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RESEARCH ARTICLE

Intra-Breed Genetic Diversity and Genetic Bottleneck Tests in a Karacabey Merino Sheep Breeding Farm Using Microsatellite Markers

Adem KABASAKAL [1(*)]

[1] Bandırma OnyediEylül University, Susurluk Vocational School, Department of Food Processing Division, TR-10600 Balıkesir - TÜRKİYE

ORCIDs: 0000-0002-6857-4380

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Abstract: In the present study, it was aimed to reveal the genetic diversity and bottleneck status of Karacabey Merino sheep with the help of 14 microsatellite markers recommended by the FAO. The study was carried out in a sheep breeding farm in Bandırma and 103 unrelated Karacabey merino sheep. The microsatellites used in this study showed high levels of polymorphism. A total of 290 alleles were detected in this study. The mean values of polymorphic information content (PIC=0.90), observed heterozygosity (Ho=0.89) and expected heterozygosity (He=0.91) were high, suggesting that the total analysed population is characterized by noticeable genetic variability. Ten out of the fourteen microsatellite markers studied had a positive F_{IS} value. The mean value of F_{IS} was 0.032. The infinite allele model (IAM), two-phase mutation model (TPM) and stepwise mutation model (SMM) in the Bottleneck software were used to check genetic bottlenecks. The L-shaped curve obtained from the analysis indicates the absence of a bottleneck in the Karacabey Merino sheep population studied.

Keywords: *Genetic bottleneck, Genetic diversity, Karacabey merino sheep, Microsatellite*

Karacabey Merinos Koyunu Yetiştirme Çiftliğinde Mikro Uydu İşaretleyiciler Kullanılarak Irk İçi Genetik Çeşitlilik ve Genetik Darboğaz Testleri

Öz: Sunulan çalışmada bir Karacabey Merinosu koyun çiftliğinde populasyon içi genetik çeşitlilik ve darboğaz durumunun FAO tarafından önerilen 14 mikrosatellit belirteç yardımıyla ortaya konması amaçlanmıştır. Bu çalışma, Bandırma'da bir koyun yetiştirme çiftliğinde akraba olmayan 103 Karacabey merinos koyunu ile yürütülmüştür. Kullanılan mikrosatellitler yüksek düzeyde polimorfizm göstermiştir. Çalışmada toplam 290 allel gözlemlenmiştir. Polimorfik bilgi içeriği (PIC=0.90), gözlemlenen heterezigotluk (Ho=0.89) ve beklenen heterozigotluk (He=0.91) değerlerine ait genel ortalamalar çalışılan populasyonların dikkat çekici düzeyde genetic çeşitliliğe sahip olduğunu göstermektedir. İncelenen on dört mikrosatellit işaretleyicinin onunda pozitif F_{IS} değerleri gözlemlenmiştir. F_{IS} değerlerinin ortalaması 0.032 olmuştur. Çalışılan ırktaki genetik darboğaz durumunun kontrolünü sağlamak Bottleneck programındaki sonsuz alel modeli (IAM), iki fazlı mutasyon modeli (TPM) ve aşamalı mutasyon modeli (SMM) kullanılmıştır. Analizden elde edilen mode-shift grafiğindeki L şeklindeki eğri, çalışılan Karacabey Merinosu koyun populasyonunun yakın zamanlarda herhangi bir genetik darboğaza girmediğini göstermiştir.

Anahtar sözcükler: *Genetik çeşitlilik, Genetik darboğaz, Karacabey merinos koyunu, Mikrosatellit*

Introduction

Türkiye has rich genetic diversity in terms of sheep breeding. Nevertheless, it is known that there have been losses in terms of farm animal genetic resources in the past 50 years. When evaluated on a world scale, 14% of the sheep breeds in the world, especially in Europe, have disappeared [1]. Despite these aforementioned adversities, issues such as the fact that Türkiye is in a suitable geography for different animal production models with its ecological and genetic richness, the role of animal production in rural development, and the protection of animal genetic resources, which have been developing in recent years, increase their importance [2,3].

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(*) Corresponding author: Adem KABASAKAL

Phone: +90 266 606 8401 Celluler phone: +90 542 343 8156 Fax: +90 266 717 0030
E-mail: akabasakal@bandirma.edu.tr

Genetic diversity is variation in the genetic material possessed by individuals in a population. Genetic diversity, calculated by characterizing statistically, includes allelic diversity, allele richness, observed and expected heterozygosity at the population level [4]. As the number of individuals in the population decreases, genetic diversity decreases, as it increases, genetic diversity increases and this is associated with high evolutionary resistance [5]. To maintain genetic diversity and variability, the number of individuals capable of effective reproduction within the population is important. Genetic bottlenecks occur when the effective population size is subject to serious reductions due to human influence, environmental effects, diseases and inbreeding. Genetic bottlenecks resulting from a founding event cause loss of genetic diversity in the population [6-8]. On the other hand, high inbreeding in small populations is one of the important causes of loss of genetic diversity [9].

Genetic drift as a result of the decrease due to the genetic bottleneck causes a decrease in the number of alleles in the population and especially the loss of rare alleles. For this reason, the occurrence of genetic bottlenecks that cause a decrease in genetic diversity should be genetically monitored [10-12]. The study of genetic diversity is especially important for the conservation and continuation of genetic resources [13]. All over the world, studies have been carried out using various molecular techniques based on DNA to reveal genetic variation in sheep breeds and to define intra and interbreed diversity. Molecular definitions for populations that are valuable genetic resources play a guiding role in conservation programs and the effectiveness of conservation activities can be tested with these studies. Genetic diversity can only be revealed in a healthy way with definitions made at the DNA level [14]. Through the molecular techniques, local gene resources, evaluation of conservation studies in public and institutes at the molecular level, genetic similarity, intrabreed difference, genetic diversity, possible bottleneck and genetic drift in populations can be determined. For this purpose, it was reported by FAO [15] that microsatellite markers can be used safely to reveal intra and interbreed genetic diversity in genetic resources.

FAO [15] has recommended over 30 microsatellite loci that can be used for genetic diversity. However, the use of such a large number of microsatellites causes high costs in genetic diversity studies [16]. Yılmaz et al.[17] tested the reliability of microsatellite panels with different loci numbers, and the reliability of panels with 8 or fewer loci was found to be low. They found that panels with 12 or more loci can be used with high reliability.

Molecular markers are genetic markers used to evaluate genetic differences between two or more individuals. They are capable of detecting polymorphism that exists in a genetically related population [16]. Microsatellites are found in large numbers in the genome, represent noncoding intron regions of DNA, and have multiple and codominant inheritance. For this reason, it is widely used in studies of detecting intra and interbreed genetic diversity and detecting bottlenecks in farm animals [6,14,18-21]. Particularly in small populations, genetic bottlenecks may cause a decrease in genetic diversity due to genetic drift and inbreeding. Models used to understand the processes that lead to reduced genetic diversity must yield consistent results. For this reason, it has been reported that the two-phase mutation model (TPM) is the most useful model to test the excess heterozygosity in bottleneck tests with microsatellites [22].

In this study, we aimed to reveal the parameters of the genetic diversity, similarity and inbreeding levels and the bottleneck status with the help of microsatellite markers of Karacabey Merino sheep bred on a farm in Bandırma, Türkiye.

Material and Methods

Ethical Statement

The study was conducted with the permission of the Balikesir University Animal Experiments Local Ethics Committee dated 27.10.2021 and numbered 2019/9-5.

Animals

The animal material of the study consisted of a total of 103 unrelated Karacabey Merino sheep in a farm operating in Bandırma, Balıkesir.

Blood Sample Collection

After taking the necessary precautions to prevent direct contact with blood, the animal to be bled was sedated, and the vein was slowly entered with a vacuum needle placed in the needle holder by slowly pressing the vena jugularis from the lateral side. Then, the blood was filled into a vacuum tube containing K3-EDTA in a controlled manner. Animal number, gender and date were written on the tubes from which approximately 10 mL of blood was collected and stored at -20°C until use.

DNA Isolation from Blood

DNA from blood samples was isolated using a commercial isolation kit (Applied Biological Materials Column-Pure Blood Genomic DNA Kit, Canada). The quantity and quality of the obtained DNA samples were checked with a NanoDrop 2000 (ThermoScientific, USA).

Microsatellite Markers Used in the Study

Fourteen microsatellite markers recommended by FAO were used in the study [15]. For use in capillary electrophoresis and fragment analysis, the forward primer of

Table 1. *Some information about the microsatellites used in thestudy*

Multiplex	Marker	Microsatellite	Primary Base Sequence		Allel Size Ranges
M1	D2	OarFCB20	F	AAATGTGTTTAAGATTCCATACAGTG	92-118
			R	GGAAAACCCCCATATATACCTATAC	
	D2	OarAE0129	F	AATCCAGTGTGTGAAAGACTAATCCAG	135-165
			R	GTAGATCAAGATATAGAATATTTTTCAACACC	
	D3	INRA0023	F	GAGTAGAGCTACAAGATAAACTTC	195-225
			R	TAACTACAGGGTGTTAGATGAACTC	
	D3	OARFCB193	F	TTCATCTCAGACTGGGATTCAGAAAGGC	96-136
			R	GCTTGGAAATAACCCTCCTGCATCCC	
	D4	INRA0132	F	AACATTTCAGCTGATGGTGGC	152-172
			R	TTCTGTTTTGAGTGGTAAGCTG	
	D4	D5S2	F	TACTCGTAGGGCAGGCTGCCTG	190-210
			R	GAGACCTCAGGGTTGGTGATCAG	
	D4	BM1818	F	AGCTGGGAATATAACCAAAGG	258-270
			R	AGTGCTTTCAAGGTCCATGC	
M2	D2	OARJMP29	F	GTATACACGTGGACACCGCTTTGTAC	96-150
			R	GAAGTGGCAAGATTCAGAGGGGAAG	
	D3	BM8125	F	CTCTATCTGTGGAAAAGGTGGG	110-130
			R	GGGGGTTAGACTTCAACATACG	
	D3	McM0527	F	GTCCATTGCCTCAAATCAATTC	165-179
			R	AAACCACTTGACTACTCCCCAA	
	D3	CSRD0247	F	GGACTTGCCAGAACTCTGCAAT	209-261
			R	CACTGTGGTTTGTATTAGTCAGG	
	D4	OARFCB128	F	ATTAAAGCATCTTCTCTTTATTTCCTCGC	96-130
			R	CAGCTGAGCAACTAAGACATACATGCG	
	D3	BM8125	F	AGTGCTTTCAAGGTCCATGC	110-130
			R	CTCTATCTGTGGAAAAGGTGGG	
	D4	HSC	F	CTGCCAATGCAGAGACACAAGA	267-301
			R	GTCTGTCTCCTGTCTTGTCATC	

each locus is labeled with a WELL-RED (D4, D3 or D2) fluorescentdye suitable for the Beckman CoulterGe XP Genetic Analysis System. Detailed information about the microsatellites used and the fluorescent dyes used in marking are given in *Table 1*.

DNA Amplificationby PCR

In the PCR stage, 0.2 mL thin-walled Eppendorf tubes were used to amplify the primer-specific regions. 10X PCR Buffer, $MgCl_2$, dNTP mix (dATP, dTTP, dGTP, dCTP), 18 fluorescently labeled microsatellite markers used in the study (Sigma, Interlab), Taq DNA Polymerase Enzyme, ~100 ng Genomic DNA and sterile PCR mix containing ddH_2O was created. In this study, the touch-down (TD) PCR technique was applied to perform DNA replication more effectively and quickly. Optimization of this PCR method is accomplished by focusing on annealing (heat of adhesion) rather than buffers used and cycling conditions. TD-PCR is widely used in studies with markers with unknown annealing temperatures. In this method, annealing degrees are arranged to change sequentially during the course of a single cycle program. Since the ratio of the target sequence that begins to be amplified in the template population increases, only the target sequence increases at decreasing temperature [23].

The PCR programs specific to the multiplex groups used for amplification of the DNA regions specific to the primers in the thermal converter are summarized in *Table 2*.

Statistical Analysis

Allele counts (Na), mean allele count (MNa), effective allele number (Ne), polymorphic information content (PIC),

Table 2. *Thermalcycler conditions according to the touchdown PCR method*

Multiplex Group	I. Denaturation	II. Denaturation	Annealing	Extension	Cycle	Final Extention
1	95°C (5 min)	95°C (40sec)	63-54°C (40 sec)	72°C (60 sec)	40	72°C (10 min)
2	95°C (5 min)	95°C (40sec)	60-50°C (40 sec)	72°C (60 sec)	34	72°C (10 min)

observed heterozygosity (Ho), expected heterozygosity (He), compliance with Hardy-Weinberg equilibrium, Wright's F- statistics (F_{IS}) [24,25] and null allele frequencies GenAlEx [26,27], POPGENE [28] and CERVUS 3.0.3 [29,30] were calculated using the programs.

The population structure was tested in the STRUCTURE program [31,32] using the clustering technique based on the Bayesian approach. In STRUCTURE analyses using independent allele frequencies and admixture model, the length value was taken as 20.000 and the Markov Chain Monte Carlo iteration number as 100.000, and the analysis was performed with 20 replications at different K values (K=2-5). The CLUMPAK [33,34] program was used to generate alignment charts from the obtained STRUCTURE results. The most appropriate cluster (cluster-K) value from the findings obtained as a result of the analysis was determined by considering the method (ΔK= m|L''(K)|/s[L(K)]) reported by Evanno et al.[35]. The STRUCTURE HARVESTER program was used to determine the cluster-K value [36]. To reveal the status of the populations in terms of genetic bottlenecks, the data set was tested using IAM (InfiniteAllel Model), SMM (Stepwise Mutation Model) and TPM (TwoPhase Mutation Model) in the Bottleneck 1.2.0.2 program [37], using Sign, Standardized and Wilcoxon tests and 1000 simulations.

Results

In this study, 290 alleles belonging to 14 microsatellite loci from the Karacabey Merino sheep breed were determined. Molecular genetic polymorphism statistics are presented in *Table 3*.

The highest number of alleles was obtained from HSC(28), and the lowest number of alleles was obtained from INRA0132(14). The overall mean of the observed heterozygosity value (0.89) was lower than the expected heterozygosity value (0.91).

The PIC values of microsatellite markers greater than 0.5 and the number of alleles greater than 4 in genetic diversity studies are an indication that these markers can be used in population genetic analyses [38,39]. The lowest PIC value obtained from the markers used in the present study was obtained from INRA0132 (0.86), and the highest was obtained from McM0527 and HSC (0.93). According to the results obtained, the average allele numbers and PIC values show that the studied breeds have high genetic diversity.

Table 3. *Polymorphism statistics of microsatellite loci*

Locus	N	Na	Ne	Ho	He	PIC	F_{IS}	HWE	F(Null)
OarFCB193	108	22	12.01	0.89	0.92	0.91	0.035	ns	0.0148
INRA0023	106	17	10.09	0.87	0.90	0.89	0.041	ns	0.0188
OarFCB20	106	21	13.03	0.93	0.92	0.92	-0.007	*	-0.0057
BM1818	106	19	10.91	0.82	0.91	0.90	0.101	ns	0.0508
INRA0132	107	14	8.00	0.84	0.87	0.86	0.043	ns	0.0171
OARAE129	108	18	10.37	0.94	0.90	0.90	-0.030	ns	-0.0158
D5S2	108	15	10.31	0.93	0.90	0.90	-0.021	***	-0.0136
CSRD0247	107	25	13.38	0.85	0.93	0.92	0.086	ns	0.0423
McM0527	106	22	14.11	0.85	0.93	0.93	0.091	ns	0.0457
HSC	106	28	14.85	0.88	0.93	0.93	0.064	ns	0.0306
OarFCB128	105	20	11.82	0.88	0.92	0.91	0.048	ns	0.0216
OarJMP29	107	26	11.82	0.88	0.92	0.91	0.045	ns	0.0214
MAF214	107	25	10.76	0.89	0.91	0.90	0.026	ns	0.0107
BM8125	108	18	10.33	0.98	0.90	0.90	-0.082	***	-0.0433
Mean		20.71	11.56	0.89	0.91	0.90	0.032		

Na:*Number of alleles,* ***Ne:****Effective number of alleles,* ***PIC:****Polymorphic information content,* ***Ho:****Observed heterozygosity,* ***He:****Expected heterozygosity,* F_{IS}*:Wright's F-statistics (According to Wright's statistics to Weir and Cockerham*[25]*,* ***HWE:****Hardy-Weinberg equilibrium,* ***F (Null):*** *Null allele frequency* P<0.05, ** P<0.01, *** P<0.001*

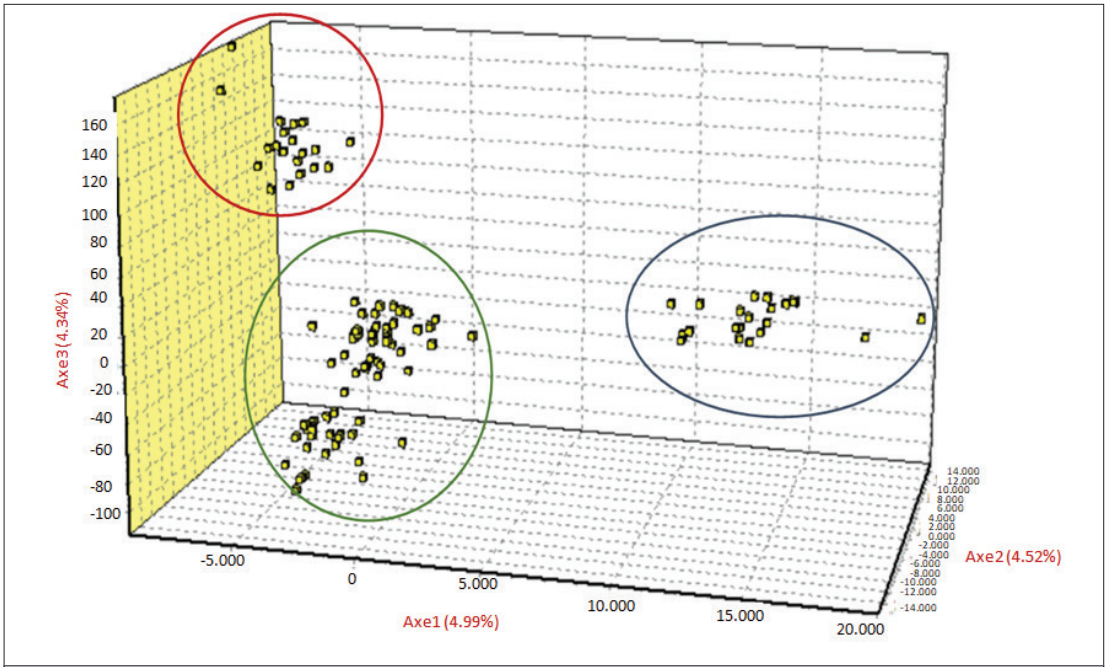

Fig 1. Graph of factorial relationship analysis (FCA) of individuals in the study

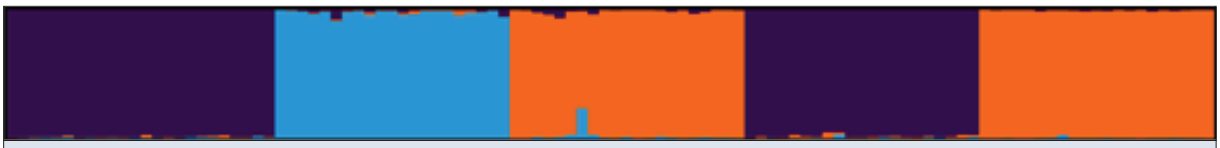

Fig 2. Structure test analysisresults in the Karacabey Merinopopulation (K=4)

F_{IS} values, which are a measure of the deviations of genotypic frequencies from Panmixia in populations, are a parameter used to determine heterozygous deficiency or excess. The overall F_{IS} value calculated for all populations in the study was 0.032. F_{IS} values, which are of great importance in terms of defining the population structure and determining heterozygosity losses in the study, varied between 0.082 and 0.101. When the general average of the F_{IS} value, which is defined as the inbreeding coefficient, is examined, it can be said that there is no loss of heterozygosity in the population. In the chi-square test, it was determined that allele distributions of 11 loci, excluding 3 microsatellite loci, did not statistically deviate from Hardy-Weinberg equilibrium.

The graph of the factorial relationship analysis (FCA) of the individuals included in the study is given in *Fig. 1*, and the STRUCTURE analysis results containing different clustering numbers (K=2-4) are given in *Fig. 2*.

The results of the factorial relationship analysis (FCA) indicate that there are 3 different groups in the studied population. Similarly, the results obtained for the STRUCTURE analysis are in agreement with the FCA results, as expected.

Findings including the estimation of posterior probabilities ([Ln Pr(X|K)]) for clustering numbers (K) and ΔK values are presented in *Table 4*.

Table 4. *Estimated posterior probabilities [Ln Pr(X|K)] and ΔK statistics*

K	[Ln Pr(X|K)]	ΔK
2	-7436.1250	-
3	-6976.8750	3.1499
4	-6616.4150	-

Particularly in the STRUCTURE analysis, it was observed that the studied populations were partially intertwined [36]. It is noteworthy that the most appropriate number of groups was 3 *(Table 4)*.

Genetic bottlenecks were investigated using the Infinite Allele Model (IAM), Stepwise Mutation Model (SMM), and Two Phase Model of Mutation (TPM) [37,40,41]. These three distinct mutation models were examined using the obtained data set *(Table 5)*.

To identify potential bottlenecks in the studied population, Mod-shift plots were obtained using allele frequency classes of 14 microsatellite loci *(Fig. 3)*. An L-shaped graph consistent with the distribution ranges of the normal frequency class is obtained from the mod-shift plot.

Table 5. *Test results according to three different mutation models forbottleneck analysis*

Mutation Model	Sign Test			Standardized Differences Test		Wilcoxon Rank Test (One Tail for H Excess)
	Hee	He	P	T2	P	P
IAM	16.44	19	0.20937	2.267	0.01168	0.00480
TPM	16.01	9	0.00577	-4.577	0.00000	0.99131
SMM	15.80	3	0.00000	-17.356	0.00000	1.00000

IAM: *The infinite allele model,* ***TPM:*** *Two-phase model,* ***SMM:*** *The stepwise mutation model,* ***Hee:*** *Expected number of loci with heterozygosity excess,* ***He:*** *heterozygosity excess*

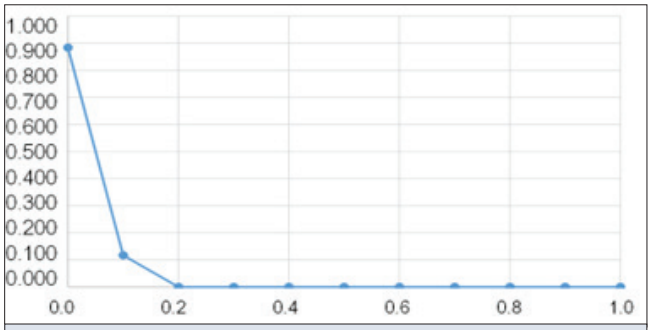

Fig 3. Mode-shift plot for bottleneck analysis in the studied population

Discussion

The variation between changes in allele number and heterozygosity has been used as a basis for statistical testing in identifying recent genetic bottlenecks in the population [37]. The PIC value indicates the probability of the presence or absence of that marker in two randomly selected individuals in a population [16]. Accordingly, the PIC value ranges from 0 to 1 and should be 0.50 for genetic diversity. Bostein et al.[42] classified the PIC value as highly informative (PIC >0.5), reasonably informative (0.5> PIC >0.25), and slightly informative (PIC <0.25). The PIC value close to 1 is desirable for high genetic diversity in that population [42]. Considering that the PIC values of microsatellite markers must be greater than 0.5 and the number of alleles must be greater than 4 to discuss genetic diversity, the results obtained in the study are quite high. As a matter of fact, as a result of the findings in the study, it is seen that the PIC value varies between 0.86-0.93 and its average is 0.90, and the average number of alleles varies between 14-28 and the average is 20.71. Considering the average allele numbers and PIC values, the population has high genetic diversity. The Na, PIC, Ho, and He values obtained in the aforementioned population were observed to be higher than the values obtained in previous similar studies on Turkish native breeds and foreign breeds [12,43-45]. High PIC value and allele number indicate that the studied population has high genetic diversity and no genetic bottleneck. It can be said that genetic diversity will continue in the future unless there are situations that will reduce genetic diversity, such as inbreeding.

While the overall F_{IS} value obtained in the study [44] was observed to be low, some studies [16,46-50] reported higher values. Although it is considered that these results are due to the difference in the number of microsatellites used in the relevant studies and the ampling methodology, attention should be paid to the loss of heterozygosity that may occur in existing populations. It was reported by Dakin and Avise [49] that null allele frequencies below 0.20 do not have a significant effect on paternity tests and determination of genetic diversity. When an allele is not oxidized by polymerase chain reaction (PCR) in heterozygous individuals, but only one allele gives a peak such as homozygous and thus causes erroneous reading, it is noteworthy that all studied loci are lower than the specified value. This indicates that the loci used in the study can be used safely.

Infinite allele models (IAMs) and stepwise mutation models (SMMs) are known to cause inconsistent results in studies using microsatellites. Therefore, the two-phase mutation model (TPM) has been reported to be the most useful model for testing heterozygous excess in bottleneck tests with microsatellites [37,41,50]. On the otherhand, it has been reported that the Wilcoxon test can be used with high confidence even in studies using a limited number of loci (<20) for bottleneck analysis [45] In the sheep population that is the subject of the research, Wilcoxon test results, which were carried out considering the TPM model, indicate that serious demographic bottlenecks do not occur.

First, Luikart et al.[41], the L-shaped graph obtained from the mode-shift plot, which graphically shows the allele frequency distribution, which is widely used in the detection of bottlenecks, shows that no genetic bottlenecks have occurred in the studied population in the recent past.

One of the limitations of this study may be the determination of the number of sheep used within the scope of the Project possibilities. However, this situation can be ignored as the situation in a single sheep farm is tried to be determined in the study.

In conclusion molecular genetic studies to identify the variation within and between populations of sheep breeds have been ongoing for a long time. Determining the relationships of individuals with each other in revealing the kinship within the same herd is very important for a healthy selection practice. In this context, this study makes important contributions to the literature. The findings obtained in the study show that the microsatellite markers used are polymorphic and can be used successfully in genetic diversity studies. The bottleneck test was used

to determine whether there was any genetic bottleneck danger as a result of increased inbreeding levels in the studied population. The findings indicated that the population did not enter any bottleneck in the recent past. As a result, the findings obtained from this study clearly revealed that the microsatellites used can be used safely in the identification of genetic diversity and detection of genetic bottlenecks in the studied Karacabey Merino population. At the same time, it can be said that the microsatellite markers used in the study can be used safely in future studies.

Availability of Data andMaterials

Data sets are not deposited in different repositories, and data from a third party were not used. The data are original, and users can get it from corresponding author (A. Kabasakal).

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CompetingInterest

The author reports no declarations of interest.

Ethical Statement

The study was conducted with the permission of the Balikesir University Animal Experiments Local Ethics Committee dated 27.10.2021 and numbered 2019/9-5.

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