GENETIC ANALYSIS OF LATVIAN DARK HEAD OLD TYPE BREEDING RAMS

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Abstract
To ensure genetic diversity of sheep production, local sheep breeds are included in genetic resource conservation programs, which increases their unique characteristics. The Latvian Dark Head old type (LTvt) sheep breed is an excellent example of how important it is to preserve genetic diversity among farm animals. The aim of the study is to evaluate the genetic diversity of LTvt breeding rams. The study included 126 LTvt breeding rams, which were genetically analysed by their year of birth (2002–2021) and paternal (ram) genealogical line, it is seven. Since 2012, the Latvian Sheep Breeders Association (LAAA) has been determining deoxyribonucleic acid (DNA) profiles by microsatellite loci lengths at the Eurofins Genomic laboratory in Germany. Fourteen microsatellite loci were used for DNA profiling. The results show that greater genetic diversity was obtained in indicators such as the number of different alleles (8.86 ± 0.601), the number of effective alleles (4.55 ± 0.476), Shannon's information index (1.66 ± 0.104) and expected heterozygosity (0.74 ± 0.037) in the group of breeding rams born between 2012 and 2016. The inbreeding coefficient values were close to zero, which indicates the absence of inbreeding. The analysed F-statistics values were negative in most loci, which indicates the excess of heterozygous genotypes in the studied population. Genetic differentiation between ram lines selected in the LTvt population was low (0.006–0.074), indicating low genetic diversity. Analysis of molecular variance showed that most of the genetic variation was between alleles within individual (93%).
Keywords: microsatellite loci, allele, genotype, genetic diversity, and variation.

Introduction

The conservation of sheep genetic resources is particularly important. Their variation is characterised by the compatibility of biodiversity conservation with sustainable agriculture, providing food for the population. Sheep genetic resources include genetic diversity that promotes their unique characteristics, such as disease resistance, ability to adapt to changing climatic conditions, and diverse productivity. Continuous cooperation at national, regional, and global levels is essential to ensure the sustainability and resilience of sheep breeds for future generations.

Although awareness of the importance of conserving animal genetic resources increases each year, their decline persists. Genetic diversity provides an opportunity to obtain or improve the disease resistance of farm animals (Jovanovic et al., 2009). Highly resilient local sheep breeds worldwide are capable of surviving extreme climatic conditions. Such breeds are easy to rear and use for small land management systems, as they require less investment in terms of costs and labour, and can lead to significant developments in sheep breeding (Molotsi et al., 2017).

Comparative genetic analysis shows that although the Latvian Dark Head sheep has similar genetic markers to other Northern European sheep breeds, it also has different genetic characteristics. Studies in the Baltic region using microsatellite loci and mitochondrial DNA have revealed both common ancestors and unique genetic diversity within this breed (Larsson et al., 2024).

The selection of the Latvian Dark Head breed is historically based on sheep breeding by ram lines. In 2014, seven breeding ram lines of this breed were identified: Skaris 0008; Edžiņš 0365; Sīgars 0005; Apollons 0302; Sīmanis 0195; Irbis 0125 and Ikars 0121. A systematic survey of the genetic resources of the Latvian Dark Head sheep breed was started in 2004. The animals had to comply with the criteria set out in the Latvian Dark Head sheep breed conservation program. In order to include typical LTvt animals in the genetic resource program in the future, a new breeding program for the Latvian Dark Head old type sheep breed was developed in 2019 (Latvjas Aitu…, 2024). In addition to all animal performance test evaluations, it introduced an evaluation based on wool growth on the head, belly, and legs.

The preservation of the Latvian Dark Head sheep includes two strategic methods. *In situ*: using support and education programs to encourage farmers to breed local animal breeds (including sheep), and *ex situ*: establishing Gene banks and cryopreserving genetic material with the aim of preserving biological material for future use. The aim of Gene bank is to preserve part of the Latvian Dark Head sheep breed as a gene reserve, without introducing other non-related breeds in future generations, and to use it as a 'mother' breed for sheep crossbreeding, as it improves fertility and milk production and is characterized by good maternal traits, a strong constitution, resistance to disease, and suitability for local conditions (Gene Bank, 2021).

Sheep farms often rely on just a few breeding rams, which can lead to a decrease in genetic diversity within ram lines as certain alleles are lost. Breeds originating from a small number of individuals often exhibit low genetic diversity. If a breeding flock initially relies on a single ram, the genetic profile of the offspring is heavily restricted by the father's limited allele pool. The loss of alleles in a population often indicates genetic drift. This is, especially true in small populations where alleles can be randomly lost by chance, particularly when the number of individuals of one sex is limited. Consequently, even neutral or beneficial alleles may fail to be passed on to offspring purely through random sampling. The loss of alleles can also result from deliberate selection goals. For example, if an individual carries alleles associated with undesirable trait like low fertility, those alleles will be passed on to subsequent generations less frequently or

eliminated entirely (David et al., 2018; Karsli et al., 2020; Ocampo et al., 2016; Tapio et al., 2010).

Materials and Methods

Microsatellite loci, or more exactly, genotype distribution, are used in population genetic calculations to determine the level of relationship between breeds, groups, and individuals (Wagner et al., 2006).

Supported by funding from the Latvian Ministry of Agriculture, the LAAA has conducted DNA profiling since 2012 using microsatellite markers based on allele fragment length analysis at Eurofins Genomics (Germany). DNA profiling allows the origin of an animal to be accurately determined and verified. During this process, the laboratory uses 14 microsatellite loci for DNA profiling, see Table 1. All of the listed microsatellite markers are widely used in sheep genetic research in analyses of allele diversity, population structure, and Hardy-Weinberg equilibrium (HWE) (Al-Atiyat et al., 2018; Saberivand et al., 2010).

Table 1

Characterization of DNA profile microsatellite loci and alleles with chromosomal localization

Microsatellite loci	Size range, bp	Primer sequences (5′ – 3′) F – forward, R – reverse	The chromosome	Expected length, bp	Allele repetition
OarFCB128	94 – 126	F: ATTAAAGCATCTTCTCTTTATTTCCTCGC R: CAGCTGAGCAACTAAGACATACATGCG	2	105	TG9
TGLA53	140 – 160	F: ATCTTCACATGATATTACAGCAGA R: GCTTTCAGAAATAGTTTGCATTCA	12	141	TG12
D5S2	186 – 196	F: GAGACCTCAGGGTTGTTGGTCAG R: TACTCGTAGGGCAGGCTGCCTG	3	194	GT16
BP33	247 – 279	F: AGGCCTCTGAATTCCTCTCC R: AACCAGCAGTCTTGCTCTCTC	18	271	AC23
MAF65	89 – 139	F: AAAGGCCAGAGTATGCAATTAGGAG R: CCACTCCTCCTGAGAATATAACATG	15	124	GT11
McM527	134 – 180	F: GTCCATTGCCTCAAATCAATTC R: GTTTCTTAAACCACTTGACTACTCCCCAA	5	164	TG16
DRB2	227 – 289	F: CTGCCAATGCAGGAGACACAAGA R: GTCTGTCTCCTGTCTTGTCTCAT	20	300	AC29
OarFCB20	87 – 129	F: GGAAAACCCCCATATATACCTATAC R: AAATGTTGTTTAAGATTCCATACATGTG	2	95	TG15
INRA49	134 – 164	F: TCCACAATCACACAGGCCA R: TGTATTAGTTTGTGTTCTTTGGC	1	135	AC8
CSRD247	209 – 283	F: GGACTTGCCAGACTCTGCAAT R: CAGCTGTGGTTTGTATTAGTCAGG	14	244	CA28
MMP9	183 – 204	F: CTTGCCTTCTCATGCTGGGACT R: GTGAGGCTGGCACTTGGTCTGGCT	13	220	AC20
OarCP49T	78 – 106	F: CAGACACGGCTTAGCAACTAAACGC R: GTGGGGATGAATATTCCTTCATAAGG	17	87	CA17
INRA172	122 – 168	F: CCAGGGCAGTAAAATGCATAACTG R: GGCTTTGCTAGCCTCTGCAAAC	22	152	GT21
BP28	209 – 241	F: CCTCCACAACACCATCCTTC R: AGGTGACAGGTGGGAGGGC	24	221	TG21

In laboratory analyses, each sample is (1) subjected to DNA extraction, (2) a multiple polymerase chain reaction is performed with appropriate primers to synthesize the region of the genome of interest, and (3) the length of the synthesized fragment is determined using a capillary electrophoresis sequencer. The laboratory provided information on the fragment lengths of each sample in all 14 loci. Information on the possible number of nucleotides repeats in the microsatellite loci according to the determined fragment length was found on the website of the University of California Regents' International Sheep Genome Consortium (Archibald et al., 2010). Using the primer sequence, the corresponding location in the sheep genome and the number of repeats at the fragment length in the database were determined. Furthermore, the locations of microsatellites loci on the chromosome were verified. The genetic analysis of the LTvt breeding ram population included in the study was performed according to their year of birth and affiliation to the paternal (ram) genealogical line, see Table 2. In total, data on 126 breeding rams born between 2002 and 2021 were analysed, dividing this period into four-year intervals. In Latvia, Apolons 0302 (46) and Skaris 0008 (27) lines of breeding rams are more commonly used on sheep breeding farms. Less represented rams are from the Edžiņš 0365 and Ikars 0121 lines.

Table 2
Number of breeding rams by year of birth and affiliation to the father (rams) line

Ram line	Ram count by birth year				Total in rams' line
	2002–2006	2007–2011	2012–2016	2017–2021	
Apolons0302	2	9	25	10	46
Edžiņš0365	0	0	0	5	5
Ikars0121	0	1	4	4	9
Irbis0125	0	2	6	5	13
Sīgars0005	1	3	8	1	13
Sīmanis0195	0	2	8	3	13
Skaris0008	1	9	7	10	27
Total by period	4	26	58	38	126

More breeding rams were included in the LTvt breeding program from 2012 to 2016 (58), but breeding rams (5) from the Edžiņš 0365 line were only included in the LTvt breeding program from 2017 to 2021. For the genetic analysis of the population, the following statistical methods were used to calculate allele frequency, heterozygosity, Wright's F–statistic (Wright, 1978), Nei's genetic distance, and the Hardy-Weinberg equilibrium test.

Population genetic diversity was characterized by several indicators that reflect allele diversity and genotype distribution. Population genetics is based on the calculation of HWE equilibrium, which indicates a constant allele and genotype frequency in a population from generation to generation, provided there are no allele mutations, migration, selection, inbreeding, or random genetic drift. The GenAlEx 6.5 software package was used to analyse genetic diversity and population structure in the animals included in the study, dividing them by birth year according to their ram line. Based on genotype and allele frequencies (N), the number of different alleles (Na), the effective number of alleles (Ne), and the observed (Ho) and expected (He) heterozygosity were calculated. Different inbreeding coefficients (F) were calculated and analysed. These help to understand the genetic structure of populations, identify the degree of inbreeding, and determine how genetic variation is distributed at the level of individual relative to its subpopulation (Fis), inbreeding coefficient among populations by year of birth (Fst), and the overall inbreeding coefficient entire population (Fit). Principal Coordinates Analysis (PCoA) was based on and calculated using standard genetic distance (Nei). The number of different alleles (Na) provides a general overview of genotype polymorphism, reflecting how many different alleles exist in a single locus. This is the basis for calculating the effective number of alleles and heterozygosity. The effective number of alleles (Ne) shows the most common alleles based on their frequency. The closer this value is to the Na, the more evenly the alleles were distributed. However, if Ne is much smaller than Na, it means that some alleles dominate, while others are very rare. Shannon's information index (I) compares genetic diversity between populations. Its value indicates the frequency of alleles, if there is one dominant allele in a locus, then I will be lower, but if all of them are common, then I will be higher. To assess the extent to which the number of heterozygous genotypes (Ho, He) in a population has decreased due to random crossing, the inbreeding coefficient (F) is calculated (Peakall & Smouse, 2009).

The aim of the study was to evaluate the genetic diversity of Latvian Dark Head old type breeding rams.

Results and Discussion

By analysing the number of alleles present in 14 loci, it was concluded that a total of 136 alleles were observed in the LTvt breeding ram population. Polymorphic genotypes were observed in 14 loci in all 7 breeding ram lines. In some loci, unique alleles were also observed, which characterize the breeding rams belonging to a certain line, for example, in loci 'OarFCB128' (allele 126), 'OarFCB20' (allele 129), 'MAF65' (allele 117), 'MAF65' (allele 123), 'MMP9' (allele 181) and 'BP28' (allele 215). Analysis of the HWE parameter showed that the LTvt breeding ram population was in equilibrium.

The evaluation of the genetic diversity parameters of breeding rams in the LTvt population by year of birth is shown in Table 3.

Over the course of the study, a total of 126 breeding rams were analysed, including their 136 alleles and 795 genotypes. Of these genotypes, 657 were heterozygous and 138 were homozygous. This confirms that the LTvt population has good genetic diversity, and it means high heterozygosity and no obvious inbreeding. Genetic diversity indices differ significantly between the birth year groups of breeding rams ($p<0.05$) by Na, I and He.

Table 3

Evaluation of genetic diversity parameters of rams in the LTvt population by birth years

Birth year of the rams	N	Na	Ne	I	Ho	He	F
		$\bar{x} \pm S\bar{x}$					
2000. – 2006.	4	3.79 ± 0.318[a]	2.96 ± 0.273	1.13 ± 0.111[a]	0.70 ± 0.075	0.60 ± 0.054[a]	–0.05 ± 0.035
2007. – 2011.	26	7.50 ± 0.693[b]	4.43 ± 0.451	1.58 ± 0.119[b]	0.70 ± 0.053	0.72 ± 0.048[ab]	0.01 ± 0.046
2012. – 2016.	58	8.86 ± 0.601[b]	4.55 ± 0.476	1.66 ± 0.104[b]	0.71 ± 0.044	0.74 ± 0.037[b]	0.03 ± 0.033
2017. – 2021.	38	7.57 ± 0.677[b]	4.33 ± 0.474	1.59 ± 0.104[b]	0.70 ± 0.037	0.72 ± 0.037[ab]	0.02 ± 0.032
Along/ Mean	126	6.93 ± 0.385	4.07 ± 0.225	1.49 ± 0.060	0.70 ± 0.026	0.70 ± 0.023	–0.02 ± 0.024

Na – number of alleles, Ne – number of effective alleles, I – Shannon's information index, Ho – observed heterozygosity, He – expected heterozygosity, F – inbreeding coefficient.

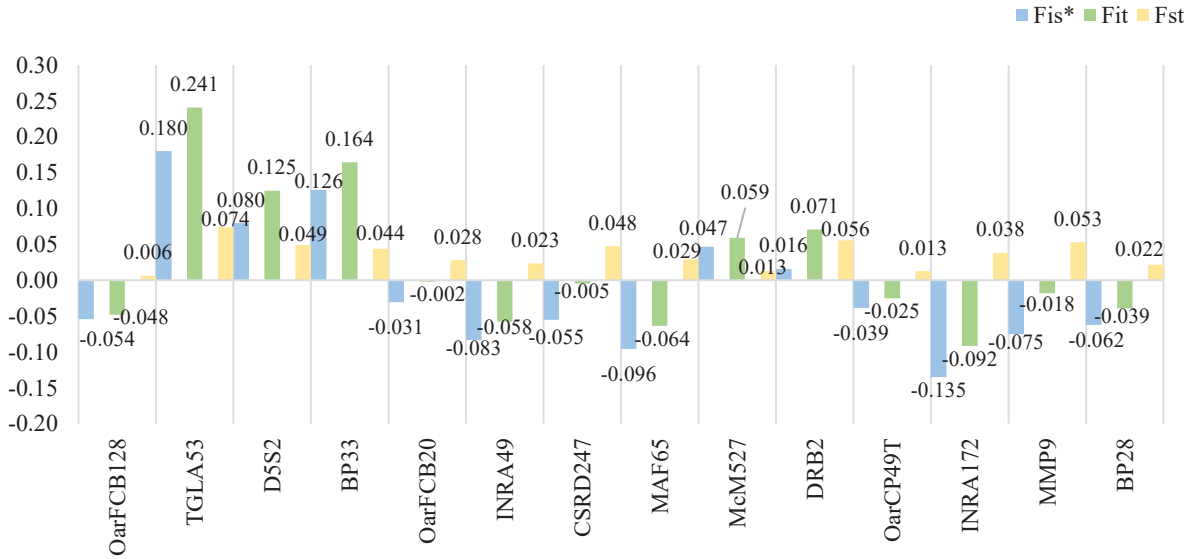
Different superscript letters within a column indicate statistically significant differences ($p<0.05$).

The group of rams born from 2000 to 2006 exhibited the lowest Na (3.79 ± 0.318), Ne (2.96 ± 0.273) and I (1.13 ± 0.111) values. This indicates reduced allelic richness, low effective allele diversity and low overall genetic diversity. However, because this group contained only four animals, and these results should be interpreted with caution. The highest Na (8.86 ± 0.601), Ne (4.55 ± 0.476), I (1.66 ± 0.104) and He (0.74 ± 0.037) values were observed in the group of breeding rams born between 2012 and 2016, indicating genotype polymorphism and higher genetic diversity, where the breeding rams in this group are genetically more diverse. The observed heterozygosity (Ho) was similar in all groups (0.70–0.71) and did not differ significantly ($p>0.05$) between studied groups. In a Lithuanian study, which also studied Latvian Dark Head breed sheep, the expected heterozygosity (He) (0.698 ± 0.173) was lower than the observed heterozygosity (Ho) (0.712 ± 0.143), which can be explained that the breed has been highly selected in flocks (Grigaliūnaite et al., 2003). The low inbreeding coefficient (F) values indicate that there is no significant inbreeding among the breeding ram groups by birth year. Consequently, no significant deficit of heterozygous genotypes was observed, suggesting that the LTvt breeding ram population is in equilibrium. The negative F value (-0.05 ± 0.035; -0.02 ± 0.024) indicates a slight excess of heterozygote genotypes, while a positive F value (0.01 ± 0.046; 0.03 ± 0.033; 0.02 ± 0.032) indicates a slight deficit of heterozygote genotypes.

Genetic differences between populations and the level of inbreeding are described by F-statistics (Fis, Fit, and Fst). They provide insight into the distribution of genetic diversity at different levels and allow us to characterize genetic differences between individuals, lines, and the population as a whole. The Fis, Fit, and Fst values of genetic differences that characterize the genetic structure of the LTvt population of breeding rams in all 14 loci can be seen in Figure 1.

Figure 1

Distribution of Fis, Fst and Fit values by loci in the ram LTvt population



**Fis – level of inbreeding within a population; Fit – total level of inbreeding across all populations; Fst – genetic differentiation between populations.*

In the LTvt breeding population, heterozygous genotypes exceeded homozygous genotypes in most loci, which was characterized by negative Fis values (from -0.135 to -0.031), but in three loci, 'TGLA53' (0.180), 'D5S2' (0.080) and 'BP33' (0.126), it is positive, where a deficit of heterozygous genotypes is observed. This is often observed in small populations or when animals with specific genotypes have been selected during the selection process. The total heterozygous genotype deficit (Fit) in the LTvt breeding ram population is slightly lower than expected, depending on the year of birth.

In the LTvt breeding poplulation rams' population, the total inbreeding coefficient (Fit) is slightly lower than the level of inbreeding within a population (Fis). The highest Fit values were observed at loci 'TGLA53' (0.241), 'BP33' (0.164) and 'DRB2' (0.071), indicating a persistent deficit of heterozygous genotypes across birth years. Conversely, 8 of the 14 analysed loci exhibited negative Fit values, indicating an excess across the ram groups of birth year.

The total genetic difference in the LTvt breeding ram population by year of birth is determined by the Fst value. If the Fst value is between 0.00 and 0.05, it is low. If it is between 0.06 and 0.15, it is medium, and if the Fst value is more than 0.16, there is a large difference between the animals in the population (Peakall & Smouse, 2009). In most loci, the Fst value varies from 0.01 to 0.07, for example, the lowest value is in the locus 'OarFCB128' (0.006), but the highest is in the locus 'TGLA53' (0.074). This means that the LTvt breeding ram population is genetically similar depending on the animal's year of birth, but there are slight differences in genetic diversity in some loci.

Research conducted by various international authors on local sheep breeds indicates that these populations typically exhibit significant allele diversity. A high number of heterozygous genotypes is often observed, indicating that there is no significant decrease in genetic diversity within breeds. The Fis values of the breeds studied by the authors ranged from 0.110 to 0.752, and negative values were also observed. When describing Fst values, the authors note that in most cases they are low (0.028–0.319), and there is a certain genetic difference between these breeds. In these mentioned studies, the total inbreeding level in relation to the entire population (Fit) ranged from 0.246 to 0.751, and therefore signs of inbreeding or frequent selection were observed in individual loci (Arora et al., 2011; Baumung et al., 2006; Ghazy et al., 2013; Hoda et al., 2009; Hoda & Ajmone, 2012; Kusza et al., 2010; Mukesh et al., 2006; Yilmaz et al., 2014). To evaluate the distribution of genetic diversity at different levels, molecular variance analysis (AMOVA) was performed (Peakall & Smouse, 2009). This helps to understand the extent to which genetic differences exist within the population and between individuals in the population. Molecular variance analysis for the whole LTvt breeding ram population by year of birth can be seen in Table 4.

Table 4

Analysis of molecular variance in ram LTvt population by birth year

Source	df	SS	MS	σ^2	%SS
Among line	3	22.86	7.62	0.04	1
Among individual	122	668.97	5.48	0.31	6
Within individual	126	613.00	4.87	4.87	93
Total	251	1304.83	…	5.21	100

df – degrees of freedom, SS – sum of squares, MS – mean square, σ^2 – estimated variance, %SS – Percentage distribution of variation.

When analysing genetic variation among all breeding ram lines depending on their year of birth, these ram lines are genetically similar across years and show slight variation, as indicated by the percentage distribution of variation (%SS) value (1%). Genetic variation between certain breeding ram lines among individual is 6%, where different individuals in the same population are genetically different, but this variation is not wide. When analysing the genetic variation between alleles within individual, this value is higher – 93%, which can be explained by the heterozygosity of the individual, where different alleles are found in the same locus. In a study carried out in Italy with a single sheep breed kept in different regions, these values were similar to the data from our study, as the highest genetic variation between individual alleles was 94.21% (Ceccobelli et al., 2016).

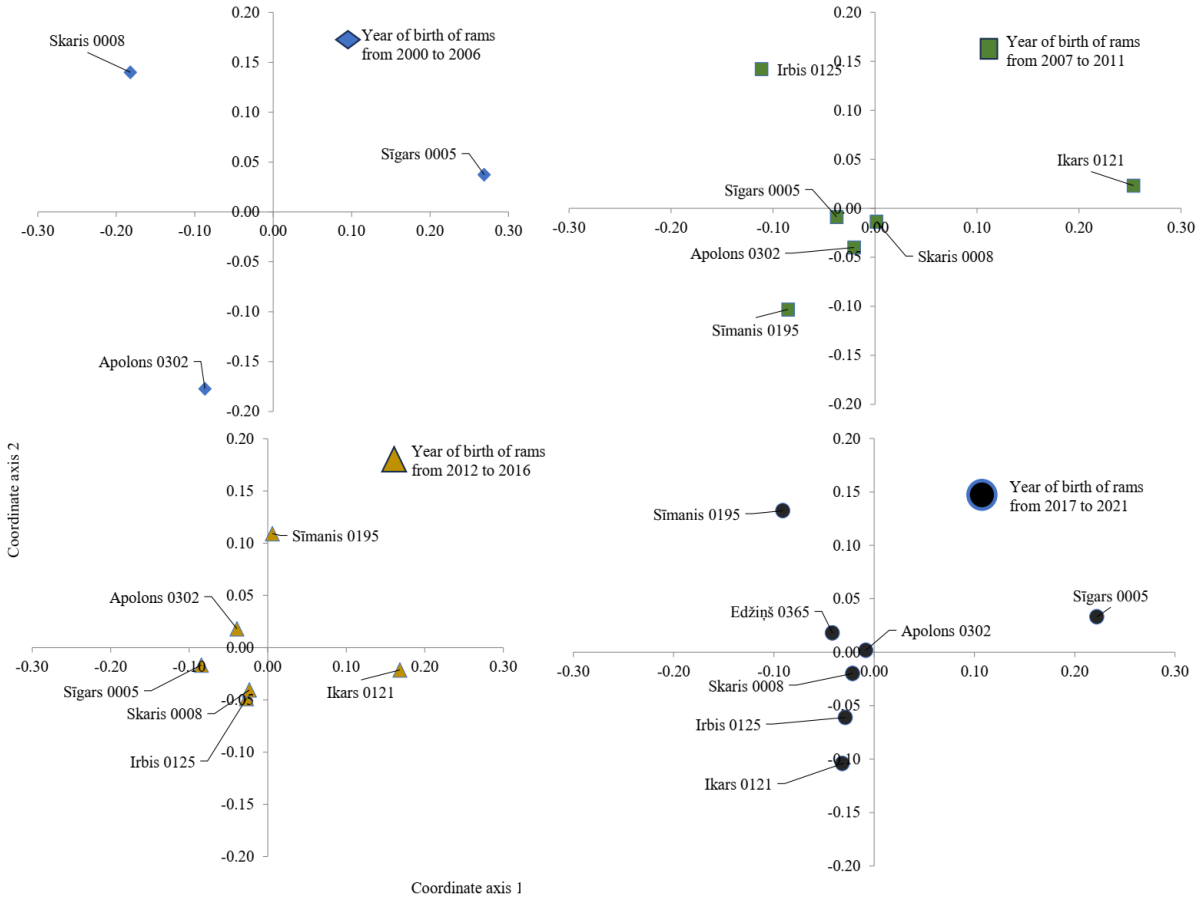
Principal coordinate analysis (PCoA) shows how animals are grouped according to similar or different alleles within a population. The position of breeding rams from a specific line within each LTvt population on the respective axis is indicated by positive or negative numbers. These determine which side of the axis (positive or negative direction) the animals from a specific line are located on. Animals with similar values are closer to each other, while those with opposite values are more different. PCoA analysis was performed for breeding rams of each line depending on their year of birth (Figure 2).

Analysis of the principal coordinates shows that the year of birth of breeding rams is not the main factor in genetic differences, but that the highest allele diversity is at the individual level. Several animals from breeding lines are located on one side of the coordinate axis, for example, animals from the Apolons 0302, Skaris 0008, and Irbis 0125 lines. The animals in these breeding ram lines are genetically similar. However, there are animals in breeding ram lines such as Ikars

0121, Sīmanis 0195, and Sīgars 0005 that are more separated from the main coordinate axis. This indicates a longer genetic distance, which is based on changes in allele frequencies in a certain individual in the year of birth of a certain breeding ram, where these alleles can disappear and reappear when mating with an ewe that may or may not have these alleles. In a study carried out in the Baltic States with local sheep breeds, the genetic characteristics of the Latvian Dark Head sheep breed were similar to the average characteristics of all breeds studied. Animals of the Latvian Dark Head breed formed the most homogeneous group, as the individuals in the group were genetically similar to each other, which can be explained by the extensive selection work carried out during the development of the breed (Tapio et al., 2005).

Figure 2

Genetic relationships among ram birth year and line in LTvt population revealed by PCoA



Conclusions

1. Breeding rams born between 2000 and 2006 had lower genetic diversity, whereas breeding rams born between 2012 and 2016 had higher genetic diversity.
2. Genetic diversity and inbreeding levels in the LTvt breeding ram population showed that most loci had an excess of heterozygous genotypes rather than a deficit, and that there was low to medium genetic variation between breeding ram lines.
3. Molecular variance analysis confirmed that LTvt breeding ram lines are very similar to each other, with the highest genetic variability occurring among the animals themselves.
4. Principal coordinate analysis reflected a similar genetic structure among LTvt ram lines, with slight changes in genetic distance observed across ram birth year periods.
5. The observed heterozygosity in all breeding ram groups by year of birth was similar, the inbreeding coefficient was close to zero, and no significant inbreeding between LTvt breeding ram lines was confirmed.

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