 - KOPR	7.308	4.802	0.811	0.772	-0.048

Table 3.10.(continued)

Flock/Breed	Na	Ne	Ho	He	Fis
FL38 - KOPR	6.077	3.718	0.804	0.713	-0.124
FL39 - SAK	7.000	4.353	0.807	0.736	-0.102
FL40 - SAK	5.462	3.837	0.801	0.716	-0.127
FL41 - SAK	6.615	4.575	0.750	0.753	0.019
FL42 - SAK	6.615	4.892	0.814	0.786	-0.037
FL43 - KOT	7.385	4.835	0.750	0.768	0.034
FL44 - KOT	7.615	5.429	0.801	0.780	-0.020
FL45 - KOT	6.923	4.585	0.776	0.768	-0.013
FL46 - KOT	7.231	5.115	0.814	0.782	-0.042
FL47 - TET	5.615	3.722	0.692	0.705	0.016
FL48 - TET	6.077	3.550	0.660	0.689	0.046
FL49 - TET	6.692	4.324	0.756	0.739	-0.009
FL50 - TET	5.385	3.505	0.827	0.700	-0.179

Within each herd of the studied breeds, the total and effective number of alleles, the observed and expected heterozygosity and the inbreeding coefficient were taken into account. The comparative analysis shows that the total number of alleles (Na) varies from 3,692 in herd FL33 (Karakachan) to 7,692 in herd FL10 (Replyan), the effective number of alleles (Ne) - from 5,429 in herd FL44 (Kotel) to 2,452 in herd FL33 Karakachan), the observed heterozygosity (No) varies from 0.829 in herd FL26 (Central Rhodope) to 0.522 in herd 33 (Karakachan), the expected heterozygosity (He) - from 0.791 in herd FL 10 (Replyan) to 0.601 in herd FL4 (Stara Zagora). The inbreeding rate (Fis) in almost all herds except FL4, FL7, FL32, FL36, FL41, FL43, FL47 and FL48 is negative, but even in the eight herds listed it is very low, indicating that there is no risk of inbreeding.

3.11. GENETIC STRUCTURE OF BREEDS

Using the program Structure v 2.3.4 the genetic structure of the population represented by 50 flocks of the 12 studied breeds was determined. Evanno's method was used to determine the most likely number of genetic clusters. As a result of its application, it was found that the most probable number of clusters are two, three, eleven and thirteen (K = 2, K = 3, K = 11 and K = 13) (Fig. 3.11.1.).

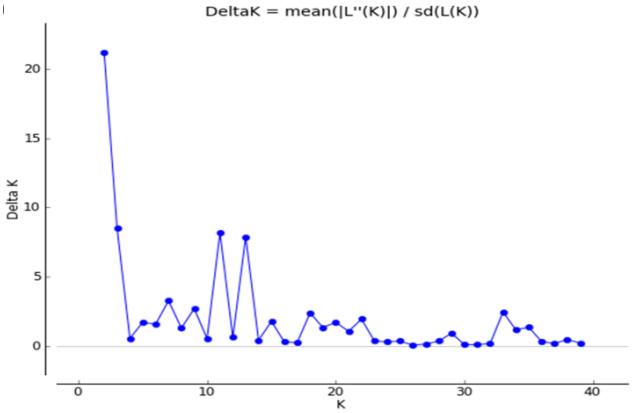


Figure 3.11.1. Number of genetic clusters in the sheep population determined based on the method of Evanno et al. (2005).

Figure 3.11.2. illustrates the genetic structure at K = 2, K = 3, K = 4, K = 8, K = 11 and K = 13 of the population of 600 sheep animals from the analyzed 50 flocks of the 12 breeds. In the graphical representation of breeds and related herds, each color represents 1 cluster, with the length of each colored segment corresponding to the percentage of each individual belonging to a particular cluster, black lines separate individuals belonging to each herd, while dotted vertical lines separate the breeds. At the level of belonging of individuals to the respective cluster over 50% (0.500) it is considered that there is a process of genetic differentiation.

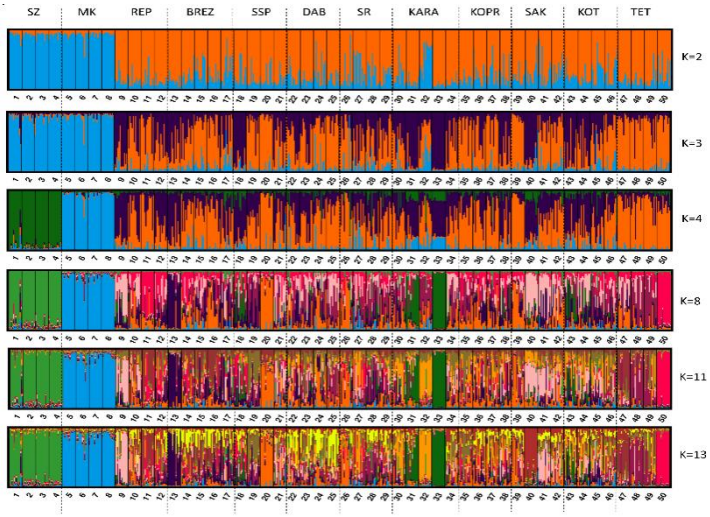


Figure 3.11.2. The genetic structure at K = 2, K = 3, K = 4, K = 8, K = 11 and K = 13 of the population of 600 sheep animals from the analyzed 50 flocks of the 12 indigenous breeds.

At K = 2, the studied population of 12 breeds of sheep showed the presence of 2 main clusters (populations), the first of which includes 10 of the analyzed breeds, and the second - breeds Stara Zagora and Local Karnobat, as the percentage of individuals of 10 breeds to the first cluster is high and is an indication that these breeds have a common ancestor and are not clearly differentiated. Evano's method showed additional lower peaks, the highest of which were reported at K = 3 and K = 11, followed by K = 13. The study is an indicator of the presence of subpopulations within the established 2 main populations at K = 2.

As can be seen from fig. 3.11.2., at K = 4 there is a process of separation of the two breeds - Stara Zagora and Local Karnobat in the second subpopulation, where % of belonging of the breeds to two different subclusters within the second cluster is clearly expressed: respectively between 82 and 95.2% for the herds of Stara Zagora and 90.8 and 96.6% for the herds of the Local Karnobat. In addition, the herds of both breeds FL1-FL4 and FL5-FL8, respectively, show a more pronounced homogeneous intra-breed structure than the other 10 breeds, which fall into the first population at K = 2, and whose structure is a mixture of both populations (clusters).

Similar results were obtained based on phylogenetic analysis and PcoA based on genetic distances, as shown in fig. 3.8.3 and fig. 3.9.1 where 3 main clusters were also observed.

The low level of genetic differentiation of breeds, with the exception of Stara Zagora and Local Karnobat, shows that phenotypic differences between the studied breeds are not accompanied by drastic changes at the genetic level, at least with respect to the studied loci. The breeds are characterized by high heterogeneity due to past and, in part, current gene flow as a result of animal exchange between breeds, which is evident at K = 4, K = 8, K = 11 and K = 13, or insufficient divergence of subpopulations from the original source.

Within several of the breeds in the first population, fragmentation was observed as a result of geographical isolation and / or the use of heterozygous breeding rams. Such is the case with the Karakachan breed, in which 2 subpopulations were observed within the breed. Two of the herds (FL31 and FL33) with area of distribution in the villages of Vlahi and Kresna, Blagoevgrad region and two (FL30 and FL32) from the region of Asenovgrad and Momchilgrad, differentiate with each other at K = 8. This shows that their individuals belong to different subclusters within the breed, which is clearly expressed in K = 11 and K = 13. The herd from the region of Smolyan (FL34) shows minimal affiliation (1% and 0.098%) to the subclusters, which differentiate the two pairs of herds of the Karakachan breed. It seems that the geographical isolation in combination with the targeted selection has led to a reduction of some alleles typical of the Karakachan breed in herds FL31 and FL33 and therefore to their pronounced genetic differentiation due to the high % of belonging to one of the subclusters (55.1% for herd FL31 and 92.8% for herd FL33).

Individuals from the FL20 herd of the Mid-Mountain breed are also differentiated from the other herds of the breed, due to the high percentage of belonging to one of the subclusters with increasing K (K = 8, K = 11 and K = 13), respectively 84.4%, 80.0% and 77.4 %. The situation is similar with the flock FL9 of the Replyan breed (65.4% at K = 13) and the flock FL13 of the Breznik breed (75.9% at K = 13) from the village of Nepraznentsi, Breznik, which are characterized by a more pronounced homogeneous structure in compared to other herds of both breeds.

The situation is similar with the FL50 herd of the Teteven breed, which also shows differentiation from the other 3 herds of this breed at K = 8, with the degree of belonging of individuals to one of the subclusters - 86.5%, and at K = 13 it is even 87.8 %. Unlike flock FL50, individuals from flocks FL48 and FL49 showed affiliation 53% and 62.6%, respectively, to another subcluster, while those from flock FL47 - to a third subcluster with 55.1% affiliation, which is another example of fragmentation within the breed due to ongoing processes of genetic differentiation.

It is interesting to note that individuals from some herds of different breeds show similar % of affiliation to respective subclusters. The presence of similarity is determined by the percentage of sharing of some evolutionarily

old alleles due to origin from a common ancestor(s), genetic exchange and their frequency in these breeds, as well as the current breeding strategies.

The differentiation of the herds, as is the case with the herds FL31 and FL32 and the herds FL30 and FL33 of the Karakachan breed and the herd FL50 of the Teteven breed, from the other herds, can be explained by "the effect of the ancestors".

3.12. PHENOTYPICAL DISTANCES BETWEEN BULGARIAN AUTOCHTHONOUS SHEEP BREEDS. CORRELATION OF PHENOTYPIC SIGNS WITH GENETIC MARKERS

3.12.1. Phenotypic distances between breeds according to the Nei-Li coefficient

The distances between the 12 sheep breeds were calculated and based on 12 phenotypic traits using the Nei-Li coefficient, as described in the Material and Methods section.

Table 3.12.1. Distances between 12 breeds of sheep based on 12 phenotypic traits using the Nei-Li coefficient.

SZ	MK	REP	BREZ	SSP	DAB	SR	KARA	KOPR	SAK	KOT	TET	
0.000												**SZ**
0.415	0.000											**MK**
0.171	0.053	0.000										**REP**
0.133	0.133	0.102	0.000									**BREZ**
0.179	0.041	0.061	0.109	0.000								**SSP**
0.291	0.076	0.102	0.053	0.083	0.000							**DAB**
0.161	0.062	0.104	0.062	0.088	0.062	0.000						**SR**
0.155	0.075	0.097	0.075	0.062	0.075	0.032	0.000					**KARA**
0.415	0.053	0.076	0.076	0.061	0.016	0.081	0.097	0.000				**KOPR**
0.133	0.133	0.171	0.076	0.179	0.133	0.028	0.075	0.171	0.000			**SAK**
0.415	0.034	0.053	0.076	0.041	0.034	0.062	0.075	0.016	0.133	0.000		**KOT**
0.034	0.221	0.171	0.133	0.109	0.221	0.104	0.124	0.291	0.076	0.221	0.000	**TET**

Table 3.12.1. shows that the greatest distances are between the Stara Zagora and the following three breeds: Local Karnobat, Koprivshtitsa and Kotel breeds (0.4146) and between the Stara Zagora and Duben breeds (0.291). The greatest phenotypic similarity was reported between the Stara Zagora and Teteven breeds. The values of the distances between the other breeds, with the exception of Koprivshtitsa and Teteven, are lower than 0.29, as Local Karnobat and Teteven, Duben and Teteven and Kotel and Teteven are characterized by

the same value of phenotypic distances between them (0.221). The distances between the other breeds are less than 0.221, which shows that the Stara Zagora breed differs in phenotypic features from the other breeds. The other breeds are very close in phenotype, with the smallest distances (0.016) between Koprivshtitsa and Kotel and between Duben and Koprivshtitsa. This is confirmed by the dendogram constructed on the basis of phenotypic features, as well as by the analysis of the main coordinates.

3.12.2. Phenotypic distances between breeds by the Neighbor-Joining method.

The dendrogram is based on the Neighbor-Joining (NJ) method and reflects the phenotypic distances between breeds based on 12 phenotypic traits.

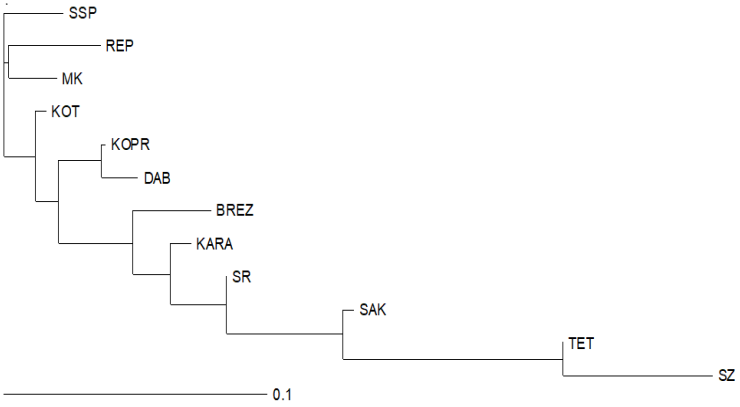


Figure 3.12.2. Dendrogram, reflecting the distances between the breeds on the basis of the reported phenotypic traits.

The dendrogram consists of 2 main clusters, as the breed Srednostaroplaninska, falls into the outer group. The first cluster includes - Replyan and Local Karnobat breeds. The second cluster includes the other 9 breeds, and it is further divided into two subclusters. The first subcluster includes the Koprivshtitsa and Duben populations, which are phenotypically most similar. The second subcluster includes 6 breeds, of which the most distant are Stara Zagora and Teteven.

3.12.3. Distances between breeds based on principal component analysis (Principle Coordinate Analysis, PcoA)

The analysis of the main components (PcoA) explains 58.44% of the total variation (Fig. 3.12.3.). The first axis explains 38.95% of the total variation and separates Breznik, Karakachanska, Central rhodope and Sakar breeds. The second axis, representing 21.94% of the total variation, shows the demarcation of the Stara Zagora and Teteven breeds. The other 6 breeds are grouped in a separate cluster, as they are not clearly separated from each other - the most similar are the Repla and Central Stara Planina, as well as Koprivshtitsa and Kotel breeds.

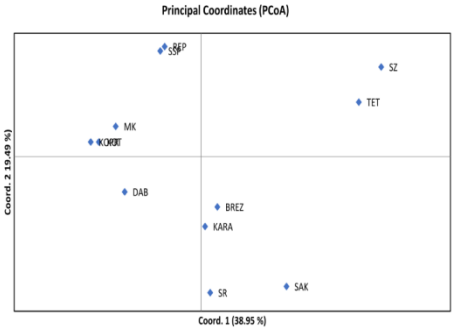


Figure 3.12.3. Analysis of the main components (Principle Coordinate Analysis, PcoA) performed on the basis of phenotypic data

3.12.4. Correlation between matrices based on genetic and phenotypic distances

A correlation analysis was performed based on the Mantel test (Mantel, 1967) to establish a link between genetic and phenotypic data for the 12 breeds of sheep. The Mantel test for correlation between matrices based on genetic and phenotypic distances, as described in the Materials and Methods section, was performed using GenAlEx to determine the existence of a correlation between genetic distance data and the distance determined on the basis of phenotypic traits.

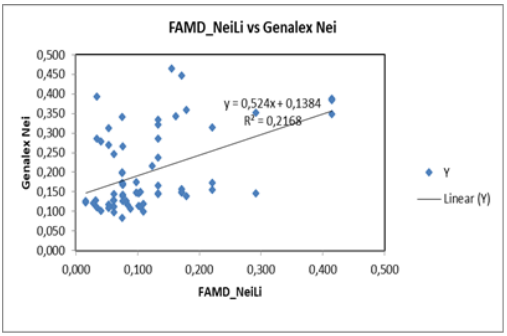


Figure 3.12.4. Mantel test for the presence of correlation between matrices for genetic and phenotypic distances in the studied breeds. * Rxy = correlation coefficient of the Mantel test. P (rxy-rand> = rxy-data) = probability of positive autocorrelation.

The matrices corresponding to the genetic and phenotypic distances calculated for the 12 breeds show a mean level of positive, statistically significant correlation (Rxy = 0.466, p = 0.03). This indicates that there is a correlation between some of the identified alleles in the analyzed 13 loci with the reported phenotypic traits. The conducted study is an indication that the used set of markers is suitable for complex assessment of sheep genetic resources in Bulgaria and future mapping of economically important QTLs based on association mapping approach.

CONCLUSIONS

The obtained results from the microsatellite and phenotypic analyses in 12 local sheep breeds in Bulgaria, within the PhD thesis allowed the formulation of the following conclusions:

1. Based on the fragment analysis, polymorphism was found in all studied microsatellite loci. A total of 228 alleles were identified in the sheep population, consisting of 600 animals, with an average of 17.54 alleles per locus. A total 129.92 alleles were found in the studied breeds with an average number of identified alleles per locus (9.99). The number of alleles varies from 6.31 in the Local Karnobat to 11.46 in the Kotel breed.

2. The relatively large number of alleles in the studied SSR loci (from 8 in D5S2 to 32 in OarCP49) showed that the set of 13 microsatellite markers used is suitable for analysis of genetic diversity in local sheep breeds in Bulgaria. The most informative are the markers HSC, INRA23, OarFCB20 and OarCP49, which stand out with the highest PIC. The highest number of alleles found - average number (15.33), effective (6.44) and population-specific (2); low heterozygous deficit (4.4%); high values of expected heterozygosity (He = 0.837) indicate that the most informative is the microsatellite marker OarCP49. The populations of Replyan and Koprivshtitsa breeds are characterized with the maximum number of alleles (18) at this locus.

3. High average level of genetic diversity was found (He = 0.78) in the studied 12 sheep populations genotyped with 13 microsatellite markers. The Duben sheep breed stands out with the highest level of genetic diversity (He=0.82), followed by the Replyan, Central Rhodope, Sakar and Kotel breeds (0.81), but the lowest value was observed in Stara Zagora (0.70). The highest Ho was reported for the Duben and Central Rhodope sheep breeds (0.81), while the lowest - in the Stara Zagora breed (0.70). The coefficient of inbreeding (Fis) varies from -0.03 in Local Karnobat to 0.1 in Karakachan breed. The mean value of Fis = 0.03 for the 12 sheep breeds is an indication of the absence of heterozygous deficiency.

4. Within the studied 12 breeds, 5 population-specific alleles were identified in 4 of the studied microsatellite loci: MAF65, OarFCB11, MAF214 and OarC49. The largest number of breed-specific alleles (2) were reported in the sample from the Replyan population at loci MAF65 and OarFCB11. Of these, allele 146 bp in OarFCB11 was presented with the highest frequency (0.083).

5. Unique alleles were reported in 11 of the studied breeds, with the exception of Local Karnobat. Their largest number is in the Stara Zagora breed (5). The latter are an indicator of a high level of allelic diversity in local sheep breeds and are a valuable "reservoir" for maintaining low levels of inbreeding.

6. Molecular variance analysis (AMOVA) showed 4.6% genetic variation between the studied populations and 95.4% between individuals within individual populations. The coefficient of genetic differentiation calculated on

the basis of this analysis (Fst = 0.046) is an indication of a low level of differentiation between breeds and is a starting point for further monitoring of the dynamics of breed development and the process of their differentiation at the genetic level.

7. The highest value of genetic distances according to Nei was found between the populations of Stara Zagora and Karakachan (DA = 0.065) and between Stara Zagora and Local Karnobat breeds (DA = 0.046), while the lowest (DA = 0.010) - between Karakachan and Kotel. The results of the principal coordinate analysis (PcoA) confirm the genetic differentiation of the Stara Zagora and Local Karnobat from other sheep breeds.

8. The analysis of the genetic structure shows the presence of 2 main populations at K = 2 in the studied sample of 12 local sheep breeds in Bulgaria, the first of which includes 10 breeds, and the second - Stara Zagora and Local Karnobat breeds. At K = 4, the Stara Zagora breed (FL1-FL4) and the Local Karnobat breed (FL5-FL8) are clearly differentiated, and their herds are characterized by a well-defined homogeneous structure in comparison to other breeds. The remaining breeds do not differentiate at increasing K and are characterized by high heterogeneity due to the ongoing process of gene flow between them. Distinct differentiation is observed for some herds of Karakachan, Teteven, Breznitsa and Replyan breeds.

9. The Stara Zagora breed has a high value of phenotypic distance in relation to the other 11 sheep breeds. The greatest distances were observed between the Stara Zagora and the following three breeds: Local Karnobat, Koprivshtitsa and Kotel breeds (0.4146) and between Stara Zagora and Duben breeds (0.291), with the greatest phenotypic similarity between Stara Zagora and Teteven breeds.

10. The correlation analysis between the genetic and phenotypic matrices based on the Mantel test shows that there is an average level of correlation (0.466) between the established genetic and phenotypic parameters. This is a basis for future comprehensive assessment of genetic resources of sheep in Bulgaria.

RECOMMENDATIONS

1. The applied set of microsatellite markers (D5S2, INRA5, MAF65, OarAE129, OarFCB11, INRA23, OarFCB20, McM527, CSRD247, HSC, MAF214, OarCP49, INRA63) can be used to characterize the genetic structure, genetic diversity and analysis of genetic processes in populations of national indigenous sheep breeds.

2. The genetic diversity in the studied populations of native breeds of sheep established by the attached set of microsatellite markers can be used effectively to preserve the gene pool of these breeds. Particular emphasis should be placed on the preservation of two of them - Local Karnobat and Stara Zagora, characterized by the highest degree of genetic differentiation, the

lowest level of observed and expected heterozygosity and due to their status of endangered breeds.

3. Given the observed strong fragmentation in 9 of the studied local sheep breeds (except for the Stara Zagora and Local Karnobat breeds, and with a few exceptions Karakachan breed) it is recommended to use breeding schemes based on individual team with strict observance of selection and exchange of breed-specific rams between herds.

CONTRIBUTIONS

1. For the first time an extended and systematic study of the genetic resources of 12 populations of local sheep breeds in Bulgaria, 7 of which are new, based on microsatellite analysis in 13 loci. The obtained results can be used as a basis for systematic observation/monitoring of the condition and development of the genetic resources of the local sheep breeds and their effective management through their application in the development and application of breeding, incl. and conservation programs.

2. For the first time, a correlation analysis was performed between the genetic and phenotypic matrices for the analyzed 12 breeds of sheep based on the Mantel test. The established average correlation between genetic and phenotypic parameters can serve as a starting point for the application of a comprehensive assessment of sheep genetic resources, including data from microsatellite analyzes and a set of phenotypic traits.

PUBLICATIONS ON THE DISSERTATION

Mihailova Y. (2021). Genetic diversity and structure of 2 indigenous sheep breeds (Kotel and Teteven) in Bulgaria using microsatellite markers. ***Biotechnology & Biotechnological Equipmen****t*. 35(1): 576-585. **IF=1.633**. **Q3** Scopus https://doi.org/10.1080/13102818.2021.1903339

CITATIONS:

1. Odjakova, T., Todorov, P., Radoslavov, G., & Hristov, P. (2022). Microsatellite Genotyping of Two Bulgarian Sheep Breeds. *Diversity*, *14*(3), 210.1, MDPI SJR 2021 = 0.654 https://www.scopus.com/sourceid/21100924379.

2. Loukovitis, D., Szabó, M., Chatziplis, D., Monori, I., & Kusza, S. (2022). Genetic diversity and substructuring of the Hungarian merino sheep breed using microsatellite markers. *Animal Biotechnology*, 1-9, SJR 2021 = 0.301 https://www.scopus.com/sourceid/14943.