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Power of different microsatellite panels for paternity analysis in sheep

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The aim of this work was to develop and to test PCR-based suitable microsatellite marker panels for paternity testing in sheep. Blood samples were collected from 212 Kivircik sheep (24 rams and their 188 offspring) raised in five farms were included in the controlled mating programme and genotyped with 20 microsatellite markers. Different combinations with multiplex microsatellite groups were established, providing seven microsatellite panels to determine the power of paternity testing. A total of 318 alleles were detected across the 20 loci investigated. The average PIC value (0.80) obtained from all loci indicated that these microsatellite panels were very polymorphic and highly informative. The highest and lowest average number of alleles were observed in Panel 3 and Panel 1, respectively, among the formed panels. Probability of identity (PI) values varied from 0.102 to 0.020. The power of discrimination (PD) values ranged from 0.678 and 0.979. Combined probability of identity (CPI) value ranged between $1.20x10^{-26}$ (Panel-7) and $7.24x10^{-6}$ (Panel-1). The lowest and highest combined probability of exclusion values were recorded for Panel-1 (0.931912000) and Panel-7 (0.999998870), respectively, as it could have been expected. According to the study results panels 4 and 5, formed with fewer microsatellite markers for paternity tests, were cheaper and more practical than the other panels in Kivircik sheep.

KEY WORDS: Combined probability of exclusion; microsatellite; paternity, ovine

In animal breeding, particularly selection of breeding stock in farm animals, it is essential not only to keep regularly updated pedigree records, but also to ensure accuracy of the parental information. Identification or validation of parents is an important key to success in animal breeding [Yilmaz and Karaca 2012]. Animal breeding programs require accurate pedigree information to provide efficient progress and to avoid

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excessive inbreeding. Pedigree errors are considered to be a common problem in livestock, particularly in small ruminants raised as flocks and giving birth to multiple offspring. In the cases that accurate information can be revealed by applying parental control methods [Crawford *et al.* 1995, Kosum 1995]. In sheep breeding, keeping records of mating and birth is crucial for obtaining reliable information on production capacity of the flock. In addition, it is important for yield records to be sustainable on the basis of pedigree. However, it is a current problem to verify the obtained results from mating practices carried out in the animal breeding programs in the field and to identify the parents accurately. It has been reported that the application of multiple microsatellite markers in parental tests may be useful thanks to the low diversity within highly related and endangered species populations [Ganai and Yadav 2005].

Studies show that pedigree errors are varied from 5% and 20% on cattle and sheep farms [Weller *et al.* 2004, Vandeputte *et al.* 2006, Crawford *et al.* 1993]. These conditions cause an incorrect estimation of genetic parameters, which is a great problem for animal breeding plans. Therefore, in order to control pedigree records it became mandatory to use highly reliable DNA-based marker systems. Microsatellites, which are widely used in paternity tests, show similarity on the basis of the entire population, whereas they show small differences between individuals. Microsatellites are valuable genetic markers due to their dense distribution in the genome, considerable variation, co-dominant inheritance and easy genotyping. In recent years they have been extensively used in parentage testing, linkage analyses, population genetics and other genetic studies [Jeffreys *et al.* 1987*,* Georges *et al.* 1988, Bruford *et al.* 1996, Montaldo and Meza-Herrera 1998, Beuzen *et al.* 2000, Goldstein and Schlötterer 2001, Un *et al.* 2001, Sancristobal *et al.* 2003]

This study was conducted in order to develop and test suitable microsatellite panels for paternity testing in sheep. The resulting information will provide an important contribution to paternity testing studies.

Material and methods

Animal resources and DNA isolation

Animal material of the study comprised a total of 212 Kivircik sheep (24 rams and their 188 offspring) raised in five farms and used in the controlled mating programme. Blood samples from five different sheep flocks were collected from the jugular vein using vacutainer tubes containing K3EDTA as an anticoagulant. A DNA isolation kit (Applied Biological Materials Inc., Canada) was used to extract genomic DNA from blood samples.

PCR and fragment analysis

Twenty microsatellite markers (Table S1) recommended by FAO [2011] were used for the microsatellite analysis of Kivircik sheep. Three multiplex groups were formed based on fragment size of 20 microsatellite loci used to amplify genomic DNA

Table 1. Microsatellite panels based on different combinations with multiplex microsatellite groups in sheep

Panel-1	Panel-2	Panel-3	Panel-4	Panel-5	Panel-6	Panel-7
INRA0005	CSRD0247	OARCFCB304	CSRD0247	OARCFCB304	OARCFCB304	OARCFCB304
OarFCB0011	MCM0527	OARFCB193	MCM0527	OARFCB193	OARFCB193	OARFCB193
DYMS1	BM8125	OARFCB20	BM8125	OARFCB20	OARFCB20	OARFCB20
MAF0065	HSC	OARAE0129	HSC	OARAE0129	OARAE0129	OARAE0129
	BM1329	BM1818	BM1329	BM1818	BM1818	BM1818
	OARFCB128	INRA0132	OARFCB128	INRA0132	INRA0132	INRA0132
	OARJMP29	OARCP34	OARJMP29	OARCP34	OARCP34	OARCP34
	MAF214	D5S2	MAF214	D5S2	D5S2	D5S2
			INRA0005	INRA0005	CSRD0247	CSRD0247
			OarFCB0011	OarFCB0011	MCM0527	MCM0527
			DYMS1	DYMS1	BM8125	BM8125
			MAF0065	MAF0065	HSC	HSC
					BM1329	BM1329
					OARFCB128	OARFCB128
					OARJMP29	OARJMP29
					MAF214	MAF214
						INRA0005
						OarFCB0011
						DYMS1
						MAF0065

Table 2. Genetic variability and paternity analysis parameters for all considered microsatellites in the studied sheep population

Locus	N	Na	Ne	Ho	He	PIC	PE	PI	PD	HWEa	F(Null)
OARFCB304	208	21	4.51	0.73	0.78	0.75	0.415	0.077	0.870	***	0.0162
OARFCB193	203	18	7.98	0.96	0.88	0.87	0.614	0.025	0.960	ns	0.0542
OARFCB20	203	22	5.11	0.86	0.81	0.79	0.475	0.056	0.810	*	0.0576
OARAE129	207	18	3.95	0.51	0.75	0.71	0.367	0.099	0.876	***	0.1991
BM1818	202	17	4.67	0.98	0.79	0.76	0.418	0.075	0.678	***	0.1214
INRA0132	206	20	9.69	0.84	0.90	0.89	0.655	0.020	0.969	ns	0.0312
OARCP34	206	11	5.10	0.93	0.81	0.78	0.449	0.062	0.912	ns	0.0749
D5S2	208	14	3.62	0.62	0.73	0.70	0.346	0.102	0.869	ns	0.0728
CSRD0247	210	21	6.43	0.85	0.85	0.83	0.538	0.040	0.944	ns	0.0048
MCM0527	210	13	5.18	0.73	0.81	0.79	0.469	0.055	0.938	ns	0.0484
BM8125	210	10	5.70	0.75	0.83	0.80	0.483	0.053	0.936	ns	0.0503
HSC	196	15	6.01	0.62	0.84	0.82	0.512	0.046	0.811	ns	0.1398
BM1329	187	11	5.65	0.75	0.83	0.80	0.483	0.053	0.888	ns	0.0363
OARFCB128	211	12	6.64	0.70	0.85	0.83	0.540	0.039	0.941	ns	0.0889
OARJMP29	210	22	8.16	0.72	0.88	0.87	0.609	0.027	0.938	ns	0.1027
MAF214	210	18	5.31	0.90	0.82	0.79	0.467	0.058	0.893	**	0.0626
INRA0005	208	17	6.09	0.83	0.84	0.82	0.519	0.044	0.924	ns	0.0049
OarFCB0011	211	12	6.47	0.90	0.85	0.83	0.536	0.040	0.946	ns	0.0359
DYMS1	211	15	5.84	0.98	0.83	0.81	0.503	0.048	0.890	**	0.0941
MAF0065	211	11	4.31	0.99	0.77	0.74	0.387	0.086	0.839	***	0.1398
Mean		15.90	5.82	0.81	0.82	0.80					

N – number of samples; Na – number of alleles; Ne – effective number of alleles; Ho – observed heterozygosity; He – expected heterozygosity; PIC – polymorphic information content; PE – probability of exclusion; PI – probability of identity; PD – power of discrimination; HWE – Hardy–Weinberg equilibrium; F (Null) –null allele frequency.
ns – non-significant; *P<0.05; ** P<0.01; *** P<0.001.

by the Polymerase Chain Reaction (PCR) in accordance with the touchdown PCR technique (Table S2). Polymerase chain reaction (PCR) was run in a 25μL reaction volume containing 100 ng DNA, 5× PCR buffer, 0.10 mM of each primer, 2.0 mM

Supplementary Table S1
Table S1. Details of considered microsatellites according to FAO [2011]

Loci		Primers	Chr. no	Allele Range	Dye
Group 1					
OarFCB304	F	CCCTAGGAGCTTTCAATAAAGAATCGG	19	150-188	D3
	R	CGCTGCTGTCAACTGGGTCAGGG			
OARFCB193	F	TTCATCTCAGACTGGGATTCAGAAAGGC	11	96-136	D3
	R	GCTTGGAAATAACCCTCCTGCATCCC			
OarFCB20	F	AAATGTGTTTAAGATTCCATACAGTG	2	93-112	D2
	R	GGAAAACCCCCATATATACCTATAC			
OarAE0129	F	AATCCAGTGTGTGAAAGACTAATCCAG	5	133-159	D2
	R	GTAGATCAAGATATAGAATATTTTTCAACACC			
BM1818	F	AGCTGGGAATATAACCAAAGG	20	248-278	D4
	R	AGTGCTTTCAAGGTCCATGC			
INRA0132	F	AACATTTCAGCTGATGGTGGC	20	152-178	D4
	R	TTCTGTTTTGAGTGGTAAGCTG			
OARCP34	F	GCTGAACAATGTGATATGTTCAGG	3	112-130	D4
	R	GGGACAATACTGTCTTAGATGCTGC			
D5S2	F	TACTCGTAGGGCAGGCTGCCTG		190-210	D4
	R	GAGACCTCAGGGTTGGTGATCAG			
Group 2					
CSRD0247	F	GGACTTGCCAGAACTCTGCAAT	14	220-247	D3
	R	CACTGTGGTTTGTATTAGTCAGG			
McM0527	F	GTCCATTGCCTCAAATCAATTC	5	165-187	D3
	R	AAACCACTTGACTACTCCCCAA			
BM8125	F	CTCTATCTGTGGAAAAGGTGGG	17	110-130	D3
	R	GGGGGTTAGACTTCAACATACG			
HSC (OLADRB)	F	CTGCCAATGCAGAGACACAAGA	20	267-301	D2
	R	GTCTGTCTCCTGTCTTGTCATC			
BM1329	F	TTGTTTAGGCAAGTCCAAAGTC	6	160-182	D2
	R	AACACCGCAGCTTCATCC			
OARFCB128	F	ATTAAAGCATCTTCTCTTTATTTCCTCGC	2	96-130	D2
	R	CAGCTGAGCAACTAAGACATACATGCG			
OARJMP29	F	GTATACACGTGGACACCGCTTTGTAC	24	96-150	D4
	R	GAAGTGGCAAGATTCAGAGGGGAAG			
MAF214	F	GGGTGATCTTAGGGAGGTTTTGGAGG	16	174-282	D4
	R	AATGCAGGAGATCTGAGGCAGGGACG			
Group 3					
INRA0005	F	CAATCTGCATGAAGTATAAATAT	10	135-149	D3
	R	CTTCAGGCATACCCTACACC			
OarFCB0011	F	GCAAGCAGGTTCTTTACCACTAGCACC	2	122-148	D2
	R	GGCCTGAACTCACAAGTTGATATATCTATCAC			
DYMS1	F	AACAACATCAAACAGTAAGAG	20	159-211	D2
	R	CATAGTAACAGATCTTCCTACA			
MAF0065	F	AAAGGCCAGAGTATGCAATTAGGAG	15	123-157	D4
	R	CCACTCCTCCTGAGAATATAACATG			

$MgCl_2$, 0.20 mM dNTPs (Applied Biological Materials Inc., Canada) and 1U of Taq DNA polymerase (Applied Biological Materials Inc., Canada).

Fragment analysis was performed using the GenomeLab™ GeXP Genetic Analysis System (Beckman Coulter, Inc., USA). Acquired data was analysed using the Beckman Coulter CEQ Fragment Analysis Software. The panels were formed by different combinations with multiplex microsatellite groups containing 20 microsatellites to determine the power of paternity testing (Tab. 1).

Supplementary Table S2
Table S2 Microsatellite *loci*, multiplex group and thermal cycling conditions according to Touchdown PCR

Loci	Dye	Multiplex Group	First Denat.	Denat.	Annealing	Extension	Cycle	Final Extention
INRA0005	D3	1	95 ºC (5 min)	95 ºC (40sec)	63-50 ºC (40 sec)	72 ºC (60 sec)	42	72 ºC (10 min)
OarFCB0011	D2							
DYMS1	D2							
MAF0065	D4							
CSRD0247	D3	2	95 ºC (5 min)	95 ºC (40sec)	60-50 ºC (40 sec)	72 ºC (60 sec)	34	72 ºC (10 min)
MCM0527	D3							
BM8125	D3							
HSC	D2							
BM1329	D2							
OARFCB128	D2							
OARJMP29	D4							
MAF214	D4							
OARCFCB304	D3	3	95 ºC (5 min)	95 ºC (40sec)	63-54 ºC (40 Sec)	72 ºC (60 sec)	40	72 ºC (10 min)
OARFCB193	D3							
OARFCB20	D2							
OARAE0129	D2							
BM1818	D4							
INRA0132	D4							
OARCP34	D4							
D5S2	D4							

Statistical analyses

The number of samples (N), number of alleles (Na), observed heterozygosity (Ho), expected heterozygosity (He), polymorphic information content (PIC), probability of exclusion (PE), combined probability of exclusion (CPE) and null allele frequency (F (Null)) estimated using Cervus version 3.0.3 [Kalinowski *et al.* 2007]. Probability of identity (PI), combined probability of identity (CPI), Hardy-Weinberg equilibrium (HWE) and effective number of alleles (Ne) were determined employing GenAlEx [Peakall and Smouse 2006]. The power of discrimination (PD) was calculated using PowerStatsV12 [Brenner and Morris 1990].

Results and discussion

A total of 318 alleles were detected across the 20 *loci* investigated. The number of alleles ranged from 10 (BM1818) to 22 (OARFCB20 and OARJMP29), with the average number of alleles amounting to 15.90. The average value of the effective number of alleles was 5.82 (Tab. 2).

The average PIC value obtained was relatively high (0.80). For all studied loci the observed (Ho) and expected heterozygosity (He) values were 0.81 and 0.82, respectively, as the overall average. The total number of alleles, number of effective alleles and PIC value derived from 20 microsatellite loci used in this study were

higher than the those reported in literature [Bolormaa *et al.* 2008, Zhang *et al.* 2010, Yilmaz and Karaca 2012]. This is an indication that the studied microsatellites exhibit a relatively high polymorphism.

The highest and lowest values in terms of the probability of exclusion (PE), which is an important parameter in paternity tests, were found in D5S2 (0.346) and INRA0132 (0.655), respectively.

Probability of identity (PI) values, also called population match probability and providing the number of individuals having the same DNA profile, ranged from 0.102 to 0.020. In this study the power of discrimination (PD) values ranged from 0.678 and 0.979. It is observed that the probability of exclusion (PE) and the probability of identity (PI) values, which are crucial parameters in paternity analysis, for all studied loci are similar to these given in research papers [Quanbari *et al.* 2007, Souza *et al.* 2012, Yilmaz and Karaca 2012]. Great power of discrimination (PD) values (>0.80) obtained for all the studied *loci*, except for BM1818, are the result of the high number of alleles, which is consistent with the literature findings [Yilmaz and Karaca 2012].

The results obtained from 20 microsatellite loci were tested using the χ^2 test of the Hardy-Weinberg equilibrium. The population was not found at Hardy-Weinberg equilibrium for investigated seven loci. This event has emerged as a natural consequence of the selection program conducted to for many years to prevent inbreeding in this population.

It is remarkable that the highest frequency was observed in the OARAE129 (0.1991) locus for null alleles, described as non-amplifying alleles due to mutations at PCR priming sites. A null allele is any microsatellite allele at a microsatellite locus, which consistently fails to amplify to detect levels in the PCR assays, and which has no significant impact on parentage analysis when the frequency is below 0.20 [Dakin and Avise 2004]. It shows that the resulting null allele frequencies were lower than the specified value of these loci, which may be safely used in paternity analysis.

Results of the panels formed from the combination of microsatellite *loci* are presented in Table 3.

Table 3. Genetic variability and paternity analysis parameters based on microsatellite panels formed with different combinations of microsatellites

Marker Panel	MNA	He	PIC	CPE	CPI
Panel-1	13.75	0.82	0.80	0.931912000	7.24E-06
Panel-2	15.25	0.84	0.82	0.996932000	1.71E-11
Panel-3	17.63	0.81	0.78	0.994573000	9.73E-11
Panel-4	14.75	0.83	0.81	0.999791000	1.24E-16
Panel-5	16.25	0.76	0.74	0.999682000	7.05E-16
Panel-6	16.44	0.82	0.80	0.999983350	1.66E-21
Panel-7	15.90	0.82	0.80	0.999998870	1.20E-26

MNA – mean number of alleles; He – expected heterozygosity; PIC – polymorphic information content; CPE – combined probability of exclusion; CPI – combined probability of identity.

The highest and lowest average number of alleles were found in Panel 3 and Panel 1, respectively, among the established panels. The highest He value was recorded in Panel 2. It was observed that PIC values are relatively high in all the established microsatellite panels. Average allele numbers (MNA) per locus for panels formed by different microsatellite combinations for the paternity analysis were higher than the literature values except for Panel 1, while He values were found to be similar to those reported in research papers [Roberts and Thomas 2003, Zhang *et al.* 2010, Yilmaz and Karaca 2012, Rosa *et al.* 2013].

Expected heterozygosity values are larger than 0.70 and it provides the power of segmentation even though individuals are close to each other. There was a linear relationship between the number of microsatellites in panels with PIC values and PE values. The relevant literature seems to support this information [Ganai and Yadav 2005, Jimenez-Gamero *et al.* 2006, Zhao *et al.* 2006, Bolormaa *et al.* 2008, Tian *et al.* 2008, Araujo *et al.* 2010, Yilmaz and Karaca 2012].

In terms of the combined probability of exclusion, the lowest and highest values are obtained in Panel 1 (0.931912000) and Panel 7 (0.999998870), respectively, as it could have been expected. When Table 3 is examined, it was found that the combined probability of identity (CPI) value ranges between $1.20x10^{-26}$ and $7.24x10^{-6}$. It was reported that the required minimum CPE value to identify the actual sire was 0.999 [Luikart *et al.* 1999, Sherman *et al.* 2004, Van Eenennaam *et al.* 2007]. In this study, CPE values obtained from Panels 4, 5, 6 and 7 have minimum amounts of exclusion reported in the literature on the subject. It can be said that Panels 4 and 5, formed with fewer microsatellite markers for paternity tests, were cheaper and more practical than the other panels according to the obtained results from the study. Although Panel 4 and Panel 5 include an equal number of microsatellites, PIC, He and CPE values in Panel 4 was higher than Panel 5. This remarkable situation showed that Panel 4 is much more suitable for use in paternity tests. Low CPE values obtained from Panels 1, 2 and 3 show that they will not give accurate results in paternity tests. CPI values (<0.01) for all the panels indicated that the used microsatellites have a high power of individual identification and have confidence intervals as those reported by Waits *et al.* [2001].

Testing of reliability of pedigree records by DNA-based paternity tests improves selection accuracy, while it also increases the rate of genetic gain. It is generally known that the exclusion probability value will increase as a result of merging multiple loci when single loci have a limited exclusion probability. When analysing literature sources it was observed that the microsatellite numbers used in paternity tests vary. However, using numerous loci for paternity tests can be expensive and time-consuming.

Therefore forming breed-specific marker panels will enable paternity tests to be cheaper and faster. Microsatellites used in the study have high genetic diversity, indicating that they can be used by creating suitable panels in paternity tests.

In the conducted study the microsatellite panels are shown to be appropriate for a high precision paternity test. It significantly contributes to the dissemination of

paternity tests as a particular service area and provides information needed to facilitate their transfer into practice.

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