Determination of Genetic Polymorphism within Güney Karaman Sheep-Breed via RAPD-PCR Method

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Abstract: Analysis of individual genotypes aims to provide information for understanding within population genetic parameters such as genetic similarity, polymorphism and heterozygosity. The aims of this research were to determine the genetic parameters within Güney Karaman sheep involved in a protection programme of gene resources by using randomly oligonucleotide primers via Randomly Amplified Polymorphic DNA (RAPD) method.

Among the primers tested, 10 most suitable ones, in terms of repeatability and scorability, were used and 1451 DNA fragments were scored. All the RAPD fragments were determined to be between 600 - 3000 bp in size. From 147 fragments, 133 were polymorphic (90.48%) and 14 fragments were monomorphic (9.52%). Within population average genetic similarity (Fragment Sharing Frequency, Fxy) and genetic distance were calculated as 0.7001 and 0.2991, respectively. The average expected heterozygosity was estimated as 0.3273 ± 0.1697.

Keywords: Güney Karaman sheep-breed, polymorphism, RAPD-PCR

Introduction

It is of great importance to determine, at DNA level through molecular techniques, the variations of farm animals that have a huge genetic potential in number and variety, to define them, to prepare schemes to preserve and develop them to this end. The majority of the sheep in Turkey are composed of domestic breeds and types that haven't been refined. Also known as Black sheep, the Güney Karaman sheep breed is raised in South and south-eastern cities, notably on the Taurus Mountains and looks very much like Karagül with its fat-tail. Its proportion is rather low in the Turkish sheep population (Öztürk, 2000).

In sheep breeding, genotype confusion has emerged in domestic sheep breeds, which constitute the gene resources, through a gradual genetic change as a result of selection on the economically important yields in certain breeds. Consequently, the present gene resources are getting lost, and the continuation of offspring is put in jeopardy with the risk of these genotypes disappearing. The disappearance of the domestic breeds means the disappearance of the distinctive traits that they bear. At this stage, it is hard or impossible to say which of these traits will be required in the future. Also, the possible traits of these gene resources, which haven't been identified yet, can be kept available with the survival of these breeds (Ertuğrul et al., 2005). One of the Turkish domestic breeds, the Güney Karaman sheep is a breed in danger of extinction. For this reason, the breed involved is brought under protection within the context of protecting gene resources with the declaration of guidelines for subsidizing animal farming (Rescript No: 2006/9) by the Ministry of Agriculture and Rural Affairs (Anonymous, 2006).

Genetic evaluation has usually started by analysing phenotypes to identify genetic influences, whereas molecular genetics often begins with known alleles or DNA sequences and then examines their influence on phenotypes. Eukaryotic genomes show considerable DNA sequence variations

(polymorphisms) between species and among individuals within a species (Beuzen et al., 2000). Studies of molecular genetics and molecular biology at DNA level have recently come to the fore so as to determine these variations in individuals and populations. Molecular techniques are utilized for determination of the genetic makeup of farm animals at DNA level and for selection studies based on a marker in amelioration studies. It is rather difficult especially in stock raising to detect the genotypes that carry the best alleles by examining the phenotypes of animals which have an economical value, and that have expensive- and difficult-to measure quantitative characteristics. In such cases, so as to determine the individuals that carry the related alleles with DNA markers, intense studies have been conducted in recent years on such quantitative characters as milk yield, resistance to diseases and fecundity (Schnabel et al., 2005). In the QTL analysis concerning quantitative characters, quantitative characters are identified in cattle, sheep and swine for commercial and experimental purposes. The markers that are connected with milk and milk components yields in dairy cattle, birth weights, horn development and preweaning growth in beef cattle, fecundity and muscle hypertrophy in sheep have been identified (Davis and DeNise, 1998).

One of the molecular techniques, the RAPD technique (Randomly Amplified Polymorphic DNA) is based on the amplification of PCR and DNA fragments of randomly spread areas in the genome by using random primers (9-10 bp). Unlike the other PCR applications, only one primer is used for DNA production both in the pattern DNA chain and in the complementary chain. It is required that the ratio of G + C primers used in RAPD techniques be 50–80% , that they not contain any palindromes and that they shouldn't be complementary to each other (Williams et al., 1990; Williams et al., 1993). Compared with other PCR applications, RAPD method is easy to apply, and more preferable because of its low cost and high and quick efficiency. Among its advantages are the sufficiency of a minimum and low quality DNA in nanogram levels and also no prerequisite information regarding DNA sequence. Moreover, the rate of polymorphism is high (Bowditch et al., 1993; Kantanen et al., 1995; Öz Aydın, 2004). It is reported that the disadvantages of this technique are low repeatability with different equipment and chemical material, and low reliability as a result of diverse results in diverse laboratories by diverse researchers. Nevertheless, RAPD technique is fit for automation under laboratory conditions (Tingey and Tufo., 1993).

Along with its widespread use in molecular biology and in many various areas, the RAPD-PCR technique is successfully used in many fields, such as the identification of genetic similarity and difference in livestock, prediction of the genetic relationship between different breeds and ecotypes, identification of wild species (Lee and Chang, 1994), study of evolution (Stepniak et al., 2002), genetic mapping (Cushwa et al., 1996), pedigre determination (Cerit, 2001), determining the sex of ovine embryos (Gutiérrea-Adán at al., 1997), and developing species-related markers. RAPD method have found application in various animal species, such as cattle and sheep (Kantanen et al., 1995; Cushwa et al., 1996; Tahmoorespur et al., 2003; Ahmed Ali, 2003; Paiva et al., 2005), goat (Li et al.,2002; Şahin, 2005), turkeys (Smith et al., 1996), chickens (Smith et al., 1996; Sharma et al., 2001), quails (Sharma et al., 2000), fish (Akhan and Canyurt, 2005), bees (Suazo et al., 1998), horses (Apostolidis, 2001) and pigs (Yen et al., 2001). This has demonstrated the efficiency RAPD as a potential genetic marker. The studies conducted with the RAPD-PCR technique have shown that this method can be successfully used in predicting genetic makeup of species and intra- and inter-population genetic parameters. This study aims to demonstrate both the applicability of RAPD-PCR method and to reveal the DNA fingerprints belonging to the population involved with the aim of identifying, at DNA level, genetic variation of Güney Karaman sheep breed, which constitutes the recent gene resources.

Material and Method

Animal Material and DNA Isolation

In the context of preservation and protection of gene resources, the blood samples that were taken from 8 male and 8 female Güney Karaman sheep, which were kept in Bahri Dağdaş International Agricultural Research and Application Center, were used. For DNA isolation, the blood samples that were taken from *Vena jugularis* of the animals by using vacuum blood tubes with sodium citrate were preserved in cold chain (+4°C), taken to the Molecular Genetic and Biotechnology Laboratory of Faculty of Agriculture in Selcuk University in the shortest possible time, and were kept at -20°C until DNA isolation were achieved. DNA isolation kit (*Bio Basic Inc., Canada*) was used for the purpose of pursuing a method that wouldn't yield different results between different blood samples and that consisted of as few

components as possible in DNA isolation. In the study, the DNA concentrations that dissolved in the 100 ul 1xTE buffer solution were read through spectrophotometer, and the concentrations were equalized with sterile pure water so as to get 20 ng/ul.

PCR Amplification

In the RAPD technique, 10-base primers, some of which had been tried before, were utilized and 10 best efficient primers (18, 19, Op15, Opm10, Opp15, Opq04, Opq06, Ra03, Ra35, Ra59) were chosen in connection with their monomorphic/polymorphic characteristics and with their productivity of sufficient number of strips and then used in RAPD analyses.

The PCR protocol was optimized as Şahin (2005) and Ahmed Ali (2003) reported, and PCR application was carried out. The DNA samples of 1 ul that were obtained as a result of DNA isolation and that were balanced so as to be 20 ng/ul each were put in PCR tubes. Reactions were triggered in thin-walled PCR tubes with a volume of 25 ul in total with 1 ul DNA+24 ul mix. The mix for PCR reaction was prepared with 14.0 ul sterile distilled water, 2.5 ul 10x Buffer pH: 8.5 (48.4 g Tris base, 10.22 ml Glacial Acetic Acid, 20 ml EDTA pH: 8.0), 2.5 ul 25 mM $MgCI_2$, 4.0 ul $dNTP_s$, (2.5 mM from each), 0.5 ul primer (50 pmol/ul) and 0.5 ul *Taq* Polymerase (*Taq Bioron 5 U/ul*). The PCR applications conducted in Thermal Cycler (*Eppendorf Master Gradient*) were exposed to 40 cycles in total- hot start at 94°C for 2 min., at denaturation level at 94°C for 50 sec., at annealing stage at 32-34°C for 55 sec. and at extension stage at 72°C for 50 sec and final extension at 72°C for 5 min. Some optimizations were made in the heat and duration units connected to the primers that were used. The RAPD fragments were separated on a 1.5% agarose gel by electrophoresis. Then it was taken out of the gel solution containing 0.5 µg/ml Ethidium Bromide, RAPD fragments were viewed in gel documentation system under UV light with the help of transilluminator. The resulting data were stored in an electronic setting. DNA Moleculer Weight Marker (100 bp Ladder, *AMRESCO*) was used as standard to determine the size range of amplified bands (bp) on which RAPD fragments moved in gel according to their molecule sizes.

Scoring and Statistical Analysis

The photographs related to RAPD fragments were scored on the basis of present/absent (1=present, 0=absent), and data matrix was formed. By using this obtained data matrix, genetic similarity between individuals, the ratio of polymorphism, the values of heterozygosity were obtained through POPGEN-1.32 pc program, and the dendogram that showed genotypical relation between individuals and PCoA (Principle Coordinates Analysis) were obtained according to the UPGMA method through NTSYS-2.1 pc (Numerical Taxonomy and Multivariate Analysis System) program. The genetic similarity between individuals (Fxy) was calculated using the formula below as defined by Nei (1987).

$$Fxy = 2\ Mxy / (Mx + My)$$

Fxy: Genetic similarity; Mxy: The number of common RAPD fragments between sheep X and Y, Mx: The total RAPD fragment number of sheep X, My: The total RAPD fragment number of sheep Y

In calculating the average value of heterozygosity (H), the formula given below was used (Nei, 1987). $H=\Sigma h/r$; In the formula; r: The number of locus, h: The expected single locus heterozygosity, which was calculated as; $h=1- \Sigma X_i^2$; In the formula; X_i^2: This is the ratio of homozygot genotypes

Where the ratio of polymorphism was calculated with a comparison of the number of observed polymorphic fragments with the total fragment number.

Results and Discussion

The RAPD-PCR fragments of 16 animals were obtained, using 10 primers. The primers used in the study and the sequences, melting temperatures (Tm), the size range of fragments (bp), the total fragment numbers (TFN), fragment numbers (FN), polymorphic fragment numbers (PFN), polymorphism (P%), the ratios of heterozygosity and standard devision (H±SD), obtained from 16 Güney Karaman breed sheep are given in Table 1.

Primers	Sequence (5^0-3^0)	Tm	Size range of fragments (bp)	TFN	FN	PFN	P (%)	H±SD
18	GGG CTA GGG T	34	700 - 3000	116	10	9	90	0.3452 ± 0.1405
19	ACC GGG AAC G	34	700 - 3000	155	16	12	75	0.2304 ± 0.1956
Op15	GAC GGA TCA G	32	600 - 3000	148	17	15	88.23	0.3158 ± 0.1914
Opm10	TCT GGC GCA C	34	800 - 3000	124	12	12	100	0.4006 ± 0.1527
Opp15	GGA AGC CAA C	32	600 - 3000	160	15	15	100	0.4055 ± 0.1166
Opq04	AGT GCG CTG A	32	700 - 3000	217	18	14	77.77	0.3104 ± 0.1960
Opq06	GAG CGC CTT G	34	600 - 3000	160	19	19	100	0.3301 ± 0.1440
Ra03	CGA TCG AGG A	32	800 - 3000	154	16	14	87.50	0.3013 ± 0.1790
Ra35	AAG CTC CCC G	34	900 - 3000	96	8	8	100	0.4121 ± 0.1422
Ra59	CGG GCA ACG T	34	900 - 3000	121	16	15	93.75	0.2946 ± 0.1571
	Total			1451	147	133		
	Average						90.48	0.3273 ± 0.1697

Table 1. Some information obtained from 16 Güney Karaman breed sheep with the 10 primers

As can be seen in Table 1, the fragments obtained with the 10-mer primers generally ranged from 600 to 3000 bp. In total 1451 fragments were obtained, of them 673 being in male individuals, 778 being in females. When the number of fragments obtained from each individual, Opq04 primer is the one which yielded the highest number of fragments, with 217, and Ra35 is the one that yielded the lowest number of primers, with 96.

Of the 147 fragments that were obtained with all the primers, 133 were determined to be polymorphic, and 14 monomorphic. The average polymorphism ratio obtained in the study was found as 90.48%. When all the fragments that could be scored were examined, the Opm10, Opp15, Opq06 and Ra35 primers proved to be the primers with the highest ratio of polymorphism (100%). The lowest polymorphism ratio (75%) was obtained from the primer 19. The 10 primers that were used and the locus of the individuals that we studied had a high level of polymorphism, which made it unnecessary to scan more diverse locus. Cushwa et al. (1996) identified the polymorphism ratio, with RAPD method, to be between 65% and 96% with a mean 85%, using 53 primers in total from 5 sheep breeds (Coopworth, Merino, Perendale, Romney and Texel) and from their crossbreds. In a study made with 17 RAPD primers on 5 Iranian sheep breeds (Arman, Balouchi, Iranblack, Kordi and Karakol), Tahmoorespur et al. (2003) reported that they obtained 10 polymorphic fragments from only three of these primers (Moh-4, Moh-13 and Moh-21) and that they found the variation within and between the markers used and the 5 Iranian sheep breeds to be rather low, and that more studies were required with more primers so as to determine, in more details, the relations within and between sheep breeds. In this study, enough polymorphic fragments (133 pcs.) to exhaustively evaluate individual genotypes of the RAPD primers that were used in Güney Karaman sheep breed. In other words, when compared with the Güney Karaman sheep breed (90.48%), the ratio of polymorphism was seen to be lower in their studies. Paiva et al. (2005) stated that polymorphism ratios in 5 Brasilian hair-sheep breeds (Santa İnes, Rabo Largo, Somali, Morada Nova and Bergamasca) were found as (%) 100, 98.15, 98.15, 94.44 and 90.74, respectively. When compared with these breeds in terms of polymorphism ratio, it displayed a rate relatively closer to Bargamasca hair sheep, but had a lower value than all the Brazilian hair sheep. As to primers, in the study made by Ahmed Ali (2003) with 19 RAPD primers in total for the identification of genetic similarity in Barki, Rahmani, Baladi and Suffolk sheep breeds raised in Egypt, though the primers no. 18 and 19 yielded no polymorphic fragments, it appeared that they could be effectively used for the identification of individual genotypes in this study. Similarly, Sharma et al. (2001) used the Opm10 and Opp15 primers, which were also used in this study, to determine the genetic variation between White Leghorn, Rhodes Island Red, Red Cornish, White Plymouth Rock and Kadaknath chicken strains. However, they reported that they couldn't obtain any polymorphic fragments with Opp15 primers. In this study, it has appeared that both Opm10 and Opp15 primers could be effectively used for identifying individual genotypes.

The heterozygosity values in polymorphic locus calculated according to Nei (1987) generally varied between 0.0615 and 0.5000, and the rate of heterozygosity on the basis of primers was changed to be between 0.2304 and 0.4121. In general, the average heterozygosity was calculated to be 0.3273 ± 0.1697. As defined by Stephen et al. (2001), in a study they made with 4 RAPD primers so as to identify the genetic

relations between 5 Tanzanian sheep ecotype, reported that the highest average heterozygosity value was the Arusha ecotype (0.203) and that the lowest heterozygosity value was in the Dodoma ecotype (0.137). When compared with these sheep ecotypes in terms of the average heterozygosity value, Güney Karaman sheep (0.3273) can be said to have a higher value. For this reason, since the higher the heterozygosity value is in a population, the wider the genetic variety of the population will be, it is understood that Güney Karaman sheep have a higher genetic variation than 5 Tanzanian sheep ecotypes. Paiva et al. (2005) stated that heterozygosity values in Snata Ines, Rabo Largo, Somali, Morada Nova and Bergamasca hair sheep by using 19 RAPD primers were found as 0.3881, 0.3857, 0.4050, 0.3929 and 0.3229, respectively. When compared with Bergamasca hair-sheep in terms of heterozygosity, Güney Karaman sheep has a little higher heterozygosity value, but a lower value when compared with other breeds.

As seen in Table 2, K9 and K10 sheep were the individuals genetically closest to each other (0.8844). The sheep, genetically the most distant from each other were K5 - K7 (0.5646), K5 - K9 (0.5646), and K5 - K15 (0.5646). In this respect, the sheep no K5 can be said to be genetically different from the rest. The genetic similarity between individuals is estimated to be 0.7009 on average; the genetic distance 0.2991 on average (Table 2). In the study made by Ahmed Ali (2003), genetic similarities between Barki, Rahmani, Baladi and Suffolk sheep breeds raised in Egypt were found to be between the values 0.8190 and 0.9570. When compared with Güney Karaman sheep breed, the genetic similarity in this study was determined to be higher between 4 Egypth sheep breeds

In Table 2, the genetic similarity values of 16 Güney Karaman sheep breeds were given in terms of 10 RAPD markers.

	K1	**K2**	**K3**	**K4**	**K5**	**K6**	**K7**	**K8**	**K9**	**K10**	**K11**	**K12**	**K13**	**K14**	**K15**	**K16**
K1	-															
K2	0.6599	-														
K3	0.6803	0.7347	-													
K4	0.7959	0.6871	0.7483	-												
K5	0.6599	0.7415	0.5850	0.6054	-											
K6	0.7687	0.6735	0.6531	0.8503	0.6599	-										
K7	0.7687	0.6054	0.6259	0.7687	0.5646	0.7687	-									
K8	0.6463	0.6327	0.6259	0.6463	0.6735	0.6735	0.6599	-								
K9	0.7551	0.6327	0.7211	0.8231	0.5646	0.7687	0.7823	0.6327	-							
K10	0.7483	0.6395	0.6871	0.8299	0.5986	0.8027	0.7347	0.6395	0.8844	-						
K11	0.8299	0.6395	0.7007	0.7755	0.6939	0.7619	0.7619	0.6667	0.7483	0.7415	-					
K12	0.7415	0.6463	0.7075	0.7687	0.5918	0.7687	0.8095	0.7007	0.7959	0.7347	0.7619	-				
K13	0.7211	0.5986	0.5918	0.6395	0.7075	0.6803	0.6803	0.8163	0.6122	0.6190	0.7959	0.6803	-			
K14	0.8027	0.6667	0.7415	0.7347	0.6531	0.7211	0.6531	0.6395	0.7075	0.7279	0.7687	0.6803	0.7143	-		
K15	0.7007	0.6054	0.6395	0.7551	0.5646	0.7415	0.7143	0.6054	0.7415	0.7347	0.6939	0.7415	0.6531	0.6939	-	
K16	0.7347	0.6259	0.6190	0.6939	0.6259	0.6531	0.7211	0.6939	0.6667	0.6735	0.7823	0.7075	0.7959	0.7143	0.7075	-

Table 2. The genetic similarity values of 16 Güney Karaman sheep breeds

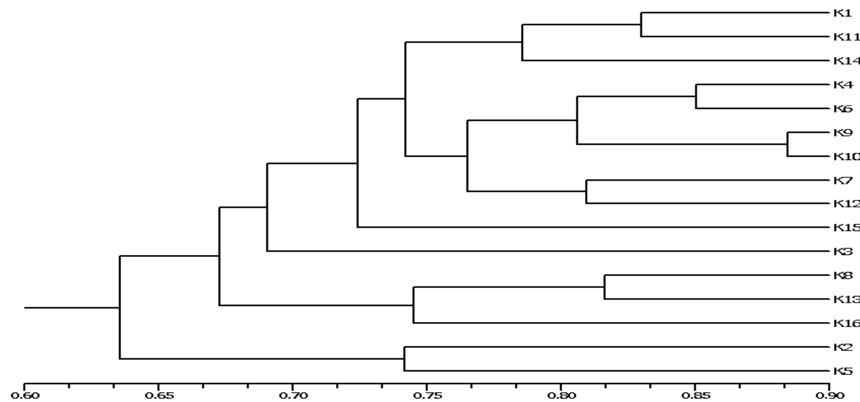

Figure 1. The UPGMA dendogram of 16 Güney Karaman sheep breeds

At the final stage of the study, the NTSYS-pc out of the RAPD fragments that could be scored and the genetic relation dendogram on which genotypic variation was displayed were formed through UPGMA method. As seen in Figure 1, 16 sheep were divided into two main groups, one with K2 and K5 individuals, and the other with all the other individuals. The individuals that made up the other group apart from K2 and K5 again were sub-grouped with K8, K13 and K16 out of them and with the remaining individuals. Again these sub-groups had their own sub-groups within themselves. From the dendogram, it can be seen that the individuals no K9 and K10 are genetically the closest genotypes. As seen in Table 2 and in Figure 1, the individuals no K2 and K5, particularly the individual no K5, can be said to be genetically different from other sheep.

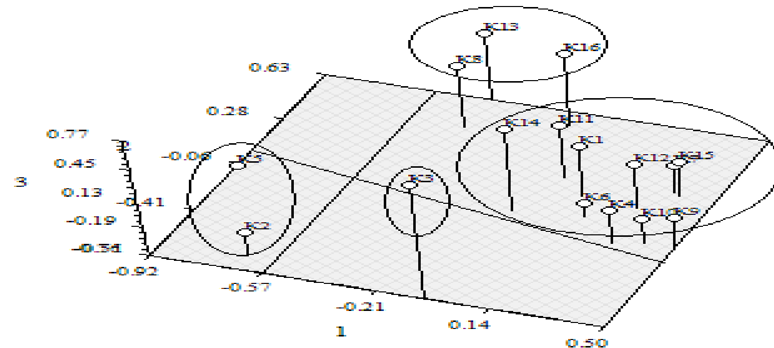

Figure 2. PCoA analysis of Güney Karaman sheep breed in terms of RAPD markers

It can be seen in the PCoA, the three dimensional vectorial analysis, the individuals no K9 and K10, which are closest to each other in genetic makeup on the dendogram are genetically related. Genetically the most distant individuals were the individuals no K2 and K5. Again during PCoA analysis, K8, K13 and K16 individuals as well as the individual no K3 are seen, on the dendogram and in PCoA analysis, to form a different group, unlike the individuals that constituted the other group outside K2 and K5.

Conclusion

As a result, it can be said that these 16 individuals, which are representative of Güney Karaman sheep, has a heterogenous nature and that this genetic variation has the potential use for purposes of improvement. In this study, the genetic similarity/distance values identified between individuals and the genetic relation dendogram on which genotypic variation is shown and PCoA analysis have shown that genotypic variation on individual basis can be obtained through RAPD-PCR method. However, with a more comprehensive study that will include all Turkish domestic sheep breeds, determination of certain

genetic parameters within and between all sheep breeds that make up the population would be more informative. Moreover, by making use of the available endemic gene resources, creating populations that have such characteristics as resistance to diseases and adverse weather conditions would be of great importance.

Acknowledgments

This research was supported by a master research project from the Coordinatory of Scientific Research Projects of Selcuk University, Turkey (Project No: 06101029). We are thankful to Bahri Dağdaş International Agricultural Research and Application Center for providing blood samples.

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