MICROSATELLITE DNA POLYMORPHISM AND ITS USEFULNESS FOR PEDIGREE VERIFICATION IN SIMMENTAL CATTLE FROM SERBIA

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The polymorphism of 11 microsatellite DNA markers (TGLA227, BM2113, TGLA53, ETH10, SPS115, TGLA126, TGLA122, INRA23, ETH3, ETH225, BM1824) was analysed in order to assess the usefulness of these markers for pedigree verification in the Serbian population of Simmental cattle. A total of 86 animals were involved in the study. Genomic DNA was extracted from sperm and blood and amplified in a multiplex PCR using primers from the commercial "StockMarks for Cattle® Bovine Genotyping Kit". Analyses were done with automated DNA sizing technology. A total of 92 alleles were detected. The mean number of alleles (n_A) per locus was 8.364, the mean value of polymorphism information content (PIC) was 0.73, and the mean frequency of the most frequent allele (FNA) was 0.372. The most polymorphic microsatellite loci were: TGLA53 (14 alleles, PIC=0.88, FNA=0.235), TGLA227 (11 alleles, PIC=0.82, FNA=0.274), INRA23 (11 alleles, PIC=0.86, FNA=0.167), BM2113 (9 alleles, PIC=0.84, FNA=0.330). Both combined power of discrimination (CPD) and combined power of exclusion (CPE) for the whole set of studied markers were 0.999. In conclusion, the polymorphism of 11 microsatellite DNA markers observed in this study indicate its usefulness for paternity testing and pedigree verification in Simmental cattle from Serbia.

Key words: cattle, microsatellites, pedigree, polymorphism, Serbia

INTRODUCTION

Microsatellite DNA markers are excellent genetic markers because of their high polymorphism and abundant distribution throughout the genome. They are generally polyallelic, and their number is almost unlimited. Nowadays, microsatellite DNA markers are used for paternity testing and pedigree verification of livestock, including cattle (Curi and Lopes, 2002; Radko, 2008; Rehout *et al*., 2006; Cervini *et al*., 2006; Tian *et al*., 2008; Ozkan *et al*., 2009; Carolino *et al*., 2009). Many authors used microsatellites for the analyses of genetic diversity in

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cattle (Čítek and Rehout, 2001; Čítek *et al*., 2006; Grzybowski and Prusak, 2004a, 2004b; Radko *et al*., 2005; Zhou *et al*., 2005; Czernekova *et al*., 2006; Zaton-Dobrowolska *et al*., 2007).

Paternity testing using microsatellites in cattle has significant advantages over the traditional methods using blood groups and protein polymorphism. The main disadvantage of traditional methods is the relatively low precision since it does not enable always the exclusion of a putative sire, even if that putative sire is not the real parent. Consequently, high frequency of incorrect cattle paternity was obtained with these methods, and the proportion of errors in sire identification ranged from few percent to as much as 23% (Bovenhuis and van Arendonk, 1991). Another disadvantage is that blood and protein analyses can not be performed retrospectively, e.g., after a sire is dead. On the other hand, the accuracy of the parentage test using microsatellite DNA markers is much higher, as the probability of detecting mistaken paternity or maternity is a function of the polymorphism of genotyped loci (Ron *et al*., 1996). In addition, any sample from an animal (e.g., hair, milk, saliva, semen) can be used, as long as it contains DNA, so genotyping can be done retrospectively, e.g., after a sire is dead, if any sample is stored.

Pedigree verification through paternity testing is necessary if we want to achieve optimal genetic progress in cattle breeding. Misidentification of parentage can lead to breeding inaccuracy, causing great financial losses in herd management and in the beef industry (Cervini *et al*., 2006). In conservation programs, pedigree recording is important for the control of inbreeding and loss of genetic variability and fitness (Carolino *et al*., 2006).

Microsatellite markers that are characterized by high polymorphism, well-balanced frequency of alleles, high values of polymorphism information content, expected heterozygosity and power of discrimination and probability of exclusion, are considered usefull for pedigree verification (Radko, 2008; Ozkan *et al*., 2009). Combined power of discrimination for all analysed loci must be greater than 0.999 for the acceptable level of the microsatellite loci in parentage testing (Vankan and Faddy, 1999; Pérez-Miranda *et al*., 2005).

Polymorphism of 11 microsatellite markers (TGLA227, BM2113, TGLA53, ETH10, SPS115, TGLA126, TGLA122, INRA23, ETH3, ETH225, BM1824) recommended by the International Society for Animal Genetics (ISAG) was already evaluated for parentage verification in other Simmental breeds, in Czech Pied and Slovakian Pied (Czerneková *et al*., 2006) and in Simmental cattle from Poland (Janik *et al*., 2001; Choroszy *et al*., 2006). However, in cattle raised in Serbia, there were no investigations of microsatellite DNA polymorphism and no report about successful establishment of paternity identification. There were only cytogenetic investigations in the past (Soldatović *et al*., 1993, 1994a, 1994b; Vučinić *et al*., 1996).

The aim of this study was to analyse the polymorphism of 11 microsatellite markers from ISAG panel (TGLA227, BM2113, TGLA53, ETH10, SPS115, TGLA126, TGLA122, INRA23, ETH3, ETH225, BM1824) and to evaluate their usefulness for pedigree verification in the Serbian population of Simmental cattle.

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MATERIAL AND METHODS

Sperm or blood samples were taken from 86 heads of Simmental cattle from Serbia. Experiments performed comply with current laws and written consent of the Scientific Ethic Committee and were done with the permission of the Local Ethic Commitee of the Faculty of Veterinary Medicine, University of Belgrade. Genomic DNA from sperm and blood was isolated with "DNeasy® Blood & Tissue Kit" (Qiagen, Valencia, CA) and kept frozen at -18ºC until further processing. Microsatellites were amplified using the "StockMarks for Cattle® Bovine Genotyping Kit" (Applied Biosystems Inc., Foster City, CA). Multiplex amplifications were performed in a programmable thermal cycler MultiGene Gradient (Labnet International Inc) in 15 μL volumes containing 1 μL of DNA, 3.0 μL of StockMarks buffer, 4.0 μL of dNTP mix (1.25 mM of each dNTP), 5.5 μL of amplification primer mix, 0.5 μL of AmpliTaq Gold DNA Polymerase (Applied Biosystems Inc., Foster City, CA), and 1.0 μL of deionized water. The sample was then amplified using PCR with the following program: the reactions were heated for 15 min at 95ºC followed by 31 cycles of denaturation (94ºC, 45 s), annealing (61ºC, 45 s), and extension (72ºC, 60 s). A final extension was carried out at 72ºC for 1 h and then at 25ºC for 2 h.

The fluorescent labeled PCR products were submitted to fragments analysis by capillary electrophoresis, with an automated sequencer ABI PRISM 310 (Applied Biosystems), using the GeneScan-350 ROX® Size Standard (Applied Biosystems), according to manufacturer's specifications. Results were read and interpreted using GeneScan® and Genotyper® software, respectively.

Eleven microsatellites (TGLA227, BM2113, TGLA53, ETH10, SPS115, TGLA126, TGLA122, INRA23, ETH3, ETH225, BM1824) recommended by ISAG for cattle paternity testing (ISAG Conference, 2008) were analysed in this study. Microsatellite markers are usefull in parentage testing and pedigree verification if they are highly informative. All measures of informativeness were estimated for the chosen markers of ISAG panel. The number of alleles (n_A), frequency of the most frequent allele (FNA), observed and expected heterozygosity (Ho and He), polymorphism information content (PIC), power of discrimination (PD) and power of exclusion (PE) were calculated for each microsatellite marker. Combined power of discrimination (CPE) and combined power of exclusion (CPE) were calculated for the whole set of studied markers. Allele frequencies, PIC, PD and PE were determined by the PowerStatsV12 freeware, Promega Corporation, USA. Ho and He, as well as calculations for Hardy-Weinberg Equilibrium were performed in Arlequine ver. 3.1 (Excoffier *et al*., 2006).

RESULTS

In the studied population of Simmental cattle from Serbia, the n_A per locus ranged from 6 (SPS115, TGLA126, ETH3, BM1824) to 14 (TGLA53), the mean n_A per locus was 8.364, and a total number of alleles was 92. The mean frequency of the most frequent allele (FNA) was 0.372 (Table 1). PIC values ranged from 0.58 to 0.88 with mean value of 0.73 (Table 2). High number of alleles and PIC values were

observed for TGLA53 (14 alleles, PIC=0.88), TGLA227 (11 alleles, PIC=0.82), INRA23 (11 alleles, PIC=0.86), BM2113 (9 alleles, PIC=0.84) and TGLA122 (9 alleles, PIC=0.70). In addition, FNA of these loci was well below 0.5, with lowest values in INRA23 (0.167), TGLA53 (0.235), TGLA227 (0.274) and BM2113 (0.330), thus are the most polymorphic and most informative markers among tested loci in this study (Table 1). These four loci (TGLA53, TGLA227, INRA23 and BM2113) showed also highest values of He and PD (Table 2). Although all loci showed PIC values over 0.5 (Table 2), the polymorphism and informativeness of ETH10 and SPS115 is lower than other loci because their FNA values were higher than 0.5 (Table 1). Both CPD and CPE were 0.999 (Table 2). In addition, Ho ranged from 0.452 (TGLA122) to 0.774 (INRA23), with mean value of 0.662, whilst He varied from 0.557 (ETH10) to 0.893 (TGLA53), with an average of 0.753. Except loci TGLA227, TGLA53 and TGLA122 that were significantly deviated from Hardy-Weinberg equilibrium ($p < 0.05$), other loci were conformed to Hardy-Weinberg equilibrium.

Table 1. Polymorphism of the 11 microsatellite markers in the Serbian population of Simmental cattle

Microsatellite marker	n_A	Allele size range (bp)	Alleles	Allele frequency	FNA
TGLA227	11	78-114	78	0.032	0.274
			80	0.274	
			82	0.032	
			86	0.016	
			88	0.097	
			90	0.194	
			92	0.097	
			94	0.032	
			96	0.129	
			98	0.081	
			114	0.016	
BM2113	9	122-142	122	0.070	0.330
			124	0.133	
			128	0.330	
			130	0.084	
			132	0.150	
			134	0.033	
			136	0.100	
			140	0.033	
			142	0.067	

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Cont. Table 1.					
TGLA53	14	150-184	150	0.103	0.235
			154	0.029	
			156	0.103	
			158	0.029	
			160	0.059	
			162	0.044	
			164	0.235	
			166	0.118	
			168	0.088	
			170	0.059	
			172	0.015	
			174	0.059	
			182	0.044	
			184	0.015	
ETH10	7	206-220	206	0.020	0.600
			210	0.060	
			212	0.040	
			214	0.600	
			216	0.120	
			218	0.100	
			220	0.060	
SPS115	6	242-254	242	0.515	0.515
			244	0.015	
			246	0.088	
			248	0.015	
			250	0.250	
			254	0.118	
TGLA126	6	114-124	114	0.375	0.438
			116	0.438	
			118	0.109	
			120	0.031	
			122	0.016	
			124	0.031	
TGLA122	9	138-162	138	0.045	0.424
			140	0.212	
			144	0.030	
			146	0.030	

Cont. Table 1.					
TGLA122 (cont.)			150	0.424	
			152	0.182	
			154	0.015	
			156	0.015	
			162	0.045	
INRA23	11	198-220	198	0.106	0.167
			200	0.045	
			202	0.030	
			204	0.076	
			206	0.121	
			208	0.167	
			210	0.106	
			212	0.152	
			214	0.167	
			218	0.015	
			220	0.015	
ETH3	6	112-124	112	0.288	0.288
			114	0.227	
			116	0.015	
			120	0.091	
			122	0.152	
			124	0.227	
ETH225	7	134-150	134	0.197	0.452
			138	0.061	
			140	0.045	
			142	0.121	
			144	0.452	
			146	0.045	
			150	0.079	
BM1824	6	176-190	176	0.015	0.364
			178	0.212	
			180	0.136	
			182	0.364	
			188	0.242	
			190	0.030	
Mean	8.364				0.372

n_A, number of alleles;
FNA, frequency of the most frequent allele.

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Table 2. Summary of heterozygosities, polymorphism information content values, power of discrimination and power of exclusion in the Serbian population of Simmental cattle

Microsatellite marker	Ho	He	p	PIC	PD	PE
TGLA227	0.733	0.851	0.00324*	0.82	0.922	0.552
BM2113	0.716	0.814	0.05005	0.84	0.920	0.387
TGLA53	0.656	0.893	0.00000*	0.88	0.939	0.393
ETH10	0.522	0.557	0.13205	0.58	0.755	0.246
SPS115	0.719	0.656	0.78688	0.60	0.806	0.485
TGLA126	0.667	0.654	0.95571	0.59	0.818	0.409
TGLA122	0.452	0.728	0.00163*	0.70	0.865	0.175
INRA23	0.774	0.889	0.26167	0.86	0.953	0.472
ETH3	0.742	0.793	0.29764	0.75	0.900	0.424
ETH225	0.594	0.686	0.05124	0.68	0.838	0.322
BM1824	0.710	0.760	0.65137	0.70	0.880	0.472
Mean	0.662	0.753		0.73	CPD=0.999	CPE=0.999

Ho – observed heterozygosity; He – expected heterozygosity; *$p<0.05$; PIC – polymorphism information content; PD – power of discrimination, CPD – combined power of discrimination; PE – power of exclusion; CPE – combined power of exclusion

DISCUSSION

The polymorphism of 11 microsatellite markers from ISAG panel (TGLA227, BM2113, TGLA53, ETH10, SPS115, TGLA126, TGLA122, INRA23, ETH3, ETH225, BM1824) was analysed in order to evaluate their usefulness for pedigree verification in the population of Simmental cattle in Serbia. Such investigations are necessary in most livestock breeds, because pedigree verification based on microsatellite markers is an indispensable step in both, conservation and selection programs. In cattle breeding the extensive application of artificial insemination (AI) causes the increase of pedigree errors. Incorrect paternity recording due to multiple inseminations by different sires could explain, at most, 20% of the paternity mistakes (Weller *et al*., 2004), but there are other factors affecting incorrect paternity assignment 1) mistakes by AI institutes in labeling semen; 2) mistakes by AI technicians in identifying semen straws; 3) the insemination of cows already pregnant by a previous insemination; 4) errors in entering the bull's herdbook number or name into the insemination record; 5) the use of natural-service bulls leading to pregnancies of previously inseminated cows which were assumed to be pregnant from the AI bull; 6) mistakes in sire identification when a cow enters the milking herd in schemes where pedigree information on milk records is obtained through the milk recording program; and 7) interchange of calves at birth (Christensen *et al*., 1982).

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The use of highly polymorphic microsatellite loci and multiplex PCR for their amplification, as well as automated genotype analysis in DNA sequencers give an almost 100% probability of parentage exclusion and ensure that the obtained results are repeatable (Radko, 2008). Paternity or parentage testing effectiveness closely depends on the level of informativeness provided by the markers (Rehout *et al*., 2006). The microsatellites are considered highly polymorphic, highly informative and effective in parentage testing if they exert high n_A, FNA<0.5, high He, PIC, PD and PE (>0.5), and CPD and CPE >0.999.

The results of microsatellite marker polymorphism observed in Simmental cattle analysed in this study, are shown in Tables 1 and 2. Overall, microsatellite DNA markers analysed in the Serbian population of Simmental cattle in this study appeared more polymorphic than in other breeds of Simmental cattle (from Poland, Slovakia, Czech Republic) (Janik *et al*., 2001; Choroszy *et al*., 2006; Czerneková *et al*., 2006). The mean n_A per locus observed in Serbian population of Simmental cattle in our study (8.364) is higher than 7.90 found in Czech Pied and 7.45 found in Slovakian Pied (Czerneková *et al*., 2006) or 7.27 found in Simmental cattle from Poland (Choroszy *et al*., 2006). Moreover, the total number of alleles we observed in Serbian population of Simmental cattle (92) is higher than Janik *et al*. (2001) and Choroszy *et al*. (2006) found in Simmental cattle from Poland for the same set of microsatellite loci (79 and 80, respectively). PIC values for 11 evaluated loci in the Serbian population of Simmental cattle ranged from 0.58 to 0.88, with the mean PIC value of 0.73, which is comparable with the results obtained for the same set of microsatellite loci in other Simmental breeds: mean PIC value was 0.757 in Czech Pied cattle, 0.642 in Slovakian Pied cattle (Czerneková *et al*., 2006) and 0.641 in Simmental cattle from Poland (Choroszy *et al*., 2006). In Simmental cattle from Serbia, He ranged from 0.557 (ETH10, 7 alleles) to 0.893 (TGLA53, 14 alleles), with average value of 0.753, which is comparable with values found in Slovakian Pied (0.650) and Czech Pied (0.764) in the study of Czerneková *et al*. (2006).

Among the 11 microsatellite loci tested in our study, four loci emerged as most polymorphic: TGLA53 (14 alleles, PIC=0.88, FNA=0.235), TGLA227 (11 alleles, PIC=0.82, FNA=0.274), INRA23 (11 alleles, PIC=0.86, FNA=0.167) and BM2113 (9 alleles, PIC=0.84, FNA=0.330). The loci that appeared most informative in Simmental cattle from Serbia in our study (TGLA53, TGLA227, INRA23 and BM2113) were also highly polymorphic in Simmental cattle from Poland (Janik *et al*., 2001; Choroszy *et al*., 2006). High polymorphism at the TGLA53 locus was also reported by Heyen *et al*. (1997) in American Simmentals. Two loci (ETH10 and SPS115) appeared less informative as FNA of these loci is higher than 0.5 (0.600 and 0.515, respectively), which is accordance with the FNA values observed for the same loci in the Simmental cattle from Poland (Choroszy *et al*., 2006). However, the PIC values of both loci are higher than 0.5 in our study so they contribute to the overall informativeness of the whole set of tested markers. In addition, CPD for the whole set of 11 tested markers was 0.999, which is the required level of discrimination in parentage analysis (Vankan and Faddy, 1999; Pérez-Miranda *et al*., 2005). The CPE, which is the exclusion probability considering all 11 ISAG markers, was greater than 0.999, indicating that these loci

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are appropriate to determine parentage in studied cattle beyond any reasonable doubt. These results indicate that whole set of 11 ISAG microsatellite markers is useful for paternity testing and pedigree verification in the studied cattle population.

The means for n_A and He (Tables 1 and 2) indicate high levels of genetic diverisity in the population studied as found in other Simmental breeds of cattle (Janik *et al*., 2001; Choroszy *et al*., 2006; Czerneková *et al*., 2006). The observed deviations from Hardy-Weinberg equilibrium at three loci (TGLA227, TGLA53 and TGLA122) could be a result of the specific selection programs or caused by high polymorphism at the same loci investigated.

In conclusion, the polymorphism of 11 microsatellite markers observed in this study indicate its usefulness for paternity testing and pedigree verification in Simmental cattle from Serbia.

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POLIMORFIZAM DNK MIKROSATELITA I MOGUĆNOST NIJIHOVE PRIMENE U VERIFIKACIJI PEDIGREA SIMENTALSKIH GOVEDA U SRBIJI

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SADRŽAJ

U ovom radu analiziran je polimorfizam 11 mikrosatelitskih DNK markera (TGLA227, BM2113, TGLA53, ETH10, SPS115, TGLA126, TGLA122, INRA23, ETH3, ETH225, BM1824) u cilju procene upotrebljivosti tih markera u verifikaciji pedigrea Simentalskih goveda u Srbiji. Analizirano je ukupno 86 grla. Genomska DNK je izolovana iz sperme i krvi. Za amplifikaciju je primenjen multiplex PCR korišćenjem prajmera komercijalnog seta "StockMarks for Cattle® Bovine Genotyping Kit". Pri analizi rezultata korišćena je tehnologija automatizovanog određivanja veličine DNA fragmenata. Ukupan broj detektovanih alela bio je 92. Srednja vrednost broja alela (n_A) po lokusu iznosila je 8,364, srednja vrednost sadržaja polimorfne informacije (PIC) bila je 0,73, dok je učestalosti najčešćeg alela (FNA) u proseku iznosila 0,372. Najveća polimorfnost uočena je kod lokusa TGLA53 (14 alela, PIC=0,88, FNA=0,235), TGLA227 (11 alela, PIC=0,82, FNA=0,274), INRA23 (11 alela, PIC=0,86, FNA=0,167), BM2113 (9 alela, PIC=0,84, FNA=0,330). Moć diskriminacije i moć isključenja celog seta proučavanih markera iznosila je 0,999. Polimorfizam uočen kod 11 mikrosatelitskih DNK markera u ovom radu ukazuje na mogućnost njihove primene u testiranju očinstva i verifikaciji pedigrea Simentalskih goveda u Srbiji.