Genetic characterization of the Akkaraman, Morkaraman and Karayaka sheep through microsatellite variation

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ABSTRACT

Genotypes have to be controlled periodically and necessary precautions should be taken in order to ensure that animal biodiversity is not lost. In this study, the genotypes of the Akkaraman, Karayaka and Morkaraman species from among Turkey's local sheep were examined at 7 different microsatellite loci. The average allele counts are close to each other and were determined as 8,905±0,483. Whereas the PIC values ranged from between 0,507-0,794 at an average of 0,705. The He and Ho values used to calculate genetic diversity were determined on average as 0,734±0,023 and 0,509±0,038 respectively. According to the Fisher-Exact test result, the populations are not in HWE (P<0.001). Whereas the F_{IS} values that show inter-population genetic diversity were determined as 0,353±0,092 (KRY), 0,328±0,095 (MRK) and 0,237±0,059 (AKK) respectively. In addition, Population Assignment and Principal Coordinate Analysis graphs were formed. When the pairwise F_{ST} and N_m, NeiuD, $^SH_{UA}$ and G Statistics values used to calculate the genetic similarities and differences are examined, it is observed that the closest relations in terms of genetics is among AKK-KRY followed respectively by AKK-MRK and KRY-MRK.

Keywords: Anatolian sheeps, Genetic diversity, Microsatellite, Polymorphism.

ABBREVIATIONS

AKK: Akkaraman, **KRY**: Karayaka, **MRK**: Morkaraman, **Na**: Number of Different Alleles, **Na (Freq >= 5%)**: Number of Different Alleles with a Frequency >= 5%, **Ne**: Number of Effective Alleles, **MNA**: Mean Number of Alleles, **I**: Shannon's Information Index, **He**: Expected Heterozygosity, **uHe**: Unbiased Expected Heterozygosity, N_m: Gene flow, F_{IT}, F_{ST}, F_{IS}: Wright's F Statistics, **Gst**: Analog of Fst, adjusted for bias., **G'stN**: Nei's standardized Gst, **G'stH**: Hedrick's standardized Gst, **NeiuD**: Nei's unbiased genetic distance, **Dest**: Jost's estimate of differentiation, ^SHU_A: Shannon's Mutual Information index, **PCoA**: Principal coordinate analysis, **HWE**: Hardy-Weinberg Equilibrium, **PIC**: Polymorphism information content

INTRODUCTION

The animal biodiversity in todays world needs to be protected from extinction and demands special attention. (Ceballos *et al.,* 2010). The similarities and differences between the genomic structure of breeds should be determined and biodiversity needs to be protected (Soysal *et al.,* 2005). It is not enough to solely detect the genetic structure of populations; these parameters should also be routinely examined according to a certain schedule since they evolve continuously and thus their genetic diversity should be protected by monitoring which breeds resemble others at which regions.

Turkey is a host to a wide genetic diversity with live migration routes due to its strategic geographical location connecting Asia, Europe and Middle East (Soysal *et al.,* 2003). The sheep breeds of Turkey namely, Akkaraman (AKK), Morkaraman (MRK) and Karayaka (KRY) have currently been specified as breeds that are local sheep (Ertugrul *et al.,* 2009). AKK is mostly in Central Anatolia. Its body is of white color with coarse-tangled wool, fatty tail and a milk yield of 50-60 kg per lactation having a body weight of 45-50 kg. MRK is bred in Eastern Anatolia. Its body is of red color with slightly better quality wool than AKK, ewes body weight ranging from 50-60kg and lactation milk yield of 80-90 kg. KRY breed is in the Black Sea region. Its body is of white color with coarse wool having a body weight of 35-40 kg in ewes with a milk yield of 30-45 kg (Yilmaz *et al.,* 2012). The breeding areas of these were shown

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in Figure (1). MRK and AKK resemble each other for having fat tails, while AKK and KRY have another similarity due to white wool colors. There is genetic relationship among breeds in accordance with the geographic proximity. But, there are different conclusions about which of these are more similar (Özdemir, 2013; Uzun *et al.,* 2006; Acan, 2012; Acar 2010). The objective of this study was to examine the genetic structures, similarities and differences of AKK, KRY and MRK sheep breeds using microsatellite markers, uncover the truth of different results of early studies.

MATERIALS AND METHODS

Sampling: A total of 90 sheep from Gumushane and vicinity were used in this study on August-November 2013, 30 each from 3 different sheep breeds (AKK, KRY and MRK). Sheep were selected carefully so that the selected sheep display the characteristics of their own breed and are genetically unrelated. Blood samples were collected in 10 ml vacutainer EDTA tubes and transported to the laboratory via cold chain. The samples were kept in a deep freezer at -80 °C until the examination time.

DNA extraction: DNA isolation from the blood samples were made via Qiagen DNeasy® Blood & Tissue kit (Cat no:69504). The estimation of the concentration and purity of DNA samples was made using UV/visible spectrophotometer (BIO-RAD, The SmartSpec Plus) by reading the optical densities at wavelengths of 260 and 280. The acquired DNA samples were stored at -20 °C until usage time.

PCR optimization and monitoring of products: The primers that had been used with high polymorphic features (Table 1) were preferred in NCBI database to determine the microsatellite loci. Initially, non-fluorescence primers were synthesized to optimize PCR conditions for microsatellite loci. Type-it Microsatellite PCR Kit (Qiagen, Cat no: 206243) was used in the study, hence only binding temperature was optimized and gradient PCR was performed. The PCR products were run on 2% agarose gel with Ethidium Bromide (1mg/100ml). The gel images were recorded using the gel documentation system (Biostep, Darkhood DH-30/32) and the thermal printer (Mitsubishi, P91D). At the end of the optimization, binding temperatures were determined as 58 °C for TGLA387, BM848, BM4505 and BM1225 loci and as 60 °C for BMC1222, OarAE16 and TGLA122 loci.

Multiplex PCR reaction: Afterwards, forward primers marked with fluorescent were subjected to two different Multiplex PCR and PCR products were mixed with each other so that the results could be read at once and 96-Well Standard Microplate was formed. Each 50 μl. PCR reaction mixture contained genomic DNA 1 μl (60 ng), 1 μl (0.2 μM) forward and reverse primers of each loci , PCR Master Mix 25 μl and ddH$_2$O 16 μl. Each group of PCRs were run for 30 cycles on Thermal Cycler as shown in the Table 1. Fragment analysis was carried out on PCR products in ABI PRISM™ 3500 Genetic Analyzer (PE Applied Biosystems, MA) device. The process was repeated for samples that yielded no results. All loci were successfully amplified.

FIG 1: Breeding areas of AKK, KRY and MRK sheep in Turkey.
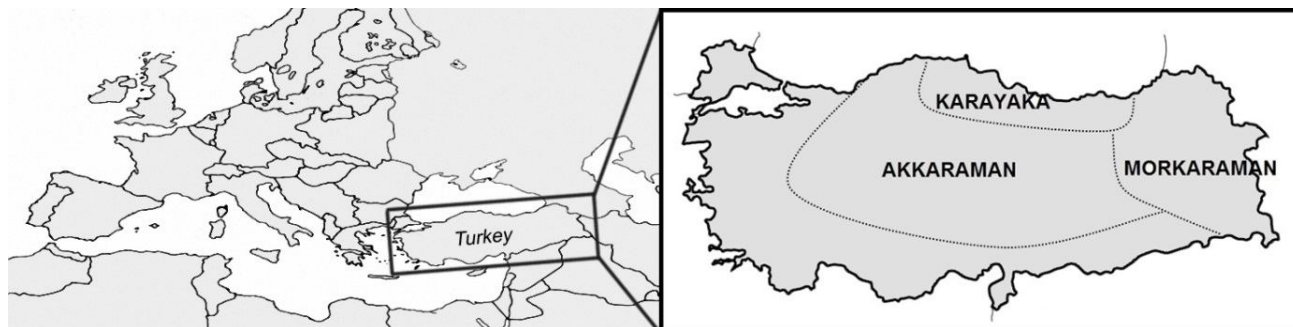
TABLE 1: Shows the PCR groups, fluorochrome dyes, binding temperatures, chromosome loci, fragment length intervals, genebank access numbers and the animal species that the SSR marker belongs to.

| PCR Groups	Fluoroch.Dye	Marker	Ann. Temp.	Chr.	Length (bp)	NCBI	Origin
1	FAM	TGLA387	58°C	20	120-174	Pr009692702	O. Aries
	VIC	BM848	58°C	15	214-294	Pr012821500	B. Taurus
	PET	BM4505	58°C	22	253-298	Pr012824667	O. Aries
	NED	BM1225	58°C	16	240-265	Pr012829797	O. Aries
2	FAM	BMC1222	60°C	13	290-320	Pr012537075	O. Aries
	VIC	OarAE16	60°C	13	84-112	Pr012487259	O. Aries
	PET	TGLA122	60°C	18	134-198	Pr012487076	O. Aries

Computational analysis: Raw data acquired as a result of microsatellite loci analysis were evaluated in GeneMarker v2.6 (Soft Genetics LLC, State Collage, PA, USA) software. The mean number of alleles of breeds and loci, the number of effective alleles, NeiuD and NeiuI, G Statistics, Principal coordinate analysis, Population assignment graph and Shannon's Information Index values were calculated using GenAlEx 6.5 (Peakall and Smouse, 2006) software. The expected and observed heterozygosis values for breeds and loci, their standard deviations and the existence of HWE were calculated via Genepop (v4.2) (Raymond and Rousset, 1995) software. Wrigth's F statistics values were calculated using FSTAT software (Goudet, 1995).

RESULTS AND DISCUSSION

The values obtained for the breeds were given in Figure 2, whereas those for the loci were given in Table 2. A total of 94 alleles were detected for 7 loci and it can be concluded that these loci are highly polymorphic. The MNA

of breeds is close to each other and mean was obtained as $8,905\pm0,483$. The highest MNA was observed in AKK ($9,714\pm0,865$). Highest MNA of loci was observed TGLA122 (13,00) and lowest in BM4505 (7,00). Whereas the PIC values for the same loci were determined to be between 0,507-0,794 with an average value of 0,705. The allele diversity is observed to be sufficient. The minimum and maximum number of effective allele is in the same loci with 5,622 and 2,186 respectively. Shannon's Information Index (I) values that represent the variation level in each population were calculated based on allele frequency values and they were found to be close to each other with an average value of $1,669\pm0,071$. The AKK ($1,686\pm0,109$) among breeds and TGLA122 (2,046) among loci were determined to be high. The H_e and H_o values used in the calculation of genetic diversity were determined on average as $0,734\pm0,023$ and $0,509\pm0,038$ respectively. Whereas the highest H_e value was observed in TGLA122 (0,812), the lowest value was
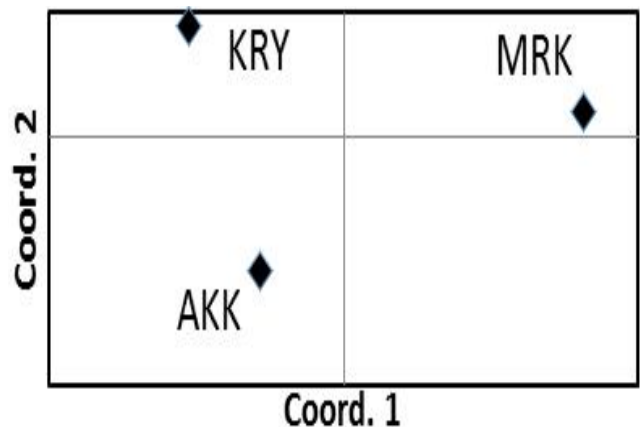
FIG 2: Na, Na Freq>=5%, Ne, I, No Private allelles and He values of AKK, KRY ve MRK breeds
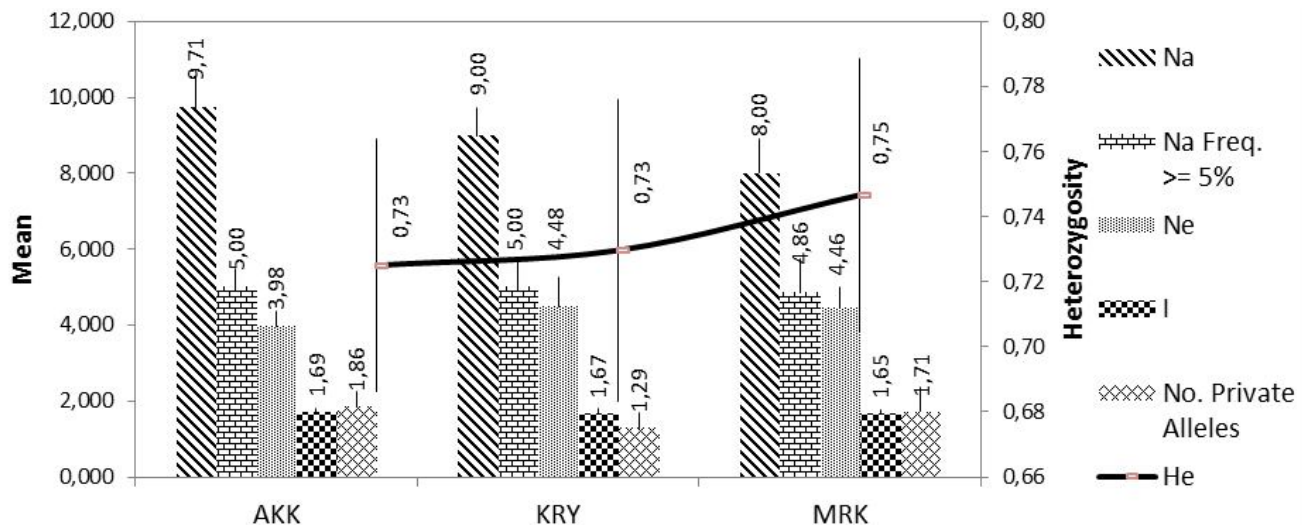
FIG 3: F_{IS} values which are indicators of interracial genetic diversity.
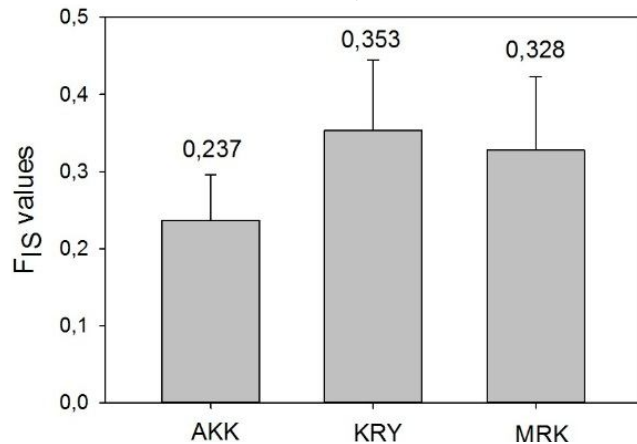
FIG 4: PCoA via Covariance matrix with data standardization along Fst values

determined in BM4505 (0,538). Whereas for H_o values, the highest value was determined as 0,653 in OarAE16 locus and the lowest value was determined as 0,204 in TG387 locus. The highest H_o and H_e values for breeds were observed respectively in AKK (0,550±0,047) and MRK (0,747±0,042) breeds. The populations are not in HWE according to the results of Fisher-Exact test (P<0.001). Whereas the F_{IS} values (Figure 3) that show the interpopulation genetic diversity were determined as 0,353±0,092 (KRY), 0,328±0,095 (MRK) and 0,237±0,059 (AKK). The F_{IT} values for loci were determined to vary between 0,736 (TG387) and 0,175 (OarAE16) (avg. 0,326±0,073), whereas the F_{ST} values were determined to vary between 0,036 (BM848, TG387, TGLA122) and 0,018 (OarAE16) (avg. 0,029±0,003). The PCoA analysis result that enables the visual interpretation of these values obtained according to F_{ST} values was given in Figure 4. The population assignment test result calculated according to allele frequency was given in Figure 5. The G-Statistics values calculated according to all loci were given in Figure 6. Pairwise unbiased genetic similarity (NeiuI) and unbiased genetic distance DS (NeiuD) values according to Nei (1978), pairwise population fst values, estimates of gene flow values (nm), pairwise population mean shannon diversity (shua) index over breeds were shown Table 3. When the pairwise F_{ST} and N_m, NeiuD, $^SH_{UA}$ and G Statistics values used to calculate the genetic similarities and differences are examined, it was observed that the highest genetic similarity was between AKK-KRY followed by AKK-MRK and KRY-MRK.
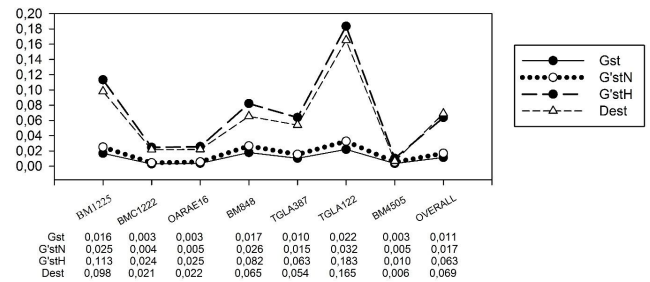
     Deviation from HWE was observed in all breeds at the end of the study. The study carried out by Koban (2004)

has put forth the values of H_o and H_e as AKK 0.66-0.78, MRK 0.69-0.76, KRY 0.74-0.79 respectively. Accordingly, HWE is observed only in KRY in these values. These results unlike from ours. The deviation from HWE of populations under human effect is normal. Because the factors of selection, migration and limited herd size can interfere with population. (Yüce and Bilgin, 2004). F_{IS} values were determined to vary between 0.23 and 0.35 showing that there is a high level of

FIG 5: Population Assignment test visualizing the genetic positions of breeds.
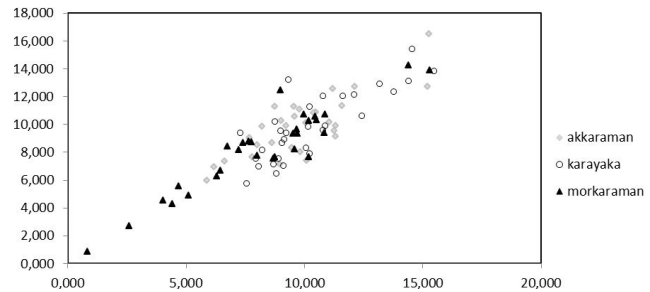
FIG 6: The G-Statistics values calculated by all loci. Gst = Analog of Fst, adjusted for bias., G'stN = G'st(Nei) = Nei's standardized Gst, G'stH = G'st(Hed) = Hedrick's standardized Gst, Dest = Jost's estimate of differentiation.

	BM1225	BMC1222	OARAE16	BM848	TGLA387	TGLA122	BM4505	OVERALL
Gst	0,016	0,003	0,003	0,017	0,010	0,022	0,003	0,011
G'stN	0,025	0,004	0,005	0,026	0,015	0,032	0,005	0,017
G'stH	0,113	0,024	0,025	0,082	0,063	0,183	0,010	0,063
Dest	0,098	0,021	0,022	0,065	0,054	0,165	0,006	0,069

TABLE 2: Allele characteristics calculated according to the all loci

Locus	N_a	N_e	I	H_0	H_e	F_{IT}	F_{ST}	PIC
BMC1225	7,667	4,502	1,675	0,619	0,773	0,225	0,032	0,741
MC1222	8,667	5,274	1,852	0,578	0,805	0,296	0,020	0,783
OarAE16	8,667	4,542	1,726	0,653	0,777	0,175	0,018	0,747
BM848	8,667	3,447	1,553	0,433	0,686	0,392	0,036	0,661
TG387	8,667	4,541	1,677	0,204	0,745	0,736	0,036	0,705
TGLA122	13,000	5,622	2,046	0,646	0,812	0,233	0,036	0,794
BM4505	7,000	2,186	1,157	0,428	0,538	0,223	0,022	0,507
Grand Mean and SE over Loci								
Mean	8,905	4,302	1,669	0,509	0,734	0,326	0,029	0,705
SE	0,483	0,334	0,071	0,038	0,023	0,073	0,003	-

TABLE 3: Pairwise unbiased genetic similarity (Nei uI) and unbiased genetic distance DS (Nei uD) values according to Nei (1978), Pairwise Population Fst Values, Estimates of Gene flow values (N_m), Pairwise population mean shannon diversity ($^SH_{UA}$) index over breeds.

	Nei uD	Nei uI	F_{ST}	N_m	$^SH_{UA}$
AKK-KRY	0,036	0,965	0,015	16,772	0,081
AKK-MRK	0,077	0,926	0,023	10,686	0,125
MRK-KRY	0,111	0,895	0,027	9,018	0,132

homozygosity in the genes as well as inbreeding. The F_{IS} results are higher in comparison with those of other researchers (Koban, 2004; Uzun, 2006; Acar, 2010; Acan, 2012). There might be many reasons for this such as; inbreeding, existence of null alleles, selection towards homozygosis and population subdivision (Wahlund effect). The reasons in this study are thought to be that the specimens are geographically close to each other (migration effect), inbreeding effect and the small size of the herds. Inbreeding is very risky, because it causes generations to weak against genetic disorders (Meszaros *et al.,* 1998; Rijks *et al.,* 2008). Hence, male stud should be included to the herd from different herds every year and genetic diversity should be preserved by preventing inbreed effects.

The average allele number in this study was determined for breeds to be between 8,00-9,71. According to the results of Acar (2010) and Acan (2012), this value varies between 9,25-9,43. The values are close to each other. Whereas the PIC values (0,507-0,794) indicate that these loci give sufficient information.

Interbreed F_{ST} values vary between 0,015-0,027 whereas G_{ST} is between 0,003-0,022 which shows a low differentiation (Wright, 1978; Nei, 1973). The high N_m value (9,01-16,77) among breeds validate this. Ligda *et al.,* (2009) has compared 10 different Greek sheep breeds in their study putting forth an average N_m value of 9,78 which is close to our result. It is smaller than the values (21,0-29,0) put forth by Hoda et al. (2012) for 3 Albanian Sheep. When the values of pairwise F_{ST}, Shannon diversity index ($^SH_{UA}$) showing genetic differentiation along with NeiuD results showing genetic distance and the results of PCoA and Population assignment test are examined, it is observed that AKK-KRY closeness is greater than that of AKK-MRK. The Nm values showing the gene transfer among populations is also in the same lines. Even though these results differ from the results of many other studies (Togan, 2011; Ozdemir, 2011) when the Neighbor-Joining tree and Principal Coordinate Analysis results of the studies carried out by Acan (2012) and Acar (2010) using microsatellite markers are examined, it is observed that the differences of these three breeds are similar to the results of this study. Again, the mtDNA haplotyping study carried out by Demirci *et al.* (2010) has put forth that AKK-KRY closeness is greater than that between AKK-MRK. These similarities determined between breeds might be due to the herd management method of the local people. When samples were being collected, we learned that AKK and KRY are bred together in some herds due to wool color similarity. This common breeding was observed to be more among rams and ewes. It can even be stated that there are many crossbreed sheep in the herds and this crossbreeding is not taken into account because of color similarity. Uzun et al. (2006) have stated that breeds with similar tail structures (fatty-thin) are genetically more close to each other. Since this is the case, it was determined in the field studies carried out that color is more efficient in crossbreeding in comparison with tail structure and hence there is more gene transfer between white colored breeds such as AKK and KRY. This may indicate that expectations of peoples are effective in the formation of new breeds. Another reason for this result might be that a breed with different colors that has mixed with previous generations can be easily distinguished phenotypically. Whereas making this differentiation is harder to same colored breeds. This will in turn lead to the mixing of breeds with similar colors bred in the same geography.

In addition, Koban (2004) has put forth in a study during which 2 AKK and KRY along with one MRK population was mixed into the herd that the highest value of the pairwise D_A results was determined as 0,311 between AKK-KRY whereas the lowest value was determined as 0,130; these values were determined between AKK-MRK as 0,255 and 0,750 and between KRY-MRK as 0,094 and 0,128. According to these results, contradictory results can be acquired between genetic distance values as a result of the comparison of different populations. Because, the genotypes in the same breed vary according to geographical regions (Stephen et al., 2003). This may put forth why the similarity between AKK-KRY is high.

In a study (Özdemir, 2013) during which estimations regarding genetic distance were made based on transferrin in blood and the hemoglobin protein polymorphisms, D_S values were determined as AKK-MRK (0.036) <AKK-KRY (0.044)<KRY-MRK (0.062). Whereas Uzun et al. (2006) have determined D_A values as AKK-MRK (0.053) <KRY-MRK (0. 143) <AKK-KRY (0.151) in loci examined via microsatellite markers. Even though in this result AKK-KRY similarity seems low, the mtDNA study carried out by the same study group one year previously has put forth that KRY is different than others but still they have a surprising maternal lineage with AKK. All these results indicate a close relation between AKK and KRY. In another mtDNA study, Pairwise Nei's D_A results are supported our results as AKK-KRY 0.023< AKK-MRK 0.10< KRY-MRK 0.23 (Yuncu, 2009).

CONCLUSION

1. Microsatellite alleles were defined in 7 loci of 3 Anatolian sheep breeds and genetic distance values, heterozygosis, F Statistics, Population assignment and PCoA graphs were formed. These results will provide a model for the genotype comparisons in future carried out by population geneticists.

2. It was determined as a result of this study that AKK-KRY similarity is greater than that of AKK-MRK. Even though this result is different than various other studies. Some similar results have also been documented.

3. When the results present study and previous studies are compared, it can be concluded that populations from the same breed can present different genetic distance values according to regions. Hence, breeds should be inspected in their regions and genetic maps should be drawn.

4. It was determined that colors play an important role in crossbreeding decision of farmers to bring different breeds together. This indicates that the cultural preferences of people play an important role in the formation of new breeds.

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