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Polymorphism Evaluation of Various Genomic Loci in the Kıvırcık Sheep Breed of Turkey

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Abstract: : In recent years regions which exhibit many genetic polymorphisms for the DNA of cattle, sheep and goats have been discovered. The aim of this study is to analyse the polymorphism of various microsatellites, which have been previously defined as polymorphism for sheep, and to evaluate the use of the Kıvırcık sheep population, which is indigenous to Turkey in relationship testing. The results have been evaluated for heterozygosity, power exclusion, matching probability, power discrimination, and paternity index.

For DNA extraction, the chelex and phenol-chloroform-isoamyl alcohol methods were applied to the blood taken from animals. After measuring the amount and degree of purity of the DNA with a spectrophotometer, 7 DNA loci were amplified by PCR. The amplification products were stained with EtBr and examined with 1.5% agarose gel electrophoresis. Using denaturated polyacrylamide gel electrophoresis and silver staining, PCR products were evaluated and phenotypes of the alleles were determined.

The OarFCB129 locus was observed to have a tendency to deviate from Hardy Weinberg Equilibrium in the general population ($P = 0.0037$; $P < 0.05$). The heterozygosity, power exclusion, power discrimination and matching probability were estimated for each locus. In 10 STR/microsatellite loci the highest heterozygosity value was 0.857 in the OarHH41 locus and the lowest value was 0.650 in the OarFCB266 locus in this study. The highest exclusion probability value was 0.71 in the OarHH41 locus and the lowest value was 0.36 in the OarFCB266 locus. The highest power discrimination value was 0.968 in the OarHH41 locus and the lowest value was 0.820 in the OarFCB266 locus. The OarFCB266, OarHH64 and OarHH51 loci have the highest values, and the OarHH41, OarFCB19 and OarFCB20 loci have the lowest values for matching probability. When all 10 loci were used together, the combined power discrimination and exclusion values were 0.0999706 and 0.999999, respectively. Among these loci, OarHH41, OarFCB19, OarFCB20, OarVH110, OarFCB128, OarFCB48 and OarFCB129 had high power discrimination and power exclusion and are recommended for use in determining relationships for their easy typing.

Key Words: Heterozygosity, genetic markers, exclusion probability, microsatellite, polymorphism, Kıvırcık.

Türkiye'deki Kıvırcık Koyun Irklarında Çeşitli Genomik Lokuslara ait Polimorfizmin Değerlendirilmesi

Özet: Son yıllarda, sığır, koyun ve keçi DNA'sında birçok yeni genetik polimorfizm gösteren bölge bulunmuştur. Bu çalışmanın amacı koyunlarda polimorfik olduğu daha önceden ifade edilen çeşitli mikrosatellitlerin polimorfizmlerinin analiz edilmesi ve bunların Türkiye yerli ırkı olan Kıvırcık koyun populasyonu için akrabalık testinde kullanılmasının değerlendirilmesidir. Sonuçlar dışlama gücü, uyuşma olasılığı, ayrımlama gücü, heterozigotluk oranı ve babalık indeksi açısından değerlendirilmiştir.

Hayvanlardan alınan kan örneklerinden fenol-kloroform-izoamil alkol ve chelex yöntemi kullanılarak DNA ekstraksiyonu yapılmıştır. Spektrofotometre ile, DNA'nın miktarı ve saflık derecesi belirlendikten sonra on DNA lokusu için PCR yöntemine dayalı amplifikasyon yapılmıştır. Amplifikasyon ürünlerinin varlığı EtBr ile boyanarak % 1,5 agaroz jel elektroforezi ile incelenmiştir. Denatüre edilmiş poliakrilamid jel elektroforezi kullanılarak ve gümüş boyama yapılarak PCR ürünleri değerlendirilerek allellerin fenotipleri belirlenmiştir.

OarFCB129 lokusunun, genel populasyonda Hardy-Weinberg dengesinden belirgin bir sapma eğiliminde olduğu gözlemlenmiştir ($P = 0,0037$; $P < 0,05$). Her lokus için beklenen ve gözlenen heterozigotluk oranı, dışlama gücü, ayrımlama gücü ve uyuşma olasılığı hesaplanmıştır. Bu çalışmada on STR/mikrosatellit lokusu içinde en yüksek heterozigotluk değeri 0,857 ile OarHH41 lokusunda gözlenirken, en düşük değer 0,650 ile OarFCB266 lokusunda görülmüştür. En yüksek dışlama olasılığı 0,71 ile OarHH41 lokusunda gözlenirken, en düşük değer 0,36 ile OarFCB266 lokusunda saptanmıştır. En yüksek ayrımlama gücü 0,968 ile OarHH41 lokusunda gözlenirken, en düşük değer 0,820 ile OarFCB266 lokusunda gözlemlenmiştir. Uyuşma olasılığında ise en yüksek değeri veren lokuslar OarFCB266, OarHH64 ve OarHH51 olurken, en düşük değerler OarHH41, OarFCB19 ve OarFCB20 lokuslarında saptanmıştır. Bu çalışmada kullanılan lokusların kombine ayrımlama ve dışlama gücü değerleri 10 lokusun tamamı kullanıldığında sırası ile 0,999706 ve 0,999999 olarak gözlenmiştir. Bu lokuslar içerisinde OarHH41, OarFCB19, OarFCB20, OarVH110, OarFCB128, OarFCB48 ve OarFCB129 lokusları yüksek ayrımlama ve dışlama gücüne sahiptirler ve kolaylıkla tiplenebilmeleri açısından akrabalık ilişkilerinin saptanmasında kullanılmaları önerilebilir.

Anahtar Sözcükler: Heterozigotluk derecesi, genetik markerler, dışlama gücü, mikrosatellit, polimorfizm, Kıvırcık.

Introduction

Endogenous sheep breeds are an important source of animal proteins and belong to the Ovis genus of the Bovidea mammalian family. The Kıvırcık, which is a native Turkish sheep breed with a long lean tail, constitutes 6.8% of the total sheep population in Turkey. The Kıvırcık breed is raised in Turkey, Greece, and Bulgaria and in Greece it is known as Thraki. It is mainly bred in the Thrace, Marmara and Aegean regions.

In every breeding and selection programme it is important to rely on parental information. The absence of determining parents and records in selections for the elimination of undesired recessive alleles in future breeding programmes is a good example. The basic fundamental of animal improvement is based on applying ideal selection methods and progeny testing, which is a method for the selection of high quality studs. The number of beneficial gene frequencies regarding the productivity characterisation of animals can be increased by this kind of selection method. However, possible erroneous parentage is a major problem in this kind of study. Some of these problems are mis-numbering, registration faults, mixing newborns, mixing sperm samples during artificial insemination and insemination of the rut females with different males. In recent years these kinds of problems have been eliminated by parentage testing according to blood type. Although blood analysis is a fast method it has certain disadvantages; the difficulty of keeping the samples and the obtaining of doubtful results contributed to the development of DNA analysis for parentage testing. DNA technology is the method which made the research of the variation among individuals possible at the level of molecular genetics (1).

Identification by DNA analysis was initially developed in order to be used in medicine, and especially in forensic medical institutions. In time this method was successfully applied to animal breeding. In particular, the development of these new methods in molecular biology has introduced new possibilities of using this method in animal breeding. One of the possibilities is to test DNA analysis and its use in parentage testing. The stable structure and easily obtainable character of DNA makes it easier to acquire the most reliable information about animals and permits identification.

Nowadays molecular genetic methods make it easier to determine the genetic information structure of animals. The main aim of this study was to research the determination of standards of population genetics and various loci which can be used in sheep parentage testing and researching the use of various loci for animal identification under proper experimental conditions.

This method, known as DNA fingerprting, which was obtained from the use of a probe with cross-hybridisation, cutting genomic DNA with the proper enzymes and obtaining individual-specific DNA band samples, including mini or microsatellite sequences in many polymorphic segments, was first used in humans (2-4) then in plants (5,6) and finally in domestic animals (7-9). The DNA fingerprint is unique to each individual; half of this fingerprint is maternal and the other half is paternal. DNA fingerprinting has found a very important and definite field of application in forensic medicine concerning the location of the source of tissue samples. In addition, these samples help to prevent doubtful parentage and confusion arising from immigrations. Microsatellites are tandem repeats, belonging to a nuclear genome. Microsatellites are variable marker candidates which are very suitable for evaluation (10).

Many numbers of polymorphic microsatellites are characterised and mapped to be used in the parentage testing of many dairy animals, such as sheep, cattle and other ruminants (11-15). For acceptability these tests require high reliability of parentage testing studies, which can be evaluated by power exclusion (16). This depends on the certainty of some estimates such as knowing the allele frequency of the population beforehand, the independence of markers in use and Hardy Weinberg Equilibrium (HWE). Microsatellites are widely used in the pedigree control of domestic animals and in studies of population genetics (14).

Allele frequencies of the populations should be determined and statistical parameters of the loci calculated for the use of microsatellites in the determination of individuals and relationships, because in the evaluation of these tests the gene frequencies of the related population are included in the estimation. Thus, if the gene frequencies exhibit differences among populations, the gene frequency of the population to which that individual belongs must definitely be examined.

In recent years regions which exhibit many genetic polymorphisms for the DNA of cattle, sheep and goats have been discovered. For each locus, DNA regions with low and high allele loci have been selected in this study. It

was thought that the differences (genetic variations) between high and low allele loci could be observed. In this way short tandem repeat (STR)/microsatellite loci which have 10 tandem repeat sequences were chosen and evaluated for use in parentage testing (Table 1). Empiric and theoretical approaches were used in the evaluation. The results were evaluated for heterozygosity, power exclusion, matching probability, power discrimination and paternity index.

The aim of this study is to analyse the polymorphism of various microsatellites, which is previously defined polymorphism for sheep, and to evaluate the use of these in relationship testing in the Kıvırcık sheep population, which is indigenous to Turkey.

Materials and Methods

Peripheral blood samples taken from 140 pure breed Kıvırcık sheep belonging to the Education, Research and Application Farm of the İstanbul University Veterinary Faculty, were used as material. Ten microsatellite loci were analysed from the samples from the Kıvırcık herds. OarHH41, OarHH64 (17,18), OarFCB19, OarFCB129, OarFCB128, OarFCB266 (18,19), OarFCB20, OarFCB48 (18,20,21), OarHH51 (18,21,22) and OarVH110 (18,21,23) microsatellites were used.

DNA isolation methods

The first method for DNA isolation was phenol-chloroform-isoamyl alcohol. DNA isolation proteins were first subtracted by using phenol-chloroform in this method. Obtaining totally pure DNA was the subsequent aim. The greatest advantage of this method is obtaining purer and more productive DNA than from other inorganic solvents. Standard steps which Sambrook et al. (24) reported were used in the application of this method. The second method was DNA isolation by Chelex®, 100. This method, which Walsh et al. (25) modified, was used for DNA extraction from sheep blood cells. The principle of this method is the denaturation of proteins and precipitation by binding them more tightly to resin. In both methods the purity level and amount of DNA on the top of the spectrophotometer mixture was determined in the ratio of $O.D._{260/280}$ (optic density). The obtained DNA extracts were rinsed under water, agarose gel electrophoresis was performed and the existence of DNA was determined under UV light. Blood samples were taken again from the sheep from which DNA was not isolated and the whole procedure was repeated. PCR products were prepared from the extracts from which DNA was isolated successfully.

PCR (polymerase chain reaction) conditions

DNA regions with low and high numbers of alleles for each locus were selected. This would make it possible to observe the differences (genetic variation) among loci with low and high alleles. The loci used in the study and the oligonucleotides of PCR primers used in proliferation of the loci and PCR proliferation conditions are given in Table 1.

The PCR mixture was prepared in a total volume of 25 µl for each sample. There were 16.45 µl of sterilised distilled water, 2.5 µl of 10 x TBE (tris borate EDTA) buffer, a 2.5 µl 0.8 µM primer pair, 0.05 µl of Taq DNA Polymerase (0.25 U) and 1.0 µl of dNTP (deoxynucleotide 3 phophate) (100 µM) in each sterilised PCR tube: 0.5 ml sterilised PCR tubes of the same quantity as the samples, were labelled and set up for spore. A 22.5 µl PCR mixture was pipetted into each PCR tube. Then 2.5 µl of template DNA (100 ng) and 2 drops of mineral oil were added to the surface of the mixture to prevent evaporation and air contact.

The tubes were put in a heat cycling machine. The heat cycling conditions determined for initial denaturation were 94 °C for 3 min, for denaturation 94 °C for 30 s, for primer binding to single strand DNA (binding=annealing) 58-63 °C for 30 s, for extension of new DNA strands 30 cycles at 72 °C for 1 min, and for the last extension 72 °C 4 min. Approximately 1.5 h later, amplification of the desired DNA regions was completed.

After amplification the PCR products were screened using underwater agarose gel electrophoresis. Samples not examined immediately were stored at -20 °C.

Screening of PCR products by agarose gel electrophoresis

After scaling 1.5 g of agarose, 100 ml of 0.5 x TBE [108 g 0.90 M tris base, 40 ml 0.5 M EDTA (di sodium ethylene diamine tetra acetic acid $2H_2O$), 55 g 0.9 M boric acid] buffer was added to agarose. In the heater it was stirred continuously until it reached boiling point and once a homogeneous mixture was obtained it was cooled

Table 1. Details of 10 sheep microsatellites.

Loci accession no	Primer sequences 5'→3'	Number of alleles	Repeat sequence	Base pair
OarHH41 (L12555)	TCCACAGGCTTAAATCTATATAGCAACC GAGCGGTGTAGTAGAAAATAGAAATCGACC	15	$(AC)_{23}$	121-149
OarFCB19 (L20003)	GGTAATGTAATTTTCAAAGGTGTGTGCG CACAGTCACTTCTCTGTCTTCTTCTC	14	$(AC)_{23}$	121-145
OarFCB20 (L20004)	AAATGTGTTTAAGATTCCATACAGTG GGAAAACCCCCATATATACCTATAC	12	$(TG)_{15}$	94-118
OarFCB48 (M82875)	GAGTTAGTACAAGGATGACAAGAGGCAC GACTCTAGAGGATCGCAAAGAACCAG	11	$(TG)_{11}$	142-164
OarFCB129 (L20005)	GCGACTTAGCAGCAGCAGCATCC CATCAAGAGATGGAGTAAAGAAGATG	10	$(CA)_{15}$	113-133
OarHH51 (U08878)	CAAATTTATAAACTGGCCTGCCGC AACTCTTGCGATTGTCTTTCGAGATTACC	9	$(CA)_{23}$	125-141
OarFCB128 (L01532)	CAGCTGAGCAACTAAGACATACATGCG ATTAAAGCATCTTCTCTTTATTTCCTCGC	8	$(GT)_{15}$	99-125
OarVH110 (L12550)	CTCTAGAGGATCACAGAGAGTCGG GCAGAAACATTTTTTTCCTTCAATATAGTTTCCC	8	$(TG)_{24}$	123-137
OarHH64 (L12588)	CGTTCCCTCACTATGGAAAGTTATATATGC CACTCTATTGTAAGAATTTGAATGAGAGC	7	$(GT)_{17}$	127-139
OarFCB266 (L01534)	GGCTTTTCCACTAGCTTTACATAGGAGTG CACCACATACCAAACACACAGCCTGC	7	$(AC)_{10}$	100-112

to 50-60 °C at room temperature. Then 5 µl from a 10 mg/ml ethidium bromide (EtBr) solution was added into the mixture. Then the gel was poured into an electrophorese cassette and polymerisation was awaited. Each PCR sample was loaded in 5 µl quantities to the gel wheels after mixing with loading buffer. The amplified products were electrophoresed in 1.5% agarose gel and stained by EtBr under 100 V, and a free current of 0.50 A for 20 min (26).

Phenotypes of alleles were determined by evaluation of PCR products after polyacrylamide gel electrophoresis and silver staining. Vertical denature polyacrylamide gel 33 cm x 42 cm x 0.4 mm in size was constructed for the protocol reported by Promega (27).

One of the most effective and best methods for naming alleles of STR/microsatellite loci is naming each allele by its base length; another method is naming alleles by their tandem repeats. In this study alleles are named by their base lengths.

Statistical estimations used in evaluating the results

For each obtained locus allele frequency, heterozygosity, power exclusion, matching probability and power of discrimination have been estimated, because evaluating the usability of a genetic marker in identifying various individual parameters (power exclusion, matching probability, power discrimination, heterozygosity, etc.) were used. Parameters of all systems were observed before making a decision about which system would be more useful for an individual's identification. To estimate these parameters the frequency and type of system observed in the related population should be known. To estimate these values the statistical procedures below were used (16,26,28-30).

1. This equation is used for determination of gene frequency (26);

$P = 2x + y / 2n$

x = Number of homozygous alleles

y = Number of heterozygous alleles

n = Number of subjects

2. Formula for standard error (26);

$sf = \sqrt{P(1-P) / 2n}$

3. Propriety of locus results to HWE was determined. Estimation of expected values;

p^2N, q^2N, r^2N, s^2N and 2pqN equations were used for homozygous and heterozygous phenotypes, respectively.

p and q: allele frequency

N: number of alleles

4. To find the connection among observed and expected frequencies X^2 analysis was applied.

X^2 = (observed – expected)^2/expected

ΣX^2 value was estimated by applying this formula (29).

5. Number of genotypes and number of alleles were subtracted to find the independency level (29).

S.D = number of genotypes-number of alleles

6. To estimate the expected heterozygosity for each locus this equation was used (16,28,30).

$h = (1-\sum P_i^2)$

P_i = allele frequency

7. Observed heterozygosity is defined by the following equation (16);

H_0 = number of observed heterozygotes/number of total subjects.

8. Exclusion probability (PE): The power of exclusion, PE, is defined as the fraction of individuals in a typical paternity case. The value of each individual case will vary. The average for a given locus is represented by the following equation (16);

$PE = h^2 (1-2hH^2)$

h = number of heterozygous,

H = number of homozygous

9. The PE for several loci is represented in the following equation (16);

$$PE = 1 - \prod_{i=1}^{n} (1 - PE_i)$$

10. Matching probability: Matching probability, also known as probability of match (pM), is the number of individuals which may be surveyed before finding the same DNA pattern in a randomly selected individual. This is represented as (16)

$$pM = \sum_{i=1}^{n} \sum_{j=1}^{n} P_{ij}^2$$

i and j = frequencies of all possible alleles: P_{ij}^2 frequencies of all possible genotypes.

11. Power of discrimination (P_d): The power of discrimination is 1-pM. The combined power of discrimination for multiple loci may be calculated by the following equation (16,26);

$$P_{d\ combined} = 1 - \prod_{i=1}^{n} (1- P_{di})$$

definition for combined discrimination formula = 1-($P_1xP_2.xP_3.x...P_n$)

n = number of loci.

12. Paternity index (PI): The paternity index reflects how many more times it is likely that the person being tested is the biological father, rather than a randomly selected individual. The typical paternity index is assigned to a locus rather than an individual case. Generally, a PI of less than 1 is indicative of being non-related. The PI is represented by the following equation (16);

PI = 1 / 2H

H: Heterozygosity

Results

In this research, blood samples used as materials were obtained from 140 healthy, non-related, male and female Kıvırcık sheep belonging to the Education, Research and Application Farm of the İstanbul University Veterinary Faculty. Gene frequencies of the OarHH41, OarFCB19, OarFCB20, OarFCB48, OarFCB129, OarHH51, OarFCB128, OarVH110, OarHH64 and OarFCB266 loci were determined from the blood samples to evaluate their efficiency in parentage testing.

Allele frequencies of the 10 microsatellite loci used in this research were estimated by numerical dispersion of each observed animal genotype. These obtained frequencies were used in determining adaptation in HWE, heterozygosity, power exclusion, matching probability

and paternity index. Each locus was compared in its own group and differences were evaluated statistically.

Number of alleles, allele frequency, and length of alleles in base pairs for each locus of individuals used in the research are given in Table 2. Some of the alleles were observed more frequently in the population. In the population for the OarHH41 locus the most frequent allele was 0.257 and the least frequent allele was 0.007 for the OarFCB19 locus. The most frequent allele was 0.311 and the least frequent allele was 0.007 for the

Table 2. Allele frequencies belonging to 10 sheep microsatellites.

Locus name	Allele size	Allele frequency	Observed allele (n)	Locus name	Allele size	Allele frequency	Observed allele (n)
OarHH41	121	0.114	32	OarFCB129	113	0.068	19
	123	0.257	72		115	0.036	10
	125	0.132	37		119	0.086	24
	127	0.057	16		121	0.039	11
	129	0.039	11		123	0.439	123
	131	0.114	32		125	0.039	11
	133	0.139	39		127	0.186	52
	135	0.032	9		129	0.007	2
	137	0.032	9		131	0.039	11
	139	0.014	4		133	0.061	17
	141	0.018	5	OarHH51	125	0.414	116
	143	0.007	2		127	0.039	11
	145	0.014	4		129	0.021	6
	147	0.018	5		131	0.014	4
	149	0.011	3		133	0.346	97
OarFCB19	121	0.100	28		135	0.086	24
	123	0.071	20		137	0.018	5
	125	0.018	5		139	0.046	13
	127	0.257	72		141	0.014	4
	129	0.225	63	OarFCB128	99	0.129	36
	131	0.036	10		111	0.161	45
	133	0.111	31		113	0.396	111
	135	0.096	27		115	0.093	26
	137	0.036	10		119	0.064	18
	139	0.029	8		121	0.104	29
	141	0.007	2		123	0.036	10
	143	0.007	2		125	0.018	5
	145	0.007	2	OarVH110	123	0.188	29
OarFCB20	94	0.021	6		125	0.247	38
	98	0.311	87		127	0.182	28
	100	0.164	46		129	0.175	27
	102	0.118	33		131	0.136	21
	104	0.075	21		133	0.045	7
	106	0.046	13		135	0.019	3
	108	0.168	47		137	0.006	1
	110	0.014	4	OarHH64	127	0.014	4
	112	0.036	10		129	0.193	54
	114	0.025	7		131	0.014	4
	116	0.014	4		133	0.339	95
	118	0.007	2		135	0.339	95
OarFCB48	142	0.136	38		137	0.086	24
	146	0.096	27		139	0.014	4
	148	0.386	108	OarFCB266	100	0.043	12
	150	0.221	62		102	0.361	101
	152	0.021	6		104	0.454	127
	154	0.025	7		106	0.086	24
	156	0.039	11		108	0.007	2
	158	0.014	4		110	0.039	11
	160	0.029	8		112	0.011	3
	162	0.014	4				
	164	0.018	5				

OarFCB20 locus; the most frequent allele was 0.386 and the least frequent allele was 0.014 for the OarFCB48 locus; the most frequent allele was 0.439 and the least frequent allele was 0.008 for the OarFCB129 locus; the most frequent allele was 0.414 and the least frequent allele was 0.014 for the OarHH51 locus; the most frequent allele was 0.396 and the least frequent allele was 0.018 for the OarFCB128 locus; the most frequent allele was 0.247 and the least frequent allele was 0.006 for the OarVH110 locus; the most frequent allele was 0.339 and the least frequent allele was 0.014 for the OarHH64 locus, and the most frequent allele was 0.454 and the least frequent allele was 0.007 for the OarFCB266 locus (Table 2).

Information about the adaptation of each locus to HWE is given in Table 3. Alleles which show HWE are inherited randomly in the population. The allele frequency of the 10 loci has been checked by the propriety to HWE by X^2 analysis ($P > 0.05$) by considering the observed genotype frequencies and expected numerical values of the same genotype (Table 3). The OarFCB129 locus has a tendency to deviate from HWE in the general population ($P = 0.0037$; $P < 0.05$).

The heterozygosity, power exclusion, power discrimination and matching probability were estimated for each locus (Table 3). In the 10 STR/microsatellite loci the highest heterozygosity value was 0.857 in the OarHH41 locus and the lowest value was 0.650 in the OarFCB266 locus. The highest exclusion probability value was 0.71 in the OarHH41 locus and the lowest value was 0.36 in the OarFCB266 locus. The highest power discrimination value was 0.968 in the OarHH41 locus and the lowest value was 0.820 in the OarFCB266 locus. The OarFCB266, OarHH64 and OarHH51 loci had the highest values and OarHH41, OarFCB19 and OarFCB20 loci had the lowest values for matching probability (Table 3). OarHH41, OarFCB19, OarFCB20, OarFCB48 and OarVH110 loci had the highest paternity index, 3.488, 3.063, 2.746, 2.408 and 2.406, respectively, in this population.

When all of the 10 loci were used together, the combined power discrimination and exclusion values were 0.0999706 and 0.999999, respectively (Table 4).

Results of DNA extraction

Chelex 100 resin chelatiser and phenol chloroform isoamyl methods were used for DNA extraction from sheep blood cells. To determine the purity of DNA obtained by the chelex and phenol-chloroform-isoamyl methods, the DNA quantity values for 100 samples were 1.200 and 1.860, respectively, in a ratio of O.D.260/O.D.280 wavelengths.

Table 3. Statistical analysis of each locus used in the study[a].

	Ho	He	P	PD	PE	MP	PI
OarHH41	0.857	0.857	0.0834	0.968	0.71	0.032	3.488
OarFCB19	0.829	0.837	0.0515	0.960	0.65	0.040	3.063
OarFCB20	0.814	0.818	0.0918	0.951	0.63	0.050	2.746
OarFCB48	0.779	0.792	0.0596	0.926	0.56	0.074	2.408
OarFCB129	0.764	0.751	0.0037[b]	0.917	0.53	0.083	2.011
OarHH51	0.679	0.691	0.0695	0.861	0.40	0.139	1.619
OarFCB128	0.786	0.770	0.0560	0.927	0.57	0.073	2.333
OarVH110	0.792	0.808	0.0998	0.943	0.59	0.057	2.406
OarHH64	0.707	0.719	0.0546	0.878	0.44	0.122	1.707
OarFCB266	0.650	0.649	0.0896	0.820	0.36	0.180	1.429

[a] Ho: Observed heterozygosity. He: Expected heterozygosity. P: HW equilibrium.
PD: Power discrimination. PE: Power exclusion. MP: Matching probability. PI: Paternity index
[b] $P < 0.05$

Table 4. Combined power exclusion and combine power discrimination of the loci used in the study.

Locus	Combined Power Exclusion (CPE)	Combined Power Discrimination (CPD)
10 Loci	0.999706	0.999999

Silver staining was used for visualisation followed by denatured vertical gel electrophoresis after the PCR applications. The greatest advantage of this method is its low cost. However, it also has some disadvantages because the gel can only be used once and colour appears on the surface besides bands because of silver accumulation in the gel.

Discussion

The chelex (resin chelatiser) method was used for DNA isolation from blood samples. The reasons for the preference of this method are that it is faster than other methods, needs very few samples, has only a few procedure steps, has a low cost, and because the isolation amplification is performed in one tube, and it has a low contamination risk regarding tube transfer and pipetting. However, because of the boiling involved in this method, DNA with a low molecular weight is obtained. Yet since the STR/microsatellite loci have short sequences they are not damaged during the boiling procedure.

Propriety of allele frequencies to HWE was checked by the X^2 test ($P < 0.05$), which has been estimated with observed genotype frequencies and expected numerical values of the same genotypes. Allele frequencies of the OarHH41, OarFCB19, OarFCB20, OarFCB48, OarHH51, OarFCB128, OarVH110, OarHH64, OarFCB266 loci were appropriate ($P > 0.05$) (Table 4) and the allele frequency of the OarFCB129 was not appropriate ($P < 0.05$) (Table 3) to HWE. Deviation from the HWE observed in the OarFCB129 sample may be a deviation from the general population.

The reason for this may be the very low numbers of some alleles belonging to this locus in the Kıvırcık sheep population, or the result of frequent selections, an insufficient number of samples, inbreeding and/or immigration. These kinds of problems can reduce the cumulative value of power exclusion for the general population in parentage testing. To prevent this, the relevant loci must be removed from the parentage testing. When this value is subtracted from the general population in this study, it will lower the value from 99.7% to 99.4%. However, because of the high number of alleles of the chosen loci exclusion power and heterozygosity were not greatly affected (Table 4).

The higher the paternity index, the higher the chances of inheritance of related allele from sire to offspring (Table 3). When the paternity index values were examined the highest values were 3.488 and 2.011 in the OarHH41, OarFCB19, OarFCB20, OarFCB48, OarVH110, OarFCB128 and OarFCB129 loci (Table 3). Matching probability is an indicator of individuals' proximity to each other. When individuals' degrees of relation increase the probability of carrying the same allele will also increase. A low value means alleles will be found to coexist at a very low frequency in the related population. The loci with the lowest matching probabilities were, in descending order, the OarHH41, OarFCB19, OarFCB20, OarVH110, OarFCB128, OarFCB48 and OarFCB129, and those with highest ones were OarFCB266, OarHH51 and OarHH64, in descending order (Table 3).

Allele frequency values were different from the study on various sheep breeds by Henry et al. (17) for the OarHH41 locus. The reason for this may be that the sheep breeds used by Henry et al. carry a different number of alleles for that locus. The allele frequency values for OarHH64 in that study are similar to the values obtained from ours. The observed values for the OarFCB19, OarFCB20 and OarFCB48 loci in this study are different from the values obtained from a similar study by Buchanan et al. (20), but are the same as regards the OarFCB129 locus. The values obtained from the OarFCB128 and OarFCB266 loci are the same as the values that Buchanan and Crawford (19) reported.

The heterozygosity value, which is estimated for the loci used in this study, is similar to the values that de Gortari et al. (31) reported. The heterozygosity obtained in the study by Thomasco et al. (15) with Uruguayan endogenous sheep breeds was different from the values for the OarFCB128 locus obtained in this study, but similar to the values for the OarHH64 and OarFCB48 loci.

Systems with high heterozygosity, power exclusion, power discrimination, paternity index and low matching probability are preferred for their usefulness in parentage testing, and these systems are used in these kinds of studies. According to the statistical parameters estimated for the Kıvırcık sheep population of the Education, Research and Application Farm of the İstanbul University Veterinary Faculty, the loci can be listed as OarHH41, OarFCB19, OarFCB20, OarVH110, OarFCB128, OarFCB48, OarFCB129, OarHH64, OarHH51 and OarFCB266 in terms of their heterozygosity. However, if a multiplex system were prepared among the loci it would

be right to choose the OarHH41, OarFCB19, OarFCB20, OarVH110, OarFCB128, OarFCB48 and OarFCB129 loci, in that order, since choosing the loci with low heterozygosity would reduce the correctness of the degree of straightness of parentage testing and would give less information. The results obtained from this study are parallel to those Bowling et al. (32) reported. In this study it was determined that loci with high heterozygosity have higher power exclusion. Using only one locus in parentage testing requires great attention to detail in parentage exclusion. The report by Heyen et al. (28) that used microsatellites for their high polymorphism in parentage testing also supports this view. This polymorphism usually occurs with the mutation of the number of tandem dinucleotide repeat sequences and these differences in the number of tandem repeats lead to the existence of new alleles.

The combined power discrimination and power exclusion values of the loci used in this study were very high (Table 4). Thus, these systems can be used as markers in DNA profiling analyses. However, instead of choosing the OarHH64, OarHH51 and OarFCB266 loci with low numbers of alleles, again choosing loci with high power exclusion will make it possible to reach a degree of straightness in parentage testing, because the expected parentage level is 99.73%. Unless a result reaches 99.73%, no definite paternity can be decided. Therefore an effort must be made to attain this percentage by raising the sample numbers for different loci.

According to the estimated parameters in this study, loci with high numbers of alleles in parentage testing have been observed to reach ideal values. Parentage exclusion and determining probability were not as high as for the other 7 loci for the OarHH64, OarHH51 and OarFCB266 loci with low numbers of alleles. In the future, studies choosing loci with high numbers of allele will elevate the heterozygosity, power exclusion, meeting probability and paternity index values to a desirable level.

The success of parentage testing depends on determining false paternity. The results obtained from this study show that there are 2 main factors in determining the reliability of evaluation of parentage testing. The first is the number of genetic markers evaluated. If 6 or 7 of the loci are taken as a genetic marker, the degree of reliability of parentage testing may increase, but it is not linear. This takes place because when the number of 'suspicious' parents and offspring increases, the parentage exclusion probability of the selected loci will decrease. Low numbers of genetic markers can be successful only if 1 or 2 suspects are investigated. The second depends on high heterozygosity (variation degree) for every genetic marker. More successful parentage testing can be performed if the genetic markers used show a high heterozygosity.

To use STR/microsatellite loci in describing individuals and determining relationships, the statistical parameters of these loci must be estimated by determining the allele frequencies of the population, because mathematical relations in which there are gene frequencies of the related population are used in the evaluation of the tests. Thus, if the gene frequencies exhibit differences among populations the gene frequency of the population the individual belongs to must be taken up, otherwise the test may result against each individual. For this reason, determining the statistical parameters by setting the gene frequencies of STR/microsatellite loci with increasing numbers of STR/microsatellite loci all over Turkey may cause an increase in the degree of reliability of parentage testing. Thus, it will afford an opportunity to compare the results with studies which have been already completed or future studies of endogenous populations. If, in addition, the DNA data of populations are constructed, it can help with the origin and structure of populations (26).

In this study single locus (monoplex) systems have been used. Among these loci, the OarHH41, OarFCB19, OarFCB20, OarVH110, OarFCB128, OarFCB48 and OarFCB129 have high power discrimination and power exclusion and are recommended for use in determining relationships for their easy typing. However, this study is limited to the area of the Education, Research and Application Farm of the İstanbul University Veterinary Faculty. A standardised multiplex system can be built by using different loci in the general area of Turkey as a continuation of this study.

These systems exhibit high polymorphism and have good amplification potential. However, closeness or shifts in the band results obtained from electrophoresis could cause a typing error as a result of having dinucleotide tandem repeats. Choosing loci with triple or quartet tandem repeats in particular will make the standardisation of parentage testing easier and will increase the degree of reliability.

References

1. Margan, U.: Prospects of the methods of bovine parentage control. Tierarztl. Wschr. 1996; 109: 1-5.
2. Jeffreys, A.J., Wilson, V., Thein, S.L.: Individual-specific fingerprints of human DNA. Nature 1985; 316: 76-79.
3. Jeffreys, A.J., Brookfield J.F.Y., Semeonoff, R.: Positive identification of an immigration test-case using human DNA fingerprints. Nature 1985; 317: 818-819.
4. Nakamura, Y., Lepper, M., O'Connel, P., Wolff, R., Holm, T., Culver, M., Martin, C., Fujumoto, E., Hoff, M. Kummlin, E, White, R.: Variable numbers of tandem repeat (VNTR) markers for human gene mapping. Science 1987; 235: 1616.
5. Dallas, F.J.: Detection of DNA fingerprints of cultivated rice by hybridization with a human minisatellite DNA probe. Proc. Natl. Acad. Sci. USA, 1988; 85: 6831.
6. Tzuri, G., Hillel, J., Lavi, U., Haberfeld, A., Vainstein, A.: DNA fingerprint analysis of ornamental plants. Plant Sci. 1991; 76: 91-97.
7. Vassart, G., Georges, M., Monsieur, R., Brocas, H., Lequarre, A.S., Christophe, D.: A sequence in M13 phage detects hyper variable minisatellites in human and animal DNA. Science 1987; 235: 683-684.
8. Georges, M., Lequarre, A.S., Castelli, M., Hanset, R., Vassart, G.: DNA fingerprinting in domestic animals using four different minisatellite probes. Cytogenet. Cell Genet. 1988; 47: 127-131.
9. Kashi, Y., Lipkin, E., Darvasi, A., Gruenbaum, Y., Beckmann, J.S., Soller, M.: Parentage identification in the bovine using deoxyribonucleic acid fingerprints. J. Dairy Sci. 1990; 73: 3306-3311.
10. Queller, D.C., Strassmann, J.E., Hughes, C.R.: Microsatellites and Kinship. Tree. 1993; 8: 285-288.
11. Hayes, H., LeChalony, C., Goubin, G., Mercier, D., Payen, E., Bignon, C., Kohno, K.: Localization of ZNF164, ZNF146, GGTA1, SOX2, PRLR and EEF2 on homologous cattle, sheep and goat chromosomes by fluorescent in situ hybridization and comparison with the human gene map. Cytogenet. Cell Genet. 1996; 72: 342-346.
12. de Gortari, M.J., Freking, B.A., Kappes, S.M., Leymaster, K.A., Crawford, A.M., Stone, R.T., Beattie, C.W.: Extensive genomic conservation of cattle microsatellite heterozygosity in sheep. Anim. Genet. 1997; 28: 274-290.
13. Jenkins, Z.A., Henry, H.M., Galloway, S.M., Dodds, K.G., Montgomery, G.W.: Comparative linkage mapping of genes on sheep chromosome 3 provides evidence of chromosomal rearrangements in the evaluation of bovidae. Cytogenet. Cell Genet. 1997; 72: 272-274.
14. Luikart, G., Biju-Duval, M-P., Ertugrul, O., Zagdsuren, Y., Maudet, C., Taberlet, P.: Power of 22 microsatellite markers in fluorescent multiplexes for parentage testing in goats (*Capra hircus*). Anim. Genet. 1999; 30: 431-438.
15. Tomasco, I., Wlasiuk, G., Lessa, E.P.: Evaluation of polymorphism in ten microsatellite loci in Uruguayan sheep flocks. Genet. Mol. Biology. 2002; 25: 1-5.
16. Kimberly A.H.: Statistical analysis STR data profiles in DNA. 2001; "http://www.promega.com/profiles/103/103_14"
17. Henry, H.M. Penty, J.M., Pierson, C.A., Crawford, A.M.: Ovine E microsatellites at the OARHH35, OARHH41, OARHH44, OARHH47, and OARHH64 loci. Anim. Genet. 1993; 24: 222.
18. Maddox, J.F., Davies, K.P., Crawford, A.M., Hulme, D.J., Vaiman, D., Cribiu, E.P., Freking, B.A., Beh, K.J., Cockett, N.E., Kang, N., Riffkin, C.D., Drinkwater, R., Moore, S.S., Dodds, K.G., Lumsden, J.M, Van Stijn, T.C., Phua, S.H., Adelson, D.L., Burkin, H.R., Broom, J.E., Buitkamp, J., Cambridge, L., Cushwa, W.T., Gerald, E., Galloway, S., Harrison, B., Hawken, R.J., Hiendleder, S., Henry, H.M., Medrano, J.F., Paterson, K.A., Schibler, L., Stone, R.T., Van Hest, B.: An enhanced linkage map of the sheep genome comprising more than 1000 loci. Genome Res. 2001; 11: 1275.
19. Buchanan, F.C., Crawford, A.M.: Ovine microsatellite at the OARFCB11, OARFCB128, OARFCB193, OARFCB266 and OARFCB304 loci. Anim. Genet. 1993; 24: 145.
20. Buchanan, F.C., Galloway, S.M., Crawford, A.M.: Ovine microsatellites at the OARFCB5, OARFCB19, OARFCB20, OARFCB48, OARFCB129 and OARFCB226 loci. Anim. Genet. 1994; 25: 60.
21. Kappes, S.M., Keele, J.W., Stone, R.T., McGraw, R.A., Sonstegard, T.S., Smith, T.P.L., Lopez-Corrales, N.L., Beattie, C.W.: A second-generation linkage map of the bovine genome. Genome Res. 1997; 7: 235.
22. Pierson, C.A., Ede, A, Crawford, A.M.: Ovine microsatellite at the OARHH30, OARHH51, OARVH54, OARCP88, OARCP93, and OARCP134 loci. Anim. Genet. 1994; 25: 294.
23. Hanarahan, V., Ede, A.J., Pierson, C.A., Hill, D.F.: Ovine microsatellites at the OARVH98, OARVH110, OARVH116, OARVH117 and OARVH130 loci. Anim. Genet. 1993; 24: 223.
24. Sambrook, J., Frisch, F.F., Maniation, T.: Spectrophotometric Determination of the Amount of DNA or RNA Appendix E: Commonly Used Techniques in Molecular Cloning. Molecular Cloning: 3A Laboratory Manual, Second Edition. Cold Spring Harbor Laboratory Press, 1989.
25. Walsh, P.S., Metzger, D.A., Higuchi, R.: Chelex100 as a medium for simple extraction of DNA for PCR-based typing from. Forensic Material. Biotec. 1991; 10: 506-513.
26. Filoğlu, G.: 7 Tetramerik STR lokusunun kriminal identifikasyonundaki önemi (Doktora Tezi). 1999; İstanbul Üniversitesi Adli Tıp Enstitüsü, Fen Bilimleri Anabilim Dalı.
27. Promega: Silver Stain Detection. Technical Manual no. 023. Madison, WI, USA, 1989.
28. Heyen, D.W., Beever, J.E., Da, Y., Evert, R.E., Green, C., Bates, S.R.E., Ziegle, J.S., Lewin, H.A.: Exclusion probabilities of 22 bovine microsatellite markers in fluorescent multiplexes for semi-automated parentage testing. Anim. Genet. 1997; 28: 21-27.
29. Guo, S.W., Thompson, E.A.: Performing the exact test of Hardy-Weinberg proportion for multiple alleles. Biometrics 1992; 48: 361-372.

30. Jamieson, A., Taylor St., C.S.: Comparisons of three probability formulae for parentage exclusion. Anim. Genet. 1997; 28: 397-400.

31. de Gortari, M.J., Freking, B.A., Cuthbertson, R.P., Kappes, M.S., Keele, J.W., Stone, R,T., Leymaster, K.A., Dodds, K.G., Crawford, A.M., Beattie, C.W.: A second-generation linkage map of the sheep genome. Mam. Genet. 1998; 9: 204-209.

32. Bowling, A.T., Eggleston-Scot, M.L., Byrns, G., Clark, R.S., Dileanis, S., Wictum, E.: Validation of microsatellite markers for routine horse parentage testing. Anim. Genet. 1997; 28: 247-257.