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Comparison of different paternity test panels in sheep

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Abstract: The aims of this study were to evaluate different paternity test panels with 17 microsatellite markers for their effectiveness in paternity analysis and to identify pedigree error rates in the Kangal Akkaraman breed of sheep. The animal material for the study consisted of 175 Kangal Akkaraman sheep, 35 rams, and their 140 offspring, raised on farms and bred using controlled mating. The panels were created according to probability of exclusion (PE) of 17 studied microsatellites. A total of 240 alleles were detected across 17 microsatellite loci. The overall mean value of the polymorphic information content (PIC) (0.78) indicated that the microsatellite panels were highly polymorphic. Probability of identity (PI) values ranged between 0.02 and 0.13. It can be said that pedigree error (2.94%) occurred at a low rate in this study. The highest combined PE values were obtained from Panel-16 (0.9999771) as expected. Panel-8 and Panel-9 met the PE value accepted in the literature (0.999). The results show that these panels are cheaper and more practical than other panels formed for Kangal Akkaraman.

Key words: Paternity test, microsatellite, probability of exclusion, sheep

1. Introduction

Accuracy of pedigree records plays a key role in estimating reliable genetic parameters in animal husbandry. In this regard, mating and birth records are especially important in small ruminants reared under extensive conditions. Pedigree information verified by paternity testing is indispensable for genetic evaluation. Pedigree errors reduce genetic progress, due to incorrect estimation of breeding value. Pedigree errors mentioned in previous studies in livestock were in the range of 10%–23% (1–4). Pedigree errors have adverse effects on animal breeding programs and make it difficult to achieve the objectives of the breeding organization involved (1,5–13). Molecular genetic techniques are very useful tools in the paternity analysis, genetic diversity studies, and genome mapping of livestock (14,15). Among these techniques, molecular genetics-based paternity tests in particular provide an important contribution to animal husbandry. Nowadays, DNA-based genetic analysis methods are being widely used in paternity testing instead of methods using blood groups, protein and enzyme polymorphisms, and the human leukocyte antigen (HLA), which have low reliability (10,16). In cases where there is doubt, paternity testing may be used as a scientific method to determine the biological father of unknown progeny in livestock and wild populations in order to obtain accurate pedigree information (17). The most commonly used DNA-based genetic analysis methods in paternity analysis are microsatellites and SNP methods (18–27).

The aim of this study was to verify the results of controlled mating in Kangal Akkaraman sheep and to demonstrate the availability of paternity test panels using microsatellites for cheap, fast, and reliable paternity testing.

2. Materials and methods

This study was conducted under the Kangal Akkaraman sheep breeding project funded by the General Directorate of Agricultural Research and Policies (GDAR).

2.1. Animal material

The animal material in the study consisted of 175 Kangal Akkaraman sheep (35 rams and 140 of their possible offspring), belonging to 35 flocks of a Kangal Akkaraman sheep breeding project in Sivas Province (Figure). Controlled hand-mating was implemented in selected farms for pedigree records.

2.2. Blood samples and DNA isolation

Using vacutainer tubes containing the anticoagulant K3-EDTA, blood samples were collected from rams during the breeding season and from their lambs at weaning (3 months after birth). Genomic DNA was extracted from the blood samples using a commercial DNA kit. A

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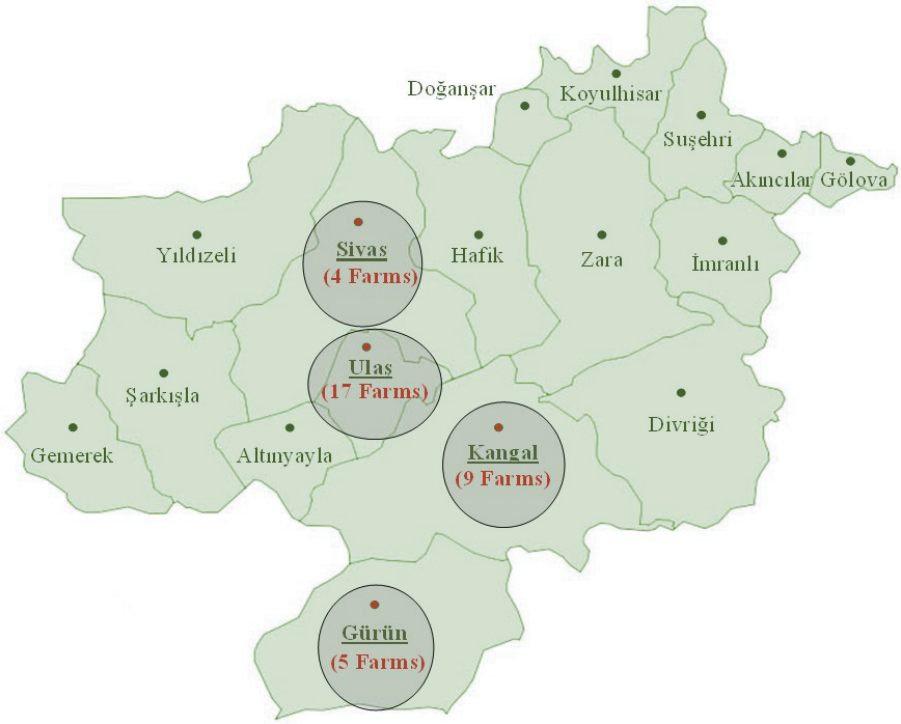

Figure. Locations of the sheep farms involved in the study.

DNA spectrophotometer (Thermo Scientific NanoDrop ND2000, Thermo Fisher Scientific, Waltham, MA, USA) was used to determine the quality and quantity of the DNA samples.

2.3. PCR and fragment analysis

Seventeen microsatellite markers (Table 1) recommended by FAO (28) and labeled with fluorescent dye (D2, D3, and D4) (Sigma® Life Science) were used for genotyping the animal material studied. Two multiplex groups were formed according to the allelic size range of the microsatellites. Polymerase chain reaction (PCR) realized according to Touchdown PCR protocol was applied in a 10-µL PCR mixture. Each amount of mixture included 0.1 µM/each primer, 0.2 mM dNTPs (Applied Biological Materials Inc., Richmond, BC, Canada), 2.0 mM $MgCl_2$, 1X PCR buffer, 1U of Taq DNA polymerase (Applied Biological Materials Inc.), and ~50 ng genomic DNA (Table 2).

Fragment analysis was performed by using the capillary electrophoresis method on the GenomeLab GeXP Genetic Analysis System (Beckman Coulter, Inc., Brea, CA, USA). Beckman GeXP fragment analysis software was used to determine fragment sizes for the microsatellite markers used.

2.4. Statistical analysis and formation of the new paternity test panels

The number of alleles per locus (Na), mean number of alleles (MNa), number of effective alleles (Ne), observed heterozygosity (Ho), expected heterozygosity (He), and Hardy–Weinberg equilibrium (HWE) were calculated using GenAlEx genetic analysis software (29,30). The polymorphic information content (PIC), probability of exclusion (PE), combined probability of exclusion (CPE), probability of identity (PI), combined probability of identity (CPI), and null allele frequencies (F[Null]) were calculated by using Cervus software, v3.0.3 (31,32).

A total of 17 paternity test panels, formed according to individual exclusion probability (PE) of microsatellites (Table 3), were analyzed according to the paternity analysis parameters.

3. Results

Genetic variability and paternity analysis results for each microsatellite locus obtained from this study are given in Table 4. A total of 240 different alleles were detected across all microsatellite markers studied. The observed number of alleles (Na) ranged from 8 (OarCP34) to 21 (HSC). The mean number of alleles was 14.12 in this study.

It can be said that the microsatellite markers used have high polymorphism, according to the Na, Ne, MNa, and PIC values obtained from the present study. The means of He and Ho for all studied loci were 0.76 and 0.80, respectively.

The highest and the lowest values of the individual PE were found in HSC (0.629) and BM1329 (0.292), respectively. The population deviated from HWE for investigated loci with the exception of OarFCB304.

Table 1. Details of considered microsatellite loci.

Primer name	Primer sequence	Chr. no.	AR (bp)	Label
OarFCB304	CCCTAGGAGCTTTCAATAAAGAATCGG	19	150–188	D3
	CGCTGCTGTCAACTGGGTCAGGG			
OarFCB193	TTCATCTCAGACTGGGATTCAGAAAGGC	11	96–136	D3
	GCTTGGAAATAACCCTCCTGCATCCC			
INRA0023	GAGTAGAGCTACAAGATAAACTTC		195–225	D3
	TAACTACAGGGTGTTAGATGAACTC			
OarFCB20	AAATGTGTTTAAGATTCCATACAGTG	2	93–112	D2
	GGAAAACCCCCATATATACCTATAC			
OarAE0129	AATCCAGTGTGTGAAAGACTAATCCAG	5	133–159	D2
	GTAGATCAAGATATAGAATATTTTTCAACACC			
BM1818	AGCTGGGAATATAACCAAAGG	20	248–278	D4
	AGTGCTTTCAAGGTCCATGC			
INRA0132	AACATTTCAGCTGATGGTGGC	20		D4
	TTCTGTTTTGAGTGGTAAGCTG			
OarCP34	GCTGAACAATGTGATATGTTCAGG	3	112–130	D4
	GGGACAATACTGTCTTAGATGCTGC			
D5S2	TACTCGTAGGGCAGGCTGCCTG			D4
	GAGACCTCAGGGTTGGTGATCAG			
CSRD0247	GGACTTGCCAGAACTCTGCAAT	14	220–247	D3
	CACTGTGGTTTGTATTAGTCAGG			
McM0527	GTCCATTGCCTCAAATCAATTC	5	165–187	D3
	AAACCACTTGACTACTCCCCAA			
BM8125	CTCTATCTGTGGAAAAGGTGGG	17	110–130	D3
	GGGGGTTAGACTTCAACATACG			
HSC	CTGCCAATGCAGAGACACAAGA	20		D2
	GTCTGTCTCCTGTCTTGTCATC			
BM1329	TTGTTTAGGCAAGTCCAAAGTC	6	160–182	D2
	AACACCGCAGCTTCATCC			
OarFCB128	ATTAAAGCATCTTCTCTTTATTTCCTCGC	2	96–130	D2
	CAGCTGAGCAACTAAGACATACATGCG			
OarJMP29	GTATACACGTGGACACCGCTTTGTAC	24	96–150	D4
	GAAGTGGCAAGATTCAGAGGGGAAG			
MAF214	GGGTGATCTTAGGGAGGTTTTGGAGG	16	174–282	D4
	AATGCAGGAGATCTGAGGCAGGGACG			

AR: Allelic range (bp) (FAO, 2011).

Table 2. Touchdown PCR conditions.

Loci	Multiplex group	First denaturation	Denaturation	Annealing	Extension	Cycle	Final extension
BM8125	1	95 °C (5 m)	95 °C (40 s)	60–50 °C (40 s)	72 °C (60 s)	34	72 °C (10 m)
CSRD0247							
HSC							
BM1329							
MAF214							
MCM0527							
OarFCB128							
OarJMP29							
BM1818	2	95 °C (5 m)	95 °C (40 s)	63–54 °C (40 s)	72 °C (60 s)	40	72 °C (10 m)
D5S2							
INRA0132							
INRA0023							
OarAE0129							
OarCP34							
OarFCB193							
OarFCB20							
OarFCB304							

Pedigree error was defined as 2.86% (4/140) based on the paternity analysis in this study.

Statistical values obtained from the paternity test panels, formed in accordance with the individual PE, are given in Table 5. The highest MNa ranged from 14.08 (Panel-12) to 19.00 (Panel-1). It is noteworthy that there is a linear relationship between the PIC and PE values obtained from the panels.

The highest and the lowest CPE values were observed in Panel-16 (0.9999771) and Panel-1 (0.8482247), respectively. In addition, it was clearly seen that microsatellites used in this study had a high power of individual identification, according to CPI values (<0.01) calculated for all panels.

New microsatellite multiplex groups were created for Panel-8 and Panel-9 with a high CPE in accordance with the Beckman Coulter Genetic Analysis System in order to enable the achievement of faster PCR reaction. These multiplex groups are given in Tables 6 and 7.

4. Discussion

The overall value of the effective number of alleles was 5.39. Although the values obtained were higher than those of some previous studies (26,33,34), they were lower than those of other previous studies (35–38). It is thought that the differences may have occurred as a result of the different breeds and microsatellites involved in the previous studies. The polymorphism information content (PIC) value obtained (0.78), an important criterion in the selection of microsatellites for the paternity tests, was significantly higher in similar studies (26,34,39–41). The PIC values obtained indicated that the microsatellite markers used can be utilized effectively in paternity analysis for this breed. The means of expected and observed heterozygosity (He and Ho) are similar to those of past studies conducted in different sheep breeds (26,33,37,38,40).

Probability of exclusion (PE) and probability of identity (PI), which are vital parameters for parentage analysis, were determined for this studied population. The values obtained for these 2 important parameters were paralleled in previous studies (25,26,42). Deviation from the HWE in the population studied is an expected result of the long-running selection program in the population, which is performed to avoid inbreeding.

The null allele was observed as a result of a lack of the microsatellite primer annealing to template DNA during the amplification stage of PCR. Dakin and Avise (43) reported that a null allele frequency value of less than 0.20 did not threaten the accuracy of the paternity test. In this context, the defined null allele frequencies indicated that the loci studied can be safely used in paternity tests for the Kangal Akkaraman breed. Pedigree errors have been reported to vary between 5% and 20% in past studies conducted in livestock (1,44). In this regard, the pedigree error rate described in this study can be considered relatively low for farm animals.

Table 3. Paternity testing panels formed with individual probability of exclusion.

Panel-1	**Panel-2**	**Panel-3**	**Panel-4**	**Panel-5**	**Panel-6**
HSC	HSC	HSC	HSC	HSC	HSC
CSRD0247	CSRD0247	CSRD0247	CSRD0247	CSRD0247	CSRD0247
	OarFCB193	OarFCB193	OarFCB193	OarFCB193	OarFCB193
		INRA0023	INRA0023	INRA0023	INRA0023
			OarJMP29	OARJMP29	OarJMP29
				INRA0132	INRA0132
					BM1818
Panel-7	**Panel-8**	**Panel-9**	**Panel-10**	**Panel-11**	**Panel-12**
HSC	HSC	HSC	HSC	HSC	HSC
CSRD0247	CSRD0247	CSRD0247	CSRD0247	CSRD0247	CSRD0247
OarFCB193	OarFCB193	OarFCB193	OarFCB193	OarFCB193	OarFCB193
INRA0023	INRA0023	INRA0023	INRA0023	INRA0023	INRA0023
OarJMP29	OarJMP29	OARJMP29	OarJMP29	OarJMP29	OarJMP29
INRA0132	INRA0132	INRA0132	INRA0132	INRA0132	INRA0132
BM1818	BM1818	BM1818	BM1818	BM1818	BM1818
MCM0527	MCM0527	MCM0527	MCM0527	MCM0527	MCM0527
	OarFCB20	OarFCB20	OarFCB20	OarFCB20	OarFCB20
		OarFCB128	OarFCB128	OarFCB128	OarFCB128
			MAF214	MAF214	MAF214
				OarAE129	OarAE129
					OarCP34
Panel-13	**Panel-14**	**Panel-15**	**Panel-16**		
HSC	HSC	HSC	HSC		
CSRD0247	CSRD0247	CSRD0247	CSRD0247		
OarFCB193	OarFCB193	OarFCB193	OarFCB193		
INRA0023	INRA0023	INRA0023	INRA0023		
OarJMP29	OarJMP29	OarJMP29	OarJMP29		
INRA0132	INRA0132	INRA0132	INRA0132		
BM1818	BM1818	BM1818	BM1818		
MCM0527	MCM0527	MCM0527	MCM0527		
OarFCB20	OarFCB20	OarFCB20	OarFCB20		
OarFCB128	OarFCB128	OarFCB128	OarFCB128		
MAF214	MAF214	MAF214	MAF214		
OarAE129	OarAE129	OarAE129	OarAE129		
OarCP34	OarCP34	OarCP34	OarCP34		
OarFCB304	OarFCB304	OarFCB304	OarFCB304		
	BM8125	BM8125	BM8125		
		D5S2	D5S2		
			BM1329		

Table 4. Genetic variability and paternity analysis parameters belonging to all considered microsatellites.

Loci	Na	Ne	Ho	He	PIC	PE	PI	HWE	F (Null)
HSC	21	8.84	0.86	0.89	0.88	0.629	2.31E-02	***	0.0184
CSRD0247	17	7.80	0.78	0.87	0.86	0.591	2.94E-02	***	0.0587
OarFCB193	17	6.55	0.74	0.85	0.83	0.541	3.93E-02	***	0.0760
INRA0023	12	6.21	0.91	0.84	0.82	0.525	4.26E-02	***	−0.0468
OARJMP29	16	6.10	0.85	0.84	0.82	0.514	4.51E-02	**	−0.0126
INRA0132	11	5.65	0.84	0.82	0.81	0.491	4.98E-02	***	−0.0175
BM1818	15	5.82	0.83	0.83	0.81	0.488	5.15E-02	***	−0.0049
MCM0527	11	5.44	0.73	0.82	0.79	0.464	5.83E-02	**	0.0564
OarFCB20	15	5.16	0.93	0.81	0.78	0.460	5.96E-02	***	−0.0781
OarFCB128	12	5.24	0.74	0.81	0.78	0.450	6.26E-02	***	0.0488
MAF214	12	5.09	0.69	0.81	0.78	0.437	6.70E-02	***	0.0757
OarAE129	16	4.26	0.70	0.77	0.74	0.407	7.63E-02	***	0.0458
OarCP34	8	4.32	0.94	0.77	0.74	0.394	8.03E-02	***	−0.1238
OarFCB304	18	3.92	0.70	0.75	0.73	0.388	8.17E-02	ns	0.0283
BM8125	16	4.23	0.71	0.77	0.73	0.384	8.90E-02	*	0.0317
D5S2	9	4.03	0.67	0.75	0.72	0.365	9.41E-02	***	0.0595
BM1329	14	2.95	0.31	0.66	0.65	0.292	1.28E-01	***	0.0373

Na: Number of allele; Ne: number of effective allele; Ho: observed heterozygosity; He: expected heterozygosity; PIC: polymorphic information content; PE: probability of exclusion; PI: Probability of identity; HWE: Hardy–Weinberg equilibrium; F (Null): Null allele frequency; ns: nonsignificant * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

Table 5. Genetic variability and paternity analysis parameters according to paternity panels.

Panels	No. of microsatellites	MNa	He	PIC	CPE	CPI
Panel-1	2	19.00	0.88	0.87	0.8482247	6.8E-04
Panel-2	3	18.33	0.87	0.86	0.9302976	2.7E-05
Panel-3	4	16.75	0.86	0.85	0.9668724	1.1E-06
Panel-4	5	16.60	0.86	0.84	0.9839124	5.1E-08
Panel-5	6	15.67	0.85	0.84	0.9918170	2.5E-09
Panel-6	7	15.57	0.85	0.83	0.9958121	1.3E-10
Panel-7	8	15.00	0.85	0.83	0.9977541	7.6E-12
Panel-8	9	15.00	0.84	0.82	0.9987879	4.6E-13
Panel-9	10	14.70	0.84	0.82	0.9993336	2.9E-14
Panel-10	11	14.45	0.84	0.81	0.9996245	1.9E-15
Panel-11	12	14.58	0.83	0.81	0.9997775	1.5E-16
Panel-12	13	14.08	0.83	0.80	0.9998651	1.2E-17
Panel-13	14	14.36	0.82	0.80	0.9999175	9.6E-19
Panel-14	15	14.47	0.82	0.79	0.9999491	8.5E-20
Panel-15	16	14.13	0.81	0.79	0.9999677	8.0E-21
Panel-16	17	14.12	0.80	0.78	0.9999771	1.0E-21

MNa: Mean number of allele; He: expected heterozygosity; PIC: polymorphic information content; CPE: combined probability of exclusion; CPI: combined probability of identity.

Table 6. Recommended multiplex groups for Panel-8.

No. of microsatellites	Multiplex group	Label	Loci	Allelic range reported by FAO, ISAG, and NCBI	Allelic range in this study (bp)	CPE
9 Microsatellites	M1	D3	OarFCB193	96–136	98–136	0.9988
		D3	INRA0132	152–172	152–174	
		D3	INRA0023	195–225	193–221	
		D3	BM1818	248–278	246–284	
		D2	OarFCB20	93–112	83–125	
		D2	MCM0527	165–187	165–187	
		D2	HSC	267–301	213–303	
		D4	OarJMP29	96–150	94–148	
		D4	CSRD0247	220–247	207–255	

CPE: combined probability of exclusion.

Table 7. Recommended multiplex groups for Panel-9.

No. of microsatellites	Multiplex group	Label	Loci	Allelic range reported by FAO, ISAG, and NCBI	Allelic range in this study (bp)	CPE
10 Microsatellites	M1	D3	OarFCB193	96–136	98–136	**0.9993**
		D3	INRA0132	152–172	152–174	
		D3	INRA0023	195–225	193–221	
		D3	BM1818	248–278	246–284	
		D2	OarFCB20	93–112	83–125	
		D2	MCM0527	165–187	165–187	
		D2	HSC	267–301	213–303	
		D4	OarJMP29	96–150	94–148	
		D4	CSRD0247	220–247	207–255	
	M2		OrFCB128	96–130	96–126	

CPE: combined probability of exclusion.

The mean number of alleles (MNa) and expected heterozygosity obtained from the panels were higher than in previous studies (12,26). The linear relationship between PIC and PE values in the present study is consistent with those in the relevant literature (12,26,42,45).

The recommended minimum CPE value for the true father to be identified with high accuracy is reported as 0.999 (44,46,47). In that respect, it can be said that Panel-8 and Panel-9 could be used with confidence for the paternity analysis, considering the CPE values obtained from these panels. There is no doubt that using a great number of microsatellite markers for paternity testing will increase CPE values. On the other hand, this would be a time-consuming and expensive paternity testing process. In light of the foregoing information, we can clearly state that Panel-8 and Panel-9 were cheaper and more practical when compared to the other panels introduced in the present study. CPI values obtained are within the acceptance limits specified by Waits et al. (48).

It has become possible to amplify DNA with microsatellite markers in a single PCR reaction using Panel-8 for the paternity test performed in Kangal Akkaraman sheep. Assessment results obtained for paternity testing panels in this study showed that Panel-8 (CPE = 0.9988) had a clear advantage in terms of reliability, speed, and cheapness for paternity test studies to be applied in the Kangal Akkaraman sheep population.

Although paternity tests are expensive and time-consuming, they are an essential practice in sheep husbandry. It is crucial that paternity test methods

used in livestock are accurate, cheap, and reliable in terms of preventing pedigree errors. The purpose of the current study was to develop paternity test panels using microsatellite markers to analyze parentage in sheep. Assessment results indicated that the paternity testing achieved high accuracy and was economical, using a relatively small number of microsatellites. Consequently, it can be clearly stated that controlled mating programs can be performed with great success under field conditions and that potential pedigree errors, which may occur for different reasons, can be prevented using DNA-based microsatellite markers. Cheap, fast, and reliable paternity test panels were developed for the Kangal Akkaraman sheep population; these panels should be tested in other breeds in order to develop their widespread and effective use in the future.

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